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Role of NF-κB in p53-mediated programmed cell death

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The tumour suppressor p53 inhibits cell growth through activation of cell-cycle arrest and apoptosis[1], and most cancers have either mutation within the *p53* gene or defects in the ability to induce p53. Activation or re-introduction of p53 induces apoptosis in many tumour cells and may provide effective cancer therapy[2]. One of the key proteins that modulates the apoptotic response is NF-κB, a transcription factor that can protect or contribute to apoptosis[3]. Here we show that induction of p53 causes an activation of NF-κB that correlates with the ability of p53 to induce apoptosis. Inhibition or loss of NF-κB activity abrogated p53-induced apoptosis, indicating that NF-κB is essential in p53-mediated cell death. Activation of NF-κB by p53 was distinct from that mediated by tumour-necrosis factor-α and involved MEK1 and the activation of pp90[rsk]. Inhibition of MEK1 blocked activation of NF-κB by p53 and completely abrogated p53-induced cell death. We conclude that inhibition of NF-κB in tumours that retain wild-type p53 may diminish, rather than augment, a therapeutic response.

p53 has been implicated in the activation of both death-receptor and Apaf-1-dependent apoptosis[1]. Signalling through death receptors such as tumour necrosis factor receptor (TNFR) generates both pro- and anti-apoptotic signals, and activation of NF-κB functions to protect cells from death under these circumstances[3]. In other situations, however, NF-κB contributes to apoptosis[3]. NF-κB is activated by a variety of signals through mechanisms that result in phosphorylation and degradation of the inhibitory IκB proteins, the best understood being activation of IκB kinase (IKK) in response to tumour necrosis factor-α (TNFα) signalling[4]. Other signalling pathways can activate NF-κB, including the RAF/MAPK pathway which leads to the phosphorylation of IkB by pp90[rsk] (ref. 5). Activation of pp90[rsk] is important for the inactivation of IκB and subsequent activation of NF-κB in response to ultraviolet light and phorbol esters (ref. 6) through a RAF-dependent but RAS-independent mechanism.

To study the pathways involved in p53-induced cell death, we generated p53-inducible Saos-2 cells, a tumour cell line that does not express endogenous p53. Induction of p53 in these cells leads to extensive programmed cell death[7] (Fig. 1a). As NF-κB is involved in the regulation of apoptosis in several systems, we examined the effect of p53 on NF-κB DNA-binding activity. A clear elevation of NF-κB DNA-binding activity was seen after expression of p53 in these cells (Fig. 1b), and antibody analysis of this complex showed that it comprised the NF-κB family members p50 and p65 (Fig. 1b). To examine the ability of endogenous p53 to activate NF-κB, we used RKO cells which contain wild-type p53 that can be activated in response to stress, such as DNA damage, or by treatment with low levels of actinomycin-D (ref. 8). Induction of p53 in these cells

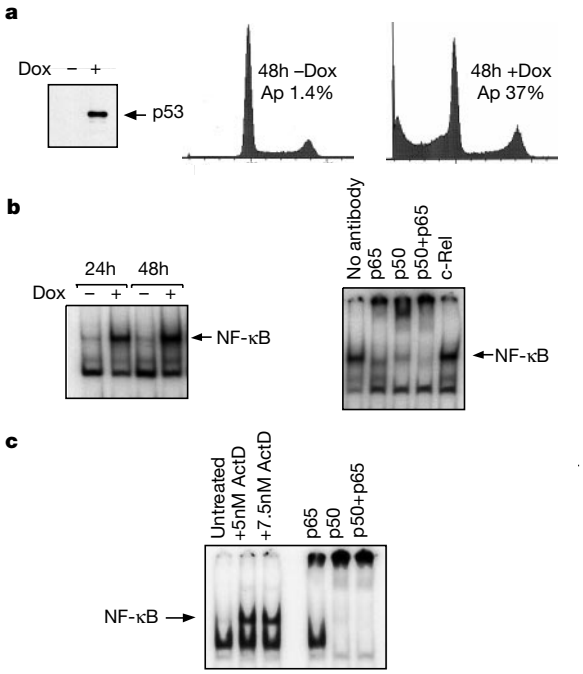

Figure 1 p53 causes activation of DNA binding by NF-κB. **a**, Treatment of p53-inducible Saos-2 cells with the tetracycline analogue doxycycline (Dox) causes apoptosis (Ap) as measured by the percentage of cells with a sub-G_1 DNA content. Three independently derived p53-inducible lines gave the same result. **b**, Dox treatment induces NF-κB, as indicated by electrophoretic mobility shift assay (EMSA), and the induced complexes are reactive with antibodies raised against the NF-κB family members p50 and p65. Three independently derived p53-inducible lines gave the same result. **c**, Treatment of RKO cells with the indicated concentrations of actinomycin-D (ActD) induces DNA binding by NF-κB. The induced complex was reactive with antibodies to the NF-κB family members p50 and p65. A faster migrating complex present in both treated and untreated cells was also found to be reactive with p50, but not p65, antibodies.

resulted in the activation of NF-κB DNA-binding activity comprising the NF-κB family members p50 and p65 (Fig. 1c).

