Identification of Myo3, a second type-II myosin heavy chain in the fission yeast *Schizosaccharomyces pombe*

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Abstract We cloned the *myo3*$^+$ gene of *Schizosaccharomyces pombe* which encodes a type-II myosin heavy chain. *myo3* null cells showed a defect in cytokinesis under certain conditions. Overproduction of Myo3 also showed a defect in cytokinesis. Double mutant analysis indicated that Myo3 genetically interacts with Cdc8 tropomyosin and actin. Myo3 may be implicated in cytokinesis and stabilization of F-actin cables. Moreover, the function of Myo2 can be replaced by overexpressed Myo3. We observed a modest synthetic interaction between Myo2 and Myo3. Thus, Myo2 and Myo3 seem to cooperate in the formation of the F-actin ring in *S. pombe*.

Key words: Fission yeast; Cytokinesis; Myosin heavy chain; Septation; F-actin ring

1. Introduction

It has been established that animal cells divide by cytokinesis through contraction of the contractile ring which is mainly composed of actin filaments [1]. Type-II myosin has been shown to exert a force for the contraction through interaction with the actin filaments [2–4]. Type-II myosin is a molecular motor composed of two identical heavy chains, two regulatory light chains, and two essential light chains [5]. However, little is known about the molecular mechanism of the formation of the contractile ring during cytokinesis.

The fission yeast *Schizosaccharomyces pombe* is an excellent organism for genetic analysis of the mechanism of cytokinesis [6]. It is a cylindrical shaped cell which divides at the middle of the cell probably by virtue of the F-actin ring similar to the contractile ring in animal cells [7,8]. It has been shown that F-actin patches are located at the growing ends of the cell during interphase. At early mitosis, the F-actin patches relocate to form the F-actin ring that surrounds the nucleus. At the end of mitosis, the possible contraction of the F-actin ring is followed by formation of the primary septum. A number of different type mutants which have defects in cytokinesis have been isolated and analyzed [9,10]. It has been reported that some of these mutations are caused by defects of the function of actin cytoskeleton-related proteins such as Cdc3 (profilin), Cdc4 (an EF-hand protein), Cdc8 (tropomyosin), and Cdc12 [11–14]. All of these genes are essential for the growth of the cells. Moreover, it has been shown that Cdc4 localizes specifically at the F-actin ring and that it associates with a 200-kDa protein which is expected to be a myosin heavy chain [12]. Very recently, it has been found that *myo2*$^+$ encodes a type-II myosin heavy chain which is colocalized with the F-actin ring [15]. It would be reasonable to expect that Cdc4 and Myo2 form a complex in the cell and cooperate in both the formation and the contraction of the F-actin ring. Although *myo2*$^+$ is also an essential gene, the terminal phenotype of *myo2* null cells is not the same as that of *cdc4* null cells. Loss of the function of Cdc4 blocks the formation of the F-actin ring [12]. In contrast, loss of the function of Myo2 leads to formation of an aberrant F-actin ring [15]. Thus, we expect that other type-II myosin(s) would also play a role in the formation of the F-actin ring. By screening an *S. pombe* genomic DNA library using the *myo2*$^+$ DNA fragment as a probe, we obtained clones which encode a novel type-II myosin heavy chain. Here we report that this *myo3*$^+$ gene product, Myo3, is required for cytokinesis under some conditions and may also be involved in stabilizing F-actin cables during interphase.

2. Materials and methods

2.1. Strains, media and genetic techniques

The *S. pombe* strains used in this study are listed in Table 1. The media used have been described previously [16]. Standard procedures for *S. pombe* genetics were carried out according to Alfa et al. [16] and Moreno et al. [17].

