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Supplementary Information

The BRUCE-ATR signaling axis is required for accurate DNA replication and suppression of liver cancer development

Running title: BRUCE promotes ATR signaling and suppresses liver tumor development

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This PDF file includes:

Supplementary materials and methods.
Figures S1- S6.

Supplementary Information

Material and Methods

Cell Culture and Transfection

Human U2OS and HEK 293T cell lines were purchased from ATCC and MEF cells were generated. Cells were cultured in DMEM high glucose medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37^{o}C in a CO2 (5%) incubator. Plasmid or siRNA transfection was mediated by Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen) following manufacturer's instruction.

Antibodies

Antibodies against BRUCE from Calbiochem (#AP1031) and Bethyl (#A300-367A); FANCD2 from Novus (#NB100-182; α-Tubulin from Sigma (#T9026); FLAG (M2) from Sigma (#A8592 and #F3165); c-Myc (#sc-40) from Santa Cruz; PRP19 from Abcam (#ab27692); ATR from Santa Cruz (#sc-515173); Phospho-ATR(Thr1989) from GeneTex (GTX128145); CHK1 (#2360) and Phospho-CHK1 (Ser345, #2348) from Cell Signaling; RPA32 (A300-244A) and Phospho-RPA32 (A300-246A) from Bethyl.

Reagents and siRNAs

Mitomycin C (#M4287), Hydroxyurea (#H8627), Aphidicolin (A0781) Cisplatin (#479306), and Diethylnitrosamine (DEN) (#N0756) from Sigma; PRP19 siRNA duplex pool from Santa Cruz (#sc-76795, GAAGUACAUUGCGGAGAAUdTdT, CGAUGCCACUAUCAGGAUUdTdT, GAUCUGCGCAAGCUUAAGAdTdT). Three BRUCE siRNAs and one control siRNA were synthesized by Dharmacon. BRUCE siRNA#1, #2, and #3 sequences are GGUACAAUCACAUCUAGCAdTdT, GACCUUAAUGGAAUCUUGUdTdT, and GUUAUGAGCUGCUUGUAGAdTdT, respectively. Control siRNA sequence is UUCUCCGAACGUGUCACGUdTdT.

Preparation of Chromatin-containing Whole Cell Lysates

Cell pellets were lysed and sonicated to elute whole cell lysates and chromatin in NETN buffer (50 mM Tris-HCI pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP40) with protease inhibitor tablets (Roche) and phosphatase inhibitors of 10 mM NaF and 50 mM β-glycerophosphate. The lysates were centrifuged at 15,000 g for 20 minutes and the supernatant was collected as cell-free extracts.

U2OS Clone #16 with Stable Expression of DOX-inducible shBRUCE

This stable cell line or inducible expression of shBRUCE was generated as previously reported (1, 2). DOX (1 μg/ml) treatment for 4 - 6 days ablated BRUCE expression.

Reconstitution of shRNA-resistant Full-length BRUCE in U2OS Clone #16

Information for this previously reported cell line can be found in the following references (1, 2).

Immunoblotting and Immunoprecipitation

Protein extracts (40-100 μg) were resolved by SDS-PAGE and transferred to nitrocellulose filter. The filter was blocked with 5% dry milk in PBST for 1 hour at room temperature, followed by incubation with primary antibody overnight at 4^{o}C or 2 hours at room temperature. The filter was then washed in PBST 5 times for 5 minutes each, followed by incubation with HRP-conjugated secondary antibody for 1 hour at room temperature. After washing with PBST, the filter was developed with ECL for 1 minute and exposed to X-ray film. Quantification of the FANCD2 large band to small band ratio (L/S) was performed with the ImageJ software. Co-immunoprecipitation was conducted in whole cell lysates. Cell extracts were first incubated with antibody for 3 hours at 4^{o}C, and then with protein A agarose beads for another 2 hours. For FLAG or c-MYC IP, cell extracts were directly incubated with FLAG M2 or c-MYC agarose beads for 3 hours at 4^{o}C. The agarose beads were washed 4 times with lysis buffer and once with high salt lysis buffer (with 500 mM NaCl), then with 1×TBS buffer. The beads were eluted with 1×SDS sample loading buffer and the elution was separated by SDS-PAGE.

