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Chronic NF- κ B activation delays RasV12-induced premature senescence of human fibroblasts by suppressing the DNA damage checkpoint response

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Abstract

Normal cells divide for a limited number of generations, after which they enter a state of irreversible growth arrest termed replicative senescence. While replicative senescence is due to telomere erosion, normal human fibroblasts can undergo stress-induced senescence in response to oncogene activation, termed oncogene-induced senescence (OIS). Both, replicative and OIS, initiate a DNA damage checkpoint response (DDR) resulting in the activation of the p53-p21^{Cip1/Waf1} pathway. However, while the nuclear factor-kappaB (NF- κ B) signaling pathway has been implicated in DDR, its role in OIS has not been investigated. Here, we show that oncogenic Ha-RasV12 promoted premature senescence of IMR-90 normal human diploid fibroblasts by activating DDR, hence verifying the classical model of OIS. However, enforced expression of a constitutively active IKK β T-loop mutant protein (IKK β ca), significantly delayed OIS of IMR-90 cells by suppressing Ha-RasV12 instigated DDR. Thus, our experiments have uncovered an important selective advantage in chronically

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activating canonical NF- κ B signaling to overcome the anti-proliferative OIS response of normal primary human fibroblasts.

Keywords

Human fibroblasts; Ha-RasV12; IKK β ca; NF- κ B; senescence; DNA damage

1. Introduction

Normal diploid mammalian cells undergo a finite number of cell divisions in culture, a phenomenon termed cellular senescence (Campisi, 2005; Campisi and d'Adda di Fagagna, 2007). Several possible mechanisms have been suggested to explain the manner in which diploid cells senesce and by which immortal cells evade senescence. In addition to telomere length and telomerase activity (d'Adda di Fagagna et al., 2003; Herbig et al., 2004; Sedelnikova et al., 2004), the pRb and p53 pathways are also involved in regulating cellular senescence through the CDKN2A locus which encodes the p16^{INK4A} and ARF proteins by alternative splicing in both human and mouse cells (Hahn and Weinberg, 2001; Serrano and Blasco, 2001; Campisi, 2005; Campisi and d'Adda di Fagagna, 2007).

Telomere shortening is not the only inducer of the senescent phenotype. Normal cells possess anti-proliferative mechanisms to counteract the consequences of oncogenic mutations, and these natural cell defenses are often disrupted during tumor development. These anti-proliferative mechanisms are often activated in response to oncogenic stress that delivers excess mitogenic signaling leading to cell growth arrest or premature cell senescence (Hahn and Weinberg, 2001). Oncogenic Ha-RasV12 (RasV12 thereafter) (Franza et al., 1986; Serrano et al., 1997; Lin et al., 1998; Woo and Poon, 2004) or Raf (Zhu et al., 1998) promote uncontrolled mitogenesis but when expressed in primary cells including normal human diploid fibroblasts (HDFs) and rodent cells they provoke a permanent cell cycle arrest with features of senescence, in the absence of telomere shortening (Serrano and Blasco, 2001; Hahn and Weinberg 2001; Campisi, 2005; Campisi and d'Adda di Fagagna, 2007). Induction of human cell senescence by oncogenic RasV12 is accompanied by increased expression of p16^{INK4a} and requires the MAPK cascade (Ras-Raf-MEK) (Serrano et al., 1997; Lin et al., 1998). These anti-proliferative responses of primary cells to activated oncogenes also explain why the formation of a transformed cell clone often depends on the properties of additional altered genes, which neutralize the anti-proliferative defense mechanism that was triggered by the primary activated oncogene (Weinberg, 1997; Hahn and Weinberg, 2001). Because it entails an essentially irreversible growth arrest, the p53 and pRb dependent senescence responses induced by either telomere shortening or oncogenic stimuli likely represents a highly conserved surveillance-like, tumor suppressive mechanism (Hahn and Weinberg, 2001; Campisi, 2005; Campisi and d'Adda di Fagagna, 2007).

Induction of senescence due to telomere erosion (d'Adda di Fagagna et al., 2003; Herbig et al., 2004) or oncogenic stress (Bartkova et al., 2006) such as that induced by RasV12 (Di Micco et al., 2006; Di Micco et al., 2007; Mallette et al., 2007) initiates a DNA double strand break (DSB) checkpoint response (DDR), which involves activation of the kinases ATM/ATR, Chk1 and Chk2 and their downstream effector p53 (Bartek and Lukas, 2007; Hemann and Narita, 2007; Di Micco et al., 2007; Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008). Previous studies showed that oncogenic RasV12 induces ARF (Palmero et al., 1998) leading to the induction of p53 phosphorylation at serine 15, a target site of ATM/ATR, and ARF which has been implicated in the modulation of NF- κ B function by repressing the transcriptional activity of the anti-apoptotic Rel (p65) NF- κ B subunit (Rocha et al., 2003; Rocha et al., 2005). Furthermore, in response to DSBs ATM activation induces the inhibitor

of NF- κ B kinase (IKK) complex (Li et al., 2001) and after the induction of DSBs, NEMO/IKK γ was also shown to associate with ATM to directly orchestrate IKK activation (Wu et al., 2006). This DNA damage-induced NF- κ B signaling response has been proposed to be necessary for cell survival during the DDR (Jansens and Tschopp, 2006; Wu and Miyamoto, 2007; Ahmed and Li, 2008; Brzóska and Szumiel, 2009).

The NF- κ B transcription factors are pivotal regulators of gene expression programs culminating in stress-like responses. They bind to DNA as hetero- or homodimers that are selectively derived from five possible subunits (RelA/p65, c-Rel, RelB, p50 and p52). All NF- κ B family members contain an N-terminal Rel homology domain that mediates DNA binding and dimerization and a nuclear localization domain. The Rel subfamily members RelA/p65, c-Rel and RelB also contain a C-terminal transactivation domain which is absent in the p50 and p52 subunits. In addition the p50 and p52 subunits are processed from precursor proteins p105 (NF- κ B1) and p100 (NF- κ B2), respectively. The p50/p65 heterodimers are bound to I κ Bs (inhibitors of NF- κ B) thereby sequestering them in the cytoplasm of most cells in the absence of a stress-like response. Kinases that directly activate NF- κ B mediate the site-specific phosphorylation of two amino terminal serines on each I κ B (serines 32 and 36 of I κ B α), which makes adjacent lysines targets for ubiquitination thereby resulting in 26S proteasome mediated I κ B degradation. NF- κ B is then free to translocate to the nucleus and bind DNA leading to the activation of a host of target genes. I κ B phosphorylation is mediated by a high molecular weight signalsome complex comprising two direct I κ B kinases, IKK α and IKK β , and two molecules of a regulatory, docking/adaptor protein, NEMO. IKK α and IKK β are serine/threonine kinases possessing an amino-terminal catalytic domain and two carboxyl-proximal interaction motifs resembling leucine zipper and helix-loop-helix domains. Activation of IKK β depends upon signal induced phosphorylation of serines 177 and 181 in its T-activation loop (Hayden and Ghosh, 2004; Karin and Greten, 2005; Scheidereit, 2006; Perkins, 2006; Perkins, 2007).

