Rapamycin reversal of VEGF-C driven lymphatic anomalies in the respiratory tract

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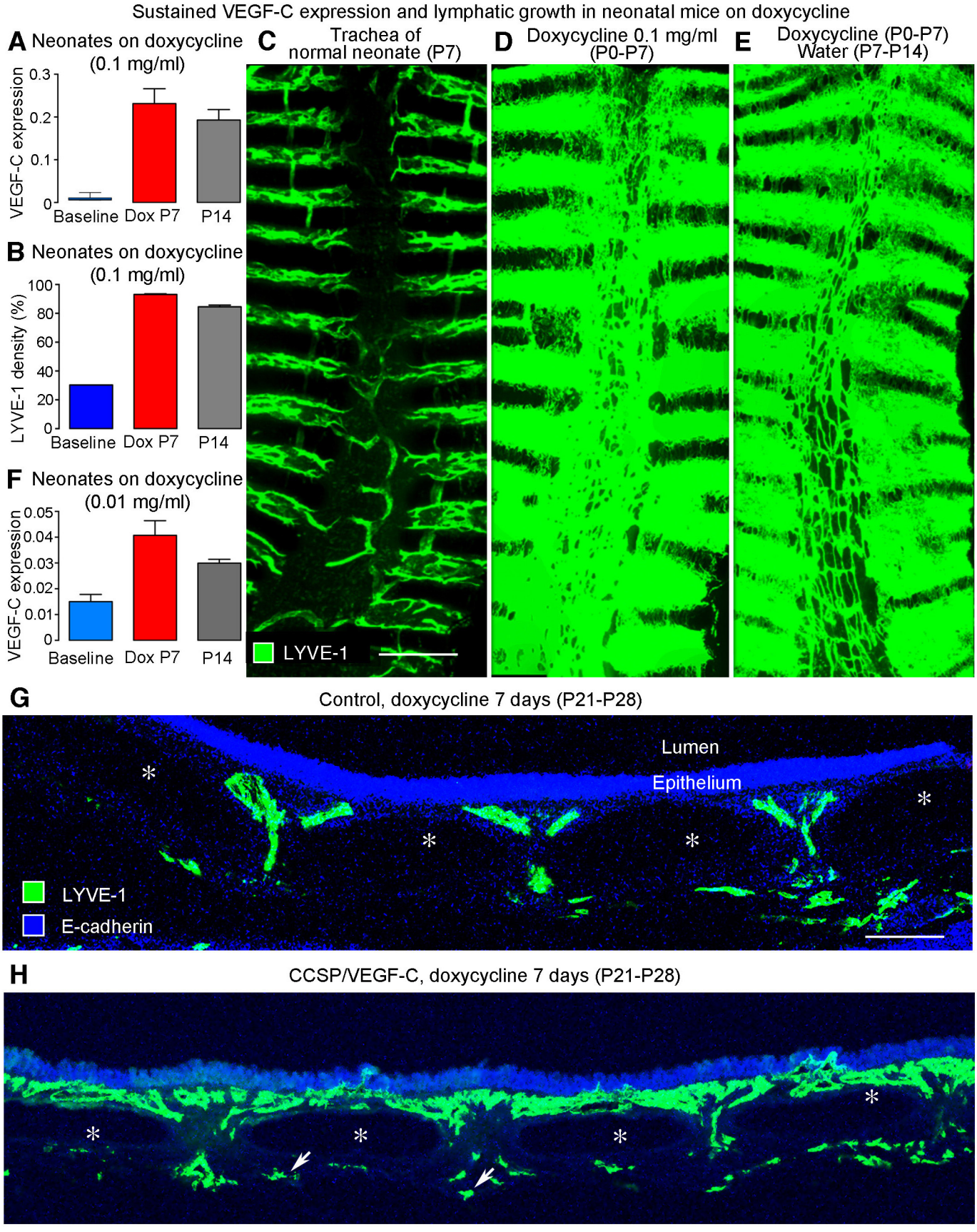
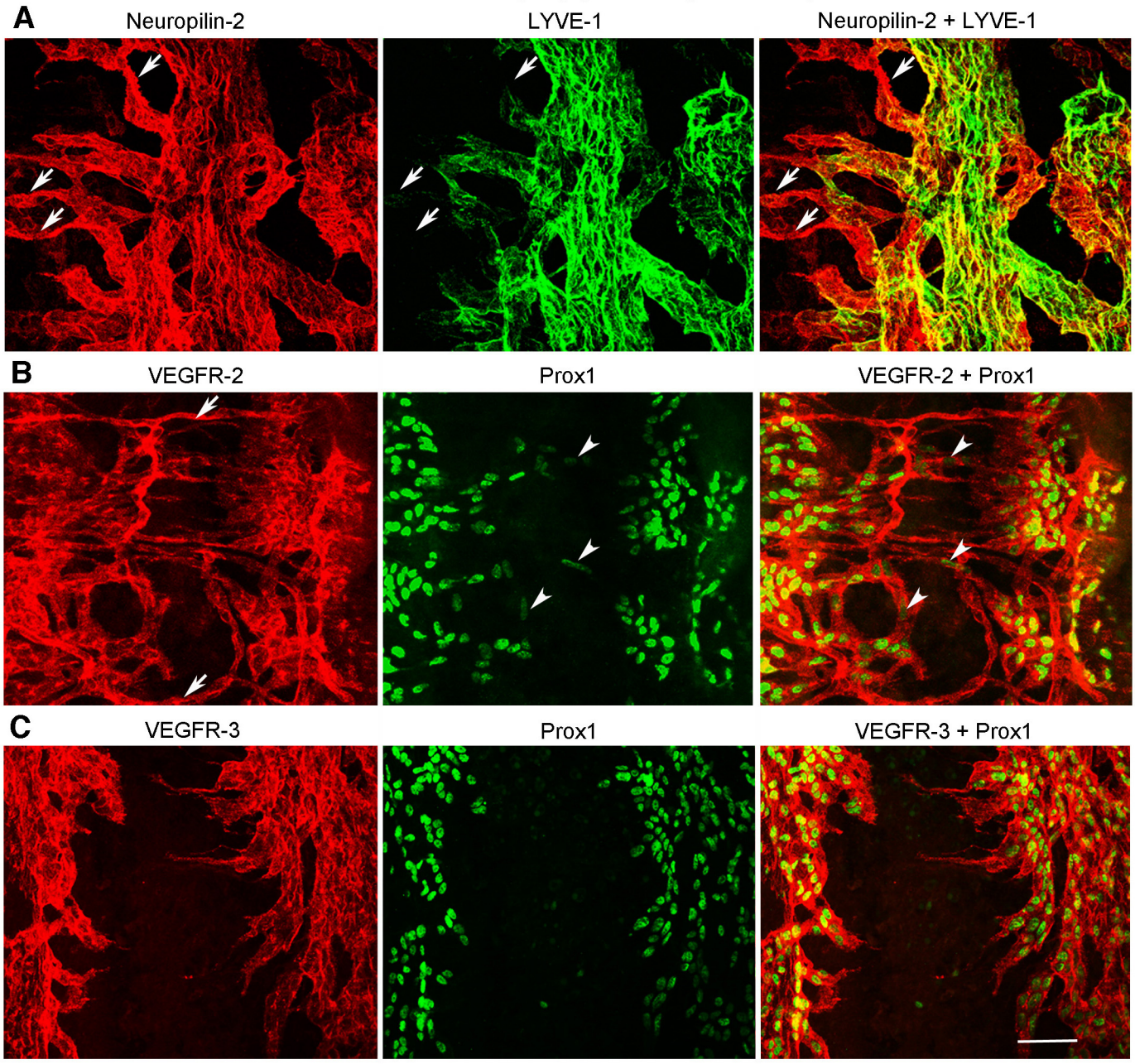
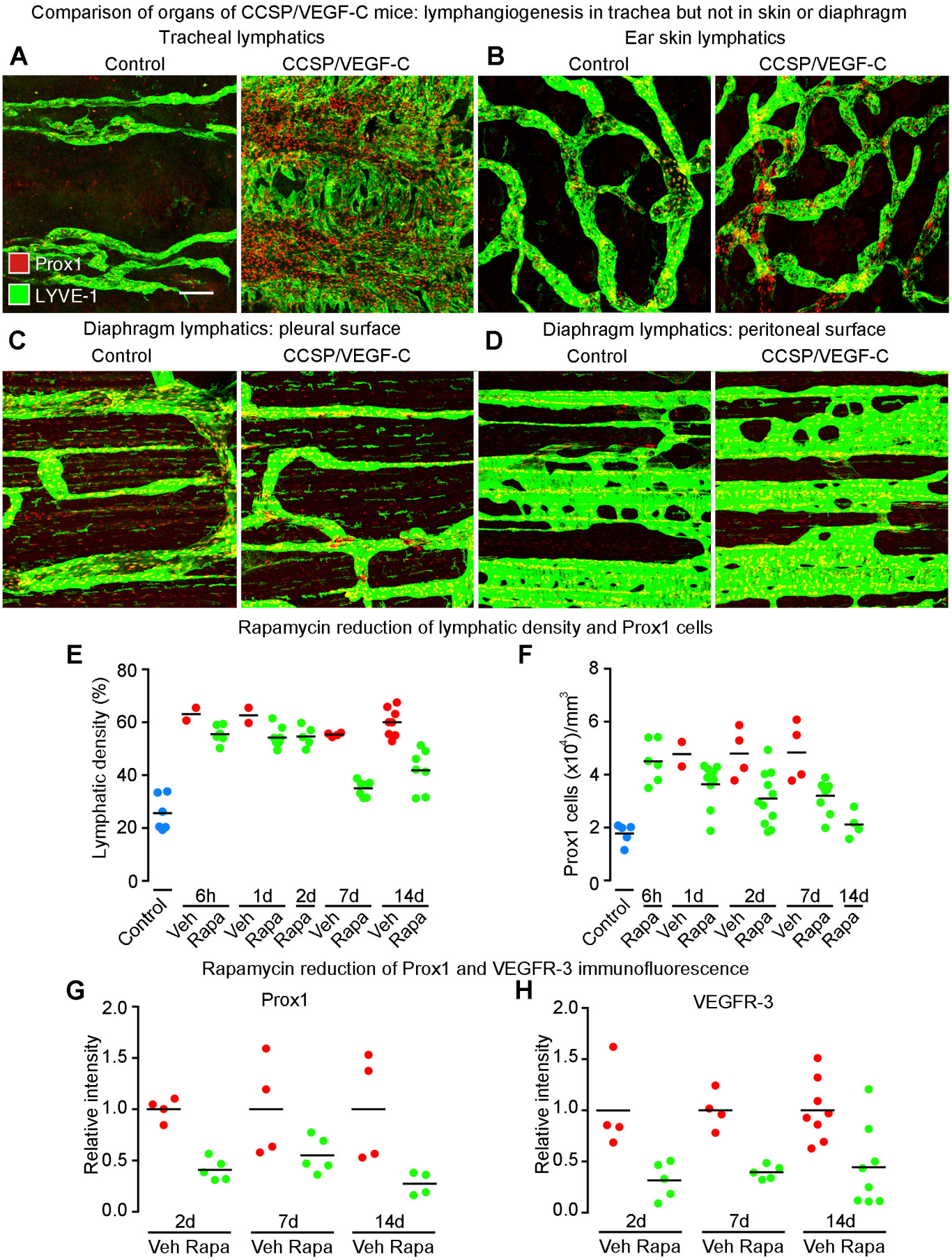
Supplemental Figures 1-5 and Supplemental Table 1



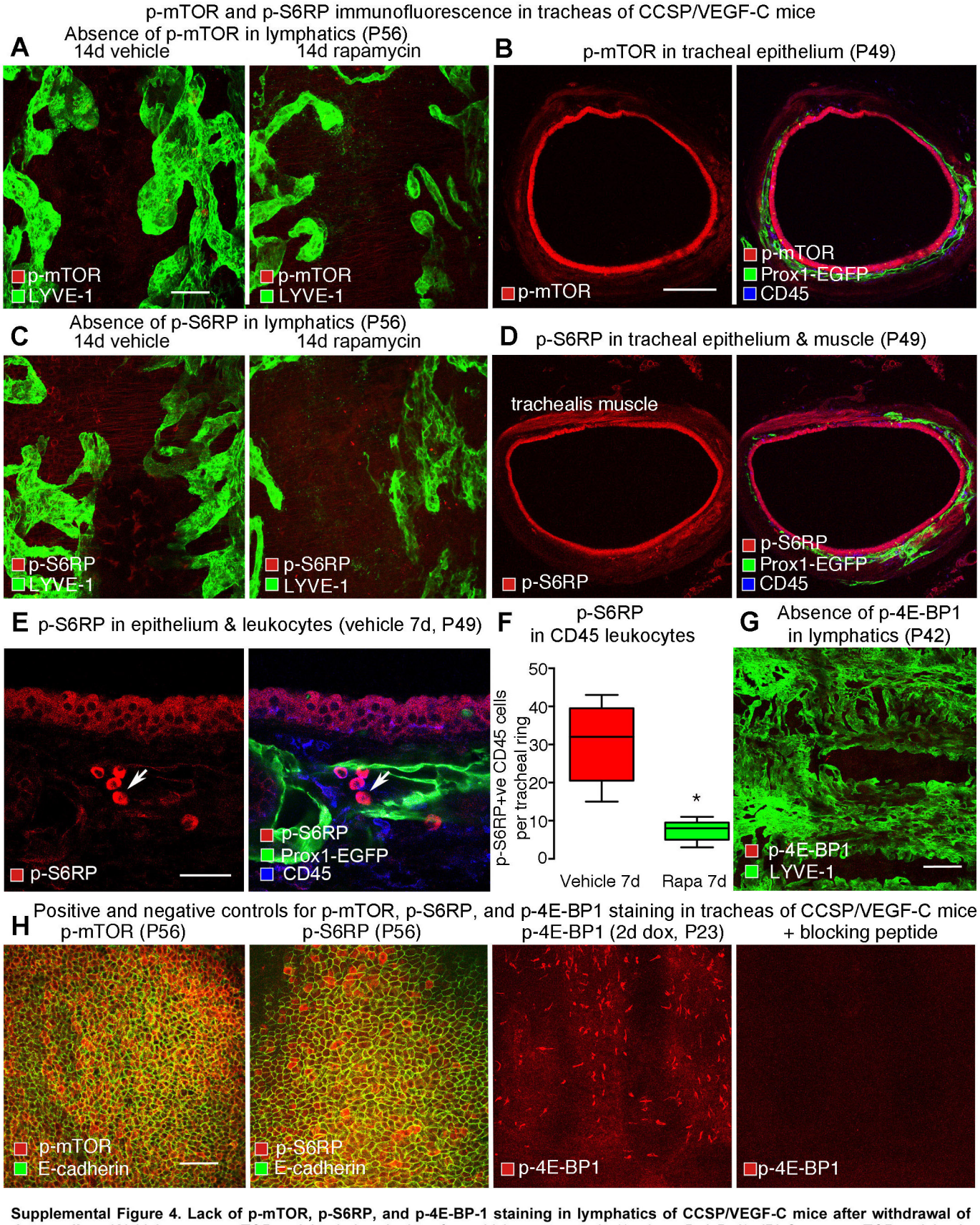
Supplemental Figure 1. VEGF-C expression and lymphatic growth in CCSP/VEGF-C mice. (A) Sustained VEGF-C mRNA expression relative to beta-actin, and corresponding lymphatic growth **(B)** in tracheas of neonatal CCSP/VEGF-C mice on doxycycline (0.1 mg/mL, 7 