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Involvement of Moc1 in Sexual Development and Survival of *Schizosaccharomyces pombe*

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Abstract

The *moc1* gene in fission yeast *Schizosaccharomyces pombe* was identified as to overcome the sterility caused by high expression of adenylyl cyclase. The *moc1* gene was found to be identical with *sds23* and *psp1*. Although *psp1* was reported to be essential for growth, *sds23* was not. To clarify this apparent discrepancy, we first assessed independently the phenotypes of the *moc1* disruptant. We confirmed the deletion mutant of *moc1* is sterile, sensitive to high salt, grew slowly at higher and lower temperature, and mutant cells were elongated. Besides those phenotypes, we here showed that viability of the *moc1* disruptant was rapidly lost at the stationary phase. We confirmed that the Moc1 protein is phosphorylated in the stationary phase. We then examined the significance of this phosphorylation of Moc1 by creating the S333A or S333D mutant type of Moc1. Interestingly while S333D mutant Moc1 is lower in inducing ability of sexual development, S333A mutant Moc1 is higher in that than wild type, suggesting that phosphorylation of Moc1 affects sexual development. The other phenotypes such as sensitivities to high salt and higher temperature and elongation of cells were not affected by mutation of S333A nor S333D. We found that the Moc1-GFP localized to both the cytosol and the nucleus during mitotic growth but accumulated in the nucleus in mating cells and then enriched in spores, and this localization shift was negatively regulated by the cAMP pathway. This and above observations suggested that the nuclear localized Moc1 can be an inducer of sexual development. Thus, in addition to the roles of *moc1*/*sds23*/*psp1* in mitosis and stress response, it is also important for survival and sexual development of fission yeast, but phosphorylation of Moc1 only affects the sexual development.

Key words: *S. pombe*; sexual development; meiosis; *moc1*

The fission yeast *Schizosaccharomyces pombe* proliferates continuously when it has abundant nutrients, but it arrests its cell cycle progression in the G_1 phase after nitrogen depletion. It then proceeds sexual development that includes conjugation, meiosis and sporulation. The sexual development that precedes meiosis is mainly regulated by three signaling pathways, namely, the cAMP pathway, the stress responsive pathway, and the pheromone signaling pathway.[1,2] The cAMP pathway transmits nutrient condition, such as glucose or nitrogen level. When glucose (or nitrogen) is abundant, the heterotrimeric type guanine nucleotide-binding protein (Gpa2) becomes activated;[3] this subsequently activates adenylyl cyclase (Cyr1) [4] to generate cAMP from ATP.[5] Cyr1 interacts with its associated protein Cap1, which plays partly a regulatory role of adenylyl cyclase and also interacts with actin.[6,7] When cAMP is abundant, it associates with the regulatory subunit Cgs1 and the catalytic protein kinase Pka1 is released.[8] Pka1 phosphorylates inhibitory the zinc-finger protein Rst2, which otherwise induces the expression of *ste11*.[9] Ste11, which regulates the transcription of many genes involved in meiosis, including *mat1-Pm*, *mat1-Mm, ste6* and *mei2*.[10] The *mei2* gene encodes an RNA-binding protein that is negatively regulated by Pat1 protein kinase.[11] Pat1 is inhibited by Mei3, whose expression is induced by the pheromone signaling pathway. These are the central regulatory system of triggering meiosis. In an alternative regulatory pathway, Pat1 can be inhibited by a truncated version of Sla1, an ortholog of the mammalian La protein.[12,13]

Overexpression of the *cyr1* gene results in sterility because cAMP level is kept high even under nutrient starved condition.[5] On the contrary, a *cyr1* mutant undergoes sexual development even under the nutrient rich condition.[5] Thus the level of cAMP controls the switch from mitotic cell cycle and meiotic cell cycle. The similar phenotype with the *cyr1* mutant was found in *sam* mutants, which undergo mating and sporulation without requiring nitrogen starvation.[14] Those *sam* mutants and suppressors were characterized in our group.[15,16]

Previously, multi-copy suppressors of the sterile strain caused by the *cyr1* over-expression were screened out.[17] Four positive clones were isolated and they were designated *moc1-4* (multicopy suppressor of overexpressed *cyr1*). The *moc2*/*ded1* gene was firstly characterized as the gene that is highly homologous to the RNA helicase *DED1* of *Saccharomyces*

cerevisiae.[17] The *moc3* and *moc4* genes were subsequently found to encode the Zn finger type proteins, but their Zn finger motifs are different.[18] The *moc1* gene was found to be identical with *sds23* which was isolated as multi-copy suppressor for mutations in PP1 and the 20S cyclosome/APC,[19] and also identical with *psp1* which was obtained as a suppressor of temperature sensitive mutant defective in cell cycle progression.[20] Both *sds23* and *psp1* were isolated through the screening in mitotically aberrant mutants,[19,20] but *moc1* was isolated as the inducer of sexual development in *S. pombe*.[17] The orthologous genes *SDS23* and *SDS24* in *S. cerevisiae* are functionally interchangeable with *S. pombe moc1*.[21] It was thought that *SDS23* and *SDS24* are involved in the antagonizing pathway against the cAMP pathway from the observation that *sds23*Δ *sds24*Δ strain produced larger vacuoles and formed pseudohypha.[21]

In this study, we first assessed the previous observation in *moc1/sds23/psp1* and then focused on the role of *moc1* in sexual development and the significance of phosphorylation of Moc1.

Materials and Methods

Strains and media. The *S. pombe* strains used in this study are listed in Table 1. *S. pombe* was grown in YES-rich medium (0.5% yeast extract, 3% glucose, 75 mg/liter adenine, histidine, leucine, uracil, and/or lysine hydrochloride) or Pombe Minimum (PM) synthetic medium,[22,23] supplemented with 75 mg/liter adenine, leucine, and/or uracil when necessary. Nitrogen-free PM medium (1% glucose without ammonium chloride) were used to culture *S. pombe* when the mating efficiency had to be measured. *Escherichia coli* strains DH5α was used for plasmid manipulation. *E. coli* was grown in LB medium (1% polypepton, 0.5% yeast extract, 1% NaCl, pH 7.2).

Table1

Plasmid construction. The plasmid pHA-moc1-U was constructed as follows: The *moc1* cDNA was amplified by PCR using 24BS-U and 24SA-U primers. The PCR product was digested with *Sal*I and *Sac*I and inserted into pALY1 which has *adh1* promoter and HA

sequence.[6] pHA-moc1S333A-U or pHA-moc1S333D-U was constructed in the similar ways by PCR using two additional sets of primers namely, moc1-SA1 and moc1-SA2, or moc1-SD1 and moc1-SD2, respectively (Table 2).

Strain construction. The *moc1*-disrupted strain was constructed as follows: An upstream non-cording region (-500 – -100bp) of *moc1* from wild type *S. pombe* SP870 genome was amplified by PCR using moc1kanF5 and moc1kanF3 primers that contain pFA6a-specific DNA sequences. Approximately a 500bp fragment from downstream of the stop codon of the *moc1* gene from SP870 was amplified by PCR using the moc1kanR5 primer that contains pFA6a-specific DNA sequence and the moc1kanR3 primer.[24] In the second PCR, *kanMX6* of pFA6a-kanMX6[24] was amplified using the first PCR products. The strain SP870 was transformed with the second PCR product and stable G418 resistant transformants were selected to obtain the *moc1*Δ strain. The proper integration of the *moc1* disruption was verified by Southern blot analysis (data not shown) and the resulting strain was named MYM1 (*moc1*Δ::*kanMX6*).

