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Supplemental Information

Mitochondrial fragmentation is crucial for *c-Myc*-driven hepatoblastoma-like liver tumors

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Supplemental Figures

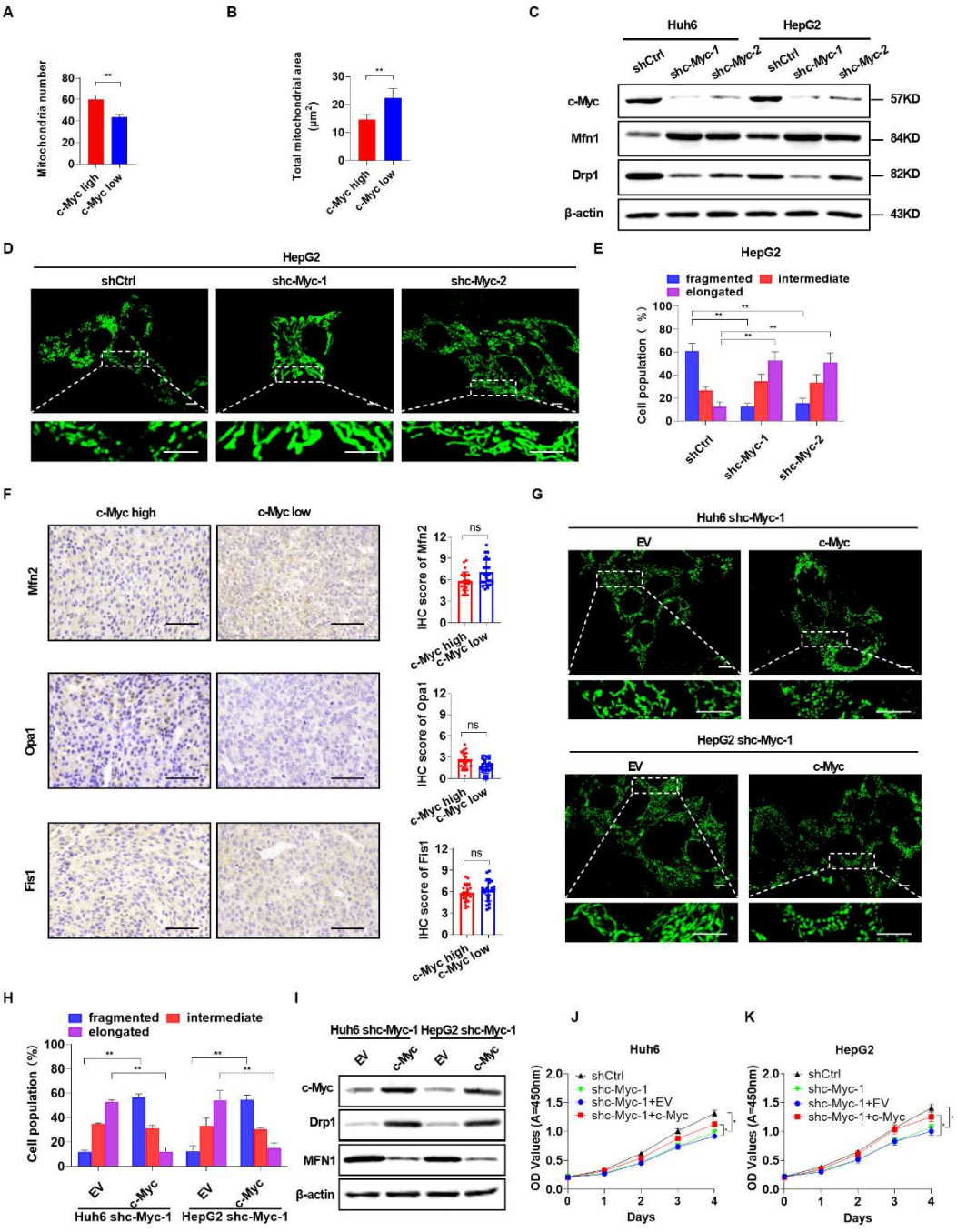

Figure S1.

(A) The number and **(B)** total area of mitochondria were analyzed from five random fields ($120\mu m^2$) in HB tissues with high (n=5) or low (n=5) expression levels of c-Myc.

