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Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase

Running title: JNK regulation of p73

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Footnotes:

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GST, glutathione-S-transferase; HA, hemagglutinin; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MEF, mouse embryonic fibroblast; MLK, mixed lineage kinase; cisplatin, *cis*-diamminedichloroplatinum; PARP, poly(ADP-ribose) polymerase; PML, promyelocytic leukaemia.

¹ E.V. Jones and A.J. Whitmarsh, unpublished data

ABSTRACT

The c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) signalling pathway is a major mediator of stress responses in cells, including the response to DNA damage. DNA damage also causes the stabilisation and activation of p73, a member of the p53 family of transcription factors. p73, like p53, can mediate apoptosis by up-regulating the expression of pro-apoptotic genes, including *Bax* and *PUMA*. Changes in p73 expression have been linked to tumor progression particularly in neuroblastomas, while in tumors that feature inactivated p53 there is evidence that p73 may mediate the apoptotic response to chemotherapeutic agents. Here we demonstrate a novel link between the JNK signalling pathway and p73. We use pharmacological and genetic approaches to show that JNK is required for p73-mediated apoptosis induced by the DNA damaging agent, cisplatin. JNK forms a complex with p73 and phosphorylates it at several Ser/Thr residues. The mutation of JNK phosphorylation sites in p73 abrogates cisplatin-induced stabilisation of p73 protein, leads to a reduction in p73 transcriptional activity, and reduced p73-mediated apoptosis. Our results demonstrate that the JNK pathway is an important regulator of DNA damage-induced apoptosis mediated by p73.

Key words: Signal transduction, phosphorylation, MAP Kinase, JNK, p73, apoptosis

INTRODUCTION

Checkpoint mechanisms exist in cells to allow the repair of damaged DNA prior to cell division, or if the damage cannot be repaired, to trigger apoptosis. Defective checkpoint and apoptotic signalling cascades can lead to the inappropriate survival of cells carrying gene abnormalities, thereby increasing the predisposition to cancer. The transcription factor p53 is frequently mutated in human cancers and is part of a family that also includes p63 and p73 [1-4]. p53 plays an important role in DNA damage induced apoptosis by up-regulating the expression of pro-apoptotic genes including *Bax*, *PUMA*, *NOXA*, and *p53AIP1* and down-regulating the expression of genes that promote cell survival [1,2].

The less well characterised p53 family member, p73, is also proposed to be important for DNA damage-induced responses [3-5]. Indeed, there is collaboration between p53 family members in apoptotic signalling. Both p63 and p73 are required for the p53 response to DNA-damage in mouse embryonic fibroblasts (MEFs) and in the central nervous system [6]. p73 also mediates p53-independent apoptosis in response to specific chemotherapeutic agents [7,8], while the ectopic expression of p73 is sufficient to induce apoptosis of cells [9,10]. Since p53 is frequently inactivated in tumours, these studies suggest that p73 status may be important in determining the cellular response to chemotherapeutic agents [7,8]. The mechanism of p73-mediated apoptosis involves the expression of certain p53 target genes [3-5,10]. For example, p73 regulates the expression of PUMA, a BH3-only protein, which promotes the activation and relocalisation of Bax to the mitochondrial membrane, resulting in the release of cytochrome c and apoptosis [10].

Understanding the role of p73 in cells is complicated by the existence of multiple isoforms including truncated $\Delta Np73$ isoforms which lack the transcriptional activation domain [3-5]. The full length and truncated p73 isoforms are expressed from different promoters [3-5]. $\Delta Np73$ isoforms are anti-apoptotic and interfere with the pro-apoptotic functions of p73 and p53 [3-5]. The relative expression levels of full-length versus ΔN isoforms of p73 may therefore be crucial for determining cell fate.

Compared to p53, the regulation of p73 activity is less well understood but is likely to be as complex. DNA damaging agents cause the stabilisation of p73 protein levels and increased

transcriptional activity mediated by the phosphorylation of p73 by the Tyr kinase c-Abl [11-13] and the Ser/Thr kinase CHK1 [14]. Similar to p53, MAPKs may also regulate p73 function. The p38 MAPK is reported to mediate the phosphorylation of p73 [15-17] leading to its interaction with the prolyl isomerase Pin1 [16] and its recruitment to promyelocytic leukaemia (PML) nuclear bodies [17]. Both these events, in addition to c-Abl-mediated phosphorylation of p73, cause enhanced p300-mediated acetylation and transcriptional activation of p73 leading to the selective up-regulation of pro-apoptotic genes [16-18].

