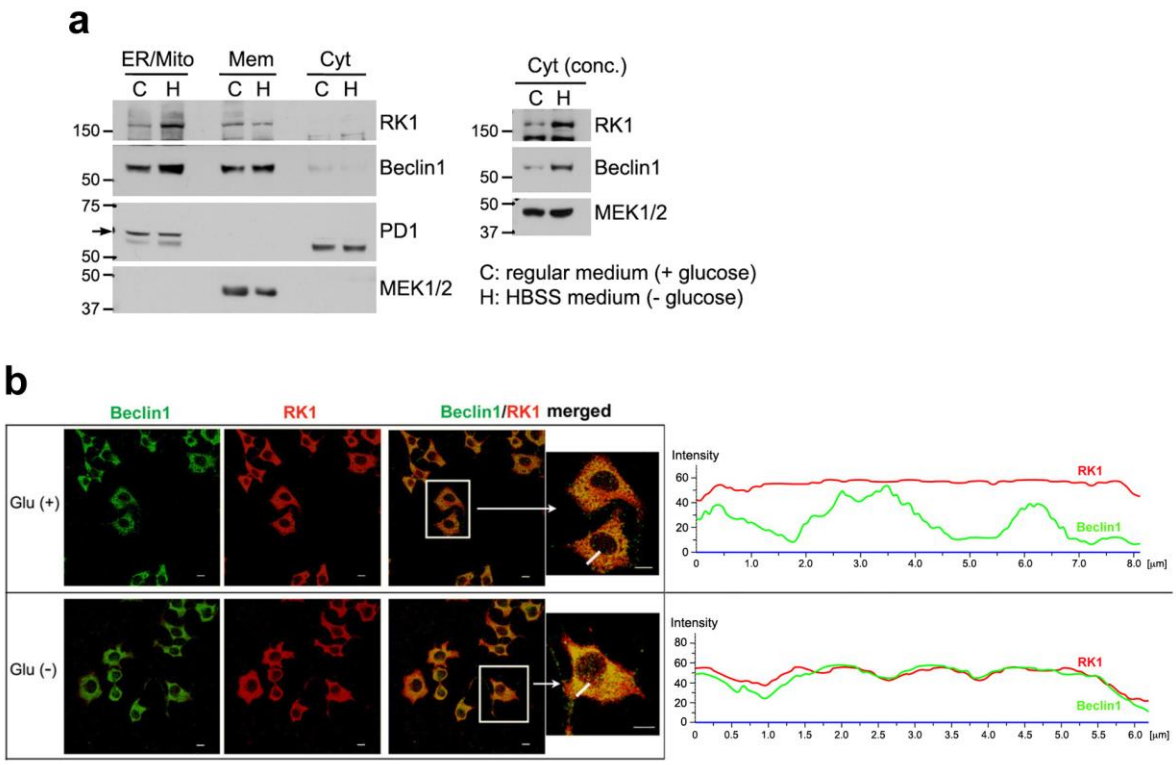
Supplementary Information

Identification of ROCK1 kinase as a critical regulator of Beclin1 mediated autophagy during metabolic stress

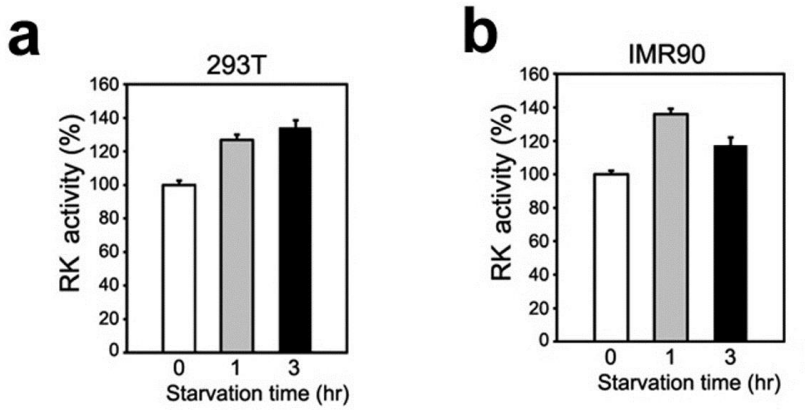
Aditi U. Gurkar[1], Kiki Chu[1], Lakshmi Raj[1], Richard Bouley[2], Seung-Hwan Lee[3], Young-Bum Kim[3], Sandra E. Dunn[4], Anna Mandinova[1,5] & Sam W. Lee[1,5*]

[1]Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Building 149 13th Street, Charlestown, Massachusetts 02129, USA. [2]Division of Nephrology, Simches Research Center, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114, USA. [3]Division of Endocrinology, Diabetes, and Metabolism, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, USA. [4]Department of Pediatrics, University of British Columbia, Vancouver, British Columbia, Canada. [5]Broad Institute of Harvard and MIT, 7 Cambridge Center, Massachusetts 02142, USA.

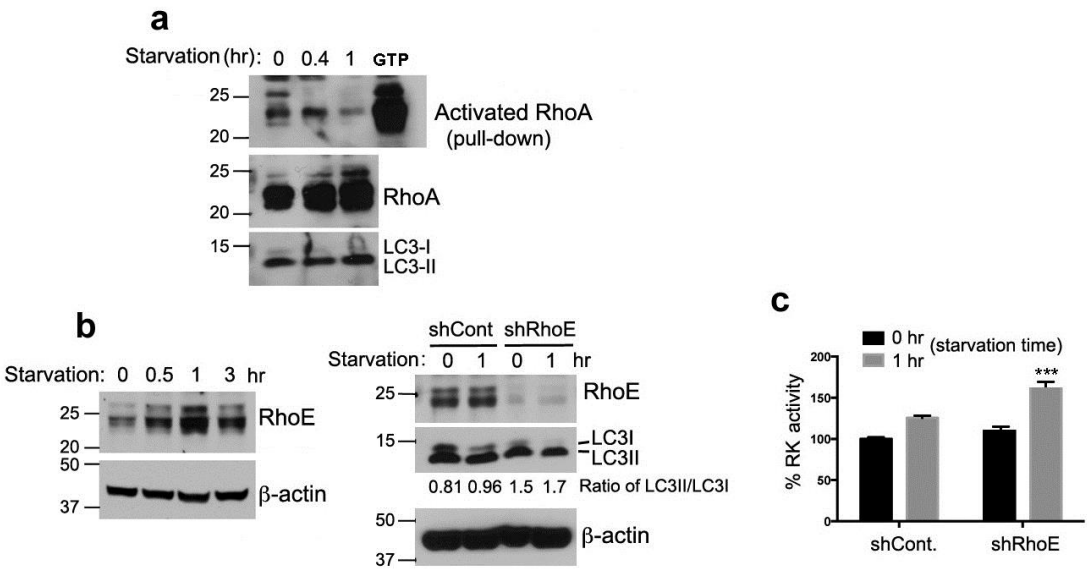


Supplementary Figure S1. Co-localization of ROCK1 with Beclin1 upon metabolic stress.

(**a**) Sub-cellular localization of ROCK1 and Beclin1 was determined by subjecting HeLa cells to differential centrifugation. Post-nuclear extracts from control or starved cells were further centrifuged to obtain ER/Mitochondrial and cytosolic fractions. Cytosolic fractions were further concentrated using Millipore concentrators. Resulting extracts were detected on two separate gels (before and after concentration) by western blotting against indicated antibodies. (**b**) HeLa cells cultured in control or starvation media for 4 h were fixed in methanol and subjected to immunofluorescence with anti-Beclin1 (green) and anti-ROCK1 (red) antibody. Representative images are shown (left panel); Scale = 10 μm. Plots of fluorescence intensity profiles for the indicated regions (white line) is shown on the right.

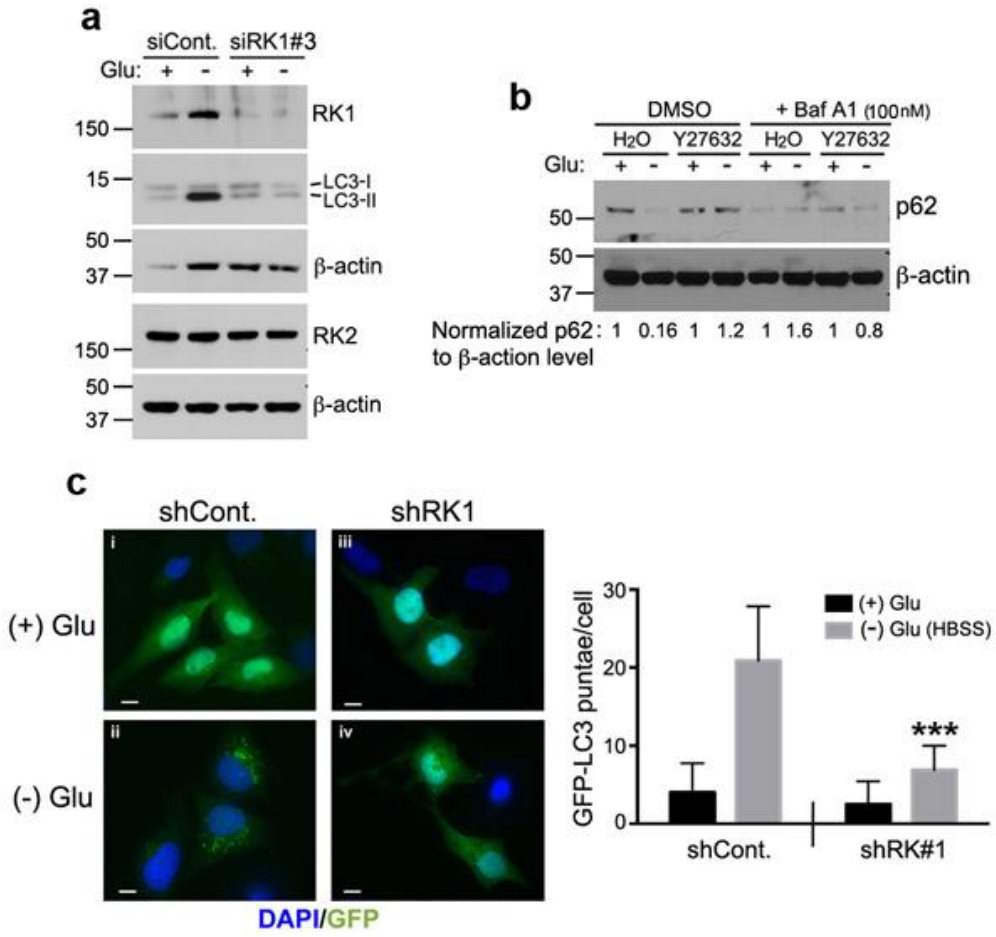


Supplementary Figure S2. ROCK1 activity is increased upon starvation.
(**a**) 293T and (**b**) IMR90 cells were grown in control (+Glu) or starved with HBSS for 1 and 3 h were lysed, extracts normalized and ROCK kinase activity was measured. Graphs represent ROCK activity in starved cells plotted as percent of ROCK activity in control (glucose-rich) cells. Mean ± s.d. is calculated from triplicate samples read at the same time (n=3).



