Supplementary Information

Supplementary Figures

Supplementary Figure 1. Characterization of perivascular cells.

A, Electron micrographs of sinusoidal vessels in the proximity of growth plate chondrocytes (ch). Osteoblasts (ob), mesenchymal cells (mc), endothelial cells (ec), red blood cells (rbc), osteoclasts (oc), and the lumen of vessels (asterisks) are marked.

B, Expression of PDGFRβ, NG2, CD146 and Nestin (magenta, as indicated) in perivascular cells (arrows) surrounding distal, mG+ sinusoidal capillaries (green) in the metaphysis of 6wk–old *Cdh5(PAC)-CreERT2* x *ROSA26-mT/mG* mice.

Supplementary Figure 2. Organization of hemospheres.

A, Maximum intensity projection of the forming hemosphere shown in Figure 2B. Right, separated channels of the area under the inset showing endothelial cells (green), non-endothelial cells (red) and nuclear staining (Hoechst, blue).

B, C, Selected confocal planes of insets in Fig. 2C or Fig. D, respectively, showing the presence of CD45+ cell populations (cyan) in small and large hemospheres, respectively. EGFP (green) labels endothelial cells, tomato (red) non–ECs and Hoechst (blue) nuclei. Note large sinusoidal vessel (green, arrowhead) inside big hemosphere (**C**) and layer of mT+ cells (arrows) lining the outer perimeter

D, E, Separated channels of the different hemospheres shown in Fig. 2C or Fig. 2D, respectively.

Supplementary Figure 3. Cells of the osteoblast lineage line larger hemospheres

Cross-sections through *Cdh5(PAC)-CreERT2* x *ROSA26-mT/mG* hemospheres and immunostaining for Runx2, Osterix, Osteopontin and N-Cadherin (cyan, as indicated). Arrows point at positive cells in the outer lining of hemospheres. mG+ (green) ECs, mT+ non-endothelial cells (red) and nuclei (blue) are labeled.

Supplementary Figure 4. Hemospheres in different skeletal elements.

A, Hemosphere in the sternum of an adult *Cdh5(PAC)-CreERT2* x *ROSA26-mT/mG* mouse with large mG+ vessel (asterisks) and a transversing arteriole (arrowhead) connected to a distal SEC protrusion (arrow). Ribs are indicated. Right panel shows higher magnification of inset.

B, Secondary ossification centre of adult femur contains hemospheres. Articular cartilage (ac) is indicated. Right panel shows higher magnification of inset.

C, Hemosphere in adult calvarium with central mG+ endothelium (arrows), CD45+ (cyan) hematopoietic cells and peripheral mT+ cells. Right panel shows higher magnification of inset.

D, Bone marrow (bm) in the *Cdh5(PAC)-CreERT2* x *ROSA26-mT/mG* vertebra with hemosphere (inset and higher magnification image on the right). CD45+ cells (cyan) are enclosed in the space between mG+ SECs and mT+ outer lining.

E, Two–photon microscopic image of a hemosphere–like structure in human distal femur near the metaphysis. Von Willebrand Factor (VWF) expression marks ECs (red), collagen (2nd–harmonic signal) highlights trabecular bone (blue) and DAPI nuclei (cyan). Arrows indicate hematopoietic cells in the space between ECs and bone.

Supplementary Figure 5. CD150+ CD48- cells in hemospheres.

A, **B**, Selected channels showing endothelial cells (green), non-endothelial cells (red), nuclear staining (Hoechst, blue), CD150 (cyan) and CD48 (magenta) signals inside of hemospheres. Images correspond to top (**A**) and bottom (**B**) panels of Figure 3A, respectively.

C, 3D reconstruction of small hemosphere containing two CD150+ (cyan) CD48- (magenta) cells. Confocal image was taken from the metaphysis of a 6wk–old *Cdh5(PAC)-CreERT2* x *ROSA26-mT/mG* mouse. mG (green) labels endothelial cells, tomato (red) non–ECs and Hoechst (blue) nuclei.

Supplementary Figure 6. Morphology of vessel-associated putative hematopoietic stem/progenitor cells inside hemospheres.

A, 3D-reconstructed confocal images of *Cdh5(PAC)-CreERT2* x *ROSA26-mT/mG* reporter mice showing that CD150+ cells (arrow, cyan) in adult hemospheres are devoid of CD48 and Lineage marker (magenta) expression. CD150+ CD48- Lin- megakaryocytes (arrowhead in central image) are readily distinguishable by their large size (arrowhead). Image in the center show selected channels of left panel. mG (green) labels endothelial cells, tomato (red) non–ECs, and Hoechst (blue) nuclei.

B-E, Electron micrograph of vessel–associated (asterisks mark lumen) putative stem/progenitor cell largely devoid of rough endoplasmic reticulum (arrowheads) and with primitive mitochondria (arrows) (**D**). Note physical interaction (black arrow) with sinusoidal endothelium (sec) (**E**). (**C**) and (**D**, **E**) are higher magnifications of inset in (**B**) and (**C**), respectively. Scale bar, 10μm (**B**), 5μm (**C**), 500nm (**D, E**).

Supplementary Figure 7. Characterization of fast-cycling hematopoietic cells inside hemospheres.

A, **B**, 3D-reconstructed confocal images of adult *Cdh5(PAC)-CreERT2* x *ROSA26-mT/mG* secondary ossification centers. mG (cyan) labeled endothelial cells, incorporated EdU (red) and Lineage marker (**A**, green) or CD48 immunostaining (**B**, green) are shown. Fast cycling cells labeled by a short EdU pulse show very little overlap with the Lin+ population, but a substantial fraction exhibits CD48 staining (arrows in **B**).

Supplementary Figure 8. Clonal expansion of transplanted cells can be observed in hemospheres.

Cells of a single fluorescent color predominate in hemospheres (indicated by dotted lines) in the adult femoral secondary ossification center at 6 days after transplantation of *Vav1-Cre* x *R26R-Confetti cells.*

Supplementary Figure 9. The fenestrated BM endothelium responds strongly to VEGF-A.

A, Electron micrograph of fenestrae (arrows) in SECs of 7wk–old femur (asterisks marks lumen). Panel on the right shows higher magnification of inset in left image.

B, Rapid ectopic vessel growth (arrows) is triggered by VEGF overexpression in the adult (12wk–old) femoral metaphysis (gp, growth plate). Bottom panels show higher magnifications of insets above. Endothelial cells have been stained by antibodies detecting CD31 (green), hematopoietic cells by CD45 (cyan), and all cells by Phalloidin (F-actin, red).