(C) Western blotting images of c-Myc, Mfn1 and Drp1 in Huh6 and HepG2 cells with

treatment as indicated. shctrl, control shRNA; shc-Myc-1 and shc-Myc-2, shRNA against c-Myc.

(**D** and **E**) Representative confocal microscopy images and morphology distribution analysis of mitochondria in HepG2 cells with or without c-Myc knockdown. Scale bars: 10 μm.

(F) Representative immunohistochemical (IHC) staining images of Mfn2, Opa1 and Fis1 in HB tissues with high (n=25) and low (n=25) c-Myc expression. Scale bar: 50μm.

(G and H) Representative confocal microscopy images and morphology distribution analyses of mitochondria in c-Myc knockdown HB cells with or without c-Myc overexpression. Scale bars: 10 μm. EV, empty vector; c-Myc, expression vector encoding c-Myc.

(I) Western blotting images of c-Myc, Drp1 and Mfn1 in HB cells with treatment as indicated.

(J and K) Cell proliferation was analyzed by CCK-8 in HB cells with treatment as indicated.

Data were expressed as mean ± SD. ns, no significant; *p< 0.05; **p< 0.01.

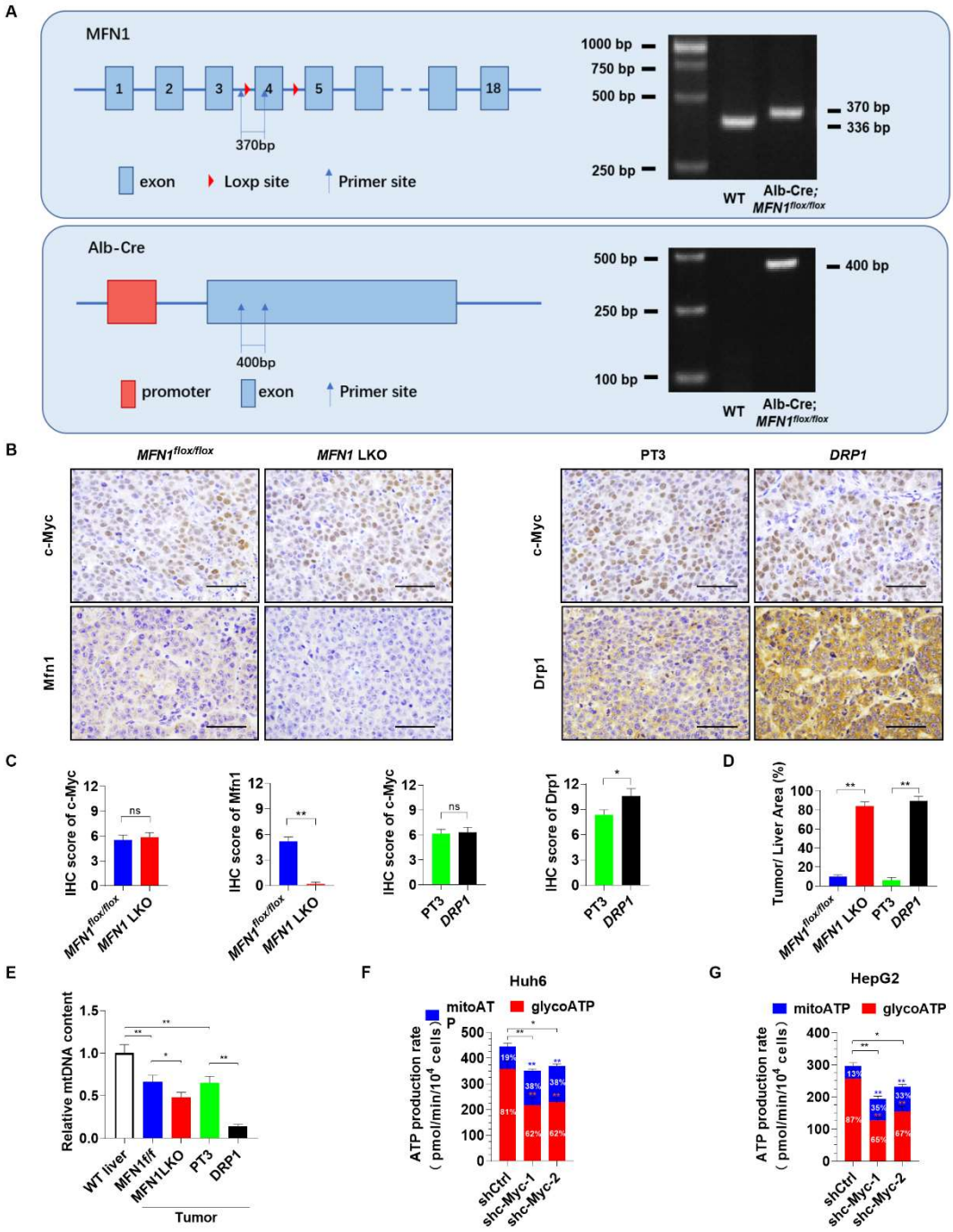

Figure S2.

(A) Schematic diagram of LoxP site in the exons of *MFN1* and Cre recombinant protease with ALB promoter. Genomic DNA extracted from the tail of the mice was used for genotype identification by performing PCR and agarose gel electrophoresis. WT, wild type.

(B) Representative immunohistochemical (IHC) staining images of c-Myc and Mfn1 in *c-Myc*

