Importance of Proteasome Gene Expression during Model Dough Fermentation after Freezing Preservation of Baker's Yeast Cells

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ABSTRACT

Freeze-thaw stress causes various types of cellular damages, survival and/or proliferation defects, and metabolic alterations. However, the mechanisms underlying how cells cope with freeze-thaw stress are poorly understood. Here, model dough fermentations using two baker's yeast strains, 45 and YF, of *Saccharomyces cerevisiae* were compared after 2-week cell preservation in a refrigerator or freezer. YF exhibited slow fermentation after exposure to freeze-thaw stress due to the low cell viability. A DNA microarray analysis of the YF cells during fermentation revealed that the genes involved in oxidative phosphorylation were relatively strongly expressed, suggesting a decrease in the glycolytic capacity. Furthermore, we found that mRNA levels of the genes that encode the components of the proteasome complex were commonly low, and ubiquitinated proteins were accumulated by freeze-thaw stress in the YF strain. In the cells with a laboratory-strain background, treatment with the proteasome inhibitor MG132 or the deletion of each transcriptional activator gene for the proteasome genes (*RPN4*, *PDR1*, or *PDR3*) led to marked impairment of model dough fermentation using the frozen cells. Based on these data, proteasomal degradation of freeze-thaw damaged proteins may guarantee high cell viability and fermentation performance. We also found that the freeze-thaw stress-sensitive YF strain was heterozygous at the *PDR3* locus, and one of the alleles (A148T/A229V/H336R/L541P) was shown to possess a dominant-negative phenotype of slow fermentation. Removal of such responsible mutations could improve the freeze-thaw stress tolerance and the fermentation performance of baker's yeast strains, as well as other industrial *S. cerevisiae* strains.

IMPORTANCE

The development of freezing technology has enabled the long-term preservation and long-distance transport of foods and other agricultural products. Fresh yeast, however, is usually not frozen because the fermentation performance and/or the viability of individual cells are severely affected after thawing. Here, we demonstrate that proteasomal degradation of ubiquitinated proteins is an essential process in freeze-thaw stress responses of *S. cerevisiae*. Upstream transcriptional activator genes for the proteasome components are responsible for the fermentation performance after freezing preservation. Thus, this study provides a potential linkage between freeze-thaw stress inputs and the transcriptional regulatory network that might be functionally conserved in higher eukaryotes. Elucidation of the molecular targets of freeze-thaw stress will contribute to advances in cryobiology, such as freezing preservation of human cells, tissues, and embryos for medical purposes and breeding of industrial microorganisms and agricultural crops that highly adapt to low temperatures.

KEYWORDS

baker’s yeast, dough fermentation, freeze-thaw stress, proteasome, *Saccharomyces cerevisiae*

INTRODUCTION

Freezing preservation is one of the most widely used methods to maintain the quality and freshness of various raw materials, ingredients, in-process foods, and end products over long periods. During the freezing process, delay in chemical and metabolic reactions prevents the loss of the original flavor, texture, and nutritional values. Microbial food spoilage is also effectively blocked under frozen conditions. Nevertheless, intracellular and extracellular ice crystal formation and the subsequent dehydration damage cellular structures and affect the frozen product's quality. Thawing is another critical phase, since frozen foods are subjected to damage by chemical and physical changes (1, 2). Fresh baker's yeast (e.g. liquid yeast and compressed yeast), which is the gas-forming ingredient in bakery products, is generally stored under refrigeration above the freezing point in order to retain the high gassing power (3–5). The breeding of baker's yeast strains that are suitable for freezing preservation may greatly improve the microbiological stability of fresh yeast.

Cell injury and adaptation under freeze-thaw stress in *Saccharomyces cerevisiae*, to which most baker's yeast strains belong, have been intensively studied over recent decades (6–8). Freeze-thawing directly or indirectly damages macromolecules including cell membranes and proteins, and leads to the generation of reactive oxygen species, as many other stresses do. Thus, the functions of heat shock proteins and antioxidant enzymes are essential for high freezing tolerance. To decrease intracellular ice crystal formation, water efflux through the aquaporins and the intracellular accumulation of trehalose and glycerol are important as well. Proline shows cryoprotective activity that is similar to those of trehalose and glycerol (9–11), although proline is not physiologically

accumulated in *S. cerevisiae* cells by freeze-thaw stress. While various protective factors and mechanisms have been analyzed as noted above, it is not yet known how *S. cerevisiae* cells effectively repair the damage by freeze-thawing, and such knowledge would make a significant contribution to the baking industry.

The ubiquitin-proteasome system is one of the major proteolytic machineries that have pivotal functions in the stress responses of eukaryotic cells (12). Proteins irreversibly damaged by stresses cannot be repaired, and they must therefore be removed by the proteasome complex after being labeled with polyubiquitin chains. The proteasome activity is at least partly controlled at the transcriptional level by a transcriptional activator Rpn4p (12, 13). Rpn4p upregulates the proteasome genes with the consensus proteasome-associated control element (PACE; 5'-GGTGGCAAA-3') sequence in the promoter region, and the proteasome degrades Rpn4p itself. This negative feedback loop (14) maintains the homeostatic expression level of the proteasome complex. However, under stress conditions in which polyubiquitinated proteins may accumulate, the *RPN4* gene expression is induced by the stress-responsive transcriptional activators Pdr1p and Pdr3p (Pdr1/3p), Yap1p, and Hsf1p (15–17). According to the evidence obtained to date, Rpn4p is required for the cellular resistance to heat shock, oxidative stress, DNA damage, proteotoxic stress, heavy metals, and other drugs, as well as for the extension of replicative lifespan (16–18). The significance of the Rpn4p-mediated proteasome gene expression under freeze-thaw stress has not yet been elucidated.

In this study, we investigated the effects of the freezing preservation of fresh yeast on model dough fermentation and the gene expression profile. Our results shed

further light on the relationship between freeze-thaw stress and the proteasomal functions in *S. cerevisiae*, which may facilitate the design of novel high-performance yeast products.

RESULTS

Effects of freezing preservation on model dough fermentation using baker's yeast strains. First, we compared the fermentation performance of two baker's yeast strains 45 and YF, which may share a common ancestor and have been used at TableMark Co., Ltd. Yeast pellets of each strain were used for the fermentation tests in a liquid medium after being refrigerated or frozen for 2 weeks. As shown in Fig. 1A, freezing did not have clear effects on the fermentation progression in 45, and the total carbon dioxide emission during 90-min fermentation using frozen pellets was 93.8% of that using refrigerated pellets. In contrast to 45, we observed slow fermentation using frozen pellets of YF (Fig. 1B). The freezing preservation of YF decreased the total carbon dioxide emission during 90-min fermentation to 85.8% of the level in the refrigerated sample. It should be noted that the negative effect of freezing in YF was observed from the initial stage (within 5 min after inoculation) of model dough fermentation. The total carbon dioxide emissions of both strains were almost the same after refrigeration (only 3.5% higher in YF on average). Consistently, the cell viability after 2-week freezing was significantly lower in YF than in 45, while refrigeration did not affect the cell viability (Fig. 1C). Based on these results, we concluded that YF is more susceptible to freezing preservation than 45.

