Supplemental Figures

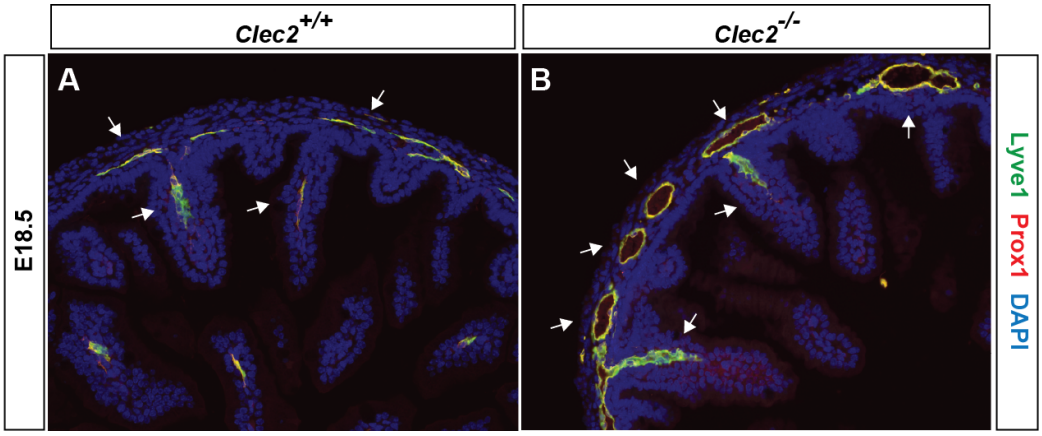


Figure S1: Lymphatic vessel growth into the intestine is normal in *Clec2* $^{-/-}$ embryos Staining of lymphatic vessels in E18.5 embryonic gut. Sections stained for lymphatic EC markers LYVE1 (green) and PROX1 (red) and with DAPI to mark cell nuclei (blue). White arrows indicate lymphatic vessels. Images are representative of two animals analyzed per genotype.

