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Award Review

Pleiotropic functions of the yeast Greatwall-family protein kinase Rim15p: a novel target for the control of alcoholic fermentation

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Rim15p, a Greatwall-family protein kinase in yeast *Saccharomyces cerevisiae*, is required for cellular nutrient responses, such as the entry into quiescence and the induction of meiosis and sporulation. In higher eukaryotes, the orthologous gene products are commonly involved in the cell cycle G_2/M transition. How are these pleiotropic functions generated from a single family of protein kinases? Recent advances in both research fields have identified the conserved Greatwall-mediated signaling pathway and a variety of downstream target molecules. In addition, our studies of *S. cerevisiae* sake yeast strains revealed that Rim15p also plays a significant role in the control of alcoholic fermentation. Despite an extensive history of research on glycolysis and alcoholic fermentation, there has been no critical clue to artificial modification of fermentation performance of yeast cells. Our finding of an *in vivo* metabolic regulatory mechanism is expected to provide a major breakthrough in yeast breeding technologies for fermentation applications.

Key words: *RIM15*; sake yeast; alcoholic fermentation; Greatwall-family protein kinase

How did sake yeast strains acquire outstanding performance of alcoholic fermentation? It is still not easy to answer this simple question. Alcoholic fermentation by the budding yeast *Saccharomyces cerevisiae* has been widely studied since Pasteur's epoch-making discovery in the nineteenth century. Numerous researchers have identified responsible *S. cerevisiae* genes, enzymes, metabolic reactions that constitute the glycolytic pathway, and their individual regulatory mechanisms. Nevertheless, there has been no critical clue to reveal the secret of sake yeast cells. Upon the entry into the 2010s, we finally obtained two keys: (i) the whole genome sequence of Kyokai no. 7 (K7),[1] a representative sake strain, which enabled us to perform genetic or genome-wide analysis of unique features of sake yeast cells; and (ii) the unique stationary phase-related physiology of fermenting yeast cells.[2] It has been long assumed that alcoholic fermentation performance is closely linked to proliferation rates because slow-growing strains or growth-inhibitory conditions often restrict alcoholic fermentation rates. It is also the fact, however, that exponential growth occurs only in the very initial stage of alcoholic fermentation.[2,3] Thus, fast-growing strains are not always good fermenters; rather, cellular physiology in the stationary phase is associated with fermentation performance (Fig. 1). Based on these keys, we successfully demonstrated that K7 and its relatives harbor a loss-of-function mutation in the *RIM15* gene, which encodes a Greatwall-family protein kinase that plays a crucial role in the control of stationary-phase cellular status, and showed that this lesion causes the high-fermentation phenotype. In this review, we describe the identification of this mutation in *RIM15* and how this lesion enhances alcoholic fermentation performance; we further consider the latest research examining the pleiotropic molecular functions of Rim15p.

I. Identification of a loss-of-function mutation in the *RIM15* gene of sake strains

To identify the genes associated with the traits during sake fermentation, we first focused on the gene expression profile. Fermenting yeast cells derived from sake strain Kyokai no. 701 (K701, a non-foaming variant of K7) or from laboratory strain X2180 (as a control strain with the non-sake strain background) were subjected to transcriptomic analysis using DNA microarrays.[3,4] It should be noted that we used X2180 as an experimental control because both K7 and X2180 are prototrophic diploids; notably, X2180 is reported to have originated by diploidization of *S. cerevisiae* S288C,[5] the strain in which the whole genome

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Abbreviations: CDK, cyclin-dependent protein kinase; CE-TOFMS, capillary electrophoresis time-of-flight mass spectrometry; Dox, doxycycline; Ensa, α-endosulfine; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; Igo1/2p, Igo1p and Igo2p; K7, Kyokai no. 7; K701, Kyokai no. 701; Msn2/4p; Msn2p and Msn4p; PKA, protein kinase A; $PP2A^{B55\delta}$, B55δ-bound protein phosphatase 2A; $PP2A^{Cdc55p}$, Cdc55p-bound protein phosphatase 2A; T6P, trehalose-6-phosphate; TORC1, target-of-rapamycin complex 1; UDPG, UDP-glucose.

