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Zng1 is a GTP-dependent zinc transferase needed for activation of methionine aminopeptidase

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Summary

The evolution of zinc (Zn) as a protein cofactor altered the functional landscape of biology, but dependency on Zn also created an Achilles' heel, necessitating adaptive mechanisms to ensure Zn availability to proteins. A debated strategy is whether metallochaperones exist to prioritize essential Zn-dependent proteins. Here, we present evidence for a conserved family of putative metal transferases in human and fungi, which interact with Zn-dependent methionine aminopeptidase type I (MetAP1/Map1p/Fma1). Deletion of the putative metal transferase in *Saccharomyces cerevisiae* (*ZNG1*; formerly *YNR029c*) leads to defective Map1p function and a Zn-deficiency growth defect. In vitro, Zng1p can transfer Zn^{2+} or Co^{2+} to apo-Map1p, but unlike characterized copper chaperones, transfer is dependent on GTP hydrolysis. Proteomics reveal mis-regulation of the Zap1p transcription factor regulon due to loss of *ZNG1* and Map1p activity, suggesting that Zng1p is required to avoid a compounding effect of Map1p dysfunction on survival during Zn limitation.

Keywords

MetAP, insertase, NME, GTPase, zinc homeostasis, nutrient limitation, COG0523, CobW, CBWD

Introduction

Zinc (Zn) is unique among the biologically essential d-block elements. Likely because it is relatively less toxic than the redox-active metal ions, Zn is an abundant enzymatic and structural cofactor. At least 10% of the proteins encoded by metazoan and fungal genomes are predicted to bind Zn (Andreini et al., 2006; Wang et al., 2018), including those responsible for fundamental cellular processes, such as transcription, translation, and posttranslational regulation. Consequences of Zn deficiency are well known. In plants, symptomatic Zn deficiency leads to poor growth and chlorosis or yellowing (Thorne, 1957), but even asymptomatic Zn deficiency can result in foods with poor Zn nutritional value (Brennan et al., 1993). Depending on the degree and duration (Prasad, 2013), Zn deficiency in humans can cause stunted growth (Wessells and Brown, 2012), compromised immunity (Bonaventura et al., 2015), and neurological defects (Chasapis et al., 2011). Zn is also a key micronutrient in hidden hunger that is estimated to affect 2 billion people (Allen et al., 2006), and, unfortunately, rising CO_2 levels are exacerbating this challenge by potentially leading to crops with increased ratios of carbohydrates to Zn (Loladze, 2014).

The biogenesis of Zn-dependent proteins needed for the proper functioning of these processes is poorly understood, especially when Zn is limiting. Assimilatory and distributive Zn transporters are essential for increasing Zn availability to proteins, but conclusive evidence for the existence of cytosolic Zn chaperones that deliver and prioritize metals to specific Zn-dependent proteins is lacking. Instead, the prevailing model is that labile metal-ligand pools exist within the cell, and proteins rely on their relative metal-binding affinity for access to the correct metal ion (Foster et al., 2014). However, this model likely does not describe the Zn-limitation situation, where inactivating metals become relatively more competitive than Zn, and both essential and non-essential Zn-dependent proteins are vying for the limiting cofactor (Barwinska-Sendra and Waldron, 2017). Indeed, during Zn limitation in the yeast *Saccharomyces cerevisiae*, less than 30% of proteinaceous Zn-binding sites are expected to be bound to Zn (Wang et al., 2018). Therefore, chaperone-mediated prioritization of essential Zn-dependent proteins would be advantageous when access becomes limiting.

Candidates for chaperone-mediated metal handling are members of the COG0523 family. These proteins are typically mis-annotated as “CobW, cobalamin synthesis protein”, an annotation derived from a cobalamin-minus phenotype in *Pseudomonas denitrificans* (Crouzet et al., 1991). Bioinformatic analyses suggest that a function in cobalamin biosynthesis is only relevant for a small subset of bacterial proteins, and the majority of family members are involved in the Zn-limitation response (Edmonds et al., 2021; Haas et al., 2009). Indeed, although there is little overlap between the response to Zn across the tree of life, members of the COG0523 family are expressed in response to poor Zn nutrition in all three kingdoms, suggesting an ancient and conserved role for this family in Zn homeostasis (Edmonds et al., 2021; Haas et al., 2009).

The molecular function of COG0523, however, has remained obscured. These proteins are members of the G3E family of GTPases (Leipe et al., 2002), which contains the closely related UreG and HypB subfamilies. UreG and HypB are nickel-binding GTPases that function in a complex of accessory factors that deliver and insert nickel during the biogenesis of nickel-dependent urease or Ni-Fe hydrogenase, respectively (Zeer-Wanklyn and Zamble, 2017). In the case of UreG, structural and biochemical characterizations are consistent with a model where conformational changes induced by GTP hydrolysis promote release of nickel from UreG and transfer to apo-urease (Soriano and Hausinger, 1999; Yuen et al., 2017). Given the shared ancestry and sequence similarity to the UreG homologs, a similar mechanism may exist for the COG0523 proteins during Zn limitation. Several studies in bacteria support this hypothesis (Blaby-Haas et al., 2012; Chandrangsu et al., 2019; Jordan et al., 2019; Nairn et al., 2016), and in the eukaryotic alga *Chlamydomonas reinhardtii*, Zn-responsive homologs ZCP1 and ZCP2 are proposed to function in metal allocation and sparing (Malasarn et al., 2013). However, even though recent molecular characterization of several COG0523 proteins is consistent with the role of these proteins as nucleotide-dependent metal chaperones (Young et al., 2021), evidence of metal transfer to a known target and demonstration of metal transferase activity is lacking, obfuscating the function of this widely distributed family of Zn-responsive proteins.

Herein, we report the bioinformatics-guided identification of a conserved physical interaction between eukaryotic COG0523 proteins and Zn-dependent methionine aminopeptidase type I

(MetAP1/Map1p/Fma1) in human and the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, leading to the hypothesis that eukaryotic COG0523 is a metal transferase for methionine aminopeptidase type I. Genetic and biochemical characterization of the COG0523 ortholog from *S. cerevisiae* supports this hypothesis, and based on functional similarity in vertebrates (co-submitted manuscript: Weiss and Murdoch et al., "Zn regulated GTPase metalloprotein activator 1 (ZNG1) regulates zinc homeostasis in vertebrates"), this orthologous group of eukaryotic proteins has been named Zng1 for Zn-regulated GTPase metalloprotein activator 1 (this nomenclature has been approved by the Saccharomyces Genome Database and the HUGO Gene Nomenclature Committee). Herein, we refer to the family as Zng1, the *S. cerevisiae* protein as Zng1p, and the *S. cerevisiae* gene as *ZNG1*. We observe that, in vitro, GTPase activity is important for metal transfer from Zng1p to Map1p. Unexpectedly, proteomics analysis of yeast mutants reveals complex impacts due to the loss of *ZNG1*, loss of methionine aminopeptidase activity, and mis-regulation of the Zn-deficiency response. Based on these results, we propose that Zng1p functions to maintain Map1p activity. This requirement for Zng1p is more acute during Zn starvation, and maintenance of Map1p activity when access to Zn is restricted ensures proper expression of proteins involved in the Zn response.

Results

Conserved interaction between Zng1p and Map1p orthologs across fungi and human

We performed a comparative genomic and molecular interaction network analysis to generate a list of putative client proteins of the COG0523 proteins in eukaryotes. There are numerous orthologous groups that may have distinct biological functions and protein targets (Figure 1A; see data file S1 for network file) (Edmonds et al., 2021; Haas et al., 2009). For instance, ZagA from *Bacillus subtilis* is proposed to function as a Zn chaperone for GTP cyclohydrolase I (Chandrangsu et al., 2019), but ZigA from *Acinetobacter baumannii* is proposed to function as a Zn chaperone for histidine ammonia-lyase (Nairn et al., 2016). Therefore, we focused on the COG0523 subfamily that appears to have evolved by vertical inheritance since the last common eukaryotic ancestor (named the Zng1 subfamily) (Figure 1B). Like most members of the superfamily, these orthologs contain conserved GTPase residues common for the G3E family of GTPases (Leipe et al., 2002) and a metal-binding motif (CxCC) (Figure S1). Additionally, the

human COG0523 homologs are regulated by Zn status (Coneyworth et al., 2012), and in a recent proteomics study, the yeast homolog was only detected after five generations in low Zn medium (Wang et al., 2018), suggesting a conserved function during Zn deficiency. A search for conserved molecular interactions resulted in a single physical interactor, methionine aminopeptidase type 1, in both human and yeast, despite over a 1,000 million years of divergence (Kumar et al., 2017) (Figure 1C; data file S2). The physical interaction was identified in four separate studies (Huttlin et al., 2015, 2017; Vo et al., 2016; Yu et al., 2008). This ribosome-associated enzyme, referred to as Map1p in yeast, MetAP1 in human, and Fma1 in fission yeast, cleaves the initiator methionine (iMet) from nascent polypeptides. Furthermore, its loss is associated with translation defects (Fujii et al., 2018) in addition to blocking N-myristoylation and some N-terminal acetylations. Methionine aminopeptidases contain a binuclear metal site analogous to urease, the target metalloenzyme of UreG, a distantly related homolog of the Zng1 family (Haas et al., 2009). Map1p is active in vitro with Zn or cobalt (Co) and was originally described as a Co-dependent enzyme, but the site is expected to bind Zn in the cytosol, since physiological levels of glutathione inhibit Co-bound Map1p (Walker and Bradshaw, 1998). Moreover, iMet-Cys-polypeptides are essential targets of Map1p in vivo (Dummitt et al., 2005), and Co-bound Map1p cannot hydrolyze iMet-Cys-polypeptides in vitro (Walker and Bradshaw, 1999).

The network also captured a negative genetic interaction between the *S. cerevisiae ZNG1* ortholog (*YNR029c*) and *MAP2,* methionine aminopeptidase type 2 (Costanzo et al., 2016). Map2p is a distant paralog of Map1p. Both enzymes cleave the iMet from nascent polypeptides that have short side chains, but they differ in substrate specificity (Chen et al., 2002). Unlike Map1p, Map2p uses manganese instead of Zn (Wang et al., 2003). Genetic analysis in *S. cerevisiae* indicates that Map1p is the dominant paralog, but Map2p has some overlap in function (Li and Chang, 1995), suggesting that the negative genetic interaction could be due to a Map1p defect in the *ZNG1* deletion strain.

