Supplementary information for

Inhibiting neddylation modification alters mitochondrial morphology and reprograms energy metabolism in cancer cells

Qiyin Zhou, Hua Li, Yuanyuan Li, Mingjia Tan, Shaohua Fan, Cong Cao, Feilong Meng, Ling Zhu, Lili Zhao, Min-Xin Guan, Hongchuan Jin, and Yi Sun

Supplementary figure legends

Figure S1. Blockage of neddylation induces mitochondrial fusion.

MDA-MB-231 (A), and SK-BR-3 (B), BEAS2B (C), and A549 (D) cells were exposed to different concentrations of MLN4924 for indicated time points, stained with MitoTracker Red, and the images of mitochondrial morphology were photographed by confocal microscope. MDA-MB-231 and SK-BR-3 cells were transfected with siRNA targeting NAEβ, stained with MitoTracker Red, and the images of mitochondrial morphology were photographed by confocal microscope (E). Scale bars: 10 μM.

Figure S2. Mitochondrial dynamics regulators mediate MLN4924-induced mitochondrial fusion.

(A, B) SK-BR-3 cells were treated with various concentrations of MLN4924 for 24 or 48h (A), or treated with 300 nM MLN4924 for indicated time periods (B) and subjected to Western Blotting using indicated antibodies. * designates a non-specific band.

(C) SK-BR-3 cells were treated with 300 nM MLN4924 for 48h and mitochondria were isolated. Cytoplasmic (CY) and mitochondrial (MT) fractions were subjected to Western Blotting with indicated antibodies. α-tubulin and Tom20 were served as loading marker for cytoplasmic and mitochondrial fractions, respectively. SE: short exposure; LE: long exposure.

(D) The DRP1 expression levels (C) in cytosol and mitochondria were normalized to α-tubulin and Tom20, respectively. Results are shown as mean ± SD (n=3). $^{**}P<0.01$, one-way ANOVA.

(E) The phosphorylated $DRP1^{S616}$ levels (C) were normalized by total DRP1 levels in indicated cellular fraction (mean ± SD, n=3). . $^{**}P<0.01$, one-way ANOVA.

(F and G) SK-BR-3 cells were transfected with indicated siRNAs against MFN1 or MFN2. Forty-eight hrs posttransfection, cells were harvested and subjected to Western Blotting with indicated antibodies. * designates a non-specific band

(H, I) SK-BR-3 cells were transfected with indicated siRNAs (H) or plasmids expressing HA-vector (I) for 24h, and then treated with 300 nM MLN4924 for another 24h, cells were then stained with MitoTracker Red, and mitochondrial morphology was photographed by confocal microscope. The interconnected filamental mitochondria are quantified (mean ± SD, n=3). . *P<0.05, **P<0.01, one-way ANOVA.

(J) SK-BR-3 cells were transfected with HA-vector or HA-DRP1 for 24h, followed by treatment with various concentrations MLN4924 for another 24h before being subjected to Western Blotting using indicated antibodies. * designates a non-specific band.

(K) SK-BR-3 cells transfected with indicated siRNAs against MFN1 or MFN2 and plasmids expressing HA-vector for 24h, treated with MLN4924 at 100 or 300 nM, followed by FACS analysis after 24h. Cells at the G2/M phase were plotted (mean ± SD, n=3).

(L) SK-BR-3 cells were transfected with either si-NC, si-MFN1#2 or si-MFN2#2 for 24h, then treated with MLN4924 at 100 or 300 nM, followed by trypan blue exclusion assay for cell viability after 48h (mean ± SD, n=3). **P<0.01, one-way ANOVA.

(M) SK-BR-3 cells were transfected with HA-vector and HA-DRP1 for 24h, then treated with MLN4924 at 100 or 300 nM, followed by trypan blue exclusion assay for cell viability after 48h (mean ± SD, n=3). **P<0.01, one-way ANOVA.

Figure S3. $SCF^{\beta\text{-}TrCP1}$ negatively regulates MFN1 level and turnover.

(A) HeLa cells were treated with various concentrations of MLN4924 for 24 or 48h, and subjected to Western blotting using indicated antibodies. *designates a non-specific band.

(B) Evolutionary conservation of β-TrCP degron motif on MFN1.

(C) SK-BR-3 cells were treated with 300 nM MLN4924 for 24h and mitochondria were isolated. Cytoplasmic (CY) and mitochondrial (MT) fractions were subjected to Western blotting with indicated antibodies. α-tubulin and Tom20 were served as loading marker for cytoplasmic and mitochondrial fractions, respectively.

(D) HEK293 cells were transfected with indicated plasmids, immunoprecipated with HA agarose beads and subjected to Western blotting to detect exogenous β-TrCP1.

(E, F) SK-BR-3 cells were co-transfected with MFN1 and increasing amounts of β-TrCP1 or β-TrCP1ΔF (E), or transfected with increasing amounts of β-TrCP1 or β-TrCP1ΔF alone (F) for 48h, respectively, cells were then subjected to Western blotting with indicated antibodies. The band density was quantified by Image J and normalized with loading control α-Tubulin. * designates a non-specific band.

(G, H) SK-BR-3 cells were transfected with eithersi-NC, or siRNA targeting β-TrCP1/2 for 48h, respectively, then incubated with CHX for indicated periods of time. Cells were collected and subjected to Western blotting with indicated antibodies (G). (H) MFN1 band density in (G) was quantified using Image J software and normalized to α-tubulin, then normalized to t = 0 time point. * designates a non-specific band.

(I) MDA-MB-231 and SK-BR-3 cells were transfected with FLAG-MFN1 or FLAG-MFN1 S85/86/90A mutant for 48 h, respectively, then incubated with CHX for indicated periods of time. Cells were collected and subjected to Western blotting with indicated antibodies.

(J, K) SK-BR-3 cells were co-transfected with β-TrCP1 and MFN1 or MFN1 S85/85/90A mutant for 48h, then incubated with CHX for indicated periods of time. Cells were collected and subjected to Western Blotting with indicated antibodies (J). (K) FLAG band density in (J) was quantified using Image J software and normalized to α-tubulin, then normalized to t = 0 time point.

(L) H1299 , H1650, and BEAS2B cells were transfected with either si-NC, or siRNA targeting β-TrCP1/2 for 48h, respectively, cells were then subjected to Western blotting with indicated antibodies. * designates a non-specific band.

(M) H1299, H1650, and BEAS2B cells were transfected with FLAG-tagged-vector or FLAG-β-TrCP1 for 48 h, and subjected to Western blotting using indicated antibodies. * designates a non-specific band.

Figure S4. Hierarchical clustering of altered metabolites.

(A, B) MDA-MB-231 cells were treated with vehicle or 300 nM MLN4924 for 24h, altered metabolites in cell extracts (A) and culture supernatant (B) with VIP > 1 are displayed with V-plots.

(C) Box plots of metabolites levels from cell extracts (3-Phosphoglyceric acid, Succinic acid, and Fumaric acid) and culture supernatant (Pyruvic acid). Results are shown as mean ± SD from three independent experiments.

(D) Flow chart of glucose metabolism and TCA cycle with metabolites altered by MLN4924 shown. VIP values with red color indicate the increase, whereas green color indicates the decrease. The step catalyzed by pyruvate kinase is marked.

Figure S5. MLN4924 inhibits mitochondrial functions.

(A-H) SK-BR-3 cells were treated with indicated concentrations of MLN4924 for 24h, and 48h, respectively, then subjected to determination of real time OCR (A, B). Basal OCR (C), ATP-linked OCR (D), proton leak OCR (E), Maximal OCR (F), non-mitochondrial OCR (G), and reserve capacity OCR (H) were calculated from data shown in A and B (mean ± SD, n=3). *P<0.05, **P<0.01, one-way ANOVA.

(I-K) SK-BR-3 cells were treated with indicated concentrations of MLN4924 for 24h, and 48h, respectively, then subjected to analysis for ATP production (I), mitochondrial membrane potential (J), mtDNA copy number (K) (mean ± SD, n=3). *P<0.05, **P<0.01, one-way ANOVA.

(L) SK-BR-3 cells were treated with indicated concentrations of MLN4924 for 24h, and 48h, respectively, and mitochondrial ROS generation was determined using the mitochondrial superoxide indicator MitoSOX-Red. Fluorescence was measured using a FACS instrument. Similar results were got from three independent experiments.

Figure S6. MFN1 is responsible for some of altered mitochondrial functions by MLN4924 in MDA-MB 231 cells.

(A-G) MDA-MB-231 cells were transfected with either si-NC or si-MFN1#2 for

48h, then treated with indicated concentrations of MLN4924 for 24h and subjected to determination of real time OCR (A). Basal OCR (B), ATP-linked OCR (C), proton leak OCR (D), Maximal OCR (E), non-mitochondrial OCR (F), and reserve capacity OCR (G) were calculated from data shown in A (mean ± SD, n=3). *P<0.05, **P<0.01, one-way ANOVA.

(H) MDA-MB-231 cells were treated with indicated concentrations of MLN4924 for 24h, then subjected to analysis for ECAR, which were simultaneously recorded to OCR as shown in Figure S6A.

Figure S7. MFN1 is responsible for some of altered mitochondrial functions by MLN4924 in SK-BR3 cells

(A-G) SK-BR-3 cells were transfected with either si-NC or si-MFN1#2 for 48h, then treated with indicated concentrations of MLN4924 for 24h and subjected to determination of real time OCR (A). Basal OCR (B), ATP-linked OCR (C), proton leak OCR (D), Maximal OCR (E), non-mitochondrial OCR (F), and reserve capacity OCR (G) were calculated from data shown in A (mean ± SD, n=3). *P<0.05, **P<0.01, one-way ANOVA.

