SUPPLEMENTARY FILE

Tonicity-responsive enhancer binding protein promotes hepatocellular carcinogenesis, recurrence, and metastasis

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SUPPLEMENTARY MATERIALS AND METHODS

Immunohistochemistry (IHC) and histology analysis

Liver tissues were fixed overnight in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. Paraffin sections were deparaffinized and dehydrated. Antigen of TonEBP and COX-2 was retrieved by citrate and peroxidase and that of Ki-67 was retrived by EDTA and peroxidase in appropriate time for each antigen. Anti-ki-67 antibodies (12202T, CST), anti-COX-2 antibodies (SP21, Thermo), and anti-TonEBP antibody [40] were used for IHC with optimized condition.

Tissue array

Human HCC patient tissue samples were collected as previously described. H&E of each patient was analyzed by histologist and representative paired hepatic tumor and adjacent non-tumor region of 296 HCC patients in formalin-fixed paraffin-embedded tissue was marked and extracted for tissue array. Then, extracted tissue was arranged, molded by using tissue microarray cassette, and solidified for tissue array analysis. The arrays were processed simultaneously for TonEBP immunohistochemistry and signal intensity was assigned to five grades (t0 to t4) using an image software (Image J).

TCGA analysis

Upper Quantile normalized FPKM (FPKM-UQ) of RNA-seq and matched clinical information of HCC cohorts was extracted from metadata of TCGA of Genomics Data Commons portal (https://gdc-portal.nci.nih.gov). With R studio, the transcript level of TonEBP and inflammatory genes was analyzed in HCC patients who have RNA-seq dataset from both tumor and matched non-tumor in GDC (n = 100). Correlation analysis between TonEBP and inflammatory genes were generated using R-derived RNA-seq and plotted with Graphpad Prizm.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen) followed by chloroform and ethanol precipitation. cDNA was synthesized by M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. After reverse transcription, Q-PCR was performed using SYBR Green I Master and LightCycler 480 II (Roche). Measured cycle threshold values were normalized with GAPDH and they were expressed as fold-over control samples. All qRT-PCR reactions were duplicated. Adequacy of GAPDH as a house keeping gene was confirmed by NormFinder.

Immunoblotting

Cell lysis for protein extraction was performed as previously described. Protein concentration was measured by BCA system (Pierce). Equal amounts of protein from each sample were separated by SDS-PAGE and immunoblotted using specific primary antibodies. HRP-conjugated secondary antibodies were used for detection. The antigen-antibody binding was detected by enhanced chemiluminescence Western blotting detection reagents (GE healthcare life sciences). Anti-YY1 antibodies(13G10, Cell Signalling Technologies (CST)), COX-2(4842, CST), anti-myc-tag(2278, CST), anti-flag antibodies (F1804, Sigma Aldrich), p300 (sc584, SantaCruz Biotechnology), LaminB (sc6217, SantaCruz Biotechnology) antibodies, anti-Hsc70 (200-301-A28, Rockland) and anti-TonEBP antibody [40] were used for immunoblotting.

ELISA

IL-1β-stimulated human prostaglandin E_2 in supernatants from HepG2 cells was analyzed with ELISA kits (R&D Systems). Prostaglandin E_2 in serum from animal experiments was analyzed with ELISA kits (Abcam)

Promoter and Reporter assay

Human COX-2 promoter fragments were inserted into pGL3 (Promega). Human genomic DNA fragment covering nucleotide positions -2,460 to +83 relative to the transcription start site of the COX-2 gene, including a TonE (TonEBP binding site) at nucleotide position -2,282, was cloned and placed in a promoter-less luciferase reporter (pGL3) to produce a COX-2 promoter reporter named "COX-2 2.5kb". TonEBP binding sites or YY1 binding sites in COX-2 promoter was mutated using cloned COX-2 promoter with primers and indicated as ΔTonE or ΔYY1. YY1 has a putative binding site at nucleotide position -546 from the transcription start site. Cells were transfected luciferase plasmid. The Renilla luciferase reporter plasmid (pRL-TK, Promega) was used as a control for transfection efficiency. Luciferase activity after 6 h of stimulation was measured using the Dual-Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activity was normalized by activity of renilla luciferase. Luciferase activity was expressed relative to pGL3 transfected cells (online supplementary figure 11A) or relative to non-treated, pGL3 transfected cells (figure 5G).

To verify the binding of miRNA-223 to the predicted site in 3-UTR region of TonEBP gene, predicting algorithms in Targetscan (www.targetscan.org) and psiCHECK-2 vector was used. 300-bp region of the predicted miR-223 binding site in 3'-UTR of TonEBP gene was cloned into a psiCHECK-2 (Promega) downstream of the Renilla luciferase–coding region. To validate the effects of miR-223, HEK293 cells were transfected miR-223 or NC, followed by transfection of a reporter construct. Luciferase activity after 48 h of transfection was measured using the Dual-Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activity was normalized by activity of renilla luciferase.

Nuclear and Cytoplasmic fractionation

Cells were harvested by using scrapper and centrifuged. The cell pellet was washed by suspension with PBS. The cell nucleus and cytoplasm were separated by using Nuclear and Cytoplasmic extraction kit (Pierce) according to manufacturer's instruction.

Electrophoretic mobility shift assay (EMSA).

A commercial kit was used: Lightshift Chemiluminescent EMSA kit (Pierce). Nuclear extracts were incubated with poly(dI:dC), binding buffer and 5′ biotinylated DNA, containing the YY1 binding site in the COX-2 promoter, at room temperature for 20 min. Samples were separated by electrophoresis for 4 h in 4% (40% 29:1 acrylamide/bis solution) gel for TonEBP and 8% gel for YY1. The detection was performed according to manufacturer's instructions.

Chromatin immunoprecipitation

ChIP was performed as previous studies. In brief, cells were crosslinked with 1% formaldehyde followed by addition of 125 mM glycine. After washing, cells were sonicated and immunoprecipitated with normal serum, anti-TonEBP and anti-YY1 (Abcam) antibodies at 4 ˚C overnight. After elution and reverse crosslinking the antibody/DNA complexes, DNA was purified by DNA purification kit (Qiagen) and analyzed by q-PCR using primer pairs covering specific region of the COX-2 promoter in duplicates.

Cell Death assays

Death of primary hepatocytes in vitro was estimated using an LDH release-based cytotoxicity assay (Promega) after incubating primary hepatocytes for 24 hr in the absence or presence of hypoxic damage. The TUNEL test was performed using the TUNEL kit (Promega) according to the manufacturer's instructions.

