Supplemental Information

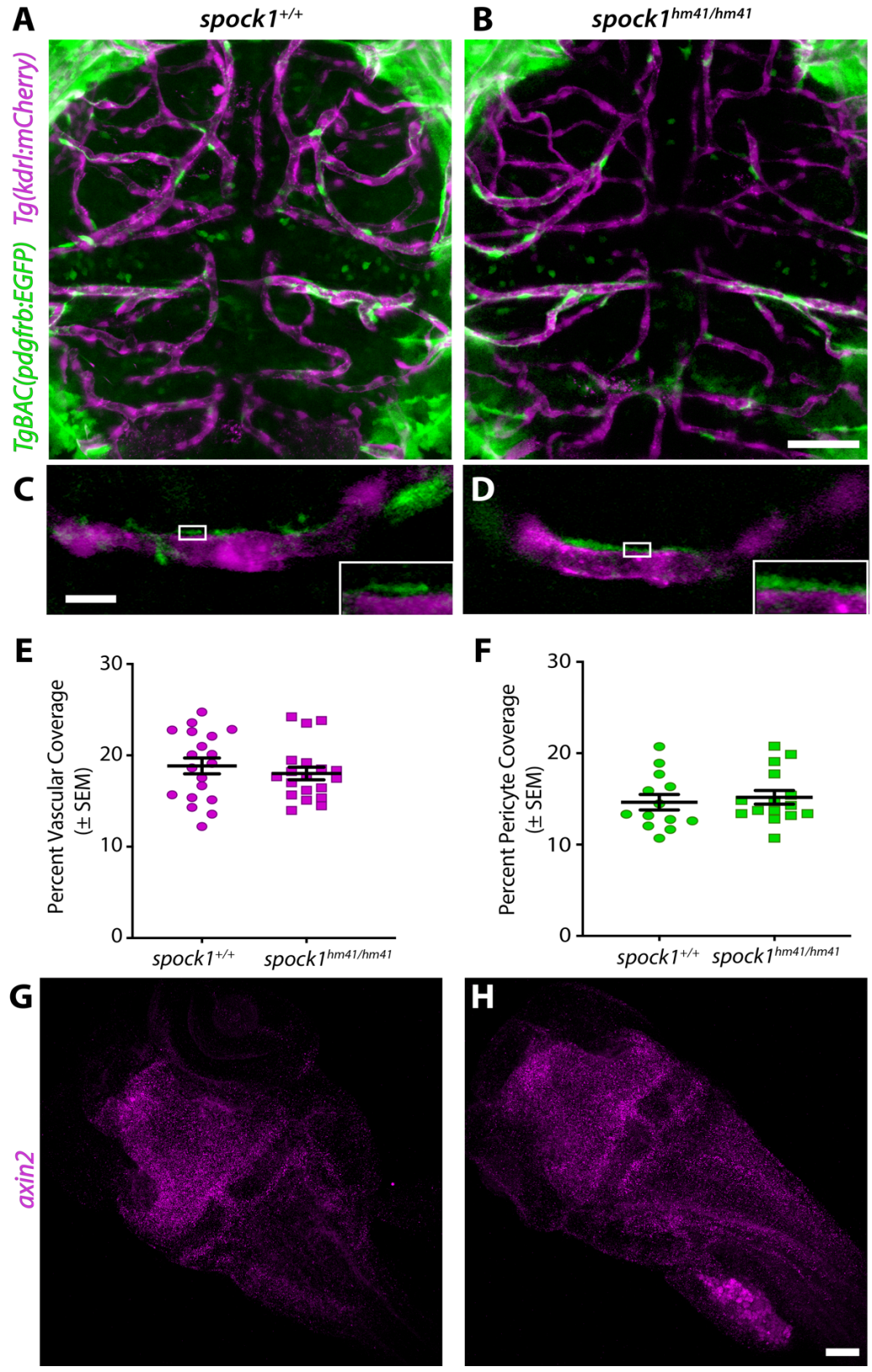

Figure S1. *Spock1*$^{hm41/hm41}$ mutants have normal vascular patterning and Wnt signaling, related to Figure 1. (**A-B**) Dorsal maximum intensity projection of 5 dpf wild type (A) and mutant

