SUPPLEMENTAL MATERIAL

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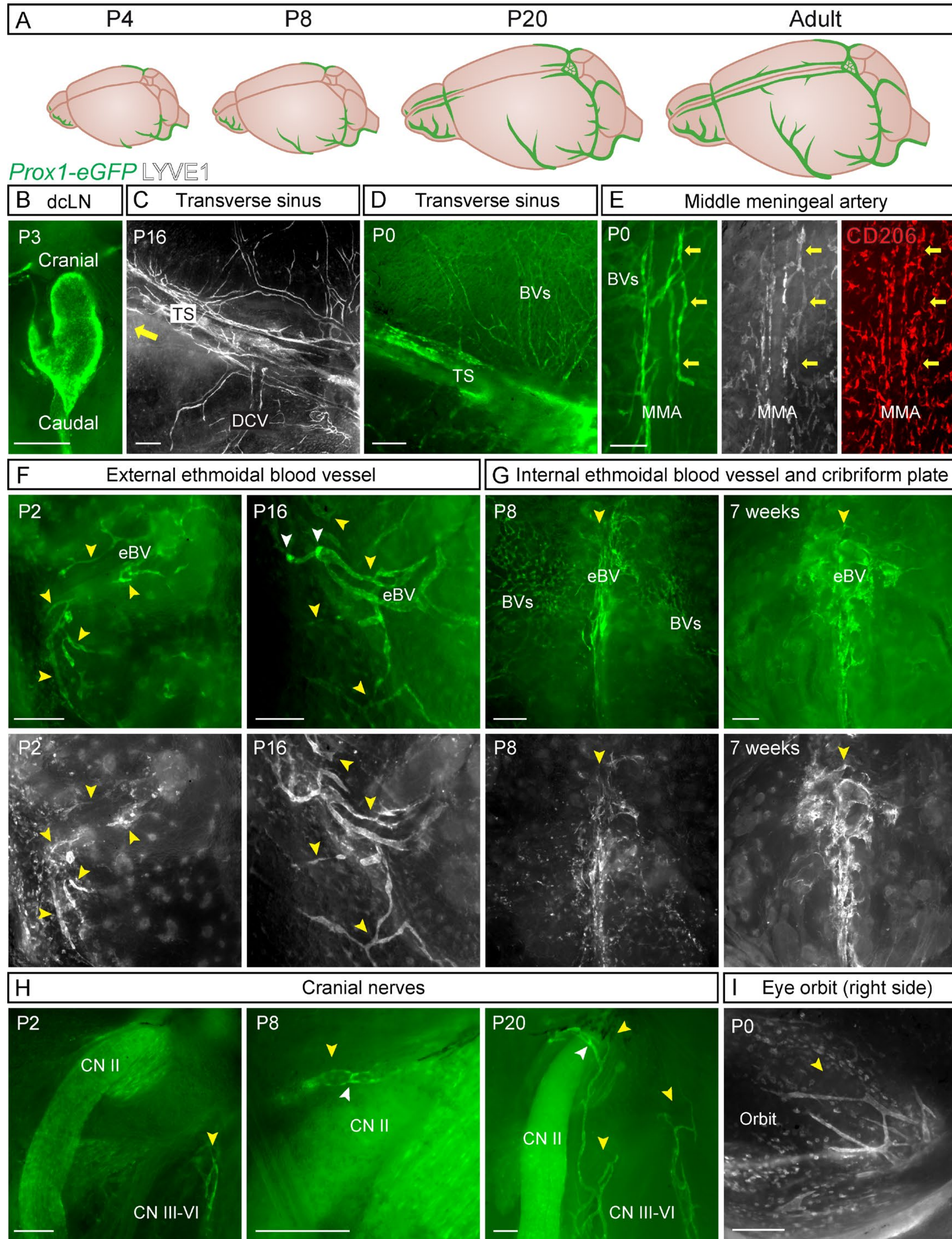

