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The canonical NF- κ B pathway differentially protects normal and human tumor cells from ROS-induced DNA damage

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Abstract

DNA damage responses (DDR) invoke senescence or apoptosis depending on stimulus intensity and the degree of activation of the p53-p21^{Cip1/Waf1} axis; but the functional impact of NF- κ B signaling on these different outcomes in normal vs. human cancer cells remains poorly understood. We investigated the NF- κ B-dependent effects and mechanism underlying reactive oxygen species (ROS)-mediated DDR outcomes of normal human lung fibroblasts (HDFs) and A549 human lung cancer epithelial cells. To activate DDR, ROS accumulation was induced by different doses of H₂O₂. The effect of ROS induction caused the G2-M phase cell cycle arrest of both human cell types. However, ROS-mediated DDR eventually culminated in different end points with HDFs undergoing premature senescence and A549 cancer cells succumbing to apoptosis. NF- κ B p65/RelA nuclear translocation and Ser536 phosphorylation were induced in response to H₂O₂-mediated ROS accumulation. Importantly, blocking the activities of canonical NF- κ B subunits with an I κ B α super-repressor or suppressing canonical NF- κ B signaling by IKK β knock-down accelerated HDF premature senescence by up-regulating the p53-p21^{Cip1/Waf1} axis; but inhibiting the canonical NF- κ B pathway exacerbated H₂O₂-induced A549 cell apoptosis. HDF premature ageing occurred in conjunction with γ -H2AX chromatin deposition, senescence-associated heterochromatic foci and beta-galactosidase staining. P53 knock-down abrogated H₂O₂-induced premature senescence of vector control- and I κ B α SR-expressing HDFs functionally linking canonical NF- κ B-dependent control of p53 levels to ROS-induced HDF senescence. We conclude that IKK β -driven canonical NF- κ B signaling has different functional roles for the outcome of ROS responses in the contexts of normal vs. human tumor cells by respectively protecting them against DDR-dependent premature senescence and apoptosis.

Keywords

NF- κ B signaling; hydrogen peroxide; DNA damage response; senescence; human cells

1. Introduction

Oxidative DNA damage results from the accumulation of reactive oxygen species (ROS) due to an imbalance between ROS production and removal by antioxidant systems. ROS cause a variety of genetic damage, mostly base lesions and DNA single strand breaks (SSBs) but also double stranded DNA breaks (DSBs). In effect, replication can stall due to DNA damage resulting from ROS in the cell, and failure to stabilize stalled replication forks can result in their collapse and ultimately genetic instability [1-2]. Oxidative damage can provoke cell cycle arrest and senescence, but it can also induce apoptosis with normal cells being more resistant to apoptosis than tumor cells [1-3].

Normal human diploid fibroblasts (HDFs) undergo a finite number of cell divisions in culture, a phenomenon termed cellular or replicative senescence. *In vitro* cell propagation causes telomere shortening, recognized as DSBs that activates a DNA damage checkpoint response (DDR), culminating in replicative senescence. In addition, human cells undergoing senescence and ageing mice accumulate DNA lesions with irreparable DSBs outside telomeres, suggesting that their accumulation may have a causal role in mammalian ageing [4-6]. DDR involves activation of the kinases ATM and Chk2 and their downstream effector p53 and its target p21^{Cip1/Waf1} [7-8]. Normal HDFs can also undergo senescence in response to oxidative stress, referred to as stress-induced premature senescence (SIPS) [8-9]. Hydrogen peroxide (H₂O₂) produces thymidine glycols that can lead to DSBs at replication forks [1] and to the appearance of DNA damage foci in HDFs [4, 9-10]. ROS accumulation was shown to accelerate HDF senescence by DDR in conjunction with the induction and stabilization of p53 and p21^{Cip1/Waf1} [11-16]. However, dependent on cell type and dosage,

H₂O₂-mediated ROS accumulation has also been reported to induce the death of normal and tumor cells [1, 3, 17-18].

NF- κ B transcription factors are critical regulators of most if not all pro-inflammatory/stress-like responses. NF- κ Bs bind to DNA as dimers of five possible subunits (RelA/p65, c-Rel, RelB, p50, p52). Archetypical p65/p50 heterodimers are cytoplasmically restrained by I κ Bs (Inhibitors of NF- κ Bs) in most cells. Canonical NF- κ B activation generally requires the phosphorylation of serines 32 and 36 in I κ B α 's signal response domain (SRD), causing its ubiquitination and subsequent proteasomal destruction, allowing p65/p50 dimers to translocate to the nucleus and activate their target genes. I κ B α SRD phosphorylation requires the IKK signalosome complex (IKK α and IKK β serine-threonine kinases, and NEMO/IKK γ , a regulatory/adaptor protein). IKK β activation by phosphorylation of its T-activating loop serines 177/181 requires NEMO and rapidly occurs in response to a host of pro-inflammatory/stress-related extracellular signals. In contrast to IKK β , IKK α activation, by phosphorylation of T-loop serines 176/180, is NEMO-independent, requires de novo protein synthesis and is mainly mediated by stimuli invoked in adaptive immune responses. *In vivo*, unlike IKK β , IKK α does not generally target I κ Bs but instead phosphorylates an I κ B-like signal SRD in NF- κ B p100, which induces its ubiquitination/proteasome-dependent processing yielding the NF- κ B p52 subunit. Thus, NF- κ B p100 functions akin to I κ B α by sequestering NF- κ B RelB in the cytoplasm and also yields NF- κ B p52, which translocates to the nucleus together with RelB, to activate other unique NF- κ B target genes. [19-20]. Some p100 also sequesters a subset of p65/p50 heterodimers, whose activation can only be initiated by IKK α [21]. IKK catalytic activity also regulates other cellular responses independent of NF- κ B activation. For example, IKK β controls anti-apoptotic, pro-inflammatory and proliferative responses by targeting p53, FoxO and other cell cycle effectors [19-20, 22].

NF- κ B is activated by moderate ROS levels in a cell-type specific manner by different mechanisms, including the classical IKK β -dependent pathway [23-24]. In addition, genotoxic stimuli producing DSBs cause ATM/NEMO-dependent IKK β -mediated canonical NF- κ B activation, which facilitates cell survival and cycle arrest thereby giving cells precious time to repair their damaged DNA before p53-dependent apoptotic pathways are invoked [25-26]. A role for IKK α in DDR invoked by oxidative stress or chemotherapeutics has also been suggested [27-28]. Collectively, these studies provided evidence that the induction of NF- κ B signaling by DNA damage is important for cell survival. However, to our knowledge little if any evidence is available on how canonical NF- κ B activity functionally contributes to ROS-induced HDF senescence responses.

To address NF- κ B's functional roles in ROS-mediated DDR in the context of normal vs. human tumor cells, we inhibited canonical NF- κ B activation in MRC-5 and IMR-90 normal HDFs and suppressed or activated NF- κ B signaling in A549 human lung tumor epithelial cells. Inhibition of canonical NF- κ B in both cell types exacerbated ROS initiated DDR by up-regulating the p53-p21^{Cip1} axis, leading to the enhanced accumulation of cells at the G2 or G2-M checkpoint. Importantly, the final physiological outcomes of ROS-induced DDR greatly differed in normal vs. human cancer cells with the former normal human diploid fibroblasts (HDFs) succumbing to premature senescence and the latter human lung cancer epithelial cells undergoing exacerbated apoptosis.

2. Materials and Methods

2.1. Cell culture and retroviral transduction

Human embryonic diploid fibroblasts (HDFs) MRC-5, originally from the National Institute for Biological standards and Controls, South Mimms, UK [29], were kept at the cell stock of

the Genetics Division, National Institute for Medical Research, London UK, and obtained from Dr. Lily Huschtscha-Holliday and used in several previous studies by our group [29-31]. IMR-90 HDFs and human epithelial lung tumor cells A549 were originally obtained from ATCC and phoenix retroviral packaging cells were a kind gift from Dr. Garry Nolan, Stanford University. All the different cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Seromed/Biochrom KG, Germany) as previously described [31]. Moloney retroviral vectors expressing a trans-dominant IκBα (S32A/S36A) super repressor (IκBα.SR) [32] and an IKKβ-targeted shRNA (IKKβ4) have been previously described [32-33]. pRetro.Super-Puro and pRS-shp53 with puromycin resistance genes have been described [34]. Young MRC-5 and IMR-90 HDFs at population doubling P23 and P14, respectively, and A549 cells were infected with high-titer amphotropic retroviruses and stably transduced cells were selected in media supplemented with either geneticin or puromycin.

To determine the relative lifespans of WT control and canonical NF-κB compromised HDFs confluent monolayers were subcultured at 1:2 or 1:4 split ratios every 2 to 4 days. To maintain equivalence of population doublings (PDLs) with each passage, a 1:2 split ratio was scored as 1 PDL (e.g., 1 passage), and a 1:4 split ratio as 2 PDLs (e.g., 2 passages) as previously described [30-32, 55]. Lifespans in Table 1 are expressed as the averages ($\pm$ standard deviation; SD) of data from two independent experiments.

2.2. Hydrogen peroxide treatment of the cells

For H₂O₂ treatments A549 and HDFs were respectively seeded at 2×10^6 and 5×10^5 cells per 10 cm dish in complete DMEM growth medium and incubated for 24 h at 37°C, 5% CO₂. Cells were treated with hydrogen peroxide (H₂O₂) (Fluka) diluted in fresh complete growth medium at 50, 100, 200, 500 and 1000 µM for 2 h at 37°C, 5% CO₂. Cells were subsequently washed twice in serum-free DMEM and incubated with fresh complete DMEM growth medium for another 24 h before harvesting cells for further analysis.

2.3. Intracellular reactive oxygen species (ROS) measurements

To quantify ROS accumulation in response to H₂O₂, adherent MRC-5 and A549 cells were pre-treated with 5 µM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (C2938, Molecular Probes, dissolved in DMSO) for 30 min at 37°C, 5% CO₂ before H₂O₂ addition. ROS levels were measured immediately after 2 h of H₂O₂ exposure in the presence or absence of 2.5 mM N-acetyl-cysteine (NAC), and 24 h-post-treatment. Cells were washed with PBS, trypsinized, resuspended in 1 ml PBS plus 0.01% albumin, 10 mM Hepes pH 7.9 and submitted to FACSCalibur analysis for green fluorescence, as an index of intracellular ROS accumulation, at excitation and emission settings of 488 and 525-550 nm (FL1), respectively.

