Appendix

Endosome maturation links PI3Kα signaling to lysosome repopulation during basal autophagy

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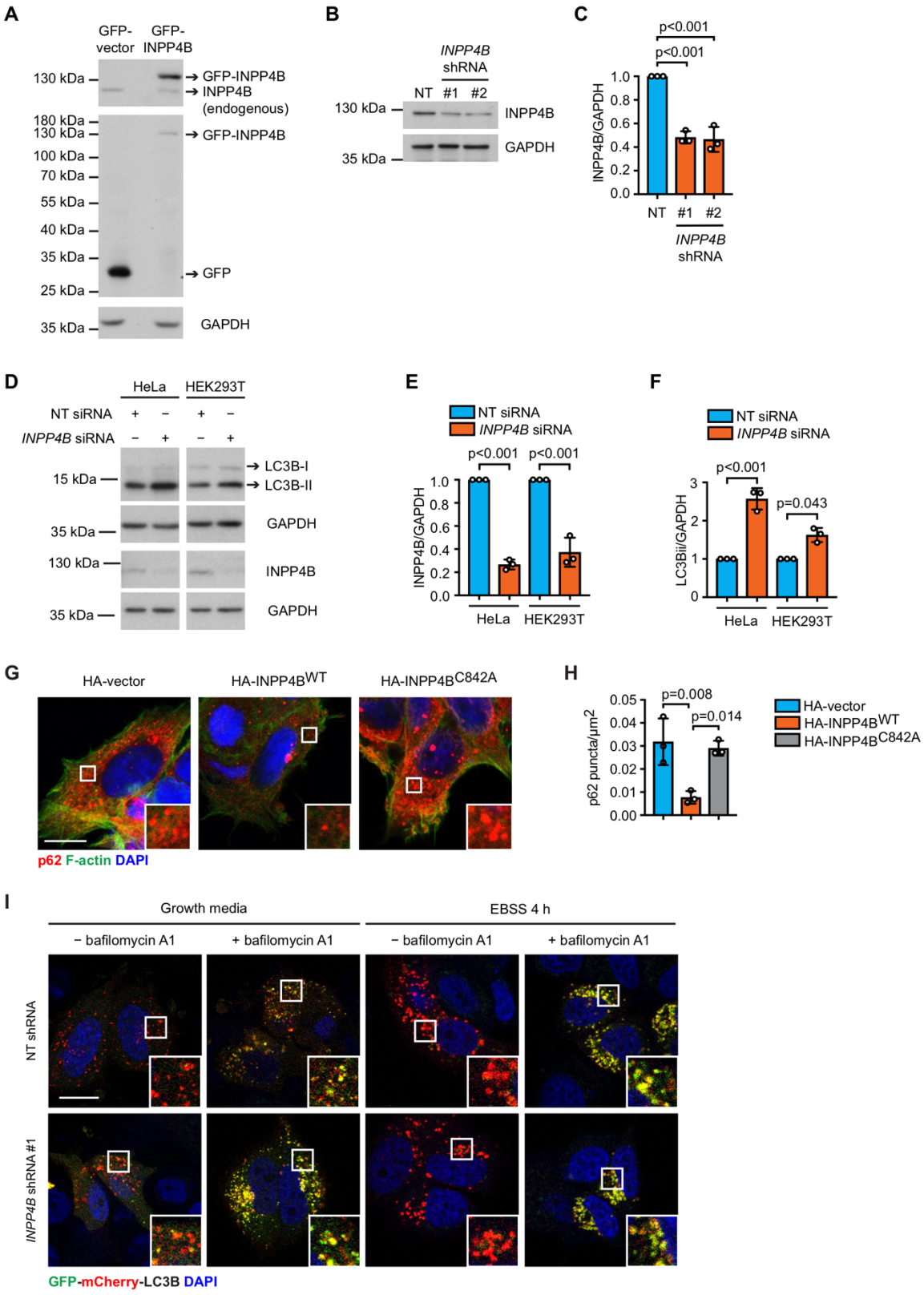
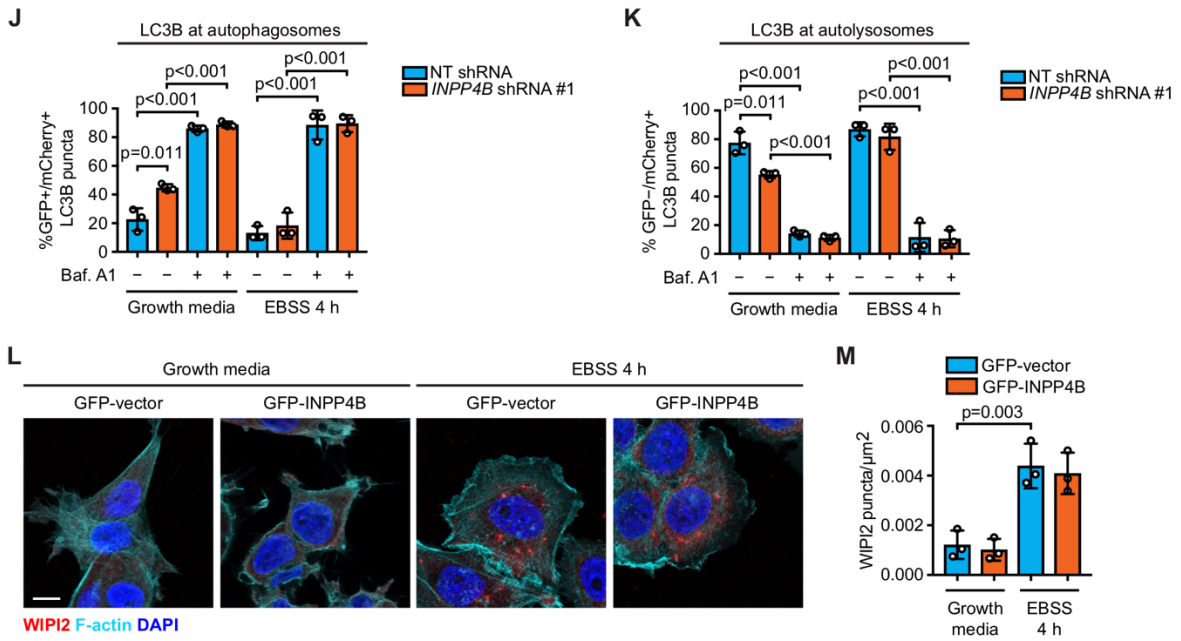

