Functional analysis of the stress-responsive transcription factor Msn2 in incorporation of amino acids in the yeast *Saccharomyces cerevisiae*

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ABBREVIATIONS

ESR	Environmental stress responses
TAD	Transcriptional activating domain
NES	Nuclear export signal
NLS	Nuclear localization signal
DBD	DNA binding domain
AZC	L-azetidine-2-carboxylic acid
OFP	*o*-fluoro-DL-phenylalanine
Can	L-canavanine
STRE	Stress-response element
MSN2-OE	*MSN2*-overexpressing
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time PCR
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
bp	Basepairs
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
TOR	Target of rapamycin
SAPK	Stress activated protein kinases
H_2O_2	Hydrogen peroxide
ROS	Reactive oxygen species
PQC	Protein quality control
PM	Plasma membrane
PMQC	Plasma membrane quality control
MVB	Multivesicular body
DUBs	Deubiquitinating enzymes

DEDICATION

This thesis indeed needs self-effort as well as the guidance and support of others, especially those who are very close to my heart.

I dedicate my humble efforts to:

Professor Dr. Raha Abdul Rahim, who ignited my research interest and passion in Genetic Engineering.

My mother and late father, who have always believed in me, whose affections, love, encouragement and prayers make me able to get such success and honor.

My family members and friends, who gave me so much motivation and support to persevere.

All my teachers, who have taught me how to love and live unapologetically.

And last but not least,

The Owner of all Knowledge in the Universe, who has saved and lifted me up from many downfalls, whose Wisdoms are always fascinating and never fail to struck me in awe.

1.0 INTRODUCTION

1.1 Stress responses in *Saccharomyces cerevisiae*

All livings cells are continuously challenged by a wide variety of stresses in their environment, such as prolonged nutrient starvation, exposure to free radicals and toxic molecules, the imbalance of osmotic pressure and pH level, and non-optimal growth temperatures (Mager & De Kruijff, 1995; Ruis & Schüller, 1995). To withstand sudden and adverse environmental changes, eukaryotic cells have developed a series of stress-responsive systems at the transcriptional, protein, and metabolic levels (Toone & Jones, 1998; Boy-Marcotte *et al.*, 1998; Estruch, 2000; Gasch *et al.*, 2000; Kandror *et al.*, 2004). External stimuli are perceived and transduced via the signal transduction pathways to cause global remodeling of gene expression, which is governed by transcriptional activators and repressors. In general, cellular stresses severely affect both transcription and translation activities, resulting in inhibition of *de novo* protein synthesis. Furthermore, the environmental fluctuations may cause protein damages, such as inhibition of enzyme activities, destabilization of cellular structures, and instability of chemical gradients, which eventually result in cell disruption. Thus, protein quality control and protein homeostasis are essential prerequisites for stress responses. Upon harsh stresses, cells also undergo the systematic downregulation of energy-producing and energy-consuming processes to enter into a quiescent state, often accompanied by a dynamic shift in central metabolic pathways that convert nutrients into energy and biomass. Cells possess tight and precise regulation systems to coordinate all the changes interconnected at those different levels.

Over the past years, extensive research advances have been made in the field of stress responses using a eukaryotic model organism, the budding yeast *Saccharomyces cerevisiae* (Causton *et al.*, 2001; Gasch, 2003). Earlier studies revealed the importance of the highly conserved stress-responsive transcription factors. Heat-shock factor 1 (Hsf1) was identified as a transcription activator that governs the expression of heat-shock proteins in response to elevated temperature (Sorger, 1990; Smith & Yaffe, 1991). A basic leucine-zipper transcription factor Yap1 is required for induction of stress-responsive genes under oxidative stress (Harshman *et al.*, 1988; Moye-Rowley *et*

al., 1989). Notably, *S. cerevisiae* has also developed the species-specific transcription factors, namely Msn2 and Msn4 (Msn2/4) (Estruch & Carlson, 1993; Martínez-Pastor *et al.*, 1996; Görner *et al.*, 1998). Msn2/4 play pivotal roles in stress responses by transcribing hundreds of stress-related genes following exposure to diverse stress conditions (Estruch, 2000; Gasch *et al.*, 2000; Hasan *et al.*, 2002; Berry & Gasch, 2008).

S. cerevisiae cells are also equipped with stress response mechanisms at the protein level to ensure protein quality at different subcellular locations, such as cytosol (Nillegoda *et al.*, 2010; Theodoraki *et al.*, 2012), the endoplasmic reticulum (Brodsky, 2012; Thibault & Ng, 2012; Gardner *et al.*, 2013; Wu *et al.*, 2014), the nucleus (Gardner *et al.*, 2005; Rosenbaum *et al.*, 2011), the mitochondria (Haynes & Ron, 2010; Baker & Haynes, 2011), and the plasma membrane (Zhao *et al.*, 2013; MacGurn, 2014; Shiga *et al.*, 2014). The protein quality control includes all processes that ensure proper protein folding and thus prevent the toxic consequences of protein misfolding (Goldberg, 2003; Turcu *et al.*, 2009). Irreversibly damaged proteins are selectively and effectively removed through proteasomal and/or vacuolar degradation systems, both of which consist of fine-tuned multiple steps including protein ubiquitination and deubiquitination (Finley *et al.*, 2012).

Intracellular metabolism is dynamically changed in response to various stresses in *S. cerevisiae*, as well as in many other organisms. When the nutrient levels (e.g. glucose and nitrogen sources) reduce, yeast cells are subjected to reprogramming the modes of energy metabolism from fermentation to respiration, a process termed diauxic shift, in order to maximize the efficiency of energy production (Gray *et al.*, 2004). Simultaneously, cells accumulate storage carbohydrates, such as trehalose and glycogen, which improve the survival rates under stress conditions and extend the life span (Fontana *et al.*, 2010). As well as carbon metabolites, nitrogen metabolites are consumed and produced in response to external stimuli; recent studies reported the significant importance of amino acids not only as building blocks of proteins but also in the control of cellular physiology (Sharma & Dietz, 2006; Takagi, 2008; Zhang *et al.*, 2017). Altogether, the interplay network among the multiple regulatory mechanisms at the transcriptional, protein, and metabolic levels protects yeast cells from

harmful conditions and allows them to adapt to the new environment (Fig. 1).

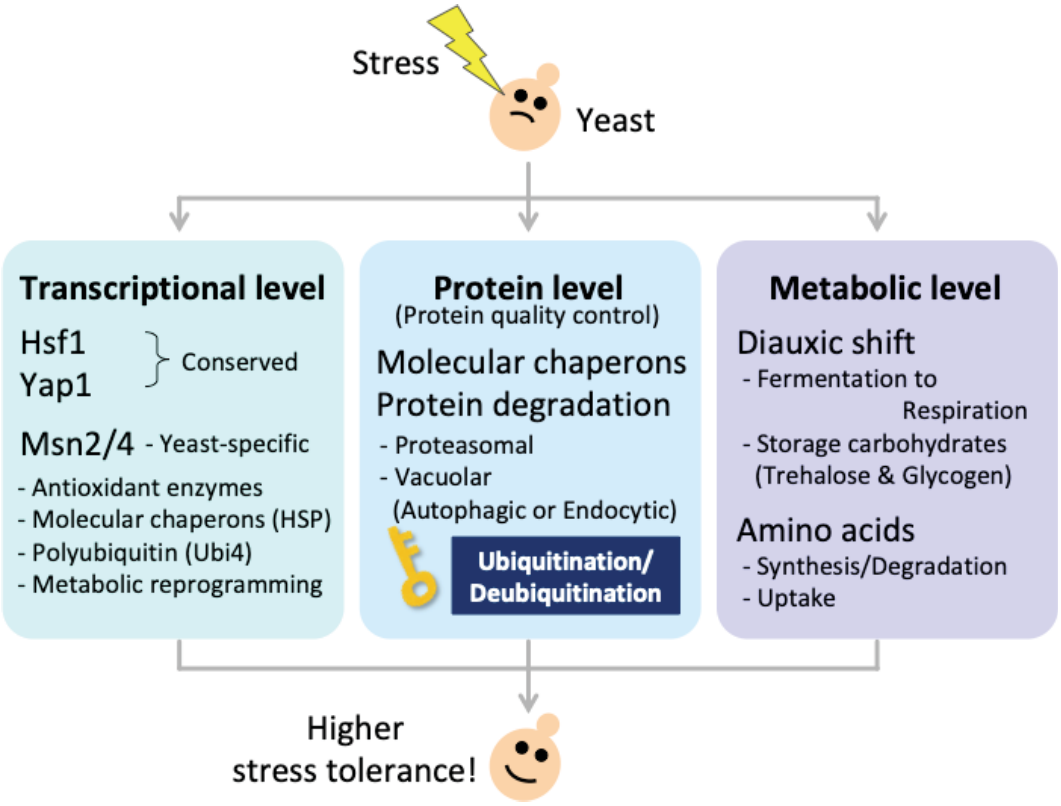

Fig. 1 The overview of stress responses in *S. cerevisiae*.

1.2 Stress-induced gene expression via transcription factors Msn2/4

Among several stress-responsive transcription factors in *S. cerevisiae*, yeast-specific Msn2 and its partially redundant paralogue Msn4 play a major role in mediating a wide range of stress responses termed the environmental stress response (ESR) (Gasch *et al.*, 2000; Causton *et al.*, 2001). ESR controlled by Msn2/4 includes responses to oxidative stress, osmotic shock, glucose starvation, high ethanol concentrations, temperature upshift, and freezing stress (Gasch *et al.*, 2000; Izawa *et al.*, 2007; Sadeh *et al.*, 2011; Sadeh *et al.*, 2012; Sasano *et al.*, 2012a; Sasano *et al.*, 2012b), and are required for both acute stress responses and cell survival during prolonged stress (Reiter *et al.*, 2013). Although Msn2/4 were first reported to be 41% identical to each other and functionally redundant (Estruch & Carlson, 1993), subsequent studies demonstrate that Msn2/4 can induce the expression of

individual genes under some stress conditions (Hohmann & Mager, 2003; Watanabe *et al.*, 2007; Berry & Gasch, 2008). Additionally, while *MSN2* is constitutively expressed, transcription of the *MSN4* gene is induced by stress in an Msn2/4-dependent manner (Gasch *et al.*, 2000). Thus, the roles of Msn2/4 are mostly overlapped but can be dissected in part. Several studies suggest the roles of Msn2 in transcriptional repression as well. The repression likely occurs via gene expression for transcription repressors or growth inhibitors. Msn2 activates the transcription of *DOT1*, which encodes a repressor of ribosome biogenesis gene (Elfving *et al.*, 2014). Transcription of *XBP1*, which encodes a repressor of cell-cycle associated genes, is also Msn2-dependent (Miles *et al.*, 2013).

Under non-stress growth conditions, Msn2/4 are phosphorylated by cAMP-dependent protein kinase A (PKA) and resided in the cytoplasm. Once yeast cells are challenged by environmental perturbations, Msn2/4 are rapidly dephosphorylated and translocated into the nucleus (Gorner *et al.*, 1998; Beck & Hall, 1999). They then bind to the stress-response element (STRE; AGGGG) sequence in the promoter region of the target genes, and subsequently activate the transcription (Boy-Marcotte *et al.*, 1998, 1999; Gasch *et al.*, 2000; Causton *et al.*, 2001). Previous studies identified functional domains of Msn2 (Fig. 2), which include the C-terminal zinc finger DNA-binding domain (DBD) (Marchler *et al.*, 1993; Schmitt & Mc Entee, 1996; Martínez-Pastor *et al.*, 1996; Moskvina *et al.*, 1998), the nuclear localization signal (NLS) region (Görner *et al.*, 1998, 2002), the nuclear export signal (NES) region (Görner *et al.*, 1998), and the imperative transcriptional activating domain (TAD) at the N terminus (Boy-Marcotte *et al.*, 2006). Besides phosphorylation by PKA, multiple upstream pathways are involved in the regulation of Msn2 and/or Msn4: the target-of-rapamycin (TOR) signaling-dependent cytoplasmic localization (Beck & Hall, 1999), Msn5 karyopherin-dependent nuclear export (Chi *et al.*, 2001; Görner *et al.*, 2002), proteasome-mediated degradation (Durchschlag *et al.*, 2004), Rsp5 ubiquitin ligase-dependent nuclear export of mRNA (Haitani & Takagi, 2008), and Rim15 protein kinase-dependent phosphorylation (Lee *et al.*, 2013).

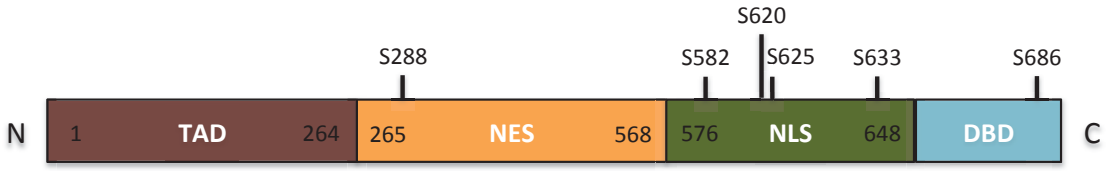

Fig. 2 Schematic representation of Msn2 functional domains.
Msn2 consists of four different domains; transcriptional activating domain (TAD), nuclear export signal (NES), nuclear localization signal (NLS) and zinc-finger DNA-binding domain (DBD). The serine residues serve as phosphorylation sites, which are responsible for the import and export of Msn2 to and from the nucleus. Msn2 binds to the STRE regulatory elements (typically AGGGG) on its target genes via DBD, while TAD involves in the activation of stress-response gene transcription.

To understand how Msn2/4 contribute to stress responses, the downstream target genes of Msn2/4 have been comprehensively investigated. First, Msn2/4 directly induce the expression of the genes encoding antioxidant enzymes, such as *CTT1* (for catalase), *SOD1* and *SOD2* (for superoxide dismutases), and *PRX1* and *TSA2* (for thiol peroxidases) (Hasan *et al.*, 2002; Drakulic *et al.*, 2005; Sadeh *et al.*, 2011). Since various kinds of stresses lead to imbalanced generation of reactive oxygen species (ROS) causing cell death, elimination of ROS by the antioxidant enzymes is an important stress response. Second, Msn2/4 activate the gene expression for stress responses at the protein level. Msn2/4 are essential in the induction of genes for heat-shock proteins, mainly including molecular chaperons (*HSP12*, sHSP-family (*HSP26* and *HSP42*), HSP70-family (*SSA1* and *SSA4*), HSP90-family (*HSP82*), and *HSP104*) (Kandror *et al.*, 2004; Eastmond & Nelson, 2006). Upon stress conditions, the polyubiquitin precursor *UBI4* gene expression is also upregulated to mark proteins for selective degradation via the ubiquitin-proteasome system (Simon *et al.*, 1999). Third, Msn2/4 triggers metabolic reprogramming in response to stress by inducing the expression of mitochondrial respiratory genes (*COX5b*, *COX17*, and *COX20*), pentose phosphate pathway genes (*SOL4*, *GND2*, and *TKL2*), trehalose synthetic genes (*TPS1*, *TPS2*, *TPS3*, and *TSL1*), and glycogen synthetic genes (*GSY1*, *GSY2*, and *GLC3*) (Estruch, 2000; Gasch *et al.*, 2000; Causton *et al.*, 2001; Sadeh *et al.*, 2011).

Due to such global effects of Msn2/4, overexpression of the *MSN2* or *MSN4* gene has been applied to the breeding of industrial yeast strains for improvement of the stress tolerance and the

fermentation performance (Cardona *et al.*, 2007; Watanabe *et al.*, 2009; Sasano *et al.*, 2012a, 2012d). During the fermentation of dough, baker's yeast cells are exposed to baking-associated stresses, such as freeze-thaw, air-drying, and high sugar concentrations. These treatments induce oxidative stress that increases intracellular reactive oxygen species levels (Kitagaki and Takagi, 2014). Yeast strains that overexpress *MSN2* have shown tolerance to oxidative stress, mainly due to high-level transcription of antioxidant genes (Sasano *et al.*, 2012d). Our lab previously reported that overexpression of *MSN2* enhanced the fermentation ability of baker's yeast in frozen dough (Sasano *et al.*, 2012a). Thus, overexpression of *MSN2* is also expected to confer tolerance to air-drying and high-sugar stresses on baker's yeast.

1.3 Ubiquitination and deubiquitination involved in stress responses

Ubiquitination is an essential posttranslational modification occurring to proteins, which regulates a variety of cellular processes such as protein degradation, protein-protein interaction, and subcellular localization (Pickart & Eddins, 2004; Grabbe *et al.*, 2011). Ubiquitination is highly dynamic and reversible, and is catalyzed by a set of three enzymes, namely the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2, and the ubiquitin ligase E3 (Fig. 3). The small, 76 amino-acid peptide ubiquitin is conjugated via a covalent isopeptide bond, linking its carboxyl-terminal glycine to a lysine residue of the substrate protein (Ciechanover & Ben-Saadon, 2004; Cadwell & Coscoy, 2005; Tait *et al.*, 2007; Vosper *et al.*, 2009; McDowell *et al.*, 2010).

Ubiquitin moieties can be subsequently linked to one of the seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), as well as to the amino-terminal methionine, resulting in the generation of mono-, poly-, or multiubiquitination (Sadowski *et al.*, 2012). The versatility of ubiquitin chains is associated with the fate of target proteins (Fig. 4); monoubiquitination modulates DNA repair, gene expression, or endosomal sorting. The two most common chain linkages, K48 and K63, command the target proteins to different outcomes; the former results in targeting proteins for proteasomal degradation, and the latter is involved in signal transduction, DNA repair, or endocytosis.

