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Supplementary Materials for

Curaxins: Anticancer Compounds that Simultaneously Suppress NF-κB and Activate p53 by Targeting FACT

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The PDF file includes:

Materials and Methods
Fig. S1. Structure and activity of curaxins.
Fig. S2. Antitumor effect of curaxin CBLC137 in xenograft mouse models of cancer.
Fig. S3. Dependence of p53 activation by curaxin on CK2 and FACT.
Fig. S4. Curaxin induction of FACT-chromatin binding.
Fig. S5. Association between FACT levels and cell growth, sensitivity to curaxins, and tumor phenotype.
Fig. S6. Suppression of IKKγ expression makes the p53 present in RCC45 cells responsive to DNA-damaging agents.
Fig. S7. Binding of curaxins to DNA without induction of detectable DNA damage.
Table S1. Physicochemical and pharmacological properties of curaxins described in the paper.
References

Materials and Methods for supplementary materials

Cells and reagents for cell treatment. Renal cell carcinoma (RCC45) and normal kidney epithelial cells immortalized by hTERT (NKE-hTERT) were described in (S 1). C8 cells were obtained from Scott Lowe (Cold Spring Harbor Laboratories, NY) and described in (S 2). HCT116 and isogenic p53-deficient cells were obtained from B. Vogelstein (Johns Hopkins Medical Institutions, Baltimore, MD). Mouse embryo fibroblasts were obtained from 16-days pregnant NIH Swiss mice as described in http://www.molgen.mpg.de/~rodent/MEF protocol.pdf. All other cell lines were obtained from American Type Culture Collection. Cell lines with p53- and NF-κB-dependent reporters were generated by lentiviral transduction and have been described (S 1). Wi38, H1299, HT1080, HCT116, HeLa and Mel7 cells were maintained in DMEM with 10% FBS and antibiotics. All other cell lines were maintained in RPMI 1640 medium and supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM Hepes buffer, 55 nM 2-mercaptoethanol, and antibiotics. The following reagents were used for cell treatment: quinacrine, 9-aminoacridine, doxorubicin, cisplatin, 5-fluorouracil, and hydrogen peroxide (all from Sigma Aldrich, Corp., St. Louis, MO). Irinotecan, oxaliplatin and sunitinib was purchased from LC Laboratories (Woburn, MA). The inhibitors 4,5,6,7-Tetrabromobenzotriazole (TBBr**,** a CK2 inhibitor) and 4,5-Dimethoxy-2-nitrobenzaldehyde (DMNB, an inhibitor of DNA-PK) were purchased from EMD Chemicals, Inc. (Gibbstown, NJ). TNFα was purchased from R&D Systems, Inc. (Minneapolis, MN). Nuclear-ID Red DNA stain was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Vectors. Vectors with p53-responsive (p21-ConA-Luc, referred to in the current work as p53-Luc) and NF-κB-responsive luciferase reporters were previously described (S 3). The pLXSN vector containing the human wt-p53 cDNA (S 3) was used as a template for PCR to generate p53 phosphorylation mutants (p53-S15A, p53-S20A, and p53-S392A) using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene Corp., La Jolla, CA) according to the manufacturer's instructions. p53 wt and generated mutants were PCR-amplified using high fidelity PCR supermix (Invitrogen Corp., Carlsbad, CA) and cloned in-frame into the XbaI and XhoI sites of the pLA-CMV-N-Flag lentiviral vector (a kind gift of Dr. Peter Chumakov, Cleveland Clinic, Cleveland OH). Full-length SSRP1 was PCR-amplified using High Fidelity PCR Supermix (Invitrogen) and cloned in frame into the XbaI and XhoI sites of the pLA-CMV-N-Flag or pLA-CMV-N-GFP vectors. All constructs were verified by sequencing. The PCR primers used were designed to introduce the corresponding restriction sites as well as start and stop codons if needed. The primers used were as follows: SSRP1 XbaI dir 5'-tata*tctaga***atg**gcagagacactg; SSRP1 XhoI rev 5'-tata*ctcgag***cta**ctcatcggatcc. Mission shRNA lentiviral vectors were purchased from Sigma Aldrich Corp. (St. Louis, MO): shControl – shRNA against GFP, shSSRP1-2 - SHGLYC-TRCN0000019270, shSSRP1-4 - SHGLYC-TRCN0000019272, shSSRP1-5 - SHGLYC-TRCN0000019273; for shSPT16-2 - SHGLYC-TRCN0000001258, shSPT16-3 - SHGLYC-TRCN0000001259, shSPT16-4 SHGLYC-TRCN0000001260. Unless otherwise indicated, shSSRP1-2 and shSPT16-4 were used. pTRIPZ lentiviral shRNAmir against SSRP1 was purchased from Open Biosystems, Inc. (Thermo Fisher Scientific, Huntsville, AL).

Lentiviral Transduction. Stocks of recombinant lentiviruses were generated and used as previously described (S 1).

Immunofluorescent staining. Cells in chamber slides were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Slides were then blocked in a solution of 5% BSA/0.1% Triton X-100 in PBS for 1 h. Anti-p65 (C-20) and anti-p53 (DO1) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-phospho-histone H2A.X (Ser-139) (Cell Signaling Technology, Inc., Beverly, MA) antibody were added at a concentration of 1 μg/ml in blocking solution (5% preimmune rabbit serum /0.1% Triton X-100 in PBS). Secondary anti-mouse Cy2- or anti-rabbit Cy5-conjugated antibodies (Sigma Aldrich Corp., St Louis, MO) were used. All washings were done with blocking solution.

Electrophoretic mobility shift assay (EMSA). Nuclear proteins were isolated as described previously (S 4). Each binding reaction contained 10 mM HEPES (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg poly(dI-dC), 0.5 μg sonicated salmon sperm DNA, 5 μg nuclear protein extract and 5'-end-[^{32}P]-labeled (2–3X10^{5} cpm) double-stranded oligonucleotide probe (either containing the NF-κB binding sequence from the Ig light chain gene promoter (Promega) or the p53 binding sequence from the p21 (Waf1) gene promoter (5'- GAACATGTCCCAACACATG TTG-3') (S 5)). DNA-binding reactions were performed at room temperature for 30 min in a final volume of 20 μl. To determine binding specificity, 200 ng of antibodies against p65, p50, p52, c-Rel, RelB (for NF-κB binding), or against p53 (Pab421) were added 30 min after the beginning of the binding reaction, and incubation was continued for an additional 30 min. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). DNA-protein complexes were separated on 6% acrylamid gel (Invitrogen Corp., Carlsbad, CA) and visualized by autoradiography.

Western blot analysis. Protein extracts were prepared by lysing cells either in TEGN buffer (10mM Tris HCl, 1mM EDTA, 10% Glycerol, 0.4M NaCl, 0.5% NP-40, pH 7.5) or in Cell Culture Lysis Reagent (Promega, Madison, WI). Extracts were spun down at 6,500Xg for 10 minutes at 4^{o}C to obtain the soluble fraction. Protein concentrations were determined with the Bio-Rad Dc protein assay kit (Hercules, CA). For total cell extracts, equal numbers of cells were directly lysed in Laemmli Gel Loading buffer and boiled for 5 min before electrophoresis. Equal protein amounts were run on gradient 4 – 20% precast gels (Invitrogen Corp., Carlsbad, CA) and blotted and transferred to Immobilon-P membrane (Millipore Corp., Billerica, MA). Membranes were blocked in 5% nonfat milk-TBS-T buffer or 3% BSA-TBS-T (for phospho-specific primary antibodies) and incubated with primary antibodies for 1.5h or 16h (for phospho-specific antibodies) at room temperature. The following antibodies were used: anti-p53, monoclonal mouse DO1; anti-p21, monoclonal mouse F-5; anti-mdm2, monoclonal mouse SMP14 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA); polyclonal rabbit CK2, polyclonal rabbit eIF2α, (both from Cell Signaling Technology, Inc., Beverly, MA), monoclonal mouse SSRP1, (BioLegend, Inc., San Diego, CA), monoclonal mouse Spt16 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal rabbit IKKγ (Cell Signaling Technology, Inc., Beverly, MA). p53 phosphorylation and acetylation were analyzed using the phospho-p53 sampler kit and the anti-acetyl-Lys382-p53 antibody, respectively, according to the manufacturer's recommendations (Cell Signaling Technology, Inc., Beverly, MA). Phospho-specific antibodies against nuclear kinases (ATM, ATR, Chk-1, Chk-2, PKR) and eIF2α were also from Cell Signaling Technology. To verify equal protein loading and transfer, anti-β-actin or anti-HPRT (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used. Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. ECL western blotting

detection reagent (GE Healthcare, Inc., Little Chalfont, Buckinghamshire, UK) was used for protein detection. Quantitation of the data was performed using QuantityOne® software from Bio-Rad Laboratories, Inc. (Hercules, CA) or Image J software from NCI.