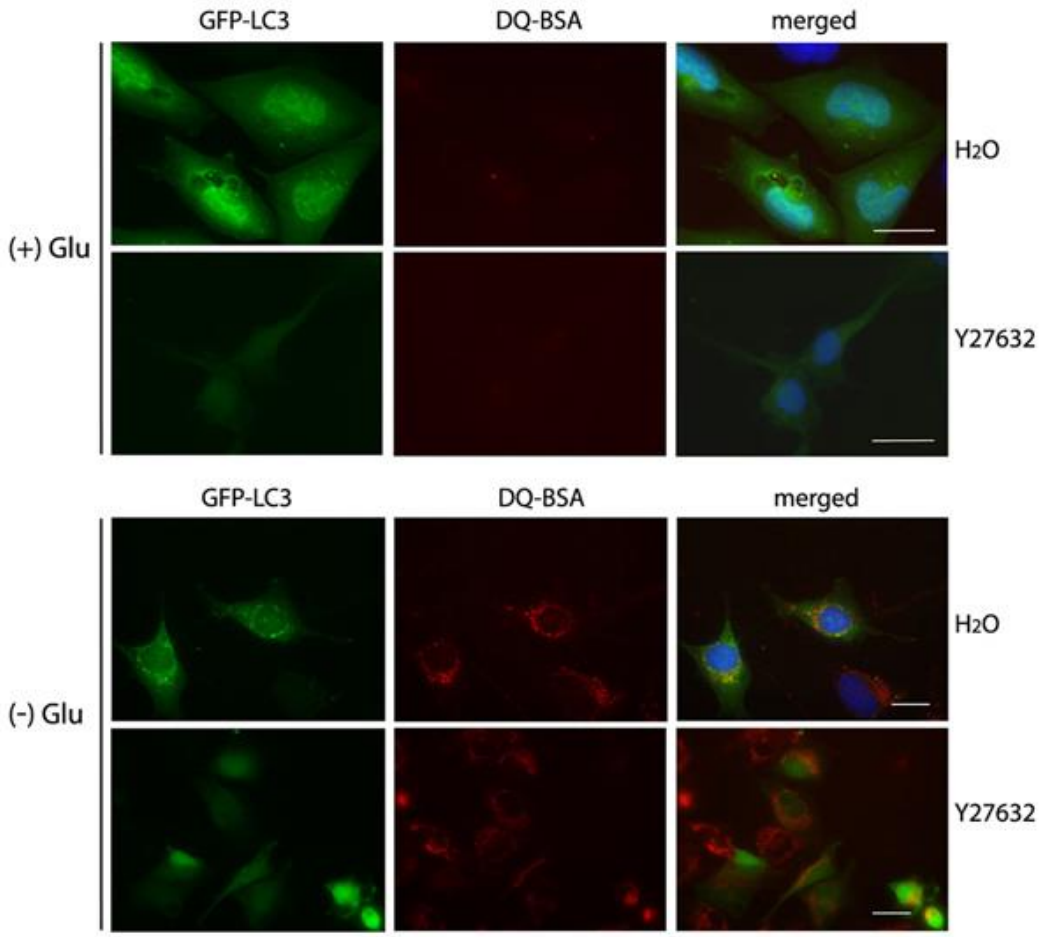
Supplementary Figure S3. ROCK1 activation upon metabolic stress is RhoA independent.

(**a**) Active RhoA assay: HeLa cells starved in HBSS for indicated time-points were lysed and active RhoA assay was performed using Rhotekin beads. Lysates from 0h were GTP loaded and used as a positive control. Inputs and IP were resolved by SDS-PAGE and blotted against indicated antibodies. (**b**) HeLa cells incubated in starvation media for indicated times were harvest and examined by western blotting (left panel). HeLa cells transfected with shCont. and shRhoE were incubated in DMEM with glucose or HBSS for 1h (right panel). Whole cell lysates were prepared and analyzed for western blotting against RhoE and β-actin. (**c**) Cell lysates from the same experiment were further used for an ELISA assay to determine ROCK activity. Graph represents % ROCK activity normalized to ROCK activity for shCont. at 0 h. Error bars represent +s.d. from triplicates read at the same time. Student t-test *** $p<0.001$



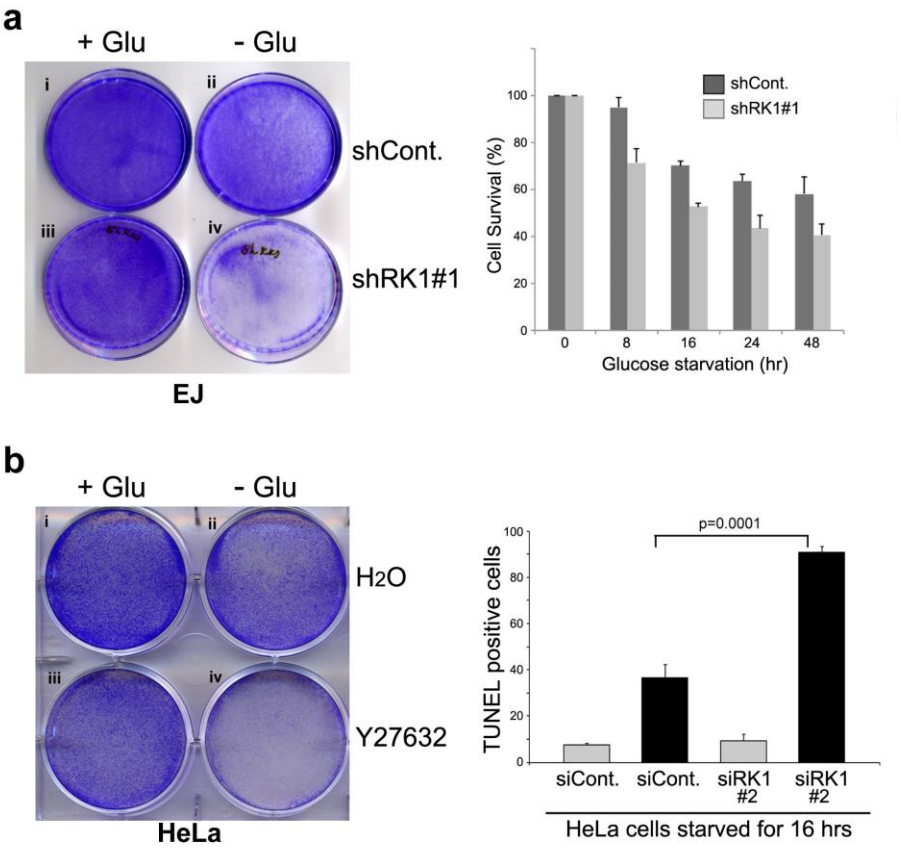
Supplementary Figure S4. The effect of ROCK1 knock-down on autophagy markers.

(**a**) HeLa cells with a transient knockdown using siCont. and siROCK1#3 for 30 h were further incubated in regular or HBSS media for 8 h. Whole cell extracts were prepared and resolved by SDS-PAGE and analyzed for the expression of RK1, LC3 and β-actin. Extracts were re-resolved to examine the expression of RK2 and β-actin. (**b**) Whole cell lysate extracts from Fig. 3c of the main text were examined for p62 and β-actin. (**c**) EJ cells (left) with a stable knockdown using shCont. or shRK1#1 were incubated in control (glucose rich) or glucose-free medium for 15 h and then treated with 0.1μM bafilomycin-A1 for an additional 60 min. Cells were fixed with 2% PFA and subjected to immunofluorescence. Representative images are shown (left) and quantification of LC3 dots plotted (right). Graph represents % of LC3 punctae/cell (mean ±SD), n=25 cells; Student t-test *** p<0.001



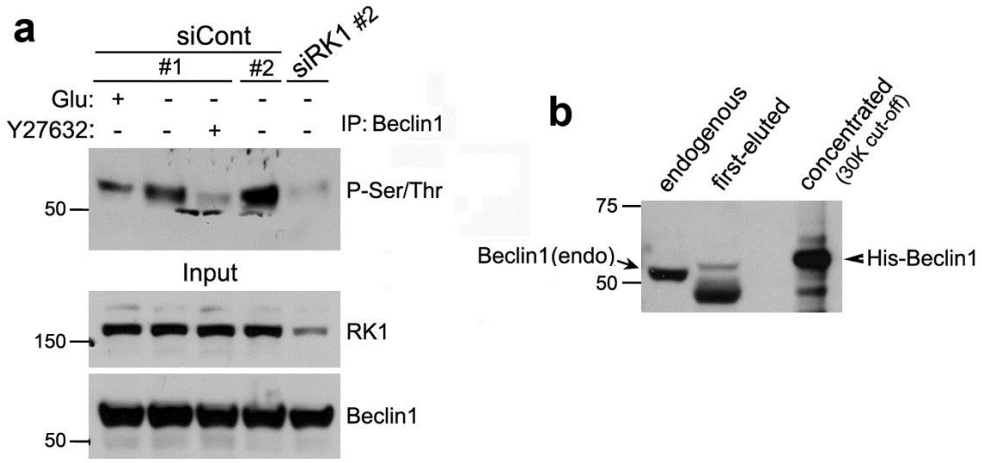
Supplementary Figure S5. Inhibition of ROCK1 activity causes defective autophagy flux.

EJ-GFP-LC3 stable cells untreated or treated with ROCK1 inhibitor Y27632 were incubated in DMEM containing DQ-BSA (10 μg/ml) for 45 min. Cells were then washed twice and incubated in DMEM for an additional 30 min, washed with HBSS and starved for 2h. Resulting cells were fixed, and co-localization of GFP-LC3 and red fluorescent of DQ-BSA was imaged. Arrows indicate co-localized GFP-LC3 and DQ-BSA red: Scale bar = 20μm.

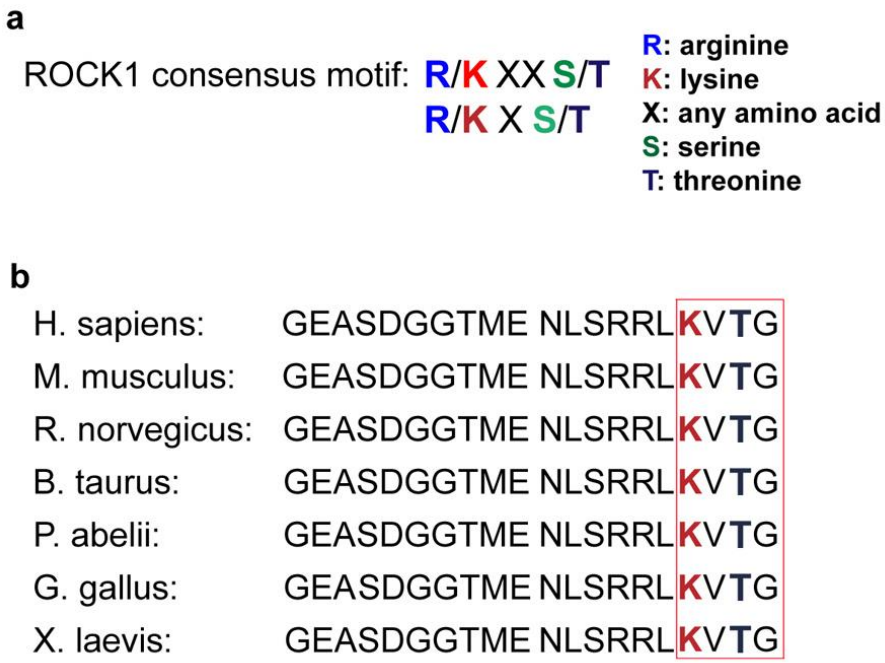


Supplementary Figure S6. Loss of ROCK1 activity during metabolic stress increases cell death.

(**a**) EJ-shCont. and EJ-shROCK1#1 cells were starved for 48 h and cell viability was assessed using crystal violet staining (left). The same EJ cells, as above were starved for indicated time points and cytotoxicity was evaluated by Alamar blue assay (right). A representative graph for cell viability is shown (with mean ± s.d), n=2, (read on same day). Experiment repeated three times. (**b**) HeLa cells treated with Y27632 cultured in nutrient rich or free medium for 24 h were stained with crystal violet to measure cell viability (left). HeLa cells transfected with RNAi oligonucleotides for ROCK1 (siRK1#2) or control (siCont.) for 36h were cultured in glucose rich or starvation medium for 16 h. Apoptosis was measured using TUNEL assay (right). Error bars indicate mean ± s.d. of triplicates, measured at the same time. Student's t-test was used for the statistical analysis.

