Magnesium uptake by connecting fluid-phase endocytosis to an intracellular inorganic cation filter

Sandra H. Klompmaker¹, Kid Kohl¹, Nicolas Fasel¹ & Andreas Mayer¹

Cells acquire free metals through plasma membrane transporters. But, in natural settings, sequestering agents often render metals inaccessible to transporters, limiting metal bioavailability. Here we identify a pathway for metal acquisition, allowing cells to cope with this situation. Under limited bioavailability of Mg²⁺, yeast cells upregulate fluid-phase endocytosis and transfer solutes from the environment into their vacuole, an acidocalcisome-like compartment loaded with highly concentrated polyphosphate. We propose that this anionic inorganic polymer, which is an avid chelator of Mg²⁺, serves as an immobilized cation filter that accumulates Mg²⁺ inside these organelles. It thus allows the vacuolar exporter Mnr2 to efficiently transfer Mg²⁺ into the cytosol. *Leishmania* parasites also employ acidocalcisomal polyphosphate to multiply in their Mg²⁺-limited habitat, the phagolysosomes of inflammatory macrophages. This suggests that the pathway for metal uptake via endocytosis, acidocalcisomal polyphosphates and export into the cytosol, which we term EAPEC, is conserved.

¹Department of Biochemistry, University of Lausanne, Ch. des Boveresses 155, CH-1066 Epalinges, Switzerland. Correspondence and requests for materials should be addressed to A.M. (email: andreas.mayer@unil.ch)
Bioavailability of nutrient metals is commonly a limiting factor for microorganisms in their natural environment. This is especially the case for unicellular organisms with limited spatial motility such as yeast. Yeast species are found on plants and in soil, habitats known to be rich in chelating agents like organic acids and tannins, which can limit the fraction of metal ions that are available for uptake across membranes. Since plasma membrane metal transporters need free ions for uptake, mitigating strategies for metal acquisition in environments with low metal bioavailability would prove beneficial.

An effective strategy involves uptake of a metal-chelate across the plasma membrane and subsequent release of the metal in the cell. This mechanism has been described for iron and, more recently, zinc. Saccharomyces cerevisiae cells can acquire iron through ARN family plasma membrane transporters for siderophores that have been produced by other fungi or bacteria. This mechanism has been described for iron and, more recently, zinc. Pra1 was identified in the fungal pathogen Candida albicans as a "zincophore", a secreted protein sequestering zinc from the host cell in order to overcome nutritional immunity during invasive growth. Pra1-mediated uptake of ferrichrome leads to its accumulation in the cytosol, where it showed slow iron release kinetics. Sit1-dependent internalization of ferrioxamine B leads to cytosolic accumulation, where it showed slow iron release kinetics. Here we investigate how Saccharomyces cerevisiae copes with limited bioavailability of metal ions, mimicking this situation by growing the cells in medium with EDTA. We find that upregulation of fluid-phase endocytosis under these conditions leads to increased transfer of extracellular solutes to the vacuole, where metal ions are accumulated and then exported into the cytosol.

### Results

#### PolyP-deficient cells are sensitive to low metal availability

Cells lacking polyP and the vacuolar polyP polymerase Vtc4 show normal growth in standard rich media such as YPD (Fig. 1a). When the bioavailability of divalent metal ions was reduced by addition of the chelator EDTA, wild-type cells only reduced their growth rate (Fig. 1b), whereas cells lacking VTC subunits (vtc1Δ, vtc4Δ) arrested growth. Cells expressing Vtc4R264A, an active-site mutant that allows the assembly of an intact yet inactive VTC complex, displayed the same growth phenotype. vtc3Δ cells were hypersensitive to high concentrations of Mn2+ and Co2+.

Here we investigate how Saccharomyces cerevisiae copes with limited bioavailability of metal ions, mimicking this situation by growing the cells in medium with EDTA. We find that upregulation of fluid-phase endocytosis under these conditions leads to increased transfer of extracellular solutes to the vacuole, where metal ions are accumulated and then exported into the cytosol.

### Methods

#### Growth and survival of wild-type and vtc4Δ cells

Cells were grown in liquid YPD or YPD supplemented with 1 mM EDTA and OD600 nm was determined at the indicated times. Capacity to form colonies was measured by plating cells grown on YPD/EDTA for the indicated periods of time. Colony-forming units (CFU) were determined, and equal numbers of cells were withdrawn and plated on solid YPD plates. Colony-forming units (CFU) were counted after 2 days of incubation at 30 °C. Live/dead cell staining was performed using the LIVE/DEAD Yeast Viability Kit (L3224, Molecular Probes, Invitrogen) following the manufacturer’s instructions. Images were acquired using a Zeiss Axio Scope.2 microscope equipped with an Axio Cam MRC5 charge-coupled device-camera (Zeiss). Differences were evaluated using Student’s t test comparing vtc4Δ and wild-type values at the respective time points; ns, non-significant; * p ≤ 0.05. The results represent the mean ± SD of 3 (a, b, d) or 5 (c) independent experiments.
Fig. 2 Amounts and chain length of polyP on YPD/EDTA. 

(a) PolyP content in wild-type cells on YPD/EDTA. Cells were grown on YPD/EDTA for the indicated periods of time, collected, and polyP content was assayed. Results represent mean ± SD of three independent experiments. Statistical differences were determined using Student’s t test comparing polyP levels from T = 0 h with the later time points and the vtc4Δ strain; *p ≤ 0.05; ***p ≤ 0.001. 

(b) Chain length. PolyP was extracted from cells growing on YPD/EDTA for the indicated periods of time. PolyP was purified from cells by glass bead rupture and phenol/chloroform extraction. Samples were treated with RNAse A and proteinase K before 30 nmol of polyP were resolved on a 15% polyacrylamide gel, followed by negative staining with DAPI. 

(c) Mother (m) and daughter (d) fractions were separated after 16 h of growth on YPD/EDTA. PolyP was extracted from cells as in (b), resolved on a 20% polyacrylamide gel and stained with DAPI. PolyP isolated from a non-separated population of wild-type cells growing logarithmically on YPD is shown for comparison. 