Comet assay. HT1080 cells at 80% confluency on 100 mm plates were treated with chemicals for 6h. As positive controls for DNA damage, UVC (20 J/m^2), gamma-rays (12Gy), doxorubicin (2 μM) and hydrogen peroxide (100 μM for 20 min at 4^oC) were used. Comet assays were performed under alkali conditions using the Comet Assay kit (Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's protocol. Analysis was performed using Demo-version of Comet IV software (Perceptive Instruments, Suffolk, UK).

DNA-binding assay. The DNA binding assay for determining the ability of compounds to alter the mobility of plasmid DNA was performed as follows: plasmid DNA was incubated in TE buffer (pH 8.0) with 5-10 μM of chemicals at room temperature for 20 min and then electrophoresed in a 0.8% agarose gel at 1.5 V/cm constant for 16 hours. Gels were stained with ethidium bromide (0.5 μg/ml) and visualized using short wavelength UV light.

Competition dialysis assay. Competition dialysis was performed as described previously (S 6). Single strand synthetic oligonucleotides of different composition were ordered from IDT, Inc. and annealed using standard protocol. Supercoiled plasmid DNA solution or synthetic oligonucleotides (in H_2O) were diluted in 200 μl BPES buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA, 185 mM NaCl, 5 mM $MgCl_2$, pH 7.0) to a concentration of 30 μM, placed into Slide-A-Lyzer MINI dialysis units with 7000 MWCO membrane (Pierce Biotechnology, Inc. Rockford, IL) and dialyzed against 800 ml of BPES buffer containing 1 μM of the ligand (quinacrine or Curaxins). Dialysis was

performed with continuous stirring for 24 hr at room temperature (22–24°C). The free ligand concentration was determined using a FluoroMax-2 fluorometer with an aliquot of the dialysate solution. The amount of bound drug was determined from fluorometer data by calculating the difference between the total concentration of drug in the dialysis unit and the free ligand concentration as described (S 6). In each experiment, every ligand sample was tested at least in duplicate. The apparent association constant, Ka, was calculated using the following equation: $Ka = Cb / Cf \times [NA]total$, where Cb and Cf are bound and free ligand concentrations, respectively, and [NA]total is the total nucleic acid concentration. In our experimental setting, Cf = 1μM and [NA]total = 30 μM. Cb = Ct – Cf, where Ct is the total concentration of ligand within the MINI dialysis unit. Ct was determined spectrophotometrically using wavelengths and extinction coefficients appropriate for each ligand.

In Vitro p53 Ser-392 kinase assay. Kinase assays with unlabeled ATP were performed as described previously (S 7). HeLa cells were treated with Curaxin compounds (0.4 μM CBLC137 or 0.16 μM CBLC100) or UVC (20 J/m^2) for 8 hours. Cells were then lysed in TNT buffer (20mM Tris HCl, pH 7.5; 200 mM NaCl, 1% Triton X-100) with protease and phosphatase inhibitors. 250 μg of total protein was used for CK2α' immunoprecipitation with 2 μg/ml anti-CK2α' antibody (Betyl Laboratories, Inc. Montgomery, TX). An equal amount of preimmune antiserum was used as a negative control. Two hours later, 20 μl of protein A-Sepharose 4B (Sigma Aldrich, Corp., St. Louis, MO) in TNT buffer was added to each sample and incubated with gentle rotation overnight at 4^oC. Immunoprecipitates were washed three times in TNT buffer with protease / phosphatase inhibitors and two times with kinase buffer (20 mM Hepes, pH 7.5, 100 mM NaCl, 50 mM KCl, 20 mM MgCl2) without these inhibitors. Kinase reactions were performed in kinase buffer using 100 ng p53 C-terminal peptide (aa. 311-393) as a substrate in the presence

of 1mM ATP. Samples were analyzed using SDS-PAGE followed by Western blotting using an anti-phospho-Ser392-p53 antibody (Cell Signaling Technology, Inc., Beverly, MA) to detect peptide phosphorylation. Equal loading of substrate was detected using an anti-p53 antibody pAb122 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Chromatin immunoprecipitation. Proteins were cross-linked to DNA at their in vivo binding positions by treating live cells with 1% formaldehyde for 10 min at room temperature. Glycine was then added to a final concentration of 0.25 mM. Cells were washed with and collected in ice cold PBS containing 0.25 mM PMSF. Upon resuspension in 2 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% SDS), samples were sonicated to fragment DNA to an average size of ~500 bp. For immunoprecipitation, an amount of chromatin containing 120 μg nucleic acid (based on absorption at 260 nm) was diluted 10-fold with ChIP dilution buffer (16.7 mM Tris-HCl pH 8.0, 0.01% SDS, 1.2 mM EDTA, 167 mM NaCl, 1.1% triton-X-100) and incubated with 50 μl of protein A/G 50% slurry that was pre-equilibrated with 1 mg/ml of BSA and 0.3 mg/ml of salmon sperm DNA for 1 hr at 4^0C with constant rotation. The supernatant was incubated with 4 μg of anti-CTD of Rpb1 subunit of RNA pol II antibody (Abcam ab817) overnight followed by addition of 40 μl of the protein A/G 50% slurry pre-equilibrated with 1 mg/ml of BSA and 0.3 mg/ml of salmon sperm DNA for another hour. Beads were collected, washed once each with low salt (20 mM Tris-HCl pH 8.0, 0.1% SDS, 2mM EDTA, 150 mM NaCl, 1% triton-X-100), high salt (20 mM Tris-HCl pH 8.0, 0.1% SDS, 2 mM EDTA, 500 mM NaCl, 1 % triton-X-100), and LiCl buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA, 250 mM LiCl, 1% NP40, 1% deoxycholate), and twice with TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA). Beads were incubated with 40 units RNAse T1 and 0.2 mg/ml proteinase K for 4 hours at 55^oC to release the immunecomplexes followed by reversal of cross-linking by treatment of bead-free supernatant at 65^oC overnight. DNA

was purified by phenol/chloroform extraction and ethanol precipitation to resuspend in 100 μl TE. Five to 10 μl of each sample was used as template for PCR using conditions of 95^0C for 1 min, 35 cycles of 95C for 45 sec, 65C for 30 sec and 72C for 30 sec. Primer pairs of IL8pF2: 5'- AAG AAA ACT TTCGTCATACTCCG-3' and IL8pR2: 5'- TGG CTTTTTATATCATCACCCTAC-3'; and IL8CF2: 5'-CTAACTGAGGTCAAGGGCTAGGAG and IL8CR2: 5'-AAGGTTTGGAGTATGTCTTTATGC were used to amplify 136 bp from the promoter and 227 bp from the coding region of the IL8 gene, respectively. For the p21 gene, 284 bp and 260 bp fragments from the promoter and coding regions were amplified using primer pairs p21prF1 5'-ACCAACGCAGGCGAGGGACT-3 and p21prR1 5'-CCGGCTCCACAAGGAACTGA-3, and p21CF1 5'-CACCTGAGGTGACACAGCAAAG-3 and p21CR1 5'- CAGTGGTGTCTCGGTGACAAAG-3), respectively.

