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Supplementary Information for

BCL-2 family protein BOK is a positive regulator of uridine metabolism in mammals.

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Supplementary text
Figs. S1 to S9
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Materials and Methods

Culture conditions for cell lines

Colorectal cancer cell lines were cultured in RPMI 1640 (Gibco, Queensland, Australia, Cat # 11875-085) containing 10% FCS (11995-065, Gibco, Thermo Fisher, Queensland, Australia) at 37ºC and 5% CO_2. HEPG2, 293T and MEF cells were cultured in normal glucose containing DMEM (11885-084, Gibco, Thermo Fisher, Queensland, Australia) at 37ºC and 10% CO_2. *Umps*$^{-/-}$ MEFs were grown in DMEM supplemented with 1µM Uridine Monophosphate (Sigma, NSW, Australia, Cat # U1752). The insect cell line *Sf*9 was grown in continuous suspension of SF900II-SFM medium ThermoFisher, Vic, Australia, Cat # 10902096) in sterile 100 mL Schott bottles with continuous shaking at 120 RPM at 27°C humid atmosphere.

For generating 5-FU resistant cell lines, initially the cells were grown in the presence of 5-FU at the lowest concentration (5 µM) till resistant clones appeared, which were expanded and exposed to higher concentration serially till the drug resistance reached 50 µM. The parental cells were grown in the absence of the drug in parallel for the same duration (approximately 8 months) as control. The resistant cells were grown in the absence of 5-FU for two weeks before using in experiments.

Yeast two-hybrid screen

For yeast two-hybrid screen, the bait *Bok* (*Rattus norvegicus*) was cloned into pGBT-9. The mouse embryonic cDNA library for the screen was prepared using HybriZAP-2.1 kit

following manufacturer's instructions (Stratagene, La Jolla, USA). The screening protocol was as described before (1).

Protein analyses

Cell transfection, protein extraction and immunoprecipitation were performed as described before (2).

RNA analysis

RNA samples were extracted using TRIzol reagent (ThermoFisher, Victoria, Australia, Cat #15596018) and cDNA synthesis was carried out using SuperScript kit (ThermoFisher, Victoria, Australia, Cat # 18091200) following the manufacturer's instructions. Droplet digital PCR (ddPCR) quantification of the absolute mRNA expression of h*BOK* was performed using the QX200 droplet digital PCR system (Bio-Rad, NSW, Australia, Catalogue #QX-200). The following primers were used for qPCR/ddPCR: *hs Bok*- F: 5' GGCGATGAGCTGGAGATGAT 3'; R: ACACTTGAGGACATC AGT CC; *hs p21*- F: 5' TGGAGACTCTCAGGGTCGAAA 3'; R: 5' GGCGTTTGGAGTGGTAGAAATC 3'; *hs Gapdh*- F: 5' GGCAAATTCAACGGCACAGT 3'; R: 5' AGATGGTGATGGGCTTCCC 3'; *mm TP53*- F: 5' CACGTACTCTCCTCCCCTCAAT 3'; R: 5' AACTGCACAGGGCACGTCTT 3'; *mm Hprt*- F:5' TGGATACAGGCCAGACTTTGTT 3'; R: 5' CAGATTCAACTTGCGCTCATC 3'.

CRISPR editing

Lentiviral particles were generated by transfecting the viral packaging constructs pCMV δR8.2, VSVg (pCAG-Eco for mouse) and the plasmid of interest at a ratio of 1:0.4:0.6, in HEK 293T cells (2). For generating Bok-specific constructs, the genomic sequence of the human Bok gene was taken from the BLAT genome database (BLAT-UCSC Genome

Bowser). An exon sequence was selected and fed into the online algorithm (http://crispr.mit.edu). Guides of up to 21 nt with minimum off-site targets were selected with the addition of 5' flanking sequences of TCCC and AAAC to the forward and reverse oligos to enable insertion in the FgH1T vector (3). The following oligos were used for CRISPR deletions cell lines: *hs Bok (exon 2)*- F: 5' TCCCCGCCACGTTGCGGTAGACGC 3'; R: 5' AAACGC GTCTAC CGC AAC GTG GCG 3'; *mm Umps (exon 1)*- F: 5' TCCCGGCGTGAC ACG ATGCCCCGC 3'; R: 5' AAACGCGGGGCATCGTGTCACGCC 3'.

Promoter methylation analysis

For methylation analysis, genomic DNA was extracted and subjected to bisulfite conversion as described previously (4). The Bok promoter region -330 bp upstream of the transcription start site was used for analysis. DNA was amplified using the following primers for NGS analysis: F: 5'-GTGACCTATGAACTCAGGAGTCAAGATATTTGTAATAATTTTAATAAATGT-3'; R: 5'-CTGAGACTTGCACATCGCAGCAAAAACTAAACTTAAAACTTAACCTTCTAA-3'. The adaptor sequences for NGS are underlined. For High Resolution Melting analysis (HRM), a 100 bp region containing 15 CpG islands was chosen and amplified using the following oligos: F: 5'- AATAATTTATTTATAATATAAAGGGTATG-3'; R: 5'-AAAAAAAACAACCCTAAAAAC-3'. HRM analysis was carried out in a magnetic induction cycler using HRM software (MIC, Gene Target Solutions, NSW, Australia).