induced models with (n=12) or without (n=12) liver specifically knockout of *MFN1*. While representative IHC staining images of c-Myc and Drp1 in mouse models induced by *c-Myc* and PT3 control plasmids (n=12) or *c-Myc* plasmid and *DRP1* expression construct (n=12). Scale bar: 50μm. $MFN1^{flox/flox}$, $MFN1^{flox/flox}$ mice hydrodynamically injected with *c-Myc* plasmid and SB transposon; *MFN1* LKO, *MFN1* liver specific knockout mice hydrodynamically injected with *c-Myc* plasmid and SB transposon; PT3, wild type mice hydrodynamically injected with *c-Myc* plasmid and PT3-EF1α empty vector, as well as SB transposon; *DRP1,* wild type mice hydrodynamically injected with *c-Myc* plasmid and PT3-EF1α-DRP1 construct, as well as SB transposon.

(C) IHC scores of c-Myc, Mfn1 and Drp1in mouse models induced as indicated.

(D) Percentage of tumor/liver area of the mice with treatment as indicated.

(E) Relative mtDNA content in mouse normal liver and the tumors induced by c-Myc from different groups as indicated.

(F and G) Glyco, Mito and total ATP production rate were measured between HB cells treated as indicated. Blue asterisks are used for Mito ATP, red asterisks are used for Glyco ATP, and black asterisks are used for total ATP (glycol ATP + Mito ATP) production rate comparisons between shctrl and shc-Myc groups. The percentage inside the bar represents the percentage of total ATP production rate generated from Glyco or Mito ATP production rate for each condition.

Data were expressed as mean ± SD. ns, no significant; *p< 0.05; **p< 0.01.

