Background and hypothesis

Cellular senescence is a state of mitotic arrest [cell cycle arrest. Mitotic arrest implies that the cells are stuck in mitosis] found in an increasing number of somatic cells as animals age (Campisi, 2013). Senescence occurs upon transcriptional induction of the p53 and p16^{Ink4a} tumor suppressors, which act respectively via the p21 and Rb tumor suppressors to permanently arrest cell division. Senescent cells remain metabolically active and do not undergo apoptosis, though they are sometimes cleared by the immune system. Because senescence can be induced by potentially oncogenic changes (K-ras activation, DNA damage) or by evidence of repeated cell division (telomere shortening) (Campisi, 2013), it presumably helps to halt tumor formation. Accordingly, p16^{Ink4a} knockout mice develop tumors more frequently than wild-type mice (Sharpless et al., 2001). However, this tumor suppression may come at a price. Senescence has been suggested to drive the progressive decline in tissue function associated with aging (Campisi et al., 2011; Kim and Sharpless, 2006).

If senescence drives aging, then it is unclear why natural selection would have favored senescence in damaged cells rather than simply activating apoptosis. A possible answer lies in the senescence-associated secretory program (SASP) (Coppé et al., 2010): senescent cells secrete a variety of autocrine and paracrine signals, including pro-inflammatory cytokines. These inflammatory signals might stimulate the immune system to clear not only the senescent cells but also neighboring cells that have undergone similar insults but have evaded senescence. Thus, senescence may suppress tumorigenesis *in trans*, whereas apoptosis would only suppress tumorigenesis in a cell autonomous fashion. However, it has proven difficult to experimentally dissect the causal role of senescence either in aging or in cancer. While p16^{Ink4a} knockout mice illustrate the effect of abolishing senescence, the field until recently lacked a model for inducing apoptosis instead of senescence. [very good introduction]

The presently considered paper (Baker et al., 2011) devises a clever trick to induce apoptosis in senescent cells. In this system (Pajvani et al., 2005) transgenic mice express the apoptotic protein caspase-8 fused to FK506-binding protein (FKBP) with an F36V amino acid substitution. The caspase remains inactive under normal conditions, but administration of a small molecule called AP20187 causes the mutant FKBP to homodimerize, activating caspase-8 and triggering apoptosis. Thus, cells expressing this fusion protein, dubbed "apoptosis through targeted activation of caspase" (ATTAC), can be selectively killed in culture or in the live animal by systemic administration of AP20187. The authors place the fusion protein, plus independently translated GFP as a marker, under the control of a partial $p16^{Ink4a}$ promoter, yielding an "INK-ATTAC" construct expressed only in senescent cells. Thus, administration of AP20187 selectively induces apoptosis in senescent cells.

This authors test this system in a BubR1-deficient mouse model they developed years earlier (Baker et al., 2004). The mouse gene *Bub1b* encodes an essential (Wang et al., 2004) spindle assembly checkpoint protein called BubR1. The $BubR1^{H/H}$ mouse (Baker et al., 2004) is homozygous for a *Bub1b* allele containing an intronic neomycin resistance cassette, which reduces BubR1 expression by ~90%. These mice experience increased rates of somatic aneuploidy, cellular senescence, and infertility. They have a median lifespan of ~6 months and a maximum lifespan of only ~15 months, though cause of death for most is unknown (Baker et al., 2004). The $BubR1^{H/H}$ mouse does not recapitulate the phenotypes of loss-of-function mutations in the human ortholog, *BUB1B*, which cause severe congenital defects and early onset cancer (Hanks et al., 2004; Matsuura et al., 2006). However, because some phenotypes in the $BubR1^{H/H}$ mice, such as cataracts and a curved spine, also appear much later in life in wild-type mice, the authors believe this is a model of accelerated aging. Previously, the authors showed that disabling senescence by

p16^{Ink4a} knockout increases lifespan and improves certain phenotypes such as spine curvature in these mice (Baker et al., 2008). In the present paper (Baker et al., 2011) the authors hypothesize that selectively inducing apoptosis in senescent cells will alleviate age-dependent phenotypes in *BubR1*$^{H/H}$ mice. [is the BubRI mouse a good model for normal aging? How does it compare to other progeroid models?]

