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Review

Direct sensing of nutrient availability by fungi

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ABSTRACT

Nutrients can be monitored directly when they bind to a sensing protein to initiate a conformational change. G protein-coupled receptors are a good example of this type of sensing and ligand binding has been proposed as the initiating sensing event that regulates other types of nutrient sensors. This review will analyse the role of two distinct types of fungal regulators that may fall in this category. Ammonium transporters are essential for various developmental processes and may respond to ammonium binding by interacting with signal transduction pathways to initiate signalling. In the nucleus, copper responsive genes are regulated by transcription factors that are proposed to undergo copper-induced intramolecular interactions that inhibit their activity. Although in each case the model of ligand initiated allosteric regulation is attractive, we do not yet fully understand the molecular basis of sensing by these molecules.

1. Introduction

Nutrient sensing allows an organism to efficiently use available nutrients to maximise growth and development and, if necessary, initiate survival strategies. Sensing nutrient availability enables appropriate transport systems and metabolic pathways required for nutrient utilisation to be expressed, and those of less available or less favoured alternatives to be repressed. The starvation-induced activation of amino acid biosynthetic genes in yeast by the transcriptional activator Gcn4 is a response to a reduction in nutrient levels (reviewed in Hinnebusch, 2005). Also important are mechanisms that monitor the need for a particular nutrient as when yeast switch from fermentative to respiratory growth requiring the increased assimilation of iron as an essential cofactor of respiratory enzymes (Haurie *et al.*, 2003). The feast and famine existence often encountered by fungi require that sensing the need and availability of nutrients is rapid and coordinated. Indirect nutrient sensing occurs when the abundance of a metabolite downstream of the nutrient itself is monitored as part of a regulatory mechanism. For example, the regulatory role of intermediates of the glycolytic pathway, such as fructose 1,6-bisphosphate, indirectly reflects the amount of metabolised glucose (Barwell *et al.*, 1971). Alternatively, direct sensing occurs when nutrient binding receptors monitor the availability of a particular nutrient.

2. Sensing at the plasma membrane: ammonium responsive transceptors

Fungi utilise a variety of nitrogen compounds. The uptake and catabolic systems required to assimilate these nutrients are controlled through the regulation of gene expression and transporter localisation. Distinct from nitrogen acquisition, ammonium, as a signal of nitrogen availability, controls a range of processes such as mating, filamentous growth and the production of secondary metabolites (Mihlan *et al.*,

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2003: Smith *et al.*, 2003; Rutherford *et al.*, 2008a). The dimorphic switch to filamentous growth that some yeast undergo in response to ammonium limitation has been well studied (Gimeno *et al.*, 1992). This process allows cells to grow away from an area where ammonium is scarce and, in a sense, forage for nutrients (Fig. 1). This developmental process occurs when cells are grown on low levels of ammonium or when utilising a non-preferred nitrogen source such as an amino acid. Under the latter conditions, ammonium remains the sensed nutrient as catabolism of a non-preferred nitrogen source results in ammonium export into the environment (Boeckstaens *et al.*, 2007). Ammonium signalling also rapidly activates the protein kinase A (PKA) pathway when ammonium is added to nitrogen starved *Saccharomyces cerevisiae* cells (Van Nuland *et al.*, 2006). The extent to which these two signalling pathways overlap is not known, however, both pathways require the activity of an ammonium transporter that has been proposed to act as an ammonium sensor.

The model yeasts *S. cerevisiae* and *Candida albicans* contain, respectively, three and two ammonium importers (Marini *et al.*, 1997; Biswas and Morschhäuser, 2005). The Mep proteins (originally identified as methylamine permeases) are members of the AmtB family of transporters that are conserved from bacteria to man. In both yeasts the high affinity Mep2 transporter is required for ammonium responsive dimorphic growth and, in *S. cerevisiae*, rapid activation of the PKA pathway (Lorenz and Heitman, 1998; Biswas and Morschhäuser, 2005; Van Nuland *et al.*, 2006). Evidence suggests that these processes are not induced by internal changes in nitrogen metabolism as the enzymes involved in ammonium assimilation have wild-type activity in a mutant lacking Mep2 (Lorenz and Heitman, 1998). Also, the rapid activation of the PKA pathway is induced by uptake of the ammonium analogue methylamine that is not metabolised (Van Nuland *et al.*, 2006). Contrary to these findings, however, a mutant lacking Gdh3 (glutamate dehydrogenase) is constitutively pseudohyphal (Wilkinson *et al.*, 1996). Whether the latter reflects mechanistic differences in signalling between pseudohyphal growth and the rapid activation of the PKA pathway remains to be seen. Ammonium sensing that is independent of ammonium metabolism has led to two distinct hypotheses of Mep2 function (Fig. 2A). In the transceptor model, the conformational changes that occur in Mep2 during ammonium transport are channelled to a relevant signalling pathway. In this way Mep2 acts as both a transporter and a sensing receptor, hence the term transceptor. Alternatively, signalling could occur in response to changes in internal pH that result from the import of ammonium (Fig. 2B).

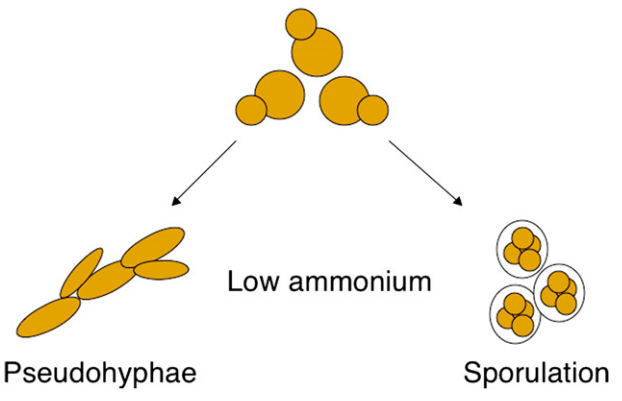

Fig. 1 – Pseudohyphal growth in yeast. The budding yeast *Saccharomyces cerevisiae* responds to nitrogen limitation by undergoing two distinct developmental processes. When glucose is available cells grow as elongated yeast and daughter cells remain attached to mother cells resulting in chains of pseudohyphal cells.

