Impaired RelA Signaling in Hepatocytes promotes the Progression of Metabolic Dysfunction-associated Steatotic Liver Disease by Remodeling Lipid Metabolism

Running title: Impaired RelA signaling promotes MASLD

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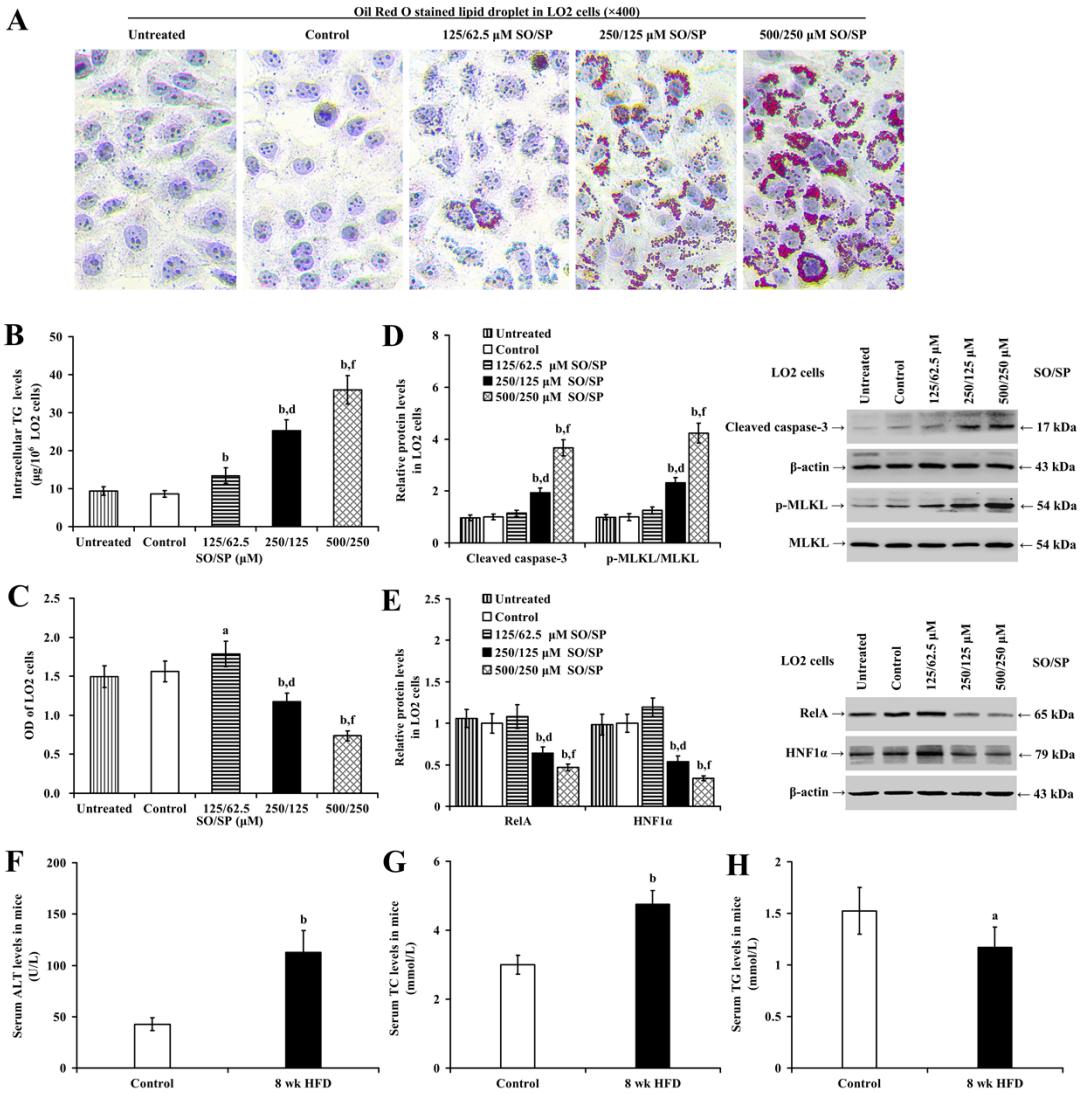
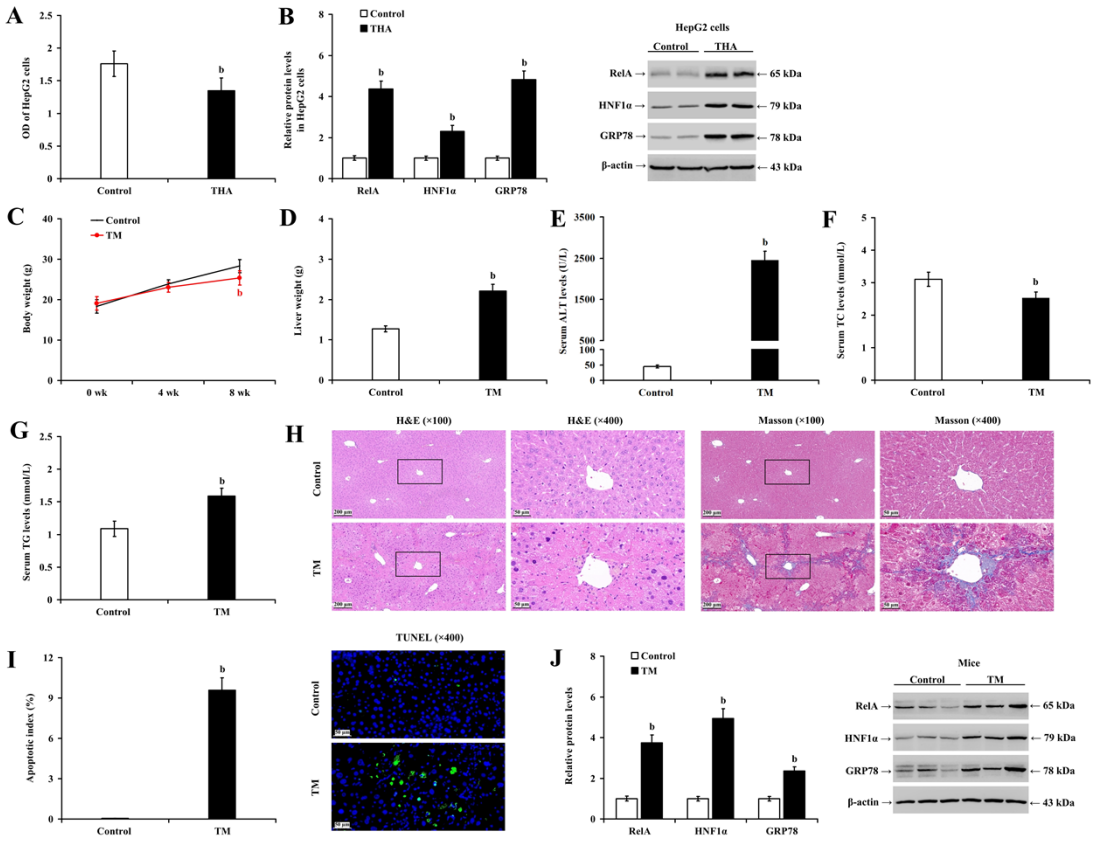
Supplementary Figures



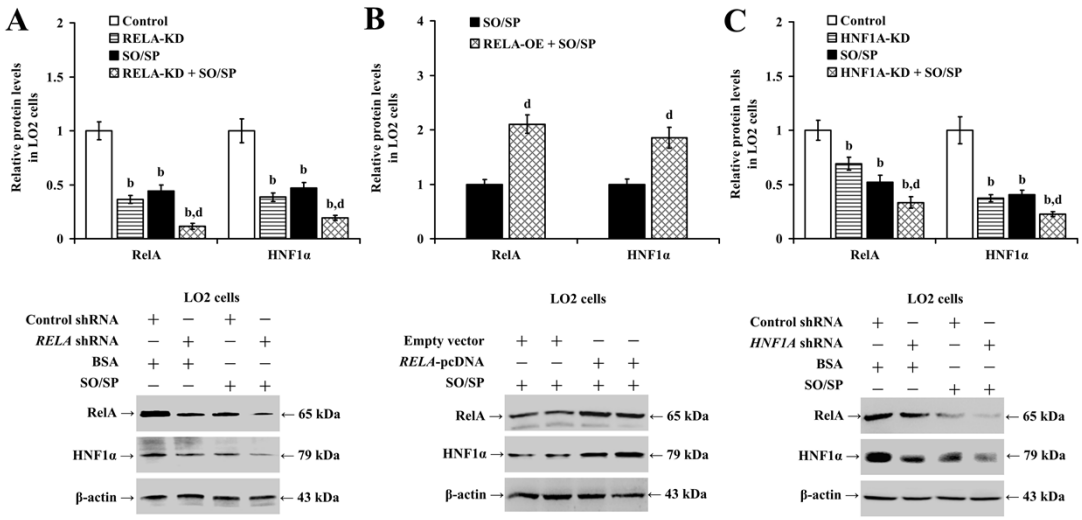
Supplementary Fig. 1. Steatotic hepatocytes exhibit reduced levels of RelA and HNF1α in MASLD. (A–E) LO2 cells were treated with SO/SP, vehicle or left untreated at indicated concentrations to induce steatosis. Under this condition, (A) lipid droplet accumulation, (B) intracellular TG levels, (C) cell viability and the

expression of (D) cleaved caspase-3, p-MLKL, and (E) RelA and HNF1α were evaluated. (F–H) Comparisons of serum (F) ALT, (G) TC, and (H) TG levels were evaluated and analyzed in mice (n=12) under indicated conditions. a P<0.05, b P<0.01, vs. control group; d P<0.01, vs. SO/SP group (125/62.5 μM); f P<0.01, vs. SO/SP group (250/125 μM). HFD: high-fat diet; SO/SP: sodium oleate and sodium palmitate; μM: μmol/L; wk: week.

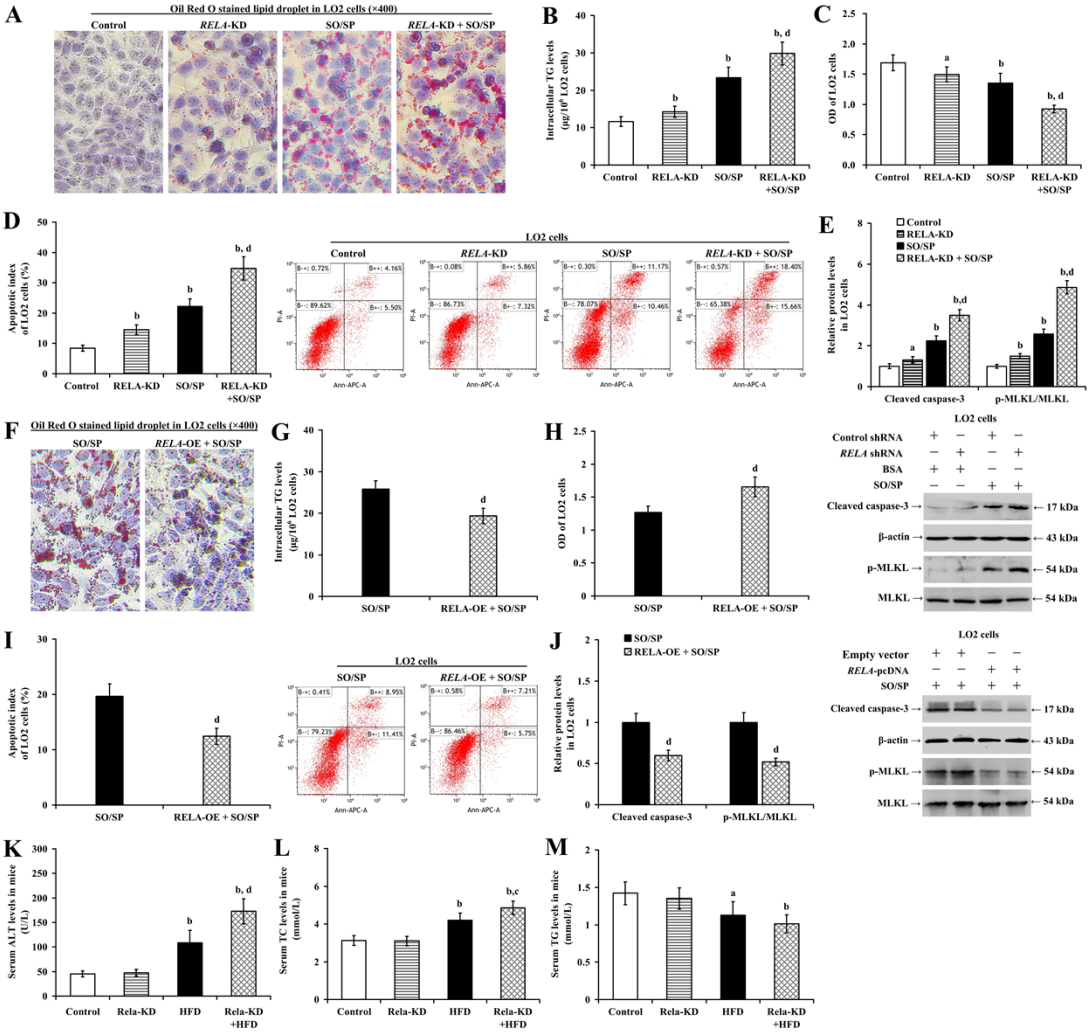


Supplementary Fig. 2. Hepatocytes without steatosis exhibit elevated RelA and HNF1α expression under ER stress. (A and B) ER stress was induced in HepG2 cells using THA (or vehicle), and (A) cell viability and (B) the expression of RelA, HNF1α, and GRP78 were evaluated. (C–K) Mice (n=12) were treated with TM or vehicle (DMSO) for 8 weeks. Under this condition, their (C) body weights, (D) liver

weights, serum levels of (E) ALT, (F) TC, and (G) TG were determined and compared. (H) Representative images of H&E (left panels), and Masson staining (right panels) of mouse livers. (I) Hepatic apoptotic index (calculated from the results of TUNEL staining). (J) Analyzed data (left panel) and representative images (right panel) of Western blot analysis showing the expression of RelA, HNF1α, and GRP78 in mouse livers under different conditions. b P<0.01, vs. control group. THA: thapsigargin; TM: tunicamycin; wk: week.

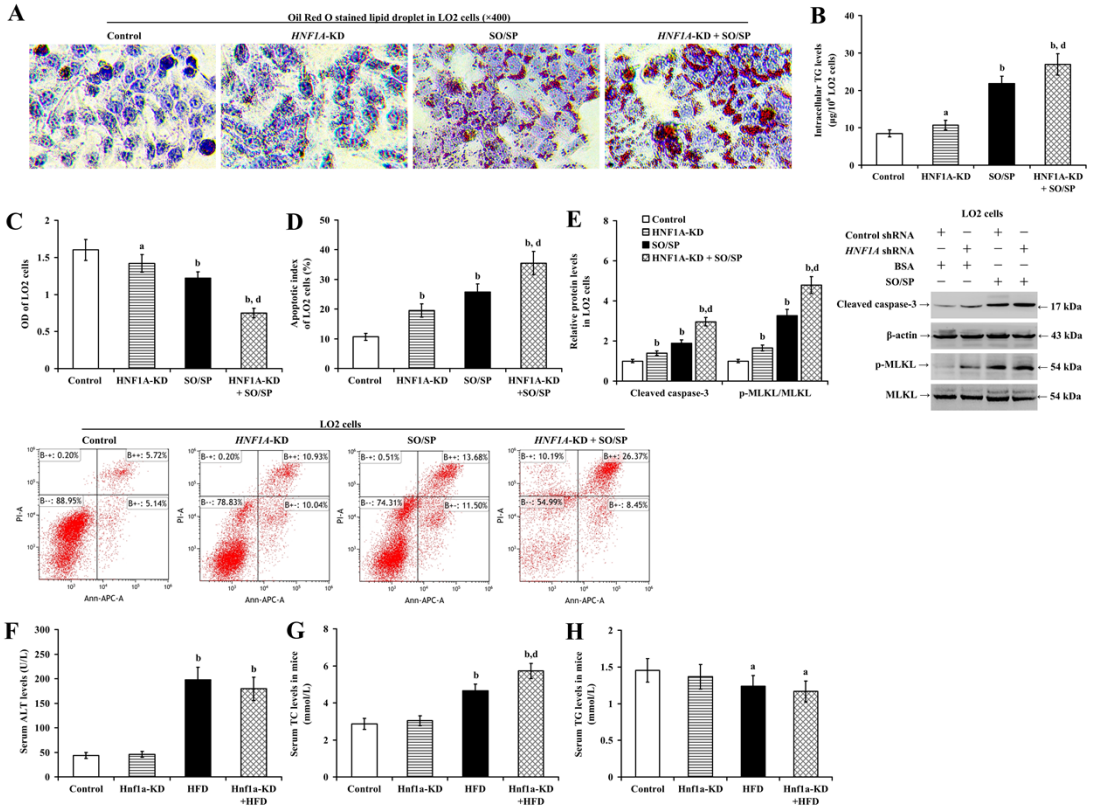


Supplementary Fig. 3. Lack of mutual transcriptional activation between RelA and HNF1α during hepatocyte steatosis. (A–C) Quantified results (upper panels) and representative images (lower panels) of western blot analysis showing the expression of RelA and HNF1α in LO2 cells under indicated conditions. b P<0.01, vs. control group; d P<0.01, vs. SO/SP group. BSA: bovine serum albumin; KD: knockdown; OE: overexpression; SO/SP: sodium oleate and sodium palmitate.



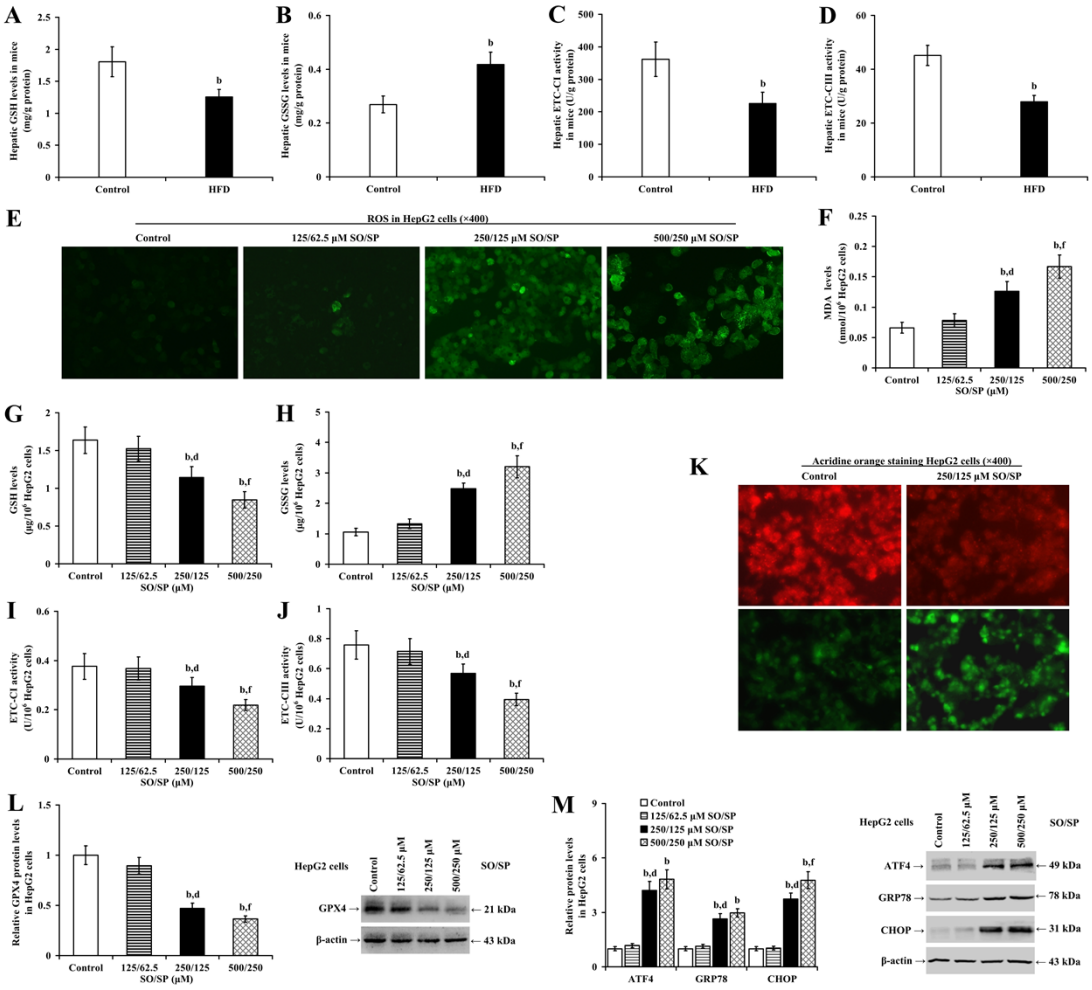
Supplementary Fig. 4. RelA mitigates hepatocyte steatosis. (A–E) LO2 cells with or without *RELA*-KD were treated with or without SO/SP. Under this condition, (A) lipid droplet accumulation, (B) intracellular TG levels, (C) cell viability, (D) cell apoptosis, and (E) the expression of cleaved caspase-3 and p-MLKL were determined and compared. (F–J) LO2 cells with or without *RELA*-OE were treated with SO/SP, and (F) lipid droplet accumulation, (G) intracellular TG levels, (H) cell viability, (I) cell apoptosis, and (J) the expression of cleaved caspase-3 and p-MLKL were determined and analyzed. (K–M) Analyzed data presenting the serum levels of (K) ALT, (L) TC, and (M) TG in mice with or without *RELA*-KD fed a normal diet or

HFD. a $P<0.05$, b $P<0.01$, vs. control group; c $P<0.05$, d $P<0.01$, vs. SO/SP group or HFD group. HFD: high-fat diet; KD: knockdown; OE: overexpression; SO/SP: sodium oleate and sodium palmitate.



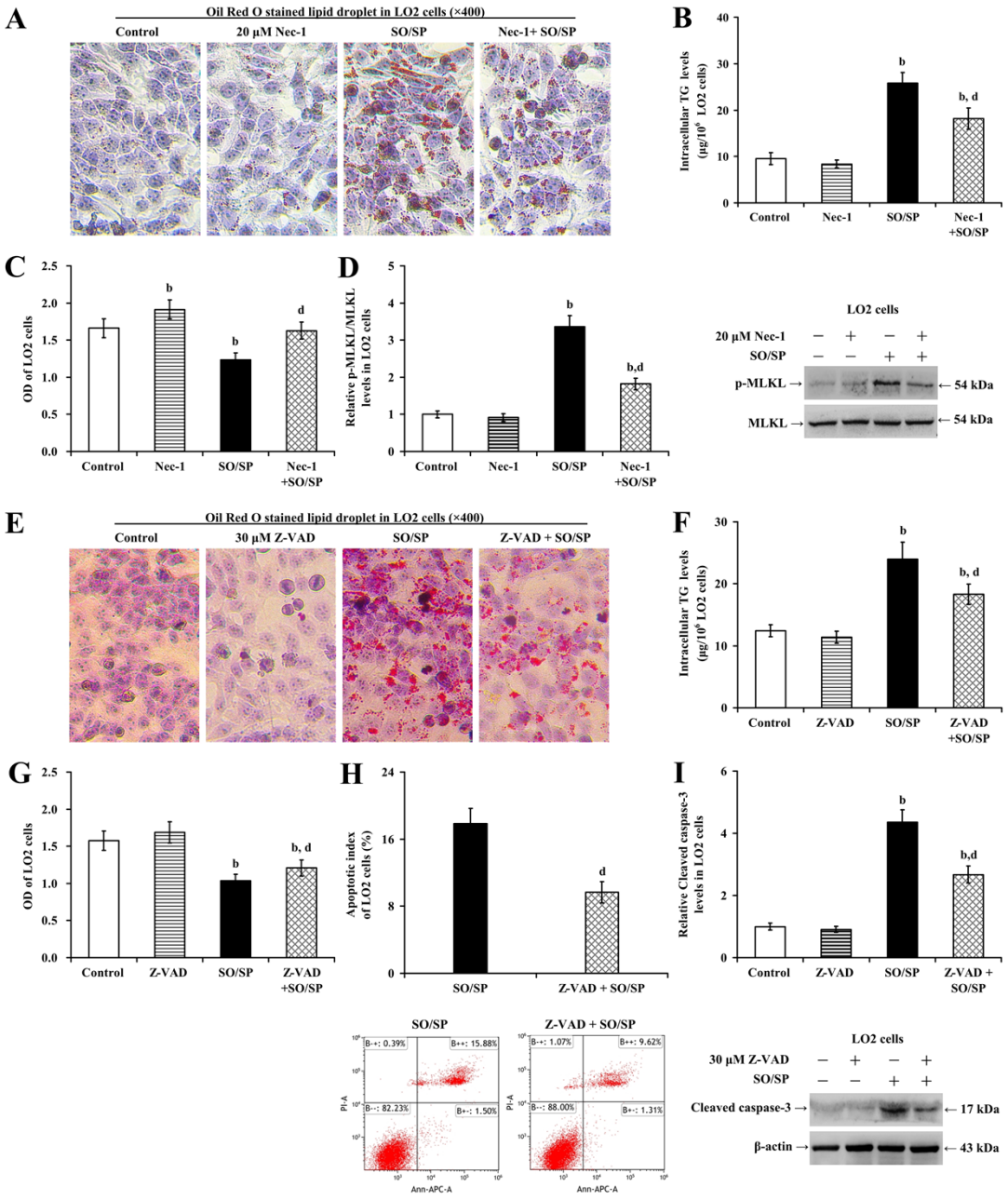
Supplementary Fig. 5. *HNF1A* knockdown exacerbates hepatocyte steatosis. (A–E) LO2 cells with or without *HNF1A*-KD were treated with SO/SP. Under this condition, (A) intracellular lipid droplet accumulation, (B) TG content, (C) cell viability, (D) cell apoptosis, and (E) the expression of cleaved caspase-3 and p-MLKL were determined and compared. (F–H) Mice (n=12) with or without *Hnf1a* KD were fed with normal diet or HFD. Their serum levels of (F) ALT, (G) TC, and (H) TG were evaluated and analyzed. a $P<0.05$, b $P<0.01$, vs. control group; d $P<0.01$, vs. SO/SP group or HFD group. BSA: albumin; KD: knockdown; HFD: high-fat diet; KD:

knockdown; SO/SP: sodium oleate and sodium palmitate.