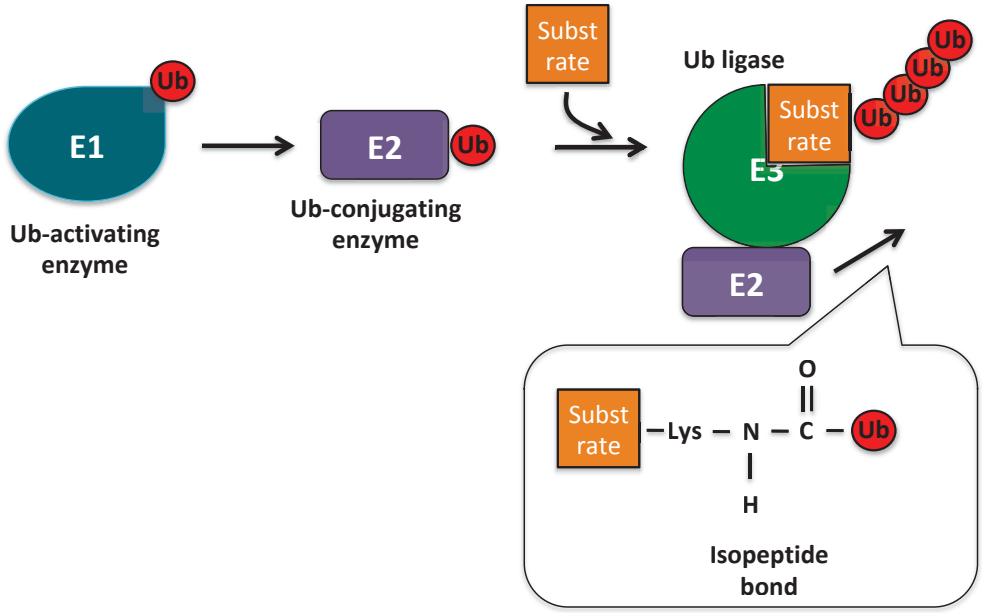

Fig. 3 Ubiquitination process.

The ubiquitination process involves a cascade of three different enzymes (E1, E2, and E3). Firstly, E1 is responsible for the activation of ubiquitin (Ub) molecule. The activated ubiquitin is then attached to the active-site cysteine residue in E1. Following that, ubiquitin is transferred from E1 to E2, and E3 aids the transfer of ubiquitin from E2 to the target protein.

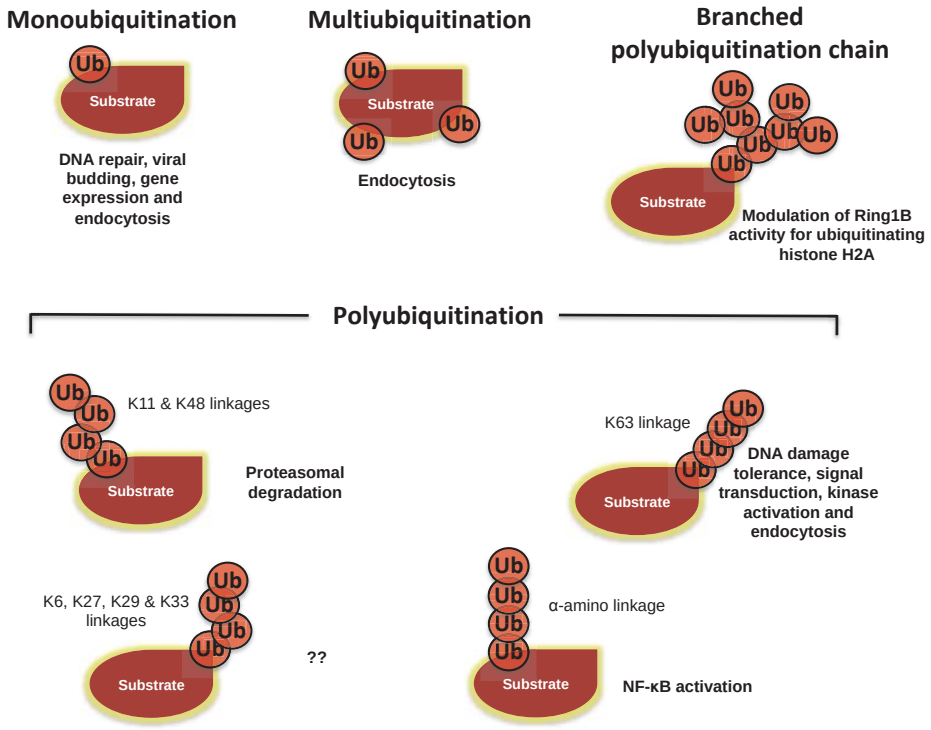

Fig. 4 Different modes of ubiquitination.

Target proteins are tagged with various forms of ubiquitination, which determine the fate of those proteins whether to go for refolding, repair, or degradation (Sadowski *et al.*, 2012).

The process of ubiquitination is reversed by a group of enzymes called deubiquitinating enzymes (DUBs), which hydrolyze the isopeptide bonds that link ubiquitin to the target proteins (Turcu *et al.*, 2009). DUBs play a similar role to that of phosphatases in a kinase/phosphatase regulatory system. Table 1 lists a total of 20 DUBs documented so far in *S. cerevisiae*, which were extensively reviewed by Finley *et al.* (2012). DUBs are classified into four families: i) the ubiquitin specific protease (USP) family, which consists of 16 members; ii) the ovarian tumor (OTU) family, with two members; and iii) the JAB1/MPN/Mov34 (JAMM) and iv) the ubiquitin carboxyl-terminal hydrolase (UCH) families, with one member each. Most of the DUBs are thiol proteases, except for Rpn11, the only zinc metalloprotease (Verma *et al.*, 2002; Yao & Cohen, 2002).

Table 1 Deubiquitinating enzymes (DUBs) in *S. cerevisiae* (Finley *et al.*, 2012).

DUB	Type	Localization/complex	Mutant phenotype[a]
Ubp1	USP	Cytoplasmic, ER	Mild
Ubp2	USP	Ubp2/Rsp5/Rup1	Pleiotropic
Ubp3	USP	Ubp3/Bre5	Pleiotropic
Doa4 (Ubp4)	USP	Endosomal, Doa4/Bro1	Ub deficient, partial ts, can^s
Ubp5	USP	Bud neck	Assorted mild phenotypes
Ubp6	USP	Proteasomal	Ub deficient, enhanced proteolysis, can^s
Ubp7	USP	Cytoplasmic	Increased prion formation
Ubp8	USP	Nuclear, SAGA	Sensitive to heat and γ-rays; partial ts
Ubp9	USP	Cytoplasmic	Mild
Ubp10	USP	Nuclear	Decreased silencing, partial cs, can^s
Ubp11	USP		Pleiotropically stress sensitive, can^s
Ubp12	USP		can^s
Ubp13	USP		Pleiotropically stress sensitive
Ubp14	USP		Elevated free ubiquitin chains, can^s
Ubp15	USP		Stress sensitive, partial ts, strong cs, can^s
Ubp16	USP	Mitochondrial	Can^s, slow growth on nonfermentable carbon
Rpn11	JAMM	Proteasomal	Essential (DUB activity not essential)
Otu1	OTU	Cdc48	Pleiotropically stress sensitive

Otu2	OTU	Ribosome associated (?)	Pleiotropically stress sensitive
Yuh1	UCH	Cytoplasmic	Acts preferentially on Rub1 (*vs.* ubiquitin)

[a]can[s], sensitive to the arginine toxic-analogue canavanine; cs, cold-sensitive; ts, temperature-sensitive
USP, ubiquitin specific protease family; JAMM, JAB1/MPN/Mov34 family; OTU, ovarian tumor family; UCH, ubiquitin carboxyl-terminal hydrolase family.

Although ubiquitin ligases and DUBs play equally important roles in numerous cellular functions, studies regarding DUBs are relatively new and slowly emerging. DUBs release ubiquitin molecules from ubiquitin-conjugated proteins before degraded, thereby replenishing the free ubiquitin pool in the cells (Fig. 5). The main key players of this process in *S. cerevisiae* are Doa4 (or Ubp4), Ubp6, and Rpn11 (Swaminathan *et al.*, 1999; Amerik *et al.*, 2000; Leggett *et al.*, 2002; Chernova *et al.*, 2003; Hanna *et al.*, 2003; Hanna *et al.*, 2007; Kimura *et al.*, 2009). Doa4 is responsible for rescuing ubiquitin from membrane proteins that are en route to be internalized within multivesicular bodies and eventually degraded in the vacuole. Meanwhile, Ubp6 and Rpn11 recover ubiquitin before the proteins are sent to proteasome for degradation. Since DUBs are proteases, it is crucial to ensure that the enzymatic activity is properly governed to prevent unintentional cleavage of non-substrate proteins. As previously reported, the transcription of *UBP6* is induced in response to reduced ubiquitin levels (Hanna *et al.*, 2007). It will be of interest to know how DUB activities are cooperatively regulated during stress responses or other cellular events.

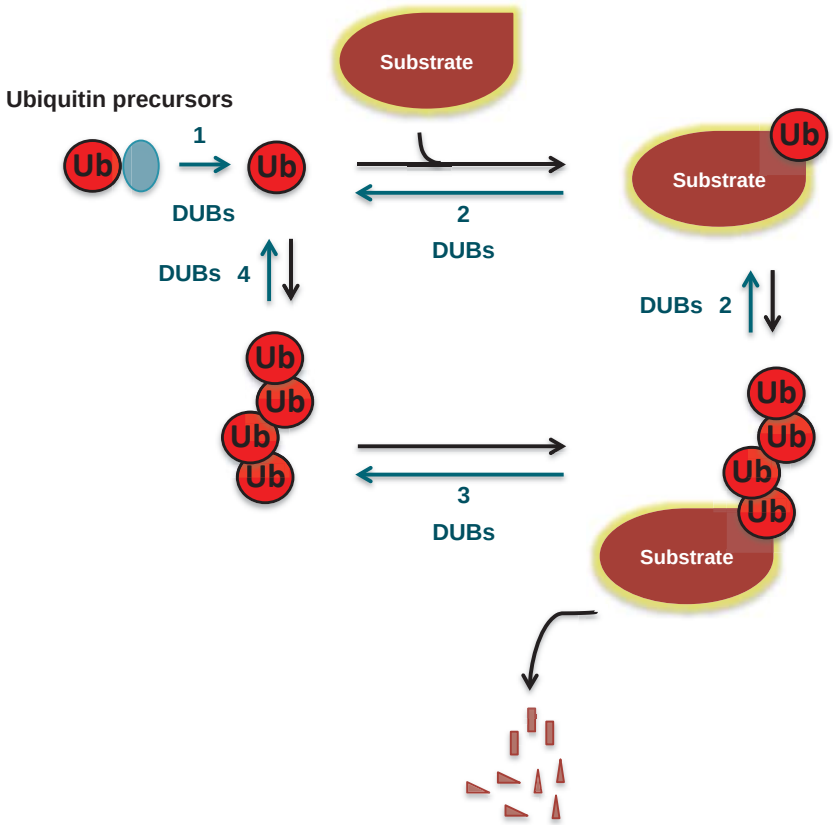

Fig. 5 Functions of DUBs in the ubiquitin pathway.
1) Ubiquitin precursors are processed. 2) Ubiquitin conjugates are rescued from another proteins, which usually found in the form of adducts to those proteins or can also be ligated to small nucleophiles. 3) Ubiquitin is cleaved from the protein conjugates en route for degradation. 4) Unanchored ubiquitin is disassembled (Amerik & Hochstrasser, 2004).

Ubiquitin homeostasis is the process of maintaining cellular levels of ubiquitin (Kimura & Tanaka, 2010). To supply sufficient ubiquitin, expression of the ubiquitin-encoding genes is the first prerequisite. In the budding yeast *S. cerevisiae,* four genes encode ubiquitin; three of them (*UBI1*, *UBI2*, and *UBI3*) are responsible for the fusions of ribosomal proteins to the carboxyl terminus of ubiquitin, while the fourth gene *UBI4* encodes a polyubiquitin precursor protein that consists of five tandem repeats of ubiquitin molecules (Özkaynak *et al.*, 1987). Ubi4 is the major source of ubiquitin under stress condition, which was highly transcribed in order to facilitate the degradation of aberrant proteins that accumulated under rigorous environments (Finley, Özkaynak, & Varshavsky, 1987). Analysis of the promoter region of *UBI4* revealed the presence of two distinct regulatory elements including heat shock elements (HSE) and STRE, which are regulated by Hsf1 and Msn2/4,

respectively (Simon *et al.*, 1999).

Ubiquitin homeostasis is also maintained by DUBs (Richter *et al.*, 2007; Kimura *et al.*, 2009) and ubiquitin degradation (Shabek & Ciechanover, 2010; Weissman *et al.*, 2013). When the Ubp6 DUB is defective, the stability of ubiquitin is challenged by fluctuating physiological conditions, resulting in a ubiquitin depletion (Chernova *et al.*, 2003; Hanna *et al.*, 2003). Ubiquitin degradation in the proteasome is suggested to occur in three different ways (Fig. 6); (i) degraded as a part of polyubiquitinated proteins, (ii) degraded together with a long, unstructured or flexible tail, and (iii) degraded after elongation of polyubiquitin chains. To globally understand ubiquitin homeostasis, which ensures the basis of stress responses and other ubiquitination-related phenomena, the key regulatory mechanisms for DUB activities and/or ubiquitin degradation should be identified.

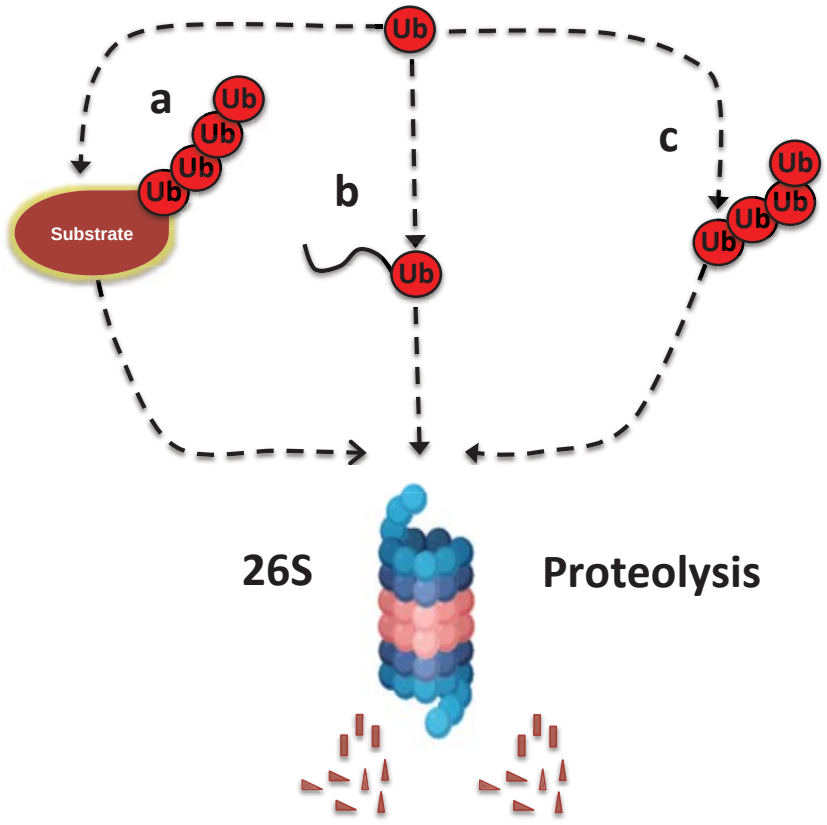

Fig. 6 Schematic representation of three different modes of ubiquitin degradation.
a) Ubiquitin is en route into 26S proteasome together with its conjugated substrate and degraded. b) Ubiquitin with an unstructured tail (>20 residues) is degraded without further modification. c) Ubiquitin monomers are targeted for proteasomal degradation, probably after ubiquitination which is catalyzed by E1, E2, and E3, and/or other adaptors (Shabek & Ciechanover, 2010).

1.4 Amino acids involved in stress responses

Among major carbon and nitrogen metabolites, our laboratory has focused on amino acids as hallmarks and mediators of *S. cerevisiae* stress responses (Takagi, 2000; Morita *et al.*, 2002; Matsuura & Takagi, 2005; Kaino *et al.*, 2008; Takagi, 2008; Nishimura *et al.*, 2010). Amino acids are essential nutrients not only as constituent of proteins but also for the growth and survival of yeast cells under stress conditions. In terms of stress-resistance activity, proline is the best studied among the 20 naturally occurring amino acids, which has cryoprotective activities in *S. cerevisiae*, as well as in many other kinds of cells (Sleator & Hill, 2001; Maggio *et al.*, 2002; Liang *et al.*, 2013). Although proline is not synthesized *de novo* in response to stress, artificial accumulation of proline by modification of proline-synthetic and -degrading enzymes in industrial baker's yeast strains increases the tolerance against freeze-thaw stress, consequently enhancing the fermentation ability of frozen dough (Kaino *et al.*, 2008; Sasano *et al.*, 2012c; Tsolmonbaatar *et al.*, 2016). Besides the freeze-thaw stress tolerance, proline confers tolerance to high osmolality, desiccation, high concentrations of ethanol, and weak acids (Takagi *et al.*, 2000; Sasano *et al.*, 2012b; Greetham *et al.*, 2014; Takagi *et al.*, 2016). Despite the importance of proline in general stress responses, the relationship between Msn2/4-mediated ESR and proline homeostasis has never been considered so far.

