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GTS1 Induction Causes Derepression of Tup1-Cyc8-Repressing Genes and Chromatin Remodeling through the Interaction of Gts1p with Cyc8p

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***GTS1* in yeast *Saccharomyces cerevisiae* is a pleiotropic gene, the expression of which affects a wide range of biological phenomena. A genome-wide transcriptional analysis identified genes upregulated in response to *GTS1* induction, most of which were found to be regulated by the Tup1-Cyc8 co-repressor. Among the upregulated genes, *FLO1* was indispensable for cell aggregation, one of the pleiotropic effects of *GTS1*. Deletion of genes encoding the chromatin remodeling complex SWI/SNF abolished *FLO1* upregulation and decreased cell aggregation, although the nuclear localization of Gts1p required for cell aggregation was not affected. *GTS1* induction caused chromatin remodeling at the *FLO1* promoter in a SWI/SNF-dependent manner on micrococcal nuclease accessibility assay. Cyc8p was detected in Gts1p-mediated protein aggregates by agarose gel electrophoresis, indicating that Gts1p inhibited Cyc8p function. These results suggest that inhibition of the Tup1-Cyc8 complex and subsequent SWI/SNF-dependent chromatin remodeling result in Gts1p-mediated gene derepression.**

Key words: *GTS1*; Tup1-Cyc8 complex; SWI/SNF complex; chromatin remodeling; protein aggregation

GTS1, isolated from a cDNA library of *Saccharomyces cerevisiae* as a clock gene candidate, is related to pleiotropic effects in yeast. These effects include cell aggregation, the unbudding period, drug resistance, heat tolerance in the stationary phase, ultradian oscillation of energy metabolism, and the regulation of dissolved oxygen levels in continuous cultures.[1–4] These Gts1p-mediated phenotypes are promoted in a gene dosage-dependent manner. A reduced phenotype was observed in the *GTS1* null mutant, whereas *GTS1* induction causes pleiotropic effects, with the exception of disturbance of ultradian oscillation, but the Gts1p-mediated molecular mechanism remains unclear.

Gts1p contains a zinc finger motif similar to that of GATA transcription factors in the N-terminal region, but in contrast to GATA transcription factors, Gts1p binds to proteins rather than to DNA.[5] In addition, Gts1p contains a poly-Q-rich region that functions as an activator,[6] and this region is found in transcriptional activators of higher organisms and acidic activators of yeast.[7]

The relationship between *GTS1* induction and pleiotropic effects suggests that Gts1p participates in global transcriptional regulation mechanisms and alters the expression of a wide variety of genes. In cultivated yeast, the amount of Gts1p changes periodically, with fluctuations greater than 4-fold, and these fluctuations in Gts1p levels are correlated with the degree of pleiotropic effects.[4,8] Therefore, the effect of Gts1p is more apparent in cells with *GTS1* induction, and *GTS1* induction is suitable in attempts to understand the Gts1p-mediated molecular mechanism. Among the pleiotropic effects, cell aggregation is a useful indicator because it is easy to detect. Hence, we investigated the molecular mechanism regulating cell aggregation, as a representative of Gts1p-mediated pleiotropic effects. In this study, genome-wide transcriptional analysis using DNA microarray was performed to analyze Gts1p-mediated signal transduction, and 19 genes were identified the expression of which was upregulated by more than 2.5-fold. Most of the upregulated genes were found to be repressed by the Tup1-Cyc8 complex, a global repressor of several diverse families of genes.[9,10] Furthermore, chromatin remodeling in the promoter of the upregulated gene and protein interaction of Gts1p with Cyc8p were identified. Our findings suggest that *GTS1* can induce gene activations that result from inhibition of the Tup1-Cyc8 complex followed by chromatin remodeling.

Materials and Methods

Strains and media. The yeast strains used in this study are listed in Table 1. The *S. cerevisiae* GPMH6 strain,[11] derived from *S. cerevisiae* strain MT8-1 (*MATa*, *ade*, *his3*, *leu2*, *trp1*, *ura3*),[12] was used as the parent strain for *GTS1* induction in DNA microarray analysis. Deletion mutant strains derived from BY4741 were generated by EUROFAN II project from EUROSCARF (Frankfurt, Germany). The yeast strain harboring endogenous *CYC8* fused with a C-terminal TAP tag-encoding sequence derived from BY4741, named CYC8-TAP, was obtained from Open Biosystems (Huntsville, AL).

Yeast transformation was performed by the lithium acetate method.[13] Transformants were selected on a selective synthetic dextrose (SD) plate (0.67% w/v yeast nitrogen base without amino acids from Difco, Detroit, MI, 2% w/v glucose, and appropriate supplements) with and without casamino acids, and were cultivated aerobically in the same medium. For DNA manipulation such as plasmid construction, *Escherichia coli* DH5α [F$^-$, φ80d*lac*ZΔM15, Δ (*lac*ZYA-*arg*F)U169, *hsd*R17 (r_K^-, m_K^+), *rec*A1, *end*A1, *deo*R, *thi*-1, *sup*E44, *gyr*A96, *rel*A1, λ^-] was used as the host in transformation. *E. coli* was grown in Luria-Bertani (LB) medium (1% w/v tryptone, 0.5% w/v yeast extract, and 0.5% w/v sodium chloride) supplemented with 100 μg mL^{-1} of ampicillin, as required.

