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Role of Dll4 / Notch in the formation and wiring of the lymphatic network in zebrafish

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Abstract

Objectives—In zebrafish embryos, sprouts from the axial vein have lymphangiogenic potential, as they give rise to the first lymphatics. Here, we studied whether Notch signaling, which regulates cell fate decisions and vessel morphogenesis, controls lymphatic development.

Methods and results—Knockdown of Dll4 or its receptors Notch-1b or Notch-6 in zebrafish impaired lymphangiogenesis. Dll4/Notch silencing reduced the number of sprouts giving rise to the string of parchordal lymphangioblasts; instead, sprouts connecting to the intersomitic vessels were formed. At a later phase, Notch silencing impaired navigation of lymphatic intersomitic vessels along their arterial templates.

Conclusion—These studies imply critical roles for Notch signaling in the formation and wiring of the lymphatic network.

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The lymphatic vasculature regulates interstitial fluid homeostasis, fat resorption, immune defense, inflammation and metastasis.[1] In mammals, venous blood vascular endothelial cells (BECs) differentiate to lymphatic endothelial cells (LECs).[1] In response to Sox18, Prox-1 induces lymphatic transdifferentiation of venous BECs.[1, 2] Additional cues must regulate lymphatic development, but their nature remains unknown. Another outstanding question is how lymphatics become wired into a stereotyped network. Deep lymphatics regularly fasciculate with other vessels and track along arteries.[1, 3] Similar to blood vessels,[4] lymphatic sprouts have tip cells with filopodia to probe guidance cues.[5] While molecules such as VEGFR-3, VEGF-C, Neuropilin-2, Ccbe1 regulate lymphatic migration,[1, 6] navigation of lymphatics remains poorly understood. Thus, the mechanisms and molecules underlying lymphatic development and wiring remain largely unknown.

Intriguingly, despite the venous origin of lymph vessels, several molecules involved in arterial BEC regulation, also regulate lymphangiogenesis. For instance, EphrinB2, an initial marker of arterial BECs,[7, 8] regulates lymphatics later in development.[1] Sox18, together with Sox7, is required for arterial differentiation and later regulates lymphatic competence.[2]. This relationship between “arterial” factors and lymphangiogenesis, as well as the anatomical congruence between arteries and lymphatics[8–11] prompted us to investigate whether Notch also regulates lymphatic development. Notch and its ligand Dll4 seemed intriguing candidates, given their role in vessel branching.[4] Using gene silencing methods in zebrafish, we revealed novel roles for Dll4/Notch signaling at multiple steps during early lymphangiogenesis.

METHODS

Zebrafish husbandry

Transgenic zebrafish lines used were *Fli1:eGFP*^{y1},[12] *Flt1:YFP*, *kdr-l:mCherry*, *Stab1:YFP*, *Fli1:DsRed*,[6] *Tp1bglob:eGFP*,[13] and intercrosses. Embryos and fish were grown and maintained as described.[6, 14] All animal experimentation was approved by the institutional ethical committee.

Morpholino injection

Morpholinos (Gene Tools, LLC, Corvallis; Supplemental Table I) were injected at the indicated doses, as described.[14] Phenotyping data are pooled data from at least 3 independent experiments, with analysis of 33 to 185 injected embryos per dose. Screening methods for evaluation of lymphatic development and functionality are detailed in Supplemental Methods.

RNA analysis

Whole-mount *in situ* hybridization of dechorionated embryos using antisense probes for the indicated genes (see Supplemental Methods) was performed as described.[14] qRT-PCR was performed on whole embryo extracts or on FACS-sorted embryo cells after *in vivo* labeling of LECs as described in Supplemental Methods.

Cell culture

Proliferation, migration and expression analyses of LECs or HUVECs are detailed in Supplemental Methods.

Statistical analysis

Each gene-specific morpholino was always compared to a control morpholino or vehicle. To determine the penetrance of the phenotype, we counted the number of embryos, exhibiting different phenotype severities, which were analyzed by Chi-square. Pairwise comparisons were performed by two-sided t-test. Asterisks represent a significance level of $P<0.05$.

RESULTS

Knockdown of Dll4 and Notch-1b/6 impairs thoracic duct formation

To explore a role for Notch signaling in lymphatic development (Supplemental Note I; Supplemental Figure I), we silenced every known zebrafish orthologue of the Notch ligands (DeltaA–D, Dll4, Jagged-1a/b, Jagged-2) and receptors (Notch-1a/b, -5, -6) as well as of the Notch activating presenilins-1/2 in *Fli1:eGFP*^{y1} zebrafish embryos, in which lymphatic, arterial and venous endothelial cells (ECs) are labeled.[12, 15, 16] Submaximal silencing conditions were used (Supplemental Note II), that did not affect general or blood vascular development (Supplemental Figure II, III). Development of the thoracic duct (TD), the first functional lymphatic formed in the trunk in-between the dorsal aorta (DA) and the posterior cardinal vein (PCV), was analyzed (for acronyms, Supplemental Note III).

Dll4 morpholino knockdown (Dll4^{KD}) inhibited TD formation. Upon injection of a morpholino affecting Dll4 mRNA splicing (Dll4^{SPL}; 10 ng), the TD failed to form at all by 6 dpf in 52% of morphant embryos, indicating that lymphatic development was completely aborted (Figure 1A,B,D). In another 27% of Dll4^{SPL} embryos, the TD formed over only 10–30% of its normal length, while in another 15% of morphant embryos, the TD formed over 30–90% (Figure 1D). Follow-up studies at 12 dpf revealed that, in embryos with intermediate defects, the TD segments that did form failed to reconstitute the entire TD and to compensate for the lymphatic failure in nearby somites (not shown). Dll4^{KD} embryos without TD at 6 dpf also failed to form a TD, even partially, at later stages (Supplemental Figure II G,H), indicating that lymphatic development was not simply delayed but aborted. Similarly, incomplete silencing of Notch-1b and, to a lesser extent, Notch-6, impaired TD formation (Figure 1C,E). Of note, their mammalian orthologues, Notch-1 and Notch-2, are expressed in LECs.[10, 17] As Notch-1b downregulation causes more penetrant lymphatic defects, only data for Notch-1b^{KD} are shown.

Similar TD defects were obtained with morpholinos, targeting the ATG of Dll4 (Dll4^{ATG}) or Notch-1b (Notch-1b^{ATG}) (not shown), but silencing of the Notch ligands DeltaA-D, Jagged-1a/b, Jagged-2 or of Notch-1a or Notch-5 (orthologue of mammalian Notch-3), did not induce lymphatic defects (not shown). Finally, inhibition of the γ-secretase complex (which proteolytically activates Notch)[18] confirmed the involvement of Notch in lymphatic development. Both morpholino knockdown of Presenilin-1 (PS-1) (but not PS-2) and

pharmacological inhibition of γ-secretase activity by DAPT[18] impaired TD formation (Figure 1F; Supplemental Figure IV Q,R; Supplemental Note II).

