ORIGINAL ARTICLE

Magnesium depletion extends fission yeast lifespan via general amino acid control activation

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Abstract
Nutrients including glucose, nitrogen, sulfur, zinc, and iron are involved in the regulation of chronological lifespan (CLS) of yeast, which serves as a model of the lifespan of differentiated cells of higher organisms. Herein, we show that magnesium (Mg2+) depletion extends CLS of the fission yeast Schizosaccharomyces pombe through a mechanism involving the Ecl1 gene family. We discovered that ecl1+ expression, which extends CLS, responds to Mg2+ depletion. Therefore, we investigated the underlying intracellular responses. In amino acid auxotrophic strains, Mg2+ depletion robustly induces ecl1+ expression through the activation of the general amino acid control (GAAC) pathway—the equivalent of the amino acid response of mammals. Polysome analysis indicated that the expression of Ecl1 family genes was required for regulating ribosome amount when cells were starved, suggesting that Ecl1 family gene products control the abundance of ribosomes, which contributes to longevity through the activation of the evolutionarily conserved GAAC pathway. The present study extends our understanding of the cellular response to Mg2+ depletion and its influence on the mechanism controlling longevity.

KEYWORDS
chronological lifespan, Ecl1 family gene, fission yeast, GAAC, magnesium

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1 | INTRODUCTION

Magnesium ions (Mg\(^{2+}\)) are the most abundant divalent cations in cells, and they play diverse physiological roles including regulating enzyme activity and stabilizing ribosomes, membranes, and genomes (Akanuma et al., 2018; Dann et al., 2007; El-Sharoud, 2004; Nureki et al., 2008). For example, Mg\(^{2+}\) mediates transition-state formation during ATP synthesis and is therefore essential for life (Gout et al., 2014; Ko et al., 1999; Uehara et al., 2010). Furthermore, the level of Mg\(^{2+}\) can affect the level of aminoacyl-tRNA (aa-tRNA) because Mg\(^{2+}\) makes complexes not only with ATP, PP\(_2\), and AMP but also with tRNA and affects the catalytic reactions of aa-tRNA synthetase (Airas, 2007). Thus, systems that control Mg\(^{2+}\) homeostasis evolved to cope with fluctuations in environmental Mg\(^{2+}\). Moreover, the effects of extracellular Mg\(^{2+}\) can be exploited for synthetic biology and brewing (Chubukov & Sauer, 2014; Tokuyama et al., 2019; Yoshizawa et al., et al.). In brewing yeast, extracellular Mg\(^{2+}\) concentration affects the incorporation of extracellular amino acids, which give sake its umami, richness, and breadth (Yoshizawa et al., 1985). In *Escherichia coli*, among various nutrient deprivation, Mg\(^{2+}\) starvation exhibits a high metabolic rate, which is related to the production of target metabolites in synthetic biology (Chubukov & Sauer, 2014). For example, Mg\(^{2+}\) starvation results in high production titers and high specific production rates of malonyl-CoA derivatives (Tokuyama et al., 2019).

In prokaryotes, it has been reported that the regulation of intracellular Mg\(^{2+}\) homeostasis occurs through mechanisms involving the Mg\(^{2+}\)-binding RNA called M-box, the Mg\(^{2+}\)-sensing mechanism of Mg\(^{2+}\) transporter MgtE, intrinsic ribosome destabilization, and the retention of Mg\(^{2+}\) by binding to ribosomes (Akanuma et al., 2018; Chadani et al., 2017; Dann et al., 2007; Ishitani et al., 2008; Nureki et al., 2008). Some studies have revealed the effects of the Mg\(^{2+}\) transporter Alr1 in budding yeast (Lim et al., 2011). The effects of Mg\(^{2+}\) on the growth and the cell cycle and mitotic spindle of fission yeast have also been reported (Sarikaya et al., 2006; Uz & Sarikaya, 2016; Walker & Dufus, 1980). However, knowledge of the molecular mechanisms that regulate the response to environmental Mg\(^{2+}\) in *Schizosaccharomyces pombe* is limited.

