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Functional analysis of recombinant human and *Yarrowia lipolytica* *O*-GlcNAc transferases expressed in *Saccharomyces cerevisiae*[§]

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_O_-linked β-_N_-acetylglucosamine (_O_-GlcNAc) glycosylation is an important post-translational modification in many cellular processes. It is mediated by _O_-GlcNAc transferases (OGTs), which catalyze the addition of _O_-GlcNAc to serine or threonine residues of the target proteins. In this study, we expressed a putative *Yarrowia lipolytica* OGT (YlOGT), the only homolog identified in the subphylum Saccharomycotina through bioinformatics analysis, and the human OGT (hOGT) as recombinant proteins in *Saccharomyces cerevisiae*, and performed their functional characterization. Immunoblotting assays using antibody against _O_-GlcNAc revealed that recombinant hOGT (rhOGT), but not the recombinant YlOGT (rYlOGT), undergoes auto-_O_-GlcNAcylation in the heterologous host *S. cerevisiae*. Moreover, the rhOGT expressed in *S. cerevisiae* showed a catalytic activity during *in vitro* assays using casein kinase II substrates, whereas no such activity was obtained in rYlOGT. However, the chimeric human-*Y. lipolytica* OGT, carrying the human tetratricopeptide repeat (TPR) domain along with the *Y. lipolytica* catalytic domain (CTD), mediated the transfer of _O_-GlcNAc moiety during the *in vitro* assays. Although the overexpression of full-length OGTs inhibited the growth of *S. cerevisiae*, no such inhibition was obtained upon overexpression of only the CTD fragment, indicating the role of TPR domain in growth inhibition. This is the first report on the functional analysis of the fungal OGT, indicating that the *Y. lipolytica* OGT retains its catalytic activity, although the physiological role and substrates of YlOGT remain to be elucidated.

***Keywords*:** *O*-GlcNAc glycosylation, *O*-GlcNAc transferase (OGT), *Yarrowia lipolytica*

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Introduction

O-GlcNAcylation, the addition of *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) to the hydroxyl group of serine or threonine residues of the target proteins, is a common post-translational modification of nuclear, cytoplasmic, and mitochondrial proteins (Torres and Hart, 1984). Majority of the proteins modified with *O*-GlcNAc belong to higher eukaryotes (Wells *et al.*, 2001; Love and Hanover, 2005; Hart *et al.*, 2007). *O*-GlcNAcylation is similar to protein phosphorylation, since both the modifications involve serine/threonine residues of the target proteins that are involved in controlling the responses to cell signaling. In addition, interplay between both the processes has also been reported (Wang *et al.*, 2008). Both the modifications may occur independently on the same amino acid residue in proteins or competitively on the adjacent residues in order to regulate the cellular signaling (Kamemura *et al.*, 2002; Yang *et al.*, 2006). *O*-GlcNAcylation regulates many cellular processes, including protein–protein interactions, protein function, localization, stability, and enzyme activity in mammals (Love and Hanover, 2005; Butkinaree *et al.*, 2010). *O*-GlcNAcylation of proteins influences a number of physiological parameters, such as memory, metabolism, and immunity by targeting various intracellular sites (Hardivillé and Hart, 2014; Yang and Suh, 2014). In plants, *O*-GlcNAc modification has been identified by the genetic screens for gibberellins (GA) response pathway (Wilson and Somerville, 1995; Silverstone *et al.*, 2007).

The process of *O*-GlcNAcylation is regulated by two enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). OGT catalyzes the addition of GlcNAc moiety from the donor substrate UDP-GlcNAc to the target protein substrates, whereas OGA catalyzes GlcNAc removal (Lubas *et al.*, 1997; Gao *et al.*, 2001). OGT is sub-divided into two domains depending on their roles in *O*-GlcNAcylation; catalytic domain (CTD) possessing glycosyltransferase activity at the C-terminus (Kreppel and Hart, 1999; Lubas and Hanover, 2000) and another domain with tandem arrays of tetratricopeptide repeat (TPR) motifs at the N-terminus (Blatch and Lässle, 1999; D'Andrea and Regan, 2003). The catalytic domains are highly conserved across most of species including humans, *Arabidopsis*, and the protist *Giardia* (Banerjee *et al.*, 2009). The TPR domain has been reported to play an important role in determining the substrate specificity of OGTs (Kreppel and Hart, 1999). The number of TPR motifs at the N-terminal region of all OGTs is variable, and has implications on protein-protein interactions involved in various cellular processes including transport of proteins, transcription, and cell cycle control (Blatch and Lässle, 1999; D'Andrea and Regan, 2003). Structural analysis of the human OGT re-

Table 1. List of plasmids used in this study

Plasmid	Description	Reference
pESC-URA	Yeast episomal expression vector containing the *GAL1* and *GAL10* promoters with *URA3*	Agilent technologies
pESU-YlOGT FL	pESC-URA for the expression of full-length of YlOGT tagged with 6 histidine residues	This study
pESU-YlOGT CTD	pESC-URA for the expression of catalytic domain of YlOGT tagged with 6 histidine residues	This study
pESU-hOGT FL	pESC-URA for the expression of full-length of hOGT tagged with 6 histidine residues	This study
pESU-hOGT CTD	pESC-URA for the expression of catalytic domain of hOGT tagged with 6 histidine resides	This study
pESU-h/YlOGT	pESC-URA for the expression of chimeric OGT protein composed of the TPR domain of hOGT and the catalytic domain of YlOGT tagged with 6 histidine residues	This study

vealed that the 'hinge' formed between the CTD and the 12th and 13th TPR is responsible for redirecting protein substrates to the active site for glycosylation (Lazarus *et al.*, 2011).