Figure S1. **Postnatal development of the meningeal lymphatic network.** (A) Schematic illustration of the location of meningeal LVs (green) in relation to the developing mouse brain. (B–I) Development of the meningeal lymphatic network as shown by *Prox1-eGFP* reporter (green) or LYVE1 immunostaining (gray). DCV, dorsal cerebellar vein; eBV, ethmoidal BV. (B) dcLN and its connected LVs at P3. (C) LVs extending into the dura mater covering the cerebellum and flanking veins branching from the TS. (D and E) PROX1-positive BVs around the TS (D) and MMA (E) at P0. (E) LYVE1 (gray) and CD206 (red) staining around PROX1^{+} BVs. Arrows point to the PROX1^{+}/weakly LYVE1^{+}/CD206^{-} ECs in the BVs. (F–I) LVs (yellow arrowheads) around the external eBV at P2 and P16 (F), internal eBV, cribriform plate and olfactory nerves (CN I) at P8 and 7 wk of age (G), CN II and nerve bundle containing CNs III–VI at P2, P8, and P20 (H), and orbit after eye enucleation (I). White arrowheads in F and H point to valves. Data shown are representative of n = 3–6 per time point and staining. Bars: (B, D, and F–I) 200 µm; (C) 400 µm; (E) 100 µm.

