Supplemental Materials and Methods

Chemicals, antibodies, and drugs

UCN-01 was the kind gift of R. Schultz, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). Cisplatin, doxorubicin and camptothecin, puromycin, and glutathione beads were purchased from Sigma-Aldrich. Propidium iodide and SB203580 were purchased from Calbiochem. Antibodies against p53, cleaved caspase 3, total and phosphorylated forms of MK2, p38 MAPK, and hsp-27 were purchased from Cell Signaling Technology (Beverly, MA). An antibody against p21 was from Santa Cruz. Antibodies against $p19^{ARF}$ and β–actin were purchased from Sigma-Aldrich; an anti-Cdc25A antibody (MS-640-P1, cocktail) was from NeoMarker (Fremont, CA); an anti-Cdc25B antibody was from Transduction Labs; anti-phospho histone H3 antibody, FITC conjugated γ-H2AX and Cy5 conjugated phospho Histone H3 antibodies and active MK2 were purchased from Upstate,

Cell culture

MEFs, U2OS, HeLa and U87MG cells were cultured in DMEM supplemented with 10% FCS and penicillin/streptomycin at 37°C in a humidified incubator supplied with 5% CO_2. $p53^{+/+}$ and $p53^{-/-}$ MEFs were a generous gift from T. Jacks (MIT). MEFs were derived from 13.5-day-old embryos and used between passages 3 and 5. $p53^{-/-}$ embryos were derived from $p53^{+/-}$ crosses maintained on a 129/SvJae background. MEFs were maintained in DMEM containing 10% fetal bovine serum supplemented with penicillin and streptomycin. The genotypes of the MEFs were determined by polymerase chain reaction (PCR) as described previously (Attardi et al., 2004) In brief, the wild-type *p53* allele was amplified using PCR primers directed against exon 6 (W5′: 5′-ACAGCGTGGTGGTACCTTAT-3′) and exon 7 (W3′: 5′-TATACTCAGAGCCGGCCT-3′), whereas the $p53^{\Delta}$ allele was amplified using a primer directed against *neo* (M5′: 5′-CTATCAGGACATAGCGTTGG-3′) and W3′.

Recombinant DNA and RNAi

siRNA duplexes consisting of 21 base pairs with a 2 base deoxynucleotide overhang were purchased from Dharmacon Research. Cells were transfected with siRNAs using oligofectamine (Invitrogen) according to the manufacturer's instructions. Cells were typically harvested for further experiments after 48 hr. $p53^{-/-}$

MEFs and p53 $^{-/-}$/ H-ras V12 stably expressing shRNA constructs were generated by lentiviral gene transfer. The RNAi hairpins were cloned into the multiple cloning site of the lentiviral transfer vector pLentiLox-3.7puro or -3.7GFP, (Rubinson et al., 2003). Amphotropic VSV-G pseudotyped lentiviruses encoding these shRNAs were packaged using standard procedures (Rubinson et al., 2003), and all subsequent infections were performed in a BL2+ facility. All transfer and packaging constructs were a kind gift from C.P. Dillon, (MIT). Targeted cells were selected in 8µg/ml puromycin for 4 days. RNAi-resistant mMK2 was generated in a two-step process. Wild-type MK2 was cloned into the MCS of modified pLXSN (kind gift of Dr. T. Benzing), and mutations in the seed region of the RNAi target sequence within MK2 were introduced sequentially using the Quickchange® method (Stratagene) using the following primers: Primer1: 5'-GGAGGAGATGACCAGTGCCTTGGCCAC**A**ATG**ACC** GTTGACTATGAGCAGATCAAGATAAAGAAG-3', and Primer2: 5'-GATGACCAGTG CCTTGGCCAC**A**ATG**ACC****A**TTGA**T**TA**C**GAGCAGATCAAGATAAAGAAGATAGAAG -3'.

Immunofluorescence and Microscopy

Cells were seeded onto 18mm^2 coverslips and either mock treated or treated with cisplatin, camptothecin or doxorubicin for the indicated times. Cells were then fixed in 3% PFA and 2% sucrose for 15 min at RT and permeabilized with 20mM Tris-HCl (pH7.8), 75mM NaCl, 300mM sucrose, 3mM $MgCl_2$, and 0.5% Triton-X-100 for 15min at RT. Slides were stained with primary antibody at 4°C overnight. Secondary antibodies were used for 3 hrs at RT. Images were collected on an Axioplan2 (Zeiss) microscope using Openlab software (Improvision).

