Pathways Change in Expression During Replicative Aging in *Saccharomyces cerevisiae*

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Running head: Gene Expression in Yeast Aging

Abstract

Yeast replicative aging is a process resembling replicative aging in mammalian cells. During aging, wild type haploid yeast cells enlarge, become sterile, and undergo nucleolar enlargement and fragmentation; we sought gene expression changes during the time of these phenotypic changes. Gene expression studied via microarrays and qPCR has shown reproducible, statistically significant changes in mRNA of genes at 12 and 18-20 generations. Our findings support previously described changes towards aerobic metabolism, decreased ribosome gene expression, and a partial Environmental Stress Response. Our novel findings include a pseudo-stationary phase, down-regulation of methylation-related metabolism, increased Nucleotide Excision Repair related mRNA, and a strong up-regulation of many of the regulatory subunits of protein phosphatase I (Glc7). These findings are correlated with aging changes in higher organisms as well as with the known involvement of protein phosphorylation states during yeast aging.

Introduction

Replicative aging in the budding yeast, *Saccharomyces cerevisiae*, is an asymmetric process that affects mother cells, but not daughter cells until late in the aging process (1, 2). In each division, the mother cell becomes a generation older, enlarges, and acquires a chitin-containing bud scar, while the daughter cell emerges at age 0 generation (g). An average mother cell of typical wild type yeast strains lives ~25g.

Dysregulation of genes has been proposed to underlie part of the aging process (3, 4). In mouse liver cells, DNA methylation diminishes through much of the life span with consequent expression of repressed genes (5-8). Large scale comparative microarray studies of mRNAs in aging animals, and in mutants considered to affect the aging process, have found many mRNA differences, especially in expression of stress related genes (9-13).

Age-related mRNA changes have also been described in yeast (14-16). Often, few ages and/or few replicate microarrays were examined. In addition, precautions to make the young controls equivalent in stress to the aged cells were difficult to perform and were handled differently in each case. A few findings run throughout the experiments, but many findings are not consistent.

The earliest yeast microarray study published (14) used three pairs of microarrays each for young and 8g yeast cells and showed that 8g cells had shifted from ethanol fermentation to gluconeogenesis and lipid metabolism. They grew the isolated aging mother cells for 30 minutes after magnetic sorting to alleviate sorting stress, but that growth may have introduced newborn daughters. The gene expression changes seen on the arrays were not extensive. For example, the only genes from aerobic alcohol metabolism and energy storage (including gluconeogenesis) found up-regulated in wild type were *PCK1* (for phosphoenolpyruvate carboxykinase) and

ADH2 (for alcohol dehydrogenase). These inductions agreed with their microchemical analyses. Metabolic changes were more evident in a *sip2* mutant strain with a shortened life span and less evident in a *snf4* mutant strain with a longer life span. Their conclusions have held up quite well through the other studies. Age-related metabolic changes are particularly interesting since yeast respond to glucose limitation with an extended life span (17, 18), an analog of the caloric restriction that extends mammalian life spans.

In another microarray-based study, much older cells were isolated using centrifugal elutriation, a method that makes it difficult to obtain a similarly stressed control population (15). Two replicate arrays were performed for young and for old cells; RNAs were prepared from a pool of samples of aged cells in an effort to average out biological variation. The mRNA differences were checked via quantitative PCR. In wild type replicative aging, many genes of the ~900-gene regulon called the Environmental Stress Response (ESR) pathway (19) changed expression in the aged cells, and the DNA double strand break repair genes were induced.

However, another group found few of the ESR genes responded in aging (16). These authors used duplicate microarray slides from magnetically sorted cells at 8-12g and 18-24g (designated 10g and 20g). They found a decrease at 10g and 20g in expression of glycolysis genes, proteasome subunit genes, and protein folding chaperones. There was more mRNA for glycogen related genes, permeases, some regulatory genes, and a few others with no known role. The magnetic sorting process used by these authors was found to induce some genes for the stress response and cell wall and repress the fatty acid metabolism and histone genes in young cells.

In our study, we used 6 to 7 microarrays each at 1g, 8g, 12g, and 18-20g, including biological and technical replication as well as dye inversion. We tested important conclusions

using quantitative real time reverse transcription PCR (qPCR). In each case, we used a log phase culture grown in parallel as the microarray control. During magnetic sorting, this log phase culture was exposed to all of the same temperature conditions in parallel with the aging culture being sorted. In our view, this study has both sufficient replication and appropriate young control cells to allow accurate conclusions about age-related gene changes. In common with others, we have found a metabolic shift and a decrease in ribosome and nucleolus gene expression. In addition, we have found evidence for a down-regulation of methylation related pathways, an up-regulation of Nucleotide Excision Repair genes, a pseudo-stationary phase, and an up-regulation of many targeting subunits of protein phosphatase I.

Methods

Aging Cell Preparation

All experiments were conducted with W303Ra, the W303 wild type in which the *RAD5* gene has been corrected by Rodney Rothstein from its mutation in W303-1A. Aging cells were prepared via magnetic sorting using Miltenyi microbeads and columns (20). The average age of at least 20 mother cells was determined after staining the bud scars with Fluorescent Brightener 28 (Sigma). We performed bud scar counting using multi-plane focusing, employing a Zeiss epifluorescence microscope. Our samples were collected from log phase 1g, 8g, 12g, and 18-20g cells; cells were never permitted to go into stationary phase of growth (<0.8 A_{600}).

Microarrays and RNA

Microarrays were printed for Genome Consortium for Active Teaching (GCAT, Davidson College, NC) at Washington University, St Louis with 70-mers corresponding to non-overlapping regions of all ORFs of budding yeast (21). Total RNA was prepared from cells at different ages via Qiagen RNeasy, in parallel with young cells sorted from the same preparation

and subjected to exactly the same conditions of temperature, etc to ensure that the control and the aging cells would have the same extent of stimulation of the Environmental Stress Response genes (19). The controls normalize the environmental response, and only the aging effects are seen. Control experiments showed that this procedure identified fewer genes as 'age-related changes in expression' than comparing all ages with a single young preparation that may have experienced small differences in protocol.

For each labeled probe preparation, we started with 50 μg of total RNA and amplified aRNA using the Ambion Amino Allyl MessageAmp II Kit and the Ambion Fragmentation Kit. For each age, there were three different RNA samples prepared and compared against matched young controls. Six to seven arrays were analyzed at each age. Three microarrays of each age set were analyzed with the dyes reversed. Arrays were scanned using an Axon Gene Pix 4000B scanner and GenePix Pro 5.1 software. The quality control report was used for all arrays; Supplementary Table 1 shows the quality control cutoff values used for all arrays in this study.

Statistical Analysis

Before running statistical models, we normalized the data using Bioconductor Software (22-24). The pre-processing included removing all empty, control, and flagged genes. Also, if the fluorescence signal was under 100 in one channel it was increased to 100; if it was under 100 in both channels, the spot was removed from the analysis. The background fluorescence was subtracted from the foreground, and a lowess smoother was used to normalize the genes for each array. If more than 50% of the array spots were missing for a particular gene, the gene was removed from the analysis.

For each gene, we fit a linear model using the limma function (22,23) in Bioconductor. After fitting the model, we were able to look at particular comparisons and contrasts (e.g., 1g vs.

18-20g). Genes that were significantly changed across the comparison according to the adjusted p-value < 0.05 by the Holm step-down method (25) were designated as significant (Supplementary Tables 2 and 3).

To identify potential functional regulons, we used three software programs. First, we imported the data into GeneSpring 7.0 (Agilent) to cluster. Our second software program was GenMAPP2 (26) plus MAPPFinder (27), which identified Gene Ontology (GO)-term-based functional groups that were changed in expression. For GenMAPP2, we selected only genes that changed at least three fold, therefore not all significantly changed genes are included in the GenMAPP2 analysis. However, the analysis can identify the most consistently up- and down-regulated pathways with high confidence. The third software we used was GOCluster (28), which uses the Gene Ontology designations for every gene to detect statistically significant cellular constituents, molecular functions, and processes that differ between samples. This software adjusts the p values for multiple sample errors using the Bonferroni correction.

To test the microarray averages for particular genes that fit our patterns of pathway regulation in aging, we used quantitative reverse transcription PCR (qPCR). The primer sets were designed to amplify a region of about 100 bp near the 3' end of the mRNA. An ABI Prism 7000 sequence detection system was used. Reactions were run in 96-well plates using the ABI standard curve method. Three replicates were performed for each concentration for the standard curve of each gene, and 4 replicates for each test sample at each age for each gene. The ABI RT kit and SYBR green reagent mixture were used. TUB1 served as our internal standard gene for the qPCR (15). After data were collected, the samples were subjected to thermal denaturation to verify that single species had been synthesized. Figure 3 and Supplementary Figure 1 show the strong correlation between the qPCR results and the average microarray results.

