# Expression of mitochondrial membrane-linked SAB determines severity of sex-dependent acute liver injury

Sanda Win[1*], Robert W.M. Min[1*], Christopher Q. Chen[1], Jun Zhang[2], Yibu Chen[3], Meng Li[3], Ayako Suzuki[4], Manal F. Abdelmalek[4], Ying Wang[4], Mariam Aghajan[5], Filbert W.M. Aung[1], Anna Mae Diehl[4], Roger J. Davis[6], Tin A. Than[1], Neil Kaplowitz[1]

**SUPPORTING DATA**

**Methods**

**Reference**

**Table S1**

**Figure S1**

**Figure S2**

**Figure S3**

**Figure S4**

**Figure S5**

**Figure S6**

**Figure S7**

# Supporting Methods

## Reagents

Antisera to P-JNK (4668), P-SRC(Tyr416) (6943), c-SRC (2109), PHB1 (2426), COXIV (4844), SIRT1 (9475) (Cell Signaling Technology), total JNK (JNK 1/2/3) (sc-571; Santacruz biotechnology, 9252; Cell Signaling Technology), GAPDH (G9295; Sigma Aldrich), β-actin (A3854; Sigma Aldrich), OTC (HPA000243; Atlas), acetyl-p53(Lys373, 382) (06-758; Millipore), ERα (E115) (ab32063; Abcam) were used. Antisera to SAB which recognizes C-terminus of SAB was developed by Abbomax and characterized in previous publication (1). The following reagents were used: APAP, D-GalN (Sigma), and mouse recombinant TNF-α (Calbiochem) were administered as described before (2). APAP was dissolved in warm PBS (55 °C) and cooled to 37 °C before intraperitoneal injection into overnight fasted mice at a dose of 150-300 mg/kg. Mice were pretreated with 800 mg/kg D-GalN dissolved in PBS by intraperitoneal injection 30 min prior to intraperitoneal injection of mouse recombinant TNF-α (12 μg/kg) in pyrogen-free PBS. ERα agonist propylpyrazole triol (PPT) (Tocris Bioscience,Ellisville, MO, USA), ERα/β receptor antagonist fulvestrant (Selleckchem), ActD (Sigma) were used. Recombinant unactivated JNK and activated P-JNK were purchased from Millipore. Reagents, plates and instruments required for Seahorse flux assay were used as described before (1, 3). Serum alanine aminotransferase (ALT) was measured by Alanine Aminotransferase (ALT or SGPT) Activity Colorimetric /Fluorometric Assay Kit (Biovision).

**Antisense oligonucleotide (ASO) and miRNA mimic**

ASO were provided by Ionis Pharmaceuticals. *P53* ASO (#1) (GCAGACAGGCTTTGCAGAAT) and scrambled control ASO (CCTTCCCTGAAGGTTCCTCC) were used. The results were confirmed with a *p53*-ASO (#2) targeting another part of the *p53* RNA sequence (GTGACTCCTCCATGGCA GTC) (Figure S5A). GalNAc-Scrambled control (CCTTCCCTGAAGGTTCCTCC) and GalNAc-*Sab* ASO (#1) (GCTGCCGCTACAGGGAATGC) were used. A parent *Sab*-ASO (#1) which is not conjugated with GalNAc was shown to protect against APAP and was confirmed with *Sab*-ASO (#2) (CCACGCTGCCGCTACAGGGA) (Figure S7, G and H). ASO was dissolved in sterile PBS and oligonucleotide concentration was determined before aliquot for storage in −80 °C. Mice received 50 mg/kg of p53/control ASO intraperitoneally on alternate days for seven doses. Mice received 2.5 mg/kg of GalNAc-*Sab*/control ASO intraperitoneally on alternate days for seven doses. Experiments were performed the day after last injection. *miR34a-5p* mimic oligo (#MIM0106) was purchased from Active Motif and transfection was performed using Lipofectamine RNAiMAX transfection reagents.

**Cell Isolation and Culture**

Primary mouse hepatocytes (PMHs) were isolated and cultured as described previously (1, 2). Three hours after plating of isolated hepatocytes, APAP (5mM) dissolved in fresh prewarmed DMEM/F-12 culture medium was added. After 8 hr of treatment, cells were double-stained with Hoechst 33258 (8µg/ml; invitrogen) and SYTOX Green (1µmol/liter; invitrogen). Quantitation of total and death cell was performed by counting a minimum of 1000 cells in 10 different fields. Necrotic cells (SYTOX Green-positive) were determined by counting the same field, as described previously (1, 2). In other experiments, PMH were rested in serum free

DMEM/F12 medium overnight and treated with actinomycin D (ActD; 0.5μg/ml)/TNF-α (20 ng/ml) for 6hr and stained with Hoechst 33258 dye and apoptotic cells were counted.

**In vivo treatment with phorone**

Overnight fasted mice received GSH depleting agent phorone (Sigma) 100mg/kg in corn oil intraperitoneally and liver samples were collected snap frozen in liquid nitrogen at 1hr and 4hr later. Liver GSH was determined by recycling assay using glutathione colorimetric assay kit (Biovision).

**In vitro treatment with Doxorubicin/Trichostatin A**

HepG2 cells (ATCC) were treated with minimal toxic dose of combination of reversible deacetylases Trichostatin A and topoisomerase II inhibitor Doxorubicin for 48hr to activate p53. 48hr later cells were rinsed with fresh culture medium to remove dead cells and cultured in fresh culture medium up to day 5. Medium was replaced every 24hr.

**Isolation of Liver Mitochondria and Cytoplasm**

Mitochondria were isolated from mouse livers by differential centrifugation as described previously (1, 2). Livers were homogenized in isolation buffer supplemented with protease and phosphatase inhibitors, without bovine serum albumin. The homogenate was centrifuged at 1000×g for 10 min, the pellet was removed, and the centrifugation process was repeated. The resulting supernatant was centrifuged at 9000×g for 10 min. The pellet, which represents the mitochondrial fraction, was washed with isolation buffer and centrifugation was repeated. The mitochondria were resuspended in RIPA buffer for Western blot analysis. The supernatant was centrifuged at 9000×g to obtain cytoplasmic fraction.