Mutants of p53 that fail to induce either apoptosis or cell-cycle arrest (p53 175His), or that retain the ability to activate cell-cycle arrest but show impaired apoptotic function (p53 175Pro) have been described[9]. Cell lines that induced comparable levels of these mutant proteins (data not shown) showed a clear correlation between the ability of p53 to activate apoptosis (Fig. 2a) and the induction of NF-κB DNA-binding activity (Fig. 2b). In contrast, inducible expression of Bax (Fig. 2c) also led to a strong apoptotic response (Fig. 2d) but failed to induce NF-κB DNA binding (Fig. 2e), indicating that the activation of NF-κB is not simply a consequence of the apoptotic process.

Other apoptotic signals, such as TNFα, also activate NF-κB[10], and we therefore compared the apoptotic pathways that were engaged by p53 activation and TNFα. We analysed the effect of expressing inhibitors of apoptosis in both p53-inducible cells and the parental p53-null Saos-2 cells treated with TNFα (Fig. 3). As expected, the broad specificity caspase inhibitor, p35 (ref. 11), efficiently rescued both p53- and TNFα-induced apoptosis. Expression of bcl2, which blocks the Apaf-1-dependent pathway[12], also inhibited p53-induced cell death as described[13], but did not have a profound effect on TNFα-induced death (Fig. 3). Expression of the NF-κB subunit p65, which leads to an increase in NF-κB activity (data not shown), efficiently protected the cells from TNFα-induced death (Fig. 3), consistent with an anti-apoptotic role for NF-κB in this pathway[3]. In contrast, expression of p65 did not significantly increase or decrease p53-induced apoptosis (Fig. 3). Furthermore, no effect was seen after transfection of other NF-κB family members, either alone or in combination (data not shown). We then examined the effect of inhibiting the activity of endogenous NF-κB in these cells using mutant forms of IκBα that either cannot be phosphorylated or cannot be ubiquitinated[14,15]. Both of these IκBα mutants inhibit NF-κB activity in the cell. Expression of the unphosphorylatable IκBα super-repressor (IκBSR) protein sensitized cells to TNFα-induced apoptosis, consistent with a role for NF-κB in the inhibition of TNFα-mediated death (Fig. 3). In contrast, expression of the IκBSR inhibited p53-induced apoptosis as efficiently as both p35 and bcl2 (Fig. 3). Western blot analysis showed that expression of the IκBSR had no effect on the levels of p53 protein expression (data not shown), and identical results were obtained using the IκB protein that cannot be ubiquitinated.

The experiments in Saos-2 cells, using inducible over-expression of exogenous p53, indicated that NF-κB is necessary for p53-induced death. To assess the role of NF-κB in mediating the apoptotic activity of physiological levels of p53, we used RKO cells that contain endogenous wild-type p53. Previous studies showed that treatment of RKO cells with low levels of actinomycin-D stabilizes p53 and induces p53-dependent cell-cycle arrest and apoptosis[16]. We established a series of RKO cell lines stably expressing the IκBSR (Fig. 4a) and verified the activity of the transfected protein by showing that these cells were no longer able to activate NF-κB in response to TNFα treatment (Fig. 4b). Previous studies have shown that p53 is a transcriptional target of NF-κB (ref. 17) and have suggested that p53 expression depends, at least partially, on NF-κB activity[18]. Mutual negative regulation of p53 and NF-κB, where each transcription factor inhibits the activity of the other by competing for a limiting pool of the co-activator p300/CBP, has also been described[19]. In RKO cells, however, expression of IκBSR did not inhibit expression or stabilization of p53 in response to actinomycin-D treatment, or transcriptional activation