Effects of freezing preservation on gene expression profiles during model

dough fermentation using baker's yeast strains. To elucidate why YF decreased cell viability and exhibited slow fermentation after freezing preservation, we analyzed the transcriptomic profiles of both strains after refrigeration and after freezing. Total RNA samples were taken from the yeast cells 30 min after the onset of fermentation, and used for a DNA microarray experiment. The differentially expressed genes between the frozen and refrigerated samples (Table S1) were subjected to a gene-ontology (GO) analysis using the GO Term Finder in the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl).

In strain 45, all of the enriched GO-term categories showed *p* values higher than 1×10^{-10}. In strain YF (Table 1), the genes that belong to five GO-term categories associated with oxidative phosphorylation and aerobic respiration were strongly expressed after freeze-thaw stress. This may reflect the impaired glycolysis and alcoholic fermentation in this strain (19). By contrast, three GO-term categories associated with proteasomal proteolysis were identified in YF as weakly expressed after freezing. As shown in Fig. 2, the expression levels of all known 33 proteasome genes, which encode 9 lid and 10 base subunits in the 19S regulatory particle and 7 α-ring and 7 β-ring subunits of the 20S catalytic core particle (12), were lower after freezing than after refrigerating in both strains. YF exhibited more prominent differences than 45 without any exception. The averaged relative expression level of all proteasome genes in YF was significantly lower than that in 45 (strain 45, 68.3 ± 9.8%; strain YF, 49.5 ± 9.6%; *t* test, $p = 2.8 \times 10^{-11}$).

These results suggested that the proteasome gene expression is more severely disturbed in YF cells during fermentation after freezing preservation. We also observed

147 that the *RPN4* gene itself, which encodes a transcription activator for the proteasome
148 genes, was apparently weakly expressed in YF (strain 45, 78.6%; strain YF, 45.3%) (Fig.
149 2). We thus speculated that the upstream transcription factors for *RPN4* (Pdr1/3p, Hsf1p,
150 and Yap1p) (15–17) are less activated in YF during fermentation after freeze-thaw stress.
151 Pdr1/3p may be likely to be affected, since the different target genes of Pdr1/3p, such as
152 the plasma membrane ATP-binding cassette (ABC) multidrug transporter genes *PDR5*
153 and *SNQ2* (20), exhibited lower mRNA levels after freezing preservation in YF, as did the
154 proteasome genes and *RPN4* (Fig. 2). Known target genes of Hsf1p or Yap1p did not
155 necessarily show similar defects (data not shown). Alternatively, degradation of *RPN4*
156 mRNA might also be enhanced in YF, which should be investigated in future.

157 **Evaluation of the proteasomal function using baker's yeast strains.** To
158 address the activities of proteasomal proteolysis, we analyzed the stress-induced
159 accumulation of ubiquitinated proteins in the baker's yeast strains. YF exhibited larger
160 amounts of ubiquitinated proteins than 45 upon heat shock, a representative stress that
161 induces protein ubiquitination (Fig. 3A), and also immediately after 2-week freezing
162 preservation (Fig. 3B). In the liquid medium for model dough fermentation, ubiquitinated
163 proteins rapidly disappeared in both strains. These results suggested that the proteasomal
164 degradation of ubiquitinated proteins under heat shock or freeze-thaw stress is impaired
165 in YF. The slow growth of YF in the presence of 75 μM MG132 (a proteasome inhibitor)
166 supported this idea (Fig. 3C).

167 It should be noted that the slow-growth phenotype was observed when the
168 MG132 concentration of the stock solution in dimethyl sulfoxide (DMSO) was 1 mM,
169 but not when it was 10 mM (data not shown). This raised the possibility that the high

concentration of DMSO in the agar plate might solely cause the slow-growth phenotype. DMSO alone did not affect the cell growth of either strain, however. Thus, the growth inhibition of YF was presumably caused by a synergistic effect of MG132 and DMSO.

Effects of the proteasomal function on model dough fermentation. We next performed model dough fermentation tests using a laboratory strain BY4741 and its gene disruptants (*rpn4*Δ, *pdr1*Δ, and *pdr3*Δ) to clarify the role of the proteasomal function and the proteasome gene expression in the acquisition of the freeze-thaw stress tolerance. When 50 μM MG132 was added to the liquid media, the fermentation performance after freezing preservation was markedly decreased (Fig. 4A). The total carbon dioxide emission during 90-min fermentation was dropped to 63.1% by the MG132 treatment.

Loss of Rpn4p or Pdr1/3p, each of which upregulates the proteasome gene expression, also impaired fermentation after freezing preservation (Fig. 4B–D). The total carbon dioxide emission during 90-min fermentation in these gene disruptants was 70–80% of the level in the wild-type strain. Accordingly, the proteasomal function and/or the proteasome gene expression through Rpn4p and Pdr1/3p are likely to be important to maintain high fermentation performance after freezing preservation.

Mutations involved in the freeze-thaw stress sensitivity of a baker's yeast strain. To identify the cause of the low freeze-thaw stress tolerance observed in YF, we performed a whole genome resequencing analysis. Nine nonsynonymous single-nucleotide polymorphisms (SNPs) were identified in the *RPN4*, *PDR1*, and *PDR3* genes of strain YF (Fig. 5A) in comparison to the S288C reference genome. We identified five of these SNPs, i.e., homozygous C221T and A1279G in *RPN4*, heterozygous C53G and A280G in *PDR1*, and heterozygous A167G in *PDR3* (corresponding to amino acid

substitutions, S74L and T427A in Rpn4p, T18R and T94A in Pdr1p, and Q56R in Pdr3p, respectively) in several other *S. cerevisiae* strains, according to SGD Align Strain Sequences (http://www.yeastgenome.org/cgi-bin/FUNGI/alignment.pl).

However, the other four heterozygous mutations, i.e., G442A, C686T, A1007G, and A1623T in *PDR3* (corresponding to amino acid substitutions, A148T, A229V, H336R, and L541P of Pdr3p, respectively), were unique to YF in our search of this database. Direct DNA sequencing of the PCR product amplified using a *PDR3* gene-specific primer pair revealed that these four nucleotide polymorphisms were located on the identical allele, suggesting a high nonsynonymous mutation speed of this unique allele. In the reciprocal hemizygosity analysis of YF shown in Fig. 5B, deletion of the $PDR3^{Q56R}$ allele did not affect the growth (see $pdr3^{WT}\Delta/PDR3^{DN}$), whereas deletion of the $PDR3^{A148T/A229V/H336R/L541P}$ allele increased the tolerance to MG132 (see $PDR3^{WT}/pdr3^{DN}\Delta$).