The JNK MAPK pathway is a major stress signalling pathway in cells that plays important roles in many cellular processes including development, apoptosis, cell growth and immune responses [19,20]. c-Abl activates the JNK pathway in response to DNA damage and JNK is involved in the apoptotic response to many genotoxic stresses [21-23]. JNK also plays an essential role in promoting c-Abl nuclear localisation via the phosphorylation of 14-3-3 proteins that sequester c-Abl in the cytoplasm [24]. However, despite strong evidence that JNK is an important regulator of DNA-damage signalling and the proposed role of JNK in p53 regulation, it is unclear whether JNK has a role in p73 function.

In this study we have examined the regulation of p73 by the JNK signalling cascade. We find that active JNK associates with p73 and phosphorylates multiple Ser/Thr residues. This leads to p73 protein stabilisation, enhanced p300-mediated acetylation of p73, and increased p73-mediated transcriptional activity and apoptosis.

EXPERIMENTAL

Materials. Cycloheximide, SP600125, SB253580, Cisplatin, Doxorubicin, Camptothecin and anisomycin were purchased from Calbiochem.

Plasmids. pSR α -HA-JNK2 α 2, pcDNA3-HA-MLK3, pGEX-cJun(1-79) were described previously [25]. pcDNA3-HA-p73 α , pRV-p300, pEBG-JNK2 α 2 and pEBG-JNK3 α 1, and *GADD45* and *Bax*-Luciferase reporters were provided by G. Melino (University of Leicester) and A. Sharrocks, C. Tournier and C. Demonacos (all from the University of Manchester), respectively. pRL-TK-Renilla was from Promega. pcDNA3-myc-p73 α was constructed by inserting full length p73 α PCR product into EcoRI/NotI sites of pcDNA3-myc. All point mutants were generated using the Quikchange kit (Stratagene) and verified by DNA

sequencing. Full length p73 α and deletion mutants were generated by PCR and inserted into BamHI/NotI sites of pGEX-4T1 (Amersham Biosciences).

Cell Culture and Transfections. Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen). U2OS cells were similarly maintained except 10% FBS was used. Wild-type and JNK-deficient MEFs were cultured as described previously [26]. H1299/p73 α cells [27] were a gift from G. Blandino (University of Rome) and were maintained in RPMI 1640 medium supplemented with 10% FBS, 400 μ g/ml G418 and 100 μ g/ml Zeocin (Invitrogen). p73 α expression was induced by the addition of 5 μ M Ponasterone A (Invitrogen) for 12 hrs. Cells were transfected using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Human JNK1/2 siRNA (AAA GAA UGU CCU ACC UUC UTT) [28] and control siRNA (AGG UAG UGU AAU CGC CUU GTT) were from MWG and used at 50nM.

Immunoprecipitations and GST pull downs. Cos-7 cells were washed with phosphate buffered saline (PBS) and then lysed in buffer containing 20 mM Tris-HCl pH7.4, 137 mM NaCl, 25 mM β -glycerophosphate, 2 mM Na-pyrophosphate, 2 mM EDTA, 1% Triton-X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 5 μ g/ml leupeptin. GST proteins were isolated by incubating lysates with glutathione-sepharose 4B (Amersham Biosciences). HA- and myc-tagged p73 α were immunoprecipitated using the appropriate antibodies bound to Protein G-Sepharose (Amersham Biosciences). Beads were washed three times in lysis buffer and bound proteins were eluted in 6x SDS loading buffer.

Immunoblotting and Immunofluorescence. Lysates of MEFs, H1299/p73 α and U2OS cells were prepared in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 10mM NaF, 5 mM EDTA, 0.5% (w/v) Deoxycholate, 0.1% SDS, 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 5 μ g/ml Leupeptin). Immunoblots were performed with antibodies to: GST (Amersham Biosciences), HA (Cancer Research UK), myc (Santa Cruz), Puma, acetyl-lysine and β -actin (all from Abcam), p73 (IMG-259, Imgenex; Ab-3, Oncogene Research Prod.), PARP p85 and phospho-cJun (Ser73) (Cell Signaling Tech.), JNK1/2 (Pharmingen), p300 (Upstate), phospho-Ser and Thr (RDI and Zymed). Immune complexes were detected with HRP-conjugated secondary antibodies (Amersham Biosciences) and enhanced chemiluminescence (Pierce). For immunofluorescence studies cells were grown on glass coverslips coated in poly-L-lysine (Sigma), fixed with 4% paraformaldehyde (Sigma), and lysed in 0.2% Triton X-100/PBS. Myc-p73 α was detected by indirect immunofluorescence

using the myc antibody and Alexa594-conjugated secondary antibody (Molecular Probes). Nuclei were visualised with DAPI stain (Molecular Probes).

Mass Spectrometry. Cos-7 cells were transfected with constructs expressing HA-p73 α with and without GST-JNK3. HA-p73 α immunoprecipitates were separated by SDS-PAGE. Bands corresponding to HA-p73 α were excised from the gel and subjected to digestion with trypsin. MS/MS analysis was performed using a QStar XL instrument (Applied Biosystems) and the data analysed using Analyst (Applied Biosystems) and Mascot (Matrix Science) software.