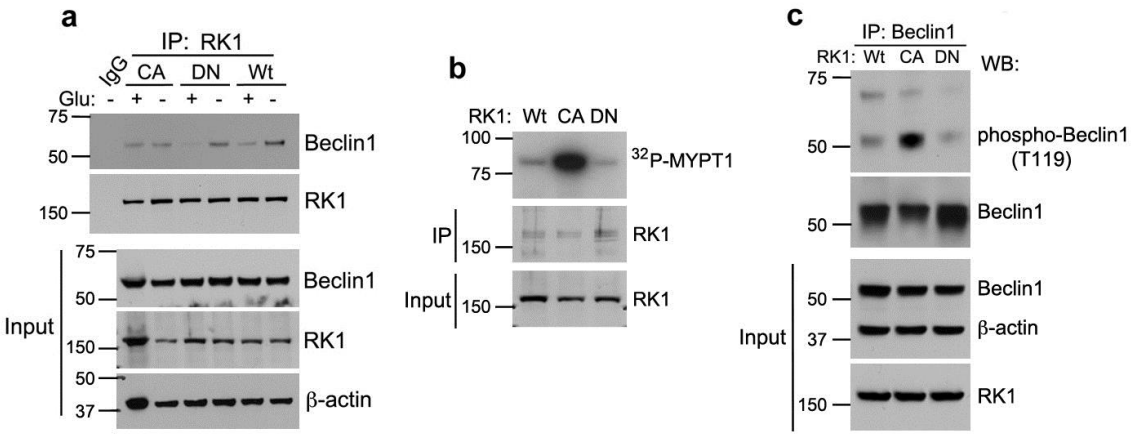


Supplementary Figure S7. **ROCK1 is required for Beclin1 phosphorylation.** (**a**) HeLa cells transfected with two different siCont., as well as, siRK1#2 were incubated in DMEM (+ Glu) or HBSS (- Glu) for 3h. Endogenous Beclin1 was immunoprecipitated using Beclin1-complexed agarose. Eluted protein was run on 7.5% gel for western blotting using phospho-Serine/Threonine specific antibody, and inputs were resolved on 4-12% gel to confirm ROCK1 knock-down. (**b**) Recombinant His-Beclin1 protein used for *in vitro* kinase assay in Figure 4. His-Beclin1 was first eluted and then concentrated using Millipore concentrator with a cut-off of 30 kDa.



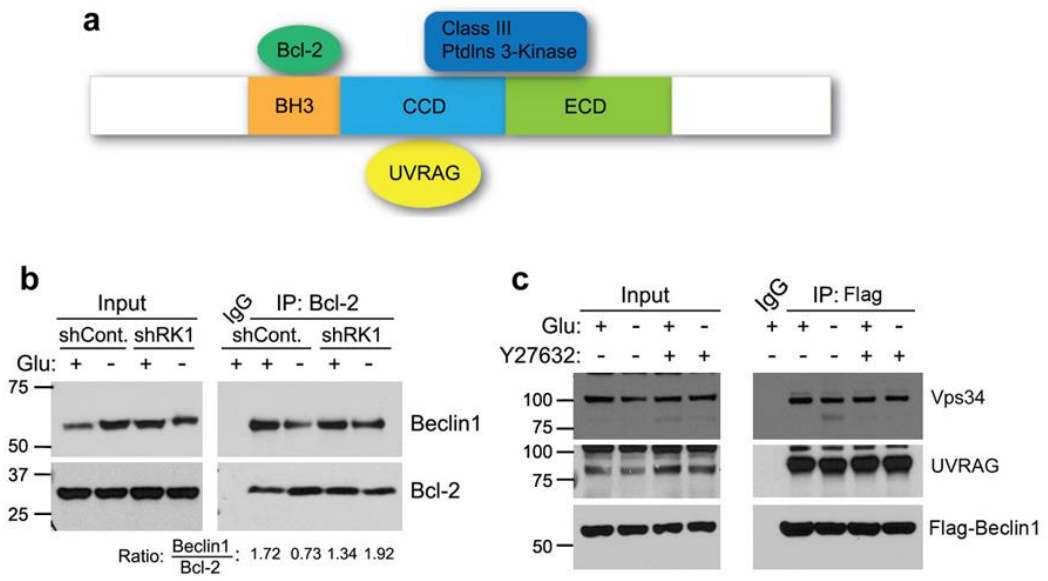
Supplementary Figure S8. ROCK1 consensus motif in Beclin1 is well conserved through species.

(**a**) Consensus sequence phosphorylated by ROCK1. (**b**) Protein sequence alignment of Beclin1 homologues from multiples species. ROCK1 phosphorylation site in Beclin1 is conserved in human, mouse, rat, cow, orangutan, chicken and frog.



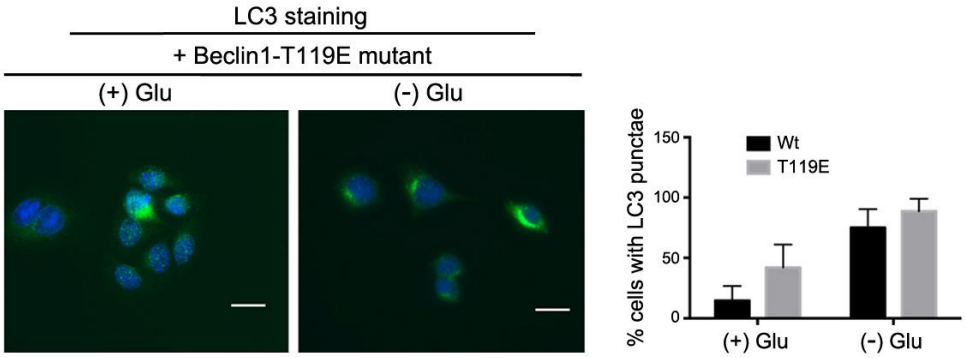
Supplementary Figure S9. **ROCK1 activation in response to metabolic stress requires Beclin1 interaction.**

(**a**) HeLa cells transfected with Myc-RK1 CA (constitutively active), DN (dominant negative) or Wt (Wild type) were incubated in nutrient rich or free media for 4h. Myc-RK1 was immunoprecipitated using Myc agarose and immune-complexes were divided in two (RK1 and Beclin1) and subjected to SDS-PAGE for western blotting. Inputs were blotted against indicated antibodies. (**b**) Myc-RK1 transfected HeLa cells were lysed and whole cell lysates prepared. RK1 was immunoprecipitated as above and used for an *in vitro* kinase assay, against recombinant MYPT1. Autoradiography is shown (top panel); RK1 IP and input was determined by SDS-PAGE (middle and lower panel). (**c**) Myc-RK1 Wt, CA and DN transfected HeLa cells were incubated in HBSS for 3h. Endogenous Beclin1 was immunoprecipitated using Beclin1-complexed agarose and resolved by SDS-PAGE and analyzed for phospho-T119 (Beclin1). The same blot was washed, re-blocked with 5% milk and re-blotted for total Beclin1 levels. Inputs were examined for Beclin1, ROCK1 and β-actin.



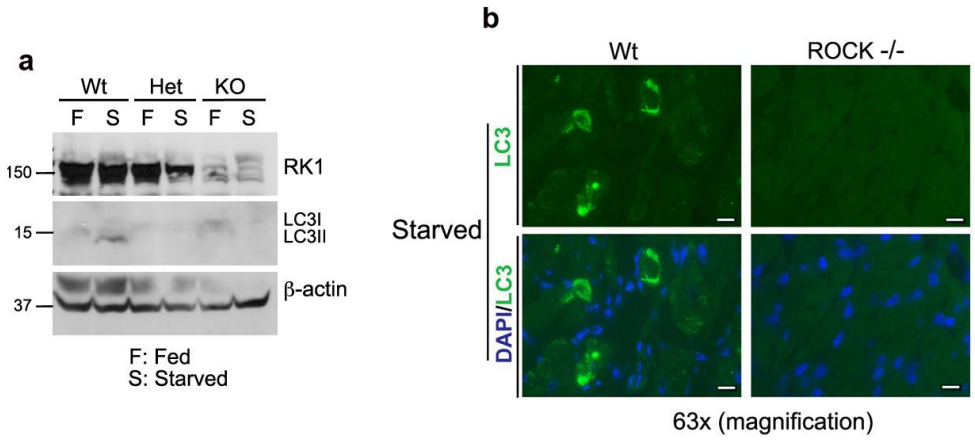
Supplementary Figure S10. **RK1 activity does not alter binding of Beclin1 to other complex core proteins, except Bcl-2.**

(**a**) Schematic representation of Beclin1 domains and its binding proteins. (**b**) HeLa cells with a transient knock-down using shCont. or shRK1#1 for 36h were incubated in glucose rich or starved (4 h) media. Endogenous Bcl-2 was immunoprecipitated, immune-complexes resolved by SDS-PAGE and blotted against Beclin1 and Bcl-2. (**c**) HeLa cells were transfected with Flag-Beclin1 for 36h and then treated with H_2O or Y27632 for an additional 8h. Treated cells were incubated in nutrient rich or free media for 4h, whole cell lysates were prepared, and immunoprecipitation was performed using Flag-agarose. Resulting proteins were eluted and western blotted against indicated antibodies.



Supplementary Figure S11. **Endogenous LC3 staining in T119E-Beclin1 transfected cells.**

HeLa cells transfected with Flag-Beclin T119E were cultured in control or HBSS media for 6 h, fixed with cold acetone and endogenous LC3 immunofluorescence was performed. Representative images are shown: Scale = 20 μm. Graph represents % of LC3 punctae cells for Beclin Wt and T119E transfected cells (mean ± s.d) (n =25).



Supplementary Figure S12. **ROCK1 KO leads to impaired LC3 processing *in vivo*.**

(**a**) Heart lysates from fed and starved Wt, Het or KO mice used for EM studies in Figure 6, were resolved by SDS-PAGE and blotted against indicated antibodies. (**b**) Higher magnification of endogenous LC3 staining from a different set of starved Wt and KO mice hearts. Scale = 10 μm

Supplementary Figure S13. Full scans of immunoblots used in the main figures.

