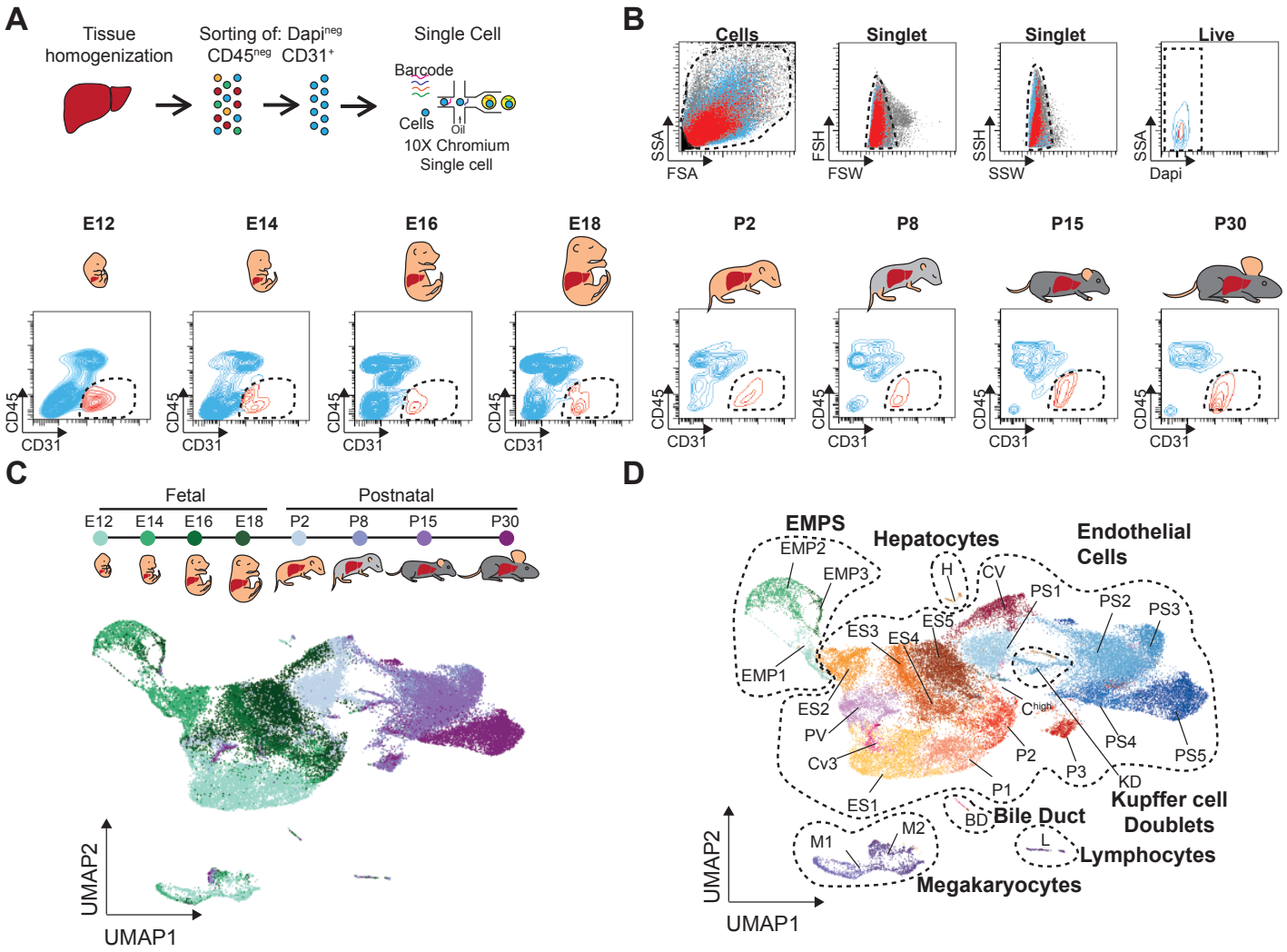
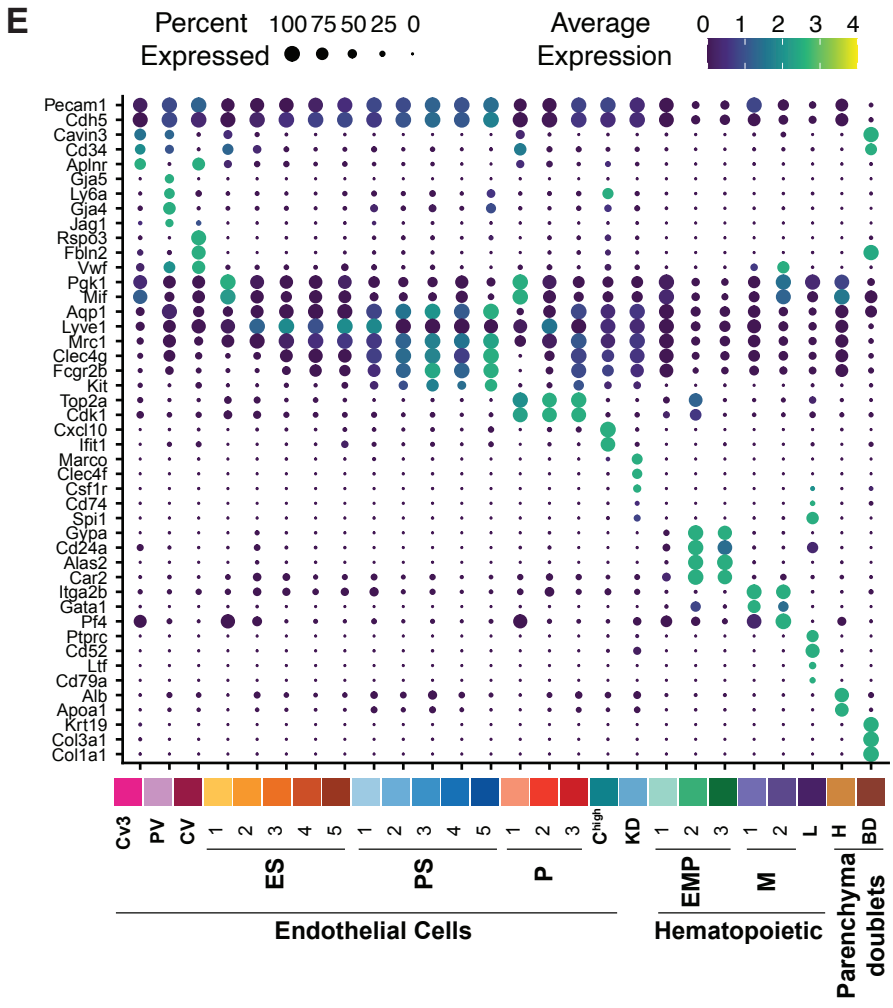
Supplemental Information

