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Abstract

The cAMP pathway in *Schizosaccharomyces pombe* is the major nutrient sensing pathway to initiate sexual development when opposite mating type cells exist. We screened out *moc1-moc4* genes that overcome a partially sterile *S. pombe* strain due to an elevation of cAMP. When we compared the strength of inducing ability of sexual development in the same *S. pombe* strain, Moc1 had highest, Moc2 had lowest, and both Moc3 and Moc4 had intermediate effects. Moc1/Sds23 and Moc2/Ded1 are known to be a potential regulator of M-phase progression and an essential RNA helicase, respectively. While Moc4 was found to be identical with a Zn-finger protein Zfs1, Moc3 (SPAC821.07c) was a novel protein containing a Zn-finger (Zn(2)-Cys(6)) motif. Deletion mutant of the *moc3* gene was constructed and its disruptant was found to be lower in mating rates and form aberrant asci. In addition, unexpectedly, a *moc3* disruptant is sensitive to $CaCl_2$ and DNA damaging agent such as MMS and UV. Those phenotypes were opposing to the phenotypes observed in a *zfs1* disruptant, and quite different from the ones in a *moc1* disruptant. Moc3 localized in the nucleus as observed in Zfs1. Moc3 binds with Moc4/Zfs1 weakly in the two hybrid system, but no other combination of Moc(s) bind each other in the same analysis. Thus, Moc3 is not only involved in sexual development, but also in ascus formation and DNA integrity in an independent manner with Moc1 and Moc2 in *S. pombe*.

Key words *Schizosaccharomyces pombe*. *moc3*. *cyr1*. meiosis

Introduction

Schizosaccharomyces pombe mates and initiates meiosis under nutritional starvation condition in its haploid state when opposite mating type cells namely, h^- and h^+ exist. These cells subsequently form ascospores after undergoing karyogamy, premeiotic DNA synthesis, meiosis I, and meiosis II (Yamamoto et al. 1997; Yamamoto 2003). Ste11, a key transcription factor, up-regulates transcription of several genes involved in conjugation, meiosis, and sporulation (Sugimoto et al. 1991). One of these upregulated genes, *mei2*, encodes a well-characterized RNA-binding protein that is absolutely required for meiosis (Watabane and Yamamoto 1994). Pat1/Ran1 kinase phosphorylates both Ste11 and Mei2 to inhibit meiosis (Li and Mcleod 1996; Watanabe et al. 1997). Phosphorylated Mei2 loses the binding ability with meiRNA (Sato et al. 2002), in turn converts Mei2 into a substrate suitable for ubiquitin-dependent proteolysis (Kitamura et al. 2001). Nitrogen starved *S. pombe* increases *mei3* transcription and then Mei3 inhibits Pat1/Ran1, thereby allowing Mei2 to initiate meiosis (Li and Mcleod 1996). These are the central regulatory system of triggering meiosis. As an alternative regulation, it was shown recently that Pat1 can be inhibited by a truncated version of Sla1, an orthlog of the mammalian La protein (Tanabe et al. 2003, 2004).

Ste11 is controlled by three signaling pathways namely, the cAMP pathway, the stress-responsive pathway, and the pheromone-signaling pathway (Yamamoto et al. 1997; Yamamoto 2003; Jeong et al. 2004a). Adenylyl cyclase, encoded by the *cyr1* gene, generates cAMP from ATP (Maeda et al. 1990; Kawamukai et al. 1991); trimeric G proteins (Gpa2, Git5, Git11) control the activity of adenylyl cyclase (Isshiki et al. 1992; Landry and Hoffman 2001) through a nutrient-sensing mechanism of the Git3 receptor (Welton and Hoffman 2000); adenylyl cyclase associated protein (Cap1) involves in regulation of Cyr1 (Kawamukai et al. 1992);

and cAMP phosphodiesterase (Pde1) downregulates the cAMP pathway by converting cAMP to AMP (Mochizuki and Yamamoto 1992). Nutrient-starved *S. pombe* cell contains lower level of cAMP so that they exit the vegetative cycle to enter the meiotic cycle (Maeda et al. 1990; Kawamukai et al. 1991), whereas experimentally high level of protein kinase A (Pka1) activity inhibits initiation of sexual differentiation (Maeda et al. 1994). The cAMP-dependent kinase holoenzyme consists of a catalytic subunit (Pka1) (Maeda et al. 1994) and a regulatory subunit (Cgs1) (Devoti et al. 1991). Pka1 regulates expression of meiosis-specific genes such as *ste11* and, as a consequence, *mei2* (Devoti et al. 1991; Sugimoto et al. 1991). *In vitro*, Pka1 phosphorylates Rst2, a zinc-finger protein that binds the upstream region of *ste11* (Higuchi et al. 2002). Low levels of intracellular cAMP during starvation decrease activity of Pka1, thereby decreasing down regulation of transcription factor Rst2 to trigger expression of *ste11* (Kunitomo et al. 2000).

A screening was set up to identify the factors that would down regulate the cAMP pathway. The multicopy suppressor designated Moc1 and Moc2 were isolated to induce sexual development in the strain that overexpressed *cyr1* (Kawamukai 1999, Goldar et al. 2005). Moc1/Sds23 is a potential regulator of M-phase progression in fission yeast (Ishii et al. 1996) and Moc2/Ded1 is a RNA helicase which involves in both sexual differentiation and mitotic cell cycle (Kawamukai 1999; Liu et al. 2002). The *moc1* deletion strain is elongated in shape, sensitive to higher and lower temperature as well as high salt and also lower in mating ratio (Isshi et al. 1996; Goldar et al. 2005). Meanwhile *moc2* is an essential gene for growth. We have two more *moc* genes namely *moc3* as an unknown gene and *moc4* as identical with *zfs1* (Kanoh et al. 1995). We report here that the Moc3 induces sexual development, binds with Moc4 and localized in the nucleus. We also show other phenotypes of a

moc3 disruptant.

Materials and methods

Strains, media, and genetic manipulation

The *S. pombe* and *S. cerevisiae* strains used in this study are listed in Table 1. Standard yeast culture media and genetic manipulations were used, as described previously (Moreno et al. 1991). *S. pombe* strains were grown in complete YEA medium (0.5% yeast extract, 2% glucose, and 0.0075% adenine) or in the synthetic minimal medium, PM (0.3% potassium hydrogen phthalate, 0.22% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts) with added appropriate auxotrophic supplements (0.0075% adenine, leucine and uracil) when required (Alfa et al. 1993). Electroporation was used to transform yeast cells (Prentice 1992). The *Saccharomyces cerevisiae* strains were maintained on rich YPD media composed of 1% yeast extract (Y), 2% bactopeptone (P), 2% dextrose (D) and 2% agar or in the SC (synthetic complete) medium lacking specific amino acids whenever needed (Burke et al. 2000). *Escherichia coli* DH5α grown in Luria broth medium (1% polypepton, 0.5% yeast extract, 1% sodium chloride) containing 50 mg/mL ampicillin, hosted all plasmid manipulations, and standard methods were used for DNA manipulations (Sambrook et al. 1989).