Lymphangiography in 7-dpf *kdr-l:mCherryRed* Dll4^{KD} embryos (in which only blood vessels express mCherryRed) revealed no drainage of fluorescent dye in the region where the TD normally forms, confirming that the lack of a GFP^{+} TD in *Fli1:eGFP^{y1}* Dll4^{KD} embryos was not due to reduced expression of GFP, but to actual absence of the vessel itself (Figure 1G,H). This assay further showed that partial TD fragments were not functional (not shown).

Notch is required for parachordal lymphangioblast string formation

We next analyzed whether silencing of Notch impaired development of the parachordal lymphangioblast (PL) cells[6] at the horizontal myoseptum, as these precursors contribute to TD formation (Supplemental Note I, Supplemental Figure I). At 52 hpf, formation of the PL string was completely formed in 53% and largely completed in 40% of embryos (Figure 2A). By contrast, in Dll4^{SPL} embryos, the PL string was completely absent in 38% and formed only in a few segments in 27% of embryos (Figure 2A). Largely comparable fractions of Dll4^{SPL} embryos exhibited similar types of PL string and TD defects (compare Figure 1D with Figure 2A), suggesting that the TD defects were, at least in part, attributable to defects in PL string formation. Imaging of lymphangiogenic structures in Dll4^{SPL} embryos using the *Stab1:YFP* line,[6] which primarily visualizes venous and lymphatic ECs, confirmed these findings (Figure 2B,C). A similar absence of the PL string was observed when using the Dll4^{ATG} morpholino (not shown), or upon knockdown of Notch-1b (Figure 2A) or Notch-6 (not shown). Since the string of PL cells forms as a result of sprouting from the PCV (Supplemental Note I; Supplemental Figure I),[6] these findings suggest that Notch signaling acts in part at very early steps.

Dll4 silencing reduces the fraction of lymphangiogenic sprouts

We then studied whether inhibition of Notch acts during branching of PL-forming secondary sprouts from the PCV (termed “lymphangiogenic” secondary sprouts denoting that they participate in the process that leads to the formation of lymphatic structures, but not blood vessels; Supplemental Note I). Whole-mount staining for *Tie2*, which marks all secondary sprouts,[19] showed a normal total number in Dll4^{KD} embryos (N=20; Figure 2D,E). However, high-resolution imaging of 4-dpf *Fli1:eGFP^{y1}* embryos revealed alterations in the proportion of venous intersomitic vessels (vISVs), connected to the PCV. In control embryos, half of the ISVs were vISVs (% of total ISVs: 54 ± 1%; N=49); by contrast, in Dll4^{SPL} embryos, 82 ± 1% of the ISVs were connected to the PCV and thus vISVs (N=97, P<0.05 versus control). Similar findings were obtained in Notch-1b^{SPL} embryos (vISVs, % of total: 69 ± 2.7%; N=27, P<0.05 versus control). Since vISVs can only be formed via connection of a secondary “angiogenic” sprout to a primary ISV (Supplemental Note I), these findings, and the observation that silencing of Dll4, Notch-1b or Notch-6 aborted PL string formation in a substantial fraction of embryos, show that a fraction of secondary sprouts, that would normally have been lymphangiogenic, were angiogenic, thereby impairing TD formation (Figure 6A,B).

We also used high-resolution video-imaging of the double transgenic reporter line *Flt1:YFPxkdr-l:mCherryRed*,[6] labeling venous cells red (CherryRed^{+}) and arterial cells yellow (YFP^{+}CherryRed^{+}) in merged images.[6] In control embryos, half of the ISVs had a red venous color and the other half had a yellow arterial color (Figure 2F,F′). In contrast, in Dll4^{KD} embryos with severe lymphatic defects, nearly all yellow arterial ISV (aISV) connections with the DA had disappeared (single white arrow in Figure 2G,G′; Supplemental Movies I, II). Thus, a supernumerary fraction of vISV-producing angiogenic sprouts is formed in Dll4^{KD} embryos at the expense of lymphangiogenic sprouts, that would otherwise go on to form the PL string.

Dll4/Notch promotes lymphatic characteristics *in vitro*

To evaluate whether Notch activation in venous ECs could induce lymphatic properties, we co-cultured human umbilical venous ECs (HUVECs, which express *NOTCH-1*, but negligible levels of *PROX-1*; not shown), with COS cells expressing DLL4 (COS^{Dll4}) or a control vector (COS^{CTR}), and analyzed by RT-PCR with human gene-specific primers the expression of lymphatic markers. Expression levels of the lymphatic markers *PROX-1*, *VEGFR3*, *LYVE-1*, and *SOX18* in COS^{Dll4}-activated HUVECs were moderately to distinctly elevated (Figure 3). Notably, expression of *EPHRINB2*, which is regulated by Notch and has been implicated in both arterial and lymphatic processes,[1, 7] and *COUP-TFII* which is expressed in both venous and lymphatic ECs,[20] were also upregulated, but levels of other blood vessel markers (*ENDOGLIN*, *VE-CADHERIN*, *CD31*) were not or only minimally affected (Figure 3). The upregulation of lymphatic markers was abolished by treatment of the cells with DAPT (30 μM; not shown).

Silencing of Dll4 impairs PL cell migration along aISVs

From 60 hpf onwards, PL cells switch to radial migration, and navigate ventrally and dorsally alongside aISVs, whereby they form lymphatic intersomitic vessels (LISVs) (Supplemental Note I). Since the TD failed to form in a fraction of Dll4^{SPL} embryos (25%) despite the presence of a partial PL string, we further explored whether Notch signaling affects LISV formation. In control embryos, LISV-PLs (PL cells that formed LISVs) migrated exclusively along aISVs, suggesting that vISVs are not permissive (Figure 4A,B). Since there were more vISVs and fewer aISVs in Dll4^{KD} embryos, migrating LISV-PLs were deprived from their arterial template and could therefore not contribute to TD formation (Figure 4C; 6C). This was the most common migration defect. Intriguingly, even when residual aISVs formed in Dll4^{SPL} embryos, LISV-PLs sometimes bypassed the aISV post, failing to turn and migrate along aISVs (Figure 4D; 6C′). Indeed, in Dll4^{SPL} embryos with a nearly complete PL string (>90% of its length; N=61), 49 ± 6% of their aISVs were not accompanied by LISV-PLs, compared to only 15 ± 4% in controls (N=29; P<0.05).

Other, much less frequent LISV defects included LISV-PLs that turned ventrally alongside the aISV, but stalled (Figure 4E; 6C”), or even, in a few cases, misrouted LISV-PLs migrating along vISVs (Figure 4F; 6C‴). *In vitro* studies revealed that Notch did not regulate LEC migration/motility, proliferation, or lymphatic capillary tube formation or sprouting (Supplemental Figure V; not shown).