Whereas the expression and activity of NF- κ B have been extensively studied following oxidative stress or during inflammation, apoptosis and transformation, the available data on potential roles of NF- κ B in ageing-related changes, and in particular during *in vitro* replicative senescence of human fibroblasts, is relatively scant (Gosselin and Abbadie, 2003). Moreover, contrary to the aging-induced up-regulation of NF- κ B binding activities in tissues (Gosselin and Abbadie, 2003; Adler et al., 2007; Kriete and Mayo, 2009), data from cultured human cells have also produced apparently conflicting observations (Dimri and Campisi, 1994; Aggarwal et al., 1995; Helenius et al., 1996; Helenius et al., 1999; Ikebe et al., 2000) and suggested a cell-intrinsic activation of NF- κ B different from the canonical pathway (Kriete et al., 2008; Kriete and Mayo, 2009).

Oncogenic RasV12 can activate multiple effector pathways that give rise to different outputs depending on the cellular context. Thus, RasV12 can induce senescence of primary cells (Serrano et al., 1997), cooperate with other genes to induce neoplastic transformation (Hahn and Weinberg, 2001), and also suppress or induce apoptosis (Cox and Der, 2003). While OIS of primary cells was shown to be due to DDR (Di Micco et al., 2006; Bartkova et al., 2006; Mallette et al., 2007; Di Micco et al., 2007) and DSBs activate NF- κ B (Jansens and Tschopp, 2006; Habraken and Piette, 2006; Wu and Miyamoto, 2007), it remains unclear whether NF- κ B activation influences the outcome of OIS.

Here we directly assessed the consequences of chronic NF- κ B activation in OIS by the classical model of oncogenic RasV12-induced senescence of IMR-90 HDFs. IMR-90 cells stably expressing oncogenic RasV12 or IKK β ca (a constitutively active IKK β T-loop mutant) or both genes together were generated by stable retroviral transduction. We found that IKK β ca delayed oncogenic RasV12-induced premature senescence of IMR-90 by suppressing the DDR triggered by oncogenic stress.

2. Experimental procedures

2.1. Cell Culture

Human diploid fibroblasts IMR-90 and amphotropic phoenix cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. To retain equivalence between passage number and population doublings (Pdl), a 1:2 split was counted as 1 Pdl and a 1:4 as 2 Pdl.

2.2. Retroviral vectors and infections

The retroviral vectors used were: pBabe-Hygro, pWZLH-Ha-RasV12 (71), CLXSN-ires-GFP (CLXSN-iG) and CLXSN-IKKβca-iG carrying a constitutively active IKKβ T-loop mutant protein of human IKKβ (denoted IKKβca). A constitutively activated human Flag-IKKβ (IKKβca) mutant in pcDNA3.1 was generated by changing the T-loop activation serines (177 and 181) to glutamic acids with a QuickStart PCR mutagenesis kit (Stratagene) (PE Massa and KB Marcu, unpublished data). The IKKβca cassette was removed from pcDNA3.1 and placed under the control of a moloney 5' LTR in CLXSN-iG by standard sub-cloning procedures. CLXSN-iG expresses a neomycin resistance gene (Neo) under the control of the SV40 promoter/enhancer (SN cassette) and IKKβca was inserted in between the retroviral LTR and an Ires-GFP sequence upstream of the vector's SN cassette (Zhang et al., 2005).

Young IMR-90 cells (P11) were infected with high-titre retroviruses carrying either oncogenic RasV12 or IKKβca or both genes together or with the corresponding control vectors, generated following transfection of amphotropic phoenix cells. Large polyclonal populations of stable retroviral transduced cells were obtained by selection in hygromycin B (HygroB) or G418 for two and three weeks, respectively. Neo-resistant IKKβca cells were infected with a RasV12-Hygro virus and submitted to selection in HygroB and G418 for an additional three weeks. HygroB- and/or G418-resistant polyclonal cell populations were used in all subsequent analyses.

2.3. Analysis of growth properties

Uninfected and retrovirus-infected IMR-90 were plated at a density of 2×10^4 cells per well in 24-multiwell plates. Cell growth was monitored by counting the cells from two wells every two days using a hemocytometer over a period of 12 days. The experiment was repeated three times and growth curves were constructed.

Exponentially growing IMR-90 cells were subjected to flow cytometric analysis using a cycle test™ plus DNA reagent kit (Becton Dickinson) according to manufacturer's instructions and analyzed on a FACScan Becton Dickinson flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Cell morphologies were observed under a Zeiss Model Axiovert S100, Germany and photographed.

2.4. Analysis of senescence-associated β-galactosidase (SA-β-Gal)

Senescent cells were identified by a histochemical stain for SA-β-Gal activity using a senescent cell staining kit (CS0030; Sigma) according to manufacturer's instructions.

2.5. Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts were prepared as described (Dimri et al., 1994). Protein concentration was determined using a Roti-Quant reagent (Roth, Germany).

2.6. Isolation of nuclei

IMR-90 cells were collected by centrifugation and washed in ice-cold PBS at 4°C. The cell pellets were lysed in TITE buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.2% Triton X-100) by incubation for 5 min on ice and disrupted by vortexing. The resulting cytoplasmic and nuclear suspension was layered over a cushion (10% sucrose in TITE buffer) and centrifuged at 800 g for 10 min at 4°C. The supernatant was removed without disrupting the nuclear pellet and the nuclei were resuspended in lysis buffer (Wolgemuth and Hsu, 1981; Reddy et al., 1996). Protein concentration was determined using a Roti-Quant reagent (Roth, Germany).

