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# Cyclin-dependent kinase Pho85p and its cyclins are involved in replicative lifespan through multiple pathways in yeast

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**Lifespan is determined by genetic factors and influenced by environmental factors. Here, we find that the phosphate signal transduction (PHO) pathway is involved in the determination of replicative lifespan in budding yeast. Extracellular phosphate does not affect the lifespan. However, deletion of *PHO80* (cyclin) and *PHO85* (cyclin-dependent kinase) genes, that is, negative regulators of the PHO pathway, shortens the lifespan, which is restored by further deletion of *PHO4* (transcriptional activator). Four of the other nine Pho85p cyclin genes are also required to maintain normal lifespan. The short-lived mutants show a metabolic profile that is similar to strains with normal lifespan. Thus, Pho85p kinase genetically determines replicative lifespan in combination with relevant cyclins. Our findings uncover novel cellular signals in longevity regulation.**

**Keywords:** cyclin; cyclin-dependent kinase; metabolome; phosphate signal transduction; replicative lifespan; yeast

The lifespan of unicellular and multicellular organisms is determined by genetic factors and significantly influenced by environmental factors, and both these factors are closely interrelated [1,2]. Calorie restriction (CR) is a major environmental lifespan determinant that can extend the lifespan of various model organisms, from yeast to monkey [3,4]. Recent studies showed that CR can improve healthspan and extend lifespan in humans [5]. The effect of lifespan extension in CR is caused by the activation of sirtuin ($NAD^+$-dependent protein deacetylases) as a genetic lifespan determinant [6]. In addition, amino acid restriction also prolongs the lifespan of yeast, fly and mouse and is associated with decreased target of rapamycin signaling pathway [7].

Research studies on lifespan-related genetic factors and pathways have considered budding yeast *Saccharomyces cerevisiae* and other model organisms for their research [8,9]. The yeast cells undergo a limited number of cell division as in the case of human primary cultured cells. These yeast cells also have a competitive advantage in the measurement of replicative lifespan, which is defined as the number of daughter cells that a mother cell can generate before dying [10]. Yeast replicative lifespan is suitable for understanding longevity *via* the aging process at the single-cell level. Using yeast as a model, the *SIR2* gene was first identified as a sirtuin gene [11], and resveratrol was discovered as a sirtuin activator, which functions as an anti-aging agent [12].

Phosphate is one of the most important macronutrients for all organisms due to its involvement in diverse physiological processes such as DNA and lipid membrane synthesis, energy transfer, protein activity, signal transduction and metabolic pathways. Phosphate homeostasis must be maintained at individual and cellular levels since its misregulation, that is,

**Abbreviations**
$^1$H-NMR, proton nuclear magnetic resonance; CDK, cyclin-dependent kinase; CR, calorie restriction; HSP70, heat-shock protein 70; PCA, principal component analysis; Pi, inorganic phosphate.

hypophosphatemia (too little phosphate) or hyperphosphatemia (too much phosphate), can cause many serious diseases and reduce lifespan [13]. In addition, mouse models with hyperphosphatemia died prematurely due to loss-of-function mutations in *Klotho* or *FGF23*. This early lethality can be rescued, and the lifespan of the mouse models can be extended by feeding them a phosphate-restricted diet [14,15]. However, how phosphate is associated with lifespan at the individual and cellular levels needs further elucidation.

The phosphate signal transduction (PHO) pathway is well investigated in *S. cerevisiae* [16,17]. Under the condition of phosphate starvation, the Pho4p transcription factor activates the expression of genes encoding secreted acid phosphatase, high-affinity phosphate transporter, and vacuolar polyphosphate polymerase [18]. Thus, this enhances the uptake of inorganic phosphate (Pi, also known as orthophosphate), and a part of the incorporated Pi is converted to polyphosphate and stored in an acidocalcisome-like vacuole. In contrast, under high phosphate condition, the Pho80p cyclin-Pho85p cyclin-dependent kinase (CDK) complex phosphorylates Pho4p [19], and the phosphorylated Pho4p was exported from the nucleus by the Msn5p/Ste21p exportin [20], followed by repression of phosphate starvation response genes. In comparison, under low phosphate conditions, the Pho80p-Pho85p CDK complex is inactivated by the Pho81p CDK inhibitor [21]. In addition to Pho80p, the other nine Pho85p cyclins (Pcls) form cyclin–CDK complexes with Pho85p and are involved in a variety of signaling pathways [22–24]. Pcl5p is required for degradation of Gcn4p (general control nonderepressible 4) transcriptional activator for amino acid starvation response; paralogous pairs of Pcl6p/Pcl7p and Pcl8p/Pcl10p regulate glycogen metabolism and storage; and Pcl1p, Clg1p (cyclin-like gene 1), and a paralogous pair of Pcl2p/Pcl9p are required for progression through the cell cycle.

