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## The yin and yang of the *Cdkn2a* locus in senescence and aging

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### Abstract

Senescence of cultured cells involves activation of the p19<sup>Arf</sup>-p53 and the p16<sup>Ink4a</sup>-Rb tumor suppressor pathways. This, together with the observation that p19<sup>Arf</sup> and p16<sup>Ink4a</sup> expression increases with age in many tissues of humans and rodents, led to the speculation that these pathways drive in vivo senescence and natural aging. However, it has been difficult to test this hypothesis using a mammalian model system because inactivation of either of these pathways results in early death from tumors. One approach to bypass this problem would be to inactivate these pathways in a murine segmental progeria model such as mice that express low amounts of the mitotic checkpoint protein BubR1 (BubR1 hypo-morphic mice). These mice have a five-fold reduced lifespan and develop a variety of early-aging associated phenotypes including cachectic dwarfism, skeletal muscle degeneration, cataracts, arterial stiffening, (subcutaneous) fat loss, reduced stress tolerance and impaired wound healing. Importantly, BubR1 hypomorphism elevates both p16<sup>Ink4a</sup> and p19<sup>Arf</sup> expression in skeletal muscle and fat. Inactivation of p16<sup>Ink4a</sup> in BubR1 mutant mice delays both cellular senescence and aging specifically in these tissues. Surprisingly, however, inactivation of p19<sup>Arf</sup> has the opposite effect; it exacerbates in vivo senescence and aging in skeletal muscle and fat. These mouse studies suggest that p16<sup>Ink4a</sup> is indeed an effector of aging and in vivo senescence, but p19<sup>Arf</sup> an attenuator. Thus, the role of the p19<sup>Arf</sup>-p53 pathway in aging and in vivo senescence seems far more complex than previously anticipated.

### Keywords

BubR1; p16<sup>Ink4a</sup>; p19<sup>Arf</sup>; spindle assembly checkpoint; premature aging; cellular senescence

### Introduction

A variety of stressful stimuli, including oncogene overexpression, telomere maintenance defects, oxidative stress, DNA damage and nutrient deprivation, lead to the onset of senescence, defined as a state of permanent cell cycle arrest.<sup>1,2</sup> Two important cell cycle inhibitors p16<sup>Ink4a</sup> and p19<sup>Arf</sup>, which are both encoded at the *Cdkn2a* locus, are expressed at high levels in senescing cultured cells and are believed to play a central role in establishing the senescent state.<sup>3,4</sup> Although both proteins block cell cycle progression when expressed above a certain level, the molecular mechanisms by which they operate are quite distinct. p16<sup>Ink4a</sup> prevents progression through G<sub>1</sub> phase by inhibiting the activity of D-type cyclins, thus rendering Rb active (Fig. 1). Short-term signaling through the p16<sup>Ink4a</sup>-Rb pathway results in a transient G<sub>1</sub> arrest, but chronic, long-term p16<sup>Ink4a</sup> induction leads to cellular senescence, which is characterized by dramatic morphological and transcriptional changes.<sup>2,5</sup> In contrast, p19<sup>Arf</sup> does not act directly on cyclins, but instead stabilizes the tumor suppressor p53.<sup>6,7</sup> This

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stabilization can lead to one of two cellular fates: apoptosis or growth arrest.<sup>8</sup> Under conditions of high stress causing cellular damage that is beyond repair, p53 induces a robust transcriptional response that mediates apoptosis and the cell is removed entirely.<sup>9</sup> However, when cycle arrest is desired to allow for repair of minor stress-related damage, p53 induces p21<sup>Cip1</sup> (referred to hereafter as p21) to transiently inhibit the activity of all cyclin-Cdk complexes present at the time stress is inflicted.<sup>10–12</sup> Long-term signaling through the p53 pathway in G<sub>1</sub> in cases where the damage is more substantial causes a more permanent cycle arrest mediated by p21 inhibition of cyclin E-Cdk2, resulting in Rb hypophosphorylation and E2F inactivation. Cells in this state are often referred to as senescent cells, but in contrast to p16<sup>Ink4a</sup>-Rb driven senescence, this arrest is reversible upon the removal of either p53 or p21.<sup>13</sup>