A
GFP-vector
GFP-INPP4B
130 kDa
GFP-INPP4B
INPP4B (endogenous)
180 kDa
130 kDa
GFP-INPP4B
100 kDa
70 kDa
55 kDa
40 kDa
35 kDa
GFP
25 kDa
35 kDa
GAPDH
B
INPP4B shRNA
NT #1 #2
130 kDa
INPP4B
35 kDa
GAPDH
C
p<0.001
p<0.001
INPP4B/GAPDH
1.0 0.8 0.6 0.4 0.2 0.0
NT #1 #2
INPP4B shRNA
D
HeLa HEK293T
NT siRNA + − + −
INPP4B siRNA − + − +
15 kDa
LC3B-I
LC3B-II
35 kDa
GAPDH
130 kDa
INPP4B
35 kDa
GAPDH
E
NT siRNA
INPP4B siRNA
p<0.001 p<0.001
INPP4B/GAPDH
1.0 0.8 0.6 0.4 0.2 0.0
HeLa HEK293T
F
NT siRNA
INPP4B siRNA
p<0.001
p=0.043
LC3Bii/GAPDH
3 2 1 0
HeLa HEK293T
G
HA-vector
HA-INPP4BWT
HA-INPP4BC842A
p62 F-actin DAPI
H
p=0.008
p=0.014
p62 puncta/μm2
0.05 0.04 0.03 0.02 0.01 0.00
HA-vector
HA-INPP4BWT
HA-INPP4BC842A
I
Growth media
EBSS 4 h
− bafilomycin A1
+ bafilomycin A1
− bafilomycin A1
+ bafilomycin A1
NT shRNA
INPP4B shRNA #1
GFP-mCherry-LC3B DAPI