Assembly and transcription of mononucelosomal template: A 199 bp DNA fragment carrying the 603 nucleosomal positioning sequence (NPS) originally isolated by Lowary and Widom (S 8) cloned downstream of the adenovirus major late promoter was isolated by PCR from a pUC18-based plasmid with the upstream primer labeled with biotin at its 5'-end. Primer sequences, PCR conditions, fragment purification, and mononucleosome assembly with bacterially expressed Xenopus histones were described previously (S 9); (S 10). Briefly, assembly reactions contained varying amounts (0.8, 1.0 or 1.2 μg) of histones (equimolar H2A/2B dimers and H3/H4 tetramers), 1 μg of 603 DNA containing a small amount (5 ng) of ^{32}P-end-labeled 603 DNA, 2M NaCl, 20 mM Tris-HCl pH 7.9, 20% glycerol, 5 mM β-ME, 0.2% NP40, protease inhibitors and 4 μg sonicated salmon sperm DNA. The salt-denatured histones were reassembled on the NPS by gradually decreasing the NaCl concentration from 2M to 0.1M through overnight dialysis against the assembly buffer. Before use in

transcription and/or EMSA experiments, the efficiency of mononucleosme assembly was tested by 4% native polyacrylamide gel electrophoresis (37.5% acrylamide:1% bisacrylamide in 10 mM HEPES, 0.2 mM EDTA, pH 8.0) as well as by testing protection of the NPS against digestion with restriction endonucleases. Mononucleosome preparations were stored in aliquots at +4^{o}C.

Transcription on the mononucleosomal templates was performed as described (S 11). Briefly, preinitiation complexes were assembled on the mononucleosomal template attached to streptavidin-coated maganetic beads with purified general transcription factors and pol II. Pol II was advanced to +21 where +1 is the transcription start site, upon incubation with 0.5 mM ApC dinucleotide, 50 μM of ATP and UTP and 1 μM α-^{32}P CTP. The stalled complexes were washed once before chasing with a solution containing 1 mM of all four NTPs with or without 5 μl (~9 pmoles) of a highly homogeneous preparation of FACT complex. The recombinant FACT used was purified by both anti-Flag and nickel affinity columns from SF9 cells using the constructs kindly provided by Dr. Danny Reinberg (New York University School of Medicine, New York, NY) and a previously described method (S 12). The transcripts were purified by phenol chloroform extraction and ethanol precipitation, resolved by denaturing gel electrophoresis, and visualized/quantified using a Phosphorimager (Molecular Dynamics) and ImageQuant software (GE Healthcare).

Binding of SSRP1 to DNA and chromatin in vitro. Two types of 5'-end [^{32}P]-labeled templates were used, a 120 bp double stranded DNA probe pML20-42 (S 13) and mononucleosomal DNA prepared as described above. Binding reactions with these two probes were performed in 20 μl for 45 min at room temperature in 20 mM Tris-HCl, pH7.6, 150 mM NaCl, 10 mM MgCl2, 5% glycerol, 0.5mM DTT 0.25 mM EDTA with 30

fmoles ($2x10^3$ cpm) of DNA probe and with 2, 4, 8 or 16 pmoles FLAG tagged-SSRP. The probe was treated with Curaxins for 10 min or with cisplatin as described previously (S 14) prior to addition of proteins. The binding reaction was resolved by electrophoresis in a 4.5% native polyacrylamide gel (29% acrylamide: 1% bisacrylamide). The gel was dried and visualized using a Phosphorimager (Molecular Dynamics).

For isolation of chromatin, HeLa cells were resuspended in PBS and aliquots of 10^6 cells were used for each binding reaction. Nuclei were isolated from each aliquot separately and lysed in no-salt buffer (S 15) followed by centrifugation of lysates at 6500Xg for 5 min at 4^oC. Pellets were washed two times with 150mM NaCl buffer. For binding reactions, chromatin pellets were resuspended in 10 mM Tris, pH7.5, 50 mM NaCl, 10 mM MgCl2, 0.5 mM EDTA, 5% glycerol, 1 mM DTT and mixed with 100ng purified SSRP1 and 5μM CBL137 for 2 hours. Reactions were then spun down at 6500Xg for 15 min at $+4^oC$. Supernatants were loaded onto gels without additional treatment; pellets were sonicated and boiled in Laemmli buffer for 5 min before loading. Electrophoresis and Western blotting with anti-SSRP1 antibodies were performed as described above.

***In vivo* evaluation of Curaxins in mouse tumor models.** All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of the Roswell Park Cancer Institute (Buffalo, NY) according to the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. The maximum tolerated dose (MTD) for oral administration, which we defined as the maximum dose not associated with mortality or persistent morbidity, was determined for single and repetitive administration of Curaxins. To begin, a pilot single dose toxicity study was performed using doses between $10xIC_{90}$ and $200xIC_{90}$ (IC_{90} as determined for *in vitro* cytotoxicity against tumor cells) with $10xIC_{90}$ representing the minimum dose expected to have

antitumor activity *in vivo*. Those compounds that were at least non-toxic at the $10xIC_{90}$ dose were tested at 3 higher doses between the $10xIC_{90}$ and $200xIC_{90}$ dose (if toxic) to more accurately define the single MTD. For single MTD studies, groups of 8 NIH Swiss or ICR mice (4 males and 4 females per group) were administered a single oral dose of Curaxins by gavage. Mice were observed for up to a week for signs of toxicity, such as excess weight loss (≥20% original weight), hunching, ruffled fur, inactivity, and persistent disturbed behavior. On days -6, 2 and 7, blood was collected from the saphenous vein to determine the effect of treatment on white blood cell populations. Since Curaxins are not administered as a single dose in in vivo efficacy studies, we determined the MTD for repetitive dosing (e.g., 5 consecutive daily treatments) using the single MTD, ½ single MTD and ¼ single MTD. Groups of NIH Swiss or ICR mice (2 males and 2 females per group) were administered a single oral dose of Curaxins by gavage daily for 5 consecutive days. Mice were observed for signs of toxicity as described above for up to 14 days. Blood was collected on Days -6, 8 and 14. In some cases, blood was collected upon euthanasia by cardiac puncture and samples subjected to serum biochemistry analysis to identify potential toxicity to liver, kidney and other organs. All parenchymal organs and bone marrow of mice treated with rMTD of CBLC137 for 3 weeks were subjected to macroscopic and microscopic histopathological analysis by a qualified pathologist (Cleveland BioLabs, Inc.).

For non-toxic Curaxins, efficacy against human tumor xenografts was determined. For these experiments, $1\text{-}3x10^6$ tumor cells grown in culture were inoculated subcutaneously into each rear flank of athymic nude mice (n=10/treatment group). For the model of pancreatic ductal adenocarcinoma, a SCID/NOD mouse with a single tumor was generated by subcutaneous transplantation of a piece of a surgical sample from a patient with pancreatic cancer (kindly provided by Dr. Repasky, Dept. of Immunology,

Roswell Park Cancer Institute, Buffalo, NY). This tumor was excised from the anesthetized mouse, washed with PBS and cut into 8-10mm^3 pieces. Recipient mice were anesthetized by isoflurane/oxygene gas mixture through a mouth mask. A 2mm cut was made on the left abdominal area with surgical scissors and ~10mm^2 area under the skin was separated from underlying tissues. A single tumor piece was placed into this "pocket" and the skin wound was closed with Reflex Clip Applier and 7mm stainless steel wound clips (Kent Scientific Corp, Gaithersburg, MD). Clips were removed 7 days after the surgery with clip-removing forceps. Upon visible/palpable presence of a tumor, tumors were measured using digital vernier calipers and tumor volume was determined using the following equation: length x $width^2/2$ where length is the largest diameter and width is the largest diameter perpendicular to the length. Tumors were measured every other day prior to the start of treatment and twice weekly once treatment commenced. Treatment was started when tumors reached ~50 mm^3. Curaxins were administered by oral gavage at rMTD following a 5 days-on/2 days-off schedule for up to 4 weeks. Mice were euthanized after 4 weeks of treatment or when at least one tumor reached a volume of 1000 mm^3. Statistically significant differences in tumor volumes of Curaxin- and vehicle-treated groups were determined using Student's t-test ($P<0.05$=significant).

p53 heterozygous mice were obtained as described (S 16). 40 male and 40 female animals were exposed to 4Gy total body gamma irradiation (^{137}Cs source) and then separated into two groups. The control and experimental groups were treated with vehicle (water) or CBLC137 (30mg/kg) was dissolved in water. Mice were treated by gavage once a day for 7 consecutive days of every month of their lifespan. Animals were observed and weighed daily and were sacrificed in cases of extensive weight loss (>15%), development of visible tumors or other signs suggesting death within 24 hours.