Figure 1

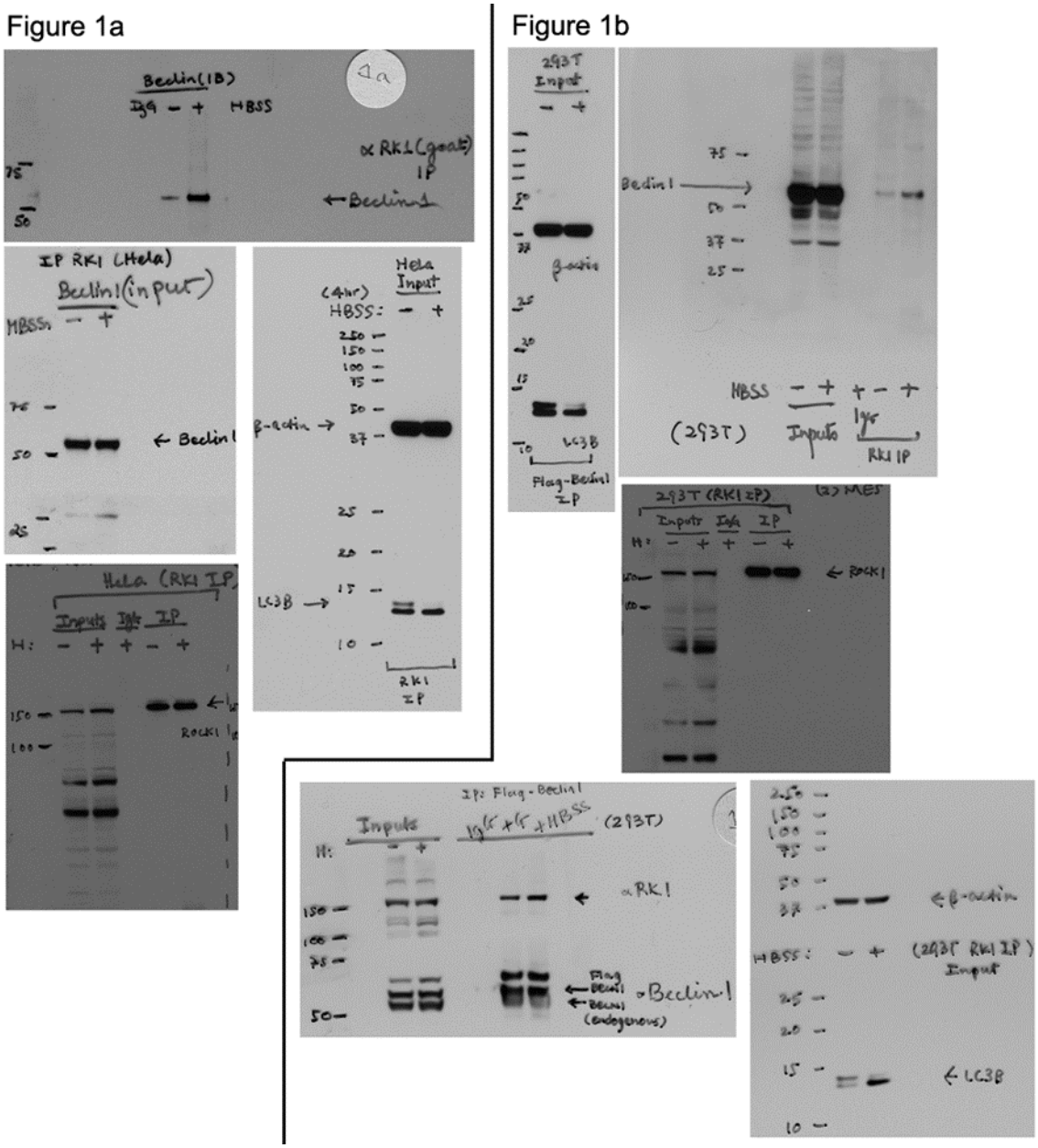


Figure 2

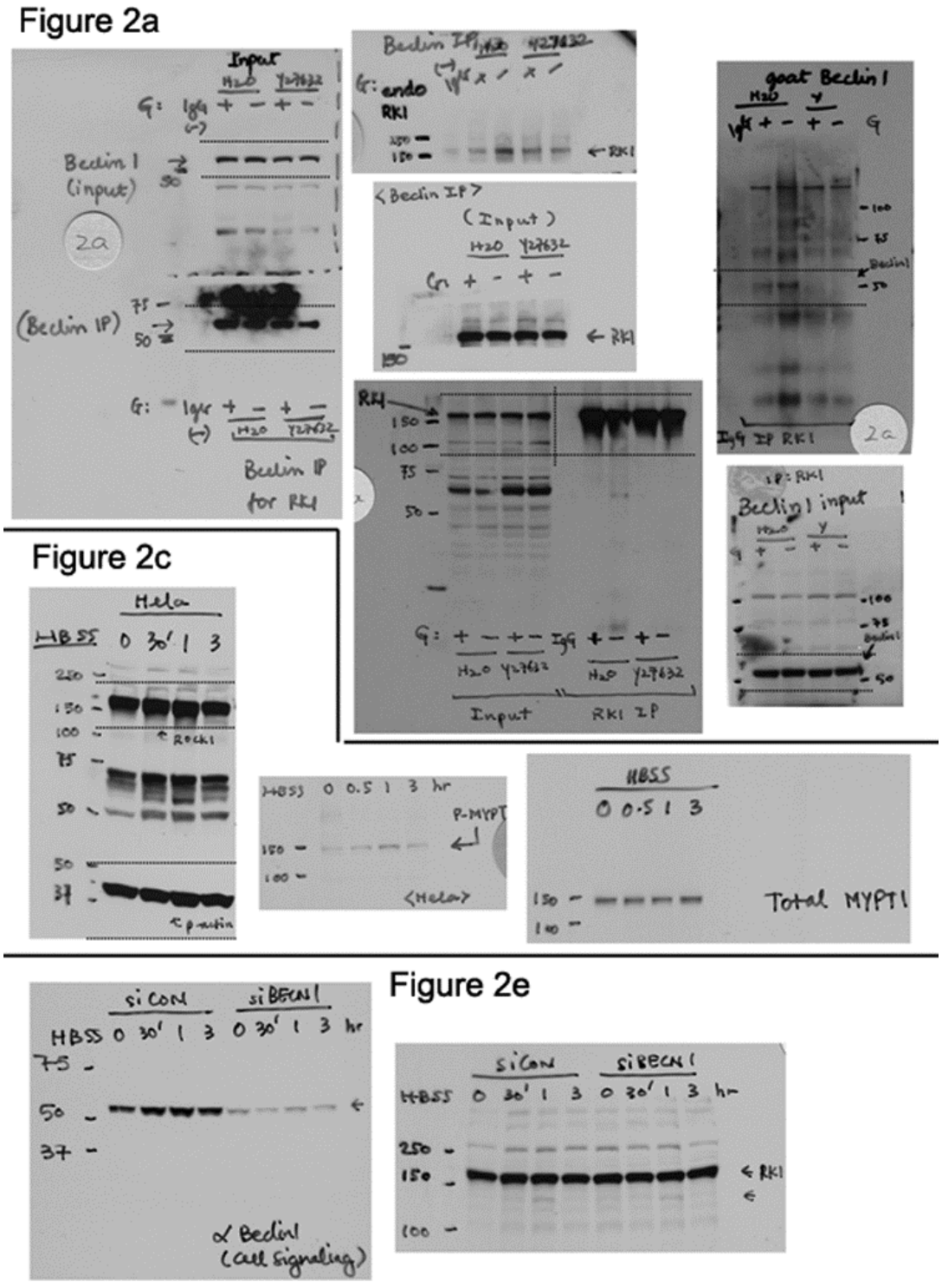
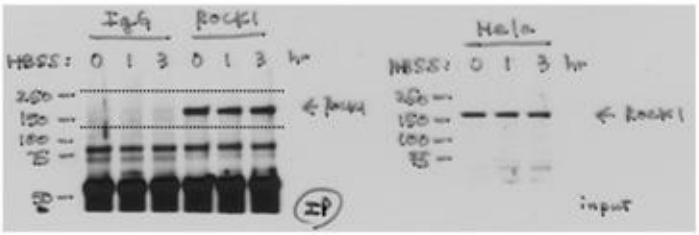
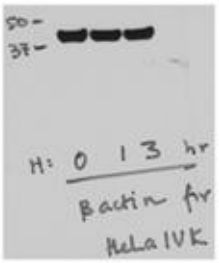
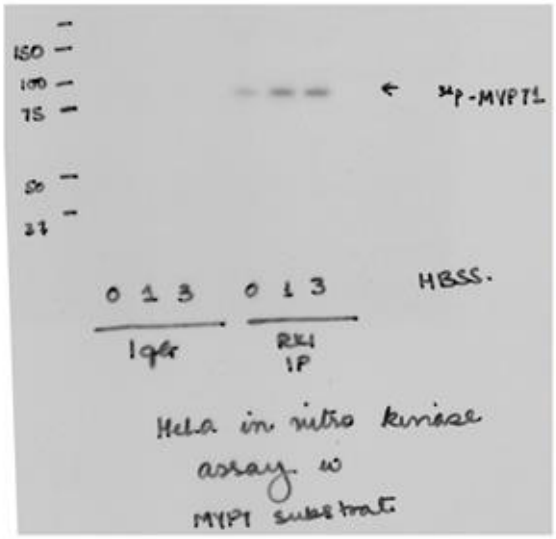


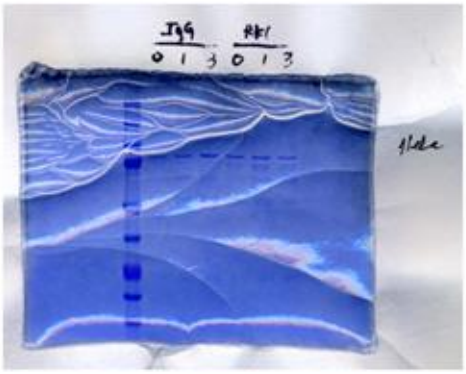
Figure 2a
Input
H2O
Y27632
Beclin 1 (input)
(Beclin IP)
Beclin IP for RKI
Beclin IP
endo RKI
(Input)
goat Beclin 1
IgG IP RKI
RKI
Beclin 1 input
Input
RKI IP
Figure 2c
Hela
HBSS 0 30' 1 3
Rock1
p-actin
HBSS 0 0.5 1 3 hr
P-MYPT
<Hela>
HBSS
0 0.5 1 3
Total MYPT1
Figure 2e
siCON
siBECN1
HBSS 0 30' 1 3 0 30' 1 3 hr
∝ Beclin1 (cell signaling)
RKI

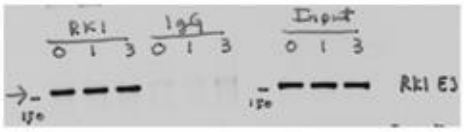
Figure 2b

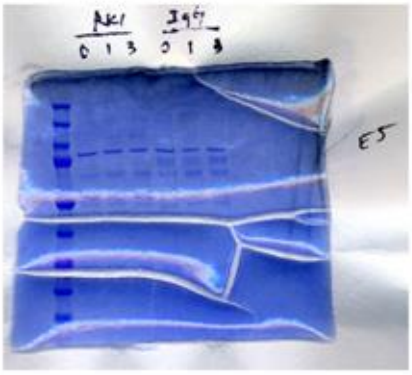

IgG
Rock1
HBSS: 0 1 3 0 1 3 hr
250
150
100
75
50
← Rock1
IP
HeLa
HBSS: 0 1 3 hr
250
150
100
75
← Rock1
input

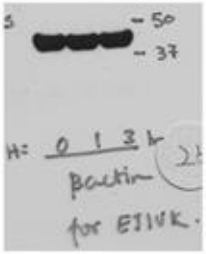

50
37
H: 0 1 3 hr
Bactin for
HeLa IVK


150
100
75
50
37
← 32p-MYPT1
0 1 3 0 1 3
HBSS.
IgG
RKI IP
Hela in vitro kinase assay w MYPT substrate