Summary and critique of methods and results

To validate their system, the authors test for expression of their construct in three different ways: GFP fluorescence, immunohistochemistry for the Flag tag on caspase-8, and qPCR for the transcript. They also test for endogenous markers of senescence by performing qPCR on other transcripts upregulated in senescent cells, and by taking advantage of the fact that senescent cells accumulate beta galactosidase and thus produce blue pigment upon X-gal exposure (Debacq-Chainiaux et al., 2009). Using these readouts, they show that their construct shows the expected dependency on senescence under a variety of cell culture and *in vivo* paradigms (Fig 1B-I). Importantly, these data also illustrate that the *BubR1*$^{H/H}$ mouse is an imperfect model of accelerated aging. While most tissues tested exhibit elevated expression of p16^{Ink4a} and INK-ATTAC in older (11 month) compared to younger (2 month) *Bub1b*$^{+/+}$ mice (Fig S2), only three tissues - adipose, skeletal muscle and eye - show higher expression of these markers in *BubR1*$^{H/H}$ compared to *Bub1b*$^{+/+}$ mice of the same age, whether at 5 months (Fig 1C) or 10 months (Fig S1B). Based on this observation, the authors restrict most of their further analyses to these three tissues. [This could also show that p16 is an imperfect marker for senescence]

The authors next consider the effects of inducing apoptosis in cells expressing INK-ATTAC, by treating *BubR1*$^{H/H}$*;INK-ATTAC* mice continuously with AP20187 from weaning and assessing them at 5 and 10 months of age. qPCR performed on RNA from the three whole tissues of interest

(adipose, skeletal muscle and eye) indicates that levels of both the INK-ATTAC transcript and several endogenous markers of senescence are reduced in the treated mice at 5 months (Fig 3B-D) and 10 months (Fig S3A-C), though in most cases not to wild-type levels, consistent with removal of a large fraction of senescent cells. Beta galactosidase activity is also decreased in adipose tissue of treated mice compared to untreated, though the authors should ideally have included a piece of wild-type tissue for comparison. In adipose tissue but not in other tissues, BrdU incorporation, a marker of apoptosis, was modestly increased (Fig 3E), consistent with apoptosis as the mechanism for clearance. [BrDU is incorporated into cells during DNA replication and is a marker for cell proliferation, not apoptosis. This experiment shows that is senescent cells are eliminated that the remaining cells proliferate at a higher rate]

The apparent reduction in the number of senescent cells in these tissues is accompanied by specific phenotypic improvements. Diameters of two muscle fibers - gastrocnemius and abdominal - are increased in treated mice (Fig 2D), though one also wonders how many muscles were measured, and why more obvious muscles such as quadriceps were not included. Treated mice also have a reduced hazard of developing spine curvature and cataracts (Fig 2B), had larger adipocytes (Fig 2G), thicker adipose tissue (Fig 2H), had increased mass in several tissues (Fig 2F) and performed 2-3 times better in various exercise tests (Fig 2E), all compared to untreated mice of the same genotype, and to treated mice lacking the *INK-ATTAC* transgene (Fig 2B and Fig S4). Across all of these functional comparisons, no wild-type controls are included for direct comparison. It would be of interest to know how the degree of phenotypic recovery observed compares to the baseline level of function in *Bub1b*$^{+/+}$ mice [this would be difficult to compare since the h/h mice age so much faster. What would be the equivalent ages of each mouse to compare?]. Disappointingly, evaluation of inflammation is also very limited: IL-6 is among the transcripts tested in qPCR (Fig 1F), but no other inflammatory markers are evaluated. Senescent cells are known to secrete pro-

inflammatory cytokines, and it has been hypothesized that the resulting inflammation drives age-related tissue degeneration at least locally, and possibly in distant tissues as well (Campisi et al., 2011). At a minimum, a histological evaluation of inflammation would have been of interest.

The authors next ask whether improvements can still be observed if mice are treated beginning at 5 months of age, rather than at weaning, and evaluated at 10 months of age. Here, the changes observed are similar to above, but smaller in magnitude. Adipocyte size and adipose tissue thickness are again increased in treated mice compared to untreated (Fig 4D-E), performance on exercise tests improved, though less than 2-fold and not all comparisons were significant (Fig 4B), and the diameter of gastrocnemius but not abdominal muscle fibers increased (Fig 4A), and a subset of tissues have significantly increased mass (Fig 4C). Beta galactosidase activity in adipose tissue is increased (Fig 4F) and some markers of senescence are reduced, though not nearly to wild-type levels (Fig 4G and Fig S7). The authors do not revisit the issues of spine curvature or cataracts in these late-treated mice, likely because a majority of untreated mice have already developed these symptoms by 5 months (Fig 2B) and it might be more difficult to reverse than to prevent such phenotypes. [good]

A caveat to all of the phenotypic evaluations in this paper, which the authors fail to note, is that *BubR1*$^{H/H}$ mice have a median lifespan of 6 months (Baker et al., 2004), yet are evaluated phenotypically at 10 months of age when only ~40% of mice are surviving (Fig S5D). Treatment does not affect survival curves in the aggregate (Fig S5D), so this selection bias applies equally to treated and untreated groups, but it still confounds interpretation of data, as it is possible that AP20187 elicits phenotypic improvements only in more mildly affected (long-surviving) and not in severely affected (short-lived) *BubR1*$^{H/H}$ mice. The late treatment is commenced at 5 months, after 83% of the mice's median lifespan has passed. This is an ambitiously late moment to begin drug

treatment, and makes the fact that improvements were observed in several phenotypes (Fig 4A-E) all the more remarkable, but again, it also selects for mildly affected individuals.

