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Transcription factor genes essential for cell proliferation and replicative lifespan in budding yeast

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ABSTRACT

Many of the lifespan-related genes have been identified in eukaryotes ranging from the yeast to human. However, there is limited information available on the longevity genes that are essential for cell proliferation. Here, we investigated whether the essential genes encoding DNA-binding transcription factors modulated the replicative lifespan of *Saccharomyces cerevisiae*. Heterozygous diploid knockout strains for *FHL1*, *RAP1*, *REB1*, and *MCM1* genes showed significantly short lifespan. ^{1}H-nuclear magnetic resonance analysis indicated a characteristic metabolic profile in the Δ*fhl1*/*FHL1* mutant. These results strongly suggest that *FHL1* regulates the transcription of lifespan related metabolic genes. Thus, heterozygous knockout strains could be the potential materials for discovering further novel lifespan genes.

1. Introduction

Lifespan is largely determined by the combined effects of environmental factors and genetics, including epigenetics [1–3]. Spontaneous and gene-targeted mutations to extend lifespan and delay aging have uncovered molecular mechanisms associated with longevity, while intrinsic genetic deficits shortening lifespan and hastening aging can result in critical problems for various organisms. In human, mutations of a single gene are known to cause premature aging disorders, like Werner syndrome, Bloom's syndrome, and Hutchinson–Gilford progeria syndrome [4].

Many genes involved in controlling aging and lifespan have been identified in a variety of model organisms, including mice, fly, and nematodes [5–7]. The budding yeast *Saccharomyces cerevisiae* has also been well investigated as a model organism for cellular aging and lifespan research because it is readily amenable to genetic analysis, especially by the use of haploid strains. It is advantageous for studying the replicative lifespan of a single cell, which is defined as the number of daughter cells that a mother cell can generate before dying [8]. Numerous studies using knockout (KO) and overexpressing yeast strains have identified lifespan-related genes involved in metabolism, environmental stress response, nutrient starvation, genomic integrity, and their signal transduction and regulation [9–11]. However, there is limited knowledge on lifespan-related information of genes involved in cell proliferation because of the convenience and usefulness of using haploid KO strains. Until now, the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) represents 1,192 of the essential genes whose deletion can lead to lethality, while overexpression of 119 genes, some of which are overlapped with the deletion-lethal genes, can also lead to cell death.

Metabolomics is a powerful tool for the elucidation of biological processes, including aging and lifespan. In a previous study, mass spectrometry-based metabolome analysis of intracellular compounds (fingerprinting) revealed a correlation between the replicative lifespan and metabolic profiles, and the prediction model of yeast lifespan was constructed based on the metabolome data [12]. The metabolic profiles presumed that *UGA3* gene encoding a transcription factor (TF) for γ-aminobutyric acid metabolic pathway genes was involved in lifespan regulation, followed by identification of the Uga3p-targeted *UGA1* and *GAD1* metabolic enzyme genes [12,13]. Furthermore, metabolome analysis of aging yeast cells showed metabolic changes at the early stage of

Abbreviations: TF, transcription factor; NMR, nuclear magnetic resonance; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; OPLS, orthogonal projections to latent structures.

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senescence, consistent with observation of human senescent cells, and such changes were caused by changing the transcript levels of the metabolic enzyme genes [14].

In the present study, we measured the replicative lifespan of heterozygous strains deleted for the essential genes that encoded DNA-binding transcription factors. Based on the results, the plausibility using heterozygous KO strains for identifying novel lifespan-related genes will be discussed.

2. Materials and methods

2.1. Strains and medium

The *S. cerevisiae* strains used in this study were derived from BY4743 (diploid) and BY4742 (haploid) [15], and are listed in Supplementary Table S1. Heterozygous diploid deletion strains were obtained from Yeast Heterozygous Essential Collection YSC1057 (Open Biosystems, AL, USA). Heterozygous strains deleted for *FHL1* and *MET4*, which were not included in the collection, and for *RAP1*, whose disruption was not verified, were constructed by a PCR-based gene disruption method [16] using oligonucleotides listed in Supplementary Table S2. A homozygous deletion mutant of *FHL1* was constructed by crossing *MAT***a** Δ*fhl1* and *MATα* Δ*fhl1* haploid segregants from the Δ*fhl1*/*FHL1* diploid. Yeast strains that have two copies of the TF genes were constructed by integration of YIp5 plasmid [17] harboring the TF genes into the original gene locus of BY4742 after digesting with a proper restriction enzyme, and validated by genomic DNA PCR analysis, as described in detail in Supplementary data. YPD medium (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% dextrose) was used for routine cultures.

