Changes in Transcription and Metabolism During the Early Stage of Replicative Cellular Senescence in Budding Yeast*

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Significance:
This is the first integrated information on transcriptome and metabolome of aging yeast cells.

Background:
Senescence is the biological process of age-related cellular and organismal deterioration in function.

Results:
The transcriptome and metabolome at early stages of replicative senescence of yeast cells were measured.

Conclusion:
The transcriptomic and metabolomic profiles drastically changed at about half of the average replicative lifespan.

Senescence is the biological process of cellular aging and organismal changes that deteriorate physiological function with the passage of time, resulting eventually in death. This deterioration is believed to be due to cumulative damage to molecular and cellular structures and by programmed alteration of gene expression. Human somatic cells have a limited capacity to divide in culture and eventually enter replicative senescence, a state of irreversible proliferation arrest, leading to tissue dysfunction (1). Biological aging is the main risk factor for human pathologies such as cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases (2), and removal of senescent cells can prevent or delay tissue dysfunction and extend the healthspan (3). In mammals, senescent cells exhibit diverse alterations in their cellular and biochemical features: an enlarged and flattened cellular morphology, an increase in the production of reactive oxygen species, senescence-associated β-galactosidase activity, and senescence-associated secretory phenotype (2, 4). Some of these age-related cellular phenomena are explained by time series gene expression profiles; the senescent process is divided into four stages (early, middle, advanced, and very advanced), with specific genes being prominently expressed at each of these stages (5).

The budding yeast, Saccharomyces cerevisiae, is a useful model for cellular aging research, and especially studies on the lifespan of a single cell, because its asymmetric division makes it easier to analyze a population of cells compared with symmetrically dividing mammalian cells. The replicative lifespan of a yeast cell is defined as the number of daughter cells that a mother cell can generate before dying (6). The median replicative lifespan of most wild-type strains is about half of the average lifespan of most wild-type strains (7). Yeast cells age as they undergo division, as do cultured mammalian cells: their size increases, their shape is altered, their cell cycle slows down, and they become sterile (8). Furthermore, the nucleus of aged yeast cells tends to be larger and/or more fragmented, and the mitochondria become dysfunctional (8, 9). The rate of protein synthesis and ribosome activity decrease linearly with age (10). In mother cells during the budding process, oxidative stress, protein aggregation, and extrachromosomal rDNA circles (self-replicating circles of ribosomal DNA) accumulate and cause senescence (9). These accumulations are relieved by calorie restriction, which helps slow aging and extend the lifespan in yeast and higher organisms (11). One mechanism by which calorie restriction extends the replicative lifespan is a metabolic

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shift from fermentation to respiration, resulting in activation of Sir2p, an NAD (nicotinamide adenine dinucleotide)-dependent deacetylase belonging to the sirtuin family that acts as a master regulator of anti-aging (12).

Several transcriptome analyses comparing old cells with young cells have shown pathway changes in expression during replicative aging (7, 13–15). In all cases, a shift from glycolysis toward gluconeogenesis and energy storage (glyoxylate cycle, lipid metabolism, and glycogen production) was observed in old cells. In addition, the expression of ribosome genes and genes involved in protein synthesis, folding, and degradation all decreased (14, 15). Besides changes in metabolic gene expression, environmental stress response genes were induced in aged cells (7), although oxidative stress gene expression did not change (14). Genes involved in DNA damage repair such as homologous recombination and nucleotide excision repair were also induced in old cells (7, 15). These transcriptome studies examined 18–20-generation-old yeast cells (i.e., approaching the end of an average lifespan) prepared by using biotin-streptavidin technology or a centrifugal elutriator (7, 14, 15). About half of the population of cells is dead by the average generation time, so the transcriptome data from the 18th to 20th generation cells may contain information from dead cells. Alternatively, the removal of old cells with an average lifespan may result in losing information from dead cells because yeast cells lyse at the end of their lifespan (7).

To our knowledge, there are few investigations focusing on the early stage of replicative aging cells. To determine when cellular aging-related behavior begins, and what change initiates the aging process, we generated transcriptomic and metabolomic profiles of yeast cells at the 1st, 4th, 7th, and 11th generation. Distinctive changes in transcription appeared after 11 generations: decreased amino acid biosynthesis and increased sugar and TCA cycle metabolism. These transcriptional changes were confirmed at the metabolite level. Moreover, the expression of stationary phase-induced genes was highly enhanced after 11 generations, despite the presence of adequate nutrients in the medium. These observations suggest that nutrient sensing and/or signaling begin to deteriorate in the early stage of replicative senescence.

**EXPERIMENTAL PROCEDURES**

**Strains and Medium—** *S. cerevisiae* strains used in this study were BY4742 (MATa,ura3Δ0,leu2Δ0,his3Δ1,lys2Δ0) (16) and X2180–1A (MATa,SLC2, mal mel gal2,CUP1) (17). Yeast extract (1%), Bacto Peptone (2%), dextrose (2%) (YPD) medium was used for routine cultures.