days, P7) and after withdrawal (7 days, P14), n = 4-7 mice/group. **(C-E)** LYVE-1-stained lymphatics in tracheas of neonatal mice under the same three conditions as in (A, B). Lymphatics expand from the normal segmental distribution **(C)** to cover almost the entire tracheal surface after doxycycline for 7 days **(D)** and change little after doxycycline is withdrawn for 7 days **(E)**. **(F)** Smaller increase in VEGF-C expression in tracheas of neonates on a lower concentration of doxycycline (0.01 mg/mL, 7 days, P7) but expression remains elevated after doxycycline is withdrawn (7 days, P14), n = 4-7 mice/group. **(G-H)** Confocal microscopic images comparing LYVE-1-stained lymphatics (green) and E-cadherin-stained epithelial cells (blue) in sagittal sections of tracheas of a normal mouse **(G)** and CCSP/VEGF-C mouse **(H)** on doxycycline (7 days, P21-P28). Asterisks mark cartilages. Arrows mark non-lymphatic LYVE-1-positive cells, probably macrophages (H). Scale bars, 500 μm in C-E, 50 μm in G-H.



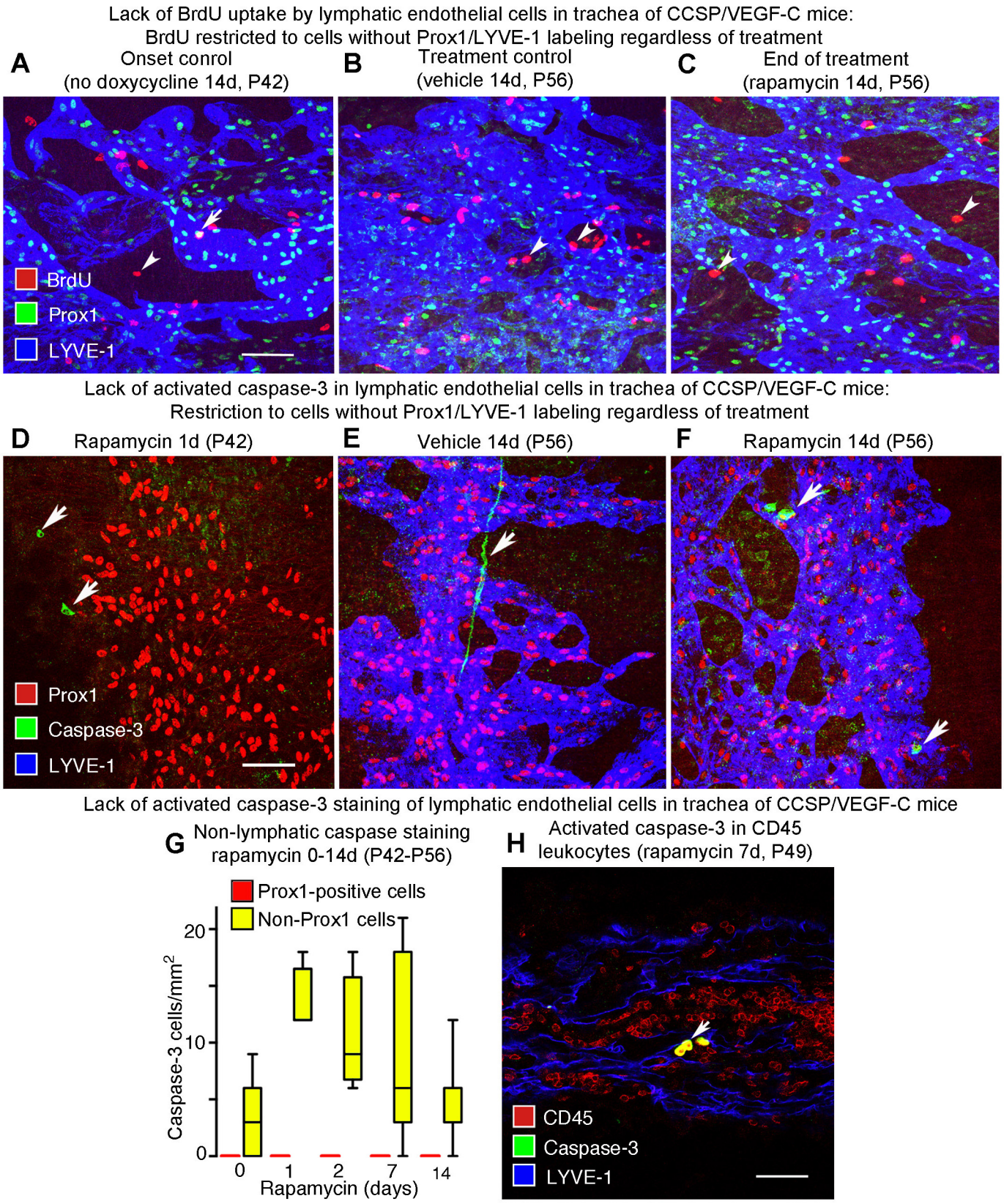
Supplemental Figure 2. Uniformly strong VEGFR-2 and VEGFR-3 in lymphatics but weak LYVE-1 in endothelial sprouts. **(A)** Confocal microscopic images of