DNA replication stress-induced loss of reproductive capacity in *S. cerevisiae* and its inhibition by caloric restriction

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Abstract

In many organisms, attenuation of growth signaling by caloric restriction or mutational inactivation of growth signaling pathways extends lifespan and protects against cancer and other age-related diseases. The focus of most efforts to understand these effects has been on the induction of oxidative stress defenses that inhibit cellular senescence and cell death. Here we show that in the model organism *S. cerevisiae*, growth signaling induces entry of cells in stationary phase into S phase in parallel with loss of reproductive capacity, which is enhanced by elevated concentrations of glucose. Overexpression of *RNR1* encoding a ribonucleotide reductase subunit required for the synthesis of deoxynucleotide triphosphates and DNA replication can suppress the loss of reproductive capacity of stationary phase cells. Reduced reproductive capacity induced by high glucose is also suppressed by excess threonine, which buffers dNTP pools when ribonucleotide reductase activity is limiting. Caloric restriction or inactivation of the AKT homologue Sch9p inhibit senescence and death in stationary phase cells caused by the DNA replication inhibitor hydroxyurea or by inactivation of the DNA replication and repair proteins Sgs1p or Rad27p. Inhibition of DNA replication stress represents a novel mechanism by which caloric restriction promotes longevity in *S. cerevisiae*. A similar mechanism may promote longevity and inhibit cancer and other age-related diseases in humans.

Introduction

Caloric restriction extends the lifespans of diverse eukaryotic organisms and protects against cancer and other age-related diseases in rodents. Most efforts to understand the underlying mechanisms have focused on the reduction in intracellular levels of reactive oxygen species (ROS) and oxidative damage to DNA and other macromolecules that occurs when conserved growth signaling pathways activated by nutrients are downregulated by caloric restriction [1]. The results of these studies are consistent with a role for oxidative damage that induces senescence and cell death as an important determinant of lifespan. However, complexities exist in the relationships between ROS and aging and age-related diseases that point to causal factors other than, or in addition to, oxidative stress and oxidative damage [2].

DNA replication stress – i.e., inefficient DNA replication - recently emerged as a primary cause of genome instability and oncogene-induced senescence (OIS) at early stages of cancer [3]. Replication stress occurs at DNA replication forks, which contain highly recombinogenic regions of unwound template DNA. Unwound DNA at replication forks is uniquely susceptible to single strand scissions that result in double strand DNA breaks [4]. Earlier studies of OIS were focused on understanding the senescence-promoting effects of ROS-induced oxidative damage to DNA and other macromolecules, which is elevated by sustained oncogenic growth signaling. However, the results of a number of recent studies revealed that in addition to inducing ROS, sustained growth signaling by oncogenes also induces DNA replication stress in preneoplastic cells, which leads to senescence in S phase [3]. How replication stress arises downstream of growth signaling by activated oncogenes remains unclear.

DNA replication stress was also recently implicated as a pro-aging factor in the budding yeast *Saccharomyces cerevisiae* chronological aging model, which shares features with OIS in mammalian cells [5]. Chronological lifespan (CLS) is assessed by measuring the reproductive capacity of cells driven into stationary phase by nutrient depletion. Reversible growth arrest (i.e. quiescence) of nutrient-depleted cells in stationary phase requires downregulation of many of the same conserved growth signaling pathways – including those that require RAS and mTOR homologues and members of the AKT/PKB family of kinases [6] – that cause OIS when inappropriately

activated in preneoplastic cells. Budding yeast cells that lose reproductive capacity in stationary phase enter into an irreversible senescent state [7] and eventually die by programmed cell death [8].

As in earlier studies of OIS in mammals and of aging in many organisms, the focus of most efforts to understand the impact of growth signaling pathways on senescence and death in the budding yeast CLS model has been on their ability to downregulate oxidative stress defenses. However, a strong correlation exists between shorter CLS and a variety of experimental manipulations that inhibit stationary phase growth arrest in G0/G1, where cells cannot develop replication stress. This includes, for example, the shorter CLS and less frequent G0/G1 arrest of stationary phase cells induced by ectopic expression of the cyclin-dependent kinase (CDK) activator Cln3p [9] or inactivation of the CDK inhibitor Sic1p [10, 11]. Both of these experimental manipulations promote entry of stationary phase cells into S phase. Increasing the concentration of glucose in medium also shortens CLS via a mechanism that depends on the AKT homologue Sch9p and results in less frequent stationary phase growth arrest in G0/G1 [10]. A strong correlation also exists between longevity in the CLS model and the increased frequency with which stationary phase cells growth arrest in G0/G1 when conserved RAS, TOR and AKT/PKB growth signaling pathways are attenuated by mutations or caloric restriction [9, 10]. Cells that are budded in stationary phase senesce [12] and/or die [10] significantly more frequently than unbudded cells, which is consistent with replication stress as a causal factor.

Based on these findings and parallels with the emerging connections between replication stress and OIS in mammalian cells, we proposed earlier that in addition to inducing oxidative stress, sustained growth signaling via conserved AKT-dependent and other growth signaling pathways induces replication stress in cells in stationary phase [9]. In this study we sought to test this hypothesis more directly. Our results show that replication stress induced by the ribonucleotide reductase (RNR) inhibitor hydroxyurea (HU) or by inactivation of genes encoding the replication-related proteins Rad27p or Sgs1p reduces the reproductive capacity of stationary phase cells via a mechanism that depends on growth signaling. Conversely, overexpression of *RNR1* encoding a subunit of RNR, which is required for the synthesis of deoxynucleotide triphosphates (dNTPs) and

DNA replication, inhibits the loss of reproductive capacity of stationary phase cells induced by sustained growth signaling. Replication stress-induced senescence and death of cells in stationary phase is inhibited by caloric restriction or by inactivation of the AKT homologue Sch9p. This represents a novel mechanism by which caloric restriction or mutational inactivation of growth signaling promotes longevity of budding yeast cells in stationary phase.

Results

Attenuation of growth signaling inhibits hydroxyurea-induced replication stress and loss of reproductive capacity in cells in stationary phase.

The CLS of budding yeast is substantially shorter in cells exposed during transitions to stationary phase to a low concentration (30 mM) of the RNR inhibitor hydroxyurea that does not inhibit the growth of cells in exponential cultures [13]. In mammalian cells, sensitivity to HU occurs specifically in S phase [14]. In budding yeast, HU-induced DNA damage and sensitivity to HU are detected in dividing cells, but not in cells arrested in G1 or G2. This contrasts with the sensitivity of budding yeast cells to the DNA damaging agent methylmethane sulfonate (MMS), which is detected in G1 or G2-arrested cells as well [15]. Therefore, sensitivity of stationary phase cells to HU likely reflects increased replication stress due to a reduction in dNTP pools required for DNA replication rather than DNA repair.

We confirmed that cells exposed to 30 mM HU as they transition from exponential growth to stationary phase suffer a substantial loss of reproductive capacity in early stationary phase (Fig. 1A and B). The reduced reproductive capacity of HU-treated cells was not caused by inhibition of entry into stationary phase, because HU-treated and control untreated stationary phase cultures achieved similar terminal densities. HU treatment also increased the fraction of cells in early stationary phase that failed to arrest in G0/G1 as indicated by the detection of buds (Fig. 1F; "2% glu"). These findings establish that in principle, replication stress can reduce the reproductive capacity of cells in stationary phase in parallel with less frequent growth arrest of cells in G0/G1. However, the increase in the fraction of budded cells (from 22% to 44%) was substantially less than the fraction of the same population of cells that exhibited a HU-

induced loss of reproductive capacity at the same time point (from 73% to 3%). Therefore, measuring the fraction of budded cells in stationary phase underestimates the fraction of cells that are undergoing replication stress.