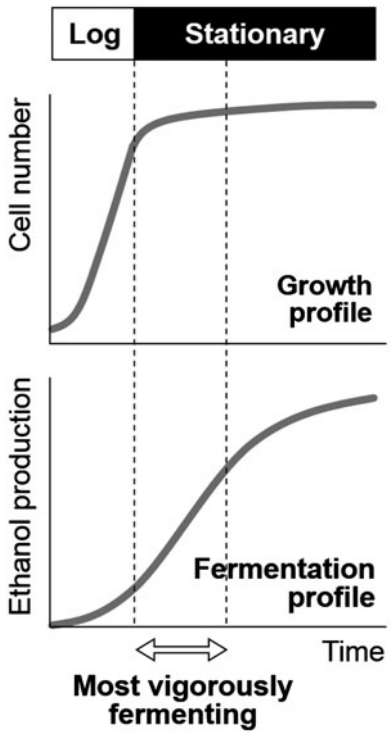

Fig. 1. Comparison of growth and fermentation profiles.
Notes: Generally, the most vigorously fermenting cells occur in the stationary phase of the growth curve.

sequence of yeast was determined for the first time.[6] By comparing the diploid strains with known genomes, we could specifically discuss the effects of their polymorphisms. Once all the differences between K7 and X2180 at the genomic DNA level (i.e., except for epigenomic differences) were known, it was theoretically possible to elucidate all phenotypes of K7 by testing the effects of individual mutations and their combinations. Thus, the whole genome analysis of K7 enabled us to adopt more rational and targeted strategies instead of laborious black-box approaches.

According to the transcriptomic analysis of fermenting cells, the target genes of transcriptional factors Msn2p and Msn4p (Msn2/4p) and Hsf1p, which play pivotal roles in the entry into quiescence (or G_0 phase) in the stationary phase,[7,8] were found to be significantly less induced in K701 than in X2180.[4,9] It was thus expected that the defect in the quiescence entry leads to the high-fermentation phenotype in K701, since quiescent cells are well characterized by low proliferative and metabolic activities.[7,8] We analyzed the gene encoding the protein kinase Rim15p, which is a prerequisite for quiescence entry and is presumed to act upstream of Msn2/4p and Hsf1p based on genetic studies.[10–12] We found a premature stop codon in the *RIM15* ORF caused by insertion of a single adenine nucleotide (*rim15*^{505insA}); this mutation was present in both alleles of the *RIM15* genes, specifically among the K7-associated sake strains, such as Kyokai nos. 6, 7, 9, and 10, and the strains bred from them (Fig. 2). This frameshift mutation was sufficient to fully abrogate the functions of Rim15p.[13] Introduction of the same mutation into the laboratory strain BY4743 (derived from S288C) remarkably increased the fermentation rate and ethanol production (from 11 vol% to 17 vol%) during 20-day sake fermentation.[13] These results identified this *rim15*^{5055insA} mutation as a novel determinant of the high alcoholic fermentation performance of sake yeast strains.[14]

II. Pleiotropic functions of Rim15p as a Greatwall-family protein kinase

To understand how loss of Rim15p enhances alcoholic fermentation, the molecular functions of Rim15p should be clarified. The name of the encoding gene represents that the locus was originally identified as one of the Regulator genes of *IME2* (Inducer of Meiosis 2), which are required for the onset of meiosis.[15] Expression of the *IME1* gene, which encodes a transcriptional activator of *IME2*, is also under the control of Rim15p.[16] Therefore, diploid strains that lack a functional *RIM15* gene are defective in meiosis and subsequent spore formation. In fact, it was reported, more than 20 years before the identification of the *rim15*^{5055insA} mutation, that K7-related sake strains exhibit a severely low sporulation efficiency, a phenotype due (at least in part) to impaired expression of the *IME1* and *IME2* genes.[17,18] It is thus worth emphasizing that the loss of the function of Rim15p affects not only alcoholic fermentation but also the other known traits of sake strains. Although introduction of a functional *RIM15* gene alone is not sufficient to restore the meiotic activity of K7,[7,18], it is expected that simultaneous expression of *RIM15* and the other responsible genes might properly enhance meiosis and sporulation, which could lead to development of novel crossbreeding techniques for sake yeast strains. A recent report of the orthologous gene in the filamentous fungus *Aspergillus oryzae* (*Aorim15*) revealed that gene's role in conidiation and formation of sclerotia,[19] suggesting the common function of fungal Rim15p in governing the balance between asexual and sexual development.

