Functional analysis of a novel endo-O-mannosidase in budding yeast, *Saccharomyces cerevisiae*

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Research aims

Glycosylation of nascent proteins is one of the most common co- and post-translational modifications. This modification is known to play a critical role in various biological processes, such as development, cell differentiation, signal transduction, cell-cell interactions, and cancer metastasis; it is also known to improve the properties of donor proteins (*e.g.,* increasing stability and half-life). The biological pathways and physiological properties of glycosylation have been extensively studied. By contrast, it is known that free-formed nitrogen-linked-glycans, designated as free N-glycans (fNgs), which are liberated from glycans on glycoproteins, are accumulated in the cytosol[(1,2)], but the biological significance of the accumulation of fNgs is unclear. To gain insight into the metabolism and accumulation of fNgs in the cytosol, we examined the generation and degradation of fNgs in various growth conditions in budding yeast cells (*Saccharomyces cerevisiae*). Unexpectedly, we found that in the mannose-containing culture (YPMan), yeast cells generated novel free glycans derived from O-linked sugar chains on the glycoproteins, suggesting that yeast cells possess a novel endo-O-mannosidase (EOM). We also found that a mutant defective in a transcription repressor, Cyc8, exhibits excessive de-mannosylation of the glycoproteins, due to the hyper-activation of EOM, and shows a severe growth defect phenotype; this is due to excessive de-mannosylation, suggesting that the EOM activity was strictly regulated by Cyc8. Our goal for this study, therefore, was the identification of the gene encoding EOM, through 1) global screening for genetic interactions with *CYC8* and 2) deletion of the whole non-essential gene. Our broader goal is to gain deeper insights into the biological and physiological significance of the generation of free glycan derived from O-glycans, designated as free O-glycans (fOGs).

Results

Construction of the doxycycline-dependent *CYC8* shutdown strain (*TetOff-cyc8-degron-DAmP*)

Previously, we found that *cyc8*Δ cells showed severe growth defects in YPMan culture, due to the EOM-dependent, excessive de-mannosylation of the cell wall proteins (Figure 1A). Therefore, we sought to identify whole-gene mutants that suppress the cell wall defect shown in *cyc8*Δ cells, because the genes encoding EOM should be found in the pool of suppressor mutants of *cyc8*Δ. Specifically, for identification of the genes suppressing the mannose-sensitive phenotype shown in *cyc8*Δ, we decided to construct a series of double mutants in a *cyc8*Δ background. To do this, we had planned to cross *cyc8*Δ mutants with all single gene deletion mutants to generate diploid cells that would contain the heterozygous double mutation (Figure 1B); then, following meiotic cell division, the resulting haploid cells possessing double mutations (*CYC8*/*cyc8*Δ *XXX/xxx*Δ) would be isolated. Unfortunately, however, it is known that heterozygous mutant cells that have mutations in the *CYC8* gene cannot enter meiosis, due to the aberrant expression of the genes involved in meiosis[3]. To overcome this obstacle, we tried to construct a strain that can shut down the expression of *CYC8* in the presence of doxycycline (Dox). Therefore, the *CYC8* promoter on the gene sequence was exchanged with a doxycycline-dependent shutdown promoter (*tetO*$_{x7}$*CYC8*$_{TATA}$ promoter). In this construct, we also inserted a CL1 degron sequence[4] and a mutation called DAmP (**D**ecreased **A**bundance by **m**RNA **P**erturbation) [5] to avoid the leaky expression of *CYC8*, even in the presence of doxycycline (Figure 1C). As shown in Figure 1D, *TetOff-cyc8-degron-DAmP* strains showed the strongest growth defect phenotype in the presence of Dox on the mannose-containing media (Ca[-URA]+Man+Dox), and also exhibited normal spore formation and normal meiotic division in the Dox-free condition (data not shown). This suggested that the *TetOff-cyc8-degron-DAmP* strain should be suitable for global screening for the genetic interactions associated with *CY8*. The construction of the series of double mutants is currently in progress.