Apoptosis and cell proliferation assays

eFluor670 cell proliferation assay: Equal densities of control and experimental cell lines (MEFs: 5000 cells/well; HCT8: 10000 cells/well) stained with the dye and seeded in four 12-Well plates simultaneously. Briefly, the cells were harvested and counted followed by aliquoting the required number of cells in a separate 10 mL tube. The aliquoted cells were then washed twice in 10 mL 1X PBS by spinning the cells at 500xg for 5 minutes at 4°C, and then resuspending the cells in 1X PBS containing 10 µM eFluor670 cell proliferation dye (Invitrogen #65-0840) for staining. The cells were stained for 10 minutes at 37°C 10 mL and excess dye was removed by adding warm complete DMEM and pelleting the cells at 500xg for 5 minutes at 4°C. The cells were resuspended again in the required volume of DMEM and seeded on 12-well plate and grown as normal. The cells were harvested and analysed by FACS after 24, 48 and 72 hours (or 72, 96, 120 hours for HCT8s).

Sulforhodamine B assay: Equal densities of control and experimental cell lines (MEFs: 500 cells/well; HCT15: 1000 cells/well) were seeded in 4 96-well plates simultaneously, to ensure that cells reach 80% confluency at the end of the experiment. Cells were then fixed (at 24-hour intervals for 4 days) by adding 100µL of 10% (w/v) trichloroacetic acid for 1 hour at 4°C, followed by washing the wells by a gentle stream of tap-water 4 times and air-dried. The fixed cells were stained with 0.057% (w/v) Sulforhodamine B stain made in 1% acetic acid for 30 minutes, and subsequently washed with 300µL of 1% acetic acid 4 times and air-dried. Once air-dried, the cell bound SRB stain was solubilised by adding 200µL of 10mM Tris pH 10 with occasional shaking for 30 minutes (5). Cell proliferation was calculated by measuring the absorbance at 510nm. All experiments were done in triplicates and repeated as 3 independent experiments.

Apoptosis analysis: Apoptosis was measured by Annexin V/PI staining as described before (6). Annexin V was either FITC labelled or Alexa Fluor-680 labelled depending on the background fluorescence in the cell.

Fluorescence microscopy

For fluorescence microscopy, Madin-Darby Canine Kidney (MDCK) cells were transiently transfected with UMPS (N-terminal GFP tagged) and rnBok (N-terminal m-Cherry tagged) constructs using Fugene HD transfection reagent (Promega, Sydney, NSW, Australia, Cat # E2311). Transfected cells were fixed with 4% paraformaldehyde. Imaging was performed on a Zeiss LSM-780 confocal microscope (Zeiss, Oberkochen, Germany) with a 63 x oil immersion objective using Zeiss ZEN software. GFP fluorescence was detected using an argon laser, while mCherry fluorescence was detected using a DPSS laser. All images are projected Z-stacks from 0.5 µM slices. Images in all experimental groups were obtained with the same settings except for detect gain adjustments that were performed to normalise saturation levels. Image J was used to process images.

Animal experiments

Both WT and *BOK*$^{-/-}$ mice (7) in C57BL/6 background used for the study were age and sex-matched and were housed at the Walter and Eliza Hall Institute animal house. All procedures in this study were approved by the institute's ethics committee. For CCl_4-induced liver fibrosis, mice were injected with 2µL/g body weight of CCl_4 mixed with mineral oil (1:1 ratio) subcutaneously into 14 days old males. Mice were sacrificed after 4 days post injection. ALT/AST measurements were done at Royal Melbourne Hospital using the ALT/AST kit as per the manufacturer's instructions. On day 4, liver sections were

harvested and fixed in 4% formaldehyde for 24h followed by staining with Hematoxyline and Eosine for observation.

For measuring 5-FU mediated apoptosis, mice were injected with 30 mg/Kg body weight of 5-FU (i.p.) or PBS injected for controls and the tissue was harvested 24 h later for TUNEL staining. All animal experiments were conducted in accordance with the approvals from La Trobe University and WEHI ethics committees.

Patient samples

De-identified, frozen colorectal tissue samples were obtained from Victorian Cancer BioBank under with the institutional approval (HEC17-103). 5 CRC primary cases had received 5-FU post-operatively and all developed resistance. All these donors have had a metastatic episode either in the lung, liver or lymph nodes. The other 7 CRC primary cases had received 5-FU post-operatively and were sensitive, i.e. no metastatic episodes. These 12 patients had received only 5-FU as treatment.

For organoid cultures, rectal and peritoneal cancer tissues were taken from patients undergoing surgery at Peter MacCallum Cancer Centre with patient informed consent and approval by the Peter MacCallum Cancer Centre Human Ethics committee (Ethics no #14/185 and ethics no #15/76 respectively). These patients were on Folfox (Folinic acid/5-FU/Oxaliplatin) adjuvant therapy. 5-FU resistance/sensitivity was tested immediately after growing the organoids in culture. The protocol for organoid culture was reported previously (8).

P53 transcriptional assay

The transcriptional activity of p53 in MEFs and HCT15s was analyzed by introducing a lentiviral construct containing the p53 transcriptional response element of *Puma (bbc3*) in

the vector pTRF1-p53-bbc3-TRE-dscGFP (9). Successful lentiviral induction was confirmed by GFP expression and positive GFP polyclonal population were sorted using a FACS sorter. The induction of p53 transcription upon treatment with various drugs (Etoposide, 5-Fluorouracil and Cytosine Arabinoside) was detected by increase in GFP fluorescence intensity by FACS analysis.

Quantitative metabolomics

For metabolite analysis, cell pellets were suspended in 100% methanol followed by repeated freeze-thawing in liquid nitrogen and sonication. The resulting supernatant was centrifuged at 12000g for 10 minutes and freeze-dried to remove methanol. The samples were dissolved in distilled and deionized water and subjected to reverse phase high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (Orbitrap Elite, Thermo Fischer). Validation of detected compounds 5-FU, FURD, FdUMP and TMP was based on detection of different specific transition ion peaks in second mass spectrum based on specific retention time of the standards used. For quantification, raw data obtained from mass spectrometry were analyzed using Skyline 3.5 software. Peak area ratio of the respective standards were used for quantitation. Data are presented as mean ± s.e.m (N=11).