While mechanisms mediating *in vitro* senescence have been well characterized, whether the same pathways are involved in promoting *in vivo* cellular senescence and aging has been unclear. Additionally, although cells with characteristics of senescence accumulate in tissues during normal aging and in tissues that exhibit certain age-related pathologies, whether senescent cells contribute directly to organismal aging remains a matter of debate.<sup>5,14</sup> Studies aimed at understanding the molecular mechanisms of cellular senescence and its role in aging have been hampered by the fact that inactivation of many members of the p19<sup>Arf</sup>-p53 and p16<sup>Ink4a</sup>-Rb pathway in mice results in early death from tumors.<sup>1,2</sup> An alternative way to determine how these pathways are implicated in organismal aging could be to utilize mutant mouse strains that develop age-related (progeroid) phenotypes at an early age. Mice that express low amounts of the mitotic checkpoint protein BubR1 develop a wide-variety of age-related pathologies affecting multiple tissue types within 3 to 5 months (Fig. 2).<sup>15–18</sup> Furthermore, the expression of both p16<sup>Ink4a</sup> and p19<sup>Arf</sup> is elevated in certain tissues susceptible to early aging in this model, specifically eye, fat and skeletal muscle.<sup>18</sup> Therefore this model seems well suited for investigating the role that p16<sup>Ink4a</sup> and p19<sup>Arf</sup> play in the establishment of these characteristics and whether *in vivo* cellular senescence is to blame.

## The Yin: p16<sup>Ink4a</sup> Drives Aging in Certain Tissues of BubR1 Mutant Mice

First, BubR1 hypomorphic mice were bred onto a p16<sup>Ink4a</sup> nullizygous background and monitored for the rate of aging. In the absence of p16<sup>Ink4a</sup>, certain, but not all, age-related characteristics of BubR1 mutant mice were delayed, including sarcopenia, cataracts, fat loss and median survival,<sup>18</sup> suggesting that p16<sup>Ink4a</sup> promotes aging only in certain tissues in response to BubR1 hypomorphism. None of the early age-related phenotypes of BubR1 hypomorphic mice were accelerated, suggesting that p16<sup>Ink4a</sup> has no anti-aging properties. One explanation for the differential corrective effect of p16<sup>Ink4a</sup> loss might be that BubR1 hypomorphism engages the p16<sup>Ink4a</sup>-Rb pathway in a tissue specific fashion. Indeed, only tissues with delayed aging when p16<sup>Ink4a</sup> is lacking, such as eye, adipose tissue and skeletal muscle, exhibit induction of this protein in response to BubR1 insufficiency.

So how might BubR1 hypomorphism trigger p16<sup>Ink4a</sup> expression? Currently, the only well established BubR1 functions are all related to mitosis. BubR1 is primarily known for its inhibition of Cdc20, a key activator of a multiprotein ubiquitin E3 ligase complex called the anaphase-promoting complex (APC/C) that regulates mitotic progression by targeting various mitotic substrates for degradation by the proteasome.<sup>19</sup> In addition, BubR1 controls the rate with which cells progress from prophase to anaphase (referred to as mitotic timing)<sup>20</sup> and mediates stable attachment of kinetochores to spindle microtubules.<sup>21</sup> As cells enter G<sub>1</sub> phase, BubR1 levels drop considerably. Whether these low residual amounts of BubR1 have additional functions outside of mitosis remains an open question. *In vitro* studies have implied a role for the BubR1-binding protein Bub3 in gene transcription through its ability to interact with certain histone deacetylases.<sup>22</sup> An attractive hypothesis would be that BubR1 might

repress the *p16<sup>Ink4a</sup>* promoter. However, this seems improbable because, unlike Bub3, BubR1 does not exert transcriptional repressive activity in vitro.

Recent data suggests that cells expressing low amounts of BubR1 that aberrantly segregate their chromosomes undergo Mek and Erk phosphorylation,<sup>23</sup> two kinases of the canonical Ras-Raf-Mek-Erk mitogenic cascade.<sup>24</sup> Oncogenic activation of this pathway is known to induce the expression of many genes, including *p16<sup>Ink4a</sup>*.<sup>25,26</sup> So perhaps BubR1 hypomorphism might activate Erk, which in turn might induce *p16<sup>Ink4a</sup>*. Whether Erk is indeed aberrantly activated and how low amounts of BubR1 trigger this response is an area requiring further exploration using hypomorphic BubR1 mice.