C, Separated mG channels of images shown in Figure 6A. Image on the left shows vessels in the metaphysis of DMSO (vehicle control) treated mice. Right shows the loss of distal vessels and the reduced caliber of capillaries after SU5416 treatment.

Supplementary Figure 10. Genetic manipulation of VEGF signalling.

Morphological changes in the metaphysis of *Flk1*$^{i\Delta EC}$ mutants compared to control littermates (12wk–old mice), visualized with the indicated markers. Dotted line marks edge of the adjacent trabecular BM. Small panels show mG+ SECs (green) and Ter-119+ (cyan) erythroid cells in separated channels of the corresponding larger image above. Note increased Ter-119+ signal in *Flk1*$^{i\Delta EC}$ mutants.

Supplementary Figure 11. Changes in oxygenation and proliferation of cells inside hemospheres after ablation of endothelial VEGFR2.

A, Increased labeling by the hypoxic cell marker pimonidazole (Pimo, green) and reduction of hemosphere-associated, fast-cycling (EdU+, red) hematopoietic cells in adult *Flk1*$^{i\Delta EC}$ mutant secondary ossification centers. ECs (endomucin, cyan) and nuclei (Hoechst, blue) are labeled.

B, Quantitation of the fraction of EdU+ (left) and Pimo+ (right) cells in control and *Flk1*$^{i\Delta EC}$ mutant hemospheres in the adult femoral secondary ossification center. Error bars, s.e.m.

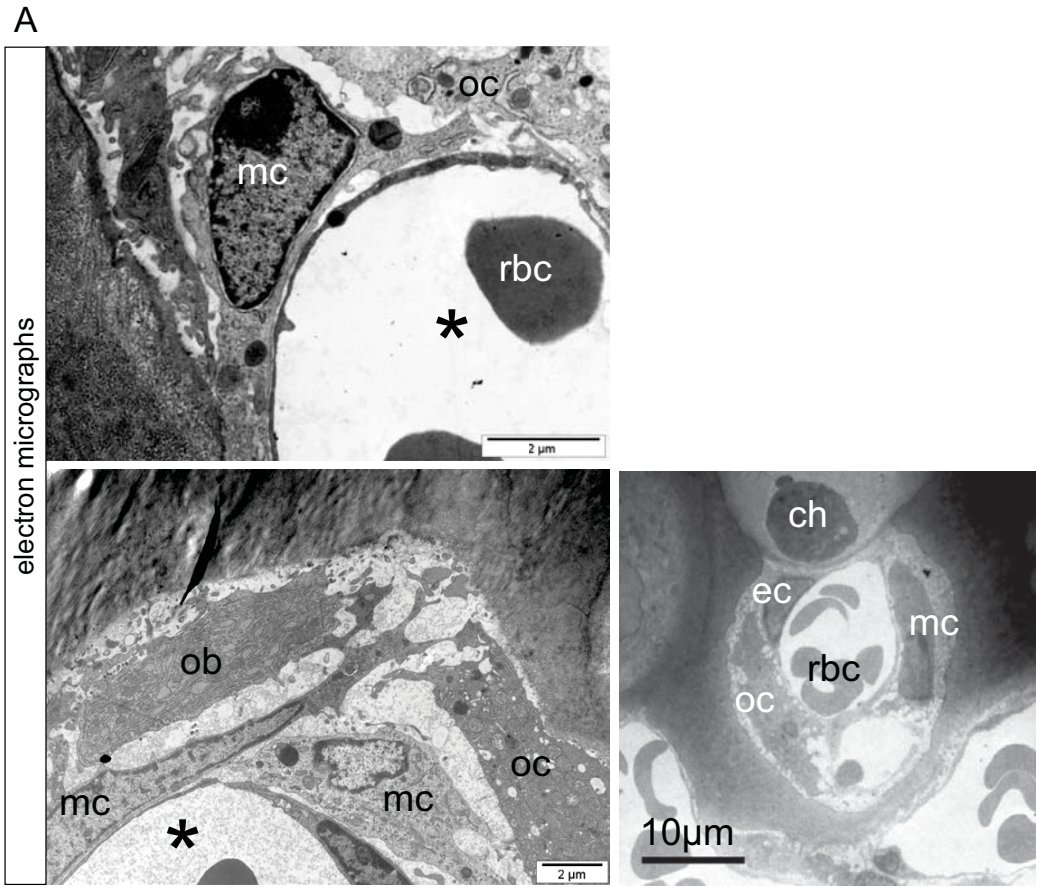
Supplementary Figure 12. Analysis of hematopietic cells in *Flk1*$^{i\Delta EC}$ mutant mice.

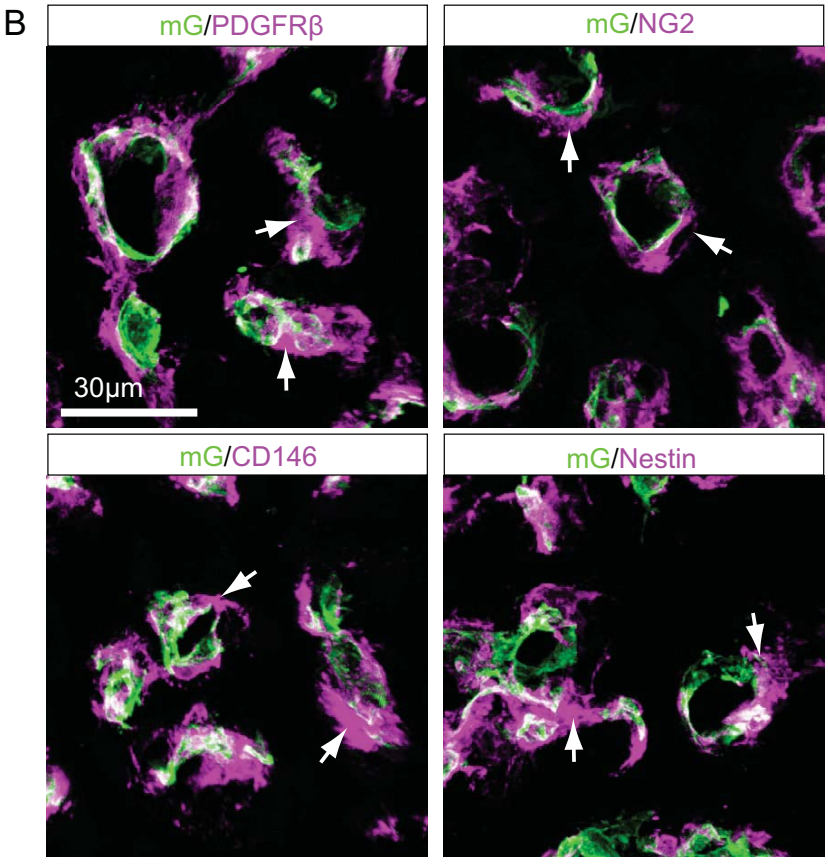
A, Multi-parametric flow cytometry of whole bone marrow from 24wk–old littermate control and *Flk1*$^{i\Delta EC}$ mutant, simultaneously stained with Lineage cocktail, c-Kit, Sca-1, Flk-2 and CD150 antibodies, as indicated. Percentage of Lin- Kit+ Sca-1+ (LSK) Flk-2- CD150+ putative HSCs in the LSK fraction is shown. Black arrows denote the hierarchy of their parental subpopulation gated on a certain antibody or combination

of antibodies. Note reduced percentage of LSK CD150+ Flk2- putative HSCs in CTX/G-CSCF-treated *Flk1*$^{i\Delta EC}$ mutants compared to littermate controls.