Homology modelling

A model of the BH3 domain of Bok was generated using the MODELLER (v9.17) program (10) using as a template the X-ray crystal structure of BAX (residues 54 to 74, PDB id 4BD6 (11)). A model of an ODCase dimer was similarly created from the crystal structure of human orotidine 5'-phosphate decarboxylase (PDBid 2QCD (12)).

Docking

Docking of the Bok BH3 domain with ODCase dimer was performed using the ZDOCK program (13). The structures of the top 2,000 scoring predictions were then refined using the YASARA program (www.yasara.org). These calculations were performed using the all-atom NOVA force field applied *in vacou* (14). Molecular interactions were re-scored on these refined structures using the ZRANK program (15). Top-scoring models were concentrated in 2 regions, i) at the interface between alpha helices 2b (16) of OCDase monomers, and ii) alongside this helix in one OCDase monomer. Only in the site 1 interaction were conserved hydrophobic residues on Bok in contact with OCDase, and had Bok-BH3 bridging both monomers.

Statistical analyses

Differences between groups were examined for statistical significance using Student's non-paired, two-tailed **t**-tests. The error bars are +/- SEM where N=3, unless stated otherwise.

SUPPLEMENTARY FIGURES

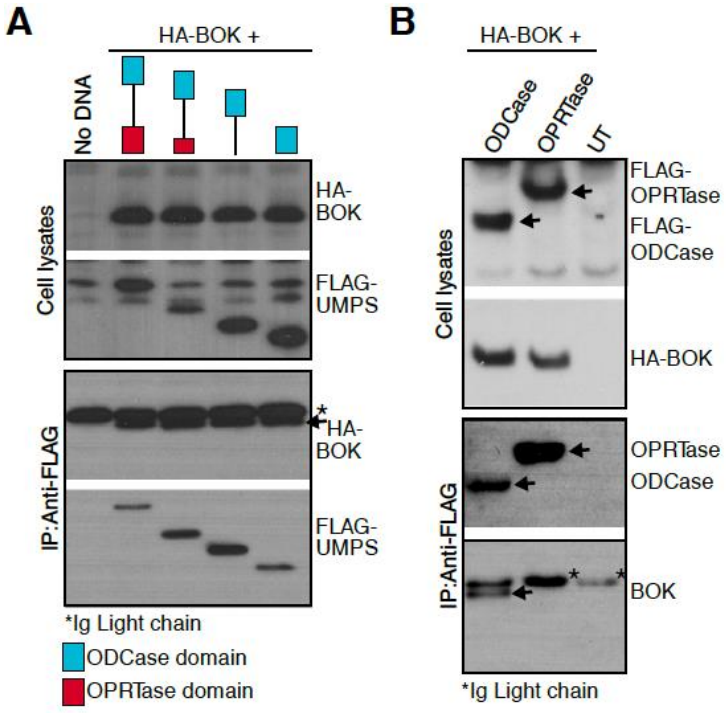

Figure S1. BOK interacts with the ODCase domain of UMPS.
(**A**). Immunoprecipitation analysis of various truncated forms of UMPS with BOK. (**B**). Immunoprecipitation analysis of BOK with the OPRTase and ODCase domains of UMPS.

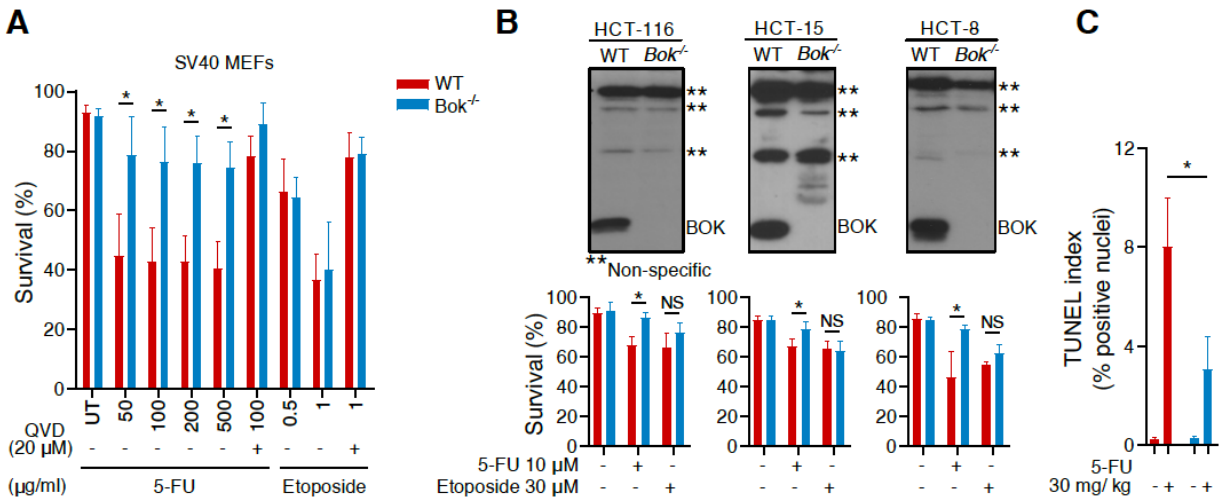

Figure S2. BOK regulates 5-FU sensitivity in MEFs and colorectal cancer cells.