In *S. cerevisiae*, Rim15p is well established as a key regulator of the entry into quiescence. Historically, the stationary phase has been the subject of limited study; this phase was simply regarded as the state in which cell proliferation ceases due to a shortage of nutrients or certain essential factors. However, a series of transcriptomic analyses of stationary-phase cells revealed that a specific gene expression program to induce quiescence is activated concurrently with growth arrest. All landmark events in the quiescence entry of *S. cerevisiae* cells, such as inhibition of growth and budding, cell cycle arrest in the G_1 phase, translational repression, autophagy induction, accumulation of storage (trehalose and glycogen) and structural (1,3-β-glucan) carbohydrates, and acquisition of stress tolerance,[7,8] are likely important to conserve nutrients and maintain viability in harsh environments. De Virgilio and his colleagues re-identified the meiotic regulator Rim15p as a protein interacting with trehalose-6-phosphate (T6P) synthase Tps1p, a protein that is essential for trehalose biosynthesis.[20] Deletion of the *RIM15* gene was reported to abrogate not only trehalose biosynthesis but also the induction of many of the landmarks described above.[10,20] Furthermore, it was found that the master regulators of nutrient signaling, protein kinase A (PKA),

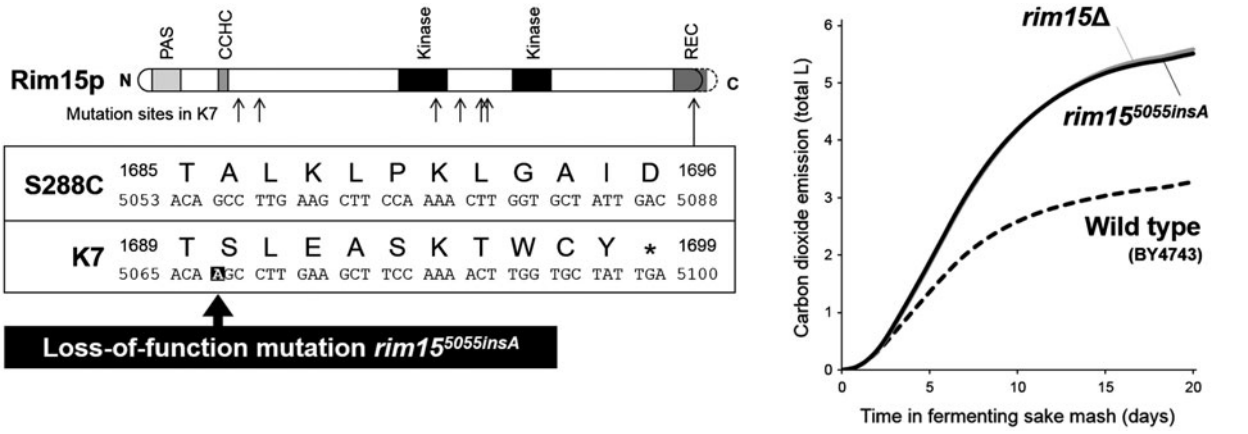

Fig. 2. The *rim15*^{5055insA} mutation found in K7 accounts for the high-fermenting phenotype.

Notes: (*Left panel*) The location of the *rim15*^{5055insA} mutation, a single adenine nucleotide insertion causing a reading frame shift near the downstream end of the ORF. PAS, CCHC, kinase, and REC represent a Per-Arnt-Sim domain, a CCHC-type zinc finger motif, protein kinase catalytic domains, and a receiver domain, respectively. (*Right panel*) The *rim15*^{5055insA} mutation (black bold line), as well as full deletion of *RIM15* (gray line), strikingly increases the fermentation rate of the laboratory strain BY4743 in sake mash compared to that of the wild-type parent (black dashed line) [modified from Watanabe et al.[13)]].

and the target-of-rapamycin complex 1 (TORC1)-Sch9p pathway, directly phosphorylate Rim15p to control its activity.[10,20,21)] Based on these experimental data, it was concluded that Rim15p induces quiescence through activation in response to nutrient starvation. Subsequent analyses of the downstream pathways suggested that Rim15p protein kinase phosphorylates the transcriptional factors Msn2/4p and Hsf1p to induce the quiescence entry genes.[11,12,22)] Consistently, the K7-related sake yeast strains with the *rim15*^{5055insA} mutation also exhibit defects in the induction of quiescence; these defects are suppressed by expression of the fully functional *RIM15* gene.[2,13)] In laboratory strains, impaired functions of Msn2/4p or Hsf1p, as well as loss of Rim15p, lead to an increase of the fermentation rate.[4,9)] These results indicate that the quiescence entry triggered by Rim15p is closely associated with the control of alcoholic fermentation in *S. cerevisiae*.