Mep2 is expressed when ammonium levels are low and localises throughout the plasma membrane (Marini *et al.*, 1997; Rutherford *et al.*, 2008b). As with many nutrient transporters, Mep2 responds to high extracellular levels of its substrate by being internalised and targeted to the vacuole (Zurita-Martinez *et al.*, 2007). In both *S. cerevisiae* and *C. albicans*, Mep2 is the most highly expressed ammonium transporter and its levels are critical for its sensing role. Reduced expression of the *CaMEP2* gene negatively affects signalling and the usually non-sensing *Ca*Mep1 can induce low levels of filamentation when expressed at higher than wild-type levels (Biswas and Morschhäuser, 2005). Likewise, increased pseudohyphal growth occurs in *S. cerevisiae* with higher Mep2 expression (Rutherford *et al.*, 2008b). In other yeasts that undergo a developmental change in response to ammonium, it is the most highly expressed member of the AmtB family that is the ammonium sensor (Smith *et al.*, 2003; Rutherford *et al.*, 2008a). Why Mep2 levels are critical for signalling is not clear. Perhaps only a fraction of expressed Mep2 is signalling-active at, for example, the growing tip so that a high level of expression ensures sufficient signalling capacity. Alternatively, if internal pH changes regulate signalling the levels of imported substrate may be important. Whatever the mechanism, an environmental condition or genetic mutation that negatively impacts on Mep2 levels or localisation will influence the cell's ability to sense ammonium.

The study of Mep2 has been aided by structural studies of bacterial AmtB proteins that have identified conserved characteristics that relate to ammonium transport (Khademi *et al.*, 2004; Zheng *et al.*, 2004; Andrade *et al.*, 2005). AmtB is a homotrimer with each monomer containing a pore through which ammonium is imported. The ammonium ion binds at an extracellular site that discriminates between ammonium and other charged ions such as sodium or potassium (Javelle *et al.*, 2008). Two phenylalanine residues form a gate that block the entrance of the pore, and mutation of the more interior of these in *Escherichia coli* AmtB results in a loss of transport suggesting that this residue has a role in substrate translocation as well as a presumed gating role (Javelle *et al.*, 2008). Within the hydrophobic pore most AmtB proteins contain two histidine residues (His_1–His_2) that are hydrogen bonded *via* their δ nitrogen atoms. In some fungal homologues, however, these histidine residues are replaced with a glutamate–histidine pair. Correctly expressed and localised *Ec*AmtB mutants with substitutions of the conserved histidine residues are transport defective establishing their importance in ammonium translocation (Javelle *et al.*, 2006).

Critical to the potential role of Mep2 as a transceptor is the extent to which it undergoes a conformational change during ammonium transport. The first structural study of AmtB

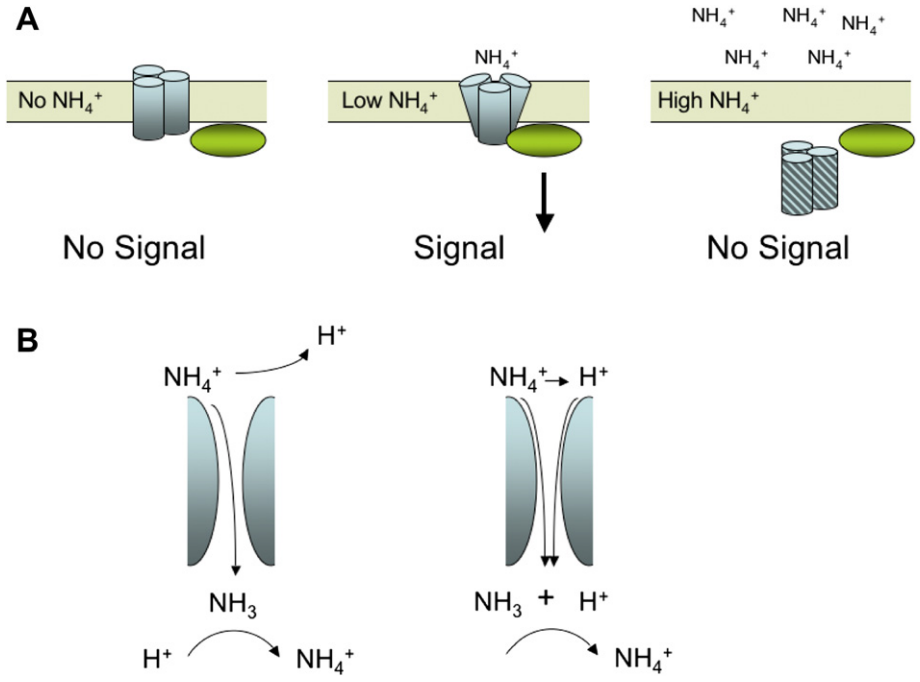

Fig. 2 — Two models of Mep2 as a sensor. (A). The transceptor model of Mep2 function. In response to low nitrogen levels Mep2 is localised to the plasma membrane. Transport of ammonium causes conformational changes in Mep2 that results in an intermolecular interaction with a signalling pathway. When ammonium levels are high Mep2 is internalised, transcription of the *MEP2* gene ceases and signalling stops. (B). Mep2 may influence cellular pH. Deprotonation of ammonium could result in ammonia gas alone being transported into the cell, which is then reprotonated and trapped in the cytosol. Alternatively, both ammonia gas and protons could be imported. Sensing may therefore reflect changes in intracellular pH that occur following ammonium uptake.

concluded that it is a channel rather than a transporter as no major structural difference was found in crystals prepared in the presence or absence of ammonium (Khademi *et al.*, 2004). The phenylalanine gate, however, suggests that transient structural changes are required to allow substrate passage, which has been supported by molecular dynamics transport simulations and estimations of substrate transport rates (Lin *et al.*, 2006; Nygaard *et al.*, 2006; Zheng *et al.*, 2004). Consistent with a fluid protein is a hydrophobic cavity in AmtB that is often found in active transporters to allow transmembrane movement during transport (Andrade *et al.*, 2005). Mutational analysis also supports conformational changes during ammonium translocation. The cytoplasmic carboxyl terminal domain of AmtB interacts with the cytoplasmic loop following the fifth transmembrane helix in its own and adjacent monomer. Deletion of this domain reduces transport efficiency whereas certain amino acid substitutions abolish transport (Inwood *et al.*, 2009). The loss of function in these latter mutants may be due to the carboxyl terminal domain adopting a conformation that prevents movement of the fifth transmembrane helix during transport (Inwood *et al.*, 2009).