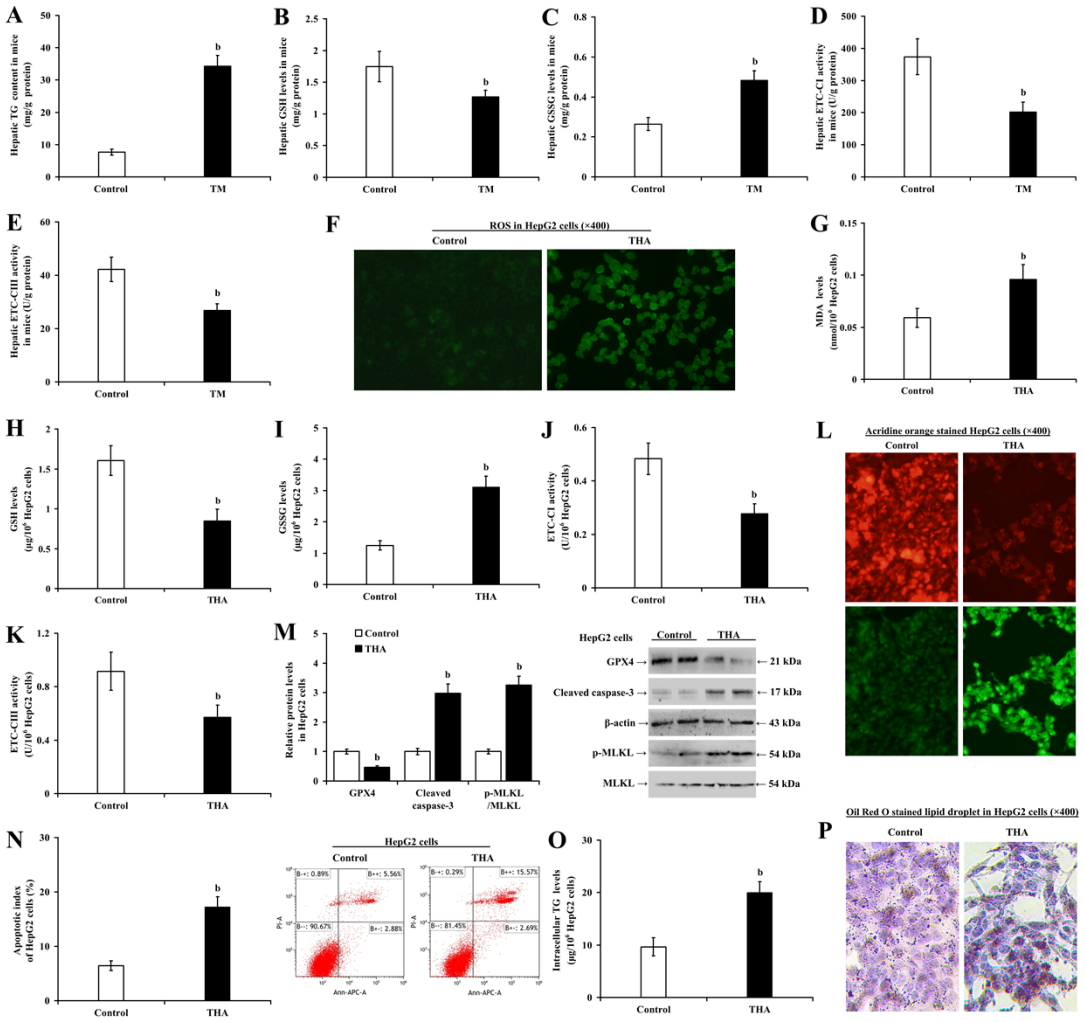
Supplementary Fig. 6. Lipid accumulation in hepatocytes exhibits lipotoxic features, potentially triggering apoptosis and necroptosis. (A–D) Analyzed data detailing the changes in (A) GSH levels, (B) GSSG levels, (C) ETC-CI activity, and (D) ETC-CIII activity in the livers of mice fed a normal diet or HFD. (E–M) HepG2 cells were treated with SO/SP at the indicated concentrations. Under this condition, (E) ROS levels, (F) MDA levels, (G) GSH levels, (H) GSSG levels, (I) ETC-CI activity, (J) ETC-CIII activity, and (K) changes in LMP (the reduction in red fluorescence and the increase in green fluorescence signify the strengthening of LMP) were determined

in the cells. (L and M) Quantified results and representative images of western blotting demonstrating the expression of (L) GPX4, (M) ATF4, GRP78, and CHOP in HepG2 cells under indicated conditions. b $P<0.01$, vs. control group; d $P<0.01$, vs. SO/SP group (125/62.5 μM); f $P<0.01$, vs. SO/SP group (250/125 μM). HFD: high-fat diet; LMP: lysosomal membrane permeabilization; SO/SP: sodium oleate and sodium palmitate; μM: μmol/L.



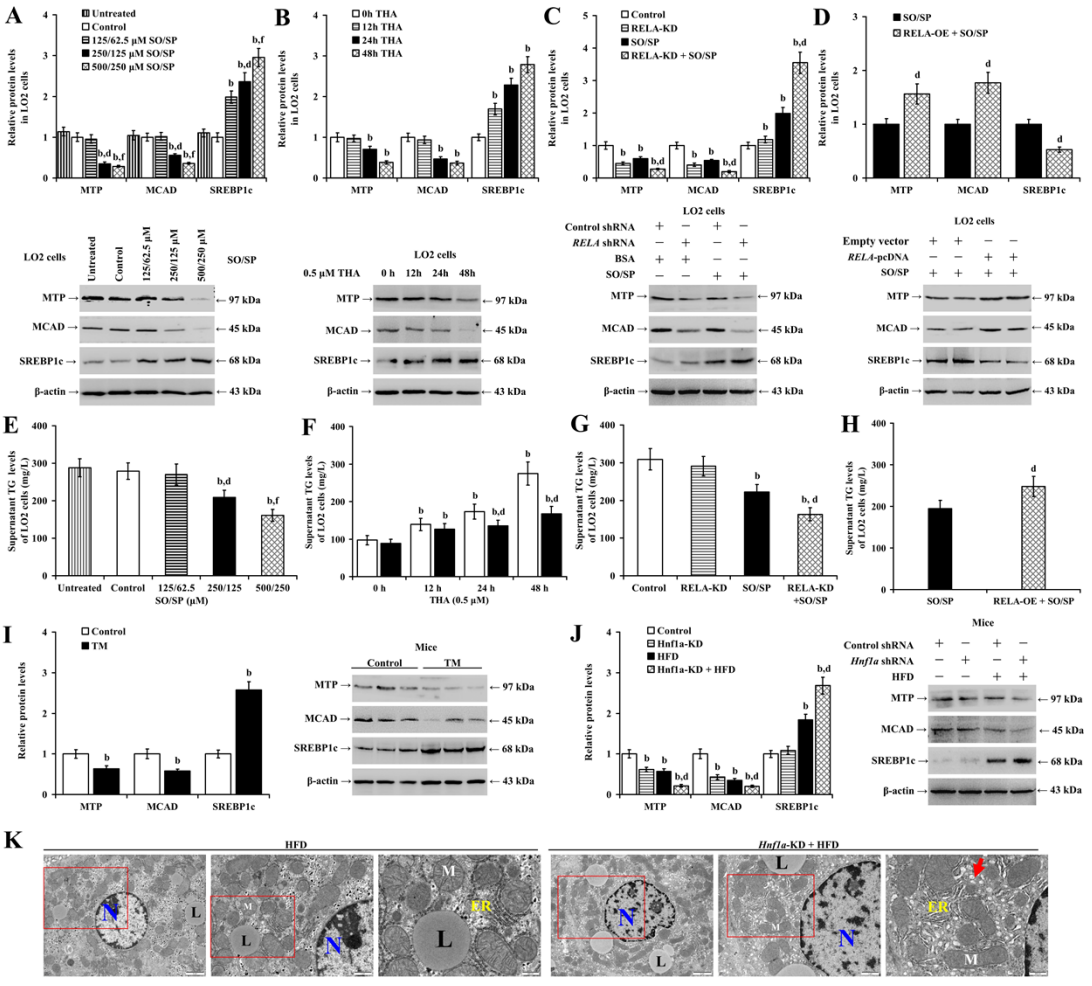
Supplementary Fig. 7. Necrostatin-1 and Z-VAD alleviate SO/SP-induced hepatocyte injury. (A–D) LO2 cells were treated with or without SO/SP in the presence or absence of Nec-1 (20 μM). Under this condition, (A) lipid droplet accumulation, (B) intracellular TG levels, (C) cell viability, and (D) p-MLKL expression were determined. (E–I) LO2 cells were treated with or without SO/SP in the presence or absence of Z-VAD (30 μM). Under this condition, (E) lipid droplet

accumulation, (F) intracellular TG levels, (G) cell viability, (H) cell apoptosis, and (I) cleaved caspase-3 expression were determined. b P<0.01, vs. control group; d P<0.01, vs. SO/SP group. Nec-1: necrostatin-1; SO/SP: sodium oleate and sodium palmitate; μM: μmol/L.



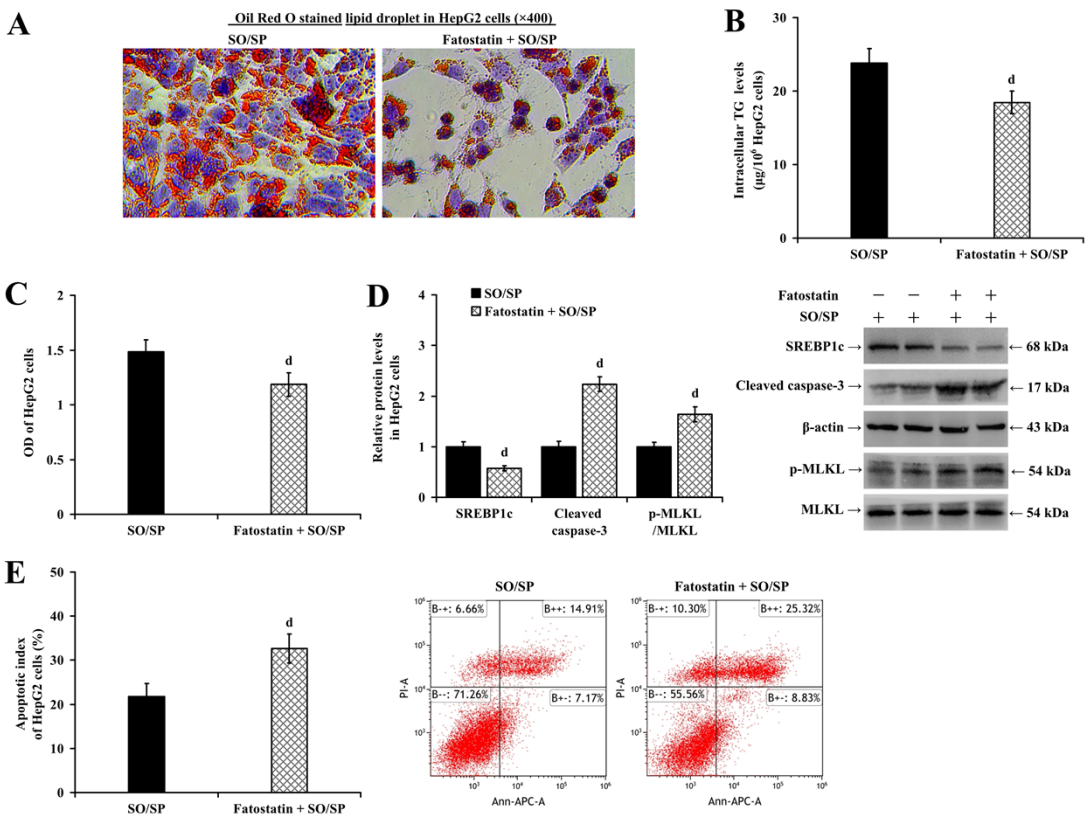
Supplementary Fig. 8. ER stress-initiated hepatotoxicity mirrors the processes involved in hepatocyte steatosis. (A–E) The mice were treated with or without TM, and the changes in (A) hepatic TG content, (B) GSH levels, (C) GSSG levels, (D) ETC-CI activity, and (E) ETC-CIII activity were measured. (F–P) In THA-treated

HepG2 cells, the changes in (F) ROS levels (green fluorescence intensity), (G) MDA levels, (H) GSH levels, (I) GSSG levels, (J) ETC-CI activity, (K) ETC-CIII activity, (L) LMP were evaluated. The expression of (M) GPX4, cleaved caspase-3, and p-MLKL, (N) cell apoptosis, (O) intracellular TG levels, and (P) lipid droplet accumulation were also determined in HepG2 cells treated with or without THA. b $P<0.01$, vs. control group. LMP: lysosomal membrane permeabilization; THA: thapsigargin; TM: tunicamycin.

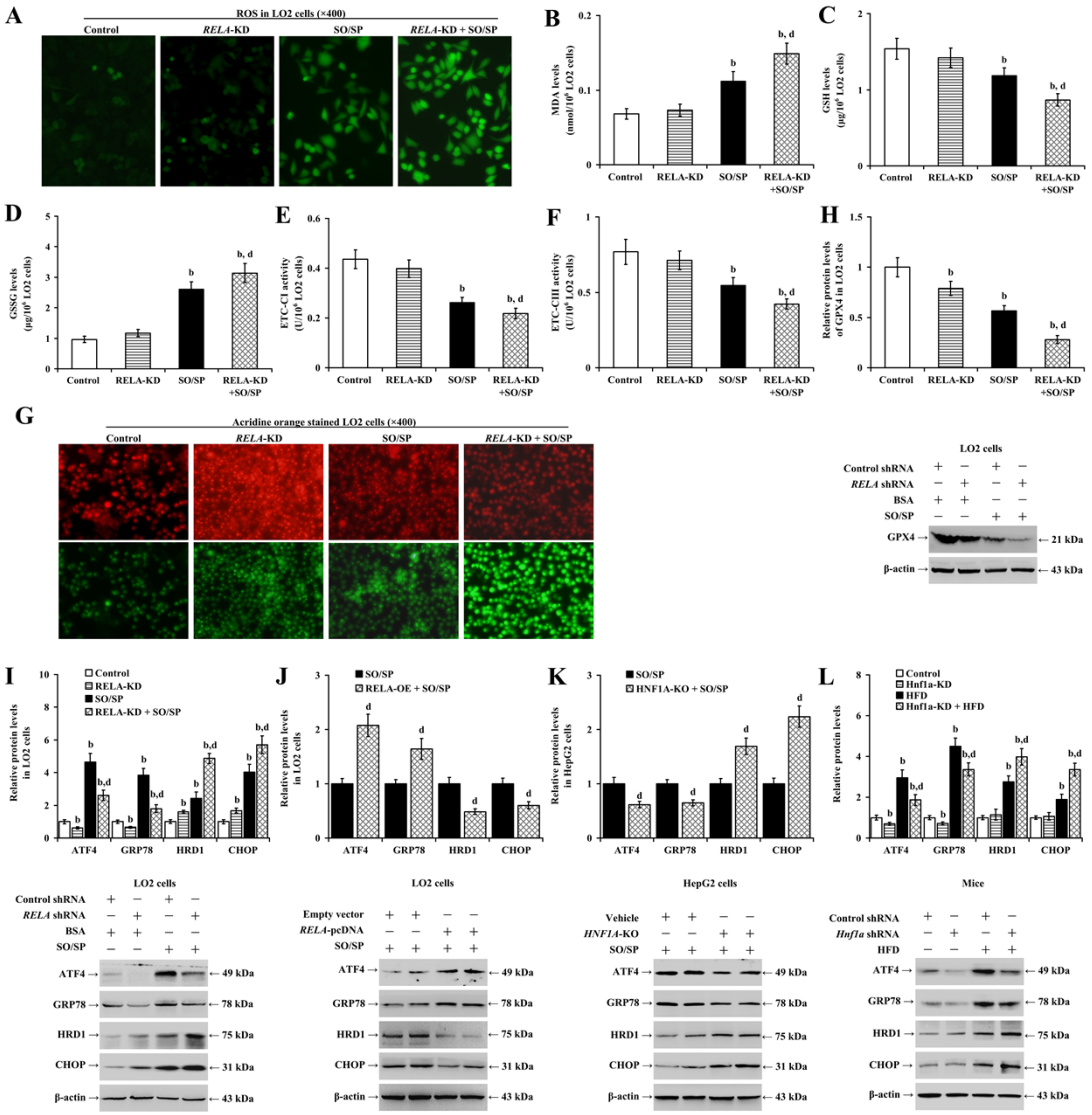


Supplementary Fig. 9. Impaired RelA signaling reprograms lipid metabolism promotes lipid accumulation in hepatocytes with steatosis. (A–D) The expression

of lipid metabolism-related proteins (MTP, MCAD, SREBP1c) in LO2 cells under the conditions of (A) SO/SP treatment, (B) THA treatment (0.5 μM), and SO/SP treatment with or without (C) *RELA*-KO or (D) *RELA*-OE. (E-H) Extracellular TG levels were measured under the conditions of (E) SO/SP treatment, (F) THA treatment, and SO/SP treatment with or without (G) *RELA*-KO or (H) *RELA*-OE. (I) Analyzed data and representative images of Western blot analysis showing the expression of lipid metabolism-related proteins in the livers from mice induced with or without TM. (J) Analyzed data and representative images of Western blot analysis showing the expression of lipid metabolism-related proteins in the livers from mice with or without *Hnf1a*-KD under normal diet or HFD. (K) Representative TEM images showing the ultrastructure of mouse liver under *Hnf1a*-KD. Red rectangles indicate magnified area. b $P<0.01$, vs. conrol group or 0 h group; d $P<0.01$, vs. SO/SP group (125/62.5 μM), the corresponding SO/SP group, or HFD group; f $P<0.01$, vs. SO/SP group (250/125 μM). BSA: bovine serum albumin; HFD: high-fat diet; KD: knockdown; OE: overexpression; SO/SP: sodium oleate and sodium palmitate; THA: thapsigargin; TM: tunicamycin; μM: μmol/L.



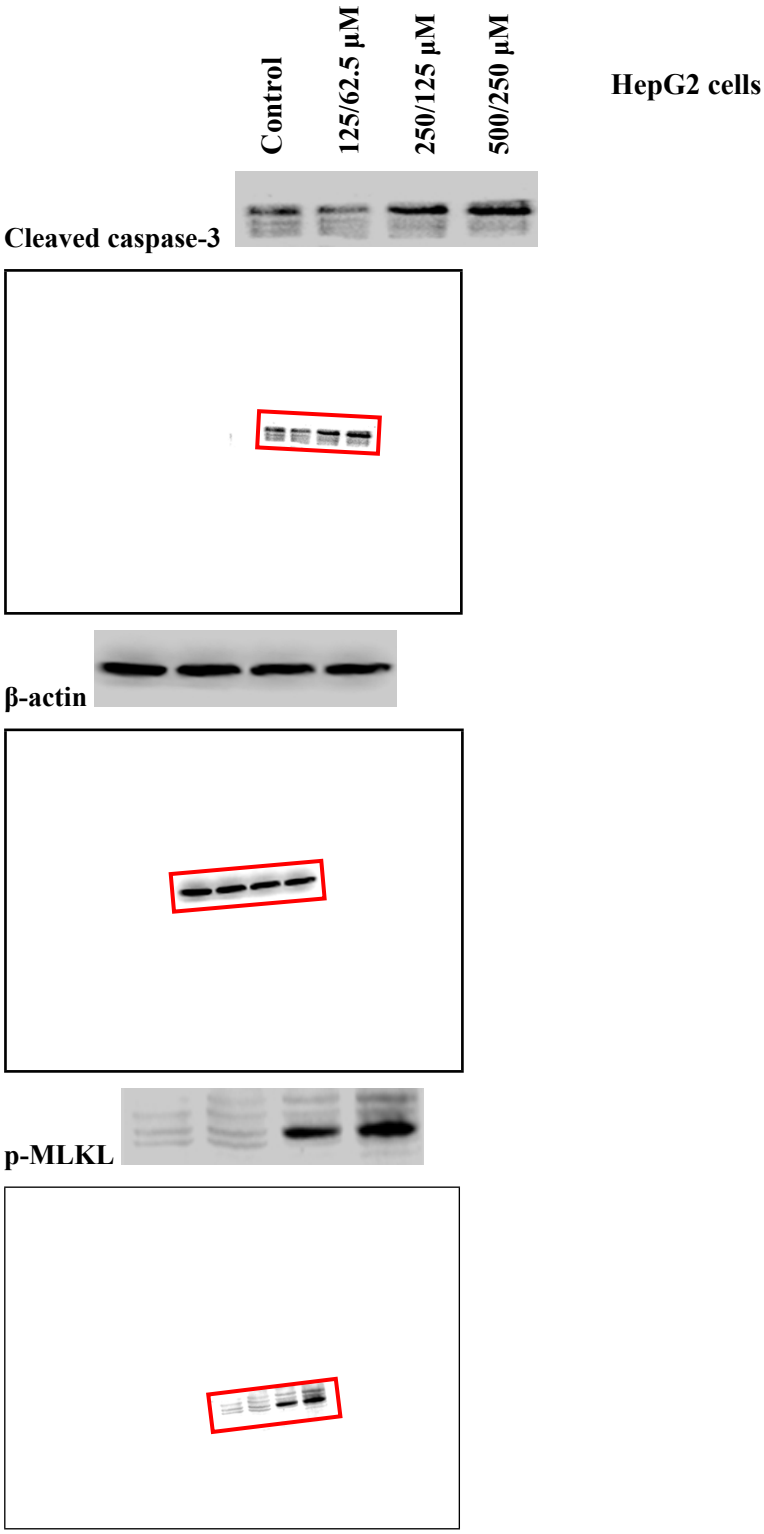
Supplementary Fig. 10. Fatostatin reduces SO/SP-induced lipid accumulation and exacerbates cell injury in HepG2 cells. (A–E) HepG2 cells were treated with SO/SP in the presence or absence of fatostatin (20 μM). Under this condition, (A) lipid droplet accumulation, (B) intracellular TG levels, (C) cell viability, (D) the expression of SREBP1c, cleaved caspase-3, and p-MLKL, and (E) cell apoptosis were evaluated. d P<0.01, vs. SO/SP group. SO/SP: sodium oleate and sodium palmitate; μM: μmol/L.



Supplementary Fig. 11. Impaired RelA signaling remodels the ER stress response and exacerbates lipotoxicity during hepatocyte steatosis. (A–H) LO2 cells with or without *RELA*-KD were treated with or without SO/SP. Under this condition, (A) ROS levels, (B) MDA levels, (C) GSH levels, (D) GSSG levels, (E) ETC-CI activity, (F) ETC-CIII activity, (G) LMP status, and (H) GPX4 expression were evaluated. (I–K) Analyzed data and representative images of western blot analysis showing the expression of ER stress-related proteins in LO2 cells treated with vehicle or SO/SP with or without (I) *RELA*-KD or (J) *RELA*-OE. (K)

Representative western blot images presenting the expression of ER stress-related proteins in HepG2 cells under the indicated conditions. (L) Analyzed data and representative images of Western blot analysis showing the expression of ER stress-related proteins in normal diet or HFD-fed mice with or without *Hnf1a*-KD. b $P<0.01$, vs. control group; d $P<0.01$, vs. SO/SP group or HFD group. BSA: bovine serum albumin; HFD: high-fat diet; SO/SP: sodium oleate and sodium palmitate.

Fig. 1D


Control
125/62.5 μM
250/125 μM
500/250 μM
HepG2 cells
Cleaved caspase-3
β-actin
p-MLKL


MLKL

Fig. 1E


Control
125/62.5 µM
250/125 µM
500/250 µM
HepG2 cells
RelA
HNF1α

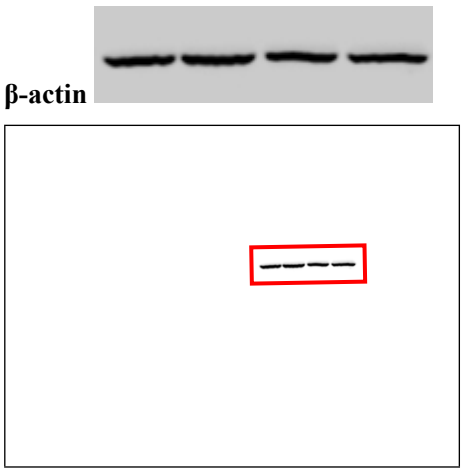
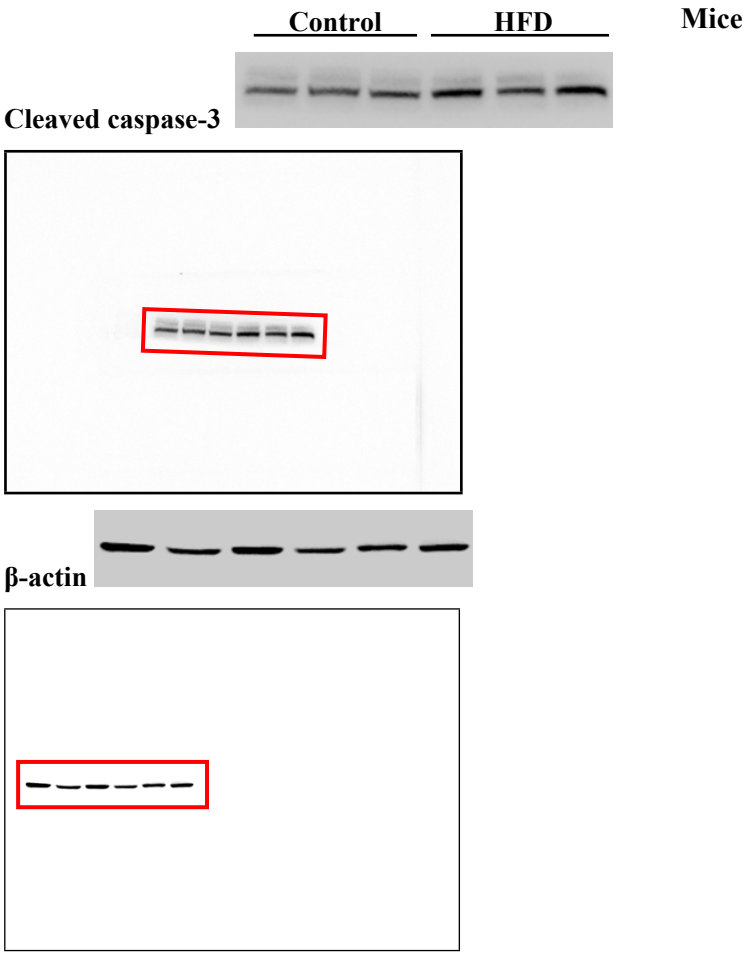
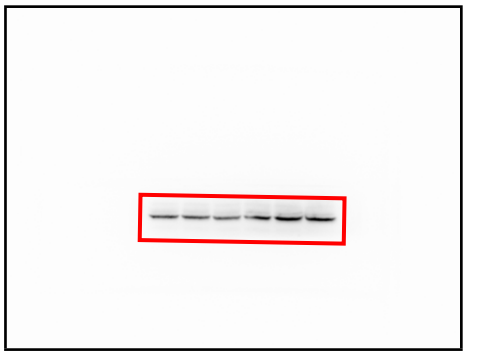


Fig. 1K



p-MLKL



MLKL

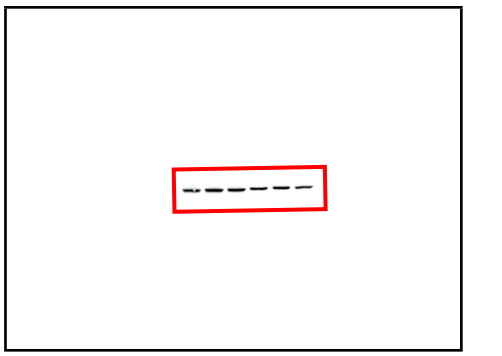
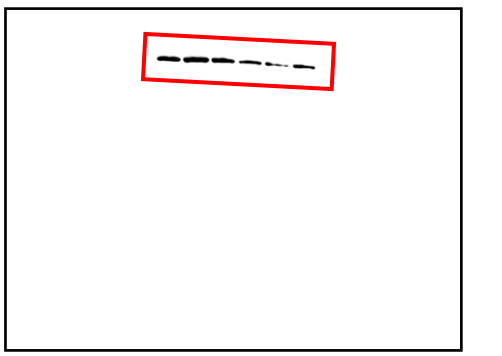


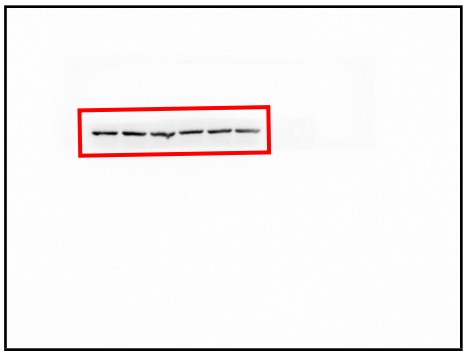
Fig 1L

Control HFD Mice

RelA



HNF1α



β-actin

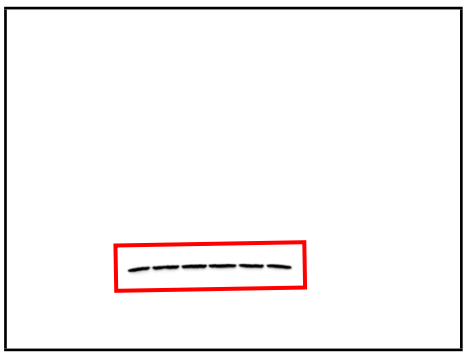
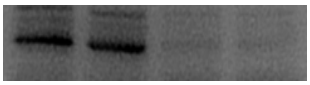


