Zinc-dependent activation of the Pho8 alkaline phosphatase in *Schizosaccharomyces pombe*

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ABSTRACT

Genome-wide analyses have revealed that during metal ion starvation, many cells undergo programmed changes in their transcriptome or proteome that lower the levels of abundant metalloproteins, conserving metal ions for more critical functions. Here, we investigated how changes in cellular zinc status affect the expression and activity of the zinc-requiring Pho8 alkaline phosphatase from fission yeast (*Schizosaccharomyces pombe*). In *S. pombe*, Pho8 is a membrane-tethered and processed glycoprotein that resides in the vacuole. Using alkaline phosphatase activity assays along with various biochemical analyses, we found that Pho8 is active when zinc is plentiful and is inactive when zinc is limiting. Although Pho8 activity depended on zinc, we also found that higher levels of *pho8* mRNAs and Pho8 protein accumulate in zinc-deficient cells. To gain a better understanding of the inverse relationship between *pho8* mRNA levels and Pho8 activity, we examined the effects of zinc on the stability and processing of the Pho8 protein. We show that Pho8 is processed regardless of zinc status and that mature Pho8 accumulates under all conditions. We also noted that alkaline phosphatase activity is rapidly restored when zinc is resupplied to cells, even in the presence of the protein synthesis inhibitor cycloheximide. Our results suggest that *S. pombe* cells maintain inactive pools of Pho8 proteins in low-zinc conditions and that these pools facilitate the rapid restoration of Pho8 activity when zinc ions become available.

Transition metals such as zinc, iron, and copper are required for the function of a wide range of proteins. As these metals can also be toxic when in excess, most organisms use a variety of mechanisms to control the acquisition, storage, distribution, utilization, and/or export of these essential metals to balance proteomic requirements for a specific metal ion with its availability (1-3).

One widely occurring mechanism to adapt to low levels of metal ions in the diet is a programmed sparing response to reduce the levels of abundant metalloproteins found within cells. These proteomic adaptations can result from changes at the transcriptional, post-transcriptional, or post-translational level, and are often accompanied by the up-regulation of compensatory proteins or pathways that do not require a metal cofactor or that use an alternative metal cofactor (4-9). In addition to conserving and salvaging metal ions for essential functions, metal sparing responses also facilitate the reallocation of metal ions inside of cells and prevent cells from synthesizing high levels of non-functional proteins, which in the absence of the correct metal ion are potentially more prone to mis-folding or mis-metalation (10,11).
In this report we used *S. pombe* as a model system to investigate how changes in cellular zinc status affected the activity of the vacuolar Pho8 alkaline phosphatase. We chose to study Pho8 as it belongs to a well characterized family of proteins that bind multiple zinc ions (12). Additionally, in other yeasts alkaline phosphatases are targeted for degradation in low zinc suggesting that these proteins may be removed as part of a zinc sparing or salvaging response (13). We show that in fission yeast Pho8 activity is dependent upon cellular zinc status, and that it is only active when zinc ions are plentiful. Unexpectedly, we also found that higher levels of *pho8* transcripts and Pho8 protein accumulate in zinc-limited cells, whereas Pho8 activity is dependent upon zinc. While at first it seems surprising that cells would synthesize and process higher levels of an inactive enzyme under conditions of zinc deficiency, we also find that Pho8 is rapidly activated by the re-addition of zinc. We propose that increased expression of Pho8 in low zinc is a proactive mechanism to enable cells to rapidly restore Pho8 activity when zinc ions are available.

### Results

**Pho8 activity in fission yeast is dependent upon zinc**

Fission yeast express two enzymes with alkaline phosphatase activity: Pho8, a membrane tethered, vacuolar enzyme that is predicted to bind one magnesium ion and two zinc ions per monomer; and Pho2, an unrelated cytosolic enzyme of unknown function (14). To examine the effects of zinc on Pho2 and Pho8 function we measured alkaline phosphatase activity in wild-type cells and in *pho2Δ* and *pho8Δ* mutants that were grown overnight in a zinc-limited minimal medium (ZL-EMM) supplemented with 0-100 μM zinc. As shown in Figure 1A, ~2-fold higher levels of alkaline phosphatase activity were detected in zinc-replete wild-type cells compared to zinc-limited cells. This activity was dependent upon Pho2 and Pho8 as the deletion of *pho2* and *pho8* nearly abolished alkaline phosphatase activity under all growth conditions, whereas deletion of *pho2* or *pho8* led to reduced, but measurable alkaline phosphatase activity. We also found that an ~10-fold increase in alkaline phosphatase activity in *pho2Δ* cells in the presence of 100 μM zinc, whereas in *pho8Δ* cells, alkaline phosphatase activity was detected in both zinc-limited and zinc-replete growth conditions. Taken together, these results indicate that Pho8 activity is dependent upon zinc and that alkaline phosphatase activity in *S. pombe* is the sum of Pho8 and Pho2 activities.

The zinc-dependent changes in Pho8 activity are independent of changes in *pho8* mRNA levels

The zinc-dependent increase in Pho8 activity could potentially result from changes in the levels of *pho8* mRNA and/or changes in the level, processing, or activity of the Pho8 protein. To determine if *pho8* gene expression was affected by zinc status, RNA blot analysis was used to examine *pho8* transcript levels in cells grown over a range of zinc levels. RNA blots were also probed for *pho2* and *zrt1*, a gene that is specifically expressed when zinc is limiting (15). In wild-type and *pho2Δ* cells higher levels of *pho8* transcripts accumulated in zinc-limited cells compared to zinc-replete (Fig. 1B and 1C), indicating that *pho8* is expressed at a higher level under a condition where the Pho8 protein is not active.

