Supplementary Materials

MATERIALS AND METHODS

Buffers. Lysis buffer consisted of 50mM Tris/HCl (pH 8.0), 0.5% NP40, 1mM EDTA, 150mM NaCl and 1mM PMSF. Buffer C 100 (BC100) included 20mM Tris/HCl (pH 7.9), 0.1mM EDTA, 10% glycerol, 100mM KCl, 4mM $MgCl_2$, 0.2mM PMSF, 1mM DTT and 0.25μg/ml of pepstatin A.

In vitro **Chk1 kinase assays.** GST-Chk1 and GST-Chk1 KD were purified from baculovirus infected Hi-5 cell lysates using GSH agarose beads. After elution with 5mM reduced glutathione (GSH), purified GST-Chk1 and GST-Chk1 KD were dialyzed in BC 100 buffer and incubated with 100 ng purified his-p53 in the mixture containing incomplete kinase buffer, 10 μM ATP, 1μCi [γ^{32}P]-ATP at 30°C for 25 minutes.

Generation and purification of phosphorylated S367 MDMX specific antibody The polyclonal anti-phosphorylated Ser-367 antibody was generated by immunizing rabbits with phosphorylated Ser-367 peptide VPDCRRTIS(P)APVVRP (Genscript Corporation, New Jersey). Serial dilutions of peptides were spotted on a nitrocellulose membrane and blotted by using anti sera. The high titer serum was first cross-absorbed by beads coupled with a non-phosphorylated form of this peptide (VPDCRRTISAPVVRP) at 4°C overnight and the affinity purification of the antibody was carried out by using the phosphopeptide column. The affinity-purified antibody was used in this study.

***In vivo* ubiquitination assay.** *In vivo* ubiquitination assay was conducted as described previously (Jin et al., 2003). Briefly, H1299 cells in 100 mm plates were transfected with His_6-ubiquitin (2μg), p53 (0.8μg), Flag-14-3-3γ (1.6μg) or c-myc-MDMX (1.6μg) expression plasmids using the TransFectin reagent (Bio-Rad). Cells were treated with 10mM MG132 for 8 hrs before harvest. 48 hrs after transfection, cells harvested from each plate were split into two aliquots, one for WB and the other for ubiquitination assays using Ni-NTA beads (Qiagen). Cell pellets were lysed and incubated with Ni-NTA beads at room temperature for 4 hrs. Proteins were eluted from beads after wash and analyzed by WB for polyubiquitination of p53 after SDS-PAGE with the monoclonal p53 antibody DO-1.

GST fusion protein association assay. The fusion proteins were expressed in *E. coli* and purified on a glutathione-Sepharose 12B column. Protein-protein association assays were conducted as reported using fusion protein-containing beads (Zeng et al., 1999). 500ng of purified His-MDMX was incubated with the GSH-sepharose 4B beads (50% slurry) containing ~1μg of GST-14-3-3γ. For peptide competition assays, peptides with the molar ratio of 1:1, 2:1 or 4:1 to His-MDMX were pre-incubated with GST-14-3-3γ on ice for 1 hr before adding His-MDMX. 30 min after incubation at room temperature, the mixtures were washed three times in lysis buffer. Bound proteins were analyzed on a 10% SDS gel and detected by WB using the anti-MDMX antibody. GST and GST-14-3-3γ levels were determined by Coomassie staining of the membrane after WB. Phosphorylated and nonphosphorylated 14-3-3 binding consensus peptides were synthesized by Quality Controlled Biochemicals Inc (Hopkinton, MA). The

phosphorylated and non-phosphorylated 14-3-3-binding peptides of MDMX were synthesized and purified by Genscript (New Jersey).

