Dissection of the relative contribution of the *Schizosaccharomyces pombe* Ctr4 and Ctr5 proteins to the copper transport and cell surface delivery functions

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The Ctr1 family of proteins mediates high-affinity copper (Cu) acquisition in eukaryotic organisms. In the fission yeast *Schizosaccharomyces pombe*, Cu uptake is carried out by a heteromeric complex formed by the Ctr4 and Ctr5 proteins. Unlike human and *Saccharomyces cerevisiae* Ctr1 proteins, Ctr4 and Ctr5 are unable to function independently in Cu acquisition. Instead, both proteins physically interact with each other to form a Ctr4–Ctr5 heteromeric complex, and are interdependent for secretion to the plasma membrane and Cu transport activity. In this study, we used *S. cerevisiae* mutants that are defective in high-affinity Cu uptake to dissect the relative contribution of Ctr4 and Ctr5 to the Cu transport function. Functional complementation and localization assays show that the conserved Met-X3-Met motif in transmembrane domain 2 of the Ctr5 protein is dispensable for the functionality of the Ctr4–Ctr5 complex, whereas the Met-X3-Met motif in the Ctr4 protein is essential for function and for localization of the hetero-complex to the plasma membrane. Moreover, Ctr4/Ctr5 chimeric proteins reveal unique properties found either in Ctr4 or in Ctr5, and are sufficient for Cu uptake on the cell surface of *Sch. pombe* cells. Functional chimeras contain the Ctr4 central and Ctr5 carboxyl-terminal domains (CTDs). We propose that the Ctr4 central domain mediates Cu transport in this hetero-complex, whereas the Ctr5 CTD functions in the regulation of trafficking of the Cu transport complex to the cell surface.

INTRODUCTION

Copper (Cu) is an essential micronutrient for aerobic organisms, in which it plays several well-established roles as a structural and catalytic cofactor for a spectrum of proteins, including cytochrome c oxidase, Cu/zinc-superoxide dismutase (Cu/Zn-SOD), and multi-Cu ferroxidases involved in iron (Fe) uptake (Balamurugan & Schaffner, 2006; Kim et al., 2008; Linder, 1991). However, when present in excess, the same redox properties that make Cu an indispensable element enable the generation of reactive oxygen species, such as hydroxyl radicals, which damage cells at the membrane lipid, protein and nucleic acid levels, and can interfere with the biogenesis of Fe–S clusters (Halliwell & Gutteridge, 1984; Macomber & Imlay, 2009). Therefore, micro-organisms must possess sophisticated mechanisms to tightly control the intracellular levels of Cu by regulating the transport of this metal across the plasma membrane.

A number of studies in *Saccharomyces cerevisiae* have allowed the identification of the proteins constituting the high-affinity Cu uptake system (K_m around 1–5 μM). To be transported into *S. cerevisiae*, extracellular Cu^{2+} is reduced to Cu^{+} by the cell surface reductases Fre1 and Fre2 (Georgatsou et al., 1997; Hassett & Kosman, 1995; Martins et al., 1998), and is transported into cells by the functionally independent and biologically redundant plasma membrane Ctr1 and Ctr3 high-affinity Cu transport proteins (Dancis et al., 1994b; Knight et al., 1996; Peña et al., 2000). The expression of Cu uptake genes, which include FRE1, CTR1 and CTR3, is activated by the Mac1 transcription factor when environmental Cu becomes...
limited (Dancis et al., 1994a; Georgatsou et al., 1997; Knight et al., 1996; Labbé et al., 1997; Martins et al., 1998). 

*S. cerevisiae* ctr1Δctr3Δ mutants exhibit Cu starvation phenotypes (Puig & Thielemans, 2002), as shown by their inability to grow in media containing non-fermentable carbon sources such as glycerol and ethanol as the sole carbon source, given the significant loss of function of the Cu- and Fe-requiring mitochondrial cytochrome c oxidase. Furthermore, ctr1Δctr3Δ cells do not grow in media with limited Fe availability because of a defect in the Cu-dependent ferroxidase Fet3, which is essential for high-affinity Fe uptake in yeast, and they are highly sensitive to oxidative stress as a result of diminished Cu incorporation into Cu/Zn-SOD.

