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Aggregation of an RNA-binding protein, not loss of heterochromatin, causes sterility of aging yeast cells

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Abstract

In yeast, heterochromatin silencing is reported to decline in aging mother cells causing sterility in old cells. This process is thought to reflect a decrease in the activity of the NAD^+-dependent deacetylase Sir2. Here, we tested whether Sir2 becomes nonfunctional gradually or precipitously during aging. Unexpectedly, silencing of the heterochromatic *HML* and *HMR* loci was not lost during aging. Old cells could initiate a mating response, however they were less sensitive to mating pheromone than were young cells due to age-dependent aggregation of Whi3, an RNA-binding protein controlling S-phase entry. Removing the poly-glutamine domain of Whi3 restored the pheromone sensitivity of old cells. We propose that aging phenotypes previously attributed to loss of heterochromatin silencing are instead caused by aggregation of the Whi3 cell-cycle regulator.

Budding yeast divide asymmetrically, and each yeast mother cell produces a finite number of daughter cells in her lifetime. This process—yeast replicative aging—has been studied for insights into aging more broadly because the processes that underlie aging in yeast might be related to factors that underlie aging in other asymmetrically dividing cells (1).

In *Saccharomyces cerevisiae*, haploid mother cells lose the ability to mate as they age (2). It was proposed that old mother cells fail to mate as a consequence of a decline of Sir2 function, which would cause loss of heterochromatic gene silencing of the auxiliary mating type loci *HML* and *HMR* (3). Loss of silencing at *HML* and *HMR* in old cells has been attributed to the redistribution of Sir proteins to the nucleolus and to a decrease in available Sir2 (4–6). Furthermore, old cells may either be either limited for the Sir2 substrate NAD+ or may be exposed to high concentrations of nicotinamide (NAM), an inhibitor of Sir2, resulting in the inactivation of Sir2 in old cells and thus sterility (7–9).

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Supplementary Materials:
Materials and methods
Supplementary figures S1–S5
Supplementary movies S1–S2
Supplementary table S1

We characterized transcriptional repression by Sir2 by testing whether transient loss-of-silencing events at *HML* might precede the complete loss of silencing attributed to the oldest cells. To study silencing at *HML* in a yeast mother cell, we monitored pedigrees of haploid cells carrying a Cre-based silencing reporter (10). The reporter uses a Cre recombinase gene inserted in place of *HMLα2*, and a fluorescent reporter inserted at a euchromatic locus elsewhere in the genome (Fig. 1A). Loss of silencing at *hmlα2Δ::CRE* induces a permanent and heritable switch from expressing red fluorescent protein to expressing green fluorescent protein (Fig. 1A), and the sensitivity of the Cre reporter approaches the sensitivity of single molecule RNA florescent *in situ* hybridization (10). We manually separated daughter cells from their mothers to analyze pedigrees in two common strain backgrounds, S288c and W303, and observed no loss-of-silencing events in dozens of pedigrees of haploids, diploids and hybrids (Fig. 1B).

To measure the frequency of silencing loss as a function of a cell's lifespan, we extended the pedigree analysis by using a microfluidic device that traps mother cells and separates their buds. We analyzed more than 1500 yeast pedigrees at single cell resolution and observed 13 loss-of-silencing events (Fig. 1C, movie S1). Furthermore, we found that a cell's age did not affect its ability to maintain silencing of *HML*, and the overwhelming majority of yeast mother cells stopped dividing without even a transient loss of silencing (Fig. 1D). As a control, when Sir2 activity in old cells was inhibited by addition of nicotinamide, all surviving cells lost silencing suggesting that old cells did not accumulate nicotinamide in amounts that inactivate Sir2 (Fig. 1E, movie S2). Similar results were obtained by analyzing a green fluorescent protein gene inserted in place of *HMLα* and with an alternative microfluidic design, indicating that the observation was independent of the reporter and the microfluidic setup used (Fig. S1). Previous studies have shown that Sir2 protein levels decrease in cells older than seven generations old, however we found no evidence of a decrease in Sir2 activity at *HML* (4). It is possible that a decrease in Sir2 levels in old cells could affect other Sir2 complexes, including the nucleolar RENT complex. Inactivating the RENT complex would decrease rDNA silencing and increase the occurrence of extrachromosomal rDNA circles in old cells, two phenotypes that have been repeatedly observed in old cells (1).

