Both Php4 Function and Subcellular Localization Are Regulated by Iron via a Multistep Mechanism Involving the Glutaredoxin Grx4 and the Exportin Crm1

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In Schizosaccharomyces pombe, the CCAAT-binding factor is a multisubunit complex that contains the proteins Php2, Php3, Php4, and Php5. Under low iron conditions, Php4 acts as a negative regulatory subunit of the CCAAT-binding factor and fosters repression of genes encoding iron-using proteins. Under conditions of iron excess, Php4 expression is turned off by the iron-dependent transcriptional repressor Fep1. In this study, we developed a biological system that allows us to unlink iron-dependent behavior of Php4 protein from its transcriptional regulation by Fep1. Microscopic analyses revealed that a functional GFP-Php4 protein accumulates in the nucleus under conditions of iron starvation. Conversely, in cells undergoing a transition from low to high iron, GFP-Php4 is exported from the nucleus to the cytoplasm. We mapped a leucine-rich nuclear export signal from low to high iron, GFP-Php4 is exported from the nucleus to the cytoplasm. We mapped a leucine-rich nuclear export signal that is necessary for nuclear exclusion of Php4. This latter process was blocked by leptomycin B. By using coimmunoprecipitation analysis, we showed that Php4 and Crm1 physically interact with each other. Although we determined that nuclear retention of Php4 per se is not sufficient to cause a constitutive repression of iron-using genes, we found that deletion of the grx4+ -encoded glutaredoxin-4 renders Php4 constitutively active and invariably localized in the nucleus. Further analysis by bimolecular fluorescence complementation assay and by two-hybrid assays showed that Php4 and Grx4 are physically associated in vivo. Taken together, our findings indicate that Grx4 and Crm1 are novel components involved in the mechanism by which Php4 is inactivated by iron in a Fep1-independent manner.

Iron is essential to the growth of the vast majority of organisms. Because of its capacity to act as both an electron acceptor and donor, iron has become an indispensable catalytic cofactor for a multitude of enzymes involved in several biological processes ranging from respiration, to the tricarboxylic acid cycle, to DNA synthesis (1). Paradoxically, excess iron is toxic because of its ability to generate hydroxyl radicals via the Fenton chemistry reaction (2). High levels of hydroxyl radicals can produce cellular damage, including direct protein or enzyme inactivation, membrane impairment because of lipid peroxidation, and oxidative DNA damage (3). These two facets of iron require that cells must establish fine-tuned mechanisms to maintain sufficient but not excessive concentrations of iron, thereby keeping the delicate balance between essential and toxic levels of iron.

In Schizosaccharomyces pombe, iron homeostasis is controlled by two iron-regulated proteins, the GATA-type transcription factor Fep1 and the CCAAT-binding factor Php4 (4, 5). When iron levels are high, Fep1 binds to GATA cis-acting elements found in the promoter regions of genes involved in iron acquisition (e.g. fip1+, fio1+, frp1+, and str1+/2+/3+) and shuts down their expression to avoid deleterious consequences of iron overload (6, 7). When iron levels are low, Fep1 is unable to bind DNA, resulting in the transcriptional induction of genes involved in iron acquisition (6, 8, 9). Analogous to S. pombe, other fungi use Fep1 orthologs to repress transcription of target genes in response to high iron. Examples include Urbs1 in Ustilago maydis (10, 11), SRE in Neurospora crassa (12), SreA in Aspergillus nidulans (13, 14), Sfu1 in Candida albicans (15), and Cir1 in Cryptococcus neoformans (16).

In the fission yeast S. pombe, one gene that is regulated in a Fep1-dependent manner is php4+; php4+ encodes a subunit of the CCAAT-binding protein complex, which includes three other subunits, denoted Php2, Php3, and Php5 (17, 18). Genes encoding Php2, Php3, and Php5 are constitutively expressed, whereas transcript levels of php4+ are induced under conditions of iron starvation and repressed under iron-replete conditions (18). Php4 is responsible for the transcriptional repression capability of the Php complex and is not required for the DNA binding activity of the complex. A genome-wide microarray analysis revealed that Php4 is capable of coordinating the repression of 86 genes in response to iron starvation (19). Among these genes, several encode proteins involved in iron-dependent metabolic pathways, such as the tricarboxylic acid cycle, mitochondrial respiration, heme biosynthesis, and iron-sulfur cluster assembly. DNA microarray analysis also showed that the gene encoding the iron-responsive transcriptional repressor Fep1 is down-regulated in response to iron deficiency in a Php4-dependent fashion. Based on these data, we proposed a model wherein tight regulation of intracellular iron levels is controlled by the interplay between Php4 and Fep1 through mutual control of each other’s expression (19).

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Down-regulation of iron-dependent pathways to optimize the utilization of limited available iron has previously been described in other organisms. When iron is limiting in *Escherichia coli*, the transcriptional repressor Fur is inactivated, leading to increased levels of RyhB, a small noncoding RNA (20). RyhB hybridizes to mRNAs encoding iron-using proteins and triggers their degradation through an RNase E-dependent process (21). In *Saccharomyces cerevisiae*, iron deprivation leads to the activation of the iron-responsive transcription factors Aft1 and Aft2 (22–27). Once activated, these two regulators induce the expression of several genes, including those encoding proteins that function in iron uptake. Additional genes are also positively regulated by Aft1 and Aft2, including CTH1 and CTH2, which encode two CCCH-type zinc finger mRNA-binding proteins that bind AU-rich elements within the 3′-untranslated region of transcripts (28, 29). Among the transcripts that Cth1 and Cth2 can bind and down-regulate in response to iron starvation, several encode iron-using proteins or enzymes that participate in biochemical pathways that use iron as cofactor (29).

As for Aft1 and Aft2, iron-responsive Cth1- and Cth2-like proteins are found mainly in *Saccharomyces* fungal species (1). The other fungal species such as *Pezizzomyces*, *Taphrinomyces*, *Basidimycota*, and *Zygomycota* appear to have a distinct pair of iron-responsive regulatory proteins that include a negative GATA-type transcriptional regulator (e.g. Fep1 and SreA) and a negative regulatory subunit of the CCAAT-binding complex (e.g. Php4 and HapX) (1).