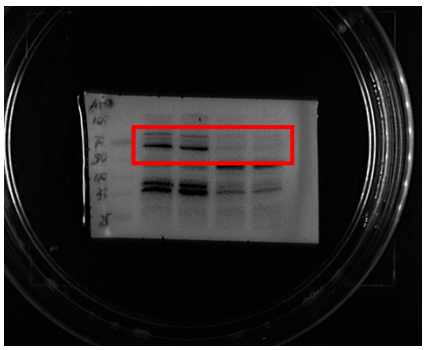
Fig. 2A

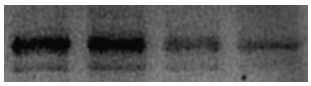
Vehicle	+	+	−	−
***RELA*-KO**	−	−	+	+
SO/SP	+	+	+	+

HepG2 cells

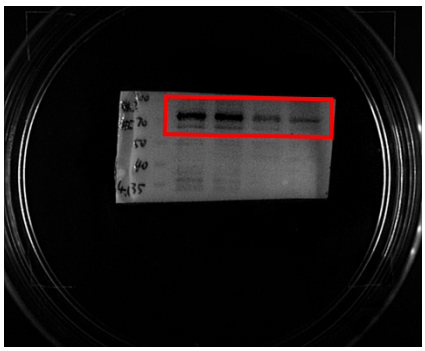


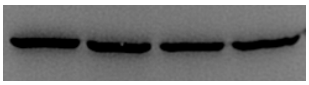
RelA





HNF1α





β-actin

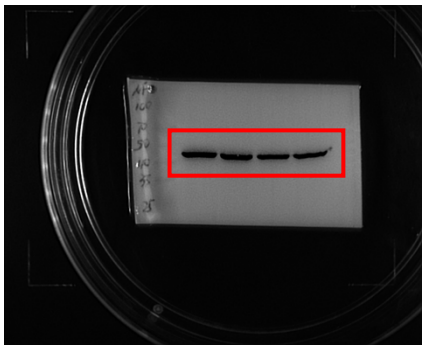


Fig. 2B

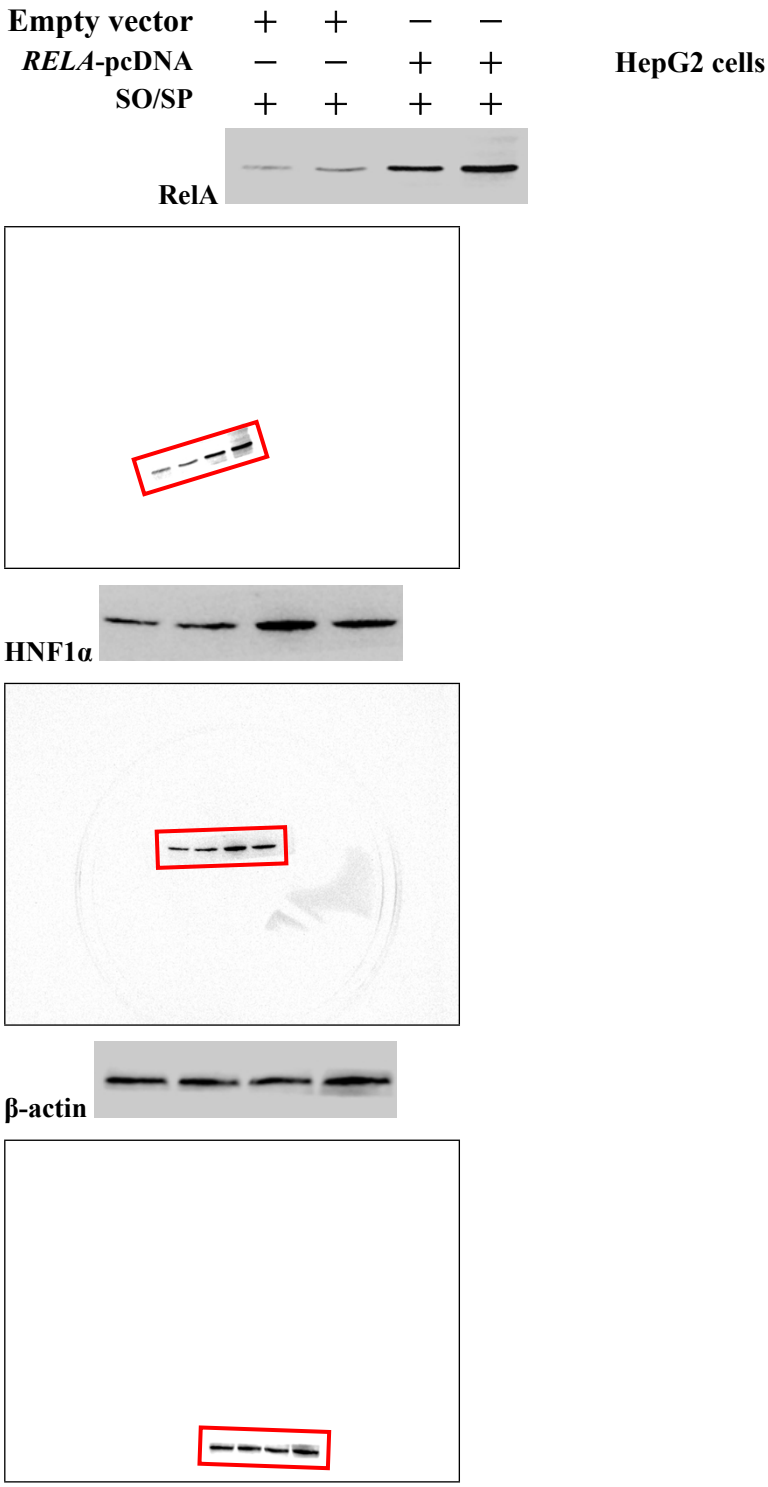
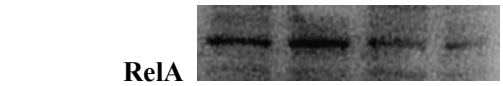


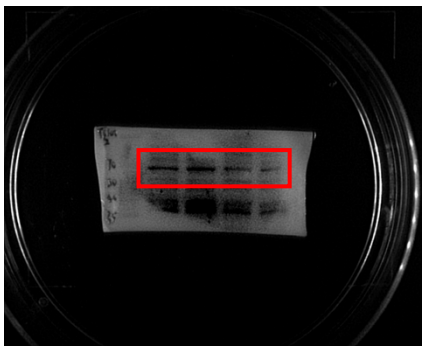
Fig. 2C

Vehicle	+	+	−	−
HNF1A-KO	−	−	+	+
SO/SP	+	+	+	+

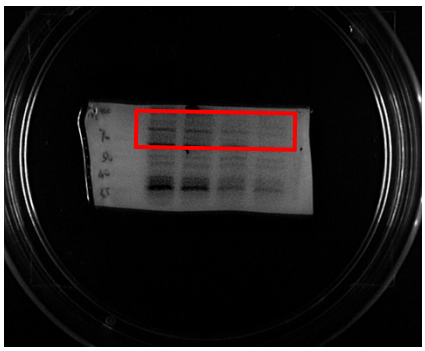
HepG2 cells

RelA





HNF1α



β-actin

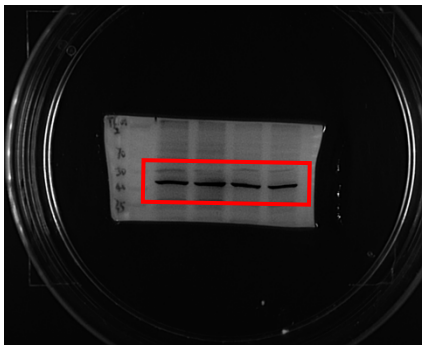


Fig. 2F

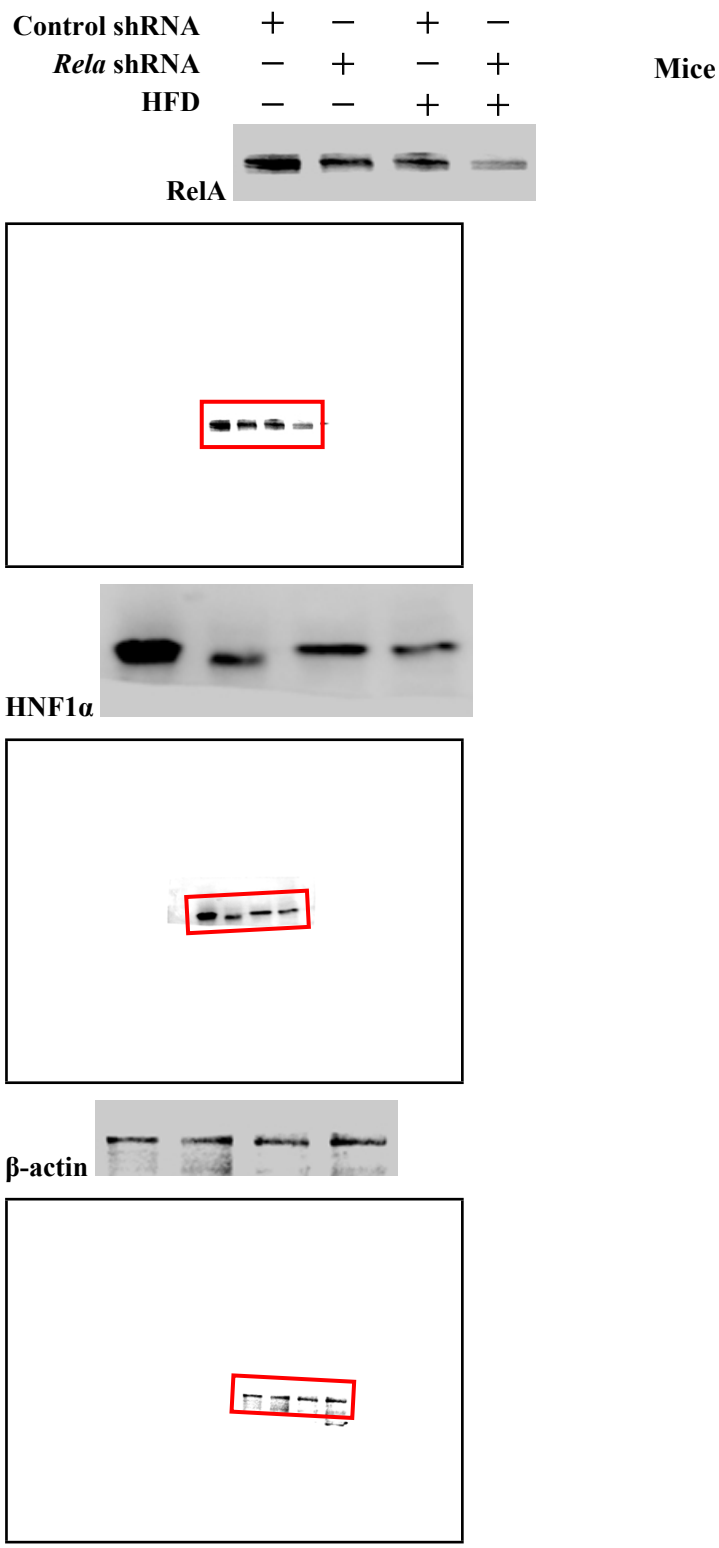


Fig. 2G

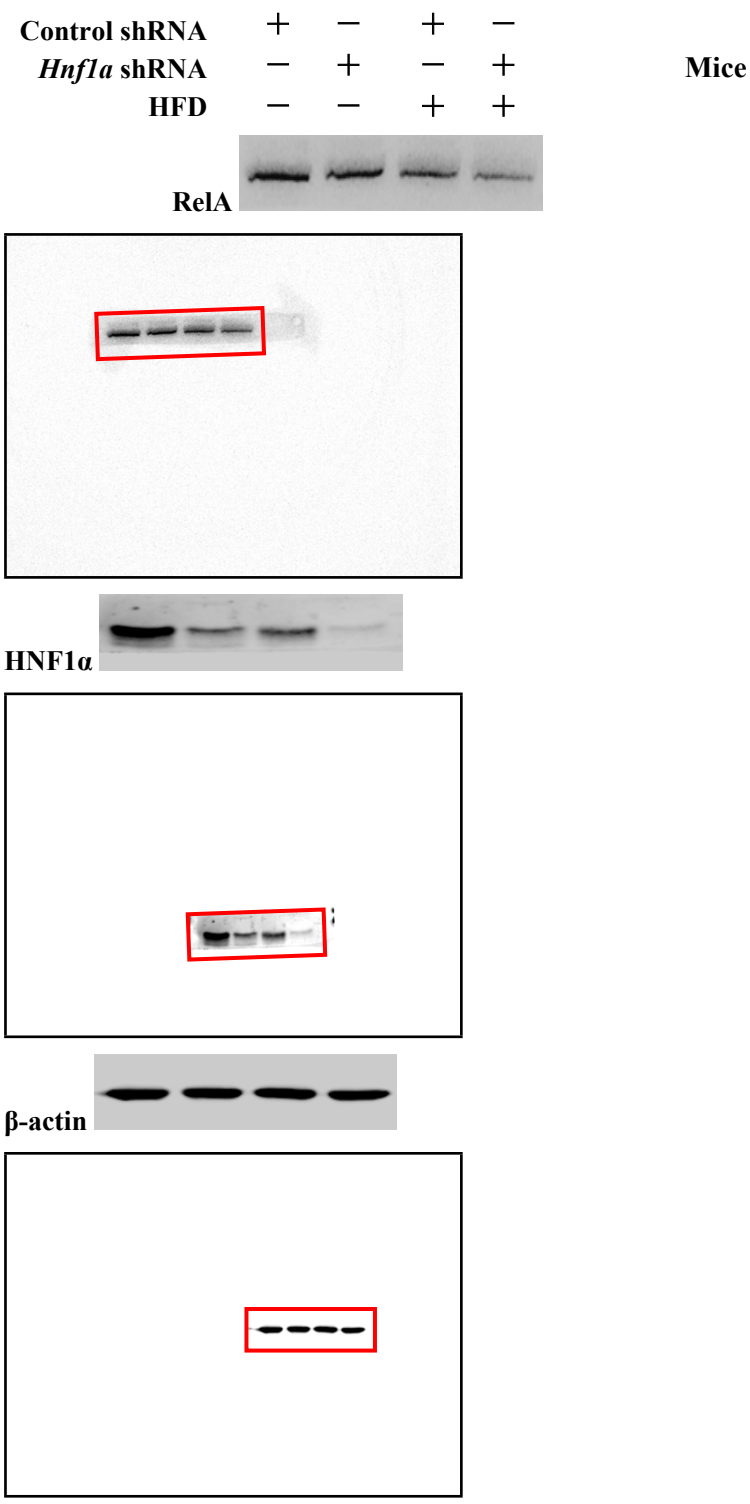
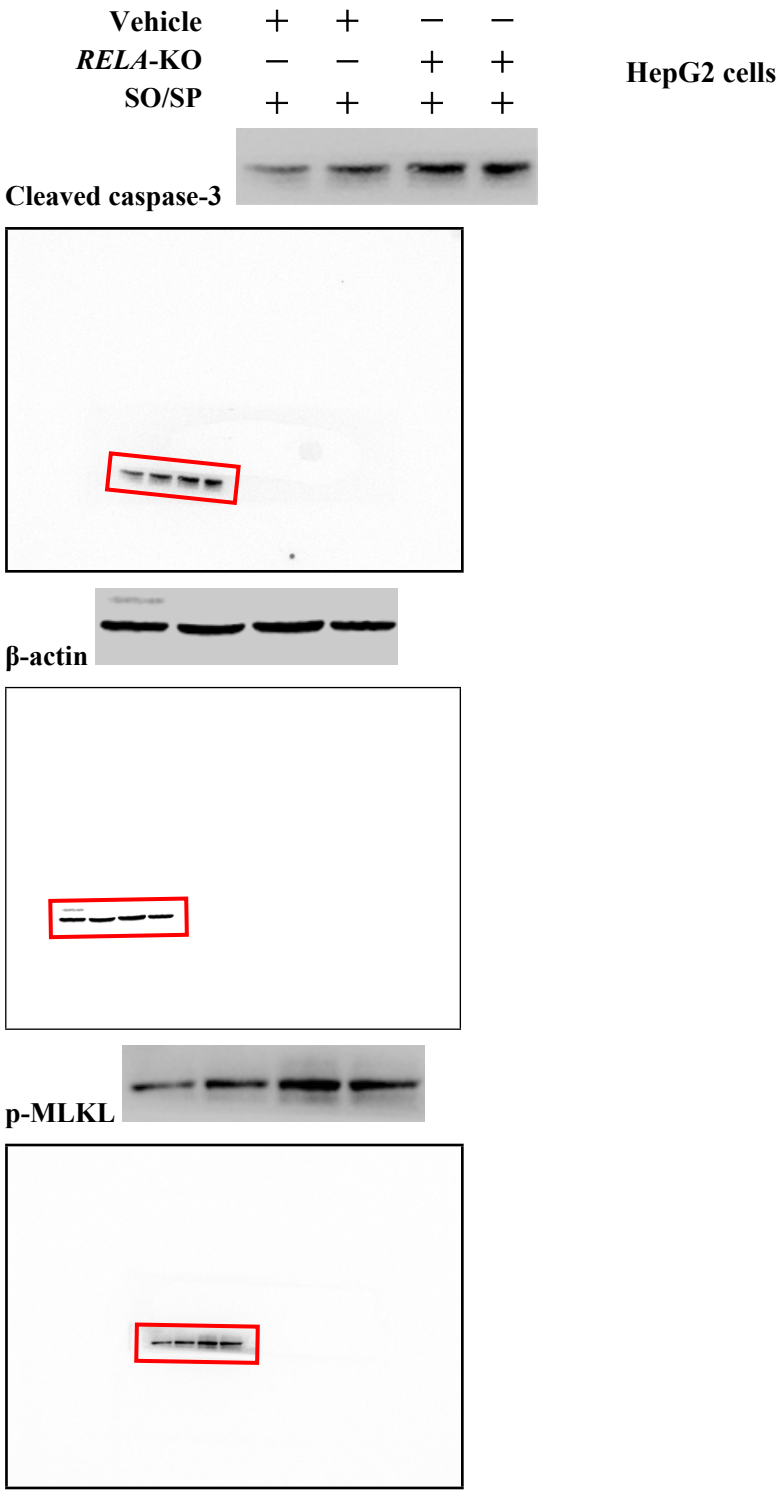


Fig. 3E


Vehicle + + − −
RELA-KO − − + +
SO/SP + + + +
HepG2 cells
Cleaved caspase-3
β-actin
p-MLKL

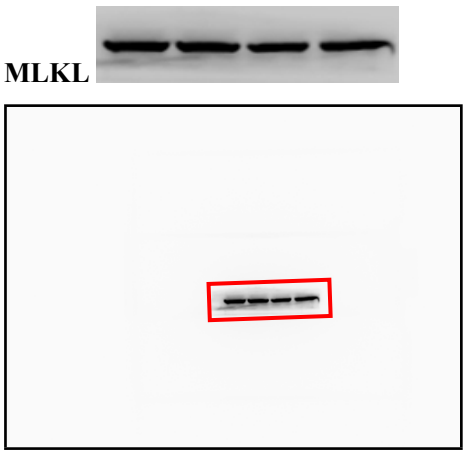
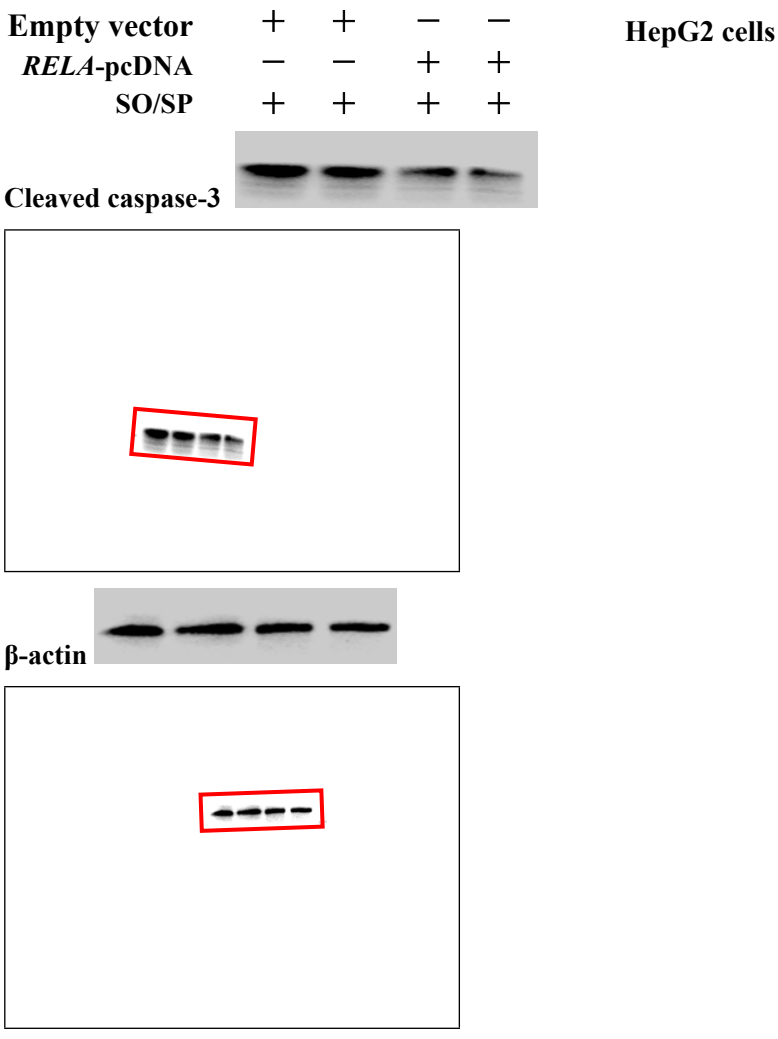
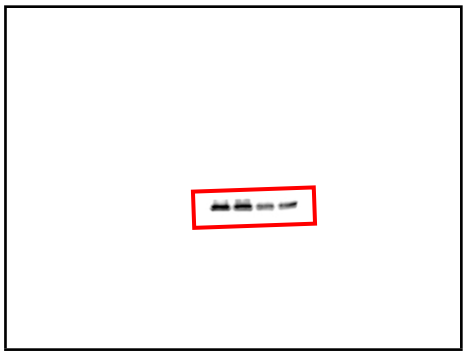

MLKL

Fig. 3J


Empty vector + + − −
RELA-pcDNA − − + +
SO/SP + + + +
HepG2 cells
Cleaved caspase-3
β-actin

p-MLKL



MLKL

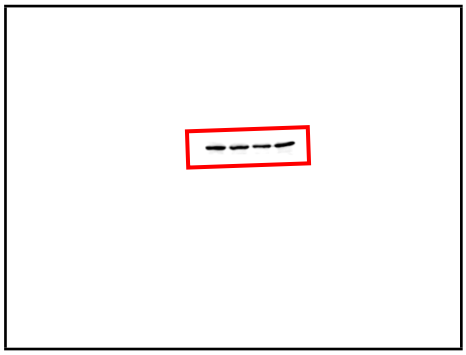
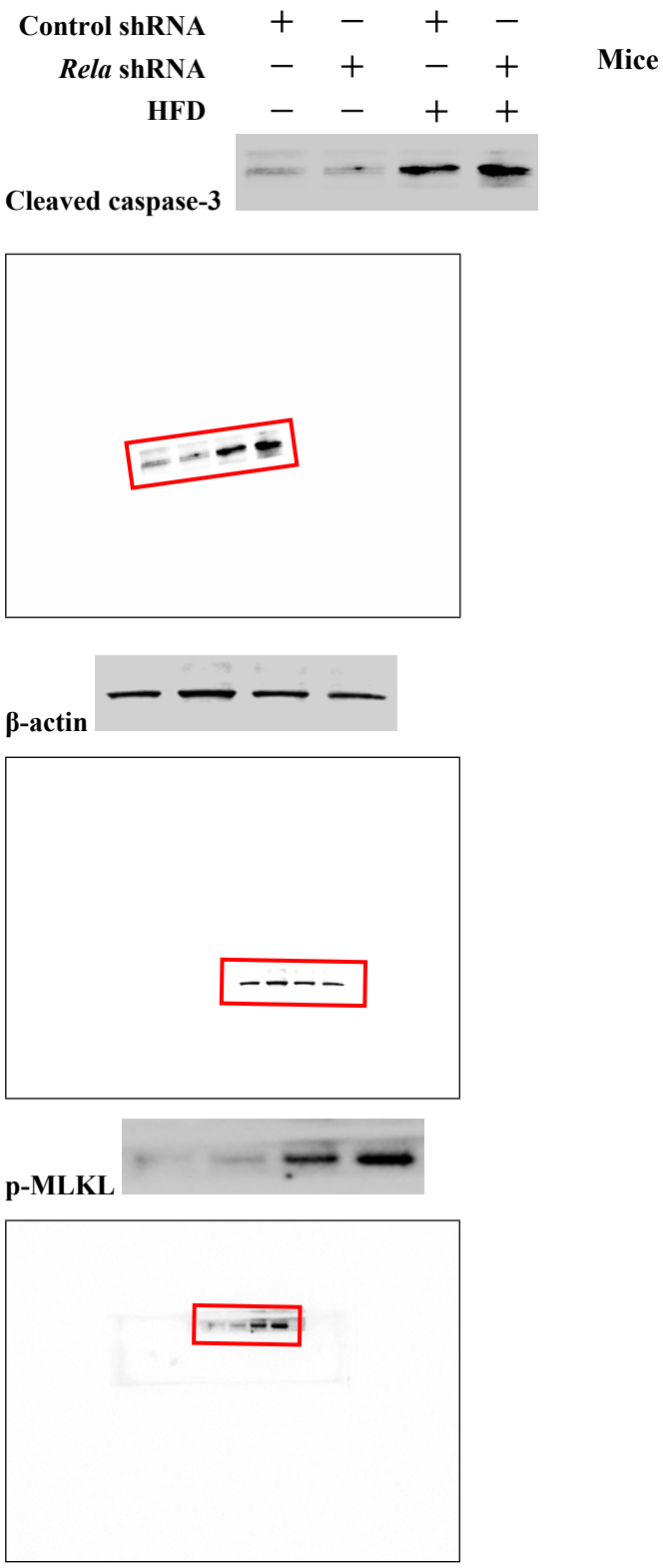


Fig. 4F


Control shRNA + − + −
Rela shRNA − + − +
HFD − − + +
Mice
Cleaved caspase-3
β-actin
p-MLKL

