JNK DEPENDENT ACUTE LIVER INJURY FROM ACETAMINOPHEN OR TUMOR NECROSIS FACTOR (TNF) REQUIRES MITOCHONDRIAL SAB EXPRESSION IN MICE

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SUPPLEMENTAL METHODS

Cells, treatment and microscopy- HepG2 cells were transfected with siControl (ON-TARGET plus Non-targeting pool, D-001810-10-05) or siSab (ON-TARGET plus SMARTpool, L-019869-00-0005, Human SH3BP5 contains target sequence: CAACAAGCCCGUCUGAGAU; GCAGAUUGGCUGAUUCAUC; GAGAGGAAUUAUU GCUGA; GUAGCAACUUUGUGUCUGA, Dharmacon, Thermo scientific) using DharmaFECT1 Transfection reagent as described in product manual and cultured for 48 hours. siRNA transfected HepG2 cells were transferred to chambered cover-slides and cultured overnight and treated with or without anisomycin 5 μg/ml for 2 hours. Cells were exposed to JC-1 dye (5 μM) for last 1 hour of anisomycin 5 μg/ml two hours treatment in 37° C, then washed and overlaid with HBSS buffer for microscopy. To evaluate the involvement of JNK activation in anisomycin-induced mitochondrial depolarization, untransfected HepG2 cells were pretreated with 420116 JNK inhibitor I,(L)-Form (5 μM) (GIBCO) for 1 hour before anisomycin exposure. CCCP (20 μM; Sigma) treated cells were used as positive control for depolarized mitochondria.

Primary cultured mouse hepatocytes (PMH) were isolated from male C57BL/6 mice 7–9 week of age and plated at 2×10^6 cell/10-cm dish. Three hours after plating, hepatocytes were washed and rested in phenol red-free and serum-free Dulbecco's modified Eagle's medium/F-12 medium (3-ml/dish) overnight (~15 h). The incubation and following treatments were carried out in a 37 °C cell culture incubator with 5% CO_2. For drug co-treatments of actinomycin D (0.5 μg/ml), working stock was added to the culture just before TNF-α (20 ng/ml) addition. After the indicated time periods, cells were washed with ice-cold Dulbecco's PBS followed by further processing.

Isolation of mitochondria and cytoplasm from PMH- Mitochondria were isolated from PMH by differential centrifugation. PMH were collected by scrapping and homogenized in H-medium (210 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.05% bovine serum albumin (w/v), plus protease and phosphatase inhibitors). The homogenate was centrifuged at 800 × g for 10 min, the pellet removed, and

the centrifugation process repeated. The resulting supernatant was centrifuged at 8,500 × g for 15 minutes. The pellet, which represents the mitochondria fraction, was washed with H-medium and the centrifugation repeated. The mitochondria were resuspended in H-medium for Western blot analysis. The supernatant was cytoplasmic fraction. The resulting preparations were stored in aliquots at -80 °C

Proteinase K digestion- For proteinase K digestion assay mitochondria pellet was suspended in homogenizing buffer without inhibitors. 100 μg of protein was incubated in proteinase K (0, 0.1, 0.5, 1 or 2 mg/ml) in 500 μl total volume at 37° C for 30 minutes. The reaction was stopped by adding 1ml of H-medium with inhibitors including phenylmethylsulfonyl fluoride (PMSF) and washed. The pellet washed 2 times and protein extracted by RIPA buffer and stored in –80° C.

Immunoprecipitation and Western blot- Primary mouse hepatocytes (PMH) were isolated and seeded in plating medium and then exposed to APAP (10 mM) completely dissolved in fresh pre-warmed DMEM/F12. At indicated time mitochondria were isolated by differential centrifugation and lysate was immunoprecipitated with mouse anti-Sab and immunoblotted with rabbit anti-P-JNK and goat-anti-Sab or lysate was immunoprecipitated with rabbit anti-P-JNK and immunoblotted with mouse anti-Sab and mouse anti-P-JNK. The association of P-JNK and Sab in the absence of APAP presumably reflects the fact that PMH are stressed under culture conditions.