2.7. Isolation of total proteins and western blot analysis

Total proteins were extracted from uninfected and retrovirus-infected IMR-90 cells with RIPA buffer as described (Kolettas et al., 2006) and protein concentration was determined using a Roti-Quant reagent (Roth, Germany). Protein samples were analyzed by SDS-PAGE followed by immunoblotting. Antibodies used were: goat polyclonal antibodies to NF- κ B p50 (sc-1190), IKK β (sc-7330), I κ B α (sc-371G), rabbit polyclonal antibodies to NF- κ B p65 (sc-372), Ha-Ras (sc-520), phospho-p53 (Ser20) (sc-18079R), Chk2 (sc-9064), phospho-Chk2 (T68) (#2661, Cell Signalling), lamin B (clone 16) and mouse monoclonal antibodies to IKK α (sc-7606), phospho-I κ B α (Ser32/36) (sc-8404), cyclin D1 (sc-20044), Cdc6 (sc-9964), p53 (DO1, sc-126), phospho-p53 (Ser15) (#9286, Cell Signaling), p21^{Cip1/Waf1} (sc-6246; sc-817; OP64 Calbiochem) and β -actin (CloneAC15, A5441; Sigma), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology). Antibody binding was detected using the ECL detection kit (GE HealthCare). Densitometric analysis was performed using Quantity One, Version 4.6 (Bio-Rad), according to manufacturer's instructions.

2.8. RNA preparation, cDNA synthesis and SYBR green real-time PCR

Total RNA was extracted from IMR-90 Neo, IMR-90 IKK β ca and from IMR-90 cells treated or untreated with 100 ng/ml with TNF α for 2 h, using the LiCl-Urea method. Total cellular RNAs (1 μ g) were converted to cDNAs with Superscript II Reverse transcriptase (Invitrogen) and cDNAs (100 ng) were submitted to SYBR green quantitative real-time PCR analysis (SYBR GreenER qPCR SuperMix Universal, Invitrogen) using 1 μ M of forward and reverse primers in a final 20 μ l master mix volume. PCR amplifications were carried out in a Rotor-gene 3000 machine (Corbett). The following oligonucleotide primer pairs were designed with BioRad Beacon Designer 2.0 software and were purchased from Invitrogen. Forward (F) and reverse (R) PCR primer sequences for each mRNA were as follows: Human I κ B α : F (5'-GCTGAAGAAGGAGCGGCTA-3') and R (5'-CTGGCTGGTTGGTGATCA-3'); Human IL-6: F (5'-GCCACTCACCTCTTCAGAA-3') and R (5'-GTACTCATCTGCACAGCTCT-3'); Human MCP-1/CCL2: F (5'-GCATGAAAGTCTCTGCCG-3') and R (5'-GAGTGTTCAAGTCTTCGGA-3'); Human GAPDH (reference/normalization control): F (5'-TGGTATCGTGGAAGGACTCA-3') and R (5'-GCAGGGATGATGTTCTGGA-3'). PCR reactions were done as follows: 50°C for 2 min; 95°C for 10 min; 50 cycles: 95°C for 15 sec, 60°C for 60 sec. PCR product melting temperature curves were derived by measuring the fluorescence during a period of warming from 72-95°C, and each primer pair produced a single species melting curve verifying their specificity. PCR product sizes were also confirmed by agarose gel electrophoresis. Relative yields of PCR products were quantified on the basis of their threshold cycle (Ct) values, which are proportional to log₁₀ mRNA copy number (Dussault and Pouliot, 2006; Contreras-Galindo et al., 2006). All cDNAs were normalized versus one another by comparing their individual glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels against the median expression level for the cDNA set with GAPDH correction also used as a normalization factor for the experimental primer data.

3. Results

3.1. Expression of NF- κ B signaling components during replicative senescence

To investigate whether the expression levels of NF- κ B signaling components changed during replicative senescence of IMR-90 cells, young IMR-90 cells were propagated continuously *in vitro* until they reached the end of their lifespan. IMR-90 senesced after culturing for ~39 - 42 population doublings (Pdl) (~P42). Cytoplasmic and nuclear extracts were extracted from asynchronous cultures of IMR-90 cells at 15-40 Pdl and analyzed for the expression of IKK α , IKK β , NF- κ B p50 and p65 subunits and I κ B α by immunoblotting. Neither cytoplasmic nor nuclear levels of these NF- κ B signaling components were altered during continuous subculturing of IMR-90 or as the cells approached senescence. No changes were observed for I κ B α , which was primarily expressed in the cytosol (Fig. 1A). NF- κ B p50 and p65 subunits were present in the cytosolic and nuclear compartments with expression levels higher in the former and with p65 being more abundant than p50. The I κ B kinases, IKK α and IKK β , were also expressed at different levels. IKK α was expressed in the cytosol and nucleus but it was more abundant in the cytoplasm. In contrast, IMR-90 expressed much lower levels of IKK β , compared to IKK α , and its expression was similar in both cytosolic and nuclear compartments (Fig. 1A), suggesting that IKK β may be the limiting factor contributing to NF- κ B activation in these cells. Moreover, cyclin D1, an NF- κ B-target gene, (Kucharczak et al., 2003; Scheidereit, 2006) was present at similar levels in the cytosolic and nuclear compartments (Fig. 1A), in keeping with NF- κ B signaling not being activated during replicative senescence of normal IMR-90 fibroblasts. To further verify these results differences IKK α and IKK β and cyclin D1 levels were also analyzed in total cell lysates extracted from IMR-90 at different Pdl (Fig. 1B). Again no changes in the expression of either IKK or cyclin D1 were detected during replicative senescence of IMR-90 fibroblasts and IKK α was expressed at much higher levels than IKK β (Fig. 1B).

3.2. Generation of RasV12- and/or IKK β ca-expressing IMR-90 cells

Next the classical model of RasV12-induced premature senescence was employed in the context of IMR-90 HDFs to investigate the effects of sustained NF- κ B activation on OIS. We generated young IMR-90 cells (P11) which stably express oncogenic RasV12, IKK β ca, or IKK β ca in combination with RasV12 by stable retroviral transduction (Fig. 2A). RasV12 was stably introduced with a retrovector that confers hygromycin resistance and IKK β ca was retrotransduced along with GFP and neomycin resistance genes in the moloney pCLXSN-iG retroviral vector with IKK β ca and GFP ORFs separated by an internal ribosome entry site (IRS). After several weeks of selection for populations of stable drug resistant cells (see Experimental procedures), total cell lysates were extracted from confluent, uninfected IMR-90, IMR-90 RasV12 (Hygro^R), or IKK β ca (G418^R) and their corresponding empty vector controls, which were immunoblotted for RasV12 and IKK β ca proteins (Fig. 2B). While all the different cell types expressed the endogenous IKK β , a second band of slightly higher molecular weight was present in IMR-90 IKK β ca and IMR-90 IKK β ca/RasV12 cells corresponding to the IKK β ca mutant protein, which was importantly expressed at comparable levels to the endogenous wild-type IKK β protein (Fig. 2B). While IMR-90 RasV12 and IMR-90 IKK β ca/RasV12 cells expressed high levels of oncogenic RasV12 protein, uninfected, vector- or IKK β ca-expressing cells expressed only very low levels of the endogenous p21^{Ha-ras} oncoprotein (Fig. 2B).

