Acidocalcisomes of *Trypanosoma brucei* and the acidocalcisome-like vacuoles of *Saccharomyces cerevisiae* are acidic calcium compartments that store polyphosphate (polyP). Both organelles possess a phosphate–sodium symporter (TbPho91 and Pho91p in *T. brucei* and yeast, respectively), but the roles of these transporters in growth and orthophosphate (P$_i$) transport are unclear. We found here that Tbpho91$^{-/-}$ trypanosomes have a lower growth rate under phosphate starvation and contain larger acidocalcisomes that have increased P$_i$ content. Heterologous expression of TbPHO91 in *Xenopus* oocytes followed by two-electrode voltage clamp recordings disclosed that myo-inositol polyphosphates stimulate both sodium-dependent depolarization of the oocyte membrane potential and P$_i$ conductance. Deletion of the SPX domain in TbPho91 abolished this stimulation. Inositol pyrophosphates such as 5-diphosphoinositol pentakisphosphate generated outward currents in Na$^+$-loaded giant vacuoles prepared from WT or from TbPHO91-expressing pho91A strains but not from the pho91A yeast strains or from the pho91A strains expressing PHO91 or TbPHO91 with mutated SPX domains. Our results indicate that TbPho91 and Pho91p are responsible for vacuolar P$_i$ and Na$^+$ efflux and that myo-inositol polyphosphates stimulate the Na$^+$/P$_i$ symporter activities through their SPX domains.

Acidocalcisomes are acidic calcium stores rich in P$_i$, PP$_\alpha$ and polyphosphate (polyP) bound to cations such as calcium, magnesium, and sodium (1). Synthesis and translocation of polyP into acidocalcisomes and the acidocalcisome-like vacuoles of *Saccharomyces cerevisiae* are mediated via the vacuolar transporter chaperone (VTC) complex, of which Vtc4 is the catalytic subunit (2, 3).

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3. The abbreviations used are: polyP, polyphosphate; VTC, vacuolar transporter chaperone; S-IP$_\alpha$, 5-diphosphoinositol pentakisphosphate; PFC, procyolic form; PFA, phosphonoformic acid; NMDG, N-methyl-D-glucamine; IP$_\alpha$, inositol hexakisphosphate; PP-IP$_4$, 5-diphosphoinositol tetrakisphosphate; FBS, fetal bovine serum.

It has been shown that rapid hydrolysis of acidocalcisome polyP occurs when trypanosomes are exposed to alkalining agents (nigericin, NH$_4$Cl) or hyposmotic stress (4). Vacuolar Pi is also a source of P$_i$ for dNTP synthesis needed for yeast DNA duplication (5) or for biosynthesis of nucleotides and phospholipids needed for cell division (6). However, how P$_i$, produced by hydrolysis of polyP, catalyzed by endo- and exopolyphosphatases (7–11), is released from these vacuolar compartments or how release is regulated is unknown.

P$_i$ is negatively charged, and it needs to be transported into or out of cells or organelles via an active transport process. P$_i$ transporters use either the transmembrane Na$^+$ (animals, fungi) or H$^+$ (plants, bacteria) gradients to drive P$_i$ transport into cells (12). P$_i$ transporters also occur in organelles, such as the mitochondrial proton/phosphate symporter (P$_i$ carrier), an inner membrane-embedded protein that translocates P$_i$ from the cytosol into the mitochondrial matrix (13), and Pho91p, which is a yeast low-affinity vacuolar sodium/phosphate (Na$^+$/P$_i$) symporter. Pho91p was proposed to export P$_i$ from the vacuolar lumen to the cytosol (14), although this has never been directly demonstrated. In a previous proteomic study of the acidocalcisomes of *Trypanosoma brucei*, we identified a P$_i$ transporter with similarity to Pho91p, localized it to the acidocalcisome by expressing the HA-tagged protein, and suggested that this transporter, TbPho91, could also be involved in returning P$_i$ to the cytosol after polyP hydrolysis (15).

As occurs with Pho91p (14) and the *Trypanosoma cruzi* Pho91 ortholog (TcPho91), which localizes to the contractile vacuole (16), TbPho91 possesses a SPX domain (named after Syg1 and Pho81, in yeast, and XPR1 in humans), and recent work has shown that SPX domains can bind inositol polyphosphates and act as sensor domains (17, 18). A particularly strong binder is 5-diphosphoinositol pentakisphosphate (5-PP-IP$_5$ or 5-IP$_7$), an inositol pyrophosphate. 5-IP$_7$ has five of the phosphates and act as sensor domains (17, 18).