Construction of a *moc3* deletion mutant

A *moc3* disruptant was created by replacing the coding sequence of *moc3* (SPAC821.07c) with the *KanMX6* cassette (Bahler et al. 1998). The deletion cassette was constructed using the recombinant PCR approach. DNA fragments of 400-500 bp and corresponding to the 5' and 3' region of the *moc3* gene were

amplified by PCR using oligonucleotides of moc3(A) and moc3 (X) or moc3 (Y) and moc3 (B) (Table 2). Both amplified fragments were used to attach with the ends of the *kanMX6* cassette by PCR. SP870 was transformed with the resulting *moc3::kanMX6* fragment. Transformants were selected with G418 (Sigma Chemical Co.). PCR and southern blot were used to confirm that the chromosomal *moc3* gene was properly replaced in the resulting transformants, one of which was named MG359.

Plasmids

Plasmid manipulation and bacterial transformation were performed using standard techniques (Sambrook et al. 1989). PCR amplified *Not*I-*Sal*I *moc1-moc4* ORFs were cloned into pSLF173L (the *LEU2* version of pSLF173), which contains the 3HA epitope (Forsburg and Sherman 1997) using oliginucleotides listed in Table 2. *Sma*I-*Sal*I sites of pBTM116 which contains LexA–DNA binding domain of Gal4p (GBD) alone with the *TRP1* auxotrophic marker and pGAD424 which bears Gal4p-DNA activating domain (GAD) alone with the *LEU2* auxotrophic marker were used for cloning *moc1-moc4* genes. Firstly *moc1, moc2, moc3* and *moc4* were amplified from pMCS24, pMCS264, pMCS33 and pMCS65, respectively, by the PCR using sense and antisenence primers listed in Table 2 that bear *Sma*I-*Sal*I sites were cloned into pBluescript II KS^{+}/-. The constructed plasmids were again digested with *Sma*I and *Sal*I restriction enzymes and were ligated into the same site of pBTM116 and pGAD424 which was previously digested with the same enzymes. pSLF272L-moc3-GFP was constructed from pSLF272L-GFP (C-terminal GFP epitope GFPS65T with *LEU2* auxotrophic marker) (Forsburg and Sherman 1997). An amplified DNA fragment which contain *Bgl*II-*Not*I sites (Table 2) was cloned into

pBleuscript *EcoR*Vsite. The resulting pBluescript-moc3 was again digested with *Bgl*II-*Not*I restriction enzymes and ligated with pSLF272L-GFP to yield pSLF272-moc3-GFP (Figure 1C).

Genomic integration of GFP epitopes

The gene for green fluorescent protein (GFP) were integrated into the genomic locus of *moc3* at the C-terminus by a PCR-based method using the pFA6a-GFP(S65T)-kanMX6 modules respectively (Krawchuk and Wahls 1999). DNA fragments of 400-500 bp and corresponding to the 5' and 3' region of the *moc3* gene were amplified by PCR using oligonucleotides of moc3 (W) and moc3 (Z) or moc3 (Y) and moc3 (B) listed in Table 2. Both amplified fragments were used to attach with the ends of the *kanMX6* cassette by PCR. SP870 was transformed with the resulting *moc3*-tagged DNA fragments. G418-resistant transformants were selected and protein expression was assessed by Western blot analysis.

Fluorescence microscopy of GFP fusion proteins

A GFP-fused *moc3* plasmid, pSLF272-moc3-GFP was used to transform wild-type SP870. Both genomically GFP-fused *moc3,* MG361 and transformants were cultured in PM at 30°C to the mid-log phase. GFP fluorescence images of living cells at x 1000 magnification were observed by a Carl Zeiss Axioskop2 microscope connected with a Hamamatsu C5985CCD camera or a Nikon Eclipse 80i microscope connected with a KEYENCE VB-6000 in the case of Figs. 5A and 5B. Cells were counterstained with Hoechst 33342 or DAPI (4,6-diamidino-2-phenylindole) to visualize the nuclei.

Conjugation and sporulation efficiency assay

Cells were grown to midlog phase in PM medium, washed with nitrogen-free or glucose-free PM medium, inoculated in PM medium with various concentrations of nitrogen and glucose, and incubated at 30ºC. After incubation for selected times, 1 ml of cell suspension was removed and sonicated gently, and the number of zygotes were counted under the microscope. Conjugation frequencies were calculated by dividing the number of zygotes (one zygote counted as two cells) by the total number of cells present.

To determine the sporulation efficiency, the wild-type and each mutant strain were incubated at 30ºC for 5 days in PM plates that contained 0.5% nitrogen and 2% glucose. A minimum of three individual colonies from each strain was resuspended in water, 1,000 cells/colony were microscopically examined for presence of ascospores, and sporulation efficiency was calculated using the following equation (Jeong et al. 2004a): Mat% = (2Z + 2A + 0.5S) / (H + 2Z + 2A + 0.5S) where Z stands for the number of zygotes, A for the number of asci, S for the number of free spores, and H for the number of cells that failed to mate.

Western blot analysis

The *moc3-GFP* genomic integrated strain, MG361 (h^{90} *ade6-210 leu1-32 moc3:GFP<<kanMX6*) was cultured to midlog phase in synthetic medium, PM at 30ºC. Cells were then shifted to nitrogen-free medium, PM-N, and cell-free extracts were prepared at indicated times. About 1 X 10^8 cells of *S. pombe* were harvested.

Pellets were washed with STOP buffer [150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3 (pH 8.0)] and stored at -80ºC. The pellets were diluted in 100 μl of dH_2O and boiled at 95ºC for 5 min. Then 120 μl of 2x Laemmli buffer [4% SDS, 20% glycerol, 0.6 M ß-mercaptoethanol, 0.12 M Tris-HCl (pH 6.8)] containing 8 M urea and 0.2% bromo phenol blue was added to the samples, which were vigorously vortexed with an equal volume of zirconia/silica beads for 3 min and then heated again at 95ºC for 5 min. The beads and large debris were removed by centrifugation at 10,000 x *g* for 15 min. Approximately equal amounts of each sample were analyzed by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel and then transferred to Immobilon transfer membranes (Millipore, Bedford, MA) using a semidry-type transfer system. For detection of GFP fusion proteins, membranes were incubated with an anti-GFP monoclonal antibody (Molecular Probes, Eugene, OR) diluted 1:3000 in TBS-T (15 mM Tris, 137 mM NaCl, 0.1% Tween20) containing 5% dry milk, washed, and incubated with horseradish-peroxidase-conjugated anti-mouse secondary antibody (Bio-Rad Laboratories, Richmond, CA) diluted 1:3000 in TBS-T containing 5% dry milk. The secondary antibodies were detected with the enhanced chemiluminescence (ECL) system as described by the manufacturer (Amersham Biosciences).