(H) SK-BR-3 cells were treated with indicated concentrations of MLN4924 for 24h, then subjected to analysis for ECAR, which were simultaneously recorded to OCR as shown in Figure S7A.

Figure S8. MLN4924 promotes glycolysis.

(A, B) SK-BR-3 cells were treated with indicated concentrations of MLN4924 for 24h, and 48h, respectively, then subjected to analysis for ECAR, which were simultaneously recorded to OCR as shown in Figure S5 A&B.

(C-F) SK-BR-3 cells were treated with indicated concentrations of MLN4924 for 24h, and 48h, respectively, then the culture media were collected to analyze glucose consumption (C), pyruvate (D), and lactate (E) production, whereas cells were collected to measure pyruvate kinase activity (F) (mean ± SD, n=3). *P<0.05, **P<0.01, one-way ANOVA.

(G) SK-BR-3 cells were transfected with either scrambled control siRNA (si-NC), two independent siRNAs targeting PKM2 (si-PKM21#1, si-PKM2#2). Forty-eight hrs posttransfection, cells were harvested and subjected to Western Blotting with indicated antibodies.

(H) SK-BR-3 cells were transfected with indicated siRNAs for 24h, and then treated with various concentrations of MLN4924 for another 24h, cells were collected to measure pyruvate kinase activity (mean ± SD, n=3). ***P*<0.01, one-way ANOVA.

(I) SK-BR-3 cells were treated with indicated concentrations of MLN4924 for 24h, cross-linked using glutaraldehyde and subjected to Western Blotting with indicated antibodies. GA, glutaraldehyde.

(J) Pyruvate kinase activity of purified human PKM2 (from elution fractions 1 and 2) in the presence of increasing concentrations of SAICAR.

(K) SK-BR-3 cells were transfected with either si-NC or si-MFN2#2 for 24h, then treated with various concentrations of MLN4924 for 72h and cell viability was detected by trypan blue exclusion assay (mean ± SD, n=3). ***P*<0.01, one-way ANOVA.

(L) SK-BR-3 cells were transfected with either si-NC or si-MFN2#2, then treated with DMSO control or 5 nM MLN4924 for 10 days.Colonies were stained and counted (mean ± SD, n=3). ***P*<0.01, one-way ANOVA.

Figure S9. Combinational targeting of neddylation and OXPHOS or PKM2.

(A, B) MDA-MB-231 and SK-BR-3 cells were treated with various concentrations of metformin for 72h and cell number was counted through trypan blue exclusion assay (mean ± SD, n=3).

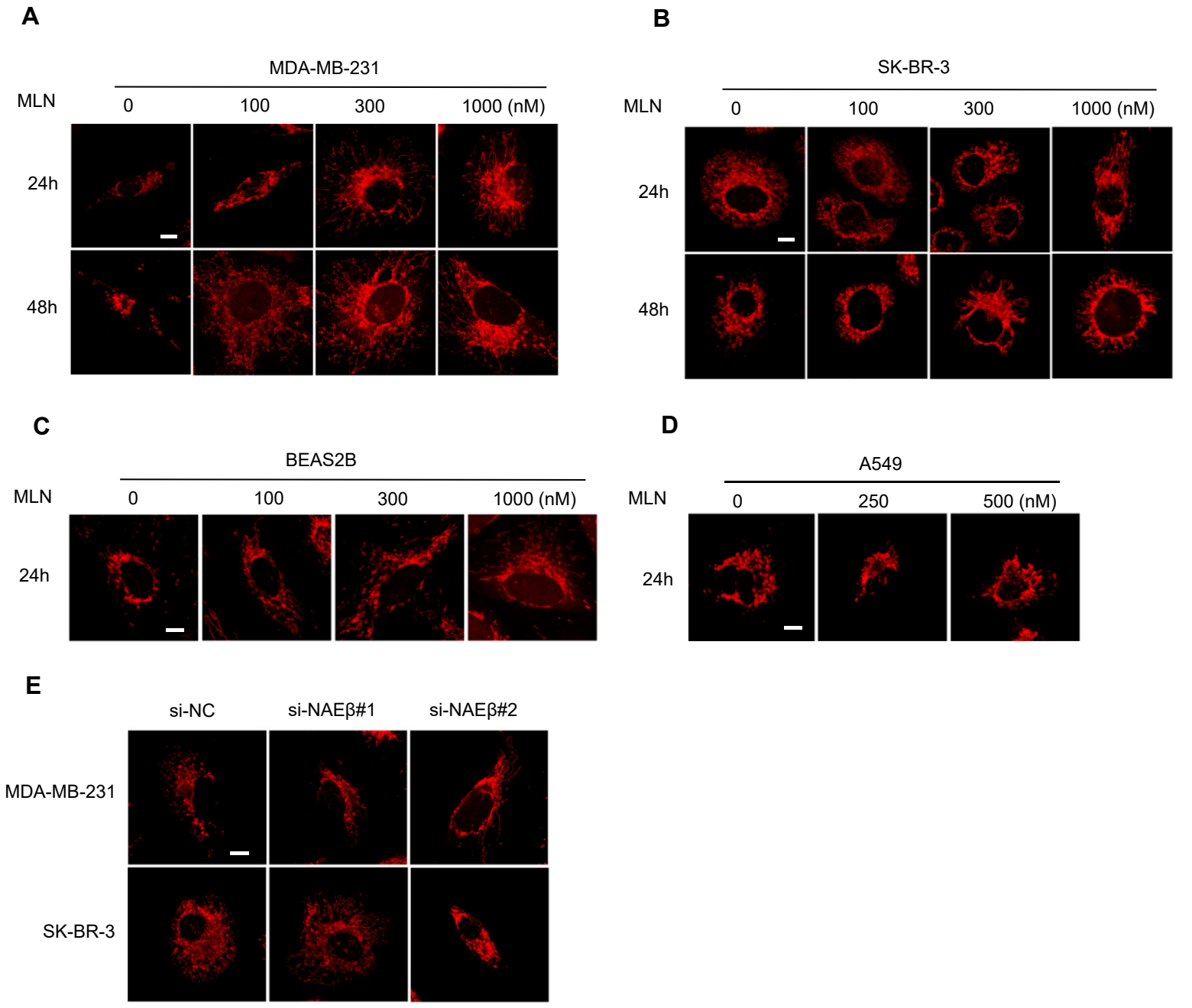
(C) SK-BR-3 cells were treated with various concentrations of MLN4924 and 2.5 mM metformin for 72h and cell number was counted through trypan blue exclusion assay (mean ± SD, n=3). ***P*<0.01, one-way ANOVA.

(D) SK-BR-3 cells were treated with 30 nM MLN4924 and 0.25 mM metformin or 0.3 μM shikonin to measure clonogenic growth (mean ± SD, n=3). ***P*<0.01, one-way ANOVA.

(E, F) MDA-MB-231 and SK-BR-3 cells were treated with various concentrations of 1 μM Shikonin for 72h and cell number was counted through trypan blue exclusion assay (mean ± SD, n=3).

(G) SK-BR-3 cells were treated with various concentrations of MLN4924 and Shikonin for 72h and cell number was counted through trypan blue exclusion assay (mean ± SD, n=3). $^{**}P<0.01$, one-way ANOVA.

