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Isolation and Characterization of the New Conditional-lethal Mutations in *byr4* of *Schizosaccharomyces pombe* by *in vitro* Mutagenesis

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Coordination of nuclear division, cytokinesis, and septation is essential for maintaining the genomic stability during the cell division cycle. *byr4* in fission yeast *Schizosaccharomyces pombe* encodes an essential gene that regulates the timing of cytokinesis and septation in a dosage-dependent manner (Song *et al.*, 1996). The knock-out of *byr4* causes cell cycle arrest in late mitosis with multiple cytokinesis and septation, while *byr4* overexpression inhibits cytokinesis and produces multinuclear cells. Since *byr4* is an essential gene, characterization of the *byr4* null phenotypes and investigation of its genetic interactions with other mutants entail technical limitations. To better characterize the functional mechanisms of *byr4* through phenotypic and genetic analyses, we generated five temperature-sensitive *byr4* mutant alleles. A truncated *byr4* with a deletion corresponding to the N-terminal 29 amino acids was randomly mutagenized by hydroxylamine *in vitro*. The mutagenized *byr4* with an N-terminal truncation was integrated into the *byr4* locus of *S. pombe* genome. Cells that formed colonies at the permissive temperature, 25°C,but could not grow at the restrictive temperatures, 18°C or 35°C, were isolated. We successfully isolated five temperature-sensitive *byr4* alleles (*KSY1-5*) that could not grow at 35°C. In the restrictive temperature, *KSY1*, *KSY3*, and *KSY5* alleles arrested cells with multiple septation while chromosome segregation was normal in these alleles. *KSY2* and *KSY4* alleles exhibited two phenotypes at the restrictive temperature: cells were arrested with multiple nuclei due to the inhibition of cytokinesis or with multiple nuclei that were separated by septum. These newly isolated *byr4* conditional alleles will be useful for the deduction of cellular processes where *byr4* functions. Genetic studies and suppressor screens of the conditional alleles can provide useful tools for the isolation of interacting proteins with Byr4p.

Key words: *byr4*, *Schizosaccharomyces pombe*, *in vitro* mutagenesis, conditional mutation

Cytokinesis divides the cytoplasm by placing the division apparatus upon the equator of the parental cell. Cytokinesis occurs in anaphase following the separation of chromosomes and is followed by septum formation in cells containing the cell wall. Premature initiation of cytokinesis before the end of nuclear division could destroy the mitotic spindles, leading to unequal segregation of chromosomes. Failure to undergo cytokinesis after nuclear division could produce polyploid multinuclear cells that are frequently observed in tumor cells. Therefore, nuclear division, cytokinesis, and septum formation must be coordinated in time and space for precise transmission of the genetic information into daughter cells. Loss of the control in these processes is directly correlated with the genomic instability that leads to the development of tumor or cancer.

We use the fission yeast *Schizosaccharomyces pombe* as an eukaryotic model system to study the mechanisms coordinating nuclear division and cytokinesis, since mitosis and cytokinesis in *S. pombe* resemble those in mammals. In addition, *S. pombe* can be readily approached with the tools of classical and molecular genetics (Moreno *et al.*, 1991). As *S. pombe* cells enter mitosis, F-actin forms a medial ring at the future site of cytokinesis and divides the cytoplasm at the end of anaphase. A primary septum is deposited after cytokinesis, and secondary septa are deposited on each side of the primary septum. Degradation of the primary septum followed by the formation of the secondary septa leads to two daughter cells (Marks and Hymes, 1985).

We originally isolated *byr4* as a multicopy suppressor of *ras1* in *S. pombe*. *byr4* encodes an essential gene that regulates the mitotic cell cycle and the timing of cytokinesis in a dosage-dependent manner

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(Song *et al.*, 1996). In detail, *byr4* overexpression inhibits cytokinesis, but the cell cycle continues leading to multinucleate cells. The knock-out of *byr4* is lethal and causes cell cycle arrest in late mitosis with multiple cytokinesis and septation (Song *et al.*, 1996). Including *byr4*, a number of other genes were identified in *S. pombe* whose mutant phenotypes suggest their functions in the control of cytokinesis. *cdc 7, cdc11, cdc14, cdc15, cdc16, plo1* and *spg1* are proposed to regulate the timing of cytokinesis and septation in *S. pombe* (Fankhauser *et al.*, 1993; Fankhauser and Simanis, 1994; Fankhauser *et al.*, 1995; Fankhauser and Simanis, 1993; Minet *et al.*, 1979; Nurse *et al.*, 1976; Ohkura *et al.*, 1995; Schmidt *et al.*, 1997: Gould and Simanis, 1997).