Specification of fetal liver endothelial progenitors to functional zonated adult sinusoids requires c-Maf induction

Jesus Maria Gomez-Salinero[1,*,#], Franco Izzo[2,*], Yang Lin[1], Sean Houghton[1], Tomer Itkin[1], Fuqiang Geng[1], Yaron Bram[3], Robert P. Adelson[4], Tyler M. Lu[1,5], Giorgio Ga. Inghirami[6], Jenny Zhaoying Xiang[7], Raphael Lis[1,5], David Redmond[1], Ryan Schreiner[1], Sina Y. Rabbany[1,4], Dan A. Landau[2], Robert E. Schwartz[3], and Shahin Rafii[1,#]


A
Tissue homogenization
Sorting of: Dapi^neg CD45^neg CD31^+
Single Cell
Barcode
Cells
Oil
10X Chromium Single cell
B
Cells
Singlet
Singlet
Live
SSA
FSA
FSH
FSW
SSH
SSW
Dapi
E12
E14
E16
E18
P2
P8
P15
P30
CD45
CD31
C
Fetal
Postnatal
UMAP2
UMAP1
D
EMPS
Hepatocytes
Endothelial Cells
EMP2
EMP3
EMP1
H
CV
PS1
PS2
PS3
ES5
ES3
ES4
ES2
PV
Cv3
C^high
P2
P3
PS4
PS5
KD
P1
ES1
BD
Bile Duct
Kupffer cell Doublets
Lymphocytes
M1
M2
L
Megakaryocytes
UMAP2
UMAP1


E
Percent Expressed 100 75 50 25 0
Average Expression 0 1 2 3 4
Pecam1
Cdh5
Cavin3
Cd34
Aplnr
Gja5
Ly6a
Gja4
Jag1
Rspo3
Fbln2
Vwf
Pgk1
Mif
Aqp1
Lyve1
Mrc1
Clec4g
Fcgr2b
Kit
Top2a
Cdk1
Cxcl10
Ifit1
Marco
Clec4f
Csf1r
Cd74
Spi1
Gypa
Cd24a
Alas2
Car2
Itga2b
Gata1
Pf4
Ptprc
Cd52
Ltf
Cd79a
Alb
Apoa1
Krt19
Col3a1
Col1a1
Cv3
PV
CV
ES
PS
P
C^high
KD
EMP
M
L
H
BD
Endothelial Cells
Hematopoietic
Parenchyma doublets

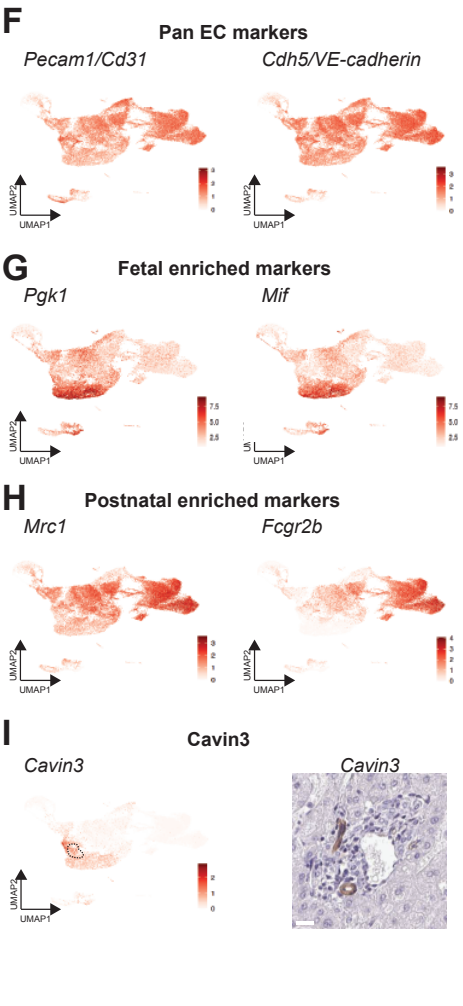

F
Pan EC markers
Pecam1/Cd31
Cdh5/VE-cadherin
G
Fetal enriched markers
Pgk1
Mif
H
Postnatal enriched markers
Mrc1
Fcgr2b
I
Cavin3
Cavin3
Cavin3
UMAP2
UMAP1

Figure S1. Identification of cell populations across multiple times during liver development using single cell RNA-seq. Related to Figure 1.

(**A**) Liver samples were mechanically homogenized in a solution of Collagenase/Dispase, and labeled with CD31 and CD45 antibodies and Dapi to identified live cells. Endothelial cells (ECs) identified as $Dapi^{neg}CD45^{neg}CD31^{+}$ were sorted and single cell RNA-seq analysis was performed using this population. (**B**) Representation of the gating strategy used to enrich the vascular population prior to performing single cell RNA-seq analysis. The ECs are identified as the red population within the dashed line that is $CD45^{neg}CD31^{+}$ at different fetal and postnatal developmental timepoints. (**C,D**) UMAP from single cell analysis of sorted liver endothelial cells (ECs; $CD45^{neg}CD31^{+}$) at fetal and postnatal timepoints: E12, E14, E16, E18, P2, P8, P15 and P30. Colors are assigned based on the sample timepoint (C) or cell cluster (D). Labeling corresponds to the different populations identified from the single cell analysis from (D): $Cavin3^{+}$: Cv3, Portal Vein: PV, Central Vein: CV, Fetal sinusoidal EC populations: FS1-5, Postnatal sinusoidal EC populations: PS1-5, Proliferating ECs: P1-3, Cxcl10 high expressing ECs (C^{high}): C, Erythro-myeloid progenitors: EMP1-3, Megakaryocytes: M1-2, Lymphocytes: L, Hepatocytes: H, Bile Duct: BD, Kupffer cell Doublets: KD. (**E**), Identification of specific markers associated to the individual populations identified in the single cell analysis. Colors were assigned as on (D), with the graphical representation of the vasculature including colors used to label each subpopulation. (**F**) The pan endothelial cell markers Pecam1/Cd31 and Cdh5/VE-cadherin were used to identify vascular populations as double positive cells for these two markers. (**G**) Identification of vascular fetal enriched markers Pgk1 and Mif, which have elevated expression during early fetal development. (**H**) During postnatal vascular development the endothelium increases the expression of genes such as Mrc1 and Fcgr2b. (**I**) $Cavin3^{+}$ population can be identified by the expression of Cavin3. Expression of Cavin3 is restricted in the human adult vasculature to the Hepatic Artery based on the data from the Human Protein Atlas (bar size = 25 μm), which were not present together in other large vessels.

