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The Nuclear Factor-κB and p53 Pathways Function Independently in Primary Cells and Transformed Fibroblasts Responding to Genotoxic Damage

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Abstract

With nuclear factor-κB (NF-κB) and p53 functions generally having disparate outcomes for cell survival and cell division, understanding how these pathways are coordinated following a common activation signal such as DNA damage has important implications for cancer therapy. Conflicting reports concerning NF-κB and p53 interplay in different cell line models prompted a reexamination of this issue using mouse primary thymocytes and embryonic fibroblasts, plus fibroblasts transformed by E1A12S. Here, we report that following the treatment of these cells with a range of stress stimuli, p53 and NF-κB were found to regulate cell cycling and survival independently.
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Introduction

During the cellular responses to cytotoxic or genotoxic agents, the p53 and nuclear factor-κB (NF-κB) pathways represent two key signaling networks that influence cell survival and cell division (1, 2). NF-κB can promote cell cycle progression and typically inhibits apoptosis, with RelA and c-Rel serving as the transcription factors that generally prevent cell death induced by diverse signals (3). p53 normally has a short half-life due to its association with MDM2 (4). Following DNA damage, p53 stability increases due to disruption of the p53/MDM2 interaction. Oncoproteins such as E1A12S or c-Myc increase p53 levels by inducing $p19^{ARF}$, which binds MDM2, thereby promoting the release of p53. The induction of p53 leads to cell cycle arrest or apoptosis, with the specific response dictated by the cell type and stimulus (5).

Deregulated NF-κB and p53 signaling is a common feature of diverse cancers (6, 7). Although genetic lesions afflicting the NF-κB pathway are rare in cancer (7), constitutive activation of NF-κB in many human cancers occurs via indirect mechanisms. In contrast, mutations in the p53 pathway are a hallmark of at least 50% of human tumors (6). The prevalence of NF-κB and p53 deregulation in cancers has prompted considerable effort into developing clinically effective inhibitors of the NF-κB pathway (8) and activators of the p53 pathway, such as nutlins. Yet, with p53 and NF-κB activation generally leading to opposing outcomes for cell survival, chemotherapeutic agents that engage both pathways, such as those inducing DNA damage (1, 2), raise important questions about integration of the two transcriptional responses. Numerous studies report that genotoxic stress results in p53 and NF-κB cross-talk, yet evidence for synergistic as well as antagonistic interactions involving multiple mechanisms underscores the controversy surrounding this issue. For example, mutual transcriptional repression models propose that the dominance of a particular pathway is controlled by the relative nuclear levels of Rel/NF-κB and p53 competing for common limited cofactors, such as p300/CBP (9-11). These models are consistent with the apoptosis that results from overexpression of p53 coinciding with NF-κB inhibition (12), or human T-cell lymphotrophic virus type I Tax inactivation of p53 requiring NF-κB activation via a dependency on p300/CREB (13). A different corepressor model suggests that $p19^{ARF}$, which activates p53 by sequestering MDM2, also induces RelA interaction with histone deacetylase repressor complexes (14). There is evidence for transcriptional coregulation, with daunomycin induction of NF-κB activating the p53 promoter (15), whereas IKK2/RelA was found to antagonize the p53 pathway in response to doxorubicin by up-regulating *mdm2* expression (16). NF-κB activation was also shown to be necessary for p53-mediated cell death (17). Finally, a study examining primary hepatocytes concluded that NF-κB and p53 regulate apoptosis independently (18).

These contradictory findings may in part reflect the use of different immortal or tumor-derived cell lines in which key growth and antiapoptotic networks that interface with the NF-κB and p53 pathways are likely to be deregulated. This prompted us to reexamine the interdependence of p53 and NF-κB signaling during the responses to genotoxic agents and other death stimuli using cells bearing defined genetic lesions within these pathways. Survival and cell cycle progression were examined in mouse wild-type (*wt*), Rel/NF-κB–deficient, and $p53^{-/-}$ primary thymocytes, mouse embryonic fibroblasts (MEF), as well as in early-passage MEFs expressing E1A12S, following exposure to γ-irradiation, serum starvation, or treatment with doxorubicin, tumor necrosis factor (TNF), or glucocorticoids. Our results show that the death of thymocytes

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lacking RelA and c-Rel in response to p53-dependent and p53-independent stimuli is normal, Rel/NF-κB–deficient MEFs undergo cell cycle arrest following irradiation or doxorubicin treatment, NF-κB activity in primary and immortal MEFs induced by DNA damage is p53 independent, impaired cell cycle arrest in $p53^{-/-}$ MEFs does not require Rel/NF-κB, and NF-κB does not influence p53-dependent apoptosis in fibroblasts expressing E1A12S. Based on these findings, we conclude that following exposure to genotoxic and cytotoxic stress, the Rel/NF-κB and p53 pathways in these cells function independently.

Results

NF-κB Signaling Is Dispensable for p53-Dependent Apoptosis in Thymocytes

Primary mouse thymocytes and fibroblasts were used to investigate Rel/NF-κB and p53 interdependence during apoptosis induced by DNA damage. Thymocytes represent a suitable cell type for this study given NF-κB and p53 signaling is dispensable for their development (19, 20), plus c-Rel and RelA are expressed in the major thymocyte population ($CD4^+CD8^+$ cells; 21) sensitive to genotoxic and cytoxic agents that induce p53 and/or NF-κB (19, 21).

First, we examined the requirement for c-Rel and RelA in p53-dependent apoptosis following γ-irradiation or doxorubicin treatment. Due to the death of $rela^{-/-}$ and $c\text{-}rel^{-/-}rela^{-/-}$ mice at ~E15 and ~E13.5, respectively (22), comparable thymocyte populations were isolated from $rag\text{-}1^{-/-}$ mice engrafted with fetal liver hemopoietic progenitors from *wt*, $c\text{-}rel^{-/-}$, $rela^{-/-}$, $c\text{-}rel^{-/-}rela^{-/-}$, or $p53^{-/-}$ E12 embryos. Cultured thymocytes were untreated, γ-irradiated (100, 250 and 500 Rad), or doxorubicin treated (5, 50, and 500 ng/mL); survival of γ-irradiated (500 Rad) and doxorubicin-treated (500 ng/mL) cells is summarized in Fig. 1A. Rates of spontaneous death were equivalent for all thymocytes, and in response to either agent, Rel/NF-κB–deficient thymocyte apoptosis was the same as *wt* cells. The dose-dependent killing of Rel/NF-κB–deficient thymocytes was also equivalent to *wt* cells (results not shown). As expected (23), $p53^{-/-}$ thymocytes were resistant to DNA damage-induced apoptosis.

We also assessed if an absence of c-Rel and RelA affected the survival of thymocytes treated with dexamethasone or TNF, agents that normally induce p53-independent apoptosis (Fig. 1B). The extent and kinetics of apoptosis were equivalent for all thymocytes following exposure to dexamethasone (10 nmol/L). The death of *wt*, $c\text{-}rel^{-/-}$, $rela^{-/-}$, and and $c\text{-}rel^{-/-}rela^{-/-}$ thymocytes in response to TNF (10 ng/mL), although marginally higher than in untreated cells, was similar for all cells. The resistance of Rel/NF-κB–deficient thymocytes to TNF-induced death was not due to reduced TNF receptor expression, as *tnfr1* mRNA levels were equivalent in all thymocytes (Fig. 1C).

Rel/NF-κB Signaling Is Dispensable for p53-Dependent Cell Cycle Arrest and Apoptosis in Primary Fibroblasts

Doxorubicin and γ-irradiation induce p53-dependent cycle arrest in primary MEFs (1). To determine if c-Rel and RelA regulate this response, the proliferation (Fig. 2A; Table 1) and survival (Fig. 2B; Table 2) of early-passage *wt*, $c\text{-}rel^{-/-}$, $rela^{-/-}$, $c\text{-}rel^{-/-}rela^{-/-}$, and $p53^{-/-}$ primary MEFs was assessed following γ-irradiation (1,000 Rad) or doxorubicin (200 ng/mL) treatment. The proportion of irradiated (1,000 Rad) *wt* and Rel/NF-κB–deficient MEFs in S phase decreased from ~14% (untreated) to ~5%. More untreated $p53^{-/-}$ MEFs were in S phase (~25%) and this was unchanged following irradiation. Doxorubicin had a more pronounced effect on the cell cycle, with <1% of the viable *wt* or Rel/NF-κB–deficient cells and ~12% of $p53^{-/-}$ MEFs in S phase (Table 1). Consistent with genotoxic damage inhibiting kinases responsible for the G_2-M transition via p53-independent pathways (24), irradiated (Fig. 2A) or doxorubicin-treated (results not shown) $p53^{-/-}$ MEFs accumulated in G_2-M. The viability of untreated and treated MEFs of all genotypes was similar (between 70% and 80%; Fig. 2B; Table 2). These data show that c-Rel and RelA do not influence primary MEF survival or p53-dependent cell cycle arrest following DNA damage.

