Supplementary information

Supplementary Methods

Cell Culture HT1080 (human fibrosarcoma) cells and DU145 (human prostrate carcinoma) cells and HCT116$^{bax-/-bak-/-}$ (human colorectal carcinoma; a gift from R. Youle) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and glutamine. MCF7 (human breast adenocarcinoma cells) and BT549 (human breast ductal carcinoma cells) were maintained in RPMI media supplemented with 10% fetal calf serum and glutamine. Cells were obtained from and characterized by ATCC. Retroviruses were produced as previously described (1). For retroviral infection HCT116$^{bax-/-bak-/-}$ cells were infected with pLXSN, pLXSN-wt BAK or pLXSN-Y108A BAK.

Chemicals and Drug Treatments All chemicals, except where specified, were purchased from Calbiochem and used at the final concentrations indicated; Camptothecin (CPT; 6μM), Etoposide (ETP; 10μM), Docetaxel (DOC; 100 nM).

Plasmid Construction BMX was PCR amplified from a cDNA clone (Origene) and cloned into the pEFIRES plasmid (2). The pEFIRES-K444Q constructs were generated using QuickChange II site directed mutagenesis Kit (Stratagene). pLSXN-WT BAK and pLXSN-Y108A expression plasmids were previously characterised (1).

Flow cytometric analysis of BAK specific immunofluorescence following drug treatment was determined incubating fixed and permeabilized cells with BAK Ab-1 antibody (AM03; Calbiochem) or mouse IgG1 (Pharmingen). Cells were washed, incubated with rabbit anti-mouse phycoerythrin secondary antibody and analysis with a CyAn ADP Analyser (Beckman). The data was then manipulated as previously described to calculate BAK-specific fluorescence (1, 3). Flow cytometric analysis of BAX activation; following drug treatment cells were fixed in 0.25% PFA/PBS for 10 minutes at room temperature. Cells were wash, permeabilized with saponin and incubated with BAX 6A7 antibody that recognizes the active monomeric form of BAX (Enzo Life Sciences). Cells were washed and incubated with rabbit anti-mouse FITC secondary antibody then analyzed by flow cytometry. Cells stained with mouse IgG (Cell Signalling) instead of primary antibody were used as a negative control to determine the % BAX positive cells. Flow cytometric analysis of cytochrome c release as performed as described (4) by incubation with cytochrome *c* antibody (clone 6H2.B4; BD Biosciences) in 3% BSA/PBS with 0.05% saponin overnight at 4˚C, then revealed with phycoerythrin secondary antibody. Detection of Annexin V

was used as a marker for apoptotic cells as described (1). In all cases 10,000 cells were analyzed per sample, three biological repeats were performed per experiments and statistical differences determine by t-test.

Western Blotting Cells were washed with PBS, harvested and lysed at 4°C. Lysis buffer contained 50mM Tris-HCL, 150mM NaCl, (pH 7.5), 2mM EDTA (pH8.0), 1% CHAPS, supplemented with protease inhibitors (Roche), and PhosSTOP phosphatase inhibitors (Roche). Protein quantitation was carried out using Bradford protein assay (Perbio Science UK Ltd). Antibodies used were as follows: BAK (ab32371; Abcam), BAK Ab-1 (AM03; Calbiochem), BAK BH3 domain (#3814, Cell Signaling Technology), BMX (BD Bioscience), BAX (Cell Signaling) GAPDH (#MAB-374, Millipore). Secondary antibodies were horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit (Dako UK, Ltd.). Reactive proteins were visualized by chemiluminescence with ECL plus (Amersham plc). All gel images were acquired at 600dpi in greyscale, cropped ensuring no important bands were excluded with at least 6 bandwidths above and below the band and figures assembled using adobe illustrator. Images are representative images of at least 3 independent experiments.

