LETTERS

Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints

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Recent studies have indicated the existence of tumorigenesis barriers that slow or inhibit the progression of preneoplastic lesions to neoplasia. One such barrier involves DNA replication stress, which leads to activation of the DNA damage checkpoint and thereby to apoptosis or cell cycle arrest[1,2], whereas a second barrier is mediated by oncogene-induced senescence[3–6]. The relationship between these two barriers, if any, has not been elucidated. Here we show that oncogene-induced senescence is associated with signs of DNA replication stress, including prematurely terminated DNA replication forks and DNA double-strand breaks. Inhibiting the DNA double-strand break response kinase ataxia telangiectasia mutated (ATM) suppressed the induction of senescence and in a mouse model led to increased tumour size and invasiveness. Analysis of human precancerous lesions further indicated that DNA damage and senescence markers cosegregate closely. Thus, senescence in human preneoplastic lesions is a manifestation of oncogene-induced DNA replication stress and, together with apoptosis, provides a barrier to malignant progression.

There are several forms of senescence. Replicative senescence, the form induced by eroded telomeres, depends on activation of the DNA double-strand break (DSB) checkpoint kinases ATM and checkpoint kinase 2 (Chk2)[7–9]. A second form of senescence induced by agents that cause DNA DSBs also depends on ATM and Chk2 (ref. 10). By contrast, oncogene-induced senescence, the form of senescence that is most likely to be associated with human precancerous lesions[3,4], has been linked to increased expression of the tumour suppressors $p16^{INK4A}$ and ARF, rather than to activation of the DNA DSB checkpoint pathway[11–13].

To examine the possibility that, in addition to $p16^{INK4A}$ and ARF, the DNA DSB checkpoint also contributes to oncogene-induced senescence, we studied human diploid cells expressing various oncogenes. The first oncogene we examined was *mos*, which was predicted to induce senescence, because, like *ras*, it activates the mitogen-activated protein kinase (MAPK) pathway[14,15]. MRC5 diploid fibroblasts were infected with a retrovirus expressing *mos* or with a retrovirus without an insert, and transiently transduced cells were selected. In these cells, overexpression of Mos protein was associated with phosphorylation of the extracellular signal-regulated kinase (ERK), indicating that the MAPK pathway was active, and also with $p16^{INK4A}$ induction, activation of the DNA damage checkpoint and senescence-associated β-galactosidase (SA-β-gal) activity (Fig. 1a, b). To determine whether senescence was causally linked to activation of the DNA DSB checkpoint, we used short interfering RNA (siRNA) to deplete ATM, the key kinase responsible for DNA DSB signalling[16]. Senescence, as assayed by both SA-β-gal activity and incorporation of 5-bromodeoxyuridine (BrdU), was suppressed after ATM depletion (Fig. 1b–d). Similar results were obtained with BJ diploid fibroblasts and with a different pool of *atm*-specific siRNA oligonucleotides (see Supplementary Figs 1, 2).

The second oncogene we studied was *cdc6*. It encodes a DNA replication licensing factor that is expressed at high levels in non-small cell lung carcinoma (NSCLC) and other tumours[17,18] and also in the MRC5 and BJ cells that overexpress Mos (Fig. 1a and Supplementary Fig. 1a). We generated MRC5, BJ and IMR90 cells expressing Cdc6 and observed activation of the DNA DSB checkpoint, as previously described[19], and also induction of senescence (Supplementary Figs 3–5). The latter was suppressed by depleting the ATM kinase or its substrate p53 and also by caffeine, an ATM inhibitor[20] (Supplementary Figs 2–5).

The third oncogene we studied was *cyclin E*, which is frequently overexpressed in human precancerous and cancerous lesions[21]. We examined our previously described U2OS cells, in which cyclin E overexpression is induced by removal of tetracycline (Tet) from the tissue culture medium. Within 2 days, this leads to activation of the DNA DSB checkpoint[1]. Similar to the findings described above for Mos and Cdc6, Cyclin E overexpression led to senescence, whose induction could be suppressed by a specific ATM inhibitor[22] (Supplementary Fig. 6).

Our results indicate that the DNA DSB checkpoint mediates oncogene-induced senescence. However, previously, senescence mediated by *ras* was shown to involve $p16^{INK4A}$ (refs 11–13). In our hands, overexpression of Mos and Cdc6 led to induction of $p16^{INK4A}$ (Fig. 1a

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and Supplementary Figs 1a, 4a), but depletion of $p16^{INK4A}$ by siRNA did not noticeably suppress senescence (Supplementary Fig. 7). Thus, the contribution of $p16^{INK4A}$ to oncogene-induced senescence might vary from oncogene to oncogene, as it does for replicative senescence, which is mediated by the DNA DSB checkpoint, but also by a parallel pathway involving $p16^{INK4A}$ (refs 7–9).