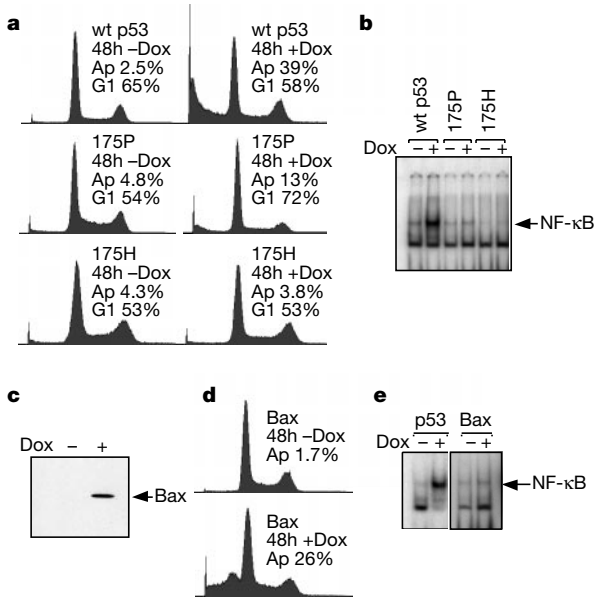

Figure 2 NF-κB activation is associated with the apoptotic activity of p53 and is not induced after cell death induced by Bax. **a**, Flow cytometry of Saos-2 cell lines containing inducible wild-type p53, or p53 mutants 175Pro and 175His. **b**, EMSA for NF-κB after induction of p53-inducible lines. **c**, Western blot of Bax in Bax-inducible Saos-2 cells after treatment with Dox. **d**, Induction of apoptosis after Dox treatment of the Bax-inducible line. **e**, EMSA for NF-κB after induction of Bax.

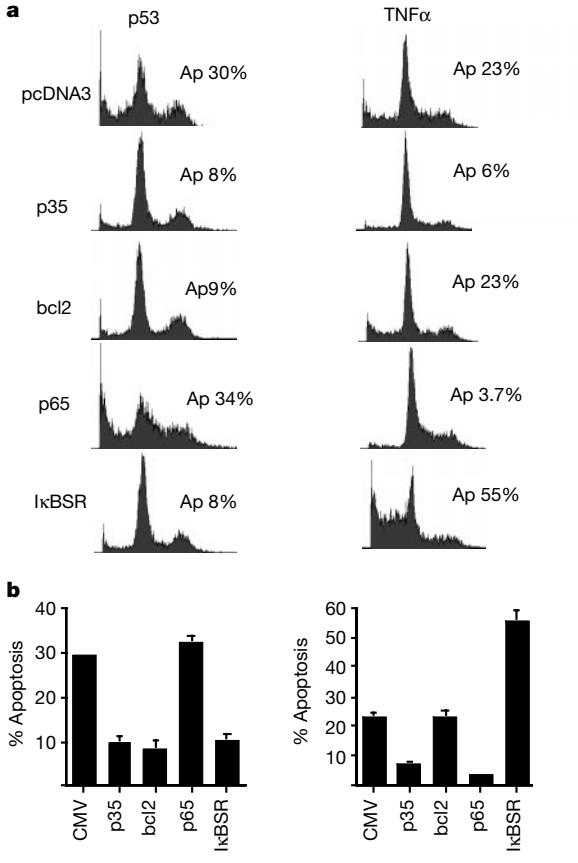

Figure 3 NF-κB is essential for p53-induced cell death. **a**, The p53-inducible cells (p53, left panel) or parental Saos-2 cells (TNFα, right panel) were transiently transfected with the indicated genes, and treated with doxycycline or TNFα, respectively. After 48 h, the transfected cells were identified by staining for co-transfected CD20 and analysed for DNA content by flow cytometry. Western analysis of these transfected cells indicated that each protein was expressed to similar levels in both cell types (data not shown). **b**, Graphical representation of several experiments as indicated in **a**.

of the p53 target gene *p21*$^{WAF1/CIP1}$ (p21; Fig. 4c). In the RKO cells expressing IκBSR, inhibition of NF-κB activity led to a significant decrease in the apoptotic response to actinomycin-D treatment, although the ability to induce cell-cycle arrest was not impaired (Fig. 4d, e). The degree of inhibition of apoptosis was identical to that seen in RKO lines containing the human papillomavirus protein, E6, which inactivates p53 function (Fig. 4e), indicating that the residual cell death seen after actinomycin-D treatment