3.3. IKK β ca but not RasV12 activated NF- κ B in IMR-90 fibroblasts

To initially investigate whether RasV12 or IKK β ca activated NF- κ B signaling in IMR-90 cells, the levels of I κ B α and phospho-I κ B α (Ser32/36) were investigated by immunoblotting in total cell lysates (Fig. 3A). Although no apparent changes in the levels of total I κ B α were detected in any cell type, p-I κ B α (Ser32/36) levels were ~5- and ~3-fold higher in IMR-90 IKK β ca and

IMR-90 IKK β ca/RasV12, respectively, compared to their control counterparts; and its expression was also lower in RasV12 cells (Fig. 3A). Thus the accumulation of p-Ik β a (Ser32/36) was due to the action of IKK β ca.

Next proteins were extracted from isolated nuclei of the different cell types and analyzed for the expression of p50, p65 and Ik β a (Fig. 3B). Expression of IKK β ca induced the nuclear accumulation of p50 and p65 subunits of NF- κ B in IMR-90 IKK β ca and also (albeit to a somewhat lesser extent) in IMR-90 IKK β ca/RasV12 cells compared to their control counterparts. No expression of Ik β a was detected in the nuclei of all the different cell types (Fig. 3B). Moreover the total levels of cytoplasmic Ik β a remained unchanged in IKK β ca cells most likely because Ik β a was simultaneously subjected to continuous IKK β ca induced degradation and canonical NF- κ B dependent re-expression. On balance, such a cycle of continuous synthesis and breakdown of Ik β a in the context of enforced IKK β ca expression contributed to the enhanced nuclear accumulation of p50/p65 heterodimers, thereby resulting in sustained canonical NF- κ B activation.

We further verified that enforced expression of IKK β ca led to sustained NF- κ B activation in IMR90-IKK β ca cells by quantifying the expression levels of several canonical, IKK β dependent NF- κ B target genes (including Ik β a, IL-6 and MCP-1/CCL2) by SYBR green real-time RT-PCR. Relative to their matched empty vector control (IMR-90 Neo), IMR-90-IKK β ca cells expressed significantly higher levels of each of these canonical NF- κ B targets, which were only somewhat lower than their relative ranges of expression observed in IMR-90 fibroblasts in response to 2 h of TNF α stimulation (Fig. 3C).

3.4. Oncogenic RasV12 promoted premature senescence of IMR-90 cells, which was blocked by IKK β ca

To study the effects of oncogenic RasV12 and IKK β ca acting singly or together, the growth rates, cell cycle profiles (Fig. 4), morphologies and the expression of senescence-associated β -galactosidase (SA- β -Gal) were investigated (Fig. 5). Analysis of the growth rates showed that overexpression of RasV12 but not IKK β ca respectively reduced the growth rates of IMR-90 at P18 and P25 as compared to their control counterparts. Specifically, the growth rate of IMR-90 RasV12 (Hygro^R) was a prolonged 52.5 h compared to IMR-90 Hygro (Hygro empty retrovector), IMR-90 Neo (CLXSN-iG) and IMR-90 IKK β ca (CLXSN-iG-IKK β ca), which had similar far faster growth rates of 28.3 h, 29.6 h and 27.5 h, respectively. Moreover IMR-90 cells co-expressing IKK β ca and RasV12 at p25 displayed an intermediate growth rate of 39.7 h, indicating that the co-expression of IKK β ca conferred a growth advantage to IMR-90 IKK β ca/RasV12 cells (Fig. 4A).

Next, cell cycle profiles were examined by flow cytometric analysis of proliferating, asynchronous cultures of each of the different IMR-90 cell populations. IMR-90 RasV12 (Hygro^R) at P18 exhibited a similar cell cycle profile to Hygro-empty vector expressing IMR-90 fibroblasts at P25 (Fig. 4B), despite a reduced growth rate observed over 12 days (Fig. 4A). In contrast, IMR-90 RasV12 cells at P25 displayed a cell cycle profile similar to senescent IMR-90 cells at P42, with most cells accumulating in the G1 phase of the cell cycle. Prolonged RasV12 expression retarded cellular proliferation of IMR-90 presumably due to excessive mitogenic signals transmitted by RasV12 (Lumpkin et al., 1986;Franza et al., 1986;Serrano et al., 1997;Lin et al., 1998). In contrast, IMR-90 IKK β ca cells exhibited a similar growth rate and cell cycle profile to IMR-90 Neo fibroblasts harboring empty CLXSN-iG retrovector. We further investigated the growth of MRC-5 Neo control and MRC-5 IKK β ca-Neo fibroblasts over a period of 12 days (after selecting for Neo resistant cell populations for up to 3 weeks as described in Materials and Methods). Under these experimental conditions, MRC-5 IKK β ca cells displayed a similar growth profile and comparable growth rates to their Neo-expressing counterparts (Fig. S1 A), which was further confirmed by flow cytometric analysis of MRC-5

Neo and MRC-5 IKK β ca (Fig. S1 B). Thus the chronic, long term activation of canonical NF- κ B signaling (i.e. for ~4-5 weeks) by IKK β ca did not visibly alter the growth of either human fibroblast strain used, IMR-90 or MRC-5. However, co-expression of IKK β ca and RasV12 resulted in an intermediate growth rate and a cell cycle profile with a higher percentage of cells in G1, somewhat between that observed in IMR-90 RasV12 and IMR-90 IKK β ca cells, and a percentage of cells in G2 similar to Neo- or IKK β ca-expressing IMR-90 fibroblasts. Taken together these data show that IKK β ca/RasV12 cells cycle at a slower rate than IKK β ca- or vector-expressing IMR-90 cells (Fig. 4) but faster than IMR-90 RasV12 cells revealing that IKK β ca conferred a growth advantage to IMR-90 cells co-expressing RasV12.

