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Effects of age on meiosis in budding yeast

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Abstract

Summary—In humans, the frequency with which meiotic chromosome mis-segregation occurs increases with age. Whether age-dependent meiotic defects occur in other organisms is unknown. Here, we examine the effects of replicative aging on meiosis in budding yeast. We find that aged mother cells show a decreased ability to initiate the meiotic program and fail to express the meiotic inducer *IME1*. The few aged mother cells that do enter meiosis, complete this developmental program but exhibit defects in meiotic chromosome segregation and spore formation. Furthermore, we find that mutations that extend replicative lifespan also extend the sexual reproductive lifespan. Our results indicate that in budding yeast, the ability to initiate and complete the meiotic program as well as the fidelity of meiotic chromosome segregation decrease with cellular age and are controlled by the same pathways that govern aging of asexually reproducing yeast cells.

Introduction

During vegetative growth, budding yeast reproduces by asymmetric division giving rise to a daughter cell, which first emerges from the mother as an outgrowth, known as a bud. The number of daughter cells a mother cell generates before its death is known as its replicative life span and is relatively uniform for a given strain (Sinclair et al., 1998). With the ongoing of replicative life, mitotic yeast cells show morphological and physiological changes such as an increase in cell size, genome instability, sterility, extension in cell cycle duration, nucleolar fragmentation, accumulation of ROS (reactive oxygen species), and loss of membrane turgescence. Several factors contribute to these changes in the cell physiology associated with senescence: damaged proteins, damaged mitochondria, redistribution of the silencing regulator proteins Sir2, Sir3 and Sir4 and the accumulation of ERCs (extrachromosomal ribosomal DNA circles). ERCs are formed through homologous recombination and are self-replicating, but they lack centromeres and therefore remain in the mother cell during cell division. Interestingly, damaged proteins or damaged mitochondria are also preferentially retained in the mother cell during mitosis. These observations have led to the suggestion that it is the accumulation of

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ERCs and damaged proteins and organelles that lead to cell death in aged cells (Sinclair and Mills, 2001).

Several genes have been implicated in regulating replicative life span in yeast. The histone deacetylase Sir2 modulates aging in virtually every organism. In budding yeast, deletion of *SIR2* severely shortens life span, whereas the presence of an additional copy of this gene increases the lifespan by approximately 50% (Kaeberlein et al., 1999). *SIR2* is thought to affect aging by preventing ERC formation through its role in repressing mitotic recombination within the rDNA locus. The rDNA localized Fob1 protein also controls life span in yeast. Fob1 prevents movement of DNA polymerases against the direction of rRNA transcription thereby reducing recombination within the rDNA (Defossez et al., 1999).

Nutrient signaling also regulates life span. The TOR (target of rapamycin) kinases are highly conserved and promote cell growth in response to favorable nutrient conditions and growth-factor signals. Budding yeast contains two TOR kinases, Tor1 and Tor2. *TOR2* is essential, but deletion of *TOR1* has been shown to increase replicative life span by approximately 20% (Kaeberlein et al., 2005). It has been proposed that it is through *TOR1* that caloric restriction delays aging and leads to an increase in life span in budding yeast. The protein kinase Sch9 is phosphorylated by Tor1 and thought to convey some of Tor1's growth promoting functions (Urban et al., 2007). Consistent with a role of caloric restriction and the TOR pathway in the regulation of replicative life span, cells lacking *SCH9* live longer (Kaeberlein et al., 2005).

The effects of age on entry into and progression through meiosis are largely unexplored. In humans, meiotic chromosome segregation errors increase with maternal age (reviewed in Hassold and Hunt, 2001). Approximately 80% of these segregation errors occur during meiosis I, and 20% result from meiosis II non-disjunction (Sherman et al., 2005). Studies on chromosome 21 non-disjunction show that only 6–10% of all trisomy 21 cases are due to errors in spermatogenesis, but meiosis I and meiosis II errors contribute equally to these male germline non-disjunction events (Sherman et al., 2005). Additionally, there is also evidence to suggest that sperm quality decreases with age (Malaspina et al., 2001; Wyrobek et al., 2006). A gradual increase in DNA damage or a reduced ability to protect germ line stem cells from free radicals has been suggested to be the basis for this decrease in sperm quality (Zhu et al., 2007). However, how replicative age affects the meiotic divisions has not been studied in detail in any organism. The ability to isolate aged yeast cells (Smeal et al., 1996) and to induce them to undergo meiosis (Honigberg and Purnapatre, 2003) enabled us to address this question.

In budding yeast four different signals are necessary for cells to enter the meiotic program. Cells must express both mating type loci, and nitrogen and glucose must be absent from the growth medium. Finally, cells must be respiration competent (Honigberg and Purnapatre, 2003). The mating-type, nutritional, and respiration signals converge at Ime1, a transcription factor governing entry into the meiotic program. This regulation occurs at multiple levels and is still largely unknown. The MATa and α genes are needed together to inactivate the transcriptional repressor Rme1 (Covitz et al., 1991), which acts at the *IME1* promoter (Sagee et al., 1998). The fermentable carbon source glucose inhibits *IME1* transcription the mechanisms of which is only partly understood (Gorner et al., 1998; Shenhar et al., 2001). Nitrogen also prevents *IME1* transcription (Sagee et al., 1998), prevents Ime1 from localizing to the nucleus (Colomina et al., 2003) and disrupts its interaction with its coactivator Ume6 (Xiao and Mitchell, 2000). The respiration state of a cell also affects *IME1* expression. Cells lacking functional mitochondria fail to express *IME1* (Treinin et al., 1993; Jambhekar and Amon, 2008), but the mechanisms whereby this occurs remain to be determined.

Once cells have entered the meiotic program, they undergo pre-meiotic DNA replication, which is followed by two rounds of chromosome segregation. During the ensuing prophase, linkages

are created between homologous chromosomes through recombination. This facilitates the correct alignment and the subsequent segregation of homologous chromosomes during the first meiotic division (meiosis I). This unusual segregation phase is immediately followed by a second division, meiosis II, which resembles mitosis in that sister chromatids are segregated. After completion of meiosis II, all four meiotic products are packaged into spores.

In this study, we adapted previously established protocols to isolate aged mother cells to examine the effects of age on the ability of cells to enter and progress through meiosis. We find that aged mother cells fail to enter the meiotic program. This inability to initiate meiosis is accompanied by a failure to induce *IME1* and other early meiotic genes. Ectopic expression of *IME1* partially suppresses the sporulation defect indicating that age inhibits meiosis in part by preventing *IME1* expression. We then describe the identification of conditions that partially alleviate the meiotic entry defect in aged cells, which enabled us to examine the consequences of cellular age on subsequent meiotic stages. When the meiotic entry defect is suppressed, aged mother cells complete the meiotic program but exhibit defects in meiotic chromosome segregation and spore formation. Finally, we show that mutations that extend the replicative life of budding yeast suppress the meiotic defects of aged cells. Our results indicate that in budding yeast, cellular age affects multiple aspects of the meiotic program and that mutations that extend the ability of cells to reproduce asexually also extend the ability to reproduce sexually.

Results

The lifespan of the SK1 strain

To determine whether age affects meiosis in budding yeast, we first determined the average and maximal lifespan of the strain SK1, which we employ to study meiosis. The average life span of the SK1 strain was similar to that of W303, a strain routinely employed in yeast aging studies (23 generations for SK1, 20 generations for W303; Figure 1A). The maximal life span of SK1 was 49 generations, slightly more than that observed in W303 (32 generations; Figure 1A). Our single cell analysis of aging SK1 cells furthermore revealed that the changes associated with very old age, such as loss of polarity, increase in cell size and cell cycle duration, become apparent 2–3 generations prior to cell death (Supplemental Figure 1; data not shown).

