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# Regulation and role of an RNA-binding protein Msa2 in controlling the sexual differentiation of fission yeast

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**Abstract**

The *msa2/nrd1* gene encodes an RNA-binding protein that negatively regulates sexual differentiation of fission yeast *Schizosaccharomyces pombe* by repressing the Ste11-regulated genes. However, it is not known how Msa2 regulates sexual differentiation, and to characterize its role, we altered the *msa2* gene by inducing point mutations and tested the resulting mutants for their ability to inhibit sexual differentiation and their suppressive effect on a temperature sensitive *pat1* mutant. Several amino acids were found to be important, including three phenylalanine residues (F153, F245 and F453) in the three consensus RNA recognition motifs (RRMs) and a threonine residue (T126) that normally functions as a phosphorylation site. Results indicated that Msa2 was negatively regulated by phosphorylation that arose from Spk1-mediated pheromone signaling. Msa2 also regulated the Ste11 protein level coordinating with Cpc2, a ribosomal-associated protein. In addition, Msa2 was detected in stress granules that co-localized with Pabp in the cytosol under conditions of glucose starvation. Msa2 may regulate the translation of Ste11, be a component of stress granules that form in response to glucose starvation, and regulate the sexual differentiation of *S. pombe*.

## Introduction

Under nutrient rich conditions, *Schizosaccharomyces pombe* exhibits haploid growth, but it initiates sexual differentiation when starved for nutrients in the presence of the mating pheromone. During starvation, *S. pombe* cells arrest at the G1 phase of the cell cycle, cells of opposite mating type ($h^-$ and $h^+$) initiate conjugation, and the resulting zygotes form four ascospores (Yamamoto 2003). Starvation induces the expression of the *ste11* gene that encodes a key factor that up-regulates the transcription of several genes involved in conjugation, meiosis, and sporulation (Sugimoto et al. 1991). Both the stress-responsive and the pheromone-responsive pathways positively regulate the transcription of *ste11*, but the cAMP-dependent protein kinase pathway negatively regulates it through the action of the Rst2 transcription factor (Higuchi et al. 2002; Kawamukai et al. 1991; Maeda et al. 1994). In addition, the TOR (target of rapamycin) pathway is involved in the nutrient starvation response to the regulation of Mei2 (Valbuena and Moreno 2010). Pat1 is an essential kinase that negatively regulates Mei2 by phosphorylation (Kitamura et al. 2001; Watanabe et al. 1997).

The *nrd1/msa2* gene negatively controls sexual differentiation in *S. pombe* and its disruption initiates sexual differentiation in the absence of nutrient starvation (Jeong et al. 2004a; Tsukahara et al. 1998). The *nrd1/msa2* gene encodes an RNA-binding protein that preferentially binds poly(U), which regulates the onset of sexual differentiation by repressing the Ste11-regulated genes until those cells experience a critical degree of starvation (Tsukahara et al. 1998). We isolated two genes, *msa1* and *msa2*, both of which encode RNA-binding proteins that are multi-copy suppressors of the *sam1* mutant, which initiates mating and forms spores on nutrient rich media (Jeong et al. 2004a; Jeong et al. 2004b; Katayama et al. 1996; Oowatari et al. 2009). Two-hybrid screening using Msa2 as bait allowed the identification of Cpc2, which is a homologue of the mammalian RACK1 protein (Jeong et al. 2004a). RACK1 is a highly conserved member of the family of WD-repeat proteins implicated in numerous signaling pathways that can interact with protein kinase C (PKC) (Ron *et al.* 1994). The RACK1 protein controls translation by interacting with ribosomes in mammals and its *S. pombe* ortholog Cpc2 was also shown to associate

98 with ribosomes in *S. pombe* (Baum et al. 2004; Coyle et al. 2009; Shor
99 et al. 2003). Interestingly, Cpc2 has previously been isolated as a
100 protein that interacts with Pat1 and is involved in the onset of sexual
101 differentiation (Jeong et al. 2004a; McLeod et al. 2000). We proposed
102 that the interaction of the negative regulator Msa2 with the positive
103 regulator Cpc2 suggests the existence of a previously unidentified
104 regulatory circuit in the sexual differentiation of *S. pombe* (Jeong et al.
105 2004a; Paul et al. 2009b).
106 Moreover, it has recently been shown that Msa2 directly binds to
107 *cdc4* mRNA and regulates its stability (Satoh et al. 2009). Cdc4
108 encodes an essential light chain of myosin and plays a crucial role in
109 cytokinesis (McCollum et al. 1995). Msa2 is phosphorylated by Pmk1,
110 which encodes a MAP kinase involved in cell integrity, and it
111 negatively regulates the binding and stabilization of *cdc4* mRNA. Thus,
112 in addition to its established role in regulating sexual differentiation,
113 Msa2 also has a role in the regulation of cytokinesis in *S. pombe* (Satoh
114 et al. 2009).
115 Here, we examined the regulation of Msa2 in the sexual
116 differentiation of *S. pombe*. Msa2 is phosphorylated under conditions
117 of nutrient-starvation, and this is dependent upon the
118 pheromone-responsive MAP kinase signaling pathway. Certain amino
119 acid sequences are necessary for Msa2 to inhibit the sexual
120 differentiation and Msa2 is a component of stress granules formed
121 under conditions of glucose starvation.

## Materials and methods

### Strains and media

The *S. pombe* strains used in this study are listed in Table 1. Standard yeast culture media and genetic manipulations were used (Alfa et al. 1993). *S. pombe* was grown in YES-rich medium (0.5% yeast extract, 3% glucose, 225 mg/liter adenine, histidine, leucine, uracil, and/or lysine hydrochloride) or Pombe Minimum (PM) synthetic medium, supplemented with 75 mg/liter adenine, leucine, and/or uracil. Nitrogen-free PM medium (0.5% glucose without ammonium chloride) was used to culture *S. pombe* when the mating efficiency was to be measured. *Escherichia coli* strain DH5α was used for plasmid manipulation. *E. coli* was grown in LB medium (1% polypeptone, 0.5% yeast extract, 1% NaCl, pH 7.2).

### Plasmid construction

The *msa2* DNA from pBluescript II KS(+)-msa2 or pBluescript II KS(+)-msa2 variant plasmid was amplified by PCR using msa2F-*Xho*I and msa2R-*Not*I primers. The PCR product was digested with *Xho*I and *Not*I and inserted into pSLF272L (Forsburg and Sherman 1997). The DNA sequences at the insertion site and at the *msa2* mutation site were verified by DNA sequence analysis.

### Site-directed mutagenesis

A ~4.7-kb DNA fragment containing the *msa2-3HA-kanMX6* sequence from the Msa2-3HA–tagged *S. pombe* strain YO1 genome was amplified by PCR using oligonucleotides msa2F-*Eco*RV and msa2R-*Spe*I (Supplementary Table 1). The DNA fragment was then digested with *Eco*RV and *Spe*I and cloned into pBluescript II KS(+) to generate pBluescript II KS(+)-msa2-3HA-kanMX6, which was used as the template for PCR-based site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Primers used for site-directed mutagenesis are listed in Supplementary Table 1. This plasmid was linearized with *Eco*RV and *Spe*I and then integrated at the genomic locus of the *msa2* gene in wild-type cells. Stable and precise integration of the tagged gene was confirmed by

158 DNA sequencing, and protein expression was confirmed by western
159 blot analysis.
160
161 Construction of an *msa2* deletion mutant
162 An *msa2* deletion mutant was created by replacing the entire coding
163 sequence of *msa2* with the *kanMX6* module (Krawchuk and Wahls
164 1999). The deletion DNA cassette was constructed using the
165 recombinant PCR approach. Five hundred bp (approximate) DNA
166 fragments corresponding to the 5' and 3' region of the *msa2* gene were
167 amplified by PCR using msa2(A) and msa2(B) or msa2(Y) and msa2(Z)
168 oligonucleotides shown in Supplementary Table 1. Both amplified
169 fragments were attached to the ends of the *kanMX6* module by PCR.
170 SP870 was transformed with the resulting *msa2::kanMX6* fragment and
171 transformants were selected with G418 (Sigma Chemical Co.). PCR
172 and southern blot analysis were used to confirm that the chromosomal
173 *msa2* gene was disrupted in the resulting transformants (YO24).
174
175 Genomic integration of epitope tags
176 Epitope tags of three hemagglutinin (3HA) and green fluorescent
177 protein (GFP) were integrated into the genomic locus of *msa2* at its
178 C-terminus using a PCR-based method with pFA6a-3HA-kanMX6 and
179 pFA6a-GFP(S65T)-kanMX6 as templates (Krawchuk and Wahls 1999).
180 Five hundred bp (approximate) fragments corresponding to the 5' and
181 3' region of the *msa2* gene were amplified by PCR using
182 oligonucleotides msa2(W) and msa2(X), or msa2(Y) and msa2(Z)
183 (Supplementary Table 1). Both of the amplified fragments were
184 attached to the ends of the *kanMX6* cassette using PCR. SP870 was
185 transformed with the resulting *msa2-3HA-kanMX6* and
186 *msa2-GFP(S65T)-kanMX6* fragments. G418-resistant transformants
187 were selected and protein expression was assessed by Western blot
188 analysis. A strain (KT150) expressing *pabp-mRFP-hphMX6* was
189 constructed in a similar way using oligonucleotides pab1 t1, pab1 t2,
190 pab1 t3, and pab1 t4 (Krawchuk and Wahls 1999).
191
192 Mating and sporulation efficiency assay
193 Cells were grown to mid-log phase in PM medium, washed twice with

nitrogen-free PM medium, and incubated in nitrogen-free PM medium at 30 °C. After incubation for the indicated times, 1 ml of the cell suspension was removed and sonicated gently, and the number of zygotes observed using a microscope was counted. The mating efficiency were calculated using the following equation:

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 (Jeong et al. 2004b).