The presence of genes encoding putative OGTs in some protists and fungal species has been reported, suggesting the presence of *O*-GlcNAc modification in simple eukaryotes. Biochemical analysis demonstrated *O*-GlcNAcylation in the protists *Giardia* and *Cryptosporidium*, suggesting thereby that *O*-GlcNAcylation is one of the most abundant post-translational modifications in some of the earliest known simple eukaryotes (Banerjee *et al.*, 2009). However, the presence of *O*-GlcNAcylation has not been documented in yeasts and filamentous fungi (Hart *et al.*, 2007). In the present study, bioinformatics analysis for identification of putative fungal OGTs was performed, which revealed the presence of several putative OGTs in different fungal phyla. The OGT from *Yarrowia lipolytica*, however, was the only putative OGT identified in the subphylum Saccharomycotina. The putative *Y. lipolytica* OGT and the human OGT were heterologously expressed as recombinant proteins in the budding yeast *Saccharomyces cerevisiae*, which lacks *O*-GlcNAcylation activity, for comparative functional analysis. The *in vitro* activity assays and phenotypic analyses were performed to examine the function of recombinant *Y. lipolytica* and human OGT proteins in *S. cerevisiae*.

Materials and Methods

Bioinformatics analysis

For the collection of OGT orthologs, the human OGT sequence (accession number O15294.3) was retrieved from the National Center for Biotechnology Information (NCBI) protein database. OGT orthologs in different fungal species were searched using BLASTP with human OGT as the query sequence. The target organisms used for the identification of fungal OGT orthologs from each subphylum were "Pezizomycotina (taxid:147538)", "Saccharomycotina (taxid:147537)", "Taphrinomycotina (taxid:451866)", "Ustilaginomycotina (taxid:452284)", "Pucciniomycotina (taxid:29000)", "Agaricomycotina (taxid:5302)", or "Early diverging fungal lineages (taxid:112252)". Multiple sequence alignment of retrieved protein sequences was performed using the CLUSTALX2 software (http://www.clustal.org/clustal2/). Thereafter, a bootstrap Neighbor-Joining (NJ) phylogenetic tree was constructed. The gap positions were excluded when the tree was inferred, and the phylogenetic tree was visualized by NJplot program (http://doua.prabi.fr/software/njplot). The protein sequence and functional information about the human OGT were retrieved from the UniProt database. The functional domains of OGT orthologs were analyzed using NCBI Conserved Domain Search database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), SMART database (http://smart.mbl-heidelberg.de/), and Pfam database (http://pfam.xfam.org/).

Strains, plasmids, and culture conditions

The yeast strains used in the study were *S. cerevisiae* Y2805 (*MATα pep4∴HIS3 prb-1.6R canl his3-20 ura3-52*) and *Y. lipolytica* CX39-74B (ATCC 32339; *MatB trp1*). The plasmids constructed during the study are listed in Table 1. *S. cerevisiae* cells were grown in a selective synthetic complete (SC-Ura or SGal-Ura) medium (0.67% Yeast Nitrogen Base without amino acids, drop-out amino acid mixture without uracil, and 2% glucose or galactose) at 30°C.

Construction of vectors for expression of recombinant OGTs in *S. cerevisiae*

To construct a suitable vector for the expression of catalytic

Table 2. List of primer sequences used in this study

Primer name	Sequence description (5′ to 3′)
YlOGT CTD *Bam*HI fwd	AAGGATCCAAAATGTCTCCTTCTCTAGCTCAAGACATCAC
YlOGT CTD 6His *Nhe*I rev	GGCTAGCCTAATGATGATGATGATGATGACCGCCCATAACAATGTCC
YlOGT FL *Bam*HI fwd	AAGGATCCAAAATGGCATTCTCTATGAAGCTGTTCG
YlOGT FL *Sma*I rev	CGTTGAGAACACCTCCATTC
hOGT CTD *Bam*HI fwd	CAGGATCCATGCAGATTGTCTGTGATTGG
hOGT FL *Bam*HI fwd	AGGATCCAAAATGGCGTCTTCCGTGGGCAA
hOGT 6His *Sal*I rev	CGCGTCGACTTAATGATGATGATGATGATGTGCTGACTCAGTGACTTC
hOGT TPR *Spe*I rev	GCTCGACTAGTGTCAGCCACAATACTGAC
YlOGT CD *Spe*I fwd	GCAGCACTAGTCTCAACGTTGGATATGTG
YlOGT CD 6His *Kpn*I rev	GTGCTGGTACCCTAATGATGATGATGATG

domain at the C-terminus of *Y. lipolytica* OGT (YlOGT CTD) in *S. cerevisiae*, the DNA fragment encoding C-terminal region of YlOGT fused with hexahistidine tag (6x-His) was amplified using the primer pair "YlOGT CTD *Bam*HI fwd"/"YlOGT CTD 6His *Nhe*I rev" (Table 2). The amplicon was cloned into the *Bam*HI/*Nhe*I sites of pESC-URA (Agilent technologies) containing *GAL1* and *GAL10* promoters, and a selective marker *URA3*. The construct generated was termed as pESU-YlOGT CTD. The N-terminal region of YlOGT amplified using the primer pair "YlOGT FL *Bam*HI fwd"/"YlOGT FL *Sma*I rev" (Table 2) and cloned into the *Bam*HI/*Sma*I sites of pESU-YlOGT CTD vector to generate the plasmid pESU-YlOGT FL, expressing the full-length YlOGT.