Since *byr4* is an essential gene, characterization of the *byr4* null phenotypes and analyses of its genetic interactions with other genes entail technical limitations. In this study, we isolated conditional-lethal alleles of *byr4* to overcome the limitations imposed. To isolate conditional alleles of *byr4*, we applied a strategy first used to create conditional alleles of actin and β-tubulin in *S. cerevisiae* (Shortle *et al.*, 1984: Huffaker *et al.*, 1988). These newly isolated *byr4* conditional alleles displayed diverse extent of the *byr4* phenotypes and will allow us to investigate the functional mechanisms of *byr4* in conjunction with other genes.

Materials and Methods

Strains and media

The *S. pombe* strains used in this study are listed in Table I. Yeast was grown in yeast extract (YE) or minimal media (MM) with required supplements at the levels of 75 mg/liter for adenine, uracil, and leucine (Moreno *et al.*, 1991). Derivatives of MM media were used as indicated, where 1% glucose was used instead of 2% glucose (MM+1% glu), 10 mM glutamate was substituted for 100 mM ammonia (MMG), or 2.5 mg/liter phloxin B (Sigma P4030) was added (MM+phloxin).

Construction of the integration plasmid

0.5 kb *sub3-5* was released from pRIP3/s by *Pst*I digestion (Maundrell, 1993). pBSK/*sup3-5* was constructed by subcloning *Pst*I digested *sup3-5* into *Pst*I digested pBSK. An incomplete copy of the *byr4* genomic fragment that includes most of the *byr4* ORF except the N-terminal 29 amino acids was obtained from *Spe*I and *Sma*I digested pS17 (Song *et al.*, 1996), and subcloned into *Spe*I and *Sma*I digested pBSK/*sup 3-5* to make pBKS1 (pBSK/*sup3-5*/*byr4*).

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

Strains	Genotypes	Source
KGY113	*h ade6-704*	K. Gould
KGY114	*h+ ade6-704*	K. Gould
KGY246	*h leul-32 ura4-d18 ade6-210*	K. Gould

Production of *byr4* mutants

pBKS1 was mutagenized *in vitro* with hydroxylamine by following Sikorski and Boeke (Sikorski and Boeke, 1991). In detail, 1 M fresh hydroxylamine (Sigma Co.) solution was made in 50 mM sodium pyrophosphate (pH 7.0), 100 M NaCl, and 2 mM EDTA. 10 μg pBKS1 DNA was incubated with 500 μl of hydroxylamine solution at 75°C. The degree of mutagenesis was titered by taking 100 μl samples at different time points of incubation (0, 30, 60, 90, and 120 min). The excess hydroxylamine was removed from the mutagenized DNA by gel filtration through Sephadex G-25 (Pharmacia Co.) spin column, and then the mutagenized DNA was concentrated by Geneclean II Kit (Bio101 Inc.). 2 μl (0.05 μg/μl) of DNA from each mutagenized time point was transformed into DH5α by electroporation. The degree of mutagenesis was examined by determining the percentage of plasmids that had lost the ampicillin resistance. Depending on different time points of the mutation, 0.5, 5.9, 12.7, 19.8, and 22.1% of the cells transformed with the mutagenized pBKS1 were ampicillin sensitive. Since the coding region for amp^r is approximately 1/6 of the plasmid sequence and not all mutations in amp^r eliminate its activity, these data suggested that most of the plasmids were mutagenized after 30 min. To obtain enough DNA for transforming yeast, two pools of mutagenized DNA were amplified in *E. coli* (DH5α) by collecting and pooling approximately 15,000 bacterial colonies.

