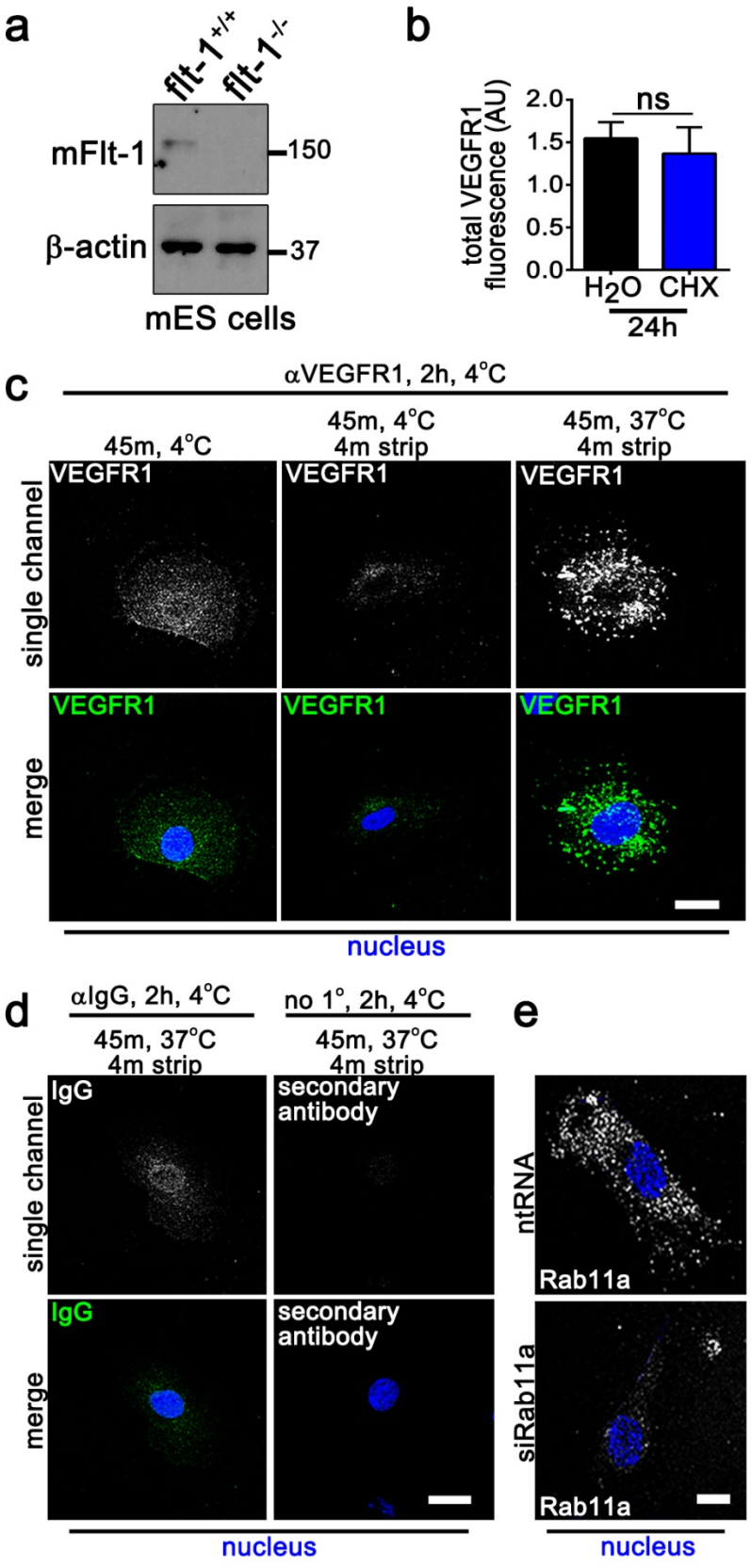
Supplementary Figure 1



Supplementary Figure 1 (linked to Figure 1). mVEGFR1 constitutively internalizes in endothelial cells.

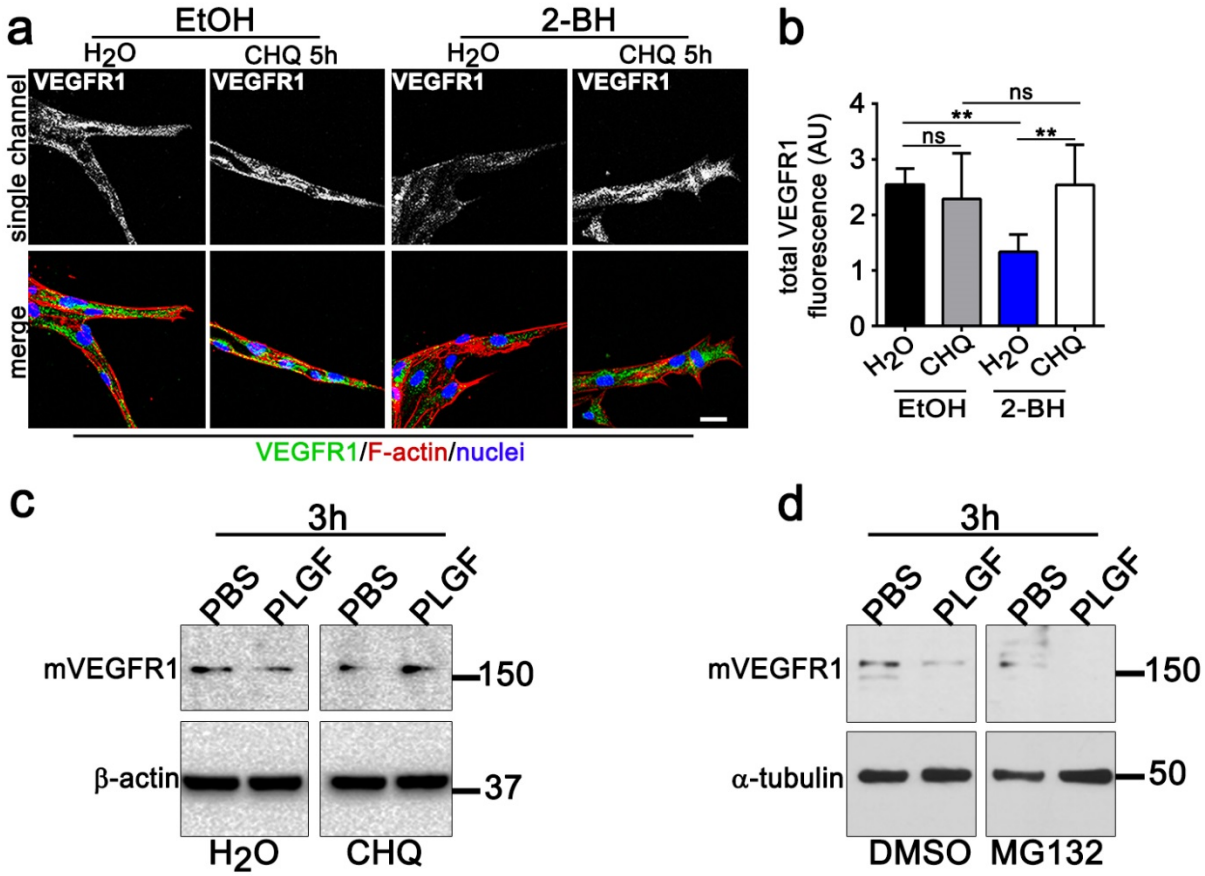
(a) Immunoblot of mFlt1 from undifferentiated mouse embryonic stem (ES) cells with indicated genotypes; 2 replicates.

(b) Quantification of VEGFR1 fluorescence intensity of HUVEC with indicated treatments via integrated density. (n=44/control; n=42/CHX). CHX, cycloheximide; 2 replicates. Statistics: Shown are means + 95% CI, student's t-test with Tukey's post-hoc range test; ns, not significant.

(c-d) Immunofluorescence of VEGFR1 from HUVEC internalization assay with indicated treatments. Strip: 0.2M acetic acid, 4min; 3 replicates.

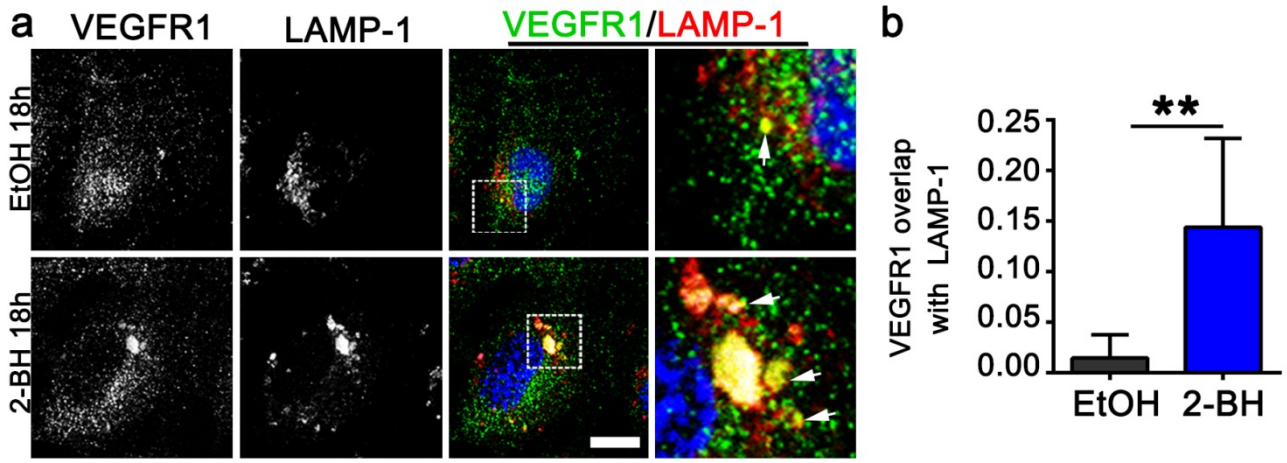
(e) Rab11a immunofluorescence in HUVEC with indicated treatments. DRAQ7 (nuclei); 2 replicates. All scale bars: 20μm.

