

ORIGINAL ARTICLE

AKT induces senescence in human cells via mTORC1 and p53 in the absence of DNA damage: implications for targeting mTOR during malignancyMV Astle¹, KM Hannan¹, PY Ng¹, RS Lee¹, AJ George^{1,2,3}, AK Hsu⁴, Y Haupt^{1,3,6}, RD Hannan^{1,5,6,7} and RB Pearson^{1,5,6,7}

¹Growth Control and Differentiation Program, Trescowthick Research Laboratories, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia; ²School of Biomedical Sciences, University of Queensland, Brisbane, Queensland, Australia; ³Department of Pathology, University of Melbourne, Melbourne, Victoria, Australia; ⁴Hematology Immunology Translational Research Laboratory, Trescowthick Research Laboratories, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia; ⁵Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia and ⁶Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Victoria, Australia

The phosphatidylinositol 3-kinase (PI3K)/AKT and RAS oncogenic signalling modules are frequently mutated in sporadic human cancer. Although each of these pathways has been shown to play critical roles in driving tumour growth and proliferation, their activation in normal human cells can also promote cell senescence. Although the mechanisms mediating RAS-induced senescence have been well characterised, those controlling PI3K/AKT-induced senescence are poorly understood. Here we show that PI3K/AKT pathway activation in response to phosphatase and tensin homolog (PTEN) knockdown, mutant PI3K, catalytic, α polypeptide (PIK3CA) or activated AKT expression, promotes accumulation of p53 and p21, increases cell size and induces senescence-associated β -galactosidase activity. We demonstrate that AKT-induced senescence is p53-dependent and is characterised by mTORC1-dependent regulation of p53 translation and stabilisation of p53 protein following nucleolar localisation and inactivation of MDM2. The underlying mechanisms of RAS and AKT-induced senescence appear to be distinct, demonstrating that different mediators of senescence may be deregulated during transformation by specific oncogenes. Unlike RAS, AKT promotes rapid proliferative arrest in the absence of a hyperproliferative phase or DNA damage, indicating that inactivation of the senescence response is critical at the early stages of PI3K/AKT-driven tumourigenesis. Furthermore, our data imply that chronic activation of AKT signalling provides selective pressure for the loss of p53 function, consistent with observations that PTEN or PIK3CA mutations are significantly associated with p53 mutation in a number of human tumour types. Importantly, the demonstration that mTORC1 is an essential mediator of AKT-induced senescence raises the possibility that targeting mTORC1

in tumours with activated PI3K/AKT signalling may exert unexpected detrimental effects due to inactivation of a senescence brake on potential cancer-initiating cells.

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Introduction

Activating mutations in the gene encoding a catalytic subunit of phosphatidylinositol 3-kinase (PI3K), PI3K, catalytic, α polypeptide (PIK3CA), or inactivating mutations in the negative regulator of PI3K signalling, phosphatase and tensin homolog (PTEN), account for approximately 30% of human sporadic tumours (Luo *et al.*, 2003; Campbell *et al.*, 2004). Furthermore, hyperactivation of an important mediator of PI3K signalling, AKT, is observed in a high proportion of human cancers, including 20–55% of breast cancers, ~60% of lung cancers, ~50% of prostate cancers and 40–70% of melanomas (Altomare and Testa, 2005). In addition to its role in PI3K/PTEN mutation-driven cancer, AKT acts as a central node in pathways activated by oncogenic mutation in genes, including those encoding EGFR, PDGFR, RAS and LKB1 (Hynes and MacDonald, 2009; Solomon and Pearson, 2009). Three homologous AKT isoforms, AKT1/PKB α , AKT2/PKB β and AKT3/PKB γ , have distinct and overlapping roles in a diverse range of fundamental cellular functions considered hallmarks of cancer when dysregulated, including cell growth and proliferation, cell survival, migration and angiogenesis (Hanahan and Weinberg, 2000; Manning and Cantley, 2007). AKT-dependent control of cell growth and proliferation is in large part mediated by mTORC1 regulation of ribosome biogenesis and protein translation (Shaw and Cantley, 2006; Hannan *et al.*, 2011).

Like the other prototypical oncogenes, RAS and MYC, PI3K is an important driver of malignant

Correspondence: Associate Professor RB Pearson, Protein Chemistry Laboratory, Peter MacCallum Cancer Centre, Locked Bag 1, A'Beckett Street, Melbourne, Victoria 8006, Australia.

E-mail: rick.pearson@petermac.org

⁷These authors contributed equally to this work.

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transformation; however, paradoxically, when hyperactivated in normal cells, all three can result in cell death, cell cycle arrest or senescence, depending on cellular context (Lowe *et al.*, 2004; Gorgoulis and Halazonetis, 2010). Well-established *in vitro* markers of oncogene-induced senescence have now been detected *in vivo* in humans, including in melanocytic naevi (Gray-Schopfer *et al.*, 2006) and neurofibromas (Courtois-Cox *et al.*, 2006). Clearly, oncogene-induced senescence must be overcome to enable tumour formation and pharmacological induction of senescence of tumour cells is being increasingly seen as a mechanism for therapeutic intervention (Gewirtz *et al.*, 2008; Ewald *et al.*, 2010; Nardella *et al.*, 2011). Thus, understanding the basis of senescence induced by individual oncogenes in normal cells and how this process is subverted in cancer cells is critical to our understanding of malignant transformation and how it can be effectively targeted.

The paradigm for oncogene-induced senescence was established by examining the growth arrest of non-transformed human and mouse cells in response to activated alleles of the RAS superfamily (Serrano *et al.*, 1997). In the case of activated RAS, cells undergo an initial hyperproliferative phase (Tremain *et al.*, 2000) that is later followed by an irreversible proliferation arrest (senescence) associated with the accumulation of cell cycle inhibitors p53, p21 and p16 and larger flattened cell morphology (Serrano *et al.*, 1997). The mechanisms contributing to the RAS-induced accumulation of p53 include increased protein stability following activation of DNA damage response (Di Micco *et al.*, 2006; Mallette *et al.*, 2007), p38MAPK (Wang *et al.*, 2002) and accumulation of promyelocytic leukemia protein (PML) (Ferbeyre *et al.*, 2000). Elevated p53 translation also contributes to the senescence phenotype induced by RAS (Bellodi *et al.*, 2010). Deletion of p53 in mouse cells is sufficient to rescue RAS-induced proliferation arrest (Serrano *et al.*, 1997); however; this is not the case in human cells where the p16 pathway is the prominent effector for proliferation arrest (Brookes *et al.*, 2002; Huot *et al.*, 2002).

Multiple lines of evidence indicate that increased PI3K/AKT signalling also induces cell senescence. Loss of PTEN, the major negative regulator of the PI3K/AKT pathway, induces senescence in mouse embryonic fibroblasts (Chen *et al.*, 2005) and mouse prostate epithelium (Chen *et al.*, 2005; Alimonti *et al.*, 2010a). PTEN deletion also induces loss of mouse hematopoietic stem cells by induction of the senescence effectors p53, p21 and p19Arf (Yilmaz *et al.*, 2006; Zhang *et al.*, 2006). Expression of constitutively active AKT induces senescence in human endothelial cells (Miyachi *et al.*, 2004), mouse embryonic fibroblasts (Chen *et al.*, 2005; Mavrakis *et al.*, 2008; Nogueira *et al.*, 2008) and mouse primary keratinocytes (Moral *et al.*, 2009). The mechanisms underlying induction of senescence by AKT are poorly defined, although the accumulation of reactive oxygen species has been implicated as playing a role (Nogueira *et al.*, 2008). A better understanding of the mechanisms of PI3K/AKT-induced senescence will be

crucial for understanding the role of dysregulated PI3K/AKT signalling in cancer.