Caloric restriction imposed by reducing the initial concentration of glucose in medium from 2% to 0.5% extends CLS in concert with a reduction in intracellular superoxide anions [16] and more frequent G0/G1 arrest of cells in stationary phase [9]. Caloric restriction also abolished the sensitivity to HU of cells in early stationary phase (Fig. 1C) in parallel with a reduction in the number of cells with detectable buds (Fig. 1F; "0.5% glu"). Inactivation of the AKT homologue *SCH9* also extends CLS in parallel with a reduction in intracellular levels of superoxide anions [10, 17] and a decrease in the frequency with which stationary phase cells fail to arrest growth in G0/G1 (Fig. 1G; see also reference 9). Compared to isogenic wild type cells (Fig. 1D), *sch9Δ* cells were also less sensitive to HU treatment during transitions to stationary phase (Fig. 1E) and less frequently exhibited buds compared to HU-treated wild type cells (Fig. 1G). We conclude that attenuation of growth signaling by caloric restriction or genetic inactivation of Sch9p-dependent growth signaling protects against the loss of reproductive capacity caused by HU inhibition of ribonucleotide reductase in cells in early stationary phase, in part by promoting growth arrest of stationary phase cells in G0/G1.

Acetic acid and other organic acids accumulate in chronological aging experiments that employ SC medium [18], and buffering medium to eliminate the resulting decline in pH extends CLS [18, 19]. This is likely due to the activation of growth signaling pathways by low pH [19]. In fact, many of the longevity-promoting effects of inactivating Sch9p and Ras2p reported previously are related to inhibition of the pro-aging effects of low pH rather than glucose. For example, in 2% glucose YPD medium, which maintains a higher pH, the CLS-extending effects of deleting *SCH9* are absent in early stationary phase cultures [10]. However, increasing the concentration of glucose in YPD medium to 10%, which approximates conditions *S. cerevisiae* cells are frequently exposed to in their natural environment, shortens CLS in a Sch9p-dependent fashion in response to glucose signaling rather than signaling by low pH [10]. To determine whether Sch9p-dependent glucose signaling impacts replication stress induced by HU when effects of low pH are

eliminated, we asked whether deletion of *SCH9* inhibits HU-induced loss of reproductive capacity in 10% glucose SC buffered to pH 6. Cells in these cultures remained sensitive to 30 mM HU (Fig 1H), although buffering the medium delayed this sensitivity until day 7 of medium depletion. In contrast, sensitivity to HU was absent in *sch9Δ* cells cultured under similar conditions (Fig. 1I). Similar results were obtained in YPD medium (Fig. 1J and K). We conclude that inactivation of Sch9p-dependent glucose signaling can inhibit replication stress induced by inhibiting ribonucleotide reductase independently of low pH.

Glucose enhances the effects of replication stress associated with the inactivation of RAD27 or SGS1 independently of pH.

The *RAD27* gene encodes the *S. cerevisiae* homologue of the conserved Fen1 5' flap endonuclease required for maturation of Okazaki fragments during DNA synthesis [20]. *rad27Δ* cells exhibit an extended S phase [21] and arrest in S phase at an elevated temperature [22]. Therefore, inactivation of *RAD27* causes replication stress, which likely contributes to the shorter CLS detected in *rad27Δ* compared to wild type cells [23, 24].

To determine whether glucose signaling enhances replication stress induced in stationary phase cells by a mechanism other than inhibiting RNR, we compared the reproductive capacity of stationary phase wild type and *rad27Δ* cells cultured in buffered SC medium that initially contained either 2% or 10% glucose. At day 7 of medium depletion, *rad27Δ* cells cultured in buffered medium prepared with 2% glucose exhibited a similar reproductive capacity compared to wild type cells. At day 13, the reproductive capacity of *rad27Δ* cells was modestly reduced compared to wild type cells under these conditions (Fig. 2A). This is consistent with prior reports that *rad27Δ* cells exhibit a shorter CLS compared to wild type cells in unbuffered 2% glucose medium [23, 24], although similar to HU experiments, buffering the medium suppressed the senescence-promoting effect of deleting *RAD27* at earlier time points. In contrast, *rad27Δ* cells cultured in buffered medium prepared with 10% glucose exhibited a substantially shorter reproductive capacity compared to wild type cells at day 7, and the reproductive capacity of *rad27Δ* cells was reduced even further by day 13 (Fig. 2A). A similar reduction in reproductive capacity was observed in *rad27Δ* compared to wild type cells cultured for

seven days in 10% glucose YPD medium, but not in YPD medium that initially contained 2% glucose (Fig. 2B). In both buffered SC medium and YPD medium, compared to wild type cells, *rad27Δ* cells also arrested growth in stationary phase more frequently with visible buds, and the fraction of budded cells in stationary phase was increased further at the higher concentration of glucose (Fig. 2C and D).

SGS1 encodes an orthologue of RecQ helicases that when defective in humans cause cancer-predisposing disorders accompanied by accelerated aging. In budding yeast, Sgs1p plays a role in responses to replication stress [25]. Although *sgs1Δ* cells have been reported to exhibit a shorter replicative lifespan (RLS) [26] and CLS [27], it has been reported by others that *sgs1Δ* cells do not exhibit a shorter CLS [23, 28]. *sgs1Δ* cells more frequently undergo adaptive regrowth in stationary phase, which complicates measurements of CLS. In fact, inactivation of Sgs1p causes a reduction in reproductive capacity in early stationary phase cells that can be masked by adaptive regrowth at later time points [28].

We next asked whether growth signaling in buffered medium containing 10% glucose would exacerbate effects on reproductive capacity associated with the defective response to replication stress in *sgs1Δ* cells. After seven days of medium depletion, the reproductive capacity of *sgs1Δ* cells cultured in buffered 2% glucose SC medium was similar to that of isogenic wild type cells. However, similar to *rad27Δ* cells (Figs. 2A and C), despite the absence of a significant change in reproductive capacity in *sgs1Δ* cells cultured under these conditions, compared to wild type cells *sgs1Δ* cells more frequently failed to arrest in G0/G1 (Fig. 2F). This is consistent with the possibility that despite the presence of replication stress in *sgs1Δ* and *rad27Δ* cells, adaptive regrowth can mask the effects of replication stress on reproductive capacity. A significant reduction in the reproductive capacity of *sgs1Δ* compared to wild type cells was in fact detected, however, when they were cultured in 10% glucose buffered medium (Fig. 2E). The reduction in reproductive capacity of *sgs1Δ* cells under these conditions was also accompanied by less frequent growth arrest of stationary phase cells in G0/G1 indicated by an increase in the fraction of visible buds (Fig. 2F). Similar effects of deleting *SGS1* on reproductive capacity were observed in 10% glucose YPD cultures of cells in a different genetic background (DBY746) (Fig. 2G). These findings establish that glucose signaling

enhances the effects of replication stress on reproductive capacity and frequency of G0/G1 arrest induced by inactivating *RAD27* or *SGS1* independently of effects of low pH. Conversely, reduced glucose signaling by a lower concentration of glucose abrogates these effects by enhancing growth arrest of stationary phase cells in G0/G1.