Protein Kinase assays and Luciferase Reporter assays. *In vitro* protein kinase assays were performed as previously described [29]. Active JNK1 was from Upstate. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega).

RESULTS

p73 is required for JNK-induced apoptosis in response to cisplatin. JNK mediates apoptosis in many cell types in response to a variety of stresses [19,20]. Several studies have implicated JNK signalling in the apoptotic response to genotoxins, including cisplatin [21-23], but its importance and the mechanisms involved remain unclear. To determine if JNK plays a role in cisplatin-induced apoptosis via p73, we utilised H1299 cells that feature the inducible expression of the p73 α isoform [27]. These cells lack functional p53 so provide a good model for observing p73-dependent signalling events. In the absence of p73 α induction, cisplatin induced the apoptosis of H1299 cells, as measured by counting condensed nuclei, while the addition of the JNK inhibitor SP600125 [30] did not affect the levels of apoptosis (Figs. 1A, B). Upon p73 α induction cisplatin treatment increased apoptosis levels and this p73 α -dependent increase was blocked by the JNK inhibitor (Fig. 1A). SP600125 has been reported to affect other protein kinases in addition to JNK [31], therefore to confirm the role of JNK, we knocked down its expression by siRNA in the H1299/p73 α cells which also lead to reduced cisplatin-induced apoptosis (Fig. 1C). The effectiveness of the siRNA for reducing JNK expression was confirmed by immunoblot analysis (Fig. 2C). These data indicate that JNK is important for p73 α induced apoptosis in H1299 cells.

JNK induces stabilisation of p73. To determine the mechanism by which JNK regulates p73 apoptotic activity we examined whether JNK was required for increased p73 protein stability in response to cisplatin in the H1299/p73 α cells. As previously reported [12], cisplatin increased p73 α protein levels (Fig. 2A, compare lanes 2 and 3; Fig. 2B, compare lanes 2 and 3; Fig. 2C, compare lanes 3 and 4). Application of either the JNK inhibitor SP600125 or transfection of cells with JNK siRNA blocked the cisplatin-induced change in p73 α stability (Fig. 2A, compare lanes 3 and 4, Fig. 2C, compare lanes 4 and 8). The JNK inhibitor caused a loss of phosphorylation of the well characterised JNK substrate c-Jun, demonstrating its effectiveness in these cells (Fig. 2A). In addition, we noted that cisplatin caused an increase in the levels of cleaved PARP p85 fragment, a measure of cellular caspase activity, and this was reduced by JNK inhibition (Fig. 2B). To determine whether JNK is required for cisplatin-induced stabilisation of endogenous p73 we employed JNK-deficient MEFs [26]. We found that p73 was stabilised by cisplatin in wild-type MEFs but not in the JNK-deficient MEFs (Fig. 2D, compare lanes 3 and 7). Furthermore, two other DNA damaging agents, doxorubicin and camptothecin, both caused p73 stabilisation in wild-type MEFs but not in the JNK-deficient MEFs (Fig. 2D, compare lanes 2 and 4 with 6 and 8). To further demonstrate that JNK could increase p73 stability we measured the half-life of transfected p73 α . U2OS cells expressing HA-tagged p73 α and either wild-type JNK or a kinase inactivated JNK were treated with cycloheximide to block new protein synthesis and the levels of p73 α measured. JNK expression significantly enhanced the stability of p73 α increasing the half-life of the protein from 2 hours to over 4 hours (Fig. 2E). This did not occur with the inactivated JNK mutant which suggests that JNK can increase p73 stability in a phosphorylation-dependent manner (Fig. 2E). Taken together these data provide strong evidence that JNK activity is required for p73 stabilisation in response to DNA damage.

p73 α is a JNK substrate. We next sought to determine whether JNK directly interacts with and phosphorylates p73. Co-expression studies in Cos-7 cells demonstrated that JNK1 co-precipitated with p73 α and the level of co-precipitation was increased in the presence of the MAPK kinase kinase, mixed lineage kinase 3 (MLK3), a potent JNK activator (Fig. 3A). We next performed *in vitro* kinase assays on a GST-p73 α fusion protein expressed in and purified from *E.coli*. All the JNK isoforms (JNK1, JNK2 and JNK3) directly phosphorylated full length p73 α (Fig. 3B). In further protein kinase assays we utilised a mammalian expression construct