The mechanism by which the DNA DSB checkpoint is activated during oncogene-induced senescence could involve DNA replication stress. Consistent with this hypothesis, overexpression of Cdc6 led to senescence in MRC5 cells that express telomerase, indicating that the induction of senescence was not mediated by short telomeres (Supplementary Fig. 8). Furthermore, in BJ cells arrested in stage G1 of the cell cycle by serum starvation, overexpression of Mos or Cdc6 did not lead to activation of the DNA DSB checkpoint or senescence (Supplementary Fig. 9). To provide more evidence that DNA replication stress is involved, we examined U2OS cells, which express cyclin E in an inducible manner, for single-stranded DNA, which is typically associated with stalled DNA replication forks, and for Chk1 phosphorylation on Ser 317 (ref. 23). Both markers scored positive (Supplementary Fig. 10a). Furthermore, the DNA damage foci in these cells, marked by H2AX phosphorylation, mapped to sites of DNA replication, as revealed by proliferating cell nuclear antigen (PCNA) staining (Supplementary Fig. 10b). Similarly, BJ cells overexpressing Mos and Cdc6 had replication protein A (RPA) foci, indicating the presence of single-stranded DNA (Supplementary Fig. 10c and data not shown).

We used DNA combing to investigate DNA replication stress further. U2OS cells expressing cyclin E in a Tet-repressible manner were pulse-labelled consecutively with the thymidine analogues IdU and CldU to distinguish ongoing DNA replication forks (a stretch of IdU label followed by CldU label) and newly-fired forks (CldU label only) from prematurely terminated forks (IdU label only). In cells that expressed normal levels of cyclin E (+Tet), most of the replication forks were ongoing or newly-fired, whereas, as early as one day after cyclin E induction (–Tet), most forks were prematurely terminated (Fig. 2a, b; Supplementary Fig. 11a, b). Premature termination of DNA replication can lead to fork collapse and formation of DNA DSBs. Indeed, overexpression of cyclin E led to the formation of DNA DSBs, but only in cells that were replicating their DNA (Fig. 2c). Similar results were obtained with BJ cells stably expressing cyclin E or Mos (Supplementary Fig. 11c–f).

The findings described here and also by d'Adda di Fagagna and co-workers[24] elucidate a previously undescribed pathway by which oncogenes induce senescence involving DNA replication stress and the DNA DSB checkpoint. We next sought to establish the relevance