In *A. nidulans*, a Php4 ortholog, denoted HapX, can trigger down-regulation of genes encoding iron-using proteins during iron deficiency (30). Interestingly, both Php4 and HapX have been shown to be regulated post-translationally by iron (19, 30). Experiments have shown that HapX dissociates from the HapE subunit of the CCAAT-binding complex (HapB-C-E) when iron is abundant (30). Using a one-hybrid approach in fission yeast, a constitutively expressed Gal4-Php4 fusion protein was shown to repress transcription as a function of iron availability when brought to a DNA promoter, revealing that Php4 has the ability to sense iron at the protein level (19). Recent data have shown to repress transcription as a function of iron availability when brought to a DNA promoter, revealing that Php4 has the ability to sense iron at the protein level (19). Recent data have shown that Php4 binds to Crm1 and is sensitive to leptomycin B (LMB), abolishing its nuclear export behavior. In the presence of LMB, although the GFP-Php4 fusion protein was localized in the nucleus under conditions of both low and high levels of iron, we observed that the protein can still be inactivated by iron, resulting in the derepression of isa1+ transcription in response to iron. Deletion of grx4+, on the other hand, led to permanent Php4 nuclear accumulation and constitutive repression of isa1+. Further analysis by bimolecular fluorescence complementation assay and by two-hybrid assays revealed that Grx4 is a binding partner of Php4. In summary, these results demonstrate that the Php4 subunit of the *S. pombe* CCAAT-binding complex translocates from the nucleus to the cytoplasm in an iron-, Grx4-, and Crm1-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Yeasts and Growth Conditions**—The *S. pombe* strains used in this study were the wild-type FY435 (h+ his7-366 leu1-32 ura4-D18 ade6-M210) and four isogenic mutant strains, fep1Δ (h+ his7-366 leu1-32 ura4-D18 ade6-M210 fep1Δ:ura4*), php4Δ (h+ his7-366 leu1-32 ura4-D18 ade6-M210 php4Δ::KanR), grx4Δ (h+ his7-366 leu1-32 ura4-D18 ade6-M210 grx4Δ::KanR), and php4Δ grx4Δ (h+ his7-366 leu1-32 ura4-D18 ade6-M210 php4Δ:loxP grx4Δ::KanR). All five strains were grown in yeast extract medium containing 0.5% yeast extract and 3% glucose that was further supplemented with 225 mg/liter of adenine, histidine, leucine, uracil, and lysine, unless otherwise stated. Strains for which plasmid transformation was required were grown in synthetic Edinburgh minimal medium lacking specific nutrients required for plasmid selection and maintenance. Cells were seeded to an A600 of 0.5, grown to exponential phase (A600 of ~1.0), and then treated with 250 μM 2′,2′-dipyridyl (Dip) or 100 μM FeSO4 or left untreated for 90 min, unless otherwise indicated. *S. pombe* grx4Δ and php4Δ grx4Δ disruption strains, as well as control strains, were cultivated in culture jars under microaerobic conditions using the GazPack EZ system (BD Biosciences). For two-hybrid experiments, *S. cerevisiae* strain L4O (Mata his3Δ200 trpl-901 leu2-3, 112 ade2 LYS2::(lexAop)_4-HIS3 URA3::(lexAop)_8-lacZ) (35) was grown in synthetic minimal medium containing 83 mg/liter histidine, adenine, uracil, and lysine, 2% dextrose, 50 mM MES buffer (pH 6.1), and 0.67% yeast nitrogen base minus copper and iron (MP Biomedicals, Solon, OH).

**Plasmids**—The pJK-194*promphp4* plasmid contains a 194-bp DNA segment of the php4+ promoter harboring multiple point mutations in the two iron-responsive GATA sequences (positions –188 to –133 and –165 to –160) found

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3 The abbreviations used are: NES, nuclear export signal; BIFC, bimolecular fluorescence complementation; DAPI, 4′,6-diamidino-2-phenylindole; Dip, 2′,2′-dipyridyl; GFP, green fluorescent protein; GSH, glutathione; LMB, leptomycin B; ORF, open reading frame; WT, wild type; MES, 2-(N-morpholino)ethanesulfonic acid; PCNA, proliferating cell nuclear antigen.
in the php4+ promoter (19). The open reading frame (ORF) encoding GFP was PCR-amplified from the pSF-GP1 plasmid (36) and inserted into the Sall and Asp718 restriction sites of pJK-194*promphp4+ to create pJK-194*promphp4+-GFP. pJK-194*promphp4+-GFP-php4+ was constructed through a three-piece ligation by simultaneously introducing a BamHI-Sall PCR-amplified fragment containing the GFP gene and a Sall-Asp718 PCR-amplified fragment bearing the php4+ ORF into a BamHI-Asp718-digested pJK-194*promphp4+ plasmid. The nmt1+ promoter up to position -1178 from the start codon of the nmt1+ gene was amplified by PCR from pREP-41X (37). Once generated, the promoter DNA fragment was inserted into the integrative vector pJK148 (38) at the SacI and BamHI sites. The construct was denoted pJKmnt41X. A DNA fragment encoding the GFP-php4+ allele was isolated from pJK-194*promphp4+-GFP-php4+ and then inserted into the BamHI and Asp718 restriction sites of pJKmnt41X to create pJKmnt41X-GFP-php4+. To generate the php4 NES mutant allele (L93A, L94A, L97A, and L100A), the plasmid pJK-194*promphp4+-GFP-php4+ was used in conjunction with the overlap expansion method (39) and the oligonucleotides 5’-CGAGGCACTGAGGCAGAGATGGCTAGCAGCTGCTGATGTAAGATTCTACTTTGG-3’ and 5’-GGCTGAGGCATCTCTGCGCTACGCTGCTGTTGTTCTCCTGTTAATTCTGCC-3’ (underlined letters represent nucleotide substitutions that gave rise to mutations) and two oligonucleotides corresponding to the start and stop codons of the ORF of php4+. The resulting PCR-amplified fragment was digested with Sall and Asp718 and cloned into pJKmnt41X-GFP, which was previously engineered by inserting the GFP gene into pJKmnt41X. The resulting construct was designated pJK-41X-GFP-php4+NESmut. All nucleotide changes were confirmed by DNA sequencing. Plasmids pSP-1178ntt-GST-GFP and pSP-1178ntt-GST-GFP-Pap1515NES533 were constructed by a strategy described previously (40). Plasmid pSP-1178ntt-GST-GFP was digested with SpeI and SstI restriction enzymes and used to receive annealed synthetic DNA fragments encoding wild-type and mutant versions of Php4 NES. Resulting plasmids were denoted pSP-1178ntt-GST-GFP-Php473NES122mut and pSP-1178ntt-GST-GFP-Php473NES122mut, respectively. Plasmid pJK-194*promphp4+-TAP-php4+ was constructed by replacing the GFP ORF in pJK-194*promphp4+-GFP-php4+ by a PCR-amplified TAP fragment isolated from pREP1-NTAP (41). The purified TAP fragment was also cloned into pJK-194*promphp4+ to create pJK-194*promphp4+-TAP. Both pJK-194*promphp4+-TAP-php4+ and pJK-194*promphp4+-TAP, respectively. The S. pombe crml1+ promoter up to -1000 from the start codon of the crml1+ gene was isolated by PCR and then inserted into the pSP1 vector (43) at the Apal and PstI sites. The resulting plasmid was denoted pSP1-1000crml1+ prom. The full-length coding region of crml1+ was isolated by PCR, using primers that corresponded to the initiator and stop codons of the ORF. Because the primers contained PstI and XmaI restriction sites, the purified DNA fragment was digested with these enzymes and cloned into the corresponding sites of pSP1-1000crml1+prom. The resulting plasmid was named pSP1crml1+. The Myc12 epitope, obtained from the pcr4+X-myc12 plasmid (44) using XmaI and SstI restriction enzymes, was subsequently inserted 3’ to the crml1+ gene in pSP1crml1+. For two-hybrid interaction assay, the complete or truncated versions of the php4+ gene were inserted downstream of and in-frame to the VP16 coding sequence using BamHI and Asp718 restriction sites found in pVP16 (35). The bait plasmid pLexA-grx4+ was created by cloning a 747-bp BamHI-PstI DNA fragment containing the full-length coding region of grx4+ into the same sites of pLexA (35).

