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A large complex mediated by Moc1, Moc2 and Cpc2 regulates sexual differentiation in fission yeast

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Running title:

Moc proteins in fission yeast

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Abstract

The sexual differentiation of *Schizosaccharomyces pombe* is triggered by nutrient starvation and is down regulated by cAMP. Screening programs have identified the *moc1/sds23, moc2/ded1, moc3,* and *moc4/zfs1* genes as inducers of sexual differentiation even in the presence of elevated levels of cAMP. To investigate possible interactions among Moc1, Moc2, Moc3 and Moc4 proteins, we first screened for individual Moc-interacting proteins using the yeast two-hybrid system and verified the interactions with other Moc proteins. Using this screening process, Cpc2 and Rpl32-2 were highlighted as factors involved in interactions with multiple Moc proteins. Cpc2 interacted with Moc1, Moc2 and Moc3, whereas the ribosomal protein Rpl32-2 interacted with all Moc proteins in the two-hybrid system. The physical interactions of Cpc2 with Moc1, Moc2 and Rpl32-2, and of Rpl32-2 with Moc2 were confirmed by co-immunoprecipitation. In addition, we revealed that each Moc protein exists as a large complex, using Blue Native PAGE. Over-expression of Moc1, Moc2, Moc3, Moc4 and Rpl32-2 resulted in the efficient induction of a key transcription factor Ste11, suggesting that all proteins tested are positive regulators of Ste11. Considering that Moc2/Ded1 is a general translation factor and that Cpc2 associates with many ribosomal proteins including Rpl32-2, it is possible that a large Moc-mediated complex, detected in this study, may act as a translational regulator involved in the control of sexual differentiation in *S. pombe* through the induction of Ste11.

Introduction

The fission yeast *Schizosaccharomyces pombe* undergoes sexual differentiation when starved of environmental nutrients. The sexual differentiation of *S. pombe* is regulated by at least four signaling pathways: the cAMP pathway, the stress responsive Sty1/Spc1 pathway, the pheromone signaling pathway, and the Tor pathway [1-4]. The cAMP pathway in *S. pombe* is the nutrient sensing pathway that initiates sexual differentiation when opposite mating-type cells co-exist [5]. When glucose (or nitrogen) is abundant, the heterotrimeric-type guanine nucleotide-binding protein (Gpa2) becomes activated via the Git3 receptor [6]. The Gpa2 protein subsequently activates adenylyl cyclase (Cyr1) to generate cAMP from ATP [5]. Cyr1 interacts with its associated protein Cap1, which plays a partly regulatory role with respect to adenylyl cyclase and also interacts with actin [7, 8]. When cAMP is abundant, it associates with the regulatory subunit Cgs1, and the catalytic protein kinase Pka1 is released [9]. Pka1 phosphorylates the zinc-finger protein Rst2, which induces the expression of *ste11,* a gene encoding a key transcription factor for many meiosis-specific genes [10]. Thus, expression of *ste11* is induced in response to a decrease in the level of cAMP and results in the initiation of meiosis.

The *S. pombe moc* (multicopy suppressor of over expressed *cyr1*) *1* to *moc4* genes have been identified as genes that overcome a partially sterile *S. pombe* phenotype caused by an elevation in cAMP [11, 12]. Among the four *moc* genes, *moc1* is the strongest inducer of sexual differentiation [12], and the Moc1/Sds23 protein in *S. pombe* is known to play important roles in stress resistance [13, 14], cell cycle [13], chronological life span [14], survival for Go cells [15] and sexual differentiation [14]. Moc1/ Sds23 has also been identified as a suppressor of *dis2* [13] and as a phosphorylated protein [16]. The Moc1 protein is localized to the cytosol during mitotic growth, but accumulates in the nucleus in mating cells and this localization shift is inhibited by cAMP [14]. Moc1 and its orthologous proteins

contain a common domain known as the cystathionine-beta-synthase (CBS) domain, which is predicted to have a multiple trafficking function for protein-protein interactions and metabolic regulation, and is found in proteins such as AMP activated protein kinase (AMPK) [17]. Moc1 and its *S. cerevisiae* orthologous proteins (Sds23/Sds24) are functionally interchangeable [17]. Moc2/Ded1 is an essential RNA helicase, which is involved in both sexual differentiation [11] and the mitotic cell cycle [18, 19], and is now known to be a general translational regulator [11, 19, 20]. Moc3, a Zn finger-type protein is localized to the nucleus and is involved in stress resistance and sexual differentiation [12]. Moc4/Zfs1 contains two Zn-finger motifs, is localized to the nucleus, and is involved in sexual differentiation and septum formation [21, 22]. Moc4/Zfs1 has also been identified as an mRNA binding and destabilizing protein in *S. pombe* [23]. While the *moc1, moc3,* and *moc4* genes are dispensable [12, 14, 21], *moc2* is an essential gene for growth [11]. However, it is not yet clear how the Moc proteins function in sexual differentiation through interactions with other unidentified proteins [12].

The possibility that these four Moc proteins might work together as part of the same complex has never been considered. Therefore, we decided to search for Moc-interacting proteins and in this study we report the isolation of Moc-interacting proteins in *S. pombe* using the yeast two-hybrid system. We then verified the relationships between the various proteins and proposed the existence of a Moc-mediated protein complex capable of regulating sexual differentiation via interactions with translational components in fission yeast.

Results

Two-hybrid screening of Moc proteins

To ascertain the relationship between the Moc proteins, we attempted to identify proteins that interact with Moc1, Moc2, Moc3 and Moc4 using the yeast two-hybrid system. By cloning

each *moc* gene into the pGBKT7 vector as bait, we conducted a large scale two-hybrid screen using an *S. pombe* cDNA library, cloned into the pGAD prey vector in *S. cerevisiae* AH109, as described in Experimental Procedures. The screened genes were verified by re-introducing them into the test strain AH109 and the genes cloned in the pGAD vector were identified by sequencing. The results of this screening process led to the identification of the following Moc1-interacting proteins: pyruvate decarboxylase, EF1α-A, glyceraldehyde 3-phosphate dehydrogenese (GAPDH), thioredoxin peroxidase, Alg9, Srp-54, Rpb3, Obr1, Sfh1, Ufd2; and the ribosomal proteins L29, L32-2, L38, S3a, S14, S16, and S20 (Table 3). We next tested whether these proteins also interacted with Moc2, Moc3, and Moc4 proteins, and we found that all Moc1-interacting proteins interacted with Moc3, while only the ribosomal protein Rpl32-2 interacted strongly with Moc1, Moc2, Moc3 and Moc4 proteins. Pyruvate decarboxylase, EF1α-A, GAPDH, thioredoxin peroxidase, Srp54 and Ufd2 interacted with Moc1, Moc3 and Moc4, whereas RNA polymerase subunit Rpb3, Alg9, Obr1 and Sfh1 interacted with Moc1 and Moc3 (Table 3). None of the proteins interacted with the GBD alone, indicating that the interactions with the different Moc proteins were specific.

In a similar-two hybrid screen using Moc2 as bait, Moc2-interacting proteins were identified as Lys3 (saccharopine dehydrogenase) and the ribosomal proteins L8, L18, L20, L27, L29, and S13 (Table 4). All of the Moc2-interacting proteins interacted with Moc3, while Lys3 and ribosomal proteins L8, L18, L29, and S13 interacted with Moc1, Moc2, and Moc3. The ribosomal protein S13 strongly interacted with Moc1, Moc2 and Moc3, and Lys3 interacted strongly with Moc2 and Moc3, but loosely with Moc1. None of the Moc2-interacting proteins interacted with Moc4, or with the GBD alone (Table 4), indicating that the interactions with different Moc proteins were specific.

Similarly, screening for Moc3-interacting proteins using the two-hybrid system identified pyruvate decarboxylase, enolase, 20S proteosome component alpha 5, EF1α-A,

GAPDH, the ribosomal protein L32-2, superoxide dismutase, GluRS [24] and Cpc2 (Table 5). All Moc3-interacting proteins interacted with Moc1, which is consistent with the results mentioned above in that all Moc1-interacting proteins interacted with Moc3. This finding suggests that Moc1 and Moc3 might form individual subunits of a putative complex. The ribosomal protein Rpl32-2 strongly interacted with all four Moc proteins, and GluRS strongly interacted with Moc1, Moc3 and Moc4, whereas Cpc2 interacted strongly with Moc1, Moc2 and Moc3. Pyruvate decarboxylase, enolase, 20S proteosome component alpha 5, EF1α-A and GAPDH interacted with Moc1, Moc3, and Moc4. None of the Moc3-interacting proteins interacted with the GBD (Table 5), again suggesting that the interactions with the different Moc proteins were specific.

Finally, Moc4-interacting proteins identified using the two-hybrid system were: GAPDH, pyruvate decarboxylase, enlace, eEF2, Ebp2, Psu1, Fba1, Crb3, SPCC74.02c (mRNA cleavage and polyadenylation specificity factor complex associated protein), and the ribosomal proteins L5, L12, L32-2, and P2B (Table 6). Among the Moc4-interacting proteins, GAPDH, pyruvate decarboxylase, the ribosomal protein L12, Psu1, Fba1, Crb3 and SPCC74.02c interacted with Moc1, Moc3 and Moc4, while Ebp2 interacted with Moc3 and Moc4. Only the ribosomal protein Rpl32-2 strongly interacted with all the Moc proteins and, except for Rpl32-2, none of the Moc4-interacting proteins interacted with Moc2 in a yeast two-hybrid system. In addition, none of the Moc4-interacting proteins interacted with the GBD (Table 6).

Interactions of Moc proteins with Cpc2 in fission yeast

The two-hybrid screen revealed that some proteins, such as Cpc2 and Rpl32-2, interacted strongly with multiple Moc proteins. We also found that Rpl32-2 interacted with Cpc2 in a two-hybrid system (data not shown). Cpc2 interacted strongly with Moc1, Moc2 and Moc3,

and Rpl32-2 interacted strongly with all Moc proteins in a yeast two-hybrid system (Table 5); therefore, we next tested the physical interactions of Cpc2 with the Moc proteins and with Rpl32-2 by co-immunoprecipitation, where the protein of interest was immunoprecipitated with a tagged antibody. Western blotting was used to identify proteins that were pulled down by interaction with the Cpc2 protein. To determine the physical interactions between Cpc2 and Moc1, Moc2 and Rpl32-2, cell extracts were prepared from the double-tagged strains: SKP6 (*cpc2-3HA*, *moc1-13Myc*), SKP2 (*cpc2-3HA*, *moc2-13Myc*) and SKP21 (*cpc2-3HA, rpl32-2-13Myc*) (Table 1). The monoclonal HA antibody was used to immunoprecipitate Cpc2-3HA, and the precipitate was then analyzed by Western blotting, first using the HA antibody and then the Myc antibody (Fig. 1 A, B, C). As shown in figure, Moc1-13Myc, Moc2-13Myc and Rpl32-2-13Myc were detected by immunoprecipitation. Equally, when Moc1-13Myc, Moc2-13Myc and Rpl32-2-13Myc were first precipitated by a Myc antibody and the precipitated proteins were analyzed by Western blotting using a monoclonal Myc antibody followed by the HA antibody (Fig. 1 A, B, C), the result showed that Cpc2-3HA was present in the anti-Myc immunoprecipitates of Moc1-13Myc, Moc2-13Myc and Rpl32-2-13Myc (Fig. 1A, B, C). These results indicated that Cpc2 interacted with Moc1, Moc2, and Rpl32-2 *in vivo*. All the experiments were conducted reciprocally and the results of the interactions were consistent in all cases. However, when we tested the co-immunoprecipitation of Moc3 and Moc4 with Cpc2, there was no co-immunoprecipitation in either case (data not shown). We did not detect any physical interaction between Moc3 and Cpc2, although they did appear to interact in the two-hybrid system.

Interactions of Moc proteins and Rpl32-2 in fission yeast

The interactions between Rpl32-2, fused to the GAL4 activation domain, and each of the Moc1, Moc2, Moc3 and Moc4 proteins, fused to a GAL4 DNA binding domain, were tested in

the two-hybrid system (Tables 3, 5 and 6). We then performed the reciprocal experiment, fusing Rpl32-2 to the GAL4 DNA binding domain and fusing Moc1 to Moc4 to a GAL4 activation domain, and again tested the interactions using the yeast two-hybrid system. The results showed that Moc1, Moc2, Moc3, and Moc4 interacted strongly with Rpl32-2 in the GAL4-based two-hybrid system (data not shown).