As our findings indicated that this uniquely mutated allele has a dominant-negative (DN) effect on the proteasomal function, it is referred to as $PDR3^{DN}$ in this study. In addition, the $PDR3^{WT}/pdr3^{DN}\Delta$ cells exhibited slightly faster fermentation after freezing preservation compared to the $pdr3^{WT}\Delta/PDR3^{DN}$ cells (Fig. 5C). The total carbon dioxide emission during 90-min fermentation using the $pdr3^{WT}\Delta/PDR3^{DN}$ strain was 6.4% lower than the level in the $PDR3^{WT}/pdr3^{DN}\Delta$ strain. The cell viability after 2-week freezing was significantly lower in $pdr3^{WT}\Delta/PDR3^{DN}$ than in $PDR3^{WT}/pdr3^{DN}\Delta$ (Fig. 5D). Based on these results, it was confirmed that the $PDR3^{DN}$ allele is at least partly associated with the slow fermentation progression and the low cell viability of YF cells after exposure to freeze-thaw stress.

DISCUSSION

The control of proteasome gene expression in eukaryotic cells is complex. Although it has been speculated that cells effectively degrade the proteins damaged by freezing and/or thawing for adaptation, the significance of the proteasome gene expression in freeze-thaw stress responses is not fully understood. In the yeast *S. cerevisiae*, the C_2H_2-type zinc-finger transcriptional activator Rpn4p is required for the basal expression of the proteasome genes with a consensus PACE sequence (12, 13). Rpn4p is highly susceptible to proteasomal proteolysis as a substrate, and it thus regulates the proteasome gene expression in a negative-feedback manner. The basis of proteasome homeostasis is probably conserved in mammalian cells; the cap'n'collar (CNC)-family bZIP transcription factor Nrf1 upregulates all proteasome subunits and mediates the recovery from inhibition of the proteasome (21–23), as yeast Rpn4p does. Thus, Rpn4p research may contribute to the elucidation of the mechanism of the human diseases that are caused by a defective Nrf1-mediated expression of the proteasome genes, such as neurodegenerative disorders.

In our study, freezing preservation led to the low expression levels of the proteasome genes in two different strains of baker's yeast, and the extent of the decrease was correlated with the defects in fermentation performance after exposure to freeze-thaw stress. This finding suggests that the proteasome gene expression is one of the major targets damaged by freeze-thaw stress, highlighting an important role of the inductive pathway for the proteasome genes for cellular adaptation. Although we first focused on the *RPN4* gene, the amino acid substitutions S74L and T427A identified in the

freezing-sensitive strain did not significantly affect the tolerance to MG132 (data not shown).

The *RPN4* gene is controlled not only by the proteasome activity but also at the transcriptional level by different transcription factors, Pdr1/3p, Yap1p, and Hsf1p (15–17). Homologous Pdr1/3p govern the gene expression of the multidrug efflux pumps for pleiotropic drug resistance, as well as that of *RPN4* (24). Although Pdr1p plays a predominant role as a master regulator in most situations, the backup and extra functions of Pdr3p are also important. The expression of *PDR3* (not *PDR1*) can be strongly induced through positive autoregulation by Pdr1/3p (25) when the activity of Pdr1p alone is deficient or insufficient. Moreover, Pdr3p and two other transcription factors (Yap1p and Hsf1p) for oxidative stress tolerance and heat shock response, respectively, comprise a transcriptional positive regulatory network to upregulate the genes that encode Rpn4p and themselves each other (16, 26). Pdr3p participates in other transcriptional processes that do not involve Pdr1p, such as retrograde response signaling and DNA damage responses (27, 28). In our present investigation, deletion of *PDR1* or *PDR3* impaired model dough fermentation after freezing preservation to the same extent as disruption of the *RPN4* gene, suggesting the importance of individual transcription factors in freeze-thaw stress responses (Fig. 6).

Although the *PDR3* mutation responsible for the dominant-negative phenotype of the freezing-sensitive baker's yeast strain has not yet been determined, two amino acid substitutions (i.e., A229V and H336R) in the conserved inhibitory domain (29) might be candidates for inhibition of the transcriptional induction activity. Alternatively, the responsible mutations might cause dominant-negative inhibition by affecting the

formation of Pdr3p homodimer and Pdr1/3p heterodimers (30), which may control the DNA binding activity.

In the proteasome gene induction pathway mediated by Rpn4p and Pdr1/3p (Fig. 6), a question remains regarding freeze-thaw stress sensing. It was recently reported that the master regulator Pdr1p is activated by the molecular chaperones Ssz1p and Zuo1p (31), both of which are members of the conserved ribosome-associated complex (RAC) (32, 33). RAC assists the biogenesis of newly synthesized polypeptide chains at ribosomes, and contributes not only to nascent protein folding but also to pleiotropic drug resistance and cold tolerance (33, 34). Thus, RAC might directly sense the nascent proteins damaged by freeze-thawing stress to upregulate the proteasome gene expression through Pdr1/3p and Rpn4p. This hypothesis should be addressed in future research.

Our present findings can be applied to the breeding of industrial strains that are optimal for the freezing preservation of fresh yeast products. Intriguingly, strain YF in this study was actually developed for superior fermentation performance in frozen doughs (data not shown), although it showed higher sensitivity to freezing preservation. Based on this finding, we should keep in mind that the stresses to be overcome during the freezing preservation of fresh yeast and during frozen dough fermentation are unlikely to be completely identical. It should be also noted that deletion of the *RPN4* gene impaired model dough fermentation after freezing preservation (Fig. 4B), but led to slightly faster fermentation after refrigerating preservation compared to the wild-type strain (Fig. S1).

Although fermentation performance of the *rpn4*Δ strain in frozen doughs was not tested in this study, we speculate that the Pdr1/3p-Rpn4p pathway might be crucial for the adaptation to freezing preservation, but not necessarily for dough fermentation. This is

consistent with our previous finding that unnecessary stress-response mechanisms should be eliminated for high-efficient fermentation (35, 36). Therefore, YF might have been selected as a strain that exhibits superior fermentation even after doughs are frozen, but the freeze-thaw tolerance as fresh yeast products of this strain is not high. If only the freezing preservation is considered, the resistance to the proteasome inhibitor MG132 in the presence of DMSO correlates with the freeze-thaw stress tolerance (Figs. 3C, 5B). The known cellular effects of DMSO on oxidative stress responses and on carbohydrate/nitrogen/lipid metabolism (37, 38) may be related to the proteasome gene expression via unknown mechanisms. Our findings thus provide clues for the development of a novel method for the breeding of freezing-tolerant industrial strains without the use of recombinant DNA technology.

MATERIALS AND METHODS

Yeast strains. Two industrial baker's yeast strains (45 and YF) used in TableMark Co., Ltd. were used in this study. 45 is a traditional yeast strain previously used in TableMark Co., Ltd., but has never been published elsewhere. YF was also developed and patented at TableMark Co., Ltd. YF has been constructed for decades through chemical mutagenesis and crossbreeding, and may partly share a common ancestor to 45, although the detailed breeding information is lost. Whole genome resequencing of YF in this study may clarify the precise origin of this strain in future. Since their fermentation abilities in frozen dough have been compared for a long time in TableMark Co., Ltd., we chose these two strains to be used in this study.

