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Molecular insights on DNA delivery into *Saccharomyces cerevisiae*☆

Shigeyuki Kawai,[a] Tuan Anh Pham,[a] Ha Thu Nguyen,[a] Hirokazu Nankai,[a] Takaaki Utsumi,[a] Yasuki Fukuda,[b] and Kousaku Murata[a,*]

[a] *Department of Basic and Applied Molecular Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan*
[b] *Chukyo Community College, Mizunami, Gifu 509-6101, Japan*

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Abstract

Understanding of the molecular system for DNA delivery into eucaryotic cells, a key to human DNA therapy, remains obscure. To understand this system, we undertook a study using the *Saccharomyces cerevisiae* model into which DNA delivery is easily assessed through competence (transformability) and for which all nonessential gene mutants (about 5000 strains) are available. We analyzed the competence of each of these mutants and identified three low-competence mutants, i.e., *sin3Δ*, *she4Δ*, and *arc18Δ*, and three high-competence mutants, i.e., *pde2Δ*, *spf1Δ*, and *pmr1Δ*. Through further studies using the six mutants, we concluded that the Arp2/3 activation machinery involving the Myo3/5p, Vrp1p, Las17p, Pan1p, and Arp2/3 complex is crucial to delivery (competence), and that high cAMP enhances competence via protein kinase A installing Tpk3p. We also propose that DNA is taken up via an endocytosis-like event, being at least partially different from well-known endocytosis.

Keywords: DNA delivery; *Saccharomyces cerevisiae*; Competence; Transformation; Arp2/3 complex; Myo3/5p; Vrp1p; P-type ATPase; cAMP; Protein kinase A

The molecular system for DNA delivery into eucaryotic cells, a key to practical human DNA therapy, remains to be clarified, even for *Saccharomyces cerevisiae*. We consider this organism suitable for studying eucaryotic DNA delivery because all nonessential gene mutants (about 5000 strains) are available and delivery is easily assessed through competence (transformability), which is expressed as colony numbers of transformants. Although competence conventionally requires artificial procedures [1–3] using lithium, electroporation, and spheroplasts, we developed simple transformation in which transformants of an order exceeding 10^2 per 1.0 μg DNA were obtained from about 10^7 viable cells by simply incubating live cells with DNA and polyethylene glycol for 1 h [4]. By analyzing the competence of all nonessential gene mutants of *S. cerevisiae* using a simple method less artificial and less injurious to intact cells than previous methods [1–3], we expected to obtain molecular insights into DNA delivery for eucaryotic cells. Here we show detail molecular insights obtained through this approach.

Materials and methods

Strains, media, and plasmids. Nonessential gene mutants (about 5000 strains), for which each nonessential gene was replaced with *kanMX4* in haploid BY4739 (*MATα leu2Δ0 lys2Δ0 ura3Δ0*) and BY4742 (*MATα leu2Δ0 lys2Δ0 ura3Δ0 his3Δ1*), were purchased from EUROSCARF. Other strains were kindly provided as follows: HA31-9c (*MATa can1-100 ade2-1 his3-11 leu2-3 112 ura3-1 trp1-1 myo3:: HIS3 myo5::TRP1*) by Dr. L.A. Pon; IDY223 (*MATa his3 leu2 ura3 trp1 ade2 las17::LEU2*) by Dr. M. Crouzet; T8-1D (*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519*), TZ81 (T8-1D *pan1-9*) by Dr. T. Zoladek; BWY1041 (*MATa his3 leu2 ura3 trp1 lys2 pan1-20*) by Dr. B. Wendland; SEY5017 (*MATa sec1-1 ura3-52 leu2-3, 112 suc2-9*) by Dr. S.D. Emr; RSY275 (*MATα sec20-1 ura3 his4*), S21PP-6C (*MATa sec21-1 ura3 leu2*), WBY6 (*MATα sec27-1 ura3 leu2 lys2 ade2*), and PC137 (*MATa tip20-5 ura3 leu2 his4 trp1 lys2 suc2-9*) by Dr. H.D. Schmitt; YWO0343 (*MATα ura3-1 his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100 prc1-1*), YWO0470 (YWO0343 *sec61-2*) by Dr. D.H. Wolf; and SEY6210 (*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel-*),

☆ *Abbreviations:* WT, wild-type strain; PE, phosphatidylethanolamine; PKA, protein kinase A; UPR, unfolded protein response.