All euthanized animals were subjected to gross pathology examination, including collection of tumors for histopathology examination.

MMTV-neu mice (Tg(MMTVneu)202Mul/J) were obtained from Jackson Lab (Bar Harbor, ME). Female mice with spontaneous palpable mammary tumors were used in the study.

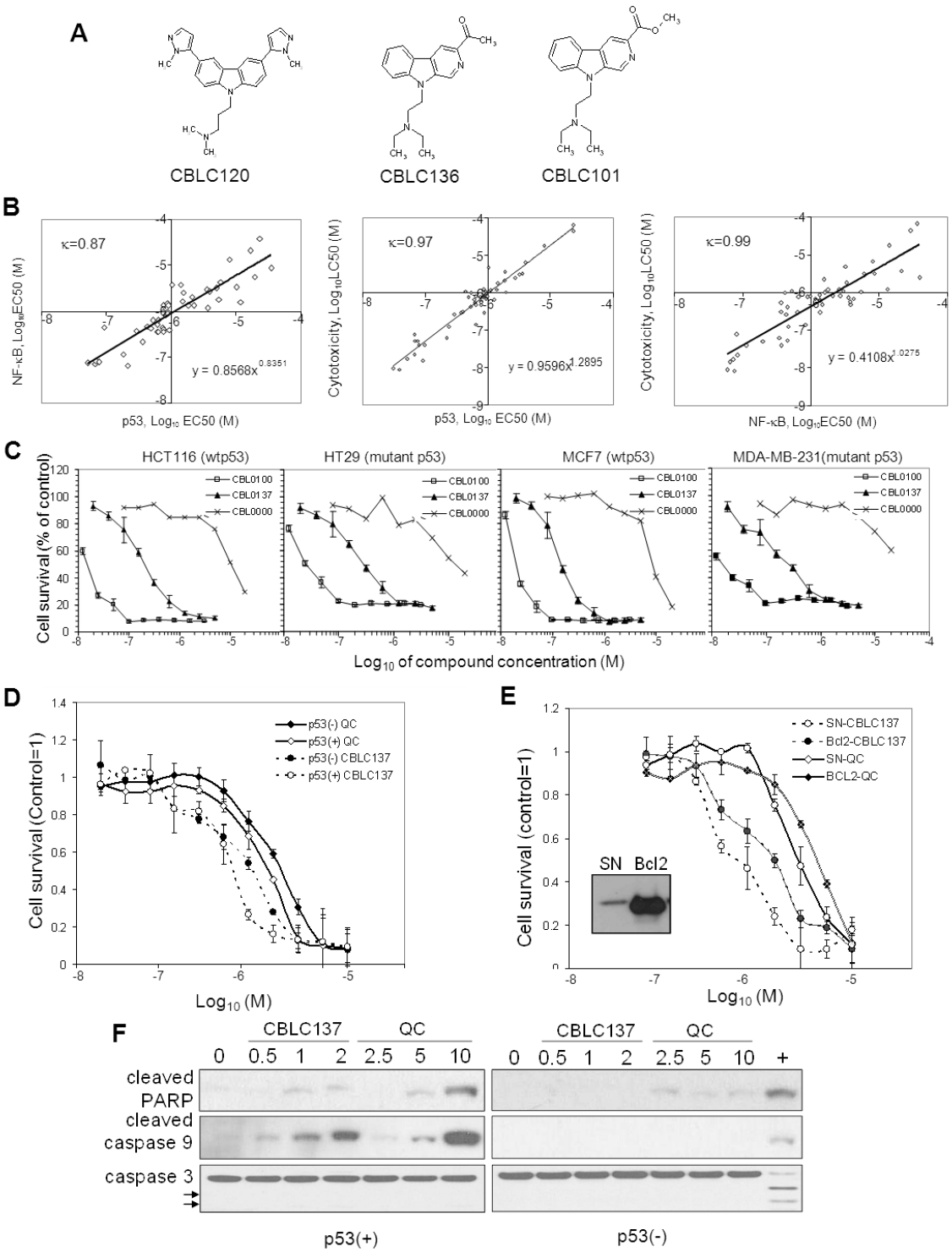

Figure S1. Structure and activity of curaxins. A. Structure of compounds with no activity in p53 and NF-κB assays used as negative controls. B. Pair-wise comparison of CX efficacy in three assays: i) p53-dependent reporter activation in RCC45 cells; ii) NF-κB dependent reporter inhibition in H1299 cells; and iii) cytotoxicity towards RCC45 cells (LC50). Each point indicates an individual Curaxin compound. Plots demonstrate near-linear dependence between logarithms of the two parameters used in each plot. Pearson correlation coefficients are shown in upper right corner of each plot. The linear regression dependencies between logs of parameter are transformed into equivalent exponential formulas. C. Cytotoxicity of CXs to tumor cell lines of different origin and p53 status. Cells were incubated with CX compounds for 24hrs, then washed and incubated in fresh medium for 72 hrs. Cell survival was assessed by methylene blue staining and extraction, followed by spectrophotometric quantitation. Percentage of viability vis-à-vis control cells treated with 0.1% DMSO is shown +/-SD. D. Toxicity of Curaxins to HCT116 cells differing in p53 status. p53 wild type ("p53(+)") and p53-/- ("p53(-)") HCT116 cells were treated with the indicated concentrations of CBLC137 or quinacrine for 1 hr. Cell survival was determined at 72 hrs by methylene blue assay. Data are mean of three replicates +/-SD. D. The anti-apoptotic factor Bcl2, does not fully protect RCC45 cells from Curaxin-induced death. RCC45 cells transduced with either LXSN (SN, empty vector control) or L-Bcl2-SN (Bcl2 expression vector) were treated with the indicated concentrations of CBLC137 for 72 hrs. Cell survival was assessed by methylene blue assay. Data are mean of three replicates +/-SD. The insert shows the level of Bcl2 protein in the tested cells. F. Appearance of apoptotic markers in HCT116 cells treated with Curaxins is p53-dependent. Western blot analysis of p53 wt or p53 -/- HCT116 cells treated for 72 hrs with the indicated concentrations of CBLC137 or quinacrine (in μM). Arrows indicate the products of caspase 3 cleavage). Cells in lanes marked "0" were untreated; cells in lanes marked "+" were treated with 20ng/ml TNF and 10μM MG132 (positive control for apoptosis).