Next to investigate the onsets of cellular senescence in each cell background, the different IMR-90 cells were propagated continuously in culture until they reached the end of their lifespan. While uninfected IMR-90 fibroblasts reached about 39 - 42 Pdl, cells infected either with the control retroviruses or with IKK β ca reached about 39 and 38 Pdl, respectively. Similarly, another human fibroblast strain, MRC-5 stably expressing either Neo or IKK β ca, were extensively propagated until the end of their lifespan, which was in the range of 58-60 Pdl. No signs of premature senescence of MRC-5 IKK β ca cells compared to their Neo-expressing control counterparts were observed (data not shown). In contrast IMR-90 RasV12 cells entered a state of permanent growth arrest at ~P23 - P25, which was about 10 Pdl after selection of the stably infected cells. However, IMR-90 IKK β ca/RasV12 cells reached ~33 - 34 Pdl, suggesting that ectopic IKK β ca expression delayed RasV12-induced premature senescence of IMR-90 fibroblasts. In addition, the morphological characteristics of IMR-90 RasV12 P25 cells resembled that of a normal senescent fibroblast phenotype with a characteristic flattened and enlarged morphology. In contrast, IMR-90 IKK β ca and IMR-90 IKK β ca/RasV12 cells presented a normal cellular morphology (Fig. 5). All the different cell types at ~P25 were also stained for SA- β -Gal expression. While IMR-90 RasV12 cells displayed strong SA- β -Gal staining similar to senescent IMR-90 cells at P39, uninfected IMR-90, vector or IMR-90 IKK β ca and IMR-90 IKK β ca/RasV12 cells were not positive for SA- β -Gal, and exhibited only background staining (Fig. 5).

3.5. Analysis of cell cycle regulatory proteins

Next to further investigate the basis for the distinctive cycle profile of each cell type we examined the expression of several essential cell cycle checkpoint regulatory proteins including cyclin D1, Cdc6, p53 and p21^{Cip1/Waf1} (Fig. 6A). Overexpression of RasV12, and to a lesser extent IKK β ca, resulted in an increase in cyclin D1 protein levels, which were sustained in the IKK β ca/RasV12 cells. Further, while RasV12 induced the expression of Cdc6 and p53, IKK β ca, acting together with RasV12, suppressed their expression to levels similar to those detected in IMR-90 IKK β ca cells; and Cdc6 expression levels in IMR-90 IKK β ca cells appeared to be comparable to their empty vector controls (Fig. 6A). In addition to the 53 kDa band, a faster migrating p53 band was also detected which was strongly expressed only in IMR-90 RasV12 cells (Fig. 6A). Importantly, enforced expression of IKK β ca suppressed both the basal and RasV12-induced expression levels of p53 in IMR-90 fibroblasts (Fig. 6A). Similar findings were obtained in IMR-90 cells expressing p65 either alone or together with RasV12 (see Supplementary Figure 2S). Further, overexpression of both RasV12 and to a lesser degree IKK β ca acting alone induced the expression of the p53 target protein, p21^{Cip1/Waf1}, the expression of which was sustained in the IMR-90 IKK β ca/RasV12 cells. Moreover, while no apparent increase in p53 levels was detected, p21^{Cip1/Waf1} levels were higher in IMR-90 IKK β ca cells than their corresponding control counterparts, suggesting that p21^{Cip1/Waf1} induction by IKK β ca was p53-independent (Fig. 6A). There was no appreciable expression of ARF in any of the different cell types. Thus, while RasV12 induced the expression of the p53 - p21^{Cip1/Waf1} axis, IKK β ca only contributed to p21^{Cip1/Waf1} induction, which has been

reported to be a target of activated canonical NF- κ B in other cells (Wuerzberger-Davis et al., 2005; Chang and Miyamoto, 2006).

To further analyze the regulated expression of cyclin D1 and p21^{Cip1/Waf1} in the IKK β ca cells in response to serum stimulation, time course experiments were performed with asynchronous sub-confluent cells. Cyclin D1 levels were higher in IKK β ca compared to vector control cells, but there were no significant fluctuations in its levels during the serum stimulation time course in either cell background, although a slight induction was observed in the control cells at 8 h following serum stimulation (Fig. 6B). Similarly, while the expression of p21^{Cip1/Waf1} was low to undetectable in IMR-90 Neo cells over a 24 h time period, it was induced in IMR-90 IKK β ca cells at 4 h, peaking at 12 h and sustained thereafter (Fig. 6B).

3.6. IKK β ca delayed RasV12-induced senescence by suppressing the DNA damage response

To determine whether oncogenic RasV12 promoted premature senescence through the activation of DDR, as previous reported (Di Micco et al., 2006; Mallette et al., 2007; Di Micco et al., 2007; Campisi and d'Adda di Fagagna, 2007) and then investigate the concomitant effects of IKK β ca on this response, we examined the expression of several protein effectors of DDR (Fig. 7). Immunoblot analysis showed that while overexpression of RasV12 triggered the phosphorylation of Chk2 at Thr68 without altering its overall levels, this effect was abrogated in IMR-90 IKK β ca/RasV12 cells (Fig. 7). Similarly, overexpression of RasV12 induced the expression of p53 and also triggered its stabilizing phosphorylations at Ser15 and Ser20. In contrast none of these effects on p53 were observed in the IKK β ca/RasV12 cells, but instead IKK β ca expressing cells appeared to destabilize p53 (Fig. 7). Taken together our data have shown that constitutive activation of canonical NF- κ B signaling delayed RasV12 oncoprotein provoked premature senescence of IMR-90 normal human diploid fibroblasts by allowing these cells to avoid the ensuing RasV12 activated DNA damage checkpoint response pathway.

4. Discussion

Oncogene-induced senescence (OIS), occurring both *in vitro* and *in vivo* has been proposed to be a safeguard against tumorigenesis (Narita and Lowe, 2005; Di Micco et al., 2007; Courtois-Cox et al., 2008; Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008). However, if cells acquire mutations in critical genes that act as breaks to tumor formation, then oncogenic RasV12, although not sufficient on its own, may contribute to transformation of normal HDFs (Hahn and Weinberg, 2001; Brookes et al., 2002; Huot et al., 2002; Campisi, 2005). In addition, normal cells possess various survival mechanisms that allow them to overcome noxious stimuli leading to DNA damage, among them being the NF- κ B signaling pathway (Habraken and Piette, 2006; Janssens and Tschopp, 2006; Wu et al., 2006; Wu and Miyamoto, 2007). IKK β is activated in a NEMO-dependent manner in response to genotoxic stimuli (Habraken and Piette, 2006; Janssens and Tschopp, 2006; Wu et al., 2006; Wu and Miyamoto, 2007), in addition to having some NF- κ B-independent effects (Perkins, 2007) and a constitutively activated IKK β T-loop mutant (IKK β ca) activates NF- κ B (Delhase et al., 1999) by phosphorylating I κ B α even in the absence of an exogenous signal (Kwak et al., 2000; Tang et al., 2003; Kucharczak et al., 2003; Lee et al., 2006; Araki et al., 2008).