Immunoprecipitation Cell lysates prepared in lysis buffer above containing 1.5mg total protein was incubated at 4˚C overnight with pre-washed protein G beads and 1µg antibody as follows: pY108 BAK antibody previously characterized (1), PTPN21 (Abgent), BAK (BH3; Cell Signaling Technologies), BMX (C-17; Santa Cruz), pY100 (Cell Signaling). Beads were pelleted and washed 3 times in lysis buffer. Precipitated proteins were eluted from the beads in 2xSDS-PAGE loading buffer. Resultant samples were analyzed by western blotting. Inputs for immunoprecipitation reactions are 10% of starting protein.

RNA Interference The siGenome Smartpools (Dharmacon) were transfected into HT1080 cells, using DharmaFECT 4 reagent (Dharmacon) according to the manufacturer's instructions at a final concentration of 30nM 48hrs prior to drug treatment and analysis by FACS. shRNA constructs against BMX, PTPN21 and a control shRNA construct (pRS; Origene) were transfected in HT1080 cells using Fugene 6 (Roche) following the manufacturer's protocol. shRNA positive cells were selected with 0.4 µg/ml puromycin.

Quantitative Real Time PCR RNA was extracted from the cells using the RNEasy kit (Qiagen) according to the manufacturer’s instructions. The quantitative real-time PCR (qRT-PCR) was performed on the DNA Engine Opticon 2 System (MJ

Research). Specific primers for each target were designed with the Primer3 Software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) spanning an Exon–Exon boundary (listed in Table S2). The DyNAmo SYBR® Green 2-step qRT-PCR Kit (Finzymes) was used in a 20µl reaction volume and PCR settings according to the manufactures protocol. Melting curves were generated at the end of the run. Relative quantification was used to determine the ratio between the quantity of the target molecule in the RNAi knockdown sample compared to the untransfected control. All values were normalised using GUS as a reference gene. ΔC(T) values were calculated as follows: ΔC(T) = Normalised C(T) untransfected control – Normalised C(T) Knockdown. Values >0 indicated an increase in expression, values <0 indicated a decrease and values equal to 0 indicated no change.

Tissue microarray, immunohistochemical analysis, and statistical analysis. These arrays included a total of 119 benign prostate tissue samples, and a total of 169 localized prostate cancer samples, 109 benign colorectal tissue samples, and a total of 100 localized colorectal carcinoma samples, 107 benign breast tissue samples, and a total of 89 localized breast cancer samples. Slides were deparaffinized with xylene and rehydrated through graded alcohol washes followed by antigen retrieval by decloaker for 3 minutes at pH 6.0. Slides were then incubated in hydrogen peroxide for 5 minutes to quench endogenous horseradish peroxidase (HRP). The slides were then blocked by incubation in normal goat serum (dilution 1:10) in PBS (pH 7.4) and subsequently incubated for 60 minutes with anti-BMX antibody (BD Bioscience). Slides were then treated with DAKO Envision system for 30 minutes. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. Immunostaining was evaluated by an independent histopathologist and graded using a two-score system based on intensity score and proportion score as described previously (5). Intensity was scored on the following scale: 0, negative; 1, weak; 2, moderate; 3, strong. Distribution of immunopositive tumor cells was scored on a scale of 0 (0%), 1 (0.1-1%), 2 (2-10%), 3 (11-33%), 4 (34-66%), and 5 (67-100%). The immunoreactivity score was determined by the sum of intensity score and proportion score. The nonparametric Wilcoxon rank sum test was conducted to determine significant difference between the benign and the tumor foci for each cancer type. The statistical analyses were carried out using Prism version 5.0 statistics software.

