Unique functions for Notch4 in murine embryonic lymphangiogenesis

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Abstract

In mice, embryonic dermal lymphatic development is a well-understood system used to study the role of genes in physiological lymphangiogenesis. The Notch signaling is an evolutionary conserved pathway that modulates cell fate decisions and shown to both inhibit and promote dermal lymphangiogenesis. Here, we demonstrate distinct roles for Notch4 signaling versus canonical Notch signaling in embryonic dermal lymphangiogenesis. At E14.5, actively growing dermal lymphatics expressed NOTCH1, NOTCH4 and DLL4, with DLL4 expression strongest and Notch active in the lymphangiogenic sprouts. Treatment of cultured LECs with VEGF-A or VEGF-C upregulated Dll4 transcripts, but differentially regulated Notch1 and Notch4 expression, and the Notch effectors of the Hes/Hey families, suggesting that VEGF-A and VEGF-C distinctly modulate Dll4/Notch signaling in the lymphatic endothelium. Mice nullizygous for *Notch4* had an increase in the closure of the lymphangiogenic fronts towards the midline which correlated with reduced vessel caliber in the maturing lymphatic plexus. Activation of Notch4 suppressed lymphatic endothelial cell migration in a wounding assay significantly more then Notch1 activation, suggesting a dominant role for Notch4 in LEC migration. Unlike *Notch4* nulls, inhibition of canonical Notch signaling by ectopically expressing a dominant negative form of MAML1 (DNMAML) in Prox1+ lymphatic endothelium suppressed lymphatic endothelial cell proliferation consistent with what has been described for the loss of lymphatic endothelial Notch1. Moreover, loss of *Notch4* did not disrupt lymphatic endothelial canonical Notch signaling. Thus, we propose that Notch4 signaling and canonical Notch signaling have distinct functions in the coordination of embryonic dermal lymphangiogenesis.

Keywords: Lymphangiogenesis, Notch, VEGF-C, dermis

Introduction

Lymphangiogenesis is the process by which new lymphatic vessels sprout off pre-existing vessels. Sprouting of new lymphatic vessels requires coordinated lymphatic endothelial cell (LEC) proliferation, directional migration, and cell-cell adhesion to form a properly patterned and functional network. In murine dorsal skin, lymphangiogenesis begins at embryonic day 12.5 (E12.5) at the side of the trunk and follows dermal blood vessel development to meet at the midline around E15.5 (Fig. 1a) [1]. Dermal lymphangiogenesis in mouse embryos is well characterized allowing for analysis of lymphatic endothelial signaling pathways, such as Notch.

The Notch family of signaling proteins consists of four cell surface receptors (NOTCH1-4) that are bound and activated by membrane-bound ligands of the Delta-like (Dll1, 4) and Jagged (Jag1, 2) families expressed on neighboring cells. Upon ligand activation, the extracellular domain of NOTCH is released which induces conformational changes that expose two proteolytic cleavage sites (TACE and γ-secretase/presenilin) and release of the intracellular cytoplasmic domain (NICD) from the cell surface [2]. In the canonical Notch signaling pathway, NICD transits to the nucleus, binds the transcriptional repressor RBPjκ, where it recruits an activation complex including Mastermind-like (MAML) and HDACs, and activates RBPjκ-dependent transcription of Notch effectors, such as those in the HES/Hey families. Notch also signals via a less well understood non-canonical RBPjκ-independent pathway that has been suggested to not require nuclear localization of NICD [2].

During development of the blood vascular system, Notch signaling is essential for arterial endothelial specification, vascular smooth muscle cell differentiation and viability, and sprouting angiogenesis [3-5]. Studies of murine retinal angiogenesis have shown that VEGF via activation VEGFR2 upregulates DLL4 expression in the filopodia-extending tip cell located at the vascular front [6,4,7,8]. Dll4 signals to the neighboring Notch-expressing stalk cell, where Notch activation downregulates VEGFR2 and VEGFR3 expression and inhibits the tip cell phenotype. During retinal angiogenesis, inhibition of DLL4 or NOTCH1 leads to a hypersprouting phenotype characterize by an increase in tip cells at the expense of the stalk cells, increased VEGFR2 and VEGFR3 expression, and decreased vascular outgrowth [6,7,9]. Although it has been shown that VEGF-C induces DLL4 in cultured LECs [10,11], the mechanisms by which Notch regulates dermal lymphangiogenesis remain to be elucidated.

We previously demonstrated that NOTCH1 and NOTCH4 are expressed in the postnatal maturing dermal lymphatics [12]. Studies of postnatal lymphangiogenesis have shown that pharmacological inhibition or genetic manipulation of Dll4/Notch1 signaling can result in both increased and decreased lymphangiogenesis [13,11]. Neutralizing antibodies against NOTCH1 or DLL4 suppressed lymphangiogenesis in the postnatal mouse ear, tail dermis and a wounding model [13]. In contrast, an inhibitory soluble DLL4 extracellular domain fused to FC (Dll4FC) stimulated lymphangiogenesis in the postnatal mouse ear [11]. In embryonic dermal lymphangiogenesis, *Notch1* deletion in LECs did not affect lymphatic branching, but increased lymphatic

vessel caliber that was proposed to be secondary to an increase in LEC proliferation and decreased LEC apoptosis [14]. More recently, it was shown that loss of one copy of *Dll4* was associated with reduced embryonic dermal lymphangiogenesis in mice [15], a phenotype opposite to that seen retinal angiogenesis [7,8]. Additional studies are needed to clarify the differences in the lymphangiogenic phenotypes observed upon disruption of lymphatic endothelial Notch signaling.

Here, we examined the roles for Notch4 and canonical Notch signaling in embryonic dermal lymphangiogenesis. We demonstrated that NOTCH1, NOTCH4 and DLL4 are expressed, and Notch signaling active in embryonic dermal lymphatic endothelium and are differentially modulated by VEGF-A and VEGF-C signaling in cultured LECs. Mice nullizygous for *Notch4* displayed an embryonic dermal lymphangiogenic phenotype consistent with increased LEC migration, while inhibition of canonical Notch signaling increased lymphatic vascular density consistent with an increased in LEC proliferation. Together, these data demonstrate that dermal lymphangiogenesis is dynamically regulated by Notch and requires both NOTCH1 and NOTCH4 functions, as well as canonical and non-canonical Notch signaling.

Materials and Methods

Cell culture/Constructs

HeLa cells were maintained in 10% FBS DMEM. Human umbilical vein endothelial cells (HUVEC) were isolated as previously described and maintained in EGM2 (Lonza) [16,17]. Neonatal human dermal lymphatic endothelial cells (HdLECs) were either purchased (Promocell) or isolated as previously described [18], and maintained on fibronectin-coated plates in EGM2-MV2 (Lonza; complete medium) supplemented with 10 ng/mL VEGF-C (R&D). To activate Notch signaling, HdLEC were lentivirally infected [19] using pCCL.pkg.wpre vector encoding N1IC, N4/int-3 or GFP. N1IC encodes the constitutively active cytoplasmic domain of NOTCH1. N4/int-3 encodes an activated *Notch4* allele generated by MMTV insertion [20]. Protein expression was confirmed by quantitative (q)RT-PCR and Western analyses of samples collected 48 hours post-infection.

HdLEC assays

VEGF-A and VEGF-C treatment of HdLECs: Confluent monolayers of HdLEC were starved overnight in 1%FBS in EBM2 (Lonza) followed by 5 hours in EBM2 and then EBM2 containing either 100ng/ml VEGF-A (R&D) or 100ng/mL VEGF-C (R&D) for an hour prior to RNA isolation. Migration Assay: $7x10^4$ HDLECs were seeded in triplicate on a fibronectin-coated 12 well plate in complete medium. The following day (0-hour time point), a scratch through the confluent monolayer was made across each well using a p200 pipet tip, and medium was changed to EBM2 containing 100ng/ml VEGF-C. Growth into the scratch was documented at 0, 4, 8, 12, and 25 hours with a Zeiss Axiovert 40 CSL inverted microscope. Cell migration rate was

determined using imageJ software [21]. All assays were performed at least 3 times.

Co-culture Notch Reporter Assay

HDLECs (90% confluent) were lipofected (Lipofectamine 2000; Invitrogen) with the Notch reporter plasmid pGL3.11CSL [12] containing 11 repeats of Notch/CSL cis-elements, and phRL-SV40 *renilla* (Promega) to normalize lipofection efficiency. HeLa cells were lipofected with pCR3 plasmids encoding either DLL4-FLAG or JAG1-FLAG with empty vector serving as a control. 24 hours after lipofection, HeLa and HdLECs were co-cultured together at a 1:1 ratio on fibronectin-coated plates in EGM2. 24hrs after co-culture, a luciferase reporter assay was performed using the Dual Luciferase Reporter Assay System (Promega) and a TD20/20 luminometer (Turner Designs). Luciferase values were normalized to Renilla values. Each condition was performed in triplicate, 4 times.

Gene Expression Analyses

RNA was isolated using the RNEasy Mini Kit (Qiagen) and reverse transcribed using the VersoTM cDNA Synthesis Kit (Thermo Fisher). qRT-PCR was performed in triplicate for each gene (Table S1), using ABsoluteTM Blue QPCR SYBR Green Master Mix (Thermo Fisher) and 7300 Real-Time PCR System (Applied Biosystems). Gene specific qRT-PCR standards were used to determine transcript levels and normalized to β-actin expression [12]. Analyses were set up in triplicate and performed at least 3 times.

