I. Supplemental Materials and Methods

Cell Lines and Transfections

Cells were grown in either Dulbecco's modified Eagle's medium (293T and tetracycline-inducible BI-1 HeLa cells) or RPMI (H322M cells) supplemented with 10% tetracycline–free FBS, penicillin (100 UI/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) and incubated at 37°C in an humidified atmosphere containing 5% CO_2. For DT40 cells we used RPMI supplemented with 10% FBS and 1% chicken serum. To induce BI-1 expression, cells were treated for 24 hrs with 1 μg/ml doxycycline. 293T cells were transiently transfected with pcDNA3.HA or pcDNA3.HA-BI-1 plasmids (Xu et al. 2008) using Lipofectamine 2000 (Invitrogen) in Opti-MEM-reduced serum medium (Invitrogen) for 24 hrs.

Recombinant Lentiviruses

Hemagglutinin (HA)-tagged version of human *BI-1* cDNA was generated by PCR and subcloned into HIV-based self-inactivating lentiviral vector pCSC-SP-PW. For *BI-1* knockdown assays, single 83-mer oligonucleotides were designed, containing an *XbaI* site at the 5' end and sense and antisense shRNA strands intermediated by a short spacer, plus a partial sequence of the H1-RNA promoter at the 3' end. Standard PCR procedures (Advantage 2 PCR kit, Clontech) were performed by using specific shRNA oligonucleotides and T3 primer plus pSuper-like 23 plasmid(Tiscornia et al. 2003) as a template to provide H1(H1 histone)-mediated shRNA cassettes with an additional *XbaI* site at the 3' end. PCR products were purified (Qiagen), digested with *XbaI*, and cloned into the 3' LTR *NheI* site of a CMV-GFP lentiviral vector as described (Tiscornia et al.

2003). The LV-shGFP construct (control) was kindly donated by O. Singer (Salk Institute, CA, USA). The validated shRNA oligonucleotide used for *BI-1* gene silencing was 5'-CTGTCTAGACAAAAAGATTATATCTGGCACTGCATCTCTTGAATG CAGTGCCAGATATAATCGGGGATCTGTGGTCTCATACA 3'. Vesicular stomatitis virus G envelope protein-pseudotyped lentiviruses were prepared and purified as described (Correa et al. 2011)

RT-PCR

Gene expression was evaluated using quantitative reverse transcriptase PCR. RNA from tissues and cells was extracted using Trizol (Invitrogen) and RNeasy Mini kit (Qiagen) respectively. RNA was reversed-transcribed with SuperScript reverse transcriptase (Invitrogen) and amplified using TaqMan Universal PCR Master mix. Expression analysis of p62 and BI-1 was performed using the following primers: BI-1 sense 5' gaccgagcaaaagagactgg 3', BI-1 antisense 5' aaggccaggatcaacatgag 3', p62 sense 5' tgtggaacatggagggaagag 3', p62 antisense 5' tgtgcctgtgctggaactttc 3'. Data were normalized relative to levels of β-actin and cyclophilin mRNAs.

Apoptosis Assays

For Annexin-V assays, cells were double-stained with fluorescein isothiocyanate-conjugated Annexin-V and propidium iodide (PI) with a kit according to the manufacturer's instructions (BioVision). The percentages of apoptotic (annexin-V positive + PI negative) and dead cells (PI+) cells were determined by flow cytometric analysis (Becton Dickinson).

Clonogenic survival assay

Cells (n = 200) were seeded in a 6-well plate and 24 hrs later treated with HBSS for 4 hrs. Cells were washed and then cultured for an additional 10 days in full-nutrient medium. Single colonies were stained with crystal violet, washed and counted.

Immunohistochemistry

Subcutaneous tumors were fixed in Z-fix and embedded in paraffin. Dewaxed tissue sections (4.0–5.0 μm) were immunostained as reported previously (Krajewska et al. 2011) using rabbit polyclonal and monoclonal antibodies to p62 (ENZO Life Sciences, Farmingdale, NY; BML-PW9860) and LC3B (Cell Signaling Technology, Danvers, MA; #3868), respectively. Application of the primary antibody was followed by incubation with goat anti-rabbit polymer-based EnVision-HRP-enzyme conjugate (Dako-Cytomation; Carpinteria, CA). DAB chromogen (DakoCytomation) was applied, yielding brown color. All slides were counterstained with hematoxylin prior to cover-slipping with DPX mounting medium (Fluka).

Intracellular Bacteria Killing Assay

For *in vitro* experiments, bone marrow cells were collected from age-matched WT and *Bi-1*-/- mice and cultured in RPMI supplemented with 20% FBS and 30% L-929 cell conditioned medium for 7 days. Adherent macrophages were collected and seeded at $3x10^5$ cells per well in RPMI containing 10% FBS in 24-well plates 1 day prior to bacterial infection. Group A *Streptococcus* (GAS) strain 5448 (serotype M1) was grown

to mid-log phase (OD_{600} = 0.5) and resuspended in RPMI 1640 + 10% FBS. In each well, ~5.4 x 10^6 CFU of bacteria was added to infect ~3.0 x 10^5 macrophages (multiplicity of infection = 18:1) in a final volume of 500 µl, and incubated at 37˚C x 30 min to allow phagocytotic uptake. Extracellular GAS were then killed by incubation in RPMI 1640 medium containing 10% FBS, penicillin G (10 µg/ml), and gentamicin (100 µg/ml). At various times, cells were washed to remove antibiotics, lysed, and dilutions plated on agar to enumerate bacterial colony-forming units (cfu). Intracellular survival was calculated as a percentage vs. the initial inoculum.

NADPH measurements

Mitochondria from wild-type and BI-1 over-expressing cells were isolated according to the method described in Sano et al (Sano et al. 2009). NADPH levels from viable mitochondria were measured with a NADPH fluorimetric assay kit (Abcam) following the manufacturer's instruction

Isocitrate dehydrogenase activity

Isocitrate dehydrogenase activity was measured using a colorimetric assay kit from Abcam according to the manufacturer's instructions.

SB-6471 activity

Compound SB-6471 was identified via a fluorescence resonance energy transfer (FRET) assay designed to identify compounds disrupting the physical interaction between donor-labeled sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) and phospholamban (PLB) in a

chemically defined reconstituted membrane system. Briefly, Ca-ATPase activity was measured by an NADH-linked, enzyme-coupled assay in reactions consisting of test compound, Ca-ATPase, MOPS (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, 1.0 mM EGTA, 1.0 mM phosphoenol pyruvate, 2.5 mM ATP, 0.2 mM NADH, 5 IU of pyruvate kinase, 5 IU of lactate dehydrogenase, 3.5 μg/mL of calcium ionophore, and $CaCl_2$. The assay was started upon addition of ATP (5 mM). Further testing in orthogonal assays for ATPase activity showed that the compound increased SERCA Ca^{2+}-ATPase activity in a concentration-dependent fashion. e time-dependence of absorbance at 340 nm was measured in a SpectraMaxPlus™ microplate reader (Molecular Devices, Sunnyvale, CA). Data were fitted using the Hill function: $V = V_{max}/[1+ 10^{-n\ (pKCa\ -\ pCa)}]$, where V is the initial ATPase rate and n is the Hill coefficient.

II. Supplemental Figure Legends

Figure S1. ***Similar levels of p62 mRNA levels in BI-1 knockout and wild-type mouse tissues.*** Heart p62 mRNA levels of wild-type [wt] and BI-1 knockout [ko] animals untreated [-] and treated [+] with rapamycin for 24 hrs were determined by Q-RT-PCR Results are expressed as % control relative to wild-type samples.

Figure S2. ***BI-1 knockout tissues have p62 inclusions.*** Brains and kidneys from wild-type and BI-1 KO mice were dissected and processed for immunostaining using an anti-p62 antibody with diaminobenzidine (DAB) colorimetric detection (brown). Specimens were counterstained with hematoxylin (blue).

Figure S3. ***BI-1-deficient tissues have reduced number of autophagosomes.*** Wild-type [wt] and BI-1 knockout [ko] mice were injected with rapamycin (1 mg/kg). After 24 hrs, heart tissue was processed for and examined by transmission electron microscopy. Representative pictures of wild-type [wt] and BI-1 knockout [ko] mouse hearts are shown as indicated. Yellow arrows indicate autophagosomes. Bar represents 1 μm scale.

Figure S4. ***BI-1 knockdown tumors have reduced autophagy.*** Tissue sections from xenograft tumors comprised of H322M cells containing sh control (*A*,*C*) or sh BI-1 (*B*,*D*) vectors were immunostained with LC3B antibody (brown) and counterstained with hematoxylin (blue).

Figure S5. ***BI-1 modulates p62 levels in Drosophila S2 cells.*** (*A*) Endogenous BI-1 was knocked down in *Drosophila* S2 cells using a synthesized dsDNA against *Bi-1* (BI-1) and *Atg1* (Atg1). Non-specific dsDNA was used as positive control (control). Cells were cultured for 2 hrs under starvation conditions (2 mg/mL glucose), lysed, normalized for protein content and subjected to SDS-PAGE/immunoblotting analysis to detect p62 levels. β-actin was used as loading control. (*B*) *Drosophila* S2 cells were transfected with empty vector, *Drosophila* [BI-1 dBI-1 flag] or human [hBI-1 HA] BI-1 vectors. After 24 hrs, levels of p62 were assessed by immunoblotting analysis. β-actin was used as loading control.

