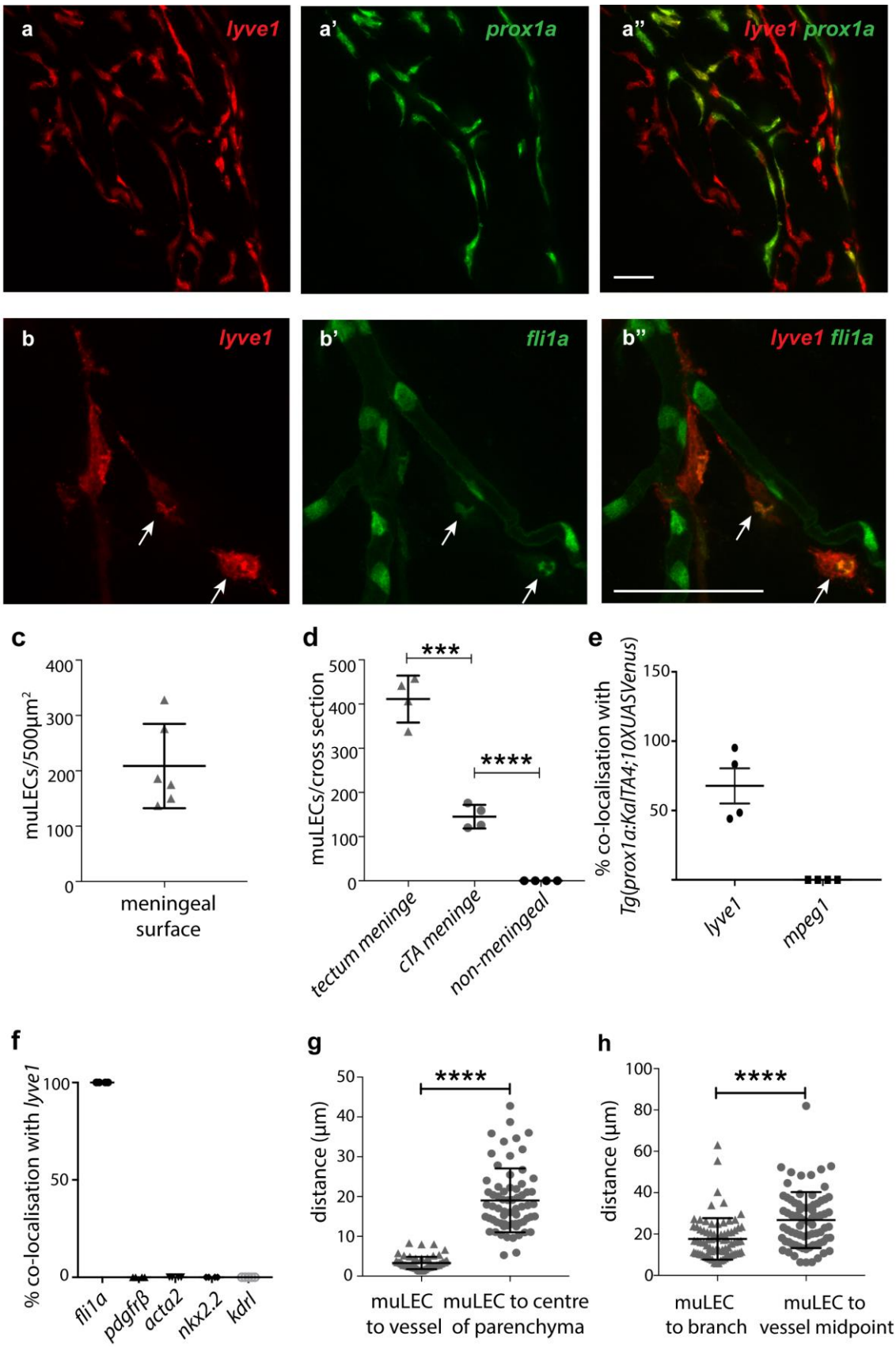

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Supplementary Figure 1

lyve1-expressing cells in the adult zebrafish meninx co-express the lymphatic marker genes _prox1a_ and _fli1a_

(**a**) Confocal images of adult zebrafish brain in *Tg(-5.2lyve1b:DsRed)*^{nz101}*; TgBAC(prox1a:KalTA4*^{uq3bh}*;10xUAS:Venus)* animals showing cells that co-express the lymphatic markers *prox1a* and *lyve1*. Expression of *prox1a* is mosaic due to the Gal4 system. Full quantification of marker co-expression can be found in **Supplementary Figure 1e**. Scale bar represents 100µm.

(**b**) Confocal images of adult zebrafish brain in *Tg(-5.2lyve1b:DsRed)*^{nz101}*; Tg(fli1a:EGFP)*^{y1} showing co-expression (arrows) of the lymphatic markers *fli1a* and *lyve1*. Full quantification of marker co-expression can be found in **Supplementary Figure 1f.** Scale bar represents 100µm.

(**c**) Quantification of muLEC density on the surface of the brain (meningeal surfaces n=6 adult brains. Error bars represent mean +/- sem, Statistical testing N/A.)

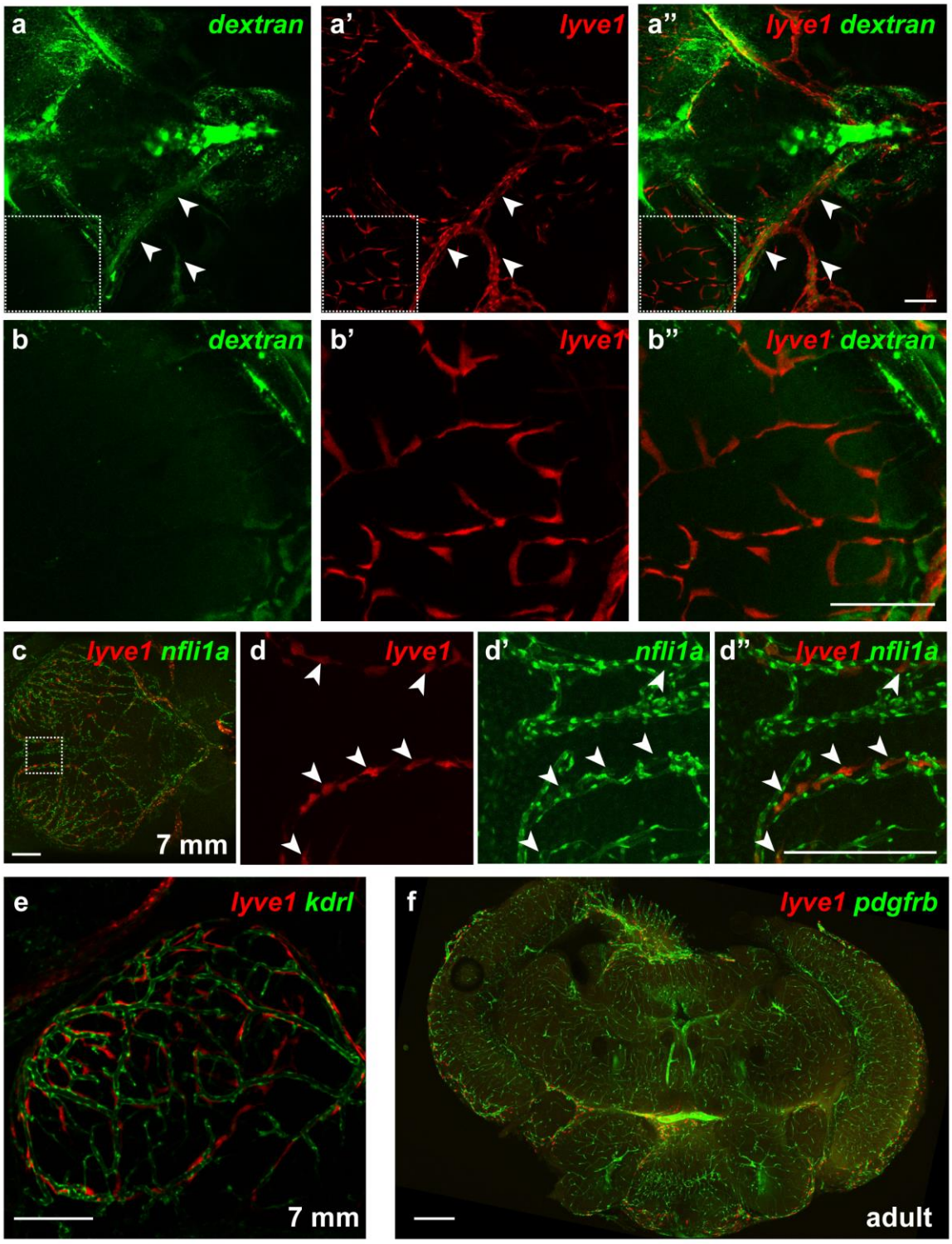
(**d**) Quantification of the number of *lyve1*-positive meningeal cells (over the surface of the tectum, or adjacent to the central arteries (cTA)) compared with non-meningeal *lyve1*-positive cells from cross sections of the adult zebrafish brain. n=4 adult brain cross sections, p<0.0001, from two-tailed student t-test (t=8.926 df=6).