**Replicative Lifespan Determination—** Replicative lifespan was assayed with minor modifications as described previously (18). Yeast cells were thawed from frozen stocks and streaked onto YPD agar plates. After 2 days, a single colony was transferred to a fresh YPD agar plate, and cells were grown overnight. A sample was spread onto a new YPD agar plate containing 10 μg/ml phloxine B. Using a micromanipulator, 48 cells were arrayed on a YPD plate and allowed to undergo one or two divisions. Virgin cells were then selected and subjected to lifespan analysis. Except during manipulation, plates were sealed with Parafilm, incubated at 30 °C during the day, and stored at 4 °C at night to avoid excessive budding. Daughter cells were removed by gentle agitation with a dissecting needle and scored every 2 h. For each of the 48 cell lines, buds from each mother cell were counted until division of living cells ceased, or cells were stained with phloxine B.

**Isolation of Old Yeast Cells—** Isolation of old cells was performed as described previously (19). Cells were grown in YPD medium to an OD600 of 1.0. 5 × 107 cells were spun, washed with 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4), and resuspended in 0.2 ml of 1× PBS. Separately, 4.0 mg of EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, IL) was dissolved in 0.15 ml of 1× PBS at room temperature and added to the cell suspension. The mixture was shaken for 15 min by vortexing at a slow setting at room temperature. Cells were then spun and washed three times with 1 ml of 1× PBS. Biotin-labeled cells were then diluted and grown in YPD until the 4th or 7th generation and then spun down and resuspended in 20 ml of cold 1× PBS. Dynabeads Biotin Binder (Invitrogen) were added, four beads per biotinylated cell. Cells and beads were rotated for 2 h at 4 °C, and then the suspension was placed in a test tube in a DynaMag-50 magnet (Invitrogen) at 4 °C. After 20 min, the supernatant was carefully aspirated, 10 ml of cold YPD was added, and the mixture was gently agitated. The cells were again placed in the sorter for 15 min, and the process was repeated seven times. For the 11th generation cells, the isolated 7th generation cells were grown in YPD for four generations, and the sorting procedure was repeated. The average bud scar count of each sorted cell population was determined by staining an aliquot with fluorescent brightener 28 (MP Biomedicals, Illkirch, France) and counting bud scars under a fluorescence microscope.

**Microarray Analysis and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)—** Total RNAs were isolated from yeast cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). Antisense RNA was synthesized and labeled with Cy3 for the 4th, 7th, and 11th generations and with Cy5 for the 1st generation. Each mixture of Cy3- and Cy5-labeled antisense RNA was hybridized on a 3D-Gene Yeast Oligo Chip 6K (Toray, Kanagawa, Japan). The hybridized array was scanned with a 3D-Gene Scanner 3000 (Toray). The average intensity of the background was subtracted from the detected signals, then the sample values were determined by staining an aliquot with fluorescent brightener 28 (MP Biomedicals, Illkirch, France) and counting bud scars under a fluorescence microscope.

**Metabolome Analysis—** Samples for quantifying intracellular metabolites in yeast were prepared as described previously (20).
with minor modifications. The dried yeast cells were resuspended in 1 ml of a single phase solvent mixture of methanol/water/chloroform (2.5:1:1, v/v/v) and 40 μl of a ribitol solution (8 mg/ml) as the internal standard, then homogenized and disrupted in a ball mill at 20 Hz for 3 min. The extraction was carried out at 37 °C for 30 min with vigorous shaking (1,200 rpm). After centrifugation at 12,000 rpm for 3 min, the supernatant (900 μl) was transferred to a new 1.5-ml microfuge tube and mixed with 400 μl of water. After centrifugation, 1 ml of the supernatant was placed in a vacuum for 4 h and dried in a freeze-dryer until dry. Derivatization of hydrophilic metabolites was carried out at 30 °C for 90 min with 100 μl of methoxyamine hydrochloride in pyridine (20 mg/ml) and subsequently at 37 °C for 30 min with 50 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide. To analyze the compounds in the growth medium, yeast cell cultures were centrifuged (13,000 rpm, 3 min) and the supernatant was filtered through a Millex-LG filter (0.20 μm, hydrophilic, polytetrafluoroethylene) (Millipore, Billerica, MA). Supernatant (20 μl) was mixed with 1 ml of methanol/water/chloroform with ribitol (1 μg/ml) and incubated at room temperature for 10 min. The hydrophilic phase was dried as described above. Derivatization was carried out with 50 μl of methoxyamine hydrochloride and 25 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide. GC-MS analysis was performed as described previously (20), independently four times for each generation. Compounds that are not distinguishable from each other by GC-MS analysis such as pyruvic acid and oxaloacetic acid are described as pyruvic acid + oxaloacetic acid.