(**A**). *Bok*$^{-/-}$ MEFs are protected against 5-FU mediated apoptosis without any general impairment in the apoptotic pathway (i.e. etoposide-mediated cell death). Cells were treated with drugs as shown indicated and cell survival was measured by annexin V-FITC/PI staining after 48 hours. (**B**). Various *Bok*$^{-/-}$ colorectal cancer cell lines are protected against 5-FU mediated apoptosis. Cell survival was measured as in (**A**). The upper panel (western blots) shows BOK deletion. Non-specific bands are indicated by **. (**C**). *Bok*$^{-/-}$ mice are significantly protected against 5-FU mediated cell death in the colonic epithelium. Error bars +/- SEM (N=3) except in figure **C** (+/- SD, n=4 for 5-FU and n=2 for PBS control) and p values: * $\leq$0.05 and **$\leq$0.005.

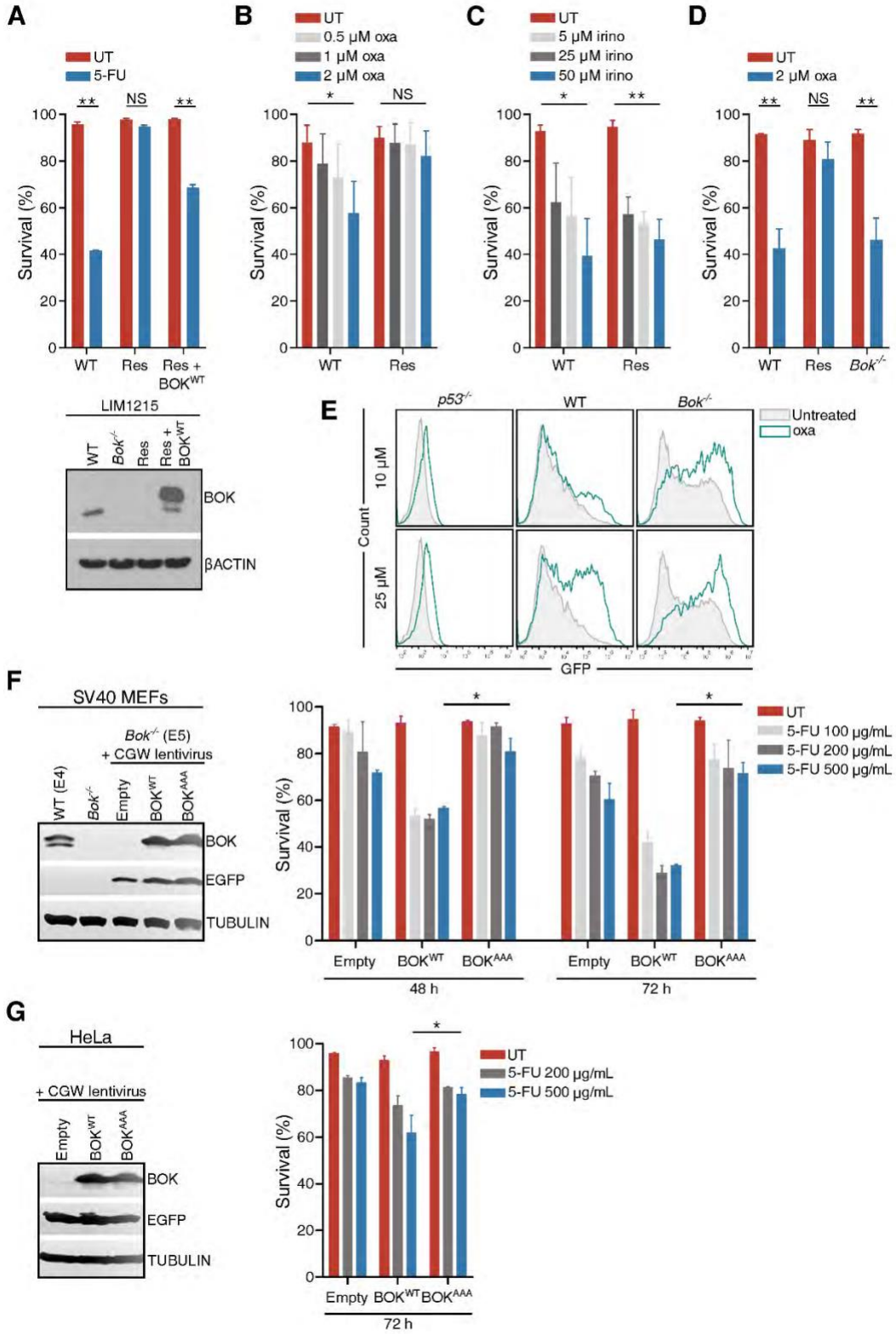

Figure S3. Bok regulates oxaliplatin sensitivity.

(A). LIM1215 (WT, 5-FU resistant and 5-FU resistant cells expressing ectopically expressing BOK) were treated with 50 µM 5-FU and cell survival was measured 72 h by annexin V/PI staining. The western blot in the lower panel shows ectopic BOK expression in 5-FU resistant cells. **(B)** and **(C)**. WT and 5-FU resistant LIM1215 cells were treated with oxaliplatin or irinotecan for 72 hours and survival was measured by annexin V/PI staining. **(D)**. WT, 5-FU resistant and *Bok*$^{-/-}$ LIM1215 cells were treated with oxaliplatin and cell survival was measured after 72 hours. **(E)**. WT or *Bok*$^{-/-}$ MEFs expressing p53-GFP reporter were treated with oxaliplatin and fluorescence was measured after 24 hours by FACS analysis. **(F)**. WT and *Bok*$^{-/-}$ MEFs reconstituted with either WT *Bok* or the AAA mutant were treated with 5-FU and survival was measured by Annexin V/PI staining. The western blot on the left shows the relative expression levels and the graph on the right shows survival. **(G)**. He La cells expressing either the WT *Bok* or the AAA mutant were treated with 5-FU and survival was measured as in **(F)**. The western blot on the left shows the relative expression levels of the transgenes. Error bars +/- SEM (N=3), p values: *$\leq$0.01, **$\leq$0.005.

