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NOTCH SIGNALING IN BLOOD AND LYMPHATIC VESSEL DEVELOPMENT

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Äidille ja isälle
To Mom and Dad

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Original publications

This thesis is based on the following original articles, which are referred to in the text by the following Roman numerals:

I   **Murtomaki A***, Uh MK*, Choi YK, Kitajewski C, Borisenko V, Kitajewski J, Shawber CJ. Notch1 functions as a negative regulator of lymphatic endothelial cell differentiation in the venous endothelium. *Development,* 140: 2365-76 (2013)

II   **Murtomaki A**, Uh MK, Kitajewski C, Zhao J, Nagasaki T, ‡Shawber CJ, ‡Kitajewski J. Notch signaling functions in lymphatic valve formation. *Development,* 141: 2446-51 (2014)

III Kangsamaksin T, **Murtomaki A**, Kofler NM, Cuervo H, Chaudhri RA, Tattersall IW, Rosentiel PE, Shawber CJ, Kitajewski J. NOTCH decoys that selectively block DLL/NOTCH or JAG/NOTCH disrupt angiogenesis by unique mechanisms to inhibit tumor growth. *Cancer Discovery*, 5: 182-97 (2015)

IV   Kofler NM, Cuervo H, Uh MK, **Murtomaki A**, Kitajewski J. Pericyte dysfunction caused by Notch deficiency results in arteriovenous malformations. *Scientific Reports*, 5: 16449 (2015).

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Abbreviations

αSMA	α-smooth muscle actin
Ad	Adenovirus
Ang1	Angiopoietin-1
BEC	Blood endothelial cell
BM	Basement membrane
CD31	PECAM/Platelet endothelial cell adhesion molecule 1
CD34	Hematopoietic progenitor cell antigen CD34
COUP-TFII	Chicken ovalbumin upstream promoter transcription factor II
CSL	CBF-1/Suppressor of hairless/Lag-1
CV	Cardinal vein
CX37	Gap-junction protein 4 (Connexin37)
DLL	Delta-like ligand
E	Embryonic day of murine development
EC	Endothelial cell
ECM	Extracellular matrix
Eph	Erythropoietin-producing hepatocellular carcinoma tyrosine kinase
Flk1	Fetal liver kinase 1 (murine VEGFR-2)
Ftl1	Fms-like tyrosine kinase 1 (VEGFR-1)
Flt4	Fms-like tyrosine kinase 4 (VEGFR-3)
FN	Fibronectin
FN-EIIIA	Fibronectin splice variant containing the EIIIA domain
FOXC2	Forkhead box C2
GFP	Green fluorescent protein
GSI	Gamma-secretase inhibitor
HBD	Heparin binding domain
HdLEC	Human dermal lymphatic endothelial cell
Hes	Hairy/Enhancer of Split
Hey	Hes-related (Hesr) with YRPW
HIF-1α	Hypoxia -inducible factor -1α
HLTS	Hypotrichosis-lymphedema-teleangiectasia syndrome
HSPG	Heparin sulfate proteoglycan
Ig	Immunoglobulin
JAG	Jagged (Notch ligand)
LD	Lymphedema distichiasis
LEC	Lymphatic endothelial cell

LYVE	Lymphatic vessel hyaluronan receptor
LS	Lymph sac
MAML-1	Mastermind-like-1
NECD	Notch extracellular domain
N1IC	Notch intracellular domain
Nrp	Neuropilin
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PlGF	Placenta growth factor
PROX1	Prospero-related homeobox-1
SMC	Smooth muscle cell
SOX18	SRY (sex-determining region Y)-box 18
Tie	Tyrosine kinase with immunoglobulin and EGF homology domains
TNR	Transgenic Notch reporter
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	Wild-type

Abstract

Mammals have two parallel vascular systems, the blood and the lymphatic vascular system. The blood vascular system is a closed system that transports oxygen and nutrients to tissues and metabolic wastes from the tissues to excretory organs. The lymphatic system is unidirectional and consists of blind-ended lymphatic capillaries, which collect excess fluid from the interstitial space and transport it first into larger caliber collecting lymphatic ducts and finally through the lymph nodes back into the bloodstream via a connection in the subclavian vein. Thus, the lymphatic system helps maintain fluid homeostasis. Like veins, lymphatic collecting ducts contain intraluminal valves that ensure unidirectional flow.

Notch signaling is an evolutionary conserved signaling pathway that mediates cell fate decisions and regulates cellular functions through its modulation of downstream targets. Using transgenic mouse models, we studied the role of Notch in embryonic lymphatic development and postnatal blood vascular development.

Lymphatic development begins at embryonic day 9.75 when a subset of endothelial cells in the cardinal vein start expressing Prox1 and other lymphatic markers and become committed to the lymphatic lineage. We show that loss of Notch during the initiation phase leads to an increase in Prox1+ lymphatic progenitor cells emerging from the cardinal vein and lymphatic overgrowth. Thus, Notch is required in the cardinal vein to limit the number of endothelial cells adopting the lymphatic endothelial fate. After lymphatic endothelial specification, lymphatic valve formation begins at embryonic day 15.5, as subsets of lymphatic endothelial cells in the lymphatic duct walls adopt a lymphatic valve fate and separate from the ductal wall cells. Valve-forming lymphatic endothelial cells express high levels of Prox1 ($Prox1^{high}$) while the ductal wall lymphatic endothelial cells express lower amounts ($Prox1^{low}$). Valve-forming lymphatic endothelial cells also express integrin-α9, fibronectin-EIIIA and Connexin37, all of which are necessary for proper valve morphogenesis. We show that loss of Notch signaling in lymphatic endothelial cells at the time of valve initiation results in a disruption of the $Prox1^{high}$/$Prox1^{low}$ expression pattern, a decrease in the number of valves, and abnormal valve morphology. Loss of Notch also resulted in reduced integrin-α9 and fibronectin-EIIIA expression in valve-forming lymphatic endothelial cells. Thus, Notch signaling is required for proper lymphatic valve development and induction of key lymphatic valve proteins during valve morphogenesis.

Blood endothelial cells interact with contractile smooth muscle cells and non-contractile pericytes, which are collectively called mural cells. Endothelial cell-mural cell interactions provide mechanical support to vessels as well as regulate many

vessel functions that are crucial for vascular integrity such as permeability, sprouting and quiescence. Notch1 is expressed in both endothelial cells and mural cells while Notch3 is restricted to vascular mural cells cells. Using a genetic approach, we show that global *Notch1* heterozygocity combined with global *Notch3* deficiency results in impaired vascular smooth muscle cell recruitment in the mouse retina leading to abnormal vascular development. We also demonstrate that biological inhibition of Notch signaling using soluble Notch1 decoys results in defective vascular smooth muscle coverage in the mouse retina. Our data show that both Notch1 and Notch3 are required for proper vascular smooth muscle cell function during vascular development and thus report a novel role for Notch1 in mural cells.

Review of the literature

1. BLOOD VASCULATURE

STRUCTURE AND EMBRYONIC DEVELOPMENT

The blood vascular system consists of the heart, arteries, veins and capillaries connecting the arterial and venous circulation. The main function of the vascular system is to deliver oxygenated blood, nutrients and hormones to tissues and remove carbon dioxide and other metabolic waste products from the tissues. The heart pumps the blood through the arteries into the capillary network where the exchange of gases and nutrient absorption occurs. The capillary network connects to the venous side, which returns the blood back into the heart. The vessels of arteries and veins consist of three distinct layers. The innermost, thinnest layer (tunica intima) consists of a single layer of endothelial cells (ECs), which are adjoined by tight and adherens junctions and surrounded by basement membrane. Endothelial cells regulate multiple blood vessel functions including blood flow and vessel permeability, as well as respond to growth factors that promote formation of new blood vessels. The middle layer (tunica media) of blood vessels consists of connective tissue, elastic fibers and vascular smooth muscle cells (VSMCs). It is the thickest layer in arteries, as the higher arterial blood pressure requires a thicker layer of VSMCs than veins to maintain vessel integrity. The outermost layer (tunica externa) is the thickest layer in veins and consists entirely of connective tissue. Larger caliber veins contain intraluminal valves that prevent the backflow of blood and help keep it flowing toward the heart. Blood capillaries consist of one layer of endothelial cells surrounded by basement membrane (BM), which allows the exchange of substances between the capillaries and tissues. Blood capillaries are supported by pericytes, which share a basement membrane with the endothelial cells and help regulate vascular functions such as permeability, sprouting and quiescence (reviewed in (Coultas et al., 2005)).

The cardiovascular system is the first functional system in a developing vertebrate embryo. In mice, the heart starts beating around embryonic day (E) 7.0. Embryonic blood vessels initially develop separate from the heart and connect to it later on. The *de novo* formation of blood vessels occurs through a process called vasculogenesis (reviewed in (Risau and Flamme, 1995)). At E7.0 common blood and endothelial cell progenitor cells called hemangioblasts aggregate and form blood islands in

the yolk sac. Subsequently, the inner hemangioblasts of the blood island become non-adherent hematopoietic progenitor cells, haemocytoblasts, while the outer hemangioblasts become the adherent endothelial progenitor cells, angioblasts. There is, however, some debate over the process and whether blood and ECs actually have a common progenitor (Eichmann et al., 2002; Fleury et al., 2015). After the initial phase of vasculogenesis, angioblasts divide and differentiate into ECs, which subsequently form a primary blood vascular plexus (reviewed in (Coultas et al., 2005)). The primary plexus is then remodeled via angiogenesis which involves sprouting as well as pruning of unnecessary connections into a mature vascular network (reviewed in (Potente et al., 2011)). Physiological vasculogenesis occurs primarily during embryonic development. Angiogenesis is active both embryonically and postnatally, for instance during wound healing and in female reproductive tissue. Both vasculogenesis and angiogenesis can occur in adult organisms in pathological settings, e.g. in tumors.

SPROUTING ANGIOGENESIS

During angiogenesis, pro-angiogenic growth factors, e.g. vascular endothelial growth factor (VEGF) stimulate pre-existing blood vessels to sprout and form new vessels (Gerhardt et al., 2003; Potente et al., 2011). One of the main triggers of angiogenesis is tissue hypoxia. In the mouse retina, hypoxic cells release pro-angiogenic growth factors, most importantly VEGF, that spread into the surrounding tissue forming a growth factor gradient (Ruhrberg et al., 2002). ECs of existing blood vessels express receptors for these growth factors, most commonly the members of the vascular endothelial growth factor receptor (VEGFR) family (Gerhardt et al., 2003). Growth factor-receptor interaction stimulates proliferation and migration of ECs toward higher concentration of a pro-angiogenic stimulus (VEGF) thus leading the angiogenic sprout to the hypoxic tissue (Gerhardt et al., 2003; Ruhrberg et al., 2002). Subsequently the newly formed vessels become lumenized, secrete a basement membrane, connect to each other through anastomosis, recruit mural cells and become a mature, quiescent vascular network.

During angiogenesis the ECs of the growing sprout are specified into tip cells and stalk cells (Gerhardt et al., 2003). Tip cells extend filopodia and express growth factor receptors that allow them to sense pro-angiogenic cues in the surrounding tissues and guide the nascent sprout toward the source of the growth factor gradient (Gerhardt et al., 2003). Stalk cells are situated behind the tip cells in the angiogenic sprouts and they are less responsive to the pro-angiogenic guidance cues (Gerhardt et al., 2003). They are proliferative and therefore responsible for the actual growth of the sprout (Gerhardt et al., 2003). In order to ensure the formation of an appropriate

amount of angiogenic sprouts, the tip cells inhibit the surrounding cells from adopting the tip cell fate through lateral inhibition, via the Notch pathway, once they have established their position (Hellstrom et al., 2007; Lobov et al., 2007; Suchting et al., 2007). However, it is thought that the tip/stalk cell identity is a very transient and plastic state and that there is constant competition between the ECs in a growing sprout for the tip cell position.

The blood vasculature of mature tissues is usually quiescent. Physiological sprouting angiogenesis in adult organisms is mainly observed during the female reproductive cycle, during tissue regeneration and wound healing making it challenging to study physiological angiogenesis outside the embryonic setting. However, the murine retina is avascular at birth and becomes vascularized in a highly consistent manner during the first three postnatal weeks, making it an excellent model for studying the different aspects of sprouting angiogenesis, including mural cell recruitment and coverage (**Figure 1**) (Gerhardt et al., 2003). During the first postnatal week, blood vessels grow from the optic nerve towards the retinal periphery forming the superficial vascular plexus. Subsequently during the next two weeks, the vessels dive perpendicularly and form the deep and intermediate vascular networks (Gerhardt et al., 2003). Observing the retinal vasculature at P5 allows the study of active sprouting angiogenesis, including determining the number of tip cells, branch points as well as mural cell recruitment. Analysis at P8 enables the study of aspects associated with a more mature network, such as arterio-venous and capillary differentiation, vascular density and mural cell coverage. At later time-points, P12-P21 the functionality of retinal network can be assessed.
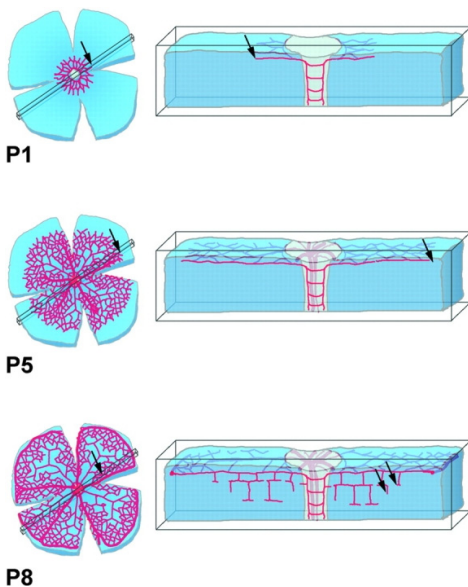
Figure 1. The postnatal vascularization of the mouse retina provides an excellent model for the study of sprouting angiogenesis. Flat-mounted retinas allow visualization and study of different aspects of angiogenesis. During the first postnatal week blood vessels grow from the optic nerve towards the retinal periphery. Upon reaching the edges, the vessels dive perpendicularly and form the deep and intermediate retinal vascular networks. Tip cells can be seen at the leading tips of the growing vessels (white arrows). Adapted from Gerhardt et al. 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. Journal of Cell Biology; 161 (6): 1163-77. © Gerhardt et al. Journal of Cell Biology. 161:1163-1177. doi:10.1083/jcb.200302047

MURAL CELLS

Mural cells have typically been divided into vascular smooth muscle cells (VSMCs) and pericytes. However, recent discoveries suggest that there is a continuum of different mural cell phenotypes rather than only two clearly distinguishable cell types (reviewed in (Armulik et al., 2011)). In this continuum pericytes represent a plastic, relatively undifferentiated mural cell population while vascular smooth muscle cells would be a more terminally differentiated and mature cell type. Pericytes have proven challenging to define conclusively by cell surface markers, many of which are common with fibroblasts and mesenchymal stem cells. In addition, pericytes display significant phenotypic heterogeneity and embryonically originate from multiple tissue types (reviewed in (Armulik et al., 2011; Gaengel et al., 2009)). In blood vasculature, contractile VSMCs are found around arteries and arterioles as well as to lesser extent around veins and venules. They wrap themselves around the blood vessels providing mechanical support and help regulate vessel tone and diameter but are separated from the endothelium by a basement membrane. In lymphatic vasculature, VSMCs are recruited to larger caliber collecting lymphatic ducts where they facilitate the movement of lymph in the collecting ducts. Both VSMCs and pericytes express PDBFR-β, but only mature VSMCs express contractile proteins like α smooth muscle actin (αSMA) and myosin heavy chain, which are used to identify the cell type (**Figure 2**). Like pericytes, VSMCs have diverse origins (reviewed in (Armulik et al., 2011)). Pericytes are defined as mural cells lining smaller caliber vessels and sharing basement membrane (BM) with the endothelial cells. They have an intimate association with the endothelial cells; the two cell types are directly connected through cellular processes they participate in and through sharing of the BM in which pericytes are embedded. Pericytes do not express contractile proteins and are typically identified as cells expressing NG2, PDBFR-β and desmin. It should be noted, however, that some VSMCs also express NG2, which is why several markers should be used to conclusively distinguish the two mural cell types.

Mural cells are crucial for the maintenance of vascular integrity. Pericytes regulate several aspects of EC biology including proliferation, EC-EC junctions, permeability and sprouting (Hellstrom et al., 2001; Stratman et al., 2009). VSMCs support larger vessels and are especially crucial for arteries, which due to high blood pressure require mechanical support. By contracting they also provide a pumping function to help move blood along in the vessels. During sprouting angiogenesis, endothelial cells recruit pericytes to the nascent sprouts and VSMCs to arteries and veins. The subsequent pericyte association with the endothelium induces basement membrane deposition and inhibits sprouting thus promoting vessel maturation and quiescence (reviewed in (Armulik et al., 2011; Stratman et al., 2009)). The central nervous system (CNS) is considered the most pericyte rich tissue and pericytes have been shown to be crucial for establishment and maintenance of the blood-brain-barrier (BBB) (Armulik et al., 2010; Daneman et al., 2010). Cerebral VSMCs are required to maintain vascular integrity in the brain. Indeed, gradual loss of brain
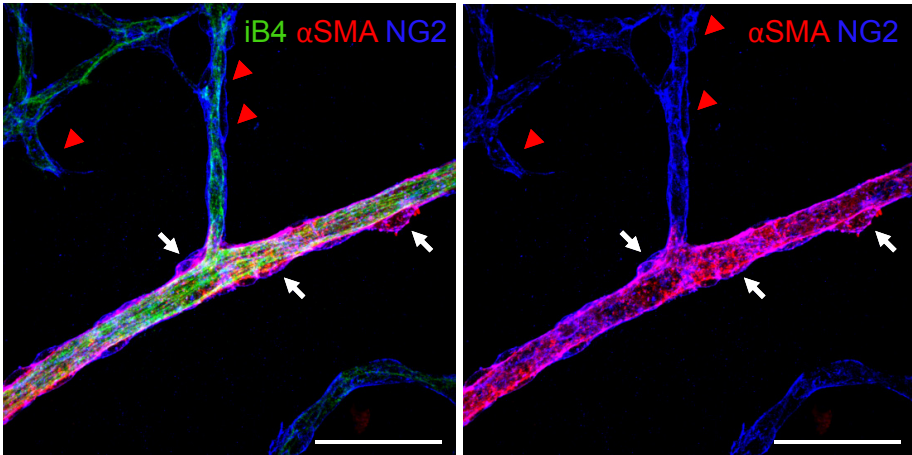
Figure 2. Localization of vascular smooth muscle cells and pericytes in the mouse retina. αSMA /NG2 positive VSMCs are only seen lining arterioles while αSMA negative/NG2 positive pericytes are found associating with both arterioles and smaller caliber vessels in a P5 mouse retina. White arrows mark double positive VSMCs and red arrowheads NG2 single positive pericytes. Blood vessels visualized by isolectin B4 (green). Scalebars 50μm.

VSMCs coverage seen in the lethal human syndrome CADASIL (cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy) causes vessel leakage and strokes (Joutel et al., 1996; Ruchoux et al., 1995).

2. LYMPHATIC VASCULATURE

STRUCTURE OF THE LYMPHATIC VASCULATURE AND LYMPHATIC ENDOTHELIAL PHENOTYPES

Due to high blood pressure on the arterial side of the blood capillary bed fluid and molecules continuously leak into the interstitial space. One of the functions of the lymphatic system is to maintain tissue homeostasis by collecting this excess fluid and macromolecules and returning them into the bloodstream. In addition, the lacteal lymphatic capillaries in the villi of small intestine absorb dietary fats and vitamins from the intestines. Finally, the lymphatic vasculature is essential for immune surveillance as it transports antigen-presenting cells into lymph nodes and provides a route for other immune cells to be transported throughout the body.