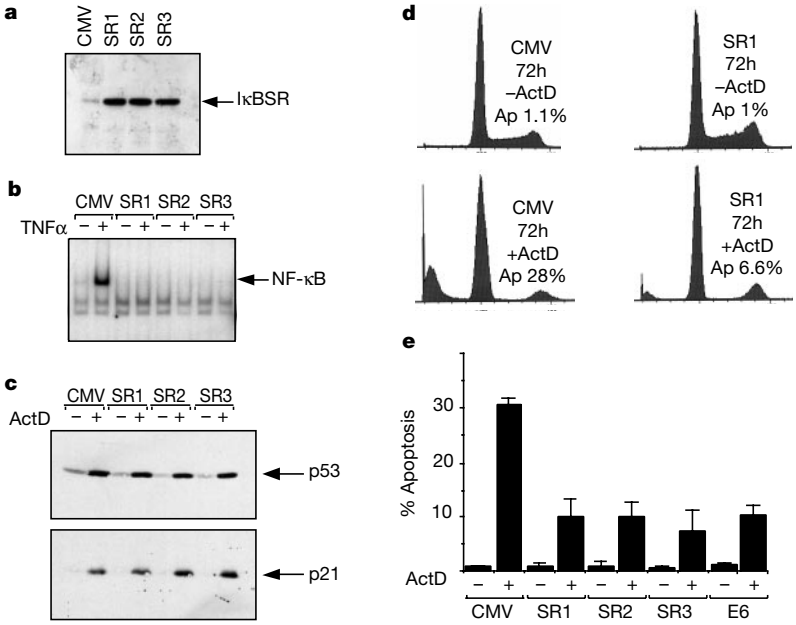

Figure 4 Inhibition of NF-κB abrogates p53-mediated apoptosis, but not cell cycle arrest. **a**, Stable expression of transfected IκBSR, determined by western blotting, in lines of RKO cells, which contain endogenous wild-type p53. **b**, Induction of NF-κB DNA-binding in response to treatment with TNFα in the IκBSR-expressing cell lines. **c**, Induction of p53 and its target gene *p21*$^{WAF1/CIP1}$ (p21) after DNA damage induced by treatment with actinomycin-D in the IκBSR cell lines. **d**,**e**, Actinomycin-D (ActD)-induced apoptosis and cell cycle arrest in cells expressing IκBSR or HPV E6.

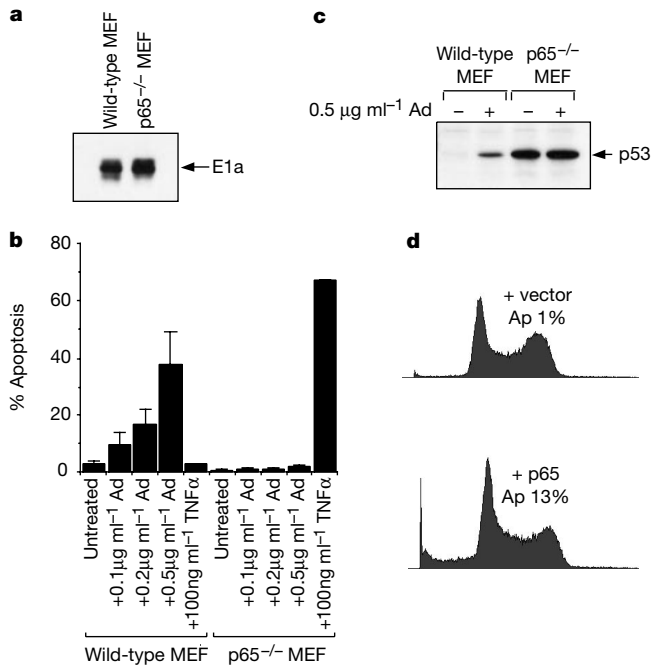

Figure 5 E1A transformed *p65*$^{-/-}$ MEFs are resistant to p53-mediated apoptosis. **a**, Expression of E1A, determined by western blotting, in wild-type and *p65*$^{-/-}$ MEFs after infection with an E1A-expressing retrovirus. **b**, The cells were treated with the indicated concentrations of adriamycin (Ad) or TNFα for 24 h. Cell populations (including floating cells) were then collected and analysed by flow cytometry. The percentage of cells with a sub-G_1 DNA content was taken as a measure of the apoptotic rate. The results of four experiments are shown. **c**, p53 expression in E1A-expressing wild-type and *p65*$^{-/-}$ MEFs. Cells were treated with or without 0.5 μg ml^{-1} adriamycin and analysed by western blotting. **d**, E1A-expressing *p65*$^{-/-}$ cells were infected with retroviral vector alone or virus expressing p65. Expression of p65 was confirmed by western blotting (data not shown). After selection the cells were analysed for DNA content by flow cytometry, the percentage of cells with a sub-G1 DNA content was taken as a measure of the apoptotic rate.

probably represents p53-independent apoptotic mechanisms.