Supplementary Figure 2



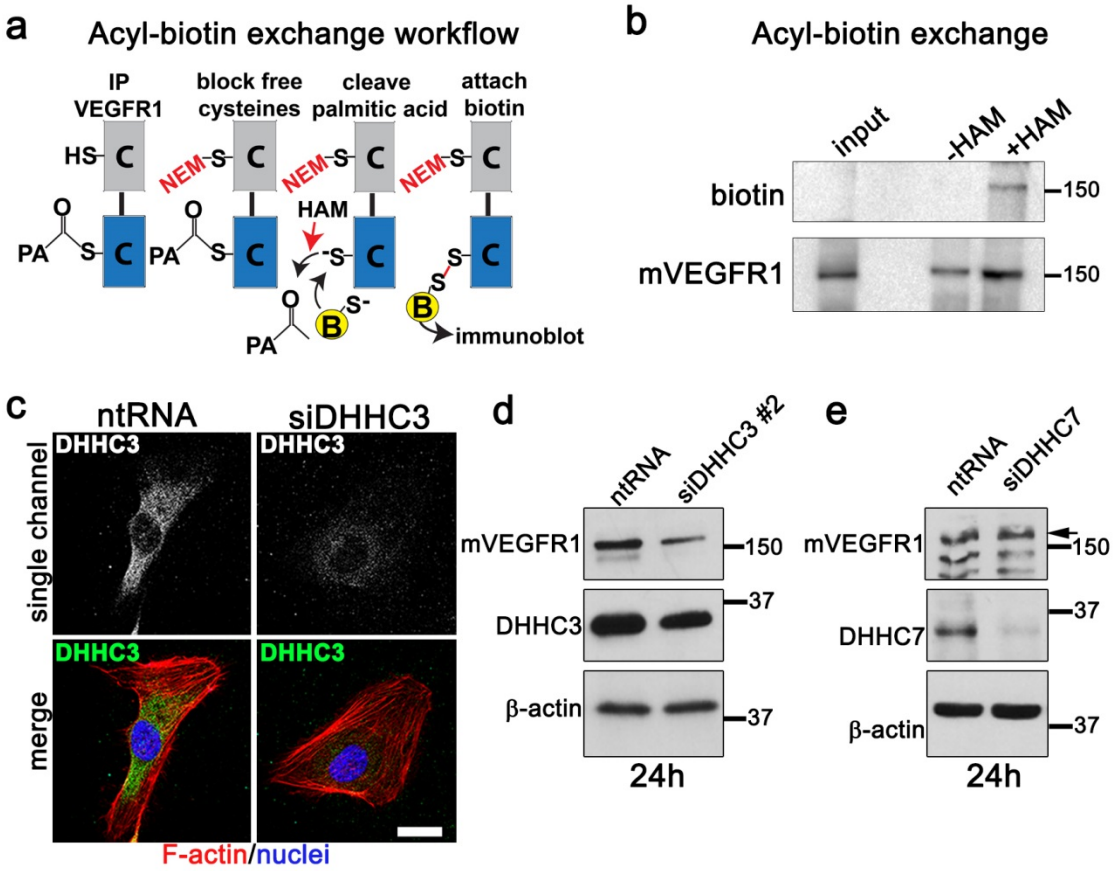
Supplementary Figure 2 (linked to Figure 2). Destabilized mVEGFR1 degrades via the lysosome.
(a-b) (a) VEGFR1 immunofluorescence of d5 HUVEC angiogenic sprouts treated with 2-BH for 12h prior to CHQ for 5h, scale bar: 25µm. (b) Quantification of VEGFR1 fluorescence via integrated density. Shown are means + 95% CI (#sprouts; EtOH/H_2O, n=6; EtOH/CHQ, n=6; 2-BH/H_2O, n=8; 2-BH/CHQ, n=5); 2-BH, 2-bromohexadecadnoic acid; CHQ, chloroquine; 2 replicates. Statistics: one-way ANOVA and pairwise comparison with post-hoc Tukey's range test. **, p≤0.01; ns, not significant.
(c-d) Immunoblot of HUVEC with indicated treatments, 3 replicates.

Supplementary Figure 3



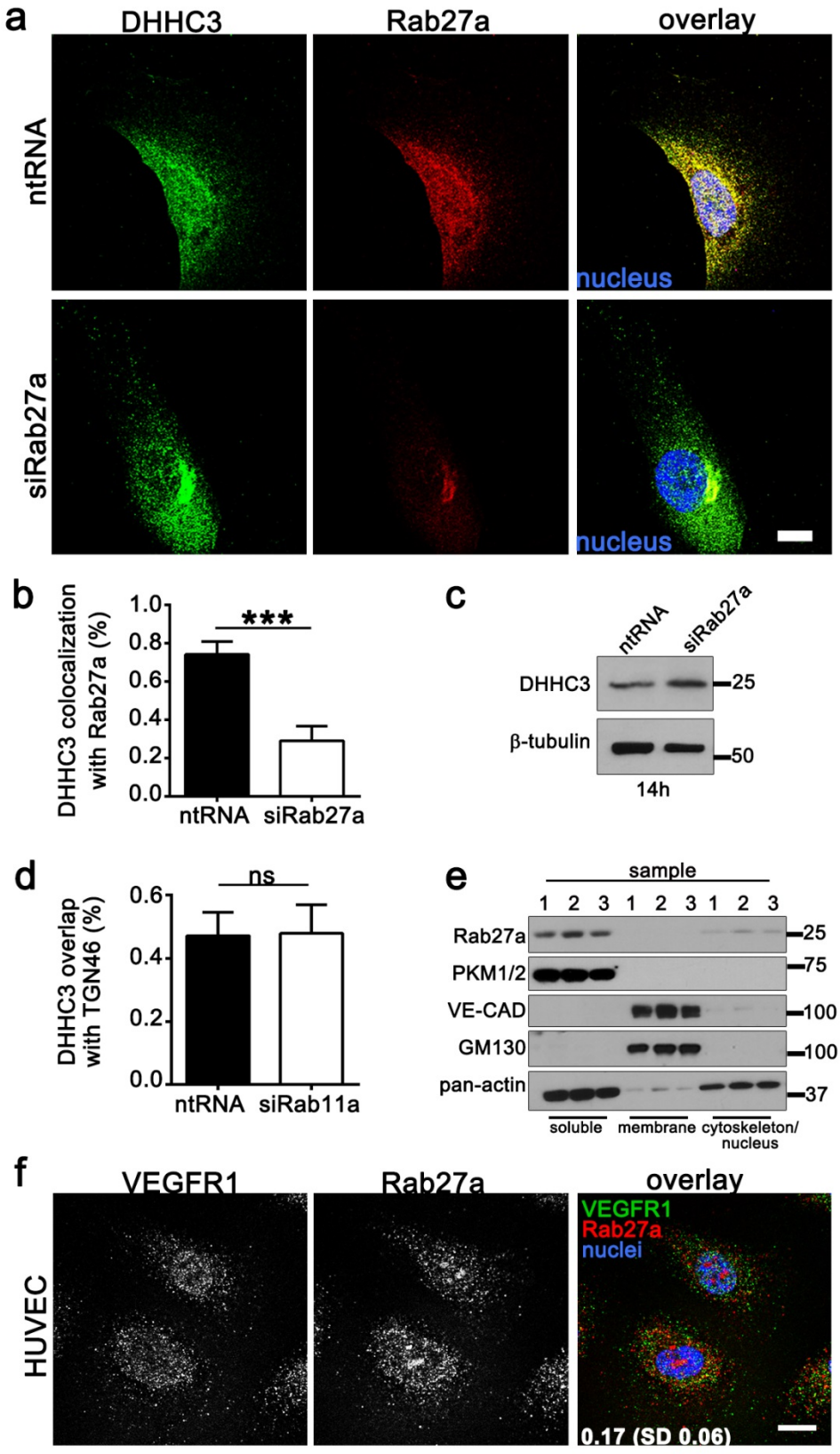
Supplementary Figure 3 (linked to Figure 3). Palmitoylation regulates mVEGFR1 localization with lysosomes.
(a-b) (a) VEGFR1 and LAMP-1 (lysosome marker) immunofluorescence of HUVEC with indicated treatments and (b) Mander's Correlation Coefficient quantification of VEGFR1 overlap with LAMP-1. (#cells: EtOH, n=38; 2-BH, n=36); 3 replicates, scale bar: 10μm.
Statistics: Shown are means +95% CI. Pairwise comparison with post-hoc Tukey's range test. **, p≤0.01.

Supplementary Figure 4



Supplementary Figure 4 (linked to Figure 4). Knockdown of the palmitoyl acetyl transferase DHHC3 reduces mVEGFR1 levels in endothelial cells.
(a) Acyl-biotin exchange workflow. B, biotin; PA, palmitic acid; NEM, N-ethylmaleimide; HAM, hydroxylamine.
(b) Acyl-biotin exchange on immunoprecipitated VEGFR1 from HUVEC with indicated treatments, then immunoblot for biotin, stripped and reprobed for VEGFR1. HAM, hydroxylamine; 2 replicates.
(c) DHHC3 immunofluorescence of HUVEC with indicated treatments for 24h; 2 replicates, scale bar: 20μm.
(d-e) Immunoblot for mVEGFR1 in HUVEC with 24h knockdown of DHHC3 (d) or DHHC7 (e); 3 replicates.