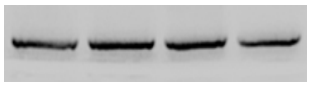
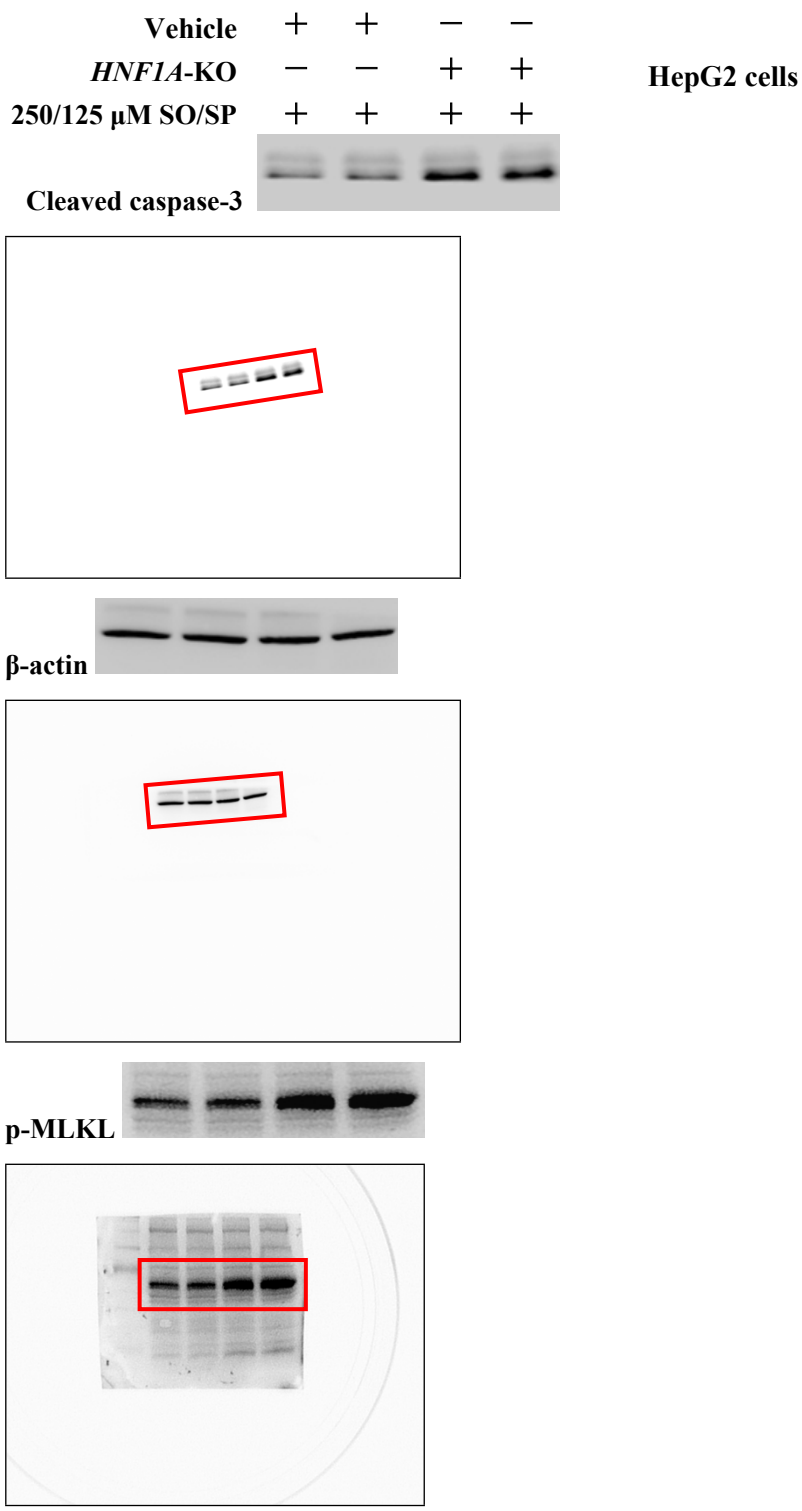
MLKL 

Fig. 5E

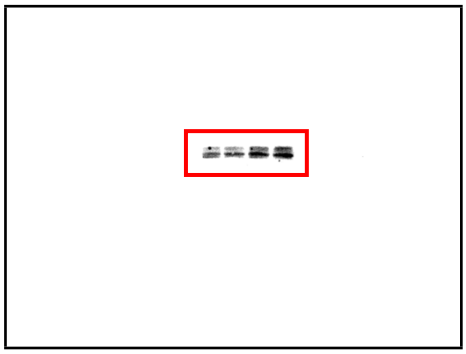

Vehicle + + − −
HNF1A-KO − − + +
250/125 μM SO/SP + + + +
HepG2 cells
Cleaved caspase-3
β-actin
p-MLKL

MLKL

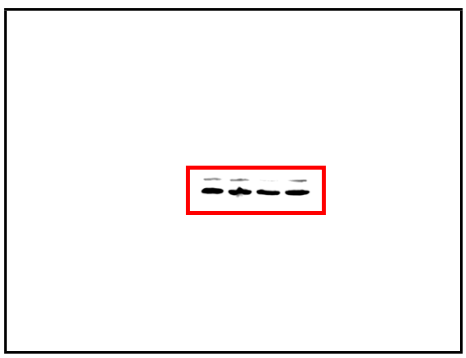
Fig. 5K

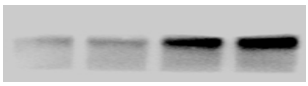
Control shRNA	+	−	+	−	
Hnf1a shRNA	−	+	−	+	Mice
HFD	−	−	+	+	

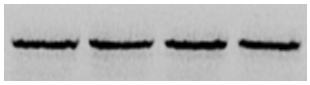
Cleaved caspase-3



β-actin



p-MLKL 

MLKL 

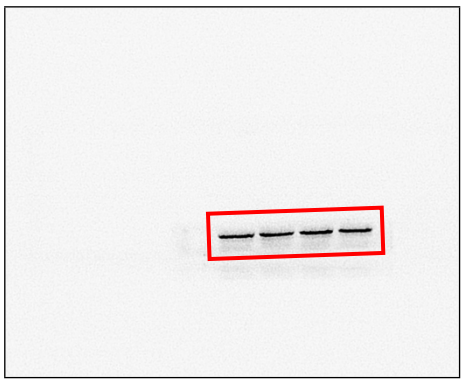


Fig. 6G

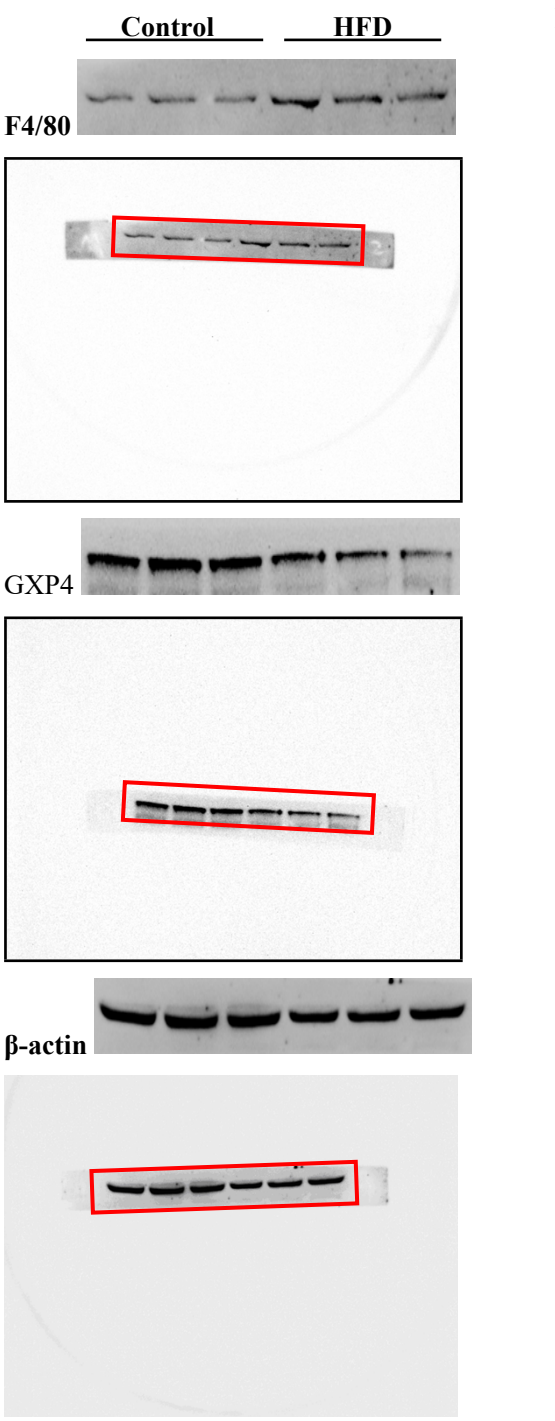
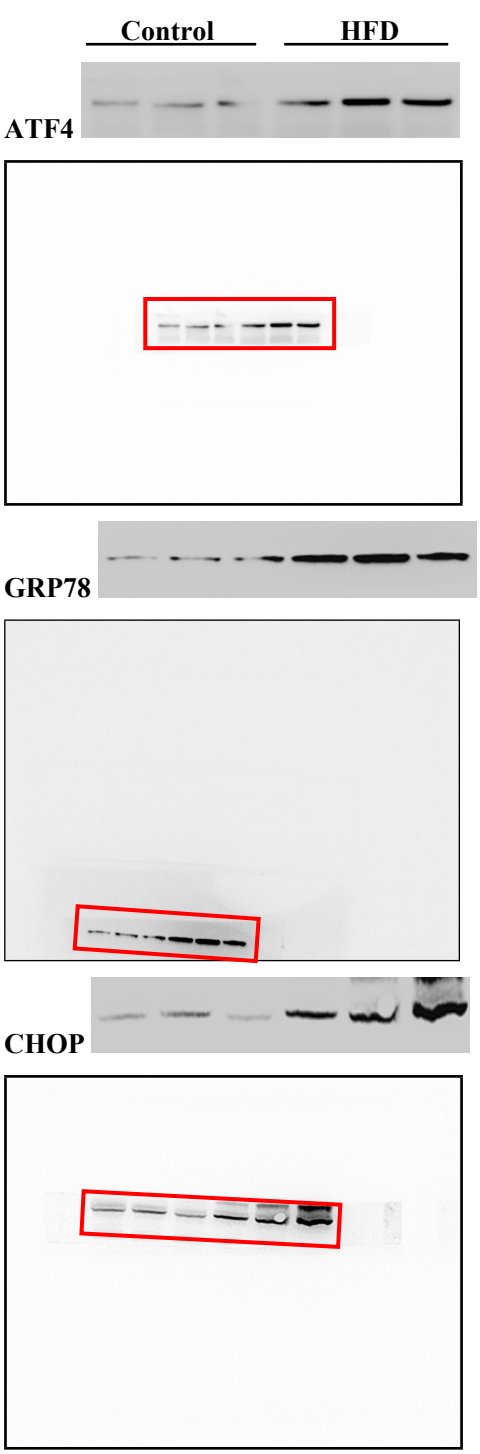


Fig. 6H



β-actin

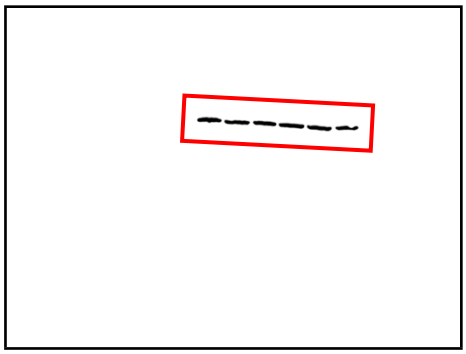


Fig. 7D

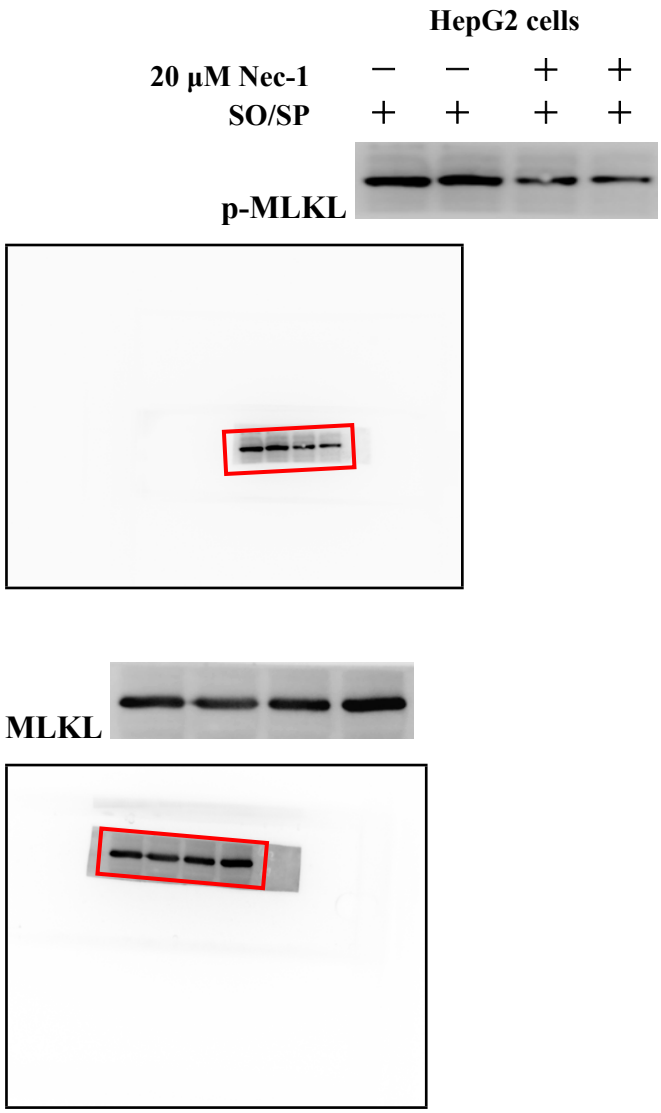


Fig. 7I

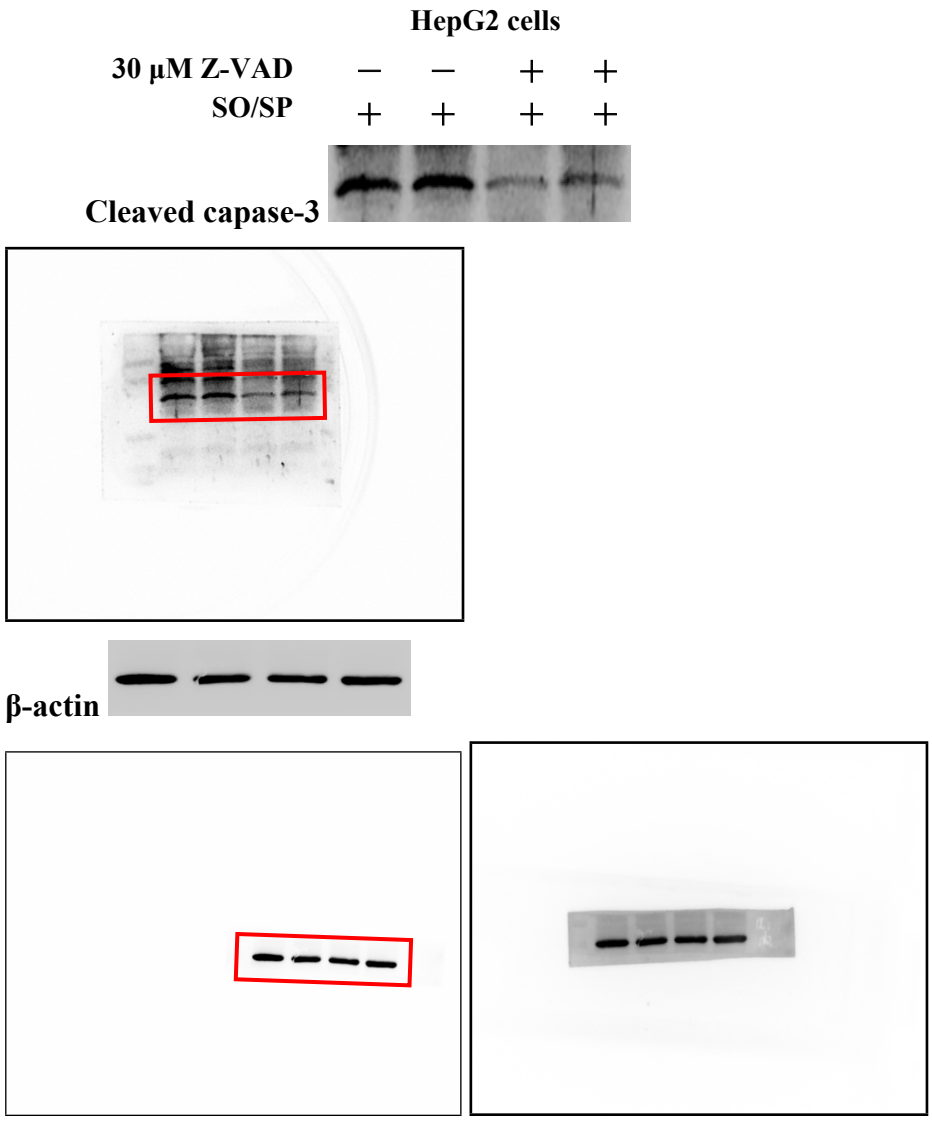


Fig. 8G

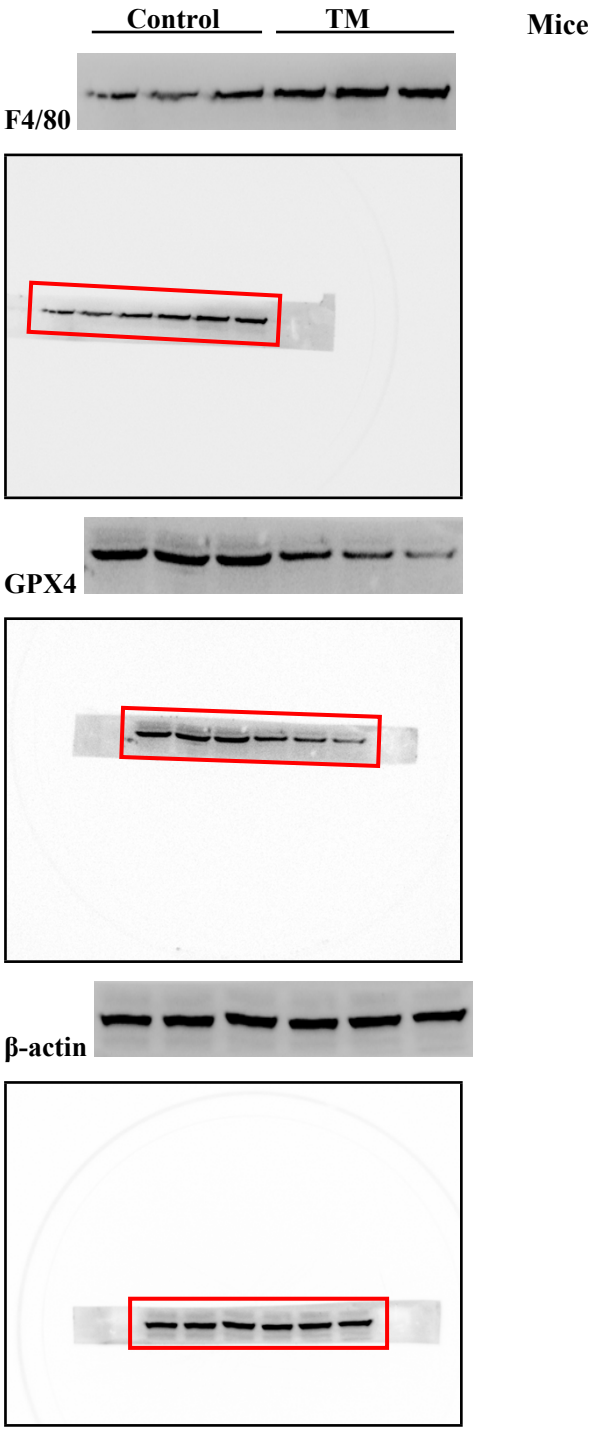
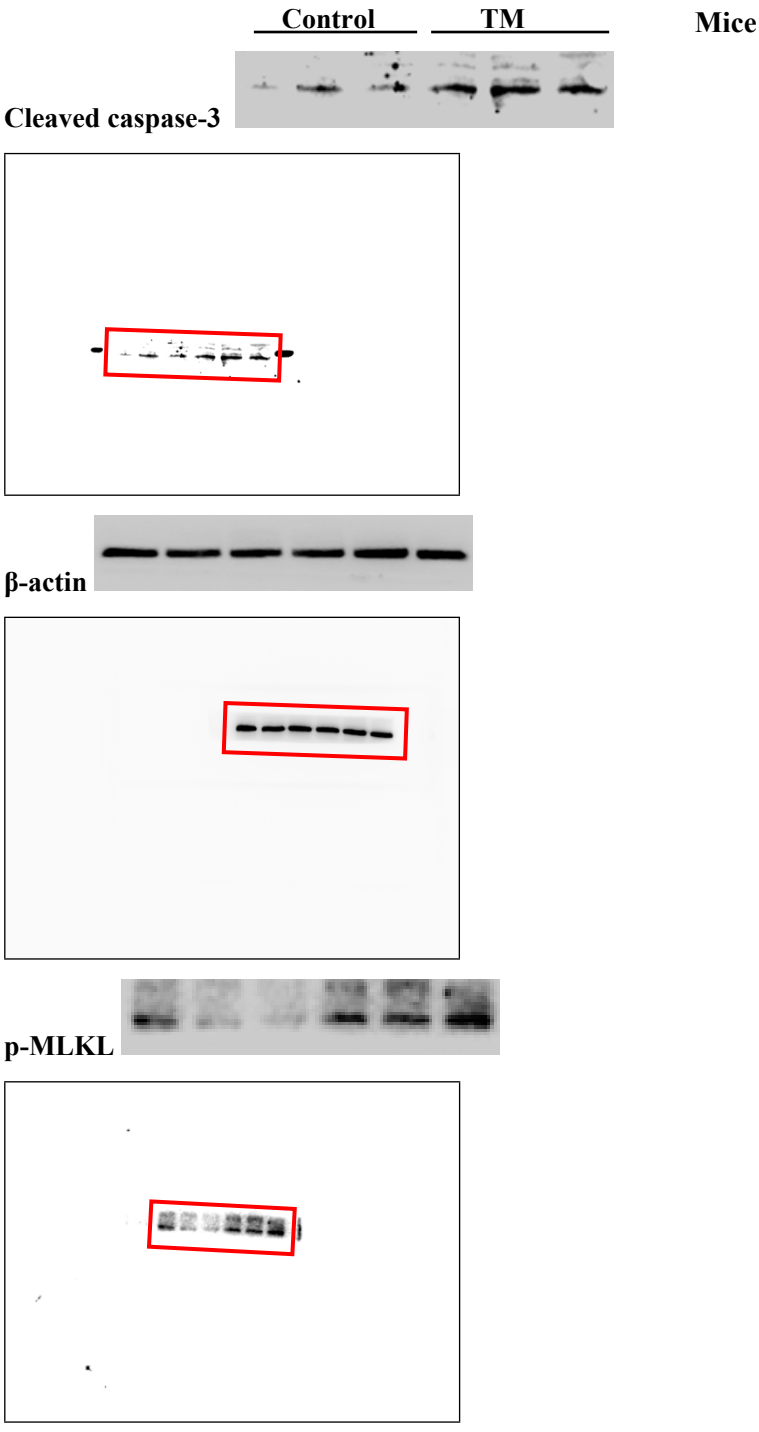
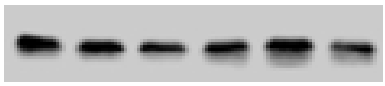


Fig. 8H



MLKL 

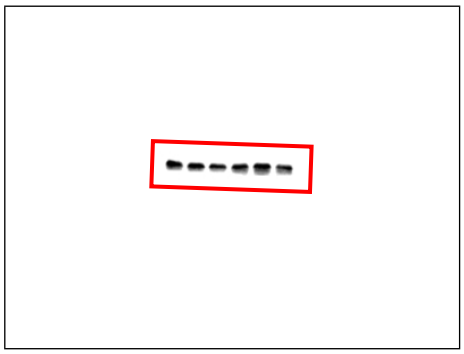


Fig. 9A


Control
125/62.5 µM
250/125 µM
500/250 µM
HepG2 cells
MTP
MCAD
SREBP1c

β-actin

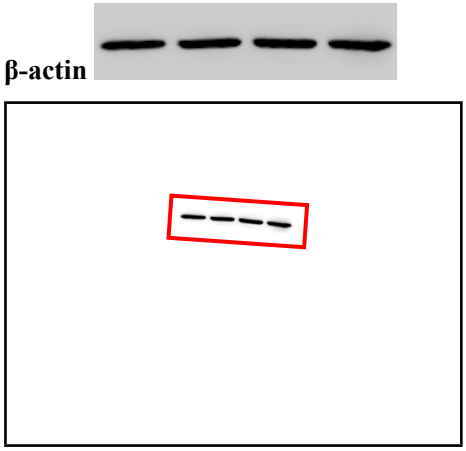
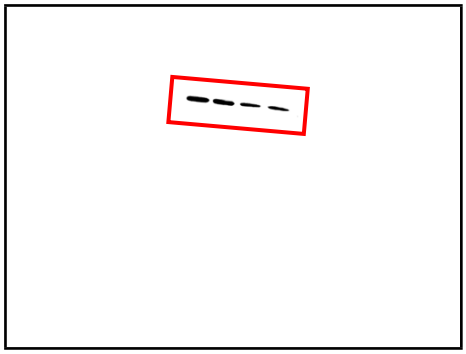


Fig. 9B

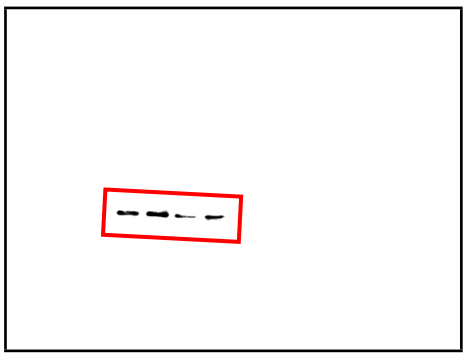
Control 48h THA

HepG2 cells

MTP



MCAD



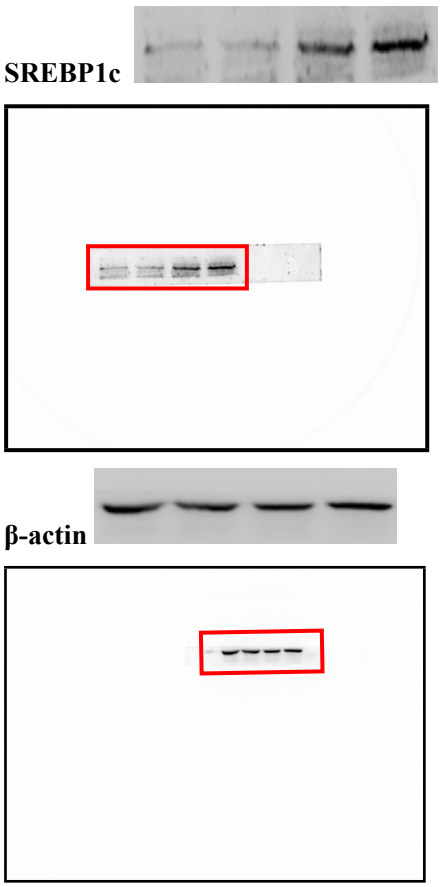
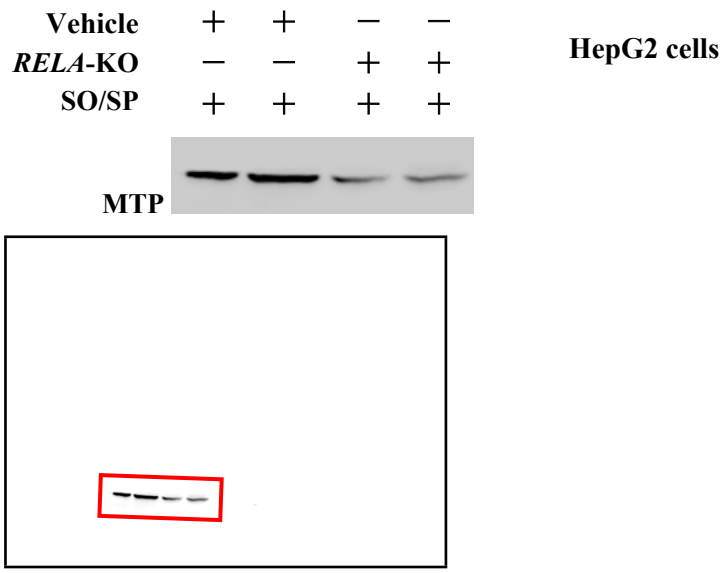


