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Crm1-Mediated Nuclear Export of the *Schizosaccharomyces pombe* Transcription Factor Cuf1 during a Shift from Low to High Copper Concentrations[▽]

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In this study, we examine the fate of the nuclear pool of the *Schizosaccharomyces pombe* transcription factor Cuf1 in response to variations in copper levels. A nuclear pool of Cuf1-green fluorescent protein (GFP) was generated by expressing a functional *cuf1*$^{+}$*-GFP* allele in the presence of a copper chelator. We then extinguished *cuf1*$^{+}$*-GFP* expression and tracked the changes in the localization of the nuclear pool of Cuf1-GFP in the presence of low or high copper concentrations. Treating cells with copper as well as silver ions resulted in the nuclear export of Cuf1. We identified a leucine-rich nuclear export signal (NES), ^{349}LAALNHISAL^{358}, within the C-terminal region of Cuf1. Mutations in this sequence abrogated Cuf1 export from the nucleus. Furthermore, amino acid substitutions that impair Cuf1 NES function resulted in increased target gene expression and a concomitant cellular hypersensitivity to copper. Export of the wild-type Cuf1 protein was inhibited by leptomycin B (LMB), a specific inhibitor of the nuclear export protein Crm1. We further show that cells expressing a temperature-sensitive mutation in *crm1*$^{+}$ exhibit increased nuclear accumulation of Cuf1 at the nonpermissive temperature. Although wild-type Cuf1 is localized in the nucleus in both conditions, we observed that the protein can still be inactivated by copper, resulting in the repression of *ctr4*$^{+}$ gene expression in the presence of exogenous copper. These results demonstrate that nuclear accumulation of Cuf1 per se is not sufficient to cause the unregulated expression of the copper transport genes like *ctr4*$^{+}$. In addition to nuclear localization, a functional Cys-rich domain or NES element in Cuf1 is required to appropriately regulate copper transport gene expression in response to changes in intracellular copper concentration.

The transition metal copper is an essential nutrient for virtually all organisms (3, 37). Normal physiological levels of copper in cells are maintained by a number of components involved in copper-sensing, uptake, trafficking, and compartmentalization (26, 35, 38). The necessity for maintaining appropriate copper homeostatic control mechanisms arises from the fact that excess copper is potentially toxic because of its ability to generate damaging free radical species that can impair cellular components, including nucleic acids, proteins, and lipids (17, 47).

In fungi, an important mechanism to maintain appropriate cellular levels of copper is to reprogram the expression of genes encoding components of the copper uptake machinery in response to changes in environmental copper levels; they are induced under conditions of copper deprivation and repressed under conditions of copper repletion (24). In the fission yeast *Schizosaccharomyces pombe*, the key copper-regulatory transcription factor that regulates this process is Cuf1 (4, 7, 25). In response to copper deficiency, Cuf1 binds to the DNA sequences 5′-D(T/A)DDHGCTGD-3′, known as copper-signaling elements (CuSEs) (4), and induces the expression of genes encoding components of the copper transport pathway, including Ctr4, Ctr5, and Ctr6 (8, 25, 55). Conversely, under conditions of copper excess, Cuf1 does not bind to the CuSEs, as determined by UV cross-linking experiments (4). Consistently, transcription of *ctr4*$^{+}$, *ctr5*$^{+}$, and *ctr6*$^{+}$ is repressed (8, 25, 55). Previously, we identified a noncanonical nuclear localization sequence between amino acids 11 and 53 within the Cuf1 N terminus (5). Its C terminus harbors a Cys-rich domain, ^{328}CysGlnCysGlyAspAsnCysGluCysLeuGlyCysLeuThrHis^{342}, that is known to play a critical role in copper sensing. When this domain is disrupted, Cuf1 fails to sense copper, giving rise to high constitutive levels of expression of *ctr4*$^{+}$ mRNA (7). We utilized a functional Cuf1-green fluorescent protein (GFP) to dissect the domains that are required for copper-dependent regulation by Cuf1 (5). Cuf1-GFP was primarily localized in the cytoplasm of cells growing under copper-replete conditions (5). In contrast, Cuf1-GFP accumulated within the nucleus of cells when trace amounts of copper were present in the medium (5). Importantly, we found that disruption of the C-terminal Cys-rich domain triggered the translocation of the mutant form of Cuf1-GFP into the nucleus under both low and high copper concentrations (5). These observations suggest that metallation of Cuf1, possibly within the C-rich domain, may induce conformational changes that mask the Cuf1 NLS and consequently block its import into the nucleus (5). In support of this proposed model, two-hybrid analyses revealed that the Cuf1 C terminus physically interacts with its N terminus in a copper-dependent manner (5). Fine structural mapping analysis revealed that at least a subset of the amino acid residues Cys-328, Cys-330, Cys-334, Cys-336, Cys-339, and His-

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342 within the C-rich domain at the C terminus of Cuf1 is required for its interaction with the N-terminal domain (5). Furthermore, we showed that copper induced the cytoplasmic retention of the N-terminal 61 amino acid residues of Cuf1 when this peptide was coexpressed as a separate molecule with the Cuf1 C-terminal domain containing the C-rich domain (5).

Signal-directed nuclear import and export are two ways to regulate the availability of transcription factors within the nucleus. Protein cargos transported from the cytoplasm into the nucleus contain nuclear localization signals (NLSs), whereas nuclear export signals (NESs) direct protein cargos from the nucleus to the cytoplasm. These localization signals are recognized by transport receptors which belong to the karyopherin family of proteins (28, 30, 50). An important group comprising the majority of NESs is composed of a short sequence (~9 to 11 amino acids) with critically spaced hydrophobic residues that are essential for protein export (34). Because leucine is a preferred residue in this group of NESs, they are referred to as leucine-rich NESs. Leucine-rich NESs are defined by the consensus Φ-X-$_{2–3}$-Φ-X$_{2–3}$-Φ-XΦ (where Φ is L, I, V, F, or M, and X indicates any amino acid residue) (23). Many proteins are exported via a leucine-rich NES, including human immunodeficiency virus Rev, protein kinase A inhibitor, and metal-regulatory transcription factor 1 (MTF-1) (40, 51). In *S. pombe*, this is also the case for the transcription factor Pap1 and for the mitogen-activated protein kinase Sty1 (16, 46). Crm1/Exportin1, which was first identified in *S. pombe*, is the cellular karyopherin receptor for proteins bearing a leucine-rich NES (13, 15, 33, 43). Crm1 binds cooperatively with RanGTP to the NES-containing cargo to form the trimeric export complex NES-Crm1-RanGTP, which then translocates through the nuclear pore. The export complex is dissociated in the cytoplasm, with the concurrent hydrolysis of Ran-bound GTP (50). A powerful tool in the discovery of Crm1 as the export receptor for leucine-rich NES-substrates was the *Streptomyces* sp. metabolite LMB (31). LMB inhibits NES-mediated export in mammalian cells and in *S. pombe* by binding directly to Crm1 and disrupting the NES-Crm1-RanGTP export-competent complex (13, 15, 21, 33).

In response to copper deficiency, Cuf1 is localized in the nucleus where it activates the transport of copper by up-regulating the expression of *ctr* genes. In this study, we show that, in cells undergoing a shift from low to sufficient copper concentrations, Cuf1 translocates from the nucleus to the cytoplasm. We determined that Cuf1 export requires the presence of a leucine-rich NES, ^{349}LAALNHISAL^{358}, within the C-terminal region of Cuf1. Disruption of the NES resulted in nuclear retention of Cuf1 regardless of copper stimulation. Moreover, mutations that impair Cuf1 NES function led to increased target gene expression, with a concomitant cellular hypersensitivity to exogenous copper. Nuclear export of Cuf1 was inhibited by LMB. Consistently, we found that nuclear exclusion of Cuf1 requires a functional *crm1*$^+$ gene. Yeast two-hybrid analysis revealed that Crm1 is a binding partner of Cuf1. Importantly, we also found that the export blocker LMB or a temperature-sensitive *crm1* mutation which induces nuclear accumulation of Cuf1 is not sufficient to cause the unresponsive expression of *ctr4*$^+$ to the presence of copper. In addition to nuclear localization, a functional Cys-rich domain or NES is required for copper transport gene regulation as a function of changes in copper levels.

MATERIALS AND METHODS

Yeast strains and media. The following *S. pombe* strains were used in this study: FY435 (*h*$^+$ *his7-366 leu1-32 ura4-Δ18 ade6-M210*) (9), JSY17 (*h*$^+$ *his7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::ura4*$^+$) (7), JSY8 (*h*$^+$ *his7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::hisG*) (5), TP113 (*h*$^+$ *leu1-32 ura4-Δ18*), and TP113-6B (*h*$^+$ *leu1-32 ura4-Δ18 crm1-809*) (kind gift of Simon Whitehall, University of Newcastle, United Kingdom). Under nonselective conditions, *S. pombe* cells were cultivated in yeast extract plus supplement (YES) medium (1). Under selective conditions, *S. pombe* cells were grown in Edinburgh minimal medium (EMM) (1) with the appropriate amino acids (225 mg/liter adenine, histidine, and uracil, unless otherwise stated); unsupplemented EMM contains 160 nM copper. When the wild-type or mutant *cuf1* alleles were expressed under the control of the *nmt1*$^+$ promoter, cells expressing these alleles were induced by the removal of thiamine from the medium. In contrast, to prevent expression of the *cuf1* alleles, cells were grown in the presence of 15 μM thiamine.

