

THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Erythro-myeloid progenitors contribute endothelial cells to blood vessels

Citation for published version:

Plein, A, Fantin, A, Denti, L, Pollard, J & Ruhrberg, C 2018, 'Erythro-myeloid progenitors contribute endothelial cells to blood vessels', *Nature*. https://doi.org/10.1038/s41586-018-0552-x

Digital Object Identifier (DOI):

10.1038/s41586-018-0552-x

Link: Link to publication record in Edinburgh Research Explorer

Document Version: Peer reviewed version

Published In: Nature

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



- 1 Erythro-myeloid progenitors contribute endothelial cells to blood vessels
- 3 Alice Plein^{1,*}, Alessandro Fantin^{1,*}, Laura Denti¹, Jeffrey W. Pollard² and Christiana Ruhrberg^{1^}
- ¹ UCL Institute of Ophthalmology, University College London, 11-43 Bath Street, London EC1V
 9EL, UK
- ⁶ ²MRC Centre for Reproductive Health, University of Edinburgh, 47 Little France Crescent,
- 7 Edinburgh EH16 4TJ, UK
- 8

2

- 9 *contributed equally
- 10
- 11 ^Corresponding author:

12 Professor Christiana Ruhrberg, Tel.: 44 (0)20 7608 4017; email: c.ruhrberg@ucl.ac.uk

13

14 The earliest blood vessels in the mammalian embryo are formed when endothelial cells 15 (ECs) differentiate from angioblasts and coalesce into tubular networks. Thereafter, the 16 endothelium is thought to expand solely by proliferation of pre-existing ECs. Here we show 17 that the earliest precursors of erythrocytes, megakaryocytes and macrophages, the yolk 18 sac-derived erythro-myeloid progenitors (EMPs), provide a complementary source of ECs 19 that are recruited into pre-existing vasculature. Whereas a first wave of yolk sac-resident 20 EMPs contributes ECs to the yolk sac endothelium, a second wave of EMPs colonises the 21 embryo and contributes ECs to intraembryonic endothelium in multiple organs, where they 22 persist into adulthood. By demonstrating that EMPs constitute a hitherto unrecognised and 23 non-redundant source of ECs, we reveal that embryonic blood vascular endothelium expands in a dual mechanism that involves both the proliferation of pre-existing ECs and 24 25 the incorporation of ECs derived from hematopoietic precursors.

26 Blood vessels distribute oxygen, nutrients, hormones and immune cells through the vertebrate 27 body and help remove waste molecules. Accordingly, functional blood vessel formation during 28 embryogenesis is a prerequisite for vertebrate life. Endothelial cells (ECs) form the inner lining of 29 blood vessels to contain the blood and its constituents. In addition, ECs serve as signalling hubs to 30 integrate tissue-derived and blood-borne signals to regulate vascular function. In the mammalian 31 embryo, the first ECs arise from mesenchymal precursors termed angioblasts that condense into 32 yolk sac vasculature and the paired dorsal aortae; this process is initiated on embryonic day (E) 33 7.0 in mice ¹. Subsequently, embryonic blood vascular endothelium is thought to expand in a 34 process termed angiogenesis, in which ECs proliferate within existing endothelium to increase 35 vascular diameter, extend sprouts into avascular tissue areas or remodel into smaller vessels by intussusceptive growth ¹. Current consensus is therefore that embryonic ECs are a self-contained 36

cell lineage that expands by proliferation without contribution from new angioblasts or circulating
 precursors. In contrast, circulating endothelial progenitors and their relationship to monocytes and
 macrophages have controversially been described in adults².

40 Monocytes and macrophages are mononuclear phagocytic cells of the innate immune system that also modulate vascular growth³. Thus, tissue-resident macrophages of the embryonic mouse 41 42 central nervous system (CNS), termed microglia, and macrophages in the zebrafish larval trunk 43 contact ECs at the tip of neighbouring vessel sprouts to promote their anastomosis into perfused 44 vessel loops ^{4, 5}. Microglia have similar roles in the injured adult zebrafish brain ⁶. Macrophages 45 also secrete VEGFA to stimulate vessel growth for nerve repair in adult mice ⁷. In contrast, a direct 46 contribution of macrophages or other myeloid cell (MC) types to embryonic vascular endothelium 47 has not been reported; thus, CRE recombinase expression under the control of the myeloid Lysm or Vav promoters does not mark embryonic blood vascular endothelium in mice^{8,9}. 48

49 Most tissue-resident macrophages arise from erythro-myeloid progenitors (EMPs) that form in the 50 extra-embryonic yolk sac during embryogenesis and also serve as precursors for erythrocytes and megakaryocytes ¹⁰⁻¹⁴. In mice, a wave of early EMPs, also referred to as primitive progenitors, 51 52 buds from yolk sac blood islands between E7.0 and E8.25 and by E9.0 differentiates into yolk sac macrophages without monocytic intermediates ^{10, 13, 15-17}. Early yolk sac macrophages also 53 54 generate tissue-resident macrophages in the embryo proper, including microglia in the brain or Langerhans cells and Kupffer cells in the epidermis and liver, respectively ¹⁶. A later wave of EMPs 55 buds from yolk sac endothelium from E8.25 onwards ^{10, 11, 14, 17}. These cells leave the yolk sac via 56 the circulation to colonise the liver ^{14, 18}, where they expand into monocytes that subsequently 57 58 colonise peripheral organs to differentiate into tissue-resident macrophages, thereby diluting the 59 pool of early EMP-derived macrophages in many organs but not the CNS^{10, 16}.

60

61 Lineage tracing with *Csf1r-iCre* identifies ECs in developing brain vasculature

To target early EMPs ^{10, 13, 15}, microglia ^{19, 20} and other differentiated MCs ²¹, we and others have 62 63 used a transgene that expresses CRE recombinase under the control of the promoter for the 64 myeloid lineage gene Csf1r (Fms), which encodes the colony-stimulating factor 1 receptor CSF1R. Microglia appear as amoeboid or ramified single YFP⁺ cells in hindbrains from mouse embryos 65 carrying Csf1r-iCre and the Rosa^{Ytp} CRE/LoxP recombination reporter, with microglia and ECs also 66 stained for isolectin B4 (IB4) (**Fig. 1a**) ¹⁹. As previously shown ⁴, the number of IB4⁺ YFP⁺ microglia 67 68 in the hindbrain subventricular zone increased between E10.5 and E11.5, when vessels fuse into 69 the subventricular vascular plexus (SVP), and decreased after SVP formation by E12.5, when they 70 move into deeper hindbrain layers (Fig. 1b). Surprisingly, Csf1r-iCre additionally targeted sporadic 71 elongated IB4⁺ cells that appeared bound into the endothelium and increased in number between 72 E10.5 and E12.5 alongside SVP expansion (Fig. 1a-c; Extended Data Fig. 1a). Csf1r-iCre-73 targeting of vessel-bound cells was not an artefact caused by spontaneous recombination of the *Rosa*^{*Yfp*} reporter or unspecific immunostaining, because *Rosa*^{*Yfp*} littermates lacking *Csf1r-iCre* also lacked YFP staining (**Fig. 1a**). Furthermore, immunostaining of *Csf1r-iCre* hindbrains carrying *CAG-Cat-Egfp* as an alternative recombination reporter or imaging of tdTomato (tdTom) fluorescence in *Csf1r-iCre* hindbrains with the *Rosa*^{*tdTom*} reporter confirmed targeting of both microglia and vessel-bound elongated cells (**Extended Data Fig. 1b,c**). The independently generated *Csf1r-Mer-iCre-Mer* transgene, in which tamoxifen treatment is required for CRE activation ²², also targeted both vessel-bound cells and microglia in E12.5 hindbrains (**Fig. 1d**).

Immunolabelling of E12.5 Csf1r-iCre;Rosa^{Yfp} hindbrains for YFP and the macrophage marker 81 82 F4/80 (ADGRE1) or the pericyte marker NG2 excluded that vessel-bound YFP⁺ cells were 83 microglia/perivascular macrophages or pericytes (Extended Data Fig. 1d,e). Instead, 84 immunolabelling for the EC-specific transcription factor ERG and surface marker PECAM1 (CD31) showed that vessel-bound, elongated Csf1r-iCre-targeted cells were ECs (Fig. 1e). Accordingly, 85 their morphology was similar to ECs targeted with CRE encoded by the Cdh5-Cre^{ERT2} transgene, 86 which is expressed in ECs but not microglia ²³ and was activated by low dose tamoxifen treatment 87 88 (Fig. 1e). Moreover, Csf1r-iCre-targeted ECs formed CDH5⁺ junctions with neighbouring non-89 targeted ECs (Extended Data Fig. 1f).

90 Immunolabelling of E11.5 Csf1r-iCre;Rosa^{Yfp} hindbrains showed that microglia were CSF1R⁺ YFP⁺, as expected, but neither YFP⁻ nor YFP⁺ ECs expressed the CSF1R protein (Extended Data Fig. 91 92 2a). To determine whether the Csf1r promoter is active in ECs despite their lack of CSF1R protein 93 expression, we used a Csf1r-Egfp transgene that faithfully reports Csf1r promoter activity as EGFP expression ^{24, 25}. However, unlike microglia, ECs were not EGFP⁺ (Extended Data Fig. 2b). 94 95 Further, the analysis of publicly available transcriptomic datasets ²⁶ showed that *Csf1r* is neither 96 expressed in E14.5 brain nor liver or lung ECs (Extended Data Fig. 2c). Finally, fluorescenceactivated cell sorting (FACS) of Csf1r-iCre-lineage traced (tdTom⁺) ECs and MCs with antibodies 97 98 for PECAM1 versus the pan-hematopoietic marker CD45 (PTPRC) showed that tdTom⁺ PECAM1⁻ 99 CD45⁺ MCs expressed *Csf1r*, whereas tdTom⁺ PECAM1⁺ CD45⁻ ECs expressed *Cdh5*, but not the 100 the myeloid marker Spi1 (Pu.1) or Csf1r (Extended Data Fig. 2d-g). Putative Csf1r expression by 101 a subset of brain ECs therefore does not explain Csf1r-iCre-mediated endothelial targeting.

102

103 Csf1r-iCre-targeted ECs and EMPs are PU.1-independent and share spatiotemporal origins

Lack of endothelial CSF1R expression suggests that *Csf1r-iCre*-targeted brain ECs arise from precursors that activate *Csf1r* prior to their incorporation into hindbrain vasculature. By examining *Csf1r-iCre;Rosa*^{Yfp} hindbrains from *Pu.1* (*Spi1*)-null mice, we excluded that *Csf1r-iCre*-targeted ECs were derived from differentiated MCs or the recently identified tissue-resident myeloid precursors of pericytes ^{4, 23}. Thus, we found that PU.1 deficiency prevented the formation of YFP⁺ F4/80⁺ IB4⁺ microglia, as expected, but did not reduce the number of YFP⁺ IB4⁺ F4/80⁻ ECs (**Fig. 1**10 **1f-h**). Microglia were also absent from the striatum of *Csf1r-iCre;Rosa*^{Yfp};*Pu.1^{-/-}* brains on postnatal 111 day (P) 0, although YFP⁺ ECs were present (**Extended Data Fig. 2h**).

As PU.1 is not required for EMP formation ²⁷, we investigated whether the formation of Csf1r-iCre-112 targeted ECs is mechanistically linked to the emergence of $Csf1r^{+}$ EMPs. E8.5 Csf1r-Eqfp yolk 113 114 sacs contained clusters of round EGFP⁺ VEGFR2⁺ cells that protruded from VEGFR2⁺ endothelium into the vascular lumen (**Fig. 2a**), consistent with previously described EMP budding ¹⁴ and prior 115 116 work showing that EMPs express Csf1r¹⁰ and Vegfr2¹². Csf1r-iCre lineage tracing similarly 117 identified round VEGFR2⁺ cells that protruded into the yolk sac vascular lumen (**Fig. 2b**), including round cells that expressed the EMP marker KIT¹⁰ and persisted in PU.1-deficient yolk sacs at E8.5 118 (Fig. 2c; Extended Data Fig. 3a,b). The endothelium of vessels in Csf1r-iCre;Rosa^{Yip} yolk sacs 119 120 additionally contained larger and flatter YFP⁺VEGFR2⁺ cells, which lacked active Csf1r expression 121 (compare Fig. 2a with 2b). These cells also lacked KIT, but were PU.1-independent (Fig. 2c; 122 **Extended Data Fig. 3b**), similar to *Csf1r-iCre*-targeted hindbrain ECs.

123 Tamoxifen-induced, CRE-mediated reporter recombination is highest approximately 6 h after and 124 ends 24 h after injecting a single tamoxifen dose into a pregnant dam ²⁸, allowing us to activate Csf1r-Mer-iCre-Mer:Rosa^{tdTom} in discrete temporal windows at E8.5, E9.5 or E10.5 before 125 126 identifying lineage-traced cells in E12.5 yolk sacs by immunostaining (Extended Data Fig. 3c). 127 Yolk sac macrophages were labelled after E8.5 induction (Extended Data Fig. 3d), consistent with their origin from early $Csf1r^+$ EMPs ¹⁰. Induction at E9.5 or E10.5 also labelled yolk sac 128 129 macrophages, likely because macrophages express Csf1r from E9.5 onwards ^{13, 15}. Induction at 130 E8.5 or E9.5 additionally yielded tdTom⁺ yolk sac ECs, whereas induction at E10.5 did not 131 (Extended Data Fig. 3d). As EMPs are present in the yolk sac at E8.5 and E9.5, but home to the 132 liver thereafter ¹⁶, their local availability makes them plausible precursors of Csf1r-iCre-labelled volk sac ECs. In agreement, tamoxifen induction of a KitCreERT2 knockin allele ²⁹ at E8.5, when KIT⁺ 133 EMPs are in the yolk sac ¹⁴, labelled VEGFR2⁺ ERG⁺ yolk sac ECs alongside macrophages 134 135 (Extended Data Fig. 3e,f).

136 A recent study showed that late wave EMPs lack Csf1r expression when they form ¹⁰. To 137 investigate whether these EMPs express Csf1r after their liver homing, we paired Csf1r-Mer-iCre-*Mer:Rosa^{tdTom}* with *Csf1r-Eqfp* mice and induced pregnant dams with tamoxifen at E10.5 before 138 analysing E11.5 liver cells via FACS to distinguish differentiated MCs and EMPs ^{10, 13, 14}. We 139 140 observed tdTom⁺ Csf1r-Eqfp⁺ cells in the KIT⁻ CD45⁺ differentiated MC population, as expected, 141 but also in the KIT⁺ CD45^{lo} progenitor population that includes EMPs (Fig. 3a,b; Extended Data 142 **Fig. 3g-I**; KIT⁺ CD45⁻ cells were neither MCs nor EMPs, because they lacked CD45, tdTom and 143 EGFP). Blood cell analysis showed that cells from the Csf1r- $Eqfp^+$ tdTom⁺ KIT⁺ CD45¹⁰ population 144 that includes EMPs was still circulating at E11.5 and therefore could access embryonic organ 145 vasculature (Fig. 3a,b).