Despite these findings, it was unclear whether p53 modulates NF-κB activation following DNA damage. Whereas γ-irradiation or doxorubicin induction of the BH3-only proapoptotic gene *puma* in primary MEFs was p53 dependent (Fig. 2C), the induction of NF-κB activity in these cells following DNA damage, assessed by a κB-regulated luciferase reporter, does not require p53 (Fig. 2D).

Inactivating the Rel/NF-κB Pathway Does Not Influence the Response of Primary $p53^{-/-}$ *Fibroblasts or Thymocytes to Genotoxic Agents*

Our findings ruled out roles for c-Rel and RelA upstream (13) or downstream (17) of p53 during apoptosis or cell cycle arrest but could not exclude that the resistance of primary $p53^{-/-}$ cells was dependent on Rel/NF-κB. This possibility was examined by inhibiting Rel/NF-κB in $p53^{-/-}$ cells using a degradation-resistant form of human IκBα (thereafter termed IκBSR; ref. 25). Retroviral delivery of IκBSR (FLAG tagged) showed that it is expressed at similar levels in primary *wt* and $p53^{-/-}$ MEFs (Fig. 3A), with both IκBSR and FLAG-IκBSR effectively inhibiting Rel/NF-κB such that the viability of TNF-treated *wt* MEFs expressing either form of IκBSR is ~30% (Fig. 3B). This is a frequency of apoptosis similar to TNF-stimulated $rela^{-/-}$ and $c\text{-}rel^{-/-}rela^{-/-}$ MEFs (see below). The TNF-induced death of $p53^{-/-}$ MEFs expressing IκBSR was less pronounced (~55%). Following γ-irradiation (1,000 Rad) or doxorubicin treatment (200 ng/mL), the viability of *wt* or $p53^{-/-}$ MEFs expressing IκBSR was equivalent to control cells (Fig. 3B). The frequency of control or IκBSR-expressing *wt* MEFs in S phase decreased from 9% (medium only) to ~2% and <0.5% following γ-irradiation and doxorubicin treatment (Fig. 3C). A similar frequency (33-36%) of untreated control and IκBSR-expressing $p53^{-/-}$ MEFs was in S phase; this remained ~30% for both populations after γ-irradiation (Fig. 3C). The reduction of $p53^{-/-}$ cells in S phase (~22%) following doxorubicin treatment was comparable between control and IκBSR-expressing populations (Fig. 3C).

We also assessed whether the canonical NF-κB pathway promoted resistance of $p53^{-/-}$ thymocytes to apoptosis. Green

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fluorescent protein–positive (GFP^+) thymocytes were isolated by flow cytometry (Fig. 3D) from *rag-1*$^{-/-}$ mice engrafted with *wt* and *p53*$^{-/-}$ hemopoietic progenitors that had been infected with control (MY-GFP) or FLAG-IκBSR–expressing (MY-GFP/IκBSR) retroviruses. Western blotting confirmed that IκBSR was expressed at similar levels in *wt* and *p53*$^{-/-}$ thymocytes (results not shown). DNA-binding assays established that nuclear NF-κB complexes containing c-Rel, RelA, and NF-κB1 (complex C2; Fig. 3E) induced by phorbol 12-myristate 13-acetate (PMA) in thymocyte controls (*wt* and *p53*$^{-/-}$ cells expressing pMY-GFP) were inhibited in pMY-GFP/IκBSR–infected cells and the expression of *A1*, a PMA-inducible NF-κB target gene (26), was blocked in *wt* and *p53*$^{-/-}$ cells expressing IκBSR (Fig. 3F). Following γ-irradiation (500 Rad), *p53*$^{-/-}$ thymocytes expressing IκBSR displayed survival profiles equivalent to those of control *p53*$^{-/-}$ cells (Fig. 3G). Therefore, the resistance of *p53*$^{-/-}$ MEFs and thymocytes to γ-irradiation or doxorubicin-induced apoptosis and cell cycle arrest is independent of the canonical NF-κB pathway.

