RRM2B Suppresses Activation of the Oxidative Stress Pathway and is Up-regulated by P53 During Senescence

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RRM2B is the DNA damage-inducible small subunit of ribonucleotide reductase, the rate-limiting enzyme in de novo deoxynucleoboside triphosphate synthesis. Although RRM2B is implicated in DNA repair and the maintenance of mitochondrial DNA content, the regulation and function of RRM2B in senescence have not been previously established. Here, we show that RRM2B is highly induced in a p53-dependent manner during senescence in primary human fibroblast IMR90 cells and is expressed at higher levels in senescent precancerous human prostatic intraepithelial neoplasm lesions compared to adjacent normal prostate glands. Paradoxically, silencing RRM2B expression leads to an increase in the level of reactive oxygen species, mitochondrial membrane depolarization, and premature senescence in a p38MAPK- and p53-dependent manner in young fibroblasts. Consistently, induction of senescence is accelerated in Rrm2b deficient mouse embryo fibroblasts. Our data demonstrate that RRM2B is induced by stress signals prior to the onset of senescence and prevents premature oxidative stress-induced senescence.

Ribonucleotide reductase (RR) consists of two large subunits, RRM1, and two small subunits, RRM2 or RRM2B (also known as p53R2).1 RRM1 expression is constant throughout the cell cycle, whereas that of RRM2 is elevated during the S-phase to boost RR activity2,3 and is degraded during the G2/M4 and G1 phases5. RRM2B is induced upon activation of the DNA damage pathway transcriptionally by p536–8 and post-translationally by ATM9. DNA damage-induced RRM2B forms an active complex with RRM1 to supply deoxyribonucleoside triphosphates (dNTPs) for DNA repair1. Mutations and deletions in the RRM2B locus are associated with human diseases, including mitochondrial DNA (mtDNA) depletion syndrome, suggesting that RRM2B plays an essential role in the maintenance of mitochondrial DNA content10. Mice with a germline homozygous Rrm2b deletion develop normally and are viable at birth but show growth retardation and early mortality due to severe renal failure8, highlighting the importance of Rrm2b in physiological function.

The tumor suppressor P53 responds to stress by inducing a repertoire of transcriptional targets that are involved in distinct biological processes, including cell cycle arrest, senescence and apoptosis, depending on the biological setting11. Senescence was first characterized in primary human fibroblasts as a state of permanent growth arrest while maintaining active metabolism and can be triggered by various signals, such as telomere shortening, oncogenic stress and DNA damage12. It has been demonstrated in vivo that senescence is a bona fide tumor suppressive mechanism and not just an “artifact” of cell culture13–16. Growing evidence indicates that senescence is far more complex than a simply irreversible cessation of cell proliferation. The senescence-associated secretory phenotype not only enforces growth arrest but also promotes tissue repair, immune clearance, aging and tumorigenesis17. Given the essential roles of p53 and the DNA damage response in both the induction of senescence and the regulation of RRM2B expression, we hypothesized that RRM2B might be functionally linked to the senescence pathway. Our results provide the first evidence that RRM2B is involved in suppressing the elevation of reactive oxidative species (ROS) levels in physiological conditions as well as those of overt oxidative stress. It is highly up-regulated in response to stress signals in a p53-dependent manner in senescent primary human fibroblasts and human precancerous lesions. Our data suggest that RRM2B is not part of senescence program itself but rather a negative modulator that impedes the activation of oxidative stress signals that prematurely induce senescence.
Results

RRM2B is induced during senescence. Although RRM2B was identified as a transcriptional target of p53, its role in the senescence pathway is not well understood. We first examined RRM2B expression in the primary human diploid fibroblast cell strain IMR90, which retains an intact DNA damage response pathway (Supplementary Fig. S1a) and undergoes replicative senescence due to telomere attrition after 50–60 population doublings. Interestingly, the RRM2B protein level was profoundly elevated along with several other senescence regulators, such as p53, p21(CPI) and p16INKA12, in late passage (LP) senescent IMR90 cells compared to early passage (EP) IMR90 cells (Fig. 1a, and Supplementary Fig. S2a). Other stress signals, such as activated oncogenes or DNA damage, can trigger senescence in fibroblasts during early passage, which is defined as “premature senescence”18. Similar to cells undergoing replicative senescence, RRM2B expression was also increased in prematurely senescent cells induced by treatment with low-dose Adriamycin or by the expression of oncogenic RASV12 (Fig. 1, b and c and Supplementary Fig. S2, b and c). Unlike RRM2B, both RRM1 and RRM2 levels were reduced upon the induction of senescence (Fig. 1, a, b and c). Furthermore, the RRM2B mRNA level was significantly higher in senescent cells compared to untreated young fibroblasts (Fig. 1d), suggesting that the induction of RRM2B is controlled, at least in part, at the transcriptional level.

