

Data Supplement To:

Title: Hepatic Regulator of G Protein Signaling 6 (RGS6) drives non-alcoholic fatty liver disease by promoting oxidative stress and ATM-dependent cell death

Red = ATM Protein; Blue = RGS6; Surface is shown for interacting AA of RGS6

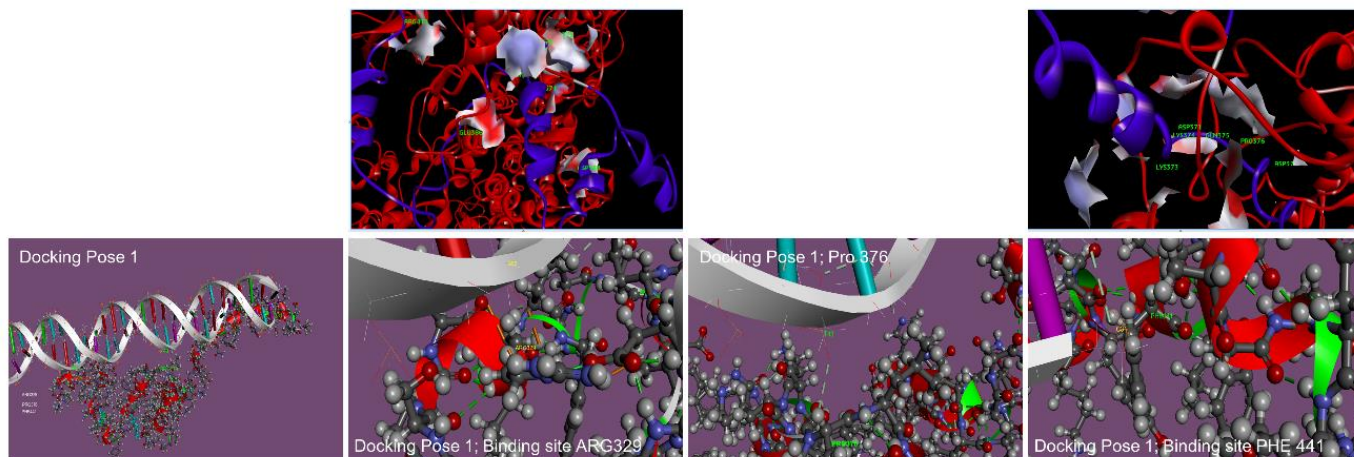


Figure S1 – *In silico* simulation of the ATM-RGS6 complex. Top images: Red = ATM protein; Blue = RGS6 protein. Key RGS6 residues predicted to support the ATM-RGS6 interaction are highlighted in green. Bottom images: RGS6/ATM complex docked on DNA.