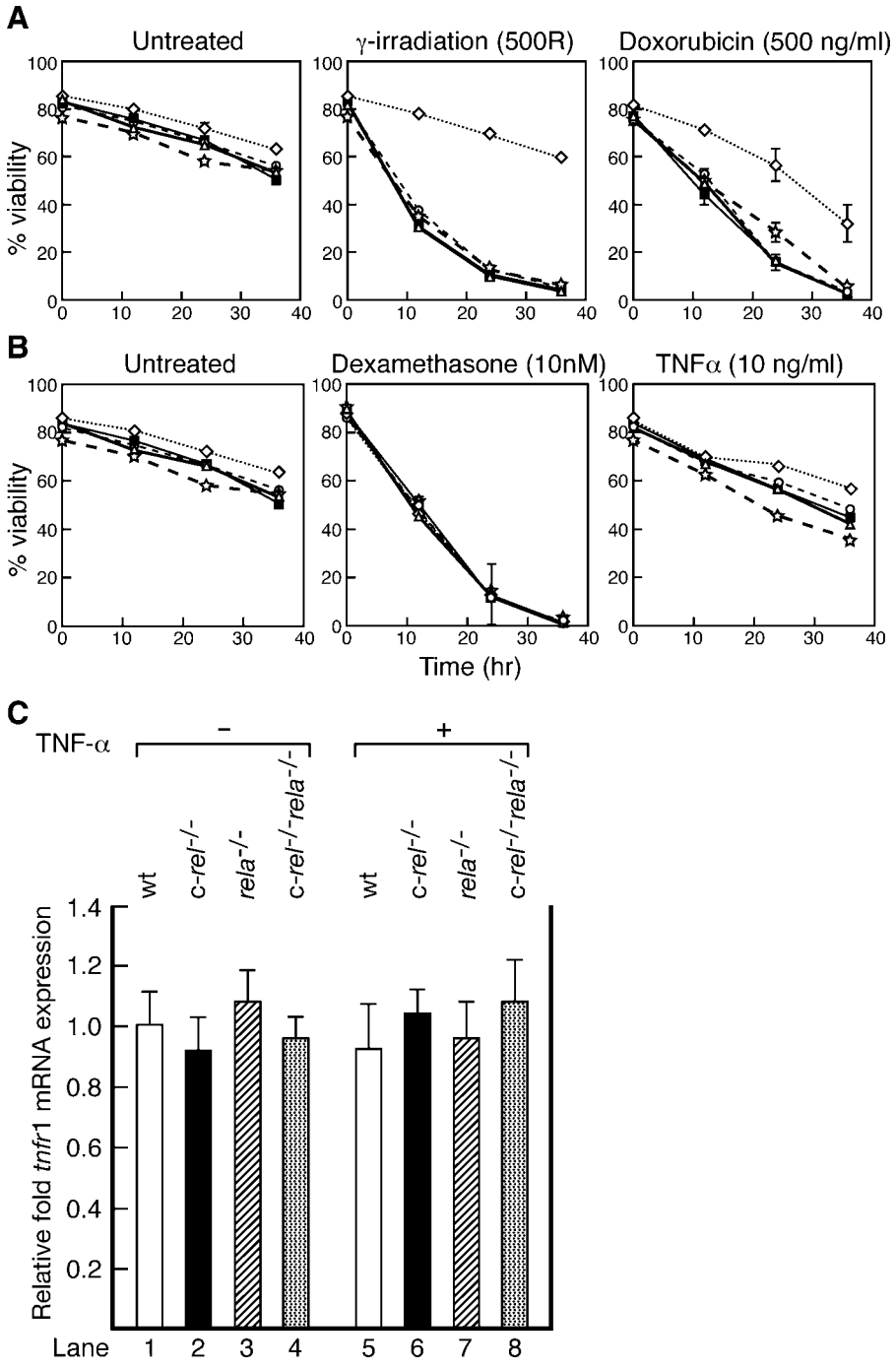

FIGURE 1. An absence of c-Rel and RelA does not alter thymocyte sensitivity to p53-dependent apoptosis. **A** and **B.** *wt* and NF-κB–deficient thymocyte apoptosis following p53-dependent and p53-independent death stimuli. *wt* (*closed square*), *c-rel*$^{-/-}$ (*open triangle*), *rela*$^{-/-}$ (*open circle*), *c-rel*$^{-/-}$*rela*$^{-/-}$ (*open star*), or *p53*$^{-/-}$ (*open square*) thymocytes were incubated in medium alone or treated with (**A**) p53-dependent apoptotic stimuli, γ-irradiation (500 Rad), and doxorubicin (500 ng/mL) or (**B**) p53-independent apoptotic stimuli dexamethasone (10 nmol/L) and TNFα (10 ng/mL). Cells were collected 12, 24, and 36 h later and stained with propidium iodide and/or Annexin V-FITC, and cell viability was determined by flow cytometry. Points, mean from separate experiments done on thymocytes of all genotypes isolated from multiple independently engrafted mice; bars, SD. **C.** *tnfr1* expression is normal in Rel/NF-κB–deficient thymocytes. *tnfr1* expression in *wt, c-rel*$^{-/-}$, *rela*$^{-/-}$, and *c-rel*$^{-/-}$*rela*$^{-/-}$ thymocytes before or following TNF stimulation (10 ng/mL) for 24 h is represented as relative to levels observed in *wt* cells. *tnfr1* levels in each RNA sample have been normalized relative to *gapdh* mRNA expression. Columns, mean of three independent experiments; bars, SD.

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p53-Dependent Genotoxic Agents Induce Apoptosis or Cell Cycle Arrest in Immortal Rel/NF-κB–Deficient MEFs

Despite failing to observe interactions between Rel/NF-κB and p53 in primary cells during DNA damage-induced apoptosis or cell cycle arrest, the relationship in transformed cells remained unclear. This was addressed using early-passage MEFs (*wt*, *rela*$^{-/-}$, *c-rel*$^{-/-}$*rela*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and *p53*$^{-/-}$) immortalized with retroviruses expressing adenovirus 5 E1A12S. In addition to initiating proliferation, E1A12S enhances MEF apoptosis via p19^{ARF}-dependent p53 stabilization following growth factor deprivation or treatment with DNA-damaging agents (27). MEFs infected with control

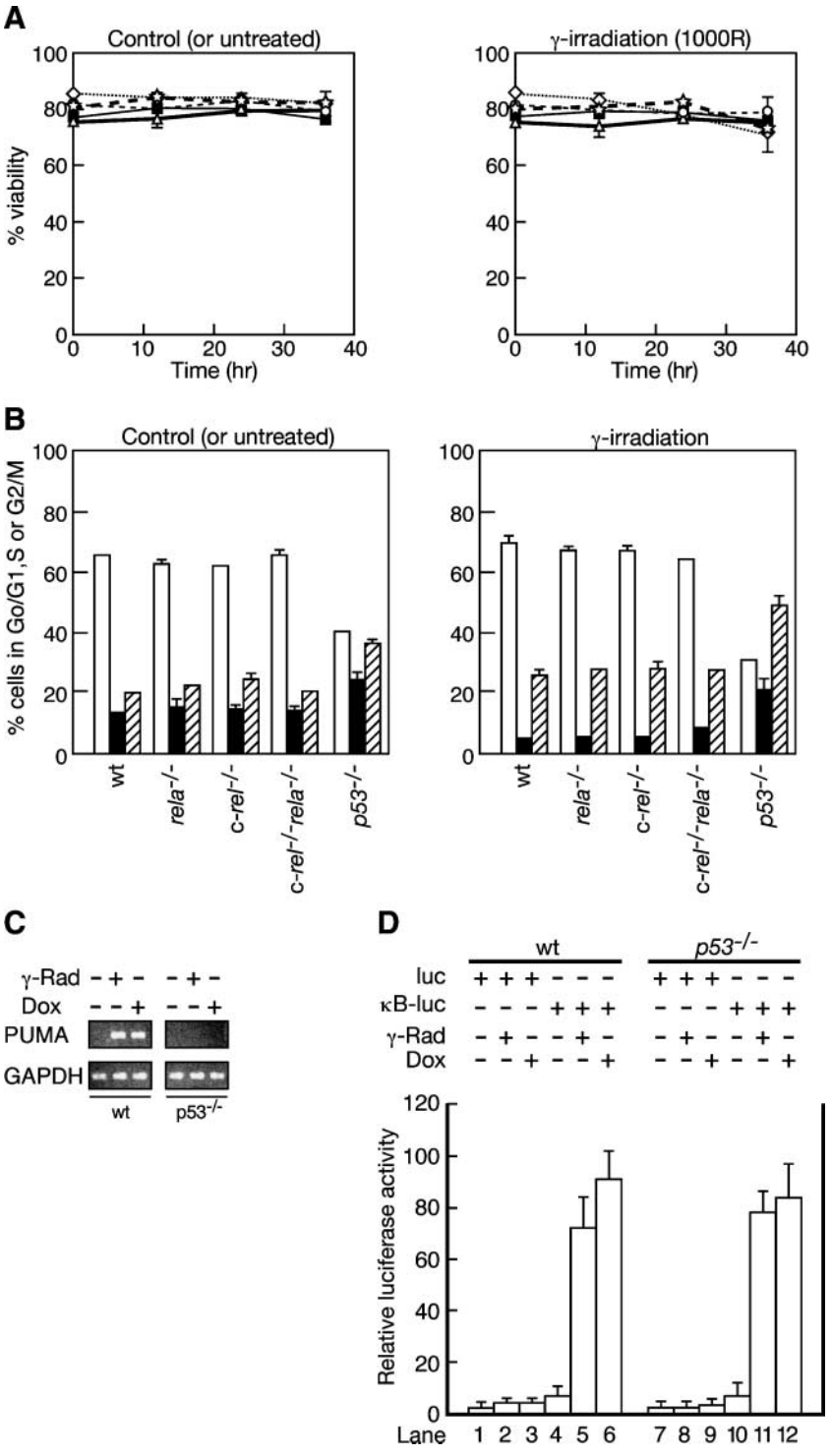

FIGURE 2. NF-κB–deficient primary MEFs undergo normal cell cycle arrest in response to ionizing radiation. Short-term passage mouse primary MEFs (*wt*, *c-rel*$^{-/-}$, *rela*$^{-/-}$, *c-rel*$^{-/-}$*rela*$^{-/-}$, or *p53*$^{-/-}$) were subjected to γ-irradiation (1,000 Rad). **A.** Viability of primary fibroblasts following γ-irradiation. Pooled adherent and nonadherent *wt* (*closed square*), *c-rel*$^{-/-}$ (*open triangle*), *rela*$^{-/-}$ (*open circle*), *c-rel*$^{-/-}$*rela*$^{-/-}$ (*open star*), or *p53*$^{-/-}$ (*open square*) cells collected before and 12, 24, and 36 h after irradiation were stained with propidium iodide and viability was determined by flow cytometry. **B.** Cell cycle in primary fibroblasts following γ-irradiation. Viable primary fibroblasts from untreated (medium alone) and irradiated (1,000 Rad) cultures were fixed after 24 h and stained with propidium iodide, and the percentage of cells in G_0-G_1 (*white columns*), S (*black columns*), or G_2-M (*cross-hatched columns*) phase was determined by flow cytometry. Columns, mean of MEFs isolated from three to five different embryos for each genotype derived from two independent experiments; bars, SD. **C.** *Puma* expression induced in primary MEFs by genotoxic stress. *puma* expression in untreated, doxorubicin-treated, or γ-irradiated (6 h after treatment) *wt* or *p53*$^{-/-}$ primary MEFs was assessed by semiquantitative real-time reverse transcription-PCR. **D.** NF-κB activity in primary fibroblasts induced by genotoxic stimuli. Following the transfection of *wt* and *p53*$^{-/-}$ primary MEFs with the reporter plasmids pluc or κB-luc, cells were either untreated or exposed to γ-irradiation (500 Rad) or doxorubicin (500 ng/mL), after which time luciferase assays were done. Promoter activity is displayed as relative luciferase units. Columns, mean luciferase activity from three sets of experiments; bars, SD.