In this study, we examined whether extracellular phosphate (an environmental factor) and the PHO pathway (a genetic factor) play a role in the regulation of replicative lifespan of budding yeast. We showed that extracellular phosphate did not affect longevity, but Pho80p cyclin and Pho85p CDK in the PHO pathway were involved in the determination of lifespan. Moreover, we identified novel lifespan-related *PCL* genes.

## Materials and methods

### Strains and media

All *S. cerevisiae* strains used in this study were derived from BY4742 strains [25] and are listed in Table S1. Deletion strains were obtained from the Yeast MATα Collection (YSC1054) (Open Biosystems, Huntsville, AL, USA). Deletion strains not included in the collection were constructed by PCR-based gene disruption method using selectable markers [26].

Synthetic high (11 mM) and low (0.22 mM) Pi media were prepared as described by Toh-e [27] and were supplemented with uracil, leucine, histidine, and lysine when required. The YPD medium (1% bacto yeast extract, 2% bacto peptone, 2% dextrose) was used as nutrient-rich medium. The Pi-depleted YPD medium was prepared by precipitating Pi as magnesium ammonium phosphate and adjusting the final pH to 5.8 with HCl [28].

### Replicative lifespan determination

Replicative lifespan was assayed with minor modifications as described previously [29]. Yeast cells were thawed from frozen stock and streaked onto an YPD agar plate. After 2 days, a single colony was spread onto an assay plate, and cells were grown at 30 °C overnight. The next day, cells were transferred again to a fresh assay plate and grown overnight. Using a micromanipulator, 48 cells were arrayed on the plate and allowed to undergo 1 or 2 divisions. Virgin cells were then selected and subjected to lifespan analysis. Except during manipulation, plates were sealed with parafilm and 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 or 96 (two sets of assays) cell lines, buds from each mother cell were counted until division of living cells ceased for at least 3 days. The measured mean and maximum replicative lifespan and the *P* value calculated using Wilcoxon rank-sum test relative to the wild-type strain BY4742 are shown in Table 1.

### Proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy metabolomics analysis

The yeast cells grown to mid-log phase were suspended in 750 μL of 0.1 M potassium phosphate buffer (pH 7.0) prepared with $D_2O$ containing 1 mM TSP (trimethylsilyl propanoic acid). The cell suspension was transferred to a 2-mL screw cap tube containing 500 μL zirconia beads, and the contents were sufficiently disrupted at 3000 r.p.m. and 4 °C using a bead smasher (Micro Smash MS-100R, TOMY). The cell extract was centrifuged at 18 000 ***g*** for 5 min, and the supernatant was collected. Acquisition of $^1$H-NMR spectra and data reduction were performed as previously described [30]. The data sets from the $^1$H-NMR analysis were judged by principal component analysis (PCA) using SIMCA-P+ 12.0.1 (Umetrics, Umeå, Sweden).

