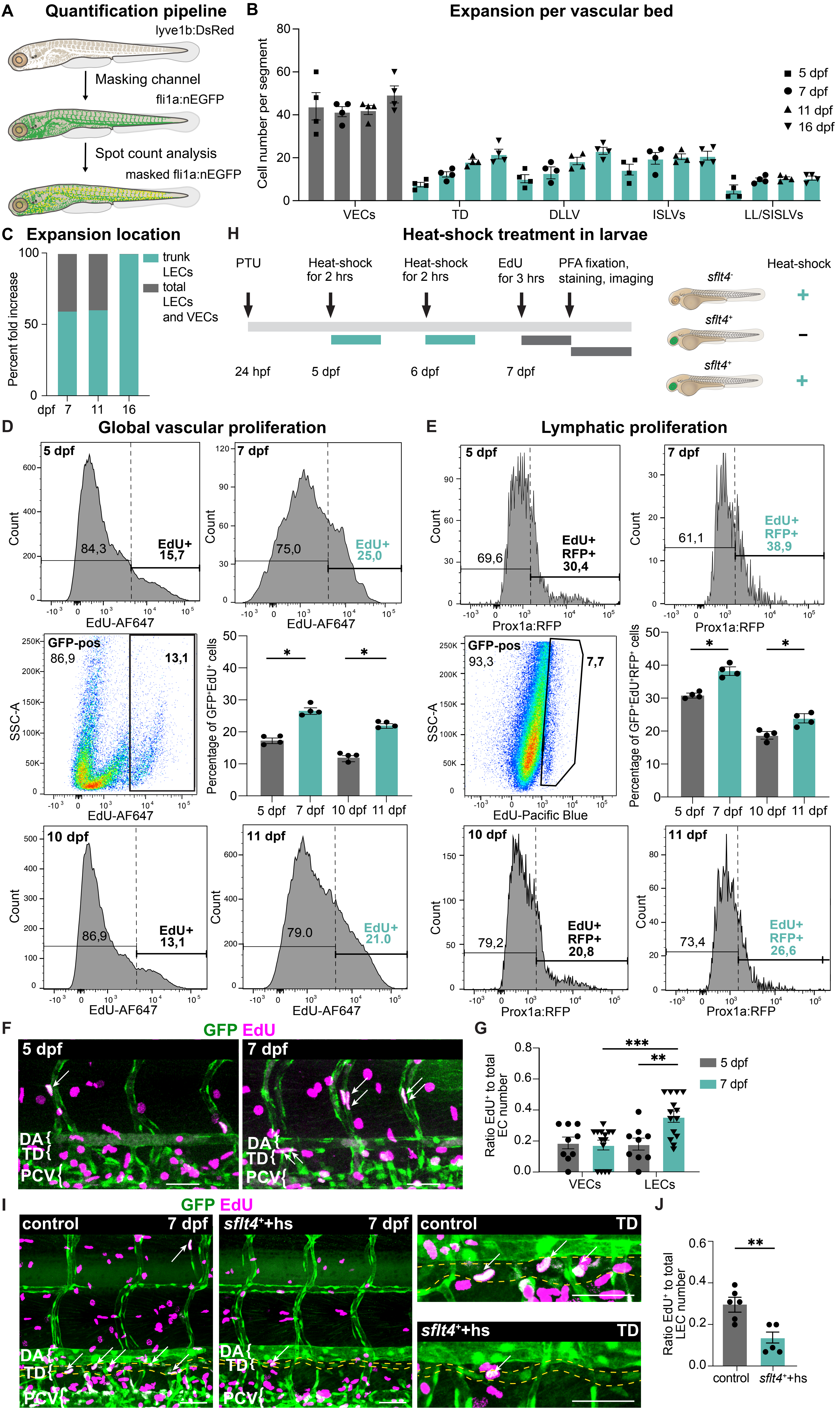
Suppl. Figure 1



Suppl. Figure 1.

(A) Schematic illustration of the quantification pipeline using surface rendering of the *Tg(-5.2lyve1b:DsRed2)*^{nz101} venous and lymphatic vasculature, channel masking into the *Tg(fli1a:EGFP)*^{y7} nuclei, and spot count analysis in the Imaris software to quantify the total number of VEC and LEC nuclei in the whole zebrafish during embryonic and larval development.

(B) Manual quantifications of VEC and LEC numbers in the different vascular beds in trunk two-somite segments (yellow boxes from **(A), (B), (C), (D)**) at 5 dpf, 7 dpf, 11 dpf and 16 dpf. n = 4 for each time point. Unpaired two-tailed Student's t-test was used for statistical analysis. ns = no significance $p > 0.05$.

(C) Contribution of lymphatic vascular expansion in the trunk (marked in teal) compared to all venous and lymphatic vascular expansion in the whole zebrafish (marked in grey) at 7 dpf, 11 dpf and 16 dpf.

(D) FACS cell cycle analysis measuring percentage of EdU-positive (AF647) cells of all GFP-positive endothelial cells by *Tg(fli1a:EGFP)*^{y7} with gating strategy, histograms and quantification at 5 dpf, 7 dpf, 10 dpf and 11 dpf. n = 4 for each time point. Mann-Whitney test was used for statistical analysis. ns = no significance $p > 0.05$, *p = 0.0286 for ECs from 5 to 7 dpf, *p = 0.0286 for ECs from 10 to 11 dpf.

(E) FACS cell cycle analysis measuring percentage of EdU-positive (Pacific Blue) cells of *fli1a*$^{+}$*prox1a*$^{+}$ LECs (RFP/GFP-double-positive) using *TgBAC(prox1a:KalTA4-4xUAS-ADV.E1b:TagRFP)*^{nim5};*Tg(fli1a:EGFP)*^{y7} background fish, showing gating strategy, histograms and quantification at 5 dpf, 7 dpf, 10 dpf and 11 dpf. n = 4 for each time point. Mann-Whitney test was used for statistical analysis. ns = no significance $p > 0.05$, *p = 0.0286 for LECs from 5 to 7 dpf, *p = 0.0286 for LECs from 10 to 11 dpf.

(F) Confocal images in the zebrafish trunk of immunostainings of endothelial cell nuclei for GFP-expressing transgenic *Tg(fli1a:EGFP)*^{y7} fish (green) and incorporated EdU (magenta) showing cell proliferation at 5 dpf and 7 dpf. White arrows indicate GFP^{+}EdU^{+} proliferating LECs in ISLVs and TD. PCV, posterior cardinal vein; TD, thoracic duct; DA, dorsal aorta. Scale bar: 100 µm.

