Elevation of cellular Mg$^{2+}$ levels by the Mg$^{2+}$ transporter, Alr1, supports growth of polyamine-deficient 
*Saccharomyces cerevisiae* cells

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The polyamines putrescine, spermidine, and spermine are required for normal eukaryotic cellular functions. However, the minimum requirement for polyamines varies widely, ranging from very high concentrations (mM) in mammalian cells to extremely low in the yeast *Saccharomyces cerevisiae*. Yeast strains deficient in polyamine biosynthesis (*spe1*, lacking ornithine decarboxylase, and *spe2*, lacking SAM decarboxylase) require externally supplied polyamines, but supplementation with as little as $10^{-3}$ M spermidine restores their growth. Here, we report that culturing a *spe1*Δ mutant or a *spe2*Δ mutant in a standard polyamine-free minimal medium (SDC) leads to marked increases in cellular Mg$^{2+}$ content. To determine which yeast Mg$^{2+}$ transporter mediated this increase, we generated mutant strains with a deletion of *SPE1* or *SPE2* combined with a deletion of one of the three Mg$^{2+}$ transporter genes, *ALR1*, *ALR2*, and *MNR2*, known to maintain cytosolic Mg$^{2+}$ concentration. Neither *Alr2* nor *Mnr2* was required for increased Mg$^{2+}$ accumulation, as all four double mutants (*spe1*Δ *alr2*Δ, *spe2*Δ *alr2*Δ, *spe1*Δ *mnr2*Δ, and *spe2*Δ *mnr2*Δ) exhibited significant Mg$^{2+}$ accumulation upon polyamine depletions. In contrast, *spe2*Δ *alr1*Δ double mutant cultured in SDC exhibited little increase in Mg$^{2+}$ content and displayed severe growth defects compared with single mutants *alr1*Δ and *spe2*Δ under polyamine-deficient conditions. These findings indicate that *Alr1* is required for the up-regulation of the Mg$^{2+}$ content in polyamine-depleted cells and suggest that elevated Mg$^{2+}$ can support growth of polyamine-deficient *S. cerevisiae* mutants. Up-regulation of cellular polyamine content in a Mg$^{2+}$-deficient *alr1*Δ mutant provided further evidence for a cross-talk between Mg$^{2+}$ and polyamine metabolism.

The polyamines putrescine (NH$_2$(CH$_2$)$_2$NH$_2$), spermidine (NH$_2$(CH$_2$)$_3$NH(CH$_2$)$_2$NH$_2$), and spermine (NH$_2$(CH$_2$)$_3$NH(CH$_2$)$_4$NH$_2$) are ubiquitous in living cells and organisms and are normally present at high concentrations (mM) (1–3). In the yeast *Saccharomyces cerevisiae*, putrescine is produced from ornithine by ornithine decarboxylase (*Spe1*; Scheme LA). Spermidine is produced from putrescine, and spermine is produced from spermidine by addition of an aminopropyl moiety from decarboxylated SAM, which is produced by SAM decarboxylase (*Spe2*; Scheme 1A). As the primary and secondary amino groups of polyamines are protonated at physiological pH, polyamines interact with negatively charged molecules such as nucleic acids, proteins, and phospholipids and influence their conformation, stability, and activity (4). The polyamines regulate a large number of cellular processes, including the enhancement of the efficiency and fidelity of translation (5). They are vital for survival of eukaryotes and are intimately involved in the regulation of eukaryotic cell growth. However, the precise modes of their action in supporting many cellular functions are not fully understood.

A high level (mM) of cellular polyamines is required for mammalian cell proliferation, and polyamine homeostasis is tightly regulated by intricate mechanisms (6). Numerous studies have demonstrated the antiproliferative effects of various inhibitors of polyamine biosynthesis or polyamine analogs that cause depletion of polyamines in mammals. The high polyamine requirement of mammalian cells is the basis for targeting the polyamine pathways in cancer chemoprevention and chemotherapies (1). Depletion of spermidine and spermine mediated by overexpression of the polyamine catabolic enzyme spermidine/spermine N$^4$-acetyltransferase 1 also caused a dramatic inhibition of protein synthesis and cell growth (7). In contrast to mammalian cells, the polyamine requirement is extremely low in the yeast *S. cerevisiae*. A *spe2*Δ mutant strain, which is unable to synthesize spermidine and spermine, grew at a nearly normal rate in medium containing $10^{-8}$ M spermidine, a condition in which the cellular spermidine content dropped as low as 0.2% of WT levels (8).

One clearly defined function of the polyamine spermidine in eukaryotes is its role as precursor of the unusual amino acid hypusine (N$^4$-4-amino-2-hydroxybutyl(lysine)) (9), which is formed by the post-translational modification of the eukaryotic translation factor eIF5A. The aminobutyl moiety of spermidine is conjugated to a specific lysine residue to form deoxyhypusine, which is subsequently hydroxylated to hypusine. Hypusine/de-
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oxypseudine is essential for the activity of eIF5A. Eukaryotic cell proliferation and animal development depend on hypusination eIF5A (10, 11). Normally, <2% of cellular spermidine is used for hypusine synthesis in eukaryotic cells. However, in a yeast spe2Δ mutant cultured in a medium containing <10⁻⁸ M spermidine, spermidine became severely limiting, and as much as ~50% of the total cellular spermidine was mobilized for hypusine synthesis. These findings suggest that hypusination of eIF5A is the most critical function of polyamines in yeast, and that, unlike mammalian cells, yeast do not require a high intracellular concentration of polyamines for growth.