(d) The signal intensity in the lanes with mother and daughter samples from (c) was quantified using ImageJ, starting at the top of the gel. The profile for polyP300 was included for comparison.
grew like wild-type because Vtc3 can be substituted for by its homolog Vtc2. The vast majority of polyP in S. cerevisiae is stored in the vacuole. However, polyP is a cell wall component in a large variety of fungi, and in S. cerevisiae evidence for and against a cell wall fraction was presented. Since a portion of the VTC complex localizes to the cell periphery, where it could potentially synthesize polyP for transfer into the periplasm, we analyzed the localization of GFP-tagged Vtc4 under metal limitation (Supplementary Fig. 1). Vacular localization was confirmed by co-localization with the marker dye FM4-64. On metal-replete YPD, the majority of GFP-Vtc4 localized to the vacuole but a significant portion was found at the cell periphery. On YPD/EDTA, GFP-Vtc4 disappeared from the cell periphery and localized exclusively to the vacuole and vacuole-associated vesicles. This observation argues against a role of the peripheral pool of VTC in metal acquisition and suggests that vacuolar rather than cell wall-associated polyP supports growth under metal limitation.

Cells experiencing starvation conditions may either die or arrest the cell cycle. We distinguished between these possibilities by subjecting cells to different periods of metal starvation on YPD/EDTA, followed by plating on metal-replete YPD medium to measure the number of colony-forming units (CFU) (Fig. 1c). During the first 4 h of adaptation to metal limitation, the number of colony-forming units of wild-type cells dropped to 75% of the initial value, that of vtc4Δ cells was reduced to 25%. We tested for the presence of dead cells by propidium iodide (PI) staining and flow cytometry (Fig. 1d). Propidium iodide is excluded from living cells but brightly stains dead cells, which lose metabolic activity that is required to maintain the selective permeability barrier of the plasma membrane. 3% of wild-type cells were PI-positive after 8 h in YPD/EDTA. Only 12% of vtc4Δ cells stained with PI at this point, even though the number of CFUs for this strain had declined by >95% over the same period. Prolonged exposure to EDTA for 24 h killed 25% of wild-type cells, but the survivors retained their capacity to form colonies, which was not the case for vtc4Δ cells. Thus, a large fraction of polyP-deficient vtc4Δ cells remain alive and metabolically active in YPD/EDTA, but they rapidly arrest growth. This suggests that polyP is necessary to preserve the capacity of cells to divide on YPD/EDTA.

**PolyP changes under limited metal bioavailability.** On medium with limiting amounts of zinc, yeast cells grow linearly instead of exponentially. This is due to the fact that mothers born on rich media continue dividing, whereas newly born daughters arrest growth in the G1 phase of the cell cycle. We observed the same behavior for wild-type yeast cells grown on YPD/EDTA (Supplementary Fig. 2). Given that polyP is crucial for growth on YPD/EDTA, we analyzed changes of polyP under these conditions. Time-course analysis showed that wild-type cells maintained their polyP levels on YPD/EDTA (Fig. 2a). PolyP chain length distribution was examined by gel electrophoresis and negative staining of the gel with DAPI. YPD-grown wild-type cells (T = 0 h) contained mostly polyP of 60–100 residues (Fig. 2b). On YPD/EDTA, long-chain polyP of >300 residues increased strongly. After 24 h of growth, the majority of the population consisted of non-dividing daughter cells. These are readily identifiable due to the fact that they carry only a single bud scar, a cell wall remnant from a preceding cell division that can be stained with Calcofluor white. In order to test whether polyP differed in dividing mothers and arrested daughters, we separated these two fractions. The inoculum (mothers) was cross-linked to biotin-fluorescein, washed and cultivated for 16 h on YPD/EDTA. Mothers and daughters were separated by purification with streptavidin-coated, magnetic Dynabeads, which adsorb preferentially biotin-labeled mothers. Fraction purity was assessed microscopically by counting fluorescein-positive and -negative cells. The mother fraction contained 35–45% of daughters, largely due to daughters that had not yet completed cytokinesis and separated from the mothers. Daughter fractions contained <10% mother cells. PolyP from daughter cells contained a higher amount of long-chain polyP, whereas short and intermediate polyP chains dominated in the mother fraction, especially if one takes into consideration that a large part of the long-chain polyP visible in the mother fraction must originate from its inevitable 35% contamination with daughter cells (Fig. 2c). If vacuolar polyP degradation is impaired, vacuoles accumulate extremely long polyP chains of >300 residues. Together with the fact that also the purified catalytic domain of the complex synthesizes very long-chain polyP, this suggests that VTC is a highly processive enzyme. The presence of shorter chains in YPD/EDTA-grown mothers can then be taken as an indication of turnover of polyP by polyphosphatases. Since only the mothers divide on YPD/EDTA, whereas daughters do not, growth on YPD/EDTA coincides with and might require polyP turnover.

**Mg** limits growth of polyP-deficient cells on YPD/EDTA. Since EDTA chelates a range of divalent metal ions, we asked which metal restricts growth of polyP-deficient strains. Cells were pre-cultured on YPD and then cultivated in YPD/EDTA for different periods of time before metal levels were determined by inductively-coupled plasma (ICP)-MS analysis. The metal content per cell decreased over time for Fe, Zn, and Mn, with no major differences between wild-type and vtc4Δ cells (Fig. 3a). This suggests that cells grew by using, at least partially, stores of these metals that are independent of polyP. Wild-type cells stabilized the levels of Mg, Cu, and Ca at 35–50% of the initial value (Fig. 3a). vtc4Δ cells showed similar stabilization for Cu and Ca, but their content of Mg kept decreasing to <10% over 24 h (Fig. 3a). Even on YPD without EDTA (T = 0 min), vtc4Δ cells had 40% less Mg than wild-type cells. This suggests that wild-type cells extract enough Mg, Ca, and Cu from YPD/EDTA to allow them to stabilize their content of these three ions. However, the stabilization of Mg levels depends on polyP. This picture was corroborated by integrating the metal content over all the cells in the culture, which allows to assay the net transfer of metals from the medium into the sedimentable, living material. Whereas cell-associated Mg and Ca increased in the pellets of wild-type cultures more than 20-fold over 24 h on YPD/EDTA (Fig. 3b), other metals showed no (Zn) or only moderate increases of <5-fold (Mn, Cu, Fe). Ca**2+** levels reproducibly spiked after 4 h (Fig. 3b), which might be due to activation of the calcineurin/Crz1 pathway, which strongly augments Ca**2+** uptake. Apart from a moderate increase in Cu, vtc4Δ cultures did not accumulate any of the tested metals, which reflects their lack of growth on EDTA.