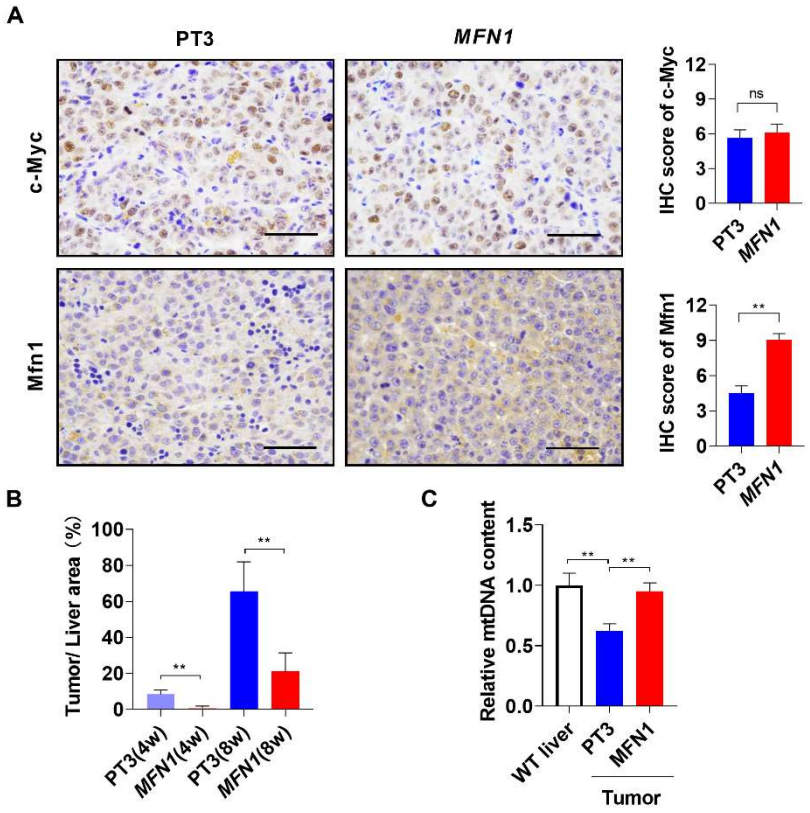

Figure S3.

(A) Representative IHC staining images and IHC scores of c-Myc and Mfn1 in mouse models induced by *c-Myc* and PT3 control plasmids (n=12) or *c-Myc* plasmids and *MFN1* expression construct (n=12). Scale bar: 50μm. PT3, wild type mice hydrodynamically injected with *c-Myc* plasmid and PT3-EF1α empty vector, as well as SB transposon; *MFN1,* wild type mice hydrodynamically injected with *c-Myc* plasmid and PT3-EF1α-MFN1 construct, as well as SB transposon.

(B) Percentage of tumor/liver area of the mice with treatment as indicated.

(C) Relative mtDNA content in mouse normal liver and the tumors induced by c-Myc from different groups as indicated.

Data were expressed as mean ± SD. ns, no significant; **p< 0.01.

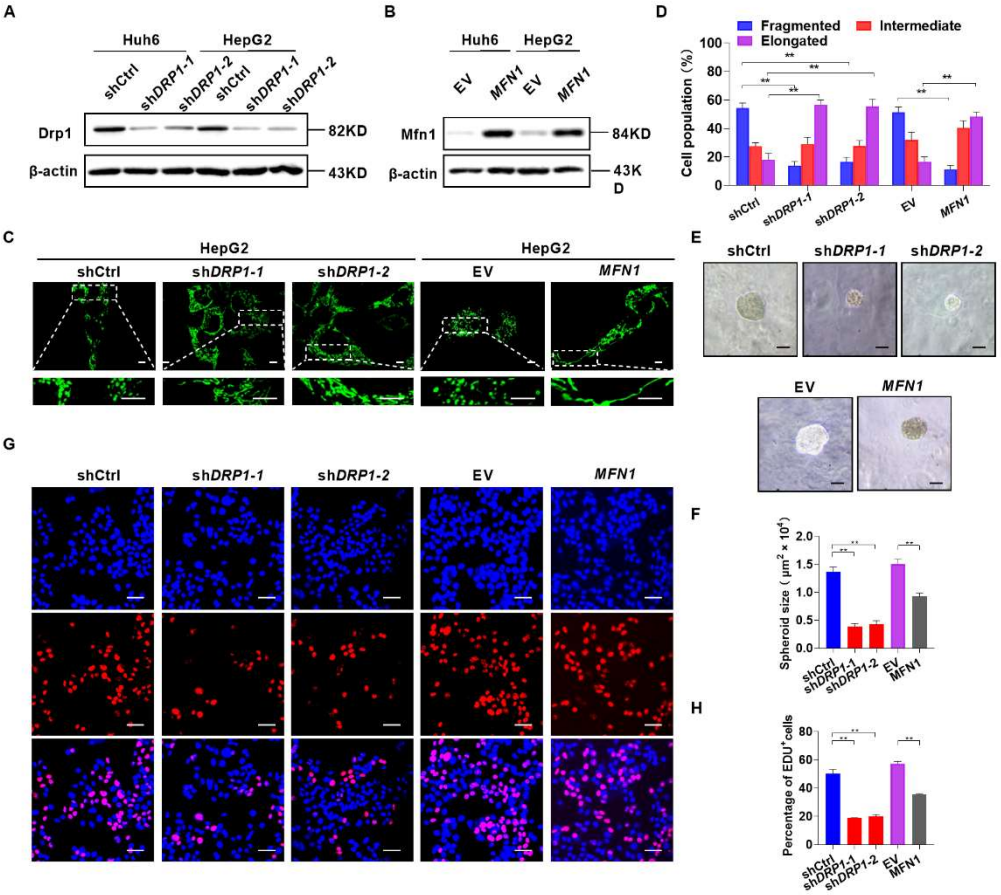

Figure S4.