Immunofluorescent Staining of DNA Replication Stress Induced DNA damage Foci
Cells cultured on coverslips in 6-well culture plates treated with DNA replication stressors were pre-extracted for 6 minutes on ice in extraction buffer (10 mM PIPES pH 6.8, 300 mM sucrose, 20 mM NaCl, 3 mM MgCl2, 0.5% Triton X-100), and then fixed with 4% PFA on ice for 15 minutes. For co-immunostaining of FANCD2 with Myc-vector or Myc-PRP19, cells were fixed in methanol at -20ºC for 20 minutes. After blocking with 3% BSA, samples were incubated with primary antibodies overnight on a shaker in a cold room for 2 hours at room temperature followed by incubation with secondary antibodies conjugated with Alexa Fluro 488 or Alexa Fluro 594 for 1 hour at room temperature. After washes, samples were examined under a Zeiss LSM 710 confocal microscope.

Hydroxyproline Assay
Hydroxyproline was measured in DEN-treated livers according to manufacturer's instructions. Kit was purchased from Sigma-Aldrich (MAK008-1KT).

Analysis of BRUCE localization to sites of DNA damage induced by I-*SceI* endonuclease
DR-95 is a human fibroblast cell line stably expressing a pDR-GFP plasmid containing a mutated GFP gene with an 18 bp I-*SceI* endonuclease cleavage site and in-frame termination codon. Cells were either subjected or not subjected to the expression of I-*SceI* endonuclease. Chromatin IP was performed by using antibodies against BRUCE and γH2AX (as control). The ChIP products were analyzed by qPCR to examine localization of BRUCE and γH2AX to DNA breaks induced by I-*SceI* endonuclease.

Immune Response RT-PCR Markers
A semi-quantitative PCR was setup to test primers and annealing temperatures. The PCR mix was made using the 1/10 cDNA solution as the template, IDT primers, and the DreamTaq PCR master mix (2X) (Thermo Fischer Scientific, #K1071). The PCR products were separated by electrophoresis on a 2% agarose gel. RT-PCR setup was setup according to the iQ™ Sybr® Green Supermix (BioRad, #170-8882). The 1/10 cDNA was used as the template. PCR conditions for the semi-quantitative and RT- PCR are as follows: initial denaturation-95°C for 1 cycle; for 40 cycles: denaturing-95°C, annealing-T_m as indicated below, extension-72°C; and an optional hold at 4°C. Gene primers were ordered from IDT and sequences are found below.

Gene	Forward Sequence: 5'→3'	Reverse Sequence: 5'→3'	T_m
GAPDH	CAA AAT GGT GAA GGT CGG TGT G	TGA TGT TAG TGG GGT CTG GCT C	57.3°C
TNFα	CTG AGG TCA ATC TGC CCA AGT AC	CTT CAC AGA GCA ATG ACT CCA AAG	57.3°C
MIP-1α	ACT CTG CAA CCA AGT CTT CTC	AGT TCC AGG TCA GTG ATG TAT TC	55°C
IL-1β	GCA CTA CAAG GCT CCG AGA TGA AC	TTG TCG TTG CTT GGT TCT CCT TGT	60°C

Immunohistochemistry Protocol
Paraffin-embedded (formalin-fixed) liver tissue was sectioned to 5-8μm thickness. Slides were deparaffinized in a series of xylene (5 mins). Slides were then rehydrated in an ethanol series (100% for 5 mins, 100% for 5 mins, 95% for 3 mins, and 70% for 3 mins). Slides were rinsed with 1X PBS for 5 mins. Antigen retrieval was performed using a solution of 0.1M Citric Acid and 0.1M Sodium Citrate. Antigen retrieval solution was boiled for 10 mins, then slides were placed in the solution in coplin jars and boiled in the microwave, 5 mins at 100% power, 5 mins at 60% power twice. During each boil, top off the antigen retrieval solution with distilled water.