* Corresponding author. Fax: +81-774-38-3767.
E-mail address: kmurata@kais.kyoto-u.ac.jp (K. Murata).

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BKY3 (SEY6210 *use1::TRP1 use1-0 layer*) by Dr. G. Fischer von Mollard. Strains were cultured at 30 °C in YPD, if necessary with 0.2 mg/ml geneticin, or SD (pH 5.0) containing appropriate nutrients. YEp13, YCp50, and pRS413 were used for transformation. Other plasmids were kindly provided as follows: *SHE4* in pRS316, YEplac195 bearing *SHE4, MYO5, MYO5* (V164I), and *MYO5* (N209S) by Dr. K. Tanaka; *SIN3* in YCp50 by Dr. D.J. Stillman; *SPF1* in YEplac195 by Dr. C. Suzuki; *PDE2* in YEp24 by Dr. A. Toh-e; and *PMR1* in B2205 by K.W. Cunningham. *ARC18* flanking the 5′-460-bp and 3′-190-bp was cloned from genomic DNA of BY4742 in YCplac33 via PCR with the following primers: 5′-TAACCGCAGGAAAAGAGATGAATTG-3′ and 5′-GA ACAAAACACAAATAAGACTTG-3′.

Check of competence. Our simple transformation was done as described elsewhere [4]. Briefly, for the first screening, cells of each mutant precultured on YPD plates were cultivated in 4 ml YPD for 5–6 h, collected, suspended in TE (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA), and incubated with YEp13 (1 μg) and polyethylene glycol 4000 (Nacalai Tesque, Japan, final 35% w/v) in 120 μl suspension for 1 h at 30 °C. Then, after the suspension was heated for 5 min at 42 °C, cells were collected and spread onto selective SD plates (method I). To further determine competence, cells were precultured overnight in YPD, transferred to 14 ml YPD to A_{600} of 0.10, and cultured to reach A_{600} of 0.30–0.60. Cells were treated as above except that (i) cell suspension was divided into two parts because two plasmids, YEp13 and YCp50, 2.5 μg, were used; (ii) after 42 °C treatment, cells were collected and supernatant was completely removed; (iii) cells were resuspended in 1.0 ml TE; (iv) from the suspension, 10 μl was taken and diluted in TE to count viable cell numbers by spreading on YPD or SD plates; and (v) cells were spread onto selective SD plates after collection (method II). In the complementation test, a mutant carrying each plasmid was precultured in SD medium to retain the plasmid. In an unpermissive condition, the cell suspension was preincubated for 1 h at 37 °C, and after the addition of DNA and polyethylene glycol, the suspension was further incubated for 1 h at 37 °C. Competence was usually assessed by two terms: (i) transformants, i.e., the number of transformants observed on selective plates; and (ii) frequency, i.e., [transformants] [viable cell number]$^{-1}$; and was expressed as times compared to that of the wild-type strain (WT). Except for the first screening, all competence was checked using method II.

Screening for low-competence mutants. Low-competence mutants showing 0–2 transformants were selected by method I and again checked by method I. The competence of mutants exhibiting 0–2 transformants, not frequency, in both duplicated assays was checked three times by method II. Mutants showing both less than 20% transformants and frequency by both two plasmids in all experiments were selected as having low competence.

Screening for high-competence mutants. Based on the first screening by method I, we selected 36 mutants with 20 times more transformants than the WT. Then, the competence of mutants was examined by method II, and 6 mutants with 10 times more transformants or frequency were selected. Finally, after another 6 examinations with method II, 3 strains were identified as described in the text. Two strains were excluded because they showed the same degree of competence as WT in 1 experiment, and another was excluded because the result of the complementation test was not as reliable as desired.