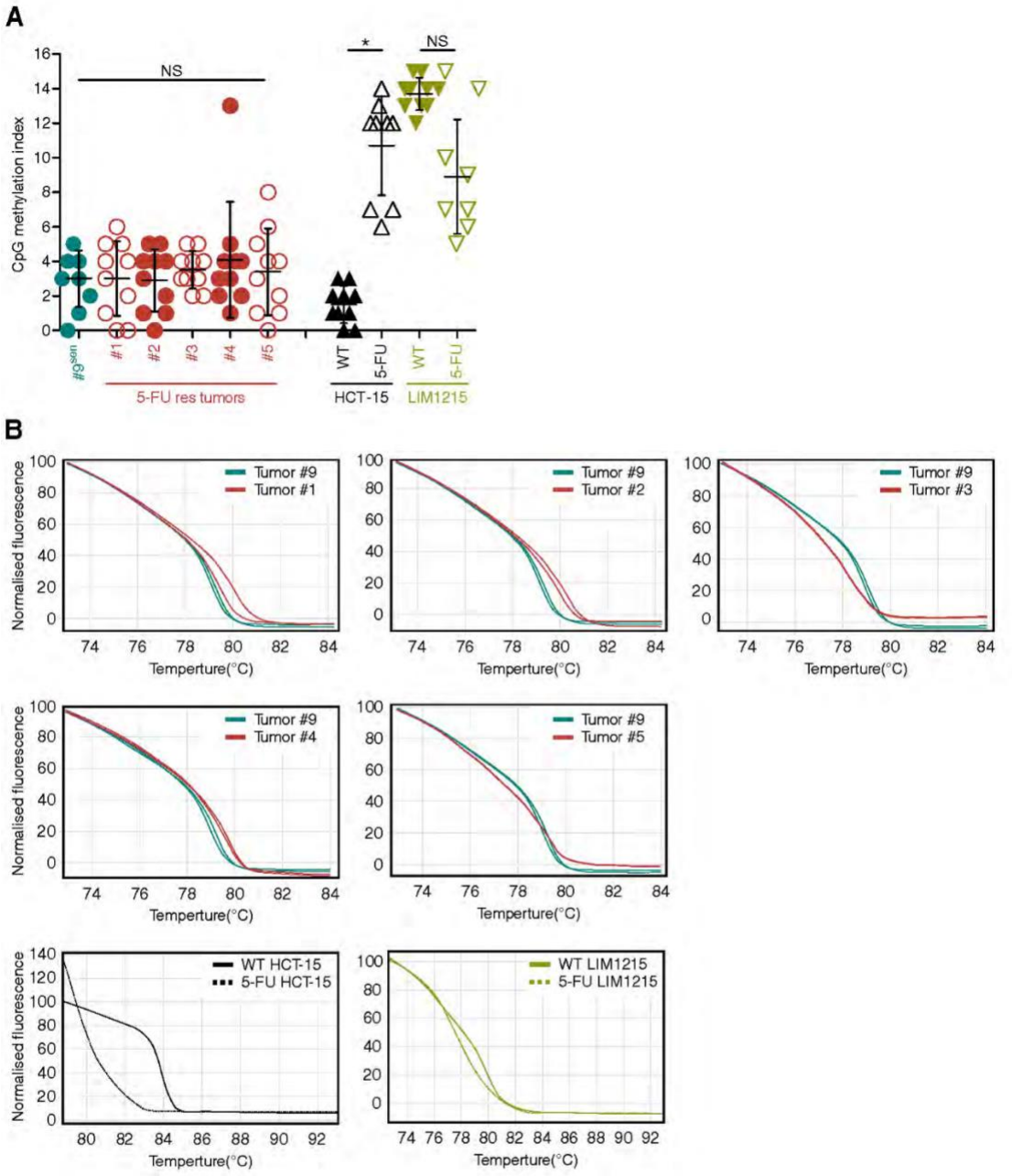

Figure S4. Bok mRNA expression in 5-FU resistance is not regulated by DNA

methylation.

(A). Bisulfite sequencing analysis of 15 CpG islands -330 bps upstream of the transcription start site on the human *Bok* promoter of various cancer tissues and cell lines. HCT-15 cell line showed a significant difference in methylation pattern between WT and 5-FU resistant cells which was verified by High Resolution Melting analysis (HRM). Data is the average of 10 independent reads of NGS. P value: *$\leq$0.001. **(B)**. HRM analysis of the samples shown in **(A)**.