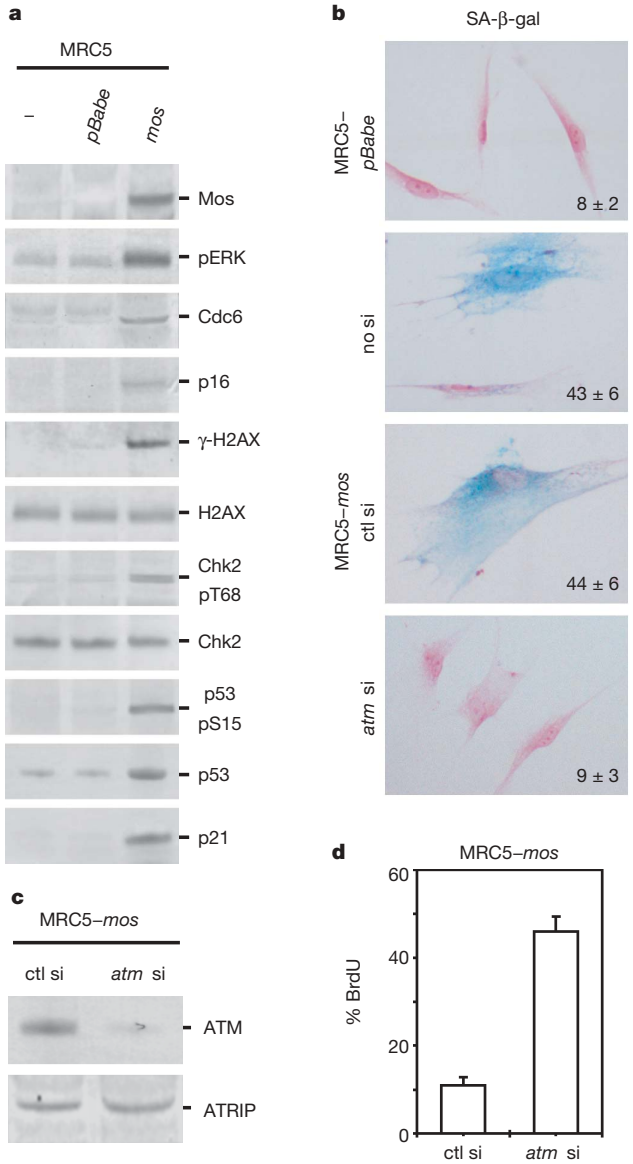

Figure 1 | Oncogene-induced senescence is dependent on the DNA DSB checkpoint. **a**, Immunoblot analysis of MRC5 diploid fibroblasts: non-infected (–), infected with empty vector (*pBabe*) or infected with a retroviral vector expressing *mos*. **b**, Images of SA-β-gal-stained MRC5 cells transfected with no siRNA (no si), control siRNA (ctl si) or siRNA specific for *atm* (*atm* si). Means and s.d. of the percentages of SA-β-gal-positive cells are indicated. **c**, Efficiency of ATM depletion. ATR-interacting protein (ATRIP) serves as a control. **d**, Means and s.d. of BrdU-positive staining for cells transfected with control or *atm*-specific siRNA.

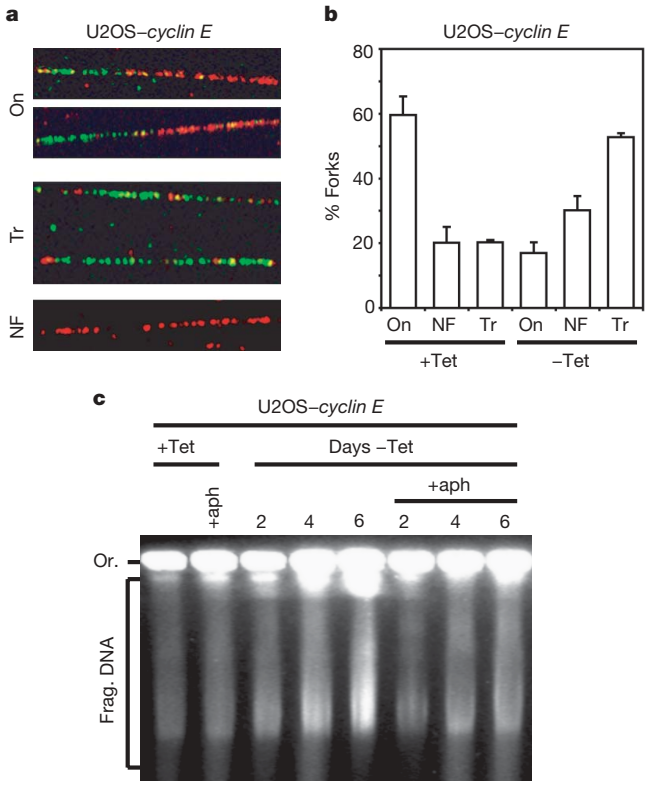

Figure 2 | DNA replication stress in cells undergoing oncogene-induced senescence. **a**, Representative DNA combing images of ongoing (On), terminated (Tr) and newly-fired (NF) replication forks. Cells were pulsed with IdU (green) and then with CldU (red). **b**, Mean and s.d. of the percentages of ongoing, newly-fired and terminated replication forks in cells with cyclin E repressed (+Tet) or induced (–Tet). **c**, Induction of cyclin E expression (–Tet) leads to DNA DSBs in cells undergoing DNA replication. aph, aphidicolin; Or., electrophoresis origin; Frag. DNA, fragmented DNA.

of this pathway in human cancerous and precancerous lesions. We first examined NSCLC, a tumour in which Cdc6 protein is overexpressed in about 50% of cases[17]. In 22 cases for which frozen sections were available from our previously described database[2,17], there was a remarkable correlation between Cdc6 overexpression, a potent DNA damage response, and SA-β-gal activity (Supplementary Fig. 12).