SUPPLEMENTARY TABLES

Supplementary Table 1. Clinical characteristics of enrolled patients with HCC who received surgical resection

Characteristics	**Patients (n=296)**
Age (mean ± S.D.)	56.6 ± 9.8
Sex (male/female)	249/47
Causes of HCC (HBV/HCV/Alcohol/Others)	234/25/26/11
Underlying liver disease (CH/LC)	63/233
Child-Pugh class: A/B/C	280/16/0
Model for End-Stage Liver Disease score	
< 10	269
≥10	27
Tumor size (cm, mean ± S.D.)	4.5 ± 3.3
Tumor number: Single/Multiple	254/42
Tumor staging, n (%)	
Very early (0)	50 (16.9)
Early (A)	216 (73.0)
Intermediate (B)	30 (19.1)
Microvessel invasion, n (%)	89 (30.1)
Metastasis, n (%)	61 (20.6)
Postoperative recurrence, n (%)	144 (48.6)
Death, n (%)	84 (28.4)
Follow up period (months, median with range)	31 (1-105)

Abbreviations: CH, Chronic hepatitis; LC, Liver cirrhosis

Supplementary Table 2. Association of non-tumor TonEBP expression with clinical parameters

Variables	**tNT0** (t0) **(n = 130)**	**tNT1** (t1 to t4) **(n = 166)**	***P* value**
Age (mean ± S.D.)	55.7±9.1	57.3±10.4	0.164
Sex (male/female)	113/17	136/30	0.243
Tumor size (cm)	3.90 ± 3.45	4.99 ± 3.19	0.005
E-S grade (I:II:III:IV)	3:67:54:6	6:58:90:12	0.039
Microvascular invasion	34	55	0.194
Lymphovascular invasion	16	33	0.082
Bile duct invasion	3	8	0.257
Recurrence	48	96	<0.001
Metastasis	20	41	0.049
Death	32	52	0.204
HBV	97	137	0.097
HCV	14	11	0.203
AFP (ng/mL)	1266±7357	4620±22295	0.071
PIVKA-II (mAU/mL)	633±2460	2978±12850	0.029
Preoperative HBV DNA (log_{10} IU/mL)	1.78±2.24	2.57±2.44	0.015
Underlying liver disease (CH/LC)	30/100	33/133	0.505
Child-Pugh class: A/B/C	121/9/0	159/7/0	0.446
Model for End-Stage Liver Disease score	5.40±4.65	5.30±3.25	0.830

Note: tNT0 and tNT1 are defined in Figure 1J; Person's Chi-Squared test was performed.

Abbreviations: E-S grade, Edmondson-Steiner grade; HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence/antagonist-II

Supplementary Table 3. Association of tumor TonEBP expression with clinical parameters

Variables	**tT0** (t0 and t1) **(n = 80)**	**tT1** (t2 to t4) **(n = 216)**	***P* value**
Age (mean ± S.D.)	56.3 ± 9.4	56.8 ±10.0	0.686
Sex (male/female)	68/12	181/35	0.801
Tumor size	4.07 ± 3.63	4.68 ± 3.22	0.163
E-S grade (I:II:III:IV)	4:46:28:2	5:79:116:16	0.003
Microvascular invasion	16	73	0.022
Lymphovascular invasion	8	41	0.065
Bile duct invasion	1	10	0.299
Recurrence	29	115	0.006
Metastasis	10	51	0.036
Death	15	69	0.025
HBV	57	177	0.045
HCV	8	17	0.558
AFP (ng/mL)	1344 ± 7703	3815 ± 19856	0.124
PIVKA-II (mAU/mL)	358 ± 789	2544 ± 11456	0.008
Preoperative HBV DNA (log_{10} IU/mL)	1.74 ± 2.21	2.41 ± 2.43	0.069
Underlying liver disease (CH/LC)	23/57	40/176	0.080
Child-Pugh class: A/B/C	73/7/0	207/9/0	0.121
Model for End-Stage Liver Disease score	5.36±4.52	5.34±3.68	0.962

tT0 and tT1 are defined in Figure 1K; Person's Chi-Squared test was performed.

Abbreviations: E-S grade, Edmondson-Steiner grade; HBV, Hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence/antagonist-II

Supplementary Table 4. TonEBP haplo-deficiency is resistant to DEN-induced liver injury

	PBS		DEN	
	***TonEBP*$^{+/+}$**	***TonEBP*$^{+/\Delta}$**	***TonEBP*$^{+/+}$**	***TonEBP*$^{+/\Delta}$**
Albumin (g/dL)	6.13 ± 0.133	5.47 ± 0.267 *	8.30 ± 0.518 $^{\#}$	6.64± 0.122 *
ALT (U/L)	29.33± 1.333	28 ± 2.309	158± 37.038 $^{\#}$	57.6 ± 12.875 *
AST (U/L)	110.67 ± 7.055	137.33 ± 14.111	192.5 ± 21.739 $^{\#}$	151.6 ± 10.074 *
LDH (U/L)	214 ± 22.983	388.27 ± 70.483	682.25 ± 97.885 $^{\#}$	477.4 ± 41.019 *
Urea (mg/dL)	101.33 ± 19.230	118.67 ± 1.333	131.5 ± 9.809 $^{\#}$	111.2 ± 5.744 *
Creatinine (mg/dL)	0.6 ± 0.040	0.61 ± 0.013	0.7 ± 0.030 $^{\#}$	0.6 ± 0.018 *
Total bile acid (mg/dL)	101.2 ± 12.427	101.87 ± 19.683	9.6 ± 2.877 $^{\#}$	18.12 ± 4.198 *
Total bilirubin (mg/dL)	0.148 ± 0.028	0.111 ± 0.003	0.1235 ± 0.011	0.1044 ± 0.008
Direct bilirubin (mg/dL)	0.197 ± 0.047	0.125 ± 0.001	0.137 ± 0.009 $^{\#}$	0.1304 ± 0.007

Note: $^{*}p<0.05$ compared corresponding *TonEBP*$^{+/+}$; $^{\#}p<0.05$ compared to corresponding PBS.

Abbreviations: ALT, Alanine transaminase; AST, Aspartate transaminase; LDH, Lactate dehydrogenase.