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Table 1. Yeast Strains Used in This Study

Strains	Genotype	Source
MT8-1	*MAT*a *ade his3 leu2 trp1 ura3*	Tajima *et al.*[12]
GPMH6	*MAT*a *ade his3 leu2 trp1*	Kuroda *et al.*[11]
GP*flo11*	GPMH6 *flo11::HIS3*	This study
GP*upc2*	GPMH6 *upc2::HIS3*	This study
GP*dan1*	GPMH6 *dan1::HIS3*	This study
GP*flo1*	GPMH6 *flo1::HIS3*	This study
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
snf2Δ	BY4741 *snf2::kanMX4*	EUROSCARF
snf5Δ	BY4741 *snf5::kanMX4*	EUROSCARF
flo1Δ	BY4741 *flo1::kanMX4*	EUROSCARF
CYC8-TAP	BY4741 *CYC8-TAP-His3MX*	Open Biosystems

Table 2. Plasmids Used in This Study

Plasmid name	Marker	Coding gene	Promoter	Tag
pHDflo1	*HIS3*	*FLO1*		
pMCG-T	*TRP1*	*GTS1*-EGFP	*CUP1*	
pM*GTS1*-EGFP	*HIS3*	*GTS1*-EGFP	*CUP1*	
pMCG1	*TRP1*	*GTS1*	*CUP1*	
pMCG2	*HIS3*	*GTS1*	*CUP1*	
pMCG3	*URA3*	*GTS1*	*CUP1*	c-myc
pMCG4	*TRP1*	*GTS1*	*GAL1*	FLAG
pYEX-BX-FLAG-GTS1	*URA3*	*GTS1*	*CUP1*	FLAG
pYEX-BX-HA-CYC8	*URA3*	*CYC8*	*CUP1*	HA

Gene disruption. Deletion mutant strains derived from the GPMH6 strain were generated by the PCR-mediated gene disruption method,[14] based on homologous recombination. The PCR products used were amplified using oligonucleotides that contained the gene-specific flanking sequence at the 5′ end and the *HIS3* marker gene-specific sequence at the 3′ end. For *FLO1* gene disruption, pHDflo1, containing 500-bp fragments of the upstream and downstream regions of the *FLO1* gene *ORF*, was constructed by ligation of homologous PCR products amplified from the MT8-1 genomic DNA into pRS403.[15] After yeast transformation with this fragment, gene disruption was confirmed by PCR using oligonucleotides annealing upstream and downstream of the *ORF* region of the specific gene.

Plasmid construction. The plasmids used in this study are listed in Table 2. The plasmid for the production of Gts1p-EGFP (enhanced green fluorescent protein) was constructed as follows: *GTS1 ORF*, amplified from the *S. cerevisiae* MT8-1 genomic DNA by PCR using oligonucleotides 5′-CTTAGTGATCAATGAGGTTTAGGAGTTCTT-CCC-3′ (*Bcl*I-compatible site of *Bam*HI underlined) and 5′-GTATC-TGTCGACGATTGTGTGTAGAAATAACCTTGTGGC-3′ (*Sal*I site underlined), and the EGFP-encoding sequence, amplified from pEGFP (Clontech, Mountain View, CA) by PCR using oligonucleotides 5′-CGGTCGCGTCGACTGTGAGCAAGGGCGAGGAGCTG-3′ (*Sal*I site underlined) and 5′-GTCGCGGAATTCTTACTTGTACAGCTCGT-CCATGC-3′ (*Eco*RI site underlined) were inserted downstream of the *CUP1* promoter in plasmid pYEX-BX (Clontech) with the *Bam*HI-*Sal*I and the *Sal*I-*Eco*RI section respectively. To change the auxotrophy, the *Hin*dIII-*Eco*RI section containing this fusion gene was blunted and transferred into the *Sma*I site of the pMW1 multicopy plasmid[16] and named pMCG-T. Plasmid pM*GTS1*-EGFP,[17] which has the same promoter and coding sequence as pMCG-T and has *HIS3* auxotrophic marker, was also used for the production of Gts1p-EGFP. The plasmid for the production of Gts1p fused with the c-myc epitope at the C-terminus was constructed as follows: the *CUP1* promoter-*GTS1 ORF* fused with the c-myc encoding sequence, amplified by PCR from pMCG1[18] using oligonuculeotides 5′-AAGCTGAATTCTC-TTTTGCTGGCATTTCTTCTAGAAGC-3′ (*Eco*RI site underlined) and 5′-ATCTTCTCGAGTTACAGGTCTTCTTCGGAAATCAAC-TTCTGTTCCTCTTGTGTGTAGAAATAACCTTGTGG-3′ (*Xho*I site underlined, c-myc-encoding sequence double-underlined) was inserted into pESC-Ura (Stratagene, La Jolla, CA) with the *Eco*RI-*Xho*I section. The resulting plasmid was named pMCG3. Plasmid pMCG1 for *GTS1* induction under the *CUP1* promoter with the *TRP1* marker was used in deletion mutant strains derived from the GPMH6 strain. pMCG2[17] for *GTS1* induction under the *CUP1* promoter with the *HIS3* marker and pMCG3 were used for *GTS1* induction in deletion mutant strains derived from BY4741. To express *GTS1* fused with the FLAG-encoding sequence at the N-terminus under the *GAL1* and the *CUP1* promoter, plasmids, pMCG4 and pYEX-BX-FLAG-GTS1 respectively[17] were used. The plasmid for the expression of *CYC8* fused with the HA-encoding sequence at the N-terminus under the *CUP1* promoter was constructed as follows: *CYC8 ORF* fused with the HA sequence was amplified from the MT8-1 genomic DNA by PCR using oligonucleotides 5′-ATGCAGGATCCATGTACCCATACGATGTT-CCAGATTACGCTGGTGGTTCTGGTGGTAATCCGGGCGGTGAA-CAAACAATAATG-3′ (*Bam*HI site underlined, HA-encoding sequence double-underlined) and 5′-AGTCAGAATTCTTAGTCGT-CGTAGTTTTCATC-3′ (*Eco*RI site underlined), and inserted into pYEX-BX with the *Bam*HI-*Eco*RI section. The resulting plasmid was named pYEX-BX-HA-CYC8.