Figure S6. ***Increased bacterial survival in BI-1 knockout bone marrow-derived macrophages.*** Bone marrow cells were collected from age-matched WT and *Bi-1*$^{-/-}$ mice and cultured for 7 days prior to bacterial infection. Group A *Streptococcus* (GAS) were added to cultures for 30 mins to allow internalization into macrophages, were extracellular bacteria were killed by addition of antibiotics to cultures. At various times, cells were washed to remove antibiotics, lysed, and various dilutions of lysate were plated onto agar. After incubation at 37°C for 1 day, bacterial colony-forming units (cfu) were enumerated. Intracellular GAS survival was calculated as a percentage relative to the initial inoculum. Experiments were performed in triplicate and repeated three times with similar results; representative experiment shown. Differences between groups were analyzed by Mann-Whitney U-test; $*P < 0.05$.

Figure S7. ***BI-1 increases number of autophagosomes.*** Stably transduced GFP-LC3 HeLa cells in which BI-1 expression was driven by a doxycycline-inducible promoter were cultured overnight with or without doxycycline, then cells were imaged using a confocal high content screening microscope. Cells were treated with various autophagy stimuli for 16 hrs [Rapamycin 25 μg/ml; tamoxifen 10 μM, E64d 10 μg/ml and glucose /FBS deprived-medium (GSD)]. Cells were fixed with 4% formaldehyde, washed and imaged (four pictures of a total of 9 wells per treatment). Representative images are shown in (*A*). The image analysis protocol performed the following steps, further illustrated in (*B*). Briefly, the nucleus of each cell was identified from the DAPI images using Acapella's standard <*Nuclei_Detection_A*> module. Next, whole cell regions were detected from the GFP images using Acapella's standard <*Cytoplasm_Detection_B*> module. GFP-LC3-labeled autophagosomes were detected within the whole cell regions defined in the previous step using Acapella's <Spot_Detection_C> module. The number of detected spots was then determined for each cell object. The values for all cells were then averaged to yield the "Average Number of Spots per Cell" (*C*) and the "Total Spot intensity per Cell" (*D*) for each well. (with $n > 7$). * represents $p < 0.05$

Figure S8. ***Autophagy does not impact cytoprotective activity of BI-1.*** Wild-type [wt] and *Atg7*$^{-/-}$ [ko] MEFs were stably transduced with empty or BI-1 encoding lentiviruses. Cells were cultured under basal conditions [NT] or for 24 hrs with various cell stress agents [TAM, tamoxifen 10 μM; Rapa, rapamycin 25 μg/ml; GSD, glucose and FBS deprivation and TG, thapsigargin 2.5 μM] in complete medium or cultured in nutrient-

depleted medium (HBSS) for 4 hrs. Cells were then stained with propidium iodide (PI) and analyzed by FACS. Results are expressed as percentage of PI^+ cells.

Figure S9. ***Analysis of UPR marker Bip (Grp78) in autophagy-deficient cells.*** Wild-type [wt] and *Atg7*$^{-/-}$ [ko] MEFs were stably transduced with empty or BI-1 encoding lentiviruses. Cells were cultured under basal conditions [NT] or for 4 hrs with either HBSS or with glucose and FBS-deprived media. Cell were then lysed and analyzed by immunoblotting using anti-BiP (Grp78) and anti-tubulin antibodies.

Figure S10. ***BI-1 does not require PERK to modulate autophagy.*** Wild-type (wt) and *Perk*$^{-/-}$ MEFs were cultured under normal nutrient conditions (basal) or for 4 hrs under starvation conditions (HBSS). Cells were lysed, normalized for total protein content, and analyzed by SDS-PAGE/immunoblotting.

Figure S11. ***Analysis of SERCA agonist compound SB-6471***. ATPase activity was measured at 37^{o}C as a function of [Ca^{2+}] in 96-well microtiter plates, using an enzyme-coupled assay in which the readout was NADH consumption. The time-dependence of absorbance at 340 nm was measured in a SpectraMaxPlus™ microplate reader (Molecular Devices, Sunnyvale, CA). Data (mean $\pm$ SD; n = 4) were fitted using the Hill function: $V = V_{max}/[1+ 10^{-n\ (pKCa\ -\ pCa)}]$, where V is the initial ATPase rate and n is the Hill coefficient.

Figure S12. ***BI-1 over-expressing cells have reduced isocitrate dehydrogenase activity.*** Isocitrate dehydrogenase (IDH) activity was measured in lysates prepared from controls and BI-1 over-expressing cells using a fluorimetric assay kit. Results are expressed as mU/min/10^6 cells (mean $\pm$ SD; n = 3). Data are statistically significant (p =0.02) as determined by t test

Figure S13. ***BI-1 over-expressing cells have increased mitochondrial NADPH.*** Mitochondria isolated from controls and BI-1 over-expressing cells were used to measure NADPH levels. Results are expressed an nM NADPH (mean $\pm$ SD; n = 3). Data are statistically significant (p = 0.001) as determined by t test

Figure S14. ***BI-1 does not change levels of mitochondrial proteins.*** BI-1-inducible HeLa cells were stimulated with doxycycline 1 μg/ml for various times ranging from 0 to 72 hrs to induce BI-1 expression. Cell lysates were prepared and normalized for total protein content. Protein levels of Hsp60, AIF, Cyclophilin D, p62, LC3, BI-1 and tubulin were assessed by immunoblotting. Tubulin served as protein loading control.

Figure S15. ***BI-1 does not alter mitochondrial mass.*** BI-1-inducible cells were stimulated with doxycycline 1 μg/ml for various times [0, dark blue; 24 hrs, orange; 48 hrs, light blue and 72 hrs, pink). Red lines represent non-stained cells. To measure

mitochondrial mass, cells were stained with nonylacridine orange [NAO, 20 nM] for 30 min at 37°C, washed with PBS, and analyzed by flow cytometry.

Figure S16. ***Elevated ATP levels in BI-1 deficient human and Drosophila S2 cells.*** (*A*) ATP levels were measured in control *vs* shBI-1 transduced H322M cells using the CellTiter-Glo® (Promega). Data represent relative luminescence units (RLU) per 10^6 viable cells (human) and 10^3 viable cells (*Drosophila*). Results are representative of three experiments, mean ± SD. ATP levels were measured in S2 cells depleted in endogenous BI-1 by siRNA experiments (*B*) under basal conditions (complete medium) and (*C*) with after 2 hrs starvation (glucose-free medium). Data represent relative luminescence units (RLU) per number of viable cells. Results are representative of three experiments, mean ± SD.

Figure S17. ***Experimental modulation of SERCA reverses BI-1-mediated reduction in bioenergetics.*** Doxycycline-inducible HeLa cells were used for all experiments, culturing cells without or with 1 ug/mL doxycycline [doxy] for 24 hrs to induce BI-1 expression. (*A*) Cells were transiently transfected with control vs SERCA plasmids or treated with the [C] DMSO (control) vs SERCA activator compound SB-6471 [10 μM] for 1 day prior to analysis. (*A*) and (*D*) Cell lysates were normalized for protein content and subjected to SDS-PAGE/immonoblotting with anti-LC3, anti-p62, anti-SERCA and anti-HA (BI-1) antibodies. ATP levels were measured in control *vs* SERCA-transfected cells (*B*) and DMSO *vs* SB-6471 [10 μM, 24 hrs]-treated (*E*) cells using the CellTiter-Glo® (Promega). Data represent relative luminescence units (RLU) per 10^6 viable cells. Activity of

mitochondrial dehydrogenases were measured using the WST-1® (Roche) kit in control [(-) doxy] and BI-1 over-expressing cells [(+) doxy] plus and minus SERCA over-expression (C) and SERCA activation (*F*). The final product (formazan) was quantified at 450 nm with an ELISA plate reader. Values were normalized for the number of viable cells based on trypan blue dye exclusion.

Figure S18. ***SERCA over-expression does not modify BI-1-mediated resistance to ER stress.*** Doxycycline-inducible HeLa cells were cultured without or with 1 ug/mL doxycycline [doxy] for 24 hrs to induce BI-1 expression. In indicated cases, cells were transiently transfected with control vs SERCA plasmids and treated with either thapsigargin (TG, 2.5 μM) or tunicamycin (TM, 10 μg/ml). *(A)* Cells were then stained with Annexin V and analyzed by FACS. Results are expressed as percentage of Annexin V^+ cells. *(B)* Cell lysates were normalized for protein content and subjected to SDS-PAGE/immonoblotting with anti-c-Jun and anti-phospho-c-Jun antibodies.