**Table 1.** Replicative lifespan measured in this study.

| Relevant genotype | Averaged lifespan ± SD | Maximum lifespan | Measured cell number | *P* value[a] |
|---|---|---|---|---|
| Synthetic high Pi medium | | | | |
| Wild-type | 25.7 ± 13.7 | 64 | 96 | – |
| *pho4* | 29.1 ± 14.5 | 65 | 96 | 1.0E-01 |
| *pho80* | 11.5 ± 6.1 | 31 | 96 | 6.8E-16 |
| *pho85* | 4.4 ± 2.0 | 9 | 96 | 6.5E-32 |
| *pho80 pho4* | 19.7 ± 9.6 | 44 | 96 | 7.2E-03 |
| *pho85 pho4* | 7.5 ± 3.2 | 16 | 96 | 1.5E-27 |
| *pcl1* | 25.3 ± 9.2 | 45 | 48 | 5.4E-01 |
| *pcl2* | 22.4 ± 9.4 | 47 | 48 | 3.9E-01 |
| *pcl5* | 15.7 ± 7.4 | 36 | 96 | 6.8E-07 |
| *pcl6* | 26.2 ± 9.0 | 48 | 48 | 2.9E-01 |
| *pcl7* | 23.1 ± 9.9 | 44 | 48 | 5.8E-01 |
| *pcl8* | 27.0 ± 10.4 | 45 | 48 | 1.8E-01 |
| *pcl9* | 24.6 ± 9.5 | 43 | 48 | 8.2E-01 |
| *pcl10* | 24.7 ± 9.8 | 44 | 48 | 8.5E-01 |
| *clg1* | 16.9 ± 7.7 | 37 | 96 | 1.2E-05 |
| *pcl2 pcl9* | 22.4 ± 10.2 | 42 | 48 | 4.2E-01 |
| *pcl6 pcl7* | 20.3 ± 8.7 | 52 | 48 | 7.9E-02 |
| *pcl8 pcl10* | 15.9 ± 6.3 | 32 | 48 | 4.4E-05 |
| *pho80 clg1* | 8.8 ± 4.7 | 23 | 48 | 1.7E-15 |
| *pho80 pcl5* | 8.0 ± 3.5 | 17 | 48 | 1.5E-17 |
| *clg1 pcl5* | 16.2 ± 6.4 | 28 | 48 | 2.7E-04 |
| *pcl5 gcn4* | 15.6 ± 7.9 | 42 | 48 | 2.7E-05 |
| *gcn4* | 24.1 ± 10.7 | 44 | 48 | 7.6E-01 |
| *pcl8 pcl10 gsy2* | 15.6 ± 7.4 | 35 | 48 | 1.5E-05 |
| *gsy2* | 23.5 ± 9.2 | 49 | 48 | 7.7E-01 |
| *ssa1* | 29.0 ± 11.5 | 49 | 48 | 6.1E-02 |
| *ssa2* | 24.6 ± 12.3 | 51 | 48 | 9.5E-01 |
| *ssa3* | 21.0 ± 10.6 | 49 | 48 | 7.7E-02 |
| *ssa4* | 22.8 ± 10.9 | 46 | 48 | 3.7E-01 |
| *ssa1 ssa2* | 24.1 ± 9.2 | 48 | 96 | 9.8E-01 |
| *ssa3 ssa4* | 19.2 ± 7.4 | 38 | 96 | 3.7E-03 |
| Synthetic low Pi medium | | | | |
| Wild-type | 26.6 ± 12.9 | 59 | 96 | – |
| *pho4* | 22.4 ± 12.9 | 69 | 96 | 5.5E-03 |
| *pho80* | 13.3 ± 6.0 | 31 | 96 | 2.9E-14 |
| *pho85* | 5.0 ± 2.1 | 10 | 96 | 9.3E-29 |
| *pho80 pho4* | 18.2 ± 7.5 | 44 | 96 | 1.2E-06 |
| *pho85 pho4* | 7.8 ± 3.2 | 13 | 96 | 4.3E-25 |
| YPD medium | | | | |
| Wild-type | 27.3 ± 9.5 | 44 | 48 | – |
| Pi-depleted YPD medium | | | | |
| Wild-type | 28.6 ± 11.1 | 46 | 48 | – |

[a] The *P* value calculated using a Wilcoxon rank-sum test relative to the wild-type strain BY4742.

## Results

### Extracellular phosphate did not affect yeast replicative lifespan

To determine whether environmental phosphate affects replicative lifespan in budding yeast, we measured the lifespan of wild-type strain BY4742 on synthetic media containing high (11 mM) and low (0.22 mM) inorganic phosphate (Pi). Mean lifespan of the wild-type strain was comparable on both high and low Pi media at 25.7 and 26.6 generations, respectively (Fig. 1A, Table 1). These lifespans were similar to wild-type mean lifespan measured on complete YPD medium, a relatively high