Yeast two-hybrid analysis

Yeast two-hybrid assay was performed by the method as described (Fields and Song 1989). The *moc1, moc2, moc3* and *moc4* genes were amplified by PCR from pMCS24, pMCS264, pMCS33 and pMCS65, respectively. The resulting fragments were cloned into the *Sma*I-*Sal*I sites of pBTM116 to create pBTM-moc (LexA'-Moc

fusion) or into pGAD424 to generate pGAD-Moc (GAL4'-Moc fusion). Translational fusions to the LexA' binding domain (BD) of pBTM116 or the GAL4' activating domain (AD) of pGAD424 were confirmed by restriction digestion and PCR amplification. The resulting constructs were transformed into *S. cerevisiae* L40 singly or in combination with the corresponding pBTM116 or pGAD424 construct by using the Li acetate-polyethylene glycol one-step transformation protocol (Gietz and Woods 1994). Transformants were selected on defined media by complementation of Trp and/or Leu auxotrophy. The resulting transformants were initially screened for ß-galactosidase activity by filter lift assay employing liquid N_2-lysed cells floated on X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside)-containing phosphate buffer. ß-galactosidase activity in liquid N_2-lysed cells was quantitatively estimated by the procedures described elsewhere (Jeong et al. 2004b).

Results

Isolation of the *moc3* and *moc4* genes

In *Schizosaccharmyces pombe*, cAMP is thought to transduce the glucose or nitrogen starvation signal within cells and to be one of the key regulators of sexual development (Maeda et al. 1990; Kawamukai et al. 1991; Landry and Hoffman 2001). When the adenylyl cyclase (*cyr1*) gene is highly expressed, *S. pombe* cells became partially sterile, because the cAMP level remains high even during the stationary phase (Kawamukai et al. 1991). We set up a screen to isolate multicopy suppressors that would overcome the sterile phenotype caused by the elevated level of cAMP. We expected to find genes that would down regulate the cAMP signaling pathway in *S. pombe*. We transformed a pWH5 based library containing the genomic DNA of *S. pombe* into MK251 (Kawamukai et al. 1991), in which *cyr1*

was over-expressed. Transformants were tested for their ability to make spores by iodine staining. By this screen we isolated four different type of a multicopy suppressor plasmid (pMCS24, pMCS264, pMCS33 and pMCS65), each with several times. We tentatively named those genes as *moc1-moc4* (multicopy suppressor of overexpressed *cyr1*) that were included in those plasmids. We sequenced partially those plasmids and found that pMCS24 contains a full length ORF named *moc1/sds23* (Ishii et al. 1996), pMCS264 contains a full length ORF named *moc2/ded1* (Kawamukai 1999), pMCS33, a 6 kb insert of inside, contains truncated *slp1*$^{+}$ and a new ORF, SPAC821.07c (Fig. 1A) designated *moc3* and pMCS65 contains a full length of the *moc4/zfs1* gene. According to the Sanger Center *S. pombe* genome sequence <http://www.sanger.ac.uk/Projects/S_pombe>, Moc3p encodes 497 aa having one zinc finger motif around 34-73 aa (Fig. 1B).

Comparison of inducing abilities of sexual development by *moc1-moc4*

We first compared *moc1, moc2, moc3* and *moc4* in their inducing abilities of sexual development in *S. pombe* MK251 strain, in which *cyr1* is overexpressed. To express those genes under the same *nmt1* promoter, we cloned all four *moc* genes in the pSLF173 plasmid as describe in Materials and Method (Fig. 1C). MK251 harboring pSLF173, pSLF173-moc1, pSLF173-moc2, pSLF173-moc3 and pSLF173-moc4 were grown in the nitrogen less liquid minimal medium and mating rates were counted. During time course study, we observed that cells gradually mates and forms zygote (Fig. 2). Over production of Moc1 induces about 75% mating within 37 hours of cultivation. Mean while over production of Moc3 and Moc4 induced about 28% mating whereas Moc2 induced only about 10% during this period. These results indicated that all Moc proteins positively induced sexual development in

different strength and Moc1 has the stronger effect than other three.

Phenotype of a *moc3* disruptant

We next characterized the role of *moc3* by making a deletion strain of *moc3* because it was not studied before. We constructed a *moc3* disruptant by using a two-step PCR method based on kanMX6 module as described in Materials and Methods (Fig. 1B). Using the *kanMX6* cassette, the *moc3::kanMX6* fragment was constructed and used to disrupt the chromosomal *moc3* gene in the homozygous haploid wild-type SP870 strain. Proper disruption was verified by southern blotting with using both *moc3* and Km^r as probes (data not shown). The resulting *moc3*Δ strain was named MG359 (*moc3::kanMX6*). MG359 grew well as the wild type, indicating that *moc3* is not an essential gene. Both wild type and MG359 strains were cultivated onto YES medium at 16ºC, 30ºC, 34ºC, 36ºC and 37ºC. But no significant difference was detected (data not shown). We then checked sporulation and mating efficiencies of *moc3*$^+$and *moc3*Δ strains. For this purpose we cultured both wild type (SP870) and *moc3*Δ (MG359) cells in minimal medium. It was observed that *moc3*$^+$ cells mates 69.6 ± 3.1 percent but *moc3*Δ cells mated 33.3 ± 3.5 percent when grown at 30ºC in 0.1% nitrogen and 1% glucose medium but the mating ratio of *moc3*Δ cells was not as low as one of a *zfs1*Δ strain, which was about 3.5 percent (Fig. 3A and 3B). The lower mating ratio in *moc3*Δ cells was completely suppressed by expression of *moc3* itself and also by *moc4*. Interestingly, the sterility of a *zfs1*Δ strain can be partly suppressed by expression of *moc3* and completely by *moc4* itself. These data indicated that Moc3 has a positive role for inducing sexual development and can partly bypass the require of *zfs1/moc4* in *S. pombe*. We also noticed aberrant asci were formed in a *moc3*Δ strain (Fig. 3C and Table 3) each of which contain one, two

or three spores. These phenotypes of MG359 was recovered by expressing *moc3* in a multicopy plasmid, but not by the vector alone (Table 3).

Moc3 involved in Ca^{++} and DNA damaging sensitivities

We then proceeded to check further phenotypes of a *moc3*Δ strain under various stress conditions. Wild type (SP870), a *moc3*Δ strain (MG359) and a *zfs1*Δ strain (JZ971) were spotted onto YES with or without chemical agents such as 0.5 M $CaCl_2$ or in lowered pH (pH 4.5). We observed that a *moc3*Δ strain is sensitive to 0.5 M $CaCl_2$ but not sensitive to lower pH. On the contrary, *zfs1*Δ cells are not sensitive to $CaCl_2$ but are sensitive to lower pH (Fig. 4). We next used several DNA damaging agents like Methyl Methanesulfonate (MMS) or exposed under Ultraviolet light (UV). We observed that the *moc3*Δ strain is sensitive to 0.01% MMS and UV light whereas a *zfs1*Δ strain is not. We also observed that 10μg/ml Thiabendazole, a destabilizing regent for microtuble, is slightly resistance to the growth of *moc3*Δ cells whereas it was sensitive to *zfs1*Δ cells (Fig. 4). These data indicate that Moc3 involves in Ca^{++} tolerance and DNA damaging response but Moc4/Zfs1 involves differently.