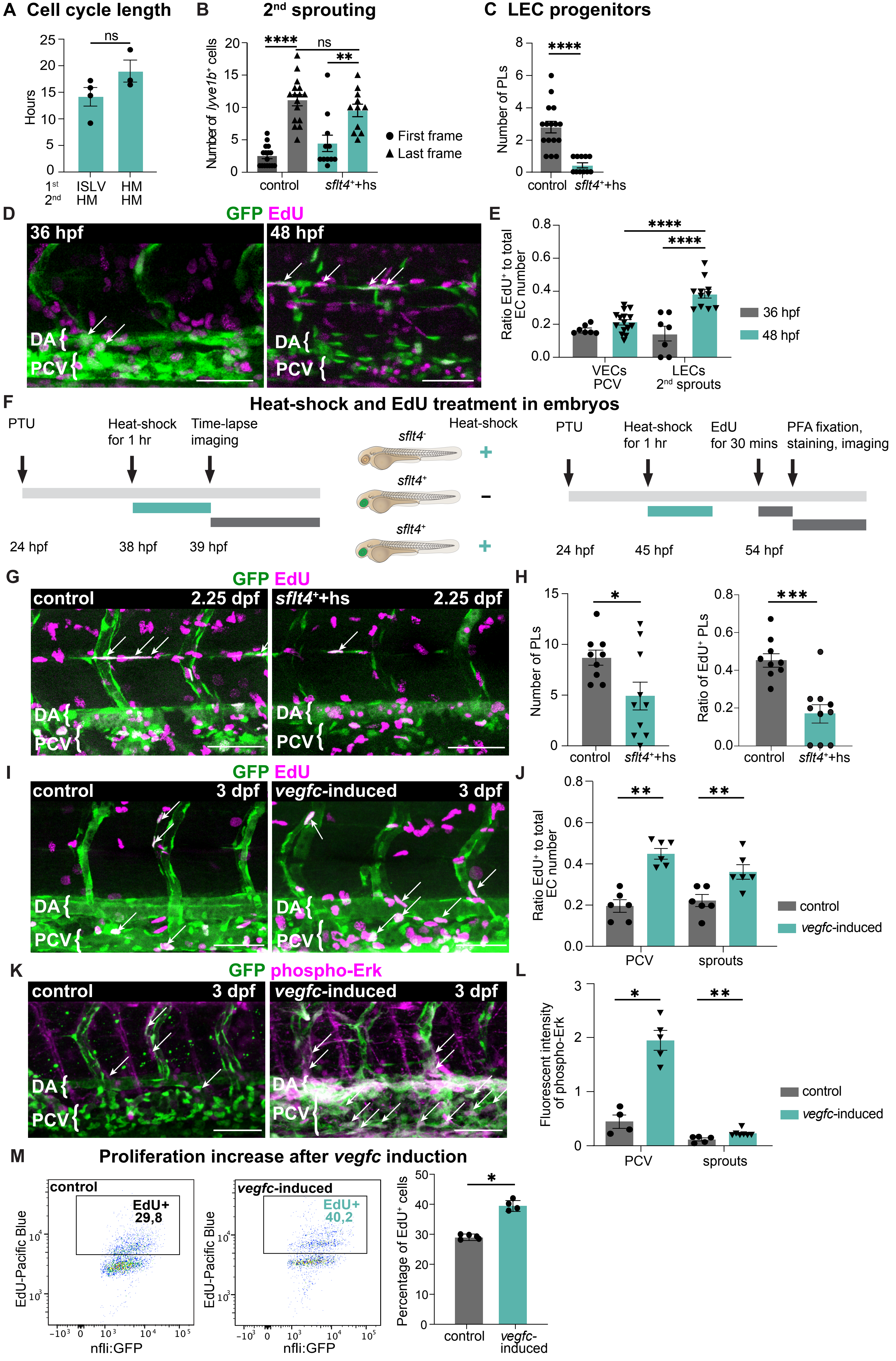
(G) Quantification of the ratio of EdU-positive endothelial cells (GFP^{+}EdU^{+}) to total EC (VEC or LEC) number at 5 dpf and 7 dpf in the trunk from (D). n = 9-13 for each time point. Two-way ANOVA test was used for statistical analysis. ns = no significance $p > 0.05$, **p = 0.0011 for LECs from 5 to 7 dpf, ***p = 0.0006 for comparing VECs to LECs at 7 dpf.

(H) Schematic illustration for the heat-shock treatment prior to EdU incubation in larvae. All embryos were treated with PTU at 24 hpf. Control and *sflt4*$^{+}$ larvae were heat-shocked for two hours at 5 and 6 dpf. *sflt4*$^{+}$ larvae without heat-shock treatment and heat-shocked *sflt4*$^-$ larvae were used as control. Larvae of all conditions were incubated with EdU for three hours prior to fixation in PFA at 7 dpf.

(I) Confocal images in the zebrafish trunk of immunostainings of endothelial cell nuclei for GFP-expressing transgenic *Tg(fli1a:EGFP)*^{y7} fish (green) and incorporated EdU (magenta) showing cell proliferation at 7 dpf without (control) and with (*sflt4*$^+$+hs) soluble Flt4 expression due to a *Tg(hsp70l:flt4,cryaa:Cerulean)*^{bns82} background. White arrows indicate GFP$^+$EdU$^+$ proliferating LECs in ISLVs and TD. Yellow dashed line marks the TD which shows formation deficiency upon Vegfc trap-induction. **On the right**, zoom-in images of the TD area are shown for both conditions. PCV, posterior cardinal vein; DA, dorsal aorta; TD, thoracic duct. Scale bar: 100 µm.

(J) Quantification of the ratio of EdU-positive LECs (GFP$^+$EdU$^+$) to total LEC number at 7 dpf in the trunk from **(I)**. n = 5-6 for each condition. Mann-Whitney test was used for statistical analysis. **p = 0.0087.

Suppl. Figure 2



Suppl. Figure 2.

(A) Quantification of cell cycle length of LECs and PLs from time-lapse videos in **Figure 2A**, with the first division in ISLV and second division in HM (n = 3) or first and second division both in HM. Unpaired two-tailed Student's t-test was used for statistical analysis. ns = no significance $p > 0.05$.

(B) Quantification of total number of sprouting *lyve1b*+ cells from time-lapse videos in **Figure 2D**. The quantification was performed at the first frame and the last frame of the movie in embryos without (control, n = 16) and with (*sflt4*$^{+}$+hs*,* n = 11) soluble Flt4 expression. Mann-Whitney test was used for statistical analysis. ns = no significance $p > 0.05$, **$p = 0.0025$, ****$p < 0.0001$.

(C) Quantification of PL number in HM from time-lapse videos in **Figure 2D**. The quantification was performed at the last frame of the movie in embryos without (control, n = 16) and with (*sflt4*$^{+}$+hs*,* n = 11) soluble Flt4 expression. Unpaired two-tailed Student's t-test was used for statistical analysis. ****$p < 0.0001$.

(D) Confocal images in the zebrafish trunk of immunostainings of endothelial cell nuclei for GFP-expressing transgenic *Tg(fli1a:EGFP)*^{y7} fish (green) and incorporated EdU (magenta) showing cell proliferation at 36 hpf and 48 hpf. White arrows indicate GFP^{+}EdU^{+} proliferating progenitor LECs in secondary sprouts or HM. PCV, posterior cardinal vein; DA, dorsal aorta. Scale bar: 100 µm.