Table 2

The tag-integrated strains were constructed as follows: Approximately a 500bp fragment of 3' region of the *moc1* gene from SP870 was amplified by PCR using moc1-W and moc1-X primers that contain pFA6a-specific DNA sequences. The downstream fragment of the *moc1* gene was amplified with the same method as used in *moc1* disruption. In the second PCR, tagging fragment was amplified using the first PCR products and pFA6a-3HA-kanMX6 or pFA6a-GFP (S65T)-kanMX6 as template.[24] The wild type *S. pombe* strain SP870 was transformed with the second PCR product and stable G418 resistant tansformants were selected to obtain the tag-integrated strain. The proper integration of the *moc1-3HA* or *moc1-GFP* (S65T) integration was verified by Western blot analysis and the resulting strain was named MYM2 (*moc1*-3HA tagging) or MYM3 (*moc1-GFP* (S65T) tagging). MYM7, MYM9 and MYM11 were constructed in the similar ways with MYM3 construction from MK251, JZ666 and JZ858 respectively. Both DY114 and MYM3 were crossed and a *cyr1* disrupted strain with having *moc1-GFP* (S65T) was obtained by random spores analysis. The resulting

strain was named MYM16. In the similar manner, both JZ633 and MYM3 were crossed and a *pka1* disrupted strain with having *moc1-GFP* (S65T) was obtained by random spores analysis. The resulting strain was named MYM19.

The *moc1* mutants were constructed as follows: Approximately a 500bp fragment of 3' region of the *moc1* gene of pHA-moc1S333A-U or pHA-moc1S333D-U which carries S333A or S333D mutation respectively was amplified by PCR using moc1-W and moc1-X primers that contain pFA6a-specific DNA sequences. The downstream fragment of the *moc1* gene was amplified with same method as in *moc1* disruption. In the second PCR, a tagging fragment was amplified using the first PCR products from pFA6a-GFP (S65T)-kanMX6 as template. The wild type SP870 was transformed with the second PCR products and stable G418 resistant transformants were selected to obtain the tag-integrated strain. The proper integration of *moc1*^{S333A}*-GFP* (S65T) or *moc1*^{S333D}*-GFP* (S65T) integration was verified by Western blot analysis and sequencing (data not shown). The resulting strains were named MYM14 (*moc1*^{S333A} mutant) and MYM15 (*moc1*^{S333D} mutant), respectively.

Western blotting. Approximately 1x10^8 cells were harvested after growth in the appropriate medium, washed twice with dH_2O, dissolved in 100 μl of dH_2O, and samples were boiled at 95˚C for 5 min. Subsequently, 120 μl of 2x Laemmli buffer (4% SDS, 20% glycerol, 0.6M β-mercaptoethanol, 8M urea, 0.12M Tris-HCl [pH 6.8]) was added and the samples were vigorously vortexed with acid-washed glass beads for 3min. The samples were sonicated for 1min and heated at 95˚C for 5min. The glass beads and large debris were removed by centrifugation at 16,000 x g for 1min (Fig. 4A lane 5 and 6 and Fig. 7). As an alternative method for protein extraction, cells were harvested and washed twice with dH_2O and then washed with Stop buffer. Subsequently, 50μl of L buffer (50mM Tris-HCl (pH 8.0), 150mM NaCl, 1% Nonidet-P40, 5mM EDTA, 10% glycerol, 1mM PMSF (BRL), and Complete Protease Inhibitor Cocktail Tablets (Roche)) were added and the sample was vigorously vortexed with acid-washed glass beads for 3min. 200 – 500 μl of L buffer was added and mixed. The glass beads and large debris were removed by centrifugation at 16,000 x g for

20min twice. Equal volume of 2xSDS buffer (0.1M Tris-HCl (pH 6.8), 0.6M β-mercaptoethanol, 4% SDS 0.2% BPB, 20% glycerol) was added to the sample and heated at 95˚C for 7min (Fig. 4 except A lane 5 and 6). Each sample were analyzed on SDS-PAGE with a 7.5% or 10% polyacrylamide gel and then transferred to Immobilon Transfer Membranes (Millipore) by using a wet-type transfer system or semidry transfer system. To detect HA fusion proteins, the membrane was incubated with an anti-HA monoclonal antibody (Santa Cruz Biotechnology, Inc.) diluted 1:3,000 in 5% dry milk in PBS-T (137mM NaCl, 8mM $Na_2HPO_4 \cdot 12H_2O$, 2.7mM KCl, 1.5mM KH_2PO_4, 0.1% Tween20). The membrane was washed by PBS-T for 15min and 5min twice per wash and then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.) diluted 1:3,000 in 5% dry milk in PBS-T. To detect GFP fusion proteins, the membrane was incubated with an anti-GFP monoclonal antibody (Roche) diluted 1:2,000 in 5% dry milk in TBS-T (20mM Tris, 137mM NaCl, 0.2% Tween20). The membrane was washed three times by TBS-T for 5min per wash and then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.) diluted 1:3,000 in 5% dry milk in TBS-T. After the membrane was washed, the secondary antibodies were detected with the ECL system as described by the manufacturer (Amersham).

Fluorescence microscopy. Cells were grown in nitrogen-free PM medium at 30˚C. Moc1-GFPs were observed in living cells by staining the cells with 4', 6-diamidino-2-phenylindole (DAPI; SIGMA) under the BX51 (OLYMPUS) microscope. Fluorescence images were taken with a digital camera DP70 (OLYMPUS) connected to the microscope.

Results

We first independently assessed the phenotypes of the *moc1* disruptant. The *moc1* disruptant was newly derived as described in Materials Methods. The *moc1* disruptant (MYM1) was viable but displayed many aberrancies including sterility, sensitivity to high salt, retarded growth at higher and lower temperatures (Fig. 1, and see Fig. 6). Cells of the *moc1* disruptant were also elongated (data not shown). The same phenotypes were observed in the previously

constructed *moc1* deleted strain [21] and also in the one reported by Ishii *et al.* [19] Beside those phenotypes, we here show that the *moc1* disruptant is lower in viability at the stationary phase (Fig. 2). The viability of the *moc1*Δ cells decreased rapidly after prolonged incubation for 2-7days after the stationary phase, while that of wild type did not decrease so much. Including this phenotype and those previously described phenotypes, those various phenotypes of the *moc1* disruptant suggested that *moc1* is at least involved in sexual development, survival, mitosis and stress response. Fig.1