To direct the integration of pBKS1 into the chromosomal copy of *byr4*, the mutagenized and amplified pBKS1 was digested with *Bam*HI that cuts approximately one third of the *byr4* coding region from the N-terminus (Orr-Weaver *et al.*, 1981; Grimm and Kohli, 1993). Linearized pBKS1 was transformed into the yeast strain KGY113 that contains the *ade6-704* allele that can be suppressed by integrated *sup3-5*. Transformation into KGY113 was performed by following the lithium chloride procedure (Moreno *et al.*, 1991). Homologous recombination occurs near the *Bam*HI site and produces a strain containing one intact and one disrupted copy of *byr4* (Fig. 1; Shortle *et al.*, 1984; Huffaker *et al.*, 1988). If the mutation on pBKS1 lies between the 5' end of the gene and the *Bam*HI site, the integrated mutation will remain in the truncated copy of *byr4* and will not be

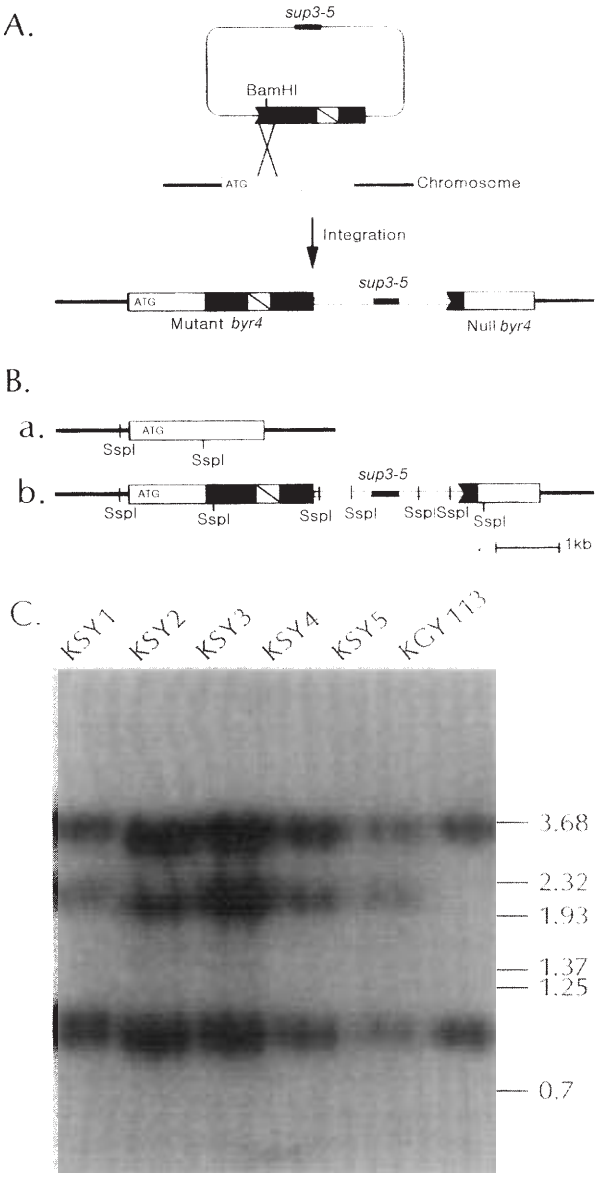

Fig. 1. Strategy used for the construction of the *byr4* conditional alleles and genomic southern analyses of the *byr4* conditional mutants. A; Schemtic illustration of the strategy for the construction of conditional alleles of *byr4*. pBKS1 (pBSK/*sup3-5*/byr4) that contains most of the *byr4* ORF except the N-terminal 29 amino acids was mutagenized by hydroxylamine, linearized with *Bam*HI, and integrated into the *byr4* chromosome locus of KGY113 by homologous recombination. The mutagenized *byr4* is intact and transcribed from the *byr4* promoter, while the wild-type C-terminus of *byr4* is nonfunctional since it lacks the promoter and initiation codon. B; Schematic physical maps of *byr4* locus in *S. pombe* (a) before and (b) after the integration of *byr4* mutant. *Ssp*I site used for genomic southern analysis is shown. C; Southern blot of the genomic DNA extracted from the *byr4* conditional alleles. KGY113 that is not integrated with mutagenized *byr4* was used as a control. The genomic DNA was digested with *Ssp*I and probed with a full *byr4*-coding sequences. Each lane contained a similar amount of DNA.

detected. If the mutation on pBKS1 lies between the *Bam*HI site and the 3' end of the gene, the integrated mutation will be present in the intact copy of *byr4* and the phenotype can be observed directly. Thus, mutations recovered by this strategy should be in the 69% of 3' *byr4* coding region beyond the *Bam*HI site.