**Measurements of Respiration in Isolated Mitochondria**

12 weeks old male and female mice liver mitochondria were isolated by differential centrifugation and resuspended in mitochondrial assay solution (MAS; 70 mM sucrose, 220 mM mannitol, 10 mM $KH_2PO_4$, 5 mM $MgCl_2$, 2 mM 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid, and 1 mM ethylene glycol tetraacetic acid, pH 7.2, at 37°C) with pyruvate and malate without protease and phosphatase inhibitors. State III, state IV and maximal respiratory capacity measurements were performed by Seahorse XF24 analyzer (1, 3). To 20µg of mitochondria in 50µl MAS supplemented with substrate with or without ATP 6µM, 50ng of ~160 U/mg P-JNK or JNK were added on ice. Pre-mixed mitochondria 20µg (50µl) were loaded into each well of XF24 cell culture microplate on ice and was spun at 3000rpm for 20min at 8°C, and then incubated at 37°C in CO2 free incubator for 15min. After incubation, each well was fed with 450µl of 37°C prewarmed MAS supplemented with substrate, and OCR was measured. The program was set to equilibrate at 37°C for 5min before measurement of basal OCR (State 2 respiration). ADP (4mM final), oligomycin (2.5µg/ml final), CCCP (4µM final) and antimycin A (4µM final) were injected from port-A, port-B, port-C or port-D and OCR was measured sequentially. State 3 respiration was determined after ADP injection. OCR after oligomycin injection was defined as State 4 respiration. The oligomycin inhibitable respiration (ADP minus oligomycin) represents oxidative-phosphorylation. CCCP induced OCR was defined as maximal respiratory capacity of mitochondria. When required, measurements were normalized to average of measurement points of the basal (starting) level of OCR of each well. Error bars in one representative experiment represent S.D of 3-5 wells. Experiments were repeated 5 times

with different mitochondria preps. Data was analyzed in groups of wells of each sample prep and statistical analysis was done by *t*-test.

**Western Blot Analysis**

Aliquots of cytoplasmic or mitochondrial extracts were fractionated by electrophoresis on 7.5, 10, or 4-20% SDS-polyacrylamide gel (Bio-Rad). Subsequently, proteins were transferred to nitrocellulose membrane using iBlot transfer (invitrogen), and blots were blocked with 5% (w/v) nonfat milk dissolved in Tris-buffered saline with Tween 20. The blots were then incubated with the primary and secondary antibodies, and detected by luminol ECL reagent (Thermo Scientific) using film or ChemiDoc (bioRad). All gels shown are representative samples from at least three experiments. Densitometry was measured from 3 separate experiments using NIH image J software.

**Quantitation of mRNA and miRNA**

Total RNA was extracted using an RNeasy RNA extraction kit (Qiagen). 0.5 μg of total RNA was reverse-transcribed using an Omniscript reverse transcription kit (Qiagen) supplemented with 10 M random hexamer (Applied Biosystems). The resulting cDNA (volume equivalent to 5–10 ng of total RNA in a reverse transcription reaction) was subjected to quantitative real-time PCR analysis with the SYBR Green PCR master mix (Qiagen) and ABI Prism 7900HT sequence detection system (Applied Biosystems). The relative quantification of mRNA expression was analyzed by the standard curve method following the manufacturer's instructions (Applied Biosystems). Three pairs of primers (TATCAACCGACGGGAGACTGA & TGCCAGTTCGT CTAGTTTCAC; AAACTAGACGAACTGGCAAAGAA & AAACTGCCGCTTGTCATCCT; AAATGCTGAACC

ATGCTACTCA & TCCTTGTGTACTAGCTCACTCC) were used to determine mouse SAB mRNA level. Data were analyzed using the SDS2.4 software (Applied Biosystems). mRNA were normalized by endogenous GAPDH expression. small RNA <200bp (including miRNA) was collected from flow through of RNA extraction procedure and concentrated by miRNeasy kit (Qiagen). Mouse miRNA primers for *miR34a-5p* (#mmu481304_mir) purchased from AppliedBiosystem were used for TaqMan Advanced miRNA Assays. miRNA was normalized by *miR-191-5p* (#mmu481584_mir) which expression was not changed in miRNA seq analysis.

**Histological Analysis**

Livers were removed and cut into 5-µm thick sections, fixed with 10% buffered formalin, embedded in paraffin. All specimens were stained with hematoxylin/eosin and evaluated under a light microscope. TUNEL staining was performed using the reagents from Roche according to manufacturer’s instructions (1, 3).

**Estrus cycle**

To determine the estrus cycle of wild type female cage mate littermates, vaginal wash with PBS was fixed immediately with equal volume of 10% neutral buffer formalin and dry on glass slides. Cells were stained with eosin to identify nucleated or un-nucleated epithelial cells and immune cells as described before (4). Liver samples from female cage mate littermates were collected at different days of estrus cycle.