2.2. DNA manipulation and cloning of the *myo3*$^+$ gene

Standard methods of DNA manipulations were carried out according to Sambrook et al. [18]. A 4.0-kbp *Bgl*II fragment containing the N-terminal region of *myo2*$^+$ was used as a probe in screening the *S. pombe Spe*I genomic DNA library [19]. Hybridization and washing of filters were performed at 50°C. After plaque purification, the insert was subcloned into pBluescript II SK$^-$ with in vivo excision and sequenced. It was revealed that a 5.9-kbp *Spe*I fragment encompassed a novel type-II myosin heavy chain gene *myo3*$^+$. Since this insert did not contain a stop codon, we repeated a similar screening procedure with the *S. pombe Eco*RI genomic DNA library (R. Arai, K. Nakano, and I. Mabuchi, manuscript in preparation) using a 156-bp *Eco*RI-*Spe*I fragment of *myo3*$^+$ as a probe. Finally, a 3.7-kbp *Eco*RI fragment which overlapped with the 3′ region of the *Spe*I fragment was isolated. By combining these cloned *Spe*I and *Eco*RI fragments, we reconstructed a 6.5-kbp fragment carrying the entire *myo3*$^+$.

2.3. Functional analysis of *myo3*$^+$

To disrupt *myo3*$^+$, we replaced a *Pst*I-*Sac*I fragment with *ura4*$^+$ or *ade2*$^+$ (Fig. 1B). A fragment produced by digestion with *Spe*I was used to transform a diploid constructed by mating strains *JY741* and *JY746*. Correct integration was verified by Southern blotting.

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Bodipy, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene

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Table 1
Strains used in this study

Strain	Genotype	Source
leu1-32	h^- *leu1-32*	Lab. stock
JY333	h^- *ade6-M216 leu1-32*	Lab. stock
JY741	h^- *ade6-M216 leu1-32 ura4-D18*	Lab. stock
JY746	h^+ *ade6-M210 leu1-32 ura4-D18*	Lab. stock
cdc3	h^+ *ade6-M210 leu1-32 cdc3-124*	M.K. Balasubramanian
cdc4	h^- *leu1-32 ura4-D18 cdc4-8*	M.K. Balasubramanian
cdc8	h^- *ade6-M210 leu1-32 ura4-D18 cdc8-110*	M.K. Balasubramanian
cdc12	h^- *ura4-D18 cdc12-112*	V. Simanis
CP18-15	h^+ *cps8-188 leu1-32*	J. Ishiguro
FMM301	h^- *ade6-M216 leu1-32 ura4-D18 myo3::ura4*$^+$	This study
FMM302	h^+ *ade6-M210 leu1-32 ura4-D18 myo3::ura4*$^+$	This study
FMM201	h^+ *ade6-M216 leu1-32 ura4-D18 myo2::ura4*$^+$	This study
FMM231	h^+ *ade6-M216 leu1-32 ura4-D18 myo2::ura4*$^+$ *myo3::ura4*$^+$	This study

To overexpress *myo3*$^+$ from the *adh* promoter, a 6.5-kbp *Pst*I-*Bam*HI fragment containing the entire open reading frame was cloned into vector pART1. *leu1-32* cells were transformed with the resultant vector (pART1-*myo3*). Single colonies of transformants were inoculated into EMM liquid medium at 30°C and fixed after incubation for 18 h for microscopic examination.

2.4. Microscopy

Staining of the cells with Calcofluor (Sigma, St. Louis, MO, USA) and Bodipy-phallacidin (Molecular Probes, Inc., Eugene, OR, USA) was performed as described [17]. The stained cells were viewed with a Zeiss Axioskop fluorescence microscope and photographed on Kodak T-MAX 400 film.

3. Results

3.1. Isolation and sequence of *myo3*$^+$

After screening about 100 000 clones of the *S. pombe Spe*I genomic library using *myo2*$^+$ as a probe, we isolated 60 positive clones. Nine of them contained a 5.9-kbp insert, the nucleotide sequence of which was similar to genes encoding the myosin heavy chain, but not identical to *myo2*$^+$. Sequence analysis of *myo3*$^+$ revealed that it encodes a 2104 amino acid protein (calculated molecular mass 242 kDa) whose N-terminal globular domain is most closely related to that of Myo2

