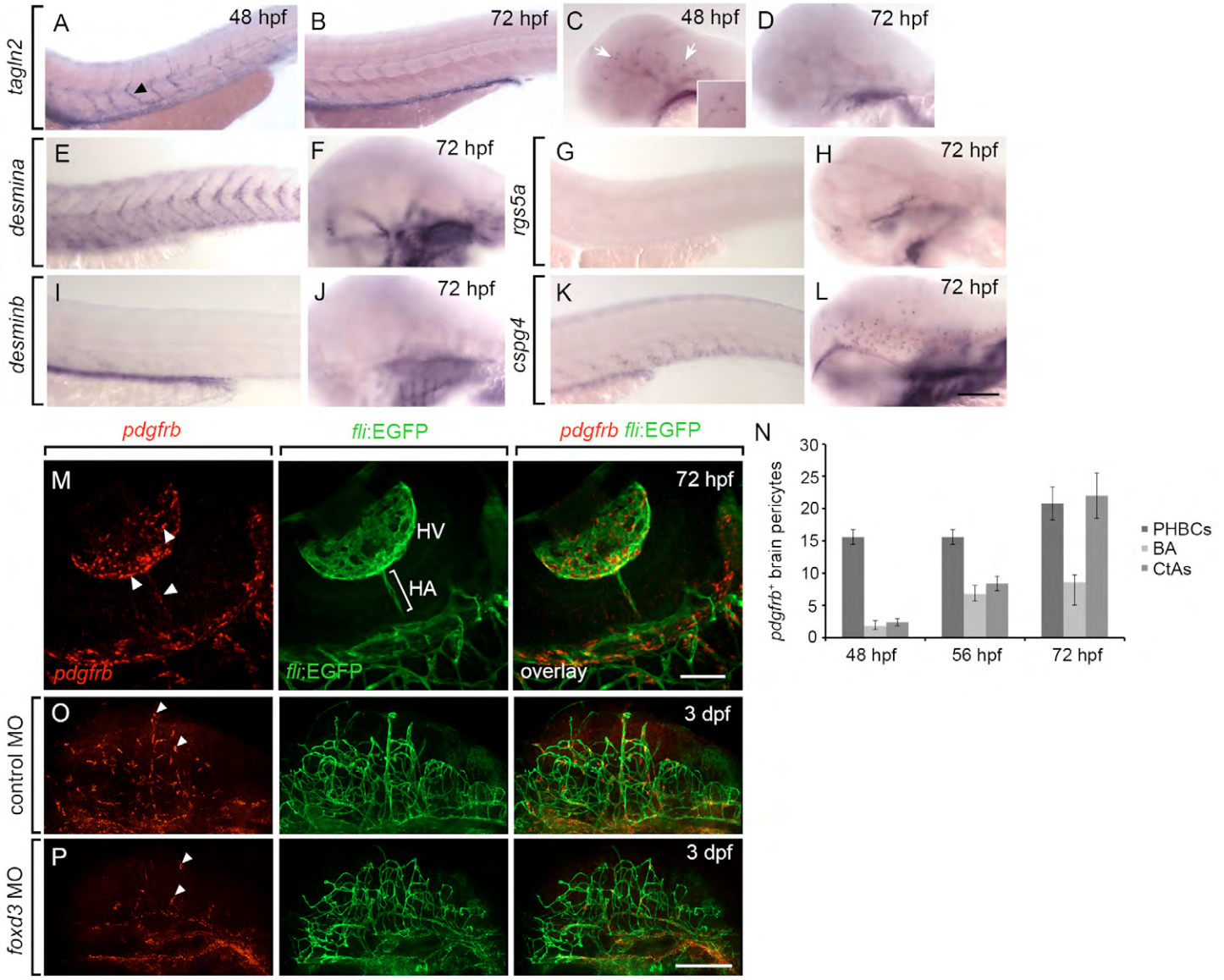

Fig. S1. Expression patterns of *tagln2* and *pdgfrb* in zebrafish embryos and larvae. (**A**-**C**) ISH for *tagln2*. (**A**,**B**) *tagln2* expression in trunk vessels at 48 hpf and 72 hpf, respectively. Arrowhead, *tagln2*$^+$ putative mural cell in ISV. (**C**,**D**) *tagln2* expression in brain vasculature at 48 hpf and 72 hpf. Arrow, *tagln2*$^+$ putative brain mural cell. Inset, high magnification view of *tagln2*$^+$ cells in the brain. (**E**-**L**) *desmina*, *desminb*, *rgs5a*, and *cspg4* expression in trunk and brain vasculature at 72 hpf. (**E**,**G**,**I**,**K**) trunk expression. (**F**,**H**,**J**,**L**) brain expression. (**M**) Fluorescent ISH showing *pdgfrb* expression in retinal pericytes at 72 hpf. Arrowheads, *pdgfrb*$^+$ retinal pericytes. (**N**) Quantification of *pdgfrb*$^+$ pericytes associated with different hindbrain vessels at various time points. (**O,P**) *foxd3* MO knock-down caused reduction in *pdgfrb*$^+$ brain pericyte number. (**O**) Control MO-injected 72 hpf larva. (**P**) *foxd3* MO-injected 72 hpf larva. Arrowheads, *pdgfrb*$^+$ brain pericytes. HA, hyaloid artery; HV, hyaloid vessels. Scale bars: (A-L) 0.5 mm, (M-P) 200 µm.