Chronological lifespan extension by overexpression of RNR1

The expression of genes encoding the RNR subunit Rnr1p and other proteins required for DNA replication are downregulated in response to the depletion of nutrients from growth medium as cells enter stationary phase [29]. To determine whether downregulation of *RNR1* as cells transition into stationary phase might induce replication stress that contributes to the senescence and/or death of stationary phase cells, we asked whether overexpression of *RNR1* encoding the large subunit of the RNR complex that catalyzes the rate-limiting step in dNTP synthesis [30] would extend CLS. When cultured in synthetic complete (SC) medium containing 2% glucose, wild type W303 cells transformed with a high copy plasmid that expresses *RNR1* exhibited a longer CLS compared to cells transformed with an empty vector (Fig. 3A). The extended CLS of cells overexpressing *RNR1* was accompanied by a reduction in the number of cells undergoing programmed cell death marked by DNA degradation that resulted in cells with a sub G1 content of DNA (Fig. 3B).

The essential function of the DNA damage and replication stress response protein Mec1p is to upregulate RNR and dNTP pools [31]. Disruption of this function by the *mec1-21* mutation causes a reduction in dNTP pools [32] and a shorter CLS [9]. A shorter CLS has also been reported for *mec1Δ* cells [27]. *mec1-21* cells transformed with an empty vector exhibited a slightly shorter CLS compared to wild type cells transformed with an empty vector, and the shorter CLS of *mec1-21* cells was suppressed by overexpression of *RNR1* (Fig. 3C). Overexpression of *RNR1* also enhanced stationary phase growth arrest in G0/G1 indicated by a reduction in cells with visible buds in stationary phase cultures of wild type or *mec1-21* cells (Fig. 3D). These findings indicate that *RNR1* expression is limiting in W303 cells in stationary phase, and limiting *RNR1* expression reduces the frequency with which cells arrest growth in G0/G1.

Sustained growth signaling by glucose enhances a requirement for RNR1 expression and

threonine in cells in stationary phase

The expression of *RNR1* and other replication-related genes could become limiting in stationary phase cells if these cells re-enter S phase after these genes have been downregulated in stationary phase. In fact, the fraction of BY4741 wild type cells in S phase increased after these cultures entered stationary phase (Fig. 4A). However, overexpression of *RNR1* did not enhance the reproductive capacity of early stationary phase cells in the BY4741 background cultured in 2% glucose SC medium (Fig. 4B), in contrast to its effect on stationary phase W303 cells cultured under similar conditions (Fig. 3). Therefore, *RNR1* expression is not limiting in BY4741 cells under these conditions.

We next asked whether enhanced glucose signaling that accelerates the loss of reproductive capacity of BY4741 cells might also increase their requirement for *RNR1* expression. The reproductive capacity of wild type BY4741 cells in early stationary phase (after ~72 hours of medium depletion) was substantially diminished in 10% glucose compared to 2% glucose SC cultures (compare Fig. 4C; "vec" with Fig. 4B; "vec"), as reported previously [10]. The reduced reproductive capacity of cells in these cultures was not related to increased non-enzymatic glycation of proteins and other macromolecules by high glucose. This is because *gal1Δ* cells, which cannot metabolize galactose, are significantly less sensitive to excess galactose compared to glucose in early stationary phase (Fig. S1), despite the ten-fold higher rate at which galactose non-enzymatically glycates proteins compared to glucose [33]. It was also not related to fewer cells achieving stationary phase in 10% glucose cultures, because after two days of medium depletion, the terminal density of 10% glucose cultures was twice that of 2% glucose cultures. Overexpression of *RNR1* restored the reproductive capacity of cells cultured in 10% glucose SC medium to levels observed in 2% glucose SC cultures (Fig. 4C). We conclude that limiting *RNR1* expression is the cause of the more rapid loss of reproductive capacity of cells in 10% glucose cultures.

The shorter CLS observed in cultures that initially contained 10% glucose occurs in parallel with a 5 to 8-fold increase in the rate at which budded compared to unbudded cells die [10]. This is consistent with the possibility that in 10% glucose cultures, limiting

RNR1 expression causes replication stress and death of cells that are more frequently driven into S phase after *RNR1* expression is downregulated in stationary phase. The fraction of cells with an S phase content of DNA in 10% glucose cultures initially declined to 7% during the first 24 hours. It then increased to 57% of the total population at 72 hours (Fig. 4D). This increase was accompanied by an increase in the fraction of cells with less than a G1 content of DNA, indicating that DNA degradation was occurring in dying cells (Fig. 4D, "sub G1"). Some cells with an S phase content of DNA detected at later time points in 10% glucose cultures may correspond to cells that were actually in G2/M but suffered DNA degradation. However, the fraction of cells with a G2/M content of DNA at 24 hours (approximately 20%), which was before DNA degradation began to occur, was substantially less than the fraction of cells with an S phase content of DNA detected at the 72 hour time point (57%). Therefore, even if all the G2/M cells in the these cultures suffered DNA degradation that resulted in an S phase content of DNA, DNA degradation in G2/M cells would account for less than half of the increase in cells with an S phase DNA content at 72 hours. Furthermore, although the fraction of budded cells in 10% glucose cultures initially decreased during the first 36 hours, a statistically significant increase ($p < 0.04$) in the fraction of budded cells occurred at later time points (Fig. 4E). Therefore, increasing the concentration of glucose increases the frequency with which cells enter S phase in stationary phase cultures, where they die due to insufficient *RNR1* activity.

In 2% glucose cultures, the increase in cells in S phase occurred after both glucose (Fig. 4F) and the nitrogen source ammonium (Fig. 4G) were completely depleted. Ammonium was also completely depleted from 10% glucose cultures after 36 hours (Fig. 4G), which coincided with the time these cultures entered stationary phase marked by the absence of a continued increase in cell number. Although the concentration of glucose initially declined as ammonium was depleted from 10% glucose cultures, it did not decline below approximately 3% during the first several days of stationary phase (Fig. 4F), which coincided with the time large numbers of cells were entering S phase.

In exponentially proliferating cells, inactivation of the *THR1* gene in the threonine biosynthesis pathway confers sensitivity to HU [34, 35], and the sensitivity of *thr1Δ* cells to

HU can be suppressed by exogenous threonine [35]. *thr1Δ* sensitivity to HU is related to the role of threonine biosynthesis in buffering dNTP pools when RNR activity is limiting [35]. Threonine biosynthesis depends on oxaloacetate, an intermediate of the tricarboxylic acid (TCA) cycle, which is repressed by glucose [36]. For example, the *MDH1* gene encoding malate dehydrogenase, which catalyzes the conversion of malate to oxaloacetate in the TCA cycle, is substantially downregulated when glucose is exhausted in 2% glucose cultures [36]. Consistent with a role for threonine biosynthesis in maintaining dNTP pools in stationary phase cells cultured in 2% glucose SC medium, *thr1Δ* cells, which are predicted to have a very short CLS [37], rapidly lost reproductive capacity within the first few days of stationary phase when cultured under these conditions, and this phenotype was suppressed by overexpression of *RNR1* (Fig. 3H). This is consistent with the possibility that in 2% glucose SC medium, threonine is required to maintain sufficient dNTP pools in BY471 cells to protect against replication stress-induced loss of reproductive capacity in stationary phase. We next asked whether the deficiency in *RNR1* expression imposed by increasing the glucose concentration to 10% might be related to inhibition of threonine biosynthesis downstream of repression by residual glucose of oxaloacetate and other TCA cycle intermediates. Consistent with this hypothesis, similar to the effect of overexpressing *RNR1* (Fig. 4C), the addition of excess threonine to 10% glucose cultures suppressed the loss of reproductive capacity observed in these cultures after a few days of medium depletion (Fig. 4I). As expected, the addition of excess threonine did not significantly impact the reproductive capacity of cells cultured in parallel in 2% glucose medium (Fig. 4J). We conclude that the inadequate levels of *RNR1* expression to maintain reproductive capacity in stationary phase cells cultured in 10% glucose medium is due to re-entry of cells into S phase combined with insufficient levels of threonine to support efficient dNTP synthesis, most likely due to repression of the TCA cycle by residual glucose.