Expression of Dll4 and Notch

Whole-mount *in situ* hybridization (ISH) in control embryos at 30 hpf, when secondary sprout formation starts, showed that Dll4 was detectable in the DA but not in the PCV (Figure 5A,B), in line with previous reports.[19, 21, 22] Notch-1b was strongly expressed in the DA (Figure 5C,D), while a much weaker signal appeared dispersed in certain endothelial cells of the dorsal part of the PCV, though the low Notch-1b signal approached the detection limit of available techniques (Supplemental Figure VI).

We also developed a new technique to isolate LECs from zebrafish embryos. When TRITC-dextran dye is injected intramuscularly in 4 week old *Fli1:eGFP*^{y1} embryos, the red dye is taken up by LECs but not BECs via pinocytosis, allowing FACS sorting of red/green LECs. By RT-PCR, low Notch-1b transcript levels were detected in these LECs (copies Notch-1b/10^5 copies β-actin: 6.9 ± 0.77, N=4). However, TRITC-dextran 'LEC labeling' is only feasible in large 4 week-old embryos, but not in small early stage embryos, precluding us from quantifying Notch-1b expression in early lymphatic development.

Dll4 and Notch-1b were also detected by ISH in primary ISVs at 30 hpf (Figure 5A–C).[21, 22] As ISH is technically challenging in embryos beyond 2 dpf, we analyzed Notch expression during LISV-PL migration in *Tp1bglob:eGFP*x*Fli1:DsRed* fish, in which all ECs are red, and cells with canonical Notch activity are green (GFP driven by a promotor containing 12 Su(H) binding sequences[13]). Imaging at the time when PL cells turn and switch to radial ventral migration revealed that the DA and aISVs are yellow in the merged image, indicating that canonical Notch signaling was active in arterial vessels, but not in LISVs or vISVs (Figure 5E).

DISCUSSION

The key finding of this study is that incomplete silencing or pharmacological inhibition of Notch impaired lymphatic development in zebrafish. Phenotypic analysis indicates that Notch signaling regulates the formation of lymphangiogenic sprouts and their descendent PL cells, which give rise to the TD (Figure 6A,B). At a later stage, Notch is required for guided migration of LISV-PLs along aISVs (Figure 6C–C‴).

Role of Notch in lymphangiogenic secondary sprout formation

Our results reveal that Notch, in addition to its role in blood vessel morphogenesis and arterial development,[4, 9] also regulates lymphatic development. Half of the Notch hypomorphant embryos failed to form a TD without later rescue, indicating lymphatic abortion rather than delay. The earliest identifiable abnormality, the increased fraction of venous ISVs, indicated a defect at the level of the secondary sprouts from the PCV, where fewer lymphangiogenic but more angiogenic sprouts developed (Figure 6A,B). Also, most embryos, surviving DAPT treatment at stages when lymphangiogenic sprouting was initiated, did not form a TD, further suggesting an early role for Notch in lymphatic development (not shown). The hypomorphant phenotypic change correlated with defective formation of the PL and TD, and could result from defects in LEC fate acquisition,

migration, proliferation, survival and/or other cellular processes contributing to sprout formation and maintenance.

How Notch signaling regulates lymphatic development remains unresolved. Based on the present and recent other studies, three possible (non-exclusive) models can be considered to explain our findings. A first explanation is that Notch silencing altered blood vessel development and, secondarily, influenced lymphatic development. Previous studies documented that arterial differentiation is impaired by inhibition of multiple Notch signaling pathways (for instance by a dominant negative Su(H)),[7] but not by selective silencing of Dll4 [21, 22]. Our imaging and marker expression analyses are consistent with these findings and reveal that initial formation and differentiation of the PCV, DA and primary ISVs all occurred normally upon incomplete silencing of Notch signaling. Thus, at least by generally accepted criteria of arterial and venous identity, these blood vessels developed normally in $Dll4^{KD}$ and $Notch\text{-}1b^{SPL}$ embryos. Nonetheless, we do not exclude the possibility that subtle alterations in arterial characteristics of the primary ISVs might have favored supernumerary connections with secondary sprouts, thereby "entrapping" sprouts that would otherwise have remained lymphangiogenic. Also, Notch silencing resulted in a greater fraction of venous than arterial ISVs; since arterial ISVs act as guidance templates for LISVS, impaired migration of the latter was indeed attributable to such a change in arterial morphogenesis. However, an outstanding question is whether the aISV changes themselves were in fact not caused by defective formation of the lymphangiogenic sprouts in the first instance. Indeed, precisely because lymphangiogenic branches failed to develop in Notch morphants, venous angiogenic sprouts formed instead, which then connected to the primary ISVs and converted them to vISVs.

A second model is that Dll4 and Notch are expressed by the same or adjacent arterial ECs within the DA and that this *cis* signaling induces the release of paracrine lymphangiogenic factors (such as EphrinB2, VEGF-D[1, 23] or an unknown signal), that indirectly instruct venous ECs of the nearby PCV to induce lymphangiogenic sprout formation in a cell non-autonomous manner. A similar indirect model was proposed to explain segregation of the DA from PCV in zebrafish.[8] Likewise, during LISV migration, release of a guidance signal from aISVs in response to Dll4/Notch signaling in arterial cells could assist navigation of LISVs to their target projection.

Finally, a third and perhaps the most appealing, but at this stage still speculative, explanation for our data is that arterial Dll4 in the DA signals *in trans* to Notch on ECs in the PCV, which lies in close juxtaposition at the time of lymphangiogenic sprouting. There are arguments in support and against this model. An argument in favour for a cell autonomous role of Notch in PCV cells is that activation of Notch by Dll4 upregulated several LEC-specific markers in venous ECs *in vitro*. Expression analysis experiments *in vivo* yielded inconclusive results. Notch-1b expression was weakly detectable in dispersed dorsal PCV cells, but only at a very low level that approached the detection limit of the techniques used. Notch-1b was also measurable by RT-PCR in isolated LECs in older embryos, but this technique could not be used during early lymphatic development. We therefore acknowledge that the Notch-1b expression results represent a limitation of this study, which precludes us

from drawing firm conclusions regarding a cell-autonomous role for Notch in lymphangiogenic sprouting.