Western blot analysis

After growth in an appropriate medium, ~1 × $10^8$ cells were harvested, washed twice with $dH_2O$, mixed in 100 µl of $dH_2O$, and boiled at 95 ºC for 5 min. Subsequently, 120 µl of 2 × Laemmli buffer (4% SDS, 20% glycerol, 0.6 M β-mercaptoethanol, 8 M urea, and 0.12 M Tris-HCl, pH 6.8) was added, and the samples were vigorously vortexed with an equal volume of zirconia-silica beads for 3 min and heated at 95 ºC for 5 min. The zirconia-silica beads and debris were removed by centrifugation at 10,000 × g for 15 min. Approximately equal amounts of each sample were analyzed by SDS-PAGE with a 10% polyacrylamide gel, and the separated proteins were transferred to a PVDF membrane, Immobilon-P (Millipore Co., Billerica, MA, USA), using either a wet-type transfer system or a semidry transfer system. To detect 3HA fusion proteins, the membranes were incubated with an anti-HA monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:3000 in 5% dry milk in PBS-T (137 mM NaCl, 8 mM $Na_2HPO_4$ 12 $H_2O$, 2.7 mM KCl, 1.5 mM $KHPO_4$ and 0.1% Tween 20), washed, and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology Inc.) diluted 1:3000 in 5% dry milk in PBS-T. To detect GFP fusion proteins, the membrane was incubated with an anti-GFP monoclonal antibody (Roche Diagnostics) diluted 1:2000 in 5% dry milk in PBS-T, washed, and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology Inc.) diluted 1:3000 in 5% dry milk in PBS-T. The secondary antibodies were detected with the enhanced

chemiluminescence (ECL) system as described by the manufacturer (GE Healthcare, Pittsburgh, PA, USA). To detect Cdc2, membranes were incubated with an anti-PSTAIRE polyclonal antibody (Santa Cruz Biotechnology Inc.) diluted 1:3000 in 5% dry milk in PBS-T, washed, and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody diluted 1:2000 in 5% dry milk in PBS-T. The secondary antibodies were detected with the ECL system (GE Healthcare).

Northern blot analysis

Total RNA was prepared and Northern blot analysis was performed as described previously (Jeong et al. 2004a; Ozoe et al. 2002). *S. pombe* cells were grown in PM medium at 30˚C to a density of ~5 x $10^6$ cells/ml. The cells were collected by centrifugation, washed with nitrogen-free PM medium, and resuspended in nitrogen-free PM medium at the same cell density. The cells were incubated for the selected times, resuspended in 1 ml of ISOGEN (Nippon Gene, Tokyo, Japan), and vigorously vortexed for 6 min with acid-washed glass beads. After centrifugation (10000 × g for 15 min at 4˚C), the supernatant was precipitated with isopropanol. Approximately 10 μg of each sample of total RNA was applied for electrophoresis using a 1% denaturing formaldehyde-agarose gel and the RNA was subsequently transferred to a hybridization membrane Hybond™-N+ (GE Healthcare) in alkaline transfer buffer (0.05 M sodium hydroxide) for 4 hours. The probes were labeled with [α-32P]-dCTP (GE Healthcare) by using the BcaBEST™ labeling kit (Takara Bio Inc., Shiga, Japan). The abundance of the transcript on the blot was analyzed by autoradiography with a BAS1500-Mac image analyzer (Fuji Film Co., Tokyo, Japan). The hybridization probes used were the 1.3-kb *Pvu*II fragment for *ste11* from pSX1 (Sugimoto et al. 1991) and the 3.5-kb *Hin*dIII fragment containing *mam2* (Kitamura and Shimoda 1991).

Fluorescence microscopy of GFP and mRFP fusion proteins

Msa2-GFP–tagged strain *S. pombe* YO3 cells were cultured in PM at 25°C to mid-log growth phase and subsequently inoculated into nitrogen-free PM. Msa2-GFP fluorescence or Pabp-mRFP images of

living cells were obtained using a digital camera DP70 (Olympus, Tokyo, Japan) connected to the BX51 microscope (Olympus, Tokyo, Japan) or a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Japan).

## Results

### Phosphorylation of Msa2

We found cultivation of *S. pombe* under nitrogen starved conditions caused decreased electrophoretic mobility of the Msa2 protein. To examine this time course, YO1 (Msa2-HA) cells were starved for nitrogen and the Msa2 protein was monitored by western blotting (Fig. 1A). Decreased electrophoretic mobility (band shift) of Msa2 was detected after nitrogen starvation for 1 h - 12 h. This band shift was eliminated after treatment of samples with λ-PPase and by prior treatment with a phosphatase inhibitor. Thus, the Msa2 band shift was probably caused by phosphorylation (Fig. 1B). To determine if the band shift could be directed by Spk1 protein kinase, a positive and essential regulator of sexual differentiation that transmits pheromone signaling (Toda et al. 1991)(Yamamoto et al. 2004), we examined a *spk1* deletion strain and found that no band shift of Msa2 was detected (Fig. 1C). In addition, the Msa2 band shift was not as large in an $h^-$ strain as in an $h^{90}$ strain. This can be explained by the absence of mating pheromone signaling, which is absent in an $h^-$ strain where the activity of Spk1 is low, but is present in an $h^{90}$ strain. Results suggest that Spk1 phosphorylates Msa2 early during nitrogen starvation.

### Effects of Msa2 mutation

Next, potential target sites for MAP kinase within Msa2 (T40, T126 and S309) that contain the consensus sequence P-X-T/S-P were mutated (T40A/D, T126A/D, and S309A/D, respectively) on the chromosome and mating efficiencies of the resulting strains were measured. The mating efficiency of the *msa2*Δ cells was higher (~25%) than the wild type (~ 7%) under the conditions examined (Fig. 2A).

The mating efficiencies of various mutants were near to the wild type level. However, this was not true for the T126D mutant, a phosphomimetic mutation of T126 in Msa2 that partly lost its ability to negatively regulate mating, while its non-phosphorylated counterpart retained that ability. T40A, T40D, S309A and S309D mutants did not affect the mating efficiency. Since wide range proteomic analysis revealed another phosphorylation site (S93) in Msa2 (Wilson-Grady et al. 2008), we tested the mutation of that site, but S93A and S93D did not affect Msa2 mating efficiency (Fig. 2A). Electrophoretic mobility shift analysis of Msa2, Msa2(T40A), Msa2(T126A), and Msa2(S309A) mutants under nitrogen starved condition showed that T126 is the major phosphorylation site of Msa2 (Fig. 2B). Double mutants of T40A and T126A or T40D and T126D showed that T126D primarily affected mating efficiency while T40D had only a modest effect (Fig. 3). Taken together, T126 of Msa2 is an important phosphorylation site within Msa2 that negatively regulates mating efficiency.

Overexpression of *msa2* or its mutant forms suppress *pat1*$^{ts}$

We next expressed various mutant forms of Msa2 on the plasmid in the *msa2*Δ strain to see suppressive effects of them on mating efficiency. The results again showed that the T126D mutant or the T40D/T126D double mutant partly lost its ability to lower mating efficiency (Fig. 3A). As shown in Fig. 3B and as found by others, the overexpression of Msa2 suppresses the temperature sensitivity of *pat1*$^{ts}$ (Tsukahara et al. 1998). However, this phenotype was abolished in the T126D mutant or the T40D/T126D double mutant, but not in the others (T40A, T40D, T126A and T40A/T126A). This confirms the mating efficiency results shown in Fig. 2 and also indicates that T126D is an inactive form of Msa2.

