Supporting Information

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SI Methods

Immunoprecipitation. Immunoprecipitations were carried as described in ref. 1. Briefly, cells were collected by scrapping, washed twice with ice-cold PBS, and lysed on ice for 30 min in NET-2 buffer [50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% (vol/vol) Nonidet P-40, 1× complete protease inhibitors (Roche), 200 U/mL RNase OUT (Invitrogen)]. The protein extracts were set to 1 mg/mL, precleared with protein A Sepharose beads (Amersham) and incubated with protein A Sepharose beads preincubated with anti-Ubc13, anti-p53 (DO1), anti-FLAG, anti-HA (F7) antibody, or isotype-matched IgGs (Calbiochem). After incubation, beads were washed 6 times in NET-2 buffer, and the immunoprecipitated material was eluted using nonreducing sample buffer (Pierce) and analyzed by Western blot.

For K63-Ub immunoprecipitations, U2OS cells were transfected as indicated in the Figs. 1*C* and 2*D* and lysed in NET-2 buffer supplemented with 6 M urea, protease inhibitors, and 2 mM N-ethylmaleamide (NEM) for 1 h at room temperature. Lysates were cleared by centrifugation (12,000 RPM for 10 min) and diluted with NET-2 to 4 M urea. Diluted lysates were incubated with 1 μg of anti-K63-Ub antibody (a kind gift from Vishva M. Dixit, Genentech) or control serum overnight at room temperature, after which 25 μL protein A Sepharose beads were added, and the reactions were incubated for additional 6 h at 4 °C. Beads were washed 2 times in NET-2 buffer supplemented with 4 M urea and 3 times in NET-2 buffer. Immunopreciptiated material was eluted by boiling in Laemmli buffer. Importantly, under these conditions, p53 complexes with other cellular proteins should be efficiently disrupted, thus eliminating the possibility of p53 being immunoprecipitated by anti-K63-Ub antibodies through a K63-ubiquitinated partner protein.

Detection of T81 Phosphorylated p53. Western blot analysis was performed using exogenously expressed WT or T81A mutant forms of p53. A total of 80 μg of TCP was loaded on SDS/PAGE. Following their blotting, membranes were blocked in 5% milk/2% BSA before incubated with the anti-pT81p53 Ab (1:150) in 5% BSA/TBST (ON/4 °C). Membranes were washed 3 times for 10 min each in TBST and probed with anti-rabbit secondary Ab (1:5,000) in 2% milk/TBST for 2 h at RT. Membranes were washed 3 times for 15 min each in TBST, washed 1 time with TBS, and exposed for 2 h.

In Vivo Ubiquitination Assays. In vivo ubiquitination was carried out as described in ref. 2. Briefly, U2OS cells were transfected with 6×His-ubiquitin and additional expression plasmids as indicated. Cells were incubated with 40 μM MG-132 for 6 h at 37 °C before harvest and lysis in urea buffer (6 M urea, 0.1 M NaH_2PO_4, 0.1 M Tris·HCl, pH 8.0, 0.05% Tween-20, and 10 mM imidazole, pH 8.0) at room temperature for 1 h. The insoluble fraction was removed by centrifugation. Equal amounts of protein were incubated with Ni-NTA agarose beads (Qiagen) overnight at room temperature. Beads were extensively washed with denaturing wash buffer (6 M urea, 0.1 M NaH_2PO_4, 0.1 M Tris·HCl, pH 8.0, 0.05% Tween-20, 20 mM imidazole, pH 8.0) and once with native wash buffer (0.1 M NaH_2PO_4, 0.1 M Tris·HCl, pH 8.0, 0.05% Tween-20, 20 mM imidazole, pH 8.0). Protein complexes were eluted in 200 mM imidazole, resolved by SDS/PAGE, and analyzed by Western blot using a combination of 2 monoclonal anti-p53 antibody (DO1 and pAb241).