(E) Quantification of the ratio of EdU-positive endothelial cells (GFP^{+}EdU^{+}) to total EC (VEC or LEC) number at 36 hpf and 48 hpf in the trunk from **(D)**. n = 7-15 for each time point. Two-way ANOVA test was used for statistical analysis. ns = no significance $p > 0.05$, ****$p < 0.0001$.

(F) Schematic illustration of the heat-shock treatment. All embryos were treated with PTU at 24 hpf. **Left**, embryo treatment for time-lapse imaging. Control and *sflt4*$^{+}$ embryos were heat-shocked for one hour before imaging from 39 hpf. *sflt4*$^{+}$ embryos without heat-shock treatment and heat-shocked *sflt4*$^{-}$ embryos were used as control. **Right**, embryo treatment for immunofluorescent staining. Control and *sflt4*$^{+}$ embryos were heat-shocked for one hour at 45 hpf. *sflt4*$^{+}$ embryos without heat-shock treatment and heat-shocked *sflt4*$^{-}$ embryos were used as control. Embryos of all conditions were incubated with EdU for 30 mins at 54 hpf prior to fixation in PFA.

(G) Confocal images in the zebrafish trunk of immunostainings of endothelial cell nuclei for GFP-expressing transgenic *Tg(fli1a:EGFP)*^{y7} fish (green) and incorporated EdU (magenta) showing cell proliferation at 2.25 dpf without (control) and with (*sflt4*$^{+}$+hs) soluble Flt4 expression due to a *Tg(hsp70l:flt4,cryaa:Cerulean)*^{bns82} background. White arrows indicate GFP$^+$EdU$^+$ proliferating PLs in HM. PCV, posterior cardinal vein; DA, dorsal aorta. Scale bar: 100 µm.

(H) Quantification of the total PL number at 2.25 dpf in the trunk HM from **(G)**. n = 7-9 for each condition. Mann-Whitney test was used for statistical analysis. *p = 0.0494 **(left)**. Quantification of the ratio of EdU-positive endothelial cells (GFP$^+$EdU$^+$) to total PL number at 2.25 dpf in the trunk HM from **(G)**. n = 9-10 for each condition. Mann-Whitney test was used for statistical analysis. ***p = 0.0007 **(right)**.

(I) Confocal images in the zebrafish trunk of immunostainings of endothelial cell nuclei for GFP-expressing transgenic *Tg(fli1a:EGFP)*^{y7} fish (green) and incorporated EdU (magenta) showing cell proliferation at 3 dpf without (control) and with *vegfc* induction due to a *TgBAC(prox1a:KalTA4)*^{uq3bh};*Tg(10xUAS:vegfc,cryaa:GFP)* background. White arrows indicate GFP$^+$EdU$^+$ proliferating LECs. PCV, posterior cardinal vein; DA, dorsal aorta. Scale bar: 100 µm.

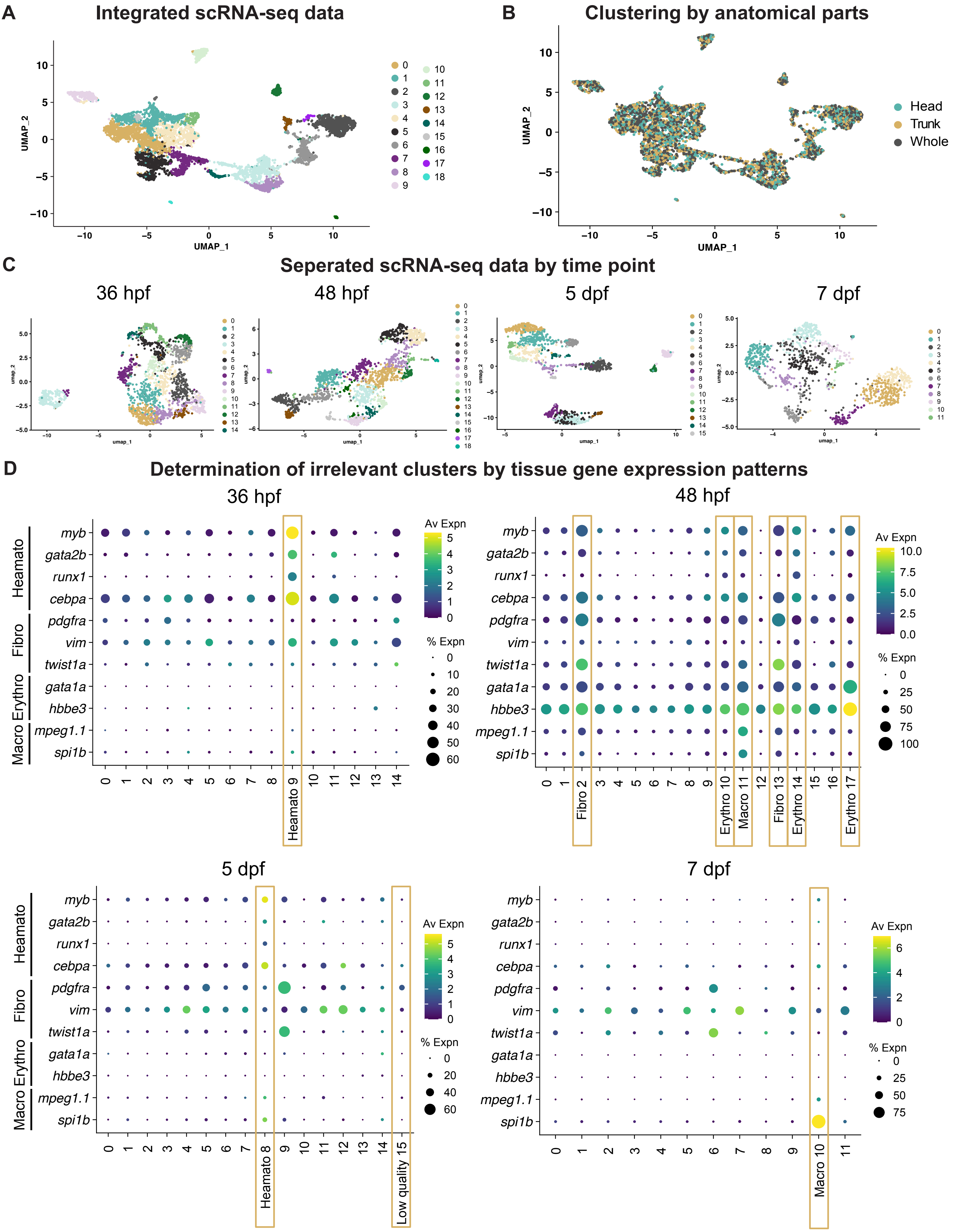
(J) Quantification of the ratio of EdU-positive endothelial cells (GFP$^+$EdU$^+$) to total EC number at 3 dpf in PCV and sprouts in the trunk from **(I)**. n = 5-6 for each condition. Multiple Mann-Whitney test was used for statistical analysis. **p = 0.0022 for PCV, **p = 0.0065 for sprouts.