146 To investigate whether *Csf1r* expression by late wave intraembryonic EMPs correlated with the 147 emergence of *Csf1r-iCre*-targeted hindbrain ECs, we induced *Csf1r-Mer-iCre-Mer;Rosa*^{tdTom} 148 embryos at E8.5, E9.5 or E10.5 and visualised tdTom expression in E12.5 hindbrains (Fig. 3c). 149 Microglia were targeted at all three stages, consistent with their origin from early Csf1r-expressing 150 EMPs and their active Csf1r expression; in contrast, hindbrain vasculature contained tdTom⁺ ECs 151 following induction at E10.5, but not E8.5 or E9.5 (Fig. 3d). Accordingly, intraembryonic Csf1r-152 *iCre*-targeted ECs emerge at a time when late wave EMPs have moved from the yolk sac into the embryo proper and have activated *Csf1r* expression ¹⁰. *Kit^{CreERT2}* induction at E8.5, when both early 153 154 and late KIT⁺ EMPs are present in the yolk sac, caused microglia targeting (**Fig. 3e,f**), suggesting 155 that microglia can still be generated around E8.5 from KIT⁺ progenitor-derived yolk sac 156 macrophages ¹¹. This approach also yielded tdTom⁺ ECs in the E12.5 hindbrain and therefore 157 corroborated that yolk sac-born EMPs can give rise to intraembryonic ECs. Lineage tracing from 158 three independent Cre alleles therefore suggests that EMPs can differentiate into ECs.

159

160 The Csf1r-iCre-targeted EMP lineage gives rise to ECs in vitro

161 The myeloid and erythroid potential of EMPs has been demonstrated through in vitro differentiation 162 assays ^{14, 30}. To investigate their endothelial potential, we FACS-isolated KIT⁻ CD45⁺ PECAM1⁻ 163 differentiated MCs and the KIT⁺ CD45^{lo} PECAM1⁻ population that contains EMPs from E12.5 *Csf1r-iCre:Rosa^{tdTom}* liver and blood, and then used these cell populations for *in vitro* differentiation 164 165 that were combined with immunolabelling for myeloid and EC markers. Both populations were 166 mostly tdTom⁺ (Fig. 4a,b). As expected ¹⁰, the EMP population was comprised of large round cells 167 with a large nucleus and little cytoplasm, whereas the MC population contained granulocytes as 168 well as monocytes in the liver and macrophages in the blood (Fig. 4a,b). We next cultured both 169 cell populations in methocult, which promotes the formation of hematopoietic colonies, but 170 additionally included a fibronectin substrate to facilitate EC differentiation. The differentiated 171 tdTom⁺ MCs from both the liver and blood persisted as single round/amoeboid cells (Fig. 4c.d) that were ERG¹⁰ VEGFR2¹⁰ (Fig. 4e,f; secondary antibody only controls in Extended Data Fig. 4a,b). In 172 173 contrast, the tdTom⁺ EMP populations from both the liver and blood formed myeloid and erythroid 174 cell colonies in suspension (Fig. 4c,d). Moreover, both the EMP populations gave rise to single 175 adherent cells, which resembled ECs due to their spindle-shaped/elongated morphology, lacked 176 myeloid cell markers and were ERG^{hi} VEGFR2^{hi}, consistent with an EC identity (Fig. 4e,f; 177 Extended Data Fig. 4b,c). In contrast, most neighbouring, adherent round/amoeboid cells 178 expressed markers indicative of MC differentiation, including CD45, F4/80 or CSF1R (Fig. 4e,f; 179 Extended Data Fig. 4c). Together, these experiments demonstrate that EMPs have endothelial 180 potential alongside their known hematopoietic capacity.

181

182 Csf1r-iCre-targeted ECs support the growth of embryonic brain vasculature

Hoxa cluster genes regulate haematopoiesis ³¹ and are upregulated in postnatal compared to adult
 ECs ³², with HOXA9 also shown to promote EC differentiation from progenitor cells in adult

ischemic disease ³³. Our analysis of published transcriptomic data ^{12, 34} revealed that several Hoxa 185 186 transcripts are enriched in E10.25 compared to E9.0 EMPs or differentiated macrophages (Fig. 187 5a). To investigate whether Hoxa-deficiency impaired the formation of EMP-derived ECs, we 188 crossed Csf1r-iCre and Rosa^{tdTom} into mice carrying a conditional null Hoxa cluster mutation (Hoxa^{fl}) ³⁵ (Extended Data Fig. 5a). Gene copy analysis showed effective gene targeting in KIT⁺ 189 190 cells from E12.5 Csf1r-iCre;Hoxa^{fl/fl} mutant compared to control livers, but the number of CD45⁺ 191 cells, including differentiated MCs, was not reduced (Extended data Fig. 5b-f). These findings 192 suggest that Hoxa genes are dispensable for MC specification from late wave EMPs. In contrast, 193 significantly fewer tdTom⁺ ECs, also derived from late wave EMPs, had formed in Csf1r*iCre:Rosa^{tdTom}:Hoxa^{tl/fl}* mutant compared to control hindbrains; moreover, SVP complexity was 194 195 significantly reduced in the mutant compared to control hindbrains (Fig. 5b-d). Although we also 196 observed 20% fewer microglia in mutant compared to control hindbrains (Extended data Fig. 5g-197 i), this unlikely contributed to the vascular defect, because even 50% fewer microglia in Csf1^{op/+} 198 mutants did not reduce SVP complexity (Extended data Fig. 5j-I). Together, these findings 199 suggest that Hoxa cluster genes promote the formation of EMP-derived brain ECs, which in turn 200 support normal brain vascular development.

201

202The EMP-derived EC population has a core endothelial transcriptional signature with203enrichment for liver EC markers

204 As Csf1r-iCre-targeted ECs appeared morphologically similar to neighbouring ECs (e.g. Fig. 1), 205 with similarly slow proliferation and overall cell cycle kinetics (Extended Data Fig. 6), we next 206 compared the gene expression signature of both EC types by RNAseq. Thus, we used E12.5 207 Csf1r-iCre;Rosa^{tdTom} embryos for FACS to separate tdTom⁺ and tdTom⁻ ECs, defined as PECAM1⁺ 208 cells lacking KIT, CD45 and CD11b (ITGAM) (Fig. 6a). Both EC populations differed by their 209 expression of the Rosa transcript, as expected (Extended Data Fig. 7a), but otherwise had largely 210 similar transcriptomes with few differentially expressed genes (Fig. 6b,c). Consistent with an 211 endothelial identity, tdTom⁺ ECs lacked markers for differentiated MCs and other non-EC lineages. 212 but expressed transcripts for core EC markers at similar levels as tdTom ECs (Fig. 6d,e). 213 Amongst the differentially expressed genes, markers typical of EC specialisation were under-214 represented in tdTom⁺ ECs, such as ephrins and their EPH receptors, which regulate 215 arteriovenous differentiation, or Slc2a1, a marker of brain EC maturation (Fig. 6c,e,f). Reverse 216 transcriptase quantitative PCR (RT-gPCR) comparisons confirmed that the brain EC maturation 217 marker *Slc2a1* was expressed at lower levels in tdTom⁺ than tdTom⁻ ECs at E12.5, consistent with 218 delayed endothelial differentiation (Extended data Fig. 7b). This observation agrees with the idea 219 that Csf1r-iCre lineage-traced ECs in the brain are derived from circulating progenitors that are 220 recruited into a vascular endothelium that is pre-formed by ECs of classical origin. Whereas Slc2a1 221 and other markers of brain ECs were under-represented in the embryo-wide tdTom⁺ EC

222 population, liver EC markers were over-represented (e.g. Oit3, Mrc1), including early markers of liver sinusoidal differentiation (*Stab2* and *Lyve1*)³⁶ (Fig. 6c,f). These observations were confirmed 223 224 by RT-gPCR (Extended data Fig. 7b.c). RT-gPCR confirmed that liver transcripts were expressed 225 at similar levels in tdTom⁺ and tdTom⁻ liver ECs (**Extended Data Fig. 7d**), thereby corroborating 226 that EMP- and non-EMP-derived ECs are overall similar. Moreover, this finding suggested that the 227 over-representation of liver EC transcripts in the total embryonic EC population reflected a 228 preferential contribution of EMP-derived vascular progenitors to liver vasculature rather than 229 altered liver EC differentiation. Immunostaining and flow cytometry of Csf1r-iCre;Rosa^{tdTom} E12.5 230 and E18.5 embryos confirmed that tdTom⁺ ECs were more prevalent in liver endothelium than 231 tdTom⁻ ECs (Fig. 6g,i; Extended Data Figs. 8 and 9a,b). Agreeing with the RT-qPCR analysis, 232 MRC1 as well as LYVE1 were observed in both tdTom⁻ and tdTom⁺ ECs within E12.5 liver 233 vasculature (Fig. 6g; Extended Data Fig. 8a). Liver ECs of two distinct origins therefore undergo 234 similar organ-specific EC differentiation.

235

236 Csf1r-iCre-targeted ECs populate multiple embryonic organs and persist into adulthood

237 Immunostaining and FACS analyses at E18.5 confirmed that Csf1r-iCre-targeted ECs contribute to 238 brain and liver, but also heart and lung vasculature (Fig. 6i; Extended Data Fig. 8 and 9a,b). 239 EMPs therefore contribute to organ vasculature at multiple sites (working model in Extended Data 240 Fig. 9c). Corresponding immunostaining and FACS analyses of adult organs showed that tdTom⁺ 241 ECs persisted in the brain, heart, lung and liver (Fig. 6h, j; Extended Data Fig. 10). Moreover, 242 tdTom⁺ ECs continued to dominate the LYVE1⁺ MRC1⁺ liver sinusoidal endothelium of adult mice 243 (Fig. 6h,j; Extended Data Fig. 10b). Accordingly, organs differ with respect to their content of 244 EMP-derived ECs, with a remarkably high contribution to liver sinusoidal endothelium in both 245 embryos and adults.

246

247 Discussion

248 The heterogeneous origin of blood vascular mural cells from distinct populations of mesodermal progenitors, hematopoietic and neural crest cells is established ³⁷. Here, we have combined 249 250 constitutive and temporally inducible lineage tracing with FACS, immunostaining, transcriptomic 251 analyses and cell culture assays to show that embryonic vascular endothelium has two major 252 origins: a classical origin via angioblast differentiation into ECs and the unexpected differentiation 253 of ECs from the EMP lineage. Multiple prior investigations have utilised the Csf1r-iCre transgene 254 together with recombination reporters to follow the embryonic myeloid lineage ^{10, 13, 15}. These 255 studies predominantly employed flow cytometry with hematopoietic markers, which precluded the 256 observation of Csf1r-iCre-targeted ECs. In contrast, we included EC markers to isolate Csf1r-iCre-257 targeted ECs alongside MCs from embryonic and adult organs. Immunostaining of tissues was

previously also used to identify Csf1r-iCre-targeted cells in the retina ²⁰, liver and colon ²¹, but 258 259 without description of EC targeting, possibly because of the close spatial proximity of ECs and 260 perivascular macrophages ^{4, 6}. By performing high resolution imaging after immunostaining for EC 261 and macrophage makers, we have overcome this limitation to demonstrate targeting of both cell 262 types alongside each other in situ. The contribution of EMP-derived ECs to yolk sac, brain, heart 263 and lung vasculature is proportionally smaller than that of ECs of classical origin, whereas EMP-264 derived ECs predominate the liver, particularly the sinusoidal endothelium. Liver endothelium was 265 previously reported to be heterogeneous in origin, with an endoderm lineage contribution of 266 approximately 15% and the remainder of the liver EC population attributed to a venous origin, i.e. 267 angiogenic ingrowth from nearby veins ³⁸. Our results suggest that liver endothelium additionally 268 contains approximately 60% EMP-derived ECs, accordingly decreasing prior estimates for liver 269 ECs of venous origin. Preferential EMP homing to the liver after their entry into the embryonic 270 circulation ¹⁶ and the dependence of liver growth on rapid vascular expansion ³⁹ may explain the 271 relatively large contribution of EMP-derived ECs to this organ. Ultimately, the discovery that EMPs 272 provide a source of ECs for organ vasculature may open up new therapeutic avenues for vessel-273 dependent organ repair and regeneration. For example, EMPs or EMP-like EC progenitors, 274 derived from human stem cells via genetic manipulation of factors such as or including Hoxa 275 genes, may be delivered systemically to support vascular growth in ischemic diseases or provide 276 angiocrine signals that stimulate tissue stem cells.

277

278 **References**

- Potente, M., Gerhardt, H. & Carmeliet, P. Basic and therapeutic aspects of angiogenesis. *Cell* **146**, 873-887 (2011).
- Hirschi, K.K., Ingram, D.A. & Yoder, M.C. Assessing identity, phenotype, and fate of
 endothelial progenitor cells. *Arteriosclerosis, thrombosis, and vascular biology* 28, 1584-1595
 (2008).
- Pollard, J.W. Trophic macrophages in development and disease. *Nature Reviews Immunology* 9, 259-270 (2009).
- 4. Fantin, A. *et al.* Tissue macrophages act as cellular chaperones for vascular anastomosis
 downstream of VEGF-mediated endothelial tip cell induction. *Blood* **116**, 829-840 (2010).
- Gerri, C. *et al.* Hif-1alpha regulates macrophage-endothelial interactions during blood vessel
 development in zebrafish. *Nat Comm* 8, 15492 (2017).
- 290 6. Liu, C. *et al.* Macrophages Mediate the Repair of Brain Vascular Rupture through Direct
 291 Physical Adhesion and Mechanical Traction. *Immunity* 44, 1162-1176 (2016).
- 292 7. Cattin, A.L. *et al.* Macrophage-Induced Blood Vessels Guide Schwann Cell-Mediated
 293 Regeneration of Peripheral Nerves. *Cell* 162, 1127-1139 (2015).

- Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R. & Forster, I. Conditional gene targeting
 in macrophages and granulocytes using LysMcre mice. *Transgenic research* 8, 265-277
 (1999).
- de Boer, J. *et al.* Transgenic mice with hematopoietic and lymphoid specific expression of Cre.
 European J Immuol 33, 314-325 (2003).
- 10. Hoeffel, G. *et al.* C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to
 adult tissue-resident macrophages. *Immunity* 42, 665-678 (2015).
- 301 11. Frame, J.M., McGrath, K.E. & Palis, J. Erythro-myeloid progenitors: "definitive" hematopoiesis
 302 in the conceptus prior to the emergence of hematopoietic stem cells. *Blood cells, molecules & diseases* 51, 220-225 (2013).
- 304 12. Mass, E. *et al.* Specification of tissue-resident macrophages during organogenesis. *Science* 305 353 (2016).
- 306 13. Gomez Perdiguero, E. *et al.* Tissue-resident macrophages originate from yolk-sac-derived
 307 erythro-myeloid progenitors. *Nature* 518, 547-551 (2015).
- McGrath, K.E. *et al.* Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and
 Provide Functional Blood Cells in the Mammalian Embryo. *Cell Reports* **11**, 1892-1904 (2015).
- 310 15. Schulz, C. *et al.* A lineage of myeloid cells independent of Myb and hematopoietic stem cells.
 311 *Science* **336**, 86-90 (2012).
- 312 16. Ginhoux, F. & Guilliams, M. Tissue-Resident Macrophage Ontogeny and Homeostasis.
 313 *Immunity* 44, 439-449 (2016).
- 314 17. Hoeffel, G. & Ginhoux, F. Fetal monocytes and the origins of tissue-resident macrophages.
 315 *Cell Immunol* (2018). doiL 10.1016/j.cellimm.2018.01.001 [Epub ahead of print]
- 18. Lux, C.T. *et al.* All primitive and definitive hematopoietic progenitor cells emerging before E10
 in the mouse embryo are products of the yolk sac. *Blood* **111**, 3435-3438 (2008).
- 318 19. Fantin, A. *et al.* NRP1 acts cell autonomously in endothelium to promote tip cell function
 319 during sprouting angiogenesis. *Blood* **121**, 2352-2362 (2013).
- Stefater, J.A., 3rd *et al.* Regulation of angiogenesis by a non-canonical Wnt-Flt1 pathway in
 myeloid cells. *Nature* 474, 511-515 (2011).
- 21. Deng, L. *et al.* A novel mouse model of inflammatory bowel disease links mammalian target of
 rapamycin-dependent hyperproliferation of colonic epithelium to inflammation-associated
 tumorigenesis. *Am J Pathol* **176**, 952-967 (2010).
- 325 22. Qian, B.Z. *et al.* CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis.
 326 *Nature* 475, 222-225 (2011).
- 327 23. Yamazaki, T. *et al.* Tissue Myeloid Progenitors Differentiate into Pericytes through TGF-beta
 328 Signaling in Developing Skin Vasculature. *Cell Reports* 18, 2991-3004 (2017).
- 329 24. Sasmono, R.T. et al. A macrophage colony-stimulating factor receptor-green fluorescent
- protein transgene is expressed throughout the mononuclear phagocyte system of the mouse.
- Blood **101**, 1155-1163 (2003).

- 332 25. Burnett, S.H. *et al.* Conditional macrophage ablation in transgenic mice expressing a Fas 333 based suicide gene. *J Leukocyte Biol* **75**, 612-623 (2004).
- 334 26. Tam, S.J. *et al.* Death receptors DR6 and TROY regulate brain vascular development. *Dev* 335 *Cell* 22, 403-417 (2012).
- 336 27. Kierdorf, K. *et al.* Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8 337 dependent pathways. *Nature Neuroscience* 16, 273-280 (2013).
- 338 28. Wilson, C.H. *et al.* The kinetics of ER fusion protein activation in vivo. *Oncogene* 33, 4877339 4880 (2014).
- 340 29. Klein, S. *et al.* Interstitial cells of Cajal integrate excitatory and inhibitory neurotransmission
 341 with intestinal slow-wave activity. *Nat Comm* 4, 1630 (2013).
- 342 30. Palis, J., Robertson, S., Kennedy, M., Wall, C. & Keller, G. Development of erythroid and
 343 myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073344 5084 (1999).
- 345 31. Alharbi, R.A., Pettengell, R., Pandha, H.S. & Morgan, R. The role of HOX genes in normal
 hematopoiesis and acute leukemia. *Leukemia* 27, 1000-1008 (2013).
- 347 32. Toshner, M. *et al.* Transcript analysis reveals a specific HOX signature associated with 348 positional identity of human endothelial cells. *PloS one* **9**, e91334 (2014).
- 33. Rossig, L. *et al.* Histone deacetylase activity is essential for the expression of HoxA9 and for
 endothelial commitment of progenitor cells. *J Exp Med* 201, 1825-1835 (2005).
- 351 34. Browning, A.C. *et al.* Comparative gene expression profiling of human umbilical vein 352 endothelial cells and ocular vascular endothelial cells. *Br J Ophthalmol* **96**, 128-132 (2012).
- 353 35. Kmita, M. *et al.* Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene 354 function. *Nature* **435**, 1113-1116 (2005).
- 355 36. Nonaka, H., Tanaka, M., Suzuki, K. & Miyajima, A. Development of murine hepatic sinusoidal
 and the endothelial cells characterized by the expression of hyaluronan receptors. *Dev Dyn* 236, 22582267 (2007).
- 358 37. Majesky, M.W. Developmental basis of vascular smooth muscle diversity. *Arteriosclerosis,* 359 *thrombosis, and vascular biology* 27, 1248-1258 (2007).
- 360 38. Goldman, O. *et al.* Endoderm generates endothelial cells during liver development. *Stem Cell* 361 *Reports* 3, 556-565 (2014).
- 362 39. Matsumoto, K., Yoshitomi, H., Rossant, J. & Zaret, K.S. Liver organogenesis promoted by 363 endothelial cells prior to vascular function. *Science* **294**, 559-563 (2001).

364

- 365 **Supplementary information** is available in the online version of the paper.
- 366

367 Acknowledgements

- 368 We thank the Biological Resources, FACS, Imaging and Genomics facilities at UCL and E. Scarpa
- 369 for technical help, D. Saur, A. Mass, D. Duboule, M. Kmita and Y. Kubota for mouse strains, M.

370 Golding for helpful discussions. This research was supported by grants from the Wellcome Trust

371 (095623/Z/11/Z, 101067/Z/13/Z), Medical Research Council (MR/N011511/1) and British Heart

372 Foundation (FS/17/23/32718).

373 Author contributions

A.P., A.F. and C.R. conceived and planned this study, analysed the data and co-wrote the manuscript. L.D. performed genetic crosses and genotyping. A.P. and A.F. performed experiments either together or replicated each other's experiments, except for the cell cycle and *Hoxa* studies, which were carried out by A.P and A.F., respectively. J.W.P. provided mouse strains. C.R. supervised the project. All authors reviewed and edited the manuscript.

379

380 Author information. Reprints and permission information is available at <u>www.nature.com/reprints</u>.

The authors declare no competing interests. Correspondence and requests for materials should be addressed to C.R. (c.ruhrberg@ucl.ac.uk).

383

Data availability. All sequence data used in this study have been deposited in the NCBI Gene
 Expression Omnibus database and are accessible through accession numbers GSExxxx.

386

387

388 Figure legends

389

390 Fig. 1: *Csf1r-iCre* lineage tracing identifies ECs in developing brain vasculature.

(a) Confocal z-stacks of hindbrains of the indicated genotypes and gestational stages, wholemount labelled for YFP and IB4, identified *Csf1r-iCre* targeting of vessel-bound cells during hindbrain vascularisation. (b) Number of YFP⁺ IB4⁺ single cells (microglia) and YFP⁺ IB4⁺ vessel-bound cells (putative ECs) per 0.72 mm²; mean \pm SD, n=3 hindbrains each. (c) Correlation between vessel area and number of YFP⁺ putative ECs; r², coefficient of determination; P < 0.01; each data point represents the value of one hindbrain.

(d) Confocal z-stack of an E12.5 *Csf1r-Mer-iCre-Mer;Rosa^{tdTom}* hindbrain after tamoxifen delivery
 on E10.5, IB4 wholemount labelled and shown including tdTom fluorescence.

(e) Confocal z-stacks of *Csf1r-iCre;Rosa^{tdTom}* and *Cdh5-Cre^{ERT2};Rosa^{tdTom}* E12.5 hindbrains,
 wholemount labelled for the endothelial markers ERG and PECAM1 and shown including tdTom
 fluorescence to demonstrate that *Csf1r-iCre* targets ECs; *Cdh5-Cre^{ERT2}* was induced with a low
 tamoxifen dose at E11.5.

403 (f-h) Confocal z-stacks of Csf1r-iCre;Rosa^{Yfp} E11.5 hindbrains on a Pu.1^{+/+} versus Pu.1^{-/-}

- 404 background, wholemount labelled for YFP and F4/80 together with IB4, show that Csf1r-iCre-
- 405 targeted ECs are PU.1-independent. The boxed area in (f) was 3D surface rendered and is shown
- 406 in (g) en face and as a lateral view starting at the plane indicated by the yellow line; the stippled
- 407 line outlines the vascular lumen (lu). (h) Quantification of YFP⁺ microglia ($Pu.1^{+/+}$ n=4; $Pu.1^{-/-}$ n=3)
- 408 and ECs ($Pu.1^{+/+}$ n=6; $Pu.1^{-/-}$ n=7); mean ± SD; each data point represents the value for one
- 409 hindbrain; n.s., non-significant, * P > 0.05 (unpaired t-test).
- 410 *Symbols*: Microglia and ECs are indicated with arrowheads and arrows, respectively. Solid and 411 clear symbols indicate the presence or absence of marker expression, respectively.
- 412 Scale bars: 20 μm (**a,d,f**), 50 μm (**e**).
- 413

Fig. 2: *Csf1r-iCre*-targeted ECs emerge concomitantly with EMPs in the yolk sac.

Confocal z-stacks of E8.5 yolk sacs from *Csf1r-Egfp* mice (**a**) or *Csf1r-iCre;Rosa^{Yfp}* mice on a *Pu.1^{+/+} versus Pu.1^{-/-}* background (**b**), wholemount labelled for VEGFR2 and EGFP (**a**) or YFP (**b**). Lateral views of 3D-rendered yolk sac vasculature starting at the positions indicated by cyan and orange lines show lineage-traced YFP⁺ VEGFR2⁺ flat cells in the vascular wall and VEGFR2⁺ round cells expressing EGFP or YFP, respectively, protruding from the vascular wall into the lumen (lu). *Symbols*: Wavy arrows indicate EGFP⁺ or YFP⁺ VEGFR2⁺ EMPs/MPs, straight arrows YFP⁺ VEGFR2⁺ ECs. *Scale bars*: 20 µm.

422

423 Fig. 3: Csf1r-iCre-targeted hindbrain ECs emerge from intraembryonic EMPs.

(a,b) A pregnant *Csf1r-Egfp;Csf1r-Mer-iCre-Mer;Rosa^{tdTom}* dam was injected with a single
tamoxifen dose on E10.5 (a) before E11.5 liver and blood cells were analysed by flow cytometry
(b) for CD45 and KIT, followed by gating the CD45^{hi} KIT⁻ differentiated MC (blue) and CD45^{lo} KIT⁺
EMP/myeloid progenitor (MP) populations (pink) for EGFP and tdTom (n=4 embryos).

428 **(c-f)** Pregnant *Csf1r-Mer-iCre-Mer;Rosa^{tdTom}* (**c**,**d**) and *Kit^{CreERT2;}Rosa^{tdTom}</sup>* (**e**,**f**) dams were injected 429 with a single tamoxifen dose on the indicated days and confocal z-stacks obtained of E12.5 430 hindbrains after wholemount staining for the indicated markers, shown including tdTom 431 fluorescence. *Symbols*: Arrows indicate tdTom⁺ ECs, arrowheads macrophages and microglia and 432 wavy arrows a cluster of tdTom⁺ ERG⁻ IB4⁻ neural progenitors, which express *Kit* at E8.5. *Scale* 433 *bars*: 20 µm.

434

435 Fig. 4: EMPs in the liver and blood give rise to ECs *in vitro*.

(a,b) FACS gating strategy for separation of the indicated cell populations, including EMP lineage
 cells, from E12.5 *Csf1r-iCre;Rosa^{tdTom}* liver (a) and blood (b) using antibodies for PECAM1, CD45
 and I/(T, (t,p)) including properties of cells with tdTage properties in the MO and EMP(MP)

438 and KIT (top), including proportion of cells with tdTom recombination in the MC and EMP/MP

439 populations and Giemsa-Wright staining of representative cells (bottom); Mo, monocyte; GC,
 440 granulocyte; MΦ, macrophage.

(c,d) Brightfield images of hematopoietic colonies formed after three days in methocult (met.); note
white and reddish colour of myeloid and erythroid colonies, respectively. (e,f) Fibronectin (FN)adherent cells after three days in methocult were immunolabelled for the EC markers ERG and
VEGFR2 and counterstained with the nuclear label DAPI. *Symbols*: Arrows indicate tdTom⁺ ECs,
arrowheads tdTom⁺ MCs. Solid and clear symbols indicate high versus low level expression,
respectively, of the indicated markers. *Scale bars*: 20 μm.

447

448 Fig. 5: *Csf1r-iCre-*targeted ECs form in a *Hoxa-*dependent mechanism and promote 449 vascularisation in the embryonic hindbrain.

450 (a) Transcriptomic analysis of the indicated cell populations, based on published RNAseq and EC 451 microarray data ^{12, 34} ($n \ge 2$), shows that *Hoxa* genes are enriched in intraembryonic EMPs; white 452 represents low and black high relative expression levels of the indicated genes.

(b) Confocal z-stacks of E12.5 littermate hindbrains of the indicated genotypes, wholemount
labelled for F4/80, RFP to visualise tdTom and with IB4. *Symbols*: Arrows and arrowheads indicate
tdTom⁺ ECs and microglia, respectively. *Scale bars*: 50 μm.

456 (c) Percentage of tdTom⁺ relative to IB4⁺ EC volume in $Hoxa^{+/+}$ (n=3) versus $Hoxa^{il/il}$ mutant (n=7) 457 hindbrains on a *Csf1r-iCre;Rosa^{tdTom}* background. (d) Number of vascular branchpoints in control 458 (pooled *Csf1r-iCre⁺;Hoxa^{+/+}* and *Csf1r-iCre⁻* of any *Hoxa* genotype; n=13) versus $Hoxa^{il/il};Csf1r-iCre$ 459 mutant (n=9) hindbrains. Mean ± SD fold change; each data point represents the value for one 460 hindbrain; * P < 0.05 (unpaired t-test).

461

462 Fig. 6: *Csf1r-iCre*-targeted ECs have a core endothelial transcription signature with an 463 increase in liver EC transcripts, and they persist in adult organ vasculature.