## Supporting Reference

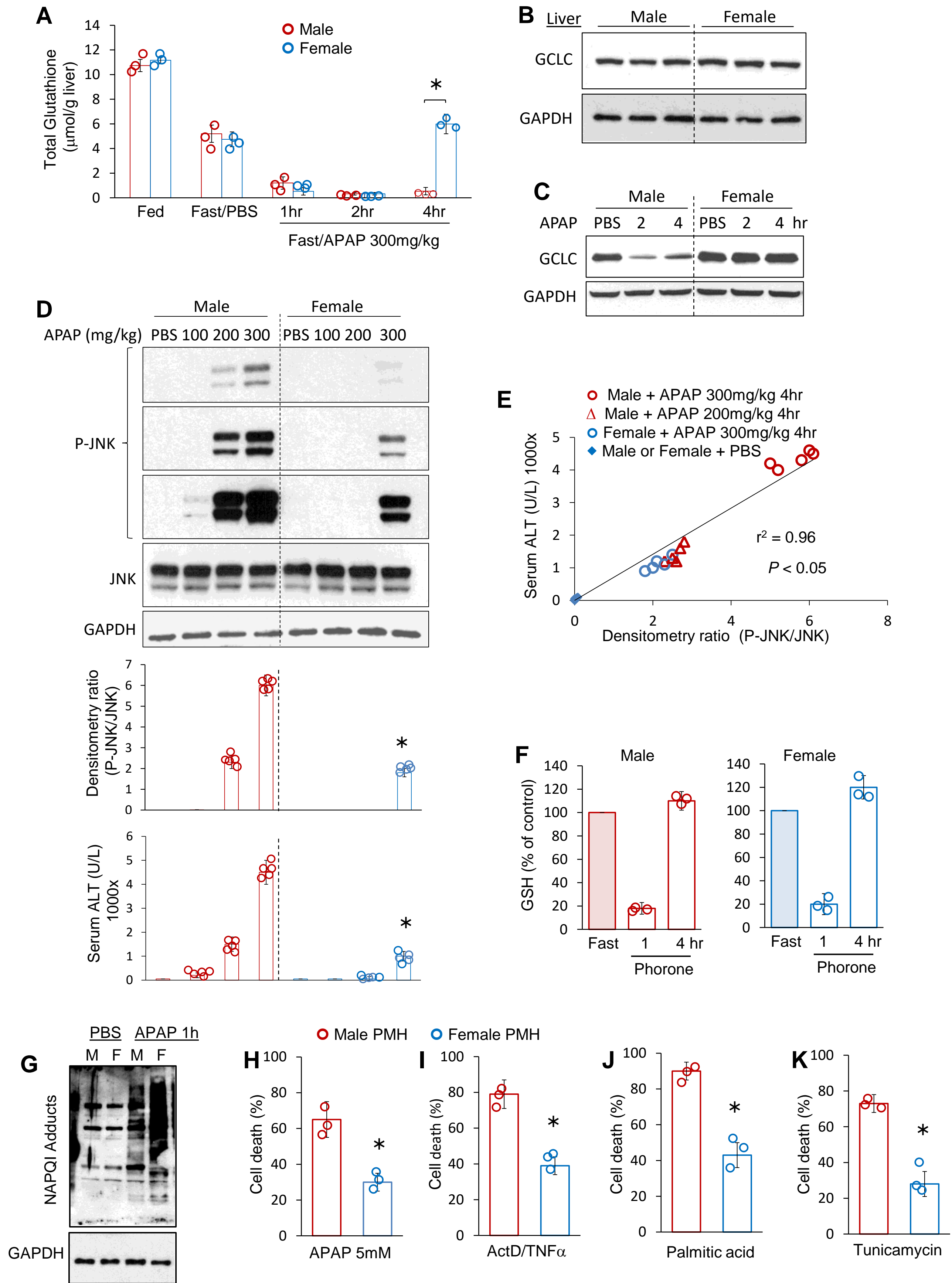
1. Win S, Than TA, Min RW, Aghajan M, Kaplowitz N. c-Jun N-terminal kinase mediates mouse liver injury through a novel Sab (SH3BP5)-dependent pathway leading to inactivation of intramitochondrial Src. *Hepatology.* 2016;63(6):1987-2003.
2. Win S, Than TA, Han D, Petrovic LM, Kaplowitz N. c-Jun N-terminal kinase (JNK)-dependent acute liver injury from acetaminophen or tumor necrosis factor (TNF) requires mitochondrial Sab protein expression in mice. *J Biol Chem*. 2011;286(40):35071-35078.
3. Than TA, Win S, Kaplowitz N. In vitro assays of mitochondrial function/dysfunction. *Clin Pharmacol Ther.* 2014;96(6):665-668.
4. Byers SL, Wiles MV, Dunn SL, Taft RA. Mouse estrous cycle identification tool and images. *PLoS One.* 2012;7(4):e35538.

## Supporting Table S1

**Table S1.** Patient characteristics

| Human | Sex | Age | BMI | E2 pg/ml | FSH miU/ml | ALT | AST |
|---|---|---|---|---|---|---|---|
| Pre-menopause | Male | 33 | 32 | 19 | - | 79 | 42 |
| | Male | 33 | 27 | 30 | - | 194 | 142 |
| | Male | 41 | 32 | 50 | - | 57 | 36 |
| | Male | 43 | 25 | 19 | - | 88 | 67 |
| | Male | 49 | 24 | 54 | - | 63 | 86 |
| | | | | | | | |
| | Female | 35 | 36 | 304 | 8 | 14 | 17 |
| | Female | 39 | 23 | 351 | 2 | 39 | 32 |
| | Female | 40 | 36 | - | - | 27 | 19 |
| | Female | 48 | 24 | - | - | 53 | 44 |
| | Female | 49 | 23 | 113 | 3 | 9 | 161 |
| | | | | | | | |
| Post-menopause | Male | 47 | 31 | 8 | - | 36 | 18 |
| | Male | 48 | 27 | - | - | 39 | 41 |
| | Male | 50 | 28 | - | - | 50 | 35 |
| | | | | | | | |
| | Female | 51 | 30 | 12 | 51 | 31 | 28 |
| | Female | 51 | 30 | <5.0 | 67 | 60 | 43 |
| | Female | 52 | 24 | <5.0 | 84 | 44 | 39 |

