Title: The many types of heterogeneity in replicative senescence

Running title: Heterogeneity in replicative senescence

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Abstract

Replicative senescence, which is induced by telomere shortening, underlies the loss of regeneration capacity of organs and is ultimately detrimental to the organism. At the same time, it is required to protect organisms from unlimited cell proliferation that may arise from numerous stimuli or deregulations. One important feature of replicative senescence is its high level of heterogeneity and asynchrony, which promote genome instability and senescence escape. Characterizing this heterogeneity and investigating its sources are thus critical to understanding the robustness of replicative senescence. Here we review the different aspects of senescence driven by telomere attrition that are subject to variation in *Saccharomyces cerevisiae,* current understanding of the molecular processes at play, and the consequences of heterogeneity in replicative senescence.

Introduction

Telomerase elongates telomeres, or the ends of linear chromosomes, and without it telomeres shorten with each cell division. As telomeres shorten, they are no longer able to prevent the ends of chromosomes from being recognized as accidental chromosomal breaks. As a consequence, cells permanently activate the DNA damage checkpoint and enter replicative senescence (d'Adda di Fagagna, et al., 2003; Enomoto, et al., 2002; Ijpma and Greider, 2003). This signaling cascade explains the correlation between average telomere length in humans and age: The number of cells in replicative senescence accumulates with age in somatic tissue of primates, in which telomerase expression is downregulated (Hastie, et al., 1990; Jeyapalan, et al., 2007). In turn, cancer precursor cells are rare cells that have bypassed replicative senescence (Shay and Wright, 2010). Hence, telomeres act as a molecular alarm clock for the enumeration of generations, and the homeostasis of many organs in humans depends on proper telomere shortening and establishment of replicative senescence. Yet the predictive power of measuring biological age or cancer risk by telomere length is limited by heterogeneity in the phenotype of replicative senescence (Blackburn, 2000; Karlseder, et al., 2002; Suram and Herbig, 2014). Even at the level of individual cells, there is great variation in the onset of senescence in response to telomere shortening. Thus, decomposing the sources and consequences of cell-to-cell variation inherent to telomeres and senescence is necessary to uncover the molecular basis of telomere control over the proliferation limit of cells.

Budding yeast, in which the phenotype of telomerase inactivation has been studied at length, constitutes a sound model to contribute to such an aim.

Replicative senescence was initially described as the proliferation limit of primary human diploid cells cultivated *in vitro* (Hayflick, 1965; Hayflick and Moorhead, 1961). This finite lifetime was shown to be an intrinsic property of cells and not a technical issue related to in vitro culture. This discovery suggested that proliferation limits at the cellular level could underlie organismal and tissue aging. Already at that time, important variation in the onset of senescence, which took place over a period of 1–3 months in these experiments, was observed (Jones, et al., 1985; Smith and Hayflick, 1974; Smith and Whitney, 1980). Two decades later, a similar heterogeneous proliferation limit was reported in *Saccharomyces cerevisiae* mutants defective in telomere elongation (Lundblad and Szostak, 1989). At the population level, replicative senescence was described as a progressive decrease in growth rate and concomitant increase in cell death, but the variability in each of these two parameters at the level of the single cell was already appreciated. Although a defect in telomere maintenance clearly caused senescence, one could only speculate about the origin of the heterogeneity, and, for example, rapid telomere shortening, increased oxidative stress, and altered gene expression due to genome-wide changes in chromatin structure were readily proposed to contribute to heterogeneity in senescence (Bahar, et al., 2006; Passos, et al., 2007).

We now have a more detailed picture of the molecular mechanisms at play when telomeres are not maintained, in particular in budding yeast, where they have been investigated at length (Wellinger and Zakian, 2012). In *S. cerevisiae,* telomerase can be inactivated by the deletion of the gene encoding one of its subunits (*EST1, EST2, EST3,* or *TLC1*), expression of a dominant negative form, repression of its expression, or a mutation affecting its catalytic activity. Without telomerase activity, and starting typically from an average length of 300–350 bp, telomeres shorten by an average of 3–4 bp per generation because the polymerases are unable to fully replicate linear DNA. This end replication problem is asymmetric in that only one of the two newly replicated telomeres is shorter than the parental telomere. On average, and when one considers successive divisions, the bulk of the telomeres shorten. When telomeres become too short, they trigger a response that is in many ways similar to the DNA damage response, activating the DNA damage checkpoint and arresting the cell in the G2/M transition of the cell cycle, which provides a mechanistic definition of replicative senescence (Teixeira, 2013). In budding yeast, although all 32 telomeres of haploid cells get