The *S. cerevisiae* laboratory strain BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0*

ura3Δ0) and its single-deletion mutants were provided by Euroscarf (Germany). Disruption of one *PDR3* allele in diploid YF was performed using a PCR-based method (39) with oligonucleotide primers PDR3-DF (5'-CGA CAA CTG CAT CAG CAG TTT TAT TAA TTT TTT CTT ATT GCG TGA CCG CAC GGA TCC CCG GGT TAA TTA A-3') and PDR3-DR (5'-GTG TCC CAT TTA CTA TGG TTA TGC TCT GCT TCC CTA TTT TCT TTG CGT TTG AAT TCG AGC TCG TTT AAA C-3') and pFA6a-kanMX4 (39) as the template. Correct disruption of the $PDR3^{WT}$ or $PDR3^{DN}$ allele was confirmed by genomic PCR and direct DNA sequencing of the PCR product. Yeast cells were routinely grown in liquid YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 30°C, unless otherwise stated.

Yeast preservation and model dough fermentation tests. For measurements of fermentation progression in model dough, yeast cells were precultured in YPD medium at 30°C and washed with distilled water. Wet yeast pellets were stored in the refrigerator (4°C) or in the freezer (-20°C) for two weeks. Approximately 0.4 g of yeast cells were inoculated into 30 mL of modified ASF medium (40), which consists of 23 g of sucrose, 2 g of urea, 1 g of ammonium sulfate, 0.8 g of magnesium sulfate, 1.6 mg of thiamine hydrochloride, 1.6 mg of pyridoxine hydrochloride, 16 mg of nicotinic acid in total 350 mL of 67 mM potassium phosphate buffer (pH 5.6), and incubated at 30°C. When the laboratory strains were used, 70 mg of histidine, 70 mg of methionine, 70 mg of uracil, and 350 mg of leucine were also included in 350 mL of modified ASF medium to complement the auxotrophy. The course of the fermentation was continuously monitored by measuring the volume of evolved carbon dioxide gas using a Fermograph II apparatus (Atto). In preliminary experiments, we observed that two-week refrigerating preservation

did not affect the fermentation rates in both strains.

A DNA microarray analysis of yeast cells during model dough fermentation. Cells were sampled 30 min after inoculation into modified ASF medium. Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen). Cy3-labeled cRNA was prepared from 50 ng of total RNA using the Low Input Quick Amp Labeling Kit (Agilent) according to the manufacturer's instructions, followed by purification with the RNeasy Mini Kit (Qiagen). By using the Gene Expression Hybridization Kit (Agilent), Cy3-labelled cRNA were fragmented at 60°C for 30 min in a reaction volume of 24 μL containing 1 × Agilent fragmentation buffer and 2 × Agilent blocking agent. After the fragmentation reaction, 25 μL of 2 × Agilent hybridization buffer were added to the sample, and cRNA was hybridized to the Agilent Technologies Yeast (v2) Gene Expression 8×15K Microarray for 17 h at 65°C in a rotating Agilent hybridization oven. After hybridization, the microarray was washed for 1 min at room temperature with Wash Buffer 1 and for 1 min at 37°C with Wash Buffer 2 of the Gene Expression Wash Buffer (Agilent), air-dried immediately, and then scanned on the Agilent DNA Microarray Scanner (Scan resolution 5 μm, TIFF file dynamic change 20 bit). The scanned images were analyzed with Feature Extraction Software 10.7.3.1 (Agilent), and the data were imported to the GeneSpring software (Agilent) and normalized by the 75-percentile shift. The microarray data were deposited to National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through the accession number GSE101071. We performed GO analysis of the genes that were differentially expressed more than two-fold between the refrigerated and frozen samples (listed in Table S1), using SGD GO Term Finder

(http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl).

Western blot analysis. For the detection of ubiquitinated proteins upon heat shock, log-phase yeast cells grown in the YPD medium at 30°C were shifted to 42°C for 15 min. For the detection of ubiquitinated proteins after freezing preservation, wet yeast pellets were stored in the freezer (-20°C) for two weeks and used for model dough fermentation as described above. Approximately, 10^8 cells were collected, suspended in 10% trichloroacetic acid, and disrupted with glass beads in a Multibead Shocker (Yasui Kikai). Proteins in the whole cell extracts were separated by SDS-PAGE (7.5% polyacrylamide), transferred to a polyvinylidene difluoride membrane, blocked with 3% powdered milk in Tris-buffered saline with Tween 20 (TBS-T) at 4°C overnight, and reacted with an anti-ubiquitin mouse antibody (P4D1; Santa Cruz Biotechnology) in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo) at 1:2,000 dilutions for 60 min. As a protein-loading control, an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit antibody (Nordic Immunological Laboratories) or an anti-Pgk1p mouse antibody (Invitrogen) in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo) at 1:10,000 dilutions was used as a primary antibody for 60 min. After several washing steps with TBS-T, the membrane was incubated for 40 min with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo) at 1:2,000 dilutions as a secondary antibody. After several washing steps with TBS-T, the target proteins were visualized by the Pierce ECL Plus Western Blotting Substrate (Thermo Scientific) and detected using the Fuji LAS4000 imager (GE Healthcare).

Whole genome resequencing analysis. The whole genome of strain YF was

analyzed by pair-end sequencing using the Illumina HiSeq 2500 sequencing system (Illumina) at Hokkaido System Science Co., Ltd. (Japan). This sequencing run yielded 45,740,770 high-quality filtered reads with 100-bp pair-end sequencing, providing approximately 308-fold genome coverage in average. The reads were mapped to the reference *S. cerevisiae* S288C genome using BWA, indexed with SAMtools, and realigned with GATK (see reference 41 for SAMtools and related tools). Duplicated read pairs were removed, and remaining reads were realigned with GATK again. The final BAM file was deposited to NCBI BioProject and is accessible through the accession number PRJNA392967. Based on this BAM file, variant calling was performed using SAMtools and BCFtools, before variant annotation and filtering were performed using SnpEff

Accession number(s). DNA microarray data of 45 and YF cells during model dough fermentation after two-week refrigeration or freezing can be accessed under GEO accession number GSE101071. Sequencing reads for the whole genome of YF can be accessed under BioProject accession number PRJNA392967.

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We declare no conflicts of interest.

REFERENCES

1. George RM. 1993. Freezing processes used in the food industry. Trends Food Sci Technol 4:134–138.
2. Li B, Sun DW. 2002. Novel methods for rapid freezing and thawing of foods – a review. J Food Eng 54:175–182.
3. Hatano S, Udou M, Koga N, Honjoh K, Miyamoto T. 1996. Impairment of the glycolytic system and actin in baker's yeast during frozen storage. Biosci Biotechnol Biochem 60:61–64.
4. Ribotta PD, León AE, Añón MC. 2003. Effects of yeast freezing in frozen dough. Cereal Chem 80:454–458.
5. O'Brien SS, Lindsay D, Von Holy A. 2008. Microbiological shelf-life studies on commercially manufactured yeast. J Food Quality 31:627–644.
6. Attfield PV. 1997. Stress tolerance: the key to effective strains of industrial baker's yeast. Nat Biotechnol 15:1351–1357.
7. Shima J, Takagi H. 2009. Stress-tolerance of baker's-yeast (*Saccharomyces cerevisiae*) cells: stress-protective molecules and genes involved in stress tolerance. Biotechnol Appl Biochem 53: 155–164.
8. Randez-Gil F, Córcoles-Sáez I, Prieto JA. 2013. Genetic and phenotypic characteristics of baker's yeast: relevance to baking. Annu Rev Food Sci Technol 4:191–214.
9. Takagi H, Iwamoto F, Nakamori S. 1997. Isolation of freeze-tolerant laboratory strains of *Saccharomyces cerevisiae* from proline-analogue-resistant mutants. Appl Microbiol Biotechnol 47:405–411.