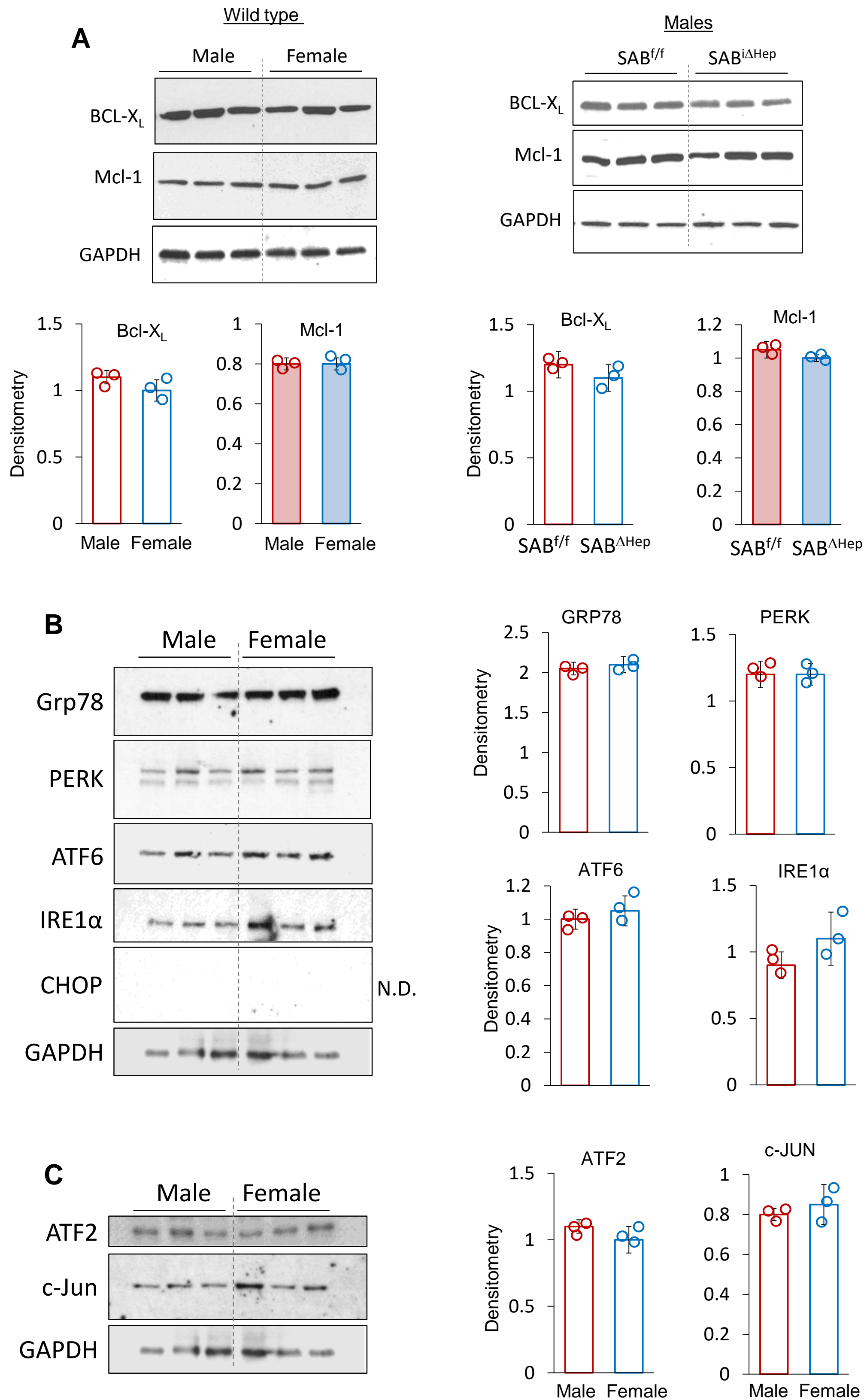
## Supporting Figure S1



**Supporting Figure S1. Total GSH, GCL, GSH re-synthesis, NAPQI adduct level, dose dependent P-JNK activation and serum ALT increase in male versus female and cell death after treatment of PMH. (A)** Hepatic GSH in chow diet fed and overnight 16hr-fasted, or 1, 2, 4 hour APAP (300mg/kg) treated C57BL/6N male and female littermates. GSH level in liver homogenate was determined by recycling assay. N = 3 mice per group. (∗) = $P<0.05$ vs male, by unpaired, 2-tailed Student's *t* test. Data are presented as the mean ± SEM. **(B)** GCLC protein level of 12 weeks C57BL/6N male versus female. N = 3 mice per group. **(C)** GCLC protein level at 2 and 4 hour after PBS or APAP 300mg/kg treatment. Representative immunoblot of 3 separate experiments. N = 3 mice per group. **(D)** Male and female littermates (12 weeks) received PBS or APAP 100, 200, 300 mg/kg i.p and liver and blood were collected at 4 hr later. P-JNK was determined by immunoblot. N = 5 mice per group. (∗) = $P<0.05$ versus corresponding male, by unpaired, 2-tailed Student's *t* test. Data are presented as the mean ± SEM. **(E)** Correlation of P-JNK and serum ALT level of male and female littermates at different doses of APAP. Statistical analysis was performed using one way ANOVA. **(F)** GSH re-synthesis capacity in male and female littermates 4hr after receiving phorone. N = 4 - 5 mice per group. **(G)** NAPQI-proteins adduct level in liver protein extract 1hr after APAP 300mg/kg was assessed using antiserum to NAPQI protein adducts provided by Dr. Laura James (University of Arkansas). Representative immunoblot of 3 separate experiments. M= male, F = female. **(H-K) Comparison of cell death in male versus female PMH.** PMH from 10-12 weeks C57BL/6N male and female littermates was treated with **(H)** APAP 5mM 8hr, (**I)** actinomycin D (0.5μg/ml)/TNF-α (20ng/ml) 6hr, **(J)** palmitic acid 1.5mM 24hr, or **(K)** tunicamycin 20μg/ml 24hr. Cell death was determined by SYTOX Green-positive staining (1μmol/liter). Total number of cells was quantitated by Hoechst 33258