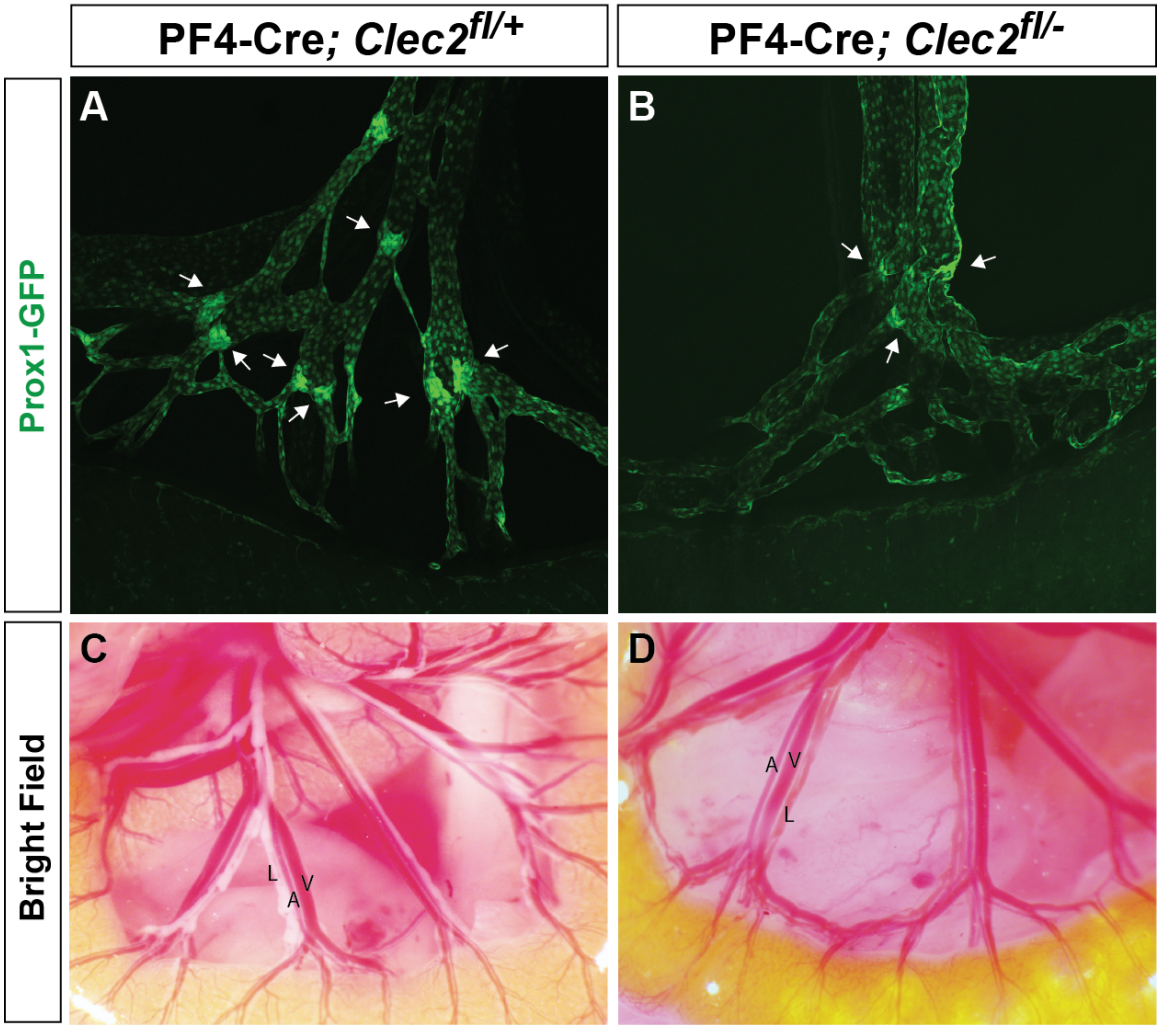


Figure S2: Lymphatic valve development is impaired in *Clec2* $^{fl/-}$; PF4-Cre+ mice. (A-B) Imaging of lymphatic valve development in neonatal *Clec2* $^{fl/-}$; PF4-Cre+ mice compared to *Clec2* $^{fl/+}$; PF4-Cre+ littermates on a Prox1-GFP BAC transgenic background is shown. Lymphatic vessels are marked by Prox1-GFP expression in LEC. White arrows indicate clusters of PROX1-high LEC that mark nascent lymphatic valves. (C-D) Bright field imaging of conditional knockout mice shows blood-filled lymphatics like those observed in constitutive CLEC2-deficient animals. A=artery, V=vein, L=lymphatic. N= 3 mice per genotype.

