Original Research Communication

Title: G protein β_5-ATM complexes drive acetaminophen-induced hepatotoxicity

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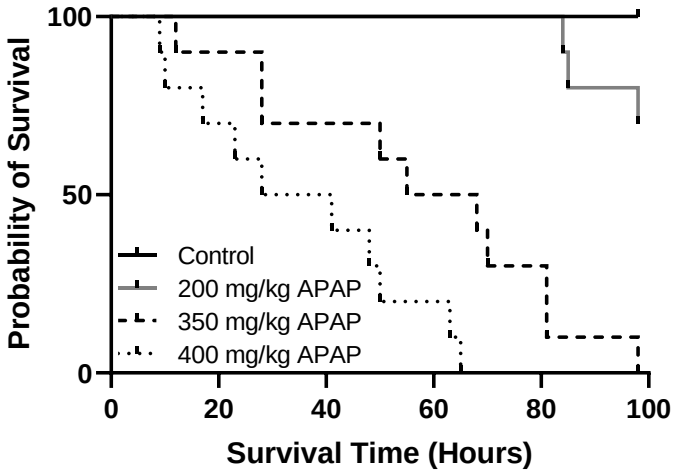


Fig. S1. Acute APAP dose response. Wild-type (WT) mice (n=10) were given a single injection of APAP (200, 350 or 400 mg/kg, i.p.) or vehicle (control). Kaplan-Meier survival curves are depicted tracking animal survival up to 96 hours post-drug administration.

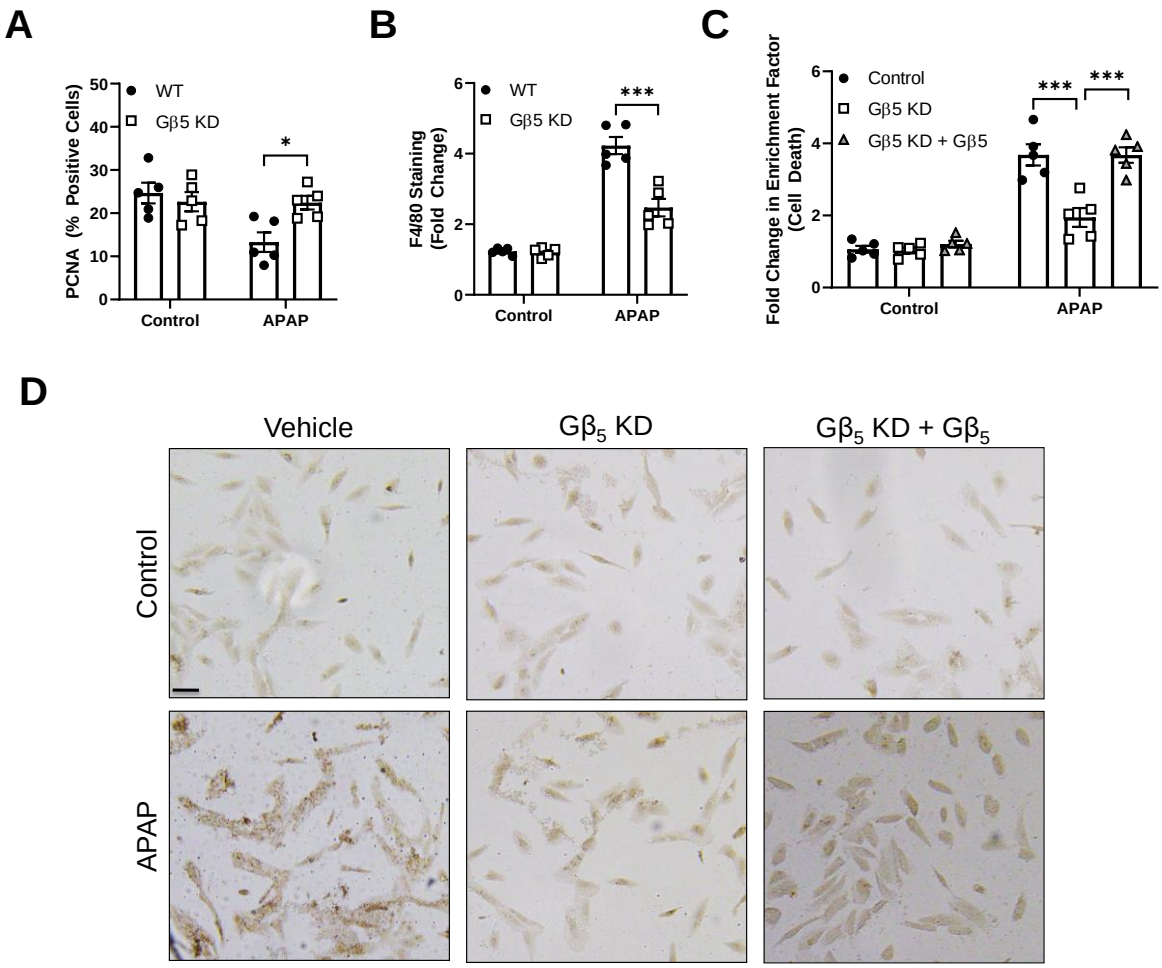


Fig. S2. $G\beta_5$ promotes inflammation and cell death and suppresses proliferation in APAP-exposed murine hepatocytes. Primary hepatocytes were isolated from WT or $G\beta_5$ KD mice and exposed to APAP in culture (5 mM). For a subset of experiments, $G\beta_5$ expression was restored via transfection. (A) Hepatocyte proliferation as determined by % total cells positive for PCNA staining (n=5). (B) Inflammation as determined by fold change in F4/80 staining (n=5). (C) Cell death (fold increase in cytoplasmic histone-associated DNA fragments) (n=5). (D) TUNEL staining (quantified in Fig. 4J) [scale bar = 100μm]. *P<0.05, ***P<0.001 via two-way ANOVA with Sidak's post-hoc test. Data are presented as mean ± SEM.