In *S. pombe* a transcription factor named Loz1 plays a central role in zinc homeostasis by inhibiting gene expression when zinc is in excess (15,16). To determine if *pho8* expression was dependent upon Loz1, we examined *pho8* transcript levels in wild-type and *loz1Δ* cells grown over a range of zinc levels. Deletion of *loz1* resulted in higher levels of *pho8* transcripts accumulating in zinc-replete cells, consistent with Loz1 facilitating the repression of *pho8* gene expression in high zinc (Fig. 2A). To test whether these changes also affected Pho8 protein level, we expressed the Pho8-GFP fusion protein from the *pho8* promoter. This fusion protein accumulated at higher levels in zinc-deficient cells in a manner that was dependent upon Loz1 (Fig. 2B). Taken together these results suggest that *pho8* is repressed in a Loz1-dependent manner when zinc is in excess, and that the growth in low zinc leads to the derepression of *pho8* gene expression, which in turn leads to higher levels of *pho8* transcripts and Pho8 protein accumulating under this condition.

To further explore the effects of gene expression on alkaline phosphatase activity, we
generated constructs to overexpress Pho8-GFP and Pho2-GFP fusion proteins from the strong constitutive phosphoglycerate kinase 1 (pgk1) promoter. Expression from the pgk1 promoter was not affected by zinc (Fig. 3A and 3B), and led to an ~16-fold increase in Pho8-GFP protein abundance in low zinc, and an ~27-fold increase in high zinc, relative to expression from the pho8 promoter (Fig. 3A). Despite the large increase in Pho8-GFP abundance, Pho8 activity remained dependent upon zinc, with growth in ZL-EMM + 100 µM Zn leading to an ~45-fold increase in alkaline phosphatase activity relative to the activity detected in cells grown with no added zinc (Fig. 3C). In contrast, over-expression of Pho2-GFP led to a large increase in alkaline phosphatase activity under all growth conditions (Fig. 3D). Taken together, these results indicate that the zinc-dependent changes in Pho8 activity are independent of changes to pho8 gene expression and transcript levels.

The processing of the Pho8 precursor is not affected by zinc status

Pho8 is synthesized as an inactive pro-form containing an inhibitory C-terminal propeptide (Fig. 4A), which in fission yeast is cleaved in a manner that is dependent upon vacuolar serine proteases Isp6 and Psp3 (17). Therefore, an alternative explanation for the zinc-dependent increase in Pho8 activity is that the processing of the pro-form of Pho8 to the mature protein is dependent upon cellular zinc status. To examine Isp6- and Psp3-dependent processing, immunoblot analysis was used to compare the forms of Pho8-GFP that accumulated in wild-type and MGF317 cells. MGF317 is a quintuple deletion strain, which is deficient in multiple proteases including Isp6 and Psp3 (18). Incubation of immunoblots with GFP antibodies revealed two major bands in wild-type cells expressing Pho8-GFP (Fig. 4B). One of these bands was ~95 kDa in size and co-migrated with the inactive, unprocessed form of Pho8 that accumulated in MGF317 cells (Fig. 4B and 4C). The other major band was ~27 kDa, which is the predicted size of the cleaved propeptide fused to GFP. Very low levels of this smaller band were detected in MGF317 cells, consistent with this latter designation.

When evaluating the effect of zinc status on the levels of the Pho8 precursor, we found that in some, but not all, experiments lower levels of pgk1-driven Pho8-GFP precursor protein were detected in zinc-replete cells, suggesting that another factor potentially affected Pho8 protein abundance (Fig. 4B). As previous studies had shown that the activity of Isp6 increases as cells enter stationary phase (19) and cells grown under zinc-limiting conditions grow more slowly than cells grown in high zinc, we repeated the above experiments using cell lysates that were prepped from cells harvested at precise cell optical densities (ODs). In both zinc-limiting and zinc-replete conditions, Pho8-GFP processing was dependent upon cell OD600 (Fig. 4D). Despite the apparent differences in processing, Pho8 activity was only affected by zinc status (Fig. 4E). Based on these results we conclude that although processing of Pho8 is dependent upon the growth phase of cells, zinc is the major factor that limits Pho8 activity in vivo.

The stability of Pho8 is not affected by zinc status

In the above experiments we were unable to detect the mature form of Pho8 following processing. To visualize the mature Pho8 we generated a new construct to express a pgk1-driven Pho8 protein containing an internal in-frame 3xHA epitope tag. The 3xHA tag was positioned so that it would remain fused to the C-terminal end of the mature Pho8 protein upon cleavage of the propeptide (Fig. 5A, Pho8-HA). We also generated a related strain expressing a truncated form of Pho8 that did not contain the 23 amino acid C-terminal inhibitory propeptide (Fig. 5A, Pho8-HA ΔC). Expression of the Pho8-HA and Pho8-HA ΔC fusion proteins in pho2Δ pho8Δ cells resulted in an ~6.5-fold and ~5-fold increases in alkaline phosphatase activity in high zinc respectively, as compared to the endogenous pho8 under this condition, indicating that both constructs were functional (Fig. 5B, pho2Δ vector).