IgG
RKI
0 1 3 0 1 3
Hela


RKI
IgG
Input
0 1 3 0 1 3 0 1 3
RKI E5
150
150


RKI
IgG
0 1 3 0 1 3
E5


50
37
H: 0 1 3 hr
Bactin
for E5IVK

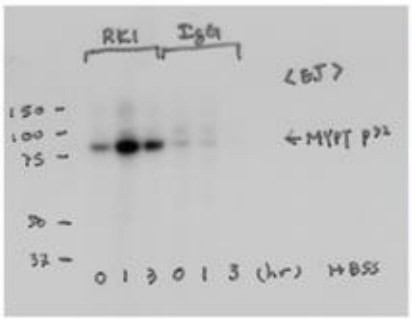

RKI
IgG
<BJ>
150
100
75
50
37
← MYPT P32
0 1 3 0 1 3 (hr) HBSS

Figure 3

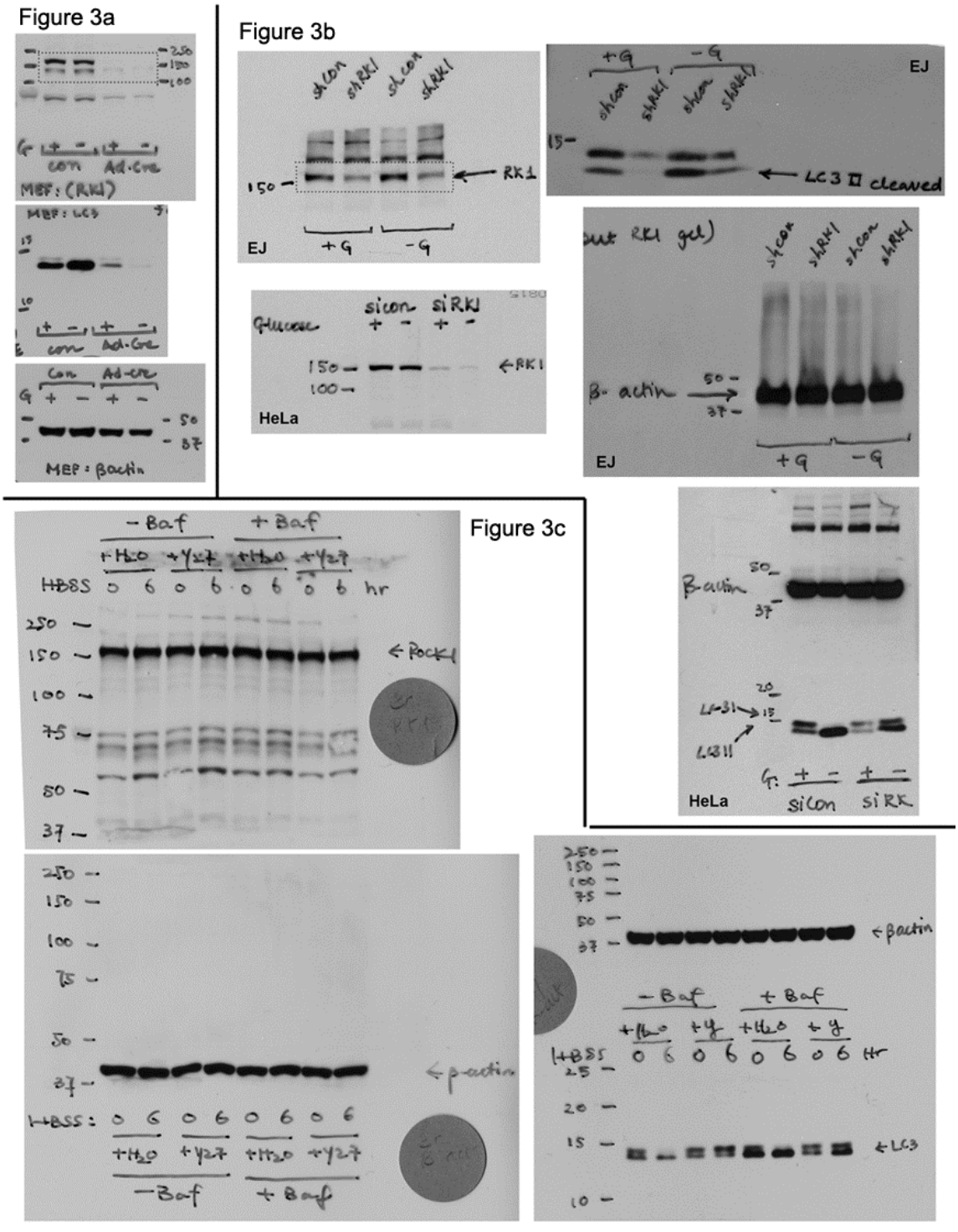


Figure 3a
MEF: (RKI)
con
Ad-Cre
MEF: LC3
MEF: βactin
Figure 3b
shCon
shRKI
RK1
EJ
+G
-G
LC3 II cleaved
HeLa
siCon
siRKI
Glucose
β-actin
Figure 3c
-Baf
+Baf
+H2O
+Y27
HBSS
Rock1
LC3I
LC3II
siRK
+βactin
+LC3

Figure 4

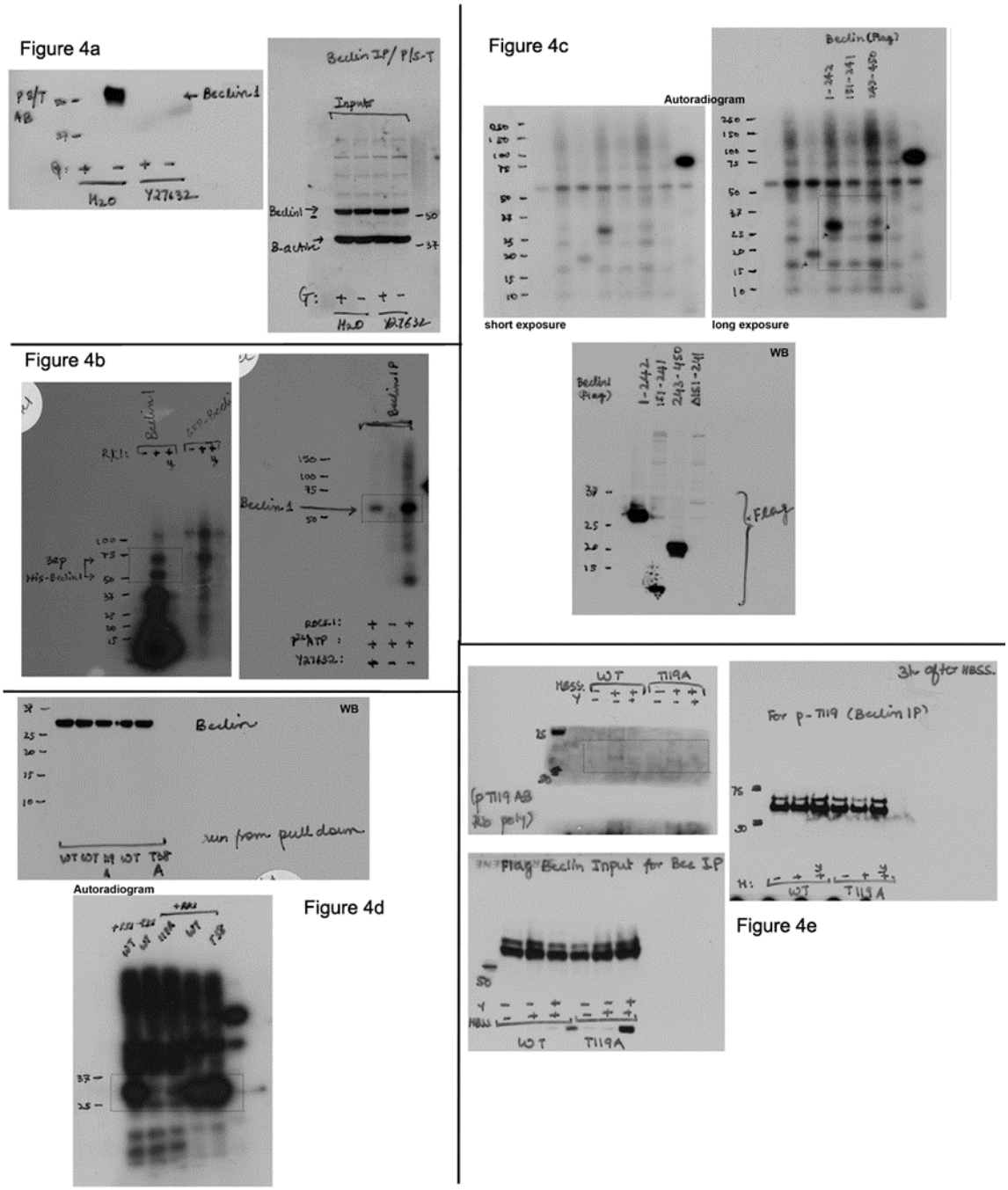


Figure 4a
Beclin 1
Beclin 1 IP/ P/S-T
Input
Beclin 1
β-actin
H2O
Y27632
Figure 4b
Beclin 1
Beclin 1 P
ROCK1:
Y27632:
Figure 4c
Autoradiogram
short exposure
long exposure
WB
Flag
Figure 4d
Beclin
WB
run from pull down
Autoradiogram
Figure 4e
WT
T119A
HBSS
(pT119 AB Rb poly)
3h after HBSS
For p-T119 (Beclin IP)
Flag Beclin Input for Bec IP

Figure 5

Figure 5a

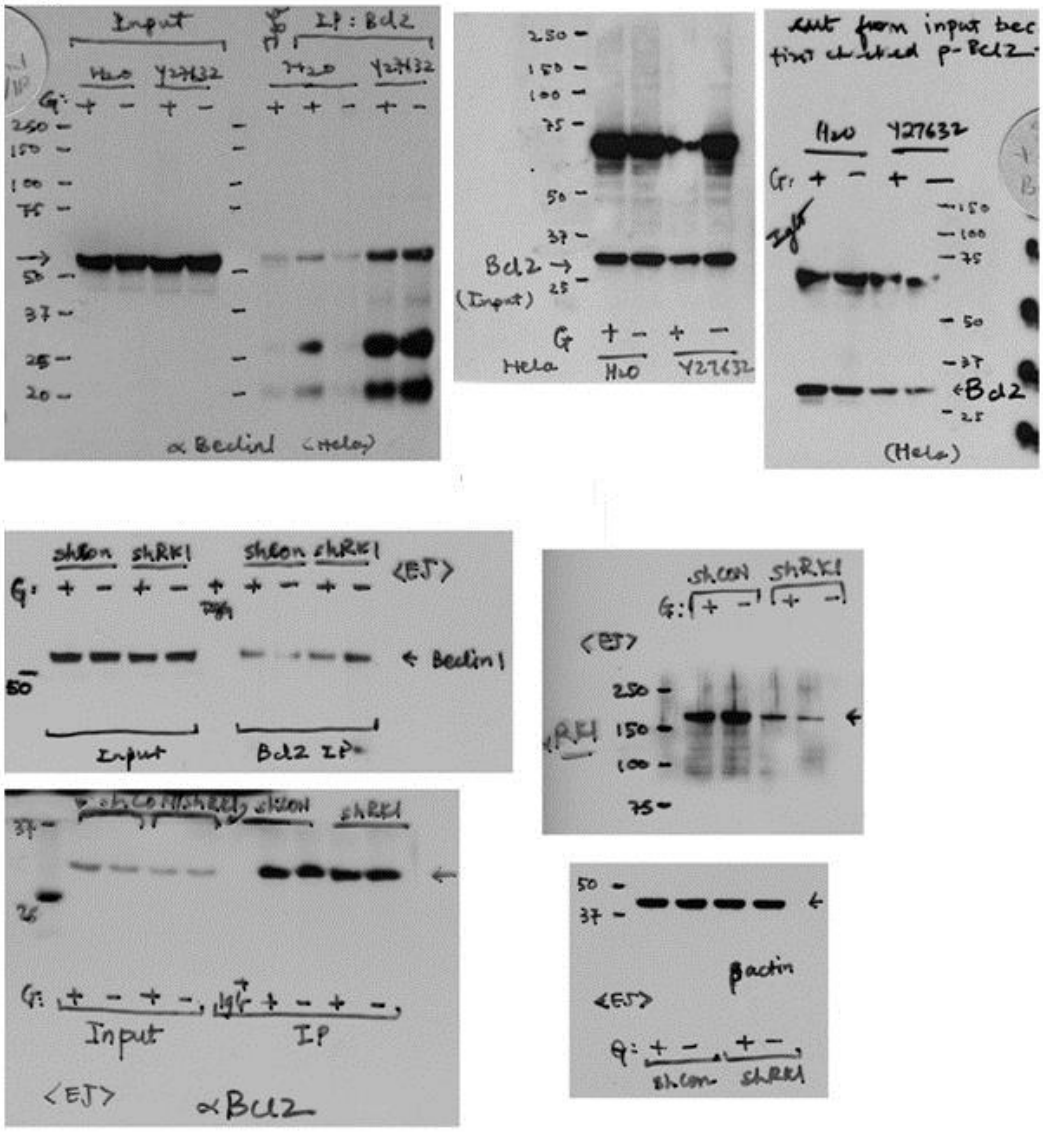
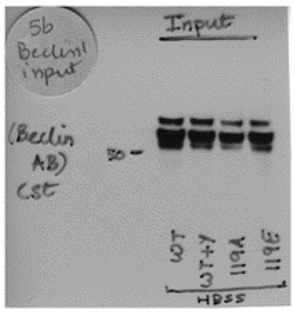
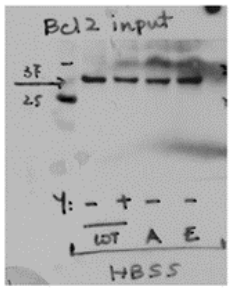
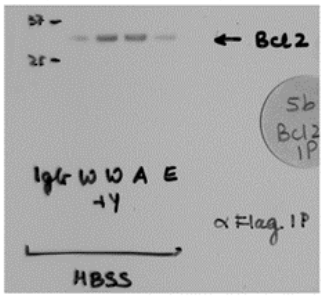


