Hep-15-0335

Receptor Interacting Protein Kinase-1 mediates murine acetaminophen toxicity independent of the necrosome and not through necroptosis.

Supplemental Methods:

Antisense treatment

Antisense (ASO) targeting mouse RIPK1, CTCCATGTACTCCATCACCA, JNK1, TGTTGTCACGTTTACTTCTG, JNK2, GCTCAGTGGACATGGATGAG, and scrambled control oligonucleotide, CCTTCCCTGAAGGTTCCTCC, were provided by William Gaarde, (Isis pharmaceuticals, Carlsbad, CA). Oligonucleotides were synthesized as 20-nt uniform phosphorothioate chimeric oligonucleotides and purified. Oligonucleotides were chimeric containing five nuclease resistant *2´-O*-methoxyethylribose-modified phosphorothioate residues on the 5´ and 3´-ends, flanking a 2´-deoxyribonucleotide/phosphorothioate region that supports RNase H-based cleavage of the targeted mRNA. Mice were injected with 50mg/kg of RIPK1 ASO, or Control ASO, every other day, for 10 days.

Hepatocyte isolation and culture

Freshly isolated hepatocytes were separated by percoll (Sigma Aldrich St. Louis, MO) centrifugation to remove debris and remaining non parenchymal cells. Three

hours after plating of the hepatocytes, APAP dissolved in fresh pre-warmed DMEM/F12 culture medium was added at different concentrations. After 18-24 hours of treatment, cells were double stained with Hoechst 33258 (8 □≡■BA糎 Invitrogen, Grand Island, NY) and Sytox Green (1 □1 D■0糎(KHCO≡Ć■

Isolation of Liver leukocytes

Liver tissues were passed through a 200-gauge stainless steel mesh in Hank's balanced salt solution. The cell suspension was centrifuged at 500g for 15 minutes, and the resulting cell pellet from was resuspended in 15 mL 35% Percoll containing 100 U/mL heparin. The cell suspension was centrifuged at 500g for 15 minutes at room temperature, and the cell pellet containing leukocytes was harvested and resuspended in 5mL red blood cell lysis solution (155 mmol/L NH4CL, 10 mmol/L KHCO3, 1 mmol/L EDTA, 170 mmol/L Tris, pH 7.3). After incubation for 5 minutes on ice, cells were washed twice in PBS.

Isolation of liver mitochondria and cytoplasm

Briefly, the livers were homogenized in H-medium (250mM sucrose, 20mM HEPES, 1mM EDTA, 1mM EGTA, plus protease and phosphatase inhibitors). The homogenate was centrifuged at 800 g for 10min twice, the resulting supernatant from the 2nd spin was centrifuged at 8,500g for 15 min, at which point the

supernatant (cytoplasm) was removed and the pellet (crude mitochondria) was washed with H-medium and the centrifugation was repeated. The mitochondria in the final pellet were re-suspended in RIPA buffer containing protease and phosphatase inhibitors for Western Blot analysis.

Western blot

Aliquots of cytoplasmic or mitochondrial extracts were fractionated by electrophoresis on Criterion and mini protean gels (Bio-Rad). Subsequently, proteins were transferred to PVDF membranes and blots were blocked with 5% (w/v) nonfat milk or 5% BSA dissolved in Tris-buffered saline with Tween 20. The blots were then incubated with the desired primary and secondary antibodies. Finally, the proteins were detected by Luminol ECL reagent (Thermo Scientific). Densitometry was done using Image J software. All gels and densitometry shown are representative samples from at least three experiments.

GSH measurements

1,2 or 4 hours following APAP treatment, total liver homogenate and mitochondrial GSH were measured using Tietze's recycling assay GSH refers to total GSH (GSH+ GSSG).

Histological analyses

Livers were removed, fixed with 10% buffered formalin, embedded in paraffin, and cut into 5 μm-thick sections. All specimens were stained with hematoxylin-eosin (H&E) and evaluated under light microscopy.

Confocal microscopy

Isolated PMH were mounted onto coverslips and treated with APAP 10mM or PBS control for 2 hrs. Cells on the coverslips were then washed once with PBS and fixed in 3.7% formaldehyde in PBS for 15 min. Cells were permeabilized with 0.5% Triton and blocked for 30 min at room temperature with 5% BSA and then incubated with a primary antibody (CoxIV and RIPK1) in 5% BSA overnight in 4 degrees. After washing three times with PBS cells were incubated with respective fluorescent-labeled secondary antibodies (Invitrogen, green, goat anti rabbit Alexa Fluor 488 for COXIV and red, goat anti mouse Alexa Fluor 594 for RIPK1) for 1 hr. The coverslips, which were washed extensively, were mounted onto slides. A Zeiss LSM 510 confocal microscope was used to obtain images. Microscopy was performed by the cell and tissue imaging core of the USC Research Center for Liver Diseases.

Statistical analysis

Statistical analyses were performed using the ANOVA and Student's t test. P value <0.05 was defined as statistically significant. The Log-rank (Mantel-Cox) test was used to calculate survival and a P value <0.05 was defined as statistically significant.

Supplemental Figure Legends

Supplemental Figure 1. Necrostatin protects against APAP toxicity in PMH.