In higher eukaryotes, researches of the orthologous protein family start from a cell cycle study reported in 2004.[23)] A *Drosophila* mutant defective in chromosomal condensation and mitotic progression in larval neuroblasts was isolated, and the mutated gene was designated as *Greatwall* (or *Gwl*) because the gene product was presumed to be involved in the protection of chromosomal structure. This Greatwall protein kinase is highly conserved among eukaryotes, and was found to be directly phosphorylated and activated by cyclin-dependent protein kinase (CDK) upon the onset of the M phase in *Xenopus* egg extracts.[24)] In turn, Greatwall activity is required for activation of the M-phase cyclin/CDK complex, thus forming a autoregulatory positive feedback loop.[24)] More recently, studies clarified the detailed molecular mechanism, whereby Greatwall phosphorylates an endogenous ligand peptide, α-endosulfine (Ensa), to inhibit B55δ-bound protein phosphatase 2A (PP2A$^{B55\delta}$), leading to activation of the M-phase cyclin/CDK complex.[25,26)] Thus, Greatwall-family protein kinases enhance G_2/M progression in higher eukaryotes, whereas Rim15p of *S. cerevisiae* is essential for the entry into quiescence that is associated with G_1 arrest. How can these homologous proteins exhibit apparently opposite roles in different species? Although *S. cerevisiae* possesses the Greatwall (Rim15p)-Ensa (Igo1p and Igo2p (Igo1/2p))-PP2A$^{B55\delta}$ (PP2A^{Cdc55p}) pathway,[27–30)] the yeast PP2A^{Cdc55p} activates the M-phase cyclin/CDK complex only in a restricted manner,[29)] instead inhibiting the S-phase cyclin/CDK complex via CDK inhibitor Sic1p.[31)] Furthermore, the same pathway has been shown to be involved in meiotic entry and the induction of stress-responsive genes in yeast.[27,28,30)] Together, these results suggest that the pleiotropic roles of Rim15p and the other Greatwall-family protein kinases likely reflect the variety of the substrate proteins dephosphorylated by PP2A$^{B55\delta}$ (Fig. 3). Thus, exploration of the target proteins dephosphorylated by PP2A^{Cdc55p} [28)] might contribute to identification of the key regulator of alcoholic fermentation under the control of Rim15p. It is worth noting that CDK activity might be one of the most promising targets, since we previously showed that sake strains with the *rim15*^{5055insA} mutation commonly exhibit a defect in G_1 arrest,[13,32)] and that artificial overwhelming enhancement of G_1/S progression raises the fermentation rate of a laboratory strain.[32)]

III. Rim15p-mediated control of alcoholic fermentation

Although the molecular functions of Rim15p as a Greatwall-family protein kinase were revealed as described above, it was still hard to clearly define how a loss of Rim15p activity leads to the high-fermentation phenotype. In our study, using laboratory fermentative conditions (20% glucose-containing YPD medium, 30 °C, static culture),[33)] both wild-type cells and *rim15*Δ cells of the laboratory strain BY4743 exhibited similar cell densities throughout the fermentation period. This observation suggested that an increase in the fermentation rate by deletion of the *RIM15* gene is not mainly attributed to a high proliferation rate. Intriguingly, biomass per cell in the wild type was found to increase during fermentation, while deletion of the