We also purified populations of aged *MATα* cells from liquid culture and analyzed them for expression of RNA from the *HMR* locus. Old cells cultured for ~20 generations showed low expression of *HMRa1* mRNA relative to cells cultured in the presence of the Sir2 inhibitor nicotinamide, establishing that Sir2-dependent silencing of auxiliary mating type loci was functional in old cells (Fig. 2A,B). In addition to analyzing silenced RNA from *HMR*, we analyzed expression of *STE3*, which encodes the a-factor pheromone receptor, is expressed in *MATα* cells and is repressed in diploids. *STE3* expression did not reflect a repressed diploid-like gene expression program that would be expected if mating-type information from *HMR* were expressed (Fig. 2A,B).

Although the *HML* and *HMR* loci are the premier context for studying Sir-based transcriptional repression, published RNA sequencing of cells lacking Sir2 has identified the full complement of genes that are subject to repression by Sir2 (13). Separately RNA sequencing data has been published from matched young and old cells, which includes RNA

expression data from these Sir2-regulated loci (14). We reanalyzed these data to ascertain whether Sir2-regulated genes show age-associated changes in transcription that could reflect loss of Sir2 function. Genome-wide, we found no evidence that Sir2-dependent gene regulation was related to aging-dependent gene regulation (Fig. 2C). Furthermore, telomeric open reading frames repressed directly by Sir2 showed no evidence of an age-dependent increase in transcription (Fig. S2). In short, we found no evidence that the transcriptional program in *sir2Δ* cells was similar to the transcriptional program in old yeast cells.

Having shown that *HML* and *HMR* were silenced during aging, we reinvestigated the sterility phenotype reported for old cells (3, 12). We treated young and old *MATa* cells with various amounts of α-factor pheromone and monitored their ability to arrest in G1 and grow a mating projection (Fig 3A). Previous experiments that identified an age-associated mating defect used assays sensitized by using a low concentration (less than 20ng/mL) of α-factor (3). Indeed, we found that old mother cells (mean age: 14.3 divisions) responded less efficiently to mating pheromone than did young cells; however, they responded efficiently with pheromone concentrations above 20 ng/mL (Fig. 3B). If the observed loss of mating depended on expression from *HML*, then deletion of *HML* would restore the sensitivity of old cells to the level of young cells, which was not the case (Fig. 3C). Young and old yeast deleted for *HMLα2* were more responsive to α-factor than were wild type cells (Fig. 3C and Fig. S3). Arrest with α-factor did not affect the stability of silencing at *HML*, indicating that the heightened sensitivity in *hmlα2Δ* mutants did not reflect transcription from *HML* during α-factor treatment (Fig. S4).

Efficient response to mating pheromone depends on arrest in the G1 phase of the cell cycle. Cells exposed to α-factor for longer than four hours escape this cell cycle arrest and become less sensitive to pheromone (15). This adaptation depends on aggregation and subsequent inactivation of Whi3, an RNA-binding S-phase inhibitor, but desensitized mothers produce daughters that are fully sensitive to α-factor (15). Old cells showed a similar asymmetric inheritance of mating competence: the daughters of old cells were more responsive to α-factor than were their mothers (Fig. 4A). To test whether aggregation of Whi3 might explain why old yeast mother cells fail to respond to α-factor, we deleted a glutamine-rich domain required for Whi3 aggregation in cells adapted to α-factor and assayed old *MATa* cells carrying this deletion for α-factor responsiveness. Indeed, deletion of the Whi3 glutamine-rich domain decreased the loss of sensitivity in old cells, indicating that Whi3 aggregation may prevent mating in old cells (Fig. 4B). Live-cell imaging of old yeast mother cells expressing a green florescent protein-tagged Whi3 indicated that old yeast cells did form aggregates of Whi3 (Fig. 4C,D and Fig. S5). Interestingly, *whi3-ΔpolyQ* strains lived slightly longer than wild-type strains, suggesting that aggregation of Whi3 might limit lifespan (Fig. 4E).