Construction of plasmids. Plasmid pJB-1178*nmt-cuf1*$^+$*-GFP* harboring the wild-type *cuf1*$^+$*-GFP* allele and plasmid pJB-1178*nmt-cuf1-M6-GFP* containing a mutant allele in which all of the five Cys residues as well as His^{342} of the C-rich motif were mutated to alanines were both described elsewhere (5). The *nmt1*$^+$ promoter region from position −1178 to position −1 with respect to the A of the initiator codon was isolated from pREP41X (14) by PCR. To create the *cuf1* mutant alleles NESmut1 (L349A and L352A) and NESmut2 (L349A, L352A, I355A, and L358A), plasmid pJB-1178*nmt-cuf1*$^+$*-GFP* was used in conjunction with the overlap extension method (19) and the oligonucleotides CUF1L349AL352A-up (5′-CAATGCAACTACAGCAGCCGCTGCAAATCATATTTCAGC-3′), CUF1L349AL352A-lo (5′-GCTGAAATATGATTTGCAGCGGCTGCTGTAGTTGCATTG-3′), CUF1L349AL352AI355AL358A-up (5′-CAATGCAACTACAGCAGCCGCTGCAAATCATGCTTCAGCTGCAGAAAAGGAAACCATAAG-3′), and CUF1L349AL352AI355AL358A-lo (5′-GGTTTCCTTTTCTGCAGCTGAAGCATGATTTGCAGCGGCTGCTGTAGTTGCATTGTTTG-3′) (underlined letters represent nucleotide substitutions that gave rise to mutations). All nucleotide changes were verified by DNA sequencing. DNA isolation and PCR were performed using standard protocols (2). To construct the plasmid for expressing GST-GFP, a 699-bp PstI-BamHI PCR-amplified DNA segment containing the *GST* open reading frame was isolated from the pGEX-4T-1 plasmid (GE Healthcare Bio-Sciences) and inserted into the PstI and BamHI restriction sites of pSP1 (5). Subsequently, the *GFP* gene was isolated by PCR from the pSF-GP1 plasmid (5). The *GFP* open reading frame, in which BamHI and SpeI restriction sites were engineered by PCR, was placed in-frame with the C-terminal region of GST. The resulting plasmid was designated pSPGST-GFP. The *nmt1*$^+$ promoter up to position −1178 from the start codon of the *nmt1*$^+$ gene was isolated by PCR and then inserted into the pSPGST-GFP plasmid at the ApaI and PstI sites. The resulting plasmid, named pSP-1178*nmt*-GST-GFP, was subsequently digested with SpeI and SstI restriction enzymes. After gel purification, the SpeI-SstI-digested pSP-1178*nmt*-GST-GFP plasmid was used to receive annealed synthetic DNA fragments encoding Cuf1^{349}NES^{358} and Pap1^{515}NES^{533}.

RNA isolation and analysis. Total RNA was extracted by the hot phenol method as described previously (20). RNase protection assays were carried out as described previously (4). Plasmids pSK*ctr4*$^+$ and pSK*act1*$^+$ (25) were used to produce antisense RNA probes, allowing the detection of steady-state levels of *ctr4*$^+$ and *act1*$^+$ mRNAs, respectively. pSK*cuf1*$^+$ was constructed by inserting a 161-bp BamHI-EcoRI fragment of the *cuf1*$^+$ gene into the same sites in pBluescript SK (Stratagene). The antisense RNA hybridizes to the region between positions +890 and +1051 upstream of the initiator codon of *cuf1*$^+$.

Microscopic analysis of Cuf1 localization. Freshly transformed cells with the wild-type or mutant *cuf1* alleles were precultivated in the presence of thiamine to an A_{600} of ~1.0. At this growth point, the cells were washed twice to remove thiamine and diluted 10-fold in EMM with bathocuproinedisulfonic acid (BCS; 100 μM), thereby allowing nuclear targeting of Cuf1. At mid-logarithmic phase, the cells were washed twice to remove BCS and then grown in the presence of 15 μM thiamine to stop gene expression. At this point, the nuclear pool of Cuf1 was analyzed under conditions of high or low copper for 0, 3, and 6 h. For treatment with LMB (catalogue no. L-2913; Sigma), cells were divided in half and treated with either 100 ng/ml LMB (in 1.4% methanol) or left untreated (with 1.4% methanol as a control), and allowed to continue growing at 30°C in the presence of 25 μM $CuSO_4$. At relevant time points after LMB treatment (if applicable), aliquots of cells were removed from each half (with or without LMB) and viewed

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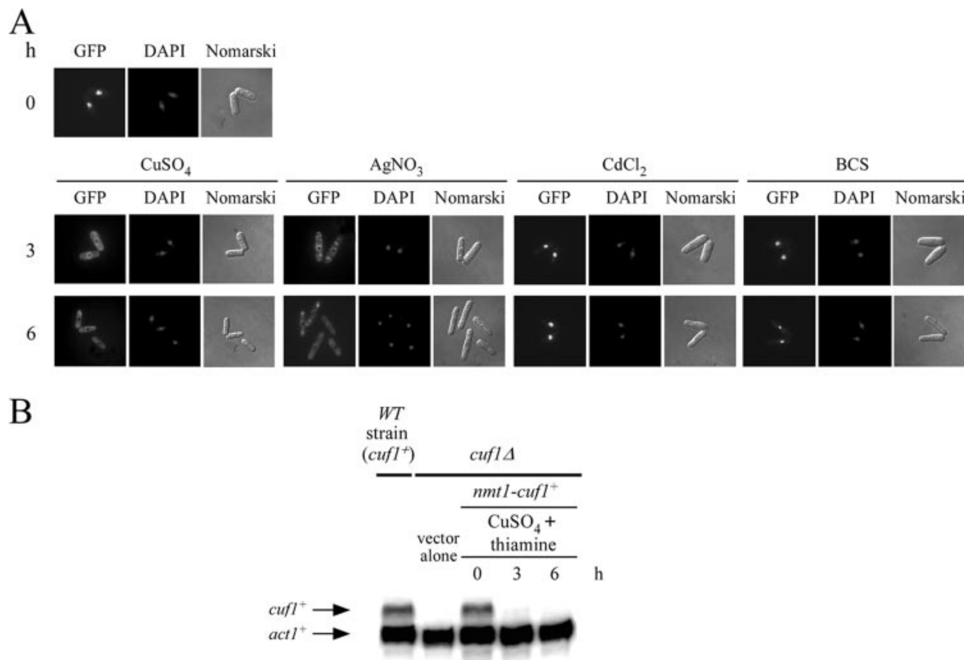

FIG. 1. Nuclear Cuf1-GFP is exported from the nucleus upon the addition of copper and silver. (A) Cells harboring a *cuf1*Δ deletion were transformed with pJB-1178*nmt-cuf1*$^{+}$*-GFP* and grown in thiamine-free medium containing BCS for 18 h. The cells (at an A_{600} of ~1.0) were then transferred to thiamine-replete medium containing 25 μM $CuSO_4$, 2 μM $AgNO_3$, 25 μM $CdCl_2$, or 100 μM BCS for 0, 3, and 6 h. Fluorescence microscopy was used to visualize the cellular location of Cuf1-GFP. The cells were treated with DAPI for nuclear DNA staining. Cell morphology was examined using Nomarski optics. For simplicity, one 0 time point (0) is shown, since the localization patterns detected from 0 h treatment for each metal ion or the copper chelator BCS were virtually identical. (B) Ten-milliliter samples were taken after 0, 3, and 6 h of thiamine and copper treatment. Fifteen micrograms of total RNA was used in the RNase protection assay for each sample. Steady-state mRNA levels of *cuf1*$^{+}$ and *act1*$^{+}$ (as an internal control) are indicated with arrows. As a positive control, *cuf1*$^{+}$ mRNA steady-state levels were determined in the isogenic wild-type (*WT*) (*cuf1*$^{+}$) strain FY435.

by direct fluorescence microscopy as described previously (6). Fluorescence and differential interference contrast images of the cells were obtained on an Eclipse E800 epifluorescent microscope (Nikon, Melville, NY) equipped with an ORCA ER digital cooled camera (Hamamatsu, Bridgewater, NJ). The samples were subjected to microscopy analysis, using a magnification of ×1,000 with the following filters: 465 to 495 nm (GFP) and 340 to 380 nm (DAPI; 4′, 6′-diamidino-2-phenylindole). The cell fields shown in this article are representative of experiments repeated at least five times.