2.4. FACS analysis, Growth rates and Clonogenic assays

Uninfected and retrovirus-infected MRC-5 and A549 cells were plated in complete DMEM growth medium and incubated for 24 h at 37°C, 5% CO₂. Cells were treated with 0 - 1000 µM H₂O₂ as described above. Twenty four hours after H₂O₂ exposure cells were trypsinized, collected by centrifugation, washed twice in ice-cold PBS and fixed in ice-cold 75% ethanol. Cells were processed using a cycle testTM plus DNA reagent kit (Becton Dickinson) according to the manufacturer's instructions and analyzed on a FACScan Becton Dickinson flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Cell growth rates were determined as previously described [31]. Briefly 2×10^4 cells were seeded in triplicate in 24-multiwell plates in complete DMEM growth medium and

incubated at 37°C, 5% CO₂. Cell growth was monitored by counting the cells from two wells every two days using a hemocytometer over a period of 12 days. Experiments were repeated multiple times in triplicate, and growth curves were constructed.

A549 cells were plated into 6-well plates (2000 or 5000 cells per well) in duplicate and incubated overnight at 37°C, 5% CO₂. Cells were treated with 0 - 1000 μ M H₂O₂ for 2 h, rinsed in serum-free medium and then incubated at 37°C in H₂O₂-free complete DMEM for 14 days. Colonies were fixed with methanol:acetic acid (3:1) and stained with 0.5 mg/ml crystal violet. Survival was calculated as a percentage, using the equation $N_T/N_U \times 100$, where N_T is the number of colonies on H₂O₂-treated plates and N_U is the number on untreated plates.

2.5. Quantification of apoptosis with annexin V/PI staining

Simultaneous flow cytometric quantification of apoptotic and viable cells was performed with an annexin V/propidium iodide kit (Annexin V-FITC/PI) (Assay Designs, Ann Arbor, Michigan, USA). A549 cells with or without exposure to up to 1000 μ M H₂O₂ were harvested, resuspended in binding buffer, incubated with annexin-FITC and propidium iodide and analyzed by flow cytometry (CyFlow ML, Partec, Germany).

2.6. Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts were prepared essentially as described [35]. Briefly, cells were scraped from dishes in PBS, pelleted, washed in hypotonic buffer (10 mM HEPES buffer, pH 7.9, 1.5 mM MgCl₂, 5 mM KCl, 1 mM PMSF, 1 mM dithiothreitol, 1 mM Na₃VO₄, 1 mM NaF), and lysed by resuspension in the same buffer with 0.1% Nonidet P-40. Cytoplasmic extracts were isolated by centrifugation at 10,000 rpm for 10 min. Nuclear pellets were washed in hypotonic buffer and resuspended in cold extraction buffer (20 mM HEPES, 25% glycerol, 450 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF), gently agitated at 4°C for 45 min, and spun at 13,000 rpm for 30 min at 4°C. Supernatants were collected, and protein concentrations determined with Roti-Quant reagent (Roth, Germany).

2.7. Isolation of total protein extracts and immunoblot analysis

Total cellular proteins were prepared and 40-60 μ g samples were submitted to SDS-PAGE followed by immunoblotting as described [31] with the following antibodies: goat polyclonal anti-NF- κ B p50 (sc-1190), IKK β (sc-7330), I κ B α (sc-371G), rabbit anti-NF- κ B p65 (sc-372), rabbit anti-phospho-p53 (Ser20) (sc-18079R), rabbit anti-phospho-p53 (Ser15) (Cell signaling #9284), rabbit anti-Chk2 (sc-9064), rabbit anti-phospho-Chk2 (T68) (#2661, Cell Signaling), rabbit anti- γ -H2AX (#07-164, Upstate Biotechnology), anti-lamin B (clone 16) (provided by Dr. S. Georgatos, University of Ioannina Medical School, Greece), rabbit monoclonal to phospho-p65 (Ser536) (#3033, Cell Signaling) and mouse monoclonal antibodies to phospho-ATM (Ser1981) (Cell signaling #4526), γ -H2AX (Millipore #05-636), p53 (DO-1, sc-126), p21^{Cip1/Waf1} (sc-817; sc-6246; Ab-1 OP64 Calbiochem), PARP-1 (sc-8007), caspase-3 (sc-7272) and β -actin (CloneAC15, A5441; Sigma). Signals were revealed using horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) and developed with an ECL detection kit (GE HealthCare).

2.8. Indirect immunofluorescence and confocal laser scanning microscopy

A549 cells were plated onto cover slips in complete growth medium and allowed to attach overnight after which cells were treated with 200 μ M H₂O₂ for 2 h and allowed to recover for another 24 h. Next cells were washed in ice-cold PBS containing Ca²⁺ and Mg²⁺ (referred to as PBS, thereafter), fixed in 4% paraformaldehyde for 20 min at RT and

permeabilized with 0.1% Triton X-100 in PBS for 3–4 min at RT. Cells were then washed 3X with PBS and blocked with 10% FCS/PBS for 30 min at RT. After the washing step, primary antibodies at 1:50 dilution were added, as indicated, for 1 h at RT. The antibodies used were: rabbit polyclonal antibodies to NF- κ B p65 (sc-372) and phospho-p65 (Ser536) (93H1, #3033; Cell Signaling, USA). The cells were washed carefully 3X with PBS and suitable FITC-conjugated secondary antibodies were added at 1:200 dilution for 1 h at RT.

To visualize the nuclei, the stained cells were treated with RNase A (1 μ g/ml) for 30 min, washed with PBS and propidium iodide was added and incubated for 10 min at RT. The stained cells were washed with PBS and mounted on glass slides. Excitation of FITC and propidium iodide was achieved using the 488 and 530 nm wavelengths, respectively. Images were collected on a Leica TCS-SP scanning confocal microscope, equipped with an argon/krypton laser and Leica TCS software.

2.9. Senescence and DNA damage marker detection

Senescence associated β -galactosidase (SA- β -Gal) staining was employed as a marker of cellular senescence. MRC-5 cells and their derivatives were plated at 1×10^4 per well of a 6-well plate 24 h prior to H₂O₂-treatment as described above. Senescent MRC-5 cells were also included in this analysis as a positive control. Prior to their SA- β -Gal staining, H₂O₂-treated MRC-5 cells were grown for 7 days. In some experiments, as a negative control for H₂O₂ mediated ROS accumulation, 2.5 mM N-acetyl-cysteine (NAC) was added 30 min prior to the 2 h of H₂O₂ treatment. MRC-5 I κ B α SR cells could not tolerate higher concentrations of NAC together with H₂O₂. Higher concentrations of NAC were toxic to MRC-5 I κ B α SR cells likely due to NAC inhibiting NF- κ B as previously described [36]. Senescent cells were detected by histochemical staining for β -galactosidase activity using a senescent cell staining kit (CS0030; Sigma) according to the manufacturer's instructions. Cells were visualized with a Nikon eclipse TS100 optical microscope fitted with a Nikon coolpix 990 camera and senescent cells were quantified by counting all cells in multiple high power fields (each 200 $\times$ magnification) with results expressed as % SA- β -Gal positive cells.

To reveal senescence-associated heterochromatic foci (SAHF), cells were grown on small round coverslips, inserted in the wells of 6-well plates, overnight at 37°C, 5% CO₂, treated with 200 μ M H₂O₂ for 2 h and after recovering for ~24 h were washed in PBS, fixed in 1% paraformaldehyde for 20 min at room temperature (RT), permeabilized in 0.2% Triton X-100 in PBS, washed in PBS and exposed to HP blocking buffer (150 mM NaCl, 15 mM HEPES-KOH pH7.4, 2 mM MgCl₂, 0.2 mM EGTA, 0.2% Triton X-100 and 0.5% fish skin gelatin) for 2 h at RT. Next cells were incubated with either murine anti-HP1 α (Euromedex Souffelweyheim, France) or rabbit anti-HP1 α and rat anti-HP1 β (M31) antibodies [37] at 1:50 dilution for 2 h followed by anti-mouse, anti-rabbit or anti-rat IgG-FITC, respectively, for 2 hr at room temperature. Cells were next treated with 1 μ g/ml RNase A for 30 min followed by propidium iodide (PI) staining for 10 min at room temperature. Antibody binding and PI staining were detected by confocal laser scanning microscopy (Leica TCS/SP).

DNA damage γ -H2AX foci were detected as above for SAHF except that cells were fixed in 4% paraformaldehyde for 20 min at RT, and then blocked in 10% FCS/PBS for 1 h. Next cells were incubated with rabbit anti- γ H2AX (#07-164, Upstate Biotechnology) (1:50 dilution) followed by anti-rabbit IgG-FITC each for 2 h at room temperature with signals captured as above for SAHF.

2.10. NF- κ B luciferase reporter assays

For NF- κ B luciferase assays, cells were seeded at 60% confluency in 24-well plates and transfected with Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Cells were transiently transfected with 1 μ g/well of NF- κ B dependent luciferase reporter plasmid 5 κ B and 0.2 μ g of pRSV- β gal. Twenty-four hours after transfection, cells were exposed to H₂O₂; and luciferase activities were assayed immediately after H₂O₂-treatment with a dual luciferase assay kit (Promega, Madison, WI, USA). Relative luciferase activities are expressed as fold induction over NF- κ B-dependent luciferase reporter alone in the absence of H₂O₂ and were normalized to the β -galactosidase activity of each sample. Experiments were repeated twice in triplicate.

2.11. Statistical analysis

Paired Student's *t* test or paired two-way ANOVA followed by Tukey multi-comparisons post test were employed as indicated in individual figures with *p* values of <0.05 considered significant with statistically analyzed data presented as means $\pm$ standard error.

3. Results

3.1. H₂O₂ activates a ROS-mediated DNA damage checkpoint response in normal and cancer cells

Increasing concentrations of H₂O₂ resulted in ROS accumulation in both normal HDFs MRC-5 and A549 lung cancer cells (Fig. 1A), reduced A549 colony formation (Fig. 1B) and also induced DDR characteristic changes in specific cellular proteins. After 2 h of H₂O₂ exposure followed by 24 h of recovery phosphorylations were detected for p-ATM(S1981), p-Chk2(T68) and γ -H2AX in both cell types (Figs 2A and B). Moreover, p-p53(Ser15), total p53 and p21^{Cip1} levels were also elevated in an H₂O₂ dose-dependent manner in both MRC-5 and A549 cells (Figs 2A and B).

