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Mfc1 is a novel copper transporter during meiosis

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Abbreviations: CAO, copper amine oxydase; Cherry, red fluorescent protein; Coppersensor-1, CS1; Ctr, copper transporter; Cu, copper; Cuf1, copper factor 1; Cuf2, copper factor 2; CuSE, copper-signaling element; FSM, forespore membrane; GFP, green fluorescent protein; Mfc1, major facilitator copper transporter 1; MFS, major facilitator superfamily; N, nitrogen; WT, wild-type

Meiosis is a specialized cell division process by which diploid germ line cells generate haploid gametes, which are required for sexual reproduction. During this process, several micronutrients are required, including copper ions. Despite important roles for copper-dependent proteins during meiosis, their mechanisms of action remain poorly understood. In a recently publication, we reported the discovery of Mfc1, the first example ever reported of a meiosis-specific copper transporter. Although Mfc1 did not exhibit any significant amino acid sequence similarities with members of the Ctr family of copper transporters, it harbored putative copper coordination motifs. Microarray data showed that *mfc1*$^{+}$ was the most highly induced of all meiotic genes detected under copper-limiting conditions. Analysis of Mfc1 localization during meiosis revealed that it localized at the forespore membrane during middle and late phases of the meiotic program. Interestingly, live-cell copper imaging using a copper-binding tracker revealed accumulation of copper ions into the forespore in wild-type cells. In contrast, mutant cells lacking Mfc1 displayed an intracellular distribution of copper ions that was dispersed throughout the ascospores without any marked preference for the forespore. We propose that Mfc1 is required to mobilize copper into the forespore, thereby providing copper to copper-requiring enzymes of the developing spores.

Redox active transition metals such as copper present a dilemma to cells. Although they are essential cofactors, they can also be cytotoxic.[1] On the one hand, copper serves as a catalytic and a structural cofactor of many enzymes but, on the other hand, due to its proclivity to change redox state, it can react with hydrogen peroxide to generate the highly toxic hydroxyl radical.[2] Consequently, it is critical that cells use homeostatic mechanisms to acquire and maintain sufficient amounts of copper while at the same time preventing its accumulation to protect the cells from its unwanted toxic effects.

The copper homeostatic machinery has traditionally been investigated in dividing cells that grow mitotically (vegetative growth). Although deficiencies in copper-dependent proteins culminate in meiotic cell developmental defects and subfertility,[3,4] copper homeostasis has been poorly studied during meiosis. Contributing reasons for that situation include: (1) paucity of mammalian germ cells, (2) unavailability of germ cell lines grown under copper depleted or copper sufficient conditions, (3) heterogeneity of mammalian germ cell populations and, (4) lack of information on the number and nature of molecules that serve as potential players, especially in response to changes in copper levels during meiosis.[5] To gain insight into the molecular basis of copper homeostasis during meiosis, we have combined the use of DNA microarrays and the fission yeast *Schizosaccharomyces pombe*. The fission yeast is one of the best-understood model systems to investigate the eukaryotic cell cycle, either the conventional mode of division (mitosis) or the meiotic cell division program (meiosis).[6] *S. pombe* is also of special interest because fission yeast cells possess similarities to mammalian cells in several respects. These include the mode of cell division (septation/medial cleavage) and the regulation of the cell-cycle (Cdc-like proteins). This latter property is especially relevant to meiosis, since it can be followed from its initiation through to generation of mature haploid cells via highly conserved meiotic proteins (Spo11, Sgo1 and Rec8).[7] In addition, *S. pombe* has become particularly attractive for the study of key molecular aspects of meiosis because conditional-growth and temperature-sensitive mutants have been developed that allow synchronization of the cells prior to entry into the meiotic program. This latter point is of paramount importance since animal models (vitamin A-deficient mice) and tissue co-cultured cells (Sertoli cells with germ cells) are not easy to synchronize prior to their entry into meiosis.[8,9]

In our recent publication, we have used the *S. pombe* model to study meiosis. We have discovered that copper was absolutely required for progression of meiosis because copper insufficient zygotes exhibited a meiotic arrest at metaphase I (**Fig. 1**).[10] During early meiosis, copper uptake is most likely ensured by the heteromeric Ctr4-Ctr5 complex as the Ctr4 protein localizes at the cell surface of developing asci and remains at the plasma membrane until the 3 h meiotic time point.[10] This preferred