Supplementary Figure 5



Supplementary Figure 5 (linked to Figure 5). Rab27a and DHHC3 but not VEGFR1 colocalize in endothelial cells.

(a-b) (a) Immunofluorescence of DHHC3 and Rab27a in HUVEC with indicated treatments 24h post-knockdown, scale bar: 10μm. (b) Mander's Colocalization Coeffcient quantification of overlap. Shown are means +95% CI. (#cells: ntRNA, n=31; siRab27a, n=20); 2 replicates.

(c) Immunoblot of DHHC3 in HUVEC with indicated treatments.

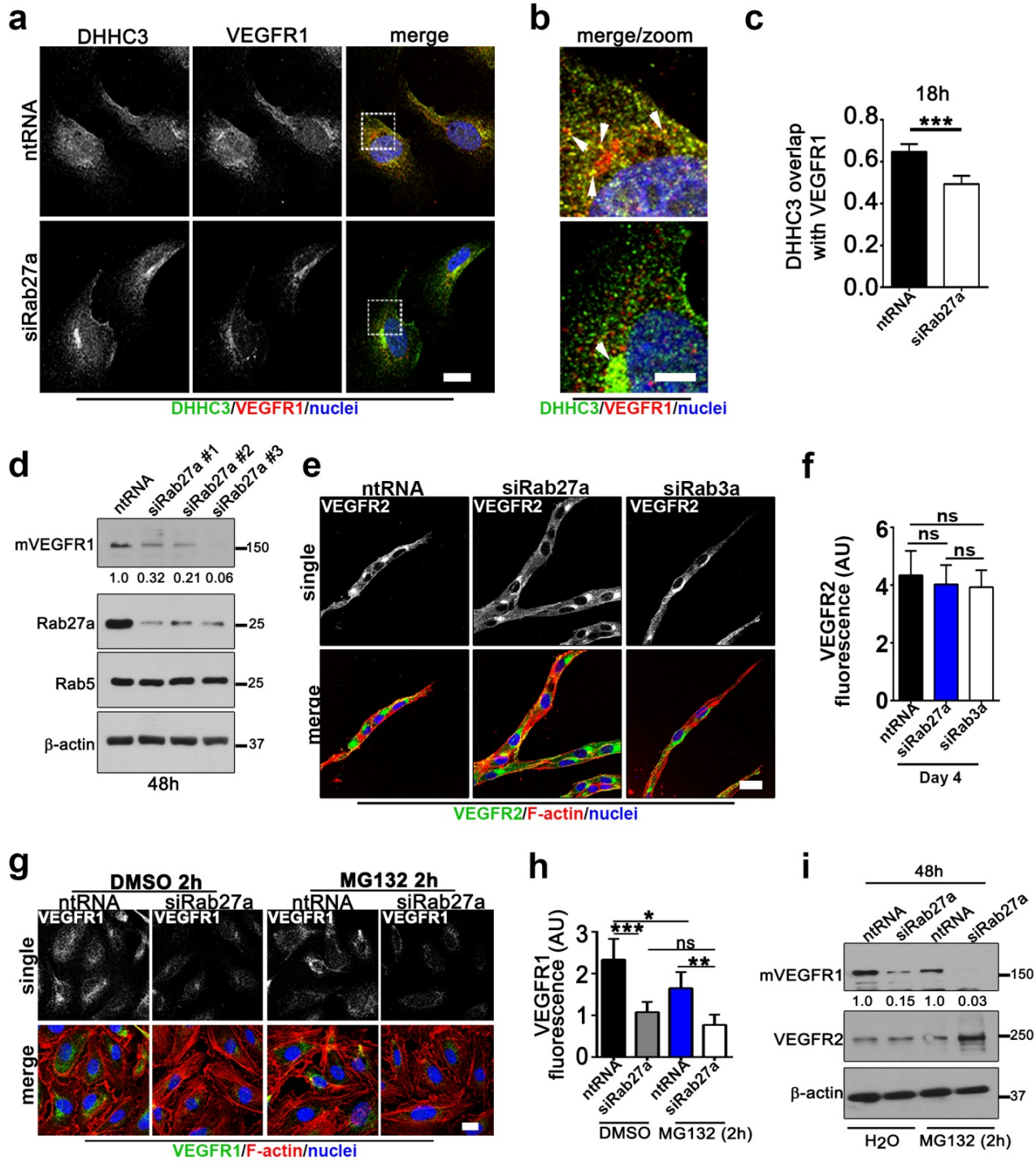
(d) Mander's Colocalization Coefficient quantification of DHHC3 and TGN46 (Golgi) overlap in HUVEC with indicated treatments. Shown are means + 95% CI. (#cells: ntRNA, n=19; siRab11a, n=27); 2 replicates.

(e) Subcellular fractionation and immunoblot of HUVEC from 3 different lots. Pyruvate kinase1/2 (PKM1/2), soluble marker; vascular-endothelial cadherin (VE-CAD), membrane and cytoskeletal marker; cis-Golgi Marker 130 (GM130), membrane marker; pan actin, soluble (G-actin) and cytoskeletal (F-actin) marker. 2 replicates.

(f) Immunofluorescence of VEGFR1 and Rab27a overlap quantified with Mander's Correlation Coefficient, +/- SD, scale bar 15μm.

Statistics: Student's t-test with post-hoc Tukey's range test. ***, $p \leq 0.001$; ns, not significant.

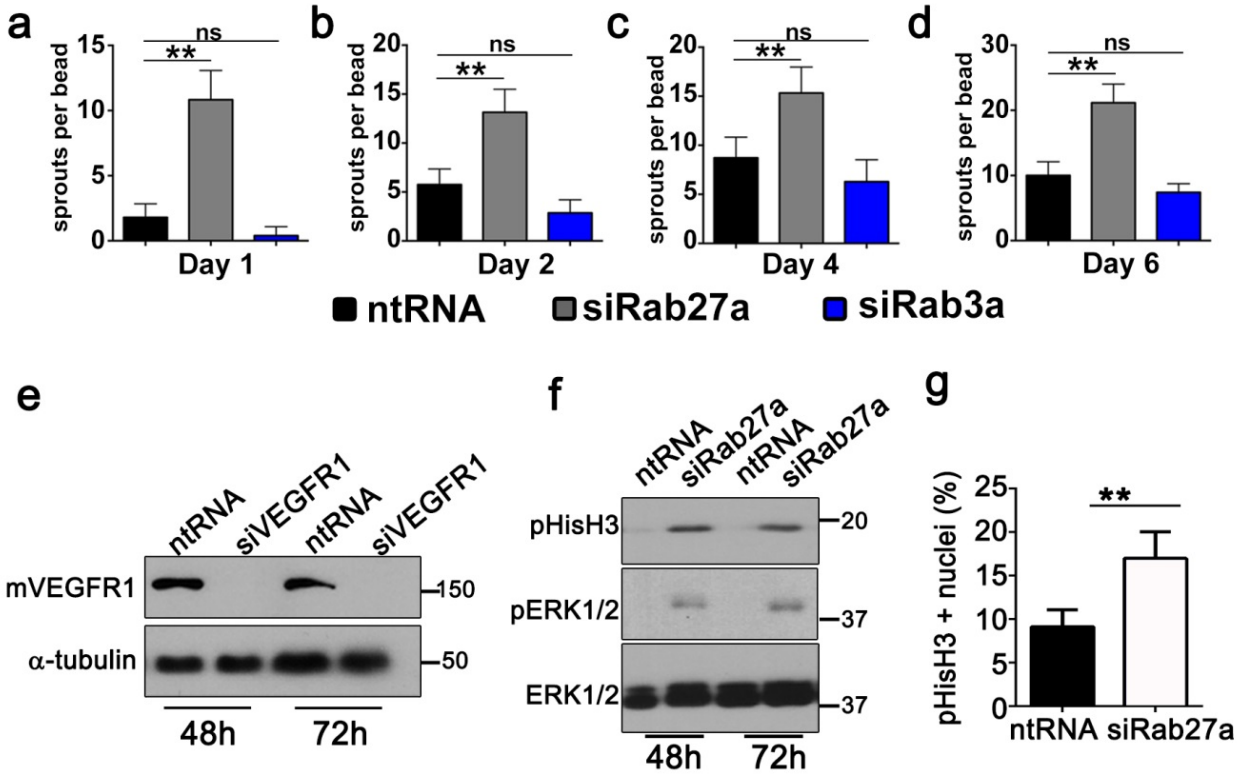
Supplementary Figure 6



Supplementary Figure 6 (linked to Figure 5). Rab27a regulates steady-state mVEGFR1 levels.