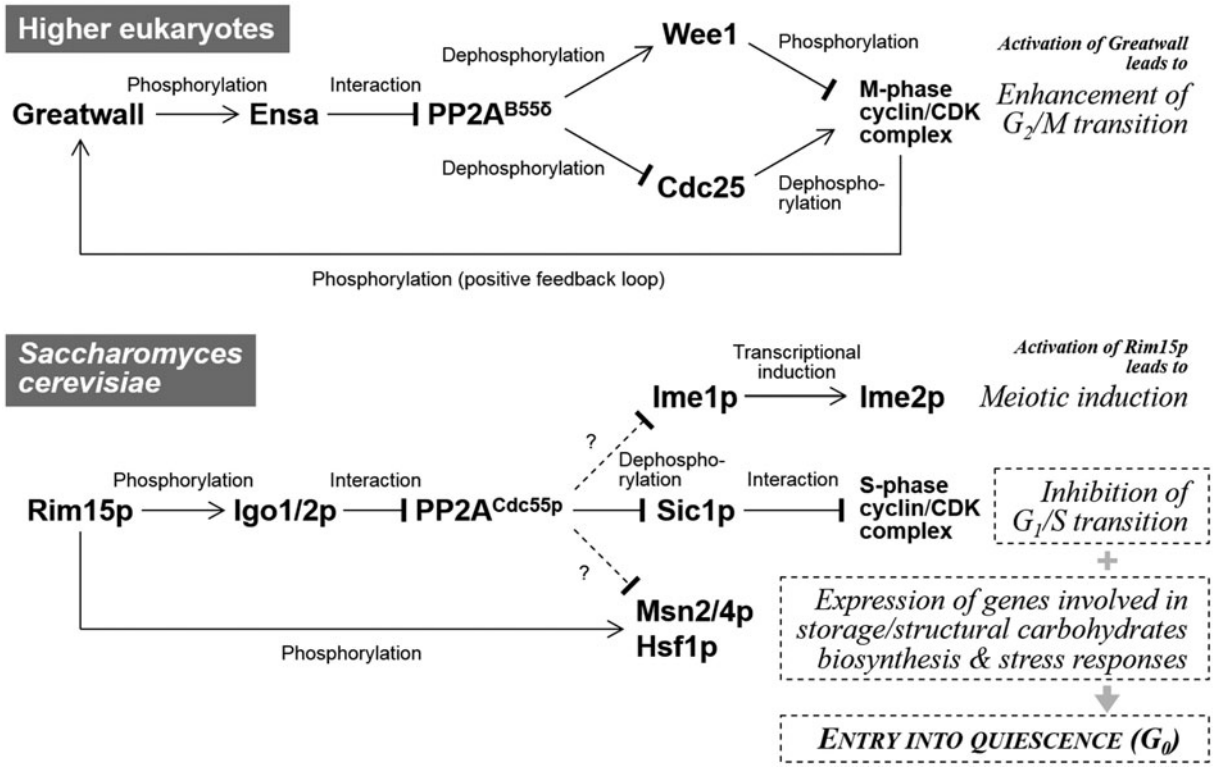

Fig. 3. Known functions of the conserved Greatwall (Rim15p)-Ensa (Igo1/2p)-PP2A$^{B55\delta}$ (PP2A^{Cdc55p}) pathway in higher eukaryotes and *S. cerevisiae*.

RIM15 gene suppressed this phenotype. A similar trend in calorie-restricted chemostat cultivations was also reported previously.[34] These experimental data raised the possibility that lack of Rim15p decreases accumulation of specific cellular carbon compounds, leading to a higher glycolytic flux and enhanced alcoholic fermentation performance.

To elucidate how the dysfunction of Rim15p affects carbon metabolism, we analyzed the intracellular metabolic profiles of the fermenting *rim15*Δ cells by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS).[33] As a result, distinctive alterations were observed in the UDP-glucose (UDPG)-dependent biosynthesis of branched-chain storage and structural carbohydrates from glycolytic products (Fig. 4). In this anabolic pathway, the glycolytic intermediate glucose-6-phosphate (G6P) is converted to glucose-1-phosphate (G1P) by phosphoglucomutases Pgm1p and Pgm2p, and the UDPG pyrophosphorylase Ugp1p catalyzes the reaction of G1P with UTP to produce UDPG. UDPG is used as a common substrate for the synthesis of trehalose, glycogen, and 1,3-β-glucan.[35] In our metabolomic analysis, deletion of the *RIM15* gene increased the G1P level but decreased the UDPG level, indicating that loss of Rim15p yields decreased Ugp1p activity. Consistently, the *rim15*Δ cells of laboratory strains, as well as the sake yeast cells encoding a defective Rim15p, exhibit decreased accumulation of intracellular trehalose and glycogen and of cell wall 1,3-β-glucan (Fig. 4).[13,33]