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Table 1. NF-κB–Deficient Primary MEFs Remain Viable after Doxorubicin Treatment

Viability	*wt*	*c-rel*$^{-/-}$	*rela*$^{-/-}$	*nfkb1*$^{-/-}$*rela*$^{-/-}$	*c-rel*$^{-/-}$*rela*$^{-/-}$	*p53*$^{-/-}$
Medium	78.7 ± 1.3	78.4 ± 1.6	76.2 ± 0.9	75.8 ± 0.8	78.4 ± 1.7	79 ± 0.7
200 ng/mL doxorubicin	74.6 ± 3.2	72.8 ± 0	71.9 ± 1.5	74 ± 1.6	74.5 ± 0.6	76.8 ± 1.7

NOTE: *wt*, NF-κB–deficient, and p53-deficient MEFs were exposed to 200 ng/mL doxorubicin for 24 h. Floating and adherent cells were combined and stained with FITC-conjugated Annexin V, and the percentage of viable cells was determined by fluorescence-activated cell sorting. The values represent the mean ± SD of duplicates from two independent experiments.

puromycin-resistant (puro) retroviruses were identical in appearance to uninfected cells, whereas E1A12S-expressing MEFs of all genotypes acquired an altered morphology (Fig. 4A), grew to higher densities, and proliferated faster.[1] Western blotting confirmed that E1A12S levels were similar in all MEF populations (Fig. 4B). The survival of control and E1A12S-expressing MEFs was assessed following γ-irradiation (1000 Rad) or doxorubicin (200 ng/mL) treatment (Fig. 4C). Enhanced apoptosis was similar in *wt* and Rel/NF-κB–deficient MEFs expressing E1A12S, with viability decreasing from ~80% (untreated) to approximately 60% to 65% and approximately 10% to 20%, respectively, in response to γ-irradiation or doxorubicin. The viability of *p53*$^{-/-}$ control and E1A12S-expressing MEFs was comparable with that of untreated cells following either treatment. In response to TNF, primary *rela*$^{-/-}$ MEFs as expected (28) underwent increased TNF-induced apoptosis. The viability of *wt* MEFs expressing E1A12S following TNF treatment was unchanged (~80%), whereas the survival of the corresponding Rel/NF-κB–deficient MEFs was reduced to between 3% and 18%, confirming that E1A12S sensitizes cells lacking Rel/NF-κB to TNF-mediated apoptosis (29). Following serum deprivation, the viability of *wt* and Rel/NF-κB–deficient MEFs expressing E1A12S dropped to approximately 25% to 30%, whereas that of *p53*$^{-/-}$ MEFs in low serum was not influenced by E1A12S.

The influence of E1A12S on MEF proliferation is summarized in Fig. 4D. Fewer *wt* or RelA-deficient MEFs underwent DNA synthesis following serum deprivation, γ-irradiation, or doxorubicin treatment. Whereas TNF did not alter DNA synthesis in *wt* cells, 2- to 3-fold more RelA-deficient MEFs underwent DNA synthesis following TNF stimulation. For *p53*$^{-/-}$ MEFs, treatments leading to DNA damage had no effect on DNA synthesis, whereas that of TNF was modest, although serum deprivation halted the cell cycle progression of *p53*$^{-/-}$ cells (Fig. 4D). E1A12S induced G_2-M arrest in *wt* MEFs following irradiation (1,000 Rad) or doxorubicin treatment (200 ng/mL), but proliferation was not markedly impaired by serum deprivation or TNF (Fig. 4D). Unlike their primary cell counterparts, following TNF stimulation, DNA synthesis in RelA-deficient cells expressing E1A12S was similar to that of transformed *wt* cells. Finally, whereas constitutive and doxorubicin-induced or γ-irradiation–induced *puma* expression in E1A12S immortalized MEFs remained p53 dependent (Fig. 4E), the induction of NF-κB activity in these cells by these genotoxic agents is p53 independent (Fig. 4F).

p53 Expression in MEFs Induced by Genotoxic Damage or E1A12S Expression Is Not Influenced by the Canonical Rel/NF-κB Pathway

A role for Rel/NF-κB in E1A12S-enhanced p53 expression and activity (27) was determined by comparing p53 levels in *wt* and Rel/NF-κB–deficient MEFs expressing E1A12S following γ-irradiation, serum deprivation, doxorubicin, or TNF treatment. p53 induction was normal in *rela*$^{-/-}$, *c-rel*$^{-/-}$*rela*$^{-/-}$, and *nfkb1*$^{-/-}$*rela*$^{-/-}$ MEFs responding to γ-irradiation (Fig. 5A) or doxorubicin (results not shown). As expected, neither TNF nor serum deprivation induced p53 in primary MEFs (results not shown). p53 was detectable in all untreated MEFs expressing E1A12S and these levels did not change in response to any stimulus (Fig. 5B).

Our findings that Rel/NF-κB transcription factors did not influence doxorubicin-induced apoptosis differed from a report showing that this agent increased apoptosis in spontaneously immortalized *ikk1*$^{-/-}$*ikk2*$^{-/-}$ MEFs by enhancing the induction of p53 through reduced IKK2/RelA-dependent *mdm2* expression (16). Although our results showed that doxorubicin-induced p53 expression in primary and E1A12S immortalized MEFs was independent of c-Rel and RelA, we examined *mdm2* expression in doxorubicin-treated MEFs. Real-time PCR quantification of *mdm2* mRNA levels in *wt* and Rel/NF-κB mutant MEFs before and after doxorubicin treatment (Fig. 5C) showed that *mdm2* mRNA levels were comparable in cells of all genotypes. Collectively, our results show that neither DNA damage-induced p53 activation nor *mdm2* expression is dependent on RelA, c-Rel/RelA, or NF-κB1/RelA and that E1A12S induction of p53 is independent of Rel/NF-κB.

Discussion

A significant body of literature indicates that the p53 and NF-κB pathways can interact via a multitude of mechanisms in transformed cells following DNA damage. We chose to determine if this pathway cross-talk is a feature of normal cells or is restricted to immortal cells. Using primary mouse thymocytes, MEFs, and low-passage E1A12S immortalized MEFs bearing defined mutations in the p53 and NF-κB pathways, we found that p53 and NF-κB signaling in these cells functions independently when activated by genotoxic stress.

To accommodate the possibility that p53-regulated and NF-κB–regulated responses to DNA damage are dependent on the cell type or stimulus, thymocytes and fibroblasts were subjected to γ-irradiation or doxorubicin treatment. For thymocytes, p53-dependent apoptosis induced by γ-irradiation was unchanged in the absence of RelA and c-Rel. Inhibition of apoptosis in irradiated *p53*$^{-/-}$ thymocytes was also unaffected by blocking

[1] Unpublished results.

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Table 2. NF-κB–Deficient Primary MEFs Undergo Normal Cell Cycle Arrest in Response to Doxorubicin Treatment

BrdUrd	*wt*	*c-rel*$^{-/-}$	*rela*$^{-/-}$	*nfkb1*$^{-/-}$*rela*$^{-/-}$	*c-rel*$^{-/-}$*rela*$^{-/-}$	*p53*$^{-/-}$
Medium	17.8 ± 4	13.9 ± 0.4	14.1 ± 0.9	9.2 ± 0.5	14.7 ± 0.8	35.5 ± 1.5
200 ng/mL doxorubicin	0	0.1 ± 0	0	0.2 ± 0.2	0	11.8 ± 1.1

NOTE: *wt*, NF-κB–deficient, and p53-deficient MEFs were treated with 200 ng/mL doxorubicin for ~20 h. Cells were then pulsed with BrdUrd for 5 h, fixed, incubated with FITC-conjugated anti-BrdUrd antibody, and analyzed by fluorescence-activated cell sorting. The percent of BrdUrd-positive MEFs represents the percentage of cells in the S phase of the cell cycle. The values represent the mean ± SD of duplicates from two independent experiments.

