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Chronological and replicative lifespan in yeast: Do they meet in the middle?

Comment on: Murakami C, et al. Cell Cycle 2012; 11:3087–96;
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Budding yeast is a preeminent model organism in studies of cellular aging pathways that are conserved in eukaryotes, including humans. There are two primary ways to query the lifespan of this organism.[1] If one asks how many times a cell can divide, the answer will be its replicative lifespan (RLS). If, on the other hand, one asks how long a cell can stay alive without dividing, the answer will be its chronological lifespan (CLS).

Budding yeast is a facultative aerobe with exceptional genetic tractability. Hence, many environmental and genetic factors are known to affect replicative and chronological lifespan. Since the context of the RLS and CLS assays is different, with dividing vs. non-dividing cells, it is not immediately obvious whether these factors should be overlapping. The results to date are ambiguous. Some well-studied interventions like dietary restriction as well as reduced TOR and protein kinase A signaling, extend both replicative and chronological lifespan.[1] However, in a quantitative comparison of gene deletions that extend lifespan in both assays, no significant overlap was observed.[2]

Further complicating the issue is the differences in methodology regarding chronological aging. When performed in synthetic-defined complete (SDC) media, it was recently shown that acidification of the medium during the growth phase accelerates mortality.[3] Lifespan is extended by buffering the culture medium to pH 6.0 or performing the experiment in rich YEPD medium, which is more refractory to acidification. Interestingly, a recent report indicates that media acidification may be a limiting component to long-term survival of non-proliferative mammalian cells as well.[4] To what extent acidification accelerates normal CLS aging mechanisms and whether it relates at all to replicative aging remain unknown.

Instead of focusing on the factors that are shared, or not, between CLS and RLS pathways, a different way to probe the relationship
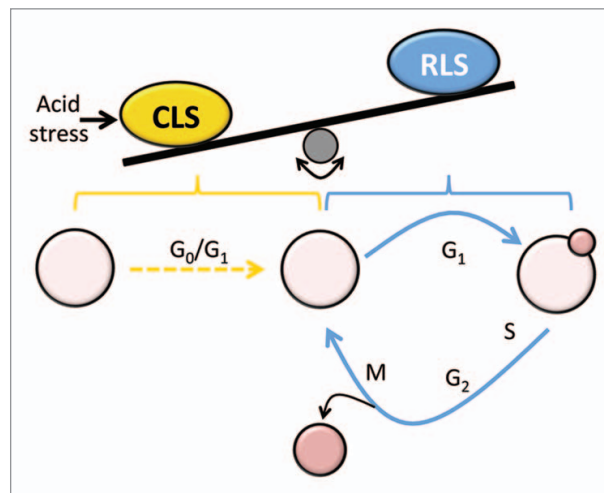

Figure 1. Schematic of the relationship between the non-dividing state of chronological aging (shown in yellow), and the dividing state during which cells age replicatively (shown in blue). Conditions such as acid stress, which accelerate chronological aging, also shorten replicative lifespan.

between CLS and RLS is to examine how one aging process affects the other. More than a decade ago, it was reported that the longer cells age chronologically, the fewer times they can divide when nutrients are restored.[5]

A new study by Murakami et al.[6] found that chronologically aged cells had a reduced replicative lifespan, confirming the earlier report.[5] In addition to replicating the initial study, in which the CLS portion of the assay was performed in YEPD, this study compared three CLS conditions: YEPD, SDC and buffered SDC, finding that replicative lifespan is dramatically shortened in the SDC conditions associated with acidification. These findings indicate that conditions associated with acidification and rapid chronological aging impact the replicative lifespan of the cells, suggesting that the consequences of acidification are related to those of slower aging in YPD and possibly replicative aging as well.

Murakami et al. went further. The CLS to RLS transition is essentially a transition from a non-dividing state, to a dividing one. Hence,

querying parameters associated with cell cycle progression ought to be pertinent for the CLS to RLS transition. Indeed, Murakami et al. found that cells with the greatest replicative potential after quiescence were smaller and arrested properly in the G_1 phase of the cell cycle, before DNA replication. These results further support the significance of G_1 control mechanisms in aging.[7,8] Why would cells that are chronologically aged have a reduced replicative lifespan? All cells in a quiescent population would be exposed to damage, either due to acidification or other causes. However, the authors note that once the population reenters a proliferative state, this damage may stay with the mother cells. This would "free up" the daughters, maximizing the fitness of the population as a whole. This model is appealing and far-reaching. Cycles of quiescent and proliferative states are the norm not only for single-celled organisms in the wild, but also for cells in animal tissues.