As previously reported, downregulation of the *UGP1* gene (note that deletion of *UGP1* is lethal) is sufficient to cause phenotypes similar to those of Rim15p-deficient cells (e.g. impaired synthesis of trehalose, glycogen, and 1,3-β-glucan).[36,37] Furthermore, we found several consensus recognition sites of transcription factors Msn2/4p and Hsf1p (known targets of Rim15p; see above) in the 5′-UTR region of the *UGP1* gene (Fig. 5). Consistently, *UGP1* mRNA expression was attenuated in the early stage of alcoholic fermentation in both BY4743 *rim15*Δ and K701 cells compared to laboratory yeast wild-type strains.[4,33] Taken together, these data suggested that a defect in Rim15p can reduce the synthesis of UDPG and downstream anabolites via the decreased activities of Msn2/4p and Hsf1p (Fig. 4). Considering that UDPG biosynthesis branches from glycolysis, it was hypothesized that blockage of the carbon anabolic pathway redirects carbon flow into glycolysis and alcoholic fermentation. As expected, downregulation of *UGP1* gene expression under the control of a doxycycline (Dox)-repressible promoter[38] markedly increased the fermentation rate in a Dox concentration-dependent manner.[33] Based on these data, impaired *UGP1* expression was identified as one of the factors involved in the high-fermentation phenotype of the Rim15p-deficient cells.

In other words, an important physiological role of Rim15p is likely induction of the *UGP1* gene to produce UDPG upon entry into quiescence. Assuming that the main significance of quiescence exists in (i) conserving nutrients, (ii) storing nutrients, and (iii) maintaining viability, *UGP1* expression alone is sufficient to realize these goals simultaneously by (i) inhibition of glycolysis, (ii) synthesis of storage carbohydrates, and (iii) synthesis of structural carbohydrates. Therefore, Rim15p-induced UDPG synthesis may be a key to the survival strategy of *S. cerevisiae* at the metabolic level. Sake yeast loss-of-function mutations that specifically occur in this pathway, including the ones in Rim15p,[13] Msn4p,[4] Ppt1p (a putative upstream activator protein phosphatase for Hsf1p),[9] and Glg2p (glycogenin, the initiator of glycogen synthesis),[33] suggest that the sake

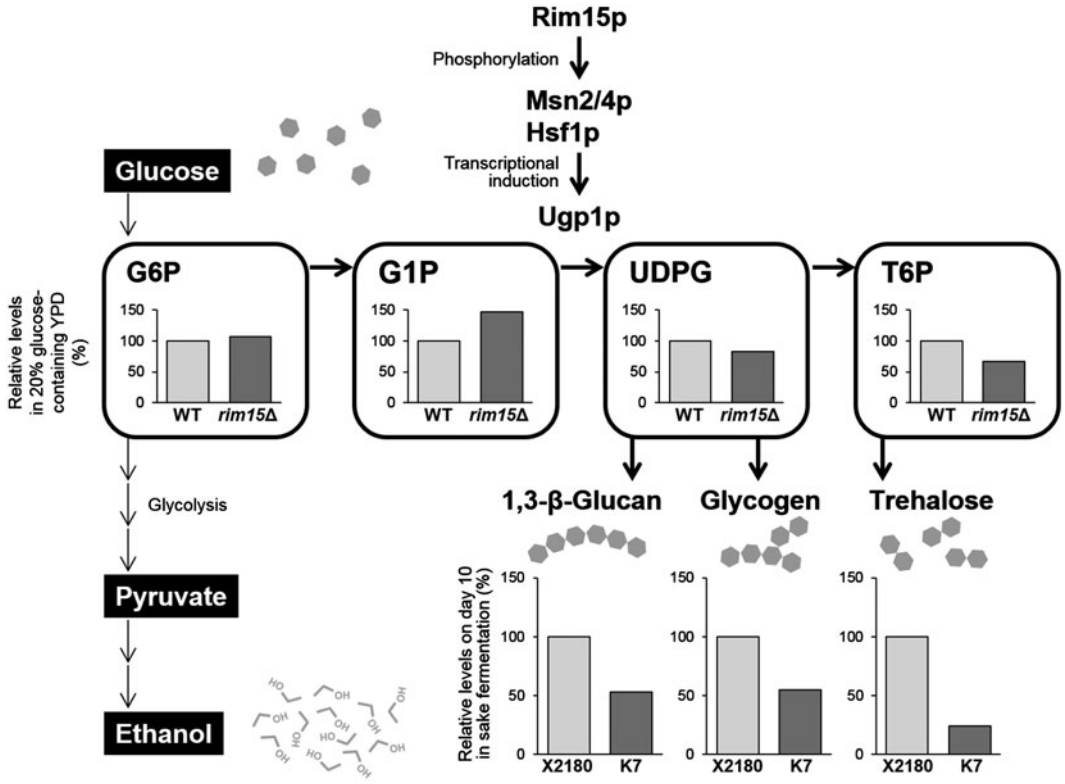

Fig. 4. The Rim15p-mediated changes in UDPG and carbohydrate anabolic pathways result in inhibition of glycolysis and alcoholic fermentation [modified from Watanabe et al.[33)]].