(H, I) Body weight of mice carrying MDA-MB-231 (n=6-10) (H) and SK-BR-3 (n=4) (I) xenografts during treatment with MLN4924, metformin or shikonin, alone or in combination.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MLN
0
100
300
1000 (nM)
24h
48h
B
SK-BR-3
MLN
0
100
300
1000 (nM)
24h
48h
C
BEAS2B
MLN
0
100
300
1000 (nM)
24h
D
A549
MLN
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24h
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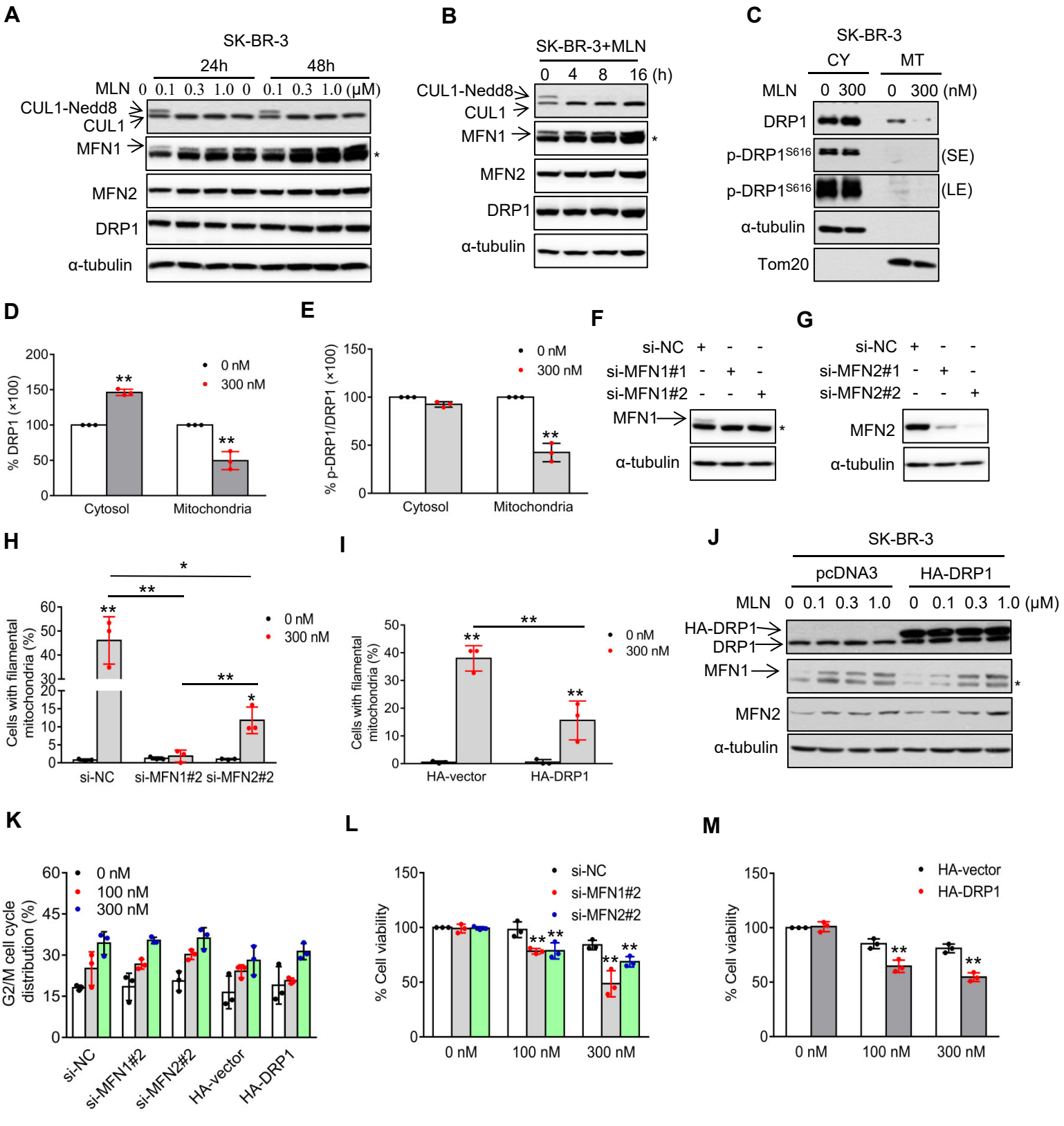
SK-BR-3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SK-BR-3
24h
48h
MLN 0 0.1 0.3 1.0 0 0.1 0.3 1.0 (μM)
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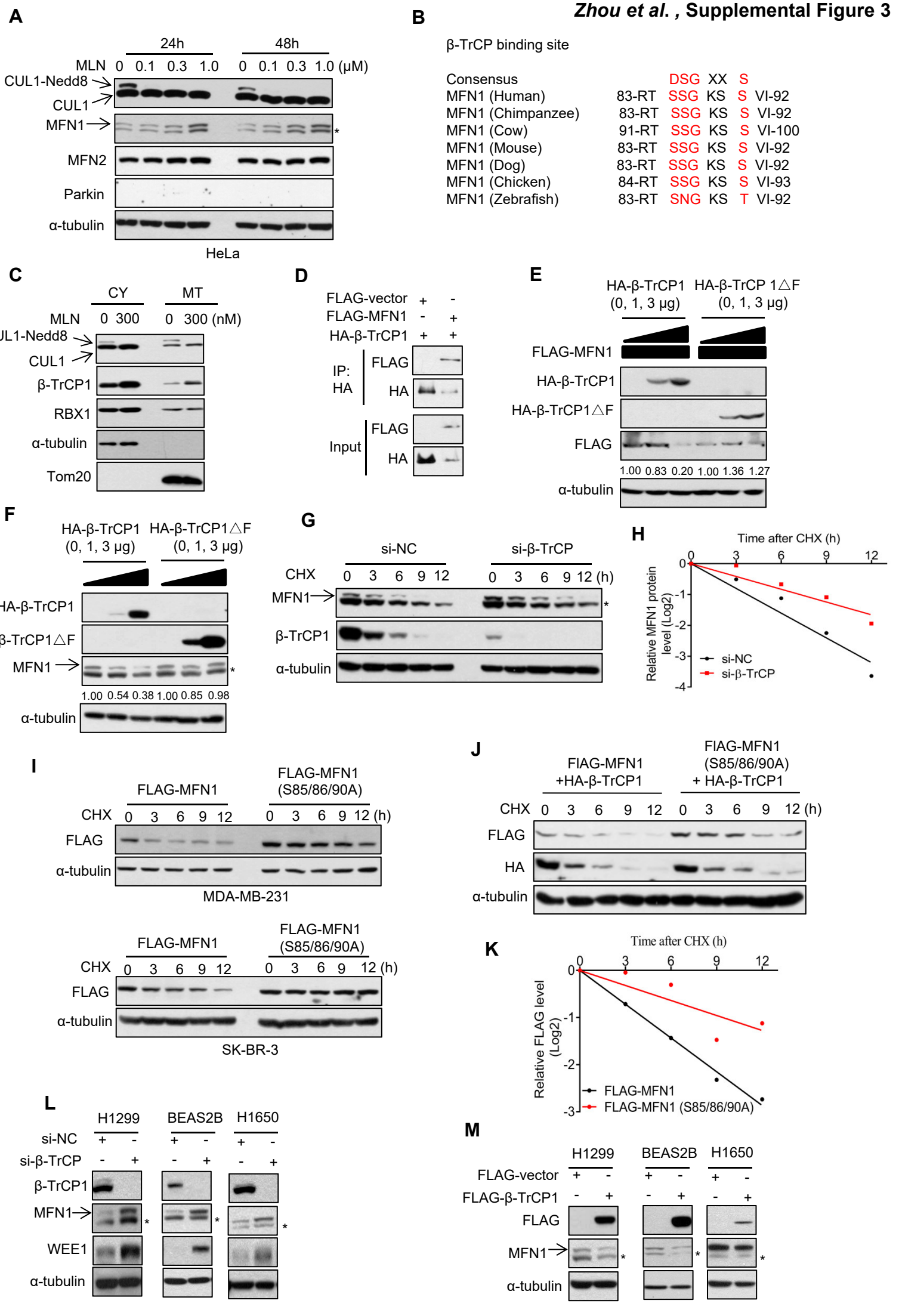
CUL1
MFN1
MFN2
DRP1
α-tubulin
B
SK-BR-3+MLN
0 4 8 16 (h)
CUL1-Nedd8
CUL1
MFN1
MFN2
DRP1
α-tubulin
C
SK-BR-3
CY
MT
MLN 0 300 0 300 (nM)
DRP1
p-DRP1S616
(SE)
p-DRP1S616
(LE)
α-tubulin
Tom20
D
% DRP1 (×100)
0 nM
300 nM
Cytosol
Mitochondria
E
% p-DRP1/DRP1 (×100)
0 nM
300 nM
Cytosol
Mitochondria
F
si-NC
si-MFN1#1
si-MFN1#2
MFN1
α-tubulin
G
si-NC
si-MFN2#1
si-MFN2#2
MFN2
α-tubulin
H
Cells with filamental mitochondria (%)
0 nM
300 nM
si-NC
si-MFN1#2
si-MFN2#2
I
Cells with filamental mitochondria (%)
0 nM
300 nM
HA-vector
HA-DRP1
J
SK-BR-3
pcDNA3
HA-DRP1
MLN 0 0.1 0.3 1.0 0 0.1 0.3 1.0 (μM)
HA-DRP1
DRP1
MFN1
MFN2
α-tubulin
K
G2/M cell cycle distribution (%)
0 nM
100 nM
300 nM
si-NC
si-MFN1#2
si-MFN2#2
HA-vector
HA-DRP1
L
% Cell viability
si-NC
si-MFN1#2
si-MFN2#2
0 nM
100 nM
300 nM
M
% Cell viability
HA-vector
HA-DRP1
0 nM
100 nM
300 nM


A
24h
48h
MLN 0 0.1 0.3 1.0 0 0.1 0.3 1.0 (μM)
CUL1-Nedd8
CUL1
MFN1
MFN2
Parkin
α-tubulin
HeLa
B
β-TrCP binding site
Consensus DSG XX S
MFN1 (Human) 83-RT SSG KS S VI-92
MFN1 (Chimpanzee) 83-RT SSG KS S VI-92
MFN1 (Cow) 91-RT SSG KS S VI-100
MFN1 (Mouse) 83-RT SSG KS S VI-92
MFN1 (Dog) 83-RT SSG KS S VI-92
MFN1 (Chicken) 84-RT SSG KS S VI-93
MFN1 (Zebrafish) 83-RT SNG KS T VI-92
C
CY
MT
MLN 0 300 0 300 (nM)
CUL1-Nedd8
CUL1
β-TrCP1
RBX1
α-tubulin
Tom20
D
FLAG-vector + -
FLAG-MFN1 - +
HA-β-TrCP1 + +
IP: HA
FLAG
HA
Input
FLAG
HA
E
HA-β-TrCP1 (0, 1, 3 μg)
HA-β-TrCP 1△F (0, 1, 3 μg)
FLAG-MFN1
HA-β-TrCP1
HA-β-TrCP1△F
FLAG
1.00 0.83 0.20 1.00 1.36 1.27
α-tubulin
F
HA-β-TrCP1 (0, 1, 3 μg)
HA-β-TrCP1△F (0, 1, 3 μg)
HA-β-TrCP1
HA-β-TrCP1△F
MFN1
1.00 0.54 0.38 1.00 0.85 0.98
α-tubulin
G
si-NC
si-β-TrCP
CHX 0 3 6 9 12 0 3 6 9 12 (h)
MFN1
β-TrCP1
α-tubulin
H
Time after CHX (h)
0 3 6 9 12
Relative MFN1 protein level (Log2)
0 -1 -2 -3 -4
si-NC
si-β-TrCP
I
FLAG-MFN1
FLAG-MFN1 (S85/86/90A)
CHX 0 3 6 9 12 0 3 6 9 12 (h)
FLAG
α-tubulin
MDA-MB-231
FLAG-MFN1
FLAG-MFN1 (S85/86/90A)
CHX 0 3 6 9 12 0 3 6 9 12 (h)
FLAG
α-tubulin
SK-BR-3
J
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FIAG-MFN1 (S85/86/90A) + HA-β-TrCP1
CHX 0 3 6 9 12 0 3 6 9 12 (h)
FLAG
HA
α-tubulin
K
Time after CHX (h)
0 3 6 9 12
Relative FLAG level (Log2)
0 -1 -2 -3
FLAG-MFN1
FLAG-MFN1 (S85/86/90A)
L
H1299
BEAS2B
H1650
si-NC + - + - + -
si-β-TrCP - + - + - +
β-TrCP1
MFN1
WEE1
α-tubulin
M
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H1650
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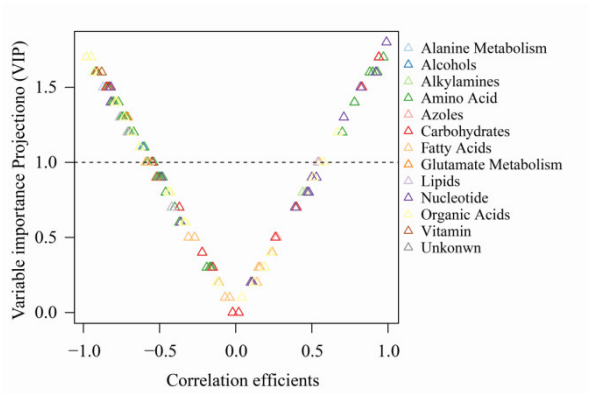
FLAG-β-TrCP1 - + - + - +
FLAG
MFN1
α-tubulin