Multivariate Analysis—GC-MS data were processed as described previously (20) and judged by principal component analysis (PCA) using the SIMCA-p + program (version 12.0.1, Umetrics, Malmö, Sweden).

RESULTS

Experimental Concepts for the Early Stage of Replicative Senescent Cells—Wild-type haploid cells of S. cerevisiae such as the BY4742 strain had a replicative median lifespan of 24 generations and a maximum lifespan of 43 generations in our experiment (Fig. 1). Transcriptome analysis of 18–20-generation-old wild-type cells was reported previously and showed that cellular aging is associated with a shift toward gluconeogenesis and energy storage and a response to genome instability (7, 15). The transcripts at this age, however, might include information from dead cells or exclude information from lysed cells. To characterize cellular aging processes in the absence of dead cells and to determine when cellular aging behavior begins, we focused on an earlier stage of replicative cellular senescence (~10 generations), when most cells are alive (Fig. 1).

We therefore assessed synchronized cultures of young cells (1st generation) and older cells (4th, 7th, and 11th generation) for changes in transcription and metabolism as cells approach senescence.

We isolated wild-type cells of designated ages by labeling the surface of mother cells with biotin and sorting the senescent cells using streptavidin magnetic beads. Bud scars on the isolated cells of designated ages were counted to determine their generation under a fluorescence microscope after staining the cells with calcofluor (Fig. 2). Cells from the 1st generation fraction had no bud scar (unbudded) or one to two bud scars, whereas cells from the 4th generation fraction had three to six bud scars. Cells from the 7th and 11th generation fractions had the highest number of bud scars, ~7 and 11, respectively. These cell fractions were used for DNA microarray and gas chromatography mass spectrometry (GC-MS) analyses, as described below.

Outline of Transcriptomic Changes in an Age-dependent Manner—We performed transcriptome analysis by probing a DNA microarray with total RNAs extracted from the 1st, 4th, 7th, and 11th generation cells. A scatter plot of the transcriptomes of cells of designated ages versus that of the 1st generation showed moderate increases and decreases in transcripts by the 7th generation and drastic up- and down-regulation after 11 generations (Fig. 3, A–C). The number of genes regulated in an age-dependent manner was compared between successive time points, using a threshold of a minimum 2-fold change in transcript level relative to the 1st generation (Fig. 3, F and G). After four generations, up- and down-regulation was observed for 538 and 557 genes, respectively, for a total of ~20% of the genes in the whole genome; few further changes were observed by the 7th generation (590 and 572 genes). Genes whose expression commonly changed at the 4th and 7th generation constituted a major group among the respective generations (blue and orange bars in Fig. 3, F and G): up-regulated genes, 85% (455/538) at the 4th generation and 77% (455/590) at the 7th generation; down-regulated genes, 79% (439/557) at the 4th generation, and 77% (439/572) at the 7th generation. Interestingly, after 11 generations, further up- and down-regulation was observed for 353 and 348 genes, respectively (red bars).

Large changes in the transcriptome from the 1st to the 4th generation, and small changes between the 4th and 7th generations, were observed as described above. The striking changes between the 1st and 4th generations seemed to reflect differ-
ences between non-budded (never experienced cell division) and budded cells, rather than a generation gap because about half of the 1st generation comprised non-budded cells but all of the 4th generation cells had budded previously (Fig. 2). To exclude transcriptional information derived from non-budded cells, we compared transcriptomic profiles of cells of designated ages versus those of the 4th generation instead of the 1st generation. The scatter plot clearly showed few changes in the transcriptome of the 7th versus 4th generation (Fig. 3D), but large changes between the 11th and 4th generation (Fig. 3E).

These data reveal that cellular senescence can be detected as changes as early as 11 generations (i.e., at about half of the median lifespan).

**Pathways That Are Transcriptionally Changed During the Early Stage of Cellular Senescence**—To explore the functions of genes whose transcript levels change with age, the up- and down-regulated genes were sorted into categories defined by the GenMAPP database. We first focused on genes whose transcript levels in old generations changed relative to the 1st generation (data not shown). Few differences in pathways between the 4th and 7th generations were found; cells in the 4th and 7th generation had accumulated the same transcripts coding for aromatic amino acid (tryptophan and phenylalanine) degradative enzymes and enzymes for the biosynthesis of sulfur amino acids (cysteine and methionine). The levels of mRNAs coding for ribosomal proteins and enzymes involved in biosynthesis of...
purine and pyrimidine were decreased after both the 4th and 7th generations, suggesting that the levels of ribosomal proteins and nucleic acids are higher in young cells than in old cells.

