理學博士學位論文

효모에서 UDP-글루코스 피로포스포릴라아제의 발현 기작과 기능

Expression mechanism and function of UDP-glucose pyrophosphorylase in *Saccharomycese cerevisiae*

2015 年 **8** 月

서울大學校 大學院

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이 대 관

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Expression mechanism and function of UDP-glucose pyrophosphorylase in *Saccharomycese cerevisiae*

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ABSTRACT

Expression mechanism and function of UDP-glucose pyrophosphorylase in *Saccharomycese cerevisiae*

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Ugp1, UDP-glucose pyrophosphorylase (UGPase), plays an important role in carbohydrate metabolism because it provides UDP-glucose (UDP-Glc) which is a pivotal metabolite in several metabolic pathways, including the biosynthesis of carbohydrate storage molecules such as glycogen and trehalose, and the formation of some cell wall components in *Saccharomyces cerevisiae*. Although many regulators have been expected to be involved in the regulation of *UGP1*, only Pho85 kinase has been reported to inhibit the *UGP1* transcription until now.

Here, this study suggests that stress-responsive transcription factors Msn2/4 play a crucial role in regulating the expression of *UGP1*. First, it is observed that Msn2/4 bind to three stress response elements in the promoter of *UGP1* depending on protein kinase A (PKA) activity. Also, the result that several stress conditions induced transcription of *UGP1* suggests that the regulation of *UGP1* mediated by Msn2/4 is involved in the general stress response.

Furthermore, the present study shows that the activity of the phosphate response (PHO) pathway affects Msn2/4-dependent regulation of *UGP1*, implying a novel link between the PKA and PHO pathway. These findings suggest that signals of the PKA, PHO and stress response pathways converge on and regulate the expression of *UGP1* via Msn2/4 in *S. cerevisiae*.

Meanwhile, the expression level of *UGP1* is required for the production of storage carbohydrate such as glycogen and trehalose. Because of the specific function of trehalose as a stress protectant, the Ugp1 expression contributes to oxidative stress response and chronological life span (CLS). Interestingly, although it was reported that the PKA pathway regulates carbohydrate metabolism negatively, modulations of Ugp1 level suppressed the typical phenotypes concerning glycogen and trehalose accumulation in the PKA-related gene mutants. Also, PKA-dependent phenotypes in anti-oxidant resistance and CLS were also alleviated via adjustment of Ugp1 level. Taken together, these results suggest that the regulation of *UPG1* may influence a variety of biological processes under the PKA pathway through adjustments of various carbohydrate levels.

Collectively, this study demonstrates that the regulation of Ugp1 level through Msn2/4 contributes to cellular homeostasis by inducing the glucose partitioning to synthesis of carbohydrates which act as defensive metabolites.

Keywords: *UGP1*, PKA, MSN2/4, PHO, glycogen, trehalose, oxidative stress, CLS
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ABBREVIATIONS

ChIP : Chromatin immunoprecipitation

CLS : Chronological life span

cAMP : Cyclic adenosine monophosphate

EDTA : Ethylenediaminetetraacetic acid

GFP : Green fluorescent protein

GDP : Guanosine diphosphate

GTP : Guanosine triphosphate

Glc-1P : Glucose-1-phosphate

Glc-6P : Glucose-6-phosphate

HRP : Horseradish peroxidase

PHO : Phosphate signal transduction

PKA : Protein kinase A

PP_i : Inorganic pyrophosphate

STRE : Stress response element

TOR : Target of rapamycin

Tre-6P : Trehalose-6-phosphate

UDP-Glc : Uridine diphosphate-glucose

UGPase : UDP-glucose pyrophosphorylase

UTP : Uridine triphosphate

CHAPTER I.

Research background and objective

1. UDP-glucose pyrophosphorylase

1.1. Properties of UGPase

UDP-glucose pyrophosphorylase (UGPase) (EC 2.7.7.9.), an ubiquitous enzyme in three kingdoms of life, plays very important roles in carbohydrate metabolism. UGPase catalyzes the interconversion of glucose-1-phosphate and UTP to UDP-glucose (UDP-Glc) and inorganic pyrophosphate in the presence of Mg^{2+} (Aksamit and Ebner, 1972) (**Fig. I-1**).

So far, structural studies on UGPase in prokaryotic (Aragão et al., 2007; Kim et al., 2010; Thoden and Holden, 2007b) and eukaryotic UGPase (Benaroudj et al., 2001; Mariño et al., 2010; McCoy et al., 2007; Quan and Xiaofeng, 2012; Roeben et al., 2006; Steiner et al., 2007) have demonstrated that the active site architecture is highly conserved despite the absence of significant amino acid sequence conservation. On the contrary, in spite of the highly conserved active site, UGPase has closely related isoforms in many species. In plant, only the monomeric form of UGPase is active (McCoy et al., 2007; Meng et al., 2009). Similarly to plants, the active UGPase of the protozoan parasites *Leishmania major* and *Trypanosoma bruccei* was reported to be monomeric (Mariño et al., 2010; Steiner et al., 2007). In several bacteria, dimeric or tetrameric form of active UGPase exists (Bosco et al., 2009; Kim et al., 2010; Thoden and Holden, 2007a). Eukaryotic UGPases in yeast and human were found to form a homo-octamer (Quan and Xiaofeng, 2012; Roeben et al., 2006).

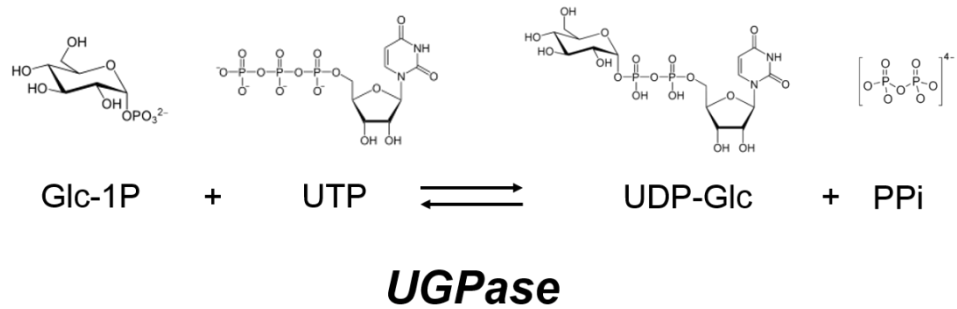


Fig. I-1. The formation of UDP-Glc

1.2. Biological functions of UGPase

Since UGPase is indispensable in overall carbohydrate metabolism, UGPase has been reported to be required for several biological activities (**Fig. I-2**). In several bacterial and protozoan pathogens, impairments of the biosynthesis of major virulence factors, lipopolysaccharide and capsular polysaccharides (Bonofiglio et al., 2005; Chang et al., 1996; Jiang et al., 2010; Mariño et al., 2010; Mollerach et al., 1998; Nesper et al., 2001; Priebe et al., 2004; Vilches et al., 2007). In plant, UGPase has reported to be essential for normal fertility (Chen et al., 2007; Woo et al., 2008). In budding yeast, over-expression or knockdown of UGPase has relevance with cell wall integrity against cell wall-perturbing agent (Daran et al., 1995; Mehlgarten et al., 2007).

1.3. Regulators of UGPase

Although the exact regulatory mechanism has not identified yet, some cues are known to regulate the expression or activity of UGPase. First, in *Sphingomonas paucimobilis*, oxidative stress elevates UGPase activity (Zhu et al., 2014). In a porcine mutant (RN^-) of AMP-activated protein kinase, UGPase is upregulated, thereby increasing synthesis of glycogen in skeletal muscle cells (Hedegaard et al., 2004). In the case of plant, level of plant UGPase can be regulated at the transcription level, according to sucrose content, light signal and phosphate deficiency (Ciereszko et al., 2001; Ciereszko et al., 2005). In the case of *S. cerevisiae*, Pho85, a yeast cyclin-

dependent kinase, regulates the expression of *UGP1* negatively through Pho4, a transcription factor in PHO pathway (Nishizawa et al., 2001).

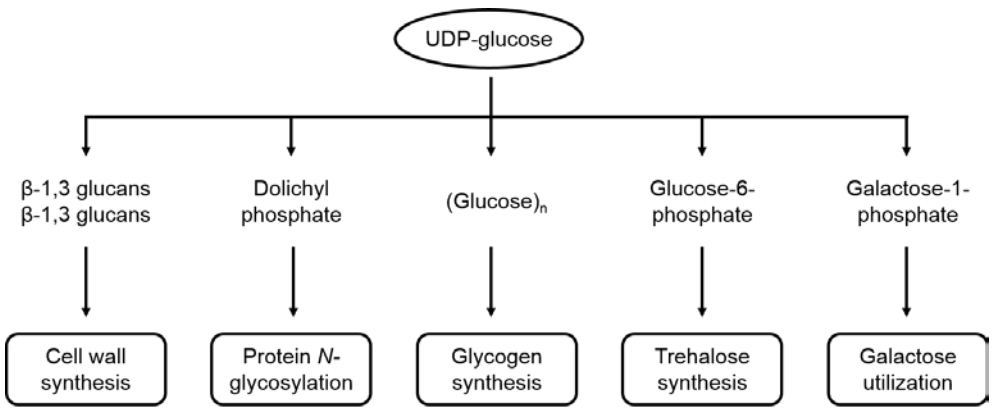


Fig. I-2. The various fates of UDP-Glc in yeast metabolism.

2. Protein kinase A (PKA) pathway

2.1. Signal transduction mechanism

Protein kinase A (PKA) is a ubiquitous serine/threonine kinase throughout the eukaryotic kingdom. In *S. cerevisiae*, PKA is a hetero-tetramer comprising two catalytic subunits (Tpk1, Tpk2 and Tpk3) and two regulatory subunits (Bcy1). In the absence of cAMP, the regulatory subunit, Bcy1, inhibits activity of Tpk1-3 kinases to phosphorylate a variety of downstream effectors by binding to those kinases. In the presence of cAMP, Bcy1 binds to cAMP instead of Tpks, which releases Tpk1-3 from Bcy1 (Cannon and Tatchell, 1987; Toda et al., 1987).

The generation of cAMP by adenylyl cyclase, Cyr1, is mediated by two parallel signaling pathways in response to glucose: (i) The yeast homologs of the Ras proteins, Ras1 and Ras2, contact directly with Cyr1 and trigger its activity. (ii) A putative G protein coupled receptor, Gpr1, and its Gα protein Gpa2 also detect extracellular glucose and stimulate Cyr1 (Santangelo, 2006; Thevelein and De Winde, 1999). Increased cAMP is hydrolyzed by low and high-affinity phosphodiesterases, Pde1 and Pde2 (Nikawa et al., 1987; Sass et al., 1986) (**Fig. I-3**).

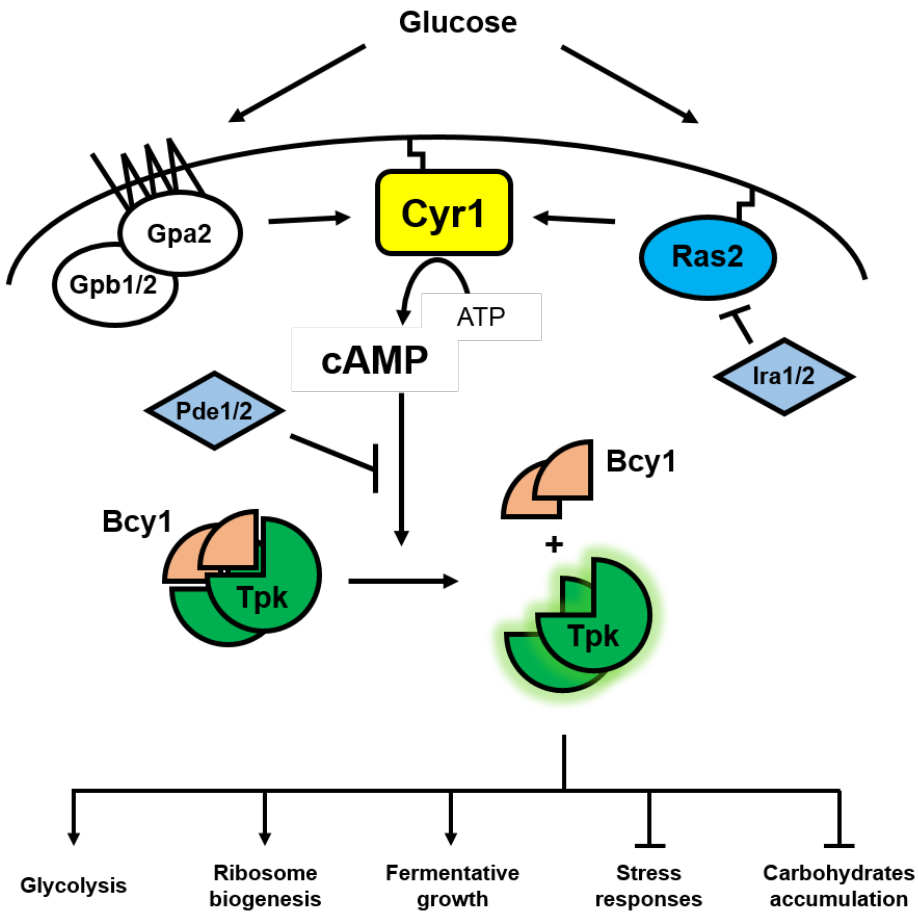


Fig. I-3. Diagram of yeast PKA signaling pathway.

2.2. PKA target systems

PKA has a broad variety of targets in yeast. In general, PKA directly phosphorylates cytosolic enzymes to alter their activities for properties that are associated with rapid fermentative growth. For example, pyruvate kinase (Portela et al., 2002), fructose-1,6-bisphosphatase (Rittenhouse et al., 1987), phosphofructokinase 2 (Dihazi et al., 2003) and trehalase (Schepers et al., 2012). Besides, current studies showed that PKA activity is closely included in autophagy and formation of P-body through direct phosphorylation of Atg1/13 and Pat1 (Ramachandran et al., 2011; Stephan et al., 2009).

On the other hand, PKA regulates expression of target genes at the transcriptional level by mediating intermediate components. Signals of PKA activates a series of genes for postdiauxic growth and stress response via phosphorylation of Rim15 and Msn2/4 (Cameroni et al., 2004; Martinez-Pastor et al., 1996; Pedruzzi et al., 2000).

2.3. Msn2 and Msn4

In *S. cerevisiae*, Msn2 and Msn4 are general stress-responsive transcription activators in charge of the transcription of ~200 genes (Causton et al., 2001; Lallet et al., 2006). Transcriptional activity of Msn2/4 is stimulated by several stress conditions such as heat, low pH, oxidant and nutrient depletion (Causton et al., 2001; Gasch et al., 2000; Hasan et al., 2002). Msn2/4 has a specific consensus binding sequence called STRE (stress response element) (Görner et al., 1998; Martinez-Pastor et al., 1996). For transcriptional

activation, Msn2/4 need to bind to STRE in the promoter of target gene. The nuclear translocalization of Msn2/4 is prerequisite to the regulation of gene transcription. It is well reported that this local transition of Msn2/4 is controlled by several nutrient sensing pathways (Beck and Hall, 1999; Schmitt and McEntee, 1996). Among them, changes of subcellular localization are largely dependent on PKA-dependent phosphorylation on Msn2/4 (Görner et al., 1998; Smith et al., 1998) (**Fig. I-4**).

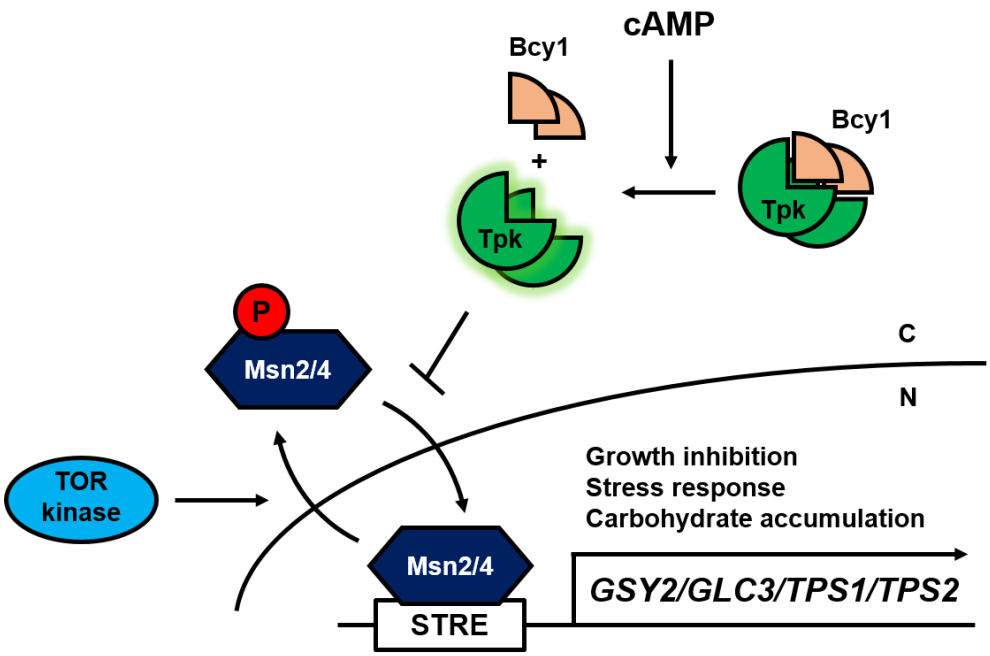


Fig. I-4. Diagram of PKA kinase control of the general stress response through Msn2/4.

3. Glycogen & Trehalose

3.1. Biosynthesis of glycogen and trehalose

The synthesis of glycogen consists of three steps; initiation, elongation and ramification. In the initiation step, 'glycogenin' produces a short α-1,4-glucosyl chain which is covalently attatched to a tyrosine residue of it, because of self-autoglucosylating activity. Then, glycogen synthase catalyzes the conversion of α-1,4-glucosidic bonds from UDP-Glc to the non-reducing end of linear α-1,4-chains of glycogen. After elongation by glycogen synthase, the linear α-1,4-glucosyl chains is catalyzed by a branching enzyme (François and Parrou, 2001) (**Fig. I-5**).

The general pathway for the biosynthesis of trehalose involves the transfer of glucose from UDP-Glc to glucose-6-phosphate to form the trehalose-6-phosphate (Tre-6P) and UDP, catalyzed by trehalose-6-phosphate-synthase (TPS). Then, trehalose-6-phosphate-phosphatase converts from Tre-6P into free trehalose (Avonce et al., 2006) (**Fig. I-5**).