(A and B) Western blotting images of Drp1 and Mfn1 in Huh6 and HepG2 treated as indicated. shctrl, control shRNA; shDRP1-1 and shDRP1-2, shRNA against DRP1; EV, empty vector; MFN1, expression vector encoding MFN1.

(C-D) Representative confocal microscopy images morphology distribution analyses of mitochondria in HepG2 cells with treatment as indicated. Scale bars: 10μm.

(**E** and **F**) High-magnification images and size of spheres formed on day 7 by HepG2 cells with treatment as indicated. Scale bar: 50μm.

(**G and H**) EdU assay was performed to evaluate proliferation ability of HepG2 cells with treatment as indicated. Scale bar: 50μ

Data were expressed as mean ± SD. **p< 0.01.

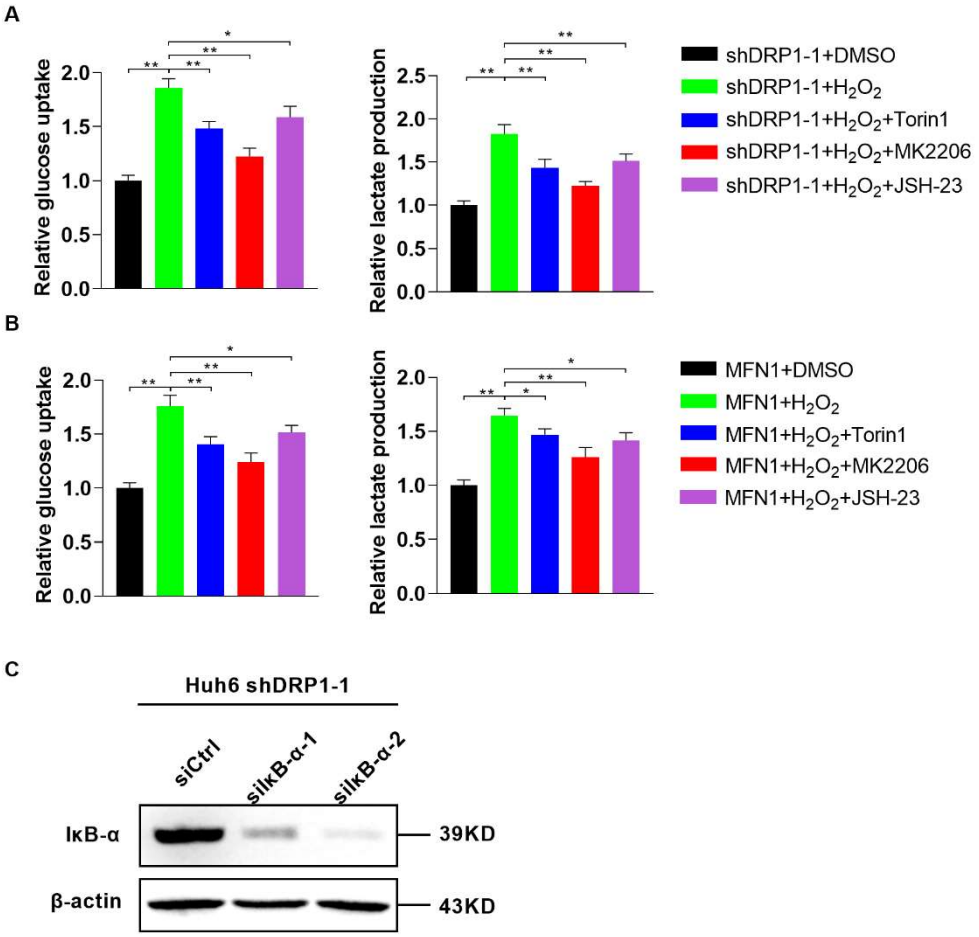

Figure S5.