Because this is effective way to measure the activity of Msa2, we tested the ability of the other Msa2 mutants to suppress the *pat1*$^{ts}$ mutant phenotype. We created the mutants located in the RNA-binding sites of Msa2 which contains four RNA binding sites each with a conserved RNA recognition amino acid sequence motif RNP1 (R/K-G-F/Y-G/A-F/Y-I/L/V-X-F/Y). The conserved phenylalanine (F) residues in the RNP1 domain of RRM1, 2, 3, and 4 in Msa2 were

replaced with alanine (F153A, F245A, F361A, F453A) and the mutants were expressed on the plasmids to test for their ability to suppress the *pat1*$^{ts}$ mutant at 32˚C (Fig. 4A). Three mutants (F153A, F245A, F453A) failed to suppress the *pat1*$^{ts}$ phenotype, but F361A suppressed it. At a higher temperature (34˚C), those suppressions were not clear (data not shown). Replacement of all four phenylalanine residues with alanine enhanced the inability to suppress the *pat1*$^{ts}$ phenotype. We also created the strains in which mutations on RRMs were integrated on the chromosome and tested their mating efficiency. Consistently, we observed that the F361A mutant is less effective than other three (F153A, F245A, F453A) mutants (data not shown). Thus, RRM1, 2, and 4 are important for the function of Msa2, but RRM3 is not. The double mutant *pat1-114* and *msa2*Δ lowered the restriction temperature of *pat1-114*, which is consistent with a negative role for Msa2 on sexual differentiation and supports a genetic interaction between *msa2* and *pat1* (Fig. 4B).

Msa2 suppresses Ste11 expression

Overproduction of Msa2 slightly reduced *ste11* gene expression and significantly reduced *mei2* gene expression (Tsukahara et al. 1998) but it does reduce the production of Ste11 protein (Fig. 5). Because *mei2* expression is dependent on Ste11 (Sugimoto et al. 1991), the results suggest that Msa2 may be involved in the translation of Ste11. Cpc2 has been reported to have multiple functions in translation because of its interactions with ribosomal proteins (Shor et al. 2003) and is also reported to interact with Msa2 (Jeong et al. 2004a). Therefore, Cpc2 may be a positive regulator of sexual differentiation by controlling the translation of the *ste11* gene. Nitrogen starvation induced *ste11* gene expression in the *cpc2*Δ strain, but Ste11 protein was not produced in a proportional manner. In addition, the expression of *mam2*, which is under the control of Ste11 (Ozoe et al. 2002), is abolished in the *cpc2*Δ strain (Fig. 5). These results suggest that Msa2 and Cpc2 have a role in the control of Ste11 possibly by a translational level.

Starvation causes the formation of Msa2 granules

While Msa2-GFP predominantly localized in the cytoplasm under nutrient rich conditions, the Msa2-GFP protein was visualized as dots in the cytoplasm after glucose starvation (Fig. 6). These dots co-localized with Pabp (a Poly(a)-binding protein) (Nilsson and Sunnerhagen 2011), a known component of stress granules, and indicates that Msa2 is a component of stress granules. The presence of Msa2 in the granules was far less abundant after nitrogen starvation and no granules were visualized with Pabp under the same condition (Fig. 6). Formation of stress granules of Msa2 and their colocalization with Pabp were seen when a lower amount of glucose (0.5 %) was used (Fig. 7A) instead of 2 % glucose (Fig. 6B) after 6 hour nitrogen starvation. Glucose concentration is the key factor for granule formation of Msa2.

As shown in Fig. 1A, insoluble proteins such as Msa2 can be extracted by boiling the samples. When the protein samples were prepared without boiling, the soluble form of Msa2 protein was not detected in the western blot of proteins prepared from cells that had undergone 8 hr of nutrient starvation (Fig. 7B). Thus, samples prepared with boiling and without boiling can be used to distinguish the insoluble and soluble forms of the protein. Under the same cell culture and sample preparation (without boiling) conditions, soluble forms of Cpc2 were detected in the western blot (Fig. 7B). Thus, Msa2 forms insoluble granules upon starvation, and Cpc2 is not detected in the insoluble granules that contain Msa2.

**Discussion**

Msa2 is an RNA-binding protein that negatively regulates sexual differentiation in *S. pombe* (Jeong et al. 2004a; Tsukahara et al. 1998), but the molecular mechanism for this is not known. Results from this and other studies suggest that Msa2 regulates Ste11 translation by cooperating with Cpc2, a ribosomal-associated WD repeat protein. This hypothesis arose from studies of the *cpc2*Δ strain that showed that the expression of *ste11* mRNA did not change so much, but that the amount of Ste11 protein decreased and was insufficient to induce its downstream target (*mam2*). Overexpression of *msa2* did not change the

expression of *ste11* mRNA so much but it decreased the amount of Ste11 protein. Because Msa2 is known to bind Cpc2, and deletion of *cpc2* is phenotypically epistatic to deletion of *msa2* (Jeong et al. 2004a), Msa2 is expected to exert its regulatory function through Cpc2. In addition, Cpc2 positively regulates the translation of Atf1, which is a transcription factor required for sexual development and the stress response (Nunez et al. 2009). Together, these results support a novel system whereby Msa2 may regulate the translation of Ste11 protein, although further investigation of this mechanism is needed. Cpc2 plays an important role in the formation of a complex mediated by Moc1, Moc2, Moc3, Moc4, and rpl32-2 proteins, which are positive regulators of sexual differentiation (Goldar et al. 2005; Kawamukai 1999; Paul et al. 2009a; Paul et al. 2009b; Yakura et al. 2006a); in addition, a positive role of glutamyl tRNA synthetase on sexual differentiation was also shown (Paul et al. 2009a). These results also suggest that the regulation of translation is important for the control of sexual differentiation in *S. pombe.*

Several amino acids important for Msa2 function were detected: phenylalanine residues F153, F245, and F453 in the consensus RNA recognition motifs (RRMs). This conclusion is derived from the fact that mutations in the RRM region downregulated the function of Msa2 and decreased its ability to suppress $pat1^{ts}$. Phosphorylation of Msa2 at a threonine residue (T126) negatively regulated its function. Others have shown that Pmk1 MAPK phosphorylates Msa2 at two target sites (T40 and T126) (Satoh et al. 2009). Here, T126 affected the role of Msa2 on sexual differentiation while T40 had only a slight effect (Fig. 2 and 3). Because T126 is located near an RNP1 domain, a mutation of T126 (but not T40) may affect the function of RNP1. Phosphorylation of T126 is dependent on the presence of Spk1 kinase, although it is unclear if it directly phosphorylates Msa2 or not. Nevertheless, as T126 is a target site for MAP kinase, we presume that Spk1 is the kinase that directly phosphorylates Msa2. Thus, in *S. pombe*, once pheromone signaling is upregulated, Spk1 is activated and phosphorylates a negative regulator of Msa2, which allows translation to proceed with the production of Ste11.

We detected Msa2 in cytoplasmic granules under conditions of

glucose starvation, but not in nitrogen starvation under higher glucose condition (2%). These granules co-localized with Pabp, suggesting that they are stress granules (Nilsson and Sunnerhagen 2011). Msa2 was also detected in an insoluble form of protein granule formed after nitrogen starvation under lower glucose condition (0.5%). Granule formation of Msa2 was clearly dependent on glucose concentration. Since starvation (glucose or nitrogen) is a signal to induce sexual differentiation, a negative regulator of sexual differentiation like Msa2 is not necessary, so it is reasonable that Msa2 may enter insoluble granules that sequester proteins and/or mRNAs that are not necessary for sexual differentiation. It is possible that these proteins and/or mRNAs may be released once a nutrient-rich condition becomes available.

Apparent orthologs of Msa2 are found in many fungi, including *S. pombe, S. cerevisiae, Kluyveromyces lactis, Megnaporthe grisea,* and *Neurospora crassa*. However, little is known about the orthologs of Msa2 in *Saccharomyces cerevisiae* and *S. pombe*. The *S. cerevisiae* ortholog MRN1/YPL184c was proposed to be involved in translational control from the results of an analysis of 40 different RNA-binding proteins (Hogan et al. 2008) and is consistent with the results found in *S. pombe*.

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**Fig. 1. Phosphorylation of Msa2 protein.**

(A) A mid-log phase culture of YO1 (*h*$^{90}$ *msa2-3HA-kanMX6*) was incubated in nutrient-rich medium (YE) or minimal medium (PM). Growing cells in PM medium were washed, resuspended in nitrogen-free PM medium at 0.5 × $10^7$ cells/ml, and incubated at 30˚C for the indicated times. Extracts of the cells were subjected to SDS-PAGE and detected by western blot with anti-HA antibody.