Appendix Figure S1: INPP4B promotes basal autophagic flux

A MCF-7 cells were transduced with lentiviral particles encoding GFP-INPP4B or GFP-vector. Cells were lysed and immunoblotted with INPP4B and GFP antibodies, and GAPDH antibodies as a loading control.

B, C MCF-7 cells were transduced with lentiviral particles encoding NT, *INPP4B* #1 or *INPP4B* #2 shRNA. Cells were lysed and immunoblotted with INPP4B antibodies, and GAPDH antibodies as a loading control (**B**). Data represent the relative INPP4B levels normalized to GAPDH, and expressed relative to NT shRNA cells which were assigned an arbitrary value of 1 (n=3 experiments) (**C**).

D-F HeLa or HEK293T cells were transfected with NT or *INPP4B* siRNA. After 24 hours, cells were lysed and immunoblotted with LC3B and INPP4B antibodies, and GAPDH antibodies as a loading control (**D**). Data represent the relative INPP4B (**E**) or LC3B-II (**F**) levels normalized to GAPDH, and expressed relative to NT siRNA cells which were assigned an arbitrary value of 1 (n=3 experiments).

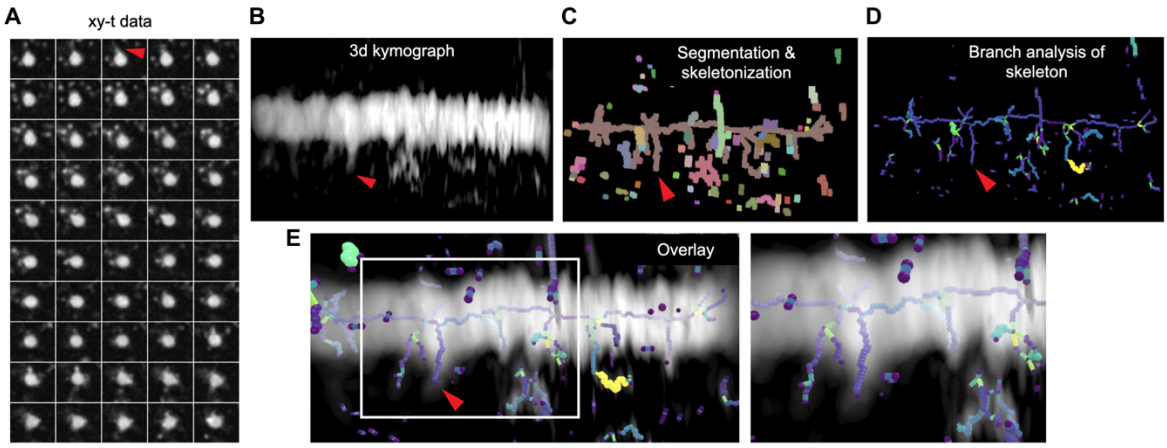
G, H MCF-7 cells were transfected with HA-vector, HA-INPP4B^{WT} or HA-INPP4B^{C842A}. After 24 hours, cells were fixed and immunostained with p62 antibodies, and co-stained with DAPI and phalloidin (**G**). Data represent the number of p62+ puncta relative to cell area (μm^2) (n=3 experiments, >20 cells/experiment) (**H**).

I-K MCF-7 cells co-expressing GFP-mCherry-LC3B and NT or *INPP4B* #1 shRNA were cultured in growth media or EBSS in the presence of 100 nM bafilomycin A1 or DMSO as a vehicle control for 4 hours, then fixed and stained with DAPI (**I**). Data represent the proportion of LC3B at autophagosomes (GFP+/mCherry+) (**J**) or autolysosomes (GFP−/mCherry+) (**K**) (n=3 experiments, >30 cells/experiment).