Chk1 kinase assays. For IP kinase assays, Flag-Chk1 was immunoprecipitated from HEK 293 cell lysates overexpressing Flag-Chk1. Cells were treated with UVC 3 hrs before harvesting and were lysed in MCLB2 (50mM Tris-HCl pH 8.0, 2mM DTT, 5mM EDTA, 0.5% NP-40, 100mM NaCl, 1μM microcystin, 1mM sodium orthovanadate, 2mM PMSF, 10μg of aprotonin per ml, 20μM leupeptin, 10mM β-glycerophosphate, 1mM sodium fluoride). Clarified lysates were incubated with monoclonal anti-Flag M2 agarose beads overnight at 4°C. Precipitates were washed twice with MCLB2 and twice with incomplete kinase buffer (50mM Tris Cl pH 7.4, 1mM DTT, 10mM $MgCl_2$). 30μl kinase reactions were carried out in the presence of incomplete kinase buffer containing 10μM ATP, 1μCi of [γ-^{32}P]-ATP, Flag-Chk1 beads and substrates. The amount of different substrates was indicated in the figure legends. Reaction mixtures were incubated at 30°C for 25 min and analyzed by SDS-PAGE. The gel was dried and ^{32}P-labeled proteins were visualized by autoradiography.

For cold kinase assays, purified GST-Chk1 or GST-Chk1 KD was incubated in the presence of incomplete kinase buffer containing 1mM ATP and substrates at 30°C for 1 hr. The amount of different substrates is indicated in the figure legends. Reaction mixtures were analyzed by SDS-PAGE followed by WB as indicated.

Subcelluar fractionation. Control or UVC treated cells were harvested and re-suspended in 3 pellet volumes of buffer A (10mM TrisCl pH7.9, 1.5mM $MgCl_2$, 10mMKCl, 0.5mM DTT, 0.2mM PMSF). Mixed manually and kept on ice for 10 mins.

Vortex the tubes for 10 second twice and span at maximal speed at 4°C for 5 mins. The supernatant was the cytoplasm fraction. Equal volume as the original cell pellet of buffer C (20mM TrisCl pH7.9, 25% glycerol, 0.42 M NaCl, 1.5mM $MgCl_2$, 10mMKCl, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF) was added to the tube after removal of the cytoplasm fraction. Mixed well and kept the tube on ice for 20 mins and span at maximal speed at 4°C for 5 mins. The supernatant was saved as the nuclear fraction. Both fractions were dialyzed in BC100 buffer (20mM TrisCl pH7.9, 20% glycerol, 1.5mM $MgCl_2$, 100mM KCl, 0.2mM EDTA, 10mM β-mercaptoethanol, 0.2mM PMSF) for 3-4 hrs at 4°C.

FACS analysis. Harvested cells in tubes were stained in 200μl of propidium iodide (PI, Sigma) staining buffer (100μg/ml PI, 30μg/ml polyethylene glycol 8000, 200μg/ml RNase A. 0.1 % Triton X-100, 0.38M NaCl) and incubated for at 37°C for 30 mins and analyzed for DNA content using Becton Dickinson FACScan flow cytometer. Data were analyzed with the multicycle software program using polynomial S-phase algorithm.

FIGURE LEGENDS

Fig. S1. Ectopically expressed MDMX interacts with 14-3-3γ but not other isoforms of 14-3-3 in HEK 293 cells. HEK 293 cells were transfected with 2 μg of GFP-MDMX along or together with a combination of 2μg of Flag-14-3-3 γ, σ or myc-14-3-3ε vector. Cells were harvested and lysed post transfection. Cell lysates (300 μg) were

immunoprecipitated with polyclonal GFP antibody followed by WB and 50 μg of lysates was used for direct WB. Proteins were detected as indicated on the right.