RNA Isolation and Analysis—Total RNA was extracted by a hot phenol method as described previously (45). RNA samples were quantitated spectrophotometrically, and 15 μg of RNA per sample were used for the RNase protection assay, which was carried out as described previously (19). To detect grx4+ mRNA levels, plasmid pSKgrx4+ was created by inserting a 191-bp BamHI-EcoRI fragment from the grx4+ gene into the same restriction sites of pBluescript SK (Stratagene, La Jolla, CA). The antisense RNA hybridizes to the region between positions +327 and +518 downstream from the A of the initiator codon of grx4+. To generate pSKVP16, a 201-bp fragment from the VP16 gene was amplified and cloned into the BamHI-EcoRI sites of pBluescript SK. Plasmids pSKisa1+, pSKphp4+, and pSKact1+ (18) were used to produce antisense RNA probes, allowing the detection of steady-state levels of isa1+, php4+, and act1+ mRNAs, respectively. A riboprobe derived from the plasmid pKSACT1 (46) was used to monitor the steady-state levels of ACT1 mRNAs in experiments using the bakers’ yeast strain L40.

Protein Extraction and Immunoblotting—Total cell lysates were prepared as described previously (18). Cell lysates were quantitated using the Bradford assay (47), and equal amounts of each sample were subjected to electrophoresis on 9% SDS-polyacrylamide gels. After electrophoresis, protein samples were electroblotted as described previously (9). The following primary antisera were used for immunodetec-
Iron Inhibition of Php4 Function

Iron Inhibition of Php4 Function—As we have previously shown (18, 19) inactivation of Php4 in response to iron is regulated at two distinct levels. First, in the presence of iron, Fep1 associates with GATA elements in the *php4* promoter to repress transcription, ensuring the extinction of most *php4* transcripts. Second, we observed that in the absence of Fep1, although *php4* mRNA levels are constitutive and unresponsive to iron for repression, the gene product (Php4) can still be inactivated by iron. Iron-mediated inhibition of Php4 results in transcriptional induction of the regulon of genes controlled by Php4 (19). To begin to characterize the mechanism by which Php4 activity is regulated by iron in a Fep1-independent manner, we developed a biological system in which *php4* and *GFP-php4* alleles were expressed under the control of a GATA-less *php4* promoter. By use of this system, we determined that *php4* mutant cells expressing *php4* or *GFP-php4* exhibited *php4* mRNA levels that were constitutively expressed under both low and high iron concentrations (Fig. 1A). As control for normal transcriptional regulation, the steady-state levels of *php4* were down-regulated when wild-type cells were grown under basal and iron-replete conditions (Fig. 1A, WT). As controls for signal specificity, cells harboring an inactivated *fep1* gene (*fep1Δ*) exhibited a constitutive transcription of *php4*, and *php4* mRNA was absent in *php4Δ* null cells (Fig. 1A). Subsequently, we examined the effect of a sustained *php4* or *GFP-php4* expression on the transcriptional profile of *isa1*, a Php4-regulated target gene (Fig. 1B). When Php4 was constitutively expressed, we observed that the repression of *isa1* occurs only under low iron conditions, suggesting
that in the absence of Fep1 or its iron-responsive cis-acting elements iron can still trigger the inactivation of Php4 (Fig. 1B).

To ensure that fusion of GFP to the N terminus of Php4 did not interfere with its function, we analyzed if iron limitation-dependent down-regulation of isa1+ gene expression was corrected by integrating a GFP-Php4+ allele expressed from both the wild-type php4+ promoter (WT GATA) or under the regulation of a GATA-less php4+ promoter (GATA-less). Transformed cells were grown under basal (−) or iron-deficient conditions (250 μM FeSO4) or with excess iron (100 μM FeSO4) (Fe). Whole-cell extracts were prepared and analyzed by immunoblotting with anti-GFP or anti-PCNA (as an internal control) antibody. M, the positions of the molecular weight standards are indicated to the left.

![Diagram](image_url)

**FIGURE 1. Php4 is inactivated in a fep1-independent manner.** A, logarithmic-phase cultures of isogenic strains FY435 (WT), AMY15 (phpΔ), and BYP10 (fep1Δ) were untreated (−) or treated in the presence of Dip (250 μM) or FeSO4 (Fe) (100 μM) for 90 min. After treatment, total RNA was prepared from each sample and analyzed by RNase protection assays. Steady-state levels of **php4** and **act1** mRNAs (indicated with arrows) were analyzed in the wild-type strain and strains lacking the php4+ or fep1+ allele. When indicated, phpΔ cells were transformed with the integrative plasmids pJK-194promphp4+ /phpΔ (GATA-less + php4+) and pJK-194promphp4+ /GFP-php4+ (GATA-less + GFP-php4+). B, aliquots of the cultures described for A were examined by RNase protection assays for steady-state levels of isa1+ mRNA. The arrows indicate signals corresponding to isa1+ and act1+ transcripts. RNase protection data shown in A and B are representative of three independent experiments. C, cells carrying a disrupted phpΔ allele were transformed with an empty plasmid (vector alone), or plasmids expressing GFP-php4+ under the control of the wild-type php4+ promoter (WT GATA), or under the regulation of a GATA-less php4+ promoter (GATA-less). Transformed cells were grown under basal (−) or iron-deficient conditions (250 μM Dip) or with excess iron (100 μM FeSO4) (Fe). Whole-cell extracts were prepared and analyzed by immunoblotting with anti-GFP or anti-PCNA (as an internal control) antibody. M, the positions of the molecular weight standards are indicated to the left.