It is important to note that while knocking out *p16<sup>Ink4a</sup>* significantly delayed the development of certain age-related diseases in BubR1 hypomorphic mice, eventually these animals exhibited progeroid features in these tissues. In addition, other tissues that develop age-related diseases that are not influenced by inactivation of *p16<sup>Ink4a</sup>*, including dermis, brain, aorta, testis and ovary, exhibit normal levels of *p16<sup>Ink4a</sup>*. These results imply that additional pathways besides *p16<sup>Ink4a</sup>*-Rb are activated in response to BubR1 insufficiency to drive the aging process. The identity of other genes and pathways that may also be activated in response to BubR1 hypomorphism remains elusive and is clearly a venue for further experimentation.

### The Yang: *p19<sup>Arf</sup>* Applies the Brakes to *p16<sup>Ink4a</sup>*-Mediated Aging

Eye, adipose tissue and skeletal muscle, all of which express high *p16<sup>Ink4a</sup>* in response to BubR1 hypomorphism, also have high *p19<sup>Arf</sup>* levels,<sup>18</sup> suggesting a coordinated activation of both genes encoded by the *Cdkn2a* locus. Inactivation of *p16<sup>Ink4a</sup>* in BubR1 hypomorphic mice restores normal *p19<sup>Arf</sup>* levels, implying that increased *p19<sup>Arf</sup>* expression is dependent on *p16<sup>Ink4a</sup>* induction. Given this dependence, it is unlikely that BubR1 controls *p19<sup>Arf</sup>* expression directly. Unexpectedly, inactivation of *p19<sup>Arf</sup>* in BubR1 mutant mice led to faster rather than slower aging, specifically in eye, adipose tissue and skeletal muscle, indicating that *p19<sup>Arf</sup>* induction in response to BubR1 hypomorphism acts to attenuate the aging process.<sup>18</sup> One possible explanation for the accelerated aging following the inactivation of *p19<sup>Arf</sup>* might be that *p16<sup>Ink4a</sup>* expression is further elevated in these tissues. Indeed, *p16<sup>Ink4a</sup>* is superinduced in skeletal muscle, fat and eye of BubR1 hypomorphic mice lacking *p19<sup>Arf</sup>*.<sup>18</sup> This, combined with the observation that BubR1 mutants lacking *p16<sup>Ink4a</sup>* have normal levels of *p19<sup>Arf</sup>*, suggests that *p19<sup>Arf</sup>* exerts its anti-aging effect, at least in part, through a currently unknown ability to negatively regulate *p16<sup>Ink4a</sup>*.

How might results obtained via the use of BubR1 hypomorphic mice combined with deficiencies in *p16<sup>Ink4a</sup>* or *p19<sup>Arf</sup>* relate to natural aging? Several mouse tissues show a substantial reduction in BubR1 during the course of natural aging,<sup>15,18</sup> suggesting that BubR1 has a potential role in the regulation of organismal aging. Critical testing of this idea would require the use of mice with forced overexpression of BubR1 via transgenes to determine if BubR1 is indeed a modulator of aging. The link between mitotic checkpoint impairment and aging is further supported by the finding that a high proportion of individuals with mosaic variegated aneuploidy (MVA) syndrome exhibit mono- or biallelic mutations in BubR1.<sup>27,28</sup> Carriers of such mutations show various progeroid features, including short lifespan, dwarfism, cataracts and facial dysmorphisms.<sup>29,30</sup> Whether these patients develop additional progeroid features seen in hypomorphic mice is not entirely clear, mainly because MVA syndrome is such a rare disorder. It will thus be imperative to develop a mouse to model human BubR1 mutations to learn more about the pathology of this syndrome and its link to premature aging. MVA patients with biallelic BubR1 mutations carry one missense mutation in the BubR1 kinase domain and a truncating mutation upstream of the kinase domain.<sup>27</sup> On the other hand, MVA patients with monoallelic BubR1 mutations harbor a truncating mutation and a non-

mutated BubR1 allele that yields subnormal amounts of wildtype BubR1 protein.<sup>27</sup> The latter finding is interesting, as it suggests that hypomorphic BubR1 alleles that produce less protein exist within the human population.<sup>28</sup> It would be interesting to identify these alleles and determine whether individuals that carry them are prone to certain age-related pathologies.