We then observed the localization of the Moc1-GFP protein. As the localization of Moc1 in the sexual development was not shown before, we made the *moc1-GFP* fusion gene and this fusion gene was integrated onto the chromosome of wild type *S. pombe* strain. When we observed the localization of Moc1-GFP in the constructed strain named MYM3, it localize in both the nucleus and cytosol during cell growth, and then it accumulated in the nucleus when cells start mating and finally it enriched in spores (Figs. 3, and 5). Thus, Moc1-GFP changes its localization during the process of sexual development. Because *moc1* was initially isolated as the suppressor of the *cyr1* over expressed strain, we thought that the localization might be affected by different condition of the cAMP levels. To know how the cAMP pathway affect the localization of Moc1-GFP, we examined its localization in *cyr1*Δ, *pde1*Δ, *pka1*Δ, and *cgs1*Δ strains, and also in the *cyr1* over expressed strain. It is known that the *cyr1* gene encodes adenylyl cyclase that generates cAMP from ATP,[5] while *pde1* encodes cAMP phosphodiesterase that generates AMP from cAMP.[25] Both *pka1* and *cgs1* encode the catalytic and regulatory subunits of protein kinase A, respectively. [8,26] Thus, it will be expected that *cyr1* and *pka1* mutations have similar effect and *cgs1* and *pde1* mutations or overexpression of *cyr1* have the opposite effect. To observe the localization in those mutants background, the *moc1-GFP* fusion gene was integrated in various strains as describe in Materials and Methods (Table 1). The proper construction of those strains was first verified by PCR and the production of the Moc1-GFP protein in all constructed strains were verified by Western blotting using a GFP antibody (Fig. 4). As the results, we found Moc1-GFP did not localize at the nucleus in *cgs1*Δ, *pde1*Δ and *cyr1*-overexpressed strains, while we found it localized in Fig.2

both the nucleus and the cytosol in wild type strain. It looks like nuclear localization of Moc1-GFP is extensively excluded in *cgs1*Δ, *pde1*Δ and *cyr1*-overexpressed strains (Fig. 3). This result indicated that an enhancement of the cAMP pathway reduced its localization of Moc1-GFP in the nucleus. But, on the contrary, Moc1-GFP can be found in the nucleus before and after mating in *cyr1*Δ and *pka1*Δ mutants. (Fig. 3). Thus, the nucleus localization of Moc1-GFP was negatively regulated by the cAMP signaling pathway. Fig.3

We next examined the significance of phosphorylation of Moc1 during different cell growth stage. As it was previously reported that Psp1 is phosphorylated during the stationary phase, [20] we independently re-examined the phosphorylation of Moc1. Samples from the strain that expressed the *moc1-HA* gene on the plasmid were taken in the different growth stage (Fig. 4B). As the result, we observed that the mobility shift of the Moc1 protein on SDS-PAGE in the samples drawn from stationary phase cultures (19-39h), but not so much in the sample from the log phase culture (12h) (Fig. 4B). This upper-shifted Moc1 was observed continuously grown cultures and those bands were downshifted by phosphatase treatment (Fig. 4B). The upper-shifted band of Moc1 was also observed in the stain in which the *moc1-HA* gene was integrated on the chromosome (Fig. 4C). This result indicated that over-expression of *moc1-HA* on the plasmid did not cause any biased observation. Thus, in fact, Moc1 is phosphorylated during the stationary phase as previously reported. Because it was reported that the site of phosphorylation is S333, we mutated this S333 site to alanine and aspartate residues to test the effect of mutation on its localization, expecting the localization of Moc1 might be changed by mutation of S333A or S333D. Prior to the observation, we first verified the band shift of Moc1 was abolished in the S333A mutation, indicating the phosphorylation of Moc1 occurs in S333 (Fig. 4D). When we observed the localization of Moc1-S333A-GFP and Moc1-S333D-GFP and compared with wild type Moc1-GFP, no significant change of their localizations was observed (Fig. 5). Thus, we concluded that phosphorylation of S333 of Moc1 does not affect its nuclear localization of Moc1. Fig.4 Fig.5

To know the significance of S333 phosphorylation further, we then expressed those *moc1* mutants in MK141 (*cyr1*-over expressed) strain to see the difference in mating inducing ability.

The plasmids that expressed wild type *moc1*, *moc1*-S333A and *moc1*-S333D were constructed as described in Materials and Methods. When those genes were expressed in MK141 and mating ratio was measured, we found that there is a slight difference in inducing ability of mating. The *moc1*-S333A gene induced mating a little higher than wild type while *moc1*-S333D induced mating a little lower than wild type *moc1*, under the condition that MK141 harboring the vector alone mated and sporulated in the lower level (Fig. 6A). Because the replacement of Ser to Asp often mimic the phosphorylated status of proteins, the result suggested that phosphorylation of Moc1 reduces the function and non-phosphorylation of Moc1 enhances the function of Moc1. Although we could repeatedly observe this difference, we could not be certain in these mutational effects because the *moc1* genes were expressed by plasmids. To test the effect of mutations much precisely, we measured the mating efficiency in the above constructed strains, in which Moc1-GFP, Moc1-S333A-GFP and Moc1-S333D-GFP were expressed on the chromosome. In those strains, the only difference is the substitution of amino acid in S333 and expression levels were same according to the results of Western blotting (data not shown). As the result, the similar tendency was observed in S333A and S333D mutations (Fig. 6B). The *moc1*-S333A induced mating a little higher and *moc1*-S333D induced mating a little lower than wild type *moc1*. Thus, mutation of S333 affect the function of *moc1* in sexual development. Fig.6

We also tested whether the stress sensitivities were changed by S333A and S333D mutations, but we could not observe the clear difference in stress sensitivities in those mutants (Fig. 1). In addition, there was no clear difference in viability loss at the stationary phase among wild type and those mutants (Fig. 2). Thus, S333 mutations only affect mating efficiency at least within the level we can observe.

As phosphorylation of Moc1 affect mating efficiency, we finally tested the possibility of the Moc1 protein might be phosphorylated under nitrogen starved condition, because nitrogen starvation is the key condition to induce mating in *S. pombe*. As the results, we found the size of the Moc1 protein is shifted up during nitrogen starvation (Fig. 7). The upper shifted band is assumed to be a phosphorylated form, but we failed to conduct the experiment to visualize the

downshift of Moc1 by a phosphatase treatment, because proteins are very unstable under nitrogen starved condition.

Fig.7

Discussion

cAMP is a key second messenger for growth control and differentiation in many different organisms ranging from prokaryotes to mammals. The level of cAMP controls the switch from mitotic cell cycle and meiotic cell cycle in *S. pombe*[5] and cAMP is necessary for cell growth in *S. cerevisise*.[27] cAMP is also known to control the cell division in *E. coli*.[28] When the level of cAMP is high in *S. pombe,* cells become elongated, and this type of change was already known to exist in *E. coli*.[29] Some common mechanism for growth control by cAMP may underlie in prokaryotes and lower eukaryotes.

In the series of studies, we have been trying to solve the molecular mechanisms how the cAMP pathway is controlled and how the cAMP pathway interacts with other pathways. For this purpose, 4 different genes named *moc1-4* were isolated as suppressors that overcome the sterility caused by high expression of adenylyl cyclase.[17, 18] Among them *moc1* is higher in inducing ability of sexual development.[18] We here showed that Moc1 in *S. pombe* plays important roles in survival and sexual development in addition to the previous findings that indicate the involvement of *moc1/sds23/psp1* in mitosis.[19, 20] The Moc1 and its orthologous proteins contain a common domain, named CBS (cystathionine-beta-synthase), which is predicted to have a multiple trafficking function for protein-protein interaction and metabolic regulations found in the protein like AMP activating kinase [http://smart.embl-heidelberg.de]. But it is not yet clear how Moc1 works in sexual development through the interaction with yet unidentified proteins. At least we know Moc1 does not interact with Moc2, Moc3, or Moc4 proteins.[18] Further analysis will be required to look for some interacting proteins with Moc1.