Another recent study also documented a cell autonomous role for Notch,[24] while a second did not.[25] In LEC cultures, Notch signaling reprogrammed lymphatic to arterial cell fate, while Prox1 counteracted this force, thereby allowing fine-tuning of the LEC fate in a delicately balanced feedback.[24] These findings should not necessarily be in contradiction to our findings, as they analyzed reprogramming of fully differentiated LECs away from their lymphatic fate, while we used venous BECs to study programming towards the LEC fate *in vitro*. As the authors of this study mention,[24] "LEC-fate may not be governed by a two-way turn ON-OFF switch, but rather by a dial switch that allows a *gradient* increase or decrease in the lymphatic cell fate force". Reconciling these and our findings, it seems that Notch levels must be tightly controlled to induce and maintain LEC fate. Low levels of Notch signaling might be required to induce lymphatic fate in venous BECs and, once differentiated into LECs, Prox1 would then secure lymphatic fate by preventing overexpression of Notch, as this would promote arterial cell fate.[24] The lower expression of Notch-1 in LECs (this study and [10, 24]) than in arterial ECs[8–11] supports this model and could also explain why incomplete Notch silencing sufficed to abrogate lymphatic but not arterial development. However, in the absence of more conclusive evidence that Notch silencing abrogates Prox1 induction in PCV cells in the zebrafish model *in vivo*, a role for Notch in programming LEC fate remains unproven. Also, Notch may regulate other processes than LEC specification in lymphangiogenic sprouting.

A recent study in mice further adds complexity to this model. Indeed, conditional inactivation of RbpJ, a mediator of canonical Notch signaling, in ECs did not alter the expression of lymphatic markers in venous ECs.[25] While these data may suggest that Notch signaling is redundant for LEC specification in mammals *in vivo*, an alternative interpretation is that Notch regulates this process via non-canonical signaling. This might also explain why we could not detect a robust signal in LECs or their precursors in the *Tp1bglob:eGFPxFli1:DsRed* line. Also, species-specific differences between mammals and zebrafish could account for some of the observations. Overall, whether Notch signaling regulates lymphatic development in a cell-autonomous manner remains to be further elucidated in the future.

Role of Notch in lymphatic migration from the PL

Notch signaling also regulated the formation of LISVs, which arise from the PL cells. Most frequently, the LISV was absent but in other rarer cases, migrating LISV-PLs stalled or became misrouted (Figure 6C–C‴). Our findings suggest that lymphangiogenic EC migration *per se* (motility) was normal. Also, we did not detect signs of lymphatic regression or retraction (not shown). It is therefore tempting to speculate that LISV defects in Notch-silenced embryos reflect impaired lymphangiogenic cell pathfinding. LISV-PLs navigated in close association along aISV templates, raising the question whether aISVs act as guidance templates for LISV-PLs, reminiscent of how *follower* axons navigate along a *pioneer* axon's pathway or how autonomic nerves use arterial tracks to reach their target.[26, 27] Hence, as fewer aISVs are present in Notch morphants because of lymphangiogenic sprouting defects,

PL cells are deprived of navigation templates and therefore cannot form LISVs normally (Figure 6C). Other observations that LISV-PLs failed to switch from tangential to radial migration or, more rarely, stalled or selected incorrect paths (Figure 6C′–C‴), are reminiscent of classic neuronal guidance defects. That arteries may act as navigation templates is evidenced by reports that autonomic nerves stall or become misrouted, when these arteries do not produce appropriate guidance cues.[27] Su(H)-dependent Notch activity was detectable in aISVs at the time when PL cells switch from tangential to radial migration alongside aISV, indicating that lymphatic navigation is regulated either non-cell autonomously or via non-canonical Notch signaling. Whether and how Notch regulates the production of turning and guidance cues for LISV-PL cells by aISVs or nearby (somitic) cells remains to be determined. Other morphant and mutant zebrafish phenotypes also suggest that LISV development requires arterial-lymphatic congruence [28].

In conclusion, this study revealed a role of Notch in lymphatic development, in part by regulating the initial steps of lymphangiogenic sprouting and PL formation. Moreover, the navigation defects of LISV-PL cells along aISVs suggest that Notch also regulates lymph vessel pathfinding along arteries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Condensed abstract

Dll4 and its receptors Notch-1b and Notch-6 are involved in the formation and wiring of the lymphatic network in zebrafish. Silencing of Dll4/Notch reduced the number of sprouts giving rise to the string of parachordal lymphangioblasts, and impaired navigation of lymphatic intersomitic vessels along arterial templates.