Impact

This study demonstrates that in the *BubR1*$^{H/H}$ mouse model, induction of apoptosis in senescent cells improves several age-dependent phenotypes in a few selected tissues. No survival benefit is seen, and unsatisfyingly, cause of death in these mice is left mysterious. The study also suffers from the troubling confounder of selection bias for surviving mice. In spite of these limitations, the study must be considered a groundbreaking effort. Prior to this study, aging researchers had seen various lines of observational evidence correlating senescence with functional decline (Campisi, 2013). The clever use of the *INK-ATTAC* system enabled this study to, for the first time ever, enact a therapeutic intervention exchanging apoptosis for senescence in order to tease out causal relationships.

The authors conclude that therapeutic interventions to inhibit or kill senescent cells could be beneficial in delaying aging and extending life. While the paper's impact should not be underestimated, this conclusion is far too ambitious for the data actually collected. Although the *BubR1*$^{H/H}$ mouse exhibits some phenotypes found in older wild-type mice, it also has molecular and organismal deficits unto itself, and according to the authors' own data, accelerated onset of senescence is seen in only three tissues (Fig 1C). Meanwhile, the short lifespan of these mice makes it impossible to evaluate the potential side effect of increased tumor formation (see Future directions). This mouse cannot rightly be considered a model of aging [why not? What would be a better model?], and therapeutic effects in this mouse should not be extrapolated to healthy mice, let alone humans.

Future directions (based on my own thinking)

Although the authors hypothesized that driving senescent cells to apoptosis would improve phenotypes in *BubR1*$^{H/H}$ mice, their conclusions are phrased much more broadly, to state that killing or inhibiting senescent cells may slow aging and extend life. These conclusions, while not justified by the present data, touch on two looming questions in the study of senescence: why would natural selection favor senescence over apoptosis, and could removal of senescent cells slow age-related tissue decline in wild-type animals [good, this second one is the most important question that was not addressed in the paper]?

The most critical additional experiment to help address these two questions is to repeat the original study in mice with a wild-type *Bub1b* genotype carrying the *INK-ATTAC* transgene. The mice would be expected to age normally when not treated with AP20187. The treated and untreated groups could be evaluated phenotypically at, say, 2 or 3 years of age instead of at 10 months, and a late-treated group could begin receiving AP20187 at, say, 1.5 years of age instead of 5 months. Effects on lifespan could also be tested. Such a study would be far more costly and time-consuming than the original experiment, as studies of natural aging in mice need to observe animals for nearly 4 years in order to reach endpoint (Harrison et al., 2009). However, this experiment is absolutely essential both to address the larger questions in senescence biology, and to tackle issues left unresolved in the original paper.

First, the authors assert that the failure of AP20187 to extend lifespan in treated mice is because *BubR1*$^{H/H}$ mice die largely of p16^{Ink4a}-independent heart disease. Much more data would be required to make this assertion convincing. The authors have never characterized the cause of

death in *BubR1*$^{H/H}$ mice, other than to note that only a small minority of them (6 in 116) have tumors on autopsy (Baker et al., 2004). The present paper shows that *BubR1*$^{H/H}$ mice suffer a cardiac arrhythmia (Fig S5A) but there is no evidence that this causes their death. The assertion that the heart disease in these mice is p16^{Ink4a}-independent is presumably based on the data indicating that neither endogenous p16^{Ink4a} nor the INK-ATTAC transgene is induced in *BubR1*$^{H/H}$ mice compared to *Bub1b*$^{+/+}$ controls culled at 5 months of age (Fig 1C). However, p16^{Ink4a} and INK-ATTAC are both induced with increasing age in *Bub1b*$^{+/+}$ mice, indicating that heart cells do undergo age-related p16^{Ink4a}-positive senescence. The fact that this senescence phenotype is not heightened in *BubR1*$^{H/H}$ mice compared to *Bub1b*$^{+/+}$ controls could be because BubR1 deficiency affects the heart in p16^{Ink4a}-independent ways - this is the authors' assumption. An alternative, troubling, possibility is that p16^{Ink4a}-associated senescence in the heart could be lethal in these animals, thus excluding affected animals from the next tissue collection. [it was known that senescence in the heart is not induced by p16, so their data makes sense, but shows that p16 is not a perfect marker for senescence] Therefore, selective ascertainment of mice still living and able to be culled for biochemical and phenotypic evaluation at fixed timepoints (5 months and 10 months) could bias the data toward mice without p16^{Ink4a}-associated senescence in the heart. One future research direction which could help to rule this out would be to perform the same biochemical tests on *BubR1*$^{H/H}$ animals found dead in the cage at daily inspection as are performed on mice culled at regular timepoints. This would be difficult, as the RNA needed for qPCR would decline in quality during the postmortem interval. GFP and beta galactosidase might be more stable markers. Regardless, the best way to address the affects of senescent cell apoptosis on heart disease is simply to repeat the experiment on *Bub1b*$^{+/+}$ mice, where the heart and other tissues develop senescence at natural rates.