Moc3p localized in the nucleus in fission yeast

We next analyzed the localization of moc3-GFP in both mitotic and meiotic cells. For this purpose we constructed GFP tagged Moc3 plasmid, pSLF272-moc3-GFP in which GFP was tagged of its C-terminus region (Fig. 1C). We also constructed a haploid wild-type strain, designed MG361, that carried a single copy of the *moc3* gene tagged with GFP (Materials and Methods). We observed that Moc3 is located

in the nucleus, both in vegetative mitotic cells and zygotic meiotic cells (Fig. 5A). Not only when we overexpressed Moc3-GFP by the plasmid, it was also located in the nucleus of *S. pombe* cells that expressed Moc3-GFP on the chromosome (Fig. 5B). The existence of a full length fusion protein of Moc3-GFP was verified by western blot analysis (Fig. 5C) in both of MG361 and the wild type strain harboring pSLF272-moc3-GFP.

Moc3p bind with Moc4p

We next assessed the relevance of four Moc proteins by checking whether these proteins interact with each other in the two-hybrid system. We used *S. cerevissiae* L40 as a host strain and used Gal4 based pBTM116 / pGAD424 expression vectors. We tested all combination among Moc1, Moc2, Moc3 and Moc4, and observed that only Moc3 and Moc4 bind each other while no other interaction was observed among four Moc proteins (Fig. 6A). We further verified Moc3p and Moc4p interaction by determining ß-galactosidase activity and found that the ß-galactosidase activity between Moc3 and Moc4 is higher than a negative control but lower than a positive interaction between Byr2 and Ras1 (Fig. 6B).

Discussion

In this study, we identified the novel protein denoted Moc3 as to involve in sexual development of *Schizosaccharomyces pombe*. In the same screening to isolate *moc3*, we isolated *moc1/sds23*, *moc2/ded1*, and *moc4/zfs1* as multicopy suppressors of a sterile strain due to over-expression of *cyr1*. We initially aimed to identify the factors that involves in sexual development specially by down regulating the cAMP

pathway, but the previous and present analyses of those isolated genes indicated that all four *moc* genes are apparently involved in many cellular process besides sexual development. It has been reported that *sds23* was isolated as a multicopy suppressor of a mutant in *dis2* encoding PP1 phosphatase (Ishii et al. 1996), *ded1* was isolated as a multicopy suppressor of a Cdc25 over produced strain or of a *cdc2* mutant (Liu et al. 2002), and *zfs1* was isolated as a multicopy suppressor of a sterile strain due to expression of *pac1* (Kanoh et al. 1995) or of a *cdc16* mutant (Beltraminelli et al. 1999). All isolated processes in other groups are the results of the screening in certain cell cycle mutants. To enter meiotic process from mitotic cell cycle, cells have to be arrested in the G1 phase. It is reasonable that any components that can assist to enter sexual development by pausing cell cycle will be included in the screening we had conducted. It may be also relevant to the role of cAMP in mitotic cell cycle. Because cAMP is thought to be involved in the regulation of Anaphase Promoting Complex (Yamada et al. 1997), any factors to oppose the cAMP effect on the function of APC are expected to be identified by our screening too. We did not obtain the other multicopy suppressors than four *moc* genes in our screening, that presumably because the plasmid containing genomic DNA we used is in a low copy number and a larger in size. We would expect to get the gene like *pde1* if we had used the other multi-copy genomic libraries.

When we compared which *moc* gene induce sexual development efficiently, we observed that *moc1* is highest and *moc2* is lowest in inducing abilities of sexual development among four *moc* genes (Fig. 2). This observation was based on the expression of four *moc* genes on the same plasmid under the strong *nmt1* promoter, so that we expected the express level is in maximum. We initially had the similar impression in that the original plasmid, pMCS24 ($moc1^{+}$) has a stronger effect on MK251 strain than other plasmids. We noticed the tendency of inducing ability of

sexual development by *moc* genes and the sterility phenotype of *moc* disruptant is anti-proportional. The *moc1*/*sds23* disruptant is very sterile but opposingly the *moc2*/*ded1* gene is essential for growth. The *moc4*/*zfs1* disruptant is partially sterile as previously reported and *moc3* deletion mutant as well. Those observations may reflect how strong each *moc* gene involves in mitotic cycle. The essential genes would be tightly involved in mitosis than the genes just involve in meiosis.

There are several interesting phenotype we observed in the *moc3* deletion mutant. The *moc3* deletion mutant forms aberrant ascus like mono, di or tri spores (Fig. 3C), indicating Moc3 has a role in proper ascus formation. Such phenotypes can be found in mutants like for examples *S. pombe chs1*Δ and *cda1*Δ strains which are defective in chitin and chitosan formation (Matsuo et al. 2004; 2005). *moc3*Δ was sensitive to $CaCl_2$, that probably reflects the role of *moc3* in Ca^{++} homeostasis. Such phenotypes can be found in the mutants like for an example *zds1*Δ cells (our observation). *moc3*Δ was sensitive to MMS and UV light, those reflect the involvement of *moc3* in DNA integrity or damage control. There are many examples that displayed sensitivity to MMS and UV light in mutants defective in DNA repair, replication or DNA integrity. As Moc3 and Moc4/Zfs1 binds together in the two-hybrid system, we expected that *moc3*Δ and *zfs1*Δ display similar response to salt stress and chemical agents. But we obtained completely opposite results in *zfs1*Δ and *moc3*Δ. Most interestingly, a spindle poison, thiabendazole is slightly resistant to *moc3*Δ cells, (Fig. 4) whereas is very sensitive to *zfs1*Δ cells. Above all the information indicates that Moc3 has an important function in surviving cells against stress and maintaining DNA integrity as well as sexual development of *S. pombe*.

Localization of Moc3 in viable cells indicated that it is localized in the nucleus of both mitotic and meiotic cells (Fig. 5A, 5B). We verified this results using a strain containing moc3-GFP on its chromosome as well as overexpression by a multicopy

plasmid (Fig. 5A, 5B). Among four Moc proteins, Moc1 and Moc2 are localized in the cytosol whereas Moc4/Zfs1 is localized in the nucleus. We initially expected that Moc proteins have some interrelationship, and in fact we observed Moc3 and Moc4 interact each other (Fig. 6) in the two hybrid system. It has also been described that Moc4/Zfs1 has two zinc finger motifs and localized in the nucleus (Kanoh 1995). But on the contrary the some phenotypes of *moc3*Δ and *zfs1*Δ are different. Thus, we think that even the interaction between Moc3 and Moc4 may have some meaning, the separate function of Moc3 and Moc4 are probably more important than a complex function of Moc3 and Moc4.

There is no apparent ortholog of *moc3* in *S. cerevisiae*, but are ones in other fungi such as in *Aspergillus* and *Neurospora*, and there is another paralogous gene (SPBC15D4.02) in *S. pombe*. Unfortunately, however, those orthologous and paralogous genes were not studied, so that no information can be obtained from those seemingly relevant genes. Thus, at present, it is difficult to predict how *moc3* contributes sexual differentiation or other cellular events. Considering the various *moc3* mutant phenotypes, the presence of a Zn finger motif in the Moc3 protein and its nuclear localization, it may be reasonable to predict that Moc3 acts at some point in the transcriptional regulation of unidentified genes involves in sexual development and some other related functions. Further study will be definitely required to assign a clear function to *moc3*.