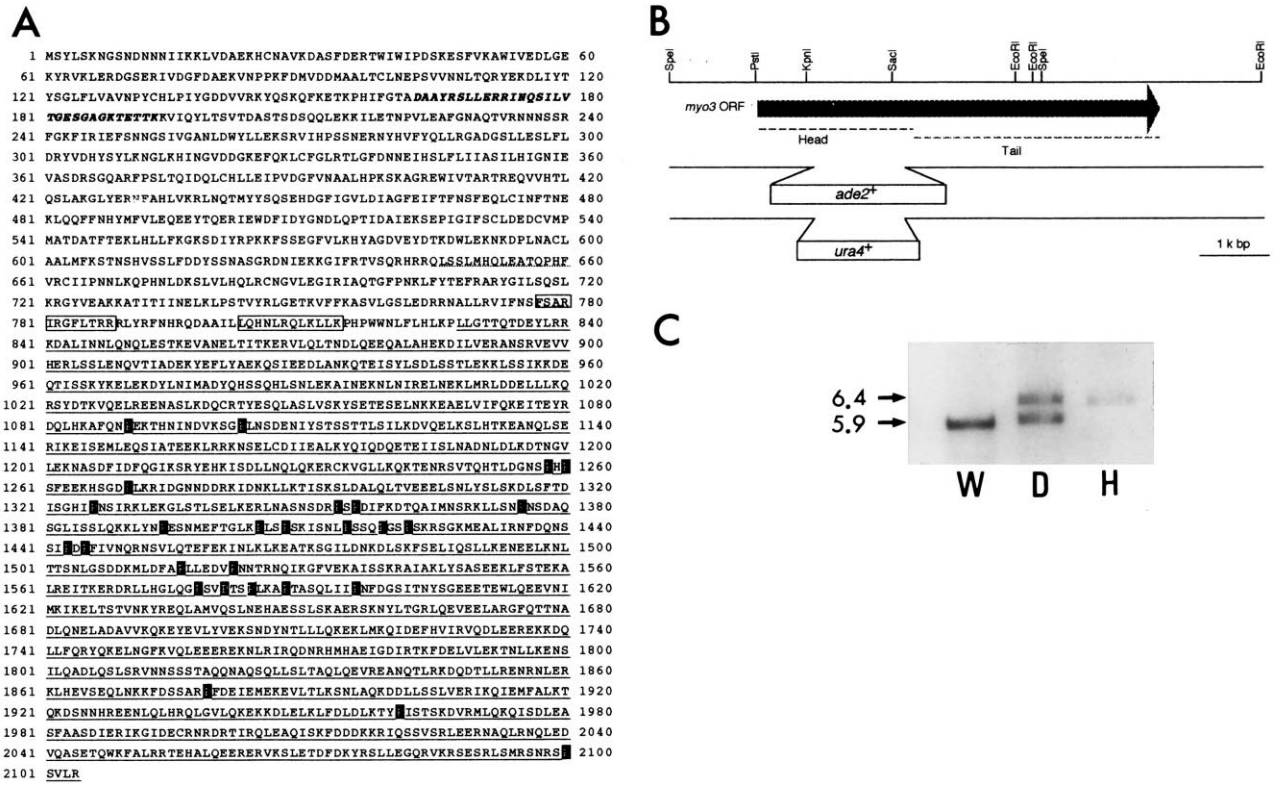

Fig. 1. Structures of the *myo3* gene and its product. A: The deduced amino acid sequence of Myo3. The putative coiled-coil region is underlined. Proline residues in the tail region are shadowed. The putative ATP-binding site is shown in italics. The putative actin-binding site is underscored with a dotted line. The putative IQ motifs are boxed. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession number AB007633. B: A restriction map of the genomic *myo3* locus. The arrow indicates the extent and orientation of the *myo3* ORF. Areas corresponding to the head and tail regions of *myo3* are shown by dotted lines. Two kinds of disruption constructs used in deleting *myo3*$^+$ are schematically shown below the arrow. C: Genomic Southern hybridization of wild-type cells, W; heterozygous diploid disruptant, D; haploid disruptant, H. The genomic DNAs were digested by *Spe*I. The probe used for hybridization was the 1.3-kbp *Kpn*I-*Sac*I fragment.