(B) Extracts of YO1, expressing Msa2-3HA, were subjected to immunoprecipitation with anti-HA antibody, and the immunoprecipitants were treated with λPPase with or without the addition of the phosphatase inhibitor mixture. The samples of the resulting reactions were separated by SDS-PAGE, and Msa2-3HA protein was detected by western blot using anti-HA antibody. Closed and open triangles indicate phosphorylated proteins and non-phosphorylated proteins, respectively. (C) YO1 (*h*$^{90}$ *msa2-3HA*), YO32 (*h*$^{-}$ *msa2-3HA*) and YO20 (*h*$^{90}$ *msa2-3HA spk1Δ*). Cells were grown in YE medium (Nutrient +) or PM medium to mid-log phase. Growing cells in PM medium were washed, resuspended in nitrogen-free PM medium at 0.5 × $10^7$ cells/ml, and incubated at 30˚C for two hours (Nutrient -). The expression of the Msa2 protein was detected by western blot with anti-HA antibody. Closed and open triangles indicate phosphorylated proteins and non-phosphorylated proteins, respectively.

**Fig. 2. The mating efficiency of *msa2* mutants.**

(A) SP870 (wild-type), YO24 (*msa2Δ*), YO1 (*msa2-3HA*), YO74 (*msa2-T40A-3HA*), YO75 (*msa2-T40D-3HA*), YO77 (*msa2-T126A-3HA*), YO78 (*msa2-T126D-3HA*), YO80 (*msa2-S309A-3HA*), YO81 (*msa2-S309D-3HA*), YO108 (*msa2-S93A-3HA*) and YO109 (*msa2-S93D-3HA*) were grown to the mid-log phase in PM medium, washed with nitrogen-free and 0.5% glucose PM medium, and incubated in PM medium with nitrogen-free and 0.5% glucose at 30˚C. After incubation for 36 hours, the numbers of zygotes were visualized and counted using a microscope. (B) YO1 (*msa2-3HA*), YO74 (*msa2-T40A-3HA*), YO77 (*msa2-T126A-3HA*) and YO80 (*msa2-S309A-3HA*) were grown in YE medium (Nutrient +) or PM medium with nitrogen-free and 0.5% glucose (Nutrient -) to mid-log phase. The proteins were subjected to SDS-PAGE and Msa2 protein was detected by western blot with anti-HA antibody. Closed and open triangles indicate phosphorylated proteins and non-phosphorylated proteins, respectively.

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**Fig. 3. Functional analysis of *msa2* with mutations in putative**

**phosphorylation sites.**

(A) YO24 (*msa2Δ*) cells harboring the plasmid containing *msa2* with the indicated mutations were grown in PM medium to mid-log phase. Each strain was inoculated into nitrogen-free PM medium containing 0.5% glucose. After incubation for 40 hours, the number of zygotes were visualized and counted using a microscope. (B) The K188-A1 (*h⁻ pat1-114*) strain harboring the plasmid containing *msa2* with the indicated mutations was spotted on PM medium and incubated at 25˚C, 34˚C or 36˚C.

**Fig. 4. Functional analysis of *msa2* with mutations in the RNA recognition motif.**

(A) *h⁻ pat1-114* strains harboring the *msa2* plasmids with the indicated mutations and KT34 (wild-type) were spotted on PM medium and incubated at 25˚C or 32˚C. (B) KT34 (wild-type), K188-A1 (*pat1-114*), YO36 (*msa2Δ*) and YO117 (*pat-114 msa2Δ*) were incubated on PM medium at 25˚C, 30˚C, and 34˚C for five days.

**Fig. 5. Expression of Ste11 in cells that overproduce Msa2 or in *cpc2Δ* cells.**

(A) SPB371 (*ste11::ste11-GFP*) cells harboring the empty vector (control) or the plasmid that over-expresses *msa2* (Msa2 O.P.) were grown in PM medium to mid-log phase. Cells were harvested, washed, and resuspended in nitrogen free PM medium containing 0.5% glucose at $0.5 \times 10^7$ cells/ml, and further incubated at 30˚C. For each time point, cells were harvested and crude extracts were prepared by the boiling SDS-beads method (Matsuo et al. 2006). Immunoblotting was performed with an anti-GFP antibody to detect Ste11 and with an anti-PSTAIRE antibody to detect Cdc2 as a loading control. (B) SPB371 (*ste11::ste11-GFP,* control), YO27 (*ste11::ste11-GFP, cpc2Δ*) were grown as described above. Ste11–GFP and Cdc2 were detected by immunoblotting with anti-GFP antibody and anti-PSTAIRE antibody. (C) SP66 (wild-type) and HT201 (*cpc2Δ*) were grown in PM medium to mid-log phase. Cells were washed, resuspended in nitrogen-free PM medium containing 0.5% glucose at $10^7$ cells/ml, and incubated for the indicated times. Total RNA was then extracted from the cells. The expression of transcripts was analyzed by Northern blot. The equality of RNA loading was confirmed by staining with ethidium-bromide (Et-Br).

**Fig. 6. Msa2-GFP forms granules in response to glucose starvation.**
KT160 (*h$^{90}$ msa2-GFP-kanMX6 pabp-mRFP-hphMX6*) cells were cultured in PM at 30℃ to the early-log phase, and harvested by centrifugation. Cells were washed twice in glucose-free PM or nitrogen-free PM (with 2% glucose), and subsequently inoculated into glucose-free PM or nitrogen-free PM (with 2% glucose). Msa2-GFP and Pabp-RFP fluorescence images were obtained using a Leica TCS-SP5 confocal laser scanning microscope.

**Fig. 7. Granule formation of Msa2-GFP and insoluble form of Msa2.**
(A) KT160 (*h$^{90}$ msa2-GFP-kanMX6 pabp-mRFP-hphMX6*) cells were grown in PM medium to mid-log phase and subsequently inoculated into nitrogen free PM medium containing 0.5% glucose. Localization of Msa2 in living cells was monitored with a fluorescence microscope at 25˚C. (B) Insoluble form of Msa2. A mid-log phase culture of YO15 (*h$^{90}$ msa2-3HA-kanMX6 cpc2-GFP-kanMX6*) was incubated in minimal medium (PM). Growing cells in PM medium were washed, resuspended in nitrogen-free PM medium at $0.5 \times 10^7$ cells/ml, and incubated at 30˚C for the indicated times. Extracts of the cells were prepared without boiling and subjected to SDS-PAGE, and proteins were detected by western blot with anti-HA antibody, anti-GFP antibody, or anti-PSTAIRE.

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

| Strain | Genotype | Source |
|---|---|---|
| SP66 | *h$^{90}$ ade6-M210 leu1-32* | (Kawamukai et al. 1992) |
| SP870 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18* | (Kawamukai et al. 1992) |
| SP870A | *h$^{90}$ ade6-M216 leu1-32 ura4-D18* | Lab. stock |
| KT34 | *h$^{-}$ ade6-M210 leu1-32 ura4-D18* | Lab. stock |
| SPKU | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 spk1::ura4* | (Yakura et al. 2006b) |
| K188-A1 | *h$^{-}$ ade6-M210 leu1-32 pat1-114* | Lab. stock |
| SPB371 | *h$^{90}$ ade6-M216 leu1-32 ura4-D18 ste11::ste11-GFP-ura4* | (McLeod et al. 2000) |
| HT201 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 cpc2::ura4* | (Jeong et al. 2004a) |
| YO1 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2-3HA-kanMX6* | (Jeong et al. 2004a) |
| YO3 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2-GFP-kanMX6* | This study |
| YO15 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2-3HA-kanMX6 cpc2-GFP-kanMX6* | (Jeong et al. 2004a) |
| YO20 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 spk1::ura4 msa2-3HA-kanMX6* | This study |
| YO22 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 spk1::ura4 msa2-GFP-kanMX6* | This study |
| YO24 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2::kanMX6* | This study |
| YO27 | *h$^{90}$ ade6-M216 leu1-32 ura4-D18 ste11::ste11-GFP-ura4 cpc2::kanMX6* | This study |
| YO32 | *h$^{-}$ ade6-M210 leu1-32 ura4-D18 msa2-3HA-kanMX6* | This study |
| YO36 | *h$^{-}$ ade6-M210 leu1-32 ura4-D18 msa2::kanMX6* | This study |
| YO74 | *h$^{90}$ ade6-M216 leu1-32 ura4-D18 msa2::msa2(T40A)-3HA-kanMX6* | This study |
| YO75 | *h$^{90}$ ade6-M216 leu1-32 ura4-D18 msa2::msa2(T40D)-3HA-kanMX6* | This study |
| YO77 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2::msa2(T126A)-3HA-kanMX6* | This study |
| YO78 | *h$^{90}$ ade6-M216 leu1-32 ura4-D18 msa2::msa2(T126D)-3HA-kanMX6* | This study |
| YO80 | *h$^{90}$ ade6-M216 leu1-32 ura4-D18 msa2::msa2(S309A)-3HA-kanMX6* | This study |
| YO81 | *h$^{90}$ ade6-M216 leu1-32 ura4-D18 msa2::msa2(S309D)-3HA-kanMX6* | This study |
| YO82 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2::msa2(F153A)-3HA-kanMX6* | This study |
| YO83 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2::msa2(F245A)-3HA-kanMX6* | This study |
| YO84 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2::msa2(F361A)-3HA-kanMX6* | This study |
| YO85 | *h$^{90}$ ade6-M216 leu1-32 ura4-D18 msa2::msa2(F453A)-3HA-kanMX6* | This study |
| YO107 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2::msa2(4FA)-3HA-kanMX6* | This study |
| YO108 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2::msa2(S93A)-3HA-kanMX6* | This study |
| YO109 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2::msa2(S93D)-3HA-kanMX6* | This study |
| YO117 | *h$^{-}$ ade6-M216 leu1-32 ura4-D18 pat1-114 msa2::kanMX6* | This study |
| KT160 | *h$^{90}$ ade6-M210 leu1-32 ura4-D18 msa2-GFP-kanMX6 pab1-mRFP-hphMX6* | This study |

