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The Mother Enrichment Program: A Genetic System for Facile Replicative Life Span Analysis in *Saccharomyces cerevisiae*

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ABSTRACT

The replicative life span (RLS) of *Saccharomyces cerevisiae* has been established as a model for the genetic regulation of longevity despite the inherent difficulty of the RLS assay, which requires separation of mother and daughter cells by micromanipulation after every division. Here we present the mother enrichment program (MEP), an inducible genetic system in which mother cells maintain a normal RLS—a median of 36 generations in the diploid MEP strain—while the proliferative potential of daughter cells is eliminated. Thus, the viability of a population over time becomes a function of RLS, and it displays features of a survival curve such as changes in hazard rate with age. We show that viability of mother cells in liquid culture is regulated by *SIR2* and *FOB1*, two opposing regulators of RLS in yeast. We demonstrate that viability curves of these short- and long-lived strains can be easily distinguished from wild type, using a colony formation assay. This provides a simplified screening method for identifying genetic or environmental factors that regulate RLS. Additionally, the MEP can provide a cohort of cells at any stage of their life span for the analysis of age-associated phenotypes. These capabilities effectively remove the hurdles presented by RLS analysis that have hindered *S. cerevisiae* aging studies since their inception 50 years ago.

THE budding yeast *Saccharomyces cerevisiae* is a popular model system for studying fundamental processes of cellular aging (reviewed in Steinkraus *et al.* 2008). Analyses over the past 50 years have led to the idea that budding yeast can be used to study three types of cellular aging. Replicative aging describes the division potential of individual cells and relies on the asymmetric cell divisions of budding yeast that yield distinct mother and daughter cells. Replicative life span (RLS) is defined as the number of times an individual cell divides before it undergoes senescence (Mortimer and Johnston 1959). Chronological aging describes the capacity of cells in stationary phase (analogous to G_0 in higher eukaryotes) to maintain viability over time, which is assayed by their ability to reenter the cell cycle when nutrients are reintroduced (Longo *et al.* 1996). Finally, budding yeast have been used to study clonal senescence, which is analogous to the Hayflick limit imposed on mammalian tissue culture cells and characterized by a finite number of times a population of cells can divide. Although wild-type yeast populations do not senesce, this phenomenon has been observed in mutant strains such as those lacking telomerase components (Lundblad and Szostak 1989; Singer and Gottschling 1994).

While genetic screens have been applied to examine clonal and chronological aging (Lundblad and Szostak 1989; Powers *et al.* 2006; Murakami *et al.* 2008), they have been limited in their application to studying replicative aging (Kaeberlein and Kennedy 2005; Kaeberlein *et al.* 2005b). This limitation arises from the arduous nature of isolating replicatively aged yeast cells. The current "gold standard" for isolating aged mother cells is by micromanipulation, where daughter cells are counted and removed after every division (Park *et al.* 2002). Although micromanipulation is currently the only method capable of accurately measuring RLS in yeast, it is severely constrained by the small number of cells that can be analyzed. Thus, genetic analysis of the regulation of RLS has been limited to a candidate gene approach (reviewed in Steinkraus *et al.* 2008).

True genetic analysis of RLS will require large populations of aged cells. However, there are two confounding issues that make isolation of aged individuals difficult. First, single-cell pedigree analysis has shown that age-associated phenotypes, such as replicative life span potential, segregate asymmetrically between mother and daughter cells, rendering age-associated phenotypes nonheritable (Egilmez and Jazwinski 1989; Kennedy *et al.* 1994). Thus, daughter cells are generally "reset" to a young state with every generation. Second, when age is measured in terms of cell divisions, an unfractionated population is predominately young. The fraction of the population at an age of *n* cell divisions

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is $\sim 1/2^n$. Individual cells that reach the median RLS, which is ~26 generations for haploid cells of the S288C strain background (Kaeberlein *et al.* 2005a), represent an insignificant fraction of the total population. In fact, it is unlikely that any cell reaches such an advanced age because nutrient depletion will limit the division potential of the population (Dickinson and Schweizer 1999).

As an alternative to micromanipulation, methods were developed to isolate aged cells from liquid cultures (Smeal *et al.* 1996; Sinclair and Guarente 1997; Chen and Contreras 2007). However, due to the exponential growth of progeny cells, these populations are technically limited to 7–12 generations before nutrient depletion interferes with replicative aging. While sequential rounds of growth and purification are possible, the inability to continuously follow an undisturbed cohort of cells prevents the measurement of RLS by these methods. Instead, purification methods are primarily used for the examination of molecular changes associated with aging cells. Unfortunately, low yields and loss of viability due to purification methods diminish their utility for analyzing phenotypes that affect cells of advanced age. As an alternative to purification from natural populations, a strategy to genetically regulate the replicative capacity of daughter cells and avoid the limits imposed by exponential growth has been described (Jarolim *et al.* 2004). While this system effectively prevents division of daughter cells, it unintentionally decreases the median RLS of mother cells to four cell divisions, thus restricting its usefulness.

Here we describe the development of a novel genetic selection against newborn daughter cells, the "mother enrichment program" (MEP), which restricts the replicative capacity of daughter cells while allowing mother cells to achieve a normal RLS. We demonstrate that upon induction of the selection, the viability of MEP strains growing in liquid culture is determined by the RLS of the initial population of mother cells. MEP cultures therefore allow the comparison of RLS between strains without the need for micromanipulation. Additionally, because MEP cultures are not subject to nutrient limitation, single-step affinity purification of aged cells can be achieved at any point during their life span. Together, these capabilities substantially resolve the technical hurdles that have made replicative aging studies in *S. cerevisiae* exceptionally challenging.

MATERIALS AND METHODS

All strains used in this work are in the S288C (BY) background. Strain and plasmid construction is described in supporting information, File S1. Oligonucleotides, strains, and plasmids described in this work are listed in Table S1, Table S2, and Table S3, respectively.

Fluctuation analysis: Twenty parallel 1-ml YEPD cultures of UCC5181 (haploid) and UCC5185 (diploid) MEP were grown to saturation overnight. Samples of each culture (~2×10^6 cells) were plated to 150-mm YEPD + 1 μM estradiol plates and incubated at 30°. Visible colonies after 48 hr were scored as estradiol-resistant mutants. Mutation rates were calculated and corrected for sampling error using the MSS-maximum-likelihood estimator method from the FALCOR fluctuation analysis calculator (http://www.mitochondria.org/protocols/FALCOR.html) (Hall *et al.* 2009).

Life span measurement by micromanipulation: Cells from logarithmically growing YEPD liquid cultures were applied to YEPD plates and incubated at 30°. Founder cells were selected for life span analysis by selecting the first daughter of a newborn daughter cell. Cells were monitored for cell divisions every 2 hr until lysis. To measure RLS on estradiol-containing YEPD plates, a portion of agar was cut away and replaced by an island of fresh YEPD agar without estradiol (Figure S1). Founding cells were isolated as described above on the YEPD island. For mother cell analysis, founding cells were allowed to divide twice before being transferred to the estradiol-containing portion of the plate for life span analysis. For daughter cell analysis, founding cells were transferred immediately to the estradiol-containing portion of the plate for life span analysis.

Liquid aging assay: Cells grown overnight in YEPD medium were diluted 1:50 to inoculate fresh YEPD cultures and incubated at 30° for 3 hr. Cultures were counted and used to inoculate 25 ml of prewarmed YEPD to a cell density of 2×10^3 cells/ml. 17β-Estradiol (Sigma, St. Louis) was added to a final concentration of 1 μM and cultures were incubated at 30° for 120 hr. At each time point, samples were taken and cells were pelleted by centrifugation at 800 × *g*. All but 100 μl of the supernatant was removed; cells were washed once with 1 ml of fresh YEPD and pelleted again. Cells were resuspended in 500 μl YEPD and plated to YEPD. Colonies were counted after 3 days incubation at 30°. Viability was calculated as CFUs per milliliter and expressed as percentage of viability compared to CFUs per milliliter at the 0- or 4-hr time points. When comparing viability between *MET15* and *met15*Δ strains, each strain was inoculated to 1×10^3 cells/ml and treated as described above, except plating was done to $Pb(NO_3)_2$ media (Cost and Boeke 1996) and colony color was scored after 5 days incubation at 30°.

Purification of aged cells: Cells were harvested from logarithmically growing YEPD cultures, washed twice in PBS, and resuspended in PBS at 5×10^7 cells/ml. Sulfa-NHS-LC-biotin (Pierce Chemical, Rockford, IL) was added to a final concentration of 3 mg/ml and incubated at room temperature protected from light on a Labquake (Thermolyne) for 30 min. Cells were pelleted, washed three times with YEPD, and used to inoculate a 500-ml YEPD culture at 2×10^4 cells/ml. Cultures were incubated 2 hr at 30° prior to the addition of estradiol to a final concentration of 1 μM. At each time point, 100 ml of the culture was harvested; cells were pelleted by centrifugation and washed twice in PBS. Cells were resuspended in 500 μl PBS and fixed with 4% paraformaldehyde for 10 min. After fixation, cells were washed twice with PBS, resuspended in 500 μl PBS, and incubated with 50 μl streptavidin-coated magnetic beads (MicroMACS, Miltenyi Biotec) for 30 min at room temperature on a Labquake. Cells were pelleted and washed twice with PBS and then resuspended in 8 ml PBS and loaded onto a LS MACS column (Miltenyi Biotec). Columns were washed with 8 ml PBS, removed from the magnetic field, and eluted with 8 ml PBS. Purified cells were pelleted and resuspended in 500 μl PBS + 1 μl fluorescein–avidin (Pierce) and incubated at room temperature for 90 min on a Labquake. Calcofluor (Sigma) was added to a concentration of 0.1 mg/ml and incubated for 5 min before cells were pelleted and washed three times in PBS. Final stained cell pellets were resuspended in 100 μl PBS and spotted to polylysine-coated microscope slides. Individual

fluorescein-avidin stained cells were imaged with a Deltavision microscope for bud scar counting.

