

Supplementary Information

iSuRe-HadCre is an essential tool for effective conditional genetics

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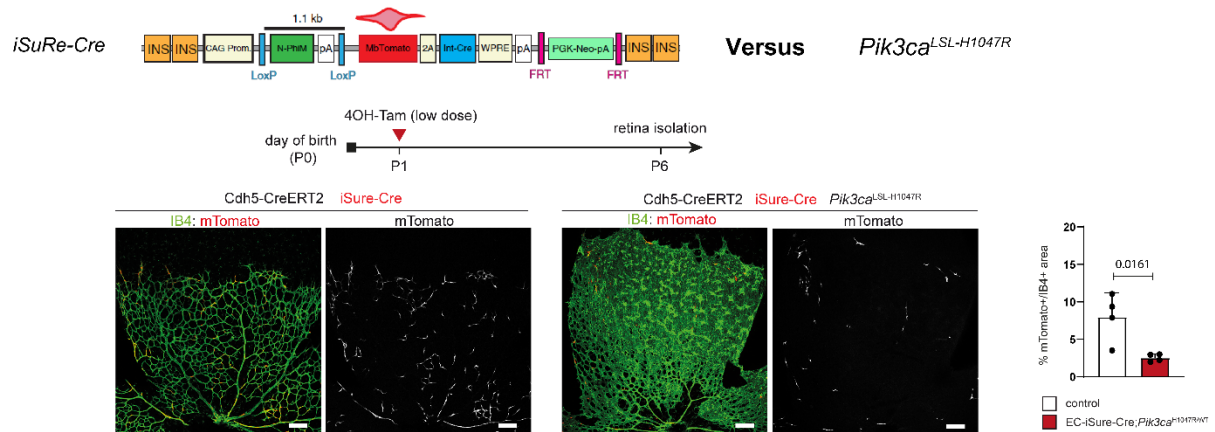
^{*}Correspondence: Rui.benedito@cnic.es

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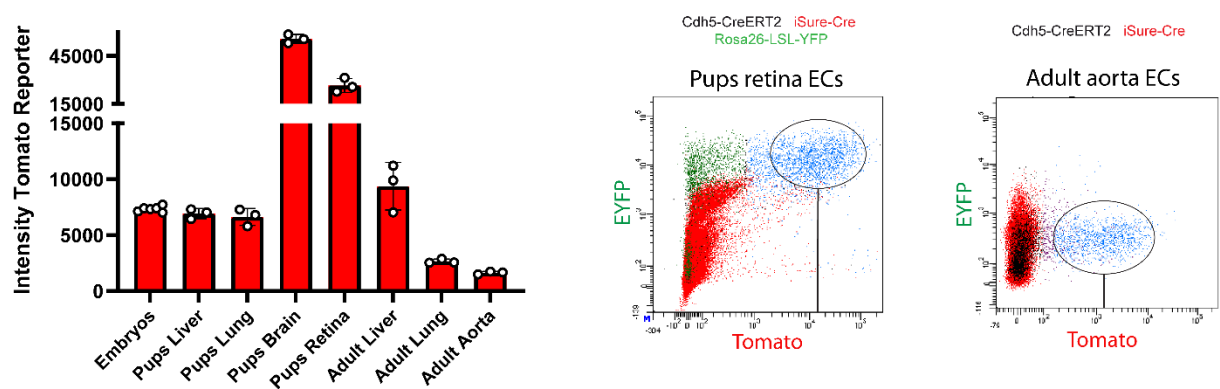
Supplementary Figures S1-S8 (Pages 2-10)

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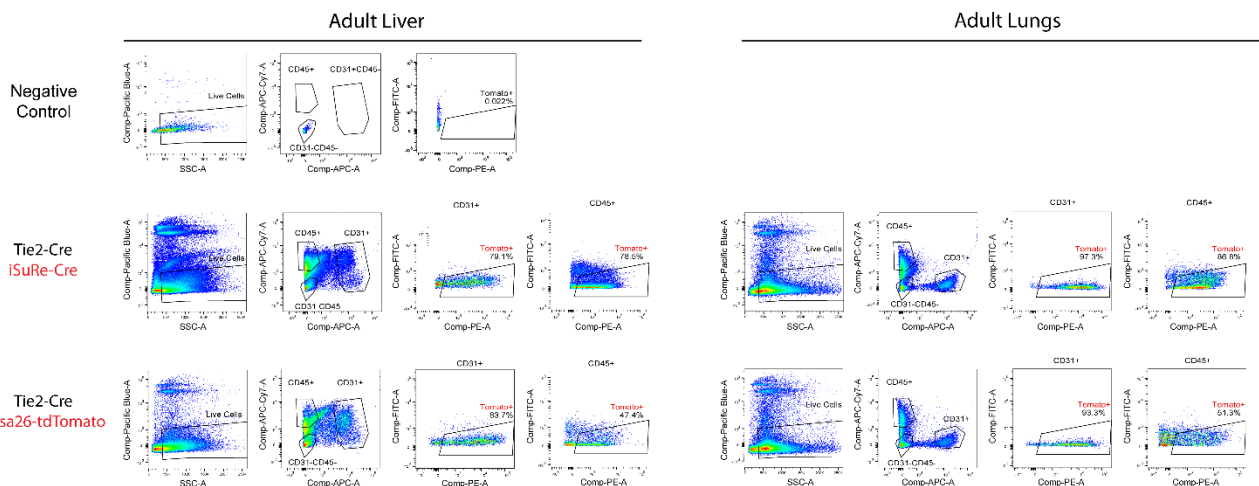
A False negatives with iSuRe-Cre



