RESEARCH ARTICLE

Figures and figure supplements

Apelin signaling drives vascular endothelial cells toward a pro-angiogenic state

Christian SM Helker *et al*

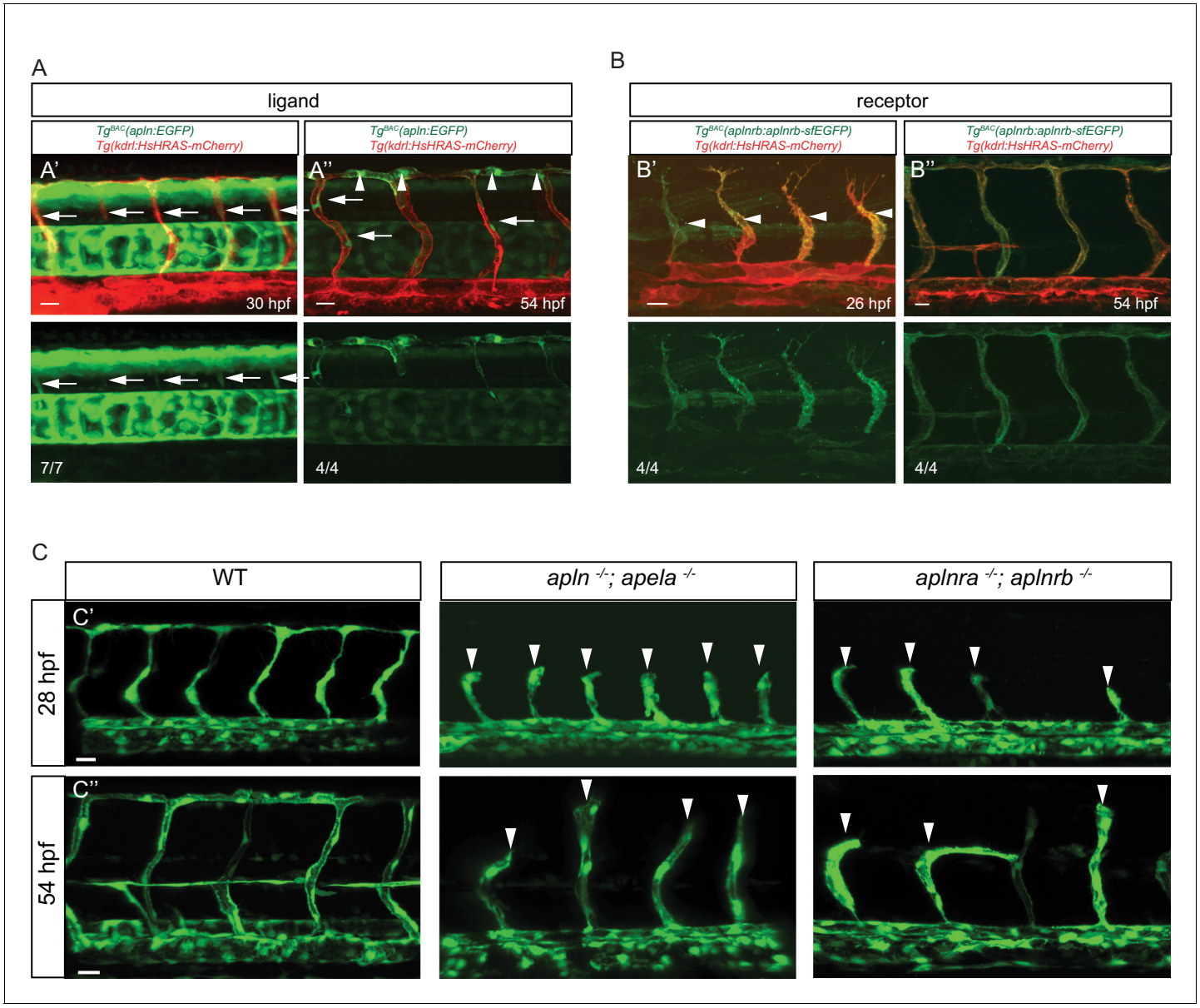

Figure 1. Apelin signaling promotes endothelial sprouting. Visualization of *apelin* and *apelin receptor b* expression using transgenic reporter lines. Confocal projection images of the trunk region of zebrafish embryos. (**A**) *Tg*^{BAC}*(apln:*EGFP) expression is detectable in growing ISVs at 30 and 54 hpf. Arrowheads point to strong *apln* expression in tip cells, while arrows point to weak *apln* expression in stalk cells. (**B**) *Tg*^{BAC}*(aplnrb:*aplnrb-EGFP) expression is detectable in sprouting ECs (arrowheads) at 26 hpf and is clearly present in the ISVs and DLAV at 54 hpf. (**C**) Inactivation of Apelin ligand and receptor genes impairs angiogenesis. Confocal projection images of the blood vasculature in the trunk region of *Tg(fli1a:EGFP)* embryos. *apln* $^{-/-}$; *apela* $^{-/-}$ as well as *aplnra* $^{-/-}$; *aplnrb* $^{-/-}$ embryos exhibit a reduction in vascular sprouting at 28 and 54 hpf. Arrowheads point to stalled ISVs. Scale bars: A', 10 µm; A'', B', C', C'', 20 µm; B'', 15 µm.