Yeast two-hybrid analysis. *Saccharomyces cerevisiae* strain L40 [*MAT***a** *his3*Δ*200 trp1-901 leu2-3,112 ade2 LYS2*::(*lexAop*)$_4$-*HIS3 URA3*::(*lexAop*)$_8$-*lacZ*] (49) was used for two-hybrid analysis. Plasmid p*LexA-*^{1}*crm1*$^{+1078}$ was constructed by cloning a 3,237-bp BamHI-PstI DNA fragment containing the entire open reading frame of Crm1 into the same sites of pLexN-a (48). The prey plasmid, p425GPD-NVP16 (5), contains the synthetic codons of the SV40 NLS that were placed upstream of and in frame to the *VP16* gene. Importantly, the SV40 NLS sequence was followed by a polylinker in which different DNA fragments of the *cuf1*$^{+}$ gene were introduced. The *cuf1*$^{+}$ allele and its derivatives were amplified by PCR using primers designed to generate BamHI and PstI sites at the upstream and downstream termini of the open reading frame. Once generated, each DNA fragment was inserted into the corresponding sites of p425GPD-NVP16. Each L40 transformant strain harboring the indicated bait and prey plasmids was tested for the association of the two fusion proteins by liquid β-galactosidase assay as described previously (5), except that cells were grown in copper-replete (25 μM $CuSO_4$) or copper-deficient (100 μM BCS) synthetic media before their disruption in lysis buffer (5). The expression of the LexA-Crm1, Cuf1-VP16, Cuf1-NESmut2-VP16, and Cuf1-M6-VP16 fusion proteins was verified by immunoblot analysis using the following antisera: polyclonal anti-LexA antibody (R990-25; Invitrogen), and monoclonal anti-VP16 antibody 1-21 (Santa Cruz Biotechnology). A monoclonal anti-3-phosphoglycerate kinase (PGK) antibody, 22C5-D8 (Molecular Probes), was used to detect PGK protein as an internal control.

RESULTS

High copper and silver levels result in nuclear export of the Cuf1-GFP nuclear pool. In previous studies, we have demonstrated that the insertion of GFP at the C terminus of Cuf1 does not interfere with its function (5, 25). Moreover, we showed that *ctr* mRNA levels in a strain expressing the *cuf1*$^{+}$ or *cuf1*$^{+}$*-GFP* allele under the control of the thiamine-regulatable promoter (designated *nmt1*$^{+}$) were regulated in a copper-dependent manner similar to that of *cuf1*$^{+}$ or *cuf1*$^{+}$*-GFP* under the control of the *cuf1*$^{+}$ promoter (5). Furthermore, we found that the *nmt1*$^{+}$ 41X promoter gave regulatable levels of *ctr* mRNA comparable to those observed with the *nmt1*$^{+}$ 3X promoter (5). Based on these previous results, we utilized the *nmt1*$^{+}$ inducible/repressible promoter system to assess the effect of copper on the nuclear pool of Cuf1. The expression of a functional *cuf1*$^{+}$*-GFP* allele under the control of the *nmt1*$^{+}$ 41X promoter (14) allowed us to induce the synthesis of Cuf1-GFP in the presence of the copper chelator BCS, thereby ensuring its nuclear sequestration (5). Subsequently, the cells were harvested, washed, and resuspended in the same media without BCS. After the addition of thiamine to repress further synthesis, we examined the effects of copper, silver, cadmium, and BCS on the subcellular localization of Cuf1-GFP (Fig. 1A, zero time point). As shown in Fig. 1A, when cells were treated for 3 and 6 h with $CuSO_4$ (25 μM) or $AgNO_3$ (2 μM), Cuf1

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accumulated in the cytoplasmic region and was absent from the nuclei, revealing a translocation of Cuf1-GFP from the nucleus to the cytoplasm. In contrast, upon treatment with $CdCl_2$ (25 μM) or BCS (100 μM) for 3 or 6 h, the Cuf1-GFP protein remained exclusively in the nuclei (Fig. 1A). The metal ion levels used were those which allowed 100% survival of *S. pombe* cells. For each metal ion tested, using concentrations which allowed 50% cell survival did not alter the Cuf1-GFP localization patterns from those shown in Fig. 1A (data not shown). Furthermore, as we observed previously (5), under all the metal ion conditions examined, GFP alone was localized in both the cytosol and nucleus (data not shown). Interestingly, treatment with silver ions also triggered export of the nuclear pool of Cuf1-GFP to the cytoplasm during a shift from low to high silver ion concentration. The electronic similarity of Ag^{1+} to Cu^{1+} suggests that reduced copper [Cu^{1+}] might be the active species that instigates the nuclear export of Cuf1-GFP. To ensure that the fluorescence observed was due to the nuclear export of preexisting Cuf1-GFP, and not due to the effect of copper on newly synthesized Cuf1 arising from a pool of stable mRNA, total RNA was extracted from cells at the time points (0, 3, and 6 h) used in these experiments. RNA from each time point was analyzed by RNase protection assay (Fig. 1B). The results showed that the *cuf1*$^+$ mRNA was completely extinguished after the addition of thiamine (3 and 6 h) compared with its level of expression observed in cells at the zero time point (Fig. 1B). Taken together, these results indicate that the subcellular localization of Cuf1-GFP is regulated in response to copper and silver through the relocalization of the transcription factor from the nucleus to the cytoplasm.

Mapping residues necessary for NES function. In light of these observations, we sought to identify amino acids in Cuf1 that could serve as an NES. Although a variety of functional NES sequences have been identified, the presence of regularly spaced hydrophobic amino acids such as leucine or isoleucine appears to be an important feature of the NES (27). Examination of the Cuf1 sequence revealed one potential NES motif, $^{349}\underline{L}AA\underline{L}NH\underline{I}SA\underline{L}^{358}$, located in its C terminus (Fig. 2A). To determine if this putative NES sequence is functional, we created two Cuf1-GFP mutants in which the first two or all of the four hydrophobic residues were replaced with alanine residues to generate Cuf1-NESmut1-GFP and Cuf1-NESmut2-GFP, respectively. Cells expressing these mutant alleles showed nuclear accumulation of Cuf1-GFP following treatment with $CuSO_4$ (25 μM) for 0, 3, and 6 h (Fig. 2B). Furthermore, these cells exhibited elevated *ctr4*$^+$ mRNA levels that were unregulated by copper (Fig. 2C). Consistent with these observations, the expression of *cuf1-NESmut1-GFP* and *cuf1-NESmut2-GFP* under the control of the thiamine-regulated *nmt1*$^+$ promoter resulted in increased sensitivity of the transformed cells to copper toxicity when grown on medium containing 25 μM $CuSO_4$ (Fig. 2D). Similarly, when the mutant alleles were expressed under the control of the constitutive *cuf1*$^+$ promoter, the transformed cells also failed to grow on medium supplemented with exogenous copper (25 μM) (data not shown). In contrast, the wild-type Cuf1-GFP fusion protein was localized in the cytoplasm following treatment of cells with 25 μM $CuSO_4$ for 3 and 6 h (Fig. 2B). Correspondingly, as shown in Fig. 2C, the *ctr4*$^+$ mRNA levels in these cells were strongly down-regulated in the presence of $CuSO_4$. Furthermore, cells expressing the wild-type allele displayed no hypersensitivity when grown in the presence of exogenous copper (Fig. 2D). We also tested whether the NES sequence found in Cuf1 is functional when fused with GST-GFP, as described for the NES of Pap1 (22). As shown in Fig. 2E, the GST-GFP protein carrying the Cuf1^{349}NES^{358} was excluded from the nucleus, whereas GST-GFP alone distributed in both the nucleus and the cytoplasm when expressed in *S. pombe*. Furthermore, LMB treatment caused diffused distribution of GST-GFP-Cuf1^{349}NES^{358} in both the cytoplasm and the nucleus. Taken together, these results demonstrate that Leu-349, Leu-352, Ile-355, and Leu-358 are critical amino acid residues in the Cuf1 NES that is essential for the nuclear export of Cuf1-GFP.

Cuf1-GFP requires the C-rich domain for copper-induced nuclear export. The NES of Cuf1 is located between residues 349 and 358 just downstream of the copper-sensing C-rich domain composed of the amino acids ^{328}CysGlnCysGlyAspAsnCysGluCysLeuGlyCysLeuThrHis^{342}. Because of the proximity of the C-rich domain to the NES sequence, we examined the role of the C-rich domain in copper-dependent nuclear export by generating a mutant allele of *cuf1*$^+$ in this region. The mutant allele (designated *cuf1-M6-GFP*), in which all of the five Cys residues as well as His^{342} were mutated to alanine residues, was expressed under the control of the *nmt1*$^+$ promoter. The mutant protein was efficiently imported into the nucleus under low copper concentrations (5; data not shown). After replacing the media and adding thiamine to inhibit further synthesis, we examined the effect of copper on nuclear exclusion of Cuf1-GFP. At the zero time point, the wild-type Cuf1-GFP fusion protein was seen in the nucleus (Fig. 3A). However, after 3 and 6 h of incubation in the presence of exogenous copper (25 μM), the wild-type Cuf1-GFP fusion protein was exported from the nucleus to the cytoplasm (Fig. 3A). Consistent with its exclusion from the nucleus, virtually no expression of *ctr4*$^+$ mRNA was detected under these copper-replete conditions (Fig. 3B). In contrast, upon copper treatment, the Cuf1-M6-GFP mutant remained in the nucleus (Fig. 3A). Moreover, cells expressing the M6 mutant protein showed increased expression of *ctr4*$^+$ mRNA (Fig. 3B) and a concomitant sensitivity to copper in a manner that parallels the magnitude of sustained expression of the *ctr4*$^+$ gene (Fig. 2D). Together, these results indicate that the C-rich domain in wild-type Cuf1-GFP is required together with the NES for the copper-induced nuclear export of the protein and for the copper-dependent extinction of copper transport gene transcription.