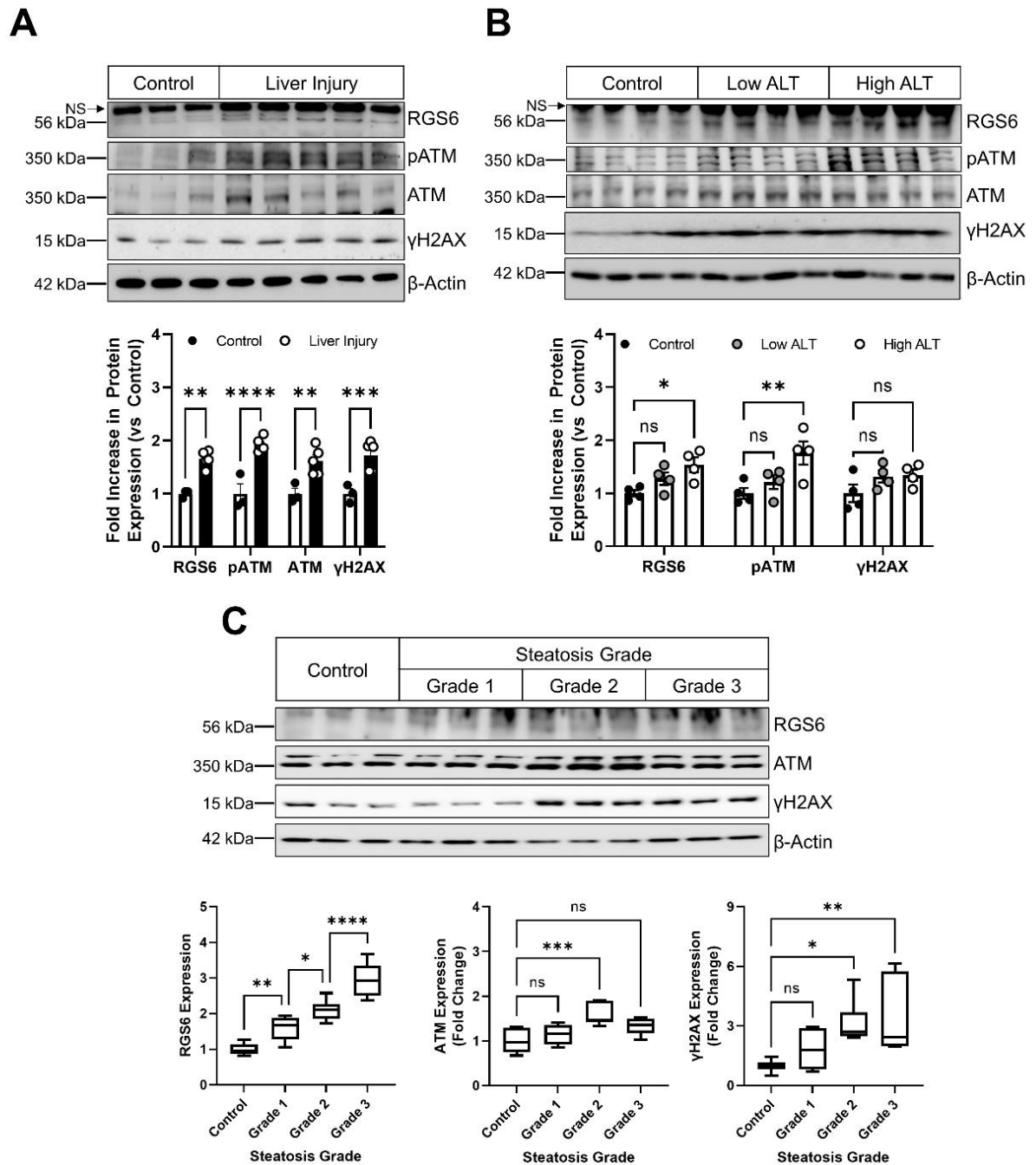


Figure S2 – RGS6 is upregulated in the livers of patients with hepatic dysfunction. (A) Immunoblotting for RGS6, pATM/ATM, and γ H2AX, in control (n=3) and liver injury (n=5) samples. (B) RGS6, pATM/ATM, and γ H2AX in liver injury samples stratified based on injury severity (high vs low ALT) (n=4). (C) Immunoblotting for RGS6, ATM, and γ H2AX in NAFLD patient samples stratified by steatosis grade (n=6). Densitometric quantification was performed, and data are presented relative to control. β -Actin is used as a loading control for all immunoblots. Data were analyzed by student's t-test or one-way ANOVA with Sidak's post-hoc test. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. ns = not significant. Data are presented as mean \pm SEM.