Approximately 22,000 transformants were saved from MM plates at 25°C, replica plated to MM+ phloxin plates, and maintained at 18 and 35°C respectively. Colonies which grew at 25°C but failed to grow at either 18° or 35°C were picked and purified as single colonies. To exclude the *sup3-5* conditionals, these colonies were checked by replica plating to MMA. Linkage of conditional phenotypes to the *sup3-5* was tested by mating the conditional transformants with KGY114 and by scoring the random spores: random spores were patched on 25°C MMA and were replica plated to MMA+phloxin at 18 or 35°C and MM at 25°C. Since no auxotrophic marker was available in the yeast strain KGY113, the isolated conditionals were crossed with the wild type KGY246 that contains three different auxotropic markers. Random spores of the diploid were scored for the conditional phenotypes and their auxotropic markers. Dominance or recessiveness of the isolated conditional mutant alleles was examined by complementation tests: crosses with the wild type strain and transformations with the plasmid-based *byr4*, pREP41/*byr4* or pREP42/*byr4*, were incubated at the restrictive temperature.

Genomic southern analyses of conditionals

Genomic DNA was prepared by bead beating with glass beads in a smash solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA), followed by phenol extractions and incubation with RNase A (Moreno *et al.*, 1991). For each southern, 5 μg of DNA was digested overnight at 37°C with *Ssp*I, electrophoresed on a 0.8% agarose gel, and transferred to a nylon membrane (Hybond; Amersham Corp.). This southern blot was probed with the entire open reading frame of the Byr4 protein prepared by PCR amplification.

Microscopic techniques

Cells were fixed by adding 1/4 volume of a freshly prepared 17% (wt/vol.) paraformaldehyde (Moreno *et al.*, 1991) and examined using phase-contrast microscope. To visualize DNA, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) before mounting and observed by fluorescence microscope. Cells were viewed and photographed using a Zeiss Axioskop with a Neofluor 63X N.A. 1.25 objective. Photographs were taken using Tmax 400 (Kodak, Rochester, NY).

Results

Isolation of the five *byr4* conditional-lethal alleles

The plasmid containing the *byr4* ORF deleted with the region corresponding to the N-terminal 29 amino acids was randomly mutagenized *in vitro* by hydroxylamine. To generate the conditional-lethal alleles of *byr4*, the truncated and mutagenized *byr4* in the plasmid pBKS1 (pBSK/*sup3-5*/*byr4*) was integrated into the *byr4* locus of *S. pombe* genome. Since the integrated *byr4* was truncated with the N-terminal 29 amino acids, the mutagenized full-length *byr4* was generated and the wild type genomic *byr4* was destroyed (Fig. 1A). We used the *sup 3-5* as a marker for integration of the plasmid, since maintenance of the *sup3-5* containing plasmid as an episome is deleterious to growth and hence it leads to integration of the plasmid (Moreno *et al.*, 1991). Presence of the *sup3-5* suppresses the *ade6-704*, an adenine auxotropic mutant allele (Moreno *et al.*, 1991). The ramdomly mutagenized pBKS1 (pBSK/*sup3-5*/*byr4*) was transformed into the *ade6-704* containing KGY113 strain, and transformants were plated on MM to select the adenine prototrophs. Most transformants form white colonies, confirming the integration of pBKS1.

From the approximately 22,000 transformants screened, five colonies were restored as temperature sensitives and one recovered as a cold sensitive. These six mutations were linked to the integrated plasmid sequences marked by *sup3-5*, suggesting that the mutations were derived from the integration of PBKS1 (pBSK/*sup3-5*/*byr4*) including mutagenized *byr4*. Since there is no other auxotropic marker except the *ade6-704* in the KGY113 strain, auxotropic markers were added to these six temperature-sensitive or cold-sensitive *byr4* mutants by crossing these mutants with KGY246. Random spores of diploids from the cross of the mutants with the wild type KGY246 were scored. During the process of mating, the cold-sensitive mutant reverted the locus to the wild type and the recovered spores were no longer cold sensitive. The five temperature-sensitive *byr4* alleles with auxotropic markers were designated *byr4-KSY1* through *byr4-KSY5*, as summarized in the Table of Fig. 2B. The temperature-sensitivity of these *byr4* mutations is shown in Fig. 2A. These mutant cells made colonies at 25°C but did not grow at 35°C, while the control wild type strain KGY246 made colonies both at 25°C and 35°C. The proper integration of pBKS1 in the *byr4* locus of these five temperature-sensitive *byr4* mutations was verified by genomic southern analyses. As deduced from the map in Fig. 1B, an extra 2 kb fragment