(K) Confocal images in the zebrafish trunk of immunostainings of endothelial cell nuclei for GFP-expressing transgenic *Tg(fli1a:EGFP)*^{y7} fish (green) and phospho-Erk (magenta) at 3 dpf without (control) and with *vegfc* induction due to a *TgBAC(prox1a:KalTA4)*^{uq3bh};*Tg(10xUAS:vegfc,cryaa:GFP)* background. White arrows indicate phospho-Erk-positive PCV and sprouts. PCV, posterior cardinal vein; DA, dorsal aorta. Scale bar: 100 µm.

(L) Quantification of the fluorescent intensity of phospho-Erk immunostaining per vessel area in x10^6 at 3 dpf in PCV and sprouts in the trunk from **(K)**. n = 4-6 for each condition. Multiple Mann-Whitney test was used for statistical analysis. *p = 0.0159 for PCV, **p = 0.0025 for sprouts.

(M) FACS cell cycle analysis measuring percentage of EdU-positive (Pacific Blue) ECs in *Tg(fli1a:EGFP)*^{y7} (GFP-positive) fish at 3 dpf without (control) and with *vegfc* induction due to a *TgBAC(prox1a:KalTA4)*^{uq3bh};*Tg(10xUAS:vegfc,cryaa:GFP)* background showing gating strategy and quantification. n = 4 for each condition. Mann-Whitney test was used for statistical analysis. *p = 0.0159.

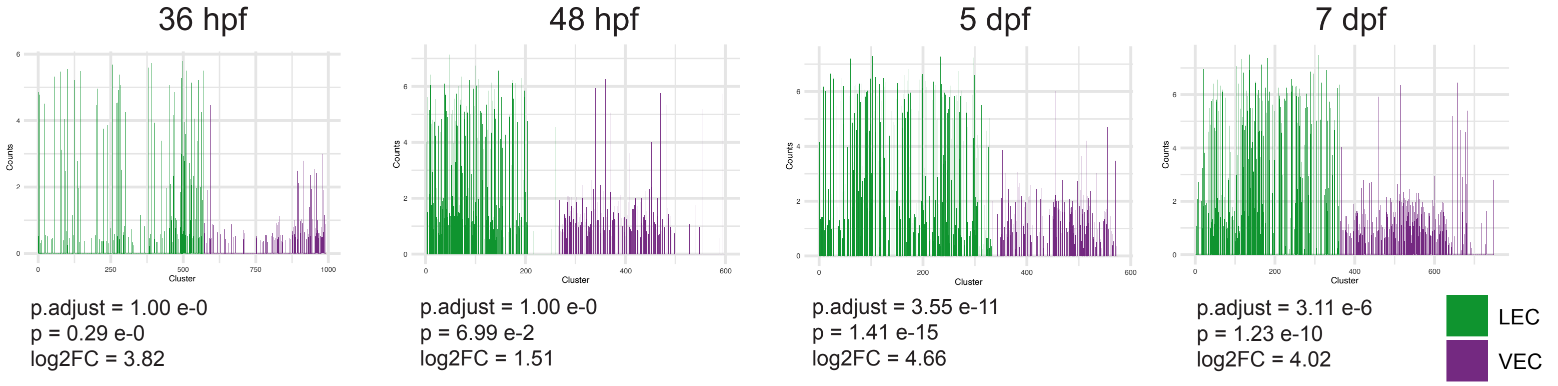
Suppl. Figure 3



Suppl. Figure 3

E

Cell counts of *prox1a* expression for the VEC and LEC clusters



36 hpf: p.adjust = 1.00 e-0
p = 0.29 e-0
log2FC = 3.82

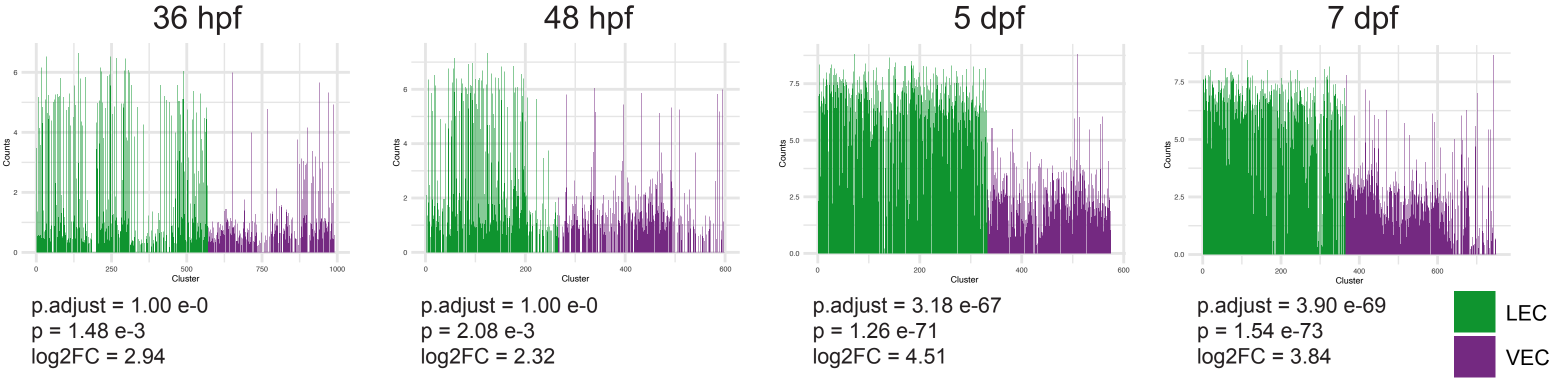
48 hpf: p.adjust = 1.00 e-0
p = 6.99 e-2
log2FC = 1.51