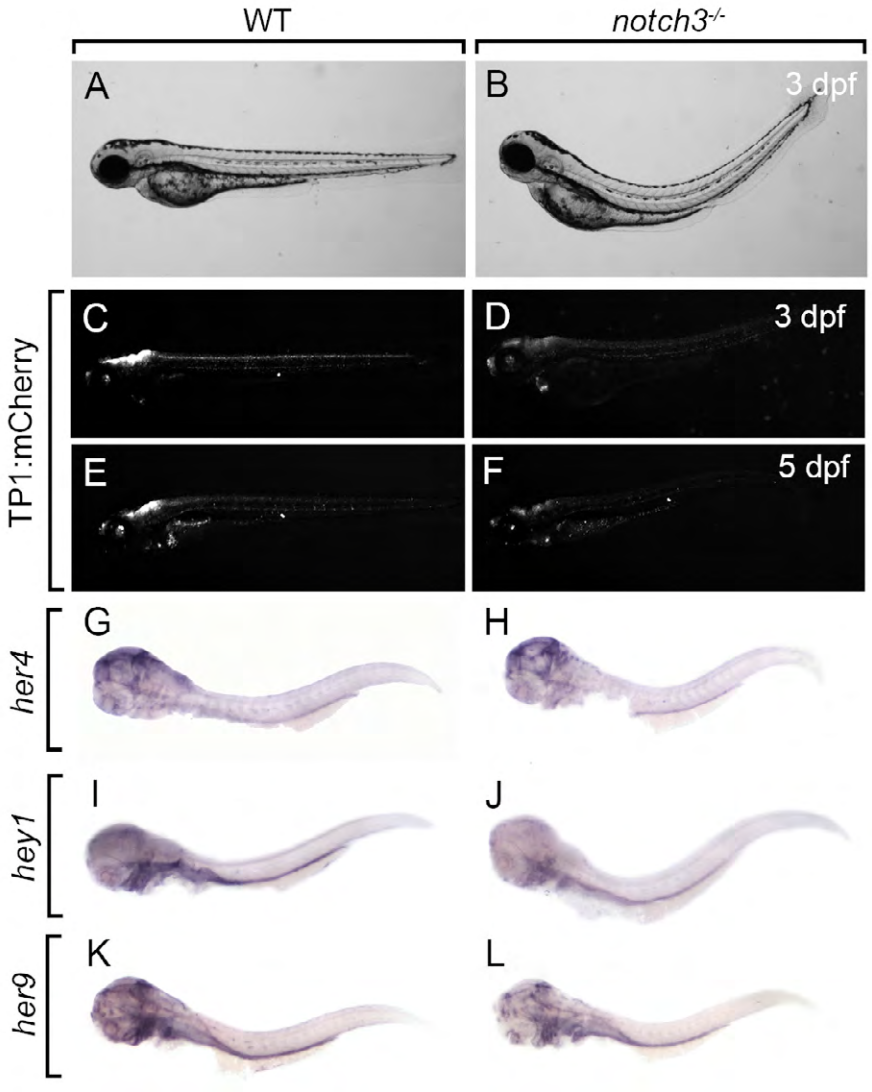

Fig. S2. *notch3*^{fh332} mutants have reduced Notch signaling activity. (**A**,**B**) Wild-type and *notch3*^{fh332} mutant larvae showing trunk curvature of the mutant at 3 dpf. (**C-F**) *notch3*^{fh332}*; Tg(Tp1bglob:hmgb1-mCherry)* (Tp1:mCherry) mutant larvae showed reduced mCherry expression compared to wild-type siblings at 3 dpf and 5 dpf. (**G,H**) Whole-mount ISH revealed similar *her4.1* expression in *notch3*^{fh332} mutant and wild-type sibling. (**I-L**) *notch3*^{fh332} mutants showed reduced *hey1* and *her9* expression compared to wild-type siblings.