Next we tested the *in vivo* interactions of Rpl32-2 with Moc1, Moc2, Moc3, and Moc4 by co-immunoprecipitation. To determine the physical interactions between Rpl32-2 and the four Moc proteins, cell extracts were prepared from the following double-tagged integrated strains: SKP22 (*moc1-3HA, rpl32-2-13Myc*), SKP25 (*moc2-13Myc, rpl32-2-3HA)*, SKP26 (*moc3-13Myc, rpl32-2-3HA)* and SKP27 (*moc4-13Myc, rpl32-2-3HA*) (Table 1). As shown in the results, only Moc2 was co-immunoprecipitated with Rpl32-2 (Fig. 2A). A Myc antibody was used to precipitate the Moc2-13Myc protein and the precipitates were analyzed by Western blotting using the monoclonal HA antibody. Conversely, the monoclonal HA antibody was used to immunoprecipitate Rpl32-2-3HA, and Moc2-13Myc was detected by a Myc antibody. Our results showed that Rpl32-2-3HA was present in the Myc immunoprecipitated sample and, reciprocally, that Moc2-13Myc was present in the HA immunoprecipitated sample (Fig. 2A), indicating that Moc2 interacts with Rpl32-2 *in vivo*. However, when we tested Moc1, Moc3, and Moc4 with Rpl32-2, no co-immunoprecipitation was observed (data not shown), in contrast to the results of the two-hybrid system.

We then tested the possible interaction of Moc1 and Moc2 by co-immunoprecipitation using the strain SKP29 (Moc1-GFP, Moc2-13Myc). A Myc antibody was used to precipitate the Moc2-13Myc protein and the precipitates were analyzed by Western blotting using the monoclonal GFP antibody. As shown in Fig. 2B, Moc1 was co-immunoprecipitated with Moc2.

Identification of the Moc complex by Blue Native PAGE

The results described above suggested the possibility of complex formation mediated by some of the Moc proteins, together with Cpc2 and Rpl32-2. To determine the nature of the putative Moc-mediated complex in fission yeast, we used Blue Native (BN) PAGE [25]. In these experiments, cell extracts were prepared from the *S. pombe* strains SKP1, SKP5, SKP7, and SKP9 that expressed Moc2, Moc1, Moc3, and Moc4 proteins, respectively. The Moc proteins were linked to a 13Myc tag at the C-terminus (Table 1). When BN-PAGE was used to separate the proteins from SKP1, a large Moc2-mediated protein complex ca. 1,000 kDa was detected by Western blotting using the Myc antibody (Fig. 3A). The proteins, separated by BN-PAGE in the first dimension, were further separated by SDS-PAGE in the second dimension and subsequently detected by a Myc antibody (Fig. 3B, C). During electrophoresis in the second dimension, the complex was separated according to the molecular masses of the individual subunits and the proteins were detected by Western blotting (Fig. 3B, C, D), which revealed a broad signal pattern ranging in size from large to small. The separation of Cpc2-3HA by second dimensional SDS-PAGE following BN-PAGE, produced a similar pattern, indicating that both proteins separate in a similar manner on a two dimensional (2-D) gel. This result also suggested that both proteins exist as complexes that range in size from high to low molecular weights. The molecular mass (ca 1,000 kDa) of the complex detected by BN-PAGE was much greater than its molecular mass (ca 100 kDa) detected by SDS-PAGE (Fig. 3E). A mass of 100 kDa for the Moc2-13Myc protein detected by SDS-PAGE is reasonable since the Moc2 protein has a mass of approximately 70 kDa and 13-Myc is approximately 20 kDa. These results indicated that the Moc2 protein exists as a large complex and associates with other proteins such as Cpc2.

A broad pattern of molecules ranging in size from large to small was also detected when proteins from the strains SKP5 (Moc1-13Myc), SKP7 (Moc3-13Myc), and SKP9

(Moc4-13Myc) were separated by BN-PAGE in the first dimension and by SDS-PAGE in the second dimension, with subsequent detection by a Myc antibody (Figs. 4A, 5A, and 6A). The double-tagged strains, SKP2 (*cpc2-3HA moc2-13Myc*), SKP6 (*cpc2-3HA moc1-13Myc*), SKP8 (*cpc2-3HA moc3-13Myc*), and SKP10 (*cpc2-3HA moc4-13Myc*), showed similar results to the single-tagged strains (SKP1, SKP5, SKP7, and SKP9) when analyzed by 2-D electrophoresis and Western blotting (Figs. 3B, 4B, 5B and 6A). The patterns for Cpc2-3HA in each strain, detected by the HA antibody, were also similar to those of the double-tagged strains (Figs. 3C, 4C, and 5C). The pattern, ranging in size from large to small, indicated the existence of a large molecule containing the Moc1, Moc2, Moc3, Moc4 and Cpc2 proteins. The pattern of 2-D analysis was quite different upon examination of a protein such as Asf1, which works as a histone chaperon and exists as a monomer of around 30 kDa (Fig. 6C). The 2-D analysis of Asf1-13Myc revealed only a small-sized protein. This control experiment confirmed that the separation of proteins by BN-PAGE functioned efficiently.

We then performed further tests to determine whether Cpc2 plays an important role in the Moc-mediated complex. To this end, we constructed various *cpc2::ura4* strains hosting the different *c-myc* tagged *moc* genes: SKP11 (*cpc2::ura4 moc1-13Myc*), SKP13 (*cpc2::ura4 moc3-13Myc*), and SKP14 (*cpc2::ura4 moc4-13Myc*). Cell extracts were prepared from these strains and the samples were loaded onto gels for first dimension separation using BN-PAGE. Gel strips were then excised and used for electrophoresis in the second dimension. Western blotting revealed that, due to the *cpc2* deletion, the Moc1- and Moc3-mediated protein complexes produced a weaker signal and were shifted towards a lower molecular weight (Figs. 4D and 5D). The results indicated that, in the absence of Cpc2, a Moc1- or Moc3-mediated large protein complex was either not formed, or was unstable in *S. pombe* cells. We constructed the strain *cpc2::ura4 moc2-13Myc,* but Western blotting failed to detect the Moc2 protein against a *cpc2*-deleted background. This result indicated that

Cpc2 is important for the existence of the Moc2 protein in *S. pombe* cells. To determine whether the stability of Moc2 is dependent on the presence of Cpc2, SKP12 (*cpc2::ura4 moc2-13Myc*) was transformed with the plasmid pSLF273-cpc2, and the proteins were analyzed by Western blotting. We were able to detect the Moc2 protein in this transformant (data not shown), which clearly indicated that, in the absence of Cpc2, Moc2 is unstable in *S. pombe* cells. It was previously reported that loss of Cpc2 did not dramatically alter the rate of cellular protein synthesis, but caused a decrease in the steady state level of variable proteins [26]. We also tested whether Cpc2 affects Rpl32-2 by 2-D analysis of the strain SKP30 (*cpc2::ura4 rpl32-2-13Myc*). The results revealed that deletion of Cpc2 lowered the total amount of protein present, but did not alter its molecular size.

Influence of Moc1 to Moc4 and Rpl32-2 proteins on the expression of Ste11

Finally, we tested whether over-expression of the Moc1 to Moc4 proteins and of Rpl32-2 induced expression of the transcription factor Ste11. Following nitrogen starvation, samples were taken from the strains that over expressed each protein at regular time intervals (Fig. 8), and Western blotting was used to monitor the level of Ste11-GFP expressed on the chromosome. Our results revealed that expression of the Ste11 was clearly induced in response to over-expression of the individual proteins Moc1, Moc2, Moc3, Moc4, and Rpl32-2 (Fig. 8). A sharp peak of Ste11 at 3hr after nitrogen starvation was observed in the wild type strain as observed before [21]. But, induction of Ste11-GFP by Moc1 gave the clearest result, consistent with the observation that, of the four Moc proteins, Moc1 is the strongest inducer of sexual development [12]. The induction of Ste11-GFP by Moc2 was observed after the 9hr time point, which may indicate up-regulation of translation. It is interesting to note that Rpl32-2 also had a positive effect on the induction of Ste11.

Discussion

In this study, we have shown that Moc1, Moc2, Moc3, and Moc4 proteins, which have been identified as positive regulators of sexual differentiation [11], exist as high molecular weight complexes, and that Cpc2 plays an important part in the formation of each complex. Figure 9 summarizes the interactions revealed in this study combined with previously reported results [14]. The interactions revealed by the two-hybrid system (shown by dashed arrows in Fig. 9) were not always detected by co-immunoprecipitation in this study. In general, co-immunoprecipitation detects stable interactions *in vivo*, while the two-hybrid system detects both stable and transient interactions. Special care is necessary to interpret protein interactions detected in the two-hybrid system, because the proteins are over-expressed in an artificial system, which does not always reflect the *in vivo* situation.

Two-dimensional (BN-PAGE and SDS-PAGE) analysis showed that the gel patterns for each Moc-mediated complex ranged in size from high to lower molecular weights, suggesting that a high molecular weight complex does exist, in some form, in each case. It is unclear whether all Moc proteins co-exist in the same complex, although it is unlikely, as the four Moc proteins do not co-exist in the same cellular compartments. Moc1 and Moc2 are mainly localized to the cytosol [11], while Moc3 and Moc4 are found mainly in the nucleus [12, 21]. Moc1 may have a chance to interact with the nuclear proteins as it migrates from the cytosol to the nucleus under starvation conditions, although this localization shift is inhibited by cAMP [14]. All proteins are translated in the cytosol, thus there will always be the chance of transient interactions between proteins that do not normally co-exist, and although Moc3 and Moc4 have been localized to the nucleus by GFP fusion analysis, this does not rule out the transient localization, or the presence of small amounts, of these proteins in the cytosol.

All Moc1-interacting proteins identified in the two-hybrid system also interacted with Moc3 (Table 3). Equally, all Moc3-interacting proteins identified in the two-hybrid system interacted with Moc1 (Table 5). These results could imply that Moc1 and Moc3 have similar characteristics to the target protein, but in fact, these two proteins have very different primary structures. Moc1 contains the cystathione beta synthase (CBS) domain that is typically found in the gamma subunit of AMP kinase [14], and Moc3 contains the Zn(2)-Cys(6) binuclear cluster, which is typically found in transcription factors. No direct interaction between Moc1 and Moc3 has been reported [12] and an explanation for the formation of a putative complex, mediated by both Moc1 and Moc3, awaits further analysis. It was shown very recently that Moc1/Sds23 interacted with PP2A-related phosphatase Ppe1 [27], but we did not screen out the related protein in our two-hybrid screening.

The two-hybrid screening, reported in this study, using *moc1, moc2, moc3,* or *moc4* genes as bait highlighted the involvement of two proteins, namely Cpc2 and Rpl32-2. Cpc2 (a RACKI ortholog) protein is a highly conserved member of the family of WD-repeat proteins, exclusively localized in the cytosol [26, 28]. It is involved in sexual differentiation and plays a role in translation through its interaction with ribosomal proteins [26]. Cpc2 has been shown to interact with Msa2, an RNA binding protein that negatively regulates sexual differentiation [28-30], Pck2, a protein kinase C ortholog [31], and Pat1 kinase, a negative regulator of meiosis [32]. Our co-immunoprecipitation study additionally revealed that Cpc2 physically interacted with Moc1, Moc2, and Rpl32-2 (Fig. 1). Co-immunoprecipitation experiments generally detect tight interactions between proteins, so we were unable to detect any interaction between Cpc2 and Moc3 by this method, although the interaction was detected in the two-hybrid system. On the contrary, Moc1 was co-immunoprecipitated with Moc2 (Fig. 2), while no interaction between them was observed in the two-hybrid system. This result strengthened the existence of a large molecule complex and the interaction is explainable by

the presence of a bridging protein such as Cpc2 between Moc1 and Moc2.

Most interestingly, the larger molecule of the Moc1- or Moc3-mediated complex shifted to the smaller-sized molecule when Cpc2 was absent (Figs. 4, 5), and the Moc2-mediated complex became undetectable (data not shown). Moc2/ded1 is an RNA helicase that functions as a general translational factor, and Cpc2 is a ribosomal associated protein; therefore, it is reasonable to think that the complex detected in this study is also associated with ribosomes. A Moc2-mediated complex, under native conditions, was clearly detected at around 1,000 kDa by BN-PAGE (Fig. 3A). This size is close to that of the small subunit (40S) of the ribosomal complex, which is estimated to be around 1,400 kDa. However, since 1,000 kDa is almost the upper size limit that can be clearly separated by BN-PAGE, the estimation of the size may not be accurate, and may reflect the size of the small subunit (40S) of the ribosomal complex. It is possible that each Moc protein is associated with the ribosomal complex through Cpc2, although more analysis will be necessary to prove this possibility. Alternatively, the large Moc1- Moc2- Moc3- or Moc4-mediated complex may link to the processing bodies (P-bodies), which is now implicated in translation repression, mRNA decay, nonsense-mediated decay, and mRNA storage [33]. In fact, very recently, an involvement of Ded1 RNA helicase in the formations of P-bodies was reported in *S. cerevisiae* [34].