Results and discussion

Identification of low- and high-competence mutants

We analyzed the competence of all nonessential gene mutants of *S. cerevisiae* using the simple method [4] detailed under Materials and methods. We roughly selected mutants with low and high competence using method I, identifying three each with low (Fig. 1A) and high (Fig. 1C) competence after assessing competence several times by method II. The three low-competence mutants were *she4Δ*, *arc18Δ*, and *sin3Δ*, where *she4Δ* and *arc18Δ* had much lower competence than *sin3Δ* (Fig. 1A). This low competence was complemented (enhanced) by introducing each of the corresponding native genes, *SHE4*, *ARC18*, and *SIN3* (Fig. 1B). We found no mutant completely lacking in competence, suggesting that genes critical for competence are essential and/or that genes involved in competence share redundant functions. The three high-competence mutants were *spf1Δ*, *pde2Δ*, and *pmr1Δ* (Fig. 1C). Their high competence was decreased by introducing each of the corresponding native genes, where although the competence of *pmr1Δ* was not higher than that of WT, introduction of native *PMR1* into *spf1Δ* decreased the competence of *spf1Δ* (Fig. 1D), suggesting that *pmr1Δ* is a mutant with high competence, but with competence lower than those of *spf1Δ* and *pde2Δ*, which agrees with screening results (Fig. 1C).

Although our approach may have missed some low- and high-competence mutants, we believe that competence (DNA delivery) of *S. cerevisiae* can be understood by using the six low- and high-competence mutants as starting points and by reassessing competence of other nonessential and essential gene mutants in relation to these six mutants. Indeed, we obtained insights into competence detailed below.

Molecular motor Myo3/5p is required for competence

she4Δ, which had the lowest competence (Fig. 1A), is deficient in endocytosis, polarization of actin patches [5,6], and mother-cell-specific *HO* expression [7]. We focused on molecular motor-type I myosins Myo3p and Myo5p, which are functionally redundunt [8], because Toi et al. [5] previously proposed that *she4Δ* phenotypes such as defects in endocytosis and actin polarization are due to the dysfunctions of Myo3/5p, and She4p functions as a molecular chaperone for Myo3/5p, based on their results that She4p interacted with Myo3/5p, and the *she4Δ* phenotypes were suppressed by mutated *MYO5*, including *MYO5* (V164I) and *MYO5* (N209S). We studied the effects of native and mutated *MYO5* on the low competence of *she4Δ* and found that *she4Δ* competence was enhanced by introducing each of native *SHE4*, mutated *MYO5* (V164I), and mutated *MYO5* (N209S), but not by native *MYO5* (Fig. 2A). In accordance with the redundant functions of Myo3/5p, a single mutant for Myo3/5p, each of *myo3Δ* and *myo5Δ*, showed no low competence, while a double mutant (*myo3Δ myo5Δ*) exhibited low competence (data not shown). We therefore concluded that the low competence of *she4Δ* is caused by the dysfunctions of Myo3/5p, i.e., that competence requires functional Myo3/5p.