(B) brains with endothelial cells marked by the kdrl:mCherry transgene (magenta) and pericytes marked by the pdgfrb:EGFP transgene (green). (**C-D**) Zoomed in view of vessels reveals diminished basement membrane between pericytes (green) and vessels (magenta) in mutants (D) compared to wild type siblings (C). This is further highlighted in the higher magnification insets, with a visible gap present in the wild type vessel (C) but not in the mutants (D). (**E-F**) Quantification of total vascular coverage of the brain (kdrl:mCherry+ area/total brain area, E) and total pericyte coverage of the vasculature (pdgfrb:EGFP+ area/kdrl:mCherry+ area, F), with each individual fish marked by a single point. (**G-H**) HCR in situ hybridization reveals similar *axin2* expression in wild type (G) and mutant brains (H), suggesting that mutant brains have normal Wnt signaling. Scale bars represent 50 μm (B, H) and 10 μm (C). T-test comparison reveals no significant difference between wild type and mutant fish for either vascular or pericyte coverage.

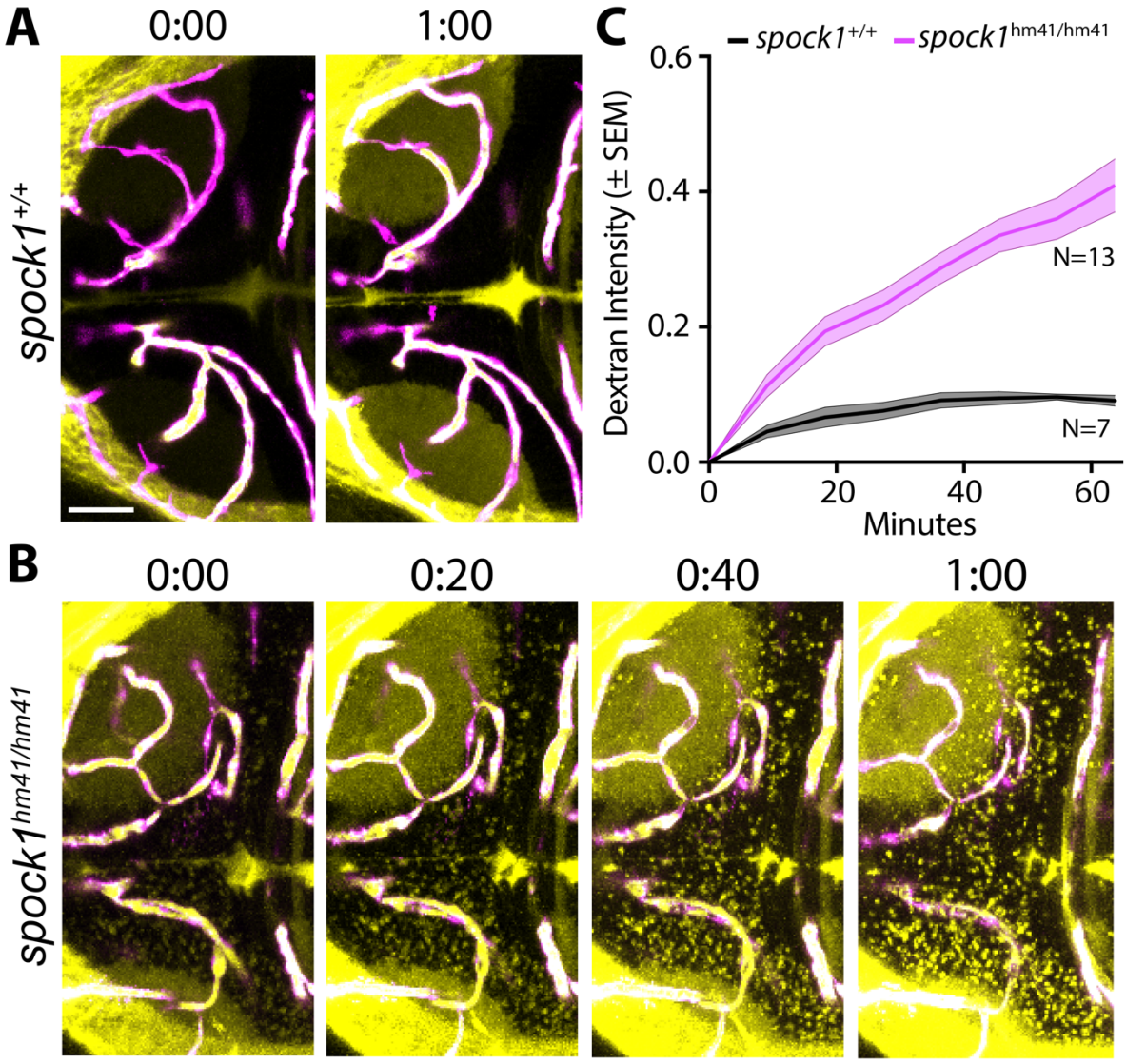

Figure S2. Time lapse imaging reveals leakage dynamics in *spock1*$^{hm41/hm41}$ mutants, related to Figure 1. (**A-B**) Dorsal maximum intensity projection of a wild type (A) and *spock1*$^{hm41/hm41}$ mutant (B) midbrain reveals steady accumulation of 10 kDa Dextran (yellow) outside of the vasculature (magenta) over the course of one hour in *spock1* mutants. (**C**) Quantification of Dextran accumulation in the midbrain parenchyma outside of the vasculature over time in wild type fish (black line) and *spock1*$^{hm41/hm41}$ mutants (magenta line). Scale bar represents 50 μm.

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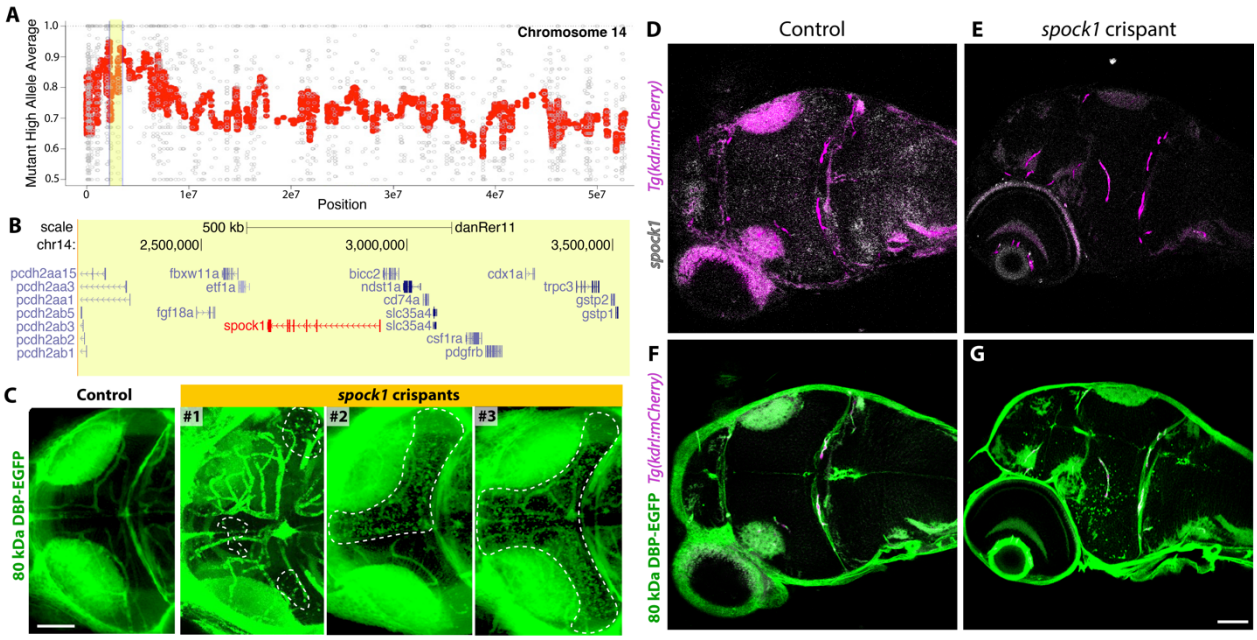