Independent of the publicly available molecular interaction data, an amino acid coevolution analysis combined with deep-learning-based structural modeling also identified a putative

complex composed of yeast Zng1p and Map1p (Humphreys et al., 2021). The structural model predicts an interaction between the N-terminal regions of the two proteins (Figure S1). Using Y2H, we detect an interaction between Zng1p and Map1p but not between Zng1p and Map2p (Figure 1D). The interaction between Zng1p and Map1p is no longer detected if the N-terminal regions of either Zng1p (trZng1p) or Map1p (trMap1p) are truncated (the first 68 amino acids of Zng1p and the first 69 amino acids of Map1p) (Figure 1E). Replacing the cysteine residues in the putative CxCC metal-binding motif of Zng1p did not affect the interaction with Map1p (Figure 1E). Based on the deep-learning structural model of the Zng1p-Map1p complex, we identified three cysteine residues belonging to Map1p that are putatively located at the interface near the Zng1p CxCC motif (Figure S1). Only substitution of C201 with serine appeared to disrupt the interaction between Map1p and Zng1p (Figure 1E), suggesting that additional structural elements in addition to the N-termini are important for the interaction.

ZNG1 *is required for Map1p activity*

Based on these analyses and published evidence that Map1p is a Zn-dependent protein in vivo (Walker and Bradshaw, 1998), we hypothesized that Zng1p is a Zn transferase, a function that is essential during Zn deficiency. Since the *S. cerevisiae map1*Δ strain has an extreme slow-growth phenotype and a *map1*Δ *map2*Δ double mutation is lethal (Li and Chang, 1995), we reasoned that a defect in Map1p biogenesis, caused by the absence of *ZNG1*, would translate to a slow growth phenotype that would be exacerbated in a *map2*Δ genetic background. We monitored the growth of *zng1*Δ and *map2*Δ *zng1*Δ mutants in Low-Zinc Medium (LZM) (Zhao and Eide, 1996). Deletion of *ZNG1* led to a growth defect in LZM, which could be rescued with 30 μM Zn (Figure 2A and 2B; Figure S2A). Although *map2*Δ resembled WT, *map2*Δ *zng1*Δ displayed an exacerbated growth defect compared to *zng1*Δ and required more Zn for growth (Figure 2A and 2C; Figure S2A). The phenotypes were reproducible on agar-solidified media (Figure 2D). These results demonstrate that when growth is dependent on Map1p function, *ZNG1* is needed for growth during Zn limitation, and by extension, indicates that *ZNG1* is required for Map1p activity when Zn is limiting.

Since LZM contains EDTA to buffer and chelate Zn, we also tested growth in CSD (a Chelex-

treated defined medium (Lyons et al., 2000)) to provide additional confidence that the growth phenotypes are specific to a deficiency in Zn. CSD without Zn supplementation is not as deficient in Zn as LZM (Wu et al., 2008), and accordingly, we did not observe a *zng1*Δ growth defect (Figure 2E; Figure S2B). However, the *map2*Δ *zng1*Δ strain displayed a growth defect in the absence of Zn, which could be rescued with 4 μM Zn (Figure 2E and 2F; Figure S2B). Growth defects were also observed in YPD with the metal chelator EDTA (Figure 2G – 2I; Figure S2C and S2D). Similar to the growth phenotypes in LZM and CSD, the *map2*Δ *zng1*Δ strain was more sensitive to Zn depletion than *zng1*Δ (Figure 2G), and supplementation with Zn rescued the EDTA sensitivity of *zng1*Δ and *map2*Δ *zng1*Δ (Figure 2J). Expression of *ZNG1* in trans rescued the *zng1*Δ and *map2*Δ *zng1*Δ growth defects (Figure 2A – 2J; Figure S2). As overexpression of Map2p can partially rescue the loss of Map1p function (Li and Chang, 1995), we also observed that expression of *MAP2* in trans rescued the *zng1*Δ and *map2*Δ *zng1*Δ growth defects (Figure 2A – 2J; Figure S2). Since Co can activate Map1p activity in vitro, we tested the ability of Co to rescue growth during Zn limitation in CSD. As shown in Figure 2K and Figure S3, Co did not stimulate growth regardless of genotype or presence of Zn, suggesting that Co cannot substitute for Zn as a nutrient in *S. cerevisiae.*

We next tested whether the absence of *ZNG1* impairs iMet cleavage, which would be expected if Zng1p is required for Map1p activity. We built a reporter construct where the N-terminal peptide of 14-3-3γ, a model methionine aminopeptidase (MetAP) substrate, is fused to glutathione S-transferase (GST). The presence of iMet, caused by a defect in MetAP activity, can be detected with an antibody that only recognizes the methionylated N-terminal fragment of 14-3-3γ (Towbin et al., 2003). An antibody that recognizes GST serves as a loading control. We observed a subtle iMet cleavage defect in the *zng1*Δ strain when grown in LZM with 10 μM Zn, which was pronounced with 1 μM Zn, but not detected with additional Zn (i.e., 100 μM Zn) (Figure 2L). With the *map2*Δ *zng1*Δ strain, where methionine cleavage is dependent solely on the activity of Map1p, defective iMet processing was observed at all Zn concentrations tested. Addition of Zn did result in partial rescue, but iMet retention was still observed with 3 mM Zn (a three-molar excess compared to the amount of EDTA present in LZM) (Figure 2M). No iMet was detected in WT or the *map2*Δ mutant (Figure 2L). These results suggest that the Zn-

deficiency-related growth defects observed with the *zng1*Δ and *map2*Δ *zng1*Δ strains are caused by a Map1p defect. Since Map1p levels increased by 25% in the *map2*Δ *zng1*Δ strain compared to WT during Zn limitation (Figure S4G), the iMet cleavage defect and growth phenotypes are not a consequence of lower Map1p abundance. Instead, these results indicate that Map1p activity decreases. Unexpectedly, although supplemental Zn could partially rescue the MetAP defect in the double mutant, a defect was still detected at 3 mM Zn (Figure 2M). In combination, these strain- and Zn-dependent phenotypes support a model where Zng1p is involved in activating Map1p. The loss of Zng1p leads to significantly less Map1p activity during low Zn, and *MAP2* serves as a back-up to compensate for the partial loss of Map1p activity due to the absence of Zng1p.

Activation of Map1p by Zn-Zng1p is dependent on GTP

Our in vivo experiments support the hypothesis that Zng1p is required for Map1p activity but do not elucidate the molecular function of Zng1p. Since purified Zng1p displays GTPase activity that is stimulated by the presence of transition metals (Figure 3A), we reasoned that Zng1p can deliver and transfer Zn to apo*-Map1p in a GTP-dependent manner. An asterisk is used to denote that after overnight treatment with EDTA, Map1p has a roughly one molar equivalent of Zn (1.33 ± 0.59), as determined by inductively coupled plasma mass spectrometry (ICP-MS) analysis. In addition to the binuclear Zn site, Map1p has an N-terminal Zn-finger domain (Zuo et al., 1995). This detected Zn in apo*-Map1p is likely bound to the Zn finger since trMap1, treated in the same way, is devoid of Zn (0.00 ± 0.10). As shown previously (Walker and Bradshaw, 1998), apo*-Map1p hydrolyzes a synthetic peptide but only if Zn is provided, suggesting that the Zn bound to the Zn finger does not participate in catalysis (Figure 3B). To test whether Zng1p can transfer Zn to apo*-Map1p, we designed an in vitro metal transfer assay where MetAP activity is measured after incubation for 3 hr at 30°C in the presence of a 10-fold molar excess of either free Zn or Zn pre-bound to Zng1p (Zn-Zng1p). We observed that Zn-Zng1p activated apo*-Map1p but only in the presence of GTP (Figure 3C). Activity was not observed with GMP-PNP, an analog of GTP that is not noticeably hydrolyzed by Zng1p (Figure 3C). Recently, ZagA, the COG0523 protein from *B. subtilis*, was proposed to function in vivo as a 5-aminoimidazole-4-carboxamide riboside 5'-triphosphate (ZTP) hydrolase (Chandrangsu et al., 2019). Like ZagA,

Zng1p can hydrolyze ZTP in a metal-dependent manner (Figure 3D and 3E), but ZTP failed to activate the metal transfer reaction (Figure 3C). These results demonstrate that GTP is needed for activation of apo*-Map1p by Zn-Zng1p. Although it was previously speculated that the GTPase activity of COG0523 proteins may be enhanced in the presence of the client protein (Jordan et al., 2019), the GTPase activity of Zng1p was not affected by the presence of Map1p (Figure 3E). Similar results were attained by measuring Map1p activity with the synthetic substrate L-Met p-nitroanilide (Figure 3F).

As previously observed for the ATPase activity of Cu-ATPases with their cognate copper chaperones compared to free Cu^{1+} (Blaby-Haas et al., 2014; González-Guerrero and Argüello, 2008), Map1p activity with Zn-Zng1p is roughly 2-fold higher compared to Zn^{2+}. Supporting a model where there is direct Zn transfer from Zng1p to apo*-Map1p, EDTA did not inhibit Map1p activity with Zn-Zng1p (Figure 3C). Indeed, in the presence of EDTA, which mimics intracellular competition, apo*-Map1p had 4-fold higher activity with Zn-Zng1p than with Zn^{2+}. Although apo-trMap1p and apo*-Map1p have similar activity when incubated with 5-fold molar excess of free Zn^{2+}, activation of apo-trMap1p by 5-fold molar excess of Zn-Zng1p was inhibited (Figure 3G). Additionally, the small increase in activity seen in the presence of GTP was abolished by EDTA, providing further support that the physical interaction between the two proteins is needed for Zn transfer from Zng1p to Map1p. However, because apo*-Map1p contains Zn^{2+} bound to the Zn-finger domain, which could not be removed with EDTA, we are unable to rule out the possibility that, in addition to the physical interaction, the Zn finger is somehow involved in activation of Map1p by Zng1p. Unfortunately, the complex between Zng1p and Map1p is stable (roughly 30% of protein is in the complex based on size-exclusion chromatography), hindering our ability to re-purify the proteins after the metal transfer assay and accurately determine Zn stoichiometry. Reflecting the promiscuity of Map1p metal dependency in vitro (Figure 3B), preloading Zng1p with a non-physiologically relevant metal ion (Co^{2+}) also activated apo*-Map1p in a GTP-dependent manner (Figure 3C).