3.2. Control mechanisms of glycogen and trehalose

A rapid accumulation of one or both of the glycogen and trehalose is caused by a wide variety of stresses such as heat, cold, oxidant, high ethanol, weak organic acid, nutrient depletion, and even copper sulfate (Hottiger et al., 1987; Neves and François, 1992; Panek et al., 1990; Parrou et al., 1997). These observations come from the fact that one to several STREs are present in the promoter of several genes in involved in glycogen and trehalose, because

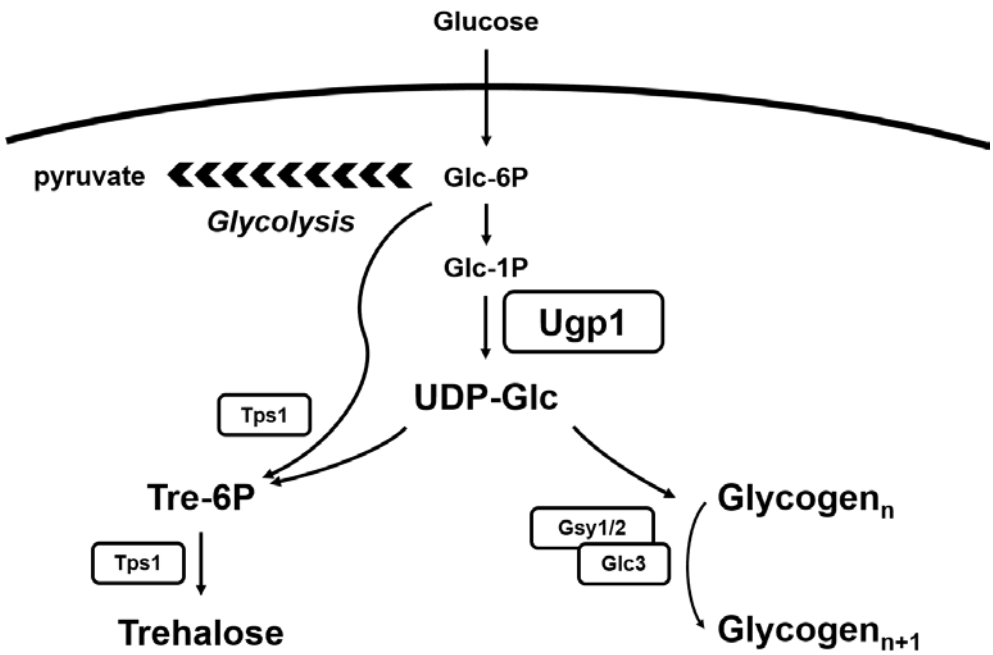


Fig. I-5. A schematic illustration of the metabolic pathways for glycogen and trehalose biosynthesis.

Msn2/4, transcription activators, bind to STRE for activation (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996; Smith et al., 1998). In addition, since Msn2/4 are under the control of nutrient-sensing pathways, synthesis of both carbohydrates are controlled by those pathways via Msn2/4 activity.

On the other hand, Pho85 which is a cyclin-dependent kinase and PAS kinase were identified to directly phosphorylate glycogen synthase, Gsy2 *in vitro* and *in vivo* (Huang et al., 1998; Rutter et al., 2002). PKA also regulates trehalose content by phosphorylating trehalase, Nth1 (Ficarro et al., 2002).

3.3. Function of glycogen and trehalose

Since both carbohydrates are found to accumulate only when carbon sources are being depleted, they have been considered as storage factors (Parrou et al., 1999; Wang et al., 2001). However, recent studies have focused on other roles of glycogen and trehalose. First, mobilization of reserve carbohydrates progression is implicated in progression of cell cycle and cell viability (Silljé et al., 1999). Especially, trehalose functions as a stress protectant against diverse stresses including heat shock, high-osmolarity, oxidative stress and dehydration (Benaroudj et al., 2001; Gadd et al., 1987; Hottiger et al., 1987; Hounsa et al., 1998; Singer and Lindquist, 1998; Tan et al., 2014). Furthermore, the role of trehalose has been reported to activate Hsf1, an essential transcriptional regulator of heat shock response in budding yeast (Bulman and Nelson, 2005; Conlin and Nelson, 2007).

3.4. Commercial value of trehalose

Trehalose has great industrial value due to its exclusive set of physical properties. Compared with other structurally similar sugars, trehalose shows high resistance to acid and α-glycosidase since the α,α-1,1-glycosidic linkage provides it with a non-reducing character (Higashiyama, 2002). Also, trehalose is more stable to wide ranges of temperatures and pH than other sugars (Richards et al., 2002). Unusually, trehalose displays both high hydrophilicity and hygroscopicity (Argüelles, 2014). Above and beyond these physical properties, trehalose has a variety of specific characters. A great number of examples suggesting trehalose stabilizes membrane, protein, DNA and cells in harsh conditions have been already reported (Aksamit and Ebner, 1972; Beattie et al., 1997; Kaoushik and Bhat, 2003; Leidy et al., 2004; Ohtake et al., 2004). In addition, the formation of foul smell (aldehyde) from food ingredients and human skin is inhibited by the addition of trehalose (Kubota, 2005). Therefore, trehalose has been continually studied and applied for commercial purposes including biotechnology, pharmaceutical applications, food and cosmetic industry because of these useful characters of trehalose (Ohtake and Wang, 2011). The details have been summarized in **Table I-1**.

Table I-1. Summary for the properties, roles and commercial use of trehalose

Physical properties	Physiological roles	Commercial uses
Non-reducing sugar	Energy and carbon reserve	Therapeutic compound
High hydrophilicity	Cell protectant against stress	Cryoprotectant
Internal chemical stability	Stabilizer of membranes, DNA and proteins	Prophylactic and stabilizer
Unable to form hygroscopic glasses	Component of the bacterial cell wall	Industrial fermentation
Absence of inner hydrogen bonds	Major compatible solute	Food sweetener
Resistance to acid hydrolysis	Sensing compound	Nutritional supplement
Elevated melting point	Virulence factor	Cosmetic additive

4. Aim of this study

Glucose is one of the most versatile sources in living cells. It is well known that the fate of internalized glucose varies from energy source to building block of cell wall. Thus, proper coordination of glucose flux is essential for the adaptive behavior in cells. In this coordination, UDP-Glc is located in the crossroad of carbohydrate metabolism. Since its production is catalyzed by UGPase (Daran et al., 1995), UGPase has been considered as a key enzyme to regulate the glucose flux. Previous investigations have shown that the regulation of Ugp1 level or activity affects sensitivity to cell wall-perturbing agents and virulence as well as the composition of carbohydrates (Coleman et al., 2006; Daran et al., 1995; Hedegaard et al., 2004; Jiang et al., 2010; Li et al., 2014; Mehlgarten et al., 2007). However, how Ugp1 is regulated remains still unclear.

This study has started to figure out the precise mechanisms that regulate UGPase in *S. cerevisiae*. First, we attempted to identify the regulatory mechanism for the control of Ugp1, the yeast homolog of UGPase. It was interesting to note that the expression of Ugp1 is dependent on PKA activity, a main glucose-sensing pathway (Santangelo, 2006). Second, this study observed the nuclear accumulation of Msn2-GFP under activation of PHO, suggesting that there is a novel connection between PKA and PHO pathway. Third, this study found that repression of Ugp1 regulates glycogen and trehalose production negatively, thereby affecting oxidative stress response

and CLS. Finally, it was observed that the regulation of *UGP1* is incorporated in the PKA-dependent regulation on diverse biological processes. The objective of this study is to provide some clues for elucidating the regulation mechanism of *UGP1*, and to make a contribution toward a better understanding about UGPase-mediated glucose partitioning in yeast.

CHAPTER II.

PKA, PHO pathway and general stresses regulate the expression of UDP-glucose pyrophosphorylase through Msn2/4 in budding yeast

1. Introduction

To respond appropriately to changes in the extracellular environment, all organisms constantly monitor surrounding conditions and transmit this information into cells for efficient resource allocation. Ultimately, the external signals are transmitted to control gene expression patterns so that cells can reprogram their metabolism to adjust the levels of several kinds of metabolites (Gasch et al., 2000). Among them, glucose metabolism plays a significant role in cellular energy production, synthesis of storage molecules and structural building blocks (François and Parrou, 2001). Since organisms must cope with cycles of glucose availability and limitation, proper glucose partitioning is necessary for maintaining cellular homeostasis (Parrou et al., 1999).

UDP-glucose (UDP-Glc) lies at a strategic point in glucose partitioning because it acts as a sole glucosyl donor for crucial metabolites (Daran et al., 1995). For example, UDP-Glc is a vital building block in the synthesis of structural and storage polysaccharides such as glycogen in animals and yeast (Alonso et al., 1995; Daran et al., 1995), cellulose in plant (Kleczkowski et al., 2004) and β-glucan in yeast (Daran et al., 1995). Disaccharides like sucrose in plant (Kleczkowski et al., 2004) and trehalose in yeast and slime mold (Bishop et al., 2002; Daran et al., 1995) are also synthesized from UDP-Glc. Besides, UDP-Glc is involved in the synthesis of glycolipids, glycoproteins and proteoglycans (Flores-Díaz et al., 1997; Herscovics and Orlean, 1993; Silbert and Sugumaran, 1995). It is also a precursor of UDP-glucuronic acid and other UDP-activated sugars used in various processes (Aw and Jones, 1984; Holden et al., 2003; Mio et al., 1998). Additionally,

the utilization of galactose in the Leloir pathway requires UDP-Glc for the synthesis of UDP-galactose (Frey, 1996).

The formation of UDP-Glc is facilitated by UDP-glucose pyrophosphorylase (UGPase), which reversibly catalyzes the interconversion of UDP-Glc and pyrophosphate from UTP and glucose-1-phosphate (Daran et al., 1995). Because of the pivotal role of UDP-Glc, UGPase has been ubiquitously found in all three kingdoms of life and considered as a key enzyme in carbohydrate metabolism in diverse organisms.

Previous investigations of UGPase have focused primarily on the regulation of its expression level. In bacteria, defective UGPase mutations result in lower levels of capsular polysaccharides, lipopolysaccharides and virulence factors, and those mutant strains show severe sensitivity to antibiotics and reduced cell invasion ability (Chang et al., 1996; Genevaux et al., 1999; Mollerach et al., 1998). In plants, substantial reduction in soluble sugars is observed when UGPase level is reduced (Borovkov et al., 1997; Spychalla et al., 1994). When the level of UGPase is upregulated in bacteria, the production of UDP-Glc and polysaccharides is enhanced (Prasad et al., 2010; Rodríguez-Díaz and Yebra, 2011). Overexpression of UGPase increases the content of starch and cellulose, resulting in enhanced vegetative growth in plants (Coleman et al., 2006; Li et al., 2014; Zhang et al., 2013). High copies of UGPase in yeast cells suppress their sensitivity to killer toxin (Mehlgarten et al., 2007). These findings suggest that the regulation of UGPase is closely related to the strategy of glucose partitioning in cells. However, the regulatory mechanisms of UGPase in cells remain largely unknown.

Msn2 and Msn4 are two redundant zinc finger transcription factors that regulate the general stress response in *Saccharomyces cerevisiae* (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). In response to various types of stresses such as oxidative stress, heat shock, osmotic stress, high ethanol concentration and nutrient depletion, Msn2/4 positively control about 200 genes (Causton et al., 2001; Gasch et al., 2000; Hasan et al., 2002; Moskvina et al., 1998). For transcription activation, Msn2/4 bind to DNA consensus sequence, the stress response element (STRE), in the promoters of target genes (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). Under normal conditions, Msn2/4 are located in the cytoplasm. However, under stress conditions, Msn2/4 are translocated into the nucleus (Görner et al., 1998). This translocation of Msn2/4 is negatively regulated by protein kinase A (PKA) pathway (Görner et al., 1998; Smith et al., 1998). Besides, other known pathways such as target of rapamycin (TOR) pathway are linked to Msn2/4 activity (Beck and Hall, 1999; De Wever et al., 2005; Kaida et al., 2002).

In this study, we found that the expression of *UGP1*, which encodes UGPase in *S. cerevisiae*, is affected by PKA activity. Among several transcription factors, Msn2/4 mainly participated in the regulation of *UGP1* by physically binding to three STREs in the promoter of *UGP1*. Correlating with the fact that Msn2/4 are stress-responsive transcription factors, several stresses elevated the *UGP1* mRNA level. Interestingly, we observed that the cellular phosphate status affects Msn2/4-dependent regulation of *UGP1*. Phosphate-deficient condition and loss of Pho85, a cyclin-dependent kinase (Huang et al., 2007), increased *UGP1* expression by inducing nuclear translocation of Msn2. Based on our results, we suggest that PKA-dependent

regulation of *UGP1* contributes to glucose partitioning in yeast cells to respond appropriately to external stimuli.

2. Materials and Methods

2.1. Yeast strains, media

Yeast strains used in this study are listed in Table S1. Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose) or synthetic complete (SC) medium lacking appropriate amino acids. For phosphate starvation, cells grown in SC medium to mid-log phase (OD_{600} = 1.0) were washed twice with distilled water, followed by incubation in phosphate starvation medium with glucose (Sung and Huh, 2007).

2.2. Plasmid and strain construction

Constitutively active $Ras2^{19V}$ or inactive $Ras2^{22A}$ mutants were generated by PCR-based mutagenesis and cloned into *Bam*HI and *Sal*I sites of pRS415ADH vector. pRS415-pr^{UGP1}-UGP1-GFP and pRS415-$pr^{UGP1(-531\sim-441\Delta)}$-UGP1-GFP were made by one-step sequence and ligation-independent cloning (SLIC) method as described previously (Jeong et al., 2012).

Table II-1. Yeast strains used in this study.

Strain	Genotype	Source
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
HY1001	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ::URA3 [pRS415-ADH-RAS2]*	This study
HY1002	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ::URA3 [pRS415-ADH-RAS2^{19V}]*	This study
HY1003	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ::URA3 [pRS415-ADH-RAS2^{22A}]*	This study
HY1183	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn2Δ::KanMX6 msn4Δ::LEU2*	This study
HY1362	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3*	This study
HY1363	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 ras2Δ::LEU2*	This study
HY1364	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 ira2Δ::KanMX6*	This study
HY1367	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 msn2Δ::KanMX6 msn4Δ::LEU2*	This study
HY1369	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 pho4Δ::KanMX6*	This study
HY1371	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 pho85Δ::KanMX6*	This study
HY1387	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 pho85Δ::KanMX6 pho4Δ::HIS3MX6*	This study
HY1478	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 yak1Δ::KanMX6*	This study
HY1479	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 snf1Δ::KanMX6*	This study
HY1493	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [pRS415-pr^{UGP1}-UGP1-GFP]*	This study

HY1494	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [pRS415-pr$^{UGP1(-531\sim-441\Delta)}$-UGP1-GFP]*	This study
HY1495	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ::URA3 [pRS415-pr^{UGP1}-UGP1-GFP]*	This study
HY1496	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ::URA3 [pRS415-pr$^{UGP1(-531\sim-441\Delta)}$-UGP1-GFP]*	This study
HY1509	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 gis1Δ::KanMX6*	This study
HY1510	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 crz1Δ::KanMX6*	This study
HY1511	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 mig1Δ::KanMX6*	This study
HY1520	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-TAP::HIS3MX6*	(Ghaemmaghami et al., 2003)
HY1521	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-TAP::HIS3MX6 ras2Δ::URA3*	This study
HY1522	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-TAP::HIS3MX6 ira2Δ::LEU2*	This study
HY1523	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 tor1Δ::KanMX6*	This study
HY1524	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 mpk1Δ::KanMX6*	This study
HY1529	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 hog1Δ::KanMX6*	This study
HY1559	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 pho85Δ::KanMX6 msn2Δ::LEU2 msn4Δ::HIS3MX6*	This study
HY1170	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-GFP::HIS3MX6*	(Huh et al., 2003)
HY1645	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-GFP::HIS3MX6 pho4Δ::KanMX6*	This study
HY1646	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MSN2-GFP::HIS3MX6 pho85Δ::KanMX6*	This study

Table II-2. Oligonucleotide primers used in this study.

Primer name	Sequence (5'→3')[a]
RAS2-F	*ATGCCTTTGAACAAGTCGAACATAAGAGAGTACAAGCTAG*CACAGGAAACAGCTATGACC
RAS2-R	*ATTAACTTATAATACAACAGCCACCCGATCCGCTCTTGGA*GTTGTAAAACGACGGCCAGT
UGP1-F2	*AAATGTTGTCGTTACTGGTAATTTGCAAATCTTGGAACAT*GGTCGACGGATCCCCGGGTT
UGP1-R1(URA)	*CGGAGAATTGAAAGTAAAAAAGGGATTGCAAGCCGCACCT*TCTGGAGGAAGTTTGAGAGG
MSN2-F2	*GTCGCAACACATCAAGACTCATAAAAAACATGGAGACATT*GGTCGACGGATCCCCGGGTT
MSN2-R1(URA)	TTATGAAGAAAGATCTATCGAATTAAAAAAATGGGGTCTATCTGGAGGAAGTTTGAGAGG
MSN4-F	*ATGCTAGTCTTCGGACCTAATAGTAGTTTCGTTCGTCACG*CACAGGAAACAGCTATGACC
MSN4-R	*TGCTCACTGCGTCTGAATGCCTTCTCACAGTCTTTACACT*GTTGTAAAACGACGGCCAGT
IRA2-F	*TCAACTAAACTGTATACATTATCTTTCTTCAGGGAGAAGC*CACAGGAAACAGCTATGACC
IRA2-R	*CATGTCGGAGTTCTTCGCAAATATACCAGAAGAAGTCACA*GTTGTAAAACGACGGCCAGT
MSN2-F	*TCATAGAAGAACTAGATCTAAAATGACGGTCGACCATGAT*CACAGGAAACAGCTATGACC
MSN2-R	*GTCTTGATGTGTTGCGACAAATTATCGCTTCTACTAAATT*GTTGTAAAACGACGGCCAGT
PHO4-F	*GAGATGAGCAAAGGAGACAGAACAAGAGTAGCAGAAAGTC*CACAGGAAACAGCTATGACC
PHO4-R	*GTCACGTGCTCACGTTCTGCTGTAGGTGACGGATGTACCG*GTTGTAAAACGACGGCCAGT

[a] Sequences in italics represent the restriction enzyme sites for cloning of the PCR products.

Table II-3. Oligonucleotide primers used in this study for plasmid construction.

Primer name	Sequence (5'→3')[a]
RAS2(G19V)-F	GTCGTTGGTGGTGTTGGTGTTGGTAAA
RAS2(G19V)-R	TTTACCAACACCAACACCACCAACGAC
RAS2(G22A)-F	GGTGGTGGTGTTGCTAAATCTGCTTTG
RAS2(G22A)-R	CAAAGCAGATTTAGCAACACCACCACC
RAS2+1200R(*Sal*I)	GACT*GTCGAC*GATGACTCTCTGCAATGTCC
RAS2-6(*Xba*I)	TCAG*TCTAGA*TTTCTGTATATCTCCTTTCAATTC
UGP1_1_*Spe*I	TGTC*ACTAGT*CCACTAAGAAGCACAC
GFP+800(*Xho*I)	GTCA*CTCGAG*ATTCGCTTATTTAGAAGTGG
pUGP1_pRS415	GGTGGCGGCCGCTCTAGAACTAGTGGATCCGATG AACGCCGTACTTCTGG
ADH1t_pRS415	GGCGAATTGGGTACCGGGCCCCCCCCTCGAGCCT ATTTGTATAGTTCATCC
pUGP1_MSN2-4	GCGGTAGCAACGTGCGTAATAAGGACTTCTCTTG GCGACTCTCGAATACC
MSN2-4_2	CATCTTCCACGGTATTCGAGAGTCGCCAAGAGAA GTCCTTATTACGCACG

[a] Sequences in italics represent the restriction enzyme sites for cloning of the PCR products.