To determine if zinc status affected the stability of the mature Pho8 protein, immunoblot analyses were performed using protein extracts prepped from pho2Δ pho8Δ and MGF317 cells expressing Pho8-HA or Pho8-HA ΔC. Two major bands were detected in pho2Δ pho8Δ cells expressing Pho8-HA (Fig. 5C). The larger band
co-migrated with the unprocessed Pho8-HA protein detected in MGF317 cells, whereas the smaller band co-migrated with the mature Pho8-HA ΔC protein. These results are consistent with the larger Pho8-HA band being the unprocessed protein and the smaller band being the mature Pho8-HA protein.

In assessing the effects of zinc status on Pho8 protein levels, we found that the mature form of Pho8-HA and Pho8-HA ΔC proteins accumulated in both zinc-limiting and zinc-replete cells (Fig. 5C). An unexpected result was that higher levels of the Pho8-HA ΔC protein were detected in zinc-limited cells, suggesting that the process of removing the inhibitory peptide leads to increased Pho8 turnover, particularly under low zinc conditions. While further studies are needed to test this hypothesis, these results further highlight that inactive forms of Pho8 accumulate within zinc-deficient cells. As a complementary approach to examine the effects of zinc binding on the stability of Pho8, we generated derivatives of Pho8-HA containing amino acid substitutions that prevented zinc from binding to the first zinc binding site (Pho8-m1), the second zinc binding site (Pho8-m2), or both sites (Pho8-m1/2). No alkaline phosphatase activity was detected in pho2Δ pho8Δ cells expressing these zinc-binding mutants, consistent with zinc binding to both sites being critical for function (Fig. 5B). When the effects of these mutations on Pho8 protein levels were analyzed by immunoblot analysis, slightly lower levels of the mature forms of Pho8-m1 and Pho8-m1/2 accumulated to lower levels in zinc-limited cells, whereas the levels of the mature Pho8-HA-m2 were not significantly altered by zinc levels (Fig. 5D). These results suggest that apo and holo forms of Pho8 accumulate in cells, and that loss of zinc from site 1 may interfere with the processing or stability of the mature Pho8 protein in low zinc conditions.

Pho8 dimerization is independent of zinc status

Both experimental and structural analyses of alkaline phosphatases from a wide range of species have shown that they exist and function as homodimers (12). We therefore next tested whether the dimerization of Pho8 was dependent upon zinc. To examine dimerization, whole cell lysates were generated from zinc-limited and zinc-replete pho2Δ pho8Δ cells that co-expressed a pho8Δ-driven Pho8-GFP fusion, and a pgkl-driven Pho8-HA fusion. The lysates were then added to protein A magnetic beads, which had been preincubated with anti-GFP antibodies. After incubation for 3 hr to allow binding, the beads were washed, and proteins eluted. To determine if the Pho8-GFP and Pho8-HA proteins physically interacted, immunoblots of the protein extracts were incubated with anti-HA antibodies. As shown in Figure 6A, Pho8-GFP and Pho8-HA proteins were detected in immunoprecipitates from zinc-limited and zinc-replete cells, consistent with the Pho8-GFP and Pho8-HA proteins forming dimers under all growth conditions.

Activation of Pho8 by zinc

As zinc did not affect Pho8 stability, processing, or dimerization, we hypothesized that the activity of Pho8 is directly affected by cellular zinc status. In fission yeast, three CDF family members Cis4, Zrg17, and Zhf1 transport zinc
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...from the cytosol into the secretory pathway (22,23). Cis4 and Zrg17 form a complex that transports zinc ions into the cis-Golgi, whereas Zhf1 plays a primary role in protecting the cytosol from zinc toxicity by transporting excess zinc ions into stores within the endoplasmic reticulum and potentially the vacuole (23,24). To determine if these transporters were required for the activation of Pho8, alkaline phosphatase activity was examined in cis4 and zrg17 deletion mutants that were grown overnight in ZL-EMM supplemented with 0-100 µM zinc (Fig. 7). As zhf1Δ cells are unable to grow in liquid medium supplemented with ≥ 10 µM zinc (22,24), these cells were grown in ZL-EMM with 0 or 1 µM zinc. Deletion of cis4 and/or zrg17 did not significantly alter alkaline phosphatase activity. Unexpectedly, a slight increase in alkaline phosphatase activity was detected in zhf1Δ cells grown in ZL-EMM with 0 or 1 µM zinc.

To further assess the role of Zhf1 in zinc delivery to Pho8 we examined alkaline phosphatase activity following a zinc shock. In zinc shock experiments, cells are pre-grown in zinc-deficient medium before being exposed to zinc (i.e. the zinc shock). As zinc-deficient cells express high levels of the Zrt1 zinc uptake system, this growth method results in a rapid influx of zinc immediately upon its addition to the growth medium (22). When a zinc shock was performed on zinc-limited wild-type cells, a rapid ~3-fold increase in alkaline phosphatase activity was detected (Fig. 8A t = 20 min). This activity was dependent upon Pho8 consistent with zinc entering the cells and being incorporated into Pho8 leading to its activation. Zinc shock also led to a rapid increase in alkaline phosphatase activity in zhf1Δ or zrg17Δ cis4Δ cells, indicating that zinc was incorporated into Pho8 in these mutants.

Potential explanations of the increase in alkaline phosphatase activity in the zhf1Δ and zrg17Δ cis4Δ strains following a zinc shock include that Zhf1 and the Cis4-Zrg17 complex have a redundant role in supplying zinc ions, and that there are other routes by which zinc ions enter the secretory pathway. To distinguish between these possibilities, we examined alkaline phosphatase activity in a cis4Δ zrg17Δ zhf1Δ triple mutant (Fig. 8A). In this strain a significant decrease in alkaline phosphatase activity was detected following a zinc shock, suggesting that Zhf1 and the Cis4-Zrg17 complex have a largely redundant role in supplying zinc ions to Pho8.