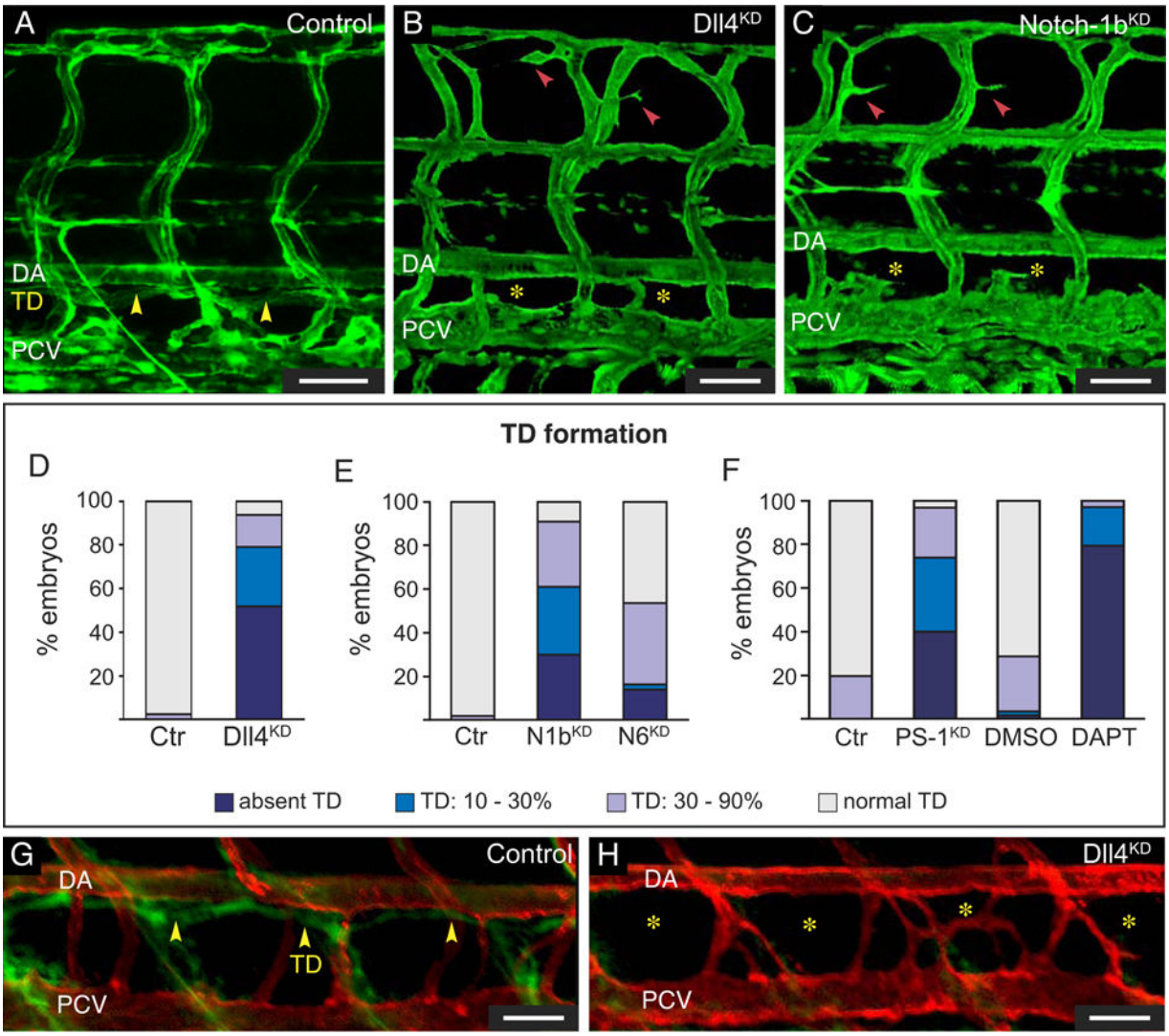

Figure 1. Role of Notch in TD formation

A–C, Confocal images of GFP^{+} vessels in *Fli1:eGFP^{y1}* embryos. Normal TD in control (yellow arrowheads; A), and absent TD in Dll4^{KD} (B) and Notch-1b^{KD} (C) embryos. Yellow asterisks: TD absence; red arrowheads: minimal hyperbranching of ISVs. **D–F**, Percentage of affected embryos in control (*N*=122 in D; 185 in E; 87 in F) or Dll4^{KD} embryos (*N*=80; 10ng Dll4^{SPL}; *P*<0.001; D); Notch-1b^{KD} (N1b^{KD}) embryos (*N*=84; 15ng Notch-1b^{SPL}; *P*<0.001; E), Notch-6^{KD} (N6^{KD}) embryos (*N*=63; 15ng Notch-6^{SPL}; *P*<0.001; E), PS-1^{KD} embryos (*N*=65; 2.5ng PS-1^{ATG1}; *P*<0.001; F), or embryos treated with DMSO (*N*=171; F) or DAPT (*N*=34; 25 μM; *P*<0.001; F). **G,H**, Lymphangiography in 7-dpf *kdr-l:mCherryRed* embryos revealed normal uptake and drainage of a green dye by the TD in the control (yellow arrowheads; G), but not in the Dll4^{KD} embryo (yellow asterisks; H). Bars: 50 μm.

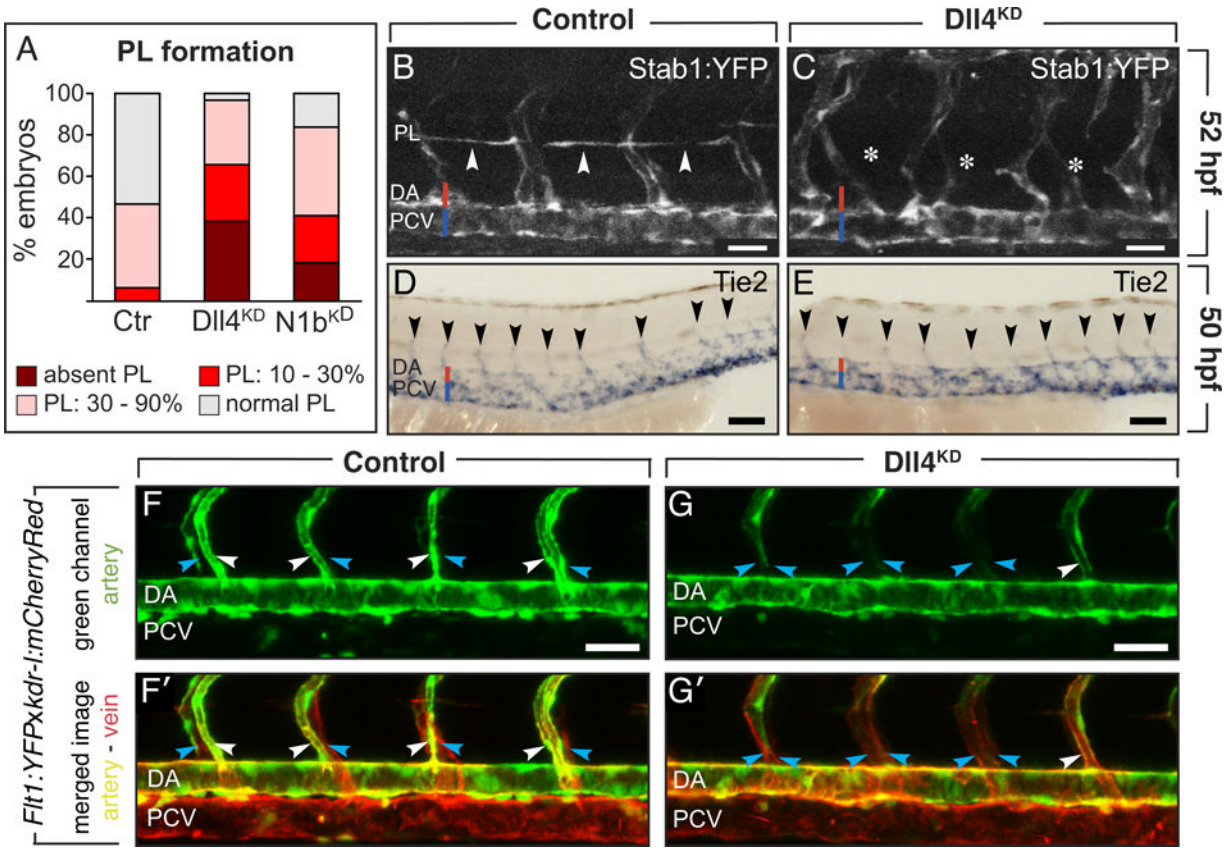

Figure 2. Silencing of Notch blocks PL and lymphangiogenic sprout formation

A, Percentage of 52-hpf embryos with affected PL in control (*N*=73), Dll4^{KD} embryos (*N*=55; 10ng Dll4^{SPL}; *P*<0.001) or Notch-1b^{KD} (N1b^{KD}) embryos (*N*=49; 20ng Notch-1b^{ATG}; *P*<0.001). **B,C**, Confocal images of 52-hpf *Stab1:YFP* embryos, showing normal PL in controls (arrowheads; B), but absence in Dll4^{KD} embryos (asterisks; C). **D,E**, Whole-mount *in situ* Tie2 staining at 50 hpf, revealing normal numbers of secondary sprouts (arrowheads) in control (D) and Dll4^{KD} (E) embryos. **F,G**, Confocal images of vessels in *Flt1:YFP*x*kdr-l:mCherryRed* embryos: *kdr-l:mCherryRed* marks venous and arterial vessels red (red channel not shown), *Flt1:YFP* labels arterial vessels green (F,G), and merged images show arterial vessels yellow and venous vessels red (F′,G′). Lateral views with left and right side ISVs partially superimposed. Imaging was at 54 hpf, when secondary angiogenic sprouts had already connected to primary ISVs, which were changing arterial to venous identity in a ventral-to-dorsal pattern. In controls (F,F′), half of the aISVs became connected by angiogenic sprouts from the PCV and acquired a venous identity, thereby losing their green arterial signal (blue arrows; F) and becoming red only (blue arrows; F′), while the other half of the ISVs remained connected to the DA and were green (white arrows; F) or yellow in the merged image (white arrows; F′). By contrast, in the Dll4^{KD} embryo (G,G′), most ISVs lost their green arterial marker (blue arrows; G; note the single white arrow), and became marked in red only (blue arrows in G′). White arrow in G,G′ denotes a residual aISV retaining its green (G) or yellow (G′) label. Bars: 50μm (B,C,F,G); 100μm (D,E).