Because the levels of NF- κ B signaling components were unaltered during replicative senescence of IMR-90 HDF, in keeping with NF- κ B's role in cell survival (Kucharczak et al., 2003; Luo et al., 2005) (Fig. 1), we investigated whether constitutive activation of NF- κ B could rescue cells from senescence. To this end, the classical model of OIS of normal HDFs was used (Serrano et al., 1997), by generating IMR-90 stably overexpressing RasV12. Secondly, because IKK β was expressed at low levels in IMR-90 cells, we also generated IMR-90 cells ectopically expressing a constitutively activated IKK β mutant protein (IKK β ca) or both genes together (Fig. 2), and propagated each of these cell populations along with their empty vector

controls until they reached the end of their lifespan. Importantly, the same IKK β ca mutant employed in our study produced physiologically relevant effects in either short-term *in vitro* (Araki et al., 2008; Penzo et al., 2009) or in more long term *in vitro* and *in vivo* experiments, wherein it was shown to specifically activate targets of the canonical NF- κ B pathway and affect the selection and development of immune effector cells (Voll et al., 2000; Denk et al., 2001; Sasaki et al., 2006; Jimi et al., 2008).

Oncogenic RasV12 did not activate NF- κ B in normal diploid fibroblasts

Previous reports showed that RasV12 and Raf activated NF- κ B in mouse NIH3T3 fibroblasts through a process that did not involve the classical I κ B α degradation pathway, DNA binding or nuclear translocation of NF- κ B, which are associated with cytokine-induced signaling (Finco et al., 1997; Norris and Baldwin, 1997; Nalca et al., 1999; Hanson et al., 2003). Others, however, showed that RasV12 induced both DNA binding and NF- κ B activity in NIH3T3 cells due to increased I κ B α degradation and NF- κ B subunit nuclear translocation (Millán et al., 2003). In contrast, NF- κ B was not activated by RasV12 in Rat-1 fibroblasts (Jo et al., 2000). Further, some studies demonstrated that oncogenic RasV12, although stimulating an NF- κ B-dependent reporter, suppressed the ability of TNF α to activate NF- κ B in NIH3T3 fibroblasts (Hanson et al., 2003), while others reported that RasV12 did not interfere with TNF α -induced activation of NF- κ B in NIH3T3 (Millán et al., 2003) or Rat-1 (Jo et al., 2000) cells. Thus, studies with established rodent fibroblasts have produced conflicting observations. While, we did not perform NF- κ B reporter assays as normal HDFs do not take up DNA in transient transfections, the data suggested that RasV12 did not induce the NF- κ B signaling pathway in IMR-90. Moreover, a comparison of the levels of phosphorylated/total I κ B α and nuclear p50 and p65 NF- κ B subunits in the IKK β ca- and IKK β ca/RasV12-expressing IMR-90 cells showed that the former expressed higher levels of p-I κ B α and nuclear p50 and p65 than the latter suggesting that RasV12 may act as a negative effector of NF- κ B signaling in this normal cell context. This observation was consistent with previous studies demonstrating that in normal human fibroblasts, suppression of the PI3K pathway, which leads to the activation of NF- κ B, was sufficient to induce senescence (Courtois-Cox et al., 2006) and PI3K-deficient MEFs could not be established, as they rapidly senesced in culture (Brachmann et al., 2005). In addition, Raf, which induced senescence in IMR-90 (Zhu et al., 1998), was found to be an extremely potent inhibitor of PI3K signaling and AKT activity, which was required for cell cycle arrest elicited by Raf activation (Westbrook et al., 2002; Menges and McChance, 2008). Whilst Ras activates both Raf-MAPK and PI3K-Akt pathways, it is conceivable that RasV12 through a negative feedback mechanism leads to the suppression of NF- κ B signaling to trigger senescence (Courtois-Cox et al., 2006; Yaswen and Campisi, 2007; Han and Sun, 2007; Courtois-Cox et al., 2008). Recently, it was demonstrated that p53 suppressed the IKK-NF- κ B pathway through suppression of aerobic glycolysis (Kawauchi et al., 2008). Hence, it follows that RasV12-mediated induction of p53 may lead to the suppression of NF- κ B signaling through suppression of aerobic glycolysis. In any event whatever the mechanism by which RasV12 may be interfering with NF- κ B activation in the context of IMR-90 normal diploid fibroblast, RasV12 did not activate NF- κ B signaling in these cells (Fig. 3).

IKK β ca circumvented the establishment of a premature senescent phenotype in response to RasV12

Flow cytometric analysis showed that IMR-90 RasV12 cells at P18 displayed a similar cell cycle profile to IKK β ca- or vector-expressing IMR-90 cells (Fig. 4). However, with prolonged culturing up to P25, sustained RasV12 expression led to a decrease in the rate of cell proliferation and induced a G1 cell cycle growth arrest similar to that exhibited by senescent IMR-90 cells at P42, consistent with previous studies (Serrano et al., 1997; Lin et al., 1998; Benanti and Galloway, 2004). In contrast, IMR-90 cells co-expressing IKK β ca and

RasV12 did not display a cell cycle growth arrest, even though they had a longer G1 phase compared to their IKK β ca counterparts (Fig. 4).