Mouse studies

Notch4 nullizygous ($N4^{-/-}$) [22], $Prox1CreER^{T2}$ [23], and $DNMAML^{fl/fl}$ [24], *CBF:H2B-Venus* (*NVR* reporter purchased from Jax Labs) [25] and *Prox1-tdTomato* lymphatic reporter (*ProxTom*); [26] mice were used for these studies. For studies using $Prox1CreER^{T2}$, tamoxifen in corn oil was administered via oral gavage (10 mg/40 g) at E12.5. 4-5 independent litters and the number of embryos analyzed presented in figure legends.

Immunohistochemistry & Imaging

E14.5 dorsal skin was dissected, fixed for 2 hours at 4°C in 4% PFA, and then incubated 2 hours at room temperature in blocking buffer (10% donkey serum, 0.3% Triton X-100, 1 x PBS). Tissues were incubated in primary antibody (Table S2) diluted in blocking buffer overnight at 4°C, and then incubated with the appropriate Alexa-Fluor secondary antibodies

(Invitrogen) diluted blocking buffer at 1:500 overnight at 4°C. Tissue was mounted using Vectashield containing DAPI (Vector Laboratories).

Images were captured using a Nikon SMZ-U Zoom 1:10 microscope and Nikon 4500 digital camera, Nikon ECLIPSE E800 microscope and NIS Elements software, Nikon DXM 1200 digital camera, and Image ProPlus v.4.01 software, a Zeiss Axioskop2 Plus and Zeiss AxioCam MRc camera with Zeiss Zen software, or an Olympus IX83 Inverted System Microscope and Olympus cellSens software. Confocal microscopy was performed with a Zeiss LSM 510 META Confocal Microscope and the LSM software. Images were analyzed with ImageJ or Adobe Photoshop. Tiled 10x images were used to quantify lymphatic and blood vascular density, distance between migration fronts, fronts per unit length, and branch-point per unit length. 20x images were used to determine lymphatic vessel caliber, distance to first branch point, number of Prox1+ cells per field, and tip cell morphology. Vascular density was determined as positive signal area divided by area. Distance between migrating fronts was determined as the mean distance between the 2 lymphatic fronts measured at multiple points (≥3) [1]. Sprouting fronts per unit length was determined as the number of sprouting fronts at the leading edge of the migration front normalized to the vertical length (posterior-anterior; Fig. 1a). Length of the sprout was determined as length from tip of sprout at lymphangiogenic front to the first branch point. Tip cell morphology was determined by counting total number of quiescent (rounded, lacking multiple filopodia) and lymphangiogenic (elongated with multiple filopodia) tip cells at the migrating front normalized to the total number of tip cells. Lymphatic vessel caliber was determined by measuring the width of lymphatic vessels in the maturing lymphatic plexus and adjacent to the first branch point away from the migrating front. Branch points per unit length in maturing lymphatic plexus was determined as the number of branch points per field normalized to the total length of lymphatic vessels per field. To measure Prox1+ LEC number, Prox1+/LYVE1+ LECs were scored and mean number per field determined. To determine the significance between control and one experimental group, a two-tailed student's t-test was used. For analyses of more than two groups, one-way analysis of variance (ANOVA) was used to determine significance. A $p<0.05$ was considered significant.

Results

Embryonic dermal lymphatics expressed NOTCH1, NOTCH4 and the Notch ligand, DLL4

In culture, human neonatal dermal lymphatic endothelial cells (HdLECs) express NOTCH1-4 and the Notch ligands, DLL4 and JAGGED1 (JAG1) [18] and that NOTCH1 and NOTCH4 are expressed P4 murine dermal lymphatic vessels [12]. Notch signaling has been shown to be active in the E15.5 dermal lymphatics [14], but it is not known which Notch proteins and ligands are expressed in dermal LECs at this time. To study the role of Notch signaling in embryonic dermal lymphangiogenesis, we determined the expression of NOTCH1 and NOTCH4 as well as the angiogenic Notch ligands, DLL4 and JAG1 (Fig. 1), as

well as Notch activity in E14.5 dorsal skin (Fig. 2). This time point is characterized by the presence of two LYVE1+ lymphatic capillary fronts migrating towards the midline which precedes a maturing lymphatic plexus (Fig. 1a). At this timepoint, two CD31+ angiogenic fronts have begun fused at the midline to form a connected blood capillary network.

Analysis of E14.5 dermal cross-section revealed the LYVE1+ dermal lymphatic endothelium expressed both NOTCH1 and NOTCH4 (Fig. 1b). Outside of the lymphatics, NOTCH1 expression was observed in the epidermis and blood endothelium, while NOTCH4 was expressed in the epidermis and a subset of LYVE1+ macrophages. Whole-mount analysis of E14.5 dermis showed that DLL4 was expressed in both in the developing lymphatics and blood vessels (Fig. 1c). Unlike the retina, where DLL4 expression is restricted to 1-2 tip cells at the angiogenic front [6-8], high DLL4 expression was observed in multiple LECs in the blunt-ended lymphangiogenic fronts. DLL4 was expressed in the arterial vessels and blood capillary network, with strongest and continuous expression observed in the large arteries and is consistent with its expression in the vasculature of the intestinal villi [10]. Unlike DLL4, JAG1 was not expressed in dermal lymphatics at E14.5 (Fig. 1c). JAG1 expression was limited to the blood vasculature, where its' expression was highest in the larger caliber arteries in a pattern consistent with vascular smooth muscle cell as well as endothelial cell expression.

To determine which LECs are actively signaling during dermal lymphangiogenesis, we evaluated the dermal lymphatics in E14.5 embryos carrying alleles for the Prox1-Tomato LEC reporter [26] and the Notch Venous Reporter [25]. Notch activity was observed throughout the lymphatic vascular plexus at both the lymphangiogenic front and the mature plexus (Fig. 2a). At the lymphangiogenic front, Notch activity was often observed in several LECs located at the tip cell positions in spiky sprouts with filopodia (Fig. 2b), consistent with the broad expression of Dll4 at the front (Fig. 1c). In the mature lymphatic plexus, the highest Notch activity was observed at sites of high Prox1 expression and numerous branch points (Fig. 2a, c). Taken together the expression data suggest Dll4 signaling via either Notch1 and Notch4 activation has a role in regulating dermal lymphangiogenic growth as well as maturation.

VEGF-C induced Dll4 expression and Notch activation in HdLECs

During sprouting angiogenesis, VEGF-A/VEGFR-2 signaling upregulates DLL4 in endothelial tip cells to activate Notch signaling in the adjacent stalk cell [6-8]. As we observed an enrichment of DLL4 and Notch activity in LECs located at the lymphangiogenic front, we determined the effect of VEGF-A and VEGF-C on Notch genes, ligands and Notch effectors in HdLECs. Responses were compared to those of blood endothelial cells, using human umbilical vein endothelial cell (HUVEC). Serum starved HdLECs or HUVECs were treated with either VEGF-A or VEGF-C and *Dll4*, *Notch1* and *Notch4* transcript levels determined after 1 hour. In HdLECs, both VEGF-A and VEGF-C induced *Dll4* (Fig. 3a). VEGF-C also induced Notch1

transcripts, and modestly reduced *Notch4* transcripts in HdLECs. Neither VEGF-C nor VEGF-A affected the expression of *Jagged1* (data not shown). To determine if increased DLL4 expression correlates with Notch activation, we determined the transcript levels of the Notch effectors, *Hey1*, *Hey2* and *Hes1* in VEGF-A and VEGF-C treated HdLECs. VEGF-A induced expression of *Hey1* and *Hes1* transcripts in HdLEC, whereas VEGF-C increased *Hey2* and *Hes1* levels (Fig. 2b). In HUVEC, only VEGF-A induced DLL4 expression, whereas VEGF-C modestly decreased *DLL4* transcripts, as well as *Notch1* and *Notch4* expression (Fig. 3a). VEGF-A also increased *Notch1* and decreased *Notch4* in HUVEC. Thus, VEGF-C specifically induced Dll4 and Notch1 expression in cultured LECs leading to Notch activation, as seen by an increase in expression of the Notch effectors, *Hey2* and *Hes1*.

As JAG1 expression was not observed in the murine dermal lymphatics, nor induced by VEGF-C, we hypothesized that DLL4 may preferentially function to induce Notch activity to LECs. To determine the capacity of LECs to respond to either DLL4 or JAG1, we performed co-culture assays in which endogenous Notch activation in HdLEC was determined with a Notch-response CSL luciferase reporter. HeLa cells were engineered to express DLL4, JAG1 or both (Fig. S1), and then seeded with HdLECs containing a CSL-luciferase reporter at a 1:1 ratio. DLL4-expressing HeLa cells upregulated Notch/CSL signaling nearly 5-fold over co-cultures using parental HeLa cells (Fig. 3c). In contrast, JAG1 modestly increased Notch signaling in HDLECs. JAG1 induction of Notch signaling was observed to be less potent than DLL4-mediated activation in four independent experiments, suggesting DLL4/NOTCH is the dominant signaling mechanism in HdLECs. Co-culture with Hela co-expressing DLL4 and JAG1 induced Notch signaling similar to the co-cultures with DLL4 alone, suggesting that JAG1 did not interfere with DLL4 signaling (Fig. 2c). In conjunction with DLL4 and JAG1 expression pattern in dermal lymphatics, this data suggests that DLL4, and not JAG1, is the primary ligand for LEC Notch signaling in embryonic dermal lymphangiogenesis.