Supplementary Table 5. TonEBP haplo-deficiency is resistant to DEN/HFD-induced liver injury

	DEN/ND		DEN/HFD	
	***TonEBP*$^{+/+}$**	***TonEBP*$^{+/\Delta}$**	***TonEBP*$^{+/+}$**	***TonEBP*$^{+/\Delta}$**
Albumin (g/dL)	4.28 ± 0.17	4.13 ± 0.33 *	6.56 ± 0.32 $^{\#}$	5.83 ± 0.26 *
ALT (U/L)	71.11 ± 14.78	43.75 ± 8.76 *	310.63 ± 42.86 $^{\#}$	243.89 ± 21.24 *
AST (U/L)	170.00 ± 11.15	136.25 ± 8.3 *	316.25 ± 36.05 $^{\#}$	186.11 ± 15.47 *
Glucose (mg/dL)	149.44 ± 13.45	110 ± 8.45	124.38 ± 10.78	143.33 ± 6.61
TG (mg/dL)	44.44 ± 2.94	48.13 ± 2.51	69.38 ± 7.26 $^{\#}$	49.44 ± 3.48 *
Cholesterol (mg/dL)	110.56 ± 10.49	96.25 ± 7.60	289.38 ± 23.24 $^{\#}$	271.11 ± 30.98
LDH (U/L)	595.67 ± 36.43	498.06 ± 37.13 *	1251.19 ± 167.21 $^{\#}$	741.94 ± 81.09 *

Note: $^{*}p<0.05$ compared corresponding *TonEBP*$^{+/+}$; $^{\#}p<0.05$ compared to corresponding DEN/ND.

Abbreviations: ND, normal diet; HFD, high fat diet; ALT, Alanine transaminase; AST, Aspartate transaminase; LDH, Lactate dehydrogenase; TG, Triglyceride

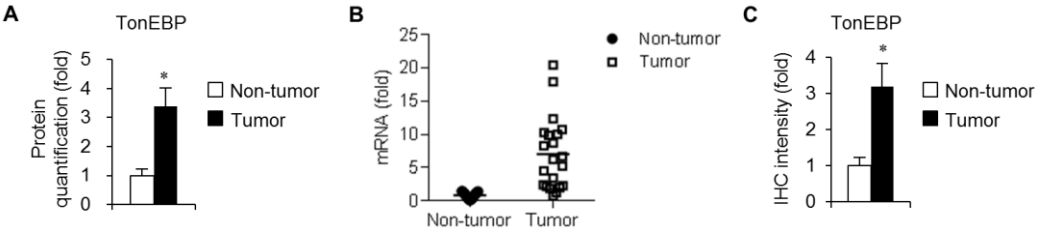
Supplementary Table 6. TonEBP expression was associated with COX-2 expression in patients with HCC

	tNT0	tNT1	Total	P value
cNT0	91	85	176	
cNT1	39	81	120	0.001
Total	130	166	296	
	tT0	**tT1**	**Total**	**P value**
cT0	47	86	133	
cT1	33	130	163	0.004
Total	130	166	296	

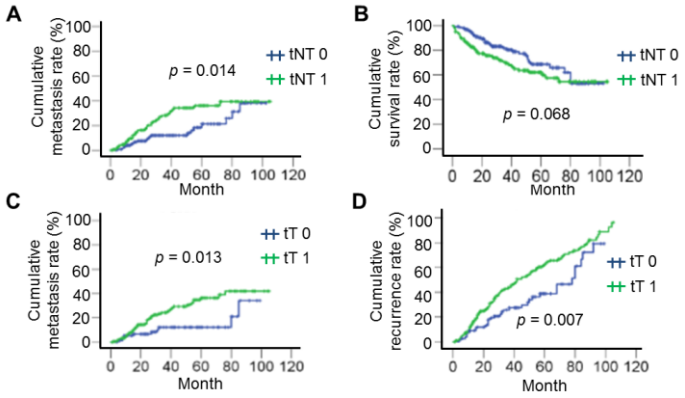
tNT0 and tNT1 are defined in Figure 1J and tT0 and tT1 are defined in Figure 1K.

COX-2 staining intensity was assigned to five grades as TonEBP (c0 – c4). COX-2 expression in Non-tumor region was stratified to cNT0 (c0, n=176) vs. cNT1 (c1-c4, n=120) and that in tumor region was stratified to cT0 (t0-1, n=133) vs. cNT1 (t2-4, n=163); Person's Chi-Squared test was performed