For the construction of a suitable vector expressing human OGT (hOGT) protein in *S. cerevisiae*, the DNA fragments encoding full length hOGT with 6x-His residues and the CTD of hOGT fused with 6x-His residues were amplified from the vector p3xFLAG-CMV-human OGT (Park *et al.*, 2010) using the primer pairs "hOGT FL *Bam*HI fwd"/"hOGT 6His *Sal*I rev" and "hOGT CTD *Bam*HI fwd"/"hOGT 6His *Sal*I rev", respectively (Table 2). Each of the amplified PCR fragments was digested with *Bam*HI/*Sal*I and then cloned into pESC-URA vector in the same sites to generate the constructs pESU-hOGT FL and pESU-hOGT CTD, respectively.

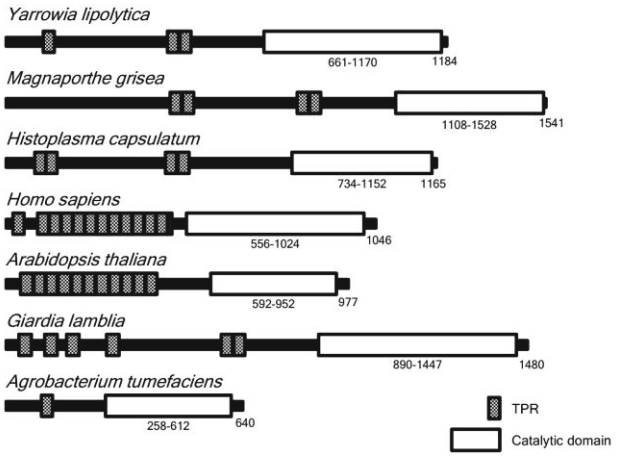

Fig. 2. Structural domain analysis of OGTs from various organisms. Schematic representation of the OGTs from selected species. The functional domains of OGT orthologs were inferred, using the NCBI Conserved Domain Search, SMART, and Pfam databases.

To construct a vector for expression of human-YlOGT chimeric protein, the TPR domain of hOGT was amplified from the vector p3xFLAG-CMV-human OGT using the primers