Endogenous peroxidase was blocked by incubating the slides in 30% H2O2 in Methanol. Slides were washed twice in PBS for 5 mins. Slides are blocked in 5% normal Goat Serum (Vector Labs, #S-1000) in PBST (made with 0.1% Triton® X-100) for one hour at room-temperature. Primary antibody incubation was done overnight at

4°C. Slides are washed twice with PBS for 5 mins. Then slides are incubated with a secondary antibody for one hour at room-temperature. Slides are then washed twice in PBS for 10 mins. Slides are incubated for 30 mins with a Vectastain® Elite® ABC solution according to the manufacturer's instructions (Vector Labs, #PK-6100). Slides are then washed twice in PBST for 5 mins. Slides are developed by DAB (Sigma, #D3939). Slides were rinsed in tap water then counterstained with hematoxylin. Slides are rinsed with tap water until water is clear then incubated in an acid rinse for 1 minute. Slides are rinsed again and incubated with a blueing solution for 1 minute. Slides are rinsed then dehydrated in an ethanol series, then rinsed in xylene. Slides were mounted and analyzed.

Primary Antibodies used in this study include: anti-ATR antibody (GeneTex, #GTX128146), anti-phospho ATR (phospho Thr1989) (GeneTex, #GTX128145), anti-phospho-RPA32 (Ser33) (Bethyl Labs, #A300-246A), anti-PRP19 (Novus, #NBP1-31354), and anti-Phospho-Histone H2A.X (Ser139) (CST, #9718), F4/80 (Santa-Cruz, sc-52664), anti-4 hydroxynonenal antibody (4-HNE; abcam, ab46545), anti-iNOS antibody (Abcam, ab15323), and alpha-smooth muscle actin (α-SMA, CST 19245T). The secondary antibody used was a biotinylated goat anti-rabbit IgG antibody (Vector Labs, #BA-1000).

ImageJ Analysis of IHC data

For Total ATR and PRP19 nuclear staining positivity was analyzed using the "Fiji" version of ImageJ software. Image was opened. Color Deconvolution was selected for images stained specifically in the nuclei. To decrease the interference of cytoplasmic staining, images that had nuclear and cytoplasmic staining, under the image pull down, RGB stack was selected under type. To decrease cytoplasmic signal, go to Image>Type>RGB stack. Once the RGB window appears, select Image>Stacks>Make Montagne then perform color deconvolution. For both nuclear-specific and other images, select the Vectors pull-down> "H DAB". The "Colour_2" image window was selected and measured. The units of intensity derived in the Results window were transferred to an Excel spreadsheet. The optimal density (O.D.) was calculated using the formula, O.D. = log (max intensity/mean intensity), where the max intensity should be 255. The average optimal density and standard deviations were calculated and graphed. The optical density of 4-HNE positive cells and F4/80 expression were quantified similarly. Upon opening the image, color deconvolution was performed. Under the Vectors pull-down> "H DAB" was selected. The "Colour_2" image window was selected and measured. The O.D. or expression were calculated using the O.D. formula mentioned above.

γH2AX Scoring

The total number of central veins were counted per liver sample. The number of central veins with positive nuclear staining surrounding the vein were counted. The percentage was then calculated: (the number of positive central veins/ total number of central veins) * 100.

Ki 67 and pATR Scoring

Slides were examined and percent nuclear positive hepatocytes per field (under 20X magnification) were counted per 100 cells.

iNOS Scoring

Slides were examined under 20X magnification and percent macrophages were calculated per field using the cell counter feature in Fiji.

Protein preparation and identification

Samples from the FLAG-IPs were concentrated in a SpeedVac and re-solubilized in 1X Laemmli sample buffer, loaded and run for 1.5 cm into a 4-12% BT minigel using MOPS running buffer. The regions of each lane between the well and the dye front were excised for trypsin digestion, peptide extraction and protein identification on a Sciex 5600+ nanoflow LC- mass spectrometry system, all as previously described (3). A full list of the proteomic analysis including all the peptide sequences have been provided in the Supplemental excel spreadsheet.