3.2. H₂O₂ differentially activated NF- κ B signaling in MRC-5 and A549 cells

Because NF- κ B is activated by oxidative insult and can protect against cell death [23-24, 38], we investigated the status of the canonical NF- κ B p50 and p65/RelA subunits with and without H₂O₂ exposure of MRC-5 HDFs and A549 cells. MRC-5 HDFs showed a modest increase in p50 nuclear translocation and a more substantial accumulation of unphosphorylated, nuclear p65 along with an increased level of phospho-p65(S536) in response to H₂O₂ (Fig. 3A). H₂O₂ did not significantly alter the expression levels of NF- κ B p50 and p65 subunits in A549 cells; but the amount and nuclear translocation of p-p65(S536) was enhanced (Fig. 3B). Immunofluorescence and confocal laser scanning microscopy of A549 cells corroborated the enhanced accumulation of nuclear p-p65(S536) in response to H₂O₂ (Fig. 3C). In addition, H₂O₂ also dose dependently increased the activity of an NF- κ B promoter-driven luciferase reporter plasmid in A549 cells (Fig. 3D).

3.3. Inhibiting canonical NF- κ B activity retarded normal HDF proliferation and exacerbated the deleterious effects of ROS accumulation

NF- κ B activity in MRC-5 HDFs was suppressed by stable transduction with an I κ B α super repressor (I κ B α .SR) retrovirus co-expressing a neomycin selection marker (Fig. 4A). I κ B α .SR is a mutant form of I κ B α in which signal response domain serine residues 32 and 36 are mutated to alanines and thus cannot be phosphorylated in response to exogenous or intracellular signals via the IKK signalsome. I κ B α .SR sequesters NF- κ B p50/p65 heterodimers, in the cytoplasm preventing their nuclear translocation and target gene activation [32, 39]. MRC-5 I κ B α .SR cell growth rate was reduced compared to vector control cells (Fig. 4B), which was also apparent for IMR-90 I κ B α .SR HDFs vs. their

matched control (Figs 5A and B). p53 and p21^{Cip1} proteins were all nuclear in confluent MRC-5 vector and MRC-5 IκBαSR cells with p53 and p21^{Cip1} proteins increased in MRC-5 IκBαSR (Fig. 4C) and IMR-90 IκBαSR cells (Fig. 5C). Importantly, stable IKKβ knock-down in MRC-5 (Figs 4D-F) and IMR-90 (Figs 5D-F) HDFs produced comparable results.

H₂O₂ treatment induced ROS levels in MRC-5 vector and IκBαSR cells (Fig. 6A) along with concomitant changes in their cell cycle profiles, with no evidence of apoptosis (see analyses of multiple FACS experiments in Fig. 6B and examples of primary FACS profiles in Fig. S1), which was also verified by analyzing low molecular weight DNA extracted from both cell types (data not shown). NAC prevented this ROS accumulation, but, MRC-5 IκBαSR cells sustained significantly higher ROS levels compared to their matched controls (Fig. 6A). Importantly, significant portions of H₂O₂ treated MRC-5 vector and MRC-5 IκBαSR cells were blocked in the G2 or G2-M cell cycle phase, and this was enhanced at 200 μM H₂O₂ in MRC-5 IκBαSR cells (Fig. 6B and Fig. S1).

Canonical NF-κB signaling impacted on p53 levels and function in H₂O₂ treated HDFs. As indicated above, p53 was entirely nuclear in proliferating (sub-confluent) vector control and canonical NF-κB compromised HDFs; but it accumulated to much higher levels in MRC-5 IκBαSR cells (Fig. 6C). Even though H₂O₂ dose-dependently induced p53, MRC-5 cells at the highest H₂O₂ dose still expressed less p53 than untreated MRC-5 IκBαSR cells (Fig. 6C). Moreover, although H₂O₂ induced a low level of the more stable p-p53(Ser20) in MRC-5, still higher levels of nuclear p-p53(Ser20) accumulated in MRC-5 IκBαSR cells in response to H₂O₂ (Fig. 6C). p21^{Cip1} was distributed in the cytoplasm and nuclei of proliferating (sub-confluent) untreated MRC-5 vector and IκBαSR cells, and its expression was particularly elevated in MRC-5 IκBαSR nuclei (Fig. 6C). In addition, 100 and 200 μM H₂O₂ induced p21^{Cip1} expression in the cytoplasm and nuclei of MRC-5 vector controls (Fig. 6C); and its expression was higher particularly in MRC-5 IκBαSR nuclei (Fig. 6C).

3.4. Suppression of canonical NF-κB signaling in normal HDFs accelerated an ROS-induced, p53-dependent senescence response

H₂O₂ treatment of proliferating MRC-5 vector control cells induced senescent cell characteristics, which were strongly enhanced in canonical NF-κB-compromised cells. H₂O₂ at 100 and 200 μM induced the expression of senescence-associated β-galactosidase (SA-β-Gal) and inhibited the proliferation of MRC-5 vector cells (Fig. 7A, upper panels and Fig. 7D, left side). MRC-5 IκBαSR cells displayed a higher percentage of SA-β-Gal positive cells in comparison to vector control cells even in H₂O₂ untreated cultures (Fig. 7A, lower panel and Fig. 7D, left side). MRC-5 vector HDFs stopped growing and entered senescence at ~passage 57. P57 is equivalent to 57 population doubling (PDLs), which were determined as previously described [29-31]. In contrast, MRC-5 IκBαSR cells entered senescence at P45 and similar results were obtained with IMR-90 IκBαSR cells (see Table 1). Exposure to 100 and 200 μM H₂O₂ strongly exacerbated the degree of SA-β-Gal accumulation in MRC-5 IκBαSR cells, while simultaneously inhibiting their proliferation (Fig. 7A, lower panel). IKKβ knock-down in MRC-5 cells also enhanced their H₂O₂ induced senescence in conjunction with inhibiting their growth in comparison to control MRC-5 cells harboring an irrelevant GL2 luciferase shRNA (Figs 7B and 7D, right side). In accord with these results, MRC-5 IKKβ^{KD} cells exhibited a ~21% reduction in their lifespan compared to MRC-5 vector control (44 vs. 56 PDLs, respectively) and similar results were obtained with IMR-90 IKKβ^{KD} cells (see Table 1). Importantly, quantification of the percentages of SA-β-Gal positive MRC-5 IκBαSR and MRC-5 IKKβ^{KD} cells in the presence or absence of H₂O₂ showed no statistically significant differences between these two cell populations (Figs 7D, left and right sides). Thus basal and IKKβ-induced canonical NF-κB activity protects HDFs against H₂O₂-induced ROS stress. In addition, exposing

either control or I κ B α .SR MRC-5 cells to H₂O₂ together with NAC substantially reduced the appearance of SA- β -Gal positive cells (Figs 7C and 7D, left side). H₂O₂ also induced the appearance of large γ -H2AX foci in early passage P28 MRC-5 HDFs, which were comparable to those present in P59 late passage senescent cells (Fig. 8A). Moreover, H₂O₂-treated P28 MRC-5 I κ B α .SR HDFs displayed more numerous, large and smaller γ -H2AX foci, which were not observed in their untreated controls (Fig. 8A). H₂O₂-treated P28 MRC-5 vector and MRC-5 I κ B α .SR cells also displayed HP1 β and HP1 α senescence-associated heterochromatic foci (SAHF) [8, 40], which were comparable to P59 senescent MRC-5 vector control cells (Fig. 8B). In contrast, H₂O₂ untreated, proliferating P28 MRC-5 and MRC-5 I κ B α .SR cells showed no evidence of SAHF (Fig. 8B).

Importantly, p53 knock-down reversed the deleterious effects of 100 and 200 μ M H₂O₂ in MRC-5 HDFs (Figs 9A and B). MRC-5 I κ B α .SR/p53^{KD} cells also reached higher cell densities in comparison to MRC-5 I κ B α .SR (Fig. 9B). P53 knock-down strongly reduced the frequency of SA- β -Gal staining in control and I κ B α .SR MRC-5 HDFs in response to ROS accumulation (Figs 9B and C). Statistical analysis of data derived from 3 independent experiments indicate that most of the exacerbated senescence phenotype of canonical NF- κ B compromised MRC-5 cells mechanistically depended on the p53 axis, as MRC-5 I κ B α .SR/p53^{KD} cells were only modestly more senescent than their MRC-5 vector/p53^{KD} counterparts (Fig. 9C).

3.5. Suppression of canonical NF- κ B signaling in A549 human tumor cells induced cell cycle arrest and exacerbated a ROS-induced apoptotic response

Blocking canonical NF- κ B activity in A549 cells by enforced I κ B α .SR expression (Fig. 10B) exacerbated the inhibitory effect of H₂O₂ on cell cycle progression. H₂O₂-treated A549 I κ B α .SR cells displayed an altered cell cycle profile characterized by a G2 or G2-M phase shift with cells in a sub-G1 peak indicative of apoptosis, as detected by PI staining (see analyses of multiple FACS experiments in Fig. 10A and examples of primary FACS profiles in Fig. S2). In addition, basal and H₂O₂-induced levels of p53 and its target protein p21^{Cip1} were enhanced in A549 I κ B α .SR cells compared to their matched vector control (Fig. 10B).

Next, we evaluated the H₂O₂-mediated apoptotic responses of A549 in more detail. Flow cytometric analysis of annexin V-PI stained cells revealed that H₂O₂ induced early and late apoptosis in a dose dependent manner in A549 I κ B α .SR cells and their matched vector control. Delayed apoptotic effects were more apparent in H₂O₂ treated control cells, and early apoptotic effects were markedly increased in I κ B α .SR A549 cells (see analyses of multiple FACS experiments in Fig. 11A and examples of primary FACS profiles in Fig. S3). Overall, I κ B α .SR exacerbated the H₂O₂-mediated apoptotic response in A549 cells, as shown by the H₂O₂ dose-dependent increase in total apoptotic cells (Fig. 11A). Moreover, H₂O₂ activated caspase-3 and induced PARP-1 cleavage in A549 I κ B α .SR and their matched control cells (Fig. 11B). The 17-20 kDa cleaved form of caspase-3, which preceded PARP-1 cleavage, and cleaved 89 kDa PARP-1 were enhanced in H₂O₂-treated A549 I κ B α .SR cells especially at the highest H₂O₂ doses (Fig. 11B). In addition, PARP-1's cleaved 89 kDa form was more abundant in the nuclear compartment along with slower migrating PARP-1 fragments (Fig. 11C). Such PARP-1 fragments have been correlated with late apoptotic-necrotic cell death [41-42]. Comparable results were obtained in IKK β ^{KD} A549 cells (Fig. 12). Loss of IKK β in A549 cell (Fig. 12A) resulted in p53 accumulation, the activation of caspase-3 and PARP-1 cleavage (Fig. 12B) and in the exacerbation of apoptosis (Fig. 12C), in response to increasing concentrations of H₂O₂.