2.3. Gel electrophoresis and western blot analysis

Cell extracts were prepared by suspending cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% NP-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/ml leupeptin, and 1 μg/ml pepstatin), followed by bead-beating. Extracts were spun by centrifugation at 1,600 *g* for 10 min at 4°C and the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was performed by standard methods with HRP-conjugated anti-GFP antibody (sc-5384 HRP, Santa Cruz Biotechnology), anti-hexokinase antibody (H2035-02, United States Biological) and anti-rabbit antibody (A6154, Sigma-Aldrich).

2.4. Quantification of mRNA

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). 1 μg of total RNA was reverse transcribed in a 20 μl reaction mixture containing MLV-reverse transcriptase (M-biotech) and 0.1 μg of oligo-dT (M-biotech) at 42°C for 1 hr. The amount of mRNA was analyzed by quantitative PCR using the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems). Primer sequences used for quantitative PCR are shown in Table S2.

2.5. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assay was carried out as described previously (Ha and Huh, 2010). For ChIP of TAP-tagged proteins, 10 μl of 50% slurry of pre-washed IgG agarose beads (GE Healthcare) was incubated with 200 μl of each lysate at 4°C for 3 hr. Input and ChIP samples were analyzed by quantitative PCR using SYBR Green (M-biotech). Relative fold enrichment was determined by calculating the ratio of target regions to *CUP1*, an internal control, using the comparative C_T method (Livak and Schmittgen, 2001). Values for untagged strains were normalized to 1. Primer sequences used for ChIP assay are shown in Table S2.

2.6. Fluorescence microscopy

Fluorescence microscopy was performed on a Nikon Eclipse Ti inverted microscope. Cells were grown in SC medium at 30°C. Image analysis was performed using the NIS-Elements AR3.1 microscopy software (Nikon) in order to determine the percentage of cells with predominately nuclear fluorescence. At least 100 cells were counted for each determination.

2.7. Statistical analysis

All quantitative data are presented as mean ± standard deviations of three independent experiments. Statistical differences were determined by Student's *t*-test with significance set at $P < 0.01$.

Table II-4. Oligonucleotide primers used in this study for quantitative real-time PCR.

Primer name	Sequence (5'→3')[a]
Primers for qPCR	
ACT1-CHK	TGACTGACTACTTGATGAAG
ACT1+1105R	ACAGAAGGATGGAACAAAGC
UGP1+1301	ACGCAAGAATCCCTCACATC
UGP1+1481R	AAATTACCAGTAACGACAAC
Primers for ChIP	
CUP1-200	TCTTTTCCGCTGAACCGTTCCAGC
CUP1+50R	GGCATTGGCACTCATGACCTTCAT
STRE-300F	GCCTCCGCGAGTCATCAGTC
STRE-300R	AAGTAAGTATTATTTATGTG
STRE-0F	ACACATATACACACTAGCTG
STRE-0R	GAATTGAATCTGTAGCAATG
STRE+300F	GGCGCTTGCAAAGTCCAAAG
STRE+300R	AGAGGACTAGGCCTTTTAAC

3. Results

3.1. The expression level of *UGP1* is dependent on PKA activity

The putative regulators of UGPase has been investigated in several organisms (Ciereszko et al., 2001; Ciereszko et al., 2005; Hedegaard et al., 2004; Nishizawa et al., 2001). However, given the physiological importance of UGPase, it is plausible that other upstream regulators of UGPase exist and have yet to be identified. To identify upstream regulators of UGPase, we examined Ugp1 level in deletion mutants of several candidate genes: *RAS2* and *IRA2* in PKA pathway (Broach, 1991), *TOR1* in TOR pathway (Cutler et al., 1999), *YAK1* in stress responses (Malcher et al., 2011), *SNF1* in AMP-activated protein kinase signaling pathway (Daniel and Carling, 2002) and *SLT2* in mitogen-activated protein kinase signaling pathway (Lee et al., 1993). A notable change in Ugp1 level was observed in PKA-related *ras2*Δ and *ira2*Δ mutants; Ugp1 level was significantly decreased in *ira2*Δ mutant and was increased in *ras2*Δ mutant (**Fig. II-1**). There was little, if any, change in Ugp1 level in other mutants.

Ras2 is a widely conserved small GTPase inducing the activation of cAMP-PKA pathway and Ira2 regulates Ras2 negatively as a GTPase-activating protein (Tanaka et al., 1991). To check whether the change in Ugp1 level is caused by the alteration of PKA activity, we constructed plasmids expressing wild-type Ras2, constitutively active $Ras2^{19V}$ or inactive $Ras2^{22A}$ mutant and

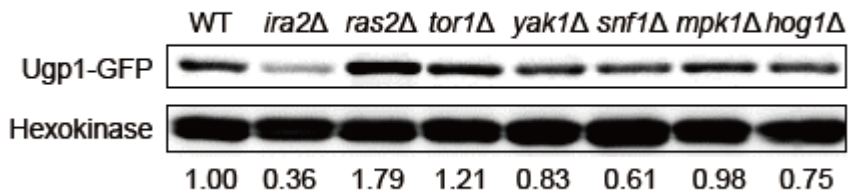


Fig. II-1. The expression of *UGP1* is dependent on the PKA pathway.

Total protein was extracted from the indicated cells, and immunoblotting was performed using a mouse anti-GFP antibody for the detection of Ugp1-GFP. Hexokinase was used as a loading control. The relative ratio of Ugp1 to hexokinase, normalized against that of wild-type (WT) cells, is shown below each lane. Data are representative of at least three independent experiments

introduced them into *ras2*Δ cells. In Ras2^{19V}-expressing cells with high PKA activity, a significant reduction of Ugp1 level was observed (**Fig. II-2A**). By contrast, in Ras2^{22A}-expressing cells with repressed PKA activity, Ugp1 level was considerably increased compared with wild-type Ras2-expressing cells. This result suggests that the expression of *UGP1* is regulated in inverse proportion to PKA activity. To confirm that PKA-dependent regulation of *UGP1* is transcriptionally controlled, we measured the *UGP1* mRNA level in the above cells. As expected, the *UGP1* mRNA level was also inversely regulated by PKA activity (**Fig. II-2B**), indicating that the expression of *UGP1* is under transcriptional control mediated by PKA activity.

3.2. The expression of *UGP1* is controlled by stress-responsive transcription factors Msn2/4

Several transcriptions factors are known to regulate genes under the control of cAMP-PKA pathway. Based on the presence of DNA binding motif in the *UGP1* promoter and functional correlation with cAMP-PKA pathway, we selected Msn2/4 (Martinez-Pastor et al., 1996), Pho4 (Nishizawa et al., 2001), Gis1 (Pedruzzi et al., 2000), Crz1 (Bodvard et al., 2013) and Mig1 (Lutfiyya et al., 1998) as candidate transcription factors that may be involved in the regulation of *UGP1*. When Ugp1 level was examined in deletion mutants of the candidate transcription factors, a notable reduction of Ugp1 level was observed only in *msn2*Δ*/4*Δ mutant (**Fig. II-3A**). We also observed that the

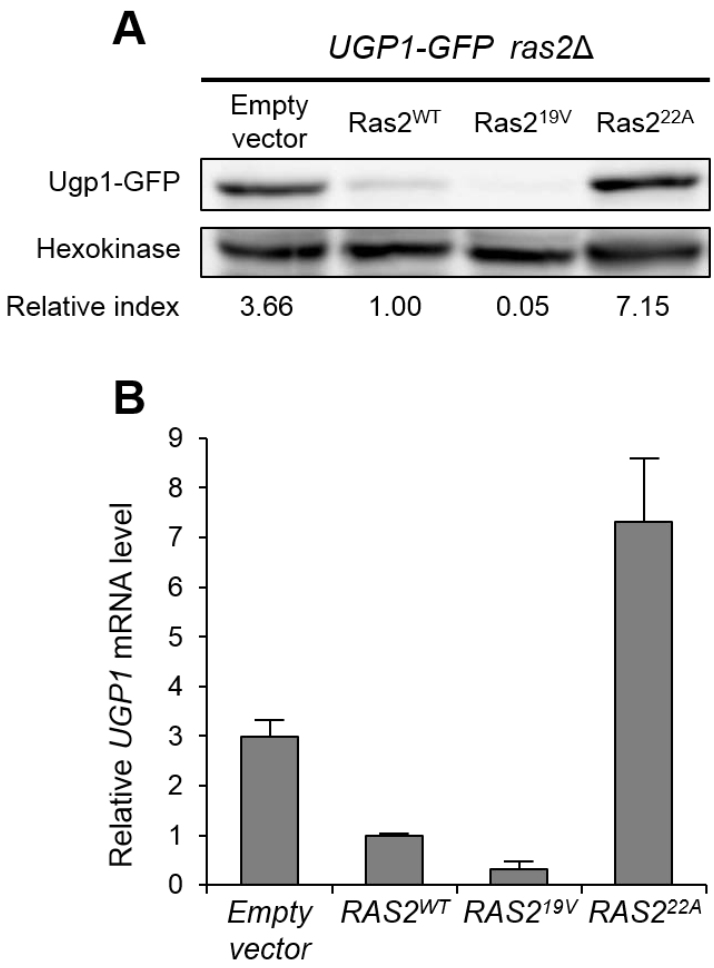

A
UGP1-GFP ras2Δ
Empty vector
Ras2WT
Ras219V
Ras222A
Ugp1-GFP
Hexokinase
Relative index
3.66
1.00
0.05
7.15
B
Relative UGP1 mRNA level
0
1
2
3
4
5
6
7
8
9
Empty vector
RAS2WT
RAS219V
RAS222A

Fig. II-2. The expression of *UGP1* is regulated inversely by PKA activity.

(A) Total protein was extracted from *ras2*Δ cells containing pRS415ADH-RAS2 ($Ras2^{WT}$), pRS415ADH-$RAS2^{19V}$ ($Ras2^{19V}$) or pRS415ADH-$RAS2^{22A}$ ($Ras2^{22A}$), and immunoblotting was performed using a mouse anti-GFP antibody for the detection of Ugp1-GFP. The relative ratio of Ugp1 to hexokinase, normalized against that of WT Ras2-expressing cells, is shown below each lane. Data are representative of at least three independent experiments. (B) Total RNA was extracted from the same cells as described in (A). Quantitative real-time reverse transcription-PCR analysis was performed to measure the mRNA level of *UGP1*. Amplification efficiencies were validated and normalized against *ACT1*. The relative *UGP1* mRNA level was calculated as the ratio of the normalized mRNA level of the *UGP1* to that of *ACT1*. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with WT Ras2-expressing cells (Student's *t*-test).

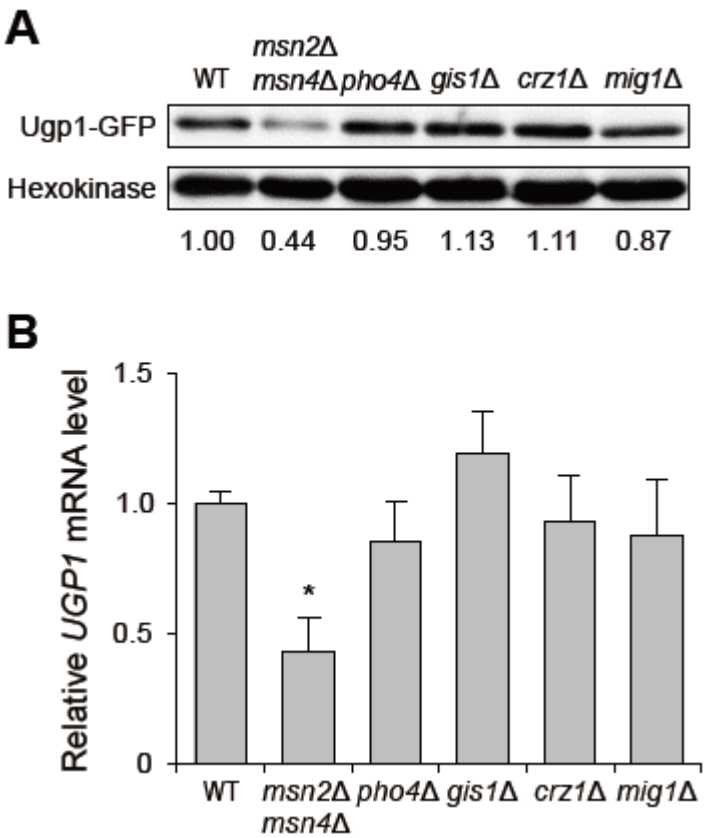

A
WT
msn2Δ msn4Δ
pho4Δ
gis1Δ
crz1Δ
mig1Δ
Ugp1-GFP
Hexokinase
1.00
0.44
0.95
1.13
1.11
0.87
B
Relative UGP1 mRNA level
1.5
1.0
0.5
0
*
WT
msn2Δ msn4Δ
pho4Δ
gis1Δ
crz1Δ
mig1Δ

Fig. II-3. Msn2/4 function as transcriptional activators of *UGP1*.

(A) Total protein was extracted from the indicated cells, and immunoblotting was performed using a mouse anti-GFP antibody for the detection of Ugp1-GFP. Hexokinase was used as a loading control. The relative ratio of Ugp1 to hexokinase, normalized against that of wild-type (WT) cells, is shown below each lane. Data are representative of at least three independent experiments. (B) Total RNA was extracted from the indicated cells, and quantitative real-time reverse transcription-PCR analysis was performed to measure the mRNA level of *UGP1*. Amplification efficiencies were validated and normalized against *ACT1*. The relative *UGP1* mRNA level was calculated as the ratio of the normalized mRNA level of the *UGP1* to that of *ACT1*. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with WT cells (Student's *t*-test).

UGP1 mRNA level was noticeably decreased in *msn2Δ/4Δ* mutant (**Fig. II-3B**). Given that Msn2/4 transcriptional activity is stimulated under stress condition (Görner et al., 1998; Schmitt and McEntee, 1996), we then measured the time course of the *UGP1* mRNA level during oxidative stress. The transcript level of *UGP1* in wild-type cells was increased more than 3-fold at 30 min (**Fig. II-4**). By contrast, no significant fluctuation in the *UGP1* mRNA level was observed in *msn2Δ/4Δ* cells. Taken together, these results suggest that the expression of *UGP1* is controlled at the level of transcription by stress-responsive transcription factors Msn2/4.

3.3. Msn2/4 regulates transcription of *UGP1* by direct binding to STREs in the *UGP1* promoter

Msn2/4 are known to bind to the consensus sequence (5′-AGGGG-3′) called STRE for the regulation of stress-responsive gene expression (Martinez-Pastor et al., 1996). We noticed that three STREs are present in the middle of the *UGP1* promoter (**Fig. II-5**). To investigate whether Msn2/4-dependent transcriptional activation of *UGP1* is mediated via these STREs, we constructed plasmids expressing Ugp1-GFP under the control of its own (*prUGP1*) or STREs-deleted promoter (S*TRE*Δ). The plasmids were then introduced into wild-type and *ras2*Δ cells and their promoter activities were analyzed by measuring the protein level and mRNA content of *UGP1*. In wild-type cells, the protein level of Ugp1 was substantially decreased under the *STRE*Δ promoter compared with that under the *prUGP1* promoter

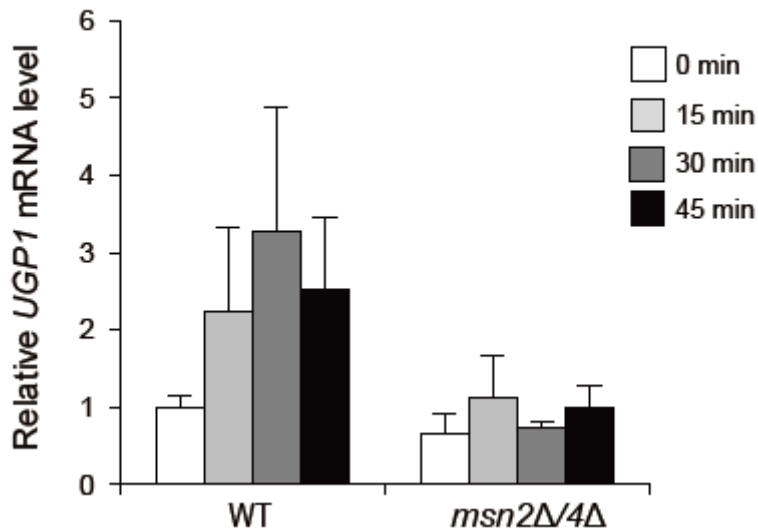


Fig. II-4. Transcriptional activation of *UGP1* is mediated by Msn2/4.

The time course of the *UGP1* mRNA level under oxidative stress was measured by quantitative real-time reverse transcription-PCR analysis. After treatment of 1 mM H_2O_2, the amount of *UGP1* mRNA from WT or *msn2Δ/4Δ* cells was determined at 15-min intervals. Total RNA was extracted from the indicated cells, and quantitative real-time reverse transcription-PCR analysis was performed to measure the mRNA level of *UGP1*. Amplification efficiencies were validated and normalized against *ACT1*. The relative *UGP1* mRNA level was calculated as the ratio of the normalized mRNA level of the *UGP1* to that of *ACT1*. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with WT cells (Student's *t*-test).

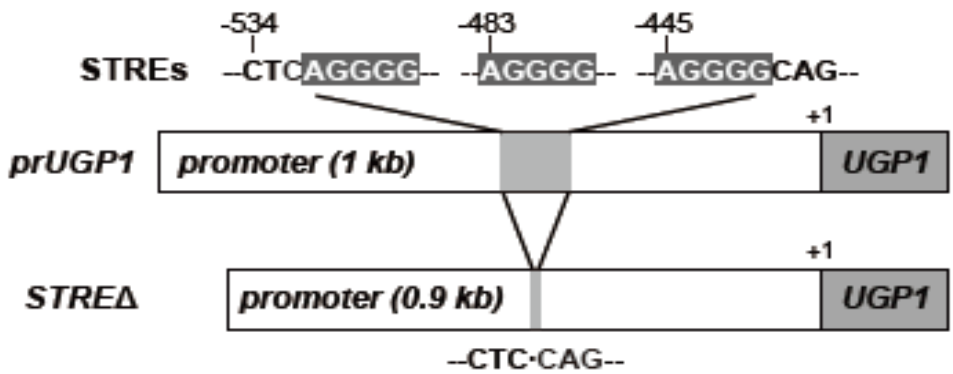


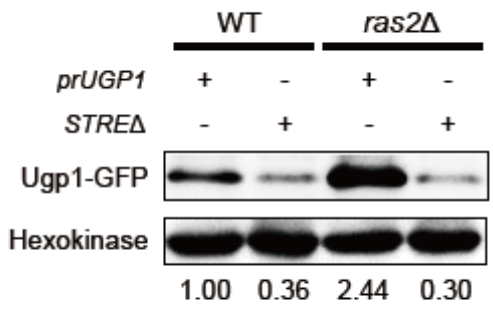
Fig. II-5. Schematic representation of part of plasmids.

Plasmids expressing Ugp1-GFP under wild-type or STREs-deleted promoter. Three STREs (5′-AGGGG-3′) located at -534, -483 and -445 are indicated in the middle of the *UGP1* promoter.

(**Fig. II-6A**). The *UGP1* mRNA level was also decreased under the *STRE*Δ promoter (**Fig. II-6B**). Remarkably, the absence of STREs in the *UGP1* promoter almost completely abolished the increase in the protein level and mRNA content of *UGP1* in *ras2*Δ cells with low PKA activity (**Fig. II-6A, B**). These results suggest that STREs in the *UGP1* promoter is necessary for transcriptional activation of *UGP1*.