2.2. Replicative lifespan determination

Replicative lifespan was assayed as described previously [14]. A sample of yeast cells grown at 30 °C on a YPD agar plate was spread onto a new YPD agar plate containing 10 μg/ml Phloxine B. Virgin daughter cells were selected and subjected to lifespan analysis. 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. This assay was performed at least twice with each strain by independent persons.

2.3. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNAs were extracted from yeast cells using an RNeasy Mini Kit (Qiagen, CA, USA). One-step RT-qPCR was carried out using a QuantiTect SYBR Green RT-PCR Kit (Qiagen) on a Thermal Cycler Dice (Takara Bio, Shiga, Japan). The expression level was normalized to that of the *UBC6* gene [18].

2.4. ^{1}H-NMR metabolomics analysis

Extraction of metabolites from yeast cells and ^{1}H-NMR analysis were performed with minor modifications as described previously [13]. Yeast cultures were grown in YPD to an OD_{600} of 1.0. The cells were collected and washed with water once. Then 75% (v/v) hot ethanol solution was added directly to the cell pellet and heated for 3 min at 80 °C. The mixture was cooled on ice and subsequently dried in rotary vacuum concentrator. The dried extracts were dissolved in 0.1 M potassium phosphate buffer (pH 7.0) in D_2O containing 1 mM sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate as a reference. The solution was then mixed by vortex, and then centrifuged to remove the undissolved materials. ^{1}H-NMR spectra were acquired at 500 MHz into 65,536 data points under the same condition as described previously.

Data reduction from ^{1}H-NMR analysis was carried out using ALICE2 for Metabolome software (JEOL RESONANCE, Tokyo, Japan). The total signal within each region with a spectral width of 0.04 ppm was integrated between 0.0 ppm and 10 ppm. The region from 4.4 ppm to 5.5 ppm that contained the residual signal from the water resonance, and the region from 1.18 ppm to 1.22 ppm and from 3.58 ppm to 3.70 ppm that were derived from ethanol, were excluded. This resulted in each spectrum being reduced to a vector of length 215.

Prediction of compounds in ^{1}H-NMR spectra was performed using Chenomx NMR Suite 7.5 software (Chenomx, Alberta, Canada). The data sets from the ^{1}H-NMR analysis were judged in all cases by orthogonal projections to latent structures (OPLS) using SIMCA-P+ 12.0.1 (Umetrics, Sweden).

3. Results

3.1. Growth property of the heterozygous TF deletion mutants

Generally, most of the lifespan-related genes have been identified and characterized by using haploid KO mutants because they make phenotypes easy to observe and investigate. Therefore, there is limited knowledge on lifespan information of genes that are essential for cell proliferation. This motivated us to evaluate lifespan of heterozygous diploid strains that were deleted for the essential genes. Although 1,192 of the essential genes are represented, we do not have a comprehensive method of replicative lifespan analysis, which is time-consuming, labor-intensive, and requires highly skilled micromanipulation. So, as representatives, we focused on essential TF genes (Table 1) because TFs implicate their regulated target genes and subsequently presume the regulatory pathways and physiological systems. We investigated all the eight essential DNA-binding TF genes, together with examples of non-DNA-binding coactivator genes, *MET4* and *NDD1*, which are essential for cell growth.

First, we confirmed lethality of the haploid deletion mutants by tetrad analysis. Each of the heterozygous diploid cells was dissected, and viable spores were assigned for the G418-resistant marker, which is derived from the KO alleles. Δ*met4*/*MET4* diploid cells did not sporulate. The G418-resistant spores from Δ*fhl1*/*FHL1* and Δ*gcr1*/*GCR1* diploid cells grew though extremely slowly, but all of the viable spores from the other heterozygous diploid cells exhibited G418 sensitivity. This shows that the TF genes, except for *FHL1* and *GCR1*, are essential for cell proliferation. We constructed and examined the diploid strain homozygously deleted for *FHL1* for following analysis.