RNA isolation and DNA microarray analysis. Yeasts grown to stationary phase were transferred to fresh medium containing 500 μM $CuSO_4$ and adjusted to an optical density of cell culture at 600 nm (OD_{600}). After 3 h of cultivation, total RNA was isolated from the cells suspended in LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris–HCl pH 7.4, and 0.2% w/v SDS) using ISOGEN-LS (Nippon Gene, Toyama, Japan) with glass bead disruption, as described previously.[19] Poly(A)$^+$ RNA was purified from total RNA using the Oligotex-dT30 ⟨super⟩ mRNA purification kit (Takara, Kyoto, Japan). The purified poly(A)$^+$ RNA was converted to double-stranded cDNA using an oligo-dT primer with a T7 RNA polymerase promoter sequence at the 5′ end. From the cDNA purified with phenol/chloroform/isoamyl alcohol, labeled cRNA was amplified by *in vitro* transcription using the T7 Megascript System (Ambion, Austin, TX) and Bio-16-UTP and Bio-11-CTP (Enzo Biochem, New York, NY), and purified on RNeasy spin columns (Qiagen, Hilden, Germany). The biotinylated cRNA under fragmentation was hybridized to oligonucleotide arrays (GeneChip S98 Arrays, Affymetrix, Santa Clara, CA), as described in the Affymetrix user's manual. The gene expression levels were determined from hybridization intensity. Microarray experiments were repeated 3 times, and the repetition included both biological and technical replicates.

Northern blot analysis and quantification. Poly(A)$^+$ RNA (1.0 μg each for *DAN1*, *FLO11*, and *HXT2*, and 1.5 μg for *FLO1*) was purified from total RNA prepared using the Oligotex-dT30 ⟨super⟩ mRNA purification kit, as described above, and separated by electrophoresis on a 1% agarose-formaldehyde gel, followed by blotting onto a positively charged nylon membrane (Hybond-N$^+$, GE Healthcare, Little Chalfont, UK). Prior to hybridization with the gene-specific probes, cross-linking of RNA to the membrane was carried out with a GS Gene Linker (Bio-Rad, Hercules, CA) at 150 mJ. DNA probes for hybridization corresponded to positions +66 to +897 for *DAN1*, +601 to +1400 for *HXT2*, +3615 to +4614 for *FLO1*, +201 to +956 for *FLO11*, and +1 to +1128 for *ACT1*. They were prepared by PCR amplification from the genomic DNA of the MT8-1 strain, and were labeled and detected with an Alkphos Direct Labeling and Detection Kit (GE Healthcare). *ACT1* was used as control for RNA loading. The band intensities after detection, corresponding to the mRNA levels, were quantified using Scion Image Software, available from the Scion Corporation (http://www.scioncorp.com/). The results were normalized to the *ACT1* mRNA level.

Measurement of cell aggregation ability. Plasmid pMCG3 or pMCG-T was used for *GTS1* induction under the *CUP1* promoter. Cell aggregation ability was determined by quantification of the unaggregated cells in the supernatant of cell culture. The yeast cell suspension (5 mL) in 10-mL test tubes was dispersed using a vortex. The tubes were agitated on a rotary shaker at 30 rpm for 10 min and allowed to stand for 5 min. Aggregation ability was measured as described previously.[18]

Fluorescence microscopy. Cells harboring plasmid (pM*GTS1*-EGFP or pMCG-T) for the production of Gts1p-EGFP under the *CUP1* promoter were cultivated in a medium containing 300 μM $CuSO_4$ and 1 μg mL^{-1} DAPI (Molecular Probes, Eugene, OR) to visualize nuclear DNA. After washing with PBS (pH 7.2), fluorescence was detected with an inverted microscope IX71 (Olympus, Tokyo). EGFP fluorescence was visualized using a U-MNIBA2 mirror unit with a BP 470–490 excitation filter, a DM 505 dichroic mirror, and a BA 510–550 emission filter (Olympus). DAPI fluorescence was visualized using a U-MNUA2 mirror unit with a BP 360–370 excitation filter, a DM 400 dichroic mirror, and a BA 420–460 emission filter (Olympus). Live images were obtained with AquaCosmos 2.0 software (Hamamatsu Photonics, Shizuoka, Japan) controlling a digital charge-coupled device camera (C4742-95-12ER, Hamamatsu Photonics).

Chromatin structure analysis. Plasmid pMCG2 was used for *GTS1* induction under the *CUP1* promoter. After cultivation in a medium containing 500 μM $CuSO_4$, yeast nuclei were isolated as described previously.[20] Micrococcal nuclease (MNase) digestion of genomic chromatin was carried out by a method described previously,[21] with slight modifications. In brief, nuclei were incubated at 37 °C with MNase (Wako Pure Chemical Industries, Osaka, Japan) for 5 min, and the reaction was stopped with SDS, EDTA, and proteinase K (Nacalai Tesque, Kyoto, Japan). The DNA purified from the MNase-treated nucleus was digested to completion with *Bsr*BI. The resulting DNA, 17 μg, was separated on a 0.5% SeaKem GTG and a 1.5% Nusieve GTG agarose gel (Cambrex Bio Science, Rockland, ME), and transferred to a nylon membrane (Hybond-N$^+$, GE Healthcare). The *FLO1* promoter and upstream region (−2,177 to −1,781) were labeled with alkaline phosphatase using an Alkphos Direct Labeling and Detection Kit (GE Healthcare) for hybridization. The intensities of the bands after detection, corresponding to the DNA concentration, were quantified with Scion Image software.