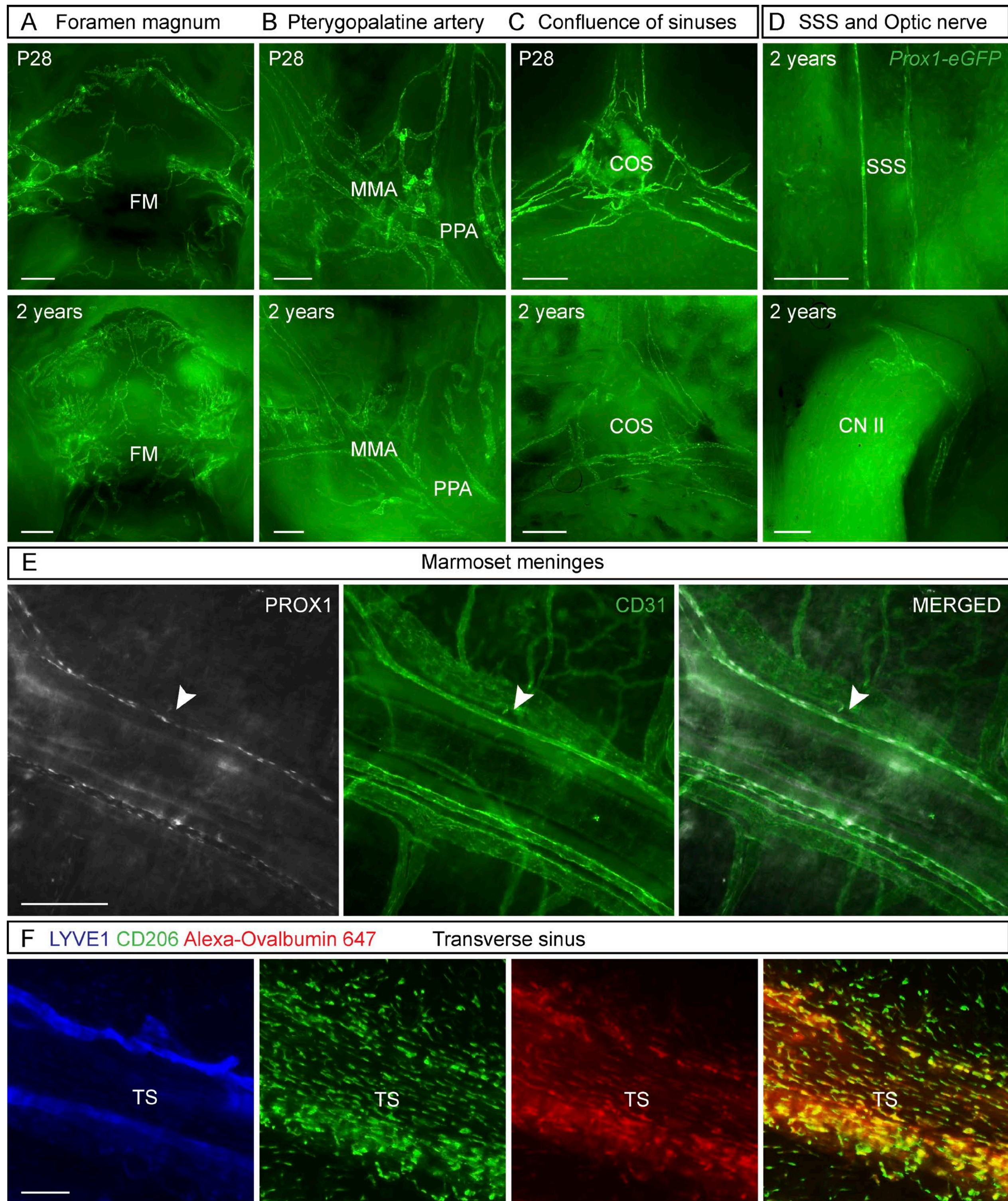

Figure S2. **Imaging of meningeal LVs in aged mice and in marmosets and phagocytosis by CD206-positive cells.** (A–D) Comparison of the meningeal LVs around the FM (A), PPA (B), and COS (C) in 1-mo-old (P28) and 2-yr-old mice. (D) Representative images of LVs in flanking the SSS and CN II at 2 yr of age. (E) Representative immunofluorescence images of meningeal LVs (arrowheads) around the marmoset MMA shown by colocalization of PROX1 (gray) and CD31 (green). Two marmoset meninges were analyzed, and LVs were found in both of them. (F) Localization of i.c.v.-injected fluorescent ovalbumin (red) and LYVE1 (LV, blue) and CD206^{+} (green) macrophage immunostaining in the TS area 2 h after injection. Data shown are representative of n = 3–6 per time point and staining if not indicated otherwise. Bars: (A and C) 400 µm; (B and E) 200 µm; (D) 150 µm; (F) 100 µm.