To gain additional evidence that the Zhf1 and the Cis4-Zrg17 complex both supply zinc ions to Pho8, alkaline phosphatase activity was examined in wild-type cells and in various mutant strains expressing the pgk1-driven Pho8-HA. In wild-type cells expressing this fusion an ~18-fold increase in alkaline phosphatase activity was detected upon the addition of zinc compared to those expressing the empty vector (Figure 8B, t = 0 + Zn). A rapid increase in alkaline phosphatase activity was also observed in zrg17Δ cis4Δ and zhf1Δ mutants, but not in the triple mutant. Taken together these results indicate that both Zhf1 and the Cis4-Zrg17 complex are able to supply zinc ions for Pho8 function.

If zinc is required for Pho8 activity, why would cells express, process, and accumulate Pho8 under conditions of zinc-deficiency? Most microorganisms are able to rapidly adapt to changes in the surrounding environment. One potential explanation for cells maintaining an inactive pool of Pho8 is therefore that as soon as zinc is available it is incorporated into Pho8 allowing rapid restoration of activity. To test this hypothesis pho2Δ pho8Δ mutants expressing the Pho8-HA ΔC from the pgk1 promoter, or Pho8-GFP from the pho8 promoter were grown overnight in zinc-limited medium before being shocked with zinc. In contrast to the previous experiments, zinc-limited cells were also incubated for 30-120 min with the protein synthesis inhibitor cycloheximide prior to the zinc shock (Fig. 9A and Fig. S1). In fission yeast the median half-life for a protein is ~12 hr (25). Incubation with cycloheximide led to a large decrease in Pho8-HA ΔC and Pho8-GFP levels, suggesting that Pho8 has a shorter half-life than most proteins in S. pombe (Fig 9A lower panel). Despite this decrease, zinc shock with 100 µM zinc led to an immediate increase in alkaline phosphatase activity, indicating that it is possible to activate Pho8 in the absence of new protein synthesis (Fig 9A and 9B upper panels). This increase was also dependent upon Cis4, Zrg17, and Zhf1 (Fig. 9C). Taken together these results are consistent with yeast maintaining an inactive...
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pool of Pho8 in low zinc, which can be rapidly activated as soon as zinc is available.

**Discussion**

In this study we investigated how changes in cellular zinc status affect the expression and activity of the fission yeast Pho8 vacuolar alkaline phosphatase. In general, increased expression of a gene in response to an environmental stress indicates that it has some important cellular function under this condition. Here we show that pho8 mRNAs and Pho8 proteins accumulate at higher levels in zinc-deficient cells, and that Pho8 is only active when zinc is restored to cells. We propose that under zinc-deficient conditions, fission yeast maintain an inactive pool of Pho8, which allows the rapid activation and restoration of Pho8 activity as soon as zinc is available.

To gain a better understanding of why high levels of pho8 mRNA accumulate in low zinc conditions, we examined pho8 transcript abundance in a loz1 deletion strain (Fig. 2). In fission yeast the transcription factor Loz1 plays a primary role in controlling zinc homeostasis by regulating zinc acquisition and zinc sparing (15,16,26). When zinc levels are high Loz1 is required for the repression of gene expression, whereas when zinc levels are low Loz1 is inactive, leading to the de-repression of its target genes. Our results revealed that higher levels of pho8 mRNA in loz1Δ mutants in high zinc, consistent with pho8 being repressed by Loz1 under this condition.

In addition to alkaline phosphatases, multiple other enzymes reside or obtain their zinc cofactor within compartments of the secretory pathway, including protein disulphide isomerases and GPI-phosphoethanolamine transferases (27,28). As some of these enzymes are essential for life, how would cells prioritize the transport of zinc to these proteins, and not to Pho8, when zinc is limiting? One possibility is that mechanisms are present in S. pombe to deliver zinc ions to essential zinc proteins. For example, in humans the zinc-dependent activation of secreted TNAP is dependent on a direct interaction with specific CDF zinc transporters (29). If related mechanisms were present in S. pombe, by prioritizing the delivery of zinc ions to other proteins in low zinc, a situation may exist where there were insufficient zinc ions for the metalation of Pho8.

An alternative possibility is that some aspect of Pho8 processing under conditions of zinc deficiency prevents it from binding its zinc cofactor, or from being activated. A notable precedent for this type of control occurs in S. cerevisiae. In this yeast, the Pho8 alkaline phosphatase is degraded in zinc-limited cells in a manner that is dependent upon the Pep4 peptidase (13). Although Pho8 protein accumulates in low and high zinc in S. pombe, we also detect multiple higher molecular weight species of Pho8 MGF317 cells. The strain MGF317 contains deletions in multiple genes including: the Psp3 and Isp6 vacuolar serine proteases; the carboxypeptidase Sxa2; the endonuclease Cdb4; and a putative di/aminopeptidase Xpa1, which is predicted to reside within the endoplasmic reticulum. At present, we do not know if the higher molecular weight Pho8 species are linked to the deletion of one or a combination of these genes. However, there are multiple mechanisms that could result in the further post-translation modification of Pho8 in the secretory pathway. For example, misfolded proteins are substrates for endoplasmic reticulum-associated protein degradation (ERAD), which leads to their ubiquitination and ultimate destruction by the proteasome (30). Recent studies in S. cerevisiae have also shown that Cot1, a vacuolar zinc transporter, is specifically ubiquitinated and targeted for degradation in low zinc conditions (31). In our current studies we are investigating the nature of these modifications and whether they are linked to Pho8 inactivity in low zinc.