Apoptosis Studies. Saos-2 cells were grown in McCoy's 5a medium supplemented with 15% FBS. Cells were nucleofected with the indicated plasmids with the help of an Amaxa nucleofector device (protocol D-024 and solution V, as recommended by the manufacturer). After 48 h, cells were permeabilized, stained with propidium iodide (PI), and analyzed by FACS to detect the subG1 population as a direct indicator of apoptotic cells harboring fragmented DNA.

Transcriptional Activation Assays. U2OS cells, transfected and treated as indicated in the legends, were subsequently transfected with the $p21^{CIP1/WAF1}$ minimal promoter-driven luciferase constructs [cotransfected with β-galactosidase (β-Gal) expression vector] and were analyzed 48 h after transfection as previously described (3).

Protein Expression and Purification from *Escherichia coli*. The human GST, GST-Ubc13, and 6×His-JNK2α2 proteins were produced growing bacteria in Luria-Bertani Broth (LB) supplemented with ampicillin at 37 °C. Typically, a fresh colony transformed with the respective DNA construct, was picked up from a solid LB plate and used to inoculate 5 mL liquid LB and incubated overnight. Next, this miniculture was diluted to 1 L LB in a 2-L flask and cultured until the optical density of the media measured at 595 nm reached a value of 0.7. Recombinant protein expression was induced with 0.1 mM of IPTG at this point. Protein expression was achieved >6 h at 25 °C for GST and GST-Ubc13 or 12 h at 37 °C for 6×His-JNK2α2. All recombinant proteins were prepared in a similar way. The affinity column used to enrich the recombinant proteins was different, as detailed below. For the liquid chromatography (LC) protocols described here, we have used a AKTA-prime fast performance liquid chromatography (FPLC) system, with chromatographic columns (XK 16), affinity resins, and other LC utilities, from GE Healthcare-Amersham Biosciences. *E. coli* expression milieu was harvested at 4 °C by centrifugation (4,000 rpm) during 30 min, using a Sorvall SLC-4000 rotor. Cells were resuspended in ice-cold lysis buffer (25 mM Tris·HCl, pH 8.0, 500 mM NaCl, and 1 tablet/L of an EDTA-free protease inhibitors mixture from Roche) and lysed by lysozyme incubation at 1 mg/mL and sonication. Next, the lysate was centrifuged with a Sorvall SS34 rotator at 15,000 rpm at 4 °C, to remove the insoluble cell debris. The resulting supernatant was loaded onto a 50 mL superloop and injected on a preequilibrated affinity column, manually packed with 20 mL either nickel-NTA (6×His-JNK2α2) or Glutathione Sepharose High Performance resin (GST and GST-Ubc13). After extensive washing (5 column volumes) with equilibration buffer (25 mM Tris·HCl, pH 8.0, 500 mM NaCl, supplemented with 2.5 mM imidazole in the case of nickel column), the bound proteins were eluted with a linear gradient of either 2.5–200 mM imidazole for 6×His-JNK2α2 or 0–50 mM reduced glutathione, pH 8.0, in the case of GST and GST-Ubc13. The elution peak was collected and dialyzed overnight against 5 L 25 mM Hepes, pH 7.6, 50 mM NaCl, and 2 mM DTT. Proteins were purified further by anion exchange, with a manually packed column containing 20 mL Mono Q Sepharose resin, using a linear NaCl gradient 0–1 M, with the same buffer conditions used to dialyse overnight. The pure proteins were concentrated by centrifugation with an Amicon Ultra concentrator (10,000 Da molecular weight cut-off from Millipore) to ≈1 mg/mL and used immediately or flash-frozen with liquid nitrogen and stored at −80 °C. Proteins purity and stability were checked by SDS/

PAGE and Coomassie-blue staining. The 6×His-JNK2α2 protein obtained was active when tested in in vitro kinase assays against a set of known JNK substrates including the N terminus of c-Jun (amino acids 1–81) (4).