Dot-plot after co-immunoprecipitation. Dot-plot analysis was performed using the CYC8-TAP strain producing endogenous Cyc8p fused with the TAP tag. This strain was transformed with pYEX-BX-FLAG-GTS1 to produce Gts1p with the FLAG tag under the *CUP1* promoter or the control plasmid. Yeast cells grown to stationary phase were transferred to fresh medium containing 500 μM $CuSO_4$. After 3 h of cultivation, 1% formaldehyde was added and incubated for 15 min for crosslinking, and the reaction was stopped by the addition of 125 mM glycine and incubation for 5 min. Collected cells were subjected to nuclear isolation and were sonicated 9 times for 30 s at 1-min intervals, on ice. After centrifugation for 15 min at 17,800 × *g* at 4 °C, the supernatant was purified with anti-FLAG (GE Healthcare) or anti-TAP resin (Open Biosystems).

Eluted proteins were spotted onto polyvinylidene difluoride membranes (Hybond-P, GE Healthcare) for Western blotting. FLAG-Gts1p and Cyc8p-TAP were detected using anti-FLAG M2 antibody (GE Healthcare) diluted 1:4,000 or anti-TAP tag antibody (Open Biosystems) diluted 1:4,000, followed by anti-Rabbit IgG HRP-linked whole antibody (GE Healthcare) diluted 1:20,000. Protein signals were visualized on X-ray film (Hyperfilm ECL, GE Healthcare) using ECL-plus (GE Healthcare) following the protocol specified by the supplier.

Agarose gel electrophoresis. Plasmid pYEX-BX-HA-CYC8 and plasmid pMCG4 were used to produce HA-Cyc8p under the *CUP1* promoter and to produce FLAG-Gts1p under the *GAL1* promoter. After 24 h of cultivation in SD medium, the transformants were transferred into a synthetic medium, in which 2% glucose in the SD medium was replaced with 2% galactose, and were cultivated for 21 h at 25 °C. A final concentration of 200 μM $CuSO_4$ was added to the medium, and the yeast cells were frozen after 15 h of cultivation. The frozen cells were subjected to glass-bead disruption with PBS buffer, containing 0.25% protease inhibitor cocktail (Sigma, St. Louis, MO) and centrifugation for 1 min at 500 × *g* at 4 °C. A final concentration of 2.5% SDS was added to the supernatant for a subsequent boil for 3 min. The resulting solution was separated on a 2% agarose-RE gel (Nacalai Tesque) containing 0.1% SDS, and electroblotted onto polyvinylidene difluoride membranes for Western blotting.

FLAG-Gts1p and HA-Cyc8p were detected using anti-FLAG M2 antibody, and anti-HA tag clone DW2 (Millipore, Billerica, MA), followed by anti-Rabbit IgG HRP-linked whole antibody respectively.

Results

Transcriptional response to GTS1 *induction and the involvement of a global repressor*

To identify the yeast genes involved in Gts1p-mediated signal transduction, the transcriptional response to *GTS1* induction was examined on a genome-wide scale by DNA microarray analysis. *GTS1* was induced under the control of the copper ion-inducible *CUP1* promoter. Cell aggregation, one of the pleiotropic effects, started within a few hours of the addition of copper ions to the medium.[18] Total RNA was extracted from the GPMH6 strain harboring *GTS1* induction and from the control strain 3 h after copper induction at the logarithmic phase. Microarray experiments were carried out to obtain the expression profiles of the cells with and without *GTS1* induction. They were repeated 3 times.

The expression of 19 genes was upregulated at least 2.5-fold with *GTS1* induction. These genes are listed in Table 3. These upregulated genes were classified into three main groups on the basis of their functions according to *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). The largest group of upregulated genes included the *DAN*/*PAU* (seripauperins) and *TIR* families. The *PAU* gene family is induced under hypoxic conditions and stress such as cold shock and encodes cell-wall mannoproteins, some of which are required for adaptation to anaerobic growth, while others are involved in sterol uptake or transport.[22,23] The second group of upregulated genes consisted of those involved in sugar transport and phosphorylation. *HXT16* and *HXT2*, encoding a high-affinity hexose transporter, are subject to glucose repression, and are controlled by the Mig1 DNA-binding repressor.[24,25] *MAL11* encodes maltose permease, a member of the hexose transporter family, and *FSP2* and *YGR287C* are predicted to encode maltase based on sequence similarity, while *RCK1* encodes a serine/threonine protein kinase. In the third group, the *FLO11* and *FLO1* gene products are cell-surface flocculins. Induced expression of these genes resulted in cell flocculation mediated by flocculin on the cell surface.[26,27] *ANB1*, a hypoxic gene that encodes the translation initiation factor eIF-5A, is repressed under aerobic conditions, similarly to members of the *PAU* gene family. *ANB1* is also regulated by the Rox1 repressor, in the same manner as *DAN1*.[28] The other genes included *SOR1* encoding sorbitol dehydrogenase, and *YNR073C* and *YJL217W*, whose functions are unknown. Northern blot analysis of the representative genes upregulated by *GTS1* induction was performed to confirm the DNA microarray analysis results (Fig. 1). *DAN1* of the *PAU* gene family, *HXT2* of the second group, and *FLO11* and *FLO1* of the third group were selected, and the transcription of these genes was analyzed. All four genes showed elevated expression in cells with *GTS1* induction, and the densitometry of the bands indicated that expression was elevated 4.3-fold for *DAN1*, 4.7-fold for *HXT2*, 5.9-fold for *FLO11*, and 11.6-fold for *FLO1*. These results confirm that the expression profiles of the representative genes were consistent with those from the microarray analysis.