Numerous studies have focused on the mechanisms and signaling pathways involved in the responses to glucose and nitrogen that affect growth, the cell cycle, sexual differentiation, autophagy, and chronological lifespan (CLS), defined as the length of time a cell can survive (Mukaiyama et al., 2010; Otsubo et al., 2017; Roux et al., 2010; Su et al., 1996; Yanagida et al., 2011; Zuin et al., 2010). Extender of chronological lifespan 1 (Ecl1) family genes play important roles in cellular responses to sulfur, zinc, iron, and amino acids (Ohtsuka & Aiba, 2017). Ecl1 family genes were first identified as extenders of CLS, and three homologous genes encoding proteins with <100 amino acid residues (ecl1\(^{\alpha}\), ecl2\(^{\alpha}\), and ecl3\(^{\alpha}\)) are encoded by the genome of *S. pombe* (Ohtsuka & Aiba, 2017; Ohtsuka et al., 2008). Sulfur depletion induces ecl1\(^{\alpha}\) expression via the transcription factor Zip1 (Ohtsuka et al., 2017). Activated ecl1\(^{\alpha}\) expression induces intracellular responses, such as CLS extension, sporulation, cell size reduction, and decreased expression of genes encoding ribosomal proteins (Ohtsuka & Aiba, 2017; Ohtsuka et al., 2017).

Although evidence indicates an association between CLS and ribosomes, there is insufficient knowledge about the involvement of Ecl1 family genes. In addition, although the transcriptional induction of Ecl1 family genes has not been observed under the starvations of zinc or iron, cellular responses to this starvation require these genes, suggesting that the products may respond to these signals post-transcriptionally (Ohtsuka et al., 2015). From these, it is expected that, although Ecl1 family genes are induced transcriptionally by various signals, the mRNA inductions may be one of the ways to activate these functions.

The induction of ecl1\(^{\alpha}\) by sulfur depletion can be observed in both prototrophic and auxotrophic strains; however, this induction can also be observed in amino acid auxotrophic strains due to amino acid depletion (Ohtsuka et al., 2019). The latter is mediated by the GATA-type transcription factor Fil1 (Ohtsuka et al., 2019). Fil1 is the homolog of Gcn4 in *Saccharomyces cerevisiae* and of ATF4 in mammals, which are basic leucine zipper (bZIP) family of transcription factors (Duncan et al., 2018). These transcription factors function in the general amino acid control (GAAC) pathway of budding yeast and in the amino acid response (AAR) of mammals (Han et al., 2018; Kilberg et al., 2012). Furthermore, the *S. pombe* genome encodes Gcn2 (ortholog of *S. cerevisiae* Gcn2 kinase that phosphorylates eukaryotic initiation factor-2 (eIF2)) and Gcn1 (ortholog of *S. cerevisiae* Gcn1 that ensures delivery of the uncharged tRNA onto Gcn2) as well as contributes to the GAAC pathway of *S. pombe* (Anda et al., 2017; Lee et al., 2015; Tarumoto et al., 2013). When *S. cerevisiae* is starved of amino acids, uncharged tRNAs accumulate, activating Gcn2 via their direct binding to Gcn2 (Zaborske et al., 2009). Activated Gcn2 phosphorylates eIF2\(\alpha\), decreasing global translation initiation efficiency, concomitant with an increased translation of GCN4 mRNA, encoding a transcriptional activator of numerous genes involved in amino acid metabolism, and the salvaging of nutrients (González & Hall, 2017; Tarumoto et al., 2013). We showed that Ecl1 family genes, one of which is induced by Fil1, are essential for survival when cells are starved of leucine (Lee; Ohtsuka et al., 2019). Despite evidence that establishes the relationship between the evolutionarily conserved GAAC/AAR pathway and Ecl1 family genes, knowledge of the GAAC pathway of fission yeast is limited.

Herein, we show that similar to amino acid depletion, Mg\(^{2+}\) depletion induces ecl1\(^{\alpha}\) through the activation of the GAAC pathway. Moreover, we conducted pharmacological and genetic studies and indicated that the extension of CLS via the activation of the GAAC pathway is closely linked to the regulation of ribosomal function.

2 | MATERIALS AND METHODS

2.1 | Strains and growth media

*Schizosaccharomyces pombe* strains are listed in Table A1. SPW054009 (pFil1) and SPW014009 (pDUAL-FFH1c) were used as fil1\(^{\alpha}\)-cloning plasmid and as a control entry vector, respectively.
(Matsuyama et al., 2004). The strains MK1 and PH1 were derived by crossing FY7876 with JY1. Cells were grown in SD medium or EMM supplemented with essential nutrients (Moreno et al., 1991). The amounts of supplemental nutrients were as follows: 40 µg/ml adenine, 60 µg/ml leucine, 480 µg/ml arginine, 360 µg/ml histidine, and 360 µg/ml lysine. EMM without MgCl₂ and synthetic SD (Ohhtsuka et al., 2017) without MgSO₄ were used as indicated. Cells were grown at 30°C.

2.2 | Mg²⁺ depletion

Cells were harvested in each medium during the logarithmic growth phase and transferred to a fresh Mg²⁺-free medium.