(A) Viability of PMH pretreated for one hour with Nec-1 or inactive control (Nec-1i) in the continued presence of APAP at different doses. Viability assessed at 24 hrs. N=3; (B) Viability of PMH treated with APAP for 2 hours at different concentrations. Subsequently media was exchanged and Nec-1 or Nec-1i was added. N=3. Results are mean ± S.D. * *p* value < 0.05 Nec1 vs Nec-1i.

Supplemental Figure 2. Necrostatin protects mice against APAP toxicity in vivo.

Mice were treated with Nec-1 (10mg/kg in DMSO, IP) 1 hour prior to APAP (500mg/kg) and euthanized at 24 hrs. (A) Serum ALT (U/L); (B) Representative liver histology (H&E). Data represents mean ± S.D. N=4 mice per group. * *p* value < 0.05 Nec-1 vs Nec-1i.

Supplemental Figure 3. GSH is depleted similarly in Control and RIPK1 treated mice.

Mice were pretreated with control ASO or RIPK1 ASO followed by APAP 300mg/kg and GSH was measured using recycling assay at indicated time points. Total GSH expressed as umoles/gr. (N=3 mice at each time point).

Supplemental Figure 4. Inaccuracies of commercially available antisera

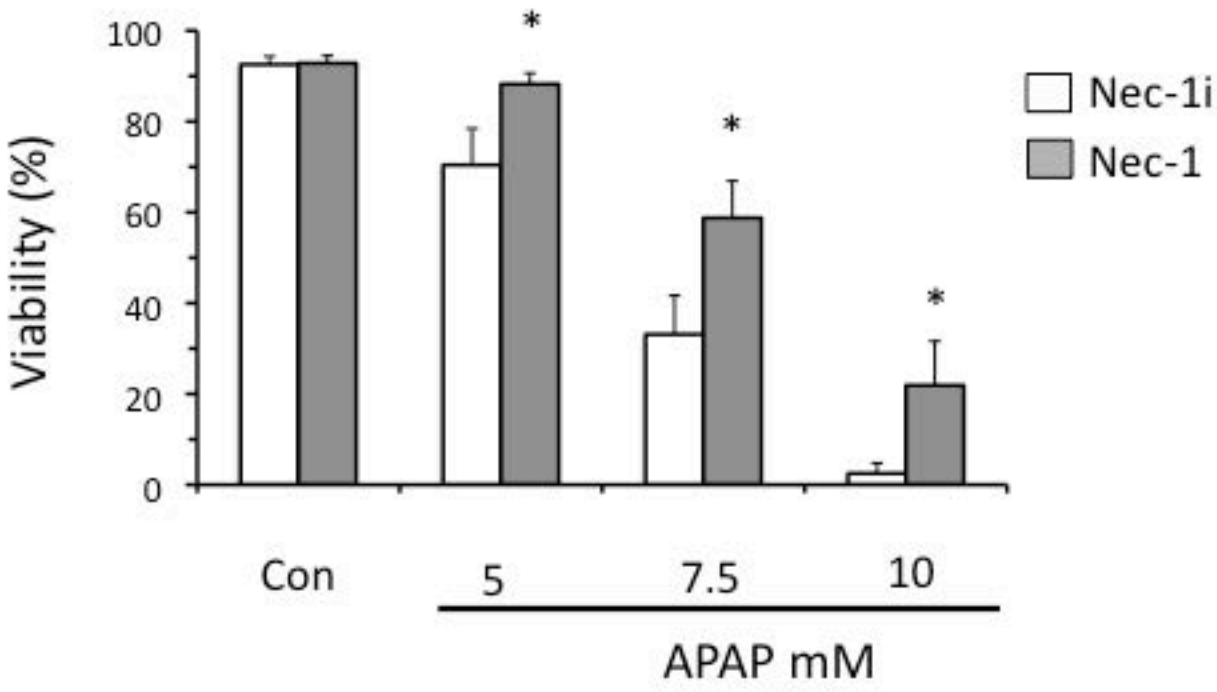
Non specificity of commercially available antibodies in liver tissue. RIPK3 immunoblots using different antisera (Imgenex (A)/ProSci(B)/Abgent(C), exhibited multiple nonspecific bands around 57 KD including in RIPK3-/- livers (D) Immunohistochemistry (using normal rabbit IgG, ProSci and Abgent) of WT and RIPK3-/- livers treated with APAP 300mg/kg for 24hr. Note positive staining of necrotic area even in RIPK3-/- liver. (N=3 per group) (E) Western blot of PMH obtained for C57B6j mice and treated with control media or 10mM APAP for 1,2 and 6hrs using RIPK3 Genentech monoclonal Ab, RIPK3 ProSci Polyclonal Ab (2 exposures) and β-actin. Note recognition of negative control, RIPK3-/- liver, by ProSci Ab indicating the band at 55KD is nonspecific (red arrows).

Supplemental Figure 5. Mdivi protects PMH against APAP in vitro.