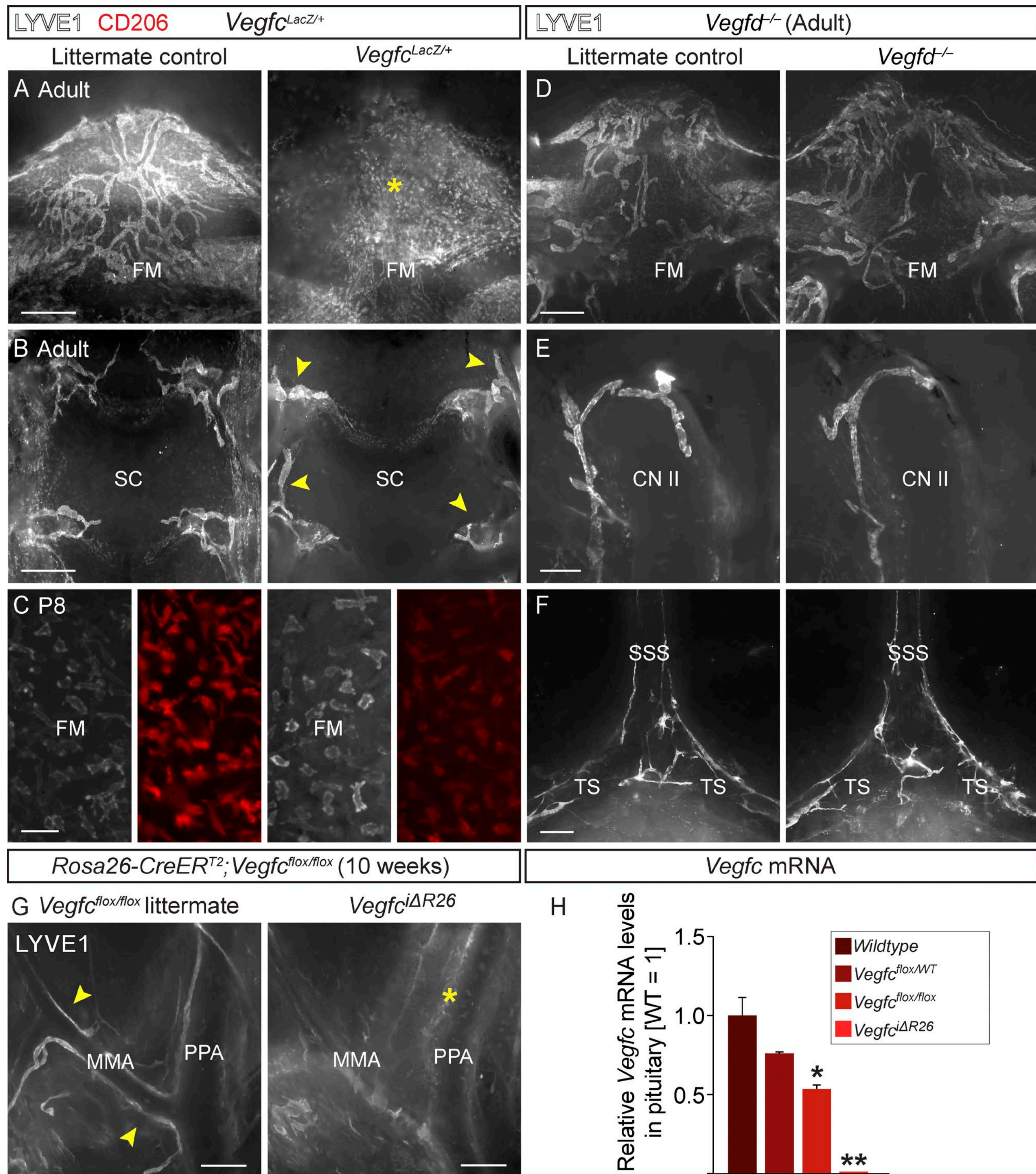

Figure S3. **VEGF-C, but not VEGF-D, is essential for normal meningeal LV development.** LYVE1 staining in *Vegfc*$^{LacZ/+}$ mice and littermate controls (n = 3, 3) in the area of the FM (A) and spinal canal (SC; B) in adult mice. (C) LYVE1 (gray) and CD206 (red) double-positive macrophages in the FM area in P8 *Vegfc*$^{LacZ/+}$ mice and littermate controls (n = 3, 3). (D–F) LYVE1 staining of adult *Vegfd*$^{-/-}$ mice and their littermate controls (n = 3, 3) in the FM (D), CN II (E), and COS areas (F). (G) Comparison of LYVE1 staining in the PPA area in *Vegfc*$^{flox/flox}$ and *Vegfc*$^{i\Delta R26}$ mice 9 wk after *Vegfc* gene deletion (n = 3, 6). (H) Quantification of *Vegfc* mRNA in WT mice (n = 3), heterozygous (*Vegfc*$^{flox/WT}$, n = 2) and homozygous (*Vegfc*$^{flox/flox}$, n = 4) gene-targeted mice, and in *Vegfc* gene-deleted mice (*Vegfc*$^{i\Delta R26}$, n = 3). Arrowheads in B and G mark remaining LV fragments. Yellow asterisks mark LYVE1-positive macrophages. Data shown are representative of two independent experiments. One-way analysis with Tukey's post hoc test was used to calculate the p-values. *, $P < 0.05$; **, $P < 0.01$. Values are expressed as mean ± SEM. Bars: (A, B, D, and F) 400 µm; (E and G) 200 µm; (C) 50 µm.

