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Short Communication

# Byr4p, a Possible Regulator of Mitosis and Cytokinesis in Fission Yeast, Localizes to the Spindle Pole Body by its C-Terminal Domains

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**Cytokinesis and septation should be coordinated to nuclear division in the cell division cycle for precise transmission of the genome into daughter cells. *byr4*, an essential gene in fission yeast *Schizosaccharomyces pombe*, regulates the timing of cytokinesis and septation in a dosage-dependent manner. We examined the intracellular localization of the Byr4 protein by expressing *byr4* as a fusion of green fluorescence protein (GFP). The Byr4 protein localizes as a single dot on the nuclear periphery of interphase cells, duplicates before mitosis, and the duplicated dots segregate with the nuclei in anaphase. The behavior of Byr4p throughout the cell cycle strongly suggests that Byr4p is localized to the spindle pole body (SPB), a microtubule organizing center (MTOC) in yeast. The presence of the Byr4 protein in the SPB is consistent with its function to coordinate mitosis and cytokinesis. We also mapped the domains of Byr4p for its proper localization to SPB by expressing various *byr4* deletion mutants as GFP fusions. Analyses of the diverse *byr4* deletion mutants suggest that the indirect repeats and the regions homologous to the open reading frame (ORF) *YJR053W* of *S. cerevisiae* in its C-terminus are essential for its localization to the SPB.**

**Keywords:** *byr4*, cytokinesis, mitosis, *Schizosaccharomyces pombe*, spindle pole body.

## Introduction

At the end of nuclear division, cytokinesis divides the cytoplasm by placing the division apparatus including actin contractile ring upon the equator of the parental cell. Premature initiation of cytokinesis before the end of nuclear division could destruct the mitotic spindle which can lead to unequal segregation and loss of chromosomes. Moreover, failure to undergo cytokinesis after nuclear division can produce polyploid multinuclear cells that are frequently observed in tumor cells. Therefore, the proper spatial and temporal control of cytokinesis is essential for the accurate transmission of genetic information, but its molecular mechanisms are not well understood. Accumulated results suggest that the signal for the initiation of cytokinesis originates from the spindle poles (Hiramoto, 1971) or from the spindle midzone (Kawamura, 1977; Cao and Wang, 1996) at the beginning of anaphase. These studies propose the essential role of the mitotic spindle in regulating the timing and orientation of cytokinesis (White and Strome, 1996).

The microtubule organizing center (MTOC) regulates the number, orientation, and arrangement of microtubules in most eukaryotic cells. In the fission yeast *Schizosaccharomyces pombe*, the spindle pole body (SPB) functions as a MTOC during mitosis and it is functionally homologous to the centrosome of animal cells (Stearns and Winey, 1997). Like the centrosome, the spindle pole body (SPB) of *S. pombe* occupies a perinuclear location in the cell, nucleates microtubule growth, and duplicates once per cell cycle (Ding *et al.*, 1997). The SPB of *S. pombe* undergoes dramatic changes during the cell cycle. The SPB duplicates during late G2 phase and the duplicated structures are connected by a bridge until the mitotic spindle forms in mitosis. As mitosis begins, the duplicated SPBs initiate intranuclear microtubules and locate themselves on the opposite sides of the nucleus to form a bipolar mitotic spindle that segregates both the chromosomes and the SPBs into the soon-to-be daughter cells (Uzawa and Yanagida, 1992).

The cytology of mitosis and cytokinesis in *S. pombe* is strikingly similar to that in higher eukaryotes and *S. pombe* can be easily approached by classical and molecular