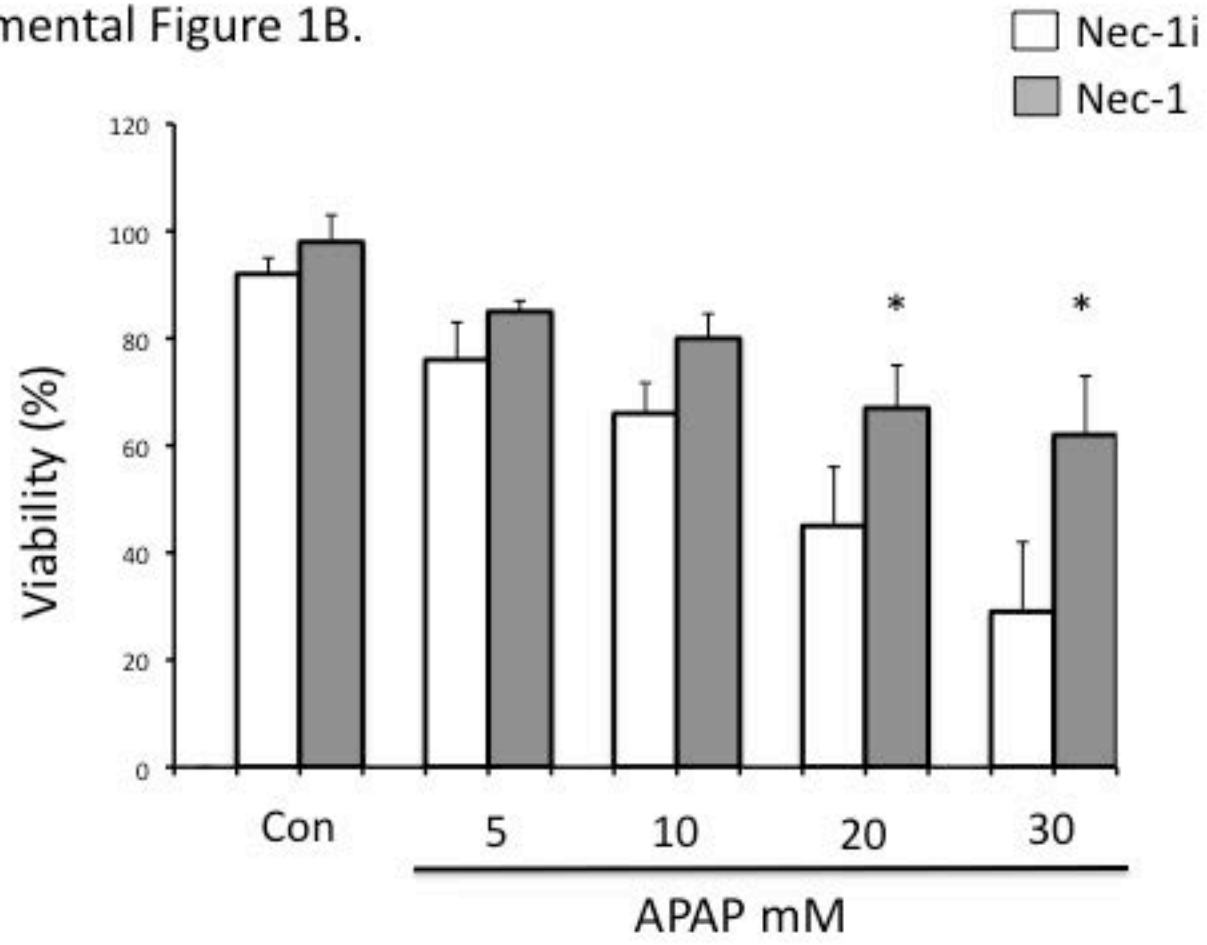
PMH were treated with indicated concentrations of APAP for 2 hrs and subsequently media was exchanged and Mdivi 50uM, was added. Viability

determined at 24hrs. N=3, * *p* value < 0.05 APAP *vs* control, # * *p* value < 0.05 *vs* control ASO. * *p* value < 0.05 Mdivi *vs* DMSO.

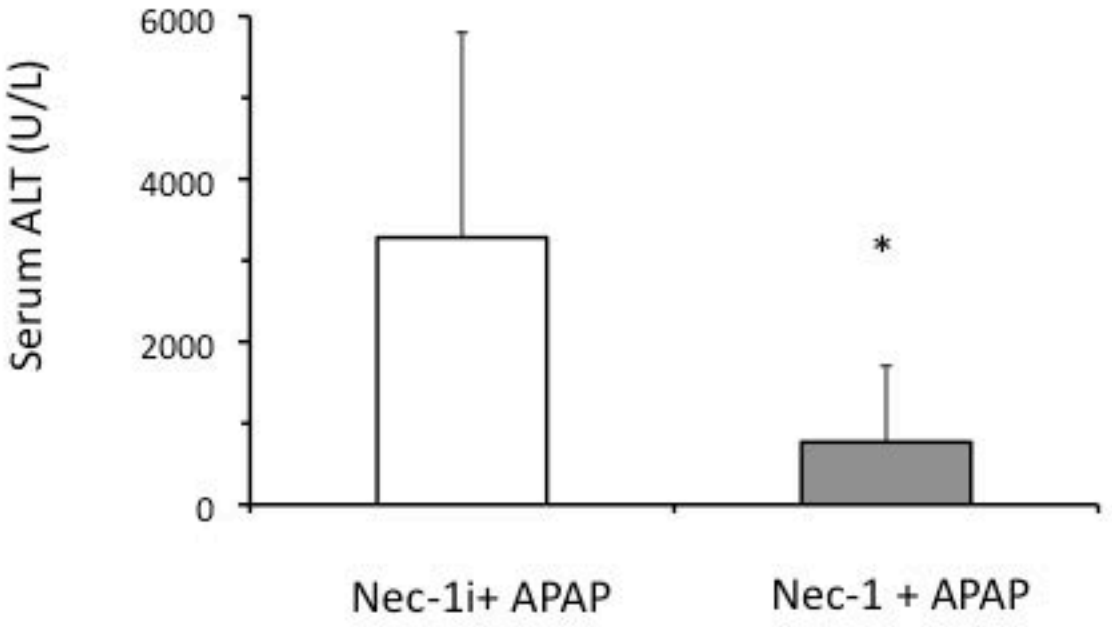
Supplemental Figure 1A.