On the basis of these results, we hypothesized that EDTA might limit growth of cells by reducing the bioavailability of Mg**2+**. In order to test this, we depleted cellular metal pools by incubating the cells in YPD/EDTA for 5 h (Fig. 3c). Subsequently, cells were diluted into YPD that had been depleted of metals by chromatography over a Chelex ion-exchange matrix, which reduced the Mg content of this medium to 10 μM (determined by ICP-MS). This medium was then supplemented with a single metal at a time. Only addition of Mg**2+** allowed cells to proliferate and the response was similar for wild-type and vtc4Δ cells. This suggests that cells grown on YPD/EDTA do not maintain a sufficiently large stock of Mg**2+** to support further cell divisions.
under Mg\textsuperscript{2+} limitation. Therefore, their growth on YPD/EDTA depends on continuous extraction of Mg\textsuperscript{2+} from this medium.

**A vacuolar Mg\textsuperscript{2+} exporter is required for Mg\textsuperscript{2+} uptake.** Several intracellular pools of Mg\textsuperscript{2+} exist, with transporters and channels controlling the fluxes between them. Alr1 mediates the majority of Mg\textsuperscript{2+} uptake at the plasma membrane\textsuperscript{43}. Cells lacking Alr1 depend on supplementation of their media with high concentrations of Mg\textsuperscript{2+} for wild-type-like growth. Overexpression of its homolog Alr2 can rescue this phenotype\textsuperscript{43,44}. The MRS2/MFM1 complex controls Mg\textsuperscript{2+} import into mitochondria\textsuperscript{45,46} and MME1 exports Mg\textsuperscript{2+} from this organelle\textsuperscript{46}. A vacuolar Mg\textsuperscript{2+} importer has not been identified, but MNR2 mediates efflux of Mg\textsuperscript{2+} from vacuoles\textsuperscript{48}.

In order to test whether a particular intracellular Mg\textsuperscript{2+} pool is essential for cells to grow on metal-limiting medium, we assayed knockout strains for these Mg\textsuperscript{2+} transporters for growth on YPD/EDTA (Fig. 4a, b). Cells lacking the plasma membrane transporter Alr1 grew only slowly on YPD and did not grow on YPD/EDTA (Fig. 4b). Absence of other known vacuolar Mg\textsuperscript{2+} transporters such as \textit{mrs2} (Zn\textsuperscript{2+}), \textit{mfm1} (Ca\textsuperscript{2+}) and \textit{mme1} (Fe\textsuperscript{3+}) grew like wild-type on both media. Interestingly, \textit{mnr2}Δ cells mimicked the phenotype of a \textit{vtc4Δ} strain. They rapidly ceased growth on YPD/EDTA but grew like wild-type on YPD/Chelex medium. In order to test whether a particular intracellular Mg\textsuperscript{2+} pool is essential for cells to grow on metal-limiting medium, we assayed knockout strains for these Mg\textsuperscript{2+} transporters for growth on YPD/EDTA (Fig. 4a, b). Cells lacking the plasma membrane transporter Alr1 grew only slowly on YPD and did not grow on YPD/EDTA (Fig. 4b). Absence of other known vacuolar Mg\textsuperscript{2+} transporters such as \textit{mrs2} (Zn\textsuperscript{2+}), \textit{mfm1} (Ca\textsuperscript{2+}) and \textit{mme1} (Fe\textsuperscript{3+}) grew like wild-type on both media. Interestingly, \textit{mnr2}Δ cells mimicked the phenotype of a \textit{vtc4Δ} strain. They rapidly ceased growth on YPD/EDTA but grew like wild-type on YPD/Chelex medium.

**Fig. 3** Metal extraction from YPD/EDTA. Time-course ICP-MS analysis. Cells were pre-cultured on YPD and transferred to YPD/EDTA. At the indicated time points, aliquots were withdrawn, cell density was determined, the cells were sedimented and their content of magnesium, iron, zinc, copper, manganese and cadmium was measured. **a** Values per OD\textsubscript{600 nm} unit of cells and **b** the values per culture volume, which allows to judge whether there is net transfer of metal from the medium into the accumulating biomass. **c** Growth on YPD/Chelex medium. Wild-type and \textit{vtc4Δ} cells were pre-cultured for 5 h on YPD/EDTA and transferred to YPD/Chelex medium supplemented with the indicated metal to final concentrations that are sufficient to support vigorous continuous growth of non-depleted wild-type cells: 0.25 μM CuSO\textsubscript{4}, 1.2 μM FeCl\textsubscript{3}, 2.6 μM MnSO\textsubscript{4}, 2.5 μM ZnSO\textsubscript{4}, 0.9 mM CaCl\textsubscript{2}, 4.2 mM MgSO\textsubscript{4}. The OD\textsubscript{600 nm} was measured at the indicated time points after transfer. **a-c** Results represent mean ± SD of at least three independent experiments. Statistical differences in **a** were determined using Student’s t test comparing \textit{vtc4Δ} and wild-type cells at each time point; *p ≤ 0.05; **p ≤ 0.005.
similarly as vtc4Δ cells, mnr2Δ cells did not efficiently accumulate Mg from the growth medium (Fig. 4d, right panel). However, mnr2Δ cells did not show the decrease in Mg content that was observed in vtc4Δ cells, showing that mnr2Δ cells could not mobilize their vacuolar Mg when needed (Fig. 4d, left panel).