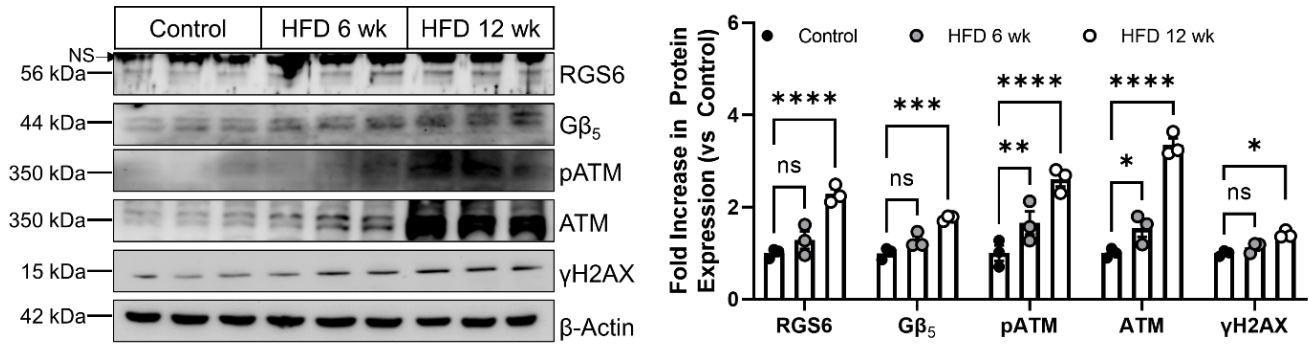


Figure S3 – Molecular characterization of livers from HFD-fed mice. Mice (n=3) were fed on a HFD for 6 or 12 weeks. Immunoblotting for RGS6, Gβ₅, pATM/ATM, and γH2AX was performed on liver tissue lysates. A representative western as well as densitometric quantification is shown. β-Actin is used as a loading control for all immunoblots. Data were analyzed by one-way ANOVA with Sidak's post-hoc test. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. ns = not significant. Data are presented as mean ± SEM.

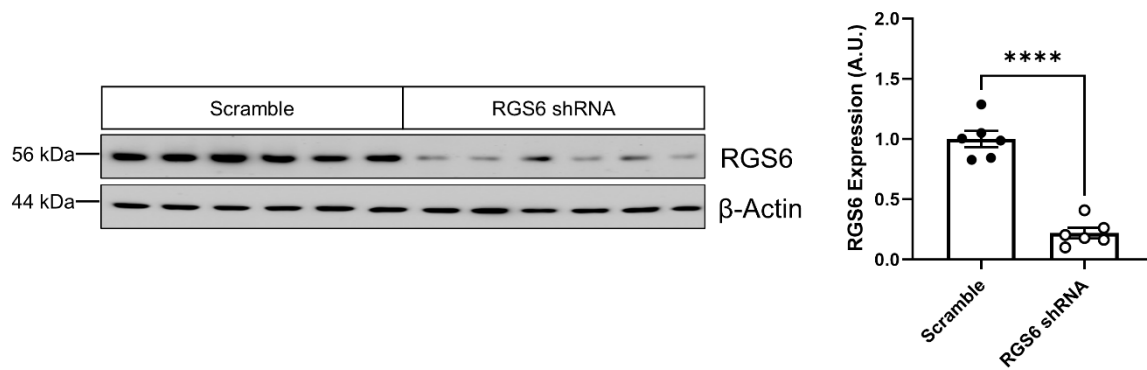


Figure S4 – Verification of RGS6 knockdown in liver. Scramble or RGS6-targeted shRNA was administered to mice (n=6) via tail vein injection, and immunoblotting for RGS6 was performed on liver tissue lysates. A representative western as well as densitometric quantification is shown. β -Actin is used as a loading control. Data were analyzed by student's t-test. **** $P < 0.0001$. Data are presented as mean \pm SEM.