Topological studies in vivo have demonstrated that the *S. cerevisiae* Ctr1 Cu transporter contains three transmembrane domains (TMDs), with the amino-terminus localizing toward the extracellular space and the carboxyl-terminus facing the cytosol (Puig et al., 2002). The extracellular amino-terminus of Ctr1 contains sequences rich in methionine residues that are arranged in Met-X-Met and Met-X-Met clusters, denoted Mets motifs. Genetic, biochemical and structural studies have revealed that the Mets motifs of Ctr1 are important for Cu acquisition when *S. cerevisiae* cells grow under Cu-deficient conditions, and that these motifs selectively bind Cu$^{+}$ ions through methionine thioester groups (Jiang et al., 2010). Indispensable residues for the function of Ctr1-mediated Cu transport include the last methionine (Met$^{127}$) of the Mets motifs, located at approximately 20 aa before TMD1, as well as the Met-X-Met motif in TMD2. The substitution of these conserved methionines with alanine or leucine completely abrogates Ctr1 function, without altering Ctr1 localization to the plasma membrane (Puig et al., 2002). Interestingly, the substitution of these conserved methionines by cysteine or histidine does not eliminate Ctr1 transport activity, suggesting that these residues directly contribute to Cu$^{+}$ conduction through the plasma membrane toward the cytosol (Puig et al., 2002). Genetic, biochemical and, more recently, structural data on the human Ctr1 protein indicate that Ctr1-like proteins oligomerize and assemble at the plasma membrane as symmetrical homotrimers with a cone-shaped pore in the centre (Aller & Unger, 2006; Dancis et al., 1994a; De Feo et al., 2009; Klomp et al., 2003; Lee et al., 2002; Peña et al., 2000; Puig et al., 2002). A conserved Gly-X$_3$-Gly motif within TMD3 is essential for the Ctr1 proteins to appropriately oligomerize (Aller et al., 2004). Studies have shown that despite the cytosolic carboxyl-terminal domain (CTD) of *S. cerevisiae* Ctr1 protein being dispensable for Cu transport activity, it is important for the post-transcriptional regulation of the protein in response to increasing exogenous Cu concentrations (Liu et al., 2007; Ooi et al., 1996; Wu et al., 2009). Ctr1 has been proposed to undergo Cu-induced degradation at the plasma membrane, as well as endocytosis and degradation at the vacuole (Liu et al., 2007; Ooi et al., 1996). More recently, the Ctr1 CTD has been implicated in the regulation of the Cu transport activity of the protein on the cell surface (Wu et al., 2009). In the presence of Cu, it has been suggested that some residues within the Ctr1 CTD trigger a conformational change which inactivates Ctr1 Cu transport activity in order to protect cells against toxic Cu levels (Wu et al., 2009). Consequently, *S. cerevisiae* cells expressing a truncated Ctr1 protein lacking amino acid residues within its CTD are more sensitive to high Cu concentrations (Wu et al., 2009).

We previously identified the *Schizosaccharomyces pombe* Ctr4 and Ctr5 proteins, structurally related to the Ctr1 family, as a high-affinity Cu transport complex (Labbé et al., 1999; Zhou & Thielemans, 2001). The heterologous expression of Ctr4 in the *S. cerevisiae* ctr1Δctr3Δ strain failed to rescue Cu transport activity, and the Ctr4 protein is trapped within the secretory pathway of the budding yeast (Zhou & Thielemans, 2001). A genetic screen using the *S. cerevisiae* ctr1Δctr3Δ strain allowed the identification of *Sch. pombe* Ctr5, which rescues both the localization of Ctr4 to the plasma membrane and its Cu-transporting activity in *S. cerevisiae* (Zhou & Thielemans, 2001). Subsequent studies in *Sch. pombe* have revealed that the expression of the ctr4$^{+}$ and ctr5$^{+}$ genes is induced in response to Cu deficiency by the Cuf1 Cu-sensing transcription factor (Beaudoin & Labbé, 2001; Zhou & Thielemans, 2001). Furthermore, unlike the *S. cerevisiae* Ctr1 and Ctr3 proteins, the Ctr4 and Ctr5 proteins physically interact with each other to form a heteromeric complex, and they are interdependent for secretion to the plasma membrane and Cu transport activity (Beaudoin et al., 2006; Zhou & Thielemans, 2001). Similar to other high-affinity Cu transport proteins of the Ctr1 family, Ctr4 and Ctr5 display amino-terminal domains (NTDs) rich in Mets motifs. Although they are not essential for the Cu transport function, the Mets motifs within the NTDs of both *Sch. pombe* proteins enhance Cu uptake efficiency in an independent yet redundant manner (Beaudoin et al., 2006). Furthermore, the recent results of a bimolecular fluorescence complementation assay strongly suggest that the assembly of a functional high-affinity Cu uptake system on the cell surface of *Sch. pombe* requires the combination of two Ctr4 molecules with one Ctr5 molecule (Ioannoni et al., 2010). Moreover, these studies have demonstrated that the Ctr4–Ctr5 complex is post-transcriptionally regulated by Cu (Ioannoni et al., 2010), with both proteins being internalized in response to high Cu concentrations and recycled back to the cell surface when Cu availability is diminished.