LMB inhibits copper-induced nuclear export of Cuf1-GFP. It is known that inhibition of Crm1 function by LMB results in the nuclear accumulation of several proteins that contain leucine-rich NESs (13, 15, 33). Because Cuf1 harbors such an NES within its C terminus, we tested the ability of LMB to inhibit copper-mediated Cuf1-GFP nuclear export. Cells were grown in low-copper medium in the absence of thiamine. Cells examined for Cuf1-GFP showed that Cuf1-GFP fluorescence colocalized with the DNA-staining dye DAPI, which was used as a marker for nuclear staining (5; data not shown). After the addition of thiamine, cells were incubated for 0, 3, and 6 h without or with 100 ng/ml LMB in the presence of exogenous copper (25 μM). After 3 and 6 h, in copper-treated cells without LMB, Cuf1-GFP was exported from the nucleus to the

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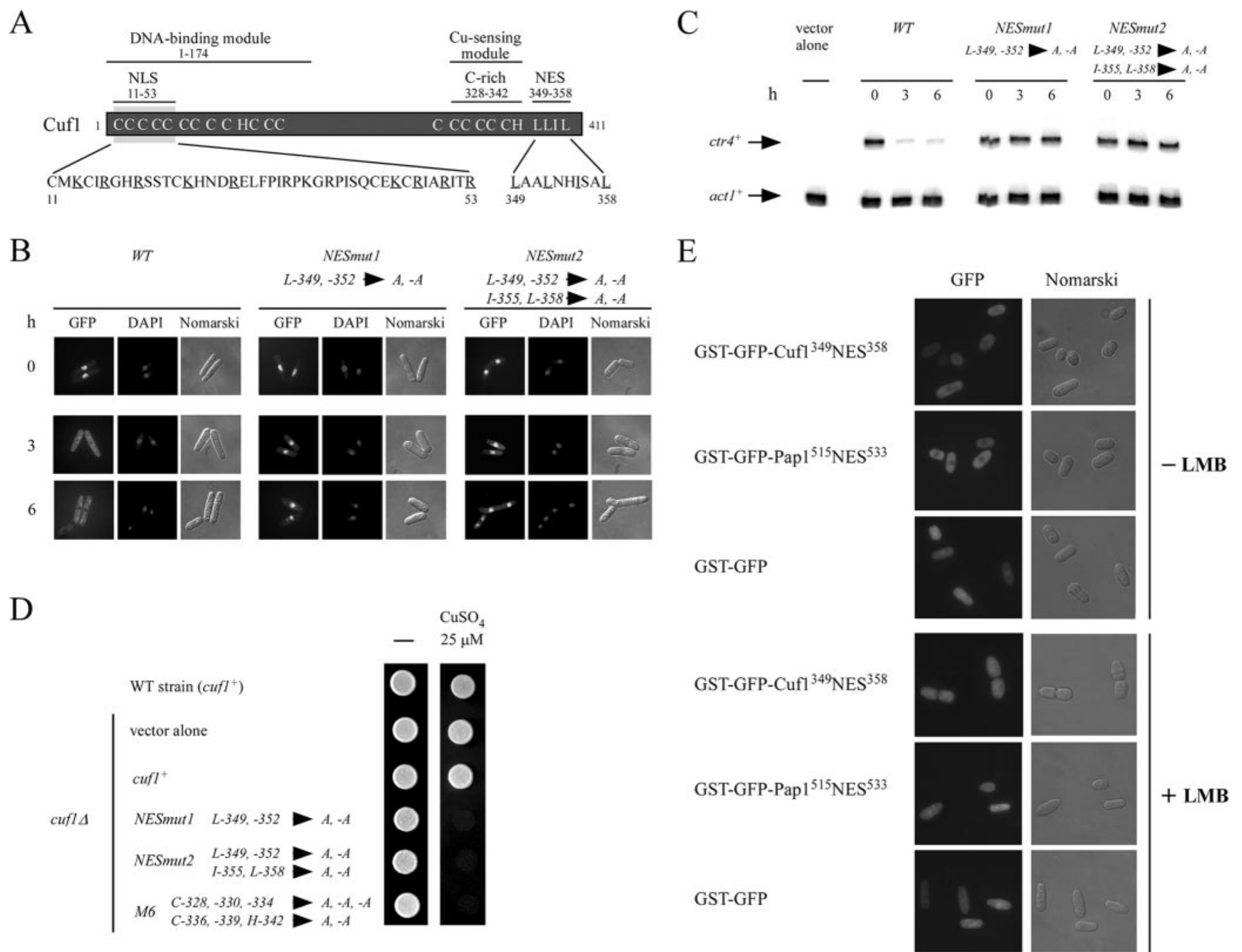

FIG. 2. Identification of a functional Cuf1 NES. (A) Schematic representation of Cuf1 showing the relative locations of the N-terminal NLS within the Cuf1 DNA-binding module, the C-terminal copper-sensing module (C rich), and a proposed C-terminal NES. The amino acid sequences of the NLS and NES are shown. Important positively charged residues (lysine and arginine) in the NLS of Cuf1 are underlined. The positions of key residues of NES are also underlined. The amino acid residues of Cuf1 are numbered relative to its initiator codon. The positions of some cysteine (C) and histidine (H) residues within Cuf1 are indicated. (B) The wild-type (*WT*) and mutant (*NESmut1* and *NESmut2*) alleles were transformed into cells harboring a *cuf1*Δ deletion. Transformed cells were grown in thiamine-free medium containing BCS for 18 h. After being transferred to thiamine- and copper-replete medium for 0, 3, and 6 h, the cells were visualized by fluorescence microscopy. DAPI staining revealed nuclear DNA, and Nomarsky microscopy was used to examine cell morphology. The two and four point mutations in Cuf1-NESmut1 and Cuf1-NESmut2, respectively, are indicated. (C) Aliquots of the cultures described for panel B were examined by RNase protection assay. Total RNA was prepared and analyzed. The arrows indicate signals corresponding to *ctr4*$^+$ and *act1*$^+$ mRNA steady-state levels. Vector alone represents JSY17 cells harboring pJB-1178*nmt* with no insert. *WT*, wild type. The results shown are representative of three independent experiments. The two and four point mutations in Cuf1-NESmut1 and Cuf1-NESmut2, respectively, are indicated. (D) Copper sensitivity phenotype resulting from expression of *cuf1* mutant alleles. *cuf1*Δ cells (JSY17) expressing the indicated *cuf1* allele were spotted at a density of 3,000 cells/5 μl onto Edinburgh minimal medium containing 0 (−) or 25 μM $CuSO_4$ and incubated at 30°C for 4 days. The isogenic wild-type (*cuf1*$^+$) strain FY435 was used as a positive control. The two, four, and six point mutations in Cuf1-NESmut1, Cuf1-NESmut2, and M6, respectively, are indicated. *WT*, wild type. (E) Shown are representative cells expressing GST-GFP-Cuf1^{349}NES^{358}, GST-GFP-Pap1^{515}NES^{533}, and GST-GFP proteins, respectively. The cells were cultivated to mid-logarithmic phase in thiamine-free medium. After two washes, the cells were incubated for 3 h in medium with thiamine and in the absence (−LMB) or presence (+LMB) of 100 ng/ml LMB. The cells were analyzed by fluorescence microscopy for GFP. The cells were also examined by Nomarski microscopy for cell morphology.

cytoplasm (Fig. 4A). In contrast, when copper-treated cells were incubated with LMB, Cuf1-GFP was observed exclusively in the nucleus (Fig. 4A). Thus, LMB inhibits the nuclear export of Cuf1-GFP. In a control experiment, when cells were starved for copper in the presence of BCS, Cuf1-GFP was observed exclusively in the nucleus (Fig. 4A, BCS). To determine whether LMB-mediated nuclear accumulation of wild-type Cuf1 results in an elevated and constitutive expression of *ctr4*$^+$ in the presence of copper, *ctr4*$^+$ mRNA levels were measured for cells treated with 100 ng/ml LMB in the presence of 25 μM $CuSO_4$. The data in Fig. 4B reveal that, while wild-type Cuf1 is retained in the nucleus, steady-state levels of *ctr4*$^+$ were still