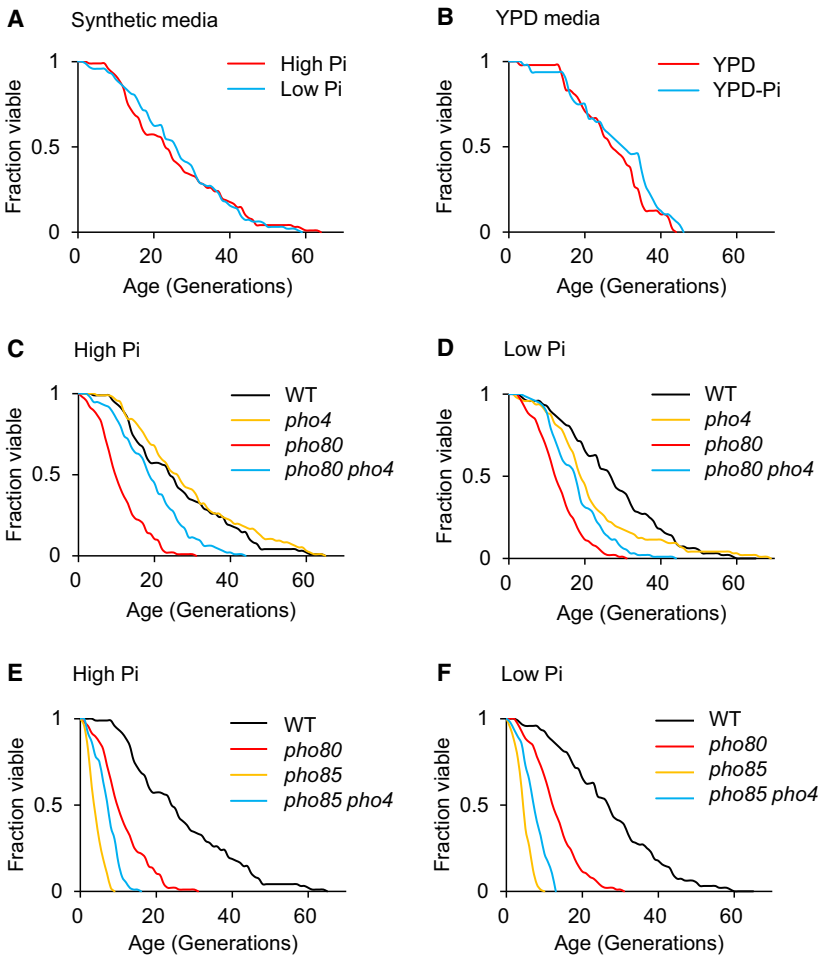


**Fig. 1.** Replicative lifespan of wild-type and mutants for the *PHO* pathway genes in high and low phosphate media. (A) Replicative lifespan of wild-type strain on synthetic media containing high (11 mM) and low (0.22 mM) inorganic phosphate (Pi). (B) Lifespan of wild-type strain on complete and Pi-depleted YPD media. (C, D) Lifespan of *pho80* and *pho4* mutants on high and low Pi synthetic media. (E, F) Lifespan of *pho85* mutants on high and low Pi synthetic media. The same data for wild-type and *pho80* mutant strains on high and low Pi media are presented in the relevant panels.

Pi concentration (about 2.5 mM), and even on Pi-depleted YPD medium (27.3 and 28.6 generations) (Fig. 1B). These results indicate that extracellular Pi did not affect replicative lifespan of wild-type yeast. Despite the comparable mean lifespan, the lifespan curve of synthetic media showed longer maximum lifespan (64 versus 44 generations in high Pi and 59 versus 46 generations in low Pi) and high cell population that died at the early stage of senescence compared to the YPD media. Since no difference of mean lifespan was observed between synthetic and YPD media, the synthetic media were used for further experiments.

## PHO pathway is involved in determining replicative lifespan

Next, we focused on determining whether the PHO pathway is involved in determining yeast replicative lifespan. We measured the lifespan of the *PHO*-deleted mutants, including *PHO4* (transcriptional activator) and *PHO80* (negative regulator), on high and low Pi media (Fig. 1C,D). Deletion of *PHO4* did not change the lifespan under high Pi condition and slightly but significantly decreased the lifespan under low Pi condition. In contrast, deletion of *PHO80* drastically shortened the lifespan both on high and on low Pi media. Deletion of *PHO4* in the *pho80* disruptant restored the lifespan to about 80% of the wild-type level under high Pi condition and about 70% of the wild-type level under low Pi condition. These results indicate that the PHO pathway regulates replicative lifespan independently of the extracellular Pi concentration and suggested that the short lifespan of the *pho80* mutant was partly due to undesirable expression of the *PHO* genes that are upregulated by unphosphorylated Pho4p. We confirmed remarkable overexpression of the *PHO84* gene, which encodes Pi transporter and is regulated by Pho4p, in the *pho80* mutant but not in the *pho80 pho4* double mutant under high and low Pi conditions (Fig. S1).

Since the *pho80* disruption mutant exhibited a short lifespan, we expected that a mutant for *PHO85*,

encoding a counterpart of Pho80p-Pho85p CDK complex, would also exhibit a short lifespan. Deletion of *PHO85* severely shortened the lifespan on high and low Pi media (Fig. 1E,F). Interestingly, the *pho85* mutant had a much shorter lifespan than the *pho80* mutant, although the two proteins formed a complex for phosphorylating Pho4p. Unlike the *pho80* mutant, the *pho85* mutant was still extremely short-lived when *PHO4* was deleted. These results indicate that Pho85p CDK has a role of replicative lifespan regulation in a Pho80p cyclin-independent manner, presumably through the other Pcl proteins. Thus, we conclude that some regulatory factors of the PHO pathway are involved in yeast replicative lifespan and that the Pho4p-regulated target genes are implicated in lifespan determination.