10. Takagi H. 2008. Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. Appl Microbiol Biotechnol 81:211–223.
11. Tsolmonbaatar A, Hashida K, Sugimoto Y, Watanabe D, Furukawa S, Takagi H. 2016. Isolation of baker's yeast mutants with proline accumulation that showed enhanced tolerance to baking-associated stresses. Int J Food Microbiol. 238:233–240.
12. Finley D, Ulrich HD, Sommer T, Kaiser P. 2012. The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. Genetics 192:319–360.
13. Dohmen RJ, Willers I, Marques AJ. 2007. Biting the hand that feeds: Rpn4-dependent feedback regulation of proteasome function. Biochim Biophys Acta 1773:1599–1604.
14. Xie Y, Varshavsky A. 2001. RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc Natl Acad Sci U S A 98:3056–3061.
15. Owsianik G, Balzi E, Ghislain M. 2002. Control of 26S proteasome expression by transcription factors regulating multidrug resistance in *Saccharomyces cerevisiae*. Mol Microbiol 43:1295–1308.
16. Hahn JS, Neef DW, Thiele DJ. 2006. A stress regulatory network for co-ordinated activation of proteasome expression mediated by yeast heat shock transcription factor. Mol Microbiol 60:240–251.
17. Wang X, Xu H, Ju D, Xie Y. 2008. Disruption of Rpn4-induced proteasome expression in *Saccharomyces cerevisiae* reduces cell viability under stressed conditions. Genetics 180:1945–1953.

445 18. Kruegel U, Robison B, Dange T, Kahlert G, Delaney JR, Kotireddy S, Tsuchiya M,
446 Tsuchiyama S, Murakami CJ, Schleit J, Sutphin G, Carr D, Tar K, Dittmar G,
447 Kaeberlein M, Kennedy BK, Schmidt M. 2011. Elevated proteasome capacity
448 extends replicative lifespan in *Saccharomyces cerevisiae*. PLoS Genet 7:e1002253.
449 19. Rolland F, Winderickx J, Thevelein JM. 2002. Glucose-sensing and -signalling
450 mechanisms in yeast. FEMS Yeast Res 2:183–201.
451 20. Decottignies A, Lambert L, Catty P, Degand H, Epping EA, Moye-Rowley WS, Balzi
452 E, Goffeau A. 1995. Identification and characterization of SNQ2, a new multidrug
453 ATP binding cassette transporter of the yeast plasma membrane. J Biol Chem
454 270:18150–18157.
455 21. Radhakrishnan SK, Lee CS, Young P, Beskow A, Chan JY, Deshaies RJ. 2010.
456 Transcription factor Nrf1 mediates the proteasome recovery pathway after
457 proteasome inhibition in mammalian cells. Mol Cell 38:17–28.
458 22. Sha Z, Goldberg AL. 2014. Proteasome-mediated processing of Nrf1 is essential for
459 coordinate induction of all proteasome subunits and p97. Curr Biol 24:1573–1583.
460 23. Bugno M, Daniel M, Chepelev NL, Willmore WG. 2015. Changing gears in Nrf1
461 research, from mechanisms of regulation to its role in disease and prevention.
462 Biochim Biophys Acta 1849:1260–1276.
463 24. Moye-Rowley WS. 2003. Transcriptional control of multidrug resistance in the yeast
464 Saccharomyces. Prog Nucleic Acid Res Mol Biol 73:251–279.
465 25. Delahodde A, Delaveau T, Jacq C. 1995. Positive autoregulation of the yeast
466 transcription factor Pdr3p, which is involved in control of drug resistance. Mol Cell
467 Biol 15:4043–4051.

26. Ma M, Liu ZL. 2010. Comparative transcriptome profiling analyses during the lag phase uncover *YAP1*, *PDR1*, *PDR3*, *RPN4*, and *HSF1* as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. BMC Genomics 11:660.
27. Devaux F, Carvajal E, Moye-Rowley S, Jacq C. 2002. Genome-wide studies on the nuclear PDR3-controlled response to mitochondrial dysfunction in yeast. FEBS Lett 515:25–28.
28. Zhu Y, Xiao W. 2004. Pdr3 is required for DNA damage induction of *MAG1* and *DDI1* via a bi-directional promoter element. Nucleic Acids Res 32:5066–5075.
29. Vermitsky JP, Edlind TD. 1994. Azole resistance in *Candida glabrata*: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. Antimicrob Agents Chemother 48:3773–3781.
30. Mamnun YM, Pandjaitan R, Mahé Y, Delahodde A, Kuchler K. 2002. The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers *in vivo*. Mol Microbiol 46:1429–1440.
31. Prunuske AJ, Waltner JK, Kuhn P, Gu B, Craig EA. 2012. Role for the molecular chaperones Zuo1 and Ssz1 in quorum sensing via activation of the transcription factor Pdr1. Proc Natl Acad Sci U S A 109:472–477.
32. Gautschi M, Lilie H, Fünfschilling U, Mun A, Ross S, Lithgow T, Rücknagel P, Rospert S. 2001. RAC, a stable ribosome-associated complex in yeast formed by the DnaK-DnaJ homologs Ssz1p and zuotin. Proc Natl Acad Sci U S A 98:3762–3767.
33. Hundley H, Eisenman H, Walter W, Evans T, Hotokezaka Y, Wiedmann M, Craig E. 2002. The in vivo function of the ribosome-associated Hsp70, Ssz1, does not require

its putative peptide-binding domain. Proc Natl Acad Sci U S A 99:4203–4208.

34. Conz C, Otto H, Peisker K, Gautschi M, Wölfle T, Mayer MP, Rospert S. 2007. Functional characterization of the atypical Hsp70 subunit of yeast ribosome-associated complex. J Biol Chem 282:33977–33984.

35. Watanabe D, Araki Y, Zhou Y, Maeya N, Akao T, Shimoi H. 2012. A loss-of-function mutation in the PAS kinase Rim15p is related to defective quiescence entry and high fermentation rates of *Saccharomyces cerevisiae* sake yeast strains. Appl Environ Microbiol 78:4008–4016.

36. Watanabe D, Zhou Y, Hirata A, Sugimoto Y, Takagi K, Akao T, Ohya Y, Takagi H, Shimoi H. 2016. Inhibitory role of Greatwall-like protein kinase Rim15p in alcoholic fermentation via upregulating the UDP-glucose synthesis pathway in *Saccharomyces cerevisiae*. Appl Environ Microbiol 82:340–351.

37. Zhang W, Needham DL, Coffin M, Rooker A, Hurban P, Tanzer MM, Shuster JR. 2003. Microarray analyses of the metabolic responses of *Saccharomyces cerevisiae* to organic solvent dimethyl sulfoxide. J Ind Microbiol Biotechnol 30:57–69.

