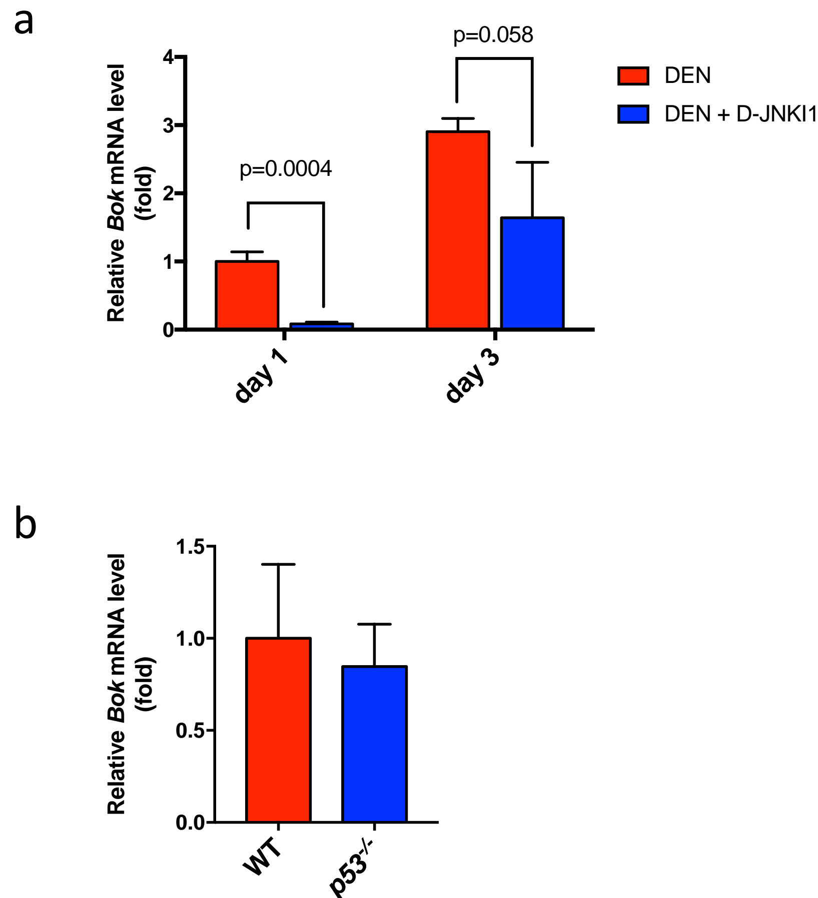
ONLINE SUPPLEMENTARY MATERIAL

Rabachini T *et al*, BOK promotes chemical induced hepatocarcinogenesis in mice

Supplementary Figures



Supplementary Figure S1. *Bok* mRNA induction by DEN is partially JNK dependent and p53 independent. (a) qPCR analysis of *Bok* mRNA expression in livers derived from WT mice pretreated with PBS or 20 mg/kg of D-JNKI1 inhibitory peptide (1) for 30 min prior to challenge with DEN (100 mg/kg) for 1 or 3 days. *Gapdh* served as reference gene. Values represent the mean ± SD (n= 3 mice per group). (b) qPCR analysis of *Bok* mRNA expression in livers derived from WT and *TP53*$^{-/-}$ mice treated with DEN (100 mg/kg) for 1 day. *Gapdh* served as reference gene. Values represent the mean ± SD (n= 3 mice per group).

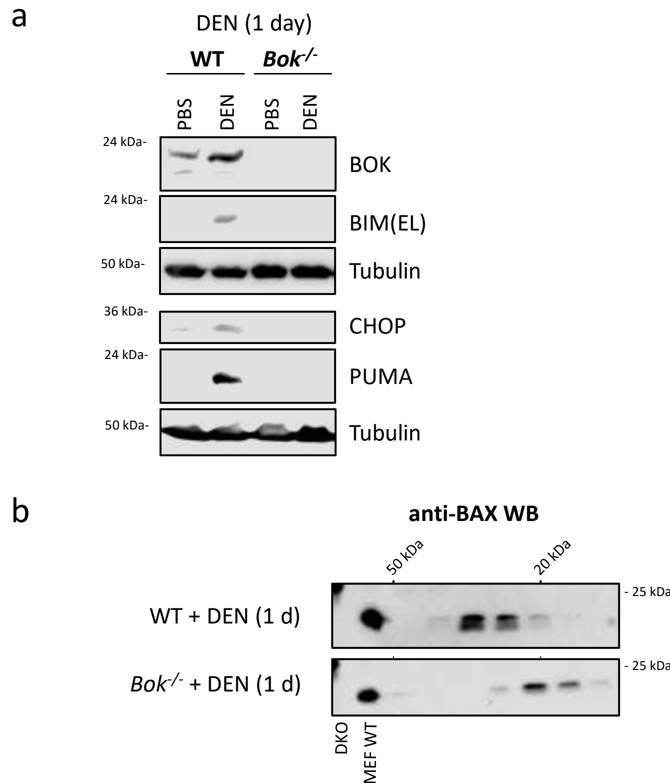


Table 1: Relation of theoretical molecular weight and its corresponding retention volume using a Superdex™200 10/300 column.