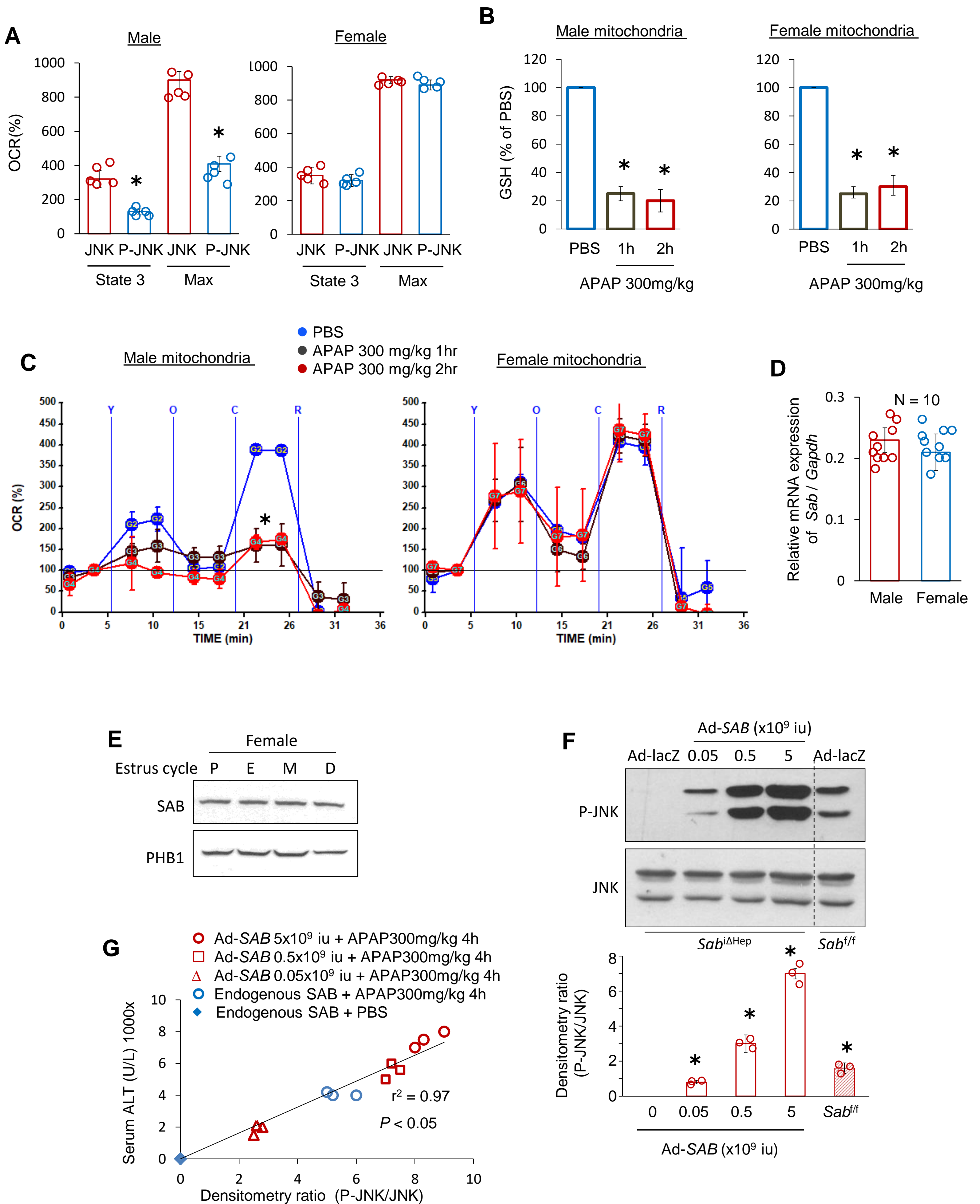
(8μg/ml). Minimum total 1000 cells in 10 different fields were counted. (∗) = $P$ <0.05 vs male of 3 separate experiments. Data are presented as the mean ± SEM.

## Supporting Figure S2



**Supporting Figure S2. Basal level of BCL-2 family proteins, ER stress response proteins and JNK substrates.** Liver homogenate from 10-12 week old C57BL/6N wild type male vs female littermates or *Sab*$^{f/f}$ vs *Sab*$^{i\Delta Hep}$ males were immunoblotted using **(A)** anti-BCL-$X_L$, MCL-1, **(B)** GRP78, PERK, ATF6, IRE1α, CHOP, **(C)** ATF2, c-JUN and GAPDH. N.D = not detected at basal. N = 3 mice per group.