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**Iron Starvation Induces Nuclear Accumulation of GFP-Php4**

To further investigate the mechanism by which Php4 activity is regulated, we examined the localization of Php4 in response to changes in iron levels. To perform these experiments, we used a phpΔ mutant strain where expression of the GFP-php4+ allele was under the control of a GATA-less php4+ promoter. Cells were grown in minimal medium (under basal conditions) to exponential phase and then treated with the iron chelator Dip (250 μM) or FeSO4 (100 μM) for 3 h. Following treatment with Dip, GFP-Php4 localized to the nucleus (Fig. 2). Consistent with this observation, GFP-Php4 fluorescence colocalized with the DNA-staining dye DAPI, which was used as a marker for nuclear staining. When cells were treated with FeSO4, GFP-Php4 was viewed primarily in the cytoplasm (Fig. 2). Before treatment (at the zero time point; minimal medium contained 74 nM iron), GFP-Php4 displayed a pancellular-fluorescence pattern (Fig. 2). As we observed previously, GFP alone was cytoplasmic as well as nuclear (52). Taken together, these results indicate that GFP-Php4 localizes to the nucleus in iron-deficient cells but is detected in the cytoplasm when cells are grown under iron-replete conditions.

The nuclear accumulation of GFP-Php4 in response to iron starvation prompted us to examine the fate of the nuclear pool of Php4 in response to variations in iron levels. To address this point, we used the nmt1+ inducible/repressible promoter system (53). Expression of an integrated GFP-php4+ allele under the control of the nmt1+ 41X promoter (37) allowed us to induce the synthesis of GFP-Php4 in the presence of Dip, thereby fostering its nuclear sequestration (Fig. 3A). Prior to nmt1+ transcriptional shut-off, basal levels of GFP-php4+ mRNA were detected in phpΔ cells (Fig. 3B, T0). Under the same low iron and thiamine-free conditions, we observed that GFP-Php4 localized in the nucleus (Fig. 3A, T0). After the addition of thiamine to repress further synthesis (Fig. 3B), we examined the effects of iron, manganese, cobalt, and Dip on the subcellular localization of GFP-Php4. As shown in Fig. 3A, when cells were incubated for 60 min with 100 μM FeSO4, GFP-Php4 was exported from the nucleus to the cytoplasm. In contrast, when cells were treated with MnCl2 (100 μM), CoCl2 (100 μM), or Dip (250 μM), there was no apparent change in the localization of GFP-Php4, with its fluorescent signal observed primarily in the nucleus (Fig. 3A). The iron-mediated nuclear exclusion of Php4 (predicted mass of 59.8 kDa) when expressed under the control of the GATA-less promoter. Immunoblot analyses revealed that GFP-Php4 is stable and present in similar relative amounts in iron-deficient and iron-replete cells, suggesting that the mechanism of Php4 inactivation was not operated through iron-regulated changes in protein synthesis or stability (Fig. 1C). On the other hand, we observed that steady-state levels of GFP-Php4 were dramatically reduced in cells grown in the presence of iron (or left untreated) when the fusion allele was under the control of the wild-type php4+ promoter (Fig. 1C). Therefore, despite a constitutively expressed GFP-Php4, either from a fep1Δ strain or a GATA-less promoter, the Php4 target gene isa1+ was only repressed under low iron conditions. These results strongly suggest that iron can trigger the inactivation of Php4 function through an additional mechanism that is independent of Fep1-mediated transcriptional repression.
GFP-Php4 resulted in a translocation of preexisting GFP-Php4 from the nucleus to the cytoplasm (and not because of a newly synthesized GFP-Php4 arising from a pool of stable mRNA) because no GFP-Php4 transcripts were detected after the addition of thiamine (30 and 60 min) compared with GFP-Php4 transcripts observed in cells at the zero time point (Fig. 3B). Furthermore, to ensure that the nuclear export of GFP-Php4 in response to iron was because of the native GFP-Php4 protein rather than products from proteolytic cleavage of the fusion peptide, total protein extracts from cells transformed with the integrative plasmid encoding GFP-Php4 were analyzed by immunoblotting (Fig. 3C). The results showed that the full-length GFP-Php4 fusion protein was detected after 30 and 60 min of Dip and iron treatments (Fig. 3C). Taken together, these data indicate that nuclear GFP-Php4 is exported from the nucleus upon the addition of iron. Consistent with its exclusion from the nucleus, GFP-Php4 was predominantly localized in the cytoplasm.

Iron-Induced Relocalization of Php4 from the Nucleus to the Cytoplasm Requires a Leucine-Rich NES—The intracellular redistribution of GFP-Php4 during a shift from low to high iron conditions shows that its subcellular localization is regulated by iron. In light of this observation, we sought to identify amino acids in Php4 that could serve as an NES. One of the characteristic features of a typical NES is the cluster of essential hydrophobic amino acids represented by the (L)X2–3(LIVFM)X2–3(L)X(L) motif (where X indicates any amino acid residue) (54). Importantly, a large number of NES regions are known to be embedded in predicted amphipathic helices (55). Examination of the Php4 sequence revealed a region containing hydrophobic residues that partially juxtapose the consensus (L)X2–3(LIVFM)X2–3(L)X(L) motif. This potential NES, **93LLEQLEML100**,
resembles the NES sequences of other proteins, except that the last hydrophobic amino acid residue is absent, being replaced by an alanine (Fig. 4A). Interestingly, however, when Php4 was subjected to a computational helical wheel prediction, the leucine residues 93, 94, 97, and 100 were regrouped on the same side of a putative amphipathic \( \alpha \)-helix, suggesting that this region could serve as an NES (Fig. 4A). To determine whether this putative NES is functional, we generated a GFP-Php4 mutant in which all of the four leucine residues were replaced with alanine residues to produce GFP-Php4 NES mutant. Cells expressing the NES mutant allele exhibited nuclear accumulation of GFP-Php4 following treatment with FeSO\(_4\) (100 \( \mu \)M) for 0 and 60 min (Fig. 4B). After the same treatment, the wild-type GFP-Php4 fusion protein accumulated in the cytoplasmic region, revealing a translocation of GFP-Php4 from the nucleus to the cytoplasm (Fig. 4B). At the zero time point or following treatment with Dip (250 \( \mu \)M) for 60 min, both the wild-type and NES mutant proteins were localized exclusively in the nucleus (Fig. 4B).