V_r (mL)	$V_{elution}$ /V_0	$\log_{10}$MW	MW (kDa)
13.00	1.548	2.052	112.7
13.50	1.607	1.952	89.6
14.00	1.667	1.853	71.2
14.50	1.726	1.753	56.6
15.00	1.786	1.653	45.0
15.50	1.845	1.554	35.8
16.00	1.905	1.454	28.5
16.50	1.964	1.355	22.6
17.00	2.024	1.255	18.0
17.50	2.083	1.155	14.3
18.00	2.143	1.056	11.4

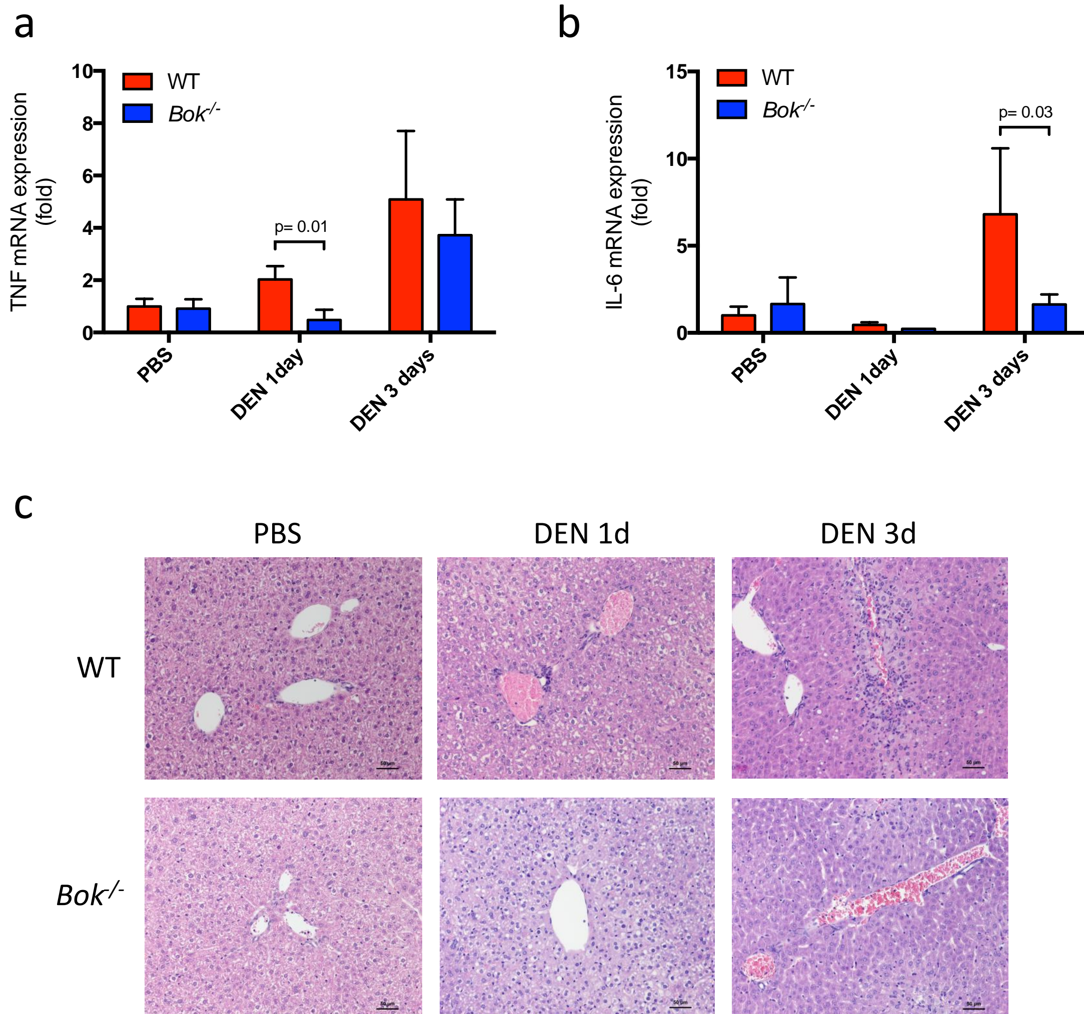
$\text{Log}_{10}\text{MW} = (V_{elution}/V_0) \times m + i$