Discussion

Replication stress inhibits the reproductive capacity of cells in stationary phase

Mutations in RecQ helicases and other DNA replication and repair proteins that induce replication stress or create defects in responses to replication stress are well-established

causes of premature aging in many organisms. Our findings indicate that in *S. cerevisiae*, replication stress-induced senescence is also a significant factor in normal aging that is promoted by growth signaling and inhibited by caloric restriction. Inhibition of replication stress-induced senescence represents a novel, largely unexplored mechanism by which caloric restriction inhibits chronological aging in this organism.

The absence of budded cells or of a continued increase in cell number when nutrients are depleted are defining hallmarks of stationary phase cultures and the basis for the yeast chronological lifespan model of aging of post-mitotic cells in complex eukaryotes. Consequently, events related to DNA replication and other aspects of cell division have not been broadly considered as factors in the senescence and death of budding yeast cells in stationary phase. However, the increase in the number of cells in S phase after entry into stationary phase (Fig. 4) establishes that contrary to long-prevailing assumptions, budding yeast cells in stationary phase frequently re-enter the cell cycle without restoration of nutrients to medium. This is consistent with a recent report that in diploid cells cultured in 2% glucose SC medium, the fraction of dead cells with an S phase content of DNA increases at later time points in chronological aging experiments [38]. It is not consistent with the argument based on the relatively small fraction of budded cells detected in stationary phase cultures that replication stress does not exert a significant impact on chronological lifespan. [39]. The fraction of cells that suffered replication stress-induced loss of reproductive capacity in early stationary phase in our experiments was substantial, and was substantially larger than the fraction of cells that exhibited detectable buds in the same cultures at the same time points (for example, Figs. 1C and 1D). Therefore, measuring the number of budded cells underestimates the fraction of cells that suffer replication stress in stationary phase.

A requirement for efficient DNA replication to avoid loss of reproductive capacity in stationary phase is consistent with the results of a prior genome-wide screen by Powers et al. for genetic determinants of chronological lifespan [37]. The results of this screen predict that the CLS of a *rnr1Δ* strain and the *rad27Δ* strain employed in our experiments is shorter than 91% of all 4759 haploid deletion strains examined in this screen. They also predict that the CLS of *sic1Δ* strains, which undergo aberrant entry into S phase [40] and

have a substantially shorter CLS compared to wild type cells [10, 11] - and of *sgs1Δ* strains is shorter than 82% (*sic1Δ*) and 75% (*sgs1Δ*) of all 4759 of these deletion strains. *lsm1Δ* cells harboring a defect in mRNA capping that causes replication stress [41] also exhibit a substantially shorter CLS compared to wild type cells [13]. The Powers et al. screen predicts that the CLS of *lsm1Δ* cells is shorter than 94% of all the strains they examined.

Growth signaling-induced replication stress and its inhibition by caloric restriction or inactivation of SCH9

The depletion of nutrients from yeast cultures triggers the downregulation of growth signaling pathways and entry into stationary phase in parallel with the reduced transcription of a large number of genes, including genes encoding Rnr1p and other proteins required for DNA replication [29]. Our findings indicate that maintaining the reproductive capacity of cells in stationary phase requires coordination of these events such that cells entering stationary phase are no longer in S phase or are blocked from entering S phase after reduced expression of *RNR1* and other replication-related genes creates suboptimal conditions for replicating DNA (Fig. 5A). The expression of *RNR1* and other replication-related genes is downregulated in response to nitrogen depletion [29]. Consequently, sustained growth signaling after nitrogen is depleted drives cells into S phase in the absence of sufficient RNR and/or other components of the replication machinery to support efficient DNA replication (Fig. 5B).

In the standard conditions employed in most chronological aging experiments (unbuffered 2% glucose SC medium), cells in stationary phase are likely driven into S phase by sustained growth signaling by low pH. This is indicated by the observations that buffering the medium to maintain a higher pH or the use of YPD medium – which also maintains a higher pH - reduces the fraction of stationary phase cells that are budded [10, 19 38] as well as the number of dead cells with an S phase content of DNA [38]. Although the senescence and death of stationary phase BY4741 cells under these conditions is not due to limiting *RNR1* expression, many other genes required for efficient DNA replication are also downregulated as cells transition into stationary phase; presumably, the product of a replication-related gene other than *RNR1* becomes limiting. In 10% glucose medium, sustained signaling by residual glucose is likely responsible for the increased fraction of

cells that enter S phase from stationary phase. Our data indicate that *RNR1* expression becomes limiting under these conditions due to a deficiency in threonine that likely leads to the depletion of dNTP pools (Fig. 4).

Caloric restriction [16] or inactivation of Sch9p [10, 17] enhance the reproductive capacity of cells in stationary phase in part by reducing intracellular levels of superoxide anions. The data reported here indicate that attenuation of growth signaling by caloric restriction or inactivation of *SCH9* also enhances reproductive capacity by promoting more frequent growth arrest of stationary phase cells in G0/G1, where they avoid replication stress. They also predict that Sch9p and other proteins can impact chronological lifespan by interacting with the cell cycle machinery. Consistent with this prediction, it was recently reported that PKA and Sch9p phosphorylate the cell cycle-regulating ubiquitin ligase Cdc34p, and expression of a mutant form of Cdc34p that mimics its constitutive phosphorylation by Sch9p significantly shortens CLS [42]. Phosphorylation of Cdc34p by Sch9p leads to destabilization of the cyclin-dependent kinase inhibitor Sic1p, which when stabilized, blocks entry into S phase. *sic1Δ* cells exhibit a short chronological lifespan in concert with accelerated entry into S phase from stationary phase [10]. Together, these findings argue that Sch9p impacts chronological aging via effects on the cell cycle machinery that lead to replication stress independently of the well-established role of Sch9p in inhibiting oxidative stress responses.

Relevance to complex eukaryotes

The loss of reproductive capacity of yeast cells in stationary phase cultures is usually considered to model events that impact the aging of quiescent, postmitotic cells of higher eukaryotes. Our findings suggest that chronological aging experiments also model events that lead to the senescence of cells in humans and other metazoans as they enter into a quiescent state, in addition to events that occur after the quiescent state is achieved. They are likely relevant to a recent report, for example, that as cultured mouse cells approach contact inhibition-induced quiescence, sustained growth signaling by oncogenic Ras induces abortive S phase entry in the absence of sufficient dNTPs to efficiently replicate DNA [43]. Similarly, the uncoordinated activation of growth signaling pathways that promote entry into S phase leads to depletion of dNTPs and genome instability in

cultured human cells transformed by ectopic expression of cyclin E or oncogenic viral proteins [44].