In this study, we focused on the ribosomal protein Rpl32-2 because it interacts strongly with all Moc proteins in the two-hybrid system. Our analyses revealed the physical interaction of Rpl32-2 with Moc2 or the ribosome associated protein Cpc2, which indicates that the Moc2 and Cpc2 proteins are associated with the ribosomal complex. This observation agrees well with previous results [26, 35]. Interestingly, Rpl32-2 of *S. pombe* was reported to exhibit a novel extra-ribosomal function by acting as a DNA binding protein and potential transcriptional regulator [36]. We investigated whether Rpl32-2 has a positive effect on the

sexual differentiation of *S. pombe*, and our results revealed that when Rpl32-2 was over-expressed in *S. pombe* cells under conditions of nitrogen starvation, expression of the key transcription factor Ste11 was increased (Fig. 8). This implied a similar role for Rpl32-2 in sexual differentiation as for the Moc proteins, which were confirmed as positive regulators of sexual differentiation and were found to induce expression of *ste11* when over-expressed (Fig. 8). Lower levels of *ste11* expression were shown in a *moc4*/*zfs1* deletion mutant [21] and a *cpc2* deletion mutant [32], both of which are sterile strains. Our preliminary data showed that *ste11* expression levels in a *moc1* mutant and a *moc3* mutant [12], which are sterile and partly sterile strains respectively, were also lowered than in wild type. Thus, all the *moc* genes, *cpc2*, and *rpl32-2* investigated in this study appear to play their roles as inducers of sexual differentiation.

These studies collectively suggest that each Moc protein exists as a large complex in fission yeast and that these proteins are involved in a regulatory network that functions through interactions with the ribosome-associated protein Cpc2 and the ribosomal protein Rpl32-2. Although all Moc proteins may not co-exist in a single complex, it is possible that a large complex mediated by Moc1, Moc2 and Cpc2 might operate as a translational regulator involved in controlling the sexual differentiation of *S. pombe* through activation of the key transcription factor Ste11.

Experimental procedures

Strains, media and genetic manipulation

The *S. cerevisiae* strain AH109 (*MATa, trp1-901, leu2-3,112 ura3-52, his3-200, gal4*Δ*, gal80*Δ*, LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3*, *GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2*, *URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-LacZ*) was maintained on YPD media composed of 1% yeast extract (Y), 2% bactopeptone (P), 2% dextrose (D) and 2% agar. Synthetic dropout media

SC-Trp, SC-Trp-Leu and SC-Trp-Leu-His were used for nutrient auxotrophy in the two-hybrid analyses. The *S. pombe* strains used in the study are listed in Table 1. Standard yeast culture media and genetic manipulations were used as described previously [37]. The *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 the addition of the appropriate auxotrophic supplements (0.0075% adenine, leucine or uracil) when required. Either LiOAc, or the electroporation method, was used to transform yeast cells [38]. *Escherichia coli* DH5α grown in Luria-Bertani (LB) medium (1% polypeptone, 0.5% yeast extract, 1% sodium chloride) hosted all plasmid manipulations using the standard methods described [39].

Plasmid construction

The bait plasmids pGBKT7-moc1 to pGBKT7-moc4 carried the *moc* genes fused to the Gal4-DNA binding domain (Gal4-BD). The *moc1, moc2, moc3* and *moc4* genes were amplified by PCR using pMCS24, pMCS264, pMCS33 and pMCS65, respectively (Table 2). The PCR products were digested with *Sma*I and *Sal*I. The digested fragments were cloned into the *Sma*I-*Sal*I sites of pGBKT7 carrying Gal4-BD, to create pGBKT7-moc1 to pGBKT7-moc4. The accuracy of the *moc1, moc2, moc3* and *moc4* gene sequences were verified from the resulting constructs.

To create pGAD424-moc1 to pGAD424-moc4, the *moc1, moc2, moc3* and *moc4* digested fragments were cloned into the *Sma*I and *Sal*I sites of pGAD424 carrying the Gal4 activation domain (Gal4-AD). The resulting constructs were confirmed by restriction digestion and PCR amplification of the respective genes.

The pGBKT7-rpl32-2 construct carried the *rpl32-2* gene fused to Gal4-BD. The

rpl32-2 gene was amplified by PCR from genomic DNA using the relevant primers (Table 2). The amplified product was digested with the restriction enzymes *Eco*RI and *Sal*I, and the digested fragment was inserted into the *Eco*RI and *Sal*I sites of pGBKT7 to create pGBKT7-rpl32-2. To create pREP1-rpl32-2, the *rpl32-2* gene was amplified by PCR from genomic DNA using the relevant primers (Table 2). The PCR product was digested by the restriction enzymes *Sal*I and *Bam*HI, and the digested fragment was inserted into the *Sal*I and *Bam*HI sites of pREP1. The plasmid construct was verified by restriction digestion and sequence analysis. Plasmid manipulation and bacterial transformation were performed using standard techniques [39].

Yeast two-hybrid screening

The yeast two-hybrid assay was performed as described previously [40]. The constructed bait and prey plasmids were introduced into *S. cerevisiae* AH109 singly, or in combination with either the pGBKT7 or pGADGH constructs, using the Li acetate-polyethylene glycol one-step transformation protocol [41]. Expression of the bait proteins (Moc1 to Mo4), fused to Gal4-BD, was verified by Western blotting with a c-Myc antibody (data not shown). The cells transformed with the bait plasmids pGBKT7-moc1 to pGBKT7-moc4 were incubated in the synthetic dropout (SC)-Trp medium for 4 days at 30°C. Cells harboring bait plasmids were re-transformed with pGADGH-cDNA and transformants were selected on SC-Leu-Trp-His + 3-AT plates. The competitive inhibitor 3-AT was used to inhibit low level expression of the yeast protein His3, and thus, to suppress background growth on medium lacking His. Similarly, the cells transformed with the plasmids pGBKT7-rpl32-2 and pGAD424-moc1 to pGAD424-moc4 were incubated on synthetic dropout SC-Trp-Leu medium for 4 days at 30°C, followed by culturing the cells in SC-Trp-Leu-His medium for 4 days at 30°C. 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.

The β-galactosidase activity was determined by filter lift assay as previously described [40]. Briefly, a sterile Whatman # 5 filter was placed over the surface of the plate. The fresh colonies that had been grown on plates at 30°C for 4 days were carefully picked, individually streaked onto filter paper in the presence of positive and negative controls, and cultured for 2 days at 30°C. The filter was then carefully lifted off the agar plate with forceps and transferred (colonies facing up) to a pool of liquid nitrogen. After the filter had frozen completely (about 30 s), it was removed and allowed to thaw at room temperature. The filter was then carefully placed, colony side up, on another filter that had been pre-soaked in a clean petri dish containing Z-buffer X-gal solution. The filters were incubated at 30°C and checked periodically for the appearance of blue color.

Strain construction

The tag-integrated strains were constructed using a PCR-based method [42, 43]. Fragments of approximately 500 bp from the 5' region of the *moc1, moc2, moc3, moc4* and *rpl32-2* genes from the *S. pombe* strain SP870 were amplified using the primer pairs: Moc1-W/Moc1-X; Moc2-W/Moc2-X; Moc3-W/Moc3-X; Moc4-W/Moc4-X; and Rpl32-2-W/Rpl32-2-X. Similarly, the 3' regions of the *moc1, moc2, moc3, moc4* and *rpl32-2* genes were amplified using the primer pairs: Moc1-Z/Moc1-Y; Moc2-Z/Moc2-Y; Moc3-Z/Moc3-Y; Moc4-Z/Moc4-Y; and Rpl32-2-Z/Rpl32-2-Y (Table 2). The amplified fragments were attached to the end of the *kanMX6* module by PCR using pFA6a-13Myc-kanMX6. The wild type strain SP870 was transformed with the tagged DNA fragments from each of the second PCR products and G418-resistant transformants were selected. Proper integration was verified by PCR and by Western blot analysis. The resulting strains were named SKP1

(Moc2-13Myc), SKP5 (Moc1-13Myc), SKP7 (Moc3-13Myc), SKP9 (Moc4-13Myc) and SKP20 (Rpl32-2-13Myc). Both the corresponding amplified fragments of Rpl32-2 were attached to the end of the *kanMX6* module by PCR using pFA6a-3HA-kanMX6. The wild type strain SP870 was transformed with the tagged DNA fragments from the second PCR product, G418-resistant transformants were selected, and proper integration was verified by PCR and by Western blot analysis. The resulting strain was named SKP24 (Rpl32-2-3HA).

The double-tagged integrated strains SKP2, SKP6, SKP8, SKP10 and SKP21 were constructed based on the YO8 (Cpc2-3HA-kanMX6) strain. The first step involved the amplification of approximately 500 bp fragments from the 5' and 3' regions of the *moc, moc2, moc3, moc4,* and *rpl32-2* genes from *S. pombe* as previously described. The PCR products were then attached to the *hphMX6* module by PCR using pFA6a-13Myc-hphMX6. The resulting tagged fragments were introduced into the *S. pombe* strain YO8. Hygromycin B-resistant transformants were selected and protein expression was analyzed by Western blotting.

The tag-integrated strains SKP11, SKP12, SKP13 and SKP14 were constructed using the *S. pombe* strain HT201 (*cpc2::ura4*). DNA fragments of approximately 500 bp corresponding to the 5' and 3' regions of the *moc1, moc2, moc3,* or *moc4* genes were amplified by PCR oligonucleotides. The second PCR amplified fragments were attached to the end of the *kanMX6* module by PCR using pFA6a-13Myc-kanMX6. In the case of the tagged strain SKP30, DNA fragments of approximately 500 bp corresponding to the 5' and 3' regions of the *rpl32-2* gene were amplified by PCR oligonucleotides. The second PCR amplified fragments were attached to the end of *hphMX6* module by PCR using pFA6a-13Myc-hphMX6. The fragments were introduced to the *S. pombe* strain HT201 (*cpc2::ura4*) and transformants were selected by G418 and Hygromycin B, respectively, and also by Western blotting. The resulting strains were named SKP11 (*cpc2::ura4*

moc1-13Myc-kanMX6), SKP12 (*cpc2::ura4 moc2-13Myc-kanMX6*), SKP13 (*cpc2::ura4 moc3-13Myc-kanMX6*), SKP14 (*cpc2::ura4 moc4-13Myc-kanMX6*), and SKP30 (*cpc2::ura4 rpl32-2-13Myc-hphMX6*).

The tag-integrated strain SKP22 was constructed based on the *S. pombe* strain MYM2 (Moc1-3HA). A DNA fragment of approximately 500 bp corresponding to the 5' and 3' regions of the *rpl32-2* gene was amplified by PCR oligonucleotides. The second PCR amplified fragments were attached to the end of the *hphMX6* module by PCR using pFA6a-13Myc-hphMX6. The fragments were introduced into the *S. pombe* strain MYM2 and transformants were selected using hygromycin B and Western blotting. The tag-integrated strains SKP25, SKP26 and SKP27 were constructed using the *S. pombe* strain SKP24 (Rpl32-2-3HA). DNA fragments of approximately 500 bp corresponding to the 5' and 3' regions of the *moc2, moc3,* or *moc4* genes were amplified by PCR oligonucleotides. The second PCR amplified fragments were attached to the end of *kanMX6* module by PCR using pFA6a-13Myc-hphMX6. The fragments were introduced into *S. pombe* SKP24 (Rpl32-2-3HA). Hygromycin B-resistant transformants were selected and protein expression was analyzed by Western blotting. The strain SKP29 (Moc2-13Myc, Moc1-GFP) was constructed based on the strain MYM3 (Moc1-GFP) in a similar manner. The tagged protein did not interfere with the normal function of each protein as judged by phenotypic observation.