There are some hits to predict the role of Moc1 by comparison with some other mutants that shows similar phenotypes with the *moc1* mutant. Cell elongation, sterility and stress sensitive phenotypes in the *moc1* disruptant are commonly found in mutants of *wis1* and *spc1*, which encode a MAP kinase kinase and a MAP kinase in the stress responsive pathway.[30] We

initially thought the relevance of Moc1 with this MAP kinase pathway because the stress sensitive phenotype of *spc1* is suppressed by over-expression of *moc1* (data not shown). In addition, the stress responsive pathway and the cAMP pathway are considered to function in an antagonizing way in sexual development, namely, activation of the cAMP pathway reduced the expression of *ste11*, while the activation of the stress responsive MAP kinase enhanced *ste11* expression.[31] Loss of viability at stationary phase found in *moc1* mutant was also found in the *cgs1* mutant [26] and the *zds1* mutant.[32] As *cgs1* encodes the regulatory subunit of protein kinase A, the activation of Pka1 reduced the viability loss at stationary phase. Loss of viability at stationary phase in the *moc1* mutant is consistent with the hypothesis that Moc1 antagonizes the cAMP pathway. Thus, four observations, ie., *moc1* is isolated as the suppressor of the *cyr1* over-expressed strain, the *moc1* disruptant is sensitive to salt, cells of the *moc1* disruptant are elongated and viability of the *moc1* disruptant was lost at stationary phase, consistently suggested that that *moc1* is antagonizing the cAMP pathway possibly through the stress responsive pathway or else.

We confirmed that the Moc1 protein is phosphorylated in the stationary phase and probably under nitrogen starved condition. We looked for the meaning of this phosphorylation by changing S333 to Asp or Ala. There was no apparent difference in localization of Moc1, sensitivity of salt and growth at higher temperature in the mutations of the phosphorylation site, except mating efficiency. As we found Moc1 localized in the nucleus during mating, we initially expected that the localization of Moc1 is affected by its phosphorylation. But we did not find any clear effect by replacement of S333 to Asp or Ala of Moc1. However we found the mating inducing ability was a slightly enhanced by S333A mutation and reduced by S333D mutation. This indicates the existence of the regulation of Moc1 function by phosphorylation. As the phosphorylation of S333 was thought to be conducted by Cdc2,[20] the regulation of Moc1 by Cdc2 during meiosis will be expected.[20]

We showed that Moc1-GFP localizes in both the cytosol and the nucleus during mitotic growth, but accumulated in the nucleus in mating cells, and then enriched in spores. This nucleus localization shift was reduced by an enhancement of the cAMP pathway. We first

expected that Moc1 is directly phosphorylated by Pka1, but we observed that Moc1 is also phosphorylated in the *pka1* mutant (data not shown), suggesting some other regulatory mechanism that controls Moc1 localization exists.

The orthologs of Moc1 can be found in eukaryotes apparent in fungi and also in plants or mammals with low but meaningful level of identity in amino acid sequences. Because no analysis of Moc1 orthologs except the ones from the budding yeast was conducted in other species, we do not know how significant their sequence similarities are. However, we believe those Moc1 types proteins have important biological functions, as we observed in *S. pombe* and *S. cerevisiae*.[21)]

In conclusions, Moc1 is a multifunctional protein that involves in not only mitosis and stress response, but also in survival and sexual development in *S. pombe*.

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Fig. 1. Temperature sensitivity and stress sensitivity in *moc1* disruptant and mutants. SP870 (wild type), MYM1 (*moc1*Δ), MYM3 (*moc1-GFP*), MYM14 ($moc1^{S333A}$-*GFP*) and MYM15 ($moc1^{S333D}$-*GFP*) cells were cultured at 30˚C in liquid medium until they reached log phase. They were concentrated to $2x10^7$ cells/ml and then diluted sequentially 5-fold (in the right-hand direction). The cells were spotted on YES plates and incubated at 30 or 36.5˚C for 3 days in the case without KCl or 4 days in the case with 1M KCl.

Fig. 2. Viability of *moc1* disruptant and *moc1* mutants. SP870 (wild type), MYM1 (*moc1*Δ), MYM3 (*moc1-GFP*), MYM14 ($moc1^{S333A}$-*GFP*) and MYM15 ($moc1^{S333D}$-*GFP*) were grown in YES medium at 30˚C. Cell numbers were monitored by cell counter (Sysmex). When cells entered early stationary phase (MYM1 is at $1x10^7$ cells /ml, and other strains are at $2x10^7$ cells /ml), cultures were spread onto YEA plate with appropriate dilution. Every 24h cells cultures were spread onto YEA plate, and plates were incubated at 30˚C. Numbers of living cells were counted by growing colonies. Viability curve was drawn as living cell numbers of first day is 100%. Square, SP870; circle, MYM1; triangle, MYM3; diamond, MYM14; upside down triangle, MYM15.

Fig. 3. Localization of Moc1-GFP in wild type, *cyr1* overexpressed, *pde1*Δ, *cgs1*Δ, *cyr1*Δ or *pka1*Δ cells. MYM3 (*moc1-GFP*), MYM7 (*cyr1* overexpressed, *moc1-GFP*), MYM9 (*pde1*Δ, *moc1-GFP*), MYM11 (*cgs1*Δ, *moc1-GFP*), MYM16 (*cyr1*Δ, *moc1-GFP*) and MYM19 (*pka1*Δ, *moc1-GFP*) were cultured in PM + adenine, leucine, and uracil or PM+ adenine and leucine, at 30˚C until mid-log phase. Cells were harvested, washed with dH_2O, and nitrogen-free PM medium and subsequently re-suspended in nitrogen-free PM medium. Cells were incubated at 30˚C. Cells were stained with DAPI and observed under the fluorescent microscope. 0hr,

before nitrogen starvation; 9hr, after 9h from nitrogen starvation.