V_0= 8.4 mL; m=-1.6731; i=4.6412

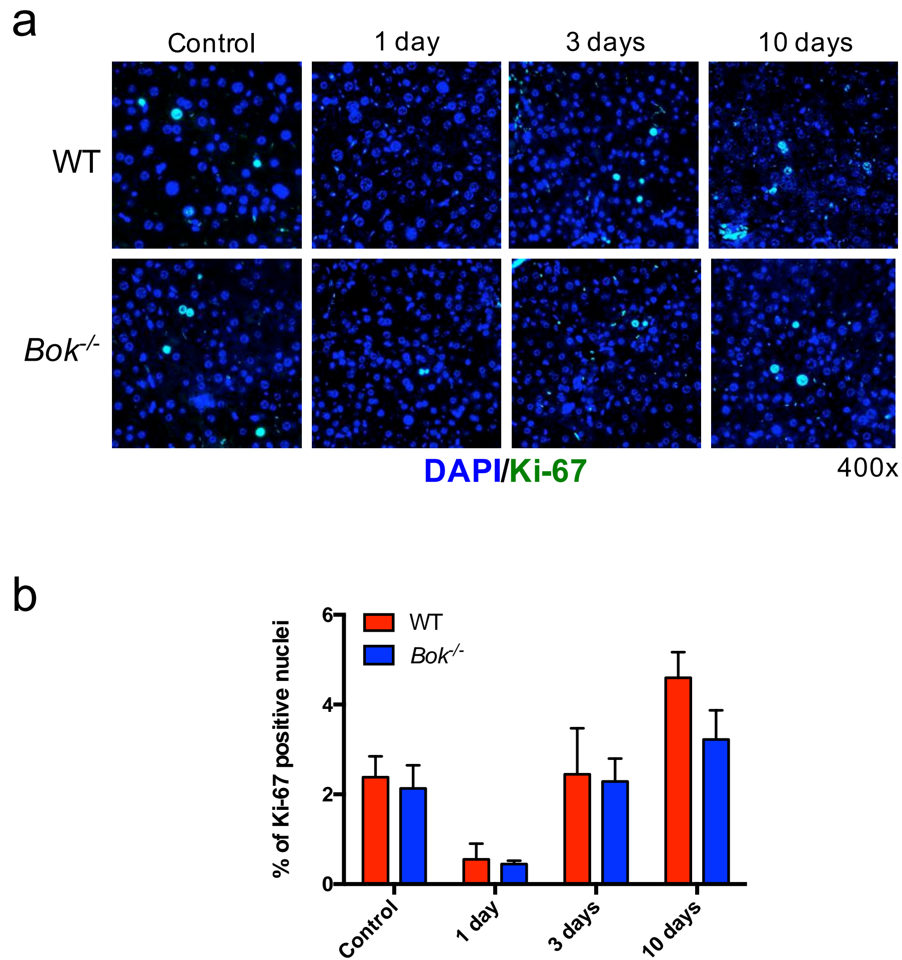
Table 2: Determination of BAX oligomer molecular weight in liver extracts from animals treated with DEN by size exclusion chromatography.

WT animals (DEN)			*Bok*-/- animals (DEN)		
V_r (mL)	Fraction Nº	MW (kDa)	V_r (mL)	Fraction Nº	MW (kDa)
13.33	24	96.8	13.39	24	94.2
13.83	25	77	13.89	25	74.9
14.33	26	61.2	14.39	26	59.6
14.83	27	48.7	14.89	27	47.3
15.33	28	38.7	15.39	28	37.6
15.83	29	30.8	15.89	29	29.9
16.33	30	24.5	16.39	30	23.8
16.83	31	19.4	16.89	31	18.9
17.33	32	15.5	17.39	32	15

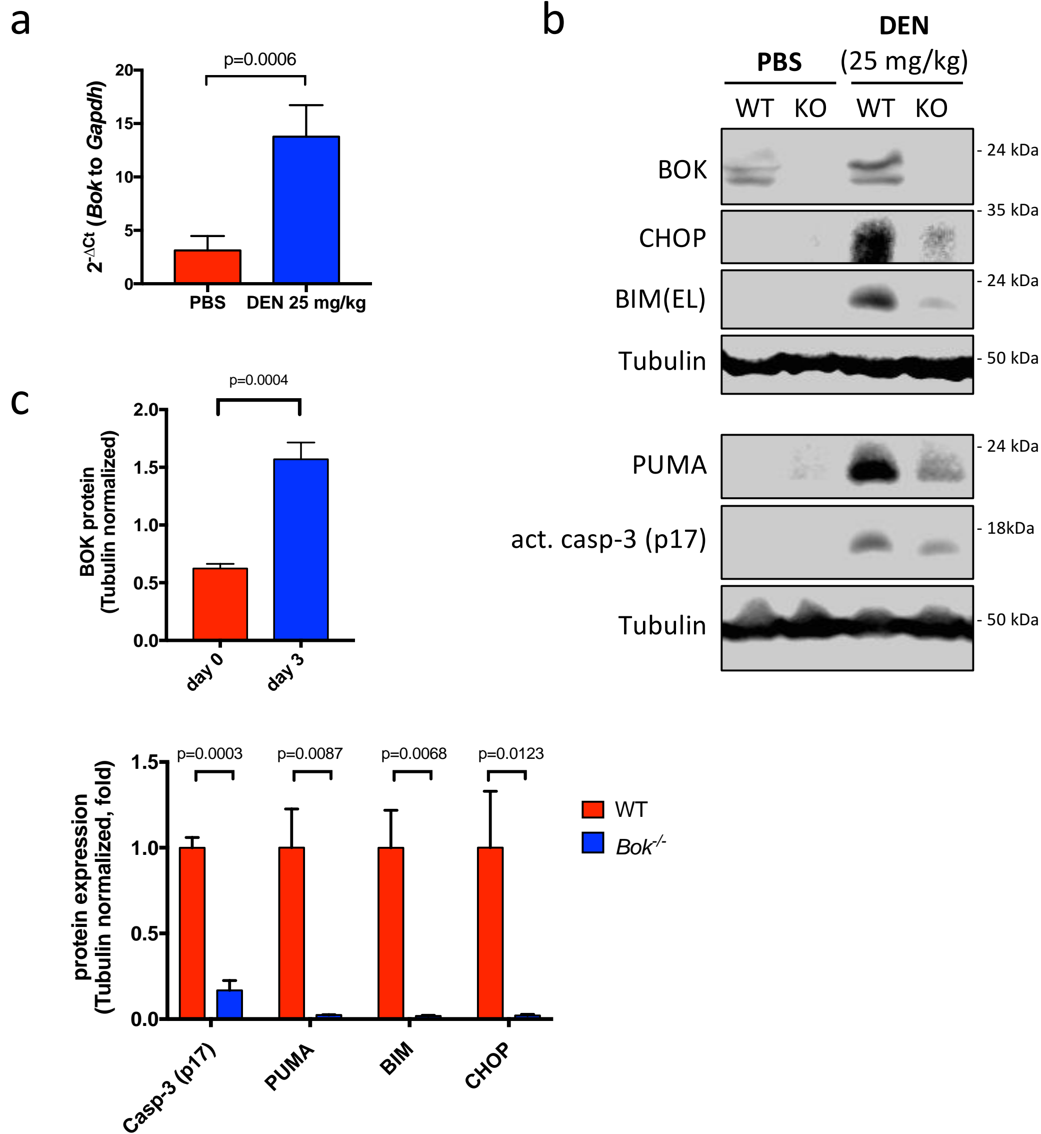
Supplementary Figure S2. (a) Western blot data of liver lysates from WT and *Bok*-/- mice treated for 1 day with PBS or DEN (100 mg/kg) and probed for the indicated proteins. (b) Size exclusion analysis of BAX oligomerization in liver extracts from DEN-treated animals. WT and *Bok*-/- mice were injected i.p. with 100 mg/kg DEN and euthanized after 24h. Total Liver extracts were prepared in IBc buffer (10 mM Tris,1 mM EGTA, 2 mM $MgCl_2$, 200 mM sucrose, 1 % CHAPS, pH 7.5, plus protease inhibitor cocktail) using a Dounce homogenizer. 8 mg of each extract were loaded onto a Superdex-200 30/100 column equilibrated with IBc buffer. Fractions of 0.5 mL were collected and further analyzed by western blot for the presence of BAX. Molecular weight of BAX oligomers was estimated using a parallel run of molecular weight markers for gel filtration chromatography (MWGF1000 Sigma, Sigma-Aldrich).