The apparent discrepancy in the minimal polyamine requirement between yeast and mammalian cells may be due to differences in the intrinsic functional roles of polyamines, or it may indicate that yeast possess a unique mechanism to compensate for polyamine deficiency. One such mechanism could be the compensatory accumulation of another cation such as magnesium (Mg²⁺), the most abundant divalent cation in cells (12, 13). Mg²⁺ is a critical cofactor for over 300 enzymes, and it also serves a structural role by stabilizing protein domains. Like polyamines, the majority of cellular Mg²⁺ is bound to negatively charged ligands such as ATP, RNA, DNA, or phospholipids (12). Functional overlap between polyamines and Mg²⁺ has been suggested by several in vitro studies. Polyamines stimulated translation in cell-free lysates when Mg²⁺ concentration was suboptimal (4, 14), suggesting that polyamines and Mg²⁺ can partially substitute for each other in protein synthesis. However, there is little information on the in vivo functional interaction between polyamines and Mg²⁺ in the regulation of eukaryotic cell growth.

The cellular content of Mg²⁺ is tightly controlled in yeast cells and remains constant over a range of 1–100 mM external Mg²⁺ (15, 16). However, when cells are cultured in low Mg²⁺ medium (<100 μM), intracellular Mg²⁺ content is substantially reduced, and growth is limited (15). Regulation of cellular Mg²⁺ is likely achieved by control of uptake systems, efflux from the cell, and sequestration within organelles (12, 13, 15) (Scheme 1A).

To mediate this regulation, yeast express five known Mg²⁺ transporters, all related to the bacterial plasma membrane Mg²⁺ transporter CorA (Scheme 1B): Alr1/Alr2 of plasma membrane, Mnr2 of the vacuolar membrane, and Mrs2/Lpe10 of the mitochondrial membrane (15, 17). The CorA superfamily of Mg²⁺ transporters share certain structural features, including two adjacent transmembrane domains near the C terminus that are connected by a short periplasmic loop. Each turn in the peptide chain represents 50 amino acids. The conserved GMN signature sequence critical for selective recognition of Mg²⁺ is indicated. PA, polyamines; ODC, ornithine decarboxylase; SAMDC, SAM decarboxylase; DeSAM, decarboxylated SAM.
Table 1
List of S. cerevisiae strains used in this study

| Strains          | Genotype                        | Ref.                                      |
|------------------|---------------------------------|-------------------------------------------|
| Y534/BY4741 (WT) | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 | Chattopadhyay et al. (8) and Giaever and Nislow (25) |
| Y535 (spe1Δ)     | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, spe1Δ::KAN6 | Chattopadhyay et al. (8) and Giaever and Nislow (25) |
| Y536 (spe2Δ)     | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, spe2Δ::KAN6 | Chattopadhyay et al. (8) and Giaever and Nislow (25) |
| DY1457 (WT)      | MATa, ade6, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, spe1Δ::KAN6 | Lim et al. (29) |
| AH1 (spe1Δ)      | MATa, ade6, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, spe1Δ::KAN6 | This study |
| AH2 (spe2Δ)      | MATa, ade6, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, spe2Δ::KAN6 | This study |
| CM200/NP10 (alr1Δ) | MATa, ade6, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, alr1::HIS3 | This study |
| AH3 (alr1Δ, spe2Δ) | MATa, ade6, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, alr1::HIS3, spe2Δ::KAN6 | This study |
| NP27 (alr2Δ)    | MATa, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, alr2::TRP1 | Pisat et al. (15) |
| AH4 (alr2Δ, spe1Δ) | MATa, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, alr2::TRP1, spe1Δ::KAN6 | This study |
| AH5 (alr2Δ, spe2Δ) | MATa, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, alr2::TRP1, spe2Δ::KAN6 | This study |
| NP174 (WT)      | MATa, ade2, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, spe2Δ::KAN6 | Pisat et al. (15) |
| AH6 (spe1Δ)     | MATa, ade2, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, spe1Δ::KAN6 | This study |
| AH7 (spe2Δ)     | MATa, ade2, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, spe2Δ::KAN6 | This study |
| NP180 (mnr2Δ)   | MATa, ade2, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, mnr2::SpHIS5 | Pisat et al. (15) |
| AH8 (mnr2Δ, spe1Δ) | MATa, ade2, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, mnr2::SpHIS5, spe1Δ::KAN6 | This study |
| AH9 (mnr2Δ, spe2Δ) | MATa, ade2, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–52, mnr2::SpHIS5, spe2Δ::KAN6 | This study |

mutation (which blocks synthesis of all polyamines) or the spe2Δ mutation (which blocks synthesis of spermine and spermidine) on cellular Mg2+ content. Interestingly, cellular Mg2+ content increased in response to polyamine depletion. Examination of double mutant strains lacking one polyamine biosynthesis gene (SPE1 or SPE2) and one Mg2+ transporter gene (ALR1, ALR2, or MNR2) indicated that Alr1 alone was required for this elevated Mg2+ accumulation. Consistent with this observation, Alr1 was found to be essential for the survival and growth of polyamine-deficient spe1Δ and spe2Δ cells. These findings provide strong evidence that yeast can specifically compensate for polyamine deficiency by up-regulating the accumulation of Mg2+ ions. The elevation of the cellular polyamine levels in the Mg2+ deficient alr1Δ mutant cultured in YPD further suggests an interaction between polyamine and Mg2+ metabolism.

**Results**

Depletion of cellular polyamines leads to an elevation of Mg2+ content in *S. cerevisiae*

We compared the growth, polyamine content, and Mg2+ content of WT yeast (Y534; BY4741) and the two polyamine biosynthesis mutants Y535 (spe1Δ) and Y536 (spe2Δ) (Table 1 and Fig. 1). In Fig. 1 (and Figs. 3 and 4), all data are color-coded (parental strains (brown), spe1Δ mutant (pink), and spe2Δ mutant (blue)). For comparison of growth, cells were initially inoculated at a very low density (0.0003), and the optical density was followed for 72 h. Cultures were regularly diluted into fresh medium to maintain log phase. In YPD rich in polyamines, the mutations had no effect on growth (Fig. 1A), as the mutants utilized polyamines supplied from the medium. Growing cultures for long periods in polyamine-free SDC led to the depletion of initial polyamine stores in spe1Δ and spe2Δ mutants.