In Vitro p53 Phosphorylation by JNK. p53 (either wild-type or Thr81Ala mutant) was produced in rabbit reticulocyte lysates and radiolabeled with [^{35}S]methionine following the manufacturer's protocols (Promega). Typically, 200 μL reticulocyte lysate expressing the p53 of interest was incubated at 30 °C for 10 min in the presence of 30 ng 6×His-JNK2α2 recombinant active protein (see above). Reaction was stopped with 500 μL cold histone H1 kinase buffer (80 mM β-glycerophosphate, pH 7.4, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, supplemented with phosphatase inhibitor cocktails 1 and 2 from Sigma and protease inhibitors) and subjected with nickel-NTA pull-down at 4 °C for 20 min in a rotating wheel. The supernatant from this reaction was further used for the in vitro binding assays described below.

In Vitro Binding Assays. Bacterially expressed and purified recombinant either GST or GST-Ubc13 were prebound to 10 μL glutathione-Sepharose (ThermoFisher) by incubation for 1 h at 4 °C on a rotating wheel, followed by 3 washes with IP buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 5 mM EDTA, 2 mM $MgCl_2$, 20 mM NaF, 100 μM sodium orthovanadate, supplemented with protease inhibitors). These beads were then mixed with either the equivalent of 25 μL reticulocyte lysate—which was programmed to express p53 proteins, labeled with [^{35}S]methionine after its phosphorylation or not by JNK—or 1 mg extracts prepared from cells overexpressing HA-tagged versions of p53, in a total volume of ≈1 mL IP buffer. For the in vitro binding assay using reticulocyte lysates, when indicated, 50 μL reticulocyte lysates programmed to express unlabeled Uev1a were used in the reaction. In corresponding control reactions 50 μL unprogrammed lysates were used. After incubation for 2 h at 4 °C on a rotating wheel, the beads were gently centrifuged, washed 3 times with IP buffer, and resuspended in 30 μL 2× sample buffer. The beads were finally boiled for 5 min and subjected to SDS/PAGE, followed by either Coomassie staining or Western blot analysis and Ponceau staining (to verify equal amounts of recombinant proteins in all samples) and subjected to either PhosphorImaging or LiCOR-based densitometry analysis.

Cell Culture, Treatments, and Antibodies. U2OS cells were obtained from the American Type Culture Collection and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin (all obtained from Invitrogen). Where indicated, cells were incubated with puromycin or cycloheximide (both obtained from Sigma and used at 100 μg/mL) for 10 min at 37 °C or exposed to UV-C irradiation (254 nm; 100J/m^2) for 30 min in a Stratalinker 2400 (Stratagene) at room temperature. JNK inhibitor (in Solution JNK inhibitor II, also known as SP600125; Calbiochem) was applied at the final concentration of 1 μM for 30 min at 37 °C before the above treatments. TAT-JIP-based JNK inhibitor (cell permeable JNK inhibitor VII; Calbiochem) was applied at the final concentration of 10 μM for 3 h at 37 °C before the above treatments. SMART pool human MAPK8, MAPK9, and controls were purchased from Dharmacon and used following the manufacturer's protocols. Antibodies used were anti-p53 (DO-1, pAb421, and FL-393; Santa Cruz Biotechnology), anti-Ubc13 (Zymed), anti-c-Jun (Santa Cruz Biotechnology), anti-phospho-c-Jun (KM-1; Santa Cruz Biotechnology), anti-HA (F-7 and Y-11; Santa Cruz Biotechnology), anti-FLAG (M2, Sigma), anti-MKK7 (rabbit polyclonal from Cell Signaling), anti-JNK1 (SC-571, Santa Cruz Biotechnology), anti-β-actin (AC-15; Sigma), and anti-α-tubulin (Sigma).

Gel Filtration Chromatography. A Superose 6 10/300 GL column (Amersham) was equilibrated with buffer containing 1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 20 mM Hepes, pH 7.4, and 150 mM NaCl. Then, 100 μL total cellular lysates dissolved in the same buffer (350 μg of total protein for exogenous p53 and 750 μg of total protein for endogenous p53) were loaded onto the column, and 0.5-mL fractions were collected and analyzed by Western blot analysis using p53 (DO-1) antibody. Approximate molecular weights of the fractions were determined using a molecular weight standard kit (Sigma).