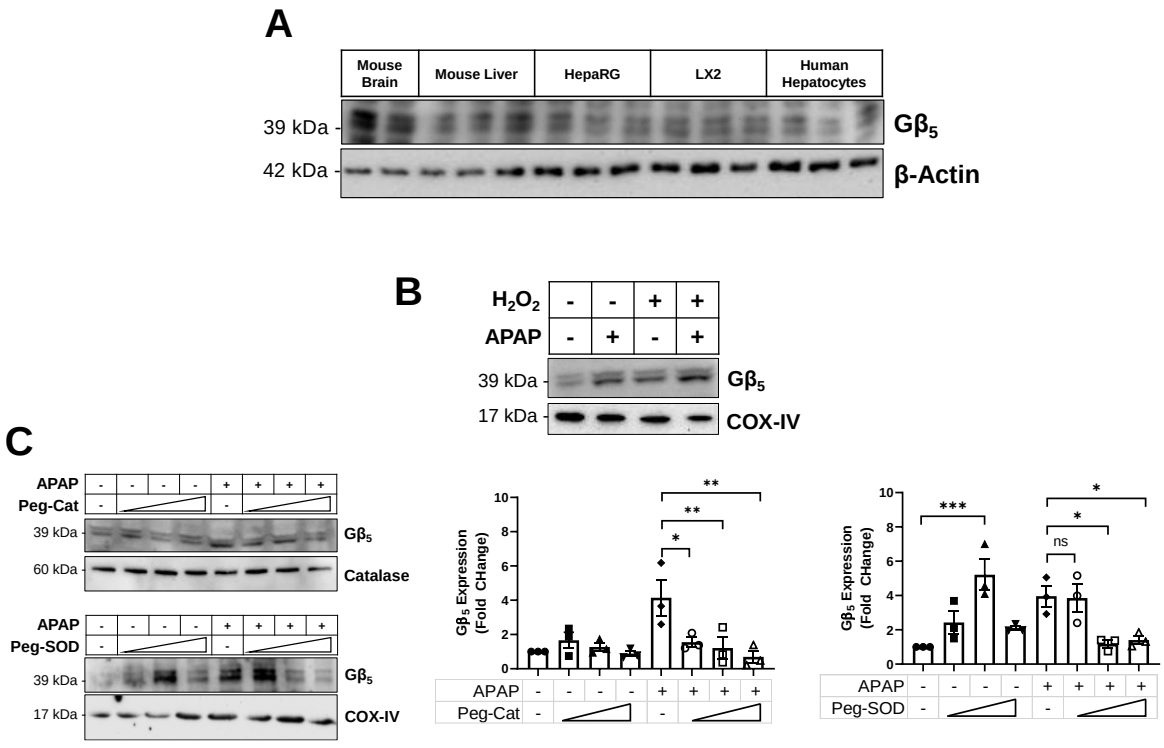


Fig. S3. ROS accumulation drives Gβ$_5$ up-regulation in HepaRG cells. (A) Gβ$_5$ expression in mouse brain and liver tissue, HepaRG cells, Lx2 cells and human hepatocytes. HepaRG cells were treated with APAP (5 mM) or hydrogen peroxide (H_2O_2; 200 mM) for 24 hours ± 1 hour pre-treatment with Peg-SOD (1000 U/mL) or Peg-Cat (200 U/mL). (A) Gβ$_5$ expression following ROS induction. (B) Gβ$_5$ expression following H_2O_2 or superoxide scavenging (n=3). Catalase or COX-IV serve as loading controls. **P*<0.05,***P*<0.01, ****P*<0.001 via two-way ANOVA with Sidak's post-hoc test, respectively. Data are presented as mean ± SEM.

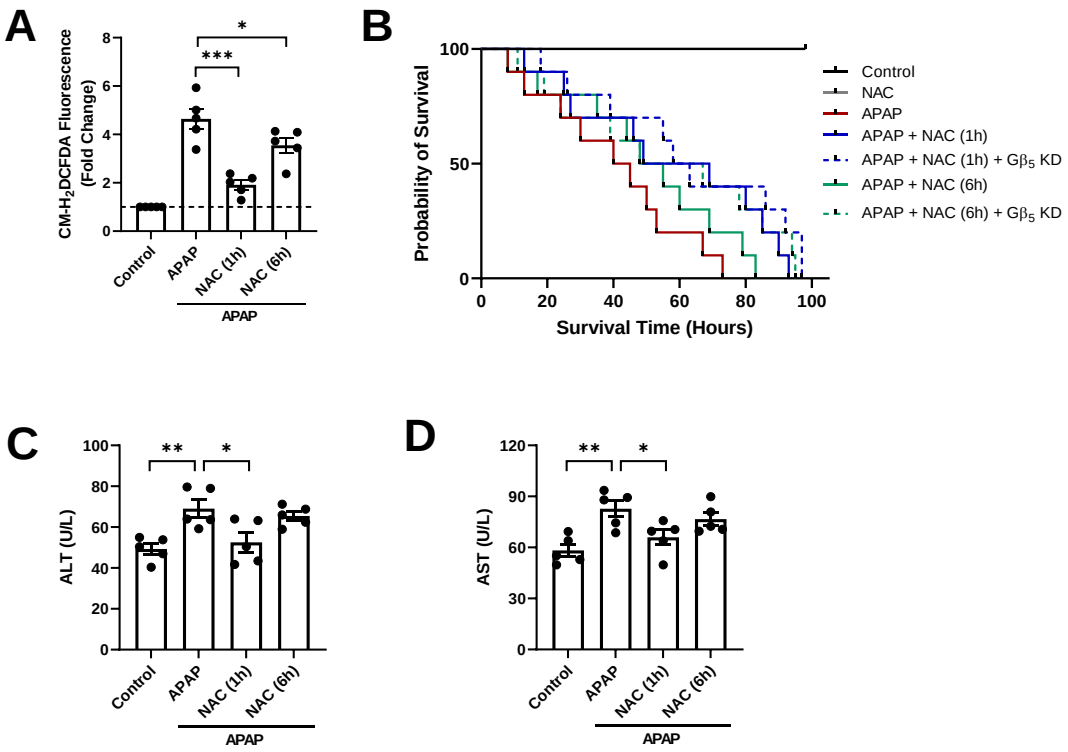


Fig. S4. NAC efficacy in treating APAP-induced hepatotoxicity is limited to a narrow window immediately after treatment. WT mice were administered vehicle (control) or APAP (350 mg/kg, i.p.) for 24 hours with or without NAC treatment (100 mg/kg, i.p.) at 1 or 6 hours post-APAP administration. (A) CM-H_2DCFDA fluorescence (total ROS, n=5). (B) Kaplan-Meier survival curve (n=10). Hepatic (F) ALT and (G) AST (n=5). **P*<0.05, ***P*<0.01, ****P*<0.001 via one-way ANOVA with Dunnett's post-hoc test. Data are presented as mean ± SEM.

