Curcumin Prevents Free Fatty Acid-Induced Lipid Accumulation *via* Targeting the miR-22-3p/*CRLS1* Pathway in HepG2 Cells by

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Supplementary Materials

Materials and Methods: Cell viability assay; Determination of miRNA expression level; Measurement of cellular reactive oxygen species level

Table S1. Additional primer sequences used in this study. **Figure S1.** Effect of curcumin on the viability of non-induced or free fatty acid (FFA)-induced HepG2 cells. **Figure S2.** The expression of miR-22-3p and *CRLS1* varied along with altered intracellular lipid level. **Figure S3.** Curcumin regulated the expression of genes involved in fatty acid β- oxidation and cholesterol metabolism. **Figure S4.** miR-22-3p repression and *CRLS1* overexpression resembled curcumin's effect on decreasing free fatty acid (FFA)-induced oxidative stress in HepG2 cells.

Materials and methods

Cell viability assay

Cell viability was determined by the MTT assay. Briefly, HepG2 cells (1×10^4 cells/well) were cultured in a 96−well culture plate overnight, followed by treatment as specified. The cells were then incubated with 0.5 mg/mL MTT (Sigma-Aldrich) for 4 h at 37°C, and culture medium was discarded. Upon continuous shaking with 150 μL of DMSO for 10 min, the optical absorbance was examined at 490 nm with a Biotek microplate reader (Winooski, VT, USA). The cell viability was calculated as the percentage of viable cells over control cells. The assay was performed three times, with six replicates for each sample.

Determination of miRNA expression level

The intracellular miRNA level was quantified using the qRT-PCR assay. The intracellular RNA was extracted as described previously and the cDNA strand was generated using a Promega's M−MLV reverse transcriptase kit (Madison, WI, USA). qPCR was then carried out using 2 μL of a properly diluted cDNA template *per* reaction with an Accurate Biology's SYBR Green Premix Pro Taq HS qPCR mix, following

the manufacturer's instructions. *U6* served as a reference gene, and the primer sets for miRNA detection were purchased from RiboBio.

Measurement of cellular reactive oxygen species level

The cellular reactive oxygen species (ROS) level was examined using a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) ROS assay kit (Beyotime, Shanghai, China) following the manufacturer's instructions. Briefly, the cells were incubated with 10 μM DCFH-DA at 37°C for 30 min and then washed three times with Opti-MEM to remove excessive probes. Upon resuspension in Opti-MEM, the cells were finally quantified for cellular ROS level with a Guava EasyCyte 6-2L flow cytometer (Millipore, MA, USA) using GuavaSoft 3.1.1 software. The cellular ROS level was presented as the green fluorescence intensity.

Table S1. Additional primer sequences used in this study.

Gene name	Forward (5' → 3')	Reverse (5' → 3')
ACOX1	ACTCGCAGCCAGCGTTATG	AGGGTCAGCGATGCCAAAC
ACADS	TCGATTGTGCTGTGAACTACG	CCAACTTGAACTGGATGACCT
ACAT1	ATGCCAGTACACTGAATGATGG	GATGCAGCATATACAGGAGCAA
HMGCR	AGAAGAAAATAAGCCGAATC	TATCCAGCGACTGTGAGC
CYP7A1	TTCTGCGAAGGCATTTGG	AGCGGTCTTTGAGTTAGAGGAG

ACOX1, acyl-CoA oxidase 1; *ACADS*, acyl-CoA dehydrogenase short chain; *ACAT1*, acetyl-CoA acetyltransferase 1; *HMGCR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *CYP7A1*, cytochrome P450 family 7 subfamily A member 1.