Proximity Ligation In Situ Assay HT1080 cells cultured on glass coverslips were treated ± CPT (6µM) for 6 hours, fixed 4% PFA and permeabilized with 0.01%

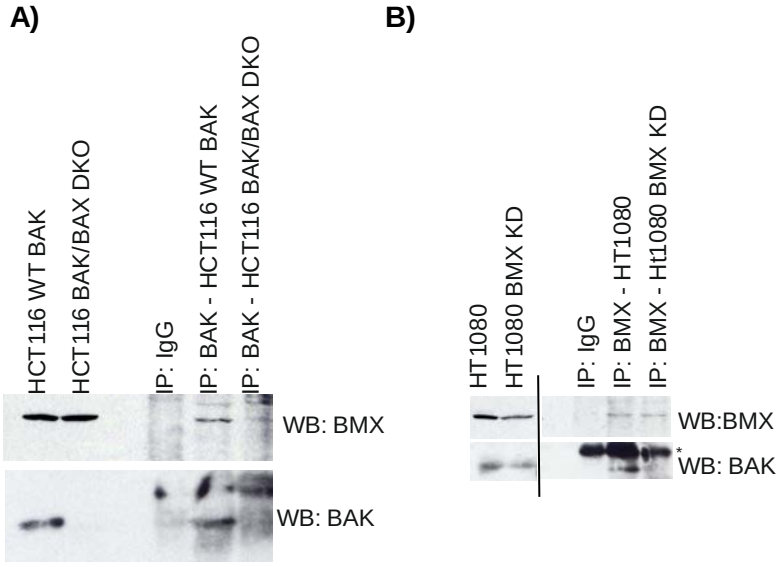
Saponin/PBS. Cells were blocked with 10% horse serum/0.2% fish skin gelatin/PBS then incubated with anti–BMX (C-17; Santa Cruz) and BAK (BH3; Cell Signaling Technologies) at room temperature in blocking solution. In situ proximity assays were performed using a Duolink II Kit (Olink Bioscience) including PLA probes for anti-Goat PLUS, and anti-Rabbit MINUS according to the manufacturer's instructions. Single color controls were stained with goat anti-rabbit Alexa568 conjugated secondary antibody and rabbit anti-goat FITC conjugated secondary antibody. Nuclei were stained with DAPI and coverslips mounted onto slides using fluormount. Images were acquired using confocal microscopy (Nikon).

In vitro kinase assay BMX was isolated by immunoprecipation from HT1080 cells in TKB lysis buffer (1% Triton-X, 20mM Tris-HCL (pH7.5), 150nM NaCl, 5mM EDTA, protease inhibitor tablet (Roche), PhosSTOP inhibitor tablet (Roche)). The immunoprecipitates were washed twice with kinase buffer (4mM MOPS pH 7.2, 2.5mM β-glycerophosphate, 1mM EGTA, 1mM EDTA, 4mM $MgCl_2$, 10mM $MnCl_2$, 0.05mM DTT and 40ng/µl BSA) and incubated with GST, GST-WT BAK or GST-Y108A BAK protein substrates produced in *E.coli* and purified using standard techniques. Reactions were performed in kinase buffer containing 20 µM ATP and 1.5 µCi of [γ-^{33}P]ATP for 30-mins at 30°C, the reactions were terminated by addition of 2× SDS sample buffer. The samples run on 15% SDS-polyacrylamide gels and phosphorylated proteins detected by phosphoimage analysis (Typhoon analyzer). For the IP-Kinase assay, prior to addition to the kinase reaction BAK was released from the GST-beads using thrombin (Sigma). Following the kinase assay the whole reaction was incubated overnight at 4˚C with magnetic protein G beads and pY108 BAK antibody. Precipitated proteins were eluted from the beads in 1xSDS-PAGE loading buffer. Resultant samples were analyzed by western blotting.

Growth Inhibition assay. MTT assay was used to for growth inhibition studies. Cells were treated with a range of drug concentrations added to quadruplicate wells for 4 days. The cell number in treated versus control wells was estimated after treatment by addition of tetrazolium MTT [3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] to the medium at a final concentration of 0.5 mg/ml for 3 hr, followed by dissolving the formazan with 100µl dimethyl sulfoxide (DMSO). The absorbance of samples at 540 nm was measured using a spectrophotometer and the IC_{50} was calculated as the drug concentration that inhibits cell growth by 50% compared with control cell growth.