A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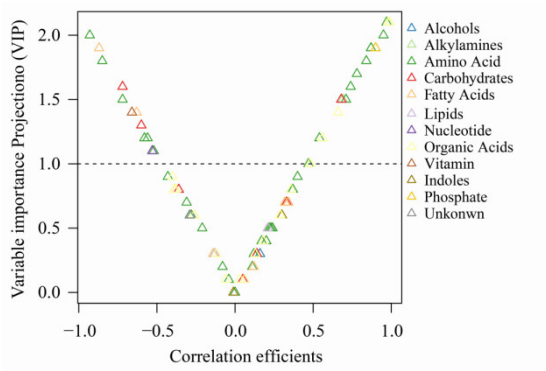
B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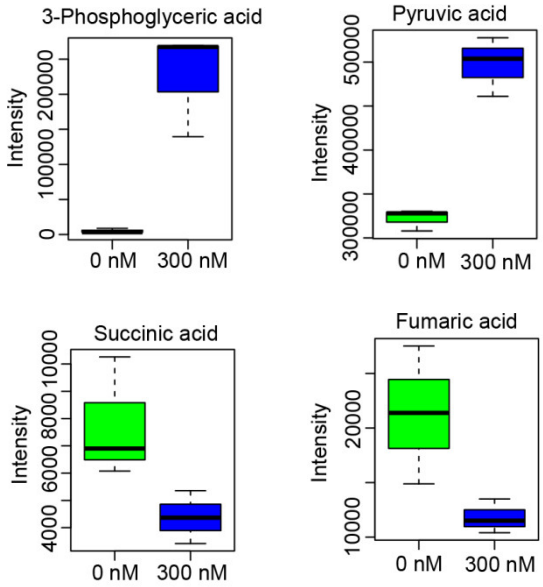
C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Pyruvic acid
Succinic acid
Fumaric acid
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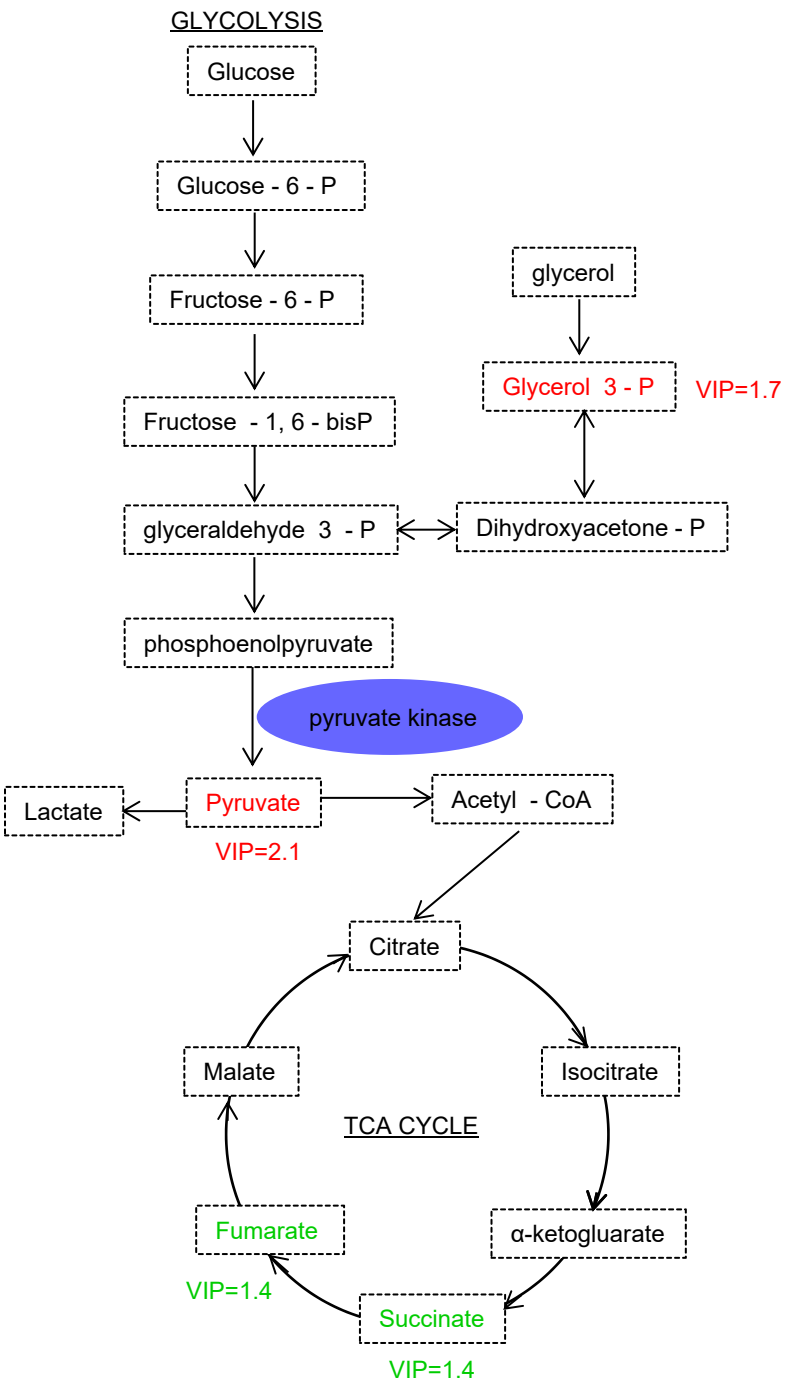
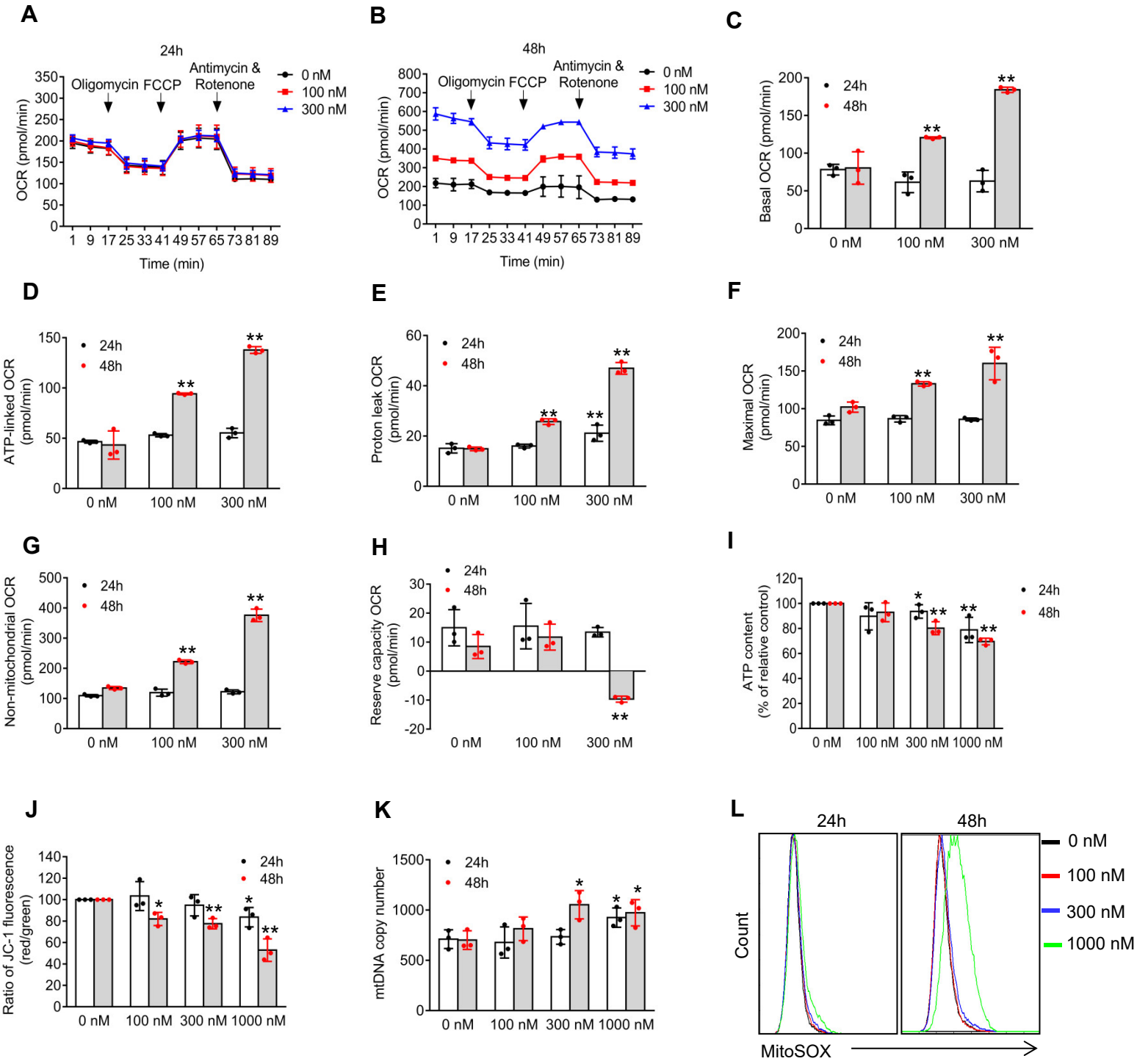
D