Next, we measured the specific growth rates of the heterozygous TF deletion mutants and the homozygous *FHL1* deletion mutant. Growth curves of these mutants showed that most of the heterozygotes showed normal growth (Supplementary Fig. S1). Specific growth rates of the heterozygous KO strains were comparable to that of the wild-type BY4743 strain in YPD medium, except that the Δ*hsf1*/*HSF1* and Δ*gcr1*/*GCR1* strains grew slightly faster, while the Δ*fhl1*/Δ*fhl1* strain grew extremely slowly (Table 2).

3.2. Identification of the TF genes that are involved in replicative lifespan

We measured the replicative lifespan of 10 heterozygous diploid strains deleted for the TF genes and the parental wild-type strain (Fig. 1 and Table 2). The heterozygous strains deleted for the *FHL1*, *RAP1*, and *REB1* genes exhibited considerably short lifespan

Table 1
The essential TF genes used in the study.

Gene name	Functions
FHL1 (Fork Head-Like 1)	Forkhead transcription factor involved in regulation of ribosomal protein gene transcription
RAP1 (Repressor Activator Protein 1)	Helix-turn-helix transcription factor involved in telomere length maintenance, chromatin silencing, and the activation of glycolytic and ribosomal protein genes
REB1 (RNA polymerase I Enhancer Binding protein 1)	Myb-family transcription factor implicated in the regulation of ribosomal RNA transcription and many genes transcribed by RNA polymerase II, as well as in centromeres and subtelomeric regions
MCM1 (MiniChromosome Maintenance 1)	α helix transcription factor of the MADS box family involved in transcription of cell-type-specific genes, cell-cycle-dependent genes and Ty elements, recombination, arginine metabolism, and osmotolerance
PDC2 (Pyruvate DeCarboxylase 2)	Transcriptional activator without overall similarity to known transcription factor families involved in activating thiamin pyrophosphate biosynthetic genes
MET4 (METhionine requiring 4)	Leucine-zipper transcriptional coactivator involved in the expression of sulfur-containing amino acids biosynthesis genes
ABF1 (ARS-Binding Factor 1)	Zinc finger transcription factor involved in transcriptional activation of carbon and nitrogen utilization genes and ribosomal protein genes, silencing, and DNA replication and repair
NDD1 (Nuclear Division Defective 1)	Transcriptional coactivator involved in nuclear division and activation of expression of late-S-phase-specific genes
GCR1 (GlyColysis Regulation 1)	Leucine-zipper transcriptional activator of glycolytic and ribosomal protein genes
HSF1 (Heat Shock transcription Factor 1)	Helix-turn-helix transcription factor of the E2F family involved in upregulating heat-shock-responsive genes

Table 2
Growth property and replicative lifespan of the heterozygous TF deletion mutants.

Allele	Specific growth rate (μ)[a]		Replicative lifespan[b]	
	Average ± SD	*P* value[c]	Average ± SD	*P* value[d]
WT	0.482 ± 0.029	–	27.7 ± 10.5	–
hsf1/HSF1	0.596 ± 0.008	6.0×10^{-3}	29.3 ± 9.3	2.0×10^{-1}
gcr1/GCR1	0.619 ± 0.028	8.9×10^{-3}	29.1 ± 8.9	2.7×10^{-1}
ndd1/NDD1	0.432 ± 0.027	1.5×10^{-1}	29.0 ± 7.9	2.4×10^{-1}
abf1/ABF1	0.424 ± 0.014	6.2×10^{-2}	28.2 ± 8.7	6.3×10^{-1}
met4/MET4	0.457 ± 0.017	3.5×10^{-1}	27.4 ± 11.6	8.8×10^{-1}
pdc2/PDC2	0.431 ± 0.020	1.1×10^{-1}	27.0 ± 9.5	7.3×10^{-1}
mcm1/MCM1	0.482 ± 0.006	9.7×10^{-1}	24.1 ± 8.6	2.2×10^{-2}
reb1/REB1	0.437 ± 0.008	1.0×10^{-1}	23.0 ± 4.8	3.3×10^{-3}
rap1/RAP1	0.440 ± 0.005	1.1×10^{-1}	21.7 ± 7.2	9.4×10^{-5}
fhl1/FHL1	0.447 ± 0.013	1.9×10^{-1}	17.3 ± 6.1	1.0×10^{-7}
fhl1/fhl1	0.122 ± 0.003	6.3×10^{-5}	17.0 ± 4.1	1.0×10^{-7}

[a] The specific growth rate was calculated using regression analysis of an exponential growth curve determined by OD_{600} measurement (n = 3).
[b] Replicative lifespan was measured for at least 96 cells of each strain.
[c] The *P* value was calculated using a Student's *t*-test relative to WT strain.
[d] The *P* value was calculated using a Wilcoxon rank-sum test relative to WT strain.