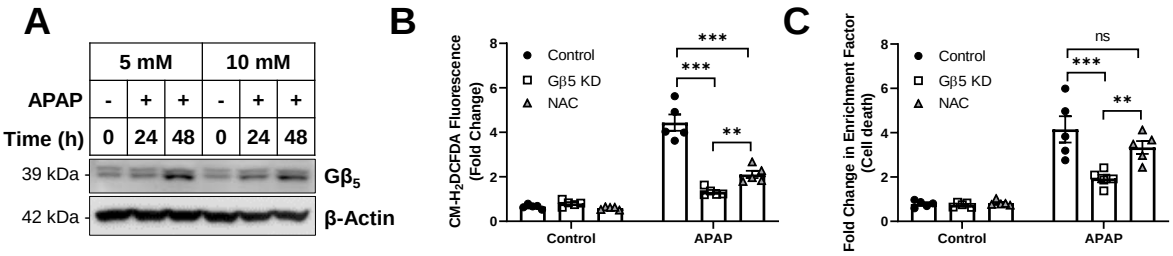
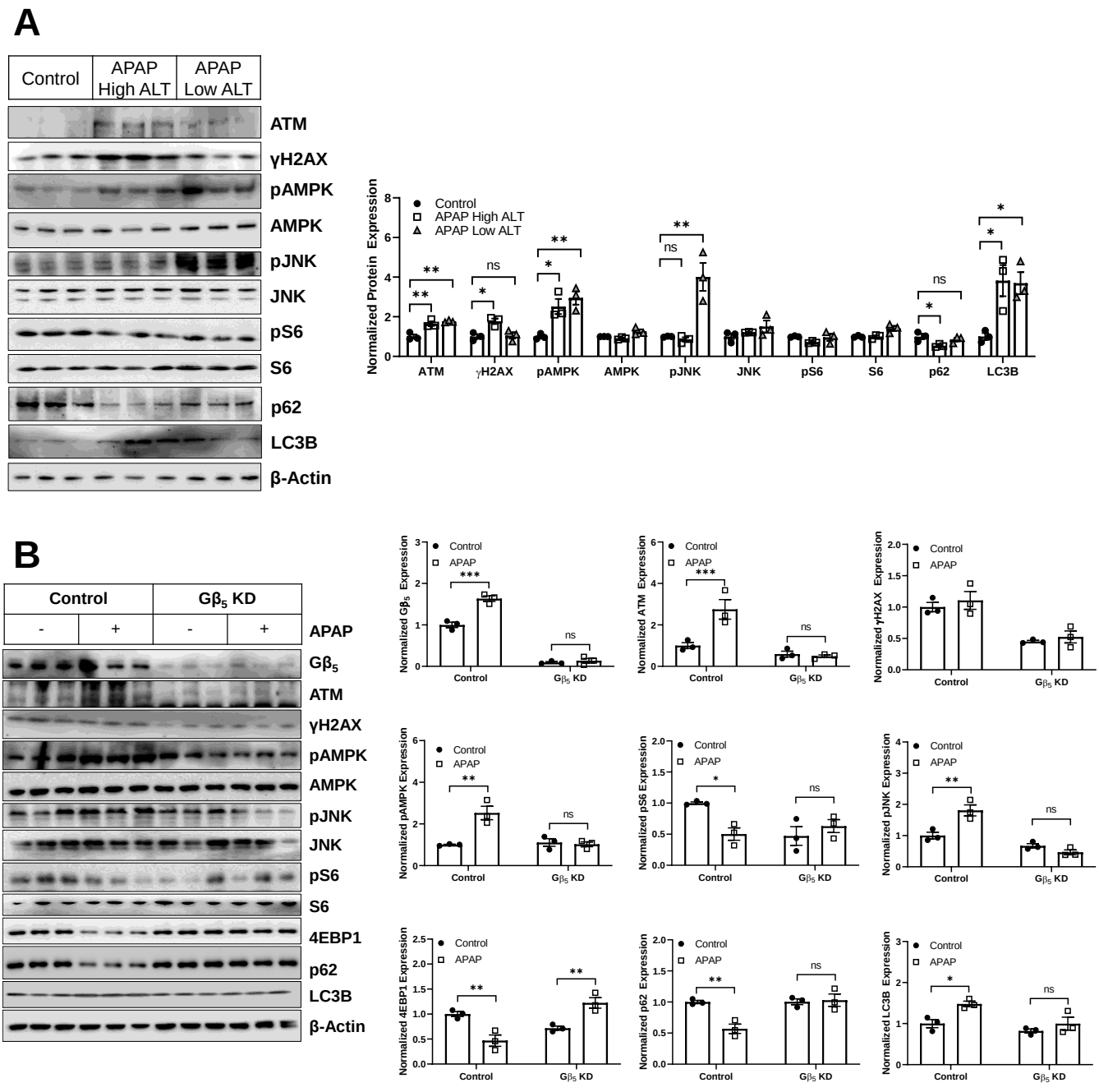


Fig. S5. Gβ$_5$ knockdown shows an improved capacity to diminish APAP-induced ROS and cell death over NAC. HepaRG cells expressing control or Gβ$_5$ shRNA were treated with APAP (5 mM or 10 mM) for 24 hours (or 48 where noted) ± NAC (5 mM). (A) Gβ$_5$ protein expression. (B) CM-H$_2$DCFDA fluorescence (total ROS, n=5). (C) Cell death (fold increase in cytoplasmic histone-associated DNA fragments) (n=5). β-Actin serves as a loading control for immunoblots. ns = not significant. ***P*<0.01, ****P*<0.001 via two-way ANOVA with Sidak's post-hoc test. Data are presented as mean ± SEM.


A
Control
APAP High ALT
APAP Low ALT
ATM
γH2AX
pAMPK
AMPK
pJNK
JNK
pS6
S6
p62
LC3B
β-Actin
Normalized Protein Expression
Control
APAP High ALT
APAP Low ALT
ATM
γH2AX
pAMPK
AMPK
pJNK
JNK
pS6
S6
p62
LC3B
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Control
Gβ5 KD
APAP
Gβ5
ATM
γH2AX
pAMPK
AMPK
pJNK
JNK
pS6
S6
4EBP1
p62
LC3B
β-Actin
Normalized Gβ5 Expression
Normalized ATM Expression
Normalized γH2AX Expression
Normalized pAMPK Expression
Normalized pS6 Expression
Normalized pJNK Expression
Normalized 4EBP1 Expression
Normalized p62 Expression
Normalized LC3B Expression

Fig. S6. $G\beta_5$ drives alterations in the ATM/AMPK/mTOR pathway and autophagy markers in primary human hepatocytes exposed to APAP. (A) Liver tissue samples from APAP-induced liver injury patients were stratified based on injury severity and probed for expression of indicated proteins (n=3). (B) Primary human hepatocytes were transfected with control or $G\beta_5$ shRNA. Cells were then treated APAP (5 mM) for 24 hours. Immunoblots were probed for expression of $G\beta_5$, ATM and its effectors, and markers of autophagy and quantified (n=3). β-Actin serves as a loading control for all immunoblots. ns = not significant. **P*<0.05, ***P*<0.01, ****P*<0.001 via one-way or two-way ANOVA with Dunnett's or Sidak's post-hoc test, respectively. Data are presented as mean ± SEM.

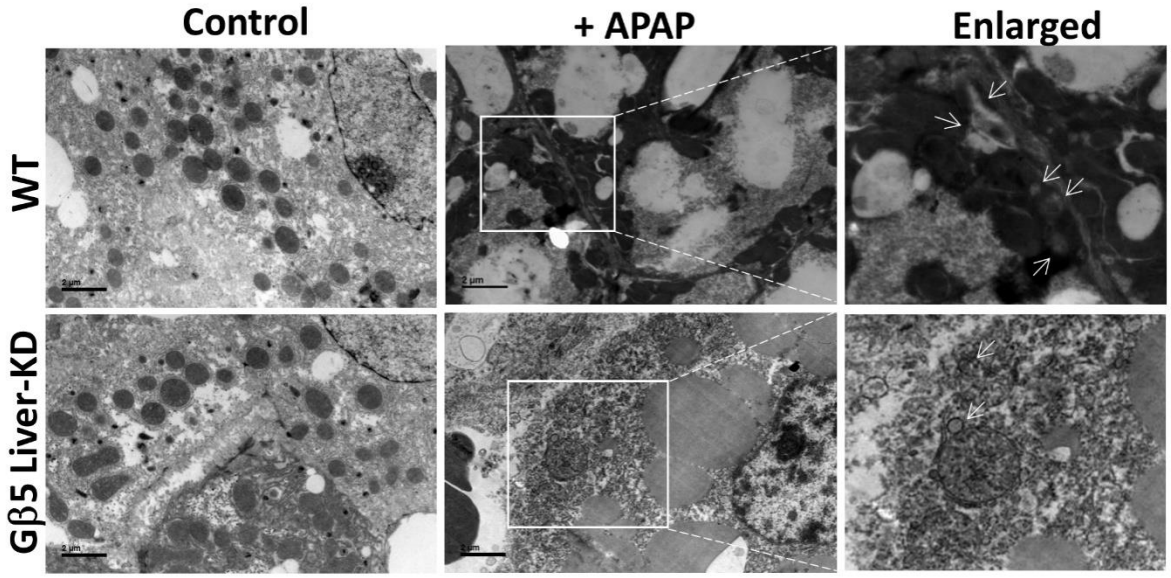

Fig. S7. $G\beta_5$ knockdown impacts autophagic flux in APAP-treated liver. Control and $G\beta_5$ KD mice were given a single APAP dose (350 mg/kg, i.p.) and liver tissue processed after 48 hours using transmission electron microscopy (TEM). Control samples (WT and $G\beta_5$ KD) showed typically dispersed mitochondria and intact nuclei. In APAP treated WT liver, the formation of large vacuoles compressed cellular organelles resulting in the formation of autophagic vacuoles (autophagosomes) [circular bulging structures around the membrane marked with white arrows]. Vacuole formation was lessened in $G\beta_5$ KD samples.