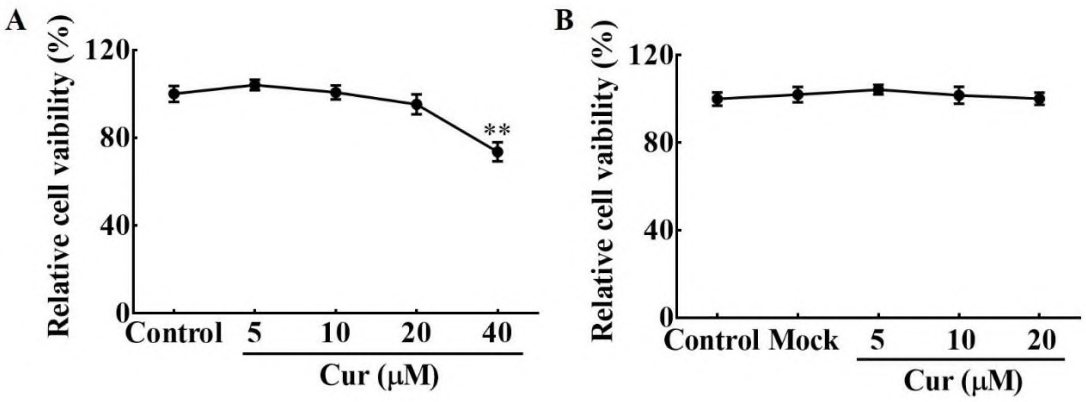

Figure S1. Effect of curcumin on the viability of non-induced or free fatty acid (FFA)-induced HepG2 cells. HepG2 cells were incubated with different amounts of curcumin (Cur) in the absence (A) or presence (B) of FFA for 24 h, and cell viability was measured by the MTT assay. The control cell viability was set as 100%, $^{**}p<0.01$, compared with control cells. Mock, FFA-induced control cells.

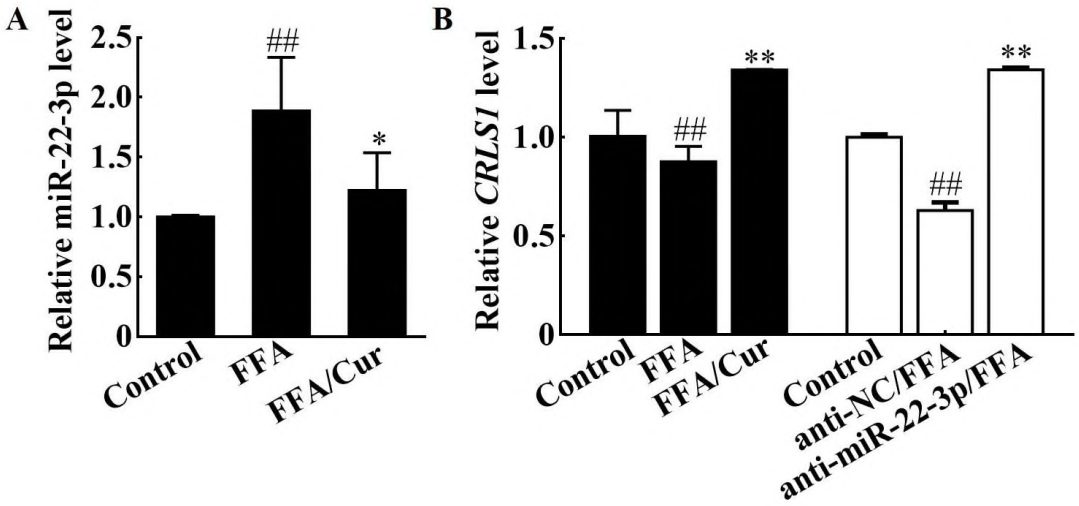

Figure S2. The expression of miR-22-3p and *CRLS1* varied along with altered intracellular lipid level. (A) Altered miR-22-3p expression upon free fatty acid (FFA) and curcumin treatment for 24 h. The miRNA expression level was set as 1.0 in control cells. $^{\#\#}p<0.01$, compared with control cells; $^{*}p<0.05$, compared with FFA-induced mock cells. (B) *CRLS1* expression was reduced upon FFA treatment, but upregulated by curcumin or miR-22-3p repression. The cells were co-treated with 1 mM FFA and various amounts of curcumin for 24 h, or transfected with 40 nM miR-22-3p inhibitor (anti-miR-22-3p) for 48 h and then stimulated with FFA for 24 h. The *CRLS1* gene expression was set as 1.0 in control cells. $^{\#\#}p<0.01$, compared with control cells; $^{**}p<0.01$, compared with FFA-induced mock cells. Cur, curcumin; anti-NC, miRNA inhibitor control.