Although our conclusions regarding whether aging impacts gene silencing stand in contrast with previous work, the methods we used were more sensitive and extensive than those available in the past. Although it would be best to repeat analyses with exactly the same strains used previously, over the years those strains have been lost, precluding a direct comparison. Nevertheless, our data establish that age-dependent loss of gene silencing is not a feature of widely used budding yeast strains.

The mechanism by which yeast deleted for *HML* show slightly decreased sensitivity to mating pheromone independent of transcription at the locus is unclear. Interruption of the silent *HML* locus could have an indirect effect on mating factor sensitivity, perhaps by inducing changes in the three-dimensional architecture of chromosome III that affect expression of genes involved in the α-factor response.

Both yeast and vertebrates are rich in RNA-binding proteins containing low-complexity prion-like domains. Unlike aggregates of typical yeast prions, Whi3 aggregates are sequestered in the mother cell during cell division (15). Aggregation during aging may be an intrinsic liability for yeast memory factors (mnemons) like Whi3 that encode memory in the form of protein aggregates. In this view, aging-induced aggregation of Whi3 would preclude alpha-factor induced Whi3 aggregation as a memory of past unsuccessful mating encounters. It is tempting to speculate that in nature yeast could benefit from a *bona-fide* differentiation between old and young cells, with some aggregates being beneficial and others not. Understanding why Whi3 aggregates form and are retained in the mother cell during mitosis may shed light on how protein aggregation influences the mitotic inheritance of cytoplasmic factors more broadly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One sentence summary

Old cells lose sensitivity to α-factor, but that phenotype wasn't caused by failure of Sir2: it was caused by aggregation of the G1/S inhibitor Whi3.