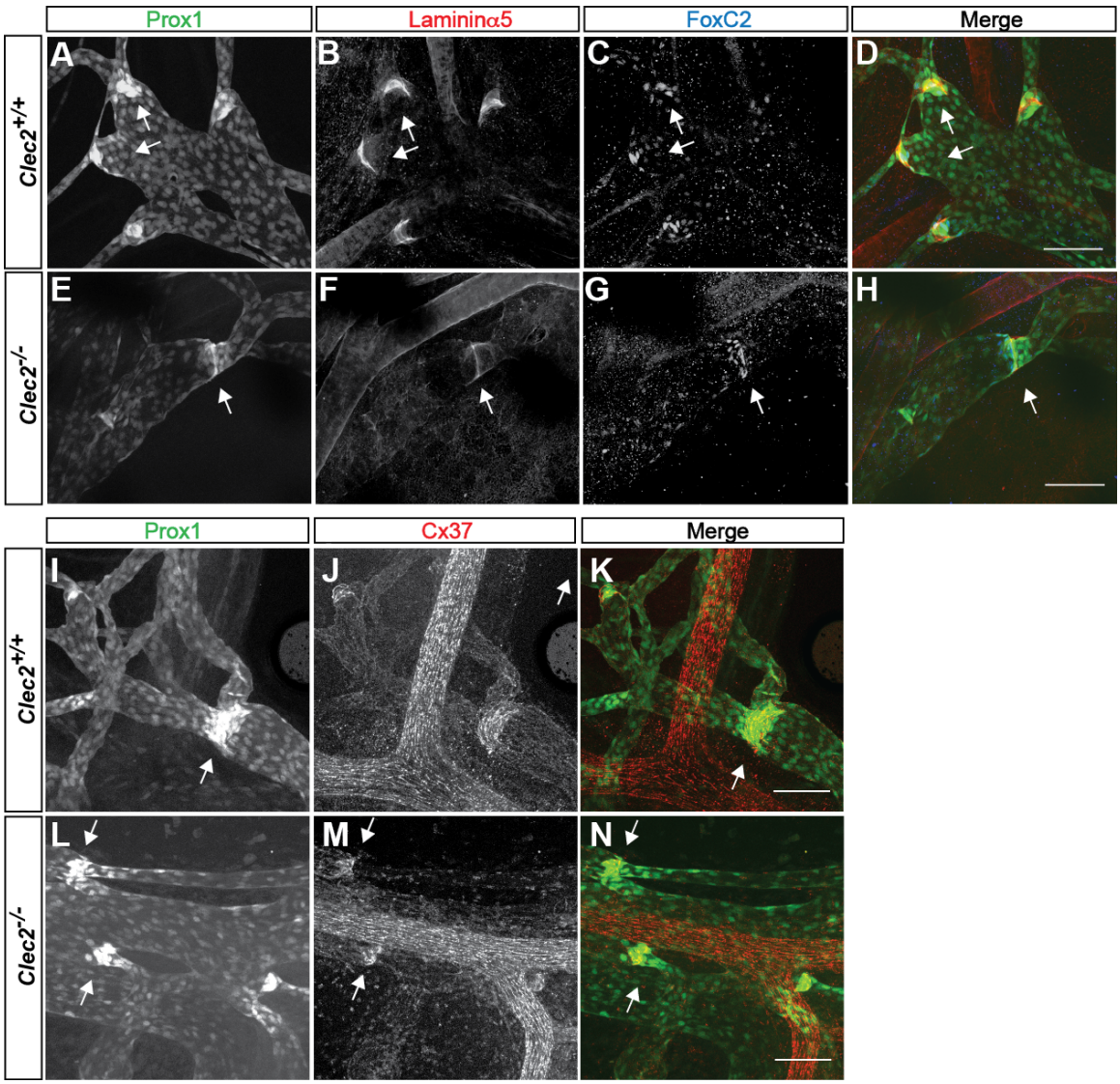


Figure S3: Molecular characterization of lymphatic valves in E18.5 *Clec2* $^{-/-}$ mice. (A-H) Confocal microscopy Z-stacks of lymphatic valves in E18.5 mice stained for PROX1 (green), Laminin α5 (red) and FOXC2 (blue). Arrows indicate lymphatic valves. Laminin α5 (red) shows valve leaflet structure, which is ring shaped in *Clec2* $^{-/-}$ mice. (I-N) Confocal microscopy Z-stacks of lymphatic valves in E18.5 mice stained for PROX1 (green) and Cx37(red). Scale bar = 100 microns