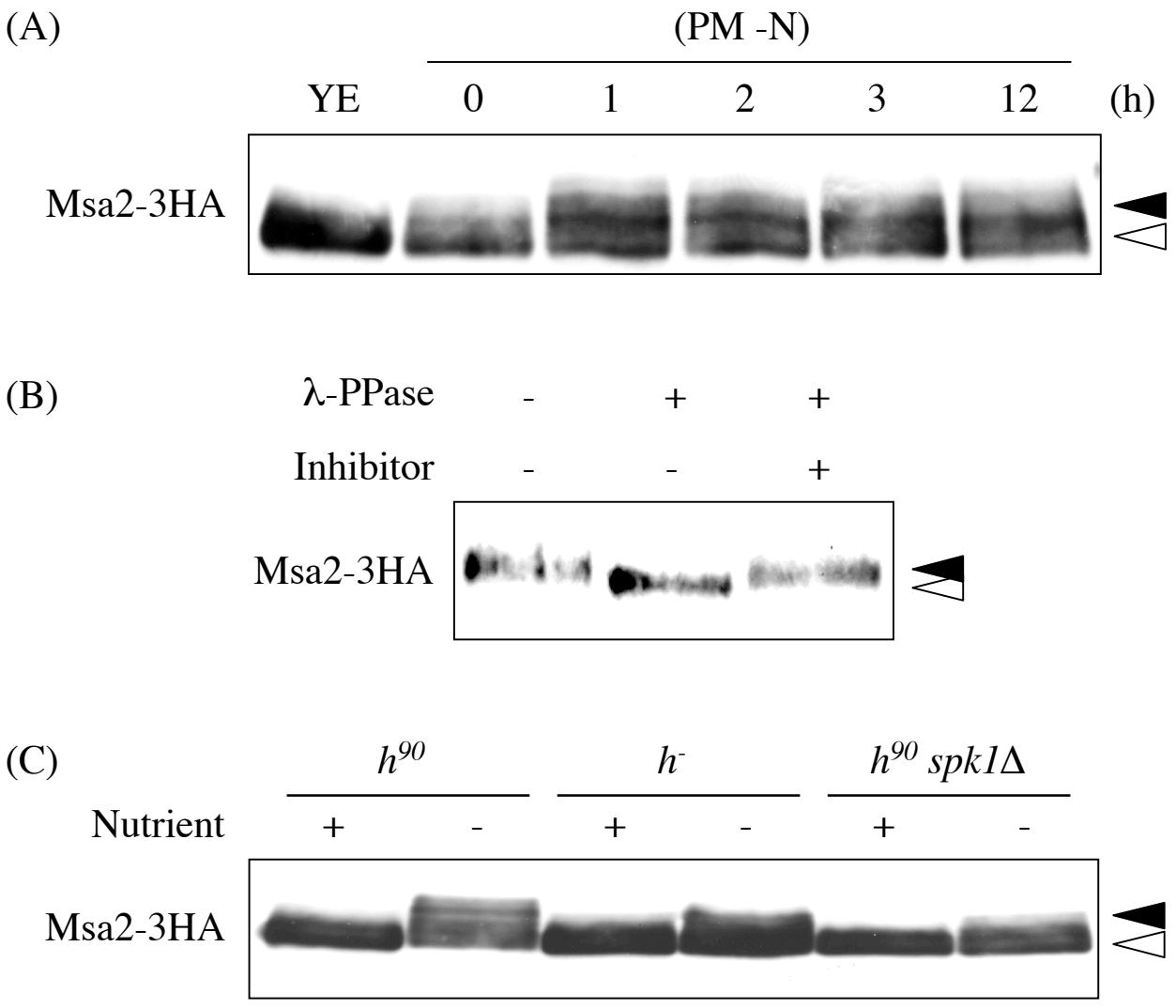
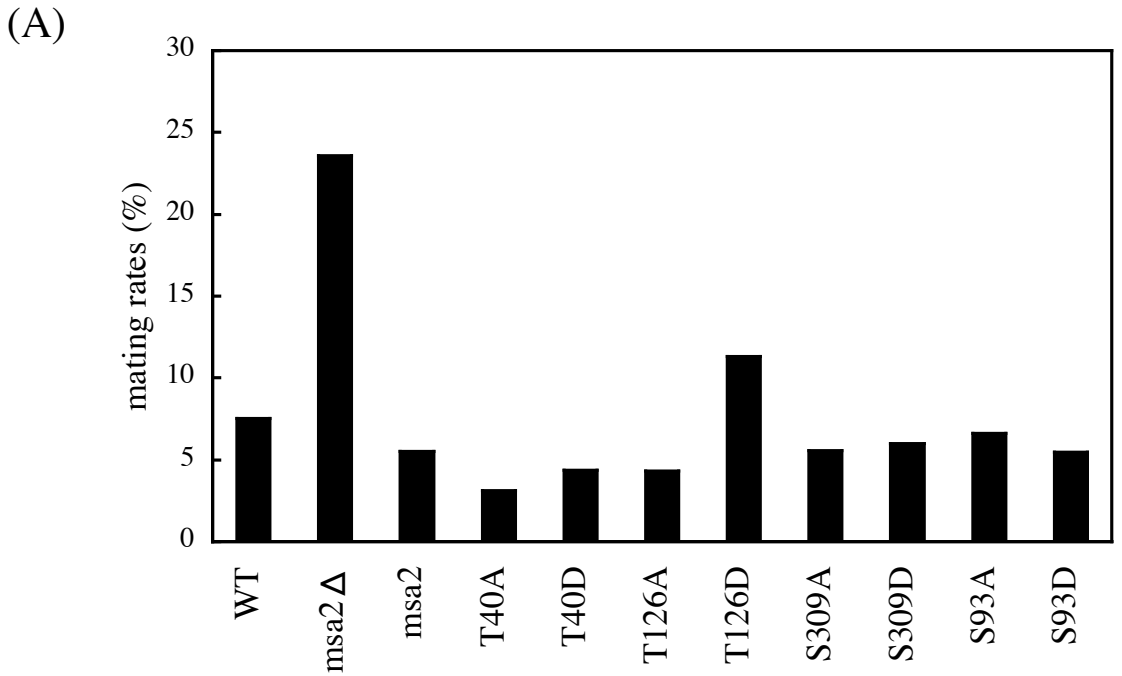

(A)
(PM -N)
YE
0
1
2
3
12
(h)
Msa2-3HA
(B)
λ-PPase
-
+
+
Inhibitor
-
-
+
Msa2-3HA
(C)
h90
h-
h90 spk1Δ
Nutrient
+
-
+
-
+
-
Msa2-3HA

Fig. 1


(A)
mating rates (%)
30
25
20
15
10
5
0
WT
msa2Δ
msa2
T40A
T40D
T126A
T126D
S309A
S309D
S93A
S93D