(a) FACS of PECAM1⁺ CD45⁻ CD11b⁻ KIT⁻ cells from E12.5 Csf1r-iCre;Rosa^{tdTom} embryos, 464 465 including gating to separate tdTom⁻ and tdTom⁺ ECs for RNAseq. (b) Graphic representation of 466 genes whose expression is significantly different (green dots) or similar (black dots) between both EC populations. (c) Volcano plot of 247 transcripts with on average > 100 counts that are 467 468 significantly different between both populations; grey and red data points represent transcripts in 469 tdTom⁻ ECs with \geq 2-fold over- or under-representation, respectively, with selected genes named. 470 (d-f) Relative expression levels in both EC populations of (d) non-EC markers typical of myeloid 471 (*Cx3cr1-Ptprc*), astrocytic (*Gfap*), smooth muscle (*Acta2*), neuronal (*Rbfox3*, *Nefl*), skeletal muscle 472 (Myog) or epithelial (Cdh1) differentiation, (e) core EC or EC maturation markers and (f) 473 representative brain or liver EC specialisation markers, shown alongside their relative expression in brain versus liver/lung ECs based on microarrays³¹. RNAseq analysis: mean of normalised 474

- 475 counts ± SD, n=3 samples each (DESeq2; Benjamini-Hochberg's multiple comparisons test for p476 value adjustment, adjP). Microarray analysis: mean ± SD, n=5 samples each (2-way ANOVA,
- 477 Bonferroni's multiple comparisons test). * P < 0.05, ** P < 0.01, *** P < 0.001, ns, non-significant.
- (g,h) *Csf1r-iCre;Rosa^{tdTom}* E12.5 (g) and adult (h) liver cryosections were labelled for the indicated
 markers and RFP to visualise tdTom, including DAPI counterstaining in (h). *Symbols*: Arrows and
 arrowheads indicate tdTom⁺ ECs and macrophages, respectively. Clear arrowheads indicate
 VEGFR2⁻ macrophages. *Scale bar*. 50 μm.
- 482 (i,j) The relative contribution of tdTom⁺ ECs to organ vasculature at E12.5 (i) and in 12-week old 483 adult mice (j) was determined by flow cytometry of *Csf1r-iCre;Rosa^{tdTom}* brain, heart, lung and liver. 484 Mean \pm SD; n \geq 4 (E12.5) and n \geq 6 (adult), with each data point representing an individual organ 485 (1-way ANOVA, Tukey's multiple comparisons test, ** P < 0.01, *** P < 0.001).
- 486
- 487

488 (online only) Methods plus associated references

489

490 Mouse strains. All animal procedures were performed in accordance with the institutional Animal 491 Welfare Ethical Review Body (AWERB) and UK Home Office guidelines. To obtain mouse 492 embryos of defined gestational age, mice were paired in the evening and the presence of a vaginal 493 plug the following morning was defined as E0.5. In some studies, we analysed adult mice, defined as more than 8 weeks of age. Mice carrying the Csf1r-iCre transgene²¹ were mated to Rosa^{Yfp 40}, 494 Rosa^{tdTom 41} or CAG-cat-Egfp ⁴² mice. PU.1 heterozygous null mice ⁴³ were mated to Rosa^{Yfp} mice 495 and then Csf1r-iCre mice to obtain Csf1r-iCre;Rosa^{Yfp};Pu.1^{-/-} embryos. Hoxa^{fl/fl} mice ³⁵ were mated 496 497 to Rosa^{tdTom} mice and then Csf1r-iCre to obtain Csf1r-iCre;Rosa^{tdTom};Hoxa^{fl/fl} embryos. Csf1r-Mer*iCre-Mer*²², *Cdh5-Cre-ER*^{T2 44} and *Kit*^{CreERT2} mice²⁹ were mated to *Rosa*^{tdTom} mice. We also used 498 Csf1r-Egfp-Ngfr/Fkbp1a/Tnfrsf6 (short: Csf1r-Egfp) mice as a reporter of Csf1r expression ²⁵ and a 499 loss of function mutation in Csf1 (Csf1^{Op})⁴⁵. All mouse strains were maintained on a mixed 500 501 background (C57BI6/J;129/Sv), with the exception of Csf1r-Mer-iCre-Mer, which was maintained 502 on a mixed FVB:C57/bl6 background. For tamoxifen induction of CRE activity, tamoxifen (Sigma) 503 was dissolved to 2 mg/ml in peanut oil and administered via intraperitoneal injection into pregnant dams. For Cdh5-Cre-ER^{T2} and Csf1r-Mer-iCre-Mer, we injected 20 µg and 1 mg tamoxifen alone. 504 respectively; for Kit^{Cre-ERT2}, we injected 3 mg tamoxifen together with 1.75 mg progesterone 505 506 (Sigma).

507 Immunolabelling. Samples were fixed in 4% formaldehyde in PBS and processed as either 508 wholemount or 20 µm cryosections. Immunolabelling was performed as described previously ⁴⁶ 509 using the following antibodies and dilutions: goat anti-CDH5 (1:200; AF1002, lot FQI0116101, R&D 510 Systems), rabbit anti-CSF1R (1:500; sc-692, lot K1212, Santa Cruz), rat anti-EMCN (1:50; sc-511 65495, lot C2917, Santa Cruz), rabbit anti-ERG (1:200; ab92513, lot GR32027 69-1, Abcam), rat 512 anti-F4/80 (1:500; MCA497R, lot 1605, Serotec), chicken anti-GFP (1:1000; GFP-1020, lot 513 0511FP12, Aves) and rabbit anti-GFP (1:500; 598, lot 079, MBL) for YFP or EGFP labelling, rabbit 514 anti-IBA1 (1:500; 019-19741, Wako Chemicals), rat anti-KIT (1:500; 553353, lot 30259, BD 515 Pharmingen), rabbit anti-NG2 (1:200; AB5320, lot 2726769, Millipore), rat anti-PECAM1 (1:200; 516 553370, lot 5205656, BD Pharmingen), rabbit anti-pHH3 (1:400; 06-570, lot 2825969, Millipore), 517 rabbit anti-RFP (1:1000; PM005, lot 045, MBL), goat anti-VEGFR2 (1:200; AF644, lot 518 COA0417021, R&D Systems). Secondary antibodies used included Alexa Fluor-conjugated goat 519 anti-chick, -rabbit or -rat IgG (Life Technologies), or, for primary antibodies raised in goat, donkey 520 fluorophore-conjugated FAB fragments of anti-chick, -goat, -rabbit or -rat IgG (Jackson 521 ImmunoResearch). Biotinylated IB4 (L2140, lot 085M4032V, Sigma) followed by Alexa-conjugated 522 streptavidin (ThermoFisher) was used to detect brain endothelial cells and microglia 4, 19. Nuclei 523 were labelled with DAPI. Images were acquired with a LSM710 laser scanning confocal 524 microscope (Zeiss) and processed using LSM image browser (Zeiss) and Photoshop CS4 (Adobe) 525 software. Three-dimensional rendering including surface rendering and the generation of virtual

526 slices for lateral views of high-resolution confocal z-stacks was carried out with Imaris (Bitplane).

527 Fluorescence-activated cells sorting (FACS) and cell culture. Tissues were mechanically and 528 enzymatically homogenised in RPMI1640 with 2.5% foetal bovine serum (ThermoFisher), 100 529 μg/ml collagenase/dispase (Roche), 50 μg/ml DNAse (Qiagen) and 100 μg/ml heparin (Sigma), 530 incubated for 5 mins with 0.5 mg/ml rat Fc block (Becton Dickinson) and labelled with a 531 combination of PE/Cy7-conjugated rat anti-PECAM1 (clone 390, cat 102418, lot B212262), FITC-532 conjugated rat anti-CD45 (clone 30-F11, cat 103108, lot B246762) or CD41 (clone MWReg30, cat 533 133903, lot B201955), APC-conjugated rat anti-KIT (clone 2B8, cat 105812, lot B217855) and 534 PerCp/Cy5.5-conjugated rat anti-CD11b (clone M1/70, cat 101227) (all Biolegend). Appropriate 535 fluorescence gate parameters were established with unstained tissue, Csf1r-iCre- or Csf1r-Egfp-536 negative tissues and fluorescence-minus-one (FMO) staining. For cell cycle analysis, cell populations were incubated with 10 µg/ml Hoechst 33342 (Sigma) for 30 mins at 37°C 47 before 537 538 labelling with PE/Cy7-conjugated rat anti-PECAM1 and performing FACS analysis. In all 539 experiments, doublets were eliminated using pulse geometry gates (FSC-H versus FSC-A and 540 SSC-H versus SSC-A), whereas dead cells were removed using SYTOX Blue (Life Technologies) 541 or LIVE/DEAD Fixable Violet (Life Technologies). Single cell suspensions were analysed using the 542 BD LSRFortessa X-20 cell analyser or sorted using the BD Influx cell sorter (BD Biosciences); 543 FlowJo software (FlowJo LLC) was used for subsequent analyses. In some experiments, a fraction 544 of each population was cytospun onto a glass slide for Wright-Giemsa staining (Sigma) followed by 545 imaging using an LSM510 microscope equipped with an AxioCam MRc camera (Zeiss). For cell 546 culture experiments, cell populations were sorted into DMEM with 100 U/ml penicillin, 100 U/ml 547 streptomycin and 20% foetal bovine serum (all ThermoFisher) before seeding the cells into a 96-548 well plate coated with 10 µg/ml fibronectin (ThermoFisher). Cells were cultured in methocult 549 (Stemcell Technologies); haematopoietic colonies were imaged using a TS100 microscope 550 equipped with a DS-5M colour camera (Nikon). Adherent cells were fixed with 4% formaldehyde in 551 PBS and then labelled for VEGFR2, ERG, CD45, F4/80 and CSF1R (see above) before imaging 552 using a Ti-E microscope (Nikon).

553 **RNAseq.** PECAM1⁺ CD45⁻ CD11b⁻ KIT⁻ ECs were isolated from E12.5 Csf1r-Cre;Rosa^{tdTom} 554 embryos and divided into tdTom⁺ and tdTom⁻ populations with the Influx cell sorter before RNA 555 was extracted with the RNeasy Micro Kit (QIAGEN). cDNA was generated and amplified using the 556 SMART-seq V4 ultra low input RNA kit (Clontech). 100 pg of amplified cDNA per sample was used 557 to prepare a library with the Nextera XT kit (Illumina) and run on the NextSeg 500 sequencer 558 (Illumina). Raw sequence data were pre-processed to trim poor quality base calls and adapter 559 contamination using Trimmomatic v.0.36.4⁴⁸ and aligned to the mouse mm10 genome with STAR v.2.5.2b⁴⁹. Mapped reads were deduplicated to reduce PCR bias using Picard v2.7.1.1 software 560 561 (http://broadinstitute.github.io/picard/), and the reads-per-transcript were then calculated using FeatureCount v1.4.6.p5 software ⁵⁰. Differential expression was performed using the BioConductor 562 563 package DESeq2 via the SARTools wrapper v1.3.2.0⁵¹.

564 Reverse transcription polymerase chain reaction (PCR). We isolated cells with the Influx cell 565 sorter (see above). We extracted RNA with the RNeasy Micro Kit and synthesised cDNA with 566 Superscript IV (ThermoFisher). Quantitative (g) RT-PCR was performed with SYBR Green on an 567 HT7900 system (Applied Biosystems) using the following oligonucleotide pairs: Actb 5'-568 CACCACACCTTCTACAATGAG-3' and 5'-GTCTCAAACATGATCTGGGTC-3'; Cdh5 569 Csf1r 5'-5'-GATGCAGATGACCCCACTGT-3' and 5'-AGGGCATCTTGTGTTCCAC-3'; 570 TGCGTCTACACAGTTCAGAG-3' and 5'-ATGCTGTATATGTTCTTCGGT-3': Spi1 5'-571 GCCATAGCGATCACTACTG-3' and 5'-CAAGGTTTGATAAGGGAAGC-3'; Hoxa11 5'-572 TCTTTGCCTCTCTCCTTCCTT-3' and 5'-TTGCAGACGCTTCTCTTTGTT-3'; Evx1 5'-573 GTGTGCTCTGGGCTCCTGT-3' and Slc2a1 5'-5'-GCCAGGGTGCCTTGAGAG-3; 574 CCCCAGAAGGTTATTGAGGAGT and 5'-ACAAAGAGGCCGACAGAGAA; Mrc1 5'-

575 ACTGGGCAATGCAAATGGAG and 5'- CCCTCAAAGTGCAATGGACA; *Oit3* 5'-576 CGTCTGCTTCCATGTCTACTG and 5'-GTGCTCACATTCATTTCGTCA. For each 577 oligonucleotide pair, a no-template control reaction was included.

578

579 *Microarray analysis.* Published microarray data ²⁶ were used to compare gene expression levels 580 in E14.5 CD45⁻ PECAM1⁺ brain versus pooled lung and liver ECs using GEO2R software (NCBI).

581 Statistical Analysis. Tissues for analysis were allocated to experimental groups according to 582 genotype and gestational age. The number of YFP⁺ ECs and YFP⁺ microglia in Csf1r-iCre;Rosa^{Yp} hindbrains (Fig. 1a,b and 1f-h) was determined in three randomly chosen 0.72 mm² regions of 583 each wholemount labelled and flatmounted hindbrain. For hindbrains in Hoxa-targeting 584 585 experiments, the number of F4/80⁺ microglia (**Extended Data Fig. 5**) and tdTom⁺ and IB4⁺ volume 586 (Fig. 5b,c) were determined from confocal z-stacks of four randomly chosen 0.18 mm² regions on 587 the lateral side of each hindbrain (Extended Data Fig. 5g). The z-stacks were surface rendered 588 with Imaris (Bitplane) to obtain the $F4/80^+$, tdTom⁺ and IB4⁺ volumes, and the $F4/80^+$ volume was 589 then subtracted from both the IB4⁺ and tdTom⁺ total volume to obtain the IB4⁺ EC and tdTom⁺ EC 590 volume before calculating the ratio of tdTom⁺ to IB4⁺ EC volume. To determine the number of 591 vascular intersections in Hoxa-targeting experiments (Fig. 5b,d), the same confocal z-stacks were 592 analysed with Imaris filament tracer after F4/80⁺ microglia were masked. For Fig. 1 and Fig. 5, all 593 counts obtained from one hindbrain were averaged to yield the value for that hindbrain; to ensure 594 unbiased interpretation of results, the genotypes were disclosed only after data collection was 595 complete. For all experiments, we calculated the mean value for at least 3 independent samples, 596 where error bars represent the standard deviation of the mean (for details, see figure legends). 597 Comparison of medians against means justified the use of a parametric test; to determine whether 598 two datasets were significantly different, we therefore calculated p values with a two-tailed 599 unpaired Student's t test; P < 0.05 was considered significant. When more than two data sets were 600 compared, we used the statistical tests indicated in the associated figure legends. Statistical 601 analyses were performed with Excel 12.2.6 (Microsoft Office) or Prism 5 (GraphPad Software).