To this end, we have analysed PI3K/AKT pathway activation-induced senescence in a normal human fibroblast model. We show that this senescence requires mTORC1-dependent accumulation of p53 involving increased p53 synthesis and stabilisation mediated by inactivation of MDM2. We have identified important differences between senescence induced by the AKT and RAS oncogenic modules. Activated PI3K/AKT signalling rapidly induces senescence independent of the hyperproliferative phase required for the effect of H-RAS^{V12}. Consistent with this finding, PI3K/AKT-induced senescence is independent of the DNA damage response that is required by H-RAS^{V12}.

Results

Hyperactivation of the PI3K/AKT pathway induces senescence in BJ-T cells

We utilised the well-characterised BJ human fibroblasts immortalised with human telomerase reverse transcriptase (BJ-T cells) (Hahn *et al.*, 1999) as a model to study the mechanisms of PI3K-induced responses in non-transformed human fibroblasts. BJ-T cells were transduced with expression constructs encoding PTEN short hairpin RNA (shRNA) or the PI3K catalytic subunit mutant PIK3CA^{E545K} (Zhao *et al.*, 2005), which is commonly detected in multiple cancer types and enhances PI3K/AKT pathway signalling (Campbell *et al.*, 2004; Samuels *et al.*, 2005). Cells with depleted PTEN protein levels or expressing PIK3CA^{E545K} exhibited increased levels of phospho-AKT and phosphorylation of the AKT substrate PRAS40 (proline-rich Akt substrate of 40 kDa) demonstrating PI3K pathway activation and consistent with a previous report (Kim *et al.*, 2007) accumulation of cell cycle inhibitors p53 and its target p21 (Figure 1a). Cells expressing PTEN shRNA or PIK3CA^{E545K} also exhibited a significant increase in cell size (Figure 1b) and senescence-associated β -galactosidase activity (SA β GAL) (Figure 1c). These results indicate that activation of PI3K activity induces multiple markers of senescence in the BJ-T human fibroblasts.

AKT is a major effector of the PI3K signalling pathway in cancer (Altomare and Testa, 2005; Franke, 2008). To examine its role in the pro-senescence response, we expressed constitutively active myristoylated AKT isoforms, myr-AKT1, myr-AKT2 and myr-AKT3, in BJ-T cells to test whether activation of AKT alone is sufficient to drive senescence. Each AKT isoform was examined as they exhibit overlapping but distinct roles in specific tumour types (Altomare and Testa, 2005; Cristiano *et al.*, 2006). BJ-T cells were also transduced with H-RAS^{V12}, to allow direct comparison with the prototypical oncogene that activates senescence pathways in non-transformed cells (Serrano *et al.*, 1997). Western blot analysis confirmed the expression of hemagglutinin-tagged myr-AKT isoforms

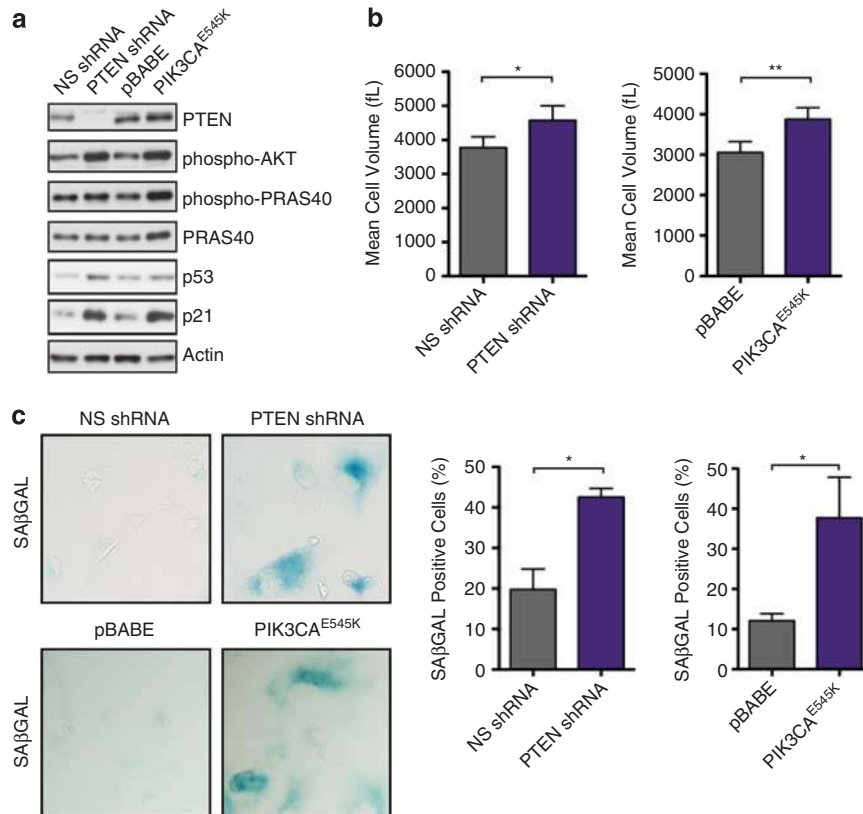


Figure 1 PTEN depletion or PIK3CA mutant expression induces senescence in BJ-T cells. (a) BJ-T cells were transduced with pGIPZ-NS-shRNA or pGIPZ-PTEN-shRNA lentivirus or pBABE or pBABE-PIK3CA^{E545K} retrovirus. Lysates of these cells were collected and immunoblotted with the indicated antibodies. Actin is used as a loading control. (b) BJ-T cells as above at day 10 post-transduction were trypsinised and analysed on a Coulter counter to obtain cell size measurements (cell volume in fL) ($n=4$, bars represent mean \pm s.e.m., * $P<0.05$, ** $P<0.01$). (c) Cells as above were fixed and incubated with SA β GAL staining solution overnight. Cells were washed, stained with DAPI and imaged for both SA β GAL and the number of nuclei. Percentage of SA β GAL-positive cells was determined for a minimum of 10 low-power magnification fields ($n=4-5$, bars represent mean \pm s.e.m., * $P<0.05$).

and H-RAS^{V12} (Figure 2a), and increased downstream signalling as indicated by elevated phosphorylation of AKT substrates GSK3 β and PRAS40. The RAS/MAPK pathway was upregulated in H-RAS^{V12}-expressing cells as demonstrated by increased levels of phospho-ERK1/2. myr-AKT-expressing cells exhibited increased p53 levels and induction of p21, but only modest increases in p16 in comparison to activated RAS.

Cells were analysed for the accumulation of senescence markers at days 10–11 post-transduction to enable direct comparison with H-RAS^{V12}, which induces SA β GAL activity between days 6 and 11 post-transduction (Serrano *et al.*, 1997; Tremain *et al.*, 2000; Young *et al.*, 2009). Cells expressing activated AKT isoforms and H-RAS^{V12} showed a significant increase in SA β GAL (Figure 2b) and cell size (Figure 2c), indicating that AKT activation is sufficient to induce cellular senescence. The formation of senescence-associated heterochromatic foci (SAHF), a marker of RAS- and DNA damage-induced senescence, is dependent on the p16 pathway (Narita *et al.*, 2003). Consistent with only a modest accumulation of p16, SAHF formation, as detected by 4',6-diamidino-2-phenylindole (DAPI) staining, was not observed in myr-AKT1-expressing

cells (or myr-AKT2/3-expressing cells, data not shown) unlike the robust induction observed with activated RAS (Figure 2d).

myr-AKT1 expression was also capable of inducing senescence in IMR90 cells, an alternative normal human fibroblast model that is wild type for human telomerase reverse transcriptase (Ouellette *et al.*, 1999; Gorbunova *et al.*, 2002). Western blot analysis confirmed the expression of hemagglutinin-tagged myr-AKT1 and H-RAS^{V12}, and increased downstream signalling as indicated by elevated phosphorylation of AKT substrates GSK3 β and PRAS40, and the RAS target, ERK1/2 (Supplementary Figure 1A). IMR90 cells expressing activated AKT1 and RAS exhibited ~60% senescent cells as detected by SA β GAL staining (Supplementary Figure 1B), and as with BJ-T cells, this was associated with increased p21 expression (Supplementary Figure 1A). IMR90 cells express higher basal levels of p16 (Beausejour *et al.*, 2003), and therefore may have a greater propensity for SAHF formation than BJ-T cells. In IMR90 cells, myr-AKT1 does moderately induce p16 levels (Supplementary Figure 1A); however, myr-AKT1 expression failed to induce SAHF formation in these cells, unlike H-RAS^{V12} (Supplementary Figure 1C), suggesting that AKT1-induced senescence is largely independent of p16.