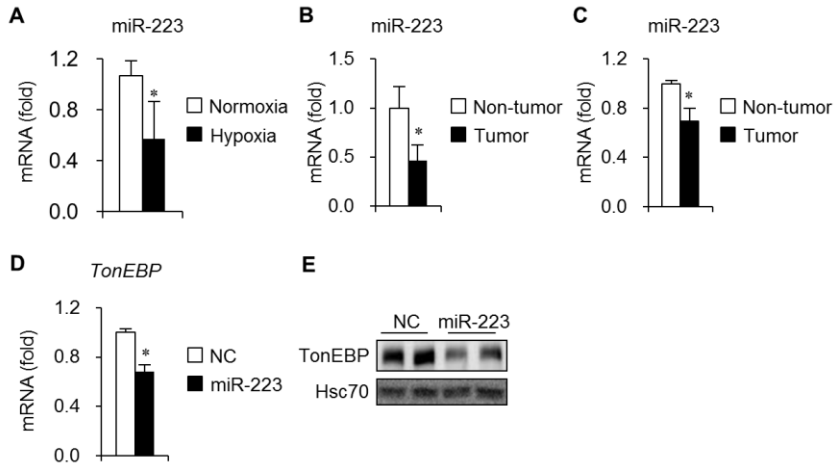
SUPPLEMENTARY FIGURES



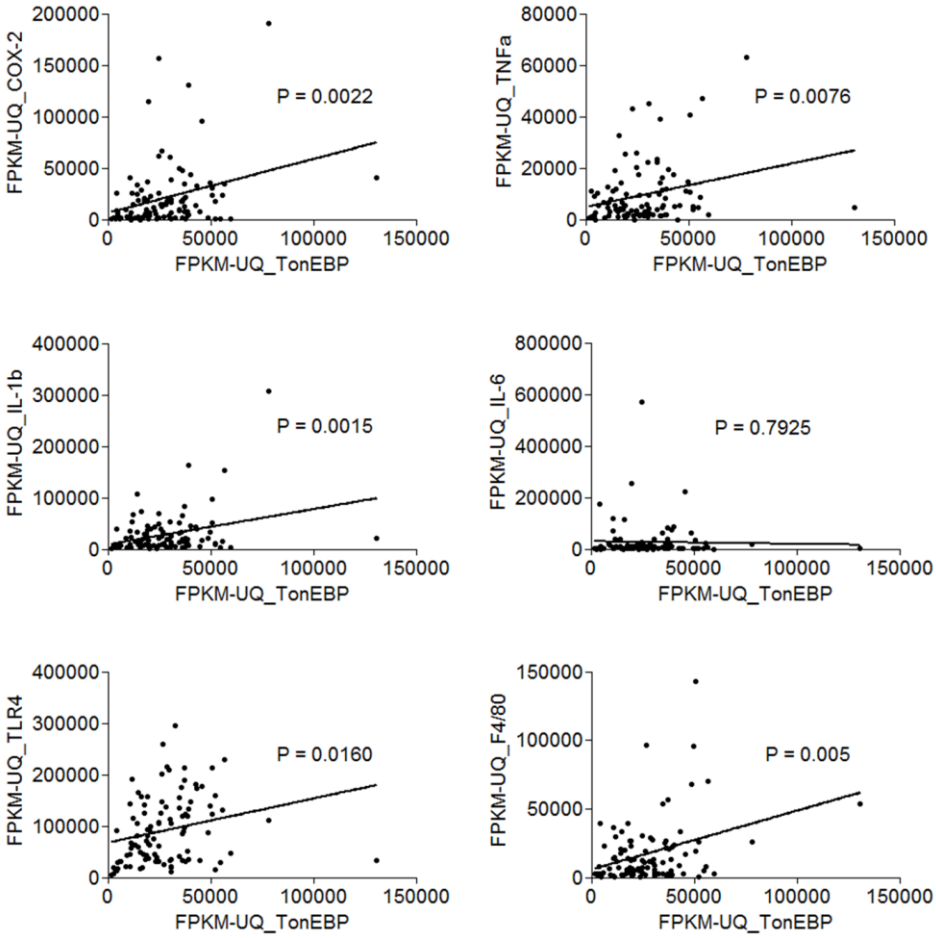
Supplementary Figure 1. Hepatic TonEBP expression is elevated in HCC. (A) Intensity of TonEBP bands in Figure 1B was measured and shown in mean + SD, n = 7. *$p < 0.05$ compared to non-tumor. (B) TonEBP mRNA was measured from a tumor and its surrounding non-tumor region in each of 25 patients. (C) Intensity of TonEBP immunohistochemical staining from figure 1E was quantified and shown in mean + SEM, n = 296. *$p < 0.05$ compared to non-tumor.



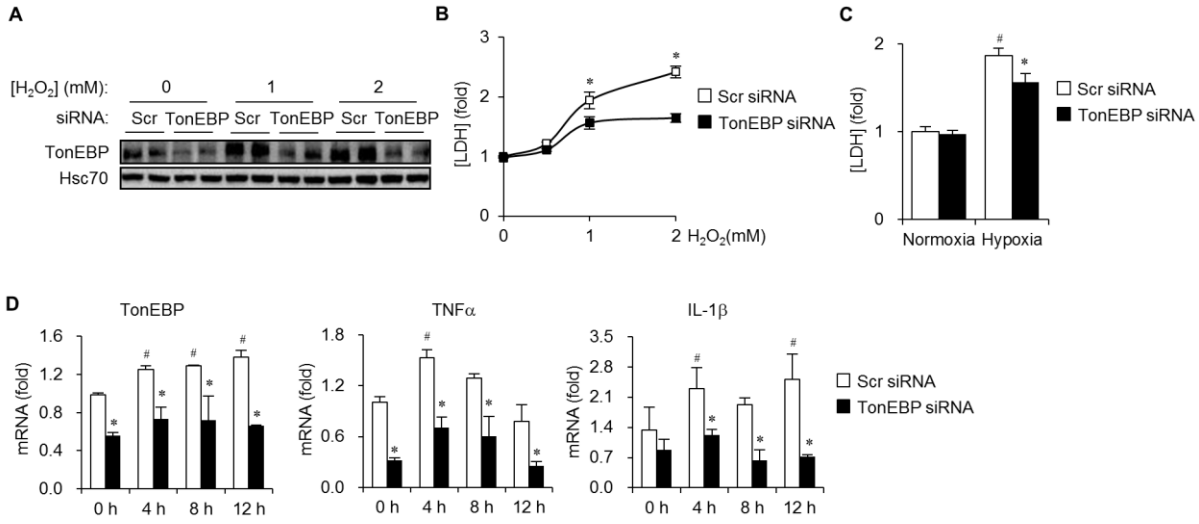
Supplementary Figure 2. Hepatic TonEBP expression is associated with poor postoperative prognosis in patients with HCC (A,B) Kaplan-Meier plot of (A) cumulative metastasis and (B) overall survival in patients whose non-tumor expression of TonEBP was t0 (tNT 0) *vs.* those patients whose non-tumor expression was higher – t1 to t4 (tNT 1). (C,D) Kaplan-Meier plot of (C) cumulative metastasis and (D) cumulative recurrence in patients whose expression of TonEBP in tumor was t0 and t1 (tT 0) *vs.* t2 to t4 (tT 1).



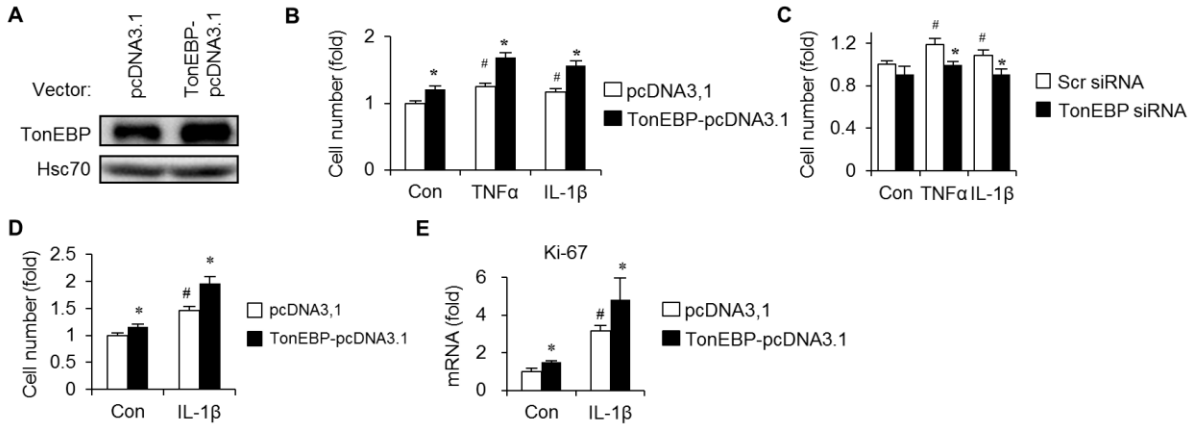
Supplementary Figure 3. Elevated expression of TonEBP in HCC was associated with a fall in the abundance of miR-223. (A) RT-qPCR analysis of miR-223 in HepG2 cells incubated for 12 h in normoxia or hypoxia (2% oxygen). Mean + SD, n = 3. *$p < 0.05$ compared to normoxia. (B) RT-qPCR analysis of miR-223 in non-tumor and tumor regions from HCC patients analyzed in figure 1G. Mean + SEM, n = 38, *$p < 0.05$ compared to corresponding non-tumor. (C) RT-qPCR analysis of miR-223 in non-tumor and tumor regions from DEN-treated animals analyzed in figure 1A. Mean + SEM, n = 18, *$p < 0.05$ compared to corresponding non-tumor. (D) RT-qPCR analysis of TonEBP in cells transfected with miRNA-223 mimic (miR-223) or non-specific control RNA (NC). n = 3. *$p < 0.05$ compared to NC. (E) Immunoblots of cells transfected with miR-223 or NC.