Supplemental Figures

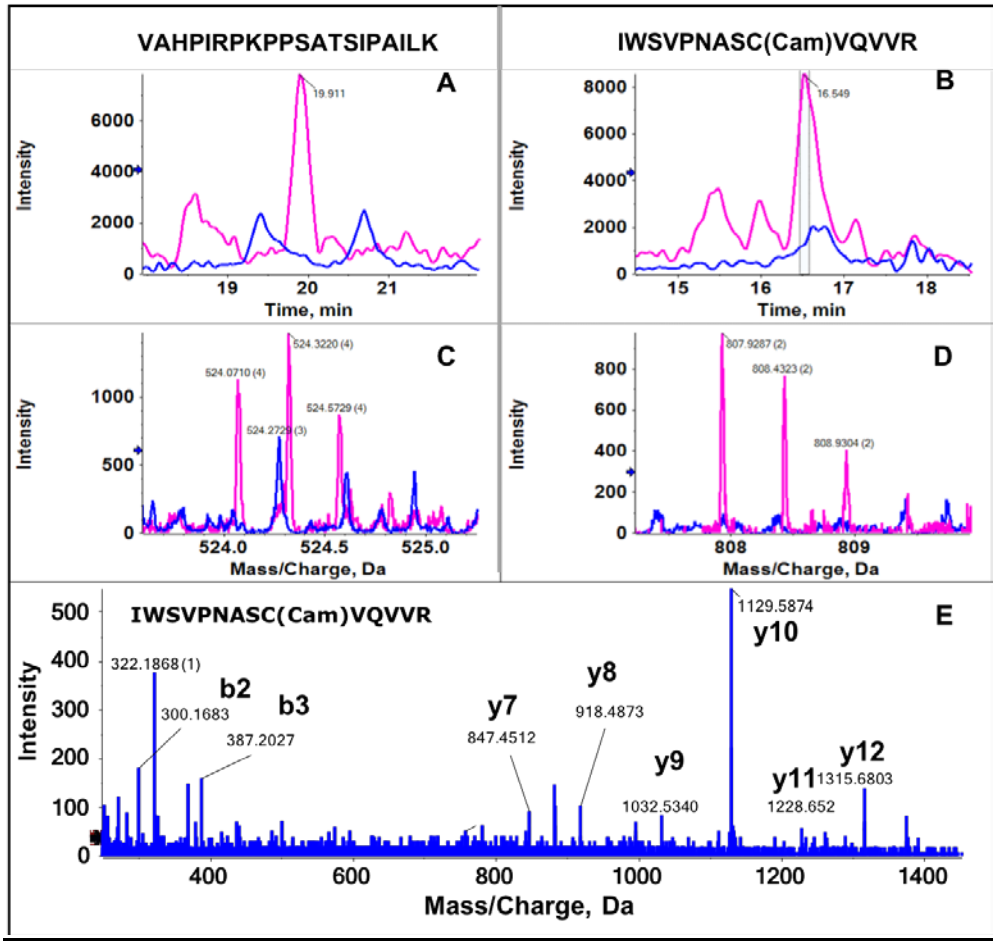

Fig. S1. Confirmation of PRP19 peptides detected in the FLAG-BRUCE IP samples. PRP19 was detected by digestion and LC-MSMS as described in the Methods section. The relative amount of the two indicated peptides in the control of IP from FLAG empty vector (blue traces) and FLAG-BRUCE IP (pink traces) samples are presented as extracted ion chromatograms **(A and B)** and as the corresponding mass spectra **(C and D)**. In both cases, the peptides are clearly detected in the BRUCE IP samples but not in the control. A representative fragmentation spectrum confirming the sequence of the IWSVPNASCVQVVR, Cam@C9 peptide is provided in panel **E**. The Identified PRP19 peptides with 99% confidence. A full list of the proteomic analysis including all the peptide sequences are provided in the Supplemental excel spreadsheet.

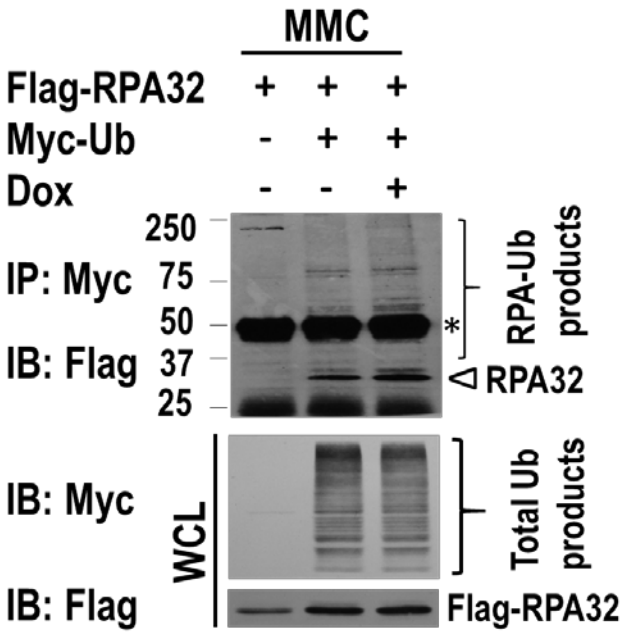

Fig. S2. BRUCE does not impact RPA ubiqutination.
shBRUCE U2OS cells transfected with Myc-Ub and Flag-RPA32 expression vectors as indicated and exposed to MMC (1uM, 24hr). Total Ub-proteins were pulled out from whole cell lysates (WCL, lower) by anti-Myc IP, from which RPA32-Ub was detected by anti-Flag IB (upper). *IgG heavy chain; open triangle: RPA32.