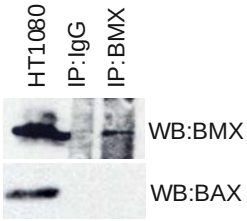
Supplementary Figure S1

Controls for co-immunoprecipitation of BAK-BMX complex. A) HCT116 cells BAX/BAK double knockout cells reconstituted +/- WT BAK were used to immunoprecipitate BAK to determine whether the interaction with BMX was specific. B) HT1080 cells with BMX knocked down using shRNA were used to immunoprecipitate BMX to determine whether the interaction with BAK was specific.



Supplementary Figure S2

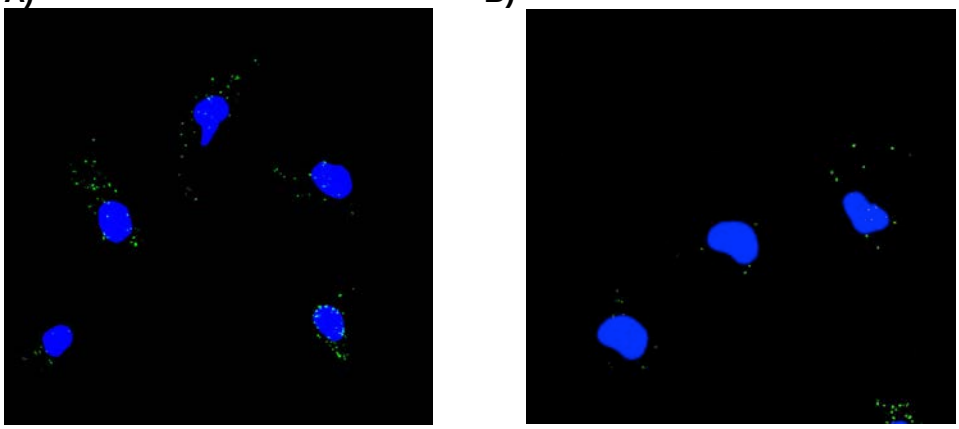
Immunoprecipitation of BMX protein from HT1080 cells did not detect BAX associated with BMX. Input for immunoprecipitation reaction is 10% starting protein.



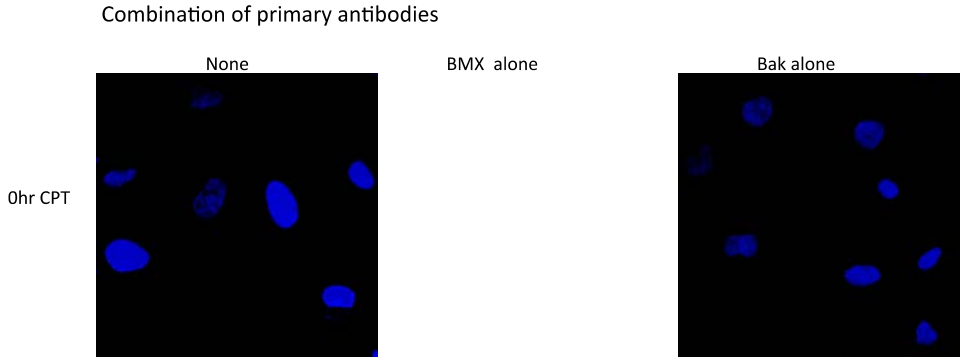
Supplementary Figure S3

Representative images of proximity ligation *in situ* assay (PLISA) in HT1080 cells. Full field images from confocal microscope. Green foci indicate BMX-BAK complexes A) without or B) following 6hrs CPT treatment (6µM). Nuclei were stained with DAPI (blue)