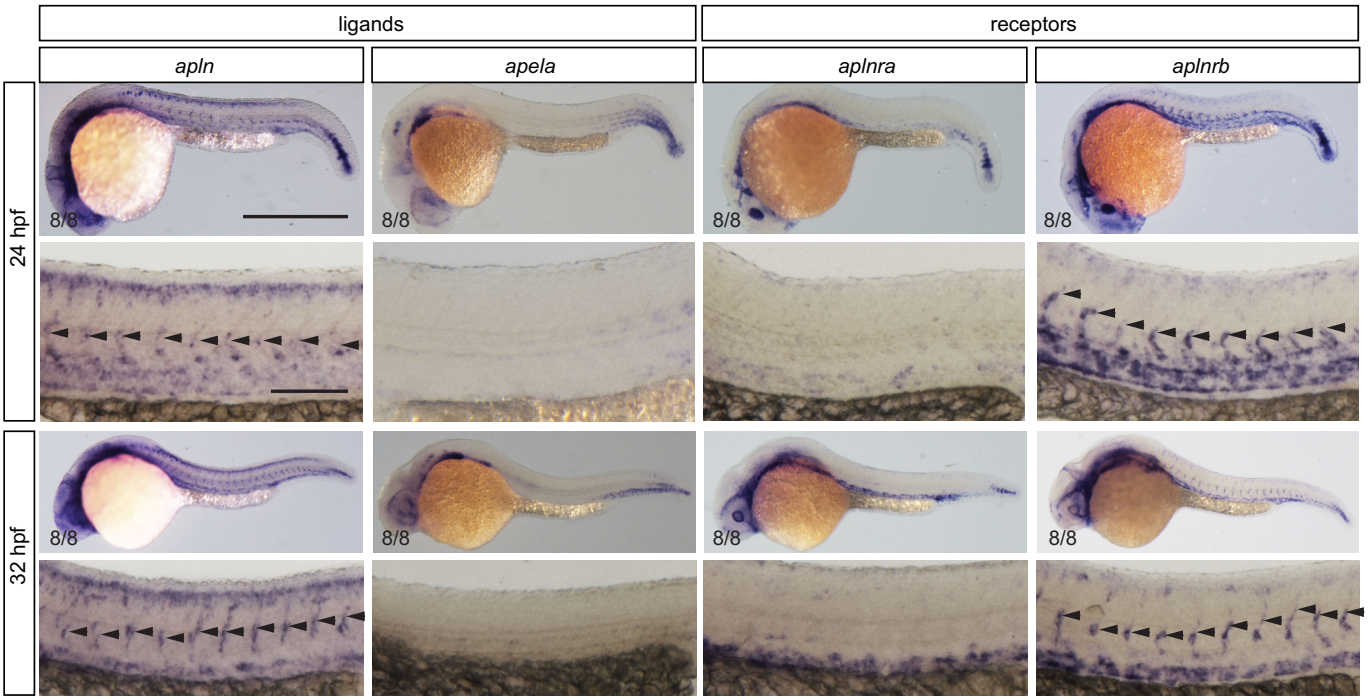

Figure 1—figure supplement 1. Expression of apelin ligand and receptor genes by in situ hybridization. Expression of apelin ligand and receptor genes at 24 and 32 hpf. *apln* is expressed in ISVs (arrowheads). *apela* is not detected in blood vessels. *aplnra* is expressed in the posterior cardinal vein. *aplnrb* is expressed in the posterior cardinal vein and ISVs (arrowheads). Scale bars: 500 μm; insets, 100 μm.

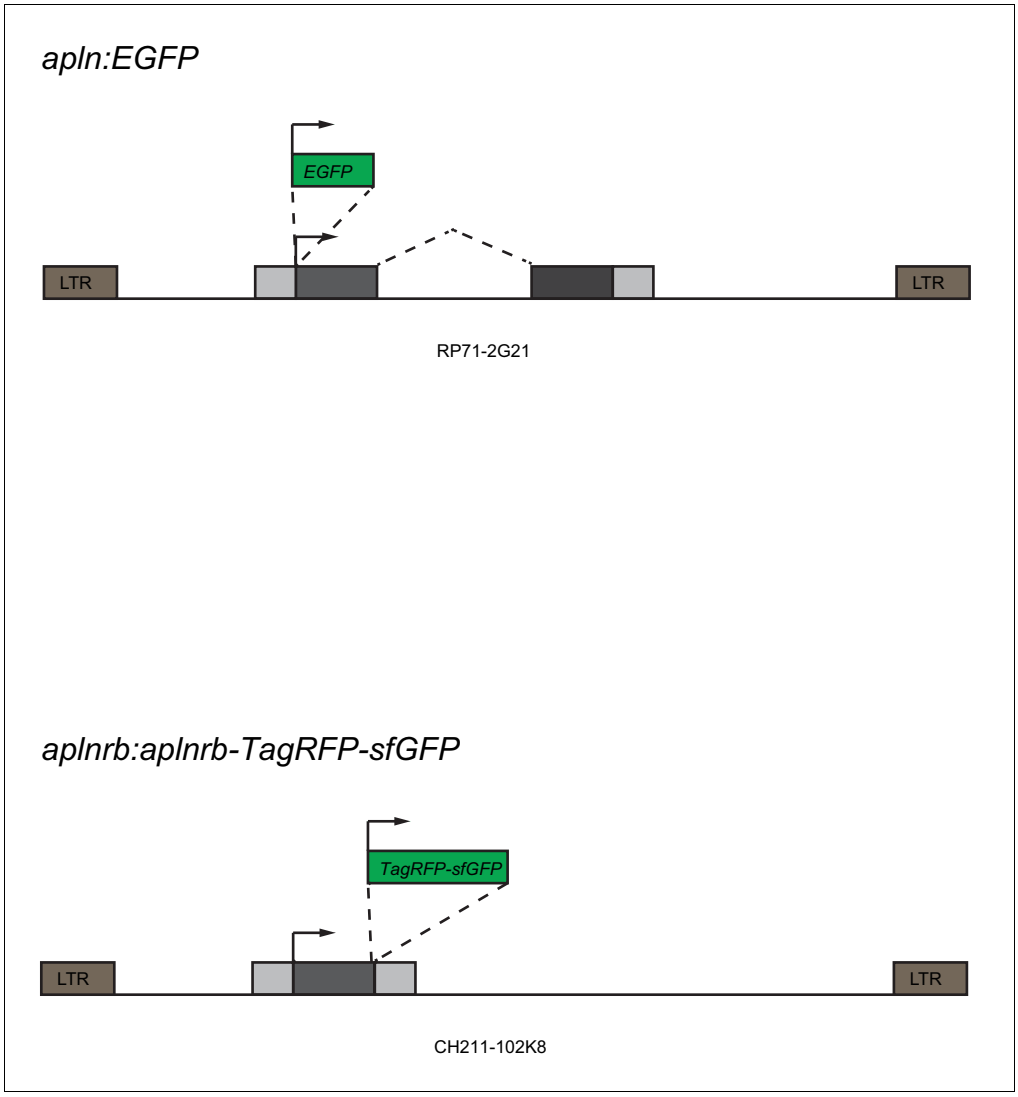

Figure 1—figure supplement 2. Generation of the *Tg*^{BAC}*(apln:EGFP) and Tg(aplnrb:aplnrb-TagRFP-sfGFP)* reporter lines. BAC engineering was used to generate the *apln:EGFP* and *aplnrb:aplnrb-TagRFP-sfGFP constructs*. The clone RP71-2G21 was engineered to replace the ATG of *apln* with an EGFP cassette. The clone CH211-102K8 was engineered to replace the stop codon of *aplnrb* with a TagRFP-sfGFP cassette. To enhance the frequency of insertion into the genome, *tol2* arms were added to the backbone of the BAC.