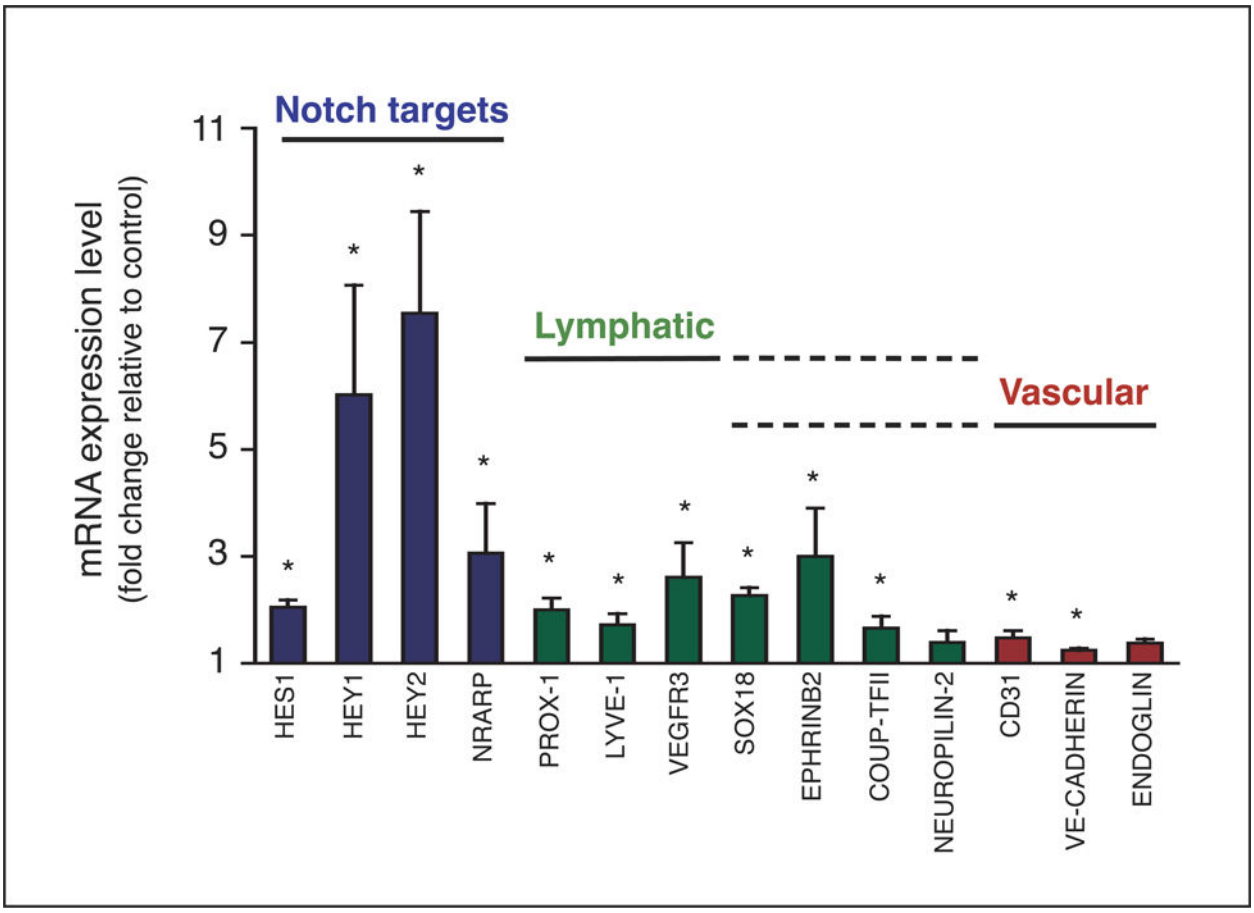

Figure 3. Notch activation by Dll4 promotes lymphatic characteristics *in vitro*

RT-PCR of HUVECs, co-cultured with COS cells expressing hDll4 (COS^{Dll4}) or control GFP (COS^{CTR}), confirming upregulation of Notch targets (*HES1*, *HEY1*, *HEY2*, *NRARP*; blue bars) and revealing enhanced lymphatic marker expression (*PROX-1*, *LYVE-1, VEGFR3*, *SOX18, EPHRINB2*, green bars), while vascular genes (*CD31*, *VE-CADHERIN*; *ENDOGLIN*; red bars) were only minimally affected. *COUP-TFII* was also upregulated. *NEUROPILIN-2* was not affected. Lymphatic/arterial and lymphatic/venous genes are marked by the overlapping dashed lines. Results are fold change in HUVEC/COS^{Dll4} co-culture versus HUVEC/COS^{CTR}. Mean±SEM; *N*=3–11; *, *P*<0.05.