These results suggest that both uptake of Mg2+ through the plasma membrane and mobilization of Mg2+ from vacuoles contribute to growth on YPD/EDTA. This is in line with the observation that vtc4Δ cells grow even slightly better than wild-type on Chelex-treated medium, which is low in Mg2+, but in which this Mg2+ is fully bioavailable and thus accessible to the plasma membrane transporters Alr1 and Alr2 (Fig. 4e). Under these conditions, plasma membrane uptake can apparently satisfy the Mg2+ requirements for growth. By contrast, growth under conditions of limited bioavailability requires vacuolar polyP and vacuolar Mg2+ export.

**Fig. 4** Role of metal transporters for growth on YPD/EDTA. a Magnesium transporter mutants were pre-cultured on YPD, transferred to YPD or YPD/EDTA, and the OD600 nm was measured at the indicated times after transfer. b Growth of Δalr1 cells was assayed as in (a). c Growth of vacuolar metal exporter mutants was assayed as in (a). d Time course ICP-MS analysis of the magnesium content of mnr2Δ cells at the indicated times after transfer from YPD to YPD/EDTA. Values per cell and per culture volume are shown. Note that the values for wild-type and vtc4Δ are the same as in Fig. 3b because mnr2Δ cells had been tested as part of the same experiments. e Growth of wild-type and vtc4Δ cells on Chelex-treated YPD. Cells were pre-cultivated in YPD, washed and transferred to YPD/Chelex. The OD600 nm was followed for one day (D1). The cells were then re-inoculated in fresh YPD/Chelex and growth was followed for another day (D2). The results represent mean ± SD of at least three independent experiments. Differences in d were evaluated using Student’s t test comparing vtc4Δ and mnr2Δ cells with wild-type cells at each time point; *p ≤ 0.05; **p ≤ 0.01. Mg2+ uptake requires fluid-phase endocytosis and retrograde traffic. Given that polyP, which is enclosed in vacuoles, facilitated Mg2+ uptake into the cytosol, we explored how Mg2+ could be brought in contact with polyP and tested fluid-phase endocytosis as a possibility. We quantified this process using the fluorescent fluid-phase endocytosis marker Lucifer yellow49. Cells were grown on YPD and YPD/EDTA for 2 and 6.5 h before adding Lucifer yellow and incubating them for an additional hour. The cells were sedimented and washed. Dye uptake was analyzed qualitatively by fluorescence microscopy (Fig. 5a) and quantified
by flow cytometry (Fig. 5b). Both wild-type and vtct4Δ cells showed significantly higher Lucifer yellow fluorescence after 3 h of growth in YPD/EDTA when compared to YPD. This difference increased further after 7.5 h on YPD/EDTA, suggesting that the cells had upregulated their capacity for fluid-phase endocytosis as a consequence of limited metal availability.

We tested the growth effects of mutations perturbing fluid-phase endocytosis (sac6Δ, vrp1Δ, end3Δ, arc18Δ, sla1Δ), vacuolar protein sorting at the endosome (vps4Δ), and autophagy (atg8Δ, Fig. 5c). Wild-type and atg8Δ cells showed robust growth during 24 h of incubation on YPD/EDTA. In contrast, cells lacking components of the endocytic machinery mimicked the growth defect of polyP-deficient cells. vps4Δ cells displayed an intermediate growth phenotype. ICP-MS analysis of the endocytosis mutants sac6Δ and vrp1Δ confirmed their defects in Mg2+ acquisition (Fig. 5d). Continuous uptake of Mg2+ should
PolyP is needed in Mg\textsuperscript{2+}-poor environments across species. We sought to test whether polyP in acidocalcisome-like organelles might act similarly in a more physiological setting and whether its requirement for Mg\textsuperscript{2+} acquisition might be conserved in evolution. To this end, we utilized macrophages, which phagocytose pathogens and finally degrade them. Some pathogens, however, evade or resist digestion and even multiply within the phagolysosomes, subverting the host cell machinery to proliferate. Access to nutrients and specifically to Mg\textsuperscript{2+} is limited within the phagosome.\textsuperscript{53–56} We utilized the trypanosomatid parasite *Leishmania major* as a model. Trypanosomatids contain acidocalcisomes that share polyP and many transporters with yeast vacuoles.\textsuperscript{57} After entering the mammalian host, *Leishmania* is taken up by macrophages, where it resides and proliferates in the phagolysosome.\textsuperscript{58} Growth in these phagolysosomes is limited by Mg\textsuperscript{2+}.\textsuperscript{59} We tested for a potential role of polyP by creating a homozygous deletion mutant of *L. major* VTC4 and a rescued version of this cell line (VTC4 2S), in which a functional VTC4 allele was re-integrated into the genome of the homozygous deletion mutant (Supplementary Figs. 3 and 4). In order to assess polyP synthesis, we cultivated the promastigote form of the parasites and quantified their polyP using the fluorescent indicator DAPI, which generates a characteristic emission at a wavelength of 550 nm (Fig. 6a). PolyP of wild-type and VTC4 2S cells peaked under low Mg\textsuperscript{2+} conditions, whereas wild-type and VTC4 2S cell numbers doubled in the presence of Mg\textsuperscript{2+}. Finally, Mg\textsuperscript{2+} is transferred from the vacuolar lumen to the cytosol via the dedicated Mg\textsuperscript{2+} exporter Mrn2.

**Discussion**

Our data identify a novel pathway for acquiring Mg\textsuperscript{2+} by fluid-phase endocytosis. Under limited metal bioavailability, the cells upregulate endocytosis in order to bring soluble metals into their acidocalcisome-like, polyP-loaded vacuole. Our results suggest that uptake via this EAPEC pathway and plasma membrane transport are not mutually exclusive but rather complementary. When Mg\textsuperscript{2+} is scarce but fully bioavailable, a condition that we mimicked through Mg\textsuperscript{2+}-depleted but EDTA-free medium, the high-affinity Mg\textsuperscript{2+}-transporters in the plasma membrane suffice to support growth. The endocytosis/polyP-dependent EAPEC route becomes essential for growth when bioavailability is low, enabling the cell to cope with this condition, which is often encountered in natural settings.\textsuperscript{60}