Polysomal Fractionation. U2OS cells were transfected and treated as indicated. Polyribosome fractionation was carried out on a continuous 10 to 40% sucrose gradient as described in refs. 5 and 6. The gradients were fractionated into 10 1.1-mL fractions by upward displacement with 60% sucrose using an ISCO Retriever 500 fraction collector equipped with a UV reader. The absorbance at 254 nm was monitored continuously. For simple Western blot analysis, fractions were diluted 5× in Laemmli buffer and boiled before loading on SDS/PAGE buffer. For the immunoprecipitation reactions, polysomal extracts were obtained by pooling the fractions corresponding to polysomes and diluting them 5× in NET-2 buffer.

1. Culjkovic B, et al. (2005) eIF4E promotes nuclear export of cyclin D1 mRNAs via an element in the 3′UTR. *J Cell Biol* 169:245–256.
2. Laine A, et al. (2006) Regulation of p53 localization and activity by Ubc13. *Mol Cell Biol* 26:8901–8913.
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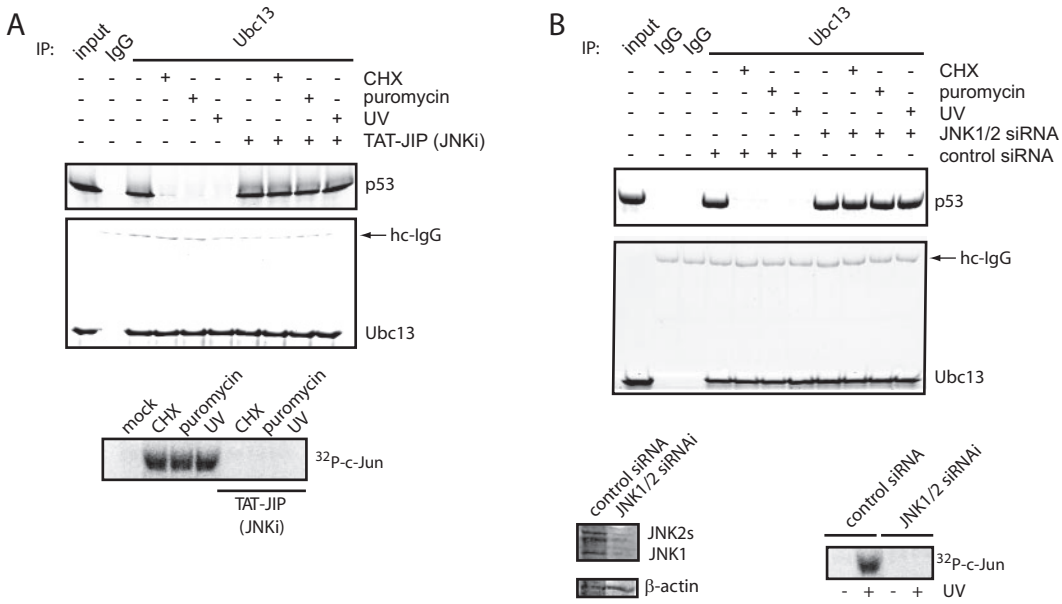

Fig. S1. JNK inhibition prevents stress-mediated Ubc13-p53 complex disruption. (*A*) U2OS cells were treated as indicated, and the interaction between endogenous Ubc13 and p53 was tested by coimmunoprecipitation. *Bottom*: JNK activation was assessed by JNK immunoprecipitation followed by in vitro kinase assay using a GST-fused N-terminal c-Jun fragment (amino acids 1–81). hc, heavy chain of IgGs. (*B*) U2OS cells were transfected with either control or JNK1 and JNK2 siRNAs and incubated for 72 h. Levels and activity of JNK under normal and stressed conditions were assessed (*bottom*). Interaction—under the indicated conditions—between Ubc13 and p53 was tested by coimmunoprecipitation analysis. hc, heavy chain of IgGs.