(**e**) Quantification of co-expression of cells from *Tg(prox1a:KalTA4*^{uq3bh}*;10xUAS:Venus)* and *lyve1* (*Tg(-5.2lyve1b:DsRed)*^{nz101}) and the macrophage marker *mpeg1* (*Tg(mpeg1:mcherry*)). 215 cells from n=4 larvae (*lyve1*) and 255 cells from n=5 larvae (*mpeg1*) were scored from confocal Z-stack images. Error bars represent mean +/- sem, Statistical testing N/A.

(**f**) Quantification of co-expression of cells in *Tg(-5.2lyve1b:DsRed)*^{nz101} and *Tg(fli1a:EGFP)*^{y1}*, TgBAC(pdgfrβ:EGFP)*^{uq15bh}, *TgBAC(acta2:EGFP)*^{uq17} and *Tg(nkx2.2a:EGFP)*^{vu16Tg}*.* 100 cells from n=5 larvae were scored from confocal Z-stack images. Error bars represent mean +/- sem, Statistical testing N/A.

(**g**) Quantification of the distance from the nucleus of muLECs to the center of the parenchyma (defined as the centre of the unlabeled space surrounded by a vascular loop) shows that muLECs are more closely associated with blood endothelial cells than the space between vessels. 77 muLEC nuclei from n=3 larvae, p<0.0001, from two-tailed student t-test (t=15.64 df=130). Error bars represent mean +/- sem.

(**h**) Quantification of the distance from the nucleus of a given muLEC to the nearest vessel branch point, compared with the distance from the nucleus of a given muLEC to the midpoint between branches of the closest vessel. muLECs are quantitatively closer to vessel branch points than vessel midpoints. 77 branch and 77 midpoints points from n=3 larvae, p<0.0001, from two-tailed student t-test (t=4.74 df=151). Error bars represent mean +/- sem.



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Supplementary Figure 2

High-molecular-weight dye can be taken up by meningeal lymphatics but not by muLECs.

(a) Dorsal confocal image of 7mm *Tg(-5.2lyve1b:DsRed)*^{nz101} zebrafish larvae following injection of FITC-labeled high molecular weight (2000kDa) dextran. Arrowheads indicate the presence of the dye in lymphatic vessels, but the dye is absent from muLECs. Images representative of data obtained from injection of n=6 larvae. Scale bar represents 100µm.

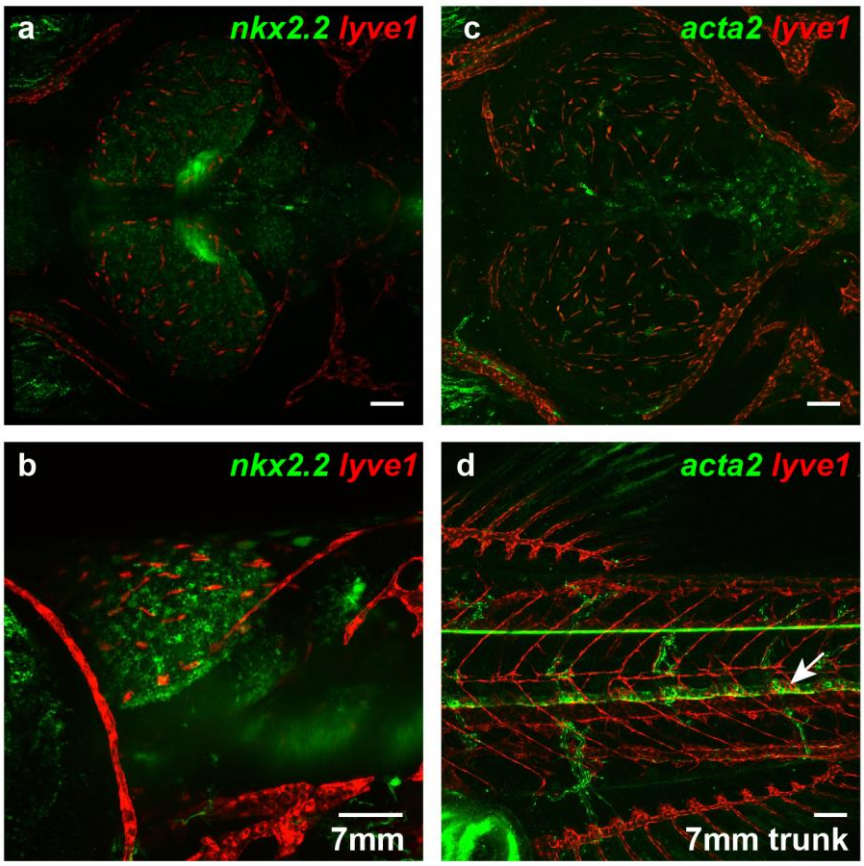
(**b**) Magnification of boxed region in (**a**) showing muLECs do not absorb high MW dextran. Images representative of data obtained from injection of n=6 larvae. Scale bar represents 100µm.

(**c**) Dorsal confocal image of a *Tg(-5.2lyve1b:DsRed)*^{nz101}*; Tg(fli1a:EGFP)*^{y1} zebrafish larvae. Image representative of n=5 larvae. Scale bar represents 100µm.

(**d**) Magnification of boxed region in (**c**) showing muLECs co-express *lyve1* and *fli1a* (arrowheads). Image representative of n=6 larvae. Full quantification of marker co-expression can be found in **Supplementary Figure 1f.** Scale bar represents 100µm.

(**e**) muLECs are closely associated with blood vessels in *Tg(-5.2lyve1b:DsRed)*^{nz101} larvae. Image representative of n=6 larvae. Scale bar represents 100µm.