in trachea of CCSP/VEGF-C mouse on doxycycline (3 days, P24) comparing the uniform distribution of staining of neuropilin-2 (red) to the more restricted distribution of LYVE-1 (green), which is weaker in lymphatic sprouts (arrows). **(B)** Uniform distribution of VEGFR-2 in blood vessels (arrows) and lymphatics, including sprouts (arrowheads), in trachea under same conditions as (A). **(C)** Uniform distribution of VEGFR-3 in lymphatics, including sprouts, but not blood vessels in trachea under same conditions as (A). Staining for Prox1 (green) in (B, C) marks lymphatic endothelial cell nuclei. Scale bar, 50 μm.



Supplemental Figure 3. Organ-specific differences in lymphangiogenesis in CCSP/VEGF-C mice and time-course of rapamycin effects on lymphangiectasia. (A-D) Lymphangiogenesis in trachea but not other organs of CCSP/VEGF-C mice. Confocal microscopic images comparing lymphatics stained for LYVE-1 (green) and Prox1 (red) in organs of normal mice and CCSP/VEGF-C mice on doxycycline (7 days, P21-P28). **(A)** Trachea has segmental lymphatics in the normal mouse but has confluent lymphatics due to widespread lymphangiogenesis in the CCSP/VEGF-C mouse. No differences in lymphatics were evident in ear skin **(B)** or diaphragm on either the pleural surface **(C)** or the peritoneal surface **(D)**. Scale bar, 200 μm. **(E-F)** Time course of lymphatic regression induced by rapamycin (green) assessed by extent of LYVE-1 staining **(E)** or Prox1-positive nuclei **(F)** in the tracheal mucosa. Controls (red) are CCSP/VEGF-C mice treated with vehicle for various durations. **(G)** Prox1 and **(H)** VEGFR-3 fluorescence intensities as in Figure 8D, E, but showing individual vehicle groups for each treatment. Each dot is the value for one mouse. *$P < 0.05$ vs. vehicle, ANOVA, n = 2-11 mice/group.