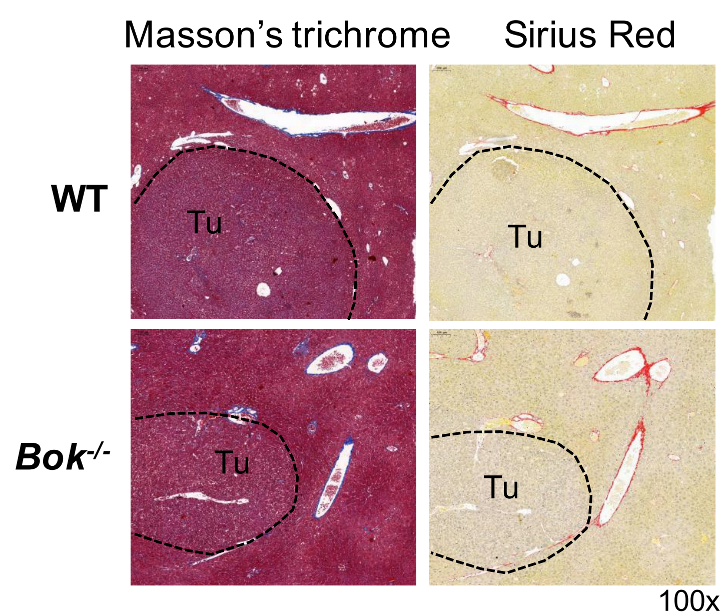
Supplementary Figure S3. *Bok*$^{-/-}$ mice present less inflammation after acute liver damage induced by DEN exposure. (a) TNFα and (b) IL-6 mRNA expression in livers from animals treated with DEN (100 mg/kg BW). *Hprt* served as reference gene. Values represent the mean ± SD (n= 3 mice per group). (c) Representative images of H&E stained liver sections from WT and *Bok*$^{-/-}$ mice after treatment with DEN (100 mg/kg) for 1 or 3 days (scale bars = 50 µm). Strong leukocyte infiltration is evident in 3 day-treated WT mice but less so in *Bok*$^{-/-}$ mice.