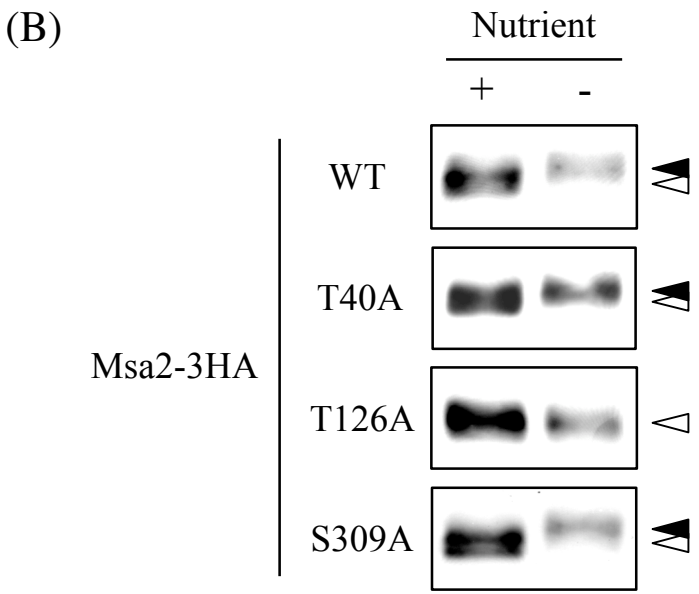
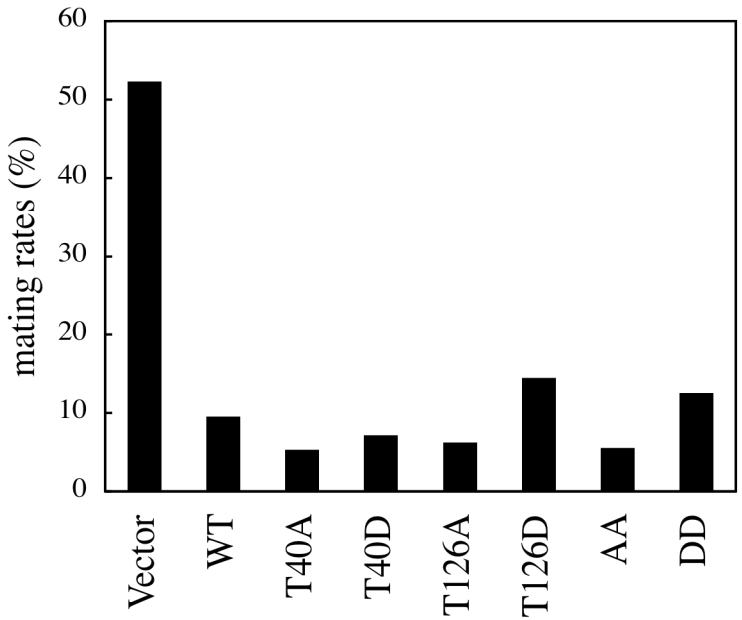
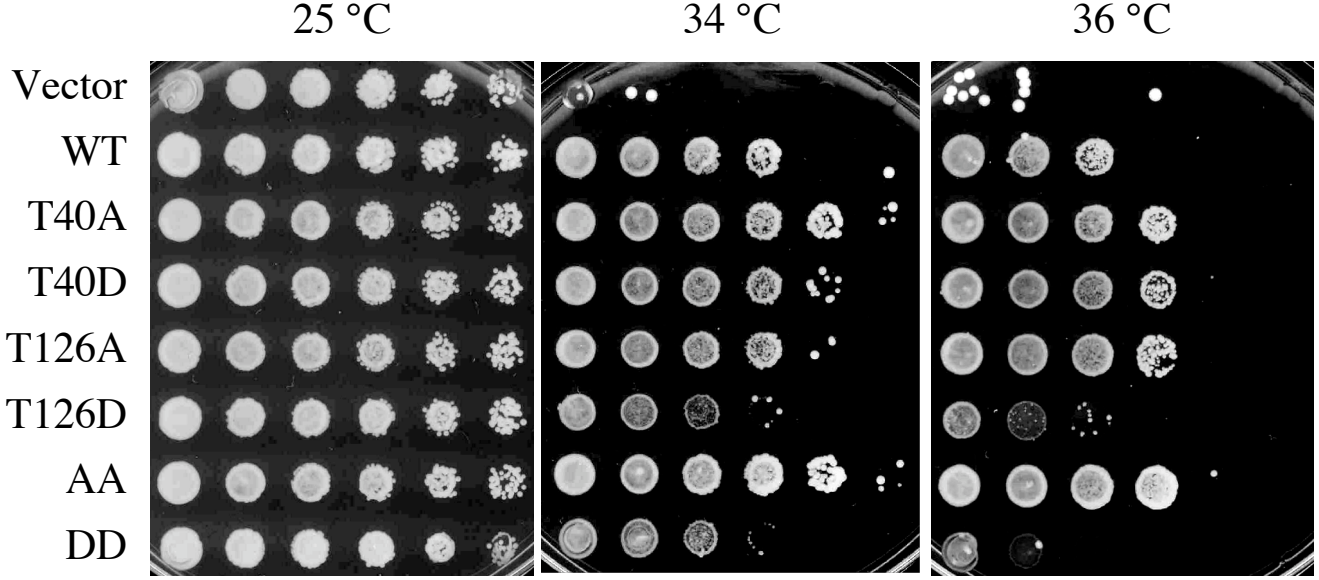

(B)
Nutrient
+
-
Msa2-3HA
WT
T40A
T126A
S309A

Fig. 2

(A)


mating rates (%)
60
50
40
30
20
10
0
Vector
WT
T40A
T40D
T126A
T126D
AA
DD

(B)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34 °C
36 °C
Vector
WT
T40A
T40D
T126A
T126D
AA
DD

Fig. 3

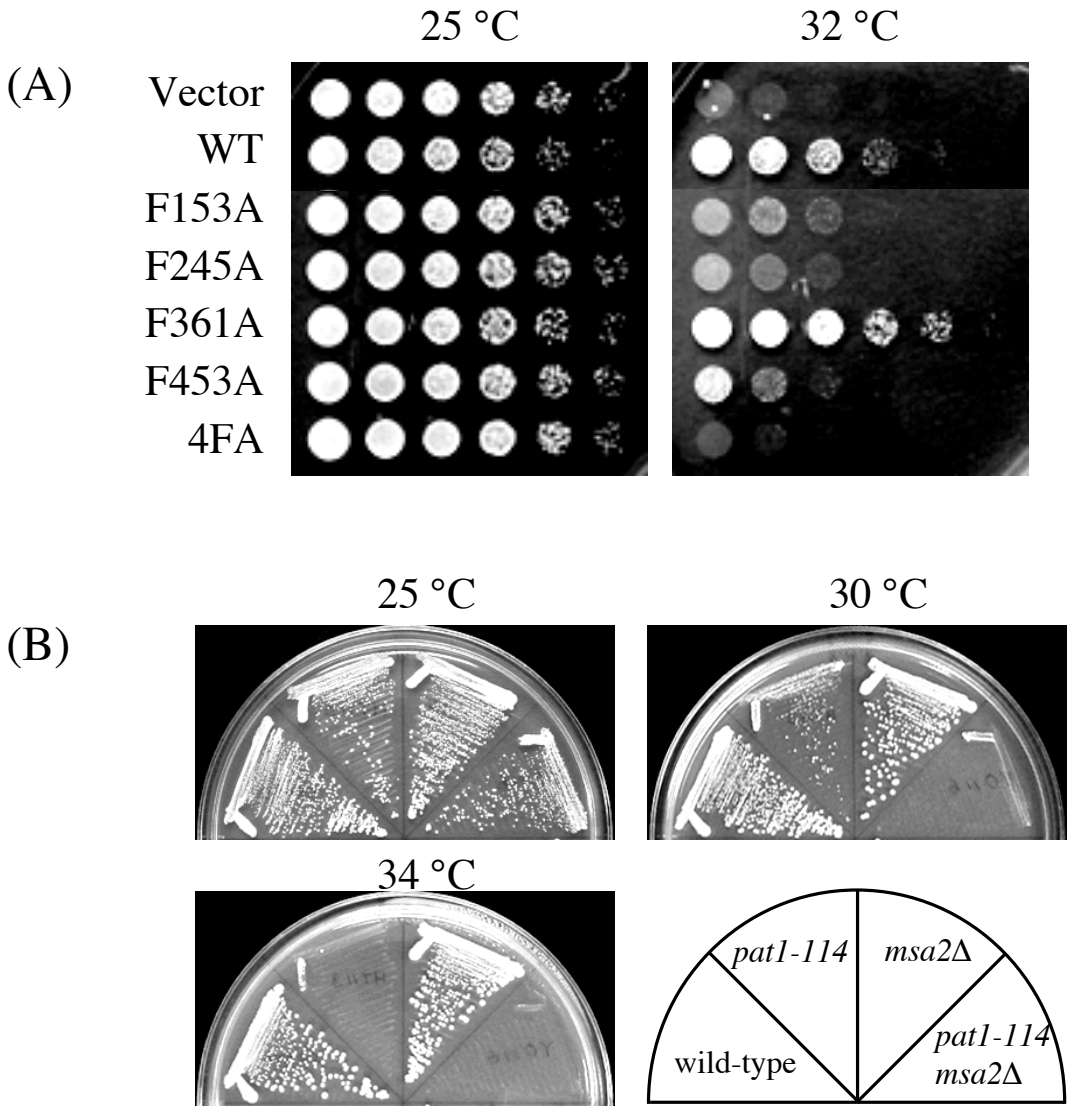
(A)
25 °C
32 °C
Vector
WT
F153A
F245A
F361A
F453A
4FA
(B)
25 °C
30 °C
34 °C
pat1-114
msa2Δ
wild-type
pat1-114
msa2Δ