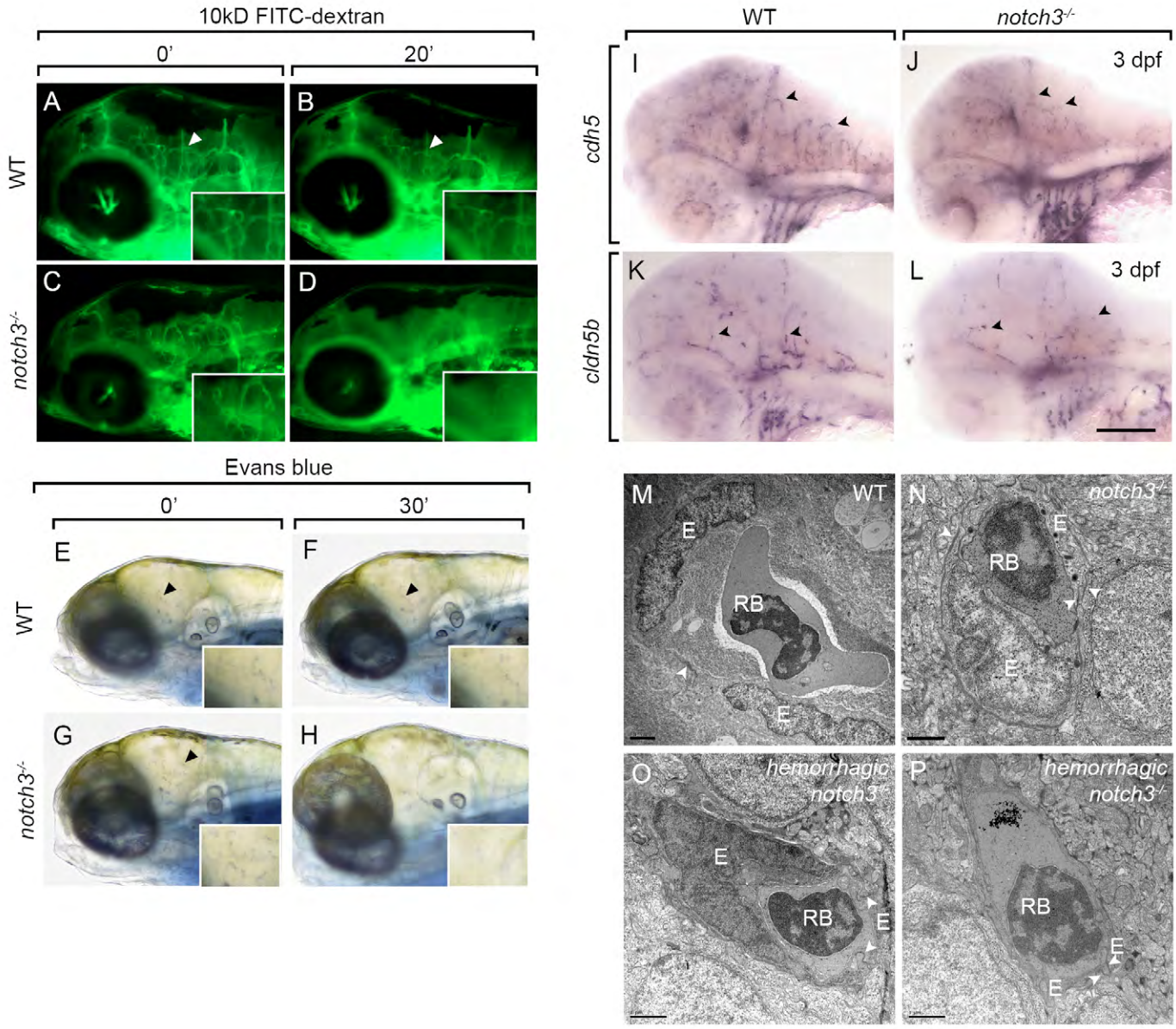

Fig. S3. Endothelial cell differentiation in *notch3*^{fh332} mutant larvae. (**A-H**) Representative results of BBB assay in 4 dpf wild-type and mutant larvae using 10KD FITC-dextran and Evans blue dye. Insets, higher magnification view of brain microvessels. (**A-D**) Mutant embryos failed to retain 10KD FITC-dextran in the brain microvessels 20 min after the injection. (**E-H**) Mutant embryos failed to retain Evans blue dye in the brain microvessels 30 min after the injection. (**I-L**) ISH showing *cdh5* and *cldn5b* expression in the brain of 3 dpf wild-type and *notch3*^{fh332} larvae. Arrowheads, endothelial expression of *cdh5* or *cldn5b*. (**M-P**) Electron microscopy images of brain microvessels in 4 dpf wild-type and *notch3*^{fh332} mutant larvae. RB, red blood cell. E, endothelial cell. Arrowheads, endothelial tight junctions. Scale bars: (**I-L**) 250 μm, (**M-P**) 1μm.