We subsequently examined preneoplastic lesions. Using frozen sections of colon adenomas, we first established a strong correlation between SA-β-gal staining and senescence markers suitable for immunohistochemistry of paraffin-embedded tissues, such as heterochromatin proteins 1α and 1γ (HP1α and HP1γ, respectively) and trimethylation of Lys 9 of histone H3 (refs 3–7, 12; Supplementary Fig. 13 and data not shown). This validation allowed us to study preneoplastic lesions for which only paraffin-embedded sections were available. In colon adenomas there was robust activation of the DNA DSB checkpoint that correlated very well with senescence (Fig. 3a and Supplementary Fig. 14). Progression to carcinoma was associated with decreased scores for both activation of the DNA DSB checkpoint and senescence. The decrease in senescence scores was more pronounced, probably due to mutations in DNA damage checkpoint genes, such as *p53* (Fig. 3a). We found induction of $p16^{INK4A}$ in both adenomas and carcinomas, but it did not correlate well with senescence (Fig. 3a). Furthermore, in those adenomas that showed heterogeneous staining, the presence of senescence correlated with activation of the DNA damage checkpoint, rather than with $p16^{INK4A}$ induction (Fig. 3b). Analysis of precancerous and cancerous lesions of the urinary bladder led to similar findings (Supplementary Fig. 14). Finally, we also found an association between DNA damage response and senescence markers in benign mouse skin papillomas generated with the two step initiation/promotion protocol (Supplementary Fig. 15).

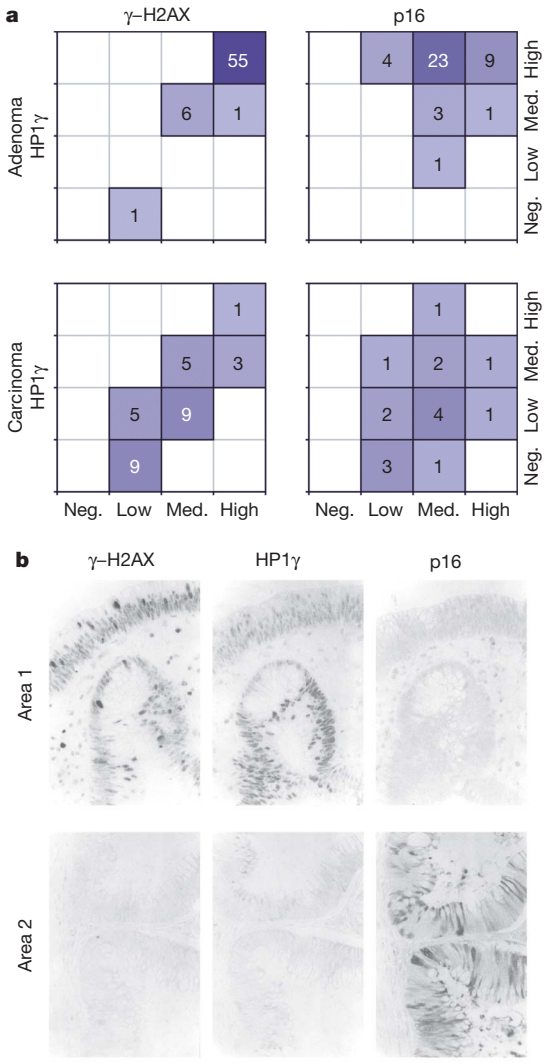

Figure 3 | Cosegregation of senescence and DNA damage response markers, but not $p16^{INK4A}$, in human precancerous and cancerous lesions. **a,** Correlation between HP1γ expression, H2AX phosphorylation (γ-H2AX) and $p16^{INK4A}$ (p16) expression in colon adenomas and carcinomas. For each marker, each lesion received a score of negative (Neg.), low, medium (Med.) or high, based on the percentage of cells scoring positive for that marker. **b**, Cosegregation of senescence and DNA damage response markers, but not $p16^{INK4A}$ induction, in different areas of the same colon adenoma.

Senescence in human preneoplasia is thought to act as a barrier to tumorigenesis[3–6]. The demonstration that oncogene-induced senescence is mediated by the DNA DSB checkpoint predicts that inhibition of the checkpoint would promote tumour progression. To test this hypothesis we established a tumour model using cells from the PDVC57 tumour line, injected subcutaneously in severe combined immunodeficient (SCID) mice and inhibiting ATM in these cells either by short hairpin (sh)RNA or with caffeine in the drinking water. PDVC57 cells have both copies of the endogenous *ras* gene mutated[25] and exhibit constitutive activation of the DNA DSB checkpoint and senescence. In tissue culture, ATM depletion by shRNA or inhibition by caffeine suppressed activation of the DNA DSB checkpoint and the induction of senescence, and led to increased invasiveness (Supplementary Fig. 16). Similarly, in tumours formed after injection of PDVC57 cells in SCID mice, depletion or inhibition of ATM suppressed activation of the DNA DSB checkpoint and senescence and led to larger and more invasive tumours (Fig. 4a–d and Supplementary Figs 17, 18).