Next, we searched pathways in which transcription changed in the 7th and 11th generation relative to the 4th generation (Table 1). This pathway analysis clearly showed that very few pathways were changed between the 4th and 7th generations, but several biological processes and metabolic pathways were strikingly enhanced or reduced after 11 generations. Cells in the 11th generation had accumulated transcripts coding for components of the sugar metabolism and TCA cycle, consistent with previous observation of a shift from glycolysis toward gluconeogenesis in old cells (7). Unlike previous reports, our pathway analysis clearly indicates that amino acid degradation pathways were enhanced and biosynthetic pathways of branched-chain amino acid (BCAA: leucine, isoleucine, and valine) were decreased.

Previous transcriptome analyses of older cells (~20 generations) reported that environmental stress response pathways were induced in aged cells (7), although oxidative stress gene expression did not change (14). Our pathway analysis, however, showed no significant change in the stress response pathways of old cells, including the oxidative stress response pathway. Similarly, we found no changes in the DNA damage repair pathways, which were reported to be induced in old cells (7, 15). Thus, the stress response and DNA damage repair pathways are not induced during the early stages of senescence. It was previously reported that ribosome gene expression decreased in 18- to 20-generation-old cells (15). However, our transcriptome analysis showed that the pathway for ribosomal protein expression was reduced by the 4th generation compared with unbudded cells but was not further reduced after another four generations; conversely, young cells have a higher level of protein synthesis that decreases even in the early stages of aging.

**Genes That Are Highly Induced by the 11th Generation**—In addition to analyzing age-dependent pathways, we focused on individual transcripts that were highly accumulated in 11th generation cells. Among 59 genes that exhibit >8-fold induc-
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By the 11th generation relative to the 1st generation, we found four genes, *SPG4*, *SNO1*, *SNZ1*, and *HSP12*, which are known to be induced during stationary phase (21, 22). The mRNA of these genes was present at more than a 5-fold higher concentration in 7th to 11th generation cells compared with 1st generation cells. We confirmed the age-dependent expression of 34 stationary phase-related genes by searching the term “stationary phase” in the *Saccharomyces* Genome Database (Fig. 4).

**TABLE 1**

Pathway analysis with transcriptomic data relative to the 4th generation

| Name*                  | No. changedb | No. measuredc | No. on MAPPd | Percent changede | Percent presentf | Z scoreg | Permute p h | Adjusted p i |
|------------------------|--------------|---------------|--------------|------------------|------------------|----------|-------------|--------------|
| **Increased 7th**      |              |               |              |                  |                  |          |             |              |
| DNA replication        | 3            | 29            | 33           | 10.3             | 87.9             | 5.96     | 0.00        | 0.37         |
| Pentose and glucuronate interconversions | 1 | 9 | 61 | 11.1 | 14.8 | 3.56 | 0.05 | 0.99 |
| 11th                   |              |               |              |                  |                  |          |             |              |
| Principle pathways of carbon metabolism | 11 | 79 | 98 | 13.9 | 80.6 | 4.46 | 0.00 | 0.40 |
| Fructose and mannose metabolism | 7 | 32 | 79 | 21.9 | 40.5 | 5.07 | 0.00 | 0.12 |
| Glycolysis/Gluconeogenesis | 7 | 47 | 65 | 14.9 | 72.3 | 3.74 | 0.00 | 0.61 |
| Butanoate metabolism   | 5            | 29            | 69           | 17.2             | 42.0             | 3.56     | 0.00        | 0.69         |
| Galactose metabolism   | 5            | 30            | 55           | 16.7             | 54.5             | 3.46     | 0.01        | 0.69         |
| Non-oxidative branch of the pentose pathway | 3 | 9 | 18 | 33.3 | 50.0 | 4.39 | 0.01 | 0.41 |
| Amino sugar metabolism | 4            | 22            | 48           | 18.2             | 45.8             | 3.51     | 0.01        | 0.72         |
| Nucleotide sugar metabolism | 3 | 14 | 39 | 21.4 | 35.9 | 3.25 | 0.01 | 0.75 |
| Valine, leucine, and isoleucine degradation | 3 | 11 | 38 | 27.3 | 28.9 | 3.85 | 0.01 | 0.61 |
| Glycine, serine, and threonine metabolism | 5 | 42 | 73 | 11.9 | 57.5 | 2.55 | 0.02 | 0.97 |
| m-Cresol degradation   | 2            | 6             | 10           | 33.3             | 60.0             | 3.58     | 0.03        | 0.69         |
| p-Cymene degradation   | 2            | 6             | 13           | 33.3             | 46.2             | 3.58     | 0.03        | 0.69         |
| Toluene degradation    | 2            | 6             | 12           | 33.3             | 50.0             | 3.58     | 0.03        | 0.69         |
| Pentose phosphate pathway | 4 | 26 | 46 | 15.4 | 56.5 | 2.89 | 0.03 | 0.95 |
| Carbon fixation        | 3            | 18            | 29           | 16.7             | 62.1             | 2.67     | 0.03        | 0.96         |
| α-Arginine alpha-ketoglutarate metabolism | 2 | 7 | 15 | 28.6 | 46.7 | 3.24 | 0.03 | 0.92 |
| Citrate cycle (TCA cycle) | 4 | 30 | 40 | 13.3 | 75.0 | 2.54 | 0.03 | 0.97 |
| Lysine degradation     | 4            | 30            | 67           | 13.3             | 44.8             | 2.54     | 0.04        | 0.97         |
| Propanoate metabolism  | 2            | 8             | 48           | 25.0             | 16.7             | 2.96     | 0.04        | 0.94         |
| Isoleucine degradation | 2            | 7             | 8            | 28.6             | 87.5             | 3.24     | 0.04        | 0.92         |
| **Reduced 11th**       |              |               |              |                  |                  |          |             |              |
| Valine biosynthesis    | 3            | 5             | 7            | 60.0             | 71.4             | 7.30     | 0.00        | 0.02         |
| Isoleucine biosynthesis | 3 | 7 | 10 | 42.9 | 70.0 | 6.04 | 0.00 | 0.06 |
| Valine, leucine, and isoleucine biosynthesis | 4 | 16 | 20 | 25.0 | 80.0 | 5.04 | 0.00 | 0.26 |
| Leucine degradation    | 2            | 4             | 6            | 50.0             | 66.7             | 5.38     | 0.01        | 0.25         |
| Pantothenate and CoA biosynthesis | 3 | 11 | 30 | 27.3 | 36.7 | 4.61 | 0.01 | 0.40 |
| Leucine biosynthesis   | 2            | 6             | 11           | 33.3             | 54.5             | 4.25     | 0.01        | 0.49         |
| Isoleucine degradation | 2            | 7             | 8            | 28.6             | 87.5             | 3.87     | 0.02        | 0.59         |
| Ergosterol biosynthesis | 2 | 19 | 31 | 15.8 | 61.3 | 3.18 | 0.03 | 0.89 |
| Phenylalanine degradation | 2 | 11 | 14 | 18.2 | 78.6 | 2.87 | 0.05 | 0.96 |