To check whether Msn2/4 physically bind to STREs in the *UGP1* promoter, we carried out ChIP assay using yeast strains in which the endogenous *MSN2* gene was modified to express C-terminal TAP fusion protein. The *UGP1* promoter region was divided into three regions (STRE-300, STRE-0 and STRE+300) to enhance the specificity of ChIP assay (**Fig. II-7**, upper panel). In wild-type cells, Msn2 bound highly to the STRE-0 region containing three STREs while it did not show significant binding to other regions (**Fig. II-7**, lower panel), suggesting that Msn2-dependent transcriptional activation of *UGP1* is mediated by physical binding of Msn2 to STREs in the *UGP1* promoter. Previously, it has been shown that STRE-dependent transcription is negatively regulated by PKA activity (Görner et al., 1998; Marchler et al., 1993). Consistent with this report, the binding of Msn2 to the STRE-0 region was considerably increased in *ras2*Δ cells with weakened PKA activity (**Fig. II-7**, lower panel). By contrast, a significant reduction in the binding of Msn2 to the STRE-0 region was observed in *ira2*Δ cells with increased PKA activity. It is likely that physical binding of Msn2/4 to STREs in the *UGP1* promoter is attributed to PKA-dependent nuclear localization of Msn2/4. In

A


WT
ras2Δ
prUGP1
+
-
+
-
STREΔ
-
+
-
+
Ugp1-GFP
Hexokinase
1.00
0.36
2.44
0.30

B

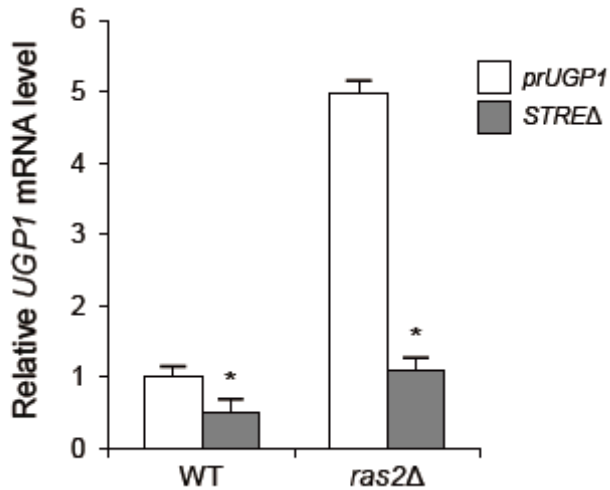

Relative UGP1 mRNA level
6
5
4
3
2
1
0
prUGP1
STREΔ
*
*
WT
ras2Δ

Fig. II-6. Binding of Msn2/4 to STREs on the promoter of *UGP1* is required for transcriptional regulation.

(A) Total protein was extracted from wild-type (WT) or *ras2*Δ cells containing pRS415-pr^{UGP1}-UGP1-GFP (*prUGP1*) or pRS415-pr$^{UGP1(-531\sim-441\Delta)}$-UGP1-GFP plasmid (*STRE*Δ), and immunoblotting was performed using a mouse anti-GFP antibody for the detection of Ugp1-GFP. The relative ratio of Ugp1 to hexokinase, normalized against that of WT cells with pRS415-pr^{UGP1}-UGP1-GFP, is shown below each lane. Data are representative of at least three independent experiments. (B) Total RNA was extracted from the same cells as described in (A). Quantitative real-time reverse transcription-PCR analysis was performed to measure the mRNA level of *UGP1*. Amplification efficiencies were validated and normalized against *ACT1*. The relative *UGP1* mRNA level was calculated as the ratio of the normalized mRNA level of the *UGP1* to that of *ACT1*. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with WT cells with pRS415-pr^{UGP1}-UGP1-GFP (Student's *t*-test).

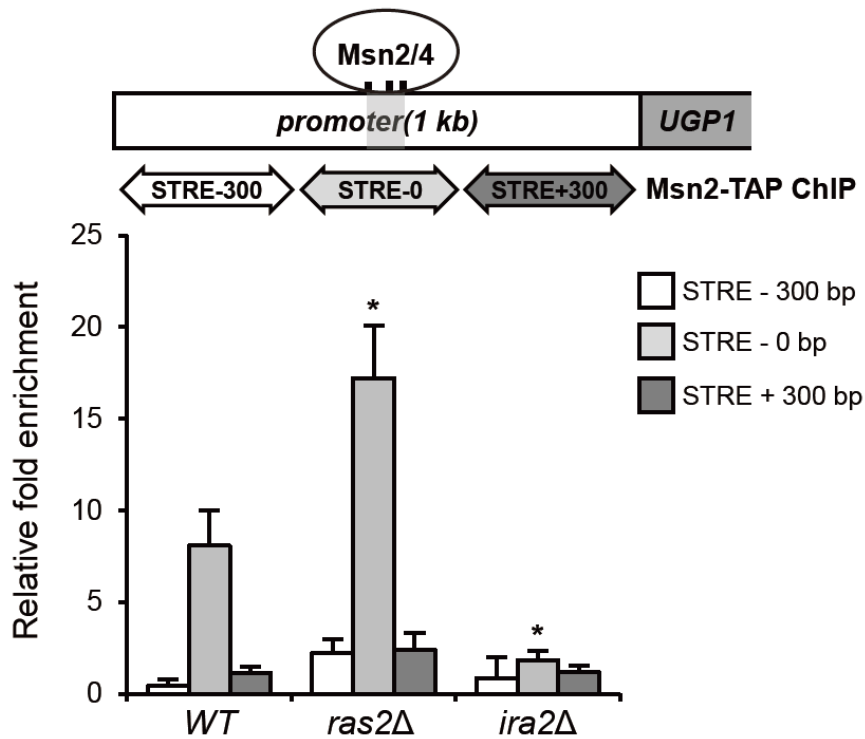


Fig. II-7. Msn2/4 bind directly to STREs on the promoter of *UGP1* for transcriptional regulation.

The degree of Msn2 binding to STRE-300, STRE-0 and STRE+300 regions (-949~-650, -649~-349 and -347~-49 bp, respectively) in the *UGP1* promoter was measured using ChIP assay in WT, *ira2*Δ and *ras2*Δ cells. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with WT cells (Student's *t*-test).

conclusion, these findings suggest that STREs in the *UGP1* promoter is necessary for Msn2/4-dependent transcriptional activation of *UGP1*.

3.4. Elevated expression of *UGP1* in response to several types of stress is mediated by Msn2/4

Several studies have shown that the expression of *UGP1* is induced under various stresses (Boy-Marcotte et al., 1999; Gasch et al., 2000; Rep et al., 2000). We wondered whether transcriptional activation of *UGP1* under various stresses is mediated by Msn2/4. To determine this, we measured the *UGP1* mRNA level under several stress conditions. Consistent with previous reports, the *UGP1* mRNA level was increased by oxidant, heat, hyperosmolarity and ethanol (**Fig. II-8**). However, the induction of *UGP1* mRNA under various stresses was almost completely lost in *msn2Δ/4Δ* cells, indicating that Msn2/4 mediate *UGP1* induction under stress conditions. Considering the role of Msn2/4, it seems that the regulation of *UGP1* is a part of the general stress response system.

3.5. Non-canonical PHO pathway positively regulates the expression of *UGP1* by driving nuclear localization of Msn2/4

It has been reported that *UGP1* is upregulated by *pho85Δ* mutation and that Pho4, a key transcription factor in PHO pathway, activates *UGP1* expression by binding to the *UGP1* promoter (Nishizawa et al., 2001). However, unlike the previous study, we could not observe the downregulation of *UGP1* in

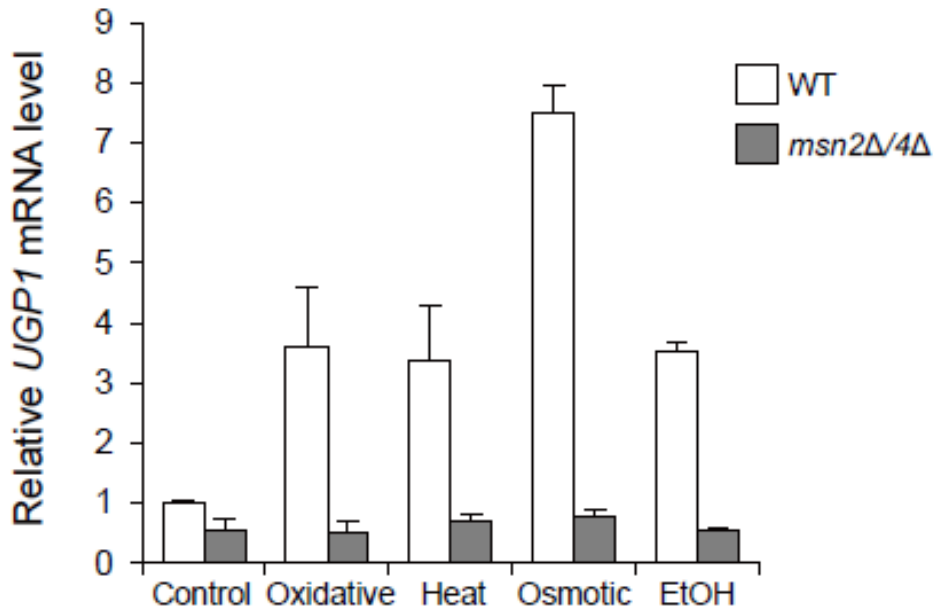


Fig. II-8. Various types of stress provoke the expression of *UGP1* through Msn2/4.

Wild-type (WT) or *msn2Δ/4Δ* cells were grown to mid-log phase and then treated with 1 mM H_2O_2 (Oxidative), 37°C (Heat), 1 M NaCl (Osmotic) or 7% ethanol (EtOH) for 30 min. Total RNA was extracted from cells and quantitative real-time reverse transcription-PCR analysis was performed to measure the mRNA level of *UGP1*. Amplification efficiencies were validated and normalized against *ACT1*. The relative *UGP1* mRNA level was calculated as the ratio of the normalized mRNA level of the *UGP1* to that of *ACT1*. Values represent the average of three independent experiments, and error bars indicate the standard deviation.

*pho4*Δ mutant (**Fig. II-3A, 3B**). To resolve the discrepancy, we examined the effect of *pho85*Δ mutation on *UGP1* expression. The protein level of Ugp1 was increased in *pho85*Δ cells (**Fig. II-9A**). The *UGP1* mRNA level was also significantly increased in *pho85*Δ cells (**Fig. II-9B**). Notably, loss of Pho4 did not affect the upregulation of *UGP1* induced by *pho85*Δ mutation, indicating that Pho4 is not involved in *UGP1* expression. By contrast, in *msn2*Δ*/4*Δ cells, lowered expression of *UGP1* was not restored by *pho85*Δ mutation (**Fig. II-9A, 9B**). These results suggest that Msn2/4, but not Pho4, act downstream of Pho85 to regulate *UGP1* expression.

Given previous reports that phosphate depletion induces UGPase expression in plants and causes Pho85 inactivation in yeast (Ciereszko et al., 2001; Kaffman et al., 1994), we postulated that phosphate starvation may increase *UGP1* expression like *pho85*Δ mutation. As expected, the protein level of Ugp1 was considerably increased under phosphate starvation (**Fig. II-10A**). The *UGP1* mRNA level was also significantly increased under phosphate starvation (**Fig. II-10B**). Phosphate starvation increased the protein level and mRNA content of *UGP1* also in *pho4*Δ cells. However, in *msn2*Δ*/4*Δ cells, we did not observe any increase in the protein level and mRNA content of *UGP1* under phosphate starvation. Thus, we conclude that the upregulation of *UGP1* under phosphate starvation is mediated by Msn2/4 but not Pho4.

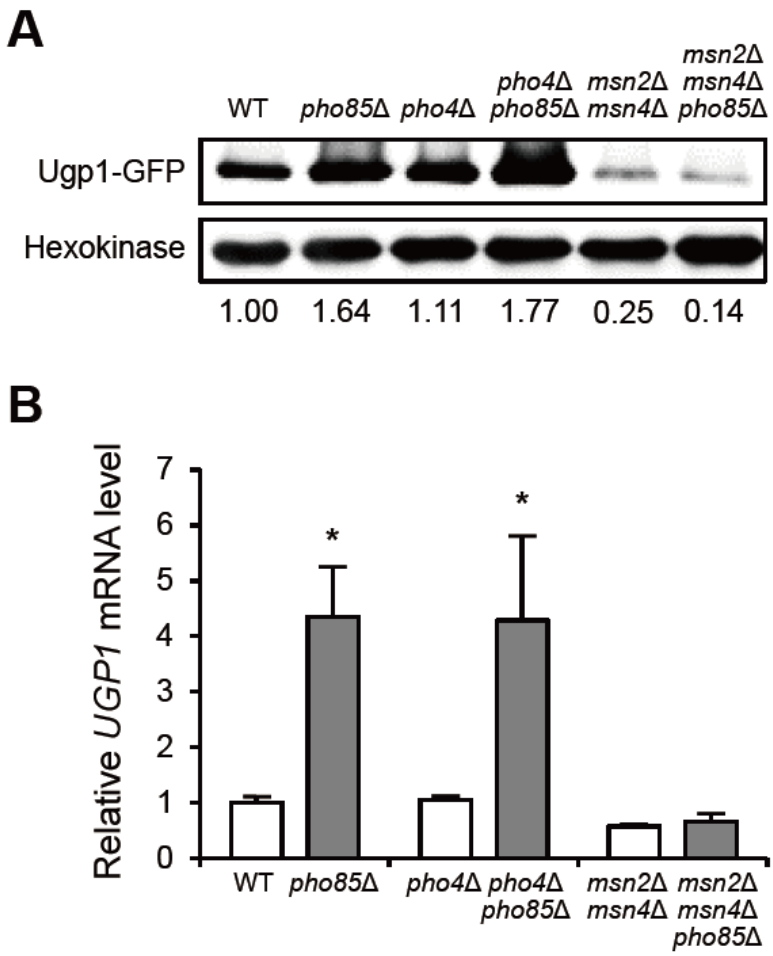

A
WT
pho85Δ
pho4Δ
pho4Δ pho85Δ
msn2Δ msn4Δ
msn2Δ msn4Δ pho85Δ
Ugp1-GFP
Hexokinase
1.00
1.64
1.11
1.77
0.25
0.14
B
Relative UGP1 mRNA level
0
1
2
3
4
5
6
7
*
*
WT
pho85Δ
pho4Δ
pho4Δ pho85Δ
msn2Δ msn4Δ
msn2Δ msn4Δ pho85Δ

Fig. II-9. Activation of PHO pathway by loss of *PHO85* leads the induction of *UGP1* in an Msn2/4-dependent manner.

(A) Total protein was extracted from the indicated cells, and immunoblotting was performed using a mouse anti-GFP antibody for the detection of Ugp1-GFP. Hexokinase was used as a loading control. The relative ratio of Ugp1 to hexokinase, normalized against that of wild-type (WT) cells, is shown below each lane. Data are representative of at least three independent experiments. (B) Total RNA was extracted from the indicated cells, and quantitative real-time reverse transcription-PCR analysis was performed to measure the mRNA level of *UGP1*. Amplification efficiencies were validated and normalized against *ACT1*. The relative *UGP1* mRNA level was calculated as the ratio of the normalized mRNA level of the *UGP1* to that of *ACT1*. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with WT cells (Student's *t*-test).

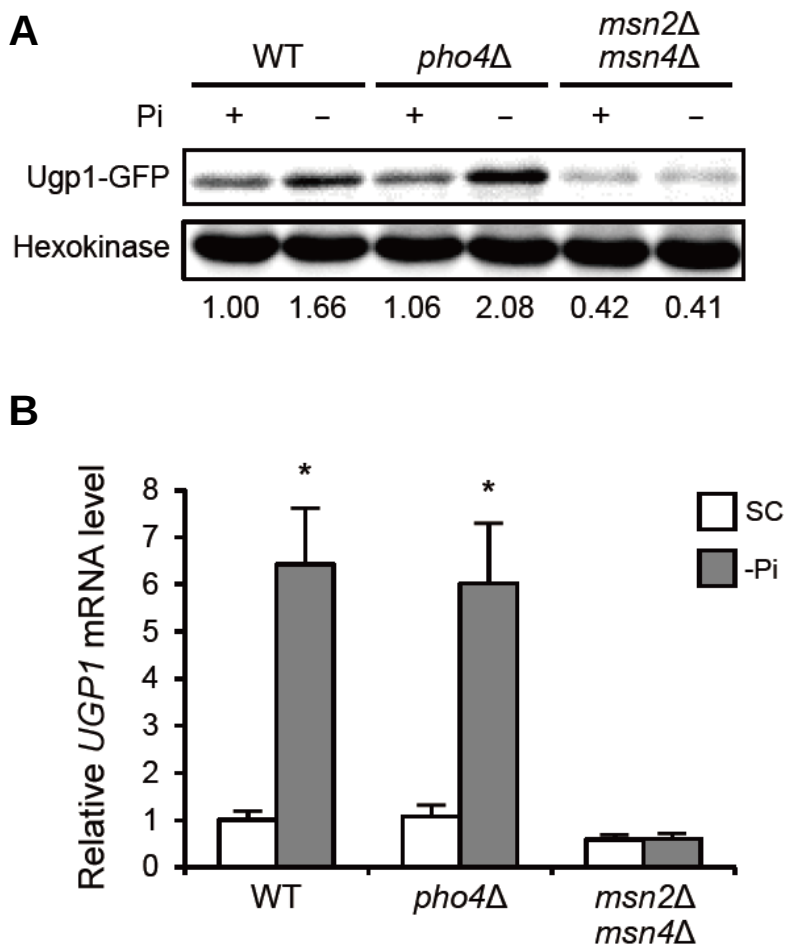

A
WT
pho4Δ
msn2Δ msn4Δ
Pi
+
–
+
–
+
–
Ugp1-GFP
Hexokinase
1.00
1.66
1.06
2.08
0.42
0.41
B
Relative UGP1 mRNA level
0
1
2
3
4
5
6
7
8
*
*
SC
-Pi
WT
pho4Δ
msn2Δ msn4Δ

Fig. II-10. Activation of PHO pathway by phosphate starvation leads the induction of *UGP1* in an Msn2/4-dependent manner.

(A) WT, *pho4*Δ or *msn2/4*Δ cells were grown in SC medium to mid-log phase and then transferred to phosphate-depleted medium for 2 h. Total protein was extracted from the indicated cells, and immunoblotting was performed using a mouse anti-GFP antibody for the detection of Ugp1-GFP. Hexokinase was used as a loading control. The relative ratio of Ugp1 to hexokinase, normalized against that of wild-type (WT) cells, is shown below each lane. Data are representative of at least three independent experiments. (B) Total RNA was extracted from the indicated cells, and quantitative real-time reverse transcription-PCR analysis was performed to measure the mRNA level of *UGP1*. Amplification efficiencies were validated and normalized against *ACT1*. The relative *UGP1* mRNA level was calculated as the ratio of the normalized mRNA level of the *UGP1* to that of *ACT1*. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with WT cells (Student's *t*-test).