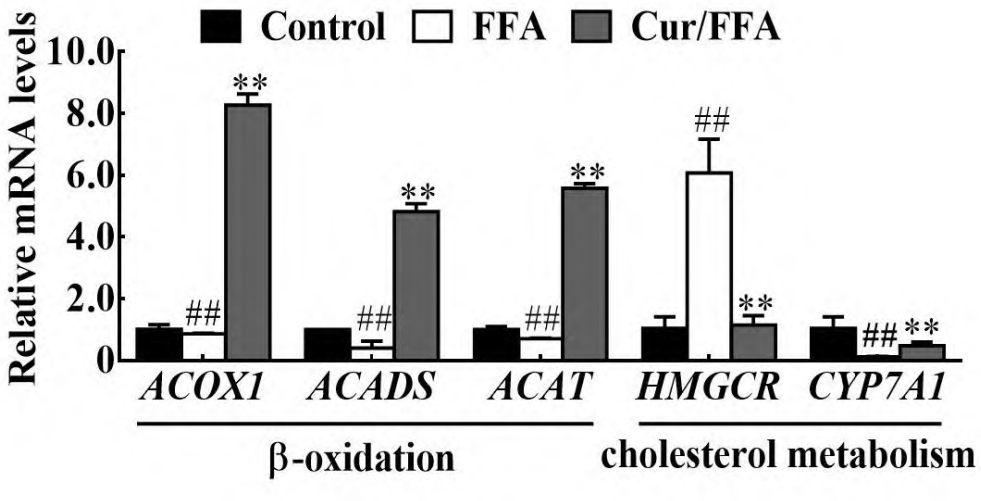

Figure S3. Curcumin regulated the expression of genes involved in fatty acid β- oxidation and cholesterol metabolism. The cells were co-treated with 1 mM free fatty acid (FFA) and 20 μM curcumin (Cur) for 24 h, followed by quantitative real–time PCR (qRT-PCR) assay. The gene expression level was set as 1.0 in non-induced control cells. $^{\#\#}p<0.01$, compared with control cells; $^{**}p<0.01$, compared with FFA-induced mock cells.

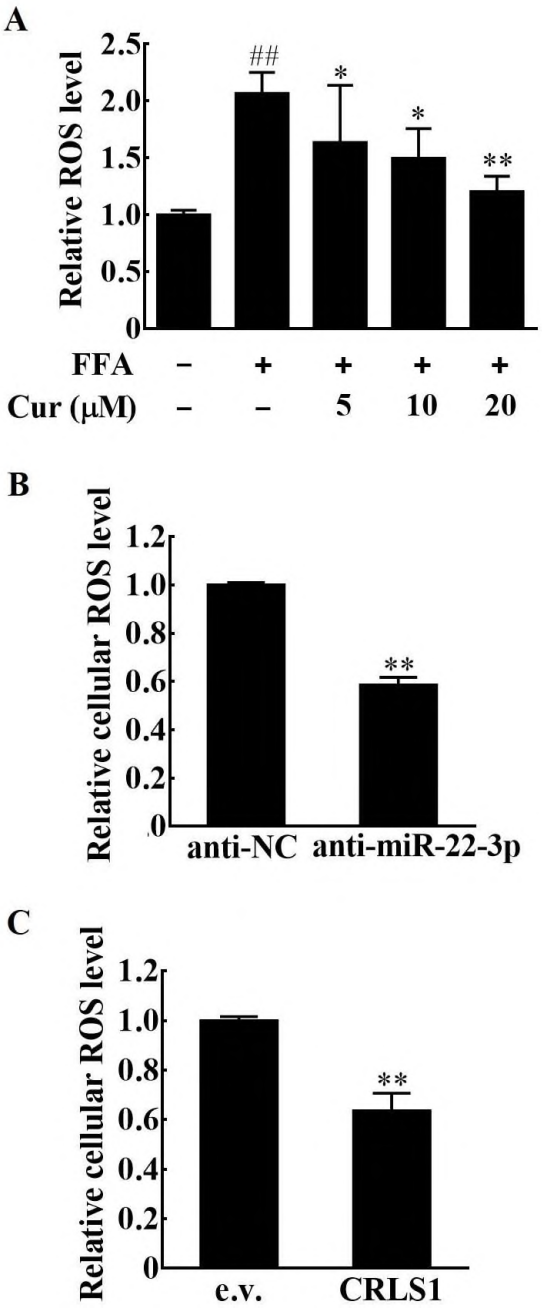

Figure S4. miR-22-3p repression and *CRLS1* overexpression resembled curcumin's effect on decreasing free fatty acid (FFA)-induced oxidative stress in HepG2 cells. (A) 24 h curcumin (Cur) treatment reduced the cellular ROS level of FFA-induced mock cells. The cellular reactive oxygen species (ROS) level was set as 1.0 in control cells. (B–C) Transfection with 40 nM miR-22-3p inhibitor (anti-miR-22-3p) (B) or the pCMV-CRLS1 vector (CRLS1) (C) for 48 h reduced the ROS level of FFA-induced mock cells. The control ROS level was set as 1.0. $^{\#\#}p<0.01$, compared with control cells; $^{*}p<0.05$ or $^{**}p<0.01$, compared with FFA-induced mock cells. anti-NC, miRNA inhibitor control; e.v., empty vector.