Western Blotting

Western blotting was performed by the simple alkali-SDS method [44] and the boiling SDS-glass bead method [45]. The cells were harvested when they reached a density of approximately $1x10^8$ cells in the appropriate medium. The harvested cells were washed twice with dH_2O and dissolved in 100 μl dH_2O, and the samples were boiled at 95°C for 5 min. Subsequently, 120 μl of 2X Laemmli buffer (4% SDS, 20% glycerol, 0.6M ß-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 acid-washed glass beads using a bead homogenizer at 2,500 rpm for 3 min. The samples were boiled at 95°C for 5 min and centrifuged at 14,000 rpm for 15 min at 4°C to remove the glass beads and large debris. An equal volume of cell extract was loaded onto SDS-PAGE using a 10% polyacrylamide gel and then transferred to Immobilon transfer membranes (Millipore, Bedford, MA, USA) using a wet-type transfer system. To block unspecific binding, the membranes were incubated in a blocking buffer (PBS containing 5% non-fat dry milk) supplemented with 0.1% Tween 20 at RT for 1 hour. To detect cMyc fusion proteins, the membrane was incubated with a Myc monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Ca) diluted 1:3000 in PBS-T (137 mM NaCl, 8 mM $Na_2HPO_4.12H_2O$, 2.7mM KCl, 1.5 mM $KHPO_4$ and 0.1% Tween 20). The membrane was washed with PBS-T for 15 min and 5 min twice per wash, and then incubated with horseradish peroxidase-conjugated anti mouse secondary IgG (Santa Cruz Biotechnology, Santa Cruz, Ca) diluted 1:3000 in 5% dry milk in PBS-T.

To detect HA fusion proteins, the membrane was incubated with an HA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Ca) diluted 1:3000 in PBS-T (137mM NaCl, 8 mM $Na_2HPO_4.12H_2O$, 2.7 mM KCl, 1.5 mM $KHPO_4$ and 0.1% Tween 20). The membrane was washed three times with PBS-T every 5 min, and then incubated with an anti-mouse secondary IgG (Santa Cruz Biotechnology, Santa Cruz, Ca), diluted 1: 3000 in 5% dry milk in PBS-T. The membrane was washed three times with PBS-T every 5 min, and then secondary antibodies were detected by the chemiluminescence (ECL) system as described by the manufacturer (Amersham, Buckinghamshire, UK).

Co-immunoprecipitation

S. pombe cells were grown in YES medium to the mid-logarithmic phase, then harvested

($2x10^8$ cells/ml) by centrifugation, and washed once with ice-cold stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, and 1 mM NaN_3 [pH 8]). The cells were then lysed in 100 µl ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 0.8% Nonidet-P40, 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor). The samples were vortexed vigorously with 0.5 mm diameter zirconia/silica beads using a bead homogenizer at 2,500 rpm for 3 min. After centrifugation (14,000 rpm for 15 min at 4°C), the protein concentration in the supernatant was estimated.

An HA monoclonal antibody and a Myc antibody were used in the immunoprecipitation of HA and Myc fusion proteins, respectively, in which 1 mg of each cell extract was incubated with 1 µg of HA antibody and 1 µg of Myc antibody for 4 hrs at 4°C. Then 40 µl of protein A sepharose beads and the same volume of protein G sepharose beads were washed 2 times with 0.5 ml lysis buffer. The cleaned protein A sepharose beads were added to the HA antibody mixture and the protein G sepharose beads were added to the Myc antibody mixture, followed by incubation with rotation for 4 hrs at 4°C.

Sepharose beads were collected by centrifugation at 14,000 rpm for 10 min at 4°C. The supernatant was discarded by aspiration and the beads were washed six to eight times using 0.5 ml lysis buffer, including protease inhibitor and PMSF. The bead pellet was suspended in 30 µl lysis buffer (including protease inhibitor and PMSF), and 60 µl of 2X Laemmli buffer (4% SDS, 20% glycerol, 0.6 M ß-mercaptoethanol, 8 M urea, and 0.12 M Tris-HCl [pH 6.8]) was added and vortexed. The suspended beads were boiled at 95°C for 5 min to dissociate the immunocomplexes from the beads. After centrifugation (14,000 rpm for 10 min at 4°C), the supernatant was collected in a new eppendorf tube and then used for SDS-PAGE and Western blotting.

Blue Native PAGE

Blue Native polyacrylamide gel electrophoresis (BN-PAGE) is a method for the isolation of intact protein complexes. The method followed the manufacturer's instructions (Invitrogen, Corp., Tokyo, Japan). Protein complexes were separated by their apparent molecular mass using this standard polyacrylamide gel electrophoresis system. In the first dimension, separation of the complexes under native conditions occurs according to their molecular mass, and in the second dimension, where electrophoresis is performed under denaturing conditions, the individual subunits of the complexes are resolved, again on the basis of their molecular mass [25].

Cells were grown in YES medium to the mid-logarithmic phase, then harvested (1 to $2x10^8$ cells/ml) by centrifugation. Cells were washed once with dH_2O and stored at -80°C. The cell pellets were dissolved with 4X Native PAGE sample buffer (25 µl) + dH_2O (72 µl) + 1 mM phenylmethylsulfonyl fluoride [PMSF] (1 µl) + protease inhibitor (2 µl). Samples were vortexed vigorously with an equal volume of acid-washed glass beads using a bead homogenizer at 2,500 rpm for 3 min. After centrifugation (14,000 rpm for 15 min at 4°C), the protein concentration in the supernatant was estimated. Approximately 50 µg protein was loaded per lane for electrophoresis.

Gel strips were cut from the BN PAGE gel, each strip was transferred individually to a 15 ml conical tube, and 5 ml of reducing solution (0.5 ml sample reducing agent (10x), 1 ml LDS sample buffer (4x) and 3.5 ml H_2O) was added to each tube. Samples were incubated for 15 min with shaking at RT and the reducing solution was then decanted. Then 5 ml of alkylating solution (1 ml LDS sample buffer (4X), 3.72 ml H_2O and 28 µl DMA) was added to each tube, incubated for 30 min at RT with shaking, and then decanted. This was followed by 5 ml of quenching solution (0.50 ml sample reducing agent (10X), 3 ml LDS sample buffer (4X), 1 ml EtOH, and 3.5 ml H_2O) being added to each tube and incubated at RT. The quenching solution was decanted and the gel strips were used for 2-D SDS-PAGE.

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Figure legends

Fig. 1. Interaction between Moc1, Moc2, or Rpl32-2 and Cpc2 *in vivo*.

A) Cell extract was prepared from fission yeast cells carrying Moc1-13Myc, Cpc2-3HA, Cpc2-3HA and Moc1-13Myc, or the un-tagged strain (wild type). **B)** Cell extract was prepared from fission yeast cells carrying Moc2-13Myc, Cpc2-3HA, Cpc2-3HA and Moc2-13Myc, or the un-tagged strain (wild type). **C)** Cell extract was prepared from fission yeast cells carrying Rpl32-2-13Myc, Cpc2-3HA, Cpc2-3HA and Rpl32-2-13Myc, or the un-tagged strain (wild type). The individual cell extract was incubated with an HA antibody and a Myc antibody. Protein A sepharose beads were added to the mixtures to co-immunoprecipitate Cpc2, and protein G sepharose beads were added to co-immunoprecipitate Moc1, Moc2 or Rpl32-2. The co-immunoprecipitates were analyzed by Western blotting using HA and Myc antibodies.

Fig 2. Interaction between Rpl32-2 or Moc1 and Moc2 *in vivo*.

A) Cell extract was prepared from fission yeast cells carrying Moc2-13Myc, Rpl32-2-3HA, Rpl32-2-3HA and Moc2-13Myc tag, or the un-tagged strain (wild type). Individual cell extract was incubated with an HA antibody and a Myc antibody. Protein A sepharose beads were added to the mixtures to co-immunoprecipitate Rpl32-2 and protein G sepharose beads were added to co-immunoprecipitate Moc2. The co-immunoprecipitates were analyzed by Western blotting using HA and Myc antibodies. **B)** Cell extract was prepared from fission yeast cell carrying Moc1-GFP, Moc2-13Myc, Moc1-GFP and Moc2-13Myc tag or the no-tagged strain (wild type). Individual cell extract was incubated with a GFP antibody and a Myc antibody. Protein protein G sepharose beads were added to the mixtures to coimmunoprecipitate Moc1 and Moc2. The coimmunoprecipitates were analyzed by Western blotting using GFP and Myc antibodies.

Fig. 3. Western blot analysis of Moc2 by Blue Native PAGE and second dimensional SDS-PAGE. **A)** Cells were extracted from *S. pombe* SKP1 (Moc2-Myc) and proteins were separated on a 4% to 16% BN-PAGE gel. Western blotting was performed using a Myc antibody (1/3000) followed by anti-mouse IgG (1/3000). The arrow indicates the complex containing the Moc2 protein. **B)** One lane was excised from the first dimensional gel and the gel strip was incubated with dissociation buffers and placed horizontally on top of the second dimension gel. A 10% SDS-PAGE was then performed in the second dimension. When the gel strip was treated with dissociation buffer, the protein complexes dissociated into their constituent polypeptides and the subunits of the protein complexes separated during second dimensional electrophoresis. Western blotting was performed following the two dimensional SDS-PAGE using a Myc antibody (1/3000), and subsequent anti-mouse IgG (1/3000). **C, D)** Two dimensional electrophoresis was performed using the *S. pombe* double-tagged strain SKP2 (Moc2-Myc, Cpc2-HA). Western blotting was performed using a Myc antibody (1/3000) and subsequent anti-mouse IgG (1/3000) [C], or an HA antibody (1/3000) and subsequent anti-mouse IgG (1/3000) [D], respectively. **E)** Western blotting with a Myc antibody (1/3000) and subsequent anti-mouse IgG (1/3000) to detect Moc2 tagged with Myc on SDS-PAGE alone.

Fig. 4. **Western blotting of Moc1 following BN-PAGE and second dimension SDS-PAGE.** Proteins were extracted from cells of *S. pombe* strains SKP5, SKP6 and SKP11, and were separated on a 4% to 16% BN-PAGE gel. Individual lanes were excised from the first dimension gel and treated with dissociation buffers, then slid into place horizontally on top of the second dimension gel for SDS-PAGE. Then, Western blotting was performed as

described in Fig. 3. **A**) *S. pombe* Moc1-13Myc tagged strain SKP5 was used for 2-D analysis. **B, C)** *S. pombe* double-tagged strain SKP6 was used for 2-D analysis. **D**) 2-D electrophoresis was performed using the *cpc2* deleted and Moc1-13Myc tagged strain SKP11. **E)** Western blotting was performed using a Myc antibody (1/3000) and subsequent anti-mouse IgG (1/3000) to detect Moc1 protein tagged with Myc in SKP5 cells on SDS-PAGE alone.

Fig. 5. **Western blot analysis of Moc3 following BN-PAGE and 2-D SDS-PAGE.**

S. pombe (SKP7, SKP8 and SKP13) cells were extracted and proteins were separated by 4% to 16% BN-PAGE and SDS-PAGE. Western blotting was performed as in Fig. 3. **A**) The *S. pombe* Moc3-13Myc tagged strain SKP7 was used for 2-D analysis. **B, C)** The *S. pombe* double-tagged strain SKP8 was used for analysis. **D**) 2-D electrophoresis was performed using the Cpc2-deleted *S. pombe* Moc3-13Myc tagged strain SKP13. **E)** Western blotting was performed using a Myc antibody (1/3000) and anti-mouse IgG (1/3000) to detect Moc3 protein tagged with Myc in SKP7 cells on SDS-PAGE alone.

Fig. 6. **Western blot analysis of Moc4 following BN-PAGE and 2-D SDS-PAGE.**

S. pombe cells with different tags (Moc4-13Myc, Cpc2-13Myc, Asf1-13Myc) and WT cells (SP870) were used for this analysis. Proteins were first separated by 4% to 16% BN PAGE. **A**, **B)** The *S. pombe* Moc4-13Myc tagged strain SKP10 was used for 2-D analysis. **C)** The *S. pombe* tagged strain Asf1 was used for this analysis. **D)** The WT strain was treated similarly as a negative control. **E)** Western blotting was performed using a Myc antibody (1/3000) and anti-mouse IgG (1/3000) to detect Moc4 protein tagged with Myc in SKP10 cells on SDS-PAGE alone.

Fig. 7. Western blotting of Rpl32-2 following BN-PAGE and 2-D SDS-PAGE.