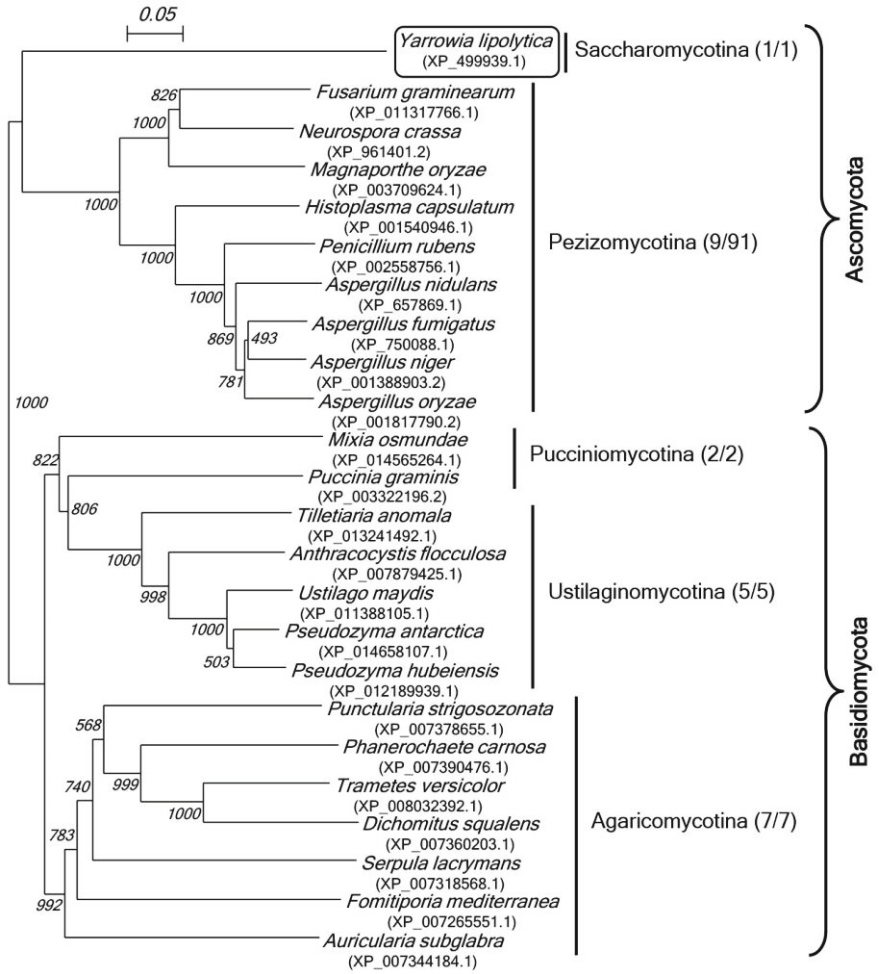

Fig. 1. Phylogenetic analysis of putative OGTs in fungi. The phylogenetic tree of the 24 putative OGT sequences was inferred using the Neighbor-Joining method implemented in CLUSTALX2, and visualized by NJplot. *Y. lipolytica* is marked with a box. The numbers of putative OGTs analyzed for phylogenetic tree among the total identified OGTs are shown in the brackets next to each phylum. In the case of Pezizomycotina, only 9 among 91 putative OGTs, which have been relatively well studied as a model organism or as an organism with medical and industrial application, were chosen for the analysis. The NCBI accession numbers of reference sequences for the 106 putative fungal OGT are listed in the supplementary data.

"hOGT FL *Bam*HI fwd"/"hOGT TPR *Spe*I rev" and digested with *Bam*HI/*Spe*I. The CTD of YlOGT was amplified from the plasmid pESU-YlOGT FL using the primers "YlOGT CD *Spe*I fwd"/"YlOGT CD 6His *Kpn*I rev", followed by digestion with *Spe*I/*Kpn*I. The two digested PCR fragments were ligated together and cloned into the *Bam*HI/*Kpn*I sites of pESC-URA vector, resulting in the construct pESU-h/YlOGT.

Western blot analysis for expression of recombinant OGTs

The generated OGT expression constructs were transformed into *S. cerevisiae* strain Y2805, according to the modified lithium acetate dimethyl sulfoxide (DMSO) protocol (Hill *et al.*, 1991). The transformed yeast cells were inoculated in SGal-Ura medium containing 2% galactose and cultivated at 28°C for 24 h. Total soluble protein fraction was extracted and analyzed by western blotting using the 6x-His mouse monoclonal antibody (Santa-Cruz). Auto-*O*-GlcNAcylation was detected using mouse *O*-GlcNAc monoclonal antibodies (RL2 or CTD110.6) (Santa-Cruz) with or without the pre-incubation with 30 mM and 100 mM GlcNAc, respectively.

In vitro OGT activity assay

The *in vitro* assay of OGT activity was carried out as described previously (Lefebvre *et al.*, 2013). Briefly, the cell lysates were incubated with 1 μCi UDP-[^{3}H]GlcNAc (American Radiolabeled Chemicals) and 1 mM casein kinase II peptide as substrates (PGGSTPVSSANMM) in the reaction buffer (50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 12.5 mM $MgCl_2$) for 90 min at 37°C. The reaction was stopped by addition of 50 mM formic acid. Thereafter, the reaction mixture was purified using Sep-Pak C18 cartridge (Waters). The presence of incorporated peptide with [^{3}H]GlcNAc was detected using a liquid scintillation counter (PerkinElmer).

Growth analysis of recombinant yeast strains expressing OGTs

Transformed yeast cells were inoculated into SC-Ura medium and grown at 28°C overnight. The pre-cultured cells were adjusted to an initial optical density at 600 nm (OD_{600}) of 1.0 in sterilized water and were serially diluted by 10 folds. The recombinant yeast cells were spotted on SC-Ura, SGal-Ura, and SGal-Ura agar plates supplemented with 0.5 μM calcofluor white, followed by incubation at 30°C for 3 days.

Results

Phylogenetic analysis to identify putative fungal OGTs

Although *O*-GlcNAc modification has been discovered in all multicellular organisms, including worms, insects, plants, and animals (Hart and Akimoto, 2009), its presence in fungi is still unknown. A fungal pathogen, *Histoplasma capsulatum* (an asexual reproductive stage of *Ajellomyces capsulatus*), which leads to a wide range of clinical symptoms from asymptomatic to fatal infections, is the only species in which *O*-GlcNAc signaling is implicated in regulating fungal morphogenesis (Gilmore *et al.*, 2013). To identify putative OGT proteins in fungi, the BLAST search followed by comparative sequence analyses were performed in the study. A total of 106 sequences of putative fungal OGTs were identified (Supplementary data Table S1), and only a single putative OGT homolog was detected in all the fungal species analyzed. Of these, the phylogenetic relationship between 24 OGTs was inferred using the Neighbor-Joining method in CLUSTALX2, and the resulting tree was visualized by NJ plot (Fig. 1).

Sequence similarities and phylogenetic analysis revealed the presence of putative OGTs in numerous fungal species. In the phylum Basidiomycota, 14 OGTs (7 in Agaricomycotina, 5 in Ustilaginomycotina, and 2 in Pucciniomycotina) were identified. In the phylum Ascomycota, 91 OGTs were identified in the subphylum Pezizomycotina. No OGT, however, was identified in the subphylum Taphrinomycotina, containing *Schizosaccharomyces pombe*, which is the earliest ascomycete lineage that diverged before separation of the Pezizomycotina and Saccharomycotina. Interestingly, the subphylum Saccharomycotina had only one putative fungal OGT in the early divergent member *Yarrowia lipolytica,* which is a dimorphic yeast with unusual physiological, metabolic, and genomic features that are quite distinctive from other yeasts (Park *et al.*, 2014; Zinjarde *et al.*, 2014). Being one of the non-conventional yeasts, *Y. lipolytica* has potential applications in industry, molecular biology, and genetics (Coelho *et al.*, 2010; Kim *et al.*, 2015; Madzak, 2015).