In this work we investigated the function of TbPho91 by deleting its gene and examining the phenotypic changes in cells. We used the two-electrode voltage clamp method to directly measure membrane electric currents stimulated by inositol polyphosphates in *Xenopus* oocytes heterologously expressing TbPHO91, with or without its SPX domain. In addition, we patch-clamped giant vacuoles of yeast expressing either WT or
SPX mutant Na⁺/P⁻ symporters. We provide evidence for the role of TbPho91 and Pho91p in P⁻ and Na⁺ release to the cytosol and for their SPX domain-mediated regulation by inositol pyrophosphates.

Results
Characteristics and localization of TbPho91

A gene (Tb427tmp.01.2950), annotated as sodium/sulfate symporter or phosphate transporter and encoding for a putative PHO91 ortholog, is present in the T. brucei genome (http://tritrypdb.org/tritrypdb/)⁴ and was named TbPHO91 (15). The full-length gDNA of TbPHO91 was cloned by PCR amplification from the Lister 427 strain of T. brucei, confirmed by sequencing and shown to be identical to the gene in the database. The orthologs identified in T. cruzi (TcCLB.508831.60) and Leishmania major (LmjF.28.2930) share 65 and 59% amino acid identity, respectively, to TbPho91. The ORF predicts a 728-amino acid protein with an apparent molecular mass of 81.4 kDa, nine transmembrane domains (Fig. 1A), an N-terminal regulatory SPX domain, and an anion-permease domain also present in other anion transporters. In previous work, we reported the endogenous C-terminal tagging of TbPHO91 with an HA tag and the localization of TbPho91-HA predominantly to the acidocalcisomes of T. brucei (15).

⁴ Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
Moreover, the sodium/sulfate symporter domain is conserved in these organisms (67% similarity and 34% identical residues).

**Phenotypic changes in** *TbPHO91* **knockout procyclic forms**

We generated knockout mutants of *TbPHO91* in procyclic forms (PCFs) by homologous recombination replacing both copies of the gene with resistance markers and isolated several clones. Southern blot analysis demonstrated that *TbPHO91* was absent in one of these clones (Fig. 2A). These *Tbpho91*−/− cells did not show significant growth defect in regular medium but showed a significant growth defect in low phosphate medium (Fig. 2B). If TbPho91 is involved in the release of P<sub>i</sub> and Na<sup>+</sup> from acidocalcisomes, a possible consequence is the accumulation of P<sub>i</sub> (and Na<sup>+</sup>) within the organelles with concomitant increase in their osmolarity. This was apparently the case as we detected higher levels of P<sub>i</sub> in the mutants with no significant changes in polyP (Fig. 2C). Immunofluorescence analysis of *Tbpho91*−/− cells using antibodies against the vacuolar pyrophosphatase (TbVP1), an acidocalcisomal marker, showed enlarged organelles (Fig. 2D). The TbVP1 signals appear as small dots in WT cells and as larger ring-like structures in the *Tbpho91* mutants. Mutant cells have increased numbers of larger acidocalcisomes (Fig. 2E), and these results were confirmed by direct transmission EM of whole cells. In contrast to WT cells, *Tbpho91*−/− cells contained larger acidocalcisomes as electron-dense spheres. G, IFA of representative *Tbpho91*−/− cells labeled with VP1 antibody showing larger acidocalcisomes than in WT cells. Bars, 1 μm. Acidocalcisomes of *Tbpho91*−/− cells display altered morphology upon phosphate starvation. WT or *Tbpho91*−/− cells were cultured in low phosphate medium (20 μM) for 24 h. WT cells have normal size acidocalcisomes throughout the cell body and a few larger ones between the nucleus and kinetoplast. *Tbpho91*−/− cells displayed a small number of abnormally large acidocalcisomes, with the largest normally observed between the nucleus and kinetoplast. Bars, 2 μm.
calciosomes (Fig. 2F). The results are consistent with an increase in the acidocalcisomes osmolarity followed by water accumulation and enlargement. Previous work has shown lower levels of polyP and PPi when TcPHO91 was down-regulated (16), but no changes were observed in pho91Δ S. cerevisiae strains (14). Immunofluorescence microscopy of Tbpho91−/− cells grown under Pi starvation conditions showed further dilation of acidocalcisomes, which appeared to fuse in larger vacuoles (Fig. 2G). In conclusion our results showing that enlargement of acidocalcisomes increased P content and decreased growth rate under Pi starvation stress are consistent with a role for TbPho91 in Pi release from acidocalcisomes.