The *Sch. pombe* Ctr4 and Ctr5 proteins contain all the domains known to be required for the Cu-transporting activity of the Ctr1 family of Cu transporters, including three TMDs, the extracellular Mets motifs, the conserved methionine residues within the NTD, the Met-X$_3$-Met motif within TMD2, and the TMD3 Gly-X$_3$-Gly motif (Fig. 1). Notwithstanding, Ctr4 and Ctr5 are unable to function independently in Cu acquisition. In this study, we used the yeasts *S. cerevisiae* and *Sch. pombe* to study the contri-
bution of Ctr4 and Ctr5 to the Cu transport activity of the heteromeric complex. The mutagenesis of the essential motifs for transport suggests that Ctr5 exerts an accessory function in the Cu transporter complex. Furthermore, the functional dissection of the Ctr4 and Ctr5 domains using Ctr4/Ctr5 chimeric proteins shows that the Ctr4 CTD exerts an inhibitory effect on trafficking the Ctr4 protein to the cell surface, and that this is rescued by the Ctr5 CTD.

**METHODS**

**Yeast strains and growth conditions.** The MPY17 strain, lacking both the Ctr1 and Ctr3 high-affinity Cu transporters (Peña et al., 1998), was used for the functional complementation assays and subcellular localization studies in *Saccharomyces cerevisiae*. The MPY17 strain was cotransformed with the centromeric p413GPD and p416GPD vectors (Mumberg et al., 1995), either empty or expressing wild-type or modified alleles of the *CTR1*, *CTR4* and *CTR5* genes. The transformed *S. cerevisiae* cells were maintained in a synthetic complete (SC) medium lacking uracil and histidine (SC-ura-his) (Kaiser et al., 1994). The *ctr4Δctr5Δ* strain, isogenic to FY435 (*h^+^ his7-366 leu1-32 ura4-Δ18 ade6-M101*) (Bezanilla et al., 1997) and to *ctr4::ura4^+^ ctr5::Kan^R^, was used for the functional complementation assays and subcellular localization studies in *Sch. pombe*. The *ctr4Δctr5Δ* strain was cotransformed with the pBPAd6 (ade6^+^) and pJK148 (leu1^+^) vectors (Beaudoin et al., 2006; Keeney & Boeke, 1994), which either were empty or expressed wild-type or modified alleles of the *ctr4^+^* and *ctr5^+^* genes. The transformed *Sch. pombe* cells were maintained in a selective Edinburgh minimal medium with all the auxotrophic requirements, except adenine and leucine (EMM-ade-leu) (Alfa et al., 1993).

**Plasmids.** *Escherichia coli* was used for the construction of recombinant plasmids. Transformants were grown at 37 °C in LBA medium with 50 μg ampicillin ml^−1^ (Sambrook et al., 1989). For expression in *S. cerevisiae*, the coding sequences of the *ctr4^+^* and *ctr5^+^* genes were PCR-amplified and cloned into the polylinker of the p413GPD and p416GPD vectors (Mumberg et al., 1995), respectively.

![Fig. 1. Notable features of *Sch. pombe* Ctr4 and Ctr5 proteins. (a) Sequence alignment and primary structural features of Ctr4 and Ctr5 proteins. The alignment was performed with CLUSTAL W2 software. Asterisks indicate the presence of an identical amino acid in both proteins, whereas dots indicate similarity between amino acids. (b) Schematic representation of the putative topology of Ctr4 and Ctr5 proteins. According to the topology displayed by the human and *S. cerevisiae* Ctr1 proteins, we propose that the amino-termini of Ctr4 and Ctr5 proteins would localize to the extracellular medium and that their carboxyl-termini would face the cytosol. Grey-shaded boxes represent the predicted locations of membrane-spanning domains (TMDs). The methionine residues located in conserved positions, which are essential for the Cu transport activity in human and *S. cerevisiae* Ctr1 proteins, are shown in bold type. The amino acid sequence number relating to the first amino acid of the protein is indicated.](http://mic.sgmjournals.org)
For GFP-tagging at the carboxyl-terminus, the coding sequences of the wild-type, mutant and chimeric ctr4<sup>+</sup> and ctr5<sup>+</sup> genes were amplified without their stop codon, and cloned into the p413GPD and p416GPD vectors, respectively, between the BamHI and EcoRI sites. The GFP coding sequence, including a stop codon, was PCR-amplified and cloned in-frame between the EcoRI and SalI sites. For expression in Sch. pombe, the pSP1-CTR4-GFP and pSP2-CTR5-GFP plasmids (Zhou & Thiele, 2001) were used as templates for the PCR amplifications. The coding sequences of the wild-type, mutant and chimeric ctr4<sup>+</sup> and ctr5<sup>+</sup> genes (with or without GFP) were cloned into the poly linker of the pBPAde6 and pJK148 vectors, respectively. In all cases, the overlap extension method (Ho et al., 1989) was followed for the mutagenesis of specific methionine residues and the construction of the Ctr4/Ctr5 chimeric proteins.