4. Discussion

Oxidative stress induces DNA damage leading to either growth arrest and senescence or apoptosis, depending on the nature and intensity of the stimulus and cell type (i.e., normal vs. tumor cells) [1-3]. Here we show that H₂O₂ induced oxidative stress initiated a DDR leading to G2 or G2-M arrest and the premature senescence of MRC-5 normal human lung diploid fibroblasts (HDFs), but instead caused the apoptosis of A549 human lung tumor epithelial cells. NF- κ B signaling was induced by these ROS-instigated responses and suppression of NF- κ B activation in conjunction with H₂O₂-induced ROS exacerbated the accumulation of both cell types in G2 or G2-M phase. However, the final physiological outcomes of the ROS responses of NF- κ B compromised MRC-5 and A549 cells were quite different. Loss of canonical NF- κ B signaling in HDFs accelerated their ROS-induced premature senescence by a p53-dependent mechanism, while NF- κ B suppression of in A549 enhanced their apoptosis.

4.1. ROS activated DDR caused the G2-M growth arrest of normal and tumor cells

H₂O₂-mediated ROS responses (Fig. 1A) activated DDR. Phosphorylated forms of ATM, Chk2, p53 and γ -H2AX in conjunction with enhanced accumulation of total p53 and p21^{Cip1} were all observed in both normal and tumor cells (Figs 2A and B). Prior studies have reported that H₂O₂ activated DDR with the formation of DNA damage foci in A549 cells [43-45] and induced γ -H2AX [4, 9, 13, 15, 43, 46] and 53BP1 [13, 15] foci in normal HDFs. In MRC-5, these H₂O₂-induced DNA damage foci are both telomere- and non-telomere associated foci and are associated with DNA damage [47-48] and cellular senescence [10]. H₂O₂ also dose-dependently induced the accumulation of MRC-5 and A549 cells in G2 or G2-M phase (Figs 6B and 10A, respectively). Earlier studies showed that ROS production peaks in the G2-M phase of the cell cycle, whereas others indicated that G2-M is a more reducing environment than G1 [3]. Induction of G2 or G2-M growth retardation in A549 is in agreement with previous studies on the effects of H₂O₂ [49-50] or ROS generation [51-52]; and the accumulation of MRC-5 in G2 or G2-M in response to H₂O₂ is in agreement with prior reports in murine [53] and human [54-55] cells.

4.2. ROS inflicted damage activated NF- κ B signaling in normal and tumor cells

NF- κ B is a redox sensitive transcription factor [23-24, 38] which is activated by DNA damage [25-26]. In normal HDFs, we find that H₂O₂ induces a gradual increase of nuclear p50, elevated levels of nuclear p65 and a modest increase of nuclear p-p65 (S536) (Fig. 3A), in agreement with previous studies employing MEFs [56-58] and HDFs [59-60] demonstrating increased NF- κ B activity in response to H₂O₂. In A549 cells, H₂O₂ induced the nuclear accumulation of p-p65 (S536) (Figs 3B and 3C) in conjunction with up-regulating NF- κ B transcriptional activity by ~2-fold (Fig. 3D). While some studies demonstrated lack of nuclear translocation and NF- κ B activity in A549 [61-62], others have demonstrated nuclear p65 expression and NF- κ B activity at various levels [63-69].

4.3. Suppression of NF- κ B signaling exacerbated the ROS-induced premature senescence response of normal human HDFs

Previous studies have established that HDFs undergo SIPS (stress-induced premature senescence) in response to various stimuli, including oxidative stress invoked by H₂O₂ via DDR [4, 9-13, 46]. Although NF- κ B has been reported to have an important role in DDR [25-26], the functional impact of NF- κ B in ROS-induced, DNA damage associated normal human fibroblast senescence remains unknown. To dissect the functional roles of NF- κ B signaling in this context, we used MRC-5 HDFs because of their well characterized DDR and formation of SAHF during replicative senescence [70-71] and inability to undergo

oncogene-induced senescence (OIS) in response to a single activated oncoprotein [29-30, 72] (and our unpublished observations).

Suppression of canonical NF- κ B signaling in MRC-5 HDFs led to a G2 or G2-M phase cell cycle block and premature senescence in response to H₂O₂ (Figs 6 and 7, respectively). HDFs expressing an I κ B α SR or deficient in IKK β expression exhibited reduced proliferation (Figs 4B and E, and Figs 5B and E) and comparable degrees of enhanced SA- β -Gal staining in response to H₂O₂ (Figs 7A,B and D); and canonical NF- κ B deficiency also correlated with higher levels of DNA damage associated γ -H2AX foci in these cells (Fig. 8A). Interestingly, statistical quantification of percent SA- β -Gal staining cells revealed that HDFs deficient in canonical NF- κ B activity were also significantly prone to premature senescence in comparison to their matched controls even in the absence of an H₂O₂ provoked ROS response (Fig. 7D). These effects were all due to ROS accumulation, because NAC abrogated the premature senescence response of control and canonical NF- κ B-compromised MRC-5 cells in the presence and absence of H₂O₂ (Figs 7C and D). We also investigated the formation of HP1 α and HP1 β because these HP1s exhibit inherent heterochromatin binding activity [37] and are recruited to various types of DNA damage foci [73]. While the detection of DNA damage foci in H₂O₂-treated HDFs [13, 15] and human endothelial cells [74] has been previously reported, our results indicate that blocking canonical NF- κ B activity in HDFs exacerbates the accumulation of HP1 α and HP1 β SAHF in response to sub-lethal concentrations of H₂O₂ (Fig. 8B).

Although ROS-mediated induction of p53 and p21^{Cip1} has been described in other HDF strains [11-15, 18], the functional contributions of endogenous NF- κ B activity for these effects have not been addressed. Herein we have shown that H₂O₂ induced the expression of the p53-p21^{Cip1} axis in both vector- and I κ B α SR-expressing HDFs, in a dose-dependent manner, which was more profound in the latter cells (Fig. 6C). Nuclear accumulations of both p53 and p21^{Cip1} were higher in MRC-5 I κ B α SR cells compared to matched controls; and the stable, nuclear p-p53(Ser20) form was observed in canonical NF- κ B deficient HDFs in response to H₂O₂ dosage (Fig. 6C). Importantly p53 knock-down dramatically reduced the accumulation of SA- β -Gal positive cells in response to H₂O₂, demonstrating that H₂O₂-induced HDF premature senescence depends on p53 (Fig. 7). Moreover, p53^{KD} also ablated the large majority of the exacerbated senescence response observed in MRC-5 I κ B α SR cells. Thus, most of the mechanism underlying the protective anti-senescence effect of canonical NF- κ B activity in HDFs involves its restraining of p53 axis activity (Fig. 7C). Previous studies using spontaneously immortalized MEFs from IKK α / β ^{-/-} and WT embryos [75] or NIH3T3-ras cells [76] revealed that canonical NF- κ B activity suppresses the p53-p21 axis. Subsequent studies showed that IKK β -mediated phosphorylation was responsible for enhanced β -TrCP1-mediated p53 degradation [77]. Further, conditional deletion of *Ikk β* in melanocytes in *Ha-Ras V12*-induced melanomas led to increased p53-p21 expression and apoptosis, and knocking-down p53 resulted in significant impairment of apoptosis associated with *Ikk β* deletion [78]. Thus, in addition to these prior studies with immortalized and cancer cells, we now show that IKK β -dependent canonical NF- κ B activity down-modulates the p53-p21^{Cip1} axis to protect normal HDFs from the senescence response invoked by ROS-induced stress.

NF- κ B's functional impact in senescence responses has been controversial with diverse effects having been reported in different physiological contexts and cell types. NF- κ B has been implicated in the induction of a senescence-associated secretory phenotype (SASP) in human skin fibroblasts (HSFs) [79-82]; but in contrast, senescent lung HDFs showed no evidence of NF- κ B activation [31, 35] and a low SASP phenotype [79, 81]. In contrast, OIS of lung HDFs, by activation of the INK4A-ARF locus [83-84] or by *Ha-Ras V12* [85], was accompanied by an increase in SASP factors [86-87]; but different functional roles have

been ascribed to canonical NF- κ B signaling in these different contexts. For example, p65 knock-down (KD) was sufficient to bypass the oncoprotein induced senescence (OIS) of human BJ fibroblasts [85]; but not in human lung IMR-90 HDFs in which the combined KD of p65 and p53 were necessary indicative of cell type-specific differences [85]. Prior to this recent work proof of functional contributions of NF- κ B in senescence responses had been lacking, because enforced expression of NF- κ B p50/p65 subunits in HSFs did not affect their proliferation despite the induction of IL-6 and IL-8 [88]; and moreover chronic activation of canonical NF- κ B in normal lung HDFs did not promote their senescence [31]; but other more recent evidence indicates that NF- κ B is activated in specific, oncogene-addicted, immortalized human mammary fibroblasts and can also promote their senescence [89]. However, others reported that transient oncogene induction in non-transformed, immortalized human mammary cells can result in elevated IL-6 production, which maintains cells in a transformed state by driving an epigenetic, positive feedback loop to simultaneously activate NF- κ B and STAT3 [90]. Thus, dependent on the immortalizing/transforming event alterations in epigenetic control can yield cells that become addicted to NF- κ B activation for their continued growth and progression towards malignancy [90-91]. Suppression of canonical NF- κ B either by p65 ablation or by the action of I κ B α SR in mouse embryonic fibroblasts (MEFs) induced genomic instability due to compromised DNA repair, leading to their accelerated immortalization. Enforced expression of I κ B α SR in telomerized HDFs also delayed their recovery from DNA damage induced by γ -irradiation [92]. Moreover, p65^{-/-} MEFs were found to exhibit a delayed recovery from DNA damage due to inefficient DNA repair. In addition, appreciably greater γ -H2AX-positive nuclei were seen in many tissues of p65^{-/-} mice in comparison with tissues from p65^{+/+} mice again due to inefficient DNA repair [92]. IKK β was also shown to regulate DSBs induced by radiation in cancer cells [93]. Finally, other recent work has shown that p65 loss in a mutant *K-Ras* mouse lung cancer model impaired tumor growth and spread in conjunction with the accumulation of apoptotic cells [94]; and ablation of IKK β has also been reported to reduce the proliferation of *K-ras*^{G12D}-induced lung adenocarcinomas in mice [95].