Abbreviations used in this paper are: g: generation, qPCR: Quantitative reverse transcription PCR, NER: Nucleotide Excision Repair of DNA, DSB: Double Strand Break, ESR: Environmental Stress Response, GO: Gene Ontology, MIAME: Minimal Information About Microarray Experiments.

Results

General Aspects of Data

Age-related changes in gene expression accumulate during mother cell aging; in our preliminary statistical analysis, we predicted and found that the variation in ratios of mRNA from older cells compared to 1g mRNA became larger as the cells aged (29). In order to make our data available for general access and analysis, we have put all genes with mRNA changes significant at the $p \leq 0.05$ with the Holm correction into Supplementary Table 3(18-20 g analysis) and Supplementary Table 2 (12g analysis); both tables are based on t-tests which compare the experimental condition to the control at 1g on a gene by gene basis. The microarray MIAME information and the raw data will be posted through the account of LLMH at Gene Expression Omnibus (30) upon acceptance of the paper for publication.

The timing of initiation of age-related changes in gene expression is later than previously appreciated. Our data indicate that for genes with $p \leq 0.05$ (again, t-tests of experimental condition versus control at 1g using the Holm correction), there are no genes with statistically significant expression changes in the 8g arrays. There is a substantial overlap between the genes significantly changed at 12g and 18-20g, as shown in Figure 1. Figure 2 shows the major categories of gene annotation that were notable among significantly changed genes in aging

yeast. These functions were determined using Saccharomyces Genome Database annotations for the genes identified as significant at 18-20g in Bioconductor.

To explore the gene functions, we began with GenMAPPII/MAPPFinder. This GO analysis software identified ribosome-related genes as the top category of age-related changes at 18-20g (Table 1) and also at 12g (data not shown). Metabolism, amino acid and nucleotide metabolism, mating-related functions, and several categories related to methylation were also significantly changed at 18-20g. Table 1 shows a z-score and two probabilities produced by MAPPFinder. A z-score of 1.96 would be consistent with a p value of 0.05. Positive z-scores indicate that the experiment found more genes changed in expression than randomly expected in the category, while negative values indicate fewer genes than expected. The permuted probability is a calculated non-parametric statistic based on 2000 permutations of the data, with gene associations randomized for each sample. The adjusted probability is adjusted for multiple testing using the Westfall-Young adjustment, which calculates the family-wise error rate for each sample. Table 1 summarizes GO categories with $p < 0.05$ for one or both types of p value. Except for DNA repair, the categories had a positive z-score meaning that the gene types were over represented among genes that changed in expression.

We also used GOCluster (28) to examine the classes of genes that had changed significantly during aging. We used the K-Means clustering with four clusters. We considered a category important if it appeared significant 50 or more times out of 200 tries. In general, the findings of this method agree with the results of GenMappII, highlighting changes in the cellular constituents affecting ribosome and nucleolus. There were important changes in transcription (especially of rRNA) and translation. Changes were strong in carbohydrate/energy metabolism

and amino acid metabolism. In addition, GOCluster's findings highlight changes in protein catabolism (especially ubiquitin-related).

We had a clear correlation between qPCR and microarray results. Figure 3 shows the correlation between average quantitative PCR results and average microarray results for specific genes of interest. The Wilcoxon rank sum test ($p=0.23$) and paired t-test ($p=0.9$, two tailed) both test the hypothesis of differences across the two types of measurements (qPCR versus microarray). The large p-values tell us that we cannot reject the null hypothesis of no difference. Correlation tests showed $p < .001$ of collecting our data set if in fact there was no correlation, so they corroborate that qPCR and microarray values provide similar information about mRNA expression. All mRNAs were found to change in the same direction with similar magnitudes in both assays, supporting the functional group changes we have identified.

In Figure 3 and Supplementary Table 3, results for *PNC1* and *SIR2* indicated that the former is increased in expression in the aging cells while the latter is not. In aging, a pseudo-diploid state is established by expression of the silent mating type locus; we have found that *IME1* is significantly induced at 12g and 18-20g as expected in a cell that acts diploid, while there is no induction of *STE12, HO*, or *RME1*, haploid-specific genes. Not all genes we have examined follow this prediction, however. For example, *SPO13,* which is thought to control aspects of the first meiotic division, is not significantly induced in 18-20g cells. In addition, HMLalpha1 and HMLalpha2 did not behave identically. The latter was significantly increased in expression, but not the former. The strong expression of *IME1* supports an effective transcription from the HML, in any case.

Ribosome and Nucleolus-Related Functions

Our first conclusion is that the most significant age-related changes affect ribosome biogenesis. Ribosomal protein genes and ribosomal RNA processing/nucleolar function genes are often co-regulated but constitute separate regulons (31, 32). Figure 4a shows the overlap between the ribosomal protein regulon with the genes we have found significantly changed that have ribosomal protein GO annotations; Figure 4b shows similar overlap for the rRNA and ribosome biosynthesis regulon.

Pseudo-Stationary Phase in Aging Cells

We propose the term pseudo-stationary phase to characterize many of the metabolic and other changes that we see as the mother cells undergo replicative aging. There are several groups of gene expression changes that support that suggestion. First, in the process of entering stationary phase, the Environmental Stress Response genes (19) respond. We see a major overlap between the genes that undergo significant age-related changes and the ESR group, as shown in the Venn diagram in Figure 5a. The ESR genes include some that are induced while others, such as the ribosomal genes, are down-regulated. We examined the issue of whether or not the aging genes are regulated in the same direction as the ESR by means of a correlation plot shown in Figure 5b, finding a strong tendency for expression to change in the same direction in aging and in stress.

Also with regard to pseudo-stationary phase, genes known to be up-regulated in stationary phase, such as the *SNZ* genes and the glycogen-related genes, are affected during aging (Table 2). One of the important genes in this group, *GSY2* encoding glycogen synthase, is regulated by Snf1 protein kinase and Glc7-Gac1 protein phosphatase I. The mRNAs for *GSY2* and its homolog *GSY1* were significantly up-regulated by 18-20g (Supplementary Table 3). As

shown for *GSY1* in Figure 6a, the graph of which is virtually identical with a graph for *GSY2,* the increase had not yet begun at 8g and is barely significant at 12g, but strong at 18-20g .

Another interesting feature of the pseudo-stationary phase involves the hexose transport proteins. In spite of the fact that glucose is still present and sufficient for logarithmic growth of the young cells in the same culture, we found mRNAs for a group of hexose transporters to be almost three-fold higher at both 12g and 18-20g (Figure 6b).

The transcription factors Msn2, Msn4, and Yap1 regulate many of these genes (19). *MSN2* is constitutive but *MSN4* is inducible. We found significant increases in expression at 18-20g for both *MSN4* and for another transcription factor that regulates some of the stress response genes (19), *YAP1* (Supplementary Table 3).

Down-Regulation of Methylation-Related mRNAs

Methylation-related genes have not been previously recognized as an important regulon in yeast aging. This lack of identification might be partly because methylation is not a GO category, an annotation problem mentioned in the MAPPFinder Analysis (Table 1). The methylation-related genes that were significantly decreased in expression are shown in Table 3. Most of these mRNAs, including *SAM1-SAM4,* were decreased in the older cells, with very low p values. Genes encoding proteins that affect both folate-derivative metabolism and S-adenosyl methionine metabolism are reduced in expression. Importantly, genes for rRNA and tRNA methylation showed decreased expression. Since there is no DNA methylation in yeast, genes for that process were not present in the study.

DNA Damage Repair Genes in Aging

Scattered DNA repair gene expression changes at 18-20g occurred, resulting in under-representation of the DNA Repair category in the MAPPFinder analysis (Table 1). Only Nucleotide Excision Repair showed several genes induced at 18-20g (Figure 7).

Protein Phosphatase I Targeting Subunit mRNAs Up-regulated

Protein kinase cascades are important in yeast aging, as has been described by several groups. We have made a related discovery, namely an up-regulation of the mRNAs for the regulatory subunits of Glc7, protein phosphatase I. Table 4 shows the effects of replicative aging on the mRNAs encoding these subunits and except for two that are related specifically to budding, they exhibit significant up-regulation. The gene for the catalytic subunit (*GLC7*) was found to be constitutive, in agreement with previous work showing the gene is constitutive during growth and is only induced in stationary phase (33).