Our findings also include that multiple zinc transporters belonging to the CDF family have a largely redundant role in supplying zinc ions to Pho8. This differs from other characterized alkaline phosphatases that require zinc transport through specific zinc transporters for their activation. For example, in humans the activation of TNAP is dependent upon zinc transport via ZnT5-ZnT6 heterodimers or ZnT7 homodimers (29). TNAP activation also depends on the presence of a di-proline motif (PP), which
is located in the second luminal loop of the ZnT5 and ZnT7 CDF family members (32). The zinc-dependent activation of Pho8 in \textit{S. cerevisiae} is also dependent upon zinc transport via the vacuolar Zrc1 and Cot1 transporters, but not two other CDF family members (Msc2 and Zrg17) which transport zinc ions into the endoplasmic reticulum (13). As Zhf1 and Cot1 both contain the PP motif, whereas Msc2 and Zrg17 do not, it has been suggested that the PP motif may also be important for the activation of alkaline phosphatases in yeast (32). Consistent with this hypothesis the PP motif is conserved in Cis4 and Zhf1 in \textit{S. pombe}. However, genetic mutations that disrupt this PP motif in Zhf1 have no effect on the zinc-dependent activation of Pho8, or Zhf1 transporter function (Fig. S2). Thus, our results indicate that the PP motif is not required for Pho8 activation in \textit{S. pombe}, at least when zinc transport occurs via Zhf1.

In summary, our results show that Pho8 activity is dependent upon cellular zinc status in \textit{S. pombe}. As Pho8 activity is also dependent upon zinc in \textit{S. cerevisiae}, this regulation appears conserved in these evolutionary distant yeasts. Despite this conservation, we find many differences in the regulation of Pho8 levels between the two yeast model systems. One of most striking differences is that in \textit{S. pombe} pho8 is expressed at higher levels under conditions of zinc deficiency, which leads to high levels of inactive Pho8 proteins accumulating inside of cells. While many metalloenzymes are regulated at a transcriptional level by metalloregulatory factors, it is unusual for cells to increase the expression of the apo-form of an enzyme when the metal cofactor is limiting. In our work we also show that the addition of zinc to zinc-limited cells leads to the rapid reactivation of Pho8, providing an explanation for why \textit{S. pombe} up-regulates pho8 expression under this condition. While the presence of pools of apo- or inactive forms of metalloenzymes inside of cells is well documented, it is not always possible to reactivate apo-forms of protein by the addition of the correct metal cofactor (34-36). While we do not yet know why some apo-proteins are able to accumulate and be reactivated while others are not, it is noteworthy that deficiencies in metals such as zinc and iron are world-wide health problems and many genetic diseases result from deficiencies in these and other metals ions (37-39). A greater understanding of the mechanisms by which apo-proteins are able to safely accumulate inside of cells, and how some proteins can be repopulated with metals ions whereas others cannot, will potentially provide more insight into the molecular and clinical consequences of metal ion deficiencies.

**Experimental Procedures**

**Yeast strains and growth conditions**

The genotypes of yeast strains used in this study are shown in Table S1. Under non-selective conditions strains were grown on yeast extract with supplements (YES) medium which contained 0.5% yeast extract, 3% glucose, 225 mg/l of adenine, uracil, and leucine. To select for the integration of plasmids, strains were grown in synthetic Edinburgh Minimal Medium (EMM), with the appropriate nutrient(s) being dropped out to allow for selection. To examine the effects of zinc status on alkaline phosphatase activity, cells were pre-grown to exponential phase in YES medium. Cells were then transferred to plastic tubes and washed twice in Zinc-Limited Edinburgh Minimal Medium (ZL-EMM), a derivative of EMM which does not contain zinc. Cells were inoculated into ZL-EMM with or without ZnCl$_2$ at an initial OD of ~0.03 and were either grown overnight or to the indicated OD$_{600}$.

**Plasmids**

The \textit{pho8} gene was amplified from \textit{S. pombe} genomic DNA by PCR and was cloned into the PstI/BamHI sites of the vector pBluescript SK (+) to generate pSK-pho8. The PstI/BamHI \textit{pho8} fragment was then subcloned into similar sites in the vector JK-pgk1-GFP to generate Pho8-GFP. JK-pgk1-GFP contains 840 bp of the \textit{pgk1} promoter inserted into the KpnI/EcoRI sites of the vector JK-GFP. A related strategy was used to generate Pho2-GFP, except that \textit{pho2} was introduced as an EcoRI/BamHI fragment into JK-pgk1-GFP. The Pho8-HA construct was generated by an overlapping PCR using primers that introduced PstI and Ascl sites at the 5’ and 3’ ends of the final overlap PCR product. Primers were designed so the final overlap PCR product
encoded the Pho8 protein containing 3 in-frame HA epitope tags inserted between amino acids 509 and 510 of Pho8. The PstI/Ascl Pho8-HA fragment replaced the PstI/Ascl pho8-GFP gene fusion in the plasmid Pho8-GFP, to generate the plasmid Pho8-HA. A plasmid Pho8-HA DC was generated using the PCR with Pho8-HA as a template. The PCR primers for this reaction were designed to introduce a STOP codon immediately after the 3 x HA coding region, which removes the last 23 amino acids of Pho8. To generate an additional pho8 vector that could be used for CoIP analysis, 406 bp of the pho8 promoter were amplified using primers containing KpnI/PstI restriction site. The KpnI/PstI-digested pho8 promoter was then used to replace the KpnI/PstI pgk1 promoter fragment in the vector Pho8-GFP. The pho8 promoter and pho8-GFP gene fusion were then released by a KpnI/SacI digest and subcloned into similar sites in the vector JK210 (ATCC). For site-directed mutagenesis, the pho8-HA gene fusion was subcloned as a PstI/AscI fragment into the vector pSK-KanMX6, a derivative of pBluescript SK (+) containing an AscI site. QuikChange mutagenesis was then used to introduce mutations in the coding sequence of Pho8 which encoded amino acid substitutions preventing zinc binding to site 1 in Pho8 (D311A, H315A), to site 2 (D352A, H353A), or to both sites. The mutated products were subcloned into the vector JK-pgk1-GFP to create Pho8-HA m1, Pho8-HA m2, and Pho8-HA m1/2 respectively. Correct clones were confirmed by DNA sequencing analysis.