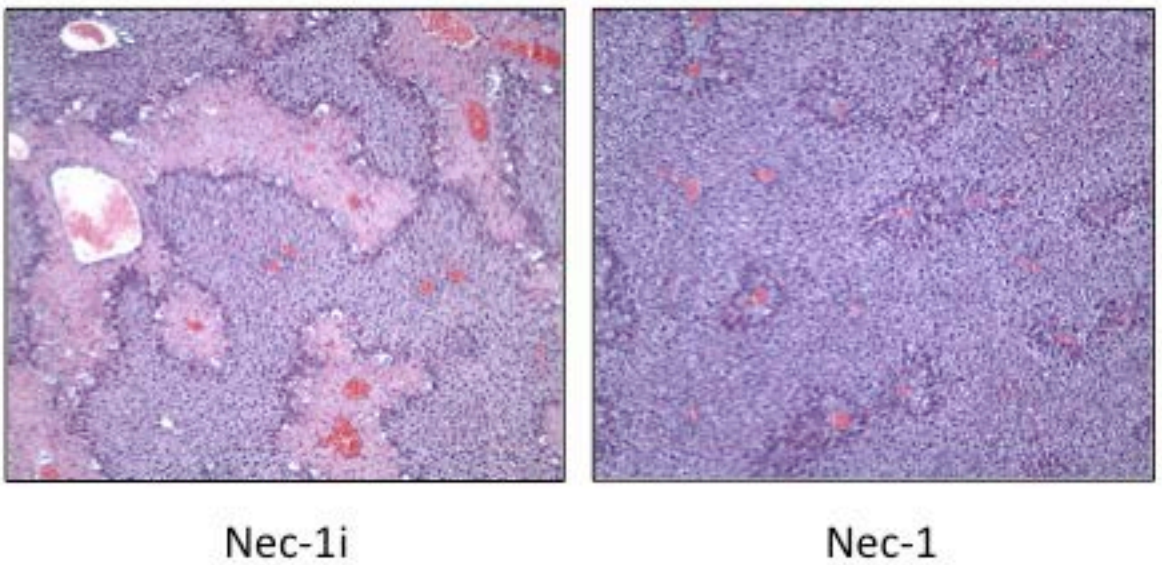
Supplemental Figure 1B.



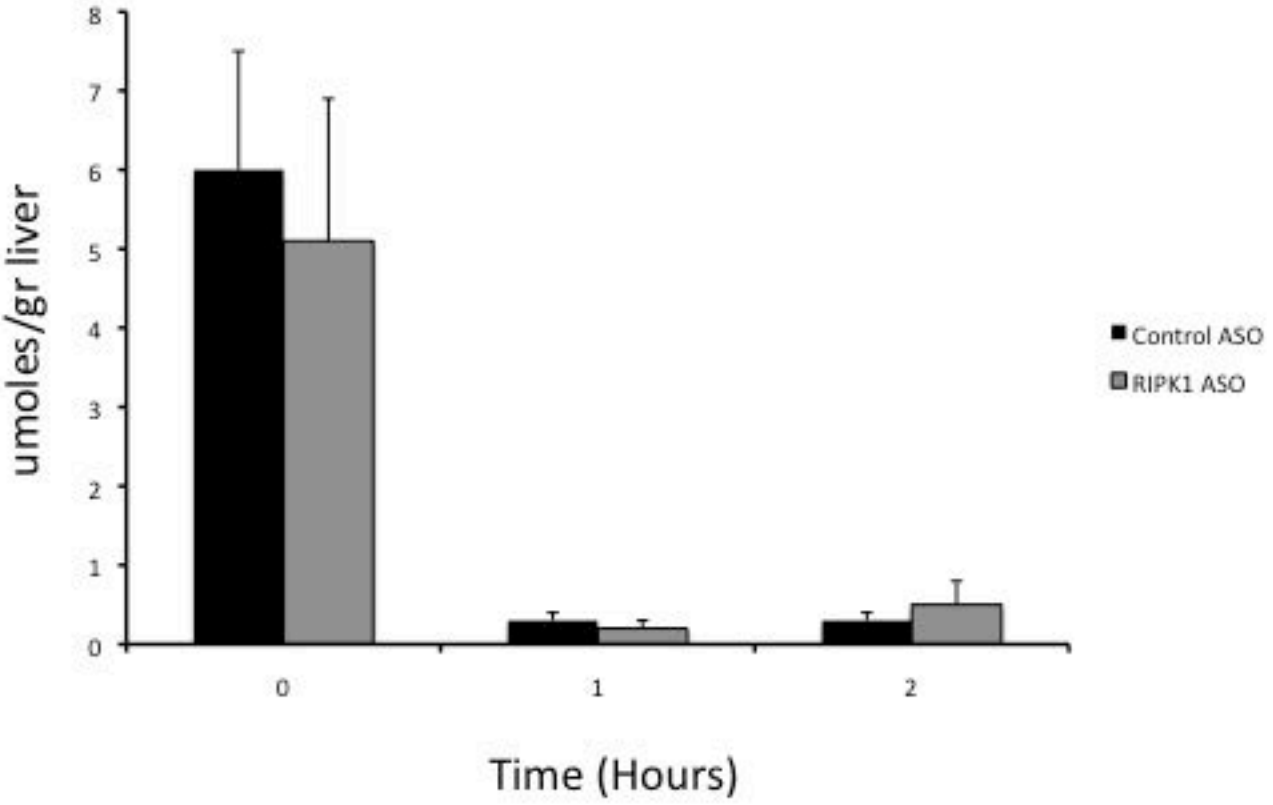
Supplemental Figure 2A.