These results indicated that NF-κB may be important in p53-mediated cell death, although NF-κB showed the expected anti-apoptotic function in response to TNFα. To investigate the generality of these unexpected observations, we used mouse embryo fibroblasts (MEFs) derived from p65 (RelA)-deficient mice[20]. The activation of endogenous p53 in MEFs results in cell-cycle arrest but does not lead to apoptosis unless the cells are transformed with an oncogene such as E1A (ref. 21). This sensitizes the cells to undergo p53-dependent apoptosis in response to chemotherapeutic agents such as adriamycin. We therefore generated wild-type and *p65*$^{-/-}$ MEFs that express E1A and confirmed equal levels of E1A expression in both cell types (Fig. 5a). E1A-expressing wild-type MEFs underwent extensive p53-dependent apoptosis after treatment with adriamycin (Fig. 5b), but showed little apoptosis in response to TNFα at this time. Although E1A can prevent activation of NF-κB in response to TNF (ref. 22), NF-κB DNA-binding activity was detected in the E1A-expressing wild-type cells, and this activity was further elevated after activation of p53 by adriamycin (data not shown), indicating that E1A does not inhibit this pathway of NF-κB activation. Analysis of E1A-expressing *p65*$^{-/-}$ MEFs confirmed previous observations that these cells are hypersensitive to TNFα-induced apoptosis (Fig. 5b). Consistent with a pro-apoptotic role for NF-κB in p53-induced cell death, however, E1A-expressing *p65*$^{-/-}$ MEFs were completely resistant to adriamycin-induced cell death (Fig. 5b). These cells resumed growth after removal of adriamycin, showing that loss of p65 provides a survival advantage under these circumstances (data not shown). In some systems, NF-κB contributes to p53 expression[18], and, to confirm that the resistance of the *p65*$^{-/-}$ cells to apoptosis was not related to a failure to express p53, we examined p53 protein levels in these cells. E1A-expressing wild-type MEFs showed detectable levels of p53 expression that were further enhanced after treatment with adriamycin (Fig. 5c). The E1A-expressing *p65*$^{-/-}$ cells consistently showed significantly higher levels of p53 expression that was not enhanced after treatment (Fig. 5c). E1A expression stabilizes p53 (ref. 23) and we propose that the tolerance to elevated expression of p53 in the E1A-expressing *p65*$^{-/-}$ MEFs reflects the resistance of these cells to p53-induced apoptosis. Finally, we showed that re-introduction of p65 into the E1A-expressing *p65*$^{-/-}$ MEFs restored the apoptotic response (Fig. 5d). In this case, as the cells already expressed high levels of p53 (Fig. 5c), further activation of p53 with adriamycin was not required to induce programmed cell death.

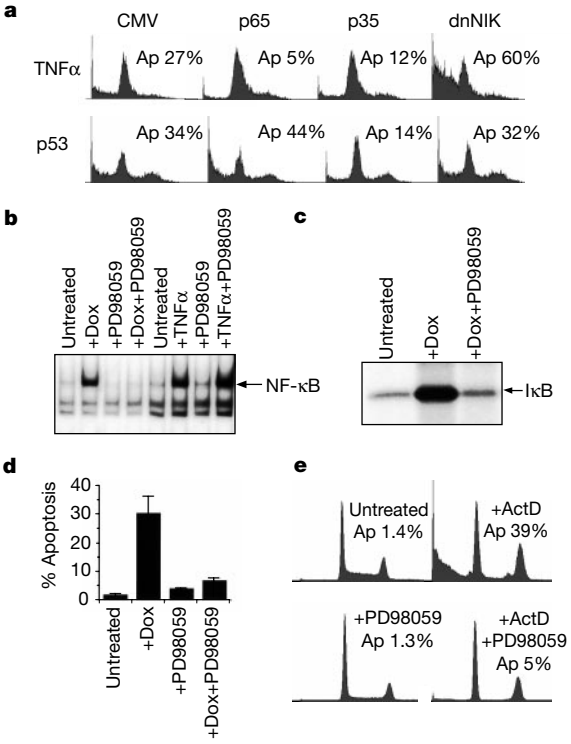

Figure 6 Signalling pathways involved in the activation of NF-κB by p53 are distinct from those engaged by TNFα and involve the RAF/MAPK pathway. **a**, Dominant-negative NIK (dnNIK) affects cell death induced by TNFα but not p53. The p53-inducible cells or parental Saos-2 cells were transiently transfected with the indicated genes, and treated with doxycycline or TNFα respectively. After 48 h, the transfected cells were identified by staining for co-transfected CD20 and analysed for DNA content by flow cytometry. **b**, Induction of NF-κB DNA binding by p53, but not by TNFα, is inhibited by treatment with the MEK1 inhibitor PD98059. p53-inducible Saos-2 cells were treated with Dox and PD98059; parental Saos-2 cells were treated with TNFα and PD98059, as indicated. **c**, p53 stimulates pp90^{rsk} activity as measured by the ability to phosphorylate IκBα (IκB), and this stimulation is inhibited by treatment with PD98059. p53-inducible Saos-2 cells were treated as indicated with Dox and PD98059 and pp90^{rsk} kinase immunoprecipitated and used to phosphorylate full-length IκBα. **d**, Treatment with PD98059 inhibits p53-dependent apoptosis induced in the p53-inducible Saos-2 cells. Cells were treated with Dox and PD98059 for 48 h, and analysed by flow cytometry. **e**, Treatment with PD98059 inhibits p53-mediated cell death, but not cell-cycle arrest, in RKO cells after treatment with actinomycin-D for 72 h.