A method to examine the effects of age on meiosis

We adapted the biotin labeling procedure developed by Smeal et al. (1996) to isolate old mother cells and induce them to enter the meiotic program (Supplemental Figure 2). Briefly, cells were labeled with biotin (Figure 1B, sort 0). After approximately 6 generations the biotin-positive cells were isolated (Figure 1B, sort 1) and grown for another 6 generations. The biotin positive cells (Figure 1B, sort 2) were then transferred into pre-sporulation medium (YPA) for 20 hours and biotin-positive cells were again isolated (Figure 1B, sort 3). The analysis of the number of bud scars on cells showed that this procedure indeed selected for older cells in the biotin-positive cell fraction (Figure 1C) and for new-born cells in the biotin-negative cell fraction (Figure 1D). The biotin positive cells isolated by this method furthermore exhibited the characteristics of aged mother cells such as enlarged and fragmented nucleoli (Figure 1E, F).

Cell viability was monitored throughout the sorting procedure, by examining the ability of cells to form colonies on YPD medium (Figure 1G). Biotin-negative cells (average and median age: 2 generations) maintained high viability throughout the procedure (Figure 1G). The viability of middle-aged cells (sort 2; average age: 9 generations) was decreased and that of the oldest cells (sort 3; average age: 13.5 generations) was 2-fold lower than that of biotin negative cells. Old yeast cells were shown to exhibit mitochondrial defects (Bonawitz et al., 2006) although whether they were respiration deficient had not been examined. It was therefore important to

ensure that the aged cells isolated by this method were still capable of respiring, as respiration incompetence would prevent entry into the meiotic program. Cells exhibited a similar ability to form colonies on medium lacking a fermentable carbon source (YEP glycerol+ethanol) as on medium containing a fermentable carbon source (YEPD; Figure 1G, H) indicating that the biotin positive cells obtained by this sorting procedure are respiration competent and therefore should be capable of entering the meiotic program.

Spore formation is impaired in aged cells

Having established a procedure to isolate aged mother cells we examined their ability to sporulate. Biotin-positive cells with a mean age of 13.5 generations and the age distribution shown in Figure 2A were transferred into sporulation-inducing medium. The comparison of the age distribution of cultures before and after sporulation showed that very old cells (cells with 23–30 bud scars) were lost during incubation in sporulation medium but cells with fewer scars survived the procedure (Figure 2B). Interestingly, we found that the old population was impaired in forming spores. After 20 hours in sporulation medium only 16.7% of cells (SD=9.3) of cultures with age distributions shown in Figure 2A had formed spores (Figure 2C; n=8 experiments). In contrast, 69.8% (SD=15) of biotin-negative cells that were isolated using the same procedure formed spores (Figure 2C; Supplemental Figure 2; n=8 experiments). Analysis of the number of bud scars in individual sporulating cells showed that the defects in sporulation became more severe with age (Figure 2D). The difference in the ability to form spores between young cells obtained from sort 1 and aged cells obtained from sort 3 was highly significant ($p=1.6\times 10^{-20}$), and so was that between sort 2 and 3 cells ($p=2.3\times 10^{-8}$).

To ensure that the biotinylation procedure and sorting did not interfere with sporulation, we labeled cells with biotin and then subjected them to the sorting procedure, except that cells were not grown between sorts (Supplemental Figure 3; see also Experimental Procedures). This generated a population of new-born cells that was biotin labeled and sorted as many times as the aged mother cells. Sporulation occurred with great efficiency (88%) in these cells (Figure 2E) and spore viability was high (92.4%). Furthermore, 90.8% of tetrads generated 4 viable spores (Figure 2F) indicating that biotinylation and the sorting procedure did not affect sporulation and spore viability. We conclude that aged cells are defective in sporulation.

The meiotic gene expression program is affected in aging cells

Analysis of the nuclear morphology of aged cells induced to enter meiosis revealed that the majority had not undergone any meiotic divisions and remained mononucleate (data not shown). To examine which aspects of the meiotic program were not occurring in old mother cells we compared the gene expression profile of old mother cells (sort 3 biotin positive cells with an average age of 16.3 generations and the age distribution shown in Figure 3B; n=2) with that of young cells (sort1, biotin positive cells with an average age of 4.46 generations and the age distribution shown in Figure 3A; n=1). We isolated RNAs from cells incubated in sporulation medium for 6 hours (the sorting and experimental procedure is outlined in Supplemental Figure 4) to allow aged cells ample time to enter and progress through the early stages of meiosis and compared the gene expression pattern of cells with that of exponentially growing new-born cells using the Gene Ontology analysis tool. As expected, transcripts that fall into the GO terms associated with sporulating cells such as sporulation, reproduction, cell differentiation and spore wall assembly were induced in young cells (Supplemental Table 1). In contrast, expression of sporulation genes was not induced in aged cells. Instead, transcripts of the GO terms associated with ribosome biogenesis, rRNA processing and nucleolar activity were up-regulated in aged cells induced to sporulated compared to exponentially growing new-born cells (Supplemental Table 1).

To determine which aspect of the meiotic program was defective in aged cells we grouped the gene expression patterns of sporulated aged and young cells according to the expression clusters defined by Primig et al. (2000). This analysis showed that genes involved in the very early stages of meiosis (Clusters 1, 2 and 3 in Primig et al., 2000) were not enriched in either cell population (Figure 3C; Supplemental Table 2). The absence of early meiotic transcripts in young cells is not surprising as we harvested cells 6 hours after transfer into sporulation medium. Transcripts in Cluster 4, which encompasses genes involved in DNA replication, recombination and Synaptonemal Complex formation and clusters 5 and 6, which is composed of genes required for the meiotic divisions were significantly enriched in young cells and underrepresented in aged cells (Figure 3C; Supplemental Table 2). A small set of genes (14) was found to be upregulated in aged cells. These belonged to cluster 7b and represented mostly genes involved in rRNA processing and ribosome biogenesis (9/14 genes; Figure 3C, Supplemental Table 2), the significance of which is at present unclear. Our gene expression analysis demonstrates that genes necessary for entry into and progression through meiosis are not expressed in aging cells, suggesting a fundamental inability to induce the developmental program necessary to produce gametes.

***IME1* is not expressed in aging cells**

The transcription factor Ime1 functions at the top of the transcriptional cascade responsible for governing entry into and progression through meiosis (reviewed in Vershon et al., 2000). As aged cells appeared to not express genes required for sporulation we wished to determine whether *IME1* was expressed in aged cells. The mCherry-encoding gene (NLS-mCherry) was placed under the control of the *IME1* promoter (Nachman et al., 2007) and expression was compared between old (biotin-positive) and new-born (biotin-negative) cells. Whereas the mCherry protein was readily detectable in new-born cells even before transfer into meiosis-inducing conditions and several hours thereafter (Figure 3D – F), the protein was not detected in cells with an average age of 13.5 generations and the age distribution shown in Figure 3E (Figure 3D – F). Consistent with an inability to enter the meiotic program, aged cells also failed to induce expression of the early meiotic gene *ZIP1* fused to GFP (Supplemental Figure 5).

To determine whether down-regulation of *IME1* expression was the sole reason for why aged cells failed to sporulate we examined the consequences of expressing *IME1* from the copper-inducible *CUP1* promoter. In the absence of copper, Ime1 was not expressed but upon addition of the metal (50 μ M) Ime1 production was induced (Figure 4A) indicating that the *CUP1* promoter is tightly regulated in the SK1 background. Biotin positive cells with one copy of *IME1* under the control of the *CUP1* promoter (average age= 15.6 generations; SD= 5.4) with the age distribution shown in Figure 4B were induced to sporulate either in the presence or absence of CuSO₄ (50 μ M). Expressing *IME1* from the *CUP1* promoter did not affect cell viability of biotin positive cells (70.21%), but improved their sporulation efficiency. Whereas only 16.75% (SD= 3.4) of aged wild-type cells formed spores, 25.58% (SD=8.4) of *CUP1-IME1*-expressing old cells sporulated (Figure 4C). This difference in sporulation was highly significant ($p=1.025\times 10^{-9}$).