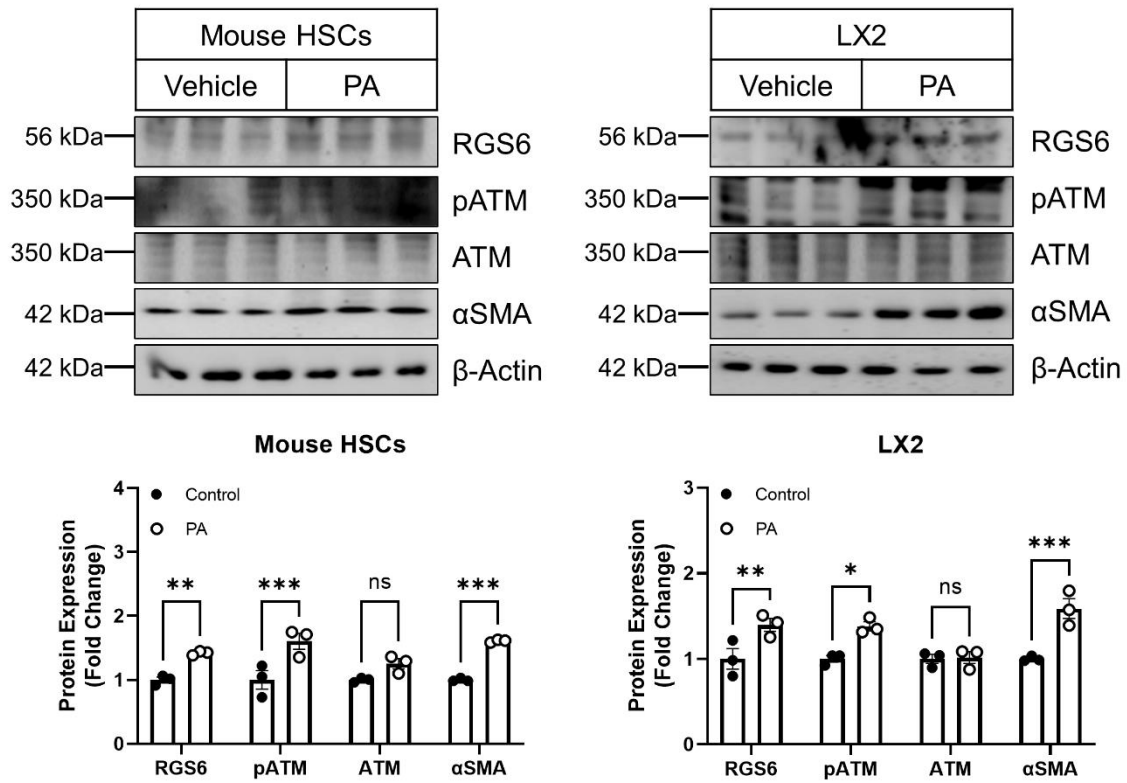


Figure S5 – Hyperlipidemia results in RGS6 up-regulation in murine and human HSCs. Cells were treated with PA (400 μ M, 24 h) 24 hours following introduction of scramble or RGS6-targeted shRNA. Immunoblotting was performed probing for RGS6, pATM, ATM and α SMA (n=3). Immunoblots as well as densitometric quantification are provided. β -Actin is used as a loading control for all immunoblots. Data were analyzed by student's t-test. * P <0.05, ** P <0.01, *** P <0.001. ns = not significant. Data are presented as mean \pm SEM.