The effect of this transcriptional increase could be to allow more of the transcription factors Msn2 and Msn4 to enter the nucleus and turn up expression of genes with STRE promoters. A number of those genes are indeed up-regulated in our data, a selection of which are shown in Figure 8. Thus, microarray analysis supports the notion developed by genetic analysis, that the protein phosphorylation cascades affecting Msn2 and Msn4 are important in yeast aging.

Discussion.

One of the novel findings of the current study was the notable decrease in mRNAs of a group of genes related to methylation. In higher eukaryotes such as rodents and humans, there is evidence that methylation of DNA decreases during aging (5-7). In addition, modification of tRNA changes in aging (34). It was not clear in these studies whether methylation decrease was protective or deleterious. However, recently it has been found that limiting the methionine in the

diet of mice extends life span, enhances stress resistance, and retards a number of age-related chemical changes (11). In *C. elegans*, a recent RNAi-based screen for aging-related genes identified a gene encoding S-AdoMet Synthase (*sams1)* as one of 23 such genes (35). They noted that down-regulation of methylation of macromolecules is an important aspect of lifespan extension by caloric restriction in *C. elegans*. It is particularly interesting to find *SAM* genes repressed in yeast replicative aging since S-Adenosyl Methionine has been found to regulate the yeast G1 Start signal (36). We have thus identified a new homology between yeast and animal aging processes.

We also found age-related effects that implicate regulation of Msn2/Msn4 transcription factors by phosphorylation. Replicative aging in yeast is regulated by the Msn2 and Msn4 transcription factors (37). Msn 2 and 4 cannot go to the nucleus to activate genes if phosphorylated. A variety of protein kinases appear capable of phosphorylating them, such as Protein Kinase A, Sch9, Snf1/Snf4/Sip1 protein kinase, and possibly the TOR pathway kinases. Of these, the Sch9 and TOR kinases appear important in matching the replicative aging process to nutritional cues (38,39).

Our finding related to these protein kinase pathways concerns protein phosphatase I (PP1), which activates Msn2 and 4 by removing phosphate. The Protein phosphatase I catalytic subunit encoded by *GLC7* is constitutive during logarithmic growth but increases in stationary phase (33), and we maintained our aging cells in logarithmic phase. As noted above, we found the catalytic subunit mRNA not to vary with mother cell age, consistent with our growth regimen and our designation of the aged metabolic state as pseudo-stationary phase. The genes for numerous regulatory protein phosphatase I subunits are inducible. Our novel contribution is the finding that five of the regulatory subunits of this phosphatase are up-regulated more than three

fold, and another is up-regulated two fold in aging yeast cells. Known targets of the two transcription factors are also up regulated, as would be expected if Msn2 and Msn4 reach the nucleus. Lin et al. (14) had found an up-regulation of one of the regulatory subunits of protein phosphatase I at 8g. We have found almost all of the regulatory subunits up-regulated (Table 4, Figure 8).

Another finding that relates to this convergence of protein kinase/phosphatase pathways involves Sip2, a myristoylated beta subunit of the protein kinase Snf1. In young cells, this protein acts as a negative regulator of nuclear Snf1 by sequestering its activating gamma subunit, Snf4, at the plasma membrane. During aging, Sip2 shifts from the plasma membrane to the cytoplasm, along with a redistribution of Snf4 from the plasma membrane to the nucleus. Deletion of *sip2* releases the activator Snf4 and increases the activity of the Snf1 protein kinase within nuclei (40). That activity in turn can phosphorylate Msn2 and 4, sending them out of the nucleus. We found that the mRNA for Sip2 is increased over two-fold in both 12g and 18g samples. This induction is predicted to have a similar effect to the up-regulation of Protein Phosphatase I activity, more of the two transcription factors could enter the nucleus. Our finding fits well with the fact that the *sip2* deletion has a shorter life span and a faster metabolic shift (40).

This protein kinase nexus may also have a connection to the genomic instability at the ribosomal DNA locus noted during aging (41,42). The *sip2* deletion has higher H3 histone kinase activity, more rDNA recombination, and loss of silencing at sites affected by histone H3 ser10 phosphorylation by Snf1, such as the *INO1* promoter and targets of the Adr1 transcription factor (38, 46). Supporting a similar change in chromatin silencing during replicative aging, we have noted significant up-regulation of several genes under the control of Adr1p, such as *ADH2,*

ALD4, GUT2, POX1, and *PEX1* (Supplementary Tables 2 and 3.) In the earlier study, deletion of the gene for Fob1, a protein that binds the replication fork block site in each rDNA repeat and prevents replication forks and transcription forks from colliding, stopped the rDNA effects of the *sip2* deletion (38). Thus, the aging effects of protein kinase cascades can extend far beyond simple metabolic regulation.

Our third significant finding relates to pseudo-stationary phase. One hallmark of stationary phase in budding yeast is the accumulation of glycogen, particularly through the approximately 10 fold induction of the glycogen synthase 2 gene, *GSY2*, during the normal logarithmic to stationary phase transition (43). This gene and many other genes related to glycogen metabolism are induced in old mother cells. Induction of glycogen-related genes had been noted before (14, 16). Early in aging, 8g cells accumulate glycogen and have increased expression of a few glycogen- related genes (14). Extensive study of stationary culture gene expression has been carried out (44), showing glycogen gene induction and stationary phase gene induction. We are intrigued by the earlier finding that experiencing stationary phase results in an advancement of the replicative aging clock (45).

We compared our age-related changes in gene expression with stationary phase expression changes found by Martinez et al. (44) and note that the two also share decreased ribosome and nucleolar gene expression. Recently, it has been found that ribosomal protein gene deletions in diploids, or deletions of one of a pair of paralogs, lengthen life span (38, 46), heightening the potential importance of the ribosomal/nucleolar down-regulation we found. In addition, SNZ stationary phase-related genes are induced at least 2.4 fold in 18-20g cells (Table 2). We have dubbed this effect ‘pseudo-stationary phase’, since the cell growth regimen keeps the cells from ever achieving a cell density that would result in stationary phase in young

cultures. In addition to the criteria of low cell density and measurable glucose in the medium, we can verify that the cells are not in real stationary phase by the lack of induction of *GLC7* (see under Protein Phosphatase below).

A typical feature of cells entering stationary phase is the diauxic shift, monitored elegantly by DeRisi et al. (47 in one of the earliest microarray studies. A change away from glycolysis and towards aerobic metabolism of ethanol and gluconeogenesis occurs early in diauxie, along with many of the gene expression changes found in the ESR. Aerobic metabolism of ethanol provides an abundance of ATP so that biosyntheses of glucose and glycogen become more feasible than under fermentation. The changes in metabolism reported by us and others for aging mother cells of yeast show them to be undergoing diauxie under conditions that do not induce diauxie in young cells, i.e. early cultures with glucose remaining in the medium.

Since the cells act as if they are running out of glucose when they are not, we were interested to find mRNAs for most of the hexose transporters were increased with aging. Koc et al. (14) had also noted an increase in transport proteins during aging, and Lin et al. (16) noted two HXT genes were up-regulated at 8g. This inaccurate detection of glucose limitation by mother cells might be related to a surface-to-volume ratio decrease as the mother cells enlarge. When the cells detect (or seem to detect) a limitation in glucose, it is likely that the cells respond by inducing the stress/diauxie pathways and preparing for stationary phase prematurely. A recent study using alpha factor arrest to regulate cell size has shown that size is related inversely to replication potential (48). We note that the recent suggestion that yeast may undergo altruistic aging (49, 50) fits well with the existence of the pseudo-stationary phase gene expression pattern because stationary phase is often followed by death in yeast. Cells that enter the pseudo-

stationary phase, we propose, can more easily sacrifice themselves for the well being of nearby young cells. In addition, the recently discovered gene sets that are important in aging and also in apoptosis (49, 50) suggest the altruistic response is readied in aging cells.

Different growth regimens could potentially result in different gene expression patterns, so it is important to note that these results apply to dilute cultures grown in liquid medium. For example, they need not be identical with the gene expression patterns to be found in cells undergoing life span studies by dissection on agar plates, essentially at infinite dilution (not near any other cells and growing at an air/liquid interface). The authors urge the gerontological community to take this caveat into consideration when applying our results to other aging studies.