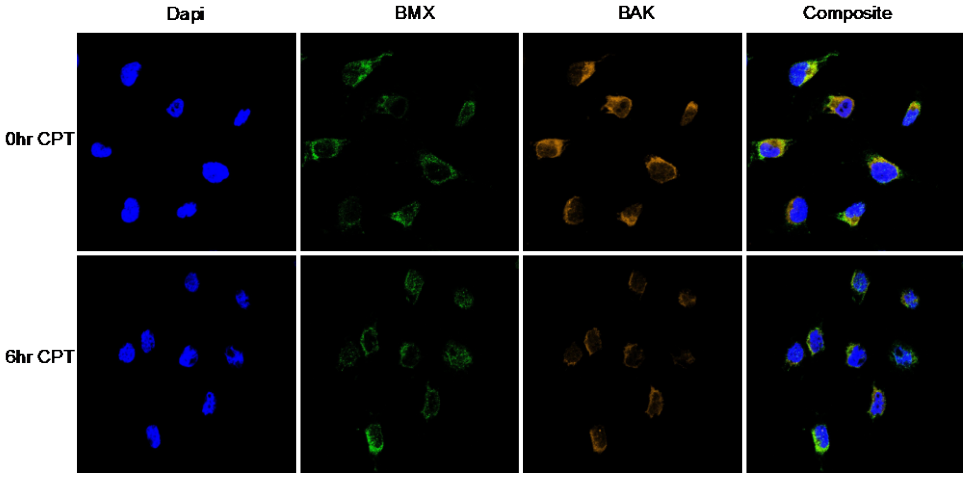
A) **B)**



C) Duolink negative controls. HT1080 cells were incubated with either no primary antibody, BMX (C-17) or BAK (BH3) antibodies alone then PLISA was performed. Nuclei were stained with DAPI (blue).

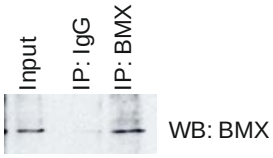


D) Duolink single color controls. HT1080 cells treated ± 6hr CPT (6µM) and stained individually for BMX (C-17; FITC) and BAK (BH3; Alexafluro 568). Nuclei were stained with DAPI (blue).



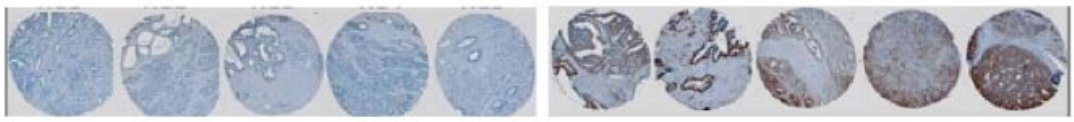
Supplementary Figure S4

Immunoprecipitation of BMX protein from HT1080 cells used in *in vitro* kinase assay (Figure 1E). Input for immunoprecipitation reaction is 10% starting protein.



Supplementary Figure S5

Representative images of BMX immunostaining in sections of prostate cancer tissue microarrays showing examples of low and high BMX reactivity in tumor tissue.

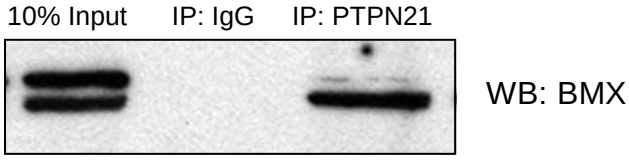


Low BMX staining

High BMX staining

Supplementary Figure S6

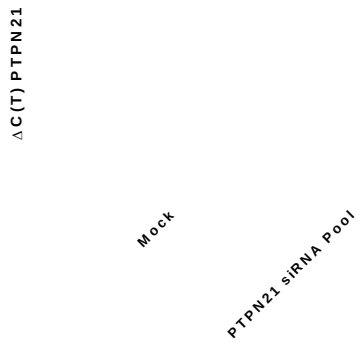
Immunoprecipitation of PTPN21 from HT1080 cells, then western blotted for BMX showing PTPN21-BMX interact in our model system.



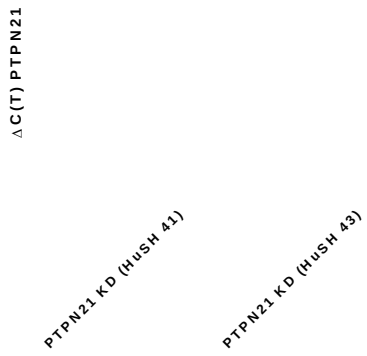
Supplementary Figure S7

Determination of PTPN21 knockdown in HT1080 cells following A) siRNA or B) shRNA treatment by qRT-PCR. Change in C(T) relative to the parental cells is plotted (for siRNA n=2, ± range; for shRNA n=3, ± SD).