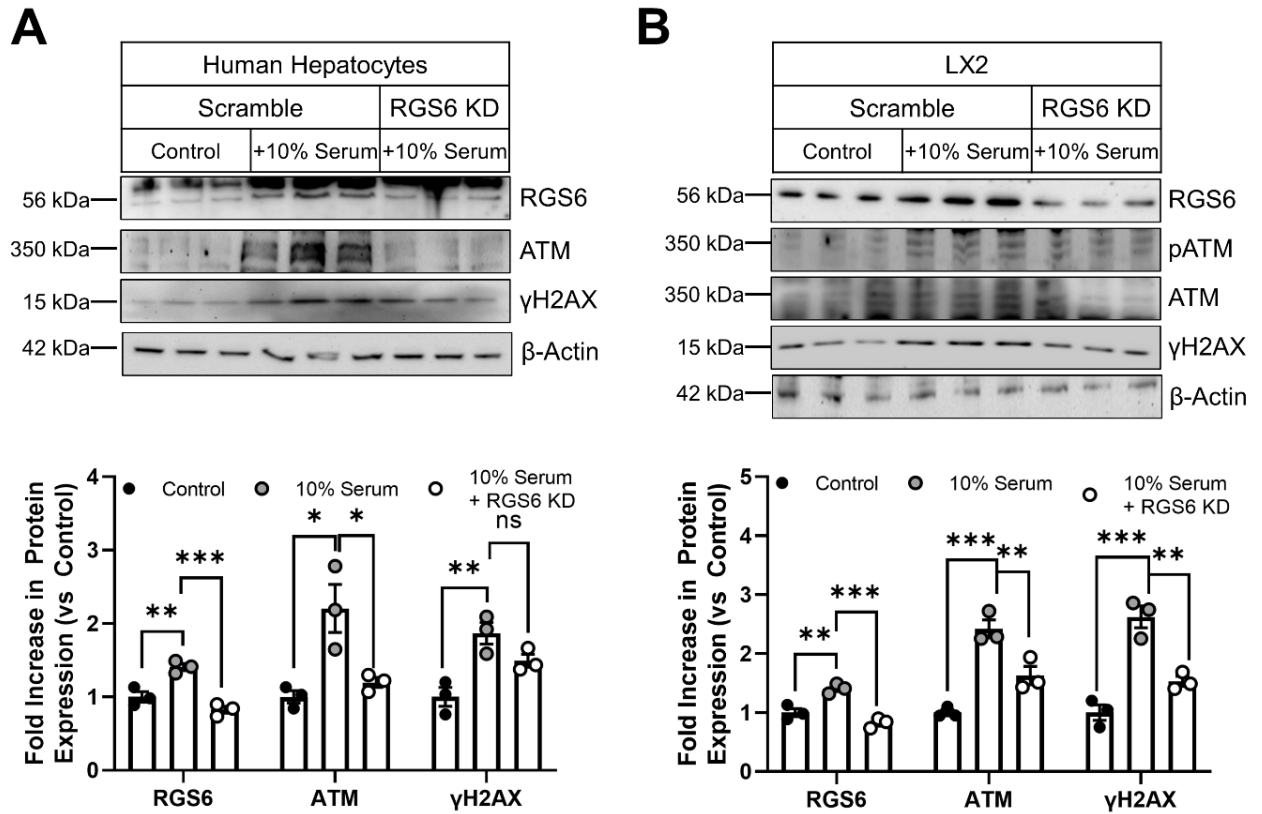


Figure S6 – RGS6 silencing ameliorates pathological molecular aberrations induced by serum from NAFLD patients. (A) Human hepatocytes (n=3) or (B) LX2 cells (n=3) were treated with 10% serum from NAFLD patients 24 hours following the introduction of scramble or RGS6-targeted shRNA. Immunoblotting for RGS6, pATM/ATM, and γH2AX was performed. Representative immunoblots with corresponding quantification are provided. Data were analyzed by one-way ANOVA with Sidak's post-hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns = not significant. Data are presented as mean \pm SEM.

