Opposing roles for p16\textsuperscript{ink4a} and p19\textsuperscript{Arf} in senescence and ageing caused by BubR1 insufficiency

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Expression of p16\textsuperscript{ink4a} and p19\textsuperscript{Arf} increases with age in both rodent and human tissues. However, whether these tumour suppressors are effectors of ageing remains unclear, mainly because knockout mice lacking p16\textsuperscript{ink4a} or p19\textsuperscript{Arf} die early of tumours. Here, we show that skeletal muscle and fat, two tissues that develop early ageing-associated phenotypes in response to BubR1 insufficiency, have high levels of p16\textsuperscript{ink4a} and p19\textsuperscript{Arf}. Inactivation of p16\textsuperscript{ink4a} in BubR1-insufficient mice attenuates both cellular senescence and premature ageing in these tissues. Conversely, p19\textsuperscript{Arf} inactivation exacerbates senescence and ageing in BubR1 mutant mice. Thus, we identify BubR1 insufficiency as a trigger for activation of the Cdkn2a locus in certain mouse tissues, and demonstrate that p16\textsuperscript{ink4a} is an effector and p19\textsuperscript{Arf} an attenuator of senescence and ageing in these tissues.

Cellular senescence is a state of irreversible growth arrest that can be induced by various cellular stressors\textsuperscript{1,2}. The Cdkn2a locus encodes two separate tumour suppressors, p16\textsuperscript{ink4a} (A001711), a cyclin-dependent kinase (Cdk) inhibitor that can block G1–S progression when present above a certain level, and p19\textsuperscript{Arf} (A001713), a positive regulator of the transcription factor p53 that integrates and responds to a wide variety of cellular stresses\textsuperscript{1,3–5}. Both p16\textsuperscript{ink4a} and p19\textsuperscript{Arf} are effectors of senescence in cultured cells\textsuperscript{6} and their levels increase with ageing in many tissues\textsuperscript{7,8}. This has led to speculation that their induction is causally implicated in \textit{in vivo} senescence and organismal ageing. However, rigorous testing of this notion has been difficult because mice that lack p16\textsuperscript{ink4a} or p19\textsuperscript{Arf} die of cancer long before they reach the age at which normal mice start to develop age-related disorders\textsuperscript{1,2}. Recent evidence in middle-aged p16\textsuperscript{ink4a} knockout mice indicates that the age-induced expression of p16\textsuperscript{ink4a} limits the proliferative and regenerative capacity of progenitor populations\textsuperscript{9–11}. Yet, whether the increased stem-cell proliferation and tissue regeneration seen in p16\textsuperscript{ink4a} knockouts actually delay onset of age-related pathologies remains unknown because of the limited animal lifespan\textsuperscript{12}.

One approach to study the role of p16\textsuperscript{ink4a} and p19\textsuperscript{Arf} in ageing would be to determine whether their respective inactivation by single gene mutations, in mouse models that develop ageing-associated pathologies at an early age, would prevent or delay premature ageing. Mutant mice with low levels of the mitotic checkpoint protein BubR1 (called BubR1 hypomorphic or BubR1\textsuperscript{H/H} mice, A003172) undergo premature separation of sister chromosomes and develop progressive aneuploidy along with various progeroid phenotypes that include short lifespan, cachectic dwarfism, lordokyphosis (abnormal curvature of the spine), sarcopenia (age-related skeletal muscle atrophy), cataracts, craniofacial dysmorphism, arterial stiffening, loss of (subcutaneous) fat, reduced stress tolerance and impaired wound healing\textsuperscript{13–15}. During the course of natural ageing, several mouse tissues show a marked decline in BubR1 protein expression, which, combined with the observation that BubR1\textsuperscript{H/H} mice age prematurely, suggests a possible role for BubR1 in regulating natural ageing\textsuperscript{14–15}. Here we show that certain mouse tissues induce p16\textsuperscript{ink4a} and p19\textsuperscript{Arf} in response to BubR1 hypomorphism. Using BubR1\textsuperscript{H/H} mice in which these tumour suppressors are lacking, we have demonstrated that p16\textsuperscript{ink4a} is an effector of cellular senescence and ageing, whereas, p19\textsuperscript{Arf} acts to suppress cellular senescence and ageing.

RESULTS

\textit{p16\textsuperscript{ink4a} inactivation increases the lifespan of BubR1\textsuperscript{H/H} mice}

To determine the requirement for p16\textsuperscript{ink4a} in the development of progeroid phenotypes in BubR1-insufficient mice, we bred BubR1\textsuperscript{H/H} mice on a p16\textsuperscript{ink4a} homozygous-null genetic background. In total, 86 BubR1\textsuperscript{H/H}, p16\textsuperscript{ink4a+/−}, 192 BubR1\textsuperscript{H/H}, 160 BubR1\textsuperscript{−/−} and 44 p16\textsuperscript{ink4a−/−} mice were generated and monitored for development of age-related phenotypes for a period of one year. Inactivation of p16\textsuperscript{ink4a} extended the lifespan of BubR1\textsuperscript{H/H} mice by 25% (Fig. 1a). Although the median lifespan of BubR1\textsuperscript{H/H} mice was extended in the absence of p16\textsuperscript{ink4a}, the maximum lifespan was not, suggesting that the condition(s) that cause(s) death was not rescued by p16\textsuperscript{ink4a} inactivation.
**ARTICLES**

**Ink4a loss blunts sarcopaenia induced by BubR1 insufficiency**

A prominent ageing-associated phenotype of BubR1<sup>H/H</sup> mice is the development of lordokyphosis<sup>13</sup>. The incidence of this phenotype was markedly reduced in BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice when compared with BubR1<sup>H/H</sup> animals (Fig. 1b, c). Furthermore, the median time to onset of lordokyphosis was three times longer in BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice compared with BubR1<sup>H/H</sup> animals (Fig. 1b, c).

**Ablation of p16<sup>ink4a</sup> in BubR1<sup>H/H</sup> mice extends lifespan and attenuates sarcopaenia.** (a) Overall survival curves for wild-type, p16<sup>ink4a</sup>−/−, BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice. The median overall survival of combined BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− is 25 weeks, a 25% extension in lifespan compared with BubR1<sup>H/H</sup> animals. We note that the p16<sup>ink4a</sup>−/−, BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− curves are all significantly different from the wild-type (BubR1<sup>+/+</sup>) curve (P<0.0001, log-rank tests). Moreover, the BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− curve is significantly different from the BubR1<sup>H/H</sup> curve (P=0.0142). (b) Incidence and latency of lordokyphosis in BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice. The curves are significantly different (P<0.0001, log-rank test). We note that no wild-type or p16<sup>ink4a</sup>−/− mice developed lordokyphosis during our one-year observation period (data not shown).