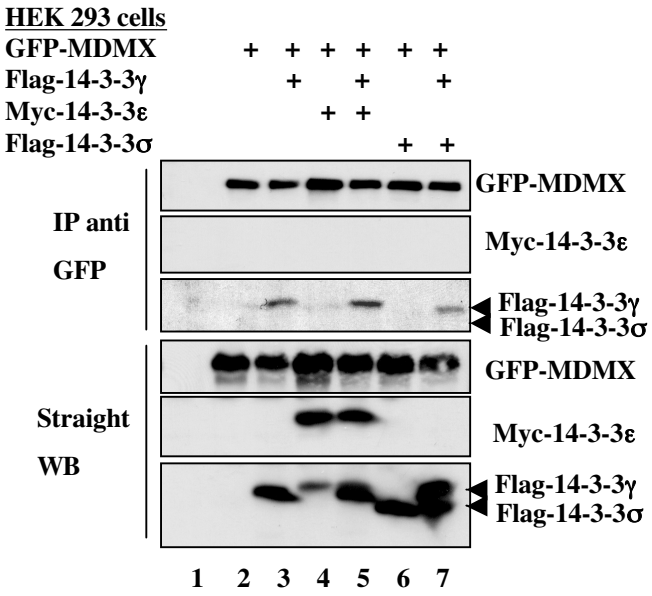
Fig. S2. Cytosolic and nuclear distribution of MDMX protein after UV irradiation. 293 cells were untreated (control) or irradiated with 30J/m^2 UVC. Cells were harvested at different time points as indicated. All samples were subjected to subcellular fractionation. 50μg of each sample was used for SDS-PAGE followed by WB to determine the amount of cytosolic or nuclear MDMX. The intensity of MDMX bands was quantified using the photoshop software and the ratio of cytosolic MDMX to nuclear MDMX at each time point was graphed.

Fig. S3. Chk1 phosphorylates p53 and MDMX *in vitro*. A.GST-Chk1 phosphorylated p53 *in vitro*. GST-Chk1 and GST-Chk1 KD were purified from baculovirus infected Hi-5 cell lysates and dialyzed against BC 100. 100ng of purified His-p53 was incubated with 500ng of GST-Chk1 or GST-Chk1 KD. Samples were subjected to SDS-PAGE. Phosphorylated proteins were detected by autoradiography. We detected the auto-phosphorylation of Chk1, but not the KD mutant. **B.** Baculovirus expressed GST-Chk1 phosphorylates MDMX. 500ng of GST0 or GST-MDMX protein was co-incubated with 500ng of GST-Chk1 or GST-Chk1 KD. After kinase reaction, samples were immunoprecipitated with the monoclonal anti-MDMX antibody. The immunoprecipitates were washed and analyzed by SDS-PAGE followed by autoradiography. After autoradiography, the same gel was re-hydrated and transferred onto PVDF membrane to detect MDMX levels by WB. The reason to conduct IP following the kinase reaction was to ensure that the phosphorylated species is MDMX. GST-MDMX was used here because his-MDMX co-migrated with GST-Chk1 on an SDS gel and thus it was difficult

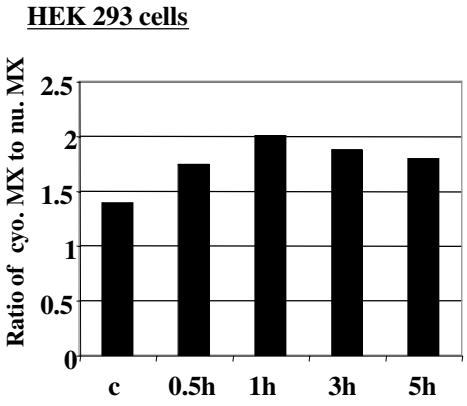
to distinguish the phosphorylated his-MDMX from auto-phosphorylated GST-Chk1 (data not shown). **C.** Generation of an antibody specific to serine 367-phosphorylated MDMX (Pi-S367 MDMX). Chemically synthesized non-phosphorylated MDMX peptides (P) or phosphorylated MDMX peptides (PS) were serially diluted and dotted on nitrocellulose membrane as indicated. After dried in air, the membrane was blotted with rabbit serum anti-phosphorylated MDMX peptide.

Fig. S4. UCN-01, the specific Chk1 inhibitor, inhibits p53 phosphorylation by Chk1 and p53 activation after UV irradiation. A. 100ng of purified His-p53 protein was incubated with 500ng purified GST-Chk1 or GST-Chk1 KD in a reaction mixture. UCN-01 was titrated as indicated. Reaction was terminated via heating at 95°C for 5 mins with 1X SDS gel loading buffer. Samples were subjected to SDS-PAGE followed by WB with anti-serine 15 phosphorylated p53 or polyclonal anti-p53 antibodies. **B.** UCN-01 inhibits p53 accumulation and p21 induction in response to UV. In lane 2 and 3, U2OS cells were irradiated with 30J/m2 and 300nM UCN-01 was added to cells in lane 3 immediately after treatment. Cells were harvested 3 hrs post treatment and lysed. 25μg of lysates was used for straight WB using antibodies as indicated on right.