Deletion of ZNG1 *leads to mis-regulation of the Zn-deficiency response*

To better understand the observed fitness defects due to the lack of *ZNG1*, we used proteomics

with Tandem Mass Tag (TMT) quantitation derived from WT, *zng1*Δ, and *map2*Δ *zng1*Δ strains grown in LZM with 1 μM or 100 μM Zn. We quantified the abundance of 4,889 proteins. Of these, 1,388 proteins displayed a statistically significant abundance change in at least 1 of 9 comparisons in a matrix between conditions and strains (Figure 4A – 4C; Table S1). Correlating with the Map1p defect observed with the reporter construct, the 100 μM Zn proteomes from WT and *zng1*Δ are the most similar followed by the 1 μM WT and *zng1*Δ proteomes (Figure 4D). The largest difference observed is between the 1 μM *map2*Δ *zng1*Δ proteome and the other samples (Figure 4D). Roughly half of the proteins (621 out of 1,388 proteins) are differentially abundant between 1 μM and 100 μM in the WT, with an additional 107 and 214 proteins that change in abundance between the two Zn conditions in *zng1*Δ and *map2*Δ *zng1*Δ, respectively (942 between the three strains) (Figure 4E). Surprisingly, over 100 proteins change in abundance in response to Zn availability (i.e., >1.5-fold change and *P* value <0.05) in the WT but not in either mutant (Figure 4E). The relatedness among the samples (quantified by PCA) is recapitulated in the cluster analysis (Figure 4F). Partitioning of proteins into clusters highlights some common protein expression patterns, such as cluster E that contains a subcluster that decreases in abundance in the WT during Zn limitation but not in *map2*Δ *zng1*Δ and a second subcluster that increases in the WT but not in the mutants (Figure 4G).

To better understand why we observe growth defects during Zn deficiency when *ZNG1* is deleted, we aimed to identify protein abundance changes that correspond with strain- and Zn-specific growth phenotypes. We reasoned that proteome changes in *zng1*Δ compared to WT, which are exacerbated in *map2*Δ *zng1*Δ compared to *zng1*Δ, may point to key impacts. Therefore, we identified proteins with abundances that are at least 1.5-fold different between *zng1*Δ and WT in the Zn-limited condition and are at least 1.5-fold different between *map2*Δ *zng1*Δ and *zng1*Δ in the Zn-limited condition, resulting in a conservative subset of 20 proteins (referred to as "subset 1" in Figure 5; Table S1). Of these, 7 are encoded by genes known to be regulated by the transcription factor Zap1p: Css1p, Vel1p, Fet4p, and Adh4p have lower abundance in the mutants, while Hnt1p, Ald3p, and Hsp12p have higher abundance (Figure 5A – 5D). Zap1p is a Zn-responsive transcription factor responsible for induction of the core Zn-deficiency response (Lyons et al., 2000; Zhao and Eide, 1997). Zap1p was reduced in abundance

by 1.3-fold in *map2*Δ *zng1*Δ compared to WT, but there was no statistically significant difference between *zng1*Δ and WT, suggesting that these strain-specific differences are not necessarily due to Zap1p abundance (Figure S5). While the magnitude of the fold change was higher between the strains grown with 1 μM Zn, the impact on protein abundance was often also observed in 100 μM Zn for both Zap1p targets and others (Figure 5C; Table S1). Without the strict fold-change requirement, an additional 15 Zap1p targets are lower in abundance in *map2*Δ *zng1*Δ compared to WT during the Zn-limited growth condition, and 10 are more abundant (Figure 5C and Table 1). The impact on the Zap1p regulon is statistically significant based on Gene Set Enrichment Analysis (GSEA) (Figure 5E and 5F). Although N-terminal processing can impact protein half-life, only 50% of these Zap1p transcription factor targets are potential substrates for MetAP, indicating that many of these abundance changes are downstream of the Map1p defect (Table S1).

These results suggest the presence of a regulatory defect caused by the absence of *ZNG1* and reduced Map1p function, which impairs the ability of the cell to properly acclimate to Zn limitation. These protein changes could be the result of either transcriptional or post-transcriptional regulation. Indeed, analysis of transcript abundance with qPCR of a subset of genes revealed different impacts. For instance, changes we observe at the protein level for Hsp12p and Hnt1p correspond with changes at the transcript level. However, *MAP1* and *ZAP1* transcript abundances increased in *zng1*Δ, while protein levels are similar to WT (Figure S5). In contrast, the transcript abundances for *ADH4*, *FET4*, and *VEL1* are unchanged in *zng1*Δ, but protein abundances are lower in WT (Figure S5). In *map2*Δ *zng1*Δ, both protein and transcript abundances are reduced for these three genes (Figure S5). These various patterns suggest that there is one or more unknown factors needed for the proper expression of these genes, which are defective in the mutant strains during Zn deficiency.

To further understand impacts due to the loss of *ZNG1* and the Map1p defect, we also compared *map2*Δ to *map2*Δ *zng1*Δ (Table S1). We identified 28 proteins that are less abundant and 60 proteins that are more abundant in *map2*Δ *zng1*Δ when grown in Zn replete. Fifty-seven proteins are less abundant, and 171 proteins are more abundant in *map2*Δ *zng1*Δ when grown in low Zn.

Supportive of a role for *ZNG1* in the regulation of some Zap1p transcription factor targets, Fet4p and Adh4p have lower abundance, while Ald3p and Hsp12p have higher abundance in *map2*Δ *zng1*Δ compared to *map2*Δ, as seen for the comparison of *zng1*Δ and WT. Mis-regulation of Adh4p and Fet4p appears to be a consequence of a transcriptional defect, which was not observed for *zng1*Δ (Figure S5). Therefore, there are complex impacts on expression at the transcriptional and post-transcriptional levels in the various mutants.

Deletion of ZNG1 *impacts the abundance of ribosome subunits*

We also used the proteomics data to determine whether *ZNG1* may be involved in processes independent of Map1p, as changes in *zng1*Δ, which are not exacerbated in *map2*Δ *zng1*Δ, could be due to other impacts. Using a 1.5-fold cutoff and a *P* value < 0.05 between the two Zn concentrations, 45 proteins have higher abundance in *zng1*Δ in the Zn-limited condition compared to WT, with no significant increase between *zng1*Δ and *map2*Δ *zng1*Δ, while 28 proteins are lower in abundance (Figure 5G and 5H; Table S1). In some cases, the response to Zn limitation in *zng1*Δ compared to WT appears to be exacerbated, while in other cases the response is muted. Examples of the former include Hpf1p, a mannoprotein and Zap1p target, and Mms22p, a subunit of the E3 ubiquitin ligase complex that is involved in replication repair (Figure 5G and 5H). Such changes suggest more stress in *zng1*Δ compared to WT, but those specific stresses are not more acute in *map2*Δ *zng1*Δ. In contrast, proteins such as the mating pheromone alpha factor and Hsp26p, a heat-shock protein with chaperone activity and Zap1p target, increase in the WT but to a significantly smaller extent in *zng1*Δ (Figure 5G and 5H). Only a single protein exhibited a reciprocal pattern: Ino1p is more abundant during Zn-replete conditions in WT but exhibits a 2.72-fold change in abundance in Zn-deplete conditions in *zng1*Δ (Figure 5A and 5G). This same pattern was observed for *map2*Δ (Ino1p more abundant when Zn replete) and *map2*Δ *zng1*Δ (Ino1p more abundant when Zn deplete), but the fold change in *map2*Δ *zng1*Δ is much lower. Ino1p is an inositol-1-phosphate synthase that converts glucose-6-phosphate to inositol-1-phosphate for use in phospholipid synthesis.

For proteins less abundant in *zng1*Δ compared to WT and with no significant decrease between *zng1*Δ and *map2*Δ *zng1*Δ, we note significant enrichment of ribosomal subunits (*P* value 2.4E-

04, Table S1). At the same time, proteins involved in nuclear export and maturation of the 60S ribosome, Bud20p (a C2H2-type Zn-finger shuttling factor that associates with pre-60S particles in the nucleus), and Nog1p (a putative GTPase that associates with pre-60S ribosomal subunits in the nucleolus and is required for their nuclear export and maturation), are higher in abundance in the mutant compared to WT. Additional proteins related to ribosome function, which are co-expressed with Bud20p, include Sda1p (involved in 60S ribosome biogenesis) and Rpp1Ap (Ribosomal stalk Protein P1 Alpha). A potential role of Zng1p in ribosome function is further supported by comparison of *map2*Δ to *map2*Δ *zng1*Δ (Figure 5I). "Structural constituent of ribosome [GO:0003735]" was the only GO term enriched in the comparison of proteins less abundant in *map2*Δ *zng1*Δ compared to *map2*Δ in low Zn (*P* value 6.2E-4). The decrease in ribosomal subunit abundance and the increase in proteins involved in biogenesis could reflect a compensatory mechanism and point to a role of Zng1p in ribosome function during Zn limitation.

Discussion

This study presents support for the existence of a conserved GTP-dependent Zn transferase and provides the basis for a model describing the activation of methionine aminopeptidase type I. Our in vivo results suggest that Map1p requires Zng1p to properly function, this dependency is more acute when access to Zn is subpar, and there are proteins needed for acclimation to Zn deficiency that rely on proper Map1p activity (either directly or indirectly). When iMet processing is solely dependent on Map1p activity (i.e., *MAP2* is deleted), we observed defective Map1p activity due to the absence of *ZNG1* even when LZM was supplemented with 3 mM Zn (Figure 2M). Therefore, Zng1p appears to play a role in Map1p biogenesis regardless of Zn status, but the resulting defect due to Zng1p's absence did not translate into an observable fitness deficit. Neither *map2*Δ *zng1*Δ nor *zng1*Δ phenocopy the *map1*Δ mutant (Figure S2), suggesting that Zng1p is involved in, but not essential for, Map1p function when Zn is plentiful. However, growth defects due to the absence of *ZNG1* during Zn limitation were apparent and reproducible in several growth media.

We propose that during Zn limitation, Map1p may no longer be able to successfully compete with the depleted Zn-buffered pool and requires the assistance of Zng1p. Unlike Map1p, Zng1p may be able to either access the depleted Zn pool or acquire Zn from a source, such as a Zn transporter or a degraded non-essential Zn protein, which is not otherwise available to Map1p. Based on our in vitro assays, we propose that after docking with Map1p, Zng1p releases Zn via GTP hydrolysis, and Zn is subsequently bound to Map1p. The conserved physical interaction between Zng1p and Map1p, along with the observations that Map1p has higher activity with Zn-Zng1p than with Zn^{2+} and that EDTA inhibits Map1p with Zn^{2+} but not with Zn-Zng1p, are supportive of a model involving direct transfer of Zn from Zng1p to Map1p. However, additional work is needed to resolve the role of GTP binding and hydrolysis and to better understand the mechanism responsible for activation of Map1p by Zng1p.