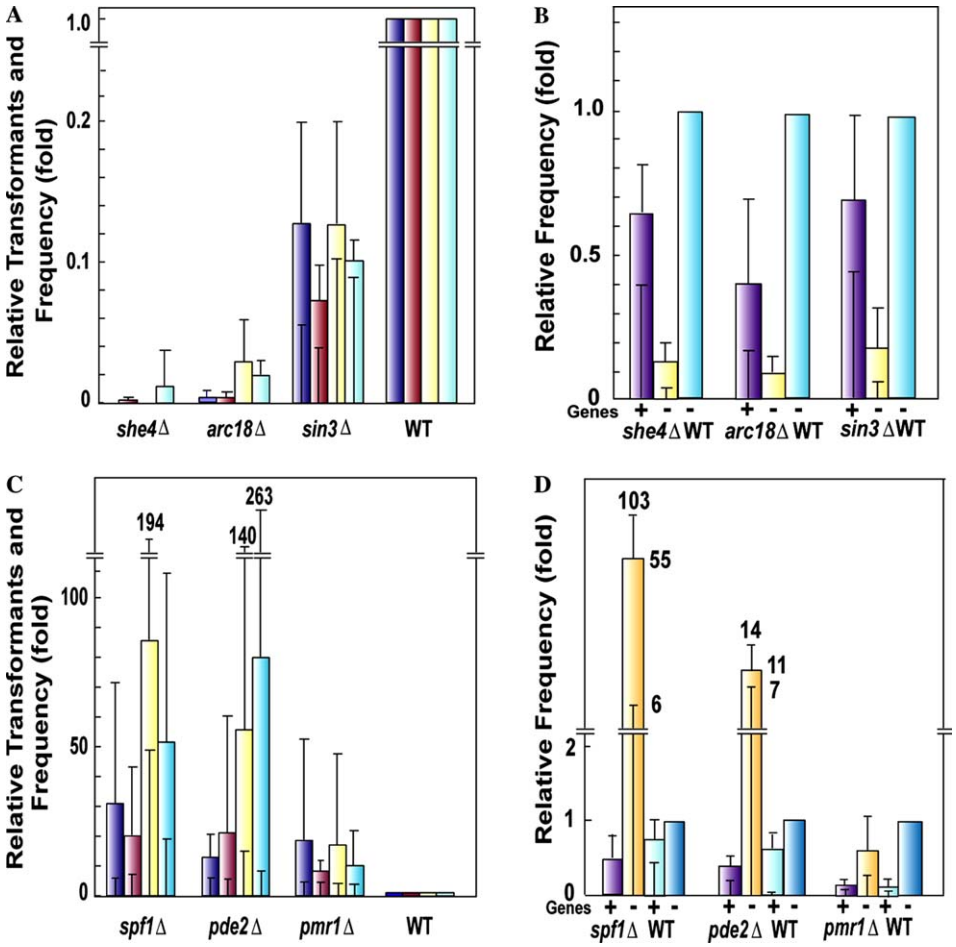

Fig. 1. Low- and high-competence mutants. (A) Relative numbers of transformants with YEp13 (2.5 μg; purple) and YCp50 (2.5 μg; red), and frequency ([transformants] [viable cell numbers]$^{-1}$) with YEp13 (yellow) and YCp50 (light blue), using WT (BY4742) as 1.0. (B) Complementation tests. Relative frequency with YEp13 (2.5 μg) of mutants and WT carrying corresponding native gene in centromeric vector (+) or vector alone (−), using each WT/vector frequency as 1.0. (C) Relative numbers of transformants and frequency as in (A). (D) Complementation tests. Relative frequency of mutants and WT carrying corresponding native genes in 2-μm vector (+) or vector alone (−), as 1.0 as in (B). Averages and minimum/maximum of three (A, B, D) and seven (C) independent experiments are shown.

Arp2/3 activation machinery is required for competence

arc18Δ, which had the second lowest competence (Fig. 1A), lacks a subunit of the Arp2/3 complex [9]. The Arp2/3 complex consists of seven subunits (Arp2p, Arp3p, Arc35p, Arc19p, Arc18p, Arc15p, and Arc40p), each of which has been shown to contribute differently to the assembly and function of the complex [9]. We thus attributed the low competence of *arc18Δ* to the dysfunction of the Arp2/3 complex, although we could not assess the competence of mutants lacking each of the subunits other than Arc18p because these mutants were not included in our mutant set.

The Arp2/3 complex functions in actin assembly [10]. Regarding the function of the complex, it has been reported that Myo3/5p, Vrp1p, and Las17p cooperate to activate the Arp2/3 complex and to control Arp2/3-mediated actin assembly [11–13], and that Pan1p also activates the Arp2/3 complex [14]. Our conclusions that the dysfunctions of Myo3/5 and the Arp2/3 complex cause low competence lead to a hypothesis that activation of the complex is required for competence. To substantiate the hypothesis, we rechecked the competence of *vrp1Δ* and checked the competence of *las17Δ* [11,13], *pan1-9Δ*, and *pan1-20Δ* [6], none of which were included in our mutant set but were kindly provided as indicated. As expected, we found that *vrp1Δ*, *las17Δ*, *pan1-9Δ*, and *pan1-20Δ* showed low competence (Fig. 2B)—a finding also confirmed by the complementation test (Fig. 2C). We therefore concluded that the functional machinery consisting of Myo3/5p, Vrp1p, Las17p, Pan1p, and Arp2/3 complex—which we tentatively term Arp2/3 activation machinery—is required for competence. Although the cooperation of Pan1p with other components (Myo3/5p, Vrp1p, and Las17p) has not been evidenced biochemically, a genetic interaction between *LAS17* and *PAN1* [14] indicates their functional cooperation as machinery. Functional redundancies of