2.3 | Measurement of CLS

To measure survival, cells were grown in indicated liquid media, sampled during each growth phase, and then plated on YE agar plates after dilution. After 4–7 days at 30°C, using colony-forming units, the numbers of viable cells in 1-ml aliquots of culture were determined and divided by cell turbidity at each sampling time (Takuma et al., 2013). Cell growth was then monitored according to turbidity determined using a Bactomonitor (BACT-550) equipped with a 600-nm filter (Nissho Electric).

2.4 | Real-time polymerase chain reaction (PCR) analysis

Real-time PCR analysis was performed as previously described (Ohhtsuka et al., 2015) using the housekeeping gene cdc2 as a control. The primers are listed in Table A1.

2.5 | Western blot analysis

Western blotting was performed as previously described (Hibi et al., 2018). To detect eIF2α phosphorylation, phospho-eIF2α (Ser52) polyclonal antibody (Thermo Fisher Cat#44-728G), eIF2α, eIF2α polyclonal antibodies (Invitrogen Cat#PA5-41916), tubulin, monoclonal anti-α-tubulin antibody (Sigma Cat#T6074), and Ecl1-HA, anti-HA monoclonal antibodies (Roche Cat#11 583 816 001) were used.

2.6 | Measurement of cellular Mg²⁺ content

JY333 cells were grown in EMM with supplements up to the exponential phase and then transferred into EMM with supplements and without Mg²⁺. The amount of free intracellular Mg²⁺ was measured using Magnesium Green (Sharikabad et al., 2001). Cells were treated at 42°C for 3 min to allow the dye to penetrate cells. We detected fluorescence using an ECLIPSE Ti microscope (Nikon) and an Attune Acoustic Focusing Cytometer (Life Technologies) with BL1-A channel. To investigate the Mg²⁺ level of cell extract, JY333 cell lysate was measured with a Metallo Assay Kit for magnesium (Metallogenics).

2.7 | Measurement of amino acid content

JY333 cells were grown in EMM with adenine and Leu up to the exponential phase and then transferred into EMM with supplements [40 µg/ml adenine and 60 µg/ml (450 µM) Leu] and Mg²⁺-free EMM with supplements [40 µg/ml adenine and 60 µg/ml (450 µM) Leu]. The amounts of intracellular and extracellular amino acids were measured via GC-MS/MS (TQ8040; Shimadzu). The cells or the liquid medium were collected at each culture time, and the amount of each amino acid was quantified. For GC-MS/MS measurements, samples containing an internal standard (amino acid mixture solution-UL-13C, 15N; Taiyo Nippon Sanso) were mixed with a solvent mixture (methanol:chloroform:water = 2.5:1:1). After centrifugation, the supernatant was transferred to a clean tube and mixed with water. After centrifugation, the resultant supernatant was lyophilized using a freeze dryer. The dried samples were resolved in pyridine and mixed with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA; GL Science) for derivatization. In this measurement, not only Leu but also isoleucine, proline, glycine, and phenylalanine were simultaneously measured.

2.8 | Sucrose density gradient sedimentation analysis

JY333 and JY333Δecls cells were grown in EMM with supplements to exponential phase (OD₂₆₀ 0.5) at 30°C with shaking and then transferred into EMM with supplements and without Mg²⁺, Leu, or sulfate. Cells were harvested with cycloheximide treatment (Pospíšek & Valášek, 2013). Sucrose density gradient sedimentation was analyzed as previously described with some modifications (Akanuma et al., 2012). Briefly, the cells were disrupted using Cryo-Press (Microtec, Japan) and cell debris was removed using centrifugation. Aliquots of the extract (20 A₂₆₀ units) were layered onto sucrose density gradients (15%–45%), which were centrifuged at 65,000 g for 14 h at 4°C (Hitachi P40ST rotor). Samples were collected using a Piston Gradient Fractionator (BioComp), and absorbance profiles were monitored at 254 nm using a Bio-Mini UV Monitor (ATTO, Japan).

2.9 | Ribozinoindole-1 (Rbin-1) treatment

To inhibit the synthesis of ribosomes, 4 µg/ml Rbin-1 was added upon media inoculation (Figures 4b and 5b). Rbin-1 was synthesized according to the method reported by Kawashima et al. (2016).
2.10 | Data analyses

Quantitative data in the figures represent the average ± standard deviation.