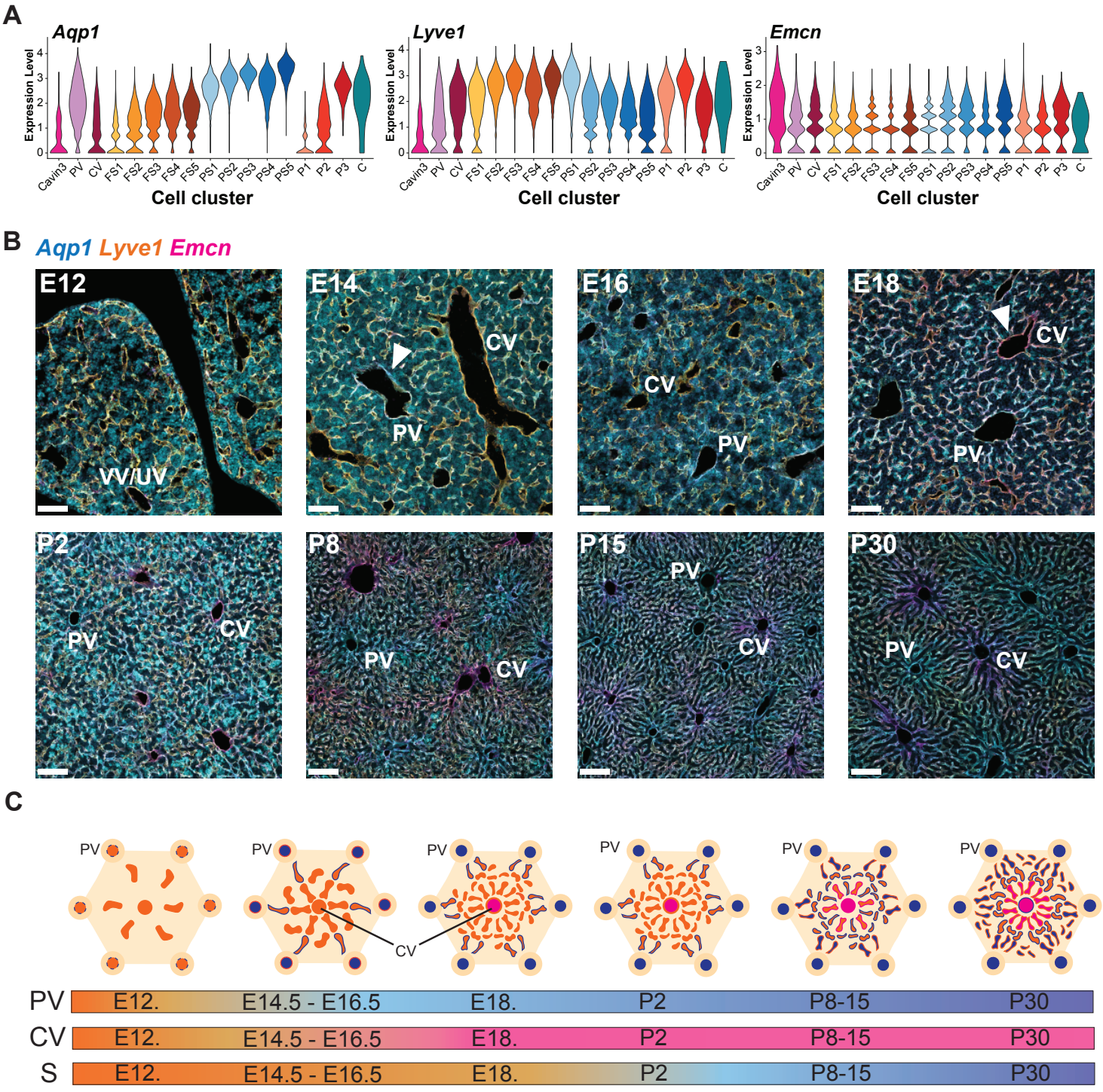

A
Aqp1
Lyve1
Emcn
Expression Level
Cavin3 PV CV FS1 FS2 FS3 FS4 FS5 PS1 PS2 PS3 PS4 PS5 P1 P2 P3 C
Cell cluster
B
Aqp1 Lyve1 Emcn
E12
VV/UV
E14
CV
PV
E16
CV
PV
E18
CV
PV
P2
PV
CV
P8
PV
CV
P15
PV
CV
P30
PV
CV
C
PV
CV
PV E12. E14.5 - E16.5 E18. P2 P8-15 P30
CV E12. E14.5 - E16.5 E18. P2 P8-15 P30
S E12. E14.5 - E16.5 E18. P2 P8-15 P30
D
E-cadherin Cyp2E1 Dapi
P2
PV
CV
P8
CV
PV
P15
CV
PV
P30
PV
CV

Figure S2. Immunofluorescence characterization of large vessel development. Related to Figure 1.

(**A**), Violin plots of the expression values of Aqp1, Lyve1 and Emcn in the different EC subpopulations. (**B**), Immunofluorescence analysis of the vascular markers Aqp1, Lyve1 and Emcn across fetal and postnatal developmental timepoints. Aqp1 emergence can be observed within the Portal Vein (PV) at E14 (arrow). Staining of the venous marker endomucin (Emcn) can be observed in the Central Vein (CV) from E18 onwards (arrow at E18). Representative images from n = 5, Bar size = 100μm. (**C**) Graphical presentation identifying the acquisition of the different markers in the Portal Vein (PV), Central Vein (CV) and Sinusoids (S). (**D**) Immunofluorescent analysis of the hepatocyte zonated Portal Vein marker E-cadherin and the centrilobular marker Cyp2E1, with nucleus stained with Dapi. Representative image of n = 5. Bar size = 100 μm.