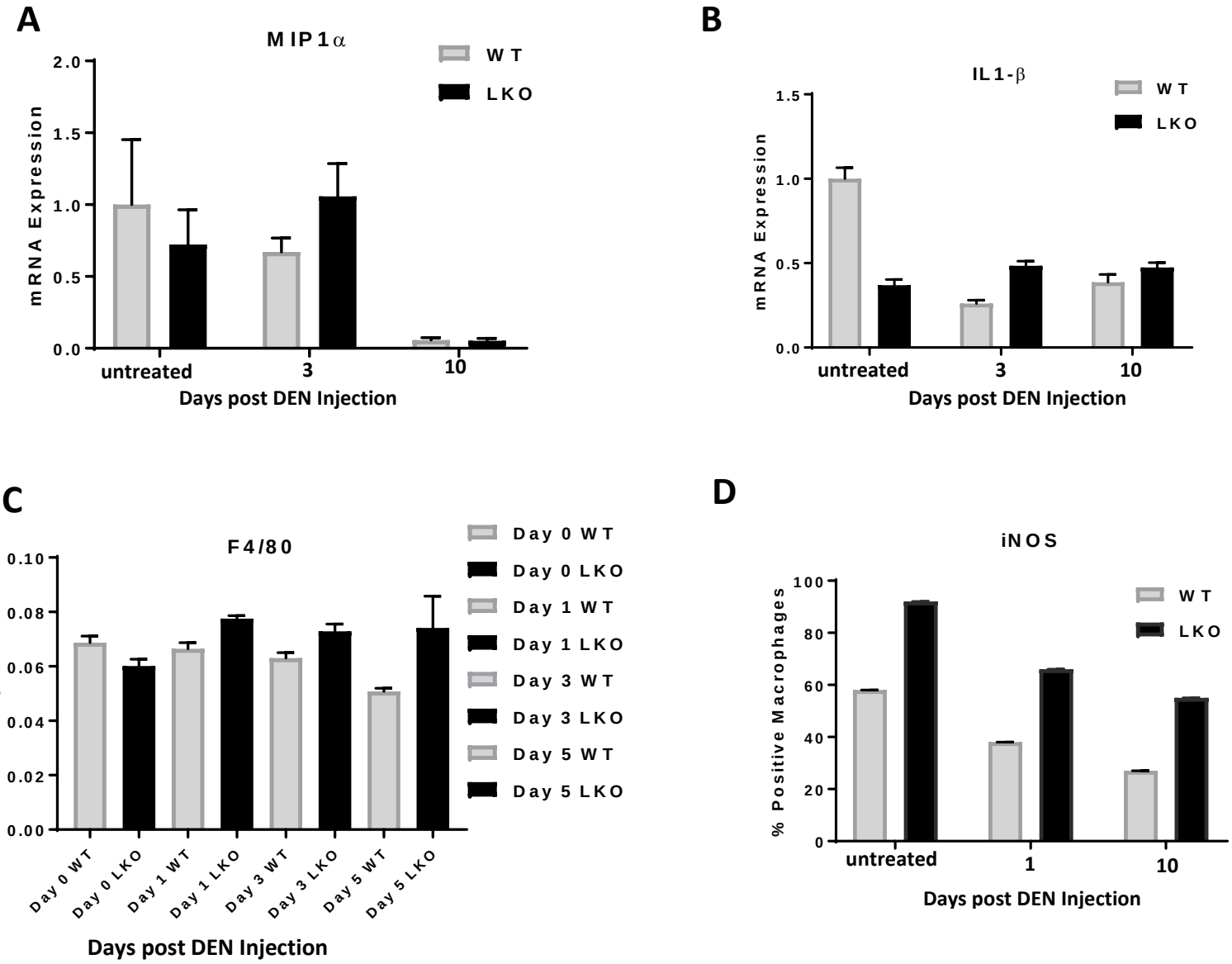

Fig. S3. Loss of hepatic BRUCE increases immune response signaling upon DEN treatment. RT-PCR analysis suggests an increase in inflammatory cytokine signaling upon DEN-treatment, demonstrated by the increase in MIP1α and IL1-β mRNA levels **(A and B)**. Quantification of immunohistochemistry staining of F4/80 demonstrates a DEN-dependent