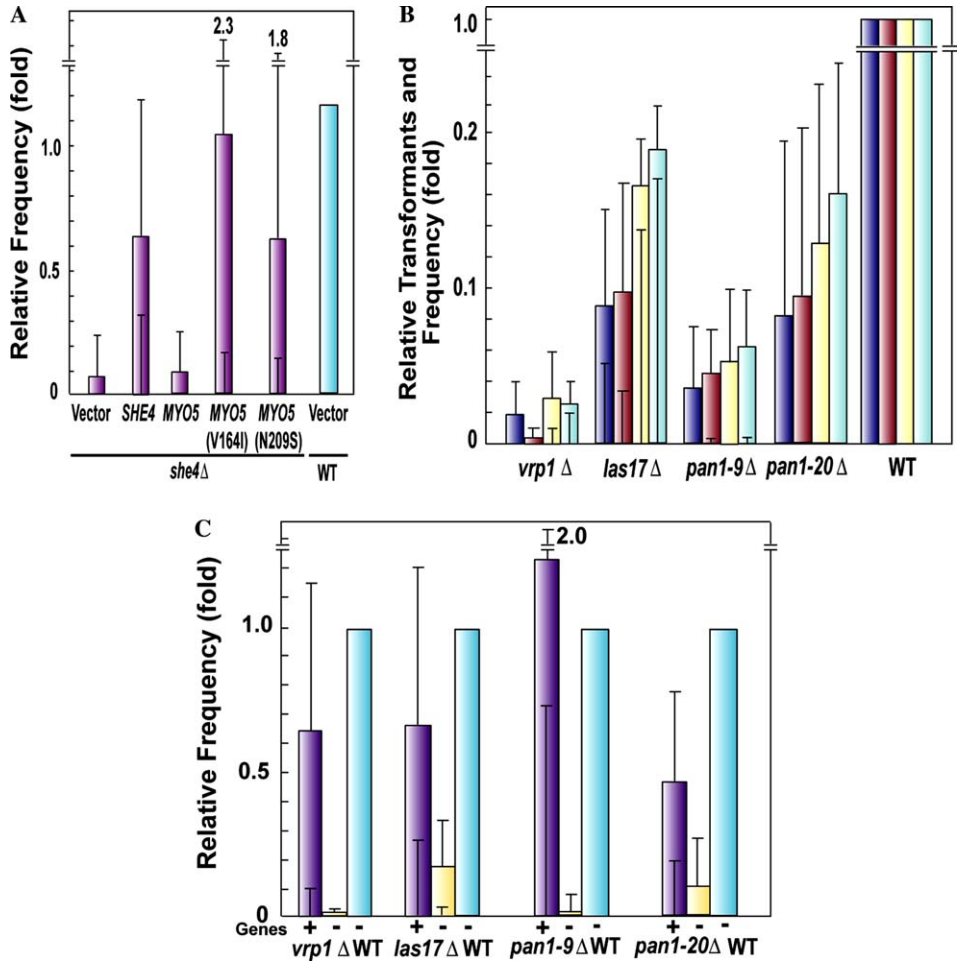

Fig. 2. Competence involves Arp2/3 activation machinery. (A) Complementation tests for *she4Δ*. Relative frequency with YEp13 (2.5 μg) of *she4Δ* carrying each gene in 2-μm vector, using each WT/vector frequency as 1.0. In every experiment, the competence of *she4Δ* carrying each vector alone and native *MYO5* was always lower than those of others. (B) The competence of mutants lacking in Arp2/3 activation machinery as in Fig. 1A, except that WT for *pan1-9Δ* and *pan1-20Δ* were T8-1D and SEY6210, and that pRS413 (2.5 μg; purple) and YCp50 (2.5 μg; red) were used for *las17Δ*. (C) Complementation tests. Relative frequency of mutants and WT as in Fig. 1B carrying corresponding native genes in centromeric vector (+) or vector alone (−), except that pRS413 (2.5 μg) was used for *las17Δ*. In every experiment, the competence of the mutant-carrying vector alone was always lower than that of the mutant-carrying native gene. (A–C) Averages and minimum/maximum of four independent experiments are shown.

the components in the Arp2/3-activation machinery [8,11–14] would explain why no mutants of the component showed a complete lack of competence.