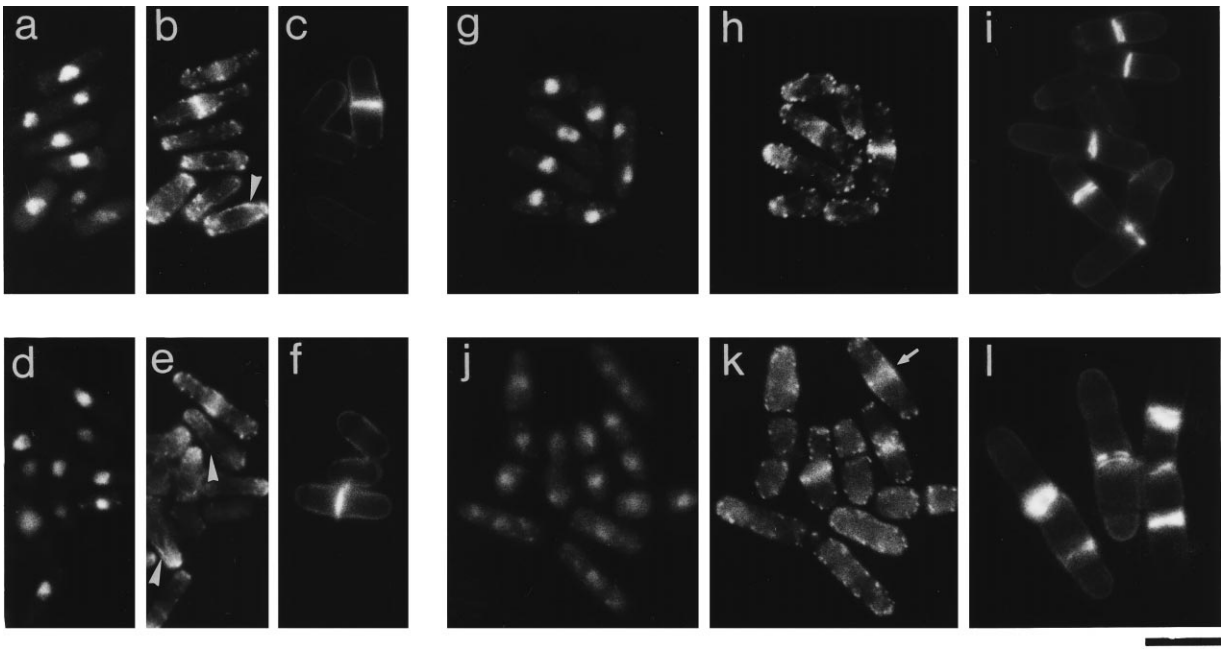

Fig. 2. The phenotype of *myo3* null cells and wild-type cells. The wild-type cells and the *myo3* null cells were grown at 30°C to mid-log growth phase, and the culture was split into two. One half was maintained at 30°C, while the other was shifted to 37°C. After 18 h, cells were fixed and stained with DAPI for chromosomal DNA (a,d,g,j), Bodipy-phallacidin for F-actin (b,e,h,k), or Calcofluor for septum materials (c,f,i,l). a–c: Wild-type cells at 30°C. d–f: Wild-type cells at 37°C. g–i: *myo3* null cells at 30°C. j–l: *myo3* null cells at 37°C. Arrow indicates broad F-actin ring. Arrowheads indicate F-actin cables. Bar, 10 μm.

(38% homology) among myosins (Fig. 1A,B). Myo3 had several structural motifs that are conserved in type-II myosin, including an ATP-binding site, an actin-binding site, and two IQ motifs (Fig. 1A). Although the tail domain of most of type-II myosin is devoid of proline residues which are expected to break the α-helix, the tail domain of Myo3 contained 27 proline residues loosely clustered in the middle part of the tail region (Fig. 1A).