Fig. 9C



MCAD

SREBP1c

β-actin

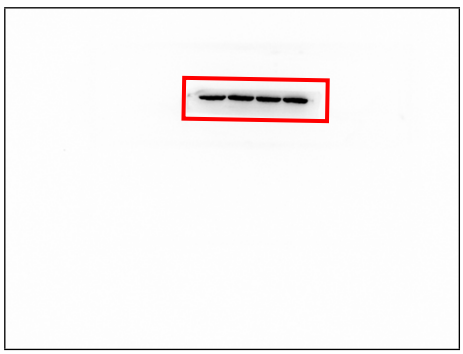
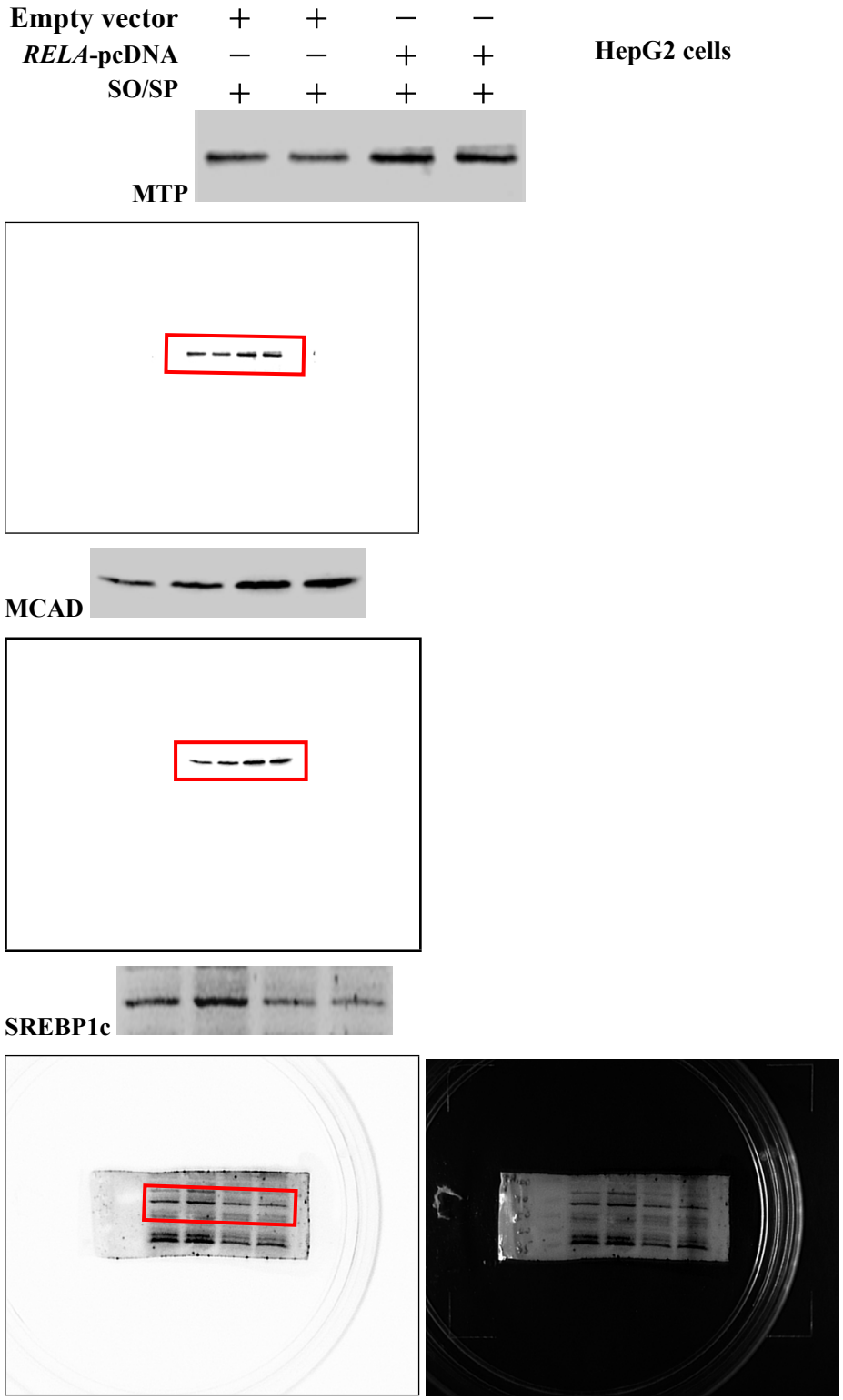


Fig. 9D



β-actin

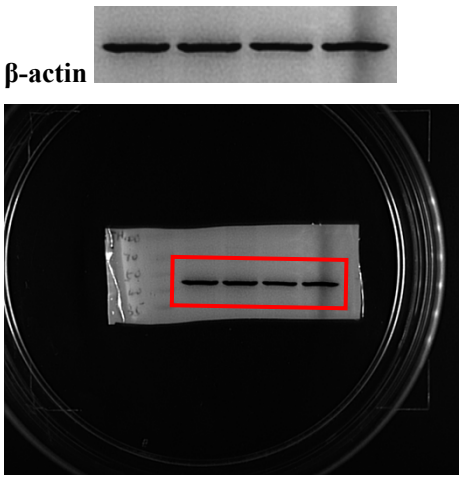
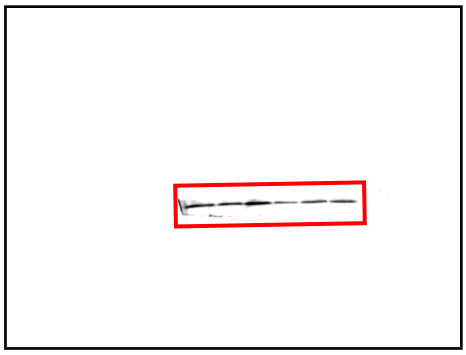


Fig. 9I

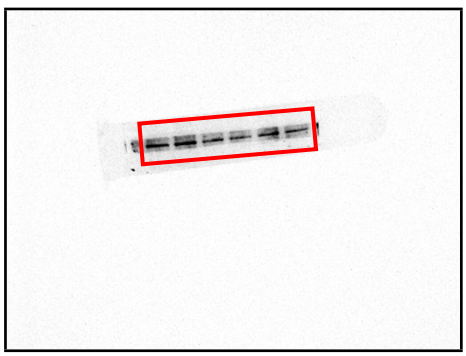
Control HFD

Mice

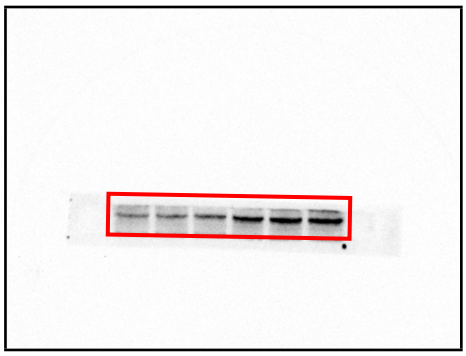
MTP



MCAD



SREBP1c



β-actin

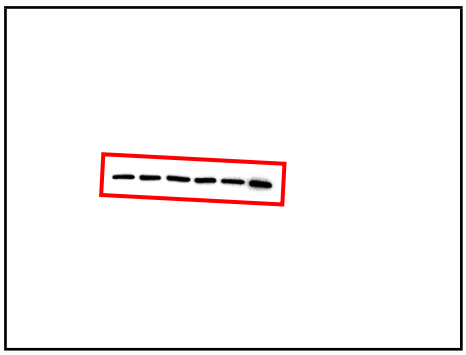
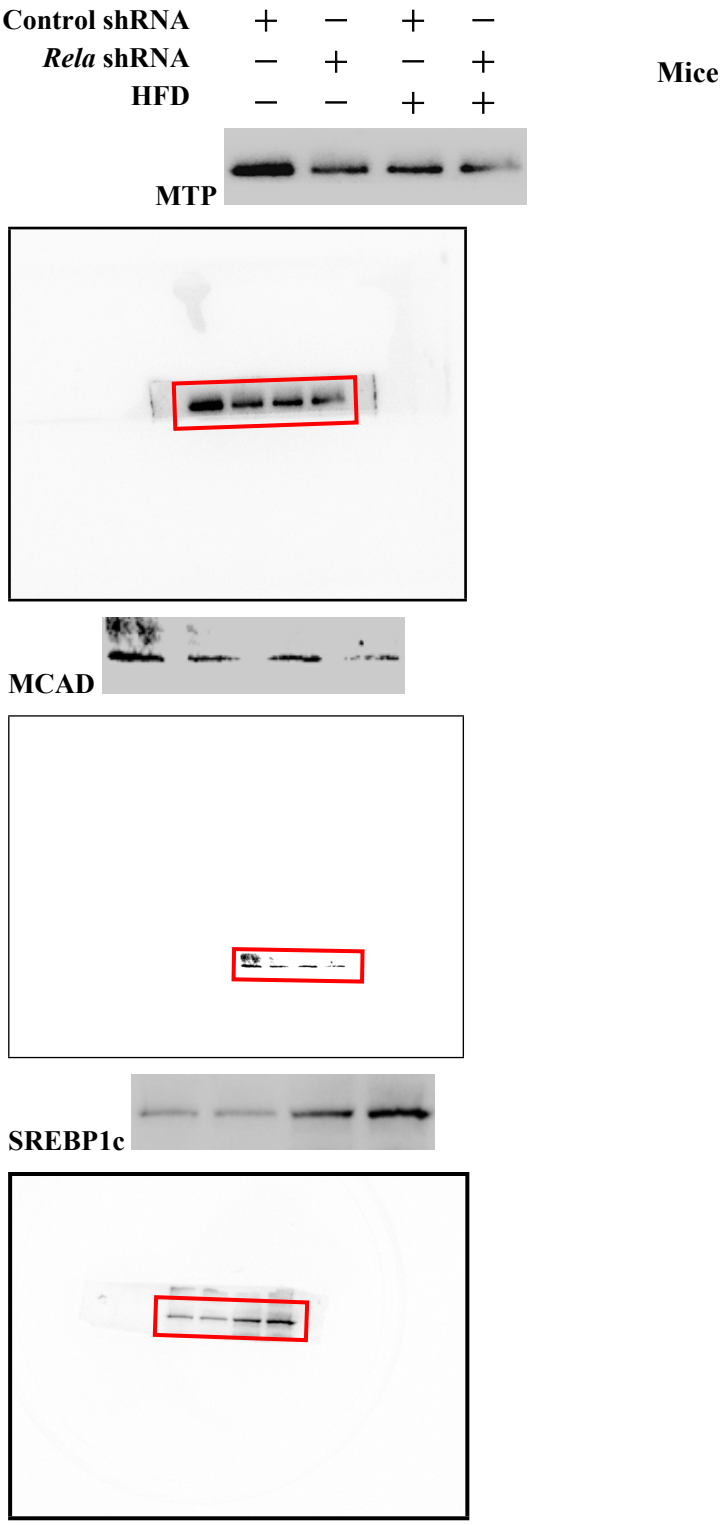
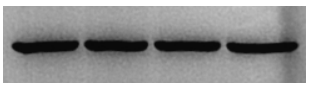


Fig. 9J



β-actin 

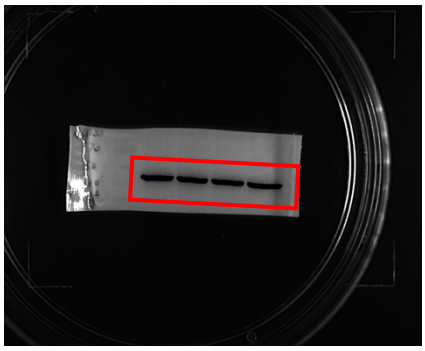


Fig. 10H

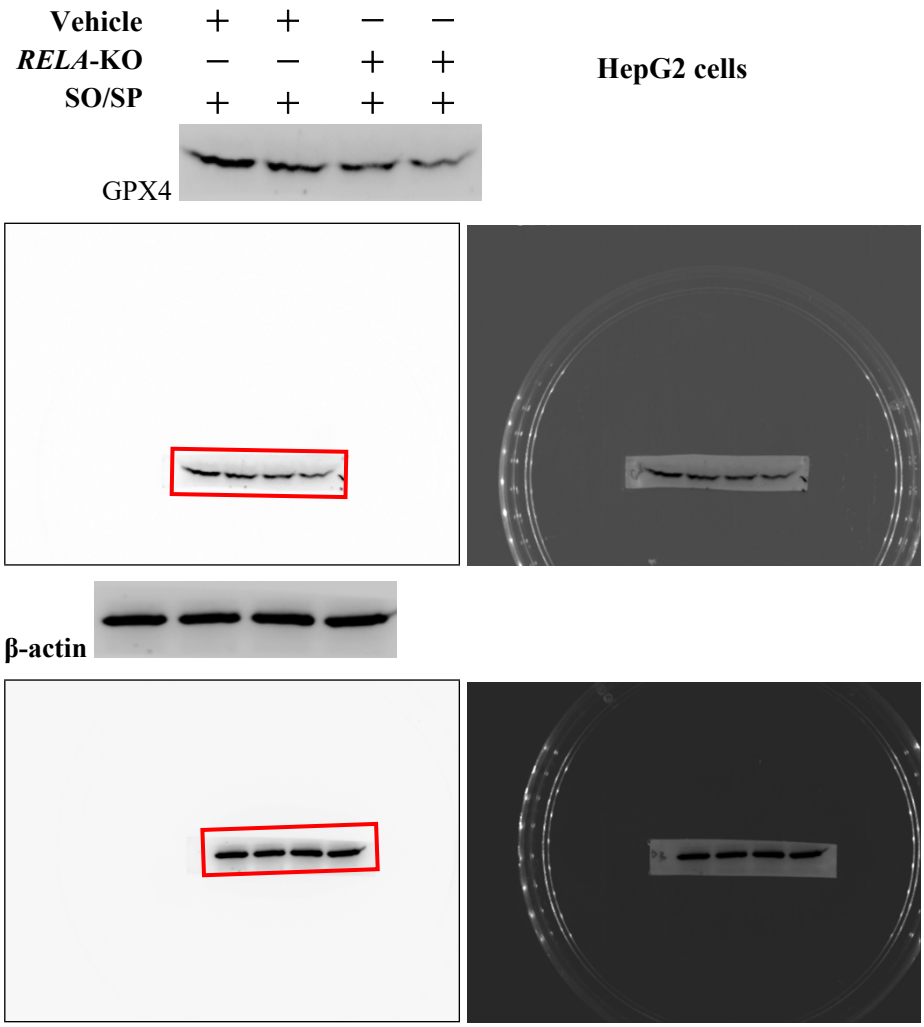
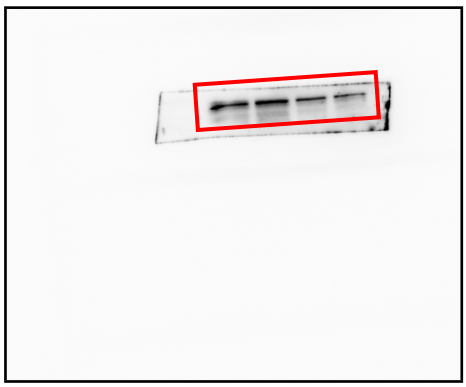


Fig. 10I

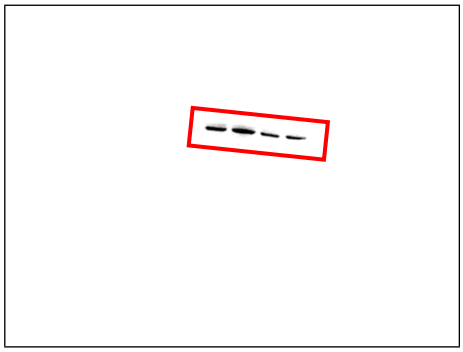
Vehicle	+	+	−	−
RELA-KO	−	−	+	+
SO/SP	+	+	+	+

HepG2 cells

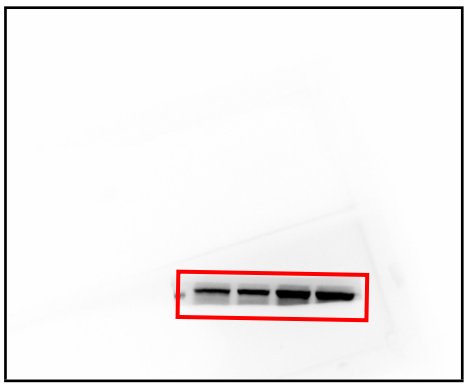
ATF4



GRP78



HRD1



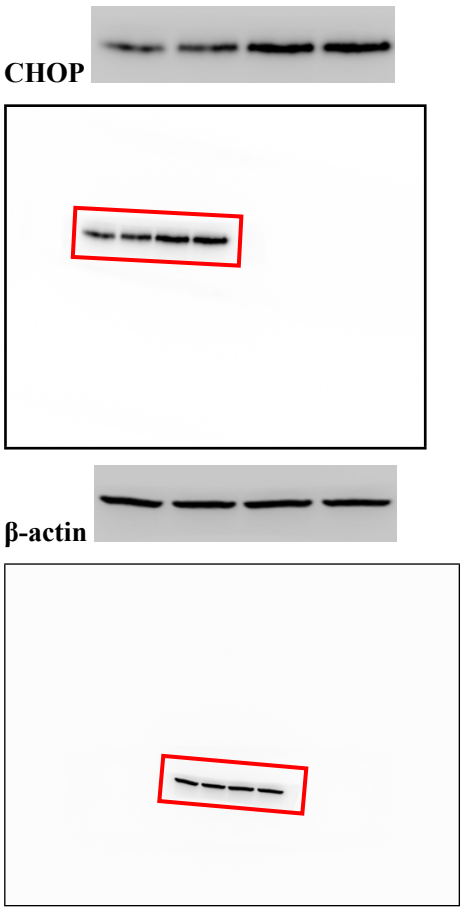
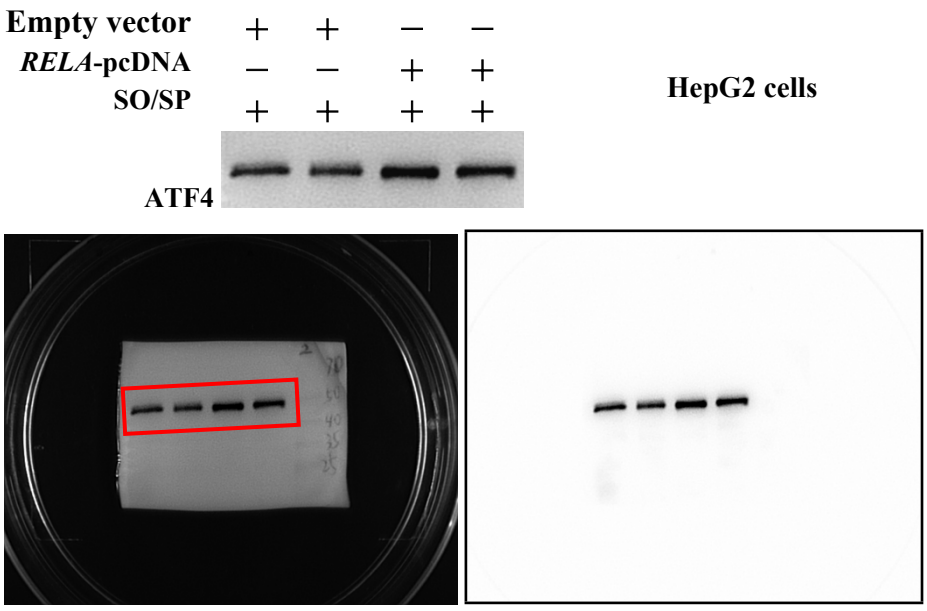
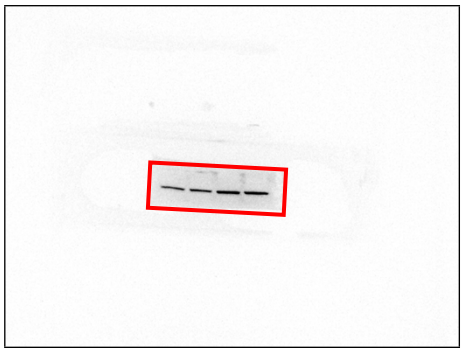


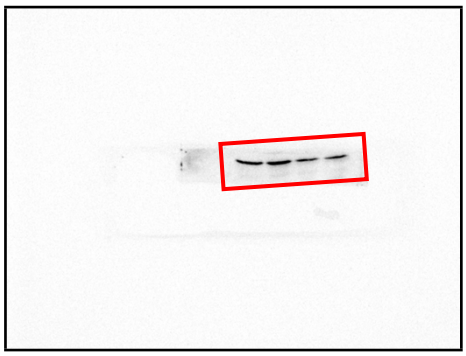
Fig. 10J



GRP78



HRD1



CHOP

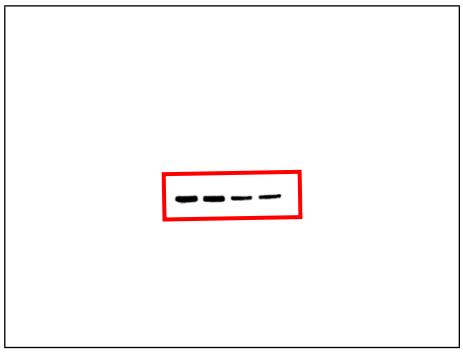
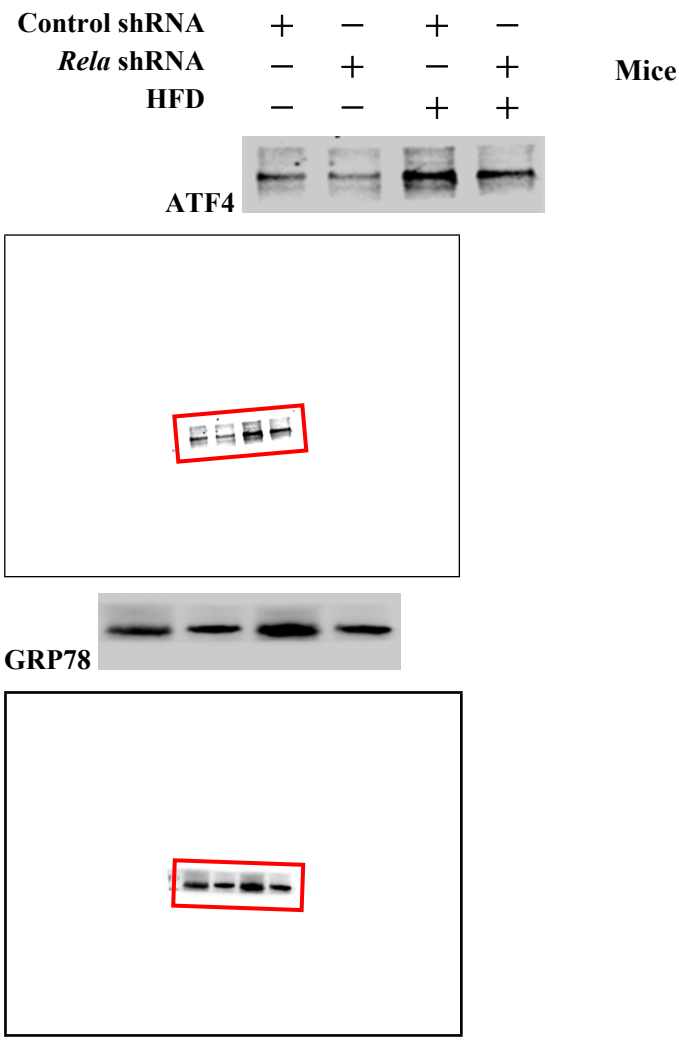
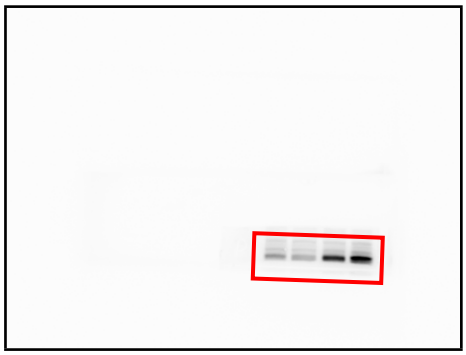