The precise molecular species that is transported by the AmtB proteins is a matter of contention and has implications for the role of Mep2 as a sensor. The AmtB family could transport the ammonium ion (NH_3^+), ammonia gas (NH_3) or both ammonia and protons (NH_3/H^+) (Fig. 2B). Differences in the net import of protons would impact on internal pH, which may regulate signalling (Boeckstaens *et al.*, 2008). Molecular dynamics simulations are consistent with the hydrophobic pore creating an energetic barrier to the transport of the ammonium ion (Lin *et al.*, 2006; Nygaard *et al.*, 2006). This is supported by the ammonium dependent rise in pH in vesicles containing bacterial AmtB, which is predicted if ammonia gas acquires a proton and is trapped in the cytosol (Khademi *et al.*, 2004). This model requires that a proton is lost from the ammonium ion and retained on the periplasmic side of the transporter. Different sites within the pore have been proposed as being the site of deprotonation (reviewed in Lamoureux *et al.*, 2010). The transport of ammonia gas, however, contradicts patch clamp experiments using oocyte expressed plant homologues in which detected currents are consistent with either the transport of the NH_4^+ ion or the co-transport of NH_3/H^+ (Mayer *et al.*, 2006). Net transport of the ammonium ion is also supported by the proton ionophore sensitivity of AmtB function (Fong *et al.*, 2007). Co-transport of NH_3/H^+ could occur with proton movement *via* an undefined route, which may include the conserved histidine residues (Lamoureux *et al.*, 2010). It is not clear if the uncertainty relating to the substrate transported by the AmtB proteins reflects differences in experimental approaches or the mechanism of transport by different homologues. This last point is particularly relevant to the fungal ammonium transporters. *Sc*Mep2 contains the conserved His_1—His_2 motif whereas the signalling-inactive *Sc*Mep1 and *Sc*Mep3 do not. Mutation of

the His_1 residue to glutamate results in ScMep2 acquiring ScMep1-like characteristics, exhibiting increased ammonium uptake and a loss of signalling capacity (Boeckstaens *et al.*, 2008). It is important to note that this $ScMep2^{H194E}$ mutant was less highly expressed than wild-type Mep2, which in itself negatively affects signalling. However, ScMep2 and ScMep1 function optimally at different pH, and ScMep2 alone is inactivated by the addition of an ionophore, suggesting each transports *via* a different mechanism (Boeckstaens *et al.*, 2008). Clearly, the mechanisms of transport by ScMep1 and ScMep2 need to be determined to fully understand the role of Mep2 as a sensor.

Mutational studies have examined the relationship between the transport and sensing roles of Mep2 and established that ammonium transport is required for signalling to occur. Mutation of a conserved aspartate residue in the second extracellular loop of Mep2 or the His_1 residue generated a protein that lacked transport and signalling activity (Marini *et al.*, 2006; Rutherford *et al.*, 2008b). Both mutant proteins were correctly expressed and localised. The correlation between transport and signalling is supported by analysis of an extensive set of site directed ScMep2 mutants (Van Nuland *et al.*, 2006). Although the localisation and levels of these mutants was not analysed, a number were identified that were both transport and signalling defective. This study also isolated mutants that uncouple transport and signalling and that support the distinction between the rapid activation of PKA and pseudohyphal signalling pathways. Mep2 mutants were identified that are transport proficient (and therefore assumed to be correctly localised and expressed), activate PKA but fail to initiate pseudohyphal growth (Van Nuland *et al.*, 2006). Strikingly, a mutation was also identified that blocks methylamine uptake but allows rapid activation of the PKA pathway. This suggests that methylamine binding alone or partial translocation is sufficient to generate the required conformational change that initiates signalling. Although found to be important for ammonium transport in the *E. coli* homologue, the ScMep2 His_2 residue is dispensable for transport but required for signalling (Rutherford *et al.*, 2008b). The need for substrate binding to the His_2 residue to initiate sensing is supported by the identification of a mutation in an adjacent residue that generates a hyperactive sensor (Boeckstaens *et al.*, 2007). Additional support for a link between transporter movement and signalling comes from analysis of mutations within the cytoplasmic carboxyl terminal domain. In both *Ca*Mep2 and ScMep2 mutations have been identified within this domain that allow transport but block signalling (Van Nuland *et al.*, 2006; Biswas and Morschhäuser, 2005). Based on the analysis of mutations in the carboxyl terminal domain of a bacterial homologue described above, the phenotypes of these mutants may reflect changes in the interactions between the carboxyl terminal tail and other cytoplasmic domains that influence the conformational changes that occur during transport.

The range of Mep2 homologues with a sensing role suggests that this type of ammonium signalling may be widespread in fungi. In some cases, homologues have been shown to restore signalling in a *S. cerevisiae* strain lacking Mep2 so that the mechanism of action of these proteins may also be conserved (Smith *et al.*, 2003; Teichert *et al.*, 2008). The weight of evidence suggests that the AmtB transporters undergo a conformational change during transport. The challenge is to determine if this movement is integral to the species that is transported, perhaps by orientating residues that allow deprotonation of the ammonium ion. Alternatively, do Mep2 conformational changes support a transceptor function? Although epistasis experiments have identified Mep2 as being upstream of regulators essential for filamentous growth, to date, no Mep2 interacting signalling partner has been identified.