Many tissues that show a decline in BubR1 with age have elevated amounts of both *p16<sup>Ink4a</sup>* and *p19<sup>Arf</sup>* in naturally aged mice.<sup>18,31</sup> Because *p19<sup>Arf</sup>* appears to counteract the pro-aging effects of *p16<sup>Ink4a</sup>*, a delicate balance between these two proteins may be required to prevent the acquisition of age-related pathologies. Young animals express relatively low amounts of both *p16<sup>Ink4a</sup>* and *p19<sup>Arf</sup>*, and therefore aging is not promoted (Fig. 3A). During the course of natural aging, the elevation of these two genes appears in concert with each other, but ultimately the balance might tip towards *p16<sup>Ink4a</sup>* and aging ensues (Fig. 3B). If the balance would be tipped more towards *p19<sup>Arf</sup>* then aging might be attenuated. Consistent with this idea is the recent observation that transgenic mice with an extra copy of both *p19<sup>Arf</sup>* and *p53* are protected from aging-associated damage and live longer than normal mice.<sup>32</sup> Under conditions where the level of *p16<sup>Ink4a</sup>* is not being balanced by *p19<sup>Arf</sup>*, organismal aging might occur at much faster rates, as evidenced by BubR1 hypomorphic mice lacking *p19<sup>Arf</sup>* (Fig. 3C). A converse to this would be that mice lacking *p16<sup>Ink4a</sup>* do not have the driving force needed for certain tissues to develop aging (Fig. 3D). In accordance with this idea, aging is delayed following the loss of *p16<sup>Ink4a</sup>* in mice with insufficient amounts of BubR1. It is important to note that this model fits within the results obtained by using BubR1 hypomorphic mice lacking *p16<sup>Ink4a</sup>* or *p19<sup>Arf</sup>*, but additional experimentation with other progeroid mouse models will be necessary to validate it.

## Linking cellular senescence in vivo to aging

It has long been suspected that senescent cells contribute to age-related pathologies.<sup>5,14</sup> However, this has been difficult to prove experimentally. Skeletal muscle and fat tissue of BubR1 hypomorphic mice, two tissues that undergo accelerated aging, accumulate vast amounts of senescent cells in a *p16<sup>Ink4a</sup>* dependent fashion, indicating that *p16<sup>Ink4a</sup>* contributes to aging by promoting cellular senescence.<sup>18</sup> The role of senescence in driving the aging process is further underscored by the observation that *p19<sup>Arf</sup>* not only delays the onset of age-related phenotypes in skeletal muscle and fat of BubR1 hypomorphic mice, but also protects cells of these same tissues from entering the senescent state.<sup>18</sup> It is important to note that the pathways that drive senescence in response to BubR1 hypomorphism in cultured cells do not necessarily represent those that occur in vivo. Senescence of BubR1 hypomorphic MEFs in culture can be completely prevented by *p19<sup>Arf</sup>* inactivation, whereas *p16<sup>Ink4a</sup>* loss has no effect. The discrepancy between the effects of the *p19<sup>Arf</sup>*-*p53* and *p16<sup>Ink4a</sup>*-*Rb* pathway on in vitro and in vivo senescence requires further validation using alternative prematurely aged mouse models, preferably those that exhibit increased rates of cellular senescence and in which both signaling pathways are engaged.