Like Lesur and Campbell (15), we found that the ribosomal protein (RP) regulon is down-regulated. We also note that the ribosome maturation regulon (Ribi or RBB), is heavily down-regulated in aging mother cells. With the current data, we cannot tell whether or not this decrease results from the slower growth experienced by older cells, but growth rate and ribosome gene expression are clearly linked (31). The mRNAs for two important proximal regulators of ribosomal gene expression, Rap1 and Ifh1 (53, 54) are significantly down-regulated at 18-20g. The Ifh1 mRNA was down-regulated by 0.54 fold compared to the young expression ($p = 0.013$ with Holm adjustment) and the Rap1 mRNA decreased 0.47 fold ($p = 0.019$ with Holm adjustment) as shown in Supplementary Table 3. However, it is likely that these are not the sole determinants of the ribosome gene expression decline because the set of ribosome genes that decrease in expression in aging are not identical with those regulated by Ifh1 in association with Rap1 (54,55). For example, we found that genes that *RPL18B* and *HYP2,* genes that were unresponsive to Ifh1, were both down-regulated to similar extents as the responsive genes such as *RPL12A, RPS11B,* and *RPL40A*.

There are connections between stress and ribosome gene expression. For example, the non-essential stress responsive genes *YAR1* and *LTV1* function in 40s ribosomal subunit production (55). The expression of these two genes is significantly decreased at 18-20g (Supplementary Table 3).

The Ribi and RP regulons are under the control of Sch9 (32), a protein kinase that has been implicated in yeast aging (37-39, 50-52). This protein kinase also interacts with the Msn2/4 pathway described above. Thus, the examination of mRNA patterns in aging yeast cells has unexpectedly led us to consider the prime importance of this covalent modification cascade involving Sch9 and Snf1 on one hand and protein phosphatase 1 on the other.

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Figure Legends.

Figure 1. Overlap in Statistically Significant Changes ($p \leq 0.05$) Among Age Classes in Microarray Data. Using Bioconductor (22-24), the p values have been adjusted for multiple testing by the method of Holm (25).

Figure 2. Functional Categories Among Statistically Significant Gene Expression Changes at 18-20g. Genes were identified by Bioconductor (22-24) as significant.

Figure 3. Correlation Between Average Expression Ratios from Quantitative RT PCR (qPCR) and Microarrays. Genes were selected from categories important in yeast aging. Average ratios of old to young mRNA for each gene, plus or minus standard deviations, are shown. Microarray data are from Bioconductor (22-24) using lowess smoothing; qPCR data are from standard curve method (ABI) with standard deviations calculated according to the ABI Guide, "Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR," available from the ABI web site. The RNA prepararations used for qPCR were from 18-20g cells for *GLC7, MF(alpha)2* given as *MF-A2*, *MET6, MIS1, PNC1, RPA12, SSB2, SUN4*, and *TRM82*. The RNA preparations assayed for *ACO1, ASF1, SIR2,* and *TYE7* were from 12g cells. The preparations assayed for *CLA4* and *MNN1* were from 8g cells.

Figure 4. Ribosome-Related Gene Expression Changes in Yeast Replicative Aging.

a. Overlap Between the RP Regulon and the Ribosomal Protein Genes that Change Significantly in Expression at 18-20g compared to 1g via Bioconductor (22-24) Analysis Using Lowess Smoothing.
b. Overlap Between the RBB Regulon and Aging Ribosomal RNA-Nucleolar Genes. The aging group included the Ribosomal rRNA Processing and Nucleolar Ribosome assembly

and export genes that change significantly in expression at 18-20g compared to 1g in Bioconductor (22-24) analysis using lowess smoothing.

Figure 5. Pseudo-stationary Phase Genes that Affect Environmental Stress Responses in Replicatively Aging Yeast. Data from Bioconductor (22-24) analysis using lowess smoothing.

a. Overlap Between Significantly Changed mRNAs in Aging and in ESR (19).
b. Age and Stress Affect ESR Genes Similarly. A sample of 25 arbitrarily chosen genes that take part in the Environmental Stress Response was tested for correspondence between expression in cells treated 45 minutes with 0.2% MMS (data from reference 19) and 18-20g cells. The log (base 2) of the expression ratio was plotted. The preponderance of the data fall in the negative/negative and positive/positive quadrants indicating similar changes in gene expression induction/repression from aging and from stress.

Figure 6. Pseudo-stationary Phase Gene Expression Patterns in Yeast Replicative Aging.

a. Expression of Glycogen Synthase *GSY1* as a Function of Age. Data from Bioconductor (22-24) analysis using lowess smoothing. The expression of the second gene for this enzyme, *GSY2,* with age is virtually identical with this graph (Supplementary Tables 2, 3).

b. Hexose Transporter mRNAs During Yeast Aging. Data from Bioconductor (22-24) analysis using lowess smoothing. The genes significantly induced during aging and averaged for this figure were: *HXT2, HXT3, HXT5, HXT6, HXT7, HXT9, HXT15* and *HXT17.*

Figure 7. Nucleotide Excision Repair mRNAs Were Significantly Induced in Old Mother Cells. The six NER Genes significantly induced ($p < 0.05$ with Holm correction at 18-20g compared to 1g) and averaged for this figure are *RAD2, RAD4, RAD7, RAD10, RAD14*, and *RAD28*.

Figure 8. Pathways of Regulation of Stress Response Genes by Protein Kinases and Protein Phosphatase I in Yeast, Showing Effects of Replicative Aging. Numbers following gene names show the expression ratio of 18-20g cells compared to 1g cells calculated in Bioconductor with lowess smoothing (22-24).

Supplementary Figure 1. Correlation Between qPCR and Microarray data. The log base 2 of the average microarray ratio with old divided by young from Bioconductor (22-24) analysis was plotted against the log base 2 of the average qPCR ratio. The R square value is high, showing that the two methods are highly correlated.

Table 1. Gene Ontology Terms Over-represented at 18-20g from MAPPFinder (26, 27). The GO name is the name of the category considered to be over-represented among the changed genes. GO is the category: Cellular location C, Pathway P, or Function F. Changed, the number of genes considered to have changed in mRNA concentration when comparing 18-20g cells to 1g cells, was limited to changes of greater than three fold. Those categories with asterisks had fewer than four genes changed and are considered less reliable. Total, the total number of genes within the GO category. Z indicates the Z score, Permuted P value indicates the probability for chance to have caused that level of representation among the total genes in the category, Adjusted P Value, the probability adjusted for the permutations of the terms.

GO name	GO	Changed	Total	z	Permuted P value	Adjusted P value
Cytosolic ribosome(sensu Eukaryota)	C	77	153	23.7	0	0
Ribosome biogenesis and assembly	P	68	221	16	0	0
Nucleolus	C	51	210	11.6	0	0
rRNA processing	P	39	160	10.1	0	0
Primary metabolism	P	228	2930	6.45	0	0.041
Mating behavior*	P	2	2	5.66	0	0.74
Amino acid metabolism	P	29	230	4.43	0	0.98
Methionine metabolism	P	8	34	4.38	0	0.98
tRNA modification	P	11	67	3.68	0	1
RNA methylation	P	4	11	4.3	0.001	0.98
Carboxylic acid metabolism	P	35	335	3.66	0.001	1
Protein modification	P	14	514	2.72	0.001	1
Methionine adenosyltransferase activity	F	2	2	5.66	0.002	0.74
rRNA modification	P	5	16	4.32	0.002	0.98
RNA polymerase complex	C	7	31	3.96	0.002	1
Nucleotide metabolism	P	17	127	3.63	0.002	1
tRNA(guanine-N7-)-methyltransferase*	F	2	2	5.66	0.004	0.74
One-carbon compound metabolism	P	5	18	3.95	0.004	1
Nucleotide transport*	P	2	3	4.47	0.005	0.98
Mating pheromone activity*	F	2	4	3.75	0.008	1
Acetate biosynthesis*	P	2	4	3.75	0.01	1
RNA methyltransferase activity	P	5	22	3.36	0.01	1
Fatty acid biosynthesis	P	4	17	3.1	0.011	1
Methyltransferase activity	F	10	76	2.71	0.015	1
DNA repair*	P	2	150	-2.4	0.02	1
Biopolymer methylation	P	5	26	2.9	0.021	1
tRNA methylation	P	3	10	3.24	0.025	1
Folic acid derivative metabolism*	P	2	11	3.02	0.029	1
rRNA methyltransferase activity*	F	2	5	3.24	0.03	1

Table 2. Induction of Genes for Stationary Phase and Starvation in Aging Yeast. Average and standard deviation of expression ratios determined using Bioconductor (22-24) with lowess-smoothed data.