FACS analysis

MEFs were transfected and treated as described. After treatment cells were washed twice in ice-cold PBS, trypsinized and fixed in 70% ethanol overnight at −20°C, permeabilized with PBS containing 0.25% Triton X-100 for 20 min at 4°C, blocked with 2% FCS in PBS, and incubated with 1µg of anti-phospho-histone H3 per 10^6 cells for 60 min on ice. Following washing, cells were incubated with FITC-conjugated secondary antibody (Molecular Probes) (diluted 1:500) for 30 min on ice, washed, and resuspended in PBS containing 50 µg/ml PI prior to analysis on a BD FACScan.

Protein production and purification

Recombinant GST-14-3-3β and ζ proteins were produced as described previously (Yaffe et al., 1997). Expression was induced in late-log phase E. coli BL-21 by addition of 0.5 mM IPTG for 4 hr at 37°C. Cells were lysed by sonication in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM DTT, 8 µg/mL pepstatin, 8 µg/mL aprotinin, and 8 µg/mL leupeptin. Fusion proteins were purified from cell lysates using glutathione beads. After extensive washing with TBS containing 0.5% NP-40 and a final wash with PBS, fusion proteins were eluted from the beads with HEPES (pH 7.2), containing 40 mM glutathione, followed by dialysis against modified 2xTBS (50mM Tris-HCl [pH 7.4], 300 mM NaCl, 1mM DTT). Protein concentrations were determined using the bicinchonic acid assay (Pierce) as recommended by the manufacturer, using BSA as the standard. Full-length GST-Chk1 in pFASTBAC was expressed in *Sf9* insect cells. Chk1 expressing cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mM DTT, 1.0% NP-40, 25mM CHAPS, 8 µg/mL pepstatin, 8 µg/mL aprotinin, 8 µg/mL leupeptin, 2 mM Na_3VO_4, 10 mM NaF, and 1 µM microcystin, and Chk1 was purified using glutathione beads. Chk1 was eluted from the beads with 40 mM glutathione in 50 mM Tris-HCl (pH 8.0) and dialyzed into kinase buffer.

14-3-3 pull down assays and Immunoblotting

MEFs were lysed in 50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1.0% NP-40, 5 mM EDTA, 2 mM DTT, 8 µg/ml pepstatin, 8 µg/ml aprotinin, 8 µg/ml leupeptin, 2 mM Na_3VO_4, 10 mM NaF, and 1 µM microcystin containing 5 µg GST-14-3-3 for 15 min at 4°C. Clarified lysates (0.5–2 mg protein) were incubated with 20 µl glutathione beads for 120 min at 4°C. Following washing, lysates and bead bound proteins were analyzed by SDS-PAGE, followed by transfer to PVDF membranes, and immunoblotting with the indicated antibodies.

Clonogenic survival assay

Cells were either mock treated or treated with increasing doses of doxorubicin or cisplatin. After 4 hr of treatment cells were washed three times with growth media and 3 three times with PBS, trypsinized and replated at a concentration of 5000 cells/ $10cm^2$ dish. After 14 days cells were fixed and stained with 0.1% crystal violet (Sigma-Aldrich). Colonies consisting of >50 cells were counted and surviving

fractions were determined by normalization against untreated cells. Experiments were performed in triplicate and are plotted as mean values with standard deviations indicated by the error bars.

Supplemental References

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Supplemental figure captions

Supplemental Figure 1. ATM and ATR are required for activation of MAPKAP Kinase-2 by DNA damaging drugs.