## p53 and Aging: a Complex Relationship

Given the importance of *p19<sup>Arf</sup>* for prevention of senescence in eye, skeletal muscle and fat in BubR1 hypomorphic tissues, it will be important to examine whether *p53* induction is required for attenuating senescence and aging of these tissues. If so, the next question that arises is which are the critical downstream *p53* targets. Various mouse studies have characterized *p53* as an effector of organismal aging.<sup>8,34,35</sup> Mutant mouse strains harboring certain *p53* mutations or expressing high levels of the *p53* splice variant *p44* show accelerated aging, presumably due to constitutive hyper-activation of *p53*.<sup>36,37</sup> Furthermore, several mouse models of premature aging, including *Zmpste24<sup>-/-</sup>*, *Ercc1<sup>-/-</sup>* and *Brca1<sup>Δ11</sup>* show elevated *p53* levels, with inactivation of *p53* in these models delaying aging.<sup>38-40</sup> However, one complication of this

idea that p53 drives aging is the finding that mice harboring an extra genomic copy of both the p53 and the p19<sup>Arf</sup> gene locus accumulate less aging-associated damage and live longer than normal mice.<sup>32</sup> Perhaps, the level of induction of p53 may influence organismal aging to vastly different degrees (Fig. 4). High/acute stressors might induce a robust p53 transcriptional response that leads to apoptosis or senescence, both of which promote tissue deterioration and aging. However, in situations where the stress is mild and more chronic, perhaps as in natural aging, p53 might trigger a long-term cell cycle arrest without the adverse consequences of cellular senescence that functionally impair neighboring cells. Conceivably, less robust p53 transcriptional responses might alleviate the impact of chronic stress, thereby perhaps extending cell functionality and thus organ function.

## BubR1 Hypomorphism: What is the Primary Lesion Causing Premature Aging?

So, what could be the nature of the primary lesion (or stress) causing the activation of senescence and aging in response to BubR1 hypomorphism? Because accurate chromosome segregation is highly dependent on BubR1, premature aging of BubR1 hypomorphic mice was initially believed to be due to the accumulation of aneuploid cells.<sup>15</sup> However, subsequent studies using other mitotic checkpoint defective mouse models did not support this hypothesis.<sup>41–43</sup> Outside of its role in the mitotic checkpoint surveillance machinery, mitotic timing and stable microtubule-kinetochore attachment, BubR1 has been implicated in mediating an adequate DNA damage response.<sup>44</sup> Specifically, BubR1 haploinsufficient MEFs were reported to be defective in  $\gamma$ H2AX phosphorylation and Parp-1 stabilization following exposure to DNA damaging agents such as doxorubicin and ultraviolet light. However, BubR1 haploinsufficient MEFs from an independent strain as well as BubR1 hypomorphic MEFs showed normal levels of  $\gamma$ H2AX and Parp-1 cleavage upon doxorubicin exposure, implying that their DNA damage repair pathways were unperturbed (Fig. 5A). This was further confirmed by data showing that survival and colony formation ability of BubR1 hypomorphic MEFs was normal upon treatment with various kinds of DNA damaging agents.<sup>15</sup> Additional agents used to promote apoptosis in cultured cells, TNF $\alpha$  and  $\alpha$ -Fas, induced Parp-1 cleavage and  $\gamma$ H2AX to a similar degree in wildtype and BubR1 hypomorphic cells (Fig. 5B). In addition, single cell gel electrophoresis, or comet, assays performed on BubR1 hypomorphic tissues with early age-related disease revealed no evidence for increased single-strand or double strand breaks, apurinic or apyrimidinic sites, or DNA adducts (Fig. 5C). Thus, although deficiencies in the DNA damage repair machinery have been linked to premature aging in mice,<sup>45</sup> there is currently no evidence for impaired DNA damage repair ability in BubR1 hypomorphic cells. Recent evidence suggests that BubR1 is implicated in the cellular response to dysfunctional telomeres.<sup>46</sup> However, the typical cellular phenotypes observed in cells deficient for adequate telomere maintenance, such as end-to-end fusions and increased rates of  $\gamma$ H2AX stabilization, are not seen in BubR1 hypomorphic MEFs, suggesting that telomere dysfunction is unlikely to underlie premature aging in BubR1 mutant mice. BubR1 is a modular protein that has various functional domains. Thus, although several lesions implicated in the aging process do not seem to apply to BubR1 hypomorphic mice, the primary lesion causing premature aging in this model remains to be identified. One approach that might provide crucial insight would be to determine which functional domains of the BubR1 protein are required for prevention of premature aging in mice.