Similar to mutations that activate oncogenes, elevated glucose activates PI3K/AKT/mTOR signaling and other oncogenic signaling pathways in cultured mammalian cells. Glucose activation of these pathways leads to the induction of cyclin D1, cyclin E and other proteins that promote entry into S phase, as well as downregulation of p27, the mammalian homologue of Sic1p [45, 46]. Elevated glucose that mimics chronic hyperglycemia in humans also induces DNA damage [47] and senescence [48] in cultured mammalian cells. In budding yeast, inactivation of the conserved AMP kinase Snf1p, which is activated by reduced levels of glucose, shortens CLS in concert with a large increase in the number of cells that fail to arrest in G0/G1 when driven into stationary phase by nutrient depletion [10]. The mammalian AMP kinase similarly drives cells into G1 in response to low glucose [49, 50]. Drugs that mimic the effects of caloric restriction by activating AMPK protect against cancer [51]. We propose that these and other effects are related in part to glucose-induced replication stress and its inhibition by caloric restriction and caloric restriction mimetics. This model and its relevance to dietary factors that promote aging and age-related diseases in humans remain to be explored.

Experimental Procedures

Strains and plasmids are described in Table 1.

Cell culture conditions: Cells were cultured at 30ºC with rotary shaking for the indicated times in YPD medium [52] or synthetic complete (SC) medium supplemented with excess amino acids and bases [53] with the exception that uracil was absent from medium in experiments that employed plasmids. SC and YPD medium contained 2% or 10% glucose as a carbon source. In experiments that employed buffered medium, citrate buffer pH 6 was added to a final concentration of 50 mM. Chronological lifespan and budded cell measurements were performed as described previously [9] and in more detail below. Reproductive capacity was assessed by spotting 10-fold serial dilutions of triplicate cultures on YPD agar plates followed by incubation at 30ºC for two or three days. Hydroxyurea was purchased from Sigma-Aldrich (St. Louis, MO).

Chronological lifespan measurements: Cells from exponentially proliferating cultures were inoculated into 50 mls. of SC medium containing 2% or 10% (weight/volume) glucose in 250 ml. flasks at an initial density of 5 X 10^7 cells/ml. and continuously cultured at 30ºC with rotary shaking for the indicated times. Aliquots removed from cultures at the indicated times were plated in triplicate on YPD agar to determine colony forming units. Data are representative of measurements made in two or more biological replicas.

Measurements of budded cells: To determine the fraction of cells with visible buds, cells in aliquots taken at each time point were pelleted by centrifugation and resuspended in water. At least 500 cells from each culture were examined for buds using a Nikon Eclipse E600 microscope with a 40X phase contrast objective. Just before examining cells, cell clumps were dissociated by sonication using a Model 60 Sonic Dismembrator sonicator (Fisher Scientific, Hampton, NH) for ten seconds at a power setting of 5.

Glucose and Ammonia measurements: Glucose and ammonia measurements were performed on aliquots of cultures at the indicated time points using a glucose oxidase (GOD) assay (Roche Diagnostics GmbH, Mannheim, Germany) and an ammonia assay kit (Sigma, St. Louis, MO) following the manufacturer's instructions.

Cell cycle analysis: Aliquots of cultures were collected at the indicated time points and cells were pelleted, washed and fixed with ethanol (70% v/v) for 30 min at 4 ºC. Cells were then resuspended in sodium citrate buffer (50 mM sodium citrate, pH 7.5), sonicated and treated with RNAse for 1 h at 50 ºC followed by subsequent incubation with proteinase K (0.02 mg/10^7 cells) for several hours. DNA was then labeled with SYBR Green (Molecular Probes/Invitrogen, Carlsbad, CA) diluted in Tris-EDTA (pH 8.0) and incubated overnight at 4 ºC. Before flow cytometry analysis, samples were diluted 1:4 in sodium citrate buffer. Flow cytometry measurements were made using a BD LSR II™ (Becton Dickinson, NJ, USA) and data analyzed with BD FACSDiva Software 6.0 (Becton Dickinson, NJ, USA). The percentage of cells in each phase of the cell cycle was determined offline with ModFit LT software (Verity Software House, Topsham, ME).

Reproducibility and statistical analysis: Spot test data are representative of data collected in three or more biological replicas. Error bars in bar graphs represent standard deviations calculated from the results of at least three independent experiments. Statistical significance was assessed where indicated using Student's unpaired *t*-test.

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Supporting information

Fig. S1. Sensitivity of *gal1Δ* cells to excess glucose or galactose.

Figure legends

Figure 1. Attenuation of growth signaling inhibits hydroxyurea-induced replication stress and loss of reproductive capacity in cells in stationary phase. A. Reproductive capacity of wild type BY4741 cells continuously exposed to 30 mM HU during three days of medium depletion. In these experiments, the potential impact of replication stress on reproductive capacity was assessed at one or two time points in stationary phase under conditions that led to a loss of reproductive capacity of less than 60% in one set of conditions. B. Reproductive capacity of BY4741 cells under the same conditions assessed by serial ten-fold dilutions of cultures spotted on YPD agar plates. Similar ten-fold dilutions were performed in subsequent experiments that assessed reproductive capacity

by spot tests. C. Reproductive capacity of BY4741 cells exposed to 30 mM HU during three days of nutrient depletion in medium that initially contained 0.5% glucose. D and E. Reproductive capacity of DBY746 wild type (D) or DBY746 *sch9Δ* (E) cells exposed to 30 mM HU during three days of medium depletion. F and G. % visibly budded BY4741 wild type cells (F) or cells in the DBY746 background (G) exposed to 30 mM HU during three days of nutrient depletion. H. Reproductive capacity of DBY746 wild type cells cultured in 10% glucose SC buffered medium for seven days in the presence or absence of 30 mM HU. I. Reproductive capacity of DBY746 *sch9Δ* cells cultured in 10% glucose SC buffered medium for seven days in the presence or absence of 30 mM HU. J. Reproductive capacity of DBY746 wild type cells cultured in 10% glucose YPD medium for seven days in the presence or absence of 30 mM HU. K. Reproductive capacity of *sch9Δ* cells in the DBY746 background cultured in 10% glucose YPD medium for seven days in the presence or absence of 30 mM HU.

Figure 2. Glucose enhances the effects of replication stress induced by inactivating *RAD27* or *SGS1*. A. Reproductive capacity after seven or thirteen days medium depletion of BY4741 and isogenic *rad27Δ* cells cultured in buffered SC medium that initially contained the indicated amounts of glucose. B. Reproductive capacity of BY4741 and *rad27Δ* cells cultured for seven days in YPD medium that initially contained the indicated amount of glucose. C and D. % visibly budded BY4741 wild type and *rad27Δ* cells cultured for seven days in buffered SC medium (C) or YPD medium (D) that initially contained the indicated amounts of glucose. E. Reproductive capacity of BY4742 wild type cells or *sgs1Δ* cells in the BY4742 background cultured for seven days in SC medium that initially contained the indicated amount of glucose. F. % visibly budded cells in the same cultures described in panel E. G. Reproductive capacity of BY4742 wild type cells and *sgs1Δ* cells in the BY4742 background cultured for seven days in YPD medium that initially contained the indicated amount of glucose.

Figure 3. High copy expression of *RNR1* extends chronological lifespan. A. CLS determined by measuring colony-forming units (cfus) of wild type (W303) cells transformed with a high copy plasmid expressing *RNR1* or the empty vector. B. DNA

content of wild type cells transformed with the plasmid expressing *RNR1* or the empty vector after five days of medium depletion. C. CLS of wild type and *mec1-21* cells transformed with the plasmid expressing *RNR1* or the empty vector. D. % visibly budded cells in the cultures described in panel C. Data in panels A – C are representative of results from two or more independent experiments.