Domain structure analysis of *Y. lipolytica* OGT

To characterize the domain organization of putative *Y. lipolytica* OGT (YlOGT), the functional domains were predicted by NCBI Conserved Domain Database, SMART, and Pfam and compared to the OGTs from other organisms (Fig. 2). YlOGT polypeptide had 1,184 amino acids and was predicted to contain three TPRs at the N-terminal region and a large CTD at the C-terminal region. The OGTs of fungi, including *Magnaporthe grisea*, *H. capsulatum*, and *Y. lipolytica*, appeared to be longer than those found in animals and plants. Interestingly, OGTs from fungi, protists, and bacteria had fewer TPR compared to their animal and plant homologs.

For a more detailed insight into the functional domains, the amino acid sequences of TPR and CTD regions of YlOGT were aligned with OGT sequences from *M. grisea* (Mg), *H. capsulatum* (Hc), and humans (Hs) using CLUSTALX2 (Fig. 3). The TPR domain of OGT is responsible for substrate recognition, and a part of C-terminal region of the TPR domain is particularly important because it forms a binding pocket for substrates (Lazarus *et al.*, 2011). It was found that a part of TPR domain was highly conserved among the three fungal species, but was quite different from human OGT (hOGT). In particular, the TPR sequences, which are believed to be responsible for controlling the access to substrates, were highly conserved within fungal OGTs (Fig. 3A). An intervening domain exists between N-terminal and C-terminal catalytic domains. Except this intervening domain, the catalytic domains at both the terminal regions were shown to be highly conserved within these four species. There was 34% sequence identity between the CTDs of *Y. lipolytica* and human OGTs (Fig. 3B). This considerably high sequence identity in the CTDs between yeast and human OGTs sup-