3 | RESULTS

3.1 | Mg²⁺ depletion induces ecl1⁺ expression

Depleting sulfur or amino acids induces ecl1⁺ expression (Ohtsuka & Aiba, 2017; Ohtsuka et al., 2017, 2019). The former occurs in prototrophic and auxotrophic strains, whereas the latter only occurs in amino acid auxotrophs. In prototrophic strain, however, histidine analog 3-amino-1,2,4-triazole, which induces GAAC, increases ecl1⁺ expression (Duncan et al., 2018). In this study, we discovered that Mg²⁺ depletion induced both ecl1⁺-mRNA and Ecl1 protein expressions (Figure 1a and b). Mg²⁺ depletion significantly induced ecl1⁺ expression in an S. pombe leucine (Leu) auxotroph (strain JY333). In contrast, although the induction of ecl1⁺ transcription by Mg²⁺ occurs in the prototrophic strain JY1, the magnitude is smaller than that of JY333. Furthermore, when a multicopy empty vector (pLB-Dblet) that contains the Leu marker is used to transform JY333, the induction of ecl1⁺ transcription by Mg²⁺ depletion is significantly suppressed (Figure 1a). These results suggest that the induction of ecl1⁺ transcription by Mg²⁺ depletion is closely related to Leu auxotrophy.

The induction of ecl1⁺ transcription also occurs by arginine (Arg), lysine (Lys), and histidine (His) depletion in the corresponding auxotrophs (Ohtsuka et al., 2019). Therefore, we investigated ecl1⁺ expression in Arg, Lys, and His auxotrophs starved of Mg²⁺ (Figure 1c). As expected, induction of ecl1⁺ expression was detected in these

**FIGURE 1** Mg²⁺ depletion induces the expression of ecl1⁺. (a) JY333, JY1, and JY333 carrying the empty vector (pLB-Dblet) cells were cultured in Edinburgh minimal medium (EMM) with each necessary supplement and then transferred to medium without Mg²⁺. Cells were harvested at 0, 2, 4, and 6 h after transfer, and the expression of ecl1⁺-mRNA was analyzed using real-time polymerase chain reaction (PCR) assays (n = 3) *p < 0.01 (Student’s t test). (b) JY333 Ecl1-HA cells were cultured in EMM with supplements and were then transferred into media without Mg²⁺ or Leu. Cells were harvested at 0, 2, 4, and 6 h after transfer, and the amount of Ecl1-HA, and the phosphorylation levels of elf2α were measured using Western blot analysis. (c) Amino acid auxotrophic strains, FY10704(28), arginine auxotroph (arg1); MK1, lysine auxotroph (lys1); and PH1, histidine auxotroph (his2), were cultured in EMM with supplements (arginine, lysine, and histidine for arg1, lys1, and his2 strains, respectively) and were then transferred to medium without Mg²⁺. Cells were harvested at 0, 2, 4, and 6 h after transfer, and the expression of ecl1⁺-mRNA was analyzed using real-time PCR (n = 3).
strains. These results suggest that Mg\(^{2+}\) response is associated with the response to the amino acid limitation in fission yeast.

3.2 | Induction of ecl1\(^{+}\) expression by Mg\(^{2+}\) depletion depends on Fil1

In S. pombe, Fil1, which is induced under conditions of amino acid depletion, directly binds the 580-bp region upstream of ecl1\(^{+}\) to induce its expression (Ohtsuka et al., 2019). Significant induction of ecl1\(^ {+}\) expression in cells depleted of Mg\(^ {2+}\) was detected in several amino acid auxotrophic strains; therefore, we investigated whether Fil1 was involved. The control strain ED668 expressed ecl1\(^{+}\) when Mg\(^ {2+}\) was depleted but not in a strain lacking fil1\(^{+}\) (Figure 2a). These findings indicate that the induction of ecl1\(^{+}\) when Mg\(^ {2+}\) is depleted is regulated by Fil1, which is active when amino acids are depleted.

Although the proteins encoded by S. pombe ecl1\(^{+}\), ecl2\(^{+}\), and ecl3\(^{+}\) have similar functions (Ohtsuka & Aiba, 2017), only the expression of ecl1\(^{+}\) was significantly induced in a Fil1-dependent manner when Mg\(^ {2+}\) was depleted (Figure 2b).

3.3 | Mg\(^{2+}\) depletion activates the GAAC pathway

Mg\(^{2+}\) depletion induces ecl1\(^{+}\) expression in a Fil1-dependent manner and therefore may activate other GAAC-related processes. Thus, we next investigated the activity of Gcn2 kinase that functions upstream of Fil1. Western blot analysis shows that Mg\(^ {2+}\) depletion, as well as Leu depletion, promotes phosphorylation of eIF2\(\alpha\) in a Gcn2-dependent manner (Figures 1b and 3a). The phosphorylation of eIF2\(\alpha\) when cells were starved for Mg\(^ {2+}\) was detected in \(\Delta\)ecl1\(\Delta\)ecl2\(\Delta\)ecl3 triple mutant (\(\Delta\)ecls) and in the parental strain (JY333) and wild-type cells (JY1; Figure 3a). These findings are consistent with the possibility that Ecl1 protein functions downstream of the GAAC pathway. Moreover, S. pombe Gcn1 is required for the phosphorylation of eIF2\(\alpha\) when Mg\(^ {2+}\) is depleted. These results indicate that Mg\(^ {2+}\) depletion activated Gcn2 in a Gcn1-dependent manner.