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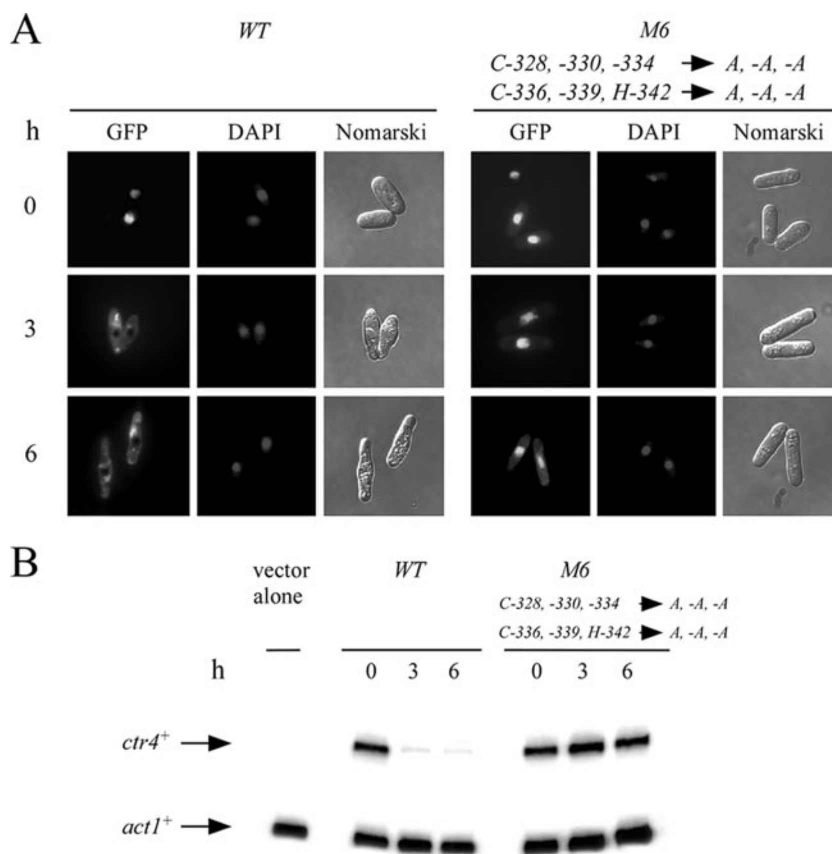

FIG. 3. The C-rich domain is required for copper-induced Cuf1-GFP nuclear export. (A) JSY17 cells expressing the wild-type *cuf1⁺-GFP* or mutant *cuf1-M6-GFP* allele were grown to mid-logarithmic phase in thiamine-free medium containing BCS. The cells were washed twice and then incubated in the presence of thiamine (15 μM) and $CuSO_4$ (25 μM). After 0, 3, and 6 h treatment, the cells were analyzed by fluorescence microscopy for Cuf1-GFP localization. Corresponding DAPI (nuclear staining) and Nomarski images are shown after each GFP panel. (B) Total RNA was prepared from aliquots of the cultures described above for panel A and then used in an RNase protection analysis to determine $ctr4^+$ and $act1^+$ (as a control) mRNA levels. The results shown are representative of three independent experiments. The six point mutations in M6 are indicated. *WT*, wild type.

repressed by copper. Thus, these results reveal that nuclear export of Cuf1 is not the main mechanism for repressing transcription of $ctr4^+$and, in fact, appears to be uncoupled from target gene regulation.

Cuf1-GFP nuclear export occurs via the Crm1 exportin. Because of the presence of a short leucine-rich hydrophobic region (^{349}LAALNHISAL^{358}) in the Cuf1 C terminus with the potential to act as a Crm1-dependent NES and the ability of LMB to inhibit the nuclear export of Cuf1-GFP, we hypothesized that Crm1 mediates the nuclear export of Cuf1. To test this hypothesis, we examined the cellular localization of Cuf1-GFP in *crm1-809* cells expressing a thermolabile Crm1 (46). Transcription of an $nmt1^+$-controlled $cuf1^+$-*GFP* gene was first induced by incubating cells in thiamine-free medium. Wild-type and *crm1-809* cells expressing $cuf1^+$-*GFP* were grown at the permissive temperature (25°C) in the presence of BCS to ensure nuclear localization of newly synthesized Cuf1-GFP, followed by the addition of thiamine to inhibit further expression. Wild-type and *crm1-809* cells were divided and then further incubated at the permissive or nonpermissive (30°C) temperature to inactivate Crm1, in the presence of 25 μM $CuSO_4$ or 100 μM BCS. After 3 h, analyses of the cells by fluorescence microscopy revealed that Cuf1-GFP was sequestered in the nucleus of copper-treated *crm1-809* cells that were grown at the nonpermissive temperature (30°C) (Fig. 5A). In contrast, at the permissive temperature (25°C), when Crm1 is functional, Cuf1-GFP accumulated in the cytoplasmic region and was absent from the nuclei of copper-treated *crm1-809* cells (Fig. 5A). In cells expressing wild-type Crm1, copper treatment resulted in the translocation of Cuf1-GFP from the nucleus to the cytoplasm under both temperature conditions (Fig. 5A). In a control experiment, Cuf1-GFP was localized in the nucleus in both wild-type and *crm1-809* mutant cells following a treatment with the copper chelator BCS. As shown in Fig. 5A, wild-type Cuf1 accumulates in the nucleus of cells expressing a temperature-sensitive *crm1-809* mutation at the nonpermissive temperature. To ascertain if the protein can still be inactivated by copper, RNA was prepared from aliquots of the cell cultures, and $ctr4^+$ mRNA levels were measured by RNase protection assays. The data in Fig. 5B show that, as observed previously with LMB, $ctr4^+$ mRNA levels were downregulated in response to copper. These data reveal that the nuclear accumulation of Cuf1 is insufficient in itself to lead to unregulated expression of Cuf1-responsive genes. Nonethe-

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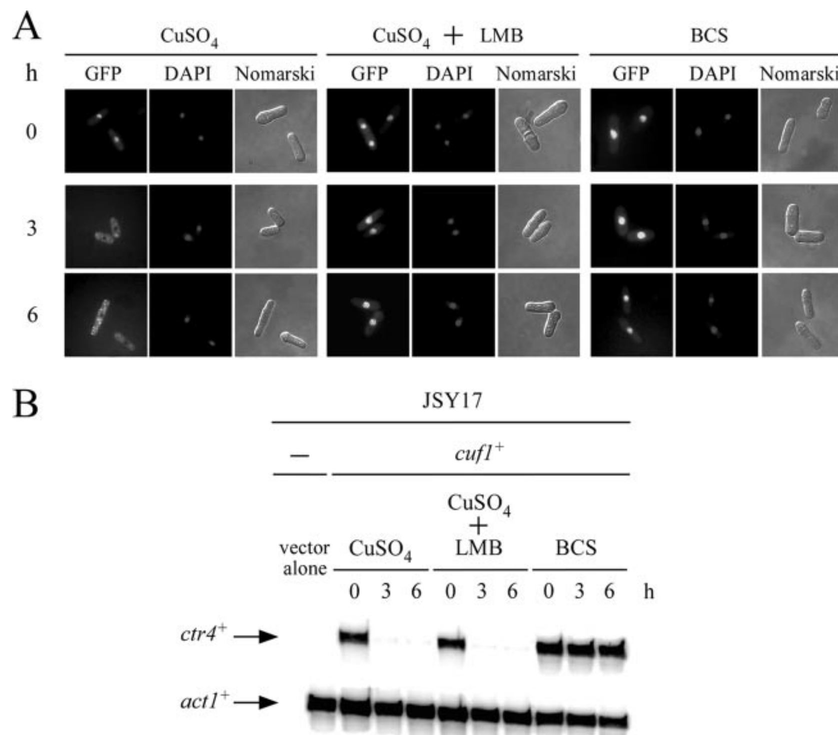

FIG. 4. LMB abrogates nuclear export of Cuf1-GFP. (A) Cells harboring a *cuf1*Δ deletion were transformed with pJB-1178*nmt-cuf1*$^+$*-GFP*. The cells were grown to mid-logarithmic phase in thiamine-free medium supplemented with 100 μM BCS. After two washes, the cells were incubated for the indicated time (0, 3, and 6 h) in medium with thiamine (15 μM) and in the presence of 25 μM $CuSO_4$, 25 μM $CuSO_4$ and 100 ng/ml LMB, or 100 μM BCS. The Cuf1-GFP fusion protein was viewed by direct fluorescence microscopy. DAPI staining, and Nomarski phase contrast images of cells expressing Cuf1-GFP are shown. (B) Total RNA was isolated from transformants of strain JSY17 harboring pJB-1178*nmt* with no insert (vector alone) or pJB-1178*nmt-cuf1*$^+$*-GFP*, and the steady-state mRNA levels of *ctr4*$^+$ and *act1*$^+$ (indicated with arrows) were analyzed by RNase protection experiments.

less, when Cuf1 is expressed in wild-type cells, its nuclear export occurs through the nuclear export receptor Crm1 in response to a shift from low to high copper concentrations.