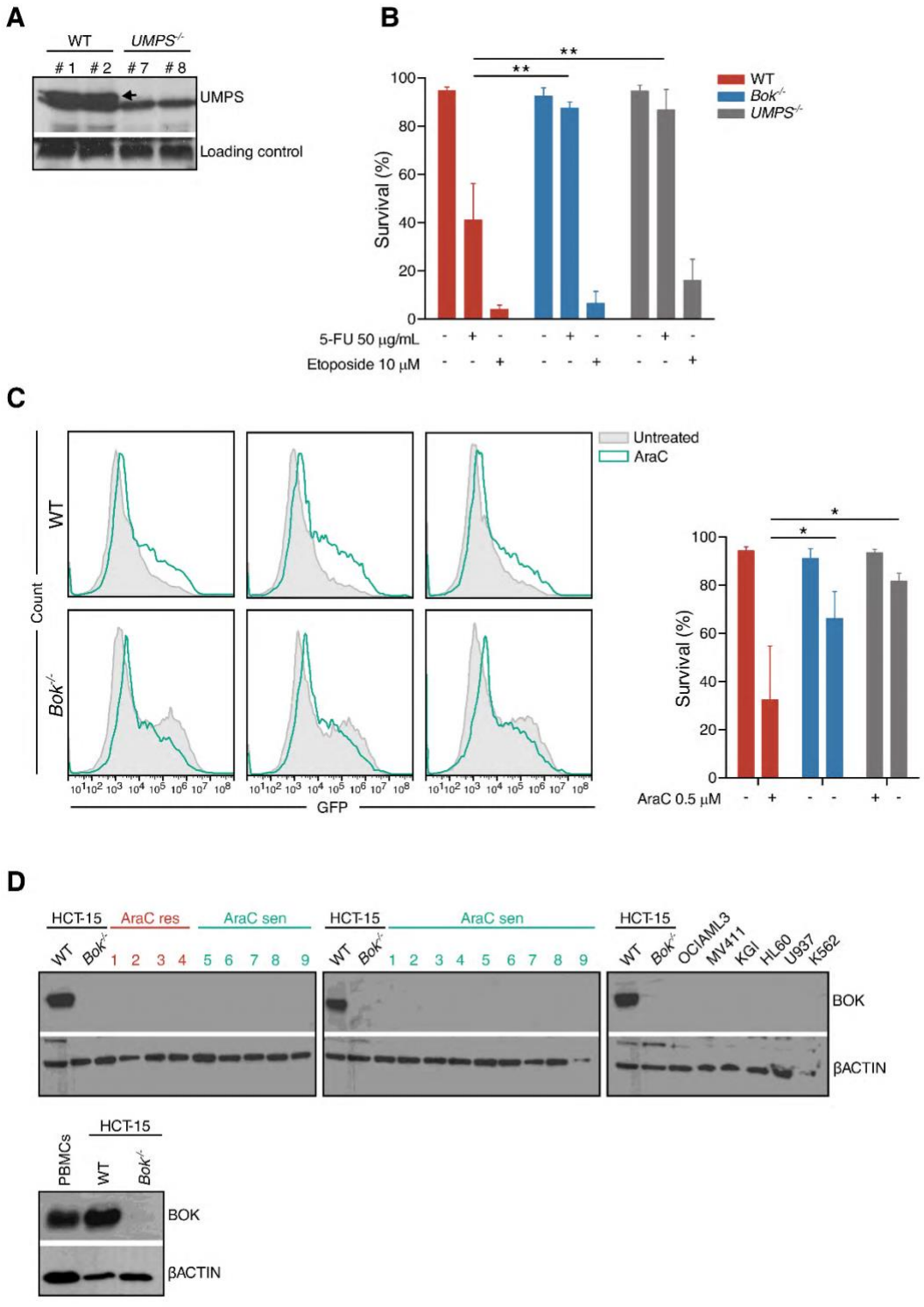

Figure S5. The UMPS pathway is the determinant of 5-FU and Cytosine arabinoside

(AraC) sensitivity.

(A). Western blot showing UMPS deletion in MEFs. **(B)**. WT, *UMPS*$^{-/-}$ and *Bok*$^{-/-}$ MEFs were treated with 5-FU or etoposide and survival was measured by Annexin V/PI staining. **(C)**. p53 response in WT and *Bok*$^{-/-}$ MEFs in response to AraC treatment (0.5μM) after 24 hours (left) and survival was measured after 48 hours (right). **(D)**. Primary AML samples and various AML lines were analyzed for Bok levels by western blot. HCT15 cells (WT and *Bok*$^{-/-}$) were used as the antibody control and PBMCs were used as control for Bok expression in hematopoietic cells. Error bars +/- SEM, N=3, p values: * $\leq$0.005 and ** $\leq$0.001.