GLYCOLYSIS
Glucose
Glucose - 6 - P
Fructose - 6 - P
Fructose - 1, 6 - bisP
glyceraldehyde 3 - P
Dihydroxyacetone - P
glycerol
Glycerol 3 - P
VIP=1.7
phosphoenolpyruvate
pyruvate kinase
Lactate
Pyruvate
VIP=2.1
Acetyl - CoA
Citrate
Isocitrate
α-ketogluarate
Succinate
VIP=1.4
Fumarate
VIP=1.4
Malate
TCA CYCLE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24h
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FCCP
Antimycin & Rotenone
0 nM
100 nM
300 nM
OCR (pmol/min)
Time (min)
B
48h
C
Basal OCR (pmol/min)
24h
48h
0 nM
100 nM
300 nM
D
ATP-linked OCR (pmol/min)
E
Proton leak OCR (pmol/min)
F
Maximal OCR (pmol/min)
G
Non-mitochondrial OCR (pmol/min)
H
Reserve capacity OCR (pmol/min)
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ATP content (% of relative control)
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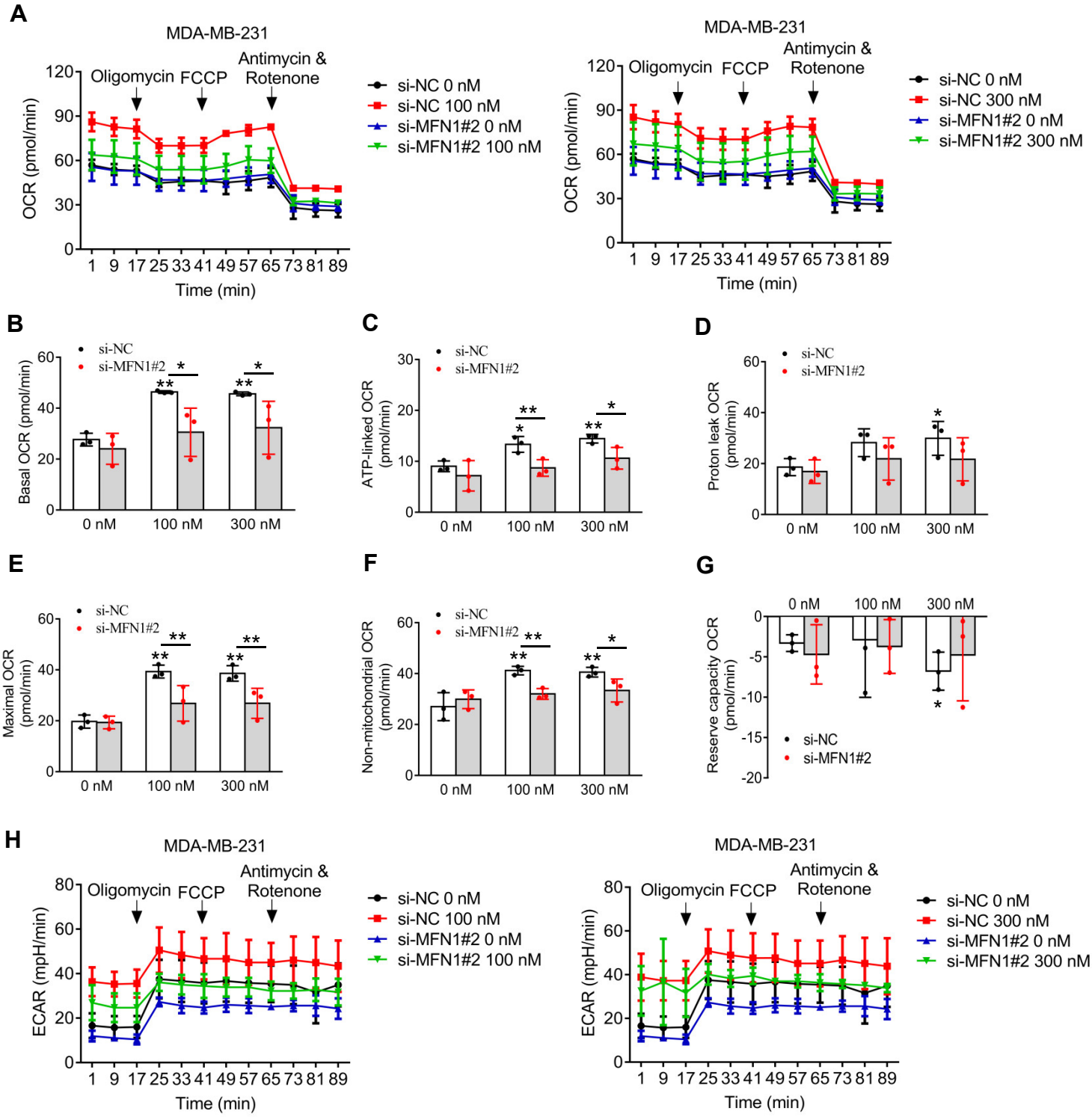
J
Ratio of JC-1 fluorescence (red/green)
K
mtDNA copy number
L
Count
MitoSOX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FCCP
Antimycin & Rotenone
OCR (pmol/min)
Time (min)
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si-MFN1#2 100 nM
si-NC 300 nM
si-MFN1#2 300 nM
B
Basal OCR (pmol/min)
C
ATP-linked OCR (pmol/min)
D
Proton leak OCR (pmol/min)
E
Maximal OCR (pmol/min)
F
Non-mitochondrial OCR (pmol/min)
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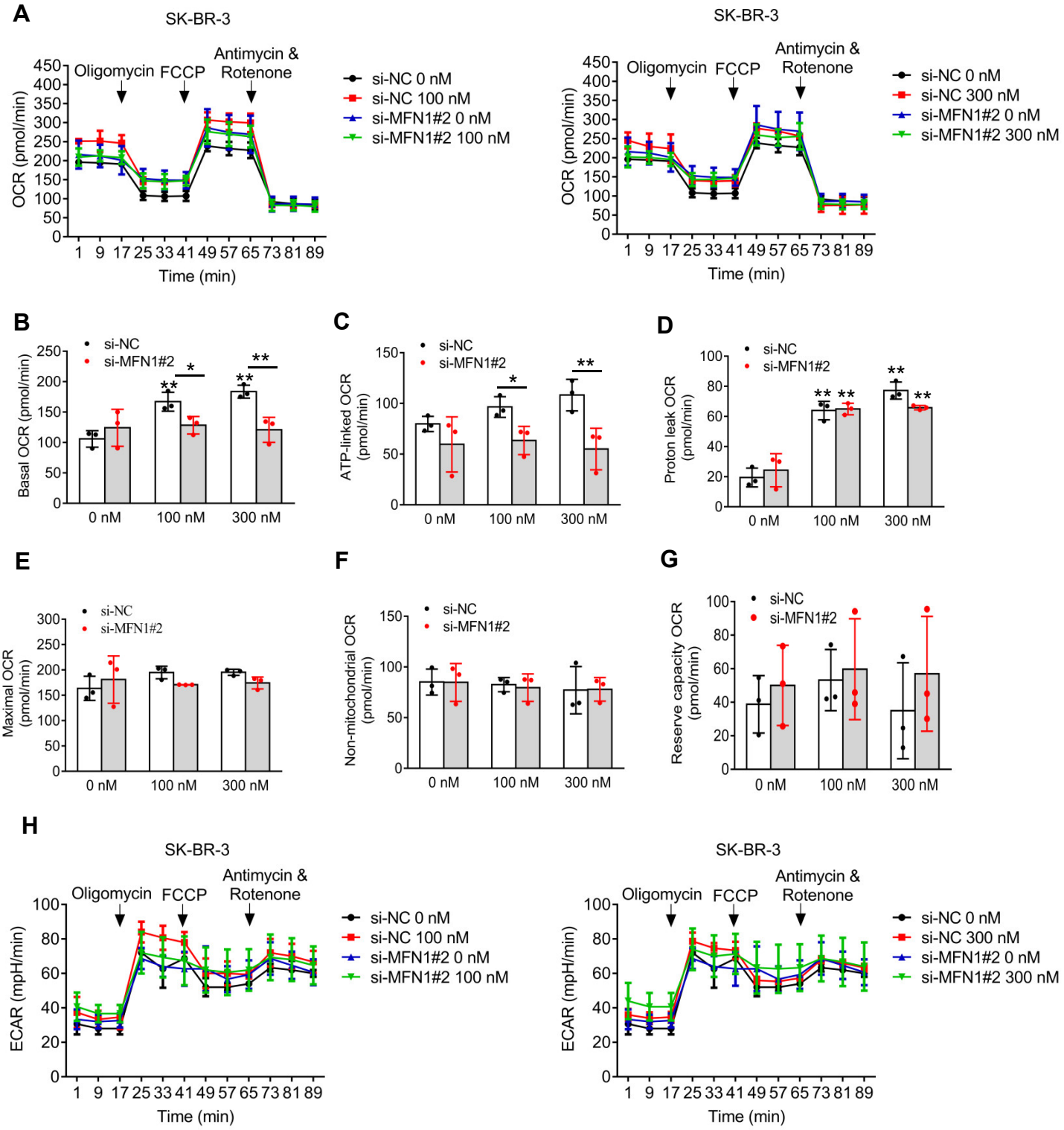
ECAR (mpH/min)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FCCP
Antimycin & Rotenone
OCR (pmol/min)
Time (min)
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si-NC 100 nM
si-MFN1#2 0 nM
si-MFN1#2 100 nM
SK-BR-3
Oligomycin
FCCP
Antimycin & Rotenone
OCR (pmol/min)
Time (min)
si-NC 0 nM
si-NC 300 nM
si-MFN1#2 0 nM
si-MFN1#2 300 nM
B
Basal OCR (pmol/min)
si-NC
si-MFN1#2
0 nM
100 nM
300 nM
C
ATP-linked OCR (pmol/min)
si-NC
si-MFN1#2
0 nM
100 nM
300 nM
D
Proton leak OCR (pmol/min)
si-NC
si-MFN1#2
0 nM
100 nM
300 nM
E
Maximal OCR (pmol/min)
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100 nM
300 nM
F
Non-mitochondrial OCR (pmol/min)
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0 nM
100 nM
300 nM
G
Reserve capacity OCR (pmol/min)
si-NC
si-MFN1#2
0 nM
100 nM
300 nM
H
SK-BR-3
Oligomycin
FCCP
Antimycin & Rotenone
ECAR (mpH/min)
Time (min)
si-NC 0 nM
si-NC 100 nM
si-MFN1#2 0 nM
si-MFN1#2 100 nM
SK-BR-3
Oligomycin
FCCP
Antimycin & Rotenone
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Time (min)
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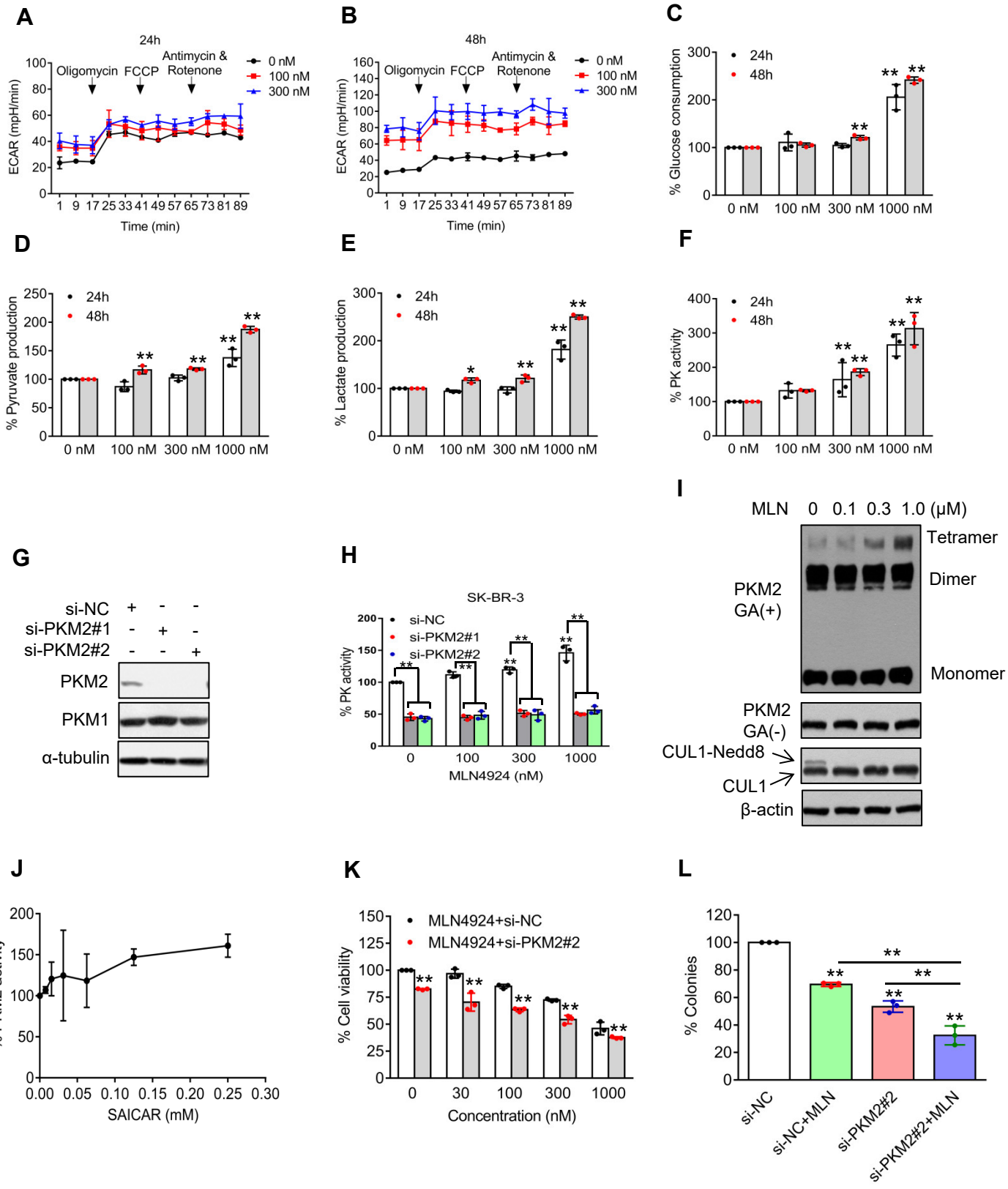
si-MFN1#2 300 nM