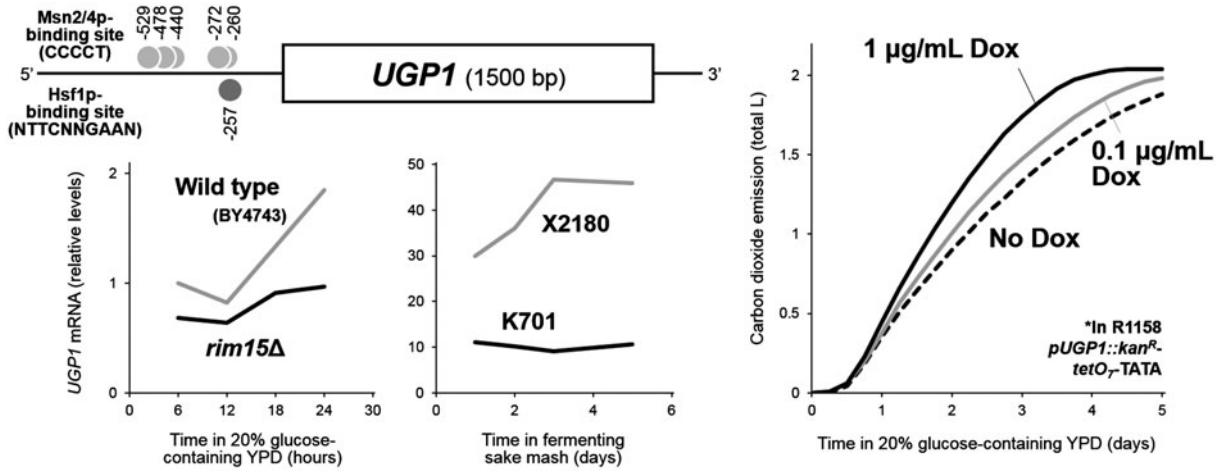

Fig. 5. The induction of *UGP1* expression mediates the effect of Rim15p activation on alcoholic fermentation.

Notes: (*Left panel*) The *UGP1* gene contains consensus Msn2/4p and Hsf1p-binding sites in its 5′-UTR sequence. While *UGP1* is upregulated in the early stage of alcoholic fermentation (gray lines), the induction is clearly inhibited in strains with lesions in the gene encoding Rim15p (a laboratory strain harboring *rim15*Δ or a sake strain carrying the $rim15^{5055insA}$ mutation; black lines). (Right panel) In the R1158 $pUGP1::kan^{R}$-$tetO_7$-TATA strain, addition of Dox decreases the expression of *UGP1* and enhances alcoholic fermentation [modified from Watanabe et al.[33)]].

strains are not derived from competitive natural environments, but are highly adapted and domesticated for artificial sake-making conditions following careful selection by sake manufacturers who pursued a yeast strain with surpassing fermentation performance.

IV. The *RIM15* gene and its modification in various yeast strains

Recent comparative genomic analyses have revealed different loss-of-function mutations in the *RIM15* gene of non-sake strains. A lineage of Σ1278b, a representative diploid laboratory strain that is often used for the study of yeast cellular differentiation (e.g. mating, sporulation, pseudohyphal growth, biofilm formation, induction of complex colony morphology), revealed heterozygosity for a nonsense allele of *rim15* (*RIM15*/$rim15^{G1216T}$).[39)] Under conditions in which limitation for fermentable carbon sources is coupled with a rich nitrogen source, haploid Σ1278b progeny harboring the wild-type *RIM15* allele display a complex colony morphology, while the haploid progeny with the