* Name indicates a gene ontology term.
* The number of genes meeting the criterion at this node.
* The number of genes measured at this node.
* The number of genes associated with this node.
* The percentage of genes meeting the criterion in this node.
* The percentage of genes measured in this node.
* The z score under the hypergeometric distribution.
* The p value calculated using a non-parametric bootstrapping approach.
* The p value using the Westfall-Young adjustment for multiple hypothesis testing.

**FIGURE 4.** Age-dependent expression of stationary phase-related genes. Stationary phase-induced and -repressed genes are shown by red or blue shading, respectively. Expression values of designated ages relative to the 1st generation based on DNA microarray analysis are represented.
Genes that are described as stationary phase-induced genes were mostly up-regulated in aged cells, and genes that are described as stationary phase-repressed genes such as CYR1, TOS6, and BAT1 were down-regulated. Ten of 34 stationary phase-related genes showed a 2-fold higher expression at the 11th generation relative to the 1st generation, and nine were higher relative to the 7th generation. RT-qPCR analysis confirmed high expression of three of the top six genes and slight expression of another two genes in the 11th generation with no change in SNO1 gene expression (Fig. 5A). Furthermore, we confirmed the age-dependent expression of 127 stationary phase genes whose mRNA levels were reported to be reproducibly detectable in stationary phase cells (22). About 40% of these stationary phase genes showed >2-fold higher expression at the 11th generation relative to the 1st generation, and nine were higher relative to the 7th generation. RT-qPCR analysis confirmed high expression of three of the top six genes and slight expression of another two genes in the 11th generation with no change in SNO1 gene expression (Fig. 5A). Furthermore, we confirmed the age-dependent expression of 127 stationary phase genes whose mRNA levels were reported to be reproducibly detectable in stationary phase cells (22). About 40% of these stationary phase genes showed >2-fold higher expression at the 11th generation relative to the 1st generation, and ~20% were higher at the 11th generation relative to the 4th generation. These observations lead to the interesting idea that the 11th generation-induced genes overlap with stationary phase genes and, therefore, a common transcription factor acts in both regulatory pathways.

In addition, after 11 generations, we observed a remarkable accumulation of transcripts for 18 of the 20 genes measured belonging to the 24-gene PAU (seripauperin) family. The gene products of the PAU family have unknown functions (23). PAU genes are highly conserved, and most of the PAU probes on the DNA microarray used cannot discriminate the respective PAU genes. However, the microarray can be used to estimate the overall expression of PAU. The transcription of 12 of 18 PAU up-regulated genes clearly increased between the 7th and 11th generations. Most PAU gene loci are located in the subtelomeric regions of chromosomes. PAU genes located both in internal regions of the chromosomes as well as those in the subtelomeric regions were induced, indicating that induction of PAU genes is independent of a particular chromosome location such as subtelomeric regions. Because the level of Sir2p protein, a telomere silencing factor, is known to be significantly reduced in replicatively aging yeast cells (24), we do not exclude the possibility that some of these increases in the PAU expression are due to the loss of Sir2p, at least for the subtelomeric set of genes.