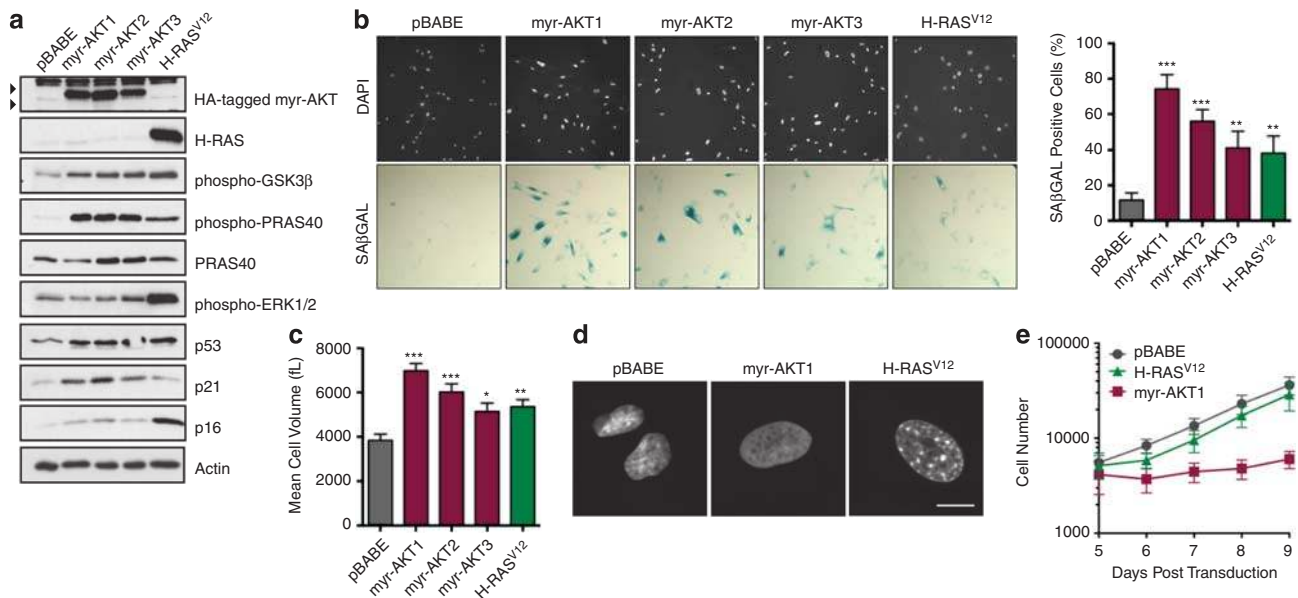


Figure 2 Activated AKT isoforms induce markers of senescence and proliferation arrest in BJ-T cells. (a) BJ-T cells were transduced with pBABE, pBABE-my-AKT isoforms or pBABE-H-RAS^{V12}. At day 10 post-transduction, cells were harvested and lysates immunoblotted with the indicated antibodies to demonstrate construct expression, activation of downstream signalling pathways and accumulation of senescence markers. Actin is used as a loading control. Black arrowheads indicate nonspecific bands from the 12CA5 anti-HA antibody. (b) Cells prepared as in (a) were fixed, incubated with SA β GAL staining solution overnight, and then stained with DAPI to visualise non-senescent cells and quantitate total cell number. The percentage of senescent cells was quantitated manually ($n=4$, bars represent mean \pm s.e.m., $**P<0.05$, $***P<0.001$). (c) BJ-T cells as above were trypsinised and analysed on a Coulter counter to determine cell size measurements (fL) ($n=6$, bars represent mean \pm s.e.m., $*P<0.01$, $**P<0.05$, $***P<0.001$). (d) To visualise SAHFs, BJ-T cells expressing myr-AKT1 or H-RAS^{V12} at day 10 post-transduction were stained with DAPI and imaged using confocal microscopy. Scale bar represents 10 μ m. (e) Proliferation of BJ-T cells expressing myr-AKT1 or H-RAS^{V12}. At 4 days post-transduction, cells were plated at equal cell number and harvested daily for cell counting on a Coulter counter over a period of 5 days ($n=5$, error bars represent mean \pm s.e.m.).

To further compare AKT- and RAS-induced senescence, we examined the time courses of the response of BJ-T cells to the expression of myr-AKT1 and H-RAS^{V12}. Analysis of cell proliferation from days 4 to 9 post-transduction, directly following puromycin selection, revealed that myr-AKT1-expressing cells exhibited markedly reduced proliferation at these early time points (Figure 2e) as did myr-AKT2/3-expressing cells (data not shown). H-RAS^{V12}-expressing cells exhibited comparable proliferation to pBABE control cells at all time points.

We next compared the SA β GAL levels and the p53, p21 and p16 responses to the expression of myr-AKT1 and H-RAS^{V12} at multiple time points (Supplementary Figure 2). myr-AKT1 expression induced a more rapid and complete induction of senescence than H-RAS^{V12} up to 11 days post-retroviral infection (Supplementary Figure 2A). At early time points following transduction (days 5 and 8), p21 expression increased rapidly in AKT-expressing cells (Supplementary Figure 2B), whereas no increase was observed in RAS-expressing cells until day 10 (Figure 2a) and day 12 (Supplementary Figure 2B). Despite the incomplete senescence response of the H-RAS^{V12}-expressing population (30–40% of cells positive for SA β GAL activity), we observed robust p16 induction at days 10–12 in these cells; however, we did not detect major p16 increases in the myr-AKT-expressing BJ-T cells.

The late induction of p21 and p16 upon expression of H-RAS^{V12} is consistent with RAS-induced senescence being associated with an initial proliferative phase and consequent DNA damage resulting in senescence (Tremain *et al.*, 2000; Di Micco *et al.*, 2006). Conversely, we found that AKT rapidly induced proliferative arrest (Figure 2e) and did not require an initial proliferative phase to promote senescence (Supplementary Figure 2A), and therefore we examined whether expression of activated AKT-induced markers of DNA damage (Figure 3). Activated AKT expression did not affect the percentage of cells exhibiting phospho- γ H2A.X foci (Figure 3a) and phosphorylation of p53 at serine 15, an indicator of ATM activation and downstream CHK2-mediated p53 stabilisation (Banin *et al.*, 1998; Hirao *et al.*, 2000) (Figure 3b). On the other hand, H-RAS^{V12} expression significantly increased both markers of the DNA damage response. Taken together, these data identify clear differences between classical RAS-induced senescence and that mediated by activated AKT that is rapid and not associated with SAHF formation or DNA damage.

myr-AKT induces a senescence-associated secretory phenotype

To further characterise the AKT-induced senescence phenotype, we examined whether, like H-RAS^{V12} (Acosta *et al.*, 2008; Coppé *et al.*, 2008), constitutively