Deleting the *IME1* transcriptional repressor *RME1* (Covitz et al., 1991) did not suppress the sporulation defect of old cells (data not shown) indicating that inactivation of mating type control of *IME1* alone is not sufficient to induce meiosis in aged cells. We conclude that the inability of aged cells to enter the meiotic program is in part due to a failure to induce *IME1* expression but this appears not to be the sole reason. Ime1 is also regulated at the posttranslational level (reviewed in Mitchell, 1994). Ageing may also affect these pathways.

Identification of conditions that suppress the meiotic entry defects of aged cells

Is the failure to enter the meiotic program the only meiotic defect of aged cells? To address this question we sought to identify conditions that allowed old cells to enter the meiotic program more efficiently. During sporulation, cells generate bicarbonate that promotes sporulation (Ohkuni et al., 1998). Consistent with this observation we found that when aged (biotin positive) mother cells were co-cultured with new-born (biotin negative) cells the sporulation efficiency of the aged cells was improved. Approximately 40% of the biotin positive cells with a mean age of 15 generations and the age distribution shown in Figure 3G induced *IME1-mCherry* (Figure 3H) compared to less than 10 percent of cells induced to sporulate under standard conditions (Figure 3E, F). Sporulation of aged cells was also significantly improved in medium in which wild-type cells had previously undergone meiosis (henceforth preconditioned medium). In preconditioned medium 50.8 % (SD=18.8) of cells with the age distributions shown in Figure 5A formed spores (Figure 5B) compared to 16.7 % (SD= 9.3) under standard sporulation conditions (Figure 2C). Finally, increasing the culture volume also improved sporulation in aged cells (data not shown).

The improved sporulation efficiency of aged cells in preconditioned medium enabled us to further examine the relationship between age and ability to sporulate. An inverse correlation appears to exist between a cell's age and its ability to sporulate. The more bud scars a cell had the less likely it was to sporulate (Figure 5C). This finding suggests that even under optimal sporulation conditions when a greater fraction of aged cells can be induced to undergo meiosis, older cells are still less likely to enter the meiotic program than younger ones.

Aged cells exhibit meiotic chromosome segregation defects

Having established conditions that allowed aged cells to sporulate more efficiently, we were able to examine whether events other than entry into the meiotic program were affected by a cell's age. We first examined the effects of age on meiotic chromosome segregation. To this end we analyzed the segregation pattern of tandem tetO repeats integrated at the *LYS2* locus of both copies of chromosome II. As these cells also express a TetR-GFP fusion, which binds to these repeats, the distribution of the TetO repeats can be analyzed in spores. When the meiotic divisions occur normally, each of the four nuclei contains a single GFP dot (Supplemental Figure 6). Meiosis I non-disjunction is evident after the completion of the first meiotic division. When chromosomes are segregated accurately, each nucleus should contain a GFP dot. When chromosomes are mis-segregated GFP dots are observed in only one of the two nuclei (Supplemental Figure 6). Meiosis I non-disjunction can also be inferred from the GFP dot distribution in tetranucleate cells resulting in an increase in tetrads harboring only two spores with GFP dots (Supplemental Figure 6). Meiosis II non-disjunction or premature sister chromatid separation is characterized by an increased presence of tetrads containing GFP dots in three of the four spores (Supplemental Figure 6). By these criteria, aged cells induced to sporulate in preconditioned medium exhibited an increased frequency in meiosis II non-disjunction or premature sister chromatid separation (Figure 5D, bottom panel). The percentage of tetrads with GFP dots in only three of the four nuclei was increased in older cells (12.8 %, SD= 2.4) compared to new-born cells (2.67 %, SD= 1.15). The combined analysis of GFP dot distribution and number of bud scars furthermore clearly demonstrated that it was cells with 15 – 21 bud scars that exhibited GFP dot segregation defects (Figure 5C). We also observed similar results using standard sporulation conditions. Meiosis I chromosome segregation appeared not affected. The percentage of tetranucleate cells with GFP dots in only two nuclei was not increased (Figure 5D, bottom panel) and neither was the percentage of bi-nucleate cells with a GFP dot in only one of the two nuclei (Figure 5D, top panel).

In agreement with the GFP dot analysis, spore viability was also reduced in aged cells. We used a dissecting microscope with fluorescence capabilities to distinguish tetrads derived from

old mother cells, which exhibit a high number of scars, from those derived from new-born cells, which have very few. Asci derived from aged and new-born cells were identified, individually isolated and dissected. Spore viability was decreased in aged cells (57%) compared to new-born cells (78%; n=200; Figure 5F, G). Our results indicate that the fidelity of chromosome segregation is decreased in tetrads derived from aged cells. Thus, budding yeast is only the second organism, after humans, where an age-dependent increase in chromosome mis-segregation has been reported.

Aged cells exhibit spore packaging defects

Our analysis of aged cells induced to undergo meiosis in preconditioned medium revealed a third meiotic defect caused by old age. We identified a significant fraction of cells that were able to progress through the two meiotic divisions but failed to package the four meiotic products into spores. This was particularly evident when we compared cellular age with the ability to package spores. The older the cell the less likely it was to package the meiotic products into spores (Figure 5E). We conclude that the ability to form spores decreases with cellular age.

Aged cells of long-lived mutants are not defective in sporulation

Several factors have been identified, which when inactivated extend the replicative lifespan of yeast. Having established that old age affects the ability of cells to enter and progress through meiosis, we wished to determine whether the factors involved in modulating the replicative lifespan of vegetatively growing yeast cells also affect meiosis in aged cells. Inactivation of the replication fork barrier protein Fob1 extends the replicative life span of yeast cells more than 2 fold by reducing ERCs formation (Defossez et al. 1999). We first determined that deletion of *FOB1* also extends the lifespan of the SK1 strain (Supplemental Figure 7A) and then examined whether deletion of *FOB1* also affected the ability of aged cells to enter meiosis. Wild-type cells and cells lacking *FOB1* with the age distributions shown in Figure 6A were induced to enter the meiotic program under standard conditions. 53.5% of *fob1Δ* cells sporulated with sporulation being particularly increased in cells with 13 – 20 bud scars (Figure 6B). The difference between aged wild-type cells and *fob1Δ* cells with the same age distribution to form spores was highly significant ($p=1.6\times 10^{-6}$). Furthermore, spore viability was equally high in new-born and old cells lacking the replication fork barrier protein (Figure 6C, D).

Overproduction of Sir2 increases the replicative life span of yeast and suppresses ERC formation (Kaeberlein et al, 1999). High levels of the NAD-dependent histone deacetylase also improved the sporulation efficiency of aged cells. Aged cells with the age distribution shown in Figure 6E sporulated with similar efficiency as new-born cells (Figure 6F). Conversely, deletion of *SIR2* led to a decrease in life span (8.87 generations Supplemental Figure 7A) and in sporulation efficiency. Biotin positive cells with a mean age of 6 generations exhibited reduced sporulation but sporulation efficiency in biotin negative cells with a mean age of 2 generations was not affected (Figure 6G, H).