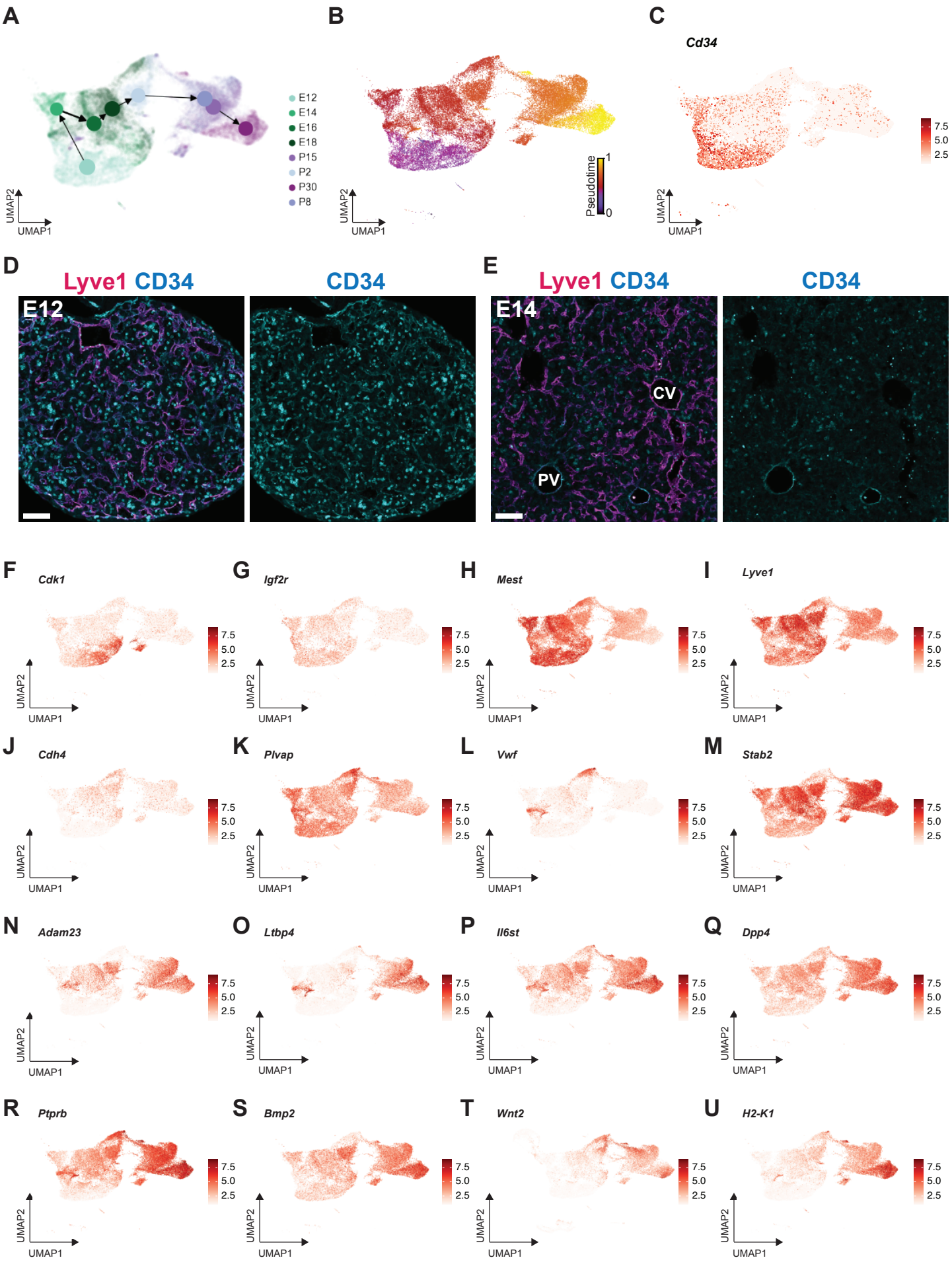

A
E12
E14
E16
E18
P15
P2
P30
P8
UMAP2
UMAP1
B
Pseudotime
1
0
C
Cd34
7.5
5.0
2.5
D
Lyve1 CD34
CD34
E12
E
Lyve1 CD34
CD34
E14
CV
PV
F
Cdk1
G
Igf2r
H
Mest
I
Lyve1
J
Cdh4
K
Plvap
L
Vwf
M
Stab2
N
Adam23
O
Ltbp4
P
Il6st
Q
Dpp4
R
Ptprb
S
Bmp2
T
Wnt2
U
H2-K1

Figure S3. Single cell pseudotime analysis defines a temporal expression of angiocrine factors in development. Related to Figure 2.

(**A**) Partition-based graph abstraction analysis of transition confidence between developmental stages. (**B**) Pseudotime prediction of gene expression transition from the EC populations. (**C**) UMAP representation of CD34 expression in the EC clusters. Higher expression levels were observed at E12. (**D-E**) Immunofluorescence analysis of Lyve1 and CD34 at E12 (D) and E14 (E) of fetal development. CD34 stained was present in all ECs at E12 whereas restricted to the Portal Vein at E14. (**F-U**) Expression of genes identified by pseudotime analysis that has significant expression changes from E12 of fetal developmental timepoints to postnatal day P30. Expression of *Cdk1, Igf2r, Mest, Lyve1, Cdh4, and Plvap* (F-K) were more enriched during fetal development, whereas *Vwf, Stab2, Adam23, Ltbp4, Il6st, Dpp4, Ptprb, Bmp2, Wnt2, H2-K1* (L-U) were more enriched postnatally.