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. **Subcellular distribution of Sab and co-immunoprecipitation of Sab and P-JNK.** (A) Immunoblots examining localization of Sab in subcellular fractions of mouse liver. W indicates whole liver homogenate. Cy indicates cytosol. M indicates mitochondria. ER indicates endoplasmic reticulum enriched post-mitochondrial pellet. (B) Immunoblots assessing submitochondrial localization of Sab after proteinase K digestion. (C) Immunoblots assessing association of Sab and P-JNK in PMH upon treatment with APAP. P indicates phosphorylation. Isolation, treatment and fractionation of PMH and immunoprecipitation are described in Methods. Results are representative of three experiments.

Fig. S2. **Effect of Sab knockdown on covalent binding of NAPQI.** Western blot of liver homogenate was prepared two hours after APAP (300 mg/kg) in Ad-shLacZ and Ad-shSab pretreated mice. Covalent binding to liver proteins was assessed using antiserum to NAPQI protein adducts provided by Dr. Jack A. Hinson of University of Arkansas.

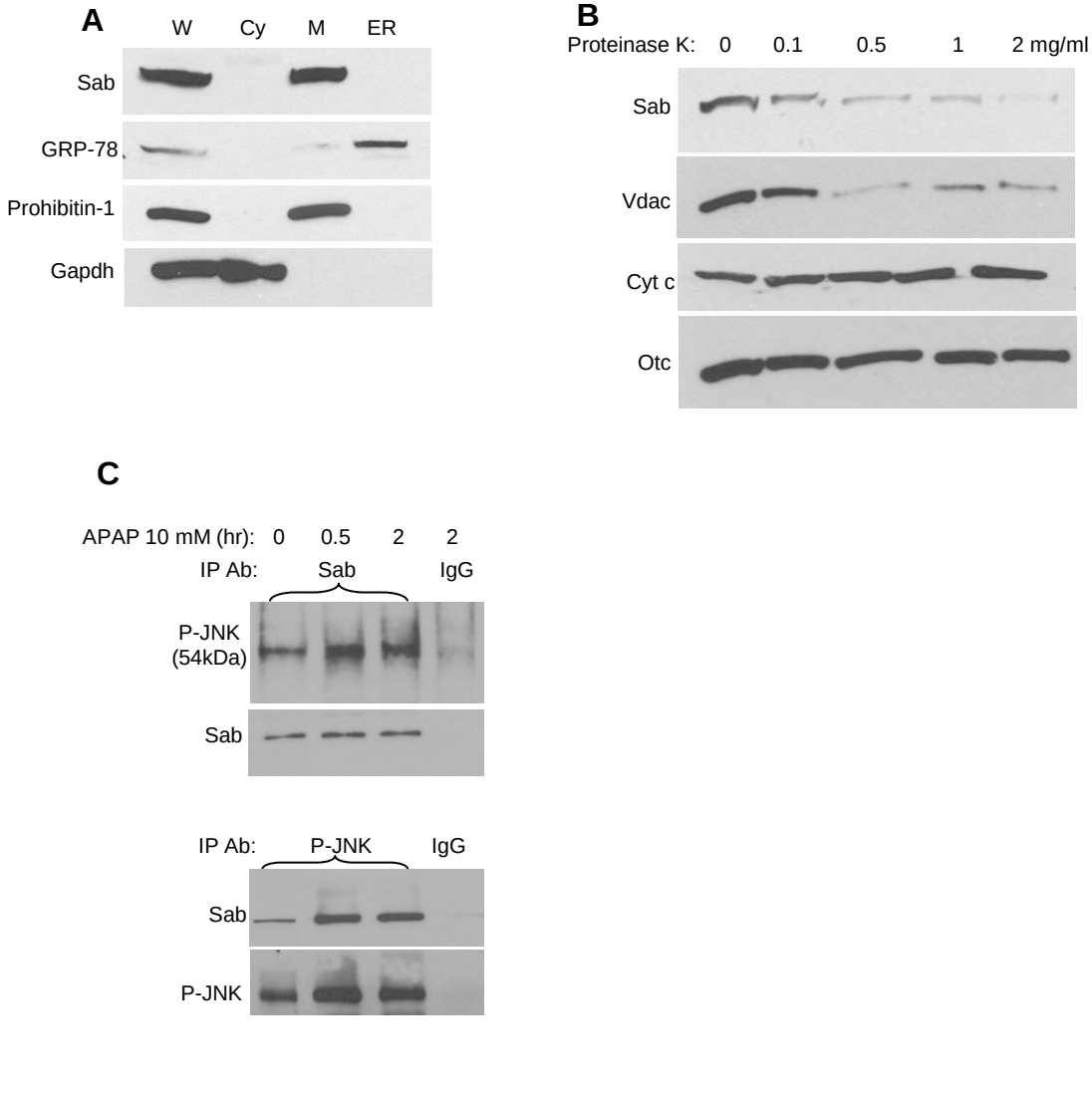
Fig. S3. **Effects of JNK inhibitor (SP600125) on GalN/TNF-α induced hepatotoxicity in C57BL/6.** Mice were pretreated with intraperitoneal injection of D-galactosamine (GalN) in PBS 15 minutes prior to injection of JNK inhibitor (SP600125) 10 mg/kg dissolved in DMSO (8.3% v/v in PBS) or equivalent amount of DMSO in PBS as vehicle control, and then, 15 minutes later mouse recombinant TNF-α was injected intraperitoneally in PBS. JNK inhibitor or vehicle was then injected intraperitoneally hourly up to 6 hours to maintain inhibitor levels. Liver injury was assessed at 6 hours after TNF-α injection. (**A**) Serum ALT. *, $p < 0.05$ versus vehicle control, n=4 per group. (**B**) Representative H&E staining of liver sections. Scale bar, 100 μm.