The divergent contributions of NF-κB to TNFα- and p53-induced cell death prompted us to examine possible differences in the signalling pathways involved in NF-κB activation. Activation of NF-κB in response to TNFα is mediated through complex formation at the TNFR and phosphorylation of the IKK complex, which in turn phosphorylates and inactivates IκB (ref. 10). Dominant-negative forms of NF-κB-inducing kinase (NIK) have been shown to block this pathway by preventing phosphorylation of IKK and degradation of IκB (ref. 24), leading to an inability to activate NF-κB. As expected, inhibition of NIK, using the dominant-negative mutant, enhanced TNFα-induced apoptosis in Saos-2 cells (Fig. 6a). Unlike the IκBSR, however, dominant-negative NIK had no effect on p53-induced death in the same cells (Fig. 6a), indicating that the pathway regulating NF-κB in the p53 response is distinct from that used by TNFα. Another pathway implicated in NF-κB activation involves activation of the RAF/MAPK cascade and phophorylation of IκB by pp90^{rsk}. In p53-inducible Saos-2 cells, treatment with the well-characterized MEK1 inhibitor, PD98059 (ref. 25), efficiently prevented activation of NF-κB DNA-binding activity in response to p53 (Fig. 6b), although the activation of NF-κB in response to TNFα remained unaffected. MEK1 functions in the RAF/MAPK pathway, which leads to the activation of multiple kinases, including pp90^{rsk}. Analysis of the kinase activity of pp90^{rsk}, using IκBα as a substrate, showed that p53 can stimulate pp90^{rsk} activity and that this is inhibited by treatment with PD98059 (Fig. 6c). Inhibition of MEK1 and pp90^{rsk} using PD98059 resulted in a complete protection of these cells from p53-induced apoptosis (Fig. 6d), although the p53-induced cell-cycle arrest was not affected (data not shown). TNFα-induced cell death was not altered in these cells in response to treatment with PD98059 (data not shown). Cell death induced by transfected p53 in U2OS and HeLa cells was also inhibited by PD98059 (data not shown), and the MEK1 inhibitor protected against apoptosis induced by activation of endogenous p53 in RKO cells (Fig. 6e). Treatment with PD98059 did not prevent activation of the p53-dependent cell-cycle arrest in RKO cells (Fig. 6e).

These results show that p53 can induce activation of NF-κB, and loss of NF-κB activity specifically abrogated the p53-mediated apoptotic response, without impinging on the ability to activate expression of target genes or induce cell-cycle arrest. NF-κB shows anti-apoptotic activities in many systems, such as the TNFα-induced apoptotic pathways, but our results reveal an unexpected pro-apoptotic activity of NF-κB in the p53-induced apoptotic response. Notably, expression of p65 alone in these cells did not induce apoptosis (data not shown), showing that NF-κB activity is

necessary but not sufficient for p53-mediated death. These results provide an interesting contrast to our study that showed that the p53-independent apoptotic activity of E2F-1 is associated with the inhibition of NF-κB activation by death receptors such as the TNFR (ref. 26). As E2F-1 can also cooperate with p53 in the induction of apoptosis, the results suggest that E2F-1 expression prevents TNFα-induced activation of NF-κB but that it does not inhibit the NF-κB activity required for p53-induced programmed cell death. Indeed, E2F-1 expression inhibits the activation of NF-κB by mediating degradation of TRAF2(ref. 26), a protein involved in transducing the signal from the TNFR. We have shown that p53 uses a different mechanism to activate NF-κB, involving the RAF/MAPK pathway and activation of $pp90^{rsk}$, which would not be affected by E2F-1. Inhibition of the RAF/MAPK pathway prevents activation of NF-κB in response to p53 but not TNFα, and efficiently abrogates p53-induced cell death, without impinging on TNFα-induced death. Our findings not only provide insight into p53 function, but may also be important in the design of therapeutic protocols that involve targeting of either p53 or NF-κB. In *p53*-null or defective tumours, inhibition of NF-κB may well be useful, leading to the potentiation of standard chemotherapeutic drugs[27]. However, in tumours that retain wild-type p53, where p53 is itself an important mediator of chemosensitivity, such therapy may be counterproductive, because targeting NF-κB is likely to result in the repression of p53-mediated tumour cell death. □

Methods

Plasmids and antibodies

The full-length human p53 sequences were cloned into the *Bam*H1 site of the pTRE vector (Clontech). The plasmids pcDNA3p35, pRcCMVp65 and CMVCD20 have been described[26]. pRcCMVIκBSR (ref. 15), CMVdnNIK (ref. 28) and pcDNA3bcl2 (ref. 29) have also been described. For western blot and EMSA analysis, we used described antibodies to detect IκBα, p50, p65 and c-rel (ref. 30), human p53- and DO-1-tagged Bax (Ab-6, Oncogene Science)[29], mouse p53 (CM1, Novacastra), E1A (M53, Pharmingen) and p21 (Ab-1 Oncogene Science). CD20 cell-surface antigen expression was achieved using a FITC-conjugated monoclonal antibody (Becton Dickinson).