Alkaline phosphatase activity assays

Alkaline phosphatase assays were performed as described previously (13) with the exceptions that 65 µl of 100 mM p-nitrophenol phosphate (PNPP) was added to 650 µl of permeabilized cells to start the reaction, and the reaction was stopped by the addition of 130 µl of 3M NaOH. Units are calculated as 1000x ΔA420/volume of cells assayed (in ml)/reaction time (in mins)/OD600 of the culture. Cell free reactions were included as blanks. Activities shown represent the average values of three independent experiments. Where appropriate, data were analyzed by a Student unpaired t-test. A p value of <0.05 was considered statistically significant.

RNA extraction and RNA blotting

Total RNA was extracted using the hot acidic phenol method (40). For this method, 5 ml of cells were harvested by centrifugation at 3500 rpm for 2 min, and cell pellets resuspended in 0.5 ml of TES solution (10 mM Tris HCl, pH 7.5, 10 mM EDTA, and 0.5% SDS). Cell suspensions were lysed by the addition of an equal volume of acid phenol and incubation for 1 hr at 65°C, with brief vortexing at 20 min intervals. Cell lysates were placed on ice for 2 min, followed by centrifugation at 12,000 rpm for 5 min. The upper aqueous layer was then transferred to a new tube and total RNA precipitated by the addition of 3 volumes of ice cold 100% EtOH. After centrifugation at 12,000 rpm for 10 min, total RNA was resuspended in 100-200 µl of 10 mM Tris pH 8.5. Total RNA (10 µg) was analyzed by RNA blot hybridization as outlined by Sambrook et al (41). 32P-labelled strand specific RNA probes were generated using the MAXIscript T7 In Vitro Transcription kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Signal intensities representing transcript abundance were visualized using GE Typhoon FLA 9500.

Protein extracts and immunoblotting

For experiments examining the processing and stability of Pho8-GFP and Pho8-HA fusions, protein lysates were prepared using a trichloroacetic acid (TCA) precipitation (42). In this method, 5 ml of cells were harvested by centrifugation for 2 min at 3500 rpm. Cell pellets were resuspended in 0.5 ml of ice-cold buffer A (20 mM Tris (pH 8.0), 50 mM NH4OAc, 0.5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride). Following the addition of 0.5 ml of ice-cold TCA and ~ 0.3 g of glass beads, cells suspensions were lysed by vortexing for 3 x 30 s, with 2 minutes on ice between each round of vortexing. Supernatants were transferred to a new tube and proteins pelleted by centrifugation for 10 min at 12,000 rpm. Pellets were resuspended in trichloroacetic acid-sample buffer (3% SDS, 100 mM Tris base pH 11, 3 mM DTT), and were boiled for 10 min. After removal of cell debris by centrifugation for 5 min at 12,000 rpm, protein extracts were separated on either 8, 10, or 12 % (wt/vol) SDS PAGE gels, followed by
immunoblotting to a PVDF membrane. Immunoblots were incubated with anti-GFP (Millipore SIGMA G1544), anti-HA (12CA5 – Millipore SIGMA), and anti-Act1 (Abcam ab3280 and Nova Biochemicals NB100-74340) primary antibodies, and IR-Dye800CW and IRDye680 IgG (LICOR) secondary antibodies. Signal intensities were analyzed using the Odyssey Infrared imaging System (LICOR).

EGS crosslinking

Protein extracts for EGS crosslinking were generated in a method that was adapted from the procedure described by previously (20). Briefly, 5 ml of yeast cells were harvested by centrifugation, washed 1x with cold dH2O, and were transferred to 2 ml tubes. Cells were resuspended in 450 µl HEGNT lysis buffer (20 mM HEPES pH=7.5, 1 mM EDTA, 10% glycerol, 0.4 M NaCl, 1% Triton X-100) containing a protease inhibitor cocktail and 1 mM phenylmethylsulphonyl fluoride (PMSF). Cells were lysed by vortexing with glass beads for 5 x 30s with 2 mins on ice between pulses. After cell beads were removed by centrifugation, cell lysates were centrifuged for 10 min at 14,000 rpm. Cell lysates were transferred to a new microfuge tube and total protein concentration measured using a Bradford Assay. For EGS crosslinking, 100 µg of protein extract was incubated with either DMSO or 1 mM EGS for 30 min at 24°C. Reactions were quenched by the addition of 50 mM glycine/0.025 mM Tris pH=7.5.