Proteins were extracted from *S. pombe* (SKP20, SKP30 and SKP21) cells and were separated by 4% to 16% BN-PAGE and subsequent SDS-PAGE. Western blotting was performed as in Fig. 3. **A)** The *S. pombe* Rpl32-2-13Myc tagged strain SKP20 was used for this analysis. **B**) 2-D electrophoresis was performed using the *cpc2*-deleted *S. pombe* Rpl32-2-13Myc tagged strain SKP30. **C, D)** 2-D electrophoresis was performed using the *S. pombe* double-tagged strain SKP21. **E)** Western blotting was performed using a Myc antibody (1/3000) and anti-mouse IgG (1/3000) to detect Rpl32-2 protein tagged with Myc in SKP20 cells on SDS-PAGE alone.

Fig. 8. Ste11 was induced by over-expression of Moc1, Moc2, Moc4 or Rpl32-2.

The Ste11-GFP tagged strain SPB371, harboring pSLF173 (L), pSLF173(L)-moc1, pSLF173(L)-moc2, pSLF173(L)-moc3, pSLF173(L)-moc4, and pREP1 or pREP1-rpl32-2 was cultured in PMA liquid medium until mid-log phase ($5x10^6$/ml). The cells were harvested, washed two times with PMA-N+0.5%G medium, and shifted to the same medium to induce mating and sporulation. Approximately $1x10^8$ cells were harvested at 0, 3, 6, 9 and 12 hours after nitrogen starvation. Western blotting was performed using the boiling SDS-glass bead method [44]. For this analysis, a GFP antibody (1/1000, monoclonal) and anti-mouse IgG (1/1000) were used to detect Ste11-GFP. Cdc2 was detected as an internal control using a PSTAIRE antibody (1/1000) and anti-rabbit IgG (1/3000).

Fig. 9. A summary of the interactions between Moc proteins, Cpc2 and Rpl32-2.

The interactions detected by co-immunoprecipitation experiments are shown by solid arrows. The interactions detected by the yeast two-hybrid system are show by dashed arrows**.** Moc3 and Moc4/Zfs1 localize to the nucleus, while the other proteins are found in the cytosol.

Moc1/Sds23 ordinarily localizes to the cytosol, but moves to the nucleus during meiosis, and this shift is inhibited by cAMP [14]. Msa2 binds with Cpc2 [28]. The involvement of the ribosome-associated protein Cpc2, and the general translation factor Moc2/Ded1, implies that a Moc-mediated complex may act as a translational regulator and may be involved in controlling sexual differentiation in fission yeast through Ste11.

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

Strain	Genotype	Source
SP870	*h^{90}ade6.210 leu1.32 ura4-D18*	[46]
MYM2	*h^{90}ade6.210 leu1.32 ura4-D18 moc1-3HA<kanMX6*	[14]
MYM3	*h^{90}ade6.210 leu1.32 ura4-D18 moc1-GFP<kanMX6*	[14]
HT201	*h^{90}ade6.210 leu1.32 ura4-D18 cpc2::ura4*	[28]
SPB371	*h^{90} ade6.216 leu1.32 ura4-D18 ste11::ste11-GFP<ura4*	[47]
YO7	*h^{90}ade6.210 leu1.32 ura4-D18 cpc2-13Myc<-kanMX6*	Lab stock
YO8	*h^{90}ade6.216 leu1.32 ura4-D18 cpc2-3HA<kanMX6*	Lab stock
YM1	*h^{-} leu1.32 ura4-D18 asf1-13Myc<kanMX6*	Lab stock
SKP1	*h^{90}ade6.210 leu1.32 ura4-D18 moc2-13Myc<kanMX6*	This study
SKP2	*h^{90}ade6.216 leu1.32 ura4-D18 cpc2-3HA<kanMX6 moc2-13Myc<hphMX6*	This study
SKP5	*h^{90}ade6.210 leu1.32 ura4-D18 moc1-13Myc<kanMX6*	This study
SKP6	*h^{90}ade6.216 leu1.32 ura4-D18 cpc2-3HA<kanMX6 moc1-13Myc<hphMX6*	This study
SKP7	*h^{90}ade6.210 leu1.32 ura4-D18 moc3-13Myc<kanMX6*	This study
SKP8	*h^{90}ade6.216 leu1.32 ura4-D18 cpc2-3H<kanMX6 moc3-13Myc<hphMX6*	This study
SKP9	*h^{90}ade6.210 leu1.32 ura4-D18 moc4-13Myc<kanMX6*	This study
SKP10	*h^{90}ade6.216 leu1.32 ura4-D18 cpc2-3HA<kanMX6 moc4-13Myc<hphMX6*	This study
SKP11	*h^{90}ade6.210 leu1.32 ura4-D18 cpc2::ura4 moc1-13Myc<kanMX6*	This study
SKP12	*h^{90}ade6.210 leu1.32 ura4-D18 cpc2::ura4 moc2-13Myc<kanMX6*	This study
SKP13	*h^{90}ade6.210 leu1.32 ura4-D18 cpc2::ura4 moc3-13Myc<kanMX6*	This study
SKP14	*h^{90}ade6.210 leu1.32 ura4-D18 cpc2::ura4 moc4-13Myc<kanMX6*	This study
SKP20	*h^{90}ade6.210 leu1.32 ura4-D18 rpl32-2-13Myc<kanMX6*	This study
SKP21	*h^{90}ade6.216 leu1.32 ura4-D18 cpc2-3HA<kanMX6 rpl32-2-13Myc<hphMX6*	This study
SKP22	*h^{90}ade6.210 leu1.32 ura4-D18 moc1-3HA<kanMX6 rpl32-2-13Myc<hphMX6*	This study
SKP24	*h^{90}ade6.210 leu1.32 ura4-D18 rpl32-2-3HA<kanMX6*	This study
SKP25	*h^{90}ade6.210 leu1.32 ura4-D18 rpl32-2-3HA<kanMX6 moc2-13Myc<hphMX6*	This study
SKP26	*h^{90}ade6.210 leu1.32 ura4-D18 rpl32-2-3HA<kanMX6 moc3-13Myc<hphMX6*	This study
SKP27	*h^{90}ade6.210 leu1.32 ura4-D18 rpl32-2-3HA<kanMX6 moc4-13Myc<hphMX6*	This study
SKP29	*h^{90}ade6.210 leu1.32 ura4-D18 moc1-GFP<kanMX6 moc2-13Myc<hphMX6*	This study
SKP30	*h^{90}ade6.210 leu1.32 ura4-D18 cpc2::ura4 rpl32-2-13Myc<hphMX6*	This study

Table 2. List of oligonucleotide primers used in this study.

pGBKT7/pGAD424 (Moc1-Moc4)	
moc1-F-*Sma*1	5'-ACTCCCGGGAATGCCTTTGTCAACTCAATC-3'
moc2-F-*Sma*1	5'-CAAACCCGGGTATGAGCGACAATGTACAGC-3'
moc3-F-*Sma*1	5'-CCTCCCGGGTATGAACCCGTATGTTTCTTATC-3'
moc4-F-*Sma*1	5'-TCTCCCGGGCATGGTTTATTCTCCTATGTC-3'
moc1-R-*Sal*1	5'-TATGTCGACTCACCGACGTTGTGTATCTAC-3'
moc2-R-*Sal*1	5'-TTTAGTCGACTTACCACCAGGATTGAGCAC-3'
moc3-R-*Sal*1	5'-CCAGTCGACTGACTGTCGTACCGTAATTCG-3'
moc4-R-*Sal*1	5'-GATGTCGACTCAAGGAGATTGCTTAATAG-3'
pGBKT7 /pREP1 (Rpl32-2)	
rpl32-2-F-EcoR1	5'-CACAGAATTCATGGCTGCTGCTGTCAATATC-3'
rpl32-2-R-*Sal*1	5'-GATGTCGACTTACTCCTGAGAGCG-3'
rpl32-2-F-*Sal*1	5'-CACGTCGACAATGGCTGCTGTCAATATC-3'
rpl32-2-R-*Bam*H1	5'-CGTGATGGATCCTTACTCCTGAGAGC-3'
Moc1 tagging primers	
Moc1-W	5'- CTTGCTGTTGTCGATGCTCA -3'
Moc1-X	5'-GGGGATCCGTCGACCTGCAGCGTACGACCGACGTTGTGTATCTACAC-3'
Moc1-Y	5'-GTTTAAACGAGCTCGAATTCATCGATTGCTAAATATTTGATGATT-3'
Moc1-Z	5'-CGATTACGCCTCTGTGATTC-3'
Moc2 tagging primers	
Moc2-W	5'-CGTGGTTTAGATATTCCC -3'
Moc2-X	5'-GGGGATCCGTCGACCTGCAGCGTACGA CCACCAGGATTGAGCAC- 3'
Moc2-Y	5'-GTTTAAACGAGCTCGAATTCATCGATGGGTTACGTGCATCTGTG-3'
Moc2-Z	5'-CATGAGCTCAAAGCCTG-3'
Moc3 tagging primers	
Moc3-W	5'-CTCGAAGTCATGCTCC-3'
Moc3-X	5'-GGGGATCCGTCGACCTGCAGCGTACGAAAGTACTGGTCGATTTAAGAC-3'
Moc3-Y	5'-GTTTAAACGAGCTCGAATTCATCGATGCTAGACAAAATCACGC-3'
Moc3-Z	5'-GCCGTGGTCGGTTCCG-3'
Moc4 tagging primers	
Moc4-W	5'-CCTAAGCTGTGCGTTCAATC-3'
Moc4-X	5'-GGGGATCCGTCGACCTGCAGCGTACGAAGGAGATTGCTTAATAGTTGCAC-3'
Moc4-Y	5'-GTTTAAACGAGCTCGAATTCATCGATGTTGTTATGCAATCTGGGTGAG-3'
Moc4-Z	5'-GATTCATGCGTATCGCATTGC-3'
Rpl32-2 tagging primers	
Rpl32-2-W	5'- CAGTCTGACCGCTTCAAG -3'
Rpl32-2-X	5'-GGGGATCCGTCGACCTGCAGCGTACGACTCCTGAGCGAACCTTAG - 3'
Rpl32-2-Y	5'-GTTTAAACGAGCTCGAATTCATCGATGGTTAAACGTGACGCAGTCG -3'
Rpl32-2-Z	5'-CGTCCTCCAGCTCAGATC -3'

Table 3. Interaction of Moc1 interacting proteins with other Moc proteins.

Moc1 interacting proteins	Systematic name	GBD	Moc1	Moc2	Moc3	Moc4
Pyruvate decarboxylase	SPAC1F8.07c	-	+	-	+	+
Elongation factor 1 α-A	SPCC794.09c	-	++	-	++	+
Glyceraldehyde-3phosphate dehydrogenase	SPBC32F12.11	-	+	-	++	+
Thioredoxin peroxidase	SPCC576.03c	-	++	-	++	+
Mannosyl transferase complex subunit Alg9	SPAC1834.05	-	+	-	+	-
Srp-54 type protein	SPCC188.06c	-	++	-	+	+
Ribosomal protein L29	SPBC776.01	-	+	-	+	-
Ribosomal protein L32-2	SPAC3H5.10	-	++	++	++	++
Ribosomal protein L38	SPBC577.02	-	+	-	+	-
Ribosomal protein S3a	SPAC22H12.04c	-	+	-	+	-
Ribosomal protein S14	SPAC3H5.05c	-	+	-	+	-
Ribosomal protein S16	SPAC664.04c	-	++	-	++	-
Ribosomal protein S20	SPCC576.09	-	+	-	+	-
RNA polymerase Rpb3	SPCC1442.10c	-	+	-	++	-
Obr1	SPAC3C7.14c	-	+	-	++	-
Sfh1	SPCC16A11.14	-	+	-	++	-
Ufd2	SPAC20H4.10	-	++	-	++	++

Positive signal is indicated by '+' and negative signal by '-'. Strength of blue color on the X-gal filter is shown by the number of plus marks.

Table 4. Interaction of Moc2 interacting proteins with other Moc proteins.

Moc2 interacting proteins	Systematic name	GBD	Moc1	Moc2	Moc3	Moc4
Ribosomal protein L8	SPBC29A3.04	-	+	+	+	-
Ribosomal protein L18	SPBC11C11.07	-	+	+	+	-
Ribosomal protein L20	SPAC3A12.10	-	-	+	+	-
Ribosomal protein L27	SPCC74.05	-	-	+	+	-
Ribosomal protein L29	SPBC776.01	-	+	+	+	-
Ribosomal protein S13	SPAC6F6.07c	-	++	++	++	-
Lys3 (Saccharopine dehydrogenase)	SPAC227.18	-	+	++	++	-

Marks represent as in Table 3.