The lymphatic vasculature consists of lymphatic capillaries, intermediate size pre-collecting ducts, larger collecting ducts and lymph nodes, which they connect to (**Figure 3**). Unlike the blood vascular system, there is no central pump in the lymphatic vasculature and flow is maintained through compression by surrounding skeletal muscles and contractile VSMCs in larger caliber lymphatic

vessels (**Figure 3**). Different parts of the lymphatic system have distinct functions, which are reflected by their specialized structures. Lymphatic capillaries which are responsible for fluid uptake, consist of a single layer of "oak-leaf" shaped lymphatic endothelial cells (LECs) that are connected by discontinuous button-like junctions. Junction proteins such as VE-cadherin are clearly localized in the junctions and absent from areas between the junctions (reviewed in (Schulte-Merker et al., 2011)). Lymph, consisting of interstitial fluid, lymphocytes and proteins, enters into capillary lumens through these gaps between the junctions as the interstitial pressure increases due to fluid accumulation. To further aid the passive entry of lymph, lymphatic capillaries are surrounded by a discontinuous basement membrane (BM) and lack mural cell coverage. LECs in lymphatic capillaries are attached to the surrounding extracellular matrix (ECM) through anchoring filaments which dilate the vessels opening the junctions during increased tissue pressure (**Figure 3**) (reviewed in (Schulte-Merker et al., 2011)). Lymph is transported from the lymphatic capillaries into pre-collecting ducts and finally to larger caliber collecting lymphatic ducts. Pre-collecting ducts display some lymphatic capillary-like and some collecting duct-like features. LECs in pre-collecting ducts exhibit the same oak-leaf shape as LECs in lymphatic capillaries but also contain intraluminal valves and sparse VSMC coverage, features usually associated with large collecting ducts (reviewed in (Schulte-Merker et al., 2011)). Unlike capillary LECs, collecting duct LECs are elongated and connected by tight, continuous zipper-like junctions with uninterrupted VE-cadherin localization along the junction. Furthermore, collecting ducts are surrounded by a continuous basement membrane and VSMCs (reviewed in (Schulte-Merker et al., 2011)). Thus, the structure of collecting ducts prevents leakage during lymph transport (continuous junctions and BM) and facilitates flow (VSMCs). Furthermore, collecting ducts contain intraluminal valves that ensure unidirectional flow of the lymph. Valves open in response to lymph pressure upstream of the valve allowing the lymph to flow. Once the lymph has passed through the valve it creates reverse flow, which pushes the valve leaflets towards each other closing the valve and thus preventing backflow. From collecting ducts lymph is transported through the lymph nodes into the thoracic duct or the right lymphatic duct and finally back into the blood vascular system through the left and right internal jugular and subclavian veins (**Figure 3**).

During embryonic development, LECs are derived from venous endothelial cells and adopt a lymphatic fate that consists of unique features different from BEC; that is, they express a distinct set of markers (Srinivasan et al., 2007). However, lymphatic marker expression varies significantly between different LEC populations depending on their developmental stage and location within the lymphatic vasculature. Transcription factor Prox1, often referred to as the lymphatic master regulator, is crucial for the initial lymphatic specification as well
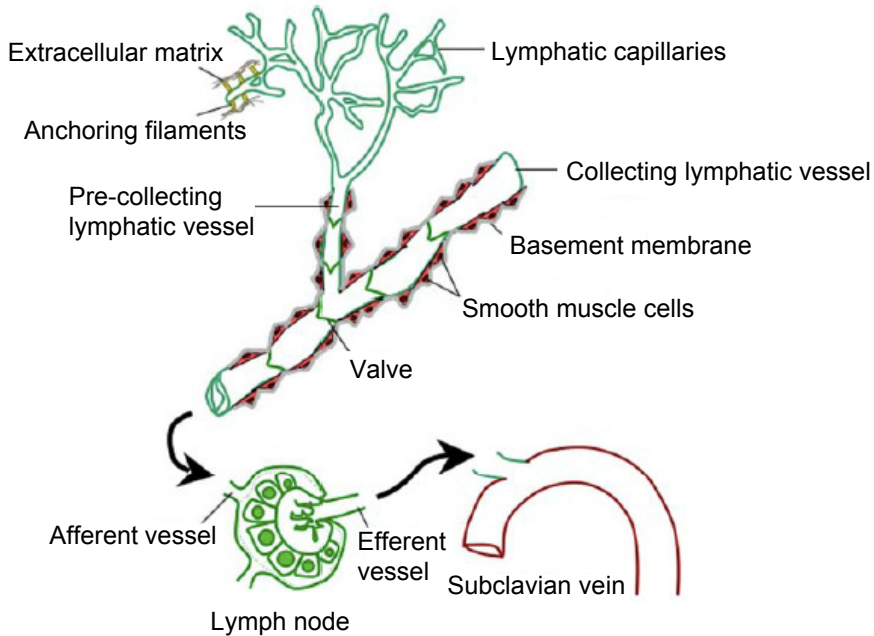
Figure 3. The lymphatic system. Lymphatic capillaries collect excess fluid from tissues and via collecting lymphatic vessels transport it back into the bloodstream. Lymphatic capillaries have a discontinuous basement membrane and no smooth muscle cell coverage to facilitate fluid absorption. Collecting vessels are lined by smooth muscle cells that provide mechanical support and move lymph forward. Intraluminal valves prevent backflow of fluid. Adapted from Maby-El Hajjami and Petrova. Developmental and pathological lymphangiogenesis: from models to human diseases. Histochemistry and Cell Biology .December 2008. 130(6): 1063-1078. © The Author(s) 2008. With permission of Springer Science+Business Media.

as maintenance of the lymphatic endothelial phenotype (Oliver et al., 1993; Oliver and Srinivasan, 2010; Wigle and Oliver, 1999). Indeed, Prox1 is expressed by all LECs throughout development and adulthood (Johnson et al., 2008). Hyaluronan receptor, LYVE1, is expressed by LECs precursors during embryonic development and transiently in collecting ducts before being dramatically downregulated in duct LECs during lymphatic maturation between E17.5-P9 (Norrmen et al., 2009; Oliver, 2004). Strong expression of LYVE1 is only maintained in lymphatic capillaries (Makinen et al., 2005). Similarly, VEGFR-3 expression is strong in all LECs during development, maintained in capillaries while down-regulated in collecting ducts during maturation (Norrmen et al., 2009). VEGFR-3 co-receptor Nrp2 is strongly expressed by a subset of LECs during development with some expression maintained in mature collecting ducts. VEGFR-2 is expressed in collecting ducts during development and strong expression is also seen in developing and mature lymphatic valves (Yuan et al., 2002). Valve-LECs are a distinct population of LECs that separate from lymphatic duct wall LECs during valve morphogenesis. Valve-LECs express high levels of Prox1, Foxc2, VEGFR-2, VEGFR-3 (also known as Flt4)

and gap-junction protein 37 (Cx37), both in forming and mature valves (Kanady et al., 2011; Norrmen et al., 2009; Petrova et al., 2004; Wirzenius et al., 2007). In addition, valve-LECs express integrin-α9 and fibronectin splice variant-EIIIA (FN-EIIIA), which are not seen in other LECs and can thus be used as valve-specific markers (Bazigou et al., 2009).

Mesenteric lymphatic vessels can be easily dissected out together with the intestines and clearly visualized as the mesentery is transparent (**Figure 4**). Therefore, the mesentery is an excellent tissue to utilize for lymphatic studies and widely used in the field. Whole-mount preparations of the mesentery allow the morphological study of mesenteric collecting ducts as well as the lymphatic valves they contain. Mesenteric collecting ducts run side by side with mesenteric arteries and veins, which can be used as controls to ensure that the observed effects are lymphatic specific.
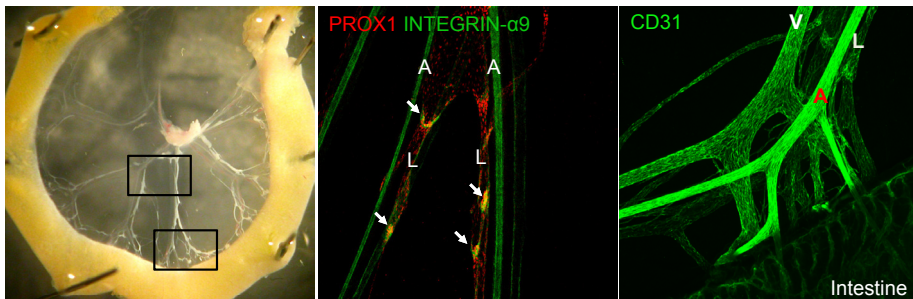
Figure 4. The mesentery can be utilized to study collecting lymphatic vessel and lymphatic valve morphology. Small intestine of a P5 mouse imaged under a light microscope. Fluorescently stained images represent examples of the structures seen in boxed areas showing mesenteric collecting lymphatic ducts containing lymphatic valves and mesenteric arteries and veins running side by side with the lymphatic vessels. White arrows mark PROX1/INTEGRIN-α9 double positive lymphatic valves. A, artery; L, lymphatic collecting duct; V, vein.

EMBRYONIC DEVELOPMENT

The lymphatic system is derived from the embryonic veins. The venous origin of lymphatic structures has been confirmed both in mice and zebrafish suggesting the process is evolutionary conserved (Srinivasan et al., 2007). In mice, lymphatic development begins around midgestation when a subset of ECs in the cardinal veins (CV) begins expressing lymphatic markers and become committed to the lymphatic lineage (**Figure 5**). Subsequently, current models propose that lymphatic vascular morphogenesis proceeds through two distinct mechanisms: "ballooning" of the LEC clusters out of the CVs and direct delamination of LECs from the CV. The ballooning LEC clusters form primitive lymph sacs while the primary lymphatic vessel network is formed through a combination of direct migration of cells from

the CV and sprouting from the lymph sacs (François et al., 2011). Similar to blood vascular development, the primary lymphatic plexus is remodeled into a mature lymphatic vasculature with capillaries and larger lymphatic collecting ducts. During maturation, valves are formed in the collecting ducts and they acquire a basement membrane and VSMCs while capillaries become highly permeable structures specialized in fluid absorption. In mice, lymphatic maturation continues during the first two postnatal weeks when the superficial capillary networks are formed. Although the lymphatic vasculature is the less studied of the two vascular systems, significant advances have been made in understanding lymphatic development and regulators during the past decade.
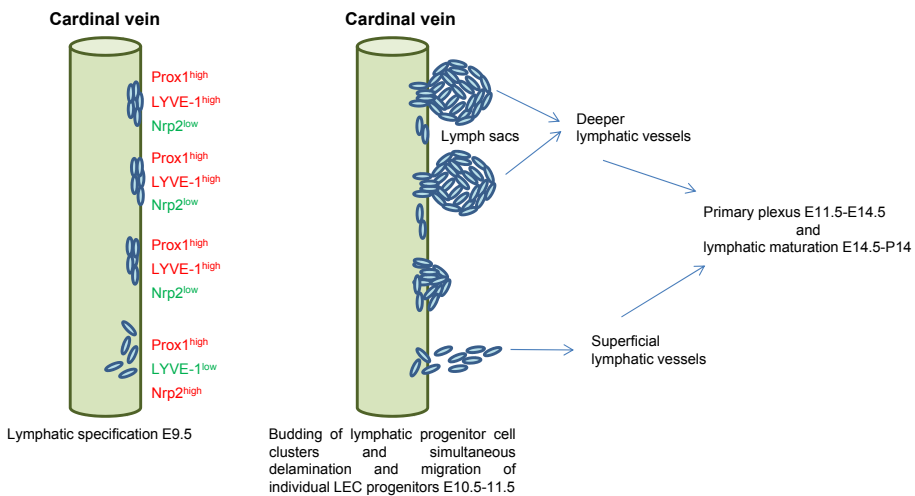
Figure 5. Lymphatic specification. Lymphatic specification occurs on the dorso-lateral side of the cardinal vein where two populations of LEC progenitors can be seen. Prox1high/LYVE-1high/Nrp2low LEC progenitors form clusters which subsequently bud off to form the lymph sacs. At the same time, individual Prox1high/LYVE-1low/Nrp2high LEC progenitors migrate out of the CV. The primary lymphatic plexus is formed between E11.5-E14.5 by a combination of sprouting from the lymph sacs and the individual progenitors delaminating from the CV. The primary plexus is then remodeled into a mature lymphatic network between E14.5-P14 through sprouting and pruning. During maturation, lymphatic capillaries and collecting lymphatic ducts acquire their specialized features and typical marker expression. Based on Francois et al. 2012. Segmental territories along the cardinal veins generate lymph sacs via a ballooning mechanism during embryonic lymphangiogenesis in mice. Developmental Biology; 364 (2): 89-98.

Early lymphatic specification: Sox18, Coup-TFII, Prox1 and LYVE1

In mice, LEC specification occurs around embryonic day 9.75 in the cardinal vein when a subset of venous endothelial cells expressing transcription factor Prox1 and hyaluronan receptor LYVE1 adopt the lymphatic phenotype (Srinivasan et al.,

2007; Wigle et al., 2002; Wigle and Oliver, 1999). Prox1 is absolutely required for lymphatic development as demonstrated by loss of LEC specification and lack of all lymphatic structures in *Prox1$^{-/-}$* mice (Wigle and Oliver, 1999). Prox1 expression maintains the lymphatic phenotype and is thus necessary in LECs beyond embryonic development: loss of Prox1 after completion of lymphatic development results in partial loss of LEC marker expression and misexpression of BEC markers by LECs and edema (Johnson et al., 2008). *In vitro* studies support a Prox1 function as lymphatic master regulator, where ectopic Prox1 expression causes upregulation of lymphatic regulators, such as SLC (Ccl21), Nrp2, podoplanin and integrin-α9 and downregulation of BEC genes, such as CD34, Nrp1 and endoglin. In embryonic development, Prox1 expression is induced by transcription factors Sox18 and Coup-TFII (Francois et al., 2008; Lee et al., 2009; Srinivasan et al., 2010). *Sox18$^{-/-}$* embryos die by E14.5 and show a complete lack of LECs while *Sox18$^{+/-}$* mice display mild edema and defective lymphatic vessel patterning demonstrating that Sox18 is crucial for normal lymphatic development (Francois et al., 2008). Coup-TFII is required for neural and cardiovascular development and Coup-TFII null mice display edema and hemorrhaging and die around E10.0 due to heart and vascular remodeling defects (Pereira et al., 1999). Endothelial specific deletion of Coup-TFII results in a dramatic reduction of Prox1+ LECs in the CV revealing its role in promoting lymphatic development. Sox18 expression is seen in the anterior cardinal vein prior to Prox1 expression and *in vitro* studies show that Sox18 binds directly to the Prox1 promoter (Francois et al., 2008). Coup-TFII is co-expressed with Prox1 in LECs in the CV and around the CV at E11.5, and in lymph sacs and lymphatic vessels later during development and postnatally (Lee et al., 2009; Srinivasan et al., 2010). As Coup-TFII binds to an evolutionary conserved binding site of Prox1 regulatory elements, it is thought to directly induce Prox1 expression (Srinivasan et al., 2010). After E13.5 Prox1 expression no longer requires Sox18 or Coup-TFII and is maintained through mechanisms that have not yet been elucidated (Srinivasan et al., 2010). Consistent with this, Sox18 expression is no longer detected in LECs after E14.5 indicating it is only transiently required for LEC specification, perhaps only to induce Prox1 expression during the initial steps of lymphatic development. However, Coup-TFII still cooperates with Prox1 to drive the expression of some LEC genes, such as *Vegfr3* and *Nrp2* (Lin et al., 2010). Prox1 and Coup-TFII have been shown to jointly regulate the formation of lymphovenous valves (Srinivasan and Oliver, 2011). Therefore, Sox18/Coup-TFII/Prox1 forms the crucial signaling axis that initiates LEC specification. While Sox18 and Coup-TFII induce Prox1 in LEC progenitors, it remains unresolved what induces Sox18 and Coup-TFII expression in endothelial cells in the CV.

LYVE1 expression is detected in the CV as early as E8.5 and its expression together with Prox1 is traditionally said to mark the initiation of lymphatic development.

However, the exact role of LYVE1 in the CV during early lymphatic specification is not clear. LYVE1 deficient mice are viable and only display mild lymphatic vessel dilation in organs with high cell turnover (Gale et al., 2007). Although the exact function of LYVE1 in the CV is unknown, LYVE1 expression seems to faithfully mark the newly specified LECs and is therefore commonly used to identify LECs during embryonic development.

Lymphatic sprouting: VEGF-C/VEGFR-3 signaling and neuropilin 2

An original model of lymphatic development posits that Prox1+/LYVE1+/VEGFR-3+ LEC progenitors are situated on the dorso-lateral side of the CV and migrate out of the CV to form the lymphatic structures. More specifically, lymph sac formation is thought to occur as the LEC progenitors migrate out of the CV towards the embryo periphery guided by VEGF-C. These LECs cluster form primitive lymph sacs while lymphatic vessels are thought to form by LEC proliferation and sprouting from the lymph sacs. Recently it has been shown that Prox1 and LYVE1 expression are not uniformly distributed along the length of the CV. Instead, at E11.5, there are condensations of LEC progenitors expressing high levels of Prox1 and LYVE1 and low levels of Nrp2 that form so called "pre-lymphatic clusters" (PLC) along the antero-posterior axis of the CV (**Figure 5**) (François et al., 2011). Francois et al. showed that LEC progenitors in these PLCs give rise to primitive lymph sacs by "ballooning" out of the CV as a cell cluster. The PLCs stay connected to the CV until E12.5 when they pinch off as the lymphatic system separates from the blood vascular system. The nascent lymph sacs envelop blood cells as they pinch off, which explains why the presence of blood cells has been noted in several studies. The Francois group also showed that there is a separate LEC precursor population in the CV that expresses lower levels of Prox1 and LYVE1 than the LEC progenitors in the PLCs but higher levels of Nrp2. These LEC progenitors delaminate directly out of the CV and migrate towards the embryo periphery and, together with the LECs sprouting out of the primitive lymph sacs, form the lymphatic vessels. In support of this model, Nrp2 is not required for the formation of lymph sacs but is necessary for lymphatic capillary formation, thus defining distinct mechanisms for each process (Yuan et al., 2002). Interestingly, recent data suggest that a large part of superficial dermal lymphatic capillaries in murine embryos is formed by cells of non-venous origin and independently of venous-derived lymphatic capillaries (Martinez-Corral et al., 2015). Martinez-Corral et al. showed that these non-venous progenitor cells start clustering at E12.5 in the embryo periphery and form lymphatic capillaries that only later, at E17.5, connect to the venous-derived lymphatic capillaries (Martinez-Corral et al., 2015). Although these non-venous progenitor cells remain to be identified,

data by Martines-Corral et al. challenges the dogma of the exclusively venous origin of the lymphatic vasculature.

The lymphatic growth factor, VEGF-C, and its receptor, VEGFR-3, are required for LEC migration during lymphatic development (Karkkainen et al., 2004; Zhang et al., 2010). VEGF-C is expressed in the lateral mesoderm of the embryo from E8.5 onwards acting as a paracrine factor forming a growth factor gradient towards which the nascent LECs expressing VEGFR-3 and its coreceptor Nrp2 migrate from the CV (Karkkainen et al., 2004). Lymphatic regulators Coup-TFII and Prox1 have both been shown to upregulate VEGFR-3 as well as Nrp2 expression (Lin et al., 2010; Srinivasan et al., 2010). VEGF-C/VEGFR-3 signaling is further required for the subsequent survival, proliferation, migration and sprouting of the LECs to form the primitive lymphatic vascular plexus (reviewed in (Alitalo et al., 2005)). VEGFR-3 is strongly expressed in all developing lymphatic vessels until E17.5 after which expression is gradually reduced in maturing collecting lymphatic ducts (Norrmen et al., 2009). High expression is maintained in lymphatic valves and capillaries (Norrmen et al., 2009). Nrp2 expression is seen in LECs in the CV from E10.0 onwards (Yuan et al., 2002) and around E11.5 in LECs migrating out of the CV (François et al., 2011).