that expresses GST-JNK3 in cells. We find that this fusion protein displays a high level of JNK activity¹, thereby removing the necessity to transfect upstream activating kinases or to treat the cells with external stresses. Using precipitates of GST-JNK3 we performed protein kinase assays on both N-terminal (amino acids 1-380) and C-terminal (amino acids 381-637) fragments of p73 α and found both fragments were phosphorylated (Fig. 3C, lanes 3 and 4). The primary sequence of human p73 α contains eleven potential JNK phosphorylation sites (Ser/Thr-Pro). Previously the phosphorylation of three C-terminal sites (Ser412, Thr442 and Thr482) has been implicated in the binding to the prolyl isomerase Pin1 and was proposed to involve p38 MAPK [16]. We mutated these three sites to Ala both individually and in combination and performed *in vitro* kinase assays. Mutation of Thr442 significantly decreased phosphorylation of the p73 α C-terminus while mutation of Ser412 partially reduced phosphorylation (Fig. 3D, compare lane 2 with lanes 3 and 4). Mutation of all three sites completely blocked phosphorylation of the p73 α C-terminus (Fig. 3D, lane 7). We identified a further site (Ser333) in the central region of p73 α by mass spectrometry analysis of immunoprecipitates from cells expressing p73 α with GST-JNK3, however mutation of this site alone had no significant effect on p73 phosphorylation *in vitro*¹. To resolve the JNK phosphorylation sites within the N-terminus of p73 α we performed further mutational analysis in combination with *in vitro* kinase assays. Mutation of Ser8 partially reduced phosphorylation of the N-terminus of p73 α , with a small further decrease when combined with mutation of Ser97 and Ser110 (Fig. 3E). We then mutated the seven potential JNK phosphorylation sites we identified in p73 α (Ser8, Ser97, Ser110, Ser333, Ser412, Thr442 and Thr482) and this mutant (p73.M7) showed a significant decrease in JNK-dependent phosphorylation compared to wild-type p73 α , although there was some residual phosphorylation of the mutant indicating that there may be additional sites (Fig. 3F). Taken together these data indicate that JNK can phosphorylate p73 α *in vitro* at a number of Ser/Thr residues located within both the N and C-termini. To determine if p73 α is phosphorylated in cells by JNK we co-expressed full length p73 α and GST-JNK3 and determined phosphorylation levels using phospho-Ser/Thr specific antibodies. We detected increased phosphorylation of p73 α in the presence of JNK (Fig. 3G). The phosphorylation site mutant (p73.M7), as expected, displayed reduced phosphorylation compared to wild-type p73 α (Fig. 3G).

Mutation of JNK phosphorylation sites in p73 prevents cisplatin-induced p73

stabilisation, transcriptional activation and acetylation. We next sought to determine if mutation of JNK phosphorylation sites in p73 α had functional consequences. Firstly, we examined the ability of the p73.M7 mutant to be stabilised by cisplatin. This mutant is not stabilised by cisplatin when compared to wild-type p73 α (Fig. 4A, compare lanes 2 and 4). Individual point mutations of the phosphorylation sites and various combinations of two or three mutations did not affect cisplatin-induced p73 α stabilisation¹ suggesting that multiple sites on p73 α need to be targeted by JNK in order to promote p73 α stability in the presence of cisplatin. Previous reports have demonstrated that acetylation of the C-terminus of p73 α by p300 contributes to p73 α activity [18]. To determine whether JNK phosphorylation of p73 regulates its acetylation we co-expressed p73 α or the phosphorylation site mutant together with p300 in the presence or absence of JNK. JNK enhanced p300-mediated acetylation of p73 α but not that of the mutant indicating that JNK phosphorylation of p73 is important for efficient acetylation of p73 by p300 (Fig. 4B, compare lanes 3 and 6). To determine the effect JNK activity on p73-mediated transcription we utilised luciferase reporter plasmids featuring the *Bax* and *GADD45* promoters, which contain well characterised p53/p73-responsive elements. Co-expression of p73 α with JNK or the JNK activator MLK3 resulted in increased transcriptional activity from both reporter constructs (Figs. 4C-E). Co-expression of an inactivated mutant of JNK did not enhance p73 α -mediated *Bax* reporter activity (Fig. 4D), while neither JNK or MLK3 expression could enhance the ability of the phosphorylation site mutant p73.M7 to activate the *Bax* reporter gene (Fig. 4E). In the absence of p73 expression the JNK pathway components had no effect on reporter gene expression¹. These data demonstrate that JNK phosphorylation of p73 α can lead to increased transcriptional activity.