**Functional complementation assays.** Transformed *S. cerevisiae* Mpy17 cells were grown to exponential phase (OD<sub>600</sub> ~1.0) in liquid SC-ura-his medium, and plated as drops (OD<sub>600</sub> 0.1, and serial 10-fold dilutions) on solid SC-ura-his medium with or without 50 μM bithiophenanthroline disulfonic acid (BPS), on rich medium with glucose (YPD) (Kaiser et al., 1994) with or without 100 μM BPS, and on rich medium with ethanol/glycerol (YPEG) (Puig et al., 2002) with or without either 100 μM CuSO₄ or 100 μM bathocuproine disulfonic acid (BCS). The transformed Sch. pombe ctr4Δctr5Δ cells were grown to the exponential phase (OD<sub>600</sub> ~1.0) in liquid EMM-ade-leu medium with 100 μM BCS, and plated as drops on a solid yeast extract plus supplements (YES) medium (Alfa et al., 1993), and a respiratory carbon source YES/glycerol/ethanol (YES-EG) medium in which glucose was replaced with 3% (v/v) glycerol and 2% ethanol. Plates were incubated at 30°C for 3–7 days and photographed.

**Fluorescence microscopy.** *S. cerevisiae* cells were grown to exponential phase in liquid SC-ura-his medium, and visualized with a confocal fluorescence microscope as described previously (Puig et al., 2002). Sch. pombe cells were grown to exponential phase in liquid EMM-ade-leu medium with 100 μM BCS, and visualized with a Nikon Eclipse E800 epifluorescent microscope equipped with a Hamamatsu ORCA-ER (charge-coupled device; CCD) camera as described previously (Beaudoin et al., 2006).

**RESULTS**

**Integrity of the Ctr4 Met-X<sub>3</sub>-Met motif is essential for the Cu transport activity of the Ctr4–Ctr5 complex in *S. cerevisiae*.

A conserved Met-X<sub>3</sub>-Met motif within TMD2 is indispensable for the Cu transport function of all the Ctr1 family of high-affinity Cu transporters studied, which includes the *S. cerevisiae* Ctr1 and Ctr3 proteins, as well as the vacuolar Ctr2 protein and human Ctr1 (Puig et al., 2002; Rees et al., 2004). Therefore, to ascertain the relative contributions of Ctr4 and Ctr5 to the Cu transport activity of the Sch. pombe Ctr4–Ctr5 complex, we performed the following assay. First, we substituted the Met<sup>223</sup> and Met<sup>227</sup> in Ctr4 and the Met<sup>136</sup> and Met<sup>134</sup> in Ctr5 with alanine residues by generating the CTR4-M223/227A and CTR5-M130/134A mutant alleles, respectively. Then, we cloned both alleles into vectors designed for constitutive expression in *S. cerevisiae* (Mumberg et al., 1995). With this strategy, we assayed protein functionality independently of the Cu regulation of the gene expression by the endogenous promoter. We expressed both mutant alleles, simultaneously or in combination with their wild-type counterpart, in the *S. cerevisiae* ctr1Δctr3Δ strain. As previously reported (Puig et al., 2002), the ctr1Δctr3Δ mutant grew neither in medium with ethanol and glycerol (YPEG) as the sole carbon source nor in medium with low Fe availability, as achieved by the addition of the Fe<sup>2+</sup>-specific chelator BPS (Fig. 2). Yeast growth was rescued if the Fe chelator was removed from YPD medium, or if excess Cu was added to YPEG medium (Fig. 2) (Puig et al., 2002). Furthermore, whereas the independent expression of CTR4 and CTR5 did not restore growth of the ctr1Δctr3Δ mutant in the presence of BPS and YPEG, their co-expression achieved growth rates similar to those obtained with *S. cerevisiae* CTR1 expression (Fig. 2) (Zhou & Thiele, 2001). Interestingly, the co-expression of wild-type CTR4 with the CTR5-M130/134A mutant allele in strain ctr1Δctr3Δ allowed growth in the presence of BPS and YPEG (Fig. 2a). This result indicates either that the Ctr5 Met-X<sub>3</sub>-Met motif is not required for the Cu transport activity of the Ctr4–Ctr5 complex, or that the Met-X<sub>3</sub>-Met motif within only one of the two proteins is required for function. The co-expression of the CTR4-M223/227A mutant allele with wild-type CTR5 indicates that the Met-X<sub>3</sub>-Met motif within Ctr4 TMD2 is absolutely necessary for ctr1Δctr3Δ growth in YPEG, and growth was strongly diminished in medium containing BPS to levels similar to those achieved by the expression of CTR4 alone (Fig. 2a). Finally, the co-expression of both mutant alleles, CTR4-M223/227A and CTR5-M130/134A, completely abrogated growth in the presence of BPS and YPEG. Taken together, these results indicate that the Met-X<sub>3</sub>-Met motif in Ctr5 is dispensable for the functionality of the Ctr4–Ctr5 complex, whereas the Met-X<sub>3</sub>-Met motif in Ctr4 is essential for Cu transport.