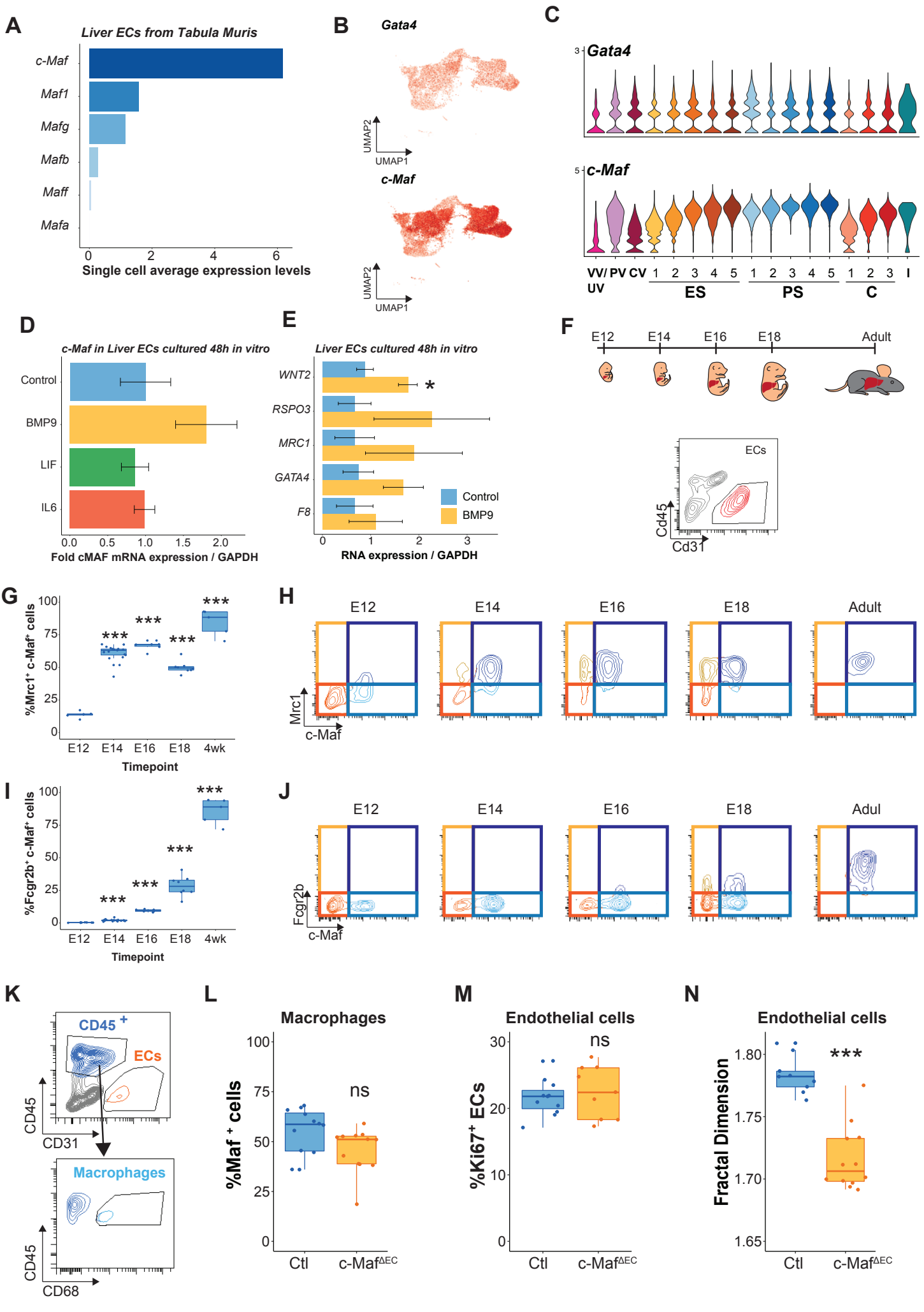

A
Liver ECs from Tabula Muris
c-Maf
Maf1
Mafg
Mafb
Maff
Mafa
0
2
4
6
Single cell average expression levels
B
Gata4
UMAP2
UMAP1
c-Maf
UMAP2
UMAP1
C
Gata4
c-Maf
VV/ PV CV UV
1 2 3 4 5
ES
1 2 3 4 5
PS
1 2 3 I
C
D
c-Maf in Liver ECs cultured 48h in vitro
Control
BMP9
LIF
IL6
0.0 0.5 1.0 1.5 2.0
Fold cMAF mRNA expression / GAPDH
E
Liver ECs cultured 48h in vitro
WNT2
RSPO3
MRC1
GATA4
F8
*
Control
BMP9
0 1 2 3
RNA expression / GAPDH
F
E12
E14
E16
E18
Adult
ECs
Cd45
Cd31
G
%Mrc1+ c-Maf+ cells
100
75
50
25
0
***
E12 E14 E16 E18 4wk
Timepoint
H
E12
E14
E16
E18
Adult
Mrc1
c-Maf
I
%Fcgr2b+ c-Maf+ cells
100
75
50
25
0
***
E12 E14 E16 E18 4wk
Timepoint
J
E12
E14
E16
E18
Adul
Fcgr2b
c-Maf
K
CD45+
ECs
CD45
CD31
Macrophages
CD45
CD68
L
Macrophages
%Maf+ cells
100
75
50
25
0
ns
Ctl
c-MafΔEC
M
Endothelial cells
%Ki67+ ECs
30
20
10
0
ns
Ctl
c-MafΔEC
N
Endothelial cells
Fractal Dimension
1.80
1.75
1.70
1.65
***
Ctl
c-MafΔEC

Figure S4. c-Maf is enriched in the liver sinusoidal cell population and sustained over time. Related to Figure 2 and Figure 3.

(**A**) Expression levels of the Maf family members in liver ECs from the Tabula Muris database. (**B**) UMAP expression of the transcription factors Gata4 and c-Maf within the present study's single cell RNAseq analysis. (**C**) Expression levels of Gata4 and c-Maf were assessed in all EC populations identified in the single cell RNA-seq analysis. (**D**) Quantitative-PCR (q-PCR) measure of c-Maf expression levels in mouse liver ECs cultured for 48 hours in the presence of Bmp9, Lif, Il6, or a negative control. (**E**) Expression of liver-enriched genes measured by q-PCR of liver ECs cultured for 48 hours in the presence of Bmp9. A *t*-test analysis was performed between controls and the other conditions, with *p<0.05. (**F**) Flow cytometry identification of endothelial cells as $CD45^{neg}CD31^{+}$ across fetal E12, E14, E16, E18 and adult mice. (**G-J**) Flow cytometry analysis of the expression levels of c-Maf - Mrc1 (G,H), and c-Maf - Fcg2b (I,J) in the liver ECs, $n \geq 5$. Colors indicate the quadrant from the flow cytometry gating. Percentage of cells identified by flow cytometry that were double positive for c-Maf and Mrc1 (H) or Fcgr2b (J) from $n \geq 5$ mice across different developmental timepoints. A *t*-test analysis between E12 and each other timepoint was performed. *** $p < 0.001$. (**K**) Flow cytometry analysis of the vasculature and myeloid $CD45^{+}$, $CD68^{+}$ compartment in mice at E16 from Control and $c\text{-}Maf^{\Delta EC}$ mice. Labels indicate identification of the different populations (**L**), Expression of c-Maf was not affected in myeloid cells after tamoxifen administration at E16. (**M**) Percentage of cells positive for Ki67 analyzed by flow cytometry at E16. T-test was not significant, $n \geq 9$. (**N**) Analysis of the change in fractal dimension of the vascular network in control and $c\text{-}Maf^{\Delta EC}$ mice at E16, $n \geq 9$. A *t*-test was used for the comparison of control and $c\text{-}Maf^{\Delta EC}$ mice, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