38. Sadowska-Bartosz I, Pączka A, Mołoń M, Bartosz G. 2013. Dimethyl sulfoxide induces oxidative stress in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 13:820–830.

39. Goldstein AL, McCusker JH. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast 15:1541–1553.

40. Atkin L, Schultz AZ, Frey CN. 1945. Influence of dough constituents on fermentation. Cereal Chem 22:321–333.

41. Li H. 2011. A statistical framework for SNP calling, mutation discovery, association

514 mapping and population genetical parameter estimation from sequencing data.
515 Bioinformatics 27:2987–2993.
516 42. Wang X, Xu H, Ha SW, Ju D, Xie Y. 2010. Proteasomal degradation of Rpn4 in
517 *Saccharomyces cerevisiae* is critical for cell viability under stressed conditions.
518 Genetics 184:335–342.

519 **FIGURE LEGENDS**

520

521 **FIG 1** Phenotypes of strains 45 and YF after refrigerating or freezing preservation. (A, B)
522 Progression of model dough fermentation. The graphs indicate the total carbon dioxide
523 emission during the model dough fermentation tests using strains 45 (A) and YF (B).
524 Light blue lines and blue lines show the means and standard deviations of three
525 independent experiments using refrigerated cells and frozen cells, respectively. Asterisks
526 represent significant differences (*t*-test, $p < 0.01$). (C) Cell viability after 2-week
527 refrigerating or freezing preservation as determined by methylene blue staining. Data
528 indicate the means and standard deviations of three independent experiments. Asterisks
529 represent significant differences (*t*-test, $p < 0.05$).

530

531 **FIG 2** Effects of freezing preservation on the proteasome gene expression. The heatmap
532 indicates the relative expression levels of individual genes, which are represented by the
533 percentages of frozen-sample data to refrigerated-sample data of the DNA microarray
534 analysis using the cells 30 min after the onset of model dough fermentation. The DNA
535 microarray experiment was performed using a single sample for each strain/condition.

536

537 **FIG 3** Assay of the proteasomal function of baker's yeast strains. (A) Ubiquitinated
538 proteins upon heat shock. Cells were incubated at 42°C for 15 min for heat shock. The
539 GAPDH level was shown as a protein loading control in Western blot analyses.
540 Representative data are shown from two repeated experiments. (B) Ubiquitinated proteins
541 after free-thaw process. Cells were frozen for two weeks and used for model dough

fermentation. Pgk1p level was shown as a protein loading control in Western blot analyses. Representative data are shown from two repeated experiments. (C) The MG132 tolerance of baker's yeast strains. Approximately 10^7 cells (OD_{600} of 1) of strains 45 and YF were serially diluted to 10^1–10^3 fold (from left to right), spotted onto YPD + DMSO (left panel) and YPD + DMSO + 75 μM MG132 (right panel) agar media, and incubated at 30°C for 2 d. Representative data are shown from two repeated experiments.

FIG 4 Role of the proteasome on model dough fermentation after freezing preservation. (A) Effects of the MG132 treatment. The graph indicates the total carbon dioxide emission during the model dough fermentation tests using the BY4741 wild-type strain in the absence (grey) and presence (orange) of 50 μM MG132. (B–D) Effects of the proteasome gene expression. The graphs indicate the total carbon dioxide emission during the model dough fermentation tests using the BY4741 wild-type strain (grey) and *RPN4* (B), *PDR1* (C), or *PDR3* (D)-disrupted strain (red). The data shown are the means and standard deviations of two or three independent experiments using frozen cells. Asterisks represent significant differences (*t*-test, $p < 0.05$). WT, wild type.

FIG 5 The dominant-negative allele of *PDR3* found in strain YF. (A) Nonsynonymous polymorphisms in the *RPN4*, *PDR1*, and *PDR3* genes of YF. Amino acid substitutions unique to YF are indicated in bold and underlined. The colored boxes indicate the previously reported sequences and domains important for their function as transcriptional factors: Deg, degrons; DBD, DNA-binding motif; ID, inhibitory domain; AD; activation domain (29, 42). (B) The MG132 tolerance of hemizygous *pdr3*Δ disruptants of YF.

Approximately 10^7 cells (OD_{600} of 1) of parental YF (*PDR3*^{WT}/*PDR3*^{DN}) and hemizygous disruptants (*PDR3*^{WT}/*pdr3*^{DN}Δ and *pdr3*^{WT}Δ/*PDR3*^{DN}) were serially diluted to 10^1–10^3 fold (from left to right), spotted onto YPD + DMSO (left panel) and YPD + DMSO + 60 μM MG132 (right panel) agar media, and incubated at 30°C for 1 d. Representative data are shown from two repeated experiments. (C) Effects of the *PDR3*^{DN} allele on model dough fermentation after freezing preservation. The graph indicates the total carbon dioxide emission during the model dough fermentation tests using the *PDR3*^{WT}/*pdr3*^{DN}Δ (grey) and *pdr3*^{WT}Δ/*PDR3*^{DN} (black) strains. The data shown are the means and standard deviations of three independent experiments using frozen cells. Asterisks represent significant differences (*t*-test, $p < 0.01$). WT, wild type; DN, dominant negative. (D) Cell viability after 2-week freezing preservation as determined by methylene blue staining. Data indicate the means and standard deviations of three independent experiments. Asterisks represent significant differences (*t*-test, $p < 0.05$).

FIG 6 The proteasome gene expression as a freeze-thaw stress response in *S. cerevisiae*.

TABLE 1 GO analysis of the differentially expressed genes between the frozen and refrigerated samples of strain YF ($p < 1 \times 10^{-10}$).

STRONGLY EXPRESSED DURING FERMENTATION AFTER FREEZING			
GO term	**Cluster frequency**	**Background frequency**	***p* value**
Oxidation-reduction process (GO:55114)	46/355 (13.0%)	162/7165 (2.3%)	1.72×10^{-20}
Energy derivation by oxidation of organic compounds (GO:15980)	36/355 (10.1%)	135/7165 (1.9%)	1.36×10^{-14}
Generation of precursor metabolites and energy (GO:6091)	38/355 (10.7%)	158/7165 (2.2%)	7.42×10^{-14}
Small molecule metabolic process (GO:44281)	85/355 (23.9%)	691/7165 (9.6%)	2.87×10^{-13}
Cellular respiration (GO:45333)	27/355 (7.6%)	88/7165 (1.2%)	3.77×10^{-12}
WEAKLY EXPRESSED DURING FERMENTATION AFTER FREEZING			
GO term	**Cluster frequency**	**Background frequency**	***p* value**
Proteolysis involved in cellular protein catabolic process (GO:51603)	43/351 (12.3%)	234/7165 (3.3%)	1.57×10^{-11}
Modification-dependent protein catabolic process (GO:19941)	41/351 (11.7%)	216/7165 (3.3%)	2.13×10^{-11}
Ubiquitin-dependent protein catabolic process (GO:6511)	41/351 (11.7%)	216/7165 (3.3%)	2.13×10^{-11}

FIG 1 (Watanabe *et al.*)