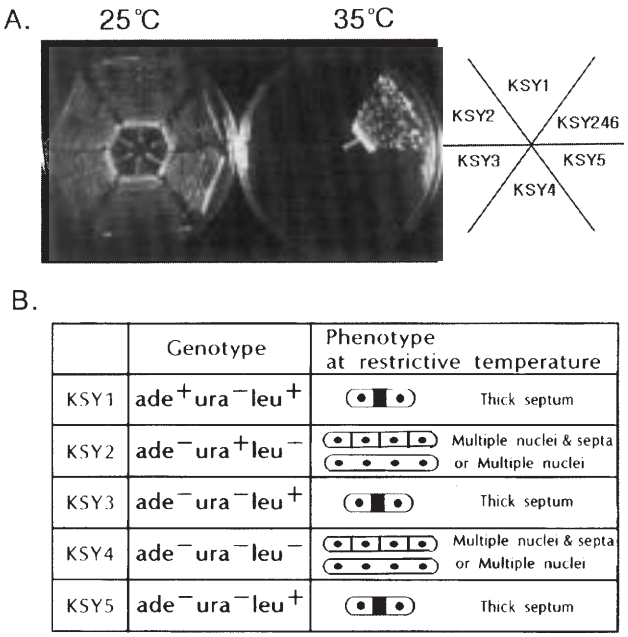

Fig. 2. Characterization of the *byr4* conditional mutants. A; Temperature-sensitivity of the *byr4* conditional mutants. Cells from the *byr4* conditional alleles were streaked on YE^{AU} plates and cultured at 25°C and at 35°C, respectively. These cells form normal colonies at the permissive temperature, 25°C, but can not form colonies at the restrictive temperature, 35°C. B; A table shows each conditional allele with genetic markers and its schematic arrested phenotypes at the restrictive temperature.

was detected with the *byr4* probe from *Ssp*I digested genomic DNA when pBKS1 was properly integrated into the *byr4* locus (Fig. 1C). The genomic southern showed that all of these mutants contain the extra 2 kb fragment, confirming the correct insertion of the mutagenized *byr4* into the genomic *byr4* locus. Linkage of the *byr4* mutations to the *sup3-5* gene and the genomic southerns of these mutations strongly suggested that the temperature-sensitive phenotypes were due to a mutation in *byr4*.

We determined whether the isolated conditional mutant alleles were dominant or recessive. First, as mentioned above, we crossed the isolated conditional mutants with the wild type KGY246 to make diploids and examined whether the conditional mutant alleles were complemented by the wild type *byr4* allele. If not reverted, the diploids from the cross of the mutants with the wild type KGY 246 showed temperature-dependent mutant phenotypes. We also examined whether the temperature-sensitivity of these mutant cells was complemented by the wild type *byr4* in the plasmid. When the mutant cells were transformed with the wild type *byr4* in the plasmid and incubated in the restrictive temperature, 35°C, none of the mutant cells form colo-

nies at 35°C (data not shown). Failure of the complementation by the wild type *byr4* in the cross or in the plasmid suggested that these five *byr4* mutations are dominant and could not be suppressed by the presence of wild type *byr4* gene (data not shown).

Phenotypic analyses of the temperature-sensitive *byr4* mutant alleles

Conditional-lethal mutations of the regulatory genes for cell division cycle block a specific stage of the cell cycle at the non-permissive temperature, leading to the arrested cells with uniform terminal phenotypes (Hartwell *et al.*, 1973). We determined the arrested phenotypes of these five temperature-sensitive *byr4* mutants, after cells were incubated for 12 hours at the restrictive temperature. The cells from *byr4-KSY1, KSY3* and *KSY5* showed the same terminal phenotypes. These cells were arrested in late mitosis with thick septa in the restrictive temperature, suggesting that they proceeded multiple septation (Fig. 3A). As visualized by DAPI staining, nuclear DNA was segregated normally into daughter-to-be cells. However, daughter-to-be cells were arrested with multiple thick septa that were not divided. These phenotypes propose that these cells were arrested in cytokinesis and septation after the nuclear division. These arrested phenotypes are different from that of the *byr4* null alleles which cause cell cycle arrest in late mitosis with multiple septa as well as unequal DNA segregation. The *byr4-KSY2* and *KSY4* mutants exhibited the same terminal phenotypes in the restrictive temperature. As shown in Fig. 3B and C, the arrested cells showed two different phenotypes. Nearly half of the cells were elongated and they contained multiple nuclei due to inhibition of cytokinesis as in the *byr4* overexpressed cells, while the other half became elongated with multiple nuclei but each nucleus was separated by undivided septum. Taken together, the newly isolated *byr4* conditional mutants displayed the arrested phenotypes that are consistent with its predicted function but are slightly different from its knock-out or overexpression phenotypes.