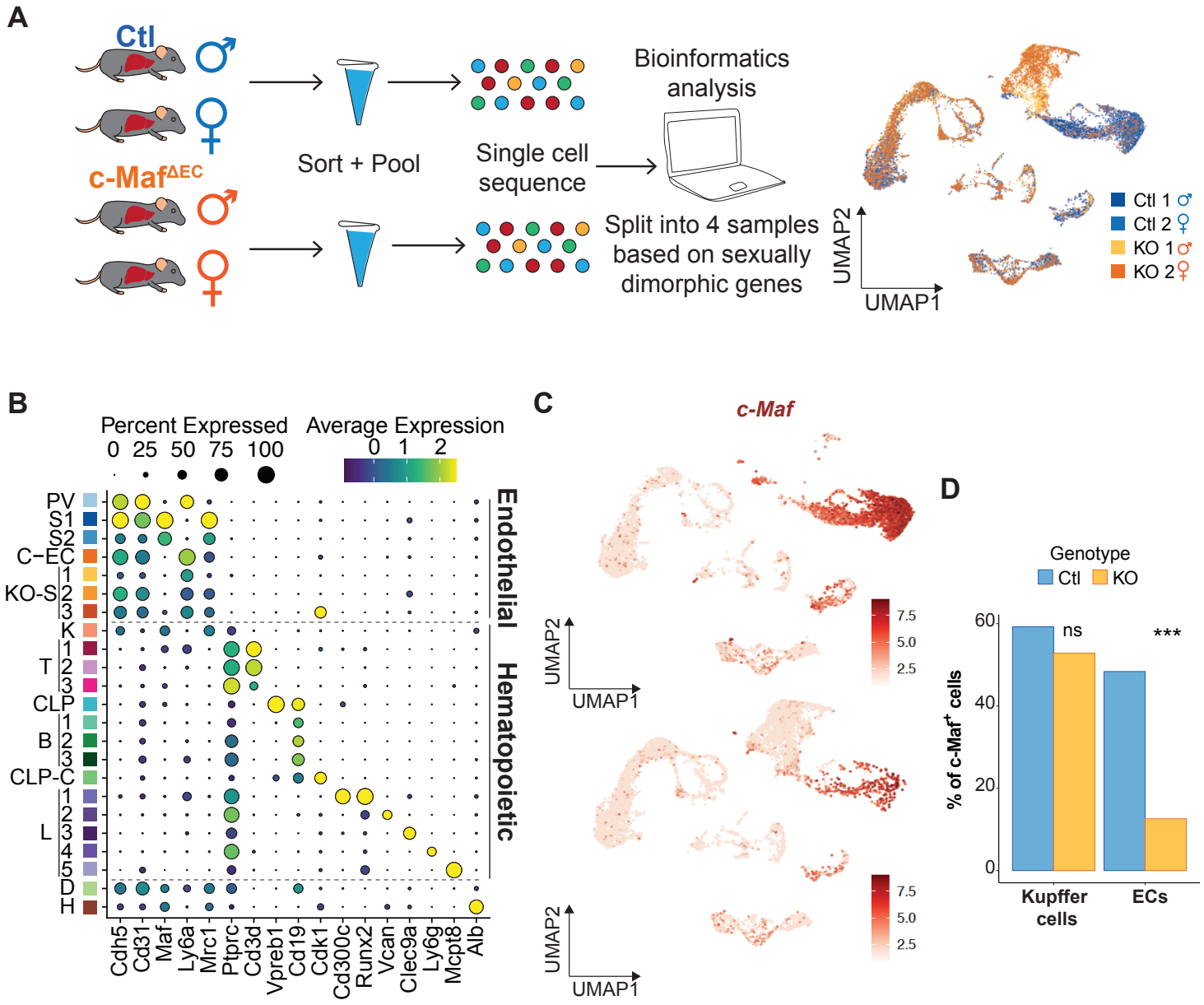

A
Ctl
c-Maf^ΔEC
Sort + Pool
Single cell sequence
Bioinformatics analysis
Split into 4 samples based on sexually dimorphic genes
UMAP2
UMAP1
Ctl 1 ♂
Ctl 2 ♀
KO 1 ♂
KO 2 ♀
B
Percent Expressed
0 25 50 75 100
Average Expression
0 1 2
PV
S1
S2
C−EC
KO-S
K
T
CLP
B
CLP-C
L
D
H
Endothelial
Hematopoietic
Cdh5
Cd31
Maf
Ly6a
Mrc1
Ptprc
Cd3d
Vpreb1
Cd19
Cdk1
Cd300c
Runx2
Vcan
Clec9a
Ly6g
Mcpt8
Alb
C
c-Maf
UMAP2
UMAP1
7.5
5.0
2.5
D
Genotype
Ctl
KO
% of c-Maf+ cells
ns
***
Kupffer cells
ECs
E
% ECs c-Maf+
***
Ctl
c-Maf^ΔEC
F
Pseudotime
***
Ctl
c-Maf^ΔEC
G
Mrc1(10³) MFI
***
Ctl
c-Maf^ΔEC
H
CFU / 30.000 cells
**
Ctl
c-Maf^ΔEC

Figure S5. c-Maf deficient ECs differentially segregate postnatally. Related to Figure 4.

(**A**) Single cell RNA-seq was performed at p15 for one male and one female mouse each from the two groups (Control and c-Maf$^{\Delta EC}$). Samples from each group were sorted and combined for the single cell analysis. Subsequently the two samples were bioinformatically split into their original four samples based on the expression of sexually dimorphic genes in each cell. All four samples were plotted in a UMAP showing the overlap of all samples. (**B**) Identification of individual populations from the scRNA-seq analysis based on expression of specific markers. Endothelial populations: Sinusoids (S1, S2), KO-Sinusoids (KO-S 1-3), Cycling (C), Portal Vein (PV); Hematopoietic populations: Common lymphoid progenitors (CLP), Cycling – CLP (C-CLP), B cells (B 1-3), T cells (T 1-3), Leukocytes (L 1-5) and Kupffer cells; Contaminant cells were identified as: Doublets (D) and Hepatocytes (H). (**C**) UMAP representation of the expression levels of c-Maf in control and c-Maf$^{\Delta EC}$ mice in all populations. (**D**) Percentage of cells positive for c-Maf in the ECs and Kupffer cell populations shows no significant changes in the Kupffer cell population but significant decreased in the EC clusters of c-Maf$^{\Delta EC}$ mice. (**E**) Flow cytometry quantification of the percentage of ECs expressing c-Maf in control and c-Maf$^{\Delta EC}$ mice, $n \geq 10$. (**F**) Pseudotime changes in the control and c-Maf$^{\Delta EC}$ mice identifying a shorter transitional stage for the c-Maf$^{\Delta EC}$ mice. (**G**) Flow cytometry quantification of the medium fluorescent intensity of Mrc1 and Fcgr2b expression levels in control and c-Maf$^{\Delta EC}$ mice, $n \geq 10$. Significance was measured using a *t*-test comparing both groups, with ***p<0.001. (**H**) CFU analysis of sorted CD45 total cells from control and c-Maf$^{\Delta EC}$ mice, n = 5. A *t*-test was used for comparisons between control and c-Maf$^{\Delta EC}$ mice in this figure, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