To confirm that Msn2/4 directly upregulates *UGP1* under phosphate starvation, we checked subcellular localization of Msn2/4 when phosphate is depleted. Because the transcription factor activity of Msn2/4 is only manifested when their dephosphorylated forms are translocated to the nucleus from the cytoplasm (Görner et al., 1998), we measured the ratio of nuclear-localized Msn2. Under normal condition, Msn2 was mainly localized to the cytoplasm and was detected in the nucleus in only approximately 12% of wild-type cells (**Fig. II-11**). When wild-type cells were starved for phosphate, approximately 80% of cells showed nuclear translocation of Msn2. *pho4*Δ cells showed Msn2 localization pattern similar to that of wild-type cells; Msn2 was predominantly cytoplasmic in SC medium but was translocated to the nucleus in phosphate-depleted medium (**Fig. II-11**). Interestingly, *pho85*Δ cells exhibited considerably increased nuclear accumulation of Msn2 even under normal condition. In *pho85*Δ cells, nuclear accumulation of Msn2 in SC medium was comparable to that in phosphate-depleted medium (**Fig. II-11**). Taken together, these results suggest that the activation of PHO pathway by *pho85*Δ mutation or phosphate depletion leads to *UGP1* upregulation in a manner dependent on Msn2/4 but not Pho4.

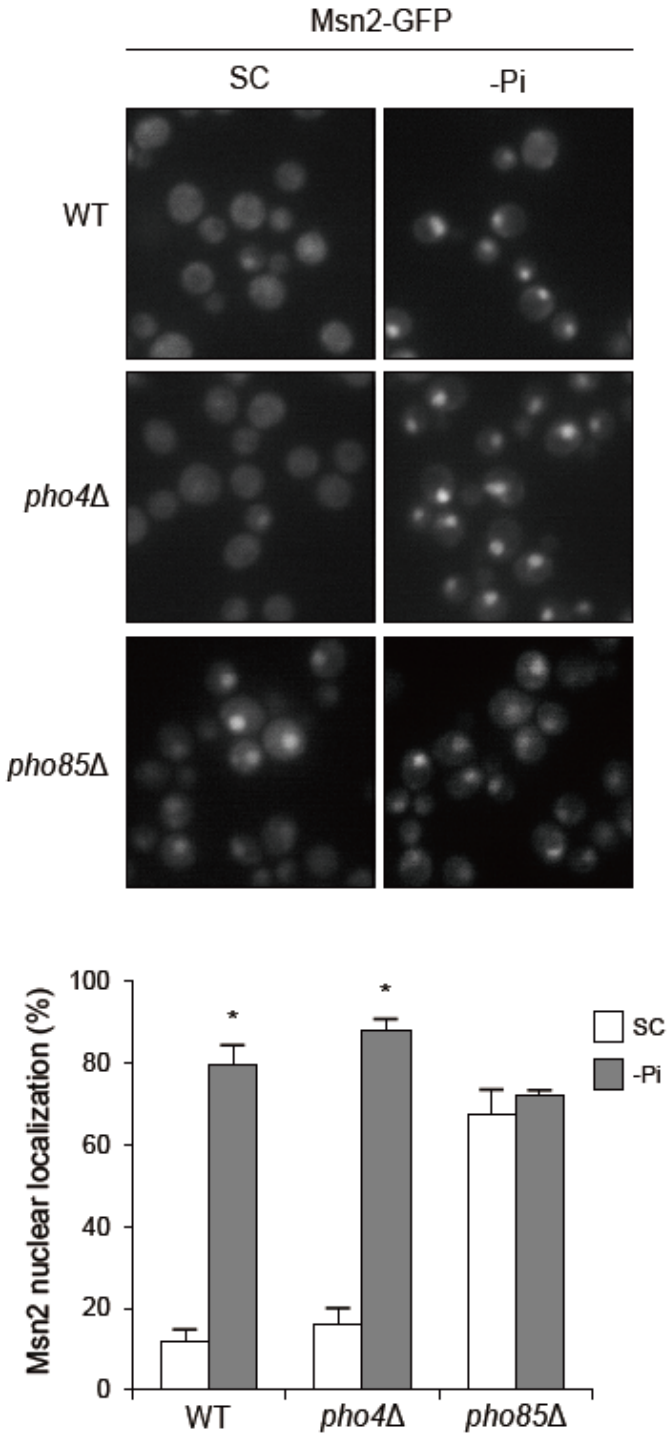

Msn2-GFP
SC
-Pi
WT
pho4Δ
pho85Δ
Msn2 nuclear localization (%)
100
80
60
40
20
0
*
*
SC
-Pi
WT
pho4Δ
pho85Δ

Fig. II-11. Activation of PHO pathway leads nuclear accumulation of Msn2/4.

WT, *pho4*Δ or *pho85*Δ cells were grown in SC medium to mid-log phase and then transferred to phosphate-depleted medium for 2 h. Subcellular localization of Msn2-GFP was analyzed by fluorescence microscopy (upper panel). The percentage of nuclear Msn2 is shown in the lower panel. Values represent the average of three independent experiments and at least 100 cells were counted for each determination. Error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with control cells grown in SC medium (Student's *t*-test).

4. Discussion

Balancing energy production and carbohydrate synthesis with limited carbon source is an important survival strategy for cell viability. UGPase plays a significant role in cellular carbon allocation and carbohydrate synthesis because the only glycosyl donor UDP-Glc is produced by UGPase (Daran et al., 1995). Nonetheless, the precise mechanism for the regulation of UGPase has remained unclear. In this study, we found that UGPase encoded by *UGP1* is controlled by PKA activity in *S. cerevisiae* (**Fig. II-1, 2**). PKA pathway has been implicated in energy metabolism and carbohydrate mobilization as the primary mediator in response to environmental changes (Santangelo, 2006). Although the relevance between PKA pathway and Ugp1 function has not been fully established yet, both PKA pathway and Ugp1 have been reported to regulate the cellular accumulation of glycogen and trehalose (Daran et al., 1997; Daran et al., 1995; Smith et al., 1998). Therefore, it seems likely that the regulation of *UGP1* is an initial step for the control of carbohydrate metabolism mediated by PKA pathway.

Msn2/4 are general stress-responsive transcription factors regulating hundreds of genes by binding to their DNA-binding motif (Martinez-Pastor et al., 1996). In this study, we found that Msn2/4 regulate the expression of *UGP1* by directly binding to the *UGP1* promoter (**Fig. II-6, 7**) and *UGP1* transcription is induced by Msn2/4 under several stress conditions (**Fig. II-**

8). Previously, it has been reported that yeast cells with overexpressed *UGP1* can overcome growth defects caused by toxins or cell wall perturbants (Mehlgarten et al., 2007). Furthermore, the overexpression of UGPase induces the production of protective molecules such as hyaluronic acid or salicylic acid 2-*O*-β-D-glucoside in other organisms (Coleman et al., 2007; Prasad et al., 2010). Taken together with previous results, our finding suggests that the regulation of *UGP1* may constitute a kind of cellular defensive tactics and the modulation of Ugp1 level may be a universal process for cells to respond to stresses.

Previous studies have shown that the addition of inorganic phosphate to phosphate-starved yeast cells enhances PKA activity regardless of cAMP level and that the deletion of *PHO85* elevates the expression of stress response genes (Hirimburegama et al., 1992; Timblin and Bergman, 1997). These results suggest that PHO pathway and PKA pathway are closely related with each other. Consistent with this notion, we observed that both phosphate starvation and loss of Pho85 induce nuclear localization of Msn2, which is a typical phenotype of cells with low PKA activity (**Fig. II-11**). Given that Pho85 is a cyclin-dependent kinase (Huang et al., 2007), it will be interesting to investigate whether Msn2/4 is directly phosphorylated by Pho85 or not. Meanwhile, depending on phosphate availability, the Pho80-Pho85 complex has been established to phosphorylate Thr^{1075} of Rim15 kinase, thereby regulating its nucleocytoplasmic distribution (Wanke et al., 2005). Since the translocation of Rim15 into nucleus is required for the

Rim15-dependent gene regulation of Msn2/4 (Pedruzzi et al., 2003), therefore, it is likely that Rim15, a nutrient signaling protein kinase, acts as a nutritional integrator between the PHO and PKA pathway.

In summary, our data suggest that signals of PKA, PHO pathway and stresses converge on and regulate transcription factors Msn2/4 in *S. cerevisiae*. Activated Msn2/4 then induce yeast UGPase, Ugp1, which plays a vital role in glucose partitioning for carbohydrate synthesis. Collectively, we propose that PKA, PHO pathway and stresses control glucose flow to the synthesis of varied carbohydrates by regulating the expression of Ugp1 (**Fig. II-12**).

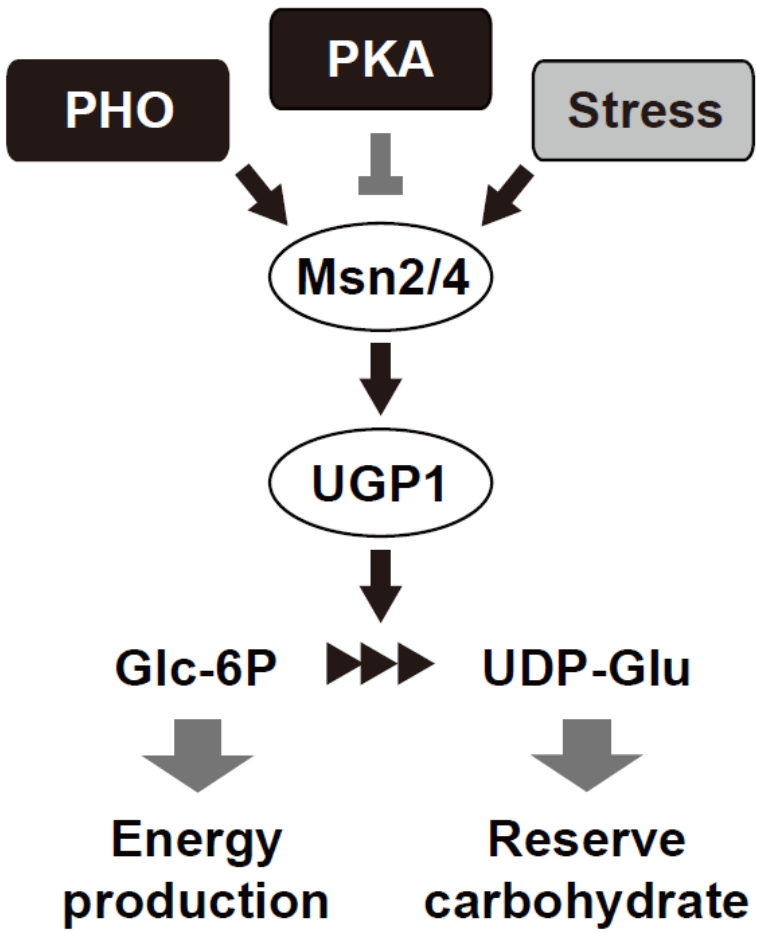


Fig. II-12. A model for transcriptional regulation of *UGP1* by Msn2/4.

Inactivation of PKA pathway, activation of PHO pathway or general stresses cause Msn2/4 to migrate into the nucleus, where they induce the expression of *UGP1*. Msn2/4-mediated regulation of *UGP1* may play a role in glucose partitioning by supplying UDP-Glc for carbohydrate synthesis.

CHAPTER III.

UDP-glucose pyrophosphorylase, Ugp1, is involved in oxidative stress response and CLS in *Saccharomyces cerevisiae*

1. Introduction

UDP-glucose pyrophosphorylase (UGPase) is found in all three kingdoms life, catalyzing the reversible formation of UDP-glucose (UDP-Glc) and pyrophosphate from glucose-1-phosphate and UTP (Aksamit and Ebner, 1972). UGPase has been considered as a crucial enzyme in carbohydrate metabolism because its product, UDP-Glc, serves as the glucosyl donor in several cellular process: the synthesis of storage carbohydrate, such as glycogen and trehalose (Daran et al., 1997; Daran et al., 1995; Hedegaard et al., 2004); the synthesis of structural carbohydrate, such as cellulose and β-glucan (Daran et al., 1995; Delmer, 1999); the synthesis of carbohydrate moiety of glycolipid and glycoprotein (Herscovics and Orlean, 1993; Sandhoff et al., 1992); the entry of galactose into glycosis (Frey, 1996); the synthesis of UDP-glucuronic acid (Aw and Jones, 1984).

Previous studies of UGPase have shown that the regulation of its expression level influences the yield of several kinds of carbohydrate. In *Lactococcus lactis*, over-expression of UGPase increases the hyaluronic acid production (Prasad et al., 2010). In hybrid poplar, soluble sugar, starch and cellulose content are increased significantly through up-regulated UGPase (Coleman et al., 2007). In *Saccharomyces cerevisiae*, repression of *UGP1*, the homolog of UGPase, induces reduction of a series of carbohydrate including glycogen, trehalose, as well as UDP-Glc (Daran et al., 1997; Daran et al., 1995). Therefore, these findings imply that the role of UGPase is

intimately related to diverse biological functions. However, these relations have not been revealed clearly in previous studies.

Glycogen and trehalose are two major forms of storage carbohydrate in *S. cerevisiae*. Although both carbohydrates have been considered as energy reservoirs, recent studies began to reveal novel biological functions. For example, glycogen levels make a great contribution to the overall fitness of yeast (Anderson and Tatchell, 2001). In addition, trehalose protects cells from stressful environment factors (Bell et al., 1992; Benaroudj et al., 2001; Gadd et al., 1987). The mobilization of glycogen and trehalose changes significantly, depending on the growth phase, nutrient conditions, and stress (François and Parrou, 2001). These changes are largely attributed to the protein kinase A (PKA) pathway (Smith et al., 1998; Winderickx et al., 1996).

In this study, we confirmed that a conditional *ugp1* null mutant in *S. cerevisiae* exhibited an impaired mobilization of glycogen and trehalose. Because of this impairment, reductions of oxidative stress resistance and chronological life span (CLS) in *ugp1* knockdown strain were observed. With complementation of Ugp1, defective phenotypes in *ugp1* knockdown cells was alleviated, suggesting the control of Ugp1 level is involved in anti-oxidant response and chronological longevity in budding yeast. Furthermore, we found that the effects of PKA activity on the assimilation of storage carbohydrate and cell viability were either enhanced or offset by adjusting the level of Ugp1. Based on above results, we suggest that regulation of *UGP1* expression plays a key role in downstream effects of the PKA pathway.

2. Materials and methods

2.1. Yeast strains, media

Yeast strains used in this study are listed in Table S1. Yeast cells were grown in YPD medium (1% yeast extract, 2% bacto-peptone, and 2% dextrose) or synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and amino acids) lacking appropriate amino acids. For hydrogen peroxide treatment, cells grown to log phase in SC medium were treated with 1 mM H_2O_2 and incubated as described in (Madeo et al., 1999).

2.2. Plasmid and strain construction

Plasmid pRS415-pr^{UGP1}-UGP1-GFP and pRS415THI4-UGP1-GFP were generated by cloning the *UGP1-GFP* sequence from yeast HY1362 into the *Spe*I and *Xho*I sites of pRS415ADH.

2.3. Chronological Life Span Determinations

All chronological life span determinations in this study were performed with cells grown in liquid SC medium lacking appropriate amino acids. Briefly, yeast cells were inoculated into 10 ml of SC media lacking leucine and grown overnight. The following day, cells were diluted into 20 ml of SC media lacking leucine to OD_{600} of 0.2 approximately. Cultures were incubated with shaking at 30ºC. 3 days after inoculation was considered as

Day 0 of chronological life span. Then, cellular viability was determined at the indicated days by colony formation unit (CFU) assay. Cell number was estimated by OD for each population and serial dilutions of different cultures were plated onto 3 YPD plates at an approximate concentration of 100 cells per plate. Plates were incubated at 30ºC for 2 days and CFU was counted.

2.4. Gel electrophoresis and western blot analysis

Cell extracts were prepared by suspending in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% NP-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg ml^{-1} leupeptin, and 1 μg ml^{-1} pepstatin), followed by bead-beating ten times for 1 min with 1 minute time intervals at 4ºC. Samples were spun by centrifugation at 1,600 g for 10 min at 4ºC and the supernatant was subjected to gel electrophoresis. SDS-PAGE and Western blot analysis were performed by using standard methods with HRP-conjugated anti-GFP IgG antibody (sc-5384, Santa Cruz Biotechnology, Inc.), anti-hexokinase antibody (H2035-2, United States Biological), and anti-rabbit antibody (A6154, Sigma-Aldrich).

2.5. Determination of glycogen and trehalose

Glycogen and trehalose content were assayed as described (Parrou and François, 1997). 10 OD of sample culture was harvested, resuspended in 0.25 ml of 0.25 M Na_2CO_3 and incubated for 4 hr at 95℃ using screw-top tubes. 0.15 ml of 1 M acetic acid and 0.65 ml of 0.2 M sodium acetate [pH 5.2] are

added to samples. For the glycogen content determination, glycogen of samples were digested by *A. niger* amyloglucosidase (A7420, Sigma-Aldrich) (1.2 U/ml) for 16 hr at 57℃ under constant agitation. For the trehalose content determination, trehalose of samples were digested by trehalase (T8778, Sigma-Aldrich) (0.05 U/ml) for 16 hr at 37℃ under constant shaking. After centrifugation for 10 min at 16,000 g, the glucose content derived from glycogen or trehalose in the supernatant was assayed using the Glucose assay reagent (G3293, Sigma-Aldrich).

2.6. Statistical analysis

All of quantitative data are presented as mean ± standard deviations of three independent experiments. Statistical differences were determined by the Student's *t*-test with significance set at $P < 0.01$.

Table III-1. Yeast strains used in this study.

Strain	Genotype	Source
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
HY1362	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3*	This study
HY1527	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ugp1Δ::HIS3MX6 [pRS415THI4-UGP1-GFP]*	This study
HY1533	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 tps1Δ::URA3 tps2Δ::KanMX6 [pRS415ADH]*	This study
HY1363	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 ras2Δ::LEU2*	This study
HY1364	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UGP1-GFP::URA3 ira2Δ::KanMX6*	This study
HY1480	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [pRS415ADH]*	This study
HY1481	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [pRS415ADH-UGP1-GFP]*	This study
HY1482	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ira2Δ::KanMX6 [pRS415ADH]*	This study
HY1483	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ira2Δ::KanMX6 [pRS415ADH-UGP1-GFP]*	This study
HY1530	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ::LEU2 [pRS415ADH]*	This study
HY1531	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ::LEU2 [pRS415ADH-UGP1-GFP]*	This study
HY1484	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pde2Δ::KanMX6 [pRS415ADH]*	This study
HY1485	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pde2Δ::KanMX6 [pRS415ADH-UGP1-GFP]*	This study
HY1486	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pde2Δ::KanMX6 [pRS415ADH]*	This study

HY1487	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pde2Δ::KanMX6 [pRS415ADH-UGP1-GFP]*	This study
HY1533	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gsy2Δ::KanMX6 [pRS415ADH]*	This study
HY1534	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gsy2Δ::KanMX6 [pRS415ADH-UGP1-GFP]*	This study
HY1647	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ::URA3 ugp1Δ::HIS3MX6 [pRS415THI4-UGP1-GFP]*	This study

Table III-2. Oligonucleotide primers used in this study.