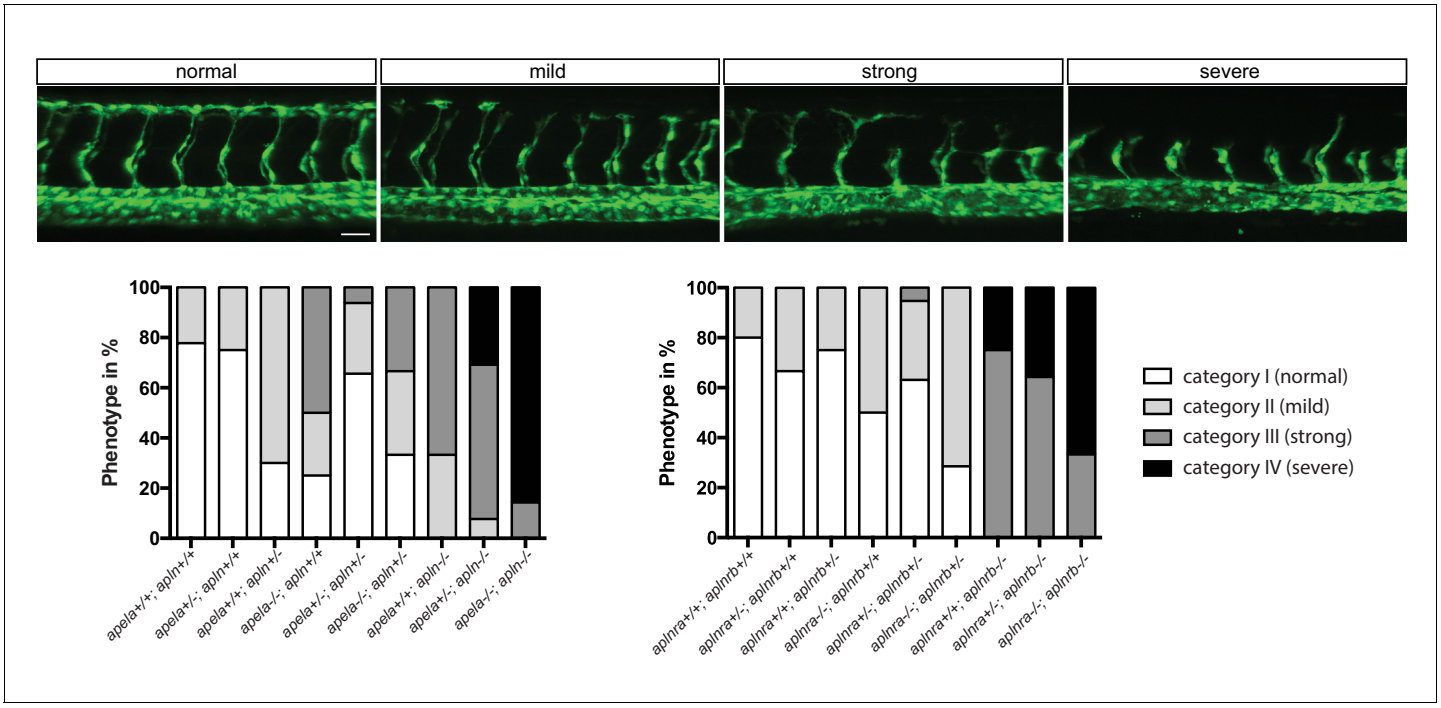

Figure 1—figure supplement 3. Quantification of angiogenic defects. Confocal projection images of the trunk region of *Tg(fli1a:EGFP)* embryos at 30 hpf. Analysis of the offspring of *apln* $^{+/-}$; *apela*$^{+/-}$ (n = 85) and *aplnra* $^{+/-}$; *aplnrb* $^{+/-}$ (n = 71) fish resulted in different phenotypic categories according to the extent of ISV development. Genotyping of single embryos revealed an additive effect for *apln* and *apela* ligands and *aplnra* and *aplnrb* receptors. Mild phenotypes correlated with loss of single ligand or receptor genes, while deficiency in *apln* resulted in stronger phenotypes than deficiency in *apela*. *aplnrb* deficiency resulted in stronger phenotypes than deficiency in *aplnra*. Deficiencies in both ligands or both receptors resulted in more severe phenotypes. Scale bar: 40 µm.

Figure 2. Apelin signaling regulates endothelial filopodia formation and endothelial cell numbers. (**A**) Still images from confocal time-lapse movies of vascular development in wild-type and *aplnra* $^{+/-}$; *aplnrb* $^{-/-}$ embryos. During sprouting, wild-type tip cells send out filopodia (arrowheads). *aplnra* $^{+/-}$;

Figure 2 continued on next page

Figure 2 continued

aplnrb $^{-/-}$ embryos exhibit smaller sprouts and fail to form filopodia. (**B**) Confocal images of the blood vasculature in 24 hpf *Tg(kdrl:HsHRAS-EGFP)* embryos injected with Ctr MO and *aplnrb* MO. *aplnrb* morphant embryos exhibit smaller sprouts and fail to form filopodia (arrowheads). (**C**) *aplnrb* morphant embryos exhibit a reduction in the number of endothelial filopodia (Ctr MO, n = 10; *aplnrb* MO, n = 15). (**D**) Confocal images of the blood vasculature of 30 and 52 hpf *Tg(fli1a:nEGFP)* wild-type and *aplnra* $^{+/-}$; *aplnrb* $^{-/-}$ embryos showing EC cell nuclei. (**E**) *aplnra* $^{+/-}$; *aplnrb* $^{-/-}$ embryos exhibit reduced EC numbers in the ISVs (30 hpf: *aplnr* +/+, n = 3; *aplnr* +/-, n = 10; *aplnr* -/-, n = 8; 52 hpf: *aplnr* +/+, n = 5; *aplnr* +/-, n = 10; *aplnr* -/-, n = 9). n.s. not significant (two-tailed t-test). Scale bars: A, D, 20 μm; B, 40 μm; B, inset 10 μm.