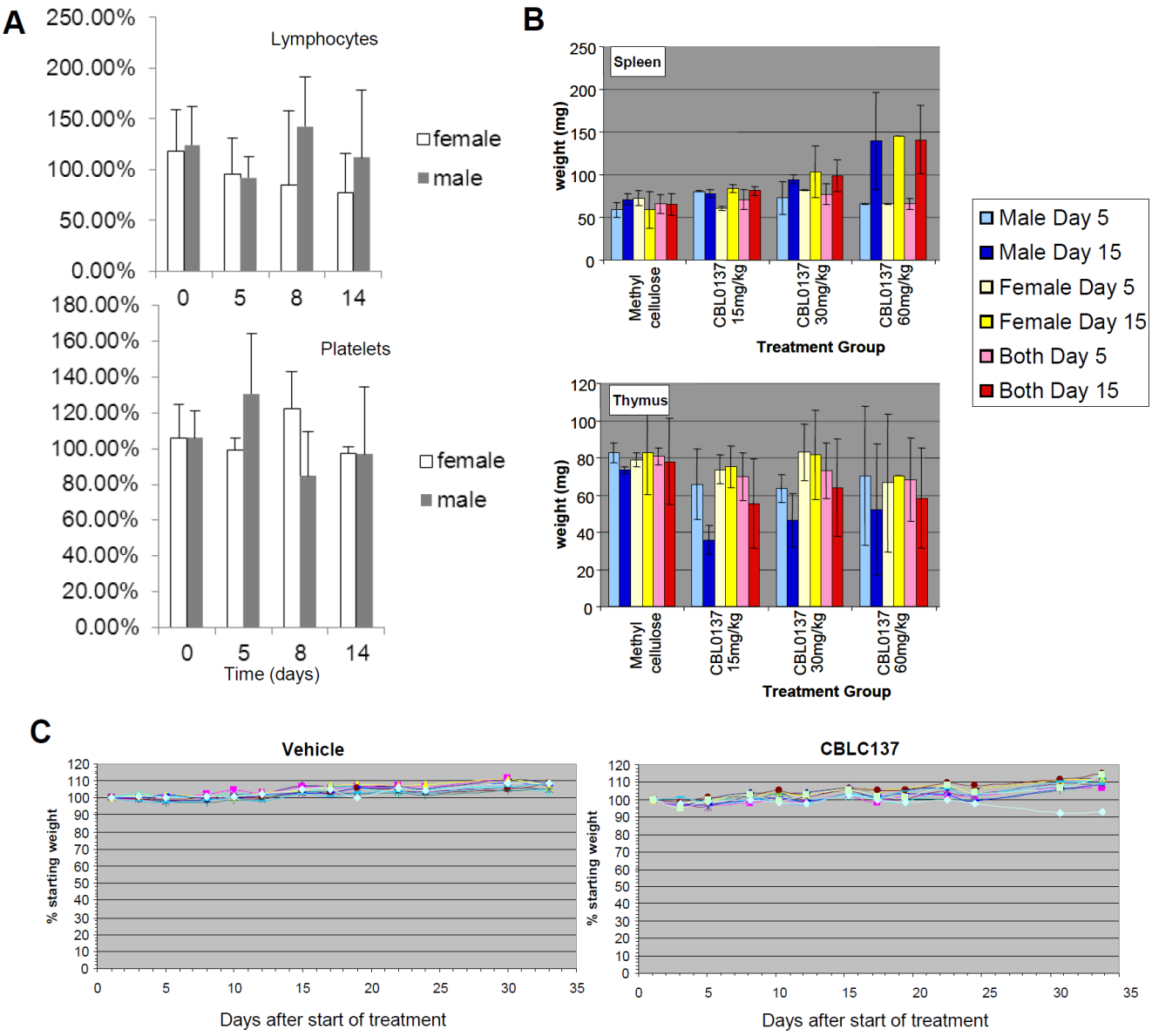

Figure S2. Antitumor effect of curaxin CBLC137 in xenograft mouse models of cancer. CBLC137 administration to mice at 30mg/kg dose did not cause side effects. A-B. 4 male and female NIH Swiss mice were treated with CBLC137 for 5 days. Mice were sacrificed at days 5 or 14. A. Lymphocytes and platelet counts in a group of CBLC137 treated mice shown as % of data in vehicle control group (0.5% mehtylcellulose)+/-SD. B. Size of spleen and thymus in different treatment groups +/-SD. No significant changes were found in these or other parameters measured in this experiment. C-D. Cage side observation (C) and weight of mice in experiment on Fig. 2A (model of RCC Caki-1). Negative control was vehicle used for solution of CBLC137, 2 0.5% methylcellulose. Positive control was 40mg/kg of sunitinib. C. Cage side observation of individual mice within each treatment group color coded as described in the box on the right. n – number of mice per group. D. Weight of individual mice within each treatment group shown as % of weigh one day before start of treatment.