NF- κ B is not the only transcriptional activator that functionally impacts on HDF premature senescence responses. Knock-down of Nrf2, another redox sensitive transcription factor, has also been reported to inhibit the proliferation and induce premature senescence of MEFs and HDFs in response to oxidative or oncogenic stress [96-97]. Loss of MTH1, a major cellular 8-oxo-dGTPase that prevents the genesis of 8-oxo-dGTP-mediated DNA lesions, was recently shown to cause HDF premature senescence by induction of oxidative DNA damage in conjunction with the p16 and p53-p21 pathways [16]. Interestingly, the MTH1 gene promoter contains NF- κ B responsive elements [98]; and it remains to be investigated whether there is a functional link between NF- κ B signaling and MTH1 in cellular senescence.

4.4. Canonical NF- κ B signaling reduced the intensity of ROS provoked tumor cell apoptosis

H₂O₂-induced DDR lead to G2 or G2-M arrest (Fig. 10) and apoptosis of A549 tumor cells by activating caspase-3 and nuclear PARP-1 cleavage (Fig. 11). Previous studies showed that H₂O₂ induced apoptosis of A549 cells [61, 99-101] and that the extent of H₂O₂-induced apoptosis was significantly reduced in cells pre-exposed to hyperoxia by induction of NF- κ B activity [100]. Our results show that suppressing canonical NF- κ B activity in A549 cells accelerated their G2 or G2-M phase accumulation (Fig. 10A and Fig. S2) in conjunction with up-regulating the p53-p21^{Cip1} axis (Fig. 10B). Moreover, inhibiting canonical NF- κ B signaling by I κ B α SR action (Fig. 11 and Fig. S3) or IKK β depletion (Fig. 12) sensitized A549 cells to H₂O₂-mediated apoptosis. In other cell backgrounds ROS has been previously

shown to induce p21^{Cip1} in the absence of p53 activation [53, 102], and can protect cells against oxidative damage [103–104].

Taken together, we have shown that compromising canonical NF- κ B signaling in normal vs. tumor cells can have radically different outcomes for ROS-induced, p53-dependent, DNA damage responses with human lung epithelial tumour cells (HTEC) succumbing more rapidly to apoptosis and normal human lung fibroblasts (HDFs) undergoing accelerated premature senescence (see graphical abstract).

Supplementary Material

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Acknowledgments

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References

- [1]. Li H, Mitchell JR, Hasty P. Mech Ageing Dev. 2008; 129(7-8):416–424. [PubMed: 18346777]
- [2]. Lu T, Finkel T. Exp Cell Res. 2008; 314(9):1918–1922. [PubMed: 18282568]
- [3]. Burhans WC, Heintz NH. Free Radical Biology and Medicine. 2009; 47(9):1282–1293. [PubMed: 19486941]
- [4]. Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, Barrett JC. Nat Cell Biol. 2004; 6(2):168–170. [PubMed: 14755273]
- [5]. Nakamura AJ, Chiang YJ, Hathcock KS, Horikawa I, Sedelnikova OA, Hodes RJ, Bonner WM. Epigenetics Chromatin. 2008; 1(1):6. [PubMed: 19014415]
- [6]. Sedelnikova OA, Horikawa I, Redon C, Nakamura A, Zimonjic DB, Popescu NC, Bonner WM. Aging Cell. 2008; 7(1):89–100. [PubMed: 18005250]
- [7]. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LV, Kolettas E, Niforou K, Zoumpourlis VC, Takaoka M, Nakagawa H, Tort F, Fugger K, Johansson F, Sehested M, Andersen CL, Dyrskjot L, Orntoft T, Lukas J, Kittas C, Helleday T, Halazonetis TD, Bartek J, Gorgoulis VG. Nature. 2006; 444(7119):633–637. [PubMed: 17136093]
- [8]. Campisi J, d'Adda di Fagagna F. Nat Rev Mol Cell Biol. 2007; 8(9):729–740. [PubMed: 17667954]
- [9]. Sedelnikova OA, Redon CE, Dickey JS, Nakamura AJ, Georgakilas AG, Bonner WM. Mutat Res. 2010; 704(1-3):152–159. [PubMed: 20060490]
- [10]. Hewitt G, Jurk D, Marques FD, Correia-Melo C, Hardy T, Gackowska A, Anderson R, Taschuk M, Mann J, Passos JF. Nat Commun. 2012; 3:708. [PubMed: 22426229]
- [11]. Chen JH, Stoeber K, Kingsbury S, Ozanne SE, Williams GH, Hales CN. J Biol Chem. 2004; 279(47):49439–49446. [PubMed: 15377661]
- [12]. Wang Y, Meng A, Zhou D. Exp Cell Res. 2004; 298(1):188–196. [PubMed: 15242773]
- [13]. Chen JH, Ozanne SE, Hales CN. DNA Repair (Amst). 2005; 4(10):1140–1148. [PubMed: 16006199]
- [14]. Duan J, Zhang Z, Tong T. Int J Biochem Cell Biol. 2005; 37(7):1407–1420. [PubMed: 15833273]
- [15]. Chen JH, Ozanne SE. FEBS Lett. 2006; 580(28-29):6669–6673. [PubMed: 17126333]

- [16]. Rai P, Onder TT, Young JJ, McFaline JL, Pang B, Dedon PC, Weinberg RA. *Proc Natl Acad Sci U S A*. 2009; 106(1):169–174. [PubMed: 19118192]
- [17]. Bladier C, Wolvetang EJ, Hutchinson P, de Haan JB, Kola I. *Cell Growth Differ*. 1997; 8(5): 589–598. [PubMed: 9149910]
- [18]. Gorbunova V, Seluanov A, Pereira-Smith OM. *J Biol Chem*. 2002; 277(41):38540–38549. [PubMed: 12140282]
- [19]. Perkins ND. *Nat Rev Mol Cell Biol*. 2007; 8(1):49–62. [PubMed: 17183360]
- [20]. Chariot A. *Trends Cell Biol*. 2009; 19(8):404–413. [PubMed: 19648011]
- [21]. Basak S, Kim H, Kearns JD, Tergaonkar V, O'Dea E, Werner SL, Benedict CA, Ware CF, Ghosh G, Verma IM, Hoffmann A. *Cell*. 2007; 128(2):369–381. [PubMed: 17254973]
- [22]. Perkins ND. *Nat Rev Cancer*. 2012; 12(2):121–132. [PubMed: 22257950]
- [23]. Gloire G, Legrand-Poels S, Piette J. *Biochem Pharmacol*. 2006; 72(11):1493–1505. [PubMed: 16723122]
- [24]. Gloire G, Piette J. *Antioxid Redox Signal*. 2009; 11(9):2209–2222. [PubMed: 19203223]
- [25]. Janssens S, Tschopp J. *Cell Death Differ*. 2006; 13(5):773–784. [PubMed: 16410802]
- [26]. Miyamoto S. *Cell Res*. 2011; 21(1):116–130. [PubMed: 21187855]
- [27]. Yamaguchi T, Miki Y, Yoshida K. *Cell Signal*. 2007; 19(10):2088–2097. [PubMed: 17644309]
- [28]. Yoshida K, Ozaki T, Furuya K, Nakanishi M, Kikuchi H, Yamamoto H, Ono S, Koda T, Omura K, Nakagawara A. *Oncogene*. 2008; 27(8):1183–1188. [PubMed: 17700524]
- [29]. Kolettas E, Rosenberger RF. *Eur J Biochem*. 1998; 254(2):266–274. [PubMed: 9660179]
- [30]. Kolettas E, Khazaie K, Rosenberger R. *Int J Oncol*. 1997; 11(5):1071–1080. [PubMed: 21528305]
- [31]. Batsi C, Markopoulou S, Vartholomatos G, Georgiou I, Kanavaros P, Gorgoulis VG, Marcu KB, Kolettas E. *Mech Ageing Dev*. 2009; 130(7):409–419. [PubMed: 19406145]
- [32]. Penzo M, Massa PE, Olivetto E, Bianchi F, Borzi RM, Hanidu A, Li X, Li J, Marcu KB. *J Cell Physiol*. 2009; 218(1):215–227. [PubMed: 18803232]
- [33]. Olivetto E, Borzi RM, Vitellozzi R, Pagani S, Facchini A, Battistelli M, Penzo M, Li X, Flamigni F, Li J, Falcieri E, Marcu KB. *Arthritis Rheum*. 2008; 58(1):227–239. [PubMed: 18163512]
- [34]. Brummelkamp TR, Bernards R, Agami R. *Cancer Cell*. 2002; 2(3):243–247. [PubMed: 12242156]
- [35]. Dimri GP, Campisi J. *Exp Cell Res*. 1994; 212(1):132–140. [PubMed: 8174635]
- [36]. Qanungo S, Wang M, Nieminen AL. *J Biol Chem*. 2004; 279(48):50455–50464. [PubMed: 15375156]
- [37]. Dialynas GK, Terjung S, Brown JP, Aucott RL, Baron-Luhr B, Singh PB, Georgatos SD. *J Cell Sci*. 2007; 120(Pt 19):3415–3424. [PubMed: 17855382]
- [38]. Morgan MJ, Liu ZG. *Cell Res*. 2011; 21(1):103–115. [PubMed: 21187859]
- [39]. Brockman JA, Scherer DC, McKinsey TA, Hall SM, Qi X, Lee WY, Ballard DW. *Mol Cell Biol*. 1995; 15(5):2809–2818. [PubMed: 7739562]
- [40]. Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. *Cell*. 2003; 113(6):703–716. [PubMed: 12809602]
- [41]. Gobeil S, Boucher CC, Nadeau D, Poirier GG. *Cell Death Differ*. 2001; 8(6):588–594. [PubMed: 11536009]
- [42]. Nozawa K, Casiano CA, Hamel JC, Molinaro C, Fritzler MJ, Chan EK. *Arthritis Res*. 2002; 4(4):R3. [PubMed: 12106502]
- [43]. Zhao H, Tanaka T, Halicka HD, Traganos F, Zarebski M, Dobrucki J, Darzynkiewicz Z. *Cytometry A*. 2007; 71(11):905–914. [PubMed: 17879239]
- [44]. Zhao H, Traganos F, Albino AP, Darzynkiewicz Z. *Cell Cycle*. 2008; 7(10):1490–1495. [PubMed: 18418078]
- [45]. Zhao H, Dobrucki J, Rybak P, Traganos F, Halicka H Dorota, Darzynkiewicz Z. *Cytometry A*. 2011; 79(11):897–902. [PubMed: 21905210]