Primer name	Sequence (5'→3')[a]
TPS1-F	*TCACATACAGACTTATTAAGACATAGAACTATGACTAC GG*CACAGGAAACAGCTATGACC
TPS1-R	*TGGTGGCAGAGGAGCTTGTTGAGCTTGATGATGTACT GTA*GTTGTAAAACGACGGCCAGT
UGP1-F	*ATGTCCACTAAGAAGCACACCAAAACACATTCCACTTA TG*CACAGGAAACAGCTATGACC
KlURA3_740R-R	*TCAACCTTAGGATCTCTACCCTTGGCGAAAAGTCCTCT GC*GTTGTAAAACGACGGCCAGT
MSN2-F2	*GTCGCAACACATCAAGACTCATAAAAAACATGGAGACA TT*GGTCGACGGATCCCCGGGTT
MSN2-R1(URA)	TTATGAAGAAAGATCTATCGAATTAAAAAAATGG GGTCTATCTGGAGGAAGTTTGAGAGG
IRA2-F	*TCAACTAAACTGTATACATTATCTTTCTTCAGGGAGAA GC*CACAGGAAACAGCTATGACC
IRA2-R	*CATGTCGGAGTTCTTCGCAAATATACCAGAAGAAGTCA CA*GTTGTAAAACGACGGCCAGT
RAS2-F	*ATGCCTTTGAACAAGTCGAACATAAGAGAGTACAAGCT AG*CACAGGAAACAGCTATGACC
RAS2-R	*ATTAACTTATAATACAACAGCCACCCGATCCGCTCTTG GA*GTTGTAAAACGACGGCCAGT

[a] Sequences in italics represent the gene-specific sequences.

Table III-3. Oligonucleotide primers used in this study for plasmid construction.

Primer name	Sequence (5'→3')[a]
THI4-600(*Sac*I)	CAGT*GAGCTC*AGTCAACTTCTTCGTTAGTG
THI4+22R	TAGCAGTAGAGGTAGCAGAC
UGP1_1_*Spe*I	TGTC*ACTAGT*CCACTAAGAAGCACAC
GFP+800(*Xho*I)	GTCA*CTCGAG*ATTCGCTTATTTAGAAGTGG

[a] Sequences in italics represent the restriction enzyme sites for cloning of the PCR products.

3. Results

3.1. A knockdown mutant of *UGP1* shows reduced carbohydrate accumulation

Since yeast UGPase encoded by *UGP1* plays an indispensable role in carbohydrate synthesis and metabolism, *ugp1* null mutant is lethal (Daran et al., 1995). To further unveil the role of UGPase, we constructed a strain with reduced Ugp1 level for a loss of function study. For the reduction of the Ugp1 level, we constructed a plasmid containing the *UGP1* coding sequence under the control of the *THI4* promoter that is repressed in media with thiamine (Praekelt et al., 1994). Previously, the *UGP1* coding sequence under the control of the *THI4* promoter has been reported to have a 95% reduction of the Ugp1 activity (Daran et al., 1997). We generated *ugp1* knockdown (*ugp1KD*) strain by using a strategy described in **Fig. III-1**. To check whether the *THI4* promoter works properly, we examined Ugp1 level in *ugp1KD* cells. As expected, in *ugp1KD* cells, Ugp1 level was reduced by ~99% compared with control wild-type cells (**Fig. III-2A**). For recovery of Ugp1 expression, a plasmid containing the *UGP1* coding sequence under the control of its own promoter was also constructed. *UGP1C* cells complemented with this plasmid showed ~56% of Ugp1 level compared with control wild-type cells. Due to a defect in the production of UDP-Glc, *UGP1*-repressed cells were

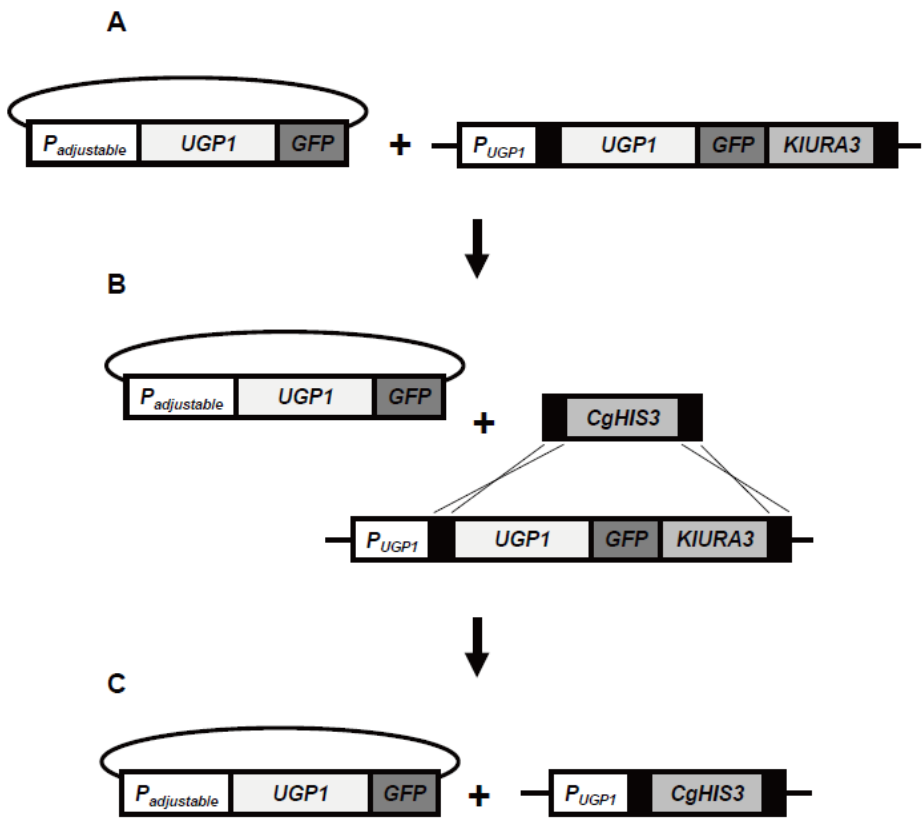


Fig. III-1. A modified method for construction of *ugp1KD* mutant.

First, yeast haploid strain expression GFP-fusion Ugp1 was prepared. (A) Then, a plasmid, pRS415THI4-UGP1-GFP, was transformed to the Ugp1-GFP expressing haploid cells. (B) After selection of transformed cells, another transformation was carried out to replace the genomic sequences (UGP1-GFP-KlURA3) of the haploid cells to cgHIS3. (C) Finally, the conditional *ugp1* mutant cells, *ugp1KD,* were selected by media selection and confirmed by colony PCR.

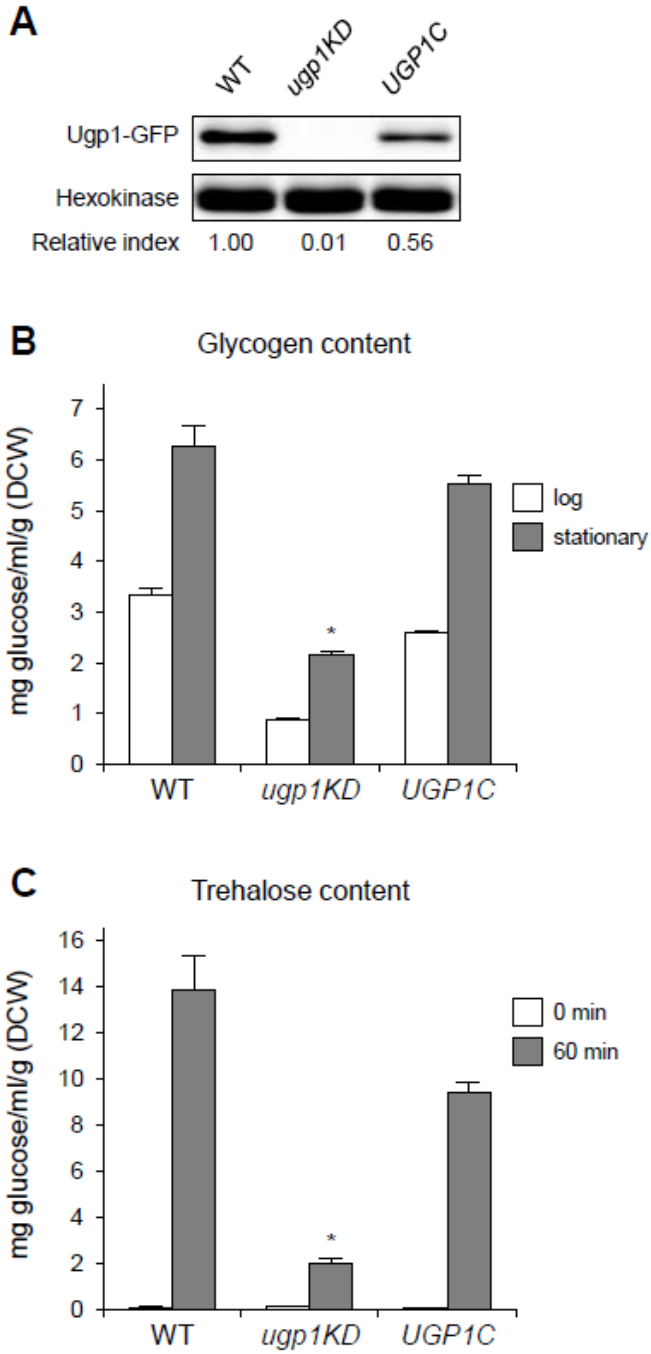

A
WT
ugp1KD
UGP1C
Ugp1-GFP
Hexokinase
Relative index 1.00 0.01 0.56
B
Glycogen content
mg glucose/ml/g (DCW)
0
1
2
3
4
5
6
7
*
WT
ugp1KD
UGP1C
log
stationary
C
Trehalose content
mg glucose/ml/g (DCW)
0
2
4
6
8
10
12
14
16
*
WT
ugp1KD
UGP1C
0 min
60 min

Fig. III-2. Properties of *ugp1KD* mutant.

(A) Yeast strains expressing Ugp1-GFP by *THI4* (*ugp1KD*), *UGP1* (*UGP1C*), or own promoter (WT) were grown to the exponential phase and extracted, followed by immunoblotting with a HRP-conjugated anti-GFP antibody. (B) Glycogen content during the log and stationary phase was measured in WT, *ugp1KD* and *UGP1C* cells. Values represent the average of three independent experiments, and error bars indicate the standard deviation. The asterisk indicate $P < 0.01$, compared with WT cells (Student's t-test). (C) Trehalose accumulation by heat stress was measured in WT, *ugp1KD* and *UGP1C* cells. Values represent the average of three independent experiments, and error bars indicate the standard deviation. The asterisk indicate $P < 0.01$, compared with WT cells (Student's t-test).

reported to have lowered levels of glycogen and trehalose (Chang et al., 1996; Genevaux et al., 1999; Mollerach et al., 1998). In order to verify the effect of *UGP1* knockdown, we determined the glycogen and trehalose content in *ugp1KD* cells. The glycogen content in *ugp1KD* cells at log phase was dropped to one third of the content in wild-type cells (**Fig. III-2B**). In yeast, glycogen builds up during the transition from log to stationary phase (Lillie and Pringle, 1980). Consistent with this, we observed a considerable accumulation of glycogen in cells at stationary phase. However, *ugp1KD* cells at stationary phase exhibited a 3-fold reduction in the glycogen content compared with control wild-type cells. The glycogen content recovered to normal levels in *UGP1C* cells. Meanwhile, since trehalose production is known to be induced under stress such as heat (Bell et al., 1992), the trehalose content of cells was determined after 37°C treatment. Induction of trehalose was notably reduced in *ugp1KD* cells, while the trehalose content was almost fully rescued in *UGP1C* cells (**Fig. III-2C**). Taken together, we confirmed that the repression of *UGP1* has a significant influence on cellular glycogen and trehalose content.

3.2. The regulation of Ugp1 level affects oxidative stress resistance of yeast cells by regulating trehalose production

Trehalose, a non-reducing disaccharide, is well known not only as a major storage carbohydrate but also as a functional molecule involved in various biological processes. Trehalose has been reevaluated as a protectant for

proteins against various stresses including heat and osmotic stress in yeast (Hottiger et al., 1987; Hounsa et al., 1998). Moreover, severe defects in cell viability under oxidative stress have been reported in trehalose-deficient mutants (Benaroudj et al., 2001). Because Ugp1 regulates cellular trehalose level as shown above, we assumed that cell survival under oxidative stress may also be influenced by Ugp1 expression. To examine this possibility, we measured the hydrogen peroxide (H_2O_2) sensitivity of wild-type, *ugp1KD*, *UGP1C* and trehalose-deficient *tps1*Δ/*2*Δ cells, which lack the subunits of trehalose-6-phosphate synthase complex, *TPS1* and *TPS2* (François and Parrou, 2001). Consistent with a previous report (Benaroudj et al., 2001), *tps1*Δ/*2*Δ cells exhibited a considerably increased sensitivity to H_2O_2 compared with wild-type cells (**Fig. III-3A**). *ugp1KD* cells with a lowered trehalose level were also highly sensitive to H_2O_2. The resistance to H_2O_2 was rescued significantly in *UGP1C* cells. Under H_2O_2 treatment, wild-type and *UGP1C* cells exhibited increased accumulation of trehalose, while *ugp1KD* and *tps1/2*Δ cells did not induce its production (**Fig. III-3B**). To determine whether trehalose works as an actual protectant against oxidative stress, we treated cells with H_2O_2 in trehalose-supplemented media. All wild-type, *ugp1KD* and *tps1/2*Δ cells showed significantly increased viability with trehalose supplementation (**Fig. III-3C**). Another non-reducing disaccharide, sucrose, had little effect on the H_2O_2 sensitivity of *ugp1KD* and *tps1*Δ/*2*Δ cells, indicating that the effect of enhanced cell survival under oxidative stress is not a general characteristic of disaccharides but rather is specific to

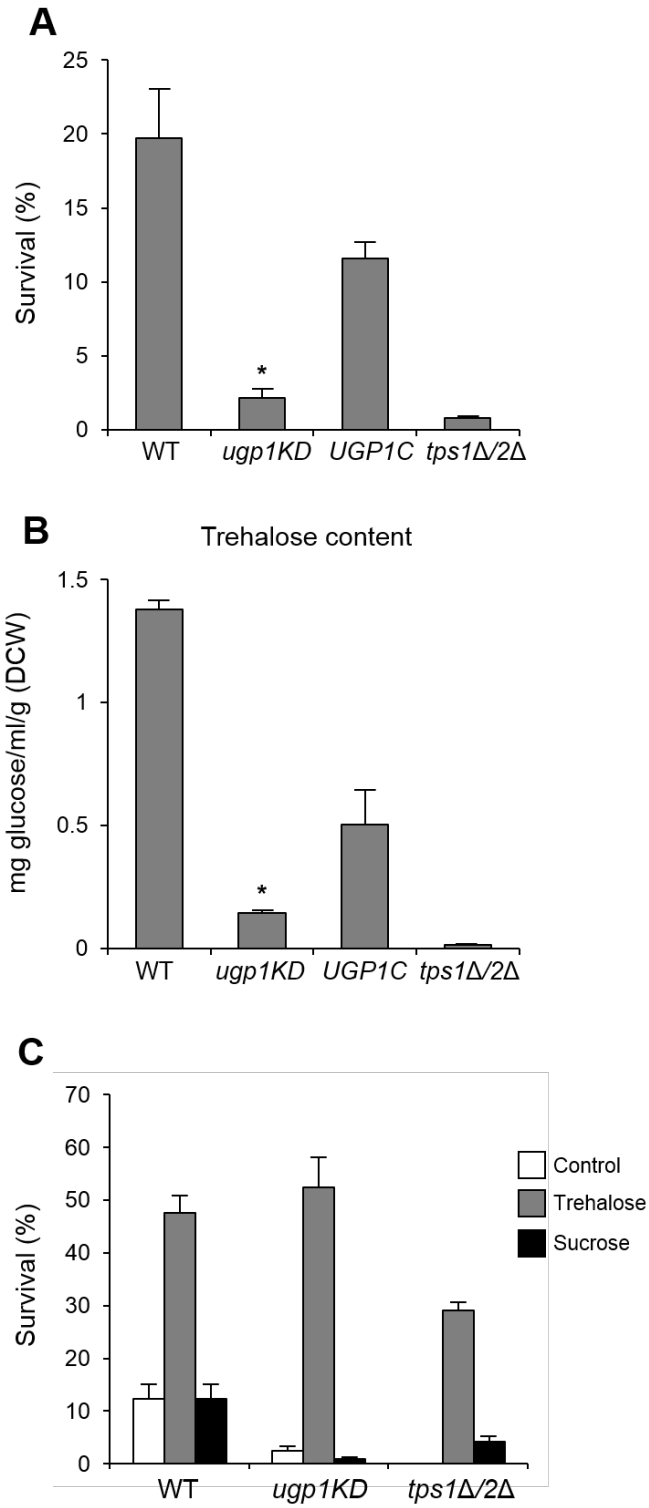

A
Survival (%)
25
20
15
10
5
0
*
WT
ugp1KD
UGP1C
tps1Δ/2Δ
B
Trehalose content
mg glucose/ml/g (DCW)
1.5
1
0.5
0
*
WT
ugp1KD
UGP1C
tps1Δ/2Δ
C
Survival (%)
70
60
50
40
30
20
10
0
Control
Trehalose
Sucrose
WT
ugp1KD
tps1Δ/2Δ

Fig. III-3. A knockdown of *UGP1* curtails anti-oxidant response through a defect in trehalose production.

(A) The survival rates in WT, *ugp1KD* and *UGP1C* cells were determined. Cells were grown to the exponential phase, and incubated with continuous shaking for 200 min after adding H_2O_2 solution to a final concentration of 1 mM. Then, the viable cells were measured by colony forming unit (CFU) assay. Values represent the average of three independent experiments, and error bars indicate the standard deviation. The asterisk indicate $P < 0.01$, compared with WT cells (Student's t-test). (B) Trehalose content in WT, *ugp1KD* and *UGP1C* cells was measured after exposure to 1 mM H_2O_2 for 1 hr. Values represent the average of three independent experiments, and error bars indicate the standard deviation. The asterisks indicate $P < 0.01$, compared with WT cells (Student's t-test). (C) WT, *ugp1KD, UGP1C* and *tps1*Δ*/2*Δ cells were pre-treated with 250 mM trehalose (Trehalose) or 250 mM sucrose (Sucrose) before treatment of 1 mM H_2O_2. After that, cells were incubated with continuous shaking for 200 min after adding H_2O_2 solution to a final concentration of 1 mM. Then, the viable cells were measured by colony forming unit (CFU) assay Cell viability of untreated cells (Control) was measured in the same way. Values represent the average of three independent experiments, and error bars indicate the standard deviation.

trehalose. These results suggest that Ugp1 can regulate oxidative stress resistance of yeast cells by mediating trehalose production.