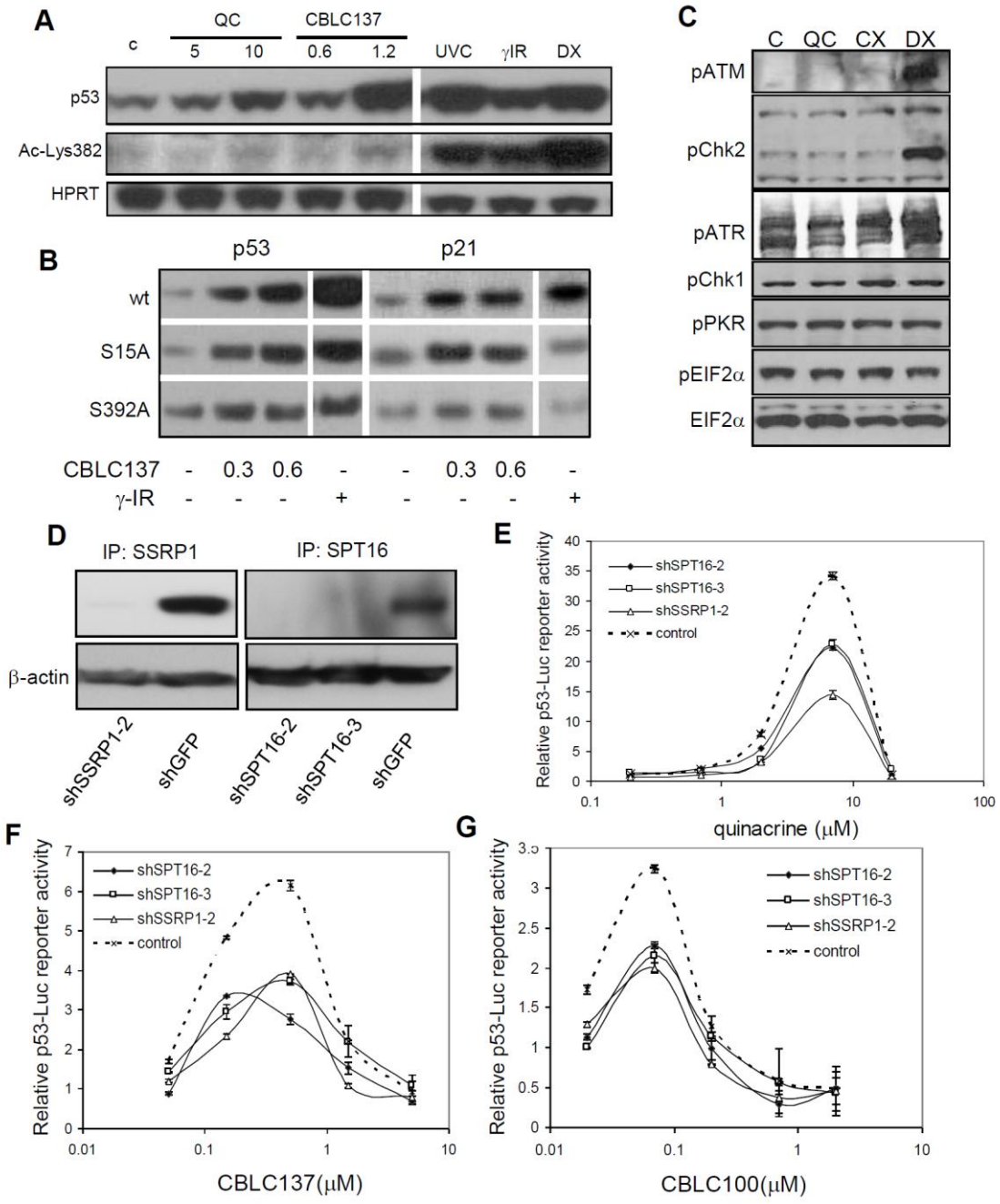

Figure S3. Dependence of p53 activation by curaxin on CK2 and FACT. A. Curaxin treatment does not induce acetylation of p53 on lysine 382. Western blots were prepared using lysates of RCC45 cells treated with the indicated concentrations of QC or CBL137 (in μM) or doxorubicin (DX, 0.5μM) for 6 hrs or with UV-C (120 J/m2) or gamma irradiation (12Gy). Blots were probed with DO1 antibody to detect "total" p53, an antibody specific for p53 acetylated on lysine 382, and anti-HPRT as a loading control.
B. Mutation of p53 leading to substitution of serine 392 with alanine prevents Curaxin-induced p53 activation. Western blotting was performed using lysates of H1299 p53-null cells transduced either with wild type (wt) p53or p53 mutants with serines 15 or 392 substituted to alanines (S15A and S392A,respectively). Cells were treated with the indicated concentrations of CBL137 or 12 Gy of gamma radiation. Cell lysates were prepared 6 hrs after treatments. C. Phosphorylation of several DNA-damage sensitive kinase targets in HT1080 cells left untreated (C), treated with quinacrine (QC, 5μM), Curaxin (CX, CBLC137, 1μM) or doxorubicin (DX, 1μM) for 2 hrs. Western blot analysis of total cell lysates was performed using antibodies specific to phosphorylated modifications of the indicated proteins. EIF2a was used as a loading control. D-G. Effect of shRNAs against FACT subunits on p53 reporter activity and expression of their target proteins. D. Level of SSRP1 and SPT16 in RCC45 cells transduced with the indicated lentiviral shRNA constructs. Western blot analysis was performed 72 hrs after transduction. E-G. p53-dependent luciferase reporter assay in RCC45-p53-Luc cells transduced with lentiviral constructs expressing the indicated shRNAs (control –shRNA against GFP) and treated with the indicated concentrations of Curaxins 72 hrs after transduction (for 16 hrs). Luciferase activity is shown relative to that in control DMSO-treated cells. Data are mean of three replicates +/-SD.

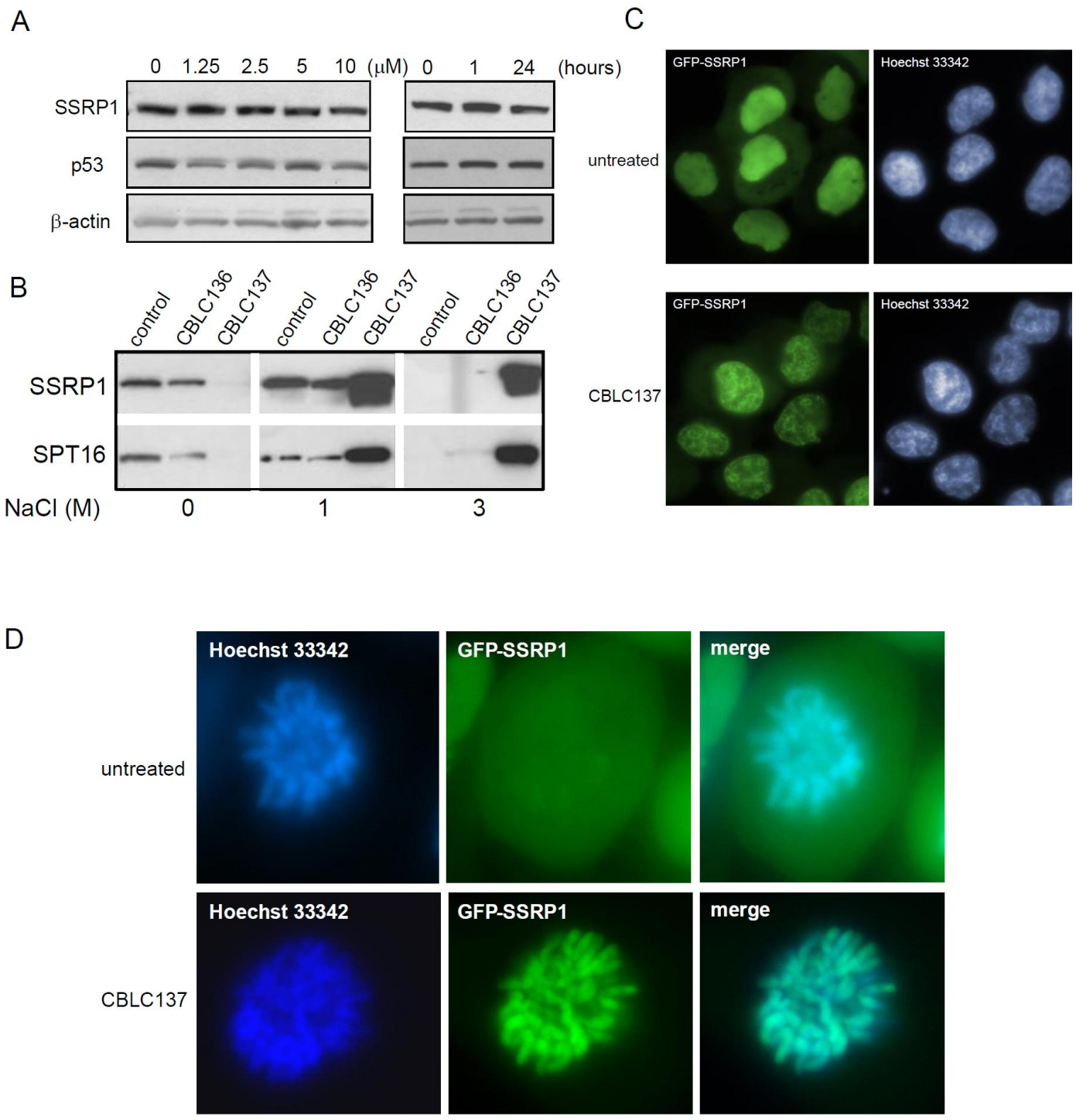

Figure S4. Curaxin induction of FACT-chromatin binding. A. An inactive structural analogue of Curaxins (CBLC136) does not induce p53 activation or reduction of the level of soluble SSRP1. Western blotting of soluble whole cell protein extracts of RCC45 cells treated with different concentrations of CBLC136 for 24 hrs (left panels) or with 10μM CBLC136 for 1 or 24 hrs. B. Chromatin trapping of SSRP1 and SPT16 proteins in HeLa cells treated with CBLC137, but not in those treated with the inactive analogue CBLC136. Western blotting of nuclear extracts isolated from Hela cells using buffers with indicated concentrations of NaCl. C-D. Association of SSRP1 with chromatin during interphase (C) and mitosis (D). Microphotographs of non-fixed HT1080 cells expressing GFP-tagged SSRP1 with or without CBLC137 treatment (1μM for 30 min). DNA was counterstained with Hoechst 33342. Images were taken at 100X oil lens.