602 References specific to the online methods

- 40. Srinivas, S. *et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into
 the ROSA26 locus. *BMC Dev Biol* 1, 4 (2001).
- 41. Madisen, L. *et al.* A robust and high-throughput Cre reporting and characterization system for
 the whole mouse brain. *Nature neuroscience* **13**, 133-140 (2010).
- Kawamoto, S. *et al.* A novel reporter mouse strain that expresses enhanced green fluorescent
 protein upon Cre-mediated recombination. *FEBS letters* **470**, 263-268 (2000).
- 43. McKercher, S.R. *et al.* Targeted disruption of the PU.1 gene results in multiple hematopoietic
 abnormalities. *The EMBO journal* **15**, 5647-5658 (1996).
- 44. Zarkada, G., Heinolainen, K., Makinen, T., Kubota, Y. & Alitalo, K. VEGFR3 does not sustain
 retinal angiogenesis without VEGFR2. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 761-766 (2015).
- 45. Yoshida, H. *et al.* The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345**, 442-444 (1990).
- 46. Vieira, J.M., Schwarz, Q. & Ruhrberg, C. Selective requirements for NRP1 ligands during
 neurovascular patterning. *Development* 134, 1833-1843 (2007).
- 47. Goodell, M.A., Brose, K., Paradis, G., Conner, A.S. & Mulligan, R.C. Isolation and functional
 properties of murine hematopoietic stem cells that are replicating in vivo. *The Journal of experimental medicine* **183**, 1797-1806 (1996).
- 48. Bolger, A.M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence
 data. *Bioinformatics* **30**, 2114-2120 (2014).
- 49. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21 (2013).
- 50. Liao, Y., Smyth, G.K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923-930 (2014).
- 51. Varet, H., Brillet-Gueguen, L., Coppee, J.Y. & Dillies, M.A. SARTools: A DESeq2- and EdgeR-Based R Pipeline for Comprehensive Differential Analysis of RNA-Seq Data. *PloS one* **11**,
- 628 e0157022 (2016).
- 629

630 Extended data figure legends:

631

632 Extended data figure 1: Endothelial *Csf1r-iCre* targeting is observed with different 633 recombination reporters and targeted ECs are distinguishable from macrophages and 634 pericytes.

(a-c) Confocal z-stacks of *Csf1r-iCre;Rosa^{Yfp}* (a) *Csf1r-iCre;CAG-Cat-Egfp* (b) and *Csf1r-iCre;Rosa^{tdTom}* (c) hindbrains at the indicated stages were wholemount labelled with IB4 and YFP
(a) or EGFP staining (b) or are shown together with tdTom fluorescence (c). In (a), the white
squares indicate areas shown in higher magnification in **Fig. 1a**. The indicated single channels are
also shown individually.

(d,e) Confocal z-stacks of E12.5 *Csf1r-iCre;Rosa*^{Yfp} hindbrains, wholemount labelled for YFP and the microglia marker F4/80 (d) or the pericyte marker NG2 (e) together with IB4 show that *Csf1riCre-targeted* vessel-bound cells are neither microglia nor pericytes. In (d), the boxed area is shown in higher magnification and as single channels adjacent to the panel. In (e), a single optical y/z cross section at the position indicated with the yellow line is displayed at higher magnification with single channels.

(f) Confocal z-stacks of a *Csf1r-iCre;Rosa^{tdTom}* E12.5 hindbrains, wholemount labelled for ERG and
 CDH5 and shown including tdTom fluorescence to demonstrate that *Csf1r-iCre* targets *bona fide* ECs that form junctions with neighbouring ECs.

549 *Symbols*: Microglia and ECs are indicated with arrowheads and arrows, respectively, pericytes with 550 double arrowheads, junctional CDH5 staining with a curved arrow. Solid and clear symbols 551 indicate the presence or absence of marker expression, respectively. *Scale bars*: 100 μ m (**a**), 20 552 μ m (**b-e**), 50 μ m (**f**).

653

654 Extended data figure 2: Endothelial *Csf1r-iCre-*targeting is not caused by endothelial *Csf1r* 655 expression and occurs independently of myeloid differentiation.

(a,b) Confocal z-stacks of E11.5 *Csf1r-iCre;Rosa^{Yfp}* (a) or *Csf1r-Egfp* (b) hindbrains, wholemount
 labelled for CSF1R and YFP or EGFP together with IB4, show lack of CSF1R protein and promoter
 activity in embryonic ECs.

659 (c) Graphic representation of relative *Cdh5* and *Csf1r* expression levels in E14.5 brain or pooled 660 lung/liver EC microarrays ³¹; n = 5 each; *** P > 0.001 (unpaired t-test).

(d-f) FACS separation of tdTom⁺ cells from *Csf1r-iCre;Rosa^{tdTom}* embryos, including (d)
 representative gating strategy to exclude dead cells and doublets in this and subsequent
 experiments and (e) sorting into PECAM1⁺ CD45⁻ ECs versus CD45⁺ PECAM1⁻ MCs for RT-qPCR
 analysis. (f) Representative gene amplification graphs for *Csf1r* versus *Actb* from tdTom⁺ MCs and

- 665 ECs; ΔRn, normalised reporter value for SYBR Green minus baseline instrument signals. (**g**) 666 Graphic representation of the fold change in amplification of the indicated genes relative to *Actb* for 667 both cell populations. Each data point represents the value of one embryo; n=3 each; * P > 0.05, 668 *** P > 0.001 (unpaired t-test).
- 669 (h) Confocal z-stacks of Csf1r-*iCre;Rosa*^{Yfp} P0 striatum on a $Pu.1^{+/+}$ versus $Pu.1^{-/-}$ background,
- 670 wholemount labelled for YFP and F4/80 together with IB4, show that *Csf1r-iCre*-targeted ECs are
- 671 PU.1-independent.
- 572 *Symbols*: Arrowheads indicate microglia, arrows YFP⁺ ECs, clear arrows YFP⁺ ECs that are 573 CSF1R⁻ and F4/80⁻.
- 674 *Scale bars*: 20 μm.
- 675

676 Extended data figure 3: Lineage tracing of yolk sac and liver EMPs.

677 (**a**,**b**) Confocal z-stacks of wholemount labelled E8.5 wild type (**a**) and Pu.1^{-/-} (**b**) yolk sacs on a

678 Csf1r-iCre;Rosa^{Yfp} background, labelled for YFP and KIT, show Csf1r-iCre-targeted KIT⁺ round

- 679 cells corresponding to EMPs/MPs and *Csf1r-iCre*-targeted KIT⁻ flat cells corresponding to ECs.
- 680 (**c-f**) Pregnant Csf1r-Mer-iCre-Mer;Rosa^{tdTom} (**c,d**) and Kit^{CreERT2;}Rosa^{tdTom} (**e,f**) dams were injected
- with a single tamoxifen dose on the indicated days; confocal z-stacks of E12.5 yolk sacs,
- immunolabelled for the indicated markers, show *Csf1r-iCre*-targeted ECs and macrophages.
- Symbols: Wavy arrows indicate EMPs, straight arrows *Csf1r-iCre*-lineage traced ECs, arrowheads
 macrophages. Solid and clear symbols indicate the presence or absence, respectively, of the
 indicated markers. *Scale bars*: 20 μm.
- (g-i) Pregnant dams were injected with a single tamoxifen dose on E10.5 (g) before analysis of
 E11.5 liver cells from *Csf1r-Egfp;Csf1r-Mer-iCre-Mer;Rosa^{tdTom}* (h; n=4 embryos) or *Csf1r-Mer-iCre-Mer;Rosa^{tdTom}* embryos lacking *Csf1r-Egfp* (i; n=4 embryos) by flow cytometry for CD45 and
 KIT; the CD45^{hi} KIT⁻ differentiated MC (blue), CD45^{lo} KIT⁺ EMP/myeloid progenitor (MP) (pink)
- and CD45⁻ KIT⁺ populations (grey) were gated further for Csf1r-Egfp and tdTom.
- 691

692 Extended data figure 4: Immunostaining controls for the analysis of cultured *Csf1r-iCre*-693 targeted cells.

The indicated cell populations were isolated by FACS from E12.5 Csf1r- $iCre;Rosa^{tdTom}$ livers or blood and cultured for three days in methocult before adherent cells were stained. In the first panel in each row, the phase contrast and DAPI images were merged. In panels 2-4 in each row, immunolabelled cells are visualised together with tdTom fluorescence, with single channels for the indicated markers shown separately in grey scale. In (**a**,**b**), adherent cells from tdTom⁺ liver MC (**a**) and EMP/MP (b) cultures were stained for ERG and VEGFR2 (top panels) or with secondary
antibodies only (bottom panels). In (c), adherent cells from tdTom⁺ blood EMP/MP cultures were
immunostained for CSF1R together with the myeloid markers CD45 (top panels) or F4/80 (bottom
panels). *Symbols*: Arrows indicate tdTom⁺ ECs, arrowheads tdTom⁺ myeloid cells. Solid and clear
symbols indicate the presence or absence, respectively, of the indicated markers. *Abbreviations*:
met, methocult; FN, fibronectin. *Scale bars*: 20 μm.

705

706 Extended data figure 5: *Csf1r-iCre-*mediated *Hoxa* ablation impairs the EMP lineage.

707 (a-f) Schematic representation of the Hoxa cluster and adjacent Evx1 gene, including position of 708 the LoxP sites used for gene targeting. (b) FACS strategy to isolate KIT⁺ cells from control (pooled 709 Csf1r-iCre⁻ or Csf1r-iCre⁺;Hoxa^{+/+}; n=14), Hoxa^{+/fl};Csf1r-iCre heterozygous (n=6) and E12.5 710 Hoxa^{1///}; Csf1r-iCre mutant livers (n = 8). (c) qPCR analysis of Hoxa gene copy number relative to 711 Evx1. Data are shown as mean \pm SD; each symbol represents the value for one individual liver; * P 712 < 0.05, *** P < 0.001 (1-way ANOVA, Tukey's multiple comparisons test). (d-f) Representative 713 FACS analysis (d) and quantification (e,f) of liver cell populations at E12.5 shows a similar number 714 of CD45⁺ or CD45⁺ CD11b⁺ cells (differentiated MCs) in control (pooled Csf1r-iCre⁻ or Csf1r-715 *iCre⁺;Hoxa^{+/+}*, n=25 for CD45⁺ and n=17 for CD45⁺ CD11b⁺) versus *Hoxa^{t/A}:Csf1r-iCre* (n=7 for 716 $CD45^+$ and n=6 for $CD45^+$ $CD11b^+$). Mean ± SD fold change in mutants compared to control; each 717 data point represents the value for one hindbrain; ** P < 0.01; ns, non-significant (unpaired t-test).

718 (g-i) E12.5 hindbrains of the indicated genotypes were immunolabelled to determine vascular 719 complexity and microglia. (g) Schematic representation of embryonic hindbrain position (left) and 720 location of the hindbrain areas i-iv used for quantification in each hindbrain (right); values for the 721 four areas in each hindbrain were averaged to obtain the value for that hindbrain; EC 722 quantifications are shown in Fig. 5c. (h) Confocal z-stacks after wholemount labelling with IB4 and 723 for RFP to visualise tdTom and F4/80 to visualise microglia; the white boxes indicate areas shown 724 in higher magnification in Fig. 5. (i) Quantification of microglia number in Hoxa^{tVII}, Csf1r-iCre mutants (n=9) versus controls (n=10, pooled Csf1r-iCre⁺;Hoxa^{+/+} and Csf1r-iCre⁻ of any Hoxa 725 726 genotype). Mean ± SD fold change in mutant compared to control hindbrain; each data point 727 represents the value for one hindbrain; ** P < 0.01 (unpaired t-test).

(j-l) Confocal z-stacks of E11.5 $Csf1^{+/+}$ and $Csf1^{+/op}$ littermate hindbrains, wholemount labelled for F4/80 together with IB4 (j) before quantification of the number of microglia (k) and vascular branchpoints (l); note that a 50% microglia reduction in $Csf1^{+/op}$ compared to $Csf1^{+/+}$ hindbrains did not reduce vascular complexity. Mean ± SD; each data point represents the value for one hindbrain, n=3 each; ** P < 0.01; ns, non-significant (unpaired t-test).

- 733 Scale bars: 200 μm (h), 100 μm (j).
- 734

735 Extended data figure 6: *Csf1r-iCre*-targeted ECs proliferate in vivo.

(**a**,**b**) Confocal z-stacks of E12.5 *Csf1r-iCre;Rosa^{tdTom}* wholemount yolk sac (**a**) or hindbrain (**b**) 736 737 after staining for the proliferation marker pHH3 and VEGFR2 or for pHH3 together with IB4, 738 respectively, and shown together with tdTom fluorescence. The areas indicated with white squares 739 are shown in higher magnification below the corresponding panel, with tdTom and pHH3 channels 740 also shown separately in grey scale. Symbols: The arrows indicate proliferating tdTom⁺ pHH3⁺ 741 ECs. Solid and clear symbols indicate the presence or absence, respectively, of tdTom 742 fluorescence. The wavy arrow indicates a tdTom⁻ pHH3⁺ neural progenitor. Scale bars: 200 μm 743 (top panels), 20 µm (lower panels).

744 (c-e) Cell cycle distribution of tdTom⁺ and tdTom ECs. (c) FACS gating strategy to isolate tdTom⁺ and tdTom⁻ PECAM1⁺ cells from *E12.5 Csf1r-iCre;Rosa^{tdTom}* embryos. (d) Graphic representation 745 746 of cell distribution based on Hoechst 33342 fluorescence as a measure of DNA content; low and 747 high staining intensity is observed in cells with a DNA ploidy of 2n, i.e. G0/G1 phase, or 4n, i.e. 748 G2/M phase, respectively; an intermediate staining intensity corresponds to S phase. (e) 749 Quantification of the proportion of tdTom⁺ and tdTom⁻ PECAM1⁺ cells in the G1. S and G2/M 750 phases based on the area of the corresponding peaks in (d). Mean \pm SD, n = 3 each; n.s., non-751 significant (paired t-test).

752

Extended data figure 7: Validation of gene expression data from RNAseq and microarray studies.