Fig. 4

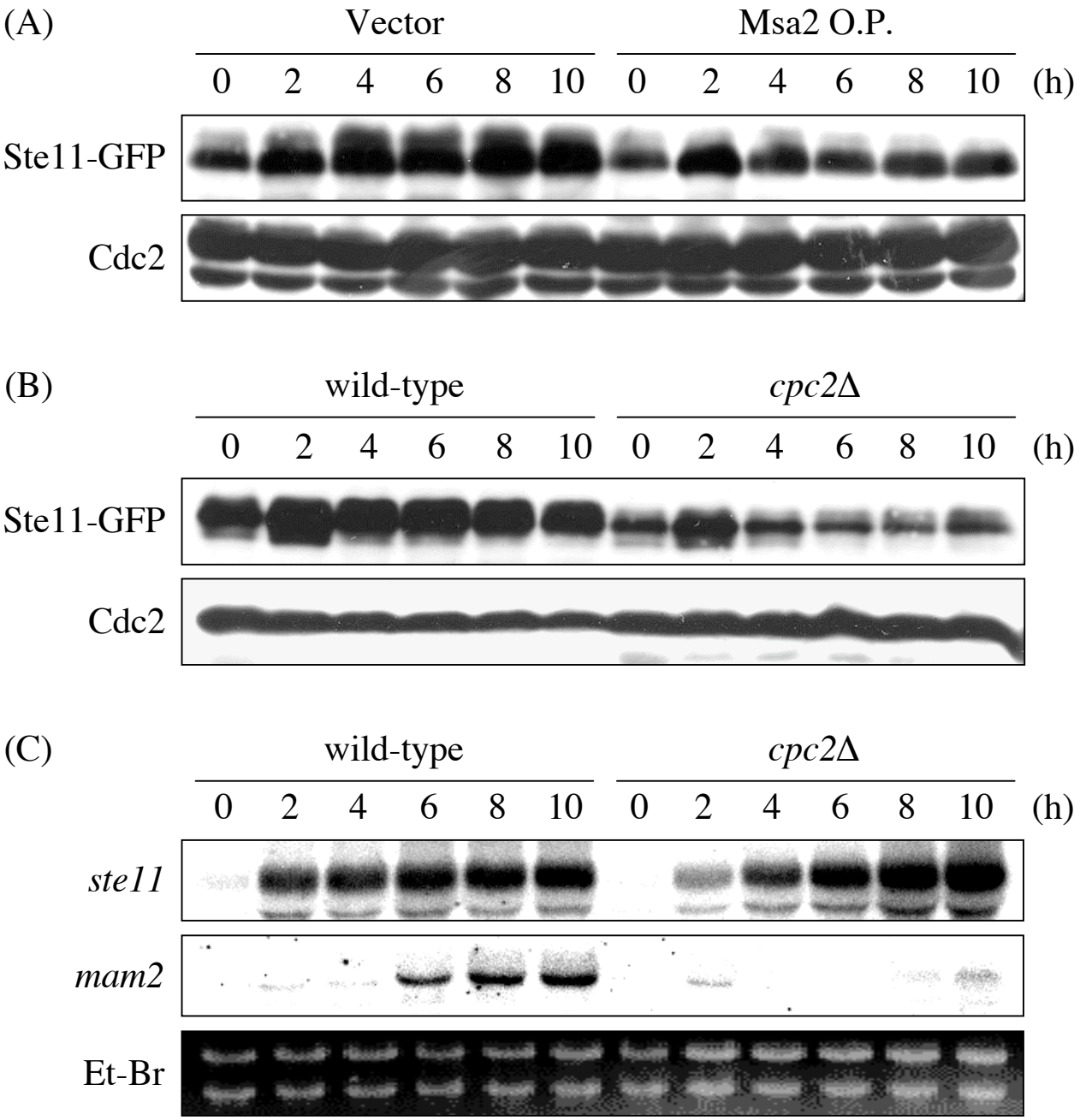
(A)
Vector
Msa2 O.P.
0 2 4 6 8 10 0 2 4 6 8 10 (h)
Ste11-GFP
Cdc2
(B)
wild-type
cpc2Δ
0 2 4 6 8 10 0 2 4 6 8 10 (h)
Ste11-GFP
Cdc2
(C)
wild-type
cpc2Δ
0 2 4 6 8 10 0 2 4 6 8 10 (h)
ste11
mam2
Et-Br