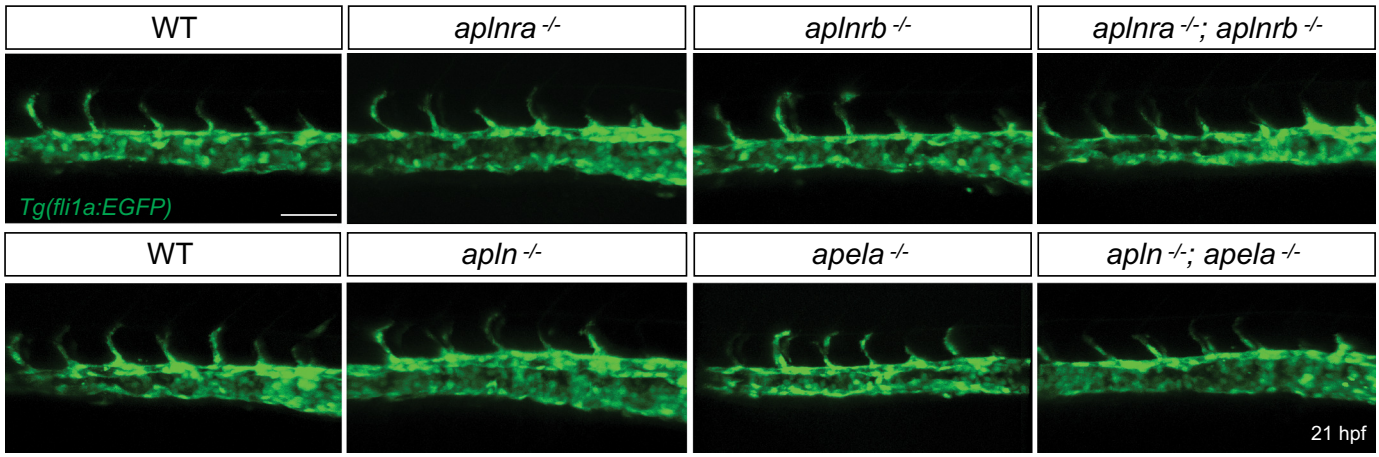

Figure 2—figure supplement 1. No obvious defects during initiation of EC sprouting in Apelin signaling-deficient embryos. Confocal projection images of the trunk region of *Tg(fli1a:EGFP)* embryos at 21 hpf. Sprouting of ECs from the DA does not appear to be altered in Apelin signaling-deficient embryos. Scale bar: 50 μm.

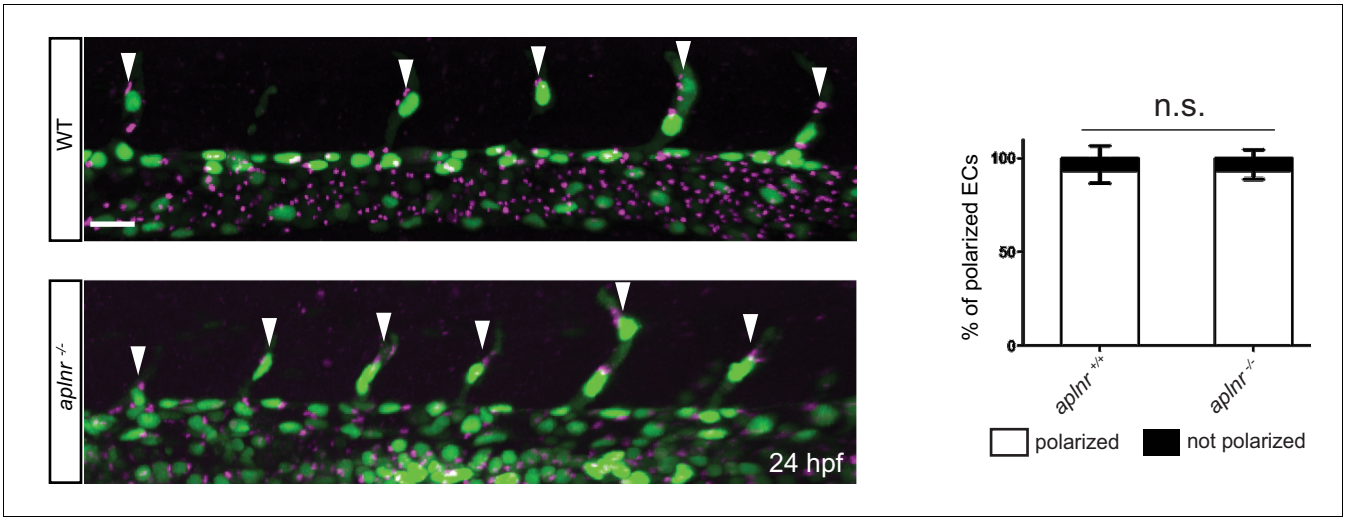

Figure 2—figure supplement 2. No obvious defects in EC polarity in Apelin deficient embryos. Confocal projection images of the trunk region of *Tg (kdrl:NLS-EGFP); Tg(fli1a:B4GALT1-mCherry)* embryos at 24 hpf. Apelin deficiency does not cause obvious defects in EC polarity (*aplnr +/+*, n = 6; *aplnr -/-*, n = 8). Arrowheads point to polarized tip cells in the ISVs. Scale bar: 20 µm.