B, Kaplan–Meier survival curve of lethally irradiated C57BL/6 mice transplanted with a limiting amount of GFP+ rescue marrow together with BM cells from control (black) or *Flk1*$^{i\Delta EC}$ mutants (red). None of the 4 mice receiving BM cells from *Flk1*$^{i\Delta EC}$ donors survived beyond day 8. In contrast, 3 out of 4 mice receiving control littermate BM were alive at 34 days after transplantation.

C, Multi-parametric flow cytometry of whole bone marrow cells from 12wk–old littermate control and *Flk1*$^{i\Delta EC}$ mutants after CTX and G-CSF treatment. BM cells were simultaneously stained with Lineage cocktail, c-Kit, Sca-1, Flk-2 and CD150 antibodies, as indicated. Percentage of Lin- Kit+ Sca-1+ (LSK) Flk2- CD150+ putative HSCs in the LSK fraction is shown. Black arrows denote the hierarchy of their parental subpopulation gated on a certain antibody or combination of antibodies. Note reduced percentage of LSK CD150+ Flk2- putative HSCs in CTX/G-CSCF-treated *Flk1*$^{i\Delta EC}$ mutants compared to littermate controls.





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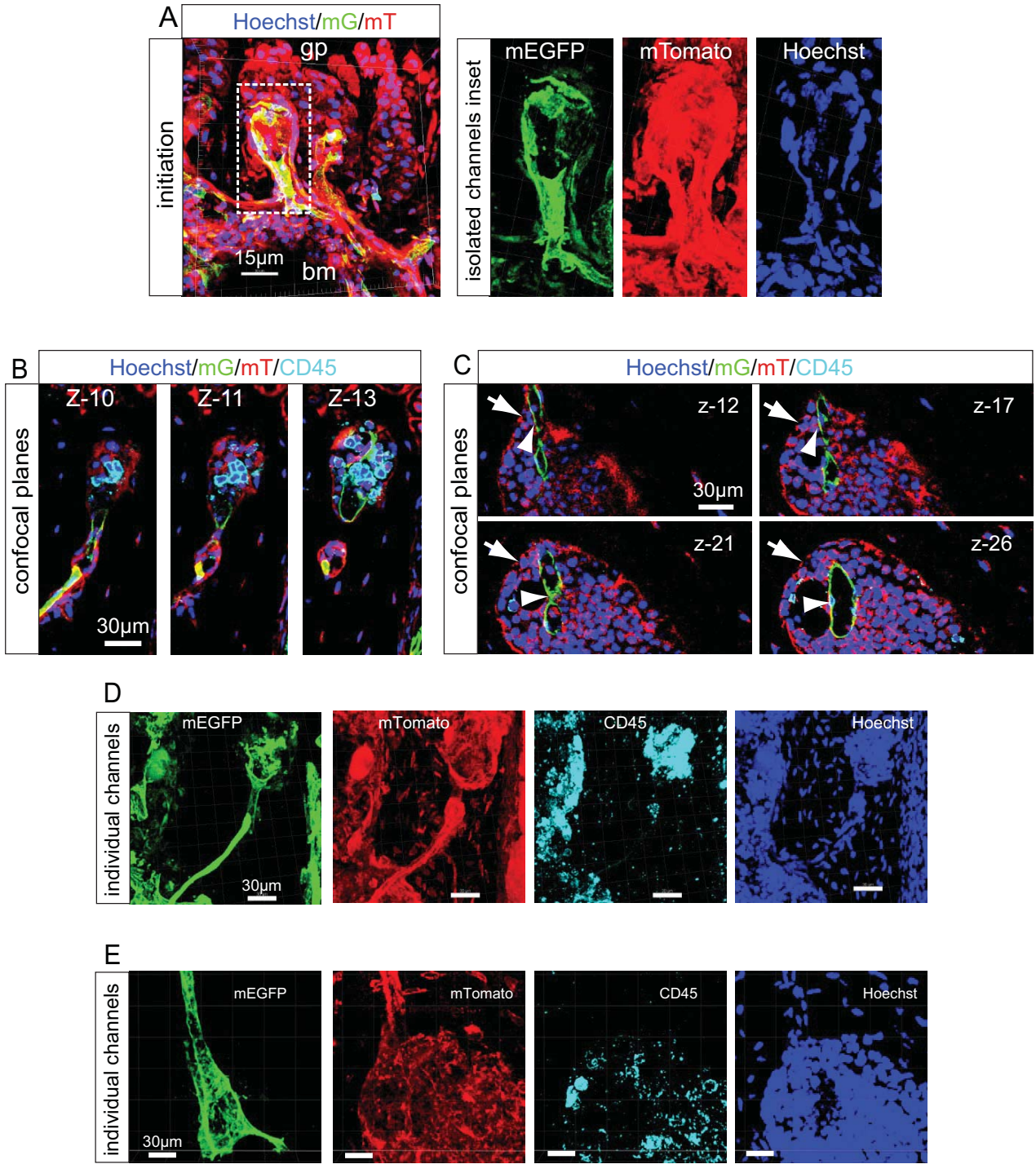