Figure 5b







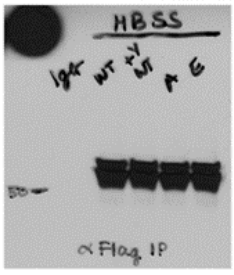
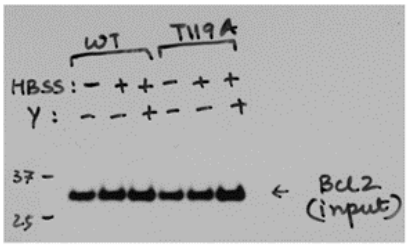
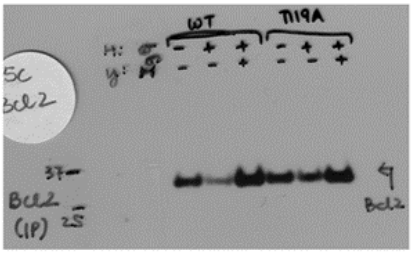
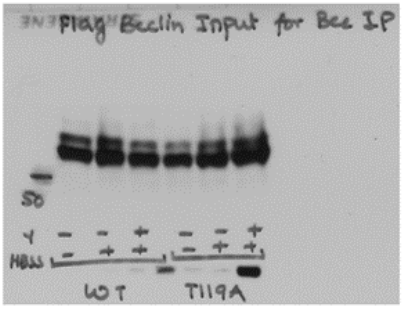
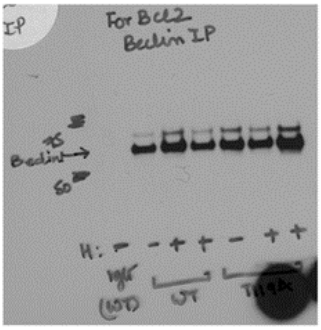


Figure 5c









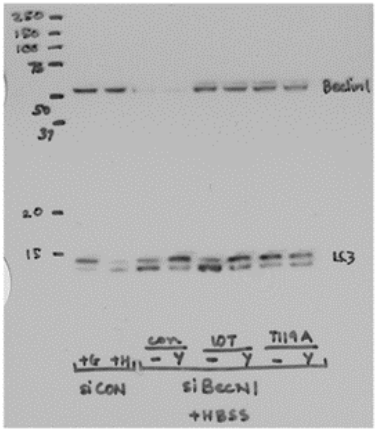


Figure 5d

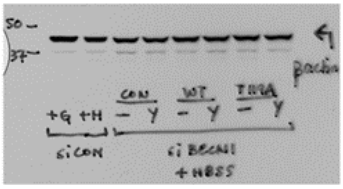


Figure 6

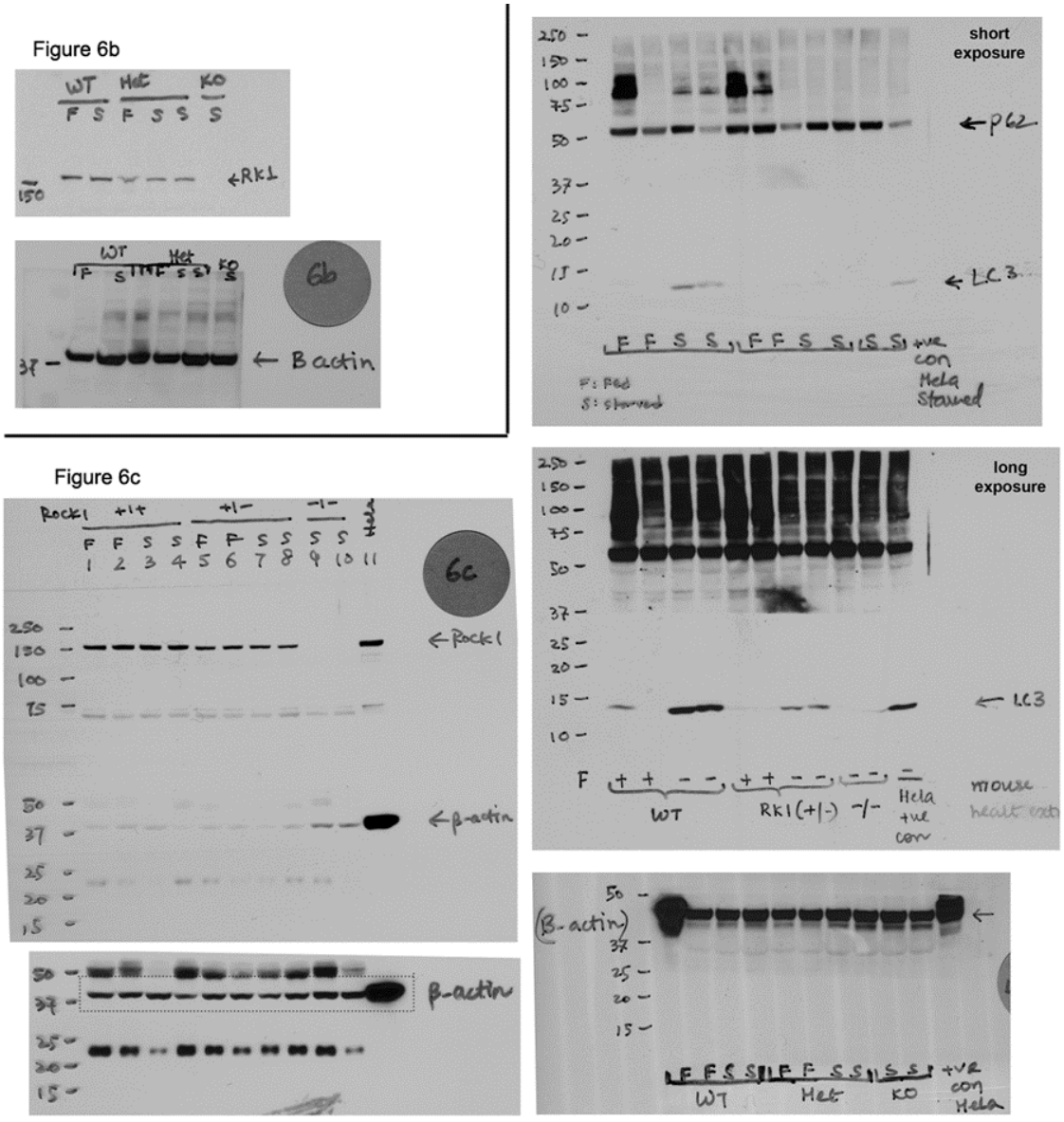


Figure 6b
WT
Het
KO
F S F S S S
Rk1
150
6b
β actin
37
short exposure
p62
LC3
F F S S F F S S S S
+ve con Hela Stawed
F: Fed
S: Starved
long exposure
Figure 6c
Rock1
+/+
+/−
−/−
6c
Rock1
β-actin
WT
Rk1(+/-)
-/-
Hela +ve con
mouse
β-actin
Het
KO

Supplementary Figure S14. Full scans of immunoblots used in the Supplementary Figures.

Figure S1

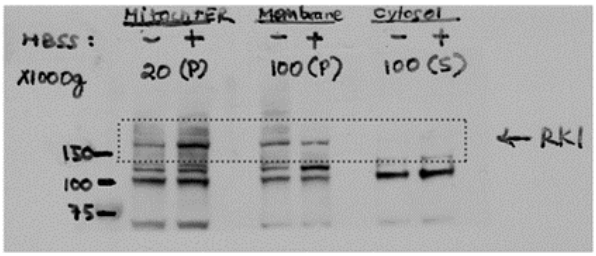
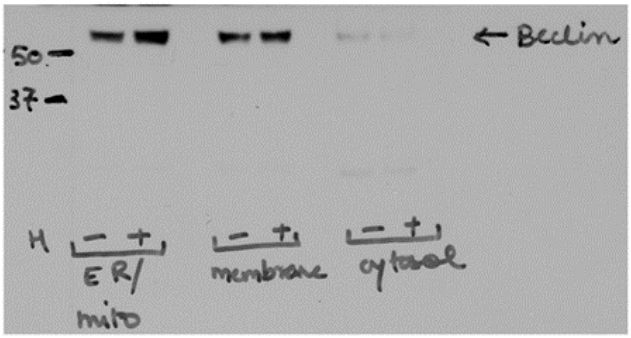
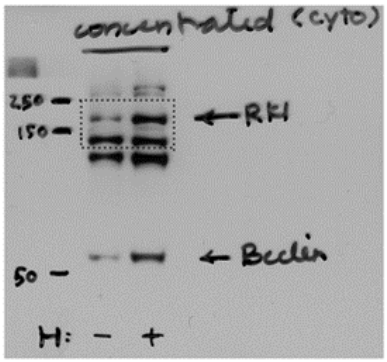
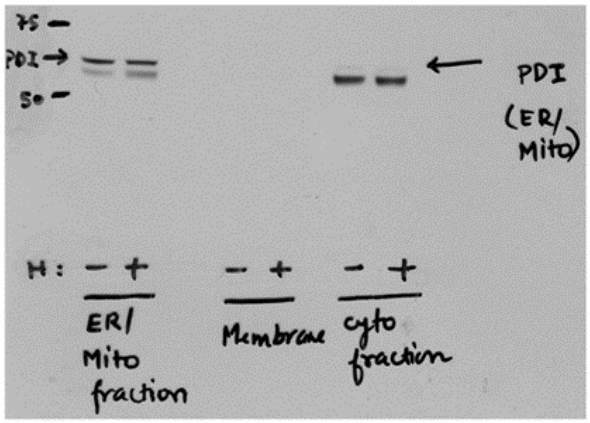
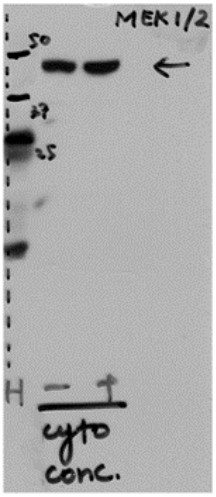


