microPublication BIOLOGY

11/23/2026 - *Open Access*

Characterization of New Alleles of *byr4*, an Essential Gene that Encodes a GAP Component for Regulated Cell Division

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Abstract

Schizosaccharomyces pombe Byr4 is an essential component of the bipartite GTPase Activating Protein (GAP) for the Spg1 GTPase that regulates the septation initiation network (SIN). When *byr4* function is lost, Spg1 becomes hyperactive and causes lethal uncontrolled septation. Here, we report the isolation and characterization of two *byr4* temperature-sensitive mutants. One *byr4* mutant phenocopies mutants in the second GAP component, Cdc16, and can be used interchangeably for cytokinesis studies. The behavior of the second mutant suggests that Byr4 not only impacts Cdc16 function but also the positive SIN regulator Cdc7, highlighting gaps in our current understanding of SIN component interplay.

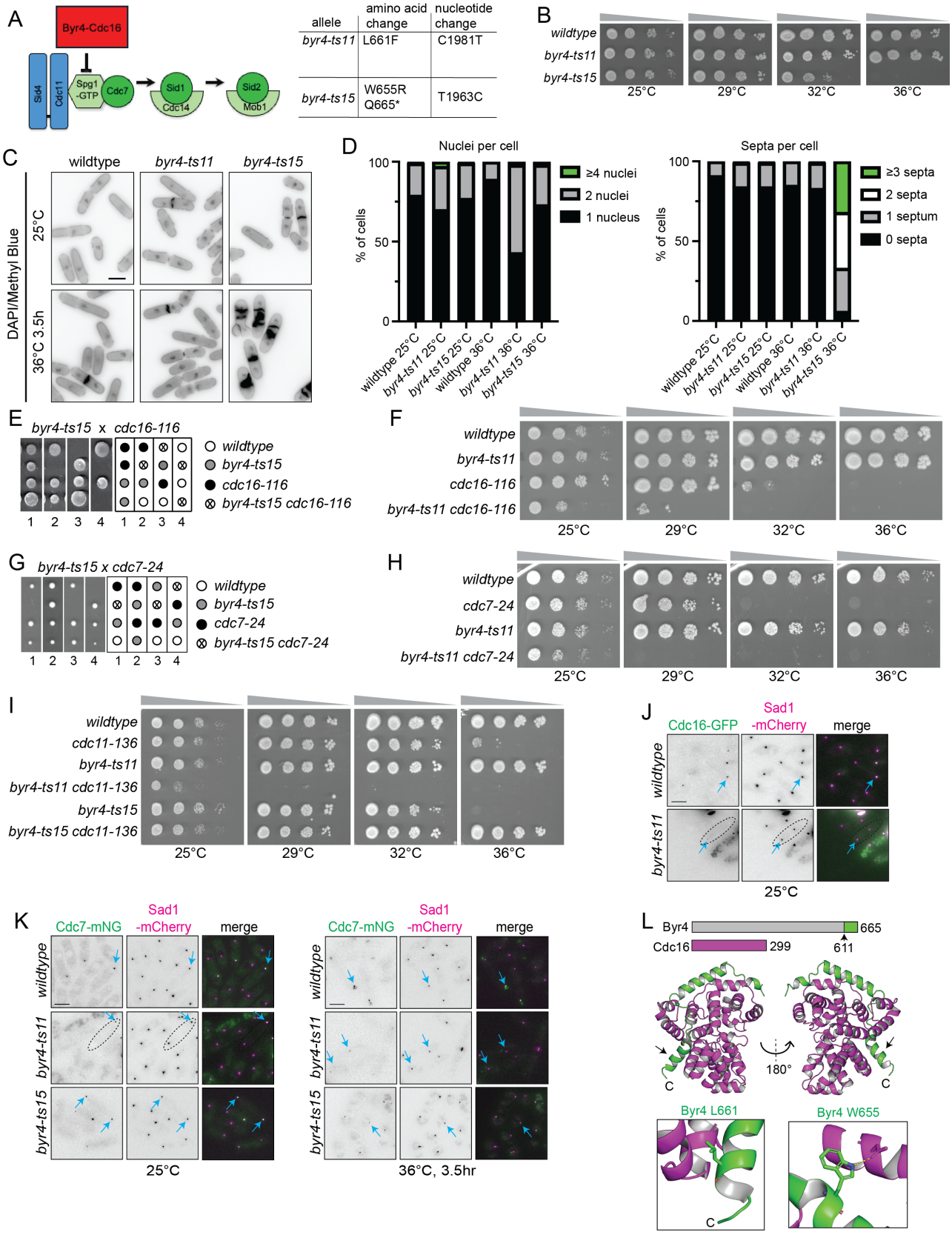

Figure 1. Characterization and comparison of *byr4* alleles:

(A) A schematic of the SIN pathway adapted from Johnson et al., 2012 (left) and the mutations encoded by each *byr4* allele (right). (B, F, H, and I) The indicated strains were grown in liquid YE media at 25°C until they reached mid-log phase and then adjusted to A_{595} = ~0.25. Next, 10-fold serial dilutions were made and 3 µL of each was spotted on YE agar plates and incubated at the indicated temperatures for 3-5 days. (C) The indicated strains were grown at 25˚C and shifted to 36˚C for 3.5 hours. Samples were collected at both temperatures and cells were fixed and stained with DAPI and

Methyl Blue prior to imaging. Scale bar, 5 µm. (D) The number of nuclei per cell (left), and the number of septa per cell (right) were quantified from the same experiments as in C. n ≥ 212 and n ≥ 207 cells for the nuclei and septa of each sample, respectively. (E and G) Four representative tetrads grown on YE plates at 25˚C from the indicated cross with schematics of the relevant genotypes. (J) Representative live-cell images of indicated strains grown at 25˚C. Scale bar, 5 µm. Blue arrows point to representative SPBs. (K) Representative live-cell images of the indicated strains grown at 25˚C (left panels) and then shifted to 36˚C for 3.5 hours (right panels). Scale bar, 5 µm. Blue arrows point to representative SPBs. (L) Schematics of Cdc16 and Byr4 proteins drawn to scale (top). Two views of a ribbon diagram of a structural model of *S. pombe* Cdc16 (magenta) bound to the C-terminus of Byr4 (green) (middle). The predicted Byr4 C-terminal helix that interacts with Cdc16 and contains the mutated residues is indicated with black arrows. The inserts below highlight the residues mutated in the *byr4* alleles. Whereas W655, mutated in *byr4-ts15*, is predicted to be involved in an interaction with a Cdc16 residue, L661, mutated in *byr4-ts11*, is not.

Description

In *Schizosaccharomyces pombe*, a signaling pathway called the septation initiation network (SIN) regulates the assembly and constriction of a cytokinetic ring (CR), as well as the coupled process of septation (reviewed in (Johnson et al., 2012; Simanis, 2015). Both deactivation and constitutive activation of the SIN are lethal. Without SIN function, *S. pombe* cells become multinucleate due to the absence of septation and cell division following mitosis and eventually lyse. Conversely, SIN hyperactivity causes the formation of multiple CRs and septa uncoupled from the nuclear division cycle.

The SIN is assembled at spindle pole bodies (SPBs) on a scaffold composed of Sid4-Cdc11 in a cell cycle-dependent manner (reviewed in (Johnson et al., 2012; Simanis, 2015) (see Figure 1A, left). SIN signaling begins at the onset of mitosis and spindle formation when the GTPase, Spg1, becomes activated at separating SPBs and recruits its effector kinase, Cdc7 (Schmidt et al., 1997; Sohrmann et al., 1998; Wachowicz et al., 2015). As anaphase proceeds, Spg1 is inactivated at one SPB with consequent loss of Cdc7 at that SPB (Grallert et al., 2004; Schmidt et al., 1997) and an increase in Cdc7 abundance at the SPB that retains active Spg1 (Garcia-Cortes and McCollum, 2009; Sohrmann et al., 1998).