Fig. S4. **P-JNK translocation to mitochondria after TNF alone or Actinomycin D and TNF treatment in PMH.** Western blot of mitochondrial and cytoplasmic fractions prepared from PMH treated with TNF, or TNF/ActD examining phospho- JNK (Thr-183/Tyr-185) (P-JNK) and total JNK, and prohibitin-1 (PHB-1) as mitochondria loading control. The exposure time is represented as triangle on the right.

Fig. S5. **Effect of siRNA knockdown of Sab on mitochondrial depolarization in HepG2 cells.** (**A**) HepG2 cells were transfected with siControl or siSab. Whole cell lysates were prepared in RIPA buffer. Immunoblot was done with indicated antibodies. Bap37 and COX IV were used as loading controls. Densitometry was measured and analyzed by NIH imageJ. (**B**) HepG2 cells with no treatment (control) or transfection with siControl or siSab were plated overnight and treated with or without anisomycin 5 μg/ml and exposed to JC-1 dye (5 μM) at 37 °C then washed and overlayed with HBSS buffer at 2 hr after anisomycin treatment. Red/ red-orange represents normal healthy polarized mitochondria due to dimerized JC-1 dye aggregated in mitochondria. Green represents monomer JC-1 dye in cytosol indicating the inability of mitochondria to take up JC-1 dye due to de-polarization. CCCP (20 μM) treated cells were used as positive control for depolarized mitochondria. HepG2 cells were pretreated with 420116 JNK inhibitor I (L)-Form (5 μM) for 1 hour before anisomycin treatment. See Supplementary Methods.