Embryonic dermal lymphangiogenic defects in Notch4 mutant mice

Prior reports have shown that loss of LEC Notch1 in mice leads to increased LEC proliferation and LECs with filopodia consistent with an increased in LEC tip cells [14]. To determine the role of Notch4 in embryonic lymphangiogenesis, we evaluated *Notch4*$^{-/-}$ mice and compared their lymphatic phenotype to that of *Notch4*$^{+/-}$ and wild-type littermates. To confirm that NOTCH4 protein is absent in the *Notch4*$^{-/-}$ dermal lymphatics, we stained P4 dermal tissue for NOTCH1 and NOTCH4. As compared to wild-type littermates, NOTCH4 expression was absent in *Notch4*$^{-/-}$ tissues, while NOTCH1 expression was unaffected (Fig. S2). Analysis of E14.5 dermal whole-mounts demonstrated that the closure of the two lymphatic fronts towards the lateral midline was increased (Fig. 4a, b), and an increase in the number of lymphatic fronts reaching towards the midline in the *Notch4*$^{-/-}$ dermis (Fig. 4c). Further analysis of the lymphangiogenic sprouts at the migration front revealed the length from

the front to the first branch-point did not differ between mutants and controls (Fig. 4d). Control tip-cell LECs that uniformly expressed LYVE1 were elongated with numerous filopodia consistent with a lymphangiogenic phenotype. In contrast, LECs in the tip cell position in *Notch4*$^{-/-}$ dermis were often rounded with reduced and blunted filopodia (Fig. 4e, f). *Notch4*$^{-/-}$ tip cells also had membrane ruffling consistent with a migratory cell fate with discontinuous LYVE1 staining on the cell surface (Fig. 4e). We next evaluated the lymphatic vessel caliber at the lymphangiogenic front and in the maturing plexus. The caliber of the vessel adjacent to the first branch-point at the front did not differ between mutant and control mice (Fig. 3g, h). However, a significant reduction of vessel caliber was observed in the maturing lymphatic plexus of *Notch4*$^{-/-}$. Although the lymphatic vessel diameter was reduced in the maturing *Notch4*$^{-/-}$ plexus, branching was similar between mutants and controls (Fig. S3). Thus, *Notch4* mutant mice had a distinct dermal lymphatic phenotype from that observed in mice with LEC *Notch1* deletion [14]. Rather than increased vessel diameter due to increased proliferation and branching due to increased sprout lymphangiogenesis, the embryonic dermal lymphatics in *Notch4* nulls had increased front closure, reduced spiky tip cells and decreased vessel caliber in the maturing plexus suggesting Notch4 suppresses LEC migration. As NOTCH4 is also expressed by the blood vasculature [22,20], we evaluated the underlying dermal blood vascular network in E14.5 *Notch4*$^{-/-}$ and *Notch4*$^{+/-}$ embryos. The density and branching of the CD31+ blood vasculature were unaffected in the *Notch4* nulls (Fig. S4) and consistent with prior studies [27,22].

NOTCH4 activation preferentially inhibited LEC migration

To determine the effects of Notch1 and Notch4 signal activation on LEC migration, HdLECs were generated to express activated forms of NOTCH1 (N1IC) or NOTCH4 (N4/int-3) a monolayer-wounding assay performed. Relative to GFP-expressing HdLEC, both N1IC and N4/int-3 expression inhibited LEC migration (Fig. 5). We next evaluated the effect of overexpressing the downstream Notch effectors HEY1 and HEY2 on HdLEC migration. Similar to Notch activation both ectopic expression of HEY1 and HEY2 suppressed migration relative to control HdLECs. Further analysis revealed that N4/int-3 was significantly a stronger inhibitor of HdLEC migration than either Notch1 activation or HEY2 overexpression at 25 hrs. This data suggests that Notch4 may signal via the downstream effector Hey1, whereas Notch1 may signal via Hey2.

Inhibition of lymphatic endothelial canonical Notch signaling increased dermal lymphatic vessel density

Notch4 has been shown to signal via CSL-dependent (canonical) and CSL-independent (non-canonical) downstream pathways [28-30]. To determine the effects of LEC specific loss of canonical Notch signaling on embryonic dermal lymphangiogenesis, we used the inducible *Prox1CreER*^{T2} driver to drive expression of a *DNMAML* transgene [24]. *DNMAML* encodes a dominant negative form of Mammalian Mastermind Like 1 (MAML1) that binds NOTCH/CSL to form an inactive

complex by blocking the recruitment of transcriptional co-activators. *Prox1CreER*^{T2} mice were crossed with *DNMAML*$^{fl/fl}$ mice to generate *Prox1CreER*^{T2}*;DNMAML*$^{fl/+}$ embryos (*DNMAML*^{LEC}) and *DNMAML*$^{fl/+}$ served as littermate controls. To circumvent the effects on early lymphatic specification caused by loss of Notch in LECs [18], tamoxifen was administered to pregnant females at E12.5, just as sprouting lymphangiogenesis begins and the dermal lymphatic phenotype analyzed at E14.5. Unlike the *Notch4* nulls, the closure of the migration fronts was no different between *DNMAML*^{LEC} and control (Fig. 6a,b). The number of sprouts along the migrating front and the length of the sprout to the first branch point were similar between mutants and controls (Fig. 6c, d). Although we did not observe overt sprouting defects, the lymphatic density was nearly 25% greater in the *DNMAML*^{LEC} compared to controls (Fig. 6e). The increase in the *DNMAML*^{LEC} dermal lymphatic density correlated with an enlargement of the lymphatic vessel caliber at the lymphangiogenic front and in the maturing plexus (Fig. 6f, g). As compared to controls, *DNMAML*^{LEC} dermal lymphatics had an increase in the number Prox1+/LYVE1+ LECs (Fig. 6h), while branching the mature plexus was unaffected (Fig. 6i). The increase in vascular density was specific to the lymphatics as blood vessel density was unchanged in *DNMAML*^{LEC} mutants (Fig. S5). Thus, we found that inhibition of lymphatic endothelial Notch signaling resulted in increased lymphatic vessel density and caliber associated with an increase in LECs, suggesting canonical Notch signaling suppressed LEC proliferation in the embryonic dermal lymphatics.

As the DNMAML protein is fused to GFP, we also determined the localization of the LECs with a loss of canonical Notch signaling as it relates to the tip cell position. Analysis of the GFP expression demonstrated that LECs that expressed DNMAML did not localize to the tip cell position, but were commonly found in LECs at penultimate position to the tip cells or in the vessel wall at both the lymphatic front and the maturing plexus (Fig. 7).

Canonical Notch signaling is unaffected in Notch4$^{-/-}$ *embryonic dermal lymphangiogenesis*

As we observed a difference between the embryonic dermal lymphatic phenotypes of *Notch4*$^{-/-}$ and *DNMAML*^{LEC} mutants, we evaluated canonical Notch signaling by introducing the *NVR* and *Prox1-Tom* alleles into the *Notch4* null background. Loss of *Notch4* did not change canonical Notch signaling observed either in the blunt or spiky lymphangiogenic sprouts (Fig. 8a), nor in the maturing lymphatic plexus (Fig. 8b). This data suggested that Notch4 is not necessary for canonical Notch signaling in the embryonic dermal lymphatics.

Discussion

Notch1 and Notch4 are expressed and Notch signaling active in the embryonic and early postnatal dermal lymphatic vasculature [14,12], suggesting a role for both these Notch family members in embryonic lymphangiogenesis. Loss of *Notch4*

was shown to exacerbate the *Notch1* null embryonic blood vascular phenotype, suggesting Notch1 and Notch4 have overlapping functions in the endothelium [22]. However, we found that loss of *Notch4* led to a distinct embryonic dermal lymphangiogenic phenotype, that was not observed in mice with deletion of *Notch1* [14], or inhibition of canonical Notch signaling in the lymphatic endothelium. At E15.5, *Notch4* null embryos displayed an increase in the closure of the lymphangiogenic fronts to the midline and reduced vessel caliber in the maturing plexus, suggesting Notch4 functioning to suppress LEC migration. In contrast, loss of LEC *Notch1* at E10.5 increased embryonic dermal lymphatic density, due to increased LEC proliferation and decreased LEC apoptosis [14]. Ectopic LEC expression of DNMAML, which inhibits canonical Notch/CSL signaling, increased the dermal lymphatic vascular density consistent with an increase in LEC proliferation and viability and similar to the *Notch1* LEC knockout phenotype. Distinct functions for Notch1 and Notch4 have been described for endothelial progenitor cells, where Dll4 signaling via Notch4 specifically induced EphrinB2 and increased proliferation and migration of cultured cells [31]. More recently, it was proposed that endothelial Dll4/Notch1 signaling induces Hey2 to suppress proliferation and tip cell formation, while Jag1 activates Notch4 to induce Hey1 and promote vessel maturation while having no effect on vascular density [32]. Taken together, we propose that Notch1 and Notch4 signaling dynamically regulate lymphangiogenesis to control both migration and proliferation/cell viability by distinct mechanisms.