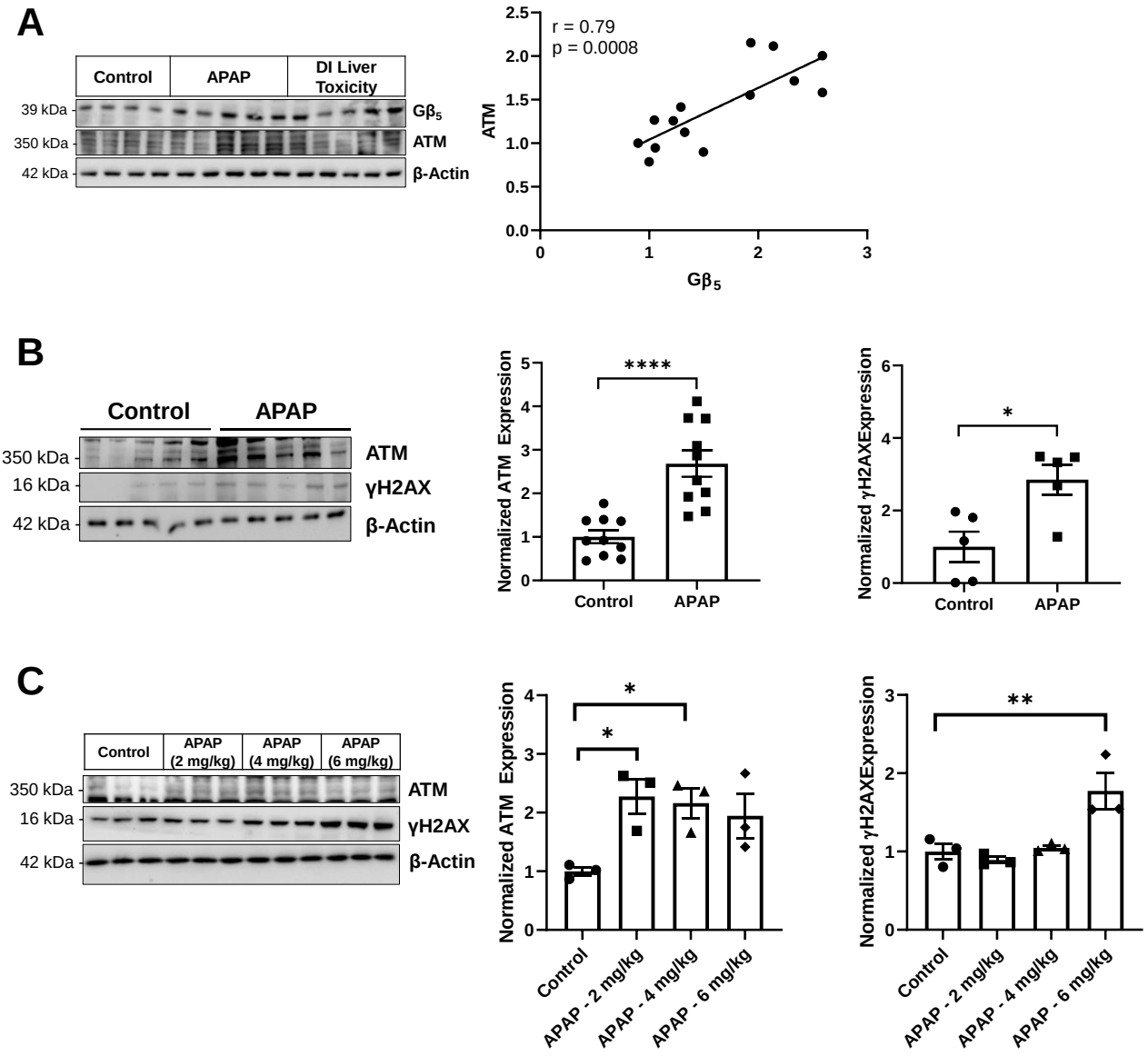

Fig. S8. ATM and Gβ$_5$ protein expression are highly correlated in liver. (A)Immunoblotting was performed for Gβ$_5$ and ATM in livers from APAP-induced liver injury and DILI. Pearson's correlation coefficient between Gβ$_5$ and ATM is displayed with corresponding *P*-value. (B)ATM and γH2AX protein expression following (B) acute (350 mg/kg, i.p., 48 hours) or (C) chronic (2-6 mg/kg, i.p., 6 weeks) APAP administration. β-Actin serves as a loading control for all immunoblots.**P*<0.05, ***P*<0.01, *****P*<0.0001 via one-way ANOVA with Sidak's post-hoc test. Data are presented as mean ± SEM.

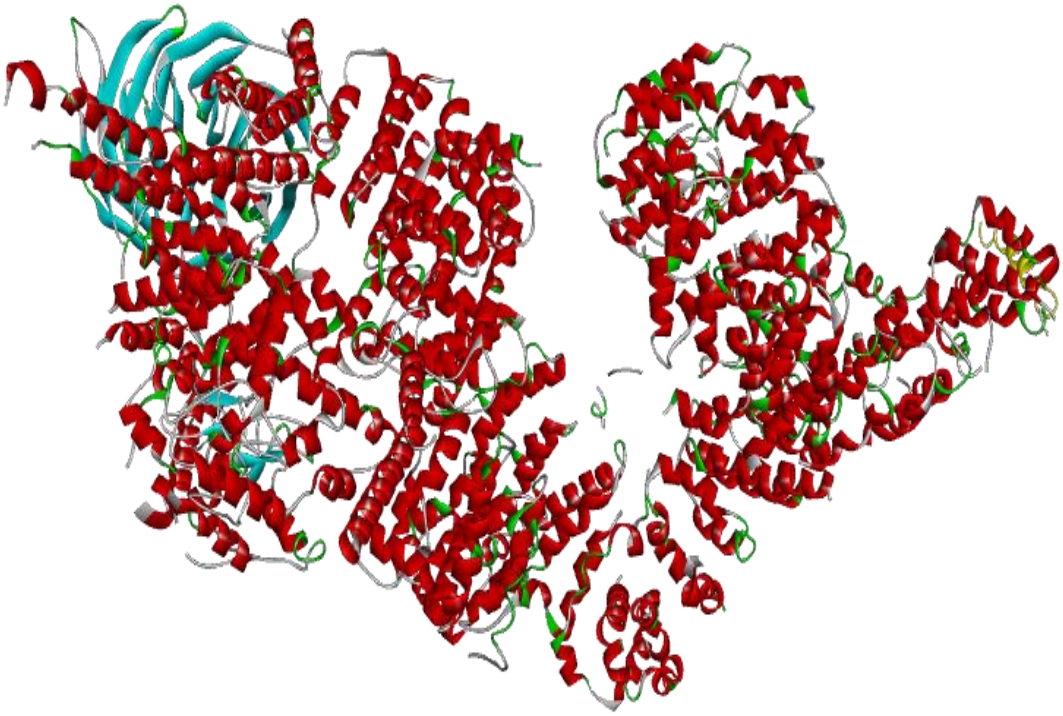

Fig. S9. Computational Modeling of a putative ATM-Gβ_5 complex. The model was generated based on previously published crystal structures for human ATM (PDB ID: 5NP1; Red) and mouse Gβ_5 (PDB ID: 2PBI; turquoise).