The finding that modulating *FOB1* and *SIR2* activity affects the ability of aged cells to sporulate suggests that it is ERC formation that hampers sporulation in old cells. To further examine whether ERC accumulation was responsible for the sporulation defect we observed in old cells, we examined the effects of high levels of Fob1, which has been previously shown to expand the rDNA array (Kobayashi et al, 1998). Furthermore, we examined the consequences of introducing additional copies of rDNA circles by transforming wild-type cells with a multi copy plasmid (2 micron) carrying an rDNA repeat (Wai et al, 2000). High levels of Fob1 reduced the sporulation efficiency of young cells (average age = 5 generations, age distribution Figure 7A) but not that of new-born cells (average age = 1.5 generations; Figure 7B). Similar results were obtained in cells carrying an rDNA-carrying high copy plasmid. High levels of

rDNA reduced the sporulation efficiency of young cells (average age = 4.3 generations, age distribution Figure 7C) but not that of new-born cells (average age = 1.8 generations; Figure 7D). We conclude that ERC formation is at least in part responsible for the sporulation defect of aged cells.

In some strain backgrounds the TOR pathway is a critical regulator of replicative aging (Kaeberlein et al., 2005). In the SK1 strain this does not appear to be the case. Deleting *TOR1* did not extend the lifespan of SK1 cells (Supplemental Figure 7B). Consistent with this result, we find that *tor1Δ* aged mother cells did not sporulate efficiently. Only 27% of biotin positive *tor1Δ* cells sporulated, whereas 88% of biotin negative *tor1Δ* cells did. We conclude that *FOB1* and *SIR2* not only modulate the replicative lifespan of yeast cells but also the ability of aged cells to sporulate.

Discussion

We have established methods to examine the effects of age on meiosis in budding yeast and thereby identified three meiotic processes that are sensitive to a yeast cell's age. (1) Entry into the meiotic program: aged cells fail to enter the meiotic program in part due to an inability to express the meiotic inducer *IME1*. (2) Spore formation: aged cells are defective in packaging the meiotic products into spores. (3) Chromosome segregation: aged cells exhibit defects in meiotic chromosome segregation. All meiotic defects present in old age cells are suppressed by mutations that repress ERC formation and extend the replicative life of budding yeast indicating that the genetic program by which age governs mitotic proliferation also controls sexual reproduction and that it does not occur in a clock-dependent manner as described for loss of heterozygosity in daughters produced by old mother cells (McMurray and Gottschling, 2003).

Age affects meiosis in multiple ways in yeast

Our results show that the key meiotic inducer *IME1* is not expressed in aged mother cells. *IME1* transcription is controlled by the simultaneous expression of both mating type loci and by nutritional and respiratory signals. Defects in respiration are not likely to be responsible for the meiotic entry defect of aged cells because these cells were able to form colonies on medium that requires cells to respire in order to grow. Whether the nutritional signals that converge on the *IME1* promoter are disrupted in aged cells is not yet known. It is however interesting to note that incubation of aged cells in medium where new-born cells had previously sporulated or co-culturing aged cells with new-born ones, partially alleviate the *IME1* expression defect. This finding raises the interesting possibility that cell-induced changes of the environment such as pH changes and/or metabolites such as bicarbonate (Ohkuni et al, 1998) suppress the age effects on *IME1* expression. Determining the molecular basis for this suppression will help elucidate the mechanisms whereby age interferes with *IME1* expression. It is furthermore important to note that *IME1* transcription is likely not the only manner in which age affects entry into the meiotic program. Ectopic expression of *IME1* only partially restored sporulation to aged cells. Nuclear entry of Ime1 and binding to promoters are also regulated by meiosis-inducing signals (Colomina et al., 2003). It is possible that old age affects these steps in meiotic entry as well.

In addition to a meiotic entry and spore formation defect, we observed that the distribution of GFP dots in tetrads produced by aged cells was abnormal. In almost 13% of tetrads produced by aged cells GFP dots were visible in only three of the four spores. This loss of GFP signal is not likely due to recombination between tetO repeats. Aged vegetatively growing cells did not exhibit a loss of GFP signals (M. B., unpublished observations). Thus, chromosome mis-segregation is likely to be responsible for the abnormal GFP dot distribution of aged cells. Indeed spore viability was also decreased in aged cells. We do not yet know the molecular

basis of this chromosome mis-segregation and whether products of aging such as ERCs affect chromosome segregation. The pattern of GFP dot distribution is most consistent with chromosome mis-segregation occurring during meiosis II or premature sister chromatid separation during the meiosis I. Premature loss of centromeric cohesion and/or defects in kinetochore – microtubule attachment could lead to such chromosome mis-segregation patterns. Given that the chromosome mis-segregation defect in aged cells is subtle, identifying the molecular basis of the defect will be challenging.

Mutations that extend life also prolong the ability to sexually reproduce

In budding yeast caloric restriction and the prevention of ERC formation have been shown to extend replicative life span. Deletion of *TOR1*, which mimics caloric restriction did not extend the life span of the *SK1* background nor did it improve sporulation in aged cells. In contrast, modulating *FOB1* and *SIR2* activity affected meiosis in aged cells. Deletion of *SIR2* or overexpression of *FOB1* decreased sporulation efficiency in young cells. Conversely, deletion of *FOB1* or overexpression of *SIR2* allowed aged cells to form gametes with a higher efficiency. Remarkably the gametes produced by these aged cells were fully viable indicating that extending life span rescues all age-related meiotic defects. This finding furthermore suggests that all age-induced meiotic defects are caused by the same *SIR2* and *FOB1* dependent mechanism.

One mechanism whereby deletion of *FOB1* or overexpression of *SIR2* extends life span is through suppression of ERC formation. ERC formation may also affect sporulation. Expression of an rDNA –containing multi copy plasmid interferes with sporulation in young cells. How ERC formation interferes with *IME1* expression, chromosome segregation or spore formation is unclear. It is however, important to note that *SIR2* overexpression and *FOB1* deletion could also promote meiosis in aged cells by means other than suppression of ERC formation as has been suggested for *SIR2* in vegetatively growing yeast cells and in other organisms (reviewed in Guarente and Picard, 2005)

Is meiosis sensitive to age in all eukaryotes?

Our studies identify budding yeast as only the second organism, after humans, where age affects germ cell formation. The age effects on meiosis in females appear to be quite different from those in budding yeast. In budding yeast, age primarily affects entry into the meiotic program. Because meiotic entry in females occurs during embryonic development, age is not a factor in meiotic entry in human females. In human males, germ cell production occurs throughout the adult life and is preceded by a number of mitotic divisions (Wolgemuth and Lele, 2002), thus resembling more the yeast meiotic program. Although germ cell quality decreases with age in males (Malaspina et al, 2001), whether entry into the meiotic program is affected by age is not known.

Like in humans, yeast exhibits age-dependent chromosome mis-segregation. In females, meiosis I non-disjunction increases with age and is due to the prolonged diplotene arrest that female germ cells experience (reviewed in Hassold and Hunt, 2001). Clearly, the replicative age dependent increase in meiotic non-disjunction observed in yeast is not related to this phenomenon. However, in males, it is not only meiosis I non-disjunction but also meiosis II non-disjunction of sex chromosomes that increases with paternal age (Griffin et al, 1995). This phenomenon could be similar to what we observed in yeast cells, where meiosis II non-disjunction and/or premature sister chromatid separation increase with age. Thus, even though there are obvious differences in germ cell morphogenesis and development, age-dependent meiosis II non-disjunction occurs in aging yeast and germ cells of older males. It will be interesting to investigate whether the molecular defects underlying age dependent meiosis II mis-segregation are similar to those observed in yeast.

Some of the genes that affect aging in *S. cerevisiae* such as *SIR2* have also been shown to modulate life span in higher eukaryotes. The mechanism by which *SIR2* affects aging in yeast is different from its mode of action in *C. elegans* or mice suggesting that the output of the pathways controlling aging vary between organisms. The machinery controlling organismal age appears however conserved with a deacetylase enzyme at the heart of this process. It will be interesting to determine whether modulating *SIR2* activity also affects reproductive age in other organisms too.