Prior studies have shown that VEGF-C induces Dll4 in LECs leading to Notch activation [10]. We found that VEGF-A and VEGF-C differentially regulated *Dll4*, *Notch1* and *Notch4* in LECs resulting in the induction of distinct downstream effectors. VEGF-C induced *Dll4*, *Notch1*, *Hes1* and *Hey2*, and suppressed *Notch4* and *Hey1* levels. In contrast, VEGF-A only induced *Dll4* and the Notch effectors, *Hey1* and *Hes1* in HdLECs. This differential response to VEGF-A and VEGF-C was specific to the LECs, as both factors induced *Dll4*, and *Notch1*, while suppressing *Notch4* expression in HUVEC. Thus, VEGF-A and VEGF-C may differentially modulation Notch1 and Notch4 expression to mediate distinct downstream effects in LECs and is consistent with the distinct dermal lymphangiogenic phenotypes observed for *Notch1* and *Notch4* mutant mice. It remains to be determined what upstream factors regulate Notch4 expression in LECs.

We observed that the dermal lymphatic phenotypes were distinct between *Notch4*$^{-/-}$ and *DNMAML*^{LEC}, suggesting Notch4 signals at least in part via a non-canonical pathway. Notch4 has been shown to signal via canonical (RBPjκ-dependent) and non-canonical (RBPjκ-independent) Notch pathways in multiple cells types [28-30]. In endothelial cells, Notch4 activation blocked LPS induced apoptosis via RBPjκ-independent upregulation of Bcl2 [28]. In mice, NOTCH4 activation in the ductal epithelium required RBPjκ for physiological alveolar development, but not for breast cancer development, suggesting Notch4 functions via both canonical and non-canonical pathway in the breast endothelium [29,30]. We observed that canonical Notch signaling was unchanged in the embryonic dermal LECs in *Notch4* nulls suggesting the Notch4 dermal lymphatic phenotype did

not occur via a RBPjκ-dependent mechanism. However, it possible that the variable phenotypes are due to differences in the penetrance of global *Notch4* loss versus a tamoxifen-induced cell mosaic expression of *DNMAML* in LECs.

In vivo and in vitro data suggest that Notch4 via Hey1 functions to suppress LEC migration. An increase in the closure of the two lymphangiogenic fronts was observed in *Notch4* mutants that correlated with reduced vessel caliber throughout the mature and lymphangiogenic plexus. This phenotype is consistent with an increase in LEC migration towards the midline. In HdLECs, ectopic Notch4 activation and HEY1 expression inhibited LEC migration significantly more than either Notch1 activation or ectopic expression of HEY2. The inhibition of LEC migration by Notch4 may occur via non-canonical Notch signaling as ectopic expression DNMAML, an inhibitor of canonical Notch did not affect the closure of the lymphangiogenic fronts. Supporting a role for non-canonical Notch4 signaling in LEC migration, loss of β-catenin signaling in LECs, which has been shown to antagonize Wnt/β-catenin signaling in stem and progenitor cells, reduced LEC migration towards the midline and increased dermal lymphatic vessel caliber [33,34]. This phenotype is opposite to that observed in *Notch4*$^{-/-}$ embryos, suggesting that Notch4 via a non-canonical signaling suppresses LEC migration.

NOTCH4 expression was not observed in the lymphatics, or surrounding cell type in the *Notch4* null using an antibody against the cytoplasmic domain of NOTCH4. It has been suggested that the *Notch4* null line analyzed here expresses a truncated extracellular NOTCH4 peptide that suppresses Notch1 signaling [35]. However, a loss of canonical Notch signaling was not observed in the lymphatics of *Notch4* mutant mice, nor the blood vasculature (data not shown), which would be predicted if Notch1 signaling was inhibited in the model. Moreover, the *Notch4* mutant dermal lymphatic phenotype is distinct from that observed in mice with *Notch1* deleted in the LECs [14], as well as the *DNMAML*^{LEC} mice presented here. However, the dermal lymphatic phenotype may be due loss of *Notch4* in non-LECs, such as macrophages and conditional *Notch4* allele needs to be develop to better understand the cell type specific requirement for NOTCH4 in lymphatic development.

Together with published data, our studies suggest that Notch1 and Notch4 distinctly in embryonic dermal lymphangiogenesis via a RBPjκ-dependent and –independent pathways. Dll4/Notch1 signaling via a canonical pathway suppressed LEC proliferation, while Notch4 signaling suppresses LEC migration possibly via a RBPjκ-dependent mechanism. Further studies into the mechanistic interaction between Notch1 and Notch4 in LECs and lymphatic development and homeostasis is necessary, as a number of therapeutics that are pan-Notch inhibitors or target specific receptors or ligands are currently in clinical trials or the research pipeline for use in the clinic.

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Authorship Contributions: AM and MKU share first authorship. AM, MKU, YM, JKK, CJS contributed to the study conception and design. Material preparation, data collection and analysis were performed by AM, MKU, JMJ, AM, JDM, CK, MG, GR, CJS. The first draft of the manuscript was written by AM, MKU and CJS, and revised by CJS.

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Compliance with ethical standards

Conflict of Interest: Jan Kitajewski has received research funding from Eisai Pharmaceuticals (CU12-3625 and UICID#084028 Eisai Ltd. Research Collaborative Agreements). All other authors declare that they have no conflict of interests.

Ethical approval: Isolation of HUVEC and HdLEC from anonymous discarded specimens and received IRB exempt status by Columbia University IRB (AAAA7338). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Mouse studies were approved by Columbia University IACUC (AC-AAAE2653, AC-AAAD0577, AC-AAAP9603, AC-AAAP0452, AC-AABB9551). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

References

1. James JM, Nalbandian A, Mukouyama YS (2013) TGFbeta signaling is required for sprouting lymphangiogenesis during lymphatic network development in the skin. Development 140 (18):3903-3914. doi:10.1242/dev.095026

2. D'Souza B, Meloty-Kapella L, Weinmaster G (2010) Canonical and non-canonical Notch ligands. Curr Top Dev Biol 92:73-129. doi:10.1016/S0070-2153(10)92003-6

3. Baeten JT, Lilly B (2017) Notch Signaling in Vascular Smooth Muscle Cells. Adv Pharmacol 78:351-382. doi:10.1016/bs.apha.2016.07.002

4. Li JL, Harris AL (2009) Crosstalk of VEGF and Notch pathways in tumour angiogenesis: therapeutic implications. Front Biosci (Landmark Ed) 14:3094-3110. doi:10.2741/3438

5. Shawber CJ, Kitajewski J (2004) Notch function in the vasculature: insights from zebrafish, mouse and man. BioEssays : news and reviews in molecular, cellular and developmental biology 26 (3):225-234. doi:10.1002/bies.20004

6. Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalen M, Gerhardt H, Betsholtz C (2007) Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445 (7129):776-780. doi:10.1038/nature05571

7. Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, Wiegand SJ (2007) Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proceedings of the National Academy of Sciences of the United States of America 104 (9):3219-3224. doi:10.1073/pnas.0611206104

8. Suchting S, Freitas C, le Noble F, Benedito R, Breant C, Duarte A, Eichmann A (2007) The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proceedings of the National Academy of Sciences of the United States of America 104 (9):3225-3230. doi:10.1073/pnas.0611177104

9. Tammela T, Zarkada G, Wallgard E, Murtomaki A, Suchting S, Wirzenius M, Waltari M, Hellstrom M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Yla-Herttuala S, Shibuya M, Pytowski B, Eichmann A, Betsholtz C, Alitalo K (2008) Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature 454 (7204):656-660. doi:10.1038/nature07083

10. Bernier-Latmani J, Cisarovsky C, Demir CS, Bruand M, Jaquet M, Davanture S, Ragusa S, Siegert S, Dormond O, Benedito R, Radtke F, Luther SA, Petrova TV (2015) DLL4 promotes continuous adult intestinal lacteal regeneration and dietary fat transport. The Journal of clinical investigation 125 (12):4572-4586. doi:10.1172/JCI82045

11. Zheng W, Tammela T, Yamamoto M, Anisimov A, Holopainen T, Kaijalainen S, Karpanen T, Lehti K, Yla-Herttuala S, Alitalo K (2011) Notch restricts lymphatic vessel sprouting induced by vascular endothelial growth factor. Blood 118 (4):1154-1162. doi:10.1182/blood-2010-11-317800

12. Shawber CJ, Funahashi Y, Francisco E, Vorontchikhina M, Kitamura Y, Stowell SA, Borisenko V, Feirt N, Podgrabinska S, Shiraishi K, Chawengsaksophak K, Rossant J, Accili D, Skobe M, Kitajewski J (2007) Notch alters VEGF responsiveness in human and murine endothelial cells by direct regulation of VEGFR-3 expression. The Journal of clinical investigation 117 (11):3369-3382. doi:10.1172/JCI24311

13. Niessen K, Zhang G, Ridgway JB, Chen H, Kolumam G, Siebel CW, Yan M (2011) The Notch1-Dll4 signaling pathway regulates mouse postnatal lymphatic development. Blood 118 (7):1989-1997. doi:10.1182/blood-2010-11-319129

14. Fatima A, Culver A, Culver F, Liu T, Dietz WH, Thomson BR, Hadjantonakis AK, Quaggin SE, Kume T (2014) Murine Notch1 is required for lymphatic vascular morphogenesis during development. Developmental dynamics : an official publication of the American Association of Anatomists 243 (7):957-964. doi:10.1002/dvdy.24129

15. Geng X, Yanagida K, Akwii RG, Choi D, Chen L, Ho Y, Cha B, Mahamud MR, Berman de Ruiz K, Ichise H, Chen H, Wythe JD, Mikelis CM, Hla T, Srinivasan RS (2020) S1PR1 regulates the quiescence of lymphatic vessels by inhibiting laminar shear stress-dependent VEGF-C signaling. JCI Insight 5 (14). doi:10.1172/jci.insight.137652