III. Supplemental References

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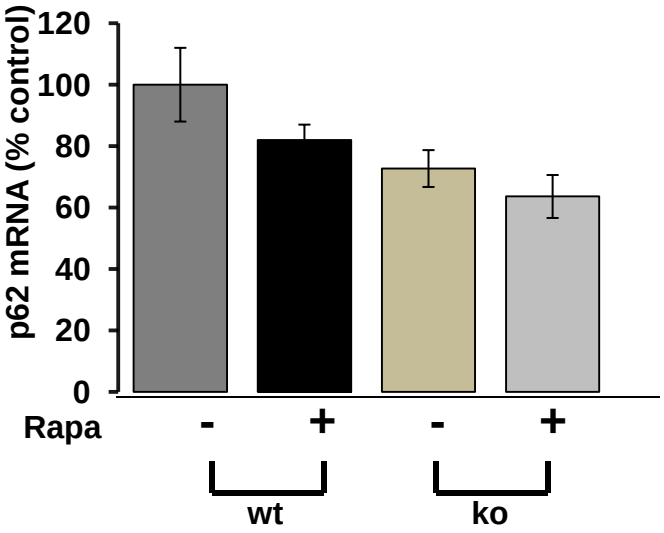
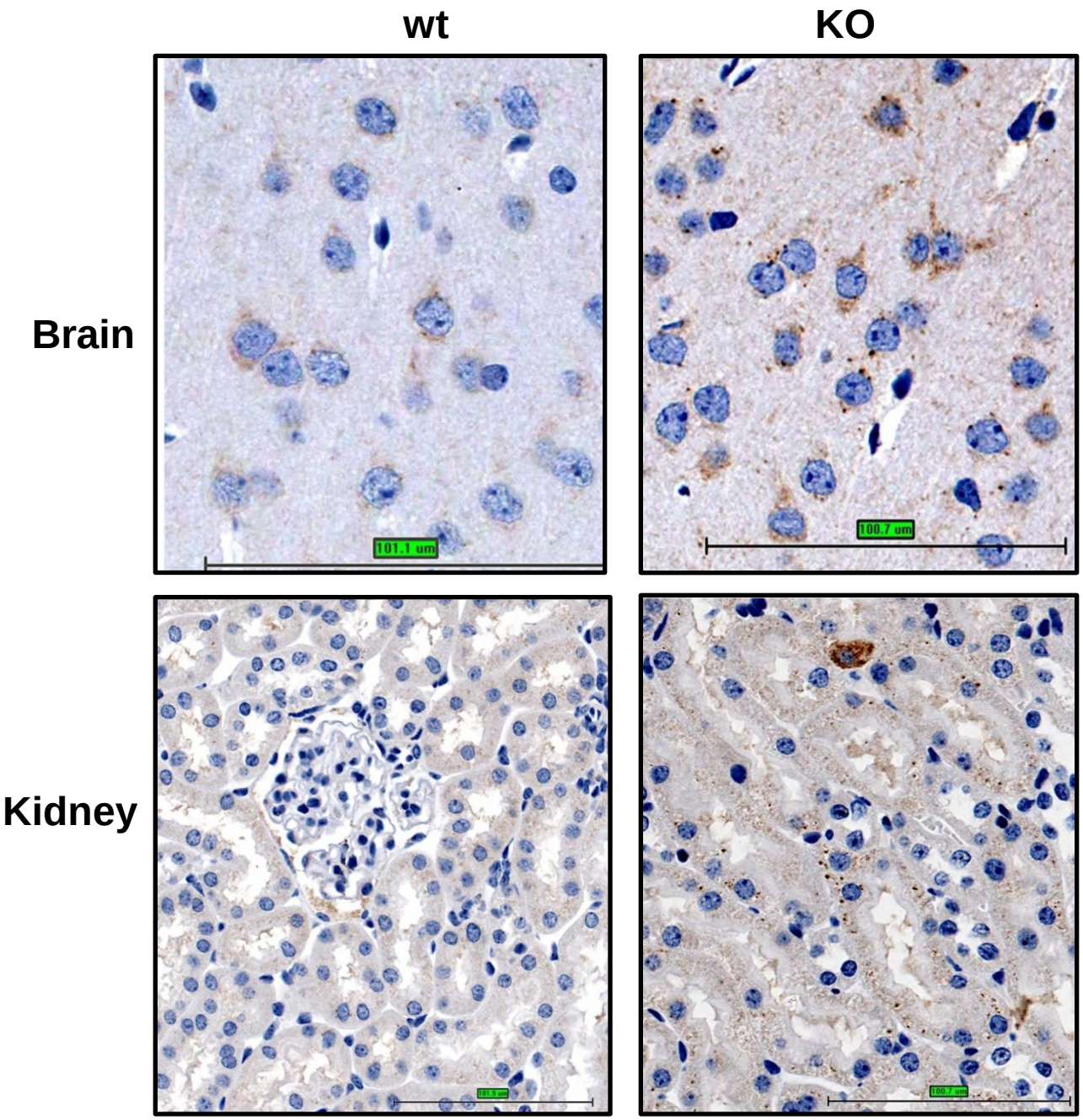
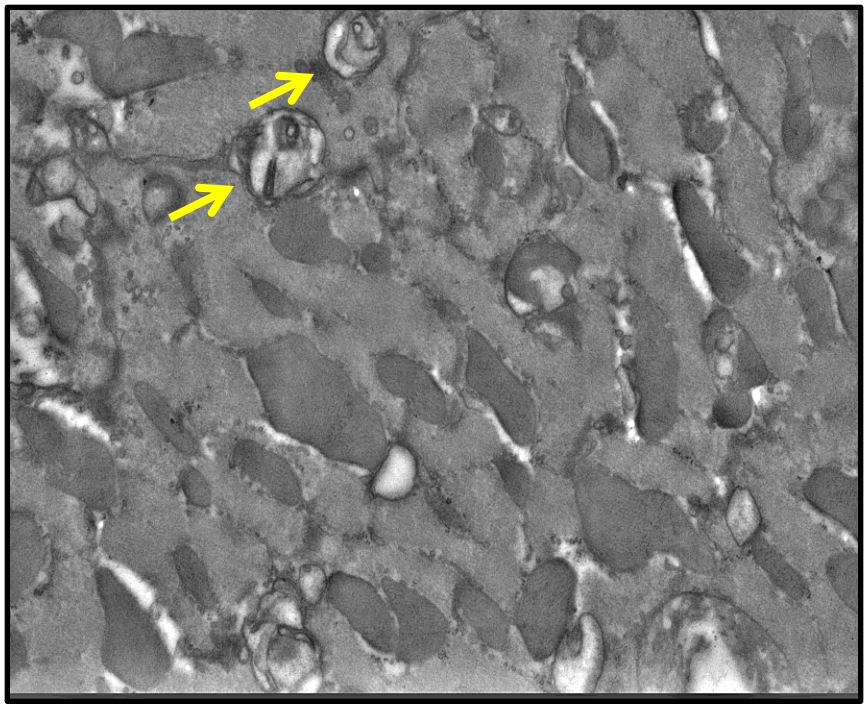

p62 mRNA (% control)
120
100
80
60
40
20
0
Rapa
-
+
-
+
wt
ko


wt
KO
Brain
Kidney
101.1 um
100.7 um

wt



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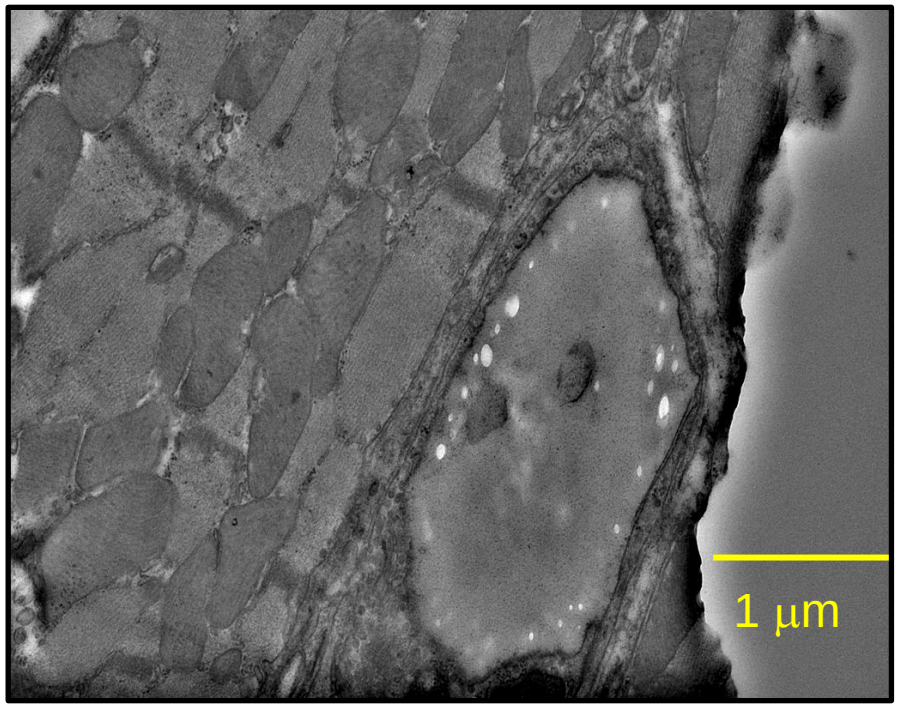