Figure 1 | RRM2B is up-regulated during replicative- and stress-induced senescence in a p53-dependent manner. (a) Western blot analysis of proteins in early passage (EP, ~20–30 PD) and late passage (LP, additional 35 PD) IMR90 cells. (b) Western blot analysis of proteins in IMR90 cells without treatment (Ctrl) or treated with 0.1 μg/ml Adriamycin (Adr) for one week. (c) Western blot analysis of proteins in IMR90 cells expressing either empty vector (Ctrl) or oncogenic RASV12. Cells were harvested 2 weeks following retroviral infection. (d) RRM2B mRNA levels from IMR90 cultures as described in (a), (b) and (c) were quantified by real-time RT-PCR. β-ACTIN or GAPDH were used as internal controls. Relative RRM2B mRNA levels from senescent IMR90 cells (■) were normalized to the values from non-senescent controls (□) (means±SEM, n=3). (e) Western blot analysis of proteins in IMR90 cells transduced with both MSCV and pLPC retroviruses expressing either vector alone, RASV12, E1A or both RASV12 and E1A. Cells were analyzed two weeks following retroviral infection. (f, g) Western blot analysis of proteins in IMR90 cells expressing pSUPER.retro.puro empty vector, non-silencing shRNA (shNS), shTP53 in (f) or shRB1 in (g) as indicated. One week following infection, cells were treated with 0.1 μg/ml Adriamycin for an additional week prior to analysis. (h, i) Western blot analysis of proteins in IMR90 cells transduced with both MSCV and pSUPER.retro.puro retroviruses expressing empty vector, shRNA, RASV12 or both shRNA and RASV12. The cells were analyzed 2 weeks following infection.
RRM2B expression was not significantly altered when the proliferation of IMR90 cells was acutely ceased by overexpressing p21CIP1 (Supplementary Fig. S3), implying that the induction of RRM2B during senescence is controlled by specific pathways and is not simply the consequence of cell cycle arrest during senescence. The induction of RRM2B depends on p53 in senescent IMR90 cells. Inactivation of both the p53 and RB pathways with the adeno-virus oncoprotein E1A overrides RASV12-induced senescence18,19. We co-expressed RASV12 and E1A in IMR90 cells and found that E1A abolished the induction of RRM2B by RASV12 (Fig. 1e). This finding further establishes the correlation between RRM2B induction and senescence under stress. To interrogate which of the two most important regulators of the senescent program20, i.e., p53 and RB, govern RRM2B expression, we expressed short hairpin RNAs (shRNAs) to silence the expression of \( \text{TP53} \) and \( \text{RB1} \), respectively. Like p21CIP1, both the basal and Adriamycin-induced expression of RRM2B was significantly curtailed upon the silencing of p53 (Fig. 1f). CDK4 expression was not influenced by p53 knockdown, demonstrating the specificity of the shTP53s (Fig. 1f). In contrast, silencing of RB1, indicated by an increase in CYCLIN E due to the activation of E2F, had no detectable effect on RRM2B in the presence or absence of Adriamycin. The p21CIP1 expression level was elevated in RB1-silenced cells (Fig. 1g), presumably due to the activation of p53 by the induction of Ser15 phosphorylation20. The mode of RRM2B regulation during chronic stress-induced senescence is reminiscent of the response to acute DNA damage in IMR90 cells (Supplementary Fig. S1, b and c). Using an identical approach, we found that the induction of RRM2B by RASV12 was also abolished by the expression of shTP53 but not shRB1 (Fig. 1, h and i). Taken together, p53 tumor suppressor exclusively regulates RRM2B expression in stress-induced senescent cells. Inactivation of \( \text{Pten} \) in the prostate of mice induces the onset of precancerous high-grade prostatic intraepithelial neoplasia (HG-PIN) and p53-dependent senescence13. Loss of \( \text{Tp53} \) in \( \text{Pten} \)-deficient prostates can fully rescue oncogene-induced senescence and convert HG-PIN to invasive prostate cancer13. To confirm the correlation between RRM2B and senescence \( \text{in vivo} \), we performed immunohistochemistry to evaluate the expression of RRM2B and p16INK4A in human PIN lesions, which display a senescent phenotype21, in comparison with adjacent normal prostate glands. PIN lesions typically showed positive staining for alpha-methylacyl-CoA (AMACR) (AMACR) and nuclear p6324 (Supplementary Fig. S4). In contrast, AMACR was undetectable in the normal prostate gland (Fig. 2, bottom panel). The expression of both RRM2B and p16INK4A in PIN lesions was strikingly higher compared to the surrounding normal prostate glands (Fig. 2). To support our finding, we searched the Oncomine expression array database for a larger sample size and found that the RRM2B mRNA level was 3.8-fold higher in PIN lesions compared to normal prostate glands (\( p=3.07E-5 \))25.