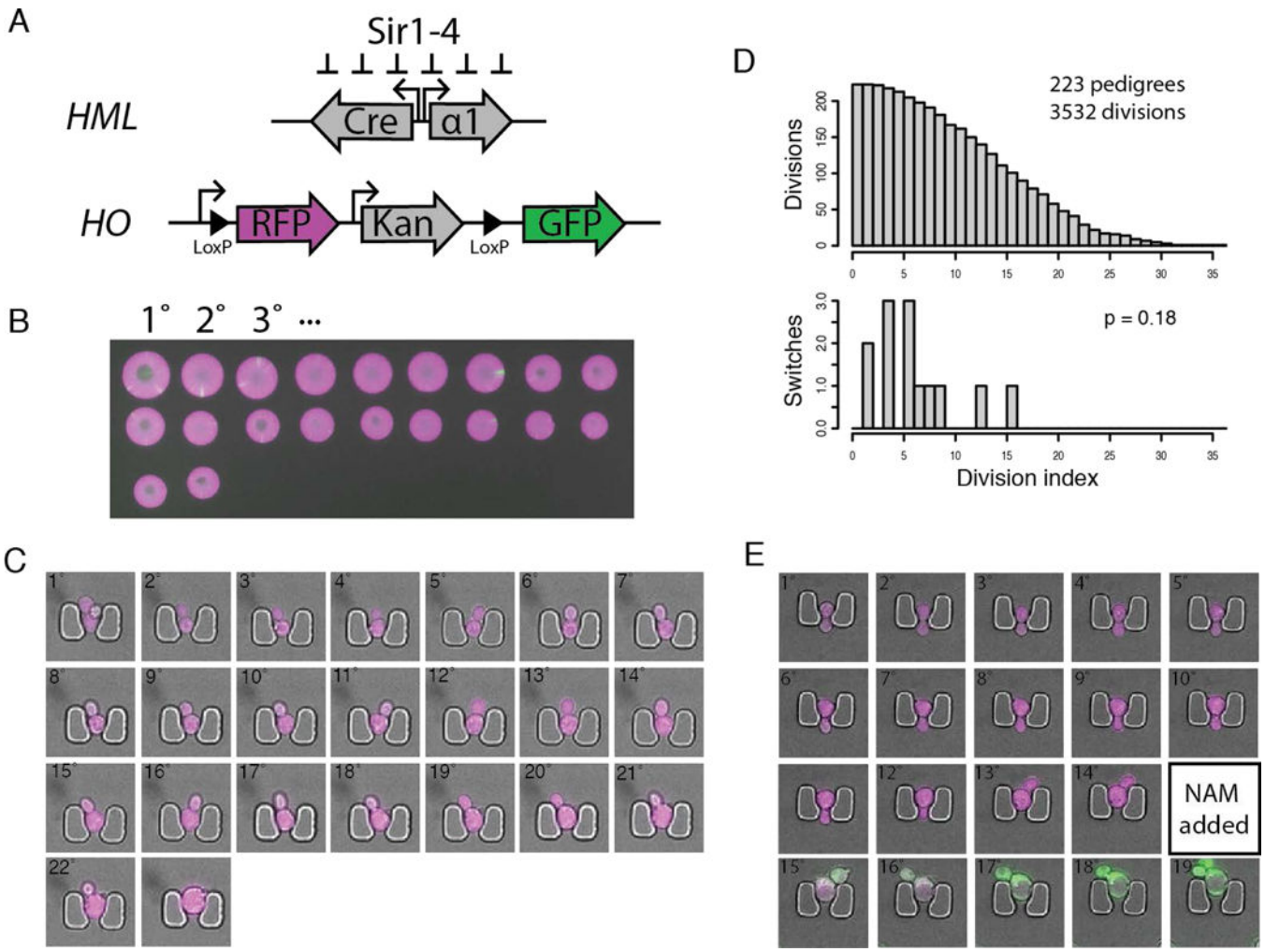

Fig 1. Loss of silencing is not a feature of yeast aging

(**A**) Schematic of the CRASH (**C**re-**R**eported **A**ltered **S**tates of **H**eterochromatin) reporter (10). (**B**) Representative pedigree of 20 sequential daughters from W303 haploid strain (JRY10774) carrying the CRASH reporter. (**C**) Frames depict each division event in a typical pedigree in the S288C haploid strain background (JRY10772). (**D**) Top - histogram of all cell-division events that occurred for 223 pedigrees from a microfluidic experiment using JRY10772. Bottom - the age of cells at the time they lost silencing is plotted as a histogram for the 13 pedigrees that lost silencing. Division index refers to the number of buds that each mother produced after the cells were loaded on the microfluidic chip, and the p-value was calculated using the Kolmogorov-Smirnov test. (**E**) Frames depict consecutive daughters of a pedigree as in (C), and nicotinamide (NAM) was added to the medium after ~24h.