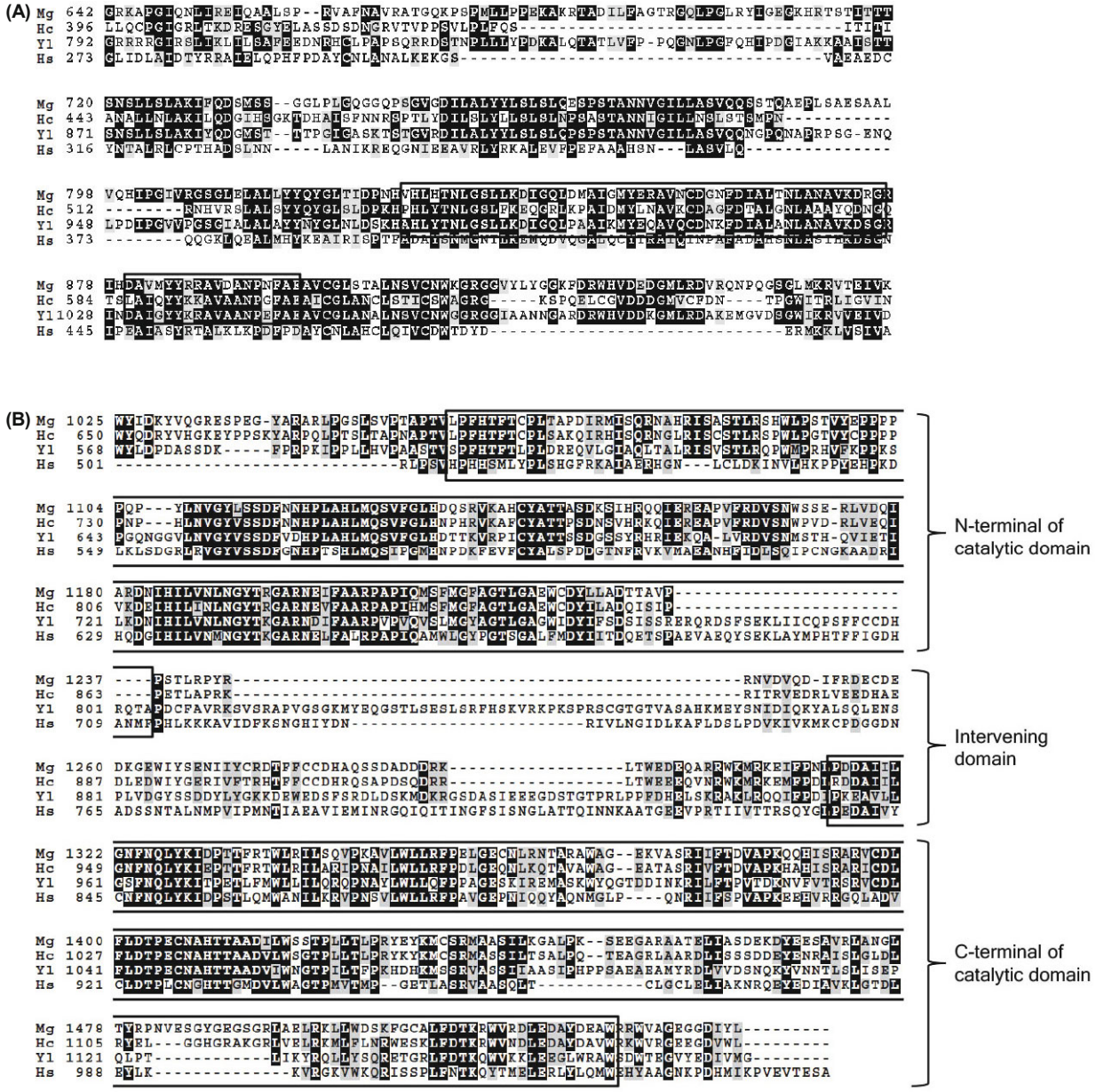

Fig. 3. Comparison of functional domains of fungal and human OGTs. Amino acid sequence alignment of the last TPR, which is responsible for controlling substrate accession (A), and catalytic domain (B) of OGTs from *Y. lipolytica* (Yl), *M. grisea* (Mg), *H. capsulatum* (Hc), and human (Hs). The last TPR motif and the two parts of catalytic domain are marked by solid boxes.

ports the notion that the catalytic domains of OGTs are conserved among diverse eukaryotes. It also indicates that the YlOGT might retain its enzymatic activity as *O*-GlcNAc transferase, similar to that of hOGT.

Expression of recombinant *Y. lipolytica* and human OGTs in *S. cerevisiae*

In order to investigate the enzyme activity for *O*-GlcNAc modification, full-length YlOGT and hOGT proteins were expressed in *S. cerevisiae* as recombinant proteins tagged with 6x-His residues at their C-terminus, and were designated rYlOGT(FL) and rhOGT(FL), respectively. In addition, the His-tagged catalytic domains of YlOGT and hOGT were expressed as recombinant proteins, as were designated rYlOGT(CTD) and rhOGT(CTD), respectively (Fig. 4A). Recombinant OGTs expressed in *S. cerevisiae* were analyzed by western blotting using anti-His antibody (Fig. 4B, (a) and (b)), revealing the successful expression of recombinant OGT proteins of expected sizes in *S. cerevisiae*. The *O*-GlcNAc antibody (RL2) was used to detect auto-*O*-GlcNAcylation in the recombinant OGT proteins. The rhOGT(FL) protein (molecular weight 117 kDa) was identified to be auto-*O*-GlcNAcylated (Fig. 4B, (c) and (d)). Some additional proteins with molecular weight of about 150 kDa were also

identified using RL2 antibody, suggesting that some yeast proteins might be *O*-GlcNAcylated by overexpressing the hOGT. In contrast, rYlOGT was not detected by RL2 antibody, suggesting that rYlOGT was not auto-*O*-GlcNAcylated. The auto-*O*-GlcNAcylation of hOGT in *S. cerevisiae* was further detected using another type of *O*-GlcNAc CTD110.6 antibody (data not shown), suggesting that the hOGT is functionally active as an *O*-GlcNAc transferase in *S. cerevisiae*.