JNK phosphorylation of p73 contributes to cisplatin-induced apoptosis. Our data indicate that JNK plays a role in p73 α -mediated apoptosis induced by cisplatin (Fig. 1). Key apoptotic targets downstream of p73 include Bax and PUMA which collaborate in the release of cytochrome c from mitochondria leading to the activation of caspases [3-5,10]. We find that in response to cisplatin, p73 α -mediated PUMA expression is blocked by the JNK inhibitor SP600125, indicating that JNK is required for p73 to mediate the apoptotic cascade (Fig. 2A). We would predict that p73 α lacking JNK phosphorylation sites would be unable to efficiently induce apoptosis. Indeed, we could not detect cisplatin-induced PARP cleavage, a measure of

cellular caspase activity, in the presence of the mutant (Fig. 4A). In addition, when U2OS cells were transfected with constructs expressing p73 α or the mutant and treated with cisplatin we observed that p73 α expression alone increased apoptosis and this was further increased in the presence of cisplatin, however cisplatin did not significantly increase apoptosis in the presence of the mutant p73 α (Fig. 4F). These data strongly support a critical role for the JNK pathway in mediating cisplatin-induced apoptosis via phosphorylation of p73 and regulation of its stability and transcriptional activity.

DISCUSSION

The JNK MAPK pathway plays pivotal roles in regulating many cellular functions. In this study we uncovered a novel role for JNK in mediating DNA-damage-induced apoptosis via p73. JNK binds to p73 and phosphorylates it at multiple sites. We demonstrate that JNK phosphorylation of p73 in response to cisplatin promotes its stabilisation, p300-mediated acetylation and transcriptional activity, which leads to PUMA expression and the activation of caspases (Figs. 2, 4). Interestingly, the JNK target c-Jun, a member of the AP-1 family of transcription factors, may also be involved in promoting p73 stability and activity [32]. Therefore, in addition to directly phosphorylating p73 to regulate its activity, JNK may be indirectly regulating p73 activity through its actions on c-Jun.

Previous reports indicate that the p38 MAPK pathway can regulate p73 [15-17] although the phosphorylation sites have not been precisely mapped. Mantovani *et al.* [16] implicate three C-terminal residues (Ser412, Thr442, Thr482) as p38 targets and propose that the phosphorylation of these sites is required for Pin1 binding and subsequent p300-mediated acetylation. It is also reported that p38 is required for p73 localisation into PML bodies [17]. Based on the use of a pharmacological inhibitor of p38, we did not find a significant role for p38 in cisplatin-induced p73 stabilisation in H1299 cells¹. It is possible that JNK and p38 may differentially regulate p73 according to the type and strength of genotoxic insult or the type of cell.

p53 and p73 target many of the same pro-apoptotic gene promoters, so p73-mediated apoptosis in response to chemotherapeutic agents may provide a way of killing tumour cells under conditions where p53 is frequently inactivated [7, 8]. The regulation of p53 and p73 by JNK

appears to have similarities but also distinct differences. Under non-stressed conditions JNK binds to p53 and targets it for ubiquitination and degradation via the 26S proteasome [33]. We have not detected any obvious effect of inactive JNK on p73 stability and indeed, only detect weak binding of JNK to p73 in the absence of an activating signal (Fig. 3A). In response to DNA damage JNK phosphorylates and stabilises both p53 and p73 ([34]; Figs. 2, 4). JNK phosphorylates p53 at a single Thr residue while p73 is phosphorylated at multiple sites ([33]; Fig. 3). In both cases additional phosphorylations by other kinases are likely to collaborate with JNK to fully regulate p53/p73 function in response to DNA damage [11-14, 35]. For example, c-Abl directly phosphorylates p73 on Tyr in response to DNA damage [11-13] but also activates the JNK pathway [21, 22], which we demonstrate here phosphorylates Ser/Thr residues on p73 (Fig. 3). It will be of interest to determine how these c-Abl/JNK-mediated signalling events are integrated to regulate p73 function.

Overall our results provide strong evidence that the JNK signalling pathway is a critical regulator of p73-mediated apoptosis in response to cisplatin. This extends our knowledge of the diverse roles this signalling pathway can play in the response of cells to stress. In addition, our work furthers understanding of the mechanisms underlying the apoptotic response to chemotherapeutic agents that are used to treat tumors.

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FIGURE LEGENDS

Figure 1. JNK is required for cisplatin-induced apoptosis via p73. SP600125 (A) and JNK siRNA (C) block p73 α -mediated apoptosis induced by cisplatin. Cells were stained with DAPI and apoptotic cells were counted based on condensed nuclei (example depicted in B; white arrows point to apoptotic cells). The data represents the mean of three independent experiments

in which at least 200 cells were counted per condition in a blind manner. (A) H1299/p73 α cells were treated overnight with or without Ponasterone A (5 μ M) to induce p73 α expression (-p73 and +p73). Cells were pretreated with SP600125 (10 μ M) for 1 hr prior to treatment with cisplatin (25 μ M) for 24 hours. (C) H1299/p73 α cells were transfected with either non-specific control siRNA or JNK1/2 specific siRNA. 24 hours post-transfection Ponasterone A (5 μ M) was added overnight to induce p73 α expression. Cells were then treated with cisplatin (25 μ M) for 24 hours and analysed as in A.