PolyP avidly binds numerous divalent metal ions and readily forms gels with them.\textsuperscript{23} PolyP accumulates in acidocalcisome-like vacuoles to concentrations that can reach hundreds of millimolar of phosphate units.\textsuperscript{20} Due to this very high concentration, we expect polyP to outcompete the chelating compounds that limited the bioavailability of Mg\textsuperscript{2+} in the surrounding medium (Fig. 6c). The acidic pH of acidocalcisomes, which is around 5, and their high concentrations of basic amino acids and polyanions, should favor this competition. PolyP should hence sequester Mg\textsuperscript{2+} from the endocytosed fluid phase and concentrate it inside these organelles. Concentration is expected to facilitate its transfer to the vacuolar Mg\textsuperscript{2+} exporter, which then makes the metal available to the cytosol. We consider polyP essentially as an intracellular ion filter that extracts metal ions from a continuous flow of media through the vacuole, which is established by vesicular traffic to and from this organelle. This sets it apart from other strategies of metal acquisition such as siderophores, which are secreted into the extracellular space and then recovered by high-affinity transporters in the plasma membrane.\textsuperscript{61}

An interesting open question is how Mg\textsuperscript{2+} that is accumulated through polyP inside vacuoles could become available for the vacuolar Mg\textsuperscript{2+}-exporter. Several properties of polyP might be relevant for this, giving rise to the following hypotheses, which are not mutually exclusive: First, polyP is a highly charged polymer that can reach 50–100 kDa and form gels. It binds Mg\textsuperscript{2+} and other metal ions. Its confinement inside acidocalcisome-like vacuoles, which are connected to the environment via vesicular import and export, is expected to create a Donnan equilibrium. Donnan equilibria are established when macromolecules with an affinity for small solutes are confined in a space that is connected to a solute reservoir through a semipermeable barrier, which allows solutes to pass but retains the macromolecules.\textsuperscript{62}
Mg²⁺ through their plasma membrane transporters (Alr1/2), they continue to grow under limited metal availability and hence keep acquiring Mg²⁺, showing shorter polyP fragments than the arrested daughters. If enzymatic hydrolysis of polyP occurred close to the vacuolar membrane, it might liberate Mg²⁺, preferentially for export. Unfortunately, the polyP degradation machinery of yeast vacuoles is poorly explored. Vacuoles contain at least three polyphosphatase activities. So far, only the membrane-associated Ppn2⁶³,⁶⁴ and the soluble Ppn1 could be identified⁶³, and their simultaneous deletion did not impair growth on YPD/EDTA (not shown). Fourth, polyP readily associates with a variety of abundant cations. Arg, Lys, K⁺, and polyamines accumulate in vacuoles also in concentrations of tens to hundreds of millimolar, maintained by dedicated vacuolar importers⁶⁵,⁶⁶. They might compete with Mg²⁺ for binding polyP, facilitating Mg²⁺ release. Fifth, polyP forms gel phases that separate adsorbed molecules from the rest of the solution. These phase transitions depend on pH, ionic strength and the type of metal ions bound⁶⁷. Continuous active vacuolar import of counter-ions could maintain their concentrations higher in proximity to their importers, which might promote preferential liberation of Mg²⁺ close to the vacuolar membrane and the vacuolar Mg²⁺ exporter. At this point, these hypotheses cannot be tested because we lack techniques to assess the distributions of short- and long-chain polyP and of the mentioned organic and inorganic cations within vacuoles with sufficient spatial resolution.

Cells might employ metal acquisition via endocytosis and polyP also for acquiring metals other than Mg²⁺. We created experimental conditions that rendered Mg²⁺ limiting, permitting an analysis focused on this metal. However, fluid-phase endocytosis is non-selective and polyP can sequester a broad range of divalent and trivalent metal ions²². Vacuoles and acidocalcisomes also contain exporters for transferring a variety of metal ions into vacuoles with sufficient spatial resolution.

In sum, whereas acidocalcisome-like organelles have frequently been considered as simple storage compartments, our results ascribe to these organelles an active role in metal acquisition for the cytosol. They identify a novel pathway for metal acquisition that utilizes an inorganic intracellular polymer, polyP, as a crucial pathway when proliferating inside macrophages. Also, polyP might be degraded inside vacuoles in order to liberate bound Mg²⁺. In line with this, mother cells, which continue to grow under limited metal availability and hence keep acquiring Mg²⁺, show shorter polyP fragments than the arrested daughters. If enzymatic hydrolysis of polyP occurred close to the vacuolar membrane, it might liberate Mg²⁺, preferentially for export. Unfortunately, the polyP degradation machinery of yeast vacuoles is poorly explored. Vacuoles contain at least three polyphosphatase activities. So far, only the membrane-associated Ppn2⁶³,⁶⁴ and the soluble Ppn1 could be identified⁶³, and their simultaneous deletion did not impair growth on YPD/EDTA (not shown). Fourth, polyP readily associates with a variety of abundant cations. Arg, Lys, K⁺, and polyamines accumulate in vacuoles also in concentrations of tens to hundreds of millimolar, maintained by dedicated vacuolar importers⁶⁵,⁶⁶. They might compete with Mg²⁺ for binding polyP, facilitating Mg²⁺ release. Fifth, polyP forms gel phases that separate adsorbed molecules from the rest of the solution. These phase transitions depend on pH, ionic strength and the type of metal ions bound⁶⁷. Continuous active vacuolar import of counter-ions could maintain their concentrations higher in proximity to their importers, which might promote preferential liberation of Mg²⁺ close to the vacuolar membrane and the vacuolar Mg²⁺ exporter. At this point, these hypotheses cannot be tested because we lack techniques to assess the distributions of short- and long-chain polyP and of the mentioned organic and inorganic cations within vacuoles with sufficient spatial resolution.