5 dpf: p.adjust = 3.55 e-11
p = 1.41 e-15
log2FC = 4.66

7 dpf: p.adjust = 3.11 e-6
p = 1.23 e-10
log2FC = 4.02

F

Cell counts of *cdh6* expression for the VEC and LEC clusters



36 hpf: p.adjust = 1.00 e-0
p = 1.48 e-3
log2FC = 2.94

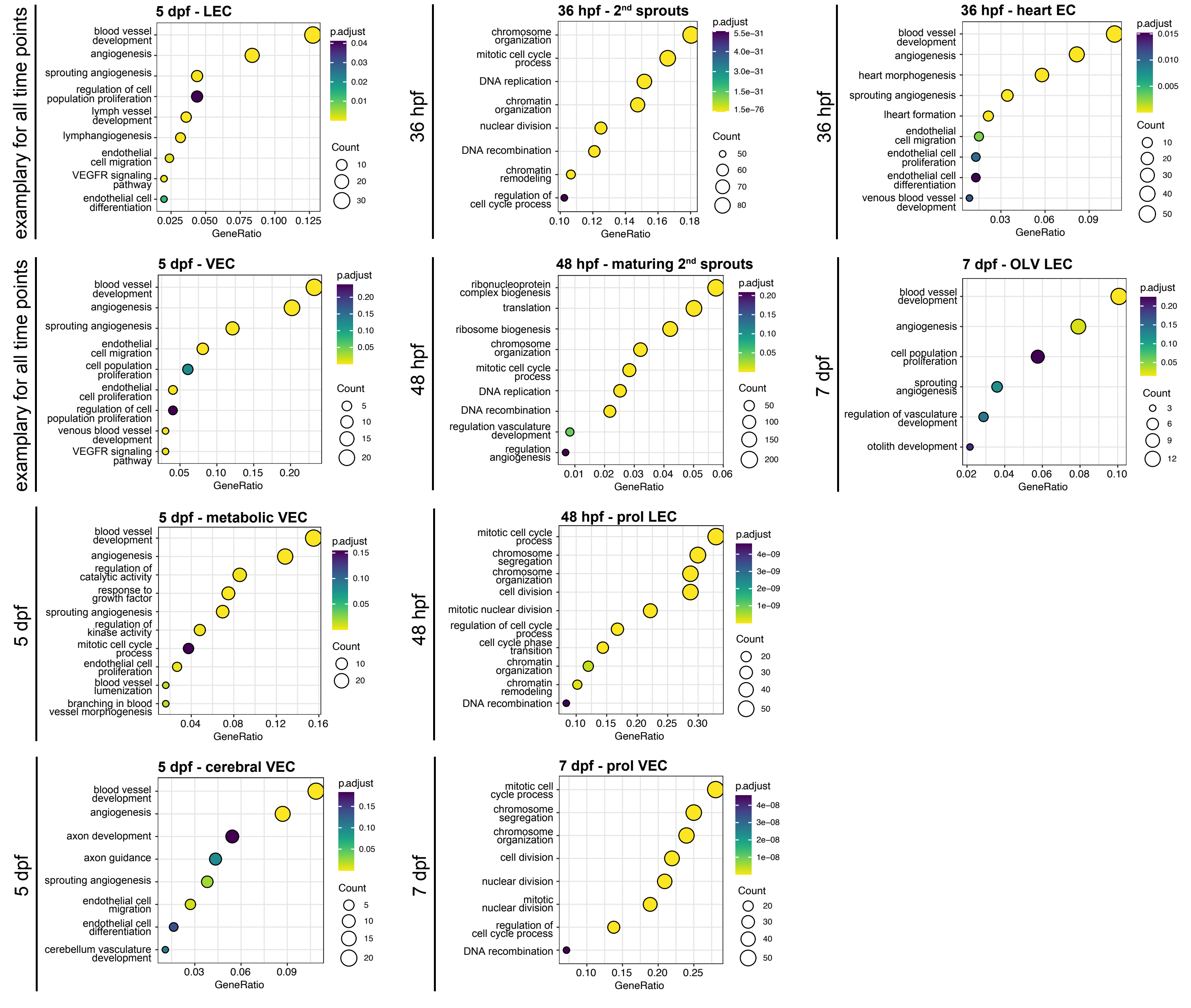
48 hpf: p.adjust = 1.00 e-0
p = 2.08 e-3
log2FC = 2.32

5 dpf: p.adjust = 3.18 e-67
p = 1.26 e-71
log2FC = 4.51

7 dpf: p.adjust = 3.90 e-69
p = 1.54 e-73
log2FC = 3.84

G

GO BP term analysis



Suppl. Figure 3.

(A) Whole scRNA-seq data set combined visualized in a UMAP landscape, before filtering out the contaminant clusters, labeled by cluster assignment.

(B) Whole scRNA-seq data set combined as in **(A)** visualized in a UMAP landscape, labeled by anatomical part cluster assignment (head, trunk, whole).

(C) Seperated scRNA-seq data sets for each developmental time point 36 hpf, 48 hpf, 5 dpf and 7 dpf visualized in a UMAP landscape, before filtering out the contaminant clusters clusters, labeled by cluster assignment.

(D) Dot plots indicating expression of key tissue markers (macrophages, erythrocytes, fibroblasts and heamatocytes) for each cluser of the seperate time point data sets as in **(C)** to determine contamination clusters. Dot size illustrates percentage of cells presenting transcript sequence counts (% Expn) and viridis scale color illustrates the average normalized expression (Av Expn) within a cluster.

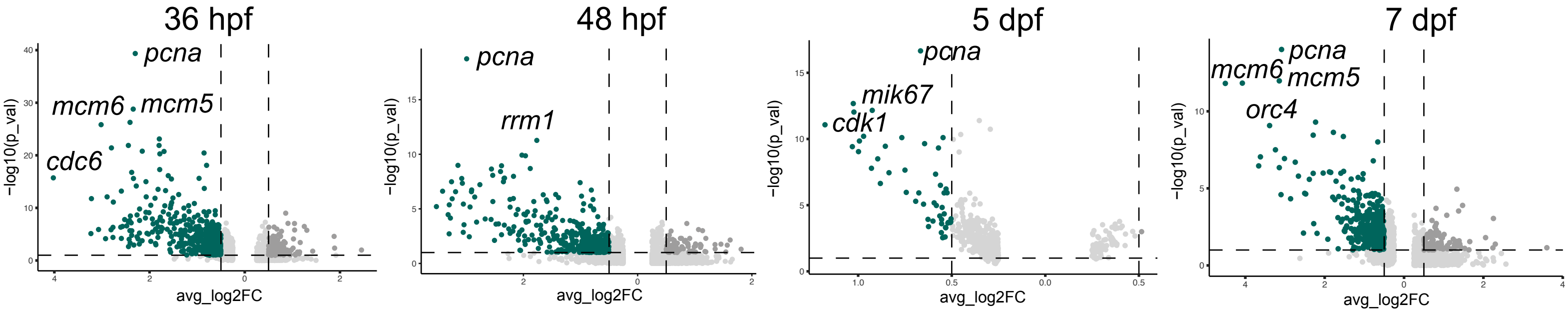
(E) Cell counts of *prox1a* expression as lymphatic marker, visualized for two clusters containing most differentiated LECs (green) or VECs (purple) at all developmental time points. Stated are adjusted p-value (p.adjust), p-value (p) and average expression log2 fold change (log2FC) between the two clusters.

(F) Cell counts of *cdh6* expression as lymphatic marker, visualized for two clusters containing most differentiated LECs (green) or VECs (purple) at all developmental time points. at all developmental time points. Stated are adjusted p-value (p.adjust), p-value (p) and average expression log2 fold change (log2FC) between the two clusters.