ECs were isolated by FACS as in **Fig. 6a** for validation of RNAseq and microarray data presented in **Fig. 6d-f.**

(a) Relative transcript levels of the *Rosa26* locus by RNAseq of E12.5 tdTom⁺ and tdTom⁻ EC populations, whose analysis is presented in **Fig. 6a-f**; mean \pm SD of normalised counts, n=3 each; ** P < 0.01 (unpaired t-test).

- (b) RT-qPCR analysis for the indicated genes in tdTom⁺ versus tdTom⁻ ECs isolated from the
 whole embryo (n=5) to validate differentially expressed genes identified via RNAseq in Fig. 6e,f.
- 762 (c) RT-qPCR analysis for the indicated genes in tdTom⁻ ECs isolated from the brain and liver (n=3
- each) to validate organ-specific transcript enrichment identified via microarray analysis in **Fig. 6f**.
- (d) RT-qPCR analysis for the indicated genes to directly compare the expression levels of brain
 and liver EC differentiation markers in tdTom⁺ versus tdTom⁻ ECs isolated from brain (n=3) or liver
 (n=5) (d) at E12.5.
- Slc2a1 was analysed as a representative brain EC-enriched transcript/differentiation marker, *Mrc1* and *Oit3* as representative liver EC-enriched transcripts. Mean ± SD of fold change; * P < 0.05, **
 P < 0.01, *** P < 0.001; ns, non-significant (unpaired t-test); ND, not detectable.

770 Extended data figure 8: *Csf1r-iCre*-targeted ECs contribute to embryonic organ vasculature.

(a) Cryosections of the indicated organs from E12.5 *Csf1r-iCre;Rosa^{tdTom}* mice were immunolabelled for the indicated EC markers together with antibodies for RFP to identify tdTom protein (top and bottom panels) or are shown with tdTom fluorescence (middle panels); single channels are shown in grey scale. The white boxes indicate the position of areas shown in higher magnification in **Fig. 6g**; note that some areas selected for higher magnification are not contained entirely within the field of view, and accordingly the boxes are not complete. *Scale bars:* 200 µm.

(b) Gating strategy for flow cytometry of dissociated cells from E12.5 *Csf1r-iCre;Rosa^{tdTom}* embryos
and a control sample lacking *iCre* after staining with antibodies for CD11b, CD41, CD45, KIT,
PECAM1 to determine the relative contribution of tdTom⁺ versus tdTom⁻ECs to vasculature in the
brain, lung, heart and liver (associated quantifications shown in **Fig. 6i**; an analogous strategy was

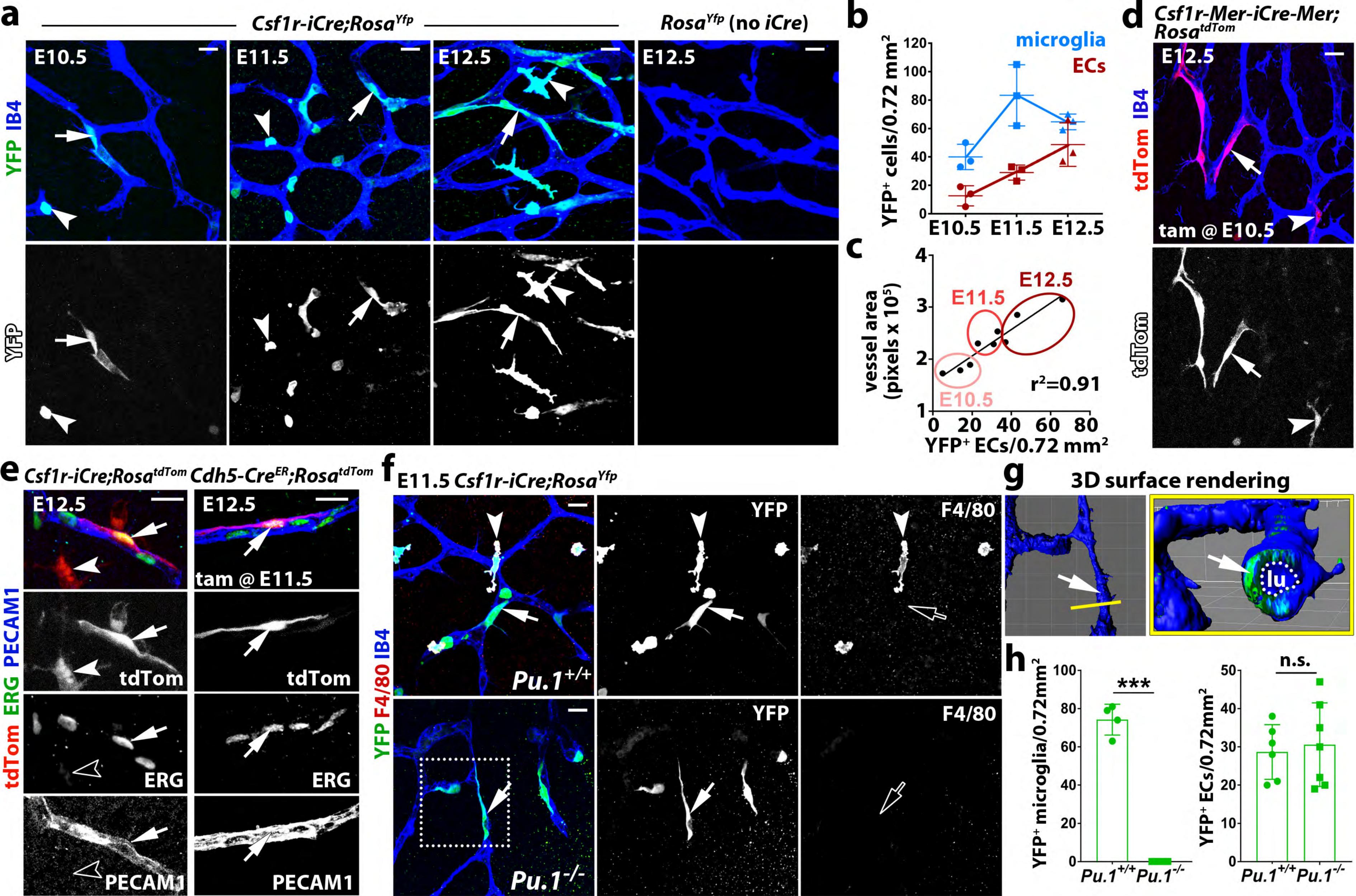
- used for the quantifications shown in **Fig. 6** and in the **Extended Data Fig. 9** b).
- 782

783 Extended data figure 9: *Csf1r-iCre*-targeted ECs contribute to organ vasculature in late 784 stage embryos.

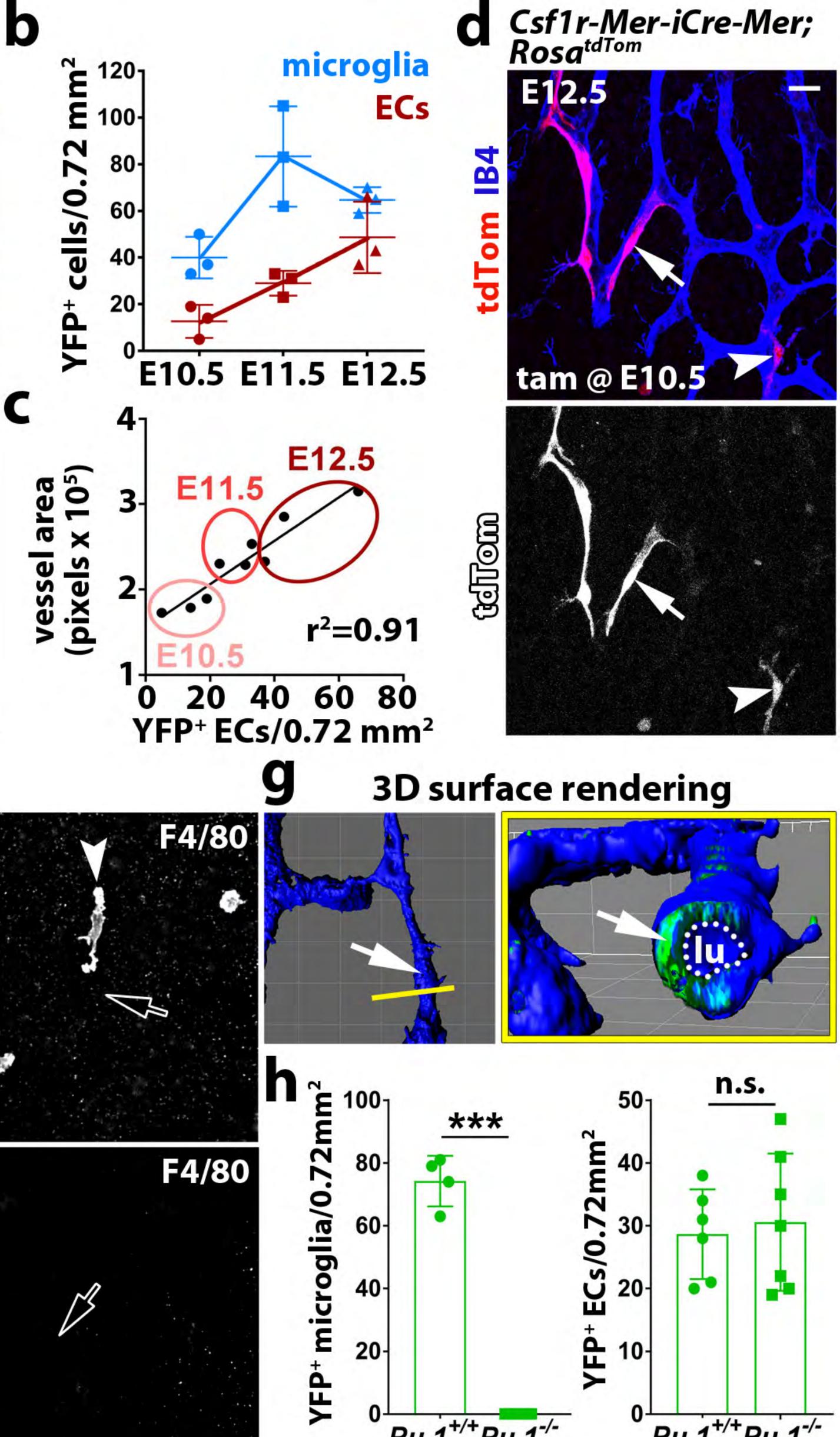
- (a) Cryosections of the indicated organs from E18.5 *Csf1r-iCre;Rosa^{Yfp}* mice were immunolabelled
 for YFP, PECAM1 and the macrophage marker IBA1; single channels are shown in grey scale. *Symbols*: Arrowheads indicate YFP⁺ and IBA1⁺ macrophages; solid and empty arrows indicate ECs
 that are YFP⁺ and lack IBA1 expression, respectively. *Scale bars*: 20 µm.
- (b) Flow cytometry of dissociated cells from the indicated organs of E18.5 *Csf1r-iCre;Rosa^{tdTom}* embryos after staining with antibodies for CD11b, CD41, CD45, KIT, PECAM1, using the gating strategy shown in the **Extended data Fig. 8a**; mean \pm SD, n = 5 each; *** P > 0.001 (1-way ANOVA, Tukey's multiple comparisons test).
- (c) Working model for the role of EMPs in generating extra-embryonic yolk sac and intra embryonic organ ECs alongside their known role in generating myeloid and
 erythrocyte/megakaryocyte cells.
- 796

797 Extended data figure 10: *Csf1r-iCre*-targeted ECs contribute to adult organ vasculature.

Cryosections of the indicated organs from 6 months old adult Csf1r- $iCre;Rosa^{Yfp}$ (**a**) or 3 months old adult Csf1r- $iCre;Rosa^{tdTom}$ (**b**) mice were immunolabelled for the indicated EC and macrophage markers together with antibodies for YFP or RFP; single channels are shown in grey scale. The white box in (**b**) indicates an area shown in higher magnification in **Fig. 6h**. *Symbols*: Arrowheads indicate YFP⁺ and F4/80⁺ macrophages; solid and empty arrows indicate ECs that are YFP⁺ and lack F4/80 expression, respectively. *Scale bars*: 20 µm (**a**), 100 µm (**b**).