Functional expression of TbPHO91 in Xenopus laevis oocytes and basic transport characteristics

We investigated the basic transport characteristics of TbPho91 by expressing it in Xenopus oocytes. Fig. 3A (upper tracing) shows the inward currents generated at holding potential (Vh = −60 mV) by addition of different concentrations of Na+ /Pi to oocytes expressing TbPHO91. The current amplitude increased in a dose-dependent manner with a threshold around 2 mM, and the results are consistent with transport of a positive charge into the cell. Negligible current changes were observed in control diethylpyrocarbonate-injected oocytes (Fig. 3A, lower tracing), probably related to endogenous Pi transporters (also called type III Na+/Pi co-transporters). The addition of 100 mM NaCl alone in the absence of Pi did not result in any significant change in current (Fig. 3C, left), whereas replacing Na+ by equimolar concentrations of K+ did not generate Pi-induced changes in currents (Fig. 3C, right), demonstrating the sodium selectivity of TbPho91. There was collapse of Vm in unclamped cells (upper panel) when Na+/Pi is taken up (upper panel), indicating depolarization.

Phosphonoformic acid (PFA) is a highly specific competitive inhibitor (20) of solute carrier SLC34 transporters (also called type II Na+/Pi co-transporters) (12). Treatment of TbPHO91-expressing oocytes with 10 mM PFA for 10 min did not impact the amplitude of the Na+ /Pi current (288.3 ± 11.7 nA before PFA and 302 ± 39.3 nA after PFA, n = 4) (Fig. 4A), suggesting that TbPho91 could belong to the solute carrier family SLC20 of transporters (also called type III Na+/Pi co-transporters). The delayed decay of the Na+ /Pi transient observed in Fig. 4A was caused by a PFA-induced slow inward current (Fig. 4B).

To analyze the voltage dependence of the transporter, we registered the currents generated in oocytes expressing TbPHO91 in response to a step change in Vh from −80 to +40 mV (Fig. 5A). The current amplitude was measured at the steady-state part of the traces. Fig. 5A (left panel) shows representative raw currents in response to the voltage step protocol when perfused in the absence (−P) or presence (+P) of 10 mM Pi or after replacing NaCl by N-methyl-D-glucamine (NMDG). Quantification of averaged currents of oocytes expressing TbPHO91 (Fig. 5A, right panel) shows a significant increase in the steady-state current at positive (280% at +40 mV) and negative (294% at −80 mV) potential in the presence of 10 mM Pi (red triangles) when comparing to control conditions without Pi (black circles). Sodium dependence is demonstrated by the lack of response when NaCl is replaced by the nonpermeable cation NMDG (blue line). These results demonstrate that both Na+ and Pi are essential for TbPho91 activation. P induces a
430% higher steady-state current at +40 mV than at −40 mV ($p < 0.05$, Student’s $t$ test, $n = 5$), indicating an outward-rectifying behavior with respect to $V_{h}$. Fig. 5B shows the anion selectivity by applying voltage pulses in the presence of either 10 mM sulfate (blue inverted triangles) or 10 mM nitrate (blue squares). No significant changes in the recorded currents were observed when compared with the controls in absence of anions.

The $N_{a}^{+}/P_i$ current showed strong dependence on the pH of the extracellular medium (Fig. 5C, left panel). Fig. 5C (right panel) shows a representative recording of currents, at different pH levels, in the presence of 10 mM $P_i$. The current amplitude in presence of 10 mM $P_i$ was increased by 318%, from $98.9 \pm 33.6$ nA at pH 7.3 to $313.7 \pm 93.7$ nA at pH 7.8 (*, $p < 0.05$, $n = 6$). On the other hand, the shift of extracellular pH to more acidic values produced a decrease by 232% in the current amplitude to $10.1 \pm 4.8$ nA at pH 6.8 (**, $p < 0.05$, $n = 6$) or by 359% to $27.5 \pm 8.3$ nA at pH 5.4 (**, $p < 0.05$, $n = 6$) at pH 6.3 (Fig. 5C, right panel), which is more typical for type II than for type III $N_{a}^{+}/P_i$ co-transporters (21).

Complete removal of extracellular calcium from the bath solution (100 µM EGTA added) tended to amplify the $N_{a}^{+}/P_i$ current, a change that, however, was not significant ($p < 0.2$, $n = 5$) (Fig. 6). Extracellular calcium concentrations above 1.8 mM were not tested, because this induced rapid death of oocytes. Taking into account its electrogenicity, low $P_i$ affinity and resistance to PFA treatment, we suggest that TbPho91 belongs to the type III $N_{a}^{+}/P_i$ co-transporters family (SLC20) (21), although in contrast to other SLC20 proteins (22, 23), $N_{a}^{+}/P_i$ currents were decreased at lower pH levels.