A
24h
Oligomycin
FCCP
Antimycin & Rotenone
0 nM
100 nM
300 nM
ECAR (mpH/min)
Time (min)
B
48h
C
% Glucose consumption
D
% Pyruvate production
E
% Lactate production
F
% PK activity
G
si-NC
si-PKM2#1
si-PKM2#2
PKM2
PKM1
α-tubulin
H
SK-BR-3
MLN4924 (nM)
I
MLN 0 0.1 0.3 1.0 (μM)
Tetramer
Dimer
Monomer
PKM2 GA(+)
PKM2 GA(-)
CUL1-Nedd8
CUL1
β-actin
J
% PKM2 activity
SAICAR (mM)
K
MLN4924+si-NC
MLN4924+si-PKM2#2
% Cell viability
Concentration (nM)
L
% Colonies
si-NC
si-NC+MLN
si-PKM2#2
si-PKM2#2+MLN

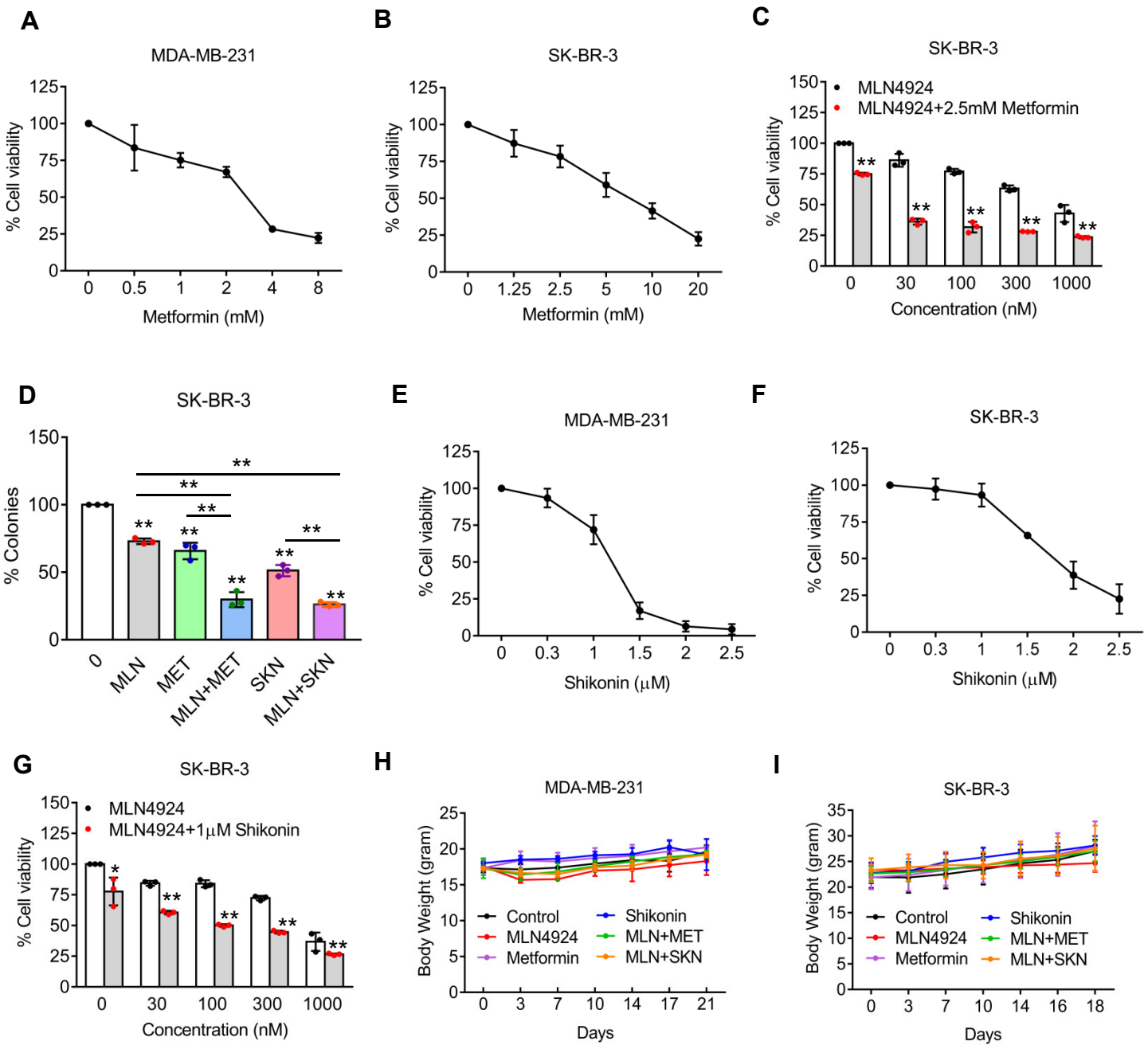

A
MDA-MB-231
% Cell viability
Metformin (mM)
B
SK-BR-3
% Cell viability
Metformin (mM)
C
SK-BR-3
MLN4924
MLN4924+2.5mM Metformin
% Cell viability
Concentration (nM)
D
SK-BR-3
% Colonies
0
MLN
MET
MLN+MET
SKN
MLN+SKN
E
MDA-MB-231
% Cell viability
Shikonin (μM)
F
SK-BR-3
% Cell viability
Shikonin (μM)
G
SK-BR-3
MLN4924
MLN4924+1μM Shikonin
% Cell viability
Concentration (nM)
H
MDA-MB-231
Body Weight (gram)
Control
MLN4924
Metformin
Shikonin
MLN+MET
MLN+SKN
Days
I
SK-BR-3
Body Weight (gram)
Control
MLN4924
Metformin
Shikonin
MLN+MET
MLN+SKN
Days