progressively shorter as the cells divide, one critically short telomere is sufficient to trigger replicative senescence (Abdallah, et al., 2009; Xu, et al., 2013). At the onset of senescence, the average telomere length is ~100–120 bp, but the shortest telomere is significantly shorter, possibly as short as ~20 bp based on mathematical modeling (Bourgeron, et al., 2015). However, the exact state and threshold length for the shortest telomere are not known, and whether the threshold is clearcut or encompasses a probabilistic range of lengths remains to be investigated. Critically short telomeres being an atypical signal, the robustness of the ensuing DNA damage response and the cell fate decision might differ from other models of DNA damage. For example, repair pathways are mostly inhibited at functional telomeres but can be activated when telomeres are no longer maintained by telomerase (Claussin and Chang, 2015). This is exemplified in the extreme case of post-senescence survivors, which are able to maintain telomeres by recombination-based mechanisms (Le, et al., 1999; Lundblad and Blackburn, 1993; Teng, et al., 2000). How short or damaged telomeres activate and maintain the DNA damage checkpoint, how cells decide to repair them, and how repair contributes to heterogeneity in replicative senescence are important questions that remain to be addressed in detail.

Here we review the different facets of heterogeneity in replicative senescence, starting with variation in proliferative potential. We then discuss the mechanisms in budding yeast known to affect and to control, at least partially, the extent of heterogeneity in senescence. Finally, we argue that heterogeneity has important consequences for senescence and other related phenomena. There are numerous points of comparison between replicative senescence in budding yeast and mammalian cells. We propose that many aspects discussed in this review could be conceptually applied to human senescence and genome instability in the early stages of tumorigenesis.

Another aging phenomenon in budding yeast, referred to as *mother cell aging* and corresponding to the limited number of daughter cells a single mother cell can produce, also imposes a proliferation limit called the *replicative life span,* which is unrelated to telomeres and telomerase (Denoth Lippuner, et al., 2014). Although mother cell aging and replicative senescence induced by telomere shortening share some properties, including heterogeneity (Knorre, et al., 2018), they have distinct mechanistic mechanisms.

Variation in proliferation potential

Proliferation potential (Box 1) is the most obvious measure for which replicative senescence appears to be heterogeneous. It was initially defined for primary human fibroblasts as the total number of population doublings achieved over several passages until the culture is no longer able to undergo at least one population doubling in 2 weeks (Smith and Whitney, 1980). In telomerase-negative budding yeast cultures, a similar definition is used: Proliferation potential represents the total number of population doublings achieved in culture from the moment telomerase is inactivated until the emergence of post-senescence survivors, which efficiently take over the culture at later stages (Lundblad and Blackburn, 1993; Lundblad and Szostak, 1989; Singer and Gottschling, 1994). Around the time survivors emerge, cell cultures reach their lowest growth rate and highest death rate.

Interclonal variation in proliferation potential

Independent telomerase-negative single cells, for example telomerase-negative spores derived from a heterozygous diploid, display distinct proliferation potentials when propagated in culture (Enomoto, et al., 2002; Lundblad and Szostak, 1989; Ritchie, et al., 1999; Singer and Gottschling, 1994). The observed variation is striking and much greater than variation in common growth that arises naturally between biological replicates. Enomoto and colleagues noted that, in this experimental setting, "this variation was a property associated with each individual spore because early or late senescence of specific spore progeny was reproducible" (Enomoto, et al., 2002). Thus, most interclonal variation in proliferation potential has a biological origin and is determined from the moment telomerase is inactive.

At the population level, replicative senescence is the result of the combination of a reduced rate of cell division and an increased rate of cell death. It is important to note that these two phenomena can be observed even shortly after telomerase inactivation and become widespread in the population later on (Enomoto, et al., 2002; Lundblad and Szostak, 1989; Ritchie, et al., 1999). Interclonal variation in proliferation potential can thus be explained by differences in either the timing or relative frequency of longer cell cycles and cell death.

Intraclonal variation in proliferation potential

Although the proliferation potential of a telomerase-negative culture initiated from a single spore is reproducible when the initial colony is used to inoculate multiple cultures, some heterogeneity in growth is also observed within a culture. Because this heterogeneity appears in cultures inoculated by a single telomerase-negative clone, it is referred to as *intraclonal variation.* For instance, when telomerase-negative cells are re-streaked on plates, especially at later passages, the colonies that form are of different sizes, which indicates that individual cells from the same culture have different growth potentials (Enomoto, et al., 2002; Lundblad and Szostak, 1989). In addition, the edges of the colonies are not smooth, which suggests that even within a colony, different cell lineages have distinct growth and mortality rates.