Figure S1a









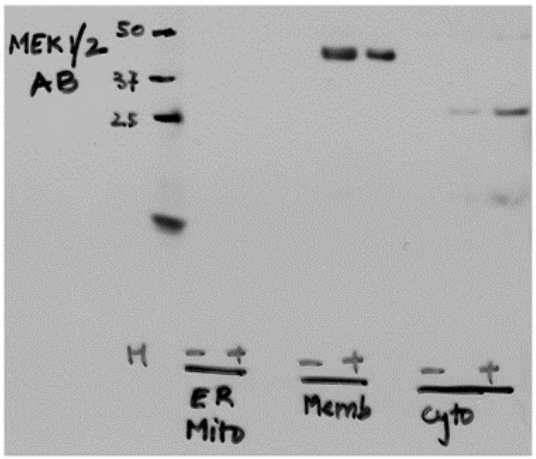
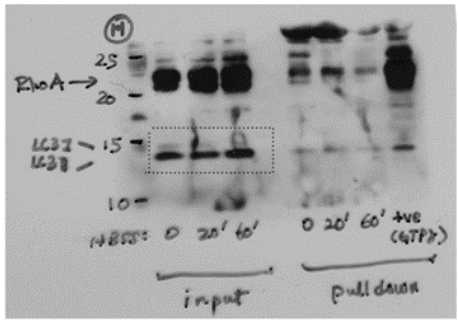
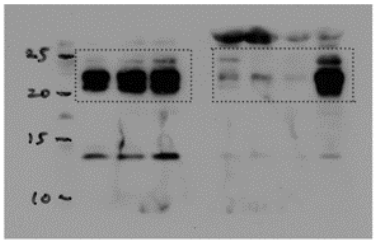


Figure S3

Figure S3a

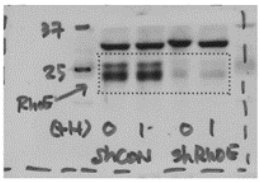


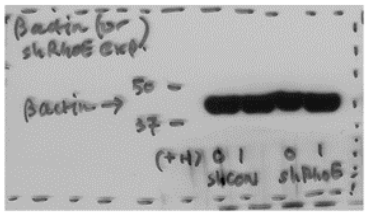
long exposure

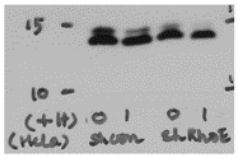


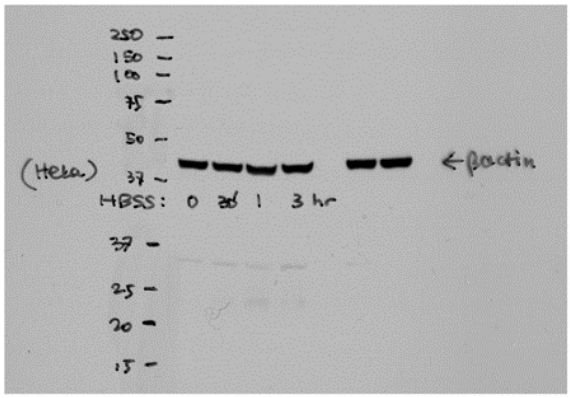
short exposure

Figure S3b

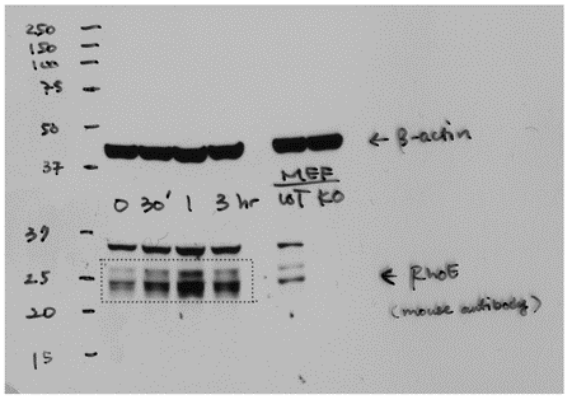






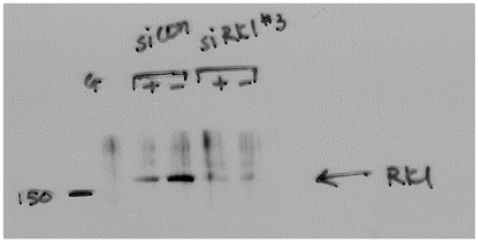


short exposure



long exposure

Figure S4



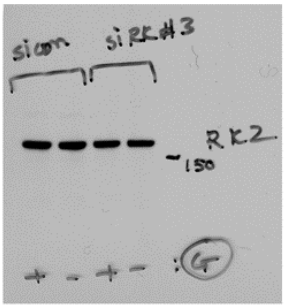
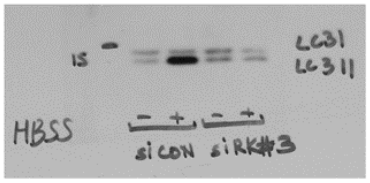
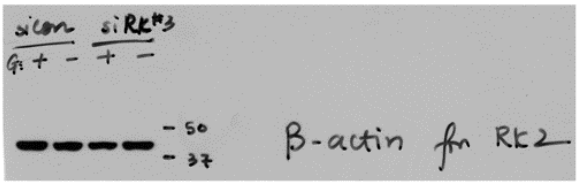


Figure S4a





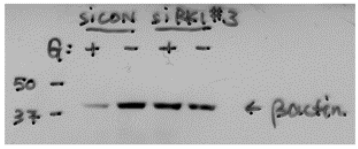
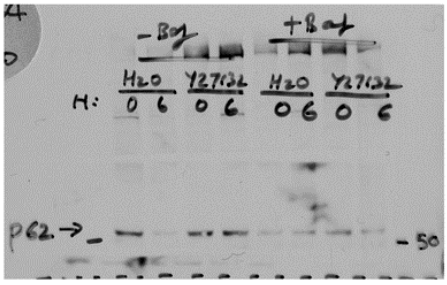