3.3. The regulation of Ugp1 level has influence on yeast CLS through trehalose accumulation

The sensitivity to oxidative stress is closely involved in CLS in yeast (Kyryakov et al., 2012). It has also been reported that glycogen and trehalose contents in cells are linked with CLS (Goldberg et al., 2009). Given that *ugp1KD* cells show decreased glycogen and trehalose contents, we hypothesized that the expression level of Ugp1 might influence CLS. To check this, we determined CLS of wild-type, *ugp1KD*, *UGP1C* and *tps1*Δ/*2*Δ cells. As expected, *tps1*Δ/*2*Δ cells had severely shortened CLS compared with wild-type cells (**Fig. III-4A**). *ugp1KD* cells also exhibited a significantly shortened CLS compared with wild-type cells, while CLS of *UGP1C* cells was comparable to that of wild-type cells. To verify whether trehalose production is related to the level of Ugp1 during the stationary phase, we determined time course of trehalose content in wild-type, *ugp1KD* and *UGP1C* cells. After entry into stationary phase, elevated trehalose level in wild-type cells steadily declines (**Fig. III-4B**). But, *ugp1KD* mutant showed an impaired trehalose production during the stationary phase while *UGP1C* cells sufficiently built up its accumulation (**Fig. III-4B**). To test if trehalose actually influences yeast longevity in this study, CLS in cells was determined after trehalose supplement at log phase. CLS of wild-type cells

and even of *ugp1KD* and *tps1*Δ/*2*Δ cells was considerably prolonged in trehalose-supplemented media (**Fig. III-4C**). Taken together, these results

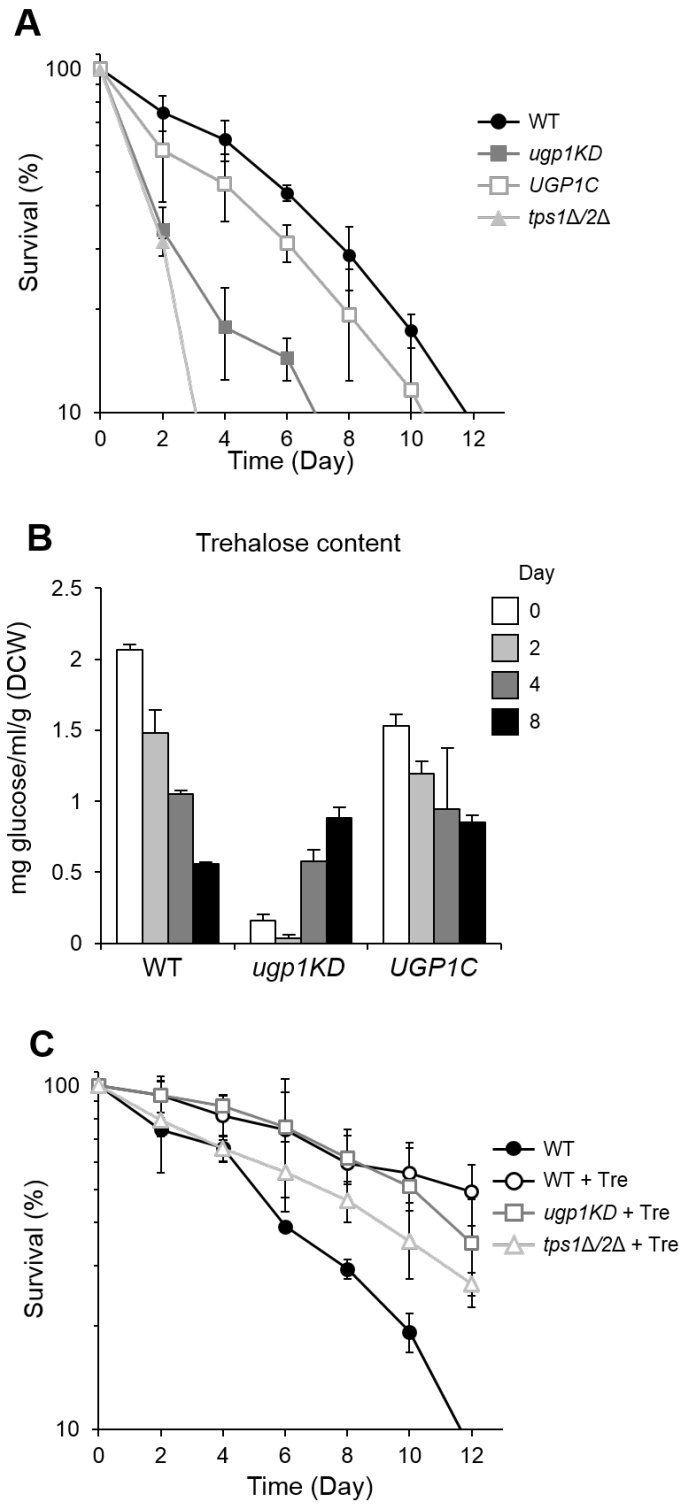

A
100
10
Survival (%)
0
2
4
6
8
10
12
Time (Day)
WT
ugp1KD
UGP1C
tps1Δ/2Δ
B
Trehalose content
mg glucose/ml/g (DCW)
2.5
2
1.5
1
0.5
0
WT
ugp1KD
UGP1C
Day
0
2
4
8
C
100
10
Survival (%)
0
2
4
6
8
10
12
Time (Day)
WT
WT + Tre
ugp1KD + Tre
tps1Δ/2Δ + Tre

Fig. III-4. Repression of *UGP1* shortens CLS through a defect in trehalose prodı

(A) WT, *ugp1KD*, *UGP1C* and *tps1*Δ*/2*Δ cells were grown to saturation for 3 days, then viable cells were measured at indicated days by CFU assay. Values represent the average of three independent experiments, and error bars indicate the standard deviation. (B) The time course of trehalose content during the stationary phase was measured at indicated days. Values represent the average of three independent experiments, and error bars indicate the standard deviation. (C) Cells of each strain were grown in SC lacking leucine medium supplemented with trehalose (Tre) to 1% at the exponential phase. CLS was measured at indicated days by CFU assay. Values represent the average of three independent experiments, and error bars indicate the standard deviation.

suggest that CLS of yeast cells has specific relevance to Ugp1-dependent regulation on trehalose content.

3.4. Modulation of Ugp1 level takes part in the PKA regulation on carbohydrate metabolism

We have already shown that Ugp1 level influences glycogen and trehalose content. Considering the fact that the PKA pathway plays a leading role in carbohydrate mobilization (Tamaki, 2007), we speculated that Ugp1 level alters according to PKA activity. To check this, Ugp1 level was examined in deletion mutants of the PKA-upstream regulator genes. A notable increase in Ugp1 level was observed in *ras2*Δ cells with low PKA activity, whereas Ugp1 level was reduced in *ira2*Δ cells with activated PKA (**Fig. III-5**). Given this result, we assumed that the modulation of *UGP1* expression may readjust carbohydrate content in PKA-related mutants. In accordance with expectations, wild-type cells containing high Ugp1 level exhibited more glycogen content than control wild-type cells. Then, the defects in glycogen production of the strains with high PKA activity such as *ira2*Δ and *pde2*Δ cells were slightly but significantly rescued by over-expression of Ugp1 (**Fig. III-6A**). By contrast, in the case of wild-type cells, knockdown of *UGP1* expression decreased glycogen content in *ras2*Δ cells (**Fig. III-6B**).

Likewise in glycogen regulation, in order to investigate whether PKA-dependent trehalose accumulation is also influenced by Ugp1 content, we determined trehalose induction by H_2O_2 in wild-type, *ira2*Δ and *tps1*Δ/*2*Δ

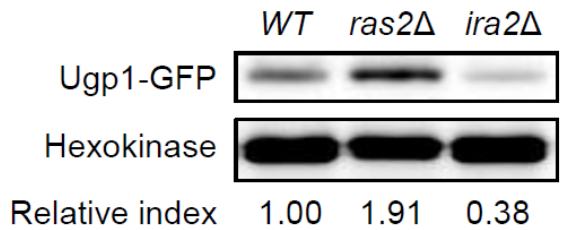


Fig. III-5. Level of Ugp1 is regulated in PKA-related gene mutants.

Total protein was extracted from the indicated cells, and immunoblotting was performed using a HRP-conjugated anti-GFP antibody. The relative ratio of Ugp1 to hexokinase, normalized against that of WT cells, is shown below each lane.

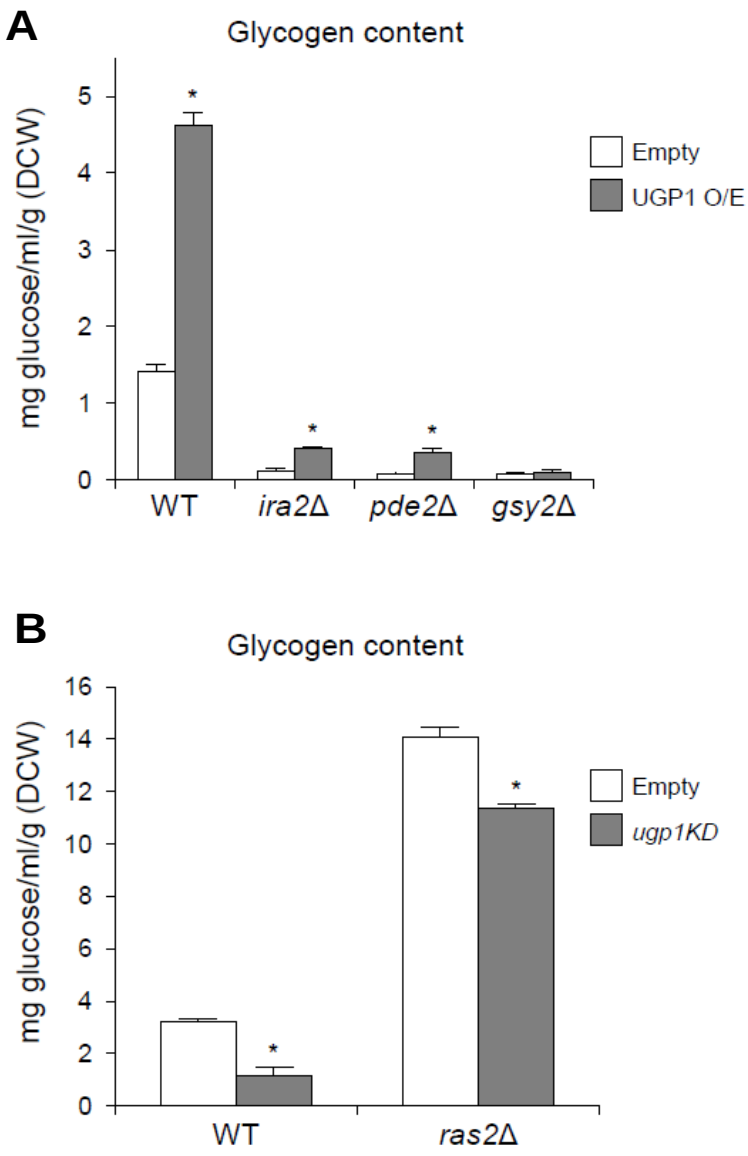

A
Glycogen content
mg glucose/ml/g (DCW)
0
1
2
3
4
5
*
*
*
Empty
UGP1 O/E
WT
ira2Δ
pde2Δ
gsy2Δ
B
Glycogen content
mg glucose/ml/g (DCW)
0
2
4
6
8
10
12
14
16
*
*
Empty
ugp1KD
WT
ras2Δ

Fig. III-6. PKA-dependent glycogen accumulation is re-regulated by modulation of Ugp1 level.

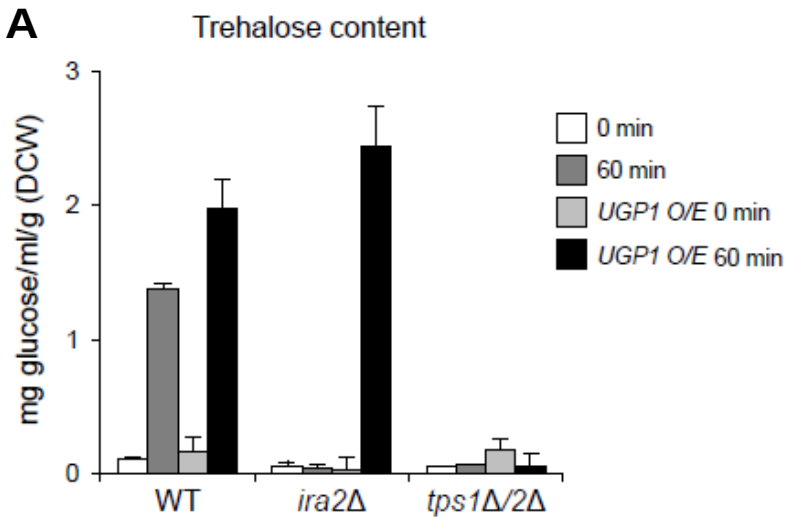
(A) Total protein was extracted from the indicated cells, and immunoblotting was performed using a HRP-conjugated anti-GFP antibody. The relative ratio of Ugp1 to hexokinase, normalized against that of WT cells, is shown below each lane. (B) The glycogen content in indicated strains with pRS415ADH (Empty) or pRS415ADH-UGP1-GFP (*UGP1 O/E*) plasmid was measured at mid-log phase. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with indicated strains containing Empty plasmid (Student's t-test). (C) The glycogen content in WT and *ras2*Δ cells with or without knockdown of *UGP1* was measured at mid-log phase. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with indicated strains id (Student's t-test).

cells with or without Ugp1 over-expression. Wild-type cells with high Ugp1 level exhibited an increased trehalose induction after H_2O_2 treatment (**Fig. III-7A**). Also, a defect of trehalose induction in *ira2*Δ cells was recovered by Ugp1 over-expression. In *ras2*Δ cells, a considerable trehalose induction under oxidative stress was observed (**Fig. III-7B**). However, Ugp1-repressed *ras2*Δ cells showed a notable decrease of trehalose level. Thus, based on these results, it is inferred that the control of Ugp1 level is implicated in PKA-dependent regulation on storage carbohydrate accumulation.

In addition, since *GSY2* encodes the major glycogen synthase in *S. cerevisiae* (Farkas et al., 1991), the results revealed a sharply reduced level of glycogen in *gsy2*Δ cells (**Fig. III-6A**). Despite this, no change in glycogen content was shown in *gsy2*Δ cells with high Ugp1 level. Likewise, induction of trehalose in *tps1*Δ/*2*Δ cells with trehalose-6-phosphate synthase deficiency was not rescued by Ugp1 up-regulation (**Fig. III-7A**). Altogether, these results suggest that the altered glycogen and trehalose accumulation through Ugp1 modulation are conducted by the existing biosynthetic machineries.

3.5. Alteration of Ugp1 level is associated with PKA-related phenotypes including oxidative resistance and CLS

Given that trehalose works as a protectant to oxidant and PKA-dependent trehalose metabolism is influenced by Ugp1 level, we assumed that the regulation of Ugp1 level may be required for PKA-dependent effects on


A
Trehalose content
mg glucose/ml/g (DCW)
3
2
1
0
WT
ira2Δ
tps1Δ/2Δ
0 min
60 min
UGP1 O/E 0 min
UGP1 O/E 60 min

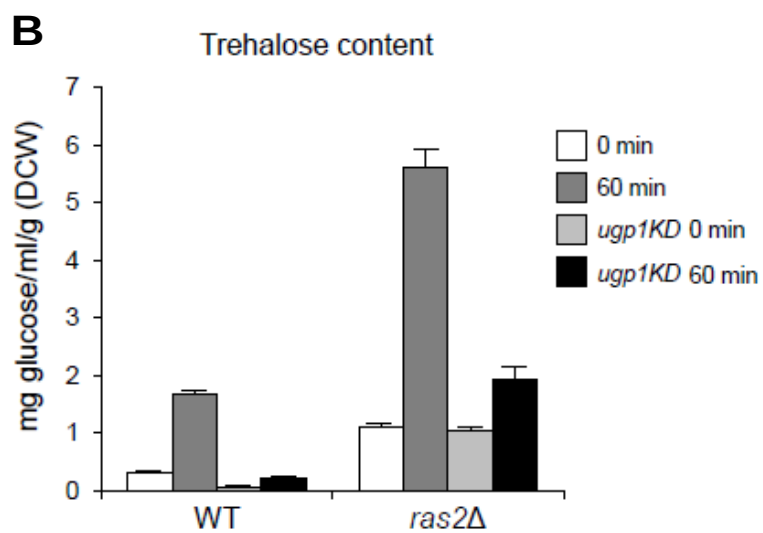

B
Trehalose content
mg glucose/ml/g (DCW)
7
6
5
4
3
2
1
0
WT
ras2Δ
0 min
60 min
ugp1KD 0 min
ugp1KD 60 min

Fig. III-7. PKA-dependent trehalose accumulation relies on Ugp1 level.

(A) WT, *ira2*Δ, and *tps1*Δ*/2*Δ cells with or without *UGP1 O/E* plasmid were grown until the exponential phase and then treated with 1 mM H_2O_2 for 1 hr, followed by determination of trehalose content. Values represent the average of three independent experiments, and error bars indicate the standard deviation. (B). Determination of trehalose content in WT, *ugp1KD*, *ras2*Δ, and *ras2*Δ*ugp1KD* cells was performed as described in (A). Values represent the average of three independent experiments, and error bars indicate the standard deviation.

oxidative stress response. To check this, we compared the effects of Ugp1 level change on the survival rate after treatment with H_2O_2 in wild-type, *ira2*Δ, and *tps1*Δ/*2*Δ cells. By over-expressing Ugp1, we observed an increase in survival rate of wild-type cells after oxidative stress and a significant alleviation of high sensitivity oo H_2O_2 of *ira2*Δ cells (**Fig. III-8A**). On the other hand, by repressing Ugp1 level, enhanced resistance of *ras2*Δ mutant was decreased as with the case of wild-type cells (**Fig. III-8B**).

We have already observed that trehalose has a significant effect on yeast CLS and the regulation of Ugp1 level plays an important role in PKA-dependent control of trehalose accumulation. Combining these results with the fact that the alteration of CLS is a typical phenotype linked to the PKA pathway (Shama et al., 1998), we speculated that the expression of *UGP1* may be a part of effects of PKA on yeast longevity. To test this, we examined the influence of Ugp1 level change on the life span in wild-type, *ira2*Δ, and *tps1*Δ/*2*Δ cells. As expected, wild-type cells with high Ugp1 level showed an extended CLS (**Fig. III-9A**). In accordance with the fact that strain with activated PKA shows a severe reduction of life span (Fabrizio et al., 2003), there was a sharp decrease in the CLS of *ira2*Δ (**Fig. III-9A**). However, *ira2*Δ cells over-expressing Ugp1 exhibited a significantly prolonged CLS. Next, we wondered whether *UGP1* knockdown could affect the CLS of strains containing low PKA activity. Consequently, the life span of *ras2Δ* cells was determined after repressing the Ugp1 expression. Although CLS of *ras2*Δ

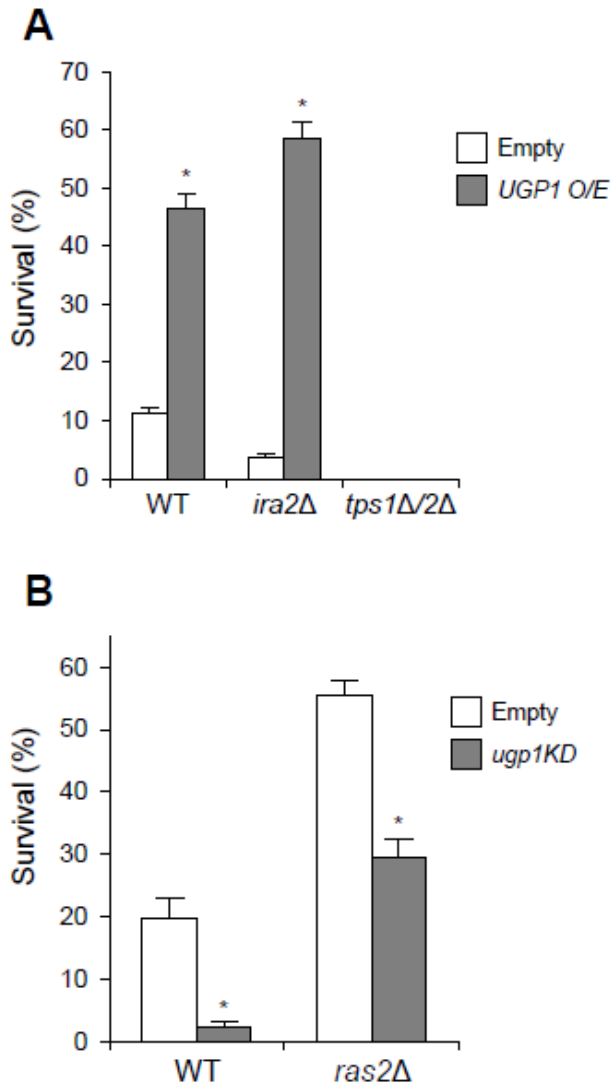

A
70
60
50
40
30
20
10
0
Survival (%)
*
*
Empty
UGP1 O/E
WT
ira2Δ
tps1Δ/2Δ
B
60
50
40
30
20
10
0
Survival (%)
*
*
Empty
ugp1KD
WT
ras2Δ

Fig. III-8. Variations of Ugp1 level affect PKA-dependent phenotypes such as high-sensitivity to oxidant.