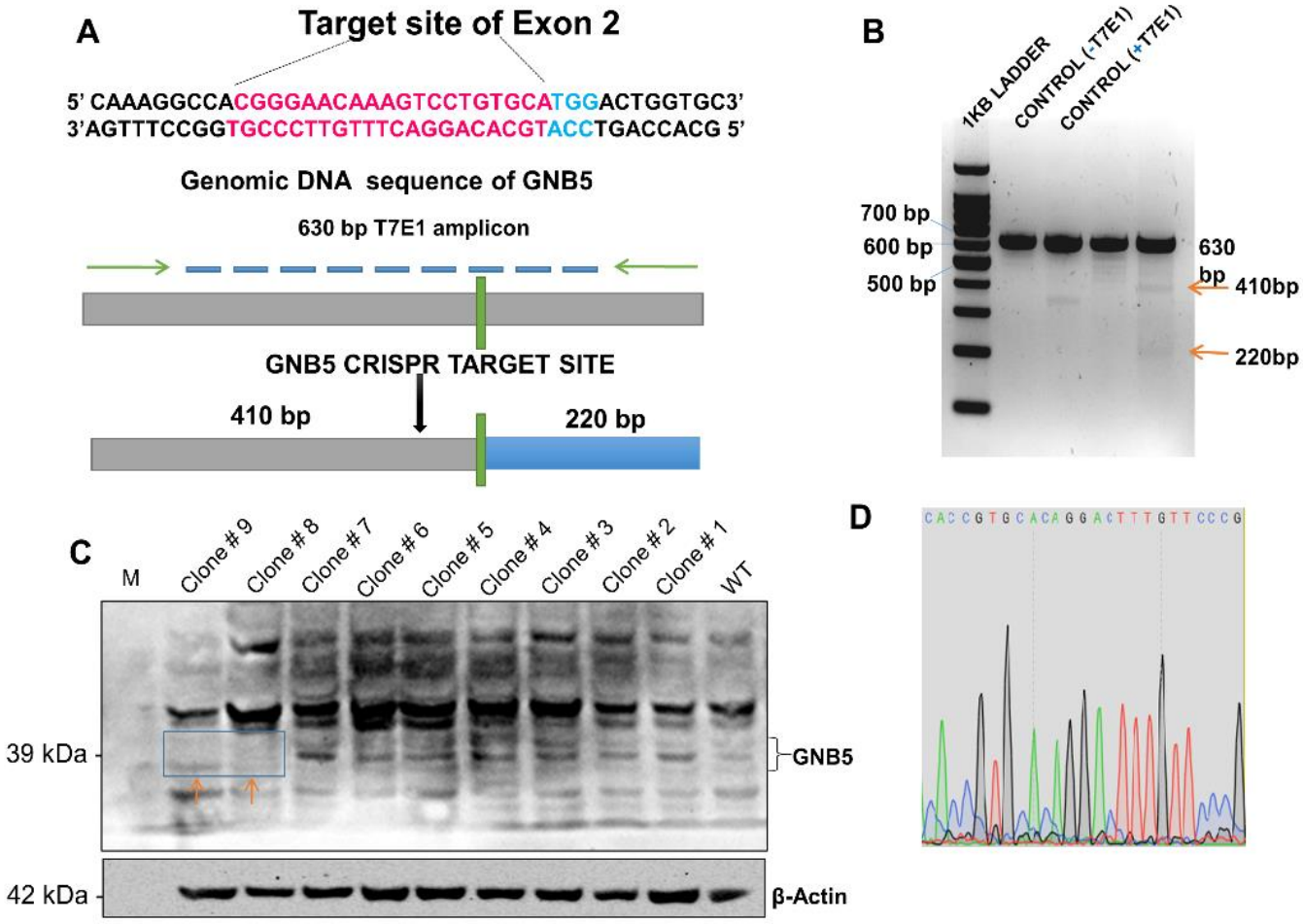

Fig. S10. Validation of Gβ$_5$ knockout in HepaRG cells via CRISPR/Cas9-dependent genomic excision. (A) Design of excision site in the *GNB5* gene overlaid with predicted products from T7E1 validation assay. (B) Results from T7E1 assay depicting expected 410 and 220 base pair (bp) products. (C) Validation of CRISPR/Cas9 clones 1-9 demonstrating successful gene knockout in clones 8 and 9. (D) Sequencing chromatogram of Gβ5 oligo which has been inserted in PX459 CRISPR plasmid.