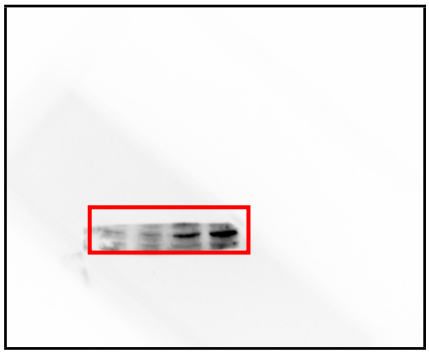
Fig. 10L



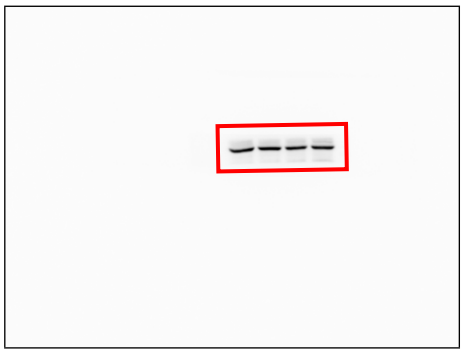
HRD1



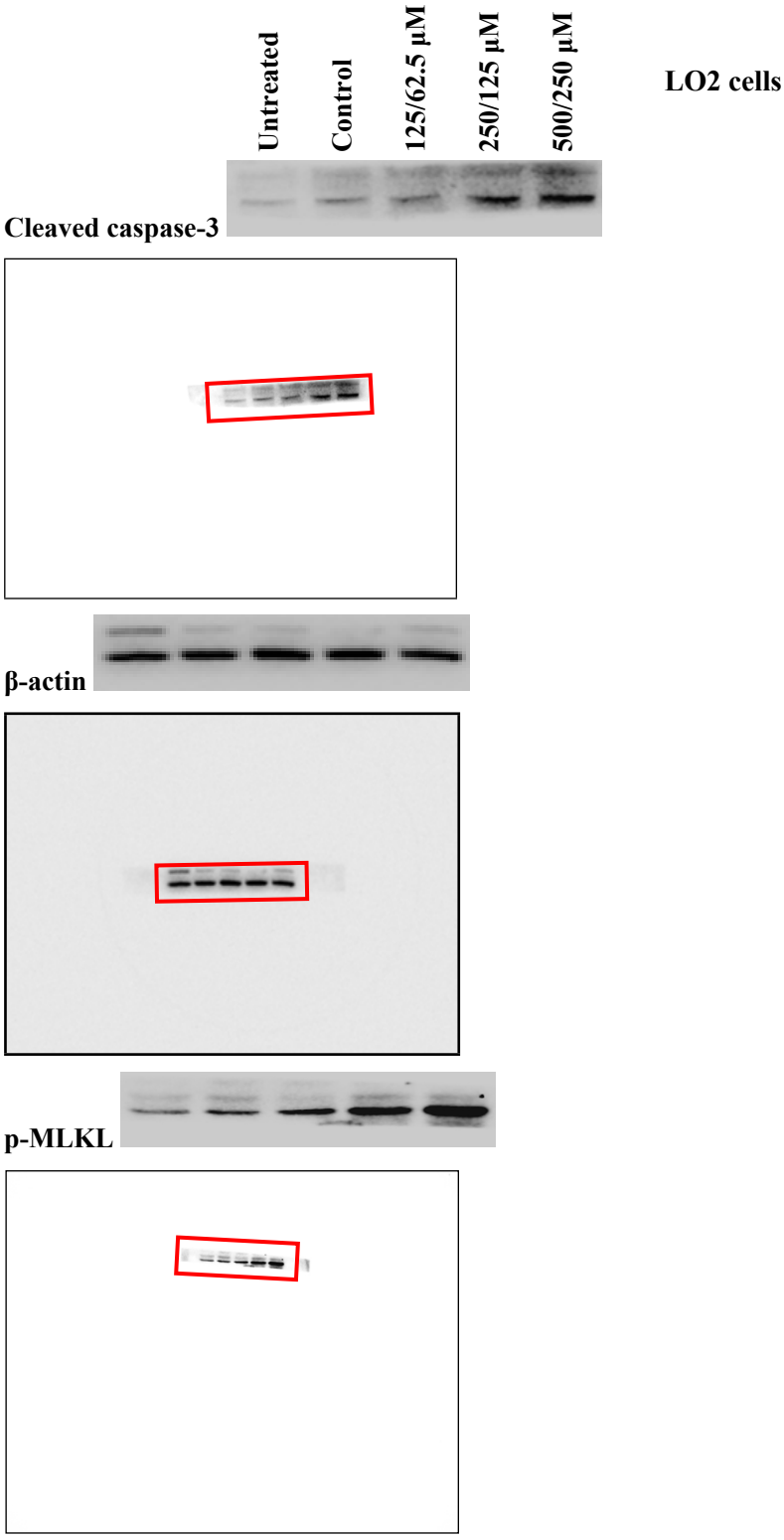
CHOP

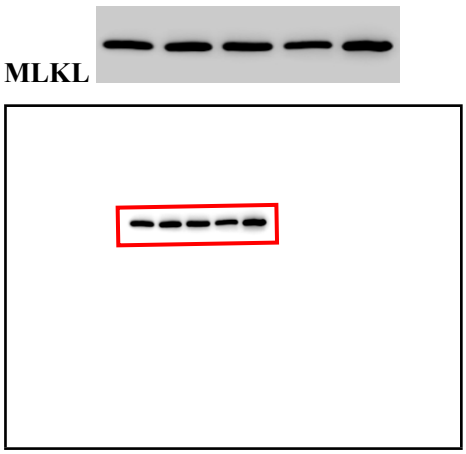


β-actin

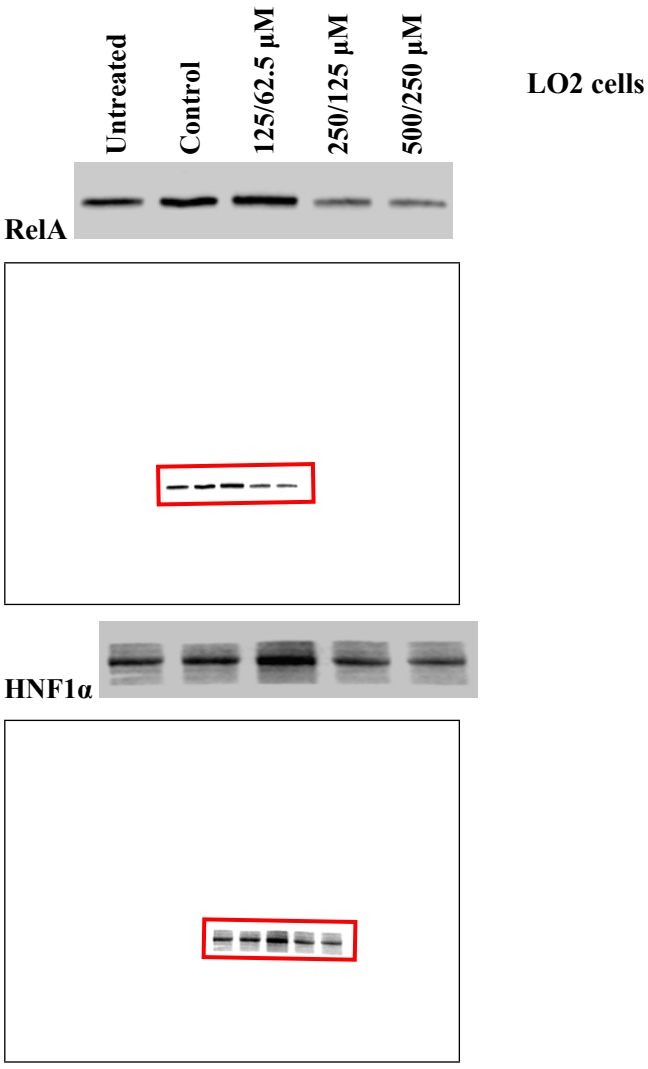


Supplementary Fig. 1D



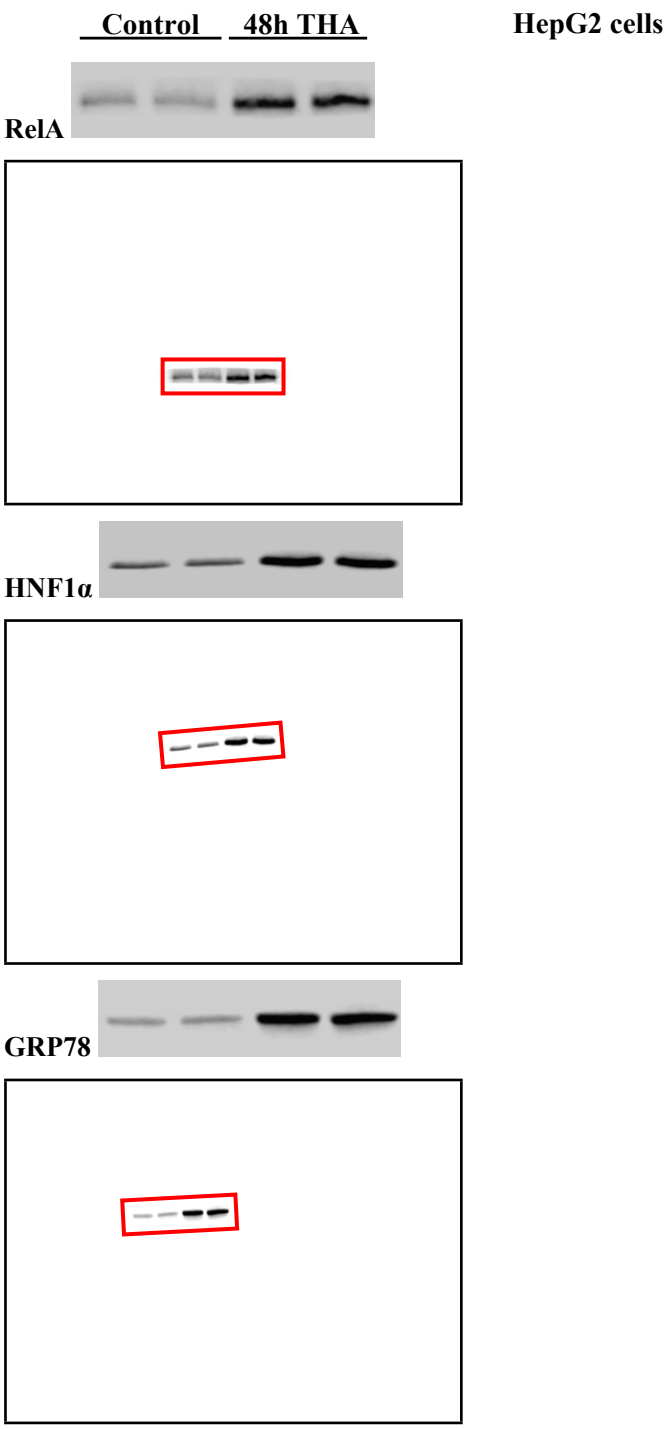


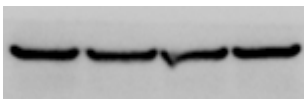
Supplementary Fig. 1E

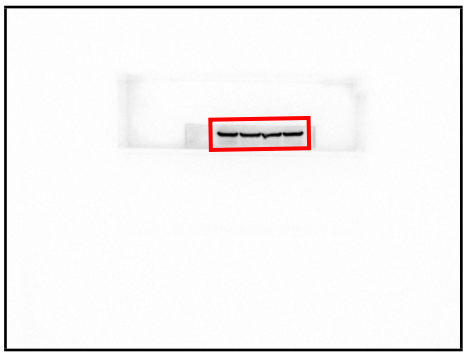


β-actin

Supplementary Fig. 2A



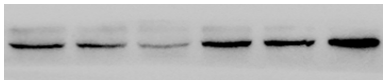
β-actin 

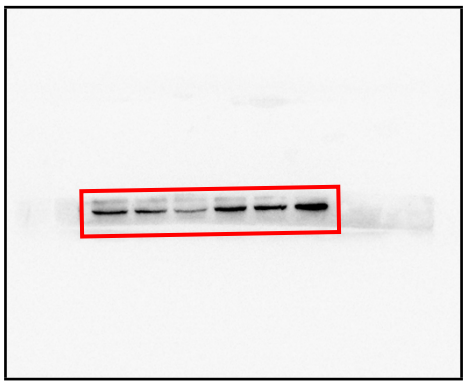


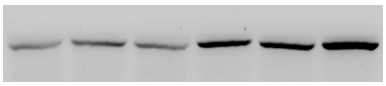
Supplementary Fig. 2J

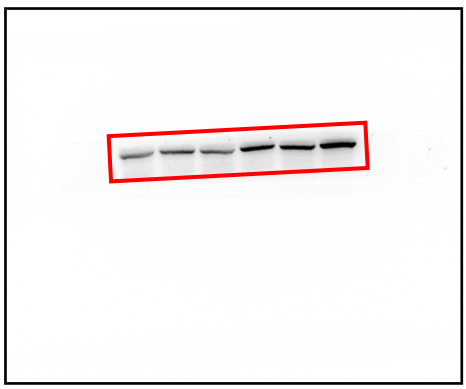
Control TM .

Mice

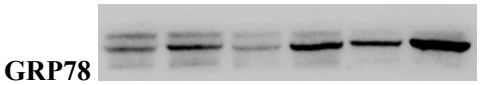
RelA 

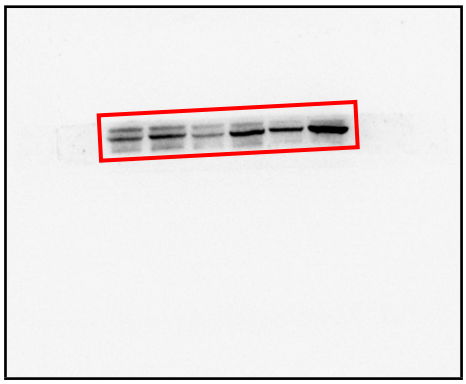


HNF1α 



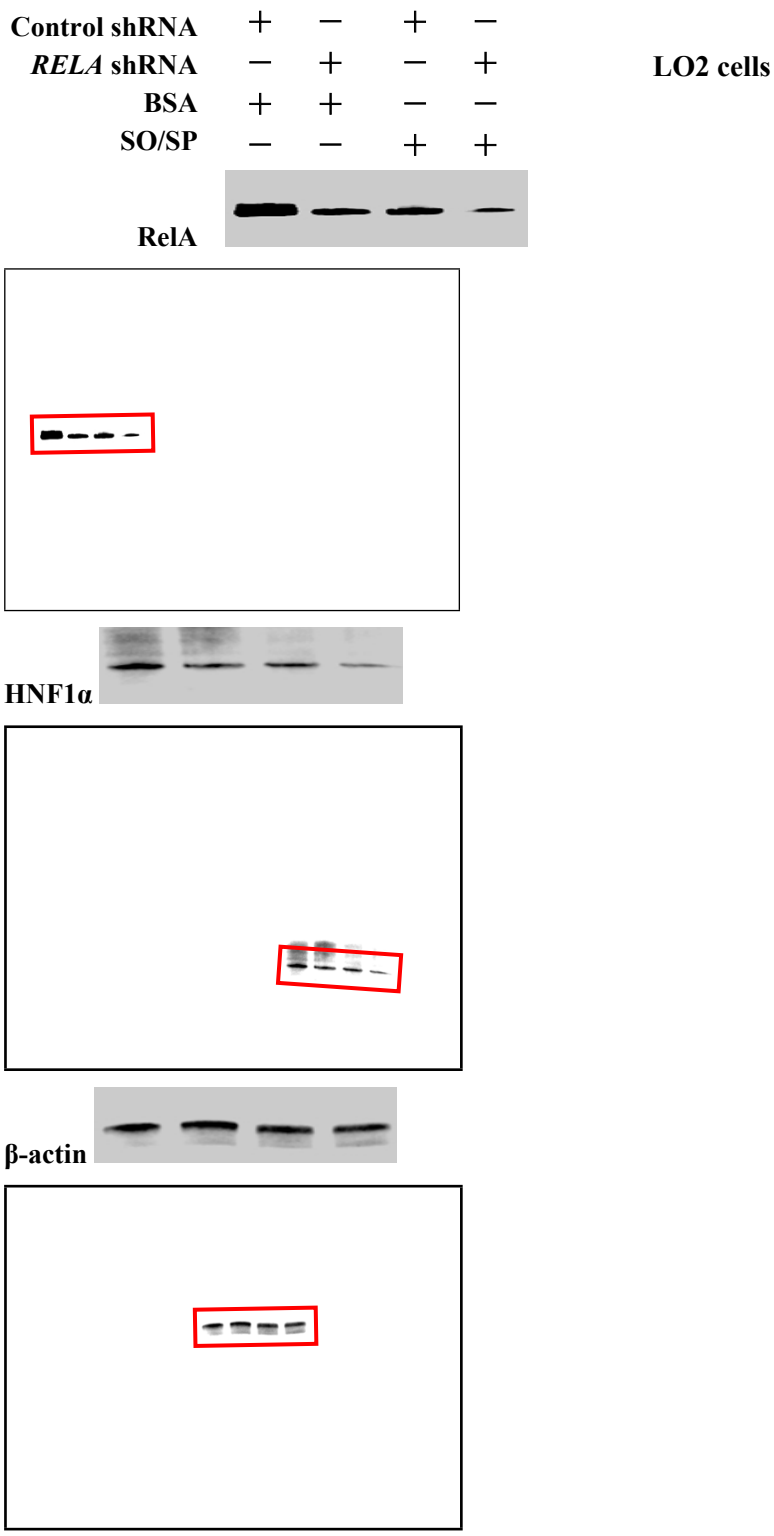
GRP78





β-actin

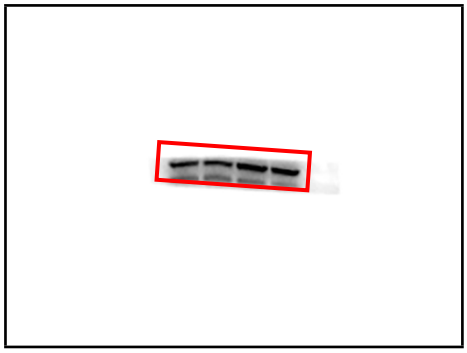
Supplementary Fig. 3A



Supplementary Fig. 2B

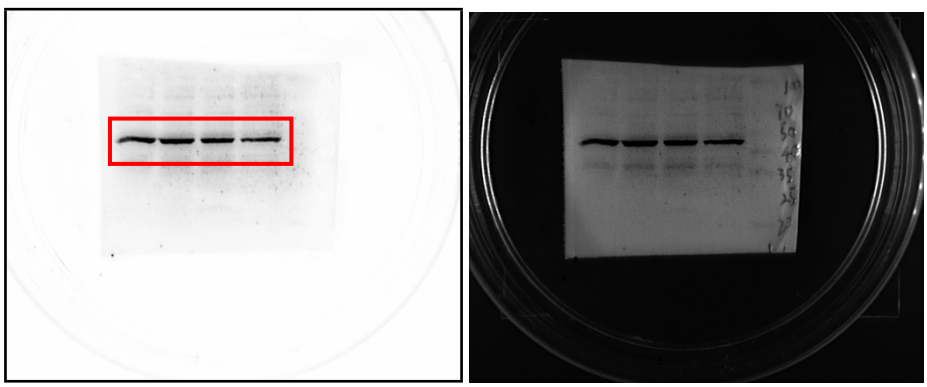
Empty vector	+	+	−	−	**LO2 cells**
***RELA*-pcDNA**	−	−	+	+	
SO/SP	+	+	+	+	

RelA

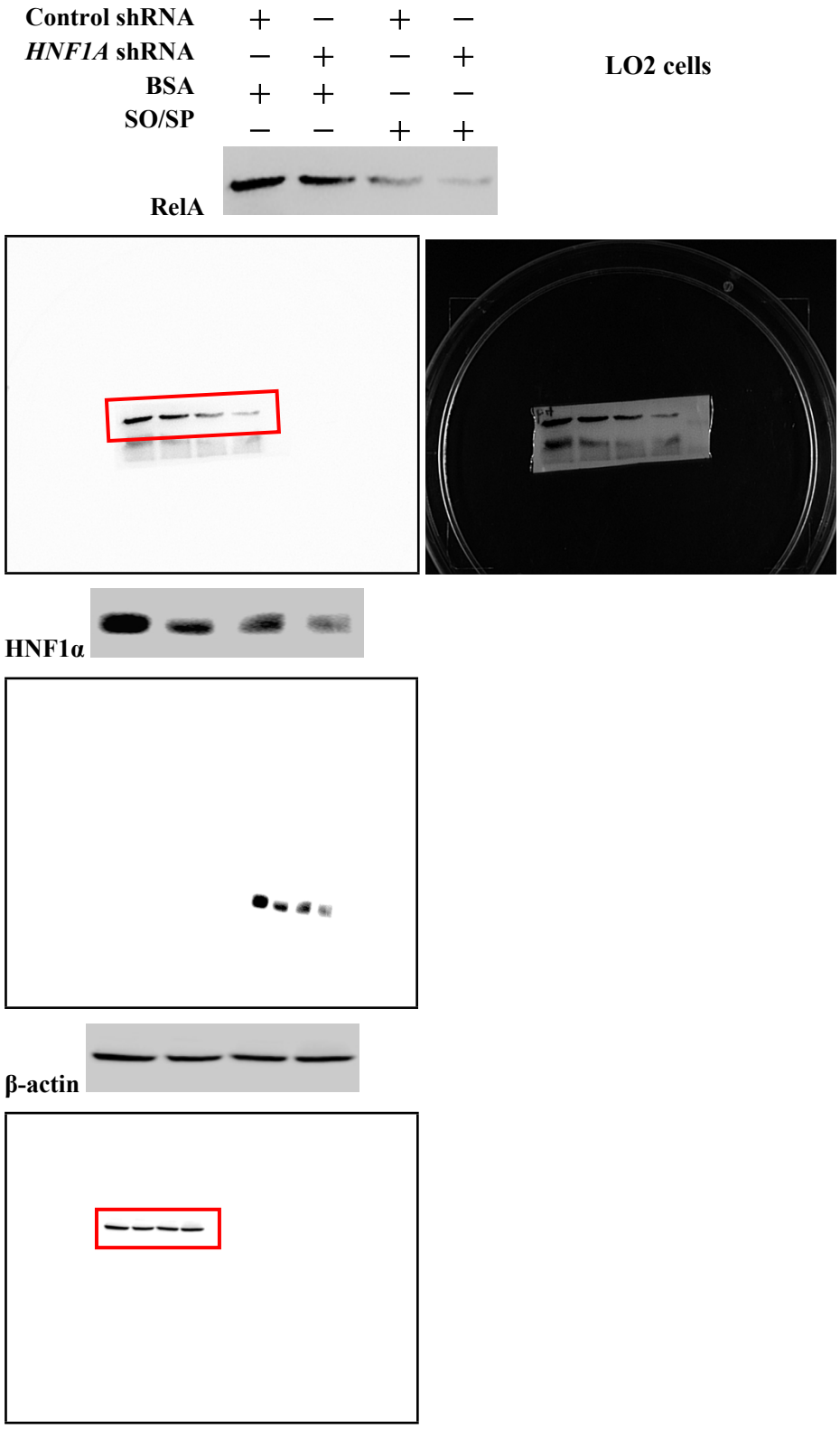


HNF1α

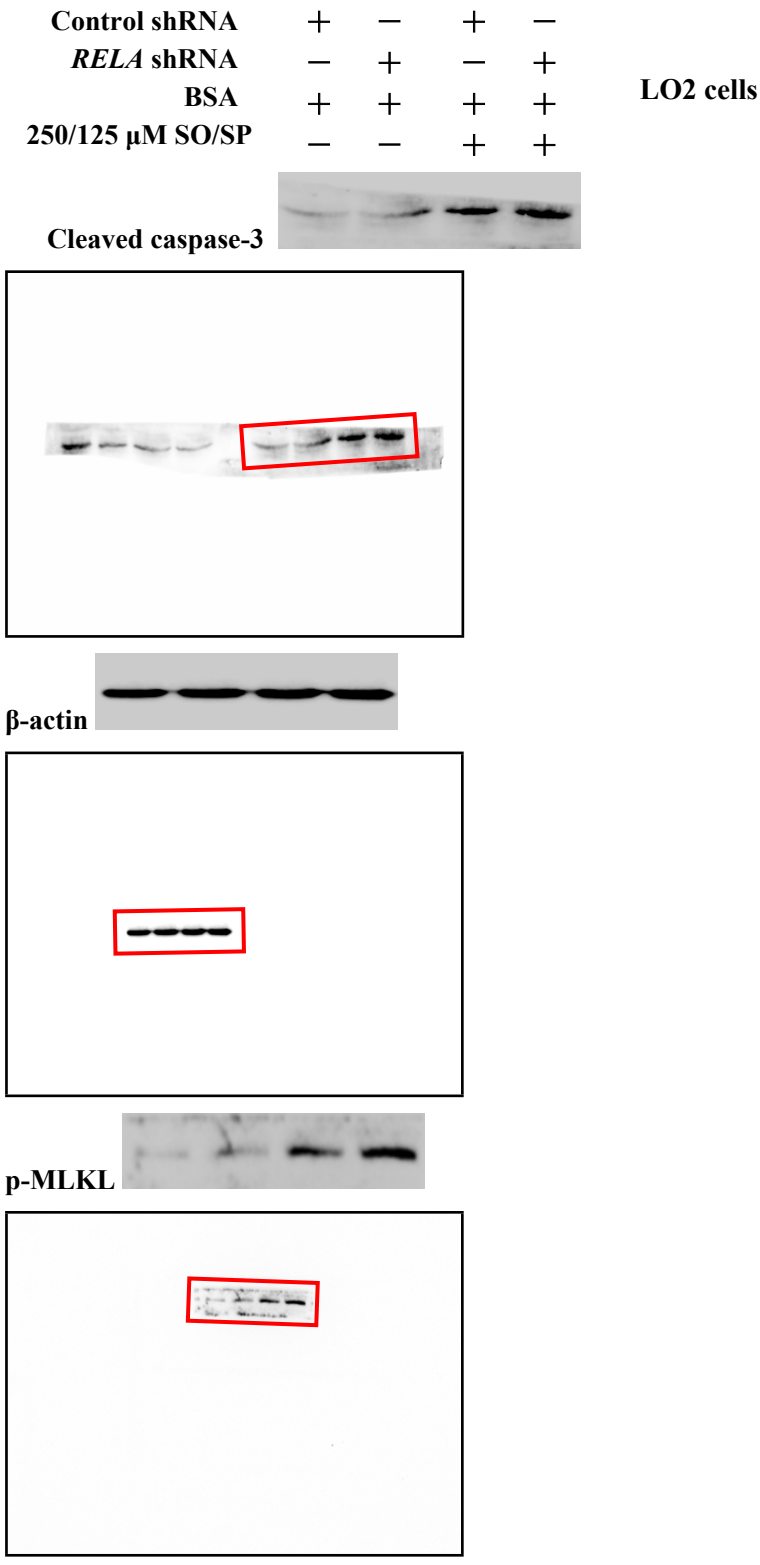
β-actin



Supplementary Fig. 3C



Supplementary Fig. 4E

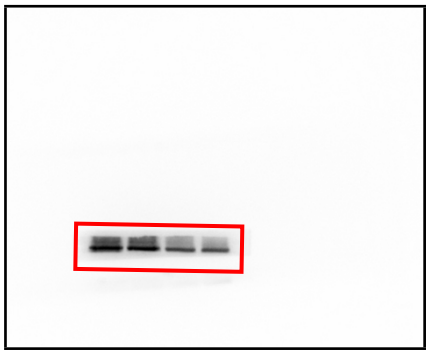


MLKL

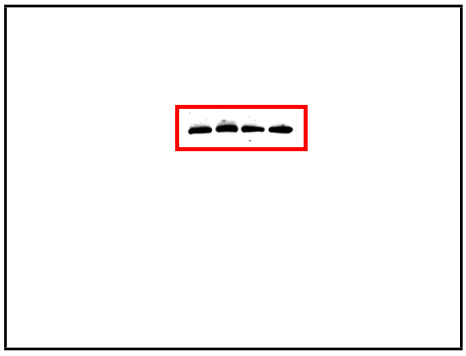
Supplementary Fig. 3J

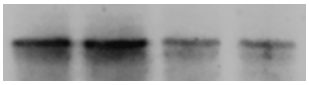
Empty vector	+	+	−	−	**LO2 cells**
***RELA*-pcDNA**	−	−	+	+	
250/125 μM SO/SP	+	+	+	+	