## Pho85p cyclin genes, *PCL5*, *PCL8-PCL10*, and *CLG1* are required for maintaining normal lifespan

The Pho85p CDK forms complexes with the ten respective Pcl proteins to mediate not only the phosphate starvation response but also various cellular events such as amino acid starvation response, glycogen metabolism, and cell cycle. As described above, in addition to *PHO80*, the other *PCL* gene(s) could be involved in replicative lifespan determination. Therefore, we measured the lifespan of single deletion mutants for the remaining nine *PCL* genes on high Pi synthetic medium. Mutants for *CLG1* and *PCL5* were moderately short-lived (Fig. 2A). On the other hand, each mutant of *PCL1*, *PCL2*, and *PCL9* (implicated in cell cycle) and *PCL6*, *PCL7*, *PCL8*, and *PCL10* (implicated in glycogen metabolism) had a lifespan similar to the wild-type (Fig. S2A,B). These results indicate that *CLG1* and *PCL5* are involved in the regulation of replicative lifespan.

To examine whether the *PHO80*, *CLG1*, and *PCL5* genes regulate replicative lifespan independently or not, we constructed their double disruptants and measured their lifespan. The *pho80 clg1* and *pho80 pcl5* double mutants had significantly much shorter lifespan than each single mutant (Fig. 2B,C). In comparison, the lifespan of the *clg1 pcl5* double mutant was similar to that of the respective single mutants (Fig. S3). These results indicate that *CLG1* and *PCL5* function independently of *PHO80* in lifespan regulation and may share the same lifespan-related pathway.

Since there are three pairs of *PCL* paralog genes, that is, *PCL2-PCL9*, *PCL6-PCL7*, and *PCL8-PCL10*, we assumed that these *PCL* paralogs might have a redundant function for lifespan regulation. Accordingly, we constructed double mutants for the respective *PCL* paralog pairs to measure their lifespan. The *pcl8 pcl10* double disruptant had a significantly short lifespan, and the *pcl6 pcl7* double mutant exhibited a slightly but not significantly shortened lifespan. In comparison, the *pcl2 pcl9* double disruptant exhibited an almost normal lifespan (Fig. 2D). These indicate that *PCL8* and *PCL10* are also involved in lifespan determination and have a redundant longevity function.

## Short-lived *pcl* mutants had a similar metabolic profile

To understand the relationship of the respective *PCL* gene function with longevity, we explored the whole metabolic profiles of the *pcl* mutants and wild-type strain using a $^{1}$H-NMR-based metabolomics analysis. A score plot of PCA of the $^{1}$H-NMR data set showed clusters of each strain analyzed and a clear separation of the clusters between short-lived strains and those with normal lifespan (Fig. 3A), indicating lifespan-related metabolic differences. To discriminate between the Pcl signal transduction pathways related to lifespan determination, the PCA using only the metabolome data set of the short-lived mutants was performed. Although we at least expected a separation of the *pho80* cluster from the other *pcl* mutants, no separation of clusters between the *pho80*, *clg1*, *pcl5* single mutants, and the *pcl8 pcl10* double mutant was observed (Fig. 3B). These data suggest a similar metabolism of these short-lived *pcl* mutants in the different signal transduction pathways.

## *SSA3*/*SSA4* chaperone genes in the Clg1p-Pho85p pathway are involved in lifespan regulation

We asked whether the signal transduction pathways including the *CLG1*, *PCL5*, and *PCL8*/*PCL10* genes regulate replicative lifespan. Since the absence of the Pcl proteins shortened the lifespan due to unphosphorylation of their substrates, the target substrates of the Pclp-Pho85p complex were examined. We, therefore, assumed that deletion of a substrate gene would shorten the lifespan if the phosphorylated form of the substrate is active for signal transduction. We also assumed that deletion of a substrate gene would suppress the short lifespan of the relevant *pcl* mutant if the phosphorylated form of the substrate is inactive for signal transduction.