Endocytosis is possibly required for uptake step of DNA

Endocytosis is a vesicular transport pathway used by eucaryotic cells to internalize plasma membrane molecules, extracellular fluid, and particles [15,16]. In *S. cerevisiae*, endocytosis has been assayed through α-factor internalization (receptor-mediated endocytosis) and the uptake of lucifer yellow (fluid-phase endocytosis) and of FM4-64 (membrane uptake), through which almost all endocytic mutants have been identified [5,6,11,13–21]. Accumulating data from eucaryotic cells including *S. cerevisiae* has led to the proposal of two models for the internalization step for endocytosis, i.e., (i) the nucleation and polymerization of actin itself provide a force pushing vesicles away from the plasma membrane, and (ii) along actin filaments, the vesicle is transported into cytosol from the membrane by molecular motor Myo3/5p, which can bind actin and possibly membrane [16]. For each case, an activation of the Arp2/3 complex, requiring Myo3/5p, Vrp1p, and Las17p, has been proposed as required [16]. Accordingly, endocytic defects have been reported in *arp2-1Δ* [17], *arp35Δ* (*end9-1Δ*) [18], *myo3Δ myo5-1Δ* [19], *vrp1Δ* [13], and *las17Δ* [11,13], and in *she4Δ* [5,6]. Pan1p is also regarded as being involved in endocytosis, because Pan1p interacts with other endocytic components such as Ent1/2p [14] and *pan1-20Δ* also showed endocytic defects [6]. Thus, from our conclusion that Arp2/3 activation machinery is required for competence, we propose that endocytosis is required for the DNA uptake step into *S. cerevisiae*. This proposition appears to be supported in part by the fact that (i) we found no

mutants completely lacking in competence in nonessential gene mutants defective in cell membrane proteins, indicating that endogenous DNA may not be transported into cell through membrane-located proteinous transporters; and that (ii) competence is enhanced temperature-dependently [4].

As a result of our reassessment of the competence of other endocytic mutants included in our set, such as *sac6Δ*, *end3Δ*, *rvs161Δ*, *rvs167Δ*, *akr1Δ*, *erg2Δ* [15,16], *sla1Δ* [20], *ip6kΔ* (*kcs1Δ*), and *ipmkΔ* (*arg82Δ*) [21], we found, however, that they showed no low competence (usual competence). The interpretation for this result is as follows: endocytosis for DNA delivery simply requires Arp2/3 activation machinery and not other well-known endocytic components, such as Sac6p, End3p, Rvs161p, Rvs167p, Akr1p, Erg2p, Sla1p, Kcs1p, and Arg82p, and therefore requires no ergosterol and inositol pyrophosphates, which are biosynthesized by Erg2p and Kcs1p/Arg82p [16,21], also indicating that endocytosis for DNA delivery differs, at least in part, from well-known endocytosis characterized previously using α-factor, lucifer yellow, and FM4-64. Indeed, in *S. cerevisiae*, the unique invagination of plasma membranes has been observed, where invagination is distinguished from that for receptor-mediated endocytosis and appears to be associated with actin patch components [22], leading us to presume that DNA may be taken up through invagination in an endocytic manner.

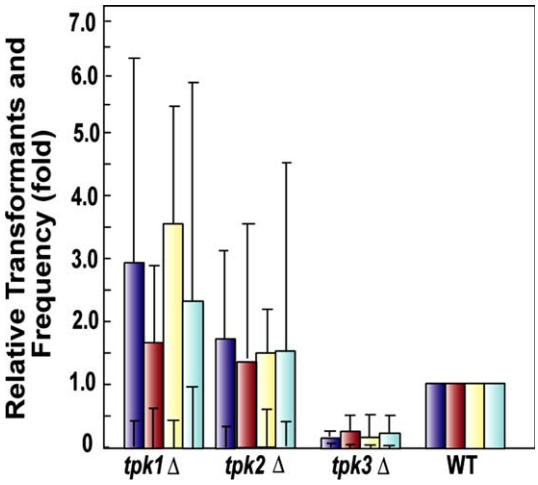

Fig. 3. Competence involves PKA. Competence of mutants deleted in each of the three catalytic subunits of PKA, *tpk1Δ*, *tpk2Δ*, and *tpk3Δ*, as in Fig. 1A. Average and minimum/maximum of four independent experiments are shown. In every experiment, the competence of *tpk3Δ*, was always lower than that of *tpk1Δ* and *tpk2Δ*.