Cells might employ metal acquisition via endocytosis and polyP also for acquiring metals other than Mg²⁺. We created experimental conditions that rendered Mg²⁺ limiting, permitting an analysis focused on this metal. However, fluid-phase endocytosis is non-selective and polyP can sequester a broad range of divalent and trivalent metal ions²². Vacuoles and acidocalcisomes also contain exporters for transferring a variety of metal ions into the cytosol. Therefore, it is possible that the same pathway and strategy that we identified here for Mg²⁺ might also be used to overcome limited availability of other metal ions. Furthermore, the EAPEC pathway might be conserved among different species because a key feature required for it, an acidocalcisome-like organelle rich in polyP and equipped with metal exporters, is found in all kingdoms of life²⁰. This is supported by our results on the proliferation of Leishmania in phagolysosomes of inflammatory macrophages. Proliferation of Leishmania in phagolysosomes is limited by Mg²⁺⁵⁹ and depends on polyP. While during growth in rich media acidocalcisomes are not connected to endocytic traffic⁶⁸–⁷¹, such a connection can be established in specific metabolic situations, which allows Leishmania acidocalcisomes to accumulate endocytic tracers⁷². Therefore, Leishmania might be able to induce the EAPEC pathway when proliferating inside macrophages. Also, Trypanosoma brucei, which causes sleeping sickness, depends on the polyP of its acidocalcisomes for virulence²⁶, although in this case, it has not yet been tested whether metal acquisition contributes to the phenotype.

In sum, whereas acidocalcisome-like organelles have frequently been considered as simple storage compartments, our results ascribe to these organelles an active role in metal acquisition for the cytosol. They identify a novel pathway for metal acquisition that utilizes an inorganic intracellular polymer, polyP, as a crucial...
element. This inorganic polymer is constrained in a specialized organelle and connected to the environment through a flow of aqueous phase generated by endocytosis and vesicular retrograde traffic. It can hence be considered as an intracellular ion filter that is dedicated to extracting Mg^{2+}, and perhaps other metal ions, from the environment, and facilitates their enrichment and transfer to the cytosol under conditions of limited bioavailability.

Methods

**Yeast strains and media.** *S. cerevisiae* strains used in this study are listed in Supplementary Table 1. Knockout strains obtained from deletion collections were re-tested by PCR. Primer sequences are listed in Supplementary Table 2. Cells were cultivated overnight at 30 °C in YPD (1% yeast extract, 2% peptone, 2% glucose) to mid-exponential growth phase and inoculated in YPD supplemented with 1 mM EDTA at 0.1 OD units ml⁻¹ (corresponds to 0.4×10^⁶ cells ml⁻¹). Since their ion content can vary moderately, new batches of yeast extract and peptone were routinely tested for the induction of a growth defect at an EDTA concentration of 1 mM. Metal-depleted YPD (YPD/Chexel) was prepared by incubating 5 g of Chelex-100 resin (Bio-Rad) per 100 ml YPD overnight on a rotating wheel at 4 °C. The resin was removed by filtration through a 0.2 μm filter and the pH was adjusted to 7 with 1 N HCl. Residual metal concentrations of YPD/Chexel were determined by ICP-MS and are listed in Supplementary Table 3.

**Parasite cell lines and media.** *L. major* MRHO/IR/75/ER was used in this study. Promastigotes were grown at 26 °C in M199 medium (Invitrogen AG) complemented with 10% heat-inactivated fetal bovine serum (FBS, Seromed), 50 μM penicillin/streptomycin (Animed), 40 mM Heps (Anamed), 0.6 mg l⁻¹ biotin (Sigma) and 5 mg l⁻¹ hemin (Sigma).

** Constructs for LmVtc4 mutant cell lines.** To target both LmjVTC4 alleles in the knockout cell line, plasmids based on the px63-HGY vector were used. 787 bp of the VTC4 3′-UTR were amplified from genomic DNA and cloned into the vector using XmaI and BglII restriction sites. 460 bp of the 5′-UTR were amplified from genomic DNA and cloned into the HindIII and SalI sites to create pLmjVTC4.k.o.-HYG. To generate plmjVTC4.k.o.-PAC, the PAC gene was released by Spel/BamHI digestion from a modified px63-PAC vector and used to replace the HGY gene in plmjVTC4.k.o.-HYG. Both plasmids were digested with BglII and HindIII and the knockout cassettes gel-purified (Promega) and used for parasite transfection. Gene replacements were verified by PCR and Southern blotting.

**VTC4 was genetically integrated and re-expressed in the L. major vtcΔ/vtcΔ cell line by replacing the HGY resistance gene of the pSsU-int construct with NEO (from px63-NEO) using Spel and XbaI. LmjVTC4 was amplified from genomic DNA and cloned into the vector using ClaI and Xmal. The intergenic region of cysteine proteinase B (CPB) 2.8 was cloned in-between LmjRHO and NEO using Xmal/Spel. The transcription cassette of pSsU-LmjVTC4-NEO was excised by PacLI/Pmel digestion, gel-purified and used for parasite transfection. Gene integration was verified by PCR and protein synthesis by Western blotting.

**Leishmania stable transfection.** A concentration of 8–12 μg linear DNA in 5 μl H₂O was used to transfect 2×10⁶ parasites. Parasites were centrifuged and washed with phosphate-buffered saline (PBS). The pellet was resuspended in 100 μl T-cell Nucleofector solution of the Amazax human T-cell Nucleofector kit (Lonza) and the DNA was added. This mixture was transferred to the Amazax cuvette and electroporated with the Nucleofector II device, program U-033. Cells were incubated for 40 min at room temperature. DAPI-PolyP fluorescence was acquired for 20 min at 420 nm excitation and 550 nm emission in a Gemini EM Microscopy microscope ( Molecular Devices). A standard curve was prepared with PolyP60. For separation of polyP chains by electrophoresis, 15–30 nmol polyP were treated with RNAse, DNase and (where indicated) with guanidinium isothiocyanate and sodium dodecyl sulfate. The sample was run on a polyacrylamide gel (acrylamide/bisacrylamide 19:1, Serva) in TBE. Gels were run overnight at 3 mA and 4 °C until the loading dye (30 μmol, 0.5% bromophenol blue, 1 mM EDTA) front migrated to 1/3 of the gel. Gels were stained in buffer with DAPI (1.5 g l⁻¹ Tris base, 2% glycerol, 7.2 μM DAPI) for 45–60 min and destained in the same buffer without DAPI for 30–45 min. Gels were exposed on a UV transilluminator to induce photobleaching, after which pictures of the gel were taken.