In the former case, Clg1p-Pho85p complex phosphorylates and activates Ssa (Stress-Seventy subfamily A) chaperones, which consist of four Ssa proteins encoded

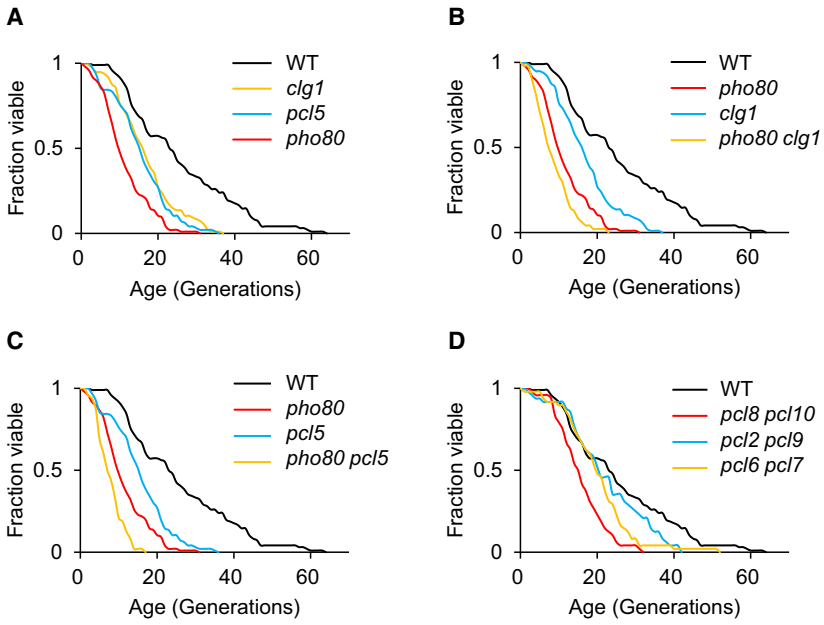


**Fig. 2.** Identification of the *PCL* genes related with longevity. (A) Replicative lifespan of deletion mutants for *CLG1*, *PCL5*, or *PHO80* genes. (B) Lifespan of mutants in combination with deletion mutations of *CLG1* and *PHO80* genes. (C) Lifespan of mutants in combination with deletion mutations of *PCL5* and *PHO80* genes. (D) Lifespan of double mutants deleted for *PCL* paralog pairs. All lifespan experiments were performed on high Pi synthetic plate. The same data for wild-type and *pho80* mutant strains are presented in the relevant panels.

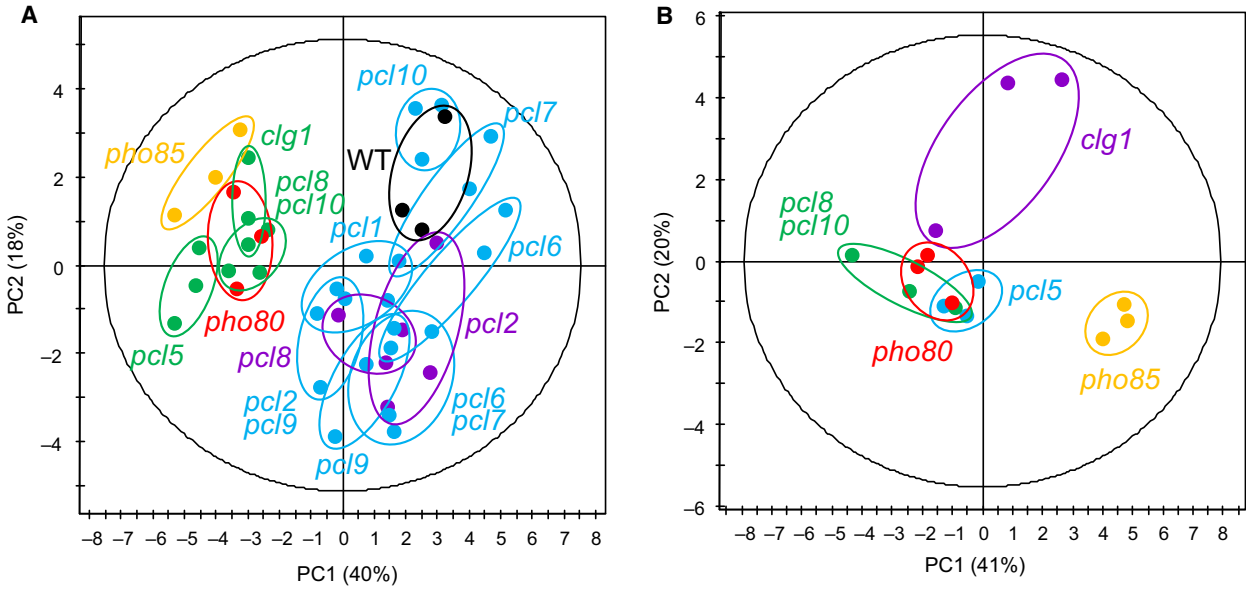


**Fig. 3.** Metabolic profiling of *PCL*-deleted mutants. (A) A score plot of PCA of the $^{1}$H-NMR metabolome data of all *PCL*-deleted mutants and wild-type. (B) A score plot of PCA of the metabolome data of the only short-lived *pcl* mutants. Cells used for the metabolome analysis were cultured in high Pi synthetic media.