Cellular proline homeostasis is mediated by the control of the biosynthesis, degradation, and incorporation of proline. Proline is synthesized from glutamate in three enzymatically catalyzed steps; γ-glutamyl kinase Pro1 catalyzes the conversion of glutamate to glutamate-5-phosphate (Brandriss, 1979). Following that, the unstable glutamate-5-phosphate is converted to glutamate semialdehyde by the γ-glutamyl phosphate reductase Pro2 (Tomenchok & Brandriss, 1987). Glutamate semialdehyde, then spontaneously cyclizes to form Δ^1-pyrroline-5-carboxylate (P5C), which is converted to proline by a P5C reductase, Pro3 (Brandriss & Falvey, 1992). Among them, Pro1 is sensitive to proline feedback inhibition, and thus, several known mutations, such as Ile150Thr and Asp154Asn, in Pro1 desensitize feedback inhibition and increase the intracellular proline content (Morita *et al.*, 2003; Sekine *et al.*, 2007). At the transcriptional level, the expression of only *PRO2* is under the general

amino acid control system (Natarajan *et al.*, 2001), and it is still unknown whether the proline-synthetic pathway genes are coordinately transcribed by a certain external stimulus. To assimilate proline as a nitrogen source, proline is degraded into glutamate via the proline oxidase Put1 and the P5C dehydrogenase Put2, both of which are mitochondrial enzymes (Brandriss, 1979). Loss of the Put1 function contributes to an increase of the intracellular proline content (Takagi *et al.*, 2000). Both the *PUT1* and *PUT2* genes are transcriptionally repressed by the mechanism known as nitrogen catabolite repression (NCR) (Hofman-bang, 1999; Georis *et al.*, 2009), and positively regulated by the transcription activator Put3 (Ann *et al.*, 1996). NCR prevents utilization of proline as a nitrogen source when preferred nitrogen compounds, such as ammonia and glutamine, are present.

In *S. cerevisiae*, there are 24 members of the amino-acid-polyamine-organocation (APC) superfamily of permease proteins whose function is to transport amino acids and other amines into the cells (Nelissen *et al.*, 1997; Jack *et al.*, 2000). Among them, Gap1, Put4, Agp1, and Gnp1 were identified as proline transporters (Andréasson *et al.*, 2004). Gap1 encodes for a high capacity transporter for all naturally occurring amino acids and is regulated by the quality of nitrogen source present in the growth medium (Grenson *et al.*, 1970; Chen & Kaiser, 2002). Put4 is required for a high-affinity transport of proline and is regulated at the transcriptional level by NCR (Xu *et al.*, 1995; Ter Schure *et al.*, 2000). On the other hand, Agp1 and Gnp1 encode for permeases with broad substrate specificity and high affinity for glutamine, respectively (Zhu *et al.*, 1996; Iraqui *et al.*, 1999). The *AGP1* and *GNP1* genes are induced by the regulation of the Ssy1-Ptr3-Ssy5 amino acid sensor complex (Didion *et al.*, 1998; Iraqui *et al.*, 1999; Forsberg *et al.*, 2001; Ljungdahl, 2009).

Structural analogues of amino acids have been widely used to analyze amino acid homeostasis. L-azetidine-2-carboxylic acid (AZC), a toxic analogue for proline, is used in both fundamental and applied researches as it is proven to be beneficial to study the cellular metabolism and the production of macromolecules in both prokaryotes and eukaryotes (Bach & Takagi, 2013). AZC is a non-protein amino acid originally found in plants and has a heterocyclic with a four-membered nitrogen ring and a carboxylic acid group on one of the ring carbon atoms. The main

difference between AZC and proline is that the former has a four-ring member while the latter has a five-ring member. AZC, as well as many other amino acid analogues, is regarded to be toxic to the cell, because it is carried into the cells through proline permeases, and competes with proline during incorporation into nascent proteins, which consequently causes protein misfolding and cell death (Fig. 7).

In this study, I focused on the relationship between the general stress-response activator Msn2 and the amino acid homeostasis in *S. cerevisiae*. The findings in this thesis may contribute to a deeper understanding of cellular stress-responsive mechanisms, as well as to construction of a hyper-tolerant yeast strain that may be useful in the broad fermentation industries.

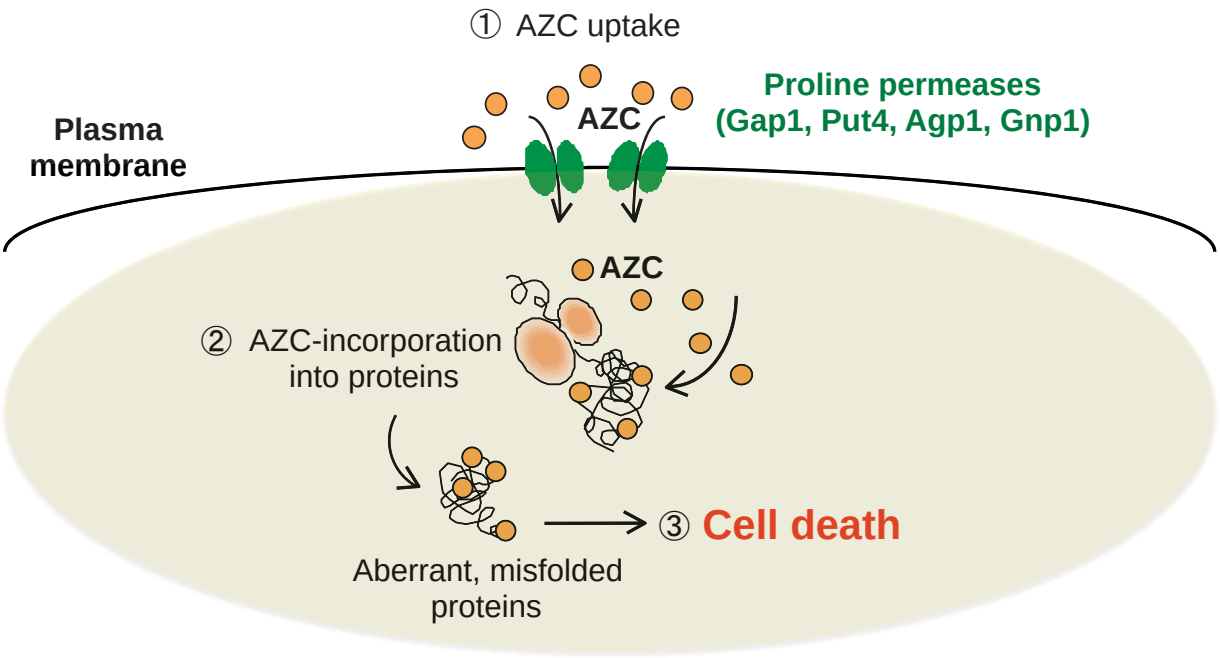

Fig. 7 Schematic representation of AZC cytotoxicity.
AZC is transported into the yeast cells via proline permeases. Following that, AZC is incorporated into proteins, causing misfolding of the proteins and thereby inhibiting the growth of the cells.

2.0 MATERIALS & METHODS

2.1 Strains and plasmids

The strains of *S. cerevisiae* and oligonucleotide primers used in this study are listed in Table 2 and 3, respectively. Gene manipulations such as gene overexpression and gene deletions were carried out through genomic integration as described previously (Janke *et al.*, 2004). For example, *MSN2* overexpression cassette was amplified via polymerase chain reaction (PCR) using a specific set of primers (MSN2_up_F and MSN2_from_1_to_534_R) and transformed into the genome of yeast cells as depicted in Fig. 8. For *MSN2* deletion, this pair of primers was used (MSN2+URA3-Fw and MSN2+URA3-Rv) to amplify the disruption cassette. For gene deletions, plasmid pUG6 harboring geneticin-resistant gene was used as a template to amplify the deletion cassettes using specific sets of primers (Fw Dmsn4-kanMX and Rv Dmsn4-kanMX, Fw Dgnp1-kanMX and Rv Dgnp1-kanMX, Fw Dubp6-kanMX and Rv Dubp6-kanMX).

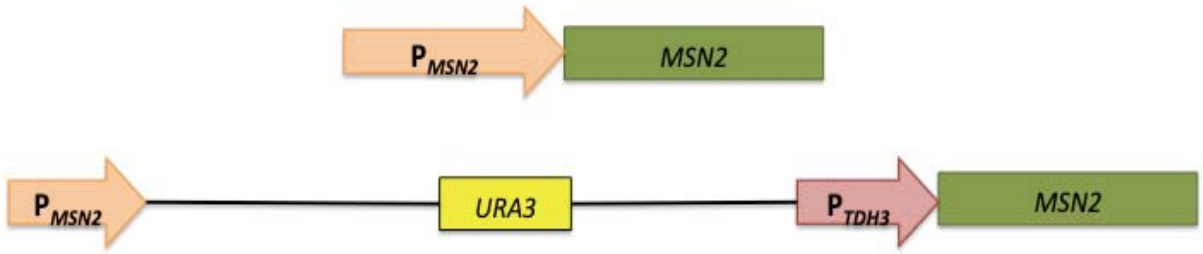

Fig. 8 Schematic diagram of how the construction of *MSN2*-overexpressing cells was carried out.

An overexpression cassette consisting of *URA3* marker (containing both native promoter and terminator of the *URA3* gene) and a constitutive, strong promoter of *TDH3* gene (P_{TDH3}) was amplified using a set of primers (MSN2_up_F and MSN2_from_1_to_534_R). This cassette was then integrated between the native promoter of *MSN2* gene (P_{MSN2}) and the open reading frame of *MSN2* gene through yeast transformation. Following that, positive clones were confirmed using another set of primers (TDH3_up_F(-132_-108) and MSN2_R2).

Table 2 List of yeast strains used in this study

Strain	Genotype	Background and/or source
WT (BY4741)	*MATa ura3 met15 leu2 his3*	EUROSCARF
MSN2-OE	BY4741 P_{MSN2}*::URA3-*P_{TDH3}	this study
Δ*msn2* Δ*msn4*	BY4741 *msn2::URA3 msn4::kanMX6*	this study
GNP1-OE	BY4741 *ura3* [pAG416GPD-GNP1]	this study
Δ*gnp1*	BY4741 *gnp1::kanMX6*	this study
MSN2-OE Δ*gnp1*	BY4741 P_{MSN2}*::URA3-*P_{TDH3} *gnp1::kanMX6*	this study
WT *GNP1-GFP*	BY4741 *GNP1-GFP::HIS3*	Invitrogen
MSN2OE GNP1-GFP	BY4741 P_{MSN2}*::URA3-*P_{TDH3} *GNP1-GFP::HIS3*	this study
Δ*ubp6*	BY4741 *ubp6::kanMX6*	this study
UBP6-OE	BY4741 *ura3 leu2* [pAG416, pAG425GPD-UBP6-HA]	this study
MSN2-OE UBP6-OE	BY4741 P_{MSN2}*::URA3-*P_{TDH3} [pAG425GPD-UBP6-HA]	this study
Δ*ubp3*	BY4741 *ubp3::kanMX4*	Open Biosystems
Δ*otu1*	BY4741 *otu1::kanMX4*	Open Biosystems
CAY29	*MATa ura3-52*	Andréasson & Ljungdahl, 2002
CAY132	CAY29 Δ*gap1*	Andréasson *et al.*, 2004
CAY140	CAY29 Δ*put4*	Andréasson *et al.*, 2004
CAY178	CAY29 Δ*agp1*	Andréasson *et al.*, 2004
CAY166	CAY29 Δ*gnp1*	Andréasson *et al.*, 2004

CAY191	CAY29 Δ*gap1* Δ*put4* Δ*agp1* Δ*gnp1*	Andréasson *et al.*, 2004
CAY29 *MSN2-OE*	*MATa ura3-52* P_{MSN2}*::URA3-*P_{TDH3}	this study
CAY132 *MSN2-OE*	CAY29 *MSN2-OE* Δ*gap1*	this study
CAY140 *MSN2-OE*	CAY29 *MSN2-OE* Δ*put4*	this study
CAY178 *MSN2-OE*	CAY29 *MSN2-OE* Δ*agp1*	this study
CAY166 *MSN2-OE*	CAY29 *MSN2-OE* Δ*gnp1*	this study
CAY191 *MSN2-OE*	CAY29 *MSN2-OE* Δ*gap1* Δ*put4* Δ*agp1* Δ*gnp1*	this study

Table 3 Oligonucleotide primers used for the construction of yeast strains

Oligonucleotide	Sequence (5' → 3')
MSN2_up_F	TTGTTTCCAGCGAAAGAGAC
MSN2_from_1_to_534_R	TGAAGTTTGAGGCGATAAATTAGT
TDH3_up_F(-132_-108)	ACGGTAGGTATTGATTGTAATTCTG
MSN2_R2	ATCAAAGGCACAGCAGACT
MSN2+URA3-Fw	GTATCTTCCTCATATTTTTCGGGAAGATCACAACAGTAGTAGC AAGGTATTTCATACGCCAAGAGGCTACGATTCGGTAATCTCCG AG
MSN2+URA3-Rv	AACAATAAGCCGTAAGCTTCATAAGTCATTGAACAGAATTATC TTATGAAGAAAGATCTATCGAATTAGTAATAACTGATATAATT AAATTG
Fw Dmsn4-kanMX	TTCGGCTTTTTTTTCTTTTCTTCTTATTAAAAACAATATAATGGG TAAGGAAAAGACTCA
Rv Dmsn4-kanMX	TAGCTTGTCTTGCTTTTATTTGCTTTTGACCTTATTTTTTTTAGA AAAACTCATCGAGCA
Fw Dgnp1-kanMX	CTTTCTCAAGTAGCTTATATAATATCAAATATTGCACATTATGG GTAAGGAAAAGACTCA
Rv Dgnp1-kanMX	TTCAAGTTTTTTTTTTTTTTTTGAATCGTGATTTCTGCTTTAGA AAAACTCATCGAGCA
Fw Dubp6-kanMX	AAAATAAGGAAATTAGCCCTACCTATCCTTGTGTTAAAATATG GGTAAGGAAAAGACTCA

Rv Dubp6-kanMX	TCCGAAAAAACTATTAATTTTGAAAAAATGAAAAATGGACTTA GAAAAACTCATCGAGCA

2.2 Growth media

To grow yeast *S. cerevisiae* cells, the following media were used; a nutrient rich (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose), a synthetic complete (SC) medium containing 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 5 g/L ammonium sulfate, 20 g/L glucose, and 2 g/L drop-out mix amino acid powder lacking uracil (for SC-Ura) or lacking L-leucine (SC-Leu), and a minimal medium synthetic dextrose (SD) containing 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 5 g/L ammonium sulfate, and 20 g/L glucose. Yeast strains were also cultured on SD agar plates containing L-azetidine-2-carboxylic acid (AZC; L-proline analogue), L-canavanine (Can; L-arginine analogue), and *o*-fluoro-DL-phenylalanine (OFP; L-phenylalanine analogue). All of these chemicals were obtained from Sigma-Aldrich. For the amino acid analysis, yeast cells were pre-cultured in SD medium containing 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 0.1% allantoin, and 20 g/L glucose, later indicated as SD(-N)+Alla. The *E. coli* recombinant strains were grown in Luria–Bertani (LB) medium (Sambrook and Russell, 2001) containing ampicillin (50 μg/mL). If necessary, 2% agar was added to solidify the medium.

2.3 Construction of strains and plasmids

E. coli transformation were carried as described previously (Sambrook and Russel, 2001). Yeast transformation was performed as described elsewhere (Schiestl and Gietz, 1989; Gietz and Schiestl, 2007). Yeast cells were pre-cultured in 5 mL YPD and grown overnight at 30°C. The main culture was prepared with the initial OD_{600} value of 0.25 and grown until the exponential growth phase at 30°C, harvested and pelleted via centrifugation. The cell pellets were resuspended in 0.1 M

lithium acetate, followed by incubation at 30°C for 15 min. The cell pellets were then resuspended with the following mixtures; i) 240 μL of PEG 4000 (50% (w/v)), ii) 36 μL of 1.0 M lithium acetate, iii) 25 μL of single-stranded carrier DNA (sheared cod and herring sperm DNA, Roche), iv) appropriate amount of plasmids or PCR-amplified fragments. The mixtures were then incubated at 30°C for 30 min, heat shock at 42°C for 15 min, centrifuged and the cells were resuspended with appropriate media and incubated at 30°C for 1-2 h before plating the cells onto appropriate media for selection. The agar plates were incubated at 30°C for 3-4 days. For selective growth of the gene-knockout transformants, 100-200 μg/mL geneticin (Santa Biocruz Biotech) was added to YPD medium.

2.4 Growth test

To determine growth phenotypes towards certain stress conditions, yeast cells were pre-cultured in the appropriate media at 30°C for overnight and inoculated into the same media the next day. The main culture was prepared with the initial OD_{600} of 0.25 and grown at 30°C until the OD_{600} value reached ~1.0. Approximately 10^7 cells were collected and serially diluted (from 10^{-1} to 10^{-4}). Following that, each dilution was spotted accordingly on the corresponding media and incubated at 30°C for several days.

2.5 Quantification of cell viability

To estimate the number of viable yeast cells during the hydrogen peroxide or AZC treatment, the cells were grown in SD medium at 30°C until the OD_{600} value reached about 1.0. Prior to or after the hydrogen peroxide or AZC treatment for 30 min, cells were harvested, appropriately diluted and plated on YPD agar plates. Colonies on the YPD plates were then counted after 3 day-incubation at 30°C.

2.6 Quantification of intracellular AZC and proline

Yeast cells were pre-cultured in SD medium containing 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 0.1% allantoin, and 20 g/L glucose (namely SD-N+Alla), at 30°C for 2 days, transferred to 200 mL SD-N+Alla medium for cultivation at 30°C with agitation, and grown until the OD_{600} reached mid-exponential phase. Following that, 50 μM AZC or proline was added to the culture. 20 OD_{600} of cell suspension were collected at 10, 30, 60, 120, and 180 min after the addition of AZC. Collected cells were washed twice with 0.9% sodium chloride and resuspended in 0.5 mL of distilled water. Intracellular amino acids were extracted by heating at 100°C for 10 min. After centrifugation (5 min at 12 000 × *g*), each supernatant was subsequently filtered using 0.2 mm Nylon-66 membrane (mdi™ India) and quantitated with the amino acid analyzer AminoTac JLC-500/V (JEOL Ltd.). Intracellular AZC concentration was represented as percent of dry cell weight (% of DCW).