Figure S3. Leaky phenotype maps to *spock1* gene on chr14:2205271-3513919, related to Figure 1. (**A**) Manhattan plot of linkage on chromosome 14 with the average mutant alleles per 20 neighbors plotted in red. The region of highest linkage is highlighted in yellow. (**B**) Genome browser view of the highest linked region to the leaky phenotype. Eight of the genes within this region were expressed in the 5 dpf bulk RNAseq data: *fgf18a, spock1, bicc2, csf1ra, ndst1a, slc35a4, gstp1* and *gstp2*. Two of these genes were differentially expressed in leaky mutants compared to wild type (*csf1ra* and *gstp2*) and *spock1* (marked in red) had several SNPs that completely segregated in the leaky fish. (**C**) Mosaic *spock1* crispants display increased leakage of the transgenic DBP-EGFP tracer outside of the vasculature (outlined by white dashed lines). (**D-E**) Dorsal view of whole mount HCR in situ for *spock1* (white) reveals expression throughout the brain of uninjected controls (D) but not in *spock1* crispants (E). (**F-G**) While controls confine the DBP-EGFP tracer (green) within the blood vessels (magenta) in the brain (F), *spock1* crispants display increased leakage into the brain parenchyma (G, outlined by white dashed line), corresponding with the loss of *spock1* expression (E). Scale bars represent 50 μm.