- [46]. Newshean S, Wukovich RL, Aziz K, Kalogerinis PT, Richardson CC, Panayiotidis MI, Bonner WM, Sedelnikova OA, Georgakilas AG. *Mutat Res.* 2009; 674(1-2):131–136. [PubMed: 18948225]
- [47]. Petersen S, Saretzki G, von Zglinicki T. *Exp Cell Res.* 1998; 239(1):152–160. [PubMed: 9511733]
- [48]. Saretzki G, Sitte N, Merkel U, Wurm RE, von Zglinicki T. *Oncogene.* 1999; 18(37):5148–5158. [PubMed: 10498864]
- [49]. Shenberger JS, Adams MH, Zimmer SG. *Am J Respir Cell Mol Biol.* 2002; 27(2):250–256. [PubMed: 12151318]
- [50]. Dandrea T, Hellmold H, Jonsson C, Zhivotovsky B, Hofer T, Warngard L, Cotgreave I. *Free Radic Biol Med.* 2004; 36(7):881–896. [PubMed: 15019973]
- [51]. Zhang Z, Huang C, Li J, Leonard SS, Lanciotti R, Butterworth L, Shi X. *Arch Biochem Biophys.* 2001; 392(2):311–320. [PubMed: 11488607]
- [52]. Zhang Z, Leonard SS, Huang C, Vallyathan V, Castranova V, Shi X. *Free Radic Biol Med.* 2003; 34(10):1333–1342. [PubMed: 12726921]
- [53]. Barnouin K, Dubuisson ML, Child ES, Fernandez de Mattos S, Glassford J, Medema RH, Mann DJ, Lam EW. *J Biol Chem.* 2002; 277(16):13761–13770. [PubMed: 11827966]
- [54]. Shackelford RE, Innes CL, Sieber SO, Heinloth AN, Leadon SA, Paules RS. *Journal of Biological Chemistry.* 2001; 276(24):21951–21959. [PubMed: 11290740]
- [55]. Moon SC, Park SC, Yeo EJ, Kwak CS. *J Med Food.* 2009; 12(3):485–492. [PubMed: 19627195]
- [56]. de Haan JB, Bladier C, Lotfi-Miri M, Taylor J, Hutchinson P, Crack PJ, Hertzog P, Kola I. *Free Radic Biol Med.* 2004; 36(1):53–64. [PubMed: 14732290]
- [57]. Taylor JM, Crack PJ, Gould JA, Ali U, Hertzog PJ, Iannello RC. *Exp Cell Res.* 2004; 300(2):463–475. [PubMed: 15475010]
- [58]. Liberatore RA, Goff SP, Nunes I. *Proc Natl Acad Sci U S A.* 2009; 106(42):17823–17828. [PubMed: 19805123]
- [59]. Canton I, Sarwar U, Kemp EH, Ryan AJ, MacNeil S, Haycock JW. *Tissue Eng.* 2007; 13(5):1013–1024. [PubMed: 17430089]
- [60]. Ryu B, Himaya SW, Qian ZJ, Lee SH, Kim SK. *Peptides.* 2011; 32(4):639–647. [PubMed: 21262307]
- [61]. Li Y, Zhang W, Mantell LL, Kazzaz JA, Fein AM, Horowitz S. *J Biol Chem.* 1997; 272(33):20646–20649. [PubMed: 9252381]
- [62]. Lakshminarayanan V, Drab-Weiss EA, Roebuck KA. *J Biol Chem.* 1998; 273(49):32670–32678. [PubMed: 9830008]
- [63]. Rahman I, Gilmour PS, Jimenez LA, MacNee W. *Mol Cell Biochem.* 2002; 234-235(1-2):239–248. [PubMed: 12162440]
- [64]. Antonicelli F, Parmentier M, Drost EM, Hirani N, Rahman I, Donaldson K, MacNee W. *Free Radic Biol Med.* 2002; 32(6):492–502. [PubMed: 11958950]
- [65]. Rahman I, Gilmour PS, Jimenez LA, Biswas SK, Antonicelli F, Aruoma OI. *Biochem Biophys Res Commun.* 2003; 302(4):860–864. [PubMed: 12646250]
- [66]. Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bouter MR, Kilty I, Rahman I. *FASEB J.* 2004; 18(15):1897–1899. [PubMed: 15456740]
- [67]. Biswas SK, McClure D, Jimenez LA, Megson IL, Rahman I. *Antioxid Redox Signal.* 2005; 7(1-2):32–41. [PubMed: 15650394]
- [68]. Hou Y, An J, Hu XR, Sun BB, Lin J, Xu D, Wang T, Wen FQ. *Int Immunopharmacol.* 2009; 9(1):120–126. [PubMed: 19038366]
- [69]. Loukili N, Rosenblatt-Velin N, Rolli J, Levrand S, Feihl F, Waeber B, Pacher P, Liaudet L. *J Biol Chem.* 2010
- [70]. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP. *Nature.* 2003; 426(6963):194–198. [PubMed: 14608368]
- [71]. Lawless C, Wang C, Jurk D, Merz A, Zglinicki T, Passos JF. *Exp Gerontol.* 2010; 45(10):772–778. [PubMed: 20117203]

- [72]. Kolettas E, Evangelou A, Gonos ES. *Anticancer Res.* 2001; 21(1A):435–444. [PubMed: 11299775]
- [73]. Luijsterburg MS, Dinant C, Lans H, Stap J, Wiernasz E, Lagerwerf S, Warmerdam DO, Lindh M, Brink MC, Dobrucki JW, Aten JA, Foustieri MI, Jansen G, Dantuma NP, Vermeulen W, Mullenders LH, Houtsmuller AB, Verschure PJ, van Driel R. *J Cell Biol.* 2009; 185(4):577–586. [PubMed: 19451271]
- [74]. Zhan H, Suzuki T, Aizawa K, Miyagawa K, Nagai R. *J Biol Chem.* 2010; 285(38):29662–29670. [PubMed: 20639198]
- [75]. Tergaonkar V, Pando M, Vafa O, Wahl G, Verma I. *Cancer Cell.* 2002; 1(5):493–503. [PubMed: 12124178]
- [76]. Sakaida T, Iwadata Y, Yamaji S, Uehara M, Noda A, Takiguchi M, Yamaura A, Hiwasa T. *Int J Oncol.* 2003; 23(4):1071–1077. [PubMed: 12963987]
- [77]. Xia Y, Padre RC, De Mendoza TH, Bottero V, Tergaonkar VB, Verma IM. *Proc Natl Acad Sci U S A.* 2009; 106(8):2629–2634. [PubMed: 19196987]
- [78]. Yang J, Splittergerber R, Yull FE, Kantrow S, Ayers GD, Karin M, Richmond A. *J Clin Invest.* 2010; 120(7):2563–2574. [PubMed: 20530876]
- [79]. Zender L, Rudolph KL. *Aging (Albany NY).* 2009; 1(5):438–441. [PubMed: 20157527]
- [80]. Adler AS, Sinha S, Kawahara TL, Zhang JY, Segal E, Chang HY. *Genes Dev.* 2007; 21(24):3244–3257. [PubMed: 18055696]
- [81]. Bhaumik D, Scott GK, Schokrpur S, Patil CK, Orjalo AV, Rodier F, Lithgow GJ, Campisi J. *Aging (Albany NY).* 2009; 1(4):402–411. [PubMed: 20148189]
- [82]. Rodier F, Coppe J-P, Patil CK, Hoeijmakers WAM, Munoz DP, Raza SR, Freund A, Campeau E, Davalos AR, Campisi J. *Nat Cell Biol.* 2009; 11(8):973–979. [PubMed: 19597488]
- [83]. Agger K, Cloos PA, Rudkjaer L, Williams K, Andersen G, Christensen J, Helin K. *Genes Dev.* 2009; 23(10):1171–1176. [PubMed: 19451217]
- [84]. Barradas M, Anderton E, Acosta JC, Li S, Banito A, Rodriguez-Niedenfuhr M, Maertens G, Banck M, Zhou MM, Walsh MJ, Peters G, Gil J. *Genes Dev.* 2009; 23(10):1177–1182. [PubMed: 19451218]
- [85]. Chien Y, Scuoppo C, Wang X, Fang X, Balgley B, Bolden JE, Premisrirt P, Luo W, Chicas A, Lee CS, Kogan SC, Lowe SW. *Genes Dev.* 2011; 25(20):2125–2136. [PubMed: 21979375]
- [86]. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, Aarden LA, Mooi WJ, Peeper DS. *Cell.* 2008; 133(6):1019–1031. [PubMed: 18555778]
- [87]. Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, Tavaré S, Arakawa S, Shimizu S, Watt FM. *Genes Dev.* 2009; 23(7):798–803. [PubMed: 19279323]
- [88]. Hinata K, Gervin AM, Zhang Y Jennifer, Khavari PA. *Oncogene.* 2003; 22(13):1955–1964. [PubMed: 12673201]
- [89]. Rovillain E, Mansfield L, Caetano C, Alvarez-Fernandez M, Caballero OL, Medema RH, Hummerich H, Jat PS. *Oncogene.* 2011; 30(20):2356–2366. [PubMed: 21242976]
- [90]. Iliopoulos D, Hirsch HA, Struhl K. *Cell.* 2009; 139(4):693–706. [PubMed: 19878981]
- [91]. Ancrile B, Lim KH, Counter CM. *Genes Dev.* 2007; 21(14):1714–1719. [PubMed: 17639077]
- [92]. Wang J, Jacob NK, Ladner KJ, Beg A, Perko JD, Tanner SM, Liyanarachchi S, Fishel R, Guttridge DC. *EMBO Rep.* 2009; 10(11):1272–1278. [PubMed: 19779484]
- [93]. Wu L, Shao L, An N, Wang J, Pazhanisamy S, Feng W, Hauer-Jensen M, Miyamoto S, Zhou D. *PLoS One.* 2011; 6(4):e18447. [PubMed: 21490922]
- [94]. Basseres DS, Ebbs A, Levantini E, Baldwin AS. *Cancer Res.* 2010; 70(9):3537–3546. [PubMed: 20406971]
- [95]. Xia Y, Yeddula N, Leblanc M, Ke E, Zhang Y, Oldfield E, Shaw RJ, Verma IM. *Nat Cell Biol.* 2012; 14(3):257–265. [PubMed: 22327365]
- [96]. Kapeta S, Chondrogianni N, Gonos ES. *J Biol Chem.* 2010; 285(11):8171–8184. [PubMed: 20068043]
- [97]. DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, Mangal D, Yu KH, Yeo CJ, Calhoun ES, Scrimieri F, Winter JM, Hruban RH, Iacobuzio-Donahue C, Kern SE, Blair IA, Tuveson DA. *Nature.* 2011; 475(7354):106–109. [PubMed: 21734707]