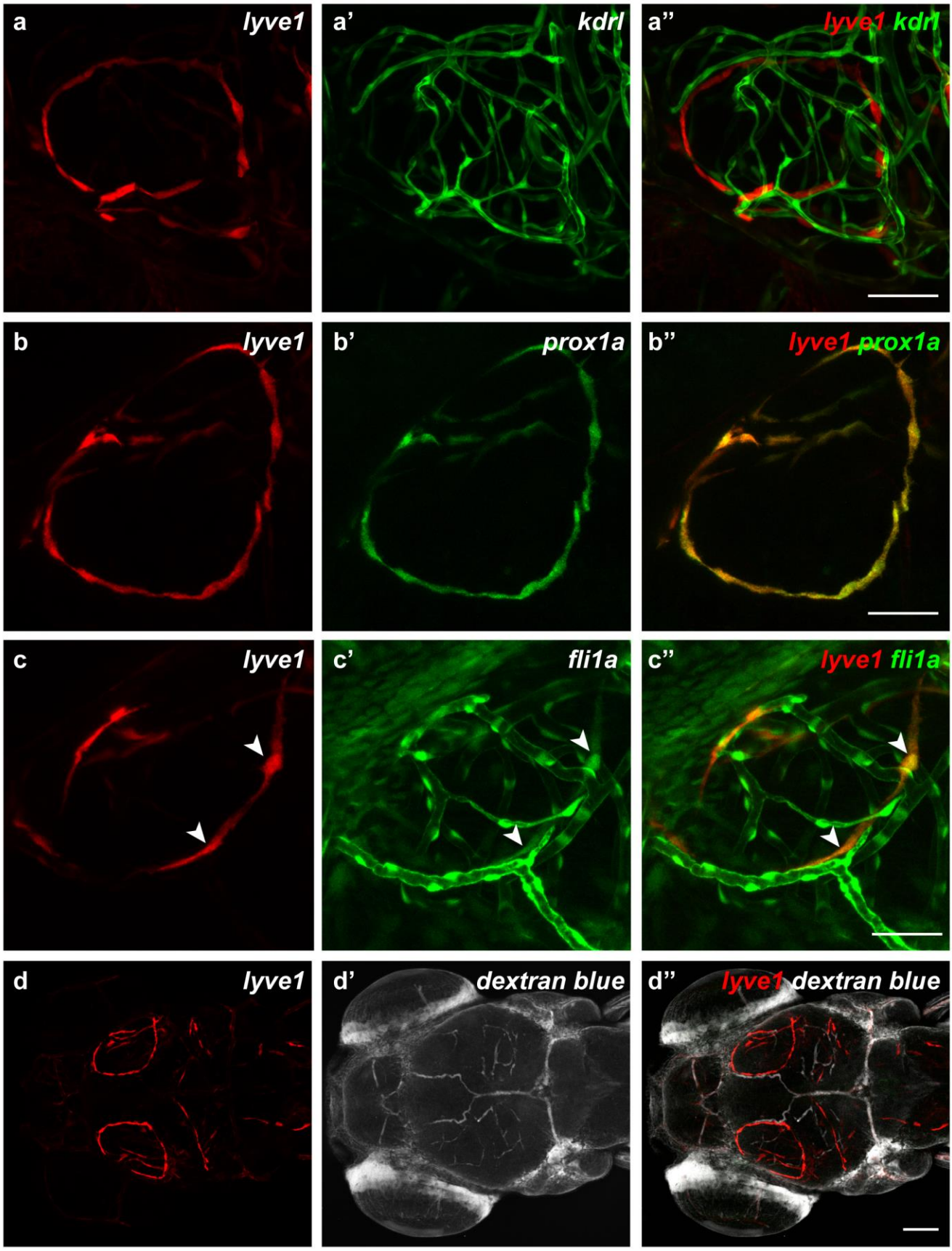
(**f**) Confocal image of a cross-section of adult (20mm stage, 3 month old) *Tg(-5.2lyve1b:DsRed)*^{nz101}*; TgBAC(pdgfrβ:EGFP)*^{uq15bh} brain showing ***pdgfrβ*** expressing pericytes are associated with blood vessels throughout the adult CNS. Scale bar represents 100µm. Full quantification of marker co-expression can be found in **Supplementary Figure 1f.** Image representative of data obtained from injection of n=3 male and female 20mm zebrafish adults.



Supplementary Figure 3

muLECs do not express markers of neuronal and/or oligodendrocyte cells and smooth muscle cells.

(a-d) Confocal images of (**a,b**) 7mm stage *Tg(-5.2lyve1b:DsRed)*^{nz101}*;Tg(nkx2.2a:EGFP)*^{vu16Tg} and (**c,d**) 7mm *Tg(-5.2lyve1b:DsRed)*^{nz101}*;TgBAC(acta2:EGFP)*^{uq17} zebrafish larvae showing a lack of co-expression of these markers (quantified in **Supplementary Figure 1f**) corresponding to neuronal/oligodendrocyte and smooth muscle respectively. Smooth muscle cells are present around the dorsal aorta in the lateral view of the trunk (arrow in **d**). Scale bars represent 100µm.



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Supplementary Figure 4

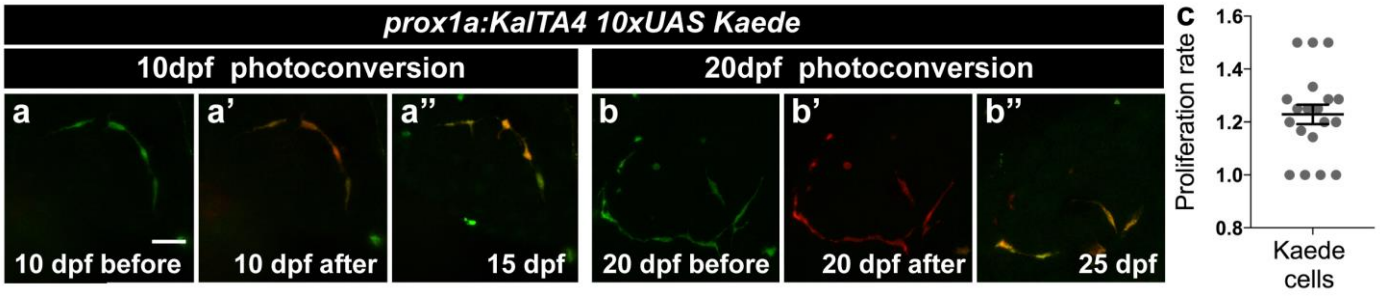
Cells of the putative lymphatic loop express lymphatic markers and are separate from the blood vasculature.

(**a**) Confocal images of a 5dpf *Tg(-5.2lyve1b:DsRed)*^{nz101}*; Tg(kdrl:EGFP)*^{s843} zebrafish midbrain showing that cells of the lymphatic loop do not co-express *lyve1* and the blood vessel marker *kdrl*. n=5 embryos. Full quantification of marker co-expression can be found in **Supplementary Figure 1f.**

(**b**) Confocal images of *lyve1*/*prox1a* positive cells of the lymphatic loop at 5dpf in *Tg(-5.2lyve1b:DsRed)*^{nz101}*; Tg(prox1a:KalTA4*^{uq3bh}*;10xUAS:Venus)* zebrafish. n=3 embryos. Full quantification of marker co-expression can be found in **Supplementary Figure 1f**.

(**c**) Confocal images of a 5dpf zebrafish head showing cells of the lymphatic loop co-express the lymphatic markers *lyve1* and *fli1a* in *Tg(-5.2lyve1b:DsRed)*^{nz101}*; Tg(fli1a:EGFP)* (arrowheads). n=5 embryos. Full quantification of marker co-expression can be found in **Supplementary Figure 1f**.