Fig. 4. Detection of Moc1-GFP and phosphorylation of Moc1 in the stationary phase. (A) The Moc1-GFP protein in wild type, *cyr1* overexpressed, *pde1*Δ, *cgs1*Δ, *cyr1*Δ or *pka1*Δ cells. Cells were grown in YES at 30˚C and crude proteins were extracted. The samples were analyzed by SDS-PAGE with a 10% polyacrylamide gel. M, Maker, Dr. Western (Oriental Yeast Co., LTD); lane 1, MYM3 (*moc1-GFP*); lane 2, MYM9 (*pde1*Δ, *moc1-GFP*); lane 3, MYM11 (*cgs1*Δ, *moc1-GFP*); lane 4, MYM7 (*cyr1* overexpressed, *moc1-GFP*); lane 5, MYM16 (*cyr1*Δ, *moc1-GFP*); lane6, MYM19 (*pka1*Δ, *moc1-GFP*). (B) phosphorylation of Moc1 in the stationary phase. TF1 (*moc1*Δ) harboring pHA-moc1-U cells were grown in PM + adenine at 30˚C until log phase (from 12h) to stationary phase (19h-39h) and crude proteins were extracted. Samples were treated by lambda protein phosphatase (NEW ENGLAND BioLabs Inc.) (+) or not (-). The samples were analyzed by SDS-PAGE with a 7.5% polyacrylamide gel. The upper arrow is the phosphorylated Moc1 and lower one is the non-phosphorylted Moc1 (C) Moc1 phosphorylatioin in MYM2 cells at the stationary phase. MYM2 (*moc1*-3HA) cells were grown in YES at 30˚C until log phase (for 15h) or stationary phase (for 36h). (D) Phosphatase treatment of the $Moc1^{S333A}$ mutant. TF1 (*moc1*Δ) harboring pHA-moc1S333A-U cells were grown in PM +adenine until log phase (from 12h) to stationary phase (23h). Crude protein was extracted and treated by lambda protein phosphatase (+) or not (-).

Fig. 5. Localization of Moc1-GFP, $Moc1^{S333A}$-GFP or $Moc1^{S333D}$-GFP in meiosis. MYM3 (*moc1-GFP*), MYM14 (*moc1*^{S333A}*-GFP*) or MYM15 (*moc1*^{S333D}*-GFP*) was cultured in PM + adenine, leucine, and uracil at 30˚C until mid-log phase. Cells were harvested, washed with dH_2O and nitrogen-free PM medium and subsequently re-suspended in nitrogen-free PM medium. Cells were further incubated at 30˚C. Cells were stained with DAPI and observed under the fluorescent microscope. Upper threes, before nitrogen starvation; middle threes, after 9h from nitrogen starvation; bottom threes, after 12h from nitrogen starvation.

Fig. 6. Mutation in *moc1* serine 333 affects mating efficiency. Cells were grown in PM medium at 30˚C until mid-log phase, washed, re-suspended in nitrogen-free PM medium at $1x10^7$ cells/ml and further incubated at 25˚C. Conjugated cells were counted at the indicated times. (A) The *moc1* or *moc1* mutants are over expressed in *cyr1* over-expressed strain MK141. MK141 (*cyr1* over-expressed) cells were transformed with pIRT5 (empty vector, square), pHA-moc1-U (plasmid carrying wild type *moc1* gene, circle), pHA-moc1S333A-U (plasmid carrying S333A mutant *moc1* gene, triangle), pHA-moc1S333D-U (plasmid carrying S333D mutant, upside down triangle). (B) Mating rate in *moc1* mutants and *moc1* disruptant. Square, SP870 (wild type); diamond, MYM3 (*moc1-GFP*); triangle, MYM14 ($moc1^{S333A}$-*GFP*); upside down triangle, MYM15 ($moc1^{S333D}$-*GFP*); circle, MYM1 (*moc1*Δ).

Fig. 7. Expression of Moc1-3HA after nitrogen starvation. MYM2 (*moc1*-3HA) was cultured in PM + adenine, leucine, and uracil at 30˚C until mid-log phase. Cells were harvested, washed with dH_2O ,and nitrogen-free PM medium and subsequently re-suspended in nitrogen-free PM medium. Cells were further incubated at 30˚C. After 0, 3, 6, 9 and 12h, 20ml cultures were withdrawn and proteins were extracted. Cells were harvested and washed twice with dH_2O, dissolved in 100 μl of dH_2O, and boiled at 95˚C for 5 min. Subsequently, 120 μl of 2x Laemmli buffer (4% SDS, 20% glycerol, 0.6M β-mercaptoethanol, 8M urea, 0.12M Tris-HCl [pH 6.8]) was added and the samples were vigorously vortexed with acid-washed glass beads for 3min. The samples were sonicated for 1min and heated at 95˚C for 5min and subjected for Western blotting.

Table 1 *S. pombe* strains used in this study

Strain	Genotype	Source
SP870	*h*^{90} *leu1.32 ade6.210 ura4-D18*	Lab. stock
DY114	*h*^{90} *leu1.32 ade6.216 ura4-D18 cyr1::ura4*	Lab. stock
MK141	*h*^{90} *leu1.32 ade6.210 ura4-D18 cyr1::pALY4* (*LEU2*)	Lab. stock
MK251	*h*^{90} *leu1.32 ade6.210 ura4-D18 cyr1::pALY5* (*ura4*)	Lab. stock
JZ633	*h*^{90} *leu1.32 ade6.216 ura4-D18 pka1::ura4*	M. Yamamoto
JZ666	*h*^{90} *leu1.32 ade6.216 ura4-D18 pde1::ura4*	M. Yamamoto
JZ858	*h*^{90} *leu1.32 ade6.216 ura4-D18 cgs1::ura4*	M. Yamamoto
TF1	*h*^{90} *leu1.32 ade6.210 ura4-D18 moc1::LEU2*	Lab. stock
MYM1	*h*^{90} *leu1.32 ade6.210 ura4-D18 moc1::kanMX6*	This study
MYM2	*h*^{90} *leu1.32 ade6.210 ura4-D18 moc1:*3HA*<<kanMX6*	This study
MYM3	*h*^{90} *leu1.32 ade6.210 ura4-D18 moc1:GFP<<kanMX6*	This study
MYM7	*h*^{90} *leu1.32 ade6.210 ura4-D18 cyr1::pALY5* (*ura4*) *moc1:GFP<<kanMX6*	This study
MYM9	*h*^{90} *leu1.32 ade6.216 ura4-D18 pde1::ura4 moc1:GFP<<kanMX6*	This study
MYM11	*h*^{90} *leu1.32 ade6.216 ura4-D18 cgs1::ura4 moc1:GFP<<kanMX6*	This study
MYM14	*h*^{90} *leu1.32 ade6.210 ura4-D18 moc1*^{S333A}*:GFP<<kanMX6*	This study
MYM15	*h*^{90} *leu1.32 ade6.210 ura4-D18 moc1*^{S333D}*:GFP<<kanMX6*	This study
MYM16	*h*^{90} *leu1.32 ade6.210 ura4-D18 cyr1::ura4 moc1:GFP<<kanMX6*	This study
MYM19	*h*^{90} *leu1.32 ade6.216 ura4-D18 pka1::ura4 moc1:GFP<<kanMX6*	This study

Table 2 Oligonucleotide primers used in this study

Primer	Sequence
24BS-U	5'-AAAGGATCCATGTCGAC (*Sal*I) AATGCCTTTGTCA-3'
24SA-U	5'-TTCGAGCTC (*Sac*I) GGCGTTTCAAATCATC-3'
moc1-SA1	5'-GCCCTCCGGCCCCAAAAA-3'
moc1-SA2	5'-TTTTTGGGGCCGGAGGGC-3'
moc1-SD1	5'-GCCCTCCGGATCCAAAAA-3'
moc1-SD2	5'-TTTTTGGATCCGGAGGGC-3'
moc1kanF5	5'-TGCCCCACTTTCCCACTTTA-3'
moc1kanF3	5'-ATCCGTCGACCTGCAGCGTACGAAGGATAGCGTTGAGGTAGGCA-3'
moc1kanR5	5'-GTTTAAACGAGCTCGAATTCATCGATTGCTAAATATTTGATGATT-3'
moc1kanR3	5'-CGATTACGCCTCTGTGATTC-3'
moc1-W	5'-CTTGCTGTTGTCGATGCTCA-3'
moc1-X	5'-GGGGATCCGTCGACCTGCAGCGTACGACCGACGTTGTGTATCTACAC-3'

Restriction enzyme sites are underlined.