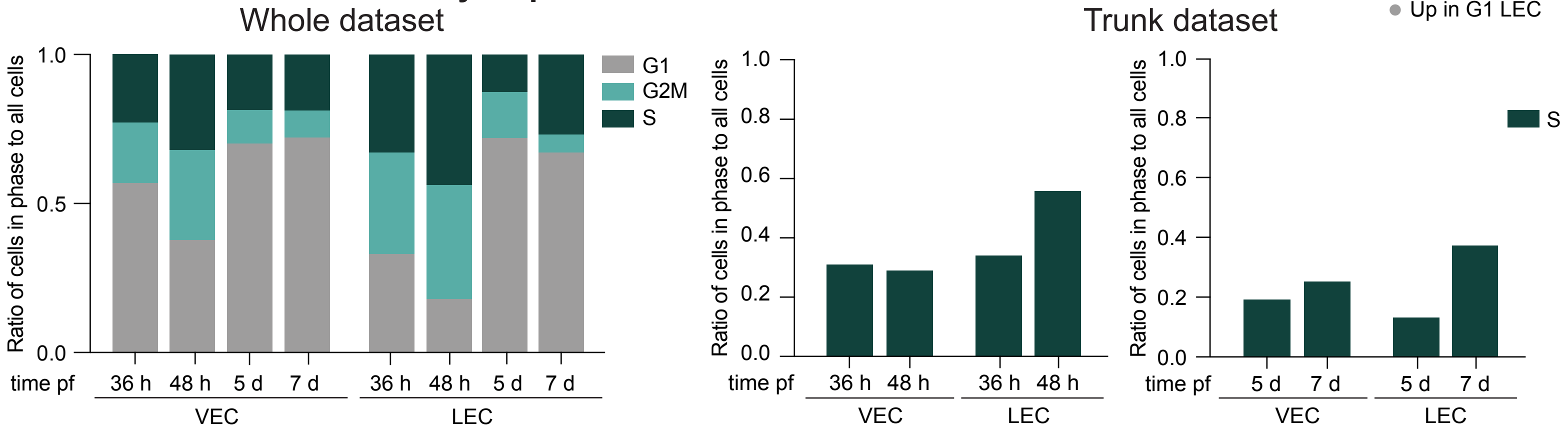
(G) GO term analysis for Biological Processes (BP) of genes enriched in the different clusters in comparison to each other of the developmental time points as in **Figure 3C**. Selected terms for enriched lymphatic or venous vessel and vasculature descriptions, angiogenetic and developmental processes, cell proliferation or other top enriched terms are shown. Y-axis represents enriched GO BP term and X-axis represents the enriched GeneRatio indicating fold enrichment. Dot size illustrates gene count number (Count) and viridis scale color illustrates adjusted p-value (p.adjust).

Suppl. Figure 4

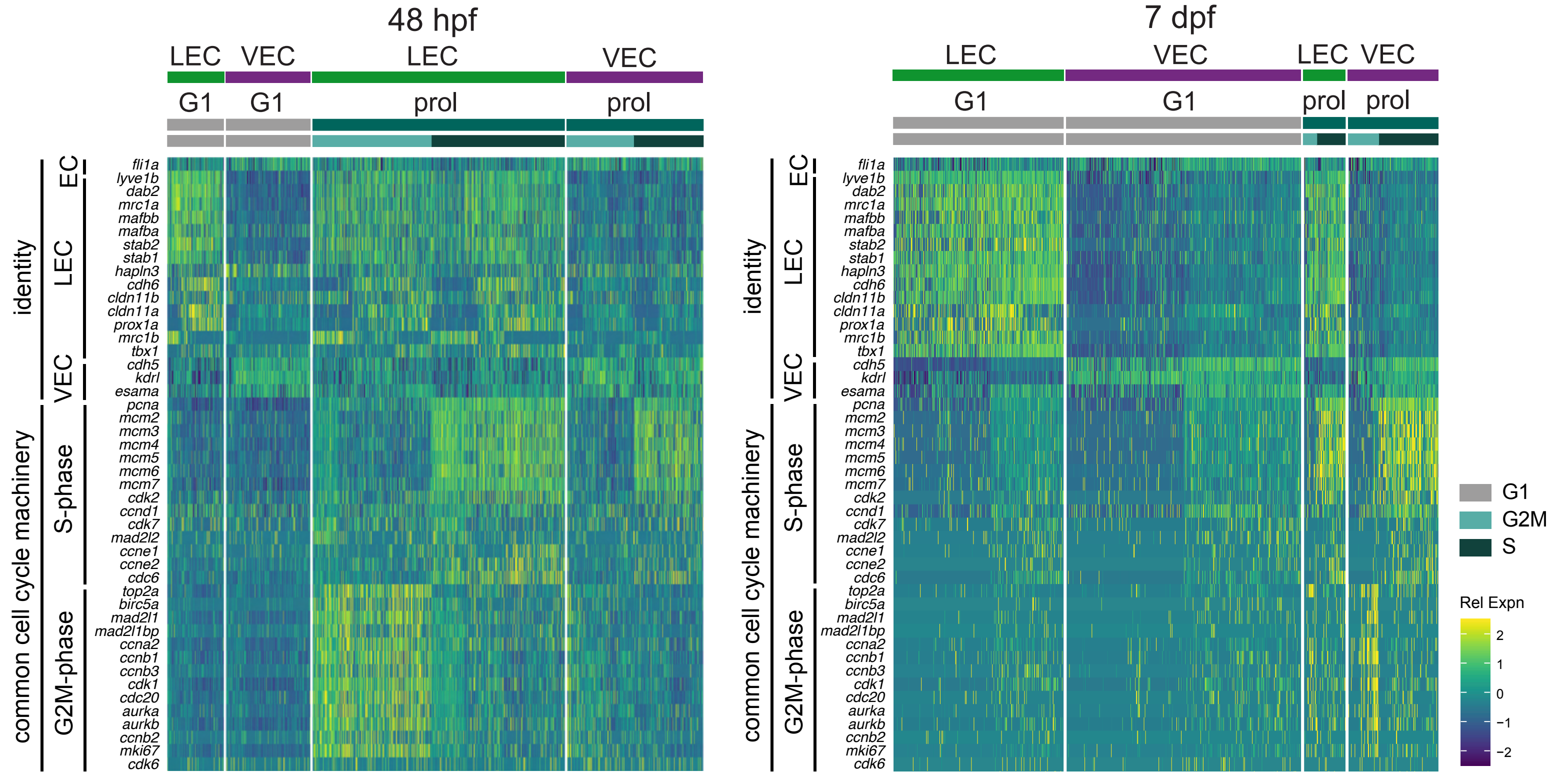
A DEG Vulcano plots of proliferating versus non-proliferating LECs