Table 3. Genes Transcriptionally Activated by *GTS1* Induction

ORF code	Gene	Induction fold	Gene description
DAN, *PAU*, and *TIR* gene family			
YJR150C	*DAN1*	8.9	Cell wall mannoprotein induced during anaerobic growth
YBR301W	*DAN3*	3.3	Putative cell wall protein
YOR010C	*TIR2*	3.0	Cold-shock induced protein
YER011W	*TIR1*	2.7	Stress induced cell wall protein
YGR294W		2.6	Similarity to PAU family
YLR461W	*PAU4*	2.6	Member of PAU family
YJL223C	*PAU1*	2.6	Member of PAU family
Sugar transport and phosphorylation			
YJR158W	*HXT16*	15.7	Hexose transporter
YGL158W	*RCK1*	5.3	Serine/threonine protein kinase
YJL221C	*FSP2*	4.0	Homology to maltase (alpha-D-glucosidase)
YMR011W	*HXT2*	3.8	High-affinity hexose transporter
YGR289C	*MAL11*	3.7	Maltose permease, member of the hexose transporter family
YGR287C		3.1	Similarity to maltase
Others			
YIR019C	*FLO11*	8.1	Cell surface flocculin, required for invasion and pseudohyphae
YNR073C		4.9	Unknown
YJR159W	*SOR1*	4.6	Sorbitol dehydrogenase
YJR047C	*ANB1*	4.1	Translation initiation factor eIF-5A, anaerobically expressed form
YAR050W	*FLO1*	3.3	Cell surface flocculin
YJL217W		2.9	Unknown

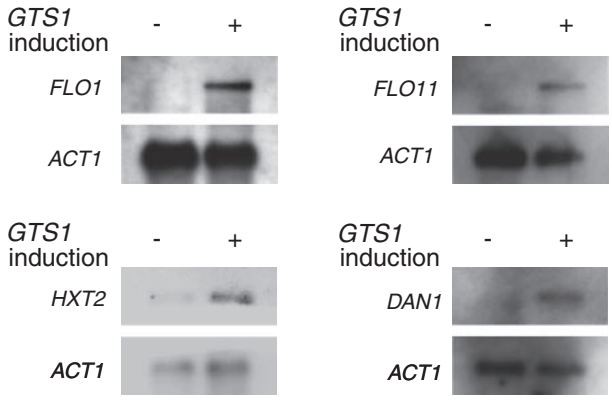

Fig. 1. Northern Blot Analysis of Cells with *GTS1* Induction.

Poly(A)$^+$ RNA was isolated, blotted, and hybridized with the indicated gene-specific probes, as described in "Materials and Methods." RNA was prepared from the cell culture of the GPMH6 strain harboring pMCG1 (for *GTS1* induction) and the control plasmid after copper induction. *ACT1* was used as RNA loading control for the various blots.

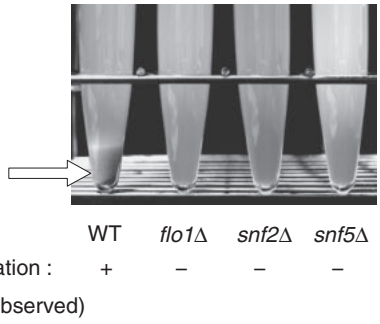

Fig. 2. Decreased Gts1p-Mediated Cell Aggregation in the Deletion Mutants.

A wild-type strain (BY4741), deletion mutants with defects in the SWI/SNF complex (*snf2Δ* and *snf5Δ*), and cell-surface flocculin (*flo1Δ*) were transformed with pMCG3 (for *GTS1* induction), and cell aggregation was observed. The cell culture was allowed in a test tube to stand for 5 min after agitation. The arrow indicates cell aggregation.

To obtain further insight into the gene upregulation mechanism by *GTS1* induction, the transcriptional regulation system of the upregulated genes in the microarray analysis was investigated. At least four genes (*DAN1*, *HXT2*, *ANB1*, and *FLO1*) were transcriptionally regulated by a common regulation system, as indicated by the results of genetic analysis.[29–32] This common transcriptional regulation requires the Tup1-Cyc8 complex, a co-repressor complex recruited to different promoters *via* specific interactions with DNA-binding regulatory proteins.[9,33] All the upregulated genes in the strain with *GTS1* induction in our analysis were also found to be upregulated in the *tup1Δ* strain, with the exception of *FLO11*.[34] In wild-type cells, these genes are considered to be repressed by the Tup1-Cyc8 complex under normal growth conditions, suggesting that *GTS1* induction can derepress genes that are repressed by the Tup1-Cyc8 complex.

SWI/SNF-dependence of cell aggregation by GTS1 *induction*

To clarify the molecular mechanism that results in pleiotropic effects by *GTS1* induction, we focused on cell aggregation, because it is representative of pleiotropic effects and of valid indicator of Gts1p-mediated signal transduction. To analyze gene products that directly cause Gts1p-mediated cell aggregation, gene disruption analysis was performed. Among the genes determined by microarray analysis, *FLO1* disruption led to abolition of cell aggregation regardless of *GTS1* induction (Fig. 2), although cell aggregation was maintained in the *flo11*, *dan1*, and *upc2* deletion mutants (data not shown). *UPC2* is involved in the anaerobic induction of the *PAU* gene family.[35] The cell aggregation ability of the *flo1Δ* strain decreased to 12.9%, the same level as the control strain without *GTS1* induction, whereas it was 72.2% in the wild-type strain with *GTS1* induction (data not shown). Furthermore, the mannose and calcium dependencies, which are characteristic of

the cell aggregation caused by Flo1p,[36] were examined. The addition of 500 mM mannose or 5 mM EDTA completely abolished cell aggregation, to the same level as the control strain (data not shown), indicating that Gts1p-mediated cell aggregation is dependent on Flo1p. Hence, in subsequent analyses, cell aggregation as phenotype and *FLO1* upregulation as transcriptional response were examined to evaluate signal transduction by *GTS1* induction.