Figure 2. JNK is required for the stabilisation of p73 in response to cisplatin. (A, B) The JNK inhibitor SP600125 blocks cisplatin-induced stabilisation of p73 α in H1299/p73 α cells. p73 α expression was induced with Ponasterone A (Pon A; 5 μ M). Cells were treated with 10 μ M SP600125 for 30 minutes prior to treatment with cisplatin (25 μ M) for 14 hours. In (A) cell lysates were immunoblotted for p73 α , PUMA, phospho-c-Jun(Ser73) (P-c-Jun), and β -actin. In (B) lysates were immunoblotted for p73 α , PARP(p85) and actin. (C) H1299/p73 α cells were transfected with either non-specific control siRNA or JNK1/2 specific siRNA. 24 hours post-transfection Ponasterone A (Pon A; 5 μ M) was added overnight to induce p73 α expression. Cells were then treated with cisplatin (25 μ M) for 14 hours. Cell lysates were immunoblotted for p73 α , JNK1/2 and β -actin. (D) Loss of DNA damage-induced p73 stabilisation in JNK $^{-/-}$ MEFs. Wild-type and JNK $^{-/-}$ MEFs were treated with camptothecin (10 μ M), cisplatin (25 μ M), or doxorubicin (1 μ M) for 8 hours and the cell lysates were immunoblotted for p73, JNK and β -actin. (E) JNK expression increases the half-life of p73 α protein. U2OS cells were transfected with constructs expressing HA-p73 α together with HA-JNK2. As a control, a kinase-dead JNK2 mutant featuring the mutations K55A and K56A (JNK2(KD)), was used to demonstrate that the effect of JNK2 expression on p73 α stability was dependent upon its protein kinase activity. Cells were treated with 10 μ g/ml cycloheximide (CHX) for the times indicated. p73 α immunoblots were analysed by densitometry. The values in the absence CHX were normalised to 100% to allow direct comparison of p73 α stability during the CHX time course under the different conditions. The mean results of three independent experiments are presented. Representative control immunoblots for HA-JNK2 and β -actin levels are shown.

Figure 3. JNK binds and phosphorylates p73. (A) Activated JNK binds to p73.

Constructs expressing myc-p73 α , GST-JNK1 and HA-MLK3 were introduced into Cos-7 cells. GST pull downs (Pellet) were analysed by immunoblotting with anti-myc antibody. Expression levels were analysed by immunoblotting cell lysates with appropriate antibodies. (B) The JNK isoforms JNK1, JNK2 and JNK3 all phosphorylate p73 α *in vitro*. *In vitro* kinase assays were performed using GST-p73 α expressed in and purified from *E.coli* and then incubated with HA-tagged JNK isoforms immunoprecipitated from transfected Cos-7 cells treated with anisomycin (5 μ g/ml; 20 mins) to activate the JNK isoforms. The products were resolved by SDS-PAGE and analysed by autoradiography. Images of the autoradiograph (upper panel), the Coomassie-stained SDS-PAGE gels (middle panel), and the levels of expression of the HA-JNK isoforms in the Cos-7 cell lysates (lower panel) are shown. *Denotes non-specific bands recognised by the HA antibody in Cos-7 lysates. (C-E) JNK phosphorylates p73 α *in vitro* at multiple sites. *In vitro* kinase assays were performed using the indicated GST-p73 α fragments and phosphorylation site mutants that were expressed in and purified from *E.coli* and then incubated with GST-JNK3 isolated from transfected Cos-7 cells. GST and GST-c-Jun(1-79) were included as negative and positive controls, respectively. The products were resolved by SDS-PAGE and analysed by autoradiography. Images of the autoradiographs (upper panels) and Coomassie-stained SDS-PAGE gels (lower panels) are shown. (F) Mutation of seven potential phosphorylation sites significantly reduces JNK phosphorylation of p73 α . GST-p73 α and GST-p73.M7 were incubated with recombinant JNK1 (Upstate) in an *in vitro* kinase assay for the indicated time course. The products were resolved by SDS-PAGE and analysed by autoradiography. Images of a representative autoradiograph (upper panel) and Coomassie-stained SDS-PAGE gel (lower panel) are shown. The results from two independent experiments were quantified and the mean values are presented. (G) JNK phosphorylates p73 α in cells. Constructs expressing myc-p73 α or myc-p73.M7 were introduced into Cos-7 cells together with GST or GST-JNK3. Anti-myc immunoprecipitates (Pellet) were analysed by immunoblotting for phosphorylated Ser/Thr residues to detect phosphorylated p73 α (Phospho-p73).

Figure 4. JNK phosphorylation of p73 regulates its stability and transcriptional activity.

