**KIMID1**, a relevant key player between endoplasmic reticulum homeostasis and mitochondrial dysfunction in *Kluyveromyces lactis*

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**INTRODUCTION**

Calcium (Ca$^{2+}$) is one of the major ions responsible for regulating cellular functions in both prokaryotic and eukaryotic organisms. Given the broad physiological roles of Ca$^{2+}$, the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) must be controlled and maintained at an appropriate value. The secretion machinery organelles, such as the endoplasmic reticulum (ER) and the Golgi apparatus, play an important role in intracellular calcium homeostasis. However, the greater part of the intracellular Ca$^{2+}$ is stored in the vacuole at a concentration in the millimolar range, which may be compared with the micromolar range of the ER (Cunningham, 2011; Pittman, 2011; Strayle et al., 1999).

Calcium homeostasis is normally achieved by a small subset of Ca$^{2+}$ transporters present in the cytoplasmic membrane and membranous organelles. Specifically, the *Saccharomyces cerevisiae* plasma membrane contains Mid1p, a putative stretch-activated nonselective cation channel component (Kanzaki et al., 1999, 2000) that interacts with a second subunit, Cch1p, to catalyse high-affinity Ca$^{2+}$ influx into cells (Locke et al., 2000; Iida et al., 1994). The gene is ubiquitous among fungal organisms; recently, a MID1 homologue from the plant *Arabidopsis thaliana* (Mca1) was cloned (Nakagawa et al., 2007).

Calcium transporters respond to the calmodulin/calciurin signalling pathway and are controlled by the transcription factor Tcn1p/Crz1p; this system maintains the [Ca$^{2+}$]$_i$ between 50 and 200 nM, although the extracellular concentrations of this ion may range from <1 to >100 mM (Eilam et al., 1985; Dunn et al., 1994; Batiza et al., 1996; Miseta et al., 1999). A wide variety of developmental and stress signals can evoke cellular responses through elevation of [Ca$^{2+}$]$_i$ (Sanders et al., 2002; Clapham, 1995; Berridge et al., 2003).

N-Glycosylation plays a crucial role in protein trafficking across the secretory apparatus to the cell surface (Conti et al., 2002), and in the recognition of misfolded proteins in the ER (O’Connor & Imperiali, 1996). Mutational analysis has shown that N-glycosylation is required for...
proper membrane trafficking and/or functioning of some membrane proteins; among them, it has been demonstrated that \textit{S. cerevisiae} \textsc{mid1} requires full glycosylation to correctly localize and assemble at the plasma membrane (Ozeki-Miyawaki et al., 2005).

The interplay between \(\text{Ca}^{2+}\) metabolism and glycosylation is largely unknown even in yeast. Our objective was to explore the role of \textit{MID1} in the yeast \textit{Kluyveromyces lactis}. We previously demonstrated that mutations in the \textit{KLOCH1} gene, encoding the Golgi \(\alpha\)-1,6-mannosyltransferase, resulted in altered calcium homeostasis. \textsc{Kloch1p} is responsible for the oligosaccharide core elongation of newly synthesized peptides by the addition of the first mannose to the \(N\)-glycan chains.

Cells carrying a mutation in the \textit{KLOCH1} gene showed a reduced expression of calcium signalling genes, such as calmodulin and calcineurin, associated with alteration in the intracellular calcium content. Besides, this \(N\)-glycosylation mutant also showed altered mitochondrial morphology accompanied by increased accumulation of reactive oxygen species (ROS) and oxidative stress (Zanni et al., 2009). In the present report, the impact of the \textit{MID1} gene on altered calcium availability was investigated in order to explore the underlying connections between the \(\text{Ca}^{2+}\) homeostasis and the secretory pathway functions that take place in \textit{K. lactis} cells when a key step of glycosylation is removed.