We next measured the levels of intracellular and extracellular amino acids (Figure 3b). The decrease in the amount of extracellular Leu was suppressed when Mg\(^ {2+}\) was depleted. At the same time, the amount of intracellular Leu decreased when Mg\(^ {2+}\) was depleted. This finding suggested that Mg\(^ {2+}\) depletion suppressed the absorption of extracellular Leu.
When we used flow cytometry to measure intracellular-free Mg\(^{2+}\) concentrations under Mg\(^{2+}\) depletion, we found that total Mg\(^{2+}\) concentration decreased in the Mg\(^{2+}\)-free medium (Figure 3c). However, microscopy indicated that the cytosolic Mg\(^{2+}\) concentration was only slightly decreased (Figure 3c). The difference in Mg\(^{2+}\) level observed in cells was also observed in the measurements of Mg\(^{2+}\) in cell extracts (Figure 3c). These results suggest that although auxotrophic fission yeast sensed this depletion through the GAAC pathway, Mg\(^{2+}\) depletion was not deleterious to cytosolic Mg\(^{2+}\) levels, possibly attributable to the cellular Mg\(^{2+}\) homeostasis.

### 3.4 Mg\(^{2+}\) depletion extends CLS partially through Ecl1 family genes

Ecl1 family genes extend CLS when ectopically overexpressed in Edinburgh minimal medium (EMM), which leads cells to long chronological lifespan, and at higher levels in SD medium (Ohtsuka & Alba, 2017; Ohtsuka et al., 2008). Similarly, Ecl1-dependent extension of CLS occurs, for example, by the depletion of sulfur and Leu (Ohtsuka et al., 2017, 2019). As previously discussed, Mg\(^{2+}\) depletion induces ecl1\(^{+}\) expression, leading us to ask whether the extension of CLS in
the SD medium occurred under this condition (Figure 4a). We found that although Mg²⁺ depletion extended the CLS of auxotrophic fission yeast, the extension of the CLS of Δecls cells starved for Mg²⁺ was less than that of the parental strain. Consistent with the level of ecl¹⁺ induction (Figure 1a), CLS extension by Mg²⁺ depletion significantly decreases in the prototrophic strain (Figure 4a). These findings suggest that the extension of CLS when Mg²⁺ is depleted partially depends on the expression of Ecl1 family genes and on the contributions of other pathways and factors.

3.5 Nutritional depletion decreases ribosome content in Ecl1 family gene-dependent manner

Depletion of Mg²⁺ activates the GAAC pathway and extends CLS. Furthermore, induction of the expression of Ecl1 family genes in cells starved of sulfur, or when they were ectopically overexpressed from plasmids, leads to the inhibition of expression of genes encoding many ribosomal proteins (Ohtsuka et al., 2017). To identify the mechanism, we analyzed the polysomes of cells subjected to nutrient depletion (Figure 4b). There were no significant differences in ribosomal abundances between JY333 and JY333Δecls cells harvested during exponential growth. This finding is consistent with the relatively weak expressions of Ecl1 family genes during the early log phase (Ohtsuka & Aiba, 2017; Ohtsuka et al., 2008). In contrast, under each condition that results in nutritional depletion, 80S monosome levels were reduced in JY333 but not in JY333Δecls cells. Moreover, the total ribosome levels seem to be reduced in JY333 but less in JY333Δecls cells. This is consistent with previous results: sulfur depletion reduces the level of expressions of many ribosomal proteins at mRNA levels in the Ecl1 family gene-dependent manner (Ohtsuka et al., 2017). These results suggest that sulfur depletion and the activation of the GAAC pathway by Leu or Mg²⁺ depletion modulate the quantity and quality of ribosomes and that Ecl1 family genes mediate these processes.