B iSuRe-Cre expression differs in ECs among stages and organs

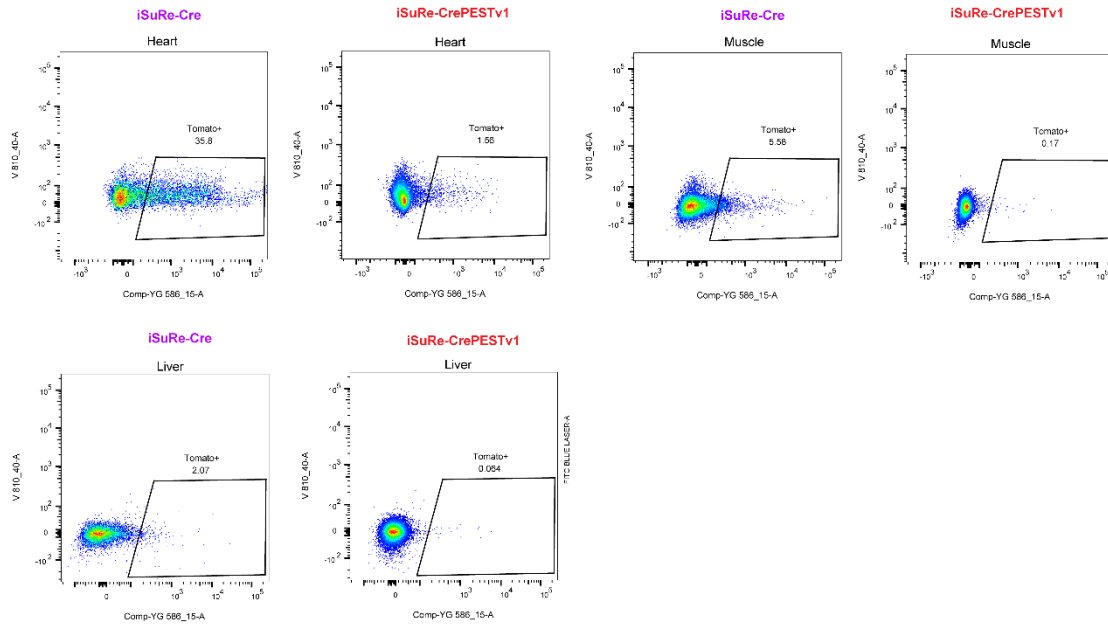


C No major iSuRe-Cre toxicity in mixed genetic background

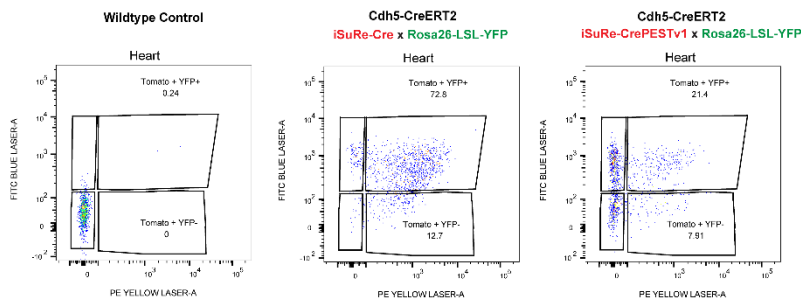


Supplementary Figure S1. Analysis of false negatives and expression or toxicity in *iSuRe-Cre* mice. (A) Mice containing the *iSuRe-Cre* genetic construct, the inducible floxed *Pi3KCA*^{H1047R} allele, and a *Cdh5-CreERT2* allele were induced with a low tamoxifen dose at P1, and retinas were collected for analysis at P6. Images show the relatively low recombination frequency of the *iSuRe-Cre* allele, with many false-negative cells having recombination of the *Pi3KCA*^{H1047R} allele and generating a strong vascular expansion/hyperdensity phenotype despite the scarcity of *iSuRe-Cre*⁺ cells. (B) FACS analysis of the variability in *iSuRe-Cre*/*MbTomato-2A-Cre* expression levels in ECs collected at distinct developmental stages and from different organs. (C) Comparative FACS analysis of the recombination frequency and intensity of *MbTomato-2A-Cre* or *TdTomato* expression in ECs and blood lineages; *MbTomato-2A-Cre* expression is non-toxic when mice are on the C57Bl6 x CD1 genetic background. Data are presented as mean values \pm s.d. For statistics, see Source Data File 1. Scale bars 150 μ m.

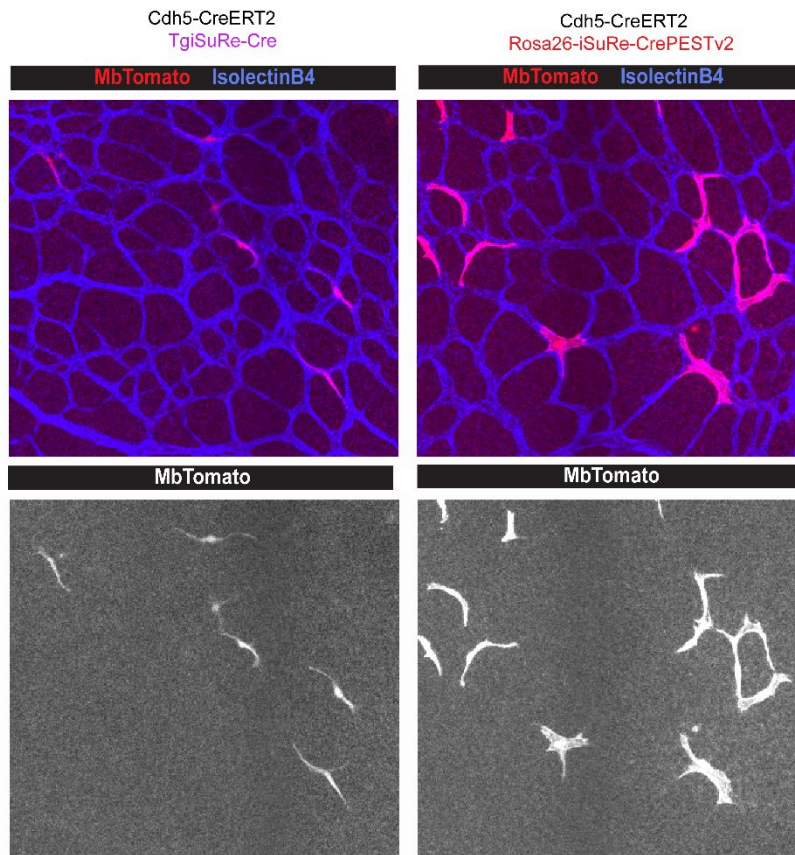
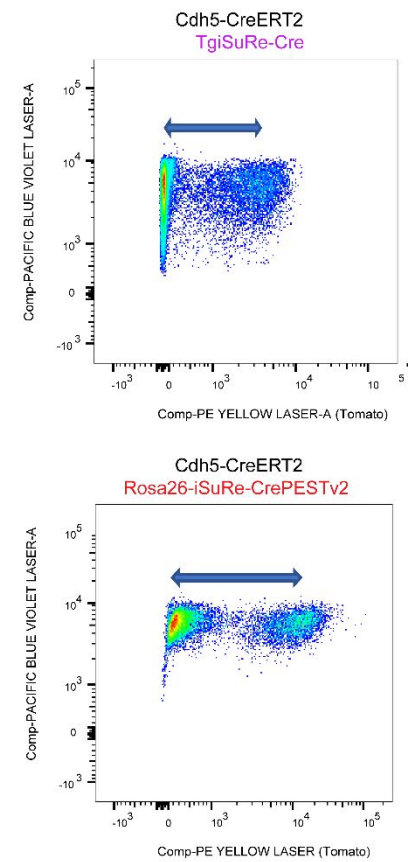
A Adult Organs Self-leakiness comparison