Modulation by inositol pyrophosphates of the $N_{a}^{+}/P_i$ conductance of TbPho91 is SPX-dependent

It has been demonstrated that the SPX domains present in the N-terminal of phosphate transporters, VTCs, and signaling proteins act as inositol polyphosphate sensor domains by binding inositol polyphosphates and stimulating polyphosphate synthesis by VTCs in yeast (17). Although different SPX domains bind inositol hexakisphosphate (IP$_6$) and inositol pyrophosphates like 5-IP$_7$, with similar affinities in vitro, it has been suggested that diphosphoinositol phosphates may be the relevant signaling molecules in vivo (17). We therefore investigated whether this was also the case with TbPho91. Preincubation of oocytes for 5–6 min with InsP$_6$ in the µM range (Fig. 7, A and B) or with 5-IP$_7$ (Fig. 8A) or 5-diphosphoinositol tetrakisphosphate (PP-IP$_4$, Fig. 8B) in the nanomolar range induced slow inward currents with subsequent amplification of the $N_{a}^{+}/P_i$–transmembrane current evoked by 10 mM $P_i$. The threshold for statistically significant amplification of the $N_{a}^{+}/P_i$ current was 100 µM for IP$_6$ (+82.5 ± 39.5%; *, $p < 0.05$, $n = 4$) (Fig. 7C). For 5-IP$_7$, the amplification threshold was 50 nM (+9.2 ± 3.5%; *, $p < 0.05$, $n = 5$), and for PP-IP$_4$, it was 1 µM (+61.7 ± 26.6%; *, $p < 0.05$, $n = 4$) (Fig. 8C). Further increases...
of the phosphate-containing ligands enhanced the amplification of the Na\(^{+}/P\(_i\) currents (Figs. 7C and 8C).

To investigate whether the phosphate-containing ligands were acting through the SPX domain of TbPho91, we expressed the protein with a deletion of this domain (TbPho91-\(\Delta\)SPX) and examined its response to 5-IP\(_7\) and PP-IP\(_4\). Neither 5-IP\(_7\) nor PP-IP\(_4\) (Fig. 8, A and B) could amplify the currents induced by 10 mM \(P_i\) addition in TbPHO91-\(\Delta\)SPX-expressing oocytes.

**SPX-dependent Na\(^{+}/P\(_i\) release from yeast vacuoles**

Acidocalcisomes have a diameter of \(~200 \text{ nm}\), and it is very difficult to do patch-clamp studies with these small organelles to test Na\(^{+}/P\(_i\) currents. Because the yeast vacuole has many similarities to the trypanosome acidocalcisome, we used giant vacuoles (up to \(~200 \text{ nm}\) in diameter) from yeast instead. This method has the additional advantage over the *Xenopus* studies that it is possible to eliminate endogenous currents by inactivation of the genes in yeasts. We prepared giant cells of *S. cerevisiae* by the spheroplast incubation method using 2-deoxy-glucose to inhibit cell wall synthesis (24). When using this method and when the giant cells are treated by moderate hyposmotic shock, only the plasma membranes are disrupted, and giant vacuoles are released. It is then possible to attach a patch pipette to isolated vacuoles and rupture the patch membrane using a high voltage pulse (Fig. 9A). The vacuolar lumen, which is connected to the pipette (whole-vacuole configuration), can then be loaded with a solution containing Na\(^{+}/P\(_i\) to monitor the currents across the membrane.

Once vacuoles were released by hyposmotic shock of giant cells, they were patched and clamped at a voltage of \(+60 \text{ mV}\). The bath solution had 10 mM Hepes, pH 7.1, containing 100 mM NaCl, 200 mM sorbitol, and 1 mM MgCl\(_2\), whereas the pipette solution contained the same mixture plus 10 mM NaHPO\(_4\)-Na\(_2\)HPO\(_4\) to detect outward currents generated by displace-
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Figure 9. IP₆ and 5-IP₇ induce SPX domain–mediated activation of Na⁺/Pᵢ currents in PHO91- and TbPho91-expressing vacuoles. A, image shows a intact giant cell of *S. cerevisiae* and a vacuole isolated after hypotonic shock showing a patch pipette attached to it. Bar, 10 μm. B, activation of Na⁺/Pᵢ outward currents in vacuoles from WT yeast after injection of IP₆ (1 mM) or 5-IP₇ (1 μM). C, effect of 1 mM 1-IP₇ on WT yeast vacuoles. D, pho91Δ vacuoles do not produce currents after application of IP₆, or 5-IP₇, E and F, complementation of pho91Δ with PHO91 (B) or TbPHO91 (F) restores vacuole response to IP₆ and 5-IP₇. G and H, complementation of pho91Δ with mutated PHO91Y22F (G) or TbPHO91Y22F (H) does not restore vacuole ability to generate Na⁺/Pᵢ currents. The data are representative of two to four independent experiments.