When a pair of telomerase-negative mother and daughter cells are separated by micromanipulation and grown independently, minor differences in proliferation potential between the two resulting cultures are observed (Xu, et al., 2013). They are, however, much smaller than the differences observed between pairs of telomerase-negative spores of the same tetrad (Figure 1A-B). In contrast, a similar experiment performed in human diploid fibroblasts revealed substantial intraclonal variation (Jones, et al., 1985), which suggests that other factors, independent of the initial telomere content, must become prominent during the course of senescence of human fibroblasts *ex vivo* (Figure 1C).

Heterogeneity at the level of single cells and individual lineages

Because proliferation potential is an aggregate endpoint measure of the whole history of a population of cells, it does not provide information on the dynamics of cell division that generate interclonal and intraclonal heterogeneity. For instance, any of the following explanations, or a combination of them, can account for the differences in colony size among subcloned telomerase-negative yeast cells:
- the average growth rates in the two colonies might differ;
- the mortality rate might be heterogeneous within a colony, higher on average in one colony than the other;
- cell cycle arrest might be more frequent in one colony than the other;
- cells might reach permanent arrest (senescence) earlier on average in one colony than the other; or

-   the lag phase in the first cell division might be longer in one colony than the other.

Previous studies have already pointed at several of these possibilities (Enomoto, et al., 2002; Ijpma and Greider, 2003; Lundblad and Szostak, 1989; Ritchie, et al., 1999). However, to fully characterize the types of heterogeneity responsible for macroscopic variation in colony size, we need to use micromanipulation methods or microfluidics-based time-resolved single-cell approaches (Churikov, et al., 2014; Ijpma and Greider, 2003; Lundblad and Szostak, 1989; Xie, et al., 2015; Xu, et al., 2015). In particular, tracking multiple independent lineages in microfluidics circuits has led to a comprehensive and detailed analysis of the dynamics of cell division from telomerase inactivation to permanent cell cycle arrest and cell death. Important variation in the number of generations an individual lineage undergoes before terminal arrest (i.e., replicative senescence) and cell death has been observed, ranging from ~10 to ~70 (median $\pm$ SD = 31 $\pm$ 13 generations, in a strain with an initial average telomere length of ~260 bp) (Coutelier, et al., 2018; Xu, et al., 2015). Although many cell lineages switch abruptly from active cell division to two or three prolonged cell cycles before cell death, others exhibit intermittent and stochastic periods of cell cycle arrest, called *nonterminal arrests,* followed by the resumption of normal cell cycles even shortly after telomerase inactivation (Churikov, et al., 2014; Xie, et al., 2015; Xu, et al., 2015) (Figure 2). The mechanisms implicated in these nonterminal arrests are discussed below.

The combination of these two types of heterogeneity, namely, variations in cell cycle durations and in the total number of generations until the onset of senescence or cell death, might be sufficient to account for all previously reported aspects of heterogeneity in the growth of telomerase-negative cell cultures and colonies.

Sources of heterogeneity

As stated above, replicative senescence combines several aspects that arrest macroscopic growth, including nonterminal arrests, increased cell cycle durations, and increased cell death. The variable timing and frequency of these events contribute greatly to the differences in heterogeneity we have reviewed. Although they are all consequences of telomerase inactivation, understanding the molecular mechanisms associated with each of these phenomena will help decipher the sources of heterogeneity in senescence. At the core of this issue is the question of whether there exists a unifying molecular definition of replicative

senescence with a set sequence of molecular and cellular events or whether telomerase inactivation unleashes multiple pathways that are called *replicative senescence* only from a phenomenological point of view.

Variation in telomere length

The dynamics of telomere shortening by the end replication problem and elongation by telomerase create intrinsic heterogeneity in telomere length distribution (Shampay and Blackburn, 1988; Teixeira, et al., 2004). The average shortening rate due to the end replication problem is around 3–4 bp per generation and appears to be independent of telomere length (Lundblad and Szostak, 1989; Marcand, et al., 1999; Nugent, et al., 1996; Singer and Gottschling, 1994). The measured value is consistent with the average shortening rate, being approximately half of the length of the 3′-overhang (Larrivee, et al., 2004; Soudet, et al., 2014). In contrast, the probability of telomere elongation by telomerase is not constant, and shorter telomeres tend to be preferred as substrates of telomerase (Britt-Compton, et al., 2009; Hemann, et al., 2001; Marcand, et al., 1997; Teixeira, et al., 2004). Telomerase processivity can be quite variable at the molecular level but does not depend on the length of undamaged telomeres (Chang, et al., 2007; Teixeira, et al., 2004). The combination of all of these molecular processes leads to a wide distribution of telomere lengths at steady state (Xu, et al., 2013). Although steady-state telomere length distribution is regulated, variation is observed across natural isolates of *S. cerevisiae* and *Saccharomyces paradoxus* (Liti, et al., 2009) and in response to external stress (Romano, et al., 2013). However, telomere length does not appear to directly affect cellular fitness when telomerase is expressed (Harari, et al., 2017). Telomerase inactivation and the initiation of replicative senescence reveal the consequences of variation in telomere length for cell proliferation.