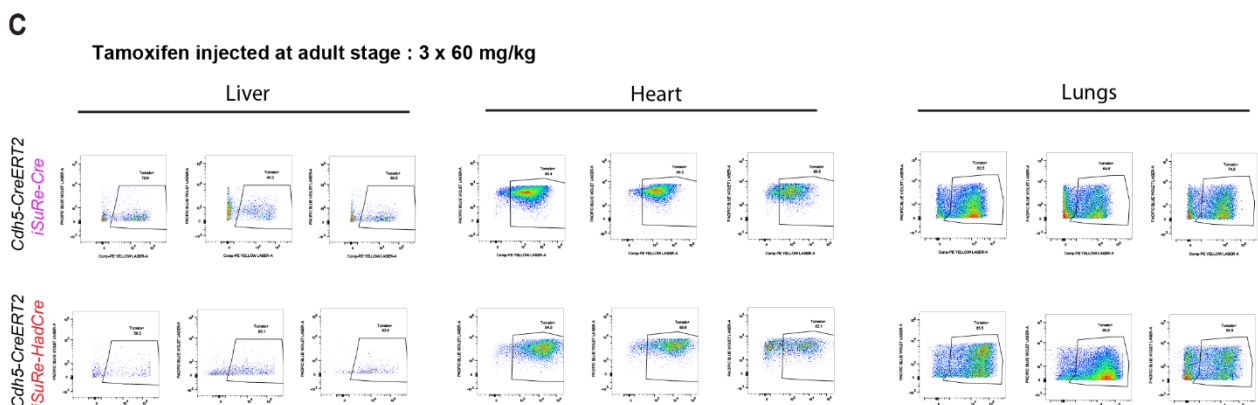
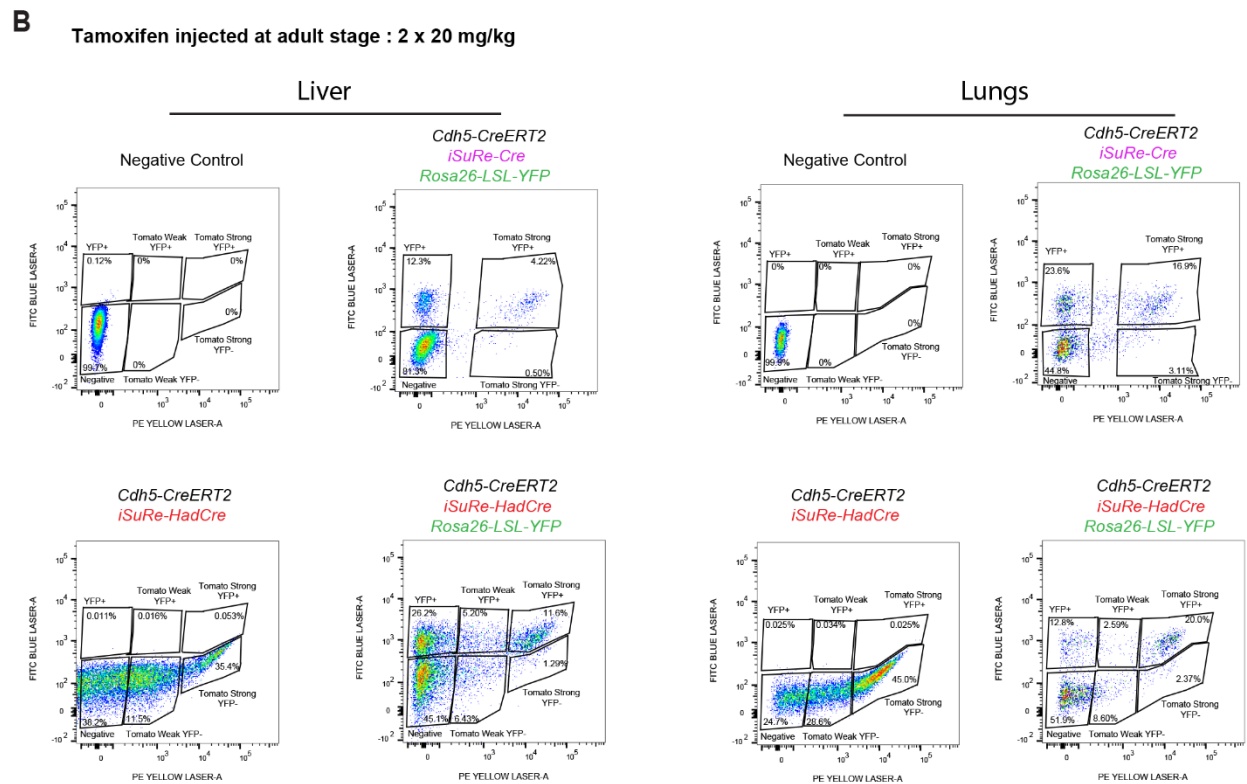
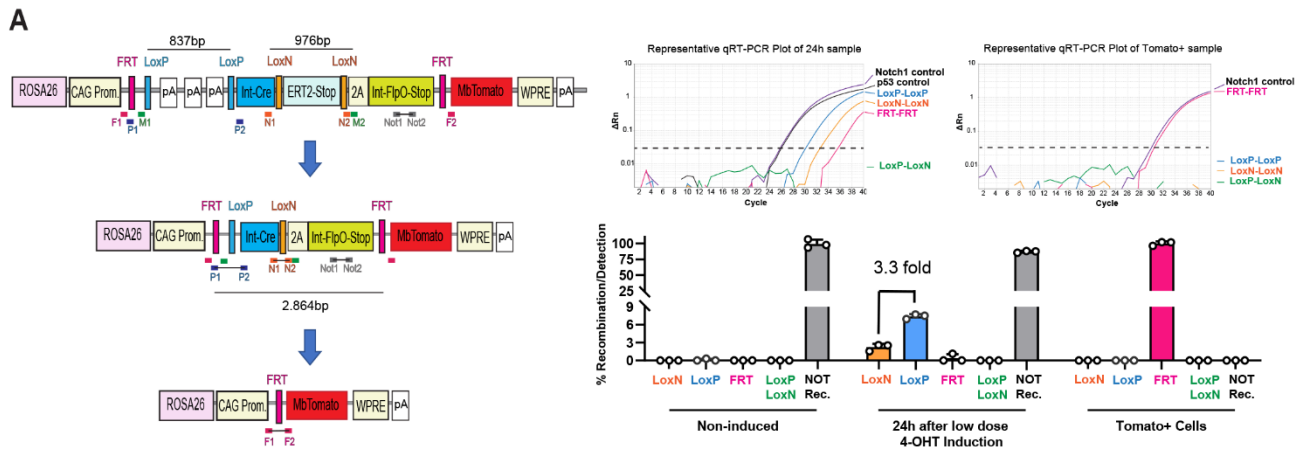
B Comparison of efficiency of induction in Heart ECs



Supplementary Figure S2. The *iSuRe-CrePEST^{v1}* allele has lower self-leakiness and recombination efficiency than the *iSuRe-Cre* allele. (A) Comparative FACS analysis of *iSuRe-Cre* and *iSuRe-CrePESTv1* self-leakiness (indexed by the frequency of MbTomato⁺ cells) in different organs and in the absence of other CreERT2 alleles or induction. **(B)** Comparative FACS analysis of the recombination efficiency of the reference *Rosa26-LSL-YFP* allele in heart ECs (Cdh5-CreERT2⁺) expressing the *iSuRe-Cre* or *iSuRe-CrePEST^{v1}* alleles. Efficiency is indexed as the percentage of MbTomato⁺YFP⁺ cells (true positives) to MbTomato⁺YFP⁻ (false positives).