16. Funahashi Y, Shawber CJ, Sharma A, Kanamaru E, Choi YK, Kitajewski J (2011) Notch modulates VEGF action in endothelial cells by inducing Matrix Metalloprotease activity. Vascular cell 3 (1):2. doi:10.1186/2045-824X-3-2

17. Funahashi Y, Shawber CJ, Vorontchikhina M, Sharma A, Outtz HH, Kitajewski J (2010) Notch regulates the angiogenic response via induction of VEGFR-1. Journal of angiogenesis research 2 (1):3. doi:10.1186/2040-2384-2-3

18. Murtomaki A, Uh MK, Choi YK, Kitajewski C, Borisenko V, Kitajewski J, Shawber CJ (2013) Notch1 functions as a negative regulator of lymphatic endothelial cell differentiation in the venous endothelium. Development 140 (11):2365-2376. doi:10.1242/dev.083865

19. Tung JJ, Hobert O, Berryman M, Kitajewski J (2009) Chloride intracellular channel 4 is involved in endothelial proliferation and morphogenesis in vitro. Angiogenesis 12 (3):209-220. doi:10.1007/s10456-009-9139-3

20. Uyttendaele H, Marazzi G, Wu G, Yan Q, Sassoon D, Kitajewski J (1996) Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Development 122 (7):2251-2259

21. Geback T, Schulz MM, Koumoutsakos P, Detmar M (2009) TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. BioTechniques 46 (4):265-274. doi:10.2144/000113083

22. Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith GH, Stark KL, Gridley T (2000) Notch signaling is essential for vascular morphogenesis in mice. Genes & development 14 (11):1343-1352

23. Srinivasan RS, Dillard ME, Lagutin OV, Lin FJ, Tsai S, Tsai MJ, Samokhvalov IM, Oliver G (2007) Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. Genes & development 21 (19):2422-2432. doi:10.1101/gad.1588407

24. Tu L, Fang TC, Artis D, Shestova O, Pross SE, Maillard I, Pear WS (2005) Notch signaling is an important regulator of type 2 immunity. The Journal of experimental medicine 202 (8):1037-1042. doi:10.1084/jem.20050923

25. Nowotschin S, Xenopoulos P, Schrode N, Hadjantonakis AK (2013) A bright single-cell resolution live imaging reporter of Notch signaling in the mouse. BMC developmental biology 13:15. doi:10.1186/1471-213X-13-15

26. Hong M, Jung E, Yang S, Jung W, Seong YJ, Park E, Bramos A, Kim KE, Lee S, Daghlian G, Seo JI, Choi I, Choi IS, Koh CJ, Kobielak A, Ying QL, Johnson M, Gardner D, Wong AK, Choi D, Hong YK (2016) Efficient Assessment of Developmental, Surgical and Pathological Lymphangiogenesis Using a Lymphatic Reporter Mouse and Its Embryonic Stem Cells. PloS one 11 (6):e0157126. doi:10.1371/journal.pone.0157126

27. Costa MJ, Wu X, Cuervo H, Srinivasan R, Bechis SK, Cheang E, Marjanovic O, Gridley T, Cvetic CA, Wang RA (2013) Notch4 is required for tumor onset and perfusion. Vascular cell 5 (1):7. doi:10.1186/2045-824X-5-7

28. MacKenzie F, Duriez P, Wong F, Noseda M, Karsan A (2004) Notch4 inhibits endothelial apoptosis via RBP-Jkappa-dependent and -independent pathways. The Journal of biological chemistry 279 (12):11657-11663. doi:10.1074/jbc.M312102200

29. Raafat A, Bargo S, McCurdy D, Callahan R (2017) The ANK repeats of Notch-4/Int3 activate NF-kappaB canonical pathway in the absence of Rbpj and causes mammary tumorigenesis. Scientific reports 7 (1):13690. doi:10.1038/s41598-017-13989-7

30. Raafat A, Lawson S, Bargo S, Klauzinska M, Strizzi L, Goldhar AS, Buono K, Salomon D, Vonderhaar BK, Callahan R (2009) Rbpj conditional knockout reveals distinct functions of Notch4/Int3 in mammary gland development and tumorigenesis. Oncogene 28 (2):219-230. doi:10.1038/onc.2008.379

31. Liu X, Luo Q, Zheng Y, Liu X, Hu Y, Liu W, Luo M, Zhao Y, Zou L (2016) NOTCH4 signaling controls EFNB2-induced endothelial progenitor cell dysfunction in preeclampsia. Reproduction 152 (1):47-55. doi:10.1530/REP-16-0132

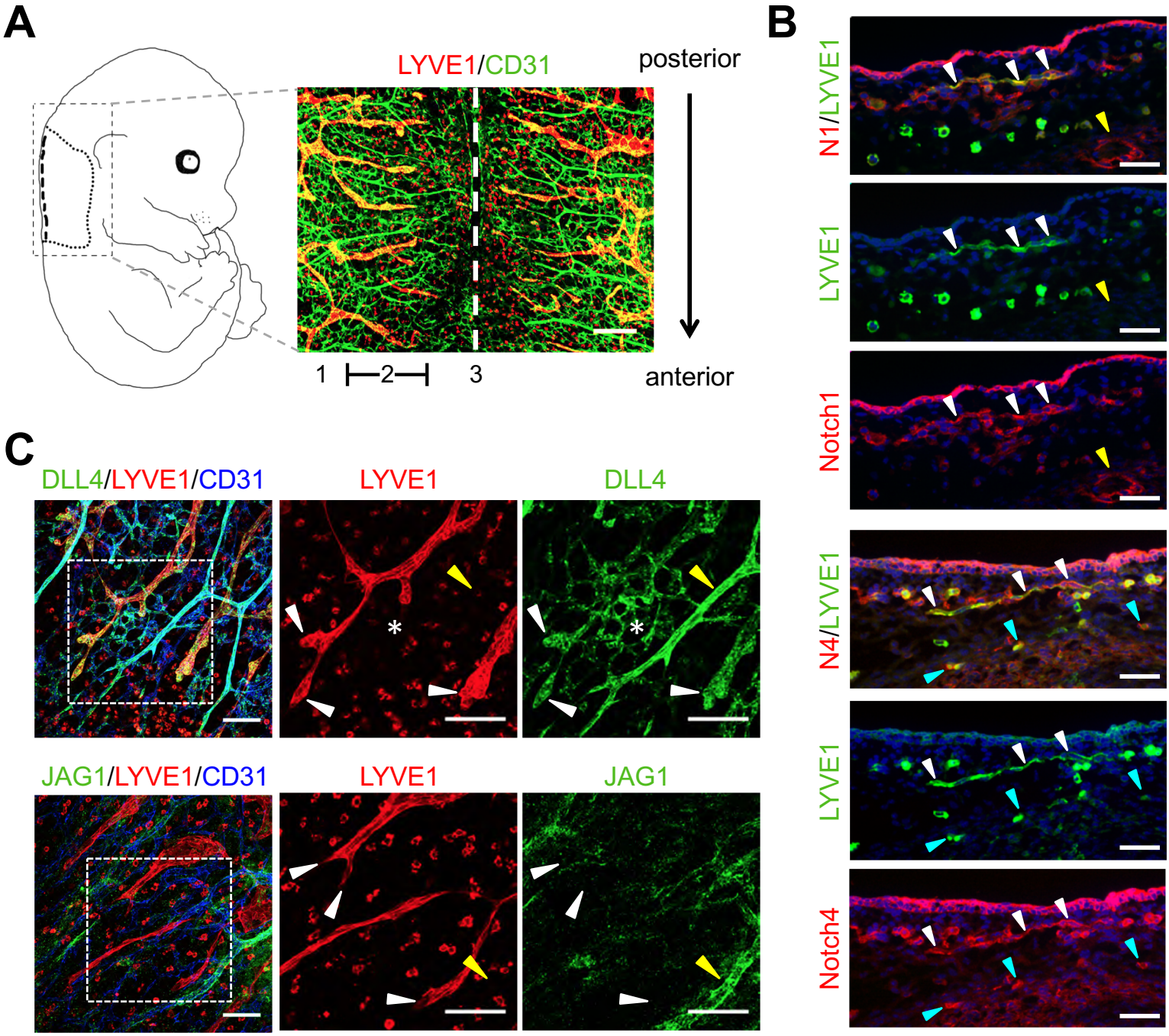
32. Pedrosa AR, Trindade A, Fernandes AC, Carvalho C, Gigante J, Tavares AT, Dieguez-Hurtado R, Yagita H, Adams RH, Duarte A (2015) Endothelial Jagged1 antagonizes Dll4 regulation of endothelial branching and promotes vascular maturation downstream of Dll4/Notch1. Arteriosclerosis, thrombosis, and vascular biology 35 (5):1134-1146. doi:10.1161/ATVBAHA.114.304741

33. Andersen P, Uosaki H, Shenje LT, Kwon C (2012) Non-canonical Notch signaling: emerging role and mechanism. Trends Cell Biol 22 (5):257-265. doi:10.1016/j.tcb.2012.02.003