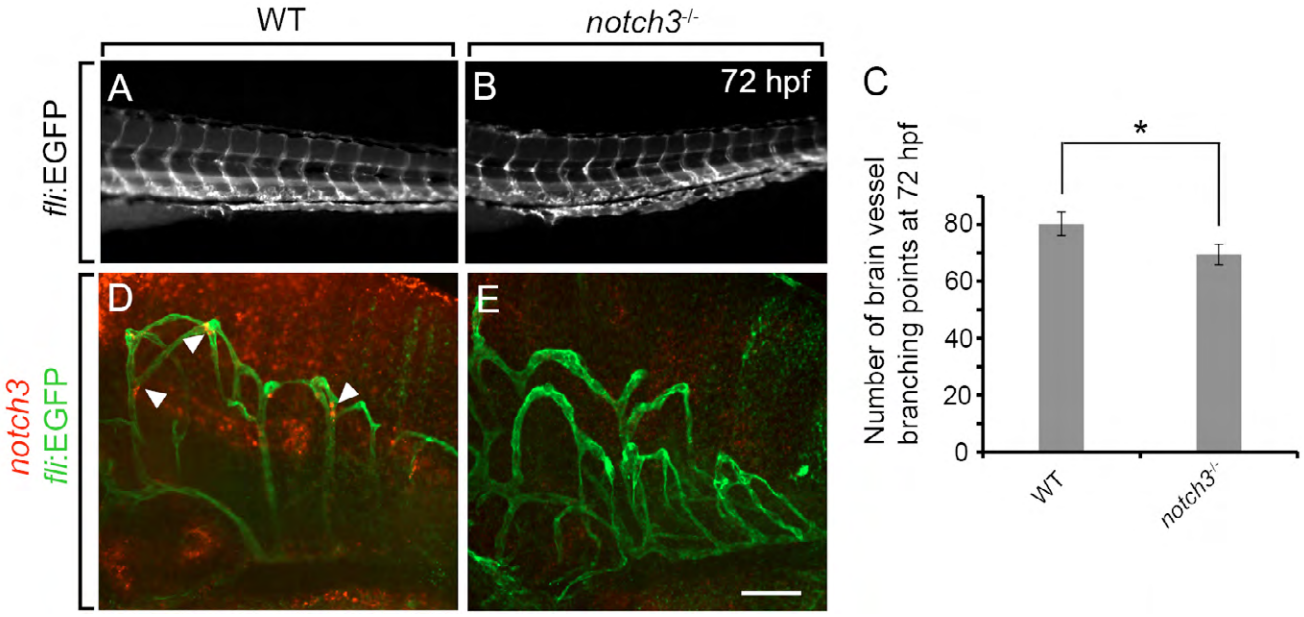

Fig. S4. Vascular morphogenesis and brain *notch3* expression in *notch3*^{fh332} mutants. (**A**,**B**) *fli1*:EGFP transgenic reporter expression showing trunk vessels in 72 hpf wild-type and *notch3*^{fh332} mutant larvae. (**C**) Quantification of vessel branch points in the brain of *notch3*^{fh332} mutant and wild-type larvae. (**D**,**E**) Fluorescent ISH showing *notch3* expression in the hindbrain. Pericyte *notch3* expression was largely depleted in the mutant. Arrowheads, *notch3*$^{+}$ pericytes. *P<0.01 by Student's 2-tailed t test. Scale bar, 200 µm.

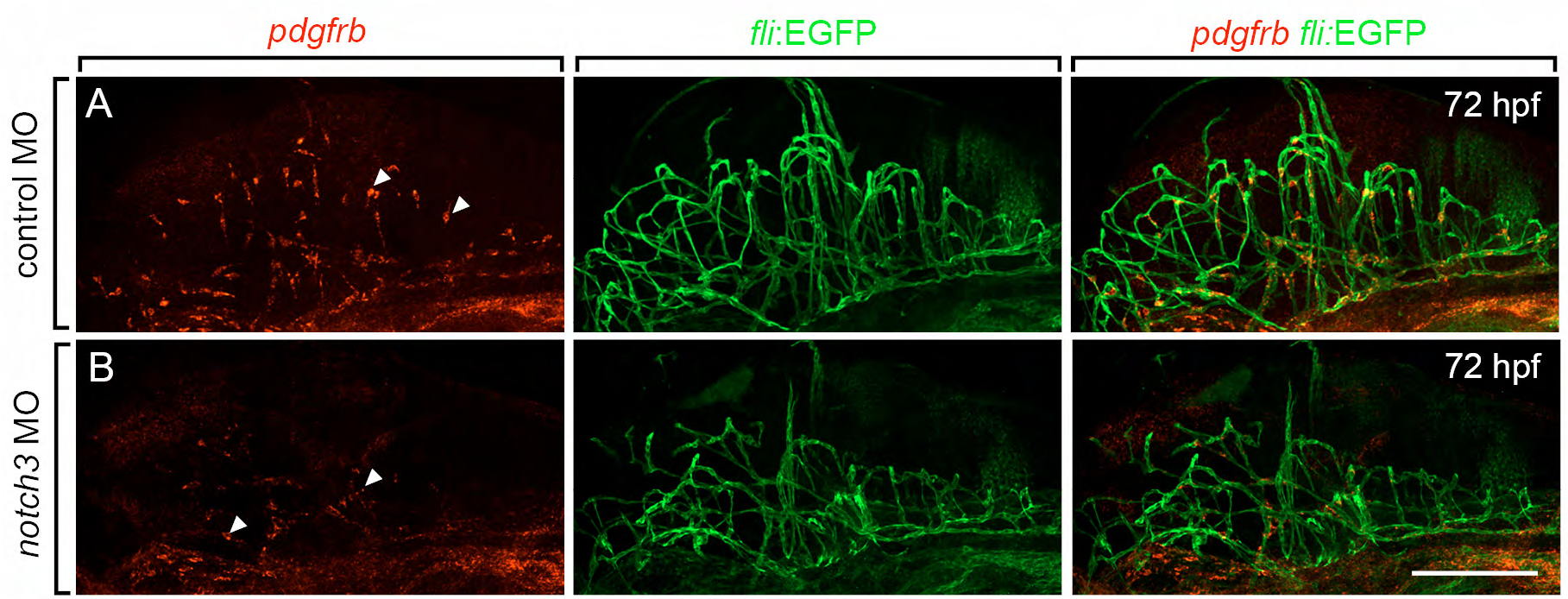

Fig. S5. *notch3* MO phenocopied the pericyte deficit phenotype in *notch3*^{fh332} mutant larvae. (**A**,**B**) Fluorescent ISH showing *pdgfrb*$^{+}$ brain pericytes in 72 hpf control and *notch3* MO-injected larvae. Arrowheads, *pdgfrb*$^{+}$ pericytes. Scale bar, 200 μm.