3. Sensing in the nucleus: copper repressed transcription factors

Copper is a cofactor for a number of key enzymes that are essential for eukaryotic life. Across eukaryotes this redox active metal forms the active site of the respiratory protein cytochrome *c* oxidase, superoxide dismutase (Sod1) that detoxifies the superoxide radical, and a family of multicopper oxidases that mediate the transport of iron across membranes. In fungi, additional copper enzymes include laccases that have a role in melanin biosynthesis and lignin degradation and amine oxidases that catalyze oxidative deamination of biogenic amines to produce ammonium. Copper uptake and distribution needs to be carefully regulated as it has a high affinity for metal binding sites within proteins and can displace other metals. Its ability to produce oxygen radicals is also potentially damaging to a cell.

Copper is transported into fungal cells in the reduced cuprous form (Cu^{1+}). In *S. cerevisiae* this requires the reduction of cupric copper (Cu^{2+}) by a cell surface reductase (Fre1) that provides the required substrate for high affinity copper transporters (Ctr1, Ctr3). The Mac1 transcription factor activates the *FRE1*, *CTR1* and *CTR3* genes when copper is limiting. A few additional Mac1 targets have also been identified by microarray studies (Gross *et al.*, 2000). Mac1 is a 46 kDa protein with an amino terminal DNA binding domain (DBD) and a carboxyl terminal activation domain. Induction of the copper acquisition genes in *S. cerevisiae* is mediated by a *cis* acting copper responsive promoter element (CuRE) that contains a core GCTC motif (Yamaguchi-Iwai *et al.*, 1997; Labbé *et al.*, 1997). Two CuREs are present in each copper responsive promoter and both are essential for Mac1 function (Yamaguchi-Iwai *et al.*, 1997; Labbé *et al.*, 1997). *In vitro* electrophoretic mobility shift assays (EMSA), using full length Mac1 or its minimal DBD, establish Mac1 binding to the CuRE. The first 41 amino acids of Mac1 contains a zinc-binding domain and mutation of three predicted zinc coordinating residues results in the loss of Mac1 DNA binding activity (Jensen *et al.*, 1998; Joshi *et al.*, 1999). *In vitro* DNA footprinting experiments are consistent with the zinc domain binding to the major groove of the CuRE core GCTC sequence and an adjacent RGRP domain (residues 36–39) interacts with upstream CuRE sequences (Jamison McDaniels *et al.*, 1999). The tandem arrangement of CuREs suggests that Mac1 acts as a dimer which is supported by Mac1 two-hybrid interactions and the detection of relevant high molecular weight EMSA complexes (Joshi *et al.*, 1999). The observed dimerisation phenotypes are

dependent on a region within the carboxyl terminal domain that is predicted to form an aliphatic helix, loss of which generates an inactive protein (Serpe *et al.*, 1999). *In vivo* footprinting studies have identified Mac1 and copper dependent changes in the occupancy of the CuREs in the *CTR3* promoter (Labbé *et al.*, 1997). This is consistent with copper regulating Mac1 DNA binding activity and is supported by *in vivo* chromatin immunoprecipitation (CHiP) studies that show a reduction in epitope-tagged Mac1 promoter occupancy when cells are switched from low to high copper media (Keller *et al.*, 2005). The loss of Mac1 binding is not due to changes in Mac1 cellular localisation or reduced levels of Mac1. Mac1-β-galactosidase and GFP fusions are localised to the nucleus, the latter in both copper replete and starved cells (Jungmann *et al.*, 1993; Jensen and Winge, 1998). Epitope-tagged Mac1 levels slightly increase on the addition of copper to cells indicating Mac1 stabilisation (Keller *et al.*, 2005). Copper sensing is likely to occur in the nucleus, as Mac1 is functional, and presumably nuclear-localised, following the inhibition of protein synthesis by cycloheximide treatment (Keller *et al.*, 2005).

Copper also regulates the transactivation function of Mac1. The carboxyl terminal domain of Mac1 contains two cysteine-rich regions that contain a terminal histidine residue. These two regions, designated C1 and C2, are potential copper binding domains *in vivo*. A $His^{279}Gln$ substitution in C1 generates a constitutively active factor that does not respond to copper (Jungmann *et al.*, 1993). Genetic screens for Mac1 mutants identified residues Cys^{264}, Cys^{266}, Cys^{271} and His^{279} in the C1 domain as being critical for sensing (Keller *et al.*, 2000). Site directed cysteine to serine substitutions in C1 also impact on copper sensing although the extent of this is dependent on the position of the mutated residues and the method used to analyse Mac1 function (Graden and Winge, 1997; Keller *et al.*, 2000). When expressed from a low copy plasmid, mutants containing serine substitutions of the Cys^{266} or Cys^{271} residues exhibit wild-type function whereas a $Cys^{264}Ser$ substitution produces a constitutively active factor. The $Cys^{266}Ser$ mutant, however, does not sense copper when expressed from a high copy plasmid. Substitutions that are predicted to induce larger structural changes in C1 ($Cys^{266}Gly$, $Cys^{271}Trp$) result in loss of copper sensing when these mutants are expressed at physiological levels (Keller *et al.*, 2000). Surprisingly, the C2 region has no role in copper sensing. When analysed alone as a Gal4 fusion, the C2 domain exhibits high copper responsive transactivation activity which is lost in the context of the whole protein (Voutsina *et al.*, 2001). A Gal4/Mac1 fusion containing double cysteine to serine substitutions in C2 responds normally to copper and a correctly expressed mutant lacking the potential copper binding residues of C2 lacks transcriptional activity (Graden and Winge, 1997; Zhu *et al.*, 1998). As well as having a role in transactivation, the C2 domain negatively impacts on the DNA binding activity of Mac1 in EMSA experiments (Voutsina *et al.*, 2001). Why some, but not all, Mac1 homologues contain the C2 domain is not clear (Fig. 3).