Spg1 signaling is opposed by an unusual GTPase-activating protein (GAP) comprised of two essential proteins, Byr4 and Cdc16 (Furge et al., 1998) (see Figure 1A, left). Temperature-sensitive mutants of *cdc16*, such as *cdc16-116*, have been widely used to hyperactivate the SIN; at their restrictive temperature, cells septate uncontrollably regardless of cell cycle stage (Minet et al., 1979; Turner et al., 2024). While the *byr4* null mutant phenocopies loss of Cdc16 (Song et al., 1996), conditional *byr4* mutants have not been reported. Here, we describe the isolation and characterization of *byr4* temperature-sensitive mutants, expanding the repertoire of reagents that are available to study the SIN, a conserved signaling pathway (Xiao and Dong, 2021).

Mutants of Byr4 were obtained using a marker reconstitution approach coupled with random mutagenesis as previously described and selected based on resistance to G418 in the agar plates (Tang et al., 2011). Two *byr4* mutants were isolated: *byr4-ts11* and *byr4-ts15*. The mutations within the *byr4* alleles were identified via whole genome sequencing. *byr4-ts11* contains a mutation that substituted leucine 661 with phenylalanine and *byr4-ts15* contains both a tryptophan 655 to arginine substitution and a premature stop codon in place of the last amino acid, Q665 (Figure 1A). The affected residues are within the last ten residues of Byr4, a region involved in the interaction with Cdc16 (Furge et al., 1999).

Serial dilution growth assays revealed that *byr4-ts11* formed colonies similarly to wildtype at all temperatures tested, while *byr4-ts15* grew less well than wildtype at 32°C and was unable to form colonies at 36°C (Figure 1B). To further characterize these strains, cells were grown in YE at 25°C and shifted to 36°C for 3.5 hours. Samples, taken at both temperatures, were fixed, stained, and imaged. At 25˚C and 36˚C, *byr4-ts11* cells had more nuclei per cell than wildtype, suggesting a mild SIN defect (Figure 1C-D). In contrast, *byr4-ts15* cells were morphologically similar to wildtype at 25˚C and displayed a predominantly multiseptated phenotype at 36°C, suggestive of a hyperactive SIN (Figure 1C-D). Consistent with hyperactivity of the SIN in *byr4-ts15* cells, we found that *byr4-ts15* was synthetically lethal with *cdc16-116* (Figure 1E). Although viable, *byr4-ts11* showed a significant negative genetic interaction with *cdc16-116*, indicative of compromised *byr4* function (Figure 1F).

cdc16-116 is synthetically lethal with *cdc7-24* from 19˚C to 36˚C (Marks et al., 1992), possibly because either Cdc7 or Byr4-Cdc16 is required for Spg1 SPB localization (Krapp et al., 2008). In contrast to the negative genetic interactions with *cdc7* mutants, *cdc16-116* suppresses *cdc11-136* (Marks et al., 1992), as expected if a negative SIN regulator is lost. We examined if the *byr4* mutants had similar genetic interactions. Like *cdc16-116*, *byr4-ts15* was synthetically lethal with *cdc7-24* at 25˚C (Figure 1G), and rescued *cdc11-136* at 36˚C (Figure 1I) on YE plates. *byr4-ts11* was not the same. Consistent with its mild SIN phenotype at 36˚C, the basis by which it retains its ts nomenclature, it showed negative genetic interactions with both *cdc7-24* (Figure 1H) and *cdc11-136* (Figure 1I).

We next tried to examine whether the localization of Cdc16 was affected by the mutations in *byr4*. *byr4-ts15* was synthetically lethal with all tagged versions of Cdc16 we tried. While we were able to recover *byr4-ts11 cdc16-GFP* cells

on YE plates at 25˚C, in liquid YE media, they were morphologically abnormal and Cdc16-GFP was only rarely detected at SPBs (Figure 1J). Thus, it is likely that both mutations within *byr4* impaired the ability of Cdc16 to localize to SPBs.

We also examined the localization of Cdc7-mNG in the *byr4* mutants. In *byr4-ts15* cells, Cdc7-mNG was detected asymmetrically during anaphase at 25˚C and at 36˚C, it was detected on most SPBs (Figure 1K), a localization pattern also observed in *cdc16-116* cells (Sohrmann et al., 1998). In contrast, in *byr4-ts11* cells at 36˚C, Cdc7-mNG was difficult to detect at SPBs (Figure 1K, right panels), a result that is also consistent with the mild SIN phenotype of *byr4-ts11* cells at this temperature.