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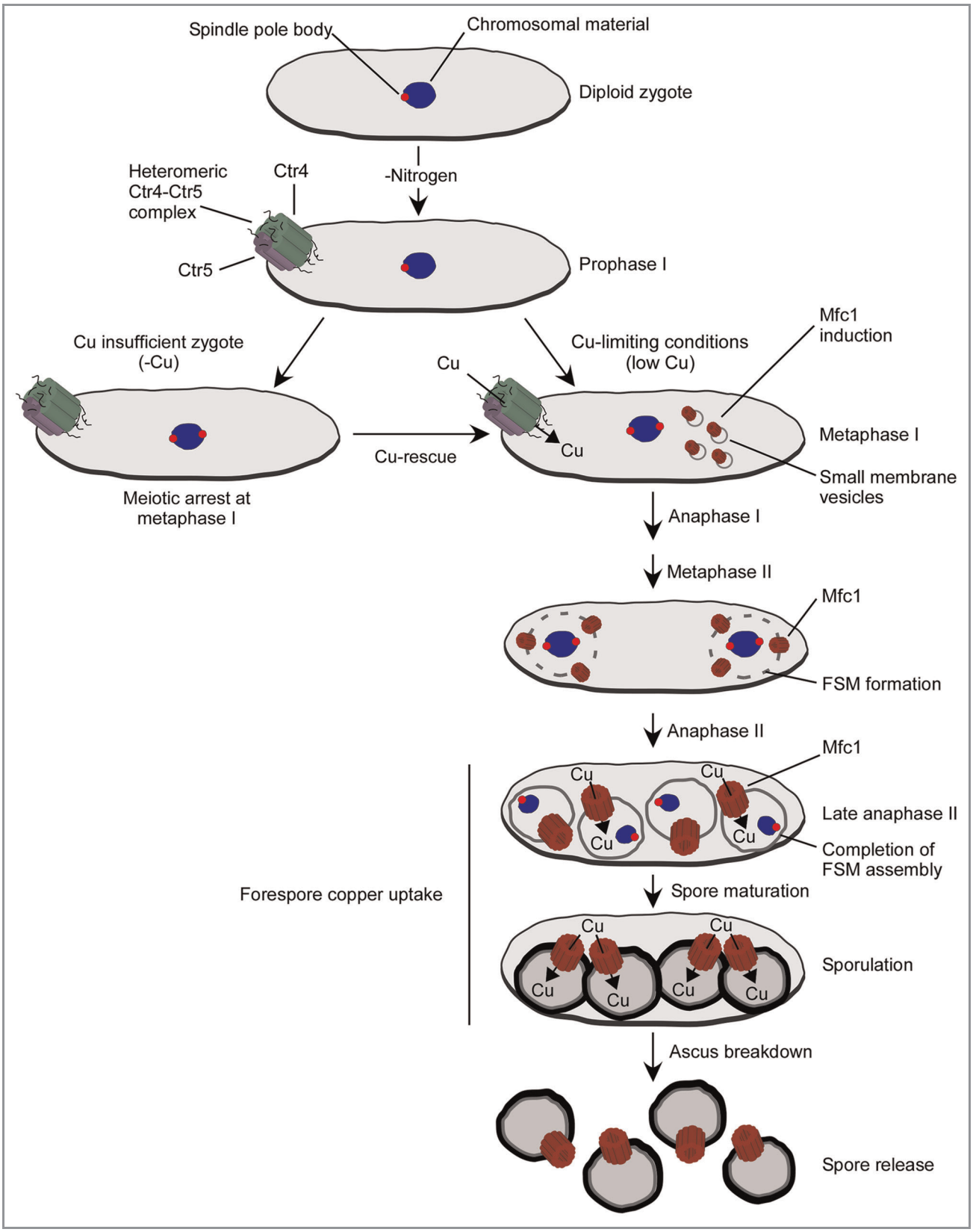

Figure 1. For figure legend, see page 120.