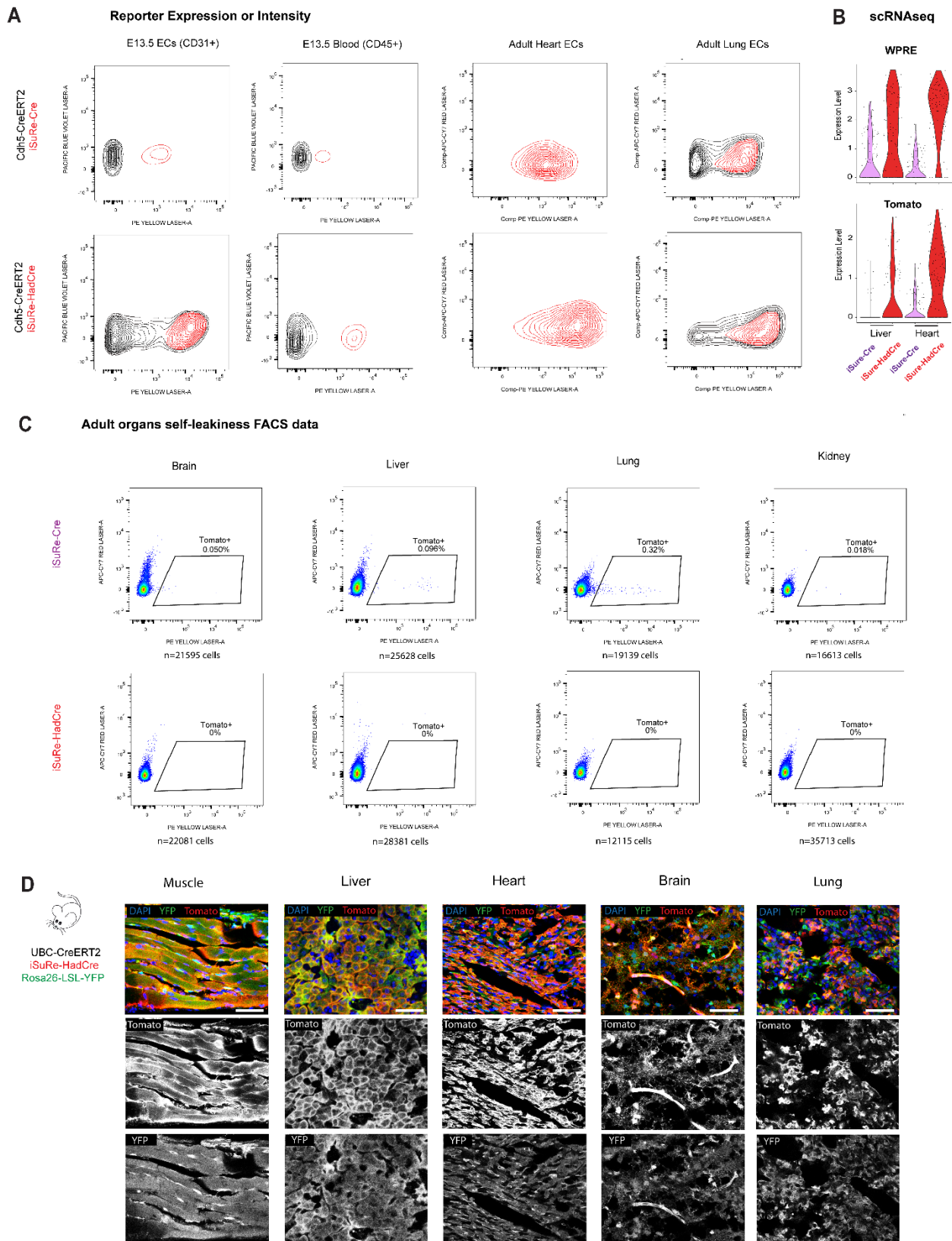
A**B**

Supplementary Figure S3. The *Rosa26-iSuRe-CrePESTv2* allele is more highly expressed than previous *iSuRe-Cre* alleles. (A) Representative confocal micrographs of P6 retinas showing higher MbTomato fluorescence intensity in ECs (marked with IsolectinB4 and *Cdh5-CreERT2*⁺) from animals expressing the *Rosa26-iSuRe-CrePESTv2* allele. **(B)** FACS plots showing higher MbTomato fluorescence intensity in postnatal day 7 liver ECs from animals expressing the *Rosa26-iSuRe-CrePESTv2* allele.



Supplementary Figure S4. Kinetics of the *Rosa26-iSuRe-HadCre* allele recombination and its efficiency in adult quiescent cells. (A) Predicted genetic recombination cascade and quantitative real time PCR with the indicated primers show how recombination between the LoxP sites is more effective than recombination

between the LoxN sites. It also shows that LoxP does not recombine with LoxN (green) and that once cells become Tomato⁺, the allele is fully recombined, and only a PCR with primers flanking the FRT site is amplified. **(B)** Comparative FACS analysis of recombination efficiency in Cdh5-CreERT2–induced liver and lung ECs reveals generally higher sensitivity of the *Rosa26-iSuRe-HadCre* allele to CreERT2 than *iSuRe-Cre*. MbTomato⁺ cells collected from iSuRe-Cre⁺ or iSuRe-HadCre⁺ animals reliably report recombination of the reference *Rosa26-LSL-YFP* reporter allele. **(C)** Comparative FACS analysis of recombination efficiency in adult quiescent ECs after three very high tamoxifen doses, showing similar recombination rates.



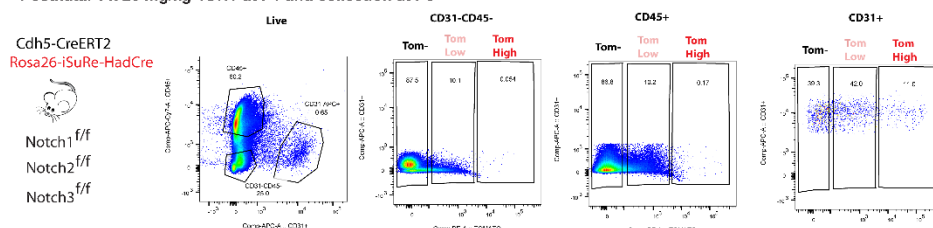
Supplementary Figure S5. The *Rosa26-iSuRe-HadCre* allele provides higher reporter expression and zero leakiness. (A) Comparative FACS analysis in different cell types and stages of development showing the higher expression of the reporter MbTomato (yellow laser axis) in *Rosa26-iSuRe-HadCre* animals. **(B)** scRNAseq analysis reveals higher expression of the *iSuRe-HadCre* allele in single cells. The WPRE and MbTomato sequences are the same in the two alleles. **(C)** Comparative FACS analysis showing that *Rosa26-iSuRe-HadCre* organs have 0% MbTomato+ cells (zero leakiness). **(D)** Histology analysis of the indicated animals showing very efficient recombination of the YFP reporter in all *iSuRe-HadCre*+/*MbTomato*+ cells. Scale Bars 50µm.