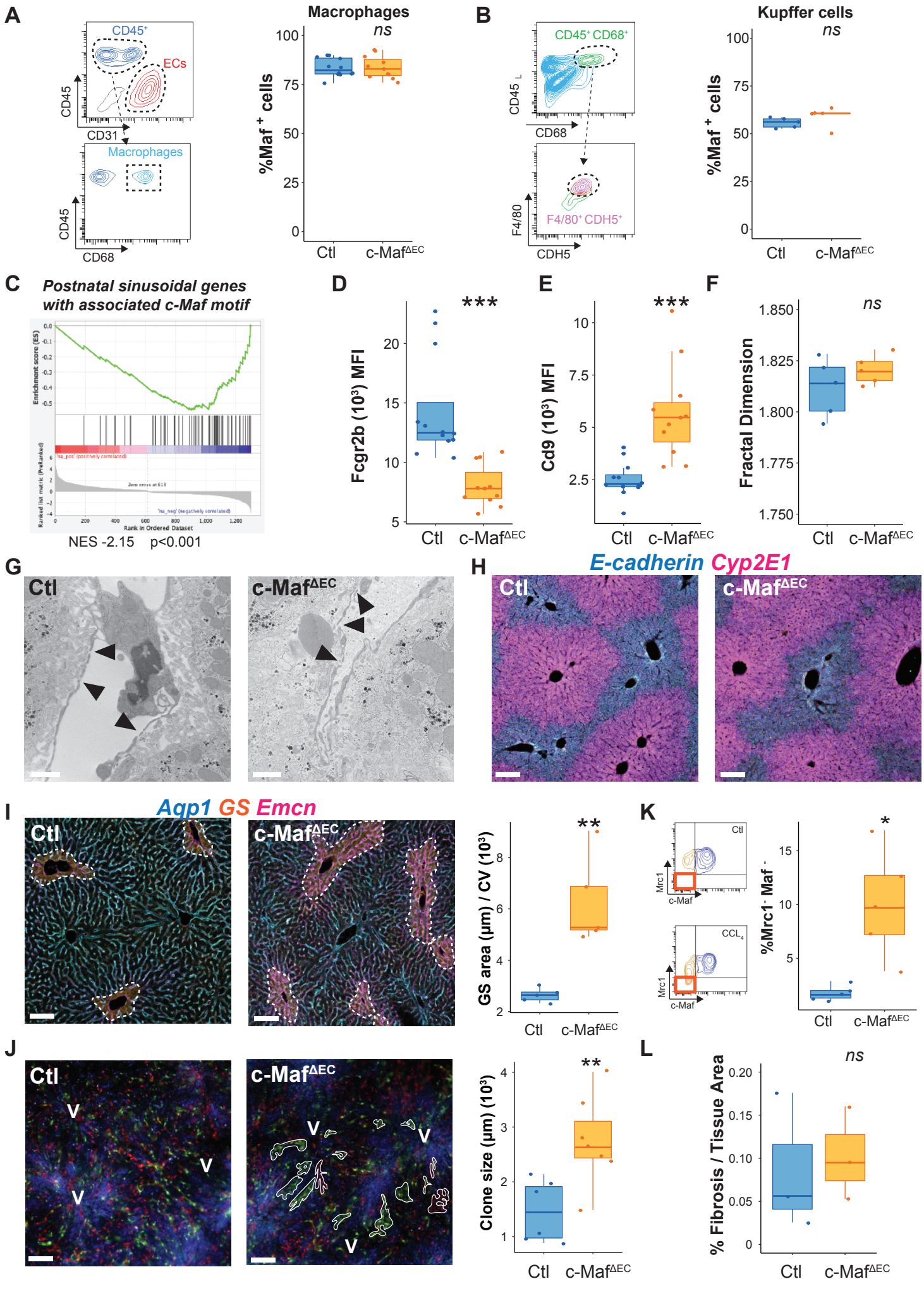

A
CD45+
ECs
CD45
CD31
Macrophages
CD45
CD68
Macrophages
ns
%Maf+ cells
100
75
50
25
0
Ctl
c-MafΔEC
B
CD45+ CD68+
CD45
CD68
F4/80
F4/80+ CDH5+
CDH5
Kupffer cells
ns
%Maf+ cells
Ctl
c-MafΔEC
C
Postnatal sinusoidal genes with associated c-Maf motif
Enrichment score (ES)
Ranked list metric (PreRanked)
Zero cross at 613
Rank in Ordered Dataset
NES -2.15 p<0.001
D
***
Fcgr2b (10³) MFI
20
15
10
5
Ctl
c-MafΔEC
E
***
Cd9 (10³) MFI
10
7.5
5
2.5
Ctl
c-MafΔEC
F
ns
Fractal Dimension
1.850
1.825
1.800
1.775
1.750
Ctl
c-MafΔEC
G
Ctl
c-MafΔEC
H
E-cadherin Cyp2E1
Ctl
c-MafΔEC
I
Agp1 GS Emcn
Ctl
c-MafΔEC
**
GS area (µm) / CV (10³)
8
6
4
2
Ctl
c-MafΔEC
K
Ctl
Mrc1
c-Maf
CCL4
Mrc1
c-Maf
*
%Mrc1- Maf -
15
10
5
Ctl
c-MafΔEC
J
Ctl
V
V
V
c-MafΔEC
V
V
V
**
Clone size (µm) (10³)
4
3
2
1
Ctl
c-MafΔEC
L
ns
% Fibrosis / Tissue Area
0.20
0.15
0.10
0.05
0
Ctl
c-MafΔEC

Figure S6. Adult vascular c-Maf deficiency is associated with a loss of the sinusoidal program. Related to Figure 5.

(**A-B**) Analysis of c-Maf expression in macrophages ($CD45^+CD68^+$) (A) and Kupffer cells ($CD45^+CD68^+F4/80^+CDH5^+$) shows no significant differences, $n \geq 11$ (A), and n = 5 (B). (**C**) GSEA analysis of the expression levels of postnatal sinusoidal genes with an associated c-Maf motif. (**D-E**) Flow cytometry quantification of the medium fluorescent intensity in the endothelial cell population of the sinusoidal marker Fcgr2b (D) and the arterial marker Cd9 (E). (**F**) Quantification of the vascular network interactome assay as the fractal dimension of the vasculature in control and c-$Maf^{\Delta EC}$ mice from n = 5 with t-test analysis between the groups showing no significant difference. (**G**) Electron microscopy of livers from Control and c-$Maf^{\Delta EC}$ mice from n = 3 showing presence of fenestration in c-$Maf^{\Delta EC}$ mice. Arrows indicate fenestrations. (**H**) Immunofluorescence analysis of the portal to centrilobular makers E-cadherin and Cyp2E1 respectively shows no changes in the overall zonation between control and c-$Maf^{\Delta EC}$ mice from n = 5. (**I**) Immunofluorescence quantification analysis of the area positive for GS staining in Control and c-$Maf^{\Delta EC}$ mice from n = 5 mice with t-test **p,0.01. (**J**) Immunofluorescence quantification analysis of clonal vascular size of Confetti mice crossed with c-$Maf^{\Delta EC}$ mice, compared to control adult mice, $n \geq 6$ with t-test **p,0.01. (**K**) Flow cytometry analysis of the expression levels of c-Maf and Mrc1 in mice treated biweekly with oil and 25% CCl_4 diluted in oil from n = 5. Data represents the percentage of cells that were double negative for Mrc1 and c-Maf in both groups with t-test *$p<0.05$. (**L**) Quantification of the percentage of fibrosis over the total tissue area from n =3 of control and c-$Maf^{\Delta EC}$ mice, t-test analysis between the groups showing no significant difference.