**METHODS**

**Yeast strains and growth conditions.** The strains used in this study were MW278-20C (MATa, ade2, leu2, ura3) and CPV3 (MATa, ade2, leu2, ura3, \textit{Kloch1}-1). Yeast strains were grown in SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids, Difco) with the appropriate auxotrophic requirements. Fivefold serial dilution from concentrated suspensions of exponentially growing cells was spotted onto YPD agar plates (1% yeast extract, 2% peptone, 2% glucose, Difco) supplemented or not with 4 mM H\(_2\)O\(_2\) (Sigma no. H1009) or 20 mM EGTA (Sigma no. E4378) and the plates were incubated at 30 °C for 3 days. For DTT (Sigma no. 43815) and Calcoflour white (CFW; Sigma no. F3543) treatments, cells were grown to exponential phase and then exposed to the perturbing agents at a final concentration of 10 mM (DTT) and 200 μg ml\(^{-1}\) (CFW) for 3 or 6 h. Untreated cultures were incubated in parallel over the same periods.

**Plasmid construction.** The \textsc{pMID1} plasmid was constructed as follows. The 2796 bp fragment containing the full ORF (1653 bp) of \textit{KIMID1} plus 1000 bp upstream and 100 bp downstream was amplified by PCR, using primers modified with the recognition site for the restriction endonuclease \textit{EcoRI}, 5'-CGGAATTCTTGTTGA-GTTGCACCCGATGC-3' and 5'-CGGAATTCCGACGGAAAACACCGACTGGC-3'. The PCR fragment encoding \textsc{Kmid1p} was cloned into the p\textit{GEM}-T Easy vector (Promega) according to the manufacturer's instructions, giving \textsc{pGEM-MID1}, and the presence of the intact gene was confirmed by DNA sequencing (MWG Biotech). The fragment was excised by \textit{EcoRI} digestion from \textsc{pGEM-MID1} and ligated into a centromeric vector (p\textit{CX18}) (Chen, 1996) linearized by the same endonuclease, to obtain plasmid \textsc{pMID1}.

**Yeast transformation.** The strains were transformed with the plasmids by electroporation (Sambrook et al., 2001). \textsc{pMID1}-positive clones were selected on URA-minus plates.

**Stress condition and viability.** Yeast cells were grown aerobically at 28 °C in liquid medium for 24 h and challenged with hydrogen peroxide. This was added directly to the growth medium to a final concentration of 20 mM. Untreated cultures were incubated in parallel over the same periods. Viability was determined by colony counts on YPD plates after 3 days of incubation at 28 °C, and was expressed as a percentage of the corresponding control culture. Values are the mean of three independent experiments with an SD of <15%.

**Ca\(^{2+}\) accumulation assays.** The method of lida et al. (1990) was generally followed. Briefly, cells of various strains were grown to approximately 2 x 10\(^{6}\) cells ml\(^{-1}\) in SD\textit{Ca100} medium at 30 °C and incubated for an additional 2 h with 185 kBq ml\(^{-1}\) \(~^{45}\text{CaCl}_2\) (1.8 kBq nmol\(^{-1}\); PerkinElmer, no. NEZ013). Samples (100 μl) were filtered with Millipore filters (type HA; 0.45 μm pore-size) presoaked with 5 mM CaCl\(_2\), and washed five times with the same solution and dried. Radioactivity retained on each filter was counted with a liquid scintillation counter. Data are means ± SD from three independent experiments.

**Fluorescence microscopy.** Exponential-phase cells grown in SD minimal medium were harvested, washed with water and then incubated with the vital dye 2-(4(dimethylaminostyryl)-N-methylpyridinium iodide (DASPMI; Invitrogen) by the method of Guthrie & Fink (2002). Chitin staining was performed by the method of Uccellietti et al. (1999). Epifluorescence microscopy was carried out with a Zeiss AxioVert 25 microscope fitted with a ×100 immersion objective and a standard filter set.