DNA is not delivered to the nucleus through the same retrograde pathway as that for killer toxin K28

It has been proposed that the killer toxin K28 polypeptide is uptaken by endocytosis, to travel to the nucleus via Golgi to ER retrograde transport in *S. cerevisiae* [23]. A basis for this proposition is that *erd1Δ* and *cne1Δ* showed resistance to the toxin [23]. We rechecked the competence of *erd1Δ* and *cne1Δ* and found

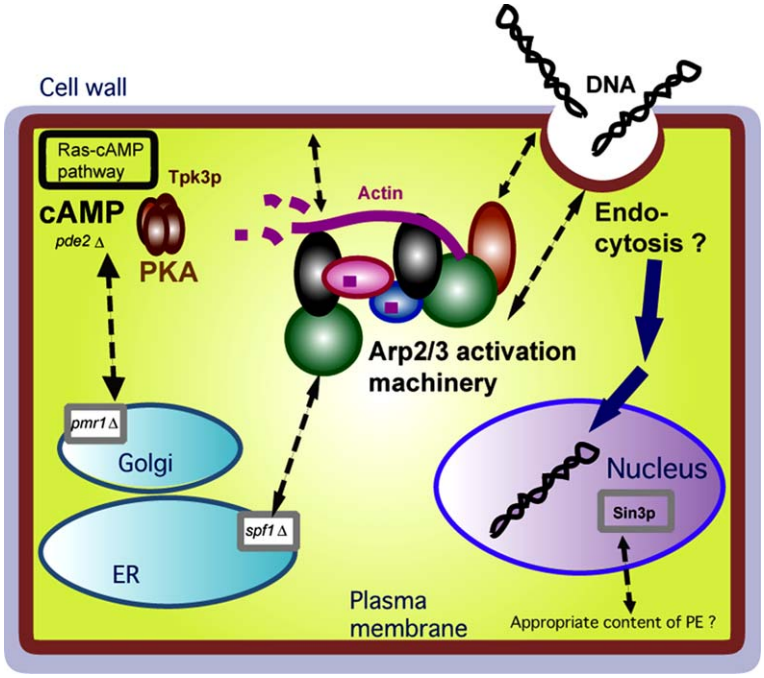

Fig. 4. Molecular insights on DNA delivery into *S. cerevisiae*. Dashed arrows indicate interactions among components involved in competence as described in the text. Arp2/3 activation machinery consists of Arp2/3 complex (green), Myo3/5p (black), Vrp1p (purple), Las17p (blue), and Pan1p (brown) [11–14].

that none of the mutants show low competence, indicating that DNA is not delivered to the nucleus via the same route traveled by killer toxin K28. We also did not detect low competence in temperature-sensitive mutants, even in an unpermissive condition (Materials and methods), such as *sec1-1Δ* [24], *sec20-1Δ*, *sec21-1Δ*, *sec27-1Δ*, *tip20-5Δ* [25], *sec61-2Δ* [26], and *use1Δ* (*use1-0 layer*) [27], where the mutants lack in retrograde- or vesicular-transport relating essential genes.

Proper content of phosphatidylethanolamine is possibly required for competence

We found another mutant, *sin3Δ*, had low competence (Fig. 1A). Sin3p is a negative regulator of early metiotic genes, the *HO* gene, the *TRK2* (low-affinity K^+ transporter) gene, and several phospholipid biosynthetic genes [28,29]. Although it is well accepted that Sin3p functions in concert with Ume6p and Rpd3p in several cases [28], *ume6Δ* and *rpd3Δ* showed no low competence, indicating that effects caused by the disruption of *SIN3* alone relate to low competence. One remarkable phenotype of *sin3Δ* is the lack of phosphatidylethanolamine (PE) in membranes [28]. It has been proposed that this compound affects membrane properties [30]. We thus attribute the low competence of *sin3Δ* to the decreased content of PE in membranes, impairing the proper function of membranes required for DNA delivery. Taken together with the above results, we suggest that PE, not ergosterol and inositol pyrophosphates, is required for competence.