Many questions remain. For example, what is the mechanistic basis for the interventions

(e.g., buffering acidification) that extend CLS, which then also extend RLS? Which aspect of G_1 control is causally related to the CLS to RLS transition? Finally, while Murakami et al. examined how CLS influences RLS, the reverse relationship is also worth examining. Do replicatively older mothers have a reduced CLS and, if so, why? While standard CLS methods cannot address this issue, a plate-based CLS assay is theoretically amenable.[9] Whatever the answers to the above questions might be,

yeast aging with its chronological and replicative flavors will continue to drive progress in the field (**Fig. 1**).

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Acetic acid and acidification accelerate chronological and replicative aging in yeast

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Yeast is widely regarded as one of the most valuable model systems to study aging and particularly the genetics of aging. Researchers have established two different methods to study yeast aging known as the replicative lifespan (RLS) and the chronological lifespan (CLS). These have led to the identification of many mammalian genes that affect aging suggesting that they will continue to shed light on the fundamental biology of aging. In spite of the clear differences underpinning the mitotic cellular potential (RLS) and the survival in the non-dividing mode (CLS), the two models are clearly regulated by partly overlapping regulatory mechanism. This idea is supported by the observation that chronologically aged diploid cells show decreased replicative lifespan proportional to the duration of the chronological aging.[1] Even though this is generally agreed to be true, very few attempts have been made to integrate both models in a comprehensive manner. Furthermore, while mutations that affect Ras-cAMP-PKA or TOR/Sch9 signaling increase both the replicative and chronological lifespan, other genes appear to affect lifespan in only one of the two models indicating that partially distinct mechanisms affect the two aging processes.

In the August 15 issue of *Cell Cycle*, Matt Kaeberlein and coworkers[2] present very interesting data, which help to fill the gap between the two aging model systems. They confirm[1] that diploid chronologically aged yeast cells

have a reduced replicative lifespan with respect to chronologically younger cells and show that pH and media composition (YPD or SDC) during the chronological aging phase, play a role in this phenomenon. *S. cerevisiae*, grown in 2% dextrose and excess amino acids, the media used in most chronological lifespan experiments, produces both ethanol and acetic acid as a normal end product of alcoholic fermentation which is accompanied by a drop in media pH to below 4.[4] It has also been demonstrated that the level of protein oxidation may be acetic acid-dependent and not simply pH-dependent.[5] In addition, intracellular acidification increases Ras signaling as well as ROS production,[6,7] linking acidification to nutrient signaling pathways. These results are paralleled by the observations that mammalian tumor cells, maintained in stationary culture, lose viability by lactate media acidification[8] indicating that acidification may have a conserved role in accelerating cellular aging. Together with previous studies, the work by Murakami et al. support two important conclusions: (1) acidification accelerates chronological aging, an effect which may be conserved in higher eukaryotes and that acetic acid does not simply function as a molecule with a toxic and "private" effect but as a carbon source that causes an expected pro-aging effect. In agreement with this conclusion are the consistent effects on lifespan of mutations in the Tor/S6K and Ras/cAMP/PKA, which are observed independently of

the presence or absence of acetic acid in the media or acidification; (2) chronological aging also promotes replicative aging underlining the existence of only one major aging process in yeast which can be measured by two different methods.[3,4]

Thus, acetic acid is likely to accelerate aging by preventing entry into a calorie restricted-like state, but similar pro-aging effects are also true for any carbon source including glucose and ethanol as suggested by previous studies.[4] Under physiological conditions, it is unlikely that acetic acid plays a central role in acidification and, thus, acetic acid and acidification should not be viewed as necessarily connected but as separate factors that can accelerate aging.[5,10]

Interestingly, the authors also find asymmetric segregation of chronologically aged cellular components. Asymmetric inheritance during cell division is of general interest and has long been debated. In budding yeast, buds show the same mitotic potential with no respect to the mother cell age. It has also been demonstrated that carbonylated proteins, DNA circles and old mitochondrial aconitase remain confined to the aging mother cell. Mechanisms implying the involvement of septin, nuclear pore segregation and the involvement of Sir2 have been postulated.[9] The authors here speculate that asymmetric inheritance during mitotic cell division may have had an evolutionary role since yeast cells cycle between dividing and non-dividing

states and the damage accumulated in the non-dividing mode may be altruistically confined to mother cells when the cell starts dividing again.