Supplemental Figure 4. Lack of p-mTOR, p-S6RP, and p-4E-BP-1 staining in lymphatics of CCSP/VEGF-C mice after withdrawal of doxycycline. (A) Little or no p-mTOR staining in lymphatics after vehicle or rapamycin (14 days, P42-P56). **(B)** Strong p-mTOR staining in epithelium (7 days, P42-P49). **(C)** Little or no staining for p-S6RP staining in lymphatics after vehicle or rapamycin (14 days, P42-P56). **(D)** Strong p-S6RP staining in epithelium and tracheal smooth muscle (7 days, P42-P49). **(E)** Strong p-S6RP staining in epithelium and CD45-positive leukocytes (arrows) (7 days, P42-P49). **(F)** Significant reduction in number of p-S6RP-positive CD45 cells after rapamycin (7 days, P42-P49). $*P < 0.05$; t-test, n = 5 mice/group. **(G)** Little or no staining for p-4E-BP1 in lymphatics after withdrawal of doxycycline (14 days, P42). **(H)** En face confocal microscopic images of the epithelium of tracheal wholemounts. Two left panels show strong p-mTOR and p-S6RP staining after withdrawal of doxycycline (28 days, P56). Two right panels show strong p-4E-BP1 staining after doxycycline (3 days, P24) with monoclonal rabbit antibody to p-4E-BP1 and absence of staining when the antibody was pre-incubated with a blocking peptide. Scale bar, 50 μm in A, C; 500 μm in B, D; 20 μm in E; 200 μm in G, H.



Supplemental Figure 5. Lack of BrdU and activated caspase-3 staining in lymphatics of CCSP/VEGF-C mice after withdrawal of doxycycline. (A-C) Few or no BrdU-labeled (red) lymphatic endothelial cells (Prox1, green; LYVE-1, blue) in onset control (no doxycycline 14 days, P42) **(A)** or after treatment with vehicle **(B)** or rapamycin **(C)** (14 days, P56). Arrow marks one lymphatic endothelial cell with Prox1 and BrdU labeling. Arrowheads mark BrdU labeling of non-lymphatic cells (Prox1/LYVE-1 negative), which are equally numerous with or without rapamycin. **(D-F)** Absence of activated caspase-3 staining of lymphatic endothelial cells (Prox1, red; LYVE-1, blue) after rapamycin for 1 day (P43) **(D)** or after vehicle **(E)** or rapamycin **(F)** for 14 days (P56). Arrows mark activated caspase staining of sparse non-lymphatic cells (Prox1/LYVE-1 negative). **(G)** Counts showing absence of activated caspase-3 labeling of lymphatic endothelial cells (Prox1-positve) but labeling of some other cells, n = 3-5 mice/group. **(H)** Positive control showing activated caspase-3 labeling of CD45-positive leukocytes (arrow) in trachea of CCSP/VEGF-C mouse treated with rapamycin (7 days, P49). Scale bar, 50 μm in A-F and H.

Supplemental Table 1: Antibodies used for Immunohistochemistry

Antigen	Host species	Vendor	Catalog #	Dilution
Lyve-1	Rabbit	AngioBio	11-034	1:500
Lyve-1	Rat	R&D Systems	MAB2125	1:200
Lyve-1	Goat	R&D Systems	AF2125	1:500
Prox1	Goat	R&D Systems	AF2727	1:500
Prox1	Rabbit	AngioBio	11-033	1:500
VEGFR-2	Goat	R&D Systems	AF743	1:200
VEGFR-3	Goat	R&D Systems	AF644	1:500
Neuropilin-2	Goat	R&D Systems	AF567	1:500
BrdU	Rat	Abcam	ab6326	1:500
Activated caspase-3	Rabbit	R&D Systems	AF835	1:500
Phospho-4E-BP1	Rabbit	Cell Signaling	2855	1:500
Phospho-S6RP	Rabbit	Cell Signaling	4858	1:500
Phospho-mTOR	Rabbit	Cell Signaling	2976	1:500
Podocalyxin	Goat	R&D Systems	AF1556	1:500
GFP	Chicken	Aves	GFP 1020	1:1000
Pecam1	Armenian hamster	Thermo	MA3105	1:500
CD45	Rat	eBioscience	14-0451-85	1:500
E-cadherin	Rat	Invitrogen	131900	1:1000