Organized chromatin structure is one of the mechanisms by which the Tup1-Cyc8 complex represses transcription.[10] Thus, it is possible that local changes in chromatin structure are associated with transcriptional activation due to *GTS1* induction. The SWI/SNF complex functions as a transcriptional activator *via* chromatin remodeling, leading to local destabilization of nucleosomes, followed by transcriptional activation.[10,37,38] To estimate the involvement of the SWI/SNF complex into Gts1p-mediated cell aggregation, deletion mutant analysis was performed. For this, *SNF2*, which is involved in primary ATPase activity as well as in the integrity of the SWI/SNF transcription activator complex,[39] and *SNF5*, which encodes a core subunit of the SWI/SNF complex,[40] were selected. In these mutant strains, Gts1p-mediated cell aggregation decreased markedly, to a level comparable to the *flo1Δ* strain (Fig. 2). Next we investigated the transcriptional levels of *FLO1* in the *snf2Δ* and *snf5Δ* strains by Northern blot analysis to determine whether the transcriptional response to *GTS1* induction is mediated by the SWI/SNF complex (Fig. 3). Transcriptional activation of *FLO1* in response to *GTS1* induction was confirmed in the wild-type strain, but was abolished in the *snf2Δ* and *snf5Δ* strains and was similar to that of the *flo1Δ* strain, suggesting that the upregulation of genes including *FLO1* by *GTS1* induction requires ATP-dependent chromatin remodeling by the SWI/SNF complex.

Nuclear localization of Gts1p is required for cell aggregation.[41] To determine the effects of gene deletion on Gts1p subcellular localization, Gts1p fused with EGFP at the C-terminus was produced in the *flo1Δ*, *snf2Δ*, and *snf5Δ* strains. The wild-type cells with induced production of the fusion protein showed the same level of cell aggregation as the cells with induced production of Gts1p alone, indicating that Gts1p fused with EGFP is functional in gene upregulation. Cell aggregation was not observed in the mutant strains (data not shown), in accordance with the data in Fig. 2. Fluorescence microscopy of these cells showed that the Gts1p-EGFP fluorescence signal overlapped with the nuclear staining signal of 4,6-diamidino-2-phenylindole (DAPI) in all strains (Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site), whereas the cells producing only EGFP showed no fluorescence in the nucleus (data not shown). This indicates that the localization of Gts1p was undisturbed by the gene deletions, and that decreased cell aggregation was caused by transcriptional defects in the mutant strains rather than by the change in subcellular localization.

Chromatin remodeling at the FLO1 *promoter by* GTS1 *induction*

The experiments described above revealed that *FLO1* upregulation by *GTS1* induction was associated with

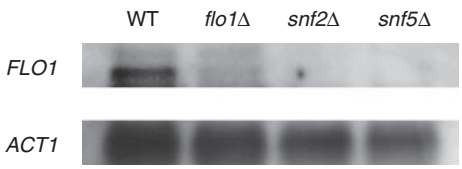

Fig. 3. SWI/SNF-Dependent Transcriptional Activation of *FLO1* by *GTS1* Induction.

Poly(A)$^+$ RNA was isolated, blotted, and hybridized with the *FLO1* gene-specific probes, as described in "Materials and Methods." RNA was prepared from the cell culture of the wild-type strain (BY4741) and BY4741-derived mutant strains (*snf2Δ*, *snf5Δ*, and *flo1Δ*) harboring pMCG2 (for *GTS1* induction). *ACT1* was used as RNA loading control for the various blots.

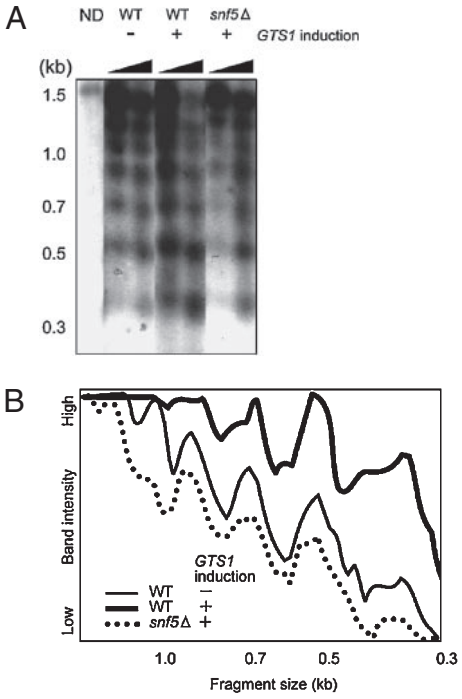

Fig. 4. Chromatin Organization and Remodeling in the Region Upstream of *FLO1*.

A, Nucleosomal mapping of MNase cleavage sites relative to a *Bsr*BI restriction site (−2,193) in the indicated strains. Lane ND is nondigested chromatin. Number at the left of the figure indicate DNA sizes (on a 1-kb scale). Black ramps at the top indicate increasing amounts of MNase (5 and 10 U mL^{-1}). Plasmid pMCG2 was used for *GTS1* induction. B, Image scan of nucleosomal mapping by MNase (5 U mL^{-1}) in A. The concentrations of the various lanes were plotted as intensity.

chromatin remodeling by the SWI/SNF complex. Hence, the effect of *GTS1* induction on the nucleosome structure at the *FLO1* promoter was investigated using isolated nuclei. Equal amounts of nuclei from the various groups were subjected to MNase digestion. The upstream region of *FLO1* was probed to resolve the nucleosome structure in the *FLO1* promoter (Fig. 4A). In the strain with *GTS1* induction, the ordered nucleosomal ladder was disrupted and shifted to shorter fragment sizes, and the chromatin structure was more susceptible to MNase digestion than the strain without *GTS1* induction (Fig. 4B), but *GTS1* induction in the *snf5Δ* strain did not result in disruption of the nucleosomal ladder, suggesting that the effect of *GTS1* induction on the nucleosome position at the *FLO1*