As previously published, the abundance of Map1p decreases during Zn limitation. This phenomenon is proposed to spare Zn (Wang et al., 2018). As such, although we see a dependency on Zng1p under Zn-replete conditions, which is masked by the presence of Map2p, Zng1p may function to compensate for the lower Zn availability and lower abundance of Map1p during Zn-deplete conditions. The process is GTP dependent, but presumably the cost is justified by the ability to maintain a lower level of Map1p and to spare Zn for use by other enzymes that do not interact with Zng1p. Based on our proteomics data, one of the major penalties for losing *ZNG1,* and consequently Map1p activity, is aberrant expression of proteins encoded by the Zap1p regulon, which likely exacerbates defects caused by Zn limitation.

Our model is based on the published assertion that Map1p uses Zn in vivo and the assumption that cofactor usage does not change during Zn limitation. Our results are consistent with the role of Zng1p as a Zn transferase, but we cannot definitively rule out the possibility that Zng1p binds and delivers Co to Map1p during Zn limitation. Our defined media do not contain supplemented Co, and supplementation with Co does not rescue growth of WT, *zng1*Δ, or *map2*Δ *zng1*Δ growth during Zn limitation (Figure 2K), which is unexpected if Map1p can use Co in vivo. Recently, based on an estimate of idealized metal availabilities in the bacterial cytoplasm, CobW, a homolog of Zng1p from *Rhodobacter capsulatus,* was predicted to bind Co over Zn in

the cell if Co is present, while homologs YeiR and YjiA from *Escherichia coli* were predicted to bind Zn over Co (Young et al., 2021). Based on published experiments with yeast Map1p, if Zng1p transfers Co to Map1p during Zn limitation, Co-Map1p would be inactivated by cytoplasmic GSH (Walker and Bradshaw, 1998). Our results indicate that Zng1p is required for Map1p activity and does not function as an inhibitor. In the case of CobW, a role as a Co chaperone is logical, since CobW is involved in the biosynthesis of the Co-dependent porphyrin vitamin B_{12}, which does not function as a coenzyme with a Zn metallocenter.

Although the identity of the metal(s) delivered by Zng1p in vivo is not yet definitively resolved, the proteomics data point to a role for Map1p and Zng1p in the protein abundance of Zap1p targets during Zn limitation. Reduced Map1p activity correlates with decreased abundance of some targets, such as Vel1p and Adh4p, while correlating with a higher abundance of other targets, such as Hsp12p and Ald3p. The absence of Zn for Zng1p, and by extension reduced Map1p activity, could be involved in regulating the Zn response during Zn starvation, where the cell transitions from acclimation to survival. Such a mechanism is reminiscent of the proposed role of mycobacterial-specific protein Y recruitment factor (Mrf) in *Mycobacterium tuberculosis* (Li et al., 2018, 2020), which is a distant homolog of Zng1p. Zn-free Mrf binds to the 30S ribosomal subunit and recruits mycobacterial-specific protein Y (Mpy), a protein that binds to and inactivates the ribosome, leading to hibernation during Zn starvation. Unlike Zng1p, however, Mrf lacks conserved GTPase residues and is not expected to have GTPase activity. This sequence divergence likely reflects loss of a metal transferase function. Indeed, the methionine aminopeptidase type 1 in *M. tuberculosis* is proposed to use iron instead of Zn (Lu et al., 2009). Whether the extant function of Mrf is the result of neofunctionalization or subfunctionalization is unknown, but a connection between Zng1p and translation in *S. cerevisiae* beyond a role in Map1p activity is supported by quantitative proteomics. Proteins involved in ribosome biogenesis and rRNA maturation decrease in abundance in the WT under Zn-deplete conditions but not in the mutants (Table S1), suggesting that Zng1p could be involved in regulating ribosome function during Zn limitation. However, we do not know to what extent such protein abundance changes are a general stress response in the mutant strains or more directly related to a biological function of Zng1p.

Given that an unbiased bioinformatic search for a candidate client protein of the Zng1 subfamily captured methionine aminopeptidase type I in both fission yeast and human in addition to *S. cerevisiae*, which we confirmed by Y2H, we propose that the function of Zng1p as a GTP-dependent metal chaperone for methionine aminopeptidase type I is conserved throughout this subfamily of eukaryotic GTPases. However, while most eukaryotes, including *Arabidopsis thaliana*, mouse, and zebrafish, have a single Zng1 ortholog, there is a gene family expansion in primates. Chimpanzees have four paralogs and humans have five nearly identical paralogs (at the amino acid level), suggesting potential cell-type-specific expression and functional tailoring that does not occur in other eukaryotes. Indeed, both human Zng1 paralogs, CBWD1 and CBWD2, were found to interact with MetAP1 but are associated with distinct expression profiles and phenotypes. CBWD1 is expressed mainly in the kidney and bladder and was recently implicated as the causal mutation in a case of congenital anomaly of the kidney and urinary tract (Kanda et al., 2020), whereas high expression of CBWD2 is prognostic for endometrial cancer (Uhlen et al., 2017). Based on our findings, these and other phenotypes may be due to aberrant iMet processing, although our study does not preclude the possibility that Zng1 proteins may interact with proteins other than methionine aminopeptidase type I.

Limitations of the study

The discovery of Zng1p and the results we have presented provide insight into the role of COG0523 proteins in metalloprotein biogenesis, in general, and Map1p activation, specifically. Many unanswered questions remain. Additional biochemical and structural characterizations are needed to understand how Zng1p activates Map1p. Although our in vitro metal transfer assays support direct transfer of Zn from Zng1p to Map1p, we were unable to determine the stoichiometry of bound Zn after the assay, and we have not quantified metal-biding affinities for Zng1p, which may differ for nucleotide-bound forms. Our study also did not resolve the role of GTP-binding and hydrolysis in Zng1p function. Some possible effects of GTP-binding and hydrolysis include shifting the metal-binding affinities of different Zng1p conformations or inducing conformational changes of Zng1p to stabilize a conformer of Map1p and facilitate movement of Zn from Zng1p to Map1p. Moreover, our conclusions are based on a two-

component model, but additional unknown proteins could be involved in this mechanism. Indeed, we see stable complex formation between Zng1p and Map1p in vitro. Since Map1p needs to bind to the ribosome to function in iMet excision (Fujii et al., 2018), stable complexation with Zng1p could actually function to inhibit iMet excision. As we do not have evidence of an inhibitory effect in vivo, additional factors are likely involved in regulating the interaction between these proteins. Our study also could not definitively prove whether Zng1p transfers Zn or Co to Map1p during Zn starvation, since Zng1p can activate Map1p with either metal in vitro. Yet, mediating cofactor substitution in response to availability is an equally exciting, although less plausible, function for Zng1p. We also note limitations with the proteomics experiments. These types of experiments are unable to differentiate direct and indirect impacts of the mutations analyzed. However, this multi-strain, multi-condition, proteome-wide experiment has generated hypotheses and additional avenues for further characterization, such as studying how the absence of *ZNG1* and reduced Map1p activity alters the expression of Zap1p targets. Although these experiments were performed with five biological replicates, we do point out that we have not provided immunoblots as confirmation of the proteome changes that we quantified.

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Author contributions

C.E.B. conceptualized the study and performed the bioinformatic analyses. C.E.B., M.P. and N.G. designed experiments. I.K.B., C.E.B and N.G. created yeast strains and generated plasmids. M.P. and N.G. performed protein purifications and in vitro experiments. N.G. performed in vivo experiments and cultured cells for the TMT-labeled proteomics. K.K.H., C.D.N. and M.L. performed the TMT-labeled proteomics. E.F.Y. performed protein purifications and Alpha-Fold protein complex annotation. J.D.H. analyzed purified proteins by mass spectrometry. C.E.B., M.P., and N.G. wrote the manuscript with comments from all authors.

Declaration of interests

Authors declare no competing interests.

Main figure titles and legends

Figure 1. Conserved interaction between Zng1 orthologs and orthologs of methionine aminopeptidase type I.

(A) Sequence similarity network of proteins containing the PF02492 domain. The location of nodes representing model eukaryotic organisms and experimentally characterized bacterial proteins are labeled with an arrow along with their putative/known biological functions and metal. The network is available as data file S1. "CBWD" refers to CBWD1-3 and CBWD5-6 from *Homo sapiens*.

(B) Inferred approximately maximum-likelihood phylogenetic tree of the eukaryotic Zng1 subfamily and related prokaryotic homologs. Leaves corresponding to proteins mentioned in the text are highlighted with a red circle. Clades are colored by shared taxonomy as in panel (A).

(C) Interaction network of Zng1 orthologs from *S. cerevisiae*, *S. pombe,* and *M. musculus,* and the paralog group from *H. sapiens*. Nodes representing Zng1 proteins and methionine aminopeptidase type I orthologs are indicated with arrows; *H. sapiens* Zng1 paralogs are indicated with a number that corresponds to CBWD1-7. Edge thickness corresponds to number of interactions between any two nodes, and edge color refers to type of interaction. The network file is available as data file S2.

(D) Y2H analysis of Zng1p and Map1p interaction. *ZNG1* was fused with DNA encoding the

Gal4 activation domain. *MAP1* and *MAP2* were fused with DNA encoding the Gal4 DNA-binding domain. SD-LW indicates SD without Leu and Trp. SD-LWHA indicates SD without Leu, Trp, His, and Ade. Self-interaction of AtAN was used as a positive control. EV, empty vector.

(E) Y2H analysis with mutant forms of Zng1p and Map1p. *ZNG1* and *MAP1* were either truncated (corresponding to Lys 69 for trZng1p and Asp 70 for trMap1p) or cysteine residues were substituted by serine. "tripC" refers to substitution of all three cysteine residues in the Zng1p CxCC motif with serine residues. Location of mutations can be found in Figure S1.

Figure 2. *ZNG1* is required for growth during Zn deficiency and for Map1p activity.

(A) X-Y scatter of culture optical density (OD) after 25 hours in LZM supplemented with the indicated concentrations of Zn. Only strains with empty vector are shown.

(B) and (C) Growth curves of strains in LZM with the indicated Zn supplementation.