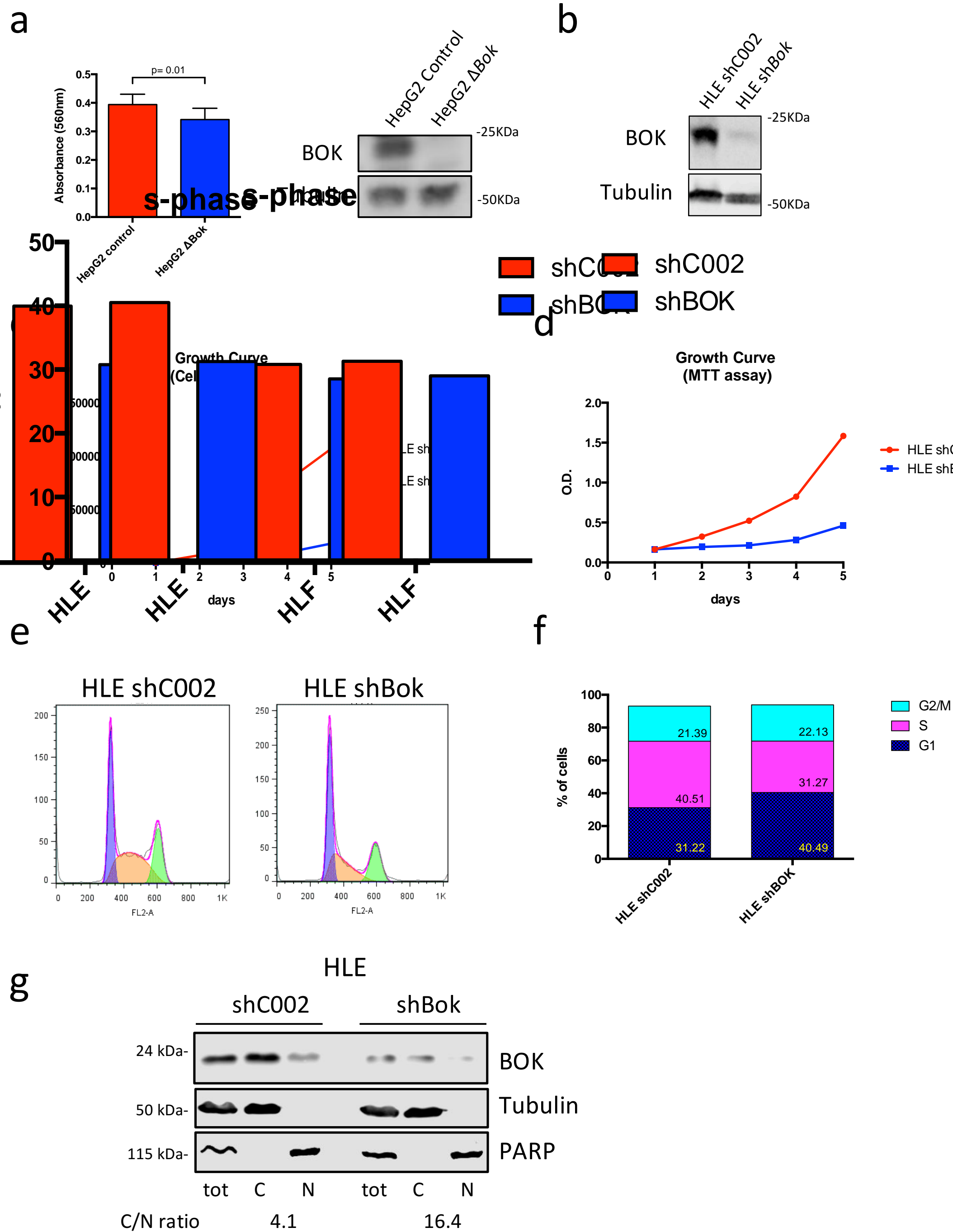
Supplementary Figure S4. (a) Representative fluorescence micrographs of Ki-67 staining and (b) quantification of proliferative cells in livers from WT and *Bok*$^{-/-}$ animals treated with 100 mg/kg BW DEN. Ki-67 index was determined by counting at least 900 cells/condition (400x magnification). Values represent the mean ± SD (n = 3 mice per group).



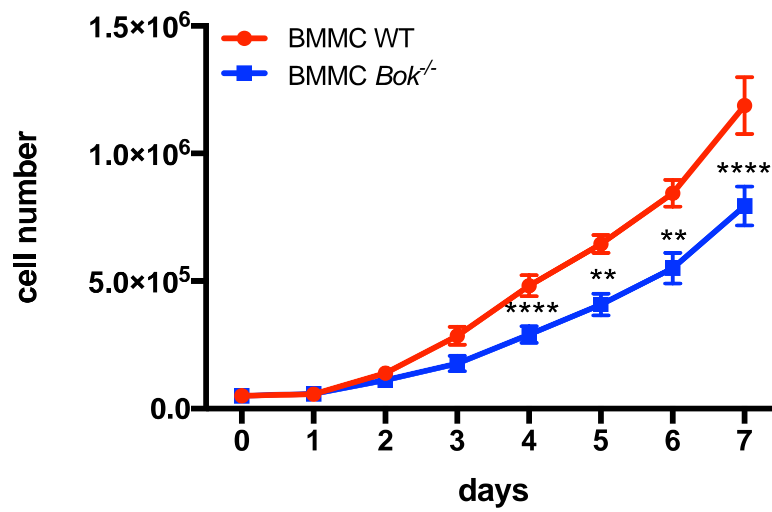
Supplementary Figure S5. (a) Adult WT mice were treated with PBS or 25 mg/kg of DEN for 3 days and *Bok* mRNA levels in their livers quantified by qPCR analysis. *Gapdh* served as reference gene. Values represent the mean ± SD (n = 4 mice per group). (b) Representative western blots of liver lysates derived from mice described in (a). (c) Quantification of western blot data using near-infrared fluorescence. Values represent the mean ± SD (n = 3 mice per group).