To further dissect the relative contributions of Met<sup>223</sup> and Met<sup>227</sup> in the Ctr4 Met-X<sub>3</sub>-Met motif to the Cu transport activity of the Ctr4–Ctr5 complex, we separately substituted the Ctr4 Met<sup>223</sup> and Met<sup>227</sup> residues with alanine. The constitutive co-expression of either the CTR4-M223A or the CTR4-M227A mutant allele in combination with wild-type CTR5 in the ctr1Δctr3Δ mutant suggests that the mutagenesis of Ctr4 Met<sup>223</sup> does not affect growth on BPS and YPEG, whereas the substitution of Ctr4 Met<sup>227</sup> strongly limited yeast growth in both BPS and YPEG media. The effect of Met<sup>227</sup> mutagenesis on the Ctr4–Ctr5 Cu transport function was only observed when cells were grown on YPEG containing the Cu<sup>2+</sup>-specific chelator BCS, or when combined with the Met<sup>223</sup> mutation (Fig. 2b). These results indicate that both methionine residues in the Ctr4 Met<sup>223</sup>-X<sub>3</sub>-Met<sup>227</sup> motif contribute to the Cu transport activity of the heteromeric complex, with a predominant role for the Met<sup>223</sup> residue.

The lack of functionality of the Ctr4–Ctr5 complex expressing a CTR4 allele mutated at its Met-X<sub>3</sub>-Met motif could either be due to a defect in the Cu transport activity on the cell surface or be the result of a defect in the delivery
of the heteromeric complex to the plasma membrane. To distinguish between these two possibilities, we determined the subcellular localization of the wild-type and the mutated GFP-tagged Ctr4 and Ctr5 proteins in the S. cerevisiae ctr1Δctr3Δ strain. As previously described (Zhou & Thiele, 2001), Ctr4 and Ctr5 were intracellularly trapped in a perinuclear structure that could correspond to the early secretory pathway when expressed without their complex counterpart (Fig. 3a, b). Furthermore, a predominant cell surface localization was observed for both wild-type proteins when simultaneously co-expressed in budding yeast (Fig. 3c, d) (Zhou & Thiele, 2001). Interestingly, the mutagenesis of the Ctr5 Met-X3-Met motif did not significantly affect the localization of Ctr4 and Ctr5 to the plasma membrane (Fig. 3e, f). Although the expression of the Ctr4-M223/227A protein slightly increased the intracellular localization of Ctr4 and Ctr5, consistent with the cell growth phenotypes, a considerable amount of both proteins still reached the cell surface (Fig. 3g, h). Finally, the co-expression of both Ctr4-M223/227A and Ctr5-M130/134A did not alter the localization of these proteins to the plasma membrane, with a significant proportion of the heterocomplex found at the cell surface (Fig. 3i, j). Taken together, these results demonstrate that the mutagenesis of the Met-X3-Met motif in Ctr4 and Ctr5 does not abrogate their localization at the surface of the cell. Furthermore, these data strongly suggest that the growth defect displayed by the cells expressing the CTR4-M223-X3-Met227 mutant alleles under low Fe and in the presence of non-fermentable carbon sources is due to a lack of Cu transport function rather than to a defect in trafficking of the heteroprotein complex to the plasma membrane.

Expression of a Ctr4/Ctr5 chimeric protein confers Cu transport activity in S. cerevisiae