To assess if the NES region of Php4 has the ability by itself to trigger nuclear export, the Php4 73–122-amino acid fragment was fused to GST-GFP, as described for the 515–533-amino acid fragment of the transcription factor Pap1, which is known to contain a functional NES (56). Likewise, we also examined the effect of mutating Leu\(_{93}\), Leu\(_{94}\), Leu\(_{97}\), and Leu\(_{100}\) to Ala on the ability of the 73–122-amino acid fragment of Php4 to export the reporter protein. This mutant was designated GST-GFP-Php4\(_{73}^{73}\)NESmut\(_{122}^{122}\). Cells expressing GST-GFP, GST-GFP-Pap1\(_{515}^{515}\)NESmut\(_{533}^{533}\), GST-GFP-Php4\(_{73}^{73}\)NESmut\(_{122}^{122}\), and GST-GFP-Php4\(_{73}^{73}\)NESmut\(_{122}^{122}\) were cultivated to mid-logarithmic phase in thiamine-free medium. The cells were washed twice and then grown in the presence of 15 \( \mu \)M thiamine to stop gene expression. After being transferred to thiamine-replete medium for 6 h, analysis by fluorescence microscopy showed that cells harboring the wild-type NES regions of Pap1 and Php4 had significantly less nuclear accumulation compared with that of the GST-GFP-Php4\(_{73}^{73}\)NESmut\(_{122}^{122}\) mutant (Fig. 5, 1st row). As a negative control, GST-GFP alone was present throughout the cells.

Generally, proteins harboring leucine-rich NES are recognized by the exportin Crm1, which mediates their nucleocytoplasmic translocation with the aid of RanGTP (57). LMB can bind covalently to a cysteine residue in Crm1 and interfere with the binding of both RanGTP and the cargo proteins, thereby preventing nuclear export (56–58). Because Php4 harbors such a leucine-rich NES within the peptidic module encompassing amino acids 73–122, we tested the ability of LMB to inhibit
Iron-mediated GST-GFP-Php473NES122 nuclear export. As shown in Fig. 5 (penultimate row), LMB treatment caused diffused distribution of GST-GFP-Php473NES122 in both the cytoplasm and the nucleus. A positive control was GST-GFP-Pap1515NES533, which is excluded from the nucleus via Crm1 (Fig. 5, 1st row), unless the exportin is inhibited by the presence of LMB (40, 56). As shown in Fig. 5, GST-GFP-Pap1515NES533 was pancellular in LMB-treated cells. Taken together, these results show that amino acids 73–122 of Php4 contain a nuclear export signal that is potentially bound by the exportin Crm1 in response to iron. Furthermore, the data reveal that the hydrophobic residues Leu93, Leu94, Leu97, and Leu100, or at least some subset of these residues, are necessary for exporting Php4 from the nucleus to the cytoplasm.

Iron-mediated Nuclear Export of Php4 Occurs through the Crm1 Exportin—To determine whether Crm1 was involved in the iron-induced nuclear export of GFP-Php4, we examined the effect of LMB on the localization of GFP-Php4 during a shift from low to high iron concentrations. Cells were grown in low iron medium in the absence of thiamine. After the addition of thiamine to stop the synthesis of new GFP-php4/H11001 transcripts, cells were incubated for 0 and 60 min without or with LMB (100 ng/ml LMB). At the zero time point, GFP-Php4 was observed in the nucleus (Fig. 6A). Likewise, when cells were starved for iron in the presence of Dip, GFP-Php4 was viewed solely in the nucleus (Fig. 6A). However, after 60 min of treatment in the presence of iron, GFP-Php4 moved into the cytoplasm, displaying a cytosolic distribution pattern (Fig. 6A). When iron-treated cells were incubated with LMB, GFP-Php4 was seen exclusively in the nucleus (Fig. 6A). Thus, iron-mediated nuclear-to-cytosolic export of GFP-Php4 is a consequence of iron-induced nuclear-to-cytosolic export of GFP-Php473NES122 nuclear export. As shown in Fig. 5 (penultimate row), LMB treatment caused diffused distribution of GST-GFP-Php473NES122 in both the cytoplasm and the nucleus. A positive control was GST-GFP-Pap1515NES533, which is excluded from the nucleus via Crm1 (Fig. 5, 1st row), unless the exportin is inhibited by the presence of LMB (40, 56). As shown in Fig. 5, GST-GFP-Pap1515NES533 was pancellular in LMB-treated cells. Taken together, these results show that amino acids 73–122 of Php4 contain a nuclear export signal that is potentially bound by the exportin Crm1 in response to iron. Furthermore, the data reveal that the hydrophobic residues Leu93, Leu94, Leu97, and Leu100, or at least some subset of these residues, are necessary for exporting Php4 from the nucleus to the cytoplasm.

Iron-induced nuclear export of Php4 is sensitive to LMB and involves an interaction between Php4 and Crm1. A, cells bearing a php4Δ deletion were transformed with pJK-nmt1X-GFP-php4Δ. The cells were grown to mid-logarithmic phase in thiamine-free medium supplemented with 250 μM Dip. After two washes, the cells were incubated for 0 and 60 min in medium with thiamine (15 μM) and in the presence of 100 μM FeSO4 (Fe), 100 μM FeSO4, and 100 ng/ml LMB (Fe + LMB) or 250 μM Dip. GFP-Php4 was visualized by direct fluorescence microscopy. Cell morphology was verified using Nomarski optics. B, php4Δ cells were cotransformed with plasmids expressing TAP alone and myc-tagged Crm1, or TAP-tagged Php4 and myc-tagged Crm1. Extracts (Total) were subjected to immunoprecipitation (IP) using IgG-Sepharose beads. The proteins bound to the IgG-Sepharose were washed, heated, loaded, and separated on SDS-polyacrylamide gels. After electrophoresis, the bound proteins were analyzed by immunoblot assay using anti-c-Myc antibody. A portion of the total cell extracts (≈2%) was included to verify the presence of the immunoblotted proteins prior to chromatography. As additional controls, aliquots of whole-cell extracts and bound fractions were probed with anti-mouse IgG antibody and anti-PCNA antibody. The positions of the molecular weight standards are indicated to the left. WB, Western blot; NS, nonspecific signal. C, aliquots of the cultures described for A were analyzed by RNase protection assay. Steady-state mRNA levels of isa1/H9001 and act1/H11001 (indicated with arrows) were probed. As a control for signal specificity, isa1Δ mRNA steady-state levels were detected in a php4Δ null strain. The results shown are representative of three independent experiments.
Iron Inhibition of Php4 Function