The growth of Y535 (spe1Δ) and Y536 (spe2Δ) in SDC declined with time and stalled after 20–40 h (Fig. 1B), and the growth defects were magnified with prolonged incubation. The content of spermidine and spermine in the mutants grown in YPD was only slightly less than that in the WT (Fig. 1C). A higher level of putrescine was observed in spe2Δ, resulting from the blockage of conversion of putrescine to spermine and spermidine to spermine in the absence of SAM decarboxylase (Spe2) (Scheme 1A). The spe1Δ cells cultured in SDC did not contain any detectable polyamines, and spe2Δ cells contained a highly elevated level of putrescine but no spermidine or spermine (Fig. 1D) as expected. The Y536 (spe2Δ) strain grew better than Y535 (spe1Δ) in SDC, suggesting that the high level of putrescine partially fulfilled the polyamine requirement.

To determine the effect of these changes in polyamine content on Mg2+ homeostasis, parallel samples of Y534 (WT), Y535 (spe1Δ), and Y536 (spe2Δ) cells were also taken for analysis of their elemental content. There was little or no difference in Mg2+ content among the three strains after growth in YPD (3.1–3.3 mg of Mg2+/g dry weight) (Fig. 1E). However, after 24-h culture in SDC, the Mg2+ content of the polyamine-deficient spe1Δ and spe2Δ cells increased substantially (by ~2.5- and ~1.6-fold, respectively) (Fig. 1F). The increase was consistently more pronounced in spe1Δ than spe2Δ cells, suggesting that the degree of Mg2+ accumulation was responsive to the severity of polyamine deficiency. To verify that this change in Mg2+ content was a consequence of polyamine deficiency and not the absence of some other component of YPD in SDC, Mg2+ content was also measured in cells cultured in SDC supplemented with 10−8 or 10−6 M spermidine (Fig. 1, G and H). The elevation in cellular Mg2+ content of the mutants was reduced by spermidine supplementation. When cultured in SDC containing 10−8 M spermidine, Mg2+ content increased ~2.1-fold in spe1Δ and ~1.4-fold in spe2Δ (Fig. 1G). We chose this concentration of spermidine, as the spe2Δ mutants can grow nearly normally in SDC containing 10−8 M spermidine when cellular polyamines were limiting (0.2% of normal level) (8). A strong increase in the Mg2+ content under this condition

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2The abbreviations used are: YPD, yeast extract–peptone–dextrose medium; SDC, synthetic dextrose complete medium; SD, synthetic dextrose medium; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; ANOVA, analysis of variance; ICP-OES, inductively coupled plasma optical emission spectrometry.
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Figure 1. Analysis of growth and polyamine and Mg\(^{2+}\) content of YS34 (WT), YS35 (spe1\(\Delta\)), and YS36 (spe2\(\Delta\)) strains. A and B, growth of yeast strains (YS34, YS35, and YS36) at 30 °C in YPD (A) and SDC (B) was analyzed over 72 h. The cells were inoculated at a starting density of 0.0003 OD\(_{600}\) and diluted down to 0.001 when the density reached an OD\(_{600}\) of −1. Data points indicate means and error bars represent S.E. (n = 4). C and D, cells were inoculated in YPD (C) and SDC (D) at 0.001, and −10–20 OD\(_{600}\) units of cells were harvested at a density of −1 OD\(_{600}\) for analyses of polyamines. The content of putrescine, spermidine, and spermine is displayed separately for each strain. E, F, G, and H, Mg\(^{2+}\) content of three strains cultured in YPD (E), SDC (F), SDC + 10\(^{-8}\) M spermidine (Spd) (G), and SDC + 10\(^{-6}\) M spermidine (H) and harvested as above is shown. The values of polyamine and Mg\(^{2+}\) content are indicated as black dots, and each bar indicates the mean and the error bars represent S.D. (n = 4). The p values were calculated by ANOVA. p values less than 0.05 were considered statistically significant: **, p ≤ 0.01; ****, p ≤ 0.0001 compared with the control. NS indicates that the difference is not significant.

suggests that Mg\(^{2+}\) elevation contributes to the nearly normal growth of the mutant. When cells were supplemented with a much higher level of spermidine (10\(^{-5}\) M), only small increases in Mg\(^{2+}\) content were observed (13.2 and 6.8% in spe1\(\Delta\) and spe2\(\Delta\), respectively) (Fig. 1H), confiriming an inverse relationship between spermidine supply and Mg\(^{2+}\) accumulation by the mutant strains.

To determine whether this effect of polyamine deficiency was specific to Mg\(^{2+}\) or reflected a more general effect on nutrient accumulation, we examined the content of potassium (K), manganese (Mn), zinc (Zn), and phosphorus (P) (Table 2). Only small variations (<13%) in potassium content in YPD, SDC, and SDC supplemented with spermidine. No consistent negative or positive effect of polyamine depletion was observed on the content of potassium, indicating that polyamine deficiency does not cause accumulation of cations in general. Like the Mg\(^{2+}\) content, the Zn\(^{2+}\) content of YS35 and YS36 cells was increased after culture in SDC (~3- and ~1.4-fold, respectively), and the elevated Zn content was reduced in cells cultured in SDC supplemented with spermidine. In contrast, the Mn\(^{2+}\) content was not elevated in response to polyamine depletion. These results suggest that Mg\(^{2+}\) and Zn\(^{2+}\) are transported by the same transporter, Alr1, but Mn\(^{2+}\) is not. A moderate increase in phosphorus content was observed in spe1\(\Delta\) cells when polyamines were depleted and cellular Mg\(^{2+}\) was substantially increased. This effect is consistent with previous reports showing a close relationship between Mg\(^{2+}\) content and phosphate accumulation by yeast (15, 24). Overall, these data indicate that a major effect of polyamine depletion is elevation of the Mg\(^{2+}\) content, perhaps mediated by a change in transporter activity.