At the molecular level, replicative senescence involves a DNA damage checkpoint arrest and may be triggered by a limited set of short telomeres reaching a critical threshold (Baird, et al., 2003; Hemann, et al., 2001; Ijpma and Greider, 2003; Kaul, et al., 2011; Zou, et al., 2004). This mechanism is compatible with the observation that independent lineages of telomerase-negative cells enter senescence after a variable number of generations. Indeed, the average length of each telomere varies between different clonal populations, which indicates that the telomere length distribution of the founder cell is unique (Shampay and Blackburn, 1988; Xu, et al., 2013). Once telomerase is inactivated, the number of generations needed for a unique

set of telomeres to shorten until they reach a critical length should thus differ for each lineage derived from a single cell.

The number of critically short telomeres required for robust and permanent arrest can significantly affect the asynchrony of senescence. While four or five short telomeres are required to stop proliferation in mammalian cells in culture (Kaul, et al., 2011), budding yeast requires just a single one. In this model, strains engineered to have a single critically short telomere in the absence of telomerase were used to show that this telomere specifically accumulates single-stranded DNA, recruits DNA damage response factors, and substantially accelerates senescence (Abdallah, et al., 2009; Fallet, et al., 2014; Khadaroo, et al., 2009), in accordance with the notion that a single short telomere is sufficient to trigger senescence. Numerical simulations suggest that the shortest telomere in a cell displays more variation in length than longer telomeres. By shortening in telomere-negative lineages, this shortest telomere triggers replicative senescence in an asynchronous manner, and substantially more asynchronously in cultures inoculated by a pair of sister cells obtained by meiosis compared to a pair of mother and daughter cells resulting from mitosis (see Figure 1 (Xu, et al., 2013)). Combined with the variability in length of the shortest telomere, the random partitioning of newly replicated telomeres, which display a slight asymmetry in length, provides another layer of intraclonal heterogeneity (Figure 3 (Bourgeron, et al., 2015; Eugene, et al., 2017; Soudet, et al., 2014)).

Thus, the initial telomere length distribution and in particular the variation in length of the shortest telomere are major sources of heterogeneity in replicative senescence, both at the population level (described as interclonal and intraclonal variation in proliferation potential) and at the level of the single cell (in the number of generations undergone by individual lineages). It would be interesting to study the contribution of telomere length distribution to heterogeneity in replicative senescence by taking advantage of the recently described yeast strains with chromosomes fused together into only one or two chromosomes (Luo, et al., 2018; Shao, et al., 2018), where telomere length would be much more limited in diversity than in a clonal telomerase-negative population.

Activation of the DNA damage checkpoint, DNA repair, and adaptation

The nonterminal arrests observed in individual lineages of telomerase-negative cells is due to activation of the DNA damage checkpoint, as evidenced using a fluorescent reporter for Rad9 phosphorylation status and by its partial suppression in a *mec1Δ sml1Δ* mutant (Coutelier, et al., 2018; Xu, et al., 2015). Although nonterminal arrests can be observed early after telomerase inactivation (<10 generations), they increase in frequency over generations, and their appearance is delayed in strains bearing longer initial telomeres, which suggests that they are correlated with telomere length. In addition, they are likely unrelated to potential noncanonical functions of telomerase, as they are also observed using a catalytic point mutant of Est2 (*est2*-D670A). Thus, we hypothesize that nonterminal arrests are caused by a critically short, damaged or dysfunctional telomere sensed by the DNA damage checkpoint. The molecular nature of the telomeric damage is still elusive, but the early timing of some nonterminal arrests seems inconsistent with progressive telomere shortening due to the end replication problem. Telomere breaks caused by replication defects and fork stalling might be a plausible alternative possibility. Because replication through the repetitive G-rich telomeric sequences is difficult (Ivessa, et al., 2002; Makovets, et al., 2004; Miller, et al., 2006; Sfeir, et al., 2009), occasional replication errors at telomeres would fit the apparent stochastic nature of nonterminal arrests. This is supported by data that implicate multiple DNA and RNA processing enzymes and replication checkpoints in the onset of senescence (Azam, et al., 2006; Balk, et al., 2013; Fallet, et al., 2014; Gao, et al., 2014; Grandin and Charbonneau, 2007; Lafuente-Barquero, et al., 2017), as discussed in (Teixeira, 2013).