increase in F4/80 expression in LKO livers as compared to WT liver samples **(C)**. Additionally, iNOS^{+} macrophages were quantified from iNOS immunohistochemical staining of DEN-treated WT and LKO livers **(D)**.

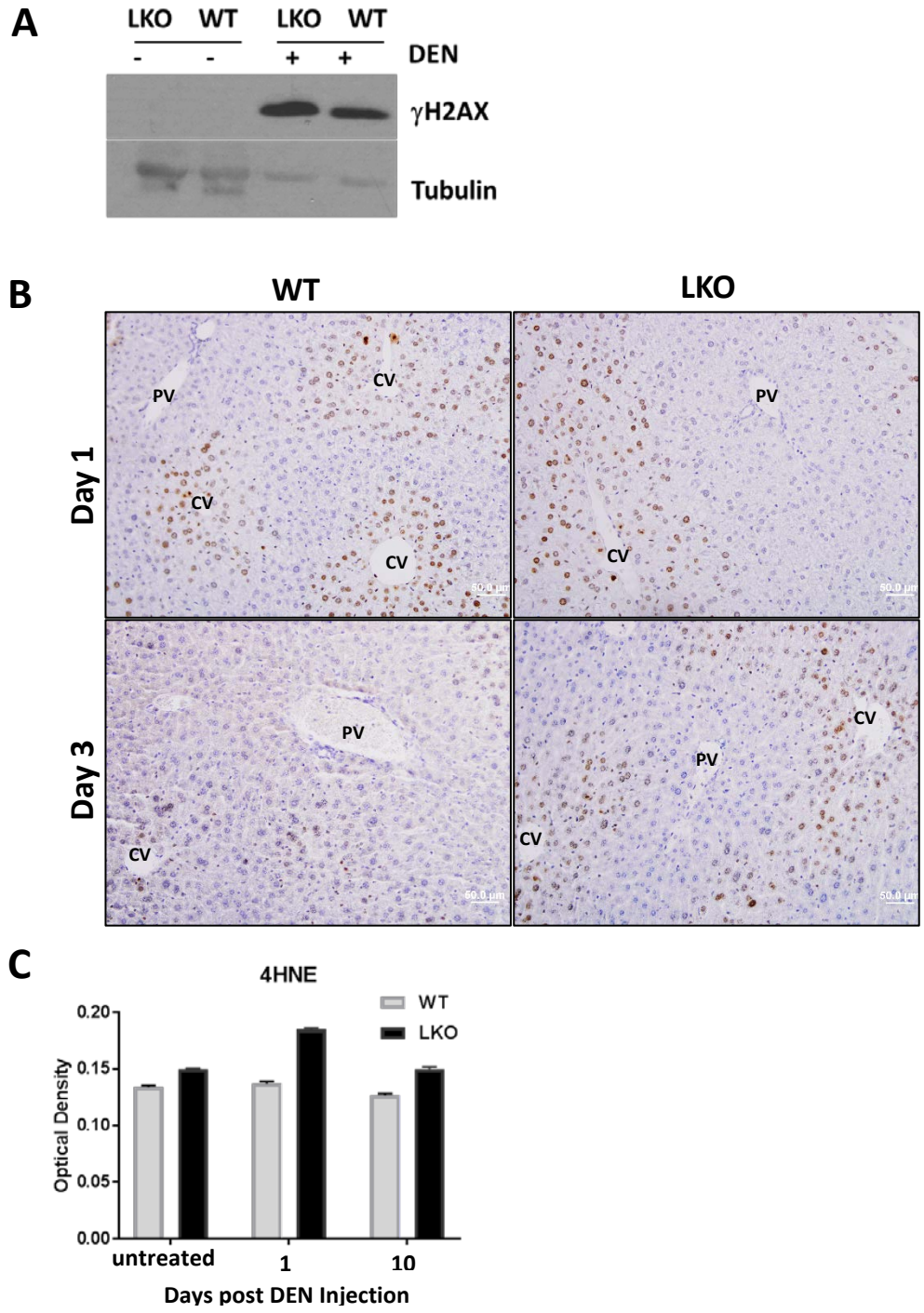

Fig. S4. Loss of hepatic BRUCE expression increases DNA damage in hepatocytes and hepatic stress upon DEN treatment.