Silencing of RRM2B triggers premature senescence in young IMR90 cells. We next sought to understand the physiological role of RRM2B in senescence by expressing shRNAs to silence RRM2B in IMR90 cells. RRM2B was efficiently silenced by three shRRM2B that target different regions of RRM2B mRNA compared to cells expressing scramble non-silencing shRNA (shNS) and an empty vector (Fig. 3a). Because RRM2B was up-regulated during senescence, we postulated that knockdown of RRM2B might impair the induction of senescence. Unexpectedly, the silencing of RRM2B triggered premature senescence (Fig. 3b) in young IMR90 cells. We introduced three point mutations (shRRM2B-mut) within shRRM2B-C (designated as shRRM2B) to interfere with the cleavage of RRM2B mRNA. ShRRM2B-mut did not silence endogenous RRM2B (Supplementary Fig. S5) and could no longer induce SA-\( \beta \)-gal activity (Fig. 3d) or suppress DNA replication (Fig. 3e).
specific silencing of RRM2B led to the progressive accumulation of multiple senescent regulators, including p53, p21CIP1 and p16INK4A, from day 7 to 19 (Fig. 3c). Furthermore, gene expression profiling by microarray analysis and Gene Ontology (GO) enrichment analysis demonstrated that genes involved in cell cycle progression, DNA damage repair and DNA metabolism were significantly down-regulated and that genes involved in the immune response were up-regulated in cells expressing shRRM2B compared to those expressing shRRM2B-mut.
expressing shRRM2B-mut (Table S3–6). This expression profile further validates certain senescent phenotypes in RRM2B-silenced IMR90 cells.

Induction of premature senescence by silencing RRM2B is mediated by p53. Knockdown of RRM2B did not further augment RASV12-induced premature senescence (Supplementary Fig. S6a–c), which is mediated by the activation of both RB and p5320, suggesting that common downstream pathways were activated by RASV12 and shRRM2B. Consistent with this view, E1A completely rescued shRRM2B-induced premature senescence (Fig. 3, g and h). Interestingly, the silencing of p53 by shRNA in shRRM2B-expressing cells (Fig. 3i) was sufficient to rescue premature senescence, as indicated by the significant reduction in SA-β-gal activity (Fig. 3j) and the increase in the replication index (Fig. 3k). Cells expressing both shRRM2B and shTP53 showed reduced expression of p21CIP1 and increased levels of RRM1 and RRM2 (Fig. 3i), whereas p16INK4A was unchanged (Fig. 3i) compared to cells expressing shRRM2B alone. In contrast, knockdown of RB1 triggered the activation of the p53 pathway (Fig 3i) and was unable to rescue shRRM2B-triggered premature senescence (Fig. 3, j and k). Taken together, the silencing of RRM2B under normal physiological conditions triggers p53-dependent premature senescence in IMR90 cells.

The expression of RRM2B in response to oxidative stress. Senescent human fibroblasts displayed aberrant mitochondrial function and biogenesis as well as an elevated level of reactive oxygen species (ROS)26–28, which, in part, mediate stress-induced senescence29. Hence, it is likely that elevated RRM2B expression during senescence is a response to increased oxidative stress. To test this hypothesis, RASV12-expressing IMR90 cells were treated with N-acetyl cysteine (NAC) to increase the intracellular antioxidant level. Treatment of RASV12-expressing cells with NAC reduced the level of RRM2B, while changes in RRM1 and RRM2 were nearly undetectable (Fig. 4a). To further demonstrate that
RRM2B expression is related to an increase of oxidative stress, IMR90 cells were transiently treated with 200 μM H₂O₂ for 2 hours and allowed to incubate for up to 96 hours. P38MAPK Thr180/Tyr182 phosphorylation was transiently induced during the H₂O₂ treatment, indicating the onset of the oxidative stress response. Unlike p53, which increased immediately, the RRM2B protein level slowly increased, starting at 20 hours (Supplementary Fig. S7a) and reaching a significantly higher level at 48 hours and 96 hours (Fig. 4b), suggesting that additional processes other than p53 accumulation are required for the induction of RRM2B by H₂O₂.