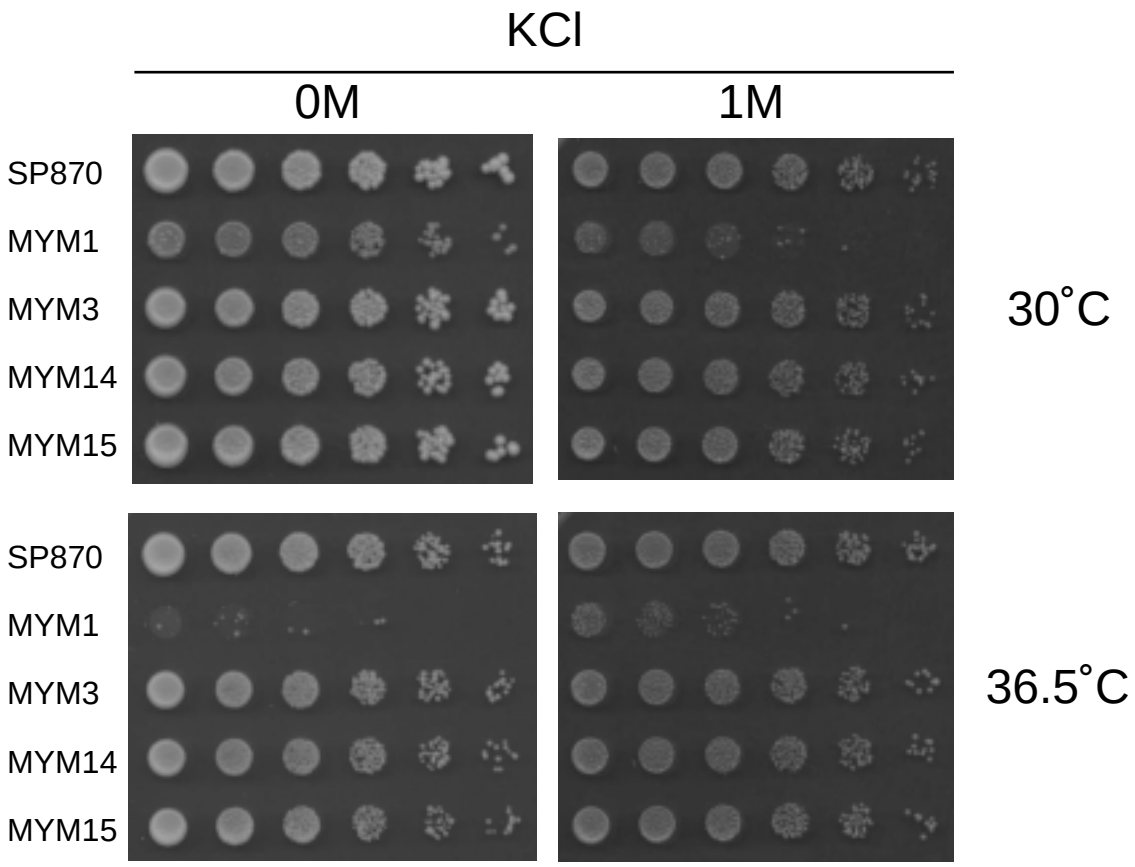

KCl
0M
1M
SP870
MYM1
MYM3
MYM14
MYM15
30˚C
SP870
MYM1
MYM3
MYM14
MYM15
36.5˚C

Fig.1

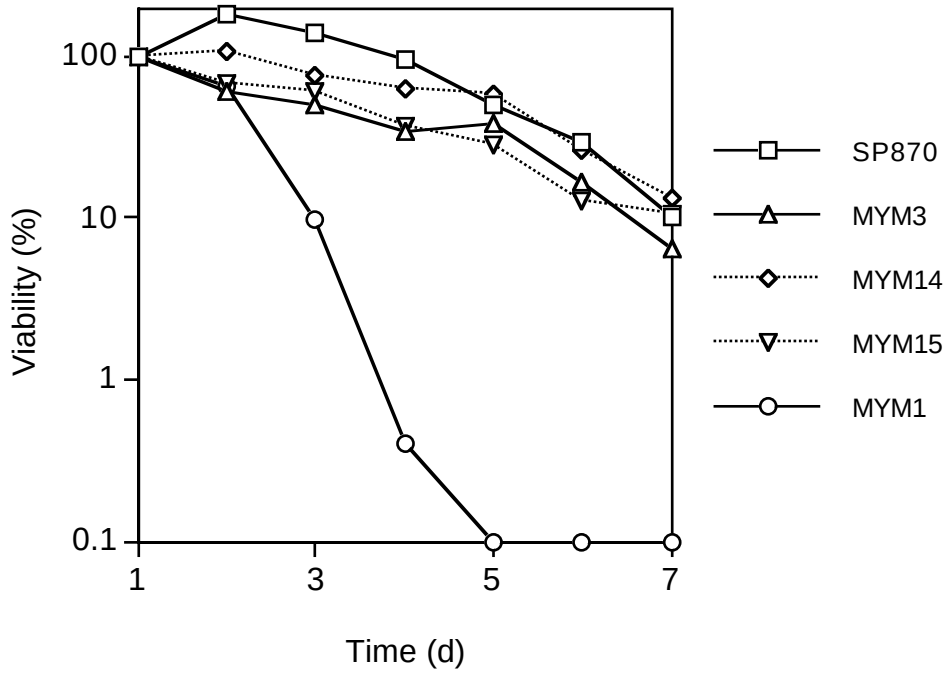


Fig. 2

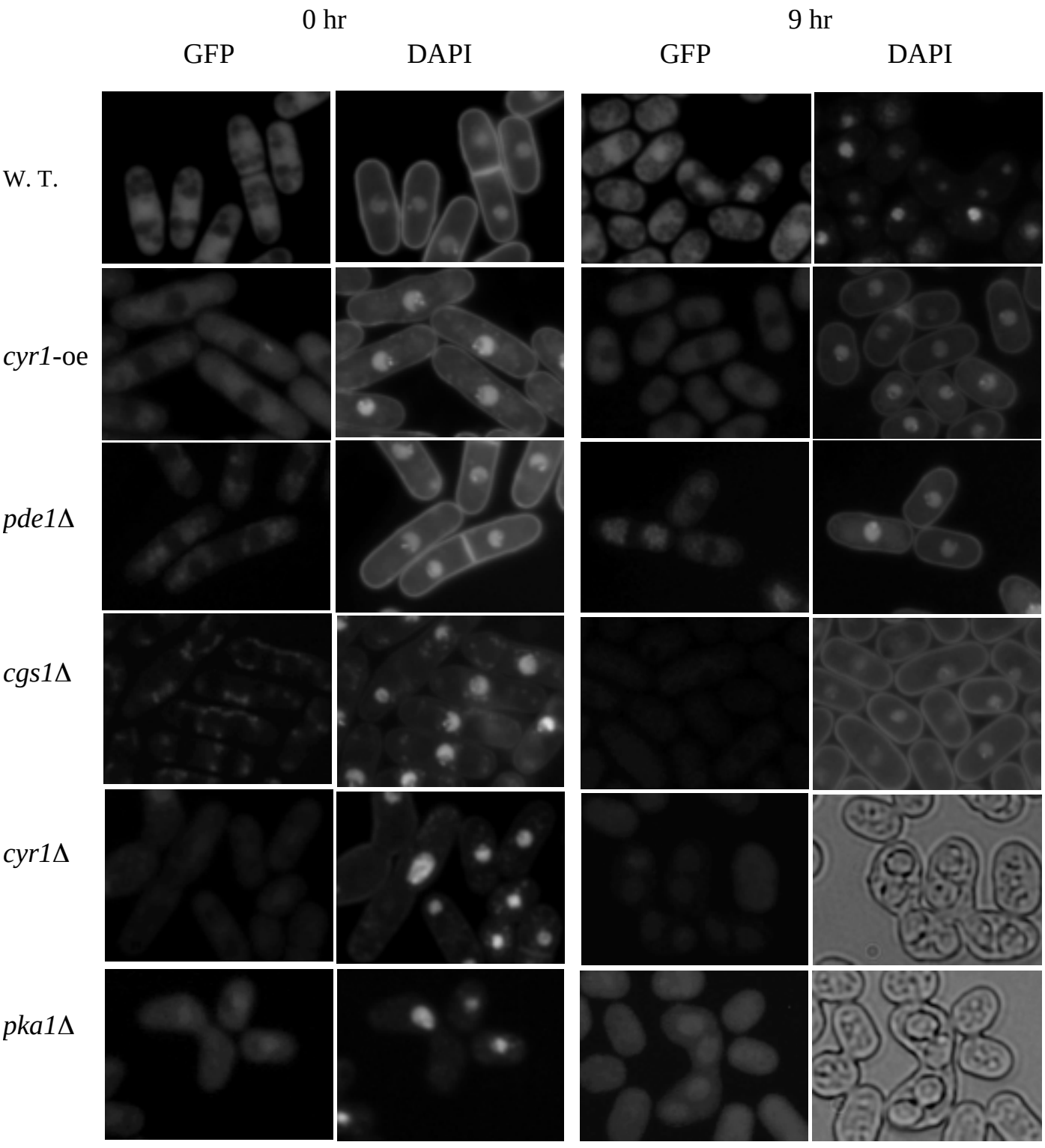