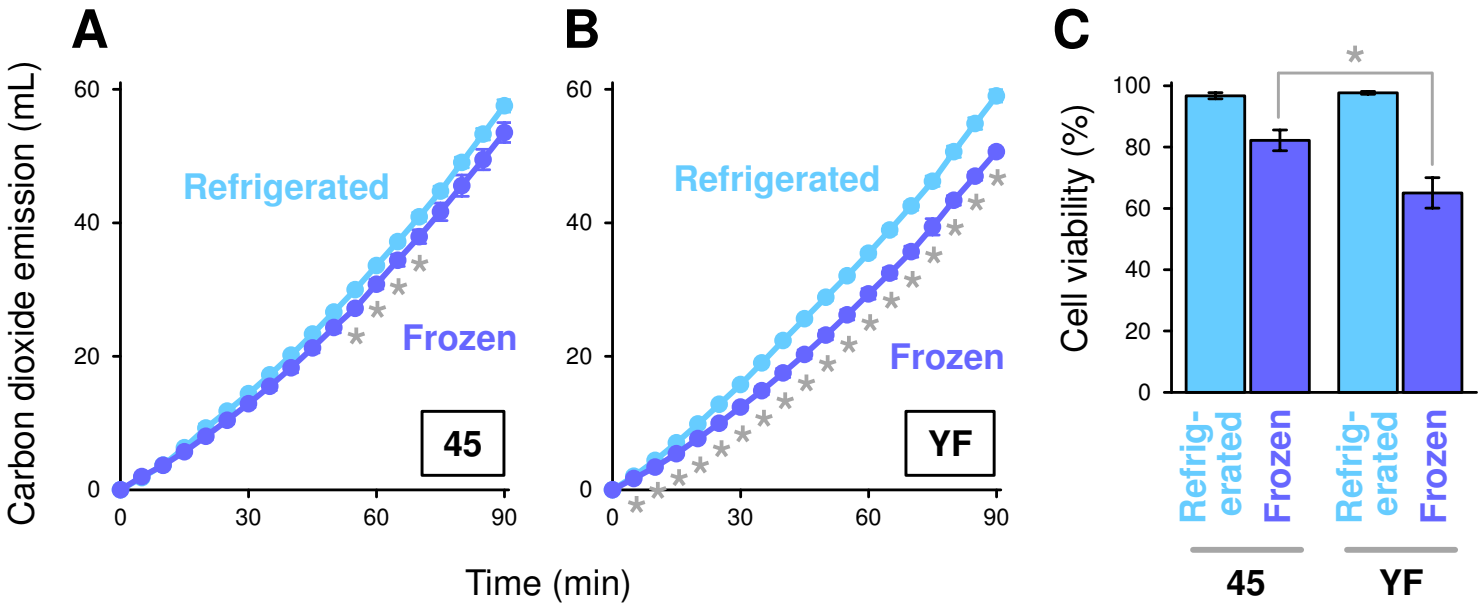

FIG 2 (Watanabe *et al.*)

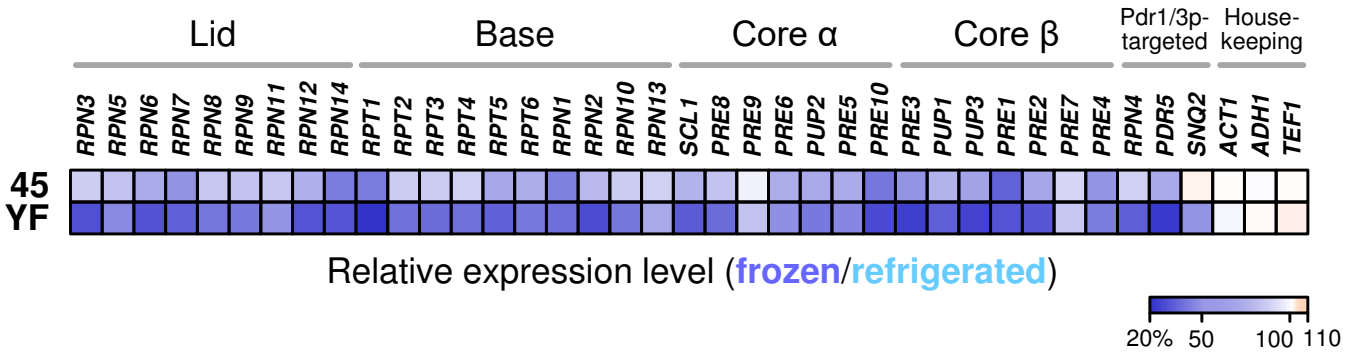

FIG 3 (Watanabe *et al.*)

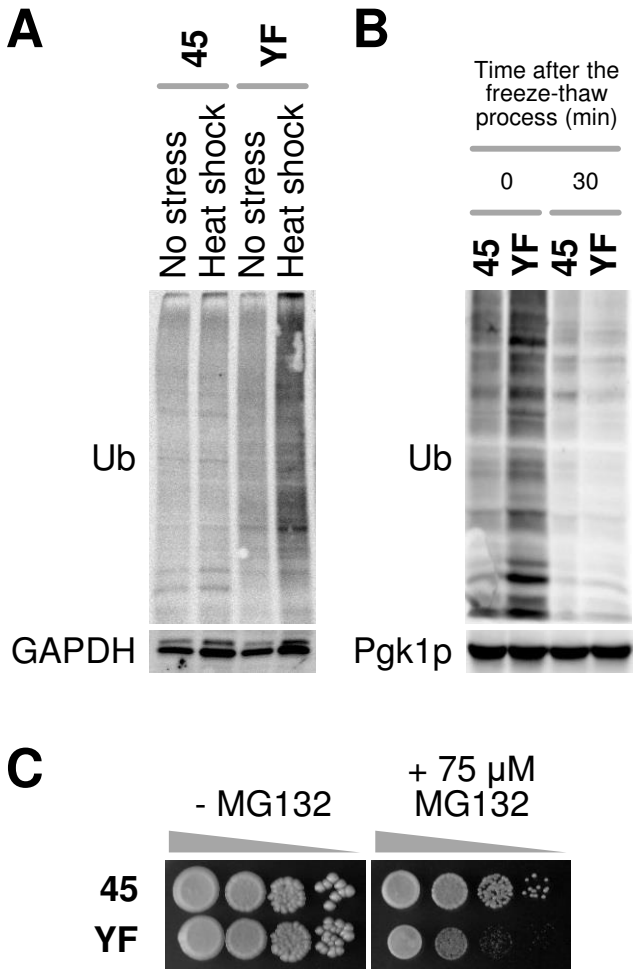

FIG 4 (Watanabe *et al.*)