(**A-D**) Differential requirements for ATM and ATR for activation of MAPKAP Kinase-2. A-T and Seckel syndrome fibroblasts, along with matched controls, were treated with 10μM cisplatin, 10μM doxorubicin, 20J/m^2 UV irradiation, or with DMSO (control) for 2 or 8 hr. Cell lysates were probed for total and activated forms of p38MAPK and MAPKAP Kinase-2 by immunoblotting as in Figure 1, with β-actin as a loading control. (A,B) ATM deficient fibroblasts (GM05849), but not their matched ATM-proficient controls (GM00637) fail to activate either p38 MAPK or MAPKAP Kinase-2 after exposure to doxorubicin, while activation of this pathway by UV was unimpaired regardless of the status of ATM. (C,D) ATR-defective fibroblasts from a patient with Seckel syndrome (GM18366), but not their matched ATR-proficient control (GM00023) fail to activate p38 MAPK and MAPKAP Kinase-2 in response to cisplatin or doxorubicin. Activation of p38MAPK/MAPKAP Kinase-2 following UV was unimpaired regardless of their ATR status.

(**E-F**) UV-induced activation of the p38 MAPK/MAPKAP Kinase-2 pathway is independent of PIKK function. U2OS cells were or were not pretreated with 20mM caffeine 30 min prior to exposure to cisplatin, doxorubicin and UV. PIKK inhibition completely prevented p38 MAPK/MAPKAP Kinase-2 activation by cisplatin and doxorubicin, but did not affect UV-mediated activation.

Supplemental Figure 2. Camptothecin activates MK2 in an ATR-dependent and ATM-independent manner.

(**A**) GM18366 ATR-defective cells from a patient with Seckel syndrome or the corresponding control GM00023 fibroblasts were treated with camptothecin (10 μM) for 2 or 8 hr. Cell lysates were probed for total and activated MAPKAP Kinase-2 by immunoblotting, and with anti-β-actin as a loading control.

(**B**) GM05849 A-T fibroblasts and corresponding control GM00637 fibroblasts were treated with camptothecin and analyzed for MAPKAP Kinase-2 activation as in panel A.

Supplemental figure 3. Lentiviral transfer vectors for shRNA delivery.

(**A**) Feature map of pLentiLox3.7.GFP. This vector drives the expression of shRNA sequences from a U6 promoter, while eGFP is driven from a CMV promoter.

(**B**) Feature map of pLentiLox3.7.Puro. This vector drives the expression of shRNA sequences from a U6 promoter, while the puromycin resistance gene is driven from a CMV promoter.

SIN LTR: self inactivating long terminal repeat; Ψ: packaging signal; cPPT: central polypurine tract; MCS: multiple cloning site; WRE: Woodchuck hepatitis B virus RNA regulatory element.

Supplemental Figure 4. Cisplatin and doxorubicin induce different cell cycle checkpoints in p53$^{-/-}$ MEFs.

(**A**) The cell cycle profile of asynchronous untransfected control p53$^{-/-}$ MEFs cells was analyzed by FACS using PI for DNA content (blue) and phospho-histone H3 staining as a mitotic marker (red).

(**B**) Following treatment with doxorubicin (10μM, 30 hr) p53-/- MEFs accumulate at the G2/M boundary. In addition, there is a mild increase in the S-phase population compared with control cells in panel A

(**C**) Following treatment with cisplatin (10μM, 30 hr) p53-/- MEFs preferentially accumulate in G1/S phases of the cell cycle.

(**D**) p53-/- MEFs treated with nocodazole (100 nM, 30 hr) accumulate in a 4N DNA containing peak with 29.5% staining strongly for phospho-histone H3.

Supplemental figure 5. Expression of an RNAi resistant MAPKAP Kinase-2 rescues the MAPKAP Kinase-2 RNAi phenotype in p53$^{-/-}$ MEFs.

(**A-C**) p53$^{-/-}$ MEFs stably expressing either control luciferase shRNA and an empty vector (panels in column A), MAPKAP Kinase-2 shRNA and an empty vector (panels in column B) or MAPKAP Kinase-2 shRNA and an RNAi resistant MAPKAP Kinase-2 cDNA (panels in column C) were untreated, or treated with 10 μM cisplatin or 10μM doxorubicin. 100ng/ml nocodazole was added 3 hrs after addition of the DNA damaging drug. Cell cycles profiles were examined 30 hr later by FACS using PI for DNA content (blue) and phospho-histone H3 staining as an indicator of mitosis (red). Expression of an RNAi resistant MAPKAP Kinase-2 cDNA (MK-2 res) in the

MAPKAP Kinase-2 depleted MEFs (C) restored both the cisplatin induced S-phase arrest and the doxorubicin induced G_2/M arrest, similar to what was observed in control luciferase shRNA-treated cells (A).