Cell lines and transfections

Tetracycline inducible cells were established by transfection into the Saos-2 TetOn line[29]. Saos-2 TetOn Bax-inducible cells have been described[29]. Transient transfections into the p53 TetOn cells were undertaken by calcium phosphate precipitation using 5 μg test plasmid and 1 μg CD20 plasmid. Clones of RKO cells expressing the IκB mutant, IκBSR, were established by transfection of 10 μg of pRcCMVIκBSR, followed by selection with 1 mg ml^{-1} G418 (Gibco BRL). At the same time, clones were also established expressing the CMV vector alone. Primary wild-type and $p65^{-/-}$ MEFs were infected with a puromycin tagged E1A-expressing retrovirus according to a described protocol[23]. Twenty-four hours after infection, the cells were selected with 2.5 μg ml^{-1} puromycin. Selected cultures were treated as appropriate with adriamycin or TNFα (100 ng ml^{-1}). Cells were further infected with either the retroviral vector pWZLHygro or pWZLHygro expressing mouse p65. Where indicated, the human cells were treated with the tetracycline analogue, doxycycline (Dox; 800 ng ml^{-1}), TNFα (5 ng ml^{-1}), actinomycin-D (5 nM) or PD98059 (100 μM) (Calbiochem).

Flow cytometry

After the treatments and times indicated, total populations of cells, including floating and adherent cells, were processed for flow cytometric analysis (FACScaliber, Becton Dickinson) as described[29]. The percentage of cells with a sub-G1 DNA content was taken as a measure of the apoptotic rate of the cell population. Cells which had been transfected with the CD20 marker were stained with a FITC-conjugated CD20 antibody, sorted for fluorescence isothiocynate fluorescence, and analysed for DNA content[29].

Protein analysis

Protein samples were harvested for western blotting after the times indicated. Lysates were normalized by Bradford assay and equal amounts of protein were electrophoresed on polyacrylamide gels. After transfer to nitrocellulose membranes, Ponceau-S staining was undertaken as a further assessment of loading and to check the fidelity of transfer. Membranes were probed with the antibodies described above using standard immunoblotting techniques.

Electrophoretic mobility shift assays

For EMSA analysis, 8×10^5 cells were seeded on 10-cm dishes and treated the following day with Dox, TNFα or actinimycin-D for the times indicated. Cells were lysed in 2 × ELB (100 mM HEPES pH 7, 0.5 M $NaCl_2$, 10 mM EDTA, 0.2% NP40, containing protease and phophatase inhibitors.). Extracts were then mixed with EMSA buffer (10 mM HEPES pH 7.5, 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.05 mg single stranded DNA and poly[dI-dC] per ml) and ^{32}P-labelled NF-κB probe (BioRad), and incubated for 30 min at room temperature. Where indicated, the reaction mix was incubated with 1 μl antiserum for 20 min at room temperature before the addition of the ^{32}P-labelled probe. Samples were resolved on non-denaturing polyacrylamide gels and visualized by autoradiography.

Kinase assay

For the $pp90^{rsk}$ kinase assays, 5×10^7 cells were plated for each data point (two 15-cm dishes) and (where appropriate) doxycycline (800 ng ml^{-1}) and PD98059 (100 μM) were added for 16 h. Cells were harvested in TNT buffer (20 mM Tris pH 7.5, 200 mM NaCl, 1% triton X-100 containing phosphatase and protease inhibitors). Samples were normalized for amount of protein. $pp90^{rsk}$ was immunoprecipitated, washed with TNT and kinase buffer (20mM HEPES pH 7.4, 2 mM MnCl, 1 mM dithiothreitol), and a kinase assay was performed using full-length IκBα as a substrate.

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Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development

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XRCC4 is a non-homologous end-joining protein employed in DNA double strand break repair and in V(D)J recombination[1,2]. In mice, XRCC4-deficiency causes a pleiotropic phenotype, which includes embryonic lethality and massive neuronal apoptosis[2]. When DNA damage is not repaired, activation of the cell cycle checkpoint protein p53 can lead to apoptosis[3]. Here we show that p53-deficiency rescues several aspects of the XRCC4-deficient phenotype, including embryonic lethality, neuronal apoptosis, and impaired cellular proliferation. However, there was no significant rescue of impaired V(D)J recombination or lymphocyte development. Although p53-deficiency allowed postnatal survival of XRCC4-deficient mice, they routinely succumbed to pro-B-cell lymphomas which had chromosomal translocations linking amplified c-myc oncogene and IgH locus sequences. Moreover, even XRCC4-deficient embryonic fibroblasts exhibited marked genomic instability including chromosomal translocations. Our findings support a crucial role for the non-homologous end-joining pathway as a caretaker of the mammalian genome, a role required both for normal development and for suppression of tumours.

Cellular DNA double strand breaks (DSBs) result from oxidative metabolism, exogenous damaging agents, or endonuclease activity[4]. Mammalian cells repair these potentially lethal or oncogenic chromosomal lesions either by non-homologous end-joining (NHEJ) in which broken DNA ends are ligated directly[5] or by homologous recombination employing a template of similar sequence[6]. The early lymphocyte-specific V(D)J recombination reaction is initiated by DSBs made by RAG endonuclease[7]. Subsequently, V(D)J recombination is completed by the ubiquitous NHEJ components, including the Ku70 and Ku80 DNA end-binding complex (Ku) and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), as well as XRCC4 and DNA Ligase IV (Lig4) which probably cooperate in ligation[4,5].