Separation of blood and lymphatic vasculature: podoplanin

The separation of the nascent lymph sacs from the CV occurs when circulating platelets in the CV aggregate at the sites between the LSs and the CV. Two haematopoietic intracellular signaling proteins, Syk and Slp76, were first identified as regulators of the process, $Syk^{-/-}$ and $Slp7^{6-/-}$ mice die perinatally and exhibit hemorrhage, blood filled lymphatics and abnormal connections between blood and lymphatic vessels (Abtahian et al., 2003). Podoplanin is a lymphatic endothelium specific membrane protein that is expressed on LEC precursors in the ballooning PLCs and was recently found to be required for the separation. Podoplanin has been shown to interact with membrane lectin CLEC-2 expressed on platelets causing platelet activation and aggregation both *in vitro* and *in vivo* (Uhrin et al., 2010). Mouse studies show that podoplanin expression and platelet aggregation is observed at the separation sites between nascent lymph sacs and the CV at E12.5 and a complete separation is achieved by E14.0. It has been suggested that the separation is caused either by the physical barrier formed by the platelet aggregate or through release of vasoconstrictive factors that sever the connections (Uhrin et al., 2010). Regardless of the exact mechanism, podoplanin is required for successful separation as podoplanin deficiency results in lack of platelet aggregation and separation of blood and lymphatic vasculatures, as demonstrated by presence of blood filled lymphatic vessels in the mutant mice (Uhrin et al., 2010).

Lymphatic maturation: Foxc2, ephrinB2 and bone morphogenetic protein 9

Once the primary lymphatic plexus has been formed through sprouting mediated by VEGF-C/VEGFR-3/Nrp2 signaling, the plexus needs to be remodeled into a mature network consisting of lymphatic capillaries and larger collecting ducts with their specialized structures and expression profiles. Transcription factor Foxc2 regulates these later maturation events during lymphatic development. In the mesentery, uniform Foxc2 expression is detected in developing LECs at E15.5(Norrmen et al., 2009). Subsequently Foxc2 expression begins to decrease in the collecting ducts between E15.5 and E16.5, as they mature and strong expression in only maintained in the developing lymphatic valves (Norrmen et al., 2009). The transient expression of Foxc2 is required for the formation of both lymphatic collecting ducts and lymphatic capillaries. Lack of Foxc2 results in a failure of the putative collecting ducts to mature and adopt their proper collecting duct phenotype characterized by presence of intraluminal valves, NG2+ mural cell coverage and downregulation of Prox1, VEGFR-3 and LYVE1 (Norrmen et al., 2009). Lymphatic capillary network formation is also defective in *Foxc2$^{-/-}$* mice (Norrmen et al., 2009). Superficial lymphatic capillary network forms postnatally through sprouting from pre-collecting vessels situated deeper in the tissues. Normal lymphatic capillaries maintain strong LYVE1 expression and lack pericyte coverage. *Foxc2$^{-/-}$* capillaries exhibit impaired sprouting and abnormal recruitment of pericytes around the lymphatic capillaries which impairs their function (Norrmen et al., 2009; Petrova et al., 2004).

Foxc2 cooperates with transcription factor NFATc1 during lymphatic maturation (Kulkarni et al., 2009; Norrmen et al., 2009). The expression pattern of NFATc1 is similar to that of Foxc2 with strongest expression seen in collecting ducts and valves (Norrmen et al., 2009). Furthermore, inhibition of nuclear translocation of NFATc1 and thus NFATc1 signaling, with cyclosporine A (CsA) results in a very similar phenotype to that observed in *Foxc2* deficient mice (Norrmen et al., 2009). Loss of NFATc1 results in embryonic lethality prior to collecting vessel formation thus making a complete analysis impossible, however, at E15.5 *NFATc1$^{-/-}$* embryos display similar tortuous and widened capillaries reminiscent of those seen in *Foxc2* mutant mice (Norrmen et al., 2009). Treating heterozygous Foxc2 mice with CsA results in a significantly more severe lymphatic phenotype compared to wild-type mice treated with CsA further suggesting that the two regulators cooperate during lymphatic maturation (Norrmen et al., 2009). The signal required for Foxc2 upregulation at E15.5 remains to be determined, however, it is known that Prox1 induces NFATc1 expression, while VEGF-C mediated activation of VEGFR-2 induces nuclear translocation of NFATc1 (Norrmen et al., 2009).

EphrinB2 and bone morphogenetic protein 9 (BMP9) have also been implicated in remodeling of the primary lymphatic plexus and collecting vessel maturation (Levet et al., 2013; Makinen et al., 2005). Mice lacking the C-terminal PDZ-

interaction domain of ephrinB2 display a phenotype similar to both Foxc2 and NFATc1 mutant mice. These mutants display hyperplastic collecting ducts that lack valves and maintain LYVE1 expression while the lymphatic capillaries show abnormal mural cell recruitment and a sprouting defect (Makinen et al., 2005). These phenotypes suggest misspecification of the lymphatic structures, that is, that both lymphatic collecting ducts and capillaries failed to acquire their distinct phenotypes. *BMP9⁻/⁻* mice display a similar phenotype with enlarged lymphatic capillaries and collecting ducts that maintain LYVE1 expression and lack valves (Levet et al., 2013). Interestingly, BMP9 was also shown to inhibit LYVE1 and induce Foxc2, Cx37 and ephrinB2 expression *in vitro*, suggesting that BMP9 mediates some of its effects by regulating the expression of these lymphatic regulators (Levet et al., 2013).

3. LYMPHATIC VALVES

EMBRYONIC DEVELOPMENT AND REGULATORS OF VALVE MORPHOGENESIS

Larger caliber lymphatic vessels (pre-collecting and collecting ducts) contain intraluminal valves that ensure unidirectional flow of the lymph. Lymphatic valves consist of two leaflets that are responsible for opening and closing the valve thus regulating flow in lymphatic vessels. The valve leaflets are made up of an ECM core surrounded by specialized LECs anchored to the core. In mice, lymphatic valve morphogenesis begins at E15.5 and four stages of valve formation can be observed (**Figure 6**) (Sabine et al., 2012). During the initiation phase, LECs expressing high levels of transcription factors Prox1 and Foxc2 cluster at the putative valve sites. Next, in the condensation phase around E16.5, constriction of the lymphatic vessels at the sites of valve formation is observed as the valve-forming LECs arrange themselves into ring-like structures and begin to lay down ECM core components, collagen type IV, laminin α5 and fibronectin splice variant EIIIA (FN-EIIIA). During the elongation phase the secretion of valve ECM components increases as the leaflet core matrix is assembled. Simultaneously, the valve-forming LECs reorient, elongate, migrate along and attach themselves to the forming leaflet core through upregulation of integrin-α9, which anchors the valve-forming LECs to the core matrix. During the maturation stage, the valve-forming LECs penetrate into the vessel lumen and form mature valve leaflets (reviewed in (Sabine and Petrova, 2014)). Lymphatic valve morphogenesis is completed during the first postnatal days and functional valves are required throughout life to prevent backflow of fluid that can often be associated with lymphedema.
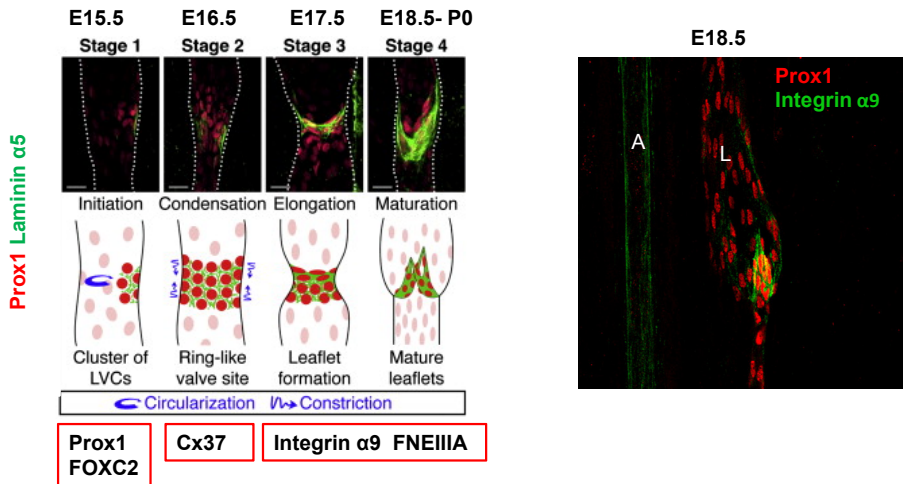
Figure 6. Stages of lymphatic valve morphogenesis. Valve morphogenesis initiates around E15.5 by clustering of Prox1high/FOCX2high LECs. During condensation phase valve-forming LECs form ring-like valve sites. This phase is accompanied by upregulation of gap-junction protein connexin37 (Cx37), which is thought to guide the process. In elongation phase valve-forming LECs reorient, elongate and start invaginating into vessel lumen forming the valve leaflets. Valve leaflet formation requires integrin α9 and fibronectin splice variant EIIIA (FNEIIIA). Valve morphogenesis is concluded by birth. Adapted from Sabine et al. 2012. Mechanotransduction, PROX1, and FOXC2 cooperate to control connexin37 and calcineurin during lymphatic valve formation. Dev. Cell; 14;22(2):430-45. Copyright license number 3620311229274.

The current model of lymphatic valve formation proposes that fluid flow, together with molecular regulators, control the process of valve morphogenesis. Mechanical forces imitating flow have been shown to induce Cx37 expression, NFATc1/ calcineurin signaling and cytoskeletal changes associated with valve-LECs *in vitro*, suggesting that flow controls the expression of valve regulators (reviewed in (Sabine and Petrova, 2014)). In addition, *in vivo* experiments suggest that initiation of valve formation in the mesenteric lymphatics correlates with the onset of lymph flow (Sabine et al., 2012). Furthermore, valves are often situated in vessel branch points and other sites with disturbed flow (Sabine et al., 2012; Wang and Simons, 2014). Together these data demonstrate that flow regulates both valve gene expression and helps determine the positions where valve morphogenesis takes place.

Clustering of Prox1^{high}/Foxc2^{high} valve-forming LECs is followed by an upregulation of gap-junction protein connexin 37 (Cx37) and nuclear accumulation of transcription factor NFATc1 in these valve-forming LECs. The induction of Cx37 and NFATc1 signaling is triggered by flow in cooperation with Prox1 and Foxc2 (Sabine et al., 2012). High Prox1 levels are required for development of lymphovenous valves (Srinivasan and Oliver, 2011). Lymphatic valve-forming LECs express higher levels of Prox1 than ductal LECs implying that high levels of Prox1 might also be required for lymphatic valve formation. However, the exact significance of high Prox1 expression observed in valve-LECs remains to be determined although Prox1

has been shown to induce several genes necessary for valve formation including Foxc2, Cx37, integrin-α9 and VEGFR-3 *in vitro* (Mishima et al., 2007; Sabine et al., 2012) Interestingly, Prox1 and Foxc2 are not uniformly expressed in all valve-forming LECs: cells upstream of the developing valves are Prox1^{high}/Foxc2^{low} while the cells downstream are Prox1^{low}/Foxc2^{high} (Sabine et al., 2012). The significance of the differential expression is unknown but it has been hypothesized that it could determine the direction of the future valve leaflets (Sabine et al., 2012).

Both *Foxc2$^{-/-}$* and *Cx37$^{-/-}$* mice display a dramatic reduction in number of valves proving their importance for valve morphogenesis (Kanady et al., 2011; Norrmen et al., 2009). Interestingly, *Foxc2$^{-/-}$* mice have no detectable Cx37 expression suggesting that lack of Cx37 is at least partially the cause for defective valve formation observed in *Foxc2$^{-/-}$* mice (Sabine et al., 2012). Cx37 regulates cell-cell communication at cell junctions and it has been suggested that the role of Cx37 is to help guide the clustered valve-forming LECs into more organized ring-like structures seen after the initiation phase. Supporting this hypothesis, valve formation arrests after the initiation phase and no ring-like structures are seen in *Cx37$^{-/-}$* mice (Kanady et al., 2011; Sabine et al., 2012). Inhibition of NFATc1/calcineurin signaling via CsA results in reduction of lymphatic valves (Norrmen et al., 2009). Furthermore, endothelial loss of calcineurin regulatory subunit Cnb1 (Cnb1^{ecKO}) inhibits the formation of well-defined valve territories with distinct boundaries between Prox1^{high} and Prox1^{low} cells (Norrmen et al., 2009; Sabine et al., 2012). The subsequent elongation phase was also disrupted in Cnb1^{ecKO} mice, as the valve-forming LECs failed to reorient and invaginate into the vessel lumen (Norrmen et al., 2009). Together these defects caused a significant reduction in the amount of mature valves observed in these mice and the valve structures that were present were poorly formed as their development had arrested (Sabine et al., 2012).

The elongation phase is characterized by increased leaflet core ECM component deposition and integrin-α9 expression in the valve-forming LECs as they invaginate into the vessel lumen. Some laminin α5 deposition is seen already at the initiation phase, however the expression becomes increasingly pronounced as valve morphogenesis progresses (Sabine et al., 2012). Deposition of fibronectin splice variant EIIIA (FN-EIIIA) coincides with the onset of expression of its receptor, integrin-α9, on valve-forming LECs around E16.5-E17 (Bazigou et al., 2009). FN-EIIIA/integrin-α9 signaling during the elongation phase is required for leaflet core matrix assembly and leaflet elongation (Bazigou et al., 2009). Embryonic endothelial-specific loss of *Itga9* (encoding integrin-α9) results in underdeveloped valve leaflets with disorganized matrix core and a similar, although milder, phenotype is seen in *FN-EIIIA* deficient mice (Bazigou et al., 2009).

Valve maturation occurs from E18.5 to early postnatal days (Norrmen et al., 2009; Sabine et al., 2012). Mature valves have two leaflets that are sufficiently

elongated to successfully close the valve and prevent retrograde flow of the lymph. Mature leaflets consist of an organized, thick ECM core. Strong expression of Prox1, Foxc2, Cx37 and integrin-α9, as well as active calcineurin signaling are maintained in LECs in mature valves (Bazigou et al., 2009; Norrmen et al., 2009; Sabine et al., 2012). Postnatal lymphatic inactivation of Cnb1 results in regression of the leaflet ECM core indicating that it is required for valve maintenance (Norrmen et al., 2009). FN-EIIIA/integrin-α9-interaction seems to be dispensable for valve maintenance as the defects observed in *FN-EIIIA*$^{-/-}$ mice gradually diminish postnatally and a transient postnatal inactivation of *Itga9* does not result in valve leaflet regression (Bazigou et al., 2009; Sabine et al., 2012).

Developing and mature valves express high levels of VEGFR-2, VEGFR-3 and ephrinB2 (Makinen et al., 2005; Wirzenius et al., 2007). The function of VEGFR-2 and VEGFR-3 in lymphatic valves is largely undetermined. However, VEGF-C/ VEGFR-2 signaling has been shown to induce nuclear translocation of NFATc1, which is required for both embryonic valve formation and postnatal maintenance (Norrmen et al., 2009), while VEGFR-3 has been suggested to cooperate with Foxc2 during lymphatic collecting duct maturation, during which valve formation also occurs (Petrova et al., 2004). Mutant mice lacking the C-terminal PDZ interaction domain of ephrinB2 (*ephrinB2*$^{\Delta V/\Delta V}$) display a complete lack of lymphatic valves (Makinen et al., 2005). Although the function of ephrinB2 in lymphatic valves is currently poorly understood, it has been shown to regulate internalization of VEGFR-3 and therefore ephrinB2 could modulate valve formation via affecting VEGFR-3 (Wang et al., 2010).

4. REGULATORS OF ANGIOGENESIS AND LYMPHANGIOGENESIS

VASCULAR ENDOTHELIAL GROWTH FACTORS AND THEIR RECEPTORS

Vascular endothelial growth factors (VEGFs) and their receptors are key regulators of endothelial cell function during vasculogenesis, angiogenesis and lymphangiogenesis. The mammalian family of vascular endothelial growth factors consists of five secreted glycoproteins VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) that bind to their cognate vascular endothelial growth factor receptors (VEGFR) 1, 2 and 3. VEGFRs are mainly expressed in endothelial cells although expression is seen in some other cell types, especially in cells of the hematopoietic lineage (Olsson et al., 2006). VEGFR-2 and VEGFR-3 signaling can promote both angiogenic and lymphangiogenic processes depending on developmental stage and context. However, VEGFR-2 most commonly induces angiogenic activity in BECs while VEGFR-3 is the main regulator of lymphangiogenesis in LECs. Instead

of promoting, VEGFR-1 restricts and modulates the angiogenic response (Ho et al., 2012) and recruits macrophages to the sites of tissue remodeling (Pipp et al., 2003). VEGFRs are transmembrane tyrosine kinase receptors that are composed of extracellular immunoglobulin (Ig) homology domains, a transmembrane domain and a C-terminal intracellular (IC) tyrosine kinase domain. Different VEGFR Ig domains convey ligand specificity and regulate dimerization and phosphorylation of the IC tyrosine residues. Upon binding of a dimeric VEGF ligand, receptor dimerization and autophosphorylation takes place followed by phosphorylation of the intracellular phosphorylation sites by other cytoplasmic tyrosine kinases (Koch et al., 2011). This triggers downstream signaling cascades eventually affecting endothelial cell behavior such as survival, proliferation, migration and sprout formation (Koch et al., 2011). The vascular endothelial growth factors have multiple isoforms that are created through alternative splicing and posttranslational processing. Different isoforms have different diffusion rates and receptor binding affinities. The VEGFs also interact with several integrins and neuropilins that have been shown to associate with VEGF receptors and modulate their function (reviewed in (Jeltsch et al., 2013; Tammela et al., 2005)).

VEGF and VEGFR-2

VEGF (VEGF-A) signaling mediated by VEGFR-2 (also known as Flk1 and KDR) is the main inducer of angiogenesis both embryonically and postnatally and promotes EC survival, proliferation, migration and sprouting as well as vascular permeability (de Vries et al., 1992; Gille et al., 2001; Hiratsuka et al., 1998; Quinn et al., 1993) (reviewed in (Ferrara and Kerbel, 2005)). Genetic deletion of VEGF leads to defects in blood island formation, EC development and subsequent embryonic lethality at E8-E9 (reviewed in (Ferrara et al., 2003)). Loss of just one copy of VEGF results in death at E11-E12 due to vascular defects demonstrating that the requirement of VEGF protein is dosage sensitive (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF is first expressed in the anterior parts of the embryo and it guides the migration and organization of VEGFR-1 and VEGFR-2 expressing cells during vascular morphogenesis (Hiratsuka et al., 2005). VEGFR-2 is also indispensable for vascular development; Vegfr2 knockout mice die at E8.5-9.5 and display a complete lack of vasculogenesis and hematopoiesis (Shalaby et al., 1995). VEGFR-2 becomes downregulated in mature blood vessels but low level autocrine VEGF/VEGFR-2 signaling has been shown to be necessary for vascular integrity even in quiescent vasculature (Lee et al., 2007). VEGFR-2 can also bind the processed form of VEGF-C and is expressed in the lymphatic endothelium (Joukov et al., 1996; Joukov et al., 1997). Activation of VEGFR-2 signaling in the lymphatics causes circumferential

growth of lymphatic vessels but is unable to induce lymphatic sprouting (Nagy et al., 2002; Saaristo et al., 2002; Wirzenius et al., 2007).

Six VEGF isoforms consisting of different amount of amino acids have been identified in humans: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ (reviewed in (Ferrara et al., 2003; Robinson and Stringer, 2001)). Of these, $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are the major variants expressed by most cell types (reviewed in (Robinson and Stringer, 2001)). In mice, three splice variants have been identified: $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$. The isoforms display different diffusion rates, as the larger variants bind to the ECM heparan sulfate proteoglycans (HSPGs) with greater affinity than the smaller ones (reviewed in (Ferrara et al., 2003)). Therefore the isoforms have different biological functions, which has been demonstrated by distinct phenotypes observed in VEGF isoform-specific knockout mice (Carmeliet et al., 1999; Stalmans et al., 2002). VEGF has also been shown to bind to Nrp1 and Nrp2 which act as coreceptors for VEGFRs (Gluzman-Poltorak et al., 2000; Soker et al., 2002). A soluble version of the VEGFR-2 (sVEGFR-2) lacking the transmembrane and intracellular domains is produced through alternative splicing (Albuquerque et al., 2009). sVEGFR-2 functions as a natural inhibitor of lymphangiogenesis by sequestering VEGF-C (Albuquerque et al., 2009). It was shown to be highly expressed in the cornea and be required for maintenance of the alymphatic cornea, similar to the way that sVEGFR-1 maintains corneal avascularity (Ambati et al., 2006). In addition, sVEGFR-2 expression was detected in the epidermis and hair follicles and it was shown to modulate postnatal growth of peripheral lymphatic capillaries (Albuquerque et al., 2009). Interestingly, sVEGFR-2 was also shown to be present in circulation, bind VEGF and promote mural cell association with vascular endothelium and vessel maturation (Lorquet et al., 2010).