Although end joining acts at telomeres (Mateos-Gomez, et al., 2015; Teixeira, 2013), homology-directed repair (HDR) is the most studied mechanism of repair acting at telomeres. HDR has mostly been examined in the context of alternative telomere maintenance mechanisms in post-senescence survivors and can be a source of rapid telomere shortening or lengthening in a single cell cycle, and consequently a source of heterogeneity in senescence (Cesare and Reddel, 2010; Le, et al., 1999; Li and Lustig, 1996; Lundblad and Blackburn, 1993; Lustig, 2003; Martens, et al., 2000). However, there is also strong evidence that in telomerase-positive cells, as well as before the onset of replicative senescence in telomerase-negative cells, HDR factors can be recruited to telomeres. This recruitment is not exclusive to telomeres progressively shortened as a result of the DNA end replication problem, as rapid telomere shortening events are detected in budding yeast strains with very long telomeres (Li and Lustig, 1996). The trigger for repair at telomeres might then result from other stochastic damage, such as replication defects.

At a mechanistic level, telomeres may be subject to sister chromatid exchange, intra- or inter-telomeric recombination, break-induced replication, rolling circle replication initiated by an intra-telomeric loop, or translocation, as shown in multiple models, including the yeasts *S. cerevisiae* and *Kluyveromyces lactis* (Claussin and Chang, 2015; Lustig, 2003; Natarajan and McEachern, 2002). Repair at telomeres is strongly repressed under normal conditions to avoid genome instability. Thus, only a subset of cells would respond to activation of the DNA damage checkpoint by attempting to repair. The cell fate decision to repair is not well understood and may depend on the exact molecular nature of the telomere damage and on the presence of factors limiting resection, an essential processing step for recombination, such as Cdc13, Stn1, or the Ku complex (Claussin and Chang, 2015; Westmoreland, et al., 2018). After homology-dependent repair of a damaged telomere, the cell presumably inactivates the DNA damage checkpoint and resumes proliferation until the next arrest. If not repaired, the telomeric damage signal should remain and the cell may stay permanently arrested in senescence or undergo adaptation after a longer period of time (see below). Therefore, repair mechanisms and their inherent inefficiency at telomeres contribute to heterogeneity in growth in telomerase-negative cells.

Arrested cells that do not successfully repair the damage may eventually adapt to DNA damage, a process that allows for cell division despite the presence of unrepaired DNA damage (Lee, et al., 1998b; Sandell and Zakian, 1993; Toczyski, et al., 1997). Telomerase-negative cells that activate the DNA damage checkpoint are also able to adapt after extended arrest (Coutelier and Xu, 2019; Coutelier, et al., 2018). Adapted cells retain a partially active checkpoint, which indicates that the initially sensed damage is still present and makes them prone to further repair or adaptation. It is important to note that after adaptation, a cell lineage still has substantial proliferation potential, which might be explained by the fact that telomeric damage is most often not deleterious or by subsequent repair of the damage (Coutelier, et al., 2018). Thus, in addition to repair, adaptation is an important mechanism of cell growth for telomerase-negative cells that experience cell cycle arrest.

Taken together, progressive telomere shortening, rapid telomere shortening, and lengthening linked to repair mechanisms and the status of the DNA damage checkpoint are the key players controlling cell proliferation when telomerase is inactive in budding yeast. Collectively these parameters can explain most cell-to-cell intra- and interclonal variation that increases as

telomeres shorten. But beyond the dynamics of cell division, these mechanisms are also intimately related to another aspect of heterogeneity in senescence: genome instability and post-senescence survival.

Cell diversity in senescence

Diversity in gene expression and beyond

Like replicative senescent mammalian cells, budding yeast also experiences a deregulation of gene expression and a reorganization of chromatin when telomerase is inactivated (Nautiyal, et al., 2002; Niederer, et al., 2016; Platt, et al., 2013). Whether this deregulated gene expression is a consequence or a cause of the heterogeneity induced by the mechanisms discussed in this review, such as a permanent activation of the DNA damage checkpoint or prolonged G2/M arrest, remains to be investigated. What seems clear is that many genes specifically expressed in senescence are expressed at very low levels. If only a few cells express each gene, each senescent cell likely expresses a distinct combinatorial set of changes, and this likely contributes to phenotypic heterogeneity.