Experimental Procedures

Strains and Plasmids

Strains used in this study are described in Supplemental Table 2 and are derivatives of SK1. Deletions of *FOB1*, *SIR2*, *RME1* and *FLO8* were generated by the PCR-based method described in Longtine et al. (1998). *SIR2* was overexpressed in cells by integrating a second copy of *SIR2* at the *URA3* locus as previously described (Kaeberlein et al, 1999)

Life Span analysis

The lifespan analysis was carried out by micromanipulation as described previously (Smeal et al, 1996) with the following modification. Cells were plated on YEP plates containing 2% glycerol at 30°C for approximately 12 hours, then streaked on YEP plates containing 4% glucose. The initial cells selected for the lifespan analysis were granddaughters, that is a cell was allowed to bud, this bud was allowed to bud again and this second bud was selected for the lifespan analysis. Selecting grand daughters for the life span analysis ensured that the cells were actively dividing when the life span analysis was started. At least 40 cells were used for each experiment. After each cell division the daughter cell was isolated and the duration of the cell cycle recorded. Cells were grown at 30°C and stored over night at 4°C. Every cell was followed until cell lysis was observed.

Expression analysis

For the control sample, SK1 cells (wild-type, 16554) were grown in YPD and harvested before they reached OD(600)=1. Experimental samples from sort1 cells (young cells, average age = 4.46 generations) were grown as showed in Suppl. Fig. 4 and transferred into sporulation-inducing condition at 30°C and harvested after 6 hrs thereafter. Sort 3 cells (old cells, average age of the two biological replicas = 16.33 generations) were grown and sorted as described in Suppl.Fig.4. Cells were then induced to sporulate by transfer into KAC medium and harvested after 6 hrs. Cells were then filtered through a filtering apparatus, the membrane with the cells was then snap frozen in liquid nitrogen and put at -80.

Total RNA was isolated from cells frozen on membrane by acid phenol extraction. Qiagen RNeasy micro kit was used for RNA purification. Labeling was done using the Quick Amp labeling kit from Agilent, according to the manufacturer's protocol. Qiagen RNeasy micro kit was used for RNA purification. Dye incorporation and yield were measured with a Nanodrop spectrophotometer. Equal amounts of differentially labeled control and sample cRNA were combined such that each sample contained at least 2.5 pmol dye. Samples hybridization was also performed according to the manufacturer's protocols. Agilent yeast expression arrays (8×15K) were used for this analysis according to the manufacturer's protocol. These expression arrays protocols are also described in details in (<http://gostat.wehi.edu.au/>). Probe intensities were median normalized and floored to the median level of the negative control probes (3xSLv1). The average log intensity values for each probe were determined and the ratio of YPD samples to experimental samples was calculated. Change in total transcript levels were determined by comparing the differential enrichment compared to YPD. Gene ontology

analysis was performed using the SGD database on Gostat (<http://gostat.wehi.edu.au/>) using the Benjamini correction for multiple hypothesis testing.

Sporulation of cells isolated by magnetic and fluorescence cell sorting

Nomenclature used to describe cell age—New-born cells are isolated as biotin negative cells and have a typical average age of 1.5 – 2 generations. Young cells are isolated as biotin positive cells in sort 1 (Supplemental Figure 2) and have a typical average age of 4 – 6 generations. Aged cells are isolated as biotin positive cells in sort 3 (Supplemental Figure 2) and have a typical average age of 13 –17 generations.

The method is a modification of that described by Smeal et al. (1996) and Chen et al (2003) and is outlined in Supplemental Figure 2. In all experiments cells lacking *FLO8* were used to avoid clumping of cells. Strains were first grown overnight on YEP plates containing 2% glycerol. Cells were subsequently grown in YEPD over night and harvested before cultures reached an OD₆₀₀ of 1.0×10^8 cells were then labeled with biotin according to the manufacturer's instructions (Pierce Chemical). Biotinylated cells were grown for six generations in YEPD at room temperature. Cells were then harvested and incubated with 75 μ l of anti-biotin magnetic beads (Miltenyi Biotechnology, Germany) per 10^8 cells for 1 hour at 4°C. Cells were washed and sorted magnetically using LS depletion columns with a QuadroMacs sorter (sort 1). Cells isolated in this manner were grown in YEPD for another six generations and biotin-positive cells were again isolated using magnetic beads. Biotin positive cells were then incubated with streptavidin-Alexa647 (Molecular Probes) for 1 hour at room temperature in the dark, washed once with PBS pH 7.4 and then sorted by fluorescence activated cell sorting (FACS) using an Aria 2 or a MoFlo FACS sorter (sort 2). Between 2 and 4×10^6 /ml sorted biotinylated cells were then grown for at least 20hrs in pre-sporulation medium (YEP+2% potassium acetate; YPA) at 30°C. Both, biotin positive and biotin negative cells were isolated using FACS (sort 3), washed twice with water and once with sporulation medium (0.3% Potassium Acetate, pH 7.0). 5×10^5 biotin positive or biotin negative cells were resuspended in 0.5 mls of sporulation medium in a 5ml polystyrene round-bottom tube and sporulated at 30°C for at least 20hrs. Unless otherwise noted cells were sporulated in 0.3% Potassium Acetate (pH 7.0) and 200 cells were examined. For the preconditioned medium experiments, wild-type new-born cells were sporulated for <20hrs with regular sporulation medium and spun down. The medium was then collected and filtered. The pH of the preconditioned medium was checked to be ~8 prior adding 0.1% potassium acetate (final volume 1ml). Meiosis with the sorted cells was then started in Erlenmeyer flask.

Other methods

The statistical analyses were performed using Fisher's Exact Test using Mstat software (Norman Drinkwater, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison). Standard deviations are shown in all graphs. To determine cell viability, 1000 biotin positive and biotin negative cells obtained from all three sorts were plated on YEPD plates, YPG (YEP +2% glycerol) or YPG+Ethanol (YEP +2% glycerol + 2% ethanol). Plates were incubated at 30°C for ~50hrs and colonies were counted. Nop1 localization was performed as described in Hochwagen et al. (2005) Bud scar staining was performed by incubating cells with Lectin-Alexa 594 at a 1:1000 dilution (Molecular Probes, 0.5mg/ml) for 1hour at room temperature. Scar counting was performed using an Axioscope 2 (Carl Zeiss Inc., Thornwood, NY; equipped with a Hamamatsu Digital Camera, Japan).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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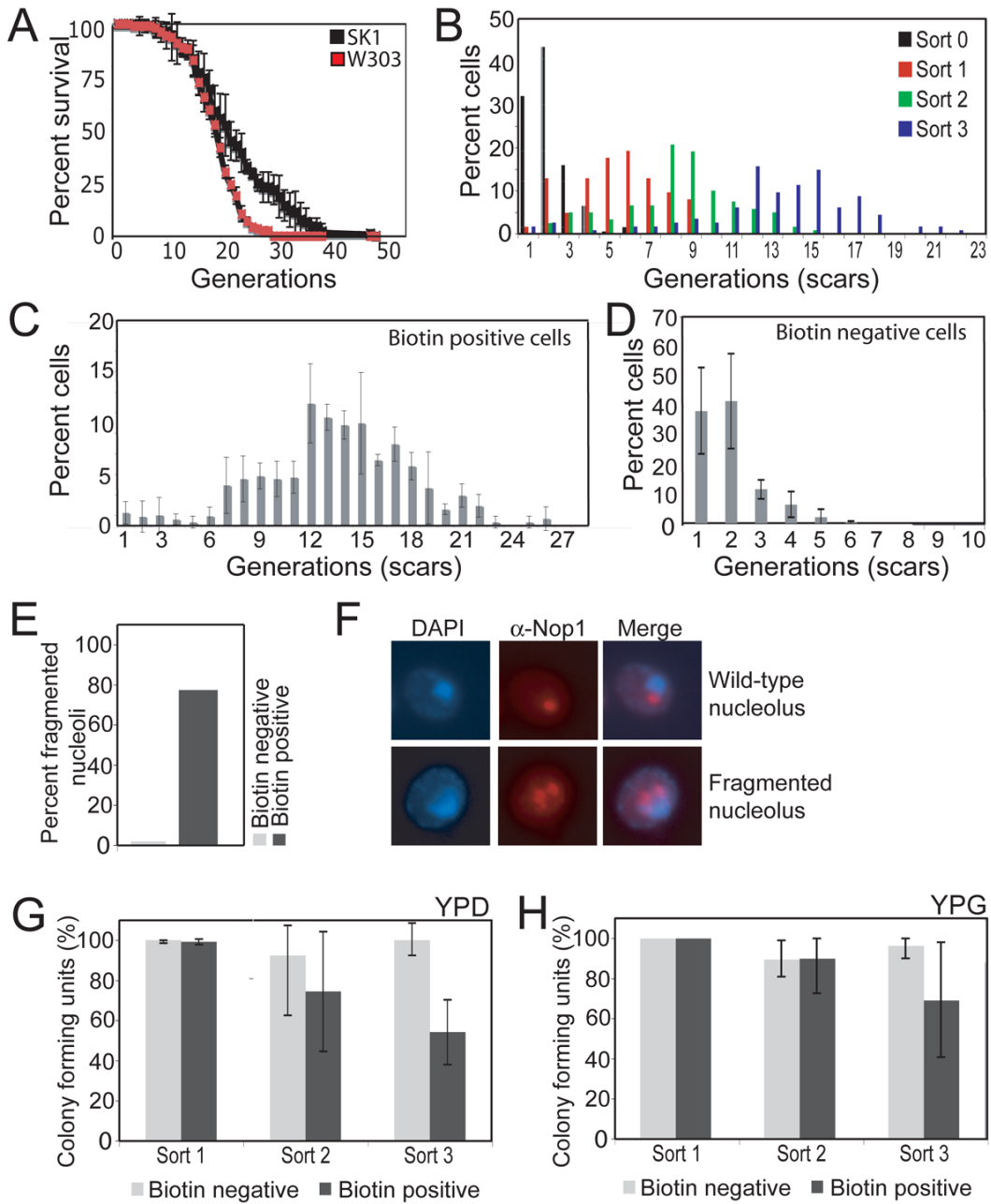