(D) Cultures of strains diluted 10^3 fold and plated on agar-solidified LZM with the indicated Zn supplementation. The image is a composite of five separate plates (distinguished with white bars) that were incubated together and imaged at the same time.

(E) X-Y scatter of culture OD after 22 hours in CSD with the indicated concentrations of Zn.

(F) Growth curves of strains in CSD with the indicated Zn supplementation.

(G) Line graph of culture OD after 18 hours in YPD with the indicated concentrations of EDTA.

(H) and (I) Growth curves of indicated strains in YPD. For ease of comparison, the growth curve for *zng1*Δ p413-GPD with 450 μM EDTA is duplicated in panel (H) and (I).

(J) Cultures of strains diluted 10^2 fold and plated on agar-solidified YPD without or with 0.45 mM EDTA plus the indicated trace metals. The image is a composite of ten separate plates (distinguished with white bars) that were incubated together and imaged at the same time.

(K) Culture OD of strains grown in CSD with the indicated concentration of Zn and/or Co at either 14- or 20-hours post-inoculation.

(L) Immunoblots of lysates from cells expressing the 14-3-3γ-GST reporter. Separate membranes were incubated with either an antibody that only recognizes unprocessed 14-3-3γ (iMet-14-3-3γ) (top) or that recognizes GST (bottom). The same membrane but with a longer exposure is shown to better visualize the detection of the 14-3-3γ fragment in samples from *zng1*Δ. Cultures were grown in LZM with either 1, 10, or 100 μM Zn.

(M) Intensity ratio of signal from immunoblots using anti-iMet-14-3-3γ antibodies normalized to signal from anti-GST antibodies in *map2*Δ *zng1*Δ grown in LZM with either 1, 10, 100, 1000, or 3000 μM Zn (n=3 biological replicates).

Full growth curves are available in Figures S2 and S3. For panels (A) – (C), (E) – (I) and (K), solid circles represent the average of 3 wells in a 96-well microplate, and error bars represent plus and minus the standard deviation of those 3 biological replicates. Individual OD measurements are shown as X's. *P* values were calculated with an unpaired Welch's t test: panels (A) and (G) between WT p413-GPD and *zng1*Δ p413-GPD (on top) and between *zng1*Δ p413-GPD and *map2*Δ *zng1*Δ p413-GPD (on bottom), panels (B) and (H) between WT p413-GPD and *zng1*Δ p413-GPD, panels (C), (F), and (I) between *zng1*Δ p413-GPD and *map2*Δ *zng1*Δ p413-GPD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, n.s. = not significant. For panel (M), two-way ANOVA with Tukey's HSD pairwise comparison test was performed. Different letters account for significant differences (*P* < 0.05). Images of full membranes corresponding to panels (L) and (M) are shown in Figure S4.

Figure 3. Transfer of Zn from Zng1p to Map1p is dependent on GTP.

(A) Metal-stimulated GTPase activity of Zng1p.

(B) Metal-dependent hydrolytic cleavage of the methionine (Met) from MSSHRWDW by apo*-Map1p.

(C) Metal transfer to apo*-Map1p (0.5 μM). Map1p activity was quantified by the amount of cleaved Met using MSSHRWDW as a substrate. The specific assay conditions are given as a table below the graph. "Zn-Zng1p" refers to 5 μM Zng1p preloaded with Zn^{2+}, while "Co-Zng1p" refers to 5 μM Zng1p preloaded with Co^{2+}.

(D) GTPase (700 μM GTP) and ZTPase (700 μM ZTP) activity of apo-Zng1p incubated without ("apo") or with the indicated metals.

(E) Release of phosphate measured simultaneously with Map1p activity during the metal transfer assays (with MSSHRWDW as the substrate) in the presence of GTP, GMP-PNP, or ZTP and the indicated assay constituents.

(F) Metal transfer to apo*-Map1p (0.5 μM). Map1p activity was quantified using L-Met-pNA as the substrate. The specific assay conditions are given as a table below the graph. "Zn-Zng1p" refers to 5 μM Zng1p preloaded with Zn^{2+}, while "Co-Zng1p" refers to 5 μM Zng1p preloaded with Co^{2+}.

(G) Activity of 1 μM Map1p or 1 μM truncated Map1p (trMap1p; where the Zn-finger domain is absent; truncated at Asp 70) in the presence of free Zn^{2+} (5 μM) or 5 μM Zn-Zng1p with addition or absence of GTP and EDTA. Map1p and trMap1p activities were measured using L-Met-pNA as the substrate.

In (A) and (B), solid shapes represent the average of three assays. Individual datapoints are shown as open shapes. Error bars represent the standard deviation of 3 assays. In (C) – (G), bars

represent the average of 3 assays. Individual datapoints are shown as X's. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ calculated with unpaired Welch's t test.

Figure 4. Deletion of *ZNG1* leads to global proteome alterations.

(A) Table of strains and conditions analyzed by proteomics in Experiment 1.

(B) Bar chart summarizing number of proteins with a statistically significant abundance change in each of 9 comparisons. NC (no change) refers to the number of proteins without a significant change in any comparison.

(C) For those proteins with a fold change (FC) greater than or equal to 1.5, the percentage that are higher or lower in each comparison is given.

(D) Principal component analysis of the proteomics samples with genotypes and Zn conditions as variables.

(E) Venn diagram comparing proteins that are differentially abundant in the different genotypes comparing limited- and replete-Zn conditions (1 μM Zn vs 100 μM Zn, ± 1.5 FC and false discovery rate (FDR) ≤ 0.05).

(F) Heatmap of proteins that are differentially abundant. Protein intensities were z-score normalized. Samples were clustered using correlation distance and complete linkage; WGCNA was performed to distinguish clusters of proteins. Letters refer to clusters in (G) and Table S1.

(G) Grey lines represent the z-score normalized protein abundance (top) or log2FC (bottom) for individual proteins while blue lines represent the mean within each cluster identified by WGCNA. For cluster E and G, subclusters were manually identified and labeled as 1 or 2.

Figure 5. Deletion of *ZNG1* and Map1p defect result in mis-regulation of the Zn response.

(A) Volcano plot of proteins differentially abundant in comparison 4 (left) and comparison 5 (right).

(B) Scatter plot of log2FC of protein abundances comparing comparisons 5 and 1. Histograms represent number of proteins.

(C) Heatmap of log2FC of the 9 comparisons for proteins encoded by the Zap1p regulon. Proteins with a statistically significant abundance difference between WT and *map2*Δ *zng1*Δ in 1 μM are labeled, and their protein abundances (average abundance of 5 biological replicates divided by the max average) are shown as a stacked line graph in panel (D). Comparison of protein and transcript abundances for selected targets can be found in Figure S5.

(E) Gene set enrichment analysis (GSEA) barcode plot comparing proteomic profiles of *zng1*Δ or *map2*Δ *zng1*Δ versus WT strains under the low Zn condition (1 μM). GSEA determines the

location of protein sets of interest in a proteome dataset by ranking proteins by their fold changes from highest (positive) to lowest (negative). A negative normalized enrichment score (NES) value of a protein set indicates that its members (e.g., Zap1p targets), are mostly at the bottom of the ranked proteome dataset and have negative fold changes.

(F) Gene set enrichment analysis (GSEA) barcode plot comparing proteomic profiles of each strain in the two Zn conditions (1 μM vs. 100 μM). A positive normalized enrichment score (NES) indicates an enrichment of Zap1p targets at the top of the ranked proteome dataset and have mainly positive fold changes.

(G) Scatter plot of log2FC of protein abundances comparing comparisons 4 and 1. Histograms represent number of proteins.

(H) Protein abundances that may be affected by a Map1p-independent impact are shown as a stacked line graph (average abundance of 5 biological replicates divided by the max average).

(I) Scatter plot of log2FC of protein abundances from Experiment 2 comparing proteins with statistically significant protein abundance changes in *map2*Δ *zng1*Δ vs. *map2*Δ with statistically significant protein abundance changes in *map2*Δ when grown in 1 μM vs. 100 μM. Histogram represent number of proteins.

Table 1. Abundance patterns for proteins encoded by the Zap1p transcription factor regulon. Corresponding intensity values and log2FC can be found in Table S1.

Expression pattern	Proteins
lower in *zng1*Δ compared to WT AND lower in *map2*Δ *zng1*Δ compared to *zng1*Δ in low Zn	Adh4p, Fet4p, Vel1p, Css1p, Lap3p, Mcd4p, Eno2p, Eno1p, Tdh1p, Zrtp1
lower in *zng1*Δ and *map2*Δ *zng1*Δ compared to WT in low Zn	Uth1p, Zps1p, Zrc1p, Tsa1p
lower in *map2*Δ *zng1*Δ in low Zn	YOR387C, Atg19p, YJL132W
lower in *map2*Δ *zng1*Δ compared to WT in low Zn	Zrt3p, Zap1p
higher in *zng1*Δ compared to WT AND higher in *map2*Δ *zng1*Δ compared to *zng1*Δ in low Zn	Hnt1p, Ctt1p, Ald3p, Hsp12p
lower in *zng1*Δ compared to WT, but higher in *map2*Δ *zng1*Δ compared to WT in low Zn	Hsp26p
higher in *zng1*Δ and *map2*Δ *zng1*Δ compared to WT in low Zn	Pst1p, Cos6p, Hpf1p
higher in *map2*Δ *zng1*Δ compared to WT and *zng1*Δ in low Zn	Prb1p, Pet10p, Gpg1p, Tkl2p
higher in *map2*Δ *zng1*Δ compared to WT in low Zn	Mpc3p, Phm7p
no significant difference between mutants and WT under low Zn	Rtc4p, Ubx6p, Sam3p, YPR003C, Ura10p, Dpp1p, Atg41p, Prc1p, Tis11p, YMR181C

679
680 **Figure 1.**

Figure 2.

Figure 3

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STAR Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Crysten Blaby-Haas (cblaby@bnl.gov).

Materials availability

Unique reagents, plasmids and strains generated in this study will be available without restrictions from the lead contact upon request.

Data and code availability

- Raw data from the TMT-based proteomics have been deposited at MassIVE Repository and are publicly available as of the date of publication. The accession number is listed in the key resource table. All other data reported in this paper will be shared by the lead contact upon request.

- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL DETAILS

Yeast strains

Yeast strains used in this study are listed in the key resource table and were derived from *S. cereviseae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). Single deletants of *ZNG1* (formerly *YNR029c*) and *MAP2* were obtained from the *S. cerevisiae* mutant collection (Giaever et al., 2002) purchased from Horizon Discovery. The *map2*Δ *zng1*Δ strain was generated by replacement of the *ZNG1* coding region with the *URA3* cassette using homologous recombination in the *map2*Δ strain. For liquid growth assays, cells were pre-grown in 50 mL

tubes to mid-log phase in 5 mL SD medium (with the appropriate amino acid selection to grow all strains in the same medium), harvested by centrifugation at 700 × g for 5 min and rinsed once with sterile 50 µM EDTA pH 8.0 and twice with sterile Milli-Q ultrapure water. Starter cultures were then diluted to an optical density at 600 nm (OD600) of 0.001 in either 200 µL LZM [Zhao and Eide, 1996], CSD (Lyons et al., 2000), or YPD supplemented with different concentrations of EDTA as indicated in the figures. Growth assays were performed in 96-well plates (non-treated polystyrene, Grenier Bio-One, Monroe, NC) at 30 °C and shaken to ensure proper aeration at 1,050 rpm with a TermoMixer F1.5 (Eppendorf). OD600 was measured on a Tecan Infinite M1000 Pro microplate reader. For spot assays on agar-solidified media, exponentially growing cells from 5 mL cultures in 50 mL tubes were collected by centrifugation 700 × g for 5 min and rinsed once with sterile Milli-Q ultrapure water. OD600 (measured in a 96-well plate (non-treated polystyrene, Grenier Bio-One, Monroe, NC, on a Tecan Infinite M1000 Pro microplate reader)) was normalized to 0.3, and cells were serially diluted. A volume of 5 µL of cells diluted 10^3 fold (LZM), 10^3 fold (CSD) or 10^2 fold (YPD) were spotted on agar-solidified medium. Plates were incubated at 30 °C and imaged at 48 hours for LZM, 26 hours for YPD, and 50 hours for CSD.

METHOD DETAILS

Sequence similarity network construction

The sequence similarity network was constructed using the EFI-EST tool (http://efi.igb.illinois.edu/efi-est/) (Gerlt et al., 2015) with an alignment score of 50. The Uniprot database was used to search for proteins matching PF02492. Nodes were collapsed by 75% sequence similarity. The network was visualized with the yFiles Organic layout in Cytoscape V3.5.0 (Shannon et al., 2003) and is available in data file S1. Clusters containing sequences that match to IPR004392 (HypB) and IPR004400 (UreG) were removed.

Phylogenetic tree reconstruction

Protein sequences corresponding to Zng1p (encoded by *YNR029c*) (*Saccharomyces cerevisiae*), SPBC15D4.05 (*Schizosaccharomyces pombe*), CBWD1 (*Homo sapiens*), and AT1G26520 (*Arabidopsis thaliana)* were used to search against UniRef90 clusters (Suzek et al., 2015) for

homologs. The constraint-based alignment tool COBALT (Papadopoulos and Agarwala, 2007) was used to build a multiple sequence alignment (MSA) of the resulting sequences. FastTree 2.1 (Price et al., 2010) on the CIPRES Science Gateway (Miller et al., 2010) was used to infer phylogeny, and iTOL's circular display mode were used to visualize the tree (Letunic and Bork, 2019). Branches with less than 0.5 bootstrap support were deleted.

Protein sequence alignments

Amino acid sequences for assessing conservation were visualized with ESPript v3.0 (Robert and Gouet, 2014).

Interaction network analysis

Interaction data curated in the BioGRID V3.5.186 database (Stark et al., 2006) was used to search for members of the Zng1 subfamily and associated protein or genetic interactions. Interactions were available for Cbwd1 from *Mus musculus*, Zng1p from *S. cerevisiae*, SPBC15D4.05 from *S. pombe*, and for CBWD1-3 and CBWD5-7 from *H. sapiens*, which are encoded by a 5-gene family (CBWD4 is designated as a pseudogene; CBWD6 and CBWD7 are encoded by the same locus in the current reference genome assembly; GenBank: GCA_000001405.28). Interactions between Zng1 interactors were also collected and included. To generate node IDs for Zng1 interactors, mapping was performed between corresponding UniProt IDs (The UniProt Consortium and Consortium, 2019) and the HOGENOM family IDs (Penel et al., 2009) to generate a table that was imported into Cytoscape V3.5.0 (Shannon et al., 2003) (the network is available in data file S2). If a corresponding HOGENOM family ID was not available, then the BioGRID ID was used for that node. The network was visualized using the yfiles Organic layout in Cytoscape. Nodes were colored by organism and edges colored by type of interaction.

Plasmid construction

Plasmid and primer descriptions are found in Table S2. For protein expression and purification using His-Tag (used in all in vitro assays, except the Map1p vs. trMap1p comparison in Figure 3G), *ZNG1* and *MAP1* were cloned into the *Nde*I site of pET11e using a Gibson Assembly

Cloning Kit (NEB) and primers YNR029c_pET11e_F / YNR029c_pET11e_R and MAP1_pET11e_F / MAP1_pET11e_R, respectively, to PCR amplify the coding regions from genomic DNA of strain BY4742. For protein expression and purification using StrepTagII (used for Map1p vs. trMap1p comparison in Figure 3G), the coding regions of *ZNG1*, *MAP1* and tr*MAP1* were amplified using primers pET11e_ZNG1_F / pET11e_ZNG1_R, pET11e_MAP1_F / pET11e_MAP1_R, and pET11e_trMAP1_F / pET11e_MAP1_R, respectively. To generate TEV-StrepTagII (for *MAP1* and tr*MAP1*) and Myc-TEV-StrepTagII (*ZNG1*) tags, Ultramer-TEV-StrepTagII-pET11e and Ultramer-3xMyc-TEV-StrepTagII-pET11e, respectively, were synthesized as ultramers by TWIST Bioscience, fused to the C-terminal end of the genes and cloned by Gibson assembly into pET11e digested with *Nde*I and N*he*I. For testing complementation, *ZNG1* and *MAP2* were cloned into p413-GPD using restriction digest. Primers YNR029c_*Xma*I / YNR029c_*Eco*RI and MAP2_*Xma*I / MAP2_*Eco*RI were used to amplify coding regions from genomic DNA (BY4742). For the 14-3-3γ-GST reporter constructs, the coding sequence of the 14-3-3γ-GST fusion was synthesized as a gBlock™ Gene Fragment from Integrated DNA Technologies (IDT) with adapters to perform Gibson assembly; the sequence codes for the first 12 amino acids of human 14-3-3γ and 217 amino acid GST protein. This synthetic fragment was cloned into the *Eco*R1 of p413-GPD by Gibson Assembly. For Y2H, *ZNG1* was amplified using primers ZNG1_Y2H_F / ZNG1_Y2H_R and cloned into the bait vector pGBKT7 (digested with *Eco*RI/*Bam*HI). *MAP1* or *MAP2* genes were amplified using primers (Table S2) and cloned into the prey vector pGADT7-AD (digested with *Eco*RI / *Bam*HI). DNA corresponding to trZNG1 or trMAP1 was amplified by using primers tr-ZNG1-Lys68_F / ZNG1_Y2H_R and tr-Map1p_F / MAP1_Y2H_R, respectively. Amino acid substitutions were made with the megaprimer strategy using primers ZNG1_Y2H_F / ZNG1-C139S_R to generate ZNG1-C139S, ZNG1_Y2H_F / ZNG1_C136S-C138S-C139S_R to generate ZNG1-C136S-C138S-C139S, MAP1-C201S_F / MAP1_Y2H_R to generate MAP1-C201S, MAP1-C292S_F / MAP1_Y2H_R to generate MAP1-C292S and MAP1-C302S_F / MAP1_Y2H_R to generate MAP1-C302S. All constructs were verified with Sanger sequencing before use.

Strain construction

The *map2*Δ *zng1*Δ strain was generated by homologous recombination using a PCR-amplified *URA3* cassette flanked by 45 bp up- and down-stream of *ZNG1* and transformed to the *map2*Δ background. Integrants were selected on SD −Ura. Each mutant was verified with colony PCR by amplification across and within the affected loci. Primers used for strain construction and plasmids for complementation studies and expression of the 14-3-3γ-GST reporter are described in Table S2.

Immunoblot analyses

Yeast strains transformed with p413-GPD bearing the 14-3-3γ-GST reporter construct were pre-grown in 250 mL flasks containing 25 mL of LZM + 100 µM $ZnSO_4$ until mid-log phase and diluted to an OD600 of 0.001 in 25 mL of LZM + 100 µM $ZnSO_4$, LZM + 10 µM $ZnSO_4$, and LZM + 1 µM $ZnSO_4$. After incubation for 17 hours at 30 °C, cells were collected by centrifugation at 700 × g for 5 min at 4 °C and frozen at -20 °C. Frozen cell pellets were sequentially resuspended in 2 M LiAc, 0.4 M NaOH, and finally in SDS-PAGE sample buffer and boiled for 5 min. Samples were centrifuged for 5 min at 11,000 x g. The soluble fraction was used for immunoblot analysis. Protein concentration was normalized between samples by Bradford assay. A total of 25 µg were loaded on SDS-PAGE (4-20% Mini PROTEAN TGX gel from BioRad). Immunoblotting was performed on PVDF membrane (0.2 µm), and antibodies used were anti-iMet-14-3-3γ antibodies (HS23) (1:1,000; NB100-407, Novus Biologicals) and anti-GST antibodies (1:1,000; NB600-326, Novus Biologicals). The secondary antibodies used were HRP-conjugated anti-rabbit (1:10,000; A0545, Sigma-Aldrich) and anti-mouse secondary antibodies (1:10,000; A9044, Sigma-Aldrich). ECL substrate (Thermo) was used, and signal detection was obtained by ImageQuant™ LAS 4000 (Amersham). The *map2*Δ*zng1*Δ strain transformed with p413-GPD bearing the 14-3-3γ-GST reporter construct was additionally grown in LZM + 1 µM $ZnSO_4$, + 10 µM $ZnSO_4$, + 100 µM $ZnSO_4$, + 1 mM $ZnSO_4$, or + 3 mM $ZnSO_4$. Biological triplicates were analyzed, and immunoblot analysis using anti-iMet-14-3-3γ antibodies and anti-GST antibodies was performed. Signal intensity of the bands were quantified by using ImageJ software, and anti-iMet-14-3-3γ antibody signals were normalized to anti-GST antibody signals used as loading controls.