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genetics (Marks and Hyams, 1985; Nurse, 1994). Also, the initiation of cytokinesis in *S. pombe* depends on the onset of mitosis as in higher eukaryotes (Marks and Hyams, 1985). These advantages make the fission yeast *S. pombe* as a useful model system to study the mechanisms coordinating mitosis and cytokinesis. Several genes for controlling the timing of cytokinesis are identified in *S. pombe* from the phenotypes of the cell division cycle mutants. They include *cdc7*, *cdc11*, *cdc14*, *spg1*, and *cdc16*·*cdc7*, *cdc11*, *cdc14*, and *spg1* are required for the initiation of cytokinesis and *cdc16* is necessary to negatively regulate the initiation of cytokinesis when the bipolar spindle has some defects (for reviews, see Fankhauser and Simanis, 1994; Gould and Simanis, 1997). The *cdc16* gene is also required for cell cycle arrest in the absence of the mitotic spindle and it shows sequence homology with *BUB2*, a spindle assembly checkpoint gene in *S. cerevisiae* (Fankhauser *et al.*, 1993). In addition, intensive homology search of *cdc16* reported that it possibly encodes a GTPase-activating protein (GAP) (Neuwald, 1997). Predicted proteins from the sequences of *spg1* and *cdc7* suggest that they encode a small GTPase and a downstream protein kinase, respectively (Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997). Therefore, the studies of *cdc16*, *cdc7*, and *spg1* suggest a model that the coordination of mitosis to cytokinesis is mediated by a possible small GTPase signal transduction pathway, where Spg1 functions as a GTPase, Cdc16 as a GAP, and Cdc7 as a downstream effector of Spg1 (Schmidt, 1997). Furthermore, Spg1p and Cdc7p localize to SPB and the Spg1p activity is required for localization of the Cdc7p *in vivo*, proposing an essential role of the SPB in signaling for the initiation of cytokinesis in fission yeast (Sohrmann *et al.*, 1998).

The *byr4* gene was identified as a multicopy suppressor of *ras1* in *S. pombe*. The *byr4* encodes an essential gene and its phenotypic studies indicate that *byr4* regulates mitosis and the initiation of cytokinesis in a dosage-dependent manner (Song *et al.*, 1996). In detail, *byr4* overexpression inhibits cytokinesis but the cell cycle continues, leading to multinucleate cells. The knock-out of *byr4* causes cell cycle arrest in late mitosis with multiple cytokinesis and septation. The *byr4* is a novel gene and the homology search of the protein sequence database only revealed a limited homology to an open reading frame (ORF) *YJR053W* of *S. cerevisiae*. The knock-out cells of *YJR053W* show no growth defects except reduced mating efficiency (Huang *et al.*, 1997), but the over-expression of *YJR053W* causes defects in nuclear division and bud separation in *S. cerevisiae* (J. Lee and K. Song, submitted). The overexpression phenotypes of the *YJR053W* suggest a possibility that *YJR053W* could be a functional homologue of the *byr4* (J. Lee and K. Song, submitted). The sequence homology between *byr4* and *YJR053W* is confined in the C-terminus of these proteins. The ORF of *byr4* also contains imperfect direct repeats of 43 amino acids at the C-terminus of the protein (Song *et al.*, 1996). The *byr4* genetically interacts with *cdc16* and *cdc7* and the Byr4 protein directly interacts with Cdc16 and Spg1 in yeast two-hybrid assays, suggesting that Byr4 also functions in a possible small GTPase pathway including *cdc16*, *spg1*, and *cdc7*, that affects the timing of cytokinesis in *S. pombe* (Jwa and Song, 1998). As an approach to understand the functional mechanisms of *byr4*, we examined the intracellular localization of the Byr4 protein. In this study, we also determined the domains of Byr4 for its proper localization by expressing various *byr4* deletion mutants as GFP fusions. The Byr4 protein is possibly localized on the spindle pole body as a GFP fusion and the C-terminal domains of the Byr4p are essential for its localization.

## Materials and Methods

***S. pombe* strain and culture** *S. pombe* strain KGY246 (*h⁻ ade2-210 ura4-D18 leu1-32*) was used in this study. Culture, manipulation, and transformation of *S. pombe* were carried out as described by Moreno *et al.* (1991).