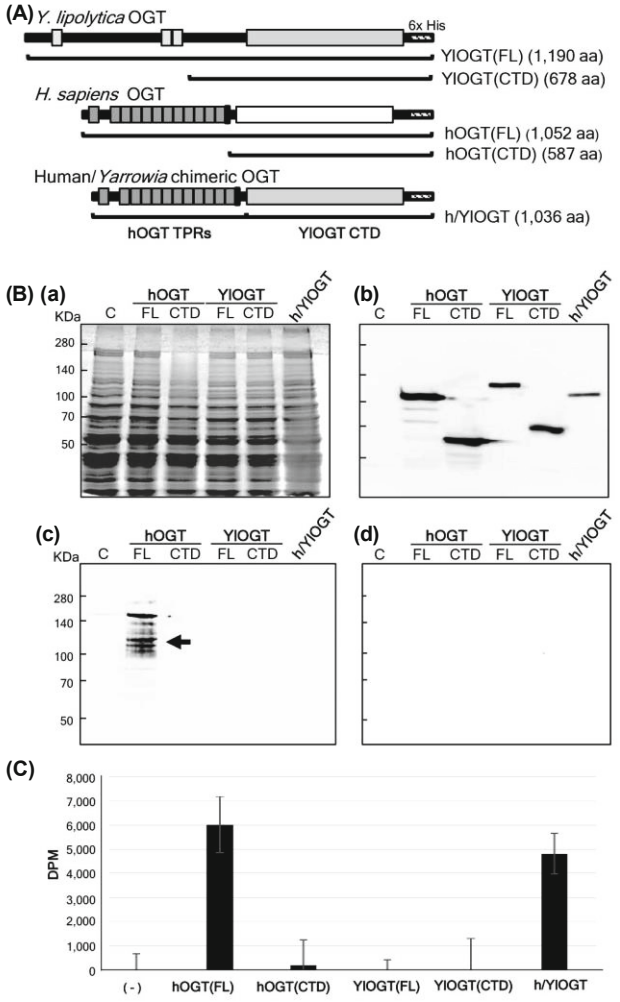

Fig. 4. Analysis of recombinant OGTs expressed in *S. cerevisiae*. (A) Schematic representation of the recombinant OGT proteins expressed in *S. cerevisiae*. Full length (FL) or catalytic domain (CTD) of *Y. lipolytica* and human OGT were expressed under the *GAL1* promoter on the episomal plasmid pESC-URA. The h/YlOGT recombinant protein was expressed as a chimeric protein composed of 495-aa hOGT TPR and 535-aa YlOGT CTD fragments. (B) Analysis of rOGTs expression by western blotting. (a) Coomassie brilliant blue staining. (b) Western blotting with anti-histidine antibody. hOGT(FL), 117 kDa; hOGT(CTD), 64 kDa; YlOGT(FL), 131 kDa; YlOGT CTD, 75 kDa; and h/YlOGT 114 kDa. Western blotting of rOGTs with anti-*O*-GlcNAc antibody (RL2) without (c) and with preincubation with the competitor 100 mM GlcNAc (d). (C) *In vitro* OGT activity assay of rOGTs expressed in *S. cerevisiae*. The incorporation of [^{3}H]GlcNAc into the CKII peptide was analyzed using scintillation counter in a reaction employing the partially purified cell extracts of the recombinant *S. cerevisiae* as the OGT source. To adjust the levels of rOGTs in the reaction, the *S. cerevisiae* cell extract expressing human hOGT(FL) was diluted 50 times, while the *S. cerevisiae* cell extracts expressing hOGT(CTD) and YlOGTs were diluted 20 times with cell lysis buffer in comparison to the cell extract expressing h/YOGT. The reaction without CKII peptide was used as a negative control for each reaction.

In vitro activity assay of recombinant OGTs

Severe degradation of rOGT proteins was observed during the purification process. Therefore, yeast cell extracts expressing the rOGTs were used as the source of OGT proteins in the study. To examine the enzyme activity of rOGTs expressed in *S. cerevisiae*, yeast cell extracts expressing rOGTs were incubated with tritiated UDP-GlcNAc (UDP-[^{3}H]GlcNAc) as the donor and casein kinase II (CKII) synthetic peptide as the target substrate. The CKII synthetic peptide, PGGSTPVS* SANMM, is the representative substrate for hOGT. Although the peptide contains multiple serine and a threonine residue, only the serine residue indicated by an asterisk (S*) undergoes glycosylation by OGT (Lazarus *et al.*, 2011). The transfer of [^{3}H]GlcNAc from the donor UDP-[^{3}H]GlcNAc to the Ser* of CKII peptide following incubation with the yeast extracts was measured by a scintillation counter, which was indicative of the level of OGT activity. *S. cerevisiae* cell extracts diluted to obtain approximately the same amount of expressed rOGTs for *in vitro* assays (Fig. 4C). The values of enzyme activity were determined by the measuring the difference of DPM (disintegration/min) values between the reactions with and without (negative control) CKII substrates.

The *S. cerevisiae* extract overexpressing rhOGT(FL), a full length human OGT, showed apparently an enzyme activity, whereas no detectable activity was observed in the yeast extract of rhOGT(CTD) containing only the catalytic domain (Fig. 4C). In case of the yeast extracts, expressing either full length or catalytic domain of rYlOGT proteins did not show any activity. It is possible that YlOGT could not recognize CKII as a substrate, since the TPR domain of YlOGT is quite different from that of hOGT and substrate recognition by OGTs is dependent on the TPR domain (Lubas and Hanover, 2000). To address this issue, a domain swapping experiment was conducted by fusing the C-terminal fragment of YlOGT containing CTD with the N-terminal fragment of hOGT containing TPR, resulting in a chimeric human-*Y. lipolytica*

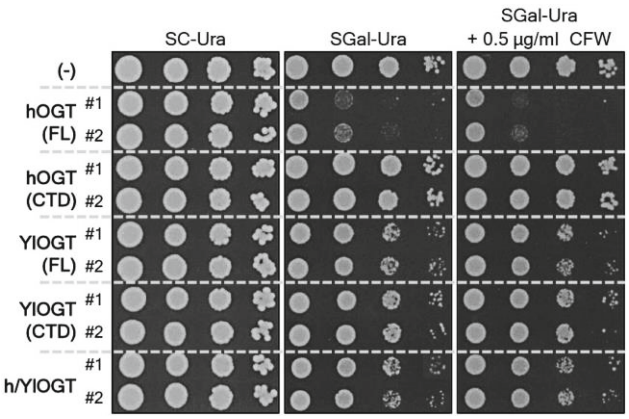

Fig. 5. Growth phenotypes of *S. cerevisiae* expressing recombinant OGTs. A series of 10-fold dilutions of yeast cells was spotted on agar plates of SC-Ura, SGal-Ura, and SGal-Ura+CFW, supplemented with calcofluor white (0.5 μg/ml), and incubated at 30°C for 3 days.