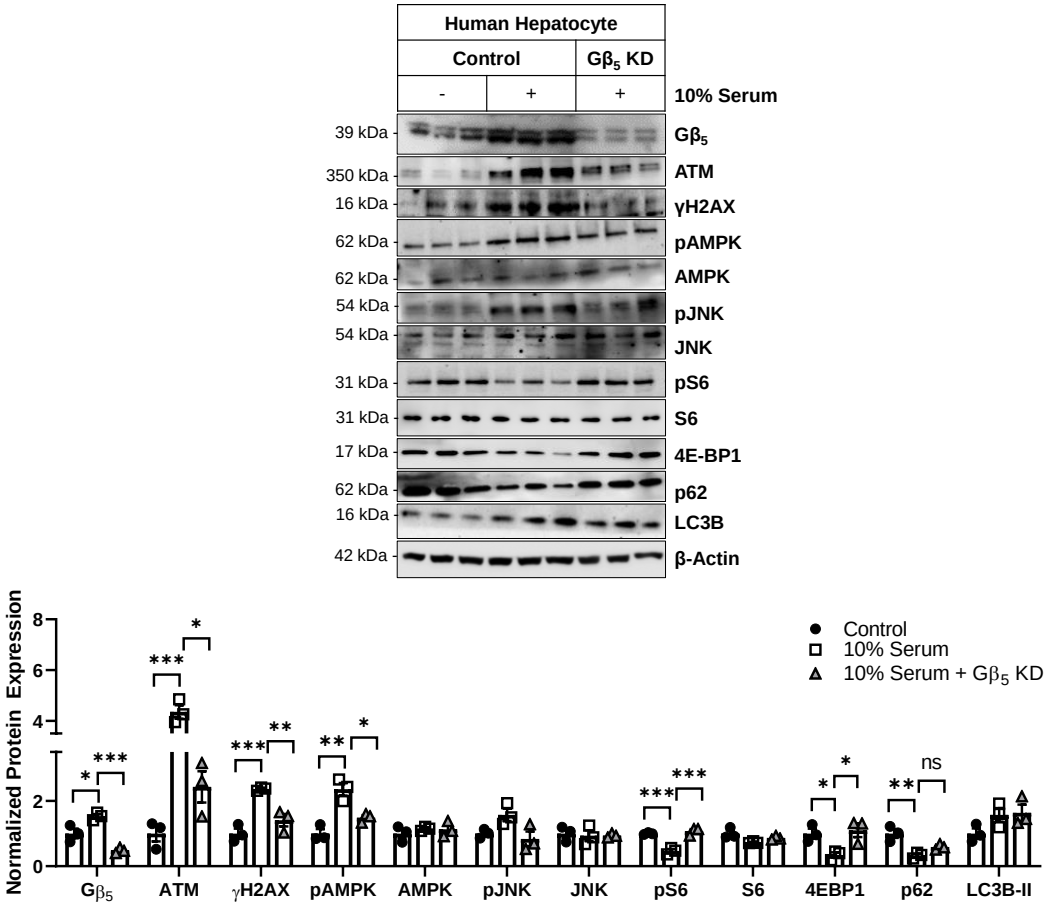

Fig. S11. Gβ$_5$ drives alterations in the ATM/AMPK/mTOR pathway and autophagy markers in primary human hepatocytes exposed to serum from APAP-induced liver injury patients. Cells were transfected with control or Gβ$_5$ shRNA. Cells were then treated with 10% serum isolated from human patients diagnosed with APAP hepatotoxicity for 24 hours. Immunoblots were probed for expression of Gβ$_5$, ATM and its effectors, and markers of autophagy and quantified (n=3). β-Actin serves as a loading control for all immunoblots. ns = not significant. *P<0.05, **P<0.01, ***P<0.001 via one-way ANOVA with Dunnett's post-hoc test. Data are presented as mean ± SEM.

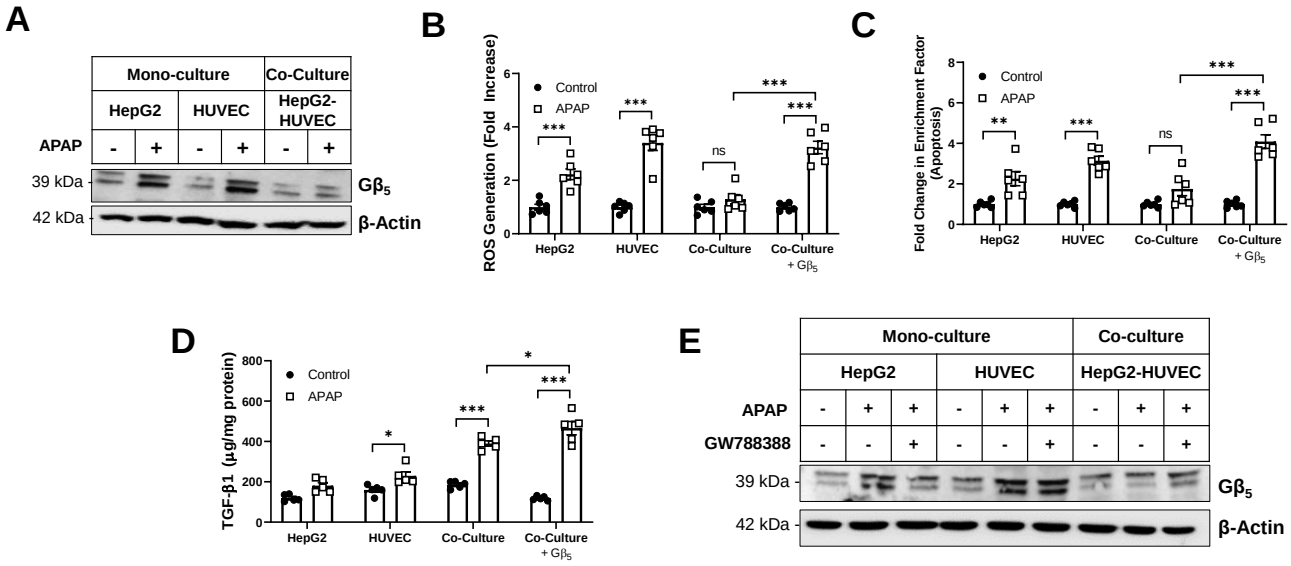

Fig. S12. Gβ$_5$ expression determines susceptibility of hepatocyte-endothelial cell co-cultures to APAP toxicity. HepG2 hepatocyte or HUVEC endothelial cell mono-cultures or co-cultures were treated with APAP (5 mM, 24 hours) after transfection with Gβ$_5$-expressing constructs. (A) Gβ$_5$ protein expression. (B) CM-H$_2$DCFDA fluorescence (total ROS, n=5). (C) Cell death (fold increase in histone-associated DNA fragments; n=5). (D) TGF-β1 content. (E) Gβ$_5$ protein expression ± TGF-β receptor 1 (TGF-βR1) inhibitor GW788388 (5 μM) pre-treatment (1 hour).β-Actin serves as a loading control for all immunoblots. ns = not significant. **P*<0.05, ***P*<0.01, ****P*<0.001 via two-way ANOVA with Sidak's post-hoc test. Data are presented as mean ± SEM.

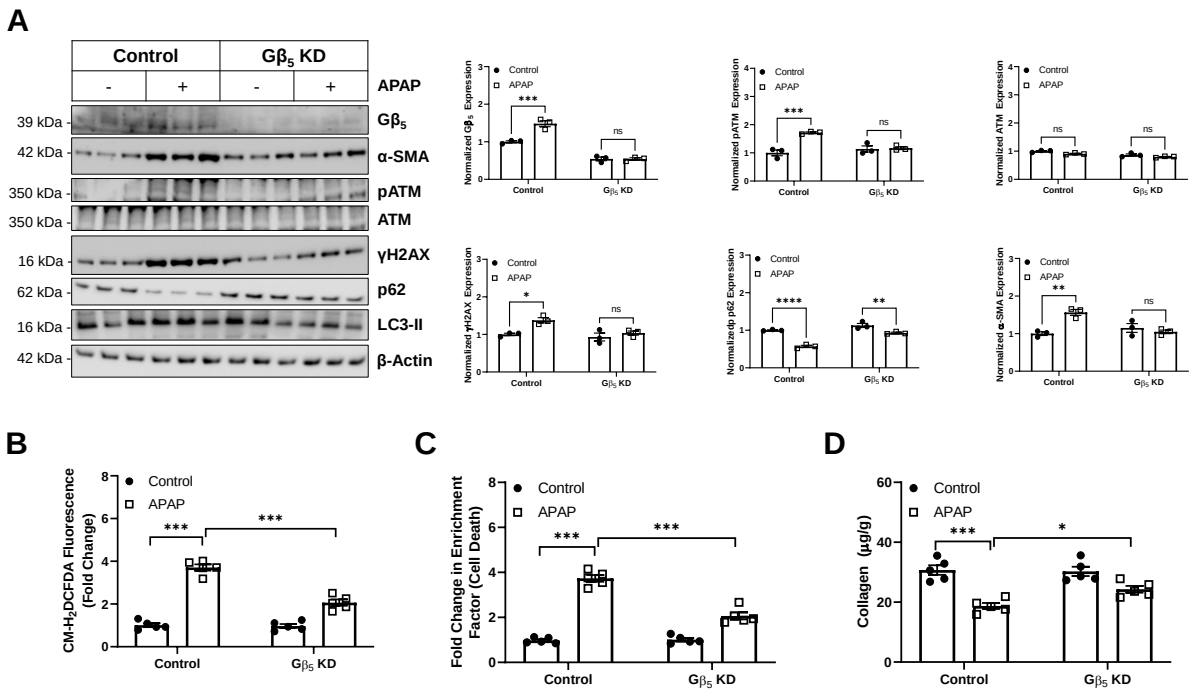

Fig. S13. Gβ$_5$ knockdown improves APAP-dependent pathological endpoints in hepatic stellate cells. LX2 cells expressing control or Gβ$_5$ shRNA were treated with APAP (5 mM) for 24 hours. (A) Immunoblots for Gβ$_5$, pATM, ATM, γH2AX, α-SMA, p62, and LC3-II were quantified (n=3). (B) CM-H$_2$DCFDA fluorescence (total ROS, n=5). (C) Cell death (fold increase in cytoplasmic histone-associated DNA fragments) (n=5). (D) Collagen content (n=5). β-Actin serves as a loading control for immunoblots. ns = not significant. *P<0.05, **P<0.01, ***P<0.001 via two-way ANOVA with Sidak's post-hoc test. Data are presented as mean ± SEM.