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Figure Legends

Fig. 1. Genomic localization and plasmid construction of the *moc3* gene. A) Localization of the *moc3* gene in pMCS33. B) Schematic diagram of genomic localization of *moc3* in the *Hind*III-*Hind*III DNA fragment. The arrow indicates the direction of *moc3*. The *moc3* gene was replaced by the *kanMX6* DNA fragment in a *moc3*Δ strain. C) Strategy for cloning *moc1, moc2, moc3* and *moc4* genes in several expression vectors, pBTM116, pGAD424, pSLF173L or pSLF173U and a strategy for cloning the *moc3* gene in the pSLF272L-GFP plasmid.

Fig. 2. Comparison of *moc* genes to induce sexual development in *S. pombe* MK251 under nitrogen starvation condition. *Schizosaccharomyces pombe* MK251 strain harboring pSLF173, pSLF-moc1, pSLF-moc2, pSLF-moc3 and pSLF-moc4 were pre-grown in PMA medium for overnight. Cells were grown into 80 ml PMA medium until mid-log phase. Then cells were harvested and washed three times with nitrogen free medium and cultured into nitrogen free medium (nitrogen, 0% and glucose 1.0%) for 45 hours. Cells were observed under microscope and mating rates among 1,000 cells were counted.

Fig. 3. Morphology of a *moc3* gene deletion mutant. A) Microscopic observation of wild type (SP870), *moc3*Δ (MG359) and *zfs1*Δ (JZ971) strains. Cells were cultured onto PM medium containing 0.1% nitrogen and 1.0% glucose and incubated at 30ºC for 3 days. Single colony was picked up and dissolved in water. After little vortex and sonication, pictures were taken. B) Three individual colonies were picked up and dissolved in water. Mating ratio of at least 1,000 cells was counted. The counts of mating rates has been plotted in the bar diagram as percentage. C) Normal spores in wild type (SP870) an aberrant ascus of two spores in *moc3*Δ (MG359).

Fig. 4. Sensitivity of a *moc3* gene deletion mutant. Wild type (SP870), *moc3*Δ (MG359) and *zfs1*Δ (JZ971) strains were spotted onto YES medium with serial dilution containing several chemical agents as indicated. Plates were incubated at 30°C for 3 days and images were taken.

Fig. 5. Subcellular localization of Moc3-GFP by live observation of cells. A) Subcellular localization of the Moc3-GFP protein was observed under microscope. GFP fusion proteins were expressed under control of the *nmt1* promoter in the vector, pSLF272-GFP. h^{90} wild-type cells (SP870) carrying each plasmids were grown in PM medium and incubated at 30°C. Cells were examined for GFP fluorescence, and the same cells were stained with the DNA-binding dye Hoechst 33342 or DAPI. B) h^{90} Moc3-GFP cells (MG361) were grown in PM medium and incubated at 30°C. Cells were examined for GFP fluorescence, and the same cells were stained with the DNA-binding dye Hoechst 33342 or DAPI. C) Western blot of SP870 harboring Moc3-GFP in a multicopy plasmid and a Moc3-GFP integrant *S. pombe* MG361 strain.

Fig. 6. Yeast two-hybrid assay. A) *S. cerevisiae* strain L40 was transformed with plasmids containing DNA binding domain (or the DNA activating domain) fused with various Moc proteins. Positive signal developing blue color was shown as '+' and negative signal as '-' symbol. The combination of Ras1 and Byr2 was used as the positive control (Ozoe et al. 2002). B) The β-galactosidase activity (in nanomoles per minute per milligram of protein) of the resulting transformants was assayed by using *0*-nitrophenyl-β-D-galactopyranoside.

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

Strain	Genotype	Source
SP870	h^{90} *ade6-210 leu1-32 ura4-Δ18*	Kawamukai
MK251	h^{90} *ade6-210 leu1-32 ura4-Δ18 cyr1::pA-LY5(ura4)*	Kawamukai
MG359	h^{90} *ade6-210 leu1-32 ura4-Δ18 moc3::kanMX6*	This study
MG361	h^{90} *ade6-210 leu1-32 ura4-Δ18 moc3:GFP<<kanMX6*	This study
JZ971	h^{90} *ade6-216 leu1-32 ura4-Δ18 zfs1::ura4*	Kanoh
L40	*MAT***a** *his3Δ200 trp1–901 leu2–3,-112 ade2* *LYS2::(lexAop)*$_4$*-HIS3 URA3::(lexAop)*$_8$*-lacZ*	Lab. Stock

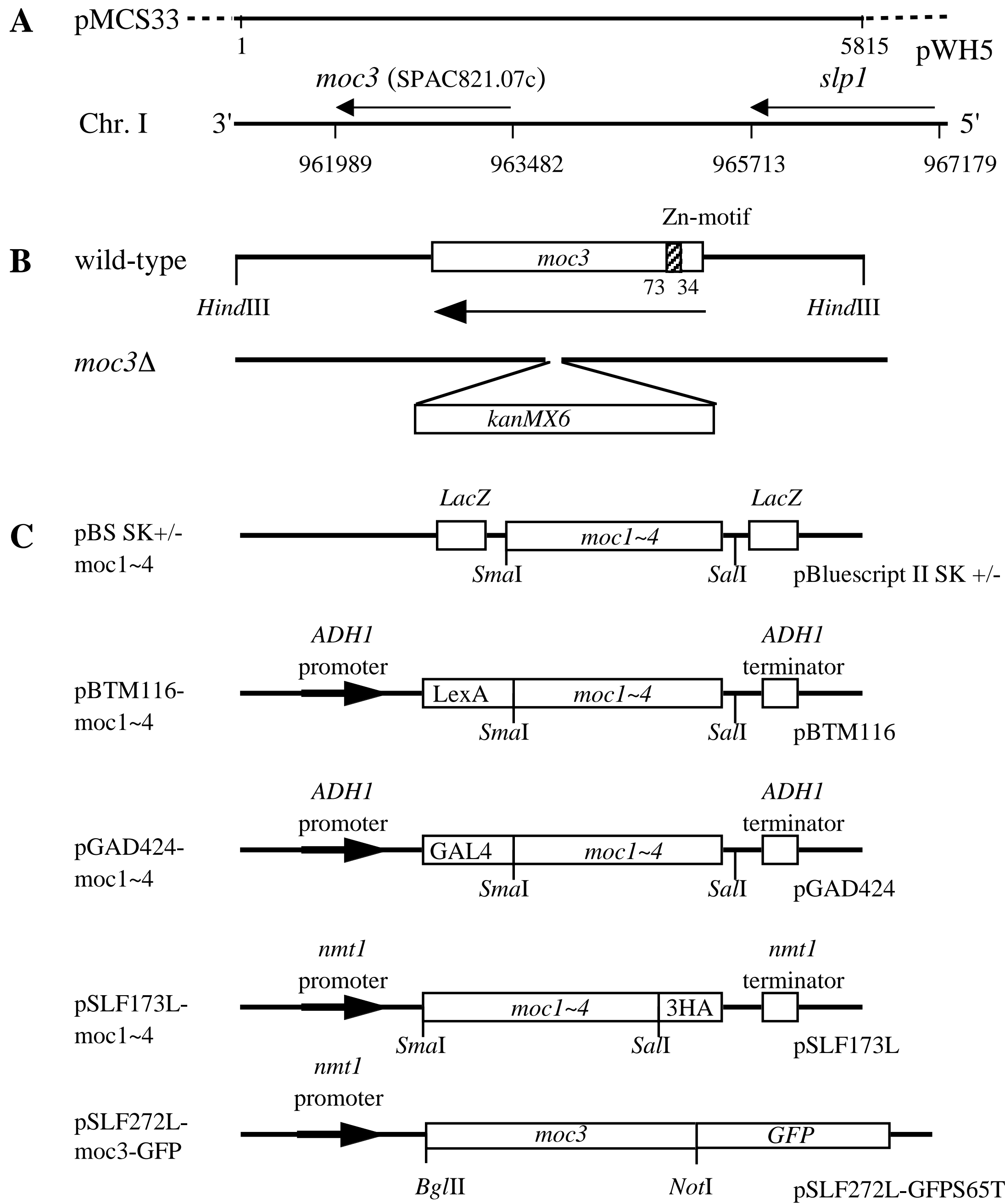
Table 2. Oligonucleotide primers used in this study