34. Cha B, Geng X, Mahamud MR, Fu J, Mukherjee A, Kim Y, Jho EH, Kim TH, Kahn ML, Xia L, Dixon JB, Chen H, Srinivasan RS (2016) Mechanotransduction activates canonical Wnt/beta-catenin signaling to promote lymphatic vascular patterning and the development of lymphatic and lymphovenous valves. Genes & development 30 (12):1454-1469. doi:10.1101/gad.282400.116

35. James AC, Szot JO, Iyer K, Major JA, Pursglove SE, Chapman G, Dunwoodie SL (2014) Notch4 reveals a novel mechanism regulating Notch signal transduction. Biochimica et biophysica acta 1843 (7):1272-1284. doi:10.1016/j.bbamcr.2014.03.015

Muley/Kim et al. Figure 1



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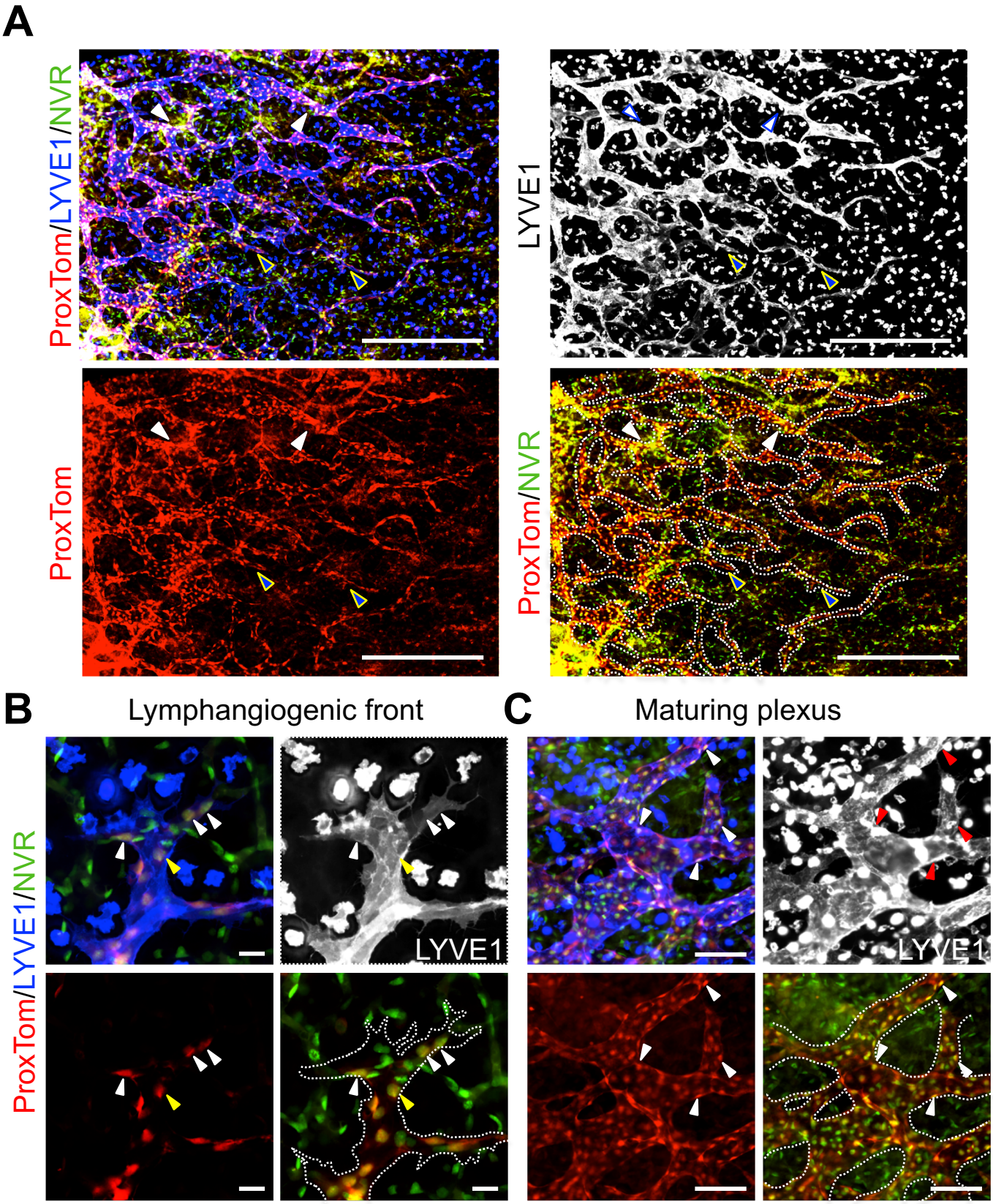

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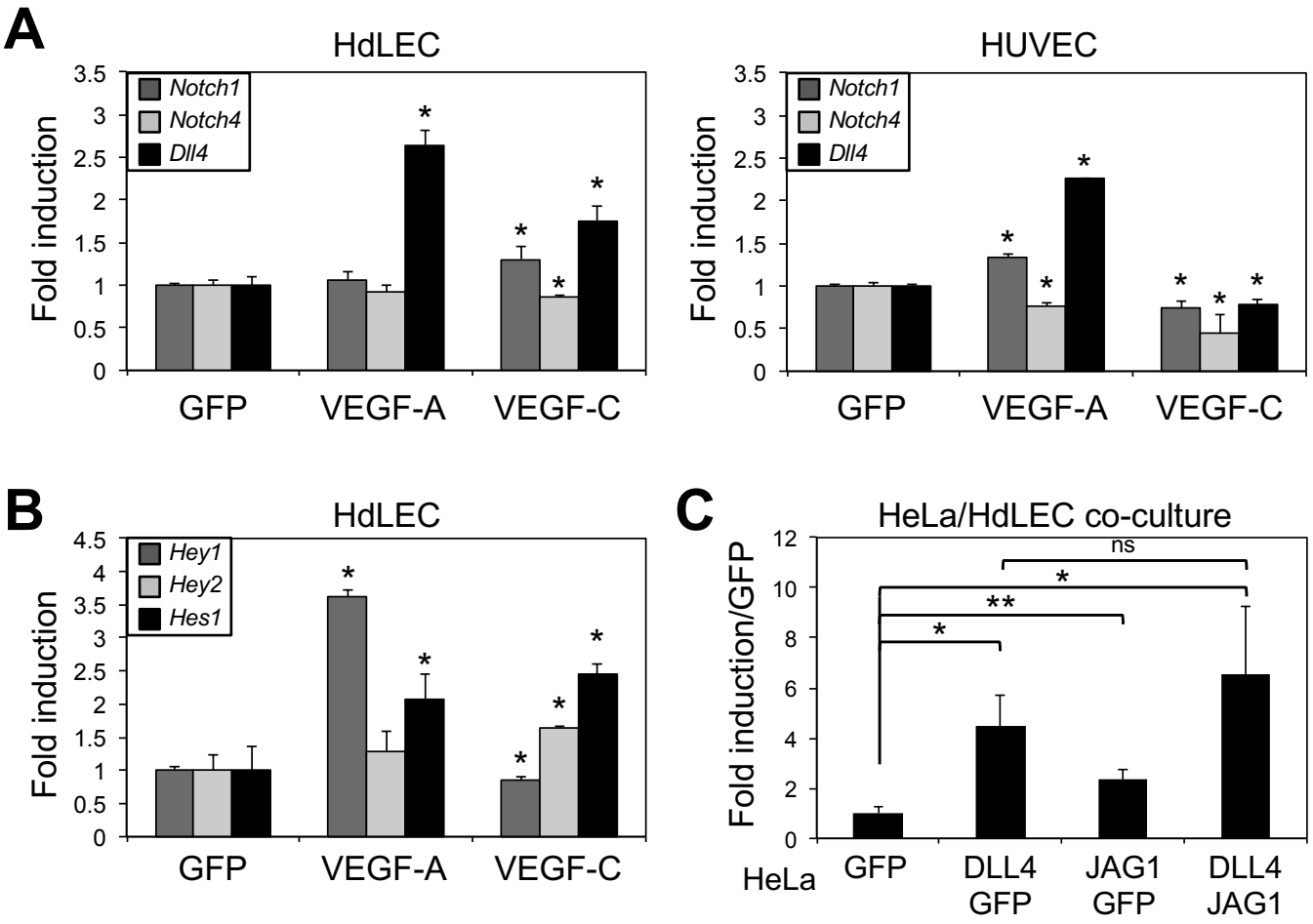