Several lines of evidence support the model that copper induces an intramolecular interaction within Mac1 that represses its function (Fig. 4). The copper responsiveness of a Gal4/Mac1 fusion is greater in the absence of the first 41 residues containing the Mac1 DBD (Serpe *et al.*, 1999). Similarly, the dimerisation of Mac1, as measured by two-hybrid analysis, is repressed by the presence of the Mac1 DBD (Serpe *et al.*, 1999). Importantly, a copper dependent two-hybrid interaction between the Mac1 amino and carboxyl terminal domains has been identified (Jensen and Winge, 1998). Such an interaction provides a mechanism through which the C2 domain represses Mac1 DNA binding activity (Voutsina *et al.*, 2001). Two studies suggest that direct copper binding to Mac1 is the initiating sensing event that could trigger a conformational change in Mac1. A Mac1–GST fusion and a GST fusion with the C1 and C2 domains purified from *E. coli* contain eight molar equivalents of copper (Jensen and Winge, 1998). The copper form of the C1–C2-GST fragment is luminescent with maximal emission at 570 nm consistent with this Mac1 fragment binding Cu^{1+} in a polycopper cluster (Jensen and Winge, 1998). In the second study, X-ray absorption spectroscopy on the copper bound C1 domain is consistent with copper binding as a tetrahedral tetracopper complex in which each copper atom is ligated to three coordinating atoms involving all the potential C1 copper binding residues (Brown *et al.*, 2002). If this occurs *in vivo* then, presumably, the C1 mutants that are active have sufficient copper binding capacity through the substituted serine residues. Alternatively, a smaller copper cluster may be sufficient to mediate Mac1 repression.

The model of Mac1 copper-induced intramolecular interaction has been supported by studies in the fission yeast *Schizosaccharomyces pombe*. Similar to Mac1, Cuf1 activates genes involved in copper acquisition under copper limiting conditions. It also represses the expression of iron uptake genes presumably as part of this process is dependent on the multicopper-containing iron oxidase Fio1 (Labbé *et al.*, 1999). Cuf1 and Mac1 are similar in size and contain some of the same features. The amino terminal zinc-binding domain and (R/K)GRP motif are conserved although the Cuf1 DNA binding domain is more homologous with a family of high copper responsive factors (Ace1/Amt1). Consequently, Cuf1

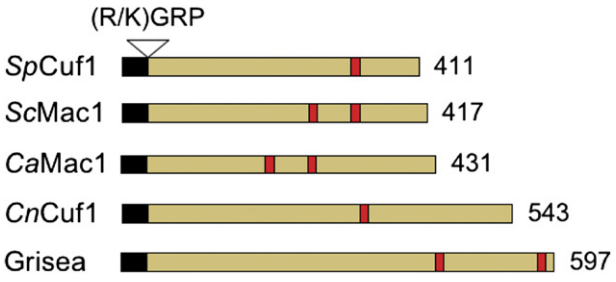

Fig. 3 – Schematic representation of a representative sample of Mac1-like fungal transcription factors. Shown are Cuf1 from *Schizosaccharomyces pombe* (*Sp*) and *Cryptococcus neoformans* (*Cn*), Mac1 from *Saccharomyces cerevisiae* (*Sc*) and *Candida albicans* (*Ca*) and Grisea from *Podospora anserina*. Conserved are the DNA binding domain (black rectangle), including the (R/K)GRP motif and potential copper binding motifs (red rectangle). In *Sc*Mac1 both copper domains contain the conserved residues Cys-X-Cys-X_4-Cys-X-Cys-X_2-Cys-X_2-His.

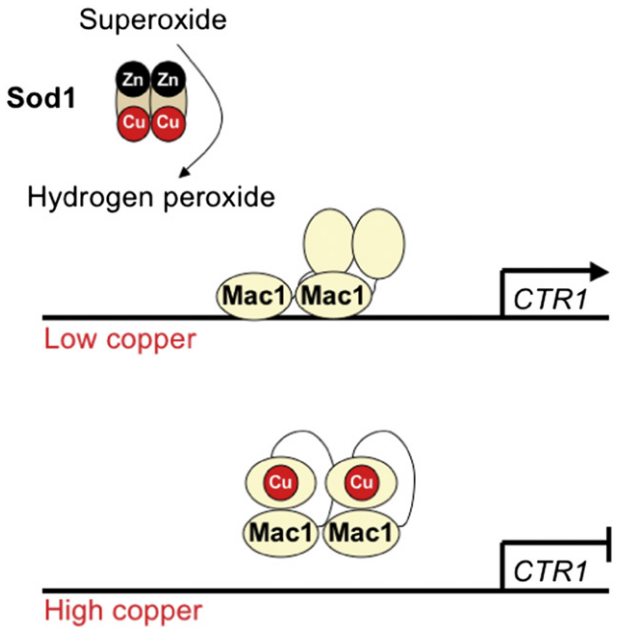

Fig. 4 – Model of Mac1 response to copper. Mac1 binds as a dimer to copper responsive genes to active transcription under low copper conditions. Catalytic active superoxide dismutase is required for the DNA binding activity of Mac1. In response to high copper an intramolecular interaction between the amino and carboxyl terminal domains of Mac1 repress both the DNA binding and transactivation functions of the factor. *In vitro* studies support the direct binding of copper to the C1 domain of Mac1 as the initiating sensing event.

binds to a copper-sensing element that is different from the Mac1 CuRE (Beaudoin and Labbé, 2001). Also conserved in the Cuf1 carboxyl terminal domain, and essential for copper sensing, is one repeat of the Mac1 C1 motif. Mutation of the potential copper binding residues in the Cuf1 C1 domain results in a factor that does not respond to copper and constitutively activates the Cuf1 regulated genes involved in copper acquisition. Mutation of an alternative cluster of four cysteine residues in the middle of Cuf1 has no effect on copper sensing supporting the allocation of C1 as the copper-sensing motif (Beaudoin *et al.*, 2003).