($P < 0.01$, Fig. 1A), while when deleted for the *MCM1* gene showed moderately short lifespan ($P < 0.05$). The other heterozygous KO strains showed normal lifespan as the wild-type strain (Fig. 1B). Therefore, we concluded that *FHL1*, *RAP1*, *REB1*, and *MCM1* were required for the replicative lifespan in yeast.

It was reported that homozygous mutation of Δ*sir2* causes a significant reduction in diploid lifespan, and Δ*sir2*/*SIR2* heterozygote has a lifespan that is intermediate between the wild-type diploid and Δ*sir2*/Δ*sir2* homozygote [19]. So, we examined the possibility of regulation of lifespan by *FHL1* in a dose-dependent manner as *SIR2*. Unexpectedly, the replicative lifespan was found to be comparative between the Δ*fhl1*/Δ*fhl1* homozygote and Δ*fhl1*/*FHL1* heterozygote ($P = 0.37$, Fig. 1C and Table 2). This was not the case of *SIR2*, and it suggested that the levels of Fhl1p are limiting for lifespan even in the heterozygote.

The level of transcription of the heterozygous deletion gene was possibly not decreased in the normal-lived KO strains. To test this possibility, we performed the RT-qPCR analysis of these TF mutant strains (Fig. 2). All of the normal-lived heterozygous strains showed less than half of transcription levels of the wild type, except that the Δ*hsf1*/*HSF1* mutant expressed about 60% of the *HSF1* transcripts of the wild-type control. Accordingly, downregulation of the designated genes seems not to affect the lifespan although it remains possible that the reduced level of transcription of these genes is still enough to sustain lifespan. We confirmed significant downregulation of the *FHL1*, *RAP1*, and *REB1* genes, and moderate downregulation of the *MCM1* gene.

3.3. Duplication of the essential TF genes did not affect lifespan

Since heterozygous deletion of the *FHL1*, *RAP1*, *REB1*, and *MCM1* genes shortened lifespan, we presumed that duplication of these genes extends lifespan. We constructed strains in which each of the essential TF genes were integrated into the original gene locus of the wild-type haploid strain, and measured the replicative lifespan (Supplementary Fig. S2). However, two copies of the four TF genes did not extend but rather slightly shortened lifespan (Fig. S2A). Similarly, two copies of the other TF genes also did not increase lifespan (Fig. S2B). Transcripts of the TF genes in the two copies strains were increased at least 1.3 fold or more, except those in the strains duplicated for *RAP1* and *MCM1* that were comparable to that of the wild-type, with unknown reason (Fig. S2C). It indicated that duplication of the TF genes did not largely affect the replicative lifespan.

3.4. Deletion of FHL1 changed ^{1}H-NMR metabolic profile

In our previous study, metabolic fingerprinting revealed a correlation between lifespan and metabolic profile [12,13]. To investigate a possibility that heterozygous deletion of *FHL1*, *RAP1*, *REB1*, and *MCM1* changes cellular metabolisms to shorten lifespan, the whole-cell metabolite levels were surveyed by ^{1}H-NMR-based metabolomic analysis. We measured the ^{1}H-NMR spectra of short-lived and normal-lived mutants and the wild-type strain independently at least four times, and calculated integral values of buckets from the spectra (Supplementary Table S4).

Since the principal component analysis of the NMR-derived metabolomic profiles did not show clear separation of the short-lived mutants from the normal-lived mutants, we reanalyzed the metabolomic profiles by OPLS algorithm, and a linear regression method, to find a correlation between the replicative lifespan and metabolome of the wild-type and the KO strains (Fig. 3). Integral values of the buckets were used as the predictor variables