An interesting finding regarding phenotypic changes in senescent budding yeast cultures concerns the activation of genes in response to environmental stresses and in oxidative phosphorylations and alterations in mitochondrial morphology (Nautiyal, et al., 2002). Mitochondrial dysfunction and oxidative stress at telomeres contribute to senescence and its heterogeneity in mammalian systems (Ahmed and Lingner, 2018; Chen, et al., 2005; Passos, et al., 2007; Sahin, et al., 2011). Thus, potential alterations in the metabolic program of senescent cells could be studied in budding yeast as well. Note that because *S. cerevisiae* can grow by either respiration or fermentation, one can expect to disentangle the causes and effects among telomeres and mitochondrial activities and uncover the origin of oxidative stress in the absence of telomerase. That said, potential mitochondrial dysfunction and the concomitant increase in reactive oxygen species in replicative senescent cells is indicative of alterations to the structure of the telomere and generalized genome instability (Fouquerel, et al., 2019; Lu and Liu, 2010; Lu, et al., 2013). This is expected to generate yet another level of diversity among populations of senescent cells: genome instability.

Senescence-specific genome instability

Global chromosomal instability increases in telomerase-negative cells over time (Hackett, et al., 2001; Ijpma and Greider, 2003; Lundblad and Szostak, 1989). A ~10-fold increase in the mutation rate of reporter genes has been reported and is accompanied by additional chromosomal rearrangements (Coutelier, et al., 2018; Hackett, et al., 2001; Hackett and Greider, 2003; Meyer and Bailis, 2007). The single-stranded DNA exposed at telomeres when they become critically short or dysfunctional may initiate this genome instability (Fallet, et al., 2014; Garvik, et al., 1995; Hackett and Greider, 2003). However, the mechanisms behind widespread genome instability due to exposed single-stranded telomeric DNA are still unclear. We propose that when telomeres are critically short, the accumulation of single-stranded DNA activates the DNA damage checkpoint and triggers repair mechanisms that generate mutations and chromosomal rearrangements. When repair fails, adaptation to the checkpoint allows for the propagation of molecular structures—such as resected telomeres, terminally deleted telomeres, repair intermediates, stalled and collapsed replication forks, or fused telomeres—that would otherwise maintain an active checkpoint and would not be inherited by the progeny. The resolution of these structures over several cell divisions may subsequently lead to widespread complex chromosomal rearrangements (Beyer and Weinert, 2016; Coutelier, et al., 2018; Hackett, et al., 2001; Hackett and Greider, 2003; Piazza and Heyer, 2019). Adaptation could also contribute to genome instability by forcing defective and asymmetric mitosis, leading to aneuploid cells (Bender, et al., 2018; Galgoczy and Toczyski, 2001; Kaye, et al., 2004).

Overall, genome instability contributes to the genetic diversity of senescent cells. In mammals, telomerase inactivation also leads to a similar mutator phenotype (Blasco, et al., 1997; Chin, et al., 1999; Lee, et al., 1998a), constitutes a potential source of genome variants at work in the early stages of tumorigenesis, and would substantiate the link between cancer incidence and aging in humans.

Post-senescence survival

The vast majority of telomerase-negative cells enter replicative senescence and die, but in most organisms studied a subpopulation can fuse; recombine their telomeres, subtelomeres, or other genetic elements; and escape growth arrest (Begnis, et al., 2018; Cesare and Reddel,

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2010; Kachouri, et al., 2009; Lundblad and Blackburn, 1993; Nakamura, et al., 1998). These post-senescence survivors quickly outcompete slow-growing and arrested senescent cells and take over the culture. The emergence of survivors is an extreme illustration of the phenotypic heterogeneity generated by telomerase-negative cells and is at the basis of telomerase-independent tumor growth, accounting for at least 5% of cancers (Barthel, et al., 2017; Cesare and Reddel, 2010). First, the time of appearance of survivors in a given telomerase-negative culture is highly stochastic and variable. Second, different types of survivors display a wide variety of growth rates, which may not be constant over time. Third, the elongation of chromosomal ends by the acquisition of subtelomeric elements and telomere sequences is also an unstable dynamic process, and a culture may contain subpopulations with different patterns of telomere and subtelomere recombination.

The exact molecular nature of the telomeres when survivors emerge is not clear. In budding yeast, the highly complex mixture found in a survivor culture hints at very heterogeneous telomeric states, defined by telomere length (Chang, et al., 2011; Grandin and Charbonneau, 2009; Lebel, et al., 2009), recruitment of factors involved in telomere processing (Claussin and Chang, 2015), and the presence of RNA–DNA hybrids (Balk, et al., 2014; Misino, et al., 2018; Yu, et al., 2014). The checkpoint status of the cell might also affect the generation of survivors (Tsai, et al., 2002). The highly variable and dynamic nature of survivors in terms of timing, cellular growth rate, and molecular pathways seems therefore intrinsically related to the heterogeneity of replicative senescence itself.