Second, the authors assert that no adverse side effects of AP20187 treatment were observed, but no data are presented to support this point, and it is impossible to know how hard the authors looked for such side effects [good]. Moreover, their treatment was conducted until 10 months of age in the *BubR1*$^{H/H}$*;INK-ATTAC* mice, whose overall sickliness might mask side effects, and until 8 months of age in *Bub1b*$^{+/+}$*;INK-ATTAC* mice, which age normally and would not be expected to have significant numbers of senescent cells by only 8 months of age, regardless of AP20187 treatment status. Treatment of *Bub1b*$^{+/+}$*;INK-ATTAC* mice for the duration of their lifespan would provide more opportunity to observe any side effects.

Third, it has been hypothesized that the SASP may suppress tumors *in trans*, potentially explaining why natural selection would have favored senescence over apoptosis (Campisi, 2013). If so, then AP20187 treatment would be expected to result in an increased rate of tumorigenesis in *INK-ATTAC* mice. Constitutive knockout of p16^{Ink4a} causes tumors, but only ~25% of mice are affected by 10 months of age, and even then, not necessarily fatally (Sharpless et al., 2001). Because p16^{Ink4a} knockout eliminates the cell-autonomous anti-tumor effects of senescence in addition to any possible anti-tumor effects exerted *in trans*, these p16^{Ink4a} knockouts place an upper bound on the increased tumor risk one might expect to see in *INK-ATTAC* mice treated with AP20187. Therefore, on the time scale of the present study, where mice were evaluated no later than 10 months of age, it is impossible to determine whether driving senescent cells towards apoptosis increases cancer risk [this could be evaluated more clearly in WT mice containing the INK-ATTAC transgene]. For this reason, the present study has done little to answer the fundamental question of why natural selection gave rise to the senescence phenomenon. Repeating the experiment with the *INK-ATTAC* transgene on a wild-type background would help to address this basic science question. All treated and untreated animals could undergo autopsy to determine whether tumors are present at the time of death.

Following wild-type mice to the end of their lifespan is a long road. In the meantime, other, quicker experiments could also help to shed light on the effects of inducing apoptosis in senescent cells.

Tumors can induce local and possibly distal DNA damage (Redon et al., 2010), and xenografts can induce a p16^{Ink4a} reporter gene in neighboring cells (Burd et al., 2013), raising the possibility that senescent cells may not only detect and restrict their own oncogenic potential, but also to detect nearby cancers and help to mount a response at the tissue level or by recruiting immune cells. Therefore, one could test the effects of senescent cell clearance on genetically programmed cancer development by crossing the *INK-ATTAC* transgene onto mouse models of human cancers, such as conditional *Brca2* knockouts (Jonkers et al., 2001). If such mice have more rapid tumor growth or mortality when treated with AP20187 than when untreated, that would further support a role for senescence in recruiting an immune response to tumorigenesis. In those mice, it would be unclear whether the senescent cells recruiting a response were driven to senescence by signals from the nascent tumors, or alternatively, developed senescence on their own. Therefore, one would also want to test the effect of senescent cell ablation on exogenous tumor growth in a xenograft model. If tumors induce nearby cells to become senescent, thus triggering an anti-tumor repsonse, then xenografts into mice expressing the *INK-ATTAC* transgene on a wild-type background should result in more rapid tumor growth and higher mortality when mice are treated with AP20187 than when they are untreated.

Suppose the above experiment reveals that xenograft tumors do grow more rapidly when host senescent cells are killed off using AP20187. This would indicate that senescent cells are able to suppress tumor growth *in trans*, but would leave open the question of how senescent cells achieve

this feat. Given the pro-inflammatory effects of senescent cells, it is likely that the immune system is involved. To test this, the xenograft experiments could be repeated after crossing the *INK-ATTAC* transgene into SCID mice, which lack a functioning immune system. If the putative ability of senescent cells to inhibit tumor growth *in trans* is mediated by immune response, then ablation of senescent cells with AP20187 in this model should not have any affect on tumor growth. If, on the other hand, tumor growth is still affected, then the effect might be mediated in part by direct paracrine signals.

Overall this is a solid paper. You correctly identified the lack of a WT control as a serious issue with the experimental design and pointed out that the BubRI mice might not reflect normal aging. You have several interesting ideas in the future directions section. I would like to see you point out potential issues with some of these experiments.

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