A

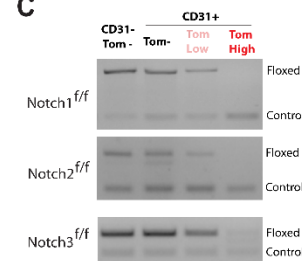
Floxed gene	<i>Notch1</i>	<i>Notch2</i>	<i>Notch3</i>	<i>Rbpj</i>	<i>Myc</i>	<i>Mycn</i>	<i>Foxo1</i>	<i>Foxo3</i>	<i>Foxo4</i>	<i>Kdr</i>	<i>Flt1</i>	<i>Dll4</i>	<i>Jag1</i>
Inter-LoxP distance (kb)	3.5	1.1	2.1	3	4.8	4.6	3.2	2.7	2.2	2.4	2.0	1.5	4.4

B

Postnatal 1 x 20 mg/kg 4OHT at P1 and collection at P6

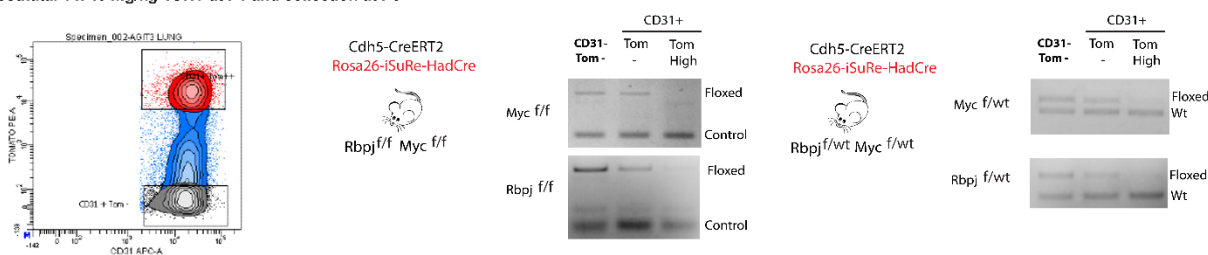


C



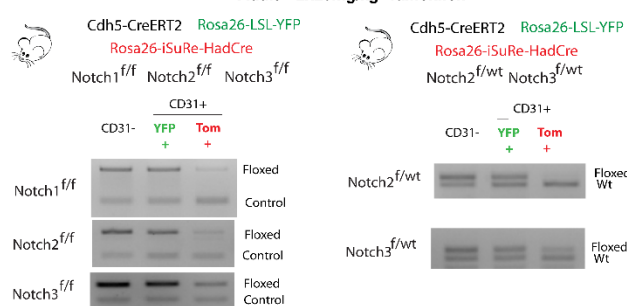
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Postnatal 1 x 40 mg/kg 4OHT at P1 and collection at P6



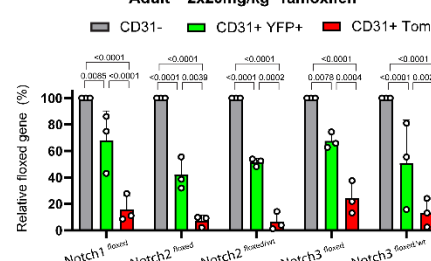
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Adult 2x20mg/kg Tamoxifen



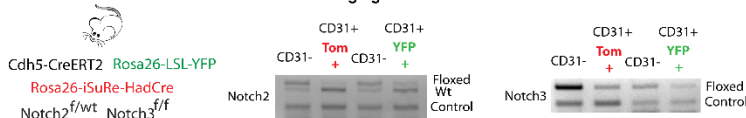
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Adult 2x20mg/kg Tamoxifen



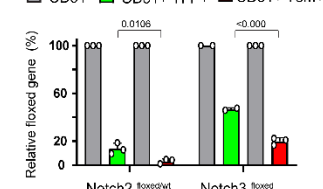
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Adult 4x60mg/kg Tamoxifen