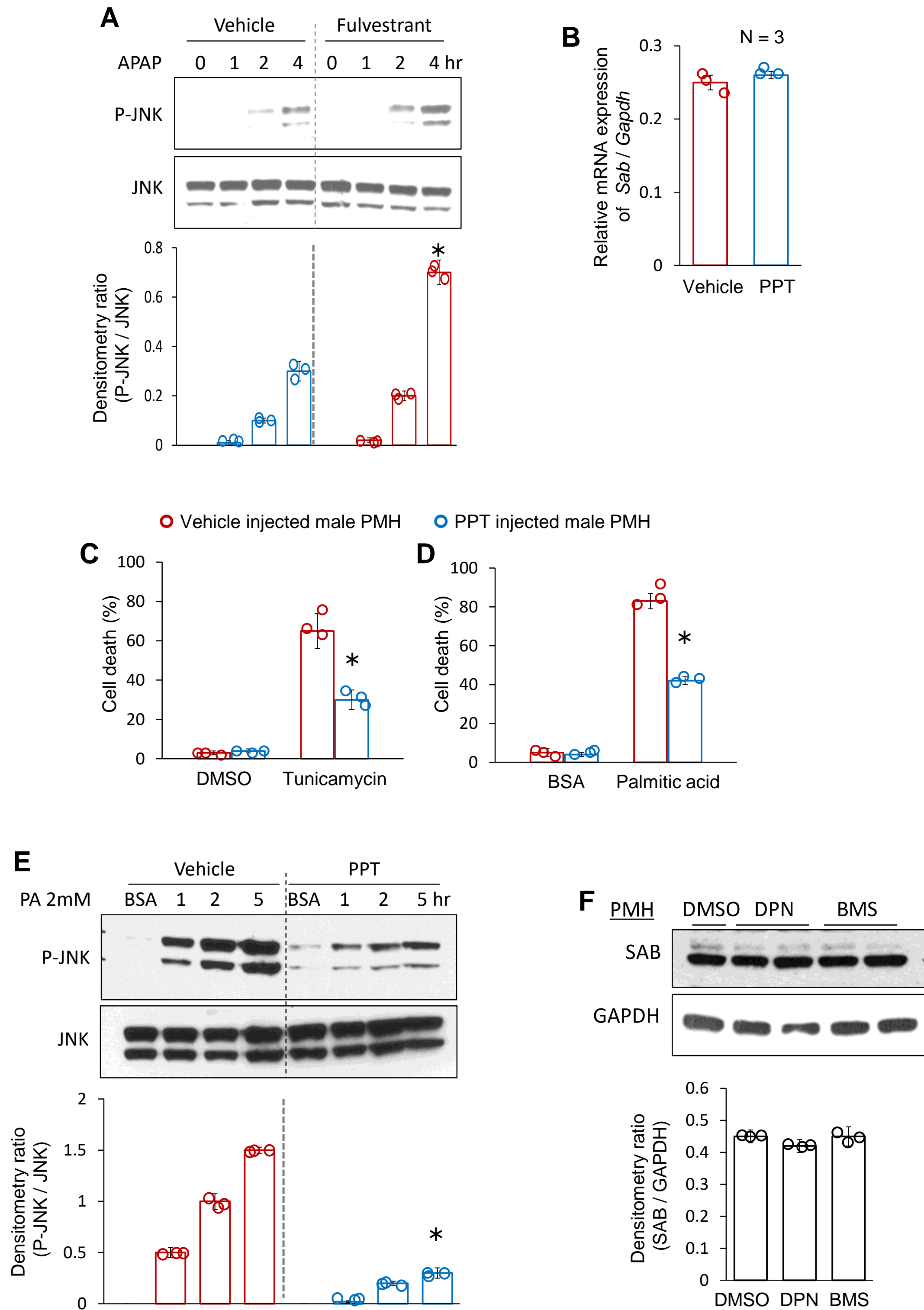
## Supporting Figure S3



**Supporting Figure S3. Direct effect of P-JNK and in vivo effect of APAP on mitochondrial function. (A)** P-JNK inhibition of mitochondrial respiration; summary of P-JNK inhibition of mitochondrial respiration shown in Figure 1E was performed 5 independent experiments. (∗) = *P* <0.05 versus JNK, by unpaired, 2-tailed Student's *t* test. Data are presented as the mean ± SEM. **(B, C)** APAP induced mitochondrial GSH depletion and inhibition of mitochondrial respiration; Mitochondrial GSH and respiration at 1 and 2hr after APAP 300mg/kg in C57BL/6N male versus female littermates was determined by recycling assay and Seahorse XF analyzer. N = 3 mice in each group. (∗) = *P* <0.05 versus PBS, by one way ANOVA. Data are presented as the mean ± SEM. **(D) Basal level of *Sab* mRNA expression in C57BL/6N male versus female was determined by qPCR.** N = 10 mice per group. **(E) SAB expression in estrus cycle;** To determine the estrus cycle of wild type female cage mate littermates, vaginal wash with PBS was fixed immediately with equal volume of 10% neutral buffer formalin and dry on glass slides. Cells were stained with eosin to identify nucleated or un-nucleated epithelial cells and immune cells. Liver samples from female cage mate littermates were collected at different days of estrus cycle. P = Proestrus; E = Estrus; M = Metestrus; D = Diestrus. **(F, G) Level of SAB expression determines P-JNK activation level and correlates with serum ALT at early time point**; **(F)** *Sab*$^{f/f}$ or *Sab*$^{i\Delta Hep}$ mice received 0.05~5x$10^9$ IU of Ad-lacZ or Ad-*SAB*. 14 days later mice were given APAP 300mg/kg. Liver homogenate collected at 4hr after APAP was immunoblotted using anti-P-JNK and JNK. Below the representative blot, the densitometry of P-JNK at different doses of Ad-*SAB* expression and endogenous level of Sab (*Sab*$^{f/f}$) are depicted from 3 separate experiments. N = 3 mice per group. (∗) = *P* <0.05 versus *Sab*$^{i\Delta Hep}$ + Ad-lacZ, by one way ANOVA. Data are presented as the mean ± SEM. **(G)** Correlation of APAP induced P-JNK level and serum

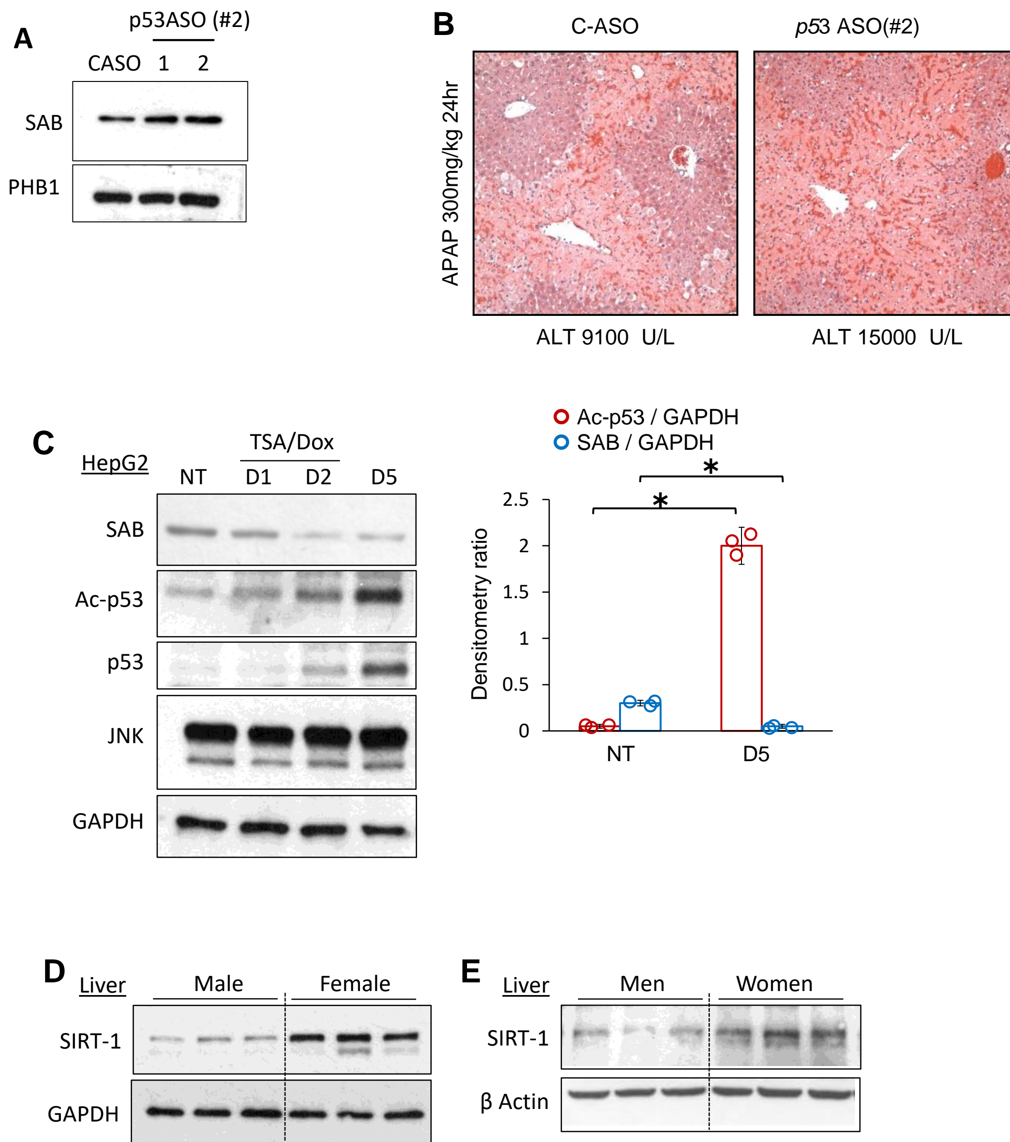
ALT level at 4 hours in various expression level of SAB. Statistical analysis was performed using ANOVA. N = 3 mice per group.