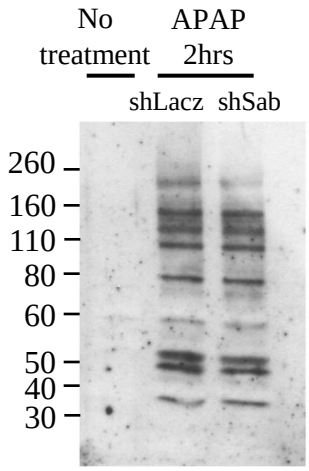
Fig. S6. **Model of JNK/Sab in hepatotoxicity.** NAPQI or TNF in the presence of inhibition of NFkB (galactosamine or actinomycin D) inhibits electron transport (ETC) promoting release of ROS from mitochondria. This leads to activation of MAP kinase kinase kinase (MAP3K), e.g. ASK-1, which in turn activates MAP2K (e.g. MKK4) which activates JNK (thin arrows). P-JNK then binds to Sab which leads to further impairment of ETC and enhanced ROS production sustaining a high level of JNK activation in a vicious cycle (thick arrows). In the case of APAP profound GSH depletion renders the mitochondria more susceptible to ROS leading to mitochondrial permeability transition (MPT) pore opening which induces a bioenergetic catastrophe causing necrotic cell death characteristic of APAP toxicity. In contrast, in the case of TNF/galactosamine or TNF/ actinomycin, the sustained JNK activation promotes a pro-apoptotic combination of events mediated by the Bcl-2 family: Bax translocation to mitochondria, inhibition of Bcl-X_L, t-Bid formation and translocation as a consequence of proteosomal degradation of cFLIP (caspase 8 inhibition).