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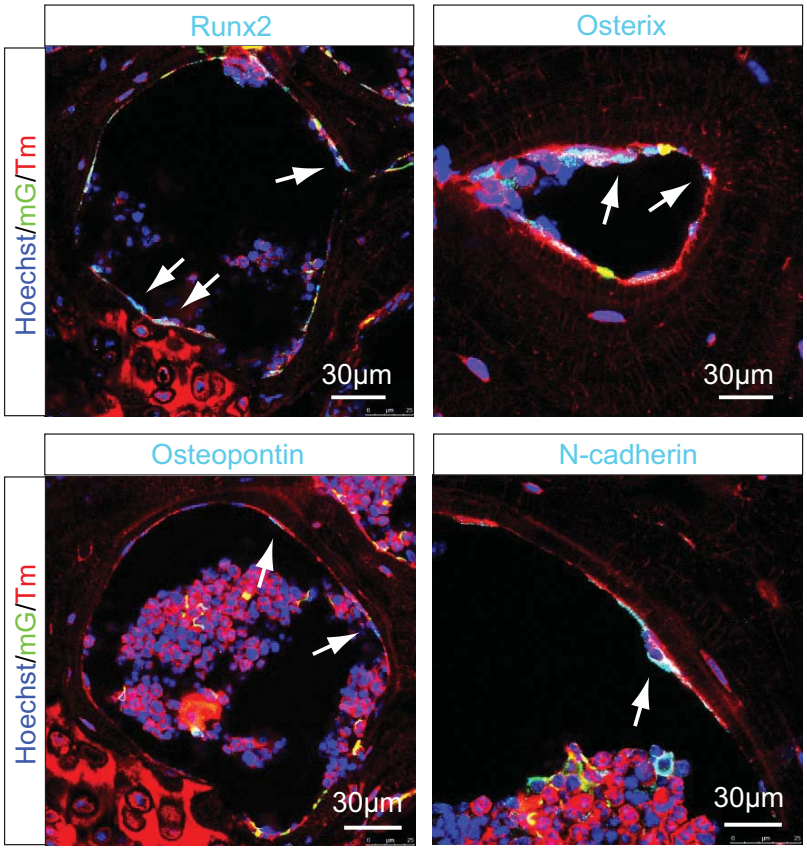

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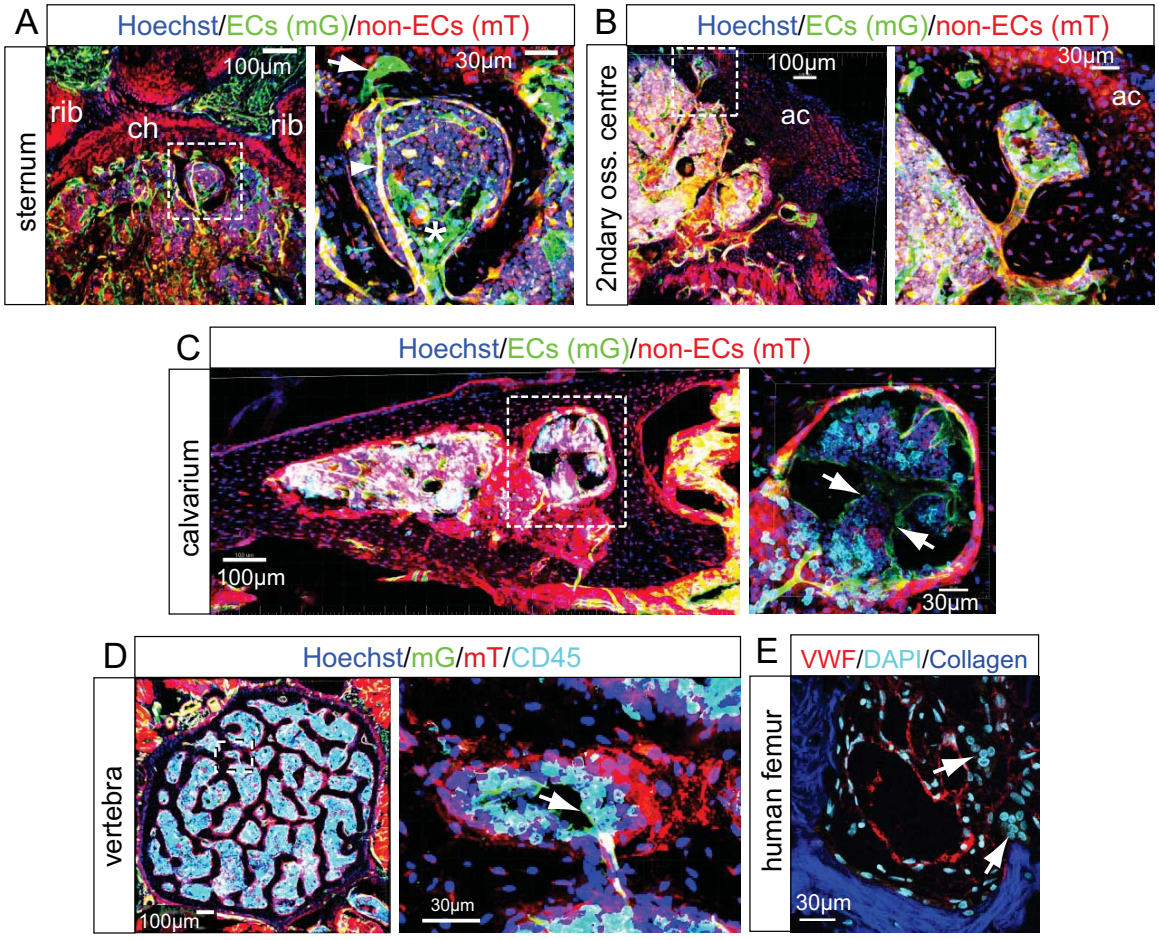

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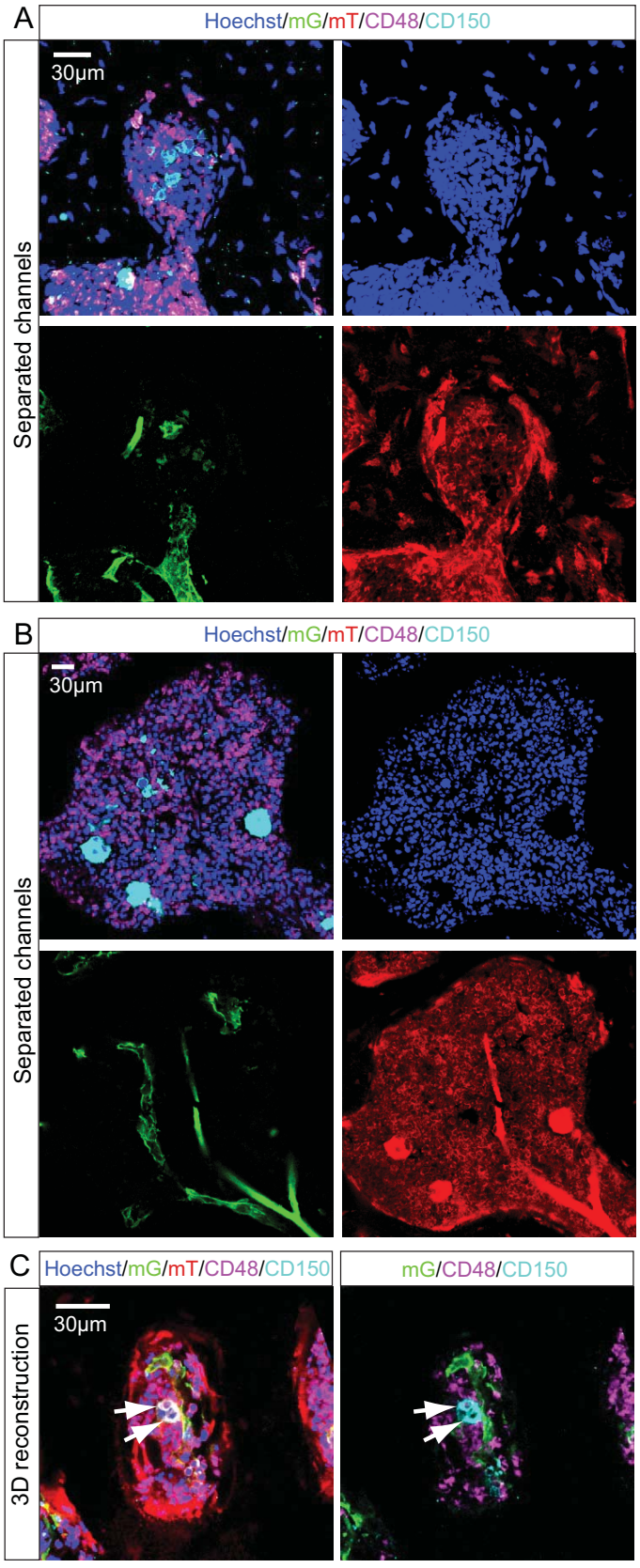