## Supporting Figure S4



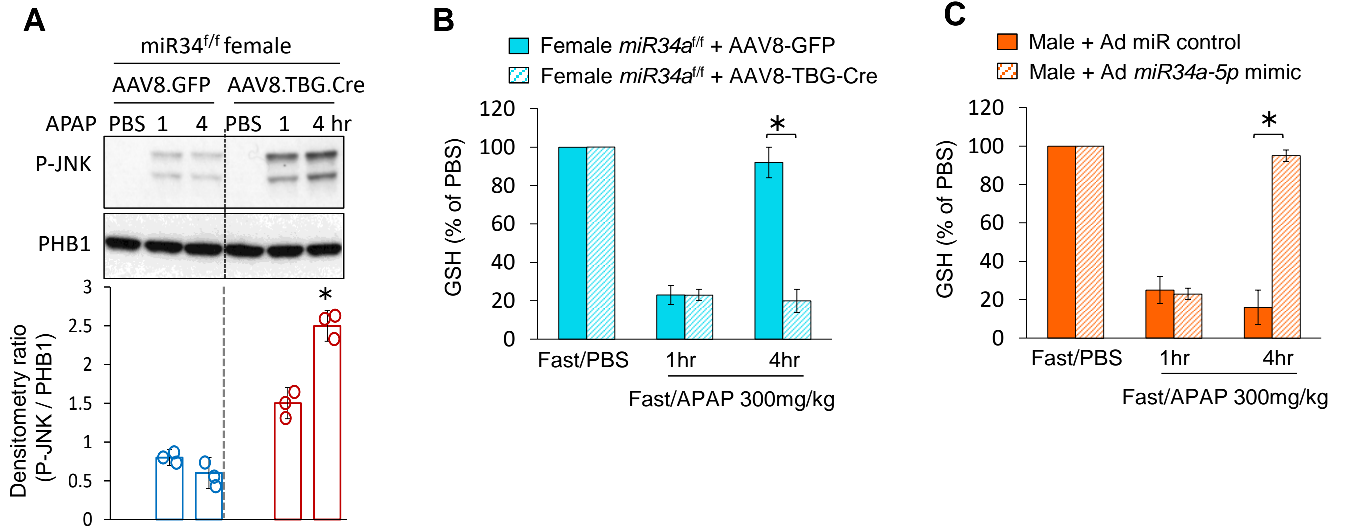
**Supporting Figure S4. ERα level modulates P-JNK activation and cell death. (A)** Mice received ERα/β antagonist fulvestrant 2 or 5 mg/kg in EtOH/Corn Oil subcutaneously (1 dose per week for 3 weeks) and PMH were isolated and treated with APAP 5mM. P-JNK level was determined by immunoblot using whole extracts which were normalized to total JNK. (∗) = $P$ <0.05 versus corresponding vehicle control of 3 separate experiments, by unpaired, 2-tailed Student's $t$ test. Data are presented as the mean ± SEM. **(B)** Mice received ERα agonist PPT 5 mg/kg/dose/day in EtOH/Corn Oil subcutaneously for 5 days per week for 2 weeks. *Sab* mRNA expression after PPT treatment was determined by qPCR. **(C, D)** PMH isolation was performed on next day after PPT treatment and treated with tunicamycin 20μg/ml 24hr or palmitic acid 2mM 16hr. Cell death was determined by SYTOX Green-positive staining (1 μmol/liter; invitrogen). Total number of cells was quantitated by Hoechst 33258 (8 μg/ml; invitrogen). Minimum of total 1000 cells in 10 different fields was counted. (∗) = $P$ <0.05 versus vehicle control of 3 separate experiments, by paired, 2-tailed Student's $t$ test. Data are presented as the mean ± SEM. **(E)** Western blot of time course of P-JNK and total JNK after exposure to palmitic acid (PA) 2mM in PMH from PPT or vehicle treated male mice. (∗) = $P$ <0.05 versus corresponding vehicle control of 3 separate experiments, by unpaired, 2-tailed Student's $t$ test. Data are presented as the mean ± SEM. **(F)** ERβ and androgen receptor agonist effect on SAB expression in PMH. PMH from C57BL/6N male were treated with 10μM of ERβ (DPN) and androgen receptor (BMS) agonist for 48hrs. Medium and reagents were refreshed every 24hr. SAB protein level in PMH from 3 mice was determined by immunoblot.