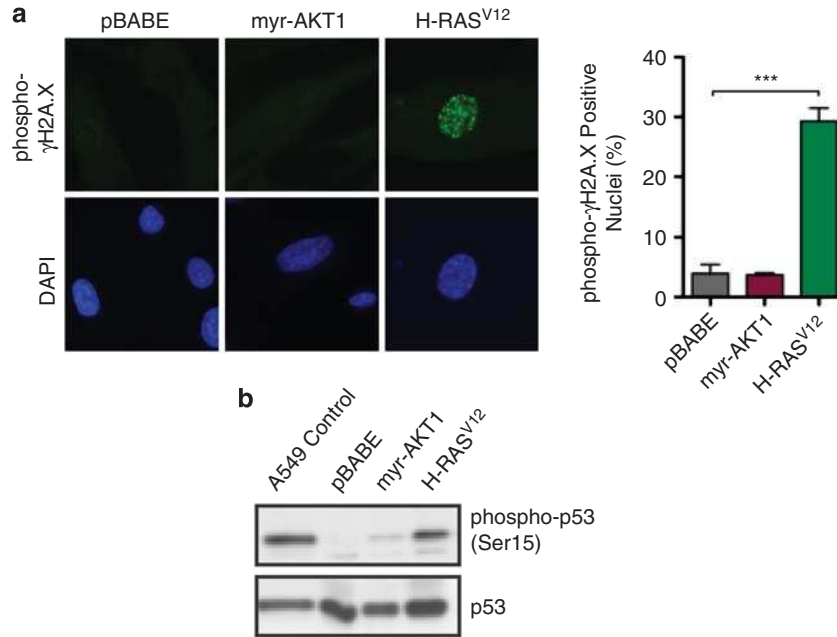


Figure 3 myr-AKT1 fails to induce markers of DNA damage. **(a)** BJ-T cells transduced with pBABE, pBABE-my-AKT1 or pBABE-H-RAS^{V12} were immunostained with phospho-Ser139- γ H2A.X. Cells were imaged and the percentage that exhibited nuclear phospho-Ser139- γ H2A.X foci was quantitated for a minimum of 200 cells per experiment ($n = 3$, bars represent mean \pm s.e.m., *** $P < 0.001$). **(b)** Lysates of BJ-T cells expressing pBABE, pBABE-my-AKT1 or H-RAS^{V12} were immunoblotted with phospho-p53(Ser15) and p53 antibodies. Lysate of γ -irradiated A549 cells were included as a positive control.

active AKT could initiate a ‘senescence-associated secretory phenotype’ or ‘SASP’, where cells markedly increase the secretion of a number of proinflammatory cytokines. The role of this SASP has not been well delineated; however, it may function in an autocrine manner to reinforce growth arrest and also promote immune system-mediated clearance of senescent cells (Freund *et al.*, 2010). Most commonly associated with the SASP is the upregulation of interleukin-6 (IL-6), IL-8 (Acosta *et al.*, 2008; Coppé *et al.*, 2008; Rodier *et al.*, 2009), and importantly IL-1 α , which can mediate a positive feedback loop, resulting in the induction of itself, IL-6 and IL-8 (Orjalo *et al.*, 2009).

As all three AKT isoforms potentially induced senescence, continuing analyses focused on the most widely expressed AKT1 isoform. Real-time PCR (RT-PCR) of known SASP factors confirmed that expression of IL-1 α and IL-1 β was upregulated in cells expressing myr-AKT1 (Figure 4a). Expression of H-RAS^{V12} also induced IL-1 α and IL-1 β as described previously (Coppé *et al.*, 2008), although to different levels than that observed with AKT. Surprisingly, IL-6 mRNA was reduced in cells expressing myr-AKT1 or H-RAS^{V12}, whereas IL-8 was only significantly upregulated in H-RAS^{V12}-expressing cells. To further investigate this lack of IL-6 induction, we determined the levels of secreted IL-1 α , IL-1 β , IL-6 and IL-8 released into the media from cells expressing myr-AKT1 or H-RAS^{V12} at day 10 post-transduction. The relative levels of secreted IL-1 α , IL-1 β and IL-8 induced by myr-AKT1 and H-RAS^{V12} (Figure 4b) reflected the mRNA expression data (Figure 4a). Furthermore, despite the paradoxically

decreased IL-6 mRNA levels detected following myr-AKT1 or H-RAS^{V12} expression, IL-6 protein levels secreted into the media were elevated fourfold, consistent with published data showing that secreted IL-6 is a major contributor to SASP (Coppé *et al.*, 2008). Although the basis of this discrepancy is unclear, IL-6 expression has been reported to be subjected to post-transcriptional regulation, including via miR-365- and mTOR-dependent regulation of IL-6 translation (Narita *et al.*, 2011; Xu *et al.*, 2011). Thus, AKT-induced senescence is characterised by an SASP, with increased secretion of IL-1 α , IL-1 β , IL-6 and IL-8.

myr-AKT-induced senescence is p53-dependent

In human fibroblasts, the proliferation arrest induced by activated RAS cannot be rescued by the inhibition of p53 activity alone (Serrano *et al.*, 1997); however, it is dependent on the presence of functional p16 (Brookes *et al.*, 2002; Huot *et al.*, 2002). Given the absence of significant p16 pathway activation and the clear differences observed between H-RAS^{V12}- and myr-AKT1-induced senescence, we examined whether AKT-induced senescence was specifically dependent on the p53 pathway.

To determine the contribution of p53 to AKT-induced senescence, we examined the induction of senescence markers in BJ-T-my-AKT cells stably expressing p53 shRNA or a control shRNA. myr-AKT failed to induce p21 expression in p53 knockdown cells (Figure 5a) or increase the cell doubling time and mean cell volume, compared with control cells

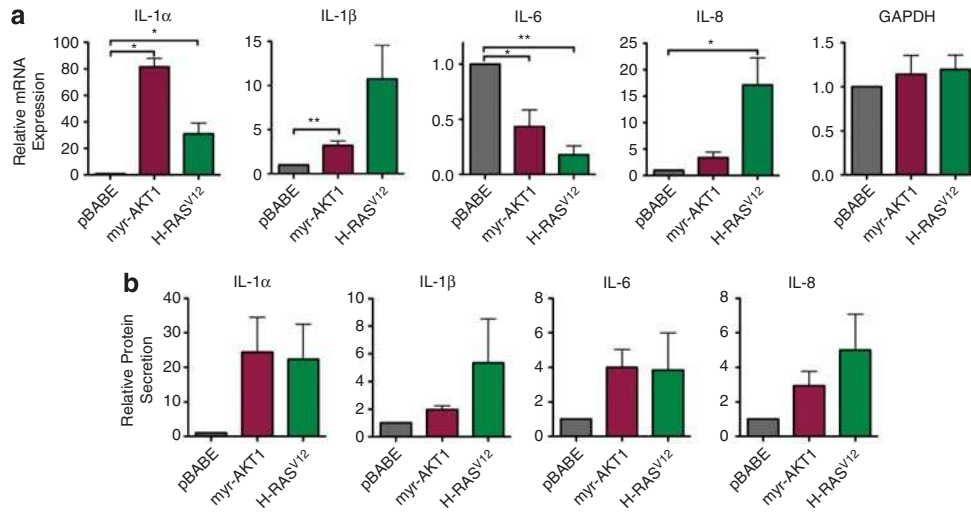


Figure 4 myr-AKT1 expression induces upregulation of senescence-associated secretory factors. **(a)** RT-PCR analysis of IL-1 α , IL-1 β , IL-6, IL-8 and GAPDH expression in BJ-T cells expressing pBABE, myr-AKT1 or H-RAS^{V12} at 10–11 days post-transduction ($n = 4$ –6, bars represent mean \pm s.e.m., * $P < 0.05$, ** $P < 0.01$). **(b)** CBA analysis of secreted IL-1 α , IL-1 β , IL-6 and IL-8 in the conditioned media from BJ-T cells expressing pBABE, myr-AKT1 or H-RAS^{V12} at 10 days post-transduction ($n = 3$, bars represent mean \pm s.e.m.).