Silencing of RRM2B triggers an elevation in ROS and the activation of the p38MAPK pathway. Without overt stress, the cells are continuously exposed to endogenous ROS, which are byproducts of normal respiration. The cellular anti-oxidation system is turned on in response to oxidative stress to prevent damage. Indeed, some of these anti-oxidation proteins are p53 targets. We previously reported that RRM2B shows anti-oxidation activity in vitro. Hence, we tested whether the silencing of RRM2B had any effect on antagonizing endogenous ROS in vivo. In fact, the ROS level was significantly increased and the mitochondrial membrane was subsequently depolarized in cells expressing shRRM2B compared to cells expressing shNS and shRRM2B-mut (Fig. 4a, c, d, and e). Treatment of shRRM2B-expressing IMR90 cells with low dose NAC (0.5 mM) reduced the percentage of β-gal-positive cells from 67.6 ± 3.6% to 52.6 ± 6.3% (p = 0.005, n = 6), suggesting that ROS partly mediates the induction of senescence in RRM2B-silenced cells. We were unable to test the impact of a higher concentration of NAC on the induction of senescence due to a cytostatic effect in IMR90 cells that occurs after long-term exposure to NAC.

It is likely that oxidative stress in RRM2B knockdown cells is derived from impaired mitochondrial function due to reduced mtDNA content. To test this hypothesis, we determined the level of the mitochondrial COX-1 gene relative to that of the nuclear β-GLOBIN gene in RRM2B-silenced IMR90 cells compared to non-silenced controls. A slight decrease in the mtDNA content was detected in shRRM2B-expressing cells compared to the control sample (Supplementary Fig. S8), suggesting that the elevation in ROS might be partially due to a loss of mtDNA content.

We further examined the signaling pathway triggered by oxidative stress, such as the phosphorylation and activation of p38MAPK, which is also an essential regulator of the stress-induced senescence program. Intriguingly, p38MAPK phosphorylation was profoundly increased when RRM2B was silenced compared to shRRM2B-mut-expressing cells (Fig. 4f). Downstream targets of p38MAPK were also activated, as indicated by the significant elevation of phosphorylated MAPKAPK-2, a substrate of p38MAPK, and the phosphorylation of HSP27, a substrate of MAPKAPK-2 (Fig. 4f). Treatment of RRM2B-deficient cells with the p38MAPK inhibitor SB203580 efficiently reduced the level of phosphorylation of both MAPKAPK-2 and HSP27 in a dose-dependent manner and also suppressed the activation of both p53 and p21CIP1. Not only did SB203580 partially reduce SA-β-gal activity (Fig. 4g), it also prevented the morphological change induced by silencing RRM2B, which was typically flat and enlarged compared to non-senescent cells (Fig. 4h).

Conversely, we overexpressed RRM2B in IMR90 cells and tested if RRM2B suppressed ROS levels in response to exogenous oxidants. Overexpression of RRM2B did not affect the basal ROS level in IMR90 cells under normal physiological conditions (Supplementary Fig. S9a), suggesting that the endogenous level of RRM2B was sufficient to suppress ROS. Interestingly, overexpression of RRM2B antagonized the increase in the level of ROS that was triggered by incubation with H₂O₂ (Supplementary Fig. S9a). Consistently, the increased expression of RRM2B had no impact on p38MAPK activity without H₂O₂ treatment but attenuated the activation of the p38MAPK pathway induced by H₂O₂, as indicated by the reduced levels of phospho-p38MAPK and phospho-MAPKAPK-2 (Supplementary Fig. S9b) compared to those in vector-expressing cells treated with H₂O₂.

**Figure 5 | Rrm2b<sup>+/+</sup> and Rrm2b<sup>-/-</sup> MEFs undergo premature senescence compared to Rrm2b<sup>+/+</sup> MEFs.** MEFs derived from different Rrm2b genotypes were generated and maintained by a defined 3T3 protocol. (a) Population doubling of MEFs was quantified 3 days after dilution and re-plating from passage 3 to 5 (means ± SEM, n = 3). □: Rrm2b<sup>+/+</sup>; ■: Rrm2b<sup>+/−</sup>; □: Rrm2b<sup>-/-</sup>. (b) Western blot analysis of known senescence regulators in MEF strains with different Rrm2b genotypes from passage 3 to 5. *: non-specific bands; ←: Rrm2b specific band. (c) A SA-β-gal activity assay was performed in MEFs with different Rrm2b genotypes at passage 5.
The induction of senescence is accelerated in mouse embryo fibroblasts (MEFs) derived from Rrm2b-deficient mice 