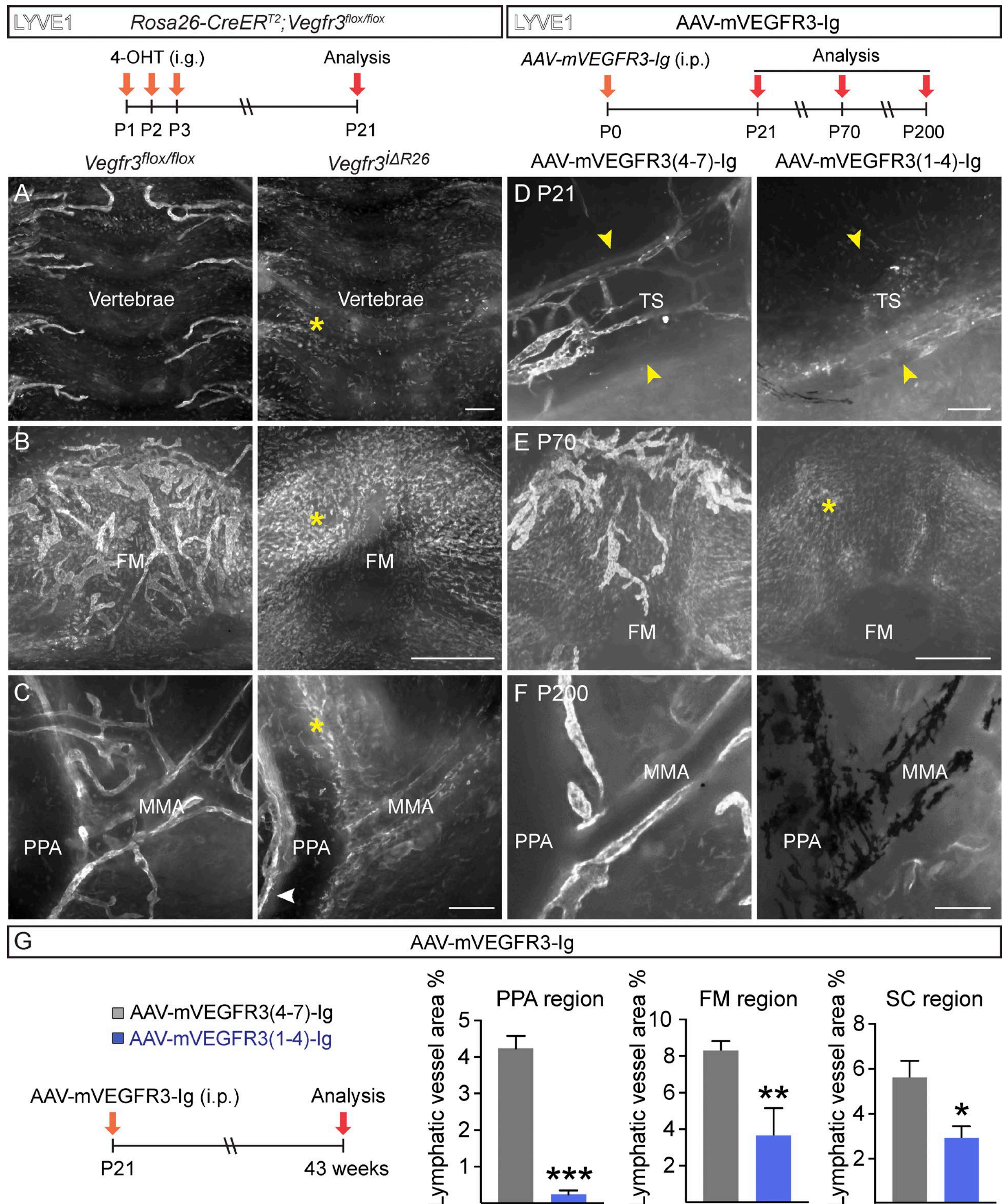

Figure S4. **VEGFR-3 is essential for meningeal LV development.** (A–F) Comparison of LYVE1 staining in the spinal canal (A), FM (B), and PPA area (C) at P21 in *Vegfr3*$^{flox/flox}$ and *Vegfr3*$^{i\Delta R26}$ mice (*n* = 9, 4) and around the TS at P21 (*n* = 3, 3; D), FM at P70 (*n* = 6, 6; E), and PPA at P200 (*n* = 3, 3; F) in mice injected at P0 with either AAV-mVEGFR3$_{1-4}$-Ig or AAV-mVEGFR3$_{4-7}$-Ig, as indicated. Arrowheads in D indicate TS width. (G) Quantification of the area covered with LVs in the FM, PPA, and SC regions of mice injected with the indicated AAVs (*n* = 7, 6). Arrowhead in C points to remaining LV fragments. LYVE1^{+} macrophages are indicated with asterisks (A–C and E). Data shown are representative of two independent experiments. A Student's *t* test was used to calculate the p-values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values are expressed as mean ± SEM. Bars: (A and D) 200 µm; (B and E) 500 µm; (C and F) 150 µm.