Fig 1F

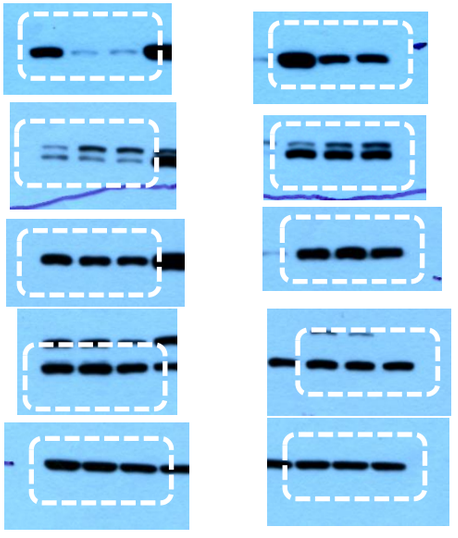


Fig 2A

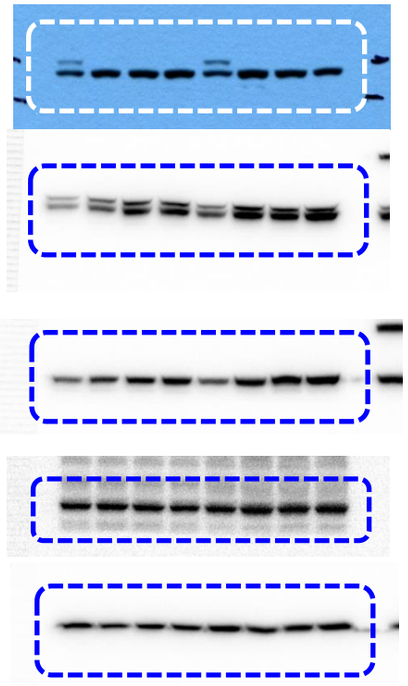


Fig 2B

Fig 2C

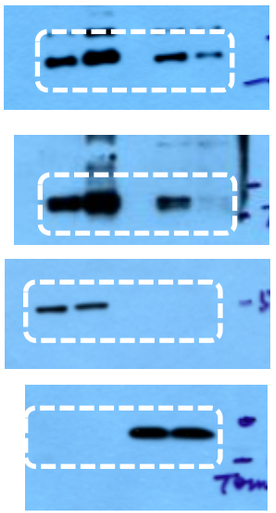


Fig 2F

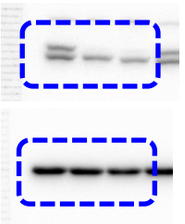


Fig 2G

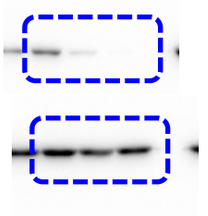


Fig 2J

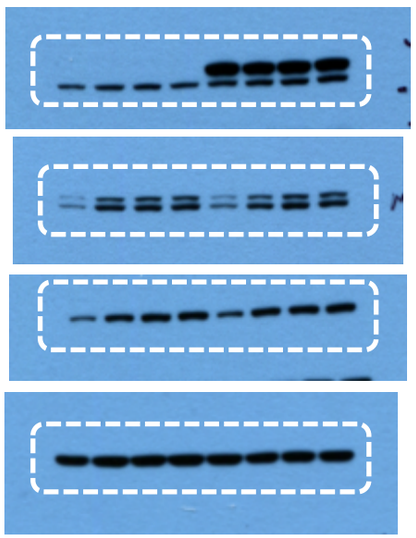


Fig 3A

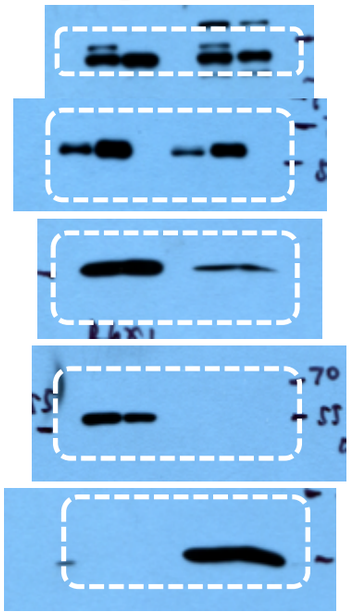


Fig 3B

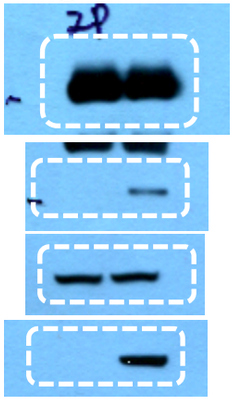


Fig 3C

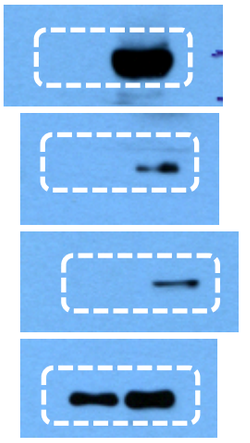


Fig 3D

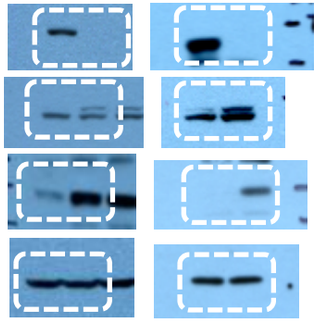


Fig 3E

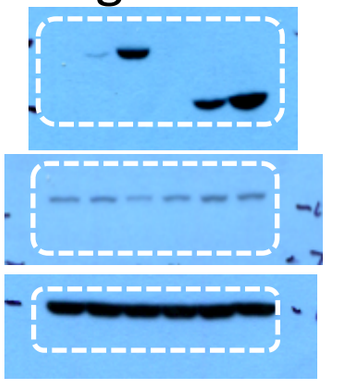


Fig 3F

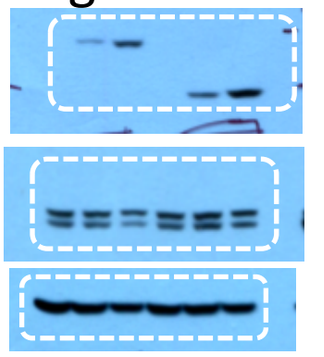


Fig 3G

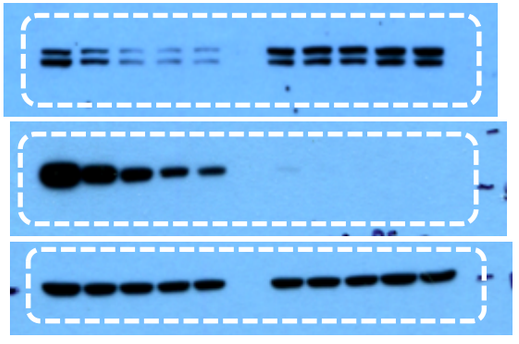


Fig 3I

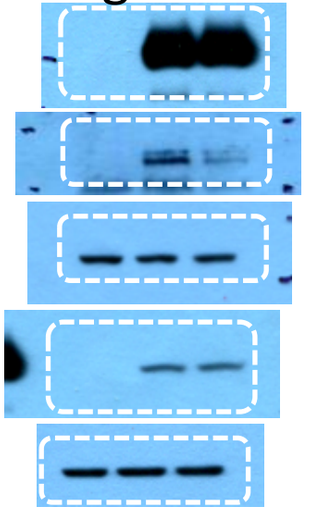


Fig 3J

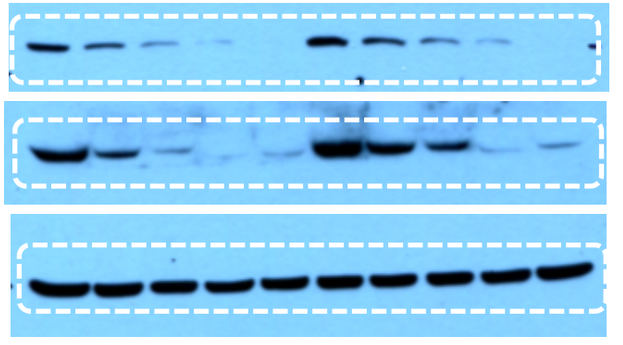


Fig 3L

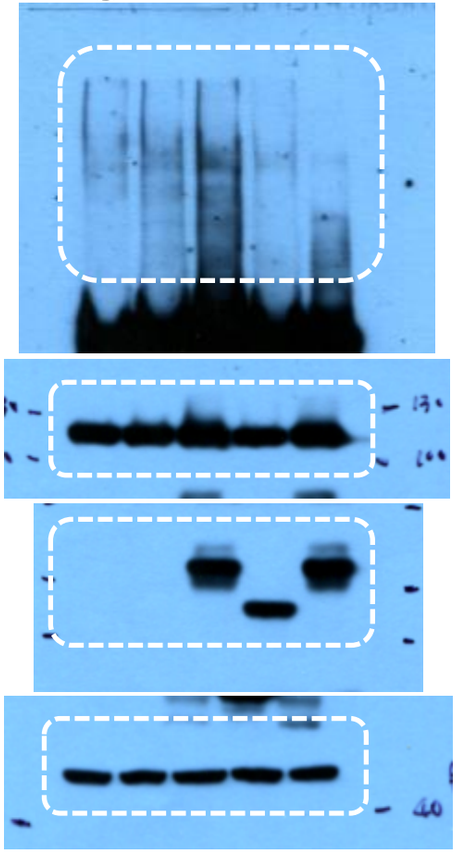