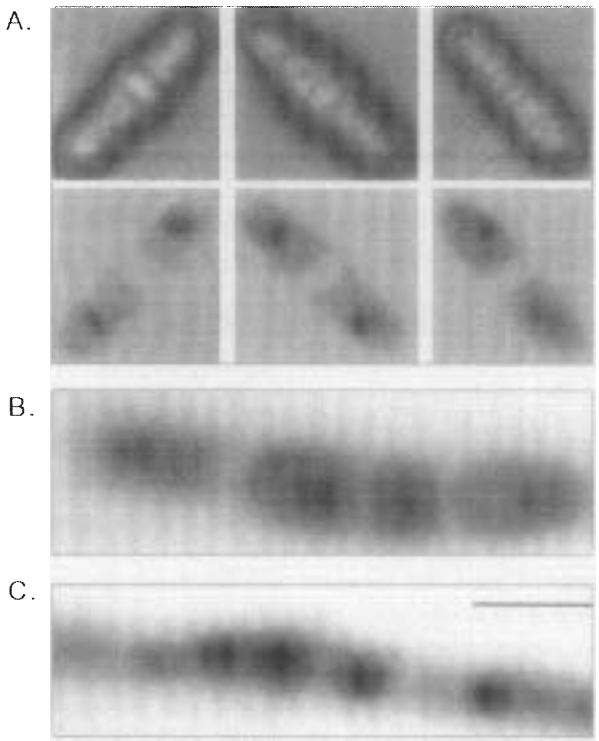

Fig. 3. Arrested phenotypes of the temperature-sensitive *byr4* mutants at the restrictive temperature. Cells incubated at the restrictive temperature, 35°C, for more than 12 hrs were fixed, and examined by phase contrast or by fluorescence microscopy after staining with DAPI. The phenotypes are assigned into 2 groups. (A) *KSY1, KSY3* and *KSY5* alleles showed thick septum. The top panels show phase contrast images and the bottoms show DNA images of the same field by DAPI staining. (B and C) *KSY2* and *KSY4* showed two marked phenotypes: (B) Some cells have multi-nuclei, and each nucleus is separated by septum. (C) The others have multi-nuclei without any septum. Bar denotes 10 μm and applies to all pictures.

Discussion

Genetic analysis provides powerful approaches to study biological processes. *S. pombe* is an excellent system to study the coordination of mitosis and cytokinesis, since we can approach the regulation of cytokinesis with the tools of classical and molecular genetics. To understand the functional mechanisms of *byr4* for the regulation of cytokinesis, we isolated five temperature-sensitive conditional-lethal alleles of *byr4* in this study. We started with the cloned *byr 4* and, through *in vitro* mutagenesis and genomic integration, generated *byr4* mutant strains. Hydroxylamine was used to randomly generate an assortment of mutations throughout the coding sequence of *byr4*. Hydroxylamine is known to react with a double-stranded target DNA to create N^4-hydroxycytosine that can pair with adenosine, and results in both C to T and G to A transition mutations (Busby *et al.*, 1982). Since hydroxylamine randomly mutagenize DNA, the level of mutagenesis of the *byr4* containing plasmid was determined by the percentage of cells that lost the ampicillin resistance. The mutagenized *byr4* with an N-terminal truncation was integrated into the genomic *byr4* locus by homologous recombination. Since the integrated *byr4* was truncated with the N-terminal 29 amino acids, the

mutagenized full-length *byr4* was generated and the wild type genomic *byr4* was destroyed (Fig. 1A). In *S. pombe*, linearized non-*ars* plasmids are strongly preferred in integrative tranformation and homologous recombination (Grimm and Kohli, 1988). To make the integration procedures favorable, we used pBSK as a non-*ars* integrating plasmid and the *sup3-5* that suppresses the *ade6-704* mutation as a selectable marker. Since maintenance of the *sup3-5*-containing plasmid as an episome is deleterious to growth, it leads to integration of the plasmid (Hottinger *et al.*, 1982; Moreno *et al.*, 1991).