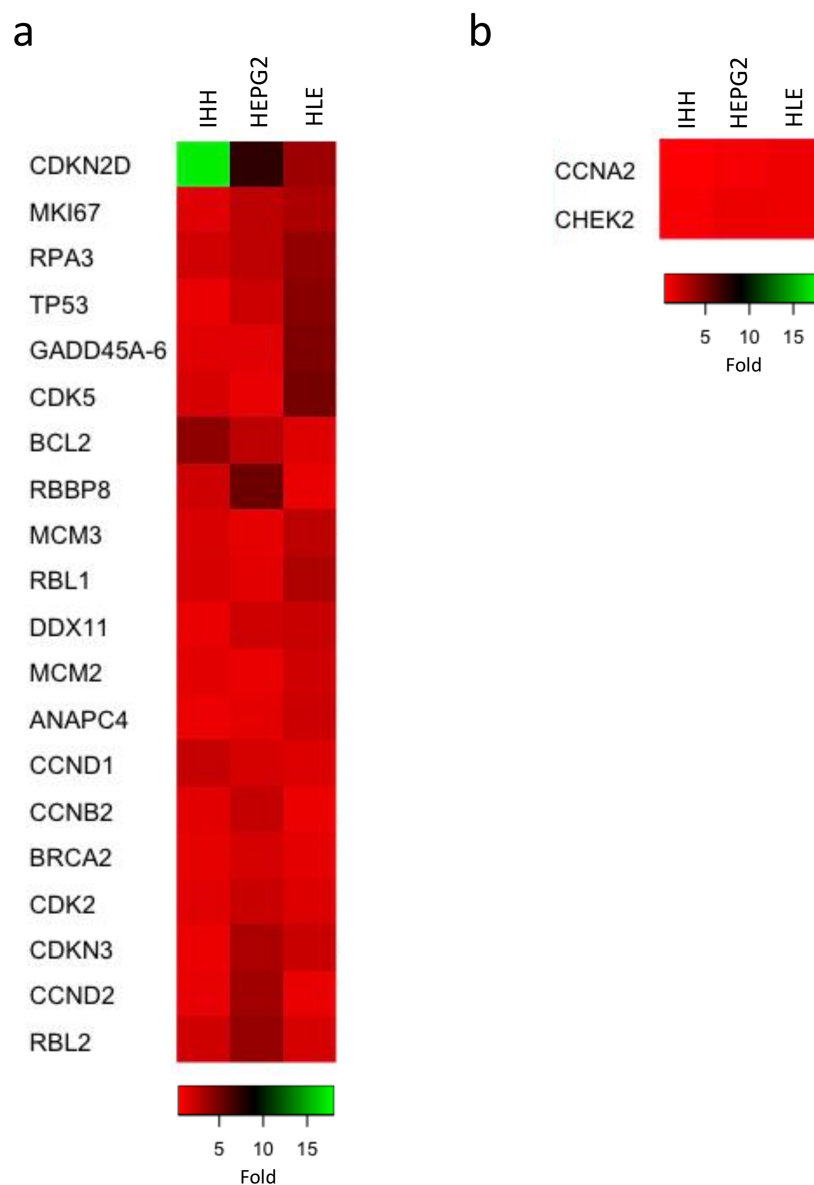
Supplementary Figure S6. HCC (Tu) containing liver sections from DEN-injected WT and *Bok*$^{-/-}$ mice were stained at the endpoint of 9 months with Masson's trichrome and Sirius Red stain to detect fibrosis.



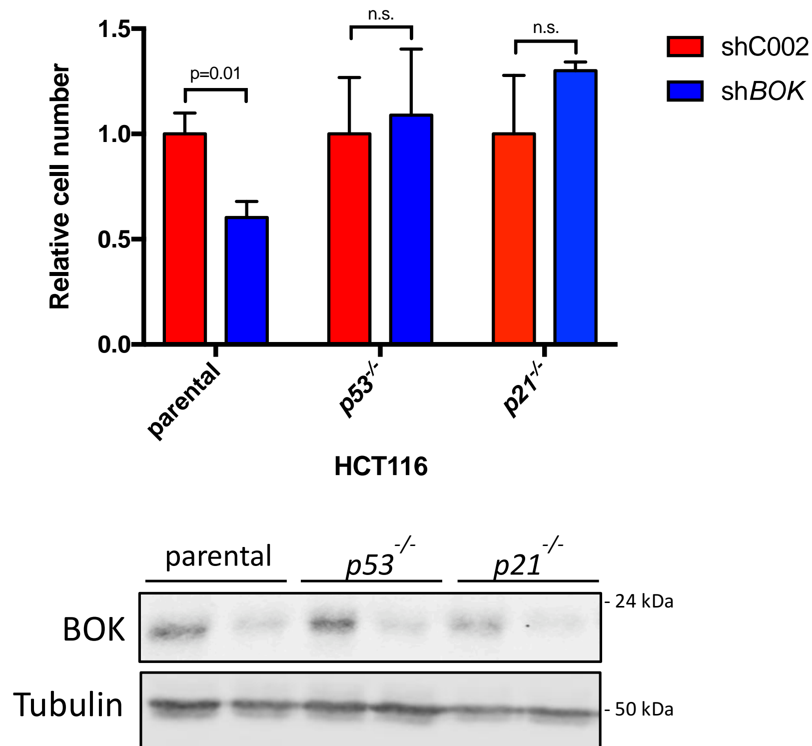
Supplementary Figure S7. *BOK* deficiency negatively affects proliferation of human HCC cell lines. (a) MTT assay showing that HepG2 with CRISPR/Cas9-mediated *BOK* disruption proliferate slower than controls. (b) Western blot analysis showing BOK downregulation by shRNA in the HCC cell line HLE. (c) Growth curves, (d) MTT assay and (e and f) cell cycle profiling of HLE cells indicating that *BOK* deficient cells proliferate slower and present less cells in S-phase than controls. (g) HLE cells with downregulated *BOK* have a increased cytoplasmic to nuclear (C/N) ratio of BOK compared to control cells.