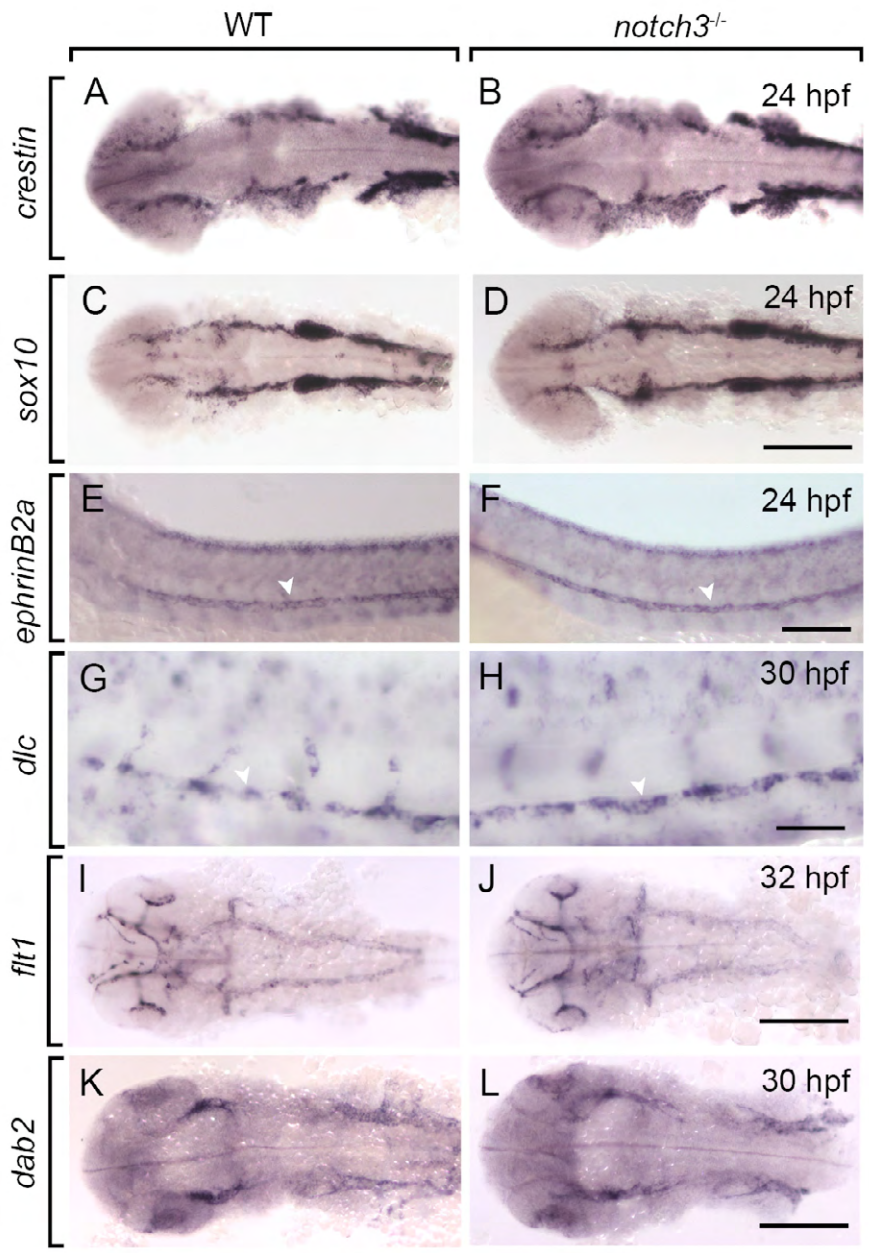

Fig. S6. Arterial-venous differentiation and neural crest development in *notch3*^{fh332} mutant larvae. (**A**-**D**) ISH showing *crestin* and *sox10* expression in 24 hpf wild-type and *notch3*^{fh332} mutant larvae. (**E**,**F**) ISH showing *efnb2a* expression in 24 hpf wild-type and *notch3*^{fh332} mutant larvae. (**G**,**H**) ISH showing *dlc* expression in 30 hpf wild-type and *notch3*^{fh332} mutant larvae. Arrowheads, DA. (**I**,**J**) ISH showing *flt1* expression in 32 hpf wild-type and *notch3*^{fh332} mutant larvae. (**K**,**L**) ISH showing *dab2* expression in 30 hpf wild-type and *notch3*^{fh332} mutant larvae. Scale bars: (**A**-**F**) 0.5 mm, (**G**,**H**) 200 µm. (I-L) 0.5mm.