**Plasmid construction** All manipulations of DNA were performed by standard methods (Sambrook *et al.*, 1989). To generate pRep41/*byr4D3* and pRep41/*byr4D4*, the *Nde*I-*Bcl*I *byr4D3* fragment and the *Nde*I-*Bam*HI *byr4D4* fragment from pRep41/*byr4* were ligated into the *Nde*I-*Bam*HI site of pRep41. pRep41/*byr4D6* was constructed by inserting the *Nde*I-*Sma*I PCR fragment of *byr4D6* into the *Nde*I-*Sma*I site of pRep41. Each of the pRep41/*byr4D2, D5, D7~D14* was made by inserting the *Nde*I-*Bam*HI digested corresponding PCR fragment into the *Nde*I-*Bam*HI site of pRep41. pRep41/*byr4D15* was constructed by replacing the *Nde*I-*Bcl*I digested fragment from the *byr4D15* PCR product with the *Nde*I-*Bcl*I digested fragment from the full-length *byr4* in pRep41/*byr4*. pRep41/*byr4D16* was made by inserting the *Nde*I-*Bcl*I *byr4D3* fragment of pRep41/*byr4D3* into the *Nde*I-*Bcl*I site of pRep41/*byr4D9*.

To detect their localizations, the full-length *byr4* ORF and its deletion mutants were fused on frame to the C-terminus of GFP (green fluorescence protein). pRep42/*gfp-byr4* was constructed by replacing the *Nde*I-*Sac*I fragment containing the entire *byr4* of pRep41/*byr4* (Song *et al.*,1996) with the *Nde*I-*Sac*I fragment of pRep42/*gfp-byr2*. pRep41/*gfp-byr4* was constructed by replacing the *Pst*I-*Sac*I fragment of pRep42/*gfp-byr4* with the *Pst*I-*Sac*I fragment of pRep41. Each of the pRep41/*gfp-byr4D1, D4~D7, byr4D12~D14* was made by replacing the *Nde*I-*Sac*I fragment of corresponding pRep41 construct with the *Nde*I-*Sac*I fragment from the pRep41/*gfp-byr4*. Each of the pRep41/*gfp-byr4D8~D11* was made by replacing the *Pst*I-*Nde*I fragment of pRep41/*gfp-byr4* with the *Pst*I-*Nde*I fragment of the corresponding pRep41 construct. Each of the pRep42/*gfp-byr4D2, D3, D15, D16* was made by replacing the *Nde*I-*Sac*I fragment of corresponding pRep41 construct with the *Nde*I-*Sac*I fragment from the pRep42/*gfp-byr4*. All oligonucleotides used to make the PCR fragment for *byr4* deletion mutants are presented in Table 1. PCR was carried out with *Ex Taq* polymerase (Takara Co.) or *pfu* polymerase (Stratagene Co.) and two independent PCR fragments were subcloned to exclude the possible error from PCR.

**Table 1.** Oligonucleotides used in PCR.

| Deletion Mutant | 5'Primer | 3'Primer |
|---|---|---|
| *byr4D2* | 5'-TCGCATATGCGTC CAGCATTAATTTCC-3' | 5'-GTAAAGACGGATCC TTGTTTATTGTTCGGC-3' |
| *byr4D5* | 5'-CGCTTTCCAATGATCA TATGAGCGTCAAAG-3' | Same as *byr4D2* |
| *byr4D6* | 5'-AACTGCAGCATATGA CTGAAGTTGAATGCTG-3' | 5'-TTAGGATCCCGGGC TAGGCGAGATAGG-3' |
| *byr4D7* | 5'-AAACAAAATCATAT GGACCCAAAAGCTTCC-3' | Same as *byr4D6* |
| *byr4D8* | 5'-GTTTCCGGAACGCATA TGAGTAAACACGCAGG-3' | Same as *byr4D2* |
| *byr4D9* | Same as *byr4D8* | 5'-ATTTTGGATCCACTACTGC GAGGTGGAGTCTGTATC-3' |
| *byr4D10* | 5'-CCACCTCGCAGCATA TGAACGAAAATTCAGG-3' | Same as *byr4D2* |
| *byr4D11* | Same as *byr4D5* | Same as *byr4D9* |
| *byr4D12* | Same as *byr4D8* | Same as *byr4D6* |
| *byr4D13* | Same as *byr4D5* | Same as *byr4D6* |
| *byr4D14* | Same as *byr4D2* | Same as *byr4D9* |
| *byr4D15* | 5'-TCTTCTCATATGCC AGAAACGTTGAAGGCT-3' | Same as *byr4D2* |

All 5'primers and 3'primers have a *NdeI* site and a *BamHI* site, respectively. The 3'primer used in *byr4D6* PCR also contains a *SmaI* site.