3.2. Disruption of *myo3*$^+$

The *myo3*$^+$ gene was disrupted by insertion of the *ura4*$^+$ marker gene (Fig. 1B). The expected deletion was confirmed by Southern hybridization (Fig. 1C). The transformed diploids were sporulated and analyzed by the random spore method. It was revealed that the *myo3*$^+$ gene was not essential for the viability of the cells at 30°C. However, the *myo3* null cells grew only slowly at 37°C in YEPD liquid medium and could not form colonies at 37°C on YEPD plates (data not shown). The morphology of the *myo3* null cells was swollen with a normal length (92%), but some cells were elongated (8%) in YEPD liquid medium at 30°C (Fig. 2g–i). Some septa seemed to be abnormal in these cells. Most of the cells in YEPD liquid medium at 37°C were swollen or elongated and multi-septated (Fig. 2j–l). The latter cells contained three (16% in total cells) or four (15%) nuclei. The F-actin rings in the cells at 37°C were sometimes broader than those in the wild-type cells (Fig. 2b,e,k). Moreover, F-actin cables were hardly seen in these cells. Calcofluor staining showed that septa in these cells were abnormally thick or irregular (Fig. 2l). Each compartment formed by the septa usually contained a single nucleus (Fig. 2j). The defect of the growth of *myo3* null cells at 37°C was suppressed by the presence of 1 M sorbitol, but not by 1 M KCl (data not shown). This result suggests the possibility that *myo3* null cells may be sensitive to

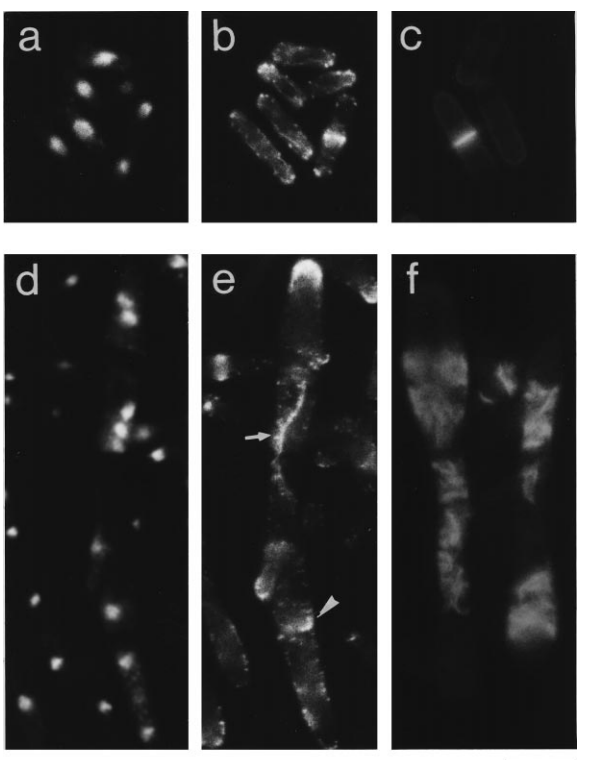

Fig. 3. Overexpression phenotype of the *myo3*$^+$ gene. Wild-type cells carrying pARTI plasmid (a–c) and those carrying pARTI-*myo3*$^+$ (d–f) were grown for 16 h at 30°C. They were fixed and stained with DAPI (a,d), Bodipy-phallacidin (c,f) or Calcofluor (b,e). Arrow indicates abnormally thick F-actin cable. Arrowhead indicates an irregular accumulation of F-actin. Bar, 10 μm.