(A) U20S cells were transfected with constructs expressing either HA-p73 or the phosphorylation site mutant (HA-p73.M7). Cells were treated with cisplatin (25 μ M) for 24 hrs

and immunoblots of the cell lysates were performed with antibodies to p73, PARP (p85) and β -actin. **(B)** JNK phosphorylation of p73 promotes its acetylation by p300. Cos-7 cells were transfected with constructs expressing GST-JNK3 and p300 together with either myc-p73 or the phosphorylation site mutant (myc-p73.M7). Cell lysates were subject to immunoprecipitation (IP) with anti-myc antibody and complexes were analysed by immunoblotting with an anti-acetyl-lysine antibody in order to detect acetylated p73 α (Ac-p73). Protein expression was analysed by immunoblotting lysates with the appropriate antibodies. We detect a band the size of p300 in lysates not transfected with the p300 expression construct, which we assume to be endogenous p300. **(C-E)** JNK regulates p73-mediated transcriptional activation of *GADD45*- and *Bax*-Luciferase reporter constructs. U2OS cells were transfected with the reporter constructs together with either GST-JNK3, GST-JNK3(K93A) (kinase-dead mutant; JNK3(KD)), or HA-MLK3 along with HA-p73 or HA-p73.M7. Luciferase assays were performed and values normalised to the activity of the control reporter, pRL-TK-Renilla. Fold induction represents luciferase activity relative to the value of HA-p73 alone. All transfections were performed in triplicate and data is presented as mean values with standard deviation. **(F)** JNK phosphorylation sites in p73 are required for cisplatin-induced apoptosis. U2OS cells seeded onto coverslips were transfected with constructs expressing myc-p73, myc-p73.M7, or empty vector. Cells were treated with or without cisplatin (10 μ M) for 24 hrs. The percentage of transfected cells (identified by immunofluorescence using anti-myc antibody) exhibiting condensed nuclei was calculated. The data represents the mean of four independent experiments in which at least 100 cells were counted per condition in a blind manner.