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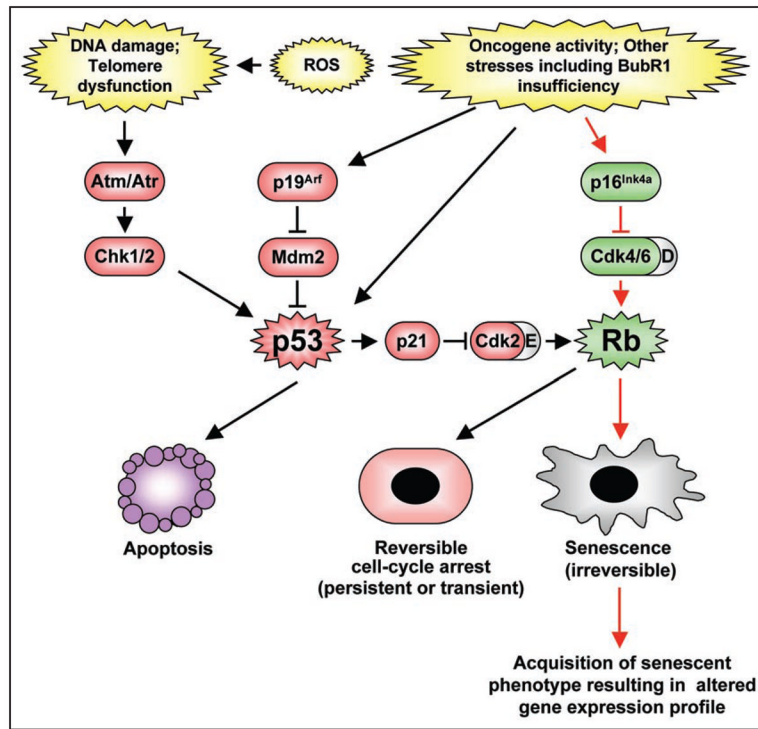
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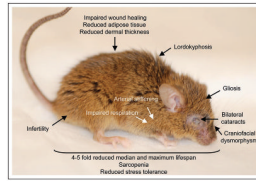
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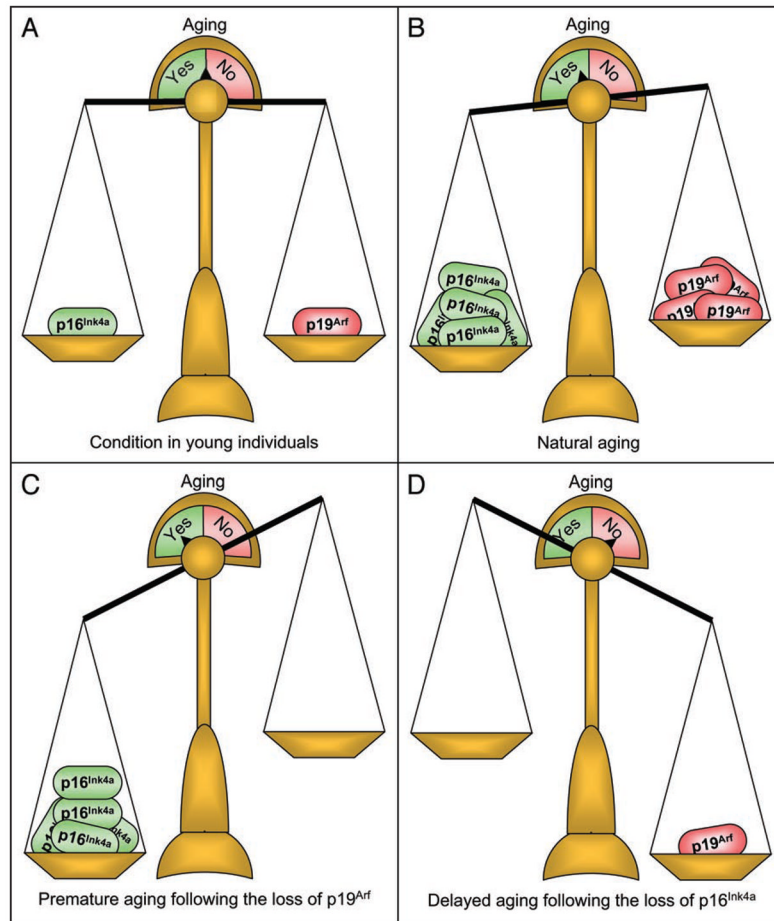