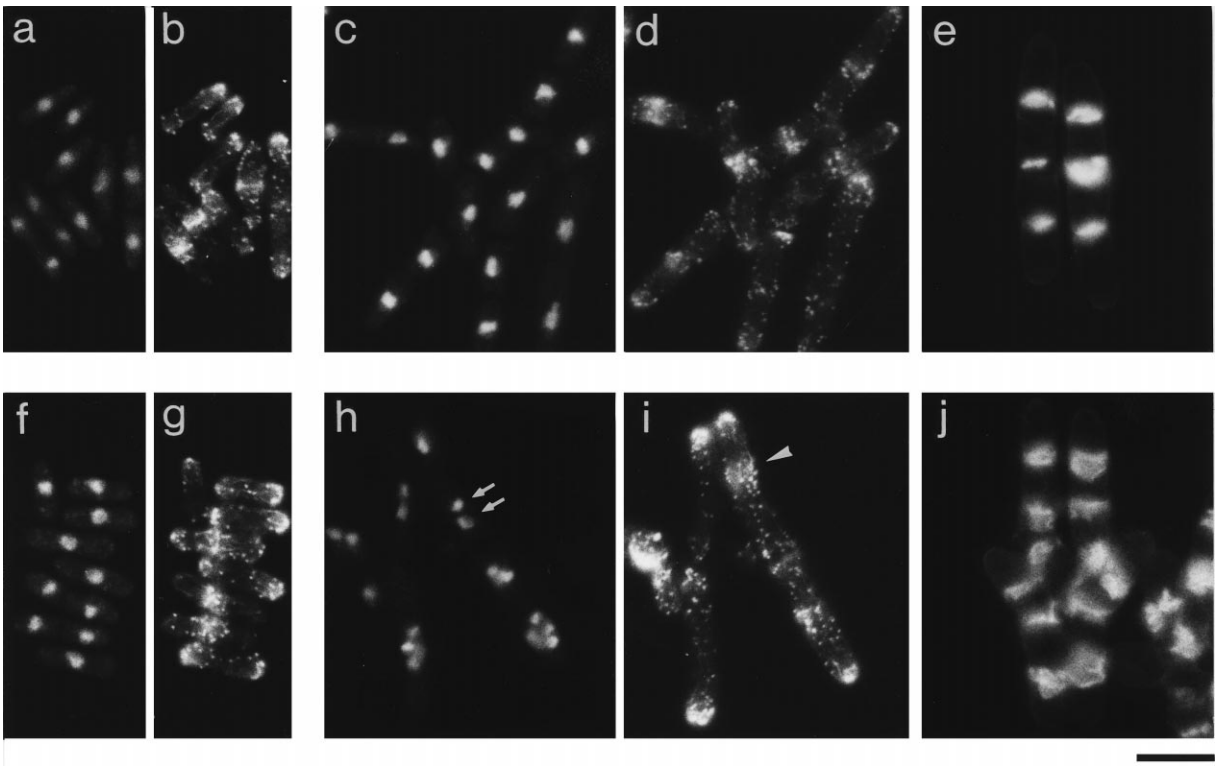

Fig. 4. The phenotype of *myo2myo3* null cells and *myo2* null cells. The *myo2myo3* null cells and the *myo2* null cells were grown in medium without thiamine at 30°C, and then thiamine was added to the medium. Cells were fixed and stained with DAPI (a,c,f,h), Bodipy-phallacidin (b,d,g,i) and Calcofluor (e,j) at 0 and 24 h after the addition of thiamine. a,b: *myo2* null cells at 0 h. c–e: *myo2* null cells at 24 h. f,g: *myo2-myo3* null cells at 0 h. h–j: *myo2myo3* null cells at 24 h. Arrow indicates pairs of closely located daughter nuclei. Arrowhead indicates an F-actin accumulation between well separated nuclei. Bar, 10 μm.

chloride ions. Another experiment showed that *myo3* null cells could not form colonies at 30°C on YEPD plates containing 0.2 M $MgCl_2$ whereas they formed colonies on plates containing 0.2 M $MgSO_4$ (data not shown). Therefore, the *myo3* null cells showed two intriguing phenotypes: temperature sensitivity and chloride ion sensitivity.

3.3. Overexpression of Myo3

It has been shown that overexpression of Myo2 leads to the appearance of multi-nucleated dumbbell-shaped cells which are similar to *cdc4*-deficient cells [15]. In order to examine the consequence of overexpression of Myo3 in the cell, wild-type cells were transformed with pART1-*myo3*. It was observed that the Myo3-overexpressing strain produced aberrant shaped cells while the shapes of the cells carrying only pART1 seemed to be normal under the same conditions (Fig. 3a–f). In the Myo3-overexpressing strain, almost all cells were elongated and enlarged in diameter, and some cells (59%) showed a multi-nucleated phenotype (Fig. 3d,e). F-actin accumulated between the separated nuclei in some of the elongated cells, although they were irregular in form. In addition, abnormally thick F-actin cables were observed in the Myo3-overexpressing cells as compared to the wild- type cells (Fig. 3b,e). It was also observed that the overexpression of Myo3 leads to abnormal septation as judged from Calcofluor staining: irregular accumulation of septum materials was seen in the cells (Fig. 3f).