Figure 4. Sustained growth signaling enhances the requirement for *RNR1* in cells in stationary phase. A. Fraction of cells in various cell cycle compartments in 2% glucose cultures determined by measuring DNA content. B and C. Reproductive capacity after three days medium depletion of BY4741 wild type cells transformed with a high copy plasmid expressing *RNR1* or the empty vector and cultured in SC medium prepared with 2% glucose (B) or 10% glucose (c). D. Fraction of cells in various cell cycle compartments in 10% glucose cultures determined by measuring DNA content. E. % visibly budded cells after depletion of 10% glucose SC medium for indicated times. Asterisk indicates statistically significant increase in the fraction of visibly budded cells at the 60 and 72 hour time points compared to the fraction at the 36h time point ($P < 0.01$). F. Concentration of glucose in medium at indicated time points in 2% glucose or 10% glucose cultures. G. Concentration of ammonium ions in medium in the same cultures. H. Reproductive capacity of *thr1Δ* cells transformed with a high copy plasmid expressing *RNR1* or the empty vector after culturing for three days in SC medium prepared with 2% glucose. I and J. Reproductive capacity of BY4741 wild type cells cultured in medium prepared with 1X or 4X threonine and 10% glucose (I) or 2% glucose (J).

Figure 5. Replication stress and senescence in budding yeast cells. A. Coordinated downregulation of growth signaling pathways in cells in stationary phase reduces replication stress and promotes longevity. B. Downregulation of some, but not all growth signaling pathways when nutrients are depleted induces replication stress that causes senescence and cell death. See text for details.

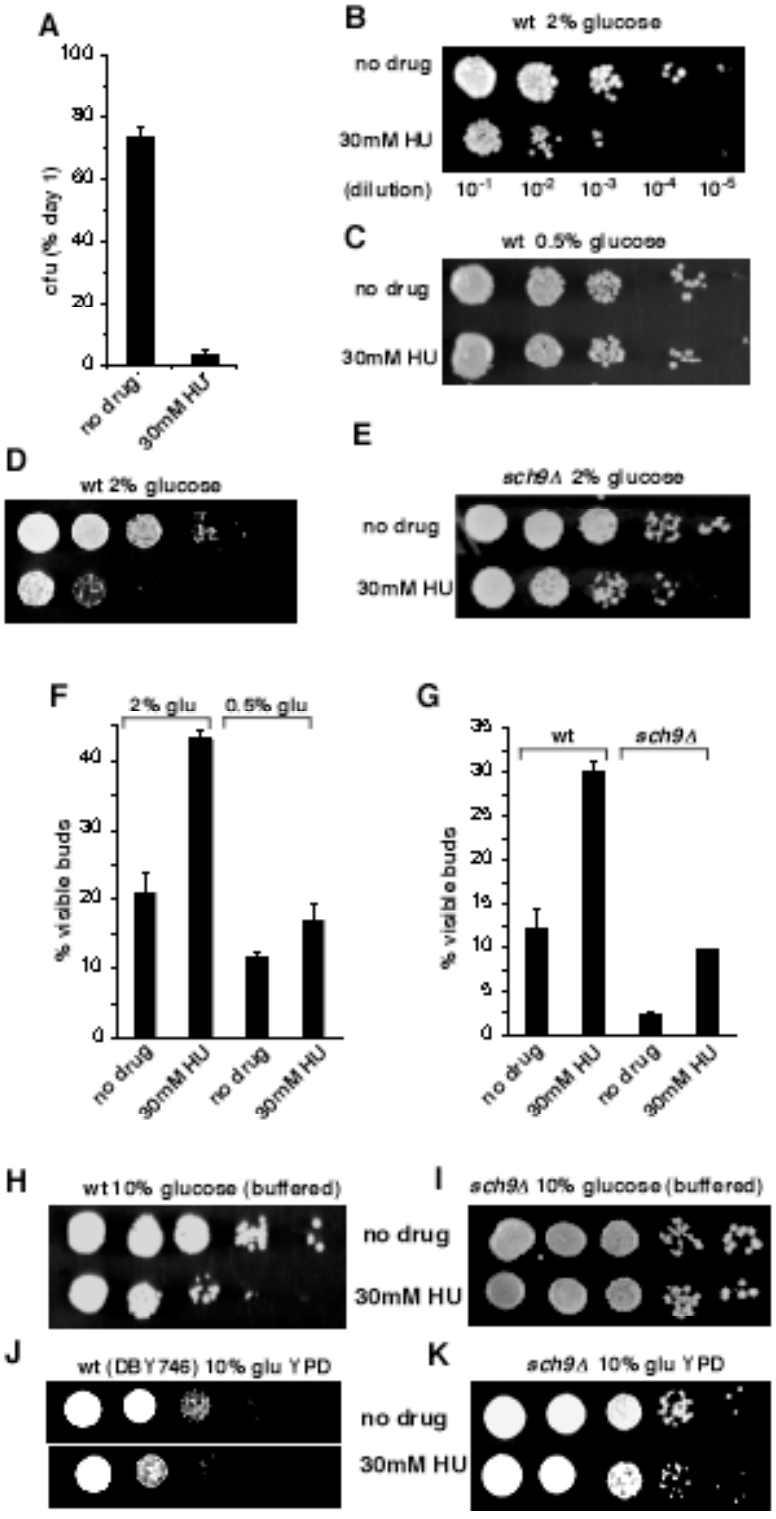
A
cfu (% day 1)
100
80
60
40
20
0
no drug
30mM HU
B
wt 2% glucose
no drug
30mM HU
(dilution)
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}
C
wt 0.5% glucose
no drug
30mM HU
D
wt 2% glucose
E
sch9Δ 2% glucose
no drug
30mM HU
F
2% glu
0.5% glu
% viable buds
40
30
20
10
0
no drug
30mM HU
no drug
30mM HU
G
wt
sch9Δ
% viable buds
30
25
20
15
10
5
0
no drug
30mM HU
no drug
30mM HU
H
wt 10% glucose (buffered)
I
sch9Δ 10% glucose (buffered)
no drug
30mM HU
J
wt (DBY746) 10% glu YPD
K
sch9Δ 10% glu YPD
no drug
30mM HU

Figure 1.