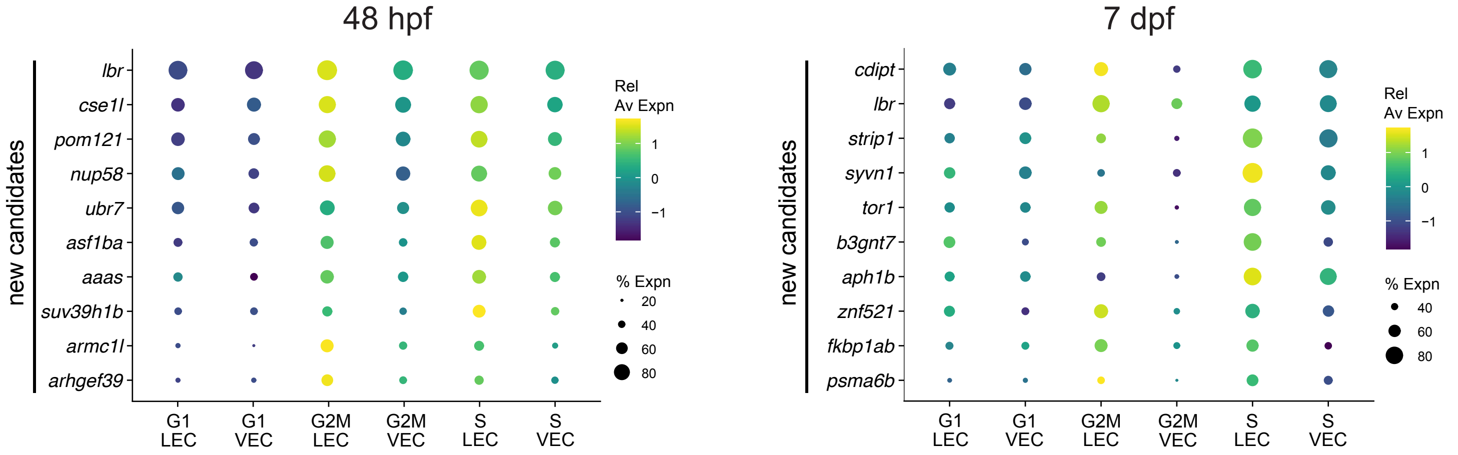
B Cell cycle phase distribution of VECs and LECs



C Common cell cycle machinery in combined VEC and LEC cluster



D LEC proliferation code in cell cycle phases



Suppl. Figure 4.

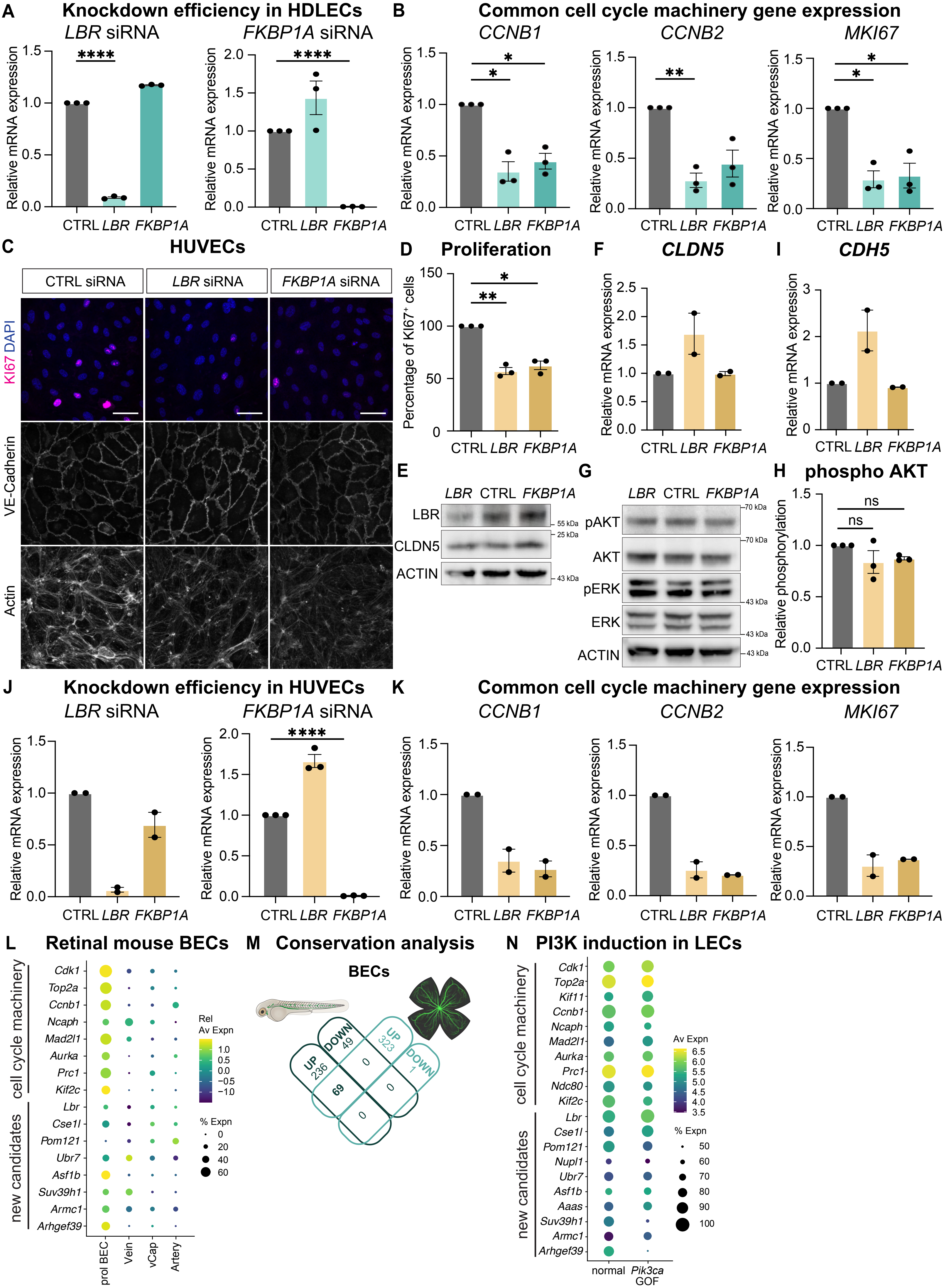
(A) Vulcano plots of differentially expressed genes (DEG) between proliferating (prol = teal) and non-proliferating (G1 = grey) LECs at the different deleopmental time points. Labeled dots represent important known common cell cycle machinery genes, significantly upregulated in prol LECs. Non-differentially expressed genes are plotted in platinum (average log2 fold change < +/-0.5 and p-value > 0.1).

(B) Proliferative state proportion of LEC and VEC populations at all developmental time points showing the ratio of cells in G1- (grey), G2M- (light teal) or S-phase (dark teal) **(left)**. Seperated transcriptomical data set of sorted trunks from *Tg(fli1a:nEGFP)*^{y7}*;Tg(-5.2lyve1b:DsRed)*^{nz101} fish only showing the ratio of LECs or VECs in S-phase (dark teal) at the different developmental time points **(middle, right)**. Population sizes are shown relative to total cell number (= 1).

(C) Heatmap showing the gene expression of endothelial, lymphatic and venous identity genes (EC, LEC and VEC) and known common cell cycle machinery regulators for S- or G2M-phase in the 48 hpf transcriptomic data set, after combining clusters from **Figure 3C** of same identity and proliferative state **(left)**. Heatmap showing the gene expression of endothelial, lymphatic and venous identity genes (EC, LEC and VEC) and known common cell cycle machinery regulators for S- or G2M-phase in the 7 dpf transcriptomic data set, after combining clusters from **Figure 3C** of same identity and proliferative state **(right)**. Cells were ordered by their assigned clusters depending on LEC or VEC identity and proliferative state (LEC = green, VEC = purple, G1 = grey, prol = teal with G2M-phase = light teal and S-phase = dark teal). Viridis scale color indicates relative normalized read counts in log-scale (Rel Expn).