# Supporting Figure S5



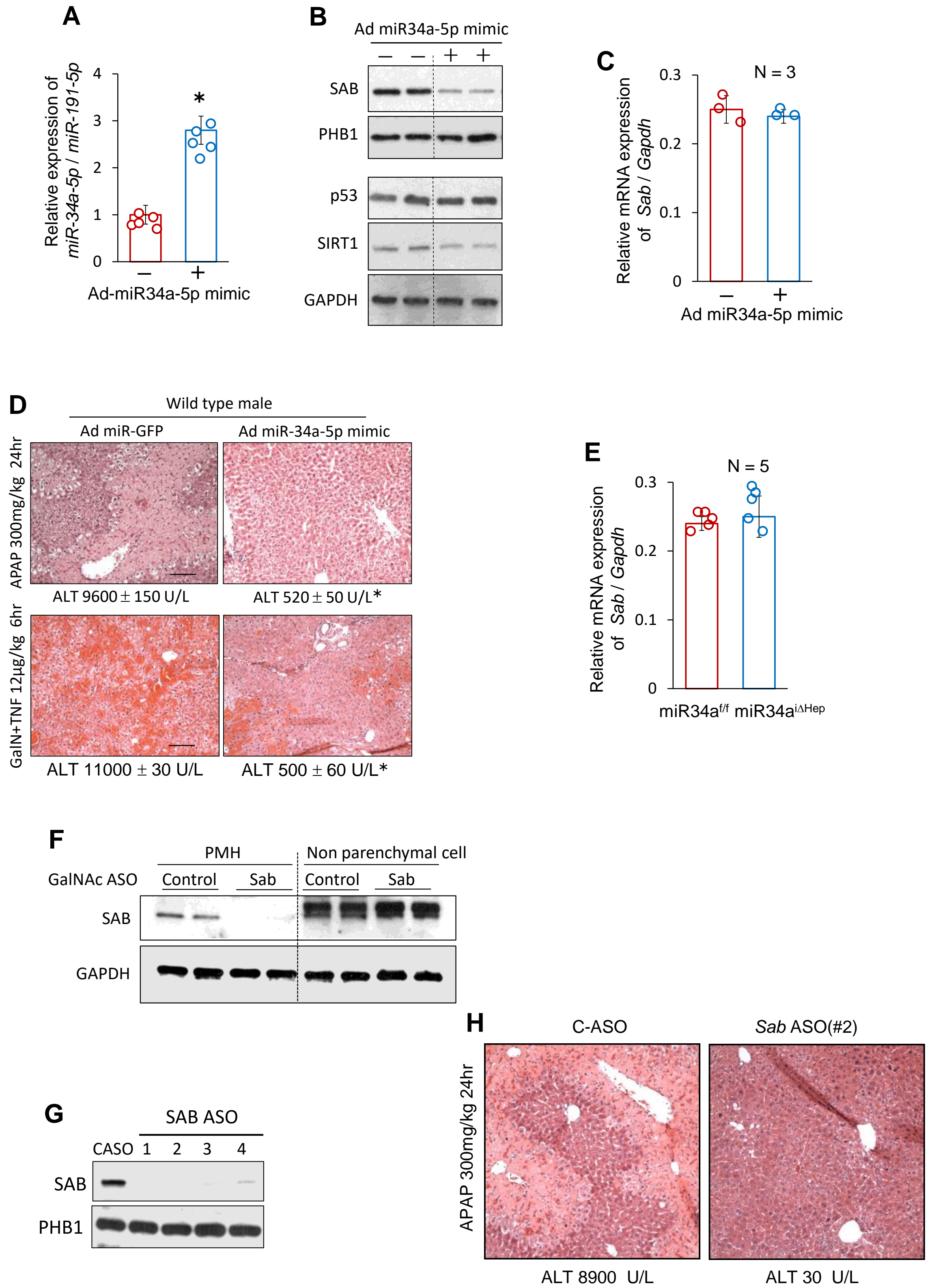
**Supporting Figure S5. P53 modulates SAB expression and hepatotoxicity. (A)** Effect of *p53* knockdown on SAB expression was confirmed by second *p53* ASO. Mice received C-ASO or *p53*-ASO (#2) as described in method. Mitochondria were isolated and SAB expression was determined by Western blot. **(B)** Effect of *p53*-ASO (#2) on susceptibility to APAP induced liver injury. *p53*-ASO (#2) similarly increased liver injury as results with *p53*-ASO (#1) shown in Figure 5. Original magnification: x20. **(C)** Increased expression and activation of p53 decreases SAB. HepG2 cells were treated with minimal toxic dose of combination of reversible deacetylases inhibitor Trichostatin A (TSA) 400nM + Doxorubicin (Dox) 400nM to increase p53 expression and activation. 48hr later cells were rinsed with fresh culture medium to remove dead cells. Cells were cultured in fresh culture medium up to day 5. Medium was replaced every 24hr. Cell lysate were collected and immunoblotted using anti-SAB, acetylated–p53, p53, JNK, P-JNK and GAPDH. Representative immunoblots of 3 separate experiments. Densitometry was determined from 3 separate experiments. NT = no treatment; D1, D2 or D5 = day1, day2, or day5. (∗) = $P < 0.05$ day 5 versus 1, by un-paired, 2-tailed Student's *t* test. Data are presented as the mean ± SEM. **(D, E)** SIRT1 expression of male versus female liver in mice and human. Immunoblots of SIRT1 in liver protein extract of 10-12 weeks C57BL/6N male and female littermates and of normal human liver. N = 3 per group.

## Supporting Figure S6



**Supporting Figure S6. (A) Effect of miR34a depletion on P-JNK activation;** miR34a was depleted in hepatocytes by AAV8-TBG-Cre injection. Two weeks after receiving AAV8-GFP or AAV8-TBG-Cre, mice were given APAP 300mg/kg and P-JNK was determined by immunoblot in mitochondrial lysates 1-4 hours after APAP. Representative immunoblots of 3 separate experiments. N = 3 mice per group. (∗) = $p < 0.05$ versus vehicle or AAV8.GFP, by un-paired, 2-tailed Student's *t* test**.** Data are presented as the mean ± SEM. **(B, C) Effect of miR34a expression on GSH recovery after APAP**; miR34a was depleted by AAV8-TBG-Cre injection to miR34a$^{f/f}$ female mice. miR34a-5p was overexpressed in liver by Ad-miR34a-5p mimic injection to C57BL/6N male mice. Mice were fasted overnight and PBS or APAP 300mg/kg i.p was given. 1 and 4 hr later liver samples were snap frozen and GSH level was determined by recycling assay. N = 3 mice per group. (∗) = $p < 0.05$ versus control, by un-paired, 2-tailed Student's *t* test. Data are presented as the mean ± SEM.

## Supporting Figure S7



**Supporting Figure S7. miRNA-34a-5p modulates SAB expression and hepatotoxicity. (A, B, C)** C57BL/6N male mice received Ad-miR-GFP or Ad-*miR34a-5p*-mimic through tail vein injection. 2 weeks later, *miR34a-5p* expression, SAB and PHB1 expression in mitochondria, p53, SIRT1 and GAPDH in liver extract were determined. *Sab* mRNA level was quantitated by qPCR. N=5 mice in each group. (∗) = $p < 0.05$ versus control, by un-paired, 2-tailed Student's *t* test. Data are presented as the mean ± SEM. **(D)** Overnight fasted mice received APAP or GalN/TNF and liver histology and serum ALT were examined at 24hr or 6hr respectively**.** N=5 mice in each group. (∗) = $p < 0.05$ versus Ad-miR-GFP, by un-paired, 2-tailed Student's *t* test. Data are presented as the mean ± SD. Original magnification: x20. Scale bars: 100μm. **(E)** Level of *Sab* mRNA of miR34a$^{f/f}$ versus miR34a$^{i\Delta Hep}$. **(F)** Selective depletion of SAB in PMH, but not in non-parenchymal cells from liver of GalNAc-*Sab* ASO versus GalNAc-scrambled control ASO treated mice. **(G)** Selective depletion of SAB by different *Sab*-ASO (#1 - #4) targeting different sequences of *Sab* mRNA. **(H)** Depletion of SAB by *Sab*-ASO (#2) protects against APAP induced liver injury similar to *Sab*-ASO (#1) shown previously. *Sab*-ASO (#1) is the parent ASO of GalNAc-*Sab*-ASO. Original magnification: x20.