Discussion

In this report, we demonstrated that under normal physiological conditions, low-level RRM2B suppresses the elevation of endogenous ROS (Fig. 6a). Silencing of RRM2B disabled the negative feedback control and led to increased oxidative stress, which triggered p38MAPK- and p53-dependent senescence (Fig. 6b). Similar to our findings, Sablina et al. previously reported that relative low level of p53 is sufficient to up-regulate several antioxidant genes in the absence of severe stress and that silencing of p53 or its antioxidant target genes under normal physiological conditions leads to an increase in the ROS level. It seems conflicting that p53 activates pro-oxidation genes in addition to anti-oxidant genes. Hence, the observed hypersensitivity to oxidative stress-induced cell death compared to wild-type MEFs expressing RASV12 (Supplementary Fig. S12). Consistent with the results from our studies of shRRM2B-expressing IMR90 cells, Rrm2b protects cells from premature senescence during early passage in MEFs.

Although our data indicate that RRM2B is essential for suppressing ROS levels under normal physiological conditions and that overexpression of RRM2B antagonizes the increase of oxidative stress in response to exogenous oxidants, the molecular mechanism underlying these functions is unknown. There are a few likely scenarios. First, the establishment of senescence requires a dynamic feedback loop between the DNA damage response and ROS production in primary human fibroblasts. Given that RRM2B has an enzymatic function to provide sufficient dNTP pools for DNA damage repair, the accumulation of DNA damage due to poor DNA repair might activate downstream pathways to trigger mitochondrial dysfunction and an elevation in ROS in RRM2B-silenced IMR90 cells. Supporting this hypothesis, Arakawa’s group previously reported that mouse embryo fibroblasts (MEFs) derived from Rrm2b−/− mice are significantly more sensitive to oxidative stress-induced cell death compared to those derived from wild-type mice. They further demonstrated that impaired Rrm2b function leads to a decrease in dNTP pools and the efficiency of DNA repair in response to oxidative stress and hypothesized that the observed hypersensitivity to hydrogen peroxide is due to the accumulation of DNA damage and an increase in senescence.
in the mutation rate. However, the impact of Rrm2b deficiency on the ROS level was not analyzed in Rrm2b-lacking MEFs. Secondly, RRM2B is implicated in mtDNA replication through the production of dNTP apart from its role in DNA damage repair12. Deficiency of RRM2B activity in cells derived from patients with mtDNA depletion syndrome results in low mtDNA content and reduced respiratory chain activities in mitochondria13. Mitochondrial dysfunction due to a lack of RRM2B activity might lead to an elevation in the ROS level that is independent of the DNA damage response. In fact, a slight decrease (~20%) in the mtDNA content was detected in RRM2B knockdown IMR90 cells compared to control cells. Although it likely contributes to the elevation in ROS, whether the minimal loss of mtDNA content is the sole mechanism underlying the increase of oxidative stress is unclear. Lastly, RRM2B might be involved in cellular redox homeostasis. The expression of RRM1 and RRM2 during stress-induced senescence was greatly reduced whereas that of RRM2B was significantly elevated, suggesting that RRM2B might have a function that is independent of ribonucleotide reduction. We previously reported that recombant RRM2B has an intrinsic catalase activity to scavenge H2O214. However, whether RRM2B is involved in the detoxification of H2O2 in vivo has not been tested. Our current study suggests that the tyrosyl radical of RRM2B is not involved in the detoxification of ROS. Hence, RRM2B might activate or inhibit another protein to provide the anti-oxidation function. Identification of RRM2B binding partners that have functions in pro-oxidation or anti-oxidation will be one approach to demonstrate a more direct role for RRM2B in ROS production.

It remains to be investigated whether the sole function of induced RRM2B is to suppress the observed elevation in ROS during senescence. Apart from RRM1, RRM2B also forms complexes with proteins involved in the DNA damage response, such as ATM, the MRE11-RAD50-NBS1 complex, ATRIP, p5314 and p2114,144. The activation of a persistent DNA damage response is associated with induction of senescence13. It is plausible that the expression of RRM2B during stress-induced senescence can modulate certain senescence phenotypes via interaction with these factors.

Taken together, our data suggest that RRM2B not only is a senescence marker but plays a critical role in preventing the oxidation of essential cellular molecules. The loss of RRM2B function likely leads to ROS-induced genomic instability, which may contribute to the onset of cancer as well as senescence-triggered pathological conditions.