**Figure 1** Ablation of p16<sup>ink4a</sup> in BubR1<sup>H/H</sup> mice extends lifespan and attenuates sarcopaenia. (a) Overall survival curves for wild-type, p16<sup>ink4a</sup>−/−, BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice. (b) Incidence and latency of lordokyphosis in BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice. (c) Images of 5-month-old wild-type, BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice. Note the profound difference in the curvature of the spine in the BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mouse. (d) Cross-sections of gastrocnemius muscles from 5-month-old wild-type, BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice. Arrowheads mark degenerated fibres and asterisks mark areas of connective tissue infiltration. Scale bar is 100 µm. (e) Quantification of the number of deteriorating (atrophic) muscle fibres in gastrocnemius muscles shown in d. Note that BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− muscles have 3-fold less atrophic fibres than BubR1<sup>H/H</sup> muscles. Data are mean ± s.d. (n=4). (f) Skinned 5-month-old wild-type, BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice demonstrating that abdominal wall thickness is visually increased in BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice when compared with BubR1<sup>H/H</sup> animals. Scale bar is 1 cm.

**p16<sup>ink4a</sup> loss blunts sarcopaenia induced by BubR1 insufficiency**

A prominent ageing-associated phenotype of BubR1<sup>H/H</sup> mice is the development of lordokyphosis<sup>11</sup>. The incidence of this phenotype was markedly reduced in BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− animals when compared with BubR1<sup>H/H</sup> mice (Fig. 1b, c). Furthermore, the median time to onset of lordokyphosis was three times longer in BubR1<sup>H/H</sup>;p16<sup>ink4a</sup>−/− mice compared with BubR1<sup>H/H</sup> animals (Fig. 1b, c).
than in BubR1<sup>H/H</sup> mice (Fig. 1b). Lordokyphosis is associated with both osteoporosis and age-related degenerative loss of muscle mass and strength (sarcopaenia) in wild-type mice of extremely advanced age<sup>16</sup>. Histological evaluation of longitudinal femur sections from kyphotic BubR1<sup>H/H</sup> mice revealed no evidence for osteoporosis (Supplementary Information, Fig. S1a, b). Histopathology on gastrocnemius and paraspinal muscles of 5-month-old BubR1<sup>H/H</sup> mice, however, revealed clear signs of skeletal muscle atrophy and degeneration (Fig. 1d and data not shown). Muscle degeneration was greatly reduced in BubR1<sup>H/H</sup> muscles lacking p16<sup>Ink4a</sup> (Fig. 1d, e). In addition, abdominal muscles of BubR1<sup>H/H</sup> mice were poorly developed, as revealed by macroscopic analysis and magnetic resonance imaging (Fig. 1f; Supplementary Information, Fig. S1c). Depletion of p16<sup>Ink4a</sup> resulted in substantial correction of this defect. These data demonstrate that p16<sup>Ink4a</sup> has a major role in establishing sarcopaenia in BubR1<sup>H/H</sup> mice.

p16<sup>Ink4a</sup> limits the regenerative capacity of β cells and has been linked to pancreatic islet atrophy and development of diabetes<sup>9,17,18</sup>, which in turn can cause muscle atrophy through accelerated degradation of muscle protein<sup>19</sup>. This prompted us to test whether the sarcopaenia observed in BubR1<sup>H/H</sup> mice might be due to β cell failure. BubR1<sup>H/H</sup> mice showed highly efficient glucose clearance in a glucose-tolerance test (Supplementary Information, Fig. S2a). Complementary blood insulin measurements indicated that insulin sensitivity was not impaired in BubR1<sup>H/H</sup> mice and showed no evidence for insulin resistance (Supplementary Information, Fig. S2b). Furthermore, overall pancreatic morphology, as well as islet size, shape and abundance were similar
in 12-month-old BubR1<sup>+/–</sup> and control mice, as verified by histology (Supplementary Information, Fig. S2c). Consistently, p16<sup>ink4a</sup> expression in the pancreas was not significantly elevated in BubR1<sup>+/–</sup> mice, compared with BubR1<sup>+/–</sup> counterparts. Thus, sarcopenia in BubR1<sup>+/–</sup> mice is unlikely to be caused by p16<sup>ink4a</sup>-mediated β cell degeneration or insulin resistance.

**BubR1 and p16<sup>ink4a</sup> levels are inversely linked in skeletal muscle**

To determine whether BubR1 may have a role in normal skeletal muscle aging, we measured BubR1 protein levels in skeletal muscle of young and old wild-type mice by western blot analysis. Gastrocnemius muscles of old wild-type mice (Fig. 2b), indicating that p16<sup>ink4a</sup> induction is an early response to BubR1 hypomorphism that precedes histological signs of sarcopenia.

Increased expression of p16<sup>ink4a</sup> with age in adult stem cells is associated with reduced tissue repair and regeneration in several mouse tissues<sup>5–12</sup>. To explore whether p16<sup>ink4a</sup>-mediated exhaustion of myogenic stem-cell potential might contribute to premature sarcopenia in BubR1<sup>+/–</sup> mice, in vitro myoblast-to-myofibre differentiation assays were performed on gastrocnemius muscles from 5-month-old wild-type, BubR1<sup>+/–</sup> and BubR1<sup>+/–</sup> p16<sup>ink4a</sup>–/– mice. In these assays, the average number of myotubes obtained per milligram of muscle tissue was about 7-fold lower in