**Figure 1.**

Senescence controlled by the p16<sup>Ink4a</sup>-Rb and p19<sup>Arf</sup>-p53 pathways. Various intrinsic and extrinsic stress-inducing signals are able to engage the tumor suppressor pathways containing p53, p16<sup>Ink4a</sup>-Rb or both. Activation of p53 triggers cell death by inducing expression of a number of downstream target genes implicated in apoptosis (e.g., Bim, Puma, Noxa, Bid, Bax and Apaf1) or a transient or permanent (though reversible) cell cycle arrest via induction of p21. On the other hand, Rb is capable of inducing an irreversible state of cellular senescence via repression of E2F target genes and alterations in chromatin structure.

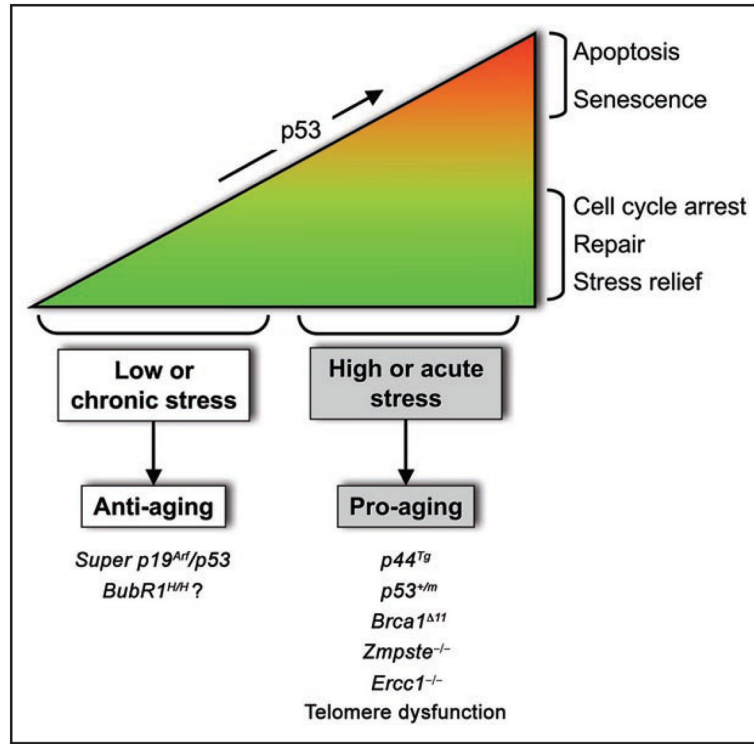


**Figure 2.** Summary of tissues affected with age-related pathologies in BubR1 hypomorphic mice. Shown is a 5-month-old BubR1 insufficient animal. Highlighted are several of the progeroid features commonly observed in this model.