Table 5. Interaction of Moc3 interacting proteins with other Moc proteins.

Moc3 interacting proteins	Systematic name	GBD	Moc1	Moc2	Moc3	Moc4
Pyruvate decarboxylase	SPAC1F8.07c	-	+	-	+	+
Enolase	SPBC1815.01	-	+	-	+	+
20S proteasome component alpha 5	SPAC323.02c	-	+	-	+	+
Elongation factor 1 α-A	SPCC794.09c	-	+	-	+	+
Glyceraldehyde-3phosphate dehydrogenase	SPBC32F12.11	-	++	-	+	+
Ribosomal protein L32-2	SPAC3H5.10	-	++	++	++	++
Superoxide dismutase	SPAC821.10c	-	+	-	+	-
Glutamyl tRNA synthetase	SPAPB1A10.11c	-	++	-	++	++
Cpc2	SPAC6B12.15	-	++	++	++	-

Marks represent as in Table 3.

Table 6. Interaction of Moc4 interacting proteins with other Moc proteins.

Moc4 interacting proteins	Systematic name	GBD	Moc1	Moc2	Moc3	Moc4
Glyceraldehyde-3phosphate dehydrogenase	SPBC32F12.11	-	+	-	+	+
Pyruvate decarboxylase	SPAC1F8.07c	-	+	-	+	+
Enolase	SPBC1815.01	-	+	-	+	+
Ribosomal protein L5	SPAC3H5.12c	-	-	-	-	+
Ribosomal protein L12	SPCC16C4.13c	-	++	-	++	+
Ribosomal protein L32-2	SPAC3H5.10	-	++	++	++	++
Ribosomal protein P2B	SPBC23G7.15c	-	++	-	-	+
Elongation factor 2	SPAC513.01c	-	-	-	-	+
Ebp2	SPAC17H9.05	-	-	-	+	+
Psu1	SPAC1002.13c	-	+	-	++	++
Fba1 (fructose-bisphosphate aldolase)	SPBC19C2.07	-	+	-	++	++
Crb3	SPAC13G7.08c	-	+	-	++	++
mRNA cleavage and polyadenylation specificity factor complex associated protein	SPCC74.02c	-	+	-	+	-

Marks represent as in Table 3.

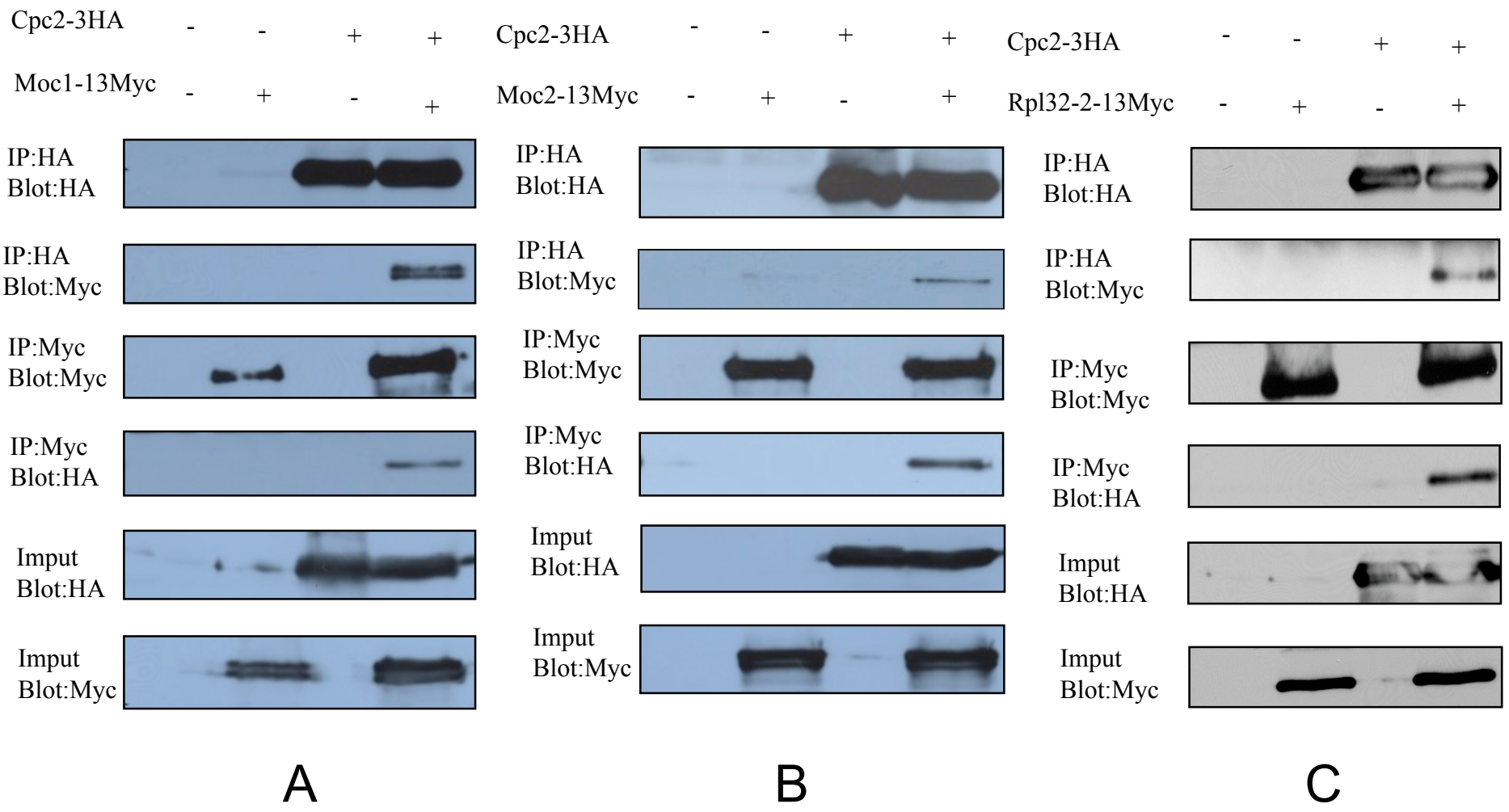
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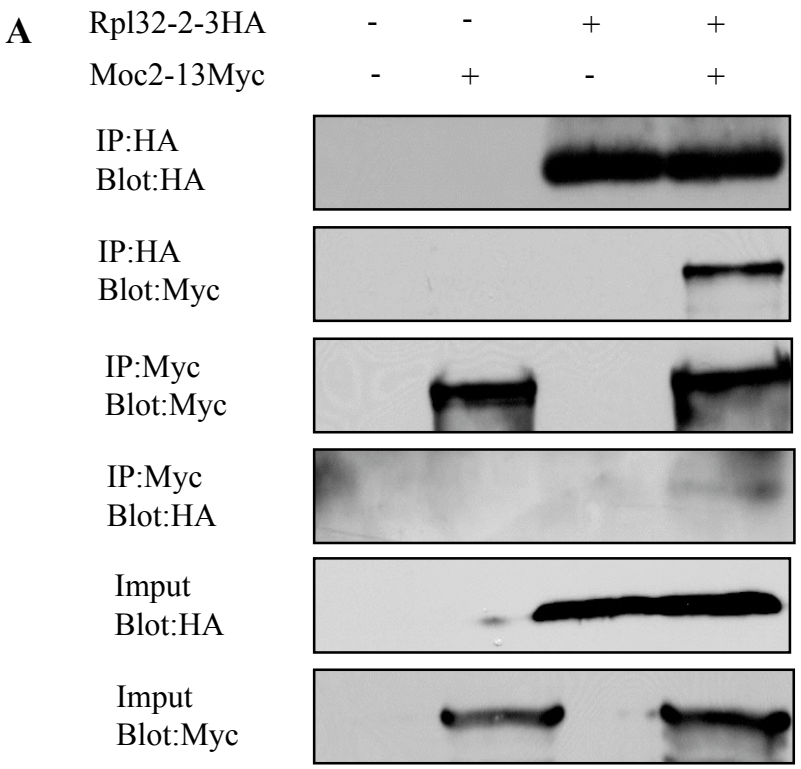
Fig. 1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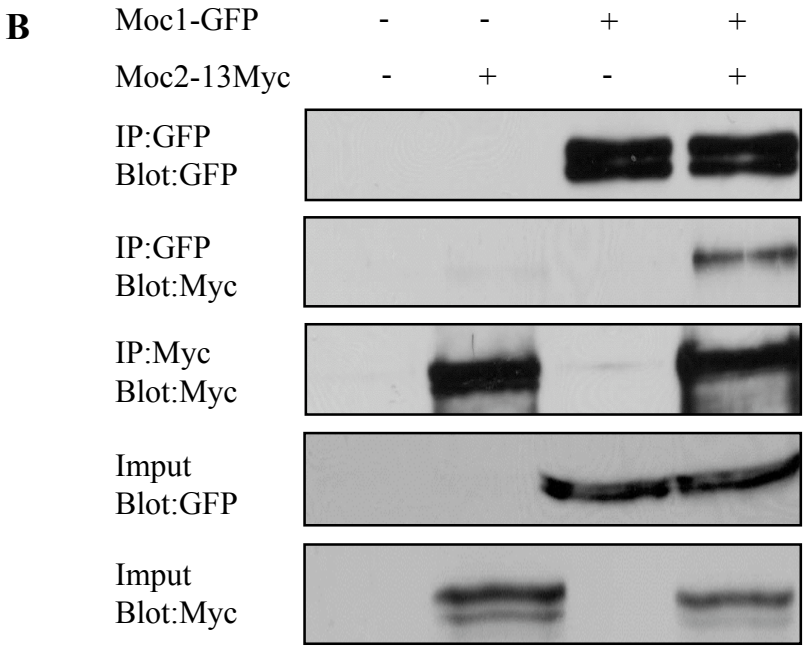
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Fig. 2.

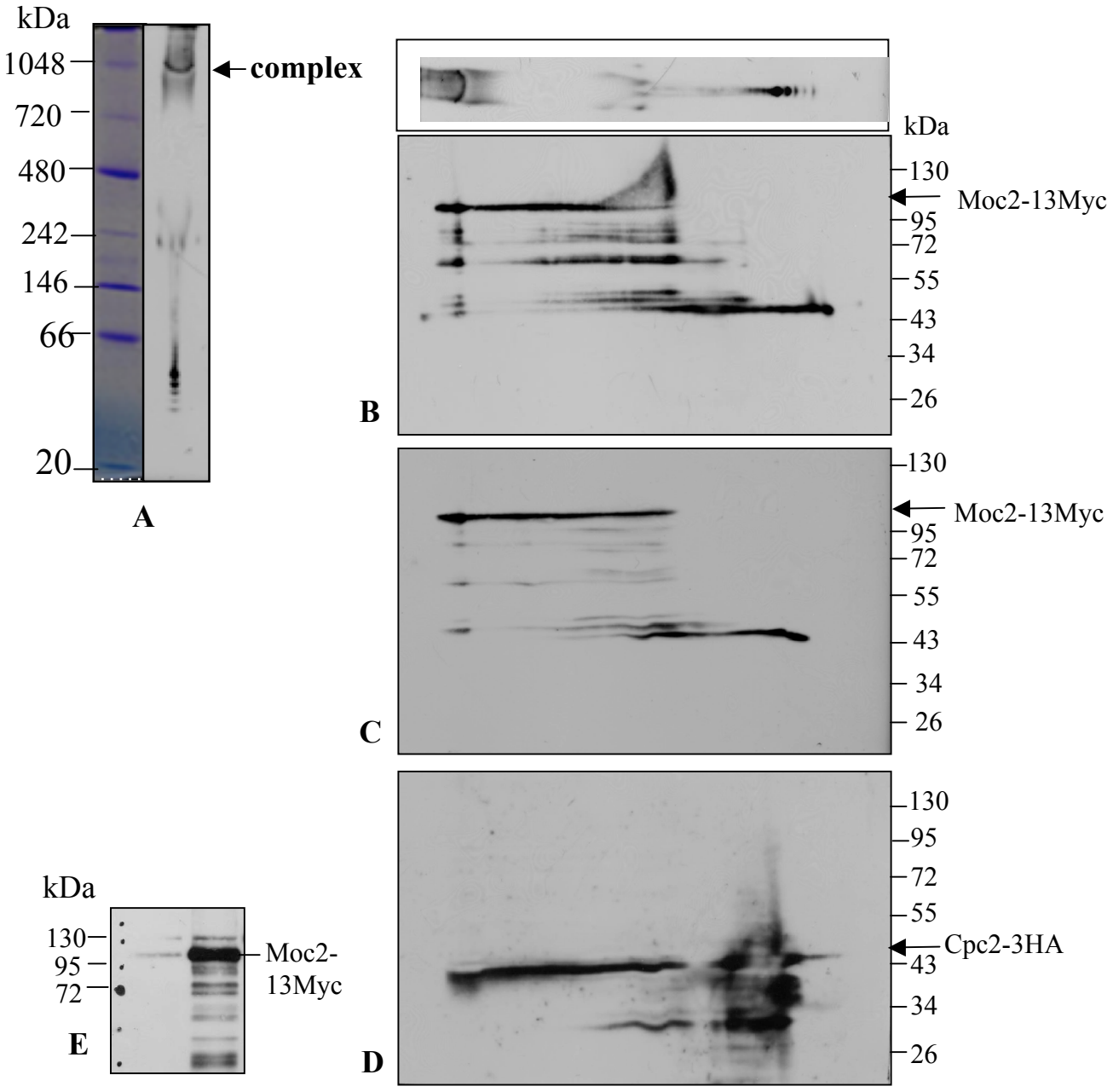
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Fig. 3.