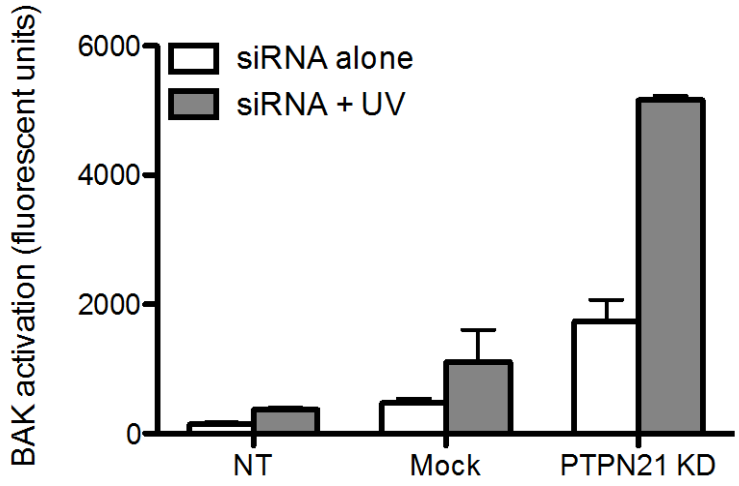
A)



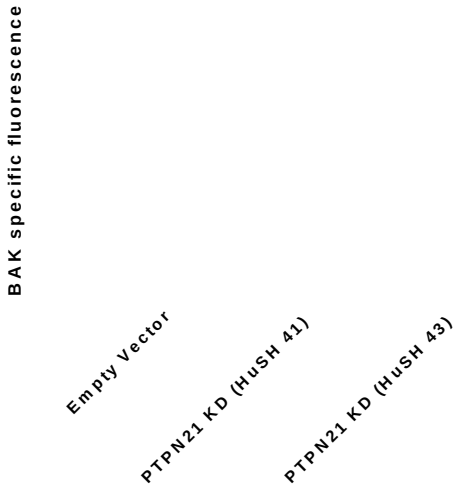
B)



Supplementary Figure S8



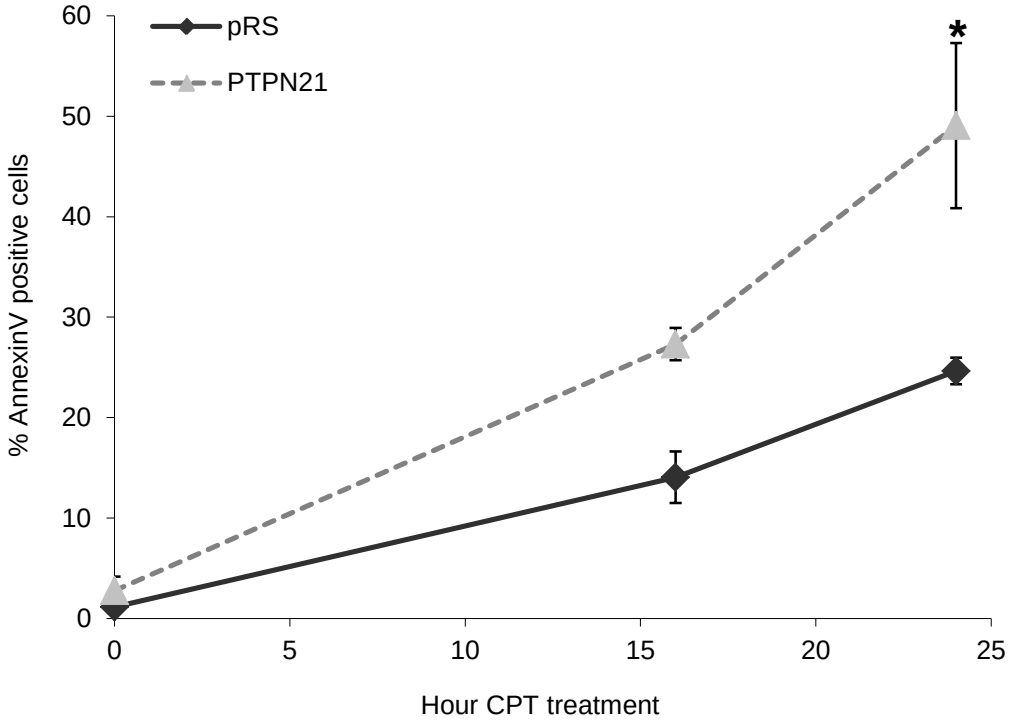
A) Knockdown of PTPN21 increases BAK activation in response to UV damage. Mean BAK activation by FACS analysis in HT1080 cells with either no-transfection (NT), mock transfection (Mock) or siRNA knockdown of non-receptor tyrosine phosphatase 21 (PTPN21) using a 30nM siRNA oligo pool 4 hours post UV 10mJ/cm^2 treatment 48h post RNAi (*n*=3, ±SEM).