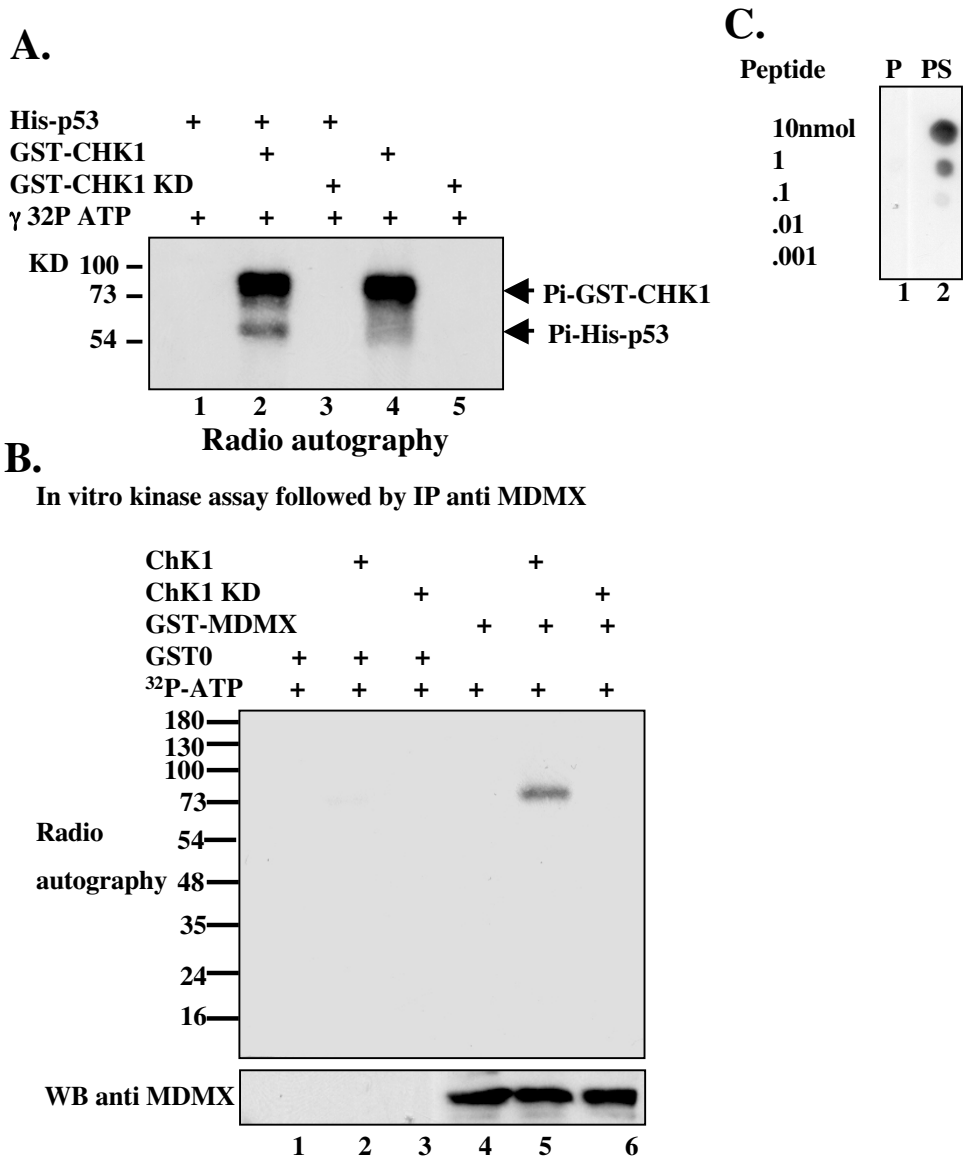
Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4