Next we generated plasmid constructs for expression of PHO91 or TbPho91 in pho91Δ cells. We cloned the genomic sequence of PHO91 or TbPHO91 in plasmid vector pRS413 that maintains a single copy per cell, and to ensure expression at endogenous levels, we also inserted the upstream promoter region of PHO91. We then compared the response of giant vacuoles from PHO91Δ complemented with exogenous PHO91 (22.2 ± 0.75 and 29.6 ± 3.6 pA for IP₆ and 5-IP₇, respectively, n = 2) or TbPHO91 (46.5 ± 8.3 and 26.3 ± 2.5 pA for IP₆ and 5-IP₇, respectively, n = 4) genes (Fig. 9, E and F) or with exogenous PHO91 and TbPHO91 genes mutated in the tyrosines ( Tyr₂²⁷) of the phosphate-binding cluster of the SPX domains (Fig. 9, G and H) described previously as important for binding to inositol polyphosphates (18). Complementation using constructs that express PHO91Y22F or TbPHO91Y22F resulted in significantly reduced or completely abolished currents induced by IP₆ or 5-IP₇ (Fig. 9, G and H). Taken together, our results show that inositol polyphosphates trigger the release of Na⁺/Pᵢ by Pho91 symporters in an SPX-dependent manner.

Discussion

We report the biochemical and electrophysiological characterization of a Na⁺/Pᵢ symporter of *T. brucei*. This transporter, which is an ortholog to *S. cerevisiae* Pho91p, localizes to the acidocalcisomes and is essential for normal growth of PCF trypanosomes in Pᵢ starvation medium. These conditions can be expected when PCF are in the intestine of the tsetse flies under starvation conditions (26) but not in the bloodstream forms (BSF) that are in contact with Pᵢ concentrations in the 1–1.4 mM range (27). The results suggest a role for Pᵢ, produced by hydrolysis of acidocalcisome polyP in cell division under Pᵢ starvation conditions, as occurs in yeast (6). Functional expression in *X. laevis* oocytes followed by two-electrode voltage clamp recordings showed that TbPho91 is a low-affinity, sodium-dependent, and Pᵢ-selective transporter. Application of nanomolar concentrations of 5-IP₇, resulted in Na⁺-dependent depolarization of the oocyte membrane potential and increase in the Pᵢ conductance. Deletion of the SPX domain abolished the stimulation produced by 5-IP₇. Expression of TbPho91 or Pho91p in giant vacuoles of pho91Δ cells allowed the determination of their role in Na⁺/Pᵢ release and their SPX-dependent regulation by inositol polyphosphates.

TbPHO91-KO PCF trypanosomes had a normal growth rate in rich medium but decreased growth in Pᵢ starvation medium. These cells did not have significant differences in the levels of polyP. This last result is in agreement with results obtained with pho91Δ strains that showed (as TbPHO91-KO cells) a slight but not significant increase in polyP (14). The results suggest that polyP levels of *T. brucei* and *S. cerevisiae* vacuoles are mainly controlled through synthesis and not through degradation and release and are in agreement with results where overexpression of the regulatory subunit of the VTC complex leads to higher polyP levels in yeast (28). The results are also consistent with the model proposed in yeast, where polyP serves as a buffer that can be mobilized during phosphate limitation to temporarily maintain internal Pᵢ levels (29). In contrast, the *T. cruzi* ortholog (TcPho91) localizes to the contractile vacuole of the parasite, and down-regulation of its expression reduces the levels of short chain polyP and PPᵢ (16), suggesting that release of Pᵢ is regulated with PPᵢ and polyP levels in this organelle. Overexpression of TcPho91 results in transfer of the protein to the plasma membrane and higher levels of PPᵢ and short-chain polyP consistent with an enhanced Pᵢ transport (16).

Acidocalcisomes of TbPHO91-KO PCF appear to fuse with each other under Pᵢ starvation, resulting in enlarged vacuoles. This phenotype suggests homotypic fusion. In this regard, it has been reported that a VAMP7 ortholog present in acidocalcisomes of *T. cruzi* has a role in their fusion to the contractile vacuole under hypotonic stress (30).