Figure 1. A method to study the effects of replicative age on meiosis in yeast

(A) The life-span of wild-type W303 (A702, closed squares) and wild-type SK1 (A727, open squares) cells was compared by single-cell analysis. The experiment was performed in triplicate using at least 40 cells for each experiment. Each cell that we analyzed was the granddaughter of the cell initially isolated at random from the population. The median life-span for W303 was 19.7 (SD=0.58) and the mean life-span 18.9 (SD=0.96). For SK1 the median life-span was 21.5 (SD=2.29) and mean life-span 22.5 (SD=0.95).

(B) The age distribution of biotin positive cells (A16554) after each sort was determined by counting the number of bud scars ($n>50$ for each experiment).

(C) The age distribution of biotin positive cells (A16554) after the third sort was determined by counting the number of bud scars ($n>50$ for each experiment).

(D) The age distribution of biotin negative cells (A16554) after the third sort was determined by counting the number of bud scars ($n > 50$ for each experiment).

(E, F) The percentage of cells (A16554) with a fragmented nucleolus as judged by Nop1 staining was determined in biotin positive cells obtained from sort 3 (E; $n = 200$ for biotin negative cells; $n = 400$ for biotin positive cells). The picture in (F) shows a cell with an intact nucleolus (top) and a cell with a fragmented nucleolus (bottom). Nop1 is shown in red, DNA in blue.

(G, H) After each sort, biotin positive and biotin negative cells (A16554) were plated on YEP + 2% glucose plates (E) or YEP + 2% glycerol plates (F) and the number of colonies that formed were counted.

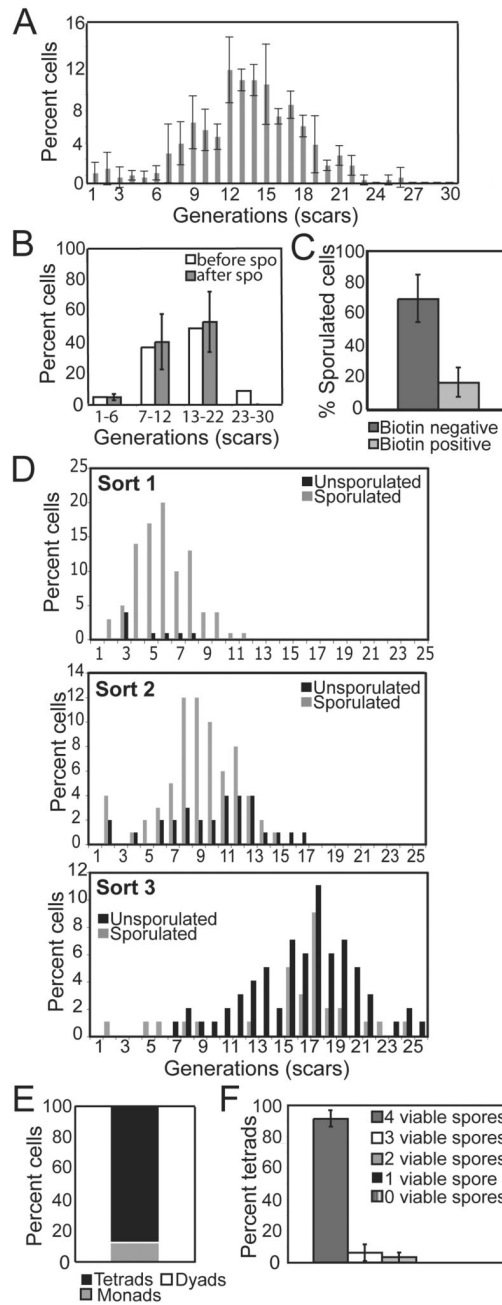


Figure 2. Aged cells fail to sporulate

(A–C) Cells were grown and sorted as described in Experimental Procedures to obtain biotin positive old cells and biotin negative new-born cells. Biotin positive cells (A16554) with the age distribution shown in (A) were induced to sporulate and the age distribution in cultures was determined before and after incubation in sporulation medium (B). The sporulation efficiency was determined 24 hours after transfer in sporulation medium (N=8 experiments) (C).

(D) Cells (A16554) were grown and sorted as described in Experimental Procedures. After sort 1 a fraction of biotin positive cells was induced to sporulate (Sort1). After 24 hours the number of bud scars per cell was determined and correlated with the cell’s ability to sporulate. The cells obtained from sort 2 and sort 3 were analyzed in the same manner (n=100). The

probability of results from different sorts being the same is as follows: Sort 1 and sort 2 $p=0.0001096$, sort 1 and sort 3 $p=1,6\times 10^{-20}$, sort 2 and sort 3 $p=2,3\times 10^{-8}$. (E, F) Biotin negative cells (A16554) were induced to sporulate. After 24 hours sporulation efficiency (C) and the percentage of monads, dyads and tetrads (D) was determined. The number of viable spores in tetrads is shown in (E) (n=240 tetrads).