Unlike Mac1, the localisation of Cuf1 changes in response to copper, being cytosolic under copper replete conditions and nuclear in low copper medium. These changes in Cuf1 cellular localisation have allowed further analysis of the model of copper-mediated intramolecular interactions as being critical for copper sensing. The Cuf1 nuclear localisation signal (NLS) consists of a series of basic residues in the Cuf1 DBD and is essential for the nuclear localisation of the protein in response to low copper (Beaudoin and Labbé, 2006). Also essential for Cuf1 retention in the cytosol are the potential copper binding residues of the C1 domain. A Cuf1–GFP mutant with alanine substitutions of these residues is localised to the nucleus under both copper replete and limiting conditions resulting in the constitutive activation of the *ctr4+* gene involved in copper uptake (Beaudoin and Labbé, 2006). Consistent with the Mac1 paradigm, these data suggest that the C1 motif responds to copper by interacting with the Cuf1 DBD to mask the Cuf1 NLS. Supporting this view is the finding that a Cuf1 DBD–GFP fusion is sequestered in the cytosol by copper when co-expressed with an epitope-tagged Cuf1 carboxyl terminus. Cytosolic sequestration of the Cuf1 DBD–GFP fusion is lost in the absence of the carboxyl terminus or mutation of the C1 domain (Beaudoin and Labbé, 2006). The Cuf1 amino and carboxyl terminal domains also interact in a standard two-hybrid assay in a copper dependent manner that requires the potential copper binding residues of the C1 domain. Copper sensing in *S. pombe* also involves copper responsive export of Cuf1 from the nucleus, which can be monitored using a Cuf1–GFP fusion under the control of a regulatable promoter (Beaudoin and Labbé, 2007). This allowed the mapping of a leucine rich nuclear export signal and identified the need for the nuclear export protein Crm1 in this process. Again, the Cuf1 C1 domain is required for copper responsive export of this factor from the nucleus. Cuf1 nuclear export is not the only mechanism of repression. Copper inactivates the activity of Cuf1 when Crm1 function is experimentally repressed so that Cuf1 remains in the nucleus (Beaudoin and Labbé, 2007). Cuf1 repression therefore occurs in two phases; inhibition of function within the nucleus followed by nuclear export.

A copper-induced conformational change that represses transcription factor function is an attractive model. However, this is by no means fully established. Although it has been attempted, Mac1 has not been isolated from yeast cells in its copper bound form due to the technical challenges of isolating a low abundance protein (Jensen and Winge, 1998). Intriguingly, Mac1 is a phosphoprotein and, *in vitro*, can only bind to the *CTR1* promoter in its phosphorylated form (Heredia *et al.*, 2001). The kinase and the conditions that regulate Mac1 phosphorylation are not known. Measurements of the *in vitro* copper binding affinity of the Mac1 C1 domain are consistent with this domain having sufficient affinity to bind any free copper that will arise once the buffering capacity of the cell is saturated (Wegner *et al.*, 2011). Conversely, a chaperone could deliver copper to Mac1, as is the case for other copper proteins, however no such chaperone has been identified. Intriguingly, the search for a Mac1 copper chaperone led to the surprising discovery that Sod1 is essential for Mac1 function (Wood and Thiele, 2009). Catalytically active Sod1 that is nuclear-localised is required for Mac1 dependent activation of *CTR1*. Sod1 does not regulate the Mac1 intramolecular interaction or the Mac1 activation domain but is essential for Mac1 binding to the *CTR1* promoter as measured by *in vivo* CHiP experiments (Wood and Thiele, 2009). Suppression of oxidative stress does not restore Mac1 function in a *sod1Δ* strain suggesting a direct role in Sod1-mediated hydrogen peroxide signalling. In this respect it is interesting to note that one of the first studies of Mac1 function demonstrated Mac1 dependent hydrogen peroxide activation of the *CTT1* catalase gene (Jungmann *et al.*, 1993). How hydrogen peroxide signalling regulates Mac1 remains to be seen but it is curious that the activity of a copper-requiring enzyme is required to activate gene expression under low copper conditions. Oxidation of regulatory cysteine residues in yeast transcription factors is an established mechanism of regulation.

Clearly, Mac1 contains several potential targets for such a mechanism of control. The challenge is to determine exactly what constitutes the copper-sensing event to which the Mac1-like regulators respond.

4. Conclusion

Nutrient sensing is a fundamental aspect of an organism's ability to perceive its environment. Identifying the molecular basis of sensing by ammonium transceptors and copper transcription factors will have implications for our understanding of a range of fungal sensors. Potential transceptors for amino acids and potassium have been described suggesting that transceptor mediated sensing may be an important mechanism of fungal sensing (Donaton *et al.*, 2003; Van Nuland *et al.*, 2006; Giots *et al.*, 2003). This is supported by the proposal that certain transceptors may have evolved into G protein couple receptors (Thevelein and Voordeckers, 2009). As with copper, levels of zinc and iron in yeast cells are sensed by metal-responsive transcription factors (Yamaguchi-Iwai *et al.*, 1995; Rutherford *et al.*, 2001; Zhao and Eide, 1997). These metals are cofactors of many essential proteins so that metal-responsive sensors indirectly affect a wide range of cellular processes. In addition to the academic interest in fungal sensing, this area of biology has many practical implications. The switch between yeast and filamentous growth is an essential aspect of the infection process of some animal and plant fungal pathogens. Likewise, the acquisition of transition metals is essential for the success of fungal pathogens as the free levels of these metals are limiting within a host. Fungal metal sensors are therefore ideal therapeutic targets as they are not conserved with higher eukaryotes. In industry, the production of metal containing enzymes, such as laccase, is ultimately dependent on relevant nutrient sensors. For these reasons, the area of fungal nutrient sensing will continue to be an area of active research.

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REFERENCES

Andrade, S.L., Dickmanns, A., Ficner, R., Einsle, O., 2005. Crystal structure of the archaeal ammonium transporter Amt-1 from *Archaeoglobus fulgidus*. Proceedings National Academy Sciences of the United States of America 102, 14994–14999.

Barwell, C.J., Woodward, B., Brunt, R.V., 1971. Regulation of pyruvate kinase by fructose 1,6-diphosphate in *Saccharomyces cerevisiae*. European Journal of Biochemistry 18, 59–64.

Beaudoin, J., Labbé, S., 2001. The fission yeast copper-sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1-like recognition sequence. The Journal of Biological Chemistry 276, 15472–15480.

Beaudoin, J., Mercier, A., Langlois, R., Labbé, S., 2003. The *Schizosaccharomyces pombe* Cuf1 is composed of functional modules from two distinct classes of copper metalloregulatory transcription factors. The Journal of Biological Chemistry 278, 14565–14577.