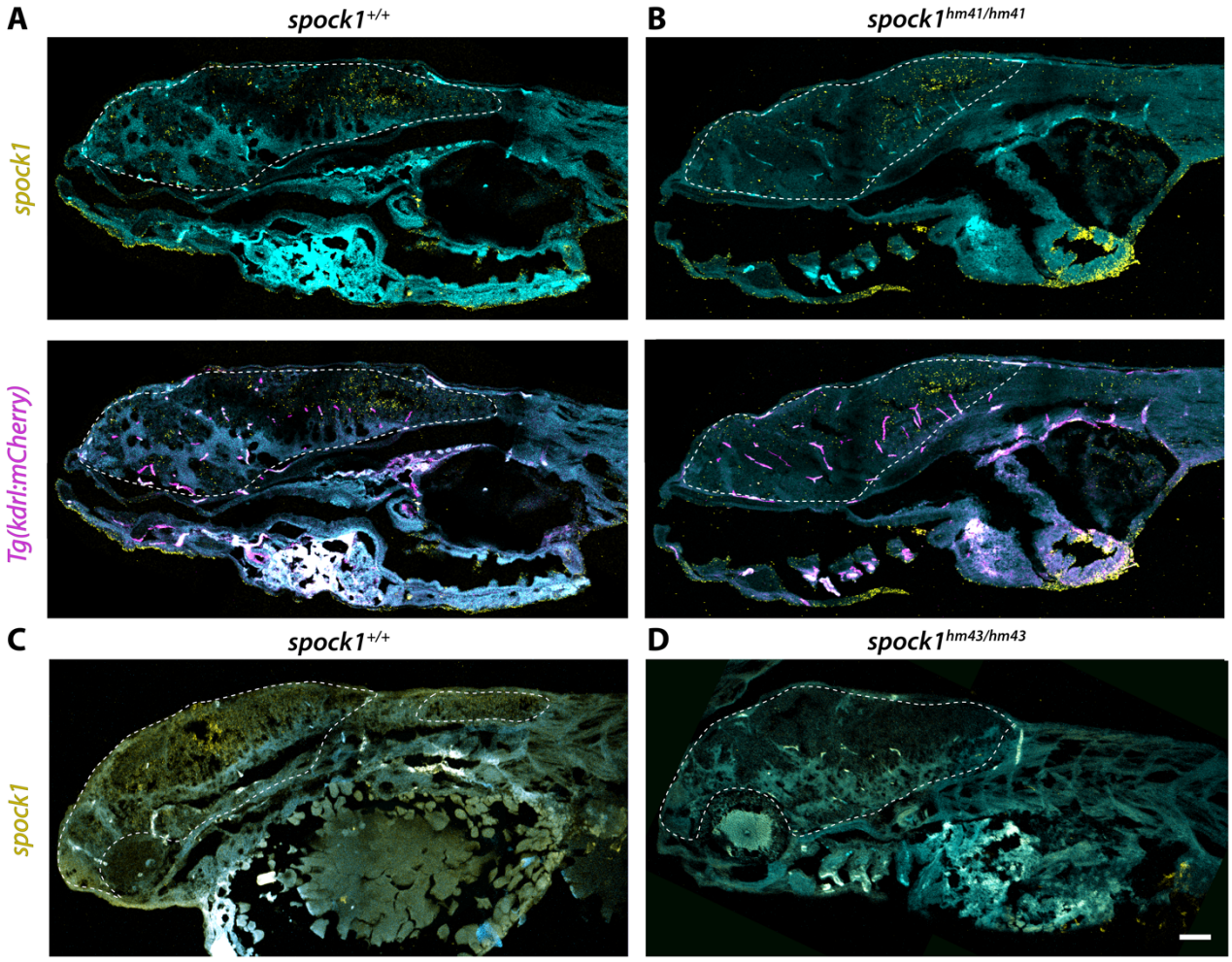

Figure S4. Expression of *spock1* is unaltered in *spock1*$^{hm41/hm41}$ mutants and absent in *spock1*$^{hm43/hm43}$ mutants, related to Figure 1. (**A-B**) *Spock1* expression (yellow) is found throughout the brain and spinal cord (outlined by a white dashed line) in wild type (A) and *spock1*$^{hm41/hm41}$ mutant fish (B) at 5 dpf. *Spock1* expression never colocalizes with vascular *Tg(kdrl:mCherry)* expression (magenta). Tissue autofluorescence is depicted in turquoise. (**C-D**) Unlike *spock1*$^{hm41/hm41}$ fish and wild type fish (C), *spock1*$^{hm43/hm43}$ mutants, which