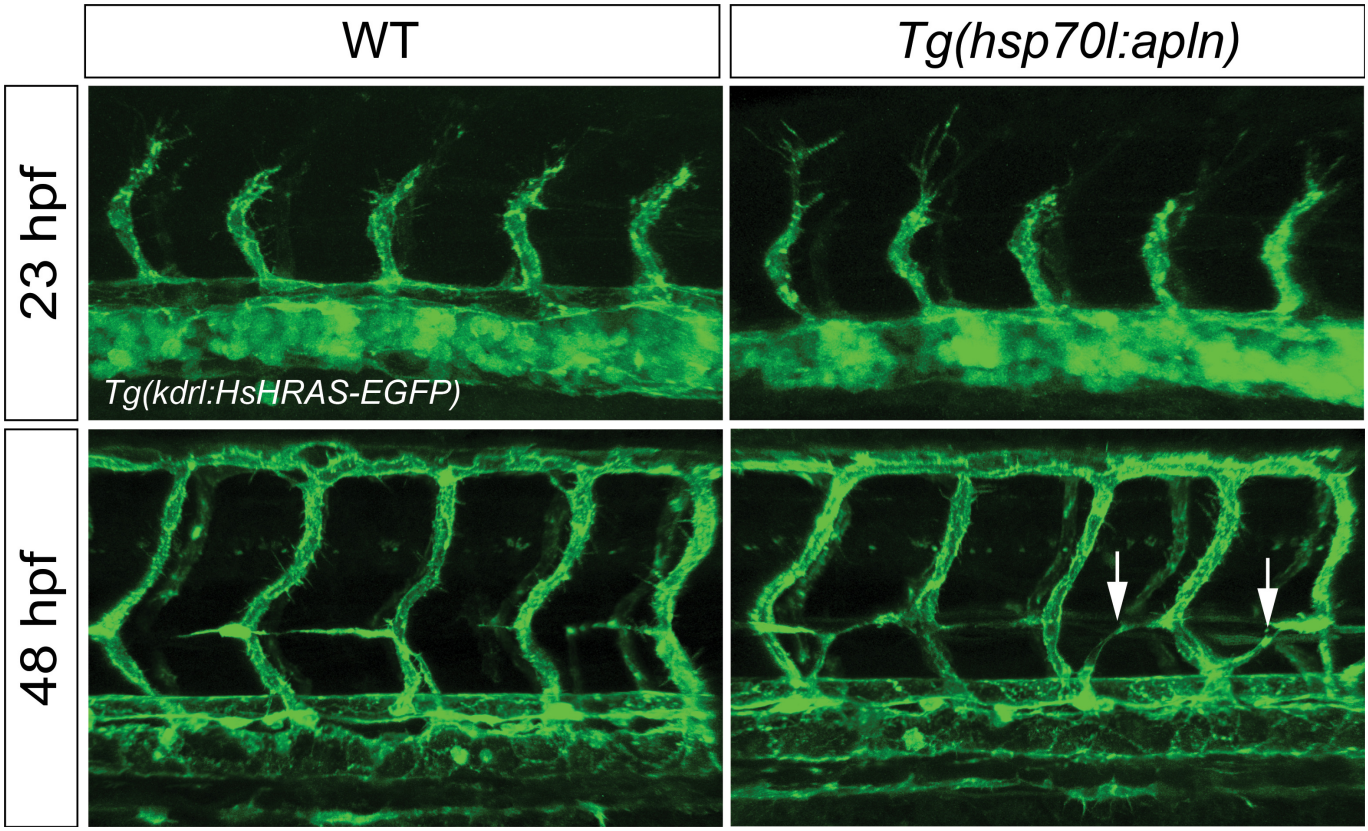

Figure 2—figure supplement 3. Overexpression of *apln* does not cause ectopic EC sprouting. Confocal projection images of the trunk region of *Tg (fli1a:LIFEACT-GFP)* embryos at 23 and 48 hpf. *apln* expression was induced by a single heatshock at 19.5 hpf for 1 hr at 39°C and analyzed at 23 hpf or heatshocked at 42 and 45 hpf for 1 hr at 39°C and analyzed at 48 hpf. Global overexpression of *apln* does not lead to ectopic vascular sprouting but mispatterning of the string of parachordal lymphangioblasts (arrows).

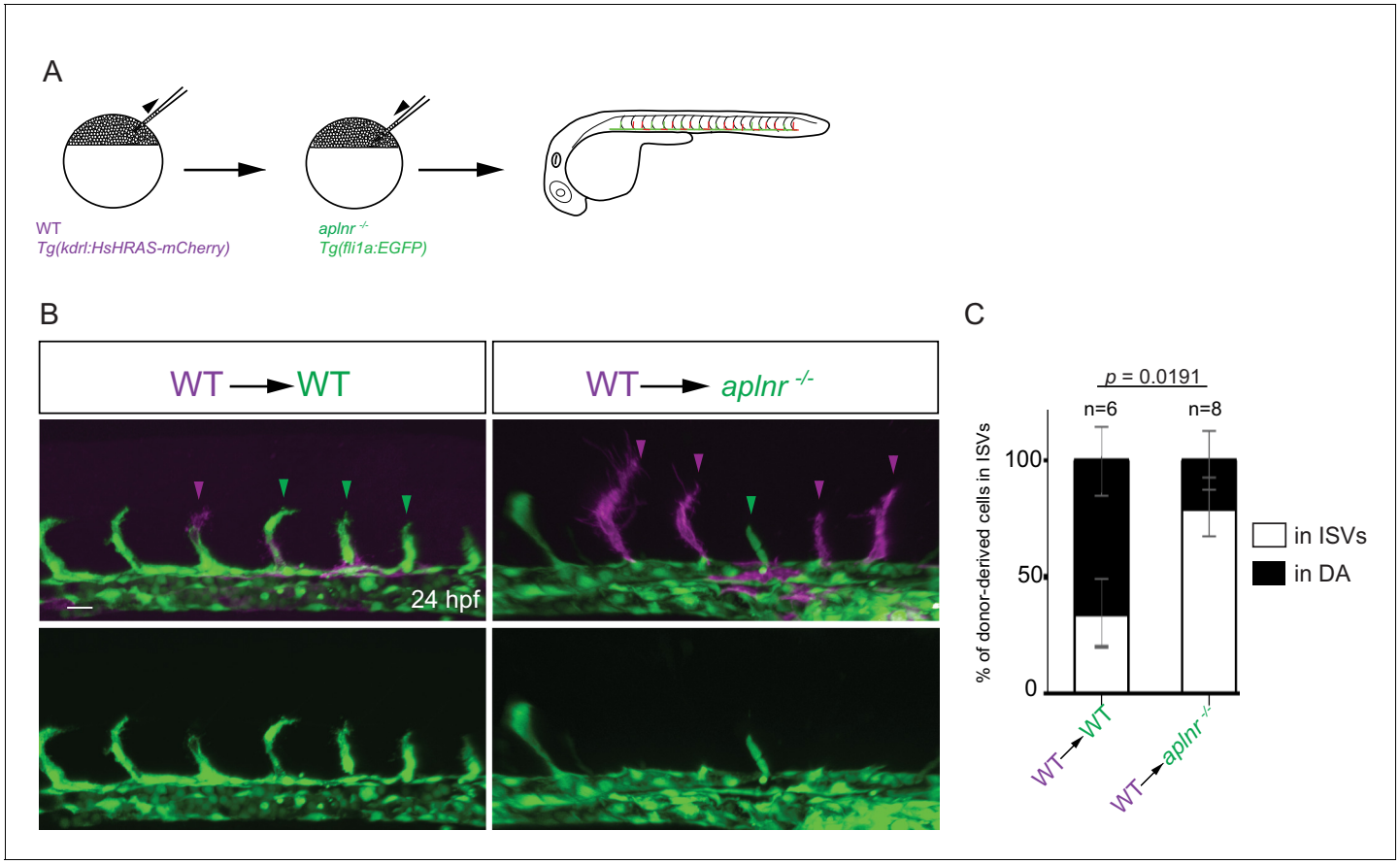

Figure 3. Apelin signaling promotes the sprouting behavior of ECs. (**A**) Experimental design: At the blastula stage, cells from *Tg(kdrl:HsHRAS-mCherry)* embryos were transplanted into host embryos obtained from *Tg(fli1a:EGFP) aplnra* $^{+/-}$; *aplnrb* $^{+/-}$ incrosses. At 24 hpf, the mosaic embryos were imaged and the donor-derived ECs scored for their position. (**B, C**) 34,5% of wild-type donor-derived ECs in wild-type hosts were found within the ISVs. 80% of wild-type donor-derived ECs in *aplnra* $^{+/-}$; *aplnrb* $^{-/-}$ hosts were found within the ISVs. Notably, wild-type ECs transplanted into *aplnr*- deficient embryos completely substituted for the lack of cells in the dorsal part of the vasculature at 54 hpf (***Figure 3—figure supplement 2***). Scale bars: B, 20 µm.