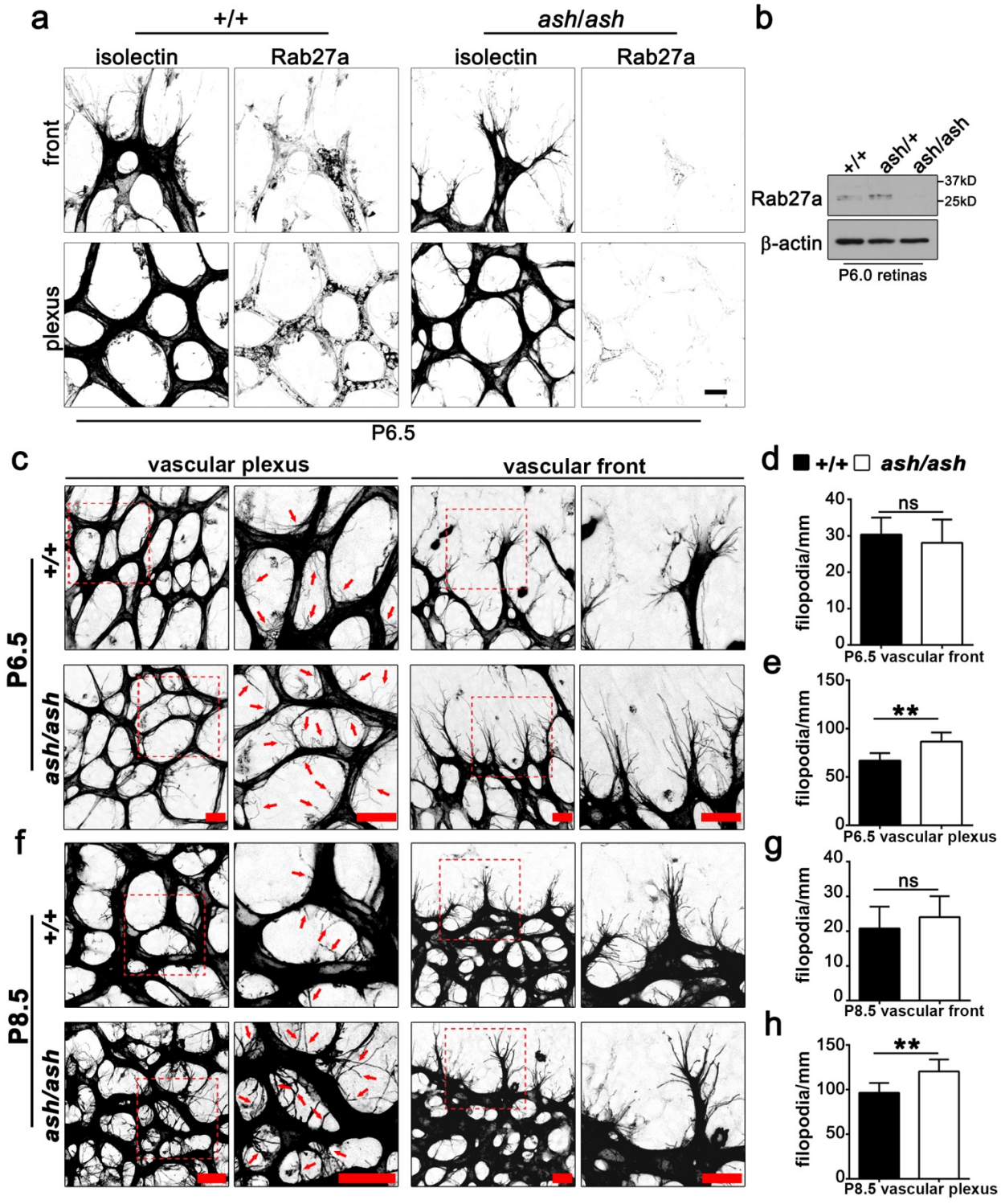
(a-c) (a-b) Immunofluorescence of DHHC3 and VEGFR1 in HUVEC with indicated treatments, scale bars: 10μm (a) and 5μm (b). (c) Mander's Colocalization Coefficient quantification of fluorescence. (#cells: ntRNA, n=33, siRab27a, n=40); 3 replicates.
(d) Immunoblot of HUVEC with indicated treatments. Values are relative mVEGR1 levels.
(e-f) (e) VEGFR2 immunofluorescence and (f) quantification of fluorescence via integrated density of d4 HUVEC angiogenic sprouts with indicated treatments. Scale bar: 20μm, (#sprouts: ntRNA, n=10; siRab27a, n=12; siRab3a, n=7); 3 replicates.
(g-h) (g) VEGFR1 immunofluorescence of HUVEC 48h post KD and (h) quantification of fluorescence via integrated density, scale bar 15μm. (# cells: DMSO/ntRNA, n=14; MG132/ntRNA, n=18; DMSO/siRab27a, n=14; MG132/siRab27a, n=16); 2 replicates.
(i) Immunoblot of HUVEC with indicated treatments. Values are relative mVEGR1 levels; 2 replicates.
Statistics: Shown are means +95% CI. One-way ANOVA and pairwise comparison with post-hoc Tukey's range test. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, not significant.

Supplementary Figure 7



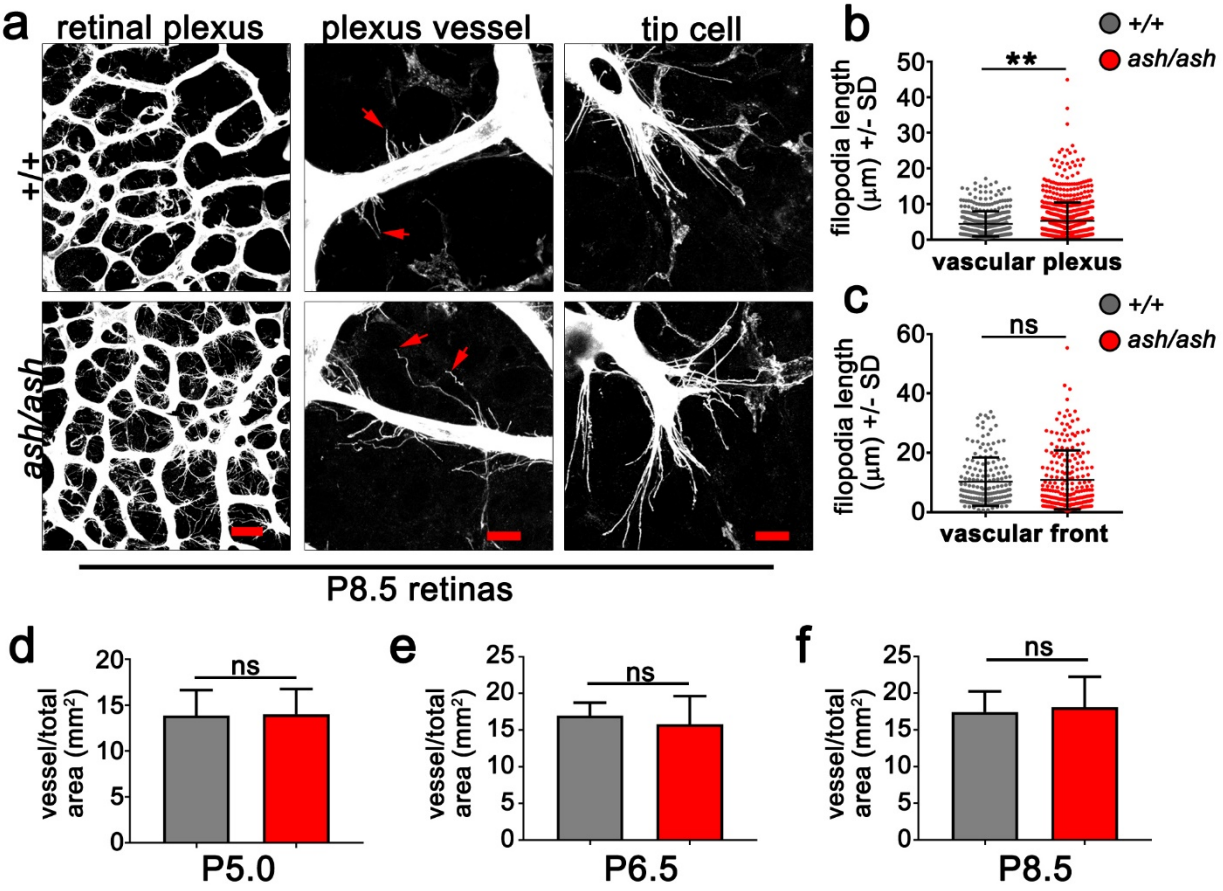
Supplementary Figure 7 (linked to Figures 6 and 7). Rab27a negatively regulates angiogenesis and increases HUVEC proliferation.
(a-d) Quantification of sprouting from HUVEC with indicated treatments on indicated days. (n=10 beads/condition/time point); 3 replicates.
(e) Immunoblot of HUVEC with indicated treatments and times; 2 replicates.
(f) Immunoblot of HUVEC with indicated treatments and times; 3 replicates.
(g) Quantification of pHisH3 (mitotic) nuclei of HUVEC 48h post-knockdown (#nuclei: ntRNA, n=682; siRab27a, n=549); 2 replicates.
Statistics: (a-d) Shown are means +95% CI. One-way ANOVA and pairwise comparison with post-hoc Tukey's range test. (g) Shown are means +SEM, Student's *t*-test. **, p≤0.01; ns, not significant.

Supplementary Figure 8



Supplementary Figure 8 (linked to Figure 8). Loss of Rab27a protein in *ash/ash* mice leads to hyperactive retinal blood vessels.
(a) Immunofluorescence of P6.5 mouse retinas for vessels (isolectin) and Rab27a of indicated genotypes; 3 replicates, scale bar: 15μm.
(b) Immunoblot of Rab27a from P6.0 retinal lysates of indicated genotypes; 4 replicates.
(c-e) (c) Immunofluorescence of P6.5 retinal vessels with indicated genotypes. Filopodia were quantified at the vascular front (d) and within the plexus (e). Red boxes, higher magnification to right. Arrows, filopodia. (#retinas: 2 independent litters: +/+, n=7*; ash*/*ash,* n=6).
(f-h) (f) Immunofluorescence of P8.5 retinal vessels with indicated genotypes. Filopodia were quantified at the vascular front (g) and within the plexus (h). Red boxes, higher magnification to right. Arrows, filopodia. (#retinas: 2 independent litters: +/+, n=6*; ash*/*ash,* n=4).
Statistics: Shown are means +95% CI. Students *t*-test. *, p≤0.05; **, p≤0.01; ***, p≤0.001; ns, not significant. Scale bars, 25μm.