Outline of Metabolic Changes Occurring in an Age-dependent Manner—The findings from the above transcriptome analysis strongly suggested that metabolic changes begin at an early stage of replicative senescence. To confirm this, 37 low molecular weight intracellular compounds (including amino acids, organic acids, and sugars) were extracted from cells of designated ages and identified and quantified using GC-MS (supplemental Table S1). PCA was performed to visualize significant effects with multivariate data of the profiles expressed as relative levels of the metabolites (Fig. 6). A scores plot where the data points were projected onto a plane defined by the first principal component (PC1) and the second principal component (PC2) showed clustering of the data points from each successive generation. The generation clusters were separated from each other in both PC directions: PC1 and PC2 accounted for 58 and 13% of the total variance, respectively (Fig. 6A). Interestingly, the variance along PC2 appeared to be correlated

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3 The abbreviations used are: qPCR, quantitative PCR; BCAA, branched-chain amino acid(s).
with the generations, with higher scores for older cells than younger cells. This indicates metabolic shifts that correlate with aging.

To identify the metabolites associated with aging, we searched the metabolites that contributed to the separation of generations along PC2 in the PCA. The relevant loading plots of PC2 represented the relative degree of correlation between the levels of each metabolite and aging (Fig. 6B). High levels of pyruvic acid and TCA cycle intermediates (oxaloacetic acid, citric acid and isocitric acid, malic acid, fumaric acid, and 2-oxoglutaric acid) positively correlated with older generations (loading on PC2 > 0.05), whereas low levels of about half of the amino acids (glycine, histidine, valine, 4-aminobutyric acid, homoserine, isoleucine, glutamic acid, leucine, tyrosine, arginine, ornithine, and methionine) negatively correlated with aging (loading on PC2 < −0.05).

We mapped the metabolic profiles of the 37 compounds measured in this study on the TCA cycle and amino acid biosynthetic pathways (Fig. 7). The concentrations of most of the metabolites varied during the early stages of senescence, as expected from PCA. Some TCA cycle intermediates increased, and about half of the amino acids decreased in concentration. Only lysine accumulated in an age-dependent fashion. Some organic acids (lauric acid, maleic acid, 2-oxoglutaric acid) and amino acids (alanine, arginine, and ornithine) did not change significantly (p > 0.05), whereas aromatic amino acids (phenylalanine, tyrosine, and tryptophan), which are biosynthesized in the shikimate pathway, decreased slightly by the 11th generation. Both the metabolomic and transcriptomic profiles revealed increased metabolite levels between generation 1 and generation 4 but exhibited little further change by generation 7. Therefore, we determined the significance of each metabolite in 7th and 11th generation cells relative to 4th generation cells; red bars and blue bars in Fig. 7 indicate a significant increase and decrease, respectively (p < 0.05). After 11 generations, significant changes were observed: enhanced TCA cycle biosynthesis and decreased amino acids biosynthesis, especially BCAA (details described below).

Additionally, intracellular glucose was reduced after 11 generations (Fig. 7). No reduction in the concentration of glucose in the cell-free culture media was expected after 11 generations because the 11th generation cell culture was prepared by exchanging the 7th generation culture medium with fresh medium and culturing for a further four generations. As expected, GC-MS analysis of the cell-free yeast cell culture medium showed no difference in glucose content before and after cultivation (Fig. 8). Accordingly, the age-dependent decrease in intracellular glucose suggested that glucose uptake decreased in senescent yeast cells. We found in DNA microarray analysis and confirmed by RT-qPCR analysis that genes encoding glucose transporters, which belong to the major facilitator superfamily that are typically highly expressed such as HXT1, HXT3, HXT4, and HXT7 (25), were notably down-regulated between the 1st and 4th generations and that low-level transcription was sustained during the early stage of senescence (Fig. 5B). Similar to PAU genes, HXT genes are highly conserved, and it is likely that the DNA microarray for HXT over-estimated gene expression. This indicates that a decrease in the ability to uptake glucose results in lower intracellular glucose despite there being sufficient glucose in the medium.