RESULTS

A Cre-*lox* mediated selection against daughter cells: To generate a population of replicatively aged *S. cerevisiae* cells, we focused on creating a system that would allow a mother cell to divide, but eliminate that potential in her daughters. We imposed several requirements on this system. First, it must accurately distinguish between mother and daughter after each cell division to avoid prematurely limiting the mother's life span. Second, it must effectively prevent daughter cells from producing progeny. This must be accomplished within a single cell division, before the daughter cell develops into a mother cell upon exiting its first mitosis. Finally, the selection must be conditional, so that a strain can be propagated. The MEP, outlined in Figure 1, achieves the stated goals by using Cre-*lox* recombination to disrupt two essential genes, *UBC9* and *CDC20*, in daughter cells (Figure 1A). We chose disrupton of essential genes as our selection mechanism to allow a combinatorial approach: By combining multiple targets we could improve the stringency of the selection. *UBC9* encodes the SUMO-conjugating enzyme, while *CDC20* encodes an activator of the anaphase-promoting complex (APC). Both are required for the degradation of mitotic cyclins and other targets vital to cell cycle progression (Sethi *et al.* 1991; Seufert *et al.* 1995; Dieckhoff *et al.* 2004), so eliminating these genes by recombination results in daughter cells that permanently arrest in M-phase. Expression of the Cre recombinase is restricted by a daughter-specific promoter derived from *SCW11* (P_{SCW11}) (Figure 1A). Daughter-specific expression from P_{SCW11} is regulated by the transcription factor Ace2, which is asymmetrically distributed to daughter cell nuclei prior to cytokinesis (Colman-Lerner *et al.* 2001; Doolin *et al.* 2001). Cre activity is also post-transcriptionally regulated by fusion to the estradiol-binding domain (EBD) of the murine estrogen receptor (Cre-EBD78). The EBD sequesters the fusion protein in the cytoplasm until estradiol is introduced, at which point the fusion protein is transported into the nucleus where Cre can act upon its *loxP* DNA substrates (Figure 1B) (Cheng *et al.* 2000).

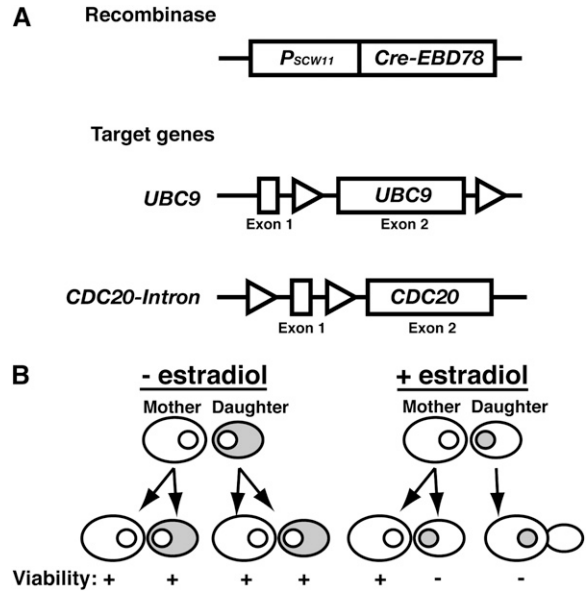

Figure 1.—Components of the MEP. (A) Diagram of the MEP components. Cre-EBD78 is a novel version of a fusion protein between the Cre recombinase from bacteriophage P1 and the estrogen-binding domain of the murine estrogen receptor that is strictly dependent on estradiol for activity (see materials and methods). P_{SCW11}-*cre-EBD78* is integrated at the *ho* locus and specifically expressed in newborn cells, using a daughter-specific promoter derived from *SCW11*. Target genes were constructed at their endogenous loci by introduction of *loxP* sites (triangles). For *UBC9*, recombination between the *loxP* sites removes exon 2, representing 92% of the coding region. For *CDC20*, an intron derived from *ACT1* and containing a *loxP* site was introduced to generate a 42-bp exon 1. Recombination between *loxP* sites removes exon 1, leaving the first in-frame start codon at methionine 197, which would generate a nonfunctional protein. (B) Illustration of the expected localization of Cre-EBD78 (shading) in response to estradiol. P_{SCW11}-*cre-EBD78* expression is restricted to the G_1 phase of daughter cells. In the absence of estradiol the fusion protein is sequestered in the cytoplasm. Upon ligand binding, the fusion protein is translocated into the nucleus (small circle), where it can act on *loxP* target sites.

To test the inducible nature of the MEP, strains were placed on media with or without estradiol (Figure 2). Cells formed robust colonies within 2 days in the absence of estradiol. By contrast, growth was severely limited in the presence of estradiol. The estradiol effect was dependent upon the presence of the MEP: Strains lacking P_{SCW11}-*cre-EBD78* or *loxP* targets grew normally in the presence of estradiol (Figure 2 and data not shown). Examination of individual cells on estradiol plates revealed that they formed microcolonies of large dumbbell-shaped cells (data not shown). This is consistent with an M-phase arrest phenotype caused by the loss of either *UBC9* or *CDC20* (Sethi *et al.* 1991; Dieckhoff *et al.* 2004). Because Cre-*lox* recombination is a reversible reaction (Sauer and Henderson 1990), we did not expect selection based on a single target to be absolute. Consistent with this idea, we observed a synergistic growth defect on estradiol in cells that contained both target genes (Figure 2). Taken together, these results indicate that the MEP is effectively activated by estradiol and that *UBC9* and *CDC20* are efficiently disrupted by the Cre recombinase.

In the course of our analyses, we occasionally encountered fast-growing cells that were resistant to estradiol-induced arrest (Figure 2). When these estradiol-resistant mutants were generated in liquid culture, their progeny quickly saturated the culture and foiled the intended purpose of the MEP. We used fluctuation analysis

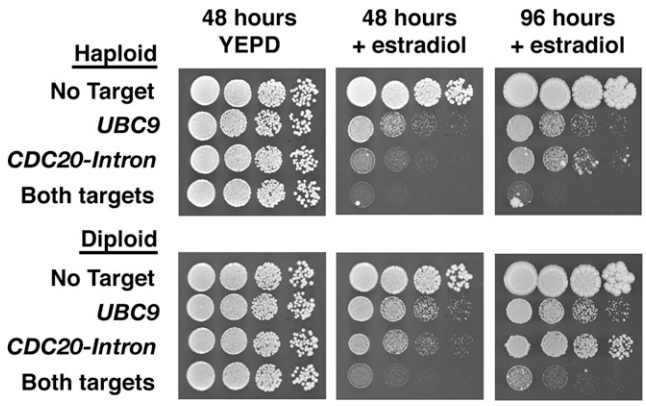

FIGURE 2.—MEP induction by estradiol. Tenfold serial dilutions of haploid and diploid MEP strains were applied to YEPD plates with or without 1 μM estradiol. Plates were incubated at 30° and photographed at the times indicated. Each strain carries P_{SCW11}-*cre-EBD78* and the indicated *loxP* target genes.

(ROSCHE and FOSTER 2000) to determine the rate at which estradiol-resistant mutations appeared in a population of cells to estimate an upper limit of the number of cells that can be analyzed with the MEP. Logarithmically growing haploid cells produced resistant mutations at a rate of 1.4×10^{-6} per cell division. This rate is ~10-fold higher than expected for forward mutation of a single locus (*i.e.*, *cre-EBD78*) (KUNZ *et al.* 1998) and is consistent with previous reports that identified multiple loci in yeast required for EBD function (GILBERT *et al.* 1993; KRALLI *et al.* 1995; MCEWAN 2001). In diploids, resistant mutations arose at a rate of 1.4×10^{-8} per division, indicating that many of the mutations were recessive. The upper boundary represented by the 95% confidence interval of these measurements (1.9×10^{-6} for haploids and 3.4×10^{-8} for diploids) provides an estimate that can be used to calculate the maximum number of cells that should be used to inoculate a liquid "aging" culture without risk of contamination by an estradiol-resistant mutant.

Validation of the MEP by single-cell analysis: To examine the specificity of the MEP, micromanipulation was used to track the fate of mothers and daughters in the absence or presence of estradiol. The median RLS of the diploid MEP strain UCC5185 in the absence of estradiol was not significantly different from that of a diploid parent strain (UCC8600) lacking the MEP components (36 *vs.* 37 generations, Figure 3A). This indicates that components of the MEP do not affect the RLS of mother cells in the absence of estradiol.