The method used in this study to make the conditional alleles of *byr4* was first developed by Shortle *et al.* in *S. cerevisiae* and was not frequently applied in *S. pombe*. Rather, plasmid shuffling is generally used to generate conditional mutants from cloned genes in *S. pombe*. However, since presence of the *byr4* as multiple copies in plasmids is lethal to cells and showed the overexpression phenotypes, plasmid shuffling could not be used to generate the conditional *byr4* mutants. We succeeded making five *byr4* conditional alleles by integrative transformation as described, but several problems were encountered. First, these mutants were unstable and the mutation locus was reverted to the wild type in an approximately 1/50 frequency, depending on the allele. Revertants were observed when these mutants were crossed with the wild type KGY246 to put the auxotropic markers. In the process of mating and sporulation, we lost the only cold-sensitive allele isolated. It would have been better if we could have used an *ade6-704* strain with auxotropic markers for pBKS1 transformantion instead of putting markers after screening the mutations. Possibly, the revert of these mutant alleles into the wild type might cause difficulties in further usages of these alleles, including suppressor screens or crosses with other mutants. In that case, these mutant alleles of *byr4* head to be recovered by PCR, and to be cloned into a targeting vector to replace the wild type genomic copy of *byr4* with these conditional alleles. Second, all the isolated *byr4* conditional alleles were not complemented by the wild type *byr4*, suggesting that these alleles are dominant mutations. A possible reason that we could isolate only dominant conditionals may be that the truncated wild type *byr4* generated by integrated transformation makes partially functional protein, and hence only dominant mutations were selected during the *byr4* conditional screen. By deletion studies, we know that the Byr4 protein deleted with the N-terminal 29 amino acid is fully functional. However, since the truncated *byr4* generated by the integrated transformation does not have any proper promoter, expression of the truncated protein is not highly plausible. Since we do not have a tool to selectively detect the truncated Byr4 protein, we can not verify whether the truncated wild type Byr4 protein is generated or not.

The arrested phenotypes of these newly isolated *byr4* conditional alleles in the restrictive temperature can be specified into two groups. One group includes the *byr4-KSY1, KSY3*, and *KSY5* alleles in which cells were arrested in late mitosis with thick septa. The chromosome segregation looks normal in these cells. These arrested phenotypes propose that the cells must have undergone normal nuclear segregation but they have defects in cytokinesis and septation. These phenotypes are different from that of the *byr4* null allele in which cells are arrested in late mitosis with unequal DNA segregation as well as multiple septation. Comparison of the phenotypes for the *byr4* null allele with these conditional alleles suggest that there are at least two separate steps in the cell cycle where wild type *byr 4* function is needed. First, *byr4* is needed to control the timing of cytokinesis and, second, *byr4* is required to regulate the septation and separation of daughter cells. Both *byr4* functions are blocked in the *byr4* null allele leading to unequal DNA segregation and multiple septation, while only the *byr4* function for septation is blocked in these conditional alleles. Isolation of the *byr4-KSY1, KSY3*, and *KSY5* conditional alleles supports the fact that the separable functions of *byr4* control cytokinesis and septation.

The *pyr4-KSY2* and *KSY4* alleles exhibited two different arrested phenotypes in the restrictive temperature as described in the Results. These cells were arrested either with multiple nuclei caused by the inhibition of cytokinesis or with multiple nuclei that were separated by septum. These arrested phenotypes suggest that these alleles blocked cytokinesis or cell separation, but cell cycle progression continued leading to multinuclear cells. The cells arrested with multiple nuclei that are separated by septum might have proceeded not only nuclear divisions but also cytokinesis and septation, although the cells were not separated. Presence of the two arrested phenotypes in these alleles is possibly due to different degree of the increased activity or different degree of the expression of the conditional *byr4* alleles in each cell. Further characterization of these alleles including detection of the nucleotide sequence changes in these alleles will give the clue for the presence of two arrested phenotypes in the cells.

The newly isolated *byr4* conditional mutants displayed the arrested phenotypes that are consistent with the function of *byr4*: regulation of cytokinesis and septation. However, the phenotypes of these

byr4 conditional alleles are slightly different from the phenotypes of its knock-out or overexpression as described, which help to deduce the separate cellular processes where *byr4* is necessary. These new *byr4* conditional alleles can provide useful tools for determining the functional mechanisms of *byr4* through genetic studies and suppressor screens.

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