L, M MCF-7 cells expressing GFP-INPP4B or GFP-vector were cultured in growth media or EBSS for 4 hours, then fixed and immunostained with WIPI2 antibodies, and co-stained

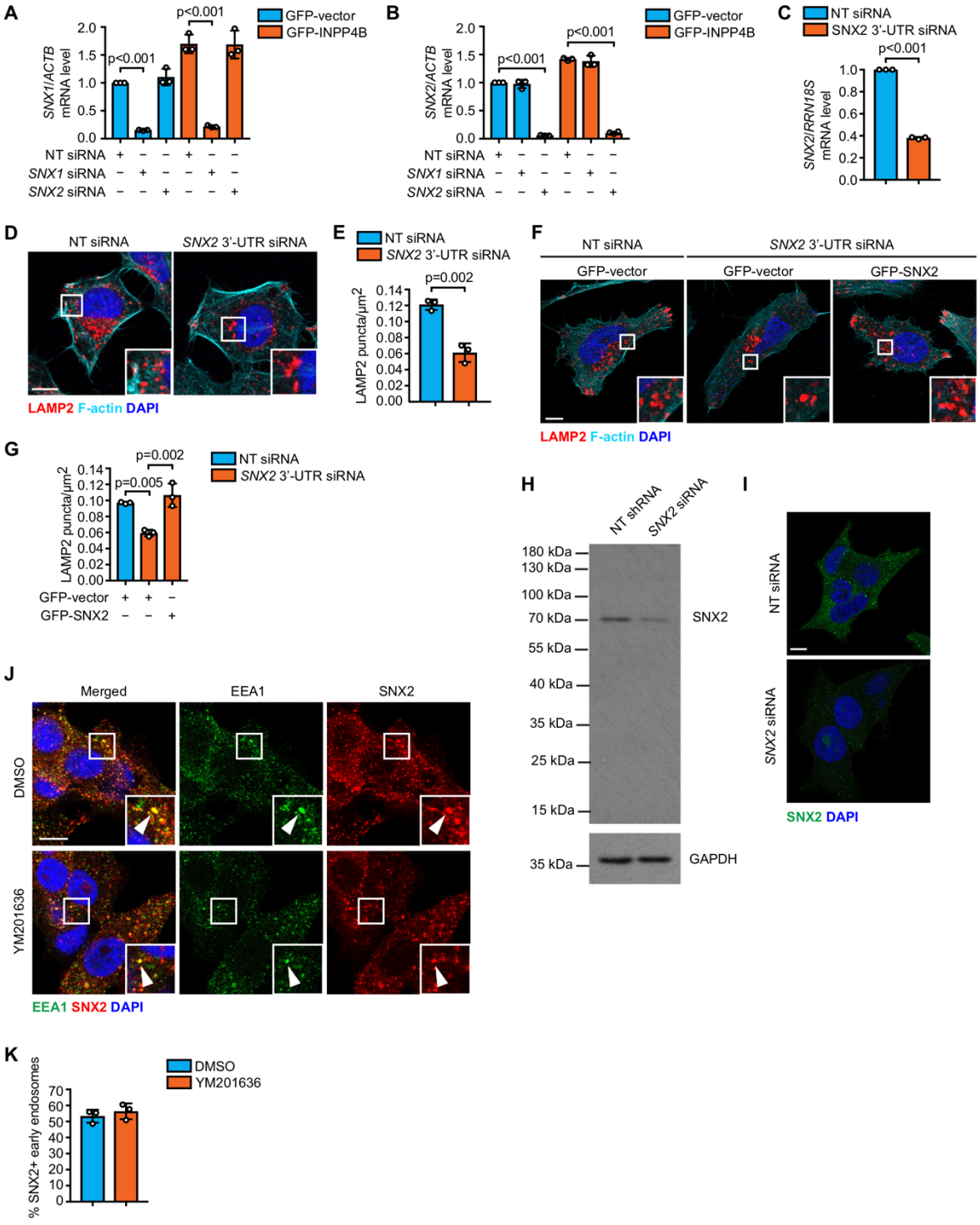
with DAPI and phalloidin (**L**). Data represent the number of WIPI2+ puncta relative to cell area (μm^2) (n=3 experiments, >50 cells/experiment) (**M**).