VEGF expression is strongly induced in hypoxic conditions by hypoxia inducible factor α1 (HIF- α1) (reviewed in (Pugh and Ratcliffe, 2003)). HIF-α1 is rapidly degraded in normoxic conditions but becomes stabilized under hypoxia. HIF-α1 then heterodimerizes with HIF-β1 and binds to hypoxia-response elements on VEGF promoter to induce expression (reviewed in (Pugh and Ratcliffe, 2003)). Subsequent secretion and diffusion of VEGF forms a gradient and activates ECs via VEGFR-2 on the already existing vessels to initiate sprouting towards the hypoxic tissue thus providing a new blood supply (Gerhardt et al., 2003; Ng et al., 2006). In sprouting angiogenesis, VEGF also induces the formation of tip cells that via expression of VEGFR-2 sense the VEGF gradient and lead the growing sprout towards the source of the growth factor (Gerhardt et al., 2003). This process has been extensively studied during postnatal vascularization of the mouse retina where VEGF gradient guides the neovessel growth from the optic nerve towards the retinal periphery. VEGF/VEGFR-2 signaling promotes angiogenesis in pathological settings as well. VEGF expression has been shown to drive tumor angiogenesis and growth as well as excessive vascular growth seen in retinopathies (reviewed in (Jeltsch et al., 2013)).

VEGF-B and PlGF

VEGF-B and PlGF are ligands for VEGFR-1 and Nrp1 (Olofsson et al., 1996; Persico et al., 1999). VEGF-B is expressed in striated muscle, myocardium and brown fat while PlGF is predominantly present in the placenta, heart and lungs (reviewed in (Olofsson et al., 1999; Salven et al., 1998)). The exact roles of these growth factors are still being studied. VEGF-B is a poor inducer of angiogenesis; however, it has been shown to regulate fatty acid uptake by ECs and thus their lipid metabolism (Hagberg et al., 2013). PlGF induces angiogenesis in various tissues although this may at least partially be due to heterodimerization with VEGF. VEGF-B knockout mice display slightly smaller hearts in one genetic background but otherwise VEGF-B and PlGF deficient mice display no obvious vascular, - or other, phenotype and have a normal lifespan indicating that both growth factors are dispensable for embryonic development (Bellomo et al., 2000). However, studies suggest that VEGF-B and PlGF might play a role in revascularization during pathological settings, especially during recovery from ischemic episodes (Bellomo et al., 2000; Carmeliet et al., 2001; Li et al., 2008; Luttun et al., 2002). In addition, VEGF-B has been shown to induce coronary artery growth in rats (Bry et al., 2010) while PlGF promotes wound healing (Failla et al., 2000; Kagawa et al., 2009). VEGF-B does not have an obvious function in tumor biology while PlGF has been suggested to drive tumor angiogenesis and growth in several tumor models although its function seems to be context-dependent (reviewed in (Bry et al., 2014)).

VEGFR-1

VEGFR-1 (Flt-1) binds VEGF, VEGF-B and PlGF with high affinity, however, ligand binding results only in weak tyrosine kinase activity (Fong et al., 1995; Huang et al., 2001). VEGFR-1 can form heterodimers with VEGFR-2 which leads to unique downstream signaling events different from both VEGFR-1 and VEGFR-2 homodimers (Huang et al., 2001). VEGFR-1 is expressed in ECs and macrophages, and it has been shown to recruit macrophages to the sites of inflammation (Zachary and Gliki, 2001). VEGFR-1 deficient mice die at E8.5 due to disorganization of the developing vasculature and endothelial cell overgrowth (reviewed in (Ferrara et al., 2003; Fong et al., 1995)). In certain settings, loss of VEGFR-1 postnatally results in increased angiogenesis as well (Ho et al., 2012). Interestingly, mice expressing a truncated form of the receptor lacking the intracellular signaling domain are viable and only display a mild phenotype (Hiratsuka et al., 1998; Niida et al., 2005; Sawano et al., 2001). These data suggest that VEGFR-1 signaling is dispensable and that VEGFR-1 mainly functions as a decoy receptor to sequester excessive VEGF and thus modulate angiogenesis. Mechanistically, in the absence of VEGFR-1, more

VEGF is available to bind and activate VEGFR-2, which promotes angiogenesis (Ho et al., 2012). Interestingly, in addition to limiting angiogenesis, VEGFR-1 has a pro-angiogenic function in vascular sprout formation and guidance from the dorsal aorta (DA) during embryonic development, evident by decreased intersomitic sprout formation seen in *flt-1*^{-/-} embryos (Kearney et al., 2002). In addition to membrane-tethered form, a soluble form of VEGFR-1 (sVEGFR-1) consisting of the extracellular domain is produced through alternative splicing (Shibuya, 2001). sVEGFR-1 has been shown to inhibit VEGF induced angiogenesis, promote vessel maturation through mural cell recruitment and maintain avascularity of the cornea (reviewed in (Ferrara et al., 2003)) (Ambati et al., 2006; Lorquet et al., 2010). As VEGFR-1 is an inhibitor of angiogenesis it has not been indicated in tumor progression, however, it might play a role in other pathologies. Most notably, elevated circulating sVEGFR-1 levels are seen in pre-eclampsia, a condition characterized with hypertension and proteinuria (Levine et al., 2004; Maynard et al., 2003).

VEGF-C, VEGF-D and VEGFR-3

VEGFR-3 (Flt4) is the main mediator of LEC survival, proliferation, migration and sprouting, but it is also necessary for embryonic vascular development (Dumont et al., 1998). VEGFR-3 is expressed by all ECs during early development but expression is downregulated, first in arteries then veins and finally expression is largely restricted to the lymphatic endothelium by birth (Kaipainen et al., 1995). In adults, the only BECs expressing VEGFR-3 are those forming fenestrated blood vessels found in endocrine organs and blood vessels in the peri-implantation uterus (Douglas et al., 2009; Partanen et al., 2000). In addition, tumor blood vessels have been shown to express VEGFR-3. VEGFR-3 binds VEGF-C and VEGF-D and both factors are proteolytically processed to increase their affinity to their receptors (Achen et al., 1998). Processing increases their affinity to VEGFR-3 while only fully processed, mature forms bind to VEGFR-2 (Achen et al., 1998; Joukov et al., 1997). VEGF-C transcripts are induced by multiple growth factors, pro-inflammatory cytokines as well as environmental stress signals, but not hypoxia (Cohen et al., 2009; Enholm et al., 1997). Unlike human VEGF-D, mouse VEGF-D does not bind to VEGFR-2 (Baldwin et al., 2001). Embryonically, VEGF-C expression is seen at the sites of lymph sac formation and later associated with lymphatic sprouting (Karkkainen et al., 2004; Kukk et al., 1996). Two VEGF-D splice variants exist and most prominent expression is seen in the heart, lung, skeletal muscle and the small intestine (Achen et al., 1998; Avantaggiato et al., 1998; Baldwin et al., 2001).

Vegfc^{-/-} mice lack all lymphatic structures and die prior to birth and even *Vegfc*^{+/-} mice display chylous ascites and lymphatic hypoplasia at birth (Karkkainen et al.,

2004). On the other hand, deletion of *Vegfd* only results in a mild phenotype with reduced lymphatic vessel density in the bronchioles (Baldwin et al., 2005). Indeed, deletion of both *Vegfc* and *Vegfd* recapitulates the phenotype seen in *Vegfc-/-* mice with no additional defects caused by deletion of *Vegfd* (Haiko et al., 2008). Even though *Vegfd* is not sufficient to compensate for the loss of *Vegfc*, *Vegfd* overexpression has been shown to promote both angiogenesis and lymphangiogenesis *in vivo* (Baluk et al., 2005; Bhardwaj et al., 2003; Byzova et al., 2002; Rissanen et al., 2003).

Vegfr3 deficient mice die by E10.5, prior to lymphatic development, due to defects in remodeling of the primary vascular plexus into a more hierarchical structure and impaired hematopoiesis (Dumont et al., 1998; Hamada et al., 2000). *Vegfr3* mutant mouse lacking the ligand-binding domain (LBD) or with an inactivating mutation in the kinase domain (TKmut) of the protein have enabled the study of its role in embryonic lymphatic development (Zhang et al., 2010). Embryos with mutated kinase domain lacked all lymphatic structures and developed severe edema. Interestingly, lymph sac formation was observed in mice that lacked the LBD of VEGFR-3 even though lymph sacs do not form in absence of VEGF-C. Also, blood vascular development was normal in both mutants suggesting that neither ligand binding nor kinase activity of VEGFR-3 is indispensable for angiogenesis (Zhang et al., 2010). A possible explanation to these phenotypes is that since both VEGFR-3 mutant proteins are still able to form heterodimers with VEGFR-2, ligand binding to VEGFR-2 could activate VEGFR-3 signaling in heterodimers and thus promote early blood vascular development and also lymph sac formation (Zhang et al., 2010). Furthermore, Nrp2 binds VEGF-C and could therefore indirectly activate VEGFR-3 signaling (Karpanen et al., 2006a; Zhang et al., 2010). It has also been shown that loss of both VEGF-C and VEGF-D does not cause defects in blood vascular development even though they are the only known ligands of VEGFR-3, raising the possibility that another ligand exists (Haiko et al., 2008). It has also been suggested that VEGFR-3 is capable of ligand-independent signaling in addition to ligand-dependent activation, and that the different signaling types have different downstream effects (Tammela et al. 2011). Indeed, data by Tammela et al. demonstrated that VEGFR-3 phosphorylation could occur in the absence of ligands *in vitro* (Tammela et al. 2011). Furthermore, postnatal endothelial-specific deletion of *Vegfr3* caused a hypersprouting phenotype, while previous data showed that VEGFR-3-blocking antibodies suppressed angiogenic sprouting in the neonatal murine retina (Tammela et al. 2008, Tammela et al. 2011). They hypothesized that in the presence of VEGFR-3 blocking antibodies ligand-independent signaling still occurs, which induces Notch signaling and inhibits sprouting (Tammela et al. 2011). In contrast, deletion of *Vegfr3* impairs both ligand-dependent and ligand-independent signaling resulting in decreased Notch signaling and hypersprouting

(Tammela et al. 2011). Mature lymphatic vessel survival appears to not require VEGFR-3 signaling as treatment with a VEGFR-3 blocking antibody has no effect on established lymphatic vasculature and only blocks formation of new lymphatic vessels (Karpanen et al., 2006b; Pytowski et al., 2005).

NEUROPILINS

Neuropilin1 and 2 are transmembrane glycoprotein receptors with a large extracellular domain and a short intracellular domain that have no independent signaling activity (Kolodkin et al., 1997). In neural cells during axon guidance, neuropilins bind class III semaphorins and function as coreceptors for Plexin signaling mediating repulsive guidance cues and both $Nrp1^{-/-}$ and $Nrp2^{-/-}$ mutant mice display defects in cranial nerve guidance (Chen et al., 2000; Giger et al., 2000; Kitsukawa et al., 1997). In the vasculature, neuropilins bind VEGFs and act as coreceptors for VEGFRs and appear to mediate attractive guidance cues. Nrp1 associates with all three VEGFRs and is capable of binding $VEGF_{165}$, $VEGF-B_{167}$, $VEGF-B_{168}$, PlGF-2, VEGF-C and VEGF-D while Nrp2 interacts with VEGFR-1 and VEGFR-3 and binds $VEGF_{145}$, $VEGF_{165}$, PlGF-2, VEGF-C and VEGF-D (reviewed in (Bagri et al., 2009)), (Gluzman-Poltorak et al., 2000; Kolodkin et al., 1997; Makinen et al., 1999; Neufeld et al., 2002; Soker et al., 1998). Nrp1 deficiency leads to defects in arterial differentiation, heart development and death by E14 while inhibition of Sema3A/Nrp1 signaling during development with blocking antibodies results in impaired lymphatic collecting duct maturation and abnormal lymphatic valve morphology (Gerhardt et al., 2004; Jurisic et al., 2012; Kawasaki et al., 1999). Mice lacking Nrp2 are viable and display only a transient lymphatic phenotype during development (Yuan et al., 2002). The distinct phenotypes observed reflect the angiogenic versus lymphangiogenic functions for VEGFRs that interact with neuropilins. Nrp1 enhances VEGF/VEGFR-2 interactions while Nrp2 has been shown to co-internalize with VEGFR-3 upon binding VEGF-C or VEGF-D (Karpanen et al., 2006a).

MOLECULAR MECHANISMS REGULATING ENDOTHELIAL CELL – VASCULAR MURAL CELL INTERACTIONS

PDGF-B/PDGFR-β

Platelet-derived growth factor B (PDGF-B) is a secreted growth factor that binds as a dimer to platelet derived growth factor receptor β (PDGFR-β). Once secreted, PDGF-B is usually

bound to the ECM heparin sulfate proteoglycans through a C-terminal retention motif (reviewed in (Andrae et al., 2008)), (Abramsson et al., 2007; Kurup et al., 2006; Ostman et al., 1991). PDGFR-β is a transmembrane tyrosine kinase receptor that dimerizes and trans-autophosphorylates upon ligand binding to activate downstream signaling cascades

Sprouting endothelial tip cells secrete PDGF-B which binds PDGFR-β expressed on mural cells (Gerhardt and Betsholtz, 2003). Binding of PDGF-B to PDGFR-β promotes pericyte proliferation and migration to the nascent sprout, which subsequently promotes vessel maturation. PDGF-B/ PDGFR-β signaling has been shown to be indispensable for pericyte recruitment as genetic or pharmacological inhibition prevents pericyte recruitment to most organs (Hellstrom et al., 1999; Lindahl et al., 1997). Indeed, both PDGF-B and PDGFR-β deficient mice die perinatally due to loss of vascular integrity and subsequent leakage and hemorrhaging caused by defective pericyte/VSMC coverage in multiple tissues (Hellstrom et al., 1999; Leveen et al., 1994; Lindahl et al., 1998; Soriano, 1994). Furthermore, mice expressing a mutated form of PDGF-B lacking the retention motif also exhibit impaired pericyte recruitment suggesting that PDGF-B has to be either cell membrane or matrix-bound in order to exert its appropriate effects on pericytes (Bjarnegard et al., 2004; Enge et al., 2002; Lindblom et al., 2003). Genetic deletion of either PDGF-B or PDGFR-β resulted in defective pericyte recruitment to the CNS and abnormal vascular permeability, the severity of which was found to directly correlate with the extent of pericyte coverage loss (Armulik et al., 2010; Daneman et al., 2010). PDGFR-β is also expressed by developing VSMCs and it is thought to play a role in VSMC maturation as they are recruited to arteries and veins (reviewed in (Armulik et al., 2011)).

Angiopoietin-1/Tie2

Angiopoietin-1 (Ang-1) is a secreted growth factor expressed by perivascular cells that binds to the Tie2 receptor tyrosine kinase expressed by endothelial cells (Davis et al., 1996; Dumont et al., 1992; Sundberg et al., 2002; Wakui et al., 2006). Both *Ang1*-/- and *Tie2*-/- mutant mice display cardiac defects, decreased mural cell coverage and loss of vessel stability leading to hemorrhaging of the microvasculature, and die by E12.5 and E14.5, respectively (Puri et al., 1995; Sato et al., 1995), (reviewed in (Gaengel et al., 2009)). The vasculature also shows defective hierarchical organization into large and small vessels suggesting impaired remodeling of the primary plexus (Patan, 1998; Sato et al., 1995). Ang-1/Tie2 signaling has been thought to mediate mural cell-endothelial cell interactions and thus promote vascular maturation and stability (Suri et al., 1996). However, recent reports have challenged the idea that loss of Ang-1/Tie2 signaling disrupts pericyte recruitment and causes the cardiovascular

defects seen in *Ang1* and *Tie2* mutant mice. Chimeric mice containing both wild-type and *Tie2*^{-/-} cells showed normal pericyte recruitment to the Tie2 negative endothelium embryonically (Jones et al., 2001). Normal pericyte recruitment was also observed in mice carrying signaling deficient Tie2 receptors (Tachibana et al., 2005). Conditional deletion of *Ang1* did not disrupt pericyte recruitment either (Jeansson et al., 2011). Overexpression of Ang-1 promotes vascular branching and remodeling of an immature vascular plexus demonstrating that Ang-1 functions in blood vessel formation (Thurston et al., 2005; Uemura et al., 2002). In light of these data it has been suggested that loss of Ang-1/Tie2 signaling causes primarily vascular defects and instability and that the loss of mural cell coverage observed in *Ang1*^{-/-} and *Tie2*^{-/-} mutant mice is secondary to endothelial cell defects. Thus, Ang-1 and Tie2 seem to not be directly involved in mural cell recruitment but are required for angiogenic sprouting/remodeling of the primary vascular plexus and stabilization of the newly formed sprouts (reviewed in (Armulik et al., 2011)).

THE NOTCH SIGNALING PATHWAY

The mammalian Notch family consists of five Delta/Serrate/Lag-2 (DSL) ligands Delta-like1 (Dll1), Delta-like3 (Dll3), Delta-like4 (Dll4), Jagged1 (Jag1) and Jagged2 (Jag2) that interact with four receptors of the Notch family, Notch1-4. The Notch proteins are single-pass transmembrane receptors consisting of an extracellular domain and an intracellular signaling domain linked non-covalently. The large extracellular domain of Notch consists of 29-36 EGF-like repeats; repeats 11 and 12 are required for interaction with Notch ligands (Cordle et al., 2008; Hambleton et al., 2004; Thomas et al., 1991; Vassin et al., 1987; Wharton et al., 1985). The ligands consist of EGF-like repeats similar to Notch with Jag ligands containing 16 and Dll ligands 8 or fewer. The Notch ligands also contain a Delta/Serrate/LAG-2 (DSL) domain, which is required for interaction and activation of Notch (Glittenberg et al., 2006; Henderson et al., 1997). Both Notch ligands and proteins are membrane-bound and therefore Notch signaling requires cell-cell contact between the ligand-presenting and receptor-expressing cell (**Figure 7**). It seems that ligands must be membrane-bound in order to efficiently induce Notch signaling as engineered soluble ligand molecules have been shown to mostly inhibit Notch signaling by competitively binding to the receptors (Dikic and Schmidt, 2010; Hicks et al., 2002). However, there are some reports of multimerized ligands able to activate Notch as well (Hicks et al., 2002). Ligand binding triggers conformational changes in Notch unmasking a cleavage site in the extracellular part. Notch is subsequently cleaved by ADAM protease(s) releasing the extracellular domain (Notch-ECD) which, together with the ligand, is endocytosed to the ligand-presenting cell (Bozkulak

and Weinmaster, 2009; Brou et al., 2000; Hartmann et al., 2002). The Notch receptor undergoes a second cleavage by a γ-secretase/presenilin complex, which releases the intracellular domain (N1IC) allowing it to translocate into the nucleus where it forms a complex with CSL (CBF1/ RBPκj, Suppressor of Hairless, Lag-1) and Mastermind-like (MAML). N1IC converts the transcriptional repressor complex CSL into a transcriptional activator and subsequently activates gene transcription through binding to CSL motifs within the promoters of target genes. Alternatively, N1IC/CSL/MAML complex can induce the expression of transcriptional repressors of the Hairy enhancer-of-split (HES) and Hairy enhancer-of-split with YRPW motif (Hey) families and thus indirectly suppress gene expression (reviewed in (Kopan and Ilagan, 2009)).
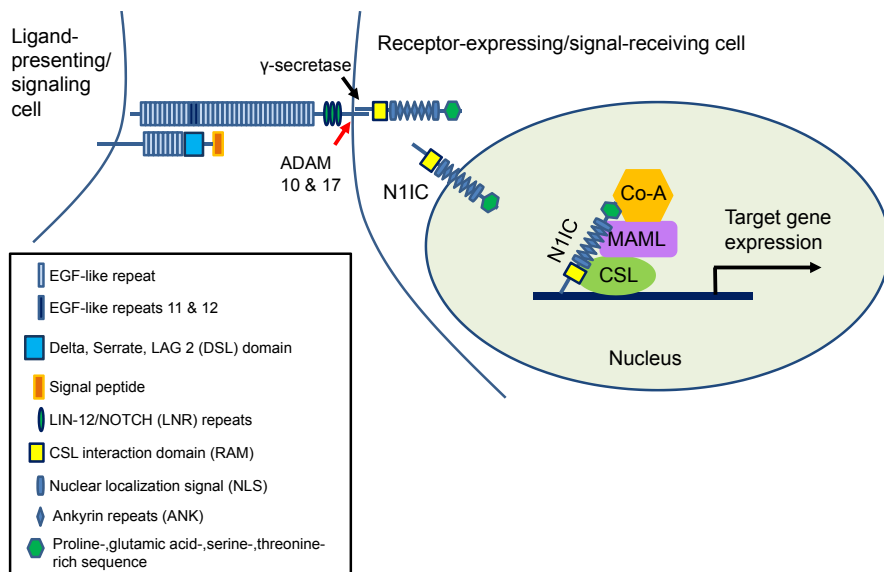
Figure 7. The Notch signaling pathway. Notch signaling requires cell-cell contact between the ligand-presenting and receptor-expressing cell. Upon ligand binding, two proteolytic cleavages take place: first, cleavage by ADAM protease(s) releases the extracellular domain and subsequently, cleavage by γ-secretase/presenilin complex releases the intracellular domain (N1IC). N1IC translocates into the nucleus where it forms a complex with CBF1/RBPκj, Suppressor of Hairless, Lag-1 (CSL), Mastermind-like (MAML) and co-activators (Co-A), which activates target gene expression.