3.4. Myo3 genetically interacts with actin and tropomyosin

We constructed double mutants between the *myo3* null strain and strains *cdc3*, *cdc4*, *cdc8*, *cdc12* or *cps8*. It has been shown that *cps8* has a mutation in the *act1*$^+$ gene that encodes actin [20]. Synthetic effects were found in *myo3cps8* and *myo3cdc8*. The *myo3cps8* cells were able to grow only in the presence of 1 M sorbitol; the cells lysed without sorbitol, while the *cps8* cells grew well without sorbitol at 25°C. It was also observed that the *myo3cdc8* cells grew only slowly at 25°C and did not grow at 28°C, while the *cdc8* cells grew at a normal rate at 30°C.

3.5. The function of Myo3 is redundant with Myo2

In order to examine the genetic interaction between *myo2*

Table 2
Number of nuclei in the compartments of the *myo2myo3* null cells and the *myo2* null cells

Cell	Number of cells counted	Number of nuclei in the compartment of the cells (%)				
		0	1	2	3	4
myo2 null	283	0	85	14	1	0
myo2myo3 null	346	0	51	42	5	2

The *myo2myo3* null cells and the *myo2* null cells were grown in medium without thiamine at 30°C, and then thiamine was added to the medium. These cells were fixed and stained with both DAPI and Calcofluor at 24 h after the addition of thiamine. Individual cells were first screened by fluorescence microscopy to examine the cell cycle stage. We selected cells that had undergone three cycles of nuclear division and counted the number of nuclei in the compartment at the end of the cell. This is because the septa in the *myo2myo3* null cells were extensively broad and distorted, and we could not precisely recognize the compartment at the middle regions of the cells.

and *myo3*, we examined whether the lethality of *myo2* null cells was suppressed by the overexpression of Myo3. It was found that the *myo2* null cells containing pART1-*myo3* were able to form colonies on EMM plates. In EMM liquid medium, almost all the cells showed a normal shape, although a small proportion of the cells were elongated (data not shown). This suggests that the function of Myo3 overlaps with that of Myo2. Next, we constructed a *myo2myo3* double disruptant containing pREP81-*myo2*, and compared its terminal phenotype with that of the *myo2* null cells containing pREP81-*myo2* in the presence of thiamine. The *myo2* null cells which were grown in the absence of thiamine were transferred to the medium containing thiamine to repress the production of Myo2. At 24 h after the addition of thiamine, elongated cells with multiple septa appeared (Fig. 4c–e). It was observed that some septa were abnormally thick or incompletely formed (Fig. 4e) and that F-actin rings seemed to be broader than those of wild-type cells (Fig. 4d). The phenotype of the *myo2myo3* null cells was similar to that of the *myo2* null cells, but some features were observed in the *myo2myo3* null cells (Fig. 4h–j). Calcofluor staining revealed that nearly all septa were severely distorted in the *myo2myo3* null cells (Fig. 4j). It was seen that pairs of daughter nuclei which were located closely to each other increased as the culture progressed (Fig. 4h). Neither F-actin rings nor septa were detected between these nuclei; on the other hand, obscure F-actin accumulation was often seen at the septum region which were located between well separated nuclei (Fig. 4h,i). At 24 h after the addition of thiamine, we counted a number of nuclei in compartments divided by septa in both strains (Table 2). It was observed that the frequency of the compartments containing multiple nuclei was higher in the *myo2myo3* null cells than in the *myo2* null cells.