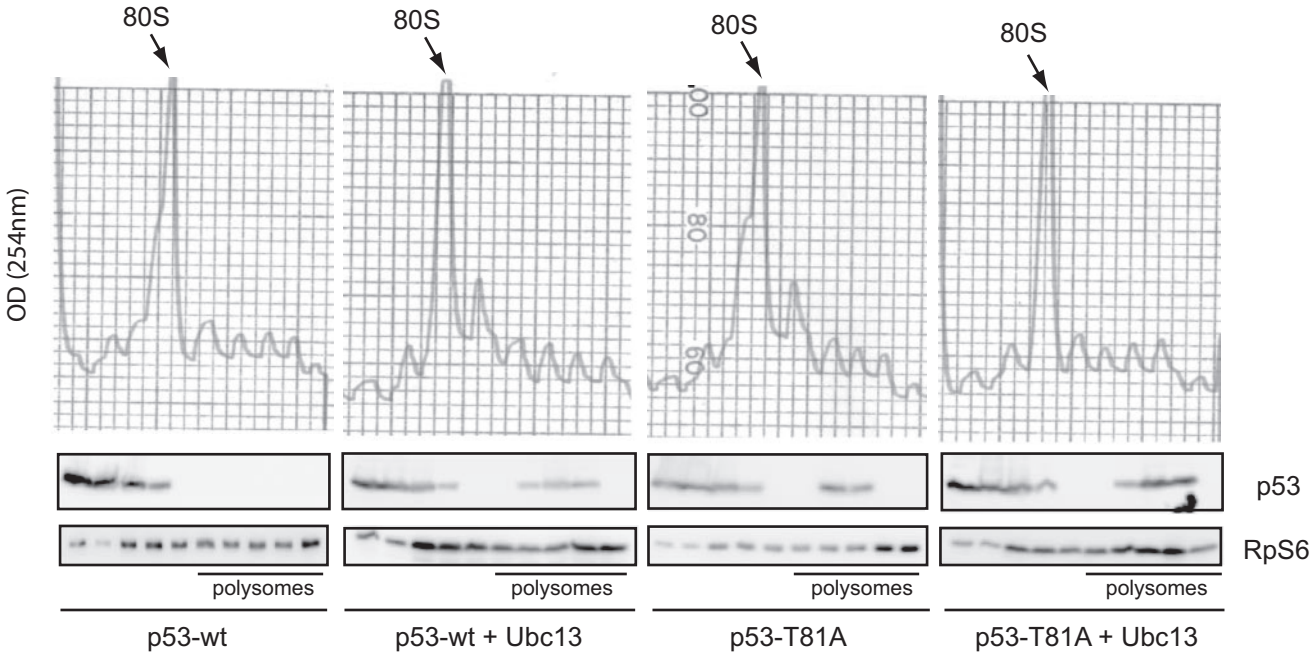

Fig. S2. Distribution of wild-type p53 and Thr81Ala p53 mutant on polysomal gradients. Cells were transfected with the indicated constructs. Ribosomes were fractionated on a continuous 10–40% sucrose gradient. UV absorption profiles of the ribosomal gradient at 254 nm are shown. Distribution of p53 either wild-type (wt) or Thr81Ala mutant (T81A) across the gradient was determined by Western blot (p53). Ribosomal protein S6 (RpS6) served as a loading control.