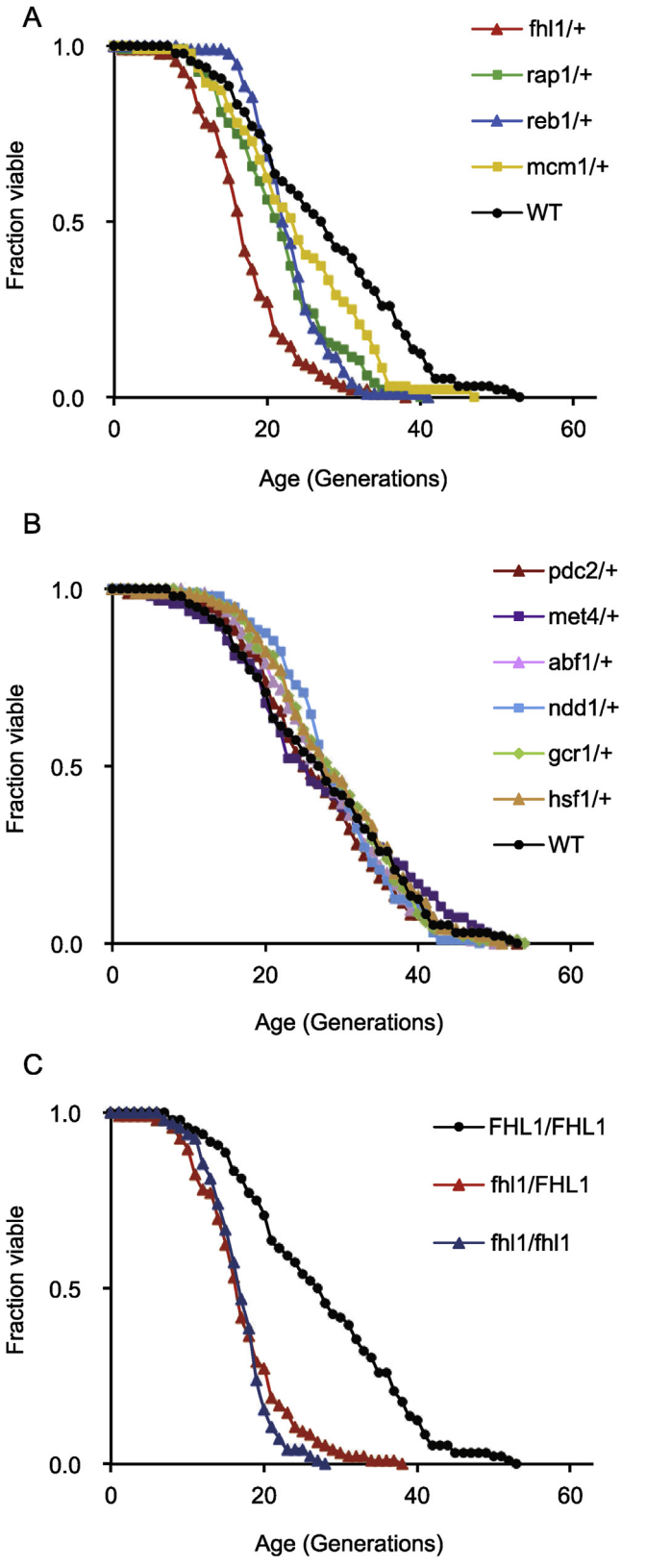

Fig. 1. Replicative lifespan of heterozygous deletion mutants for the essential TF genes and homozygous *FHL1* deletion mutant. Lifespan of heterozygous deletion mutants of *FHL1*, *RAP1*, *REB1*, and *MCM1* genes (A) and the others genes (B), and Δ*fhl1*/Δ*fhl1* homozygote (C). Replicative lifespan was measured for at least 96 cells of each strain. Averaged lifespan is shown in Table 2.

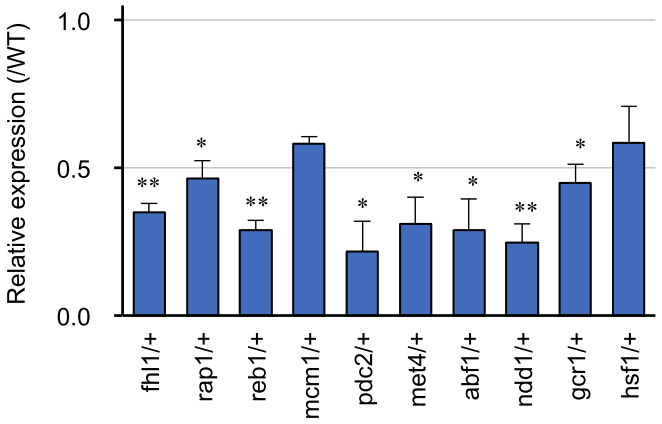

Fig. 2. RT-qPCR confirmation of the decreased transcriptional levels of the heterozygously deleted TF genes. RT-qPCR measurement was performed independently for three times. *, $P < 0.05$; **, $P < 0.01$ *versus* the wild-type strain by Student's *t*-test.