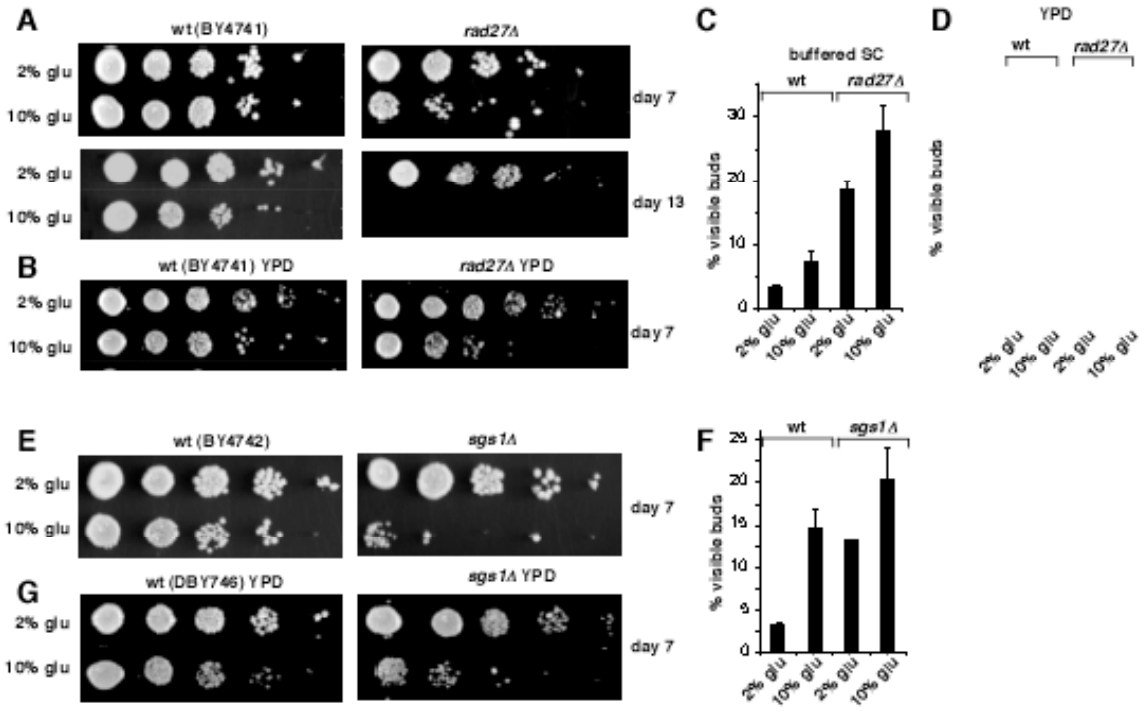
A
wt(BY4741)
rad27Δ
2% glu
10% glu
day 7
2% glu
10% glu
day 13
B
wt (BY4741) YPD
rad27Δ YPD
2% glu
10% glu
day 7
C
buffered SC
wt
rad27Δ
% visible buds
2% glu
10% glu
2% glu
10% glu
D
YPD
wt
rad27Δ
% visible buds
2% glu
10% glu
2% glu
10% glu
E
wt (BY4742)
sgs1Δ
2% glu
10% glu
day 7
G
wt (DBY746) YPD
sgs1Δ YPD
2% glu
10% glu
day 7
F
wt
sgs1Δ
% visible buds
2% glu
10% glu
2% glu
10% glu

Figure 2.

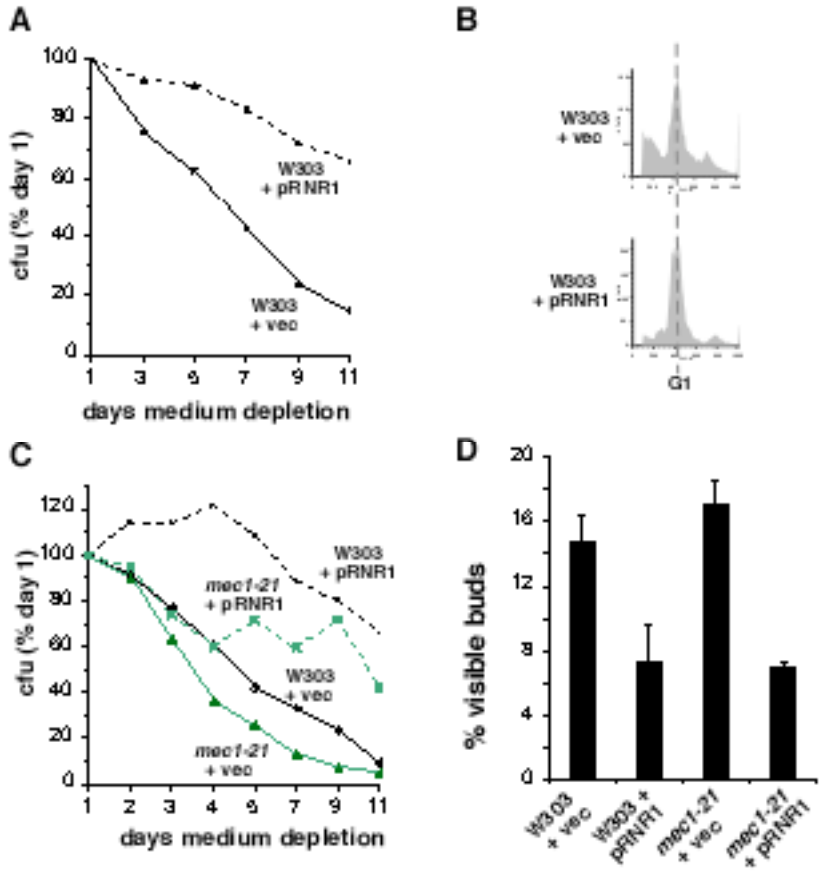
A
cfu (% day 1)
100
80
60
40
20
0
1
3
5
7
9
11
days medium depletion
W303
+ pRNR1
W303
+ vec
B
W303
+ vec
W303
+ pRNR1
G1
C
cfu (% day 1)
120
100
90
80
60
40
20
0
1
2
3
4
5
7
9
11
days medium depletion
W303
+ pRNR1
mec1-21
+ pRNR1
W303
+ vec
mec1-21
+ vec
D
% visible buds
20
16
12
8
4
0
W303
+ vec
W303 +
pRNR1
mec1-21
+ vec
mec1-21
+ pRNR1

Figure 3.