- [98]. Nakabeppu Y. *Mutat Res.* 2001; 477(1-2):59–70. [PubMed: 11376687]
- [99]. Kazzaz JA, Xu J, Palaia TA, Mantell L, Fein AM, Horowitz S. *J Biol Chem.* 1996; 271(25): 15182–15186. [PubMed: 8662947]
- [100]. Franek WR, Horowitz S, Stansberry L, Kazzaz JA, Koo HC, Li Y, Arita Y, Davis JM, Mantell AS, Scott W, Mantell LL. *J Biol Chem.* 2001; 276(1):569–575. [PubMed: 11034997]
- [101]. Geiser T, Ishigaki M, van Leer C, Matthay MA, Broaddus VC. *Am J Physiol Lung Cell Mol Physiol.* 2004; 287(2):L448–453. [PubMed: 15121637]
- [102]. Chung YW, Jeong DW, Won JY, Choi EJ, Choi YH, Kim IY. *Biochem Biophys Res Commun.* 2002; 293(4):1248–1253. [PubMed: 12054510]
- [103]. O'Reilly MA. *Antioxid Redox Signal.* 2005; 7(1-2):108–118. [PubMed: 15650400]
- [104]. Chen W, Sun Z, Wang XJ, Jiang T, Huang Z, Fang D, Zhang DD. *Mol Cell.* 2009; 34(6):663–673. [PubMed: 19560419]

Fig. 1.

Effects of H₂O₂-induced ROS levels on cell growth. (A) Accumulation of endogenous ROS in response to H₂O₂ in MRC-5 and A549 cells. Proliferating cells treated with increasing concentrations of hydrogen peroxide (H₂O₂) (0 - 1000 μM) for 2 h were analyzed by flow cytometry for endogenous ROS levels as described in Materials and Methods. **p*<0.01 and ***p*<0.001 for H₂O₂-treated cells compared to untreated controls (H₂O₂ = 0) for each experimental series were determined by Student's *t*-test. (B) Effect of H₂O₂ dose on colony A549 formation. Media was changed on the indicated cells every two days and colonies were fixed and stained with crystal violet after 14 days. Colonies were counted and expressed as percentage of the total number of cells plated. Results shown are means ± SEM of 3 experiments each performed in triplicate. In panel B, ***p*<0.01, ****p*<0.001 for H₂O₂-treated cells compared to untreated controls (H₂O₂ = 0) were determined by Student's *t*-test.

Fig. 2.

H₂O₂ activated DDR in normal and cancer cells. (A) MRC-5 and (B) A549 cells were treated with increasing concentrations of H₂O₂ as indicated and total extracts were analyzed for the expression of p-ATM(Ser1981), γ-H2AX, p-Chk2(T68), Chk2, p-p53(Ser15), total p53 and p21^{Cip1}, with β-actin serving as a reference control. Samples were run on the same gel and blotted on the same filter, but were then probed separately with antibodies specific for p-ATM and γ-H2AX, p-Chk2(T68), total Chk2, p-p53(Ser15), total p53, p21^{Cip1} and γ-H2AX. Representative results of several independent experiments are shown.

Fig. 3.

Expression and subcellular localization of canonical NF- κ B subunits in response to H₂O₂. Immunoblot analysis for the expression of NF- κ B p50, p65 and p-p65(S536) or β -actin and lamin B in cytoplasmic and nuclear extracts of untreated and H₂O₂-treated (A) MRC-5 and (B) A549 cells. Results are representative of several independent experiments. (C) Detection of p65 and phospho-p65 in untreated and H₂O₂-treated A549 cells. Proliferating A549 cells were plated on coverslips in 6-well plates and allowed to attach overnight. Half of the cells were treated with 200 μ M H₂O₂ for 2 h and half were left untreated. Cells were washed in PBS and stained with specific antibodies to detect p65 and p-p65(S536), which were visualized and photographed with a laser scanning confocal microscope as described in Materials and Methods. Experiments were performed twice in duplicate with comparable results. (D) Luciferase reporter assays to determine NF- κ B activity in untreated and H₂O₂-treated A549 cells. Each value represents mean $\pm$ SEM from 3 independent experiments each done in triplicate, which were performed as described in Materials and Methods. * $p < 0.01$ and ** $p < 0.001$ for H₂O₂-treated cells compared to untreated controls (H₂O₂ = 0) were determined by Student's t-test.

Fig. 4.

Inhibition of canonical NF- κ B activity retards the proliferation of MRC-5 fibroblasts in conjunction with enhancing p53 and p21 levels. (A) MRC-5 HDFs were stably transduced with an empty retroviral vector as a control or with the same retrovector carrying an NF- κ B super repressor I κ B α SR. Cells were selected in G418 and total lysates extracted and immunoblotted for I κ B α and β -actin as a reference control. (B) MRC-5 I κ B α SR cells had a significantly prolonged growth rate compared to their matched vector control (39.28 h vs. 25.36 h, respectively). Growth curves of MRC-5 vector control and I κ B α SR cells at P26 were constructed as described under experimental procedures. Data points represent the mean $\pm$ SEM of triplicate measurements [$*p < 0.05$, comparing MRC-5 I κ B α SR cells to control (MRC-5 vector) values by Student's *t*-test] (C) Immunoblots of p53, p21^{Cip1} and β -actin or lamin B proteins in cytoplasmic and nuclear extracts of confluent vector and I κ B α SR-expressing MRC-5 fibroblasts at P27. (D) MRC-5 were stably transduced with retrovectors delivering a control GL2 control shRNA or an IKK β shRNA (IKK β ^{KD}). Cells were selected in puromycin and total cell lysates extracted and analyzed for IKK β protein levels by immunoblotting. (E) Growth curves of MRC-5 GL2 and IKK β ^{KD} cells were constructed as described in Materials and Methods. MRC-5 IKK β ^{KD} cells had a significantly prolonged growth rate compared to their matched vector control (38.0 h vs. 25.1 h, respectively). Data points represent the mean $\pm$ SEM of triplicate measurements [$*p < 0.05$ for MRC-5 IKK β ^{KD} cells compared to control (MRC-5 GL2) values by Student's *t*-test]. (F) Immunoblotting for p53, p21^{Cip1} and β -actin in confluent, serum-stimulated MRC-5 GL2 and IKK β ^{KD} cells.

Fig. 5.

Inhibition of canonical NF- κ B signaling reduces IMR-90 normal human fibroblast proliferation. (A) Expression of an NF- κ B super repressor I κ B α SR was enforced in IMR-90 HDFs by stable retroviral transduction. Total proteins were extracted from G418-resistant confluent cultures and analyzed for the expression of I κ B α by immunoblotting with β -actin as a protein reference control. (B) IMR-90 I κ B α SR cells exhibited a prolonged growth rate of ~34 h vs. a faster ~26 h growth rate for their matched vector control. Growth curves of IMR-90 vector control and I κ B α SR cells at P14 were constructed as described in experimental procedures. Data points represent the mean $\pm$ SEM of triplicate measurements, * p <0.05, for IMR-90 I κ B α SR cell numbers relative to control (vector) values by Student's t -test. (C) Immunoblots of p53, p21^{Cip1} or β -actin and lamin B proteins in cytoplasmic and nuclear extracts of confluent vector and I κ B α SR-expressing IMR-90 fibroblasts at P15. (D) IMR-90 HDFs were stably transduced with retroviruses conferring puromycin resistance and co-expressing either a GL2 control or IKK β (IKK β ^{KD}) targeted shRNA. Cells were selected in puromycin and total cell lysates were analyzed for IKK β or β -actin expression by immunoblotting. (E) Growth curves of IMR-90 GL2 and IKK β ^{KD} cells were constructed as >described in Materials and Methods. MRC-5 IKK β ^{KD} cells had a significantly prolonged growth rate compared to their matched vector control (33.3 h vs. 24.2 h, respectively). Data points represent the mean $\pm$ SEM of triplicate measurements * p <0.05 for IMR-90 IKK β ^{KD} cells relative to their matched IMR-90 GL2 control values by Student's t -test. (F) Immunoblotting for p53 and p21^{Cip1} or β -actin in confluent, serum-stimulated IMR-90 GL2 and IKK β ^{KD} cells.

Fig. 6.