Senescence was initially identified as a response of tissue culture cells to eroded telomeres or activated oncogenes[13], but its relevance to human cancer was unclear until its recent demonstration in preneoplasia[3–6]. The fact that senescence is more common in preneoplasia than in neoplasia indicated that senescence might serve as a barrier to oncogenesis. However, the molecular basis leading to induction of senescence in preneoplastic lesions was not defined and at least in melanocytic lesions did not involve telomere erosion or $p16^{INK4A}$ induction[3]. Our results indicate that senescence, which, like apoptosis, serves as a tumorigenesis barrier in preneoplastic lesions, is induced by the DNA damage checkpoint in response to DNA replication stress (Fig. 4e).

METHODS

Manipulation of tissue culture cells. Human diploid IMR90 and MRC5 embryonic lung and BJ foreskin fibroblasts were infected at passages 8, 28 and 21, respectively, with a replication-incompetent retrovirus (*pBabe*) expressing human *mos* or *cdc6* or *cyclin E* and a hygromycin-resistance marker or with a control retrovirus expressing only the hygromycin-resistance marker. Stable clones (MRC5-*cdc6*, IMR90-*cdc6*, BJ-*cyclin E*) were selected with hygromycin (100 μg ml^{-1}, Invitrogen) 48 h after infection over 3 weeks. Transiently transduced clones (MRC5-*mos*, BJ-*mos* and BJ-*cdc6*) were selected 24 h after infection for 2 days and examined 2 days later. U2OS cells expressing Tet-repressible cyclin E (ref. 1) were also examined. We used antibodies specific for the following proteins: Mos (clone P-19, Santa Cruz); pERK (ref. 25); Cdc6 (ref. 17); $p16^{INK4A}$ (clone F-16, Santa Cruz); γ-H2AX, H2AX, Chk2 pT68, Chk2, p53, p21, ATRIP (ref. 2); ATM (GeneTex); Actin (clone AC-15, Abcam); RPA (clone 34-19, GeneTex); p53 pS15, cyclin E, Cdk7 (ref. 1); and Chk1 pS317 (ref. 26). Some cells were fixed with 1% paraformaldehyde, processed for SA-β-gal activity and counterstained with nuclear fast red[27]. Cells were transfected with control siRNA (luciferase, Dharmacon) or with an siRNA pool specific for *atm* (ATM siGENOME, Dharmacon, sequences: 5′-CUA ACA AAC AGG UGA UAU AUU-3′, 5′-AAA GGC CCA AGC UCC UCC UUU-3′) after selection was completed (for the stable clones) or 2 days after selection was completed (for the transiently transduced clones). 72 h after after siRNA transfection, the cells were assayed for SA-β-gal activity and BrdU incorporation[27].

DNA combing was performed as described previously[28] with slight modifications. Briefly, U2OS cells expressing Tet-repressible cyclin E were grown in the presence or absence of Tet for 1 day and then pulsed-labelled with 20 μM IdU for