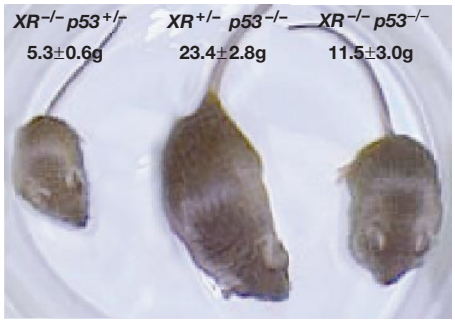
Mice deficient in XRCC4 (or Lig4) die during late embryonic development[2,8,9]. XRCC4-deficient embryos display extensive apoptotic death of newly generated, postmitotic neurons throughout the developing nervous system[2]. In addition, progenitor (pro)-lymphocyte development is arrested due to impaired V(D)J recombination. XRCC4-deficient embryos are also growth-retarded and their fibroblasts exhibit decreased proliferation and premature senescence in culture[2]. The precise cause of embryonic death is unknown, but the massive apoptotic neuronal phenotype is associated with defective NHEJ and may be a checkpoint response to eliminate nascent neurons with DSBs that have not been repaired[10]. Normally, DSBs lead to stabilization and activation of p53, followed by cell cycle arrest or apoptosis depending on cell type and/or physiological context[3].

Mice containing a mutation that inactivates the *p53* gene (either heterozygous, $p53^{+/-}$, or homozygous, $p53^{-/-}$) are relatively normal, though $p53^{-/-}$ mice become cancer-prone at about 5 months of age[3]. To test potential involvement of p53 in XRCC4-deficient phenotypes, we bred $XRCC4^{+/-}$ mice with $p53^{-/-}$mice and then bred progeny to generate the various $XRCC4^{-/-}$ cohorts against all three p53 genotypes. We have not observed a live $XRCC4^{-/-}$

a

Genotype	$XR^{+/-}$ $p53^{+/-}$	$XR^{+/-}$ $p53^{-/-}$	$XR^{+/-}$ $p53^{+/+}$	$XR^{-/-}$ $p53^{+/+}$	$XR^{-/-}$ $p53^{+/-}$	$XR^{-/-}$ $p53^{-/-}$
Expected	60	81	14	7	44	47
Actual	88	74	10	0	17	33

b



c

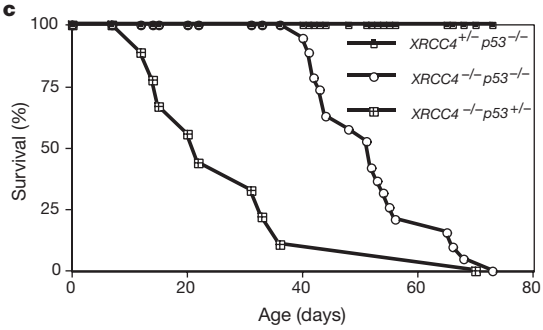


Figure 1 Effects of p53-deficiency on survival of $XRCC4^{-/-}$ mice. **a**, p53 deficiency rescues embryonic lethality. Genotypes of offspring from $XRCC4^{+/-}p53^{+/-} \times XRCC4^{+/-}p53^{+/-}$, $XRCC4^{+/-}p53^{+/-} \times XRCC4^{+/-}p53^{-/-}$, and $XRCC4^{+/-}$ $p53^{-/-} \times XRCC4^{+/-}$ $p53^{-/-}$ crosses. Shown are relevant genotypes for $XRCC4^{+/-}$ and $XRCC4^{-/-}$ offspring. 11 pups died within 2 days of birth without genotyping and were not included. **b**, Average weights of 5-week-old $XRCC4^{-/-}p53^{+/-}$ ($n = 3$), $XRCC4^{+/-}$ $p53^{-/-}$ ($n = 10$) and $XRCC4^{-/-}p53^{-/-}$ ($n = 10$) mice and a representative photograph of littermates. **c**, Percentage survival of $XRCC4^{+/-}p53^{-/-}$ ($n = 30$), $XRCC4^{-/-}$ $p53^{+/-}$ ($n = 9$) and $XRCC4^{-/-}$ $p53^{-/-}$ ($n = 19$) mice versus age (in days). 95% of $XRCC4^{-/-}p53^{-/-}$ mice developed pro-B ($B220^{+}CD43^{+}CD4^{-}CD8^{-}Thy1^{-}$) lymphomas. $XRCC4^{+/-}$ data points represent the death or killing of terminally ill mice. XR, XRCC4.