Beaudoin, J., Labbé, S., 2006. Copper induces cytoplasmic retention of fission yeast transcription factor cuf1. Eukaryotic Cell 5, 277–292.

Beaudoin, J., Labbé, S., 2007. Crm1-mediated nuclear export of the *Schizosaccharomyces pombe* transcription factor Cuf1 during a shift from low to high copper concentrations. Eukaryotic Cell 6, 764–775.

Biswas, K., Morschhäuser, J., 2005. The Mep2 ammonium permease controls nitrogen starvation-induced filamentous growth in *Candida albicans*. Molecular Microbiology 56, 649–669.

Boeckstaens, M., André, B., Marini, A.M., 2007. The yeast ammonium transport protein Mep2 and its positive regulator, the Npr1 kinase, play an important role in normal and pseudohyphal growth on various nitrogen media through retrieval of excreted ammonium. Molecular Microbiology 64, 534–546.

Boeckstaens, M., André, B., Marini, A.M., 2008. Distinct transport mechanisms in yeast ammonium transport/sensor proteins of the Mep/Amt/Rh family and impact on filamentation. The Journal of Biological Chemistry 283, 21362–21370.

Brown, K.R., Keller, G.L., Pickering, I.J., Harris, H.H., George, G.N., Winge, D.R., 2002. Structures of the cuprous-thiolate clusters of the Mac1 and Ace1 transcriptional activators. Biochemistry 41, 6469–6476.

Donaton, M.C., Holsbeeks, I., Lagatie, O., Van Zeebroeck, G., Crauwels, M., Winderickx, J., Thevelein, J.M., 2003. The Gap1 general amino acid permease acts as an amino acid sensor for activation of protein kinase A targets in the yeast *Saccharomyces cerevisiae*. Molecular Microbiology 50, 911–929.

Fong, R.N., Kim, K.S., Yoshihara, C., Inwood, W.B., Kustu, S., 2007. The W148L substitution in the *Escherichia coli* ammonium channel AmtB increases flux and indicates that the substrate is an ion. Proceedings National Academy Sciences of the United States of America 104, 18706–18711.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., Fink, G.R., 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. Cell 68, 1077–1090.

Giots, F., Donaton, M.C., Thevelein, J.M., 2003. Inorganic phosphate is sensed by specific phosphate carriers and acts in concert with glucose as a nutrient signal for activation of the protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Molecular Microbiology 47, 1163–1181.

Graden, J.A., Winge, D.R., 1997. Copper-mediated repression of the activation domain in the yeast Mac1p transcription factor. Proceedings National Academy Sciences of the United States of America 94, 5550–5555.

Gross, C., Kelleher, M., Iyer, V.R., Brown, P.O., Winge, D.R., 2000. Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. The Journal of Biological Chemistry 275, 32310–32316.

Haurie, V., Boucherie, H., Sagliocco, F., 2003. The Snf1 protein kinase controls the induction of genes of the iron uptake pathway at the diauxic shift in *Saccharomyces cerevisiae*. The Journal of Biological Chemistry 278, 45391–45396.

Heredia, J., Crooks, M., Zhu, Z., 2001. Phosphorylation and Cu+ coordination-dependent DNA binding of the transcription factor Mac1p in the regulation of copper transport. The Journal of Biological Chemistry 276, 8793–8797.

Hinnebusch, A.G., 2005. Translational regulation of GCN4 and the general amino acid control of yeast. Annual Review of Microbiology 59, 407–450.

Inwood, W.B., Hall, J.A., Kim, K.S., Fong, R., Kustu, S., 2009. Genetic evidence for an essential oscillation of transmembrane-spanning segment 5 in the *Escherichia coli* ammonium channel AmtB. Genetics 183, 1341–1355.

Jamison McDaniels, C.P., Jensen, L.T., Srinivasan, C., Winge, D.R., Tullius, T.D., 1999. The yeast transcription factor Mac1 binds to DNA in a modular fashion. The Journal of Biological Chemistry 274, 26962–26967.

Javelle, A., Lupo, D., Zheng, L., Li, X.D., Winkler, F.K., Merrick, M., 2006. An unusual twin-his arrangement in the pore of ammonia channels is essential for substrate conductance. The Journal of Biological Chemistry 281, 39492–39498.

Javelle, A., Lupo, D., Ripoche, P., Fulford, T., Merrick, M., Winkler, F.K., 2008. Substrate binding, deprotonation, and selectivity at the periplasmic entrance of the *Escherichia coli* ammonia channel AmtB. Proceedings National Academy Sciences of the United States of America 105, 5040–5045.

Jensen, L.T., Winge, D.R., 1998. Identification of a copper-induced intramolecular interaction in the transcription factor Mac1 from *Saccharomyces cerevisiae*. EMBO Journal 17, 5400–5408.

Jensen, L.T., Posewitz, M.C., Srinivasan, C., Winge, D.R., 1998. Mapping of the DNA binding domain of the copper-responsive transcription factor Mac1 from *Saccharomyces cerevisiae*. The Journal of Biological Chemistry 273, 23805–23811.

Joshi, A., Serpe, M., Kosman, D.J., 1999. Evidence for $(Mac1p)_2$ DNA ternary complex formation in Mac1p-dependent transactivation at the *CTR1* promoter. The Journal of Biological Chemistry 274, 218–226.

Jungmann, J., Reins, H.A., Lee, J., Romeo, A., Hassett, R., Kosman, D., Jentsch, S., 1993. MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. EMBO Journal 12, 5051–5056.

Keller, G., Gross, C., Kelleher, M., Winge, D.R., 2000. Functional independence of the two cysteine-rich activation domains in the yeast Mac1 transcription factor. The Journal of Biological Chemistry 275, 29193–29199.

Keller, G., Bird, A., Winge, D.R., 2005. Independent metalloregulation of Ace1 and Mac1 in *Saccharomyces cerevisiae*. Eukaryotic Cell 4, 1863–1871.

Khademi, S., O'Connell 3rd, J., Remis, J., Robles-Colmenares, Y., Miercke, L.J., Stroud, R.M., 2004. Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 Å. Science 305, 1587–1594.

Labbé, S., Zhu, Z., Thiele, D.J., 1997. Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. The Journal of Biological Chemistry 272, 15951–15958.