3.6 Inhibition of ribosome synthesis rescues the loss of the viability of Δecls cells starved of Leu

If the decrease in the abundance of ribosomes described above was critical for extending CLS, we reasoned that the CLS of Δecls cells will be extended when the levels of ribosomes are pharmacologically reduced. We therefore measured CLS of Δecls cells starved of Leu (GAAC activation) in the presence of ribozinoindole-1 (Rbin-1), which reduces ribosome biosynthesis via inhibition of midasin (Mdn1) (Chen et al., 2018; Kawashima et al., 2016). Rbin-1 partially but significantly increased the survival of Δecls cells after Leu was depleted from these media (Figure 5a), indicating that reduction in ribosome abundance was essential for extending CLS. Moreover, we were intrigued to find that Rbin-1 treatment significantly reduced the amounts of 80S ribosomes in exponentially proliferating Δecls cells (Figure 5b). Because Rbin-1 inhibits the maturation of 60S ribosomes (Chen et al., 2018; Kawashima et al., 2016), it may also inhibit the formation of 80S ribosomes that require the mature 60S. A smaller Rbin-1-mediated decrease in the abundance of 80S ribosomes occurred when the medium was depleted of Leu. This result supports the conclusion that regulation of ribosome biosynthesis, likely leading to the reduction in 80S ribosomes, contributed to maintaining cell survival when the GAAC pathway was activated in response to nutrient depletion.

4 DISCUSSION

The cellular responses identified here are summarized in Figure 6. Mg²⁺ is transported into the cell via the transporter SPBC27B12.12c (Uz & Sarikaya, 2016). When the extracellular Mg²⁺ concentration is low, the intracellular Mg²⁺ concentration slightly decreases (Figure 3c). This might lead to reduced levels of tRNA aminoacylation because Mg²⁺ affects the catalytic reactions of aa-tRNA synthetase (Airas, 2007). Increases in uncharged tRNAs activate Gcn2 kinase in a Gcn1-dependent manner, culminating in the phosphorylation of eIF2α (Figure 3a). This, in turn, stimulates the activation of the transcription factor Fil1 and induces Fil1-dependent ecl¹⁺ expression (Figure 2). Induction of the expression of Ecl1 family genes via the GAAC pathway contributes to the maintenance of survival and extends CLS possibly by regulating the abundance of ribosomes (Figures 4 and 5).

Here, we show that cells starved of amino acids, as well as Mg2+, lead to activation of the GAAC pathway. Further, we present data indicating that a reduction in the abundance of ribosomes is critical for extending CLS. The decrease in ribosomes is thought to lead to decreased growth and metabolism, which is expected to contribute to survival because of reduced nutrient consumption when environmental nutrients are limited. In yeasts, similar to the induction of sporulation, long-term survival in the stationary phase (e.g., the extension of CLS) contributes to the conservation of genetic information in an environment that has fluctuating levels of nutrients. One of these response processes, the GAAC pathway, is conserved in fungi and in mammals as the AAR. Although confirmed homologs of Ecl1 family genes were not identified in mammals when using protein BLAST search, there is a possibility that similar, but less well-conserved, genes, which mediate analogous processes that regulate lifespan may exist in higher organisms.

Recently, it was reported that the circadian clock controls translation through GAAC in Neurospora crassa (Karki et al., 2020). It has been known that UV also affects GAAC in fission yeast (Anda et al., 2017), and it is becoming clear that GAAC is involved in numerous stimulatory responses other than amino acids, including the Mg²⁺ response that was investigated in this study.

4.1 The response to Mg²⁺ depletion

Here, we show that the induction of the expression of ecl¹⁺ was associated with the depletion of Mg²⁺ and amino acids, leading to
activation of the GAAC pathway, especially in auxotrophic strains (Figures 1a and 3a). Thus, Mg$^{2+}$ depletion may activate the GAAC pathway by directly inhibiting the aminoacylation of tRNA and by inhibiting the uptake of amino acids.

Activation of the GAAC pathway caused by Mg$^{2+}$ depletion was observed when the prototrophic strain JY1 was cultured in the absence of exogenous amino acids (Figures 1a and 3a). This induction may be caused by decreases in aa-tRNA synthetase activities when the intracellular Mg$^{2+}$ concentration was reduced. Aa-tRNA synthetases promote aminoacylation in the presence of high Mg$^{2+}$ concentrations compared with a low Mg$^{2+}$ concentration (Airas, 2007). Here, we detected a slight decrease in the level of intracellular Mg$^{2+}$ when Mg$^{2+}$ was depleted from the medium (Figure 3c). This reduction may affect the activities of these enzymes.

Additionally, we found that in amino acid auxotrophs, Mg$^{2+}$ depletion induced ecl1$^+$ expression approximately 20–100 times versus <5 times in the prototrophic strain JY1 (Figure 1). Therefore, the four- to 20-fold induction can be attributed to the reduced uptake of amino acids specific to the auxotrophic strains, which we consider to correspond to activation of the GAAC pathway when Mg$^{2+}$ is depleted from the medium. These results are consistent with our findings here that reduced Leu uptake by the Leu auxotroph occurred when cells were cultured in a medium depleted of Mg$^{2+}$ (Figure 3b). Moreover, a study using Brewers’ yeast indicates a positive correlation between the amounts of Mg$^{2+}$ in the environment and those of intracellularly incorporated amino acids (Yoshizawa et al., ). These findings suggest that the control of amino acid uptake by the extracellular Mg$^{2+}$ level is conserved in these yeasts.