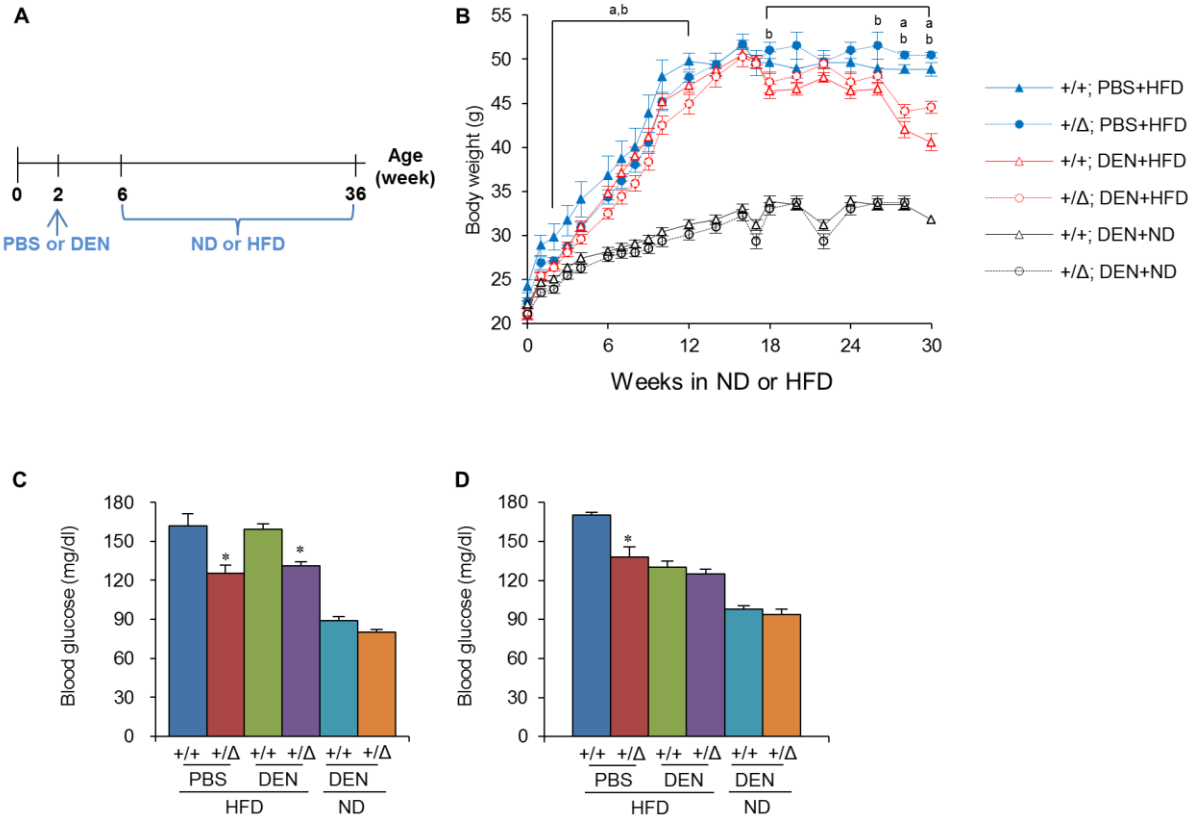
Supplementary Figure 4. Expression of TonEBP is associated with inflammation in patients with HCC. TonEBP vs. inflammatory gene transcript abundance in tumors from 100 HCC patients. Data were obtained from the Genomic Data Commons portal using Upper Quantile normalized RNA-seq FPKM (FPKM-UQ).