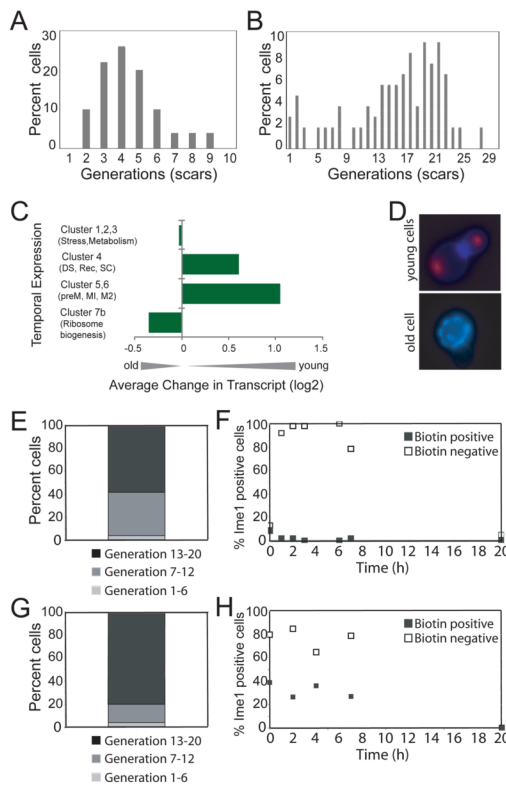


Figure 3. Aged cells do not enter the meiotic program

(A–C) Wild-type cells (A16554) were grown and sorted as described in Experimental Procedures. Cells sorted either once (young cells, age distribution shown in (A)) or three times (old cells; age distribution shown in (B)) were induced to sporulate for 6 hrs to determine their gene expression pattern. (C) shows average changes in transcript levels for genes identified as up-regulated during meiosis in Primig et al. (2000). Grouped clusters identified by Primig et al. are shown on the y axis. Abbreviations: DS = DNA synthesis, Rec = Recombination, SC = sister chromatid cohesion, preM = pre-meiotic, MI = Meiosis I, M2 = meiosis II.

(D – H) Cells carrying the *NLS-mCherry* fusion under the control of the *IME1* promoter (A18489) were grown and sorted as described in Experimental Procedures and induced to sporulate in standard sporulation medium. The presence of mCherry was analyzed at the indicated times in biotin positive cells with the age distribution shown in (E) and biotin negative cells (F). (D) shows an old cell that fails to express *IME1-NLS-mCherry* and a new-born cell that does. Bud scars are shown in blue, *IME1-NLS-mCherry* fusion in red.

(G, H) Cells carrying the *IME1-NLS-mCherry* fusion (A18489) were grown and sorted as described in Experimental Procedures. Then the biotin positive (median age= 16 generations; age distribution in G) and biotin negative populations were combined and induced to enter meiosis (H). The ability to express mCherry was determined for each population at the indicated times (n>100).

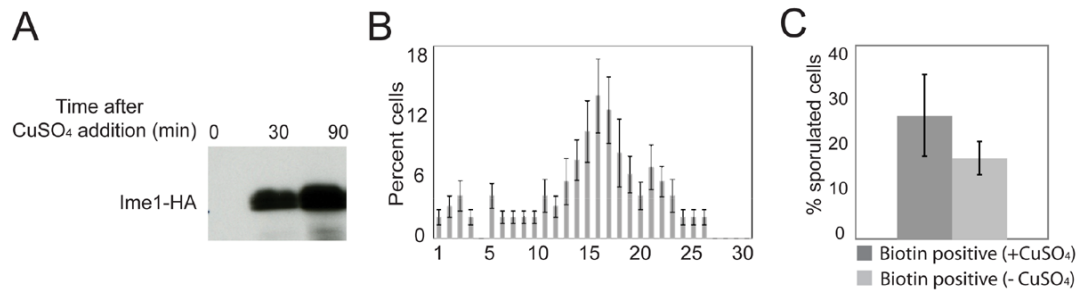


Figure 4. Overexpression of *IME1* partially suppresses the sporulation defect of aged cells
 (A-C) Cells carrying *IME1* under the control of the *CUP1* promoter (*pCUP1-IME1*; A22260) were grown in YPA for 12 hours, washed and then transferred to sporulation medium (0.3 % KOAc, 0.02% raffinose) containing 50 μ M of CuSO₄. $\sim 8 \times 10^7$ cells were used for protein extraction (TCA) for each time point. (B, C) Cells heterozygous for the *pCUP1-IME1* fusion (A22764) were grown as described in Experimental Procedures to obtain biotin positive old cells (mean age = 15.73 generations, SD= 4.96) with the age distributions shown in (B) were either transferred to sporulation-inducing conditions either in the presence (+CuSO₄) or absence (-CuSO₄) of CuSO₄. The percentage of sporulated cells was determined after 24 hours. $P=1.025 \times 10^{-9}$.

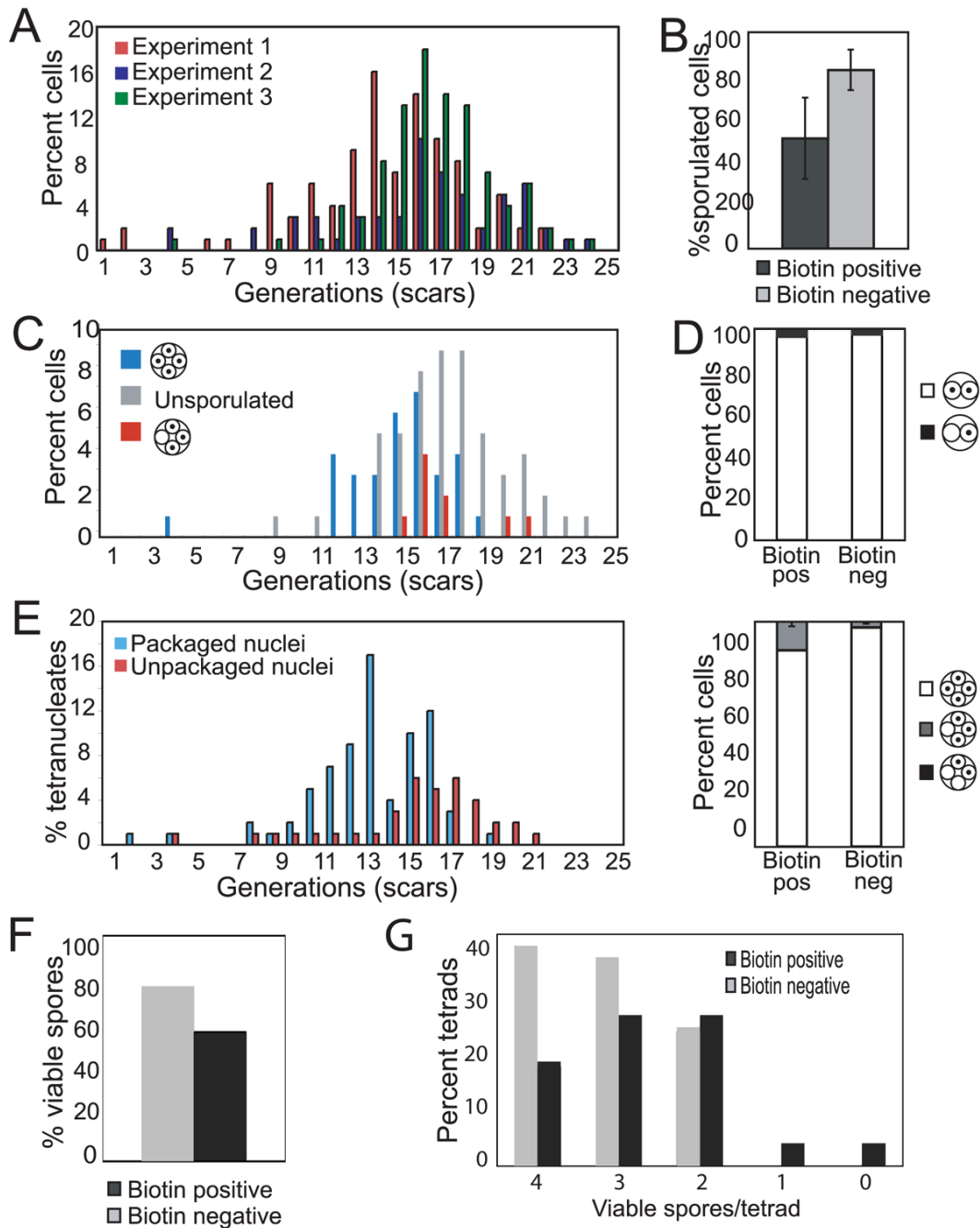


Figure 5. Increased meiosis II non-disjunction in aged cells

(A–D) Cells carrying GFP dots at *LYS2* on both copies of chromosome II (homozygous *LYS2*-GFP dots; A16554) were grown and sorted as described in Experimental Procedures. Biotin positive cells with the age distribution shown in (A) and biotin negative cells were induced to sporulate in preconditioned medium. After 24 hours overall sporulation efficiency (B; $n > 100$ cells) and the number of bud scars per cell was determined and correlated with the cell's ability to sporulate (C; $n = 95$). Unsporulated cells are shown in grey, sporulated cells with a wild-type distribution of GFP dots are shown in blue and cells with GFP dots in only three of the four nuclei are shown in red. The overall GFP dot distribution in biotin positive and biotin negative cells during both Meiosis I and Meiosis II are shown in (D; $n = 100/\text{experiment}$; binucleate