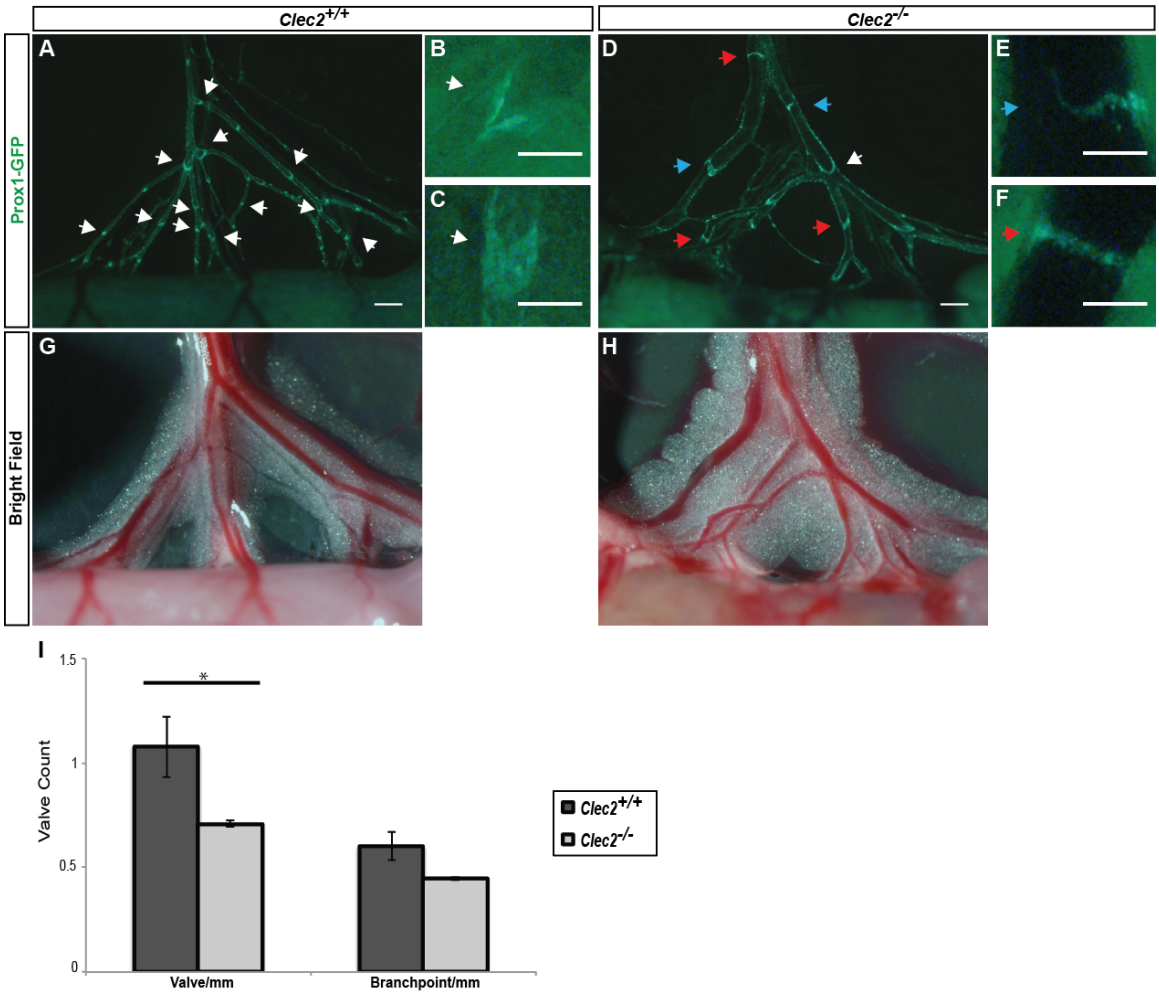


Figure S4: Analysis of lymphatic valves in 5 week old *Clec2* $^{-/-}$ mice. (A-F) Fluorescent imaging of PROX1-GFP expression in lymphatic ECs and lymphatic valves in (A-C) Control and (D-F) *Clec2* $^{-/-}$ mice that survived to 5 weeks of age. Lymphatic valves in Control mice are mostly V-shaped (white arrows) whereas *Clec2* $^{-/-}$ valves are immature in structure with ring-like shape (red arrows) or asymmetric leaflets (blue arrows). Scale bar (A,D)= 500 microns. Scale bar (B,C,E,F)= 25 microns. (G-H) Bright field imaging showing the presence of blood in lymphatic vessels of *Clec2* $^{-/-}$ mouse but not *Clec2* $^{+/+}$ mouse. (I) Quantitation of lymphatic valves per mm of vessel length and branchpoints per mm of vessel length. N=2 mice per genotype. All values expressed as means +/- SEM. *p<0.05.