Generation of mutant strains with a combined deletion of a polyamine biosynthesis gene and a Mg\(^{2+}\) transporter gene

As yeast responded to polyamine deficiency by increasing Mg\(^{2+}\) accumulation, we suspected that this increase was essential to maintaining viability and growth. If so, inactivation of one of the required Mg\(^{2+}\) transporters might prevent Mg\(^{2+}\) accumulation and compromise the growth of polyamine-deficient cells. To examine this possibility, we constructed a set of double mutant strains combining the spe1\(\Delta\) or spe2\(\Delta\) mutation with a mutation in one of the three Mg\(^{2+}\) transporters (Alr1, Alr2, or Mrt2) (Scheme 1, A and B), known to be important in the regulation of cytosolic Mg\(^{2+}\) concentration (15). Double...
Efficient Mg$^{2+}$ uptake by Alr1 is essential for survival and growth of polyamine-deficient cells

To determine the effect of lack of a Mg$^{2+}$ transporter in polyamine deficiency, we first compared the growth of each of the spe1Δ and spe2Δ mutants derived from the WT DY1457 and the two mutants, CM200 (alr1Δ) and NP27 (alr2Δ), and NP180 (mn12Δ) in standard SDC (Fig. 3). SDC is polyamine-free and was chosen to maximally display the growth defect resulting from deletion of SPE1 or SPE2. In addition, the Mg$^{2+}$ concentration of SDC (4 mM) is lower than that required for optimum growth of alr1Δ strains (15, 16) while still allowing measurable growth. Thus, SDC should reveal any growth defects resulting from novel synthetic interactions. As previously observed for the Y534, Y535, and Y536 series of strains (Fig. 1), loss of SPE1 in each case caused a more pronounced growth defect than loss of SPE2 (Fig. 3, A and C). Each spe1Δ mutant lacked all polyamines, and each spe2Δ mutant contained only putrescine at a highly elevated level, confirming the knockout of SPE1 or SPE2, respectively (Fig. 3, D–F).

Of the three double mutant strains, AH3 (alr1Δ spe2Δ), AH4 (alr2Δ spe1Δ), and AH5 (alr2Δ spe2Δ), only AH3, lacking both Alr1 and Spe2, displayed an obvious synthetic phenotype; its growth was much reduced from that of alr1Δ (CM200) (Fig. 3B).

Table 2

| Medium       | Strain         | K     | Mg    | Mn    | P     | Zn    |
|--------------|----------------|-------|-------|-------|-------|-------|
|              | mg/g dry weight| mg/g dry weight| mg/g dry weight| mg/g dry weight| mg/g dry weight| mg/g dry weight|
| YPD          | Y534 (WT)      | 23.39 ± 2.37 | 3.16 ± 0.22 | 0.0042 ± 0.0006 | 26.81 ± 1.95 | 0.3878 ± 0.0526 |
| YPD          | Y535 (spe1Δ)   | 20.70 ± 0.74 | 3.23 ± 0.16 | 0.0043 ± 0.0004 | 26.26 ± 2.28 | 0.4194 ± 0.0677 |
| YPD          | Y536 (spe2Δ)   | 22.27 ± 0.96 | 3.25 ± 0.23 | 0.0042 ± 0.0004 | 27.00 ± 2.32 | 0.4114 ± 0.0664 |
| SDC          | Y534 (WT)      | 19.82 ± 1.87 | 3.00 ± 0.13 | 0.0062 ± 0.0012 | 22.14 ± 0.37 | 0.2014 ± 0.0222 |
| SDC          | Y535 (spe1Δ)   | 20.18 ± 3.13 | 7.53 ± 0.85 | 0.0029 ± 0.0016 | 27.66 ± 0.63 | 0.6058 ± 0.0571 |
| SDC          | Y536 (spe2Δ)   | 21.07 ± 2.11 | 4.84 ± 0.19 | 0.0034 ± 0.0012 | 26.48 ± 1.62 | 0.2757 ± 0.0394 |
| SDC + Spd (10^{-4} M) | Y534 (WT)      | 20.99 ± 1.07 | 3.00 ± 0.13 | 0.0066 ± 0.0005 | 23.38 ± 0.62 | 0.1816 ± 0.0436 |
| SDC + Spd (10^{-4} M) | Y535 (spe1Δ)   | 21.84 ± 2.98 | 6.34 ± 0.60 | 0.0060 ± 0.0012 | 26.10 ± 4.89 | 0.2741 ± 0.0102 |
| SDC + Spd (10^{-4} M) | Y536 (spe2Δ)   | 19.06 ± 0.32 | 4.07 ± 0.12 | 0.0067 ± 0.0008 | 23.88 ± 0.46 | 0.1403 ± 0.0345 |

Figure 2. Validation of parental strains and spe1Δ and spe2Δ mutant strains by PCR. The status of SPE1 and SPE2 genes was determined in six sets of isogenic strains marked by bars above the strain names. Knockout of the SPE1 or SPE2 gene was confirmed by the presence of a PCR product with a knockout primer set and the absence of a PCR product using an ORF primer set. From each of the four sets of PCR reactions (four panels), one main product (either a knockout PCR product or an ORF PCR product) was generated, consistent with each genotype. The positions of 1-kb ladder DNA standards flanking the PCR products are marked on the right side of each panel.

Figure 3. Comparison of the growth of parental strains and spe1Δ and spe2Δ mutant strains. Each strain was transformed with a pFL38 plasmid expressing SPE1 or SPE2 (giving the indicated constructs) and cultured in YPD (top) or SDC (bottom) with 10^{-20} OD_{600} units of Y534, Y535, and Y536 cells cultured in different media. The values (mg/g dry weight) are means ± S.D. (n ≥ 4). Spd, spermidine.
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### Table 3