The RLSs of cells on media containing estradiol were also measured. Typically an RLS measurement begins with newborn daughter cells that, after a single division, become mother cells. A modification of the RLS protocol was made to measure the RLS of naive (*i.e.*, no prior exposure to estradiol) mother and daughter cells by constructing plates that contained an island lacking estradiol and a larger zone containing 1 μM estradiol to perform the RLS analysis (Figure S1).To generate naive mother cells, newborn daughters were allowed to complete two divisions on the island lacking estradiol before transfer to the RLS zone. Naive mother cells displayed either of two responses upon exposure to estradiol: Approximately 15% of the mothers showed a terminal M-phase arrest phenotype and died quickly (data not shown), which is consistent with these young mothers having experienced elimination of *CDC20* or *UBC9* by Cre-*lox* recombination. This response to estradiol was not observed in the parent strain UCC8600 that lacks the MEP (median RLS = 35, n = 59, data not

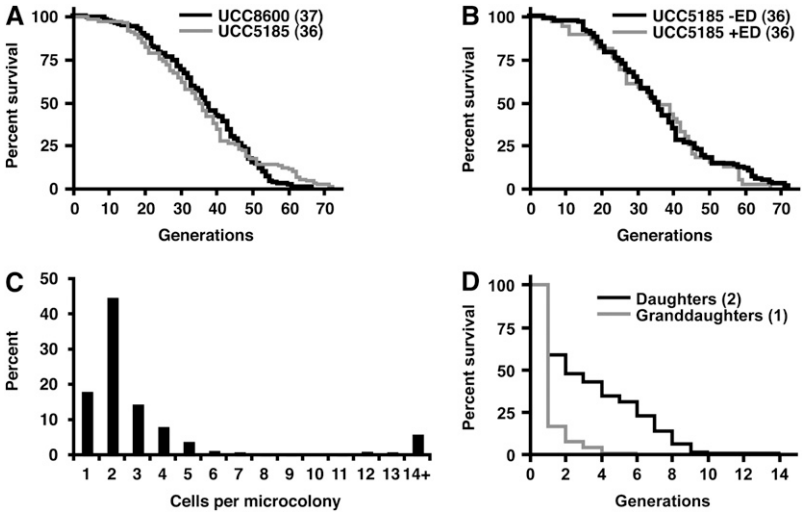

FIGURE 3.—Validation of the MEP by microdissection. (A) Survival curves of diploid parent (UCC8600, n = 147, median = 37) and MEP (UCC5185, n = 90, median = 36) strains generated by micromanipulation on YEPD without estradiol. Median life spans are not significantly different (Mann–Whitney nonparametric t-test, P = 0.3904). (B) Survival curves of MEP strain UCC5185 generated by micromanipulation on YEPD with 1 μM estradiol (+ED, n = 39, median = 36) or without it (−ED, n = 90, median = 36). The survival curve for +ED includes only naive mother cells that did not exhibit rapid M-phase arrest upon transfer to estradiol. (C) The distribution of the number of cells per microcolony generated by daughter cells born during +ED RLS analysis presented in B. (D) Survival curves of UCC5185 naive daughters (n = 118, median = 2) transferred to YEPD + 1 μM estradiol and their resulting progeny (granddaughters, n = 269, median <1). The two leaky daughters have been censored from the analysis. In each case, the RLSs of cells that failed to complete a single division were scored as 1 generation.

shown), indicating that these early deaths were not a general effect of estradiol on yeast. We postulate that M-phase arrest in naive mothers—individuals that were recently daughter cells—is the result of perdurance of Cre-EBD mRNA or protein. For the other 85% of mother cells, no terminal M-phase arrest was observed and these mothers had life spans that were indistinguishable from those of cells grown in the absence of estradiol (median RLS = 36 generations, Figure 3B), indicating that the majority of mother cells were unaffected by estradiol throughout their life spans. Furthermore, this result suggests that there is no loss of mother–daughter asymmetry in expression from P_{SCW11} in aging cells.

By contrast, the effect of estradiol on the daughters born to this cohort of mother cells was profound. All 472 daughters born on estradiol had a severely limited replicative capacity and formed microcolonies of M-phase arrested cells. The majority failed to complete more than a single division (Figure 3C and Figure S2). We saw a negative correlation between age of the mother cell and division potential of the daughter (Nonparametric correlation, $P < 0.0001$) (Figure S3), supporting the conclusion that the effectiveness of the MEP does not deteriorate with age. Thus, upon induction of the MEP, most mother cells live a normal life span in the presence of estradiol, while the majority of their daughters succumb to Cre-mediated recombination and arrest within a single generation.

Approximately 8% of daughter cells on estradiol formed microcolonies of >10 M-phase arrested cells. These microcolonies could be the result of phenotypic lag in the MEP selection; it may take several cell divisions to effectively deplete Cdc20 or Ubc9 after deletion of the genes in some daughter cells. Alternatively, microcolonies could be the result of individuals that escaped the MEP selection and became productive mother cells, a phenomenon we term "leakiness." To distinguish between these possibilities, we determined the fate of naive daughter cells upon exposure to estradiol. Naive daughter cells were isolated on an island lacking estradiol and immediately transferred to the RLS zone. Of 120 naive daughter cells transferred, 29 arrested in M-phase and never divided. The remaining 91 cells completed their first division and developed into mother cells. With the exception of 2 individuals, these newly formed mothers displayed an aberrant cell cycle, with an average division time of 3.1 hr and a prolonged G_2–M phase. Their median RLS was two generations and ended with a terminal M-phase arrest (Figure 3D), indicating that these cells were succumbing to Cre-*lox* recombination. We monitored the fate of progeny cells ("granddaughters") born to these mothers and found 61% of granddaughters arrested without completing a single division (median RLS <1 generation, maximum = 4, $n = 269$, Figure 3D). We conclude that the vast majority of daughter cell divisions are the result of phenotypic lag rather than leakiness.

Two naive daughter cells of 120 (1.7%) failed to display an M-phase arrest upon exposure to estradiol and became mother cells that divided at a normal rate and achieved normal life spans. These cells produced daughters that responded to estradiol and quickly arrested, indicating that they were "leaky" mothers that had escaped the MEP selection rather than estradiol-resistant mutants. At this rate of leakiness, we expect an average mother cell to generate 0.6 viable progeny throughout her life span. However, the leakiness rate is likely to be lower in daughters that are born in the presence of estradiol. For example, in the experiment where naive daughters were transferred to estradiol, 41% arrested without completing >1 division (Figure 3D). In contrast, 63% of daughter cells born on estradiol media during the measurement of naive mother cell RLS arrested without completing >1 division (Figure 3C). This indicates that the replicative capacity of daughter cells born in the presence of estradiol is very low.

Aging cells in liquid cultures via the MEP: Given its successful performance on solid medium, we next examined the effectiveness of the MEP in liquid culture, where it has its greatest potential as a resource for studying yeast replicative aging. To avoid generation of estradiol-resistant mutants during the experiment, 25 ml YEPD cultures containing 1 μM estradiol were innoculated at a cell density of 2×10^3 cells/ml. We found that cultures at this starting cell density do not exceed $\sim 10^6$ cells/ml after 120 hr of incubation and thus should not impinge on the growth of mother cells through nutrient depletion (data not shown). Samples of cells were harvested at regular intervals, washed, and plated to media lacking estradiol. Formation of colonies on these plates demonstrated that the MEP was reversible; once estradiol was removed, if a mother cell still had replicative capacity, her daughters divided normally to form a colony.

MEP cells demonstrated a rapid loss of viability within the first 4 hr of exposure to estradiol (Figure 4A). This was expected since daughters constituted approximately half of the initial inoculum. These daughter cells are immediately arrested by estradiol, leaving only a cohort of mother cells in the viable population. After arrest of the daughter population, there was a 16-hr period with little change in viability, demonstrating a low hazard rate of death in young mothers (Machin 2006). This phase was followed by a rapid decline in viability over ~48 hr, indicating an increasing hazard rate in the aging population. This change in the hazard rate in liquid culture parallels the changes in hazard rate observed when measuring the RLS of MEP strains on solid media containing estradiol (Figure 3A and Figure S4) and is stereotypical for survival curves in yeast and other species (Pohley 1987; Kennedy *et al.* 1994). These similarities suggest that cells aging in liquid culture exhibit the same pattern of age-associated

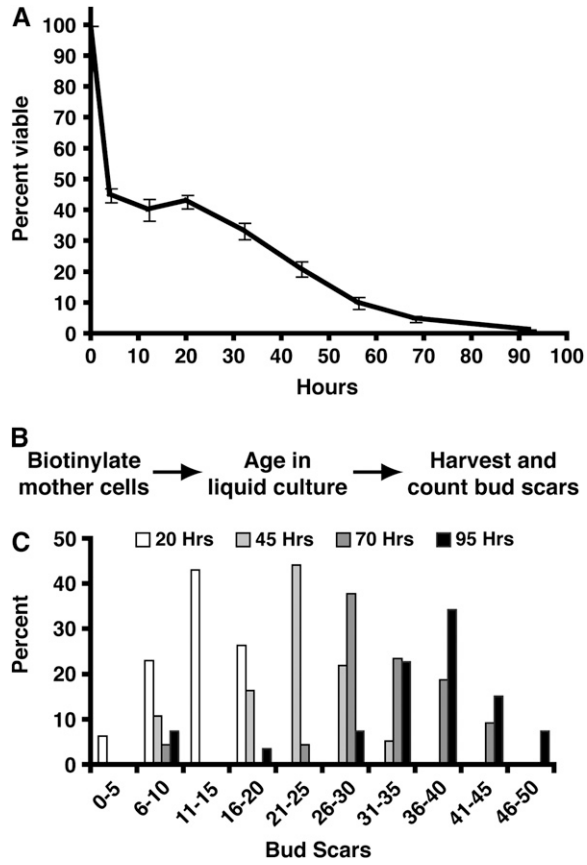

Figure 4.—Aging cells in liquid culture with the MEP. (A) Viability curve of diploid MEP strain UCC8848 in liquid YEPD with 1 μM estradiol, normalized to the 0-hr time point. Viability as represented by CFUs per milliliter was monitored by harvesting samples at the indicated time points and washing and plating cells to media lacking estradiol. Values represent the median of 12 independent cultures with 95% confidence intervals indicated by error bars. (B) A flow chart of steps for purifying and measuring age of founding mother cells from a liquid MEP culture. LHC-Biotin was covalently cross-linked to the cell walls of logarithmically growing UCC5185 cells. Cells were incubated in the absence of estradiol for 2 hr to allow all labeled cells to become mothers, and estradiol was then added to a final concentration of 1 μM. At intervals, samples were removed for purification with streptavidin-coated magnetic beads and stained with fluorescein-avidin and calcofluor white for bud scar counting. (C) The distribution of bud scars on purified cells is presented after binning into five-generation intervals: 20 hr, $n = 30$, mean = 12; 45 hr, $n = 18$, mean = 22; 70 hr, $n = 21$, mean = 31; 95 hr, $n = 26$, mean = 35.

changes in hazard rate as individual cells aging on solid media.