Figure 1 (See previous page). A model for copper transport during meiosis in *S. pombe*. A zygote under low nitrogen (N) and copper (Cu) conditions proceeds through meiosis. In contrast, a copper insufficient zygote displays a meiotic arrest at metaphase I. At early stages of meiosis (prophase and metaphase I), copper uptake is mediated by the heteromeric Ctr4-Ctr5 complex (green and violet cylinders). During metaphase I, while the Ctr4-Ctr5 heterocomplex disappears from the cell surface, Mfc1 (brown cylinders) is first detected in small membranous vesicle structures within the cytoplasm of zygotic cells. These membranous vesicles appear to fuse to subsequently form the forespore membrane (FSM). After FSM closure, Mfc1 resides at the FSM where it persists until matured spores are released from the ascus. Blue, chromosomal material; red, spindle pole body; gray, FSM.

location of the Ctr4-Ctr5 copper-transport system coincides primarily with the start of meiosis and the premeiotic S-phase and recombination. However, when middle meiosis is initiated, Ctr4 expression is abolished. To follow up on this observation, microarrays were hybridized with probes derived from RNA isolated from copper-starved vs. copper-replete meiotic cells. At middle meiosis, analysis of gene expression profiling data identified several uncharacterized genes, including the *SPAPB1A11.01* gene, which was highly transcriptionally induced in copper-starved cells. We named this novel *SPAPB1A11.01* gene *mfc1*$^+$. Further investigations revealed that *mfc1*$^+$ exhibited a distinct temporal expression profile when compared with that of *ctr4*$^+$, since it was expressed after 3 h of meiotic induction. The levels of *mfc1*$^+$ mRNA reached a maximum within 5 h, coinciding with meiotic divisions. Thereafter, the expression profile of *mfc1*$^+$ was relatively sustained with only a slight decrease over time being observed.[10]

Under conditions of copper starvation, the meiotic transcriptional program revealed that *ctr4*$^+$ and *mfc1*$^+$ transcript levels were induced at distinct times during meiosis. Whereas the deletion of the *cuf1*$^+$-encoded copper-dependent transcription factor (*cuf1*Δ mutant) impaired the induction of *ctr4*$^+$, transcriptional activation of *mfc1*$^+$ was unaffected. This observation suggested the existence of a distinct transcriptional regulator of induction of *mfc1*$^+$ in response to copper starvation.[10] This result was also consistent with the fact that only a single, inverted weak putative copper-signaling element (CuSE) was identified in the promoter region of *mfc1*$^+$.[10,11] Based on these observations, it can be suggested that the DNA sequence requirement for copper starvation-dependent induction of *mfc1*$^+$ is different than that of the *ctr*$^+$ genes, including *ctr4*$^+$, *ctr5*$^+$ and *ctr6*$^+$. Global transcriptome and deletome profiles of seven meiosis-specific transcription factors that are known to be essential for successful meiotic differentiation and sporulation have consistently shown that the *mfc1*$^+$ gene was not regulated by Rep1, Mei4, Cuf2, Atf21, Atf31, Rsv1 and Rsv2 (unpublished data).[12] Again, these results represent compelling arguments in favor of the interpretation that an uncharacterized meiotic regulator is responsible for copper-dependent regulation of the *mfc1*$^+$ gene.

During middle meiosis, a Cherry epitope-tagged Mfc1 protein was first detected on membranes of small intracellular vesicles that are thought to originate from the endoplasmic reticulum and Golgi apparatus network.[10,13] These intracellular vesicles that are known to fuse for the assembly of the forespore membrane (FSM) carry integral transmembrane proteins needed for the FSM maturation and function. One of the vesicle-trafficking pathways involves many proteins, including Spo14 and Spo20. Based on sequence homology data, *spo14*$^+$ encodes an ortholog of *S. cerevisiae* Sec12, which is a GDP/GTP exchange factor for the GTP-binding protein Sar1 that is required for vesicle trafficking from the ER to the Golgi.[14] The Spo20 protein is highly similar to *S. cerevisiae* Sec14, which is a phosphatidyl choline/phosphatidyl inositol-transfer protein.[15] In budding yeast, Sec14 is essential for vesicle budding from the Golgi apparatus.[16] According to a proposed model, after fusion of membrane vesicles to form FSM, the Mfc1-Cherry fluorescent protein was observed in the surroundings of FSM. Indeed, Mfc1-Cherry-associated fluorescence produced four fluorescent circle-like structures that corresponded to precursor spores.[10] Mfc1 is expressed exclusively during meiosis, whereas other FSM resident proteins such as Psy1 are expressed during the two types of cell cycle programs.[13] When Psy1 is expressed in mitotically growing cells, it localizes to the plasma membrane.[13] However, when cells undergo meiosis, Psy1 disappears from the plasma membrane after the first meiotic division and relocalizes to the nascent FSM as the second meiotic division starts. The relocation of Psy1 from the plasma membrane to FSM may use a distinct intracellular route as opposed to Mfc1. Further analysis of genes (*eg. spo14*$^+$, *spo20*$^+$) that encode putative components of vesicle-trafficking pathways will be required to decipher the relocation mechanism of Psy1 and Mfc1 during the "switching-on" of de novo membrane formation at the FSM.