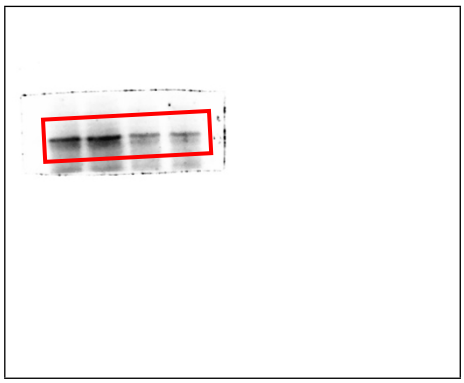
Cleaved caspase-3



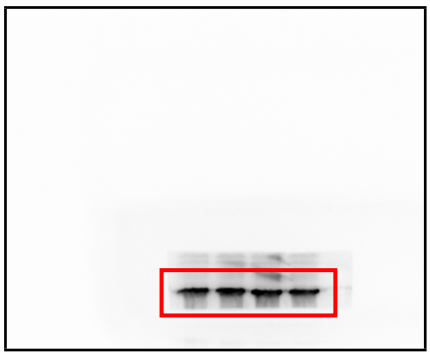
β-actin



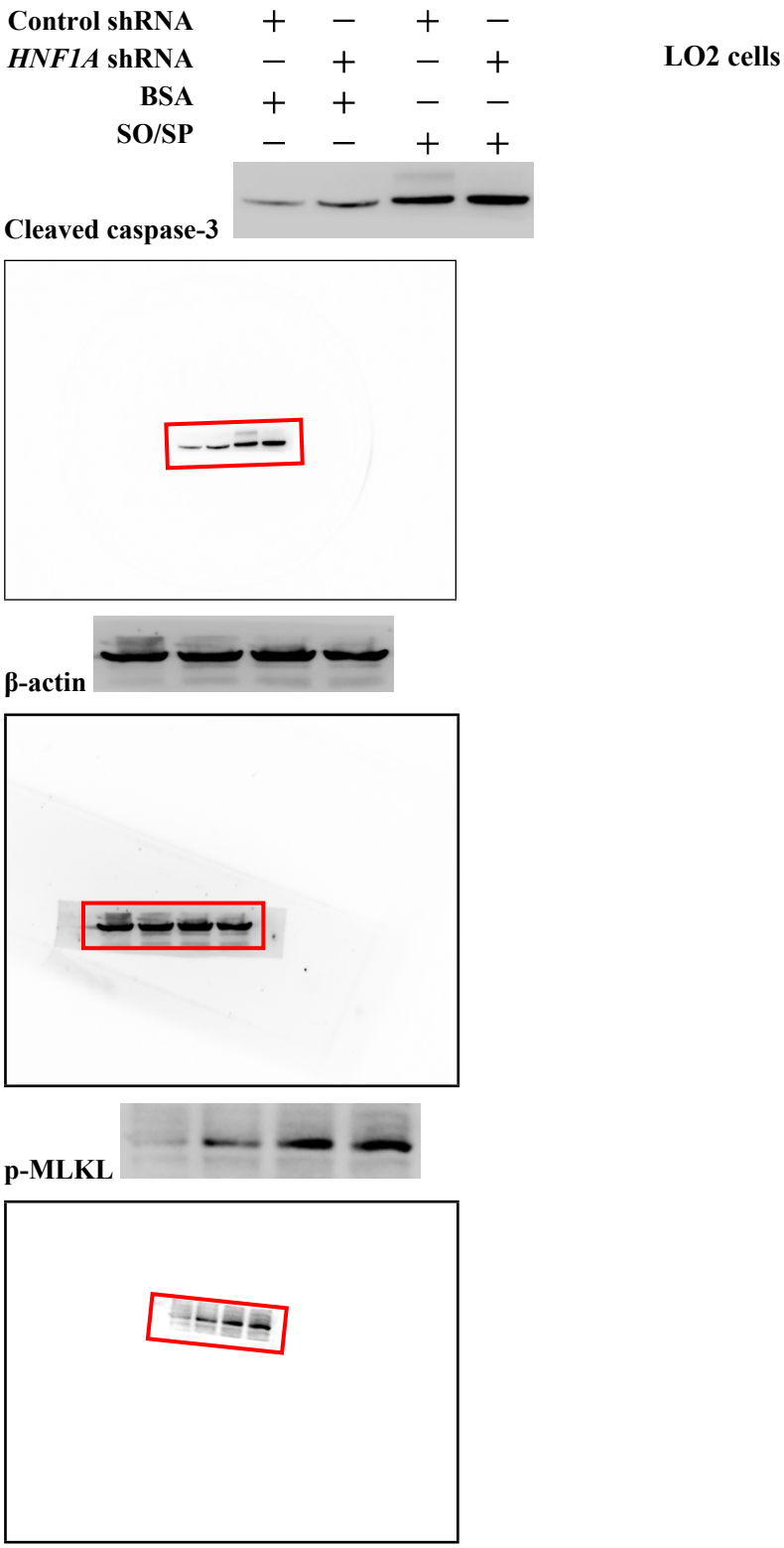
p-MLKL 

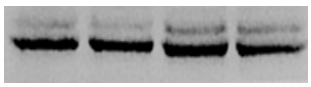


MLKL 

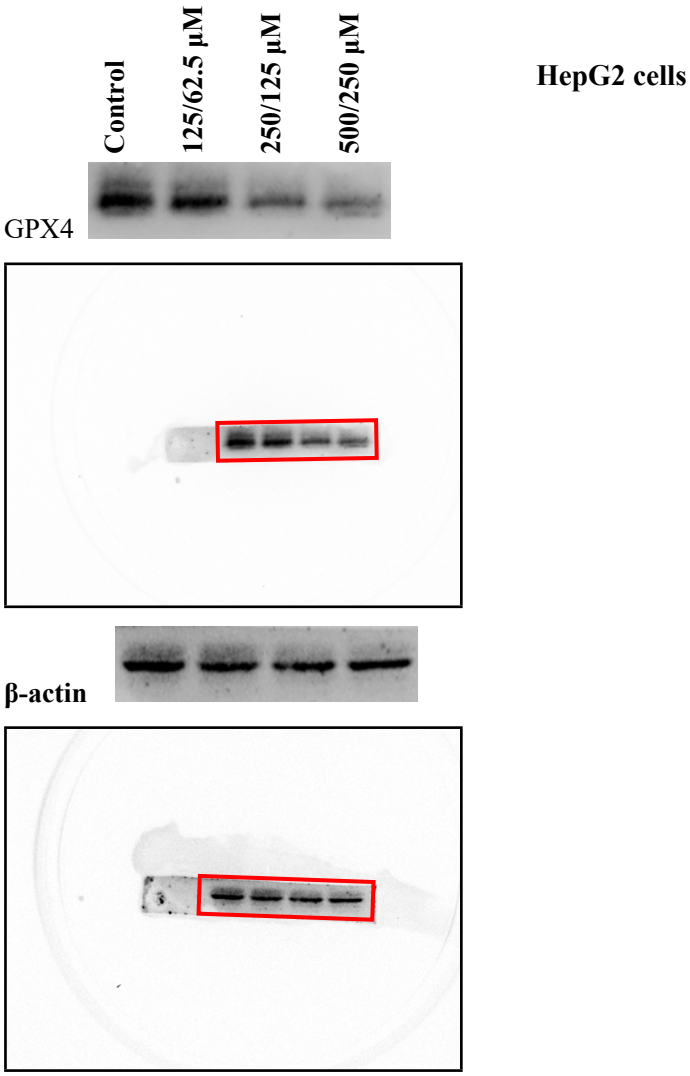


Supplementary Fig. 5E

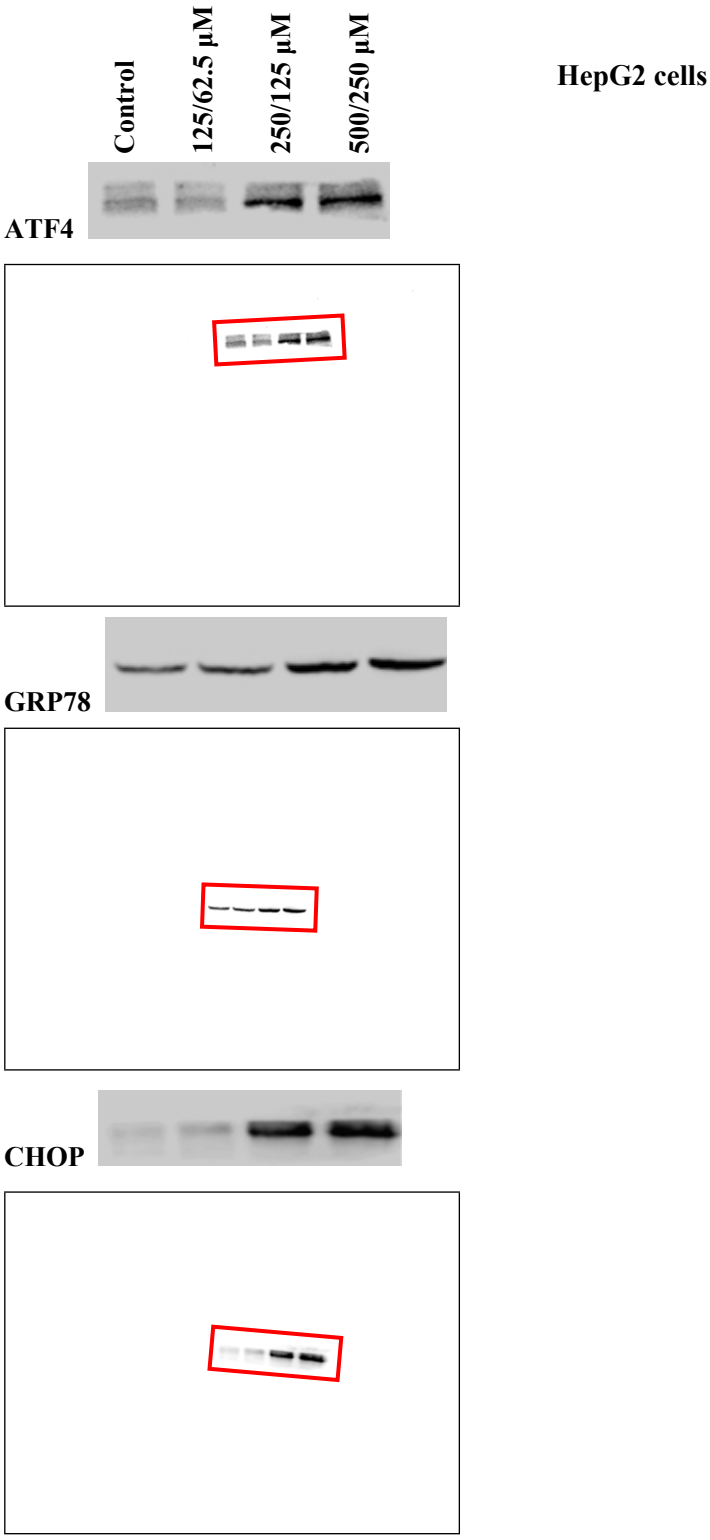


MLKL 

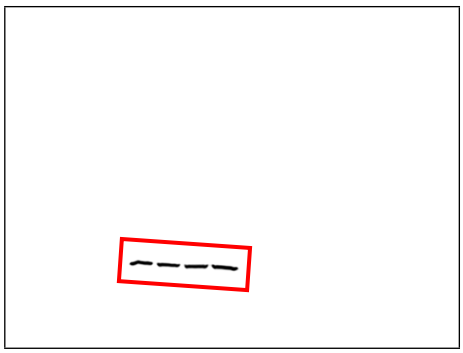
Supplementary Fig. 6L



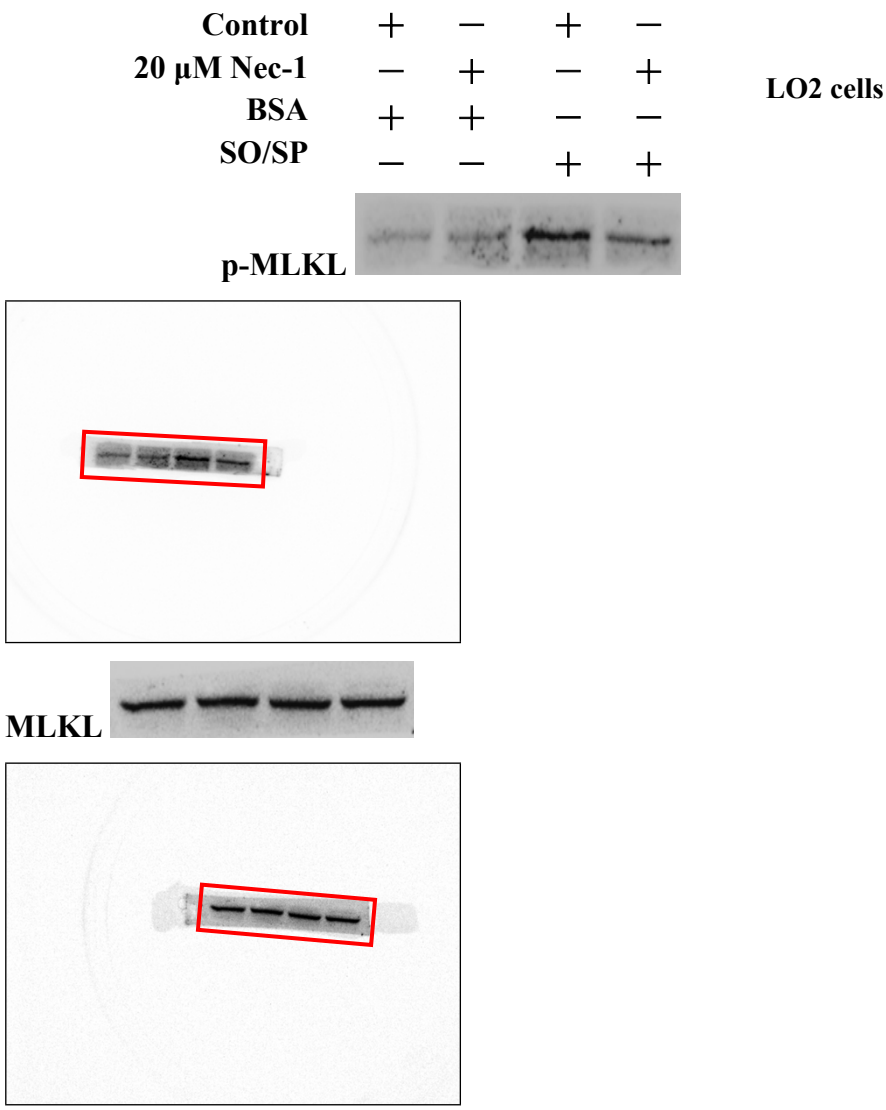
Supplementary Fig. 6M



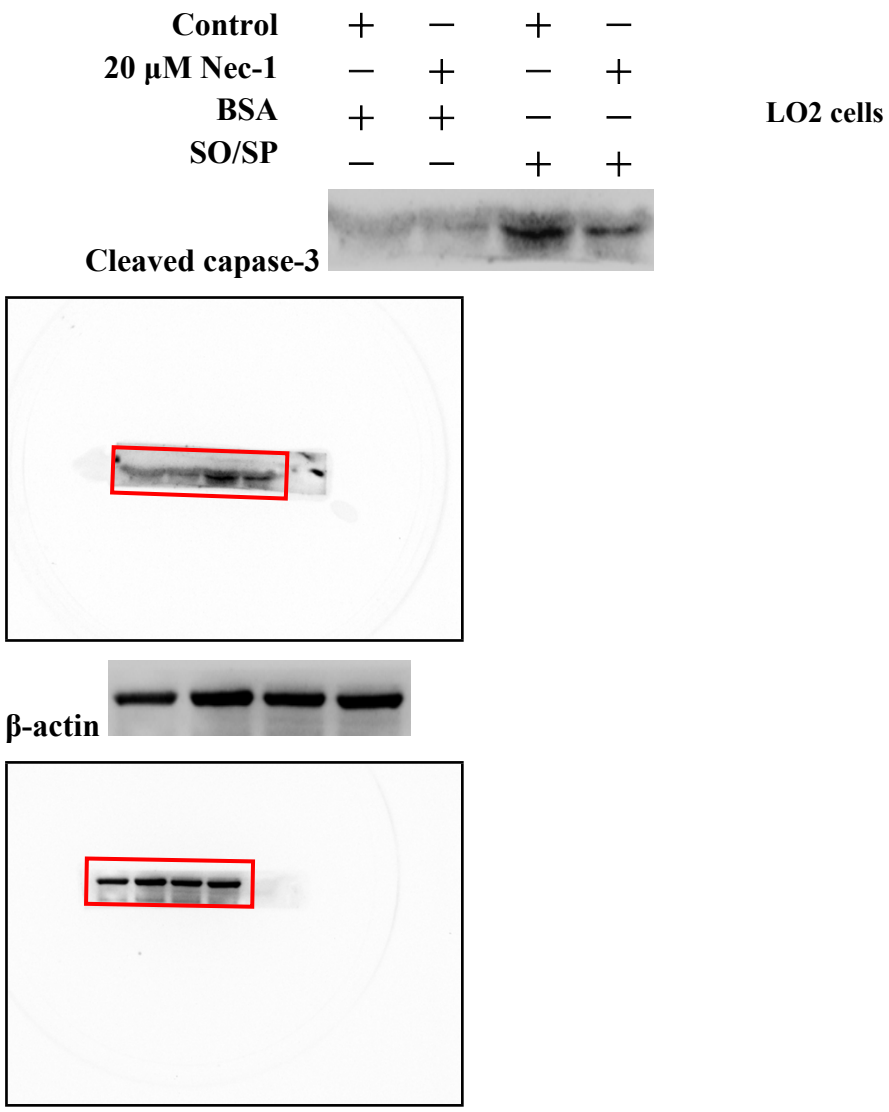
β-actin



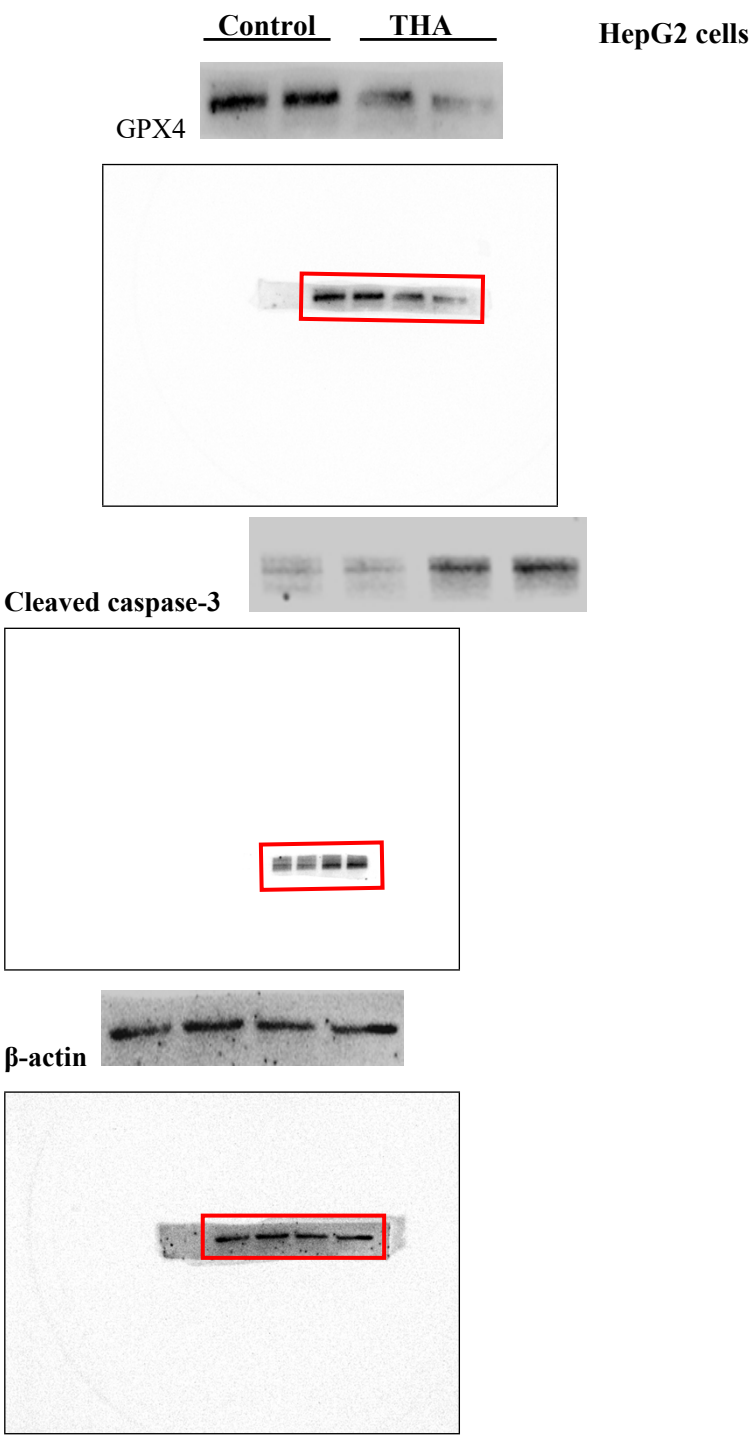
Supplementary Fig. 7D



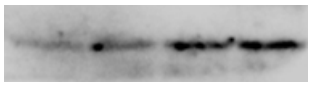
Supplementary Fig. 7I

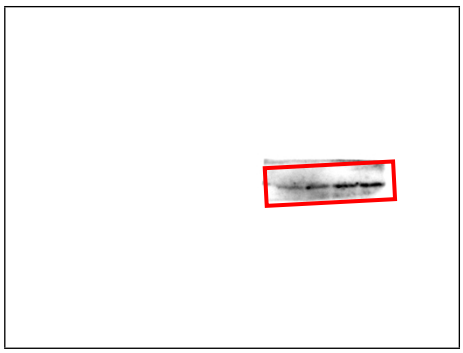


Supplementary Fig. 8M

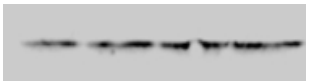


p-MLKL

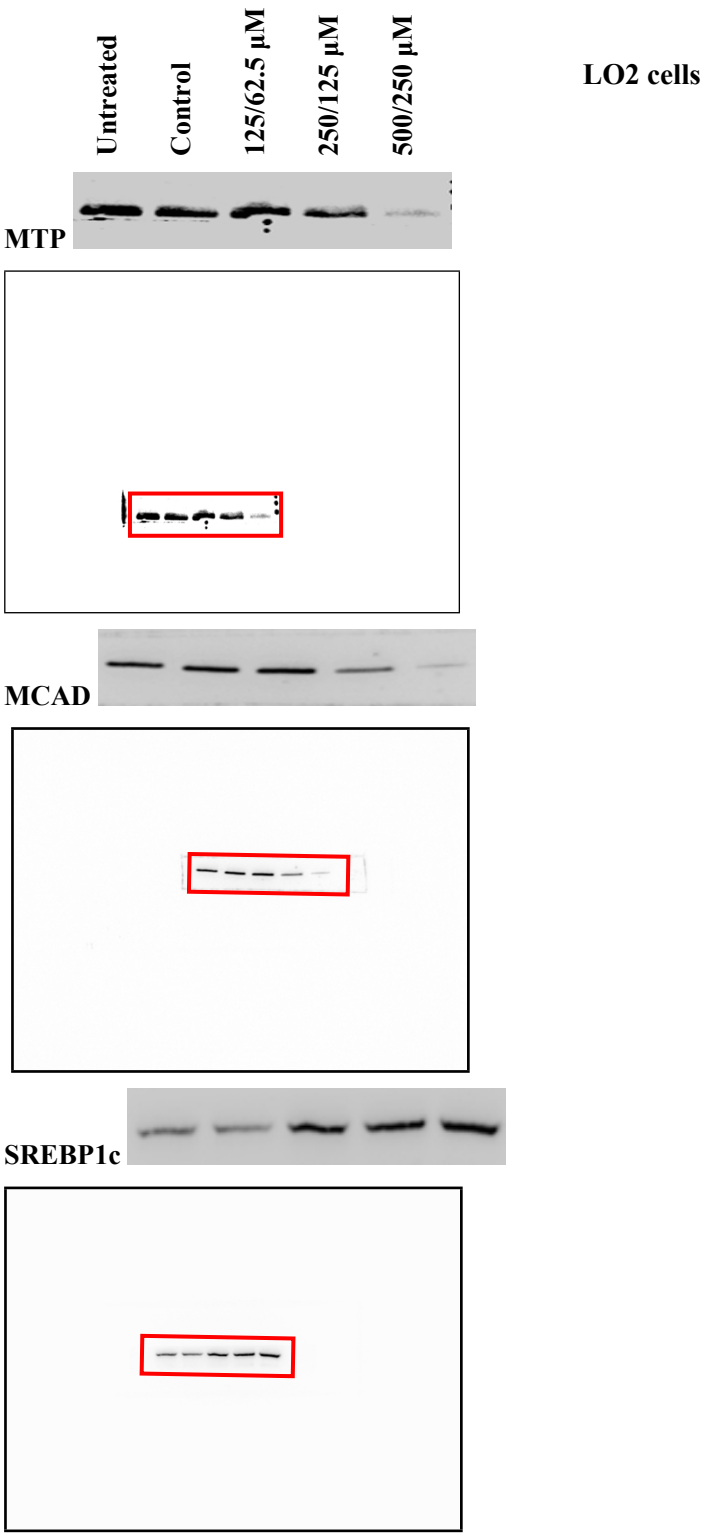




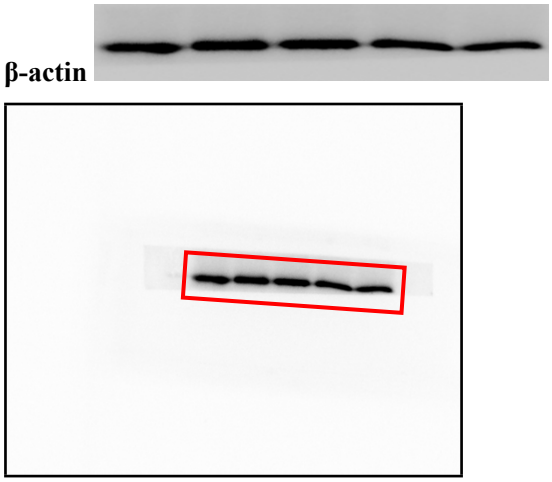
MLKL



Supplementary Fig. 9A



β-actin

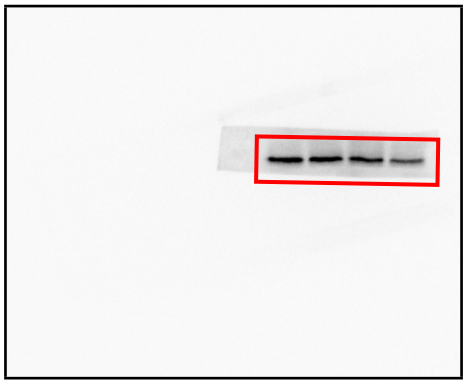


Supplementary Fig. 9B

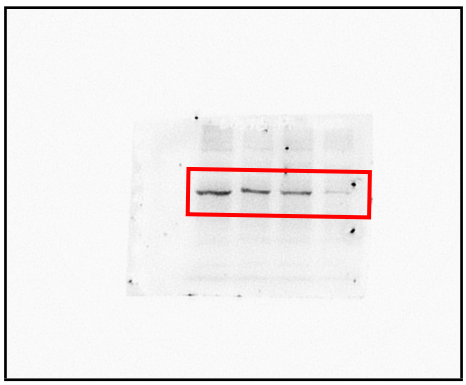
0.5 μM THA 0h 12h 24h 48h

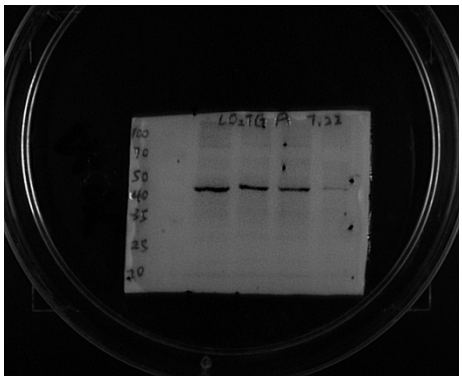
LO2 cells

MTP

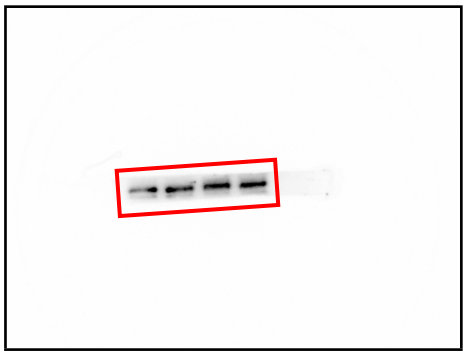


MCAD

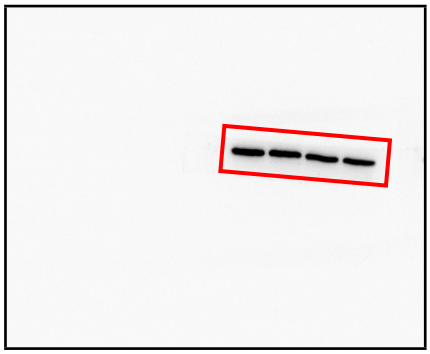




SREBP1c



β-actin

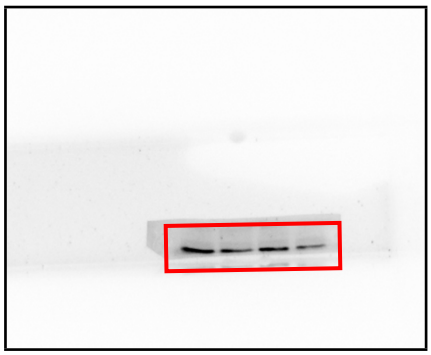


Supplementary Fig. 9C

Control shRNA	+	−	+	−
RELA shRNA	−	+	−	+
BSA	+	+	+	+
250/125 μM SO/SP	−	−	+	+

LO2 cells

MTP



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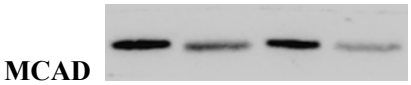
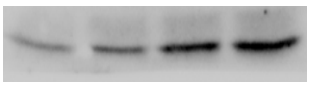
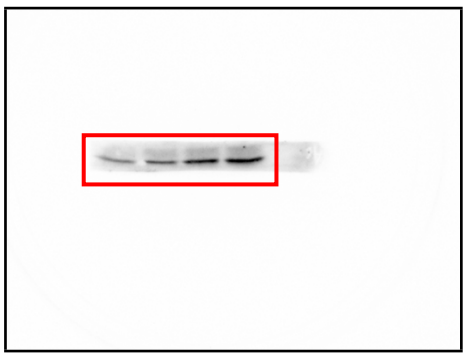
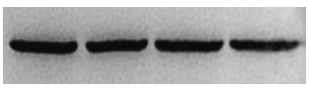