**Overexpression of the *gfp-byr4* and its deletion mutants in *S. pombe*** KGY246 was transformed with *byr4* and its deletion constructs subcloned into pRep/*gfp*. Cells were grown to mid-log phase in the presence of thiamine. The expression was induced from the *nmt1* promoter by washing and growing in thiamine-free media to mid-log phase for 14–25 h.

**Microscopy** To observe the GFP signal, induced cells were collected by centrifugation, rapidly fixed with 20% ethanol, washed twice with PBS (pH 8.0), and resuspended in PBS (pH 8.0). To visualize DNA, the fixed cells were stained with 1 $\mu$g/ml DAPI (4'-6'diamidino-2-phenylindole, Sigma). Photographs were taken using a camera connected to Zeiss Axioscope with ×100 objective onto TMAX 400 (Kodak, Rochester, USA). For immunofluorescence microscopy, cells were fixed and permeabilized by following the method of Song *et al.* (1996). Cells were immunostained with affinity purified polyclonal anti-Byr4 antiserum followed by Texas-red conjugated anti-rabbit goat IgG (Molecular Probes, Inc.). For sandwiching to amplify the signal, Texas-red conjugated anti-rabbit goat IgG was followed by Texas-red conjugated anti-goat donkey IgG (Molecular Probes, Inc.).

## Results and Discussion

**Localization of the Byr4 protein** We determined the intracellular localization of *byr4* in two ways. First, we tried immunofluorescence microscopy with the polyclonal antiserum specific for the Byr4p. Byr4p was detected as a very tiny dot in the nuclear periphery in interphase cells, but the signal was too weak to be monitored or to be convinced. We tried the sandwiching method using two layers of secondary antibodies to amplify the signal without success (Pringle *et al.*, 1989).

Localization of the Byr4 protein was confirmed and detected by the overexpression of Byr4p as a green fluorescence protein (GFP) fusion. We fused the full-length *byr4* ORF to the C-terminus of the GFP (pRep41/*gfp-byr4*). The expression of the GFP-Byr4 fusion protein was under the control of *nmt1* promoter that induces the expression in the absence of thiamine in the media (Basi *et al.*, 1993; Maundrell, 1993). Before the examination of the GFP-Byr4 localization in *S. pombe*, we tested whether the GFP-Byr4 fusion protein is fully functional to complement a *byr4* null allele. Since *byr4* is an essential gene, the *byr4* knock-out strain (CA103) only exists as a diploid, where one allele of *byr4* is replaced with the *ura4* gene (Song *et al.*, 1996). We transformed pRep41/*gfp-byr4* into CA103 and analyzed random spores to isolate $ura^+$ haploids. We could isolate $ura^+$ haploids since the *byr4::ura4* allele was complemented by *gfp-byr4* on the plasmid (data not shown). In addition, when the GFP-Byr4 fusion protein was overexpressed, the phenotypes typical of the *byr4* overexpression were observed, including inhibition of cytokinesis and septation (Fig. 1F). These results assure that the GFP-Byr4 fusion is fully functional. When the expression of the GFP-Byr4 fusion protein was induced, it was monitored as a single dot on the nuclear periphery in interphase cells (Fig. 1B). When we observed this dot on the nuclear periphery throughout the cell cycle, it duplicated before mitosis and each of the duplicated structures was segregated with each dividing nucleus (Figs. 1C & 1D). Since two duplicated structures were not in the same focal plane, one dot looked clearer than the other one (Figs. 1C & 1D). The behavior of this dot throughout the cell cycle strongly suggests that this structure represents the spindle pole body (SPB). Also, cells induced with the GFP-Byr4 underwent the normal cytokinesis and septation as shown in Fig. 1E, suggesting that the localization of Byr4 is functional for the cell cycle progression. In cells shown in the Figs. 1B to 1E, the GFP-Byr4 protein was modestly overexpressed with 14 h, induction to examine normal cell cycle pattern. However, when induction was prolonged for over 16 h, most cells became multinucleated, displaying the typical *byr4* overexpression phenotypes. The overexpressed GFP-Byr4 protein also located on the SPB in each nuclear boundary of the multinucleate cells (Fig 1F). When the cells overexpressing only the GFP were examined, no localized signal or cell cycle abnormalities were observed (Fig. 1A), affirming the specificity of GFP-Byr4 localization.