Experiments in *Xenopus* oocytes allowed the characterization of TbPho91 and revealed its SPX-dependent activation by IP₆ and 5-IP₇. One of the drawbacks of using these cells is the difficulty of making deletion mutants. This is essential to suppress endogenous currents that could interfere with currents...
caused by the transporter under investigation (24). This problem sometimes is solved by overexpression of the exogenous transporter, but a more adequate solution is to use giant vacuoles of *S. cerevisiae* that have been deprived of the endogenous transporter (24). An additional advantage of this system is that once patched, vacuoles could be loaded with Na⁺/P_i, and the currents detected indicate their release to the cytosolic site. Using this technique we directly demonstrate for the first time that Pho91 and TbPho91 release Na⁺ to the cytosolic site.

Until now inositol pyrophosphates have been shown to stimulate only one SPX domain–controlled process: polyP synthesis with an EC_{50} of 300–500 nM. However, our results showed that under similar conditions 5-IP₇, but not 1-IP₇, was able to generate currents in giant vacuoles of *T. brucei* harboring either TbPho91 or Pho91p. This differential stimulation of a polyP synthesis and P_i release by the VTC complex of isolated yeast vacuoles. 5-IP₇ directly stimulates this activity.

In conclusion, we have characterized a Na⁺/P_i symporter of *T. brucei* and revealed that inositol pyrophosphates stimulate its activity and that of its yeast ortholog, through their SPX domains. The symporters were shown to be involved in the translocation of polyP and P_i to the cytosolic site of the vacuoles. 5-IP₇ directly stimulates this activity.

## Experimental procedures

### Chemicals and reagents

Phusion high-fidelity DNA polymerase and the Gibson Assembly² cloning kit were from New England Biolabs (Ipswich, MA). Guinea pig polyclonal antibody against TbVP1 was described previously (31). Alexa-conjugated secondary antibodies were purchased from Invitrogen (Thermo Fisher Scientific). The primers were purchased from Integrated DNA Technologies (Coralville, IA). All other reagents of analytical grade were purchased from Sigma–Aldrich. 5-IP₇, 1-IP₇, and PP-IP₄ were synthesized as described previously (17).

### Cell cultures

*T. brucei* Lister 427 strain PCF were cultivated at 28 °C in SDM-79 medium (32) supplemented with 10% heat-inactivated fetal bovine serum and hemin (7.5 mg/ml). For growth in low phosphate medium, the cells were grown in SM medium (32) produced without phosphate and supplemented with heat-inactivated fetal bovine serum (FBS). FBS phosphate content was measured using the Malachite Green assay. Addition of 1% or 5% FBS resulted in a final P_i concentration of 20 or 100 μM P_i, respectively.

### Yeast strains

We used *S. cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). To generate *pho91Δ*, we amplified the sequence of KanMX4 resistance marker containing homology to the 100 nucleotides upstream and downstream of *PHO91* coding sequence using the primers listed in Table 1. BY4741 was transformed with the resulting amplicon via the standard lithium acetate technique (33) and selected for growth on YPD plates supplemented with G418. Constructs for yeast comple-
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Preparation and isolation of giant yeast vacuoles

Preparation of giant yeast from WT and pho91Δ mutants was done as described previously (24) with some modifications. Briefly, mid-log phase yeast were centrifuged; resuspended in 0.1 M Tris-HCl, pH 7.2, and 5 mM DTT; and incubated on a shaker at 50 strokes/min for 10 min. The cells were centrifuged again, washed once with distilled water supplemented with 1 mM DTT, and resuspended in 0.1 M Tris-HCl, pH 7.2, 1 mM DTT, 1 mM sorbitol, and 1 mg/ml Zymolase. The cells were incubated on the shaker for 30 min at room temperature. Once the cells were confirmed to fully convert to spheroplasts, they were centrifuged, and the pellet was carefully resuspended in 6 ml of YPD medium supplemented with 1 mM sorbitol and 0.05% 2-deoxy-D-glucose. The cells were then incubated at 25 °C overnight on a shaker at 50 strokes/min. Giant vacuoles were prepared from giant spheroplasts by hypotonic shock in the recording chamber of the patch-clamp apparatus by incubating the spheroplasts in 10 mM Hepes, pH 7.1, containing 100 mM KCl, 100 mM sorbitol, and 1 mM MgCl2. Then we raised osmolarity to prevent disruption of vacuoles by keeping them in 10 mM Hepes, pH 7.1, containing 100 mM NaCl, 200 mM sorbitol, and 1 mM MgCl2. Patch-clamp recordings were performed on vacuoles attached to the poly-l-lysine–coated chamber. Solution inside the micropipette was 10 mM Hepes, pH 7.1, containing 100 mM NaCl, 200 mM sorbitol, 1 mM MgCl2, 5 mM NaH2PO4, and 5 mM Na2HPO4, whereas bath solution was the same without the addition of NaH2PO4 and Na2HPO4.