Co-Immunoprecipitations

25 mls of yeast cells were grown overnight in ZL-EMM supplemented with 0 or 100 µM zinc. Following centrifugation for 2 min at 3500 rpm, cells were washed twice in phosphate buffered saline (PBS), before being resuspended in 1 ml of cold lysis buffer (20 mM Tris HCl pH 8.0, 100 mM NaCl, 1% Triton-X100, 2 mM EDTA) containing 1 mM PMSF. Cell suspensions were lysed by vortexing in the presence of zirconium beads for 5 x 1 min, with 2 min on ice between pulses. Zirconium beads were removed by centrifugation for 1 min at 3500 rpm, and cell lysates centrifuged at 14,000 rpm for 5 min. Cell lysates containing soluble proteins were transferred to a new microfuge tube and total protein concentration measured using a Bradford Assay. For immunoprecipitations, 500 µg of cell lysates were incubated with protein A dynabeads prebound with 2 µg anti-GFP antibodies (Abcam ab290) for 3 hr at 4°C. Dynabeads were washed 3x in lysis buffer and bound proteins eluted by boiling in 50 µl SDS-PAGE gel loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% bromophenol blue, 100 mM DTT).

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Conflict of Interest:
The authors declare that they have no conflicts of interest with the contents of this article.

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**Footnotes:** The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
Figure 1. Effects of zinc on alkaline phosphatase activity and gene expression. (A) Wild-type, \( \text{pho2}\Delta, \text{pho8}\Delta, \) and \( \text{pho2}\Delta \text{pho8}\Delta \) cells were grown overnight in ZL-EMM supplemented with 0, 1, 10, or 100 µM zinc and were assayed for alkaline phosphatase activity. Activity is the mean of three independent repeats with the error bars representing standard deviations. (B) RNA blot analysis was performed using total RNA from wild-type, \( \text{pho2}\Delta, \) and \( \text{pho8}\Delta \) cells that were grown overnight in ZL-EMM supplemented with 0, 1, 10, or 100 µM zinc. RNA blots were probed for \( \text{pho8}, \text{pho2}, \) and \( \text{zrt1} \) mRNAs, with ribosomal RNAs shown for a loading control. (C) Relative levels of \( \text{pho8} \) transcripts normalized to ribosomal RNAs. Data are shown as the mean ± SD from three independent replicates. \( p \) values were determined using the two-tailed unpaired student's t-test. **\( p<0.01. \)
Figure 2. Pho8 is regulated in a manner that is dependent upon Loz1.
(A) RNA blot analysis was performed using total RNA from wild-type, loz1Δ, and loz1Δ cells expressing a functional Loz1-GFP fusion protein. Cells were grown overnight in ZL-EMM supplemented with 0, 1, 10, or 100 µM zinc. RNA blots were probed for pho8 and zrt1 mRNAs. Ribosomal RNAs are shown for a loading control. (B) Immunoblot analysis of protein extracts prepped from pho8Δ and pho8Δ loz1Δ cells containing the empty vector or expressing Pho8-GFP from the pho8 promoter. Cells were grown overnight in ZL-EMM supplemented with 0 or 100 µM zinc. The positions of the molecular weight markers in kDa are shown on the left. The lower panel shows the mean ratio of Pho8-GFP/Act1 levels from three independent repeats with error bars representing standard deviations. p values were determined using the two-tailed unpaired student's t-test. *p<0.05, **p<0.01
Zinc-dependent alkaline phosphatase activity

Figure 3. Effects of overexpression of *pho8* and *pho2* on alkaline phosphatase activity. (A) Immunoblot analysis of protein extracts prepped from *pho8Δ* cells expressing the indicated fusion proteins following growth in ZL-EMM supplemented with 0, 1, 10, or 100 µM zinc, or the empty vector following growth in ZL-EMM + 100 µM zinc. Protein extracts prepped from *pho8Δ* cells expressing *pgk1*-Pho8-GFP were diluted by 5-fold. The positions of the molecular weight markers in kDa are shown on the left and an arrow indicates the full length Pho8-GFP protein. The lower panels show the mean ratio of Pho8-GFP/Act1 levels from three independent repeats with error bars representing standard deviations. **p<0.01, ns, not significant. (B) Immunoblot analysis were performed as described in panel A, with the indicated strains. (C) Wild-type, *pho2Δ*, and *pho2Δ pho8Δ* cells bearing the empty vector, and *pho8Δ*, and *pho2Δ pho8Δ* cells expressing Pho8-GFP were assayed for alkaline phosphatase activity following growth overnight in ZL-EMM supplemented with 0, 1, 10, or 100 µM zinc. Activity is the mean of three independent repeats with the error bars representing standard deviations. (D) Wild-type, *pho2Δ*, and *pho2Δ pho8Δ* cells bearing the empty vector, and *pho2Δ*, and *pho2Δ pho8Δ* cells expressing Pho2-GFP were grown and assayed for alkaline phosphatase activity as described in panel C.
Figure 4. The processing of Pho8 is dependent upon growth phase and not zinc. (A) Schematic diagram of Pho8-GFP highlighting the positions of the transmembrane domain (TM), cleavage site, C-terminal propeptide, and GFP. (B) Wild-type cells bearing the empty vector and wild-type and MGF317 cells expressing Pho8-GFP were grown for 16 hr overnight in ZL-EMM supplemented with 0 (-Zn) or 100 µM (+Zn) zinc, and protein extracts prepped for immunoblot analysis. Immunoblots were incubated with anti-GFP antibodies and anti-Act1 (Actin) antibodies for a loading control. The positions of the molecular weight markers in kDa are shown on the left. (C) Alkaline phosphatase activity was assayed in the indicated strains following growth for 16 hr in ZL-EMM supplemented with 0 or 100 µM zinc. Each is the mean of three independent repeats with the error bars representing standard deviations. (D and E) pho8Δ and pho2Δ pho8Δ cells expressing Pho8-GFP were grown overnight in ZL-EMM supplemented with 0 (-Zn) or 100 µM (+Zn) zinc and were harvested at OD600 of ~4.0, ~6.0, and ~8.0. Protein extracts preparation and immunoblot analysis were performed as described above (D) or cell lysates were assayed for alkaline phosphatase activity as described above (E).
Zinc-dependent alkaline phosphatase activity