ID	name	Avg 1g	SD 1g	Avg 8g	SD 8g	Avg 12g	SD 12g	Avg 18g	SD 18g
YFL059W	*SNZ3*	1.0206	0.1409	1.4388	0.4298	1.9820	0.9638	2.5410	0.6001
YOR027W	*STI1*	0.8995	0.1094	0.8503	0.9322	1.6204	0.6621	2.6528	1.4236
YER150W	*SPI1*	1.0165	0.0692	0.9432	0.1495	2.3104	1.0157	4.3893	1.1000
YNL333W	*SNZ2*	1.1314	0.1958	1.5657	0.7702	2.2121	0.8372	2.5176	0.7519
YLR258W	*GSY2*	1.0700	0.0376	0.9123	0.1156	1.6803	1.3599	4.5849	2.7983
YGL208W	*SIP2*	1.0132	0.1467	0.9834	0.5962	2.0004	0.5360	2.3379	0.9386

Table 3. Significant Age-Related Decreases in Methylation Gene Expression. Average and standard deviation of expression ratios determined using Bioconductor (22-24) with lowess-smoothed data.

ID	**Gene**	Avg 1g	SD 1g	Avg 8g	SD 8g	Avg 12g	SD 12g	Avg 20g	SD 20g
YPL266W	*DIM1*	0.9674	0.0660	1.2129	0.2104	0.4266	0.2607	0.2483	0.1193
YPL208W	*RKM1*	1.0586	0.0655	0.9247	0.1542	0.7579	0.2366	0.5472	0.2434
YNL062C	*GCD10*	1.0467	0.0877	1.0311	0.3832	0.8174	0.1976	0.5618	0.1055
YDR120C	*TRM1*	1.1122	0.1534	1.1094	0.3552	0.4883	0.3867	0.3513	0.2045
YKR056W	*TRM2*	0.9938	0.0356	0.9280	0.1527	0.5037	0.3987	0.4116	0.1401
YDL112W	*TRM3*	0.9671	0.1570	1.0034	0.1911	0.8412	0.5165	0.3133	0.0819
YML014W	*TRM9*	0.9503	0.0968	1.0219	0.3355	0.6265	0.3903	0.3871	0.0900
YOL124C	*TRM11*	0.9816	0.0500	1.2151	0.2408	0.6500	0.5144	0.1238	0.0373
YDR165W	*TRM82*	0.9570	0.0979	0.8098	0.2685	0.3978	0.1213	0.2439	0.1018
YNR046W	*TRM112*	1.0357	0.1442	1.2315	0.3727	0.4733	0.1150	0.4064	0.1435
YBR261C	*YBR261C*	1.0225	0.0603	1.2230	0.2258	0.5189	0.1713	0.3420	0.1090
YGR001C	*AML1*	0.9295	0.1812	0.9957	0.4376	0.4452	0.3190	0.1506	0.0610
YIL064W	*YIL064W*	1.0370	0.0565	1.1154	0.3571	0.3845	0.2100	0.2185	0.0680
YIL110W	*MNI1*	1.0687	0.1258	1.0836	1.4986	0.4454	0.1369	0.3463	0.0939
YDR465C	*RMT2*	0.9797	0.0713	0.8974	0.1249	0.4237	0.3451	0.2617	0.0560
YCR047C	*BUD23*	1.0770	0.1300	1.1256	0.3104	0.6436	0.2390	0.4019	0.1939
YLR180W	*SAM1*	1.0262	0.0893	1.0127	0.6826	0.4334	0.2381	0.1613	0.0503
YLDR502C	*SAM2*	1.0172	0.1139	0.7624	0.3788	0.2925	0.1215	0.1305	0.0549
YPL274W	*SAM3*	0.9487	0.0676	0.7908	0.2100	0.4396	0.1377	0.3823	0.0960
YPL273W	*SAM4*	0.8874	0.1417	0.8453	0.6518	0.4326	0.1802	0.3810	0.1343
YOL052C	*SPE2*	1.0254	0.0725	0.8776	0.3301	0.6228	0.1887	0.4469	0.1611
YER091C	*MET6*	0.9488	0.1522	0.8233	0.5831	0.4652	0.2225	0.2368	0.0983
YBR081W	*MIS1*	1.0015	0.0428	0.9123	0.3840	0.6090	0.3430	0.2399	0.0914
YGR264C	*MES1*	0.9286	0.0837	0.8257	0.1616	0.2504	0.1901	0.1401	0.0591
YOR201C	*MRM1*	1.0907	0.1094	1.1826	0.3216	0.4784	0.2272	0.6020	0.2406

Table 4. Induction of Many Regulatory Subunits of Protein Phosphatase I in Yeast Aging. Average and standard deviation of expression ratios from Bioconductor (22-24) using lowess-smoothed data.

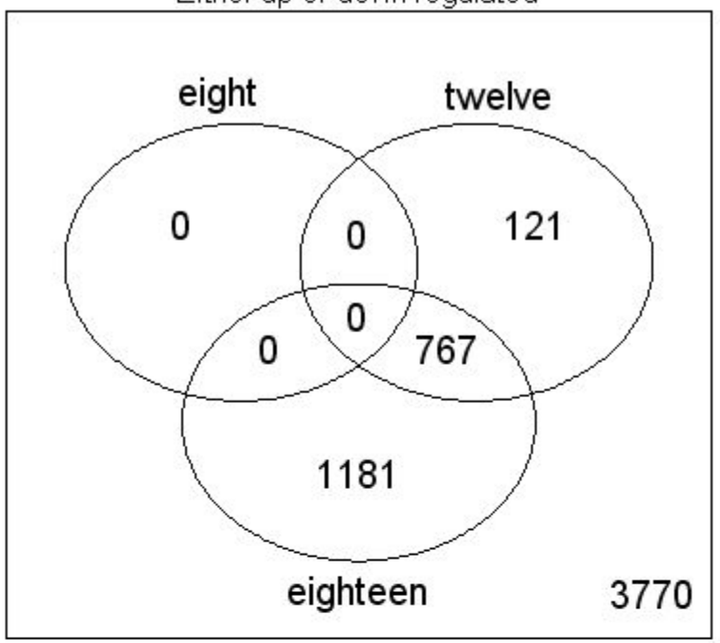
Name	**Subunit encoded**	Avg 1g	SD 1g	Avg 8g	SD 8g	Avg 12g	SD 12g	Avg 20g	SD 20g
GLC7	Catalytic	1.059	0.153	1.030	1.305	1.204	0.558	1.018	0.106
SDS22	Regulatory	1.139	0.260	1.179	0.918	2.918	1.677	3.862	1.345
REG1	Regulatory	1.072	0.145	1.273	0.542	1.550	0.281	2.427	1.648
REG2	Regulatory	1.122	0.139	0.885	0.235	4.630	1.735	7.707	2.054
SIP5	Regulatory	0.976	0.085	0.863	0.373	1.636	0.730	1.510	0.416
GAC1	Regulatory	1.027	0.107	0.779	0.165	2.497	1.352	3.367	3.328
GLC8	Regulatory	1.023	0.143	0.982	0.561	1.736	0.622	3.262	0.585
SHP1	Regulatory	0.976	0.066	1.119	0.402	1.869	1.293	2.077	0.264
BUD14	Reg, budding	0.981	0.100	0.963	0.284	0.845	0.132	0.866	0.321
BNI4	Reg, budding	1.042	0.111	0.993	0.077	1.118	0.332	1.004	0.545

Supplementary Table 1. GenePix Pro 5.1 Microarray Quality Control Measurements. All arrays used satisfied the criteria in the table below. Bkgrd is background intensity of the microarray. Included are measures of the signal strength, slide background strength compared to signal, non-roundness of spots, background variability across and down the slide, signal saturation of spots, spots not found by the software, and spots designated as bad due to smearing or wrong positioning.