Cellular localization of the GFP-Byr4 protein on the SPB could explain why Byr4p was not an abundant protein and why part of the Byr4 protein was not soluble even in the buffer containing nonionic detergent, 0.2% Triton X-100 (Song *et al.*, 1996). It also explains the technical difficulties of the Byr4 immunofluorescence localization

**Fig. 1.** Localization of the Byr4 protein in the cells overexpressing GFP-Byr4. Wild-type strain KGY246 containing pRep41/*gfp-byr4* (B to F) or pRep41/*gfp* (A) was incubated in thiamine-free media for 14 h at 29°C. Cells were then collected, fixed, and stained with DAPI. Left panels show the DNA and right panels show the GFP signal of GFP-Byr4 (A to E). For F, the top panel shows the DNA and the bottom panel shows the GFP signal of GFP-Byr4. The bar denotes 10 μm. A. Interphase cells overexpressing GFP only; B. Interphase cells, each with one nucleus and one SPB; C. An interphase cell with one nucleus and the duplicated SPBs; D. An anaphase cell with two segregated nuclei and SPBs; E. A septated cell; F. A multinucleated cell in which cytokinesis was blocked by the Byr4p overexpression.

since the SPB is a tiny, compact, and protein-rich structure in the nuclear periphery.

Presence of the Byr4 protein in the SPB is consistent with its function to coordinate mitosis and cytokinesis, since the critical role of MTOC in determining the time and position of the cleavage plane has been demonstrated in other eukaryotes (Cao and Wang, 1996). Previously, we showed that *byr4* genetically and directly interacted with a possible small GTPase pathway including *cdc16*, *spg1*, and *cdc7*, that regulates the timing of cytokinesis in *S. pombe* (Jwa and Song, 1997). Cellular localization of the Byr4 protein on the SPB also supports the interactions of the Byr4p with this small GTPase signal transduction pathway, since Spg1p and Cdc7p localize to the SPB. Localizations of the key regulatory proteins of cytokinesis in *S. pombe*, Spg1, Cdc7, and Byr4 on the SPB may provide an important insight into how the link between the mitotic apparatus and the cytokinesis machinery is established to ensure proper coordination of mitosis and cytokinesis. The *S. pombe* equivalent of mitosis promoting complex (MPF), Cdc2p/Cdc13p kinase, and components of the 20S cyclosome/anaphase promoting complex (APC), localize to the spindle pole body, or its mammalian equivalent, the centrosome (Alfa *et al.*, 1990; Tugendreich *et al.*, 1995). Co-localization of the regulators of mitosis with the proteins that are required for the onset of cytokinesis and septum formation provides the potential for coordinating their activities during mitosis. Localizations of the Spg1p, Cdc7p, and Byr4p on the SPB also support the essential role of MTOC in signaling for the initiation of cytokinesis in eukaryotes.

**Domains of the Byr4p for its proper localization** We determined the domains of the Byr4 protein necessary for its localization to SPB by expressing various *byr4* deletion mutants as fusions of the GFP. We generated sixteen *byr4* deletion mutants and fused them individually to the C-terminus of the GFP (pRep41/*gfp-byr4D1~D16*) as shown in Fig. 2. The possible functional domains of Byr4p, including the imperfect direct repeats and the regions with high sequence similarity to *YJR053W* from *S. cerevisiae*, are present in the C-terminus of the protein. In addition, the *byr4D1* mutant complements the *byr4* null allele (Song *et al.*, 1996). Therefore, we constructed diverse deletion mutants of the C-terminus to examine the roles of these possible domains in the Byr4p localization. As expected, truncation of one third of the N-terminal Byr4p (Byr4D1) did not affect on its localization. Further truncations from the N-terminal boundary of the Byr4D1 indicated that the Byr4D5 contained the minimal N-terminal boundary for its localization. Various further deletions from the C-terminus of the Byr4D1 determined the C-terminal boundary for its localization and the Byr4D7 included minimal C-terminal boundary. Comparisons of the Byr4D5 and the Byr4D7 suggested that one repeat and one region homologous to *YJR053W* are minimal domains for the Byr4p localization. To prove this, the *byr4D12* was constructed by further trimming of the *byr4D7*, but the *byr4D12* could not be localized on the SPB, suggesting the middle part of the protein between the *Bam*H1 and *Bcl*1 sites in the sequence map contributed to its localization when there is no C-terminal second repeat.