List of PCR primers used for validation of spe1Δ and spe2Δ strains

| Primer set | Primer | Sequence | Product size |
|------------|--------|----------|--------------|
| **SPE1 ORF primer set** | Spe1F3 | GATATTTCTGAGTAAGGATCTCCGCG | 737 bp |
| | Spe1R3 | AGAAGACGGTGTGATTGTTGTTCA |  |
| **SPE2 ORF primer set** | Spe2F3 | GACAAGAAGCAACTGGAAC | 565 bp |
| | Spe2R3 | GCAAGTACCTCCTGATGTTGTTCA |  |
| **Primer set to check SPE1 deletion** | Spe1.5UTR.F3 | CTCTCAGGCTTCTGTTGAGT | 1,854 bp |
| | Spe1.5UTR.R4 | AGGACCGCAGTAGTGGATTCA |  |
| **Primer set to check SPE2 deletion** | Spe2.5UTR.F3 | GTAACAGGCTTCTGCTGCTGTTGA | 1,811 bp |
| | Spe2.3UTR.R3 | CTCTCAGGCTTCTGCTGCTGCTG |  |
| **Primer set for the spe1Δ::KanMX4 cassette** | Spe1.3UTR.R4 | CTTCTCAGGCTTCTGCTGCTGCTG | 2,510 bp |
| | Spe1.3UTR.R4 | AGGACCGCAGTAGTGGATTCA |  |
| **Primer set for the spe2Δ::KanMX4 cassette** | Spe2.5UTR.F3 | CAAAGAACGGTGTGATTGTTGTTCA | 2,429 bp |
| | Spe2.3UTR.R3 | AGGACCGCAGTAGTGGATTCA |  |

*SPE2* in each of these strains, putrescine was much higher in AH3 (Fig. 3E) than in other *spe2Δ* mutants. The inability of AH3 (alr1Δ spe2Δ) to enhance Mg2⁺ levels might have caused a compensatory overaccumulation of putrescine. Expression of *SPE2* in AH3 using pFL38/SPE2 restored polyamine content (Fig. 3E, tan bars) and cell growth (Fig. 3B, tan line) and complemented the Mg2⁺ accumulation defect, indicating that these phenotypes were a specific consequence of the *spe2Δ* mutation.

In contrast to Alr1, lack of Alr2 did not impair the growth of the *spe1Δ* or *spe2Δ* mutants (Fig. 3C). Similar growth between AH5 (alr2Δ spe2Δ) and AH2 (spe2Δ) (Fig. 3, A and C) was observed, and the growth of AH4 (alr2Δ spe1Δ) appeared to be even higher than that of AH1 (spe1Δ). These data indicates that, unlike Alr1, Alr2 is not required for growth of polyamine-deficient cells. The significant elevation of the Mg2⁺ content in the polyamine-deficient AH4 (alr2Δ spe1Δ) and AH5 (alr2Δ spe2Δ) (Fig. 3I) is in accordance with the minor contribution of Alr2 to Mg2⁺ homeostasis (20, 21).

We also examined the effects of deletion of SPE1 or SPE2 in an *mnr2Δ* background (Fig. 4). As Mnr2 is required for the release of vacuolar Mg2⁺ stores under Mg2⁺-deficient conditions (15), we suspected that this transporter might release Mg2⁺ from storage vacuoles to increase cytosolic Mg2⁺ concentration in response to polyamine deficiency and enhance growth of *spe1Δ* or *spe2Δ* mutants. However, no notable differences in the growth (Fig. 4, A–B) and the polyamine patterns (Fig. 4, C–D) were observed between the polyamine synthesis-deficient mutants derived from WT(NP174) and the *mnr2Δ* mutant (NP180). The growth and the polyamine patterns of AH8 (mnr2Δ spe1Δ) and AH9 (mnr2Δ spe2Δ) were similar to those of AH6 (spe1Δ) and AH7 (spe2Δ), respectively (Fig. 4, A and B), an indication that Mnr2 is not required for tolerance to polyamine deficiency. Interestingly, the *mnr2* mutation did substantially increase total cellular Mg2⁺ content over the levels observed in *spe1Δ* or *spe2Δ* single mutants (Fig. 4, E and F, compare AH8 with AH6 and AH9 with AH7). Although direct measurement of the vacuolar Mg2⁺ content has not been made, this increase is probably due to elevated uptake of Mg2⁺ by Alr1 in the *spe1Δ* or *spe2Δ* mutants and the increased sequestration of Mg2⁺ in the vacuoles of polyamine-depleted *mnr2Δ* cells (AH8 and AH9) (15). The observation that loss of Mnr2 function did not inhibit growth of polyamine-deficient strains argues that this store is not normally utilized to compensate for polyamine deficiency. Taken together, these results indicate that Alr1, but not Alr2 or Mnr2, is required for the elevated accumulation of Mg2⁺ in polyamine-deficient cells and that this response is essential for survival.

### Effects of Mg2⁺ supply on polyamine metabolism

Because the above data revealed an inverse relationship between polyamine and Mg2⁺ content in polyamine-depleted *S. cerevisiae* cells, we wondered whether Mg2⁺ deficiency would lead to an increased polyamine accumulation. To address this question, we compared growth and Mg2⁺ and polyamine content of DY1457 (WT) and CM200 (alr1Δ) strains cultured in YPD supplemented with different concentrations of Mg2⁺ (0–200 mM). Mg2⁺ supplementation was necessary for normal growth of the *alr1Δ* mutant, as the Mg2⁺ concentration of YPD is quite low (~500 μm) (26). The growth of DY1457 was not enhanced by Mg2⁺ supplementation and was slightly inhibited with increasing concentration of Mg2⁺ (>30 μm) (Fig. 5A). The exogenously supplied Mg2⁺ had little influence on cellular Mg2⁺ content of WT cells (Fig. 5B, brown bars). In contrast, the growth of the *alr1Δ* mutant was severely inhibited in YPD but increased with supplemented Mg2⁺ and was restored to the WT level at 200 mM Mg2⁺ (Fig. 5A, red line). The Mg2⁺ content of the *alr1Δ* mutant cultured in standard YPD was ~1.43 mg/g dry weight (46% of WT) (Fig. 5B). Mg2⁺ supplementation increased the Mg2⁺ content of CM200 (alr1Δ) to 2.1 mg/g dry weight at 200 mM Mg2⁺ (Fig. 5B, red bars) but did not restore it to WT levels. Inability of the *alr1Δ* to fully restore the Mg2⁺ content is probably due to inefficient Mg2⁺ uptake preventing the refill of all the intracellular stores, which represent up to 80% of the total Mg2⁺ content (15). However, our results indicate that the minimal Mg2⁺ requirement for normal growth was met in the *alr1Δ* cells at this level by supplementation with 200 mM Mg2⁺.
Strikingly, the total polyamine content of Mg\(^{2+}\)-deficient CM200 \((alr1\Delta)\) cells cultured in standard YPD was ~67% higher than that of WT DY1457 cells (Fig. 5C). This heightened level decreased as the Mg\(^{2+}\) concentration in the medium increased and returned to the WT level at 100 mM Mg\(^{2+}\), a level that almost completely suppressed the \(alr1\Delta\) growth defect.
Thus, yeast responded to Mg²⁺/H⁺ deficiency by increasing polyamine content. The elevation in polyamine content in the Mg²⁺/H⁺-deficient alr¹/H⁻ cells suggests an interrelationship between Mg²⁺/H⁺ and polyamine metabolism. A slight decrease in total polyamines was also detected in DY1457 with increasing Mg²⁺ supplementation. There was also a differential decline in the relative levels of spermine in both DY1457 and CM200 as the Mg²⁺ level in the medium increased, suggesting effects of Mg²⁺ on cellular polyamine metabolism.