**Northern blot analysis.** Total RNA of \textit{K. lactis} strains was extracted by the hot phenol method (Schmitt et al., 1990) from cultures grown on SD medium to exponential phase (about 6 x 10\(^7\) cells ml\(^{-1}\)). RNA was quantified by absorbance (A\text{260}) and separated by denaturing agarose electrophoresis. After electrophoresis RNA was transferred to nylon membranes and hybridized with \(^{32}\text{P}\)-labelled random-primed probes (Roche). All the probes were PCR-amplified from the \textit{K. lactis} DNA genome. The fragment of 1653 bp containing the \textit{KIMID1} gene was obtained with primers 5'-GCGATGATGTCGATATCAG-3' and 5'-CGGAATTCGACGAGGAGAAAACCCGACTGGC-3'. The 1300 bp PCR product of the \textit{KIMID1} gene was obtained using primers 5'-CAAGATGATGGATACGTA-3' and 5'-GGGAATTCGACGAGGAGAAAACCCGACTGGC-3'. The optimal parameters determined for PCR were 5% \textit{Cna1} and 30 s; and 25 cycles for both the \textit{KIMID1} and \textit{KCNB1} genes.

**Semiquantitative RT-PCR.** Total RNA extracted as above was subjected to TURBO DNase treatment according to the manufacturer’s instructions (Ambion). Reverse transcription was performed using a Promega Reverse Transcription System with \(3\-\text{P}\)-labelled random-primed probes (Roche). All the probes were PCR-amplified from the \textit{K. lactis} DNA genome. The fragment of 1653 bp containing the \textit{KIMID1} gene was obtained using primers 5'-CAAGATGATGGATACGTA-3' and 5'-GGGAATTCGACGAGGAGAAAACCCGACTGGC-3'. The optimal parameters determined for PCR were 5% \textit{Cna1} and 30 s; and 25 cycles for both the \textit{KIMID1} and \textit{KCNB1} genes.
**RESULTS**

**Increased KIMID1 gene dosage is able to suppress calcium-related defects in Kloch1-1**

The aim of this study was to shed light on the molecular mechanisms that link glycosylation and calcium homeostasis in Kloch1-1 cells. We hypothesized that the impaired calcium metabolism of this N-glycosylation mutant strain could be caused by a defective Mid1p/Cch1 calcium membrane channel. In fact, in *S. cerevisiae* it has been reported that Mid1p requires full glycosylation for correct localization and assembly at the plasma membrane (Ozeki-Miyawaki et al., 2005). A Northern blotting analysis was performed in *K. lactis* cells lacking mannosyltransferase activity, revealing a decreased level of the KIMID1 transcript with respect to wild-type cells (Fig. 1). Therefore, we wondered whether an increase in KIMID1 gene dosage could relieve the phenotypes shown by the mutant cells. Accordingly, the KIMID1 gene was cloned in the pCXJ18 centromeric vector, resulting in plasmid pMID1, as KIMID1 on a multicopy vector was toxic to the cells (not shown).

After transforming Kloch1-1 cells with pMID1 calcium homeostasis was examined. A sensitivity assay to EGTA was performed; this cationic chelator makes calcium unavailable in YPD medium, resulting in lack of growth for cells with impaired calcium homeostasis, such as the Kloch1-1 mutant. As shown in Fig. 2(a), cells carrying the mutation in the OCH1 gene were able to grow in the presence of EGTA when transformed with pMID1.

We previously reported that the N-glycosylation mutant is sensitive to hydrogen peroxide (H$_2$O$_2$), a strong ROS generator (Zanni, et al., 2009). In order to explore whether oxidative stress in Kloch1-1 cells can be influenced by modulating calcium homeostasis, the response to this oxidant was analysed. No sensitivity to H$_2$O$_2$ was observed in the mutant strain harbouring plasmid pMID1 (Fig. 2a).
compared with 93% of the wild-type counterpart (Fig. 2b). Notably, the survival rate increased up to 68% in Kloch1-1 cells carrying plasmid pMID1.