In summary, this is a valuable study solidifying the overlap between yeast replicative and chronological aging and providing strong evidence for the role of acetic acid and acidification as accelerators of the yeast aging process, which may be more relevant to mammalian aging than expected.

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Cyclin D1 goes metabolic: Dual functions of cyclin D1 in regulating lipogenesis

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Recent findings have revolutionized thinking in terms of how D-type cyclins control diverse cellular processes including development, cellular proliferation and carcinogenesis. The D-cyclin consists of three members with overlapping functions, cyclin D1, cyclin D2 and cyclin D3.[1] Biochemically, D-type cyclins function in late G1 phase as catalysts for cyclin-dependent kinases 4 and/or 6 (CDK4/6). D-type cyclin production is generally enhanced by mitogenic stimuli, and enrichment of the D-cyclins initiates the cell cycle engine. Binding of cyclin D1 to CDK4/6 induces kinase activity and promotes cell cycle progression through phosphorylation of the retinoblastoma tumor suppressor protein, RB, thereby suppressing the ability of RB to attenuate cell cycle advancement. As such, elevated cyclin D1 expression in model systems drives unchecked cellular proliferation and promoting tumor growth.[2] High levels of cyclin D1 are in fact associated with numerous human malignancies, including both breast cancer and hepatocellular carcinoma. Moreover, a variant of cyclin D1 that arises from alternative splicing of the CCND1 transcript, gives rise to a highly oncogenic form of the protein (cyclin D1b), which is associated with aggressive tumor phenotypes.[3] Given the importance of D-cyclins in controlling the phenotypes associated with human cancers, this aspect of cyclin D function has been widely studied and is well understood.

While the pro-proliferative actions of cyclin D1 are largely mediated by CDKs, it is clear that the D-cyclins harbor a number of critical, CDK-independent functions. Strikingly, unbiased biochemical analysis revealed that a major fraction of endogenous cyclin D1 is found in association with transcription factors.[4] Subsequent analyses demonstrated that cyclin D1 is found at promoters and is a key mediator of selected transcription factor functions. The ability of cyclin D1 to regulate transcription appears to underpin major in vivo activity; exemplifying this, the retinal hypoplastic phenotype of the cyclin D1-knockout mouse results from loss of cyclin D1-mediated Notch signaling. The finding that cyclin D1-controlled transcriptional regulation controls in vivo phenotypes is consistent with a litany of previous studies identifying cyclin D1 as a regulator of nuclear receptors. Cyclin D1 associates with and modulates function of the androgen receptor (AR),[5] estrogen receptor alpha (ER),[6] PPAR-gamma,[7] thyroid hormone receptor beta (TR-B) and multiple nuclear receptor co-regulators. Morever, cyclin D1 can regulate androgen and estrogen metabolism in the liver, further implicating the protein as a major effector of hormone action.[8]

In a new study by Hanse and colleagues,[9] cyclin D1 was identified as a critical mediator of de novo hepatic lipogenesis, manifest by both CDK-dependent and CDK-independent mechanisms. Initial studies demonstrated that cyclin D1 inhibits lipogenesis in primary rat hepatocytes, and was associated with altered lipogenic gene expression programs that are distinct from the role of cyclin D1 in facilitating injury-induced hepatocyte proliferation. The underlying mechanisms hinge upon two distinct actions of cyclin D1. First, cyclin D1 negatively regulates ChREBP (carbohydrates response element-binding protein) expression and activity in a manner dependent on CDK4 function. The ChREBP transcription factor is typically activated by high glucose and promotes expression of genes whose functions are important for mediating hepatic lipogenesis. By contrast, cyclin D1 binds to and suppress the function of HNF4α (Hepatocyte nuclear factor 4 alpha), a member of the nuclear receptor superfamily that influences liver function. Cyclin D1 suppresses binding of HNF4α to chromatin at regulatory regions of target genes associated with lipogenesis, and the impact of cyclin D1 was further confirmed by the observation that cyclin D1 knockdown enhanced both HNF4α activity and lipogenesis. Finally, the relationship between liver regeneration and the lipogenic response was examined with a focus on cyclin D1 activity; as expected, injury introduced by partial hepatectomy induced cyclin D1 expression and hepatocyte cell cycle advancement. Notably, injury-induced cellular proliferation was associated with a concomitant suppression of lipogenic gene expression.