Supplemental Figure S1

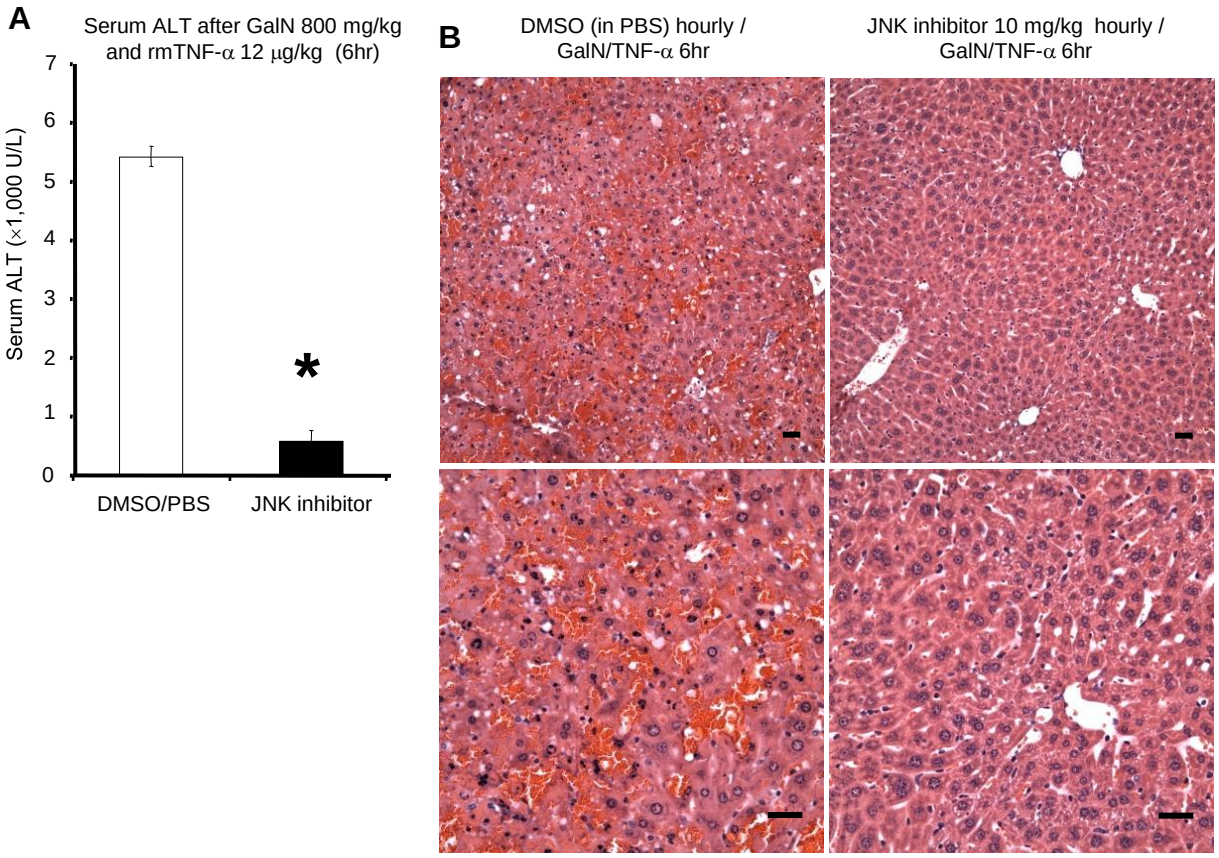


Supplemental Figure S2

Anti- APAP antiserum (covalent adducts)