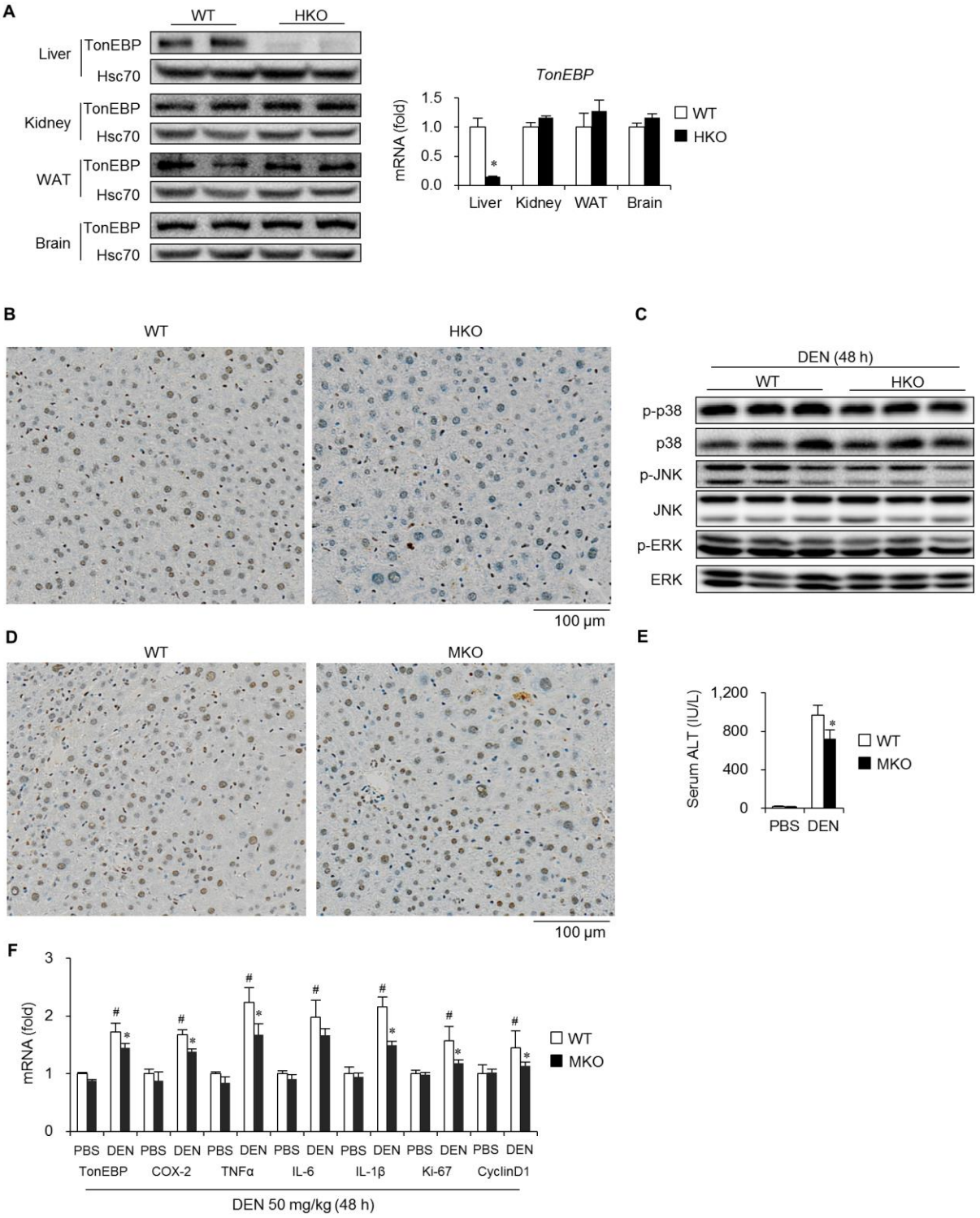
Supplementary Figure 5. TonEBP promotes oxidative stress-induced cell death and inflammation (A) Immunoblot analyses of HepG2 cells transfected with TonEBP-targeting siRNA or non-specific scrambled (Scr) siRNA followed by a 24 h treatment with 0 to 2 mM H_2O_2 as indicated. (B) HepG2 cells were transfected with siRNA followed by a treatment with H_2O_2. LDH was measured from cultured medium and concentration was expressed relative to treatment with no H_2O_2. Mean ± SD, n = 3. *$p < 0.05$ compared to TonEBP siRNA. (C) Cells transfected with siRNA were treated for 24h with hypoxia (2% oxygen) or normoxia. LDH was measured from cultured medium. *$p < 0.05$ compared to corresponding Scr siRNA. #$p<0.05$ compared to corresponding normoxia. (D) RT-qPCR analyses of TonEBP, TNFα, or IL-1β in cells transfected with siRNA followed by treatment with 1 mM H_2O_2 for up to 12 h, as indicated. Mean + SD, *$p < 0.05$ compared to corresponding Scr siRNA. #$p<0.05$ compared to corresponding 0 h.



Supplementary Figure 6. TonEBP-induced paracrine factors promote cell proliferation (A) HepG2 cells transfected with pcDNA3.1 or TonEBP-pcDNA3.1 were immunoblotted 48 h later. (B) Cells were transfected as in A. 24 h later TNFα (10 ng/ml), IL-1β (20 ng/ml), or vehicle (Con) were added, and then, the cells were cultured for an additional 24 h followed by trypsinization and cell counting. Mean + SD, n = 4. *$p < 0.05$ compared corresponding pcDNA3.1. #$p < 0.05$ compared corresponding Con. (C) Cells were transfected with siRNA. They were treated with TNFα or IL-1β and counted as in B. (D) Cells transfected as in A were seeded on a permeable support. The transfected cells were co-cultured above a bed of non-transfected for 24 h. IL-1β was added to the medium and the cells cultured for an additional 48 h before counting of the non-transfected cells. (E) The non-transfected cells in online supplementary figure 3D were analyzed for Ki-67 mRNA by RT-qPCR.