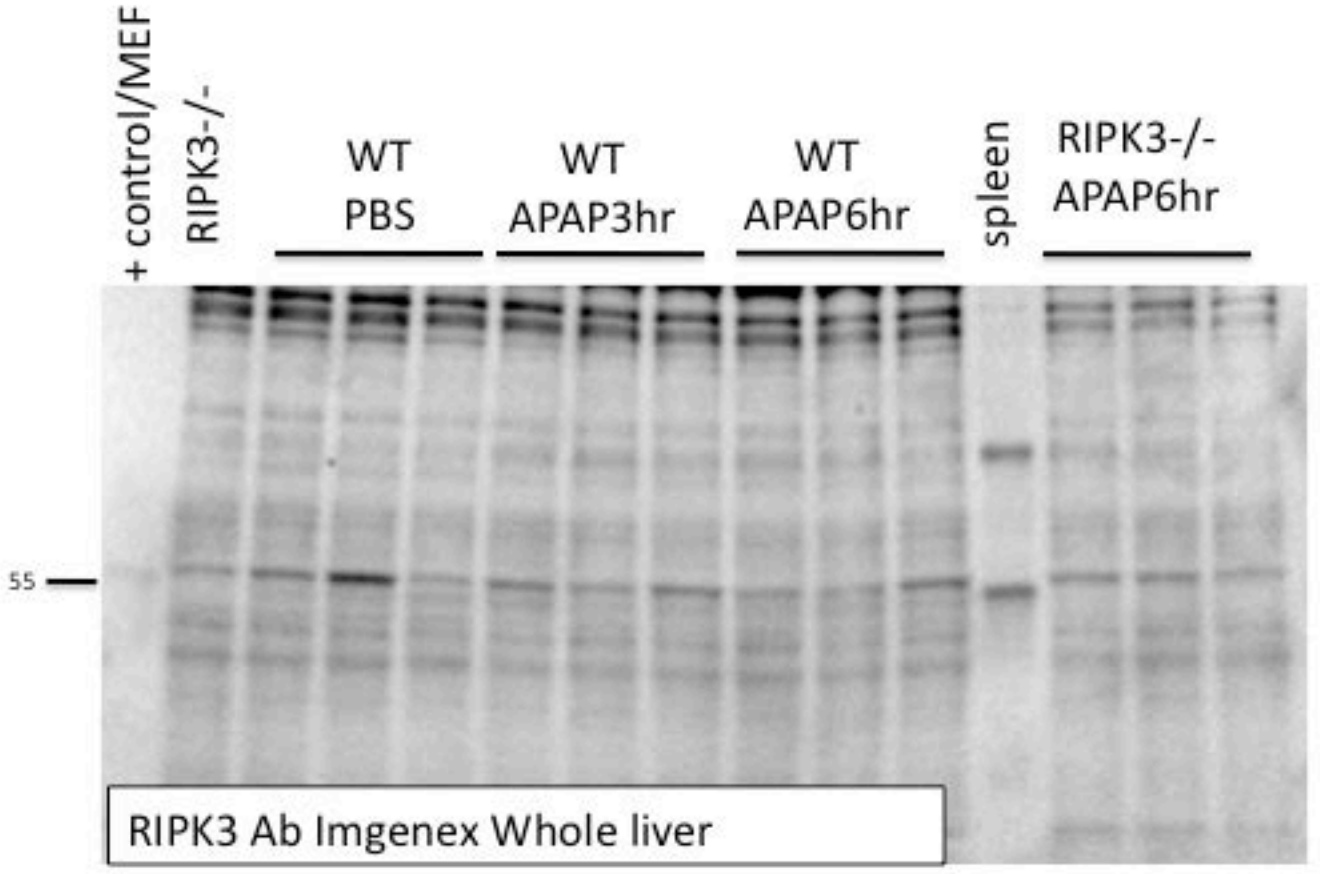
Supplemental Figure 2B.