Increased cAMP enhances competence via protein kinase A

pde2Δ, which lacks high-affinity cAMP phosphodiesterase [31], showed high competence (Fig. 1C). In *pde2Δ*, basal cAMP is elevated [32]. *pde1Δ* lacking low-affinity cAMP phosphodiesterase where the cAMP level is not affected [32] showed no high competence. We therefore predicted that elevated basal cAMP enhances competence. Tpk1p, Tpk2p, and Tpk3p are redundant catalytic subunits of protein kinase A (PKA), which is a major cAMP target in the Ras–cAMP pathway [32]. As shown in Fig. 3, *tpk3Δ* exhibited lower competence than *tpk1Δ* and *tpk2Δ*, suggesting that the Ras–cAMP pathway, where Tpk3p is installed in PKA, enhances competence, and that the pathway involving Tpk1p or Tpk2p in PKA decreases competence. We failed to determine the target of PKA, however, because we detected no effect on the competence of *msn2Δ*, *msn4Δ*, *rim15Δ*, or *sok2Δ*, in which known transcription factors controlled by PKA are disrupted [32]. The genome-wide transcriptional profiling of mutant deleted individually in *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* [33] should aid in understanding how cAMP and PKA influence competence. Our findings on the competence of *tpk1Δ*, *tpk2Δ*, and *tpk3Δ* should also facilitate an understanding of the individual functions of the three catalytic subunits, especially of Tpk3p, whose function has not been well assigned by genome-wide transcriptional profiling [33].

High competence of pmr1Δ and spf1Δ is not caused by dysfunction of cell wall structure, increased expressions of calcineurin-dependent genes, and unfolded protein response

It has been proposed that Spf1p, as an endoplasmic reticulum-located P-type ATPase, is involved in Ca^{2+} homeostasis in ER [34,35]. Pmr1p is also Golgi-located Mn^{2+}/Ca^{2+} P-type ATPase [36]. Disruption of *PMR1* or *SPF1* thus alternates ER-Golgi functions, resulting in a wide variety of phenotypes including dysfunction of the cell wall structure, elevated expression of calcineurin-dependent genes, and the activation of unfolded protein response (UPR) [34–36].

We could not attribute the high competence of *pmr1Δ* and *spf1Δ* to the dysfunction of the cell wall structure, being a lack of glycosylation of cell wall proteins (mannoprotein) and the consequent lack of negative-charged phosphomannose [37], because we could not confirm high competence in mutants [37] deficient in N-linked (*mnn9Δ* and *och1Δ*), O-linked glycosylation (*kre2Δ*), and phosphomannosylation (*mnn6Δ*). In *pmr1Δ* and *spf1Δ*, the expression of specific genes such as *FKS2*, *PMC1*, and *ENA1* is elevated calcineurin-dependently [35], leading to the hypothesis that high competence is caused by the high expression of calcineurin-dependent genes. This hypothesis appeared to be denied, however, because (i) WT (grown in the presence of 30 μg/ml calcineurin inhibitor, cyclosporin A) and (ii) *crz1Δ* and *cnb1Δ* showed no low competence, where the expression of calcineurin-dependent genes is perturbed in the presence of cyclosporin A [35] and in *crz1Δ* and *cnb1Δ* [38]. In *spf1Δ*, but not in *pmr1Δ*, UPR is constitutively activated [35]. Although we assumed that UPR increased competence in at least *spf1Δ*, this supposition was also ruled out because (i) *hac1Δ* and *ire1Δ* showed no high competence, where *hac1Δ* is a UPR-deficient strain and *IRE1* is a key component in UPR; and (ii) WT also exhibited no high competence when grown in the presence of 1 μg/ml tunicamycin or 4 mM DTT, both of which activate the UPR of the WT to the same degree as *spf1Δ* [35].

The double mutants *tpk1^{w}Δpmr1Δ*, *spf1Δarp2-33Δ*, and *spf1Δarc40-40Δ* have been shown to be lethal [39,40], where *tpk1^{w}Δ* has elevated cAMP [39], suggesting a relationship between cAMP and Pmr1p, and between Spf1p and the Arp2/3 complex. These genetic interactions may be associated with the mechanism of competence.

Perspectives

As detailed above, we obtained molecular insights on *S. cerevisiae* competence (DNA delivery into this

organism) (Fig. 4). Many questions remain to be clarified, however, e.g., how Arp2/3 activation machinery participates in the DNA uptake step, probably via an endocytosis-like event, how the event can be distinguished from other endocytosis, and how DNA travels to the nucleus. We have not yet elucidated how high cAMP increases competence via PKA, and why *pmr1Δ* and *spf1Δ* show high competence. This report is, to the best of our knowledge, the first detailing molecular insights on DNA delivery into *S. cerevisiae*, a suitable point for elucidating the system of this organism and those of other eucaryotic cells.

Acknowledgments

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