Cuf1 interacts with the Crm1 exportin. To determine if Cuf1 can form a complex with the *S. pombe* Crm1, we carried out two-hybrid analysis using the full-length *crm1*$^+$ gene fused to the *LexA* coding region as bait and three different constructs of the *cuf1*$^+$ gene fused to the coding region of the VP16 activation domain as prey. As shown in Fig. 6, under conditions of copper excess, coexpression of the full-length wild-type Cuf1 fused to VP16 with the LexA-Crm1 fusion protein produced significant levels of β-galactosidase activity (~58 Miller units), indicating a physical interaction between these proteins. However, in the presence of the copper chelator BCS, no significant β-galactosidase activity was measured. We examined the role of the Leu-rich NES (^{349}LAALNHISAL^{358}) in the Cuf1 C-terminal region in its interaction with Crm1 by performing site-directed mutagenesis to replace the three leucines (Leu-349, -352, and -358) and the isoleucine (Ile-355) with alanines. This mutant was designated Cuf1-NESmut2-VP16. When Cuf1-NESmut2-VP16 was tested for its interaction with LexA-Crm1 by two-hybrid analysis, no significant β-galactosidase activity was detected regardless of copper stimulation (Fig. 6). On the other hand, in the presence of copper we detected a weak but reproducible interaction between LexA-Crm1 and Cuf1-M6-VP16 (~9 Miller units) (Fig. 6). However, this interaction appears to be insufficient for the nuclear export of Cuf1-M6 upon copper treatment, as the Cuf1-M6 mutant remained in the nucleus (Fig. 3A). To ensure that the fusion proteins were expressed in the transformed cells, immunoblot analyses of protein extracts were performed using anti-LexA and anti-VP16 antibodies (Fig. 6C). Although we consistently detected the Cuf1-VP16 fusion proteins used in this study by immunoblotting, we were unable to detect the VP16 polypeptide alone, perhaps owing to its low predicted molecular mass of ~8 kDa.In a control experiment, the DNA binding domain of LexA did not interact with Cuf1-VP16 fusion protein. Likewise, no interaction was detected between the VP16 activation domain and the chimeric LexA-Crm1 molecule. In conclusion, these results show that the NES of Cuf1, ^{349}LAALNHISAL^{358}, is required for the interaction of Cuf1 with Crm1.

DISCUSSION

We and others have previously demonstrated that *S. pombe* can distinguish between conditions of copper sufficiency and copper starvation and adjust accordingly the expression levels of copper transport genes (4, 7, 8, 25, 55). These changes are dependent on the Cuf1 transcription factor and reflect modifications in the activity of Cuf1. Recently, we have shown that *S. pombe* can respond to copper overload by inhibiting the nuclear entry of Cuf1 (5). We proposed a model in which

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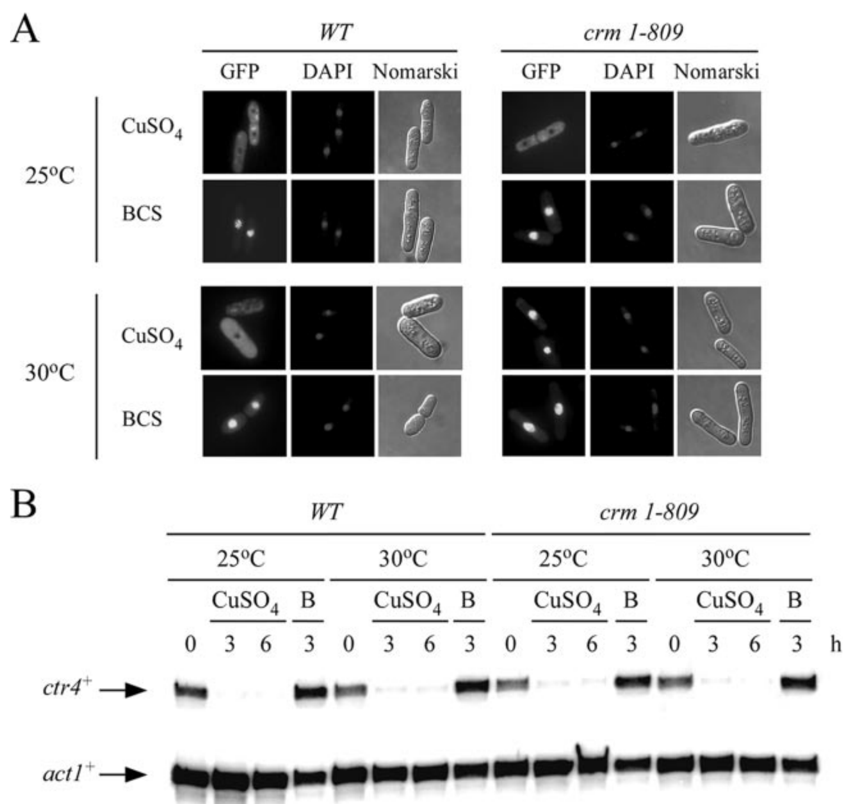

FIG. 5. Nuclear export of Cuf1-GFP is inhibited in *crm1-809* cells at the nonpermissive temperature. (A) Strains TP113 (*crm1*$^+$) (*WT*, wild type) and TP113-6B (*crm1-809*) were transformed with pJB-1178*nmt-cuf1*$^+$*-GFP*. To examine GFP fluorescence, the transformed strains were grown at 25°C to an A_{600} of ~1.0 in thiamine-free medium in the presence of BCS. The cultures were then washed twice and resuspended in medium supplemented with 15 μM thiamine to repress protein synthesis. Cultures were divided into four treatment groups: the presence of 25 μM $CuSO_4$ or 100 μM BCS at either the permissive (25°C) or nonpermissive (30°C) temperature. After 3 h, cells were subjected to fluorescence microscopy to visualize Cuf1-GFP fusion protein. As controls, nuclear DNA was visualized by DAPI staining and cell morphology by Normarski optics. (B) Total RNA was extracted from each culture described for panel A and at an additional time point, which was 6 h in the presence of copper. The mRNA steady-state levels of *ctr4*$^+$ and *act1*$^+$ (as a control) were analyzed by RNase protection assay. The results shown are representative of three independent experiments. B, BCS; *WT*, wild type.

metallation of Cuf1 triggers intramolecular conformational changes that mask its NLS, thereby preventing its nuclear import (5). The existence of a mechanism for the copper-dependent regulation of Cuf1 subcellular localization raises a number of questions. An important question is how the nuclear pool of Cuf1 is inactivated during a shift from low to high copper concentrations. In this study, we show that the nuclear pool of Cuf1 is regulated at two distinct steps. First, a blockage of Cuf1 nuclear export with LMB or with a temperature-sensitive *crm1* mutant did not disrupt copper transport gene down-regulation as the concentration of copper increased. This suggests that a primary mechanism for repressing *ctr* transcription would consist of a Cu ion-mediated inactivation of the nuclear pool of Cuf1, thereby preventing its action. Second, we also show that the nuclear pool of Cuf1 is exported from the nucleus to the cytoplasm in cells undergoing a transition from conditions of copper deficiency to copper sufficiency. The exportation of Cuf1 to the cytoplasm is likely to serve as a cellular defense mechanism to further ensure that no copper transport gene expression will take place in excess-copper conditions. The nuclear export of Cuf1 is directed by a leucine-rich C-terminal NES which resembles the NES sequences of other proteins, except that an additional alanine residue is present between the last two large hydrophobic amino acids (Ile-355 and Leu-358). Interestingly, however, the amino acid next to this penultimate residue (Ala-357) is a serine (at position 356), which is known to be the optimal amino acid in that position for high-affinity interaction with the nuclear export receptor Crm1 (11). The Cuf1 NES (^{349}LAALNHISAL^{358}) is located 7 amino acids downstream of the putative copper binding C-rich motif ^{328}CQCGDNCECLGCLTH^{342}, within the C-terminal region of Cuf1. Mutation of the three leucine residues and the isoleucine within the NES motif resulted in the constitutive nuclear localization of Cuf1. A concomitant increase in *ctr4*$^+$ mRNA levels was observed in copper-replete cells expressing the NES mutant alleles *cuf1-NESmut1* and *cuf1-NESmut2*. These cells were hypersensitive to copper in a manner that paralleled the magnitude of sustained *ctr4*$^+$ gene expression. Because these mutant proteins are unresponsive to copper for repression of target gene expression, cells need a prolonged exposure and the effect of continued copper uptake by the copper transporters to develop a copper sensitivity phenotype. This phenotype led us to hypothesize that mutations in Leu-349, -352, and -358 and Ile-355 may alter the copper-depen-

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FIG. 6. *S. pombe* Crm1 interacts with Cuf1 as shown by two-hybrid assay. (A) Schematic diagrams of LexA DNA-binding domain and fusions with full-length Crm1 protein. The indicated bait molecule was coexpressed with the VP16 activation domain or different Cuf1-VP16 fusion proteins. The black rectangle indicates that the Leu-349, Leu-352, Ile-355, and Leu-358 residues in the NES of Cuf1 were mutated to alanines, generating the Cuf1-NESmut2 mutant. The amino acid sequences of the Crm1 and Cuf1 proteins are numbered relative to their initiator codons, respectively. (B) The constructs diagrammed in panel A were coexpressed in the *S. cerevisiae* strain L40 grown in the presence of exogenous $CuSO_4$ (25 μM) or under copper-deficient conditions (100 μM BCS). Liquid β-galactosidase activities (indicated in Miller units) were assayed in the L40 strain using an integrated $(lexAop)_8$-*lacZ* reporter. Each sample was assayed in triplicate. (C) Whole cell extracts were prepared from aliquots of the cultures described for panel B and were analyzed by immunoblotting using either anti-LexA or anti-VP16 antibody. As an internal control, total extract preparations were probed with anti-PGK antibody. −, VP16 alone; B, BCS; M, molecular markers.

dent inhibition of Cuf1 by exposing the putative *trans*-activation domain, leaving it poised to direct the continued expression of *ctr4*$^+$. This hypothesis is supported by the data obtained by yeast two-hybrid analysis showing that a chimeric protein containing the N-terminal 61 amino acids of Cuf1 fused to the LexA DNA binding domain failed to physically interact with C-terminal residues 62 to 410 of Cuf1, which lacks the NES motif, fused to the VP16 activation domain (J. Beaudoin and S. Labbé, unpublished data). In contrast, using the same two-hybrid assay, these chimeric proteins were shown to physically interact with each other when the NES motif is present in the Cuf1 C terminus (5). Alternatively, this may also suggest that residues within the Cuf1 NES are involved in copper coordination, and mutation of these residues would inhibit the physical interaction between the N and C termini of Cuf1 in the presence of excess copper.