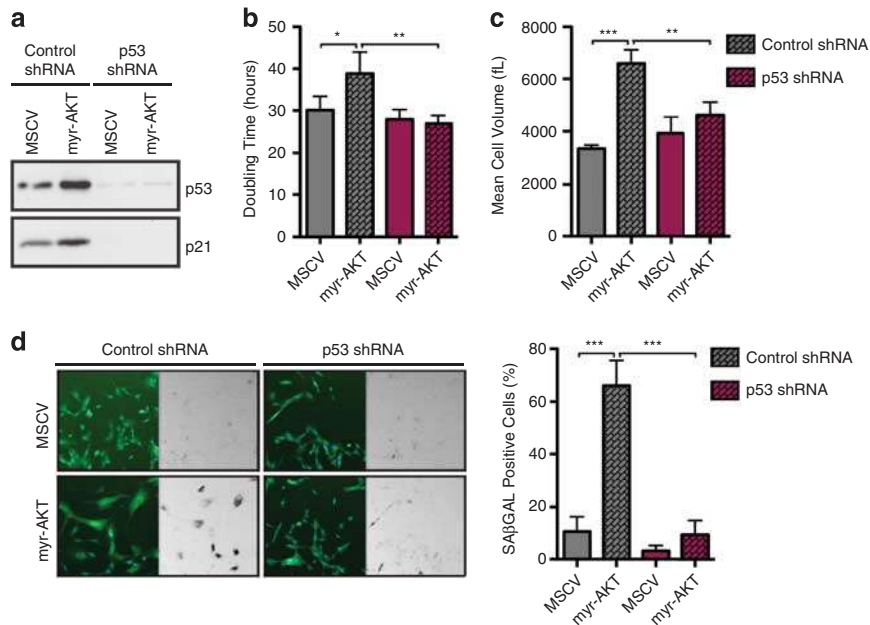


Figure 5 AKT-induced senescence is p53-dependent. **(a)** BJ-T cells were transduced with retrovirus encoding control or p53 shRNA. These cell lines were then transduced with MSCV or MSCV-my-AKT. Lysates were harvested at day 10 post-transduction and immunoblotted with p53 and p21 antibodies. **(b)** Control or p53 shRNA expressing BJ-T cell lines transduced with MSCV or MSCV-my-AKT. Cells were plated at equal cell number and harvested 3 days later. Cell numbers were used to determine population doubling time ($n = 4$, bars represent mean \pm s.e.m., * $P < 0.05$, ** $P < 0.01$). **(c)** Cells as in **(b)** were analysed on the Coulter counter to obtain cell size measurements ($n = 3$, bars represent mean \pm s.e.m., ** $P < 0.01$, *** $P < 0.001$). **(d)** Cells as in **(b)** were analysed for SA β GAL activity. Cells were imaged under brightfield for SA β GAL- and DAPI-stained nuclei, and the cellular autofluorescence was imaged to indicate cell morphology. The percentage of cells that stained positive for SA β GAL was determined ($n = 4$, bars represent mean \pm s.e.m., *** $P < 0.001$).

expressing myr-AKT and the control shRNA (Figures 5b and c). SA β GAL positivity was significantly reduced in BJ-T-my-AKT/p53 stable knockdown cells as compared with control BJ-T-my-AKT cells (Figure 5d). We also determined that AKT could not induce p21 or SA β GAL activity in BJ-T cells expressing SV40 large T antigen (Hahn *et al.*, 1999), consistent with

the ability of SV40 large T antigen to repress p53 and retinoblastoma activity (data not shown).

We also examined the effect of acute p53 knockdown during induction of myr-AKT1 expression. BJ-T cells co-expressing p53 shRNA and myr-AKT1 were analysed for proliferation from day 5 post-transduction, and for apoptosis markers and the percentage of cells in

the S phase at day 10 post-transduction (Supplementary Figure 3). Acute p53 knockdown, (confirmed by immunoblot analysis (Supplementary Figure 3A), rescued the reduced proliferation of myr-AKT1-expressing cells to that of control cells (Supplementary Figure 3B). The major effect of p53 knockdown was to increase the percentage of cells in the S phase (Supplementary Figure 3C), with minimal effect on apoptosis observed by Annexin V and propidium iodide positivity (Supplementary Figure 3D).

Taken together, these results indicate that p53 knock-down specifically regulates the induction of senescence markers by activated AKT and, unlike the case of H-RAS^{V12} (Serrano *et al.*, 1997), is sufficient to rescue cell proliferation. These results are intriguing given that previous reports demonstrate that AKT promotes the activity of the E3 ubiquitin ligase MDM2 (Ogawara *et al.*, 2002), the important negative regulator of p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Post-translational modifications, protein–protein interactions and/or alterations to cellular localisation of either p53 or MDM2 can block the p53:MDM2 interaction resulting in p53 stabilisation (Meek and Knippschild, 2003).

To investigate the mechanism by which p53 accumulates in AKT-expressing BJ-T cells, p53 stability was

assessed by [³⁵S]Met/Cys pulse-chase analysis. Over the 60 min chase period, [³⁵S]Met/Cys-labelled p53 reduced at a slower rate in myr-AKT cells compared to pBABE cells, indicating that myr-AKT expression decreased p53 turnover (Figure 6a). Importantly, a 45% increase in p53 synthesis was observed in the 30 min pulse period in myr-AKT-expressing cells compared with control cells (Figure 6b), with no change in relative p53 transcript levels detected (Supplementary Figure 4A). Furthermore, rapid lysis of pBABE- or myr-AKT-expressing cells plus or minus the proteasome inhibitor MG132 treatment indicated that AKT-expressing cells had reduced levels of polyubiquitinated p53 in the steady state (Figure 6c). These results indicate that p53 accumulation in AKT cells results from both a reduction in MDM2-mediated p53 ubiquitination and degradation, plus enhanced p53 translation.

Our results are in contrast to previous reports that AKT phosphorylation of MDM2 promotes its nuclear localisation (Mayo and Donner, 2001; Mayo *et al.*, 2002) and enhances MDM2 ubiquitin ligase activity, resulting in p53 destabilisation (Ogawara *et al.*, 2002). To determine if the reported AKT/MDM2 pathway is functional in BJ-T cells, we assessed MDM2 phosphorylation at Ser166 and cellular localisation. BJ-T cells already express appreciable levels of