completely lack the 5' UTR and start codon of *spock1*, do not have any detectable *spock1* expression (yellow) in the brain (D). Tissue autofluorescence is depicted in turquoise. The remaining signal in the yolk and blood vessels is due to tissue autofluorescence. Scale bar represents 50 μm.

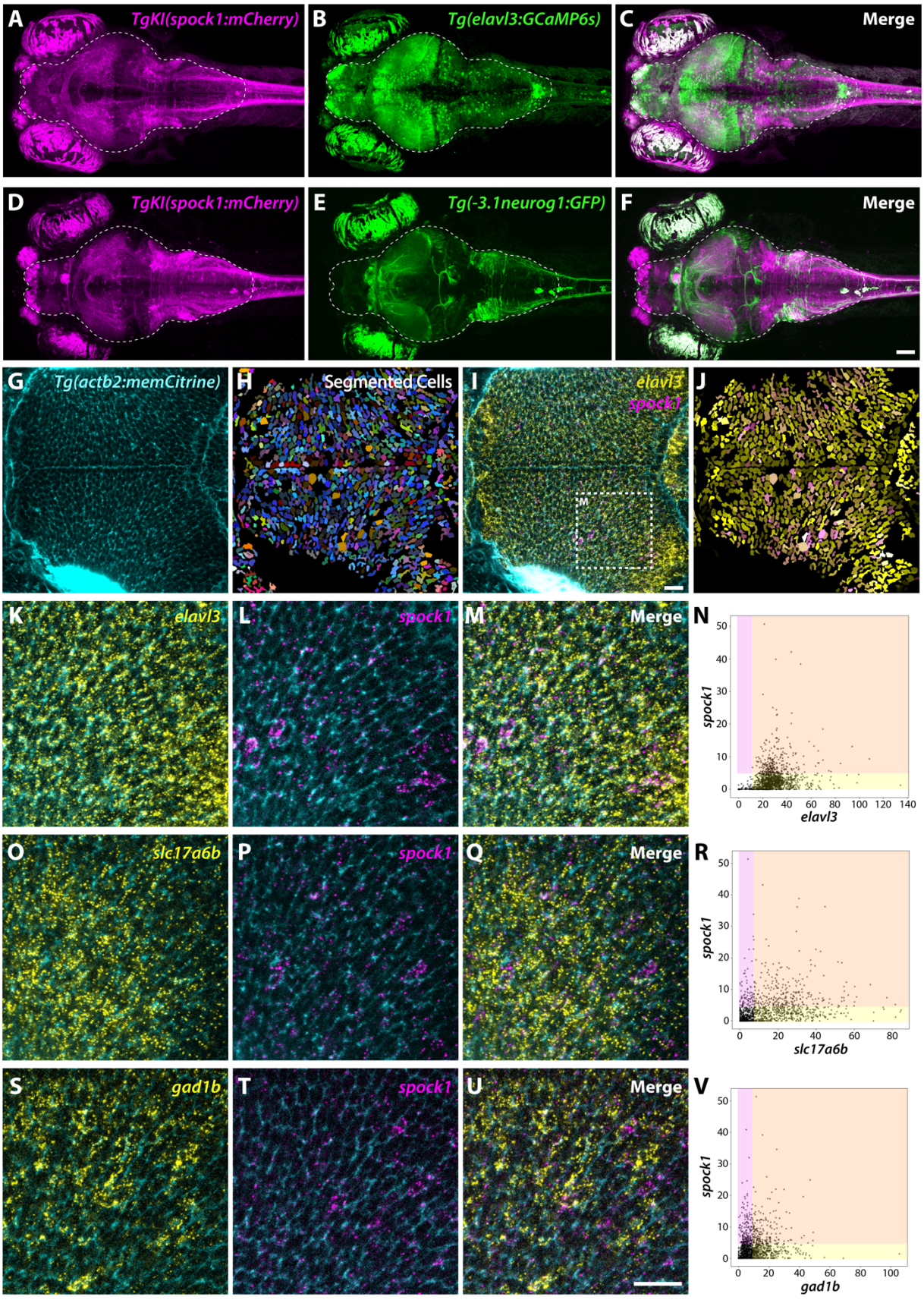
A
TgKI(spock1:mCherry)
B
Tg(elavl3:GCaMP6s)
C
Merge
D
TgKI(spock1:mCherry)
E
Tg(-3.1neurog1:GFP)
F
Merge
G
Tg(actb2:memCitrine)
H
Segmented Cells
I
elavl3
spock1
M
J
K
elavl3
L
spock1
M
Merge
N
spock1
elavl3
O
slc17a6b
P
spock1
Q
Merge
R
spock1
slc17a6b
S
gad1b
T
spock1
U
Merge
V
spock1
gad1b