Supplementary Figure S8. *Bok*$^{-/-}$ bone marrow-derived mast cells proliferate slower than WT controls. Values represent the mean ± SD from 3 independent mast cell cultures (each derived from an individual mouse) per genotype. ** P<0.005, ****P<0.0001.



Supplementary Figure S9. (a) Heat map showing genes upregulated or (b) downregulated in cells with *BOK* expression downregulated by CRISPR/Cas9 (IHH and HepG2) or shRNA (HLE).



Supplementary Figure S10. BOK deficiency affects proliferation of HCT116 isogenic cell lines in a *p53/p21* dependent-manner. (a) Cells were seeded in 6-well plates and counted after 7 days. Values represent the mean ± SD (n = 3). Western blot indicating BOK downregulation in HCT116 parental, *p53*$^{-/-}$ and *p21*$^{-/-}$.

Supplementary Table S1. Primers used for qPCR reactions

mmHprt1	Fw 5’-TGGATACAGGCCAGACTTTGTT -3’
	Rev 5’- CAGATTCAACTTGCGCTCATC -3’
mmGapdh	Fw 5’- CCTCGTCCCGTAGACAAAATG -3’
	Rev 5’- TGAAGGGGTCGTTGATGGC -3’
mmBok	Fw 5’- CCACAGACAAGGAGCTGGT -3’
	Rev 5’- TAGCCAAGGTCTTGCGTACA -3’
mmBim	Fw 5’- GAGTTGTGACAAGTCAACACAAACC -3’
	Rev 5’- GAAGATAAAGCGTAACAGTTGTAAGATAACC -3’
mmPuma	Fw 5’- ATGCCTGCCTCACCTTCATCT -3’
	Rev 5’- AGCACAGGATTCACAGTCTGGA -3’
mmIL-6	Fw 5’- ACAAGTCGGAGGCTTAATTACACAT -3’
	Rev 5’- TTGCCATTGCACAACTCTTTT -3’
mmTNF	Fw 5’- ATGAGAAGTTCCCAAATGGC -3’
	Rev 5’- CACTTGGTGGTTTGCTACGAC -3’

Supplementary Table S2. Sequence of the primers used to create the guide RNA 1, 2 and 3 targeting human *BOK*.

gRNA1	Fw 5'-**caccg**GATCTCGGCGGCGAAGACCG-3'
	Rev 5'-**aaac**CGGTCTTCGCCGCCGAGATC**c** -3'
gRNA2	Fw 5'-**caccg**AAAGGCGTCCATGATCTCGG-3'
	Rev 5'-**aaac**CCGAGATCATGGACGCCTTT**c** -3'
gRNA3	Fw 5'-**caccg**GTCTGTGGGCGAGCGGTCAA-3'
	Rev 5'-**aaac**TTGACCGCTCGCCCACAGAC**c**-3'

Targeted area in the human *BOK* gene

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                                                                          ◄ gRNA3
                                                                         AA CTG GCG AGC GGG TGT CTG
                                                      ◄ gRNA2
                                                 GG CTC TAG TAC CTG CGG AAA
                                      ◄ gRNA1
                                 GC CAG AAG CGG CGG CTC TAG
  1 atg gag gtg ctg cgg cgc tcc tcg gtc ttc gcc gcc gag atc atg gac gcc ttt gac cgc tcg ccc aca gac aag gag ctg gtg gcc cag gcc aag gcg ctg
    tac ctc cac gac gcc gcg agg agc cag aag cgg cgg ctc tag tac ctg cgg aaa ctg gcg agc ggg tgt ctg ttc ctc gac cac cgg gtc cgg ttc cgc gac
1    M   E   V   L   R   R   S   S   V   F   A   A   E   I   M   D   A   F   D   R   S   P   T   D   K   E   L   V   A   Q   A   K   A   L
    >>.......................................................................................................................................>

103 ggc cgg gag tac gtg cac gcg cgg ctg ctg cgc gcc ggc ctc tcc tgg agc gcg ccc gag cgt gcc gcg ccg gtc ccg gga cgc ctg gct gag gtg tgc gcg
    ccg gcc ctc atg cac gtg cgc gcc gac gac gcg cgg ccg gag agg acc tcg cgc ggg ctc gca cgg cgc ggc cag ggc cct gcg gac cga ctc cac acg cgc
35   G   R   E   Y   V   H   A   R   L   L   R   A   G   L   S   W   S   A   P   E   R   A   A   P   V   P   G   R   L   A   E   V   C   A
    >.................................................................................................................................>>

205 gtg ctg ctg cgc ctg gg
    cac gac gac gcg gac cc
69   V   L   L   R   L
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Supplementary materials and methods

Cell Culture

Primary C57BL/6 WT and *Bok*$^{-/-}$ mouse embryonic fibroblasts (MEF) immortalized with SV40 LT/pSG5 were generated as previously described (2). At least three independent lines were generated for each genotype and cultured in DMEM supplemented with 5% FCS and penicillin (100 IU/mL)/streptomycin (100 µg/mL). Immortalized human hepatocytes (IHH) were a kind gift from Dr. T. Brunner (Konstanz, DE). IHH were maintained in IMDM media supplemented with 2 mM L-glutamine, 10% FCS and penicillin/streptomycin. HepG2 and HLE human hepatocarcinoma cell lines were maintained in DMEM supplemented with 10% FCS and penicillin/streptomycin. All cell lines were cultivated under a 5% CO_2 humidified environment at 37°C.