Data information: Data is presented as mean ± SD. The insets at the lower right of each image are higher power regions of the boxed areas. Scale bar is 10 μm in **G**, **I**, **L**. p values determined by one-way ANOVA with Tukey post hoc test in **C**, **H**, **J**, **K**, **M**, or by two-tailed unpaired t test in **E**, **F**.



Appendix Figure S2: Rapid imaging and analysis workflow used to quantify lysosome reformation

A-E Spinning disk movies of LAMP1-mCherry signals were projected as maximum intensity from 3 z-planes (**A**). 3-dimensional kymographs were constructed from *x*, *y* and time dimensions (**B**), then subjected to segmentation and skeletonization (**C**). Branch points from the parent lysosome indicate lysosome reformation (**D**). Overlay between 3-dimensional kymographs and skeletons (**E**). Lysosome reformation event is indicated with red arrows. The inset at the bottom right is a higher power region of the boxed area.



Appendix Figure S3: Validation of SNX2 siRNA depletion

A, B MCF-7 cells expressing GFP-INPP4B or GFP-vector were transfected with NT, *SNX1* or *SNX2* siRNA. After 24 hours, RNA was extracted and two-step quantitative RT-PCR was performed using primers for *SNX1* (**A**) or *SNX2* (**B**), and expression was normalized to *ACTB*. Expression was determined using the ΔΔCt method and expressed relative to GFP-vector;NT siRNA cells which were assigned an arbitrary value of 1 (n=3 experiments).

C HeLa cells were transfected with NT or *SNX2* siRNA. After 24 hours, RNA was extracted and two-step quantitative RT-PCR was performed using primers for *SNX2*, and expression was normalized to *RRN18S*. Expression was determined using the ΔΔCt method and expressed relative to NT siRNA cells which were assigned an arbitrary value of 1 (n=3 experiments).

D, E HeLa cells were co-transfected with NT or *SNX2* 3'-UTR siRNA. Cells were fixed and immunostained with LAMP2 antibodies, and co-stained with DAPI and phalloidin (**D**). Data represent the number of LAMP2+ puncta relative to cell area (μm^2) (n=3 experiments, >50 cells per experiment) (**E**).

F, G HeLa cells were co-transfected with NT or *SNX2* 3'-UTR siRNA, and GFP-vector or GFP-SNX2. Cells were fixed and immunostained with LAMP2 antibodies, and co-stained with DAPI and phalloidin (**F**). Data represent the number of LAMP2+ puncta relative to cell area (μm^2) (n=3 experiments, >20 cells per experiment) (**G**).

H MCF-7 cells were transfected with NT or *SNX2* siRNA, then lysed and immunoblotted with SNX2 antibodies, and GAPDH antibodies as a loading control.

I MCF-7 cells were transfected with NT or *SNX2* #1 siRNA, then fixed and immunostained with SNX2 antibodies, and co-stained with DAPI.

J, K MCF-7 cells were treated with 5 μM YM201636 or DMSO as a vehicle control for 4 hours. Cells were fixed and immunostained with SNX2 and EEA1 antibodies, and co-stained with DAPI (**J**). Data represent the percentage of SNX2+ early endosomes (n=3 experiments, >50 cells/experiment) (**K**).

Data information: Data is presented as mean ± SD. The insets at the bottom of each image are higher power regions of the boxed areas. Scale bar is 10 μm in **D**, **F**, **I**, **J**. p values determined by one-way ANOVA with Tukey post hoc test in **A**, **B**, **G**, or by two-tailed unpaired t test in **C**, **E**.