Conclusions

Characterization of the diversity of deregulated processes affecting heterogeneity is more advanced in the field of mother cell aging, with mechanisms such as the accumulation of extrachromosomal rDNA circles, asymmetric segregation of protein aggregates, or dysfunctional mitochondria and vacuole being well documented (Denoth Lippuner, et al., 2014; Knorre, et al., 2018). In the field of telomere-induced replicative senescence, an exhaustive characterization of the contribution of deregulated cellular processes would help in formulating an integrative model of replicative senescence that would encompass all levels of heterogeneity.

In sum, although telomerase inactivation deterministically initiates the process, the route to replicative senescence is filled with hurdles that generate cell-to-cell variation and subsequent intra- and interclonal variation. Deciphering the causes of heterogeneity in replicative senescence would improve understanding of the robustness of the senescent state and the mechanisms at work in escaping it, at both the molecular and systems levels, not only in pathological situations like cancer but also from the perspective of evolution.

Acknowledgments

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Box

Box 1: On population doublings and generations

It is important to note that proliferation potential is defined for a population of cells, and the unit should be *population doublings,* which is equal to $Log_2(Nf/Ni) = Log_2(Nf) - Log_2(Ni)$, where Ni and Nf are the initial and final numbers of cells in the population, respectively. Because the structure of the population is heterogeneous (i.e., not all cells within the population divide at the same rate, and some are arrested or dead), the number of population doublings can deviate substantially from the number of generations or divisions that individual cells actually undergo. Proliferation potential measured in population doublings is also different from the *average* number of divisions/generations, as cells compete and the fittest are selected. Conversion between population doublings and the average number of divisions/generations is in most cases nontrivial and nonlinear. Only in the case of a perfectly homogeneous population of cells in terms of generation time is the number of population doublings equivalent to the number of divisions/generations. To determine the actual number of divisions/generations cells undergo, we have to use more precise approaches, such as lineage tracking using microfluidics devices.

Figure legends

Figure 1:

Differences in proliferation observed in pairs of sister telomerase-negative cells after budding yeast meiosis (A), budding yeast mitosis (B), and human fibroblast mitosis (C).
In theory, the lengths of telomeres in a pair of mother and daughter cells after mitosis are similar (but not equal, see Figure 3). In contrast, telomere lengths are more frequently different among meiotic products because of independent segregation of chromosomes in meiosis. Proliferation potential in the progenies of telomerase-negative pairs of cells from a meiotic product is thus more variable than for telomerase-negative pairs of mitotically divided

mother and daughter cells (A–B) (Enomoto, et al., 2002; Xu, et al., 2013). In contrast, in human fibroblasts, the onset of senescence is variable among pairs of daughter cells derived from mitosis (Jones, et al., 1985) (C). This suggests that replicative senescence of human cells is accompanied by events that might amplify heterogeneity. – and + indicate telomerase negative and telomerase positive, respectively.
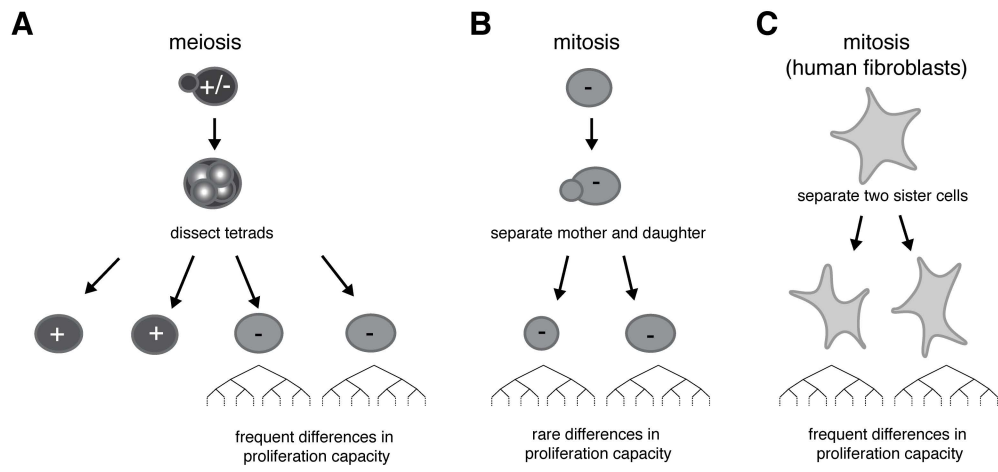
Figure 2:

Heterogeneity at the level of single-cell lineages.