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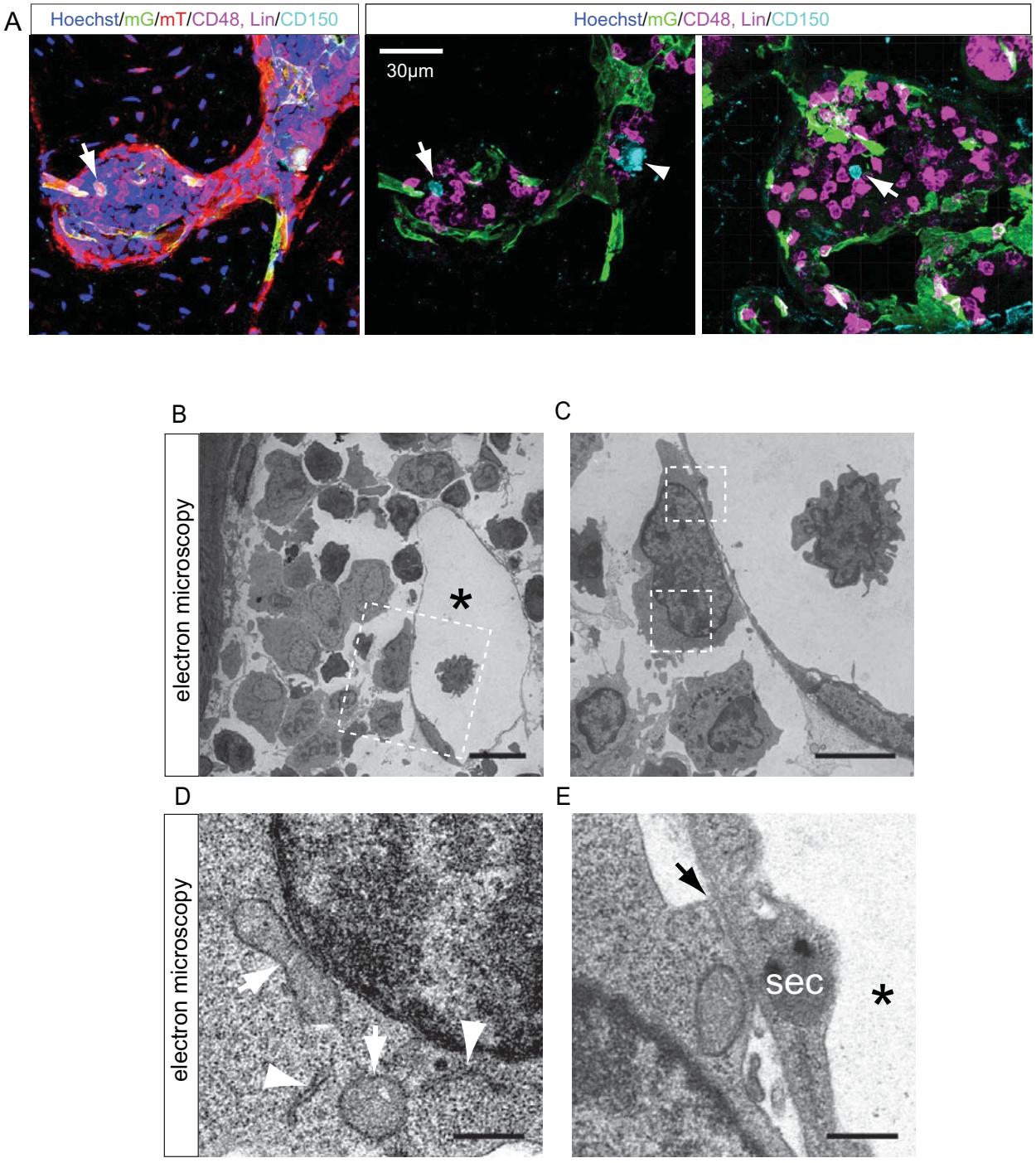