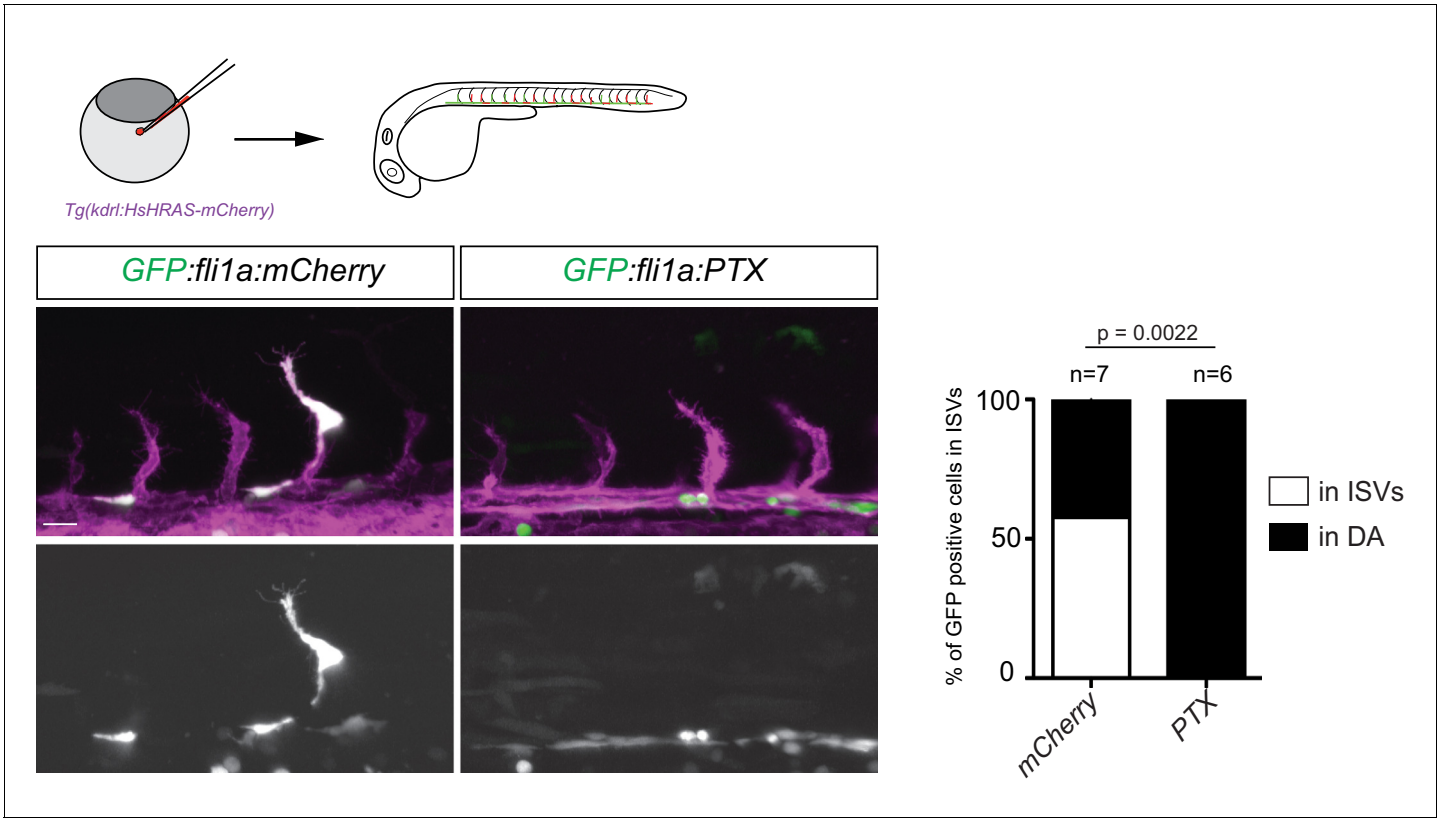

Figure 3—figure supplement 1. Overexpression of PTX phenocopies loss of Apelin signaling. Confocal projection images of the trunk region of *Tg (kdrl:HsHRAS-mCherry)* embryos at 24 hpf. PTX was overexpressed by injecting a bidirectional *fli1a* promoter driving PTX and GFP at the same time. ECs expressing PTX were detected only in the DA while mCherry expressing control ECs were also detected in the ISVs. Scale bar: 20 μm.