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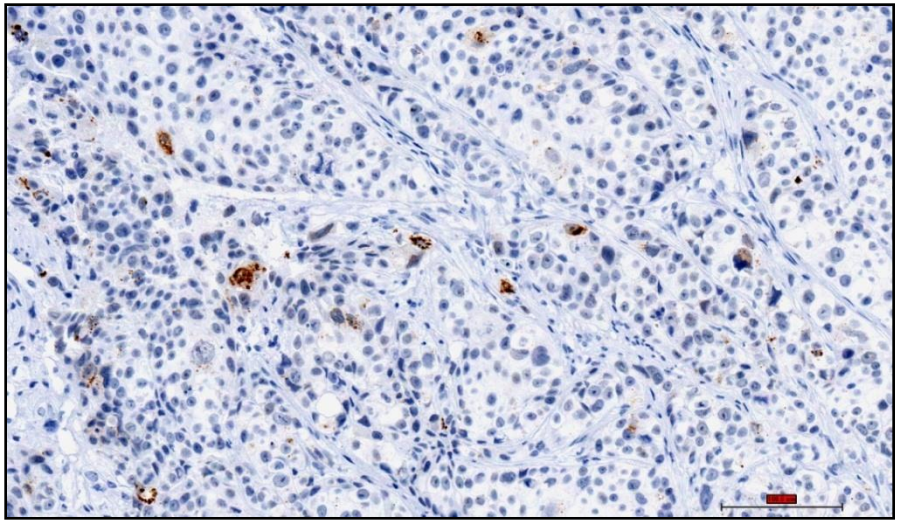

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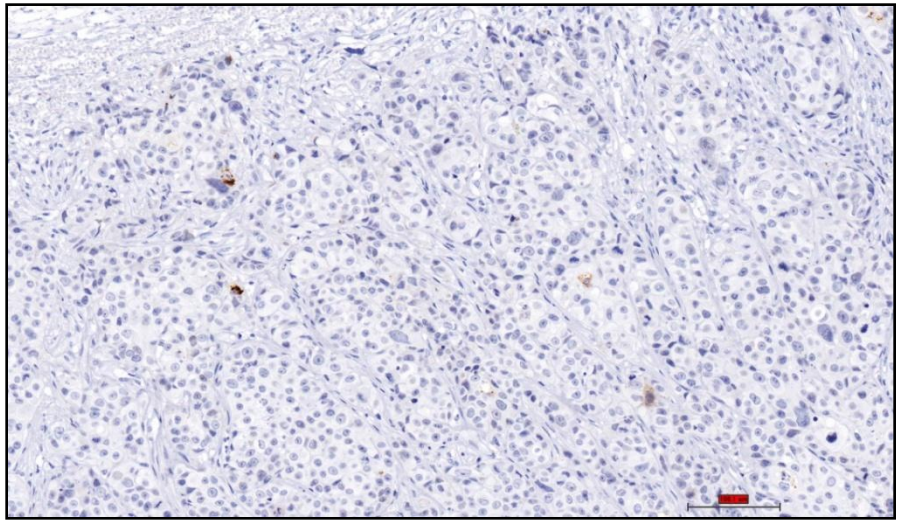
B



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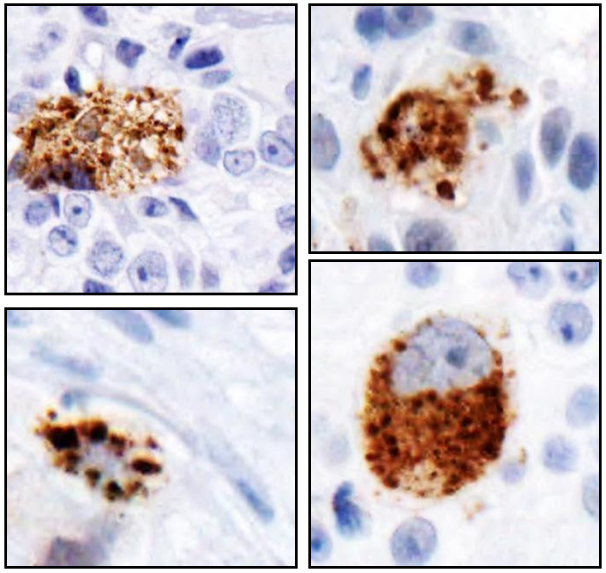
C



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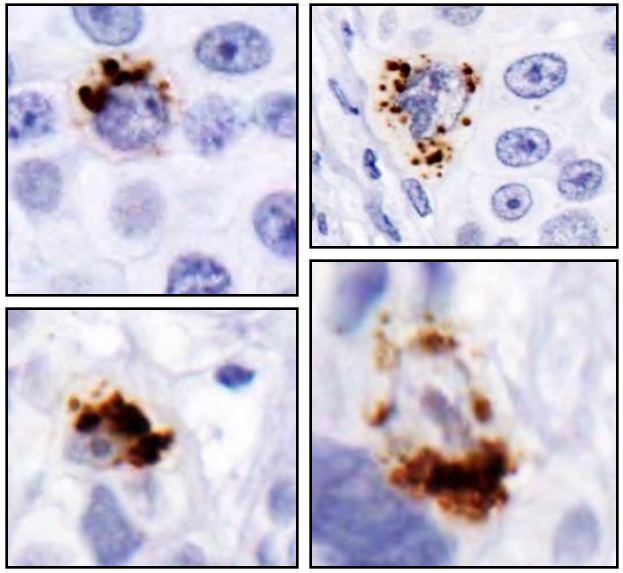