Overall, these findings indicate that the *byr4-ts15* mutant can be used interchangeably with *cdc16* mutants to remove Spg1 GAP activity when that is desired. The combination of genetic and imaging results indicate that the *byr4-ts11* mutant has more complex defects. Overall, the behavior of this mutant is consistent with the findings that a reduction in SIN function caused by mutations in either *cdc7* or *mob1* can rescue the lethality of *cdc16* deletion (Fournier et al., 2001; Salimova et al., 2000), and implicate the Byr4 C-terminus not only in interacting with Cdc16 but also a positive SIN component, the identity of which is yet unknown. This conclusion underscores that gaps remain in our understanding of the spatial and regulatory interplay between SIN components at SPBs that deserve further study.

Methods

Yeast Methods

S. pombe strains were grown in yeast extract (YE) medium (Moreno et al., 1991). Strain construction and tetrad analyses were accomplished through standard methods (Forsburg and Rhind, 2006). YE plates at 36°C, YE-hygromycin B, and YE-G418 plates (Geneticin, 100 µg/mL, Thermo Fisher Scientific cat# 11811031; hygromycin B, 125 µg/mL, Gibco cat# 10687010) were used for selection of strains when appropriate. For growth assays, strains were grown overnight in YE at 25°C to A_{595}=0.3-0.6. Cells were then adjusted to A_{595} = 0.25 and a series of 10-fold dilutions were made. 3 µL of each dilution were spotted onto YE plates and incubated at a variety of temperatures for 3-5 days. Each growth assay was performed twice.

Tagged strains were generated by adding sequences encoding mNeonGreen and resistance cassette *HpHMX6* at the 3' end of the endogenous *cdc7* open reading frame using a pFA6 cassette as previously described (Bahler et al., 1998). Successful tagging of strains was verified by whole cell PCR.

Identification of *byr4* mutations

A 10 mL YE culture of each strain was grown to saturation overnight in a 25°C dry shaker. 8-10 optical density (A_{595}) samples of each strain were collected by centrifugation and rinsed once in sterile PBS buffer. Supernatants were removed by aspiration and pellets were resuspended in 500 µL of DNA/RNA shield (ZYMO Research). The samples were then sent to be sequenced by Plasmidsaurus (Eugene, OR).

Oxford Nanopore whole-genome reads from mutant and control (wild type) samples were quality assessed with NanoComp (De Coster et al., 2018) and normalized to matched sequencing depth by random downsampling with seqtk (Shen et al., 2024) to the lowest observed coverage. Reads were aligned to the *Schizosaccharomyces pombe* PomBase all-chromosomes reference genome using minimap2 (-ax lr:hq) (Li, 2018), then coordinate-sorted and indexed with SAMtools (Li et al., 2009). Small variants were called independently per sample using Clair3 in ONT mode with the dna_r10.4.1_e8.2_400bps_sup@v5.2.0 model, configured to output gVCFs for all contigs with intermediate long-read phasing enabled (Zheng et al., 2022). Sample gVCFs were jointly genotyped with GLnexus (Yun et al., 2021) using a Clair3-specific configuration and processed with BCFtools (Danecek et al., 2021). Candidate mutant-specific variants were defined as homozygous alternate in the mutant and homozygous reference in wild type, with depth ≥10 and genotype quality ≥10 in both samples. A broader review set additionally retained mutant homozygous-alternate sites for which the wild-type genotype was missing. Candidates were annotated to the nearest PomBase GFF3 gene feature and visually reviewed in IGV (Robinson et al., 2011).

Microscopy methods

To visualize nuclei and septa, cells were grown overnight in YE to log phase at 25°C, shifted or not to 36.5°C for 3.5 hours, and fixed in ice-cold 70% ethanol. Samples were then washed in phosphate-buffered saline three times and stained with 1 mg/mL Methyl Blue (Millipore Sigma) and 4',6-diamidino-2-phenylindole (DAPI). A single Z-slice was obtained using a Zeiss Axio Observer inverted epifluorescence microscope with an AxioCam 503 mono camera and a Zeiss Plan Apochromat 63x oil (1.46 nA) objective. Representative images were formatted using ImageJ (Schindelin et al., 2012).