2.7 Quantitative real-time PCR (qRT-PCR) analysis

Yeast cells were cultivated in SD medium at 30°C until they reached exponential growth phase (OD_{600} ~1.0). The cells were harvested and collected prior to and after the addition of 50 μM AZC, and cell extracts were prepared by vortexing with glass beads. Total RNA was isolated by using RNeasy Mini Kit (Qiagen) and 1 mg of RNA was used as a template for the cDNA synthesis via reverse transcription reaction using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Transcript levels of the targeted genes were assessed with quantitative real-time PCR (qRT-PCR) using SYBR Green PCR master mix (Applied Biosystems). qRT-PCR was performed using LightCycler96 (Roche Diagnostics) according to the following cycles; i) initial heating at 50°C for 2 min, ii) 95°C for 10 min, iii) repeated 95°C for 15 s, 60°C for 1 min for 40 times, iv) for dissociation curve, 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Specific pairs of primers (as listed in Table 4) were used to amplify approximately 60-70 nucleotides of DNA fragments of the target

open reading frames (ORFs). The threshold cycle (C_t) of each gene was normalized to a housekeeping gene *ACT1*, and the differences of the threshold cycles (ΔC_t) between these two were considered as relative expression levels. The fold changes in respect to mRNA transcription levels were estimated from the exponential values of the differences of the relative ΔC_t values between the samples and the control treatments ($\Delta\Delta C_t$) (Livak & Schmittgen, 2001).

Table 4 Oligonucleotide primers used to amplify genes for real-time PCR

Oligonucleotide	Sequence (5' → 3')
ACT1-1-F	CACCAACTGGGACGATATGGA
ACT1-2-R	GGCAACTCTCAATTCGTTGTAGAA
MSN2-RT-Fw	TGCGTCCGTTATTGCGAAA
MSN2-RT-Rv	CCTCTGTCAAAAATGGCATATCAT
CTT1-RT-Fw	CGCCGCTCCATACCAGAAT
CTT1-RT-Rv	CGGTGGAAAAACGAACAAGAC
GAP1-5-F	TTGGTGCCTCCTCTGTGGAT
GAP1-6-R	CCGTGAGTCTTAATGGCAATGA
PUT4-8-F	TCCGTCGATGGCGATAGC
PUT4-9-R	CGCGACTGCAAACCTTGTTT
AGP1_RealTime_157_F	CTTCTGCCGTGCGTAGGTTT
AGP1_RealTime_217_R	TTCATCCTGTGGACCCTGATC
GNP1_RealTime_1079_F	GCTGGCTATGACTGCCAGTGA
GNP1_RealTime_1140_R	CTTTGCCGCCGATGGA
UBP1-RT-Fw	GCAGAGGGGAACTATGCGTC
UBP1-RT-Rv	TCATCATCGGATGTCACGCC
UBP2-RT-Fw	TGGGAGAATCCAGAGCAAGG
UBP2-RT-Rv	TGAGGTTCTGCGTCCAAAGG
UBP3-RT-Fw	ATGGCCTCTCCAAACAGTGG
UBP3-RT-Rv	AGTAGCCGAAGAACCGTTGC

DOA4-RT-Fw	CCTCTACATTCGCCTGACCC
DOA4-RT-Rv	CCAAAGATGTGGCGTTTCCG
UBP5-RT-Fw	TCTGTCAGTGAAACAGCGCA
UBP5-RT-Rv	TGTTGGGACTTGCGGACTAC
UBP6-RT-Fw	TTGACCCAAGTCCCAAGTGC
UBP6-RT-Rv	TGGACTCTTCGCCAGACAAG
UBP7-RT-Fw	CGTTTCGTTGTCCATCACGG
UBP7-RT-Rv	GTCCGGAATGTTTTCGCAGC
UBP8-RT-Fw	TTGCCAAATGCCAAGGAAGTC
UBP8-RT-Rv	GCCCTCAAACACAGTATGCAC
UBP9-RT-Fw	AGCGTGAGATGTTGAACGGG
UBP9-RT-Rv	AGTCCGACCATACGTTCTGC
UBP10-RT-Fw	AGCGTGGTGGTTCATGAGG
UBP10-RT-Rv	TACCGTCTGGTTGCTTGCAG
UBP11-RT-Fw	AGCGGAGTCAAACGGAATTTG
UBP11-RT-Rv	AACAGGTAAAGGCCGTAGCG
UBP12-RT-Fw	CCGAAAAACAAGACGGGAGG
UBP12-RT-Rv	TCAACGCAGACATCGAGAGG
UBP13-RT-Fw	CGGAGGCATTATCACGCAAG
UBP13-RT-Rv	TCCATAGGGCATTGAGTCGG
UBP14-RT-Fw	TTTCCCGAACCTGAAGTGCC
UBP14-RT-Rv	CGTACGGTTTAGCGGTCGAA
UBP15-RT-Fw	ACAAAGGGAGGAATCGAGCC
UBP15-RT-Rv	GTGGGTCGTAGCTCTTGACG
UBP16-RT-Fw	TGCAAAAGGCGACAATCCTAC
UBP16-RT-Rv	TTCGTGTAAGGGGGCAAGTG
RPN11-RT-Fw	TGACACGGGCGCATTGATAA
RPN11-RT-Rv	AAGCCTGTGTTGGAGGTTGT
OTU1-RT-Fw	CCCGTCAAGTTCAATGACGC

OTU1-RT-Rv	ATGGCTCCACCCCAAGATTC
OTU2-RT-Fw	TGGAGCATACAGCTCAATGGG
OTU2-RT-Rv	GCTAATGGGGCAGTCGAAAAC
YUH1-RT-Fw	AATGCGTGCGGATTGTATGC
YUH1-RT-Rv	AAATTGTCCAAGTCGGAGCC
UBI4-RT-Fw	TTGTGCTAAGGCTAAGAGGTGG
UBI4-RT-Rv	GTTATCGATCGTGTCGGAGG

2.8 Fluorescence microscopy

Yeast cells harboring *GNP1* tagged with GFP (Thermo Fisher Scientific) were cultivated in SD liquid medium and grown until exponential growth phase at 30°C, collected and harvested by centrifugation. The cells were observed immediately without fixation under a fluorescence microscope Axiovert 200M (Carl Zeiss). Cellular images were captured with HBO 100 microscope illuminating system (Carl Zeiss) and processed using AxioVision Rel. 4.8 software (Carl Zeiss).

2.9 Western blotting

Whole cell extracts were prepared by harvesting approximately 3×10^8 cells by centrifugation, resuspended in 10% trichloroacetic acid (TCA), disrupted with glass beads in a Multi-beads shocker (Yasui Kikai) and lysed by heating in SDS sample buffer (50 mM Tris-HCl [pH8.0], 2% SDS, 0.0125% BPB, and 2.25% glycerol) for 5 min at 98°C. Total cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10-15% polyacrylamide gels. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) and blocked with 5% skim milk (wt/vol) in 1 × TBST buffer (20 mM Tris-HCl [pH7.6], 150 mM NaCl, and 0.1% Tween-20) at 4°C for overnight. The procedure was continued to probe the membranes with a mouse monoclonal anti-GFP (Roche) or a mouse monoclonal anti-ubiquitin (P4D1, Santa Cruz) and followed by a mouse IgG HRP-conjugated

secondary antibody (GE Healthcare). After proper washing with 1 × TBST buffer, Western blot signals were detected by Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences) and visualized using the ImageQuant LAS 4000 (GE Healthcare Life Sciences).

3.0 RESULT

3.1 Involvement of the stress-responsive transcription factor gene *MSN2* in the control of amino acid uptake in *Saccharomyces cerevisiae*

3.1.1 Tolerance to toxic amino acid analogues in *MSN2*-overexpressing cells

To understand whether Msn2 is involved in amino acid homeostasis, I first tested the growth phenotypes of the *MSN2*-overexpressing cells in the presence of several toxic amino acid analogues on SD agar plates. Msn2 is well known as a general stress transcription factor in yeast *S. cerevisiae*. As demonstrated previously (Sasano *et al.*, 2012d), overexpression of *MSN2* markedly increased the resistance to 2 mM hydrogen peroxide, which causes oxidative stress to yeast cells (Fig. 9). Using the same strain, it was exhibited that the overexpression of *MSN2* decreased the resistance of yeast cells to azetidine-L-carboxylic acid (AZC), *o*-fluoro-DL-phenylalanine (OFP), and L-canavanine (Can), which are the toxic analogues of L-proline, L-phenylalanine, and L-arginine, respectively (Fig. 10). When I tested another nitrogen source allantoin, instead of ammonium sulfate, as a nitrogen source in the synthetic minimum medium, *MSN2*-overexpressing cells similarly showed the hypersensitivity to AZC, OFP, and Can. The impaired resistance was observed not only in the laboratory yeast strain BY4741 (Fig. 10A), but also in an industrial baker's yeast strain (Fig. 10B). These results suggest a novel role of Msn2 in amino acid incorporation.

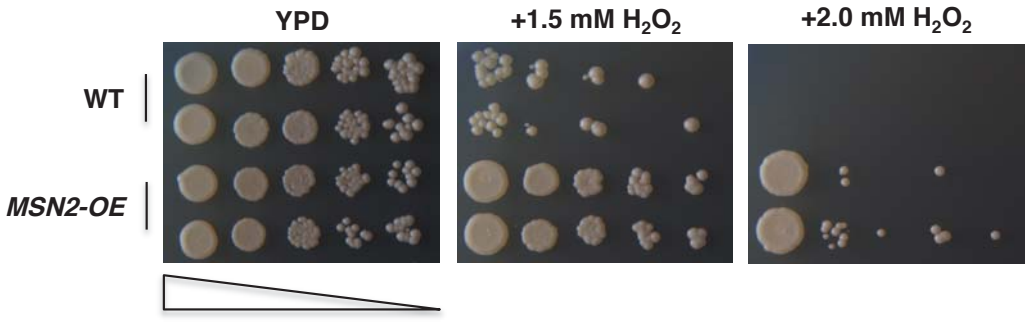

Fig. 9 Overexpression of *MSN2* conferred tolerance towards oxidative stress (hydrogen peroxide).
The cells were grown until the exponential phase, serially diluted and spotted on the minimal media containing different concentration of hydrogen peroxide (H_2O_2).

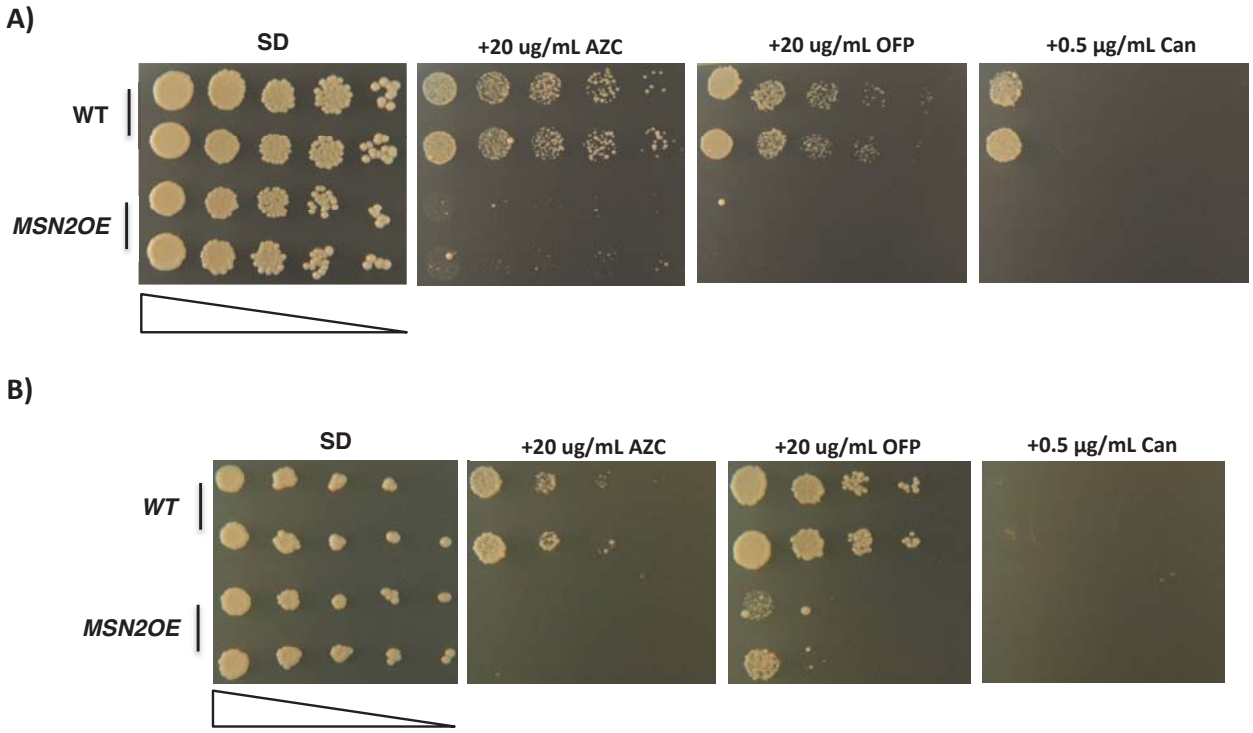

Fig. 10 Growth phenotype of laboratory and industrial baker's strains on toxic amino acid analogues.

After the wild-type and *MSN2*-overexpressing cells in the laboratory strain BY4741 (A) and an industrial baker's strain (B) were cultivated in minimal SD media until the cells reached the exponential growth phase, a serial dilution of 10^{-1} to 10^{-4} were spotted on the media in the absence (control) and presence of toxic amino acid analogues.

3.1.2 Survival of *MSN2*-overexpressing cells in the presence of AZC

Msn2 increases not only the cell growth but also the cell survival under different kinds of environmental stresses, including oxidative stress (Gasch *et al.*, 2000; Sadeh *et al.*, 2011; Sadeh *et al.*, 2012; Sasano *et al.*, 2012d). Consistent with these reports, overexpression of *MSN2* in the laboratory strain BY4741 enhanced cell survival under hydrogen peroxide treatment (Fig. 11). In contrast, the strain carrying deletions of both *MSN2* and *MSN4* showed a lower survival rate after 120-min hydrogen peroxide treatment.

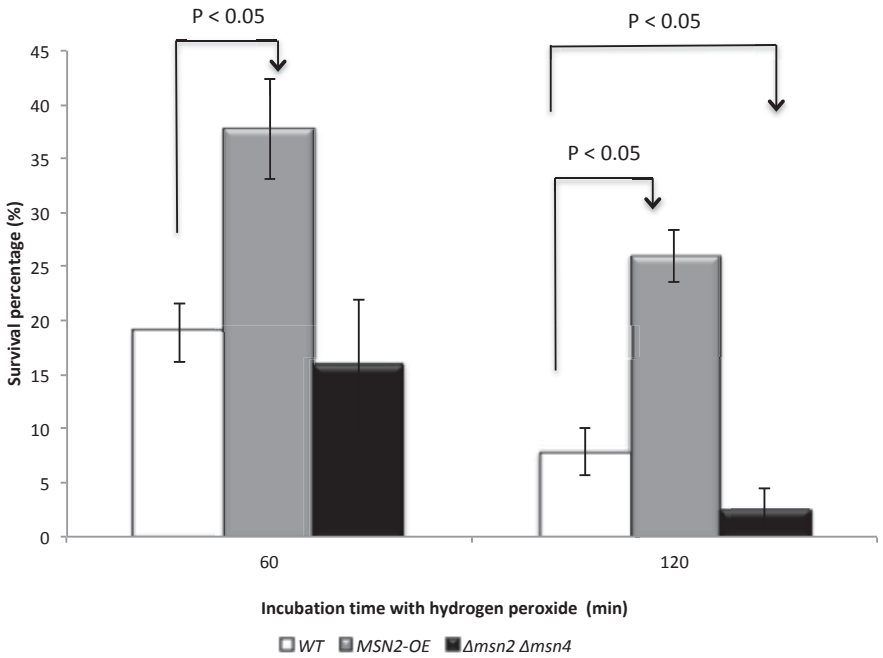

Fig. 11 Overexpression of *MSN2* conferred tolerance against oxidative stress (hydrogen peroxide).

Survival percentage of *MSN2*-overexpressing cells in the presence of oxidative stress (hydrogen peroxide). The cells were grown until reached the exponential growth phase, followed by the addition of 2 mM hydrogen peroxide. The cells were then serially diluted and plated on YPD agars accordingly.

As shown in Fig. 12, the survival rate of *MSN2*-overexpressing cells decreased dramatically by 70% after the 30-min AZC treatment. In contrast, only 30% of the wild-type cells lost their viability under the same treatment, indicating a higher tolerance of the wild-type cells to AZC. This result suggests that overexpression of *MSN2* negatively controls both the growth and survival of yeast cells in the presence of amino acid analogues. It is also noted that disruption of *MSN2* and its paralogue *MSN4* did not exacerbate the sensitivity of the cells toward AZC by the 60-min treatment.

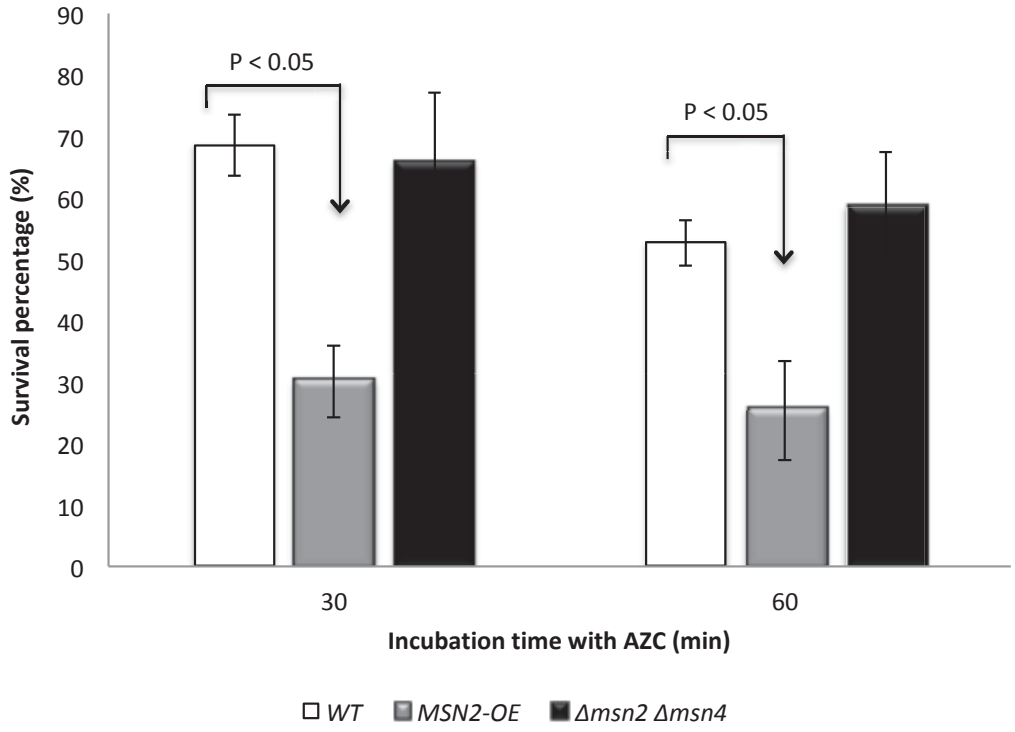

Fig. 12 Survival percentage of *MSN2*-overexpressing cells in the presence of AZC.
The cells were grown until reached the exponential growth phase, followed by the addition of 50 μM AZC. The cells were then serially diluted and plated on YPD agars accordingly.