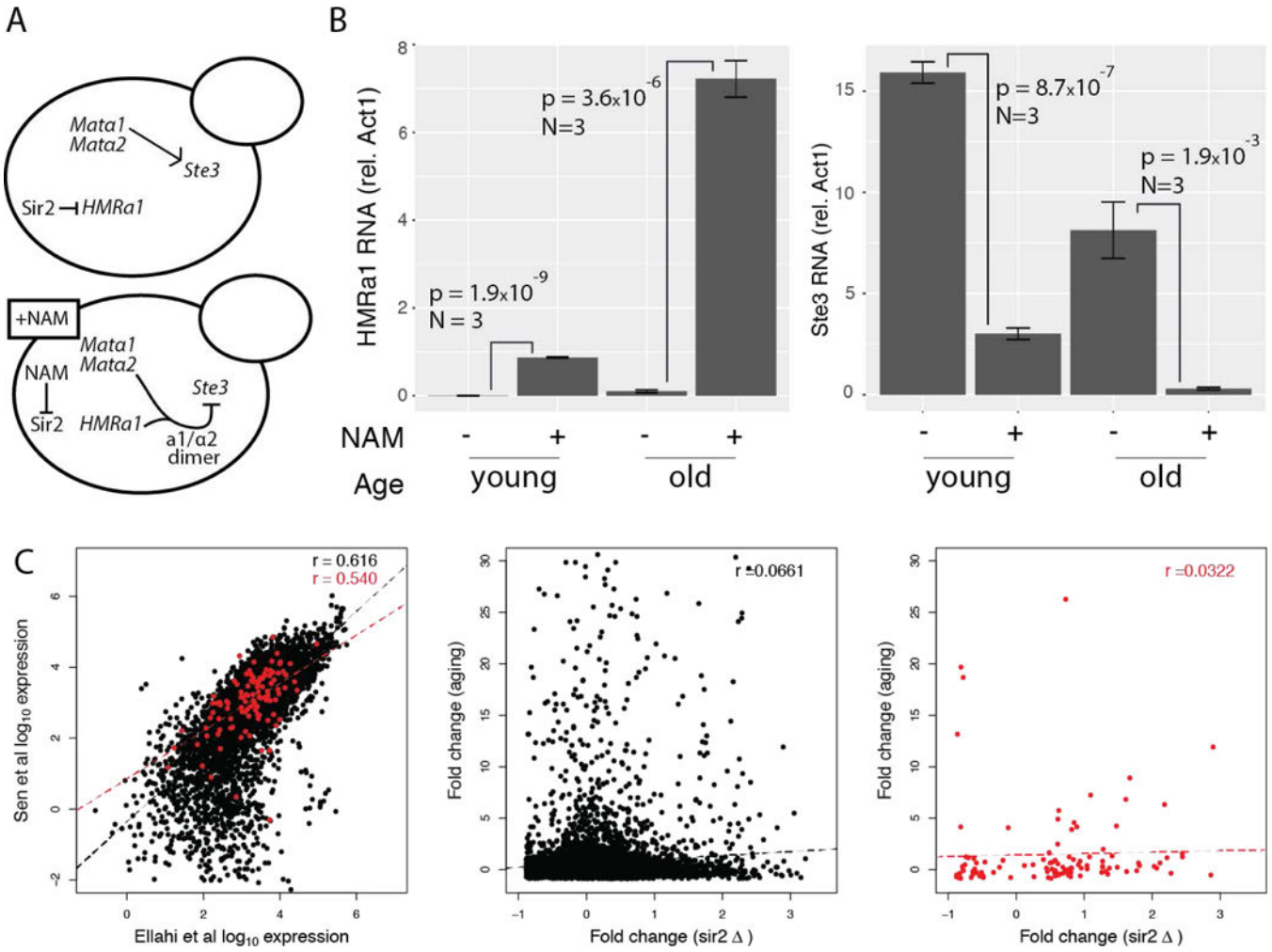

Fig 2. Mating pathways genes did not show diploid-like RNA expression in old cells

(**A**) Schematic of mating-factor receptor regulation for a *MATα* haploid strain. (**B**) RT-qPCR for the Sir2-regulated *HMRa1* RNA and the mating-type regulated *STE3* mRNA. To account for difficulty in handling small numbers of cells (10^6–10^7 old cells were prepared for RT-qPCR), we restricted our analysis to direct comparison of age-matched samples where the RNA preparation conditions were directly comparable. P-values were calculated by two-tailed T-test. (**C**) Reanalysis of published RNA sequencing data. At left, young cells from two different publications (13, 14) showed repeatable RNA expression. Red dots indicate genes that are regulated by Sir2 as defined by Ellahi *et al* (13). At center, fold-change in expression is plotted for old cells from Sen *et al* (14) compared to matched young cells and for *sir2Δ* cells from Ellahi *et al* (13) compared to a matched wild-type control strain. At right, the subset of genes from the center panel that were identified as Sir regulated by Ellahi *et al* are plotted.