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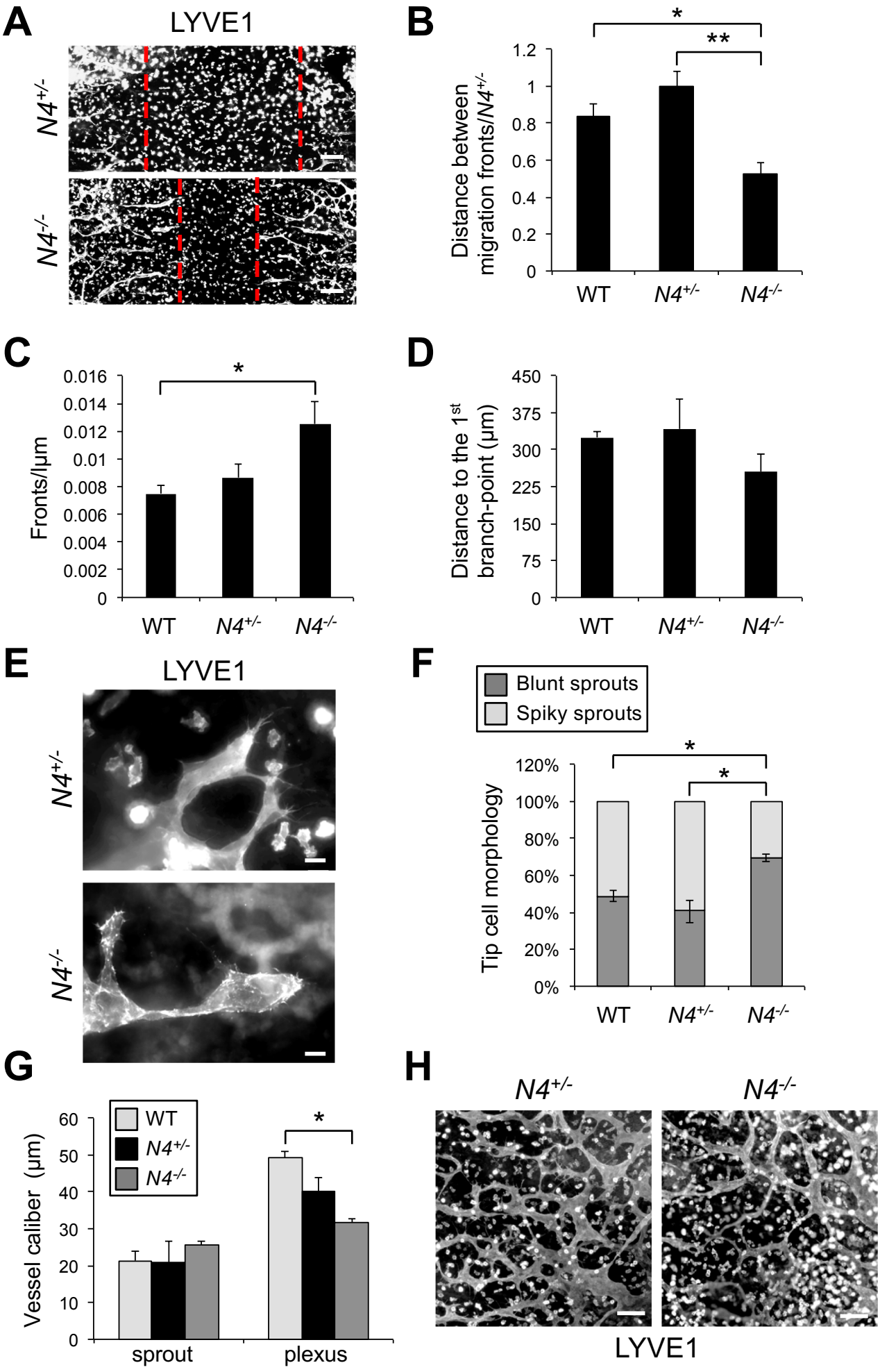

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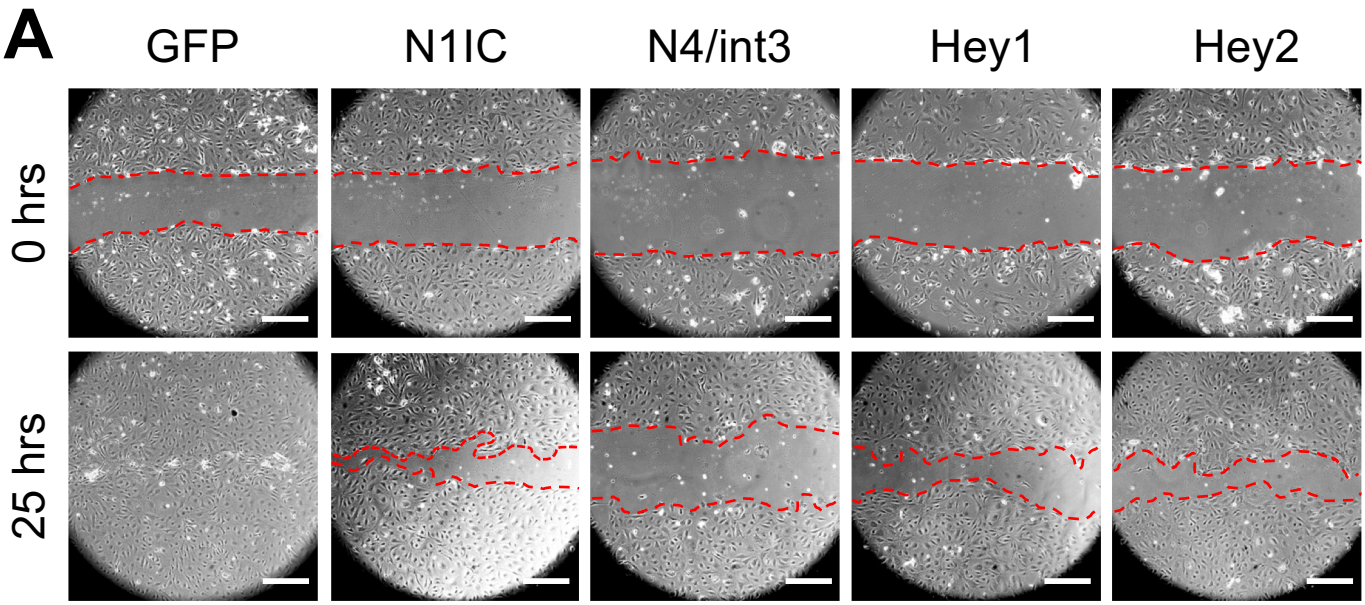