3.1.3 Incorporation of AZC and proline in *MSN2*-overexpressing cells

To test the possibility that the excess Msn2 enhances the cellular incorporation of amino acid analogues, I quantified intracellular AZC concentrations after the addition of AZC into the log-phase culture. As shown in Fig. 13, *MSN2*-overexpressing cells exhibited higher intracellular AZC levels than wild-type cells throughout the 3-hour treatment. The AZC contents in both wild-type and *MSN2*-overexpressing cells were maximized 30 min after the addition, and then gradually decreased. Thus, the increased sensitivity to AZC in *MSN2*-overexpressing cells is at least partly associated with the enhanced uptake of non-metabolized AZC into the cells. Overexpression of *MSN2* increased not only AZC (Fig. 13B) but also proline 30 min after the addition (Fig. 13C). It is noted that the proline levels were strikingly lower than the AZC level probably because the excess proline was quickly metabolized after incorporation into the cells.

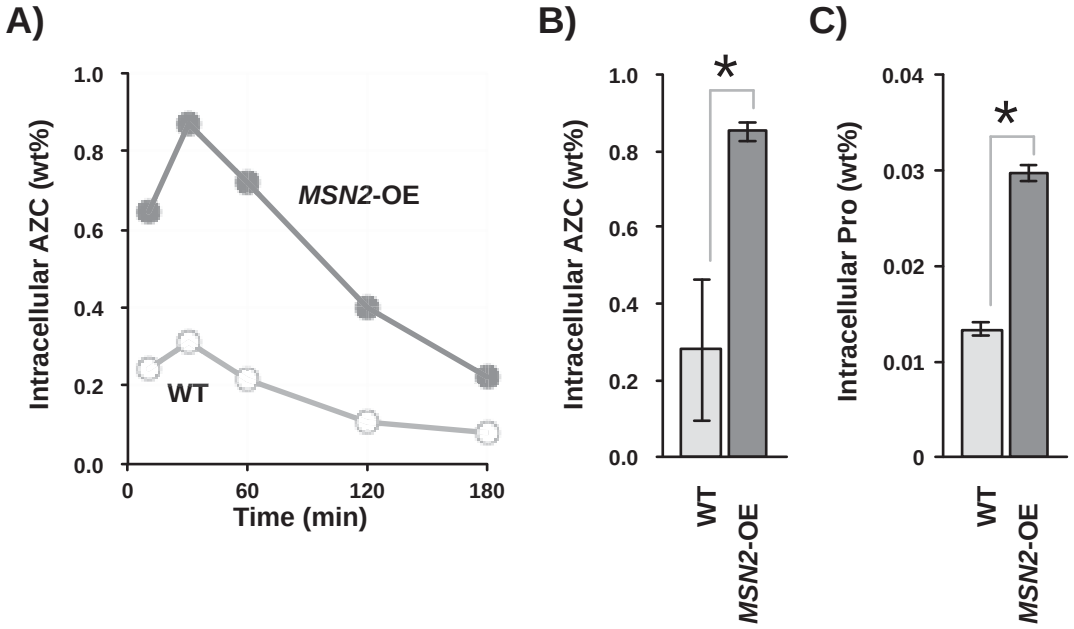

Fig. 13 Intracellular levels of AZC or proline in *MSN2*-overexpressing cells.
A) Changes in intracellular AZC levels in BY4741 wild-type and *MSN2*-OE cells after the addition of 50 μM AZC to SD-N+Alla medium. Shown here is the representative data from three independent experiments. B) Intracellular AZC levels in BY4741 wild-type and *MSN2*-OE cells 30 min after the addition of 50 μM AZC to SD-N+Alla medium. Data represent the mean ± standard deviation (SD) from three independent experiments. Asterisks indicate significant difference (*t* test, $P < 0.05$). C) Intracellular proline levels in BY4741 wild-type and *MSN2*-OE cells 30 min after the addition of 50 μM proline to SD-N+Alla medium. Data represent the mean ± standard deviation (SD) from three independent experiments. Asterisks indicate significant difference (*t* test, $P < 0.05$).

3.1.4 Proline permease genes responsible for the enhanced incorporation of AZC in *MSN2*-overexpressing cells

Amino acids and their analogues are incorporated into yeast cells by the active amino acid permeases on the plasma membrane. AZC, the analogue for L-proline, is transported across the plasma membrane by the proline permeases Gap1, Put4, Agp1, and Gnp1 (Andréasson *et al.*, 2004; Sasaki & Takagi, 2013). To identify the permeases responsible for the enhanced uptake of AZC in *MSN2*-overexpressing cells, I examined the growth phenotype of yeast cells with deletion of the *GAP1*, *PUT4*, *AGP1*, or *GNP1* gene in the presence of AZC (Fig. 14). It is noted that the AZC tolerance was lower in the CAY29 genetic background used in this experiment than in the BY4741

background, although a slight difference in growth under AZC between the wild-type and *MSN2*-overexpressing cells was observable. As previously reported, the quadruple deletion of the permease genes markedly increased the cell resistance towards AZC. Among the permease genes, a single deletion of the *GNP1* gene markedly eliminated the toxicity of AZC. In the CAY29 *MSN2*-OE background, the quadruple deletion also increased the resistance of cells to AZC, and Δ*gnp1* exhibited the most prominent effect among single deletions of the permease genes. This result suggested that Gnp1 plays a predominant role in the incorporation of AZC under the condition used. Since the growth of the *GNP1*-deleted strains was weaker than that of the quadruple disruptants in the presence of AZC, the three other permeases, i.e., Gap1, Put4, and Agp1, may have redundant roles in AZC uptake.

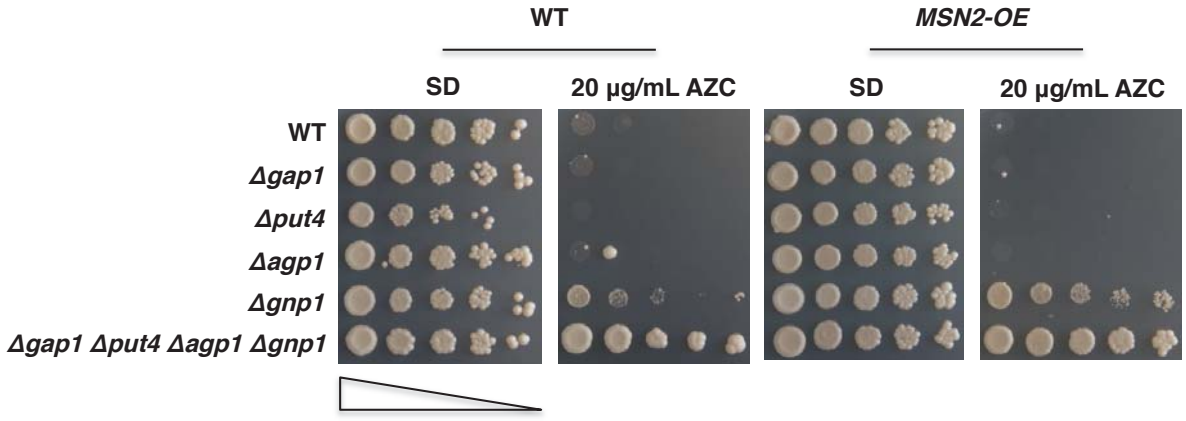

Fig. 14 Growth phenotype of *S. cerevisiae* CAY29 strains lacking each or all of the proline permease genes on AZC-containing media.

Deletion of *GNP1* was also carried out in the strains with the BY4741 genetic background (Fig. 15A). In accordance to the results presented in Fig. 14, deletion of *GNP1* mitigated the AZC toxicity in both the wild-type and *MSN2*-overexpressing strains. Furthermore, overexpression of *GNP1* reduced the AZC resistance in the wild-type cells (Fig. 15). Deletion or overexpression of the *GNP1* did not affect the resistance to other amino acid analogues (OFP or Can); this is consistent with the fact that Gnp1 is not involved in the transport of phenylalanine (incorporated by Gap1, Agp1, and Bap2) and arginine (incorporated by Can1) (Ljungdahl and Daignan-Fornier, 2012). This result

suggests the idea that Gnp1 selectively transports proline and its analogue AZC. It is also noted that disruption of *MSN2* did not affect the growth phenotype probably due to the homologous gene *MSN4* and other genes with redundant functions. As shown in Fig. 15B, Gnp1 also has a negative role in cell survival under AZC treatment; deletion of *GNP1* suppressed the low survival rate of the *MSN2*-overexpressing strain, and overexpression of *GNP1* in the wild-type cells decreased the survival rate. Together, it is hypothesized that Msn2 positively regulates Gnp1 in the incorporation of AZC.

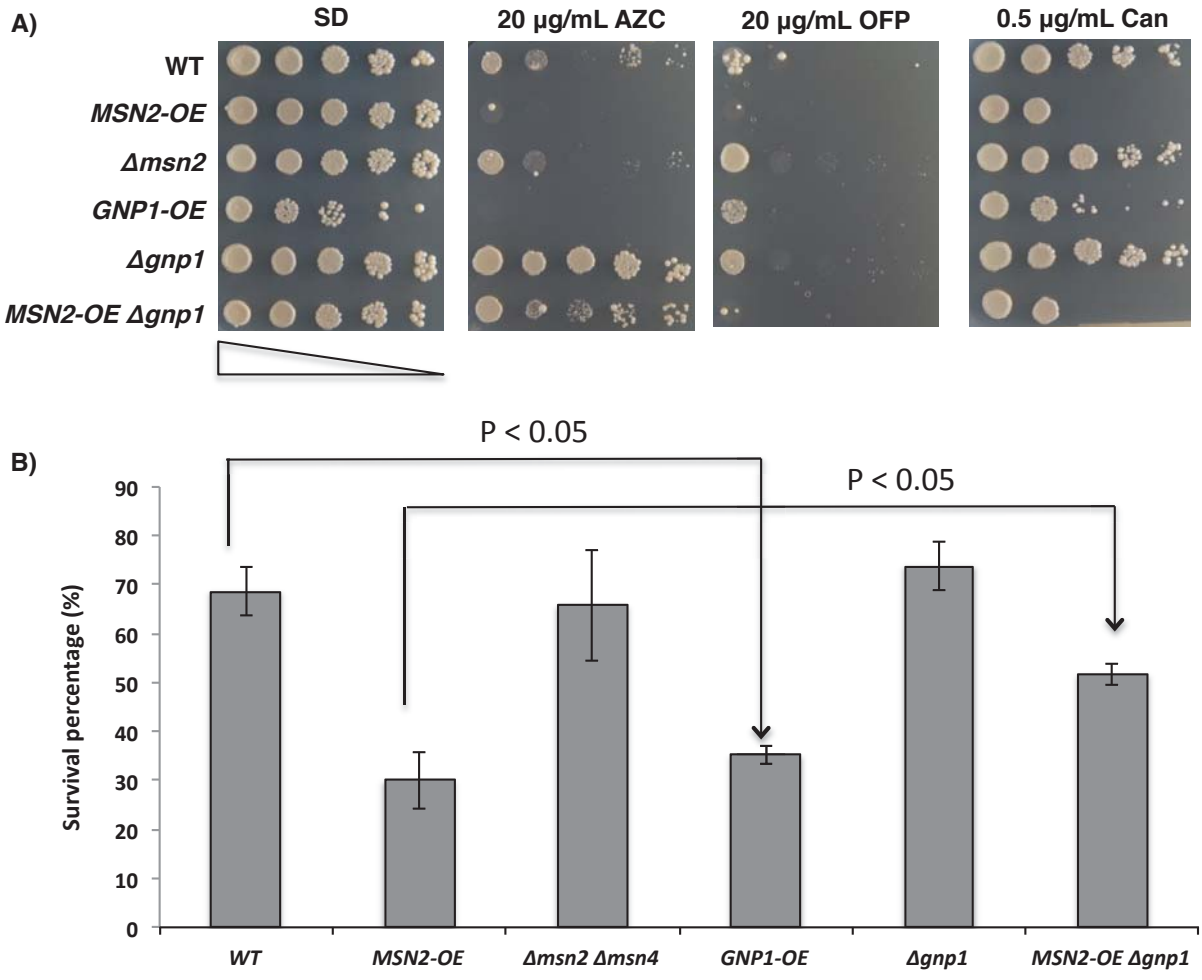

Fig. 15 Role of Gnp1 in the cell growth and survival in the presence of AZC.
A) Growth phenotype of *S. cerevisiae* strain lacking or overexpressing *GNP1*. B) Survival percentage of the strains under the treatment of 50 μM AZC for 30 min.

3.1.5 Transcription of proline permease genes in *MSN2*-overexpressing cells

To test the possibility that Msn2 directly activates the transcription of the *GNP1* gene, I focused on the transcriptional levels of the proline permease genes in *MSN2*-overexpressing cells. Based on the *in silico* analysis of the promoter sequences of the proline permease genes (*GAP1*, *PUT4*, *AGP1* and *GNP1*) using the Yeastract database (http://www.yeastract.com/index.php), *GAP1*, *PUT4*, and *AGP1* contained the stress-response elements (STREs; AGGGG), typical binding sites of Msn2/4, in the 1000 bp upstream of the coding sequence (Fig. 16). In contrast, the promoter region of the *GNP1* gene did not contain the STRE sequences. As shown in Fig. 17A, when *MSN2* was overexpressed, the transcription level of a representative Msn2-targeted gene *CTT1* (encoding a catalase) with multiple STREs in the promoter was upregulated 100- or 20-folds in the absence or presence of AZC, respectively. On the other hand, none of the levels of *GAP1*, *PUT4*, *AGP1*, and *GNP1* mRNA transcripts was significantly upregulated under overexpression of *MSN2*, regardless of the absence or presence of AZC (Fig. 17B). Thus, it is unlikely that Msn2 enhances the incorporation of proline via transcriptional activation of the *GNP1* or the other proline permease genes.

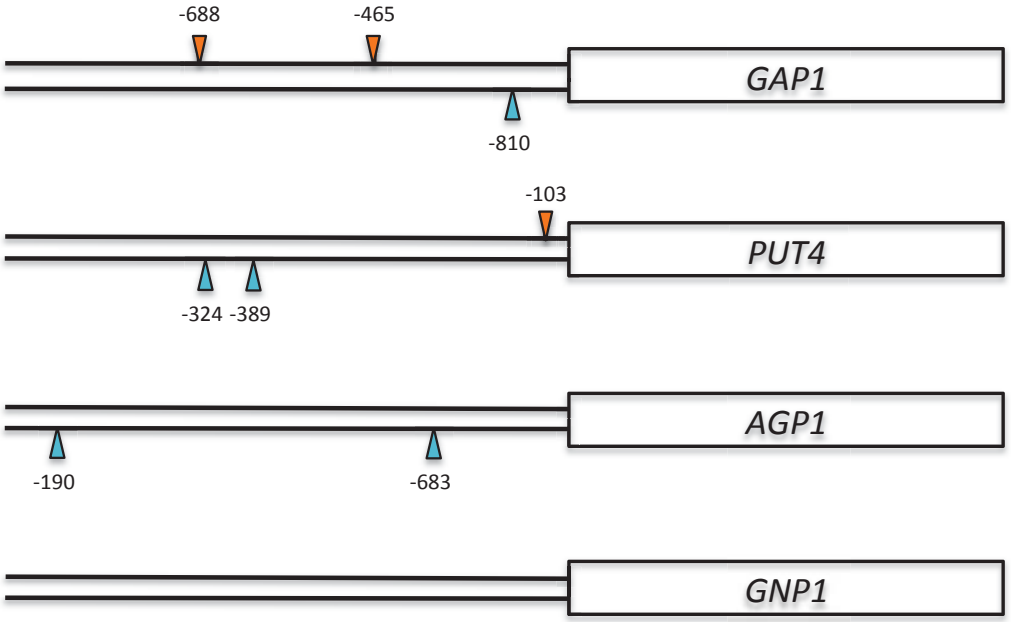

Fig. 16 I*n silico* analysis of the promoter sequences (1000 bp upstream of the coding sequence) of the proline permease genes (*GAP1*, *PUT4*, *AGP1*, and *GNP1*).
Orange triangles indicate STREs (AGGGG) found on the 5'-3' strands, while blue triangles indicate STREs found on the complementary strand of each gene.