Fig. 3

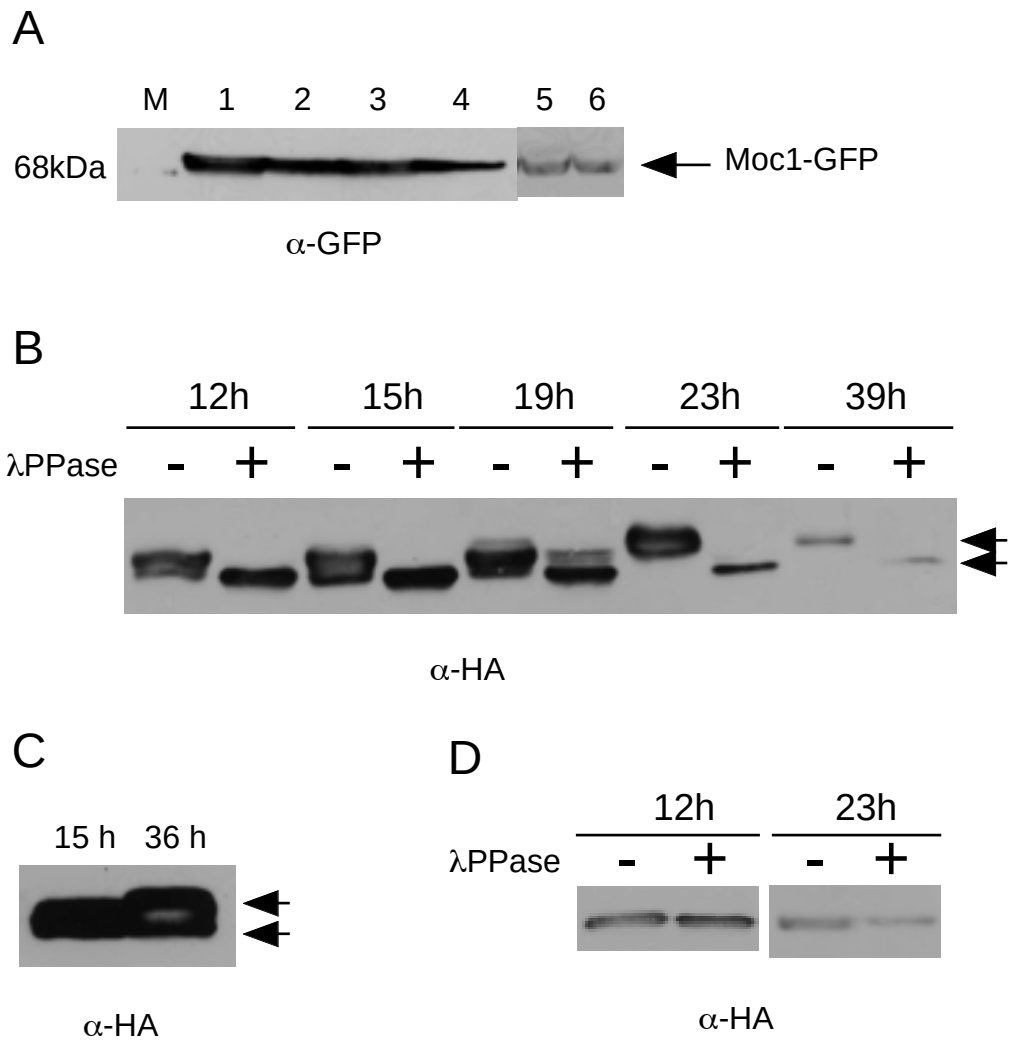

A
M 1 2 3 4 5 6
68kDa
Moc1-GFP
α-GFP
B
12h 15h 19h 23h 39h
λPPase - + - + - + - + - +
α-HA
C
15 h 36 h
α-HA
D
12h 23h
λPPase - + - +
α-HA