Supplemental Figure S3



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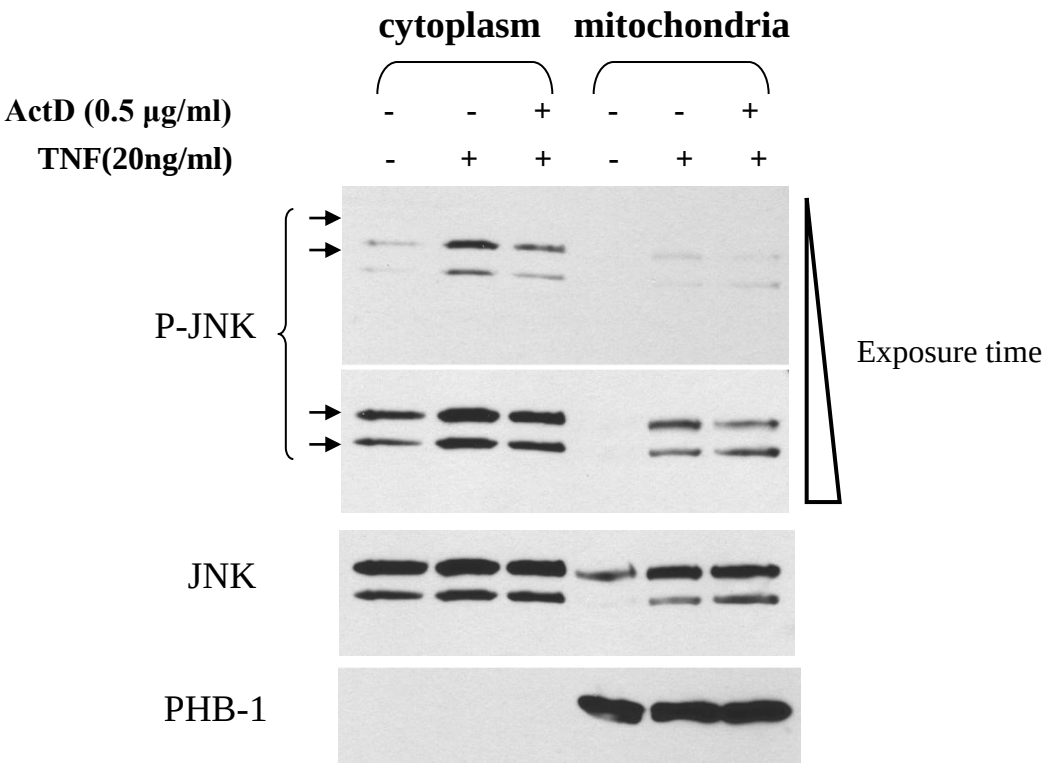
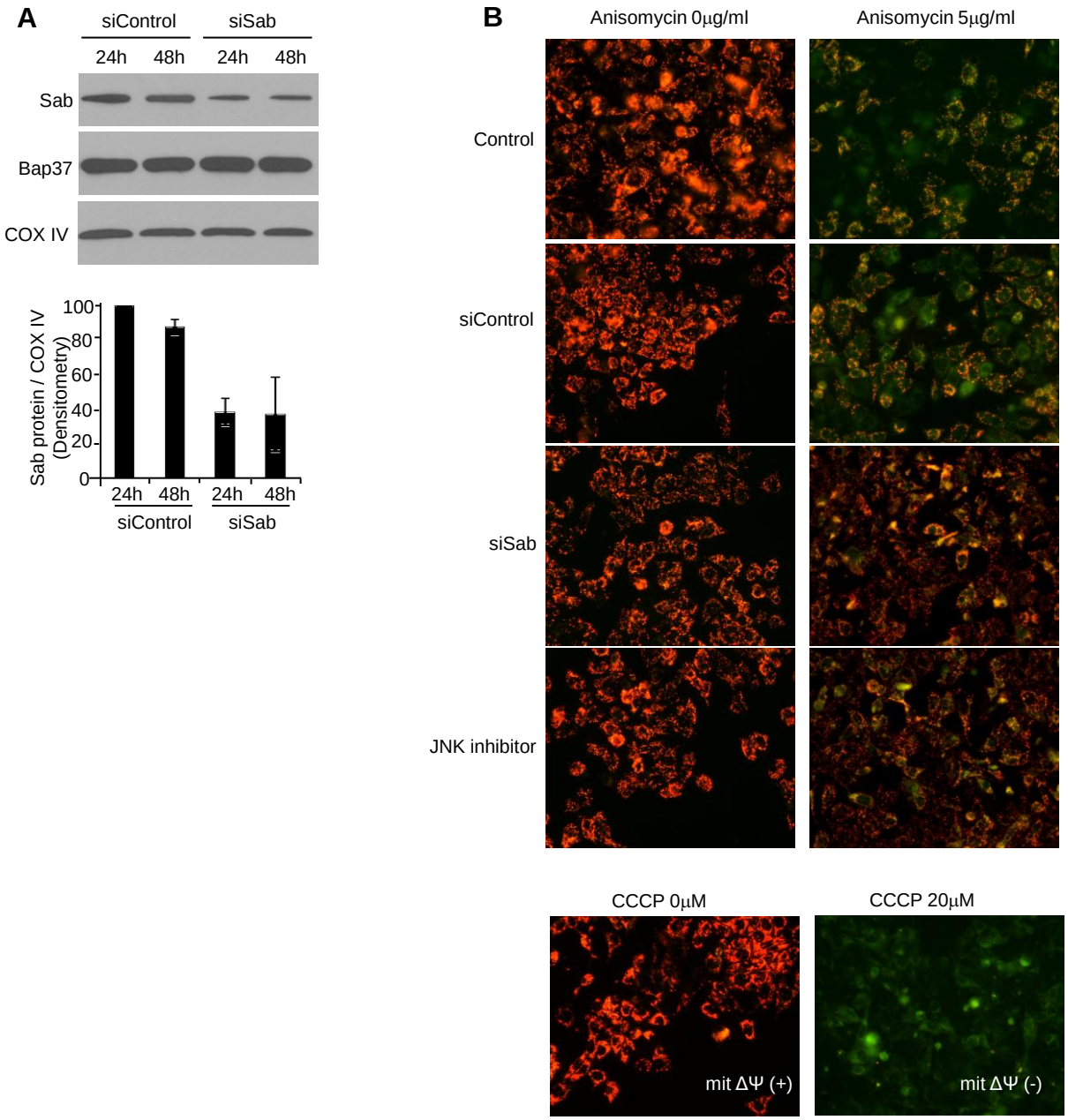


Supplemental Figure S5



Supplemental Figure S6