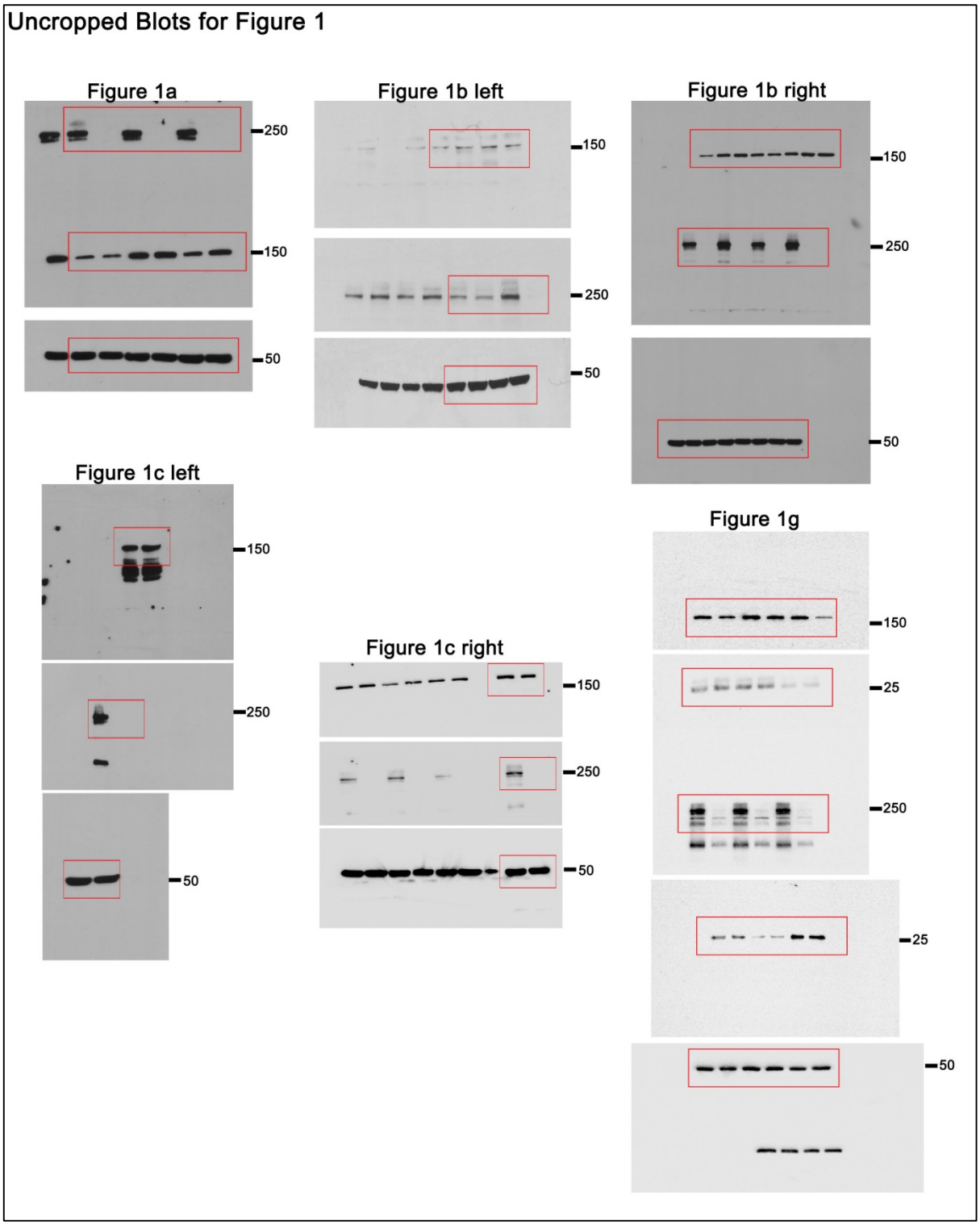
Supplementary Figure 9



Supplementary Figure 9 (linked to Figure 8). Retinal vessels from *ash/ash* mice have longer plexus filopodia and model for regulation of mVEGFR1 stability. **(a-c)** (a) Immunofluorescence of filopodia in P8.5 retinal vessels of indicated genotypes (left, low magnification plexus, scale bar, 25µm left column; middle, high magnification plexus; right, high magnification tip cells, scale bars 10µm, middle and right columns). (b-c) Quantification of filopodia length from vascular plexus (b) and vascular front (c). (#filopodia: plexus: +/+, n=396; *ash/ash*, n=827; front: +/+, n=175; *ash/ash*, n=220); 2 independent replicates.

Statistics: Shown are means +95% CI. One-way ANOVA and pairwise comparison with post-hoc Tukey's range test. **, p≤0.01; ns, not significant.

Supplementary Figure 10 (linked to all Figures). Uncropped Western blots.



Uncropped Blots for Figure 2

Figure 2c

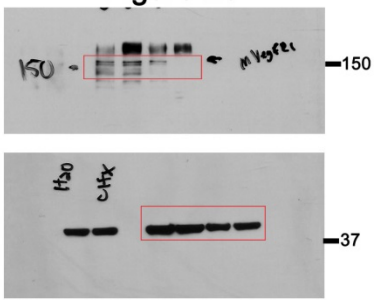


Figure 2g

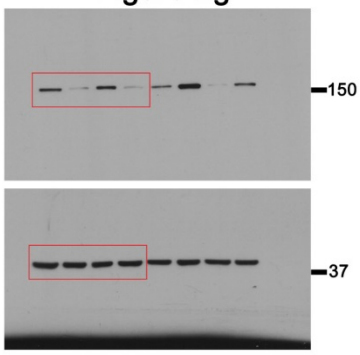
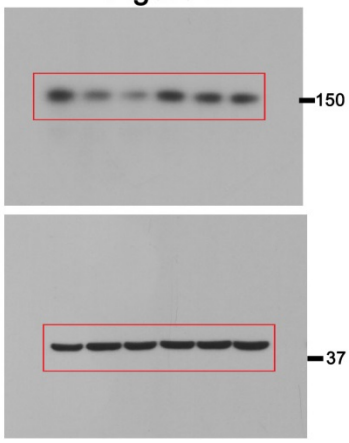
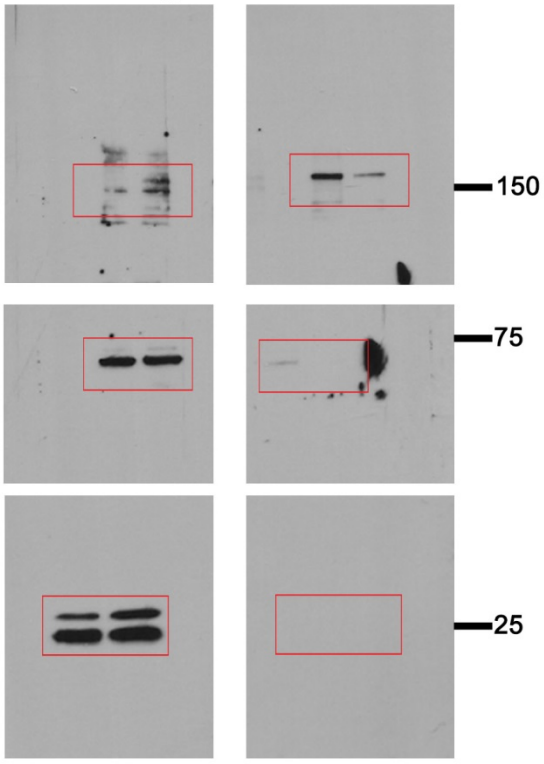