Combined, these findings suggest that altered metabolic function during liver regeneration may be attributed to more than alteration of hepatic mass, but may be controlled by the induction of cyclin D1-mediated transcription regulation. As the study establishes a new link between cell cycle regulation and hepatic metabolism, the implications of these cyclin D1 functions for liver development,

homeostasis and cancer should be explored. On balance, these studies provide further impetus for discerning the cellular and biological impact of cyclin D1-mediated transcriptional regulation.[9]

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A role for protein phosphatase 4 in regulating non-homologous end-joining

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DNA double strand breaks (DSBs) are the most deleterious form of DNA damage as unrepaired or misprocessed DBSs can lead to genomic instability and ultimately cancer.[1] To counter the deleterious nature of DSBs, cells have developed a number of pathways which repair DSBs with the two most prominent being non-homologous end joining (NHEJ) and homologous recombination (HR). The cellular response to DSBs requires efficient recognition of the damaged DNA, signal transduction pathways, activation of cell cycle checkpoint controls and repair pathway selection. At the heart of the cellular response to DSBs is the DNA damage response (DDR), which mediates DNA damage-induced signal transduction via activation of a large number of protein kinases.[2] Phosphorylation mediated by these protein kinases is required for the transmission, and coordination of the DDR and the direct targets include the sensors of the DSBs, DDR signaling mediators, checkpoint control transducers and effectors, repair proteins, histones and chromatin modifiers.

While the role of protein kinases on the repair process have been well-documented, the role of serine/threonine protein phosphatases (PPs) in the DDR and the DSB repair process have only recently started to be uncovered. A number of PPs have been implicated in regulating the phosphorylation status of DDR proteins and HR, including PP1, PP2A, PP4, PP6 and Wip1.[3] Direct substrates include initial sensors of the DSB (Ku70/Ku80), DDR signaling mediators (BRCA1), checkpoint control transducers and effectors (CHK1, CHK2 and p53), repair proteins (RPA), histones (γH2AX) and the DSB activated kinases (ATM, DNA-PKcs and ATR) implicating that reversible phosphorylation mediated by PPs plays an important role in the cellular response to DSBs.

In a report by Liu et al. in *Cell Cycle*, the Xu group continued their studies on the role that protein phosphatase 4 (PP4) plays in DSB repair as they had previously shown that PP4 is required for HR.[4,5] As a number of NHEJ factors are phosphorylated in response to DSBs, in this study the Xu group determined if PP4 played a role in NHEJ. Using established in vivo NHEJ assays, the authors showed that the PP4 catalytic subunit (PP4c) and its catalytic activity are required for NHEJ. The PP4 holoenzyme occurs in different assemblies of the catalytic subunit with one or more regulatory subunits. Knockdown of each of the PP4 regulatory subunits with specific siRNAs showed that PP4R2, but not PP4R1, PP4R3α or PP4R3β-containing, holoenzymes are involved in the regulation of NHEJ.

Using a large-scale immunoprecipitation assay, followed by mass spectrometry analysis to identify the PP4 substrate which plays a role in NHEJ, the authors identified KAP1 (KRAB-associated protein (1) as an interactor of PP4c as well as PP4R2. KAP1 was identified as a transcriptional co-repressor but was later found to be rapidly phosphorylated by ATM at serine 824 (S824) in response to DSBs.[6]

Phosphorylation of KAP1 at S824 impacts repair of DSBs within heterochromatin by promoting chromatin relaxation to allow repair proteins access to the DSB.[7] The authors next assessed if KAP1 was a substrate of PP4. Knockdown of PP4c or PP4R2 resulted in an increase in KAP1 phosphorylation at S824 following DNA damage. PP4 dephosphorylated KAP1 in vitro further implicating that KAP1 is a PP4 substrate. Finally, knockdown of KAP1 resulted in a decrease in NHEJ but co-depletion of PP4 and KAP1 did not have a synergistic effect on NHEJ suggesting that PP4 and KAP1 are in the same NHEJ epistasis group. Together, the data implicates that PP4's ability to regulate NHEJ is through its ability to regulate KAP1 phosphorylation.

Many new interesting questions are raised by this study: (1) does PP4 play a role in all end-joining pathways or a specific one; (2) KAP1 dephosphorylation by PP4 should result in chromatin condensation, is this important for NHEJ; (3) a recent manuscript also found that PP4 dephosphorylates KAP1 at S824 but this was due to a holoenzyme with PP4R3β8, is dephosphorylation of KAP1 by different PP4 holoenzymes regulated; (4) PP4c knockdown results in a greater decrease in NHEJ than KAP1 implicating that PP4 may have other NHEJ substrate(s), what are these substrates and what role do they play in regulating NHEJ. Together, it will be of great interest to continue to identify the role(s) that PPs play in regulating NHEJ.

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