Discussion

In this study, we present strong evidence that yeast cells accumulate excess Mg²⁺ to maintain viability upon depletion of cellular polyamines (Scheme 1A). This increase in the cellular Mg²⁺ content of polyamine-deficient spe1Δ and spe2Δ cells was reversed by the addition of spermidine in the medium (Fig. 1), suggesting a direct response to polyamine availability. Given that the cellular Mg²⁺ content is normally maintained within a narrow range in yeast (15), even in medium containing high Mg²⁺ (100–200 mM), the observed increases in cellular Mg²⁺ content (1.6–2.5-fold) in polyamine-deficient cells represent a marked deviation with likely functional significance.
The vital importance of elevated Mg\(^{2+}\) accumulation for polyamine-deficient yeast cells was indicated by the severe synthetic growth defect of an alr1Δ spe2Δ mutant, which was not able to elevate cellular Mg\(^{2+}\) content (Fig. 3) in response to polyamine deficiency. Our data further demonstrate that the polyamine depletion–induced Mg\(^{2+}\) accumulation is mainly dependent on the plasma membrane Mg\(^{2+}\) transporter Alr1 and not on its isof orm Alr2 or the vacular Mg\(^{2+}\) transporter Mnr2. This study presents firm *in vivo* evidence for the functional interaction between Mg\(^{2+}\) and polyamines in the regulation of *S. cerevisiae* growth.

The elevation of cellular Mg\(^{2+}\) in polyamine-deficient cells is not due to a general increase in cation uptake, as other cations such as K\(^+\) or Mn\(^{2+}\) did not increase in response to polyamine depletion. Although the Zn\(^{2+}\) content was also increased in response to polyamine depletion (Table 2), the increased Zn\(^{2+}\) content was far too low to compensate for the cation loss from polyamine depletion. The elevated Mg\(^{2+}\) would be sufficient to maintain the charge balance of polyamine-deficient cells. Furthermore, there are no reports suggesting that Zn\(^{2+}\) can substitute for polyamines in macromolecular synthesis. These findings corroborate the specificity of the Mg\(^{2+}\) function in supporting the growth of polyamine-depleted *S. cerevisiae* cells.

The specificity of Alr1 toward other diver tal cations is not well-understood. Direct measurement of competitive inhibition of Mg\(^{2+}\) transport by other metals has not been conducted in yeast as no radioactive Mg\(^{2+}\) isotope is commercially available. The increased sensitivity of an Alr1-overexpressing strain toward other metals (La\(^{3+}\), Co\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), and Zn\(^{2+}\)) suggested a possibility of transport of these other metals by Alr1 (20). However, our metal content analysis data (Table 2) imply that Mg\(^{2+}\) and Zn\(^{2+}\), but not Mn\(^{2+}\), are transported by Alr1. Further studies are needed using the Alr1-overexpressing strain as well as the alr1Δ strain to clarify the substrate specificity of Alr1.

In addition to the apparent regulatory effect of cellular polyamines on Mg\(^{2+}\), an inverse relationship between polyamines and Mg\(^{2+}\) was also observed in the Mg\(^{2+}\)-transport–deficient alr1Δ cells cultured in YPD (Fig. 5). In these cells, the total polyamine content increased in response to Mg\(^{2+}\) deficiency. These findings suggest that the polyamine pathway enzymes and/or polyamine transporters are regulated by cellular or exogenous Mg\(^{2+}\) concentration. Future investigations to identify and elucidate the specific polyamine pathways regulated by the Mg\(^{2+}\) level are warranted.

Despite the vital importance of Mg\(^{2+}\) in cellular physiology, the mechanism of Mg\(^{2+}\) homeostasis in eukaryotic cells, not to mention its functional interactions with polyamine homeostasis, is poorly understood. Although the yeast CorA family Mg\(^{2+}\) transporters have diverged considerably from the bacterial transporter CorA, they share common structural features, including two transmembrane domains connected by a short loop near the C terminus and a signature motif, GMN, that is important for Mg\(^{2+}\) selectivity (Scheme 1B). A cryo-EM structure of the *Thermotoga maritima* CorA suggested a mode of its regulation by intracellular Mg\(^{2+}\) concentration (27); at high Mg\(^{2+}\) concentration, Mg\(^{2+}\) is bound to cytoplasmic N-terminal domains of the CorA homopentamer, and this binding induces a closed conformation, whereas loss of Mg\(^{2+}\) binding in a low-Mg\(^{2+}\) environment reverses it to an open channel. The acidic residues involved in binding cytosolic Mg\(^{2+}\), located at the subunit interfaces of the pentamer, were identified (28).

Chemical cross-linking and the split-ubiquitin assay data (22) suggest that Alr1 and Alr2 also form homo- or heterooligomers and that, like CorA, they function as pentamers. Residues that participate in Mg\(^{2+}\) binding at the regulatory sites are conserved in prokaryotic CorA proteins (28) and in Alr1 (29), suggesting conservation of this mechanism. The CorA and Alr1/Alr2 proteins are functionally interchangeable, as overexpression of CorA partially restored the growth of an alr1Δ strain (16).