Excessive de-mannosylation disrupts the cell wall integrity pathway

Our previous study showed that the *cyc8*Δ mutant exhibits severe cell wall defect phenotypes (e.g., temperature, calcofluor-white [CFW] sensitivity, and Congo red [CR] sensitivity). Based on this fact, we suspected that one major cell wall integrity pathway,

the MAPK pathway, would be impaired, owing to the excessive de-mannosylation of the sensor protein for MAPK localized to the cell surface (Figure 2A). To test this hypothesis, we first tried to examine the phosphorylation status of one of the signal transduction intermediators, Mpk1, using anti-phospho-Mpk1 antibodies. As shown in Figure 2B, irrespective of the carbon source in the culture media, the MAPK pathway in wild type cells was activated by the addition of the cell wall stressor, CFW. By contrast, the MAPK pathway in *cyc8*Δ cells was slightly, but constitutively, activated in YPMan culture. We next examined the O-glycosylation status of Wsc1, a sensor protein for the MAPK pathway, from 0 to 6 h after exchanging the old media with fresh YPGlc or YPMan. Only when *cyc8*Δ cells were cultured with YPMan media, slight but significant mobility shifts in the Wsc1-HA band were observed (Figure 2C; Low-Mw Wsc1-HA). This mobility shift was due to the excessive de-mannosylation of the stem region, which is located in the extracellular space and is highly mannosylated (Figure 3). We also observed reductions of stable Wsc1-HA on the cell surface of *cyc8*Δ cells (data not shown). Taken together, these observations lead us to conclude that the EOM-dependent, harsh de-mannosylation in *cyc8*Δ cells causes dysfunctions in the stress-sensing capability of Wsc1. From these data, we proposed a model for the dysfunction of the MAPK pathway in *cyc8*Δ cells, as shown in Figure 3. In *cyc8*Δ cells cultured with YPMan, as a result of the under-glycosylation of Wsc1, unstable Wsc1 causes dysfunctions in the MAPK pathway under stress conditions. Therefore, *cyc8*Δ cells cultured with YPMan, display the severe cell wall defect phenotype.

Conclusion

Analysis of the phenotype of *cyc8*Δ not only revealed the regulatory mechanisms of the novel molecule endo-O-mannosidase, but also revealed a part of its physiological function. However, the details of the biological significance and molecular functions of EOM-dependent fOG generation remain unclear. Our future studies, which will include global screening for the gene encoding EOM, as well as for the genes involved in the generation and regulation of fOGs, will help to address these concerns.

References

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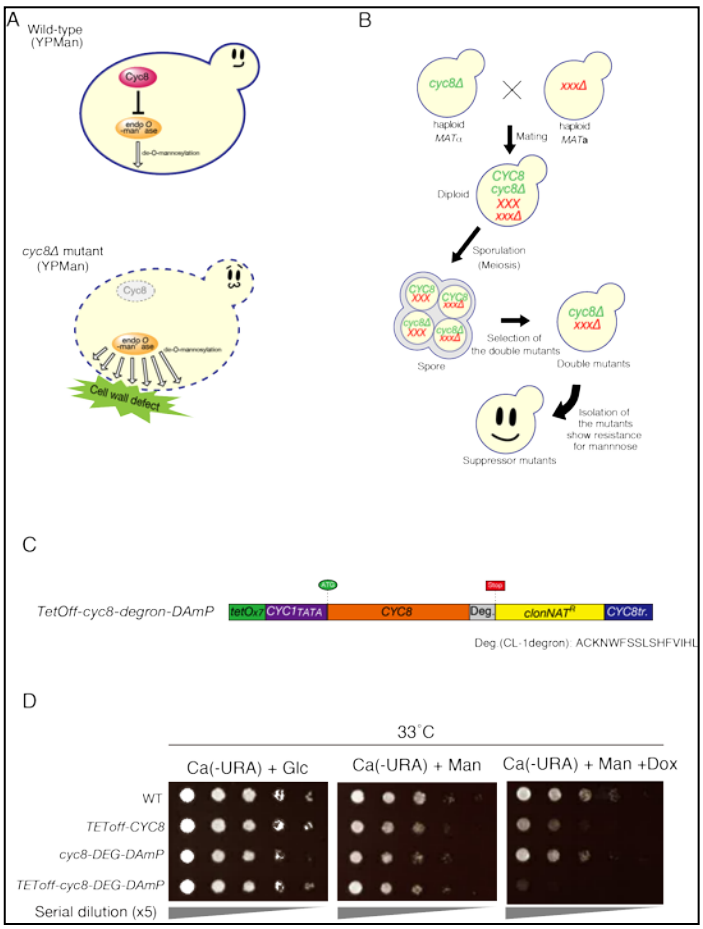