We next determined whether individual mother cells in a liquid MEP culture continued to divide throughout the experimental time course. Many of the cell wall components that a cell is born with remain intact throughout its life span (Ballou 1982). We took advantage of this attribute and cross-linked biotin to the cell wall of the starting population of mother cells (Smeal *et al.* 1996). The biotin tag permitted sampling and purification of founder cells at intervals throughout the time course. Purified cells were stained with fluorescein-avidin, to confirm that an individual cell was part of the initial mother population, and with calcofluor white to count bud scars to determine replicative age (Figure 4B) (Park *et al.* 2002). Both the median and the maximum age of the founding mother population increased throughout the time course and were similar to values obtained by micromanipulation (Figure 4C and data not shown). Thus, individual mothers can achieve a normal RLS in the presence of estradiol in liquid cultures.

The MEP can distinguish between mutants with altered RLS in liquid cultures: The results presented above indicate that aging of a MEP strain in liquid culture recapitulates the characteristics of replicative aging on solid medium. To further examine the relationship between RLS and viability in liquid culture, we constructed MEP strains deleted for either *SIR2* (UCC8836) or *FOB1* (UCC526), which are well characterized for their effects on yeast RLS (Defossez *et al.* 1999; Kaeberlein *et al.* 1999; reviewed in Steinkraus *et al.* 2008). Consistent with previous studies using haploid cells (Kaeberlein *et al.* 2005a), these mutations affect RLS on solid media in the diploid MEP background (Figure 5A, −69% (*sir2*Δ) and +56% (*fob1*Δ) change in median RLS compared to UCC5185). To compare viability of mutant strains to wild type within a single culture, we took advantage of a colony color phenotype in *MET15* mutants (Cost and Boeke 1996). First, we constructed diploid MEP strains that were homozygous *MET15* or *met15*Δ. Estradiol cultures were inoculated with an equal number of cells of each type, and both viability and *MET15* status were monitored by plating to solid media containing Pb^{+2}. We observed no effect of the *MET15* allele on viability under these conditions (data not shown). Next, we compared a wild-type *MET15* diploid with a *sir2*Δ *met15*Δ strain. We observed a rapid decline in viability in the *sir2*Δ strain, with no evidence of a low hazard of death in young cells (Figure 5B). This was consistent with the hazard rate observed by measuring RLS on solid media (Figure 5A). The reduction of ~60% in median viability of the *sir2*Δ strain compared to wild type was also consistent with the reduction in RLS observed on solid media. These results indicate that viability in liquid culture can easily distinguish between strains with normal and short life spans.

We next examined the viability in liquid culture of a long-lived *fob1*Δ strain. We found that the *fob1*Δ strain showed an increase in median viability in liquid culture of ~21% compared to a cocultured wild-type strain (Figure 5C). While the magnitude of this increase was smaller than that observed on solid media, the *fob1*Δ strain yielded significantly higher viability than wild type at each time point between 32 and 68 hr (paired *t*-test, $P < 0.01$). These results indicate that viability of MEP

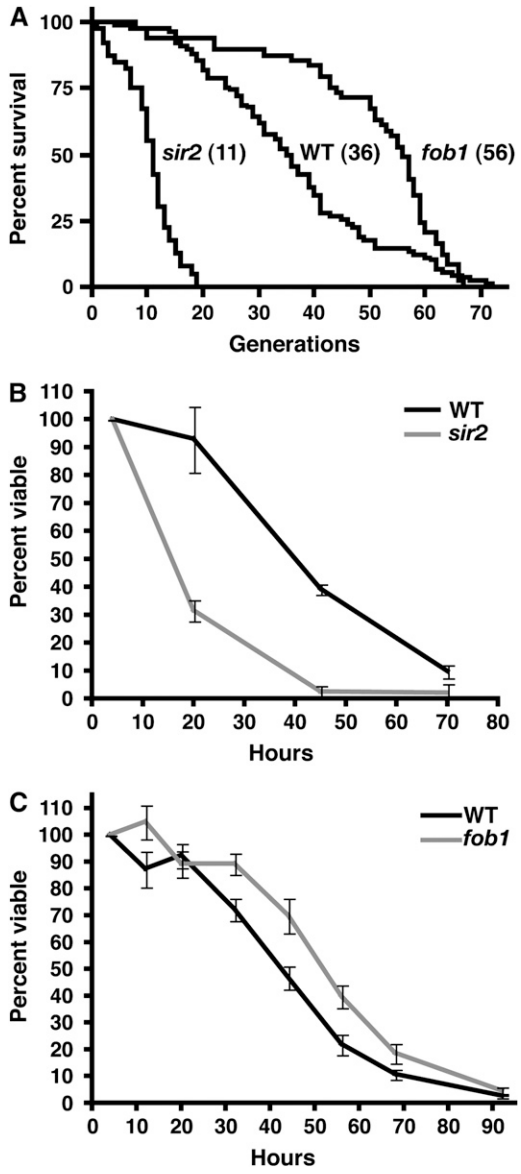

Figure 5.—Differences in RLS measured with the MEP. (A) Survival curves of diploid MEP strains generated by micromanipulation on YEPD without estradiol (wild-type UCC5185, $n = 90$, median = 36; *fob1*Δ UCC526, $n = 49$, median = 56; *sir2*Δ UCC8836, $n = 40$, median = 11). (B) Viability of wild-type *MET15* (UCC8848) *vs. sir2*Δ *met15*Δ (UCC8849) diploid MEP strains in liquid culture measured as described in Figure 4A. Values represent the median of three independent cultures normalized to the 4-hr time point, with error bars indicating 95% confidence intervals. (C) Viability of wild-type *vs. fob1*Δ diploid MEP strains in liquid culture. Values represent the median of six wild-type *MET15* (UCC8848) *vs. fob1*Δ *met15*Δ (UCC8850) and six wild-type *met15*Δ (UCC8861) *vs. fob1*Δ *MET15* (UCC8863) cultures, with error bars indicating 95% confidence intervals.

strains in liquid culture can be used to distinguish between strains with normal and long life spans. Additionally, they indicate that life span in liquid culture is regulated by some of the same genetic pathways that regulate RLS on solid media.

DISCUSSION

Benefits of the MEP compared to other systems: The MEP provides a new tool that greatly expands the potential for applying *S. cerevisiae* genetic, biochemical, and cell biological approaches to examine the fundamental processes of replicative aging. Previously, methods for isolating replicatively aged yeast cells for analysis have followed two complementary approaches. The gold standard is based upon following single cells by micromanipulation (Park *et al.* 2002). This method allows the accurate determination of an individual's life span, but only ~100 cells can be followed in an experiment by a single researcher. Micromanipulation is the method by which all genetic analyses of replicative life span have been carried out (Steinkraus *et al.* 2008). However, because of the arduous nature of the assay, most genetic analyses have followed a candidate gene approach; only recently was an unbiased genetic screen attempted (Kaeberlein and Kennedy 2005; Kaeberlein *et al.* 2005b). Given the magnitude of effort required for this screen, it is unlikely to be duplicated with varying environmental conditions or genetic backgrounds—elements that are known to critically affect the aging process (Lin *et al.* 2002; Kaeberlein *et al.* 2005a). The MEP has overcome the limitations of measuring RLS by micromanipulation: It is easy to perform and does not require continuous monitoring. As shown here, it can readily distinguish between genetic alterations that either increase or decrease life span.

The complement to micromanipulation is purification of aged cells from liquid cultures (Smeal *et al.* 1996; Sinclair and Guarente 1997; Chen and Contreras 2007). These methods are not applicable to the measurement of RLS, but instead provide larger populations for the analysis of age-associated phenotypes. Unfortunately, the age of cells in liquid cultures is limited by the exponential proliferation of young progeny cells. Consequently, single-step purification strategies typically yield cells that are only 7–12 generations old. Purification strategies are also confounded by low yields, contamination by daughter cells, and extensive sample processing in PBS or other buffers. While older populations can be obtained through successive rounds of growth and purification, or the addition of a FACS sorting step (Chen and Contreras 2007), the problems facing purification are compounded with each round. The MEP has overcome the limitations imposed by progeny cells by efficiently eliminating daughter cell propagation. Aging of a cohort can be carried out for several days in a single culture that begins at a low cell density (*e.g.*, ~10^3 cells/ml, Figure 4A). Additionally, because the MEP is rapidly reversible upon removal of estradiol, purification of aged cells from their inviable progeny is not necessary for colony-based assays that monitor viability or other age-associated phenotypes.

A similar approach to eliminating daughter cells by restricting *CDC6* expression to mother cells has been described (Jarolim *et al.* 2004). While daughter cell division is effectively prevented, the median RLS of mother cells is reduced by ~75%. This has precluded its use in examining age-associated phenotypes. In contrast, the MEP is successful at preserving the normal RLS of mother cells (Figure 3B).

Limitations of the MEP: The MEP shares limitations common to other genetic selections. First, the individual components of the MEP must be introduced into a strain of interest. The MEP as presented here has been created in the S288C strain background, which is the basis for many comprehensive strain collections that are available [*e.g.*, the nonessential gene deletion set (Winzeler *et al.* 1999)]. With the large molecular and genetic toolkits that have been built upon this strain background, studies of replicative aging in yeast should advance rapidly.