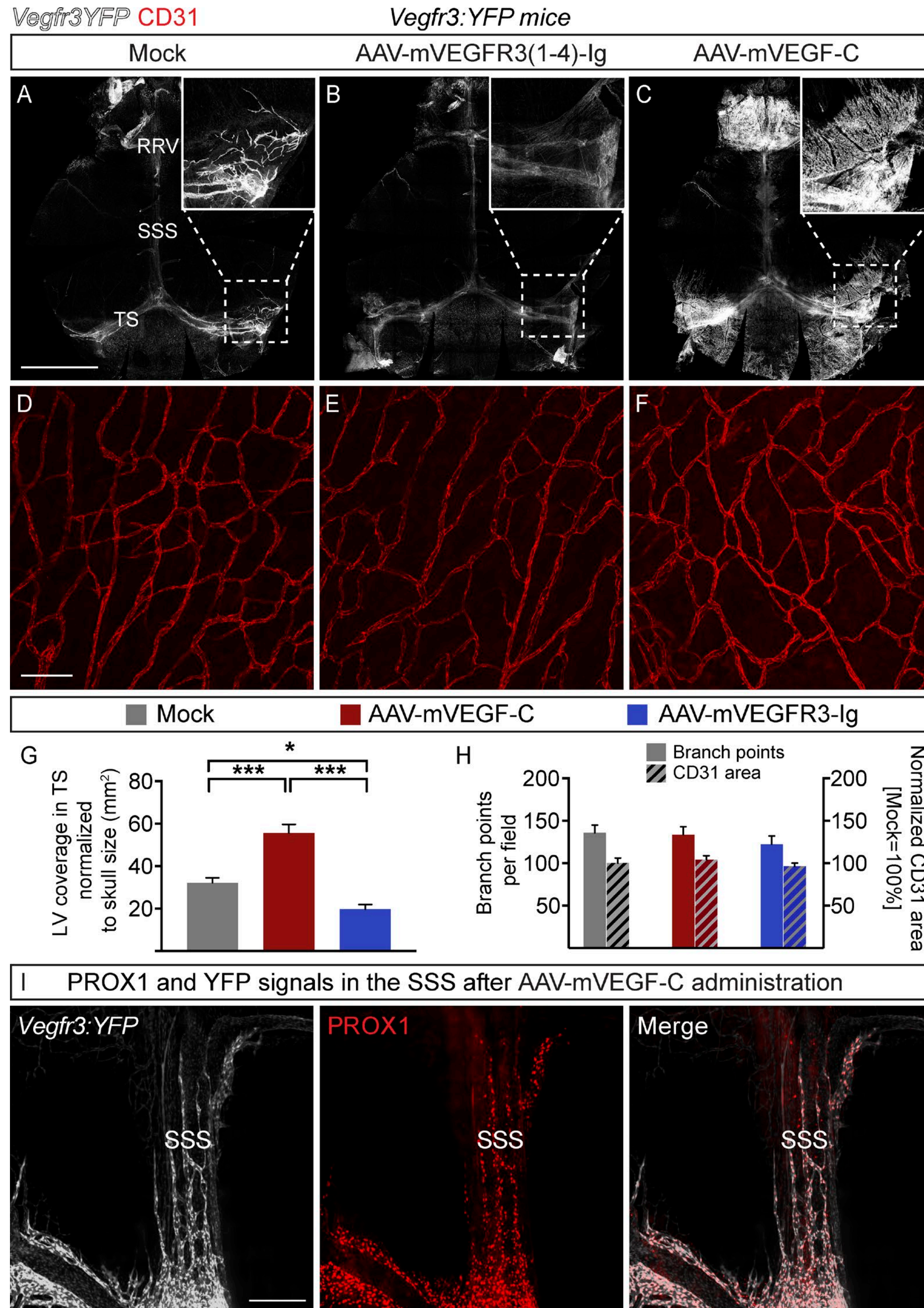

Figure S5. **Effects of inhibition or stimulation of VEGF-C–VEGFR3 signaling on the growth of meningeal LVs.** Analysis of dura mater LVs in *Vegfr3:YFP* mice at P14, after i.c.v. injection of PBS (mock, *n* = 5), AAV–mVEGFR3$_{1-4}$-Ig (*n* = 5), or AAV–mVEGF-C (*n* = 6) at P7. (A–C) *Vegfr3:YFP* signal (gray) around the TS, SSS, and RRV, with a close-up view of the TS area. (D–F) Representative images of dura mater BVs stained for CD31 (red). (G) Quantification of LV coverage of the TS, normalized to skull size. (H) Quantification of meningeal BV branch points per field and of BV area, normalized to PBS-injected mice. (I) Representative close-up images showing the lymphatic hyperplasia induced by AAV–mVEGF-C and the PROX1 (red) and *Vegfr3:YFP* signal (gray) colocalization (86.3% ± 6.0) around the SSS region. Data shown are representative of at least two independent experiments. One-way ANOVA test and Tukey's multiple comparision test were used to calculate the p-values. *, P < 0.05; ***, P < 0.001. Values are expressed as mean ± SEM. Bars: (A–C) 0.5 cm; (D–F) 100 µm; (I) 200 µm.