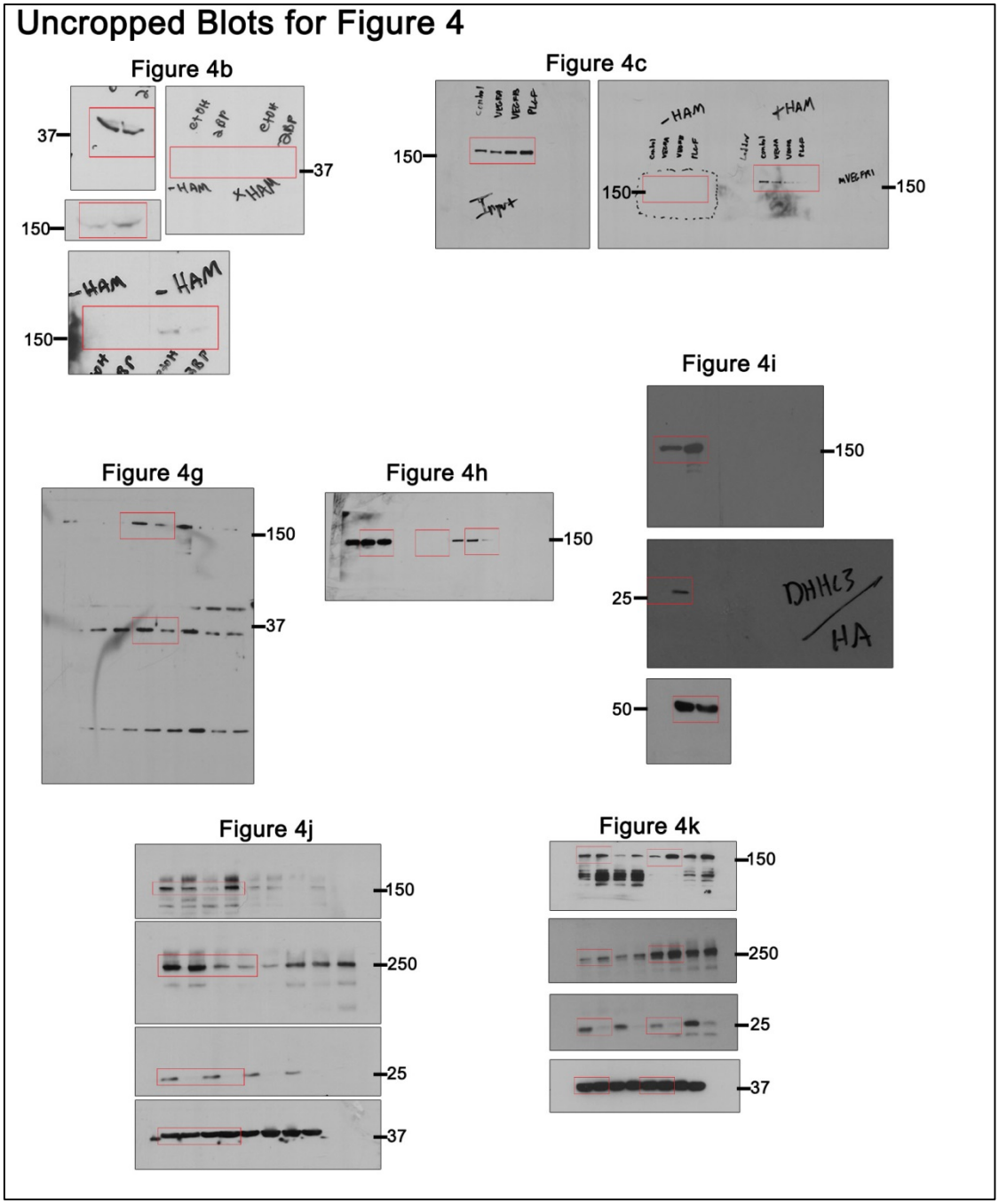
Figure 2i



Uncropped Blots for Figure 3

Figure 3e

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Uncropped Blots for Figure 5

Figure 5c

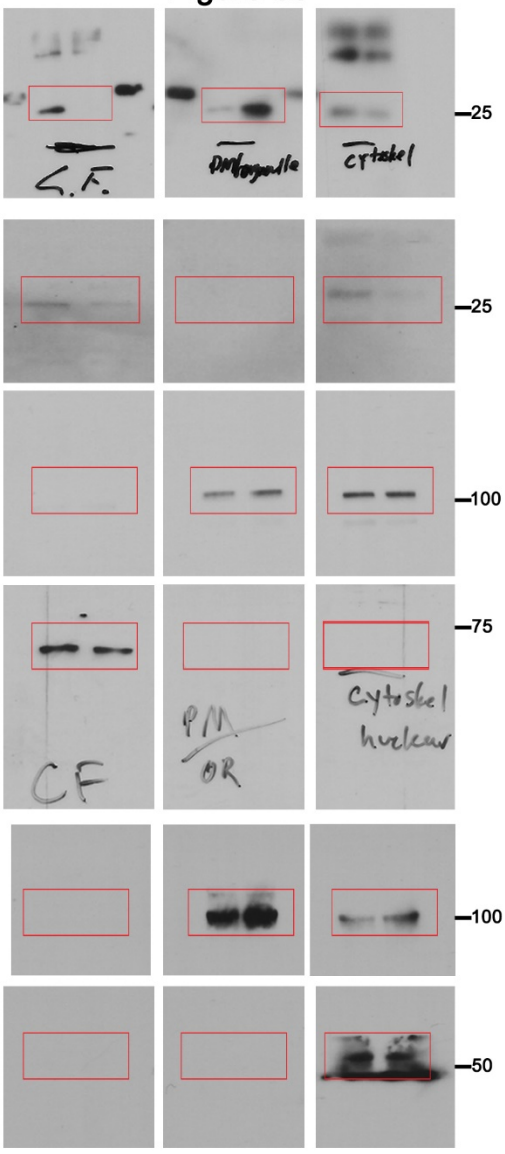


Figure 5f

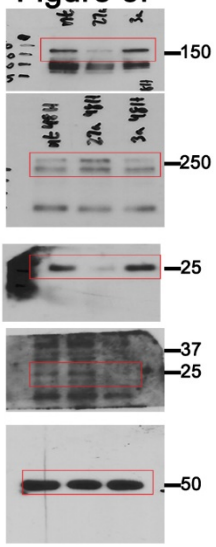


Figure 5l

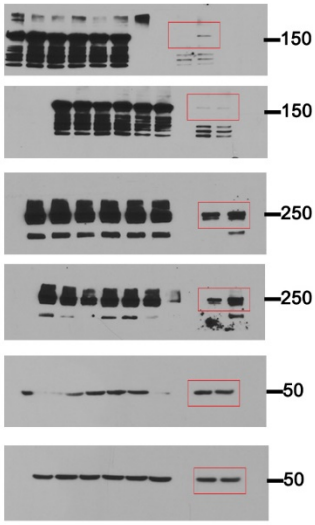
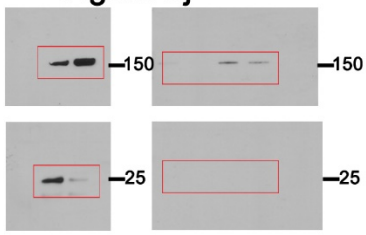
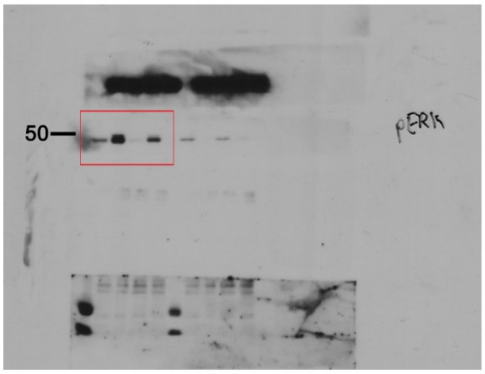


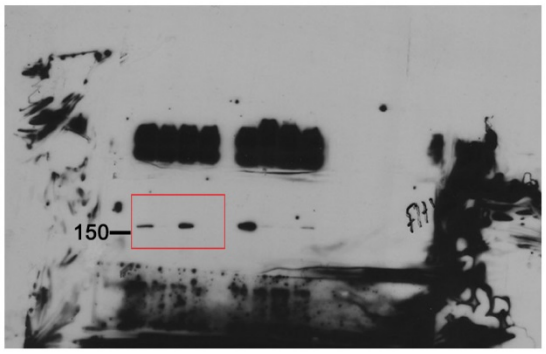
Figure 5j

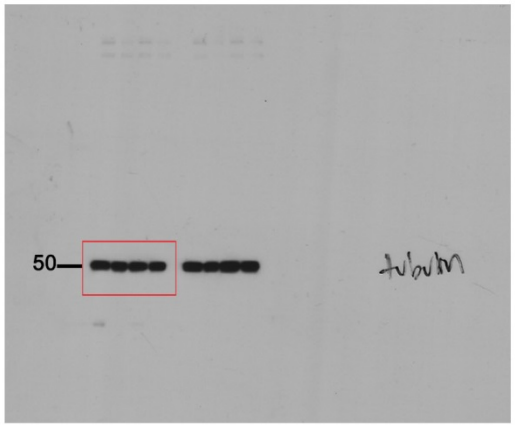


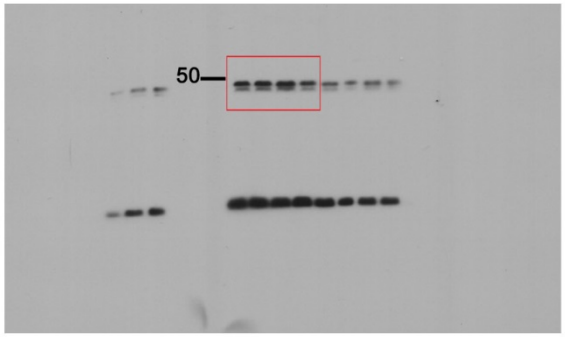
Uncropped Blots for Figure 7

Figure 7c









Uncropped Blots for Figure 8

Figure 8h

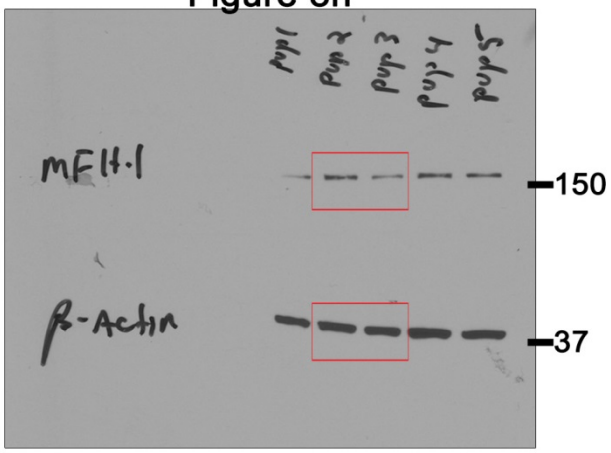


Figure 8i

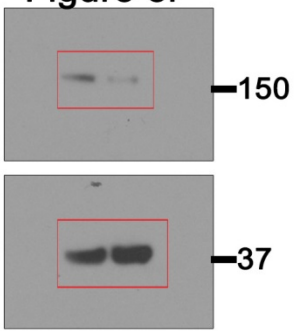
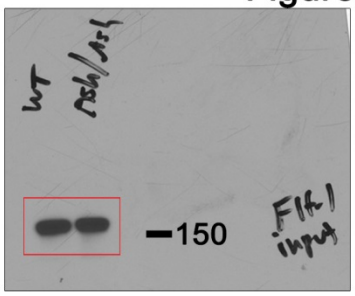
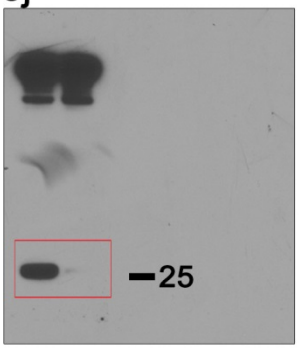