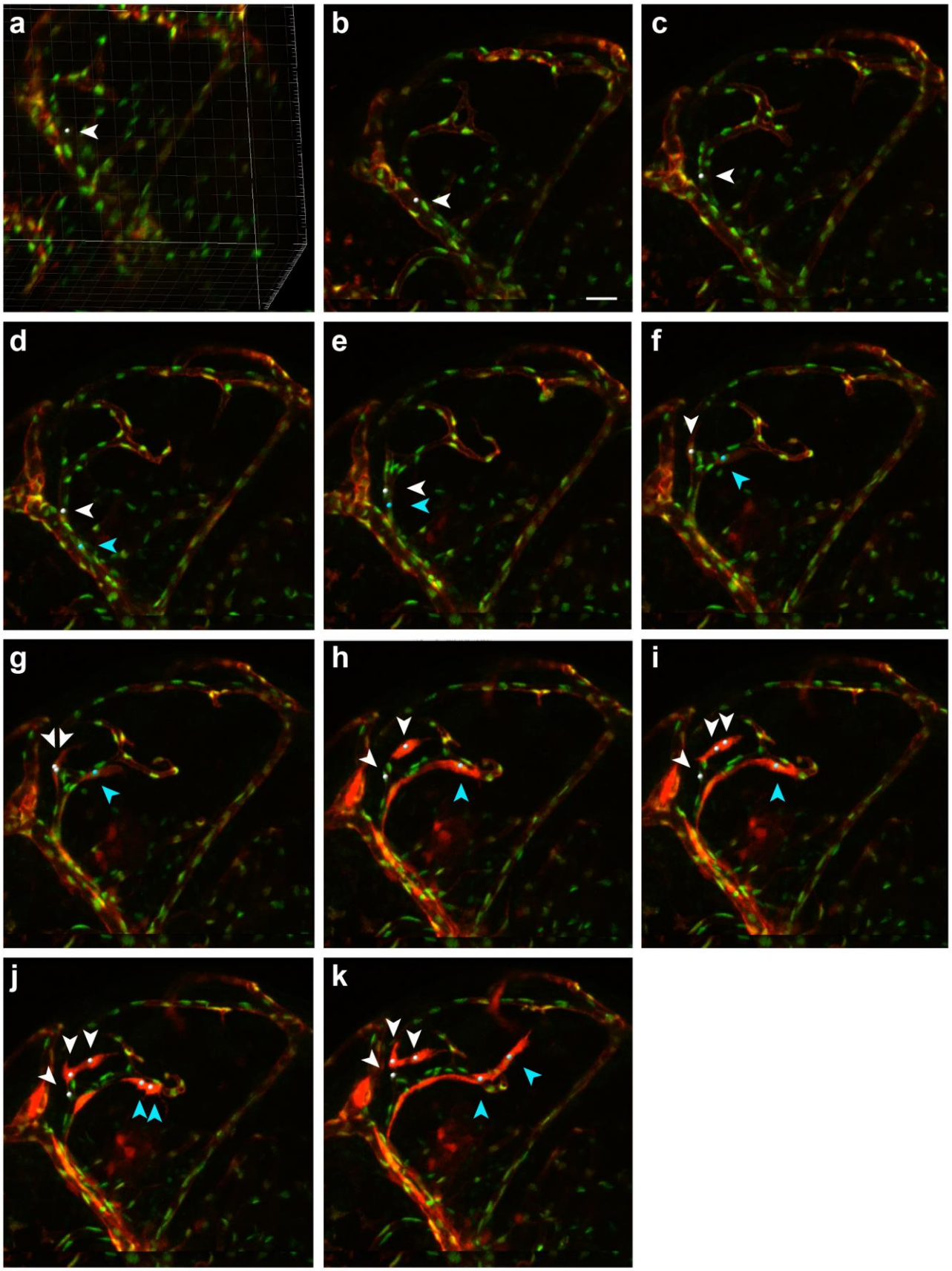
(**d**) Confocal images of 5dpf *Tg(-5.2lyve1b:DsRed)*^{nz101} embryo injected with dextran into the blood vasculature. The *lyve1* positive cells do not contain dextran demonstrating they are separate from the blood vasculature. Scale bars represent 100μm. Representative of analysis from n=4 embryos.



Supplementary Figure 5

Kaede photoconversion demonstrates that the lymphatic loop gives rise to the meningeal muLEC population.

(**a-b**) Confocal images of *Tg(prox1a:KalTA4)*^{uq3bh}; *Tg(10xUAS:Kaede)*^{s1999t} photoconverted at 10dpf and reimaged at 15dpf (**a**) and photoconverted at 20dpf and reimaged at 25dpf (**b**). Scale bars represent 50µm. For the 10-15 dpf stages n=34 cells from 7 larvae were examined and for the 20-25dpf stages n=29 cells from 3 larvae were analysed. Positive lineage tracing was confirmed in all cases. (**c**) Quantification of the average increase in photo-converted cell number over 5 days (proliferation rate, cell number 5 days after conversion/0 days after conversion) data combined for 5-20 dpf experiments, n=19 larvae overall. Error bars represent mean +/- sem, Statistical testing N/A.



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Supplementary Figure 6

Still images of a *Tg(-5.2lyve1b:DsRed)^{nz101};Tg(fli1a:nEGFP)^{y7}* zebrafish embryo from Supplementary Movie 2.

(**a-k**) Images are representative of n= 5 movies.

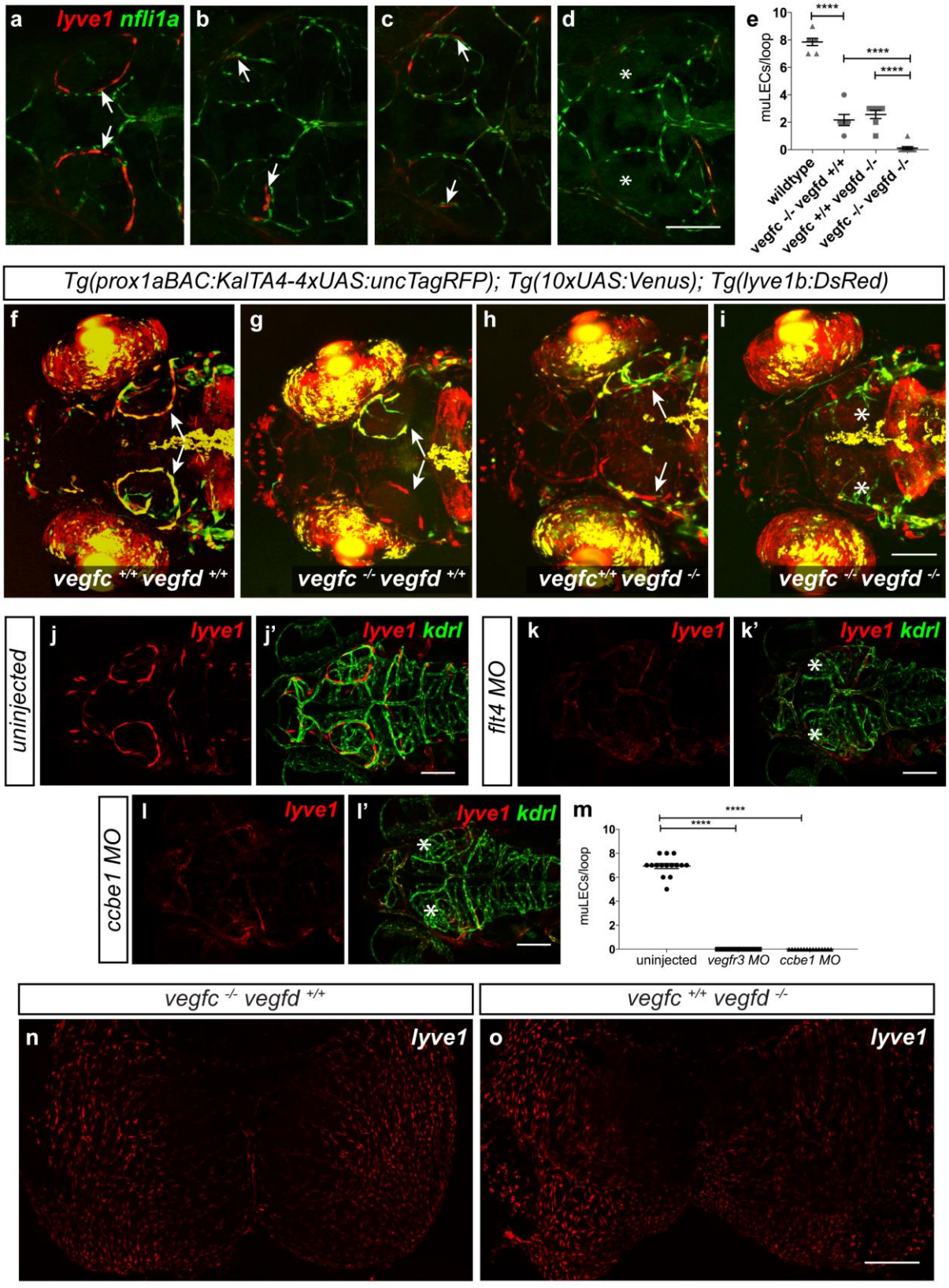
(**a**) Tilted view showing the labeled endothelial nucleus in the choroidal vascular plexus (arrowhead).