B) Knockdown of PTPN21 using 2 different shRNA sequences increases BAK activation in response to camptothecin damage. Mean BAK activation by FACS analysis in HT1080 cells with stably transfected and puromyocin selected for shRNA knockdown of non-receptor tyrosine phosphatase 21 (PTPN21) 4 hours post treatment with 6µM camptothecin (*n*=3, ±SD).

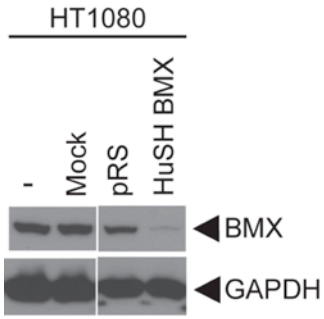
Supplementary Figure S9

Mean percentage of cells undergoing apoptosis as determined by Annexin V positivity by FACS analysis of HT1080 cells stably transfected with shRNA HuSH43 to knockdown PTPN21 expression over a 24 hour time course of CPT treatment (n=3, ± SEM, * p<0.05).



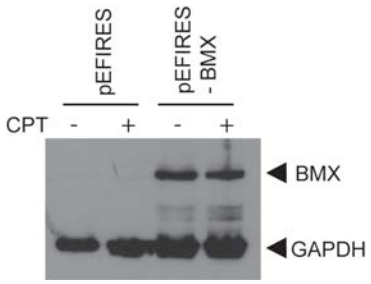
Supplementary Figure S10

Silencing of BMX expression in HT1080 cells. Western blot of BMX from HT1080 cells either untreated (-), treated with transfection reagent only (mock), transfected with empty vector (pRS) or HuSH BMX vector. GAPDH acted as a loading control. Samples were from the same blot but not from adjacent lanes.



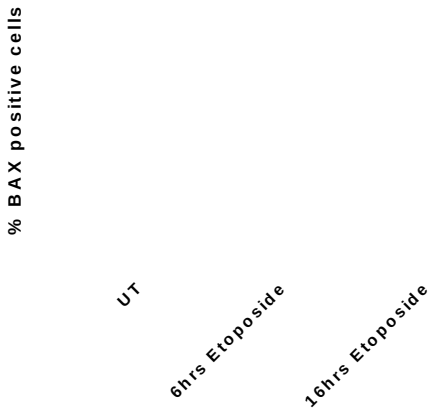
Supplementary Figure S11

BMX over-expression in HT1080 cells. Western blot of BMX expressed in HT1080 cells that were transfected with empty vector (pEFIRES) or a BMX expression plasmid (pEFIRES-BMX). Treatment of cells with camptothecin (6µM) had no marked effect on BMX levels. At this exposure endogenous levels of BMX are at the threshold of detection, whereas BMX levels are greatly elevated by over-expression.