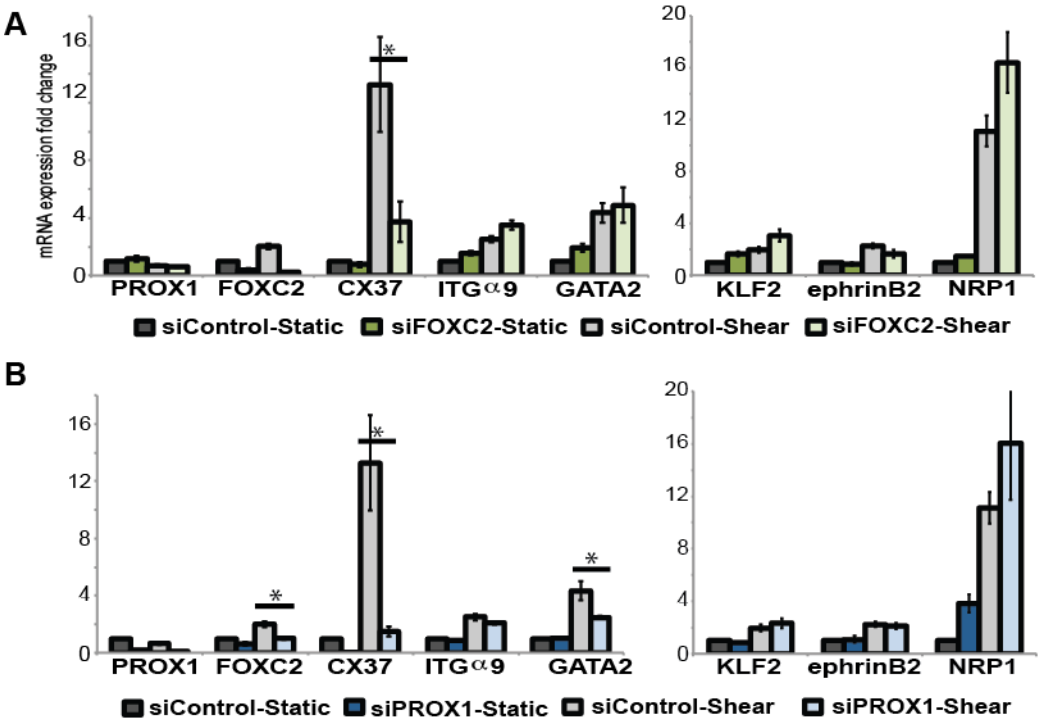


Figure S5: FOXC2 and PROX1 are required for LEC valve gene expression in response to lymphatic shear. Changes in LEC valve gene expression program induced by lymphatic fluid shear following siRNA knockdown of *FOXC2*. Gene expression was measured following transfection with siFoxC2 or control siRNA ("siControl") in static LEC or LEC exposed to lymphatic flow for 24 hours. N=4 independent experiments. (B) Changes in LEC valve gene expression induced by lymphatic fluid shear following siRNA knockdown of *PROX1*. Studies were performed as described in (A). N= 4. (C-D) All values are means +/- SEM. *p<0.05.