Gfp4 is Required for Iron Inhibition of Php4 Activity in S. pombe—To gain additional insight into the mechanism by which Php4 is inactivated by iron in a Fep1-independent manner, we sought to identify cellular components involved in mediating iron inhibition of Php4 activity. In S. cerevisiae, it is known that when iron concentrations are low, the transcription factor Aft1 translocates in the nucleus and induces expression of its target genes (59, 60). Conversely, in response to iron-replete conditions, Aft1 moves from the nucleus to the cytoplasm, where it remains inactive (61). Aft1 is an activator, and in contrast, S. pombe Php4 is a repressor; however, both are active under conditions of iron deficiency and inactive in the presence of iron. Recent studies have provided clues with respect to iron-dependent inhibition of Aft1 (31–34, 62). One aspect is the requirement of the monothiol glutaredoxins Grx3 and Grx4 to allow Aft1 to sense iron excess (32, 33). Loss of both glutaredoxins results in constitutive nuclear localization of Aft1 and permanent expression of its target genes irrespective of iron status. To test if the S. pombe monothiol glutaredoxin Grx4 plays an important role for iron-dependent inhibition of Php4 function, we insertionally inactivated the grx4Δ allele in cells grown under micro-aerobic conditions. To validate that transcripts of grx4Δ were absent in the grx4Δ deletion strain, the steady-state mRNA levels of grx4Δ were analyzed by RNase protection assays. The results shown in Fig. 7A indicate that no grx4Δ transcript was observed in the grx4Δ null strain. As a control, grx4Δ transcripts were detected as a doublet in the wild-type strain (Fig. 7A). Interestingly, we repeatedly observed a low but significant increase (2.2-fold) of grx4Δ transcript levels in iron-starved cells (Fig. 7A). Although our data do not allow us to establish the reason for this result and the detection of the grx4Δ transcripts as a doublet, it corroborates data obtained previously by microarray experiments (19). To assess the effect of the absence of Grx4 on Php4 function, we carried out RNase protection analysis to examine the relative expression of isa1Δ in grx4Δ cells. In the presence of Dip, isa1Δ mRNA levels were repressed (Fig. 7B). In untreated and iron-replete cells, isa1Δ transcript levels remained very low and approximately equal to those observed in iron-starved cells (Fig. 7B). As expected, in wild-type cells, isa1Δ transcript levels were repressed only in the presence of Dip (Fig. 7B). The fact that isa1Δ transcripts were invariably repressed in grx4Δ cells indicated that the Php4 repressor failed to respond to iron. To further investigate this aspect, we measured isa1Δ mRNA levels in pphp4Δ grx4Δ double mutant cells. We determined that isa1Δ expression was no more constitutively repressed in the absence of Php4. As a control, we observed that isa1Δ mRNA levels were permanently expressed in a pphp4Δ single mutant strain, irrespective of cellular iron status (Fig. 7B).

Based on these results, we therefore wondered if the constant repression of isa1Δ measured in the grx4Δ mutant strain was intrinsically associated with a constitutive Php4 nuclear retention. To address this point, we used the nmt1Δ transcriptional shut-off system that allowed us to extinguish the biosynthesis of GFP-Php4 and to subsequently examine the ability of preexisting GFP-Php4 molecules to translocate from the nucleus to the cytoplasm in response to iron. Strains with indicated genotypes (Fig. 7C) were cultured under iron-limiting conditions in the

was blocked by LMB, suggesting that the exportin Crm1 plays a critical role in the nuclear export mechanism of GFP-Php4.

To assess if Php4 is a cargo of the karyopherin Crm1, we analyzed the ability of these proteins to associate with each other. The coding sequence of TAP was inserted upstream of and in-frame with the php4Δ gene. Furthermore, 12 copies of the c-myc epitope were fused downstream of and in-frame with the crm1Δ coding sequence. Plasmids expressing the tagged Php4 and Crm1 were cotransformed into a php4Δ deletion strain. As a control, we also coexpressed Crm1-myc12 with TAP alone in php4Δ cells. The cells (at an A600 of 0.7) were grown in thiamine-free medium containing Dip for 3 h. Subsequently, the cells were transferred to thiamine-replete medium containing 100 μM FeSO₄ for 15 min. Total lysates from these cells were immunoprecipitated using IgG-Sepharose beads that selectively bind TAP or TAP-tagged proteins. Analysis of the proteins bound to the beads by immunoblotting with anti-c-Myc antibody showed that Crm1-myc12 and TAP-Php4 associated in vivo as Crm1-myc12 was significantly enriched by the immunoprecipitation of TAP-Php4 (Fig. 6B, upper panel). In contrast, only a background signal was observed when Crm1-myc12 was probed in the bound fraction of control cells expressing only untagged TAP (Fig. 6B, upper panel). To assess TAP or TAP-Php4 steady-state levels, immunoblot analysis of the protein preparations and coimmunoprecipitation reactions was performed using anti-IgG antibody (Fig. 6B, middle panel). To ascertain the specificity of the immunoprecipitation experiments, the total cell lysates and immunoprecipitates (or bound fractions) were analyzed by immunoblotting using an antibody directed against PCNA, a soluble protein like the Crm1-myc12 and TAP-Php4 fusion proteins. As shown in Fig. 6B (lower panel), PCNA was present in the total cell extracts but not in the immunoprecipitates. Collectively, these results reveal that immunoprecipitation of Crm1-myc12 with anti-IgG antibody can be accomplished only when it is coexpressed with TAP-Php4, therefore revealing the formation of a heteroprotein complex between Crm1-myc12 and TAP-Php4.

A prediction for a negative regulator with a Crm1-dependent NES is that target gene repression should accompany Crm1 inactivation regardless of growth conditions. Concerning Php4, one can envision that, even in the presence of iron, its target genes become constitutively repressed when Crm1 is inactivated by LMB. To determine whether LMB-mediated nuclear accumulation of GFP-Php4 results in a constitutive repression of isa1Δ in the presence of iron, isa1Δ mRNA levels were measured in cells treated with LMB. The results in Fig. 6C revealed that, although GFP-Php4 was retained in the nucleus (Fig. 6A), steady-state levels of isa1Δ were still derepressed in the presence of iron. As control, the isa1Δ mRNA levels were derepressed in iron-treated cells without LMB. As control for constitutive transcriptional repression, the isa1Δ transcripts were constantly repressed following treatment of cells with 250 μM Dip (Fig. 6C). Thus, these data show that nuclear export of Php4 from the nucleus to the cytoplasm is not the main mechanism for inducing transcription of isa1Δ and, in fact, appears to be uncoupled from target gene regulation.
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**FIGURE 7. Effects of disruption of Grx4 on Php4 localization and expression of isa1**

A. Isogenic strains FY435 (WT) and AMY34 (grx4Δ) were grown to mid-logarithmic phase under micro-aerobic conditions. Cultures were incubated in the absence (−) or presence of Dip (250 μM) or FeSO4 (100 μM) for 90 min. Total RNA was isolated and analyzed by RNase protection assay. grx4Δ and act1Δ mRNA levels are indicated by arrows. B, strains FY435 (WT), AMY34 (grx4Δ), AMY50 (php4Δ grx4Δ), and AMY15 (php4Δ) were cultured as described for A. After total RNA extraction, the isa1Δ and act1Δ mRNA levels (indicated by arrows) were analyzed by RNase protection assay. Each culture in A and B was assayed three times (independent experiments). C. php4Δ and php4Δ grx4Δ strains were transformed with pJKpmm14X-GFP-php4Δ. Cells were grown to mid-logarithmic phase in the absence of thiamine. During the last 3 h when thiamine was withdrawn, cells were treated with Dip (250 μM). Cells were washed and then grown for 60 min in 250 μM Dip or 100 μM FeSO4. Cells were analyzed by fluorescence microscopy for GFP and examined by Nomarski microscopy for cell morphology.