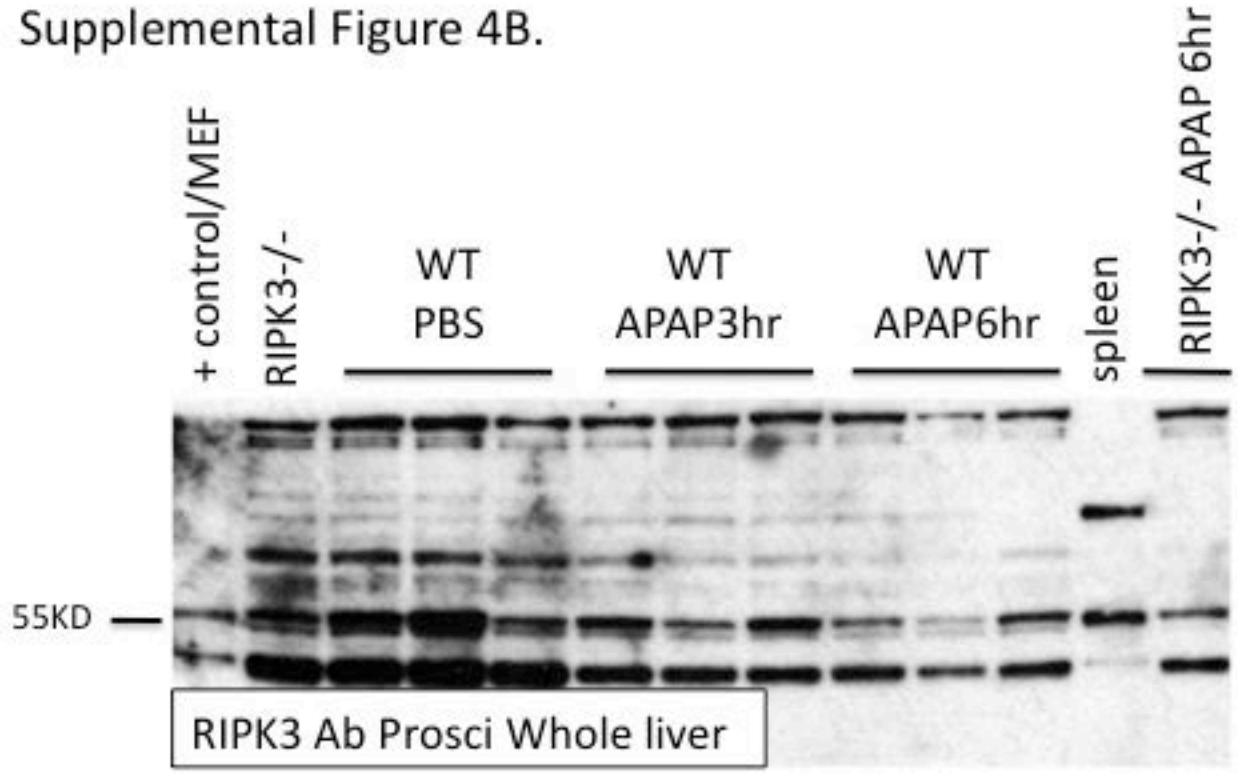
Supplemental Figure 3.