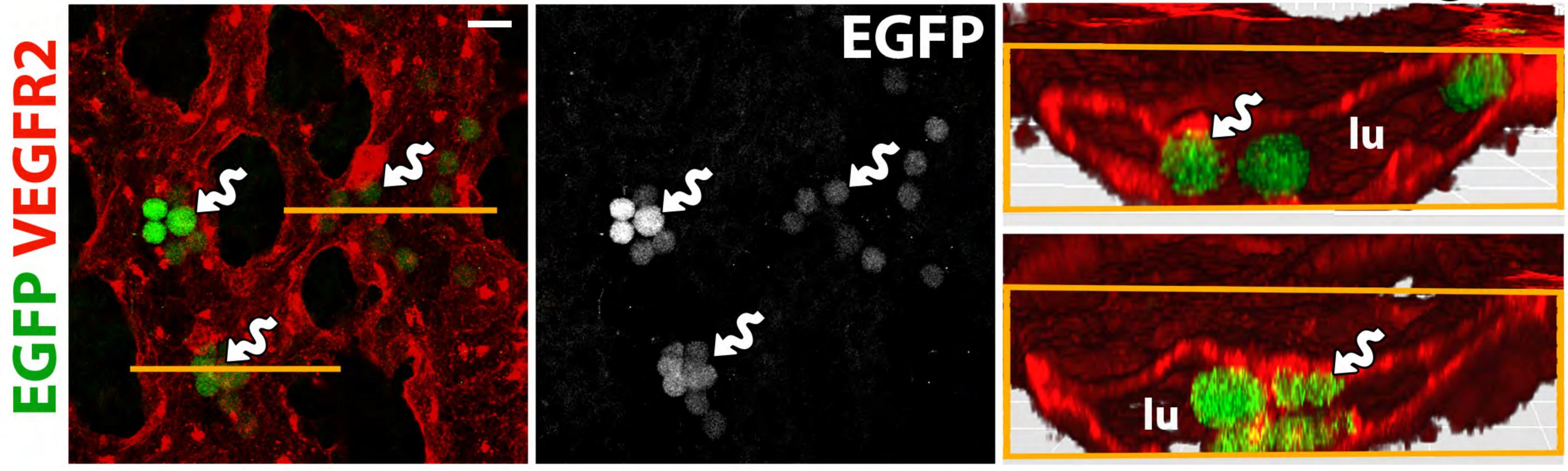


PECAM1



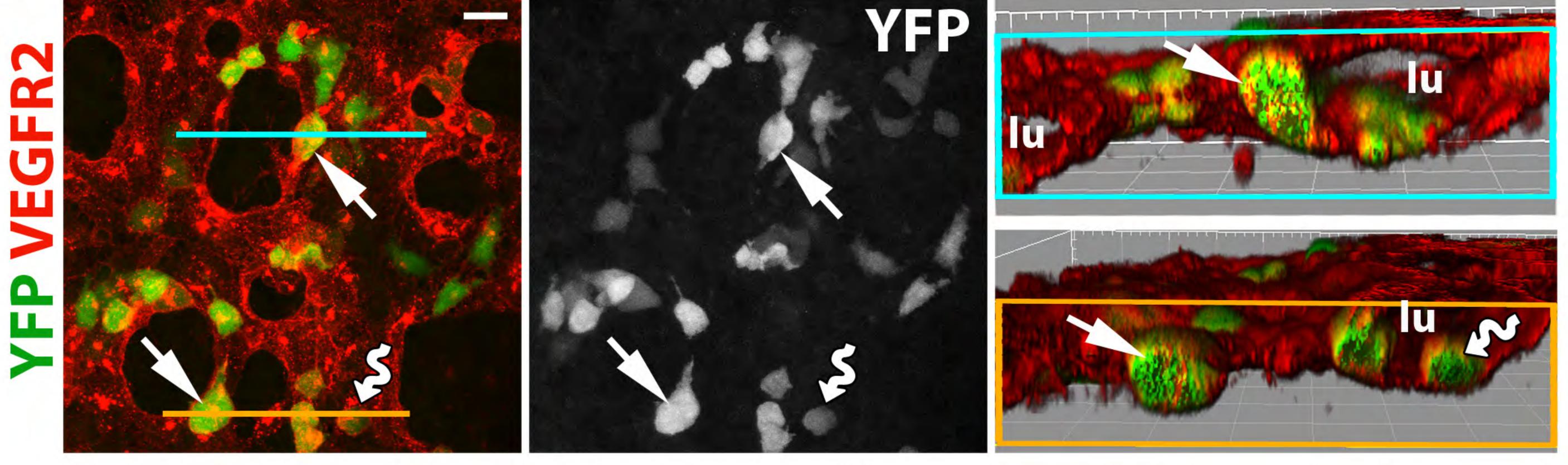
a E8.5 Csf1r-Egfp

3D rendering



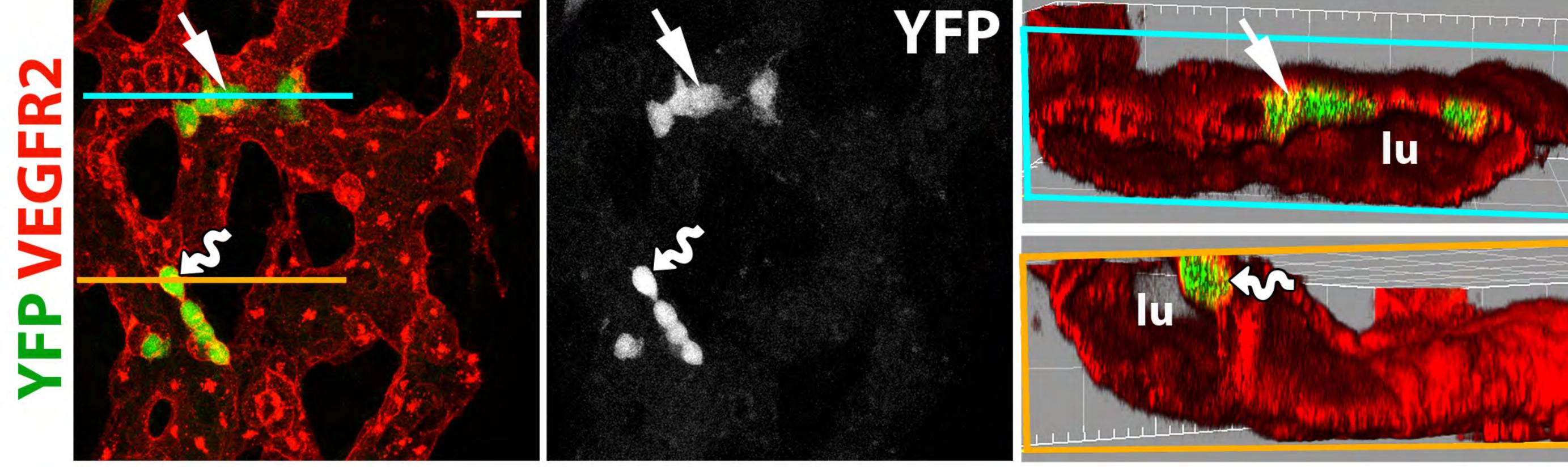
E8.5 Csf1r-iCre;Rosa^{Yfp} D

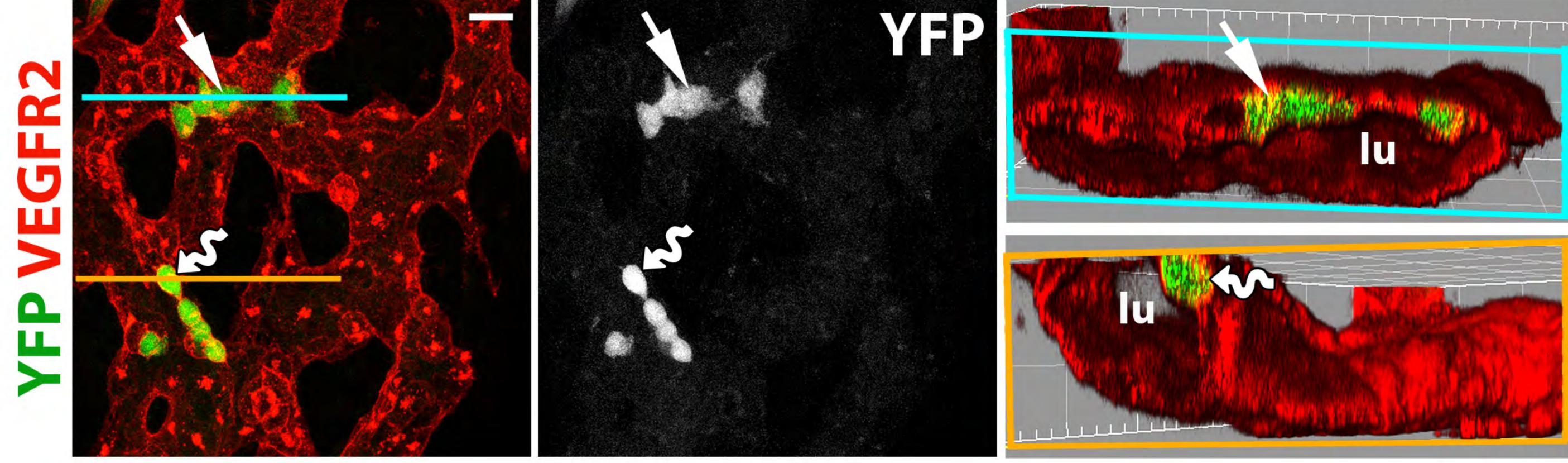
3D rendering

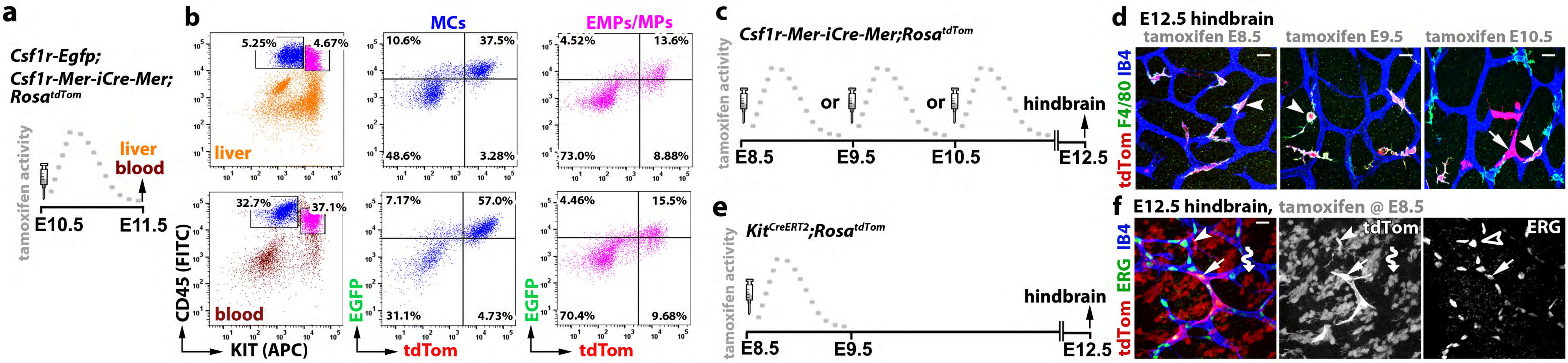


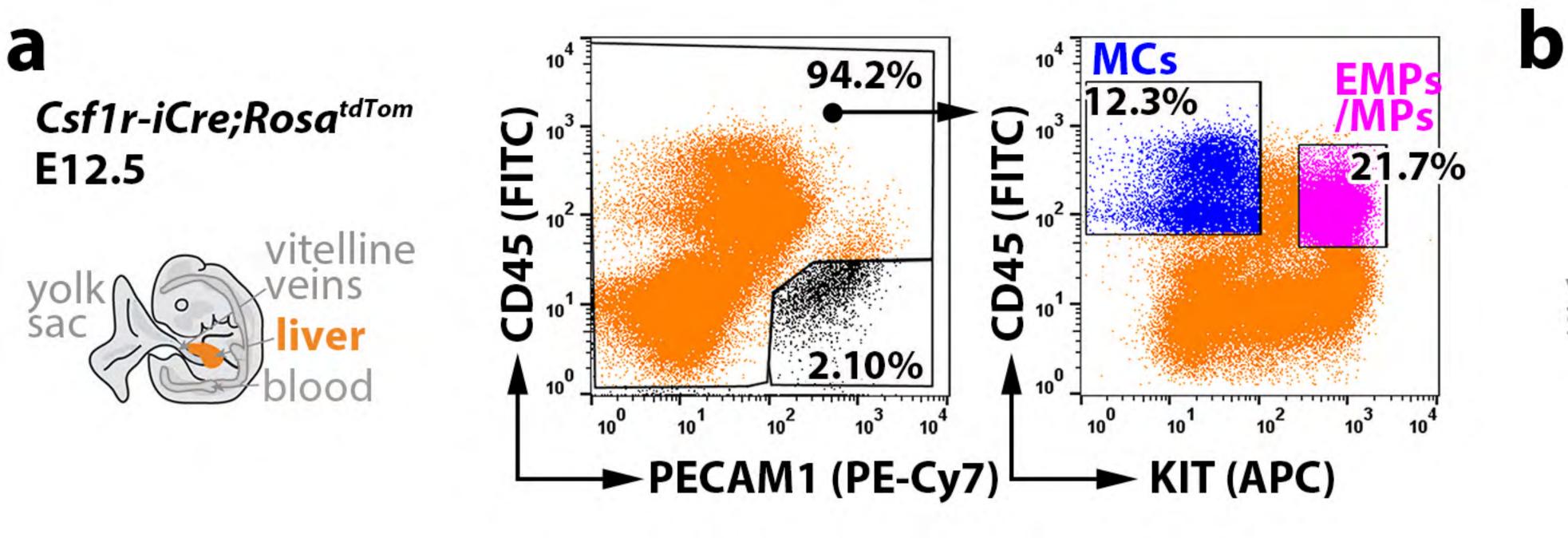
E8.5 Pu.1^{-/-};Csf1r-iCre;Rosa^{Yfp}

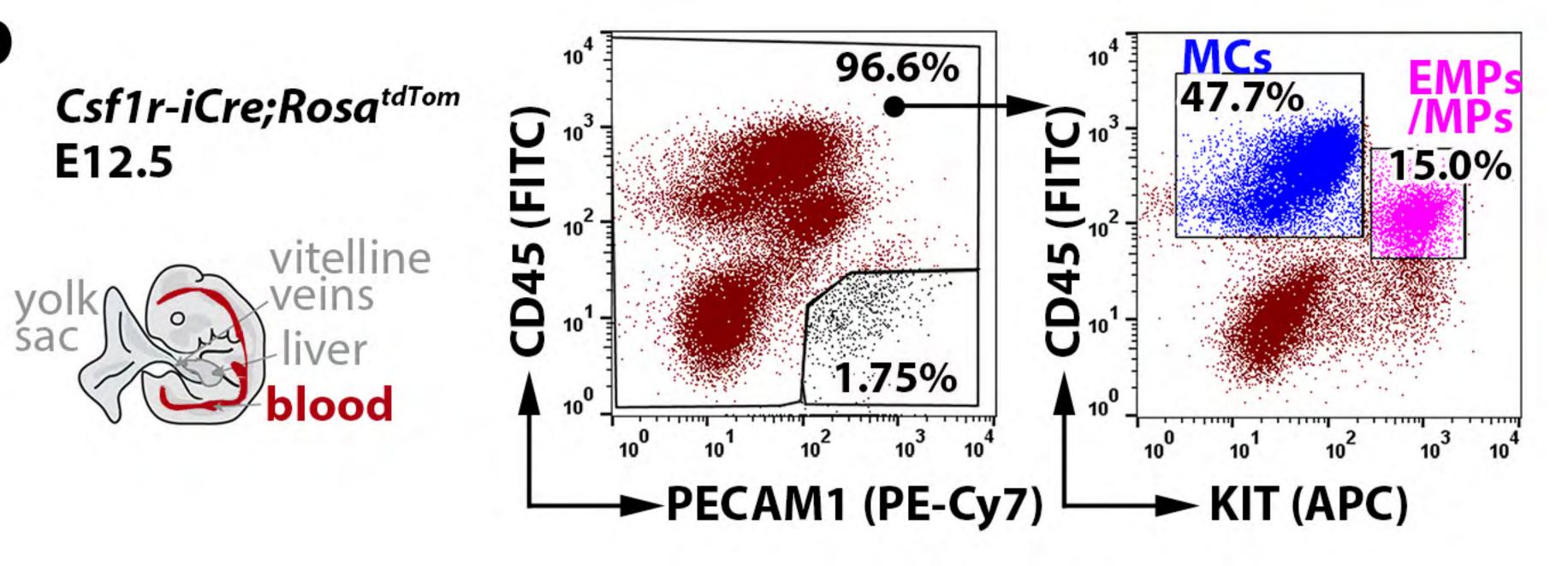
3D rendering

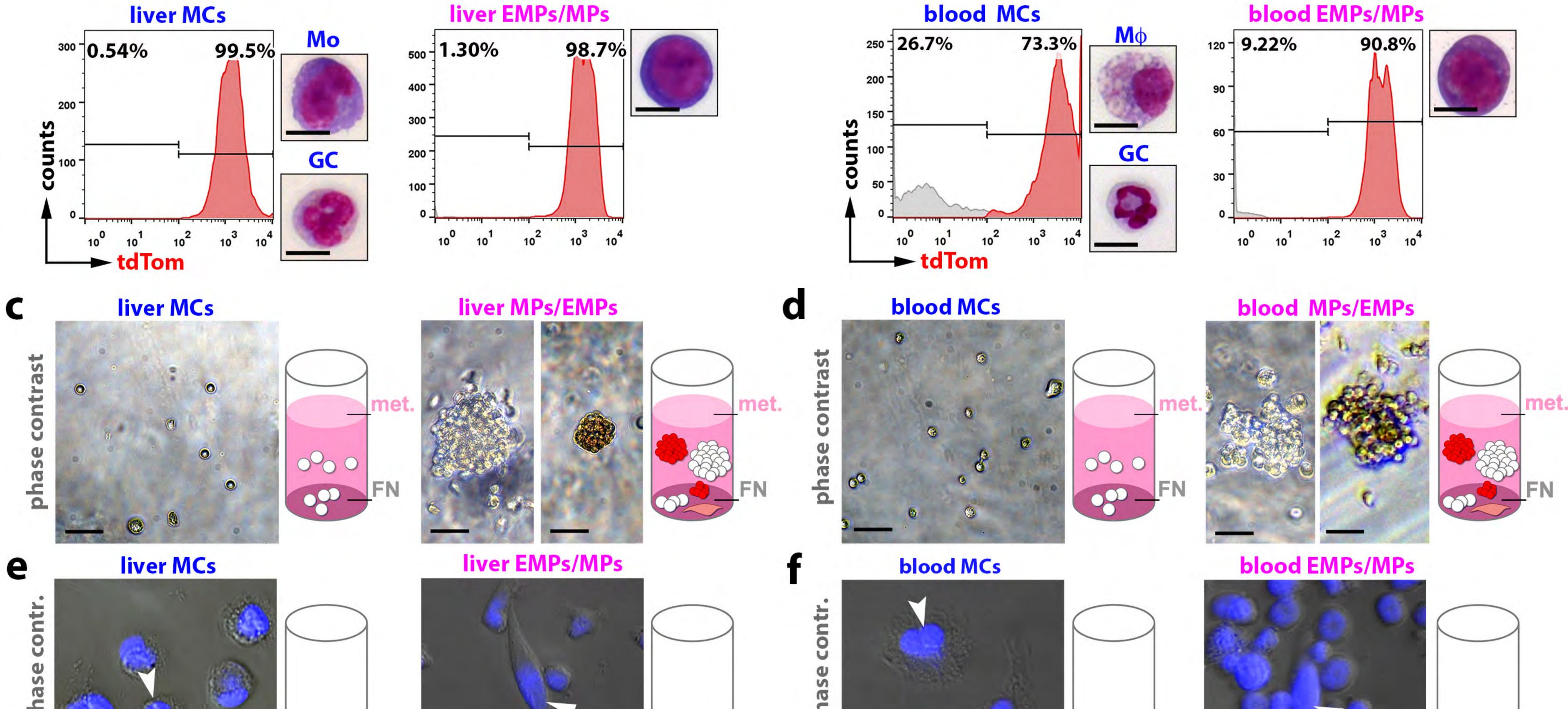




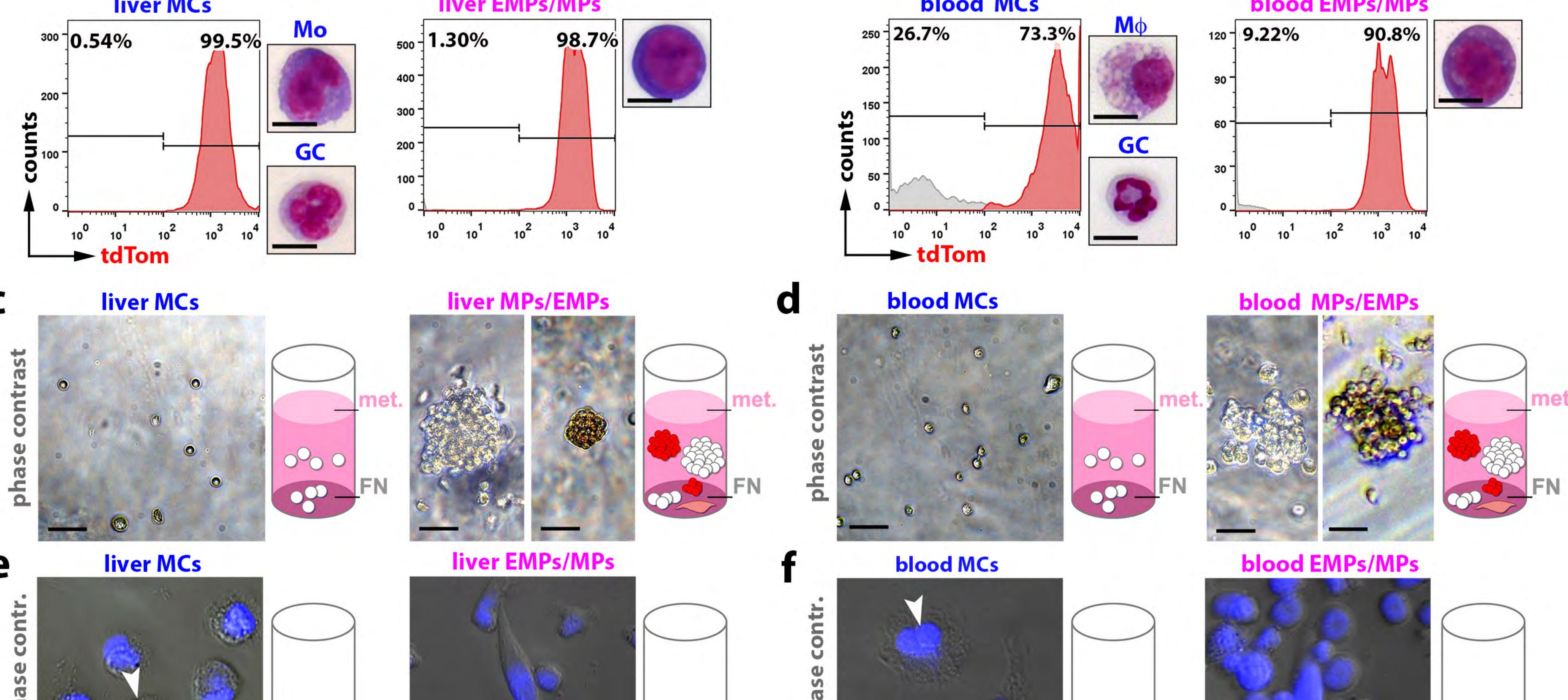