(A-B) Relative glucose uptake and relative lactate production were measured in Huh6 cells treated as indicated. shctrl, control shRNA; sh*DRP1-1*, shRNA against *DRP1*; *MFN1*, expression vector encoding *MFN1*.

(C) Western blotting images of IκB-α in Huh6 treated as indicated.

Data were expressed as mean ± SD. *p< 0.05; **p< 0.01.

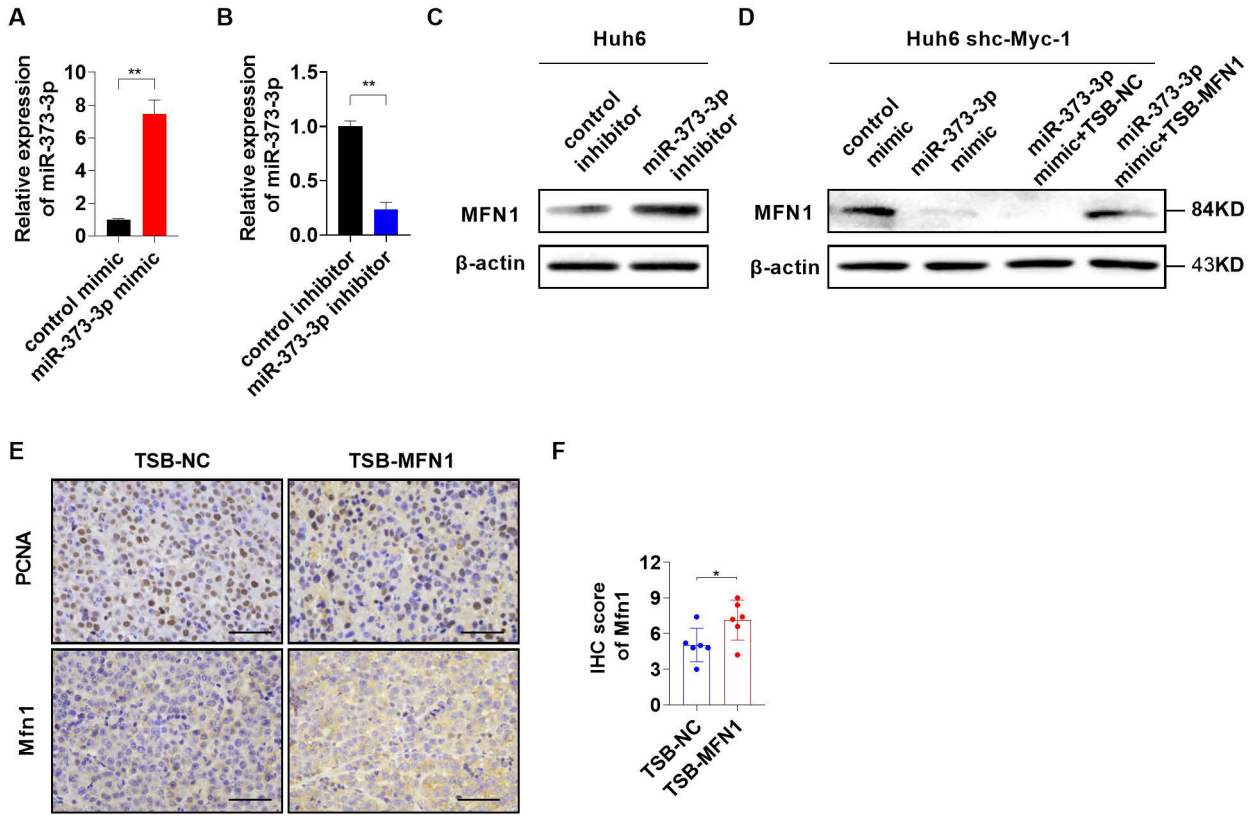

Figure S6.

(A and B) Quantitative real-time reverse transcription PCR (qRT-PCR) analyses for expression levels of miR-373-3p in Huh6 treated as indicated.

(C and D) Western blotting images of Mfn1 in Huh6 cells treated as indicated. shc-Myc-1, shRNA against c-Myc, TSB-NC, negative control of miRNA target site blocker; TSB-MFN1, a target site blocker which selectively prevents miR-373-3p binding to the 3'UTR of MFN1.