Figure S5. *Spock1* is expressed by a subset of post-mitotic neurons, related to Figure 2. (**A-C**) *Spock1* expression (A, magenta) is found in a subset of active *elavl3*+ postmitotic neurons (B, green) throughout the 5 dpf brain (outlined by white dashed line). Iridophore pigment cells in the eyes are autofluorescent in both channels, and not indicative of *spock1* expression. (**D-F**) *Spock1* (D, magenta) is not highly expressed by actively dividing *neurog1*+ neurons (E, green) in the 5 dpf brain (outlined by white dashed line). (**G-J**) Workflow of segmentation and quantification of *spock1* co-expression with other neuronal genes. Using *Tg(actb2:mem-Citrine)* membrane-labelled fish (G), we segmented individual cells in the brain (H), and then quantified expression levels of *spock1* (magenta) and other neuronal markers, like *elavl3* (I-J, yellow) throughout the entire midbrain. Boxed region in I marks region of brain used for zoom ins (M). (**K-N**) All *spock1* (L) expressing cells were co-expressing the post-mitotic neural marker *elavl3* (K-N). (**O-R**) Some glutamatergic neurons (O), marked by *slc17a6b* expression, express *spock1* (P-R). (**S-V**) Some GABAergic neurons (S), marked by *gad1b* expression, express *spock1* (T-V). Scale bars represent 50 μm (F) and 20 μm (U).