Fig 6G

Fig 6I

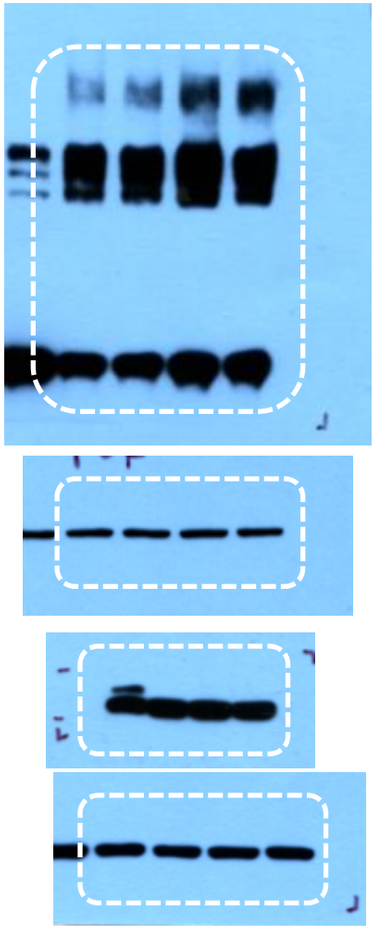


Fig 6J

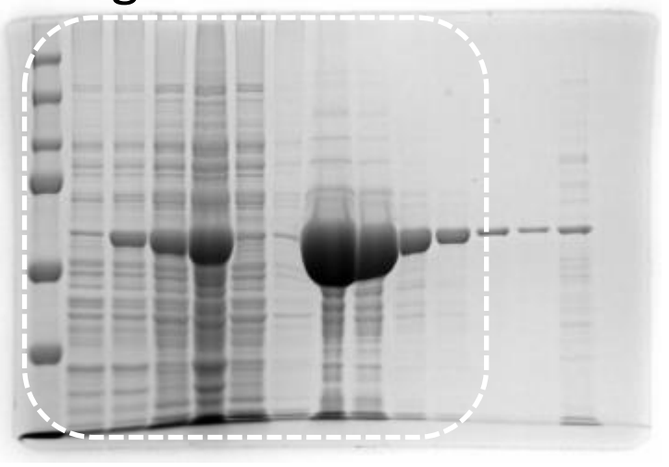


Fig S2A

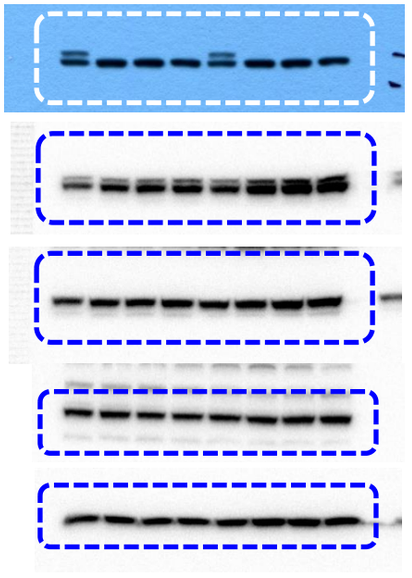


Fig S2B

Fig S2C

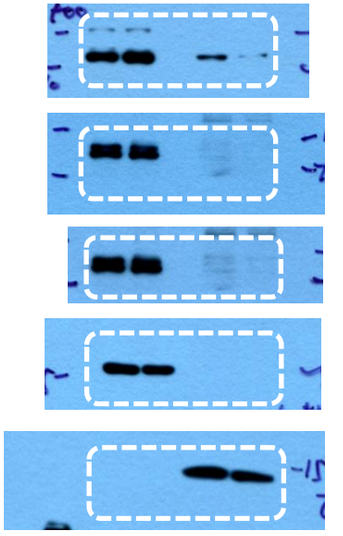


Fig S2F

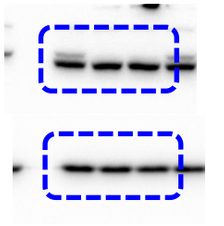


Fig S2G

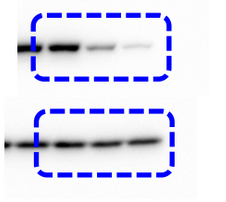


Fig S2J

Fig S3 A

Fig S3 C

Fig S3 D

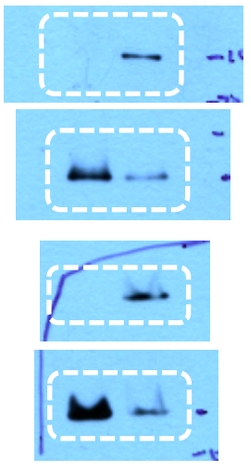


Fig S3 E

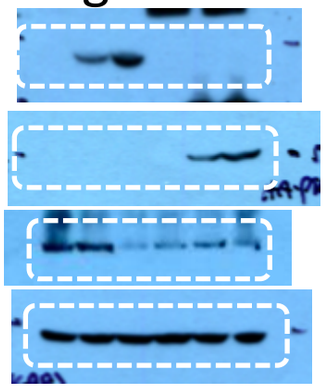


Fig S3 F

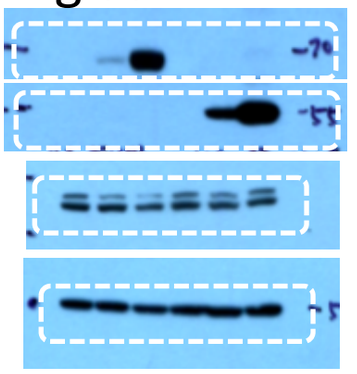


Fig S3 G

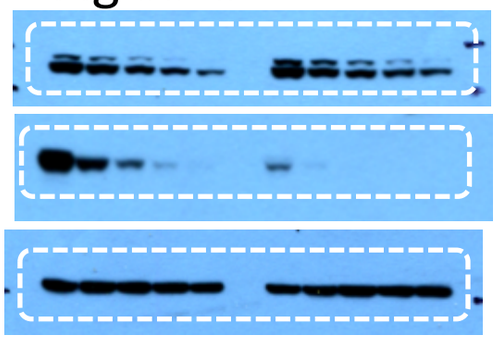


Fig S3 I

Fig S3 J

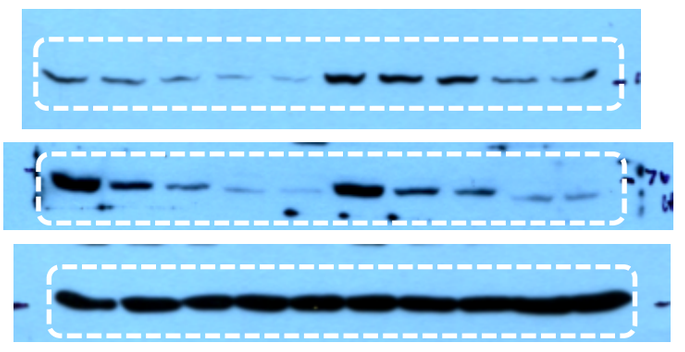


Fig S3 L

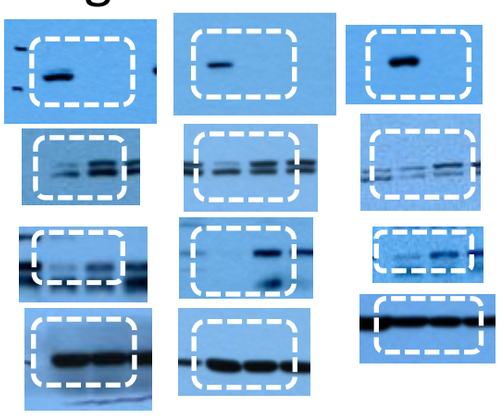


Fig S3 M

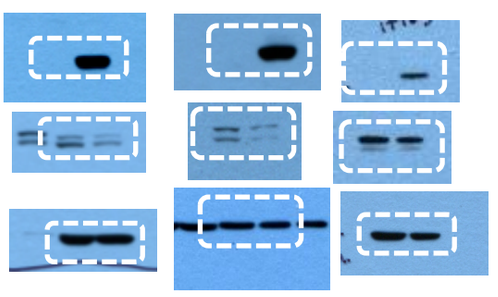


Fig S8G

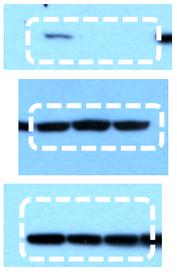


Fig S8I