**Figure 3 p16<sup>ink4a</sup> disruption attenuates selective progeroid features of BubR1 hypomorphic mice.** (a) Incidence and latency of cataract formation in BubR1<sup>+/+</sup> and BubR1<sup>+/–</sup> p16<sup>ink4a</sup>–/– mice as detected by the use of slit light after dilation of eyes. The curves are significantly different (P < 0.0001, log-rank test). We note that no wild-type or p16<sup>ink4a</sup>–/– mice developed cataracts during this observation period. (b) Subcutaneous adipose layer thickness of p16<sup>ink4a</sup>–/–, BubR1<sup>+/+</sup> and BubR1<sup>+/–</sup> p16<sup>ink4a</sup>–/– mice at 2 and 5 months of age. Data are mean ± s.d. (n = 4 male mice for each age per genotype). A two-tailed Mann-Whitney test was used for statistical analysis. (c) qRT–PCR analysis for relative expression of p16<sup>ink4a</sup> in a variety of 2-month-old tissues from BubR1<sup>+/+</sup> and wild-type mice. Values were normalized to GAPDH, and relative fold is to 2-month-old wild-type samples. Data are mean ± s.d. (n = 3 male mice for each tissue, with triplicate measurements taken). (d) Western blots of eye and fat extracts from 2-month-old BubR1<sup>+/+</sup> and BubR1<sup>+/–</sup> mice probed with anti-p16<sup>ink4a</sup> antibody. Anti-tubulin antibody served as loading control. Uncropped images of the scans are shown in Supplementary Information, Fig. S6b, c.
Figure 4 p16<sup>ink4a</sup> induction in BubR1<sup>hi3</sup> mice promotes cellular senescence. (a) Relative expression of BubR1 in gastrocnemius, subdermal adipose, fat deposits and eyes from 2-month-old wild-type, p16<sup>ink4a</sup>–/–, BubR1<sup>hi3</sup> and BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/– mice as determined by qRT-PCR. Values were normalized to GAPDH. Relative fold is to 2-month-old wild-type samples. Data are mean ± s.d. (n = 3 male mice per genotype, with triplicate measurements taken per sample). We note that ablation of p16<sup>ink4a</sup> was unable to increase the amount of BubR1 present in either wild-type or BubR1 hypomorphic mice. (b) IAT of 5-month-old wild-type, BubR1<sup>hi3</sup> and BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/– mice stained for SA-β-galactosidase activity. Scale bar is 2 mm. (c) Relative expression of senescence markers in gastrocnemius muscles of 2-month-old wild-type, p16<sup>ink4a</sup>–/–, BubR1<sup>hi3</sup> and BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/– mice analysed by qRT-PCR. Data are mean ± s.d. (n = 3 male mice per genotype). Values were normalized to GAPDH. Relative fold expression is to 2-month-old wild-type muscle. (d) Analysis of replicative senescence in skeletal muscle and fat of 2-month-old wild-type, BubR1<sup>hi3</sup> and BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/– mice by analysing in vivo BrdU incorporation. Data are mean ± s.d. (n = 3 males per genotype).

BubR1<sup>hi3</sup> mice than in wild-type mice (Fig. 2c). By contrast, only a 2-fold reduction in myotube formation was observed in BubR1<sup>hi3</sup> mice lacking p16<sup>ink4a</sup>. To confirm these data, 5-month-old wild-type, BubR1<sup>hi3</sup> and BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/– mice were challenged to regenerate muscle fibres by injection of cardiotoxin, a 60-amino-acid polypeptide that causes acute injury by rapidly destroying muscle fibres<sup>25</sup>. Consistent with our in vitro data, muscle regeneration was overtly delayed in BubR1<sup>hi3</sup> mice, but not in BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/– counterparts (Fig. 2d). Collectively, these data indicate that p16<sup>ink4a</sup> promotes senescence in BubR1<sup>hi3</sup> mice, at least in part, by impairing muscle regeneration.

p16<sup>ink4a</sup> loss attenuates ageing in selective BubR1 hypomorphic tissues

Loss of p16<sup>ink4a</sup> caused a modest, yet significant, delay in the latency of cataract formation in BubR1<sup>hi3</sup> mice (Fig. 3a). Aged skin is characterized by reduced dermal thickness and subcutaneous adipose tissue, both of which are observed in BubR1<sup>hi3</sup> mice at young ages<sup>13</sup>. At 2 months of age, BubR1<sup>hi3</sup>, BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/–, and p16<sup>ink4a</sup>–/– mice had similar amounts of subdermal adipose tissue (Fig. 3b). As expected, the mean thickness of the subcutaneous adipose layer decreased notably in 5-month-old BubR1<sup>hi3</sup> mice. This decline was not accompanied by increased fat storage in liver tissue (Supplementary Information, Fig. S2e). The decrease in subcutaneous fat was much less severe in age-matched BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/– mice (Fig. 3b), indicating that p16<sup>ink4a</sup> is, at least in part, responsible for loss of subcutaneous adipose tissue in BubR1<sup>hi3</sup> mice. Tolerance of anaesthetic stress was also greatly improved in BubR1<sup>hi3</sup>,p16<sup>ink4a</sup>–/– mice (Supplementary Information, Table S1), as was adipose tissue deposition (Supplementary Information, Fig. S2f). However, several progeroid symptoms seen in BubR1<sup>hi3</sup> mice remained unchanged following loss of p16<sup>ink4a</sup>, including dwarfism, dermal thinning, arterial wall stiffening and infertility (Supplementary Information, Table S1 and data not shown). No progeroid phenotypes of BubR1<sup>hi3</sup> mice were aggravated by p16<sup>ink4a</sup> loss.

The differential corrective effects of p16<sup>ink4a</sup> disruption on individual progeroid phenotypes suggest tissue-specific differences in engagement of the p16<sup>ink4a</sup> pathway in the cellular response to BubR1 deficiency. BubR1<sup>hi3</sup> tissues in which p16<sup>ink4a</sup> loss causes a significant...
delay of premature ageing, such as eye and (subdermal) adipose tissue, showed strong induction of p16\(^{i64a}\) expression in response to BubR1 hypomorphism (Fig. 3c, d; Supplementary Information, Fig. S6b, c). BubR1\(^{123\text{c}}\) tissues in which p16\(^{i64a}\) inactivation has a discernible correlative effect, such as dermis, brain, aorta, testis and ovary, did not exhibit significant p16\(^{i64a}\) induction (Fig. 3c and data not shown). Furthermore, mutant tissues that are not subjected to premature ageing, including lung, pancreas, colon and liver\(^{15}\), maintained low p16\(^{i64a}\) expression levels. Together, these data demonstrate that p16\(^{i64a}\) is activated in a subset of tissues in BubR1\(^{123\text{c}}\) mice, where it contributes to progeroid phenotypes.