Given that oxidative stress and ROS accumulation play a pivotal role in mitochondrial morphology and biogenesis, mitochondrial functionality was then observed in these strains by DASPMI staining. As shown in Fig. 2(c), in most of the mutant cells carrying plasmid pMID1 the mitochondrial tubular network appeared almost identical to that of the wild-type counterpart; the punctate phenotype, the result of the mutation in the KLOCH1 gene, was detectable only in a few cells.

**KIMID1 is able to modulate calcium signalling and cell wall organization**

We previously reported that the Kloch1-1 mutant strain displays a significantly thicker and disorganized cell wall with respect to the wild-type (Uccelletti et al., 2006). To analyse the effect of KIMID1 overexpression on cell wall-related defects, we analysed sensitivity to Congo red, a molecule known to interfere with cell wall organization (Kopecka & Gabriel, 1992). As shown in Fig. 3(a), the presence of pMID1 conferred upon Kloch1-1 cells the ability to grow on medium containing Congo red (Fig. 3a). In parallel, CFW fluorescent dye was also employed to investigate the cell wall structure because of its ability to specifically bind chitin (Roncero & Durán, 1985). After CFW staining, wild-type cells showed the usual chitin deposition localized only at the bud scars. By contrast, in the mutant strain, fluorescence was abnormally distributed across the entire cell wall (Uccelletti et al., 2000). Moreover, we observed a chitin distribution at the bud emergence sites in Kloch1-1 cells transformed with pMID1 (Fig. 3b). Both the size and the shape of the cells were almost indistinguishable from those of the wild-type.

Since calcineurin overexpression restores the cell wall defects in the mutant cells (Zanni et al., 2009), we attempted to determine whether an increased dosage of KIMID1 was able to induce the cell wall integrity (CW1) pathway by modulating the expression of calcineurin in the mutant strain. Indeed, the transcription of the regulatory subunit of calcineurin, KICNB1, showed a twofold increase in Kloch1-1 cells transformed with pMID1 in comparison with the wild-type counterpart (Fig. 3c); similar results were also observed for the catalytic subunit, KICNA1 (results not shown).

**Perturbed ER homeostasis in K. lactis is responsible for reduced transcription of KIMID1**

In *S. cerevisiae* cells, Mid1p, together with Cch1p, is required for the high-affinity Ca2+ influx system; expression analysis of the *K. lactis* CCH1 gene was therefore performed in Kloch1-1 cells. As shown in Fig. 4(a), the mutant strain showed a twofold KICCH1 transcript reduction, which reverted to the wild-type level when the KIMID1 gene was overexpressed. Given that both components of the single calcium influx system were downregulated in the mutant cells, calcium accumulation was evaluated. Surprisingly, a 10-fold increase in calcium uptake was observed in the mutant strain with respect to the wild-type. This increase disappeared in the mutant cells transformed with vector pMID1 (Fig. 4b). On the other hand, in previous work we found a reduced cystolic calcium content in Kloch1-1 cells, as demonstrated by lower FURA-2 AM fluorescence (Zanni et al., 2009). In order to explain these conflicting results, a possible alteration of vacuolar calcium homeostasis was hypothesized. The expression of the KIPMC1 gene encoding the vacuolar calcium pump was therefore investigated. Indeed, the mutant cells exhibited an eightfold increase in
the KlPMC1 transcript compared with the wild-type. This alteration was completely relieved by restoring KIMID1 expression in the mutant cells (Fig. 4c).

In S. cerevisiae, a calcium cell survival (CCS) pathway is activated under ER stress, whereby calcineurin activity is inhibited, leading to calcium accumulation (Bonilla et al., 2002). We therefore investigated whether this mechanism could also occur in K. lactis strains. Since in Kloch1-1 cells an increased calcium uptake is associated with a down-modulation of calcineurin, a possible ER homeostasis perturbation was investigated. To this end, a Northern blotting analysis directed to an ER stress marker gene was performed. In particular, we found that the amount of the KIKAR2 transcript rose up to 2.4-fold in the Kloch1-1 strain with respect to the wild-type (Fig. 5a). In addition, we made use of the reducing agent DTT, a well-known ER stressor. It has been demonstrated that this molecule can cross membranes and promote accumulation of misfolded proteins in the ER by preventing disulfide bond formation, thus triggering the ER stress response (Simons et al., 1995; Cox et al., 1993). Effectively, when DTT was added to the medium, the mutant cells exhibited a growth-sensitive phenotype (data not shown). Taken together these findings support the idea that the CCS pathway might be activated in Kloch1-1 cells.