Primer	Sequence
pBTM116 / pGAD424	
moc1-F-*Sma*I	5`-ACTCCCGGGAATGCCTTTGTCAACTCAATC-3`
moc2-F-*Sma*I	5`-CAAACCCGGGTATGAGCGACAATGTACAGC-3`
moc3-F-*Sma*I	5`-CCTCCCGGGTATGAACCCGTATGTTTCTTATC-3`
moc4-F-*Sma*I	5`-TCTCCCGGGCATGGTTTATTCTCCTATGTC-3`
moc1-R-*Sal*I	5`-TATGTCGACTCACCGACGTTGTGTATCTAC-3`
moc2-R-*Sal*I	5`-TTTAGTCGACTTACCACCAGGATTGAGCAC-3`
moc3-R-*Sal*I	5`-CCAGTCGACTGACTGTCGTACCGTAATTCG-3`
moc4-R-*Sal*I	5`-GATGTCGACTCAAGGAGATTGCTTAATAG-3`
pSLF173	
moc1-F-*Not*I	5`-AAACGCGGCCGCATGCCTTTGTCAACTCAA-3`
moc2-F-*Not*I	5`-ACAAGCGGCCGCATGAGCGACAATGTACAG-3`
moc3-F-*Not*I	5`-CCCGCGGCCGCATGAACCCGTATGTTTCTTATC-3
moc4-F-*Not*I	5`-GTTTGCGGCCGCATGTCTCGACCTCAAGTA-3`
pSLF272 (L)-GFP	
BglII-M3-F	5`-CTTAGATCTTATGAACCCGTATGTTTCTTATC-3`
NotI-M3-R	5`-ATTGCGGCCGCAAAGTACTGGTCGATTTAAG-3`
Disruption and tagging primers	
moc3-(A)	5`-GCCGCCTTATCACTCGCTAATGTCGAGG-3`
moc3-(X)	5`-GGGGATCCGTCGACCTGCAGCGTACGAGCTTGAGAAAAGCGCGC-3`
moc3-(Y)	5`-GTTTAAACGAGCTCGAATTCATCGATGCTAGACAAAATCACGC-3`
moc3-(B)	5`-CGTAGATGGAAGGCCTATTC-3`
moc3 (W)	5`-GCTATGGTCGTTCGTGAAC- 3`
moc3 (Z)	5`-GGGATCCGTCGACCTGCAGCGTACGAAAGTACTGGTCGATTTAAG- 3`

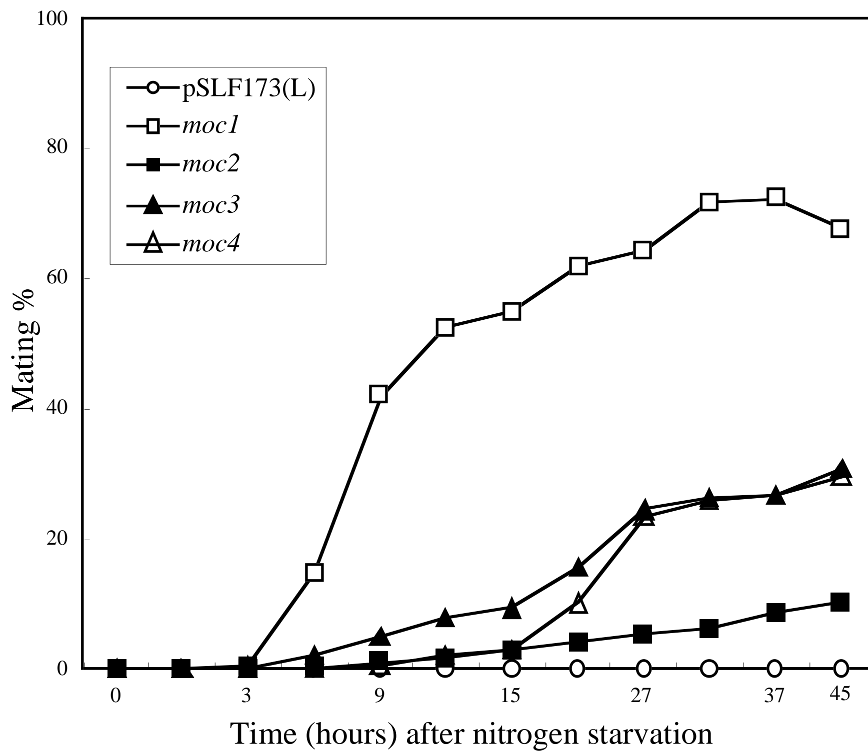
Table 3. Aberrant asci of a *moc3* disruptant

Strain	Plasmid	% tetra	% tri	% di	% mono	Total (%)
SP870	-	69.46 ± 1.64	-	-	-	69.46 ± 1.64
MG359	-	20.36 ± 2.06	3.44 ± 1.41	2.54 ± 1.25	1.25 ± 0.26	27.69 ± 4.46
MG359	pSLF173(U)	23.12 ± 3.62	2.62 ± 1.31	3.22 ± 0.75	0.51 ± 0.50	29.48 ± 3.93
MG359	pSLF173(U)-moc3	65.67 ± 7.78	-	-	-	65.67 ± 7.78

SP870 and MG359 were patched on PM medium containing 0.1% nitrogen and 1.0% glucose at 30°C for 3 days. MG359 was transformed with pSLF173(U) and pSLF173(U)-moc3 and was grown in PMAL medium containing 0.1% nitrogen and 1.0% glucose at 30°C for 3 days. Spore formation was estimated by counting under the microscope. At least 1,000 cells were counted in each case. Values were the means and standard deviations calculated from three independent experiments. Tetra, tri, di and mono refers to spores with 4, 3, 2 and 1 asci respectively. Total refers to the sum of all sporulated cells.