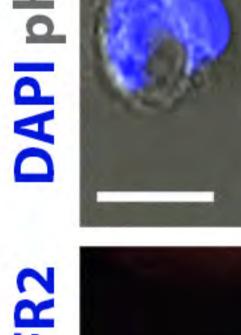




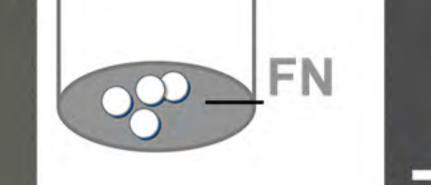


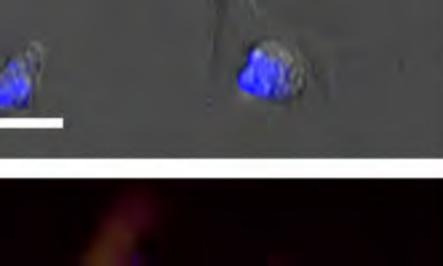


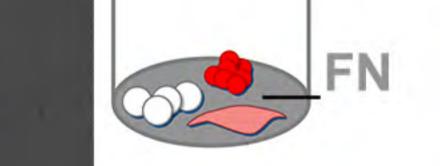










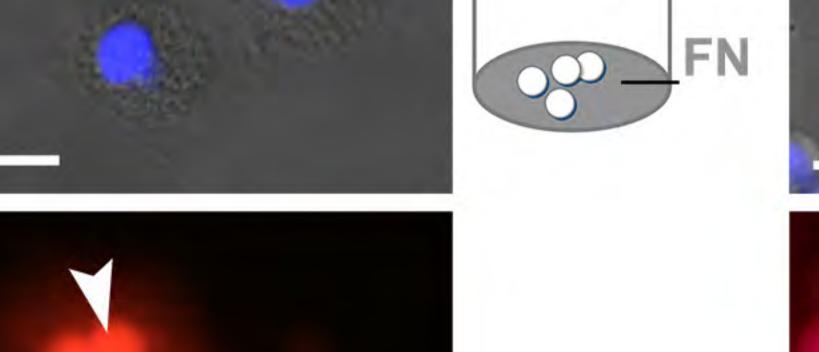


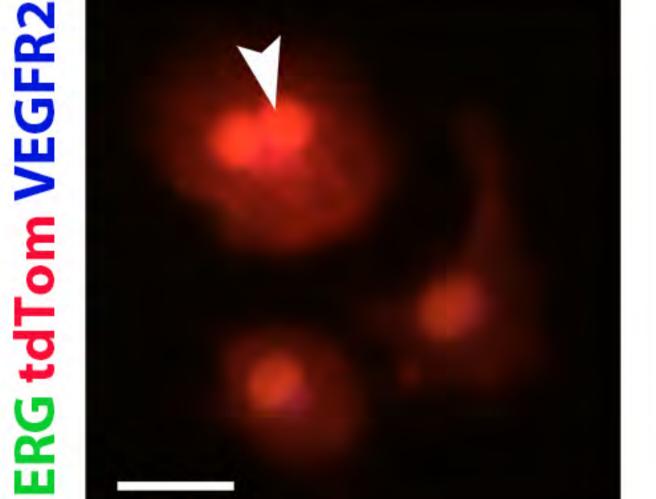


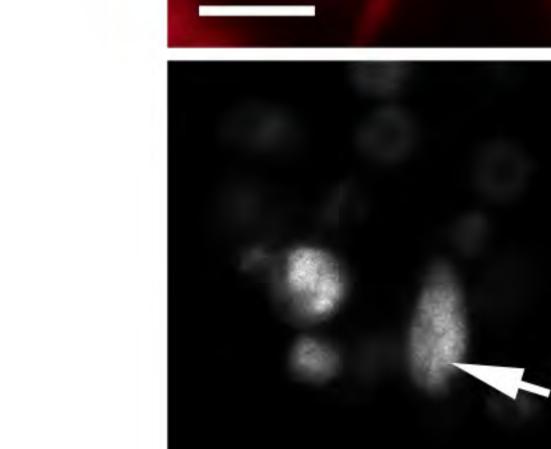
ph

DAPI



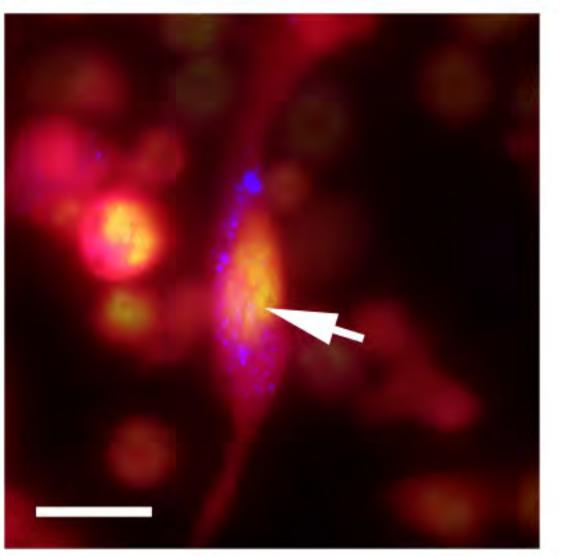


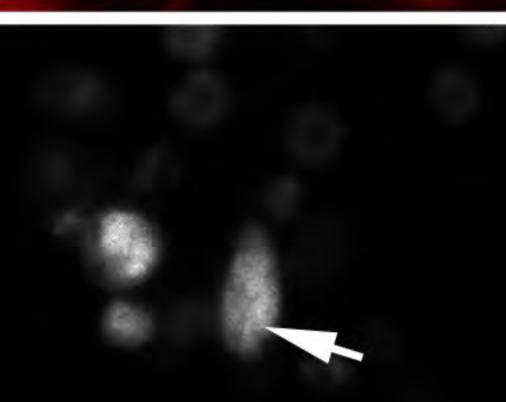


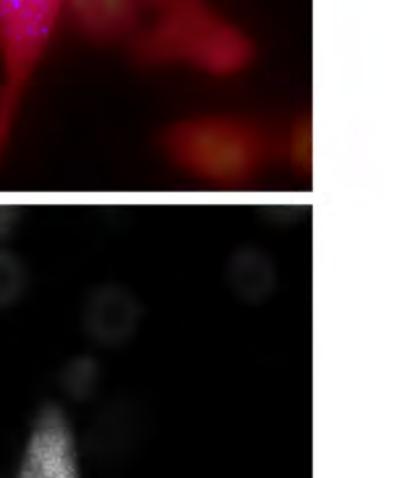




FN

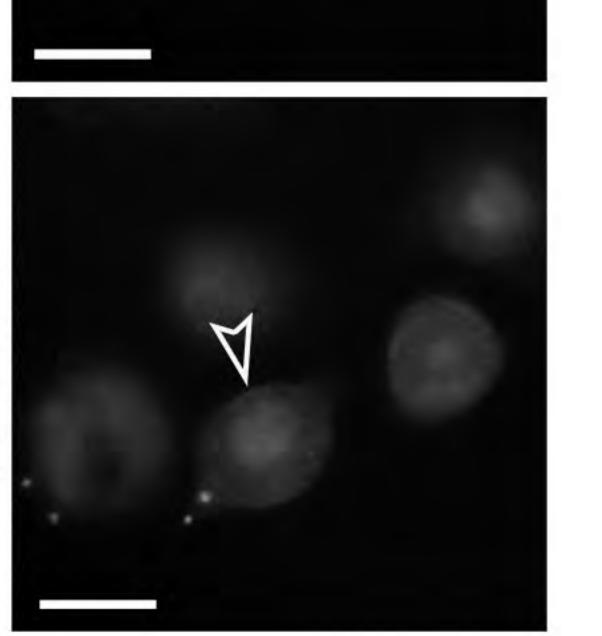












M