(**b,c**) First cell (grey render, arrow) exits the choroidal plexus vessel and migrates dorsally along another pre-existing vessel.

(**d-f**) Second cell (blue render, arrow) exits vessel and migrates dorsally along pre-exiting vessel.

(**g-i**) First cell divides (arrowhead) and one daughter cell divides again (arrow).

(**i,j**) Second cell (blue) divides (arrowhead) and continues to migrate (**k**, arrowhead). Scale bar represents 50µm.



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Supplementary Figure 7

muLEC sprouting is dependent upon Vegfc, Vegfd, Ccbe1 and Vegfr3 signaling.

(**a-d**) Dorsal confocal images of 5dpf *Tg(-5.2lyve1b:DsRed)*^{nz101} ; *Tg(fli1a:nEGFP)*^{y7} embryos showing the lymphatic loop that forms in wildtype embryos (**a**, arrows, representative image based on analysis of n=7 embryos) is reduced in *vegfc*^{hu5055} (**b**, arrows, n=6 embryos) and *vegfd*^{uq7bh} (**c**, arrows, n=6 embryos) single mutants, but is absent in *vegfc*^{hu5055}/*vegfd*^{uq7bh} double mutants (**d**, asterisk, n=9 embryos). Scale bars represent 100µm.

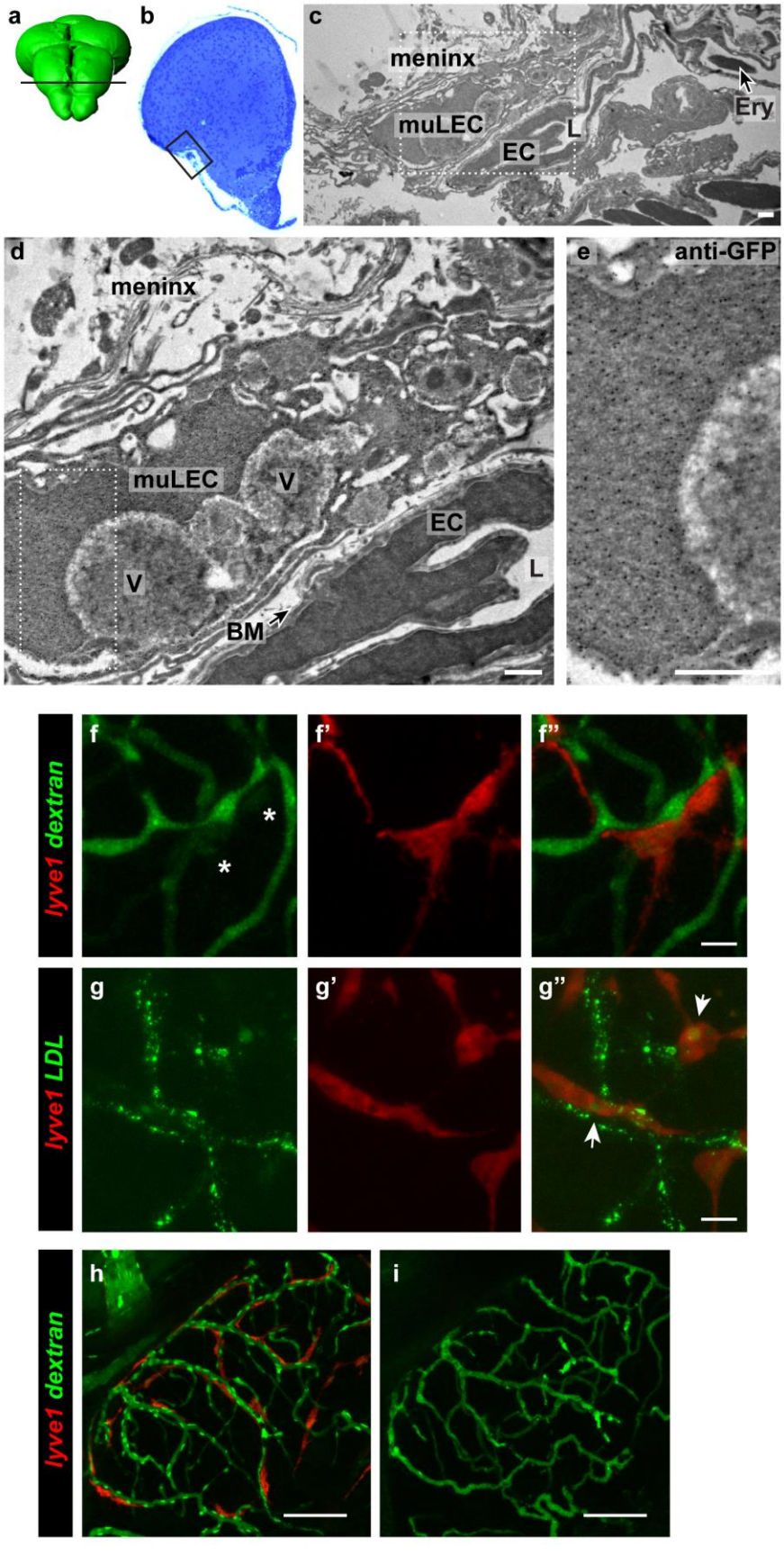
(**e**) Quantification of the number of muLECs present in a single putative lymphatic loop at 5dpf in wildtype (n=7 embryos), *vegfc*^{hu5055} (n=6 embryos), *vegfd*^{uq7bh} (n=6 embryos) single mutants and *vegfc*^{hu5055}/*vegfd*^{uq7bh} double mutants (n=9 embryos). Error bars represent mean +/- sem; **** p<0.0001, from one way ANOVA (F (3, 25) = 166.6).

(**f-i**) At 5 dpf, the *lyve1*/*prox1a* positive cells in *Tg(-5.2lyve1b:DsRed)*^{nz101}*; Tg(prox1a:KalTA4, 4xUAS:uncTagRFP)*^{nim5}*; Tg(10xUAS:Venus)* have formed a lymphatic loop in wildtype embryos (**f**, n=6 embryos), however in *vegfc*^{hu5055} (**g,** n=6 embryos) and *vegfd* (**h**, n=6 embryos) mutants, the development of the lymphatic loop is reduced (arrows). In *vegfc*^{hu5055} / *vegfd*^{uq7bh} double mutant embryos (**i**, n=6 embryos) the lymphatic loop is completely absent (asterisks). Scale bars represent 100µm.

(**j-l**) The *lyve1* lymphatic loop structure (control, **j**) is absent in *vegfr3* morphant (n=15 embryos) (**k**) and (**l**) *ccbe1* morphant (n=15 embryos) embryos at 5dpf (asterisks).

(**m**) Quantification of the number of muLECs present in a single putative lymphatic loop at 5dpf in uninjected controls (n=15 embryos), *vegfr3* morphant (n=15 embryos) and *ccbe1* morphant (n=15 embryos). **** p<0.0001, from two-tailed student t-test (t=33.62 df=28). Scale bars represent 100µm.

(**n,o**) Dorsal confocal images from 24 mm *Tg(-5.2lyve1b:DsRed)*^{nz101} *vegfc*^{hu5055} (**n**) and *vegfd*^{uq7bh} (**o**) mutants showing the presence of either ligand is sufficient for development of the muLECs. Representative image based on analysis of n=4 adult brains. Scale bars represent 200µm.



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Supplementary Figure 8

Mature muLECs display a distinctive ultrastructure and take up LDL.

(**a,b**) Schematic diagram indicating the region used for immuno-electron microscopy.

(**c**) Low magnification immuno-electron micrograph image showing overview of imaged region in Fig. 4a,b. Scale bar represents 1µm.