Figure 1. Construction of the doxycycline-dependent shutdown strain, *TetOff-cyc8-degron-DAmP*. (A) *cyc8*Δ cells show the cell wall defect phenotype in the mannose-containing culture condition, due to excessive EOM activity. (B) Strategy for the construction of ~4,000 double mutants in a *cyc8*Δ background, via mating and meiosis. (C) Schematic structures of *TetOff-cyc8-degron-DAmP*. (D) Study of the growth phenotype of various TetOff-cyc8 mutants via spotting assay.

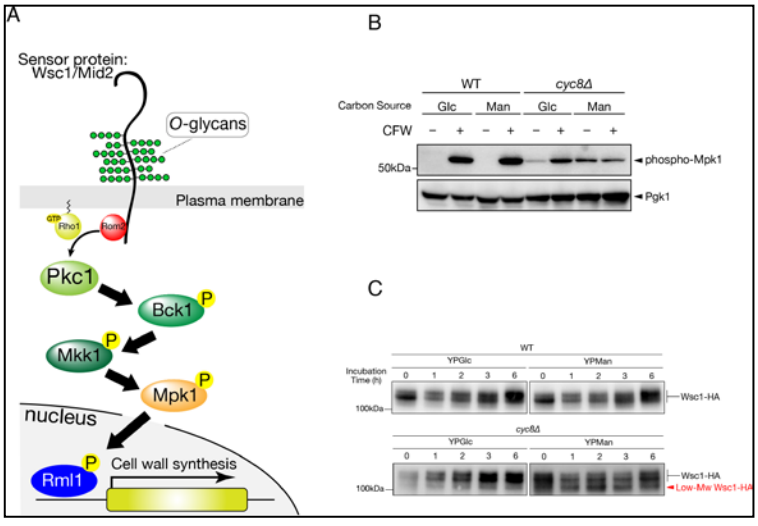

Figure 2. EOM-dependent, excessive de-mannosylation of Wsc1 disrupts the MAPK pathway. (A) The MAPK pathway mediates cell wall integrity under cell wall stress conditions. (B) Activation status of the MAPK pathway, confirmed by the phosphorylation of Mpk1, using anti-phosphorylated Mpk1 antibody (C) Glycosylation status of Wsc1 in wild type and *cyc8*Δ cells, in YPGlc or YPMan culture media.

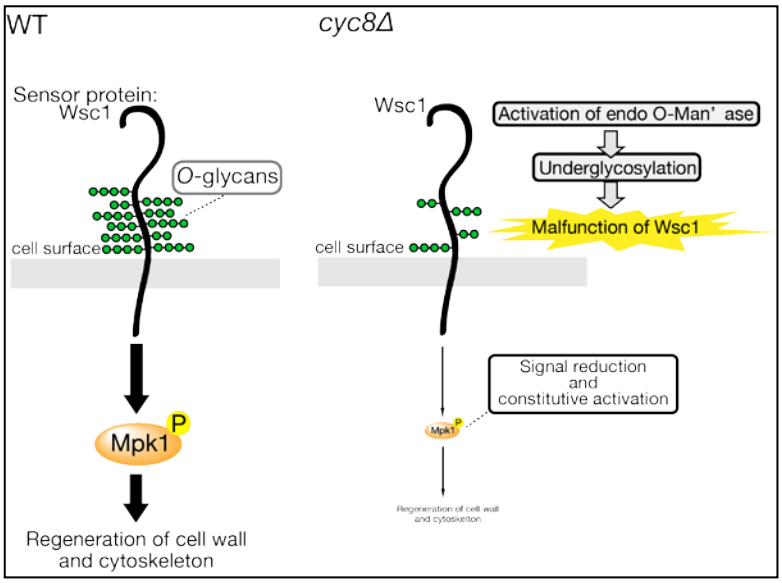

Figure 3. A model for the impairment of the MAPK pathway in *cyc8*Δ cells cultured with mannose.