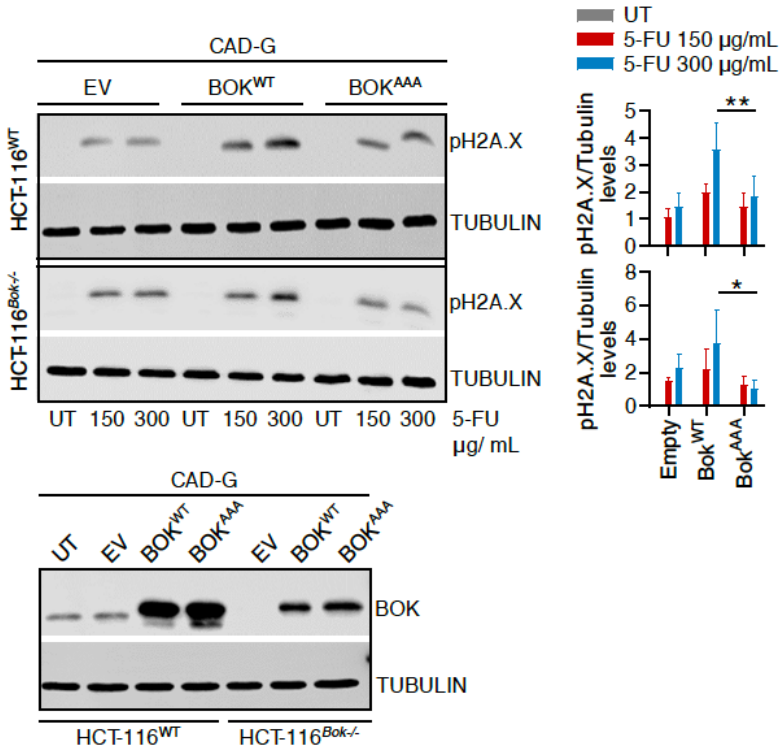

Figure S6. 5-FU mediated p53 induction and genotoxic stress is BOK-dependent. HCT116 (WT, top panel and *Bok*$^{-/-}$, middle panel) cells expressing either the wild type or the BH3 domain mutant of BOK were treated with 5-FU and phospho-Histone 2A levels were measured by western blot. The relative quantitation of pH2A.X levels is shown in the graphs on the right. The expression levels of both the WT and the mutant BOK are shown in the panel at the bottom. Error bars +/- SD, n=3, p values: * $\leq$0.005 and ** $\leq$0.001.

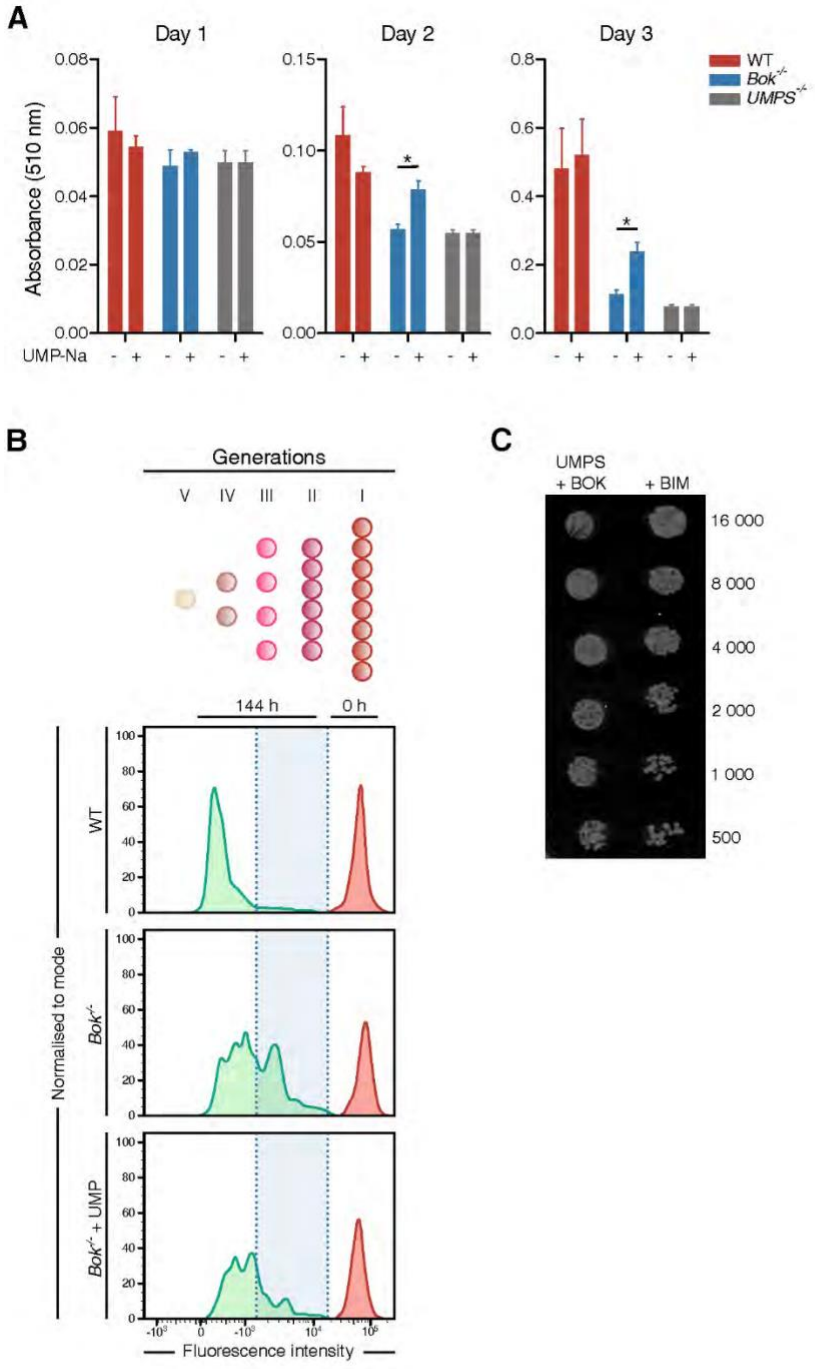

Figure S7. The proliferation defect in *Bok*$^{-/-}$ cells could be partially restored by UMP supplementation in the medium.

(A). *WT, Bok*$^{-/-}$ and *UMPS*$^{-/-}$ MEFs were grown in the absence or in the presence of 1 mM UMP and cell proliferation was measure by sulforhodamine assay. Note that UMP supplementation is obligatory for the *UMPS*$^{-/-}$ cells and yet their proliferation is severely impacted, probably due to inefficient cellular transport of UMP. **(B)**. *WT, Bok*$^{-/-}$ MEFs were surface stained with eFluor670 and proliferation was measured in the absence or in the presence of 1mM UMP by FACS analysis. The schematic shows how with each cell division, the surface fluorescence is reduced by half. **(C)**. *S. cerevisiae* strain W303*α* (*MATα {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15} [phi*$^{+}$*)* was complemented for uracil deficiency by mouse UMPS and co-expressed either moues BIM (negative control) or BOK and growth was monitored by serial dilution on synthetic medium. Error bars +/- SEM, N=3, p values * $\leq$0.01.