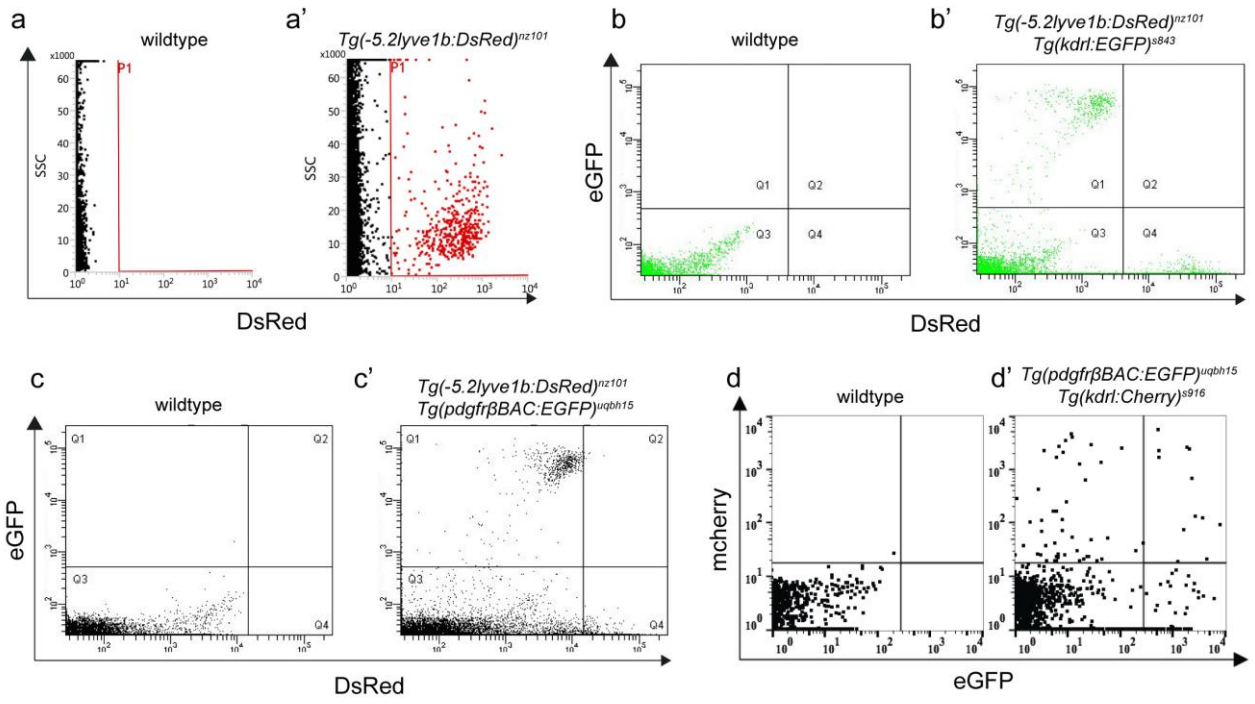
(**d**) Higher magnification of image shown in (**c**). Scale bar represents 1µm. ***** = prominent vacuoles or inclusion bodies consistent with confocal imaging in **Figure 1** and panel **g** below, L=Lumen, EC= endothelial cell, BM=basement membrane.

(**e**) High magnification of muLEC cell showing positive immunostaining by anti-gfp antibody. Scale bar represents 1µm.

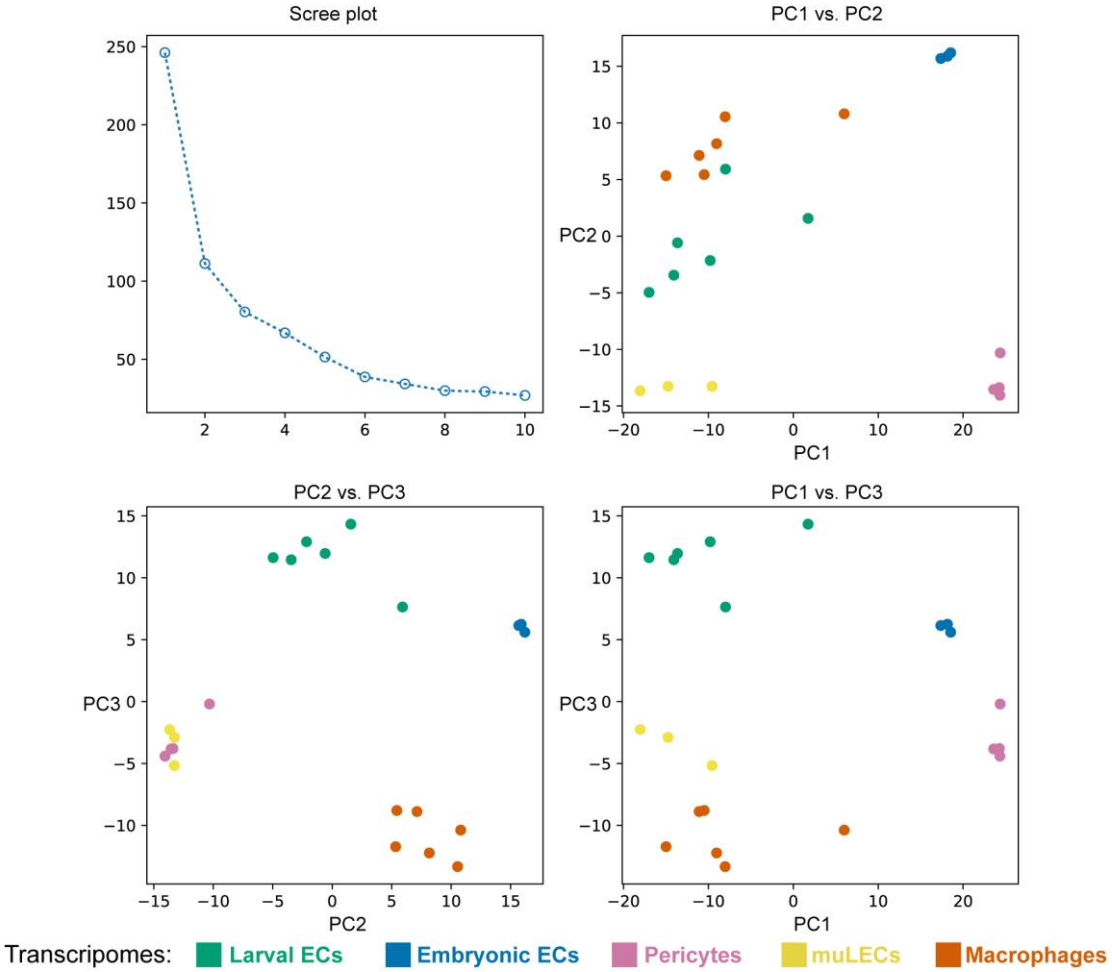
(**f**) High molecular weight dextran is not observed in muLECs (asterisks) 3 hours post injection into the blood stream of *Tg(-5.2lyve1b:DsRed)*^{nz101} larvae. n=63 muLECs were analysed in detail by scoring confocal z-stacks from n= 3 larvae.

(**g**) Alexa 488-labelled acetylated LDL is observed in the endothelial cells and the inclusion bodies (arrows) of 61 +/- 11 % of muLECs 3 hours post injection into the blood vasculature. Scale bars represent 20µm. n=72 muLECs were analysed in detail by scoring confocal z-stacks from 3 embryos.

(**h,i**) Three hours post injection of high molecular weight dextran, there is no obvious vascular leakage in wildtype (h) or *vegfc*^{hu5055} / *vegfd*^{uq7bh} double mutant larvae (i) which lack muLECs. Scale bar represents 50µm. n=3 larvae were analysed in detail by scoring confocal z-stacks.



e Principle Component Analysis



Supplementary Figure 9

muLECs can be FAC-sorted and separated from blood endothelial cells and pericytes.