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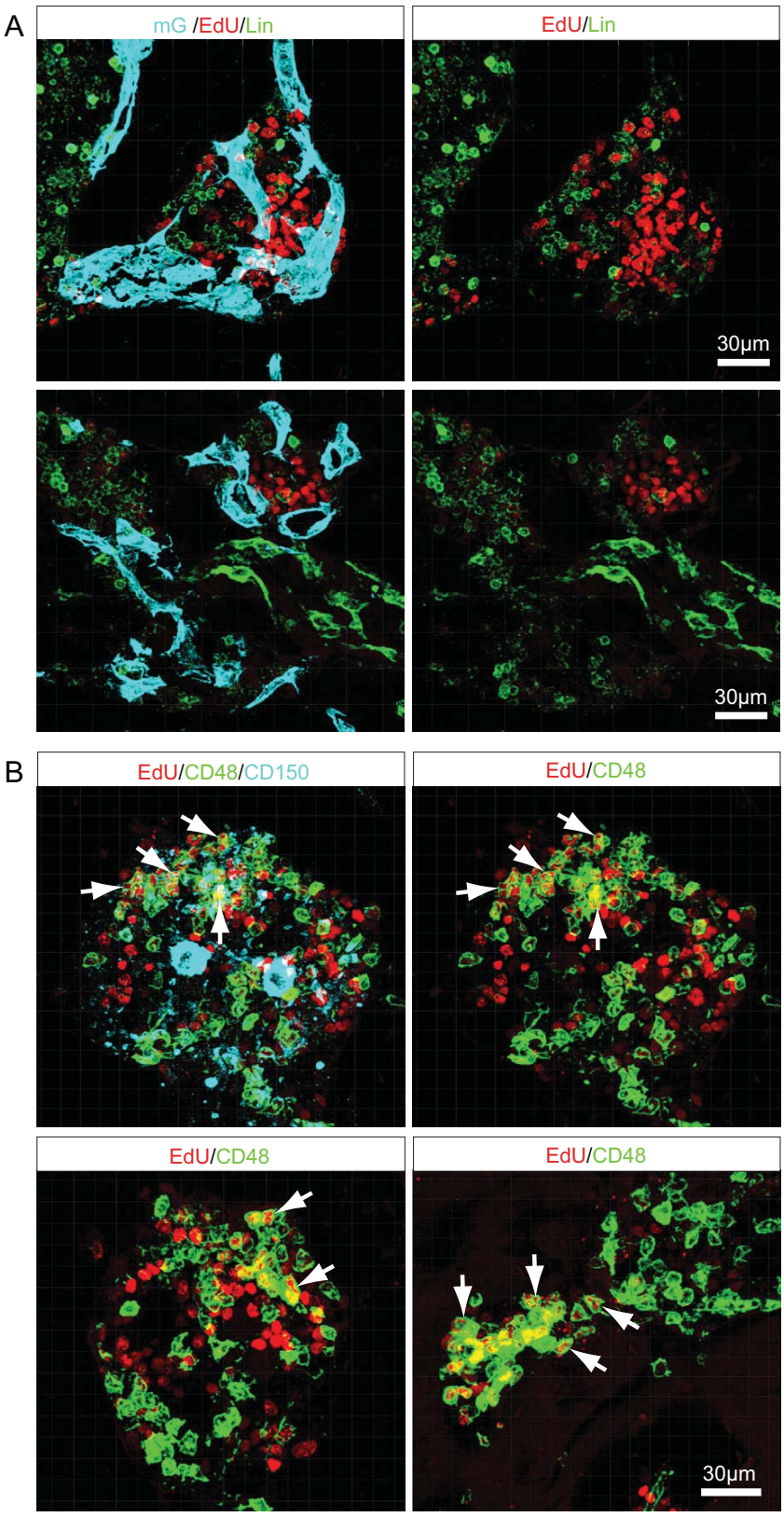

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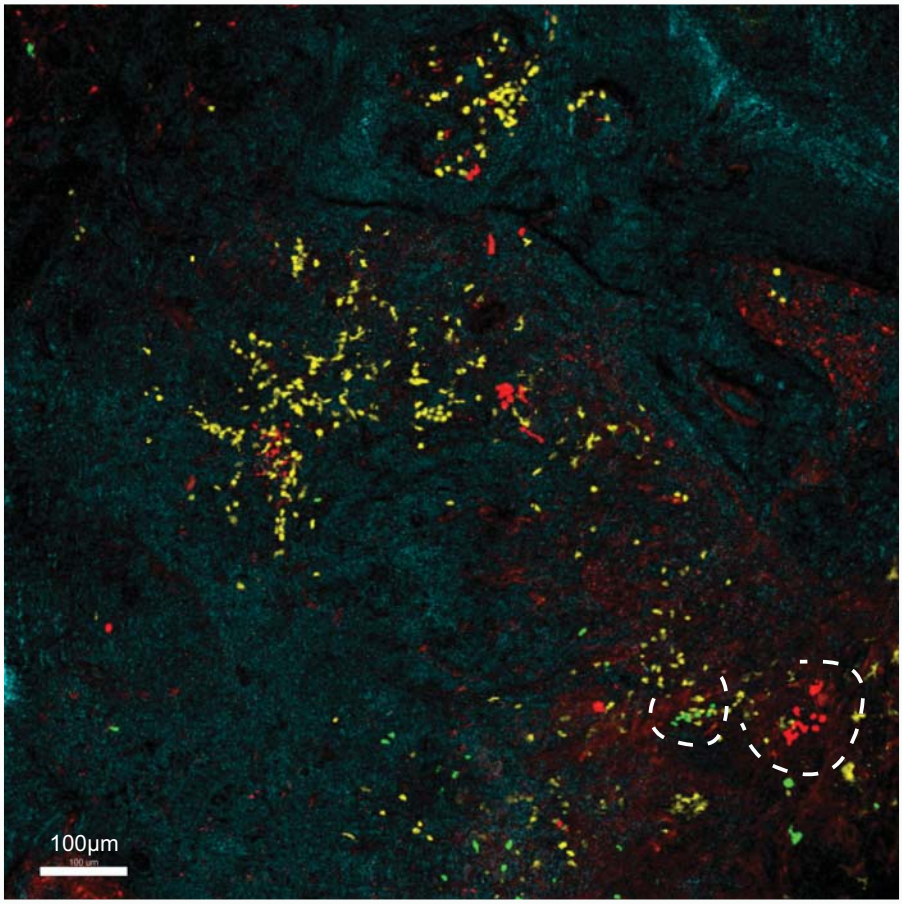