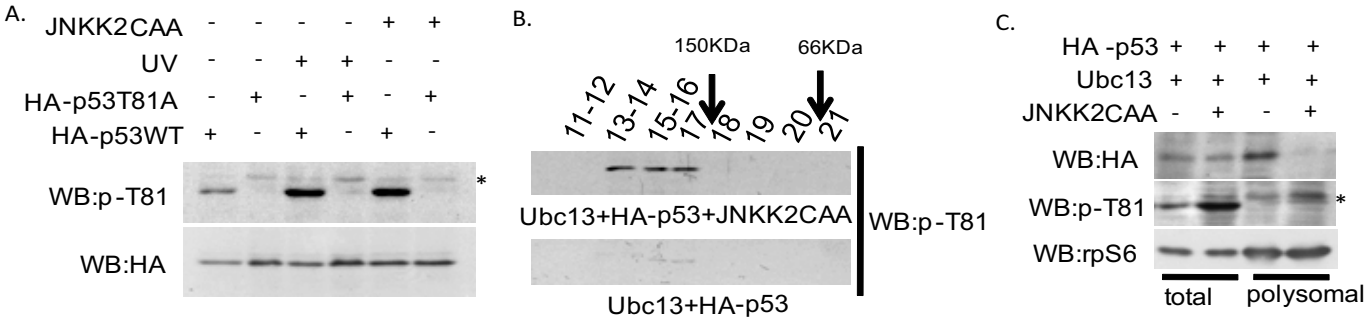

Fig. S3. Distribution of T81 phosphorylated p53 following JNK activation. (*A*) Saos2 cells transfected with HA tagged WT or T81A p53 construct were UV irradiated (100 J/m^2 for 30 min), or cotransfected wiith JNKK2^{CAA} construct. Expression levels of exogenous p53 (WB:HA) and the phosphorylation of p53 on residue Thr-81 (WB:*p*-T81) was determined by Western blot analysis. (*B*) Lysates of U2Os cells transfected as indicated were fractionated on the size exclusion column. Distribution of p53 phosphorylated on the residue Thr-81 (WB:*p*-T81) was monitored by Western blot analysis. (*C*) U2Os cells were transfected as indicated, and the levels of exogenous p53 (WB:HA) and the phosphorylation of p53 on the residue Thr-81 (WB:*p*-T81) in total and polysomal extracts was monitored by Western blot analysis. rpS6 served as a loading control. *- indicates non specific band.

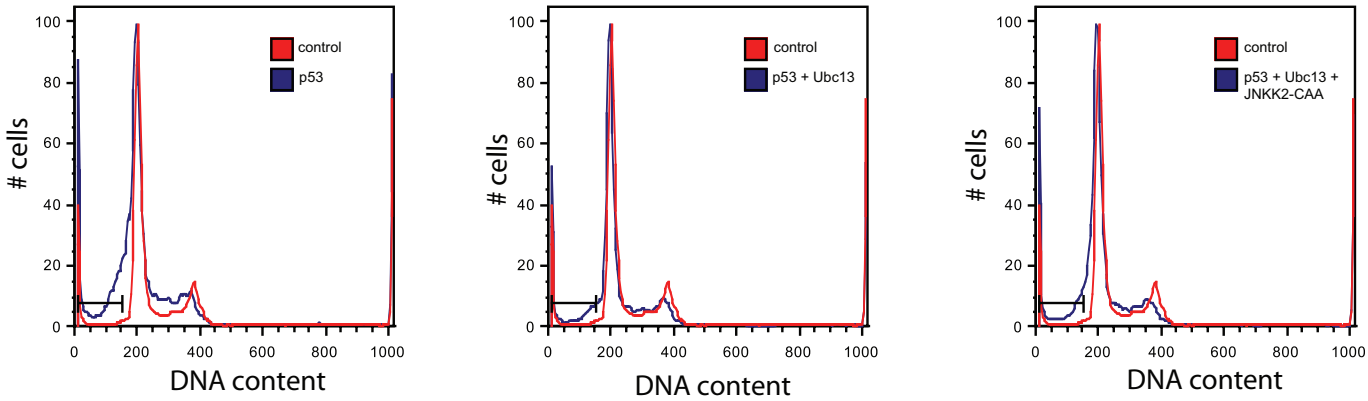

Fig. S4. The JNK-Ubc13-p53 axis affects cellular apoptosis. Saos-2 cells were nucleofected with the indicated plasmids. Forty-eight hours after transfection, cells were harvested. Cell cycle profiles, determined by staining DNA with propidium iodide followed by fluorescence-activated cell sorter analysis.