Analogous to Cuf1, the *S. cerevisiae* Aft1 and mammalian MTF-1 are two metal-regulatory transcription factors that also contain a leucine-rich NES (40, 54). Aft1 is the major iron-responsive transcriptional activator in baker's yeast (39, 41, 52, 53). Under conditions of iron starvation, Aft1 is nuclear and activates the expression of genes encoding products involved in iron acquisition and intracellular iron distribution (54). Conversely, under iron-replete conditions, Aft1 is returned to the cytoplasm and the expression of genes encoding components of the iron regulon is extinguished. This nuclear exclusion of Aft1 occurs via an NES-like sequence that contains two leucine residues (Leu-199 and Leu-201) (54). Similar to results with Cuf1, mutation of these leucine residues within the NES resulted in the constitutive transcriptional activation of *FTR1*, an Aft1 target gene, thereby altering the iron-dependent inactivation of Aft1. MTF-1 plays an essential role in activating metallothionein gene transcription in response to changes in zinc and cadmium levels (18, 36). Under resting conditions,

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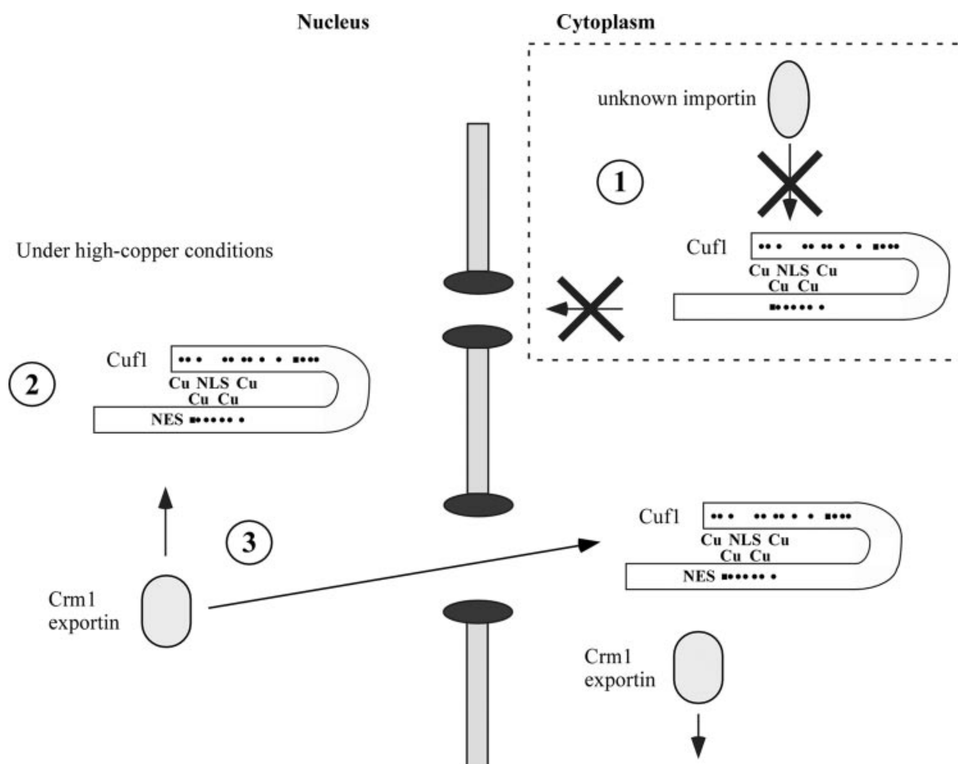

FIG. 7. Proposed model for copper-dependent nuclear-to-cytosolic export of Cuf1. Under conditions of copper starvation, Cuf1 is delivered to the nucleus, activating target gene transcription. Under conditions of copper excess, three distinct steps are proposed to take place. First, as previously shown (5), cytoplasmic Cuf1 is retained in the cytoplasm through a copper-dependent intramolecular interaction between the Cuf1 N and C termini that masks the NLS, blocking its interaction with importin and subsequent entry into the nucleus (step 1). Second, in response to "copper shock," metallation of Cuf1 induces intramolecular conformational changes that would prevent binding of Cuf1 to the CuSE, inhibiting its transactivation function (step 2). Third, to further ensure that no expression of the target gene takes place in excess-copper conditions, the Crm1 exportin interacts with Cuf1 via its accessible NES, leading to the exportation of the transcription factor to the cytoplasm (step 3).

MTF-1 is located predominantly in the cytoplasm, while treatment of cells with zinc or cadmium causes the nuclear translocation of MTF-1 (40, 42). Dual localization of MTF-1 is conferred by a classical NLS that facilitates its nuclear localization and an NES that promotes its nuclear exclusion. Nuclear export of human MTF-1 occurs via the NES sequence ^{336}LCLSDLSLL^{344}, which is located in the central region of the protein (40). Within this region of MTF-1 lies an acidic activation domain (40). Analogous to results with Cuf1 and Aft1, mutations within the NES result in the nuclear accumulation of MTF-1. However, in contrast to results with Cuf1 and Aft1, the MTF-1-NES mutant fails to activate transcription from the metallothionein-I promoter (40). It is possible that loss of activity of the mutant is due to an impaired function of the acidic MTF-1 activation domain. Alternatively, nucleocytoplasmic trafficking of MTF-1 might be required to ensure activation, perhaps by allowing an essential posttranslational modification of the protein in the cytoplasm.

We have shown that Cuf1 is imported into the nucleus in response to low levels of copper, creating a nuclear pool of the metal-regulatory transcription factor (5). Conversely, excess copper inhibits its entry into the nucleus. We proposed a model wherein metallation of Cuf1 induces an inhibitory conformational change that masks the Cuf1 NLS, blocking its interaction with importin and subsequently preventing its import into the nucleus (5). In this study, we define additional steps of the regulatory pathway that involves copper regulation by Cuf1. Specifically, we investigate the fate of the Cuf1 nuclear pool in the presence of excess copper. We propose a model (Fig. 7) in which the binding of copper to Cuf1 induces conformational changes that allow a copper-dependent interaction between the N-terminal and the C-terminal regions of Cuf1 (5). This intramolecular interaction promotes the shutoff of the nuclear pool of Cuf1, resulting in down-regulation of copper transport gene expression. To further ensure that no expression of the copper transport genes will occur in the presence of copper, the inactive form of Cuf1 is subsequently transported to the cytoplasm by making an association with the Crm1 exportin via its NES. The Cuf1-Crm1 complex moves through the nuclear pore into the cytoplasm, where it is dissociated. Interestingly, it has been demonstrated that in humans, *S. cerevisiae*, and *S. pombe*, RanBP3, Yrb2, and Hba1, respectively, act as adaptor molecules that stabilize the interaction between Crm1-RanGTP and cargo substrates for nuclear export (10, 12, 29, 32, 44, 45). Although some *S. pombe* NES-containing proteins do not require Hba1 for exportation, the possibility exists that this Ran-binding protein (Hba1) assists Crm1 to export Cuf1 in response to copper. Further studies will allow us to identify additional components required for the Cuf1 nucleocytoplasmic trafficking in response to changes in environmental copper levels.