p16\(^{i64a}\) loss attenuates \textit{in vivo} senescence

BubR1 is a putative E2F-regulated gene\(^{21}\) and loss of p16\(^{i64a}\) leads to increased E2F transcriptional activity\(^{22}\). Accordingly, attenuation of ageing in skeletal muscle, fat and eye may be the result of increased BubR1 gene expression. However, this is unlikely as BubR1 transcript levels in these tissues were not affected by loss of p16\(^{i64a}\) (Fig. 4a). As p16\(^{i64a}\) is an effector of cellular senescence, p16\(^{i64a}\) deletion may delay ageing in hypomorphic mice by decreasing senescence. As shown in Fig. 4b, BubR1\(^{123\text{c}}\) adipose tissue expresses high levels of senescence-associated (SA)-\(\beta\)-galactosidase, a marker of cellular senescence\(^{23}\). SA-\(\beta\)-galactosidase staining was much lower in adipose tissue of BubR1\(^{123\text{c}}\) mice (Fig. 4b). Skeletal muscles of 2-month-old BubR1\(^{123\text{c}}\) mice did not stain positive for SA-\(\beta\)-galactosidase but expressed high levels of several other senescence-associated genes, including Igfbp2, Nrg1, Mmp13 and PAI-1\(^{13\text{c}}\) (Fig. 4c). Expression of these markers was decreased markedly in skeletal muscles of age-matched BubR1\(^{123\text{c}}\) mice. A key feature of senescence is loss of proliferative potential. In \textit{vivo} 5-bromo-2-deoxyuridine (BrdU) labelling showed that 2-month-old BubR1\(^{123\text{c}}\) mice had much lower percentages of cycling cells in skeletal muscle and fat than wild-type mice (Fig. 4d). These reductions were less profound in

### Figure 5

\(p19^{\text{em}}\) is elevated in BubR1 hypomorphic tissues with high p16\(^{i64a}\). (a) Skeletal muscles of wild-type and BubR1\(^{123\text{c}}\) mice of various ages were analysed for \(p19^{\text{em}}\) expression by qRT–PCR. All values were normalized to GAPDH. Data are mean ± s.d. (\(n = 3\) mice were used per genotype and age group). (b) Relative expression of \(p19^{\text{em}}\) in various tissues of 2-month-old BubR1\(^{+/+}\) and BubR1\(^{123\text{c}}\) mice as measured by qRT–PCR. Data are mean ± s.d. (\(n = 3\) males per genotype). All values were normalized to GAPDH. Relative expression is to wild-type samples. (c) Western blots of eye and fat extracts from 2-month-old BubR1\(^{+/+}\) and BubR1\(^{123\text{c}}\) mice probed with anti-\(p19^{\text{em}}\) and anti-p15\(^{i64b}\) antibodies. Anti-tubulin antibody was used as a loading control. Uncropped images of the scans are shown in Supplementary Information, Fig. S6b, c. (d) Relative expression of \(p19^{\text{em}}\) in skeletal muscle (gastrocnemius), subdermal adipose, fat deposits and eyes of 2-month-old wild-type, BubR1\(^{123\text{c}}\) and BubR1\(^{+/+}\) mice as determined by qRT–PCR. Data are mean ± s.d. (\(n = 3\) males per genotype). All values were normalized to GAPDH. Relative expression is to wild-type samples.
BubR1 hypomorphism causes cellular senescence in adipose tissue and skeletal muscle through a p16Ink4a-dependent mechanism. As p16Ink4a inactivation attenuates both senescence and ageing in these tissues, the mechanism by which BubR1 hypomorphism accelerates the ageing phenotypes may involve p16Ink4a-induced senescence.
p19\textsuperscript{arf} is elevated in BubR1\textsuperscript{H/H} tissues with high levels of p16\textsuperscript{ink4a}

Besides p16\textsuperscript{ink4a}, p19\textsuperscript{arf} is expressed at increased levels in many tissues of wild-type mice with advanced age, including skeletal muscle (Fig. 5a). Although p19\textsuperscript{arf} is an established effector of senescence in cultured mouse embryonic fibroblasts (MEFs), its role in senescence and ageing in the context of the whole organism has not been clarified\textsuperscript{1,2}. To explore the role of p19\textsuperscript{arf} in BubR1-mediated ageing, we analysed its relative expression in tissues from 2-month-old BubR1\textsuperscript{H/H} and BubR1\textsuperscript{+/+} mice. Increased p19\textsuperscript{arf} expression was consistently observed in BubR1\textsuperscript{H/H} tissues that were subjected to premature ageing and had high p16\textsuperscript{ink4a} levels, including skeletal muscle, (subdermal) adipose tissue and eye, but not in tissues that developed age-related pathology in a p16\textsuperscript{ink4a}-independent fashion (Fig. 6a, b). Six-week-old BubR1\textsuperscript{H/H}, p19\textsuperscript{arf}/- mice had significantly smaller fibres in gastrocnemius and abdominal muscle, (subdermal) adipose tissue and eye, but not in tissues that were subjected to BubR1 hypomorphism. Cataract formation was also significantly accelerated in BubR1\textsuperscript{H/H} mice (Fig. 6c, d). Furthermore, the mean thickness of the subcutaneous adipose layer was significantly smaller in BubR1\textsuperscript{H/H} mice than in BubR1\textsuperscript{+/+} mice (Fig. 6e). This was confirmed by weighing inguinal adipose tissue (IAT, Fig. 6e). Furthermore, the mean thickness of the subcutaneous adipose layer was significantly smaller in BubR1\textsuperscript{H/H}, p19\textsuperscript{arf}/- mice than in BubR1\textsuperscript{+/+} mice (0.07 versus 0.11 mm; P < 0.0001, two-tailed Mann-Whitney test). Other progeroid features of BubR1\textsuperscript{H/H} mice seemed to be unchanged by p19\textsuperscript{arf} inactivation (Supplementary Information, Fig. 5b and Table S1). Taken together, these data indicate that p19\textsuperscript{arf} acts to delay ageing in response to BubR1 hypomorphism.

One possible explanation for the pro-ageing effect observed in BubR1\textsuperscript{H/H} mice may involve increased expression of p16\textsuperscript{ink4a}. Consistent with this idea, p16\textsuperscript{ink4a} levels in skeletal muscle, fat and eye increased markedly when p19\textsuperscript{arf} was knocked out in BubR1\textsuperscript{H/H} mice (Fig. 6f, g; Supplementary Information, Fig. 5b).

p19\textsuperscript{arf} disruption accelerates ageing in BubR1\textsuperscript{H/H} mice