H

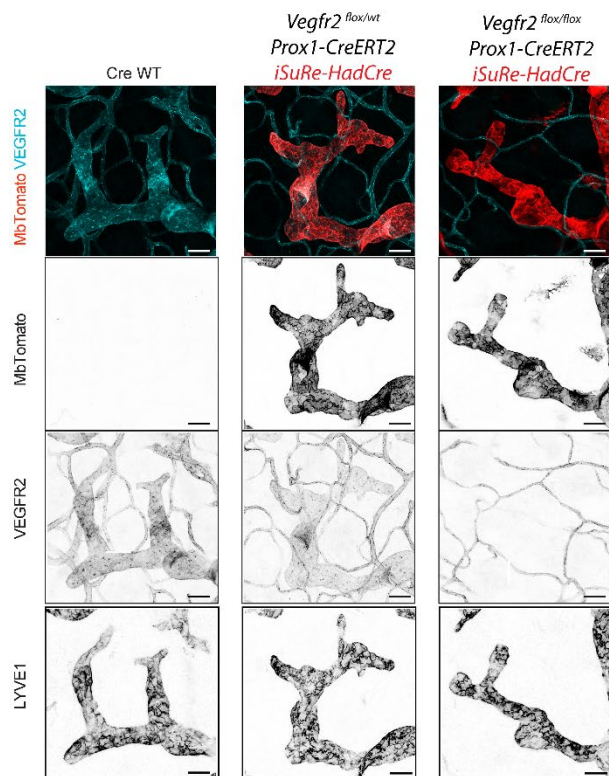
Adult 4x60mg/kg Tamoxifen



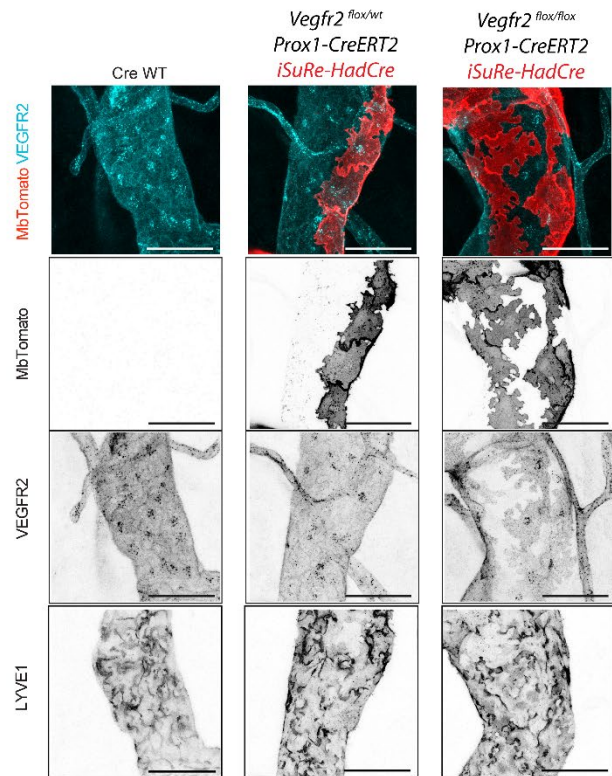
Supplementary Figure S6. The *Rosa26-iSuRe-HadCre* allele enables efficient deletion of multiple floxed alleles in postnatal and adult cells. (A) Distances between LoxP sites for all the floxed genes used in this study. **(B)** FACS plots with gating strategy used to isolate cells from the indicated animals. Control cells were CD31⁻, Tom⁻ (non-endothelial, Cdh5-CreERT2⁻), and CD31⁺ cells were gated based on zero, low or high MbTomato content. Only MbTomato^{high} cells were specific to the endothelial lineage (CD31⁺, Cdh5⁺) and had recombination of the *iSuRe-HadCre* allele. **(C)** Semi-quantitative DNA PCR with primers detecting the floxed sequence and a reference wildtype sequence as control. The gels show a pronounced deletion of *Notch1*, *Notch2*, and *Notch3* floxed genes only in cells that had recombination of the *iSuRe-HadCre* allele (CD31⁺ MbTomato^{high}). **(D)** FACS plots with gating strategy used to isolate cells from animals with the indicated genotype. The lower PCR band served as an internal control. The PCR gels show pronounced deletion of *Rbpj* and *Myc* floxed genes only in CD31⁺ MbTomato^{high} cells. **(E-H)** Adult mice were induced for 2 or 4 consecutive days. CD31⁻ (Cdh5⁻, negative control, non-induced), CD31⁺YFP⁺, and CD31⁺MbTomato⁺ cells were collected and DNA extracted. Gel pictures show representative semi-quantitative PCR results, and charts show relative quantification. Data are presented as mean values \pm s.d. For statistics, see Source Data File 1.

A

3x1mg (120mg/kg) Tamoxifen at P21-23, full recombination
Collection of tissues for immunostaining 5 weeks after

**B**

2x50µg (38mg/kg) 4-OHT at P1/P2, mosaic recombination
Collection of tissues for immunostaining 3 weeks after



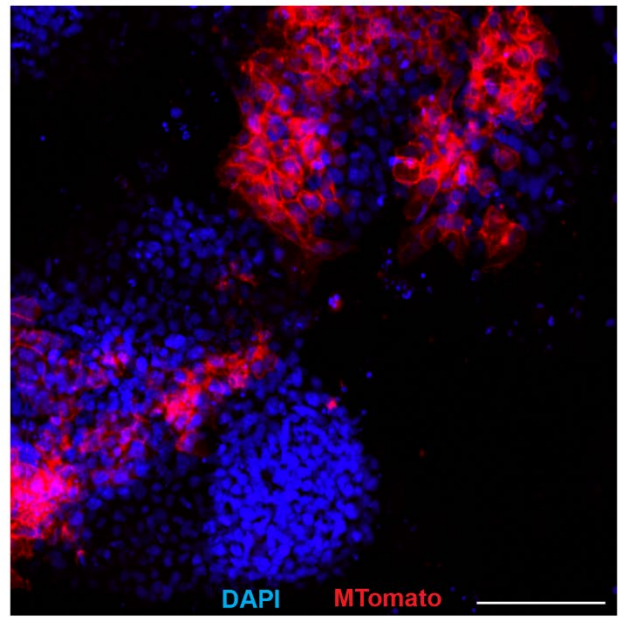
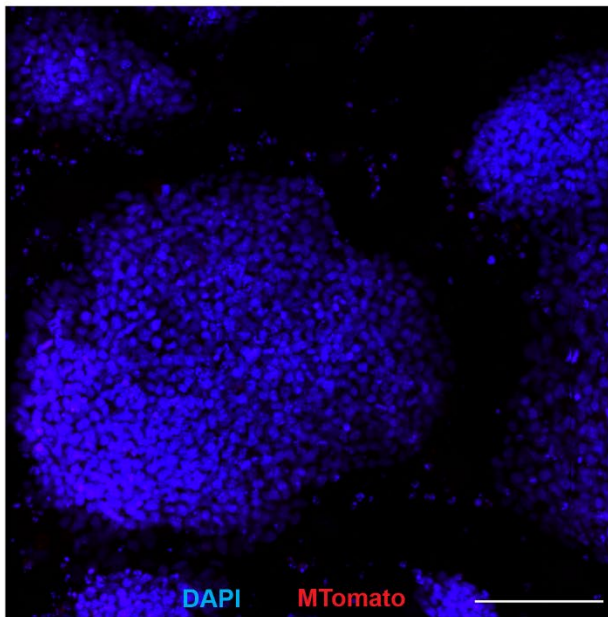
Supplementary Figure S7. Effective gene deletion in lymphatic endothelial cells with iSuRe-HadCre. (A, B) Representative confocal micrographs of skin vessels of control and *Vegfr2* floxed mice induced as indicated and stained with anti-VEGFR2 that labels both lymphatic (PROX1+LYVE1+) and blood endothelial cells (PROX1- LYVE1-). Anti-LYVE1 stains lymphatic endothelial cells. At high recombination rates (A), all lymphatic endothelial cells recombine the *iSuRe-HadCre* allele and delete VEGFR2. At low recombination rates (B), a mosaic of cells expressing *iSuRe-HadCre* is induced. MbTomato⁺ cells lose VEGFR2 expression, whereas the neighbouring cells retain the gene expression despite expressing *Prox1-CreERT2* and being induced with the same dose of tamoxifen. Scale Bars 50µm.

A

Mouse ES cells **Ubc-CreERT2** **iSuRe-HadCre**

Without 4-OH-Tamoxifen

4-OH-Tamoxifen (0,01 μ M) for 4h, remove and wait 72h

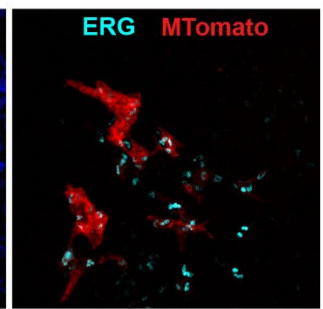
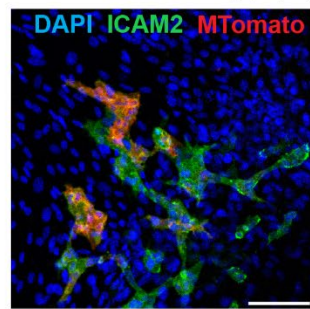
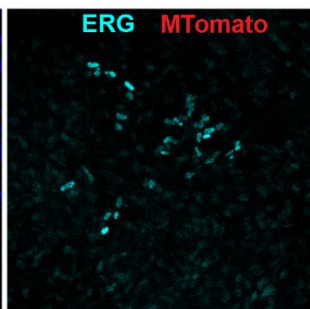
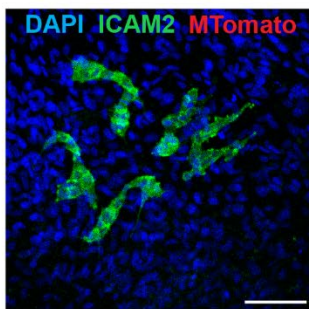