(**D**) Expression levels of MAPKAP Kinase-2 in the three cell lines used in panels A-C above was examined by immunoblotting. β-actin is shown as a loading control.

Supplemental figure 6. MAPKAP Kinase-2 and Chk-1 phosphorylate Cdc25A in vitro.

GST-tagged Cdc25A was expressed in HEK 293T cells and affinity purified using GSH beads. Samples were phosphorylated *in vitro* in identical 30 μl kinase reactions containing either 0.3 μM Chk1 or 0.1 μM MAPKAP Kinase-2 for 20 min at 30^{o}C. (**A**) Samples were analyzed by SDS-PAGE, transferred to nitrocellulose, and examined by autoradiography, revealing equivalent Cdc25A phosphorylation by Chk1 and MAPKAP kinase-2. (**B**) Equal substrate loading was shown by subsequently immunoblotting the membranes using an anti-GST antibody.

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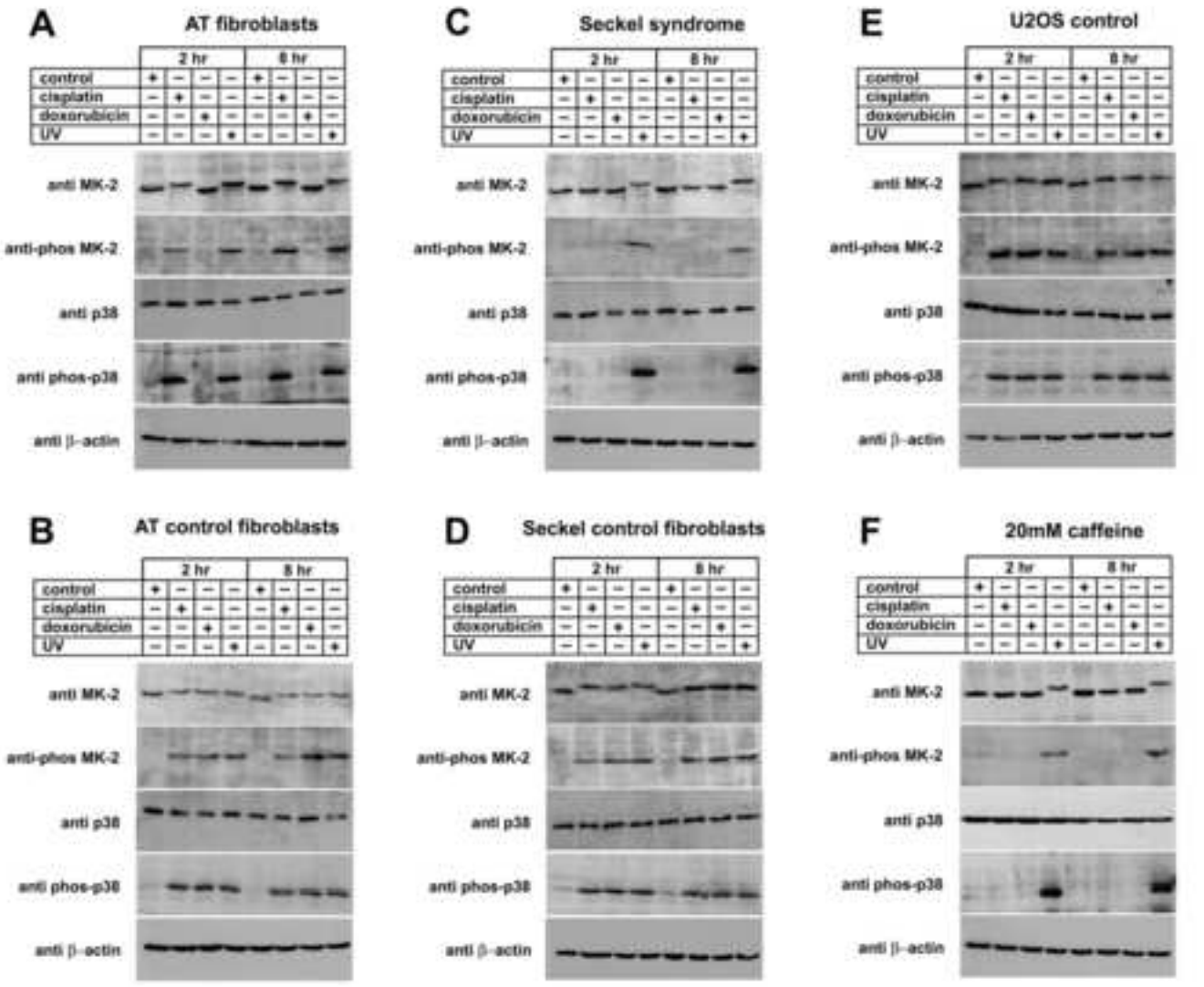