To determine whether p19\textsuperscript{arf} also acts as an effector of ageing in BubR1\textsuperscript{H/H} mice, 41 BubR1\textsuperscript{H/H}, p19\textsuperscript{arf}/- mice were generated and monitored for development of age-related phenotypes. Surprisingly, lordokyphosis developed at a significantly faster rate in BubR1\textsuperscript{H/H}, p19\textsuperscript{arf}/- mice than in BubR1\textsuperscript{+/+} mice (Fig. 6a, b). Six-week-old BubR1\textsuperscript{H/H}, p19\textsuperscript{arf}/- mice had significantly smaller fibres in gastrocnemius and abdominal muscles than age-matched BubR1\textsuperscript{+/+} mice (Fig. 6c; Supplementary Information, Fig. S4a), indicating that muscle wasting was accelerated in the absence of p19\textsuperscript{arf}. Cataract formation was also significantly accelerated when p19\textsuperscript{arf} was absent (Fig. 6d). Skinned 6-week-old BubR1\textsuperscript{H/H}, p19\textsuperscript{arf}/- mice showed overt reductions in deposition of adipose tissue (Fig. 6b). This was confirmed by weighing inguinal adipose tissue (IAT, Fig. 6e). Furthermore, the mean thickness of the subcutaneous adipose layer was significantly smaller in BubR1\textsuperscript{H/H}, p19\textsuperscript{arf}/- mice than in BubR1\textsuperscript{+/+} mice (0.07 versus 0.11 mm; P < 0.0001, two-tailed Mann-Whitney test). Other progeroid features of BubR1\textsuperscript{H/H} mice seemed to be unchanged by p19\textsuperscript{arf} inactivation (Supplementary Information, Fig. 5b and Table S1). Taken together, these data indicate that p19\textsuperscript{arf} acts to delay ageing in response to BubR1 hypomorphism.
explanation for these results may be that genetic manipulation of p19arf sequences changes the normal regulatory balance in the Cdkn2 locus, thereby increasing p16ink4a expression. However, disruption of p19arf had no appreciable effect on p16ink4a levels in tissues undergoing p16ink4a-independent ageing or lacking age-related pathologies (Fig. 6g), arguing against this possibility.

**Inactivation of p19arf increases cellular senescence**

Next, we investigated whether p19arf loss accelerates ageing in BubR1H/H mice through increased senescence. Indeed adipose tissue of 6-week-old BubR1H/H;p19arf–/– mice showed much higher SA-β-galactosidase activity than that of corresponding BubR1H/H mice (Fig. 7a). Furthermore, two senescence-associated genes, Igfbp2 and Nrg1, which are expressed at increased levels in gastrocnemius muscles of 6-week-old BubR1H/H mice, were further elevated in corresponding muscles of age-matched BubR1H/H;p19arf–/– mice (Fig. 7b). In vivo BrdU incorporation showed that cell proliferation in skeletal muscle and fat was considerably lower in BubR1H/H;p19arf–/– mice than in BubR1H/H mice (Fig. 7c). Collectively, these data indicate that p19arf induction in BubR1H/H mice functions to prevent or delay senescence and provide further evidence for the notion that in vivo senescence promotes ageing.

**Distinct in vivo and in vitro effects of p16ink4a and p19arf inactivation on senescence**

Previously, we have shown that BubR1H/H MEFs express high levels of p16ink4a and p19arf and age prematurely13. To determine the effects p16ink4a and p19arf on cellular senescence in these MEFs, we stained BubR1H/H;p16ink4a–/– and BubR1H/H;p19arf–/– MEFs for SA-β-galactosidase. Inactivation of p19arf caused a marked decrease in senescence in BubR1H/H MEFs, whereas inactivation of p16ink4a had no effect (Supplementary Information, Fig. S5a, b). Consistently, the percentage of cycling cells was greatly increased in BubR1H/H;p19arf–/– MEFs, but not in BubR1H/H;p16ink4a–/– MEFs (Supplementary Information, Fig. S5c, d). Furthermore, BubR1H/H;p19arf–/– MEFs grew considerably faster than BubR1H/H MEFs, but BubR1H/H;p16ink4a–/– MEFs did not (Supplementary Information, Fig. S5e, f). Immunoblotting showed that the p19arf–53 pathway remained highly active in BubR1H/H;p16ink4a–/– MEFs, similarly to BubR1H/H MEFs, whereas it was inactive in BubR1H/H;p19arf–/– MEFs (Supplementary Information, Fig. S5h, g). Together, these data demonstrate that the effects of p16ink4a and p19arf ablation on in vivo senescence in skeletal muscle and fat of BubR1H/H mice are not recapitulated by their effects on in vitro senescence in BubR1H/H MEFs.

**Figure 8** Ablation of p16ink4a accelerates lung tumorigenesis in BubR1 insufficient mice. (a) Percentage of mice with tumours at time of death as a function of time for p16ink4a–/–, BubR1H/H and BubR1H/H;p16ink4a–/– mice. Biopsies were performed on moribund animals and all tissues were screened for tumours. Tumour tissues were collected and processed for histological confirmation. The BubR1H/H;p16ink4a–/– curve is significantly different from the BubR1H/H curve with P = 0.0027 (calculated using a log-rank test). (b) Tumour spectra of p16ink4a–/–, BubR1H/H and BubR1H/H;p16ink4a–/– mice. (c) As in a but for p19arf–/–, BubR1H/H and BubR1H/H;p16ink4a–/– mice. There is no significant difference between the curves of BubR1H/H;p19arf–/– and p19arf–/– mice using a log-rank test.
p16\(^{\text{Ink4a}}\) loss synergizes with BubR1 insufficiency in lung tumorigenesis

\(\text{BubR1}^{\text{H/H}}\) mice show progressive and severe aneuploidy but rarely develop tumours\(^1\). Activation of p16\(^{\text{Ink4a}}\) or p19\(^{\text{Arf}}\) in response to BubR1 hypomorphism may act to suppress tumorigenesis. To test for this possibility, \(\text{BubR1}^{\text{H/H}}\) mice lacking p16\(^{\text{Ink4a}}\) or p19\(^{\text{Arf}}\) were monitored for tumour formation. Live \(\text{BubR1}^{\text{H/H};p16^{\text{Ink4a/-}}}\) and \(\text{BubR1}^{\text{H/H};p16^{\text{Ink4a/-}};p19^{\text{Arf/-}}}\) mice showed no overt tumours, but biopsy of moribund or dead animals revealed that \(\text{BubR1}^{\text{H/H};p16^{\text{Ink4a/-}};p19^{\text{Arf/-}}}\) mice had significantly more tumours than \(\text{BubR1}^{\text{H/H}}\) mice (Fig. 8a). Eight out of nine \(\text{BubR1}^{\text{H/H};p16^{\text{Ink4a/-}}}\) tumours were lung adenocarcinomas, a type of tumour observed in only one \(\text{BubR1}^{\text{H/H}}\) mouse and none of the \(\text{p16}^{\text{Ink4a/-}}\) mice (Fig. 8b). Sarcomas, the most prevalent tumour type in \(\text{p16}^{\text{Ink4a/-}}\) mice, were rare in \(\text{BubR1}^{\text{H/H};p16^{\text{Ink4a/-}};p19^{\text{Arf/-}}}\) mice and not present in \(\text{BubR1}^{\text{H/H}}\) mice. Thus, the effect of BubR1 insufficiency is synergistic with that of p16\(^{\text{Ink4a}}\) loss during tumorigenesis in lung epithelial cells, but not in other cell types (see Supplementary Discussion). \(\text{BubR1}^{\text{H/H};p19^{\text{Arf/-}}}\) and \(\text{p19^{Arf/-}}\) mice, however, had overlapping tumour-free survival curves (Fig. 8c), indicating that BubR1 insufficiency and p19\(^{\text{Arf}}\) loss do not synergize in tumorigenesis.