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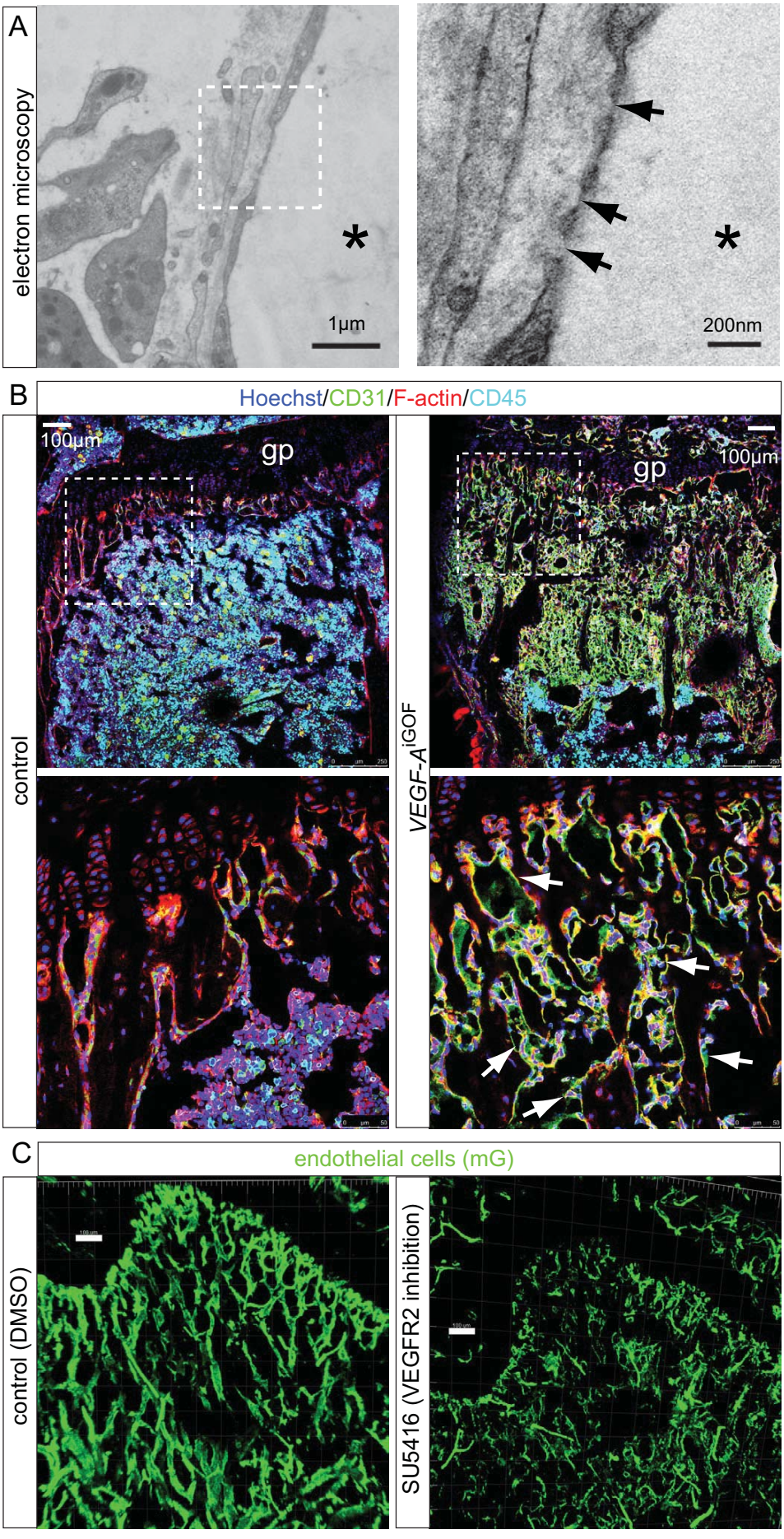

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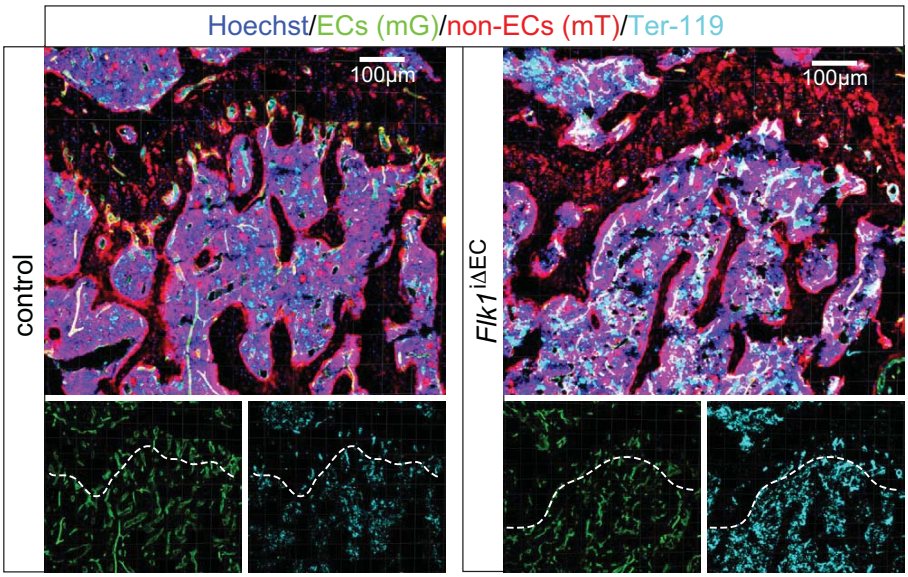

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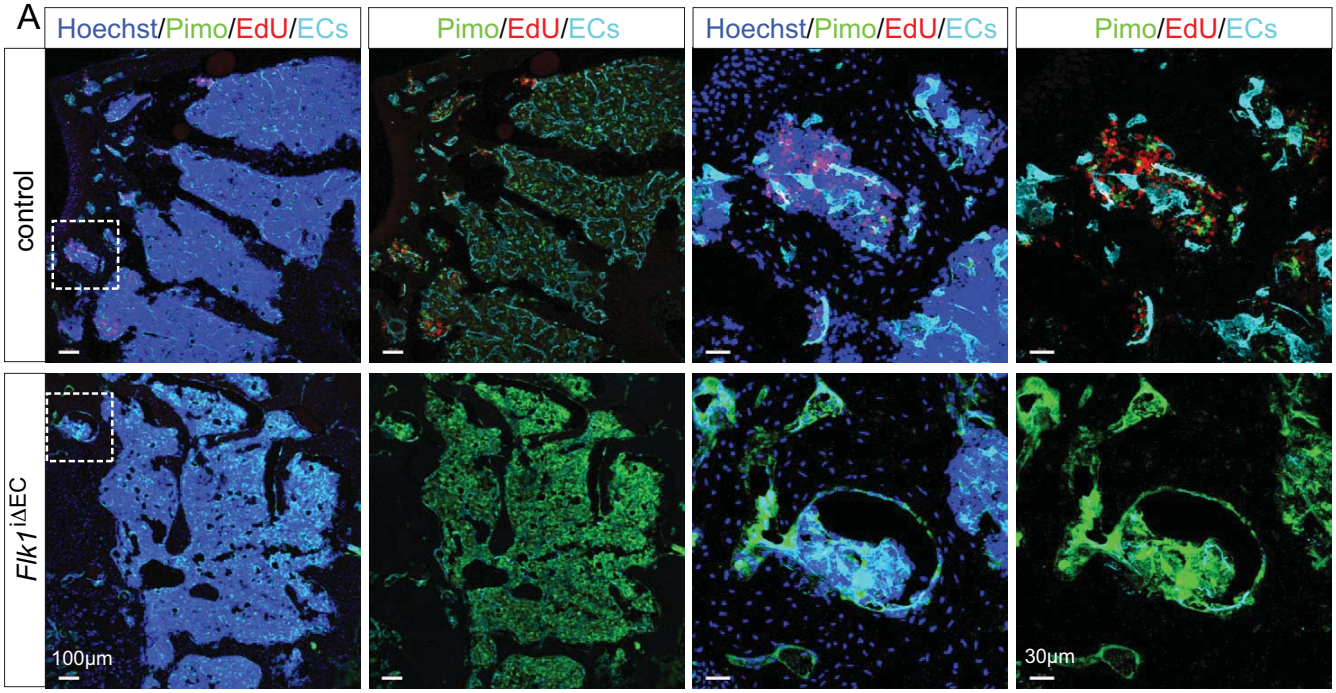