OGT, designated as h/YlOGT (Fig. 4A). The recombinant fusion OGT was expressed with the expected size of 114 kDa in *S. cerevisiae* (Fig. 4B, (a) and (b)). Although auto-*O*-GlcNAcylation of the chimeric OGT was not detected in the western blotting with RL2 antibody (Fig. 4B, (c) and (d)), h/YlOGT showed an enzyme activity in the *in vitro* assay at a relatively comparable level to that of rhOGT(FL) (Fig. 4C), suggesting that the CTD of YlOGT might retain its catalytic activity for *O*-GlcNAcylation.

Phenotypic analysis of recombinant *S. cerevisiae* expressing YlOGT or hOGT

The effect of overexpression of recombinant OGTs on the growth of *S. cerevisiae* was examined (Fig. 5). The yeast cells overexpressing rOGTs were spotted onto the SC-Ura and SGal-Ura agar plates, followed by incubation at 30°C for 3 days. The overexpression of full-length hOGT severely inhibited the growth of *S. cerevisiae* (Fig. 5, SGal-Ura plate). The overexpression of the full-length YlOGT or chimeric h/YlOGT also resulted in marginal growth inhibition. No growth inhibition, however, was observed with the overexpression of only CTD in both human and *Y. lipolytica* OGTs, suggesting that full-length OGT is required for growth inhibition. It could also be speculated that functionally active human and chimeric OGTs overexpressed in *S. cerevisiae* may compete for UDP-GlcNAc, which is the substrate for nascent chitin biosynthesis, resulting in growth inhibition by disturbing the cell wall integrity (Cabib *et al.*, 2008). The phenotypic analysis of yeast expressing rOGT in the presence of the cell wall disturbing reagent calcofluor white, however, did not show remarkable differences in growth patterns (Fig. 5, SGal-Ura+CFW). This raises the possibility that *O*-GlcNAc modification of yeast proteins by hOGT, as indicated in Fig. 4C, may exert negative effects on their functions pertaining to the growth of yeast.

Discussion

In the present study, bioinformatics analysis revealed that most members of the subphylum Saccharomycotina have lost the OGT genes, and that the gene is present only in the members of early divergent lineages, such as *Y. lipolytica* (Fig. 1). The presence of a well-conserved catalytic domain in YlOGT suggested towards its catalytic activity as *O*-GlcNAc transferases. A large variation, however, in the number of TPR in YlOGT was observed, along with sequence divergence from other OGTs of higher eukaryotes, indicating the presence of different modes of target protein recognition (Figs. 2 and 3). *In vitro* and *in vivo* functional analyses of YlOGT overexpressed in *S. cerevisiae*, in comparison with hOGT, suggested that YlOGT may possess catalytic activity as *O*-GlcNAc transferases (Figs. 4 and 5). For investigation of the physiological role of OGTs in *Y. lipolytica*, a YlOGT null mutant strain was generated and examined for the changes in growth pattern under different conditions such as high temperature, high osmotic shock, cell wall disturbance, and oxidative stress (Supplementary data Fig. S1). Diverse forms of cellular stresses and tissue injuries resulted in enhanced *O*-GlcNAcylation in animal cells and, thus, it was concluded that *O*-GlcNAc regulates numerous intracellular proteins involved in the cellular stress responses (Groves *et al.*, 2013). The deletion of *YlOGT* gene in *Y. lipolytica*, however, did not bring about apparent changes in the growth patterns under the most conditions tested in this study. Our results strongly indicate that the *Y. lipolytica* OGT retains its catalytic activity. However, further studies are required to understand the physiological functions of the *Y. lipolytica* OGT in detail.

O-GlcNAcylation modulates cell signaling and other cellular processes through an intricate interplay with protein phosphorylation, and serves as a key sensor of nutrients by linking the hexosamine biosynthetic pathway to cellular signaling (Butkinaree *et al.*, 2010). A recent study reported the presence of sites for the *O*-linked mannose addition (*O*-Man glycosites) on nuclear, cytoplasmic, and mitochondrial proteins in *S. cerevisiae* and *S. pombe* (Halim *et al.*, 2015). It was found that the nucleocytoplasmic *O*-Man glycosites were located on proteins at positions resembling that of the *O*-GlcNAcylation process in higher eukaryotes, indicating that the yeast-specific *O*-Man glycosylations may play important biological functions similar to the *O*-GlcNAcylation in higher eukaryotes. The observation that the overexpression of hOGT severely inhibited the growth of *S. cerevisiae* might reflect aberrant *O*-GlcNAcylation of some yeast proteins involved in normal growth (Fig. 5). It will be interesting to identify protein targets of *O*-GlcNAcylation by hOGT, in comparison with the natural targets of *O*-Man glycosylation.

In conclusion, in the present study, the putative fungal OGTs based on the sequence similarity of domain structures were identified, revealing the conservative features of catalytic domains along with the specific configuration of TPR domains within fungal species. Although the activity of these fungal OGTs has not yet been demonstrated, it is likely that some fungal OGTs have specialized functions *in vivo*. As the first report on the functional analysis of the fungal OGTs, activity of *Y. lipolytica* OGT was compared with that of the human OGT through heterologous expression of the proteins in *S. cerevisiae*. This study also provided information about the structure domains of putative fungal OGTs. Besides, the resources established during this study, such as a functional analysis system employing *S. cerevisiae* as an expression system, are expected to provide useful tools for the detailed functional studies of fungal OGTs.

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