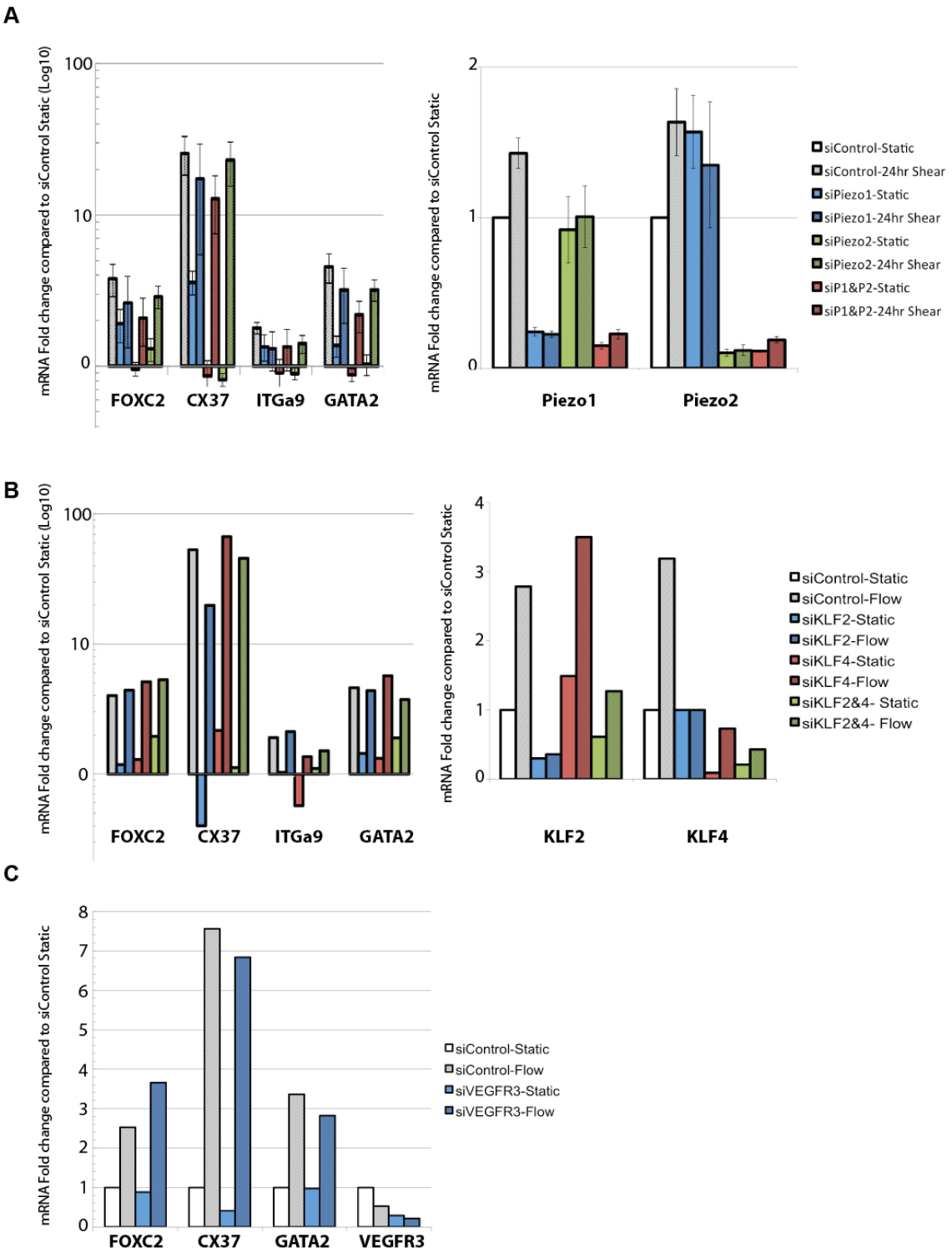
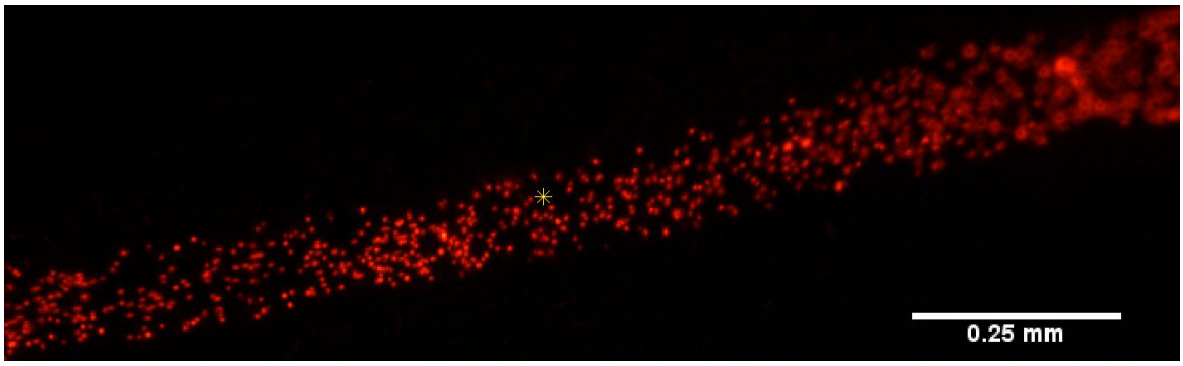
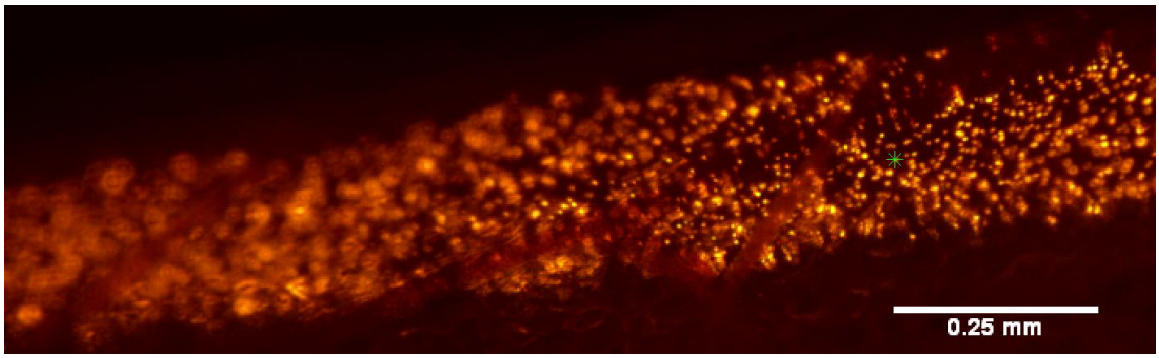