(A) Many telomerase-negative cell lineages, defined here as consecutive cell divisions upon telomerase inactivation until irreversible cell cycle arrest, undergo senescence in a single switch from proliferative to arrested state. (B) In contrast, other cell lineages are likely subject to accidental damage at telomeres that triggers the DNA damage checkpoint and DNA repair mechanisms. The frequent failure of repair mechanisms, combined with the relative ease of bypassing checkpoints by adaptation, then leads to a cascade of genome instability and a multiplicity of cell fates. Modified from (Coutelier, et al., 2018).
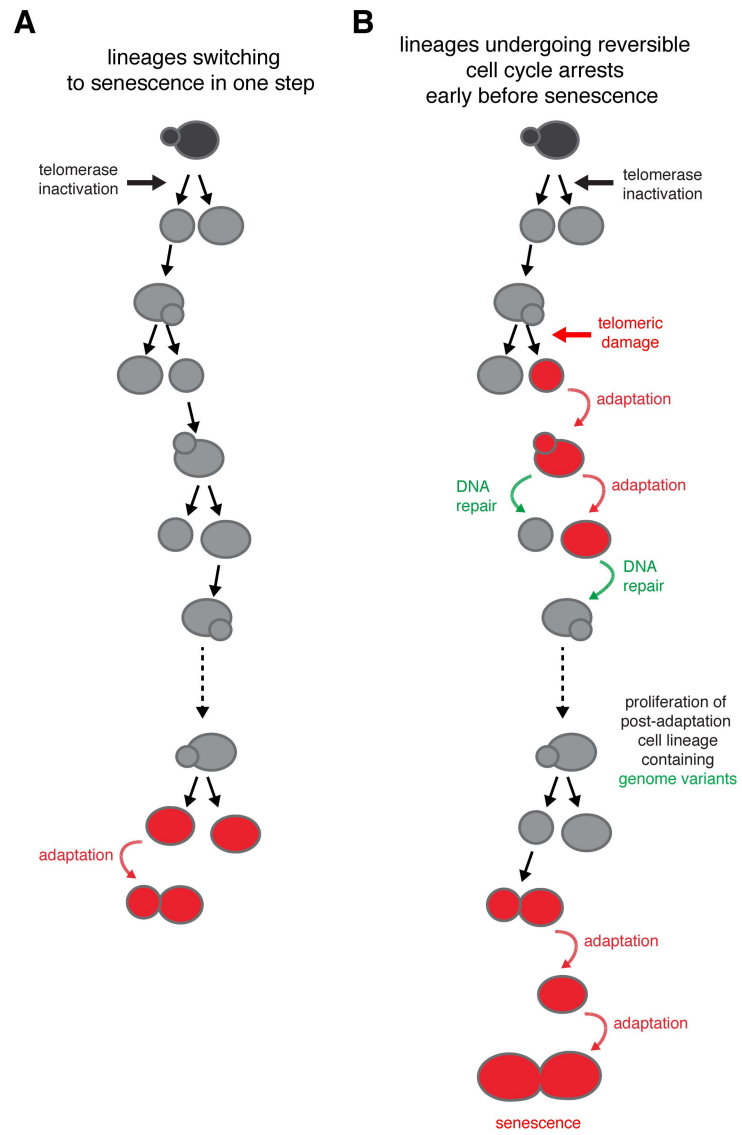
Figure 3:

The DNA end replication problem and progressive telomere shortening contain an intrinsic mechanism that generates length asymmetry (modified from (Soudet, et al., 2014)). Telomeres end with a 3′-overhang of 5–10 nucleotides in *S. cerevisiae.* Passage of the replication fork leads to two newly replicated telomeres of different lengths. The telomere replicated by the lagging strand machinery naturally bears a 3′ overhang by the removal of the last RNA primer of the Okazaki fragment, whereas the telomere replicated by the leading strand machinery requires additional resection and fill-in steps to regenerate a 3′-overhang.

A meiosis

dissect tetrads

frequent differences in
proliferation capacity

B mitosis

separate mother and daughter

rare differences in
proliferation capacity

C mitosis
(human fibroblasts)

separate two sister cells

frequent differences in
proliferation capacity

Xu and Teixeira Figure 1

A

lineages switching
to senescence in one step

telomerase
inactivation

adaptation

B

lineages undergoing reversible
cell cycle arrests
early before senescence

telomerase
inactivation

telomeric
damage

adaptation

DNA
repair

adaptation

DNA
repair

proliferation of
post-adaptation
cell lineage
containing
genome variants

adaptation

adaptation

senescence

Xu and Teixeira Figure 2

parental
telomeres

3'

5'

5-10 nt

semi-conservative DNA replication

lagging telomere

3'

5'

leading telomere

3'

5'

Xu and Teixeira Figure 3