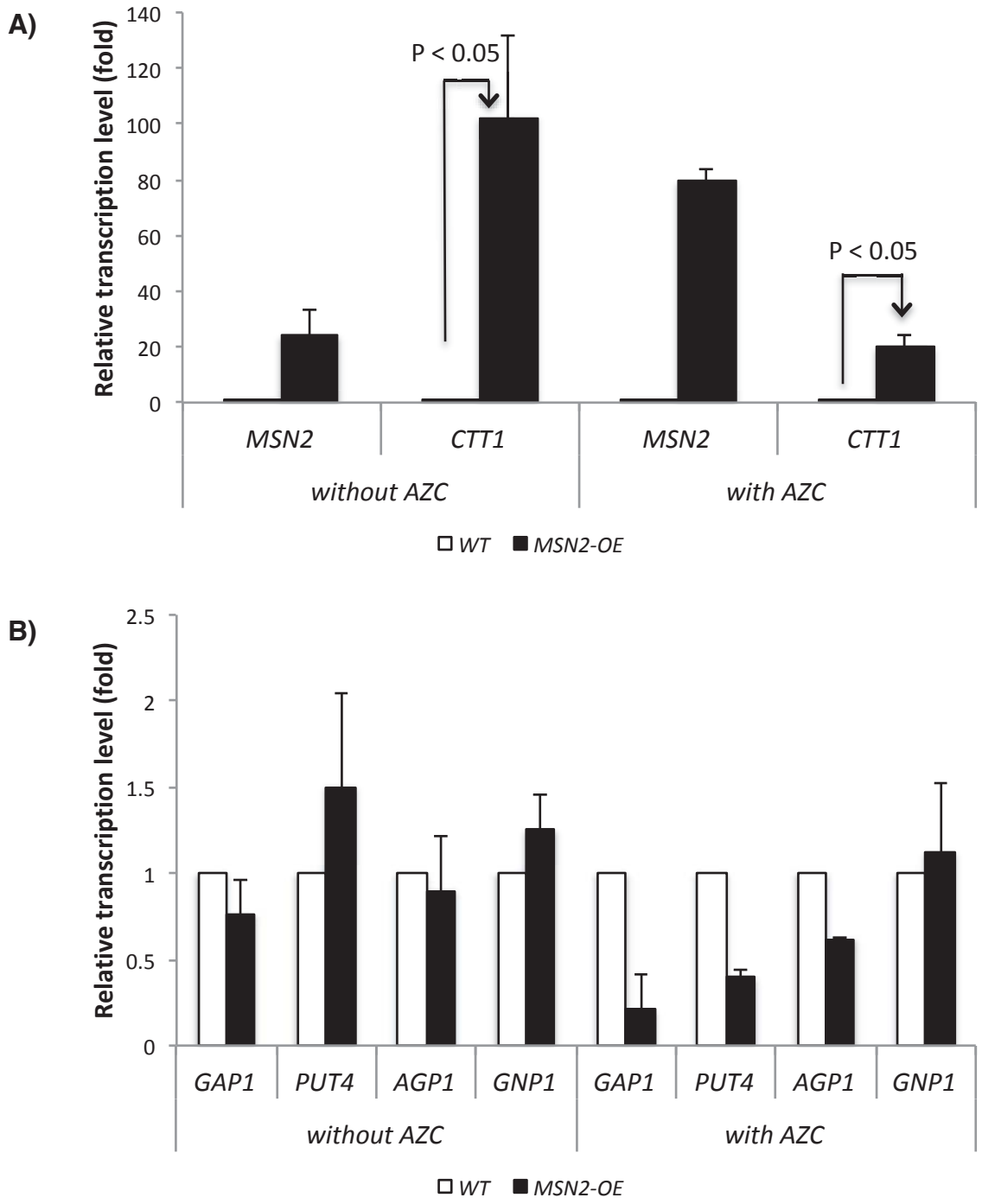

Fig. 17 Relative transcription level of genes encoding for proline permeases in the absence and presence of AZC.

The cells were grown in SD media until reached the exponential growth phase, harvested A) prior to and B) after 30-min exposure to AZC. Transcript levels were analyzed by quantitative real-time PCR (qRT-PCR) using cDNA prepared from the harvested cells. The fold-changes were normalized with the housekeeping gene, *ACT1* transcript levels.

3.1.6 Protein level and localization of Gnp1 in *MSN2*-overexpressing cells

Although the transcription levels of *GNP1* and the other proline permease genes were not significantly changed in *MSN2*-overexpressing cells, Gnp1 might be regulated by Msn2 in a posttranscriptional manner. To analyze the protein abundance of Gnp1, yeast cells expressing Gnp1 tagged with GFP at the carboxyl terminus were used. As a result, I detected a higher level of Gnp1-GFP in the *MSN2*-overexpressing cells (Fig. 18). Gnp1-GFP signals disappeared after 30-min treatment with AZC, supporting the observation that intracellular AZC levels decreased after 30 min from the addition of AZC (Fig. 18).

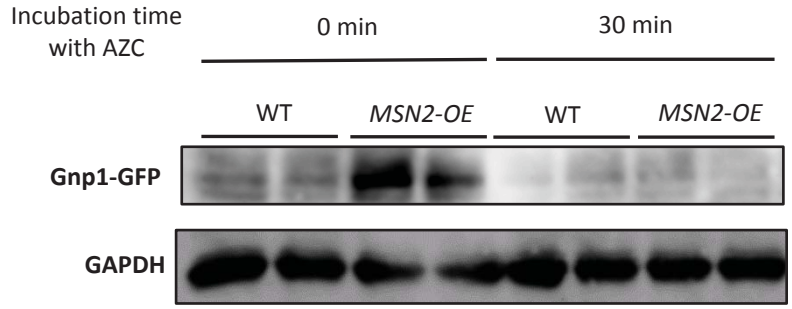

Fig. 18 Gnp1 protein levels in wild-type and *MSN2*-overexpressing cells.
Cell lysates were extracted from yeast cells grown in SD media harvested prior to and after the addition of 50 μM AZC for 30 min. Gnp1 protein tagged with GFP was detected by Western blotting using anti-GFP antibody, and GAPDH was used as a loading control. Blots are representative from three independent experiments.

Since the Gnp1-GFP protein level was elevated under the overexpression of *MSN2*, I speculated that Gnp1 in *MSN2*-overexpressing cells is more strongly distributed on the plasma membrane (Fig. 19). In wild-type cells, Gnp1-GFP was accumulated largely in the interior of the cells, suggesting endocytosis and degradation normally occurred in the vacuoles. On the other hand, most of Gnp1-GFP signal was observed to localize on the plasma membrane of the *MSN2*-overexpressing cells. This data supported the idea that the impaired tolerance to AZC in *MSN2*-overexpressing cells is caused by the enhanced plasma-membrane localization of Gnp1. It is likely that Msn2 negatively regulates endocytosis and/or degradation of Gnp1 at the posttranslational level.

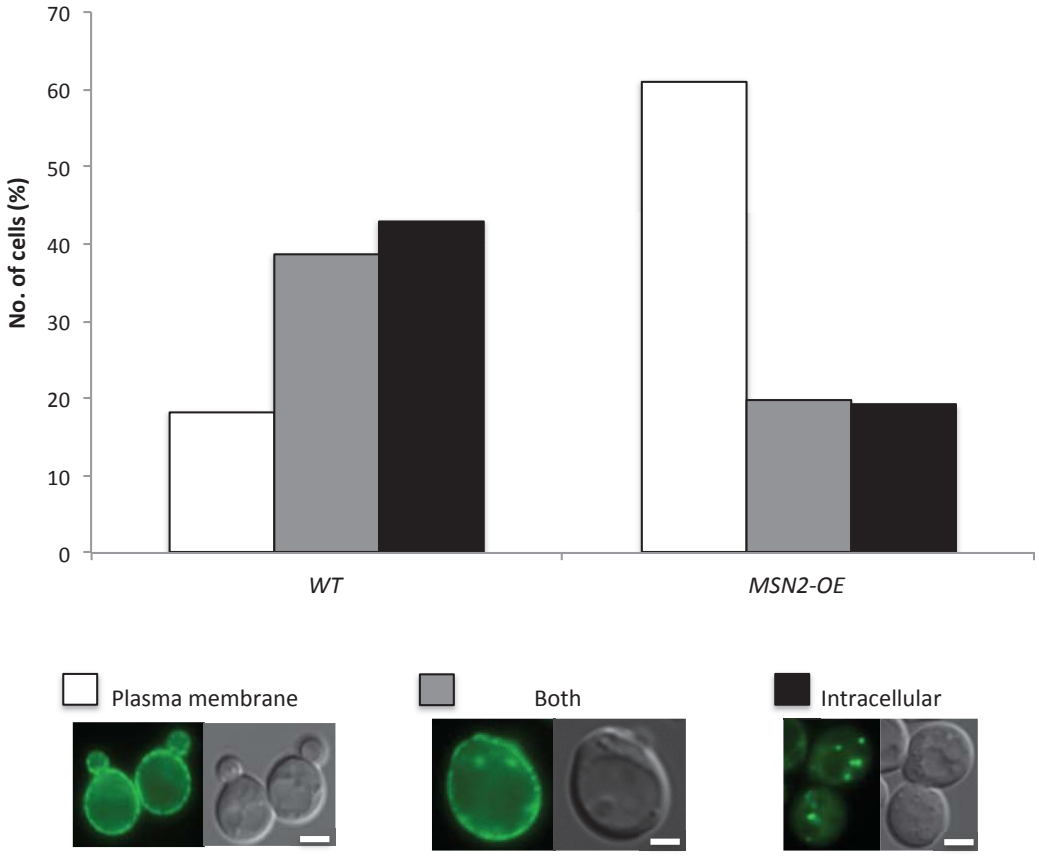

Fig. 19 Localization of Gnp1 in wild-type and *MSN2*-overexpressing cells.

Fluorescent microscopic analysis of Gnp1-GFP in BY4741 wild-type (WT) and *MSN2*-overexpressing (*MSN2*-OE) cells. Observation of more than 100 cells each was repeated three times. Cell morphology was observed through differential interference contrast. Bar: 5 μM.

3.2 Effects of deubiquitination enzyme gene *UBP6* on the Msn2-mediated control of the amino acid permease Gnp1 in *Saccharomyces cerevisiae*

3.2.1 Ubiquitinated proteins and free ubiquitin in *MSN2*-overexpressing cells

Based on the data described above, it was presumed that Msn2 inhibits degradation of Gnp1 at the posttranslational level. Previous studies revealed that ubiquitination and deubiquitination are the keys to the control of endocytic degradation of plasma membrane-localized permeases (Springael & André, 1998; Crosas *et al.*, 2006; Saksena *et al.*, 2007; Wolf & Petroski, 2009; Kimura *et al.*, 2009; Kimura & Tanaka, 2010; Jones *et al.*, 2012; MacGurn, 2014). It is also well known that Msn2 induces the transcription of the *UBI4* gene, which encodes polyubiquitin, under stress conditions (Simon *et al.*, 1999). Thus, I first tested the global effects of overexpression of *MSN2* on the abundance of ubiquitinated proteins and free ubiquitin (Fig. 20). The whole cell extracts were subjected to SDS-PAGE, and the blotted membrane was incubated with an anti-ubiquitin antibody. As a result, ubiquitinated proteins were detected and their abundance was remarkably increased by the AZC treatment in wild-type cells. This suggested that AZC-incorporated proteins are misfolded and then degraded. Notably, I found that the level of ubiquitin-protein conjugates in *MSN2*-overexpressing cells was higher than that in wild-type cells in the absence of AZC (Fig. 20A and B). Moreover, the free ubiquitin level in *MSN2*-overexpressing cells was reduced compared to wild-type cells in the presence of AZC (Fig. 20A and C). These two phenomena were also observed in *Δubp6* cells. This result suggested that the overexpression of *MSN2* leads to a reduced or an impaired activity of DUBs including Ubp6, resulting in an increase in ubiquitinated proteins with a depletion of ubiquitin monomers.

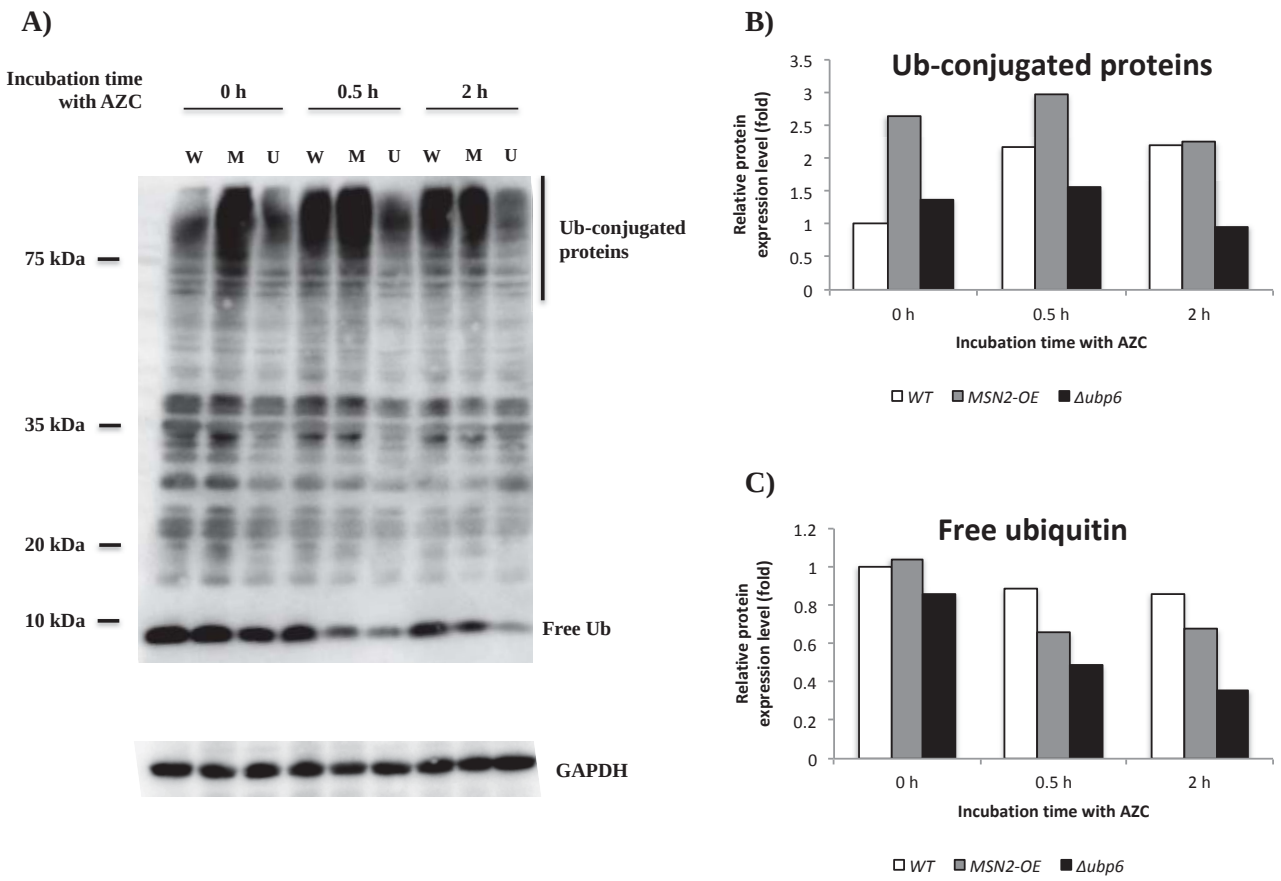

Fig. 20 Western blot analysis of ubiquitin in yeast cells.

A) *S. cerevisiae* wild-type (W), *MSN2*-overexpressing (M), and *Δubp6* (U) strains were cultured to the exponential growth phase in liquid SD medium at 30°C and were harvested prior to and after the addition of 50 μM AZC for 0.5 h and 2 h. Proteins extracted from the whole cell extract were electrophoresed on SDS-PAGE, transferred to the blots, and free ubiquitin (Ub) and Ub-conjugated proteins were detected by using anti-ubiquitin antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. Molecular mass standards are shown on the left. Shown here is the representative data of three independent experiments. The relative quantification of B) Ub-conjugated proteins and C) free Ub were normalized to the loading control GAPDH, and compared to the wild-type strain at 0 h.

I also tested the possibility that *MSN2* overexpression suppresses the expression of the ubiquitin-coded genes (*UBI1*, *UBI2*, and *UBI3*) by qRT-PCR. As shown in Fig. 21, there were no significant differences in the levels of the *UBI1-3* mRNA transcripts between wild-type and *MSN2*-overexpressing cells, although the *CTT1* gene encoding catalase was remarkably induced by *MSN2* overexpression. Therefore, it was concluded that low level of free ubiquitin in *MSN2*-overexpressing cells is not due to the suppression of *UBI1-3* gene expression.

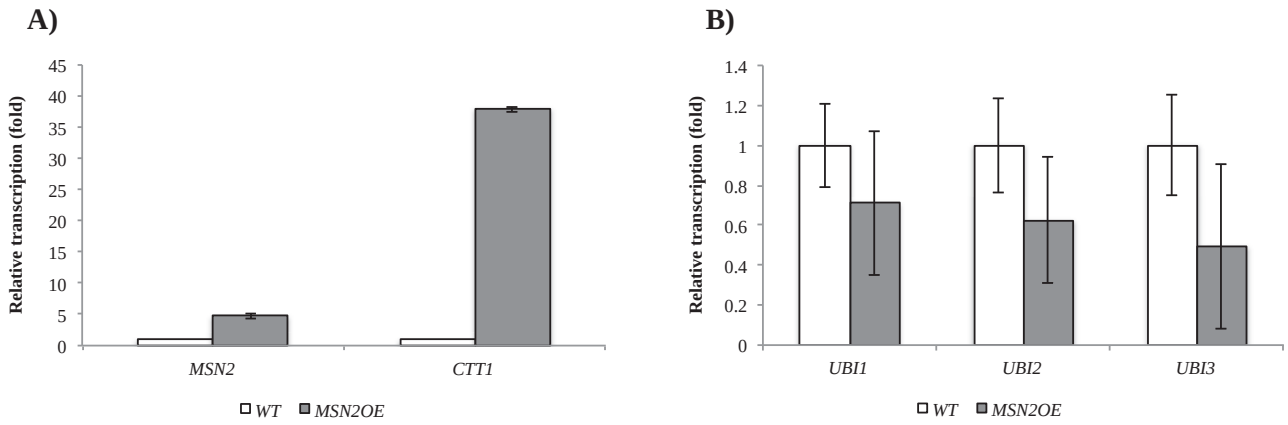

Fig. 21 Transcriptional analysis of ubiquitin-encoding genes in yeast cells.
S. cerevisiae wild-type and *MSN2*-overexpressing strains were cultured to the exponential growth phase in liquid SD medium at 30°C and harvested. RNA was extracted and used as a template to synthesize cDNA. Transcript levels were analyzed by quantitative real-time PCR (qRT-PCR). The fold-changes were normalized with the housekeeping gene *ACT1*. *CTT1*, which encodes for catalase, serves as a positive control.