**PolyP quantification for time-course analysis of L. major promastigotes was performed as follows**. Cells were pelleted and washed with 50 mM HEPES (pH 7.5), 150 mM KCl buffer and then suspended in 400 μl DAPI buffer (20 μM DAPI) on a polycrylamide gel (acrylamide/bisacrylamide 19:1, Serva) in TBE. Gels were run overnight at 3 mA and 4 °C until the loading dye (30 μmol, 0.5% bromophenol blue, 1 mM EDTA) front migrated to 1/3 of the gel. Gels were stained in buffer with DAPI (1.5 g l⁻¹ Tris base, 2% glycerol, 7.2 μM DAPI) for 45–60 min and destained in the same buffer without DAPI for 30–45 min. Gels were exposed on a UV transilluminator to induce photobleaching, after which pictures of the gel were taken.

**Microscopy.** Images were taken with an UltraView Vox spinning disk confocal microscope (Perkin Elmer-Cetus) connected to an inverted microscope (Carl Zeiss) with a 100× oil immersion objective with a numerical aperture of 1.41 and an Hamamatsu C9100-50 camera (Hamamatsu, Japan). FM4-64 was excited at 561 nm and imaged using a 705W/90 nm band pass filter. Lucifer yellow was excited at 406 nm and imaged using a 527W/55 nm band pass filter. Calcium fluorescence was excited at 406 nm and imaged using a 445W60 nm band pass filter. GFP and fluorescein were excited at 488 nm and imaged using a 527W/55 nm band pass filter. Images were processed with ImageJ and Adobe Photoshop software.

**Vacuole staining with FM4-64.** Cells were grown overnight to early logarithmic phase in YPD and inoculated in YPD or YPD-EDTA. 0.25 OD units were stained with 10 μM FM4-64 (in dimethyl sulfoxide) in 1 ml of the respective medium for 30 min at 30 °C. Cells were washed twice, chased for 45 min at 30 °C and analyzed with a confocal microscope.

**Lucifer yellow internalization assay.** A total of 0.25 OD₅₀ units were removed from the culture, pelleted and resuspended in 90 μl of the respective medium. Lucifer yellow was added from a 10× stock solution to a final concentration of 2.3 mM. Cells were incubated for 75 min at 30 °C before the dye was removed by 5
washes with 1× PBS. Fluorescence was acquired on a FACSCanto flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star). Lucifer yellow was excited with the 403 nm laser and detected in the AmCyan channel (BP 525/20).

Macrophage infection. Bone marrow cells were obtained by flushing the femurs and tibiae of naive female C57BL/6 mice (Harlan). The harvested cells were differentiated into bone marrow-derived macrophages (BMDM) for 5–7 days using complete DMEM supplemented with L929 conditioned media at 37 °C. Differentiated BMDM were cultured in DMEM medium supplemented with 1% penicillin/streptomycin, 10% FCS and 1× HEPES at 37 °C and 5% CO2 for 2–12 h before being infected with late stationary phase promastigotes at a ratio of 1:5 (macrophage: Leishmania). Macrophages were incubated with parasites for 6 h at 37 °C on culture coverslips (Thermomax) in 24-well plates (500′000 macrophages per well). Non-phagocytosed parasites were removed by 2 washes with 1× PBS and fresh DMEM medium was added to the wells. Cells were fixed after 24, 48, and 72 h with 4% PFA for 15–20 min and stained with Diff-Quik (Dade Behring). Phase contrast pictures were taken with a Leica microscope and the number of amoebastigotes quantified in >100 macrophages per cell line.

Data availability. All source data are available from the corresponding author upon request.