Methods

Plasmids. The pSUPER.retro.puro or pSUPER.retro.neo + gfp retroviral vectors (OligoEngine, Seattle, WA, USA) were used to express shRNA. The inserted oligonucleotide sequences are listed in Supplementary Table S1. The pBABE-puro-H-RASV12, pLPC and pLPC-12s E1A vectors were obtained from Dr. Scott Lowe (OligoEngine, Seattle, WA, USA) using a previously described procedure45. RASV12, pLPC and pLPC-12s E1A vectors were obtained from Dr. Scott Lowe (OligoEngine, Seattle, WA, USA) were used to express shRNA. The inserted plasmids

Retroviral production and infection. Retroviruses were generated as previously described. IMR90 were first infected with the FV-G pseudotyped retrovirus pWZL-Neo-EcoR that expressed the ecotropic receptor and then selected with 4 µg/ml G418. The derived cells were transduced with ecotropic retrovirus expressing cDNA or shRNAs and selected with 2 µg/ml puromycin (Sigma-Aldrich) or 100 µg/ml hygromycin (EMD Chemicals, Gibbstown, NJ, USA) prior to all assays.

Senescence associated β-galactosidase (SA-β-gal) activity assay. Cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 minutes and washed with PBS twice before staining with 1 mg/ml 5-bromo-4-chloro-3-indolyl-B-D-galactoside (Research Products International Corp., Mount Prospect, IL, USA) solution that contained 5 mM K4[Fe(CN)]6, 5 mM K3[Fe(CN)]6, 150 mM NaCl, 2 mM MgCl2, 7 mM CaCl2, and 25 mM NaHPO4, pH 6.0 overnight at 37°C. Blue staining was visualized by light microscopy and photographed. A total of at least 100 cells were scored from each sample.

Bromodeoxyuridine (BrdU) incorporation assay. Cells were fixed in methanol-acetone (1:1, vol/vol) 24 h after the addition of BrdU (10 µM), treated for 10 min with 1.5 N HCl, and stained for 1 h with a BrdU antibody (IBIS, Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by anti-mouse AlexaFluor 594 and goat anti-mouse AlexaFluor 647 (anti-rabbit and anti-mouse). DNA was visualized by light microscopy (Nikon Eclipse Ti) (Sigma-Aldrich). A total of at least 100 cells were scored from each sample.

Antibodies and protein detection. We raised rabbit antibodies to a synthetic RRM2B peptide (acetyl-perPEAALGDLQDDERscAmido; amino acids 4-18) conjugated to keyhole limpet hemocyanin, and the anti-sera were purified by affinity chromatography using the synthetic RRM2B peptide coupled to agarose beads (custom-made by Rockland Immunochemicals, Gilbertsville, PA, USA). Proteins were detected using primary antibodies against human RRM2B (as described above), mouse Rmz2b (ProSci Incorporated, Poway, CA, USA), RRM1 (United States Biological, Swampscott, MA, USA), PM2, mouse p[15S]P, human p[16S], mouse p[16S], p[17S], p[21S], H-RAS, E1A, GAPDH, CDK4, CYCLIN E (Santa Cruz Biotechnology, Santa Cruz, CA, USA), human p53 (EMD Chemicals, Gibbstown, NJ, USA), mouse p53 (Leica Microsystems Inc, Buffalo Grove, IL, USA), MDM2 (mouse monoclonal antibody clone 2A10, provided by Dr. Arnold Levine), β-ACTIN (Sigma-Aldrich), phospho-p38 MAPK (Thr180/Tyr182), p38MAPK, phosphor-MAPKAPK-2 (T222), MAPKAPK-2, phospho-HSP27(Ser82) and HSP27 (Cell Signaling Technology, Inc., Danvers, MA, USA) using a previously described procedure44.

Measurement of reactive oxygen species. Cells were harvested by trypsinization, resuspended in PBS containing 2.5 µM CM-H2DCFDA (Invitrogen) and incubated at 37°C for 30 minutes. Cells were collected by centrifugation, washed twice with PBS and analyzed by flow cytometry. To measure the total superoxide level, cells were collected as described above and stained with 2.5 µM DHE (Invitrogen) in PBS containing 5 mM sodium pyruvate at 37°C for 40 minutes. The samples were then immediately fixed on ice, and centrifuged. Cell pellets were washed with ice-cold PBS three times before analysis by flow cytometry.

JC-1 staining and analysis. Cells were incubated with 0.77 µM JC-1 (Invitrogen) for 20 minutes at 37°C and washed twice with Hank’s balanced salt solution (Invitrogen). Cells were then resuspended in PBS containing 10 µg/ml tetramethylrhodamine-conjugated DAPI (Molecular Probes) and analyzed by fluorescence microscopy (Nikon Eclipse Ti) (Sigma-Aldrich). A total of at least 100 cells were counted for each sample from five randomly selected fields were analyzed by Zeiss LSM AIM4CL software (Carl Zeiss MicroImaging, LLC).