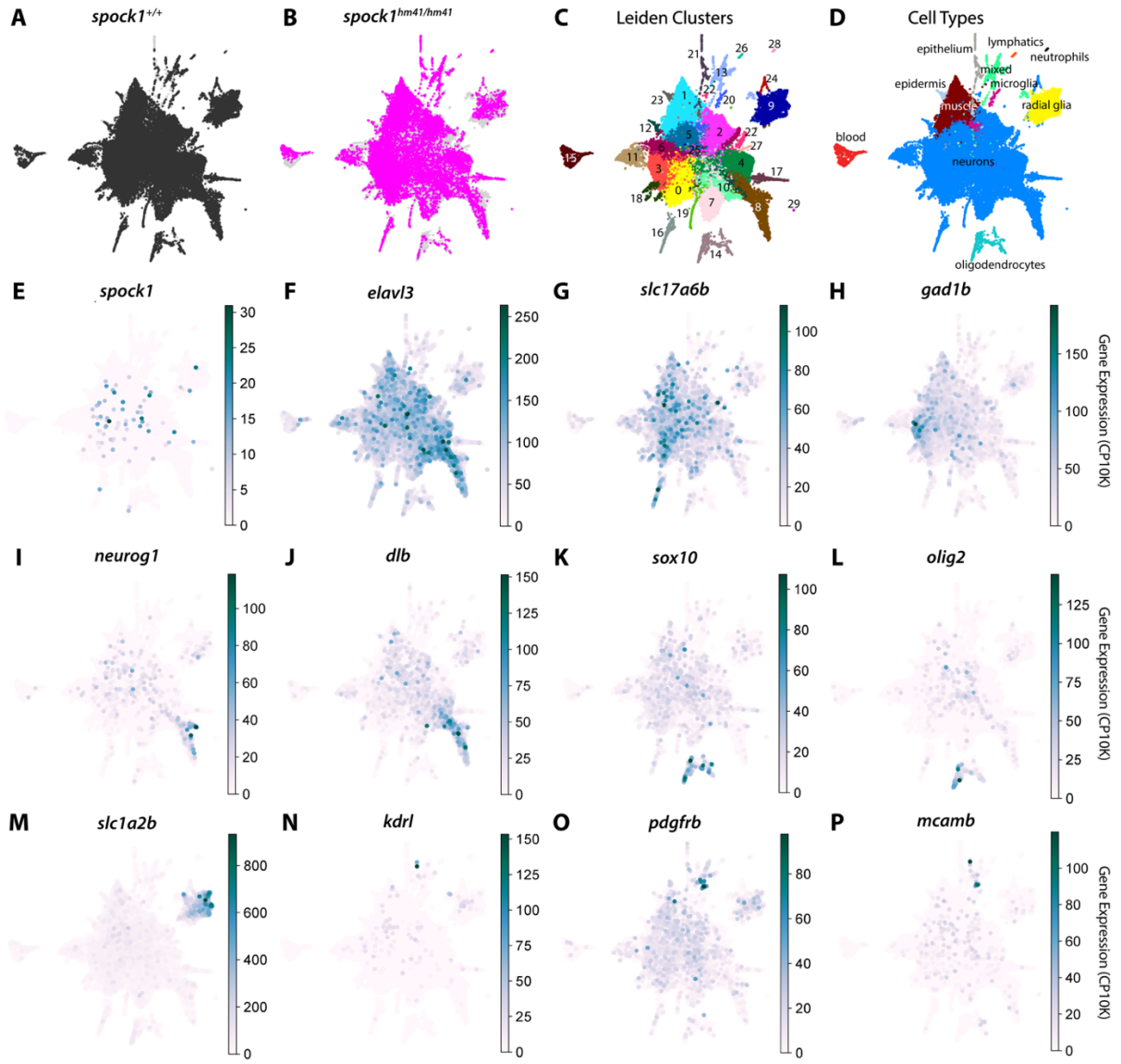

Figure S6. scRNA-seq captures all neurovascular cells in both mutant and wild type larvae, related to Figure 6. (**A-B**) UMAP of the whole brain dataset shows overlap between wild type (A) and *spock1*$^{hm41/hm41}$ mutant (B) cell type coverage, indicating that no cell type is absent in the mutant background. (**C**) UMAP with individual Leiden clusters annotated by color. (**D**) UMAP of the total data set separated by annotated cell type, with the vast majority of sequenced cells being neuronal (blue). Our data does capture blood cells (red), microglia (magenta), oligodendrocytes (aqua), radial glia (yellow), and other cells. (**E-P**) Gene expression plots for *spock1* (E), *elavl3* (F) marking postmitotic neurons, *slc17a6b* (G) for glutamatergic neurons, *gad1b* (H) for GABAergic neurons, *neurog1* (I) and *dlb* (J) for dividing neurons, *sox10* (K) and *olig2* (L) for oligodendrocytes, *slc1a2b* (M) for radial glia, *kdrl* (N) for vascular endothelial cells, *pdgfrb* (O) for pericytes and neurons, and *mcamb* (P) marking both endothelial cells and pericytes. These gene expression

plots reveal that *spock1* (E) is primarily expressed by postmitotic neurons and absent from the vascular cells (N-P).