Protein purification

Recombinant Zng1p and Map1p were overexpressed in BL21(DE3) *E. coli* cells. Cultures were grown aerobically at 37 °C in Luria Broth (LB) until the OD measured at 600 nm (in a 96-well microplate with a Tecan Infinite M1000 Pro microplate reader) reached 0.5 – 0.7. Protein overexpression was then induced at 18 °C with 0.15 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 18 h. For His-tagged protein purification, cells were collected by centrifugation and resuspended in B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) supplemented with 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM imidazole, 5% glycerol, 1 tablet of complete protease inhibitor cocktail (Roche), 10 µg mL^{-1} benzonase, 0.5 mg mL^{-1} lysozyme and 0.05 mM TCEP. After homogenization, chemical lysis was performed on ice for 45 minutes. Residual cell debris were removed by centrifugation at 20,000 × g for 25 min. The supernatant was then loaded onto a Ni^{2+}-NTA column (Thermo Scientific) pre-equilibrated with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM imidazole, 5% glycerol and 0.05 mM TCEP. The column was washed with buffers containing increasing concentrations of imidazole (30 mM and 60 mM), and the proteins were finally eluted using a buffer supplemented with 250 mM imidazole. Elution fractions were analyzed by SDS-PAGE. The protein concentration was determined based on the UV absorption at 280 nm measured on a NanoDrop UV-Vis spectrophotometer. Gel filtration analysis was performed on a Superdex 200 HR 10/300 GL column (GE Healthcare) that was previously calibrated with protein standards. The column was equilibrated with 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5% glycerol and 0.05 mM TCEP. Before performing any activity assay, proteins were treated with TEV protease for 3 h at 4 °C and loaded on Ni^{2+}-NTA to remove the TEV protease and the poly-histidine tag. The flowthrough was analyzed by SDS-PAGE. For StrepTagII-tagged protein purification, cells were collected by centrifugation and resuspended in a lysis buffer consisting of 75 % B-PER/25 % Y-PER (Thermo Scientific), 30 mM HEPES pH 8.0, 300 mM NaCl, 1 mM TCEP, cOmplete protease inhibitor cocktail (Roche), 10 µg mL-1 benzonase, 1 mg mL-1 lysozyme and Biolock (IBA Lifesciences). The resulting lysate was centrifuged at 15,000 rpm for 45 min at 4 °C to remove cellular debris. Supernatant was loaded on Strep-Tactin® Sepharose® resin (IBA Lifesciences) pre-equilibrated with lysis buffer. The resin was washed with 5 column volumes (5 CV) of 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and proteins were eluted with 100 mM Tris-HCl pH 8.0, 150 mM NaCl,

1 mM EDTA, 2.5 mM desthiobiotin. StrepTagII from Zng1p-Myc was cleaved by TEV protease overnight at 4 °C. Map1p-StrepTagII, trMap1p-StrepTagII, and cleaved Zng1p-Myc were further purified by size exclusion chromatography on a Superdex 200 HR 10/300 GL column (GE Healthcare) and eluted in 50 mM HEPES pH 8.0, 300 mM NaCl, 1 mM TCEP and 5 % glycerol. Protein-containing fractions of > 90% purity were collected and stored at -80 °C until further analysis. Purified proteins were then treated with EDTA (1 mM, 3 h, at 4 °C). EDTA was subsequently removed by buffer exchange using 30-kDa cutoff Amicon centrifugal filter units (Millipore).

GTPase activity

GTPase activity was measured using the Malachite Green (MG) colorimetric assay (Motomizu et al., 1984) with Tween 20 to stabilize the dye complex (Itaya and Ui, 1966). The assay was performed as previously described for YeiR, a homologous protein from *E. coli (Blaby-Haas et al., 2012).* Briefly, the reaction mixture contained 1 µM of apo-protein, 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 5 µM of metal ($ZnSO_4$, $NiCl_2$, $MnCl_2$ or $CoCl_2$) and varying concentrations of GTP (from 5 to 700 µM). The mix was incubated at 30 °C for 150 min, while the MG reagent was prepared using 1 mL of 0.045% Malachite Green (prepared in 0.1 N HCl), 250 µL of 7.5% ammonium molybdate and 20 µL of 11% Tween 20. This mixture was incubated at room temperature for 45 min. After the incubation, 40 µL of MG reagent was added to 160 µL of each sample. After briefly shaking, sodium citrate was added at a final concentration of 3.5% to block the reaction. The absorbance was measured at 630 nm with a Tecan Infinite M1000 Pro microplate reader. ZTP hydrolysis was measured as for GTP, except that a single concentration of ZTP (700 µM) was used. The blank for each assay corresponds to the reaction mix without the enzyme. The amount of phosphate released per minute was calculated from a standard plot built using phosphate standards. The data were analyzed with Origin 9.0.

Methionine aminopeptidase (MetAP) assays

MetAP activity was measured spectrophotometrically, using either the octapeptide MSSHRWDW (Walker and Bradshaw, 1998) or L-methionine p-nitroanilide (L-Met pNA). For assays with the octapeptide, the assay buffer was composed by 50 mM Tris-HCl, pH 8.0, 200 µM

MSSHRWDW, 0.5 μM apo-protein, 0.1, 5, 10, 50, 100, 500, or 1000 μM of metal ($ZnSO_4$, $NiCl_2$, $MnCl_2$, or $CoCl_2$) in a total volume of 200 μL. Released methionine was detected by the addition of 50 μL of a ninhydrin solution (30 mM ninhydrin, 15% acetic acid), as described in Towbin, et al. (Towbin et al., 2003). Microplates were incubated for 5 minutes at room temperature, and the absorbance was measured at 490 nm. The blank for each assay was the reaction mix without the enzyme. The methionine produced per minute was calculated based on a standard curve generated with L-methionine. For the L-Met pNA assays, MetAP activity was measured as described in Tan and Konings (Tan and Konings, 1990) with the following modifications. The enzyme activity was determined spectrophotometrically in a 96-well plate using a Tecan Infinite M1000 Pro microplate reader. The MetAP reaction mix was composed by 0.5 μM of apo-protein, 1 mM L-Met p-NA, 50 mM Tris-HCl, pH 8.5, and 0.1 mM metal ($ZnSO_4$, $NiCl_2$, $MnCl_2$ or $CoCl_2$) in a total volume of 200 μL. This mixture was incubated at 30 °C for 150 minutes. The reaction was quenched by adding 80 μL of 15% acetic acid, and the absorbance was measured at 405 nm. The blank for each assay corresponds to the reaction mix without the enzyme. The p-nitroaniline produced per minute was calculated based on a standard curve generated with pure p-nitroaniline.

Metal transfer assays

Metal ion preloading was performed by incubating 5 μM of apo-Zng1p overnight at 4 °C with 50 μM of $ZnSO_4$ or $CoCl_2$. Unbound metal was then removed by buffer exchange using 30-kDa cutoff Amicon centrifugal filter units (Millipore). To perform the assay, Zng1p (with or without preloading of metal) was added to the MetAP reaction mixture, as described above for the MetAP activity assay, in the presence or absence of 1 mM EDTA. After 25 minutes, 700 μM GTP, GMP-PNP, or ZTP was added, and the production of either methionine (MSSHRWDW as substrate) or p-nitroaniline (L-Met pNA as a substrate) was monitored for 3 h at 30°C on a Tecan Infinite M1000 Pro microplate reader. The blank for each assay was the reaction mix without the enzymes, metals, and EDTA.

Whole proteome sample preparation and TMT labeling

Strains were pre-grown in 250 mL flasks containing 25 mL of LZM + 100 µM $ZnSO_4$ until mid-log phase. At which point the cultures were diluted to an OD600 of 0.001 in 25 mL of either LZM + 100 µM $ZnSO_4$ or LZM + 1 µM $ZnSO_4$. After incubation for 17 hours at 30 °C, cells were collected by centrifugation at 700 × g for 5 min at 4 °C, flash frozen with liquid nitrogen, and kept at -80 °C until proteomic analysis. Five separate cultures were grown for each strain and each condition, resulting in a total of 30 samples. Each cell pellet was resuspended in 8 M urea and transferred to 2 mL pre-filled Micro-Organism Lysing Mix glass bead tubes. Tubes were shaken using a Bead Ruptor Elite bead mill homogenizer (OMNI International, Kennesaw Georgia) at speed 5.5 for 45 sec. After bead beating, the lysate was immediately placed in an ice block before centrifuging at 1,000 × g for 10 min at 4 °C. A volume of 200 µl was transferred into a 2 mL tube and DTT was added to reach a concentration of 10 mM. Samples were sonicated, vortexed, and centrifuged, then incubated at 60 °C for 30 min with constant shaking at 800 rpm. Samples were diluted with 100 mM ABC and 1 mM $CaCl_2$, and trypsin was added at 50:1 (w/w) ratio, incubated at 37 °C for 3 hours, and snap frozen. Peptides were purified using C18 SPE desalting columns and dried to 100 µL. A total of 80 µg of peptide per sample were used for TMT labeling, while 10 µg from each sample were combined for the universal pool. All samples were dried. For TMT labelling, peptides were reconstituted in 500 mM HEPES, pH 8.5 to 5 µg/µL, and a pH above 7.4 verified prior labeling. TMT 16plex reagents were reconstituted to 20 µg/µL concentration in anhydrous acetonitrile. Peptides were mixed with TMT reagents in 1:2 (w/w) ratio. Reactions were incubated at 25 °C for one hour with shaking in a Thermomixer set at 400 rpm. Reaction was quenched with 3 µL of 5% hydroxylamine and incubated at room temperature for 15 min at 400 rpm. Samples were diluted to 2.5 mg/ml with 20% acetonitrile. Aliquots of 2 µg from each sample were combined for a pre-mix QC test, snap-frozen, and dried down. Labeled peptides from a same TMTplex were combined and dried down. Prior to MS analysis, peptide samples were purified using MicroSpin C18 Silica columns (The Nest Group, SEM SS18V). Columns were activated sequentially with 100% acetonitrile, H_2O, and 2% acetonitrile/0.1% FA. Peptides were resuspended in 2% acetonitrile/0.1% FA, loaded on the resin columns, washed twice with 2% acetonitrile/0.1% FA, and sample eluted with 80% acetonitrile/0.1% FA. Samples were dried and reconstituted with 3% acetonitrile/0.1% FA. Sample concentrations were adjusted to 0.1 µg/µl and analyzed by LC-MS/MS.