(x-variables), while the replicative lifespan was used as the response variable (y-variable). In the scores plot of OPLS, the variance along horizontal axis appeared to be correlated with the replicative lifespan, with lower scores for the short-lived mutants than normal-lived strains (Fig. 3A). We noted low score of Δ*fhl1*/*FHL1* heterozygote. The scatter plot indicated levels of six bins above the associated chemical shift value negatively correlated with longevity (Fig. 3B). These results suggest that Fhl1p regulates metabolism related to lifespan.

We predicted the compounds represented in each KO strain with their concentration from ^{1}H-NMR spectra using Chenomx NMR Suite software, and explored those correlated to lifespan. The chemical shift values contributed to separation along the lifespan axis in the scatter plot. So far, we predicted an increased concentration of lysine and cadaverine (decarboxylation product of lysine) that was observed in Δ*fhl1*/*FHL1* heterozygote (Fig. 3C). This suggested lysine and cadaverine as candidate compounds, important for short lifespan of the Δ*fhl1*/*FHL1* heterozygote.

4. Discussion

Involvement of genes essential for cell proliferation in the replicative lifespan of budding yeast was investigated. We measured the replicative lifespan of heterozygous diploid strains deleted for the essential TF genes. It was found that the *FHL1*, *RAP1*, *REB1*, and *MCM1* genes were required for the replicative lifespan. Overexpression of any of the TF genes did not show any change in lifespan. ^{1}H-NMR metabolomics analysis showed that cellular metabolism of the TF KO strains was correlated with the replicative lifespan and heterozygous deletion of *FHL1* that clearly shifted into a distinctive metabolism state, probably related to the short lifespan. The yeast lifespan-regulating TFs have mammalian homologues: FOXs for Fhl1p [20], hRap1 for Rap1p [21], Myb for Reb1p [22], and SRF for Mcm1p [23], included in diverse biological processes, but did not describe aging and lifespan.

In this study, *FHL1* was first shown to be a lifespan-regulating gene. Fhl1p activates transcription of ribosomal protein (RP) genes under rich nutrient condition [24] and deletion of some *RP* genes extends the replicative lifespan on YPD rich medium [25]. These are inconsistent with our observation that deletion of *FHL1* shortened lifespan. Therefore, Fhl1p target genes, instead of the *RP* genes, would positively regulate longevity. The *Saccharomyces* Genome Database reported that Fhl1p binds to the promoter region of 365 genes [26,27]. We found that two of them, when deleted, are related to shortening of the lifespan, including nucleosome-