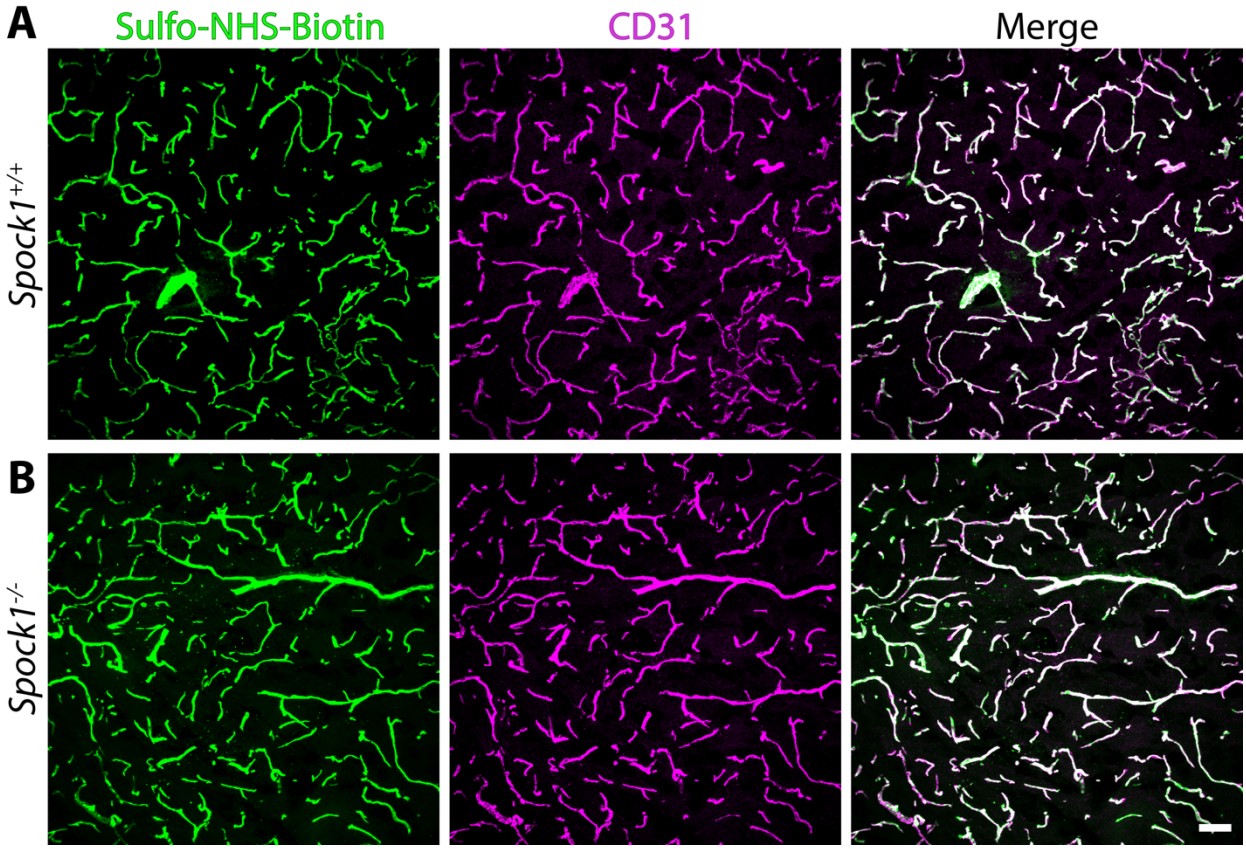

Figure S7. Adult *Spock1*$^{-/-}$ mice recover BBB function, related to Figure 5. (**A-B**) Tracer leakage assays in adult wild type (A) and *Spock1*$^{-/-}$ knockout mice (B) reveals that both genotypes confine the injected Sulfo-NHS-Biotin tracer (green) within the CD31+ vasculature (magenta), indicating a functional BBB. Scale bar represents 50 μm.