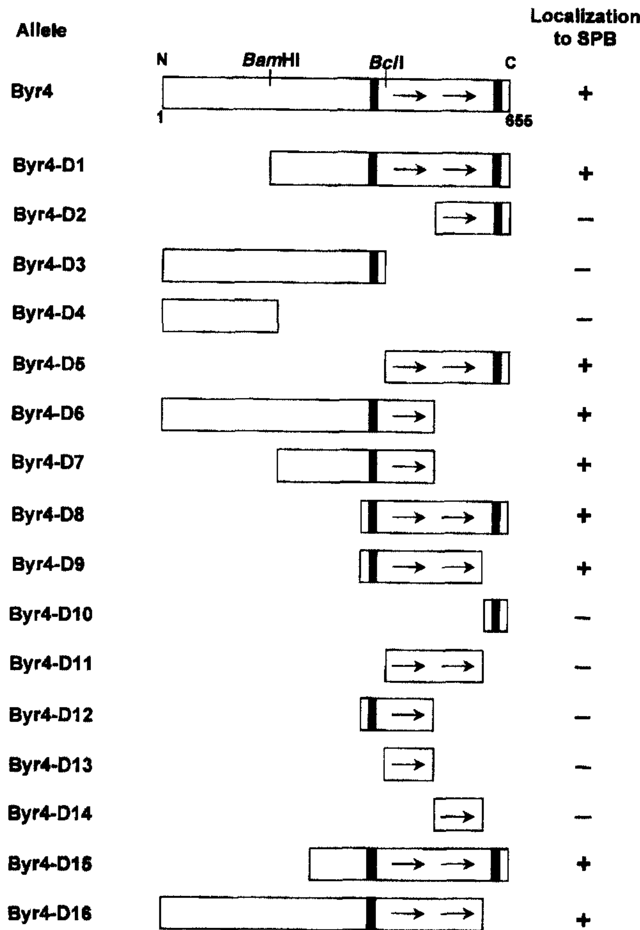


**Fig. 2.** Schematic illustration of *byr4* and its deletion mutants. The arrows represent the imperfect direct repeats of 43 amino acids. The black boxes represent regions of high sequence similarity to *YJR053W* from *S. cerevisiae*. The entire *byr4* ORF and its deletion mutants were fused to the C-terminus of the GFP. Symbol "+" represents its localization to the SPB, while symbol "–" represents no specific localization.

However, in the presence of both repeats, this middle part of the protein was not necessary for localization, as shown in the *byr4D9*. Among sixteen deletion mutants generated, *byr4D1*, *byr4D5*, *byr4D6*, *byr4D7*, *byr4D8*, *byr4D9*, *byr4D15*, and *byr4D16* were localized to the SPB as GFP fusions (Fig. 2). When these mutants were overexpressed with prolonged induction, the phenotypes typical of the *byr4* overexpression were observed including inhibition of cytokinesis and septation (data not shown). However, not all the deletion mutants of the *byr4* that exhibited the *byr4* overexpression phenotypes localized on the SPB, suggesting that the Byr4p domains for localization do not directly coincide with the domains for overexpression phenotypes. Our laboratory cloned *YJR053W* of *S. cerevisiae* and expressed it as a GFP fusion (submitted). The GFP fusion of *YJR053W* of *S. cerevisiae* is also localized to the SPB, providing another evidence that the domains including the indirect repeats and the regions homologous to *YJR053W* of *S. cerevisiae* are essential for its localization to the SPB.

In summary, we examined the intracellular localization of the Byr4 protein and mapped the domains for its localization by expressing various *byr4* deletion mutants as GFP fusions. The Byr4 protein localizes as a single dot on the nuclear periphery in interphase cells and the behavior of this dot throughout the cell cycle strongly suggests that it is the SPB. Analyses of the diverse *byr4* deletion mutants suggest that the indirect repeats and the regions homologous to *YJR053W* of *S. cerevisiae* in its C-terminus are essential for its localization to the SPB.

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