(A) Western blot analysis confirms an increase in γH2AX phosphorylation in DEN-treated LKO mouse livers upon a single day of DEN exposure. **(B)** The IHC staining pattern of γH2AX is specific to the central vein and midzone areas of the liver. Upon 3 days post-DEN exposure, the Bruce LKO liver exhibits increased γH2AX staining, indicative of increased DNA damage, suggesting a lack of efficient DNA repair. **(C)** Quantification of immunohistochemical staining of 4-hydroxynonenal (4-HNE) in WT and *Bruce* LKO livers post-DEN treatment.

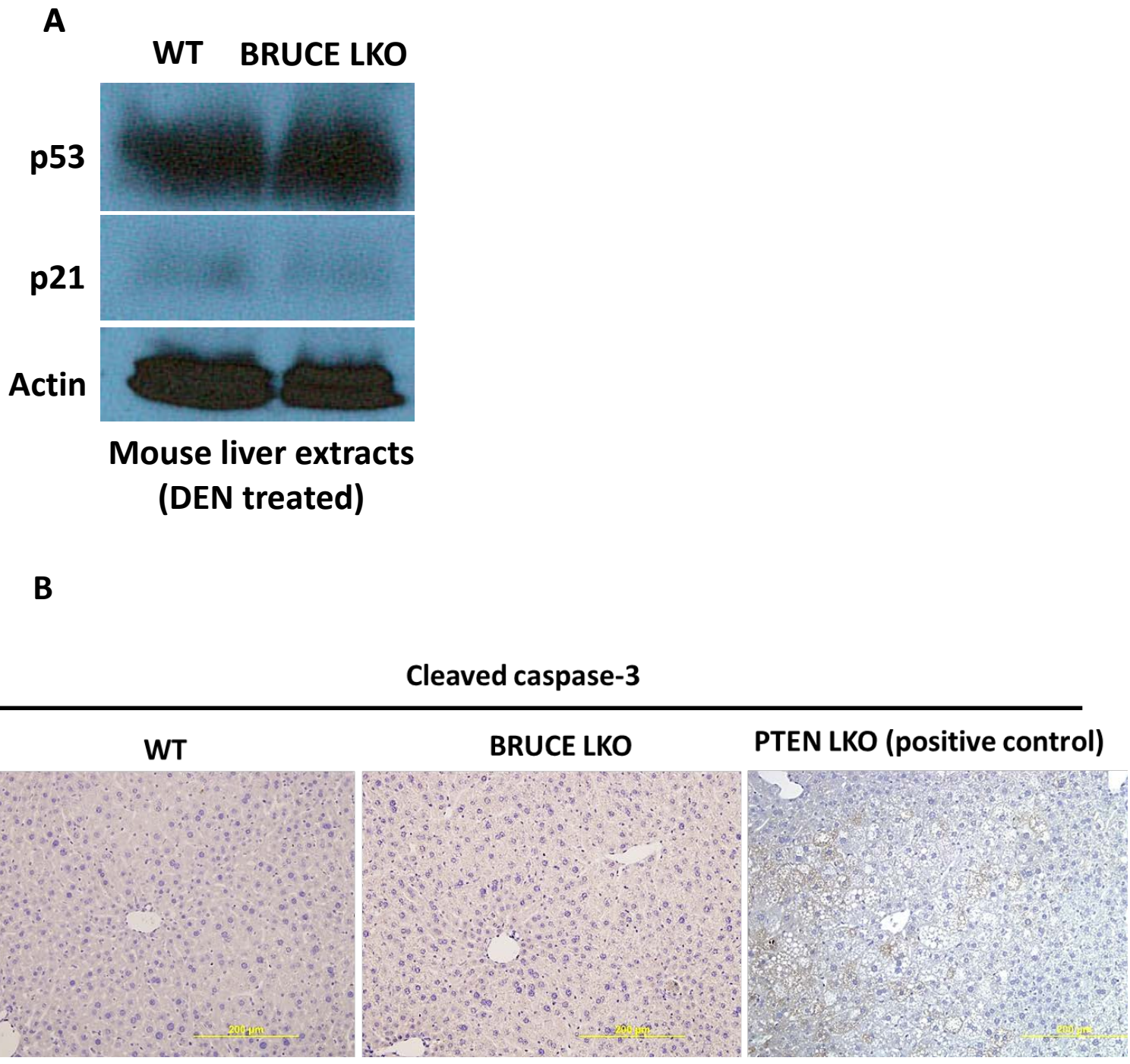

Fig. S5. Loss of hepatic BRUCE expression did not result in changes in p53 status or apoptosis upon DEN treatment. Western blot analysis of liver protein extracts from DEN-treated mice shows no difference in the activation of p53 or its target p21 in WT and liver-specific *Bruce* KO livers **(A)**. Immunohistochemistry for cleaved caspase-3 shows no difference in caspase-3 activation (active form) in the liver tissues of WT and liver-specific *Bruce* KO. We included a positive control of a liver-specific PTEN KO sample (available in our lab, data not published) **(B)**.

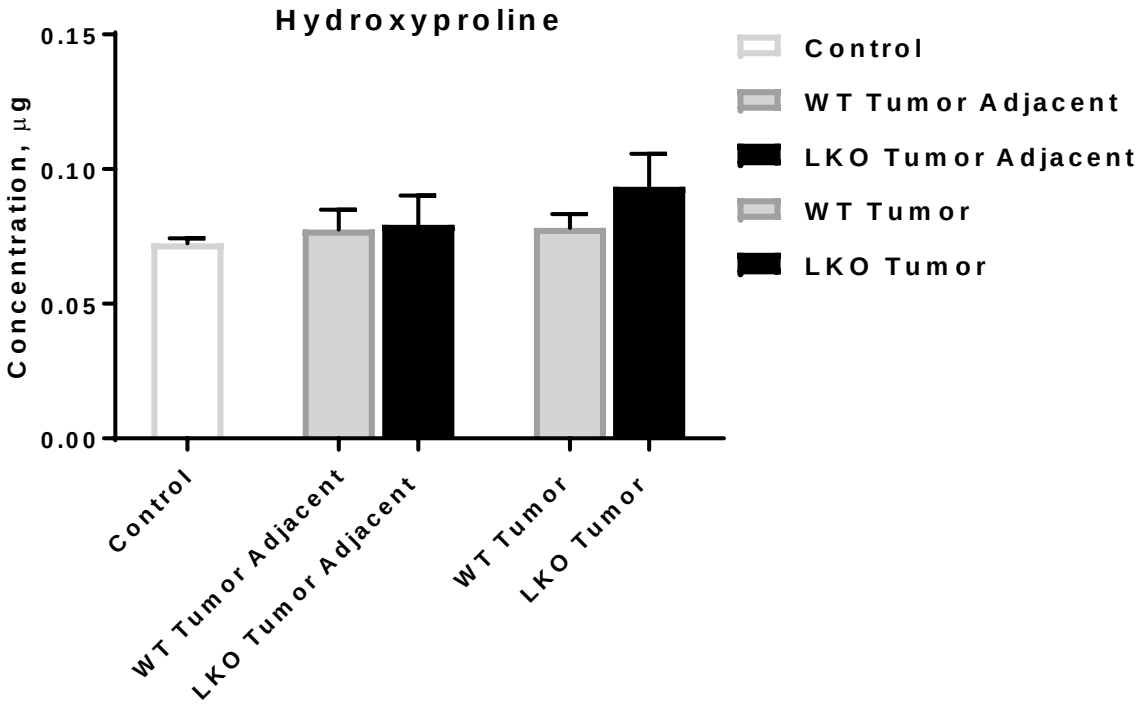

Fig. S6. Loss of hepatic BRUCE expression increases hepatic fibrosis. A non-treated WT control mouse liver sample was used as a control for measuring the hydroxyproline concentration levels in the liver. Additionally, 3 WT and 3 LKO non-tumor adjacent and tumor samples were examined for hydroxyproline levels, as well. The data is demonstrated above, further demonstrating exacerbated fibrosis in *Bruce* LKO mouse livers over WT.

Supplemental Spreadsheet: Full proteomic information for BRUCE IP and control samples