Table S1. **Quantification of LYVE1 fluorescence in the indicated figures**

Figure	Genotype	Age	Location	Group 1 ± SEM, *n* (control)	Group 2 ± SEM, *n* (experimental)	Test	P
5 A	$Vegfc^{LacZ/+}$	P12	FM	2.641 ± 0.557, 3	0.052 ± 0.031, 3	Welch's *t*	<0.05
5 B	$Vegfd^{-/-}$	P12	FM	2.749 ± 0.411, 3	1.750 ± 0.566, 3	Unpaired *t*	0.2264
5 C	$Vegfc^{i\Delta R26}$	P21	FM	0.013 ± 0.004, 3	0.009 ± 0.002, 3	Unpaired *t*	0.4391
5 D	$Vegfc^{i\Delta R26}$	10 wk	FM	0.134 ± 0.028, 3	0.009 ± 0.003, 6	Welch's *t*	<0.05
7 A	$Vegfr3^{i\Delta R26}$	P21	TS	0.101 ± 0.009, 9	0.006 ± 0.001, 4	Welch's *t*	<0.0001
7 C	AAV-mR3-Ig	P70	COS	7.195 ± 0.201, 3	0.200 ± 0.058, 3	Unpaired *t*	<0.0001
9 A	$Vegfr3^{i\Delta R26}$	Adult	TS/COS	0.083 ± 0.003, 4	0.013 ± 0.003, 4	Unpaired *t*	<0.0001

The meningeal LV area was chosen based on PROX1/LYVE1 double staining in both pups and adults.