Sequence analysis

The analysis of TbPho91 sequence (gene ID Tb427tmp. 01.2950) was performed using Geneious® 10.2.3 software for alignments and BLASTp for searching homologous sequences. Information on number of transmembrane domains and other general information available for this sequence was obtained from TriTrypDB (34).

Molecular constructs for TbPho91 mutant cell lines

For TbpH091 knockout construction in PCF, one TbpH091 allele was knocked out by replacement with a puromycin selectable marker. This cassette was obtained by PCR using primers shown in Table 1 containing 100–120 nucleotides from the 5′- and 3′-UTRs flanking regions of the TbpH091 ORF and the pPOTv6 vector (35) as template. The second allele was knocked out using the same strategy by replacement with a blasticidin selectable marker. The linear constructs were used for transfection of PCF and selection of stable resistant clones. Cell transformations were done as described previously (2). Following each transfection, resistant cells were selected and cloned by limiting dilution in SDM-79 medium containing 10% FBS with appropriate antibiotics (1 μg/ml puromycin, 5 μg/ml blasticidin S) in 24-well plates. Integration of the constructs into genomic DNA of each transfecant was verified by PCR and Southern blot analysis.

Southern blot analysis

This was done as described previously (2). Briefly, we extracted genomic DNA from T. brucei and digested 3 μg of DNA with EcoRI and Ndel (probe for 5′-UTR) or HindIII and NotI (probe for coding sequence). Digestion products were resolved by electrophoresis in 0.8% agarose gels, and the DNA was transferred to Zeta-probe blotting membranes (Bio-Rad) by capillarity using 400 mM NaOH. The membranes were hybridized with a radiolabeled TbpH091 probe, generated by PCR using primers listed in Table 1 to generate probes for the 5′-UTR and coding sequence of the gene, and labeled with [α-32P]dCTP using random hexanucleotide primers and the Klenow fragment of DNA polymerase I (Prime-a-Gene labeling system; Promega). The membranes were analyzed with a PhosphorImager.

Fluorescence and EM

Immunofluorescence assays with T. brucei PCF trypanosomes were done as described previously (2). Antibodies against TbVP1 were used at a dilution of 1:100. Imaging of whole T. brucei PCF and determination of morphometric parameters were done as described previously (36).

P, and polyP quantification

106 cells/ml were washed twice in buffer A with glucose (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 50 mM Hepes, pH 7.3, and 5.5 mM glucose) and immediately resuspended in 100 μl of ice-cold 0.5 M perchloric acid. Lystrate was incubated on ice for 5 min and centrifuged at 15,000 × g for 5 min at 4 °C to remove cell debris. The supernatant was transferred to a new tube, and the pH neutralized with 0.8 mM KOH, 0.8 mM KHCO3. Precipitated KClO4 was removed by centrifugation at 15,000 × g for 5 min, and the supernatant was transferred to a new tube. The final volume of the solution was adjusted to 200 μl. One-half was used for phosphate quantification, and the other half was used for polyP determination. P, and polyP levels were determined using the Malachite Green assay (37) with some modifications. In case of polyP, the levels were determined from the amount of P, released upon treatment with an excess of recombinant S. cerevisiae exopolyphosphatase. PolyP extracts were incubated for 1 h at 37 °C with 50 mM Tris-HCl, pH 7.4, 2.5 mM MgSO4, and 3000–5000 units of purified yeast rPPX. One unit corresponds to the release of 1 pmol of P, min at 37 °C. Malachite Green reagent mix was prepared at least 10 min before use, by mixing 0.045% Malachite Green in water with 4.2% ammonium molybdate in 4 mM HCl at a 1:3 ratio, respectively, and the solution was filtered with syringe filter units. After mixing samples with reagent, we immediately read absorbance at 660 nm. Absorbance values that were within the standard curve done with dilutions of 250 μM KH2PO4 were
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adjusted for a dilution factor and used to determine final Pi, or polyP concentration.

Preparation and maintenance of oocytes

*X. laevis* oocytes were purchased from Xenocyte™ (Dexter, MI) and used as standard heterologous expression system for the study of cloned Na+/Pi co-transporters. Stage IV–V surgically collected oocytes were manually defolliculated and devitellinized with collagenase (1 mg/ml) for 1 h at room temperature and then maintained in filtered Barth’s solution (containing 88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 0.41 mM CaCl₂, 2.4 mM NaHCO₃, 0.33 mM Ca(NO₃)₂, 10 mM Hepes, plus 50 μg/ml gentamicin, pH 7.4) at a density of less than 100/60-mm plastic Petri dish. The Barth’s solution was replaced daily.