Fig. 5

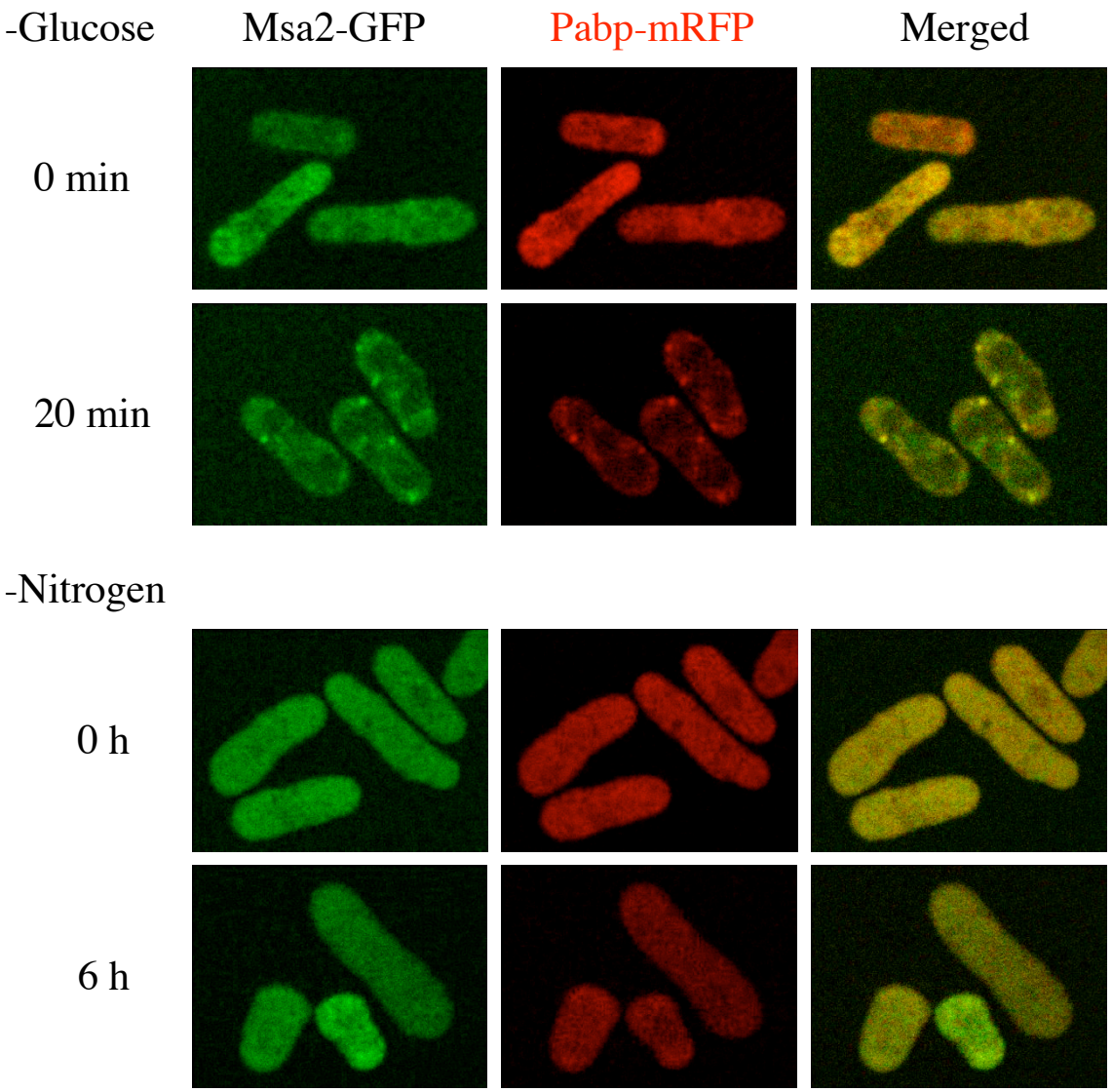


Fig. 6

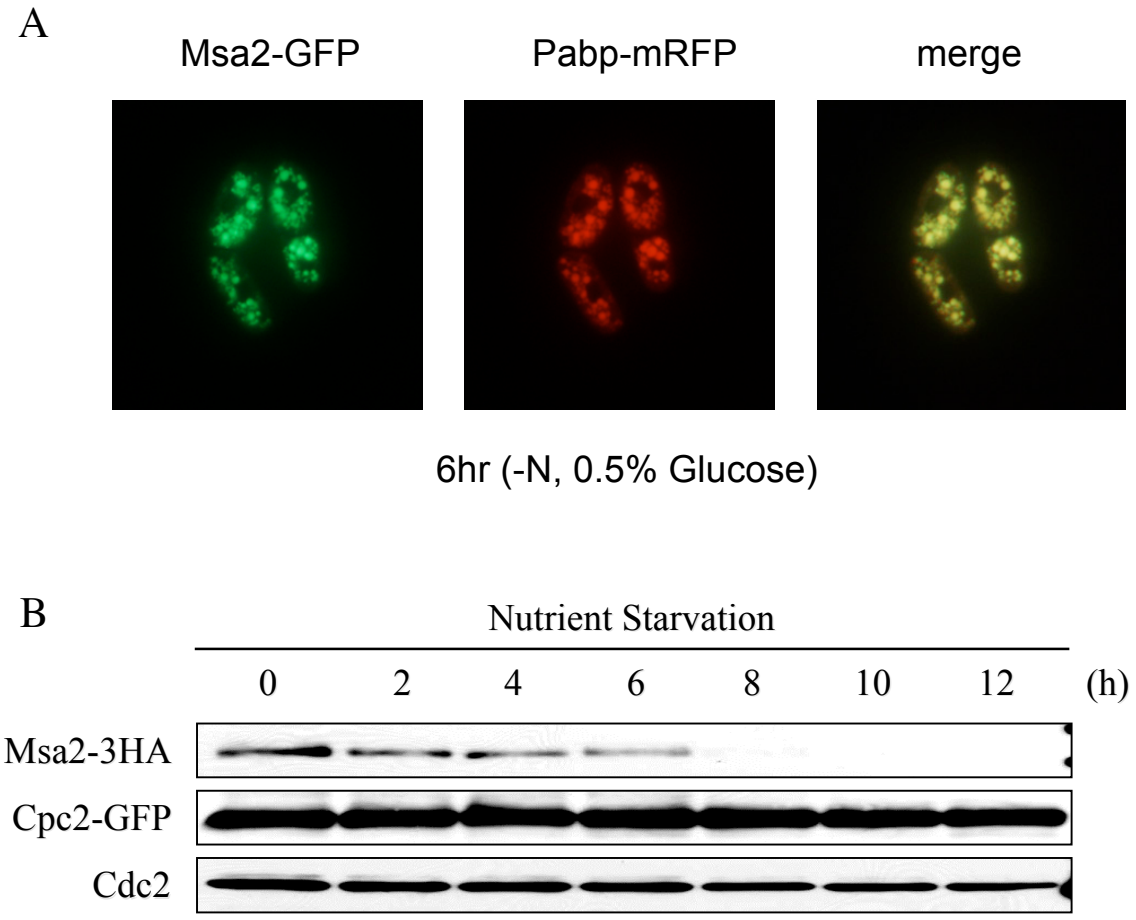
A
Msa2-GFP
Pabp-mRFP
merge
6hr (-N, 0.5% Glucose)
B
Nutrient Starvation
0
2
4
6
8
10
12
(h)
Msa2-3HA
Cpc2-GFP
Cdc2

Fig. 7

**Supplementary Table 1. Oligonucleotide primers used in this study**

| Primer name | Sequence (5' to 3') |
|---|---|
| pSLF272L | |
| msa2-F-*Xho*I | TTCCTCGAGTATGTCTTCTAGCAGTCCC |
| msa2-R-*Not*I | TAGCGGCCGCCAATATTGGAGTTTGT |
| | |
| Site-directed mutagenesis | |
| msa2-F-*Eco*RV | GTTGATATCTGCTTGTTTCATTCGC |
| msa2-R-*Spe*I | CGGTTTGGAACTAGTAGACAATAATG |
| msa2-F-T40A | GGTCTCGCTACACCAAACGCACCGCATGCTCTAC |
| msa2-R-T40A | GTAGAGCATGCGGTGCGTTTGGTGTAGCGAGACC |
| msa2-F-T40D | GGTCTCGCTACACCAAACGACCCGCATGCTCTAC |
| msa2-R-T40D | GTAGAGCATGCGGGTCGTTTGGTGTAGCGAGACC |
| msa2-F-T126A | GGTAACCTTCCTCCCAATGCCCCTATTGATGAG |
| msa2-R-T126A | CTCATCAATAGGGGCATTGGGAGGAAGGTTACC |
| msa2-F-T126D | GGTAACCTTCCTCCCAATGACCCTATTGATGAG |
| msa2-R-T126D | CTCATCAATAGGGTCATTGGGAGGAAGGTTACC |
| msa2-F-S309A | GGGGTATCCTCCCCCAGCCCCGGTTCTCCAAAAAC |
| msa2-R-S309A | GTTTTTGGAGAACCGGGGCTGGGGGAGGATACCCC |
| msa2-F-S309D | GGGGTATCCTCCCCCAGACCCGGTTCTCCAAAAAC |
| msa2-R-S309D | GTTTTTGGAGAACCGGGTCTGGGGGAGGATACCCC |
| msa2-F-S93A | TCACGTGTTACCGCTCCTAATGTTGCTAAC |
| msa2-R-S93A | AGCAACATTAGGAGCGGTAACACGTGAAAC |
| msa2-F-S93D | TCACGTGTTACCGATCCTAATGTTGCTAAC |
| msa2-R-S93D | AGCAACATTAGGATCGGTAACACGTGAAAC |
| msa2-F-F153A | CTACCTGAGAAAAATTGTGCCGCTATTTCCTTTTTGGATCCGAGC |
| msa2-R-F153A | GCTCGGATCCAAAAAGGAAATAGCGGCACAATTTTTCTCAGGTAG |
| msa2-F-F245A | CTGAACGAAATATAGCTGCTGTTCATTTTTTGAACATTGCAGCTG |
| msa2-R-F245A | CAGCTGCAATGTTCAAAAAATGAACAGCAGCTATATTTCGTTCAG |
| msa2-F-F361A | CTTCAGGAAAAACACATTTGTGCCGTAACTTTTGTTGACCCTG |

| | |
|---|---|
| msa2-R-F361A | CAGGGTCAACAAAAGTTACGGCACAAATGTGTTTTTCCTGAAG |
| msa2-F-F453A | GAATTGCGCTGCTGTTAACTTTACGTCGTTGGC |
| msa2-R-F453A | CGTAAAGTTAACAGCAGCGCAATTCTTTTCGCG |

Disruption and tagging

| | |
|---|---|
| msa2(A) | CCTTTCCACATCATTCAAG |
| msa2(B) | GGGGATCCGTCGACCTGCAGCGTACGAGACCGAAATCAATAACTG |
| msa2(W) | GGGTAAGCATAGCGGCCCTC |
| msa2(X) | GGGGATCCGTCGACCTGCAGCGTACGAAATATTGGAGTTTGTTGGC |
| msa2(Y) | GTTTAAACGAGCTCGAATTCATCGATCAGCGCTTTGAGTTCATATC |
| msa2(Z) | GATTCGTCAATATTGTTTGC |
| msa2-R-check | CACCGTTGAAGTCTTCAGC |
| pab1 t1 | GCTGTTCAATATGGTGCCAC |
| pab1 t2 | CGTCGACCTGCAGCGTACGACTCAGTGAAGCCAGGCTCTT |
| pab1 t3 | CGAGCTCGAATTCATCGATGTGAATGACATTTCTATGAAAGTT |
| pab1 t4 | GCTCATTGGTCATCTGAACG |
| pab1 chk | TTAAGGATCTGGTTCGGGAC |

1