(E) Representative immunohistochemical (IHC) staining images of PCNA and MFN1 in subcutaneous xenograft tumors. Scale bar: 50μm.

(F) IHC scores of MFN1 in subcutaneous xenograft tumors.

Data were expressed as mean ± SD. *p< 0.05; **p< 0.01.

Supplemental Tables

Table S1. Distribution of HB patients' characteristics

Variable	All patients, n=50
Age	
<5 years	44
>=5 years	6
Gender	
Female	30
Male	20
Tumor size	
<5.0 cm	21
>=5 cm	29
Differentiation grade	
well	18
Moderately	18
Poor	14
pretext stage	
I+ II	23
III+ IV	27
Survival	
Dead	13
Alive	37

Table S2. Sequences of primers, siRNA, miRNA mimic and inhibitor, TSB, and mRNA sequence for shRNA

Primer name		Sequences
1. Primers for real-time PCR:		
c-Myc	forward primer:	CATACATCCTGTCCGTCCAAG
	reverse primer:	GAGTTCCGTAGCTGTTCAAGT
DRP1	forward primer:	GGAGACTCATCTTTGGTGAAGAG
	reverse primer:	AAGGAGCCAGTCAAATTATTGC
MFN1	forward primer:	TGGCTAAGAAGGCGATTACTGC
	reverse primer:	TCTCCGAGATAGCACCTCACC
GAPDH	forward primer:	GGAGCGAGATCCCTCCAAAAT
	reverse primer:	GGCTGTTGTCATACTTCTCATGG
miR-373-3p	RT primer:	CTCAACTGGTGTCGTGGAGTCGGCA
	forward primer:	TCGGCAGGCTTCACGAAGCTAA
	reverse primer:	GGTGTCGTGGAGTCGGCAATTC
miR-200a-3p	RT primer:	CTCAACTGGTGTCGTGGAGTCGGCA
	forward primer:	TCGGCAGGATTGTGACAGACCA
	reverse primer:	GGTGTCGTGGAGTCGGCAATTC
miR-4319	RT primer:	CTCAACTGGTGTCGTGGAGTCGGCA
	forward primer:	TCGGCAGGAGGGACTCGTT
	reverse primer:	GGTGTCGTGGAGTCGGCAATTC
miR-302c-3p	RT primer:	CTCAACTGGTGTCGTGGAGTCGGCA
	forward primer:	TCGGCAGGATTCACGAAGGTAC
	reverse primer:	GGTGTCGTGGAGTCGGCAATTC
miR-3129-5p	RT primer:	CTCAACTGGTGTCGTGGAGTCGGCA
	forward primer:	TCGGCAGGCGTCATCACATCTC
	reverse primer:	GGTGTCGTGGAGTCGGCAATTC

2. Primers for gene cloning		
DRP1	forward primer:	CCGGAATTCTAGCCAGTCTCCACAT
	reverse primer:	CGCGGATCCGGCCCCGTGTTTTCAG
MFN1	forward primer:	ACGAATTCCTTGCCACCATGGCAGA
	reverse primer:	ACCTCGAGTGTTAGGATTCTTCATT
3. Primers for DRP1 promoter construct		
(-1894/+103)DRP1	forward primer:	TAGGTACCACTCCTGACCTCAAGTG
(-961/+103)DRP1	forward primer:	TAGGTACCTCCTGCTTCTGCTTCCTA
(-514/+103)DRP1	forward primer:	TAGGTACCCAGGAGAATGGCGTGA
(-244/+103)DRP1	forward primer:	TAGGTACCTAGCCTTTGACTAGAGC
(-11/+103)DRP1	forward primer:	TAGGTACCAGGAAGGAGGCGAACT
	reverse primer:	GAAGATCTGGAGCTTGTTTATGACA GGAA
4. Primers for DRP1 promoter site-directed mutagenesis		
(-961/+103) DRP1 mutation forward primer:		CCTCCCGGCAGGCCAAAAGCTGCG AGGGAGGTG
(-961/+103) DRP1 mutation reverse primer:		CACCTCCCTCGCAGCTTTTGGCCTG CCGGGAGG
5. Primers for ChIP in the DRP1 promoter		
DRP1	forward primer:	CTCCGCTCCAGAACTACAAC
	reverse primer:	TACCAACAGTACCGGAATGC
6. mRNA sequence for shRNA and siRNA sequences		
shDrp1-1	mRNA sequence	GCTACTTTACTCCAACTTATT
shDrp1-2	mRNA sequence	CGAGATTGTGAGGTTATTGAA
shc-Myc-1	mRNA sequence	CTGAGACAGATCAGCAACAA
shc-Myc-2	mRNA sequence	GGAAACGACGAGAACAGTTGA
shctrl	mRNA sequence	UUCUCCGAACGUGUCACGU
siIκBα-1		5′-GCUAUUCUCCCUACCA AGCU-3′