Figure S6: No role for mechanosensitive proteins in lymphatic shear-induced valve gene upregulation in LEC. (A) Changes in LEC valve gene expression program induced by lymphatic fluid shear following siRNA knockdown

of mechanosensitive ion channels *PIEZO1* and *PIEZO2*. Gene expression was measured following transfection with siPIEZO1 or siPIEZO2 or siPIEZO1+2 or control siRNA in static LEC or LEC exposed to lymphatic flow for 24 hours. Gene expression fold change compared to siControl-Static condition plotted on log10 scale (left) and gene knockdown plotted as fold change (right). N=4 independent experiments. (B) Changes in LEC valve gene expression following siRNA knockdown of mechanosensitive transcription factors *KLF2* and *KLF4*. Gene expression was measured following transfection with siKLF2 or siKLF4 or siKLF2+4 or control siRNA in static LEC or LEC exposed to lymphatic flow for 24 hours. Gene expression fold change compared to siControl-Static condition plotted on log10 scale (left) and gene knockdown plotted as fold change (right). N=2 independent experiments. (C) Changes in LEC valve gene expression program induced by lymphatic fluid shear following siRNA knockdown of mechanosensitive RTK *VEGFR3 (FLT4)*. Gene expression was measured following transfection with siVEGFR3 or control siRNA in static LEC or LEC exposed to lymphatic flow for 24 hours. Gene expression fold change compared to siControl-Static condition is plotted. N=2 experiments.



Movie S1: Lymphatic Flow in control animals is pulsatile with net forward displacement and a short period of reversal. 1 micron fluorescent microparticles were injected into lymph node and tracked as they flow through downstream efferent lymphatic vessel. Representative movie of one contraction cycle in control Clec2 $^{fl/fl}$ mouse.



Movie S2: Lymphatic Flow in Clec2 knockout mice is pulsatile with net backward displacement. 1 micron fluorescent microparticles were injected into lymph node and tracked as they flow through downstream efferent lymphatic vessel. Representative movie of one contraction cycle in conditional knockout Clec2 $^{fl/fl}$; PF4-Cre mouse that exhibited blood-filled lymphatic vessels.