4. Discussion

4.1. Myo3 is a novel type-II myosin in S. pombe

myo3$^+$, which encodes a novel type-II myosin heavy chain in *S. pombe*, was isolated and characterized. The structurally conserved domains of the type-II myosin heavy chain, such as the ATP-binding site, the actin-binding site, and IQ motifs, were also conserved in Myo3. However, we cannot decide whether both of the two IQ motifs of Myo3 are functional, since the first IQ motif of Myo3 (FSARIRGFLTR) is considerably different from the consensus sequence (IQXXXRGXXXR) (X indicates an arbitrary amino acid [21]). The α-helical coiled-coil structure of the type-II myosin heavy chain is required for dimerization and usually contains no proline residue which breaks the α-helix structure [22]. The type-II myosin heavy chains in yeasts have some proline residues in the tail region: *Saccharomyces cerevisiae* MYO1 has six proline residues [23] and *S. pombe* Myo2 has nine [15]. We found that the tail region of Myo3 contained 27 proline residues which are mainly located in the central part of the tail region. However, these proline residues may not prevent the dimerization of Myo3; the tail of Myo3 except for the proline-rich part shows a heptad repeat pattern and is likely to form α-helical coiled-coil structures, as judged by a computer program, MTIDK [24].

4.2. Myo3 is involved in cytokinesis

It was observed in the *myo3* null cells that F-actin rings were malformed around the nuclei during mitosis at a restrictive temperature or a high concentration of chloride ions. It was also revealed that overexpression of Myo3 produced multi-nucleated elongated cells. These observations suggest that Myo3 is involved in cytokinesis. It was previously reported that Myo2 is localized in the F-actin ring and is also involved in its formation [15]. Although *myo2*$^+$ is an essential gene, it was demonstrated here that the function of Myo2 can be replaced by overexpressed Myo3. It seems that Myo2 and Myo3 cooperate in the formation of the F-actin ring, while the function of Myo2 may be more important than that of Myo3. In addition, it has been reported that the function of Cdc8 tropomyosin is also required for the formation of the F-actin ring [13]. Recently, we observed that the function of Cdc8 is essential for the formation of F-actin cables in interphase cells and that Cdc8 is localized in the F-actin cables (Arai et al., manuscript in preparation). These data may indicate that Cdc8 functions to stabilize F-actin bundle structures. The *cps8* gene product is an actin containing a mutation at a position neighboring the hydrophobic plug. This region is likely to be important for the polymerization of actin, since F-actin is unstable in *cps8* cells [20]. We demonstrated that *myo3* genetically interacts with both *cdc8* and *cps8*. Moreover, F-actin cables were hardly detected in the *myo3* null cells at 37°C. In contrast, aberrant F-actin cables were seen in Myo3-overproducing cells. Therefore, it is possible that Myo3 is involved in the proper formation and the stabilization of F-actin bundle structures, that is the F-actin ring and the cable, in the cell.

S. cerevisiae has one type-II myosin heavy chain, MYO1, which is not essential for cell proliferation. *myo1*-disrupted cells grow slowly and undergo incomplete separation of daughter cells after cytokinesis [23]. Moreover, the cells show abnormal chitin distribution and cell wall organization [25]. Thus, MYO1 has been considered to be involved in cell separation rather than in cytokinesis, and may also be involved in delivery to septum of cell-wall components. Therefore, the function of *S. pombe* Myo3 may be different from that of *S. cerevisiae* MYO1.

The double disruptant *myo2myo3* showed more serious defects in cytokinesis than the single disruptants. This result supports the above view that Myo2 and Myo3 cooperate in the formation of the F-actin ring. However, we cannot compare the phenotypes of the double disruptant and of *cdc4* null cells, since the experimental conditions are different. The *cdc4* null cells are generated by germination of *cdc4* null spores [12]. On the other hand, we first constructed the *myo2myo3* double disruptant carrying pREP81-*myo2*, and then repressed the expression of *myo2*. A possibility remains that Myo2 is not completely deleted in the cells even 24 h after the addition of thiamine. Thus, further analysis of the relationship between Myo2 and Myo3, and that between these myosin heavy chains and Cdc4, is necessary to understand more precisely the roles of myosin II in cytokinesis in *S. pombe*.

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