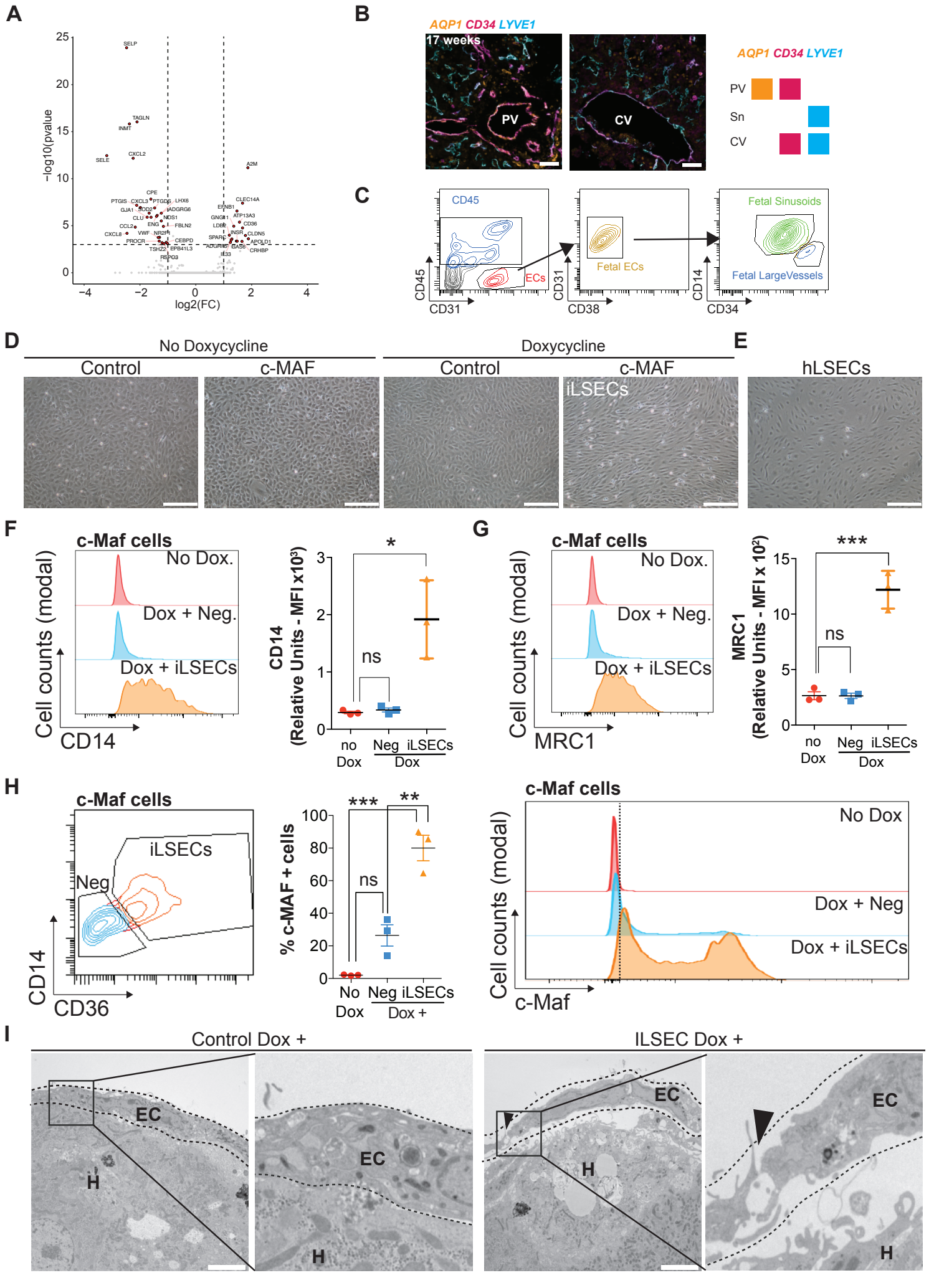

A
B
C
D
E
F
G
H
I
AQP1 CD34 LYVE1
17 weeks
PV
CV
Sn
−log10(pvalue
log2(FC)
CD45
CD31
CD38
CD14
CD34
ECs
Fetal ECs
Fetal Sinusoids
Fetal LargeVessels
No Doxycycline
Doxycycline
Control
c-MAF
iLSECs
hLSECs
c-Maf cells
No Dox.
Dox + Neg.
Dox + iLSECs
Cell counts (modal)
CD14
CD14 (Relative Units - MFI x10³)
MRC1
MRC1 (Relative Units - MFI x 10²)
ns
no Dox
Neg
iLSECs
Dox
Dox +
CD36
% c-MAF + cells
No Dox
Dox + Neg
c-Maf
Control Dox +
ILSEC Dox +
EC
H

Figure S7. Overexpression of c-Maf in endothelial cells (iLSECs) induces the expression of surface markers associated to human liver sinusoidal cells. Related to Figure 6 and Figure 7. (**A**) Volcano plot comparing the Portal Vein and Central Vein gene signatures. (**B**) Immunofluorescent analysis of a 17-week human liver sample label with Aqp1, Cd34, and Lyve1 allows the identification of the Portal Vein, sinusoids. and Central Vein. Boxes represent the labeling of the gene at a particular population. Bar size = 50 μm. (**C**) Flow cytometry expression analysis of vascular markers in a 17-week human liver sample shows the expression of CD14 and CD34 by liver ECs at this fetal developmental stage. At 17 weeks of human fetal development, we could not observe the population of plasma cells $CD45^{neg}CD31^{+}CD38^{+}$. (**D**) ECs transduced with a lentiviral control vector or c-Maf inducible vector co-cultured with or without doxycycline for seven days. Representative image of n=3. Bar size = 250 μm. (**E**) Representative image of human liver EC culture *in vitro* after isolation with CD144 (VE-Cadherin – CDH5) beads, n=1. Bar size = 250 μm. (**F-G**) Quantification of CD14 (F) and MRC1 (G) expression levels by flow cytometry of iLSECs compared to negative (non-reprogrammed) cells. n=3 (different donors). *t*-test analysis, *p<0.05, ***p<0.001. (**H**) Identification of iLSECs as $CD36^{+}$ $CD14^{+}$ by flow cytometry. Expression of c-Maf in iLSECs compared to negative ($CD36^{-}$ $CD14^{-}$) cells or cells cultured without doxycycline. Representative image of n=3. (**I**) Electron microscopy analysis of the co-culture of hepatocytes with control ECs or iLSECs. Arrows indicate the existence of empty cavities in the iLSECs, but not the controls, that emulate fenestrations. White bar represents 1 μm.