The second limitation is that mutations in MEP strains can arise that allow a mutant cell to avoid the selection. In addition to *cre-EBD78* itself, genes involved in localization of the EBD (reviewed in McEwan 2001) or multidrug resistance (Gilbert *et al.* 1993; Kralli *et al.* 1995) are potential sources of estradiol resistance. We found that estradiol-resistant mutations arise at a rate of 1.4×10^{-6} per cell division in haploid cells and 1.4×10^{-8} per cell division in diploid cells. These rates, adjusted depending on the target age of the final population, can serve as a guide to determine how many cells can be evaluated in an experiment.

The third limitation is based on the observation that while the daughter cells and their progeny arrest irreversibly in M-phase, they do not immediately senesce. They can remain metabolically active in this arrested state, continuing to grow without dividing for ~24 hr before lysis (data not shown). Upon exposure to estradiol, an average daughter cell produced 6.9 progeny cells (daughters and subsequent generations). If an average mother cell generates 36 daughter cells, this translates to ~248 inviable progeny cells during her life span. We have been able to avoid nutrient limitation when measuring RLS by inoculating at low cell density (10^3–10^4 cells/ml). The actual starting density can be adjusted depending on the target age for the experiment; a higher initial inoculum can be used without risk of nutrient depletion when analysis is done after 1 or 2 days of aging.

Comparing RLS to viability in liquid culture: For the past 50 years, RLS in *S. cerevisiae* has been measured using the metric of cell divisions rather than time, on the basis of the observation that perturbations such as temperature change drastically alter division rate without affecting the median number of generations (Muller *et al.* 1980). This metric also introduces an essential element for micromanipulation, since it allows one to interrupt a life span analysis nightly by moving the cells to 4°. Survival curves obtained by this method generally conform to a Gompertz distribution similar to that observed when monitoring survival in protected metazoan populations (Figure 3A and Kennedy *et al.* 1994; Kirkwood 2005).

With the MEP, we show that RLS can now be examined as a function of time. As long as the cell division rate remains constant within a cohort of mother cells, viability in liquid culture will be directly proportional to RLS. Because the MEP avoids fluctuations in temperature and nutrient availability, this assumption is generally true. We showed that with advancing time, mother cells in the original population advance in age (Figure 4C). When the life spans of three strains (wild type, *sir2*Δ, and *fob1*Δ) were examined by micromanipulation and MEP, we found that in both situations the median life span was changed in the same way: *sir2*Δ was much shorter, and *fob1*Δ was significantly longer, than wild type (Figure 5). However, the magnitude of the difference was somewhat smaller when measured with the MEP (*sir2*Δ, −69% *vs.* −60%; *fob1*Δ, +56% *vs.* +21%, for micromanipulation and MEP, respectively).

We offer a few explanations that could contribute to differences between life span measured using micromanipulation *vs.* the MEP. First, there may be intrinsic differences between growth on solid medium and that in liquid that affect the aging process. Second, cell division rate may change with increasing replicative age. Consistent with previous reports, we found that when cells approach the end of their life span on solid media, the time between cell divisions increases (Mortimer and Johnston 1959; Muller *et al.* 1980; and Figure S5, A and B). Differences in how division rates change with age between wild-type and mutant strains could lead to distortion of the MEP viability curves. While cell division rate (in terms of fitness) has been determined for the nonessential yeast gene deletion set for logarithmically growing (young) cells (Deutschbauer *et al.* 2005), age-dependent changes to these rates have not been systematically examined. Finally, the intrinsic cell division rate of mutants may differ, so that the relationship between time and age is not standard between wild-type and mutant strains. While there is no difference in division rate between young wild-type and *fob1*Δ cells (Chen and Contreras 2007 and data not shown), this may be an important consideration when examining other mutations that regulate RLS.

Further potential of the MEP: Restriction of the replicative capacity of progeny cells both eliminates nutrient depletion in liquid cultures and effectively enriches for aging mother cells within a population. Thus, populations of aged cells can be generated at any point in their life span without the need for sequential rounds of purification (Figure 4C). By simply eliminating the losses inherent in sequential purifications, this advance improves *S. cerevisiae* as a model system for studying the molecular changes associated with aging. The uninter-

rupted aging of a large cohort of cells also unlocks the possibility of examining phenotypes, such as spontaneous genetic mutation, that occur at very low frequencies. By solving the limitations of both RLS measurement and aged cell purification, the MEP unlocks the burgeoning yeast molecular genetic toolbox for the examination of cellular aging. Given that a number of the processes of cellular aging may be conserved among eukaryotes (Smith *et al.* 2008; Steinkraus *et al.* 2008), the MEP offers a new opportunity for developing a greater depth of understanding of these processes.

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GENETICS

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The Mother Enrichment Program: A Genetic System for Facile Replicative Life Span Analysis in *Saccharomyces cerevisiae*

Derek L. Lindstrom and Daniel E. Gottschling

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FILE S1

SUPPORTING MATERIALS AND METHODS

Strain and plasmid construction:

Deletions of *sir2*Δ and *fob1*Δ were constructed by one-step gene replacement with drug resistance markers (GOLDSTEIN and MCCUSKER 1999) in the haploid MEP strains UCC5179 and UCC5181, which were subsequently mated to generate heterozygous deletions in diploid strains UCC8836 and UCC526, respectively. To allow mating of *sir2*Δ mutants, strains were transformed with pRS314-SIR2 (BEDALOV *et al.* 2001) before mating and the diploid strain was subsequently cured of the plasmid.

Construction of P_{scw11}-*cre-EBD78*:

The *GAL* promoter driving expression of a Cre-EBD fusion protein (CHENG *et al.* 2000) on plasmid pFvL113 was replaced by gap repair with a 1 Kb promoter region of *SCW11* (generated by PCR from p126SCW using oligonucleotides CreScwF and CreScwR) to create pDL01. Upon introduction of this plasmid into reporter strain UCC8612 carrying *loxP*-flanked *ADE2*, we found 100% of transformants had lost *ADE2* through Cre-mediated recombination, indicating high recombinase activity in the absence of estradiol. In order to create a version of Cre-EBD that displayed strict dependence on estradiol for activity, we used error-prone PCR mutagenesis to generate mutations within *cre-EBD* as described in (WILSON and KEEFE 2001) using oligonucleotides CreF and EbdR. These PCR products were co-transformed along with pDL01 (gapped by restriction digest with *Stu*I and *Spe*I) into UCC8612 to isolate candidate mutants by gap repair. Transformants that yielded unsectored white colonies on media lacking estradiol were patched to YEPD + 1 μM estradiol to screen for induction of recombinase activity. Candidates that gave robust induction based on color were recovered by plasmid rescue and further characterized. The lead candidate, pDL20/*cre-EBD78* displayed an *ADE2* recombination rate of 1.68 x 10^{-4} per cell division in the absence of estradiol. After a two hour exposure to 1 μM estradiol, *ADE2* was lost in ~53% of cells. Sequencing identified four missense mutations within the *cre* domain and an additional four within the *EBD* domain. The *cre-EBD78-NATMX* cassette was amplified by PCR with oligonucleotides HOpolyF and HOPolyR and subcloned into the *Eco*RI site of the HO-poly-HO vector (VOTH *et al.* 2001) generating pDL12. This vector was used for integration of *cre-EBD78-NATMX* at the *ho* locus after restriction digestion with *Not*I.

Construction of *loxP* target genes:

The 5' *loxP* site in *UBC9* was introduced by homologous recombination of a *loxP-KANMX-loxP* cassette generated from pUG6 (DELNERI *et al.* 2000) using oligonucleotides UBC9lox5F and UBC9lox5R in the diploid strain UCC8600. Excision of *KANMX* was induced with Cre-EBD expressed from pDL01, and strains were sporulated to verify viability

of the *loxP* allele and generate the haploid strain UCC8701. The 5' *loxP* site for *CDC20* was constructed as described above using oligonucleotides CDC20loxF and CDC20loxR to generate strain UCC8611.

To introduce 3' *loxP* sites, a double-stranded oligonucleotide (Bamlox1 + Bamlox2) containing the *loxP* sequence flanked by 4-bp single-stranded 5' overhangs was subcloned into the *Bam*HI site of pAG32 (GOLDSTEIN and MCCUSKER 1999) to generate pDL03(+) and pDL03(-) containing either *loxP* orientation. The *loxP-HPHMX* cassette from pDL03(-) was amplified using oligonucleotides CDC20lox3F and CDC20lox3R and integrated into UCC8611 by homologous recombination.

To introduce a different selectable marker along with the 3' *loxP* site at *UBC9*, a *loxP-LEU2* PCR product was amplified from pRS305 (SIKORSKI and HIETER 1989) with oligonucleotides RS+loxP-Not1 and RS-Not1 and subcloned into pDL03(-) by digestion with *Not*I to create pDL26(-). The *loxP-LEU2* cassette was integrated into UCC8701 by homologous recombination of a PCR product generated with oligonucleotides UBC9lox3F and UBC9lox3R using pDL26(-) as a template to generate strain UCC8697.