The results obtained from the mutagenesis of the Met-X3-Met motifs located in TMD2 suggest that Ctr4 plays a major role in the mechanism that transports Cu across the plasma membrane lipid bilayer, whereas the Ctr5 Met-X3-Met motif does not directly participate in Cu transport activity. Previous data have demonstrated that the extracellular Mets motifs of both proteins contribute to efficient Cu assimilation by the Ctr4–Ctr5 heteromeric complex (Beaudoin et al., 2006). In order to investigate the relative contributions of the different Ctr4 and Ctr5 protein domains to the Cu transport activity of the heteromeric complex, we constructed chimeric proteins between Ctr4 and Ctr5, and tested the chimeras for Cu transport activity. A crucial point in this approach was the choice of the most appropriate position to perform the sequence swap between Ctr4 and Ctr5 in order to minimize any potential alteration of the overall transporter structure. We swapped the protein sequences at two conserved positions which are essential in the S. cerevisiae Ctr1 protein (Puig et al., 2002). The first swap position localizes to the methionine residue located 20 aa before TMD1, which is Met^{122} in Ctr4 and Met^{31} in Ctr5 (Fig. 1). The second swap point was located in the last methionine residue of the Met-X3-Met motif of both proteins, which is Met^{227} for Ctr4 and Met^{134} for Ctr5 (Fig. 1). Therefore, we divided each protein into three distinct regions: the extracellular NTD [Ctr4(1–122) and Ctr5(1–31)], which contains the Mets motifs; the central region [Ctr4(122–227) and Ctr5(31–134)], which extends over TMD1, the cytosolic loop and TMD2, including the Gly-X3-Gly motif; and the CTD [Ctr4(227–318) and Ctr5(134–173)], which covers TMD3, including the Gly-X3-Gly motif and the intracellular carboxyl-terminus (Fig. 1).
Thereby, we obtained six different Ctr4/Ctr5 chimeric proteins, three with a Ctr5 central region, denoted Ctr455, Ctr554 and Ctr454; plus three additional chimeras with a Ctr4 central region, denoted Ctr544, Ctr445 and Ctr545 (Fig. 4a, b). To assess their function in Cu transport, we used the growth assay in the S. cerevisiae ctr1Δctr3Δ strain, as described above. As shown in Fig. 4(c), none of the chimeras with a Ctr5 central region was able to rescue the growth defect of the ctr1Δctr3Δ strain in BPS or YPEG medium. This result is consistent with the essential function of the central domain of the Ctr4 protein, which contains the key Met-X3-Met motif that is not present in these chimeric proteins (Fig. 4a). An identical result was obtained for the Ctr544 chimera (Fig. 4d), probably due to the lack of a Ctr5 counterpart. One interesting finding for the chimeric proteins containing the central region of Ctr4 and the Ctr5 CTD, i.e. Ctr445 and Ctr545, was that they fully complemented the growth defect of the ctr1Δctr3Δ cells in BPS or YPEG medium, and did not require any Ctr4 or Ctr5 counterpart for the function (Fig. 4d). Taken together, these data demonstrate that the expression of a Ctr4/Ctr5 chimeric protein suffices to complement the growth defects of the S. cerevisiae ctr1Δctr3Δ strain, perhaps because it comprises the essential functional properties separately contained in the individual Ctr4 and Ctr5 polypeptides.

To ascertain whether the distinct functionality obtained for the expression of the different Ctr4/Ctr5 chimeric proteins in the S. cerevisiae ctr1Δctr3Δ strain resulted from either a Cu transport malfunction or a defect in the delivery of the protein to the plasma membrane, the chimeric proteins were epitope-tagged with GFP at the carboxyl-terminus and their subcellular localization was ascertained by fluorescence microscopy. As a control, wild-type Ctr4-GFP and Ctr5-GFP were separately or simultaneously expressed (Fig. 5a–d). All of the Ctr4/Ctr5 chimeric proteins that did not complement the ctr1Δctr3Δ strain (Ctr455, Ctr554, Ctr454 and Ctr544) displayed an intracellular localization pattern (Fig. 5e, f, h and j), and the Ctr4/Ctr5 chimeras complementing the ctr1Δctr3Δ strain (Ctr445 and Ctr545) localized to the cell surface, similar to the result observed for co-expression of wild-type Ctr4-GFP and Ctr5-GFP (Fig. 5c, d, g, i). These results show that the functionality of the Ctr4/Ctr5 chimera directly correlates with its subcellular localization in S. cerevisiae. Furthermore, these data strongly suggest that the information for the correct delivery of the Ctr4–Ctr5 complex to the S. cerevisiae plasma membrane for Cu transport function is contained in both the Ctr4 central domain and the Ctr5 CTD, which are present in the Ctr4–Ctr5 heteromeric complex, and also in chimeric proteins Ctr445 and Ctr545.

The S. cerevisiae Ctr1 protein possesses two motifs which are indispensable for Cu acquisition and which contain key methionine residues, the Met-X3-Met motif, and the extracellular Met 127 located at approximately 20 aa before TMD1 (Puig et al., 2002). As shown above, the mutagenesis of the Met-X3-Met motif in Ctr4 abolishes the Cu transport function of the Ctr4–Ctr5 complex in S. cerevisiae. However, as previously reported (Beaudoin et al., 2006), the mutagenesis of the extracellular Ctr4 Met122 residue, which is the equivalent of Met127 in Ctr1, does not eliminate the Cu transport activity of the Ctr4–Ctr5 complex, perhaps due to the redundant function of the corresponding extracellular methionine residues in Ctr5 (Met129-X-Met131). Here, we show that a single protein,
Ctr445 (and also Ctr545), is able to complement the defects of the S. cerevisiae ctr1Δctr3Δ strain (Fig. 4d). If these Ctr4/Ctr5 chimeric proteins assembled as homotrimers, as previously reported for other Ctr1 family members (Aller & Unger, 2006; Lee et al., 2002; Peña et al., 2000), we would expect the mutagenesis of the Met 122 residue within the NTD of Ctr445 to completely eliminate its Cu transport activity. Indeed, the CTR445-M122A mutant allele does not allow the ctr1Δctr3Δ cells to grow in YPEG or in medium with low Fe availability (Supplementary Fig. S1). This result suggests that the Met122 residue is essential for the Cu transport activity of Ctr445 in S. cerevisiae.