Integrating Metabolic and Transcriptional Profiling in the TCA Cycle and BCAA Biosynthetic Pathway—The observation that transcripts coding for components of the TCA cycle, and that TCA cycle metabolites accumulated in 11th generation cells, led us to examine the relation between the level of TCA cycle intermediates and the transcript level of the gene coding the enzyme that catalyzes the corresponding reaction in the TCA cycle. Pyruvic acid and three neighboring TCA cycle intermediates (oxaloacetic acid, citric acid, and isocitric acid) significantly increased after 11 generations. Citrate synthase catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate and is the rate-limiting enzyme in the TCA cycle (26, 27). Interestingly, the transcript levels of the CIT1, CIT2, and CIT3 genes, which encode citrate synthase, are increased in senescent cells according to DNA microarray analysis (Fig. 9A), and the CIT1 up-regulation was confirmed by RT-qPCR analysis (Fig. 5C). This may explain the increase in intracellular citric acid and isocitric acid by the 11th generation. The high level of oxaloacetic acid by the 11th generation might be caused by up-regulation of the PYC1 gene, which encodes...
pyruvate carboxylase that converts pyruvate to oxaloacetate (28), although the PYC2 gene, a paralog of PYC1, was downregulated. Down-regulation of the LAT1 gene, which encodes a component of the pyruvate dehydrogenase complex that catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA (29), might contribute to the accumulation of pyruvic acid after 11 generations. These data indicate that the accumulation of several TCA cycle intermediates is under the transcriptional control of the corresponding metabolic genes.

The accumulation of TCA cycle intermediates suggests that TCA cycle biosynthesis might be enhanced by aging, resulting in a higher respiration rate in aging cells. However, we did not find a gross up-regulation of oxidative phosphorylation genes, although several COX (cytochrome c oxidase) genes were up-regulated. This indicates that yeast cells do not shift toward respiration with aging.

We also confirmed the role of transcriptional regulation in the decrease of many amino acids, especially BCAA (Fig. 5D). Analysis of the products of the BCAA biosynthetic pathway showed significantly decreased levels of isoleucine, valine, and leucine at the 11th generation, although the concentration of pyruvic acid, the initial metabolite of this pathway, increased.

FIGURE 7. A metabolic map of central carbon and amino acid metabolism during cellular aging. Bar graphs indicate the amount of the metabolite relative to the 1st generation. For each metabolite, blue and red bars represent a significant decrease and increase, respectively, relative to the level in the 4th generation.
gradually with aging (Fig. 9B). Transcript levels of most of the BCAA biosynthetic pathway genes decreased, as suggested by the above pathway analysis with transcriptomics. This clearly indicates that the low level of BCAA at the 11th generation is caused by down-regulation of the BCAA biosynthetic pathway genes. A decrease in glutamic acid and γ-aminobutyric acid was observed after 11 generations (Fig. 7). This decrease can be explained by reduced mRNA levels of the \textit{GLT1} gene, which encodes glutamate synthase, the enzyme that catalyzes the synthesis of glutamate from glutamine and α-ketoglutarate (30), and by increased mRNA levels of the \textit{GAD1}, \textit{UGA1}, and \textit{UGA2} genes, which encode components of the glutamate degradation pathway (31, 32). Alternatively, there could also be post-transcriptional changes in enzyme stability or activity that led to the change of amino acids as well as TCA cycle intermediates observed in metabolomics analysis.

A decrease in amino acid concentrations can be explained by reduced transcript levels of the corresponding amino acid biosynthetic genes, rather than by down-regulation of the amino acid transporter genes (whose transcription did not change with aging). We examined the expression of the \textit{GCN4} gene that encodes a transcriptional activator of general amino acid biosynthetic genes. The expression of \textit{GCN4} was comparable between designated ages of wild-type cells (Fig. 5E). Next, we compared senescence-associated expression in wild-type and \textit{GCN4} deletion mutant strains (data not shown). Deletion of \textit{GCN4} decreased the transcription of amino acid biosynthetic genes at the 11th generation compared with the 1st generation. Thus, \textit{GCN4}-independent reduction of amino acid biosynthetic gene transcription during replicative senescence was observed, suggesting that other transcription factors regulate age-dependent expression of amino acid biosynthetic genes.

Because intracellular amino acids decreased significantly in 11-generation-old cells, we analyzed the nutrients in the culture medium. Most nutrients were not depleted even after 11 generations, as described above (Fig. 8), suggesting that yeast cells exhibit decreased nutrient sensing and/or signaling by the 11th generation.

**DISCUSSION**

We performed transcriptional and metabolic profiling of yeast cells at early stages of senescence (4th, 7th, and 11th generation). Previous transcriptomic studies had analyzed older cells close to the median replicative lifespan (18th to 20th generation) (7, 13–15). The transcriptional profiles showed remarkable up- and down-regulation of gene expression after 11 generations. The 11th generation cells had increased levels of pyruvic acid and TCA cycle intermediates and decreased levels of amino acids, especially BCAA. An apparent relation was observed between metabolites and transcripts of the corresponding metabolic genes. Furthermore, high expression of \textit{PAU} family and stationary phase-induced genes was found after 11 generations, even though the yeast cells were cultivated under aerobic conditions, and the growth medium contained sufficient nutrients. These changes are presumably early indications of replicative senescence.