In order to understand whether the altered calcium homeostasis and mitochondrial dysfunction taking place in the mutant strain were caused by ER stress per se or by defective glycosylation, we challenged K. lactis wild-type cells with DTT. This treatment not only resulted in reduced transcription of KIMID1, but also led to down-modulation of the calmodulin transcript (Fig. 5b). In addition, in the wild-type cells under ER stress the transcriptional profile of KlPMC1 was upregulated, similarly to Kloch1-1 cells, although not to the same extent (Fig. 5c). DASPMI staining after DTT treatment was also performed in wild-type cells. After only 3 h this reducing agent provoked a dotted mitochondrial network, nearly identical to that of the Kloch1-1 counterpart, in 42% of the cells (Fig. 5d). This phenotype became more severe after 6 h of treatment, affecting 67% of the cell population. By contrast, long-term exposure was not sufficient to cause an alteration of mitochondrial functionality in 92% of wild-type cells overexpressing the KIMID1 gene (Fig. 5d).

The cell wall and the ER stress responses are coordinately regulated in S. cerevisiae (Krysan, 2009). Recently it has been demonstrated in K. lactis that DTT treatment affects the clustering of the cell wall sensor Wsc1p by breaking the disulfide bridges (Dupres et al., 2011) and therefore inducing the CWI pathway. We therefore investigated whether the observed effects of DTT on both calcium homeostasis and mitochondrial functionality could be related to its action on the cell wall. To this end, cells were treated with CFW as a cell wall structure-perturbing agent and the modulation of calcium signalling was evaluated. This treatment was not able to induce the ER stress response, as the cells did not show enhanced transcription of KIKAR2. Northern blotting analysis revealed only a slight decrease in the KIMID1 transcript (Fig. 6), while no variation was observed in the case of KICMD1 (not shown), suggesting that cell wall stress is not the major component responsible for the down-modulation of calcium signalling in Kloch1-1 cells.

**DISCUSSION**

Eukaryotic proteins that enter the secretory pathway in the ER are glycosylated by distinct sets of enzymes that catalyse...
the formation of either N- or O-linked glycans. Glycosylation can influence the folding of proteins, and their biological activity and half-life. The Och1 proteins of yeasts are α-1,6-mannosyltransferases that initiate a distinct branch in the N-glycan core, thereby providing the platform for the subsequent formation of a large poly-mannosylated outer chain (Nakayama et al., 1992; Bates et al., 2006; Uccelletti et al., 2006). The Kloch1-1 mutant of K. lactis shows hypersecretory capabilities, altered mitochondrial functionality linked to calcium homeostasis, as well as cell wall defects (Uccelletti et al., 2006; Zanni et al., 2009). In the present study we investigated the role of MID1 as a key player in the calcium-related defects that are found in the mutant cells.

In S. cerevisiae the glycoprotein Mid1p is one component of the plasma membrane Mid1/Cch1 Ca\(^{2+}\) channel (Iida et al., 1994). Mid1 is probably activated by membrane stretching and mediates the subsequent opening of the Cch1 channel, thus leading to localized Ca\(^{2+}\) influx. On the other hand, there are indications that in some cases Mid1p alone is able to form a functional Ca\(^{2+}\) channel without the contribution of Cch1p (Kanzaki et al., 1999, 2000; Courchesne & Ozturk, 2003). In addition, a full glycosylation of Mid1p is required for proper localization of the calcium uptake system at the plasma membrane (Ozeki-Miyawaki et al., 2005). Based on this, our initial aim was to investigate the functioning of MID1 in the K. lactis glycosylation mutant.