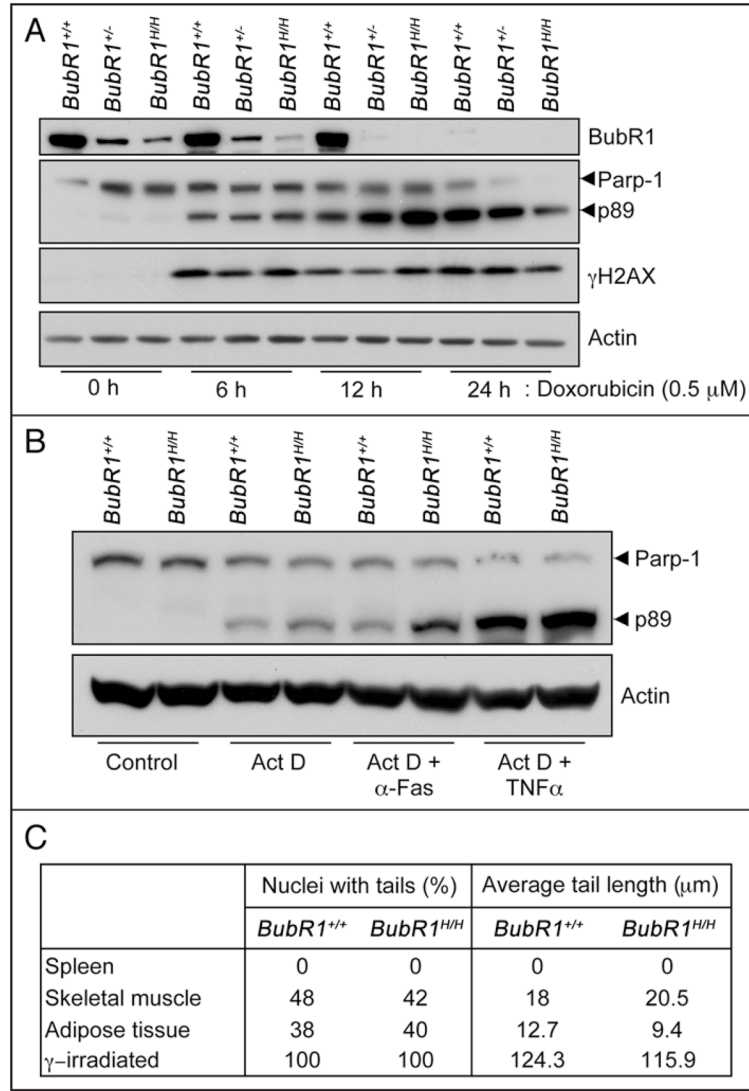


**Figure 3.**

The *Cdkn2a* locus and aging. (A) In young mice, the amount of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> is quite low and in balance. (B) As animals age, p16<sup>Ink4a</sup> and p19<sup>Arf</sup> accumulate in the same tissue, with p19<sup>Arf</sup> attempting to counter-balance the pro-aging effects of p16<sup>Ink4a</sup>. With time, the balance shifts towards a state of aging. (C) In scenarios where p19<sup>Arf</sup> is not present to counteract p16<sup>Ink4a</sup>, the absolute levels of p16<sup>Ink4a</sup> increase dramatically, further promoting a pro-aging microenvironment. (D) When p16<sup>Ink4a</sup> is absent, p19<sup>Arf</sup> does not accumulate. Aging is delayed and the induction of p19<sup>Arf</sup> is not required.



**Figure 4.** Working model for regulation of aging by the p19<sup>Arf</sup>-p53 pathway. Various stresses engage and stabilize p53. Acute situations mobilize an intense p53 response, leading to apoptosis or cellular senescence and the establishment of early aging. Chronic/low amounts of stress on the other hand, might activate p53 to provide a transient cell cycle arrest that provides an opportunity to repair damage or relieve cellular stress, thereby improving cell function in the presence of stress. Stress encountered with natural aging might engage a response similar to that of chronic stress.



**Figure 5.** BubR1 deficient cells have normal DNA damage responses. (A) Passage 5 mouse embryonic fibroblasts (MEFs) of the indicated genotypes were treated with doxorubicin (Dox) for the indicated times as described.<sup>44</sup> Cell lysates were generated and western blotting was performed as described.<sup>47</sup> Blots were probed for BubR1, Parp-1 and  $\gamma$ H2AX. Position of p89, the major degradation product of Parp-1 is indicated. Antibodies for BubR1,<sup>15</sup> Parp-1,<sup>44</sup> and  $\gamma$ H2AX<sup>48</sup> were as described. Actin was used as a loading control. These results suggest an intact response to doxorubicin-induced damage in MEFs expressing low amounts of BubR1. (B) Additional DNA damage-induced cell death analysis in BubR1 hypomorphic cells. Passage 5 MEFs were treated with actinomycin D (Act D), or a combination of Act D and anti-Fas or TNF $\alpha$  and compared to non-treated MEFs as described.<sup>49</sup> These blots suggest that signals that activate apoptosis are interpreted similarly in wildtype and BubR1 hypomorphic MEFs. (C) Comet assay results from 5-month-old tissues of wildtype and BubR1 hypomorphic mice. Comet assays were performed according to manufacturer's protocol (Trevigen). In this assay, both the percentage of nuclei demonstrating DNA damage as well as the amount of damage reflected by tail length, are equal irrespective of genotype, suggesting that BubR1 hypomorphic mice

exhibit a normal DNA damage response to in vivo stresses.  $\gamma$ -irradiation was used as a positive control in the Comet assays.