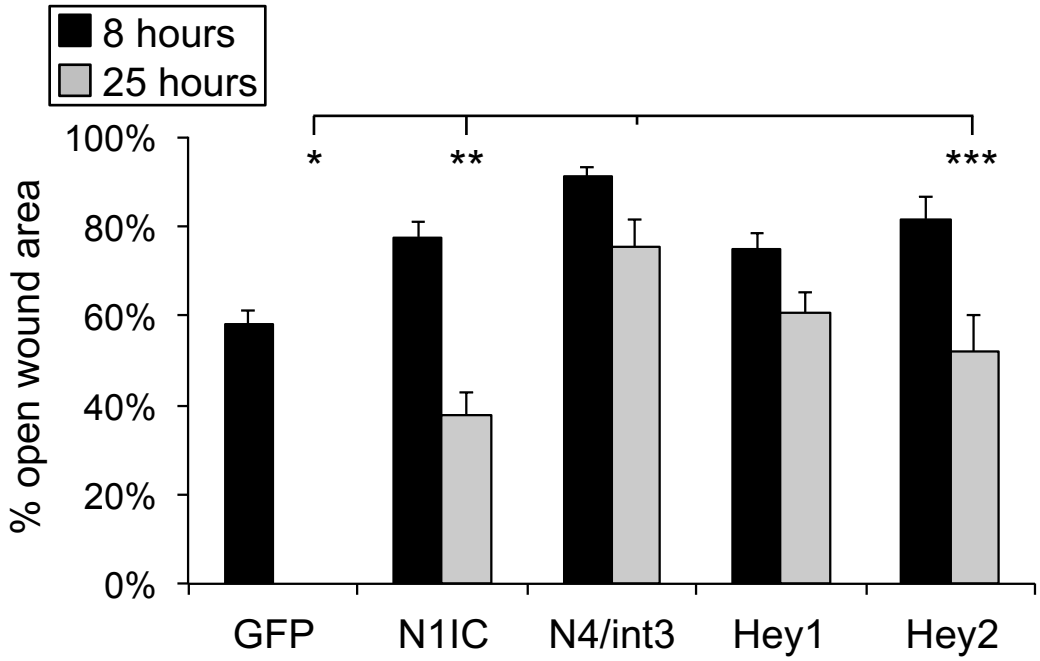

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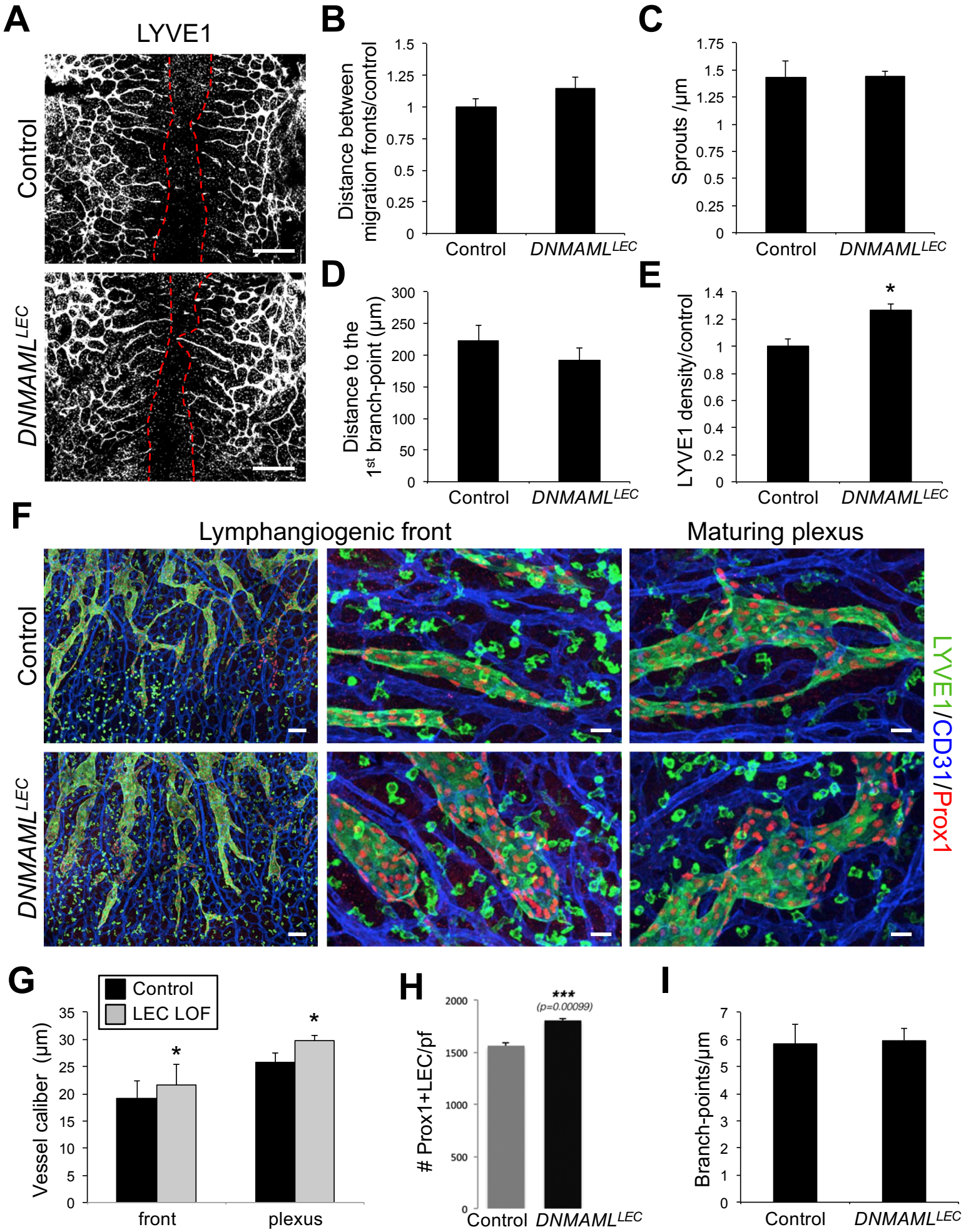

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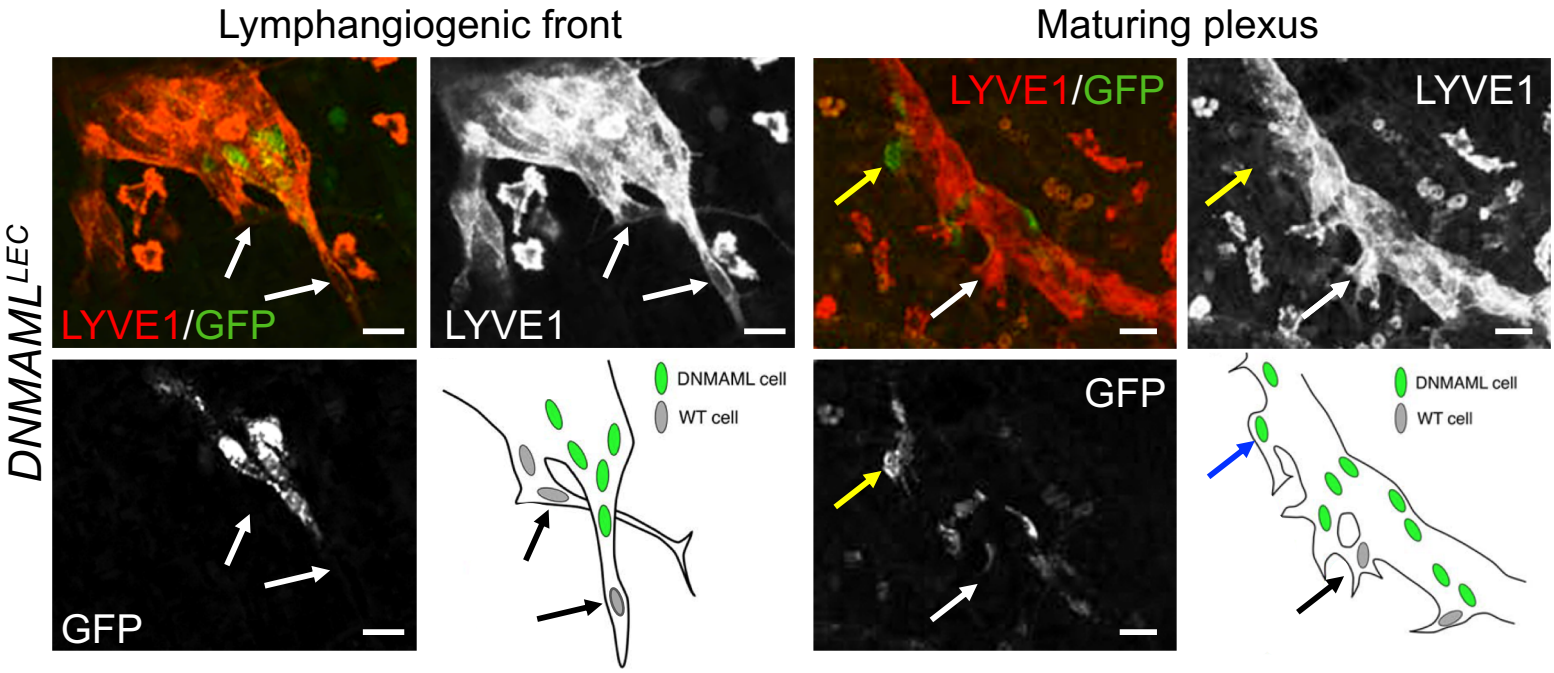
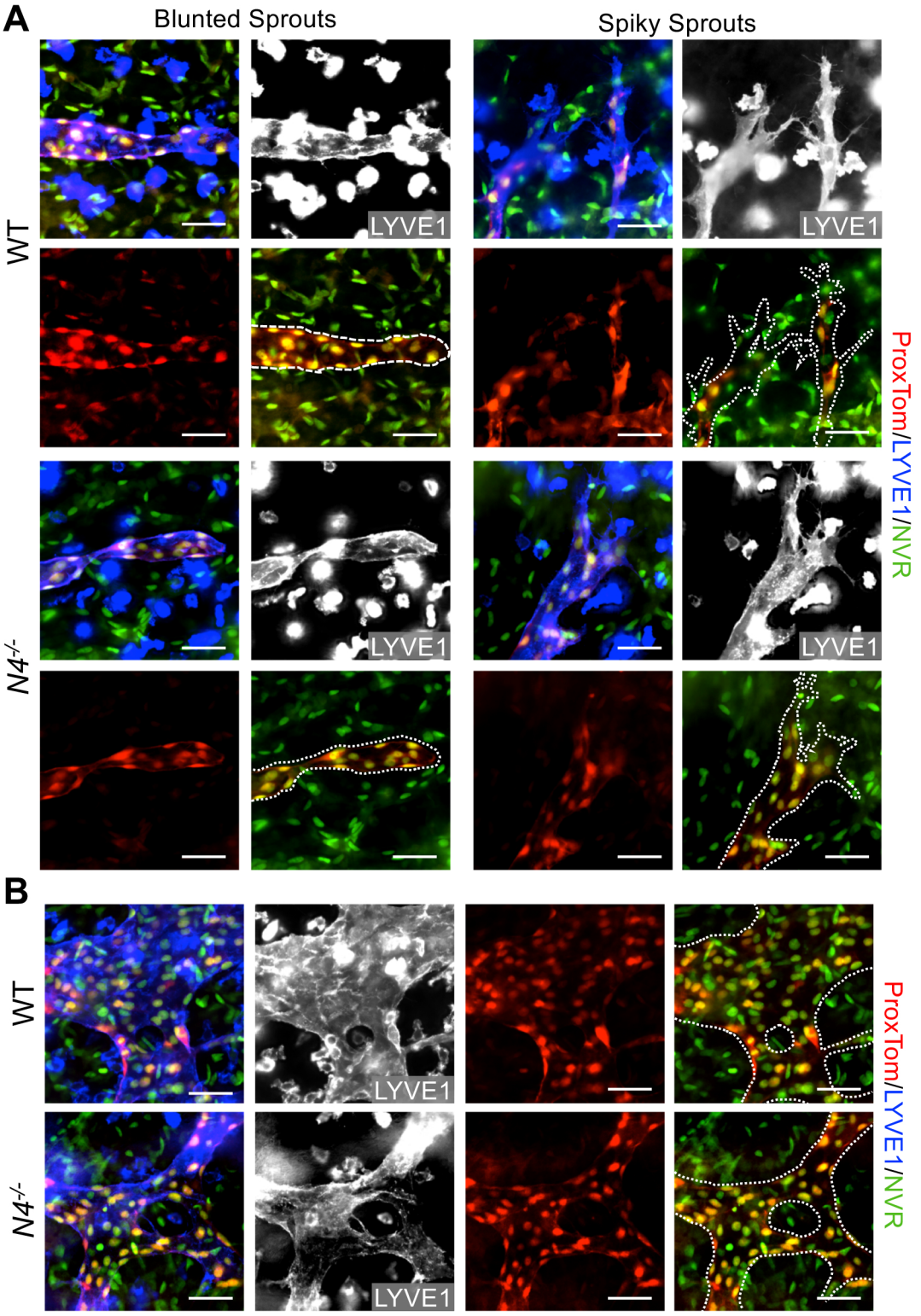
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