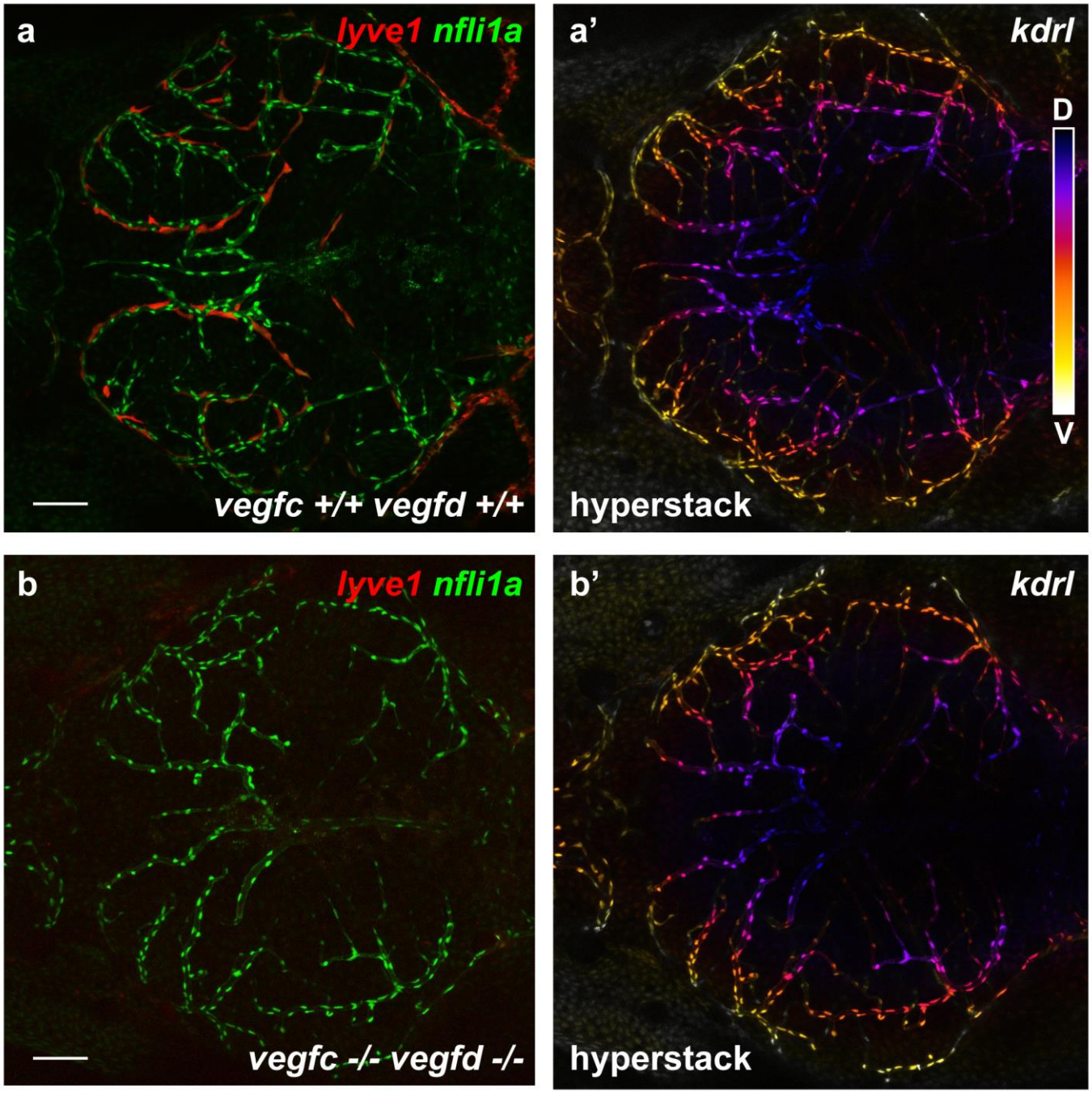
(**a**) Representative plots of FAC sorted cells from adult brains in unlabeled wildtype and *Tg(-5.2lyve1b:DsRed)*^{nz101} positive samples.

(**b**) Representative plots of FAC sorted *EGFP* positive cells in unlabeled wildtype and *Tg(-5.2lyve1b:DsRed)*^{nz101}; *Tg(kdrl:EGFP)*^{s843} positive samples.

(**c**) Representative plots of FAC sorted *EGFP* positive cells in unlabeled wildtype and *Tg(-5.2lyve1b:DsRed)*^{nz101}*; TgBAC(pdgfrβ:EGFP)*^{uq15bh} positive samples.

(**d**) Scree plot of variance (eigenvalue versus component number) and principle component comparisons from **Fig. 5b** shown in 2-dimensional plots (PC1 vs PC2, PC2 vs PC3 and PC1 vs PC3), clear separation of muLECs from control samples is observed.

(**e**) Representative plots of FAC sorted *EGFP* positive cells in wildtype and *Tg(pdgfrβBAC:EGFP)*^{uq15bh}; *Tg(kdrl:mCherry)*^{s843} positive samples.

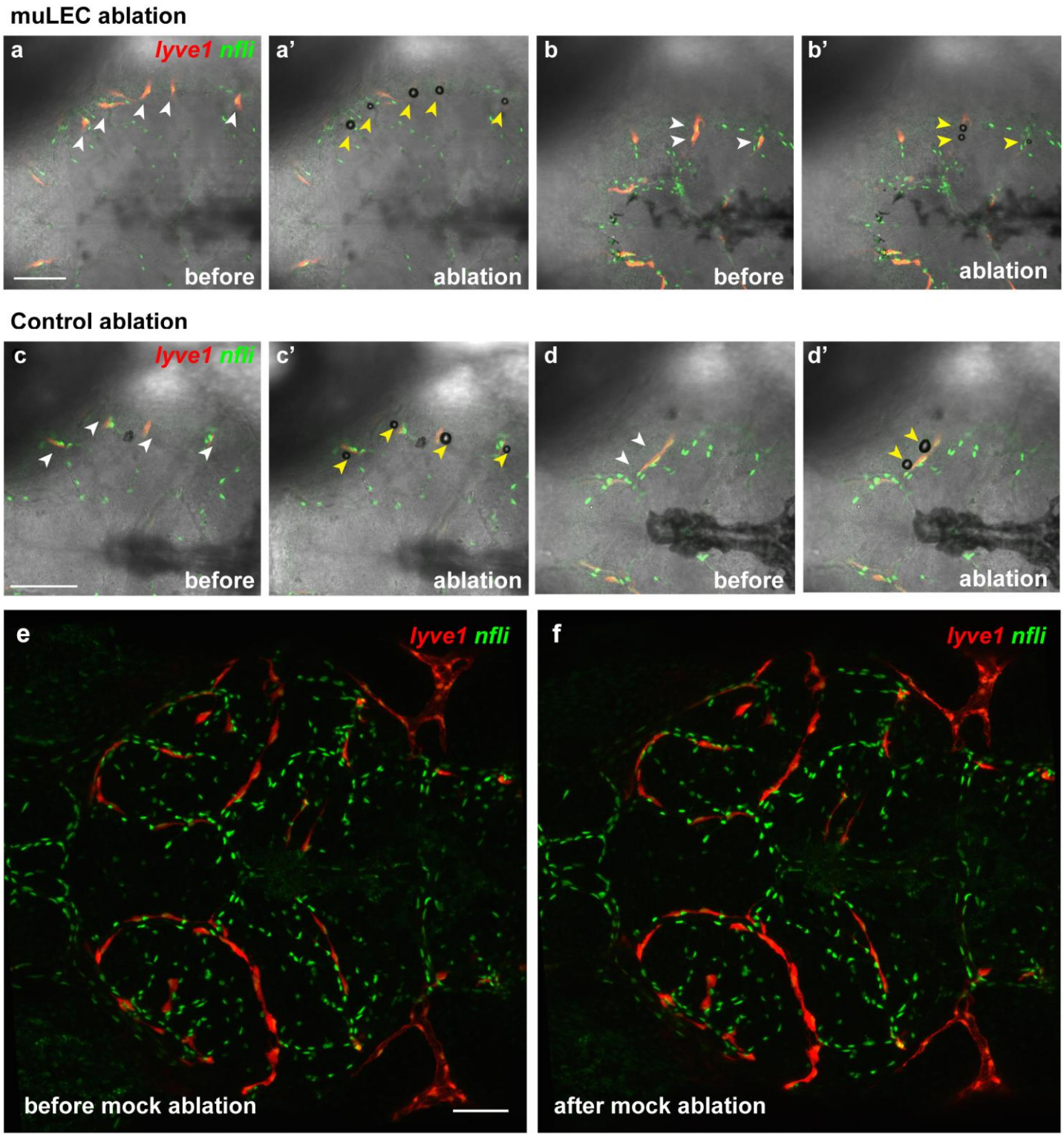


Supplementary Figure 10

***vegfc*^{hu5055}*vegfd*^{uq7bh} double-mutant larvae have reduced meningeal BEC nuclei compared to wild-type larvae.**

(**a**) Dorsal confocal image (**a**) and hyperstack (**a'**) of z-stacks from the *Tg(-5.2lyve1b:DsRed)*^{nz101}*;Tg(fli1a:nEGFP)*^{y7} signal with darker shades representing z-stack slices closer to the objective, showing *vegfc*^{hu5055}*/vegfd*^{uq7bh} double mutant larvae (**a**, representative images of n=8 larvae