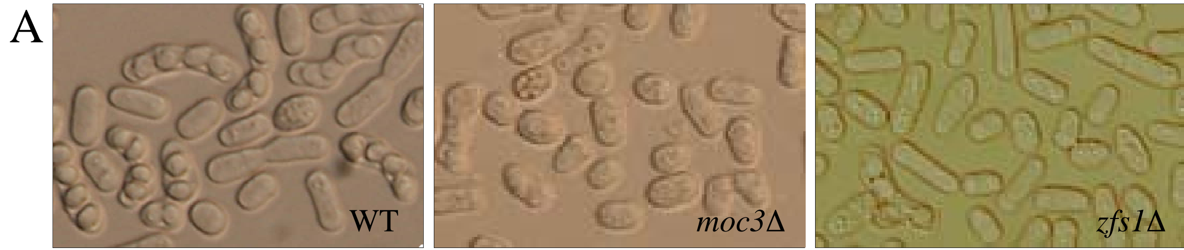


Goldar *et. al.* Fig.1

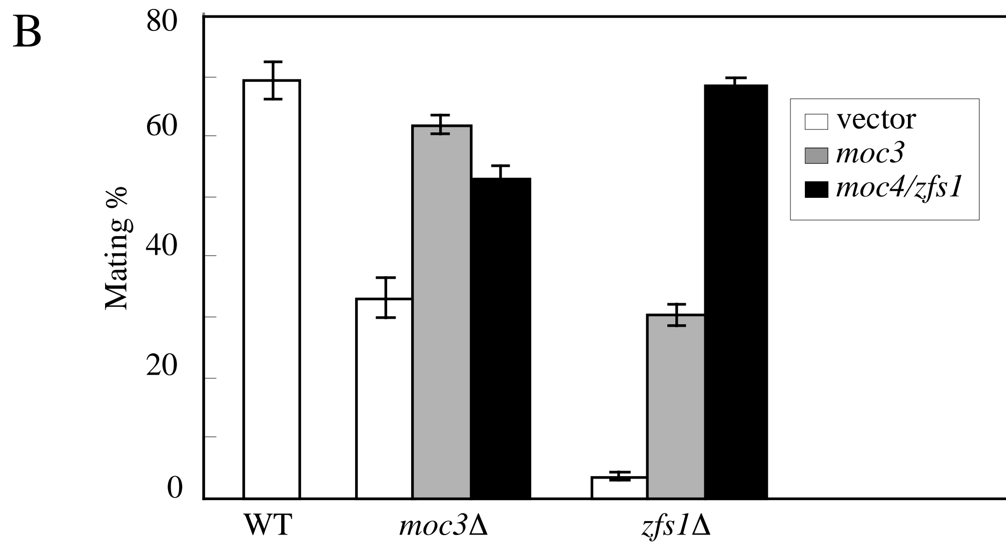


Goldar *et. al.* Fig.2

A

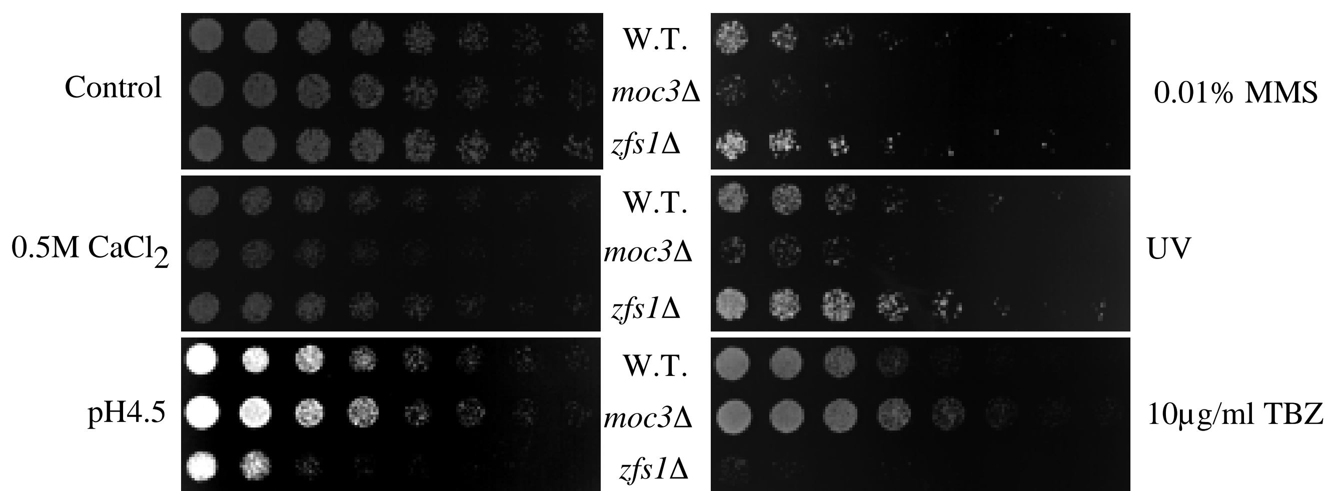


B

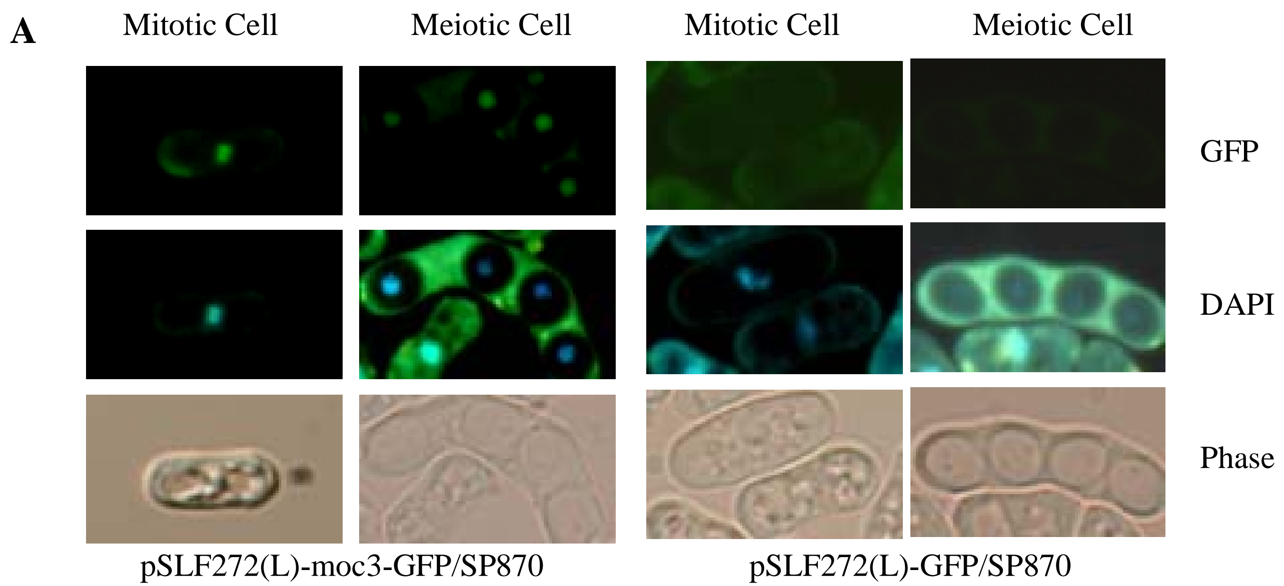


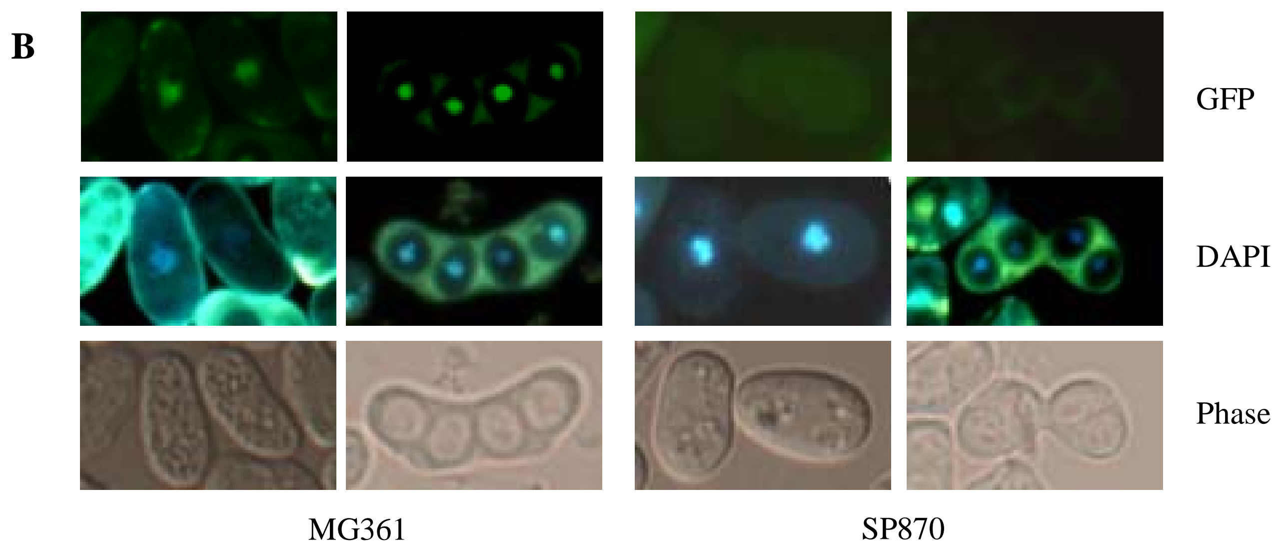
C

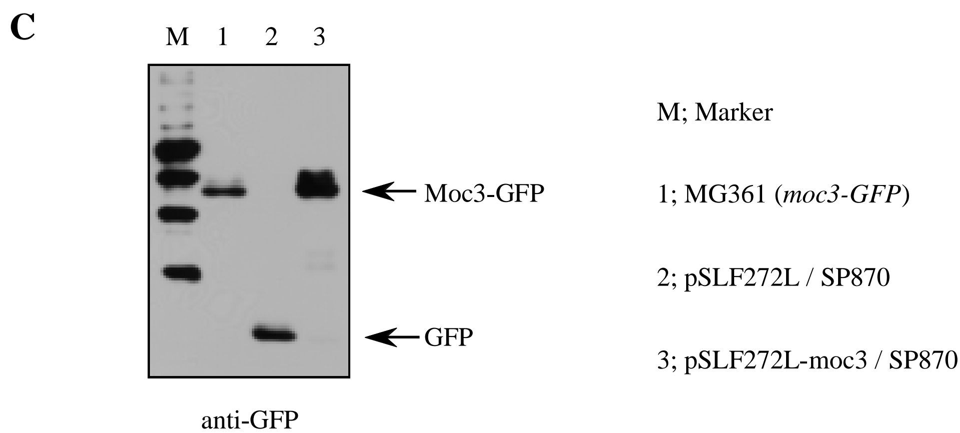