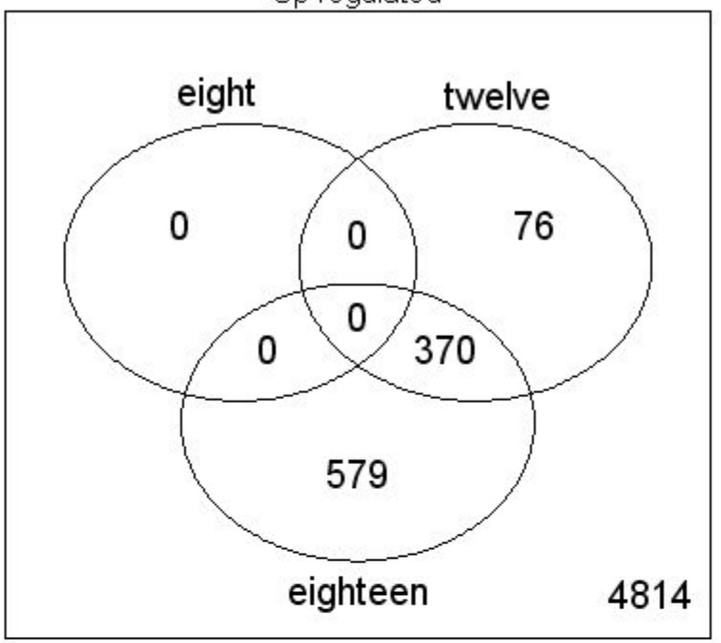
Median signal to bkgrd	Mean of median bkgrd	Median signal to noise	Median %>bkgrd+1SD	Feature variation	Bkgrd variation	Saturated	Not found	Bad spot
>2.5	<500	>4	>90	<0.5	<1.2	<3.3%	<18%	<7%