The Notch signaling pathway is an evolutionary conserved signaling pathway that regulates various aspects of embryonic development and tissue regeneration in adults. Notch mediates cell-fate decisions in a highly context dependent manner and can thus either promote or inhibit proliferation, cell death and activation of various differentiation programs through its downstream targets. Notch signaling is indispensable for normal embryonic development as well as maintenance of proper

cell fates in many adult tissues. Indeed, abnormal expression of Notch ligands and/ or proteins has been implicated in tumor growth and progression and several other pathological conditions in humans (reviewed in (Andersson et al., 2011)).

Notch in blood vasculature

The importance of Notch signaling for mammalian vascular development has been demonstrated using several transgenic mouse lines. Blood endothelial cells express Notch1, Notch4, Dll1, Dll4 and Jagged1 and *Notch1$^{-/-}$, Dll1$^{-/-}$, Dll4$^{-/-}$* and *Jagged1$^{-/-}$* mice die between E9.5 and E12.5 due to vascular remodeling defects (Hrabe de Angelis et al., 1997; Krebs et al., 2004; Krebs et al., 2000; Xue et al., 1999). *Dll4* haploinsufficiency causes severe vascular defects and embryonic lethality in certain inbred backgrounds indicating that it is required in dosage dependent manner for normal vascular development (Duarte et al., 2004; Gale et al., 2004). Notch1 expression is detected at E7.5 in the embryonic mesoderm and in the endothelium from E8.5 onwards. In addition, strong expression is seen in presomitic mesoderm and neuroectoderm and Notch1 has been shown to be indispensable for somite polarization and segmentation as well as neural development (Conlon et al., 1995; Del Amo et al., 1992; Reaume et al., 1992). Notch4 displays an endothelial cell specific expression pattern both during development and adulthood (Shirayoshi et al., 1997; Uyttendaele et al., 1996). *Notch4* null mice display no vascular phenotype developmentally suggesting Notch1 is able to compensate for the loss of Notch4 in endothelial cells (Krebs et al., 2000). Although *Notch4$^{-/-}$* mice do not exhibit a vascular phenotype embryonically, loss of *Notch4* combined with *Notch1* heterozygocity or deficiency results in a more severe phenotype than *Notch1* heterozygocity or loss alone indicating that the two Notch proteins genetically interact (Krebs et al., 2000). Postnatally, Notch4 has been implicated in pathological angiogenic settings. Overexpression of constitutively active form of Notch4 is associated with arteriovenous malformations (Murphy et al., 2014).

Dll4 has been shown to be the key ligand required for blood vascular development in mice (Gale et al., 2004; Krebs et al., 2004; Suchting et al., 2007). Dll4 expression is first seen in the dorsal aortae and endocardium at E7 and later during development and adulthood in the arterial vasculature where it functions as a ligand for Notch1 and Notch4 (Shutter et al., 2000). Jagged1 is first detected at E8.5 in endothelial cells and heart tube (Lindsell et al., 1995; Myat et al., 1996). Notch1/Dll4 signaling has been shown to regulate arterial-venous specification by promoting arterial fate (Duarte et al., 2004; Kim et al., 2008; Trindade et al., 2008). Dll1 expression is restricted to the arterial endothelium where it is first seen at E13.5, and it is required for maintenance of arterial phenotype later in development and postnatally (Lawson et al., 2001; Limbourg et al., 2007; Lobov et al., 2007; Sorensen et al., 2009).

During sprouting angiogenesis in the postnatal mouse retina, Notch signaling functions to limit the number of "tip cells" and thus sprouts that form in response to VEGF released by the underlying astrocyte network (**Figure 8**) (Hellstrom et al., 2007). According to the current model, VEGF induces Dll4 expression in tip cells which in turn activates Notch signaling in the adjacent endothelial cells and through lateral inhibition prevents them from adopting the tip cell fate (Hellstrom et al., 2007; Lobov et al., 2007; Suchting et al., 2007). These cells with active Notch signaling become "stalk cells" instead, which are more proliferative than the chemotactic tip cells guiding the growth of the sprout. Supporting the model, it was shown that genetic or pharmacological disruption of Dll4/Notch signaling leads to excessive tip cell and sprout formation and increased vascular density in the mouse retina (Hellstrom et al., 2007). Although the role of endothelial Jagged1 is less studied, it seems that Jag1/Notch signaling normally promotes angiogenesis as endothelial-specific loss of *Jagged1* results in impaired angiogenesis in the mouse retina (Benedito et al., 2009). Notch signaling also regulates the expression of VEGF receptors and it is thought that the tip and stalk cells display different expression profiles due to their different Notch signaling status (Shawber et al., 2007; Suchting et al., 2007; Tammela et al., 2008). Tip cells express Dll4 and therefore have no or little Notch signaling and express high levels of both VEGFR-2 and VEGFR-3 making them highly responsive to VEGF (Hellstrom et al., 2007; Tammela et al., 2008). In the stalk cells, however, Notch signaling suppresses VEGFR-2 and induces VEGFR-1 expression making them less responsive to VEGF thus preventing them from forming excessive sprouts as loss of VEGFR-1 results in excess tip cell formation in the mouse retina (Gerhardt et al., 2003; Shawber et al., 2007; Taylor et al., 2002). In addition, Notch1 has been shown to induce ephrinB2, which positively regulates internalization of, and therefore signaling through VEGFR-2 (Sawamiphak et al., 2010). Interestingly, the effect of Notch signaling on VEGFR-3 appears to be more context-dependent as it has been shown to both induce and repress VEGFR-3 expression (Shawber et al., 2007; Tammela et al., 2008), (reviewed in (Benedito et al., 2012; Thomas et al., 2013) This may be due to Notch regulating VEGFR-3 both directly by binding to the VEGFR-3 promoter and indirectly by regulating the expression of other factors, such as the Hes and Hey transcription factors, that suppress VEGFR-3 expression (Shawber et al., 2007). VEGFR-2 and VEGFR-3 have been shown to form heterodimers that preferentially localize in tip cells (Nilsson et al., 2010). In conclusion, Notch and the VEGFRs interact in multiple ways to regulate sprouting angiogenesis and the working model will definitely need to be updated as new data emerge.
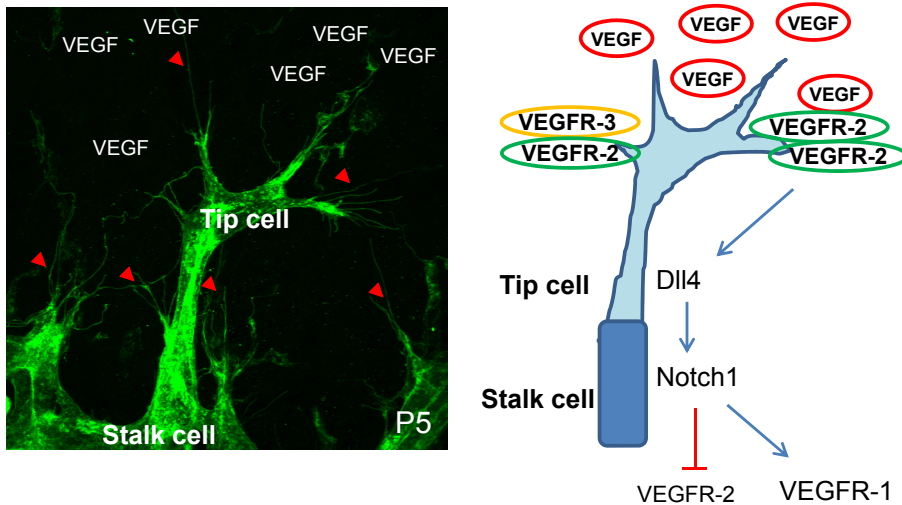
Figure 8. Notch signaling regulates tip cell/stalk cell determination during sprouting angiogenesis.
Tip cells are specialized endothelial cells that express VEGF receptors and utilize filopodia to detect the environmental cues and guide the growing sprout towards the VEGF gradient. Cell situated behind tip cells are stalk cells, that are not chemotactic but instead proliferate to ensure the growth of the sprout. Blood vessels in a P5 mouse retina visualized with isolectin-B4 (green). Red arrowheads mark filopodia.

Notch in mural cells

Notch signaling between ECs and mural cells as well as between adjacent mural cells regulates vascular maturation. Mural cells express Notch1, Notch2, Notch3 and Jagged1 and to date Notch3 and Jagged1 have been shown to be required for normal mural cell function *in vivo* (Domenga et al., 2004; Joutel et al., 1996; Kitamoto et al., 2005; Liu et al., 2010b; Varadkar et al., 2008; Wang et al., 2012). Global *Jag1* null mice die due to vascular remodeling defects but mice with endothelial specific deletion of *Jag1* display defects in VSMC maturation and recruitment demonstrating that endothelial Jagged1/VSMC Notch-signaling is crucial for EC/VSMC interactions and normal VSMC coverage (Benedito et al., 2009; High et al., 2008). Furthermore, Notch3 expression in VSMCs is induced and maintained by endothelial Jagged1 *in vitro* (Domenga et al., 2004; Liu et al., 2009; Liu et al., 2010b). *Notch3* deficient mice are viable and display normal mural cell coverage during early development; however, postnatal arterial VSMC maturation is impaired resulting in abnormal VSMC morphology, enlarged arteries and dysregulated cerebral blood flow (Domenga et al., 2004). Furthermore, reduced coverage is seen in the adult *Notch3*^{-/-} retinas suggesting that Notch3 might be important for maintenance of mural cell coverage (Liu et al., 2010b). *In vitro*, both Jagged1 expression and Notch3 signaling have been shown to induce expression of contractile proteins in VSMCs and thus promote maturation towards a contractile phenotype (Liu et al., 2009).

In addition, both Notch1 and Notch3 have been shown to induce expression of PDGFR-β in cultured VSMCs although only N1IC was shown to bind directly to the promoter of PDGFR-β (Jin et al., 2008).

In humans, mutations in NOTCH3 cause CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), a lethal adult-onset condition characterized by loss of cerebral mural cell coverage and subsequent vessel instability, which leads to repeated small strokes (Joutel et al., 1996). On cellular level, granular osmiophilic material (GOM) and NOTCH3 extracellular domain have been shown to accumulate in the cell membrane of VSMCs, which is thought to gradually cause degeneration and loss of the VSMCs in the brain (reviewed in (Ayata, 2010; Joutel and Tournier-Lasserve, 2002)). In mice, both loss and ectopic expression of Notch1 and Notch4 in the endothelium have been associated with arteriovenous malformations (AVMs) (Krebs et al., 2004; Krebs et al., 2010). AVMs cause arterial blood to flow directly into veins through shunts, thus bypassing the capillary plexus, which leads to high blood pressure in veins and an increased risk of hemorrhaging. Interestingly, brain AVMs and venous malformations display disorganized and reduced αSMA positive mural cell coverage suggesting that defective mural cell recruitment or function might contribute to the formation of vascular malformations (Chen et al., 2013; Walker et al., 2011). Both Notch1 and Notch3 are expressed in mural cells and Notch3 has already been implicated in mural cell function *in vivo*, however, the possible role of Notch1 or genetic interaction with Notch3 in regulating mural cell biology remains to be determined.

Notch in lymphatic vasculature

Although Notch has been more extensively studied in the blood vasculature, more recent work has showed that Notch also regulates lymphatic endothelial cell functions, such as lymphangiogenic sprouting in physiological and pathological settings. Notch1 and Notch4 are expressed in some lymphatic vessels in mice, as well as extratumoral lymphatics in humans (Shawber et al., 2007). Work by Geudens *et al.* in zebrafish first demonstrated that a decrease in Dll4 levels or its receptors Notch-1b or Notch-6 resulted in reduced lymphatic sprouting in zebrafish (Geudens et al., 2010). Subsequently, two groups working with mice published their opposing results leaving the function of Notch in murine lymphatic development somewhat undetermined. Niessen et al. demonstrated that inhibition of Notch signaling by blocking antibodies against Notch1 or Dll4 lead to defective lymphatic sprouting postnatally and wound healing thus concluding that Notch promotes lymphangiogenesis (Niessen et al., 2011). On a molecular level, inhibition of Notch

reduced LYVE1 expression in cultured HDLECs and ephrinB2 expression *in vivo* but had no effect on VEGFR-3 (Niessen et al., 2011). On the other hand, Zheng et al. generated and utilized a Dll4 decoy (Dll4-Fc) to inhibit Notch signaling and showed that treatment with the decoy stimulated postnatal lymphatic sprouting suggesting that Notch normally functions to limit lymphangiogenesis the same way Dll4/Notch1 signaling has been shown to limit angiogenic sprouting (Hellstrom et al., 2007; Zheng et al., 2011). Dll4-Fc treatment did not affect VEGFR-2 or VEGFR-3 expression levels in cultured HDLECs although a reduction in ephrinB2 levels was detected (Zheng et al., 2011). Authors of both reports hypothesize that Notch regulates lymphatic sprouting through indirect regulation of VEGFRs but do not identify the mechanism. Therefore, further studies are required to elucidate the effect of Notch on postnatal lymphangiogenesis. Furthermore, to date, the function of Notch during different steps of embryonic lymphatic specification has not been studied in mice.

Aims of the study

As Notch regulates cell-fate determination in multiple settings during development and is a key regulator of blood vascular development, we sought to define roles of Notch signaling in regulating specification events during embryonic lymphatic development. Mural cells express both Notch1 and Notch3 and Notch3 is known to be necessary for mural cell function although the exact mechanism remains to be determined. We thus conducted evaluations to elucidate the role of Notch in endothelial cell-mural cell interactions during postnatal angiogenesis.

The specific aims were:
I.   To investigate the possible role of Notch1 during early lymphatic specification.
II.   To study the possible function of Notch signaling in valve morphogenesis.
III. To determine the role of Notch in vascular smooth muscle cells during sprouting angiogenesis

Materials and methods

Materials and methods used are described below. Transgenic mouse lines are listed in Table 1 with the original references. Antibodies and the applications they were utilized in are listed in Table 2. Cell lines are listed in Table 3 and adenoviruses in Table 4. The publications in which mouse lines, antibodies, cell lines and viruses were used are indicated with Roman numerals.

Table 1. Mouse lines

Mouse line	Description	Source or Reference	Used in
C57BL/6	Inbred laboratory mouse strain	NCI	II, IV
Notch1$^{+/-}$	Notch1 disrupted by *neo* cassette insertion into exon coding for EGF repeat 32	(Swiatek et al., 1994)	I, III
Notch1$^{fl/fl}$	Exon 1 flanked by loxP sites and deleted after cre expression resulting in gene inactivation	(Yang et al., 2004)/ JAX Labs	I, II
N1IC	Transgene expressing Notch1-IC preceded by a floxed STOP codon inserted into *Ef1alpha* locus. Expression induced by cre	(Buonamici et al., 2009)	I
Notch3$^{+/-}$	Exons coding for EGF repeats 8-12 deleted resulting in gene inactivation	(Krebs et al., 2003)	III
DNMAML	Expresses a dominant negative, GFP tagged MAML protein. Transgene inserted into ROSA26 locus preceded by loxP flanked STOP codon. Inhibits canonical signaling by all Notch receptors upon cre expression (**Figure 9**)	(Tu et al., 2005)	I, II
Prox1Cre^{T2}	Transgene expresses both Prox1 and CreER^{T2} where Prox1 is normally expressed	(Srinivasan et al., 2007)	I, II
Prox1^{GFPcre}	GFPCre cassette inserted into Prox1 locus, expresses a GFPCre fusion protein	(Srinivasan et al., 2010; Srinivasan and Oliver, 2011)	I
TNR (Transgenic Notch Reporter)	Expresses GFP in response to canonical Notch signaling	(Duncan et al., 2005)/ JAX Labs	II
ROSA$^{mT/mG}$	Express red fluorescence (mT) prior to Cre activity and GFP (mG) after	(Muzumdar et al., 2007)/ JAX Labs	II
ROSA:LacZ$^{fl/fl}$	Express lacZ where Cre is expressed	(Soriano, 1999)/ JAX Labs	I, II
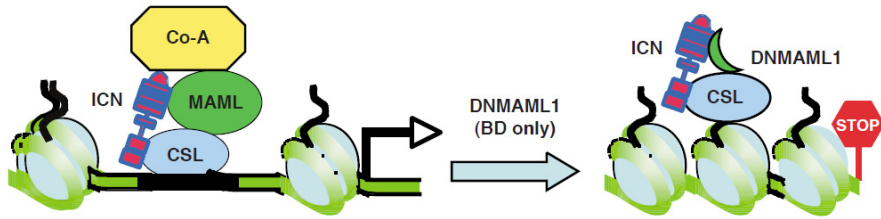
Figure 9. Molecular mechanism of Notch inhibition by DNMAML. During Notch signaling, Mastermind-like is normally recruited to the transcription factor CSL together with the intracellular domain of Notch (ICN) and co-activators (Co-A) to transform the transcriptional repressor CSL into a transcriptional activator. The DNMAML protein only contains the N-terminal basic domain (BD) responsible for interaction with ICN and CSL but lacks transcriptional activation domains (TADs) required for Notch signaling. Thus, DNMAML inhibits canonical signaling all four Notch receptors. As CSL is able to bind to DNA in the presence of DNMAML this Notch inhibition does not interfere with transcriptional repression function of CSL. Adapted from McElhinny, et al. 2008. Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways. Oncogene; 27(38): 5138-47. Copyright license number 3620330062847.