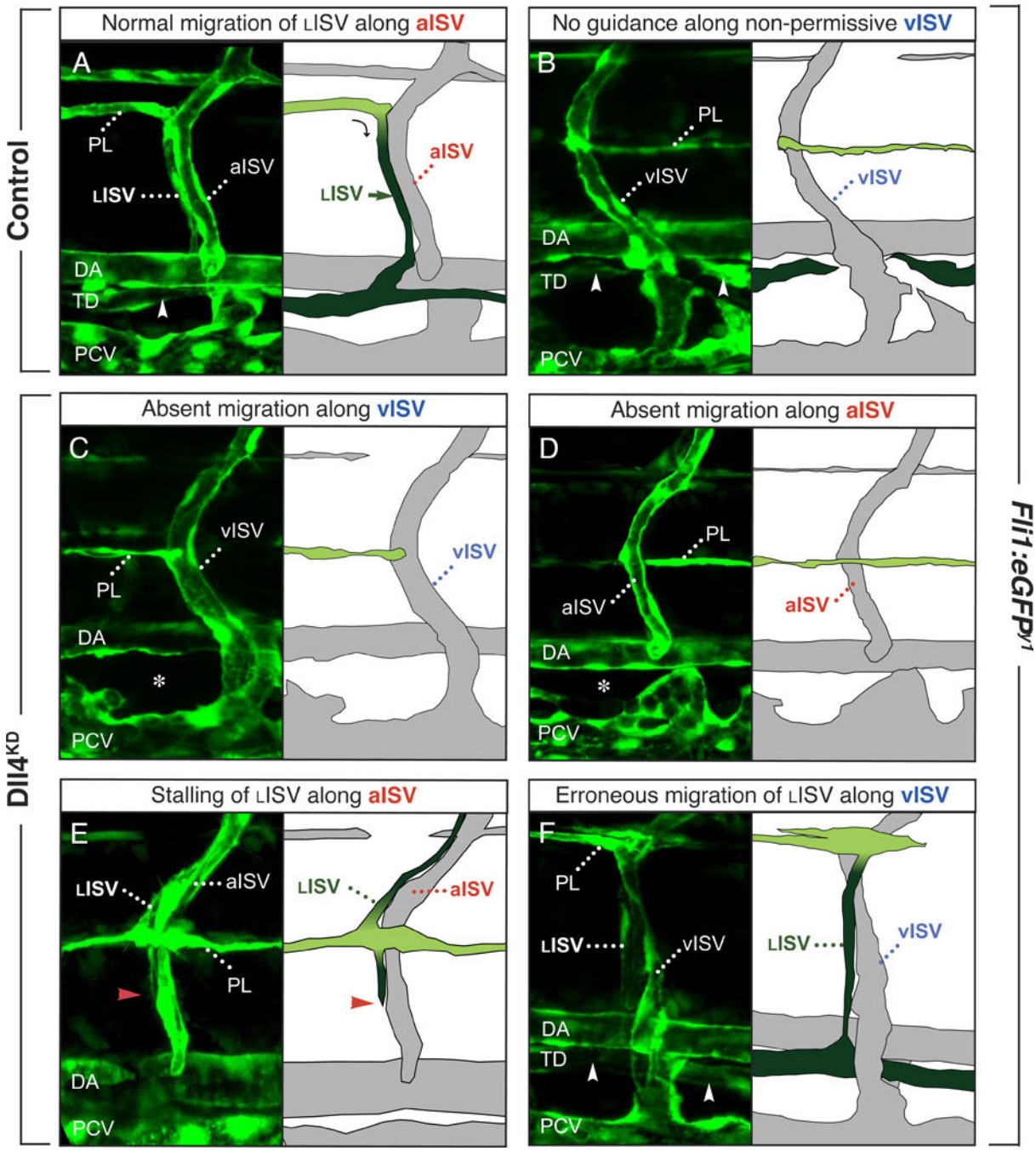

Figure 4. Incomplete silencing of Notch perturbs lymphatic navigation

Confocal images with accompanying schematic redrawing of the navigation routes of LISVs along aISVs or vISVs in 4-dpf control (A,B) and Dll4^{KD} (C–F) *Fli1:eGFP*^{y1} embryos. Permanent lymphatic structures (LISV, TD) are dark green; transient lymphangiogenic structures (PL) are light green. **A,B**, In control embryos, LISV-PLs navigate alongside aISVs and establish a continuous TD (arrowhead). Note how LISVs "creep" over their aISV guidance templates (A). LISV-PLs never navigate along vISVs in control embryos (B). **C–F**, Navigation defects in Dll4^{KD} embryos. **C**, In a large fraction of morphant somites, LISV-

PLs lack migration templates because fewer aISVs develop. As LISV-PLs do not normally migrate along vISVs, no TD was formed in these somites (asterisks). **D**, In other morphant somites, LISV-PLs bypassed the point of turning at the aISVs, and failed to switch to radial migration. **E**, In a small fraction of somites, LISV-PLs accomplished to make the turn and switched to radial migration, but then stalled (red arrowhead denotes the arrested tip of a navigating LISV). **F**, In most $Dll4^{KD}$ embryos, vISVs were not permissive to guide LISV-PLs, but, occasionally, LISV-PLs erroneously navigated alongside a vISV.