NF-κB activation. p53-dependent cell cycle arrest in primary fibroblasts following γ-irradiation was unchanged by inhibiting NF-κB, as was the failure of *p53*$^{-/-}$ MEFs to undergo S-phase arrest. Similar findings were made in both cell types treated with doxorubicin. Consistent with p53-dependent responses to genotoxic stress functioning independently of NF-κB, the canonical pathway was also dispensable for the induction of p53 and *mdm2* expression. Conversely, NF-κB activation in MEFs after γ-irradiation or doxorubicin treatment was not influenced by p53. Further support for the independence of these pathways in primary cells during apoptosis comes from our findings on the survival of primary MEFs treated with TNF. Although our results are supported by a report showing that p53-regulated and NF-κB–regulated hepatocyte apoptosis is independent (18), this relationship may not apply to all cell types. Furthermore, while excluding an interdependence of c-Rel or RelA and p53 following genotoxic stress, we cannot dismiss such a role for RelB or NF-κB2, both transcriptional regulators of the alternate NF-κB pathway (8).

Results described here show that RelA differentially regulates survival in primary fibroblasts and thymocytes following TNF stimulation. *rela*$^{-/-}$ MEFs display increased sensitivity to TNF-induced apoptosis, whereas neither RelA nor c-Rel is necessary to protect thymocytes from this death stimulus. This finding differs from the increased TNF-dependent death reported for *ikkb*$^{-/-}$ thymocytes *in vivo* (30), a difference that may reflect a c-Rel/RelA–independent role for IKKβ in the survival of TNF-activated thymocytes.

Unlike *wt* MEFs, the frequency of RelA-deficient MEFs in S phase following TNF stimulation increases 2- to 3-fold. Although untreated *wt* and *rela*$^{-/-}$ MEFs have equivalent doubling times,[2] the TNF-induced increase of *rela*$^{-/-}$ MEFs in S phase may reflect RelA serving as a negative regulator of TNF-induced cell division as described for keratinocytes (31). However, we propose an alternate explanation. With ~2-fold more TNF-activated *rela*$^{-/-}$ MEFs in S phase coinciding with reduced viability (~80% of *wt* cells versus ~35-40% of *rela*$^{-/-}$ MEFs at T24), *rela*$^{-/-}$ MEFs in S phase may be more resistant to TNF-induced apoptosis than *rela*$^{-/-}$ cells at other stages of the cell cycle and as a consequence are enriched. This proposal predicts that in fibroblasts, RelA-mediated resistance to TNF-induced apoptosis is dependent on the phase of the cell cycle. Consistent with this model, TNF treatment of *rela*$^{-/-}$ MEFs, while resulting in proportionally more cells in S phase, also selectively reduced viable cells in G_0-G_1 (results not shown). This finding is supported by studies showing fibroblasts synchronized in G_0-G_1 by serum starvation, arrested in G_1 by an inducible Rb mutant or at the G_1-S boundary by aphidicolin, all undergo enhanced TNF-induced apoptosis (32, 33). Despite p53 not directly regulating TNF-induced cell death (34), *p53*$^{-/-}$ MEFs were less sensitive to TNF-induced apoptosis than *wt* cells when NF-κB activation was blocked. With more primary *p53*$^{-/-}$ MEFs in cycle, this result further supports a role for RelA in selectively promoting the resistance of primary fibroblasts in S phase to TNF-induced cell death. Although *rela*$^{-/-}$ MEFs immortalized by E1A12S divide at rates similar to those of *p53*$^{-/-}$ MEFs, the frequency of these cells in S phase following TNF stimulation did not increase despite being more sensitive than primary *rela*$^{-/-}$ MEFs to TNF-induced apoptosis. This indicates that E1A12S transformation renders cells at all stages of the cell cycle sensitive to TNF-induced apoptosis when NF-κB function is inhibited.

Our experiments using short-term passage E1A12S-transformed MEFs also failed to establish functional links between the NF-κB and p53 pathways. Following genotoxic stress, NF-κB did not modulate p53-dependent apoptosis or cell cycle arrest nor was constitutive p53 expression affected. Similarly, TNF-induced apoptosis resulting from NF-κB inhibition, although heightened by E1A12S expression, was not directly affected by the loss of p53. The difference between our findings using low-passage immortal polyclonal MEFs and the many published reports using different transformed cell lines may simply highlight how genetic changes that frequently occur in established cell lines modulate the regulation of the p53 and NF-κB pathways. Nevertheless, we acknowledge that our findings may not apply to every oncoprotein or all types of transformed cells. Given that many signaling networks interface with the NF-κB and p53 pathways, with hindsight it is not surprising that transforming events affecting an unrelated pathway could modulate cellular functions in a way such that the p53 and NF-κB pathways now seem to interact during apoptotic responses. Our findings and other published work together highlight the need to exercise caution in predicting how the regulation of p53 and NF-κB might be coordinated in cancers treated with DNA-damaging agents, particularly without knowledge of the underlying transforming events reshaping the global signaling network within the cell.

Materials and Methods

Mice

p53$^{-/-}$ and *c-rel*$^{-/-}$ mice (C57BL6 background) have been described previously (19, 35). The *rela*$^{+/-}$, *nfkb1*$^{-/-}$*rela*$^{+/-}$, and *rela*$^{+/-}$*c-rel*$^{-/-}$ parental strains used to generate *rela*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and *rela*$^{-/-}$*c-rel*$^{-/-}$ embryos were derived

[2] S. Gerondakis, unpublished results.

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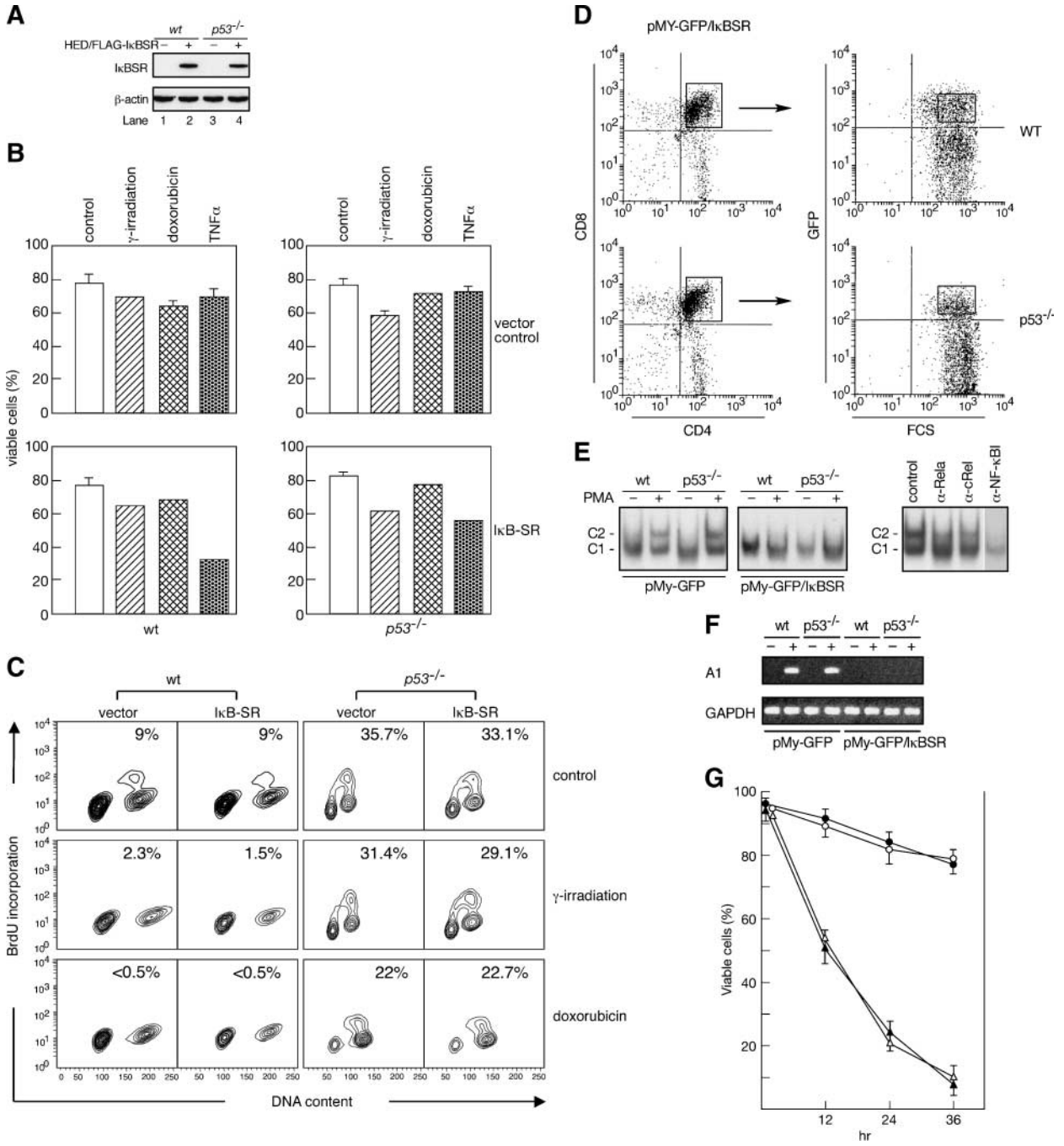