DISCUSSION

Here, we report that inactivation of p16\(^{\text{Ink4a}}\) attenuates the development of age-related pathologies in \(\text{BubR1}^{\text{H/H}}\) tissues with elevated p16\(^{\text{Ink4a}}\). This shows that induction of p16\(^{\text{Ink4a}}\) by cellular stress resulting from BubR1 insufficiency drives the development of age-associated phenotypes, and provides direct evidence for a causal involvement of this tumour suppressor in organismal ageing. Importantly, skeletal muscle and fat, two tissues that are subject to p16\(^{\text{Ink4a}}\)-dependent ageing when BubR1 levels are low, have higher numbers of replicating cells and show decreased expression of senescence-associated proteins in the absence of p16\(^{\text{Ink4a}}\). These observations support the notion that p16\(^{\text{Ink4a}}\) contributes to age-associated pathologies through accumulation of senescent cells. This is a significant finding as evidence that cellular senescence promotes ageing has thus far been largely circumstantial\(^2\)–\(^9\).

\(\text{BubR1}^{\text{H/H}}\) mouse tissues, in which p16\(^{\text{Ink4a}}\) is elevated, also have increased p19\(^{\text{Arf}}\). However, p19\(^{\text{Arf}}\) inactivation accelerates rather than delays ageing in these tissues, indicating that this tumour suppressor provides anti-ageing activity. This seems surprising, considering that p19\(^{\text{Arf}}\) is an effector of senescence in cultured cells\(^2,\)\(^6\). However, the recent observation that transgenic mice carrying an extra copy of both p19\(^{\text{Arf}}\) and p53 are protected from ageing-associated damage and live longer than normal mice\(^2,\)\(^8\), is consistent with our finding that p19\(^{\text{Arf}}\) has anti-ageing activity in \(\text{BubR1}^{\text{H/H}}\) mice. It has been proposed that the p19\(^{\text{Arf}}\)-p53 pathway in response to low, chronic stress, may primarily induce genes that promote cell survival and repair, thereby extending lifespan\(^11\)–\(^13\). High, acute types of stress, on the other hand, may accelerate ageing by triggering a more robust p53 response, causing irreversible cell-cycle arrest and/or apoptosis\(^12\)–\(^21\). Therefore, one possibility is that p19\(^{\text{Arf}}\) may elicit a p53 transcriptional response that provides protection against cellular stress resulting from BubR1 hypomorphism, thus delaying the onset of cellular senescence. The observation that skeletal muscle and fat from \(\text{BubR1}^{\text{H/H}}\) mice lacking p19\(^{\text{Arf}}\) accumulate more senescent cells is consistent with this idea. Strong additional support for the conclusion that p19\(^{\text{Arf}}\) has anti-ageing activity is provided by our unpublished observations indicating that \(\text{BubR1}^{\text{H/H}}\) mice lacking p53 phenocopy those lacking p19\(^{\text{Arf}}\). Two observations reported here suggest that p19\(^{\text{Arf}}\) may exert its anti-ageing effect, at least in part, through negative regulation of p16\(^{\text{Ink4a}}\) expression. First, inactivation of p19\(^{\text{Arf}}\) in \(\text{BubR1}^{\text{H/H}}\) mice resulted in increased p16\(^{\text{Ink4a}}\) expression in skeletal muscle, fat and eye, three tissues that have high p19\(^{\text{Arf}}\) levels and are subjected to accelerated ageing. Second, inactivation of p16\(^{\text{Ink4a}}\) prevented the induction of p19\(^{\text{Arf}}\) in these \(\text{BubR1}^{\text{H/H}}\) tissues. How p19\(^{\text{Arf}}\) attenuates p16\(^{\text{Ink4a}}\) expression remains to be addressed.

Although inactivation of p16\(^{\text{Ink4a}}\) significantly delays the development of certain ageing-associated phenotypes in \(\text{BubR1}^{\text{H/H}}\) mice, it does not completely prevent them. Furthermore, other progeroid phenotypes are not affected by loss of p16\(^{\text{Ink4a}}\). These findings suggest that BubR1 hypomorphism engages other progeroid effectors in addition to p16\(^{\text{Ink4a}}\). The identity of these effectors is currently unclear and remains to be established. Striking similarities exist between the progeroid phenotypes of \(\text{Bmll}\) knockout and \(\text{BubR1}^{\text{H/H}}\) mice\(^9\). The molecular basis of this similarity is unclear, although, based on the known roles of each protein, it is unlikely that BubR1 and Bmll are functionally connected. However, it is possible that downstream pathways that respond to stress resulting from Bmll loss and BubR1 hypomorphism are shared.

METHODS

Generation of compound mutant mice. \(\text{BubR1}^{\text{H/H}}\) mice were generated as described previously\(^3\), p16\(^{\text{Ink4a}}\) and p19\(^{\text{Arf}}\) knockout mice have been generated previously and were acquired from the Mouse Models of Human Cancers Consortium located at the National Cancer Institute, Frederick\(^3,\)\(^4\). All mice were on a mixed 129 \(\times\) C57BL/6 genetic background. They were housed in a pathogen-free barrier environment for the duration of the study. Experimental procedures involving the use of these laboratory mice were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. Prism software (GraphPad Software) was used for generation of all survival curves and for statistical analyses.

Collection and analysis of tumours. Moribund mice were killed and all major organs were screened for overt tumours using a dissection microscope. Tumours that were collected were processed by standard procedures for histopathology. A Fisher’s exact test was used to compare tumour incidence proportions across the genotypes for mice that developed tumours. Board-certified pathologists assisted in the histological evaluation of tumour sections.