**Figure 5. Pho8 stability is not affected by zinc status.** (A) Schematic diagram of Pho8-HA and Pho8-HA ΔC highlighting the positions of the transmembrane domain (TM), 3 x HA epitope, cleavage site, and C-terminal propeptide (blue box). (B) Alkaline phosphatase activity was assayed in the indicated strains that were grown in ZL-EMM supplemented with 0 or 100 µM zinc to an OD$_{600}$ of ~5. Activity is the mean of three independent repeats with the error bars representing standard deviations. (C) The indicated strains were grown in ZL-EMM supplemented with 0 or 100 µM zinc to an OD$_{600}$ of ~5, and protein extracts prepped for immunoblot analysis. Immunoblots were incubated with anti-HA antibodies and anti-Act1 antibodies for a loading control. (D) pho8Δ pho2Δ cells bearing the empty vector, Pho8-HA, or the indicated Pho8-HA zinc binding mutants were grown overnight in ZL-EMM supplemented with 0 (-Zn) or 100 µM zinc (+Zn) to an OD$_{600}$ of ~5. Protein extracts preparation and immunoblot analysis were performed as described above. The lower panel shows the mean ratio of mature to unprocessed Pho8-HA.
proteins from three independent repeats with error bars representing standard deviations. *p<0.05, ns, not significant.

**Figure 6. The dimerization of Pho8 is not dependent upon zinc status.** (A) *pho2Δ pho8Δ* cells expressing *pgk1*-driven Pho8-HA or co-expressing *pho8*-driven Pho8-GFP and *pgk1*-driven Pho8-HA grown in ZL-EMM to an OD_{600} of ~5. Cell lysates were incubated with anti-GFP-bound Protein A beads and Pho8 co-immunoprecipitation complexes analyzed by immunoblotting. The right panel shows the mean output signal for Pho8-HA relative to the input signal from three independent repeats with error bars representing standard deviations. (B) *pho2Δ pho8Δ* cells bearing an empty vector or Pho8-HA were grown in ZL-EMM to an OD_{600} of ~5. Protein lysates from these cells were incubated with DMSO or EGS for 30 mins and Pho8 dimerization complexes analyzed by immunoblotting. (C) EGS crosslinking and immunoblotting were performed as described in panel B using protein lysates prepared from *pho2Δ pho8Δ* cells bearing an empty vector, Pho8-HA, Pho8-HA E478K, or Pho8-HA G475S. (D) Alkaline phosphatase activity was assayed in *pho2Δ pho8Δ* cells expressing Pho8-HA, Pho8-HA E478K, or Pho8-HA G475S that were grown in ZL-EMM supplemented with 0, 1, 5, 10, 100, or 200 µM zinc to an OD_{600} of ~5. The inset shows the results for Pho8 E478K and E475S mutants on an expanded scale. Activity is the mean of three independent repeats with the error bars representing standard deviations.
Figure 7. Alkaline phosphatase activity in CDF family mutants under steady state growth conditions. Alkaline phosphatase activity was assayed in the indicated strains that were grown in ZL-EMM supplemented with 0, 1, 10, or 100 µM zinc to an OD₆₀₀ of ~5. Activity is the mean of three independent repeats with the error bars representing standard deviations. **p<0.01, *p<0.05. ND = Not determined.
Figure 8. Zhf1 and the Cis4-Zrg17 complex have a redundant role in the activation of Pho8. (A) The indicated strains were grown overnight in ZL-EMM to an OD$_{600}$ of ~5 ($t$ = -0). Cells were then exposed to 100 µM zinc and were harvested for alkaline phosphatase activity assays at the indicated time points. Activity is the mean of three independent repeats with the error bars representing standard deviations. (B) The indicated strains expressing Pho8-HA or the empty vector were subject to zinc shock as described above and alkaline phosphatase activity assayed at the indicated time points. Activity is the mean of three independent repeats with the error bars representing standard deviations.
Figure 9. Activation of Pho8 by zinc. \textit{pho2Δ pho8Δ} cells expressing the \textit{pgk1}-driven Pho8-HA ΔC (A) or \textit{pho8}-driven Pho8-GFP (B) were grown overnight in ZL-EMM to an OD\textsubscript{600} of ~5. Cells were preincubated with 0, 0.1, or 1 mM cycloheximide for 2 hr (A) or for 30 min (B), before being exposed to 100 \textmu{}M zinc (t=0). Cells were then harvested for alkaline phosphatase activity assays or immunoblot analysis at the indicated time points. Alkaline phosphatase activity and immunoblot analyses were performed as described in Figure 3. (C) The indicated strains expressing Pho8-HA ΔC were treated with cycloheximide for 2 hr, shocked with 100 \textmu{}M zinc, and assayed for alkaline phosphatase activity as described above.
Zinc-dependent activation of the Pho8 alkaline phosphatase in *Schizosaccharomyces pombe*

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