3.2.2 Transcriptional analysis of DUB genes in *MSN2*-overexpressing cells

In *S. cerevisiae*, 20 DUBs (Ubp1 to 16, Otu1, Otu2, Rpn11, and Yuh1) have been reported to date (Finley *et al.*, 2012). To test the possibility that the overexpression of *MSN2* impairs DUBs through their transcriptional control, the transcriptional levels of DUB genes were analyzed in both wild-type and *MSN2*-overexpressing cells (Fig. 22). Although the *CTT1* gene, which is a representative target gene of Msn2, was markedly induced in *MSN2*-overexpressing cells, not only the transcription of *UBP6* but also other DUB genes did not show any significant changes in *MSN2*-overexpressing cells. These results suggested that Msn2 is not involved in inhibition of the cellular DUB activity via transcriptional repression of DUB genes.

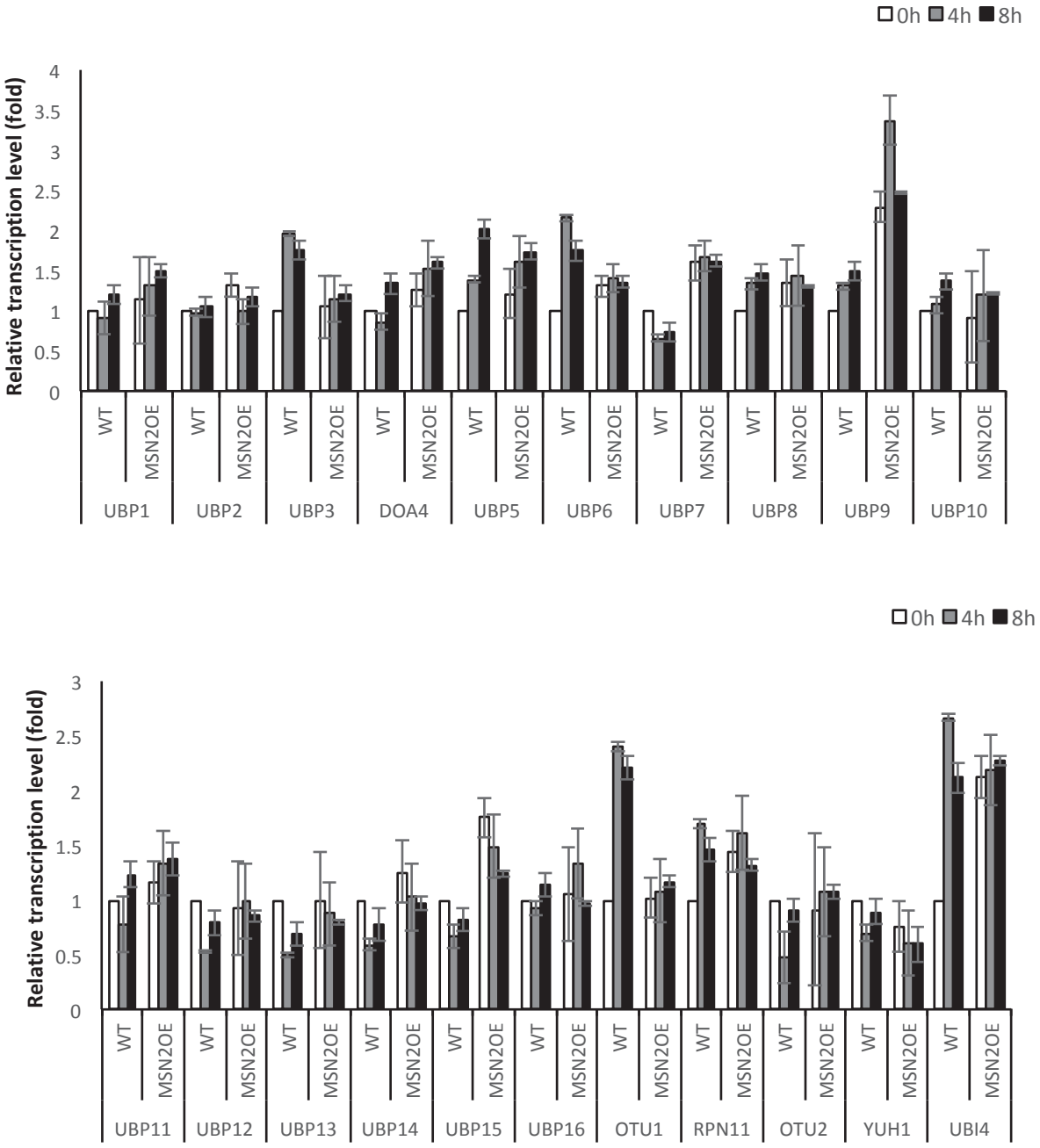

Fig. 22 Transcriptional levels of yeast deubiquitinating enzyme genes in the absence and presence of AZC.

The cells were grown in SD media until reached the exponential growth phase, harvested before and after the addition of 50 μM AZC up to 8h. Transcript levels were analyzed by quantitative real-time PCR (qRT-PCR) using cDNA prepared from the harvested cells. The fold-changes were normalized with the housekeeping gene, *ACT1* transcript levels. *UBI4* encodes for polyubiquitin, a ubiquitin precursor protein and served as a positive control.

3.2.3 Growth of DUB-deleted cells in the presence of amino acid analogues

Although it is unlikely that overexpression of *MSN2* directly regulates the transcription of the DUB genes, the deletion of the DUB genes might mimic the phenotype of the *MSN2*-overexpressing cells. As shown in Fig. 23, the absence of some DUB genes, such as *UBP6*, *UBP3*, and *OTU1*, impaired cell growth in the medium containing amino acid analogues. Among them, the deletion of *UBP6* gene most strikingly conferred the sensitivity towards AZC, OFP, and Can. The single disruption of *UBP3* or *OTU1* decreased the resistance to a lesser extent. It is also worth noting the previous reports that ubiquitin monomers were depleted in yeast cells in response to the loss of Ubp6 (Chernova *et al.*, 2003; Hanna *et al.*, 2007), just as observed in *MSN2*-overexpressing cells in my experiments.

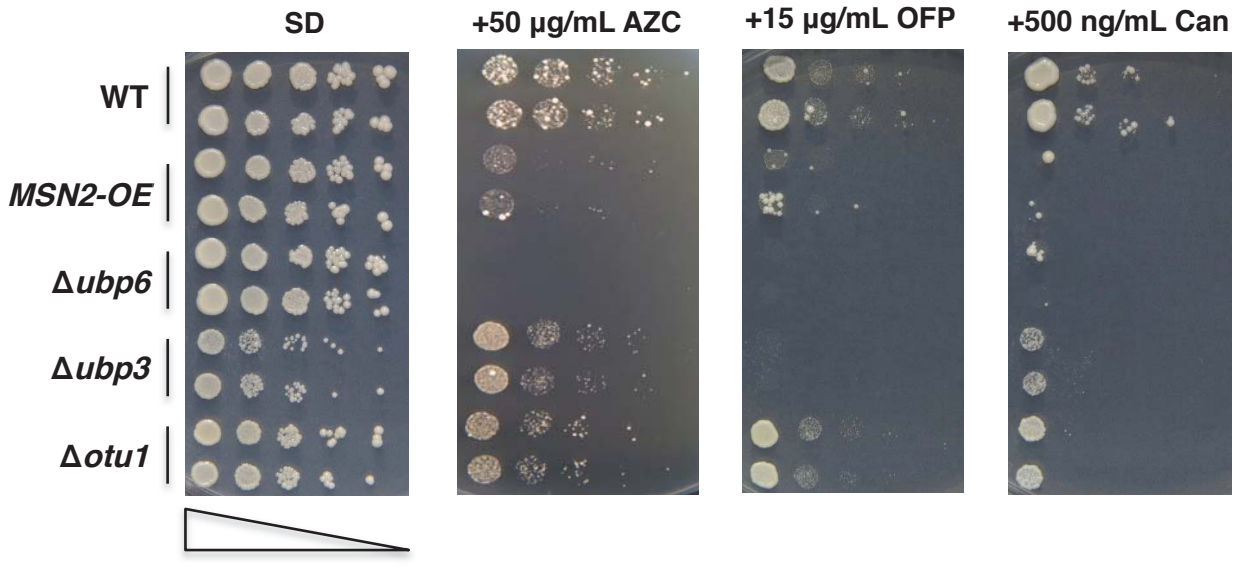

Fig. 23 Growth phenotype of cells lacking *UBP6*, *UBP3*, or *OTU1* genes on media containing amino acid analogues.
The yeast cells were cultivated in minimal SD media until the cells reached the exponential growth phase, a serial dilution of 10^{-1} to 10^{-4} were spotted on the media in the absence (control) and presence of toxic amino acid analogues.

3.2.4 Genetic interaction between overexpression of *MSN2* and deletion of *UBP6*

As described above, the overexpression of *MSN2* and the deletion of *UBP6* commonly led to impaired resistance towards amino acid analogues. However, it is still unclear whether these mutant cells showed similar phenotypes via similar or different mechanisms. To address this, I tested the combined effects of overexpression of *MSN2* and deletion of *UBP6* on AZC tolerance (Fig. 24). At low concentrations of AZC, *Δubp6* cells overexpressing *MSN2* showed similar growth as *Δubp6* cells. Because I did not test AZC concentrations lower than 5 μg/mL, I could not rule out the possibility that Msn2 and Ubp6 might affect AZC resistance via two independent mechanisms.

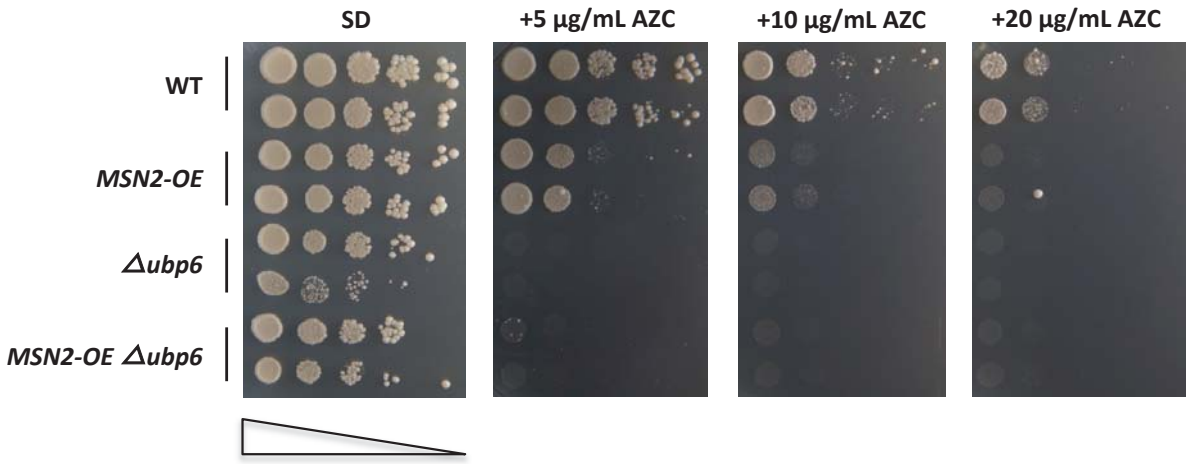

Fig. 24 Growth phenotype of WT, *MSN2-OE*, *Δubp6*, and *MSN2-OE Δubp6* cells on AZC-containing media

The yeast cells were cultivated in minimal SD media until the cells reached the exponential growth phase, a serial dilution of 10^{-1} to 10^{-4} were spotted on the media in the absence (control) and presence of different concentrations of AZC.

3.2.5 Genetic interaction between overexpression of *MSN2* and overexpression of *UBP6*

Assuming that the loss of function of *UBP6* is responsible for the weak tolerance towards toxic amino acid analogues in *MSN2*-overexpressing cells, constitutively high expression of *UBP6* might be able to complement the growth inhibition. To support this idea, the *UBP6* gene was overexpressed in both wild-type and *MSN2*-overexpressing cells. As predicted, the overexpression of *UBP6* alleviated the toxicity of AZC, OFP, and Can in *MSN2*-overexpressing cells (Fig. 25).

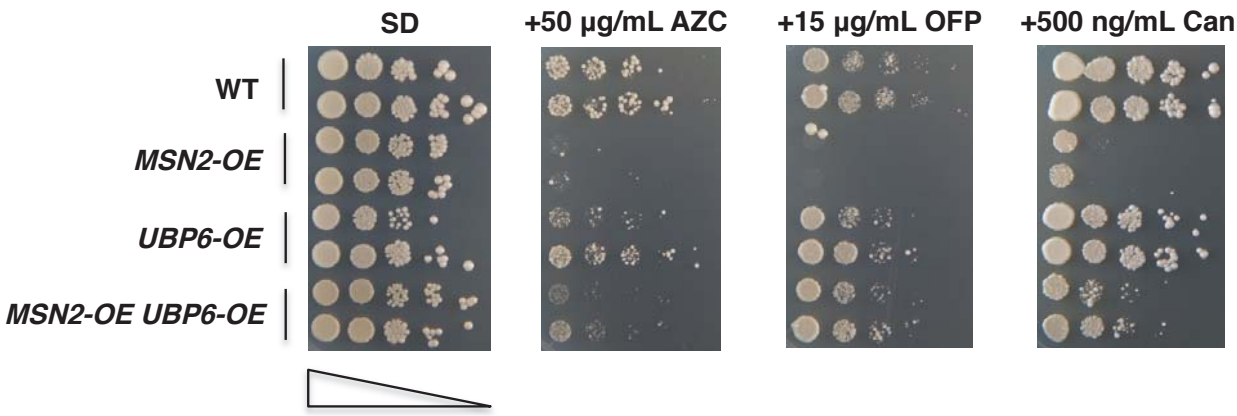

Fig. 25 Growth phenotypes of WT, *MSN2-OE*, *UBP6-OE*, and *MSN2-OE UBP6-OE* cells on media containing amino acid analogues.

The yeast cells were cultivated in minimal SD media until the cells reached the exponential growth phase, a serial dilution of 10^{-1} to 10^{-4} were spotted on the media in the absence (control) and presence of toxic compounds.

3.2.6 Protein level and localization of Gnp1 in *Δubp6* cells

The results described above suggested that the overexpression of *MSN2* (i) impairs the function of DUBs and (ii) inhibits the endocytosis and degradation of Gnp1, leading to a decreased resistance to AZC, through unknown mechanism. To test whether the loss of DUB is required to cause the defect in degradation of Gnp1, I examined the protein level of Gnp1-GFP in *Δubp6* cells. As shown in Fig. 26, *Δubp6* cells exhibited higher abundance of Gnp1-GFP than wild-type cells, just as *MSN2*-overexpressing cells did. The Gnp1-GFP signals also disappeared after 30-min treatment with AZC in *Δubp6* cells, as well as in the wild-type and *MSN2*-overexpressing cells.

Furthermore, fluorescent microscopic observation revealed that most of Gnp1-GFP signals were localized on the plasma membrane in *Δubp6* cells, just as in *MSN2*-overexpressing cells (Fig. 27). This result reinforces my speculation that overexpression of *MSN2* impairs the function of DUBs by unknown mechanism, leading to inhibition of the endocytic degradation of amino acid permeases.

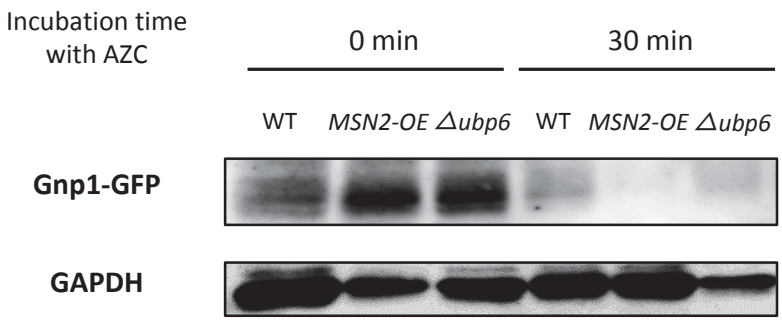

Fig. 26 Gnp1 protein level in wild-type, *MSN2-OE*, and Δ*ubp6* cells.

Cell lysates were extracted from yeast cells grown in SD media harvested prior to and after the addition of 50 μM AZC for 30 min. Gnp1 protein tagged with GFP was detected by Western blotting using anti-GFP antibody, and GAPDH was used as a loading control. Blots are representative from three independent experiments.