analysis top panel; tetranucleate analysis bottom panel). Note that in 43% of aged cells had a daughter cell attached to it. In the event that this daughter cell sporulated (40%) the GFP dot segregation pattern was not examined.

(E) Aged mother cells (A16554) were isolated and induced to sporulate in preconditioned medium. The ability of cells to package the four meiotic products into spores was correlated with the number of bud scars. The results represent data from three independent experiments (n=112).

(F, G) Tetrads obtained from aged mother cells (biotin positive cells; average age =15.72 SD= 5.53; A16554) were identified and dissected using a fluorescence/dissecting microscope. Percent of viable spores (N= 200 spores) is shown in (F) and the number of viable spores per tetrad (N=50 tetrads) in (G).

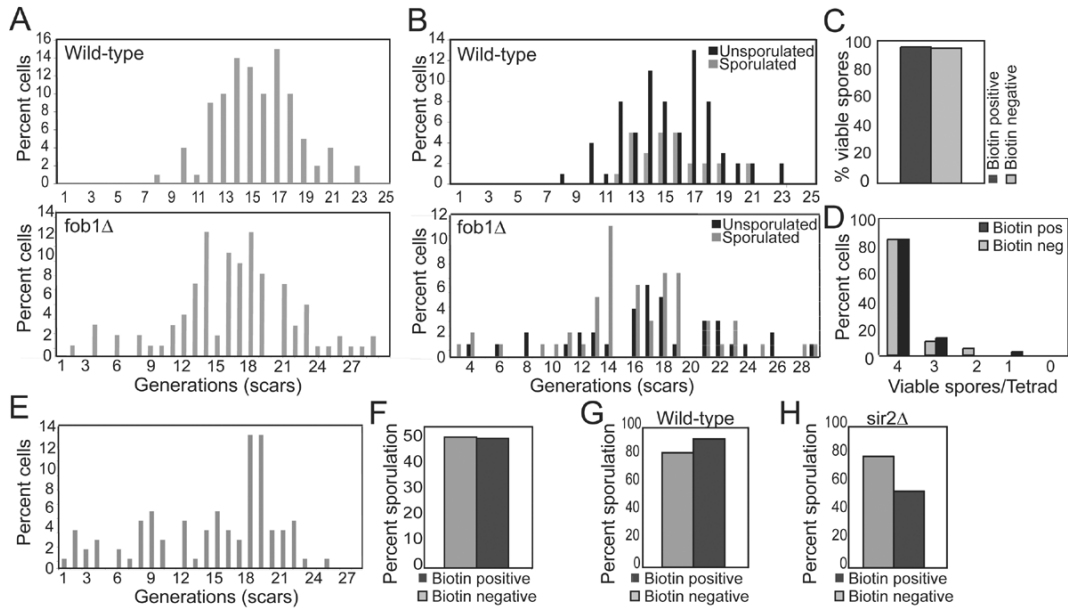


Figure 6. A mutation that extends the replicative life span suppresses the sporulation defect of aged cells

(A–D) Wild type cells (A16554) and cells lacking *FOB1* (A18319) were grown and sorted as described in Experimental Procedures. Biotin positive cells with the age distribution shown in (A) and biotin negative cells were induced to sporulate. After 24 hours the number of bud scars per cell was determined and correlated with the cell's ability to sporulate (B; n=100). Tetrads obtained from *fob1Δ* cells were dissected to determine spore viability (C; n=160) and the number of viable spores in tetrads (D, n=40 tetrads).

(E, F) Cells overexpressing *SIR2* (A20375) were grown and sorted as described in Experimental Procedures. Biotin positive cells with the age distribution shown in (E) and biotin negative cells were induced to sporulate for 24 hours to determine the number of sporulated cells (F; n>100).

(G, H) Wild type (A16554; G) and *sir2Δ*(A20782; H) cells were sorted one time by FACS and induced to sporulate. Biotin positive (median age 6 generations) and biotin negative cells (median age 2 generations) were sporulated for 24 hours to determine the number of sporulated cells (n>100).

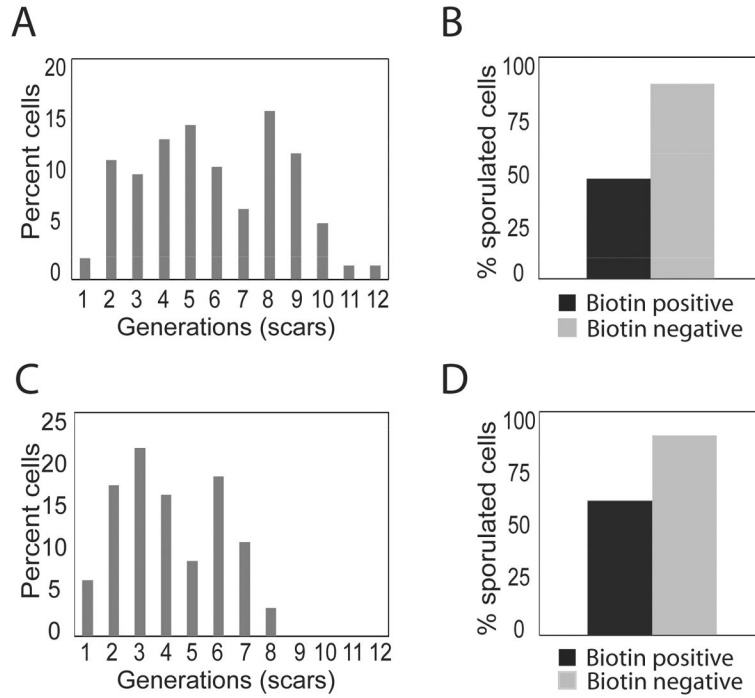


Figure 7. *ERCs* decrease the ability of the cells to enter meiosis

(A, B) Biotin positive cells harboring a 2 μ plasmid carrying the *FOBI* gene (A22532) were first grown in medium lacking uracil, labeled with biotin, then grown in presporulation medium (YEP+2% potassium acetate; YPA) for few generations and sorted one time to obtain young and new-born cells. Biotin positive young cells with the age distribution shown in (A) and biotin negative new-born cells were induced to sporulate for 24 hours to determine the number of sporulated cells (n>100) (B).

(C, D) Cells harboring a 2 μ plasmid carrying the 5S and the 35S rDNA repeats (A22611) were first grown in medium lacking leucine, labeled with biotin, then grown in presporulation medium (YEP+2% potassium acetate; YPA) for few generations and sorted one time to obtain young and new-born cells. Biotin positive young cells with the age distribution shown in (C) and biotin negative new-born cells were induced to sporulate for 24 hours to determine the number of sporulated cells (n>100) (B).