FIGURE 3. Blocking NF-κB activation in *wt* and *p53*$^{-/-}$ MEFs and thymocytes does not alter survival or cell cycle responses to genotoxic stimuli. **A.** The expression of FLAG-tagged human IκBSR in *wt* and *p53*$^{-/-}$ MEFs infected with control (puro only) or FLAG-IκBSR (puro/IκBSR) retroviruses was monitored by Western blotting. **B.** Response of *wt* and *p53*$^{-/-}$ MEFs expressing the IκBSR to ionizing radiation, doxorubicin, and TNF. Control and IκBSR-expressing *wt* and *p53*$^{-/-}$ MEFs were treated with TNFα (10 ng/mL) and doxorubicin (200 ng/mL) or γ-irradiated (1,000 Rad), and viability was determined 24 h later. Columns, mean of three independent experiments; bars, SD. **C.** DNA synthesis in *wt* and *p53*$^{-/-}$ MEFs expressing IκBSR following genotoxic stress. *wt* and *p53*$^{-/-}$ MEFs infected with control or IκBSR-expressing virus that were untreated or subjected to γ-irradiation or doxorubicin treatment were pulsed with BrdUrd, fixed, and stained with FITC-anti-BrdUrd antibody and propidium iodide to detect cells in S phase (*Y axis*) or in the G_0-G_1 and G_2-M phases (*X axis*), respectively. These data are representative of three independent experiments. **D.** Isolation of *wt* and *p53*$^{-/-}$ thymocytes expressing IκBSR. $CD4^+CD8^+$ thymocytes expressing high levels of GFP were isolated from mice engrafted with hemopoietic progenitors infected with MY-GFP/IκBSR virus. **E.** IκBSR inhibition of NF-κB activation in thymocytes. Electrophoretic mobility shift assay was done on nuclear extracts from untreated or PMA-activated (10 ng/mL) GFP^+ *wt* and *p53*$^{-/-}$ thymocytes ($Ly5.2^+$) isolated from $Ly5.1^+$*rag-1*$^{-/-}$ mice engrafted with hemopoietic progenitors infected with MY-GFP (control) or MY-GFP/IκBSR virus. The C1 complex comprises p50NF-κB1 homodimers and C2 represents PMA-inducible NF-κB heterodimers comprising p50NF-κB1, RelA, and c-Rel. These data are representative of three independent experiments, each done using nuclear extracts isolated from thymocytes derived from different mice. **F.** PMA induced *A1* expression in *wt* and *p53*$^{-/-}$ thymocytes. *A1* expression in untreated or PMA-activated (2 h) *wt* and *p53*$^{-/-}$ thymocytes transduced with pMY-GFP (control virus) or pMY-GFP/IκBSR was assessed by semiquantitative real-time reverse transcription-PCR. **G.** Inhibiting NF-κB activation does not alter the survival of γ-irradiated *wt* or *p53*$^{-/-}$ thymocytes. *wt* (*triangles*) and *p53*$^{-/-}$ (*circles*) thymocytes carrying the MY-GFP control (*open symbols*) or MY-GFP/IκBSR (*closed symbols*) virus were irradiated (500 Rad), and survival was monitored at 12-h intervals over 36 h. Points, mean of three independent experiments each done in triplicate; bars, SD.

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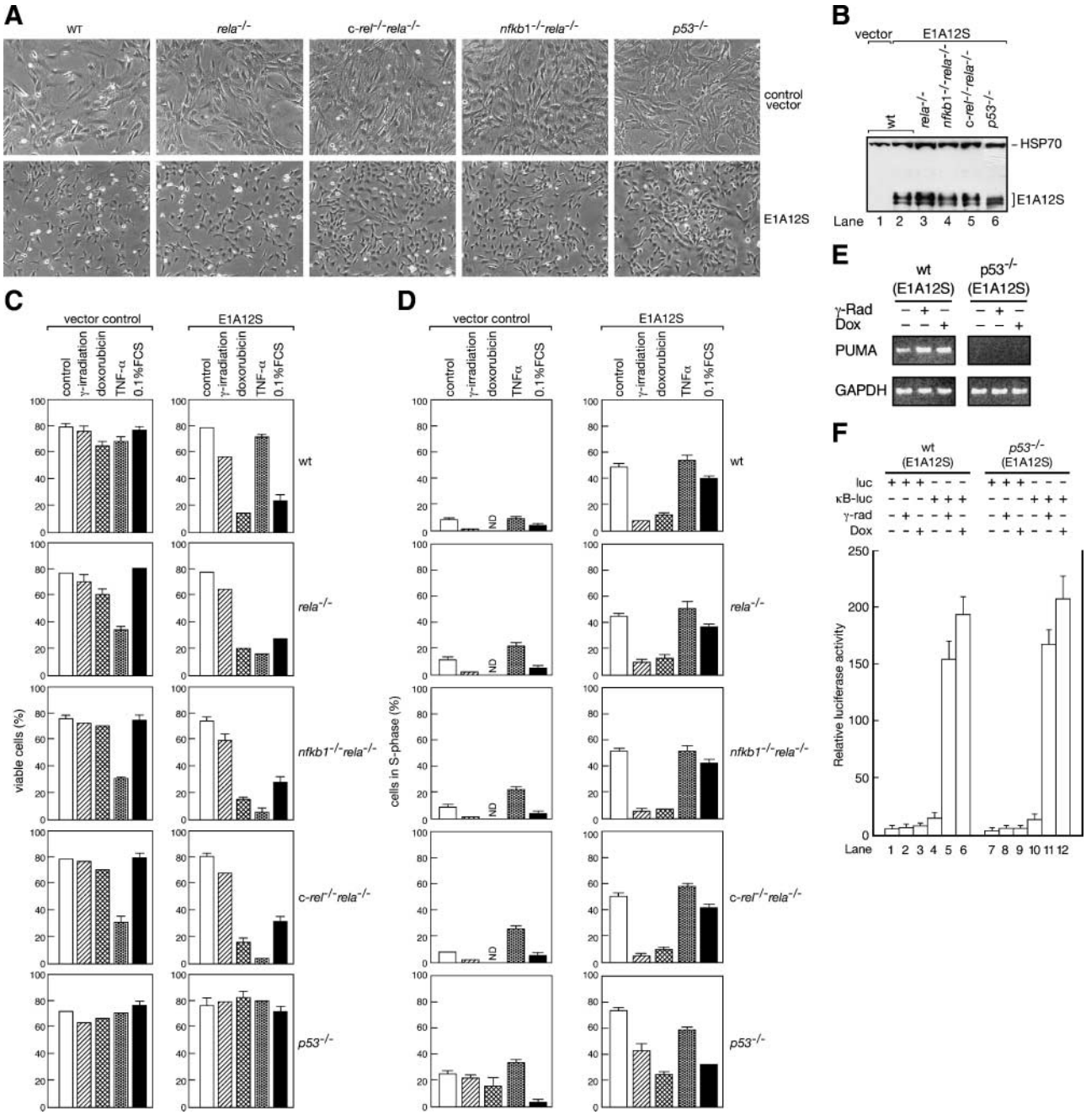