Accumulation of IMR-90 RasV12 cells in the G1 phase of the cell cycle could be indicative of growth arrest or senescence. Additional evidence for a premature senescent phenotype was obtained by using the most widely accepted feature of senescent cells, the presence of SA- β -Gal activity. IMR-90 RasV12 cells at P25 stained for SA- β -Gal gave an intense signal similar to that detected in senescent IMR-90 cells at P39, whereas IMR-90 IKK β ca at P25 or vector-expressing IMR-90 at P25 gave a negative signal and IMR-90 IKK β ca/RasV12 cells at P25 gave a weak or negative signal (Fig. 5). Collectively, these data showed that RasV12 provoked premature senescence of IMR-90 cells, but this was prevented in the presence of IKK β ca. Previous studies showed that in short-term experiments using adenoviral vectors, overexpression of c-Rel induced premature senescence of normal human keratinocytes (Bernard et al., 2004) and HDFs (Zdanov et al., 2007). Recently we showed that enforced NF- κ B activation by IKK β ca transiently blocked cell proliferation but this effect disappeared by up to 3 weeks of culture of immortalized or primary MEFs, and although the cells recovered their normal growth rate we did not continue to examine the effects of chronic NF- κ B activation in response to other physiological responses which effect cellular lifespan (Penzo et al., 2009). A similar transient growth suppressive effect was also recently reported for primary human fibroblasts, TIG-3, employing the same strategy, but no evidence of their long term phenotype was provided (Araki et al., 2008). However, others showed that while overexpression of NF- κ B subunits p50/p65 induced premature senescence of normal human keratinocytes through the induction of p21^{Cip1/Waf1} (Seitz et al., 2000), they failed to do so in HDFs (Hinata et al., 2003). In agreement with the latter report, enforced IKK β ca expression leading to chronic activation of canonical NF- κ B signaling in IMR-90 (Fig. 3) or in MRC-5 HDFs did not induce premature senescence or growth arrest (unpublished data and Fig. S1). Importantly, while IKK β ca acting alone was not sufficient to extend the lifespan or immortalize IMR-90 or MRC-5 fibroblasts (unpublished data), it delayed senescence of RasV12-expressing IMR-90 fibroblasts thus acting in a dominant fashion over the senescent promoting effects of sustained RasV12 expression.

However, it should be mentioned in this context that not all HDF strains respond to RasV12 by undergoing OIS. For example, human neonatal foreskin (Benanti and Galloway, 2004) or embryonic fibroblasts, MRC-5, (unpublished data) did not senesce upon stable overexpression of oncogenic RasV12. Thus, neither the tissue of origin nor the cultured-induced stress can account for the differences in response to RasV12 expression, although p16^{INK4A} has been suggested to be a key determinant of RasV12-induced senescence of HDFs that could explain some of these different outcomes (Serrano et al., 1997; Lin et al., 1998; Brookes et al., 2002; Beauséjour et al., 2003; Benanti and Galloway, 2004; Voorhoeve and Agami, 2004).

Enforced IKK β ca expression altered the effects of RasV12 on effectors of cell cycle progression

Analysis of cell cycle regulatory proteins in confluent cells showed that overexpression of RasV12 induced the expression of cyclin D1 and Cdc6 but also of p53 and its target protein p21^{Cip1/Waf1}. Cyclin D1 was up-regulated in IMR-90 IKK β ca cells but to lesser degree than observed in their IMR-90 RasV12 counterparts (Fig. 6A). Cdc6 levels were similar in IMR-90 IKK β ca cells compared to empty vector controls; and although Cdc6 expression was induced by RasV12, it was back down to control levels in IMR-90 cells co-expressing IKK β ca/RasV12. In contrast, enforced expression of IKK β ca suppressed both the basal and RasV12-induced levels of p53. However, the expression of p21^{Cip1/Waf1} in IKK β ca cells was higher than in corresponding empty vector control cells, but lower than in IMR-90 RasV12 cells. In fibroblasts co-expressing IKK β ca/RasV12, the expression of these cell cycle regulatory

proteins were reduced in comparison to their levels in IMR-90 RasV12 cells but were similar to the levels detected in IMR-90 IKK β ca fibroblasts, with the exception of p21^{Cip1/Waf1}. Thus, RasV12 induced the expression of cell cycle regulatory proteins, in agreement with its mitogenic role (Serrano et al., 1997; Lin et al., 1998; Benanti and Galloway, 2004), but at the same time activated the p53-p21^{Cip1/Waf1} pathway, most likely due to the transmission of excessive mitogenic signals (Fig. 5), which appears to be the main axis (Lou and Chen, 2005; Braig and Schmitt, 2006; Di Micco et al., 2006; Bartkova et al., 2006; Di Micco et al., 2007), but not the only one (Serrano and Blasco, 2001; Campisi, 2005; Mallette et al., 2007; Campisi and d'Adda di Fagagna, 2007) operating in OIS of HDFs. Interestingly, RasV12 induced both p53 and a faster migrating p53 band, as detected by the monoclonal antibody DO1, which most likely corresponded to Δ p53 described previously (Rohaly et al., 2005). Δ p53 displays differential activity resulting in the up-regulation of p21^{Cip1/Waf1} but not of Mdm2 or Bax (Rohaly et al., 2005). Moreover, the induction of Δ p53 by RasV12 was of significance as Δ p53 has been shown to attenuate S-phase progression in part by inducing p21^{Cip1/Waf1}, and also by acting as an essential component of the ATR-intra-S phase checkpoint (Rohaly et al., 2005). Thus both, p53 and Δ p53 may have contributed to RasV12-mediated induction of p21^{Cip1/Waf1} in IMR-90 cells.

In agreement with the results presented here (Fig. 6), oncogenic RasV12 expression was previously shown to induce the expression of Cdc6, (a positive cell cycle regulator), resulting in an increase in the active replicons of oncogene-induced senescent cells, suggesting that RasV12 via Cdc6 induced hyper-proliferative signals (Di Micco et al., 2006). Thus, oncogene expression leads to hyper-proliferation and DNA hyper-replication causing an accumulation of DNA damage and activating an S-phase-specific DNA damage checkpoint response (DDR) (Di Micco et al., 2006). Further, our previous collaborative studies showed that Cdc6 overexpression was sufficient to induce DDR activation and senescence (Bartkova et al., 2006), although it may have additional oncogenic functions in mice (Gonzalez et al., 2006), but not in normal or telomerized human MRC-5 fibroblasts (unpublished data; Bartkova et al., 2006). The elevated levels of cyclin D1 observed here are in keeping with previously ascribed roles for activated NF- κ B in fostering cell proliferation (Kucharczak et al., 2003; Scheidreit, 2006). However, p53-independent, higher levels of p21^{Cip1/Waf1} in IKK β ca cells may reflect a dual role of IKK β ca or activated NF- κ B as a negative or positive effector of cell cycle progression depending on the physiological context and other extracellular stimuli. For instance, the elevated expression of p21^{Cip1/Waf1} in IKK β ca cells may act as a break in cell cycle progression, by activating the pRB pathway (Campisi, 2005; Campisi and d'Adda di Fagagna, 2007) in response to stress stimuli, thereby giving cells the time to repair their damaged DNA and continue to proceed through the cell cycle.