Adjusted p-value (Holm) < 0.05

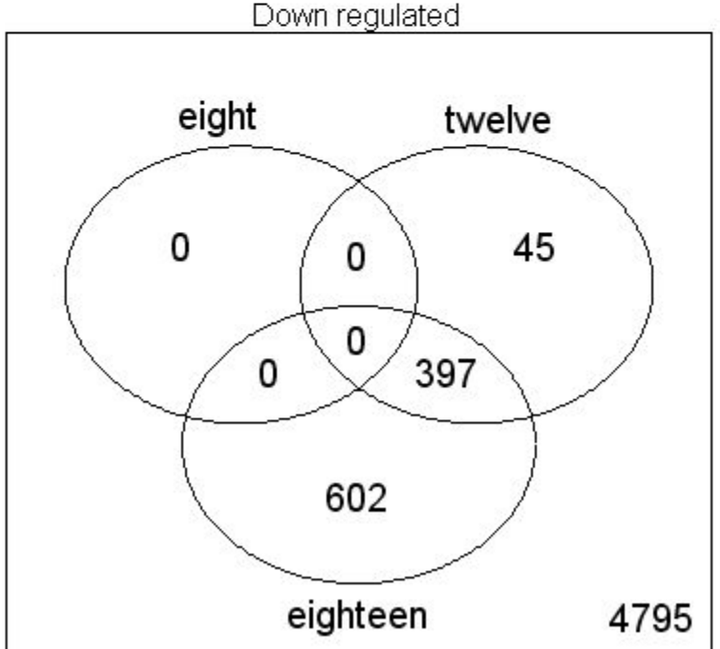
Either up or down regulated



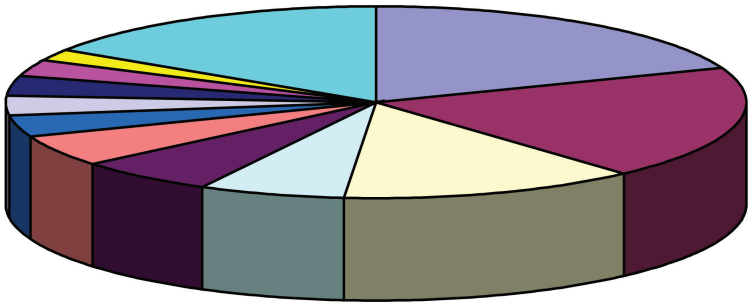
Up regulated



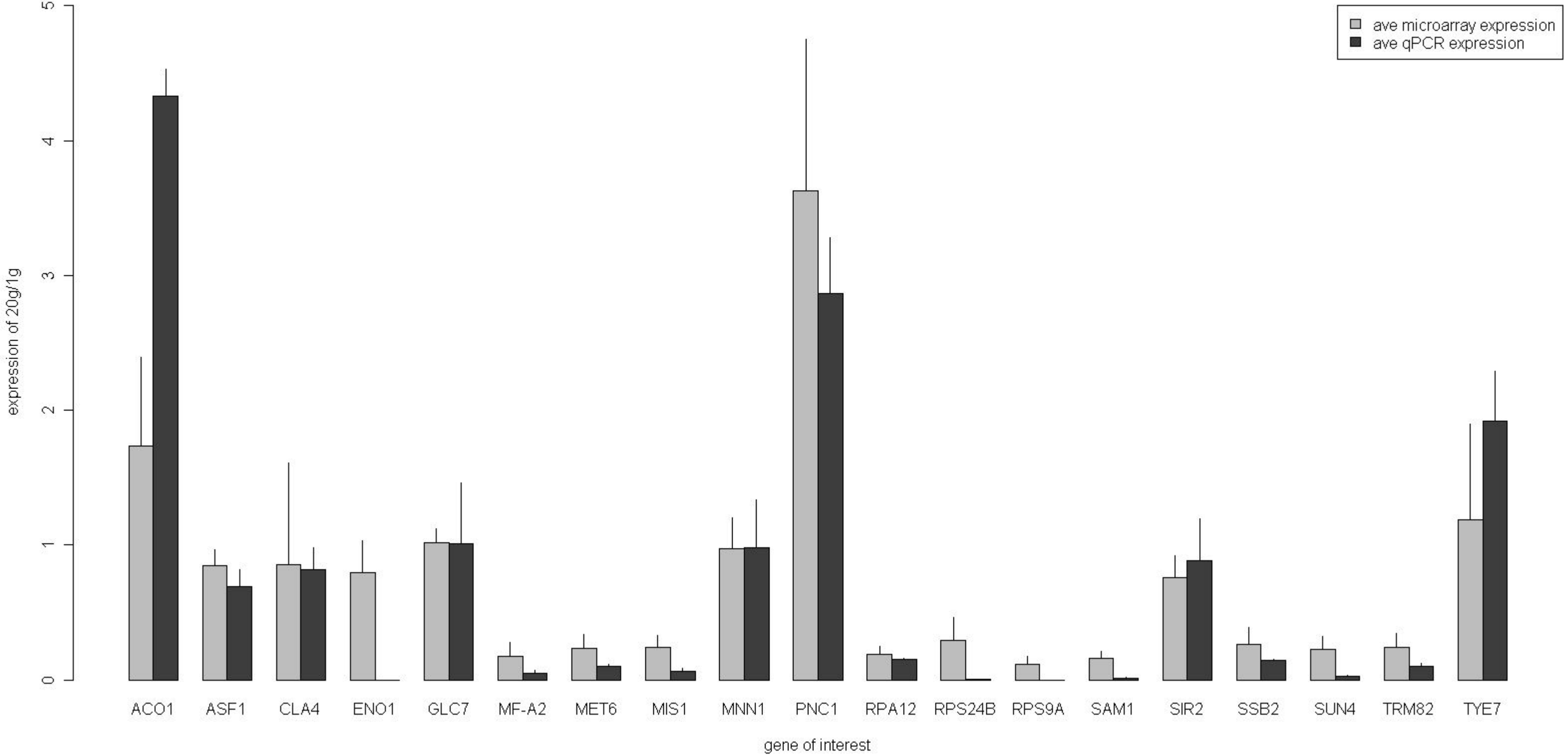
Down regulated

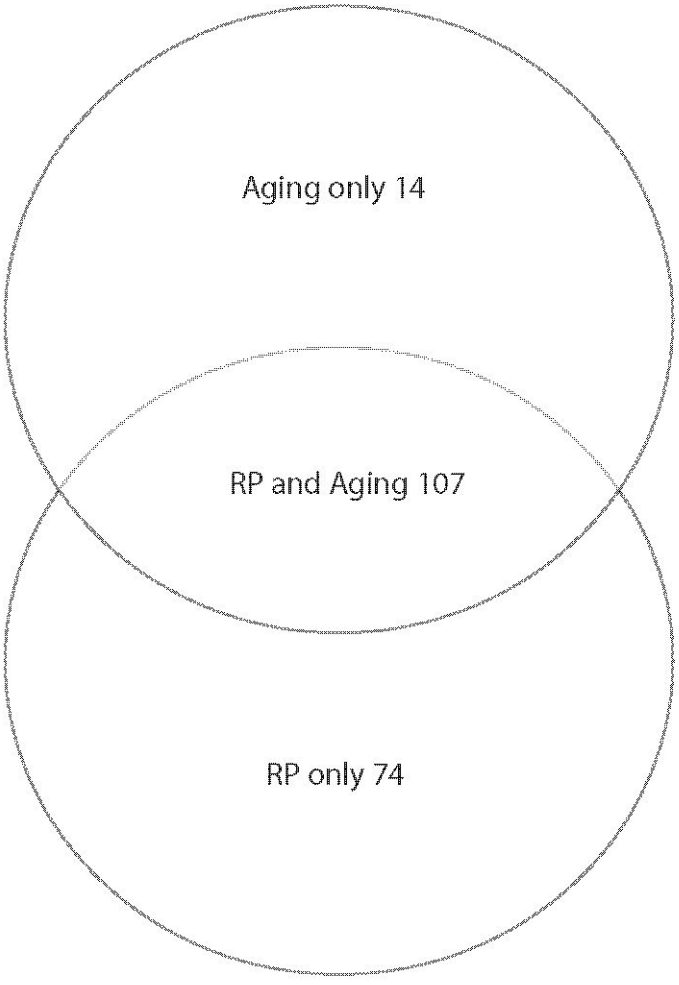


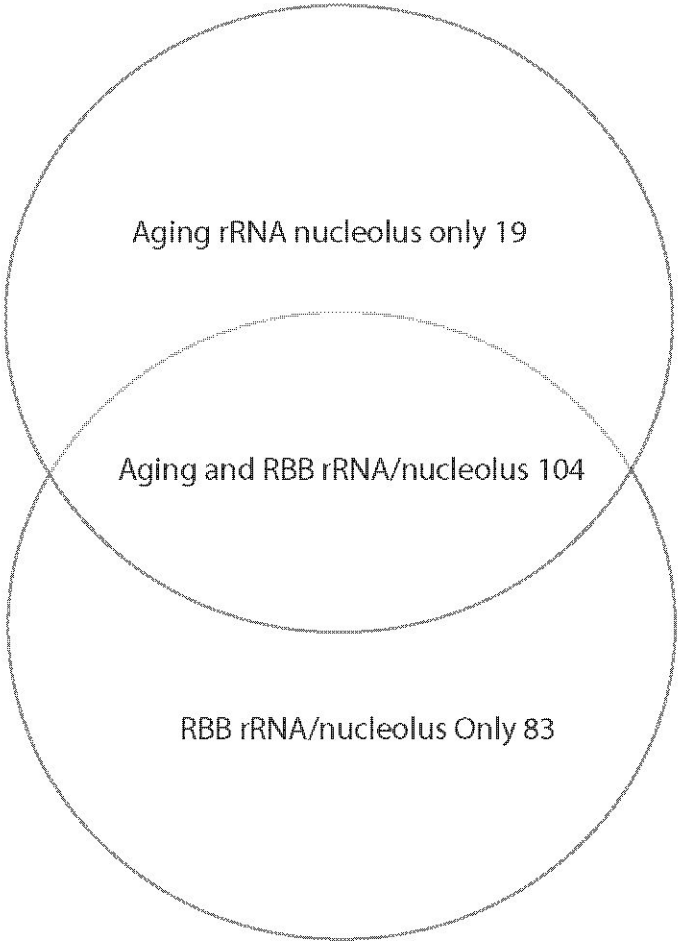
Significant mRNA Changes in Aging

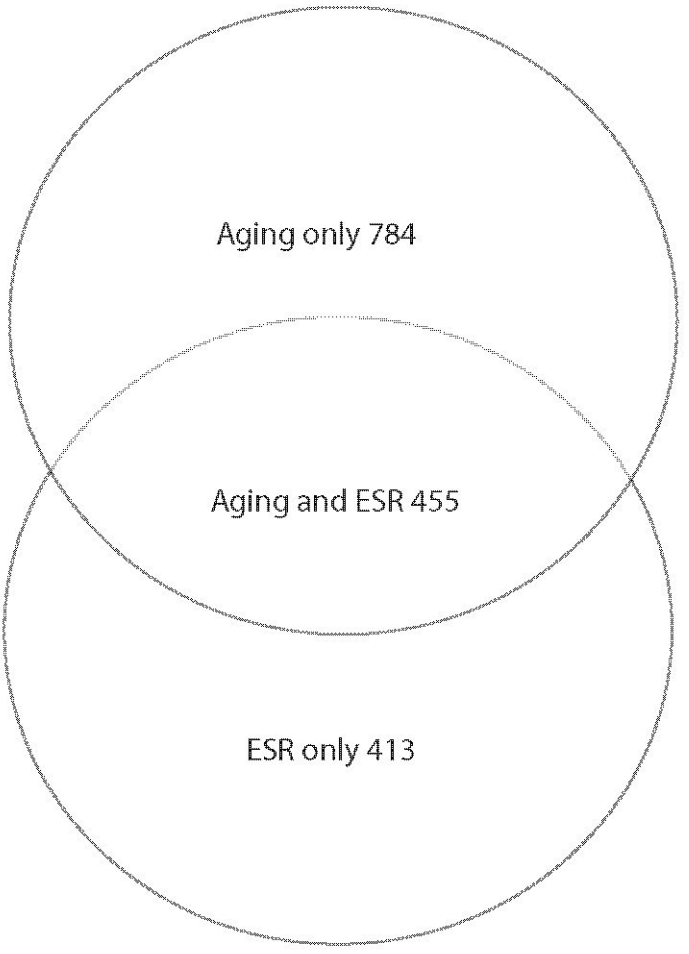


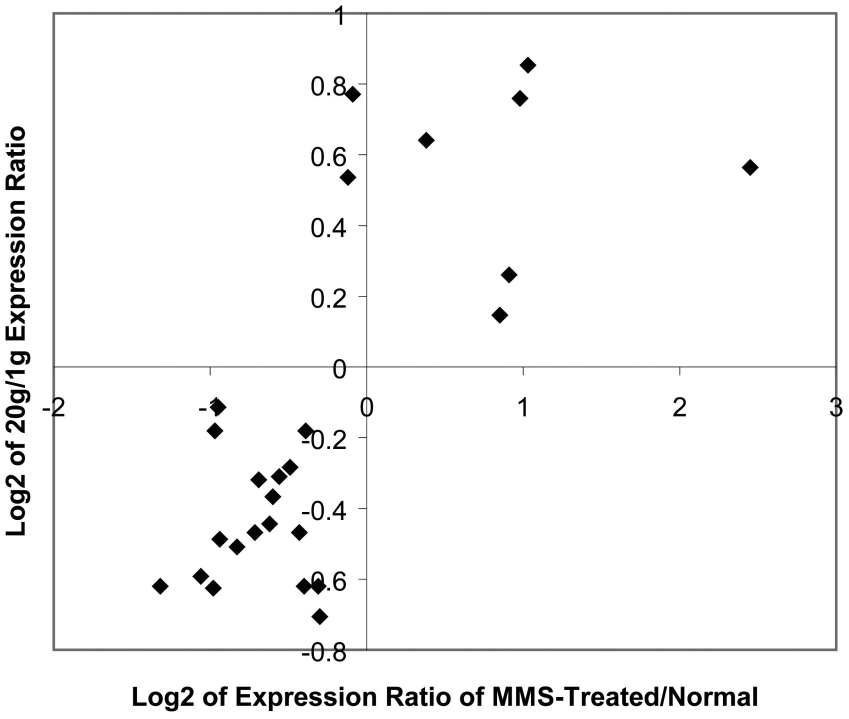
Nucleolus/ribosome	Carbohydrate metabolism	Transcription&Translation
Golgi & ER	Mating	N anabolism
Cell wall,Osmotic	Transport	Stress, HS, Chaperones
Methylation	DNA Replication, Repair	Other

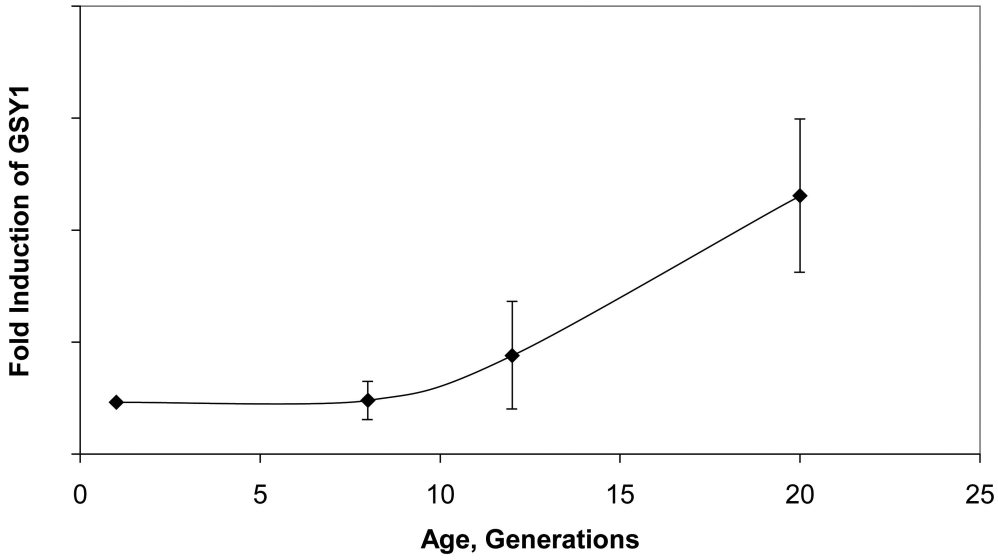

ave microarray expression
ave qPCR expression
expression of 20g/1g
0
1
2
3
4
5
ACO1
ASF1
CLA4
ENO1
GLC7
MF-A2
MET6
MIS1
MNN1
PNC1
RPA12
RPS24B
RPS9A
SAM1
SIR2
SSB2
SUN4
TRM82
TYE7
gene of interest