Figure 8j





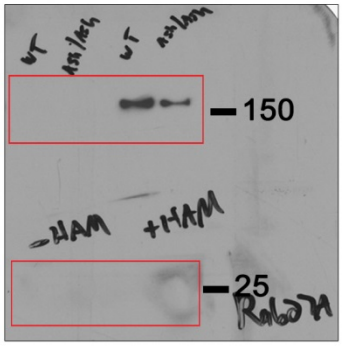
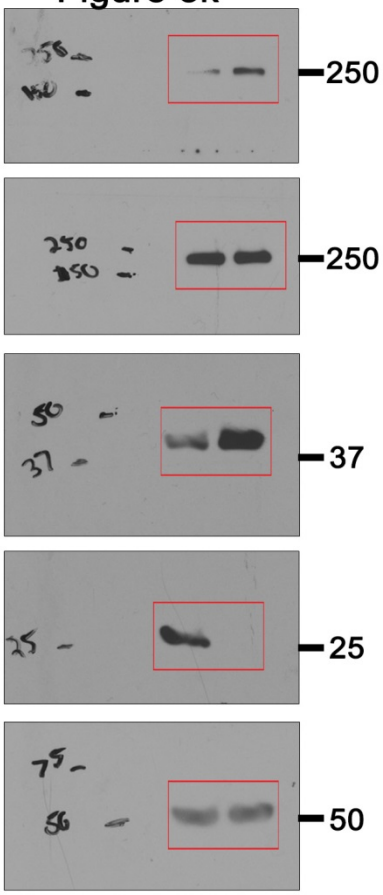
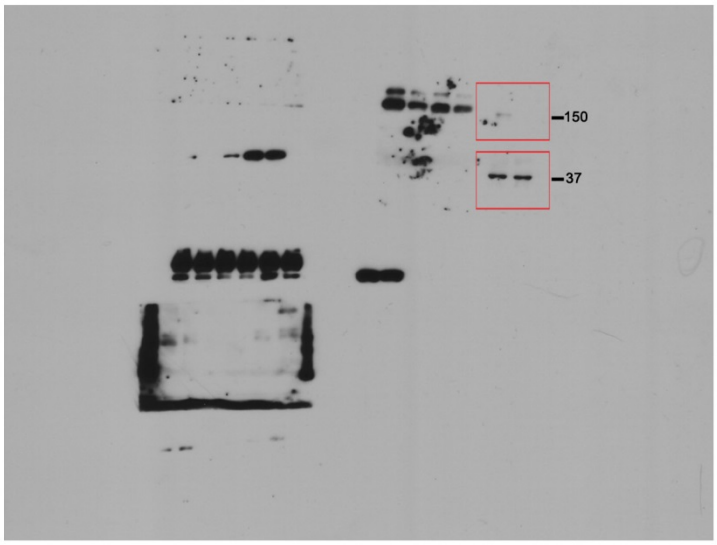


Figure 8k



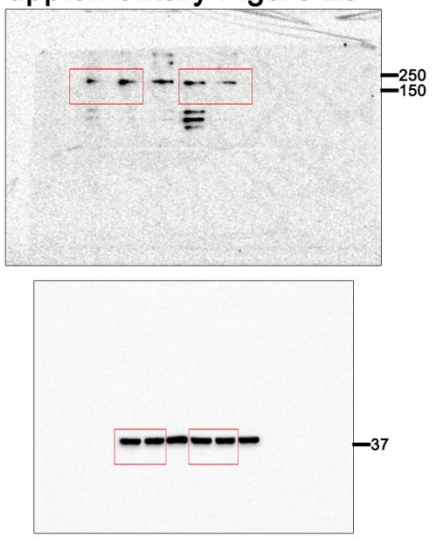
Uncropped Blots for Supplementary Figure 1