Appendix Table S1: Antibodies and dyes

AKT(pan) (IB – 1:1000)	Cell Signaling Technologies	Cat # 4691
CD63 (IF – 1:200)	DSHB	Cat # H5C6
GAPDH (IB – 1:500,000)	ThermoFisher Scientific	Cat # AM4300
GFP (IB – 1:50,000)	Roche	Cat # 11814460001
GFP (IF – 1:500)	Invitrogen	Cat # A10262
GFP (IEM – 1:500)	Abcam	Cat # ab6556
GST (IF - 1:500)	Invitrogen	Cat # 71-7500
HA (IB – 1:1000, IF – 1:1600)	Cell Signaling Technologies	Cat # 3724
Hrs (IB – 1:1000)	Cell Signaling Technologies	Cat # 15087
INPP4B 3D5 (IB – 1:1000, IF – 1:50)	(Fedele et al., 2010)	N/A
LAMP1 (IF – 1:100)	DSHB	Cat # G1/139/5
LAMP2 (IF – 1:100)	DSHB	Cat # H4B4
LC3B (IB – 1:1000)	Cell Signaling Technologies	Cat # 2775
Phospho-AKT^{S473} (IB - 1:1000)	Cell Signaling Technologies	Cat # 4058
Phospho-mTOR^{S2448} (IF – 1:100)	Cell Signaling Technologies	Cat # 2971
Phospho-S6K^{T389} (IB – 1:2000)	Cell Signaling Technologies	Cat # 9234
S6K (IB – 1:2000)	Cell Signaling Technologies	Cat # 9202
SNX2 (IB – 1:2000, IF – 1:100)	Invitrogen	Cat # PA5-83367
SQSTM1/p62 (IF – 1:500)	Abcam	Cat # ab56416
Ubiquitin (IF – 1:500)	Enzo Life Sciences	Cat # BML-PW8810-0500
WIPI2 (IF – 1:500)	Abcam	Cat # ab105459
Anti-mouse HRP-conjugated (IB – 1:10,000)	Millipore	Cat # AP308P
Anti-rabbit HRP-conjugated (IB – 1:10,000)	Millipore	Cat # AP307P
Donkey anti-mouse IgG (H+L) Alexa-Fluor 488-conjugated (IF – 1:500)	Life Technologies	Cat # A-21202
Donkey anti-mouse IgG (H+L) Alexa-Fluor 555-conjugated (IF – 1:500)	Life Technologies	Cat # A-31570

Goat anti-mouse IgG1 Alexa-Fluor 555-conjugated (IF – 1:500)	Life Technologies	Cat # A-21127
Goat anti-mouse IgG1 Alexa-Fluor 647-conjugated (IF – 1:500)	Life Technologies	Cat # A-21240
Goat anti-mouse IgG2a Alexa-Fluor 488-conjugated (IF – 1:500)	Life Technologies	Cat # A-21131
Donkey anti-rabbit IgG (H+L) Alexa-Fluor 488-conjugated (IF – 1:500)	Life Technologies	Cat # A-21206
Donkey anti-rabbit IgG (H+L) Alexa-Fluor 555-conjugated (IF – 1:500)	Life Technologies	Cat # A-31572
DAPI (IF – 1:1000)	Sigma	Cat # D9542
Phalloidin Alexa-Fluor 647-conjugated (IF – 1:500)	Life Technologies	Cat # A22287

Appendix Table S2: Oligonucleotides

Human *ATCB* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00095431
Human *ATP6V1C1* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00015022
Human *ATP6V0D1* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00018116
Human *CTNS* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00046914
Human *LAMP1* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00070994
Human *M6PR* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00026894
Human *PIKFYVE* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00035231
Human *RRN18S* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00199367
Human *SNX1* (qRT-PCR)	Sigma	KiCqStart primer pair #1
Human *SNX2* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT02307648
Human *TPP1* (qRT-PCR)	Qiagen	QuantiTect GeneGlobe ID # QT00097363