Table 2. Antibodies

Antigen	Source and Catalogue #	Application	Used in
α-tubulin	Sigma-Aldrich, T6074	WB	I, II
β-Galactocidase	Abcam, Ab9361	IHC	I
CD31	BD Pharmingen, 553370	IHC	I, II
CD34	BD Pharmingen, 550537	IHC	I
CD34-PE	BD Pharmingen, 5550619	FACS	I, II
Coup-TFII	RnD Systems, PP-H7147-00	IHC	I
Cx37	Alpha Diagnostics, CX37A11-A	WB	II
Dll4	RnD Systems, AF1389	IHC	I
Endomucin	Santa Cruz, sc-65495	IHC	I
Ephrin B2	RnD Systems, AF496	IHC	I
FN-EIIIA (clone FN-3E2)	Sigma-Aldrich, F6140	IHC	II
FN-EIIIA (IST9)	Abcam, Ab6328	WB	II
Fibronectin (total)	Abcam, Ab23750	WB	II
GFP	Invitrogen, A-11122	IHC	I
GFP	Abcam, Ab6556	IHC	II
Isolectin-B4	Vector Laboratories, B-1205	IHC	IV
Integrin β1α9	Abcam, Ab27947	FACS	II
Integrin-α9	RnD Systems, AF1057	IHC	II
Jagged1	RnD Systems, AF599	IHC	II
LYVE1	eBioscience, 14-0443	IHC	I
LYVE1	RnD Systems, AF2089	IHC	I

Antigen	Source and Catalogue #	Application	Used in
NG2	Millipore, AB5320	WB	III
NG2	RnD Systems, MAB6689	IHC	III
Notch1 (extracellular)	RnD Systems, AF1057	IHC,WB	I, II
Notch1 (intracellular)	Abcam, Ab8387	IHC	I
Notch2	Abcam, Ab8926	IHC	I
Notch3	Abcam, Ab60087	IHC,WB	I
Notch4	J.Kitajewski, RB2-2	IHC	I
Nrp1	Abcam, Ab81321	IHC	I
Nrp2	RnD Systems, AF2215	IHC	I
Podoplanin	Angiobio, 11-0033	IHC	I
Prox1	Angiobio, 11-002	IHC	I, II
Prox1	Millipore, AB5475	IHC	I
αSMA-Cy3	Sigma, C6198	IHC	III, IV
αSMA	Sigma, A2547	IHC	I
VEGFR-2	RnD Systems, AF644	IHC	I
VEGFR-3	RnD Systems, AF743	IHC	I, II

Table 3. Cell lines

Cell line	Description	Source or Reference	Used in
HdLEC	Primary human dermal lymphatic endothelial cells	Isolated by Uh, M.K.	I, II
HdLEC-pccl-N1IC	HdLEC line expressing constitutively active intracellular domain of human Notch1	(Murtomaki et al., 2013)	I
HdLEC-pccl-Hey1	HdLEC line expressing Hey1	(Murtomaki et al., 2013)	I
HdLEC-pCCL.pccl-Hey2	HdLEC line expressing Hey2	(Murtomaki et al., 2013)	I
HdLEC-pccl-GFP	HdLEC line expressing GFP	(Murtomaki et al., 2013)	I
HdMVEC	Primary human dermal microvascular endothelial cells	Lonza	I
HUVEC	Primary human umbilical vein endothelial cells	Isolated by Uh, M.K.	I

Table 4. Recombinant adenoviruses

Virus	Description	Source or Reference	Used in
AdGFP	Adenovirus that encodes GFP	(Tung et al., 2009)	II
AdLacZ	Adenovirus that encodes β-galactosidase	(Shawber et al., 2007)	I, II
AdHey1	Adenovirus that encodes Hey1	(Murtomaki et al., 2013)	II
AdHey2	Adenovirus that encodes Hey2	(Murtomaki et al., 2013)	II
AdN1IC	Adenovirus that encodes constitutively active intracellular domain of human Notch1	(Shawber et al., 2007)	I, II
AdN4/int3	Adenovirus that encodes a truncated form of Notch4 consisting of the transmembrane and intracellular domain, and is constitutively active	(Shawber et al., 2007)	II

Table 5. Primers used in qRT-PCR

Amplicon	Forward primer	Reverse primer	Used in
β-actin	5' CGA GGC CCA GAG CAA GAG AG 3'	5' CTC GTA GAT GGG CAC AGT GTG 3'	I, II
Cx37	5' GGT GGG TAA GAT CTG GCT GA 3'	5' GGC CGT GTT ACA CTC GAA AT 3'	II
Dll4	5' CGG GTC ATC TGC AGT GAC AAC 3'	5' AGT TGA GAT CTT GGT CAC AAA ACA G 3'	I
FN-EIIIA+	5' TTG ATC GCC CTA AAG GAC TG 3'	5' ACC ATC AGG TGC AGG GAA TA 3'	II
FN-EIIIA-	5' GGT AAC ACC CAT TCC TGC AC 3'	5' CCT GAT ACA ACC ACG GAT GA 3'	II
Hey1	5' ACG AGA ATG GAA ACT TGA GTT C 3'	5' AAC TCC GAT AGT CCA TAG CAA G 3'	II
Hey2	5' ATG AGC ATA GGA TTC CGA GAG TG 3'	5' GGC AGG AGG CAC TTC TGA AG 3'	II
Integrin – α9	5' CGG AAT CAT GTC TCC AAC CT 3'	5' TCT CTG CAC CAC CAG ATG AG 3'	II
Jagged1	5' GCT TGG ATC TGT TGC TTG TGT GAC 3'	5' ACT TTC CAA GTC TCT GTT GTC CTG 3'	I
LYVE1	5' TAG CTT TGA AAC TTG CAG CTA TG 3'	5' TCA ACA AAT GGT TCA GTT TCT GTA G 3'	I
Notch1	5' CTC ACC TGG TGC AGA CCC AG 3'	5' GCA CCT GTA GCT GGT GGC TG 3'	I, II
Notch2	5' CAG TGT GCC ACA GGT TTC ACT G 3'	5' GCA TAT ACA GCG GAA ACC ATT CAC 3'	I
Notch3	5' CGC CTG AGA ATG ATC ACT GCT TC 3'	5' TCA CCC TTG GCC ATG TTC TTC 3'	I
Notch4	5' GGT GAC ACC CCT GAT GTC AG 3'	5' AGC CTG GCA GCC AGC ATC 3'	I, II
Podoplanin	5' CCC AGG AGA GCA ACA ACT CAA C 3'	5' CTC GAT GCG AAT GCC TGT TAC 3'	I
Prox1	5' ACG TAA AGT TCA ACA GAT GCA TTA C 3'	5' CCA GCT TGC AGA TGA CCT TG 3'	I
VEGFR-2	5' GGA CTG GCT TTG GCC CAA T 3'	5' CTT GCT GTC CCA GGA AAT TCT G 3'	I
VEGFR-3	5' GAG ACC TGG CTG CTC GGA AC 3'	5' TCA GCA TGA TGC GGC GTA TG 3'	I

Isolation of HUVECs and HdLECs/cell culture (I, II)

Human dermal microvascular endothelial cells (HdMVEC, Lonza) were maintained in EGM-2 MV medium. Human umbilical vein endothelial cells (HUVEC) were isolated from neonate umbilical veins by collagenase digestion as previously described (Jaffe et al., 1973) and maintained in EGM-2 Endothelial Cell Growth Medium (CC-3162, Lonza).

Human dermal lymphatic endothelial cells (HdLEC) were isolated from neonate foreskins. Foreskins were digested in DMEM low glucose medium (Gibco) supplemented with Ca^{2+}/Mg^{2+}, 2% fetal bovine serum, 1X pen-strep (100U penicillin and 100U streptomycin, Invitrogen) and 1mg/ml collagenase A (Roche) for 30min at 37°C in a rotating incubator. Subsequently, an equal volume of "Buffer A" (PBS supplemented with 5mg/ml bovine serum albumin and 0.6% Acid citrate dextrose solution) was added and tissue was homogenized with a cell scraper to release cells. The procedure was repeated three times. The solution containing the cells was filtered through a 100μm cell strainer, centrifuged at 1500rpm for 5min at 4°C and resuspended in Buffer A. Magnetic Dynabead system (Invitrogen) was used to separate CD31+ cells from the solution according to the manufacturer's instructions. The cells were expanded and first positively sorted for podoplanin (Angiobio) and then negatively sorted for CD34 (Pharmingen) to remove any remaining blood endothelial cells. Resulting CD31+; podoplanin+; CD34-HdLECs were maintained on fibronectin-coated plates in EGM-2MV BulletKit (Lonza) with 10ng/ml VEGF-C (RnD) and used before passage 7.

Adenoviral infection of cells (I, II)

$1x10^6$ HdLECs were infected in suspension at a multiplicity of infection (MOI) of 20 in low serum (2%) media for 1h at 37°C gently rotating the plates manually every 15min (Shawber et al., 2007). Cells were subsequently plated in growth medium and RNA and protein isolated 48h after infection. $1x10^6$-$3x10^6$ of early passage HdMVECs were infected in suspension at 25 pfu/cell for 1h at 37°C, grown in growth medium and RNA and protein isolated 48h after adenoviral infection. Transgene expression was validated by Western blot analysis and qRT-PCR.

Generation of stable HdLEC cell lines by lentiviral transfection (I)

For Notch activation studies in HdLECs, 293T cells were transfected with 10μg of lentiviral vectors pccl-GFP, pccl-N1IC, pccl-Hey1 or pccl-Hey2. 48h after transfection the supernatant produced by the 293T cells containing the lentiviral particles was

collected (Tung et al., 2009). It was then passed through a 0.45µm filter and used to replace the normal growth medium of target cells. RNA and protein were isolated 48h after infection. Transgene expression or knockdown was validated by western blot analysis and/or qRT-PCR.

Activation of endogenous Notch signaling in HdLECs with EDTA (I)

Confluent plates of HdLECs were first treated with 200nM of compound E overnight at 37°C to quench endogenous Notch signaling and get an easily detectable induction. The following day, compound E was removed and cells washed with PBS. Subsequently, endogenous Notch signaling was activated in HdLECs by treating with 10mM EDTA for 15min at 37°C. Medium was then replaced with normal HdLEC growth medium and RNA isolated at multiple time-points. DMSO was used as a control.

Gene expression analysis by qRT-PCR (I, II)

RNA isolation from cultured cells was performed using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). Isolated RNA was treated with DNase1 for 30min prior to using it as a template for reverse-transcription PCR. RT-PCR was performed with Verso™ Reverse Transcriptase kit according to the manufacturer's instructions (Thermo Fisher). Quantitative Real-Time PCR (qRT-PCR) was performed in triplicate for each sample using Sybr Green Master Mix (ABI) or ABsolute™ Blue qPCR SYBR Green ROX Mix (Thermo Fisher) and the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). Primers used are listed in Table 5. Specific standards for each gene of interest were created by cloning PCR amplicons into pDrive. β-actin (I, II) qRT-PCR was used to normalize samples. Data was analyzed using Microsoft Excel.

Protein expression analysis by Western blot (I, II)

Cell lysates were isolated from cultured cells using cold TENT lysis buffer (50mM Tris pH8.0, 2mM EDTA, 150mM NaCl, 1% Triton X-100) containing Halt™ Protease Inhibitor Cocktail (Pierce). 20µg-40µg of total cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 30min in PBS with 0.1% Tween, 2% milk and 2% BSA at RT on a rotator, and subsequently incubated overnight at 4°C with primary antibodies diluted in blocking buffer (Table 2). The next day, membranes were incubated for 30min at

room temperature with the appropriate HRP-conjugated secondary antibodies in blocking buffer at 1:5000. Proteins were detected using Amersham ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences).

Flow cytometry (II)

Adenovirally infected HdLECs were detached by scraping, blocked in PCN buffer containing 5% FBS and incubated with anti-$\alpha9\beta1$ antibody (Abcam, Table 2) diluted in PCN buffer for 30min at 4°C. Cells were washed three times in PCN buffer followed by incubation with anti-rabbit-APC diluted in PCN buffer at 1:200 for 20min at 4°C. After three washes with PCN buffer cells were passed through filter tops into polystyrene tubes. Ten thousand cells were analyzed with BD FACSCaliburTM flow cytometer and CellQuestPro acquisition software.

Transgenic mouse models (I, II)

All procedures were performed according to approved protocols and following guidelines established by the Columbia University Institutional Animal Care and Use Committee. In order to activate or inactivate gene expression in mouse embryos, cre-carrying driver mice were bred to mice carrying transgenes or floxed target genes. Recombination was induced by tamoxifen delivery either via oral gavage or intraperitoneal injection to the pregnant females. For early embryonic studies, 5mg/40g or 7mg/40g tamoxifen was administered at E9.75, E10.5, E13.5 or E14.5 (I). For later studies, 10mg/40g was administered at E13.5 or E15.5 (II). Tamoxifen was dissolved in corn oil. Embryonic time-points were estimated from the day when vaginal plug was first observed and the day when plug was observed was considered embryonic day 0.5. As controls, single transgenic or wild-type littermates were used. None of the single transgenic mice displayed any obvious phenotype. Two to six litters were analyzed for each time-point.

Notch inhibition using Notch1 decoys and analysis of retinal angiogenesis (IV)

P2 C57BL/6 mouse pups were injected subcutaneously with 2.5 x 10^8 focus forming units (ffu) of adenoviruses encoding different Notch1 decoys or Fc as a control. A total volume of 50 μl per pup was injected. Eye balls were isolated at P5 and retinal vasculature and mural cells visualized via fluorescent staining as described below.

Blood sample was collected from each pup at the time of sacrifice and Western blot analysis performed to verify decoy protein expression.

Immunohistochemistry of retinas (III, IV)

For analysis of angiogenic sprouting and mural cell recruitment P5 mouse pups were sacrificed and eyes collected. The eyes were fixed in 4% paraformaldehyde (PFA) for 2h at 4°C. Retinas were dissected out, rinsed with wash buffer (0.1% Triton-X-100 in PBS) and incubated in blocking solution (0.5% Triton-X-100-1% BSA) two hours at room temperature or overnight at 4°C. Subsequently, the retinas were incubated overnight at 4°C in blocking solution containing biotinylated Isolectin-B4 (1:50 dilution, Vector Laboratories) and additional primary antibodies (anti-αSMA, anti-NG2, anti-CD31). The following day retinas were washed several times with wash buffer and then incubated with Alexa Fluor-488, -594 or -647 conjugated secondary antibodies (1:500 dilution, Invitrogen) in blocking solution overnight at 4°C or for 2 hours at room temperature. Retinas were washed several times with wash buffer, post-fixed with 4% PFA for 15 minutes at room temperature and washed several times with PBS. Finally, retinas were mounted on slides with mounting media containing 90% glycerol in 0.1M Tris pH8 and 1μg/ml of Hoechst nuclear stain (Sigma).

β-galactosidase staining (II)

Dorsal skins of E18.5 embryos were isolated and fixed in 0.2% glutaraldehyde, 2mM $MgCl_2$ and 5mM EGTA in 100mM phosphate buffer for 30min at room temperature followed by several washes with wash buffer (2mM $MgCl_2$, 0.02% NP-40 and 0.01% deoxycholate in 100mM phosphate buffer). Samples were stained overnight at 37°C with solution containing 1mg/ml X-gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$, 0.02% NP-40 and 0.01% deoxycholate in 100mM phosphate buffer. After several washes with wash buffer, samples were post-fixed with 4% PFA for 15min at RT and then washed several times with PBS before mounting on glass slides with 85% glycerol.

Immunohistochemistry of mesenteries (I, II)

Embryonic intestines were collected preserving the mesenteries intact at E16.5, E17.5 and E18.5. Samples were pinned down on silicon-coated plates with insect pins (Fisher Scientific), fixed with 4% PFA 2h, washed several times with wash

buffer (0.3% Triton-X-100 in PBS) at 4°C and blocked with donkey blocking solution (5% donkey serum, 0.2% BSA and 0.5% Triton-X-100 in PBS) for 2h at room temperature or overnight at 4°C. Samples were then incubated with primary antibodies (Table 2) diluted in blocking solution overnight at 4°C, washed several times with wash buffer and again incubated overnight with Alexa Fluor 488, 594 or 647 secondary antibodies (Invitrogen) diluted at 1:500 in 0.5% BSA in 0.5% Triton-X-100. Following several washes with wash buffer, samples were post-fixed with 4% PFA for 5min at room temperature and mounted on glass slides with mounting medium containing containing 90% glycerol in 0.1M Tris pH8 and 1μg/ml of Hoechst nuclear stain (Sigma). Mouse-on-mouse (MOM) detection kit (Vector laboratories) was used with mouse monoclonal antibodies.

Immunohistochemistry of cryo- and paraffin sections (I)

For cryosectioning, E9.75, E10.5, E13.5 E14.5 and E16.5 embryos were collected, fixed with 4% PFA overnight at 4°C, immersed in 30% sucrose overnight at 4°C and embedded in OCT. 5μm and 30μm sections were cut, dried, acetone-fixed, blocked 1h at room temperature (3% BSA and 2% appropriate serum in PBS) and incubated with primary antibodies (Table 2) diluted in blocking solution. The following day sections were washed with PBS, incubated with Alexa Fluor secondary antibodies (Invitrogen) diluted at 1:1000 in blocking solution for 1h at room temperature and mounted with Vectashield containing DAPI (Vectorlabs, H-1200).

For paraffin sectioning, embryos were isolated at E14.5, fixed overnight at 4°C in 4% PFA, dehydrated and embedded in paraffin. 5μm sections were cut, deparaffinazed and rehydrated followed by sodium citrate antigen retrieval. Sections were blocked, incubated in appropriate primary antibodies, washed and primary antibodies detected with appropriate biotinylated antibodies (Vector Laboratories) and visualized with either Vectastain Standard ABC Elite Kit (Vector Labs) or streptavidin-conjugated Alexa Fluor 488 or 594 secondary antibodies (Invitrogen). Mouse-on-mouse (MOM) detection kit (Vector labs) was used with mouse monoclonal antibodies. Hematoxylin counterstain was performed on slides stained colorimetrically.

Image acquisition (I, II, III, IV)

Images of fluorescently labeled, wholemount mesenteries and retinas were acquired with a laser scanning confocal Zeiss LSM 510 Meta microscope and LSM software and a Nikon A1R confocal laser scanning microscope and NIS Elements software.

Cryosections and paraffin-embedded sections imaged using a Nikon SMZ-U Zoom 1:10 microscope and Nikon 4500 digital camera, Nikon ECLIPSE E800 microscope, Nikon DXM 1200 digital camera and Image ProPlus software or Nikon A1R confocal laser scanning microscope and NIS Elements software. ImageJ and Adobe Photoshop were used for image processing.

Quantification (I, II)

ImageJ was used for all quantification. In embryo cross sections and wholemounts, number of Prox1+ cells was determined by manually counting Prox1+ cells. Threshold for Prox1+ was set for control samples first and applied to all slides analyzed. In cross sections, number of Prox1+ cells within the CV and lymph sacs (LS) was normalized to CV and LS circumference, respectively. Number of Prox1+ cells emerging from the CV was normalized to area analyzed. LYVE1+ LS and vessel luminal area were normalized to area quantified. In embryo wholemounts, the number of Prox1+ cells within the CV and emerging from the CV was normalized to area analyzed.

In the mesenteries, Prox1high cells were manually counted and normalized to μm duct length to quantify number of Prox1high cells relative to duct length. Threshold for Prox1high cell was determined for control ducts first and the applied to all images analyzed. The percentage of Prox1high cells located in valve clusters was determined by manually counting Prox1high cells in and outside of clusters. A cluster was defined as a group of Prox1high cells in which nuclei appeared to be physically in close contact with each other. Percentage of Prox1high cells within the Prox1high clusters reorienting at least 45° to the duct wall was also determined by manually counting the cells that had reoriented and the cells that had not reoriented. Prox1/integrin-α9/FN-EIIIA triple-positive valves were counted in 10x pictures and the number normalized to the amount of ducts in each picture. Integrin-α9 and FN-EIIIA-positive signal at the sites of valve formation was measured by first setting the threshold for control valves and applying it to all images. Valve sites were defined as clusters of Prox1high cells. Integrin-α9 and FN-EIIIA signal was normalized to Prox1high signal at valve formation sites.

Statistical analysis (I, II)

Statistical significance was determined using two-tailed student's t-test and p-values less than 0.05 were considered significant. Error margins indicate either standard error of mean or standard deviation, as indicated. All data represents three or more independent experiments unless stated otherwise.