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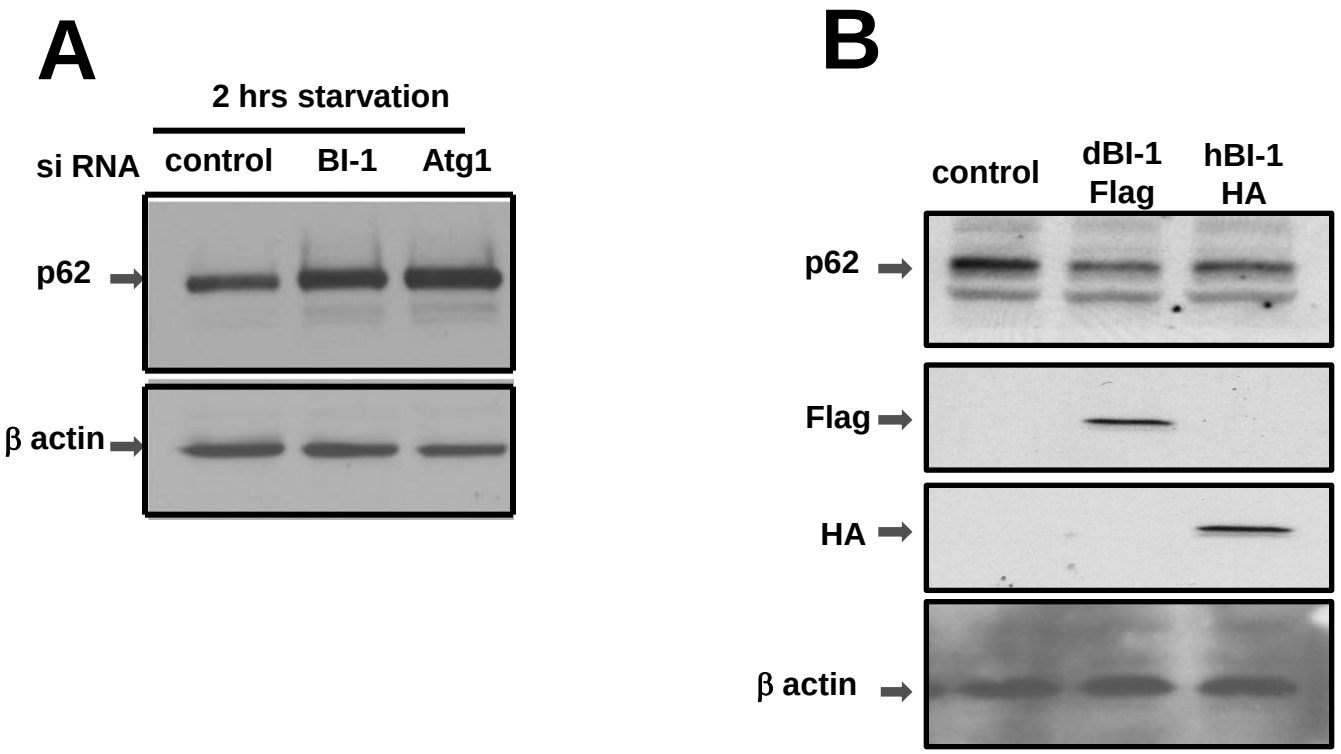
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Atg1
p62
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B
control
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Flag
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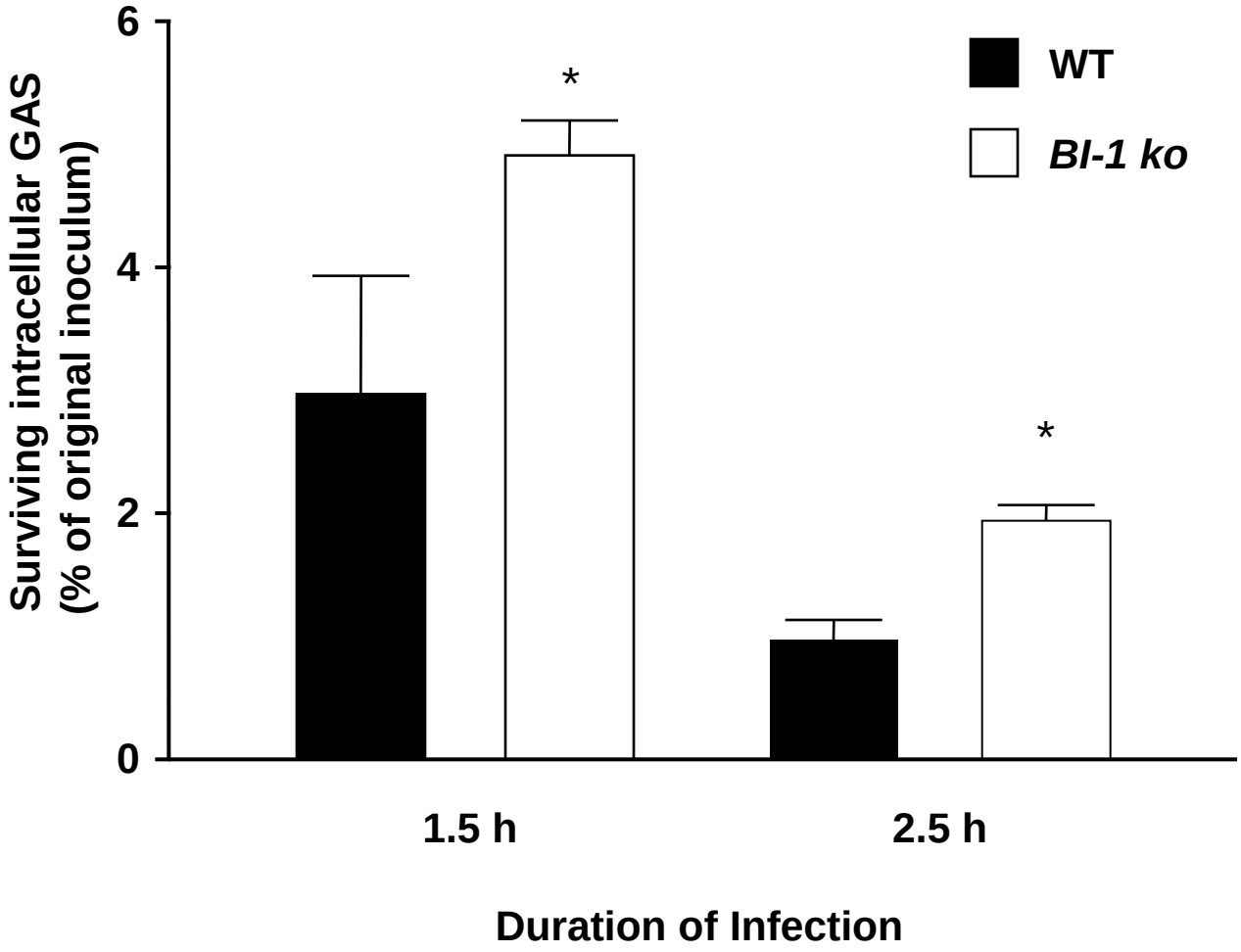
Surviving intracellular GAS
(% of original inoculum)
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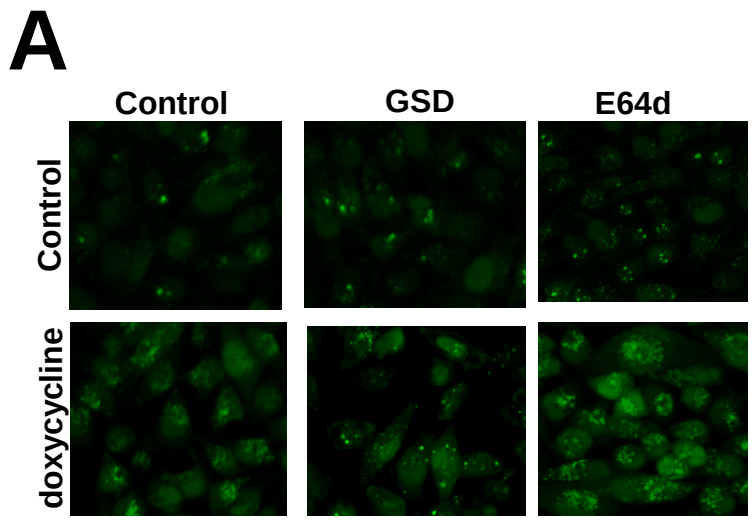
4
2
0
*
*
WT
BI-1 ko
1.5 h
2.5 h
Duration of Infection