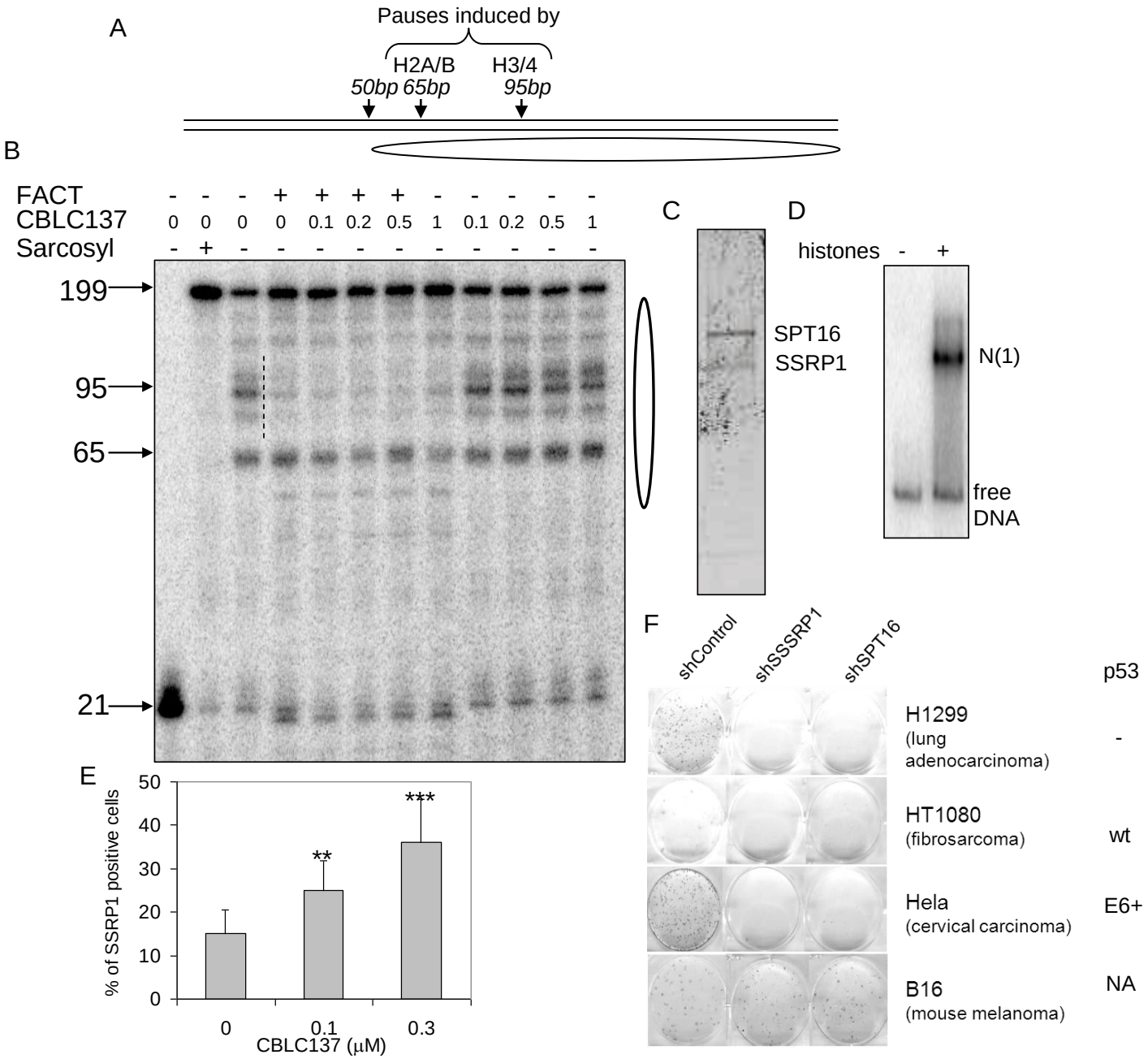

Figure S5. Association between FACT levels and cell growth, sensitivity to curaxins, and tumor phenotype. A-D. Curaxin treatment does not inhibit transcription from a nucleosomal template or FACT nucleosome destabilizing activity in an in vitro system. A. Scheme showing the nucleosome positioning signal (NPS) on a 199 bp template. The cluster of nucleosomal positions is shown by an oval. B. Curaxin CBLC137 does not interfere with FACT-mediated removal of the nucleosomal barrier to RNA pol II elongation. As described in the Materials and Methods, pol II stalled at +21 (lane 1), was advanced with 1 mM NTPs through the downstream nucleosome at the indicated concentrations (in μM) of CBLC137 in the presence (+) or absence (–) of recombinant FACT (10 pmoles). The dashed line indicate the areas where pol II pausing is relieved in the presence of FACT. Sarcosyl was used as a positive control to produce a complete run-off reaction on nucleosome-free DNA. Arrowheads on the left indicate the lengths of transcripts. C. Coomassie blue-stained gel showing the FACT subunits purified from insect SF9 cells that were used in B. D. Autoradiogram of labeled DNA before and after assembly of the mononucleosome (-histones and +histones, respectively) on the 199 bp DNA template shown in A. The band corresponding to the mononucleosome is labeled N(1). E. The proportion of SSRP1-expressing cells is increased when cells are cultured in the presence of CBLC137. HT1080 cells transduced with shSSRP1-2 vector were selected with puromycin for 72 hours and then replated into drug-free medium or medium containing the indicated concentrations of CBLC137 for 24 hours. Five days later, immunofluorescent staining with anti-SSRP1 antibodies was performed on survived cells and the number of SSRP1-positive cells was assessed by fluorescent microscopy. Data are mean of 5 fields of view +/-SD. ** corresponds to $p<0.01$, *** corresponds to $p<0.001$ (t-test). F. Knockdown of FACT subunits is toxic for tumor cells independently on p53 status. Colony assay on cells transduced with lentiviral shRNAs and selected on puromycin. Status of p53 in cells us shown on the left: (-) – deleted, wt – wild type, E6 – expression of E6 protein of HPV, destabilizing p53. Mouse B16 cells were used to normalized transduction efficiency (mouse FACT subunits are not targets of the used shRNAs)

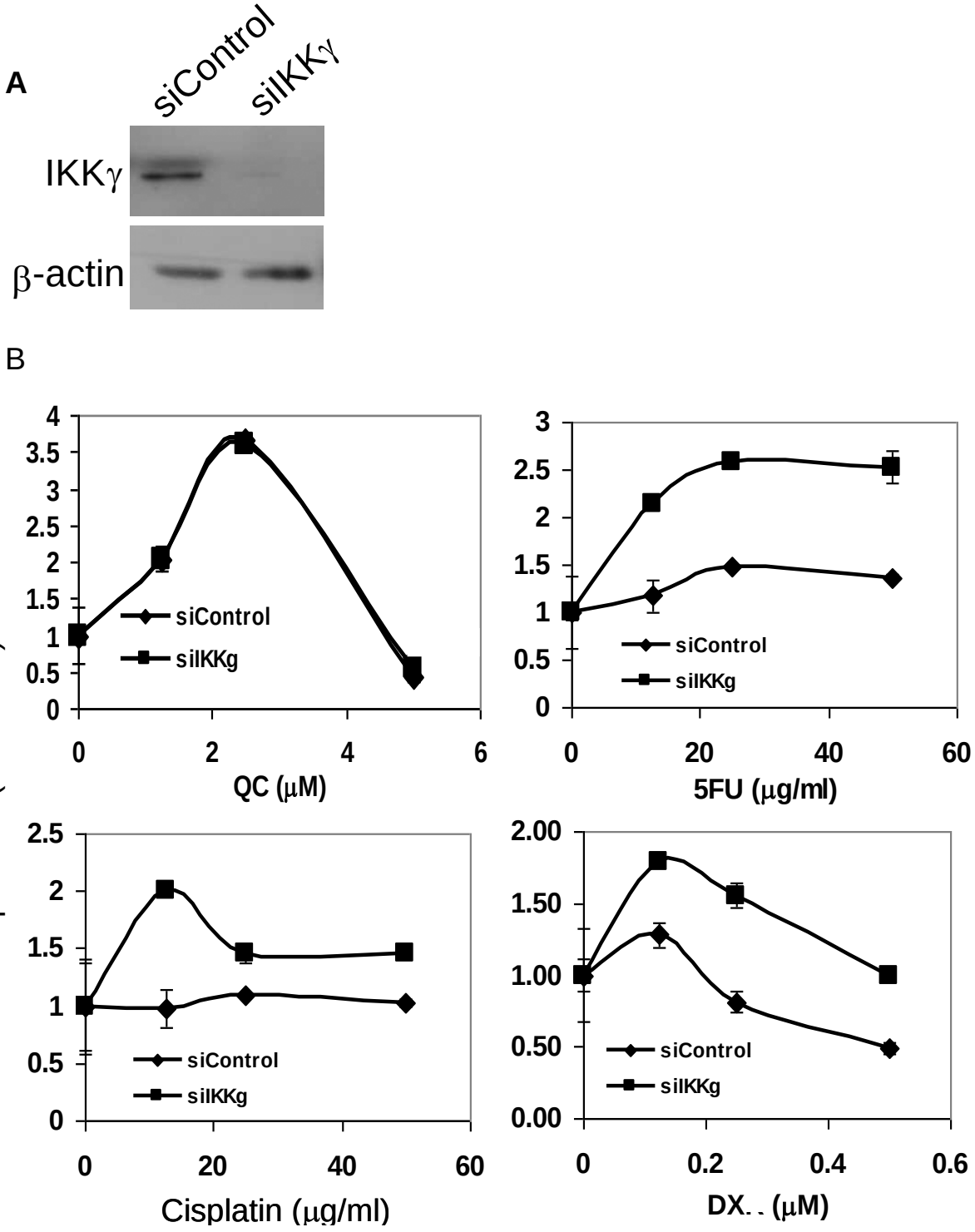

Figure S6. Effect of curaxins on NF-kB. Suppression of IKKγ expression makes the p53 present in RCC45 cells responsive to DNA-damaging agents. A. Knockdown of IKKγ expression in RCC45-p53-Luc cells by transfection of siRNA to IKKγ. Western blotting was performed on cell lysates prepared 72 hrs after transfection of siControl or siIKKγ. B. Knockdown of IKKγ in RCC45 cells allows a p53 transcriptional response to DNA damage. p53-dependent luciferase reporter assay in RCC45-p53-Luc cells 72 hrs after siRNA transfection treated with QC or different DNA-damaging agents at the indicated concentrations for 16 hrs. 5FU – 5-fluorouracil, DX – doxorubicin. Luciferase activity is shown relative to that in cells treated with 0.1% DMSO (set at 1.0). Data are mean of three replicates +/-SD.