Supplementary Figure 1a

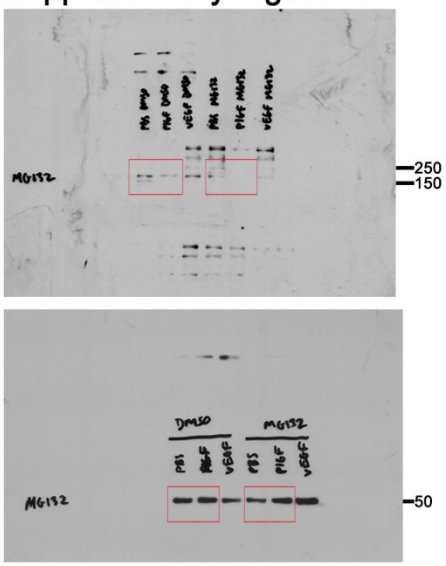


Uncropped Blots for Supplementary Figure 2

Supplementary Figure 2c

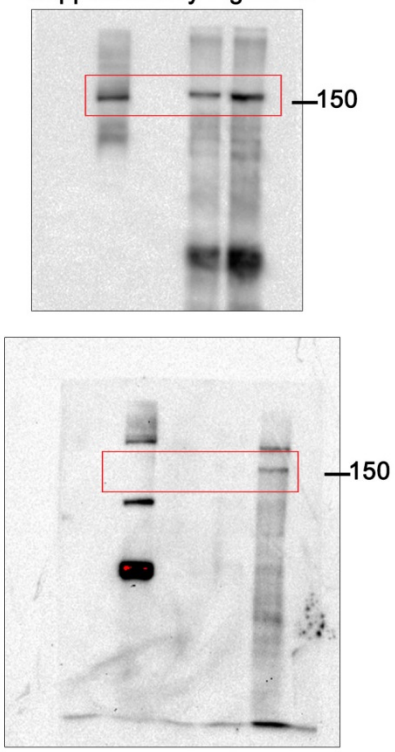


Supplementary Figure 2d

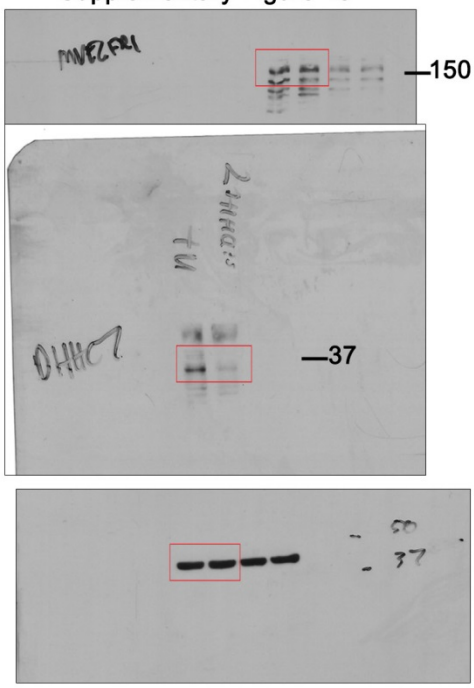


Uncropped blots for Supplementary Figure 4

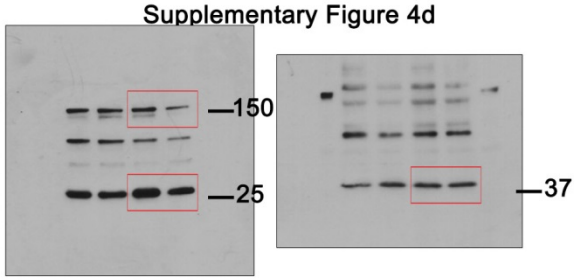
Supplementary Figure 4b



Supplementary Figure 4e

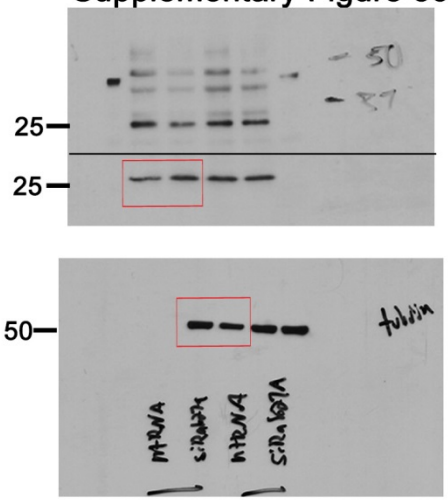


Supplementary Figure 4d

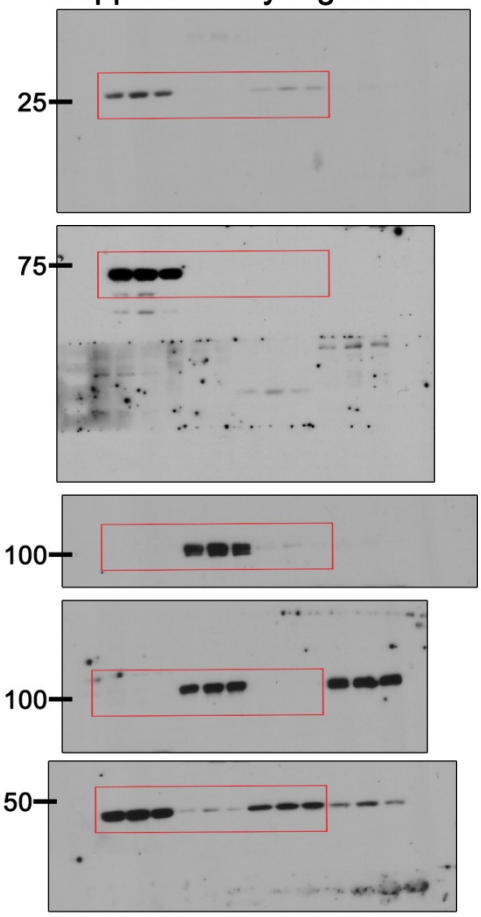


Uncropped Blots for Supplementary Figure 5

Supplementary Figure 5c

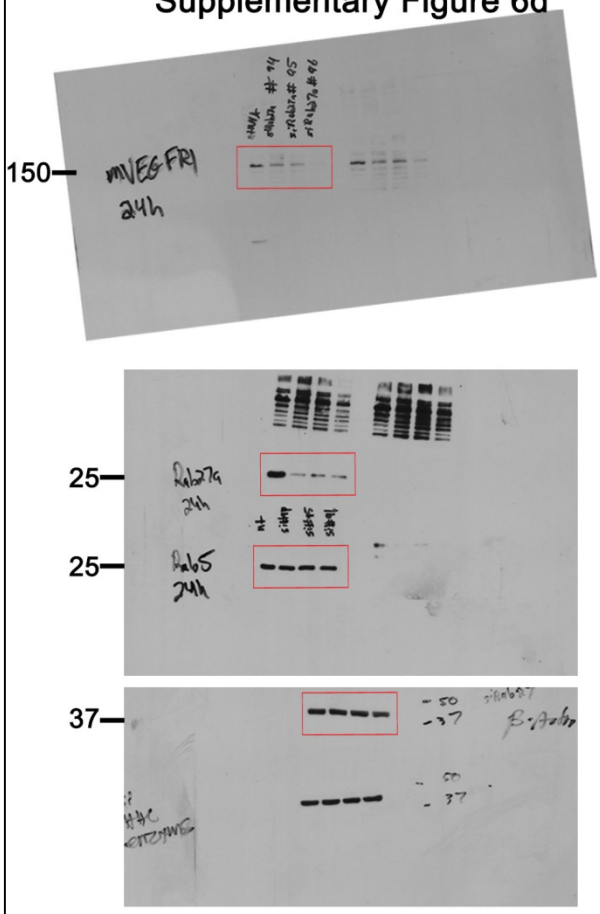


Supplementary Figure 5d

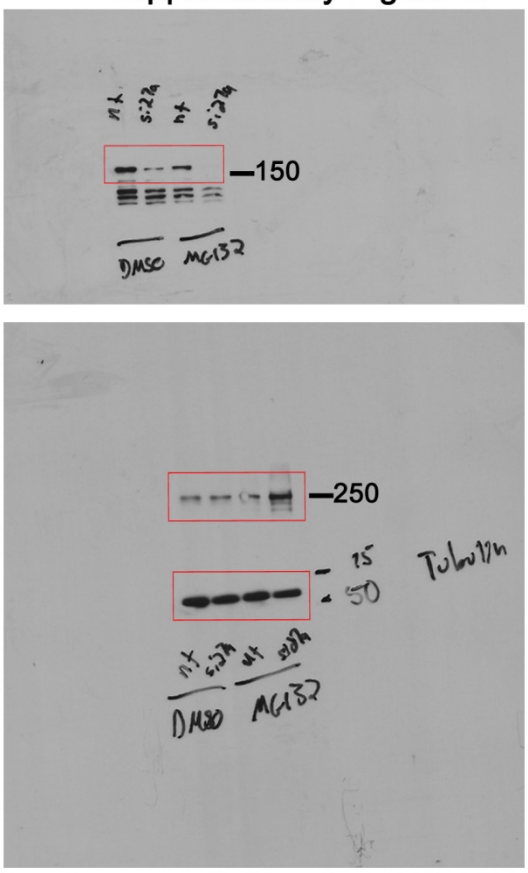


Uncropped Blots for Supplementary Figure 6

Supplementary Figure 6d



Supplementary Figure 6i



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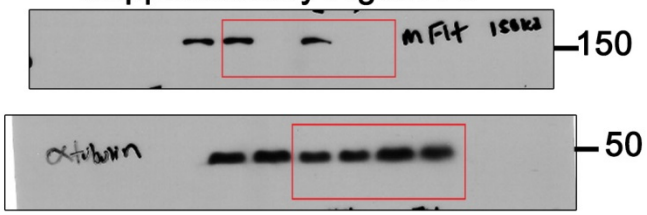
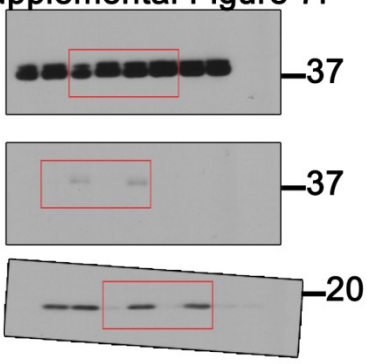
Supplementary Figure 7e

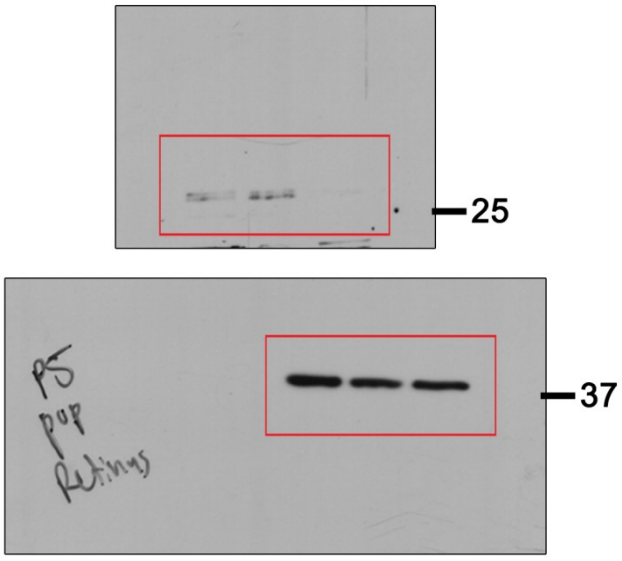


Supplemental Figure 7f



Uncropped Blots for Supplementary Figure 8

Supplementary Figure 8b



Supplementary Table 1. List of antibodies used for this study.

Antibody	Company	IB Titer	IF Titer
PRIMARY			
α-tubulin	Sigma	1:10000	-
β-actin	CST	1:10000	-
DHHC3	Abcam	1:4000	-
DHHC3	SCBT	1:2000	1:750
Erk1/2 (4695)	CST	1:5000	-
p-Erk1/2 (4370)	CST	1:1000	1:500
GM130 (ab-52649)	Abcam	1:2000	-
HA	CST	1:5000	-
P-Histone H3 (9701)	CST	1:1000	1:1000
LAMP1	Abcam	-	1:2000
LYPLA1 (APT1)	Abcam	1:2000	-
PKM1/2 (3186S)	CST	1:1000	-
P-VEGFR2 Y1175 (2478)	CST	1:500	-
PECAM	Abcam	-	1:500
Rab3a (MN1250)	Thermo Scientific	1:750	-
Rab4/14 (sc-376243)	SCBT	1:1000	-
Rab5	SCBT	1:500	-
Rab11 (sc-166912)	SCBT	1:2000	1:750
Rab27a (sc-81914)	SCBT	1:1000	1:500
Streptavidin HRP (N100)	Life Technologies	1:5000	-
TGN46	Abcam	-	1:2000
VE-Cadherin (sc-28644)	SCBT	1:1000	-
VEGFR1 (ab-32152)	Abcam	1:2000	1:500*
Alexa-fluor 488-VEGFR1 (ab-195253)	Abcam	-	1:50
VEGFR2 (2479)	CST	1:5000	1:1000
ZDHHC21 (ab-103755)	Abcam	1:750	-
SECONDARY			
Anti-mouse IgG, HRP-linked (secondary)	CST	1:5000	-
Anti-rabbit IgG, HRP-linked (secondary)	CST	1:5000	-
Goat anti-mouse 488	Life Technologies	-	1:1000
Goat anti-mouse 594	Life Technologies	-	1:1000
Goat anti-rabbit 488	Life Technologies	-	1:1000
Goat anti-rabbit 594	Life Technologies	-	1:1000

*A titer of 1:200 was used for Flt-1 staining in the retina

Abbreviations:

CST= Cell Signaling Technologies

SCBT= Santa Cruz Biotechnology

Supplementary Table 2. List of Inhibitors used for this study.

Inhibitors	Concentration	Company	Target
2-Bromohexadecanoic Acid (2-BH)	20 µM in EtOH	Sigma	Palmitoyl-acetyl transferases
Chloroquine (CHQ)	10 µg/mL in H2O	Sigma	Lysosome
Cycloheximide (CHX)	500 µg/mL in H2O	Sigma	Ribosome
Dynasore	1µM in DMSO	Santa Cruz	Dynamin
MG132	5µM in DMSO	Calbiochem	Proteasome
Palmostatin B (Pal-B)	10 µM in DMSO	Calbiochem	Acyl-protein thioesterases

Supplementary Table 3. List of small interfering RNA knockdown probes used for this study.

siRNAs	Catalogue #	Company	Pmol/10^6 cells	Target species
APT1	S20410	ThermoFisher	100	Human
DHHC3	S27899	ThermoFisher	125	Human
DHHC3 #2	S27900	ThermoFisher	125	Human
DHHC7	S31109	ThermoFisher	125	Human
Flt-1	SI00031465	Qiagen	75	Human
Non-targeting	SI03650318	Qiagen	varies	none
Rab4a	S11677	ThermoFisher	100	Human
Rab11a	S16702	ThermoFisher	100	Human
Rab27a#1*	SI02662744	Qiagen	100	Human
Rab27a#2**	S11695	ThermoFisher	-	Human/mouse
Rab27a # 3	S11494	ThermoFisher	100	Human/mouse

*Used for all human cell work in 2D and 3D.

**Used for mouse aortic ring assay (200pmol) and to validate effects of Rab27a-1 probe. Used at 100pmol/10^6 cell for Rab27a knockdown validation in Supplementary Figure 6d.