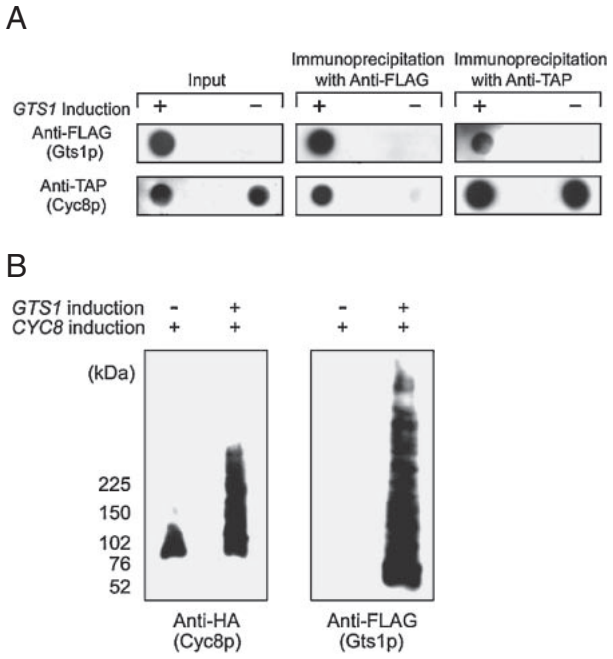

Fig. 5. Co-Immunoprecipitation of Cyc8p with Gts1p and Detection of Protein Aggregates on Agarose Gel.

A, Dot-plot analysis after co-immunoprecipitation was performed. The yeast strain producing endogenous Cyc8p with the TAP tag was transformed with pYEX-BX-FLAG-GTS1 (for *GTS1* induction) and with the control plasmid. After purification with anti-FLAG and anti-TAP resin followed by dot-plot, FLAG-Gts1p and Cyc8p-TAP were detected with FLAG and TAP antibodies respectively. B, Agarose gel electrophoresis and subsequent Western blotting were performed for detection of protein aggregates. Yeast was transformed with pMCG4 (for FLAG-Gts1p production) and pYEX-BX-HA-CYC8 (for HA-Cyc8p production), and with the control plasmid and pYEX-BX-HA-CYC8. FLAG-Gts1p and HA-Cyc8p were detected by FLAG and HA antibodies respectively.

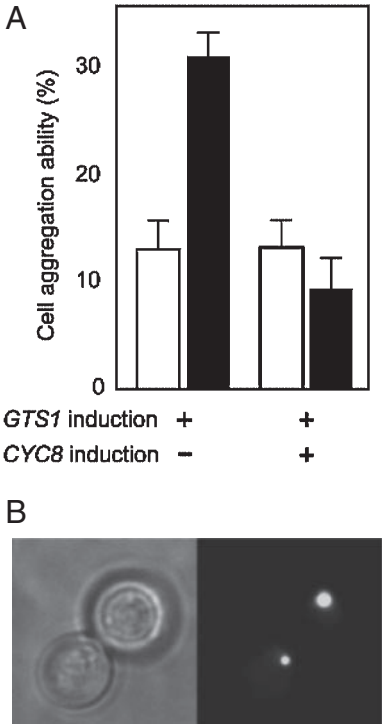

Fig. 6. Effects of *CYC8* Expression on the Cell Aggregation Ability and Subcellular Localization of Gts1p.

A, Cell aggregation was measured before (white bar) and 3 h after *GTS1* induction (black bar). Each experiment was performed in triplicate, and error bars indicate standard deviation. B, Subcellular localization of Gts1p-EGFP in the yeast producing both Gts1p-EGFP and HA-Cyc8p was observed by fluorescence microscopy. Left and right show phase contrast and fluorescent images respectively. Plasmids pMCG-T and pYEX-BX-HA-CYC8 were used to produce Gts1p-EGFP and HA-Cyc8p respectively.

promoter is to be attributed to the SWI/SNF complex, and that chromatin remodeling activity is required for *FLO1* upregulation by *GTS1* induction.

The involvement of Cyc8p in Gts1p-mediated protein aggregates

To determine the derepression mechanism of genes by *GTS1* induction, we examined the possible interaction of Cyc8p with Gts1p by co-immunoprecipitation. The strain producing endogenous Cyc8p fused with the TAP tag was used as host, and was transformed with *GTS1* induction plasmid or the control plasmid. After cross-linking with formaldehyde, nuclear lysate was purified with anti-FLAG or anti-TAP resin for subsequent dot-plot. The results for Western blotting indicated that Gts1p was co-purified with Cyc8p, and *vice versa* (Fig. 5A), suggesting interaction between Gts1p and Cyc8p. Moreover, *GTS1* induction is known to cause Gts1p-mediated protein aggregates, whose molecular weight cannot be measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), but can be measured by agarose gel electrophoresis.[42] Hence, to determine whether Gts1p-mediated protein aggregates involve Cyc8p, agarose gel electrophoresis was performed. The yeast strains producing both Cyc8p and Gts1p, and producing only Cyc8p, were used in this experiment. After separation on an agarose gel, the mobilities of Gts1p and Cyc8p in the cell lysate were investigated by Western blotting. In the strain with *GTS1* induction, Gts1p was detected as a smeared band, showing the formation of protein aggregates, as previously reported.[42] In the strain with *GTS1* induction, Cyc8p was detected as a smeared band of a higher molecular weight, although the smeared band of Cyc8p was not detected in the strain without *GTS1* induction (Fig. 5B). This result suggests that protein aggregates of Cyc8p were formed in association with *GTS1* induction.