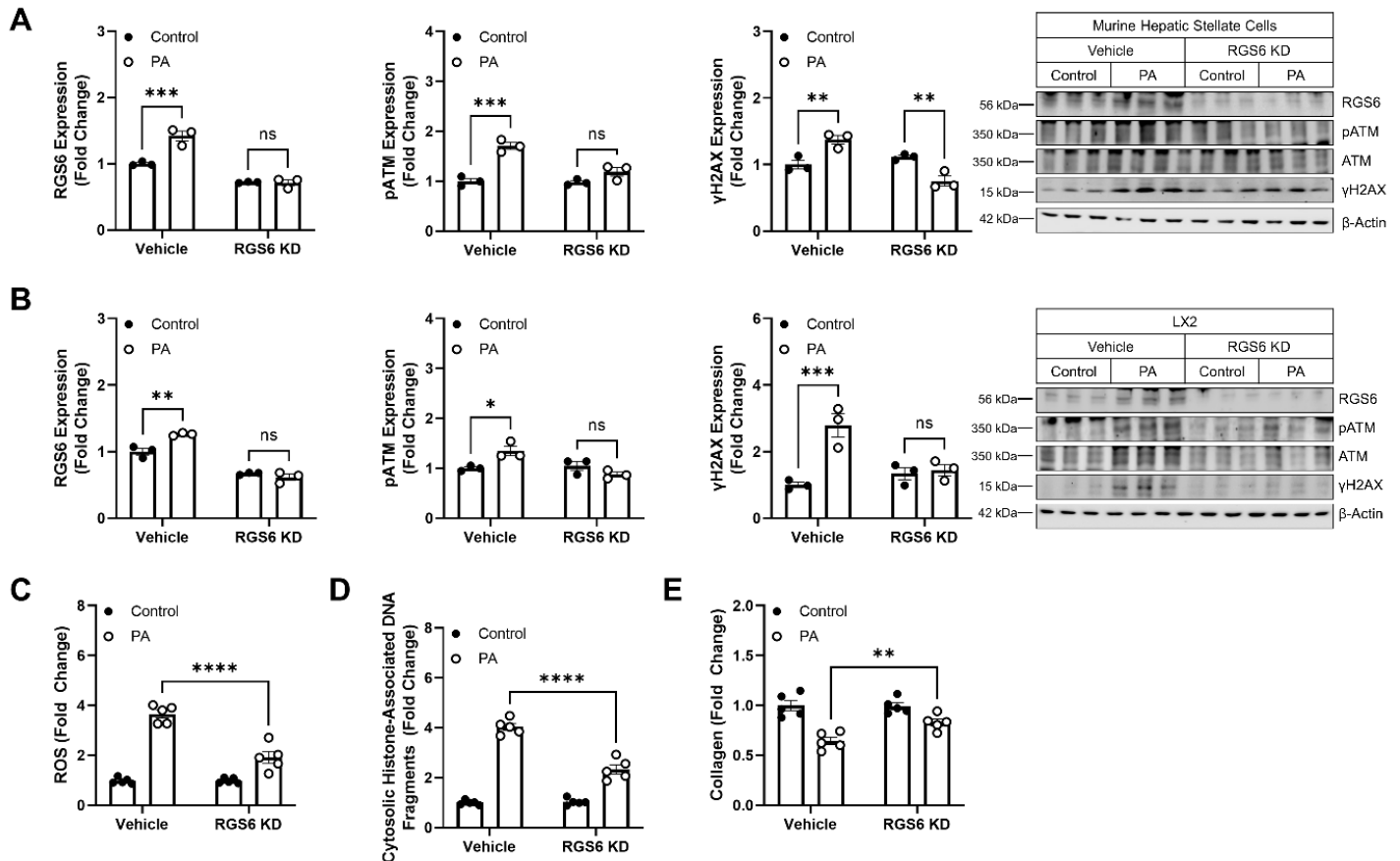


Figure S7 – RGS6 promotes PA-dependent cytotoxicity and fibrotic remodeling in hepatic stellate cells (HSCs). Cells were treated with PA (400 μ M, 24 h) 24 hours following the introduction of scramble or RGS6-targeted shRNA. (A) Immunoblotting for RGS6, pATM/ATM, and γ H2AX in murine HSCs. Representative immunoblots with corresponding quantification are provided (n=3). (B) Immunoblotting for RGS6, pATM/ATM, and γ H2AX in human HSC cell line LX2. Representative immunoblots with corresponding quantification are provided (n=3). (C) CM-H₂DCFDA fluorescence (n=5); (F) cell death (cytoplasmic histone-associated DNA fragments; n=5); and collagen production (n=5) in PA-treated LX2 cells. Data were analyzed by two-way ANOVA with Sidak's post-hoc test. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. ns = not significant. Data are presented as mean \pm SEM.

Table S1: Reagent List

Company	Location	Reagent
Sigma Chemical	St. Louis, MO, USA	N-acetyl cysteine (NAC)
		Ru360
		Cyclosporin A
		CM-H ₂ DCFDA
		Oil Red O
		Palmitic acid (PA)
		Polyethylene glycol-catalase (Peg-Cat)
Addgene	Watertown, MA, USA	pLenti CMV Puro DEST cloning vector
		pMD2.G VSV-G envelope expressing plasmid
		psPAX2
Abcam	Cambridge, UK	Masson Trichrome Stain
		N-acetyl cysteine (NAC)
		KU-55933
		Protein A/G sepharose
Thermo Fisher Scientific	Waltham, MA, USA	Sirius Red
		MitoSox
Invitrogen	Carlsbad, CA, USA	Phusion Hot Start II High-Fidelity PCR Master Mix (F-565S)
Takara Bio	Kyoto, Japan	Platinum Taq DNA Polymerase High Fidelity
Sisco Research Laboratory	Mumbai, India	pMD20-T vector
		Hematoxylin
		Eosin
		Other regular chemicals

Table S2: Antibody List

Company	Location	Antibody	Catalog #	Dilution
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)
		Mouse Secondary-HRP	ab97023	WB (1:2000), IHC (1:500)
		Rabbit Secondary-HRP	ab97051	WB (1:2000), IHC (1:500)
		RGS6	ab128943	WB (1:1000), IHC (1:200)
		GFP	ab290	WB (1:1000), IP (1:200)
Cell Signaling Technology	Danvers, MA, USA	Actin	ab8227	WB (1:1000)
		α-SMA	19245S	WB (1:1000)
		CD68	76437	IHC (1:300)
Millipore	Burlington, MA, USA	GFP	2956	WB (1:1000), IP (1:200)
		Gβ5	ABS1062	WB (1:800), IHC (1:200)
Thermo Fisher Scientific	Waltham, MA, USA	ATM	MA123152	WB (1:1000), IHC (1:250)
		IRS pS307	PA1-1054	WB (1:1000)
		IRS pY941	44-820G	WB (1:1000)