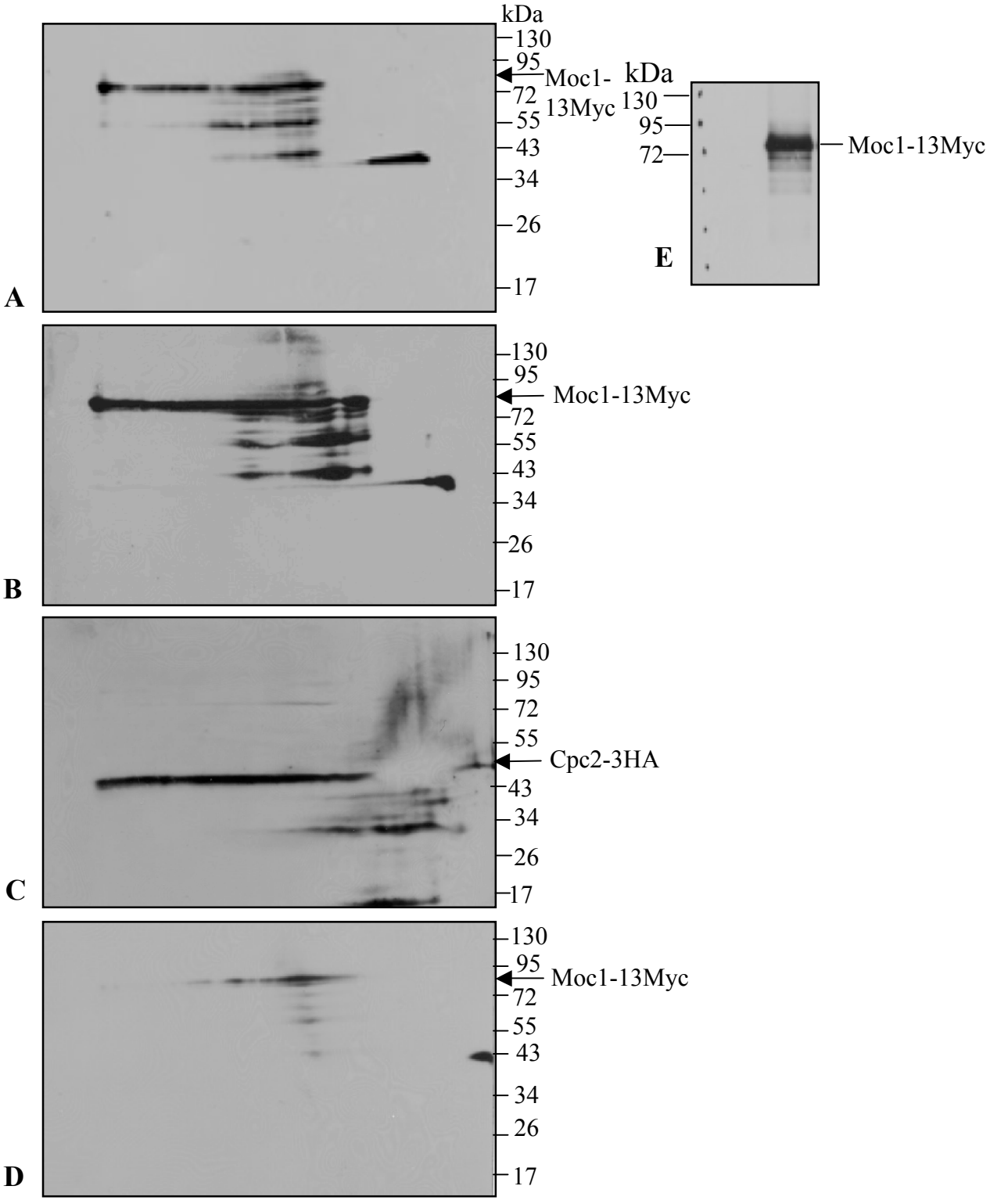
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Fig. 4.

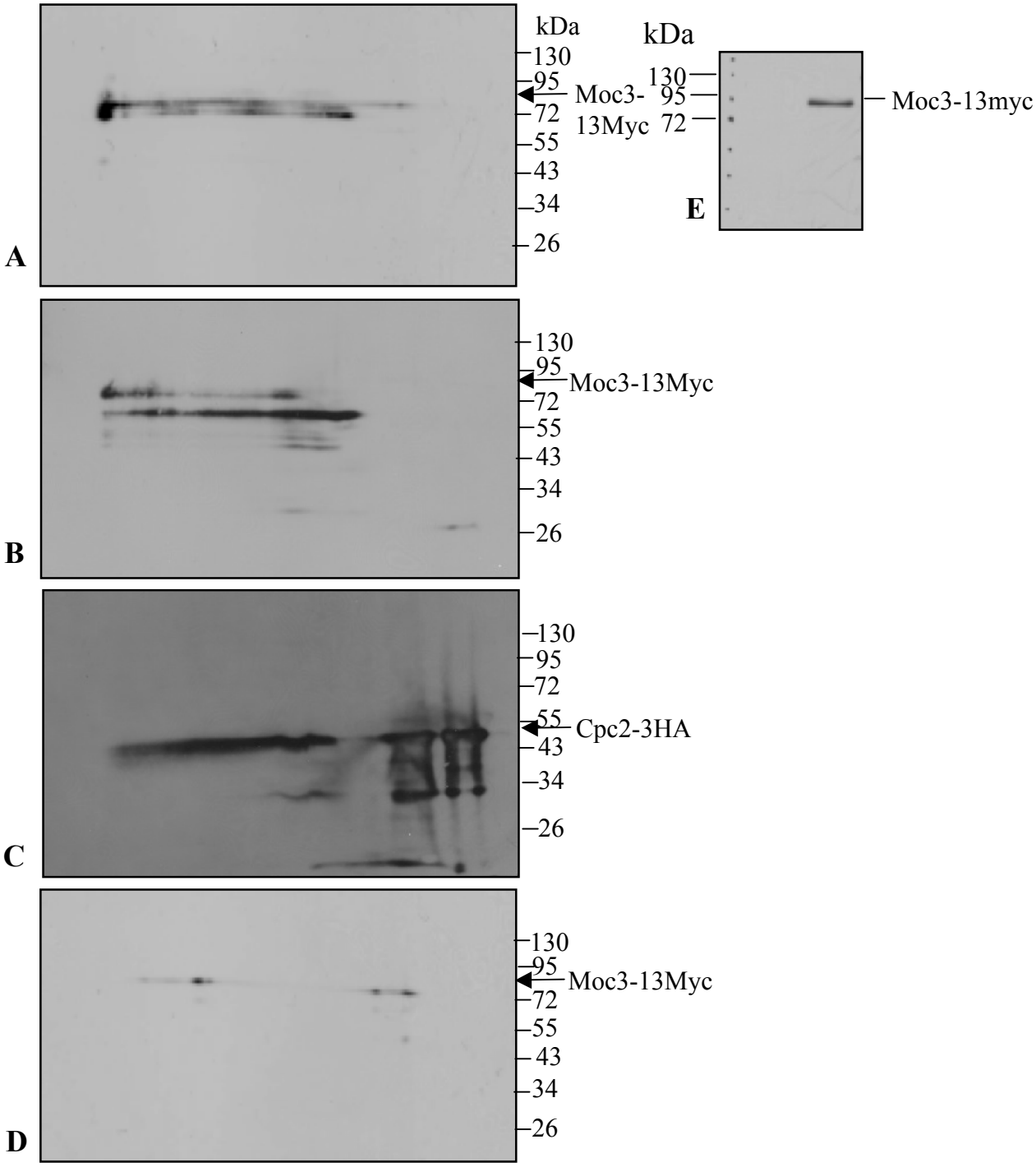
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Fig. 5.

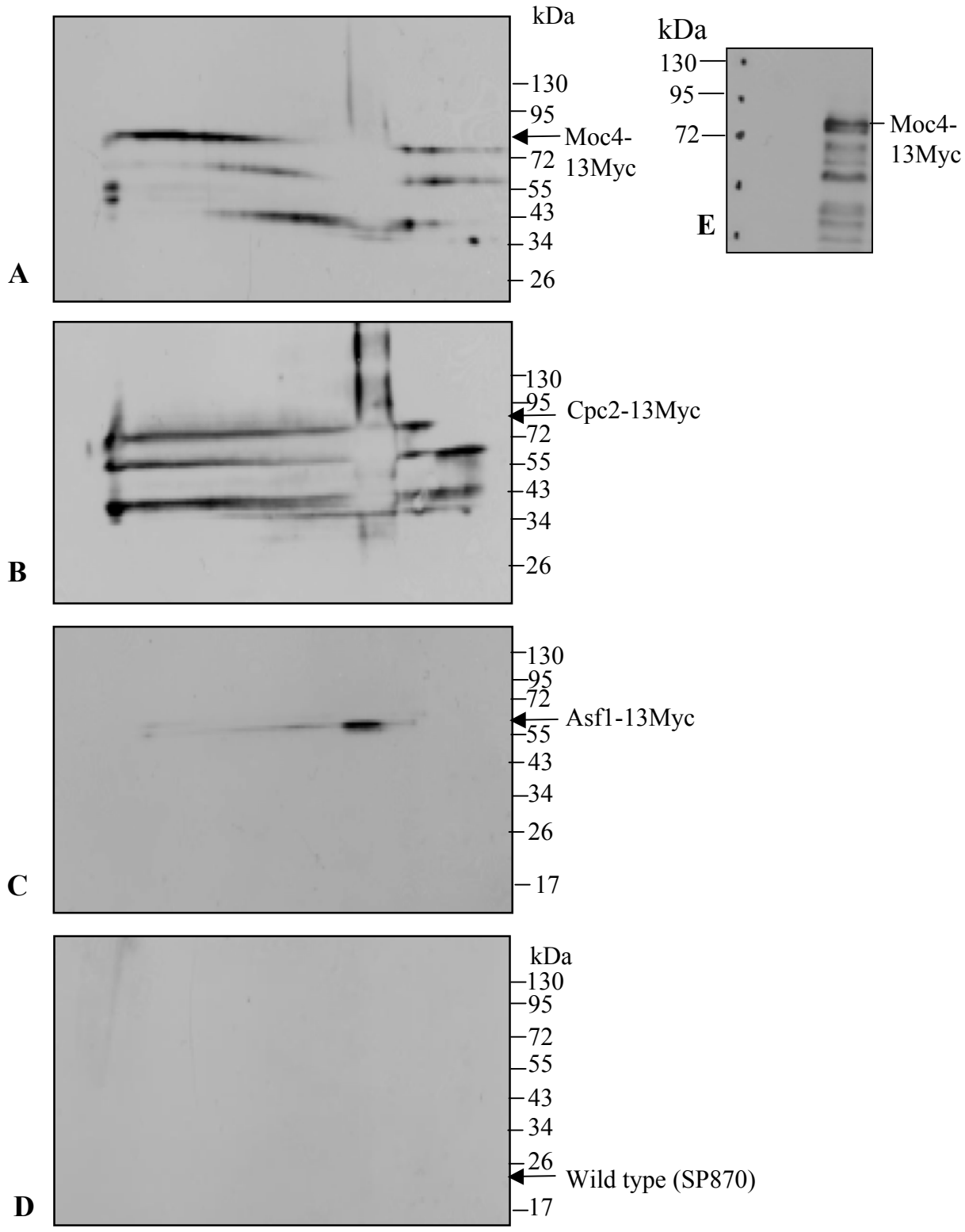
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Fig. 6.