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REFERENCES

1. **Alfa, C., P. Fantes, J. Hyams, M. McLeod, and E. Warbrick.** 1993. Experiments with fission yeast: a laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, New York, NY.
3. **Balamurugan, K., and W. Schaffner.** 2006. Copper homeostasis in eukaryotes: teetering on a tightrope. Biochim. Biophys. Acta **1763:**737–746.
4. **Beaudoin, J., and S. Labbé.** 2001. The fission yeast copper-sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1-like recognition sequence. J. Biol. Chem. **276:**15472–15480.
5. **Beaudoin, J., and S. Labbé.** 2006. Copper induces cytoplasmic retention of fission yeast transcription factor Cuf1. Eukaryot. Cell **5:**277–292.
6. **Beaudoin, J., J. Laliberté, and S. Labbé.** 2006. Functional dissection of Ctr4 and Ctr5 amino-terminal regions reveals motifs with redundant roles in copper transport. Microbiology **152:**209–222.
7. **Beaudoin, J., A. Mercier, R. Langlois, and S. Labbé.** 2003. The *Schizosaccharomyces pombe* Cuf1 is composed of functional modules from two distinct classes of copper metalloregulatory transcription factors. J. Biol. Chem. **278:**14565–14577.
8. **Bellemare, D. R., L. Shaner, K. A. Morano, J. Beaudoin, R. Langlois, and S. Labbé.** 2002. Ctr6, a vacuolar membrane copper transporter in *Schizosaccharomyces pombe*. J. Biol. Chem. **277:**46676–46686.
9. **Bezanilla, M., S. L. Forsburg, and T. D. Pollard.** 1997. Identification of a second myosin-II in *Schizosaccharomyces pombe*: Myp2p is conditionally required for cytokinesis. Mol. Biol. Cell **8:**2693–2705.
10. **Castillo, E. A., A. P. Vivancos, N. Jones, J. Ayte, and E. Hidalgo.** 2003. *Schizosaccharomyces pombe* cells lacking the Ran-binding protein Hba1 show a multidrug resistance phenotype due to constitutive nuclear accumulation of Pap1. J. Biol. Chem. **278:**40565–40572.
11. **Engelsma, D., R. Bernad, J. Calafat, and M. Fornerod.** 2004. Supraphysiological nuclear export signals bind CRM1 independently of RanGTP and arrest at Nup358. EMBO J. **23:**3643–3652.
12. **Englmeier, L., M. Fornerod, F. R. Bischoff, C. Petosa, I. W. Mattaj, and U. Kutay.** 2001. RanBP3 influences interactions between CRM1 and its nuclear protein export substrates. EMBO Rep. **2:**926–932.
13. **Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90:**1051–1060.
14. **Forsburg, S. L.** 1993. Comparison of *Schizosaccharomyces pombe* expression systems. Nucleic Acids Res. **21:**2955–2956.
15. **Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida.** 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature **390:**308–311.
16. **Gaits, F., G. Degols, K. Shiozaki, and P. Russell.** 1998. Phosphorylation and association with the transcription factor Atf1 regulate localization of Spc1/Sty1 stress-activated kinase in fission yeast. Genes Dev. **12:**1464–1473.
17. **Halliwell, B., and J. M. Gutteridge.** 1992. Biologically relevant metal ion-dependent hydroxyl radical generation. FEBS Lett. **307:**108–112.
18. **Heuchel, R., F. Radtke, O. Georgiev, G. Stark, M. Aguet, and W. Schaffner.** 1994. The transcription factor MTF-1 is essential for basal and heavy metal-induced *metallothionein* gene expression. EMBO J. **13:**2870–2875.
19. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
20. **Kohrer, K., and H. Domdey.** 1991. Preparation of high molecular weight RNA. Methods Enzymol. **194:**398–405.
21. **Kudo, N., N. Matsumori, H. Taoka, D. Fujiwara, E. P. Schreiner, B. Wolff, M. Yoshida, and S. Horinouchi.** 1999. Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proc. Natl. Acad. Sci. USA **96:**9112–9117.
22. **Kudo, N., H. Taoka, T. Toda, M. Yoshida, and S. Horinouchi.** 1999. A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor Pap1. J. Biol. Chem. **274:**15151–15158.
23. **Kutay, U., and S. Guttinger.** 2005. Leucine-rich nuclear-export signals: born to be weak. Trends Cell Biol. **15:**121–124.
24. **Labbé, S., J. Beaudoin, D. R. Bellemare, and B. Pelletier.** 2002. Regulatory responses to copper ions in fungi, p. 571–587. *In* E. J. Massaro (ed.), Handbook of copper pharmacology and toxicology. Humana Press, Totowa, NJ.
25. **Labbé, S., M. M. O. Peña, A. R. Fernandes, and D. J. Thiele.** 1999. A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*. J. Biol. Chem. **274:**36252–36260.
26. **Labbé, S., and D. J. Thiele.** 1999. Pipes and wiring: the regulation of copper uptake and distribution in yeast. Trends Microbiol. **7:**500–505.
27. **la Cour, T., L. Kiemer, A. Molgaard, R. Gupta, K. Skriver, and S. Brunak.** 2004. Analysis and prediction of leucine-rich nuclear export signals. Protein Eng. Des. Sel. **17:**527–536.
28. **Lange, A., R. E. Mills, C. J. Lange, M. Stewart, S. E. Devine, and A. H. Corbett.** 2007. Classical nuclear localization signals: definition, function, and interaction with importin alpha. J. Biol. Chem **282:**5101–5105.
29. **Lindsay, M. E., J. M. Holaska, K. Welch, B. M. Paschal, and I. G. Macara.** 2001. Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export. J. Cell Biol. **153:**1391–1402.
30. **Mosammaparast, N., and L. F. Pemberton.** 2004. Karyopherins: from nuclear-transport mediators to nuclear-function regulators. Trends Cell Biol. **14:**547–556.
31. **Nishi, K., M. Yoshida, D. Fujiwara, M. Nishikawa, S. Horinouchi, and T. Beppu.** 1994. Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. J. Biol. Chem. **269:**6320–6324.
32. **Noguchi, E., Y. Saitoh, S. Sazer, and T. Nishimoto.** 1999. Disruption of the YRB2 gene retards nuclear protein export, causing a profound mitotic delay, and can be rescued by overexpression of XPO1/CRM1. J. Biochem. (Tokyo) **125:**574–585.
33. **Ossareh-Nazari, B., F. Bachelerie, and C. Dargemont.** 1997. Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science **278:**141–144.
34. **Pemberton, L. F., and B. M. Paschal.** 2005. Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic **6:**187–198.
35. **Puig, S., and D. J. Thiele.** 2002. Molecular mechanisms of copper uptake and distribution. Curr. Opin. Chem. Biol. **6:**171–180.
36. **Radtke, F., R. Heuchel, O. Georgiev, M. Hergersberg, M. Gariglio, Z. Dembic, and W. Schaffner.** 1993. Cloned transcription factor MTF-1 activates the mouse *metallothionein* I promoter. EMBO J. **12:**1355–1362.
37. **Rees, E. M., and D. J. Thiele.** 2004. From aging to virulence: forging connections through the study of copper homeostasis in eukaryotic microorganisms. Curr. Opin. Microbiol. **7:**175–184.
38. **Rutherford, J. C., and A. J. Bird.** 2004. Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. Eukaryot. Cell **3:**1–13.
39. **Rutherford, J. C., S. Jaron, and D. R. Winge.** 2003. Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. J. Biol. Chem. **278:**27636–27643.
40. **Saydam, N., O. Georgiev, M. Y. Nakano, U. F. Greber, and W. Schaffner.** 2001. Nucleo-cytoplasmic trafficking of metal-regulatory transcription factor 1 is regulated by diverse stress signals. J. Biol. Chem. **276:**25487–25495.
41. **Shakoury-Elizeh, M., J. Tiedeman, J. Rashford, T. Ferea, J. Demeter, E. Garcia, R. Rolfes, P. O. Brown, D. Botstein, and C. C. Philpott.** 2004. Transcriptional remodeling in response to iron deprivation in *Saccharomyces cerevisiae*. Mol. Biol. Cell **15:**1233–1243.
42. **Smirnova, I. V., D. C. Bittel, R. Ravindra, H. Jiang, and G. K. Andrews.** 2000. Zinc and cadmium can promote rapid nuclear translocation of metal response element-binding transcription factor-1. J. Biol. Chem. **275:**9377–9384.
43. **Stade, K., C. S. Ford, C. Guthrie, and K. Weis.** 1997. Exportin 1 (Crm1p) is an essential nuclear export factor. Cell **90:**1041–1050.
44. **Taura, T., H. Krebber, and P. A. Silver.** 1998. A member of the Ran-binding protein family, Yrb2p, is involved in nuclear protein export. Proc. Natl. Acad. Sci. USA **95:**7427–7432.
45. **Taura, T., G. Schlenstedt, and P. A. Silver.** 1997. Yrb2p is a nuclear protein that interacts with Prp20p, a yeast Rcc1 homologue. J. Biol. Chem. **272:**31877–31884.
46. **Toone, W. M., S. Kuge, M. Samuels, B. A. Morgan, T. Toda, and N. Jones.** 1998. Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. Genes Dev. **12:**1453–1463.
47. **Valko, M., H. Morris, and M. T. Cronin.** 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. **12:**1161–1208.
48. **Vojtek, A. B., J. A. Cooper, and S. M. Hollenberg.** 1997. Searching for interacting proteins with the two-hybrid system II, p. 29–42. *In* P. Bartel and S. Fields (ed.), The yeast two-hybrid system: a practical approach. Oxford University Press, New York. NY.
49. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
50. **Weis, K.** 2003. Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. Cell **112:**441–451.
51. **Wen, W., J. L. Meinkoth, R. Y. Tsien, and S. S. Taylor.** 1995. Identification of a signal for rapid export of proteins from the nucleus. Cell **82:**463–473.

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52. **Yamaguchi-Iwai, Y., A. Dancis, and R. D. Klausner.** 1995. AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. EMBO J. **14:**1231–1239.
53. **Yamaguchi-Iwai, Y., R. Stearman, A. Dancis, and R. D. Klausner.** 1996. Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. EMBO J. **15:**3377–3384.
54. **Yamaguchi-Iwai, Y., R. Ueta, A. Fukunaka, and R. Sasaki.** 2002. Subcellular localization of Aft1 transcription factor responds to iron status in *Saccharomyces cerevisiae*. J. Biol. Chem. **277:**18914–18918.
55. **Zhou, H., and D. J. Thiele.** 2001. Identification of a novel high affinity copper transport complex in the fission yeast *Schizosaccharomyces pombe*. J. Biol. Chem. **276:**20529–20535.

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