Quantitative real time polymerase chain reaction (qRT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen), and cDNA was generated according to the manufacturer’s instructions (Invitrogen). qRT-PCR was performed in the ABI Prism 7900 HT Sequence Detection System (Life Technologies Corporation, Carlsbad, CA, USA) in a 20-µl mix containing cDNA synthesized from 3 ng of total RNA, 5 µM of each primer and ABI SYBR Green PCR Master Mix (Life Technologies Corporation). Relative gene expression was calculated with the ΔΔCt method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-ACTIN as an internal control. Primer sequences are listed in Table S2.

Statistical analysis. We performed Student’s t tests with two-tailed distribution and two-sample equal variance in Excel (Microsoft, Redmond, WA, USA).

Immunohistochemistry staining. Five-micrometer-thick sections were prepared from formalin-fixed, paraffin-embedded tissue and deparaffinized in xylene followed by 100% ethanol. The samples were then quenched in 3% hydrogen peroxide, pretreated to promote antigen retrieval by steam in DIVA buffer (Biocare Medical, Concord, CA, USA) for 20 minutes and incubated in Protein Block (Dako Corporation, Carpinteria, CA, USA) for 5 minutes. The slides were incubated with a primary antibody against RRM2B (custom-made by Rockland Immunocchemicals), p16INK4A, p19ARF, p21CIP1, H-RAS, E1A, GAPDH, CDK4, CYCLIN E (Santa Cruz Biotechnology, Santa Cruz, CA, USA), human p53 (EMD Chemicals, Gibbstown, NJ, USA), mouse p53 (Leica Microsystems Inc, Buffalo Grove, IL, USA), MDM2 (mouse monoclonal antibody clone 2A10, provided by Dr. Arnold Levine), β-ACTIN (Sigma-Aldrich), phospho-p38 MAPK (Thr180/Tyr182), p38MAPK, phosphor-MAPKAPK-2 (T222), MAPKAPK-2, phospho-HSP27(Ser82) and HSP27 (Cell Signaling Technology, Inc., Danvers, MA, USA) using a previously described procedure44.