Our transcriptomic and metabolomic analyses clearly indicate that replicative senescence of yeast cells begins around the 11th generation, which is about half of the average replicative lifespan. Because yeast cells begin to die by the 10th generation,
as shown in Fig. 1, this generation appears to be the start point for cellular senescent behavior. It was reported that 20-generation-old cells exhibited enhanced gluconeogenesis and energy storage (7); therefore, 11-generation-old cells might be just starting to switch sugar metabolisms, consistent with previous observations during the early stage of aging (13, 14). In human diploid fibroblasts, the replicative senescence process was divided into four stages (early, middle, advanced, and very advanced) by profiling cellular senescence phenotypes and mRNA expression patterns (5). Reactive oxygen species levels increased in the early stage and were drastically elevated after the middle stage. A low level of senescence-associated β-gal activity was evident in middle stage cells and thereafter gradually increased. Gene expression profiling revealed four distinctive modules: module G1 (doubling times, 2 days), G2 (doubling times = 2–7), G3 (doubling times = 3–20), and G4 (doubling times = 10–30). Gene expression during each module governs each stage of senescence, supporting the development of the associated senescence phenotypes. These findings are consistent with our observation in yeast cells that transcriptional changes of metabolic enzyme genes caused the corresponding metabolic changes. Module G1 was prominently enriched for genes that are related to cell cycle and DNA repair, indicating active cell proliferation. This behavior of human cells is typi-
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cally similar to that of young yeast cells. Module G2 included genes that are related to metabolic and tRNA processes. Although we do not know the correspondence of replicative senescence stages between yeast and human cells, the 11th generation in yeast cells might correspond to the middle stage of aging of human cells.

Transcriptomic changes at the early stages of senescence imply the existence of transcription factors that regulate gene expression at this stage. High expression of stationary phase-induced genes after 11 generations indicated that transcription factors induce gene expression during stationary phase and regulate the transcription of senescence-induced genes. For example, Msn2p/Msn4p and Gis1p in the Rim15p protein kinase pathway and Adr1p, Cat8p, and Mig1p in the Snf1p protein kinase pathway are thought to be transcription factors that regulate stationary phase-induced genes (33, 34). Transcription of the MSN2, MSN4, GIS1, and CAT8 genes was not regulated during the early stage of senescence, but the ADR1 activator gene was up-regulated 4-fold, and MIG1 repressor gene was down-regulated 2-fold at the 11th generation relative to the 1st generation. Thus, Adr1p and Mig1p might be required for the induction of age-associated genes, although we do not exclude the possible existence of an unidentified transcription factor for senescence-specific induction. Using our transcriptome data, we analyzed the age-associated transcription of 189 genes encoding DNA-binding transcription factor, which were searched in the Saccharomyces Genome Database. Thirty-eight of these transcription factor genes were up-regulated >2-fold in 11th generation cells, and twenty-six were down-regulated; these genes might include senescence-associated transcription factor genes.

What deterioration begins during the early stage of senescence in yeast? We found that 11th generation cells highly express stationary phase genes that are induced by nutrient deprivation (33). One possible trigger of cellular senescence is that a nutrient-sensing and/or signaling pathway degrades with aging. The transcriptional regulation of ADR1 and MIG1 is unknown; however, the observed changes in their transcription may suggest that the Snf1p signaling pathway deteriorates during the early stage of senescence, even in medium containing sufficient nutrients, resulting in induction of stationary-phase genes. PAU genes are induced by anaerobiosis through an unknown transcription factor(s) (35). Up-regulation of PAU genes causes degeneration of the oxygen-responding pathway. Tupd1 corepressor is required for transcriptional repression of PAU by oxygen. In contrast, Rox1p, a DNA-binding repressor involved in oxygen-dependent repression of other target genes such as ANB1, is not required (36). TUP1 and CYC8, two subunits of Tup1p-Cyc8p corepressor complex, were slightly down-regulated in our microarray experiment (70% at the 11th generation relative to the 1st generation). This may be one reason why PAU genes are up-regulated in senescent cells.

Another possible mechanism triggering cellular senescence is mitochondrial dysfunction. Expression of the CIT genes, which are controlled by retrograde transcription factors Rtg1p and Rtg3p, drastically increased after 11 generations. Retrograde regulation is triggered by mitochondrial dysfunction, and the signal is transduced from mitochondria to the nucleus (37).

Yeast petite mutants lacking mitochondrial DNA up-regulate TCA cycle enzyme-encoding genes, including CIT genes (38). Mitochondrial fragmentation begins to occur in cells after four generations and increases as cells age, and mitochondria are severely fragmented with no tubular structure at the 11th generation (39). These observations are consistent with our observations of aging cells at the 11th generation. We propose that age-associated mitochondrial dysfunction is one cause of transcriptional and metabolic changes at the early stages of replicative senescence.

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