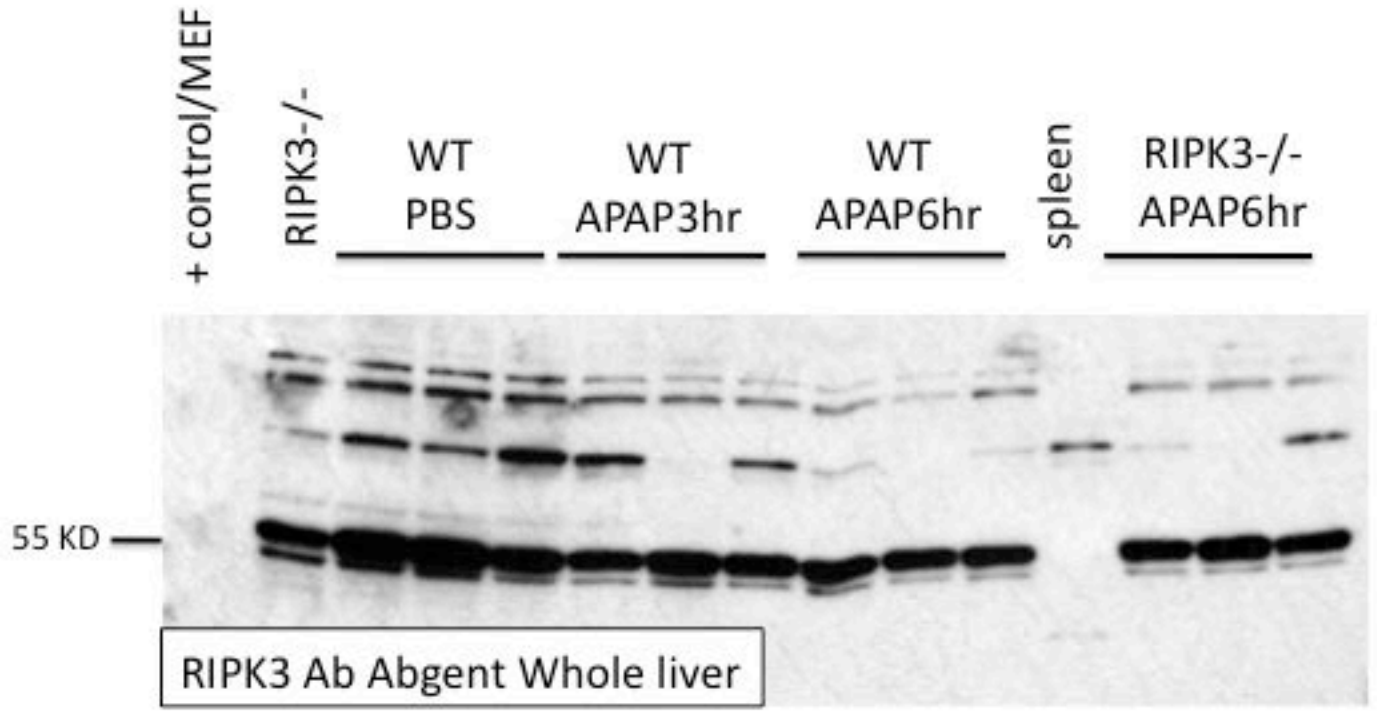
Supplemental Figure 4A.



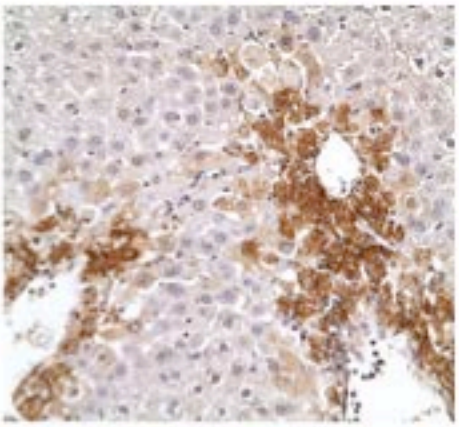
Supplemental Figure 4B.



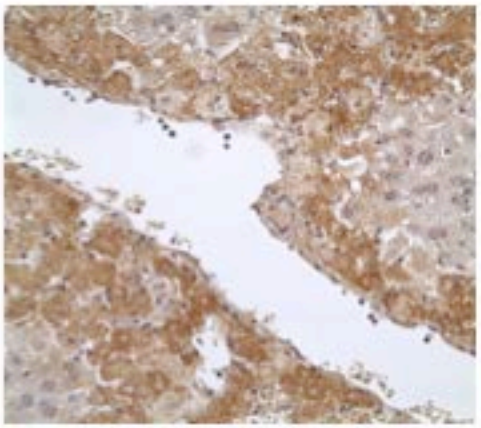
Supplemental Figure 4C.



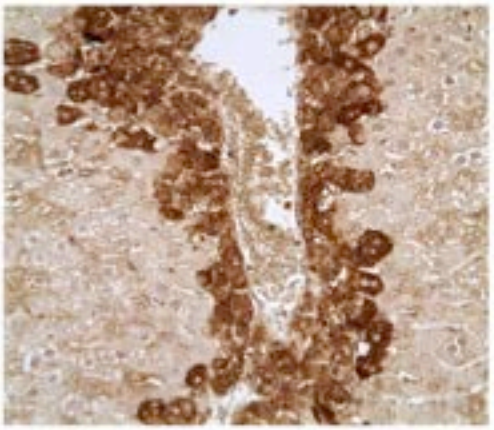
Supplemental Figure 4D.