Our observation that polyamine deficiency increases Mg\(^{2+}\) accumulation via Alr1 suggests that this protein may be regulated directly or indirectly by cytosolic polyamine concentration. At least three possible models might explain this effect. First, polyamines might bind directly to Alr1 and inhibit its activity in a manner similar to cytosolic Mg\(^{2+}\) (27). Polyamines might interact directly with the Mg\(^{2+}\)-binding sites to mimic the effect of Mg\(^{2+}\), or they may bind to other sites within the Alr1 cytoplasmic domains. Second, polyamines might bind directly to the Mg\(^{2+}\) channel pore itself to block it, as has been observed for some potassium channels (28). A third mechanism for increased Mg\(^{2+}\) accumulation might be the induction of expression of Alr1 mRNA and/or protein by a polyamine-dependent regulatory mechanism. Comparison of Alr1 mRNA levels in polyamine-replete and polyamine-deficient *S. cerevisiae* cells by quantitative RT-PCR did not reveal up-regulation of Alr1 expression upon depletion of polyamines (data not shown). This mechanism is also less likely than the others, considering that Alr1 transport activity was enhanced under Mg\(^{2+}\) deficient conditions, but the induction of the Alr1 protein or its mRNA was not observed (29).

A functional interaction between polyamines and Mg\(^{2+}\) has been suggested by several previous studies. In a reticulocyte lysate freed of polyamines by gel filtration, polyamines enhanced translation at suboptimal concentrations of Mg\(^{2+}\) and beyond the level achieved by high Mg\(^{2+}\) alone, suggesting that polyamines can at least partly substitute for Mg\(^{2+}\) (4, 14). Evidence for their functional interaction *in vivo* was also reported in *S. cerevisiae* (30) and mammalian cells (31) in which polyamine overloading caused cellular toxicities by displacement of cellular Mg\(^{2+}\). The growth inhibition was attributed to excessive accumulation of polyamines leading to a concomitant decrease in cellular Mg\(^{2+}\). It was suggested that replacement of ribosome-bound Mg\(^{2+}\) by accumulated polyamines inactivated ribosomes, leading to inhibition of protein synthesis and cell growth, but no underlying molecular mechanism was identified. Our study reveals an essential functional requirement for Mg\(^{2+}\) content elevation in polyamine-deficient cells, a biological situation opposite to that of cellular toxicity caused by excess polyamines (30). Although the two studies complement each other in supporting the concept of functional interplay between polyamines and Mg\(^{2+}\), our study uniquely reveals a mechanism of up-regulation of cellular Mg\(^{2+}\) content involving Alr1 upon depletion of polyamines and offers an explanation for the extremely low polyamine requirement for *S. cerevisiae*. 
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growth. In addition, this study provides new evidence that polyamine pathways are regulated by Mg$^{2+}$ supply (Fig. 5).

The observations that spe1Δ and spe2Δ strains can survive and grow with very low amounts of cellular polyamines and that cellular Mg$^{2+}$ accumulation is up-regulated in polyamine-deficient cells support the notion that much of the polycationic polyamine requirement of yeast can be fulfilled by Mg$^{2+}$. In SDC free of polyamines, the growth of spe1Δ and spe2Δ strains was arrested upon prolonged incubation in SDC despite increased Mg$^{2+}$ content (Figs. 1, 3, and 4). Obviously, Mg$^{2+}$ cannot totally substitute for polyamines for polyamine-specific functions, including the fine-tuning of translation (5) and the elf5A hypusine modification. Thus, growth of spe1Δ and spe2Δ strains in SDC arrests when cellular polyamines and hypusinated elf5A fall below the minimum threshold levels. Accumulation of reactive oxygen species and various morphological changes, including large cell size, large vacuoles, and apoptotic cell death, were reported in a S. cerevisiae spe2Δ strain under conditions of an extreme depletion of polyamines (32, 33). However, we found no little changes in cell sizes and viability of the spe1Δ and spe2Δ mutants after 24-h culture in SDC, when cells were harvested for the analysis of Mg$^{2+}$ and polyamines. This suggests that elevation of Mg$^{2+}$ in our mutants was not associated with the loss of viability and other cellular changes that accompany extreme polyamine depletion. Instead, the elevated Mg$^{2+}$ sustains the growth and viability of the spe1Δ and spe2Δ mutants in medium containing very low spermidine (10$^{-8}$ M).

The fact that mammalian cells, unlike yeast, require a high level of polyamines for growth suggests either that there are mammalian-specific processes intrinsically dependent on a high polyamine concentration or that mammalian cells lack a mechanism for Mg$^{2+}$ accumulation in response to polyamine depletion. Mg$^{2+}$ homeostasis is poorly understood in mammalian cells, although a number of putative Mg$^{2+}$ transporters have been described (34). In addition to the human mitochondrial CorA protein Mrs2, these include novel families unrelated to CorA, such as SLC41, TRPM6/7, MagT1, NIPA, MMgT, and HIP14 families. From a screening of a Jurkat cell library, MagT1 and its homolog TUSC3 were identified as cell-surface Mg$^{2+}$ transporters that complement the ara1Δ mutation (35). As their structures are unrelated to the CorA superfamily, they are not likely to respond to polyamine depletion in a similar manner as does Alr1. Although intricate mechanisms of polyamine homeostasis have been established in mammalian cells, there is poor understanding as to how polyamine pathways interact with other cellular pathways such as metal metabolism and homeostasis. Parenthetically, cellular iron levels were reported to regulate many polyamine pathway proteins in several mammalian cells (36). Future investigations will be directed toward further exploration of the interplay between Mg$^{2+}$ and polyamine homeostasis in S. cerevisiae and in mammalian cells.