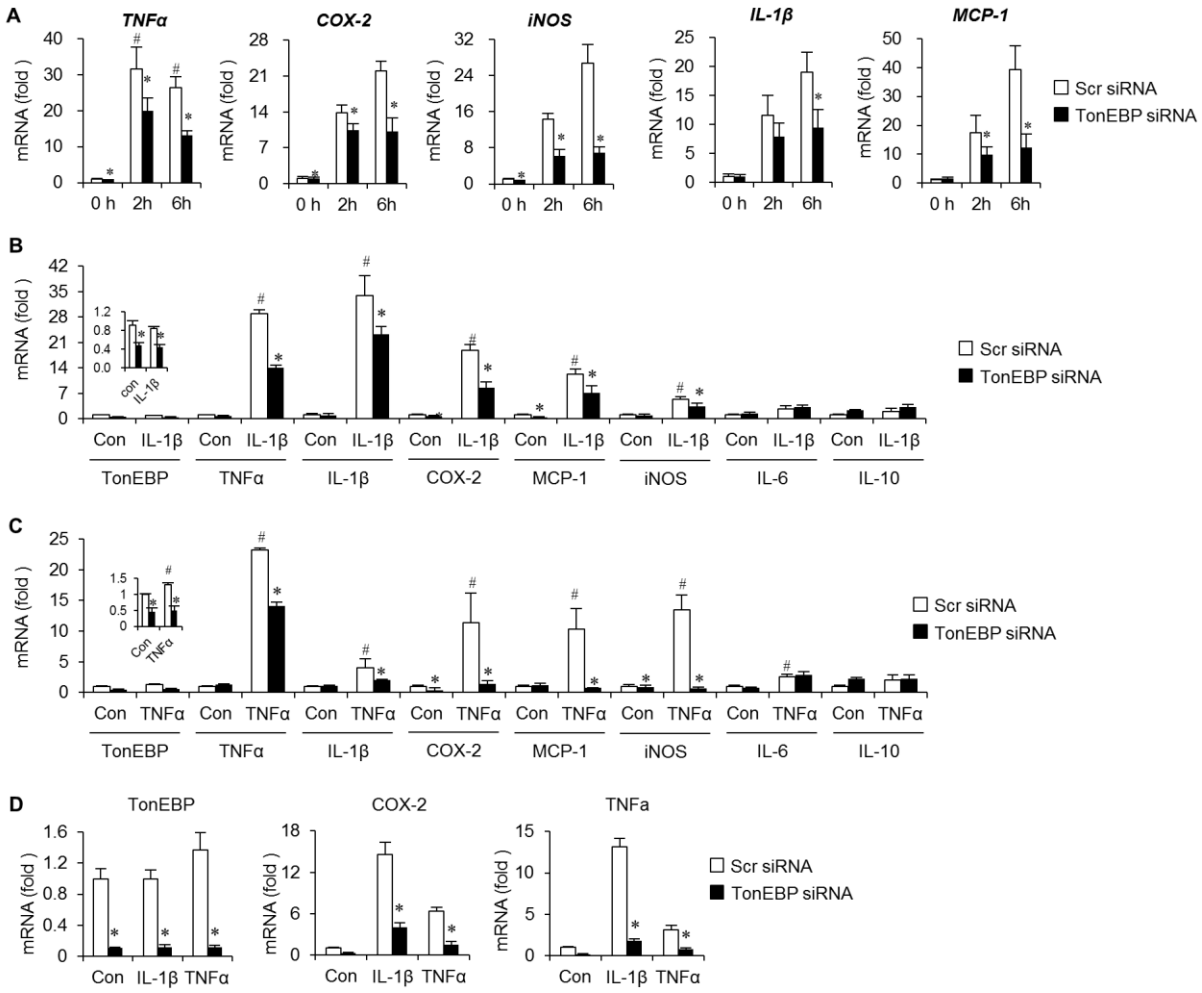


Supplementary Figure 7. Body weight and blood glucose in animals treated with DEN and fed with HFD (A) Experimental scheme of DEN (diethylnitrosamine) or PBS (phosphate-buffered saline: vehicle) injection and feeding with HFD (high fat diet) or ND (normal diet) in *TonEBP*$^{+/\Delta}$ mice and their *TonEBP*$^{+/+}$ littermates. (B) Body weight during the 30 week with HFD (n = 15) or ND (n = 12). Mean ± SEM. +/+: *TonEBP*$^{+/+}$ mice. +/Δ: *TonEBP*$^{+/+}$ mice. p<0.05: a, +/+ vs. +/Δ in PBS + HFD; b,+/+ vs. +/Δ in DEN + HFD; c, PBS + HFD vs. DEN + HFD in +/+. (C,D) Serum blood glucose levels after 24 weeks (C) or 30 weeks (D) of HFD or ND. Mean + SEM. *p < 0.05 compared to corresponding +/+.

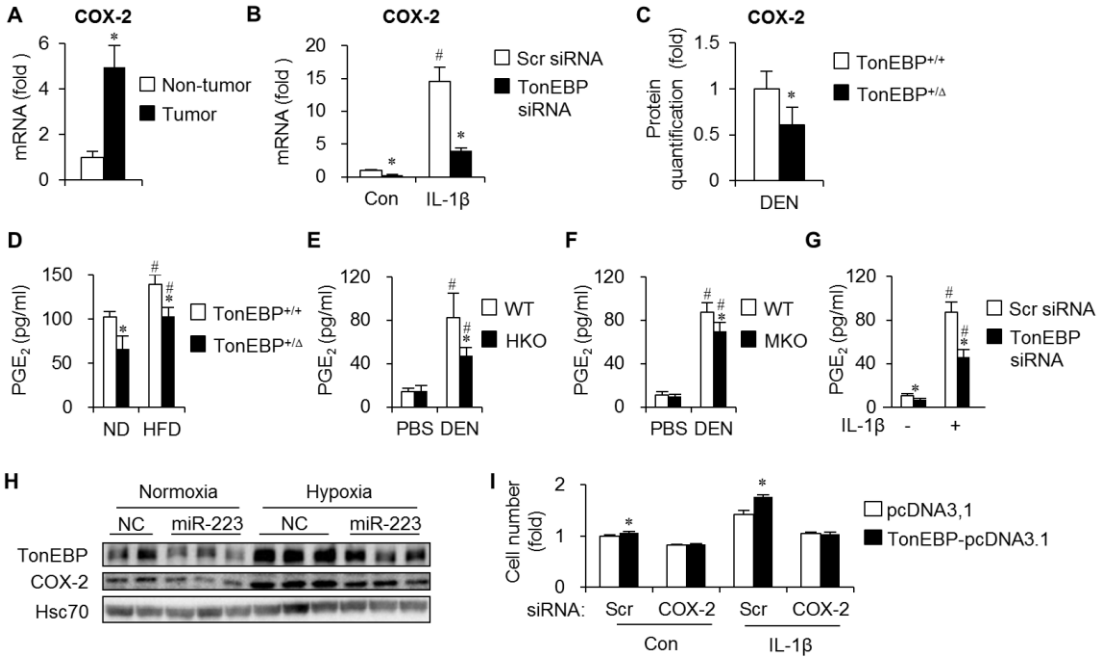


Supplementary Figure 8. Signaling and hepatic inflammation in hepatocyte- and myeloid-specific TonEBP deficiency (A) Livers, kidneys, white adipose tissues (WAT), and brains from 10 week old *TonEBP*$^{fl/fl}$*; Albumin cre*$^{+/-}$ mice (HKO) and their *TonEBP*$^{fl/fl}$*; Albumin cre*$^{-/-}$ (WT) littermates were analyzed by immunoblotting (2 animals from each genotype) or RT-qPCR (n = 7). *p < 0.05

compared to corresponding WT. (B) Immunohistochemical images of TonEBP in hepatic tissues from WT or HKO mice. (C) Animals were treated with DEN. 48 h later, livers were immunoblotted for p38, JNK, and ERK, and their phosphorylated forms (p-p38, p-JNK, and p-ERK). 3 animals were used from each genotype. (D) Immunohistochemical images of TonEBP in hepatic tissues from WT or MKO mice. (E) Serum ALT was measured 48 h after injection with PBS (n = 5) or DEN (n = 7) into *TonEBP*$^{fl/fl}$*; Lys-M cre*$^{+/-}$ mice (MKO) and their *TonEBP*$^{fl/fl}$*; Lys-M cre*$^{-/-}$ (WT) littermates. Mean + SEM, *$p < 0.05$ compared to corresponding WT. (F) RT-qPCR analyses of inflammatory genes and proliferation markers from livers.