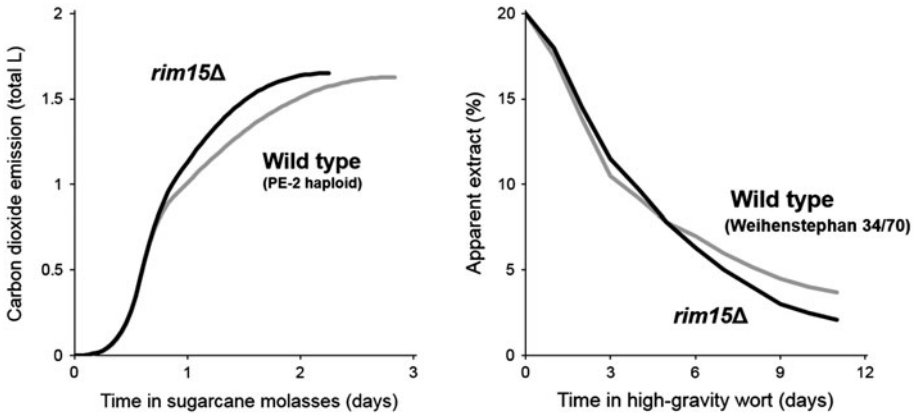

Fig. 6. Effects of deletion of the *RIM15* gene in industrial strains.
Notes: (*Left panel*) Deletion of *RIM15* increases the fermentation rate and shortens the fermentation period in molasses fermentation using a strain derived from bioethanol yeast PE-2 [modified from Inai et al.[43)]]. (*Right panel*) Deletion of *RIM15* enhances sugar consumption in high-gravity wort fermentation using *S. pastorianus* strain Weihenstephan 34/70 [modified from Oomuro et al.[45)]].

rim15^{G1216T} allele do not exhibit this phenotype. Although the precise role of Rim15p in determining colony morphology is still unknown, Rim15p might be somehow involved in cellular responses to nutrient changes, as in meiosis or in quiescence entry. Another example is the frameshift mutation *rim15*^{459insCA} found in the haploid strain DBVPG6765, which is regarded as closely related to vineyard strains.[40)] This mutation leads to a decrease in sporulation efficiency and an increase in the fermentation rate in synthetic grape must,[40,41)] consistent with previous studies. As this mutation is not described in any industrial wine strains, we cannot conclude at present that the *rim15*^{459insCA} mutation indeed contributes to the enological traits in practical winemaking. Even if such mutated alleles are heterozygous in industrial strains, loss of heterozygosity by mitotic gene conversion may facilitate the full potential of the *rim15* mutation to enhance the fermentation performance. Taken together, these data suggest that loss of Rim15p may enhance the utility of domesticated yeast strains in research or in industrial settings where the yeast are maintained in artificial or man-made habitats; such lesions may prevent highly sensitive environmental responses (cellular or colony morphological changes, meiotic genomic rearrangement, and metabolic remodeling including the control of alcoholic fermentation), thus stabilizing phenotypes favorable for human purposes.

Might deletion of *RIM15* be applied in non-sake yeast strains? To fulfill increasing demands for alternative energy sources, the development of more efficient technologies for bioethanol production is required. We tested the effects of Rim15p loss in PE-2, a strain representative of the industrial strains used for bioethanol production in Brazil.[42)] A haploid strain derived from PE-2 was deleted for *RIM15* and subjected to fermentation tests in sugarcane molasses.[43)] Deletion of *RIM15* significantly increased the fermentation rate and shortened the fermentation period (Fig. 6). This observation is an intriguing result because the molasses fermentation conditions used (the main carbon source is sucrose; 35 °C) were different from sake-making conditions (the main carbon source is glucose derived from rice starch; usually less than 15 °C). This observation suggested that this method can be widely used for improvement of the fermentation rates of a variety of industrial yeast strains. For example, this technique was applied in the brewing of lager-type beers using the bottom-fermenting yeast *S. pastorianus*, which is a polyploid interspecific hybrid of *S. cerevisiae* and *S. eubayanus*.[44)] The deletion of the *S. cerevisiae*-type *RIM15* genes in *S. pastorianus* enhanced the depletion of sugars in fermenting high-gravity wort, corresponding to an increase of the fermentation rate (Fig. 6).[45)] It should be noted that *RIM15* deletion also enhanced alcoholic fermentation using maltose by the strain that does not belong to *S. cerevisiae*. The effects of deletion of genes orthologous to *RIM15* in other species should be elucidated in future work to understand the novel conserved role of Greatwall-family protein kinases in the control of carbon metabolism. Moreover, the decreased cell viability due to a loss of Rim15p might not always be beneficial, for example, in repetitive use of yeasts or under harsh fermentative conditions, both of which often occur in production of bioethanol or lager-type beers. Recently, we successfully modified the promoter of the *RIM15* gene of a laboratory strain to increase fermentation rates specifically under stress conditions.[46)] The advances in our studies of the regulatory genes for alcoholic fermentation are expected to contribute to the development of a novel custom-made yeast breeding technology that may permit the sophisticated optimization of the fermentation performance of specific strains under specific conditions, leading to innovation and further evolution in the fermentation industry.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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