Blocking canonical NF- κ B activity in HDFs enhanced ROS levels and their accumulation in G2 or G2-M in conjunction with enhancing their levels of p53 and p21 in response to H₂O₂. (A) Accumulation of intracellular ROS in response to increasing concentrations of H₂O₂. Proliferating MRC-5 vector and MRC-5 I κ B α SR fibroblasts treated with increasing concentrations of H₂O₂ (0 - 200 μ M) in the presence or absence of NAC for 2 h or instead allowed to recover for 24 h after H₂O₂ exposure were analyzed by flow cytometry for endogenous ROS levels as described under Materials and Methods. Values represent the mean $\pm$ SEM of results from multiple experiments each performed in triplicate. Results were analyzed by paired two-way ANOVA, followed by Tukey post-hoc comparisons. Significant H₂O₂ dose-dependent increases in the percentages of ROS positive MRC-5 vector and MRC-5 I κ B α SR HDFs are indicated in comparison to their H₂O₂ untreated controls (* p <0.01). Significant decreases in ROS accumulation in NAC treated cells are also noted (# p <0.05). Finally, significant increases in the percentages of ROS positive MRC-5 I κ B α SR cells at 100, 200 mM H₂O₂ relative to their in vector controls at the same H₂O₂ concentration are also indicated (* p <0.05). (B) Proliferating MRC-5 vector and MRC-5 I κ B α SR cells were treated with increasing concentrations of H₂O₂ (0 - 200 μ M) for 2 h and then analyzed 24 h later by flow cytometry using a CycleTestTM Plus DNA reagent kit. Values represent the mean $\pm$ SEM of three independent experiments. Results were analyzed by paired two-way ANOVA, followed by Tukey post-hoc comparisons. Statistically significant differences in the cell cycle profiles of MRC-5 vector and I κ B α SR cells at each H₂O₂ dose in comparison to their untreated controls (H₂O₂ = 0) are indicated (* p <0.05). Statistically significant increases or decreases in the cell cycle profile of MRC-5 I κ B α SR cells in comparison to MRC-5 vector controls at the same H₂O₂ doses are also highlighted in the right side of Panel A (# p <0.05). (C) Immunoblotting for p53, p-p53(Ser20), p21^{Cip1}, lamin B and β -actin, expression in cytoplasmic and nuclear extracts of proliferating MRC-5 control and I κ B α SR fibroblasts. Data are representative of several independent experiments.

Fig. 7.

Suppressing canonical NF- κ B signaling accelerates the senescence response of MRC-5 HDFs in response to H₂O₂ treatment. SA- β -Gal staining was performed on the indicated proliferating cell populations with and without prior exposure to increasing H₂O₂ doses as described in Materials and Methods. Representative examples of SA- β -Gal staining observed in 3 independent experiments are shown in Panel A-C. (A) MRC-5 vector and MRC-5 I κ B α SR cells, (B) MRC-5 GL2 and IKK β ^{KD} cells. (C) MRC-5 vector and I κ B α SR cells with and without exposure to H₂O₂ as in Panel A but in conjunction with NAC to suppress ROS accumulation. (D) Comparisons of the percentages of SA- β -Gal positive cells in different populations of MRC-5 cells quantified from 3 independent experiments. Black bars in the left and right sides of Panel D show the percentages of SA- β -Gal stained MRC-5 vector control, MRC-5 I κ B α SR and MRC-5 GL2 cells with and without prior H₂O₂ treatment. Gray bars on the left side of Panel D quantify the effects of NAC on MRC-5 vector control and I κ B α SR cells with and without H₂O₂ exposure; and the gray bars in the right side of panel D show the percentages of senescent MRC-5 IKK β ^{KD} cells with and without prior H₂O₂ exposure. Symbols directly above individual bars refer to statistical comparisons between that specific bar's H₂O₂ dose and the same cells without H₂O₂ exposure. All data were analyzed by paired two-way ANOVA, followed by Tukey post-hoc comparisons and are presented as means $\pm$ SEM (* p <0.05, ** p <0.02, *** p <0.01, ## p <0.02, ### p <0.01). The senescence responses of MRC-5 I κ B α SR and MRC-5 IKK β ^{KD} cells (left and right sides of Panel D) showed no significant differences.

Fig. 8.

DNA damage and senescence marker detection in untreated and H₂O₂-treated proliferating MRC-5 vector and IκBαSR cells. (A) Formation of γ-H2AX foci: MRC-5 vector and MRC-5 IκBαSR HDFs at P28 and senescent MRC-5 vector cells at P59 were plated on small round coverslips in 6-well plates, media was replaced the next day and cells were treated with 200 μM H₂O₂ (except senescent cells) or left untreated and then stained 24 h later with specific antibodies to detect γ-H2AX foci, which were visualized and photographed under a laser scanning confocal microscope. Experiments were performed twice in duplicate with essentially the same results. (B) SAHF formation: MRC-5 vector and MRC-5 IκBαSR cells at P28 and senescent MRC-5 vector cells at P59 were plated on cover slips and treated with H₂O₂ as described in (A) and then stained 24 h later with specific antibodies to detect HP1β and HP1α foci, which were visualized and photographed under a laser scanning confocal microscope. Experiments were performed twice in duplicate and at least three cover slips of each cell type were examined with essentially the same results.

Fig. 9. p53 knock-down delays the premature senescence of control and H₂O₂-treated MRC-5 and MRC-5 canonical NF-κB compromised cells. (A) Two days post infection with a high titer RS-Puro-p53^{KD} retrovirus, cells were analyzed for p53 and β-actin expression by immunoblotting. (B) Untreated and H₂O₂-treated MRC-5 carrying an empty vector or IkBaSR and their corresponding p53^{KD} cells were stained for SA-β-Gal expression. Cells were allowed to recover for 4 days post-H₂O₂ treatment. Images shown are representative of three independent experiments. (C) Comparisons of the percentages of SA-β-Gal positive cells quantified from 3 independent experiments. Results were analyzed by paired two-way ANOVA, followed by Tukey post-hoc comparisons with all values presented as mean ± SEM. Symbols above specific connected bars represent the following statistically significant differences: *p<0.05, **p<0.02; ***p<0.01, ####p<0.01.

Fig. 10. Suppressing canonical NF-κB activity in A549 cells enhances their G2 or G2-M phase cell cycle block and accumulation of p53 and p21^{Cip1} in response to H₂O₂ treatment. (A) A549 cells were treated with increasing concentrations of H₂O₂ (0 - 1000 μM) and cell cycle profiles were determined by flow cytometry with a cycle testTM plus DNA reagent kit. Values are the mean ± SEM of three independent experiments. Results were analyzed by paired two-way ANOVA, followed by Tukey post-hoc comparisons. Statistically significant differences in the cell cycle profiles of A549 vector and IκBαSR cells in response to H₂O₂ dose in comparison to untreated controls (H₂O₂ = 0) are indicated (*p<0.05). Statistically significant increases or in the percentages of A549 IκBαSR cells residing in each cell cycle phase in comparison to A549 vector controls at the same H₂O₂ doses are also highlighted in the right side of Panel A (#p<0.05). (B) Left side: Western blotting showing IκBα expressed by A549 vector control and A549 IκBαSR cells. Right side: Immunoblots representative of several independent experiments showing expression of p53 and p21^{Cip1} in response to increasing concentrations of H₂O₂ in A549 vector and A549 IκBαSR cells. β-actin served as a protein loading reference control.

Fig. 11.

Suppression of canonical NF- κ B signaling sensitizes A549 cells to H₂O₂-mediated apoptosis. (A) A549 vector and A549 I κ B α SR cells were treated with increasing concentrations of H₂O₂ (0 - 1000 μ M) and subjected to Annexin V/PI staining followed by flow cytometric analysis to quantify apoptosis. Values are the mean $\pm$ SEM of multiple independent experiments. Results were analyzed by paired two-way ANOVA, followed by Tukey post-hoc comparisons. Statistically significant differences in the viability of A549 vector and I κ B α SR cells at each H₂O₂ dose in comparison to untreated controls (H₂O₂ = 0) are indicated (* p <0.05). Statistically significant increases or decreases in the viability profiles of A549 I κ B α SR cells in comparison to A549 vector controls at the same H₂O₂ doses are also highlighted in the right side of Panel A (# p <0.05). Both early apoptotic (EA) and late apoptotic-necrotic cells (LA-N) were quantified. FACS gating of cell subpopulations is shown in Fig. S3. (B) Immunoblot analysis for the expression of caspase-3 and PARP-1 in total cell extracts as indicated. (C) Immunoblot analysis of PARP-1 in cytoplasmic and nuclear extracts prepared from untreated and H₂O₂-treated A549 Vector and A549 I κ B α SR cells. β -actin served as a reference control. Immunoblots in (B) and (C) for PARP-1 and caspase-3 were overexposed to detect the cleaved forms of the corresponding proteins. Results are representative of several independent experiments.

Fig. 12.

Generation and characterization of IKK β -knockdown A549 cells. (A) A549 cells were stably transduced by a puromycin resistance retrovirus harboring either a GL2 control or an IKK β targeted shRNA (IKK β ^{KD}). Cells were selected in puromycin and total cell lysates extracted from 12 clones were randomly selected for analysis of IKK β expression by immunoblotting. One specific A549 IKK β ^{KD} clone with >80% IKK β ablation was chosen for subsequent experiments. (B) Immunoblotting for p53, caspase-3, PARP-1 and β-actin proteins in H₂O₂-treated A549 GL2 and A549 IKK β ^{KD} cells. (C) A549 GL2 and A549 IKK β ^{KD} cells were treated with increasing concentrations of H₂O₂ (0 - 1000 μM) and subjected to Annexin V/PI staining followed by flow cytometric analysis to quantify apoptosis. Values are the mean ± SEM of several independent experiments each performed in triplicate. Results were analyzed by paired two-way ANOVA, followed by Tukey post-hoc comparisons. Significant differences in percentages of live and apoptotic A549 vector and A549 IKK β ^{KD} cells induced by increasing H₂O₂ concentrations in comparison with untreated controls (H₂O₂ = 0) for each experimental series are indicated (*p<0.05). Significant increases or decreases in the percentages of A549 IKK β ^{KD} apoptotic or live cells respectively in comparison to control (GL2) values at the same H₂O₂ concentration are also shown (#p<0.05).

Table 1Life spans of control and canonical NF- κ B-compromised HDFs *

Cell Type	Lifespan (Population Doublings; PDL $\pm$ SD)
MRC-5 Vector	57 $\pm$ 2
MRC-5 I κ B α SR	45 $\pm$ 1.4
MRC-5 GL2	56 $\pm$ 1.5
MRC-5 IKK β ^{KD}	44 $\pm$ 2.1
IMR-90 Vector	38.5 $\pm$ 2.1
IMR-90 I κ B α SR	29 $\pm$ 1.4
IMR-90 GL2	37.5 $\pm$ 2.1
IMR-90 IKK β ^{KD}	28.5 $\pm$ 2.1

* Life spans are expressed as the number of population doublings (PDLs) before cells stopped proliferating. PDLs were determined as described in Supplementary Materials and Methods. Data shown are the averages of two independent experiments.