Analysis of progeroid phenotypes. Bi-weekly, mice were screened for the development of overt cataracts by examining dilated eyes with a slit-light. Incidence of lordokyphosis was checked bi-weekly. Mice that showed lordokyphosis for three consecutive monitoring periods were determined to have this condition. Various skeletal muscles were collected and processed for histology as described previously\(^20\)–\(^23\). Fibre diameter measurements were performed on cross sections of gastrocnemius and abdominal muscles from 6-week-old male mice (\(n = 3\) mice per genotype). A total of 50 fibres were measured per muscle using a calibrated computer program (Olympus MicroSuite Five). Dissection, histology and measurements of dermal and adipose layers of dorsal skin were performed as described previously\(^20\). Values represent an average of four males. Analysis of arterial wall stiffening was performed as described previously\(^24\). Measurements of body weight and IAT were performed on 6-week-old males (\(n = 3\) per genotype). Oral glucose tolerance tests were performed on 5-month-old male mice (\(n = 5\) per genotype) as described by the Jackson Laboratory (www.jax.org). Insulin measurements were performed as described previously\(^27\).

Quantitative real-time PCR. Total RNA was extracted from tissues using a Qiagen RNeasy RNA isolation kit according to the manufacturer’s protocol.Transcription into cDNA was performed using random hexamers and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. All PCR reactions used SYBR green PCR Master Mix (Applied Biosystems) to a final volume of 12 \(\mu\)l, with each cDNA sample performed in triplicate in the ABI PRISM 7900 Sequence Detection System (Applied Biosystems) according to the protocol of the manufacturer. All experiments were performed on organs/tissues from at least

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three different animals in each age group and genotype. The expression of genes was normalized to GAPDH. Sequences of primers used for qRT–PCR of p15INK4b (ref. 38), p16INK4a (ref. 39), p19ARF (ref. 28), BubR1 (ref. 40), Mnp1-13 (ref. 41), PAI-1 (ref. 42), Igfbp-2 (ref. 43), and GAPDH (ref. 44) were as published. Additionally, sequences for Ngn1 were: forward, CATGGTTAACATGGGAAATGGCC; reverse, CCACAAATGCTCAGCTGGAAGATG A. Statistical differences were determined using an unpaired two-tailed t test.

Analysis of satellite-cell function. Analysis of in vivo satellite-cell function were carried out as described previously. Briefly, mice anaesthetized with avertin (200 mg kg−1, I.P.) were given a single 50-µl injection of cardiotoxin (10 µM; Calbiochem) into the gastrocnemius muscle. After this injection, the skin incision was closed with a nylon suture. Mice were allowed to recover and then were analysed at both 5 and 18 days post-injection by routine histology. Isolation and culture of skeletal muscle satellite cells were performed as described previously. Briefly, hindlimb muscles of 5-month-old mice were removed and trimmed of excess connective tissue and fat. Minced muscles were subjected to several 15-min rounds of digestion at 37 °C in incubation medium (50% DMEM high glucose (GIBCO)/50% F-12K (CellGro)/168 µl ml−1 collagenase type II (Worthington)/0.04% Trypsin (GIBCO)). Once fully digested, cells were successively filtered through 70- and 40-µm strainers, collected by centrifugation at 300g for 5 min and resuspended in propagation medium (DMEM high glucose/15% FCS (GIBCO)/glutamine (CellGro)/penicillin-streptomycin (CellGro)). After seven days in culture, differentiation medium (propagation medium with 2% FCS) was applied and cells were fixed seven days after transfer to the medium. Myotube formation was quantified using the total number of myotubes for each sample normalized to the muscle mass extracted.

Magnetic resonance imaging. Magnetic resonance images with a 7-tesla scanner (Bruker) were obtained in 2%-isofluorane anaesthetized mice using a spin-echo method as described previously. Digital images were analysed with the Metamorph software (Visitron, Universal Imaging). The ratio of muscle area (paraspinal and chest or abdominal wall muscles) to total body cross-section was measured at the distal thorax and mid-abdomen levels.

In vivo BrdU incorporation. At 24 and 6 h before tissue collection, male mice of various genotypes were injected intraperitoneally with 200 µl of BrdU (10 mg ml−1; Sigma) in PBS. Mice were anaesthetized (avertin, 200 mg kg−1, I.P.) and successively perfused (transcardially) with PBS and 10% formalin. Organs were collected and embedded in paraffin. Five-µm sections were prepared and stained for BrdU according to the manufacturer’s protocol (BD Pharmingen). The percentage of BrdU-positive cells was determined by counting total and BrdU-positive nuclei in 10 non-overlapping fields at x40 magnification (n = 3 mice per genotype).

Generation and culture of MEFs. MEFs were generated as described previously. BrdU incorporation assays on MEFs were performed according to the manufacturer’s protocol (BD Bioscience). Growth curves were generated as described previously. Three independent MEF lines for each genotype were analysed in both experiments.

SA-β-galactosidase staining. Adherent MEFs were stained with a SA-β-galactosidase activity kit according to manufacturer’s protocol (Cell Signaling). Nuclei were stained with Hoechst to determine percentages of cells positive for SA-β-galactosidase activity. The percentage of SA-β-galactosidase-positive cells was the total number of cells positive for SA-β-galactosidase activity divided by the total number of cells (n = 3 independent MEF lines for each genotype at each passage). Adipose tissue preparations were stained for SA-β-galactosidase activity, as described previously.

Western blot analyses. Western blot analyses were carried out as described previously. Antibodies for senescence-associated proteins were as described. The antibody for p15INK4b was a gift from M. Barbacid.


Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Figure S1 Screening of BubR1+/+ and BubR1H/H mice for osteoporosis and analysis of BubR1+/+, BubR1H/H and BubR1H/H/p16Ink4a−/− mice for abdominal muscle volume and fat disposition. (a) Haematoxylin and eosin-stained longitudinal sections of femurs from a 1-year-old wild-type, a 1-year-old BubR1 hypomorphic and a 35-month-old wild-type mouse. Scale bar = 100 µm. (b) Quantitation of relative width of the cortical wall to total bone width in sections from a. For each genotype, three male mice were used and 40 random measurements were taken. Note the clear reduction in bone mass and density in the old wild-type animal, while there is no difference between the 1-year-old samples. Asterisks indicates p value of < 0.0001 compared to 12-month-old wild-type using a two-tailed Mann-Whitney test. Error bars are s.d. (c) Analysis of abdominal muscle and fat tissue of 5-month-old wild-type, BubR1H/H, and BubR1H/H/p16Ink4a−/− mice by MRI. Representative cross-sectional MRI images are shown. Areas of muscle (M), fat (F), and the spinal cord (S) are indicated. Note the substantial increase in fat and muscle in BubR1H/H/p16Ink4a−/− mice.
Figure S2  Analysis of pancreatic beta-cell function, young skeletal muscle histology, and fat deposition of BubR1 hypomorphic mice (a-c) BubR1 hypomorphic mice have normal pancreatic beta-cell function. Changes in blood glucose (a) and insulin (b) levels after oral glucose administration to 5-month-old BubR1+/+ and BubR1H/H mice (n = 5 males for each genotype). Error bars are s.d. (c) Hematoxylin and eosin stained pancreas sections of 1-year-old BubR1+/+ and BubR1H/H mice. Arrowheads mark β-cell islets. Scale bar = 100 µm. (d) Muscles of 3-week-old BubR1H/H appear normal. Cross-sections of gastrocnemius muscles from 3-week-old wild-type and BubR1H/H mice stained with hematoxylin and eosin. Scale bar = 100 µm. (e) BubR1 hypomorphic mice do not have fatty livers. Hematoxylin and eosin stained liver sections of 5-month-old BubR1+/+ and BubR1H/H mice. Scale bar = 100 µm. (f) Quantitation of inguinal adipose tissue in 6-week-old BubR1+/+, BubR1H/H and BubR1H/H/p16Ink4a–/– mice. IAT is expressed as percentage of total body weight. Three males of each genotype were used. Note that BubR1H/H mice lacking p16Ink4a have improved fat disposition.
Figure S3  