Supplemental Table 1: Reagent List

Company	Location	Reagent
Sigma Chemical	St. Louis, MO, USA	Acetaminophen Ru360 Cyclosporin A CM-H2DCFDA Oil Red O Leupeptin Torin 1 MG132 Polyethylene glycol-superoxide dismutase- (Peg-SOD) Polyethylene glycol-catalase (Peg-Cat) Collagenase IV Type I collagen MHY1485
Addgene	Watertown, MA, USA	pLenti CMV Puro DEST cloning vector pMD2.G VSV-G envelope expressing plasmid psPAX2 PX459 CRISPR system plasmid
Tocris Biosciences	Bristol, UK	GW788388
Abcam	Cambridge, UK	Masson Trichrome Stain N-acetyl cysteine (NAC) KU-55933 Acridine Orange Stain Protein A/G sepharose Sirius Red
Thermo Fisher Scientific	Waltham, MA, USA	MitoSox Phusion Hot Start II High-Fidelity PCR Master Mix (F-565S)
Invitrogen	Carlsbad, CA, USA	Platinum Taq DNA Polymerase High Fidelity
Takara Bio	Kyoto, Japan	pMD20-T vector
Sisco Research Laboratory	Mumbai, India	Hematoxylin Eosin

Supplemental Table 2: Antibody List

Company	Location	Antibody	Catalog #	Dilution
Santacruz Biotech	Dallas, TX, USA	αTubulin	SC58667	WB (1:1000)
		Cytochrome C	SC13156	WB (1:800)
		β-Actin	SC47778	WB (1:1000)
Abcam	Cambridge, UK	PCNA	ab29	WB (1:1000), IHC (1:200)
		F4/80	ab6640	WB (1:1000), IHC (1:200)
		ATM	ab78	WB (1:800)
		p-ATM	CST45265	WB (1:1000)
		γH2AX	ab124781	WB (1:1000)
		γH2AX	ab26350	WB (1:1000)
		4EBP1	ab2606	WB (1:1000)
		Lamp-1	ab24170	WB (1:1000)
		LC3-II	ab48394	WB (1:1000)
		COX-IV	ab202554	WB (1:1000)
		Mouse Secondary-HRP	ab97023	WB (1:2000), IHC (1:500)
		Rabbit Secondary-HRP	ab97051	WB (1:2000), IHC (1:500)
Cell Signaling Technology	Danvers, MA, USA	SQSTM1/p62	ab56416	WB (1:1000)
		Ubiquitin	ab179434	WB (1:1000)
		SAPK/JNK	9252S	WB (1:1000)
		p-SAPK/JNK	9255S	WB (1:1000)
		Catalase	129805	WB (1:1000)
		β-Actin	4970	WB (1:1000)
		α-SMA	19245S	WB (1:1000)
		AMPK	2532S	WB (1:1000)
		pAMPK	2531S	WB (1:750)
		pS6	2211S	WB (1:1000)
		S6	2217S	WB (1:1000)
		4EBP1	9644	WB (1:1000)
Millipore	Burlington, MA, USA	Gβ5	ABS1062	WB (1:800), IHC (1:200)
Thermo Fisher Scientific	Waltham, MA, USA	ATM	MA123152	WB (1:1000)

Supplemental Table 3: Assay Kit List

Company	Location	Assay	Catalog #
Abcam	Cambridge, UK	Ca^{2+} Flux Assay Kit	ab102505
		Albumin Assay Kit	ab235628
		Collagen Assay Kit	ab222942
		Hydroxyproline Assay Kit	ab222941
		Mitochondrial isolation Kit	ab110170
		Terminal deoxynucleotidyltransferase dUTP Nick-End Labelling (TUNEL) kit	ab206386
Promega	Madison, WI, USA	Mitochondrial ATP: ToxGlo™	G8000
Thermo Fisher Scientific	Waltham, MA, USA	TGF-β1 ELISA	88-8350-22
Roche	San Francisco, CA, USA	Cell Death Detection Kit	C755B93
Erba Mannheim	London, UK	ALT	120207
		AST	120204
		Triglycerides	120211

Supplemental Table 4: Cell Line List

Company	Location	Cell Line	Catalog #	Culture Conditions
Merck & Co.	Kenilworth, NJ, USA	HepaRG	MMHPR116	37°C incubator at 5% CO_2 in William's E Medium with GlutaMAX™ Supplement (Thermo Fisher Scientific) with 10% FBS (Gibco)
Himedia Laboratories	Mumbai, Maharashtra, India	HUVEC		37°C incubator at 5% CO_2 in DMEM + 10% FBS
Gift from Dr. Jiaur Rahman Gayen, CDRI, Lucknow, India		HepG2		37°C incubator at 5% CO_2 in DMEM + 10% FBS
Gift from Dr. Suvro Chatterjee, Vascular Biology Lab, Life Sciences Division, AU-KBC Research Centre, Anna University, Chennai 600044, Tamilnadu, India		Lx2		37°C incubator at 5% CO_2 in DMEM + 3% FBS

Supplemental Table 5: Primer List

#	Names of Primer	Sequence of Primer
1	GNB5 Human full length Forward	5'- ATACTCGAGATGGCAACCGAGGGGCT -3'
2	GNB5 Human full length Reverse	5'-ATAAAGCTTGGCCCAGACTCTGAGGGTAT-3'