Decreased Gts1p-mediated cell aggregation ability in the CYC8 *co-expression strain*

To determine the functional relationship between Gts1p and the Tup1-Cyc8 complex, the effects of *CYC8* expression on Gts1p-mediated cell aggregation and the subcellular localization of Gts1p were investigated. Although the strain expressing only *GTS1* showed clear cell aggregation after *GTS1* induction, cell aggregation ability by *GTS1* induction was decreased by *CYC8* induction (Fig. 6A). On the other hand, the nuclear localization of Gts1p did not change regardless of *CYC8* expression (Fig. 6B). This suggests that endogenous Cyc8p was initially inhibited by Gts1p, and that inductively produced Cyc8p rescued the inhibition.

Discussion

GTS1 is a pleiotropic gene that affects a variety of biological phenomena in a gene dose-dependent manner. In continuous cultures, the synchronization between the

periodic fluctuation of the *GTS1* transcript and the degree of pleiotropic effects has been elucidated.[4,8] The interaction of Gts1p with the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, regulates metabolic oscillation in yeast,[43] but the molecular mechanism of the pleiotropy of *GTS1* remains unclear. Generally, there is a limit to the signal transduction analysis of an individual effect related to pleiotropy. *GTS1* induction leads to clear pleiotropic phenotypes, allowing easy detection of Gts1p-mediated signal transduction. Hence, we performed a genome-wide analysis of gene expression in response to *GTS1* induction. Nineteen genes, including hypoxic genes, sugar-transporter-encoding genes, and flocculation-inducing genes, were found to be upregulated by at least 2.5-fold. The gene expression pattern shown in Table 3 indicates that the Tup1-Cyc8 complex, a global co-repressor, is involved in the transcriptional response to *GTS1* induction. Hence, it is suggested that *GTS1* induction can derepress the genes repressed by the Tup1-Cyc8 complex, and that derepression can result in *GTS1*-mediated pleiotropy.

Cell aggregation is a representative phenotype of the pleiotropic effects of *GTS1* induction, and is useful for estimating Gts1p-mediated signal transduction. Gene disruption analysis of the upregulated genes showed that transcriptional activation of the dominant flocculation-inducing gene, *FLO1*,[44] caused cell aggregation in response to *GTS1* induction (Fig. 2). Moreover, cell aggregation was inhibited by mannose but not by glucose, confirming Flo1p-dependent cell aggregation. This is consistent with a previous study, indicating that Gts1p-mediated cell aggregation is *FLO1* dependent.[45] Hence, we focused on *FLO1* upregulation leading to cell aggregation among the various Gts1p-mediated pleiotropic effects.

The Tup1-Cyc8 complex is reported to affect chromatin organization by direct contact between its repression domain and the N-termini of under-acetylated histones H3 and H4.[46] Derepression of the Tup1-Cyc8-repressing genes involves chromatin remodeling by an antagonist, the SWI/SNF complex.[37,47] In deletion mutant analysis, Gts1p-mediated cell aggregation and *FLO1* transcription were significantly decreased in deletion mutants with a defect in the SWI/SNF complex, indicating that a functional chromatin remodeling complex is required for activation of genes, including *FLO1* (Figs. 2 and 3), whereas localization of Gts1p was not affected in the SWI/SNF-deficient mutants (Supplemental Fig. 1). An MNase accessibility assay indicated that *GTS1* induction resulted in disruption of the nucleosomal ladder over the *FLO1* promoter, but *SNF5* deletion diminished the effect of *GTS1* induction, suggesting that the nucleosome array is dependent on the chromatin remodeling activity of the SWI/SNF complex (Fig. 4).

Gts1p has a characteristic poly-Q and -QA region at the C-terminus, and this region is believed to induce protein aggregation.[42] Cyc8p also has a poly-Q and -QA region, which is highly homologous to the C-terminus of Gts1p. Structural analysis suggests that formation of protein aggregates might occur by amide stacking between glutamine residues.[48] Hence, it is suggested that the Gts1p-mediated inhibition mechanism that works against the Tup1-Cyc8 complex is related to the formation of protein aggregates through the poly-Q and -QA region. As previously reported,[42] *GTS1* induction caused a smeared band on agarose gel, and the formation of Gts1p-mediated protein aggregates was detected. However, in the case of Cyc8p, a smeared band was also detected with *GTS1* induction, but no smearing was detected without *GTS1* induction (Fig. 5B), suggesting that Gts1p-mediated protein aggregates involve Cyc8p through the poly-Q and -QA region. The Poly-Q and -QA region in Gts1p is believed to be indispensable for the formation of protein aggregates, and indeed, Gts1p lost the ability to form Gts1p-mediate protein aggregates without the poly-Q and -QA region at the C-terminus (data not shown). Moreover, Gts1p and Cyc8p were co-purified by co-immunoprecipitation (Fig. 5A), indicating that Gts1p interacted with Cyc8p. The results shown in Fig. 6 indicate that Gts1p is related with the inhibition machinery that works against Cyc8p.

The results reported here provide an understanding of the molecular mechanism involved in the transcriptional response to *GTS1* induction. *GTS1* induction derepressed the Tup1-Cyc8-repressing genes and caused chromatin remodeling at the *FLO1* promoter in a SWI/SNF-dependent manner. Moreover, Gts1p-mediated protein aggregates involving Cyc8p and functional interaction between Gts1p and Cyc8p were identified. Our findings suggest that *GTS1* induces pleiotropic effects, including cell aggregation, by regulating Tup1-Cyc8-mediated gene repression, followed by SWI/SNF-dependent chromatin remodeling in the nucleus.

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