Table S3: Assay Kit List

Company	Location	Assay	Catalog #
Abcam	Cambridge, UK	Ca ²⁺ Flux Assay Kit	ab102505
		Collagen Assay Kit	ab222942
		Hydroxyproline Assay Kit	ab222941
		Mitochondrial isolation Kit	ab110170
		Mitochondrial Membrane Potential Assay Kit	ab113852
		Terminal deoxynucleotidyltransferase/UTP Nick-End Labelling (TUNEL) kit	ab206386
		NAD ⁺ /NADH assay kit	ab65348
		Lipid Peroxidation (MDA) Assay Kit	ab23347
		Liver Lactate/Pyruvate Assay kits	ab65331/ ab65342
Roche	San Francisco, CA, USA	Cell Death Detection Kit	C755B93
Erba Mannheim	London, UK	ALT	120207
		AST	120204
		Triglycerides	120211
Biomedical Research & Clinical Application (BMR)	Buffalo, NY, USA	Fatty acid oxidation (FAO) assay	E141

Table S4: 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)
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

Table S5: Primer List

#	Names of Primer	Sequence of Primer
1	RGS6 Human isoform 2 full length in pEGFP-N1 Forward XhoI	5'ATACTCGAGATGGCTCAAGGATCCGG3'
2	RGS6 Human isoform 2 full length in pEGFP-N1 Reverse HindIII	5'ATAAAGCTTGGAGGACTGCATCAGGC3'
3	RGS6 Human Isoform 2 deletion 30-150aa overlapping Forward	5'GCAAAATTGAATATGAAGCAGAAAACCTTAGC3'
4	RGS6 Human Isoform 2 deletion 30-150aa overlapping Reverse	5'TCTGCTTCATATTCAATTTTGCAGTAAACGA3'
5	RGS6 Human Isoform 2 deletion 255-315aa overlapping Forward	5'CAATCAGGAAATTGTGGGACATAGAGATGAG3'
6	RGS6 Human Isoform 2 deletion 255-315aa overlapping Reverse	5'ATGTCCCACAATTTCCCTGATTGGTTGGCTGA3'
7	RGS6 Human Isoform 2 Short, deletion 1-139aa Forward	5'ATACTCGAGATGCAAAATAAAGCAAGGCTG3'
8	RGS6 Human Isoform 2 Short, deletion 1-139aa Reverse	5'ATAAAGCTTGGAGGACTGCATCAGGC3'
9	RGS6 Human Isoform 2 deletion 301-400aa overlapping Forward	5'ACCCTTTGATAAACCTGGATTCTCACAGC3'
10	RGS6 Human Isoform 2 deletion 301-400aa overlapping Reverse	5'GAATCCAGGTTTATCAAAGGGTCATATTC3'
11	RGS6 Human Isoform 2 deletion 376-456aa overlapping Forward	5'TTAAGAAACAAGGAAAGTCGCTGGCGGGC3'
12	RGS6 Human Isoform 2 deletion 376-456aa overlapping Reverse	5'AGCGACTTTCCCTTGTTTCTTAAGATCTTG3'
13	RGS6 Human Isoform 2 deletion 326-456aa overlapping Forward	5'GCAAAGAGCCCCGAAAGTCGCTGGCGG3'
14	RGS6 Human Isoform 2 deletion 326-456aa overlapping Reverse	5'AGCGACTTTCGGGCTCTTTGCTCATCTC3'
15	RGS6 Human Isoform 2 Point mutation D379Y overlapping Forward	5'CAACCCCTACAGTATGTGGC3'
16	RGS6 Human Isoform 2 Point mutation D379Y overlapping Reverse	5'TCTTGCCACATACTGTAGG3'
17	RGS6 Human Isoform 2 Point mutation D379V Forward	5'AACCCCTACAGTTGTGGCC3'
18	RGS6 Human Isoform 2 Point mutation D379V Reverse	5'CTCTTGCCACAACCTGTAG3'
19	RGS6 Human Isoform 2 Point mutation D348Y Forward	5'CAGGTGGGGCGGTACCAGTTT3'
20	RGS6 Human Isoform 2 Point mutation D348Y Reverse	5'TAGAAACTGGTACCGCCCCA3'
21	RGS6 Human Isoform 2 Point mutation D371Y Forward	5'GCTGGCTGTCCAATATCTTA3'
22	RGS6 Human Isoform 2 Point mutation D371Y Reverse	5'GTTTCTTAAGATATTGGACAGCC3'
23	RGS6 Human Isoform 2 Point mutation K374I Forward	5'CCAAGATCTTAAGATACACCC3'
24	RGS6 Human Isoform 2 Point mutation K374I Reverse	5'CCTGTAGGGTTGTATCTTAAG3'
25	RGS6 Human Isoform 2 Point mutation R419I Forward	5'GTCAAAGATGGAGGGATATATACAT3'
26	RGS6 Human Isoform 2 Point mutation R419I Reverse	5'GGCGTCTTCAAATGTATATATCCCTCCATC3'
27	RGS6 Human isoform 2-point mutation N401V overlapping Forward	5'AAGTGCAATCGTCCTGGATT3'
28	RGS6 Human isoform 2-point mutation N401V overlapping Reverse	5'GAGAATCCAGGACGATTGCA3'
29	RGS6 Mouse isoform2 full length Forward XhoI	5'-ATACTCGAGATGGCTCAGGGTCCGGG-3'
30	RGS6 Mouse isoform2 full length Reverse HindIII	5'ATAAAGCTTGGAGGACTGCATCAGGC3'