Figure 1

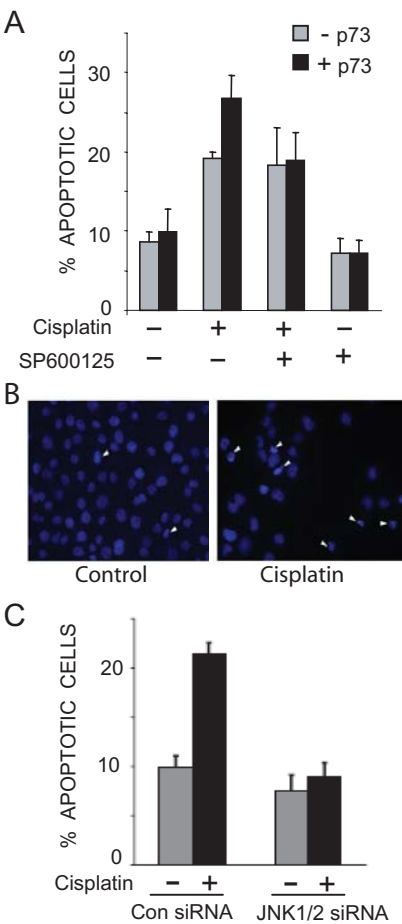


Figure 2

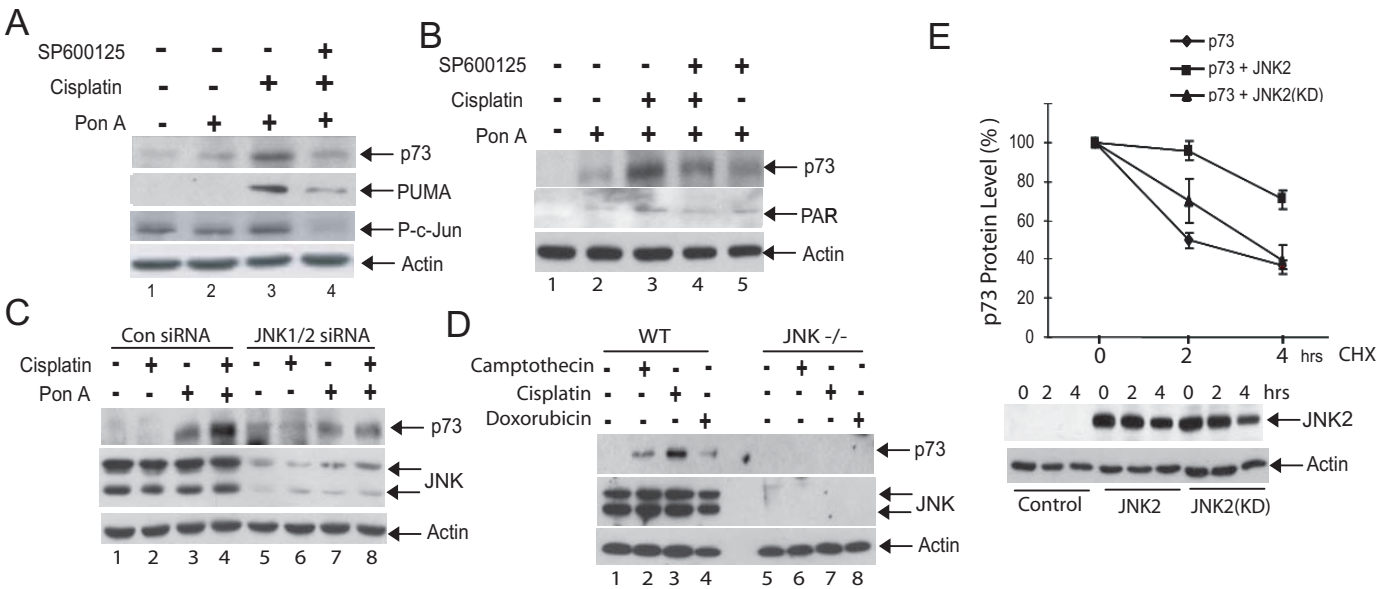


Figure 3

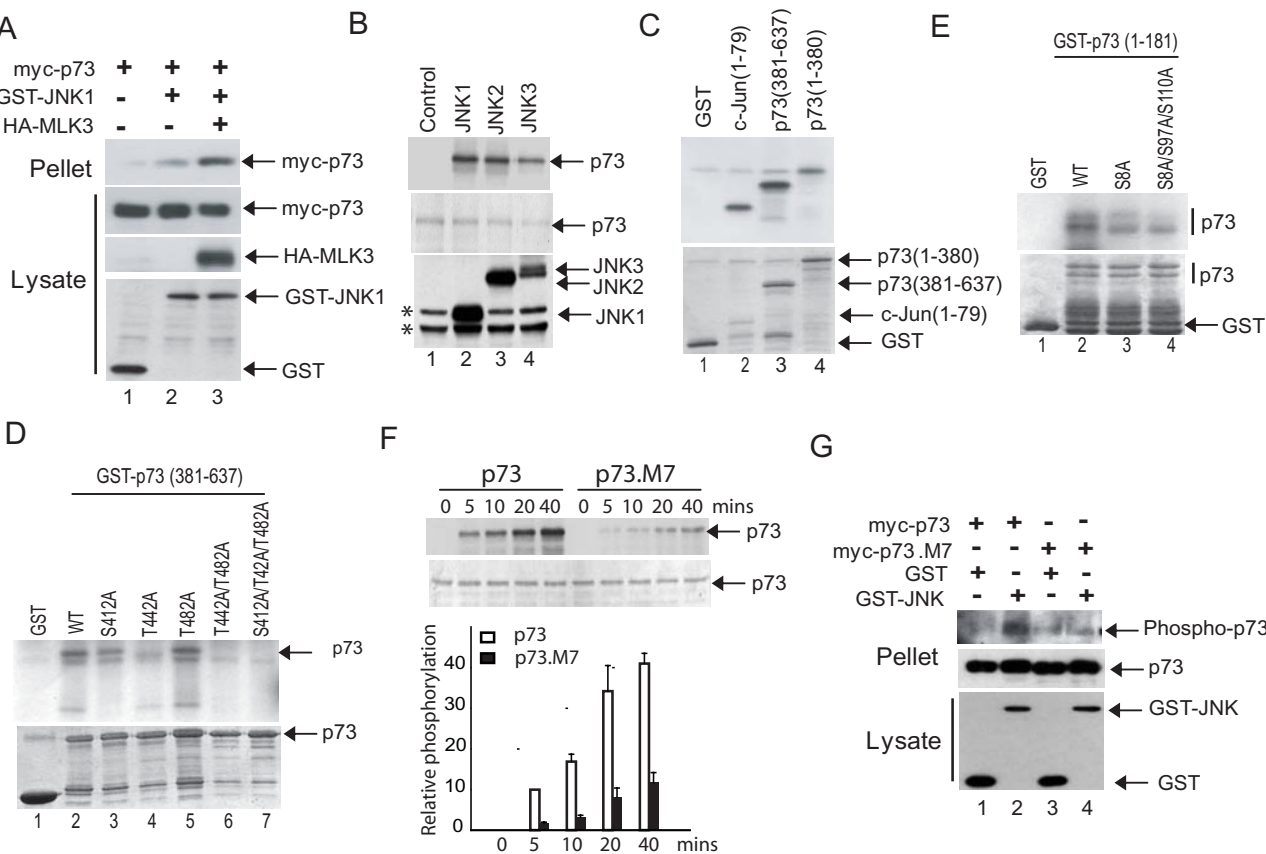


Figure 4