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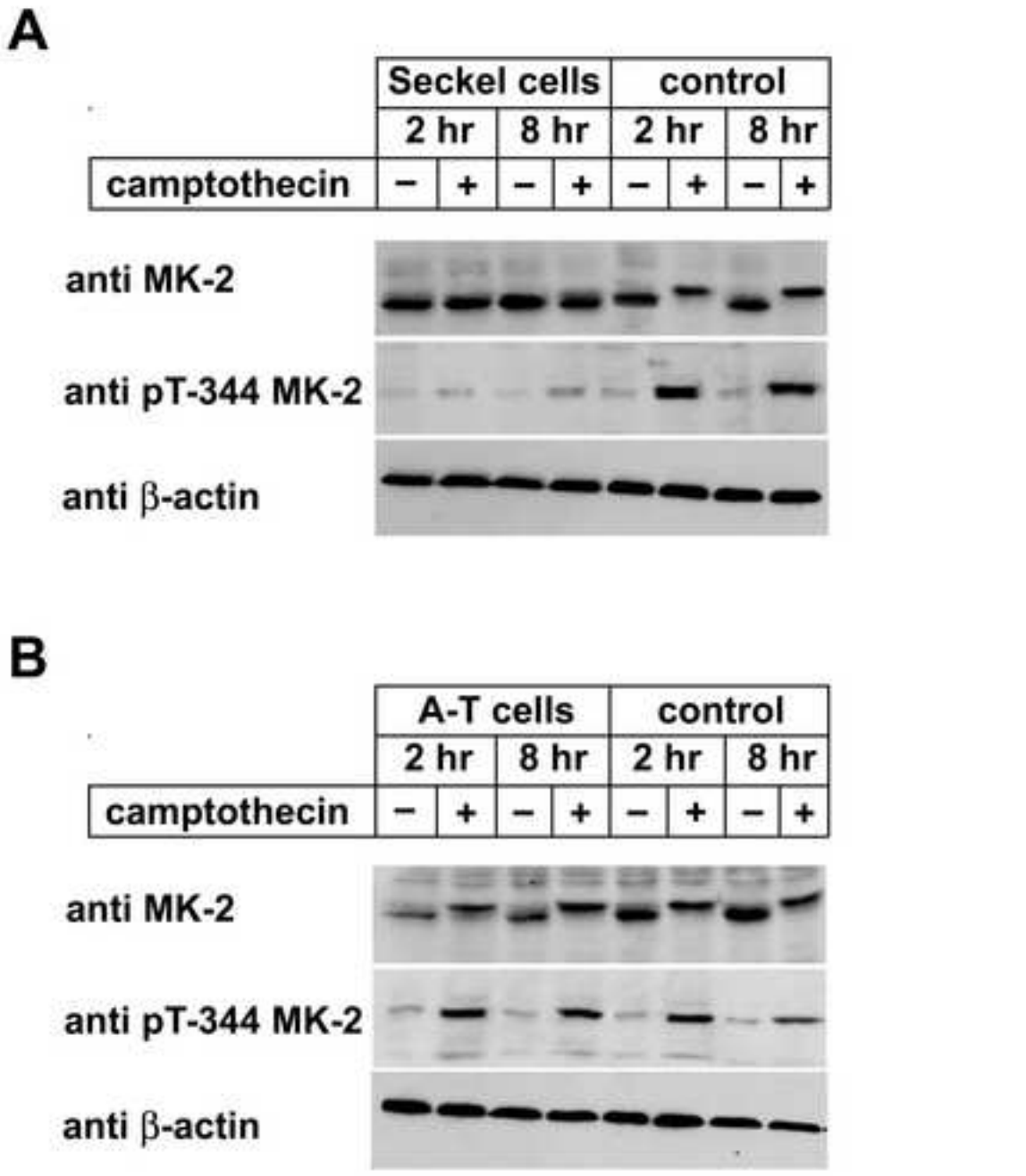