Sano_FigS7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GSD
E64d
Control
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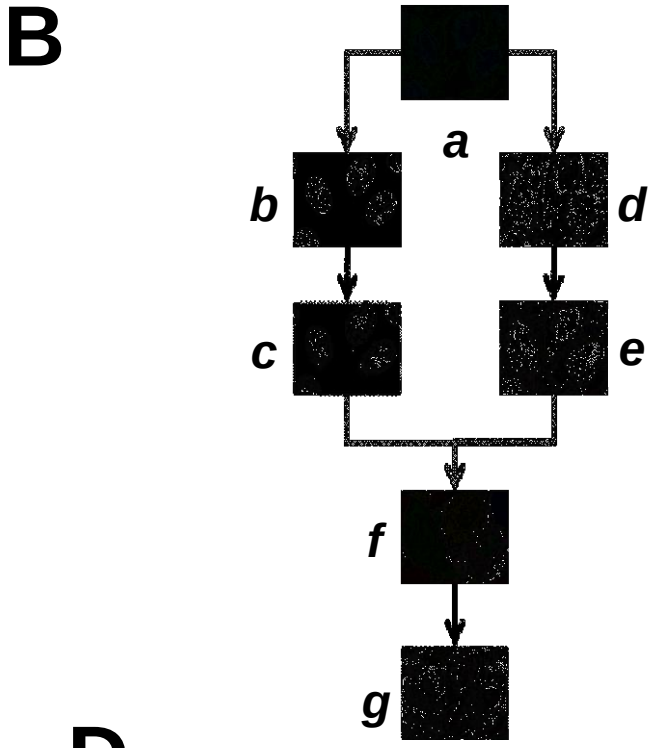
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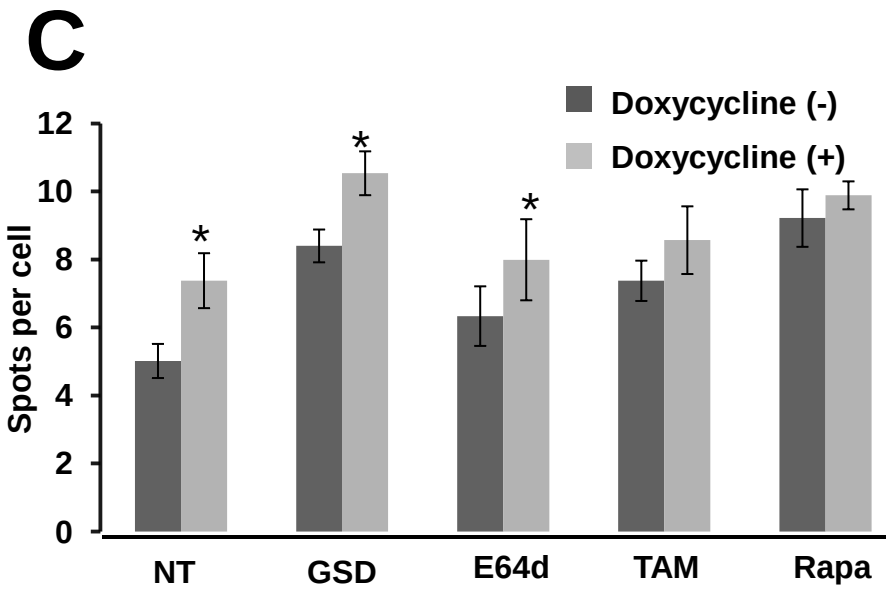
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NT
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TAM
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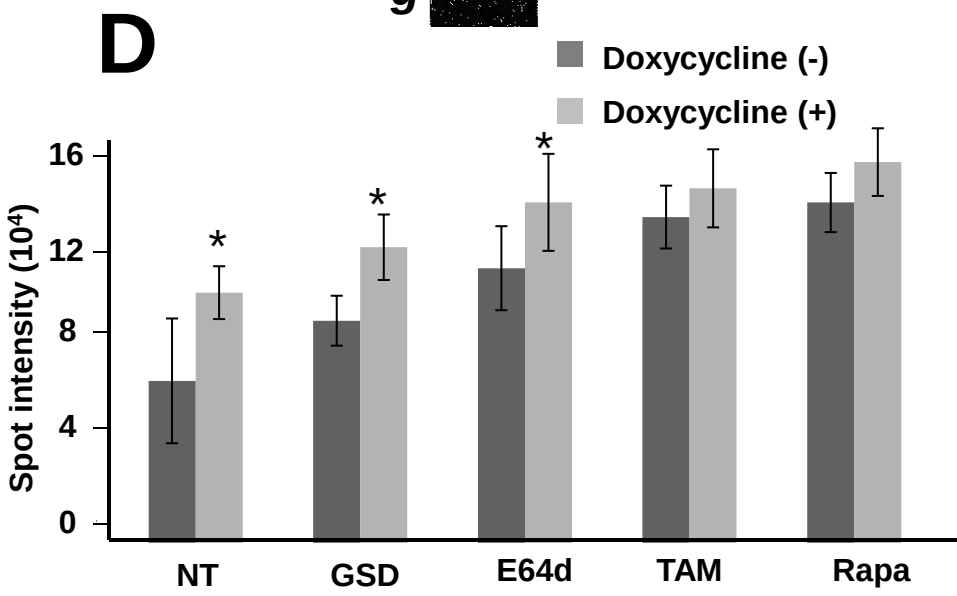
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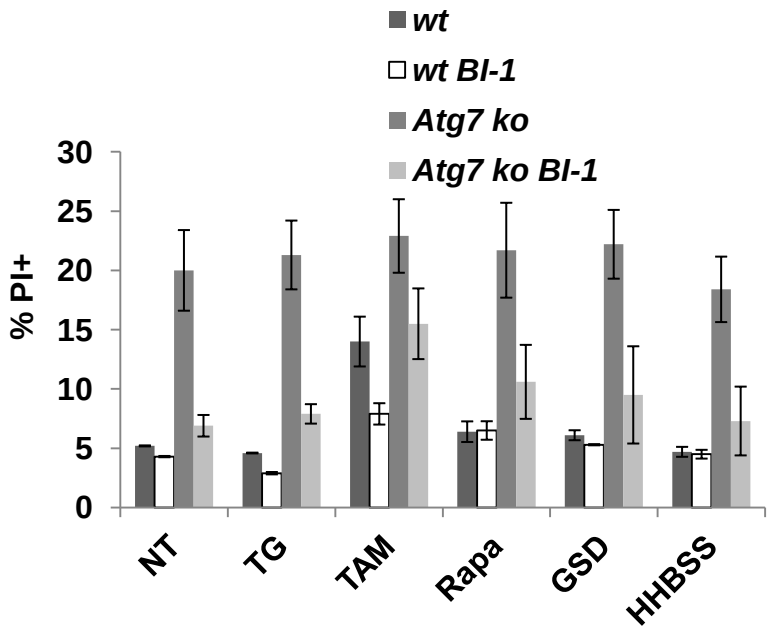
wt
wt Bl-1
Atg7 ko
Atg7 ko Bl-1
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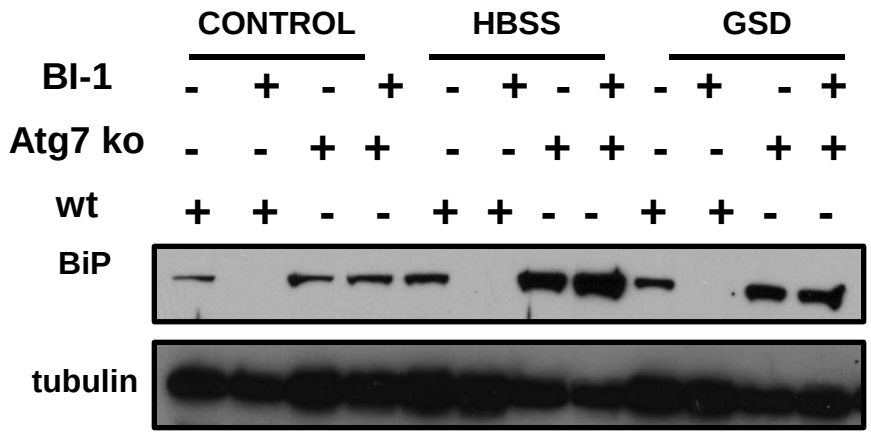
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HBSS
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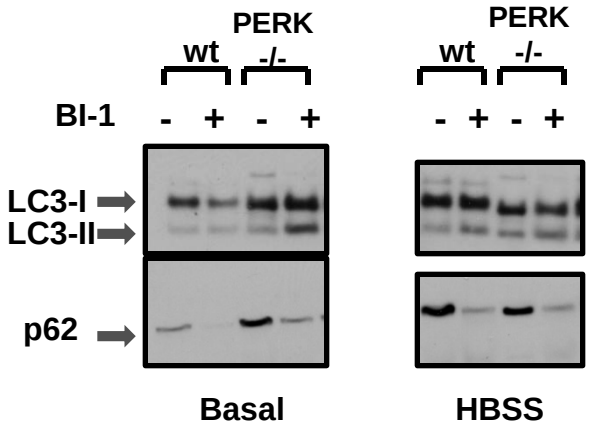
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-/-
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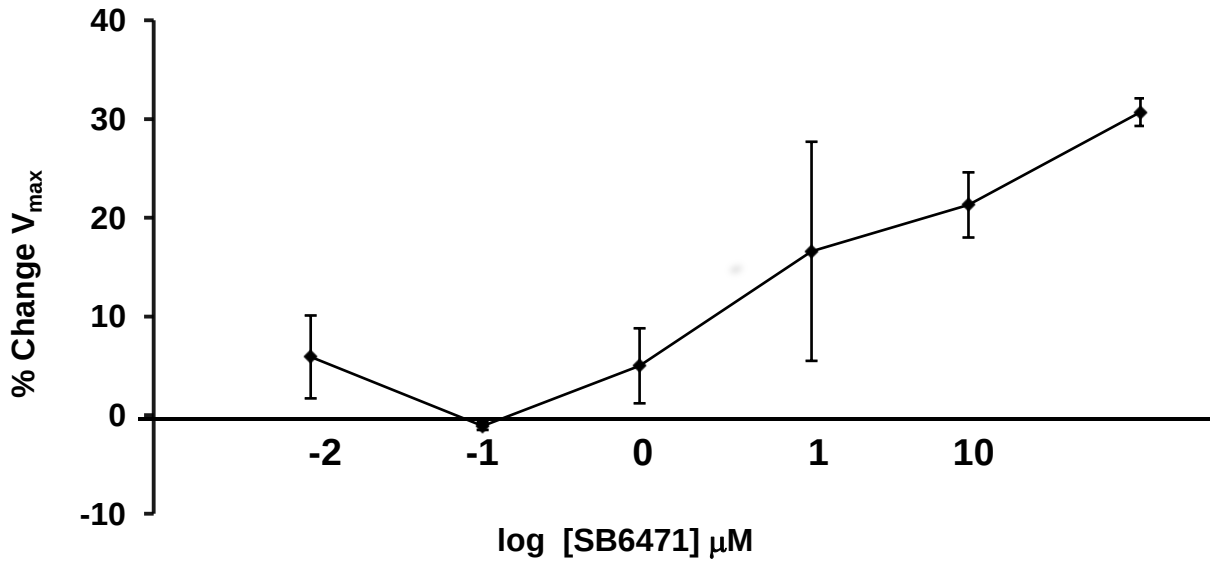
LC3-I
LC3-II
p62
Basal
HBSS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-10
% Change V_{max}
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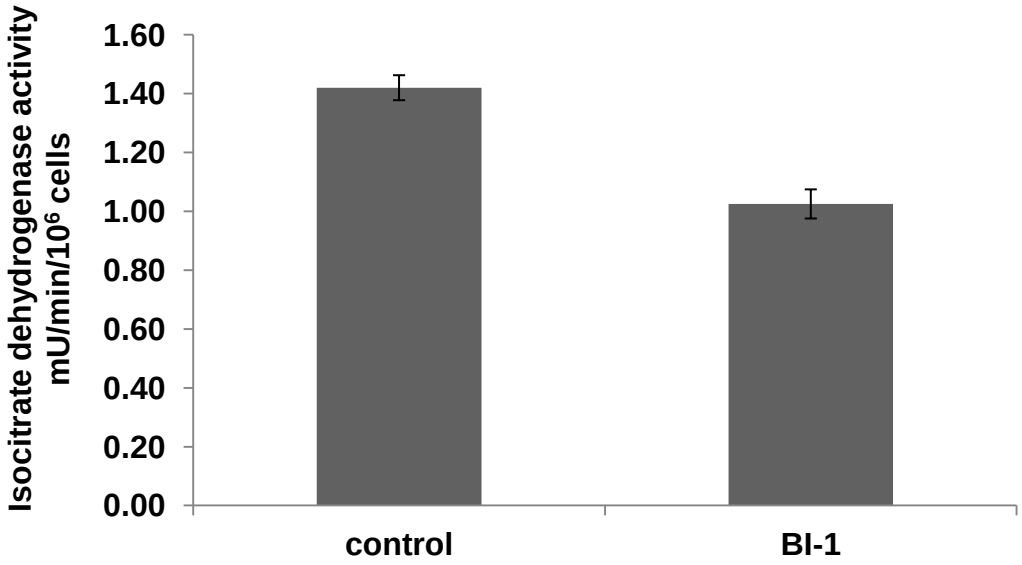
-1
0
1
10
log [SB6471] μM