Figure S4b



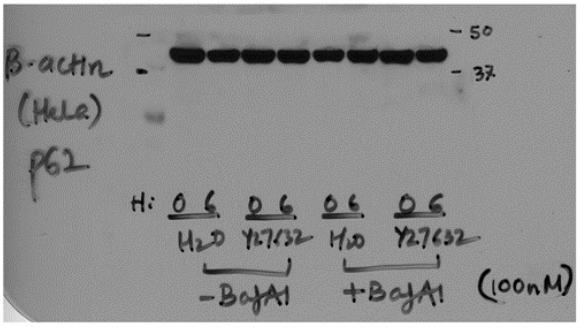


Figure S7

Figure S7a

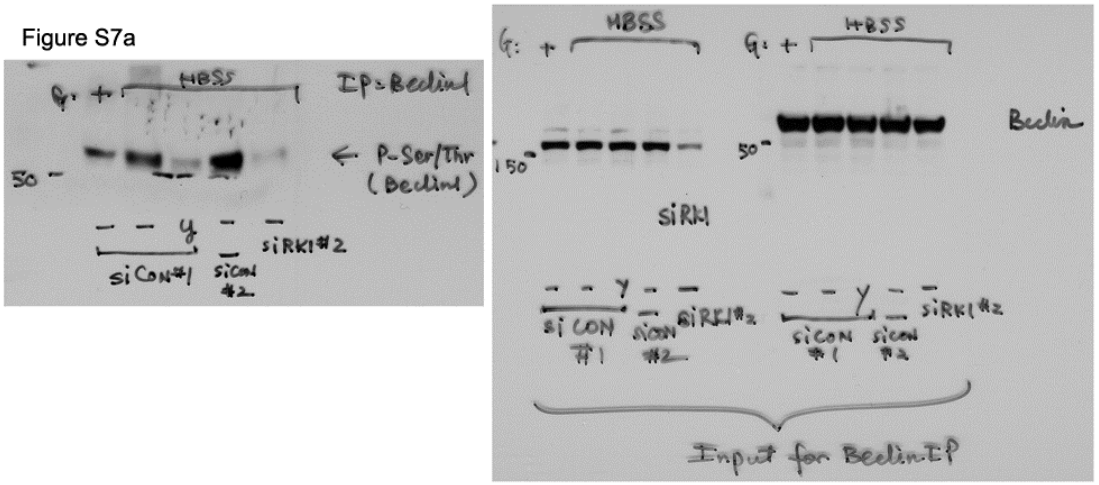


Figure S7b

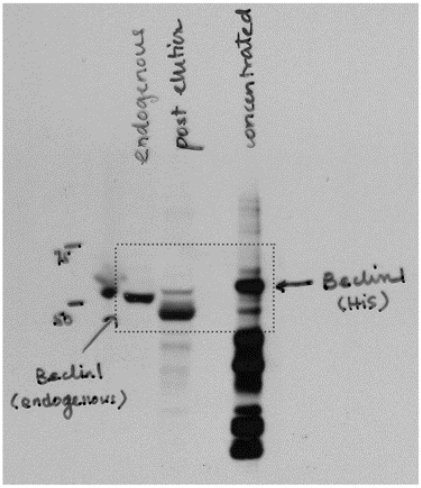


Figure S7b

Figure S9

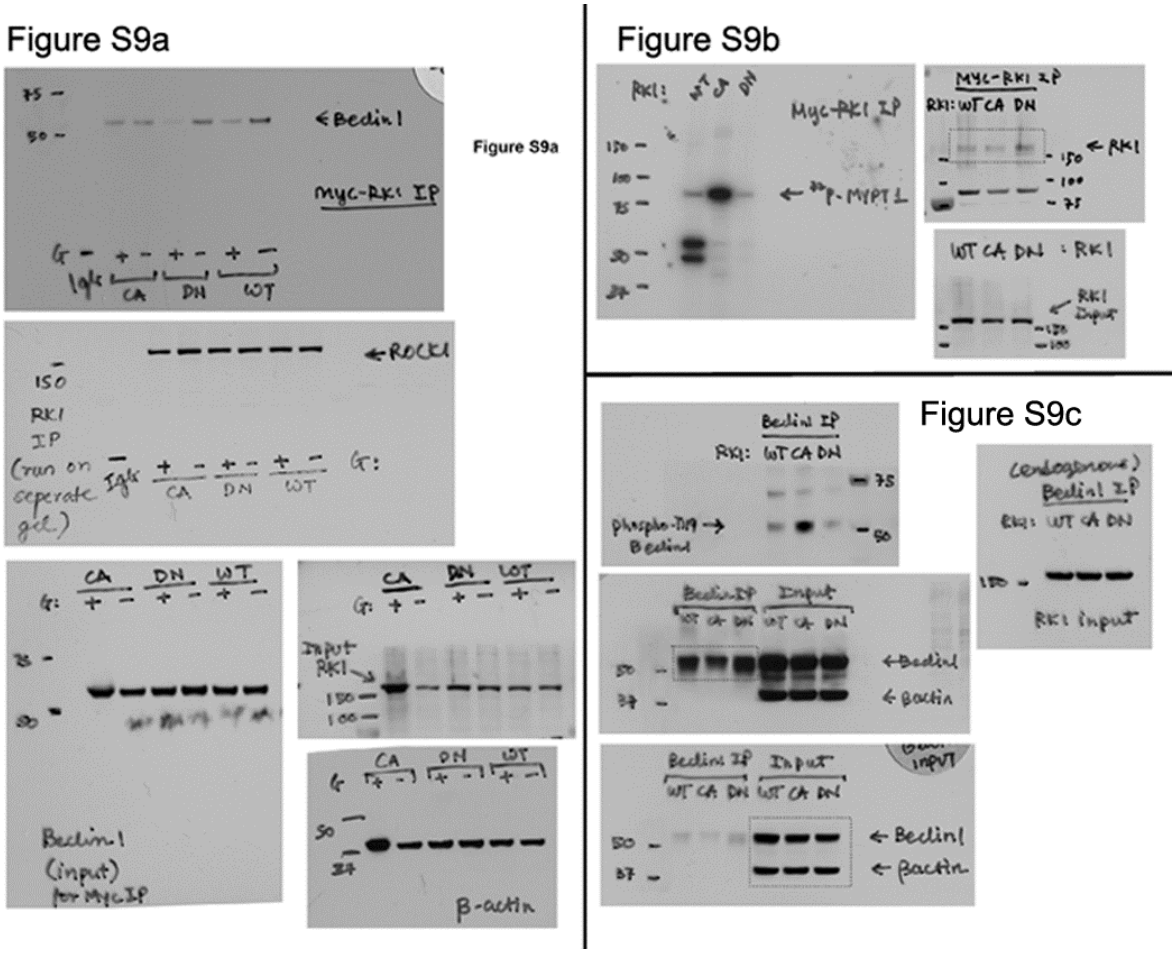


Figure S9a
Beclin 1
Myc-Rk1 IP
IgG
CA
DN
WT
ROCK1
Rk1 IP
(run on seperate gel)
Beclin-1 (input) for Myc IP
Input Rk1
β-actin
Figure S9a
Figure S9b
Rk1: WT CA DN
Myc-Rk1 IP
32P-MYPT1
Myc-Rk1 IP
Rk1: WT CA DN
Rk1
Rk1 Input
Figure S9c
Beclin IP
Rk1: WT CA DN
phospho-Thr Beclin
(endogenous) Beclin1 IP
Rk1: WT CA DN
Rk1 input
Beclin IP
Input
Beclin
β-actin
Beclin1
β-actin

Figure S10

Figure S10b

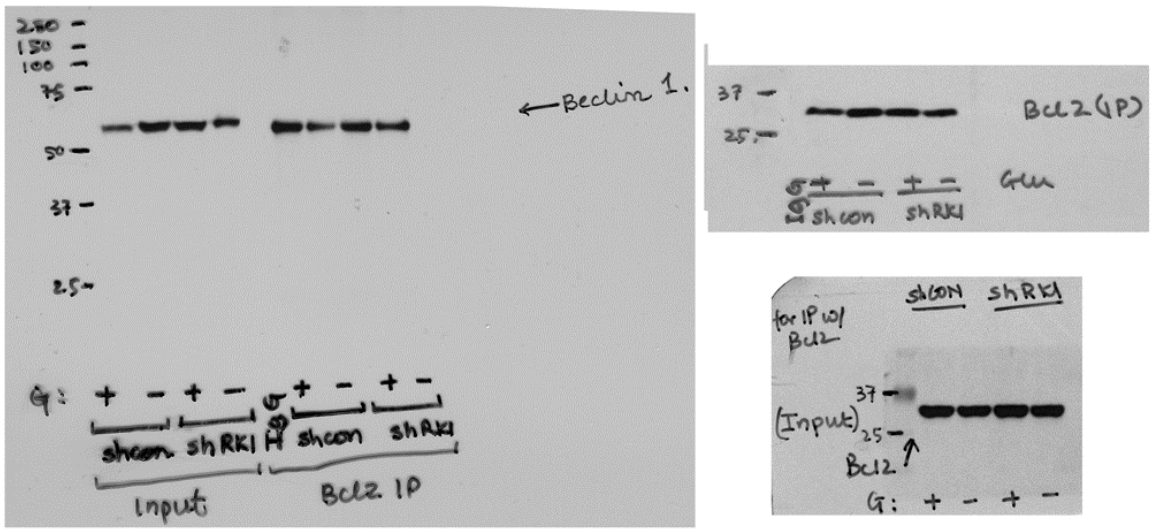


Figure S10c

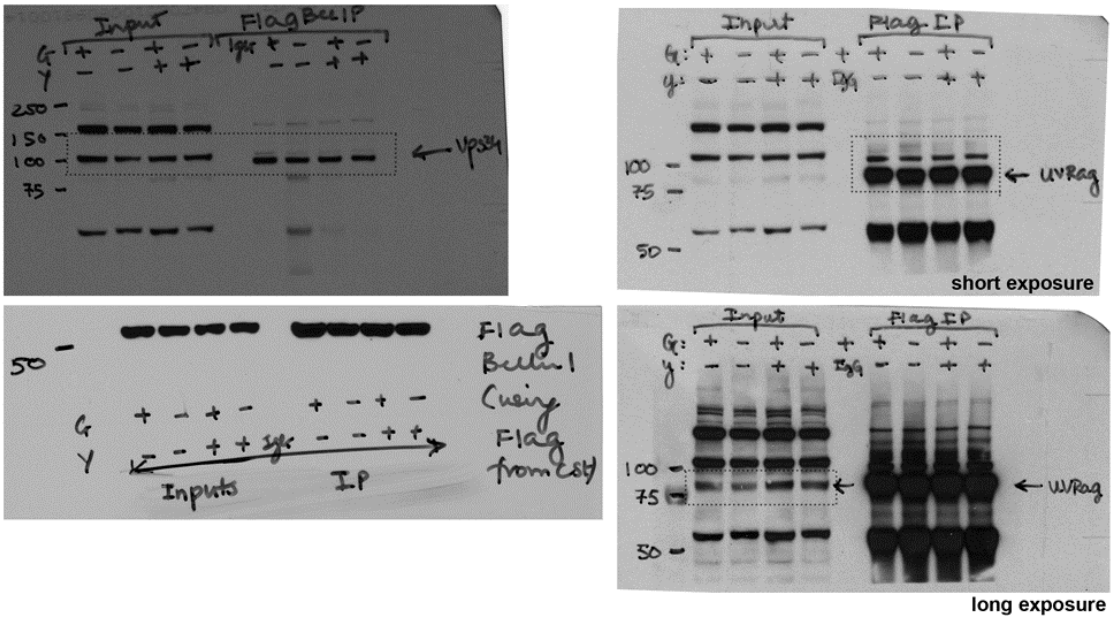
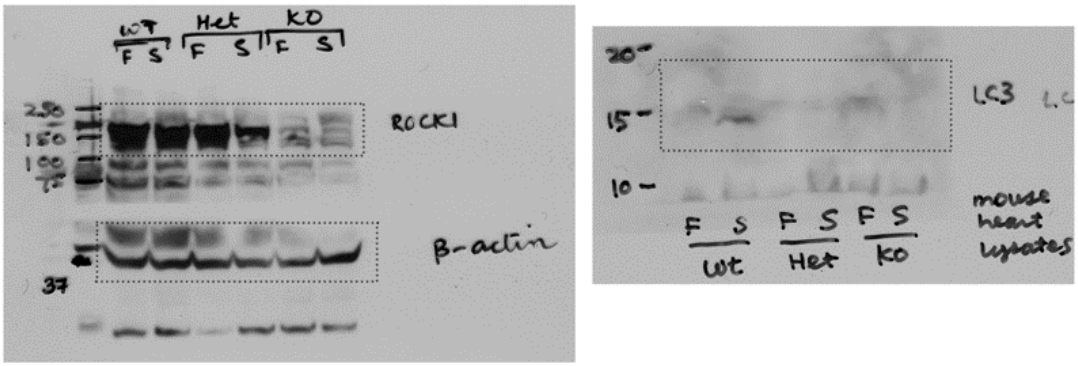


Figure S12

Figure S12a



Supplementary Table S1. ROCK1 associated polypeptides identified in response to nutritional stress.

Protein name	# of unique peptides	Involved in
Beclin1	43	Autophagy
PKM2	26	Glycolysis
XRCC6	24	DNA repair
STK38	21	Ser/Thr kinase-MAP3K signaling

Supplementary Methods

siRNAs and stable cell lines. For human Beclin1 RNAi experiments the targeting sequences were as follows: L-010552-00-0005 (Dharmacon), and siCON: D-001810-10-20 (Dharmacon). For human ROCK1 knockdown experiments, the targeting sequences are as follows: shROCK1#1, 5′-GTACTTGTATGAAGATGAC-3′; siROCK1#2, SI02622102 + SI02622095 (Qiagen); and siROCK1#3, 5′-GCCAAUGACUUACUUAGGA-3′ (Dharmacon). All Stars Negative Control siRNA (Qiagen) was used as siCont. and 5′-UAGCGACUAAACACAUCAA-3′ (Thermo Fisher Scientific) as siCont.#2. EJ cells stably expressing ROCK1 shRNA were generated by annealing the shRNA to the BamH1 and Xho1 sites of the pBabe-U6 vector that harbors a puromycin resistance gene. The pBabe-ROCK1 transfectants were selected with 2 μg/ml of puromycin and pooled for further use. In addition, for certain experiments, stable ROCK1 knockdown EJ cells were transfected with GFP-LC3 and selected with G418. Pooled stables were cultured in the presence of both puromycin and G418 and used for immunofluorescence experiments.

ROCK ELISA assay. The assay was performed according to the manufacturer's protocol (Cell Biolabs) with a few changes. Cells incubated in HBSS media for varying times were harvested in lysis buffer. Equal amount of protein (total volume 90 μl) using Bradford was determined and added to the wells. Kinase reaction was initiated using 10 μl of 10X Kinase Reaction buffer (with DTT and ATP). After 60 min, the reaction was stopped by adding 50 μl of 0.5 M EDTA. The wells were then washed and incubated with anti-phospho-MYPT1 antibody for an additional 60 min. Secondary antibody was added to washed wells for 60 min, after which substrate solution was added to each well and absorbance was measured at 450nm.

Crystal Violet staining. Cells incubated in DMEM or HBSS as indicated in figure

legends were washed with ice-cold PBS and fixed with 1% PFA for 10 min. Fixed cells were stained with crystal violet for 30 min, washed extensively, dried and scanned.

TUNEL staining. To assess cell death by TUNEL assay, cells were harvested by trypsinization, washed with ice-cold PBS and recovered by centrifugation at 300g. Cell pellets were fixed in 2% paraformaldehyde-containing phosphate buffered saline for 16 hrs. Permeabilization and enzymatic labeling with fluorescein conjugated-dUTP was performed according to the manufacturer's protocol (Roche, IN). The percentage of cells that incorporated the fluorescence-conjugated dUTP was determined by flow cytometry.

DQ-BSA assay. Autophagy flux was analyzed using DQ^{TM} Red BSA (self-quenched red BODIPY dye conjugated to BSA) as described previously[61]. Briefly, EJ GFP-LC3 stable cells were incubated in DMEM media containing DQ-BSA (10 μg/ml) for 45 min. Cells were then washed twice and incubated in DMEM for an additional 30 min, washed with HBSS and starved for 2h. Resulting cells were fixed, and co-localization of GFP-LC3 and red fluorescence of DQ-BSA was imaged using Zeiss VivaTome microscope.

Mouse Genotyping. The genotype of ROCK1 progeny was determined by PCR amplification of genomic DNA extracted from tail biopsies. The primers were (5'-CCTGATGAGCAACTATGACGTGCC-3') and (5'-GTACAGTACAGGAGCTACAAAATTAG-3') for wild-type and (5'-TATAGAGAGCTGTGAAGAGG-3') and (5'-GTACAGTACAGGAGCTACAAAATTAG-3') for mutant.

Supplementary References

61 Vázquez, C.L**.**, & Colombo, M.I. Assays to assess autophagy induction and fusion of autophagic vacuoles with a degradative compartment, using monodansylcadaverine (MDC) and DQ-BSA. *Methods Enzymol.* **452,** 85-95, (2009).