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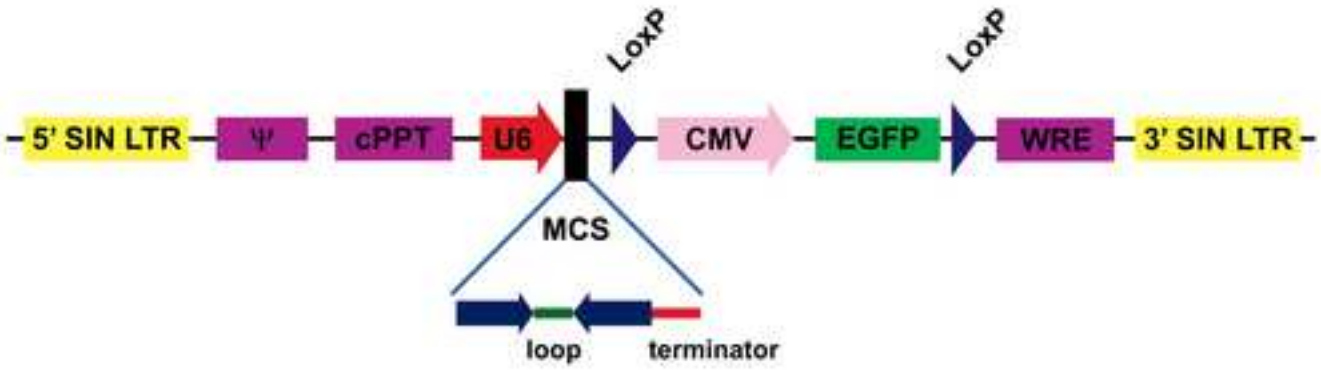
pLentiLox3.7.GFP



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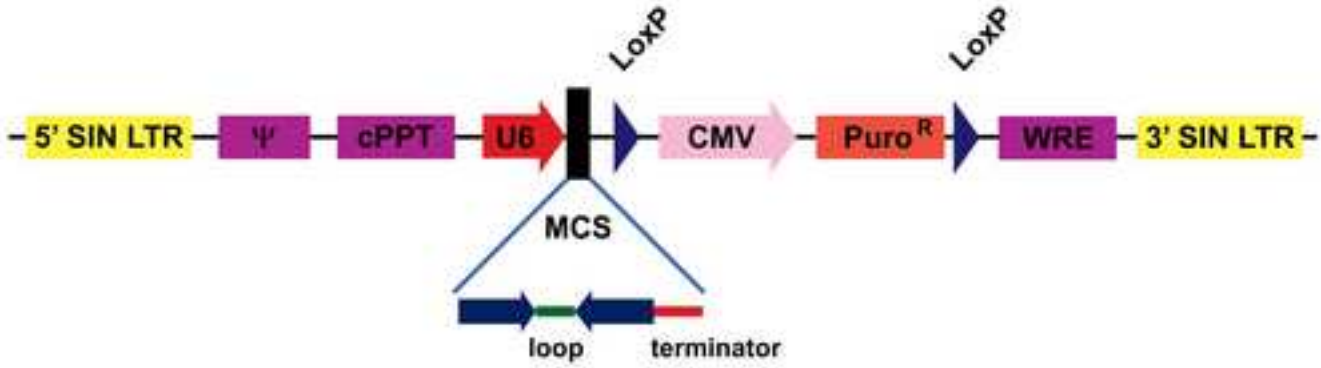