Received: 24 November 2016 Accepted: 24 October 2017

Published online: 01 December 2017

References

1. Boynton, P. J. & Greig, D. The ecology and evolution of non-domesticated Saccharomyces species. Yeast 31, 449–462 (2014).
2. Scallert, A. Antimicrobial properties of tannins. Phytochemistry 30, 3875–3883 (1991).
3. Cos, P. et al. Proanthocyanidins in health care: current and new trends. Curr. Med. Chem. 11, 1345–1359 (2004).
4. Rieuwerts, J. S., Thornton, I., Farago, M. E. & Ashmore, M. R. Factors influencing metal bioavailability in soils: preliminary investigations for the use of a Saccharomyces cerevisiae magnesium transport system confers resistance to aluminum ion. J. Biol. Chem. 275, 1051–1062 (2010).
5. González-Guerrero, M., Escudero, V., Saéz, Á. & Tejada-Jiménez, M. Transition metal transport through Arn1p and its metabolism in Saccharomyces cerevisiae. J. Biol. Inorg. Chem. 15, 1051–1062 (2010).
6. Uittenweiler, A., Schwarz, H., Neumann, H. & Mayer, A. The vacuolar transporter chaperone complex, regulates polyphosphate synthesis and phosphate homeostasis in yeast. J. Biol. Chem. 291, 22622–22275 (2016).
7. Lander, N., Ulrich, P. N. & Docampo, R. Trypanosoma brucei vacuolar transporter chaperone 4 (TbVtc4) is an acidocalcisome polyphosphate kinase required for in vivo infection. J. Biol. Chem. 288, 34205–34216 (2013).
8. Smith, S. A. et al. Polyphosphate modulates cellular copper and other trace metal homeostasis in yeast. J. Biol. Chem. 288, 5152–5161 (2013).
9. Yun, C.-W. et al. Desferrioxamine-mediated Iron Uptake in Saccharomyces cerevisiae. J. Biol. Chem. 283, 5152–5159 (2008).
10. Yun, C.-W. et al. Desferrioxamine-mediated Iron Uptake in Saccharomyces cerevisiae. J. Biol. Chem. 283, 5152–5159 (2008).
11. Froissard, M. et al. Trafﬁcking of siderophore transporters and protein traffic networks differentially affecting metal tolerance: a genomic phenotyping study in yeast. Genome Biol. 9, R67 (2008).
12. Chesi, A., Kilaru, A., Fang, X., Cooper, A. A. & Gitler, A. D. The role of the parkinson’s disease gene PARK9 in essential cellular pathways and the manganese homeostasis network in yeast. PLoS ONE 7, e34178 (2012).
13. Rees, E. M., Lee, J. & Thiele, D. J. Mobilization of intracellular copper stores required for in vivo infection. J. Cell. Sci. 127, 1721–1729 (2014).
14. Rees, E. M., Lee, J. & Thiele, D. J. Mobilization of intracellular copper stores by the ctr2 vacuolar copper transporter. J. Biol. Chem. 279, 54221–54229 (2004).
15. Blaby-Haas, C. E. & Merchant, S. S. Lysosome-related organelles as mediators of metal homeostasis. J. Biol. Chem. 289, 28129–28136 (2014).
16. Docampo, R., de Souza, W., Miranda, K., Rohloff, P. & Moreno, S. N. J. Acidocalcisomes - conserved from bacteria to man. Nat. Rev. Microbiol. 3, 151–161 (2005).
17. Docampo, R. & Moreno, S. N. J. Acidocalcisomes. Cell. Calcium 50, 113–119 (2011).
18. Van Wazer, J. R. & Callis, C. F. Metal complexing by phosphates. Biochim. Biophys. Acta. 721, 394–398 (1982).
19. Froissard, M., Gerasimaitė, G., C. & Schweczke, J. Localization of polyphosphate in vacuoles of Saccharomyces-Cerevisiae. Arch. Microbiol. 116, 275–278 (1978).
20. Tissier, F., Steen, H. W. & Van Steveninck, J. Localization of polyphosphates in Saccharomyces fragilis, as revealed by 46-diamidino-2-phenylindole fluorescence. Biochim. Biophys. Acta. 1051, 394–398 (1982).
21. Rees, E. M., Lee, J. & Thiele, D. J. Mobilization of intracellular copper stores by the ctr2 vacuolar copper transporter. J. Biol. Chem. 279, 54221–54229 (2004).
52. Arlt, H., Reggiori, F. & Ungermann, C. Retromer and the dynamin Vps1
54. Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. Unravelling the
55. Mann, F. M., VanderVen, B. C. & Peters, R. J. Magnesium depletion triggers
63. Kumble, K. D. & Kornberg, A. Endopolyphosphatases for long chain inorganic
49. Wiederkehr, A., Meier, K. D. & Riezman, H. Identi
NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-01930-5
ARTICLE
46. Gregan, J. et al. The mitochondrial inner membrane protein Lpe10p, a
48. Pisat, N. P., Pandey, A. & MacDiarmid, C. W. MNR2 regulates intracellular
57. Docampo, R. & Huang, G. Acidocalcisomes of eukaryotes.
65. Wiemken, A. & Dürr, M. Characterization of amino acid pools in the vacuolar
61. Haas, H. Molecular genetics of fungal siderophore biosynthesis and uptake: the
68. Scott, D. A., Docampo, R., Dvorak, J. A., Shi, S. & Leaman, R. D. In situ
70. Mullin, K. A. et al. Regulated degradation of an endoplasmic reticulum membrane protein in a tubular lysosome in Leishmania mexicana. Mol. Biol. Cell. 12, 2364–2377 (2001).
71. Huang, G. et al. Adaptor protein-3 (AP-3) complex mediates the biogenesis of acidocalcisomes and is essential for growth and virulence of Trypanosoma brucei. J. Biol. Chem. 286, 36619–36630 (2011).
72. Vannier-Santos, M. A. et al. Impairment of sterol biosynthesis leads to phosphorus and calcium accumulation in Leishmania acidocalcisomes. microbiology 145 (Pt 11), 3232–3220 (1999).
73. Cruz, A., Coburn, C. M. & Beverley, S. M. Double targeted gene replacement for creating null mutants. PNAS 88, 7170–7174 (1991).
74. Price, H. P. et al. Myristoyl-CoA:protein N-myristoyltransferase, an essential enzyme and potential drug target in kinetoplastid parasites. J. Biol. Chem. 278, 7206–7214 (2003).
75. Azevedo, C., Livermore, T. & Saiardi, A. Protein polyphosphorylation of lysine residues by inorganic polyphosphate. Mol. Cell. 58, 71–82 (2015).
76. Desoufghé, Y., Neumann, H. & Mayer, A. Organelle size control - increasing vacuole content activates SNAREs to augment organelle volume through homotypic fusion. J. Cell. Sci. 129, 2817–2828 (2016).
77. Kulakova, A. N. et al. Direct quantification of inorganic polyphosphate in microbial cells Using 4'-6-Diamidino-2-Phenylindole (DAPI). Environ. Sci. Technol. 45, 7799–7803 (2011).
78. Meerpoht, H. G., Lohmann Matthes, M. L. & Fischer, H. Studies on the activation of mouse bone marrow-derived macrophages by the macrophage cytotoxicity factor (MCF). Eur. J. Immunol. 6, 213–217 (1976).

Acknowledgements
We thank Andrea Schmidt and Florence Prevel for assistance; Deborah F. Smith (Imperial College of Science, London, UK) for providing us with the pX63-HYG plasmid; Toni Aebischer (University of Tübingen, Germany) for the pSSU-int vector; and Toshikazu Shiba (Regenetiss Inc., Japan) for polyP60. Yeast strains DY1457 WT and Δalr1 Toshikazu Shiba (Regenetiss Inc., Japan) for polyP60. Yeast strains DY1457 WT and Δalr1 were kindly provided by David Eide (University of Wisconsin-Madison, USA) and DT1Y165 co22a by Dennis J. Thiele (Duke University School of Medicine, Durham, USA). This work was supported by grants from the SNF (CRS115_170925 and FNS 3100A0-144258) and the ERC (FP7-233458) to A.M., by grants from the SNF to N.F., and by EU COST Action CM0801 (SER C09.0045) to N.F. and A.M.

Author contributions
S.H.K.: Conceived and performed the experiments and interpreted the data. K.K. and N.F.: Contributed the reagents and materials. S.H.K., K.K., and N.F.: Performed the experiments. K.K.: Performed the labeling and imaging experiments. S.H.K.: Contributed to the writing of the manuscript. N.F.: Contributed to the writing of the manuscript.

Additional information
Supplementary Information accompanies this paper at doi:10.1038/s41467-017-01930-5.

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2017