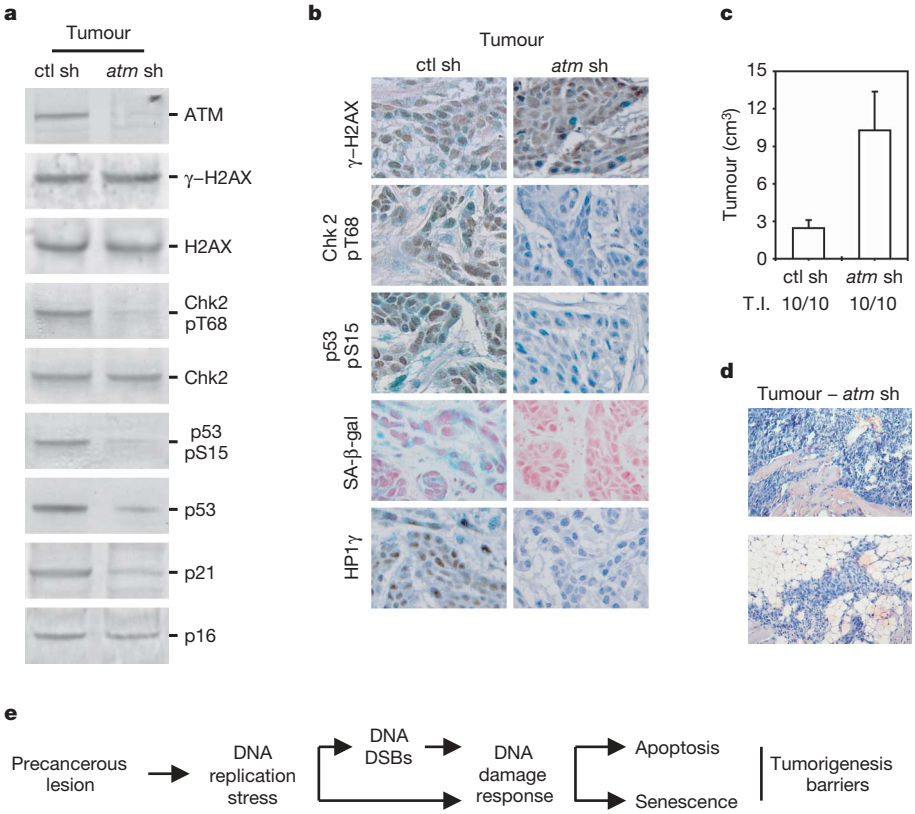

Figure 4 | Oncogene-induced senescence acts as a barrier to invasiveness and tumorigenesis. a, Immunoblot analysis of protein extracts prepared from tumours formed by PDVC57 cells stably expressing control shRNA (ctl sh) or *atm*-specific shRNA (*atm* sh). **b**, Immunohistochemistry and SA-β-gal analysis. **c**, Tumour volume (mean and s.d.) and tumour incidence (T.I.). T.I. indicates whether tumours formed in all injected sites. **d**, Histochemistry showing invasion of PDVC57 cells expressing *atm*-specific shRNA (dark blue) in muscle tissue (pink; upper panel) and adipose tissue (clear; lower panel). **e**, Model for senescence and apoptosis as tumorigenesis barriers induced by activated DNA damage checkpoints in human precancerous lesions.

15 min, incubated in regular medium for 30 min, and then labelled with 20 μM CldU for 15 min. The cells were then harvested and lysed on glass slides in spread buffer. The DNA was denatured and stained with rat anti-BrdU/CldU (OBT0030F, Immunologicals Direct; 1:200) and mouse anti-IdU/BrdU (clone B44, Becton Dickinson; 1:50) primary antibodies.

For pulsed-field gel electrophoresis, U2OS cells expressing Tet-repressible cyclin E and BJ fibroblasts were treated with aphidicolin for 8 h and then allowed to grow for an additional 20 h (U2OS) or 40 h (BJ) in the absence or presence of aphidicolin. 1×10^6 cells were inserted into agarose plugs and subjected to pulsed-field gel electrophoresis[29].

Analysis of preneoplastic and neoplastic human lesions. Our databases of preneoplastic and neoplastic lesions have been described[1,2,17]. Frozen sections of colon adenomas were from patients who had received no therapy before removal of the lesion. Immunohistochemistry was performed using optimally titrated antibodies specific for the following proteins: HP1α (15.19s2, Upstate); HP1γ (42s2, Upstate); histone H3 methylated on K9 (07-442, Upstate); $p16^{INK4A}$ (16P04, NeoMarkers); Ki67, γ-H2AX, Chk2 pT68 (refs 1, 2). Each lesion was scored by an experienced pathologist as negative (no or only scattered stained cells, fewer than 2%), low (heterogeneous staining, with at least 20% of the section showing 2–10% positive cells), medium (at least 20% of the section showing 11–50% positive cells) or high (at least 20% of the section showing greater than 50% stained cells).

Mouse model for senescence as tumorigenesis barrier. PDVC57 cells were infected with a retrovirus expressing shRNA specific for *atm* (Open-Biosystems, shRNA sequence: 5′-TGC TGT TGA CAG TGA GCG CGC AGG GTC AGT CAA CAG ATT ATA GTG AAG CCA CAG ATG TAT AAT CTG TTG ACT GAC CCT GCA TGC CTA CTG CCT CGG A-3′) or a control retrovirus and stably infected cells were selected over 3 weeks. To assay tumour progression, 10 SCID mice were injected subcutaneously at two different sites on their abdomen with 2×10^6 PDVC57 cells expressing shRNA specific for *atm* or with control cells[30]. Two weeks later the mice were killed, the tumour volume was measured and the tumours were used to prepare protein extracts and for immunohistochemistry analysis and SA-β-gal staining. In a second experiment the mice were killed 3 weeks after subcutaneous injection and tumour invasion was monitored by histochemistry.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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