Goldar *et. al.* Fig.3



Goldar *et. al.* Fig.4







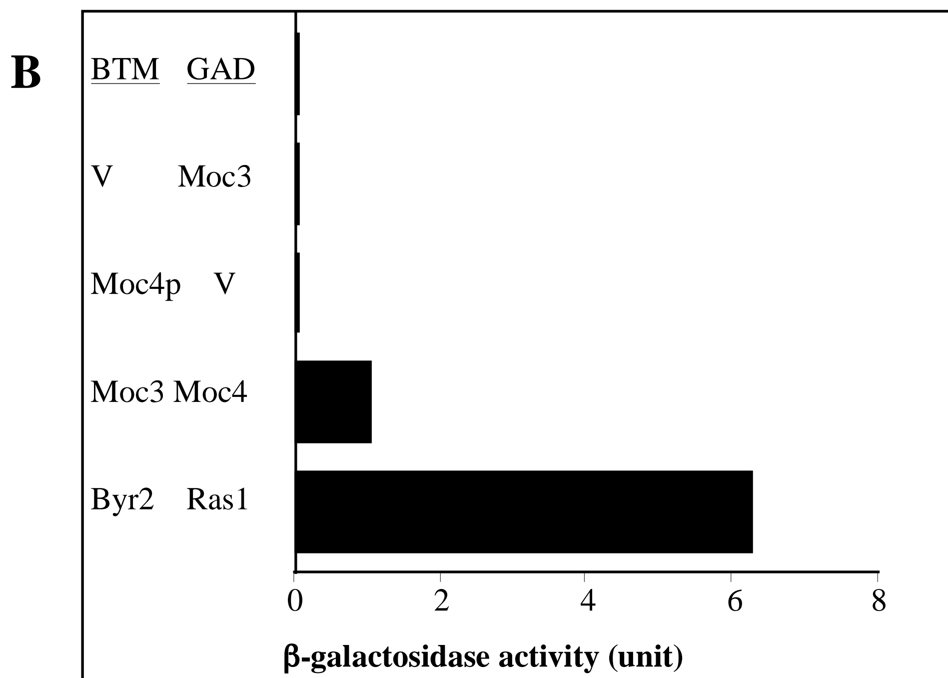
Goldar *et. al.* Fig.5

Interaction between Moc proteins

A

DNA-binding	DNA-activating	Color development
pBTM116	pGAD424	-
Moc1	Moc2	-
Moc1	Moc3	-
Moc1	Moc4	-
Moc2	Moc3	-
Moc2	Moc4	-
Moc3	Moc4	+
Byr2	Ras1	++

B



Goldar *et. al.* Fig.6