Results and discussion

1. Notch functions as a negative regulator of lymphatic endothelial cell differentiation in the venous endothelium (I)

Previous studies have established that lymphatic endothelial cells differentiate from the venous endothelium in the cardinal vein and that Prox1 is crucial for the process (Srinivasan et al., 2007). However, Prox1 is only expressed in a subset of endothelial cells in the CV: the ECs that do not express Prox1 maintain their venous identity. Although it has been shown that Sox18 and Coup-TFII induce Prox1 expression and are required for lymphatic differentiation (Francois et al., 2008; Lee et al., 2009; Srinivasan et al., 2010), it is not known whether another signaling pathway is required to further suppress lymphatic endothelial fate and/or promote venous endothelial identity in the cells that do not adopt the lymphatic fate. Notch signaling mediates cell fate decisions during development and importantly, is required for arterio-venous specification during remodeling of the primary vascular plexus (Duarte et al., 2004; Kim et al., 2008; Lawson et al., 2001; Trindade et al., 2008). Therefore, we hypothesized that Notch mediates the decision between venous and lymphatic fate in the CV during lymphatic specification.

We found that Notch1 and Jagged1 were expressed in the ECs in the CV at E9.75 and E10.5, at the time when lymphatic specification occurs. Their expression patterns were punctate and overall weaker than in the adjacent aortic endothelium. Interestingly, we often observed stronger and more continuous staining on the ventral side and weaker and more punctate on the dorsal side of the CV. We also detected Notch activity in some of the Notch1 expressing cells in the CV at E9.75 using a transgenic Notch reporter (TNR) mouse, which expresses GFP in response to CSL-dependent Notch signaling. Again, the Notch activity detected in the CV was weaker and more discontinuous than in the aorta. Co-staining for Notch1 and Prox1 revealed that the Prox1+ LEC progenitors often resided on the dorsal side that had less Notch1 expression and that Prox1+ LECs migrating out of the CV had no or very low Notch1 expression. On the other hand, the Prox1+ cells still residing in the CV were sometimes Notch1+ and the cells expressing high levels of Notch1 were Prox1 negative. The expression patterns suggest that fully differentiated LECs have little or no Notch1 while the ECs maintaining their venous identity also maintain Notch1 expression. Both Notch1 and Jagged1 expression levels in the CV at E9.75 and E10.5 were weaker than what was observed in the adjacent aortic

endothelium and the same Notch1 pattern was observed in embryonic mesenteries at E16.5 and E17.5. It has been shown in T-cell development that different levels of Notch signaling can activate different target genes (Liu et al., 2010a). Thus, it is possible that different levels of Notch1 observed translate into different gene expression profiles in endothelial cells. Furthermore, Notch function may not be solely limited to arterial endothelial fate as has been previously suggested. Indeed, we observed that ectopic Notch activation in Prox1+ LEC progenitor cells did not result in arterial marker expression, suggesting that Notch mediated arterialization in ECs might be restricted to a certain developmental window. Alternatively, once LECs express Prox1 they are no longer susceptible to Notch-induced arterial fate.

To further study the role of Notch in lymphatic specification, we both inactivated and activated Notch signaling in LEC progenitors. We utilized a tamoxifen responsive, lymphatic endothelial specific $Prox1CreER^{T2}$ mouse line that drives cre-expression in Prox1+ cells without disrupting Prox1 expression from the locus (Srinivasan et al., 2007) in combination with a floxed Notch1 allele ($N1^{fl/+}$)(Yang et al., 2004). To disrupt Notch1 signaling we crossed $Prox1CreER^{T2};N1^{fl/+}$ males to $N1^{fl/+}$ females. In order to inhibit canonical signaling through all four Notch proteins, we crossed $Prox1CreER^{T2}$ driver mice to mice carrying a transgene encoding a dominant-negative form of MAML (DNMAML) (Tu et al., 2005). DNMAML binds to N1IC-CSL complex but is unable to recruit co-activators thus inhibiting canonical signaling through all four receptors (**Figure 9**). To ectopically activate Notch signaling, we crossed $Prox1CreER^{T2}$ drivers to mice carrying a transgene encoding constitutively active Notch1 intracellular domain ($N1IC$) (Buonamici et al., 2009). Loss of $Notch1$ or Notch signaling through DNMAML expression in LEC progenitor cells at E9.75, the time of lymphatic specification, resulted in increased numbers of ECs committing to the lymphatic lineage. We observed an increase in the number of Prox1+ cells remaining in and emerging from the CVs of the mutant embryos, as well as enlarged lymphatic sacs and vessels at E14.5. We occasionally observed connections between podoplanin positive vessels and the CV indicating a defect in blood and lymphatic separation. These data show that Notch suppresses Prox1 expression in the CV to ensure that only a subset of ECs in the CV adopts the lymphatic fate. We next included $Prox1^{GFPCre/+}$ mice which carry a GFPcre expression cassette in one Prox1 allele, resulting in $Prox1$ haploinsufficiency and embryonic lethality (Srinivasan et al., 2010; Srinivasan and Oliver, 2011). We crossed $N1^{fl/fl}$ mice with $Prox1^{GFPCre/+}$ mice to determine whether loss of one copy of $Notch1$ would rescue the lethality caused by $Prox1$ haploinsufficiency as could be predicted since we hypothesized that Notch1 suppresses Prox1. Indeed, we observed predicted numbers of $Prox1^{GFPCre/+};N1^{fl/fl}$ pups and an increase in Prox1+ LEC progenitors compared to $Prox1^{GFPCre/+}$ pups indicating that loss of one copy of $Notch1$ in the lymphatic progenitors was able to rescue lethality caused by $Prox1$ haploinsufficiency.

Ectopic activation of Notch1 in Prox1+ progenitors at E9.75 or at E10.5 lead to

edema, blood filled lymphatic vessels and embryonic lethality by E15.5. Upon closer examination of *Prox1CreER^T2;N1IC* embryos, we observed poorly formed, blood filled lymph sac like structures that had reduced podoplanin, LYVE1 and VEGFR-3 and spotty Prox1 expression at E14.5. The number of Prox1+ ECs detected in the mutant embryos was significantly reduced compared to controls, suggesting that Notch1 suppresses Prox1 expression *in vivo*. Using *in vitro* analysis, we showed that ectopic activation of Notch1 in cultured HdLECs via adenoviral infection resulted in suppression of Prox1 transcripts and protein. VEGF-C/VEGFR-3 signaling is the main pathway mediating LEC migration ((Karkkainen et al., 2004; Zhang et al., 2010), thus it was surprising to see that LEC progenitors expressing no detectable levels of VEGFR-3 still emerged from the CV in *Prox1CreER^T2;N1IC* embryos. VEGFR-3 co-receptor Nrp2 has been shown to promote VEGF-C-guided LEC migration *in vitro* and recently, a Nrp2^{high}/LYVE1^{low} population of LECs has been identified that migrate directly out of the CV and form peripheral lymphatics (François et al., 2011; Karkkainen et al., 2004; Xu et al., 2010). As Nrp2 expression was unchanged in *Prox1CreER^T2;N1IC* embryos it is possible that Nrp2 is able to mediate LEC migration, even with dramatically reduced VEGFR-3 levels. It is not known whether the Nrp2^{high}/LYVE1^{low} cells also express VEGFR-3 or are able to migrate without VEGFR-3. Our *in vitro* data showed that Notch1 transiently induces VEGFR-3 in HdLECs followed by repression of VEGFR-3, likely mediated by the later Notch1 induction of the transcriptional repressors Hey1/Hey2. An alternative explanation to the migration seen in *Prox1CreER^T2;N1IC* embryos is that the transient VEGFR-3 induction by N1IC was sufficient to promote LEC progenitor migration out of the CV. Alternatively, VEGFR-3 levels below antibody detection are sufficient for LEC progenitor cell migration from the CV.

Sox18 and Coup-TFII are expressed in the CV and induce Prox1 expression in LEC progenitors between E9.75 and E13.5 (Francois et al., 2008; Lee et al., 2009; Srinivasan et al., 2010). We explored the possibility that Notch might regulate Prox1 via regulation of one of these transcription factors. Using cultured HdLECs, we demonstrated that Notch did not regulate Sox18 expression *in vitro*. However, the effect of Notch on Sox18 expression *in vivo* remains to be determined. As data from blood vasculature suggest that Notch downregulates Coup-TFII (You et al., 2005), we analyzed Coup-TFII expression pattern in wild-type embryos at the time of lymphatic specification, E10.5, and *Prox1CreER^T2;N1IC* embryos at E12.5 and E13.5 in which Notch activation was induced at E10.5. In wild-type embryos at E10.5, Notch activity and Coup-TFII expression patterns in the CV did not overlap but were observed in neighboring cells suggesting that Notch indeed suppresses Coup-TFII in the CV. Following tamoxifen administration and Notch activation at E10.5, *Prox1CreER^T2;N1IC* embryos revealed a significant reduction of Coup-TFII expression both in venous endothelium and presumptive lymphatic endothelium at E12.5 or E13.5.

It has been shown that Coup-TFII regulates Prox1 expression within a developmental window between E9.75 and E13.5 (Srinivasan et al., 2010). In order to determine if Notch regulation of Prox1 was limited to the same developmental window, we administered tamoxifen at E13.5 or E14.5 and analyzed control and *Prox1CreER^{T2};N1IC* embryos at E16.5 or E18.5. Tamoxifen administration at E13.5 resulted in a mild lymphatic phenotype in about 40% of the *Prox1CreER^{T2};N1IC* embryos. Administration at E14.5 resulted in *Prox1CreER^{T2};N1IC* embryos that were indistinguishable from control embryos and had similar levels of Prox1 and podoplanin expression as controls. Therefore, we found that Notch regulates Prox1 between E9.75 and E13.5 and not beyond. As this is the same developmental window for Coup-TFII regulation of Prox1, it is likely that Notch inhibits Prox1 through suppression of Coup-TFII.

Together these data suggest that Notch1/Jag1 signaling in a subset of cardinal vein ECs during lymphatic specification inhibits Prox1 signaling via suppression of Coup-TFII. Notch-mediated inhibition of Prox1 leads to suppression of lymphatic differentiation in these ECs and maintenance of venous identity, while lack of Notch signaling in the adjacent cells results in Coup-TFII expression, Prox1 induction and adoption of lymphatic fate. Therefore, Notch signaling restricts the amount of ECs that differentiate into LECs, thus ensuring proper lymphatic development. Recent work has showed that Notch functions in postnatal lymphatic sprouting in both physiological and pathological settings (Niessen et al., 2011; Zheng et al., 2011). As we discovered that Notch no longer suppresses Prox1 after E13.5, our findings are not in conflict with Notch functioning in Prox1+ lymphatic endothelium later in development, such as sprouting lymphangiogenesis.

2. Notch signaling functions in lymphatic valve formation (II)

During lymphatic valve morphogenesis a subset of LECs in the lymphatic duct wall undergoes changes in gene expression, cytoskeletal rearrangements, reorients and differentiates into a distinct population of cells referred to as valve-forming LECs. These valve-forming LECs secrete a specialized valve ECM, assemble the leaflet core and form valve leaflets (Bazigou et al., 2009; Sabine et al., 2012). Prox1, Foxc2, integrin-α9, fibronectin splice variant EIIIA (FN-EIIIA) and gap junction protein 37 (connexin37, Cx37) are highly expressed in valve-forming LECs and all, other than Prox1, have been shown to be required for normal lymphatic valve formation (Bazigou et al., 2009; Kanady et al., 2011; Norrmen et al., 2009; Petrova et al., 2004; Sabine et al., 2012). Prox1 expression is higher in valve-LECs (Prox1^{high}) than LECs in the duct wall (Prox1^{low}) and Prox1 is necessary in a dosage-dependent manner for lymphovenous valve formation (Srinivasan and Oliver, 2011), suggesting that

high Prox1 levels seen in valve-forming LECs are similarly required for lymphatic valve formation. Although previous studies have established the importance of these valve regulators for valve morphogenesis, it has not been known what triggers their expression and how a subset of LECs adopts the valve-LEC fate while the other LECs remain in the duct walls. As Notch mediates binary cell fate decisions in various developmental settings, including during lymphatic specification, we explored the hypothesis that Notch functions in the differentiation events associated with lymphatic valve formation.

We found that Notch1 was expressed in the developing mesenteric lymphatic vessels and valves. At E16.5 Notch1 was uniformly expressed throughout the mesenteric lymphatics. At E17.5, highest Notch1 expression was seen in, and immediately around, the putative valves. By E18.5, Notch1 expression was enriched in lymphatic valve-LECs and only weak expression was seen elsewhere in the duct. Using a transgenic Notch reporter mouse that expresses GFP in response to canonical Notch signaling we observed Notch signaling activity in valve-forming LECs at E17.5 and E18.5. At both time points, Notch activity was also seen outside the putative valves, however, the strongest signal was seen in reoriented valve-forming LECs. We sought to determine which ligand induces signaling through Notch1 during valve formation. Despite our efforts to stain for Jagged1, Dll1 and Dll4, we were unable to obtain results that would identify a Notch ligand that might function in lymphatic valve formation (unpublished data by Murtomaki A., data not shown). The enrichment of Notch1 and Notch activity to lymphatic valves as valve morphogenesis proceeds suggested that Notch functions in valve formation.

To assess Notch1 function in lymphatic valve formation, we crossed male mice carrying the lymphatic endothelium-specific *Prox1CreER^{T2}* driver (Srinivasan et al., 2007) and one copy of a floxed allele of Notch1 (*N1^{fl/+}*) (Yang et al., 2004) to *N1^{fl/+}* females. We administered tamoxifen at E15.5, the time when valve morphogenesis begins, and analyzed valve morphology and marker expression in mesenteric lymphatics at E18.5, by this time, most valves are mature. At E18.5, *Prox1CreER^{T2};N1^{fl/fl}* (LOF) embryos were externally indistinguishable from control littermates. Upon examining the mesenteric lymphatics, we observed an increase in the amount Prox1^{high} cells in LOF mesenteries. Occasionally, large expansions of Prox1^{high} cells were seen in LOF mesenteries instead of typical, well-defined valve clusters.

In control mesenteries, valve-forming Prox1^{high} LECs clustered and reoriented at least 45° perpendicularly to the duct wall. However, even when Prox1^{high} LECs clustered in LOF mesenteries, they often failed to reorient. We observed a significant reduction in the number of LECs reorienting at the putative valve sites. The valves that were seen in the LOF mesenteries often displayed abnormal morphology and poorly organized leaflets. As valve leaflet organization requires integrin-α9

and FN-EIIIA (Bazigou et al., 2009), we analyzed the expression pattern of these valve markers. We observed reduced integrin-α9 expression in LOF mesenteries compared to control mesenteries. The overall amount of lymphatic valves defined as clusters of Prox1^{high} cells, albeit poorly organized, was similar in both LOF and control mesenteries, suggesting valve initiation still occurs.

As we did not observe a complete loss of valves in *Prox1CreER^{T2};N1$^{fl/fl}$* embryos, we hypothesized that another Notch protein might be expressed in developing valves and compensate for the loss of Notch1. To explore this we inhibited canonical Notch signaling through all four receptors in the lymphatic endothelium by crossing the *Prox1CreER^{T2}* drivers to mice carrying a transgene expressing a dominant-negative form of MAML (DNMAML) (Tu et al., 2005). We administered tamoxifen at E13.5 or E15.5 and analyzed mesenteries at E18.5. The *DNMAML$^{fl/+}$* transgene expresses GFP upon recombination and therefore the recombination efficiency was assessed by GFP staining. GFP signal was only observed in the lymphatic endothelium of *Prox1CreER^{T2};DNMAML$^{fl/+}$* (DNMAML) embryos. *Prox1CreER^{T2};DNMAML$^{fl/+}$* embryos displayed a more severe phenotype than *Notch1* LOF embryos. The expected Prox1 expression pattern with valve-LECs being Prox1^{high} and duct LECs Prox1^{low} was seen in control mesenteries. However, the pattern was disrupted in *Prox1CreER^{T2};DNMAML$^{fl/+}$* mesenteries with both E13.5 and E15.5 tamoxifen administration with significantly less Prox1^{high} LECs located in clusters. Furthermore, LECs observed in clusters at putative valve sites displayed abnormally rounded nuclei instead of the elongated nuclei seen in control valve-LEC clusters. The overall number of Prox1/integrin-α9/FN-EIIIA triple-positive valves was also reduced in *Prox1CreER^{T2};DNMAML$^{fl/+}$* mesenteries compared to control mesenteries. Even when putative valves were observed, they were morphologically abnormal displaying poorly formed leaflets and only expressing one of the valve markers analyzed, integrin-α9 or FN-EIIIA. Indeed, we observed significant reduction in FN-EIIIA expression by Prox1+ valve-LECs in the *Prox1CreER^{T2};DNMAML$^{fl/+}$* mesenteries compared to control mesenteries. We were not able to reach significance for loss of integrin-α9 largely due to the small number of valves that expressed integrin-α9 in the *Prox1CreER^{T2};DNMAML$^{fl/+}$* embryos.

As lymphatic valve proteins integrin-α9 and FN-EIIIA were reduced in *Notch1* LOF and *Prox1CreER^{T2};DNMAML$^{fl/+}$* mesenteries, respectively, we sought to determine if Notch regulates their expression. We adenovirally infected cultured HdLECs to express constitutively active forms of Notch1 (N1IC) or Notch4 (N4/int3) intracellular domains, the latter included to determine whether Notch4 functions in valve morphogenesis in addition to Notch1. We included Cx37, calcineurin regulatory subunit Cnb1, and Foxc2 in our target gene analysis as they are expressed in valves and required for valve formation (Kanady et al., 2011; Norrmen et al., 2009; Petrova et al., 2004). Both N1IC and N4/int3 induced integrin-α9 and FN-

EIIIA transcripts, although the effect of N4/int3 on FN-EIIIA transcript levels was less robust. Only N1IC induced integrin-α9 surface expression while both N1IC and N4/int3 induced FN-EIIIA protein levels. The lack of N4/int3-induced integrin-α9 surface expression might be due to its lower signaling activity compared N1IC, which is seen in all experiments as a lower induction of Notch targets, Hey1 and Hey2. The transcriptional repressors Hey1 and Hey2 did not affect integrin-α9 transcript levels. Both N1IC and N4/int3 strongly induced Cx37 transcript and protein expression. N1IC or N4/int3 induced Foxc2 transcripts slightly (1.5-fold) while neither affected the transcript levels of Cnb1.

Our data demonstrate that Notch functions in multiple steps of lymphatic valve morphogenesis. Differentiation and clustering of Prox1high cells at the putative valve sites is the first stage of valve formation. We observed increased numbers of Prox1high cells and lack of clustering in LOF and *Prox1CreER^T2^;DNMAML^fl/+^* mesenteries, respectively, suggesting that Notch might function to restrict the amount of LECs that become valve-forming LECs and/or promote clustering. In this model, lymphatic ducts must generate distinct Prox1high cells in order to initiate valve formation; in the absence of Notch activity developing lymphatic ducts have dysregulated Prox1 levels, and valve formation is impaired. As these processes are still poorly understood, the exact mechanism for Notch function during valve initiation stage remains to be determined.

After the first stage, valve-forming LECs form a ring-like structure and reorient. We observed defects in Prox1high cell reorientation within the putative valve sites in LOF mesenteries. Cx37 functions in gap-junction formation and, valve-LECs in *Cx37^-/-^* mice fail to reorganize into a ring-like structure and reorient (Kanady et al., 2011; Sabine et al., 2012). We showed that Notch strongly induced Cx37 transcript and protein levels in cultured HdLECs. Therefore, it is possible that the reorientation defect seen in LOF valves results from loss of Cx37 and impaired cell-cell communication. Cx37 expression levels in *Notch1* LOF and *Prox1CreER^T2^;DNMAML^fl/+^* mesenteries still need to be determined in order to further elucidate the issue.

Upon reorientation valve-LECs penetrate into the vessel lumen, elongate, assemble the ECM core and leaflets. Integrin-α9 and FN-EIIIA are required for valve ECM core assembly and leaflet elongation and the loss of either results in abnormal, shortened valve leaflets and disorganized valve matrix core (Bazigou et al., 2009; Sabine et al., 2012). We observed poorly formed and disorganized valve leaflets as well as reduction in integrin-α9 and FN-EIIIA expression in *Notch1* LOF and *Prox1CreER^T2^;DNMAML^fl/+^* mesenteries. The fact that valve formation was severely impaired in Notch mutant mesenteries, leading to a significant reduction in number of Prox1/integrin-α9/FN-EIIIA-triple positive valves in *Prox1CreER^T2^;DNMAML^fl/+^* mesenteries, suggests that Notch controls several aspects of valve formation.