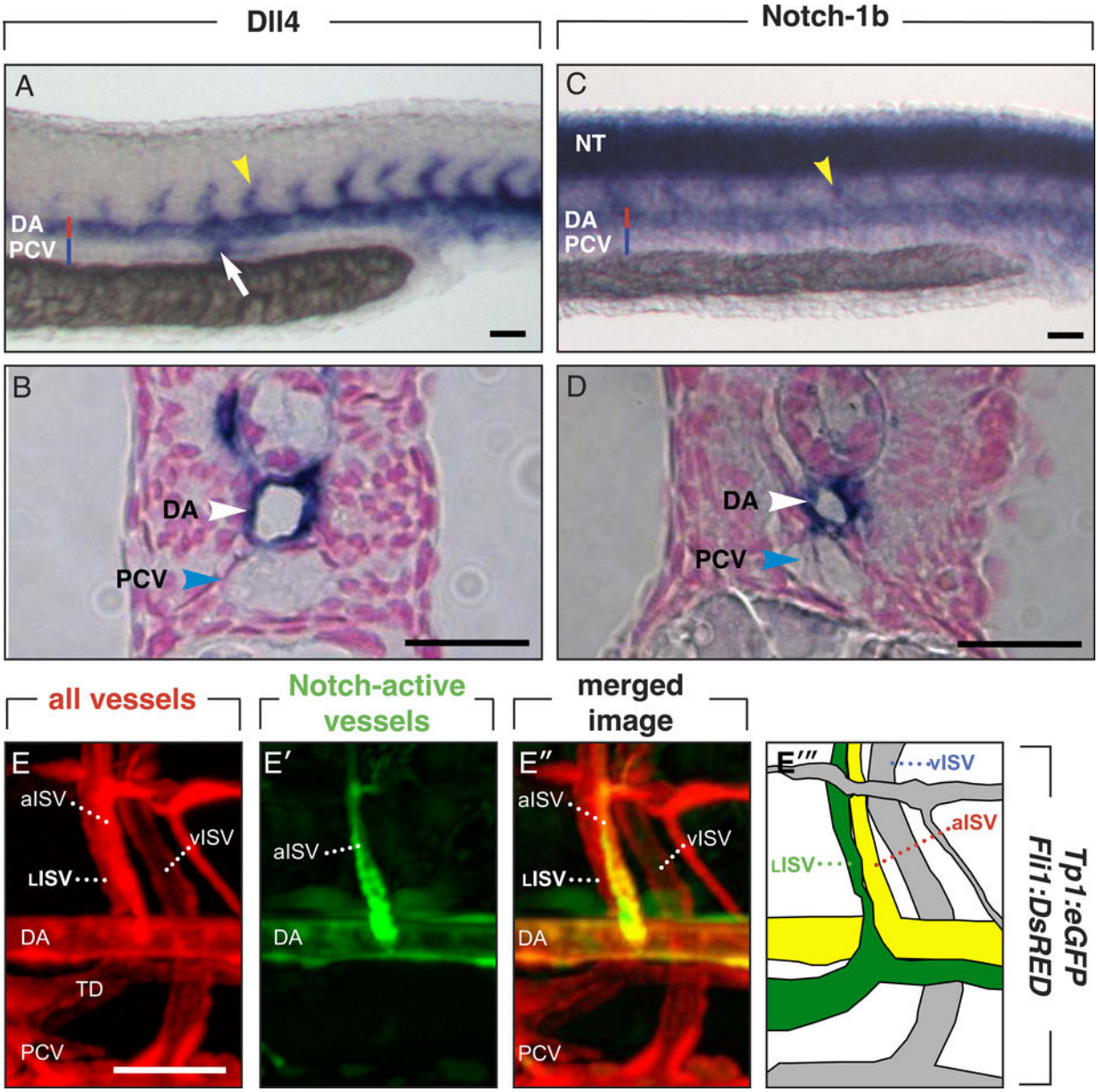

Figure 5. Expression of Dll4./.Notch-1b

A–D, Whole-mount embryos at 30 hpf, when lymphangiogenic sprouting occurs, stained for Dll4 (A,B) or Notch-1b (C,D); panels B,D show cross-sections of the respective embryos. Primary ISVs are indicated by yellow arrowheads (A,C). **A,B**, Dll4 expression was detected in the DA and primary aISVs, pronephric duct (white arrow; A). **C,D**, Notch-1b is strongly expressed in the neural tube (NT), DA and primary aISVs. **E**, Confocal images of *Tp1bglob:eGFP*x*Fli1:DsRed* embryos, in which *Fli1:DsRed* marks blood and lymph vessels in red (E) and *Tp1bglob:eGFP* labels cells with activated canonical Notch activity in green (E′). The merged image shows arterial vessels (DA; aISV) with active Notch in yellow (green-red), while the LISV are only red (E”). In the schematic representation, the lymphatic structures are indicated in green, Notch-activated vessels in yellow, and other vessels in grey

(E‴). Representative images of arterial activation of Notch in a 6-dpf embryo are shown (for technical reasons), but similar data were obtained at 60 hpf. Bars: 50 μm.

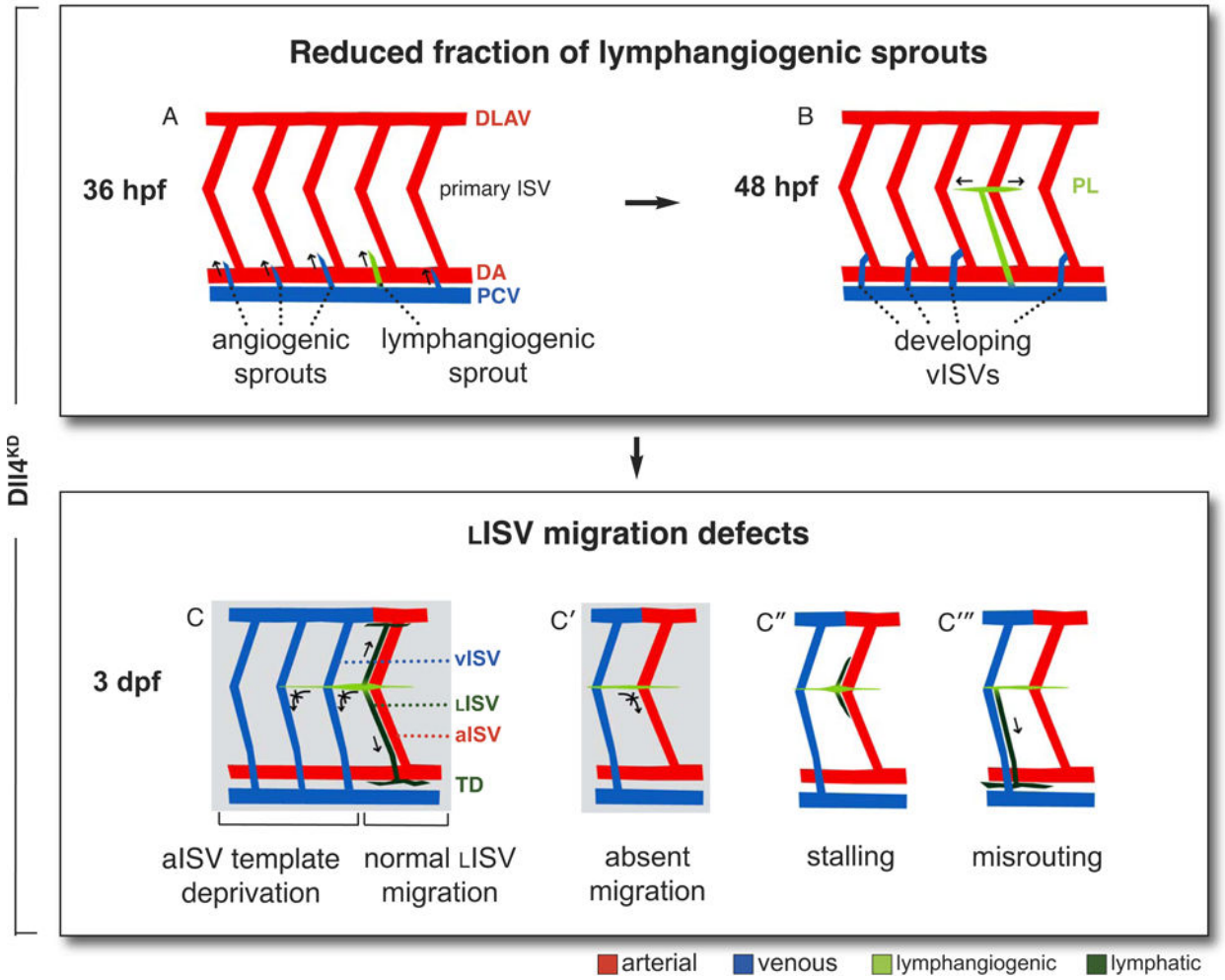

Figure 6. Schematic model of Notch in lymphatic development

Scheme, illustrating the different lymphatic defects in Dll4^{KD} embryos (normal lymphatic development, Supplemental Figure I). Permanent lymphatic structures (LISV, TD) are dark green; transient lymphangiogenic structures (lymphangiogenic secondary sprouts; parachordal lymphangioblasts) are light green. **A,B**, REDUCED FRACTION OF LYMPHANGIOGENIC SPROUTS, resulting in underdevelopment or absence of the PL string, with accompanying overrepresentation of angiogenic secondary sprouts. **C–C‴**, LISV MIGRATION DEFECTS: **C**, As a result of vISV overrepresentation, LISV-PLs are deprived of their normal aISV guidance template. **C′–C‴**, LISV formation is further impaired by additional navigation defects, most frequently because LISV-PLs cells bypass their turning point and never initiate ventral radial migration (C′), or occasionally make the turn but then stall (C”). More rarely, navigating LISV-PLs become misrouted along vISVs (C‴). The most frequent defects are boxed in grey.