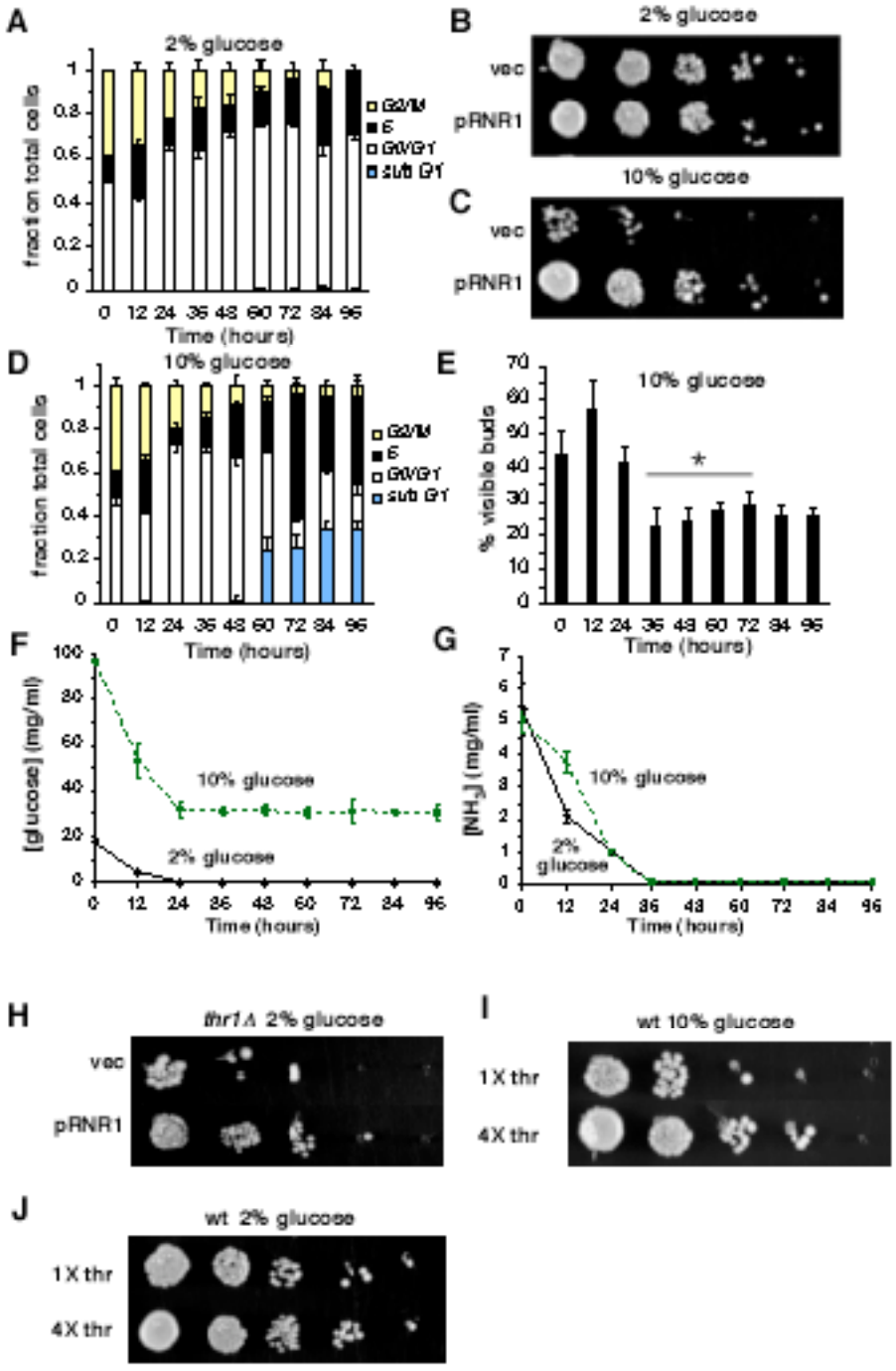
A
2% glucose
fraction total cells
G2/M
S
G0/G1
sub G1
Time (hours)
B
2% glucose
vec
pRNR1
C
10% glucose
vec
pRNR1
D
10% glucose
fraction total cells
Time (hours)
E
10% glucose
% visible buds
Time (hours)
F
[glucose] (mg/ml)
10% glucose
2% glucose
Time (hours)
G
[NH3] (mg/ml)
10% glucose
2% glucose
Time (hours)
H
thr1Δ 2% glucose
vec
pRNR1
I
wt 10% glucose
1X thr
4X thr
J
wt 2% glucose
1X thr
4X thr

Figure 4.

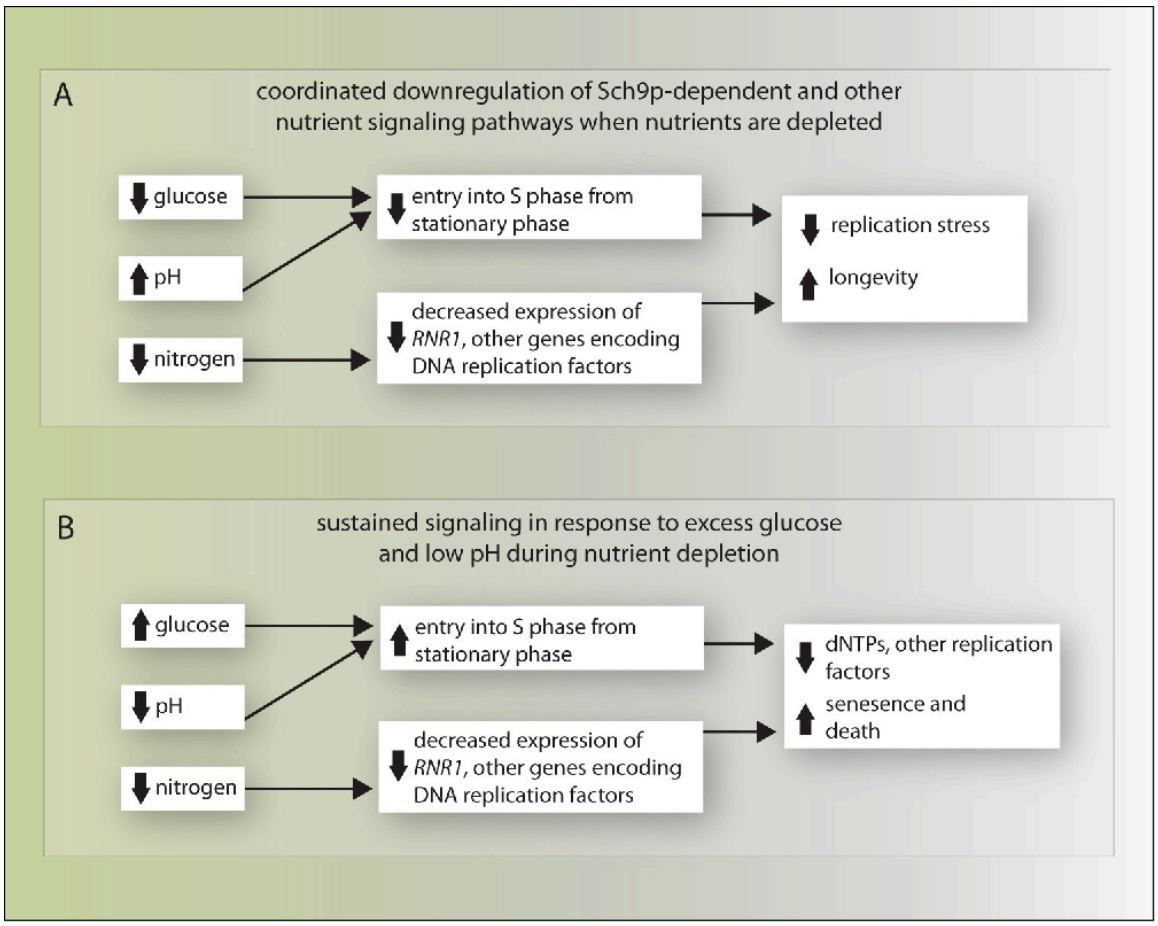
A
coordinated downregulation of Sch9p-dependent and other nutrient signaling pathways when nutrients are depleted
glucose
pH
nitrogen
entry into S phase from stationary phase
decreased expression of RNR1, other genes encoding DNA replication factors
replication stress
longevity
B
sustained signaling in response to excess glucose and low pH during nutrient depletion
glucose
pH
nitrogen
entry into S phase from stationary phase
decreased expression of RNR1, other genes encoding DNA replication factors
dNTPs, other replication factors
senesence and death

Figure 5.

Table 1. Strains and plasmids employed in this study

Strain	Genotype	Source
DBY746	*MATa leu2-3,112 his3Δ1 trp1-2889 ura3-52*	V. Longo
DBY746 *sch9Δ*	*MATa leu2-3,112 his3Δ1 trp1-2889 ura3-52 sch9::URA*	V. Longo
BY4741	*MATa his3Δ leu2Δ met15Δ ura3Δ*	Open Biosystems
BY4741 *rad27Δ*		ATCC
BY4741 *sgs1Δ*		ATCC
BY4741 *gal1Δ*		Research Genetics
W303-1A	*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3 can1-100*	Bruce Stillman
Y604 (W303 *mec1-21*)	*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3 can1-100 mec1-21*	
Plasmids	**Description**	**Source**
YEP24	2μ based *URA3* selectable shuttle vector	ATCC
YEP24-RNR1	RNR1 cloned with its native promoter into YEP24	Elizabeth Vallen