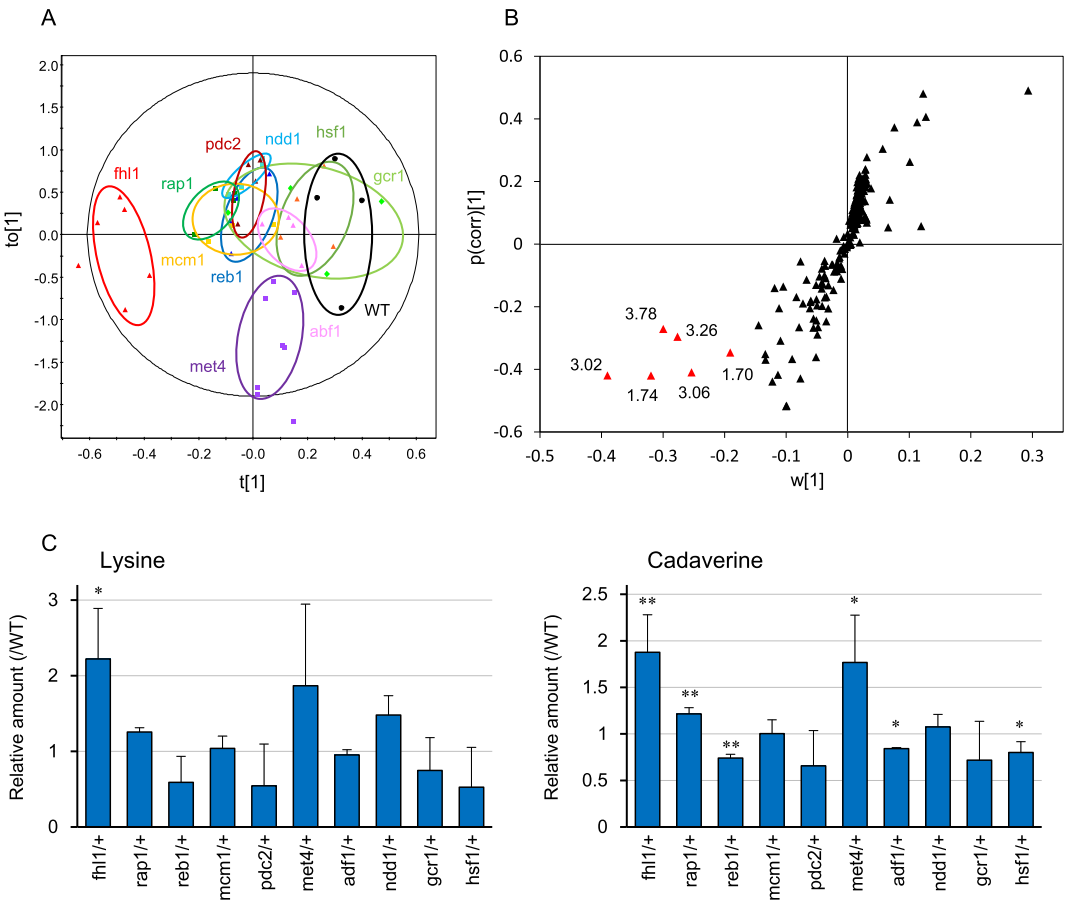

Fig. 3. OPLS analysis of ^{1}H-NMR metabolomics data from heterozygous TF mutants. A, score plot of OPLS. Each point represents an individual batch from wild type (WT) and the mutants deleted for designated gene. B, scatter plot of OPLS (loading (w[1]) vs. correlation (p(corr)[1]). Plots with the chemical shift value (median between bin ranges) correlated with short lifespan are indicated in red. C, lysine and cadaverine contents predicted from the ^{1}H-NMR spectra of the TF mutants. Data are expressed as a ratio relative to wild type. *, $P < 0.05$; **, $P < 0.01$ *versus* the wild-type strain by Student's *t*-test (n = 4–8). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

associated factors, such as *ASF1* [28] and *VPS71* [29]. Down-regulation of these genes in Δ*fhl1*/*FHL1* heterozygote might lead to a shorter lifespan. Heterozygous *FHL1* deletion altered the cellular metabolism and seemed to increase the content of lysine and cadaverine. Previously, we reported that lysine was accumulated in yeast senescent cells [14]. Therefore, increased content of lysine might be related to the short lifespan of Δ*fhl1*/*FHL1* mutant even though we did not find metabolic genes associated with the lifespan and catalysis of lysine and cadaverine amongst the Fhl1p-targeted genes.

Rap1p, Reb1p, and Mcm1p were also implicated in the regulation of longevity. They, unlike Fhl1p, did not regulate metabolism although Rap1p was found to activate transcription of the glycolytic and ribosomal protein genes [30,31]. Mcm1p was also observed to be involved in the control of arginine metabolism [32]. Rap1p and Reb1p were bound to the telomeric and subtelomeric regions and have a role in subtelomeric gene silencing, telomere length regulation, and chromosome end protection [33–35]. In the aged cells, transcriptional silencing at the subtelomeric regions was compromised [36] and loss of Set2p histone methyltransferase reduced the unstable telomeres to extend replicative lifespan [37]. Deletion of *RAP1* and *REB1* may shorten the lifespan by disrupting the telomere structure. *MCM1* is involved in cell-cycle-dependent transcription, recombination, Ty transcription, and osmotolerance [38–41], and these cellular processes could be related to lifespan.

In the present study, we found four novel genes essential for cell proliferation and replicative lifespan by using heterozygous deletion mutants. Only 18 essential genes were previously shown to regulate the replicative lifespan even though 1,192 of the essential genes were represented. Overexpression and conditional mutation of the essential genes were used for investigating the regulation of replicative lifespan [42–44], although two copies of the essential TF genes did not affect lifespan. Only one report indicated the effect of heterozygous deletion of the essential *NAR1* gene on longevity [45]. Thus, the use of yeast heterozygous diploid strains, as shown in this study, might enable discovering the unidentified lifespan genes. This is advantageous because heterozygous deletion of the essential genes effectively decreased transcript levels of the designated genes and heterozygous diploid strains were easily obtained from the company.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.067.

Transparency document

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