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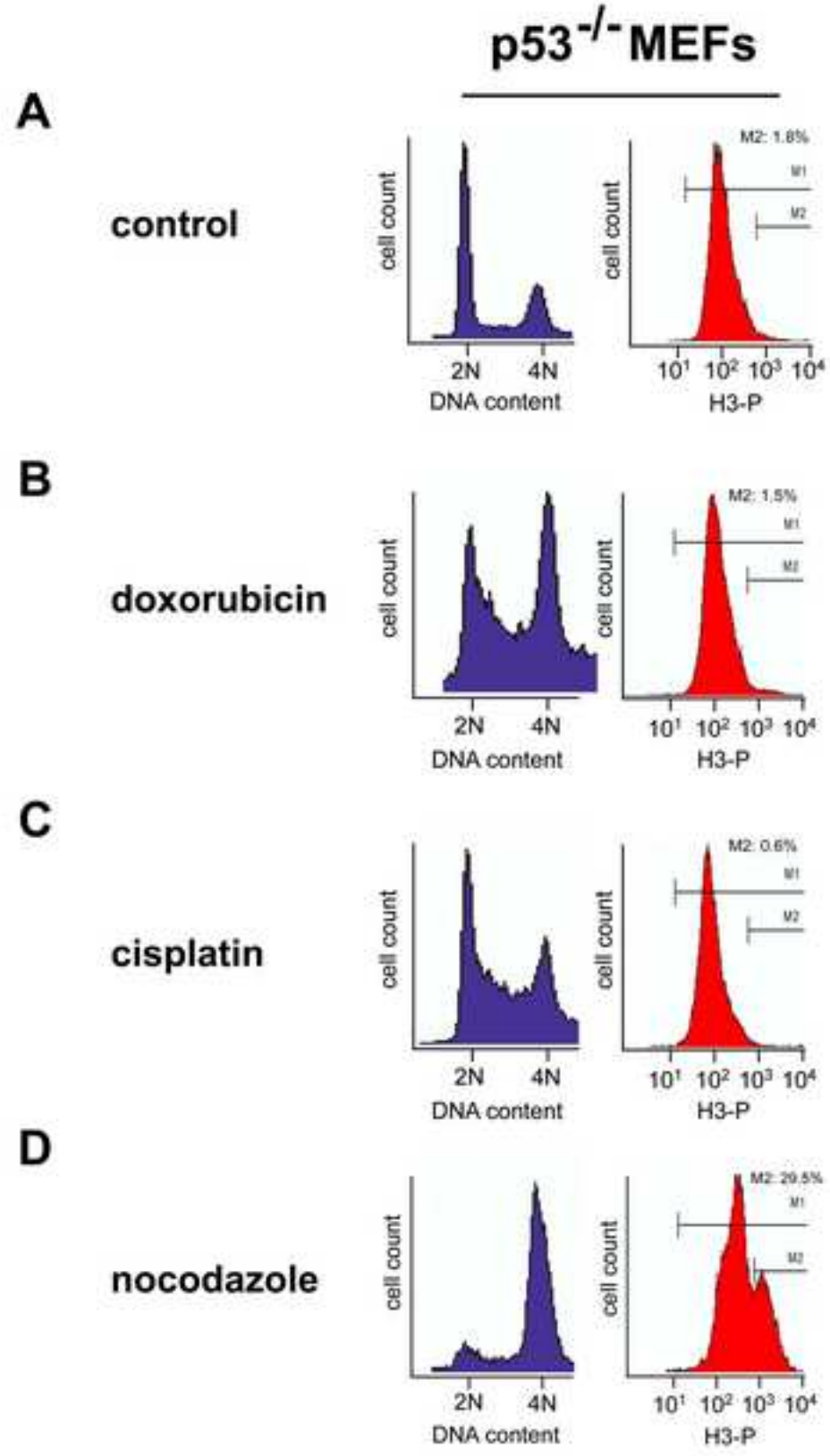

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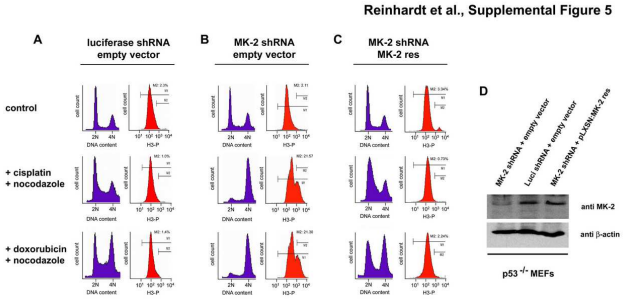

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