by *SSA1*, *SSA2*, *SSA3*, and *SSA4*, and *SSA1*/*SSA2* and *SSA3*/*SSA4* are paralogs [31]. The single disruptants for *SSA1* and *SSA2* had a normal lifespan, but that for *SSA3* and *SSA4* had a slightly short lifespan (Fig. 4A,B). The *ssa1 ssa2* double mutant had a normal lifespan, whereas the *ssa3 ssa4* double mutant had a lifespan as short as the *clg1* mutant. These results indicate that the Ssa3p/Ssa4p molecular chaperone pathway that is regulated by Clg1p is involved in lifespan regulation.

In the latter case, Pcl5p-Pho85p complex phosphorylates and facilitates degradation of Gcn4p transcriptional activator for amino acid starvation response [32] and Pcl8p/Pcl10p-Pho85p complex induces degradation of Gsy2p (glycogen synthase 2) [33]. When the candidate substrate genes, *GCN4* and *GSY2*, were

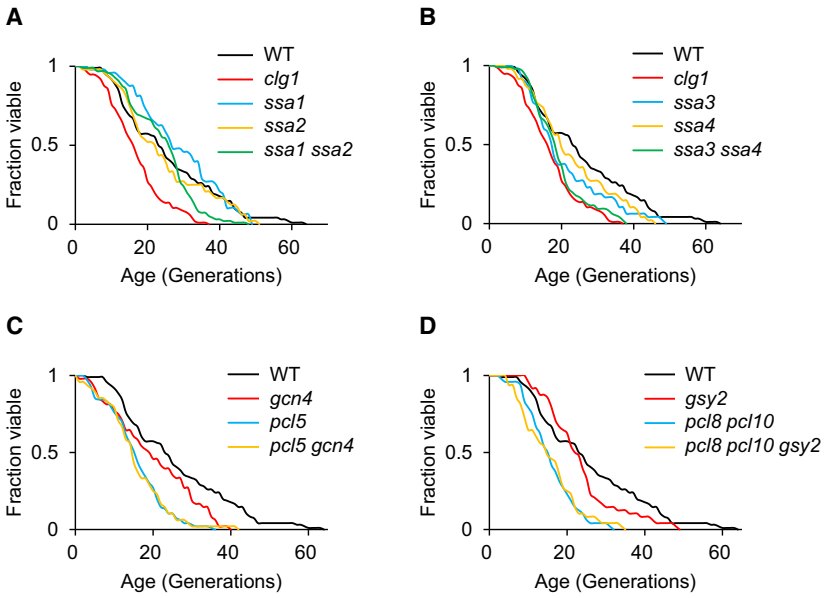


**Fig. 4.** Identification of the Pcl target substrate genes related with longevity. (A) Replicative lifespan of mutants in combination with deletion mutations of *SSA1* and *SSA2* genes. (B) Lifespan of mutants in combination with deletion mutations of *SSA3* and *SSA4* genes. (C) Lifespan of mutants in combination with deletion mutations of *PCL5* and *GCN4* genes. (D) Lifespan of mutants in combination with deletion mutations of *PLC8, PCL10*, and *GSY2* genes. All lifespan experiments were performed on high Pi synthetic plate. The same data for wild-type and *clg1* mutant strains are presented in the relevant panels.

disrupted in the *pcl5* single and *pcl8 pcl10* double mutants, respectively, the lifespans remained still short (Fig. 4C,D). In contrast, the single disruptants for *GCN4* and *GSY2* in the wild-type strain exhibited a normal lifespan. These indicated that the amino acid starvation response and glycogen synthesis pathways were not involved in lifespan regulation and that *PCL5* and *PCL8*/*PCL10* regulate replicative lifespan through the other signal transduction pathways.

## Discussion

In this study, we have examined whether the extracellular phosphate (an environmental factor) and the PHO pathway (a genetic factor) play a role in the regulation of replicative lifespan in yeast. We uncovered that the environmental organic and inorganic phosphates did not affect the lifespan, whereas the PHO pathway genes including *PHO80* (cyclin) and *PHO85* (CDK) regulate the lifespan. In addition, the other Pho85p cyclin (*PCL*) genes also play a role in lifespan regulation through multiple pathways. These findings in yeast highlight new physiological roles of Pcl proteins in the determination of replicative lifespan and also serve as a precursor to understanding lifespan regulation by CDK cyclins in eukaryotes.