B

Mouse ES cells **Cdh5-CreERT2** **iSuRe-HadCre** after differentiation on OP9 to ECs

Without 4-OH-Tamoxifen

4-OH-Tamoxifen (0,1 μ M) for 4h, remove and wait 48h



Supplementary Figure S8. Mouse ES cells from iSuRe-HadCre mice as an *in vitro* model for effective conditional genetics. (A) Representative confocal micrographs of the indicated ES cells showing the nuclei (DAPI+) and the membrane Tomato protein in induced cells (iSuRe-HadCre+), 72h after induction with a low dose of 4-OHT. (B) Representative confocal micrographs of ES cells with the indicated genotype co-cultured with OP9 cells for 8 days. After six days of coculture, ES cells differentiate into ECs: ICAM2+ (EC surface) and ERG+ (EC nuclei) and the addition of a relatively low dose of 4-OHT for 4h at day 6 of differentiation, was able to induce recombination of the *iSuRe-HadCre* allele and expression of the MbTomato reporter specifically in Cdh5+, ICAM2+, ERG+ endothelial cells. Scale bars 100 microns.

Supplementary Table 1. Genotyping primers

Name of mouse line	Expected size fragment	Genotyping oligonucleotides
<i>Tg(PAC-Cdh5-CreERT2)^{1Rha}</i>	Allele band: 550bp	Cdh5 Trans F: GGAGGCTGGAAAGTAGAGCA CreM R: TCCCTGAACATGTCCATCAG
<i>Tg(BAC-Myh11-CreERT2)^{1Soff}</i>	Allele band: 287bp WT band: 200bp	Rev Cre: AGT CCC TCA CAT CCT CAG GTT Rev Myh11: AAC TCC ACG ACC ACC TCA TC Myh11 Fwd: TGA CCC CAT CTC TTC ACT
<i>Tg(Sox2-Cre)</i>	Allele band: 165bp WT band: 207bp	SOX2 WT Fwd: CTT GTG TAG AGT GAT GGC TTG A SOX2 Common: TAG TGC CCC ATT TTT GAA GG SOX2 Transgene Fwd: CCA GTG CAG TGA AGC AAA TC
<i>Tg(iSuRe-Cre)</i>	Allele band: 260bp WT band: 104 bp	Sv40 pA F: CCCCTGAACCTGAAACATA AVT MTomato R: CCTTGCTCACCATGGTCTTG Chr17 R: GTGTCTGTACCAGTTGGTTTG Chr17 R: GCTCTGCATGTTGCAAGAAA
<i>Tg(Tie2-cre)</i>	Allele band: 500bp	Ts: GGG AAG TCG CAA AGT TGT GAG TT C2: CTA GAG CCT GTT TTG CAC GTT C
<i>iSuRe-CrePESTv1</i>	Allele band: 1kb	H2B F: CGAGGGTACTAAGGCCATCA CUB end R: GGAATTCGGGGATCCCTAT
<i>Rosa26-iSuRe-CrePESTv2</i>	WT band: 250bp Allele band: 373bp	Rr711:GCACTTGCTCTCCCAAAGTC Rr713:CTTTAAGCCTGCCCAGAAGA ERT2 Fwd: TCCTCATCCTCTCCACATC PEST Rev: atcatcctgctcctccacct
<i>Rosa26-iSuRe-HadCre</i>	WT band: 250bp Allele band: 354bp	Rr713:CTTTAAGCCTGCCCAGAAGA Rr711:GCACTTGCTCTCCCAAAGTC iCreERT2 F: ATCGCATTCTTGCAAAAAGT FlpO trans and seq Rev II: catccagcacaggtaggtca
<i>R26-LSL-EYFP</i>	WT band: 250bp Allele band: 350bp	Rr711:GCACTTGCTCTCCCAAAGTC Rr712: GGGCGTACTTGGCATATGAT Rr713:CTTTAAGCCTGCCCAGAAGA Rr714:GCGAAGAGTTTGTCTCAACC
<i>Gt(ROSA)26Sor^{tm14}(CAG-tdTomato)Hze</i>	WT band: 250bp Allele band: 350bp	Rr711:GCACTTGCTCTCCCAAAGTC Rr713:CTTTAAGCCTGCCCAGAAGA CAG PCR F: CGGGGTCATTAGTTCATAGCC CAG PCR R: CACCTCGACCATGGTAATAGC
<i>Gt(ROSA)26Sor^{tm2}(Pik3ca*)Dsa</i>	WT band: 330bp Allele band: 630bp	aIMR4349: CTCTGCTGCCTCCTGGCTTCT aIMR4350: CGAGGCGGATCACAAGCAATA XpGL3:TGAATAGTTAATTGGAGCGGCCGCAATA