Hence, in auxotrophic strains, it is considered that both the events activate the GAAC pathway: Mg$^{2+}$ depletion can directly inhibit the aminoacylation of tRNA and inhibit the uptake of amino acids (Figure 3b). On the other hand, (a) phosphorylation of eIF2α was also found in wild-type (JY1) cells (Figure 3a), (b) auxotrophy significantly changed the level of induction of ecl1$^+$ (Figure 2), and (c) the induction of ecl1$^+$ was dependent on Fil1 (Figure 2), suggesting

**FIGURE 4** Mg$^{2+}$ depletion extends CLS partially through Ecl1 family genes
(a) JY1, JY333, and JY333Δecl1/2/3 cells were cultured in synthetic SD medium (2% glucose) with or without Mg$^{2+}$ (n = 3). Cell growth data are shown in Figure A1. (b) JY333 and JY333Δecl1/2/3 cells were cultured in EMM with supplements and then transferred to media without Mg$^{2+}$, Leu, or sulfate. Cells were harvested at exponential phase (OD$_{600}$ 0.5) and 2 days after transfer (-Mg, -Leu, -S). Crude cell extracts were sedimented through a 10% to 40% sucrose gradient as described in Materials and Methods. The 40S, 60S, and 80S peaks are indicated in each profile.
that there is an unknown mechanism for Fil1 activation other than eIF2α phosphorylation.

Interestingly, Atf4, a functional homolog of Fil1 in mammals, is activated by tetracycline, which is a strong Mg^{2+} chelator, and this effect is dependent on the Mg^{2+} concentration (Brüning et al., 2014; Xiao et al., 2008). If tetracycline acts as a Mg^{2+} chelator and contributes to the decrease in intracellular-free Mg^{2+} concentrations, the decrease in Mg^{2+} may contribute to the activation of Atf4 through the activation of the AAR, consistent with our findings for fission yeast. Further, Mg^{2+} depletion affects protein and amino acid metabolism in rats (Nemoto et al., 2006; Uehara et al., 2010), indicating that the link between Mg^{2+} depletion and AAR response may not be limited to yeast.

### 4.2 Expression of Ecl1 family genes is associated with the abundance of ribosomes

In bacteria, ribosomes are inactive when nutrients are depleted, and cells consequently enter stationary phase, during which the majority of 70S ribosomes dimerize to form translationally inactive 100S ribosomes, or alternatively, YfiA inactivates 70S monomers (El-Sharoud, 2004; Polikanov et al., 2012; Yoshida & Wada, 2016). Further, the number of ribosomes decreases in this phase (Akanuma et al., 2016). In budding yeast, the accumulation of 80S monosomes occurs in a Δdom34 mutant, although a stalled 80S monosome generally dissociates into subunits, which is mediated by Dom34:Hbs1 in wild-type cells (Guydosh et al., 2017; Inada, 2012; Shoemaker et al., 2010; Tsuboi et al., 2012). Here, we show that although we did not detect ribosomal dimers, a decrease in ribosomal abundance was detected during nutrient depletion in S. pombe wild-type cells and in bacteria (Figure 4b; Akanuma et al., 2016). In Δecls cells cultured under nutrient depletion, 80S monosomes accumulated, which is similar to the phenotype of the Δdom34 mutant, indicating that Ecl1 family genes may be involved in the dissociation of the ribosome. Thus, the death of Δecls cells cultured under nutrient depletion might be explained by stalled monosomes bound to mRNA that cannot dissociate into subunits, which is required for ribosome recycling and continued protein synthesis. In Δecls cells, a decrease in the abundance of ribosomes equivalent to that of the parental strain was not
detected. Ecl1 family genes regulate the expression of ribosomal proteins (Ohtsuka et al., 2017), and therefore, this decrease may be partially caused by transcriptional regulation. Therefore, Ecl1 family genes may regulate the dissociation of ribosomes into subunits, as well as the abundance of ribosomes.

Leu depletion led to a significant accumulation of 80S ribosomes and a decrease in polysomes in Δecls cells compared with those of the parental strain (Figure 4b). In contrast, when Mg2+ was depleted, the differences found in Δecls cells were smaller compared with those of cells subjected to Leu depletion. These findings are consistent with the observation that extension of CLS because of Leu depletion mainly depends on the expression of Ecl1 family genes (Ohtsuka et al., 2019) and only partially when Mg2+ was depleted (Figure 4a). These findings suggest that the GAAC pathway and others that respond to Mg2+ may be involved in the regulation of ribosomal abundance and CLS.