For live cell imaging, strains were grown at 25°C in YE overnight to mid-log phase prior to shifting to 36°C for 3.5 hours. Live-cell representative images were deconvolved and projected with maximum intensity. All imaging experiments were repeated twice.

AlphaFold3 structural prediction

Protein structure predictions were generated with the AlphaFold3 server (Abramson et al., 2024) and visualized using the PyMOL molecular graphics system (version 3.0, Schrodinger, LLC).

Reagents

Strain	Genotype	Source
KGY184	*cdc16-116 h*$^{-}$	Lab stock
KGY246	*ade6-M210 leu1-32 ura4-D18 h*$^{-}$	Lab stock
KGY694	*cdc16-116 leu1-32 h*$^{+}$	Lab stock
KGY735-3	*byr4-ts11:kanMX6 cdc7-mNG:hphMX6 sad1-mCherry:natMX6 ade6-M210 leu1-32 ura4-D18 h*$^{+}$	This study
KGY802-3	*byr4-ts15:kanMX6 cdc7-mNG:hphMX6 sad1-mCherryMX6 natR ade6-M210 leu1-32 ura4-D18 h*$^{-}$	This study
KGY1120-3	*byr4-ts11:kanMX6 cdc7-24 ade6-M21X leu1-32 ura4-D18 h*$^{-}$	This study
KGY1121-3	*byr4-ts11:kanMX6 cdc11-136 ade6-M210 leu1-32 ura4-D18 h*$^{-}$	This study
KGY1137-3	*byr4-ts15:kanMX6 cdc11-136 ade6-M210 leu1-32 ura4-D18 h*$^{-}$	This study
KGY11610	*byr4-ts15:kanMX6 ade6-M210 leu1-32 ura4-D18 h*$^{-}$	This study
KGY11617-2	*byr4-ts11:kanMX6 ade6-M210 leu1-32 ura4-D18 h*$^{+}$	This study
KGY1986-2	*byr4-ts11:kanMX6 cdc16-116 ade6-M210 leu1-32 ura4-D18 h*$^{?}$	This study
KGY6188	*cdc11-136 ade6-M210 leu1-32 ura4-D18 h*$^{-}$	Lab stock
KGY6628	*cdc7-24 ade6-M21X leu1-32 ura4-D18 h*$^{-}$	Lab stock
KGY2764-2	*byr4-ts11:kanMX6 cdc16-linkerGFP:kanMX6 sad1-mCherry:natMX6 ade6-M210 ura4-D18 leu1-32 h*$^{+}$	This study

Acknowledgements: The *byr4* mutants were isolated by Tang Xie, when he was a research fellow in the Balasubramanian laboratory as part of a larger marker-reconstitution mutagenesis project. We are grateful to Pombase for all of the annotation that provides such rapid access to pertinent gene, protein, and interaction data.

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Funding: This work was supported by NIH grant R35GM131799 to K.L.G.

Conflicts of Interest: The authors declare that there are no conflicts of interest present.

Author Contributions: Lillias Tureau: formal analysis, investigation, visualization, writing - review editing. Lesley A. Turner: investigation, visualization, formal analysis, supervision, writing - review editing, validation, writing - original draft. Caroline M. Duncan: formal analysis, investigation, visualization, writing - review editing. Alaina H. Willet: data curation, writing - review editing, validation, visualization, supervision. Mohan K. Balasubramanian: conceptualization, project administration, resources, supervision, writing - review editing. Jean-Philippe Cartailler: formal analysis, investigation, methodology, writing - review editing. Kathleen L. Gould: conceptualization, funding acquisition, project administration, resources, supervision, validation, writing - original draft.

Reviewed By: Anonymous

Nomenclature Validated By: Anonymous

History: Received November 8, 2026 **Revision Received** November 17, 2026 **Accepted** November 23, 2026 **Published Online** November 23, 2026 **Indexed** December 7, 2026

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Citation: Tureau L, Turner LA, Duncan CM, Willet AH, Balasubramanian MK, Cartailler JP, Gould KL. 2026. Characterization of New Alleles of *byr4*, an Essential Gene that Encodes a GAP Component for Regulated Cell Division. microPublication Biology. 10.17912/micropub.biology.002514