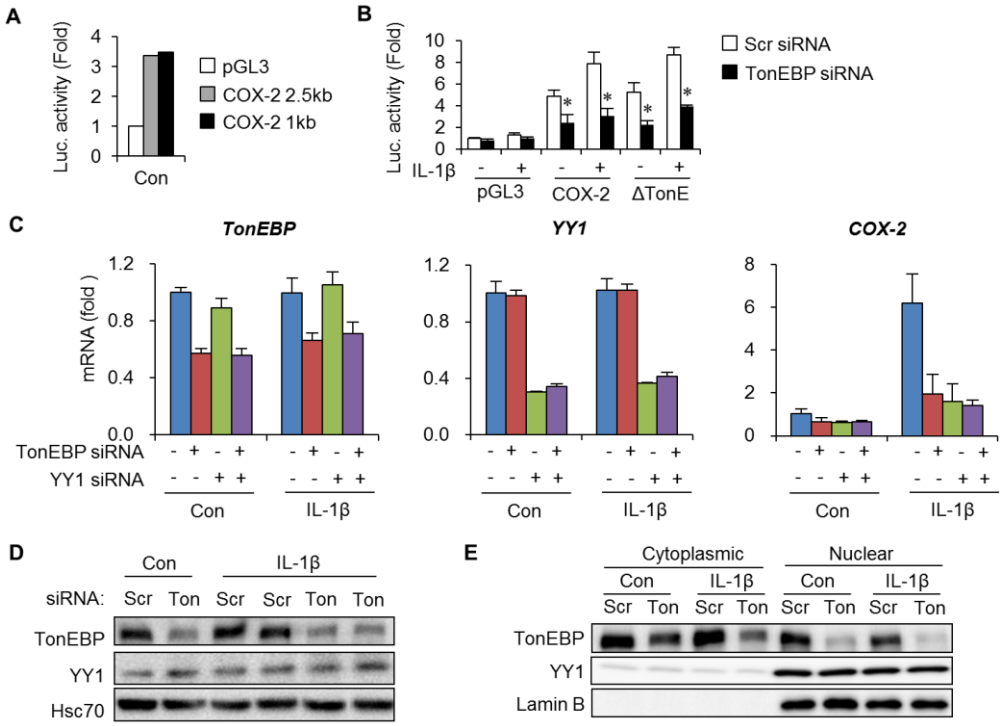


Supplementary Figure 9. TonEBP deficiency attenuates inflammation in hepatocytes (A–C) RT-qPCR analyses of inflammatory genes in HepG2 cells transfected with siRNA. (A) The transfected cells were treated for up to 6 hr as indicated with LPS (500 ng/ml). (B,C) The transfected cells were treated for 6 h with (B) IL-1β (20 ng/ml), (C) TNFα (10 ng/ml), or vehicle only (Con). (D) AML-12 cells were transfected with siRNA and treated for 6 h with 10 ng/ml of IL-1β or 80 ng/ml of TNFα. mRNA was analyzed as above. Mean + SD, n = 4. *$p < 0.05$ compared to Scr siRNA. #$p < 0.05$ compared to corresponding 0 h or Con.



Supplementary Figure 10. TonEBP stimulates COX-2 expression and PGE_2 production. (A) RT-qPCR of COX-2 in non-tumor and tumor region from HCC patients analyzed in figure 1G. Mean + SEM, n = 38, **p* < 0.05 compared to corresponding non-tumor. (B) HepG2 cells transfected with siRNA were treated for 6 h with IL-1β or vehicle (Con). COX-2 mRNA was measured by RT-qPCR. Mean + SD, n = 4. **p* < 0.05 compared to corresponding Scr siRNA. #*p*<0.05 compared to corresponding Con. (C) Intensity COX-2 bands in Figure 5C was quantified and shown in mean + SD. n = 6. *p < 0.05 compared to $TonEBP^{+/+}$. (D) Serum PGE_2 levels in $TonEBP^{+/\Delta}$ or $TonEBP^{+/+}$ animals fed with ND (n = 7) or HFD (n = 8) from Figure 3. Mean + SEM. **p* < 0.05 compared to corresponding $TonEBP^{+/+}$. #*p* < 0.05 compared to corresponding ND. (E, F) Serum PGE_2 levels in PBS- (n = 5) and DEN-treated animals (n = 6) from (E) figure 4B – D and (F) supplementary figure 8C,D. (G) HepG2 cells transfected with siRNA were treated with IL-1β as indicated. PGE_2 was measured from culture medium. n = 4. Mean + SD, **p* < 0.05 compared to corresponding Scr. $^{\#}p$ < 0.05 compared to corresponding -. (H) Cells transfected with miR-223 or NC as in Figure 1L were subjected to a 24 h hypoxia or normoxia. TonEBP, COX-2, and Hsc70 were visualized by immunoblotting. (I) Cells transfected as in supplementary figure 6A followed by transfection of scr siRNA or COX-2 siRNA were seeded on a permeable support. The transfected cells were co-cultured above a bed of non-transfected for 24 h. IL-1β was added to the medium and the cells cultured for an

additional 48 h before counting of the non-transfected cells



Supplementary Figure 11. TonEBP-dependent stimulation of COX-2 requires transcription factor YY1. (A) HepG2 cells were transfected with promotorless vector (pGL3), COX-2 promoter reporter with 2.5 kb of sequence containing a TonEBP binding site (COX-2 2.5kb) or with 1 kb of sequence without TonEBP site (COX-2 1kb). Luciferase activity was measured and expressed relative to pGL3 transfected cells. Means of 2 independent experiments are shown. (B) HepG2 cells were transfected with siRNA followed by a second transfection with pGL3, COX-2 2.5kb (COX-2), or a mutant COX-2 promoter reporter whose TonEBP binding site was inactivated (ΔTonE). Luciferase activity was measured after treatment without or with IL-1β. Mean + SD, n = 3. **p*<0.05 compared to corresponding Scr. (C) Cells were transfected with various combinations of TonEBP-targeted, YY-1-targeted, and Scr siRNA (-) as indicated. They were treated with IL-1β and RT-qPCR was performed to analyze TonEBP, YY1, and COX-2 mRNA. Mean + SD, n = 4. (D) Cells transfected with siRNA were treated for 1 h with IL-1β and immunoblotted. Ton, TonEBP. (E) Cells were transfected and treated for 2 h with IL-1β. After fractionation into cytoplasmic and nuclear fractions, they were immunoblotted.