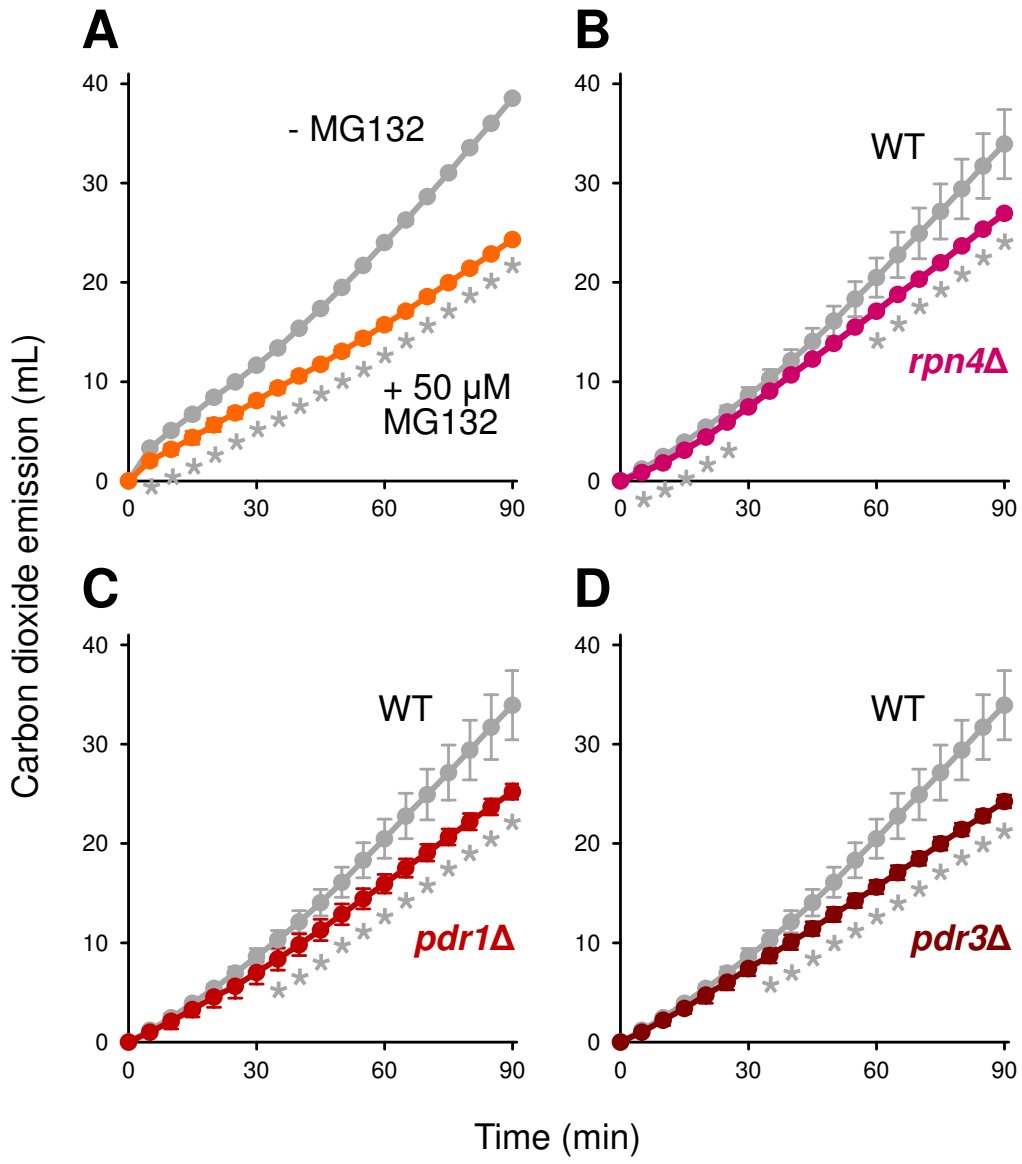

FIG 5 (Watanabe *et al.*)

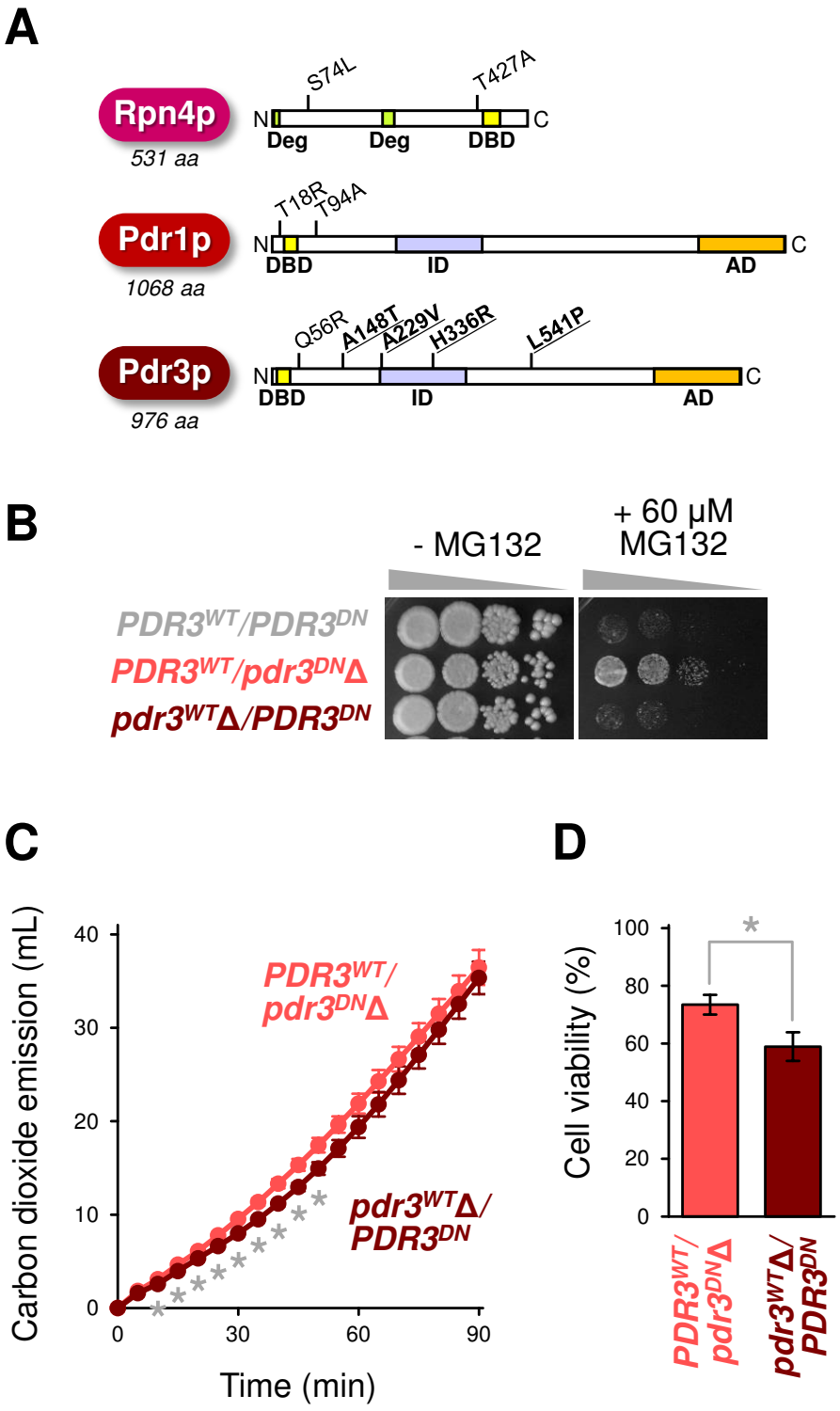

FIG 6 (Watanabe *et al.*)