Expression in Sch. pombe corroborates the results obtained in S. cerevisiae

To ascertain whether the results obtained via heterologous expression in S. cerevisiae cells were also applicable to Sch. pombe, we expressed the distinct ctr4+ and ctr5+ alleles in a ctr4Δctr5Δ fission yeast mutant. As previously reported (Zhou & Thiele, 2001), the simultaneous expression of ctr4+ and ctr5+ is required for Sch. pombe growth in media containing non-fermentable carbon sources, such as glycerol and ethanol (YES-EG) (Fig. 6). Growth of the ctr4Δctr5Δ strain in YES-EG medium was fully rescued by the addition of excess Cu (Fig. 6a). Similar to our results in budding yeast, the co-expression of the CTR4-M223/227A mutant allele with wild-type ctr5+ did not support Sch. pombe ctr4Δctr5Δ growth in YES-EG (Fig. 6a). Localization of a Ctr4-M223/227A-GFP fusion protein in the ctr4Δctr5Δ fission yeast mutant co-expressing wild-type ctr5+ indicated that it was mostly trapped intracellularly (Fig. 7d). Furthermore, the mutagenesis of the Met130 and Met134 residues in Ctr5 had no effect on Sch. pombe growth in YES-EG (Fig. 6a). Taken together, these results demonstrate that the Met-X3-Met motif in protein Ctr4 is essential for the functionality of the Ctr4–Ctr5 complex on the cell surface of Sch. pombe, whereas the Met-X3-Met motif in Ctr5 is dispensable for function.
To ascertain whether the expression of a single Ctr4/Ctr5 chimeric protein is also sufficient to promote Cu transport activity in *Sch. pombe*, we expressed the CTR445 chimera, fused to GFP, in a ctr4Δctr5Δ strain. As Fig. 6(b) illustrates, CTR445 complemented the growth defects of the *Sch. pombe* strain in YES-EG. Furthermore, protein localization studies demonstrated that CTR445-GFP reaches the plasma membrane in *ctr4Δctr5Δ* *Sch. pombe* cells without requiring any accessory counterpart (Fig. 7e). Taken together, these data strongly suggest that the CTD of Ctr4 inhibits the delivery of the protein to the cell surface in *Sch. pombe*. The substitution of the Ctr4 CTD with the Ctr5 CTD, or the simultaneous expression of *ctr5*Δ, leads to the localization of Ctr4 to the cell surface of *Sch. pombe* cells, where it mediates high-affinity Cu transport.

**DISCUSSION**

The Ctr1 family of Cu transporters mediates high-affinity Cu uptake in eukaryotic organisms. Recent structural studies have demonstrated that the human Ctr1 complex assembles as a symmetrical homotrimer with a cone-shaped pore in the centre (Aller & Unger, 2006; De Feo et al., 2009). The conserved TMD2 Met-X3-Met motif, which is essential for function, is located at the narrowest end (~8 Å) of the Ctr1 pore toward the extracellular face of the lipid bilayer (De Feo et al., 2009). The close proximity between the Met-X3-Met motifs from different Ctr1 monomers leads to the formation of disulfide bonds in cross-linking experiments when a methionine residue at the Met-X3-Met motif is substituted by a cysteine (De Feo et al., 2009). These results, and the in vivo functionality of the *S. cerevisiae* Ctr1 Met256-X3-Cys260 mutant (Puig et al., 2002), suggest that Cu⁺ simultaneously coordinates to the methionine thioether residues from different Ctr1 monomers during metal ion passage through the pore. The results of genetic studies with *S. cerevisiae* Ctr1 are also consistent with the cooperation between different monomers of the assembled complex during Cu transport. Whereas *S. cerevisiae* cells expressing a Ctr1 protein with a substitution of either the extracellular Met¹²⁷ residue or the Met²⁶⁰ in the Met²⁵⁶-X3-Met²⁶⁰ motif for alanine are unable to transport Cu, yeast cells co-expressing both mutants display significant Cu uptake capacity (Puig et al., 2002). The *Sch. pombe* Cu transporter, which is composed of two distinct Ctr1-like proteins (Ctr4 and Ctr5), is an excellent system to dissect the contribution of each monomer to the Cu transport complex. Although both proteins contain all known critical motifs described for the Ctr1 family of high-affinity Cu transporters, they are unable to function independently in Cu acquisition (Zhou & Thiele, 2001). Recent bimolecular fluorescence assays in *Sch. pombe* point to the assembly of two Ctr4 molecules in combination with one Ctr5 molecule (Ioannoni et al., 2010). According to this ratio, the mutagenesis of the Ctr4 Met-X3-Met motif would leave a single motif in the complex, whereas the substitution of the Ctr5 Met-X3-Met motif would leave two functional motifs. Given that the mutagenesis of the Ctr4 Met-X3-Met motif abolishes Cu transport, whereas Ctr5 Met-X3-Met mutagenesis does not, we propose that cooperation between at least two functional Met-X3-Met motifs is necessary for Cu transport across the Ctr4–Ctr5 complex at the plasma membrane.