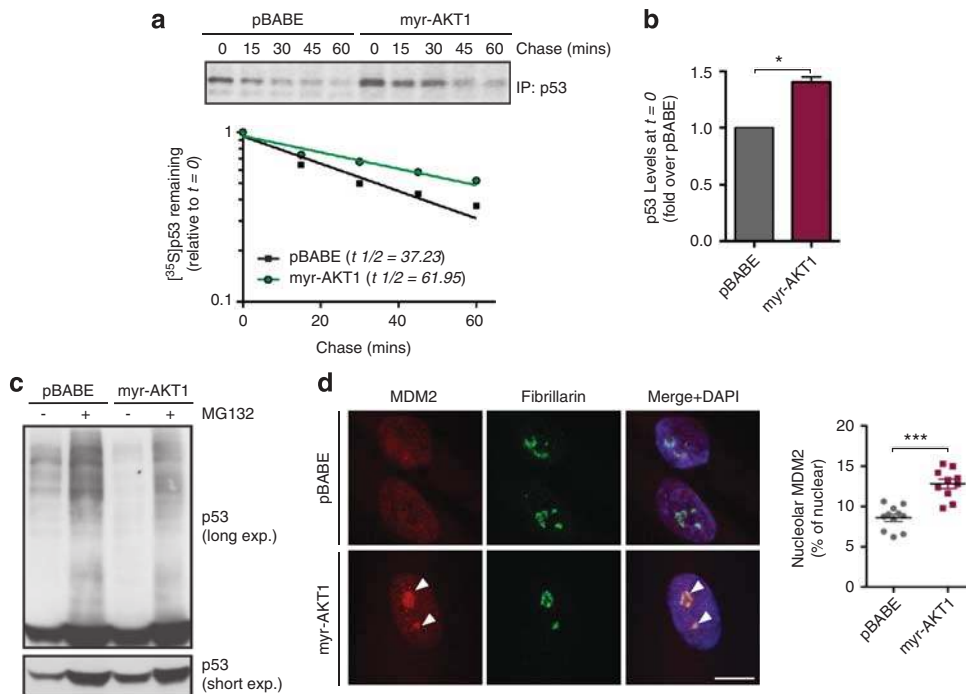


Figure 6 MDM2 activity is reduced with the expression of AKT. **(a)** BJ-T cells expressing pBABE or pBABE-my-AKT1 were subjected to [³⁵S]Met/Cys pulse-chase analysis. Lysates were collected at various chase times and p53 immunoprecipitations (IPs) performed. Complexes were resolved by SDS–PAGE and gels exposed to phosphorimager storage screens. p53 band intensity was quantified using the ImageQuant software (GE Healthcare, Uppsala, Sweden). p53 half-life determination values were standardised to $t=0$ for the particular cell population. p53 half-life, which was determined using regression analysis, is indicated ($n=2$). **(b)** The amount of [³⁵S]Met/Cys-labelled p53 following a 30 min pulse period ($t=0$) was quantitated ($n=3$, bars represent mean values \pm s.e.m., $*P<0.05$). **(c)** BJ-T cells expressing pBABE or pBABE-my-AKT1 were treated with MG132 (30 μ M) for 3 h and subjected to rapid lysis in SDS–PAGE reducing buffer. Lysates were run on SDS–PAGE and immunoblotted with p53 antibodies. **(d)** Cells as in **(a)** were fixed, immunostained with MDM2 (red) and fibrillarin (green) antibodies and co-stained with DAPI (blue). Cells were imaged at the same laser attenuation. Scale bar represent 10 μ m. The total nucleolar MDM2, as a percentage of total nuclear MDM2, was quantitated ($n=10$ –11 fields, $***P<0.001$).

phospho-Ser166-MDM2, but enforced expression of myr-AKT did not increase these levels any further (Supplementary Figure 4B). The migration of phospho-Ser166-MDM2 was altered, suggesting that AKT may promote alternative post-translational modifications of MDM2. Fractionation experiments demonstrated that AKT expression induced a slight increase in nuclear and cytoplasmic MDM2 levels (Supplementary Figure 4C).

To investigate why p53 ubiquitination was markedly reduced despite slightly elevated nuclear MDM2 levels, we examined MDM2 subnuclear localisation. Nucleolar accumulation of MDM2 has been reported to be associated with reduced MDM2 activity (Weber *et al.*, 1999; Ashcroft *et al.*, 2000), due to its sequestration into inactive complexes. Confocal analysis revealed a robust increase in the accumulation of MDM2 in the nucleolus of AKT-expressing cells (Figure 6d). Next, we examined if induction of the tumour suppressor ARF contributes to MDM2 nucleolar localisation. ARF expression can be induced by oncogenic insult and mediates p53 stabilisation by sequestration of MDM2 in the nucleolus (Weber *et al.*, 1999). ARF protein could not be detected in myr-AKT-expressing cells (Supplementary Figure 4D), although it was observed in response to MYC, a known activator of ARF (Zindy *et al.*, 1998), indicating that MDM2 nucleolar sequestration is ARF-independent. Accumulation of the PML protein has been shown to contribute to p53 stabilisation (Ferbeyre *et al.*, 2000) via its nucleolar sequestration (Bernardi *et al.*, 2004). However, we found no change in the expression levels of PML or any increase in PML nucleolar localisation in BJ-T cells expressing AKT as compared with control cells (data not shown). Taken together, these results indicate that AKT induces DNA damage-independent p53 stabilisation via ARF- and PML-independent nucleolar sequestration of MDM2.

mTORC1 activity is essential for AKT-induced senescence

mTORC1 is an important mediator of PI3K/AKT signalling to protein synthesis, cell growth and proliferation (Manning and Cantley, 2007). To determine the contribution of enhanced mTORC1 signalling to AKT-induced senescence, BJ-T cells transduced with myr-AKT or control pBABE were treated with the mTORC1-selective inhibitor, rapamycin and markers of senescence analysed. Upon treatment with rapamycin, the percentage of AKT cells positive for SA β GAL was significantly reduced (Figure 7a). Rapamycin treatment also dramatically reduced AKT-induced effects on cell size (Figure 7b), and the SASP (Figure 7c), indicating that mTORC1 activity is critical for PI3K/AKT-driven senescence.

Given that we have shown a marked effect of inhibiting mTORC1- on AKT-induced senescence, together with the fact that mTORC1 is an important regulator of protein synthesis (Proud, 2007) and a previous report links mTORC1 to the regulation of p53 translation rates (Lee *et al.*, 2007), we examined the

effect of rapamycin on the AKT-induced accumulation of p53. We found that inhibition of mTORC1 by rapamycin treatment ablated p53 accumulation and the expression of the p53 effector and transcriptional target p21 (Figure 7d). This reduction in multiple markers of senescence with rapamycin did not correspond with an increase in proliferation (Supplementary Figure 5); however, this result is expected given the observed cytostatic effect of rapamycin on fibroblasts transduced with control pBABE.

Taken together, our data are consistent with a model where elevated PI3K/AKT signalling to mTORC1 in non-transformed human fibroblasts leads to increased p53 synthesis. In combination with MDM2 nucleolar sequestration and reduced p53 ubiquitination, this results in the robust accumulation of p53 and cellular senescence (Figure 8).

Discussion

Oncogene-induced senescence is an important mechanism for protection from tumourigenesis *in vivo* and current research suggests that it may be possible to activate senescence-inducing pathways for cancer therapy (Collado and Serrano, 2010; Lin *et al.*, 2010). Here we demonstrate that activation of the PI3K/AKT pathway, one of the most commonly upregulated signalling modules in human tumours, rapidly induces senescence in human fibroblasts. We demonstrate that depletion of p53 levels via shRNA-mediated knockdown or inhibition of its activity via stable expression of SV40 large T antigen bypasses the senescence response. Thus, p53 signalling represents an important potential barrier to PI3K/AKT-driven tumourigenesis and activation of AKT in normal cells is likely to provide selective pressure for loss of p53 function. We find that AKT enhances both p53 translation and protein stability, and that AKT-induced p53 accumulation and downstream senescence is dependent on mTORC1 activity.

AKT fails to induce DNA damage

p53- and retinoblastoma-dependent oncogene-induced senescence has been best characterised in response to activated RAS signalling in mouse and human fibroblasts (Serrano *et al.*, 1997; Ferbeyre *et al.*, 2002), where increased p53 expression is dependent on an initial hyperproliferative phase induced by activated RAS followed by accumulation of DNA damage (Di Micco *et al.*, 2006; Mallette *et al.*, 2007). Importantly, here we show that PI3K/AKT-induced senescence proceeds via a different mechanism to RAS. It occurs rapidly, and is independent of DNA damage. The rapid cell cycle arrest induced by AKT hyperactivation implies that these cells are far less likely to escape senescence than cells with hyperactivating mutations in RAS; thus, suggesting that somatic mutations in AKT are unlikely to be the initial mutation in the multistep progression to tumourigenesis.