1. Guittet, O. et al. Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in
response to DNA damage and in proliferating cells. J. Biol. Chem. 276, 40647–40651 (2001).
2. Engstrom, Y. et al. Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. J. Biol. Chem. 260, 9114–9116 (1985).
3. Eriksson, S., Grauland, A., Skog, S., Thelander, L. & Tribukait, B. Cell cycle-dependent regulation of mammalian ribonucleotide reductase. The S-phase correlated increase in subunit M2 is regulated by de novo protein synthesis. J. Biol. Chem. 259, 11695–11700 (1984).
4. D’Alessio, J. et al. Cyclin F-Mediated Degradation of Ribonucleotide Reductase M2 Controls Genome Integrity and DNA Repair. Cell 149, 1023–1034 (2012).
5. Chabes, A. L., Pfeifer, C. M., Kirschner, M. W. & Thelander, L. Mouse ribonucleotide reductase R2 protein: a new target for anaphase-promoting complex-Cdh1-mediated proteolysis. Proc. Natl. Acad. Sci. U. S. A. 100, 3925–3929 (2003).
6. Nakano, K., Balint, E., Ashcroft, M. & Vousden, K. H. A ribonucleotide reductase gene is a transcriptional target of p53 and p73. Oncogene 19, 4283–4289 (2000).
7. Tanaka, H. et al. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature 404, 42–49 (2000).
8. Kimura, T. et al. Impaired function of p53SR2 in Rrm2b-null mice causes severe renal failure through attenuation of dNTP pools. Nat. Genet. 34, 440–445 (2003).
9. Chang, L. et al. ATM-mediated serine 72 phosphorylation stabilizes ribonucleotide reductase small subunit p53R2 protein against MDM2 to DNA damage. Proc. Natl. Acad. Sci. U. S. A. 105, 18519–18524 (2008).
10. Bourdon, A. et al. Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. Nat. Genet. 39, 776–780 (2007).
11. Vousden, K. H. & Lane, D. P. p53 in health and disease. Nat. Rev. Mol. Cell Biol. 8, 275–283 (2007).
12. Kuijlaar, T., Michaloglou, C., Mooi, W. J. & Peper, D. S. The essence of senescence. Genes Dev. 24, 2463–2479 (2010).
13. Chen, Z. et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature 436, 725–730 (2005).
14. Collado, M. et al. Tumour biology: senescence in premalignant tumours. Nature 436, 642 (2005).
15. Michaloglou, C. et al. BRAF/E600-associated senescence-like cell cycle arrest of human naevi. Nature 436, 720–724 (2005).
16. Braig, M. et al. Oncogene-induced senescence as an initial barrier in lymphoma development. Nature 436, 660–665 (2005).
17. Rodier, F. & Campisi, J. Four faces of cellular senescence. J. Cell Biol. 192, 547–556.
18. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88, 593–602 (1997).
19. Ruley, H. E. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304, 602–606 (1983).
20. Chicas, A. et al. Dissecting the unique role of the retinoblastoma tumor suppressor in cancer. Cell 138, 376–387.
21. Acosta, J. C. et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 133, 1006–1018 (2008).
22. Kuijlaar, T. et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell 133, 1019–1031 (2008).
23. Collado, M. & Serrano, M. Senescence in tumour: evidence from mice and humans. Nat. Rev. Cancer 10, 51–57.
24. Zynger, D. L. & Yang, X. High-grade prostatic intraepithelial neoplasia of the prostate: the precursor lesion of prostate cancer. Int. J. Clin. Exp. Pathol. 2, 327–338 (2009).
25. Tomlins, S. A. et al. Integrative molecular concept modeling of prostate cancer progression. Nat. Genet. 39, 41–51 (2007).
26. Moiseeva, O., Bourdeau, V., Roux, A., Deschenes-Simard, X. & Ferbeyre, G. Mitochondrial dysfunction contributes to oncogene-induced senescence. Mol. Cell. Biol. 29, 4495–4507 (2009).
27. Passos, J. F. et al. Feedback between p21 and reactive oxygen production is necessary for cell senescence. Mol. Syst. Biol. 6, 347 (2010).
28. Passos, J. F. et al. Mitochondrial dysfunction accounts for the stochastic heterogeneity in telomere-dependent senescence. PLoS Biol. 5, e110 (2007).
29. Lee, A. C. et al. Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. J. Biol. Chem. 274, 7936–7940 (1999).
30. Zdanow, S., Debaco-Chaiñiaux, F., Remacle, J. & Toussaint, O. Identification of p38MAPK-dependent genes with changed transcription abundance in H2O2-induced premature senescence of IMR-90 HERT2 human fibroblasts. FEBS Lett. 580, 6455–6463 (2006).
31. Sablina, A. A. et al. The antioxidant function of the p53 tumour suppressor. Nat. Med. 11, 1306–1313 (2005).
32. Liu, X., Xue, L. & Yen, Y. Redox property of ribonucleotide reductase small subunit M2 and p53R2. Methods Mol. Biol. 477, 195–206 (2008).
33. Frixiat, C., Dewelle, J., Remacle, J. & Toussaint, O. Signal transduction in H2O2-induced senescence-like phenotype in human diploid fibroblasts. Free Radic. Biol. Med. 33, 1334–1346 (2002).
34. Freund, A., Patil, C. K. & Campisi, J. p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. Embo J. 30, 1536–1548 (2011).
35. Wang, W. et al. Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. Mol. Cell. Biol. 22, 3389–3403 (2002).
36. Cuenda, A. et al. SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. 364, 229–233 (1995).
37. Kumar, S., Jiang, M. S., Adams, J. L. & Lee, J. C. Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. Biochem. Biophys. Res. Commun. 263, 825–831 (1999).
38. Todaro, G. J. & Green, H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17, 299–313 (1963).
39. Zindy, F. et al. Myc signaling via the AFB tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev. 12, 2424–2433 (1998).
40. Kamiyo, T. et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell 91, 649–659 (1997).
41. Powell, D. R. et al. Rapid development of glomerular injury and renal failure in mice lacking p33R2. Pediatr. Nephrol. 20, 432–440 (2005).
42. Thelander, L. Ribonucleotide reductase and mitochondrial DNA synthesis. Nat. Genet. 39, 703–704 (2007).
43. Xue, L. et al. Wild-type p53 regulates human ribonucleotide reductase by protein-protein interaction with p33R2 as well as hRrm2 subunits. Cancer Res. 63, 980–986 (2003).
44. Xue, L. et al. Ribonucleotide reductase small subunit p53R2 facilitates p21 induction of G1 arrest under UV irradiation. Cancer Res. 67, 16–21 (2007).
45. Kuo, M. L., den Besten, W., Thomas, M. C. & Sherr, C. J. Arf-induced turnover of the nuclear neucleosomin-associated SUMO-2/3 protease Senp3. Cell Cycle 7, 3378–3387 (2008).