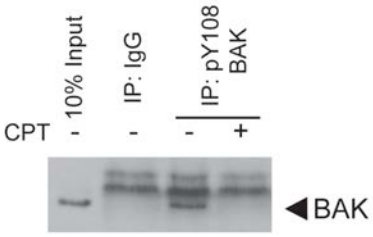
Supplementary Figure 12

Knockdown of BMX in HT1080 cells using shRNA had no effect on BAX conformational change in response to etoposide treatment. Mean BAX activation determined by FACS analysis using 6A7 conformation specific antibody 6 and 16 hours post drug treatment (*n*=3, ±SD)



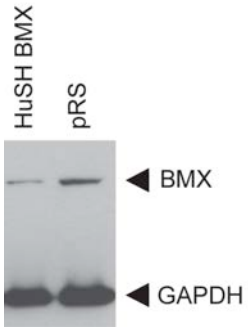
Supplementary Figure 13

A) BAK Y108 dephosphorylation in DU145 prostate cancer cells.



DU145 cells were treated ± 6 μM camptothecin (CPT) for 6 hrs then harvested. Lysates were immunoprecipitated with either IgG or pY108 BAK antiserum followed by western blotting to detect BAK. Without damage, BAK phosphorylated at Y108 was precipitated but following CPT treatment a decrease in the amount of phosphorylated BAK pulled down was observed, in-line with previous findings in other cells lines. Input was from the BAK IP that had not been treated with CPT.

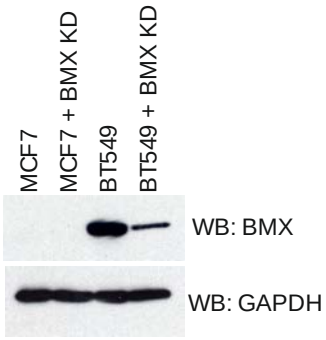
B) Stable silencing of BMX in DU145 prostate cancer cells



DU145 cells were transfected with empty vector (pRS) or a Hush Vector for BMX (Origene). Transfected cells were selected with puromycin at 0.4 μg/ml and cultures expanded. Knockdown of BMX protein levels was revealed by western blot, GAPDH served as a loading control.

Supplementary Figure 14

A) MCF7 and BT549 cells were transfected with empty vector (pRS) or a Hush Vector for BMX (Origene). Transfected cells were selected with puromycin at 0.4 µg/ml and cultures expanded. Knockdown of BMX protein levels was revealed by western blot, GAPDH served as a loading control.



B) Knockdown of BMX in both MCF7 and BT549 cell lines using shRNA increased BAK activation and apoptotic sensitivity as determined by Annexin V positivity in response to etoposide and docetaxel treatment. Mean BAK activation determined by FACS analysis 4 hours post drug treatment (*n*=3, ±SD) and mean percentage of cells undergoing apoptosis as determined by Annexin V positivity by FACS analysis 24 hour times post drug (n=3, ±SD, *p<0.05).

Supplementary Table1

	STS (μM)	**CPT (μM)**	**ET (μM)**
pRS	0.016	0.017	0.815
BMX KD	0.007	0.009	0.069

Cellular IC_{50} values for chemotherapeutic agents with BMX knockdown. Mean IC_{50} values determined by MTT assay for HT1080 cells with BMX expression knocked down using shRNA (BMX KD), control cells are expressing empty vector (pRS) in response to 96 hours camptothecin (CPT), staurosporin (STS) or etoposide (ET) treatment.

Supplementary Table 2 Primer sequences used for qRT-PCR.

GUS Fwd	AAACGATTGCAGGGTTTCAC
GUS Rve	CTCTCGTCGGTGACTGTTCA
PTPN21 Fwd	GAGAATGATGCTGGTGCAGA
PTPN21 Rve	CATTCAGACTGCGCCACTTA

Supplementary References

1. Fox, J. L., Ismail, F., Azad, A., Ternette, N., Leverrier, S., Edelmann, M. J., et al. Tyrosine dephosphorylation is required for Bak activation in apoptosis. EMBO J 2010;29:3853-68.
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