Although a down-modulation of both KIMID1 and KICCH1 genes occurred in the mutant cells, a striking increase in calcium uptake was found. Notably, a co-regulation of the

![Fig. 5. ER stress and calcium signalling in K. lactis cells.](http://mic.sgmjournals.org)
two subunits seems to exist in K. lactis. In fact, the wild-type cells carrying pMID1 showed an increased transcription of the KlCCH1 gene (unpublished results). We can thus hypothesize that the reduced transcription of the two subunits in Kloch1-1 cells involves a compensatory mechanism in response to the activation of the channel. In S. cerevisiae, the activity of the high-affinity calcium system (HACS), composed essentially of the Mid1 and Cch1 proteins, is stimulated in wild-type strains overexpressing the vacuolar Ca^{2+}-ATPase Pmc1p (Locke et al., 2000). Notably, overexpression of KIPMC1 also occurs in Kloch1-1 cells.

Increased dosage of KlMID1 in Kloch1-1 cells was able, through the modulation of calcium signalling, to suppress mitochondria-related defects. Moreover, we showed that cell wall alterations in the N-glycosylation mutant were restored by overexpressing MID1. This strongly suggests that the mechanism responsible for the synthesis and the deposition of chitin could be influenced by Mid1p, probably by increasing calcineurin activity and thus inducing the CWI pathway.

Surprisingly, we found that the down-modulation of KlMID1 that occurred in the mutant strain is probably to be ascribed to ER stress rather than to the impaired glycosylation per se. In fact, Kloch1-1 cells showed sensitivity to DTT and increased levels of KAR2 transcript as compared with wild-type cells. In addition, under ER stress conditions, Kloch1 cells showed a reduction in KlMID1 mRNA and a concomitantly altered mitochondrial functionality.

Notably, in the yeast Pichia pastoris, down-modulation of the MID1 transcript also occurs when the cells are treated with DTT or the unfolded protein response (UPR) is activated by overexpression of the transcription factor HAC1 (Graf et al., 2008). It has recently been reported that downregulation of expression of the nucleotide sugar transporters of the Golgi apparatus also induces ER stress in mammalian cells (Xu et al., 2010). Whether this is also accompanied by altered calcium homeostasis deserves further investigation.

To date, the involvement of Ca^{2+} signalling in a multitude of cellular pathways has been well documented (Berridge et al., 2003); however, little is known about the role of Ca^{2+} signalling in restoring ER homeostasis once ER stress has been triggered. Yeasts respond to ER insults by initiating Ca^{2+} influx across their plasma membrane through the high-affinity Ca^{2+} channel, assembled with the subunits Cch1 and Mid1; the latter has been suggested to be the one responsible for mediating the influx (Bonilla et al., 2002; Hong et al., 2010). We can hypothesize that in K. lactis cells a similar mechanism occurs, although the functioning of an additional Ca^{2+} channel/transporter cannot be excluded rather than the involvement of Mid1p and Cch1p, which are even down-modulated in the Kloch1-1 background. Currently, several reports propose the existence of a calcium influx system, not yet identified, that could operate as a substitute when the well-characterized HACS is inactivated (Cui et al., 2009; Groppi et al., 2011).

Intriguingly, it has been reported that fully glycosylated Mid1p is present at the plasma membrane and also at the ER membrane, where it may function to release Ca^{2+} from the ER under stress (Yoshimura et al., 2004). Recently, discrete sites of close apposition between ER and mitochondria that facilitate inter-organelle calcium and phospholipid exchange have also been characterized in yeast (Kornmann et al., 2009). We speculate that in K. lactis cells, Mid1p is required for, or participates in, these interactions to ensure proper Ca^{2+} homeostasis between the ER and mitochondria. Further studies will be performed to elucidate the role of KlMID1 in ER/mitochondria homeostasis in K. lactis cells.

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