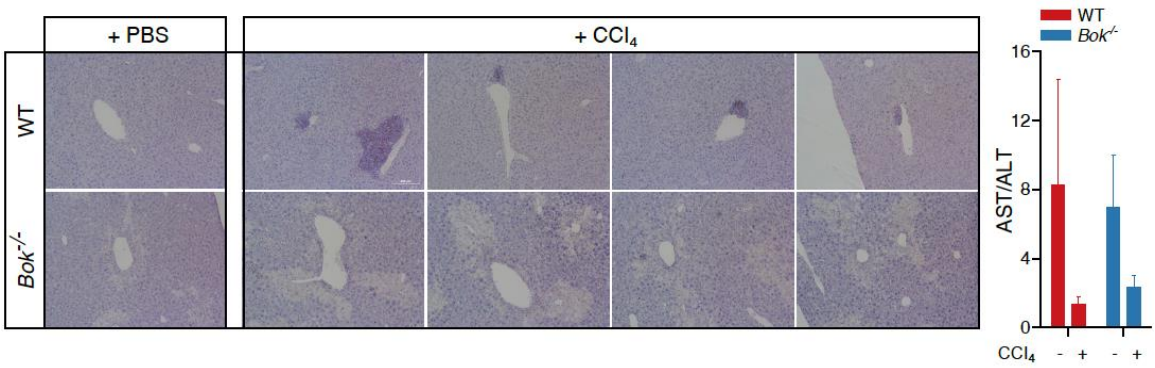

Figure S8. Proliferation defect in *Bok*$^{-/-}$ liver tissues

Wild type and *Bok*$^{-/-}$ were injected with CCl_4 and liver sections on day 3 were analyzed by histology. The liver damage was comparable in both genotypes as evident from increased ALT in CCl_4 treated mice, yet the recovery was severely impaired in *Bok*$^{-/-}$ mice compared to wild type. Error bars +/-SD, n=4.

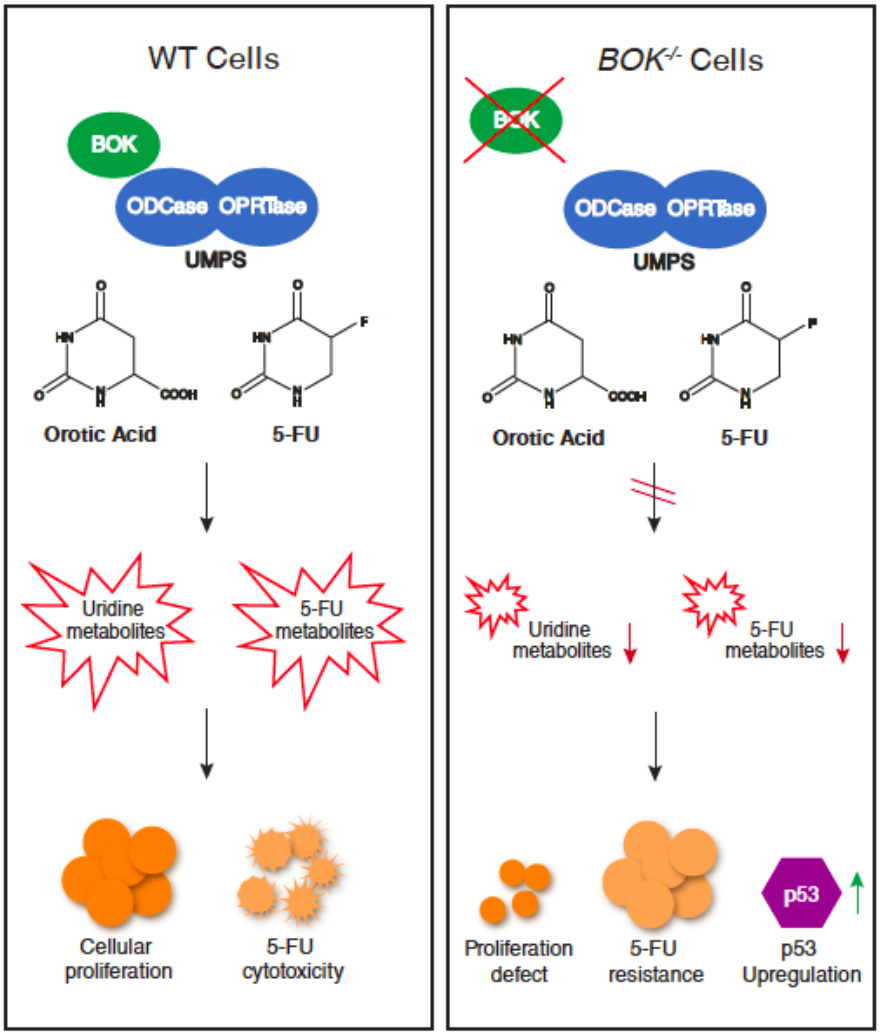

Figure S9. Model explaining how BOK regulates cell proliferation, p53 and chemo-sensitivity.

In BOK-proficient cells (left), through interaction with UMPS, it increases uridine biosynthesis, which promotes cell proliferation. However, these cells are sensitive to 5-FU due to chemo-conversion into cytotoxic derivatives by increased UMPS activity. In BOK-deficient cells (right), decreased UMPS activity leads to reduced uridine biosynthesis, increased basal level p53 activity and cell proliferation defect. However, these cells are defective in chemo-conversion and therefore resistant to 5-FU and fail induce p53 beyond the basal level.

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