FIGURE 4. NF-κB–deficient MEFs expressing E1A12S remain sensitive to p53-dependent apoptotic stimuli. *wt, rela*$^{-/-}$, *c-rel*$^{-/-}$*rela*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and *p53*$^{-/-}$ MEFs infected with control (pHED) or E1A12S-expressing (pHEDE1A12S) retroviruses were enriched using puromycin selection. **A.** Morphology of MEFs infected with control and E1A12S-expressing retroviruses. **B.** Expression of E1A12S in virally transduced MEFs. Cell lysates isolated from *wt* cells infected with control virus (*lane 1*) or *wt* (*lane 2*), *rela*$^{-/-}$ (*lane 3*), *nfkb1*$^{-/-}$*rela*$^{-/-}$ (*lane 4*), *c-rel*$^{-/-}$*rela*$^{-/-}$ (*lane 5*), and *p53*$^{-/-}$ (*lane 6*) MEFs infected with pHEDE1A12S were subjected to Western blotting using antibodies for adenovirus 5 E1A and HSP70. These data are representative of multiple Westerns, including blots, in which cells of all genotypes were infected with control virus. **C.** Viability of MEFs expressing E1A12S following treatment with p53-dependent and p53-independent apoptotic stimuli. *wt, rela*$^{-/-}$, *c-rel*$^{-/-}$*rela*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and *p53*$^{-/-}$ MEFs infected with control or E1A12S-expressing retroviruses were untreated, serum deprived (0.1% FCS), γ-irradiated (1,000 Rad), or stimulated with doxorubicin (200 ng/mL) or TNF (10 ng/mL). Twenty-four hours later, adherent and nonadherent cells were pooled and cellular viability was determined. Columns, mean of three independent experiments; bars, SD. **D.** S-phase profiles of MEFs expressing E1A12S in response to p53-dependent and p53-independent apoptotic stimuli. Cell cycle profiles of control and E1A12S-expressing MEFs described in **C** were measured by monitoring BrdUrd incorporation and DNA content. The data are representative of three independent experiments done in duplicate. **E.** *Puma* expression in E1A12S immortalized *wt* or *p53*$^{-/-}$ MEFs. Following γ-irradiation or doxorubicin treatment (6 h), *puma* expression was determined by semiquantitative real-time reverse transcription-PCR. **F.** NF-κB activity in E1A12S immortalized MEFs induced by genotoxic stimuli. Luciferase assays were done on low-passage-number E1A12S immortalized *wt* and *p53*$^{-/-}$ MEFs transfected with pluc or κB-luc that were untreated or exposed to γ-irradiation (500 Rad) or doxorubicin (500 ng/mL). Promoter activity is displayed as relative luciferase units. Columns, mean luciferase activity of three sets of experiments; bars, SD.

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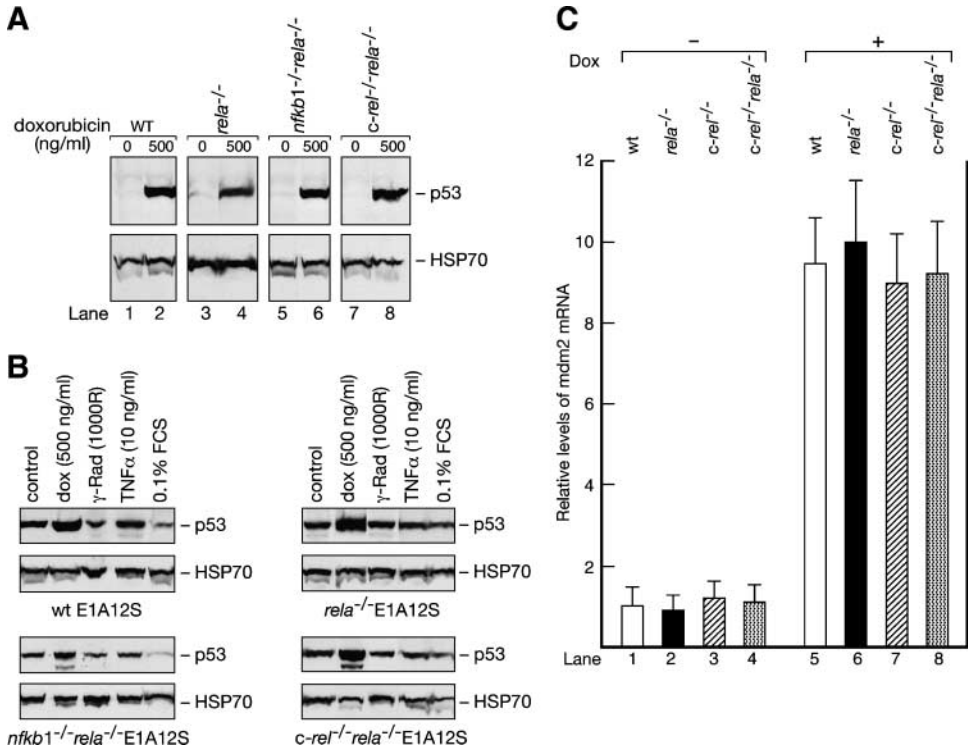

FIGURE 5. An absence of NF-κB function does not alter cellular levels of p53 induced by apoptotic stimuli. **A.** p53 expression in doxorubicin-treated primary MEFs. Whole-cell lysates from primary *wt* and NF-κB–deficient (*rela*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and *c-rel*$^{-/-}$*rela*$^{-/-}$) MEFs treated with doxorubicin (500 ng/mL) were subjected to Western blotting using anti-p53 and HSP70-specific antibodies. These data are representative of three independent experiments. **B.** p53 expression in E1A12S immortalized MEFs. Low-passage-number *wt* and NF-κB–deficient (*rela*$^{-/-}$, *nfkb1*$^{-/-}$*rela*$^{-/-}$, and *c-rel*$^{-/-}$*rela*$^{-/-}$) MEFs immortalized with E1A12S were untreated, γ-irradiated (1,000 Rad), serum deprived (0.1% FCS), or treated with doxorubicin (200 and 500 ng/mL) or TNF (10 ng/mL) and, 24 h later, examined for p53 expression as described in **A**. The data are representative of three independent experiments. **C.** Doxorubicin induction of *mdm2* in primary MEFs lacking NF-κB function. *mdm2* expression in *wt*, *c-rel*$^{-/-}$, *rela*$^{-/-}$, and *c-rel*$^{-/-}$*rela*$^{-/-}$ MEFs before or following doxorubicin treatment (500 ng/mL) for 24 h is represented as relative to levels observed in untreated *wt* cells. *mdm2* levels in each RNA sample have been normalized relative to *gapdh* mRNA expression. Columns, mean of three independent experiments; bars, SD.

from mice backcrossed onto C57BL6 (n = 9-10 backcross generations) and have been described elsewhere (35). wt and knockout alleles for *rela, nfkb1, c-rel*, and *p53* were distinguished by PCR of DNA samples from tail biopsy or embryos. All animal experimentation described in this study has been approved by the Melbourne Health Research Directorate in accordance with the guidelines for animal experimentation outlined by the National Health and Medical Research Council of Australia.

Preparation of MEFs and Thymocytes

Mouse embryonic cell suspensions generated from E12 or E13 embryos were placed into DMEM (Life Technologies) supplemented with 4.5 g/L glucose, 13 μmol/L folic acid, 1.25 μmol/L L-asparagine, 50 μmol/L β-mercaptoethanol, and 10% fetal bovine serum, and 24 h later, nonadherent cells were removed and adherent cells were trypsinized and then replated and grown for an additional four doublings before freezing. Thymocytes of all genotypes (*wt, rela*$^{-/-}$, *c-rel*$^{-/-}$, *c-rel*$^{-/-}$*rela*$^{-/-}$, and *p53*$^{-/-}$) were generated by engrafting sublethally irradiated (300 Rad) *rag-1*$^{-/-}$ mice with E12 fetal liver hemopoietic progenitors (1×10^6 to 4×10^6) as described (35). Flow cytometry was used to isolate $CD4^+CD8^+$ thymocytes expressing high levels of GFP from mice engrafted with hemopoietic progenitors infected with pMY-GFP or pMY-GFP/FLAG-IκBSR.