Preparation of protein lysates

Frozen liver samples were homogenized in modified RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1% Triton X-100) containing Complete protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, CH), 1 mg/mL pepstatin and phosphatase inhibitors (2 mM sodium orthovanadate and 50 mM sodium fluoride) for 15 seconds using a pestle and motor mixer (VWR Pellet Mixer – VWR International GmbH, Dietikon, CH). After centrifugation at 14000 x g for 10 min, supernatant was collected and protein concentration was determined using a BCA assay (Fisher Scientific, Reinach, CH). Samples were boiled in Lämmli buffer containing 100 mM DTT before separation on denaturing SDS-PAGE gels and transfer to PVDF membranes (Immobilon-FL, Merck Millipore, Zug, CH).

CRISPR/Cas9-mediated *BOK* disruption

The *BOK* genomic locus was targeted using CRISPR/Cas9 technology producing IHH and HepG2 cell lines devoid of BOK protein expression. The lentiCRISPR v2

plasmid was a gift from Feng Zhang (Addgene plasmid #52961) (3). We designed three guide RNAs (gRNA1, gRNA2, gRNA3) targeting the exon 2 of human *BOK* using the publicly available service at http://crispr.mit.edu (Supplementary Table S2). HEK293T cells were transfected with the plasmids lentiCRISPR v2, pMD2GVSV-G, psPAX2 using X-tremeGENE HP DNA Transfection Reagent (Roche). Viruses were harvested after 24 h, filter-sterilized and freshly added to semi-confluent cultures of IHH or HepG2 cells in the presence of 8 µg/mL polybrene, followed by 3 weeks of selection with 2 µg/mL puromycin. The IHH line targeted with the gRNA3 exhibited the most consistent downregulation of BOK (4) and sequencing of subclones IHH1D10 and IHH2C12 confirmed a frameshift insertion destroying the *BOK* open-reading frame.

shRNA mediated downregulation of *BOK*

Mission^{TM} lentiviral clones expressing short hairpin RNA (shRNA) targeting human Bok (5'-CCGGCCGCTTCCTGAAGGCTGCCTTCTCGAGAAGGCAG CCTTCAGGAAGCGGTTTTTG) and non-targeting shRNA control (shC002), were purchased from Sigma-Aldrich. BOK re-expression was performed with CAD-G-Whiz lentiviral vector, kindly provided by Dr. M. Tschan (Bern, CH). Lentivirus production was performed as previously described (5). Briefly, Mission^{TM} plasmids or CAD-G-Whiz were transiently co-transfected with packaging plasmids pMD2GVSV-G and pCMVδR8.2 into HEK293T cells using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics). Viral supernatant was harvested 48 h later and freshly used to transduce HLE cells for 24 h in the presence of 8 µg/mL polybrene (Sigma-Aldrich). Selection of transduced cells was performed with 1 µg/mL puromycin for two weeks until resistant lines were obtained.

DEN-induced liver damage in the presence of D-JNKI1

A >97% pure acetate salt of the D-JNKI1 inhibitory peptide (H-Gly-D-Arg-D-Lys-D-Lys-D-Arg-D-Arg-D-Gln-D-Arg-D-Arg-D-Arg-D-Pro-D-Pro-D-Arg-D-Pro-D-Lys-D-Arg-D-Pro-D-Thr-D-Thr-D-Leu-D-Asn-D-Leu-D-Phe-D-Pro-D-Gln-D-Val-D-Pro-D-Arg-D-Ser-D-Gln-D-Asp-D-Thr-NH2) (GL Biochem Shanghai Ltd, Shanghai, CN) was administered i.p. at 20 mg/kg, 30 min prior to DEN (100 mg/mL) treatment. Mice were sacrificed 3 days later and their livers analyzed.

Generation of bone marrow derived mast cells.

Total bone marrow isolated from femora of WT *Bok*$^{-/-}$ mice was cultured for four weeks in RPMI-1640 AQmedia™ supplemented with 10% Fetal calf serum, penicillin/ streptomycin, 50 µM 2-mercaptoethanol and 10% of WEHI-3B cell conditioned medium as a source of murine IL-3 (≥ 200 pg/ml final concentration, as determined by ELISA). The purity of CD117^{+}FcεRI^{+} mast cells was between 95-99% as determined by flow cytometry.

Colony formation assay

Cells expressing vector control or vectors downregulating *BOK* expression were seeded in 6 well-plates at a density of $1x10^3$ cells/plate. One week later, cells were fixed with 4% paraformaldehyde (PFA) for 5 min, washed with PBS and stained with 0.5% crystal violet.

Gene expression profiling of cell cycle regulatory genes

Total RNA isolation, cDNA synthesis and qPCR were performed as described in the Materials and Methods section of the main text. Cell cycle regulatory genes expression was assessed using the Human Cell Cycle Primer Library (Biomol GmbH, Hamburg, DE). The Ct value of each gene obtained from individual experiments was

used to calculate its expression value. Heatmaps were prepared using the package *gplots* (6) in the R version 3.2.1 (7).

References

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