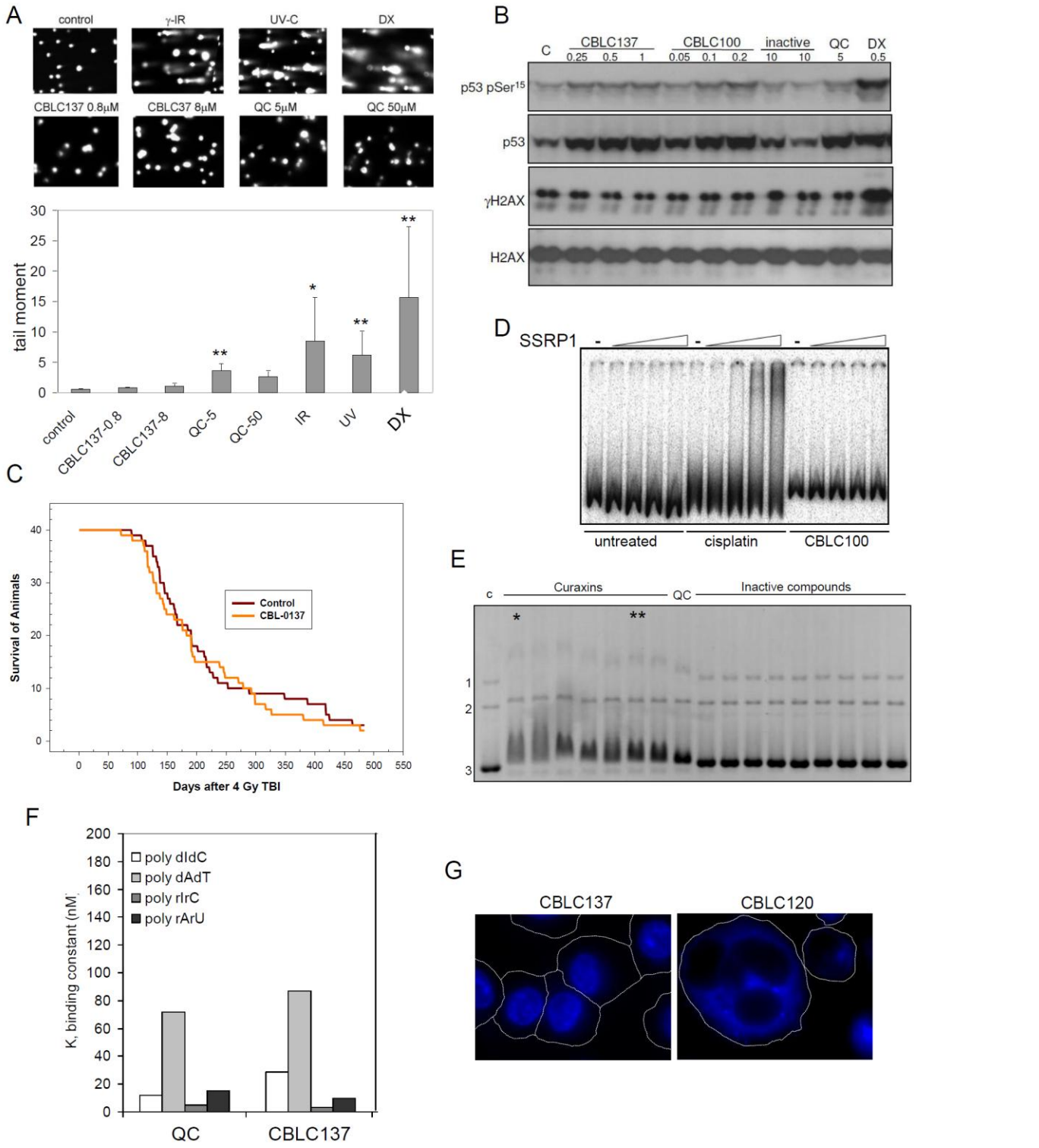

Figure S7 Binding of curaxins to DNA without induction of detectable DNA damage. AComet assays performed using HT1080 cells left untreated (control) or treated with γ-irradiation (12 Gy), UV-C (120 J/m2), doxorubicin (DX, 1 μM), or the indicated concentrations of CBLC137 or QC (in μM) for 6 hrs. The assay was run under alkali conditions to reveal single- and double-strand breaks. The bar graph presents tail moments of >10 cells (Comet Assay IV software from Prospective Instruments). Asterisks indicate values found to be significantly different from control by Kruskal-Wallis One Way Analysis of Variance on Ranks (* $p<0.05$, ** $p<0.01$). B. Western blot analysis of total cell lysates from HT1080 cells left untreated (C) or treated with indicated concentrations of compounds for 6 hrs. C. Curaxin-137 does not promote tumor formation in non-lethally irradiated p53 heterozygous mice. Mice were exposed to 4 Gy total body irradiation (TBI) at 5 weeks of age and then treated with solvent or CBLC137 daily for 1 week each month. D. Curaxins do not induce binding of FACT to purified linear DNA fragment. EMSA was performed using a ^{32}P-labeled probe consisting of a purified 120 bp DNA fragment treated with either cisplatin or CBLC100 in the presence or absence of recombinant SSRP1. Binding of CBLC100 to the DNA fragment is indicated by its altered migration. E. Curaxin compounds that activate p53 cause retardation of plasmid DNA migration in an agarose gel. EMSA of Curaxins or structurally similar inactive compounds ncubated with plasmid DNA for 10 min in water. 1 – relaxed, 2 – linear, and 3 – supercoiled plasmid DNA. c – control untreated cells, * -CBLC100, QC – quinacrine, ** -CBLC137. F. Binding of Curaxins to double-stranded oligonucleotides of different compositions. Competitive dialysis assays with the indicated double-stranded oligonucleotides were performed as described in Materials and Methods. G. CBLC137 is localized in the nuclei of cells, while CBLC120 in cytoplasm. Hela cells were treated with 2μM CBLC137 or CBLC120 . Images were acquired 10 min later. Both compounds are naturally fluorescent in UV light. Cell shapes are shown by white dotted lines (copied from phase contrast photographs).

Supplementary Table S1. Physicochemical and pharmacological properties of curaxins described in the paper.

Compounds		CBLC137	CBLC100
MW*		336.45	374.45
LogP*		2.839	3.31
pK*		10.2	8.5
LogD*	-6.5	-0.26	1.77
LogD*	-7.4	0.2	0.51
PSA*		51.1	42.31
H-bond*	Donors	1	0
H-bond*	Acceptors	4	4
Metabolic** stability	Compound left after 1hr incubation with human liver microsome fraction (%)	77	51
Solubility**	water, pH7.4 (M)	10.8X10(-3)	47X10(-6)
oral repetitive MTD	daily>14d (mg/kg)	30	5

* - calculated values
** - studies done by Covance Laboratories Ltd (not included in Material and Methods)

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