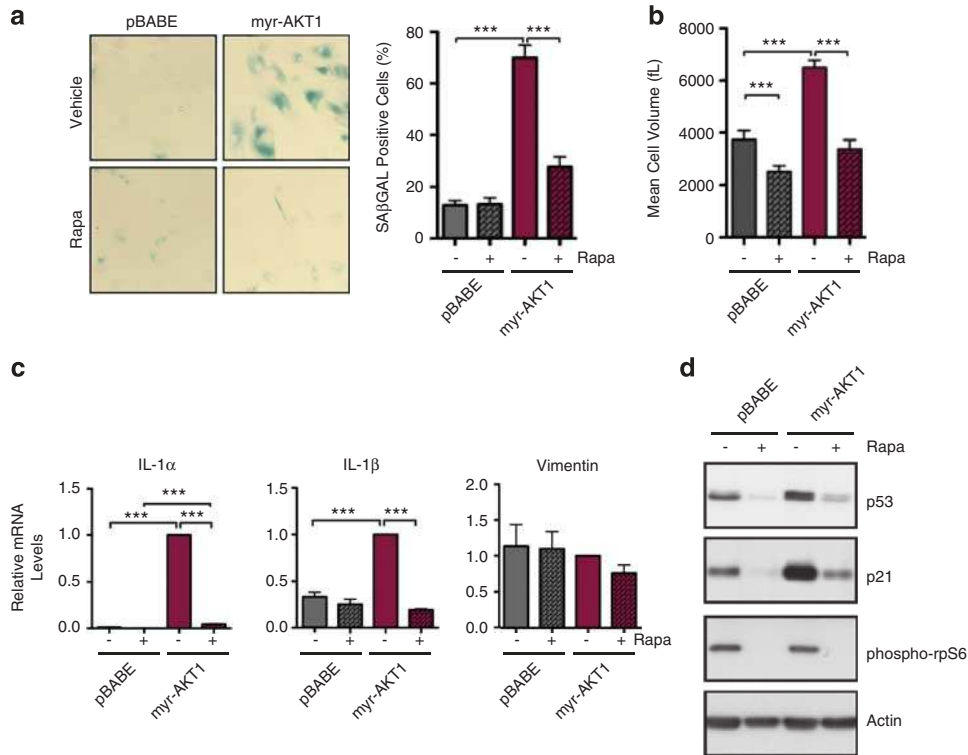


Figure 7 AKT-induced senescence is dependent on mTORC1 activity. (a) BJ-T cells were transfected with pBABE or pBABE-my-AKT1 and treated with either vehicle or 20 nM rapamycin for 11 days. Cells were fixed, incubated with SAβGAL staining solution overnight, and then stained with DAPI to visualise non-senescent cells and quantitate total cell number (DAPI not shown). The percentage of senescent cells was quantitated manually ($n=4-7$, bars represent mean \pm s.e.m., $***P<0.001$). (b) BJ-T cells as above were trypsinised and analysed on a Coulter counter to obtain cell size measurements (fL) ($n=4-6$, bars represent mean \pm s.e.m., $***P<0.001$). (c) RNA was harvested from cells as in (a). RT-PCR analysis was performed for IL-1 α , IL-1 β and vimentin expression. Values are expressed relative to myr-AKT1-Rapa ($n=4$, bars represent mean \pm s.e.m., $***P<0.001$). (d) Cell lysates were prepared from BJ-T cells as in (a) and immunoblotted with the indicated antibodies. Actin is used as a loading control.

AKT-induced senescence occurs independent of p16 activation of SAHFs

In addition, we demonstrate that, unlike RAS, AKT fails to induce high levels of p16 or SAHFs in either BJ-T or IMR90 cells. Although the levels of p16 have been shown to be an important determinant for RAS-induced senescence (Benanti and Galloway, 2004), our data indicate that p16 is unlikely to play a role in AKT-induced senescence. Rapid induction of senescence without signs of DNA damage, p16 accumulation or SAHF formation has similarly been reported for the oncogenic fusion protein RUNX1-ETO (Wolyniec *et al.*, 2009). The p16-dependent alterations to chromatin structure, detected as SAHFs, are thought to promote the irreversibility of the cell cycle arrest due to stable silencing of proliferative genes (Narita *et al.*, 2003). It is not clear as to how the absence of SAHFs would affect the maintenance of the senescence phenotype of AKT cells *in vivo*. However, here we demonstrate that both AKT and RAS induce a robust senescence-associated secretory phenotype, which may function to maintain senescence (Freund *et al.*, 2010). Identification of these distinctly different mechanisms for p53-dependent senescence induction by active RAS and AKT reinforce the concept that specific oncogenic signalling modules may target distinct mediators of senescence.

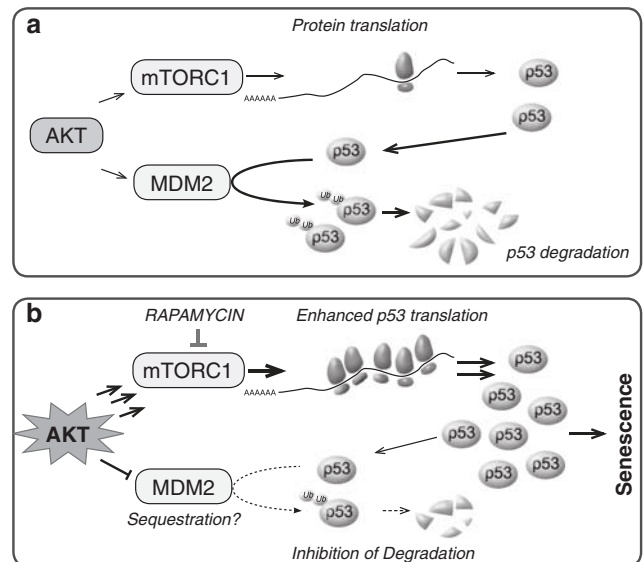


Figure 8 Proposed mechanism of AKT-induced senescence. (a) Physiological levels of AKT signalling lead to the activation of MDM2 at levels that maintain low p53 protein expression. (b) Chronic hyperactivation of AKT results in mTORC1-dependent increases in p53 translation and simultaneously stimulates MDM2 sequestration within the nucleolus, inhibiting p53 ubiquitination to further enhance p53 accumulation leading to cellular senescence.

AKT induces senescence via post-transcriptional regulation of p53

Activated PI3K/AKT signalling results in marked stabilisation of p53 protein. This was unexpected given that AKT has been reported to promote the activity of the E3 ubiquitin ligase MDM2, the important negative regulator of p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). However, in human fibroblasts, we found that active AKT signalling reduces p53 ubiquitination and this corresponds to increased nucleolar sequestration, and hence inactivation of MDM2. Our data indicate that this nucleolar sequestration is independent of ARF and PML, and thus is likely to involve binding to other proteins, which localise to the nucleolus either constitutively or under conditions of stress such as nucleophosmin (Kurki *et al.*, 2004) and multiple ribosomal proteins, including RPL5, RPL11 and RPL23 (Lohrum *et al.*, 2003; Bhat *et al.*, 2004; Dai and Lu, 2004; Dai *et al.*, 2004). These binding events are invariably associated with p53 stabilisation (Zhang and Lu, 2009). It is tempting to hypothesise that while transient activation of AKT by growth factors can lead to active MDM2, p53 degradation and cell survival, chronic activation of the pathway, such as that following oncogenic mutation within the signalling network, results in nucleolar sequestration and inactivation of MDM2, and accumulation of p53 with a subsequent induction of senescence.

PI3K/AKT pathway activation and p53 alterations cooperate in human cancer

Consistent with p53-induced senescence acting as a major 'brake' for PI3K/AKT-driven human cancer, mutational analysis of primary human tumours has demonstrated significant associations between PTEN or PIK3CA, and p53 alterations in colorectal cancer (Nosho *et al.*, 2008), gastric cancer (Oki *et al.*, 2005), non-small-cell lung cancer (Andjelkovic *et al.*, 2011) and bladder cancer (Puzio-Kuter *et al.*, 2009). Furthermore, concomitant alterations in these pathways often result in poor patient outcome (Catasus *et al.*, 2009; Puzio-Kuter *et al.*, 2009; Andjelkovic *et al.*, 2011). These reports, together with our data, raise concerns for the rational design of pro-senescence therapies to combat cancers driven by activating mutations in the PI3K/AKT signalling network, as aggressive tumours will commonly have previously subverted p53 signalling. In this context, therapeutic activation of p53-independent pro-senescence pathways will thus be required, such as targeting the E3 ligase Skp2, which acts via regulation of p21, p27 and Atf4 (Lin *et al.*, 2010), or inducing p16.

mTORC1 inhibitors prevent AKT-induced senescence: implications for cancer therapy

Importantly, our results demonstrate that mTORC1 is the critical mediator of induction of p53-dependent senescence by AKT in non-transformed human fibroblasts. Treatment of cells expressing active AKT with the allosteric mTORC1 inhibitor rapamycin reduced

p53 and p21 to control levels and prevented senescence. Previous reports indicate that mTORC1 activation downstream of AKT will promote p53 accumulation via regulation of p53 translation as in *PTEN*^{-/-} (Alimonti *et al.*, 2010b) and *Tsc1*^{-/-} mouse embryonic fibroblasts, (Lee *et al.*, 2007). Our results in human cells are also consistent with the emerging idea that the levels of mTORC1 activity determine the outcome of p53 activation in cells, where low mTORC1 activity in the context of genotoxic stress or MDM2 activation favours cellular quiescence over senescence (Korotchkina *et al.*, 2010; Leontieva and Blagosklonny, 2010).