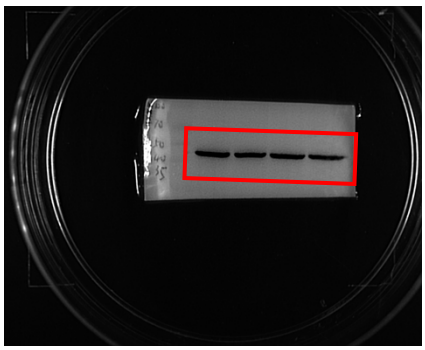
SREBP1c





β-actin



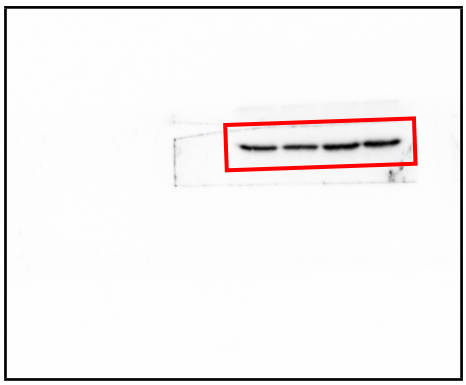


Supplementary Fig. 9D

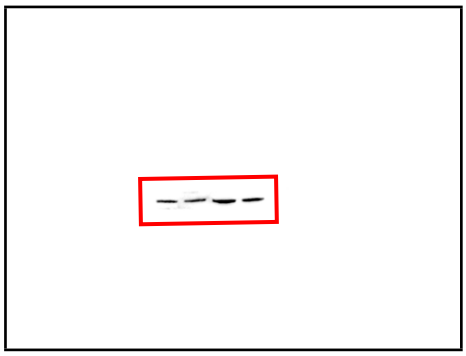
Empty vector	+	+	−	−
***RELA*-pcDNA**	−	−	+	+
250/125 μM SO/SP	+	+	+	+

LO2 cells

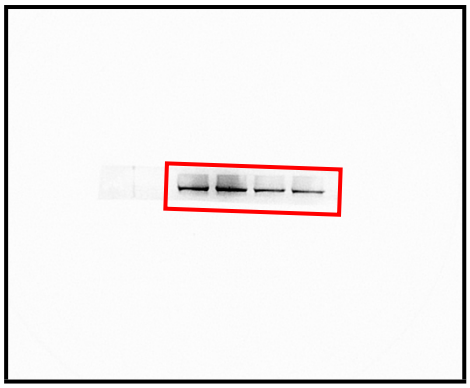
MTP



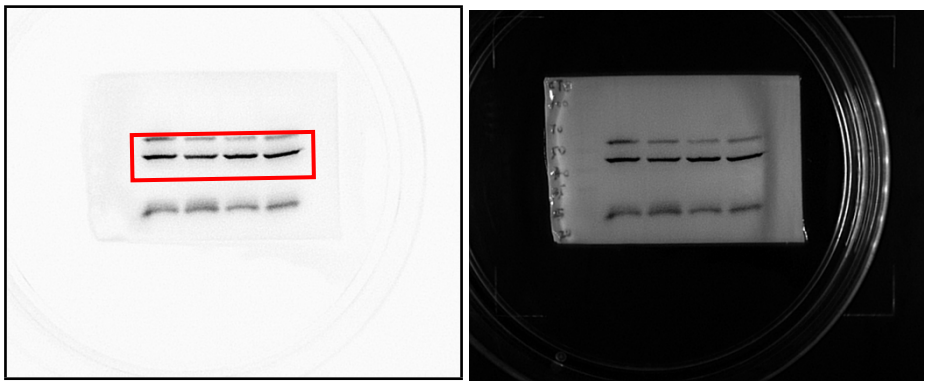
MCAD



SREBP1c



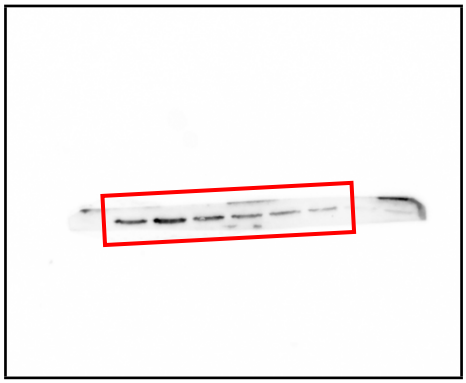
β-actin



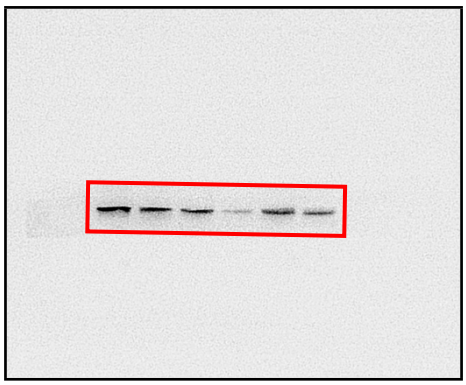
Supplementary Fig. 9I

Control TM Mice

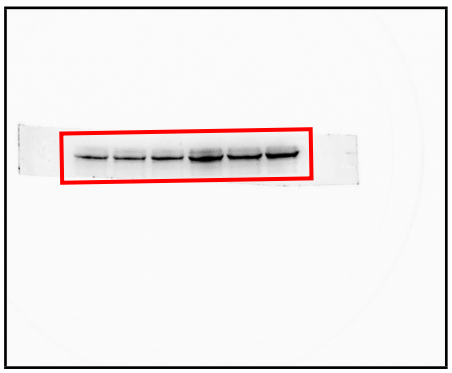
MTP



MCAD



SREBP1c

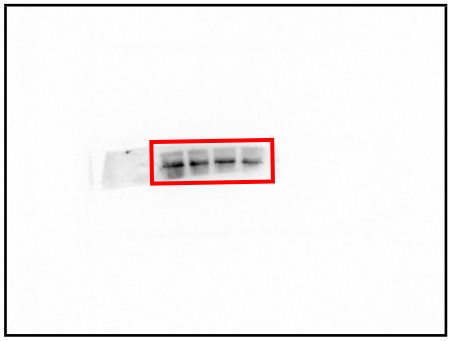


β-actin

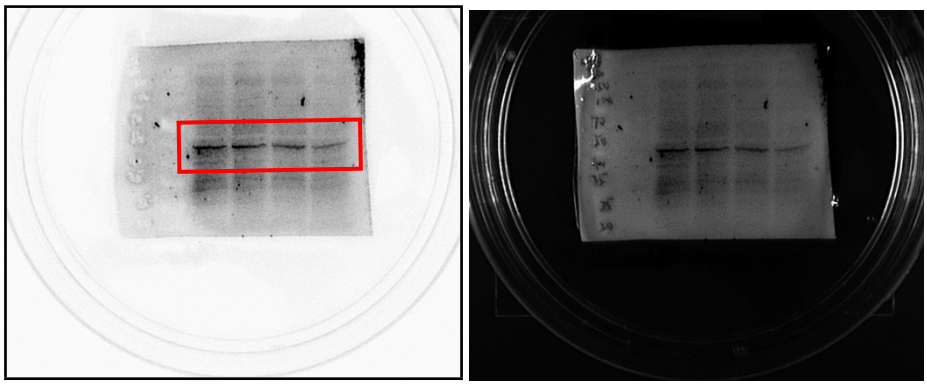
Supplementary Fig. 9J

Control shRNA	+	−	+	−	Mice
Hnf1a shRNA	−	+	−	+	
HFD	−	−	+	+	

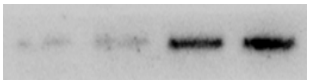
MTP

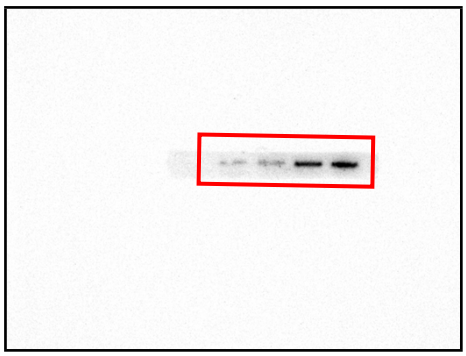


MCAD

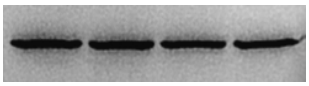


SREBP1c





β-actin



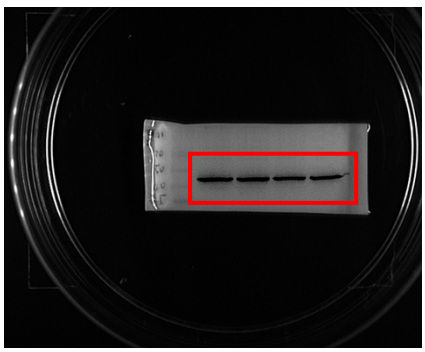
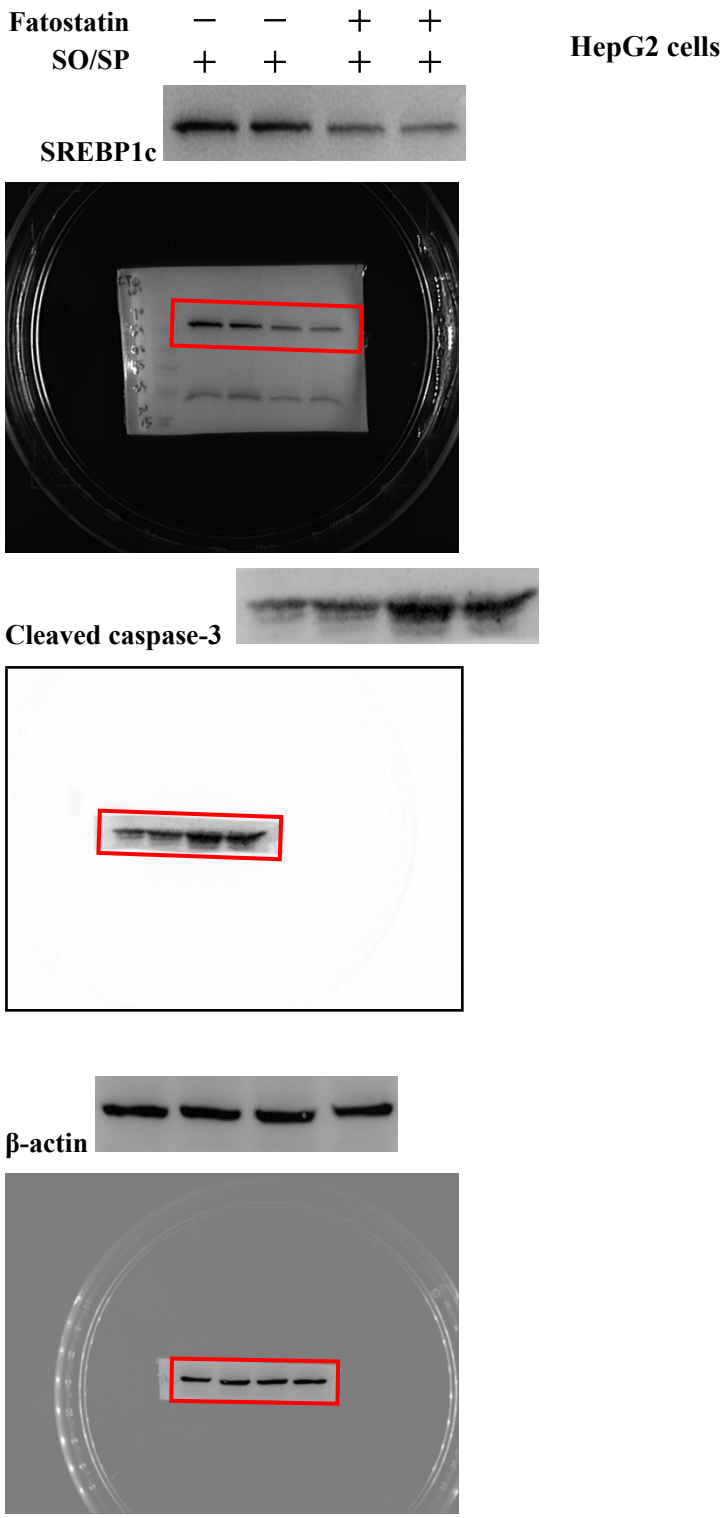
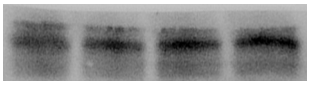
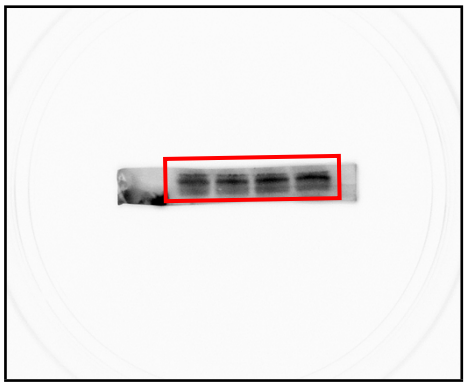
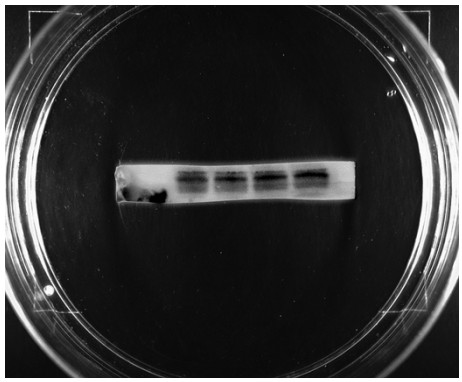


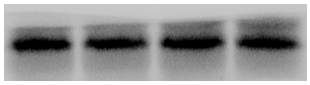
Fig. 9D

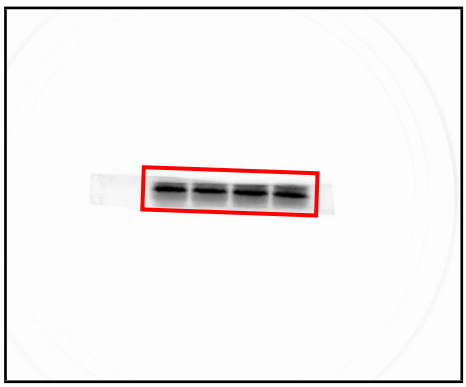

Fatostatin − − + +
SO/SP + + + +
HepG2 cells
SREBP1c
Cleaved caspase-3
β-actin

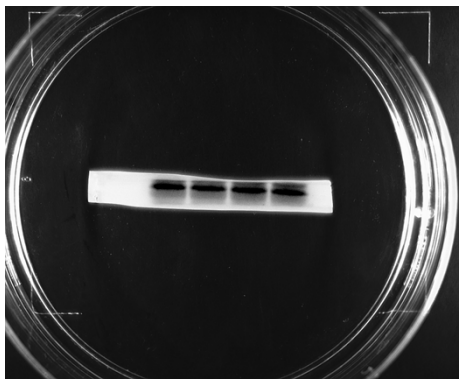
p-MLKL 





MLKL 



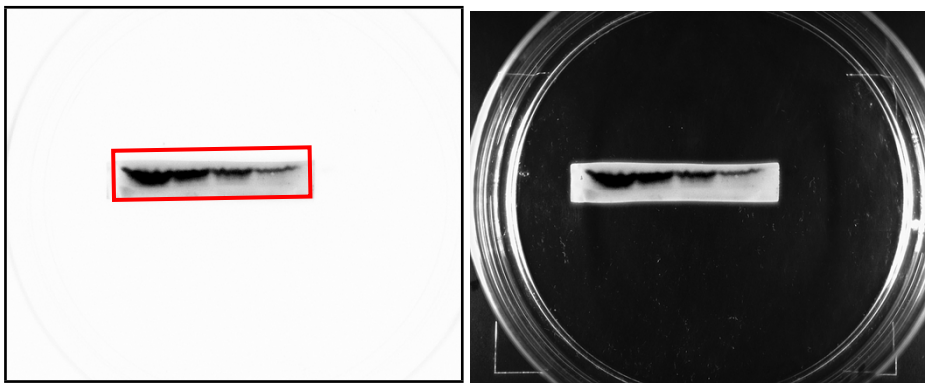


Supplementary Fig. 11H

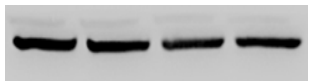
Control shRNA	+	−	+	−
RELA shRNA	−	+	−	+
BSA	+	+	−	−
SO/SP	−	−	+	+

LO2 cells

GPX4



β-actin

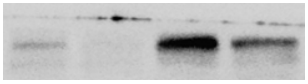


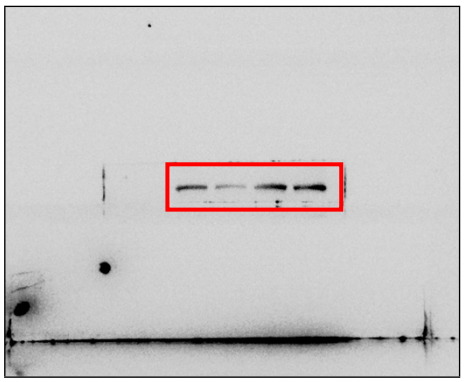
Supplementary Fig. 11I

Control shRNA	+	−	+	−
RELA shRNA	−	+	−	+
BSA	+	+	−	−
SO/SP	−	−	+	+

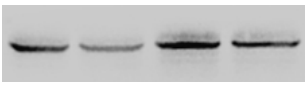
LO2 cells

ATF4

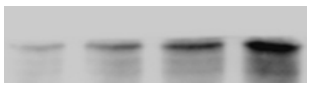


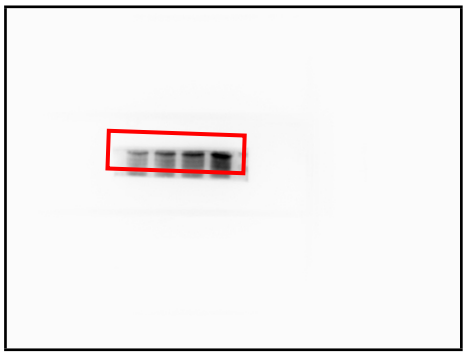


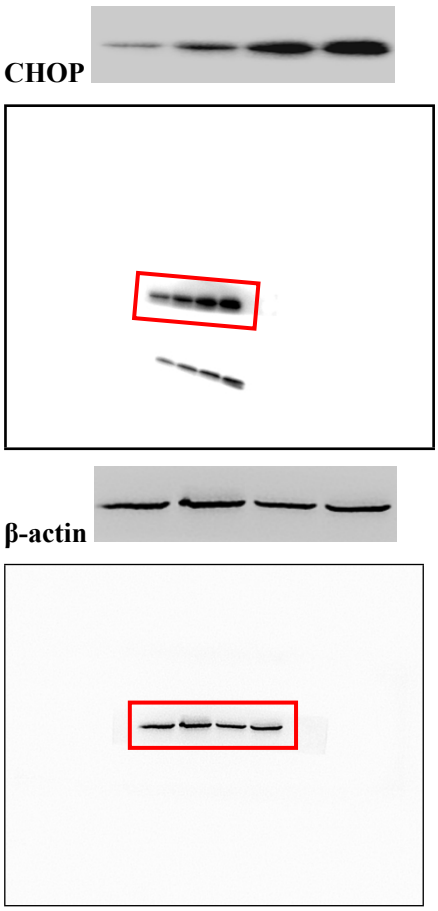
GRP78



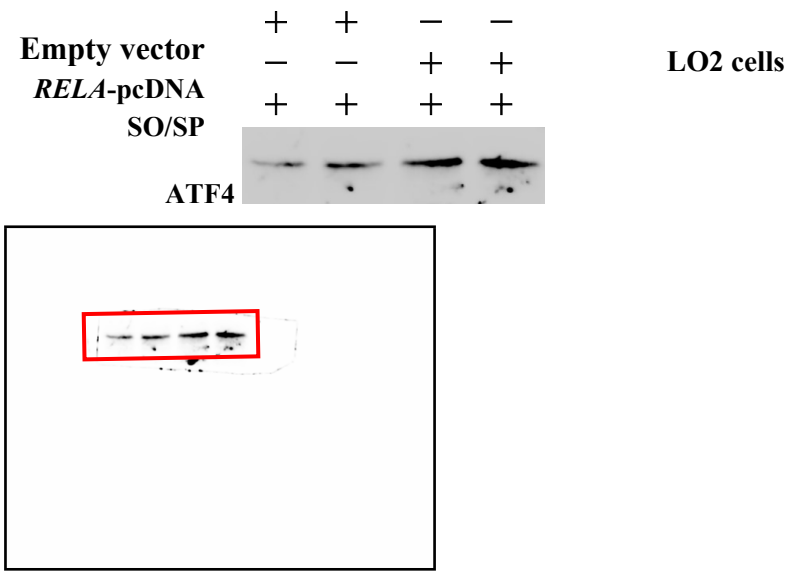
HRD1







Supplementary Fig. 11J



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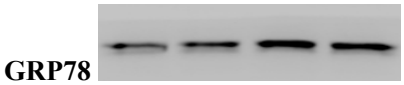

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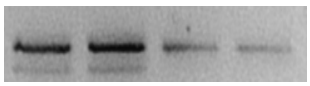
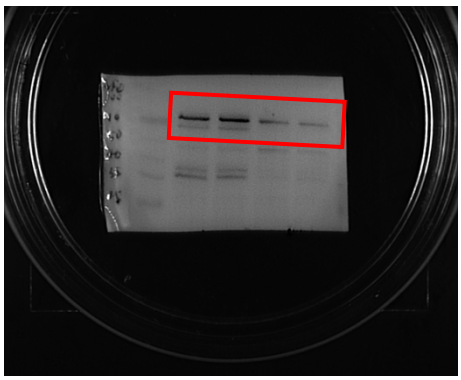
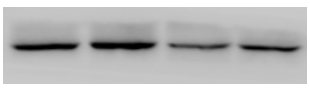
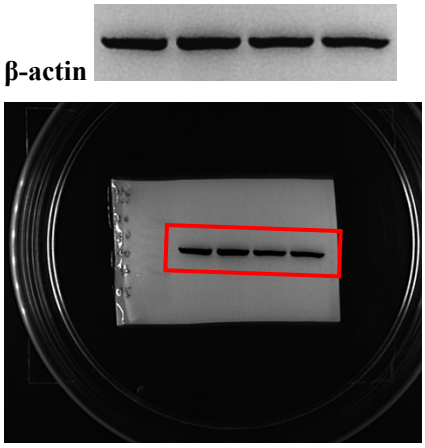




CHOP



β-actin

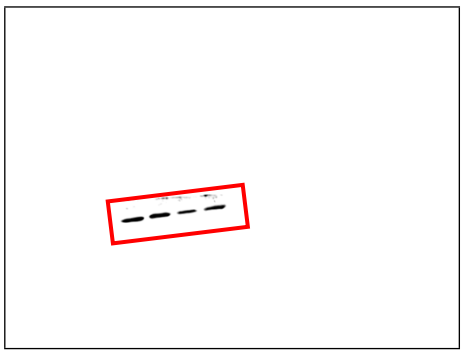


Supplementary Fig. 11K

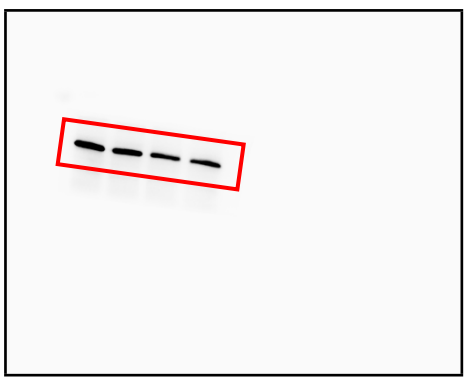
Vehicle	+	+	−	−
HNF1A-KO	−	−	+	+
SO/SP	+	+	+	+

HepG2 cells

ATF4

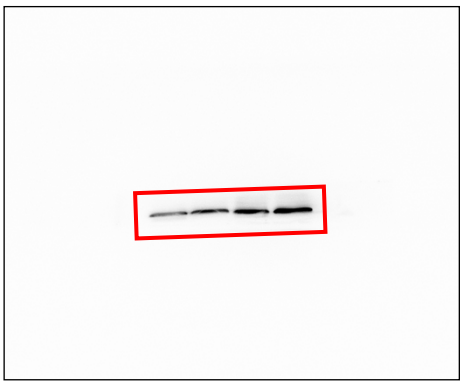


GRP78



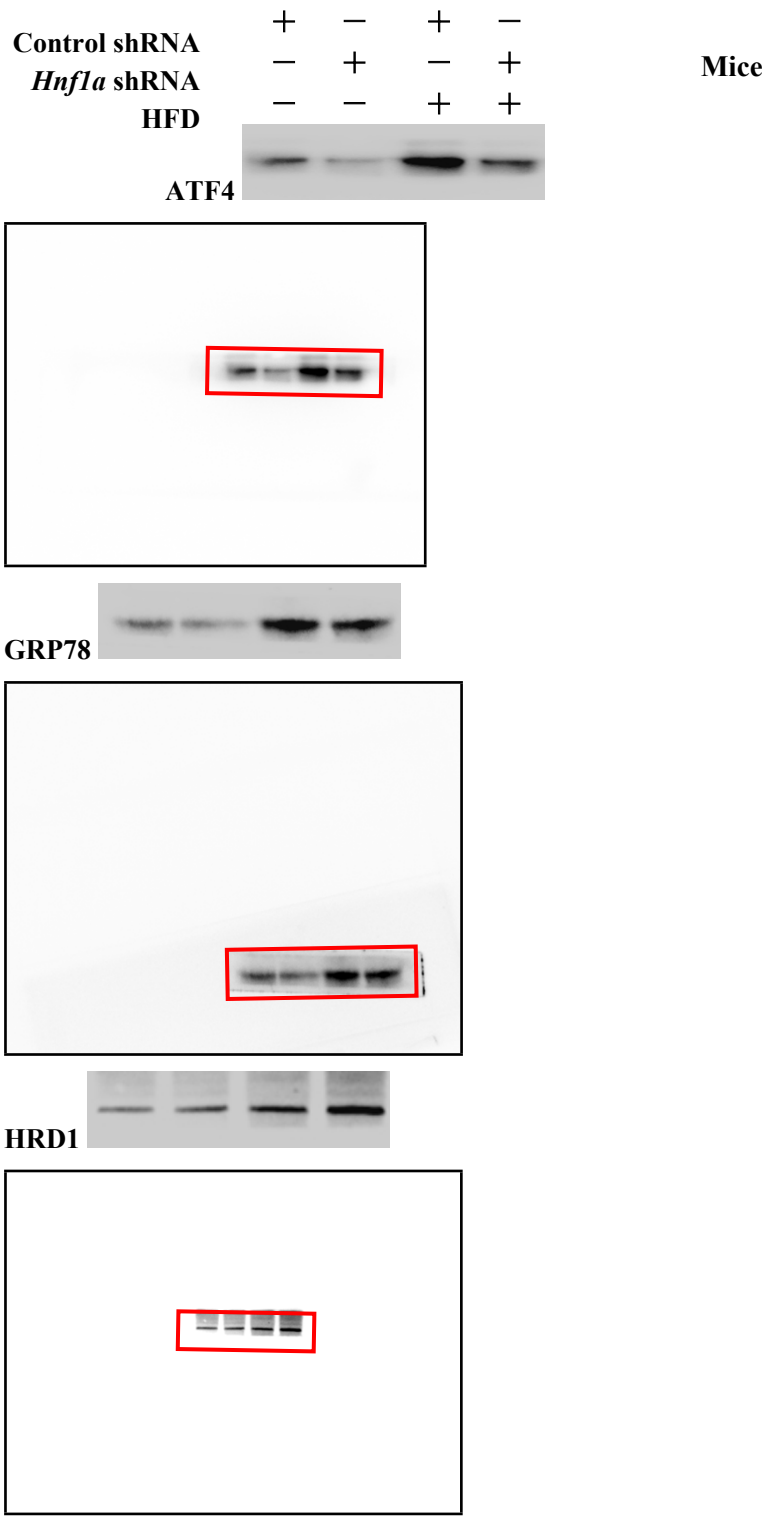
HRD1

CHOP

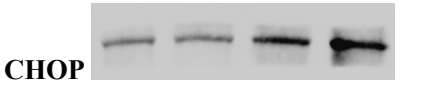


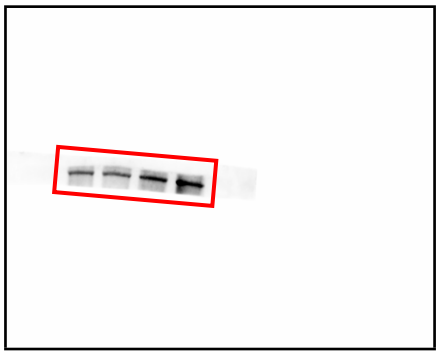
β-actin

Supplementary Fig. 11L



CHOP





β-actin