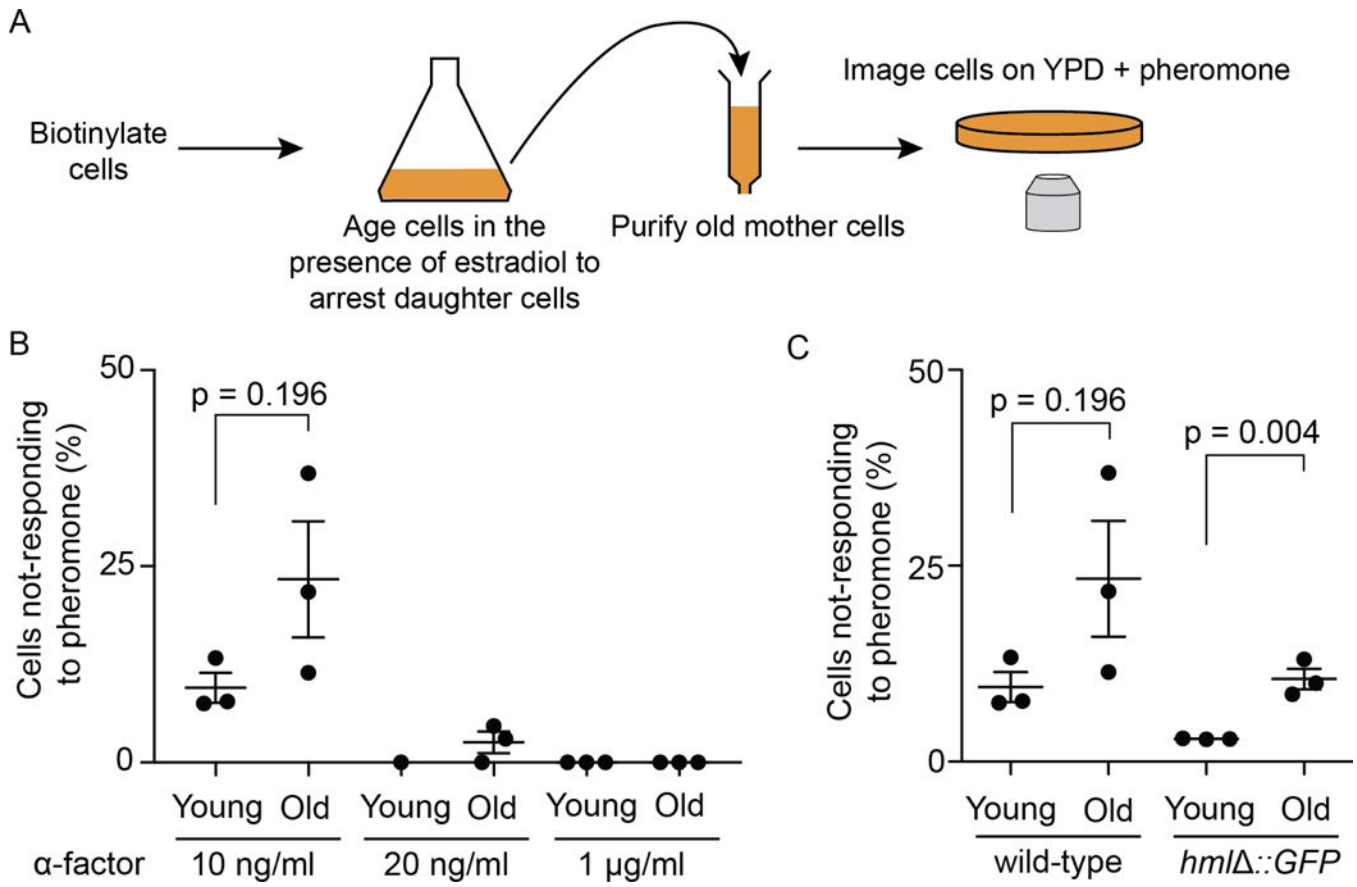

Fig 3. Old cells required a higher pheromone dose than young cells to form a mating projection

(**A**) Schematic of the experimental approach (**B**) Young and old (on average 14 divisions old) *MATa* cells (yYB4172) were purified from 2 hour and 20 hour cultures, respectively, and their response to pheromone was assayed on agar pads containing indicated α-factor concentrations. Fraction of cells not-responding to α-factor (at 10 ng/ml) increased with age, however all cells responded to higher concentrations of α-factor. (**C**) Young and old cells of yYB6829 (*hmlΔ)* strain were tested for pheromone response to α-factor (10 ng/ml). Both wild type and *hml*Δ mutant cells lost pheromone sensitivity to a similar extent with age, however *hmlΔ* cells were more sensitive to pheromone than the corresponding wild-type cells (yYB4172, data from Fig. 3B repeated for comparison. All the plots show mean values ± SEM, dots represent independent experiments (n≥30 cells). P values were calculated by two-tailed T-test.