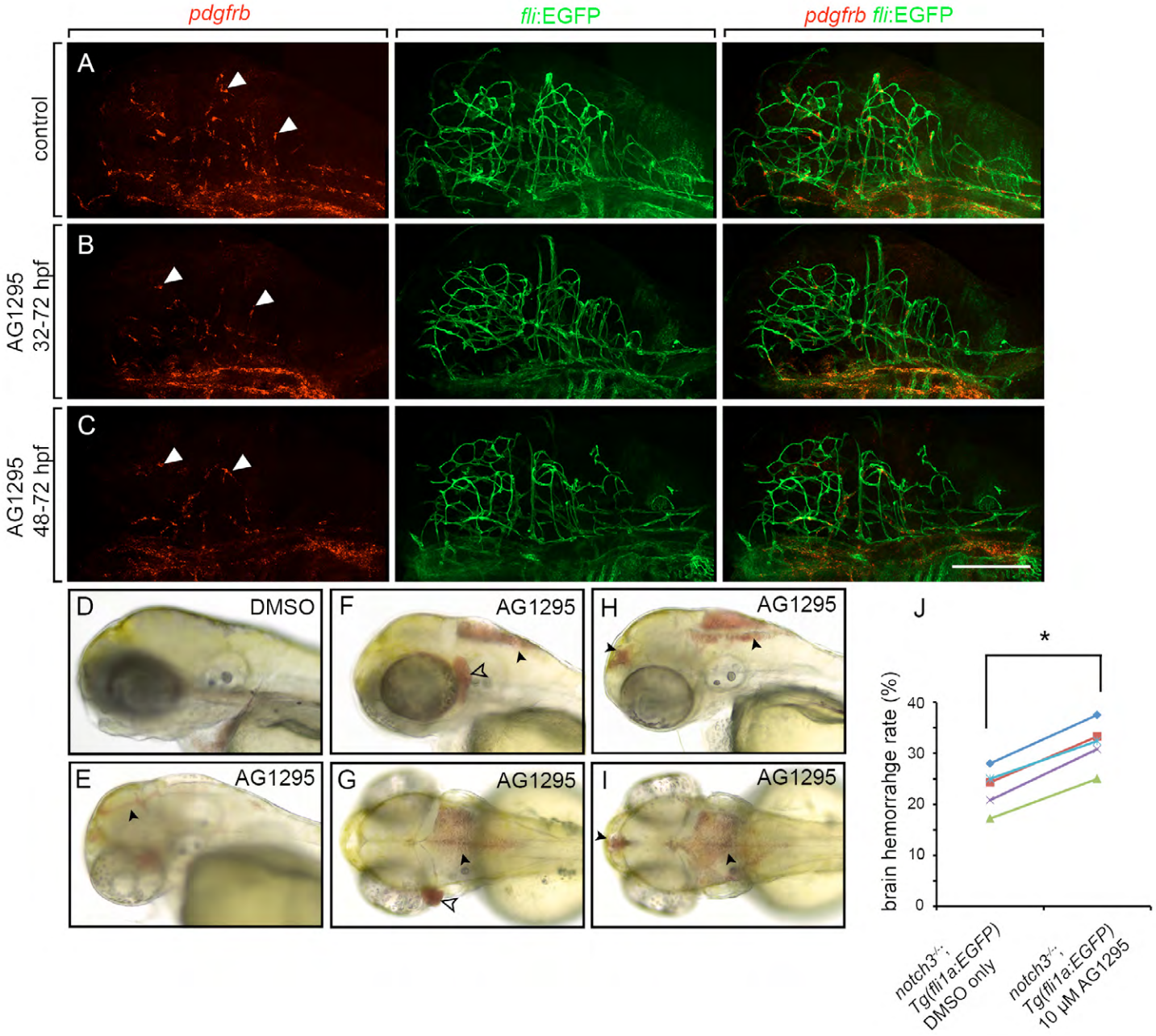

Fig. S7. Inhibition of Pdgfr-β activity produces a pericyte deficit and brain hemorrahge. (**A**) *pdgfrb*$^{+}$ brain pericytes in DMSO treated control larvae. (**B**,**C**) Larvae treated with 25 µM AG1295 from 32-72 hpf, and from 48-72 hpf, respectively, showed a reduction in *pdgfrb*$^{+}$ brain pericyte number. Arrowheads, *pdgfrb*$^{+}$ brain pericytes. Scale bar, 200 µm. (**A-C**) All images show confocal projections of whole brains, viewed from lateral. (**D-I**) Brain hemorrhage in AG1295 treated zebrafish larvae. (**D**) DMSO treated control at 3 dpf. (**F-I**) 25µm AG1295 treated larva at 3 dpf. (**E**,**F**,**H**) Lateral views. (**G**,**I**) Dorsal views of (**F**,**H**), respectively. (**J**) *notch3*^{fh332} homozygous mutants showed a more penetrant hemorrhage phenotype when treated with 10 µM AG1295 (n=5, *P<0.05, by paired t-test). Arrowheads, blood pooling at the brain ventricle; open arrowhead, blood pooling behind the eye.