(D) Dot plots indicating expression of new candidate genes (new candidates) from the LEC proliferation code in the 48 hpf transcriptomic data set for LEC and VEC clusters ordered by cell cycle phase (G1, G2M, S) **(left)**. Dot plots indicating expression of new candidate genes (new candidates) from the LEC proliferation code in the 7 dpf transcriptomic data set for LEC and VEC clusters ordered by cell cycle phase (G1, G2M, S) **(right)**. Dot size illustrates percentage of cells presenting transcript sequence counts (% Expn) and viridis scale color illustrates the relative average normalized expression (Rel Av Expn) within a cluster.

Suppl. Figure 5



Suppl. Figure 5.

(A) qRT-PCR analysis of cultured HDLECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing decreased *LBR* expression in *LBR* siRNA-treated HDLECs in comparison to CTRL siRNA-treated HDLECs, confirming efficient knockdown. Data represent mean relative mRNA expression, presented normalized to CTRL siRNA-treated HDLECs. $n = 3$ per group, p-values were obtained using one-sample t-test. ns = no significance $p > 0.05$, $****p < 0.0001$ for *LBR* **(left).** qRT-PCR analysis of cultured HDLECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing decreased *FKBP1A* expression in *FKBP1A* siRNA-treated HDLECs in comparison to CTRL siRNA-treated HDLECs, confirming efficient knockdown. Data represent mean relative mRNA expression, presented normalized to CTRL siRNA-treated HDLECs. $n = 3$ per group, p-values were obtained using one-sample t-test. ns = no significance $p > 0.05$, $****p < 0.0001$ **(right).**

(B) qRT-PCR analysis of cultured HDLECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing decreased *CCNB1, CCNB2 and MKI67* expression in *LBR* siRNA- and *FKBP1A* siRNA-reated HDLECs in comparison to CTRL siRNA-treated HDLECs, confirming cell proliferation reduction. Data represent mean relative mRNA expression, presented normalized to CTRL siRNA-treated HDLECs. $n = 3$ per group, p-values were obtained using one-sample t-test. ns = no significance $p > 0.05$, $*p < 0.05$, $**p < 0.01$.

(C) Representative images of immunofluorescence stainings of KI67 (magenta) and DAPI (blue) **(upper panel)**, VE-Cadherin (grey) **(middle panel)** or Actin (grey) **(lower panel)** in HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA. Scale bars: 50 µm.

(D) Quantification of KI67-positive HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA from immunofluorescence stainings in **(A, upper panel)**, showing reduced cell proliferation upon siRNA treatment. Data represent mean percentage compared to all cells, presented normalized to CTRL siRNA-treated HUVECs. $n = 3$ per group, p-values were obtained using one-sample t-test. ns = no significance $p > 0.05$, $*p = 0.0115$, $**p = 0.0061$.

(E) Western blots of cultured HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing decreased CLDN5 protein in *FKBP1A* siRNA-treated HUVECs in comparison to CTRL siRNA-treated HUVECs. $n = 2$ per group.

(F) qRT-PCR analysis of cultured HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing unaltered to increased *CLDN5* expression upon siRNA. treatment in comparison to CTRL siRNA-treated HUVECs. Data represent mean relative mRNA expression, presented normalized to CTRL siRNA-treated HHUECs. $n = 2$ per group.

(G) Western blots of cultured HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing unaltered phospho AKT protein upon siRNA treatment in comparison to CTRL siRNA-treated HUVECs. n = 3 per group.

(H) Western blot intensity quantification of cultured HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA in **(G)** showing unaltered basal AKT phosphorylisation (phospho AKT) upon siRNA treatment in comparison to CTRL siRNA-treated HUVECs. Data represent mean relative phosphorylation, measured by densitometry normalized to GAPDH and CTRL siRNA-treated HUVECs. n = 3 per group, p-values were obtained using one-sample t-test. ns = no significance p > 0.05.

(I) qRT-PCR analysis of cultured HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing unaltered to increased *CDH5* expression upon siRNA treatment in comparison to CTRL siRNA-treated HUVECs. Data represent mean relative mRNA expression, presented normalized to CTRL siRNA-treated HUVECs. n = 2 per group.

(J) qRT-PCR analysis of cultured HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing decreased *LBR* expression in *LBR* siRNA-treated HUVECs in comparison to CTRL siRNA-treated HUVECs, confirming efficient knockdown. Data represent mean relative mRNA expression, presented normalized to CTRL siRNA-treated HUVECs. n = 2 per group **(left).** qRT-PCR analysis of cultured HDLECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing decreased *FKBP1A* expression in *FKBP1A* siRNA-treated HUVECs in comparison to CTRL siRNA-treated HUVECs, confirming efficient knockdown. Data represent mean relative mRNA expression, presented normalized to CTRL siRNA-treated HUVECs. n = 3 per group, p-values were obtained using one-sample t-test. ns = no significance p > 0.05, ****p < 0.0001 **(right).**

(K) qRT-PCR analysis of cultured HUVECs treated with *LBR*, *FKBP1A* or control (CTRL) siRNA showing decreased *CCNB1, CCNB2 and MKI67* expression in *LBR* siRNA- and *FKBP1A* siRNA-reated HUVECs in comparison to CTRL siRNA-treated HUVECs, confirming cell proliferation reduction. Data represent mean relative mRNA expression, presented normalized to CTRL siRNA-treated HUVECs. n = 2 per group.

(L) Dot plots indicating expression of conserved genes from the LEC proliferation code, including common cell cycle machinery regulators (cell cycle) and new candidate genes (new candidates), in BECs from mouse retinas (Chavkin *et al*., 2022) ordered by assigned cluster identity (prol BEC = proliferating BEC, Vein, vCap = venous capillaries, Artery). Dot size illustrates percentage of cells presenting transcript sequence counts (% Expn) and viridis scale color illustrates the relative average normalized expression (Rel Av Expn) within a cluster.

(M) Venn diagram of gene number found differentially expressed in embryonic zebrafish proliferating LECs and proliferating BECs from mouse retinas, as well as conserved gene number.

(N) Dot plots indicating expression of conserved genes from the LEC proliferation code, including common cell cycle machinery regulators (cell cycle) and new candidate genes (new candidates), in dermal normal proliferating LECs (normal) and proliferating mutant *Pik3ca* GOF LECs (*Pik3ca* GOF) from ear skin of juvenile mice (Petkova *et al*., 2023). Dot size illustrates percentage of cells presenting transcript sequence counts (% Expn) and viridis scale color illustrates the average normalized expression (Av Expn) within a cluster.