Unlike CR, the limitation of extracellular phosphate did not affect replicative lifespan of wild-type yeast strain in the examined phosphate concentration (0.22 mM Pi). However, this observation in yeast is different from those observed in mammals. In humans, hypophosphatemia, which is defined as a serum phosphate level of less than 0.81 mM, can cause serious disorders and may also contribute to death [13]. Replicative aging in yeast seems to be adaptive to the environment with low concentration of phosphate, presumably because intracellular phosphate homeostasis is highly maintained by the PHO pathway. This is because the deletion of *PHO4*, a positive regulator of phosphate starvation response, shortened replicative lifespan under low phosphate condition but not under high phosphate condition.

Since the deletion of *PHO80* drastically reduced replicative lifespan and the further deletion of *PHO4* restored the lifespan, the undesirable expression of phosphate starvation response genes that are transcriptionally activated by Pho4p could reduce the lifespan of *pho80* mutant. The Pho4p target genes encode secreted acid phosphatases, high-affinity phosphate transporters, and vacuolar polyphosphate polymerases [18] and might be candidate genes for the short lifespan of *pho80* mutant. It was reported that *Klotho*-knockout mice exhibited premature aging and short lifespan and that an additional knockout of sodium phosphate cotransporter *NaPi2a* restored prolonged survival [34]. This indicates that the phosphate transporter gene such as *PHO84* is a plausible candidate gene for yeast aging. Interestingly, the lifespan of *pho4 pho80* double disruption mutant was not recovered completely to the wild-type level. This implies that Pho80p-Pho85p CDK has a substrate other than Pho4p that may influence the lifespan independently of the PHO pathway. Such candidate target substrates of Pho80p-Pho85p CDK may be a transcriptional repressor Rim101p, which is required for alkaline growth and ionic stress tolerance [35,36] and a PAS family

protein kinase Rim15p, which is required for oxidative damage prevention [37,38]. A future study will identify a novel longevity gene among the phosphate starvation response genes and a novel Pho80p-Pho85p CDK substrate associated with longevity.

We have identified the *SSA3* and *SSA4* genes as lifespan-related genes that work in the Clg1p-Pho85p pathway. Ssa3p and Ssa4p are molecular chaperone proteins comprising members of the heat-shock protein 70 (HSP70) family [39]. The HSP70 is highly conserved in a wide variety of organisms and involved in prolonging the lifespan of fly and mice [40,41]. In humans, molecular chaperones are induced by environmental stress and play a protective role against stresses and age-related diseases [42]; the HSP70 protein induction was reduced in cells of most aged humans but not of centenarians [43]. Thus, yeast Ssa3p and Ssa4p molecular chaperones may behave as anti-aging factors through phosphorylation by Clg1p-Pho85p. Unlike the *SSA3* and *SSA4* genes, the *SSA1* and *SSA2* genes, the other *SSA* subfamily members of the *HSP70* family, were not related to lifespan regulation. The four *SSA* isoforms are functionally redundant to some degree as expression of at least one family member is essential for cell growth [39]. Meanwhile, Ssa3p and Ssa4p, but not Ssa1p and Ssa2p, are necessary for response to oxidative stress and cell wall damage [44], thus probably determining the specificity of lifespan regulation. Alternatively, Clg1p-Pho85p might discriminate Ssa3p and Ssa4p from Ssa1p and Ssa2p and activate the only Ssa3p and Ssa4p to maintain the lifespan. In this study, we could not identify the lifespan-related factors regulated by the Pcl5p, Pcl8p, or Pcl10p cyclins in combination with Pho85p CDK. In our future work, we hope to identify a target substrate of these Pcl proteins that serves as a longevity factor and, thereby, uncover a signal transduction pathway and an environmental signal in longevity regulation.

## Author contributions

YM conceived and supervised the study; YM designed experiments; TN, TMa, and TMo performed experiments; TN analyzed data; TN and YM wrote the manuscript.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
**Fig. S1.** Undesirable expression of the *PHO84* gene in the *pho80* mutant.
**Fig. S2.** Replicative lifespan of the normal-lived *PCL*-deleted mutants.
**Fig. S3.** Replicative lifespan of mutants in combination with deletion mutations of the *CLG1* and *PCL5* genes.
**Table S1.** Strains used in this study.