Table S6: Amino acid interaction from ATM-RGS6 simulation

Protein	ATM	RGS6	Frequency of interaction during simulation
Position of Amino Acids	ASP2822	LYS374	21
	ARG2912	ASP379	22
	GLU2778	ARG419	24
	ARG1973	ASP348	27
	ARG2792	ASP379	33
	ARG2792	ASP371	39

Table S7: Clinical Data for control, NAFLD (steatosis grade 1-3) and NAFLD + DM patients whose liver tissue was utilized for the human datasets generated in this study. [BMI, body mass index; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; FBG, fasting blood glucose; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; HbA1c, hemoglobin A1c]

	Controls	Steatosis (Grade 1-3)	Steatosis + Diabetes Mellitus	P-value
<i>N</i>	8	24	18	-
BMI (kg/m ²)	20.98 ± 0.62	28.12 ± 0.53	28.63 ± 0.68	P<0.0001
Fat mass (kg)	17.74 ± 0.82	21.01 ± 0.76	20.91 ± 0.84	P=0.064
Alcohol (g/day)	4.87 ± 0.89	3.21 ± 0.24	3.19 ± 0.16	P=0.009
Cholesterol (mg/dl)	161.38 ± 8.77	174.1 ± 4.57	179.11 ± 4.98	P=0.18
LDL (mg/dl)	91.38 ± 2.96	103.46 ± 3.86	108.7 ± 4.68	P=0.13
HDL (mg/dl)	51.23 ± 1.84	49.54 ± 1.91	47.79 ± 1.95	P=0.61
TG (mg/dl)	97.89 ± 3.04	151.9 ± 3.9	152.47 ± 5.76	P<0.0001
AST (IU/l)	19.88 ± 0.86	30.73 ± 1.01	32.58 ± 1.54	P<0.0001
ALT (IU/l)	15.94 ± 0.68	33.71 ± 1.13	37.54 ± 1.54	P<0.0001
FBG (mg/dl)	92.55 ± 2.88	111.35 ± 3.34	111.93 ± 3.48	P=0.0069
Insulin (μ IU/ml)	6.59 ± 0.3	11.1 ± 0.63	12.32 ± 0.59	P<0.0001
HOMA-IR	1.61 ± 0.11	3.09 ± 0.084	3.12 ± 0.11	P<0.0001
C-Peptide (ng/ml)	1.48 ± 0.096	2.92 ± 0.058	3.08 ± 0.078	P<0.0001
HbA1c (%)	5.34 ± 0.12	5.91 ± 0.98	6.13 ± 0.076	P=0.0002