**FIGURE 8. Php4 and Grx4 physically interact in S. pombe.** Cells harboring a php4Δ grx4Δ double deletion were cotransformed with integrative vectors encoding VN alone and grx4Δ-VC or VN-php4Δ and grx4Δ-VC. Cells coexpressing the indicated fusion alleles were grown to an A600 of 0.9. At this log phase, cultures were treated with 250 μM Dip or 100 μM FeSO4, for 3 h and then visualized for BiFC signals by fluorescence microscopy (top). Nomarski optics (bottom) was utilized to monitor cell morphology. For simplicity, cells cotransformed with VN alone and grx4Δ-VC were taken from iron-deficient cells because the fluorescent images from iron-supplemented cells were identical.

absence of thiamine. After the addition of thiamine, cells were incubated for 0 and 60 min in the presence of exogenous iron (100 μM). In a php4Δ single deletion strain, GFP-Php4 was expelled from the nucleus, exhibiting fluorescence in the cytoplasm of cells (Fig. 7C). In contrast, in iron-treated php4Δ grx4Δ double deletion cells, GFP-Php4 was retained in the nucleus (Fig. 7C). In control experiments, when php4Δ or php4Δ grx4Δ cells were starved for iron, GFP-Php4 was viewed exclusively in the nucleus (Fig. 7C). Collectively, the results reveal that a functional grx4Δ gene is required for iron-dependent inactivation of Php4 activity. Furthermore, the results show that the presence of Grx4 is essential for Crm1-mediated export of Php4 in response to iron-replete conditions.

**Php4 and Grx4 Physically Associate in S. pombe**—To determine whether Php4 can form a complex with the S. pombe glutaredoxin Grx4, we used a BiFC approach in fission yeast. The BiFC method is based on the finding that N- and C-terminal subfragments (VN and VC) of the yellow fluorescent protein can only reconstitute a functional fluorophore when they are brought together into a tight contact by a pair of interacting proteins. The two partner proteins are fused to the nonfluorescent subfragments (VN and VC), and the interaction of the fusion proteins can be monitored by fluorescence microscopy (63). In this study, VN and VC were fused to the N terminus of Php4 and to the C terminus of Grx4, respectively. Integrative plasmids expressing the tagged (VN-php4Δ− and grx4Δ-VC) coding sequences were cotransformed into php4Δ grx4Δ mutant cells. These cells were grown to logarithmic phase and then starved for iron during 3 h. Microscopic analysis showed BiFC signals, indicating that complexes were formed between Php4 and Grx4 (Fig. 8). Furthermore, VN-Php4-Grx4-VC fluorescent complexes were seen primarily in the nuclei of cells (Fig. 8). In contrast, when cells were incubated in the presence of exogenous iron, BiFC signals accumulated in the cytosol and were to a great extent excluded from the nucleus (Fig. 8). Importantly, only cells cotransformed with plasmids encoding both fusion proteins (VN-Php4 and Grx4-VC), but not cells cotransformed with either plasmid alone, were fluorescent (Fig. 8 and data not shown).

Grx4 Interacts with the C-terminal Region 152–254 of Php4—Using two-hybrid analysis, the physical association between Php4 and Grx4 was investigated further. Coexpression of the full-length Php4 fused to VP16 with the LexA-Grx4 fusion protein produced high levels of β-galactosidase activity (1643 ± 156 Miller units), indicating a strong interaction between these proteins (Fig. 9A). To gain insight into the specific region of Php4 that interacts with Grx4, we constructed two chimeric VP16-Php4 molecules, denoted VP16-1Php4254 and VP16-1Php4218 (Fig. 9A). When the C-terminal region of Php4 was truncated to position Asp254, although the β-galactosidase activity decreased by 52% compared with the full-length Php4, a clear transactivation of the reporter gene expression was
observed (Fig. 9A). However, when the C terminus of the VP16-1Php4254 fusion protein was further truncated to position Arg218, the β-galactosidase activity was negligible (154 Miller units) compared with the full-length Php4. Based on these results, we used the region of Php4 corresponding to amino acids 1–254 in subsequent analyses. Truncations were created from the N-terminal end, generating four chimeric proteins. When the β-galactosidase activity of VP16-54Php4254 and VP16-152Php4254 coexpressed with LexA-Grx4 was assayed, comparable levels of activity relative to the VP16-1Php4254 fusion protein were detected (Fig. 9A). However, when the N-terminal region of 1Php4254 was further deleted to position Glu188, the β-galactosidase activity was considerably decreased by 79% (16623 Miller units). Additional deletion to position Gly219 completely abolished the activity of the reporter gene (Fig. 9A). To ensure that the fusion proteins were expressed in the cotransformed cells, immunoblot analyses of protein extracts were carried out using anti-LexA and anti-VP16 antibodies (Fig. 9B). Although we consistently detected LexA alone and the full-length LexA-Grx4 fusion protein, we were unable to detect the VP16 polypeptide alone and the last two VP16-188Php4254 and VP16-219Php4254 fusion proteins. This may be due to their very low predicted molecular weights. Given this situation, we assayed the levels of mRNA expression of VP16 alone and the full-length LexA-Grx4 by RNase protection experiments. The results showed that the VP16 used without or with the wild-type or mutant php4 constructs were clearly expressed, with transcripts detected for each prey construct (Fig. 9C). As shown in Fig. 9D, the interaction between the full-length LexA-Grx4 and VP16-Php4 fusion proteins using two-hybrid analysis was not modulated by iron. The full-length LexA-Grx4 and VP16-Php4 chimeric proteins that specifically associate with each other gave high constitutive steady-state levels of lacZ mRNA.
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as assayed by RNase protection experiments under conditions of both low and high concentrations of iron. We therefore conclude, on the basis of these data, that the monothiol glutaredoxin Grx4 can associate with the CCAAT-binding factor Php4 through its C-terminal region encompassing amino acid residues 152–254.