3	GNB5 Human WD40-deletion Forward	5'- ATACTCGAGATGGCAACCGAGGGGCT -3'
4	GNB5 Human WD40-deletion Reverse	5’-ATAAAGCTTGGTCTTCATGACAAACTGC-3’
5	GNB5 Human 7WD40-deletion Forward	5'- ATACTCGAGATGGCAACCGAGGGGCT -3'
6	GNB5 Human 7WD40-deletion Reverse	5’-ATAAAGCTTGGCCCAGACTCTGAGGGTAT-3’
7	GNB5 Human 1-52aa. deletion Forward	5'- ATACTCGAGATGAAGACCAGAAGGACCCTCA -3'
8	GNB5 Human 1-52aa. deletion Reverse	5’-ATAAAGCTTGGCCCAGACTCTGAGGGTAT-3’
9	GNB5 Human 56-200 aa. deletion Forward	5'- ATACTCGAGATGGCAACCGAGGGGCT -3'
10	GNB5 Human 56-200 aa. deletion Reverse	5’-ATAAAGCTTGGCCCAGACTCTGAGGGTAT-3’
11	GNB5 Human 103-300 aa. deletion Forward	5'- ATACTCGAGATGGCAACCGAGGGGCT -3'
12	GNB5 Human 103-300 aa. deletion Reverse	5’-ATAAAGCTTGGCCCAGACTCTGAGGGTAT-3’
13	GNB5 Mouse full length Forward	5’-TATGCTAGCATGTGCGAT CAGACC TTCCT-3’
14	GNB5 Mouse full length Reverse	5’-TATAGATCTTGCCCA AACTCTTAGGGTGT3’
15	GNB5 Sense Strand (ex2) CRISPR	5’- CACCGTGCACAGGACTTTGTTCCCG-3’
16	GNB5 Antisense Strand (ex2) CRISPR	5’- AAACCGGGAACAAAGTCCTGTGCAC-3’
17	T7E1 Assay Forward	5’- AAAGGGAATAATTTTATGTAGT-3’
18	T7E1 Assay Reverse	5’- CCCAAATTATGAAGTTCTATAA-3’
19	GNB5 Human Q50H point mutation overlapping Forward	5'- AGGCCCTGGGGCACTTTGTCAT -3'
20	GNB5 Human Q50H point mutation overlapping Reverse	5’- TCTTCATGACAAAGTGCCCCAG -3’
21	GNB5 Human K54T point mutation overlapping Forward	5'- CAGTTTGTCATGACGACCAGA -3'
22	GNB5 Human K54T point mutation overlapping Reverse	5’- GGTCCTTCTGGTCGTCATGAC -3’
23	GNB5 Human R56G point mutation overlapping Forward	5'- TGTCATGAAGACCGGAAGGAC -3'
24	GNB5 Human R56G point mutation overlapping	5’- TTGAGGGTCCTTCCGGTCTTC -3’

	Reverse	
25	GNB5 Human W107R point mutation overlapping Forward	5'- CATGCCCTGCACGAGGGTGAT -3'
26	GNB5 Human W107R point mutation overlapping Reverse	5'- ATGCCATCACCCTCGTGCAG -3'
27	GNB5 Human D241A point mutation overlapping Forward	5'- CACATGAATCTGCCATCAAC -3'
28	GNB5 Human D241A point mutation overlapping Reverse	5'- GACACTGTTGATGGCAGATTC -3'
29	GNB5 Human Y305D point mutation overlapping Forward	5'- TGGATACAATGATGACACTAT -3'
30	GNB5 Human Y305D point mutation overlapping Reverse	5'- CGTTGATAGTGTCATCATTG -3'

Supplemental Table 6: Clinical summary data for all liver injury patient samples utilized for the study

Patient Demographics		All patients	Hepato-cellular Injury	Cholestatic Injury	APAP Injury	Mixed Injury
	n	31	10	7	6	8
	Age (y)	29-68	31-62	49-57	43-61	29-68
	Gender (F/M)	16/15	5/5	3/4	2/4	6/2
	ALT (U/L)	256.5 ± 16.9	301.9 ± 43.2	249.2 ± 30.8	260.9 ± 28.7	208.7 ± 19.2
	AST (U/L)	269.2 ± 19.4	189.2 ± 24.6	382.1 ± 38	327.9 ± 30.4	214.6 ± 17.6
	TBIL	7.5 ± 0.25	6.9 ± 0.46	8.3 ± 0.46	8.1 ± 0.64	7.2 ± 0.42

Severity, n(%)						
	Mild	12 (38.71)	4 (40)	2 (28.57)	2 (33.33)	4 (50.0)
	Moderate	15 (48.39)	4 (40)	3 (42.86)	4 (66.67)	4 (50.0)
	Severe	4 (12.90)	2 (20)	2 (28.57)	0 (0)	0 (0)
	Fatal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Supplemental Table 7: Individual patient history for all liver injury patient samples utilized for the study

Patient	Sex	Age	Patient history	Liver Injury Category	ALT (U/L)	AST (U/L)	TBIL
1	Male	54	Vascular congestion, periportal chronic nonspecific inflammatory cell infiltration, early fatty liver changes	Hepatocellular	190.68	156.76	5.7
2	Male	49	Drug induced liver damage	Cholestatic	356.68	526.67	6.9
3	Female	56	Sinusoidal congestion, cholestatic injury	Cholestatic	299.12	310.57	9.4
4	Female	61	Steatohepatitis	Hepatocellular	510.59	311.32	9.1
5	Female	38	Early fatty liver changes, h/o taking antidepressants	Hepatocellular	283.39	273.68	6.5
6	Male	31	Fatty liver	Hepatocellular	421.76	242.78	6.2
7	Male	29	Obese and hypertension, fatty liver	Mixed	218.16	210.56	6.7
8	Female	33	H/O taking antipsychotics, fatty liver	Mixed	233.76	223.45	8.2
9	Male	59	Pericardial injury, ventricular hypertrophy, fatty liver changes	Mixed	174.89	157.75	9.1
10	Male	68	Fatty liver, death due myocardial infarction	Mixed	243.71	190.67	6.8
11	Male	41	Early fatty liver changes	Cholestatic	201.47	413.56	7.9
12	Female	45	H/O taking NSAIDs and antidepressants, fatty liver	Mixed	199.48	276.59	5.4
13	Female	40	Early fatty liver changes, left ventricular hypertrophy, atheroma and h/o taking antidepressants	Mixed	135.38	203.41	7.5
14	Male	49	Cirrhosis of liver and h/o taking acetaminophen	APAP	378.98	406.84	5.9
15	Male	55	Hepatocellular injury	Hepatocellular	-	-	-

16	Male	32	Suicidal hanging, fatty liver	Mixed	157.38	168.47	6.1
17	Female	67	Fibrosis of liver and h/o taking NSAIDs	Mixed	306.45	300.51	7.8
18	Male	52	Cholestatic injury with cardiac fibrosis	Cholestatic	310.39	378.64	10.4
19	Female	38	Hepatic malignancy	Hepatocellular	411.39	199.67	4.6
20	Male	55	Steatohepatitis	Hepatocellular	331.66	167.86	8.4
21	Male	49	Alcoholic hepatitis	Hepatocellular	279.90	146.57	7.6
22	Female	62	Hemochromatosis	Hepatocellular	157.70	103.41	6.8
23	Female	48	Sinusoidal congestion and drug induced changes	Cholestatic	271.45	478.08	7.3
24	Male	39	Hepatitis B	Cholestatic	135.37	233.79	8.6
25	Male	57	Cirrhosis of liver	Cholestatic	169.90	333.66	7.8
26	Male	45	Steatohepatitis - acetaminophen	APAP	207.69	414.46	10.6
27	Female	61	Steatohepatitis - acetaminophen	APAP	311.65	319.75	8.9
28	Male	53	Cirrhosis of liver and taking acetaminophen	APAP	199.68	214.67	7.4
29	Male	51	Steatohepatitis - acetaminophen	APAP	241.58	295.67	8.1
30	Female	49	Hepatocellular injury	Hepatocellular	129.69	100.53	7.1
31	Female	43	Cirrhosis of liver and taking acetaminophen	APAP	225.74	315.78	7.6

Supplemental Table 8: Amino acid interactions from ATM-Gβ_5 interaction study

GNB5 (B chain amino acids)	No. of Interactions
B:GLN50	37
B:LYS54	32
B:ARG56	57
B:TRP107	29
B:ASP241	27
B:TYR305	24

ATM (A chain amino acids)	No. of Interactions
A:ALA2525	28
A:ARG2526	23
A:ASP2214	22
A:ASP1693	21
A:PRO2518	20

A:CYS2021	19