Isocitrate dehydrogenase activity
mU/min/10^6 cells
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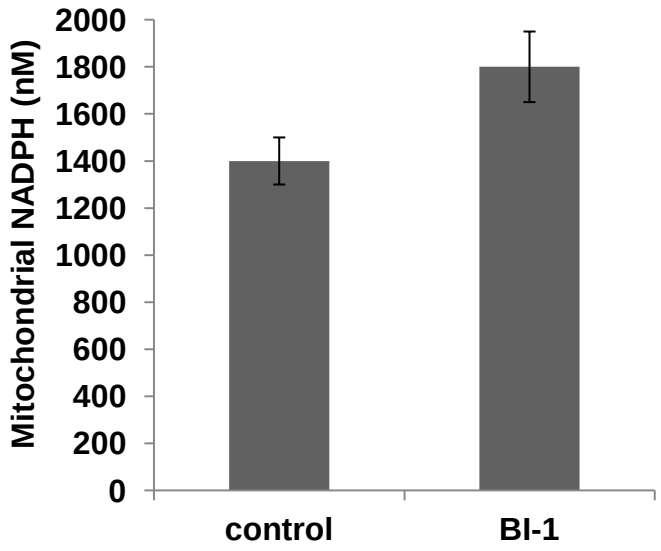
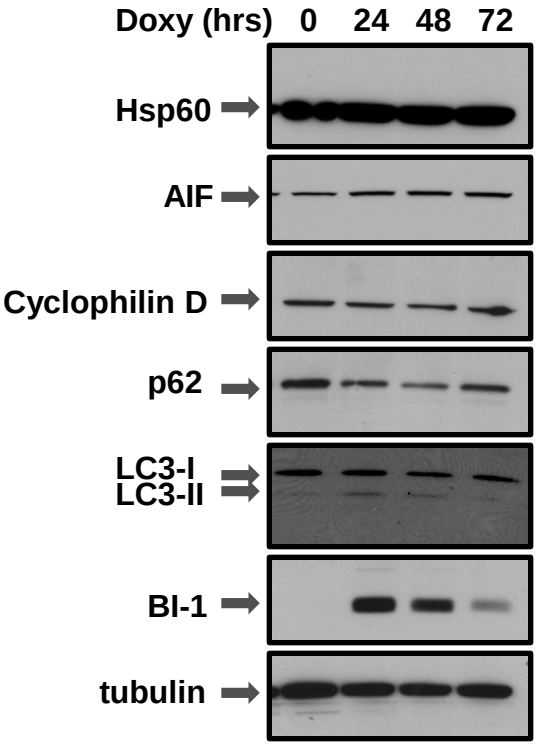
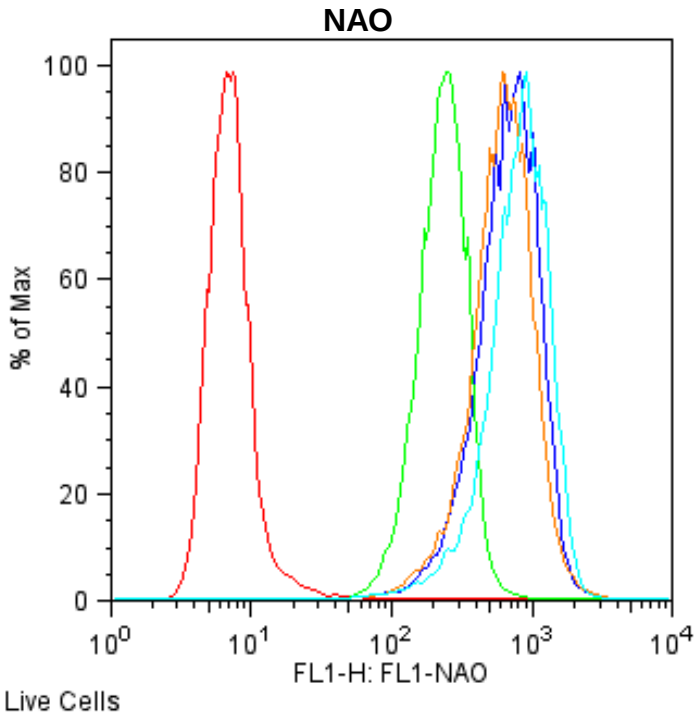
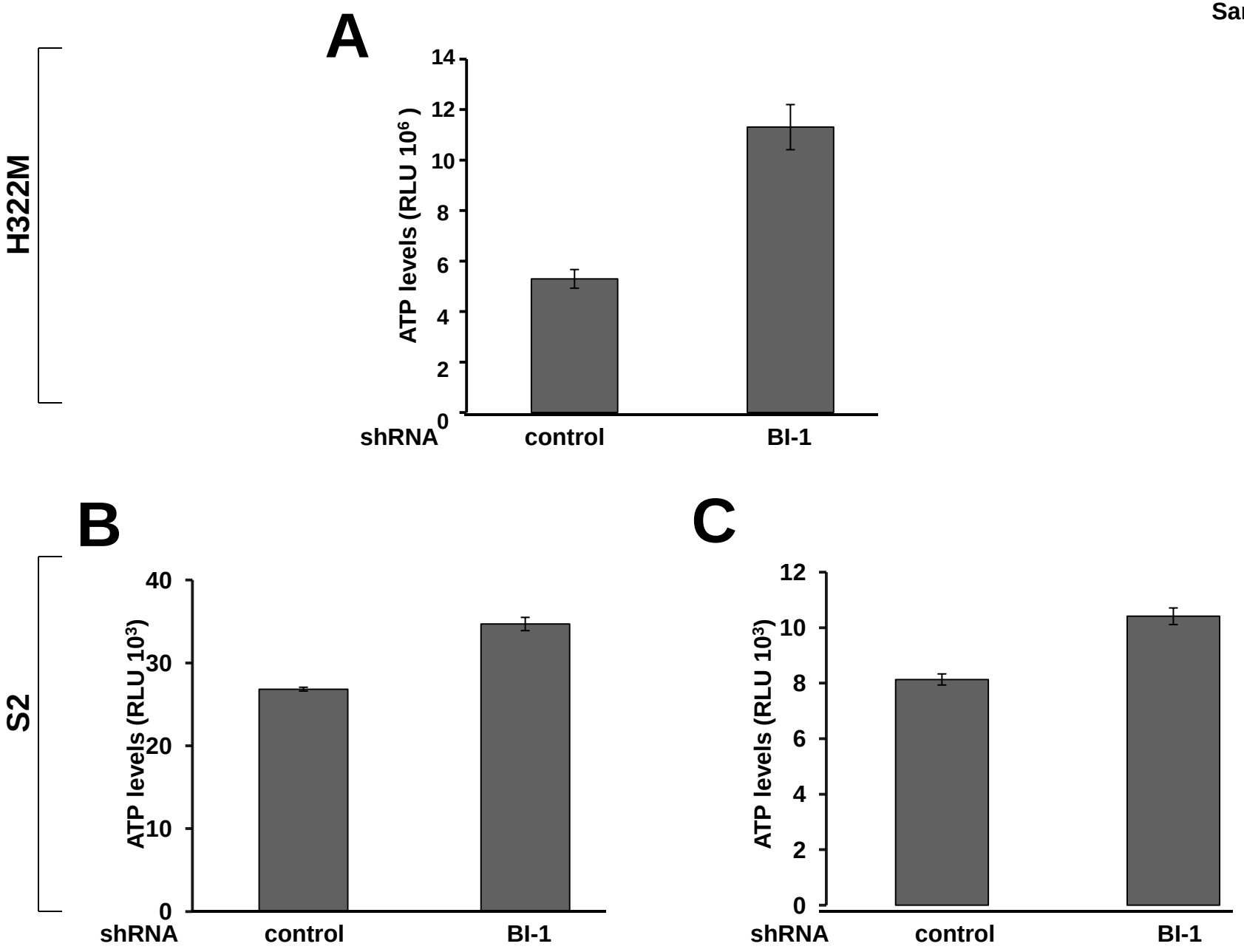
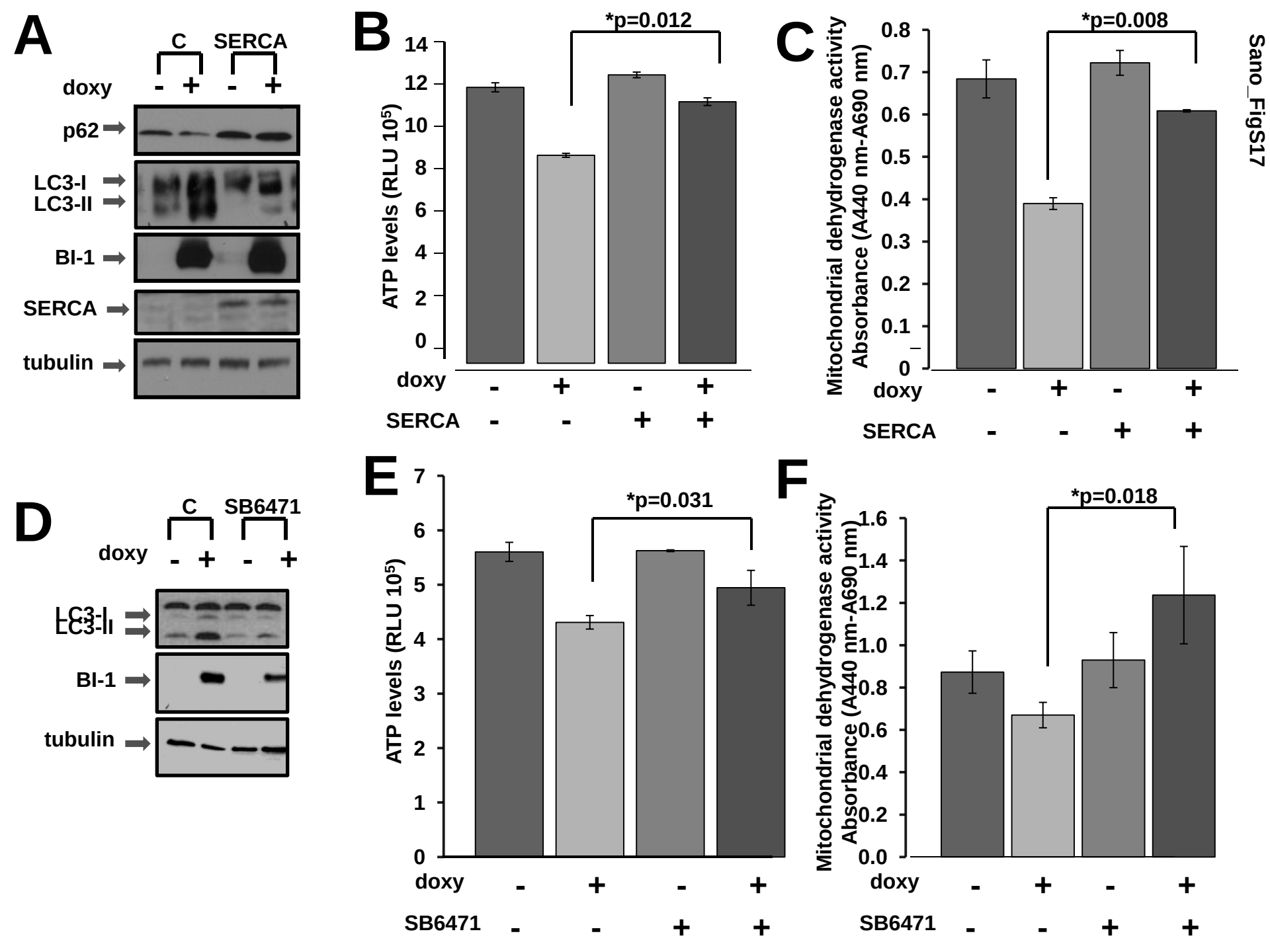
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
control
BI-1