**DISCUSSION**

As part of the coordinated response to iron deprivation, *S. pombe* expresses the Php4 subunit of the heteromeric CCAAT-binding complex (18). Php4 associates with the Php2/Php3/Php5 heterotrimer to reprogram the cell for iron use. This is achieved by down-regulating expression of genes encoding iron-using proteins. Conversely, when cells undergo a transition from conditions of iron deficiency to iron sufficiency, it is critical that Php4 stop functioning, so that iron-binding proteins can participate to sequester iron and perform their enzymatic reactions. Previous studies have revealed that, when iron is abundant, Php4 is inactivated by different mechanisms (18, 19). One mechanism operates at the transcriptional level (18). Our group determined that php4+/gene expression is under the control of the iron-regulatory transcription factor Fep1; its expression is repressed under iron-replete conditions and induced under conditions of iron starvation. A second mechanism operates at the post-translational level (19). Using a one-hybrid approach, we found that a constitutively expressed Gal4-Php4 hybrid protein is able to repress transcription in response to iron deficiency when brought to a DNA promoter. However, this Php4-mediated repression was annihilated when iron was replete, revealing an iron inhibition of Php4 activity.

To begin to understand the mechanism by which Php4 activity is regulated, we engineered a biological system that allowed us to unlink iron-dependent behavior of Php4 protein from its transcriptional regulation by Fep1, and we examined the localization of Php4 in response to changes in iron levels. Using this system, we showed that Php4 is a nucleo-cytoplasmic shuttling protein. Php4 accumulates into the nucleus in response to low levels of iron. In contrast, on imposition of iron-replete conditions, Php4 relocalizes from the nucleus to the cytoplasm in a process that is dependent on a leucine-rich NES, as well as the export receptor Crm1. The Php4 NES (\(\text{LLEQLEML}^{100}\)) closely resembles experimentally verified leucine-rich NES of other proteins (55). Although the last hydrophobic leucine residue is absent compared with the generally accepted consensus (L)\(X_2\)-(LIVFM)\(X_2\)-(L)\(X\)-(LI) motif (54), its location in the predicted Php4 secondary structure is highly favorable for a nuclear export activity via the Crm1 pathway. Four observations support an NES function for 93–100-amino acid region of Php4. First, the predicted Php4 secondary structure obtained using the JPred program (64) shows that the NES is located in an \(\alpha\)-helical secondary structure with the Leu\(^{93}\), Leu\(^{94}\), Leu\(^{97}\), and Leu\(^{100}\) residues stacked on one side of the \(\alpha\)-helix, which is a clear tendency for NES (55). Second, it has been suggested that \(\alpha\)-helical NES regions are often flanked by other structural elements, which in most cases are \(\alpha\)-helices. Therefore, several NES regions would be packed up against another \(\alpha\)-helix, creating \(\alpha\)-helical bundles or globular domains (55). Interestingly, the Php4 NES, which is located in a putative \(\alpha\)-helix, is predicted to be located downstream to another \(\alpha\)-helical domain (located between amino acids 70 and 89). Thus, one can envision that both \(\alpha\)-helices are able to stack together because they are predicted (using the Paircoiled2 program (65)) to be part of a coiled-coil fold (amino acids 73–111). Third, a hallmark of experimentally verified NES is the prevalence of negatively charged amino acids, which results in isoelectric point (pI) values between 4 and 5 (55). Given that the anchor position, denoted P0, was assigned to the last hydrophobic residue in each NES and that the accepted NES region covers 15 and 10 amino acid residues on each side of P0, we determined that the Php4 NES has a pI of 4.25. This low pI value matches with >80% of characterized NES (55). Fourth, as found for the Pap1 NES, the Php4 NES functions as a transferable NES sequence when fused with a reporter protein. Although the Php4 NES robustly induced nuclear export of a reporter protein, the process was not modulated by iron.4 As a result, our interpretation is that the Php4 intrinsic determinants involved in controlling iron-dependent nuclear export of the protein should be located outside of the NES region. Nonetheless, taken together, our results are consistent with the 93–100-amino acid region of Php4 functioning as an NES in *S. pombe*.

Analogous to Php4, the *S. cerevisiae* transcription factor Aft1 shuttles between the nucleus and the cytoplasm in response to iron levels (59, 61). A proposed model suggests that iron leads to the dimerization of Aft1, and the protein complex is exported from the nucleus by the exportin Msn5 (61). Although *S. pombe* has a putative Msn5 ortholog (SPAC328.01c), we found that Php4 nuclear export rather depends on the nuclear receptor Crm1. Consistently, we determined that use of LMB, a specific inhibitor of Crm1, blocks Php4 nuclear export. Using a coimmunoprecipitation approach, we also showed that Crm1 associates with Php4. This suggests a different mode of recognition between Crm1 and Php4 compared with the mechanism by which Aft1 is recognized by Msn5 because, among the four exportins that have been identified in yeast, each one has a different mechanism of cargo recognition (66).

Our results showed that iron is a necessary requirement for nuclear export of Php4. How might this occur? Export of Php4 from the nucleus may occur from direct iron binding. Using *in vitro* surface plasmon resonance for molecular interaction analysis, it has been shown that iron causes the release of HapX from the HapB-HapC-HapE complex (30). Based on this study, it has been suggested that iron may directly modulate the binding affinity of HapX for the CCAAT-binding complex. Given that HapX and its putative orthologs share three conserved cysteine-rich regions, it has been proposed that they may represent potential iron-binding sites (1, 30). Although the amino acid residue configurations of the cysteine-rich motifs in HapX-like proteins are different compared with the conserved iron-binding motif identified in the GATA factor Sre1 (67), they may bind iron. The caveat to this possibility in *S. pombe* is that Php4 has only one conserved cysteine-containing region, which shows only 28% similarity with the second cysteine-rich region of HapX. Furthermore, we observed that only two of the four

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4 A. Mercier and S. Labbé, unpublished data.
Iron Inhibition of Php4 Function

Iron response in *S. pombe*

1. *php4* 
   
2. *Php4* function is inactivated by *Grx4*
   
3. *Php4* is exported from the nucleus by *Crm1*

**FIGURE 10. Proposed model for iron-dependent inactivation of Php4.** During a shift from low to high iron levels, three distinct steps are envisioned to take place. Step 1, Fep1 specifically interacts with GATA elements within the *php4* promoter, inhibiting target gene transcription. Step 2, Grx4 exerts an iron-dependent inhibitory effect on Php4 function, leading to Php4 inactivation. Step 3, Grx4-mediated inactivation of Php4 leads to its recruitment by the exportin Crm1, and its subsequent exportation from the nucleus to the cytoplasm.

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