(A). Cells of indicated strains with pRS415ADH (Empty) or pRS415ADH-UGP1-GFP (*UGP1 O/E*) plasmid were grown to exponential phase, and treated with 1 mM H_2O_2 for 200 min. Then, the rates of cell survival were measured by CFU assay. Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with indicated strain containing Empty plasmid (Student's t-test). (B) The survival rates of WT and *ras2*Δ cells with or without knockdown of *UGP1* were determined as described in (A). Values represent the average of three independent experiments, and error bars indicate the standard deviation. Asterisks indicate $P < 0.01$, compared with indicated strain (Student's t-test).

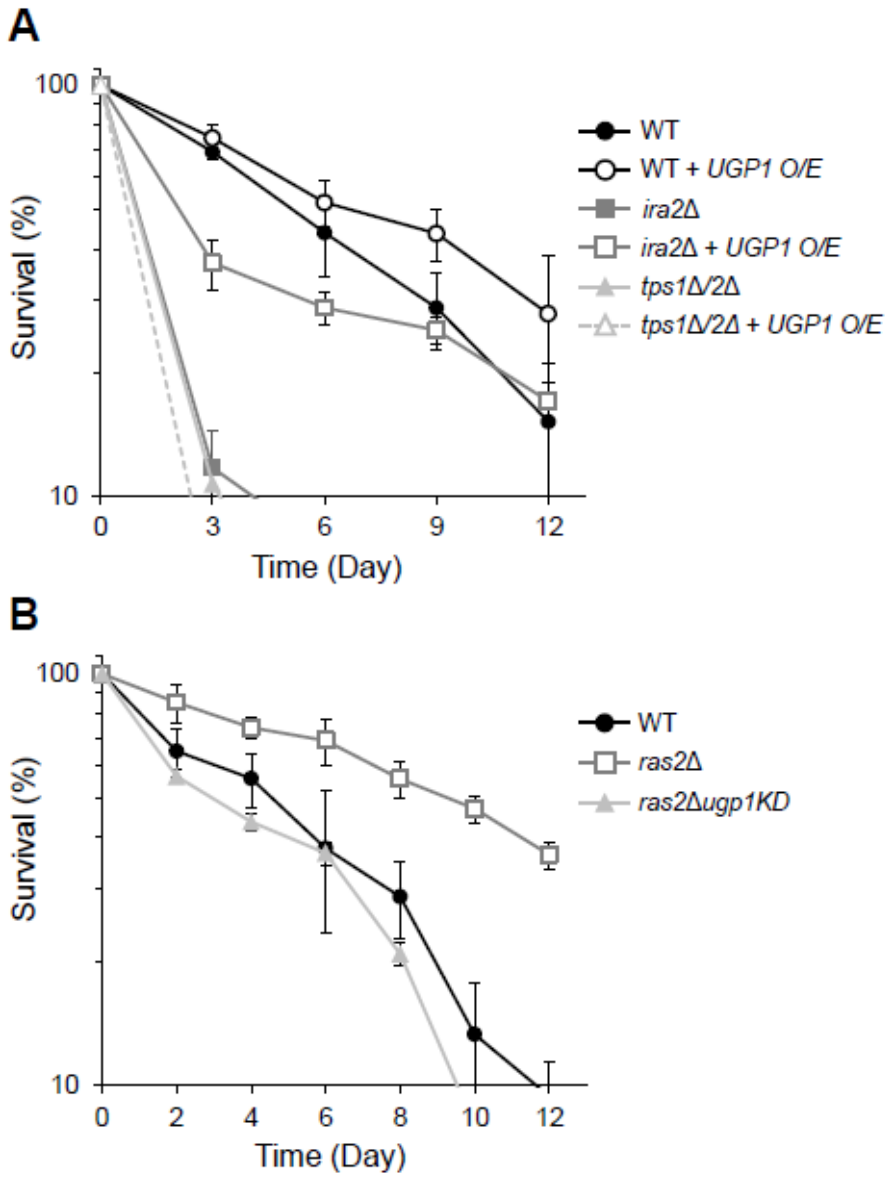

A
100
10
Survival (%)
0
3
6
9
12
Time (Day)
WT
WT + UGP1 O/E
ira2Δ
ira2Δ + UGP1 O/E
tps1Δ/2Δ
tps1Δ/2Δ + UGP1 O/E
B
100
10
Survival (%)
0
2
4
6
8
10
12
Time (Day)
WT
ras2Δ
ras2Δugp1KD

Fig. III-9. Variations of Ugp1 level affect PKA-dependent phenotypes such as CLS.

(A) Cells of indicated strains with or without *UGP1 O/E* plasmid were grown to saturation for 3 days, then the live cells were measured at indicated days by CFU assay. Values represent the average of three independent experiments, and error bars indicate the standard deviation. (B) CLS of WT, *ras2*Δ, and *ras2*Δ*ugp1KD* was measured as described in (A). Values represent the average of three independent experiments, and error bars indicate the standard deviation.

cells was extended, *ras2*Δ*ugp1KD* strain showed a declined CLS comparing to that of wild-type (**Fig. III-9B**).

In the case of *tps1*Δ*/2*Δ mutant, modulation of Ugp1 level has no effect on the resistance under oxidative stress or CLS (**Fig. III-8A, 9A**). These results indicate that the effects of Ugp1 modulation depend on alterations in trehalose production. Therefore, by adjusting trehalose content, the regulation of Ugp1 level is involved in PKA-related phenotypes like oxidative resistance and CLS.

4. Discussion

Since UGPase produces UDP-Glc, a biosynthetic precursor of carbohydrate and its derivatives (Daran et al., 1995), it has been considered a key enzyme for glucose partitioning. Previous studies with the conditional *ugp1*Δ mutant strain demonstrated that reductions of not only UDP-Glc but also storage and structural carbohydrate were observed in the conditional mutant (Daran et al., 1997). Here, we constructed a new conditional *ugp1*Δ mutant (*ugp1*Δ*KD*) through another method and confirmed the similar defects in production of glycogen and trehalose (**Fig. III-2**).

Recently, glycogen and trehalose have been identified as versatile molecules which respond to surrounding conditions (François and Parrou, 2001). In particular, trehalose works as a protectant of protein integrity against not only heat and dehydration but also oxidant (Bell et al., 1992; Benaroudj et al., 2001; Gadd et al., 1987). In this study, we demonstrated that the regulation of *UGP1* expression is required for oxidative stress response and CLS due to the Ugp1-dependent control of trehalose assimilation (**Fig. III-3, 4**). Taking into consideration that the supply of UDP-Glc is necessary for biosynthesis of carbohydrates, the regulation of *UGP1* is expected to have numerous novel connections with other biological processes.

We observed that variations of Ugp1 level could clearly alter the anti-oxidant sensitivity and CLS of wild-type and mutants with PKA activity due

to readjustment of storage carbohydrate (**Fig. III-8, 9**). Since the mobilization of the protectant trehalose is largely dependent on the PKA pathway (François and Parrou, 2001), it seems plausible that the regulation of Ugp1 expression may be included in the defense responses mediated by the PKA pathway (**Fig. III-10**). Furthermore, owing to the diverse physiological function of carbohydrate, studying the *UGP1* regulation might contribute to understanding the pleiotropic effects of the PKA pathway.

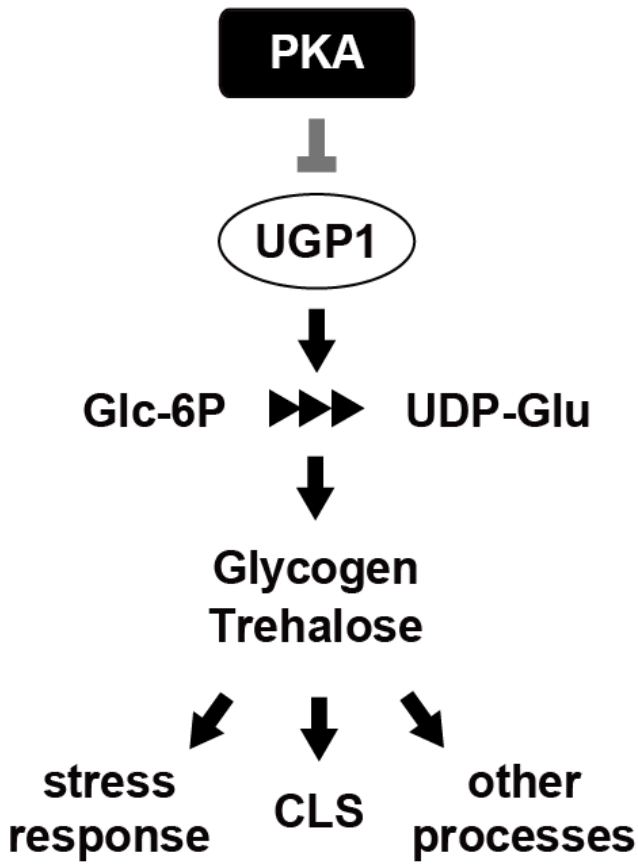


Fig. III-10. A schematic diagram for the versatile effects of the *UGP1* expression.

The expression level of *UGP1* influences the production of glycogen and trehalose. In this study, not only oxidative resistance but also chronological life span in budding yeast is also dependent on Ugp1 level through the altered level of these carbohydrates. Based on the fact that the *UGP1* regulation is able to suppress the PKA-dependent phenotypes, this regulation might be linked to other PKA-related processes such as cell wall integrity and cell adhesion.

CHAPTER IV.

Conclusion

Conclusion

All living organisms have evolved the strategies to metabolize carbon source for proper adaptation against the ever-changing circumstances. For that, glucose partitioning is important because it coordinates the fate of intracellular glucose. Since UDP-Glc is utilized as a sole donor of a variety of carbohydrates including glycogen, trehalose, N-glycosylation, β-glucan and activated UDP-sugars (Daran et al., 1997; Daran et al., 1995), it has been considered that UDP-Glc is a key intermediate in glucose partitioning. The formation of this core metabolite is produced only by UGPase catalyzing the synthesis of UDP-Glc and pyrophosphate from UTP and glucose-1-phosphate (Daran et al., 1995). Therefore, many researchers have believed that UGPase plays an active role in carbon metabolism involving glucose partitioning, and numerous studies have been carried out in diverse organisms.

Of course, the effects of reduction or over-expression of UGPase on carbohydrate accumulation or cell wall integrity have been reported in plant, bacteria, yeast and so on (Borovkov et al., 1997; Chang et al., 1996; Coleman et al., 2006; Genevaux et al., 1999; Mehlgarten et al., 2007; Mollerach et al., 1998; Prasad et al., 2010; Rodríguez-Díaz and Yebra, 2011; Spychalla et al., 1994; Zhang et al., 2013). Despite the importance of UGPase, the mechanism for the exact regulation of UGPase is largely unknown. This study suggests that the expression of yeast UGPase is controlled by the PKA-dependent

manner. According to PKA activity, Msn2/4 directly activated the transcription of *UGP1* via the binding to STRE sites in the promoter of *UGP1* (**Fig. II-1, 2, 3, 4, 6 and 7**). Due to the character of Msn2/4 which are activated under general stress conditions, the elevation of both *UGP1* mRNA and protein level was observed in several conditions such as heat, hydrogen peroxide, hyper-osmolarity and ethanol depending on Msn2/4 (**Fig. II-8**). In addition, previous studies have investigated that the conditions activating the PHO pathway including phosphate starvation and loss of PHO85 induce the expression of UGPase in yeast and *Arabidopsis thaliana* (Ciereszko et al., 2001; Nishizawa et al., 2001). Interestingly, these conditions also increased the levels of *UGP1* mRNA and protein through the Msn2/4-dependent regulation, but rather a traditional Pho85 downstream transcription factor Pho4 (**Fig. II-9 and 10**). Based on the findings that activation of the PHO pathway accumulates Msn2/4 into nucleus (**Fig. II-11**), the present study suggests there is a novel linker to interchange signals between the PHO and PKA pathways.

It is established that signals of the PKA pathway have influences on a wide variety of biological processes such as autophagy, cell differentiation, apoptosis and mitochondrial respiration (Cameron et al., 1988; Stephan et al., 2009; Thevelein and De Winde, 1999). Recent studies showed that trehalose works as a novel mTOR-independent autophagic enhancer in neuronal cells in transgenic mice (Sarkar et al., 2007; Zhang et al., 2014). This study shows that the altered levels of Ugp1 actually drove glucose flow into the

production of storage carbohydrate such as glycogen and trehalose (**Fig. III-2**). Moreover, because of reduced trehalose production, the *UGP1* knockdown strain displayed significant defects in oxidative resistance and CLS (**Fig. III-3 and 4**). Combining these results with the fact that the modulations of Ugp1 level alleviated a set of typical phenotypes regarding carbohydrate accumulation, sensitivity to oxidant and CLS in the PKA-related gene mutants (**Fig. III-6, 7, 8 and 9**), it is possible that regulations of carbohydrate underlie some of the existing PKA-dependent effects.

In that Ugp1 level affects adjustment of carbohydrate content, it is interesting to note that the final products of UDP-Glc are of merchantable quality. For example, sucrose and cellulose contents increased in the transgenic plant over-expressing UGPase (Coleman et al., 2007; Coleman et al., 2006). Also, it is reported that the production of industrial biopolymers including gellan gum and hyaluronic acid is enhanced by the additional expression of UGPase in microorganisms (Prasad et al., 2010; Zhu et al., 2013). Especially, when it comes to commercial value, applications of trehalose in industry are outstanding. Nowadays, trehalose is being used for a range of purposes such as preservation of food and cosmetic products, deodorization, stabilization of therapeutic compounds and flavoring (Higashiyama, 2002; Kubota, 2005; Ohtake and Wang, 2011; Richards et al., 2002). Therefore, our studies may serve as an inspiration for the more efficient production of biomass in the bioengineering field.

There are many problems awaiting solution in the research with UGPase. For example, although it has been reported that the phosphorylation of Ser^{11} is required for the Ugp1-dependent glucose partitioning into β-glucans and glycogen (Grose et al., 2007; Rutter et al., 2002; Smith and Rutter, 2007), little is known about the interaction between the control of expression by Msn2/4 and phosphorylation of Ser^{11}. Also, though yeast PAS kinases, Psk1 and Psk2, are known as the kinase of Ugp1 (Rutter et al., 2002; Smith and Rutter, 2007), there is still another possibilities that other kinases like PKA and Pho85 are in competition with PAS kinase. Nevertheless, this study is worth of notice and expected to contribute to better understandings of cellular strategies for proper glucose partitioning.

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초 록

효모에서 UDP-글루코스 피로포스포릴라아제의 발현 기작과 기능

효모에서 UDP-글루코스 피로포스포릴라아제는, 글리코겐과 트레할로즈 생합성과 세포벽 구성과 같이 다양한 물질대사에 중요한 대사물인 UDP-글루코스를 생성하기 때문에 탄수화물 대사에서 핵심적 역할을 담당한다. 많은 상위 조절자들이 *UGP1* 유전자의 조절과 관련된 것으로 예상됨에도 불구하고, 현재까지 Pho85 인산화효소만 *UGP1* 유전자의 전사를 억제시키는 것으로 보고되어 있었다.

본 연구에서는 스트레스-반응성 전사인자 Msn2/4 가 *UGP1* 유전자의 발현을 조절하는데 중요한 역할을 맡고 있음을 제시한다. 우선, Msn2/4 가 단백질 인산화효소 A (PKA) 의 활성에 따라, *UGP1* 유전자의 프로모터에 있는 세 개의 스트레스 반응 요소들에 결속되는 것을 확인하였다. 또한, 여러 스트레스 조건이 *UGP1* 의 전사를 조절했던 결과는 Msn2/4 에 의한 *UGP1* 유전자 조절이 일반적인 스트레스 반응과 관련되어 있다는 점을 시사한다. 더욱이, 본 연구는 인산 반응 경로 (PHO) 의 활성이 Msn2/4-의존적 *UGP1* 의 조절에 영향을 준다는 점을 제시하며, 이는 PKA 와 PHO 경로 사이를 이어주는 새로운 연결고리를 암시한다. 이러한 결과들은 효모에서 PKA, PHO 와 스트레스 반응 경로에 의해 전송된 신호들이 수렴되어 Msn2/4 에 의한 *UGP1* 유전자 발현을 조절한다는 점을 제시한다.

한편, *UGP1* 유전자의 발현은 글리코겐과 트레할로즈 같은 저장성 탄수화물의 생산에 필수적이다. 스트레스 보호제로서 작용하는 트레할로즈의 특정 기능 때문에, Ugp1 단백질 발현은 산화적 스트레스 반응과 연대순 수명에 기여한다. 흥미롭게도, PKA 경로가 탄수화물 대사를 역으로 조절하는 것으로 알려져 있음에도 불구하고, Ugp1 단백질 양의 조절은 PKA 경로 관련 유전자 돌연변이들에서 나타나는 글리코겐과 트레할로즈의 축적에 대한 전형적인 표현형들을 억제하였다. 또한, 항산화제 저항성과 연대순 수명에서의 PKA-의존적 표현형들이 Ugp1 단백질 양 조절에 의하여 완화되었다. 종합하면, 이러한 결과들은 *UGP1* 유전자 조절이 다양한 탄소화물 양의 조절을 통해 여러가지 생물학적 프로세스들에 영향을 미칠 수 있음을 시사한다.

총괄하여, 본 연구는 Msn2/4 에 의한 Ugp1 단백질 양의 조절이 방어적인 대사물질들로서 작용하는 탄수화물들의 합성으로의 포도당 분할을 유도함으로써 세포의 항상성에 기여한다는 것을 제안한다.

주요어: UGP1, PKA, MSN2/4, PHO, 글리코겐, 트레할로즈, 산화적 스트레스, CLS

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