To construct the *CDC20-Intron* allele, an *HPHMX* cassette with no *loxP* site was introduced into UCC8611 by homologous recombination of a PCR product from pAG32 (GOLDSTEIN and MCCUSKER 1999) generated with oligonucleotides CDC20_hphF and CDC20_hphR to create UCC3813. A 5' *loxP-CDC20*-HPHMX cassette was PCR amplified from UCC3813 with oligonucleotides CDC20notF and CDC20notR and subcloned into the *Not*1 site of pRS313 (SIKORSKI and HIETER 1989) to create pEH5. An *ACT1* intron sequence containing a *loxP* site was amplified from pLND4 with oligonucleotides CDC20_ACT1_F and CDC20_ACT1_R and subcloned into the *Bst*EII site of pEH5 to create pEH6. A *Not*I-*Xho*I fragment from pEH6 containing the *loxP-CDC20-Intron-loxP-HPHMX* cassette was used to replace the *cdc20*Δ*::KANMX* allele by homologous recombination in UCC8723 to yield strain UCC8779. Plasmid pDL25 was constructed by amplifying *CDC20* from genomic DNA using oligonucleotides CDC20NotF and CDC20NotR. The PCR product was digested with *Not*I and subcloned into the *Not*I site of pRS316.

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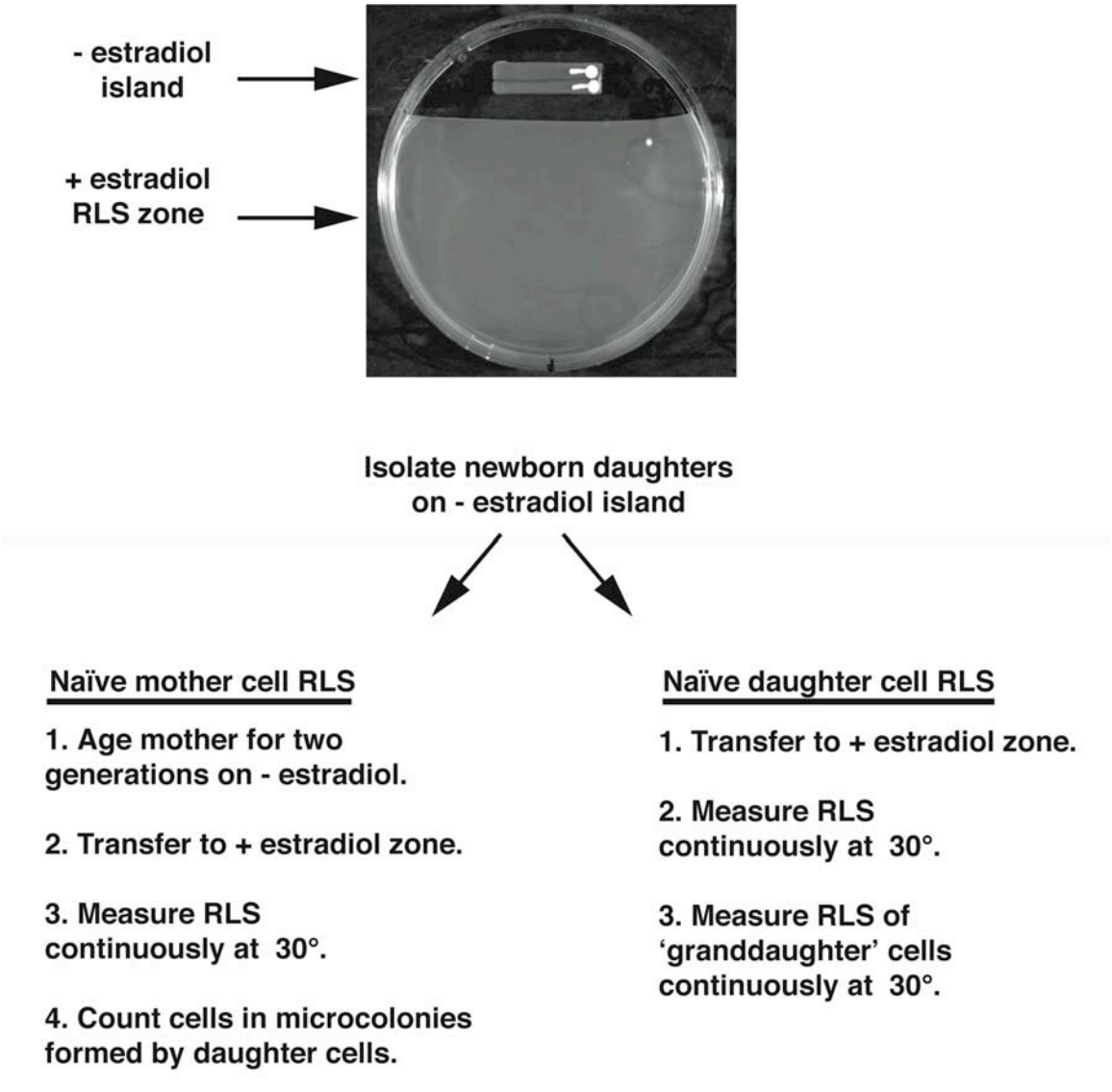

FIGURE S1.—Diagram of the experimental approach for measuring naïve mother and daughter RLS on media containing estradiol.

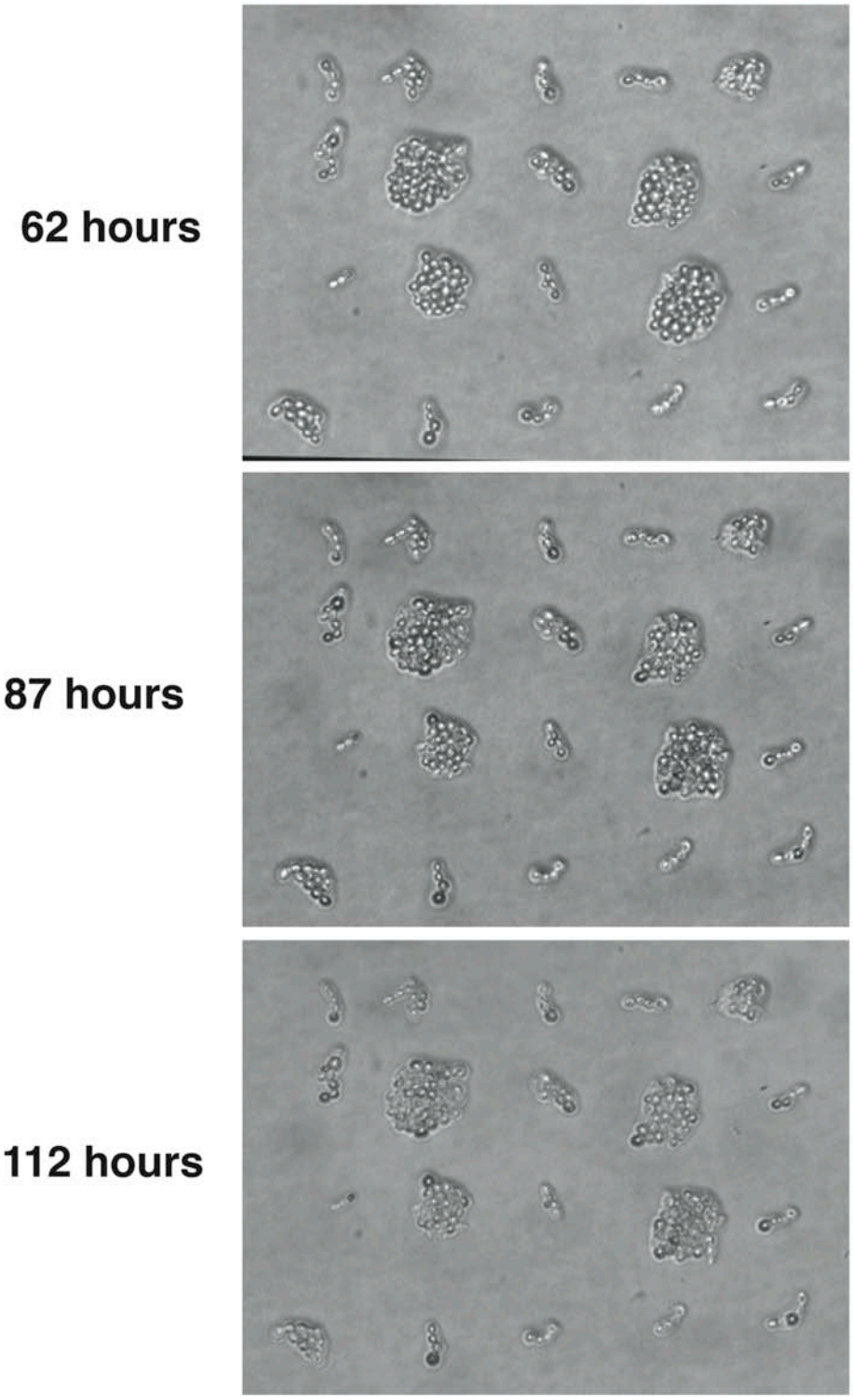

FIGURE S2.—Photographs of microcolonies formed by daughter cells born on estradiol during the life span measurement of naïve mother cells. Each photograph displays daughters #1-20 born to a single mother cell, arrayed from top left to bottom right. Times indicate hours after the mother was first placed on estradiol media (equivalent to 35, 60, and 85 hours after the birth of daughter #20). By 62 hours, each microcolony consisted of M-phase arrested cells. Microcolonies showed no change in cell number between 87 and 112 hours.