To further dissect the contribution of the distinct Ctr4 and Ctr5 protein domains to the assembly of an active heteromeric complex on the cell surface, we constructed six different Ctr4/Ctr5 protein chimeras and assayed for Cu transport functionality. Only two chimeras, CTR445 and CTR545, were able to rescue the respiratory defect of a yeast strain defective in high-affinity Cu uptake without a Ctr4 or Ctr5 counterpart. These data suggest that both Ctr4 and Ctr5 NTDs are compatible with a functional chimera, and this result is consistent with the redundant and independent functions of the Ctr4 and Ctr5 amino-terminal Mets.

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**Fig. 5.** Subcellular localization of the Ctr4 and Ctr5 protein chimeras in *S. cerevisiae*. The *S. cerevisiae* *ctr1Δctr3Δ* deletion mutant was cotransformed with combinations of vector, CTR4, CTR4-GFP, CTR544-GFP, CTR445-GFP, CTR5-GFP, CTR6-GFP, CTR545-GFP, CTR455-GFP and CTR454-GFP, as indicated in the different panels. Representative images of four separate experiments are shown. Cells were grown and analysed as in Fig. 3.
motifs in efficient Cu transport (Beaudoin et al., 2006). Previous results have also revealed that the simultaneous mutagenesis of both extracellular methionine residues within Ctr4 and Ctr5 NTDs is necessary to abolish Cu transport function in Sch. pombe, whereas the substitution of one does not abrogate Cu transport (Beaudoin et al., 2006). Consistent with these results and with previous data on the lack of functionality of the yeast Ctr1-Met_{127} mutant (Puig et al., 2002), the single substitution of Met_{122} for alanine eliminates the Cu transport function of the Ctr4_{45} chimera, but not that of the Ctr4–Ctr5 complex. Taken together, these studies suggest that a single methionine-rich NTD is necessary and sufficient for Ctr4–Ctr5 Cu-mediated transport.

The functionality of the Ctr4_{45} and Ctr5_{45} chimeras highlights the fact that only the Ctr4 central domain and the Ctr5 CTD are appropriate for an independent functional chimera. The central domain comprises the TMD1, the cytosolic loop, and the TMD2 containing the Met-X_{3}-Met motif. The lack of cell surface localization for Ctr5, expressed without a Ctr4 counterpart, as compared with the peripheral localization of the Ctr5_{45} chimera, suggests that the amino acid composition of the Ctr5 central domain abrogates the delivery to the plasma membrane, probably as a result of defects in the assembly of the trimer complex. Regarding Ctr4–Ctr5 CTDs, Ctr4 is trapped during secretion when expressed without a Ctr5 counterpart, whereas the Ctr4_{45} chimera localizes to the plasma membrane and functions in Cu transport. These results suggest that the Ctr4 CTD, which is composed of TMD3 and the cytosolic amino-terminal tail, exerts a negative effect on the delivery of the protein to the cell surface that is antagonized by the Ctr5 CTD.

Yeast cells expressing the Ctr4_{45} chimeric protein show a significant growth defect, as compared with cells expressing the wild-type Ctr4–Ctr5 heteromeric complex, when the Cu availability of a medium containing non-fermentable carbon sources is decreased by the addition of the Cu chelator BCS (data not shown). This observation is consistent with a slight defect in Ctr4_{45} functionality as compared with the Ctr4–Ctr5 native complex, and suggests that the Ctr4/Ctr5 chimeric proteins do not retain all the biological properties of the wild-type Ctr4–Ctr5 complex. Although most of the structural and functional character-
Fig. 7. Subcellular localization of the Ctr4 and Ctr5 protein mutants and chimeras in Sch. pombe. The Sch. pombe ctr4Δctr5Δ deletion strain was cotransformed with vector, CTR4-GFP, CTR5, CTR5-GFP, CTR4-M223/227A-GFP, CTR445-GFP and CTR544-GFP, as indicated. Transformant cells were grown and analysed as described. Transformant cells were grown and analysed as previously described (Beaudoin et al., 2006). Representative images of four separate experiments are shown.

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