Labbé, S., Peña, M.M., Fernandes, A.R., Thiele, D.J., 1999. A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*. The Journal of Biological Chemistry 274, 36252–36260.

Lamoureux, G., Javelle, A., Baday, S., Wang, S., Bernèche, S., 2010. Transport mechanisms in the ammonium transporter family. Transfusion Clinique et Biologique 17, 168–175.

Lin, Y., Cao, Z., Mo, Y., 2006. Molecular dynamics simulations on the *Escherichia coli* ammonia channel protein AmtB: mechanism of ammonia/ammonium transport. Journal American Chemical Society 128, 10876–10884.

Lorenz, M.C., Heitman, J., 1998. The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. EMBO Journal 17, 1236–1247.

Marini, A.M., Soussi-Boudekou, S., Vissers, S., André, B., 1997. A family of ammonium transporters in *Saccharomyces cerevisiae*. Molecular Biology of the Cell 17, 4282–4293.

Marini, A.M., Boeckstaens, M., Benjelloun, F., Chérif-Zahar, B., André, B., 2006. Structural involvement in substrate recognition of an essential aspartate residue conserved in Mep/Amt and Rh-type ammonium transporters. Current Genetics 49, 364–374.

Mayer, M., Dynowski, M., Ludewig, U., 2006. Ammonium ion transport by the AMT/Rh homologue LeAMT1;1. Biochemistry Journal 396, 431–437.

Mihlan, M., Homann, V., Liu, T.W., Tudzynski, B., 2003. AREA directly mediates nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi*, but its activity is not affected by NMR. Molecular Microbiology 47, 975–991.

Nygaard, T.P., Rovira, C., Peters, G.H., Jensen, M.Ø., 2006. Ammonium recruitment and ammonia transport by *E. coli* ammonia channel AmtB. Biophysical Journal 91, 4401–4412.

Rutherford, J.C., Jaron, S., Ray, E., Brown, P.O., Winge, D.R., 2001. A second iron-regulatory system in yeast independent of Aft1p. Proceedings National Academy Sciences of the United States of America 98, 14322–14327.

Rutherford, J.C., Lin, X., Nielsen, K., Heitman, J., 2008a. Amt2 permease is required to induce ammonium-responsive invasive growth and mating in *Cryptococcus neoformans*. Eukaryotic Cell 7, 237–246.

Rutherford, J.C., Chua, G., Hughes, T., Cardenas, M.E., Heitman, J., 2008b. A Mep2-dependent transcriptional profile links permease function to gene expression during pseudohyphal growth in *Saccharomyces cerevisiae*. Molecular Biology of the Cell 19, 3028–3039.

Serpe, M., Joshi, A., Kosman, D.J., 1999. Structure–function analysis of the protein-binding domains of Mac1p, a copper-dependent transcriptional activator of copper uptake in *Saccharomyces cerevisiae*. The Journal of Biological Chemistry 274, 9211–9219.

Smith, D.G., Garcia-Pedrajas, M.D., Gold, S.E., Perlin, M.H., 2003. Isolation and characterization from pathogenic fungi of genes encoding ammonium permeases and their roles in dimorphism. Molecular Microbiology 50, 259–275.

Teichert, S., Rutherford, J.C., Wottawa, M., Heitman, J., Tudzynski, B., 2008. Impact of ammonium permeases mepA, mepB, and mepC on nitrogen-regulated secondary metabolism in *Fusarium fujikuroi*. Eukaryotic Cell 7, 187–201.

Thevelein, J.M., Voordeckers, K., 2009. Functioning and evolutionary significance of nutrient transceptors. Molecular Biology and Evolution 26, 2407–2414.

Van Nuland, A., Vandormael, P., Donaton, M., Alenquer, M., Lourenço, A., Quintino, E., Versele, M., Thevelein, J.M., 2006. Ammonium permease based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast. Molecular Microbiology 59, 1485–1505.

Voutsina, A., Fragiadakis, G.S., Boutla, A., Alexandraki, D., 2001. The second cysteine-rich domain of Mac1p is a potent transactivator that modulates DNA binding efficiency and functionality of the protein. FEBS Letters 494, 38–43.

Wilkinson, B.M., James, C.M., Walmsley, R.M., 1996. Partial deletion of the *Saccharomyces cerevisiae* GDH3 gene results in novel starvation phenotypes. Microbiology 142, 1667–1673.

Wegner, S.V., Sun, F., Hernandez, N., He, C., 2011. The tightly regulated copper window in yeast. Chemical Communications 47, 2571–2573.

Wood, L.K., Thiele, D.J., 2009. Transcriptional activation in yeast in response to copper deficiency involves copper–zinc superoxide dismutase. The Journal of Biological Chemistry 284, 404–413.

Yamaguchi-Iwai, Y., Dancis, A., Klausner, R.D., 1995. AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. EMBO Journal 14, 1231–1239.

Yamaguchi-Iwai, Y., Serpe, M., Haile, D., Yang, W., Kosman, D.J., Klausner, R.D., Dancis, A., 1997. Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to

upstream regulatory sequences of *FRE1* and *CTR1*. The Journal of Biological Chemistry 272, 17711–17718.

Zhao, H., Eide, D.J., 1997. Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in *Saccharomyces cerevisiae*. Molecular and Cellular Biology 17, 5044–5052.

Zheng, L., Kostrewa, D., Bernèche, S., Winkler, F.K., Li, X.D., 2004. The mechanism of ammonia transport based on the crystal structure of AmtB of *Escherichia coli*. Proceedings National Academy Sciences of the United States of America 101, 17090–17095.

Zhu, Z., Labbé, S., Peña, M.M., Thiele, D.J., 1998. Copper differentially regulates the activity and degradation of yeast Mac1 transcription factor. The Journal of Biological Chemistry 273, 1277–1280.

Zurita-Martinez, S.A., Puria, R., Pan, X., Boeke, J.D., Cardenas, M.E., 2007. Efficient Tor signaling requires a functional class C Vps protein complex in *Saccharomyces cerevisiae*. Genetics 176, 2139–2150.