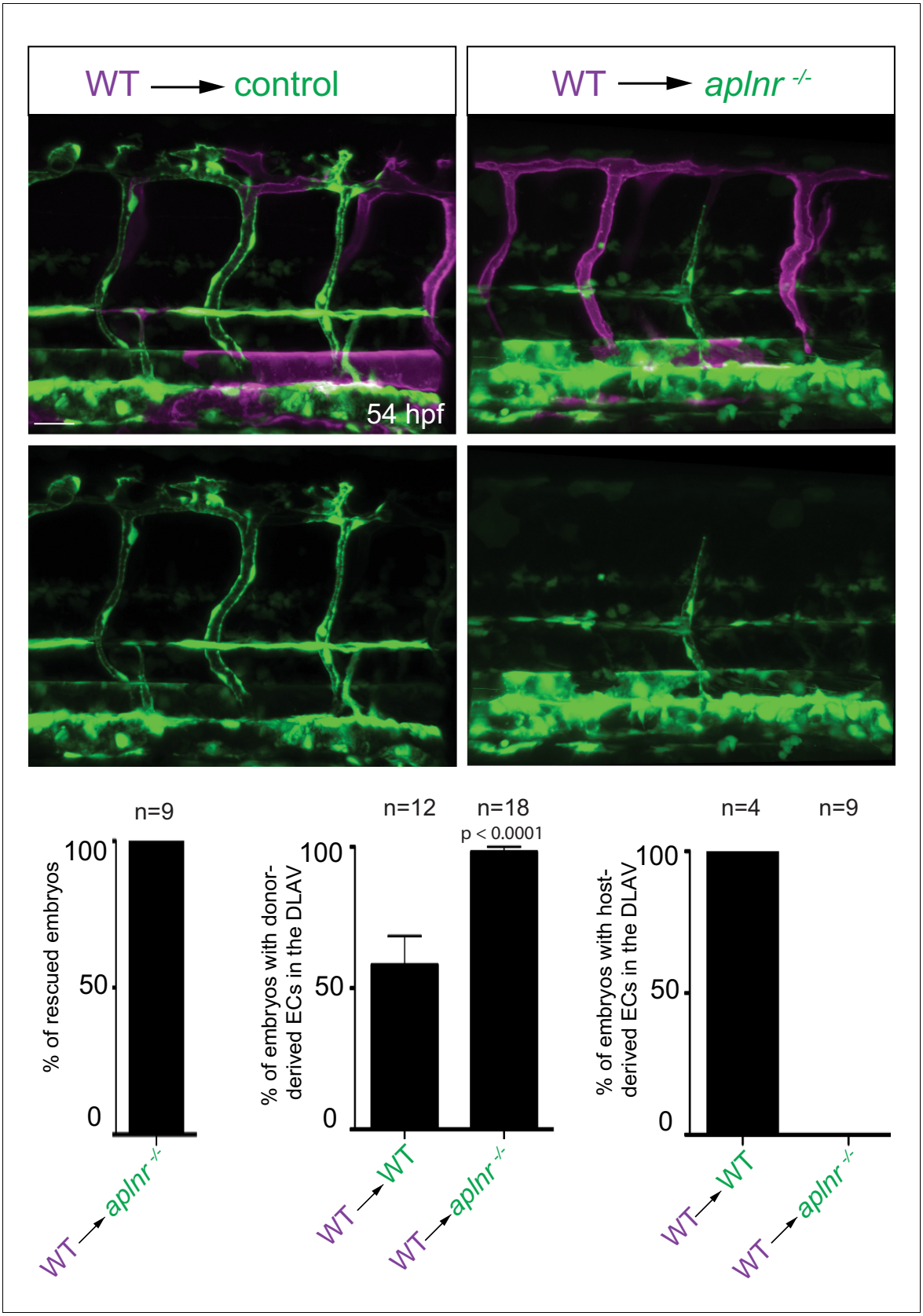

Figure 3—figure supplement 2. Apelin signaling functions cell-autonomously in ECs. Confocal projection images of the trunk region of *Tg(fli1a:EGFP)* embryos at 54 hpf. At the blastula stage, cells from *Tg(kdrl:HsHRAS-mCherry)* embryos were transplanted into host embryos obtained from *Tg(fli1a:*

Figure 3—figure supplement 2 continued on next page

Figure 3—figure supplement 2 continued

EGFP) aplnra $^{+/-}$*; aplnrb* $^{+/-}$ incrosses. At 54 hpf, the mosaic embryos were imaged and the donor-derived ECs scored for their position. Notably, wild-type ECs transplanted into *aplnr* deficient embryos completely substituted for the lack of cells in the dorsal part of the vasculature at 54 hpf (***Figure 3—figure supplement 1***). Scale bar: 30 µm.

Figure 4. Apelin signaling functions downstream of Notch signaling in endothelial cells. (**A - D**) Confocal projection images of the blood vasculature in the trunk region of *Tg(flt1:tdTomato)* (**A**) and *Tg(fli1a:EGFP)* (B–D) animals at 54 (B–D) and 72 (**A**) hpf. (**A**) Injection of a *dll4* morpholino leads to an increase in *Tg*^{BAC}*(apln*:EGFP) expression. (**B**) Loss of Apelin function can block excessive endothelial sprouting in *dll4* morphants. (**C, E**) Angiogenic response in *aplnrb* $^{-/-}$, *plxnd1* $^{-/-}$, and *aplnrb* $^{-/-}$*; plxnd1* $^{-/-}$ embryos (arrowheads) (n = 95). (**D, F**) Angiogenic response to *bmp2b* overexpression in *aplnr*

Figure 4 continued on next page

Figure 4 continued

$^{+/-}$ and *aplnr* $^{-/-}$ embryos (arrowheads). (**E**) Genotype of embryos for *aplnrb* after sorting them according to phenotype. (**G**) RT-qPCR analysis of *APELIN* mRNA levels in HUVECs treated with DAPT for 24 hr. Blocking Notch signaling with DAPT induces *APELIN* expression. (**H**) RT-qPCR analysis of *APELIN* mRNA levels in HUVECs cultured on DLL4 to activate Notch signaling. Activating Notch signaling represses *APELIN* expression. Arrowheads point to ectopic sprouts. n.s. not significant (two-tailed t-test). Ct values can be found in ***Figure 4—source data 1***. Scale bars: A, C, 20 µm; B, 15 µm; D, 30 µm.

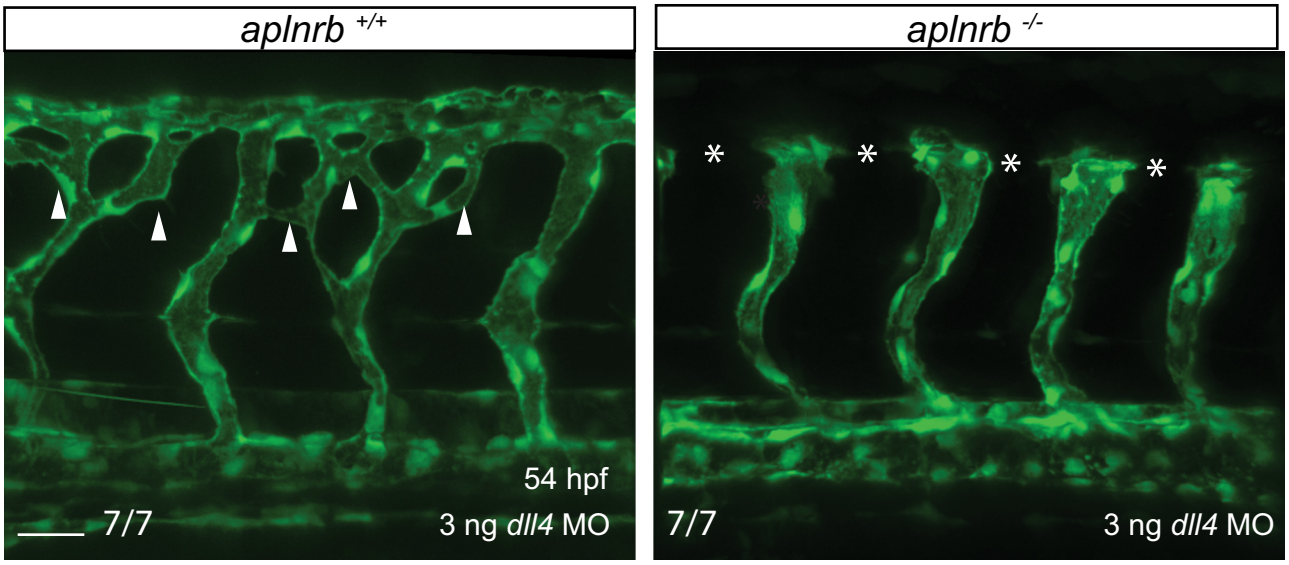

Figure 4—figure supplement 1. Apelin signaling functions downstream of Notch signaling in ECs. Confocal projection images of the trunk region of *Tg(fli1a:EGFP)* embryos at 54 hpf. Loss of *aplnrb* function can rescue excessive endothelial sprouting in *dll4* morphants. Arrowheads point to ectopic sprouts and asterisks indicate missing DLAV fragments. Scale bar: 30 µm.