Over the past years, studies have suggested the presence of independent activities for copper transport within cells.[17] Frequently, cells possess both primary and secondary active transporters for the same substrate. Whereas primary transporters bind ATP and require ATP hydrolysis for transport activity, secondary transporters are driven by proton-motive or gradient forces.[18] Thus far, copper is known to be transported across membranes by at least two types of transmembrane proteins. These are the P-type ATPases and the Ctr (channel-like) proteins.[1] Based on computer analysis, Mfc1 is predicted to be related to the major facilitator superfamily (MFS) of transporters, which is the largest group of secondary membrane transporters.[19] Mfc1 contains 19 Met residues, four pairs of which are present in the potential copper coordination arrangements Met-X-Met or Met-X_2-Met.[20] Furthermore, Mfc1 has 7 Cys residues scattered throughout the protein that are potential copper-binding ligands. These observations suggest that Mfc1 may bind copper through these amino acid residues, and this may also be an important feature related to its activity. In the context of a global analysis of protein localization in *S. pombe*, a GFP epitope-tagged *mfc1*$^+$ allele has been artificially expressed in cells proliferating in mitosis.[21] In these cells, the *mfc1*$^+$*-gfp* gene product localized at the plasma membrane. To evaluate the potential contribution of Mfc1 in copper acquisition, *ctr4*Δ *ctr5*Δ cells expressing the *mfc1*$^+$*-gfp* gene under control of a heterologous mitotic promoter were assayed for respiratory competence and results compared with a wild-type strain harboring the *ctr4*$^+$ and *ctr5*$^+$ genes. *ctr4*Δ *ctr5*Δ cells failed to grow on a nonfermentable carbon source due

to their inability to provide copper to the copper-requiring cytochrome *c* oxidase enzyme. When Mfc1-GFP was expressed at the plasma membrane in mitotic cells grown in a medium containing 2 μM $CuSO_4$, copper was delivered to cytochrome *c* oxidase, allowing cells to grow on respiratory carbon sources.[10] Furthermore, ^{64}Cu uptake assays in *ctr4*Δ *ctr5*Δ cells expressing the *mfc1*$^{+}$*-gfp* allele revealed that these cells had the property to transport ^{64}Cu with high affinity in a manner that was consistent with their capacity to utilize nonfermentable carbon sources for growth.[10]

In meiosis, the subcellular localization of Mfc1 at FSM suggested that Mfc1 was an intracellular transporter that mediated copper uptake into the forespore. This finding suggested the presence of a pathway by which copper is transported into this compartment where copper is required for copper-dependent enzyme activities. This proposed function of Mfc1 is supported by the fact that deletion of the *mfc1*$^{+}$ gene (*mfc1*Δ/Δ) resulted in a strong reduction in copper amine oxidase 1 activity, a cuproenzyme found in the forespore.[10] Furthermore, as shown by experiments using Coppersensor-1 (CS1),[22] analysis of live-cell labile copper in a *mfc1*Δ/*mfc1*Δ mutant revealed that labile copper pools were mainly detected outside the forespores.[10] In contrast, wild-type cells (*mfc1*$^{+}$/*mfc1*$^{+}$) exhibited an intense punctuate fluorescence corresponding to the presence of labile copper pools within the forespores. The fluorescent detection of labile copper pools in forespores in *mfc1*$^{+}$/*mfc1*$^{+}$ cells adds further weight to the argument favoring a role for Mfc1 in mediating copper uptake into forespores. As all of the proteins identified in copper transport in yeast have higher eukaryote homologs, it is not surprising that we have recently found a putative mouse ortholog of Mfc1 (denoted as mMfc1). Using typical copper acquisition assays, our preliminary results have revealed that the mMfc1 protein functionally complements yeast cells defective in copper transport (unpublished data). Consequently, our discovery of the involvement of Mfc1 in copper transport in *S. pombe* opens the way for further investigations of the role of Mfc1 and its putative homologs in physiology and copper homeostasis in gamete biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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