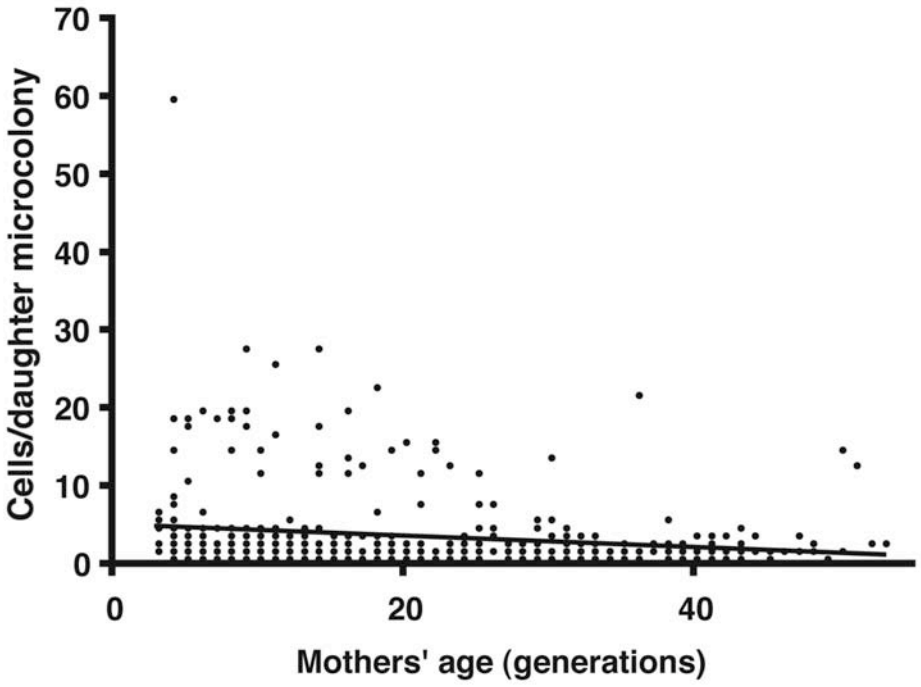

FIGURE S3.—A scatter plot comparing daughter's birth order (reflecting the age of the mother cell) to the number of cells in the daughter-cell microcolony (a measure of the daughter's proliferative capacity). The plot represents 472 daughter cells born on estradiol during the RLS analysis of naïve mothers. A best-fit line indicates negative correlation between mother's age and daughter's proliferative capacity.

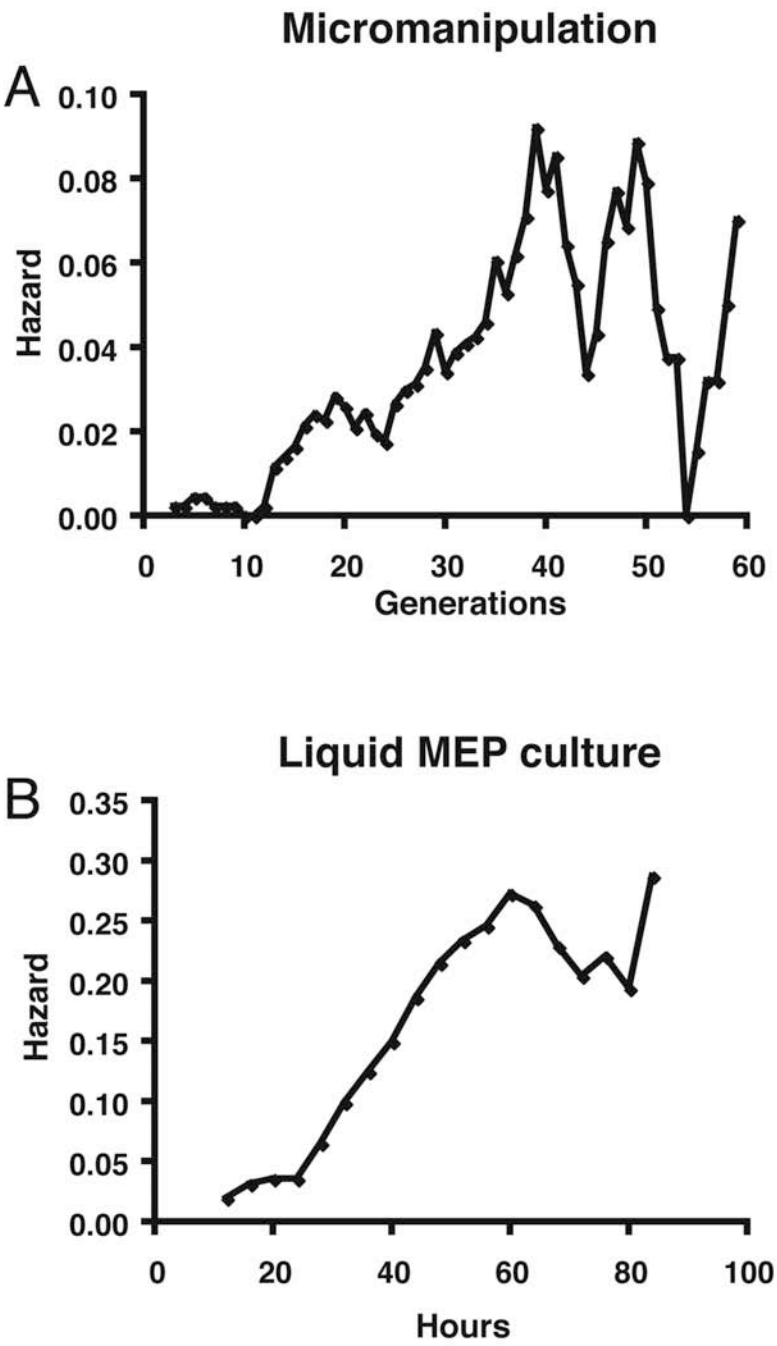

FIGURE S4.—(A) Hazard rate plot (# deaths/# individuals at risk) of UCC5185 RLS measured on estradiol media by micromanipulation, smoothed over an average of five neighbors. Hazard plots of wild type yeast populations typically display a low hazard rate at young ages, followed by an exponentially increasing rate. At very old ages, the small number of individuals observed results in large variations in hazard rate. (B) Hazard rate plot of UCC8848 viability measured in liquid estradiol media, smoothed over an average of five neighbors. To allow smoothing, intermediate values were estimated for four hour intervals based on a linear change in viability between sampling points.

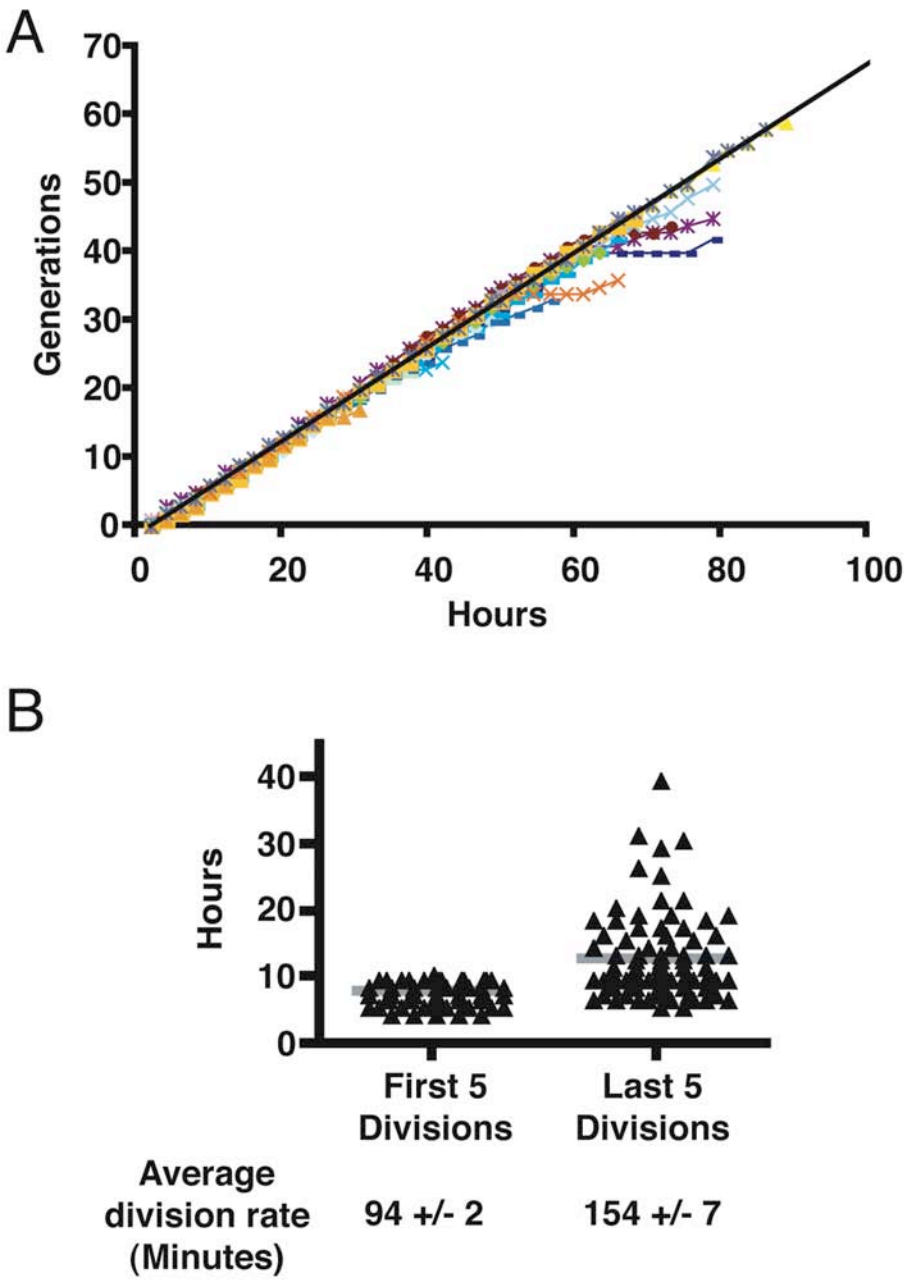

FIGURE S5.—(A) Division rates of 22 individual mother cells (UCC5185) during RLS measurement on estradiol media. A linear best-fit trendline (black, R=0.9992) based on the first 20 generations indicates a constant division rate of 87 minutes. Individual mothers approaching the end of their RLS often fall away from the trendline, indicating prolonged cell division times. (B) Time required for individual mothers to complete their first five divisions, and last five divisions before senescence. Each group represents a combined analysis of four pedigrees of UCC5185 (two on YEPD, two on estradiol media, n=99). Crossbars indicate the mean value of each group. The average division rate in minutes represents the mean value divided by five divisions.