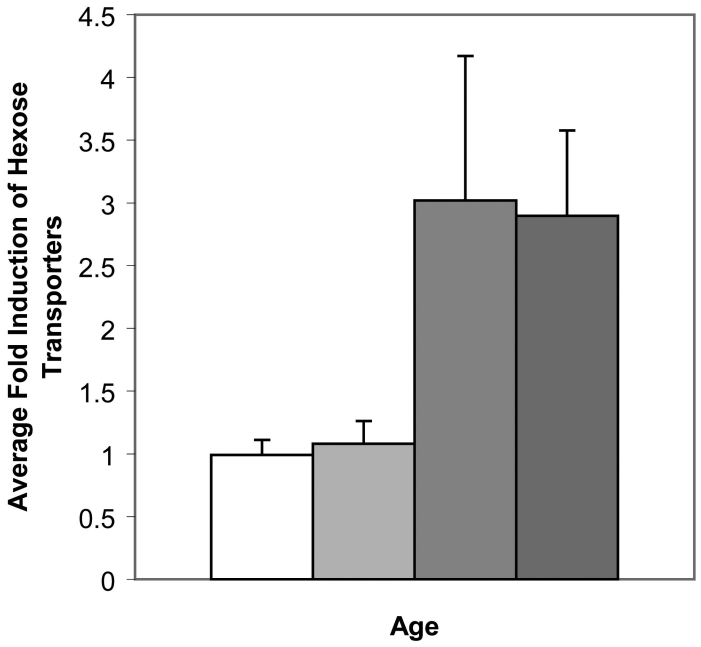

Aging only 14
RP and Aging 107
RP only 74

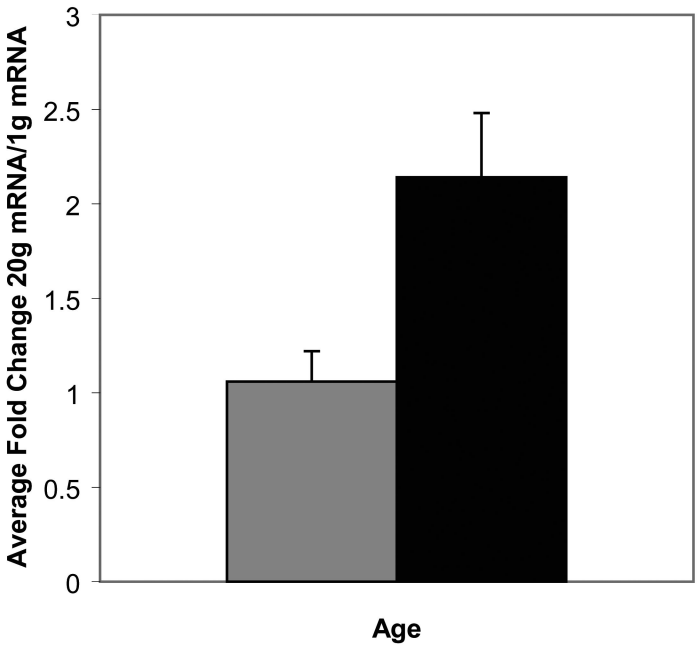

Aging rRNA nucleolus only 19
Aging and RBB rRNA/nucleolus 104
RBB rRNA/nucleolus Only 83

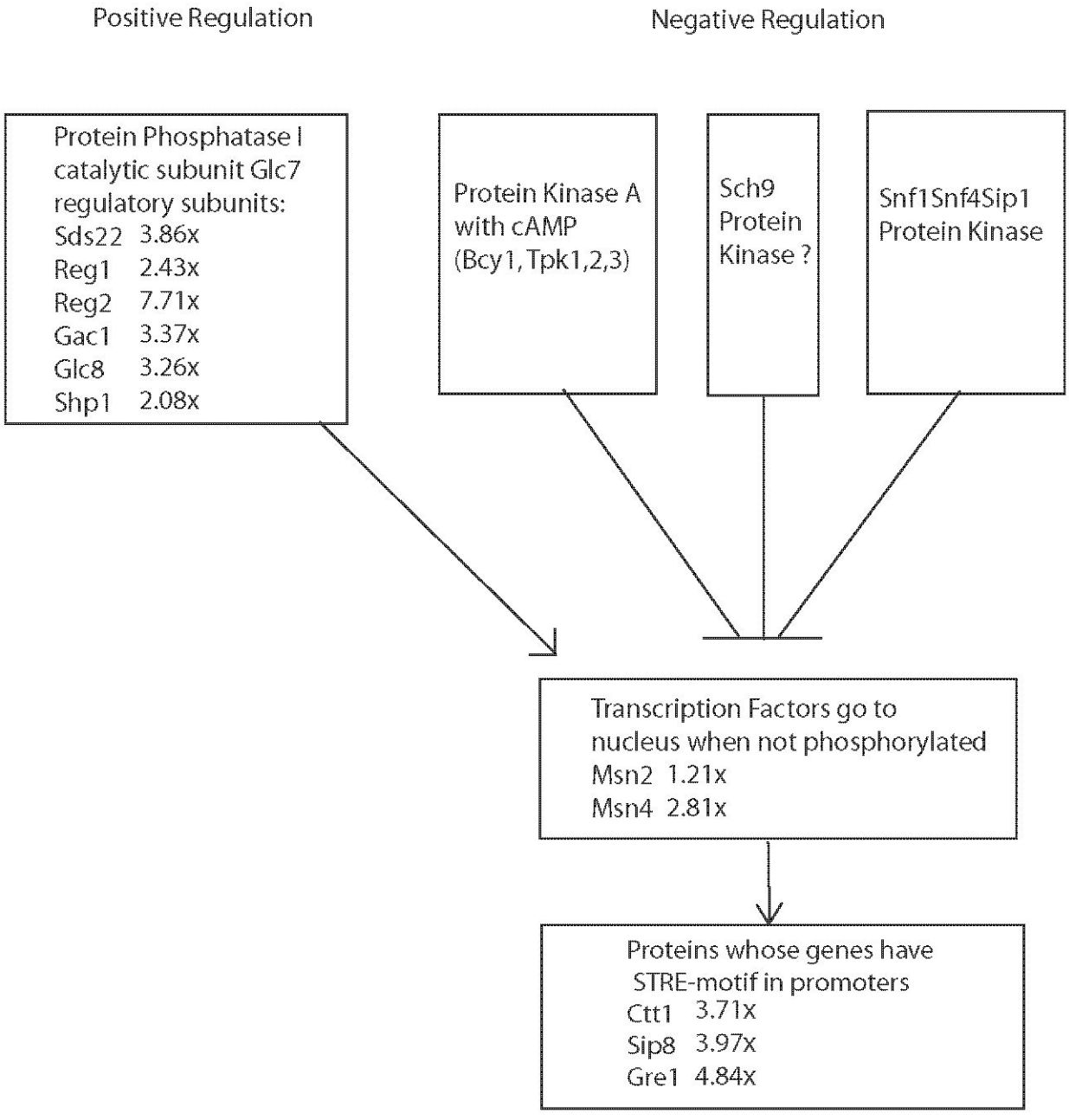

Aging only 784
Aging and ESR 455
ESR only 413


Log2 of 20g/1g Expression Ratio
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-2
0
1
2
3
Log2 of Expression Ratio of MMS-Treated/Normal


Fold Induction of GSY1
0
5
10
15
20
25
Age, Generations


Average Fold Induction of Hexose Transporters
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Age


Average Fold Change 20g mRNA/1g mRNA
3
2.5
2
1.5
1
0.5
0
Age


Positive Regulation
Negative Regulation
Protein Phosphatase I
catalytic subunit Glc7
regulatory subunits:
Sds22 3.86x
Reg1 2.43x
Reg2 7.71x
Gac1 3.37x
Glc8 3.26x
Shp1 2.08x
Protein Kinase A
with cAMP
(Bcy1, Tpk1,2,3)
Sch9
Protein
Kinase ?
Snf1Snf4Sip1
Protein Kinase
Transcription Factors go to
nucleus when not phosphorylated
Msn2 1.21x
Msn4 2.81x
Proteins whose genes have
STRE-motif in promoters
Ctt1 3.71x
Sip8 3.97x
Gre1 4.84x