5 | CONCLUSION

In conclusion, we demonstrated that Mg2+ depletion activated Gcn2 and the transcription of genes encoding components of downstream pathways via the transcription factor Fil1. Although the induction of ecl1+ caused by Mg2+ depletion is small in prototrophic JY1 cells, it is significantly high in auxotrophs (Figure 1a and c). Furthermore, we observed that Mg2+ depletion sufficiently
activated the GAAC pathway in auxotrophic fission yeast, which is highly convenient and generally used for experiments. The extension of lifespan when nutrients are restricted occurs in evolutionarily diverse organisms (Fontana et al., 2010; Kapahi et al., 2017; Mirzaei et al., 2014), and its regulation is influenced by the regulation of ribosomal abundance (MacInnes, 2016; Ohtsuka & Aiba, 2017; Steffen et al., 2008). Our results also indicated that GAAC activation correlates with decreased ribosomal abundance and CLS extension. Although the extension of lifespan mediated by the GAAC pathway and AAR is the focus of several studies (Gallinetti et al., 2013; Mittal et al., 2017; Santos et al., 2016), Ecl1 family genes, which are regulated by Fil1, are major regulators of CLS during Leu depletion (Ohtsuka et al., 2019). Although homologs of Ecl1 family genes have not been found in higher organisms, their functional homologs may be conserved in these organisms as components or downstream effectors of the GAAC pathway/AAR. The identification of the mechanism by which cells adjust to the depletion of environmental nutrients, such as Mg²⁺, may provide further insights into CLS.

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CONFLICT OF INTEREST
None declared.

AUTHOR CONTRIBUTIONS
Hokuto Ohtsuka: Conceptualization (equal); Funding acquisition (equal); Investigation (equal); Writing-original draft (equal).
Mikuto Kobayashi: Data curation (equal); Investigation (equal).
Takafumi Shimasaki: Data curation (equal); Investigation (equal).
Teppeli Sato: Data curation (equal); Investigation (equal).
Genki Akanuma: Data curation (equal); Investigation (equal); Methodology (equal).
Yaoyayo Kitaura: Data curation (equal); Investigation (equal); Methodology (equal).
Yoko Otsubo: Investigation (equal); Methodology (equal).
Akira Yamashita: Methodology (equal); Supervision (supporting).
Hirofumi Aiba: Conceptualization (lead); Funding acquisition (lead); Project administration (lead); Supervision (lead); Writing-review & editing (equal).

ETHICS STATEMENT
None required.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this published article.

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APPENDIX 1

Figure A1  Effects of Mg\(^{2+}\) depletion on growth. The state of growth of each strain at the time of CLS measurement is shown in Figure 4.

APPENDIX 2

TABLE A1  Yeast Strains and PCR Primers

| Fission yeast strains | Genotype |
|-----------------------|----------|
| **Schizosaccharomyces pombe** |          |
| JY1                   | h\(^+\)   |
| JY333                 | h\(^-\) leu1-32 ade6-M216 |
| JY333 Ecl1-HA         | h\(^-\) leu1-32 ade6-M216 ecl1::ecl1-HA-kan\(^r\) |
| JY333Δ/2/3            | h\(^-\) leu1-32 ade6-M216 ecl1::kan\(^r\) ecl2::kan\(^r\) ecl3::kan\(^r\) |
| FY10704               | h\(^+\) arg1 |
| FY7876                | h\(^-\) leu1-32 lys1 his2 |
| MK1                   | h\(^-\) lys1 |
| PH1                   | h\(^+\) his2 |
| ED668                 | h\(^+\) leu1-32 ade6-M216 ura4-D18 |
| ED668Δfil1            | h\(^+\) leu1-32 ade6-M216 ura4-D18 fil1::kan\(^r\) |
| ED668Δgcn1            | h\(^+\) leu1-32 ade6-M216 ura4-D18 gcn1::kan\(^r\) |
| YT3360                | h\(^-\) leu1-32 ura4-D18 gcn2::ura4\(^+\) |

| Real-time PCR primers | Sequence |
|-----------------------|----------|
| **ecl1**              | TTTTGACACGTGTGGAGC |
|                       | TGTGTAAGCAGCTGGAGCG |
| **ecl2**              | GGAACACCACACTTGGAGC |
|                       | GAACAGCAAGCTTGGAGCG |
| **ecl3**              | GCTATGTTGAACTTCATCG |
|                       | GAGAATCTCGAGAAGGAGC |
| **cdc2**              | CCGAATTTCCAAACTGAGG |
|                       | GTATAGTCCCGAAAGGAGC |