<i>Pik3ca</i>^{tm1.1Waph}	<i>WT band: 553bp</i> <i>Allele band: 601bp</i>	19F: TTGGTTCCAGCCTGAATAAAGC 20R: GTCCAAGGCTAGAGTCTTTCCG
<i>Notch1</i>^{flox/flox} mice	<i>WT band: 445bp</i> <i>Flox band: 500bp</i>	Notch1 lox F: CTGAGGCCTAGAGCCTTGAA Notch1 lox R: TGTGGGACCCAGAAGTTAGG
<i>Notch2</i>^{flox/flox} mice	<i>WT band: 202 bp</i> <i>Flox band: 240 bp</i>	Notch2 rv: GAG CCT TTT CCC CAT ATT CC Notch2 Fwd: CAA CCC CAG ATA GGA AGC AG
<i>Notch3</i>^{flox/flox} mice	<i>WT band: 150bp</i> <i>Flox band: 190bps</i>	Notch3 LoxP1 F: tggctatgaggacaaaagg Notch3 LoxP1 R: ctgcctctagcactgggtt
<i>Rbpj</i>^{flox/flox} mice	<i>WT band: 200bp</i> <i>Flox band: 350bp</i>	Rbpj lox F: ATAATTTGCCAAGCCAAAGC Rbpj lox R: GCTCCCCACTGTGTGAAC
<i>Myc</i>^{flox/flox} mice	<i>WT band: 315bp</i> <i>Flox band: 370bp</i>	Myc Flox RB F: gacaggatgtgaccgattc Myc Flox RB R: gctcagtctccggctatcac
<i>Mycn</i>^{flox/flox} mice	<i>WT band: 200bp</i> <i>Flox band: 220bp</i>	N-Myc 1: GTCGCGCTAGAAGAGCTGAGAT N-Myc3: CACAGCTCTGGAAGGTGGGAGAAAGTTGAAGCGTCTCC
<i>Dll4</i>^{flox/flox} mice	<i>WT band: 400bp</i> <i>Flox band: 500bp</i>	Dll4 lox F: GTGCTGGGACTGTAGCCACT Dll4 lox R: TGTTAGGGATGTCGCTCTCC
<i>Jag1</i>^{flox/flox} mice	<i>WT band: 401bp</i> <i>Flox band: 453bp</i>	Jag4716: AGG TTG GCC ACC TCT AAA TC Jag5032: GCA AGT CTG TCT GCT TTC ATC
<i>Foxo1</i>^{flox/flox} mice	<i>WT band: 115bp</i> <i>Flox band: 149bp</i>	Foxo1 primer A: GCTTAGAGCAGAGATGTTCTCACATT Foxo1 primer B: CCAGAGTCTTTGTATCAGGCAAATAA Foxo1 primer D: CAAGTCCATTAATTCAGCACATTGA
<i>Foxo3</i>^{flox/flox} mice	<i>WT band: 100bp</i> <i>Flox band: 138bp</i>	Foxo3 primer A: ATTCCTTTGGAAATCAACAAAACCT Foxo3 primer B: TGCTTTGATACTATTCCACAAACCC Foxo3 primer D: AGATTTATGTTCCCACTTGCTTCCT
<i>Foxo4</i>^{flox/flox} mice	<i>WT band: 343bp</i> <i>Flox band: 555bp</i>	Foxo4 primer A: CTTCTCTGTGGGAATAAATGTTTGG Foxo4 primer B: CTAATTCAAGGACAAGGGTGACAG Foxo4 primer D: TGAGAAGCCATTGAAGATCAGA
<i>Kdr</i>^{flox/flox} mice	<i>WT band: 200bp</i> <i>Flox band: 220bp</i>	Flk lox F3: GCCTGCAGGTTCTGGTTTG Flk lox Rev1: GCTGTGACATCTGGGTAGAG
<i>Flt1</i>^{flox/flox} mice	<i>WT band: 120bp</i> <i>Flox band: 180bp</i>	VEGFR1 lox F: CCT GCA TGA TTC CTG ATT GGA VEGFR1 lox R: GCC TAA GCT CAC CTG CGG
Control for semi-quant. PCRs	<i>WT band: 104bp</i>	R: GTGTCTGTACCAGGTTGGTTTG F: GCTCTGCATGTTGCAAGAAA

Supplementary Table 2. Quantitative real-time PCR primers

(used in Sup. Fig. S4A data)

LoxN Flank N1: CCACTGCCGCTAGCTAATAA

LoxN Flank Probe: TGCTGGAAGATGGCGATCCGATAA

LoxN Flank N2: CCGTAACCTGGATAGTGAACA

LoxP Flank P1: GGCCGAATTCGATCTAGCTT

LoxP Flank Probe: TTATTAGGTCCCTCGACCTGCAGC

LoxP Flank P2: GGCCCTAAGAAGTTCCTATTCTC

FRT Flank F1: TCACCATGGTCTTGGAGAAAC

FRT Flank Probe: TTGATTTGATACCGCGGGCCCTAA

FRT Flank F2: TGCTGGTTATTGTGCTGTCT

LoxP-LoxN Flank M1: CGACGTCACCGCATGTTA

LoxP-LoxN Flank Probe: AGTGGAGAGGGCAGAGGAAGTCT

LoxP-LoxN Flank M2: GGGCCCTAAGAAGTTCCTATTC

FlpO Not1: CTGTACCAGTTCCTGTTCTG

FlpO Probe: TCTTGATGTCGCTGAACCTGCCG

FlpO Not2: CTTGTCTTGGTCTCGGTCAC

Notch1 genomic P1: CGTCCAAGATGCCTTCCTTAT

Notch1 genomic Probe: TGGAATCTGGAACGAACAACACCTCC

Notch1 genomic P2: GGGCCCTTATTCCTTGTTGA

P53 genomic P1: CTGTGGATGGGACAAAGAAGA

P53 genomic Probe: AGAGCAGAAAGGGACTTGGGCTTT

P53 genomic P2: TCCAGACTTCCTCCAGAAGATA

Supplementary Table 3. Antibodies

ANTIBODIES	APPLICATION	DILUTION	SOURCE	IDENTIFIER
Goat anti-GFP	IF	1:200	Acris Antibodies	Cat# R1091P
Chicken anti-GFP	IF	1:200	Abcam	Cat# ab13970
Rabbit anti-DsRed	IF	1:400	Clontech	Cat# 632496
Rabbit anti-RFP-CF594	IF	1:400	Biotium	Cat#20422
Mouse anti-V5-FITC	IF	1:100	ThermoFisher	Cat# R963-25
Mouse anti-V5-Alexa488	IF	1:100	Biorad	Cat#MCA1360A488
Rabbit Anti-V5	IF	1:200	Abcam	Cat# ab9116
Rabbit anti-ERG-AF-647	IF	1:200	Abcam	Cat# ab196149
Rabbit anti-ERG (EPR3863)	IF	1:200	Abcam	Cat# ab110639
Rat anti-Ki67e660	IF	1:200	eBioscience/Thermo	Cat# 50-5698-82
Rat anti-Endomucin	IF	1:200	Santa Cruz Biotechnology	Cat# SC-53941
Rat anti-Endomucin	IF	1:200	Santa Cruz Biotechnology	Cat# SC-65495
Rat anti-CD31	IF	1:200	BD Biosciences	Cat# 553370
Rat anti-p21 (monoclonal)	IF	1:10	CNIO	
Rat anti-VEGFR2/KDR	IF	1:50	BD Pharmingen	Cat# 666307
Rabbit anti-Myc	IF	1:200	Millipore	Cat# 06-340
Rat anti-RBPJ	IF	1:50	Cosmobio	Cat# SIM-22RBP2
Goat anti-Esm1	IF	1:200	R&D systems	Cat# AF1999
Rabbit anti-FOXO1	IF	1:200	CellSignalling Technology	Cat# 2880S
Rat anti-FLT1	IF	1:200	ImClone	Cat# MF-1
Rat anti-ICAM2	IF	1:200	BD Biosciences	Cat# 553325
Rat anti-CD31-APC	FACS	1:200	BD Pharmingen	Cat# 551262
Rat anti-CD45-APC-Cy7	FACS	1:200	BD Pharmingen	Cat# 557659
Donkey anti-rat Alexa-647	IF	1:400	Abcam	Cat# ab150155
Donkey anti-goat Alexa-488	IF	1:400	Invitrogen	Cat# A-11055
Donkey anti-rabbit Alexa-594	IF	1:400	Jackson Immunoresearch	Cat# 711-587-003
Donkey anti-rabbit Alexa-647	IF	1:400	Jackson Immunoresearch	Cat# 711-607-003
Biotin-SP Donkey anti-goat	IF	1:200	Jackson Immunoresearch	Cat# 705-065-003
Biotin-SP Donkey anti-rat	IF	1:100	Jackson Immunoresearch	Cat# 712-067-003