cRNA production

Full-length (Tb427tmp.01.2950), truncated *TbPHO91* (*TbPHO91-ΔSPX*), which was obtained by removal of the 606-nucleotide sequence of *TbPHO91* encoding the N-terminal putative SPX domain (residues 1–202), and *PHO91* (GenBank™ accession number NM_001183190) ORFs were amplified with PrimeSTAR HS DNA polymerase (Clontech) from *T. brucei* or *S. cerevisiae* genomic DNA, respectively, by PCR using the corresponding gene specific primers flanking a T₇ promoter, Kozak consensus sequence, or polyT₃₀ sequence as indicated in Table 1. The PCR products were gel-purified using QIAquick gel extraction kit (Qiagen) according to the manufacturer’s instructions, and the double-stranded nucleotide sequences were confirmed by sequencing at GENEWIZ (University, Stanford, CA). Required pH of ND96 was adjusted either with NaOH or HCl. The effects of anions (PO₄³⁻, SO₄²⁻, and NO₃⁻) and inositol polyphosphates were studied by their titrations and NO₃⁻ and isositol polyphosphates were studied by their

Electrophysiology

The standard two-electrode voltage-clamp technique was used, as described previously (38). Briefly, oocytes were placed in a small volume (200 μl) perfusion chamber (RC-3Z; Warner Instruments, Hamden, CT) and superfused with ND96 solution at room temperature. Intracellular recording electrodes made of borosilicate glass capillaries with tip resistance of ~3 MΩ were backfilled with 3 mM KCl. For best temporal resolution and minimization of errors KCl–agarose bridges connected with Ag/AgCl ground electrodes were used. Pho91 activities were evaluated by whole cell voltage clamp in an Oocyte Clamp OC-725 amplifier (Warner Instruments). Recordings were filtered at 500 Hz, digitized at 16-bit 5 kHz using a Digidata 1440 (Axon Instruments, Molecular Devices), and analyzed using PClamp 10 software (Axon Instruments). All experiments were performed at ~60 mV holding potential. Steady-state currents were recorded in the range from −80 to +40 mV during a 1-s period with repeatable 20-mV steps. Responses to Pi, and other anions were always examined at holding potential in the presence or absence of sodium. For I/V curve construction, currents were normalized at ~80 mV. Each experiment was done with at least four oocytes from two different frogs. The quality criteria used for evaluation of cells suitable for electrophysiological recording were a resting membrane potential lower than −30 mV, a leak current smaller than 100 pA, and a resting membrane potential at the end of experiment lower or equal to those at its start.

All recordings were obtained at room temperature. The oocytes were bathed in ND96 buffer bath solution (96 mM NaCl, 2 mM KCl, 5 mM MgSO₄, 1 mM CaCl₂, 2.5 mM Hepes, pH 7.5) with continuous perfusion speed of ~2 ml/min. We used a perfusion valve control system VC-6 (Warner Instruments) for fast local application of modified solutions and drugs as needed (indicated under “Results”) at 2 ml/min speed. Low calcium solutions were prepared by adding of Ca²⁺ and EGTA at proportions, calculated with MaxChelator software (Stanford University, Stanford, CA). Required pH of ND96 was adjusted either with NaOH or HCl. The effects of anions (PO₄³⁻, SO₄²⁻, and NO₃⁻) and isositol polyphosphates were studied by their addition to ND96 with subsequent pH readjustments. High potassium and sodium-free solution (NDX) were prepared by equimolar substitution of sodium with potassium and NMDG, respectively. Phosphate solution was made by mixing of 300 mM stock solutions of mono- and dibasic sodium phosphates until pH 7.4 was obtained.

For the yeast giant vacuole experiments, currents were recorded in whole-vacuole patch-clamp mode. Recording electrodes were prepared as described above and treated with Sigmacote (tip resistance of ~15 MΩ) and then backfilled with recording solution. The recording pipette was applied to the vacuolar membrane for a few minutes to establish gigaseal formation (~10 GΩ). Whole-vacuole configuration was achieved by application of a series of ZAP pulses (1.3 V, 50 ms). Before starting the recording we let the pipette solution diffuse into the vacuole for 5 min. All recordings were performed at Vₚ₀ = +60 mV. An Axopatch 200b amplifier was used for current registration, and the data were filtered at 1000 Hz, digitized with Digidata 1550A (Axon Instruments), and analyzed offline using PClamp 10 software.

Statistical analysis

All of the values are expressed as means ± S.D. unless indicated. Significant differences between treatments were compared using unpaired Student’s *t* tests. Differences were considered statistically significant at *p* < 0.05, and *n* refers to the number of independent biological experiments performed. All
statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

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