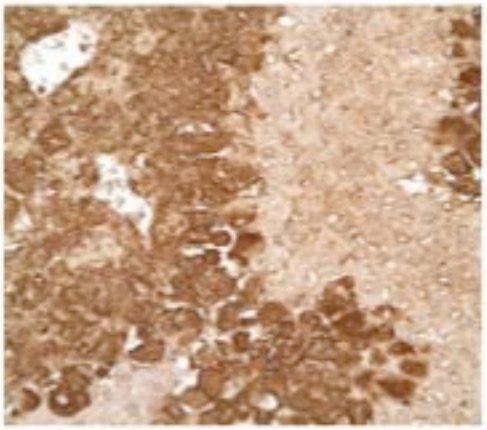
RIPK3KO APAP ABGENT AB



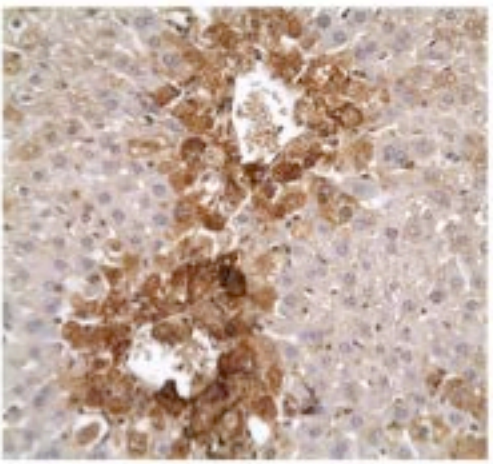
WT APAP ABGENT AB



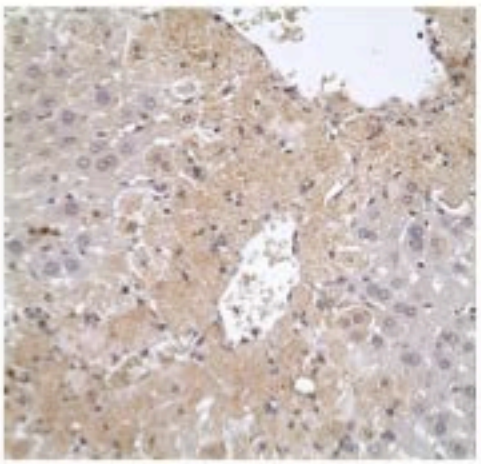
RIPK3KO APAP PROSCI AB



WT APAP PROSCI AB

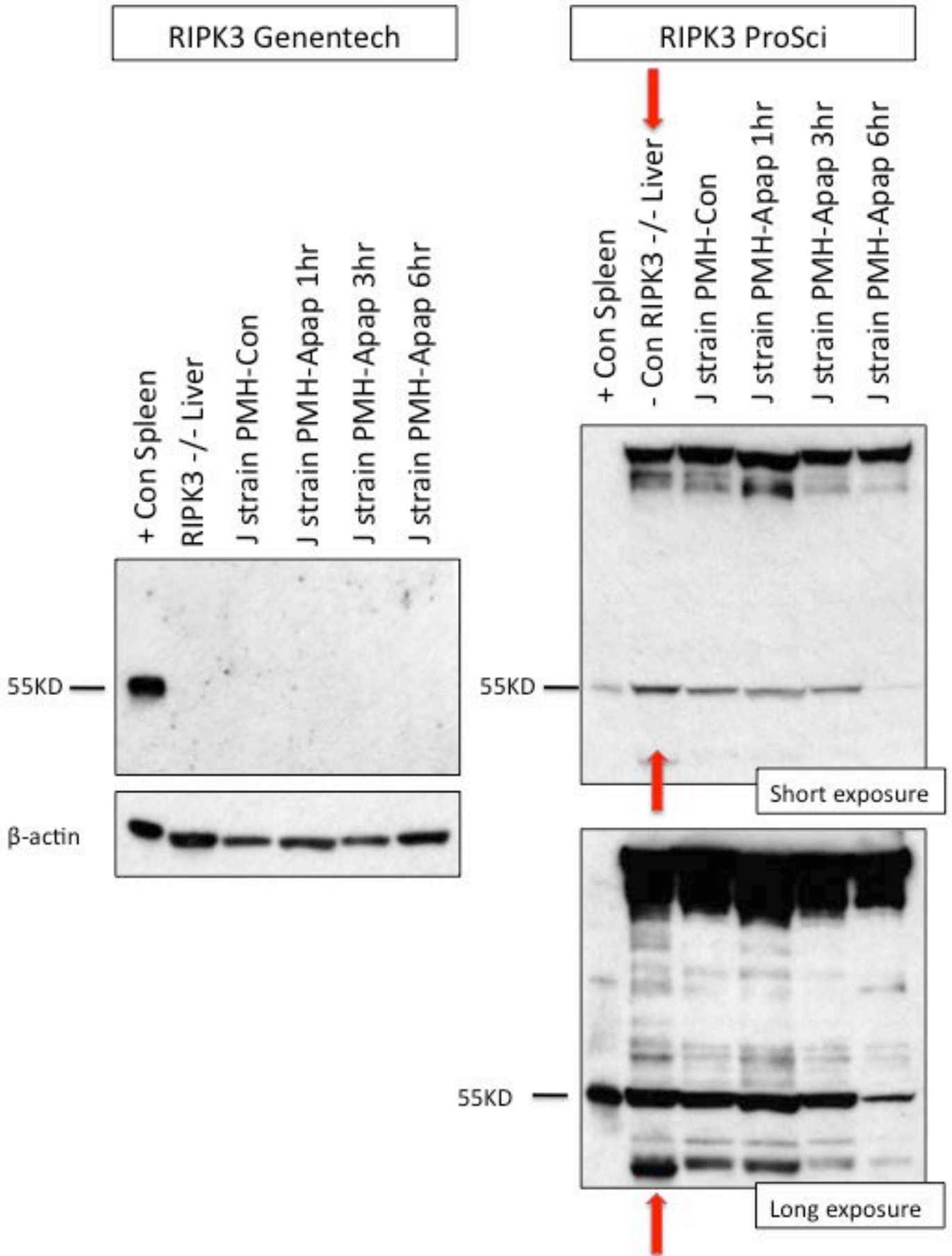


RIPK3KO APAP RABBIT IgG



WT APAP RABBIT IgG

Supplemental Figure 4E.



Supplemental Figure 5.