Cell Stimulations

Fibroblasts and thymocytes were subjected to γ-irradiation (100, 250, 500, or 1,000 Rad) or treated with TNF (10 ng/mL; Sigma-Aldrich), doxorubicin (200 and 500 ng/mL; Sigma-Aldrich), or dexamethasone (10 nmol/L; Sigma-Aldrich).

Western Blotting

Expression of IκBαSR and E1A12S was monitored by Western blotting using monoclonal anti-FLAG and anti-adenovirus type 5 E1A antibodies (M58; PharMingen), respectively. Levels of p53 protein were detected using a monoclonal anti-p53 antibody (Mab122).

Retrovirus Production and Infections

Viral stocks used for MEF infections were generated by transfecting Phoenix cells (2.5×10^6) seeded into 6-cm dishes with 20 μg of retroviral plasmid using calcium phosphate precipitation in the presence of 25 μmol/L chloroquine. Ten hours after transfection, cells were placed in fresh medium, and 2 d later, supernatants were collected, filtered (0.45-μm filter; Millipore), snap frozen, and stored at −70°C. MEFs to be

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infected were plated at a density of 1.5×10^6 cells in six-well plates 24 h before infection. Viral supernatants supplemented with 4 μg/mL polybrene were added to MEFs and the plates were centrifuged for 1 h at 2,000 rpm at 30°C. Two days later, MEFs were placed in puromycin (3 μg/mL) containing medium for 48 h and then either used immediately for experimentation or frozen and stored in liquid nitrogen. Helper-free virus stocks of MY-EGFP and MY-EGFP/FLAG-IκBSR were generated by transient transfection of BOSC23 cells. Approximately 10% of cultured fetal liver hemopoietic cells were routinely GFP^+ following two rounds of infection. These infected hemopoietic cells (5×10^5 per mouse) were engrafted into sublethally irradiated (300 Rad) *rag-1*$^{-/-}$ mice.

Cell Viability Assays

To assess thymocyte survival, cells obtained from the thymus of *rag-1*$^{-/-}$ mice reconstituted 6 to 8 wk earlier were cultured at a starting concentration of 1×10^5 in DMEM supplemented with 4.5 g/L glucose, 13 μmol/L folic acid, 250 μmol/L L-asparagine, 50 μmol/L β-mercaptoethanol, and 10% fetal bovine serum. At various times following γ-irradiation, doxorubicin, dexamethasone, or TNFα treatment, cell viability was determined using flow cytometric analysis of cells stained with propidium iodide or FITC-conjugated Annexin V. MEF viability determined by using propidium iodide uptake essentially as described for thymocytes involved pooling detached and adherent MEFs collected by trypsinization at various times following treatment.

Cell Cycle Analysis

MEFs were plated at a density of 0.7×10^6 cells per 6-cm dish and subjected to various treatments 24 h later. Twenty-four hours after treatment, cells were pulsed with 10 μmol/L bromodeoxyuridine (BrdUrd; Sigma) for 4 h, harvested, and fixed in 90% cold ethanol. DNA synthesis and DNA content were analyzed using flow cytometry (35). On fixation, cells were treated with 2 N HCl and 0.5% Triton X-100 for 30 min at room temperature. After one wash with 0.1 mol/L $Na_2B_4O_7$, cells were incubated with a FITC-conjugated anti-BrdUrd antibody (Becton Dickinson) in 0.1 mL PBS/2% FCS/0.5% Tween 20 for 30 min at room temperature. Cells were then washed once with PBS/2% FCS and resuspended in 0.1 mL of PBS/2% FCS containing 10 μg of propidium iodide. DNA synthesis was assessed by flow cytometry as the amount of BrdUrd incorporated by 10,000 to 20,000 viable cells using CellQuest software. The percentage of cells in G_0-G_1, S, and G_2-M phase was determined using propidium iodide staining and the ModFit program (Verity Software House, Inc.).

DNA Constructs

The 12S form of E1A generated by PCR from the plasmid CMVE1A12S (36) was cloned into pHEDME2SV (37). pHEDME2SV/IκBSR and pHEDME2SV/FLAG-IκBSR comprise the coding region for human IκBα (with or without an NH_2-terminal FLAG tag), with alanine substituted for serine at positions 32 and 36 (25), cloned into pHEDME2SV. The retroviral vector pMY-GFP/IκBSR was generated by inserting the cDNA for human IκBαSR that had an NH_2-terminal FLAG tag into pMY-EGFP (35). The plasmids pluc and κB-luc have been described previously (38).

Luciferase Assays

Early-passage primary MEFs or E1A12S immortalized MEFs seeded in 10-cm dishes at a density of 2×10^6 cells were transiently transfected with equivalent amounts of DNA using Superfectamine (Promega), and 24 h later, cells were either left untreated or exposed to γ-irradiation (500 Rad) or doxorubicin (500 ng/mL) for a further 12 h, after which time luciferase assays were done using the dual-luciferase reporter system (Promega). All results represent luciferase assays normalized against a control *Renilla* luciferase reporter, pRL-TK (Promega). The amounts of plasmid used were as follows: pRL-TK, 1 μg; luc, 5 μg; and κB-luc, 5 μg.

Electrophoretic Mobility Shift Assays

A [^{32}P]dATP end-labeled probe corresponding to the mouse *c-rel* κB3 site (5′-GCGGGAAATCCCCC-3′) was incubated with 1 μg of nuclear extract prepared from thymocytes infected with pMY-GFP or pMY-GFP/IκBSR that were either unstimulated or activated for 60 min with PMA (10 ng/mL). The composition of the DNA-binding complexes was determined by incubating nuclear extracts with antibodies specific for NF-κB1, c-Rel, or RelA for 20 min before adding the radiolabeled DNA. All samples were fractionated on 5% nondenaturing polyacrylamide gels, which were then dried and exposed to autoradiography at −70°C.

Real-time PCR

Total RNA was isolated from the following: thymocytes (*wt, c-rel*$^{-/-}$*, rela*$^{-/-}$, or *c-rel*$^{-/-}$*rela*$^{-/-}$) that were untreated or stimulated with 10 ng/mL TNF for 12 h; primary MEFs (*wt, c-rel*$^{-/-}$*, rela*$^{-/-}$, and *c-rel*$^{-/-}$*rela*$^{-/-}$) that were unstimulated or activated for 24 h with 500 ng/mL doxorubicin; *wt* and *p53*$^{-/-}$ primary MEFs subjected to doxorubicin or γ-irradiation; untreated, irradiated, or doxorubicin-activated *wt* and *p53*$^{-/-}$ MEFs immortalized with E1A12S; and *wt* and *p53*$^{-/-}$ thymocytes transduced with pMY-GFP or pMY-GFP/IκBSR retroviruses that were untreated or activated with PMA (10 ng/mL). Endogenous *tnfr1* and *mdm2* mRNA expression was measured using SYBR Green real-time PCRs as described (35). *tnfr1* and *mdm2* mRNA levels were normalized by taking an aliquot of each sample and doing quantitative PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression to compensate for input RNA variation and inefficiencies in cDNA synthesis. Semiquantitative PCR done as described previously (39) was used to detect *A1* and *puma* mRNA expression. Primer sets used for real-time PCR were *tnfr1* (forward, 5′-TGAGTGAGACACACTTCCAG-3′; reverse, 5′-AACAGCACCGCAGTACCTGA-3′; ref. 40), *mdm2* (forward, 5′-CCAACATGTCTGTGTCTACC-3′; reverse, 5′-CTTGCTGACTTACAGCCACT-3′; ref. 41), *A1* (forward, 5′-CAAATCTGGCTGGCTGACTTTTC-3′; reverse, 5′-CAAGTGCTGATAACCATTCTCGTC-3′; ref. 42), *puma* (forward, 5′-ATGGCCCGCGCACGCCAGG-3′; reverse, 5′-CCGCCGCTCGTACTGCGCGTTG-3′; ref. 43), and *gapdh* (forward, 5′-TTCACCACCATGGAGAAGGC-3′; reverse, 5′-GGCATGGACTGTGGTCATGA-3′; ref. 35).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Nuclear Factor-κB and p53 Pathways Function Independently in Primary Cells and Transformed Fibroblasts Responding to Genotoxic Damage

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