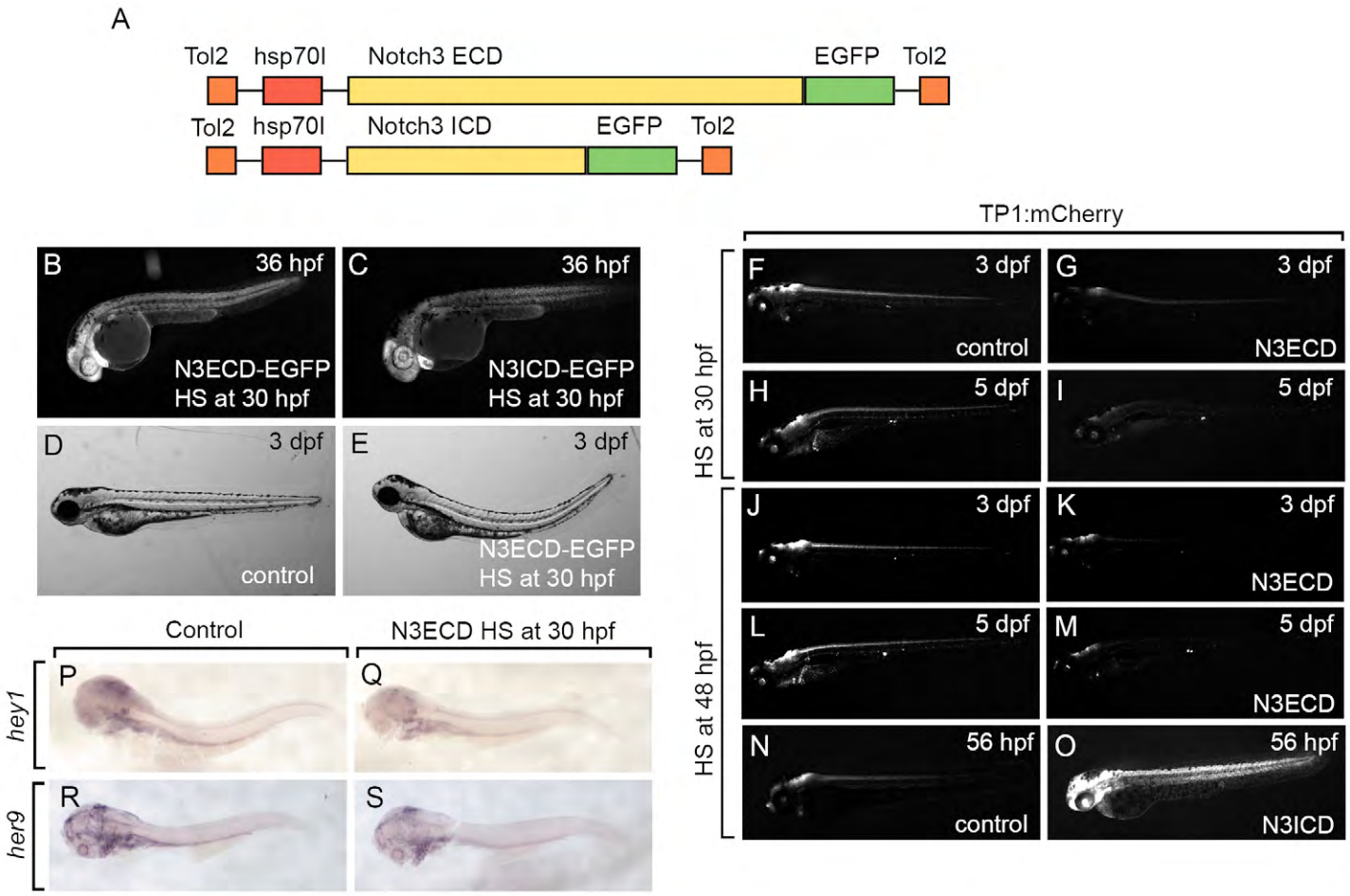

Fig. S8. Organization and transgene expression for *Tg(hsp70I:Notch3ECD-EGFP)* and *Tg(hsp70I:Notch3ICD-EGFP)* transgenic constructs. (**A**) Diagram depicting the organization of *Tg(hsp70I:Notch3ECD-EGFP)* and *Tg(hsp70I:Notch3ICD-EGFP)* transgenic constructs. (**B**,**C**) Heat shock of *Tg(hsp70I:Notch3ECD-EGFP)* embryos (**B**) and *Tg(hsp70I:Notch3CD-EGFP)* embryos (**C**) at 30 hpf induced robust EGFP expression. (**D**,**E**) Heat shock of *Tg(hsp70I:Notch3ECD-EGFP)* embryos at 30 hpf resulted in trunk curvature at 3 dpf. (**F-M**) Heat shock of *Tg(hsp70I:Notch3ECD-EGFP)*; *Tg(Tp1bglob:hmgb1-mCherry)* embryos at 48 hpf or 30 hpf caused significant reduction in mCherry intensity at 3 dpf and 5 dpf. (**N**,**O**) Heat shock of *Tg(hsp70I:Notch3ICD-EGFP)*; *Tg(Tp1bglob:hmgb1-mCherry)* embryos at 48 hpf resulted in significantly increased mCherry fluorescent intensity at 56 hpf. (**P-S**) *Tg*(*hsp70I:N3ECD-EGFP)* larvae heat-shocked at 30 hpf showed reduced *heyl* and *her9* expression compared to control group.