The dependence of AKT-induced senescence on mTORC1 activity has direct implications for the use of mTORC1 inhibitors as anticancer agents. Inhibitors such as RAD001 and CCI-779 have delivered limited therapeutic benefit as single agents (Brachmann *et al.*, 2009). This lack of effect has been attributed, at least in part, to the inactivation of mTORC1-dependent negative feedback of PI3K/AKT signalling (O'Reilly *et al.*, 2006) and/or the activity of the RAS pathway (Carracedo *et al.*, 2008). Our data provide another level of potential concern where mTORC1 inhibition may relieve the senescence 'brake' in p53 wild-type cells and promote PI3K-dependent transformation. Targeted therapies are now being designed and tested that will alleviate the pro-tumourigenic effects of mTORC1 inhibition. These include mTOR active site inhibitors that will target both mTORC2-dependent AKT activation and mTORC1-dependent growth (Feldman *et al.*, 2009; Dowling *et al.*, 2010; Hsieh *et al.*, 2010), dual PI3K/mTOR inhibitors (Bhatt *et al.*, 2010) or the use of the MDM2 antagonist Nutlin-3a in combination with mTOR inhibition (Alimonti *et al.*, 2010b).

In summary, we demonstrate that constitutive PI3K/AKT pathway activation in normal human cells results in the accumulation of p53 and rapid induction of cellular senescence. Consequently, inactivation of the senescence response is likely to be critical at the early stages of PI3K/AKT-driven tumourigenesis, and consistent with this model, PTEN or PIK3CA mutations are significantly associated with p53 mutation in human tumours. Importantly, we demonstrate that mTORC1 is an essential mediator of AKT-induced senescence, providing further insight to aid in the rational design of cancer therapeutic strategies to target tumors with dysregulated PI3K/AKT signalling.

Materials and methods

Plasmid constructs

Murine stem cell virus (MSCV) plasmids encoding myr-AKT isoforms are as described previously (Chan *et al.*, 2011). pBABE-puro, pBABE-puro-myr-AKT2, pBABE-puro-myr-AKT3 and pBABE-puro-PIK3CA^{E545K} were from Addgene Inc. (Cambridge, MA, USA). pBABE-myr-AKT1 was directly subcloned from MSCV-myr-AKT1. pBABE-puro-H-RAS^{V12} was a gift from Patrick Humbert (Peter MacCallum Cancer Centre, Melbourne, VIC, Australia). pGIPZ-PTEN shRNA and

pGIPZ-NS shRNA were from Open Biosystems (Huntsville, AL, USA).

Cell lines

BJ-T cells, a gift from Robert Weinberg, and derivatives were cultured in Dulbecco's modified Eagle's medium:M199 media at a ratio of 4:1 (v/v) with 15% fetal calf serum and 2 mM L-glutamine (Hahn *et al.*, 1999, 2002) and maintained in 5% CO₂ at 37°C. Cells expressing pBABE or MSCV constructs were enriched by puromycin selection (1 µg/ml) (Sigma-Aldrich, St Louis, MO, USA) or fluorescence-activated cell sorter, respectively. Rapamycin (20 nM) (Calbiochem, Gibbstown, NJ, USA) was added 1 day following infection and replaced every two days.

Western blotting and antibodies

Cells were lysed in western solubilisation buffer (0.5 mM EDTA, 20 mM HEPES (pH7.9), 2% sodium dodecyl sulfate (SDS)). Lysates were mechanically sheared and a DC assay (BioRad, Hercules, CA, USA) utilised for protein concentration determination. Proteins were visualised using horse radish peroxidase-conjugated secondary antibodies (BioRad) and Western Lightening Chemiluminescence Plus (Perkin-Elmer, Covina, CA, USA). MDM2 (SMP14), p16, p21 (C-19), p53 (DO-1) and H-RAS antibodies were from Santa Cruz Technology (Santa Cruz, CA, USA). Actin and phospho-Ser139-γH2A.X antibody were from Millipore (Billerica, MA, USA). Fibrillarlin antibodies were from Abcam (Cambridge, MA, USA). The 12CA5 (HA-tag) antibody was purified in-house. All other antibodies were from Cell Signalling Technology (Beverly, MA, USA).

SAβGAL staining

Cells were stained for SAβGAL as described previously (Debacq-Chainiaux *et al.*, 2009) and co-stained with DAPI (Molecular Probes, Invitrogen, Carlsbad, CA, USA), before imaging on a Olympus BX-51 or BX-61 microscope (Olympus America Inc., Center Valley, PA, USA), or a Cellomics imaging platform (Thermo Fisher Scientific, Waltham, MA, USA). SAβGAL cells and total nuclei were counted manually using the public domain ImageJ software (NIH, Bethesda, MD, USA; Abramoff *et al.*, 2004).

Immunofluorescence

For phospho-Ser139-γH2A.X, staining cells were fixed in 95% ethanol/5% acetic acid, permeabilised in 0.1% Tx-100/phosphate-buffered saline for 2 min and blocked for 30 min in 3% bovine serum albumin/phosphate-buffered saline. For MDM2/fibrillarlin co-staining, cells were fixed in methanol for 5 min at -20°C and blocked with 5% goat serum/phosphate-buffered saline for 30 min. Antibodies were diluted in blocking buffer and incubated with cells for 1 h. Cells were imaged on an Olympus BX51 epifluorescence or Leica SP5 confocal microscope (Leica, Wetzlar, Germany). Quantitation of relative nucleolar MDM2 localisation was performed using MetaMorph (Molecular Devices, Sunnyvale, CA, USA).

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RNA extraction and RT-PCR

RNA was extracted using an RNA extraction kit (Biolone, Alexandria, NSW, Australia). ³²P-labelled riboprobe was added to samples before column purification to allow RNA recovery calculation. RNA was normalised for cell number for cDNA production using random hexamers primers (Promega, Madison, WI, USA) and Superscript III (Invitrogen). Primer sequences are listed in Supplementary information.

Cytokine bead array for the analysis of IL secretion

Cells were plated at 50 000–100 000 in 150 µl of media per well of a 96-well plate. After 24 h, the media were removed, cleared by centrifugation and stored at -80°C. The level of secreted IL-1α, IL-β, IL-6 and IL-8 was determined using a custom-made Millipore Milliplex Human Cytokine Multiplex Immunoassay Kit following the manufacturer's instructions.

p53 pulse-chase analysis

Cells were incubated in Cys- and Met-free Dulbecco's modified Eagle's medium containing 15% fetal calf serum for 1 h, and then 200 µCi Express ³⁵S Protein Labelling Mix (Perkin-Elmer) was added for 30 min. Cells were washed with media, and incubated for the designated periods and harvested (50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, complete protease inhibitors; Roche Applied Science, Castle Hill, NSW, Australia). P53 was immunoprecipitated using p53 antibody (0.3 µg) and Protein A-Sepharose (Amersham Biosciences, Piscataway, NJ, USA). Following washes, samples were run on SDS-polyacrylamide gel electrophoresis (PAGE), gels dried, exposed to a Phosphorimager screen and imaged on a Typhoon (GE Healthcare).

Statistical analysis

All statistical tests were performed in GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). The analysis was performed using paired *t*-tests or repeated measures analysis of variance with Bonferroni post-test.

Conflict of interest

The authors declare no conflict of interest.

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Author/s:

Astle, MV;Hannan, KM;Ng, PY;Lee, RS;George, AJ;Hsu, AK;Haupt, Y;Hannan, RD;Pearson, RB

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