Figure 5. Apelin signaling positively regulates EC metabolism. (**A - B**) Extracellular acidification aate (ECAR) (**A**) and oxygen consumption rates (OCR) (**B**) in siScr and *APLN+APLNR* siRNA-treated HUVECs under basal conditions and in response to oligomycin, fluoro-carbonyl cyanide phenylhydrazone

Figure 5 continued on next page

Figure 5 continued

(FCCP) and antimycin A (AA)/rotenone. (**A**) Reduced basal and maximal glycolytic activity in *APLN+APLNR* siRNA-treated compared to siScr-treated HUVECs. (**B**) No significant difference in oxygen consumption in *APLN+APLNR* siRNA-treated compared to siScr-treated HUVECs. (**C**) Reduced c-MYC levels in *APLN+APLNR* siRNA-treated compared to siScr-treated HUVECs. (**D**) RT-qPCR analysis of *PFKFB3* mRNA levels in *APLN+APLNR* siRNA-treated compared to siScr-treated HUVECs. (**E**) Confocal projection images of the blood vasculature in the trunk region of a 54 hpf *Tg(kdrl:HsHRAS-mCherry)* animal injected with an *EGFP:fli1a:pfkfb3* plasmid. Arrowheads point to formed DLAV fragments while asterisks indicate missing DLAV fragments. (**F**) Quantification of the rescue of the DLAV fragment by mosaic *pfkfb3* overexpression in *aplnrb* $^{-/-}$ embryos. n.s. not significant (two-tailed t-test). Scale bar: E, 50 μm.

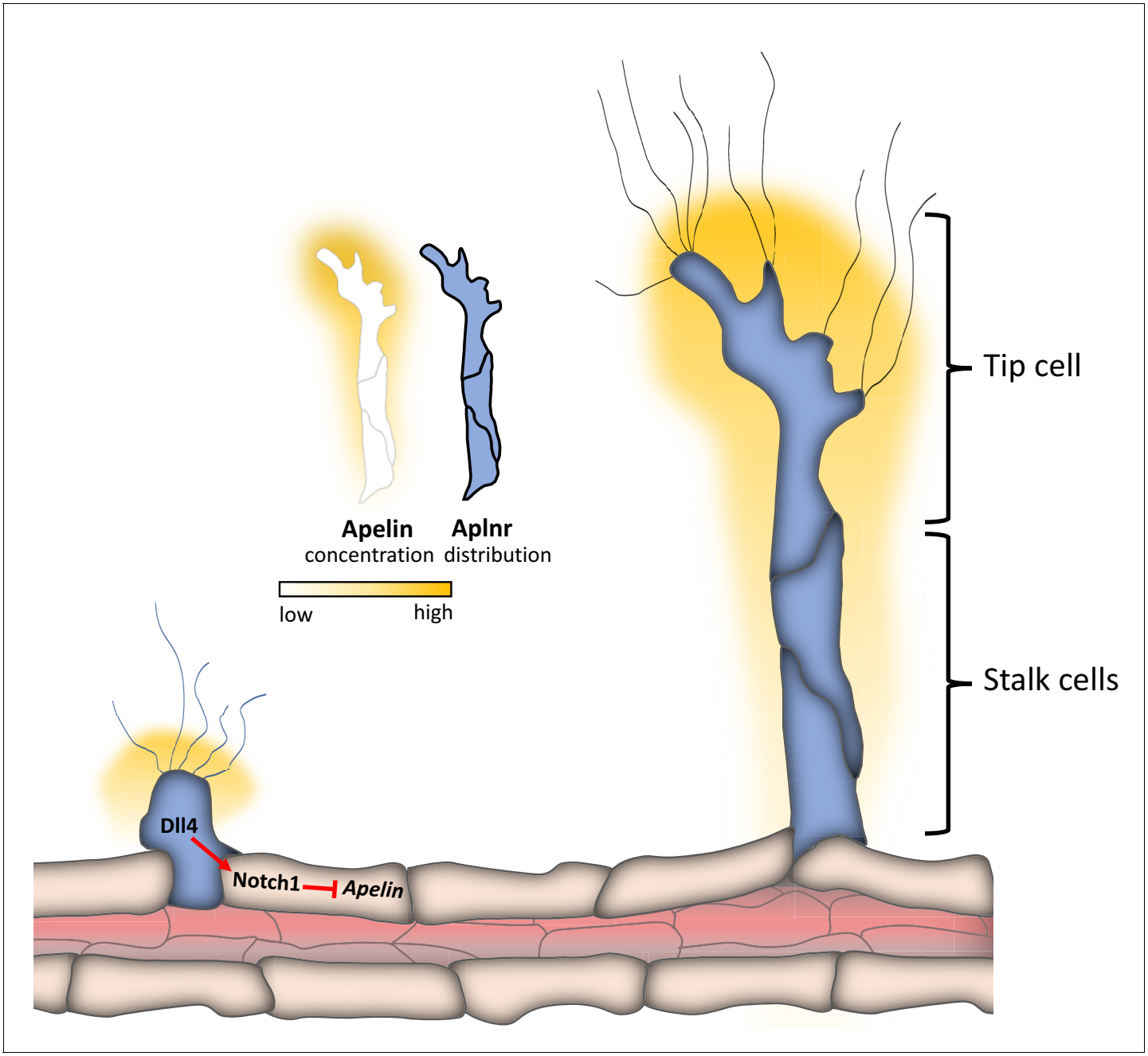

Figure 5—figure supplement 1. Schematic model. Schematic model of the developing blood vessels in the zebrafish trunk depicting the expression of Apelin and Aplnr and the relationship between Notch signaling and *Apln* expression during ISV sprouting.