Fig. 4

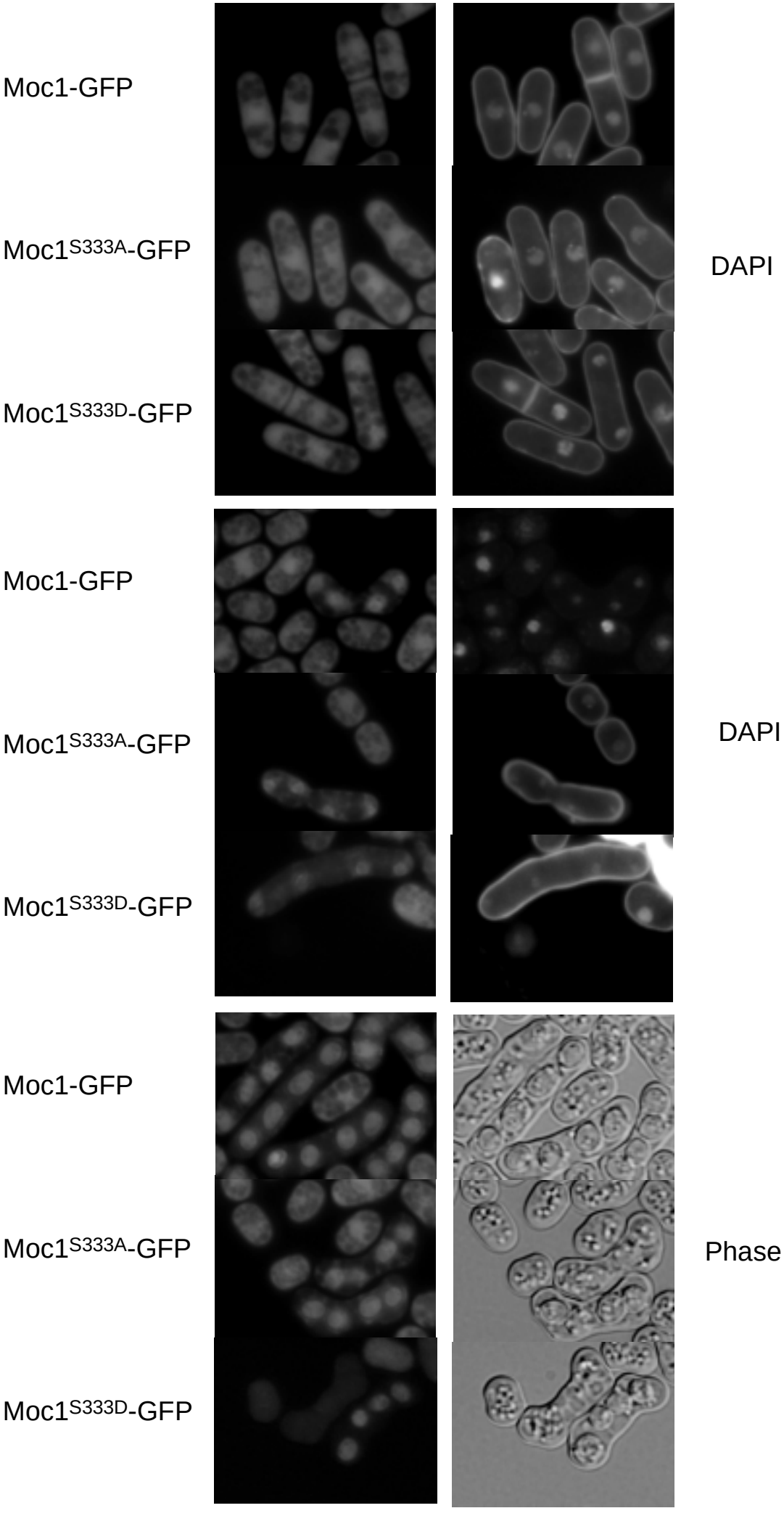
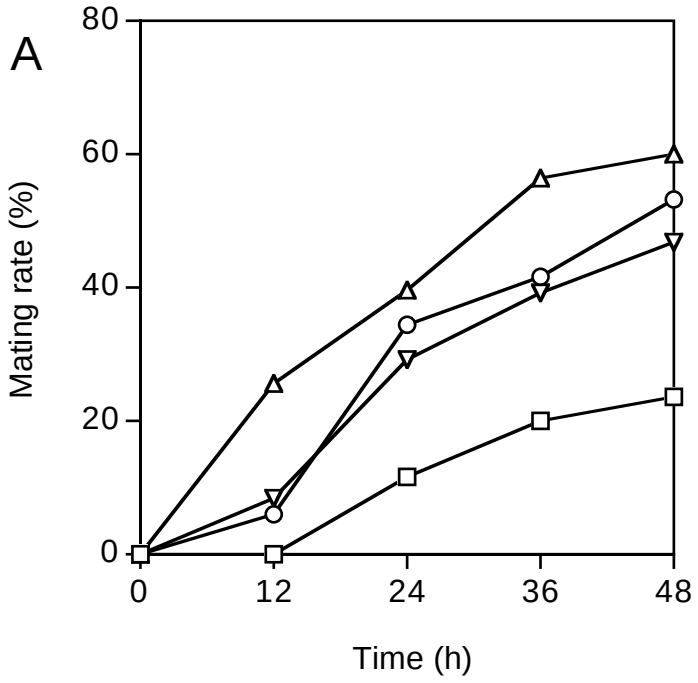

Moc1-GFP
Moc1S333A-GFP
Moc1S333D-GFP
DAPI
Moc1-GFP
Moc1S333A-GFP
Moc1S333D-GFP
DAPI
Moc1-GFP
Moc1S333A-GFP
Moc1S333D-GFP
Phase

Fig. 5


A
80
60
40
20
0
Mating rate (%)
0
12
24
36
48
Time (h)

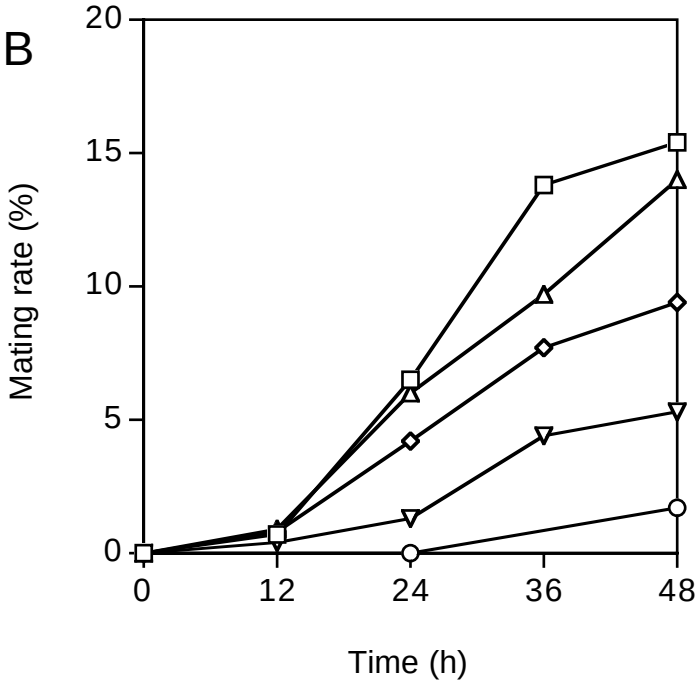

B
20
15
10
5
0
Mating rate (%)
0
12
24
36
48
Time (h)

Fig. 6

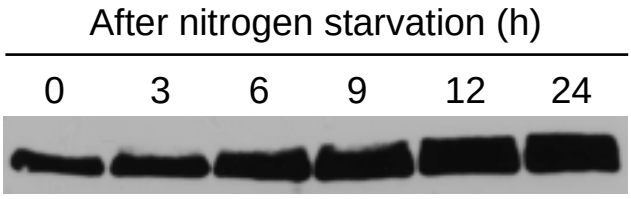


Fig. 7