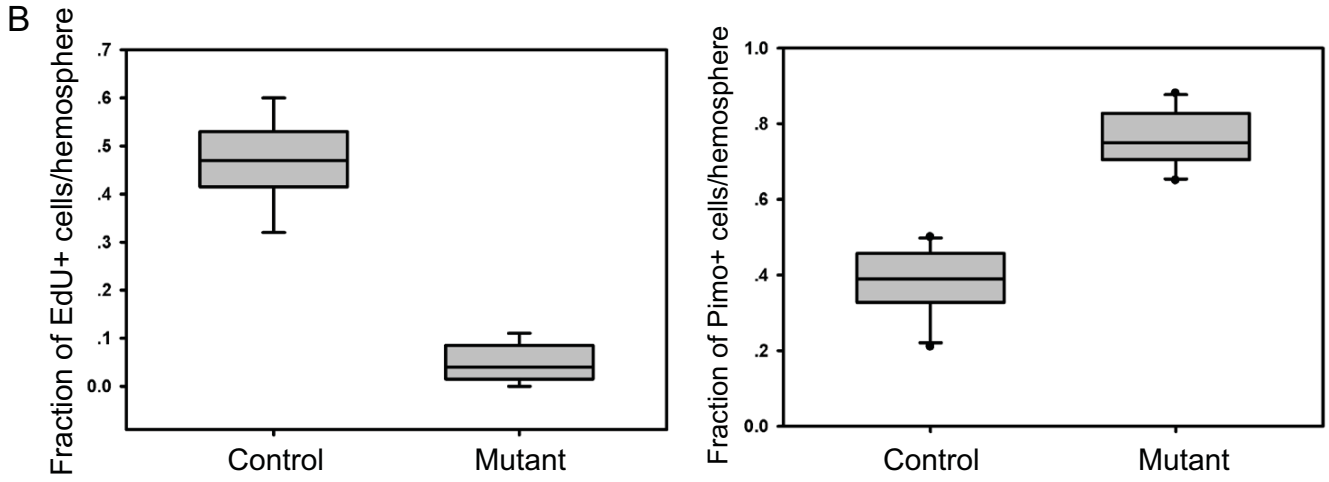

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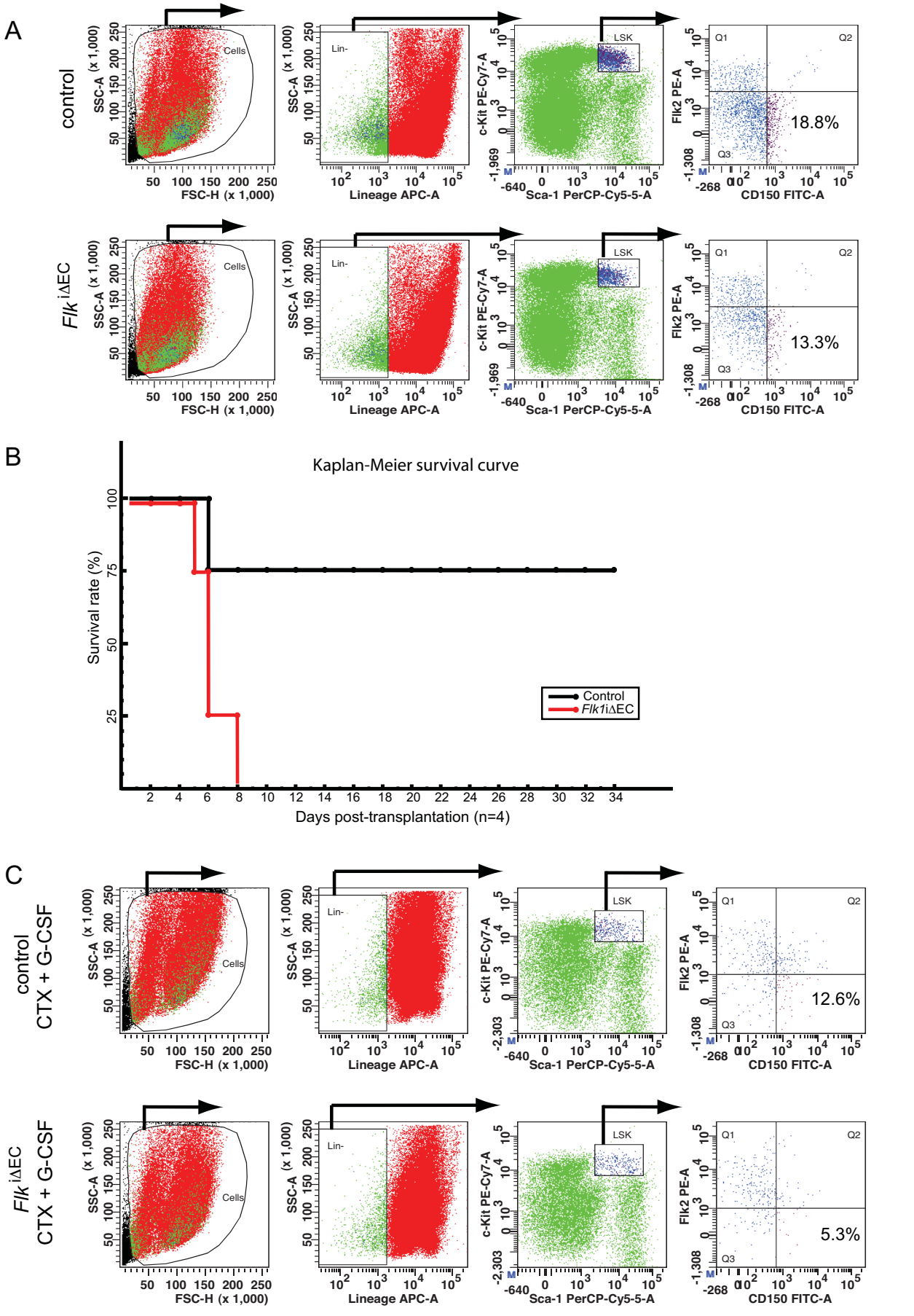

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