**p15Ink4b** is not induced in response to BubR1 hypomorphism.  

(a) Relative expression of **p15Ink4b** in different tissues of 2-month-old **BubR1^{H/H}** and **BubR1^{H/H}** mice as determined by qRT-PCR (n = 3 males for each genotype). All values were normalized to **GAPDH**. Relative expression is to wild-type samples. Error bars are s.d.

(b) Relative expression of **p15Ink4b** in gastrocnemius muscles of wild-type and **BubR1^{H/H}** males at various ages as measured by qRT-PCR (n = three muscles per genotype and age group). Values were normalized to **GAPDH**. Relative fold expression is to 2-month-old wild-type values. Error bars are s.d.
Figure S4. Ablation of p19Arf in BubR1 hypomorphic mice increases muscle wasting but has no impact on lifespan. (a) Cross sections of gastrocnemius and abdominal muscles from 6-week-old BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>/p19<sup>Arf</sup>–/– mice. Sections were stained with hematoxylin and eosin. Note that fibers diameters are typically smaller when p19Arf is lacking. Scale bar = 100 µm. (b) Overall survival curves for wild-type, p19Arf–/–, BubR1<sup>H/H</sup>, and BubR1<sup>H/H</sup>/p19<sup>Arf</sup>–/– mice. We note that BubR1<sup>H/H</sup> and BubR1<sup>H/H</sup>/p19<sup>Arf</sup>–/– curves are not statistically different using a log-rank test.
**Figure S5**  In vivo and in vitro effects of p16\(^{\text{Ink4a}}\) and p19\(^{\text{Arf}}\) ablation on cellular senescence are dissimilar. (a) and (b) Percentages of SA-beta-galactosidase positive cells in P3, P5 and P7 MEF cultures of the indicated genotypes. For each genotype and passage, three independent MEF lines were scored for SA-beta-galactosidase staining. Error bars are s.d. (c) and (d) Percentages of cycling cells in P7 MEF cultures of the indicated genotypes as measured by BrdU incorporation. Three independent MEF lines were used for each genotype. Error bars are s.d. (e) and (f) In vitro growth curves of P7 MEF cultures of the indicated genotypes. On day 0, 1.5 x 10^5 cells were seeded in duplicate and counted for five consecutive days thereafter in three independent lines of each genotype. Compared to wild-type MEFs, the proliferative capacity of both BubR1\(^{\text{H/H}}\) and BubR1\(^{\text{H/H/p16\^{Ink4a}-/-}}\) MEFs was greatly reduced but that of BubR1\(^{\text{H/H/p19\^{Arf}-/-}}\) was not. Lines represent three independent MEF lines per genotype. Error bars are s.d. (g) Western blots of extracts from P7 MEFs of the indicated genotypes probed for p16\(^{\text{Ink4a}}\), p19\(^{\text{Arf}}\), p53 and p21. Extracts from three independent MEF lines (1-3) were loaded for each genotype. (h) Western blots of extracts from P3, P5, and P7 MEFs of the indicated genotypes were probed for p16\(^{\text{Ink4a}}\), p19\(^{\text{Arf}}\), p53 and p21. Blots are representative of three independent MEF lines of each genotype. Actin was used as a loading control.
Figure S6  Uncropped images of the western blots shown in: (a) Fig. 2a, (b and c) Fig. 3d and Fig. 5c, (d) Fig. 6f. Blots were cut horizontally into two portions prior to antibody incubations. Arrowheads indicate specific bands shown in cropped images.
Figure S6 continued
### Table S1

Analysis of progeroid phenotypes in $BubR1^{H/H}$ mice lacking $p16^{Ink4a}$ or $p19^{Arf}$

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<th>Aging Characteristic</th>
<th>Effect in $BubR1^{H/H}$/$p16^{Ink4a−/−}$ mice compared with $BubR1^{H/H}$ mice [p value]</th>
<th>Effect in $BubR1^{H/H}$/$p19^{Arf−/−}$ mice compared with $BubR1^{H/H}$ mice [p value]</th>
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<td>Worsened (++) [p &lt; 0.0001]</td>
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<td>Respiratory function</td>
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<td>N.D.</td>
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Supplementary Discussion

The only discernible adverse effect of p16\textsuperscript{Ink4a} inactivation in BubR1 hypomorphic mice was an acceleration of lung tumorigenesis. The simplest explanation for this effect would be that p16\textsuperscript{Ink4a} is induced in BubR1 hypomorphic lung tissue as part of a tumor-suppressive mechanism triggering senescence of cells that are at risk for neoplastic transformation. We extensively screened BubR1 hypomorphic lungs for the presence of premalignant lesions, but none were found (data not shown). This precluded us from testing whether p16\textsuperscript{Ink4a} levels are indeed elevated in such lesions. Alternatively, as aneuploidy has been shown to promote tumorigenesis in certain mouse tissues\textsuperscript{1}, it is conceivable that the numerical chromosome instability resulting from BubR1 insufficiency cooperates with p16\textsuperscript{Ink4a} loss in lung tumorigenesis. Our data demonstrating that p16\textsuperscript{Ink4a} inactivation accelerates lung tumorigenesis in BubR1 hypomorphic mice provides a first example of synergy between a mitotic checkpoint gene defect and a cancer gene mutation. Our observation that BubR1 insufficiency does not cooperate with p19\textsuperscript{Arf} loss in tumorigenesis, suggests that the genetic context or microenvironment in which mitotic checkpoint gene defects promote tumorigenesis is limited.

Reference