TABLE S1

Oligonucleotides used in this study

Name	Sequence
CreScwF	AGCTCTAGTACGGATTAGAAGCCGCCGAGCGGGTGACAGCTTAATTACTTGTGTCTTG
CreScwR	TTAACACTCAGATAATGGTTTTAAGTAAAGTGTACAGGATGATGGTTTTCTATTAGAT
Bamlox1	GATCCTCTAGAATAACTTCGTATAATGTATGCTATACGAAGTTATG
Bamlox2	GATCCATAACTTCGTATAGCATACATTATACGAAGTTATTCTAGAG
RS+loxP-Not1	GAAAAAAGCGGCCGCCATAACTTCGTATAGCATACATTATACGAAGTTATGATTGTACT GAGAGTGCACC
RS-Not1	AAGGAAAAAAGCGGCCGCCTGTGCGGTATTTCACACCG
CreF	CCTGTACACTTTACTTAAAACC
EbdR	CACGAAATGTTCAGCACTAC
HOPolyF	CGAGGAATTCCATGATTACGCCAAGCGCGC
HOPolyR	CGAGGAATTCGCAGATTGTACTGAGAGTGC
UBC9lox5F	AAGGTAAGTAGTAGTTTTCCTCCTTTTATGCTTACATTGCATAGGCCACTAGTGGATCTG
UBC9lox5R	TTGTTAGTATACCGCTGGATGAAATTGTGTATGCCTACCAGCTGAAGCTTCGTACGC
UBC9lox3F	ATCTTTCCCATTCTTCCTCCTTTTGTACTTTATTTAACTAGGTGACACTATAGAAC
UBC9lox3R	TAATGTTATTTTGCTCTATTTTTGTTACACAACGACATAATACGACTCACTATAGG
CDC20loxF	ATTGGAAAGAAACCCAAAAATATAGAAATCGTCCATTCGCATAGGCCACTAGTGGATCTG
CDC20loxR	TAGTCTTCTTTGTAATACTTGTCTTTTGATATTTCTGCACCAGCTGAAGCTTCGTACGC
CDC20lox3F	TTAAAGAACCCACACACCACACGCGCGAGAACTGGGAGGGTAGGTGACACTATAGAAC
CDC20lox3R	TTCTTCCAAGGCCTAAATTTTGTTATTTGCTAAATTTCTCAATACGACTCACTATAGG
CDC20_hphF	TTAAAGAACCCACACACCACACGCGCGAGAACTGGGAGGGCACCCGGCCAGCGACATGGA
CDC20_hphR	TTCTTCCAAGGCCTAAATTTTGTTATTTGCTAAATTTCTCATAGGCCACTAGTGGATCTG
CDC20notF	TCACGCGGCCGCGTACCCTACATACTACATATTTC
CDC20notR	TCACGCGGCCGCTTCCTTATCGATTCTACAGC
CDC20_ACT1_F	CCAGAAAGCTCTAGAGATAAGGGAAATGCAGCAATTAGCGGTATGTTCTAGCGCTTGCAC
CDC20_ACT1_R	AGCTTTGTTGGGGACGCAATAGAAAGTACAGAACGGTTACCTAAACATATAATATAGCAA

TABLE S2

Yeast strains used in this study.

Strain	Genotype
UCC526	*MAT**a**/MATα ade2::hisG/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2 ura3Δ0/ura3Δ0 trp1Δ63/trp1Δ63 MET15/met15Δ:: ADE2 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX fob1Δ::KANMX/fob1Δ::KANMX*
UCC3813	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 loxP-CDC20-HPHMX*
UCC5179	*MAT**a** ade2::hisG his3 leu2 lys2 ura3Δ0 trp1Δ63 hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX*
UCC5181	*MATα ade2::hisG his3 leu2 trp1Δ63 ura3Δ0 met15Δ::ADE2 hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX*
UCC5185	*MAT**a**/MATα ade2::hisG/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2 ura3Δ0/ura3Δ0 trp1Δ63/trp1Δ63 MET15/met15Δ::ADE2 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX*
UCC8600	*MAT**a**/MATα ADE2/ade2Δ::hisG his3Δ1/his3Δ200 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 ura3Δ0/ura3Δ0 TRP1/trp1Δ63 MET15/met15Δ0 CAN1/can1Δ::pMFA1-HIS3, pMFα1-LEU2*
UCC8611	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 loxP-CDC20*
UCC8612	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 can1Δ::pMFa1-HIS3, pMFα1-LEU2 loxP-ADE2-loxP-HPHMX rga1*
UCC8650	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hoΔ::P_{SCW11}-cre-EBD78-NATMX*
UCC8697	*MATα his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 loxP-UBC9-loxP-LEU2*
UCC8701	*MATα his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 loxP-UBC9*
UCC8723	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 cdc20Δ::KANMX [pDL25]*
UCC8740	*MAT**a** his3Δ1 leu2Δ0 ura3Δ0 hoΔ:: P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2*
UCC8779	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 loxP-CDC20-Intron-loxP-HPHMX*
UCC8836	*MAT**a**/MATα ade2::hisG/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2 ura3Δ0/ura3Δ0 trp1Δ63/trp1Δ63 MET15/met15Δ::ADE2 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX sir2Δ::KANMX/sir2Δ::KANMX*
UCC8848	*MAT**a**/α ADE2/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2Δ0 trp1Δ63/ trp1Δ63 ura3Δ0/ura3Δ0 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2*
UCC8849	*MAT**a**/MATα ade2::hisG/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2 ura3Δ0/ura3Δ0 trp1Δ63/trp1Δ63 met15Δ::ADE2 /met15Δ::ADE2 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX sir2Δ::KANMX/sir2Δ::KANMX*
UCC8850	*MAT**a**/MATα ade2::hisG/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2 ura3Δ0/ura3Δ0 trp1Δ63/trp1Δ63 met15Δ::ADE2/ met15Δ::ADE2 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-*

	cre-EBD78-NATMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX fob1Δ::KANMX/fob1Δ::KANMX
UCC8852	*MAT**a** his3 leu2 lys2Δ trp1Δ63 ura3Δ0 hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-CDC20-Intron-loxP-HPHMX*
UCC8853	*MAT**a** his3 leu2 lys2Δ ura3Δ0 hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-CDC20-Intron-loxP-HPHMX loxP-UBC9-loxP-LEU2*
UCC8857	*MAT**a**/MATα ADE2/ade2::hisG his3Δ1/his3 leu2Δ0/leu2 LYS2/lys2D0 TRP1/trp1Δ63 ura3D0/ura3D0 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX*
UCC8858	*MAT**a**/MATα his3Δ1/his3 leu2Δ0/leu2 ura3Δ0/ura3Δ0 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2*
UCC8859	*MAT**a**/MATα his3/his3 leu2/leu2 LYS2/lys2Δ TRP1/trp1Δ63 ura3Δ0/ura3Δ0 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX*
UCC8860	*MAT**a**/MATα ADE2/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2 ura3Δ0/ura3Δ0 TRP1/trp1Δ63 MET15/met15Δ::ADE2 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2*
UCC8861	*MAT**a**/MATα ade2::hisG/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2 ura3Δ0/ura3Δ0 trp1Δ63/trp1Δ63 met15Δ::ADE2/ met15Δ::ADE2 hoΔ::P_{SCW11}-cre-EBD78-NATMX/hoΔ::P_{SCW11}-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX*
UCC8863	*MAT**a**/MATα ADE2/ade2::hisG his3/his3 leu2/leu2 LYS2/lys2Δ ura3Δ0/ura3Δ0 trp1Δ63/trp1Δ63 hoΔ::SCW11pr-cre-EBD78-NatMX/hoΔ::SCW11pr-cre-EBD78-NatMX loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPHMX loxP-UBC9-loxP-LEU2/loxP-UBC9-loxP-LEU2 fob1Δ::KANMX/fob1Δ::KANMX*

TABLE S3

Plasmids used in this study

Plasmid	Source	Notes
pDL01	This work	P_{SCW11}-*cre-EBD-NATMX* CEN ori ampR
pDL03(-)	This work	*loxP-HPHMX* CEN ori ampR
pDL12	This work	*cre-EBD78-NATMX* in the HO-poly-HO integration vector
pDL20	This work	P_{SCW11}-*cre-EBD78-NATMX* CEN ori ampR
pDL25	This work	*CDC20* subcloned into *Not*1 site of pRS316
pDL26	This work	*loxP-LEU2* CEN ori ampR
pEH5	This work	*loxP-CDC20-HPHMX HIS3* CEN ori ampR
pEH6	This work	*loxP-CDC20-Intron-loxP-HPHMX HIS3* CEN ori ampR
pLND4	Lazar Dimitrov	*loxP* site inserted into *ACT1* intron on plasmid pRX1 (CHENG *et al.*)[a]
pFvL113	Fred van Leeuwen	*HIS3* from pSH62 (CHENG *et al.*)[a] replaced with *NATMX*
p126SCW	Daniel Lockshon	P_{SCW11}-*GFP HIS3* CEN ori ampR

(a) CHENG, T. H., C. R. CHANG, P. JOY, S. YABLOK and M. R. GARTENBERG, 2000 Controlling gene expression in yeast by inducible site-specific recombination. Nucleic Acids Research **28:** E108.