siIκBα-2		5′-GCUGCCCUAUGAUGACUGU-3′
sictrl		5′-UUCUCCGAACGUGUCACGUTT-3′
7. Primers for genotyping		
MFN1 (mouse)	forward primer:	AGCAGTTGGTTGTGTGACCA
	reverse primer:	TTGGTAATCTTTAGCGGTGCTC
Cre (mouse)	forward primer:	TGGCAAACATACGCAAGGG
	reverse primer:	CGGCAAACGGACAGAAGCA
8. miRNA mimic and inhibitor		
miR-373-3p mimic	sense:	GAAGUGCUUCGAUUUUGGGGUGU
	antisense:	ACCCCAAAAUCGAAGCACUUCUU
control mimic	sense:	UUCUCCGAACGUGUCACGUTT
	antisense:	ACGUGACACGUUCGGAGAATT
miR-373-3p	inhibitor:	ACACCCCAAAAUCGAAGCACUUC
control	inhibitor:	CAGUACUUUUGUGUAGUACAA
9. Primers for mtDNA measurement		
ND1	forward primer:	CTAGCAGAAACAAACCGGGC
	reverse primer:	CCGGCTGCGTATTCTACGTT
16S rRNA	forward primer:	CCGCAAGGGAAAGATGAAAGAC
	reverse primer:	TCGTTTGGTTTCGGGGTTTC
HK2	forward primer:	GCCAGCCTCTCCTGATTTTAGTGT
	reverse primer:	GGGAACACAAAAGACCTCTTCTGG
10. Sequence of Target Site Blocker		
TSB-MFN1		TGAAATCTGGTTAAAAGCACTTT
TSB-NC		TAACACGTCTATACGCCCA

Table S3. Primary antibodies used for Western bloting and immunohistochemistry

Antibody	Company (Cat. No.)	Working dilutions
MFN1	abcam (ab104585)	WB: 1/1000 IHC:1/150
MFN2	abcam (ab101055)	IHC:1/100
DRP1	abcam (ab56788)	WB: 1/1000 IHC: 1/150
FIS1	abcam (ab156865)	IHC: 1/10000
OPA1	abcam (ab90857)	IHC: 1/400
c-Myc	Proteintech（67447-1-lg）	WB: 1/500 IHC: 1/300
c-Myc	Proteintech（10828-1-AP）	Chip:1/50
AKT	Cell Signaling Technology (9272)	WB: 1/1000
p-AKT	Cell Signaling Technology (4051)	WB: 1/600
β-actin	Beijing TDY BIOTEC CO., Ltd.	WB: 1/3000
NF-kB	abcam(ab7970)	WB: 1/1000
IKB α	abcam(ab76429)	WB: 1/1000
LMNB1	Beijing TDY BIOTEC CO., Ltd.	WB: 1/2000
p-IKK	Santa Cruz Biotechnology (C-23470-R)	WB:1/1000
p70S6K	Cell Signaling Technology (9202)	WB: 1/1000
p-p70S6K(Thr389)	Cell Signaling Technology (9234)	WB: 1/1000
PCNA	abcam (ab18197)	IHC:1/15000

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Accession No.	Platform	HB Sample No.	Patient Ethnicity	PMID	Source URL
GSE133039	Illumina HiSeq 2500 (Homo sapiens) [HG-U133_Plus_2]	66	-	32240714	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133039
GSE75271	Affymetrix Human Genome U133 Plus 2.0 Array	50	Hispanic Caucasian Asian African American	27775819	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75271

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