Because enforced IKK β ca expression forestalled a RasV12 hyperproliferative state leading to premature senescence, we investigated whether the RasV12 induced DDR response (Di Micco et al., 2006; Bartkova et al., 2006; Mallette et al., 2007; Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008) was antagonized by IKK β ca. In IMR-90 cells RasV12 induced the expression of proteins involved in DDR, including phospho-Chk2 (T68) and phospho-p53 (Ser15 and Ser20), leading to p53 stabilization. No induction of these DDR marker proteins was detected in IMR-90 IKK β ca cells, and of particular significance their expression was markedly reduced in cells expressing IKK β ca or co-expressing IKK β ca/RasV12 compared to RasV12 cells. Importantly, IKK β ca suppressed both the basal and RasV12-induced levels of p53, and in addition it appeared to destabilize p53 protein, in agreement with recent studies (Xia et al., 2009). However even though the onset of RasV12 induced cellular senescence was significantly delayed by the presence of IKK β ca, the degree of sustained activation of canonical NF- κ B signaling afforded by constitutive IKK β ca expression, together with destabilization of p53, was not sufficient to fully rescue IMR-90 from RasV12-induced premature senescence. We speculate in this context that the accumulation of irreparable DSBs induced by oncogenic

stress in combination with replicative senescence (Sedelnikova et al., 2004) may have eventually exceeded the capacity of IKK β -mediated NF- κ B signaling to shift the balance towards repair and cell survival (Bartek and Lucas, 2006).

In conclusion, we have carefully examined the long term effects of chronic NF- κ B activation on oncogene induced cellular senescence and uncovered the surprising observation that this form of cellular senescence is delayed by chronic NF- κ B activation. This leads us to posit that prolonged, chronic NF- κ B activation can provide cells with the time to repair their damaged DNA, thereby allowing them to escape Ras induced premature senescence. Interestingly, because pro-inflammatory chronic NF- κ B activation *in vivo* has also been linked to cancer development or progression (Karin and Greten, 2005), we further speculate that OIS/DDR suppression by sustained canonical NF- κ B activation in cells could subsequently provide an intrinsically conducive environment, which facilitates the selection of other epigenetic or genetic changes leading to cellular immortalization and full malignant conversion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Expression of NF- κ B signaling pathway components during replicative senescence of IMR-90.

(A) Cytoplasmic and nuclear extracts, ranging from 15 to 40 Pdl, were analyzed for the expression of IKK α , IKK β , NF- κ B p50 and p65 subunits, I κ B α and cyclin D1, or lamin B and β -actin. (B) Total cell lysates were analyzed for the expression of IKK α , IKK β and cyclin D1 or β -actin by immunoblotting.

Fig. 2.

Generation of RasV12- and/or IKK β ca-expressing IMR-90. (A) Infection scheme of IMR-90 with the replication defective recombinant retroviruses. (B) Total cell lysates extracted from uninfected IMR-90 and from HygroB- and/or Neo-resistant IMR-90 cells carrying either RasV12 and/or IKK β ca or their corresponding control vectors were analyzed for the expression of Ha-RasV12 and IKK β ca proteins or β -actin by immunoblotting.

Fig. 3. IKKβca but not RasV12 activated canonical NF-κB signaling. (A) Total cell lysates extracted from uninfected IMR-90 and from HygroB- and/or Neo-resistant IMR-90 cells carrying either RasV12 and/or IKKβca or their corresponding control vectors were analyzed for the expression of phospho-IkBα (Ser32/36), IkBα or β-actin. (B) Proteins extracted from isolated nuclei of uninfected IMR-90 and from HygroB- and/or Neo-resistant IMR-90 cells carrying either RasV12 and/or IKKβca or their corresponding control vectors were analyzed for the expression of p50, p65 and IkBα, and lamin B by immunoblotting. (C) The relative expression levels of several canonical NF-κB target genes (IkBα, IL-6 and MCP-1/CCL2) were determined by SYBR green real-time RT-PCR in IMR-90 Neo, IMR-90 IKKβca and in IMR-90 cells

stimulated with 100 ng/ml TNF α for 2 h. Fold change values are relative to empty vector control (IMR-90 Neo) cells grown under the same conditions. Results shown are representative of several experiments with variation being no more than 5-10%.

Fig. 4.

Analysis of growth properties of RasV12- and/or IKK β ca-expressing IMR-90 fibroblasts. (A) HygroB- and/or Neo-resistant IMR-90 fibroblasts expressing either RasV12 and/or IKK β ca or their corresponding control vectors were plated into 24-multiwell plates and counted every two days. The experiment was performed twice in duplicates and growth curves were constructed. (B) Flow cytometric analysis of all the different IMR-90 at P25. IMR-90 RasV12 at P18 and senescent IMR-90 at P42 fibroblasts were included in the analysis. The percentages of cells in corresponding phases of the cell cycle are indicated.

Fig. 5. Morphologies and SA- β -Gal expression in RasV12- and/or IKK β ca-expressing IMR-90. Morphologies and SA- β -Gal staining, as a marker for cellular senescence, of senescent IMR-90 (P39) fibroblasts and uninfected or HygroB- and/or Neo-resistant IMR-90 expressing either RasV12 and/or IKK β ca or their corresponding control vectors.

Fig. 6. Analysis of cell cycle regulatory proteins in RasV12- and/or IKKβca-expressing IMR-90 fibroblasts. (A) Total cell lysates extracted from normal IMR-90 and from HygroB- and/or Neo-resistant IMR-90 expressing either RasV12 and/or IKKβca or their corresponding control vectors were analyzed for the expression of selected cell cycle regulatory proteins or β-actin by immunoblotting. (B) Time course of expression of cyclin D1 and p21^{Clp1}/Waf1 in sub-confluent proliferating cultures of IMR-90 Neo and IMR-90 IKKβca fibroblasts following serum stimulation of the cells for 0 - 24 h.

Fig. 7.

Analysis of proteins involved in DNA damage checkpoint response in RasV12- and/or IKKβca-expressing IMR-90 fibroblasts. Total cell lysates extracted from HygroB- and/or Neo-resistant IMR-90 expressing either RasV12 and/or IKKβca or their corresponding control vectors were analyzed for the expression of phospho-Chk2 (Thr68), total Chk2, phospho-p53 (Ser15 and Ser20) and total p53 or β-actin by immunoblotting.