Offline fractionation of peptides and preparation of proteome samples for TMT-labelled proteomics

Labeled peptides were separated using an off-line high pH (pH 10) reversed-phase (RP) separation with a Waters XBridge C18 column (250 mm x 4.6 mm column containing 5 µm particles and a 4.6 mm x 20 mm guard column) using an Agilent 1200 HPLC System. The sample loaded onto the C18 column was washed for 15 min with Solvent A (10 mM ammonium formate, adjusted to pH 10 with ammonium hydroxide). The LC gradient started with a linear increase of Solvent B (10 mM ammonium formate, pH 10, 90:10 acetonitrile:water) to: 5% Solvent B over 10 min, 45% Solvent B over 65 min, and then a linear increase to 100% Solvent B over 15 min. Solvent B was held at 100% for 10 min, and then was changed to 100% Solvent A, this being held for 20 min to recondition the column. The flow rate was 0.5 mL/min. A total of 96 fractions were collected into a 96 well plate. The high pH RP fractions were then combined into 24 fractions using the concatenation strategy previously reported (Wang et al., 2011) excluding CHAPS containing wells. Peptide fractions were dried down and re-suspended in nanopure water at a concentration of 0.075 µg/ µL for mass spectrometry analysis using an Q Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Scientific) system as described below.

Mass-spectrometry based analysis of samples for TMT-labelled proteomics

All peptide samples were analyzed using an automated constant flow nano LC system (Agilent) coupled to a Q Exactive Orbitrap (Thermo Fisher Scientific). Electrospray emitters were custom made using 150 µm o.d. x 20 µm o.d. x 20 µm i.d. chemically etched fused silica. An on-line 4-cm x 360 µm o.d. x 150 µm i.d. fused-silica capillary analytical column (3 µm Jupiter C18) was used. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid acetonitrile (B) operated at 300 nL/min with a gradient profile as follows (min:%B); 0:5, 2:8, 20:12, 75:35, 97:60, 100:85. The LTQ Orbitrap Velos mass spectrometer was operated in the data-dependent mode acquiring higher-energy collisional dissociation (HCD) scans (R=7,500, 5 x 104 target ions) after each full MS scan (R=30,000, 3 x 106 target ions) for the top ten most abundant ions within the mass range of 300 to 1800 m/z. An isolation window of 2.5 Th was

used to isolate ions prior to HCD. All HCD scans used normalized collision energy of 45 and maximum injection time of 1000 ms. The dynamic exclusion time was set to 60 s and charge state screening was enabled to reject unassigned and singly charged ions.

Peptide identification and quantification for TMT-labelled proteomics

For peptide identification, MS/MS spectra were searched against a decoy *S. cerevisiae* protein database as well as a contaminants database containing human keratin and trypsin sequences, using the algorithm SEQUEST (Eng et al., 1994). Protein FASTA databases were derived from UniProt (Yeast_UniProt_2015-10-21). Search parameters included: no enzyme specificity for proteome data, trypsin enzyme specificity with a maximum of two missed cleaves, ± 50 ppm precursor mass tolerance, ± 0.05 Da product mass tolerance, carbamidomethylation of cysteine residues, TMT labeling of lysine residues, and peptide N-termini as fixed modifications. Allowed variable modifications were oxidation of methionine. MSGF+ (Kim et al., 2008) spectra probability values were also calculated for peptides identified from SEQUEST searches. Measured mass accuracy and MSGF spectra probability were used to filter identified peptides to <0.4% false discovery rate (FDR) at spectrum level and <1% FDR at the peptide level using the decoy approach (Elias and Gygi, 2010). TMT reporter ions were extracted using the MASIC software (Monroe et al., 2008) with a 20 ppm mass tolerance for each expected TMT reporter ion as determined from each MS/MS spectrum. Relative peptide abundances were determined from the TMT reporter ion intensities in each MS/MS spectrum.

Proteomics data analysis for TMT-labelled proteomics

The WT, *zng1*Δ and *map2*Δ *zng1*Δ strains were grown and processed together (Experiment 1). A second experiment with the *map2*Δ and *map2*Δ *zng1*Δ strains was also performed (Experiment 2). Therefore, the comparison between WT, *zng1*Δ and *map2*Δ *zng1*Δ strains is treated in separate analyses from the *map2*Δ and *map2*Δ *zng1*Δ strain comparison. For Experiment 1, peptide counts were rolled up to protein values by summing peptides that belong to each protein in each of the 30 samples (five biological replicates for each strain and condition). Peptide counts that could belong to multiple proteins were grouped under a single name representing the proteins within the group. Protein counts were then normalized between TMT pools by internal

reference scaling (IRS) methodology and trimmed mean of M value (TMM) correction. Background intensities (potentially due to peptide co-isolation between channels) were estimated based on protein intensity values for Zng1p in *zng1*Δ and *map2*Δ *zng1*Δ, Map2p in *map2*Δ *zng1*Δ, and Ura3p in WT and *zng1*Δ. Protein abundance in each sample was calculated over the total protein abundance of all samples. Similar to as previously published (Paulo et al., 2016), a background level of 3% was estimated, and proteins below this threshold are considered to be below the threshold of detection. For Experiment 2, the bioinformatic analysis was identical to experiment 1 except only the *map2*Δ and *map2*Δ *zng1*Δ strains grown in LZM + 1 µM and 100 µM $ZnSO_4$ were analyzed with edgeR. After rolling up peptide counts to protein values in each of the 20 samples (five biological replicates for each strain and condition), and IRS-TMM normalization, pairwise comparisons between strains and conditions were carried out with an exact negative binomial test using edgeR to calculate logFC and *P* values.

Gene expression analysis by quantitative PCR (qPCR)

WT, *zng*1Δ, *map2*Δ and *map2*Δ *zng1*Δ strains were pre-grown in 250 mL flasks containing 25 mL of LZM + 100 µM $ZnSO_4$ until mid-log phase. At which point the cultures were diluted to an OD600nm of 0.001 in 25 mL of either LZM + 100 µM $ZnSO_4$ or LZM + 1 µM $ZnSO_4$. After incubation for 17 hours at 30 °C, cells were collected by centrifugation at 700 × g for 5 min at 4 °C, flash frozen with liquid nitrogen, and stored at -80 °C. Total RNA were extracted using RNeasy Mini Kit (Qiagen), treated for an hour with Turbo DNAse (Ambion), and total RNA was further purified using a Quick-RNA MiniPrep kit (Zymo Research). Absence of DNA contaminant was verified by PCR amplification. cDNA synthesis was performed using 1 µg of total RNA and oligo(dT) primers, RiboLock RNase inhibitor (Thermo Scientific) and RevertAid Reverse Transcriptase kit (Thermo Scientific). cDNA synthesis was confirmed by PCR amplification. qPCR was performed using the iTaq Universal SYBR® Green Supermix (BioRad) and run on a BioRad CFX96 Real-Time System instrument with gene-specific primers reporter in Table S2. Gene expression normalization was performed using the 2-ΔCT method using *ACT1* as reference gene. Biological triplicates were used for each strain and condition, and technical replicates were made for each target gene.

Yeast 2-hybrid assays

pGBKT7 bait plasmids containing *ZNG1* or *ZNG1* mutants were co-transformed into the yeast strain Y2H-Gold with the pGADT7-AD prey vectors containing *MAP2*, *MAP1* or *MAP1* mutants, and clones selected on SD -Leu -Trp. Three independent clones were used per interaction and grown in SD -Leu -Trp until saturation. Five microliters of ten-fold dilutions were spotted on agar-solidified SD -Leu -Trp, SD -Leu -Trp -His -Ala, LZM -Leu -Trp and LZM -Leu -Trp -His -Ala medium. Plates were incubated at 30 °C and imaged at 3 days.

Determination of metal levels by inductively coupled plasma mass spectrometry (ICP-MS)

A volume of 50 µL of 1 µM apo*-Map1p and apo-trMap1p, prepared as described above, were incubated with 17.5 µL of ICP-MS-grade 69% nitric acid (1017992500, Sigma-Aldrich). After overnight digestion at room temperature, samples were diluted to a final nitric acid concentration of 2% (v/v) with Milli-Q-grade water. Fresh buffer used to prepare the apo proteins was treated the same way and measured in parallel. Zn content was determined by ICP-MS on a NexION 350D (PerkinElmer) calibrated with an environmental standard mix (N9307805, PerkinElmer), instrument metal calibration standard (N9301721, PerkinElmer), and ^{89}Y and ^{115}In as internal standards (M1-ISMS-25, Elemental Scientific). ^{66}Zn and ^{68}Zn levels were determined using a Helium (He) collision mode with kinetic energy discrimination (KED) to estimate the total content of Zn in the samples. The average of 5 technical measurements was integrated for each sample.

QUANTIFICATION AND STATISTICAL ANALYSIS

Local support values for branches in the phylogenetic tree reconstruction were calculated with a Shimodaira-Hasegawa test with 1000 replicates, which is a test built into the FastTree software run on CiPRES. Statistical analyses used in this study for comparison between two groups (growth curves and biochemical assays) were conducted using unpaired Welch's t-test. Two-way ANOVA followed by Tukey's HSD pairwise comparison test was used for band density comparison in Figure 2M. Statistical tests used for each experiment are also described in the respective figure legends. Data is shown as the average of replicates ± standard deviation (SD). Each replicate is an independent assay (n = 3) for in vitro experiments or a separate culture for in

vivo experiments (n=3) and are shown as individual datapoints in addition to the average and SD. For proteomics analysis, five biological replicates (separate cultures) were analyzed (n = 5). Pairwise comparisons between samples were performed with an exact negative binomial test using the R package edgeR (Robinson et al., 2010) to generate logFC and *P* values. Cutoff values of logFC |1.5| and *P* value <0.05 were applied on differentially expressed proteins. GO-term analysis was performed using the R package clusterProfiler (Yu et al., 2012). A *P* <0.05 was considered significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, n.s. = not significant.

Supplemental Excel table titles and legends

Table S1. Quantification of protein abundances based on Tandem Mass Tag (TMT) labeling and calculated fold changes, related to Figures 4 and 5. Raw data is available from https://massive.ucsd.edu/ProteoSAFe/private-dataset.jsp?task=14d4d1d4fb5b456db690414e67e9e016.

Table S2. Plasmid descriptions and oligonucleotide (primer) sequences used in this study, related to the STAR Methods.

Data file S1. Sequence similarity network file, related to Figure 1A. Provided as a Cytoscape file. Cytoscape is freely available from https://cytoscape.org/.

Data file S2. Molecular interaction network file, related to Figure 1D. Provided as an annotated Cytoscape file. Cytoscape is freely available from https://cytoscape.org/.

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