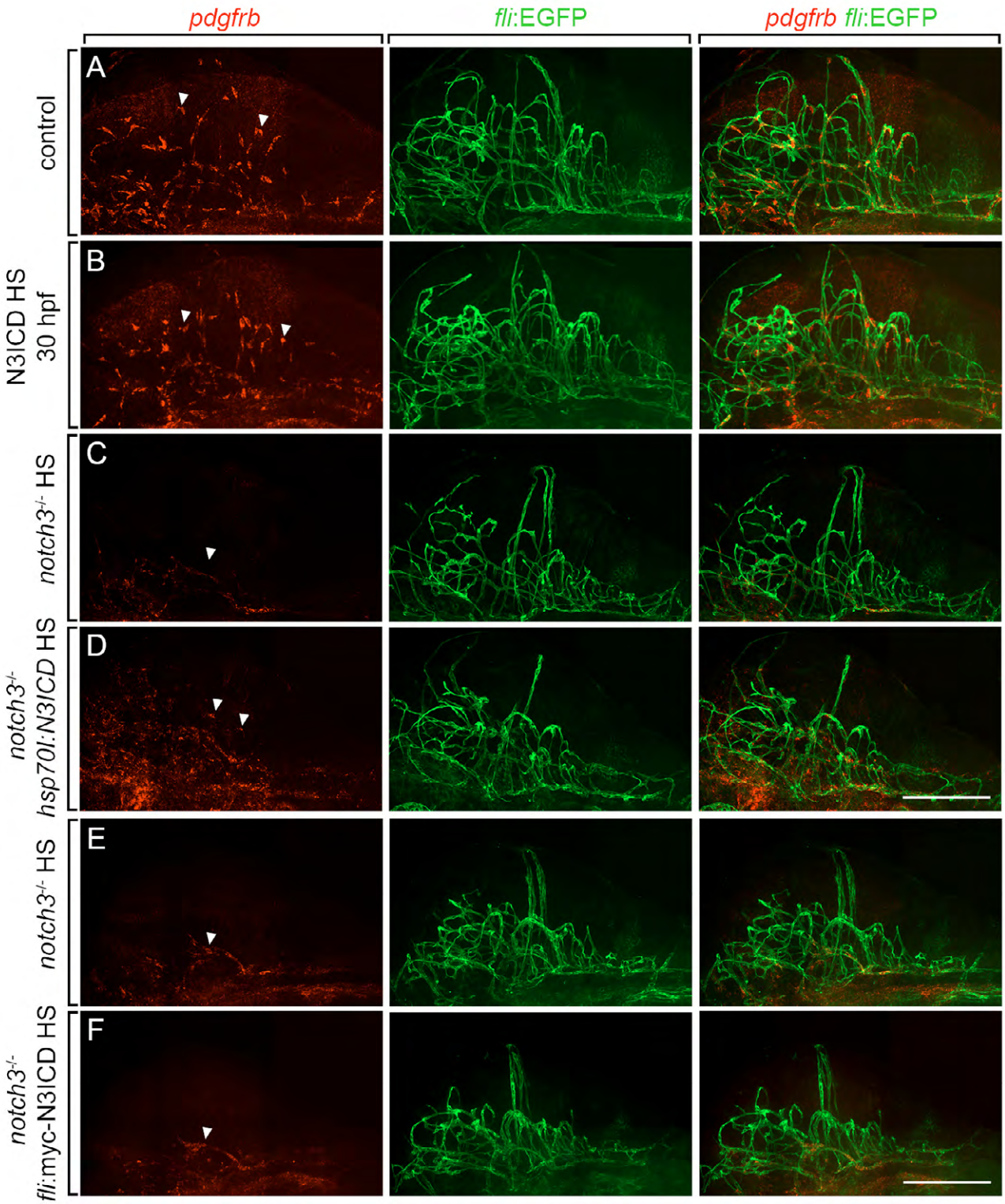

Fig. S9. Notch3 is insufficient to induce ectopic brain pericyte specification and forced Notch3ICD expression in the endothelium failed to rescue the pericyte deficit in *notch3*^{fh332} larvae. (**A**,**B**) Heat shock of *Tg(hsp70I:Notch3ICD-EGFP)* embryos at 30 hpf did not result in increase of brain pericyte number at 72 hpf. (**A**) Non-transgenic control larvae. (**B**) *Tg*(*hsp70I:N3ICD-EGFP)* larva heat shocked at 30 hpf. (**C**,**D**) Transient N3ICD overexpression partially rescued the brain pericyte deficit phenotype in *notch3*^{fh332} mutant larvae at 72 hpf. (**C**) *notch3*^{fh332} mutant mock-injected control. (**D**) *notch3*^{fh332} mutant injected with *hsp70I:N3ICD-GFP* construct and heatshocked at 48 hpf. (**E**,**F**) Transient *N3ICD* expression in endothelial cells failed to rescue the brain pericyte deficit phenotype in *notch3*^{fh332} mutant larvae. (**E**) *notch3*^{fh332} mutant mock-injected control. (**F**) *notch3*^{fh332} mutant injected with *fli1:myc-Notch3ICD* construct and heat shocked at 48 hpf. Arrowheads, *pdgfrb*$^{+}$ brain pericytes. Scale bar, 200 μm.

Table S1. Brain hemorrahge phenotype in *notch3*^{fh332} larvae, *notch3* MO treated larvae, *foxd3* MO treated larvae, and AG1295 treated larvae.

Genotype or treatment	Hemorrahge rate
notch3^{fh332}	10% (n = 30)
	6.25% (n = 32)
	12.5% (n = 40)
	19.5% (n= 87)
notch3^{fh332};*Tg(fli1a:EGFP)*	25% (n = 56)
	24.4% (n = 45)
	19.4% (n = 31)
	28.9% (n = 76)
	12.5% (n = 48)
control MO	0 (n = 56)
	1.4% (n = 72)
	0 (n = 34)
notch3 MO	33.3% (n = 66)
	31.6% (n = 57)
	42.2% (n = 45)
AB; 25 µM AG1295 30 hpf to 72 hpf	8.6% (n = 35)
	10.7% (n = 28)
AB; 25 µM AG1295 48 hpf to 72 hpf	4.8% (n =21)
	10.8% (n = 37)
Tg(fli1a:EGFP) 25 µM AG1295 30 hpf to 72 hpf	25% (n =60)
	23.1% (n= 39)
	31.0% (n =42)
Tg(fli1a:EGFP) 25 µM AG1295 48 hpf to 72 hpf	16.7% (n =30)
	19.3% (n = 31)
	25% (n =20)
control MO	0 (n = 48)
	0 (n = 52)
	0 (n = 63)
foxd3 MO	15.8% (n = 76)
	11.1% (n = 63)
	6.25% (n= 64)
notch3^{fh332};*Tg(fli1a:EGFP)* raised at 28°C	23.5% (n = 34)
	18.8% (n = 32)
	24.1% (n=29)
notch3^{fh332};*Tg(fli1a:EGFP)* raised at 32°C	55.6% (n = 36)
	50% (n = 36)
	66.7% (n=27)

notch3^{fh332} ;*Tg(fli1a:EGFP)* DMSO only, 30 hpf to 72 hpf	28% (n = 25)
	24.3% (n =37)
	17.2% (n=29)
	20.8% (n=24)
	25% (n=28)
notch3^{fh332};*Tg(fli1a:EGFP)* 10 µM AG1295 30 hpf to 72 hpf	37.5% (n = 48)
	33.3% (n = 27)
	25% (n=32)
	30.8% (n=26)
	32.4% (n=34)