Experimental procedures

Yeast strains and cell growth assay

The S. cerevisiae strain WT Y534 (BY4741) and the two knockout strains Y535 (spe1Δ) and Y536 (spe2Δ) generated by a S. cerevisiae gene deletion project (25) were kindly provided by Dr. Herbert Tabor (NIDDK, National Institutes of Health). Other strains with a deletion of Mg$^{2+}$ transport–related genes (alr1Δ, alr2Δ, and mnr2Δ) and new strains generated in this study are listed in Table 1. Cells were routinely cultured in YPD or SDC with or without Mg$^{2+}$, G418, or spermidine as indicated in specific experiments. YPD is rich in polyamines (putrescine, spermidine, and spermine, ~0.3, 2.2, and 0.4 mM, respectively) and low in Mg$^{2+}$ (26). Standard SDC contains 4 mM Mg$^{2+}$ but no polyamines. For the selection of Ura$^+$ transformants, uracil-dropout medium (SD–Ura) was used. Because the polyamine requirement for optimal growth of spe1Δ and spe2Δ strains is extremely low, ultrapure water was used, and special care was taken to avoid any environmental contamination of polyamines in SDC. To compare the growth of different strains in SDC, each strain was patched on YPD plates, and freshly grown patches of cells were inoculated in SDC at 0.001 or 0.0003 OD$_{600}$ and cultured at 30 °C with shaking. Cell density was measured using a spectrophotometer at 600 nm every 2 h during the day. To follow growth over a 72-h period, cells were repeatedly diluted in the same fresh medium to OD$_{600}$ 0.001 or 0.003 when the density reached ~1 OD$_{600}$.

Generation of spe1Δ and spe2Δ strains

The SPE1 and SPE2 knockout cassettes were prepared using genomic DNA of Y535 (spe1Δ) and Y536 (spe2Δ), respectively, as templates. The primers used for amplifying the spe1Δ::KanMX4 cassette were CATTTCCTCTTCTTGCTGTTGCT and TGGGCGTGTGTAAGTTGTT. The primer sets for amplifying the spe2Δ::KanMX4 cassette were CCAGATATGTAAGCTTCTCATC and GGCCACAACCTTTTGAGCATC. PCR was performed using the Easy-A 2X Master Mix (Agilent) with the following program: 94 °C for 5 min, denaturation at 94 °C for 0.5 min, annealing at 58 °C for 1 min, an extension reaction at 72 °C for 2.5 min for 30 cycles, and a final extension reaction at 72 °C for 5 min. Yeast transformation with the purified PCR products (~2.51 and ~2.429 kb, respectively for spe1Δ::KanMX4 and spe2Δ::KanMX4) was carried out with the Yeast Transformation kit (Sigma-Aldrich), according to the manufacturer’s instructions, using 1 μg of linear DNA. The transformed clones were isolated on YPD plates containing 500 μg/ml G418.

Isolation of genomic DNA and PCR confirmation of genotypes of parental and spe1Δ and spe2Δ mutant strains

Cells were cultured in YPD to 1 OD at 600 nm and harvested. Genomic DNA was isolated from the cell pellets following a published protocol (37). Briefly, the cell pellets (10 OD$_{600}$ units) were resuspended in 0.2 ml of 200 mM LiOAc, 1% SDS solution and heated at 70 °C for 5 min. 600 μl of 100% EtOH was added, and DNA and cell debris were spun down at 15,000 × g for 3 min. The pellets were washed with 70% EtOH. After removal of all EtOH, DNA was extracted by resuspension in 0.1 ml of Tris-EDTA buffer, and the DNA concentration was measured using a NanoDrop ND-100 spectrophotometer. PCR was performed as described above using 0.1 μg of genomic DNA, a knockout primer set or an open reading primer set (Table 3), and Jump-
Start REDTaq ReadyMix Reaction Mix (Sigma-Aldrich) according to the manufacturer’s instructions.

**Construction of pFL38/SPE1 and pFL38/SPE2 plasmids**

To check the reversal of the phenotypes of the spe1Δ and spe2Δ strains, pFL38 plasmids encoding SPE1 and SPE2 were reintroduced into the corresponding null strains. The recombinant plasmids were constructed by GenScript USA Inc. by synthesis of SPE1 and SPE2 genes (each ORF with 200 bp 5’-UTR and 3’-UTR) and subcloning into pFL38, and the transformants were selected on SD-Ura plates.

**Analysis of cellular Mg²⁺ content by inductively coupled plasma optical emission spectrometry (ICP-OES)**

WT and most mutant cells were inoculated at a density of 0.001 in 10–20 ml of the indicated medium, and exponentially growing cells (1 or less than 1 OD₆₀₀) were harvested at ~24 h after inoculation. For slow-growing mutant strains, a higher inoculum density and a larger culture volume were used to obtain ~10–20 OD₆₀₀ units of cells in 24 h. Cells were harvested by centrifugation, transferred to preweighed Eppendorf tubes, and washed once with 1 mM EDTA and twice with ultrapure water, and the washed cell pellets were frozen on dry ice. The total Mg²⁺ content was measured by ICP-OES as described previously (38).

**Determination of yeast polyamine content**

*S. cerevisiae* cells were cultured, harvested, and frozen, in parallel or similarly to those for Mg²⁺ analysis, as described above. Polyamines were extracted from the cell pellets by resuspension in 1.2 M perchloric acid, repeated vortexing with glass beads, and incubation on ice. The extracted amines and the internal standard (1,7-diaminoheptane) were derivatized using dansyl chloride, and the dansylated polyamine derivatives were analyzed in duplicate by reverse-phase HPLC as described previously (39). The polyamine content was normalized against cell proteins determined using the Pierce BCA protein assay dye reagent after dissolving the perchloric acid precipitates in 0.1 N NaOH.

**Statistical analysis**

All values are presented as means of four or more biological replicates (n) as indicated in the legends. The contents of Mg²⁺ and polyamines are expressed as mean ± S.D., and the optical densities of cells are expressed as mean ± S.E. Differences between samples were compared using analysis of variance (ANOVA) and were considered statistically significant at p < 0.05. All statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software, San Diego, CA).

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