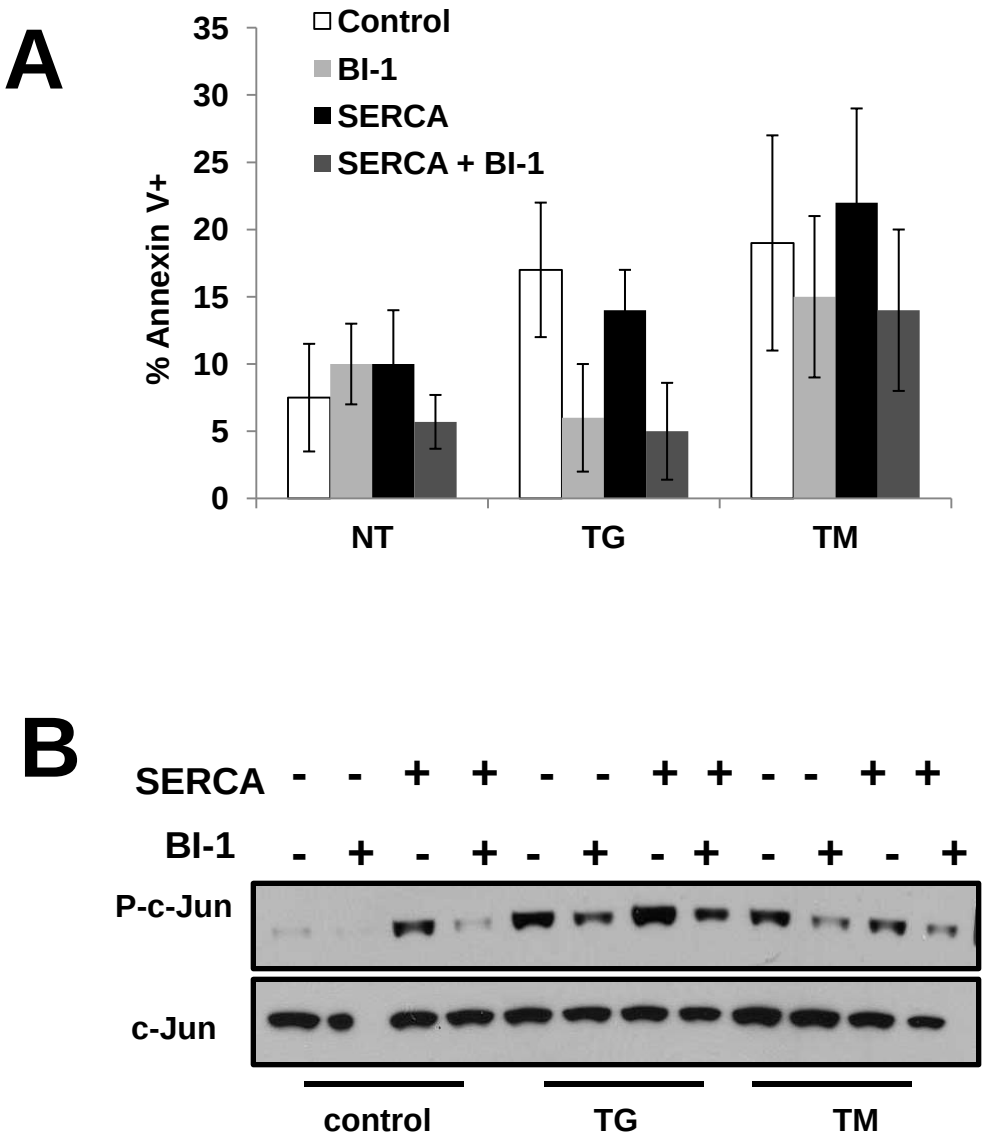

Mitochondrial NADPH (nM)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
control
BI-1


Doxy (hrs) 0 24 48 72
Hsp60
AIF
Cyclophilin D
p62
LC3-I
LC3-II
BI-1
tubulin


NAO
100
80
60
40
20
0
% of Max
10^0
10^1
10^2
10^3
10^4
FL1-H: FL1-NAO
Live Cells


A
H322M
ATP levels (RLU 10^6)
0
2
4
6
8
10
12
14
shRNA
control
BI-1
B
S2
ATP levels (RLU 10^3)
0
10
20
30
40
shRNA
control
BI-1
C
ATP levels (RLU 10^3)
0
2
4
6
8
10
12
shRNA
control
BI-1


A
C
SERCA
doxy
- + - +
p62
LC3-I
LC3-II
BI-1
SERCA
tubulin
B
*p=0.012
ATP levels (RLU 10^5)
14
12
10
8
6
4
2
0
doxy - + - +
SERCA - - + +
C
*p=0.008
Mitochondrial dehydrogenase activity
Absorbance (A440 nm-A690 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
doxy - + - +
SERCA - - + +
Sano_FigS17
D
C
SB6471
doxy
- + - +
LC3-I
LC3-II
BI-1
tubulin
E
*p=0.031
ATP levels (RLU 10^5)
7
6
5
4
3
2
1
0
doxy - + - +
SB6471 - - + +
F
*p=0.018
Mitochondrial dehydrogenase activity
Absorbance (A440 nm-A690 nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
doxy - + - +
SB6471 - - + +


A
Control
BI-1
SERCA
SERCA + BI-1
% Annexin V+
35
30
25
20
15
10
5
0
NT
TG
TM
B
SERCA - - + + - - + + - - + +
BI-1 - + - + - + - + - + - +
P-c-Jun
c-Jun
control
TG
TM