analysed), which have reduced meningeal BEC number when compared to size matched (5.7mm) wildtype siblings (**b**, n=7 larvae). Scale bar represents 100μm.



Supplementary Figure 11

Visual confirmation of muLEC ablation and mock ablation.

(a) Single z-slices from a dorsal confocal image of a 10dpf *Tg(-5.2lyve1b:DsRed)*^{nz101}*;Tg(fli1a:nEGFP)*^{y7} larvae

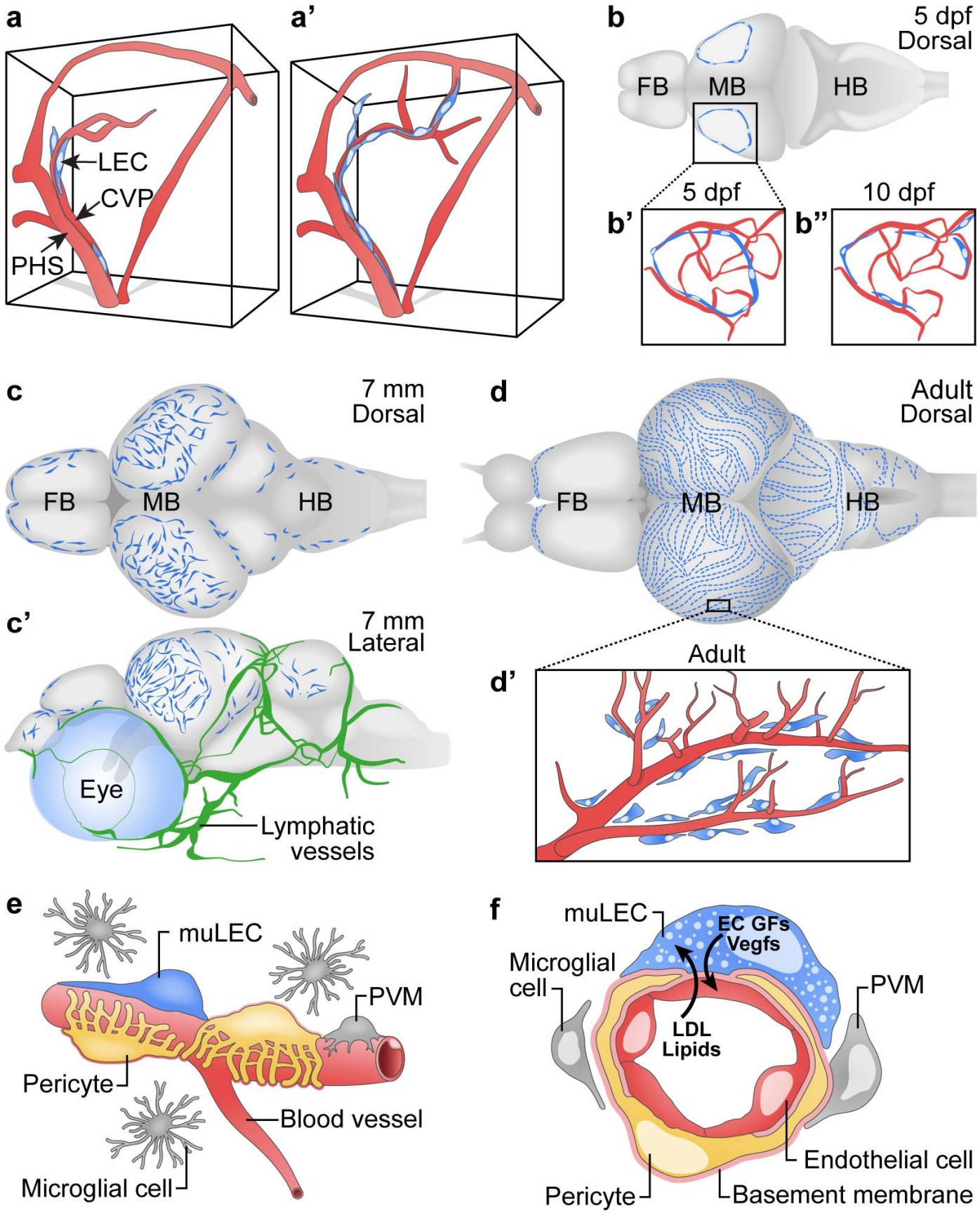
before and after targeted ablation of muLECs. Ablation can be confirmed by direct observation of cell death, with “bubbles” which disperse post ablation. Scale bar represents 50μm. Representative images of n=27 larvae analysed.

(**b**) Second example of single z-slices from a dorsal confocal image of 10dpf *Tg(-5.2lyve1b:DsRed)^{nz101};Tg(fli1a:nEGFP)^{y7}* larvae before and after ablation of muLECs. Ablation can be confirmed by direct observation of cell death. The analysis in **Fig. 7** used the same approach to verify cell ablations for n=27 embryos in total.

(**c**) Example of single z-slices from a dorsal confocal image of 10dpf *Tg(-5.2lyve1b:DsRed)^{nz101};Tg(fli1a:nEGFP)^{y7}* larvae before and after mock ablation in which cells adjacent to the muLECs are targeted. Ablation confirmed by direct observation and this approach was used in **Fig. 7** for analysis of n=15 larvae. This is the same larvae as in Fig. 7g,h. Scale bar represents 50μm.

(**d)** Second example of single z-slices from a dorsal confocal image of 10dpf *Tg(-5.2lyve1b:DsRed)^{nz101};Tg(fli1a:nEGFP)^{y7}* larvae before and after mock ablation in which cells adjacent to the muLECs are targeted. This is the same larvae as above and in Fig. 7g,h.

(**e** and **f**) 10 dpf *Tg(-5.2lyve1b:DsRed)^{nz101};Tg(fli1a:nEGFP)^{y7}* larvae before (**e**) and after (**f**) mock ablation. Representative of n=15 larvae analysed. Scale bar represents 100μm.



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Supplementary Figure 12

Schematic summary of muLEC development and function

a. Mural lymphatic endothelial cells (muLECs) emerge from the choroidal vascular plexus (CVP) at 54 hpf (a) and sprout to the periphery of the midbrain at 96 hpf (a').

b. The resulting lymphatic vascular loop in the midbrain (MB) begins to undergo a transition to a mesenchymal morphology between 5 (b') and 10 dpf (b'')

c - d. muLECs (blue) are present on the midbrain, fore brain (FB) and hind brain (HB) in 7 mm larvae (c) and expand over the adult brain (d). c' and d' provide lateral views and indicate the meningeal lymphatics (green, c') and muLEC location relative to blood vessels (red) (d').

e and **f**. Three dimensional (e) and cross sectional schematic representation of the blood vasculature and mural cells at the zebrafish meninx. muLECs (blue) secrete vascular growth factors and take up LDL from the bloodstream. PVM= perivascular macrophage.