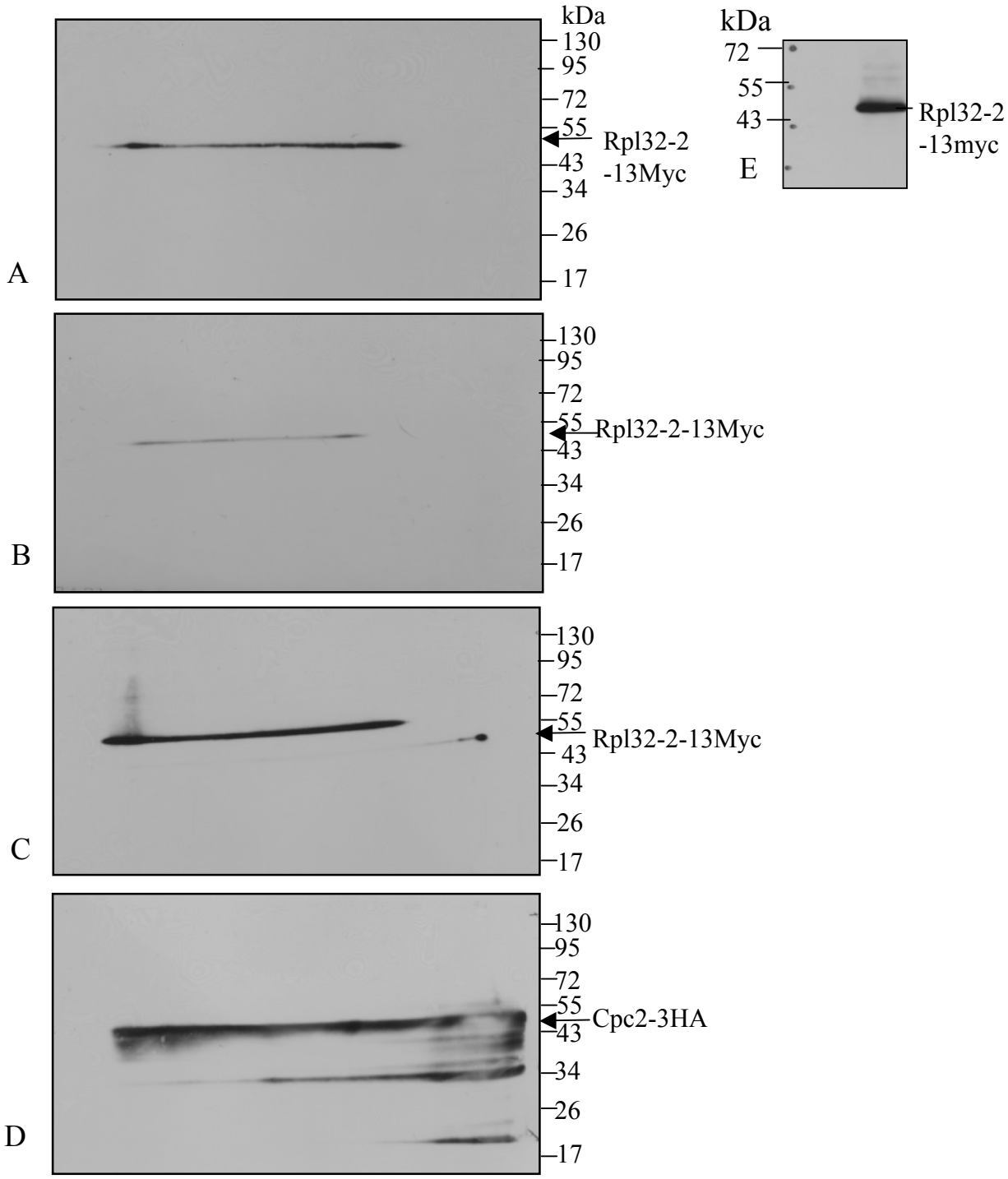
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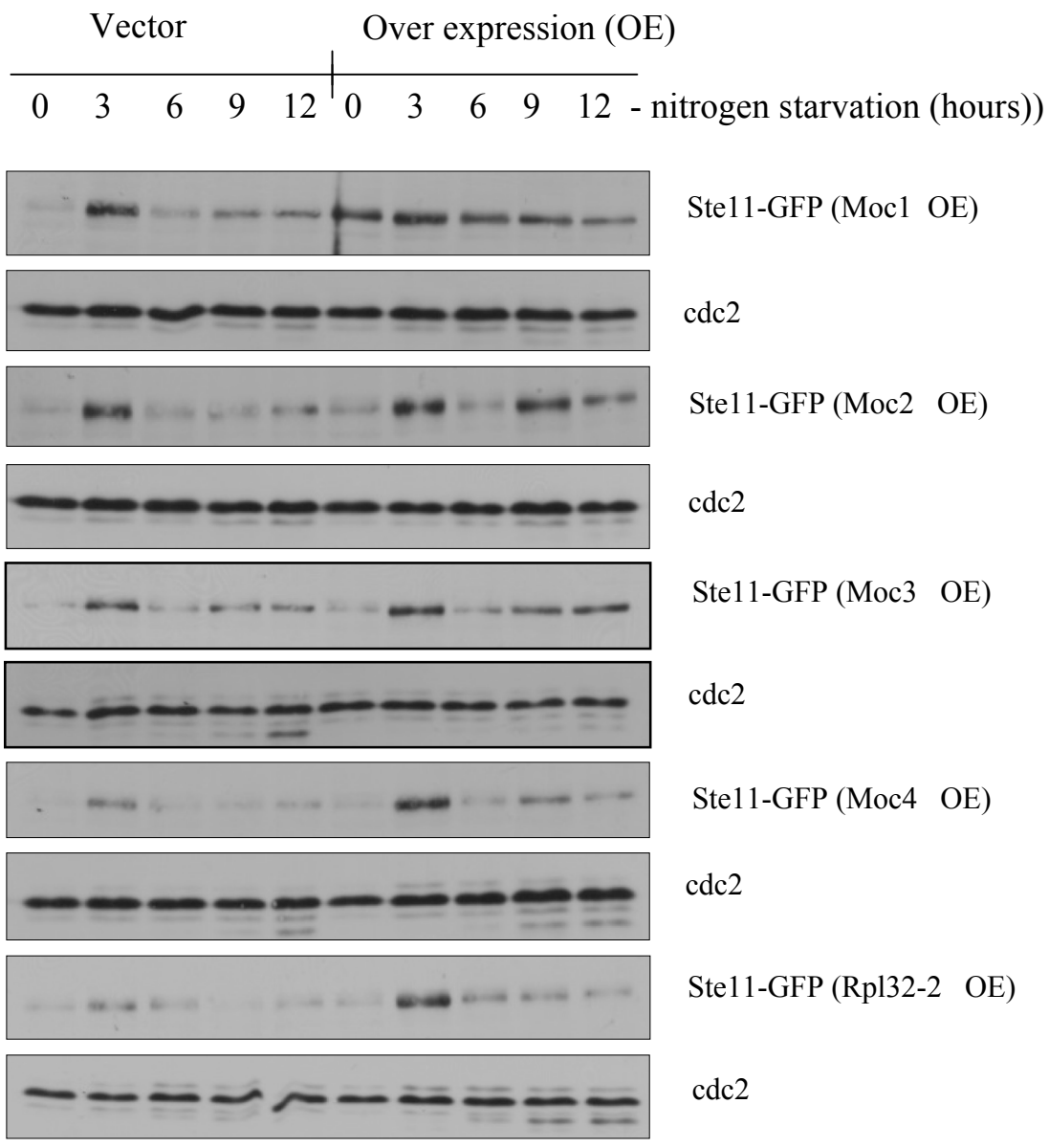
Fig. 7.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Ste11-GFP (Moc2 OE)
cdc2
Ste11-GFP (Moc3 OE)
cdc2
Ste11-GFP (Moc4 OE)
cdc2
Ste11-GFP (Rpl32-2 OE)
cdc2

Fig. 8.

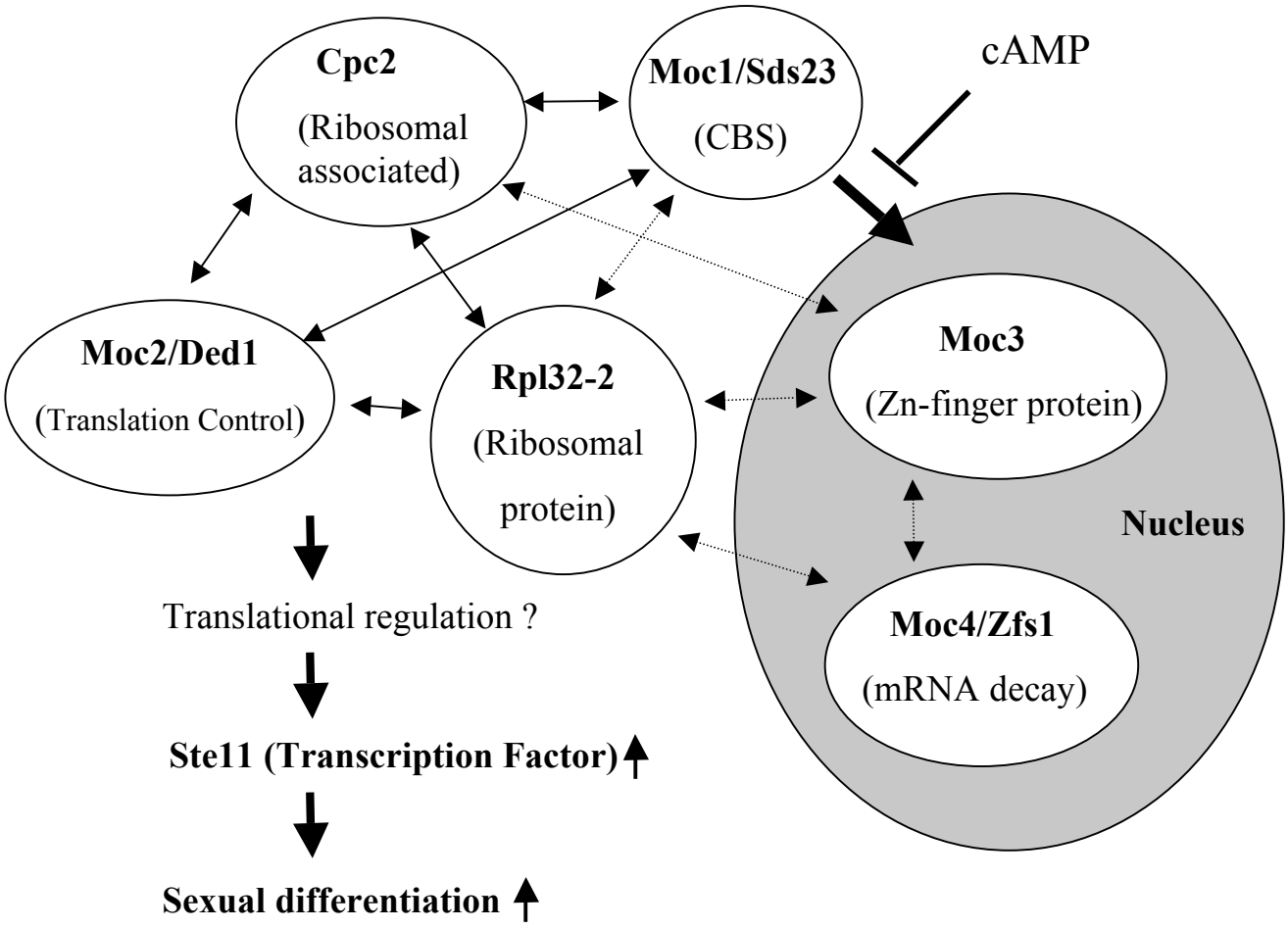
Cpc2
(Ribosomal associated)
Moc1/Sds23
(CBS)
cAMP
Moc2/Ded1
(Translation Control)
Rpl32-2
(Ribosomal protein)
Moc3
(Zn-finger protein)
Nucleus
Moc4/Zfs1
(mRNA decay)
Translational regulation ?
Ste11 (Transcription Factor)
Sexual differentiation

Fig. 9.