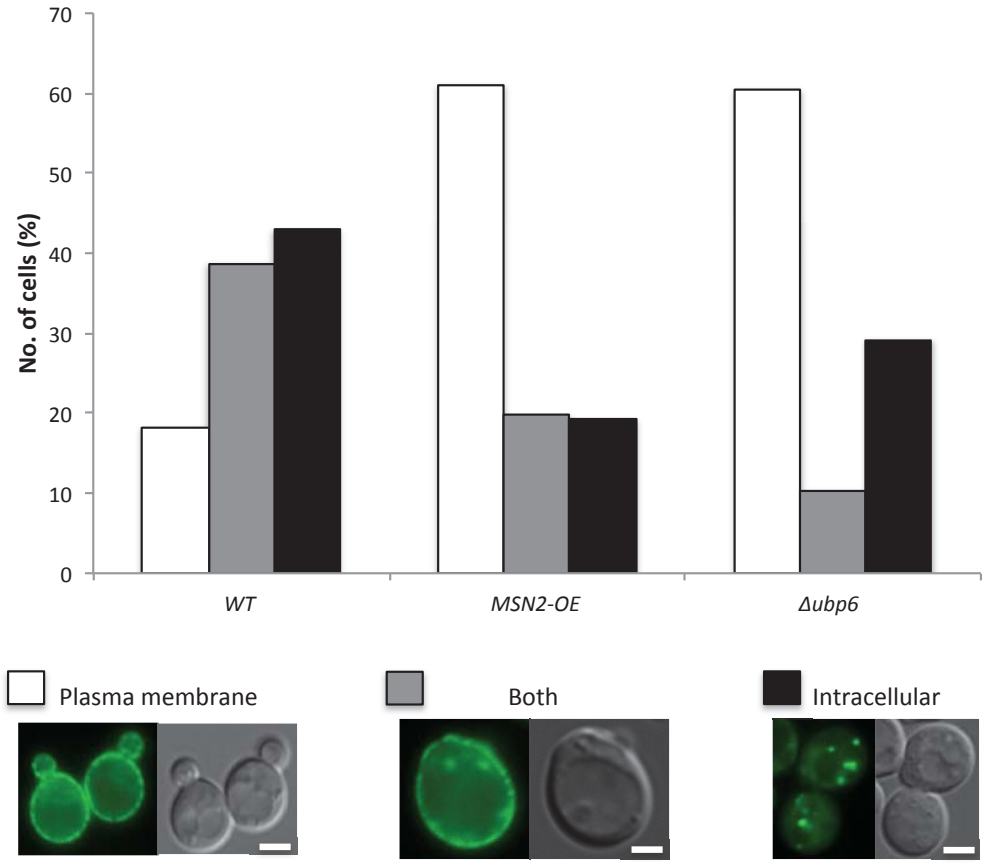

Fig. 27 Localization of Gnp1 in wild-type, *MSN2-OE*, and Δ*ubp6* cells.

Fluorescent microscopic analysis of Gnp1-GFP in BY4741 wild-type (WT), *MSN2*-overexpressing (*MSN2*-OE), and *ubp6*-deleted (*Δubp6*) cells. Observation of more than 100 cells each was repeated three times. Cell morphology was observed through differential interference contrast. Bar: 5 μM.

4.0 DISCUSSION

4.1 Msn2, a pleiotropic player in *S. cerevisiae* stress responses

In the budding yeast *Saccharomyces cerevisiae*, the transcription activator Msn2 and its partially redundant homologue Msn4 govern pleiotropic stress responses at the transcriptional, protein, and metabolic levels. At the same time, the amino acid proline also acts as a cellular protectant against various kinds of stresses. In this study, I focused on the relationship between the functions of Msn2 and proline homeostasis, and found that Msn2 functions in the control of endocytic degradation of a proline permease Gnp1 through the impaired DUB activity (Fig. 28). The data presented shed light on a novel role of Msn2 in the control of ubiquitin homeostasis, which may be responsible for intracellular amino acid homeostasis and for stress responses.

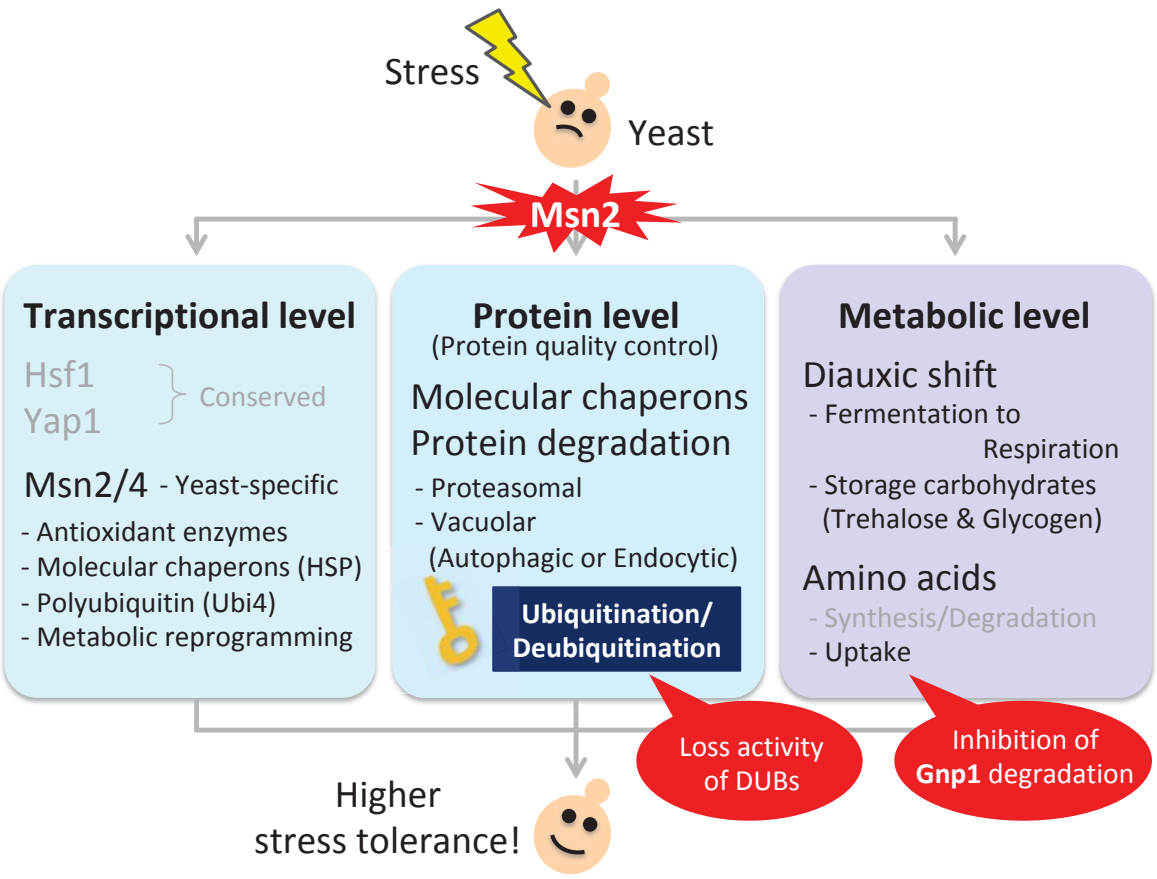

Fig. 28 Schematic diagram depicting the summary of the findings obtained in this study.
Gray letters indicate stress responses that are not directly mediated by Msn2.

4.2 Possible roles of Msn2 in the control of amino acid homeostasis

In this study, I focused on the relationship between stress-induced transcription activator Msn2 and proline homeostasis. The findings suggest that the excess Msn2 inhibits endocytic degradation of the proline permease Gnp1 (Fig. 19). Based on the fact that *MSN2*-OE cells exhibited the decreased resistance not only to AZC but also to OFP and Can (Fig. 10), Msn2 may be involved in the global control of amino acid uptake. Thus, it is speculated that *S. cerevisiae* cells incorporate extracellular amino acids in response to stress to maintain intracellular amino acid homeostasis.

How does the excess Msn2 inhibit endocytic degradation of Gnp1? Ubiquitination is an essential step for the endocytic degradation of plasma membrane-localized transporters (Lauwers *et al.,* 2010). Since the Nedd4-family ubiquitin ligase Rsp5 is involved in the control of Gnp1 localization (Sasaki and Takagi, 2013), the excess Msn2 might negatively regulate Rsp5-mediated protein ubiquitination. As previously reported, the nutrient-sensing target-of-rapamycin complex 1 (TORC1) signaling inhibits the kinase Npr1, which in turn inactivates the adaptor protein for Rsp5 (MacGurn *et al.,* 2011). Considering that Msn2/4 are also under the control of TORC1 signaling (Beck and Hall 1999), the excess Msn2 might induce the expression of *NPR1* or the associated inhibitor genes for Rsp5-dependent protein ubiquitination (Fig. 29). The Msn2-targeted gene responsible for the inhibition of Rsp5 might be the key to understanding the novel amino acid-based stress response in *S. cerevisiae* and other eukaryotic cells.

4.3 Effects of Msn2 on DUB functions

Msn2 plays one of the most important roles in yeast stress responses by activating the transcription of hundreds of required genes. In this study, however, I did not conclude that Msn2 regulates DUB genes transcriptionally. It is still unclear how overexpression of *UBP6* partially alleviates the cytotoxicity of amino acid analogues in *MSN2*-overexpressing cells (Fig. 25), but this observation raises two possibilities. First, overexpression of *MSN2* reduces or impairs the DUBs activity, through Ubp6 which could be supported by my results that numerous ubiquitinated proteins

were detected in *MSN2*-overexpressing cells even in the absence of AZC (Fig. 20) and that both Δ*ubp3* and Δ*otu1* cells as well as Δ*ubp6* cells were sensitive to AZC, OFP, and Can (Fig. 23). Secondly, the excess level of *UBP6* may not be mediated by Msn2; instead, it functions independently to enhance the degradation of misfolded proteins in yeast cells, therefore increasing the cell resistance towards amino acid analogues (Fig. 25).

Msn2 might activate the gene expression for unidentified repressors of the DUB genes (Fig. 29). For instance, Rfu1 (regulator of free ubiquitin chains 1) was identified as a protein which inhibits the activity of the ubiquitin hydrolase Doa4 (Kimura *et al.*, 2009). Assuming that Msn2 induces the expression of repressors of the DUBs in a similar way, overexpression of *MSN2* might cause the repression of the DUBs activity. Msn2 may regulate the Rfu1-like protein(s), leading to a decreased activity or dysfunction of DUBs. Additionally, I should examine whether ubiquitin overexpression could reverse AZC cytotoxicity and resume Gnp1 internalization in *MSN2*-overexpressing cells.

4.4 DUB-mediated control of the endocytic degradation of Gnp1

In this study, loss of Ubp6 led to the inhibition of endocytic degradation of Gnp1 (Figs. 26 and 27). How does Ubp6 affect the endocytosis and membrane trafficking of Gnp1? First, deubiquitination of Gnp1 by Ubp6 might be required for the endocytic degradation of Gnp1. Assuming that Ubp6 is directly involved in the deubiquitination of Gnp1, this result may represent a novel role of Ubp6. Ubp6 is often associated with proteasomal degradation, where it rescues ubiquitin from ubiquitin-protein conjugates before the target is degraded (Elsasser *et al.*, 2002; Leggett *et al.*, 2002; Shi *et al.*, 2016). In contrast, plasma membrane proteins are deubiquitinated by another DUB Doa4, which releases ubiquitin from membrane proteins before they are being internalized within multivesicular bodies en route for vacuolar degradation (Finley *et al.*, 2012). Earlier studies suggested that Doa4 is also associated with proteasomal degradation (Swaminathan *et al.*, 1999; Amerik *et al.*, 2000). The substrate specificity of Ubp6 and Doa4 toward Gnp1 might be the key to understand the molecular mechanism. Second, ubiquitin homeostasis mediated by Ubp6 might be required for

endocytic degradation of Gnp1. The ubiquitin molecules released by Ubp6 are used for ubiquitination of other target proteins. Thus, the ubiquitin depletion caused by defective Ubp6 might lead to impaired ubiquitination of Gnp1, which is required for the endocytic degradation. To discriminate these two models, the ubiquitination status of Gnp1 should be intensively examined under conditions where *UBP6* is deleted or *MSN2* is overexpressed.

Among the four yeast proline permeases reported so far, only Gnp1 predominantly incorporated AZC especially under excessive levels of Msn2 (Fig. 14), suggesting that Gnp1 has higher affinity to AZC than to proline. Since disruption of the other three proline permeases further decreased the AZC tolerance, Gap1, Put4, and Agp1 are also likely to incorporate AZC to a lesser extent. Under physiological conditions, it is speculated that Msn2 commonly mediates the regulation of proline permeases to control proline uptake.

4.5 Contributions to the fermentation industries

Cellular proline homeostasis is mediated by the control of biosynthesis, degradation, and incorporation of proline. The activity of the γ-glutamyl kinase Pro1 in proline biosynthesis is subject to feedback inhibition by proline, and thus, several known variants of Pro1, such as Ile150Thr and Asp154Asn, have been shown to desensitize the proline feedback inhibition and increase the intracellular proline content (Morita *et al.*, 2003; Sekine *et al.*, 2007). Such proline accumulating strains increased the survival rate compared with that of the wild-type strains after exposure to various kinds of stress conditions including freezing, desiccation, oxidative stress, and high ethanol concentrations (Takagi, 2008). Meanwhile, excess Msn2 inhibiting the endocytic degradation of Gnp1 found in this study may contribute to the fermentation industries as well, as yeast cells overexpressing *MSN2* could increase the uptake of proline from the extracellular medium. By combination of these two strategies, overexpression of *MSN2* and *PRO1* mutants might be promising for improving stress tolerance of industrial yeast cells. In addition, co-overexpression of *MSN2* and *UBP6* may increase the cell resistance towards proteotoxic stress caused by the intracellular accumulation of misfolded

proteins.

I propose here a novel role of the global stress-response transcription factor Msn2 in the control of intracellular uptake of amino acids (Fig. 29). Together with the known effects of Msn2 – such as upregulation of antioxidant enzyme genes, protein quality control via molecular chaperones, and reprogramming carbon metabolism – the Msn2-mediated uptake of amino acids is expected to contribute to the global stress responses that allow the unicellular microorganism *S. cerevisiae* to adapt to various environmental changes. The observations in this study may contribute to a deeper understanding of cellular stress-responsive mechanisms, as well as to construction of stress-tolerant yeast strains that would be useful in a broad range of fermentation industries. How i) Msn2 and DUBs control the activity of amino acid uptake and ii) proline and other amino acids protect the cells are challenging but crucial problems to be addressed in future.

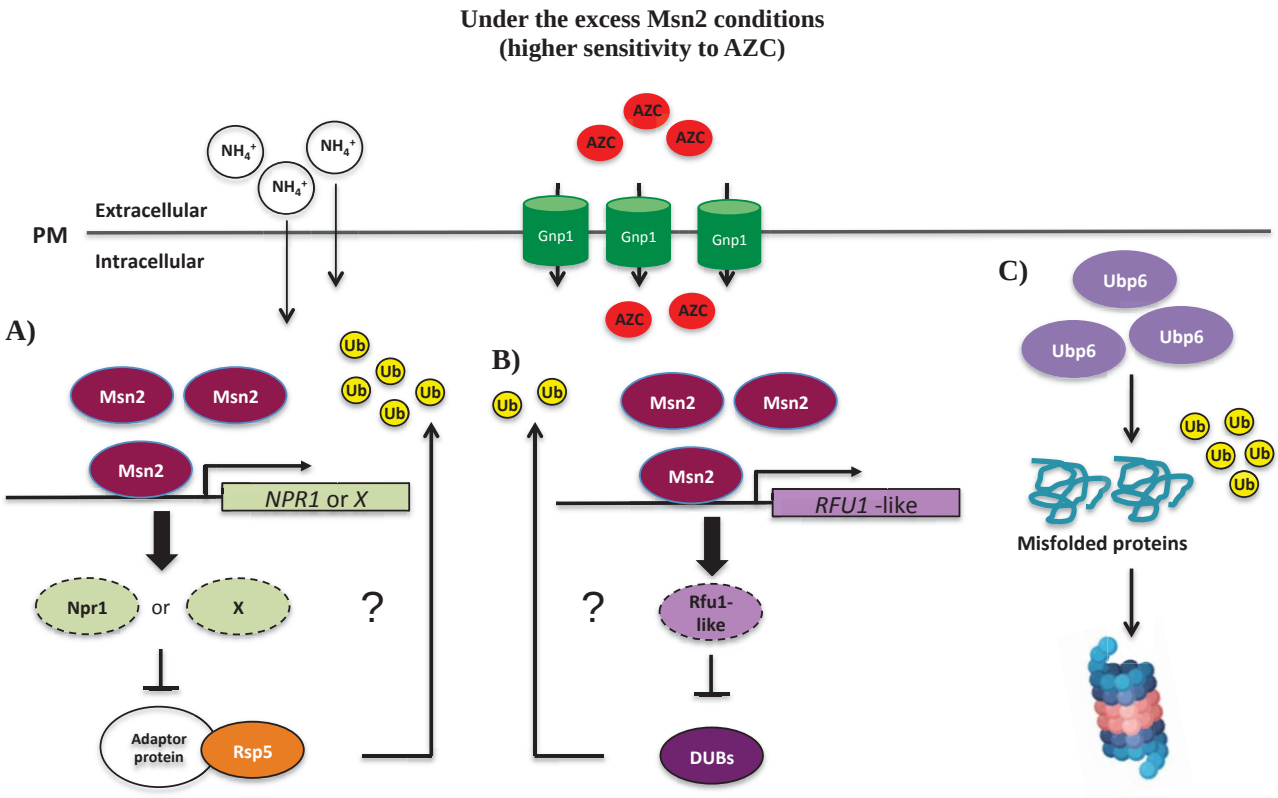

Fig. 29 Proposed model of Msn2-mediated regulation of Gnp1 endocytic degradation.
Excess Msn2 inhibits endocytic degradation of Gnp1 by unknown mechanisms. There are at least three plausible mechanisms discussed in this study. A) Overexpression of *MSN2* might induce the expression of *NPR1* or the associated inhibitor genes for Rsp5-dependent protein ubiquitination, therefore Gnp1 could not be ubiquitinated and endocytosed. B) Secondly, high level of Msn2 may activate the gene expression of DUB repressors such as Rfu1-like protein(s), causing a reduced or loss of function of DUBs. C) In the yeast cells overexpressing *UBP6*, excess Ubp6 may enhance the deubiquitination and accelerate the degradation of misfolded proteins by unknown mechanisms, for example, the proteasome-mediated mechanism in the *MSN2*-independent manner, leading to tolerance towards proteotoxic stress caused by the intracelullar accumulation of misfolded proteins.

5.0 ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious, The Most Merciful.

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7.0 LIST OF PUBLICATIONS

Noreen Suliani binti Mat Nanyan, Daisuke Watanabe, Yukiko Sugimoto, Hiroshi Takagi (2019). Involvement of the stress-responsive transcription factor gene *MSN2* in the control of amino acid uptake in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, **19**, foz052. doi:10.1093/femsyr/foz052

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