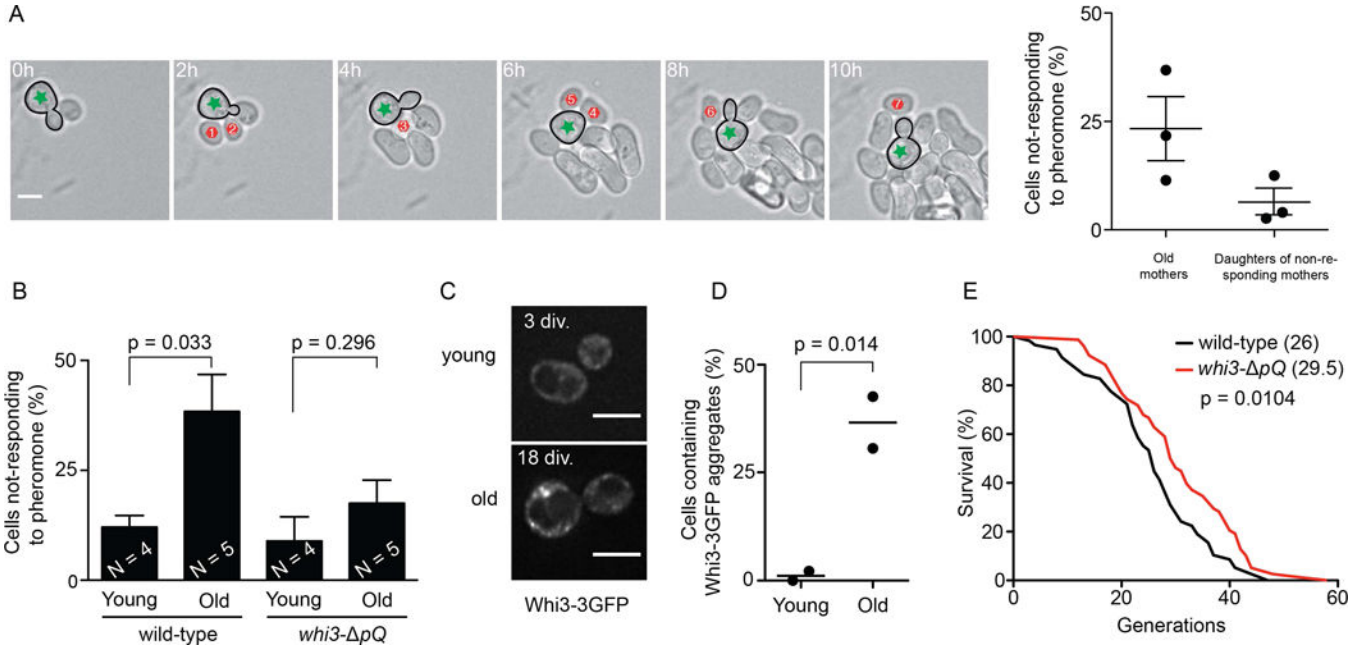

Fig 4. Formation of Whi3 aggregates contributes to the loss of pheromone sensitivity with age

(**A**) Left: example sequence showing old mother cell and her progeny exposed to α-factor (10 ng/ml). An old mother cell (green asterisk) buds instead of responding to pheromone, but her daughters arrest in G1 and form mating projections. Right: quantification of pheromone response of first 3 daughters of pheromone-insensitive old mothers of yYB4172 from figure 3B. (**B**) Old and young *MATa* cells were exposed to α-factor (10 ng/ml). Pheromone insensitivity increased with age in the wild-type strain (3.2-fold between young and old cells) while this effect was reduced in *whi3-ΔpQ* cells (1.9-fold increase). Young cells were ~5 divisions old, and old cells were between 15 and 20 divisions old. Bars show mean value ± SEM (young cells total n > 200, old cells total n > 170). P-values were calculated by unpaired two-tailed T-test. (**C**) Whi3 forms aggregates in old cells. (**D**) Quantification of the fraction of cells containing Whi3-3GFP aggregates in young and aged yeast cells. Each dot represents an independent experiment and p-value indicates one-tailed T-test. **(E)** Survival curves of wild-type and whi3-ΔpQ strains (wild type yYB14326 n = 58, whi3-ΔpQ yYB14325 n = 78 cells). Deletion of the glutamine-rich domain of Whi3 extends lifespan. P-value was calculated with the Log-rank (Mantel-Cox) test.