To conclude, we propose that Notch regulates several aspects of valve morphogenesis. Disruption of Prox1high/Prox1low pattern and clustering defects

demonstrate that Notch functions during the initial phases to separate valve-LECs from the ductal LECs and promote their clustering at the putative valve sites. Furthermore, our data suggest that Notch promotes reorientation possibly through regulation of Cx37 in valve-LECs and subsequent leaflet formation through regulation of integrin-α9 and FN-EIIIA. We observed strong Notch1 expression and activity in mesenteric valves at E18.5, when valve morphogenesis is mostly completed, suggesting a potential role in valve maintenance. Whether Notch is required for maintenance of mature valves, remains to be determined. The more severe phenotype observed in *Prox1CreER^{T2};DNMAML^{fl/+}* mice compared to *Notch1* LOF mice indicated that another Notch receptor functions in valve formation. Our *in vitro* data suggest that Notch4 is a likely candidate, however, *in vivo* studies are required to verify this. Finally, a ligand for Notch during valve morphogenesis is yet undetermined.

3. Disruption of Notch signaling impairs vascular smooth muscle cell recruitment during sprouting angiogenesis in the mouse retina (III, IV)

Blood vessel maturation occurs during angiogenesis and depends on mural recruitment. Contractile vascular smooth muscle cells are recruited to arteries, arterioles, veins and venules, where they provide structural support for these larger caliber vessels and regulate vessel diameter and blood flow by contracting (reviewed in (Armulik et al., 2011)). Endothelial Jag1/mural cell Notch3 signaling is required for postnatal VSMC maturation and maintenance of mural cell differentiation in mice (Benedito et al., 2009; High et al., 2008; Ruchoux et al., 2003). *Notch3^{-/-}* mice still display αSMA+ mural cell coverage during early postnatal life, suggesting that initial recruitment of VSMCs occurs in these mice (Domenga et al., 2004). It should be noted that although the VSMCs detected in these mutant mice are αSMA+, they fail to express two other markers used to identify fully mature VSMCs, smoothelin and SM22, suggesting that VSMC maturation might also be compromised in *Notch3^{-/-}* mice (Domenga et al., 2004). Considering that Notch1 is expressed in VSMCs and that Notch induces the expression of an important signaling protein in vascular mural cells, PDGFR-β, it was plausible to hypothesize that Notch1 regulates VSMC function during sprouting angiogenesis (Jin et al., 2008; Kitamoto et al., 2005; Liu et al., 2009). We asked whether Notch1 has overlapping or distinct functions from Notch3 in VSMCs. That is, we explored the hypothesis that Notch1 with Notch3 may promote VSMC recruitment, differentiation or homeostasis.

To evaluate Notch1 function in VSMCs in the context of *Notch3* deficiency, *Notch1* and *Notch3* mutations were combined in mice. It should be noted that Notch1 is

expressed in both endothelial cells and VSMCs, whereas Notch3 is restricted to the vascular mural cells (Del Amo et al., 1992; Joutel et al., 2000; Kitamoto et al., 2005; Sweeney et al., 2004). *Notch3$^{+/-}$* mice were crossed to *Notch1$^{+/-}$;Notch3$^{+/-}$* mice and the retinal vasculature of all the resulting genotypes was analyzed (wild-type, *Notch3$^{+/-}$*, *Notch3$^{-/-}$*, *Notch1$^{+/-}$*, *Notch1$^{+/-}$;Notch3$^{+/-}$* and *Notch1$^{+/-}$;Notch3$^{-/-}$*). *Notch3$^{+/-}$* mice were used as controls as they were phenotypically identical to their wild-type littermates. Compared to *Notch3$^{+/-}$* controls, all other genotypes displayed increased number of tip cells and vascular density at P5 in the retinas, when the superficial plexus is actively undergoing robust angiogenic growth (Figure 1 in IV, generated by Kofler N). Hypersprouting observed in *Notch1$^{+/-}$* mice was found, consistent with reduced endothelial Notch signaling which limits tip cell and sprout formation during retinal angiogenesis (Hellstrom et al., 2007; Suchting et al., 2007). *Notch3$^{-/-}$* mice have been reported to display reduced vascular outgrowth from the optic nerve towards the retinal periphery (Liu et al., 2010b), which we also observed (Figure S2 in IV, generated by Kofler N). When total retinal endothelial content was normalized to vascularized area, retinal vascular density in *Notch3$^{-/-}$* mice was increased despite reduced outgrowth. It is not clear why *Notch3$^{-/-}$* retinas display reduced vascular growth towards retinal periphery as endothelial growth and sprouting still occurs as evident by increased number of tip cells and overall endothelial content. Thus, it is possible that reduced vascular growth towards the retinal periphery is due to defective directional guidance of growing endothelial sprouts and not reduced vascular plexus growth. As Notch3 is restricted to mural cells these data suggest that Notch3 signaling in mural cells regulates endothelial cell behavior and vascular patterning during sprouting angiogenesis. Reduced vascular outgrowth from the optic nerve towards the periphery was also observed in *Notch1$^{+/-}$;Notch3$^{+/-}$* and *Notch1$^{+/-}$;Notch3$^{-/-}$* mice, providing further evidence for the role of *Notch3* in regulating vascular growth in the retina.

To evaluate Notch1 and Notch3 function in VSMCs, arterial αSMA+ VSMC coverage in control and mutant retinas was evaluated. In the mouse retina, αSMA+/NG2-double positive VSMCs represent a more mature, contractile mural cell population, mainly observed around arterioles and to a lesser extent around venules (Hellstrom et al., 1999). *Notch3$^{-/-}$* arterioles showed reduced αSMA+ VSMC coverage as previously reported (Liu et al., 2010b). Interestingly, *Notch1$^{+/-}$* and *Notch1$^{+/-}$;Notch3$^{+/-}$* retinas displayed a discontinuous αSMA staining pattern. However, the αSMA staining intensity was comparable to that observed in control retinas where VSMCs were observed. *Notch1$^{+/-}$;Notch3$^{-/-}$* arterioles displayed the most dramatic phenotype; the αSMA expressing mural cell coverage was significantly reduced when compared to *Notch3$^{-/-}$* arterioles (Figure S3 in IV).

We found that loss of one copy of *Notch1* combined with loss of *Notch3* resulted in a significantly more severe phenotype than *Notch1* haploinsufficiency or *Notch3*

nullizygocity alone. Moreover, the significantly reduced αSMA+ VSMC coverage in *Notch1*^{+/-}*;Notch3*^{-/-} retinas correlated with a more severe vascular hypersprouting phenotype compared to *Notch3*^{-/-} retinas. Importantly, loss of one *Notch1* allele alone resulted in altered VSMC coverage suggesting a role for Notch1 in VSMC organization. Together these data suggest that both *Notch1* and *Notch3* are required for proper VSMC function during sprouting angiogenesis. It should, however, be noted that Notch1 is also expressed in the endothelium and therefore the severe hypersprouting phenotype seen in *Notch1*^{+/-}*;Notch3*^{-/-} retinas was likely due to a combination of endothelial and mural cell dysregulation. Although our data strongly suggest that Notch1 functions in VSMCs, it would be beneficial to analyze mice with a mural cell-specific deletion of *Notch1* combined with loss of *Notch3* in order to verify the mural cell-specific function. Furthermore, it should be determined how reduced Notch1 signaling affects VSMCs. Our data showed reduced αSMA+ VSMC coverage in the mutant retinas, however, it cannot be concluded whether this was due to reduced VSMC recruitment or reduced αSMA+ expression by the VSMCs that had been recruited. If it is the latter, then the significant reduction in Notch signaling in *Notch1*^{+/-}*;Notch3*^{-/-} retinas may have resulted in a failure in VSMC differentiation where the mural cells are more pericyte-like. As fully mature VSMCs express smoothelin and SM22 in addition to αSMA, it would be helpful to establish the expression pattern of these proteins in *Notch1*^{+/-}*;Notch3*^{-/-} retinas in order to determine if VSMC maturation is compromised in these mutant mice.

Another approach to study the role of Notch in mural cells is through the use of biological inhibitors that target Notch function in vascular cells. We studied the role of Notch signaling in retinal angiogenesis and mural cell differentiation using such inhibitors. Kangsamaksin T. and Shawber C. designed and generated soluble Notch1 decoys that can be delivered *in vivo* via adenoviral infection and interfere with ligand/receptor interaction to inhibit Notch signaling. These Notch1 decoys consist of different regions of EGF-like repeats of NOTCH1 fused to human IgG Fc (**Figure 10**). Notch/CSL reporter assay was utilized to determine the inhibitory capabilities of the decoys. Through these reporter assays, Notch1 decoys were identified that act as pan-ligand inhibitors or specifically inhibit either DLL/NOTCH or JAG/NOTCH signaling. Decoys consisting of EGF-like repeats 1-24 ($N1_{1-24}$ decoy) or 1-36 ($N1_{1-36}$ decoy) inhibited signaling through both DLL and JAG class ligands. Notch1 decoy consisting of EGF-like repeats 1-13 ($N1_{1-13}$ decoy) preferentially inhibited DLL induced Notch1 signaling while decoy consisting of EGF-like repeats 10-24 ($N1_{10-24}$ decoy) interfered with JAG/NOTCH signaling.
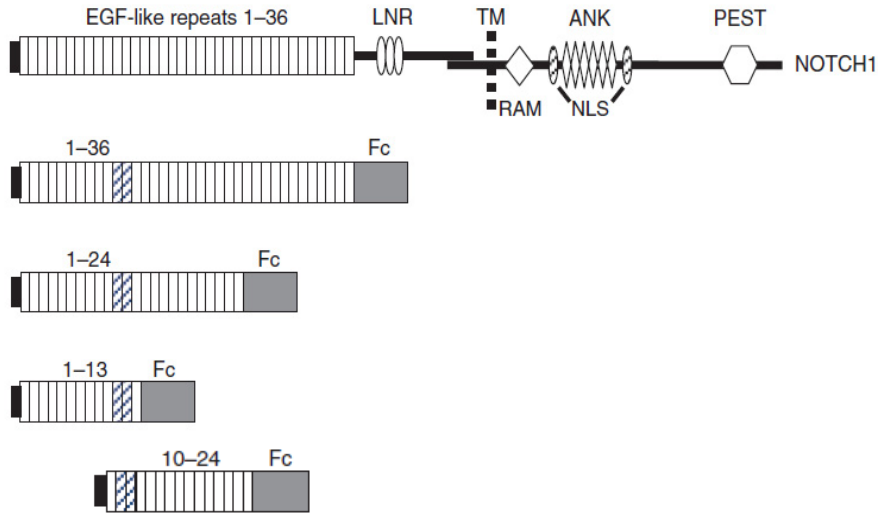
Figure 10. Design of the Notch1 decoys. The Notch1 decoys consist of EGF-like repeats of human NOTCH1 fused to human IgG Fc. In all variants, EGF-like repeats are fused in frame with human IgG Fc. EGF-like repeats number 11 and 12 required for receptor/ligand interaction are marked with shading. LNR, LIN-12/NOTCH repeats; TM, transmembrane domain; RAM, CSL interaction domain; ANK, ankyrin repeats; NLS, nuclear location signal; PEST, proline-,glutamic acid-,serine-, threonine-rich sequence. Adapted from Kangsamaksin et al. 2015. NOTCH decoys that selectively block DLL/NOTCH or JAG/NOTCH disrupt angiogenesis by unique mechanisms to inhibit tumor growth. Cancer Discovery; 5(2); 182-97.

To ensure the decoys would be expressed and secreted by cells, 293T cells were transfected and total lysates and supernatants analyzed. All decoys were secreted into media by 293T cells. Of the two pan-ligand inhibitors, $N1_{1-24}$ was selected for further studies due to its better secretion into media. Co-immunoprecipitation studies further confirmed that $N1_{1-13}$ decoy physically interacts with DLL1 and DLL4 but not JAG1 and JAG2, and $N1_{10-24}$ decoy with JAG1/2 but not DLL1/4. $N1_{1-24}$ decoy co-immunoprecipitated with both DLL and JAG class of ligands. None of the decoys interacted with the NOTCH1 itself.

To determine how the Notch1 decoys affect angiogenesis they were evaluated using an *in vitro* angiogenesis assay that incorporates HUVEC-coated dextrin beads embedded in fibrin, where growth factors secreted by a fibroblast feeder layer induces sprouting of the HUVECs into the surrounding fibrin gel. HUVECs were infected with lentivirus encoding Fc (control), $N1_{10-24}$, $N1_{1-13}$ or $N1_{1-24}$ decoys and sprout formation evaluated on day 7. The HUVECs expressing Fc sprouted from the beads, branched and formed lumenized vessels day 7. Compared to the control, $N1_{1-13}$ decoy expressing HUVECs displayed a hypersprouting phenotype characterized by increased growth and branch points. This result is in line with studies showing that inhibition of DLL4 induced Notch signaling results in an increased tip cell and sprout formation and subsequent vascular overgrowth. In contrast, $N1_{10-24}$ and $N1_{1-}$

$_{24}$ decoys impaired sprouting of the HUVECs as evident by stunted sprouts and a reduced numbers of branch points observed. Thus, inhibition of JAG induced Notch signaling had an antiangiogenic effect. Interestingly, the antiangiogenic effect, - at least in this assay, was more potent than the proangiogenic effect caused by inhibition of DLL4/Notch signaling when using the pan-ligand inhibitor $N1_{1-24}$ decoy.

To determine how the Notch1 decoys affect angiogenesis *in vivo*, we assessed their activity using a postnatal murine retina as a model for vascular growth. Adenoviruses encoding different decoys or Fc control were delivered to P2 neonates via a subcutaneous injection, which led to hepatocyte infection by the adenoviruses and subsequent production and secretion of the Notch1 decoys into the bloodstream. All Notch1 decoys were detected in the bloodstream of the pups at the time of sacrifice. Compared to the Fc retinas, both retinas from $N1_{1-13}$ and $N1_{1-24}$ decoy expressing mice displayed a hypersprouting phenotype at P5, while $N1_{10-24}$ decoy retinas showed reduced vascular density (Figure 3D and E in III). We conclude that, during murine retinal angiogenesis, the pan-ligand $N1_{1-24}$ decoy functions more like a DLL4 inhibitor than a JAG1 inhibitor based upon the sprouting phenotype, which differs from the activity observed in the *in vitro* sprouting assay. The hypersprouting phenotype seen in retinas of $N1_{1-13}$ decoy expressing mice is consistent with previous studies that have demonstrated the pro-sprouting effects of DLL4 blockade (Hellstrom et al., 2007). Postnatal endothelial-specific loss of *Jagged1* has been shown to cause reduced vascular density in the mouse retina and thus the antiangiogenic effect observed in the $N1_{10-24}$ decoy retinas is in line with previous findings (Benedito et al., 2009).

As JAG1 is known to regulate VSMC maturation and recruitment (High et al., 2008), αSMA⁺ VSMC coverage was evaluated in the retinas isolated from Notch1 decoy treated mouse neonates. Retinas from $N1_{1-13}$ decoy treated mice displayed comparable VSMC coverage and organization when compared to Fc control retinas. In contrast, $N1_{10-24}$ decoy treated mice displayed reduced αSMA⁺ VSMCs along arterioles, consistent with previous work implicating Jag1/Notch in VSMC maturation. Interestingly, the pan-ligand inhibitor $N1_{1-24}$ decoy also caused reduced αSMA⁺ VSMCs, showing that while it causes hypersprouting by inhibiting Dll4/Notch1 signaling in the endothelial cells of the newly formed sprouts, it also affects VSMC function likely via inhibition of Jagged1 mediated Notch signaling.

Conclusions

Proper function of both blood and lymphatic vasculature is essential for normal development and health. For a long time, the lymphatic vasculature has been less studied than the blood vascular system. However, work by numerous investigators during the past decade has greatly increased our understanding of lymphatic development. In addition to definitely proving the venous origin of lymphatic system, several lymphatic regulators have been identified using transgenic mouse models. While new information has accumulated, it has become clear that the molecular mechanisms regulating lymphatic development and lymphangiogenesis are extremely complex.

The Notch signaling pathway has been shown to mediate cell-fate decisions in various developmental settings as well as regulate cellular functions in numerous adult tissues. Notch has been extensively studied in the blood vascular system and shown to be required for normal vascular development. Recent work has identified Notch as a novel regulator of postnatal lymphatic growth where it can either promote or inhibit lymphangiogenesis, depending on the tissue and context.

In the present study, we establish Notch as a novel regulator of embryonic lymphatic specification. Notch expression downregulates Prox1 in a subset of endothelial cells in the cardinal vein thus suppressing the lymphatic fate and maintaining venous fate in these cells. In contrast, Prox1 expression is induced in the cells lacking Notch activation which then go on to differentiate into lymphatic endothelial cells and migrate out of the cardinal vein. Although our results elucidate the molecular mechanism determining how a subset of venous endothelial cells adopt the lymphatic fate while others maintain their venous fate, it remains to be determined how Notch itself is regulated in the cardinal vein. Specifically, is Notch initially expressed throughout the cardinal vein and subsequently turned off in a subset of cells to allow lymphatic specification? If so, how is this achieved? It is possible that Notch signaling activity is restricted to certain cells through ligand availability, however, further studies are required to determine the exact mechanism.

Our data show that Notch no longer regulates Prox1 expression after E14.5, as has been reported before (Srinivasan et al., 2010). Therefore, it was not surprising to detect Notch1 expression in Prox1-expressing lymphatic endothelial cells during later embryonic development. Our studies demonstrate that Notch promotes lymphatic valve morphogenesis through regulation of valve-LEC clustering, reorientation and expression of known valve regulators, integrin-α9, fibronectin splice variant EIIIA and gap-junction protein connexin37. Like the initial lymphatic specification in the cardinal vein, lymphatic valve development also involves a specification event

during which a subset of LECs adopts a novel, valve-LEC fate. Considering the role of Notch in mediating binary cell-fate decision, it would be tempting to suggest that Notch mediates the separation of valve-LECs from the lymphatic duct wall LECs. However, our results do not demonstrate this. Instead, our data suggest that Notch promotes several steps of valve morphogenesis after the initiation phase, possibly by first guiding the clustering and reorientation of valve-LECs through upregulation of connexin37 in the valve-LECs and subsequently promoting valve leaflet formation by inducing integrin-α9 and fibronectin splice variant EIIIA. As Notch seems to regulate later steps of valve morphogenesis, further studies are required to determine the mechanism through which valve-LECs differentiate into a separate population. Future studies will hopefully also further elucidate how Notch interacts and/or cooperates with the other regulators to promote valve morphogenesis.

Finally, we show that *Notch1* in addition to *Notch3* is required for normal VSMC function during sprouting angiogenesis and that perturbed Notch signaling leads to abnormal vascular development in the postnatal mouse retina. Proper endothelial cell-mural cell interactions are crucial for vascular integrity and there are human conditions associated with impaired mural cell function. The human stroke syndrome CADASIL is an autosomal dominant disorder caused by mutations in *NOTCH3* and associated with progressive loss of cerebral VSMC coverage followed by loss of vascular integrity causing repeated strokes. As the condition is progressive, it is possible that another signaling mechanism is initially able to partially compensate for the abnormal function of Notch3. Considering that CADASIL is associated with progressive loss of VSMCs and that *Notch3* null mice display normal mural cell coverage at birth, it is possible that Notch1 is sufficient for the initial recruitment and/or maturation of VSMCs and that Notch3 is required for the maintenance of VSMC coverage later in life. Alternatively, only one copy functional copy of NOTCH3 may be necessary for early VSMC events. The Notch1 decoys presented in publication III inhibit ligand interaction with all Notch proteins and therefore we cannot separate the effects of impaired Notch1 and Notch3 activation on VSMCs in our decoy studies. However, as the Notch1 decoys are ligand-specific, they will undoubtedly provide a useful tool for studying effects of ligand-specific inhibition *in vitro* and *in vivo*.

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