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Investigation

Perturbation of kinetochore function using GFP-binding protein in fission yeast

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Abstract

Using genetic mutations to study protein functions *in vivo* is a central paradigm of modern biology. Single-domain camelid antibodies generated against GFP have been engineered as nanobodies or GFP-binding proteins (GBPs) that can bind GFP as well as some GFP variants with high affinity and selectivity. In this study, we have used GBP-mCherry fusion protein as a tool to perturb the natural functions of a few kinetochore proteins in the fission yeast *Schizosaccharomyces pombe*. We found that cells simultaneously expressing GBP-mCherry and the GFP-tagged inner kinetochore protein Cnp1 are sensitive to high temperature and microtubule drug thiabendazole (TBZ). In addition, kinetochore-targeted GBP-mCherry by a few major kinetochore proteins with GFP tags causes defects in faithful chromosome segregation. Thus, this setting compromises the functions of kinetochores and renders cells to behave like conditional mutants. Our study highlights the potential of using GBP as a general tool to perturb the function of some GFP-tagged proteins *in vivo* with the objective of understanding their functional relevance to certain physiological processes, not only in yeasts, but also potentially in other model systems.

Keywords: fission yeast (*Schizosaccharomyces pombe*); kinetochore protein; GFP-binding protein (GBP)

Introduction

Kinetochores are one of the largest and most functionally intricate molecular machines, which provide the fundamental link between chromosomal domains termed centromeres and spindle microtubules in all eukaryotic cells (Westermann and Schleiffer 2013). This link is essential for precise chromosome segregation during cell division and thus ensures that DNA is correctly transmitted from one generation to the next. Kinetochores fulfill these key functions by attaching and orienting sister chromatids to spindle microtubules and recruiting spindle assembly checkpoint (SAC) components when the tension or occupancy resulting from chromatid-microtubule attachment is absent, which induces a mitotic delay (London and Biggins 2014b). As multiprotein assemblies, the biochemical composition of kinetochores is highly conserved in evolution from yeast to humans, although the underlying centromere DNA sequences they bind are considerably diverse in different organisms (Meraldi *et al.* 2006; Westermann and Schleiffer 2013). The number of the kinetochore proteins examined to date is at least 100, among them approximately 30 are core structural components, while others are transiently residing units and only play accessory or regulatory roles (Westermann *et al.* 2007; Fukagawa and De Wulf 2009; Perpelescu and Fukagawa 2011; Takeuchi and Fukagawa 2012).

A hallmark of centromeric chromatin in all eukaryotes is the presence of nucleosomes that contain the essential H3 variant CENP-A (named as Cnp1 in the fission yeast *Schizosaccharomyces pombe*), which replaces canonical H3 in nucleosomes that wrap centromeric DNA (De Rop *et al.* 2012; Westhorpe and Straight 2013; Fukagawa and Earnshaw 2014). The kinetochore is often conceptually divided into the inner kinetochore and the outer kinetochore based on their positioning relative to centromeric DNA or chromatin. In vertebrates, the inner kinetochore consists of roughly 17 additional CENPs proteins that bind centromeric chromatin, these proteins are collectively known as the constitutive centromere-associated network (CCAN) (Foltz *et al.* 2006; Izuta *et al.* 2006; Okada *et al.* 2006; Hori *et al.* 2008). Analogously, most of these proteins are conserved in *S. pombe* and form the Mis6-Sim4 complex (comprising mainly Mis6/CENP-I, Sim4/CENP-K, Mis15/CENP-N, Mis17/CENP-U, Mal2/CENP-O, and Dad1) (Takahashi *et al.* 2000; Jin *et al.* 2002; Pidoux *et al.* 2003; Hayashi *et al.* 2004; Liu *et al.* 2005; Fukagawa and De Wulf 2009). In vertebrates, a ten-subunit assembly known as the KMN network (comprising KNL1, MIS12, and NDC80 complexes, designated KNL1C, MIS12C, and NDC80C, respectively) establish the outer kinetochore, which is responsible for direct binding to microtubules (Cheeseman *et al.* 2006; DeLuca *et al.* 2006; Petrovic *et al.* 2016; Weir *et al.* 2016; Pesenti *et al.* 2018). A similar complex called NMS (named after its components Ndc80 complex, Mis12 complex, and human KNL1 homolog Spc7) is also present in fission yeast (Liu *et al.* 2005). The connection between the inner and outer kinetochore is mediated by at least two recruiters, CENP-C and

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CENP-T (Cnp3 and Cnp20, respectively, in *S. pombe*), which bind to the Mis12 complex and Ndc80 complex, respectively (Hori *et al.* 2008; Gascoigne *et al.* 2011; Schleiffer *et al.* 2012; Nishino *et al.* 2013; Petrovic *et al.* 2016).

Historically, a major discovery approach for kinetochore proteins has taken advantage of the power of random mutagenesis-based forward genetic screening, followed by genetic mapping and gene cloning in both budding and fission yeast. For example, *cut* (cell untimely torn), *dis* (defective in sister chromatid disjoining), *mal* (minichromosome altered loss), *mis* (minichromosome stability), *mlo* (missegregation and lethal when overexpressed), and *nda* (nuclear division arrest) mutants were generated in 1980s and 1990s from screens for defective chromosome transmission in *S. pombe* (Toda *et al.* 1983; Ohkura *et al.* 1988; Hagan and Yanagida 1990; Takahashi *et al.* 1994; Fleig *et al.* 1996; Javerzat *et al.* 1996). Because almost all core structural kinetochore protein-encoding genes are essential genes in fission yeast, these kinetochore protein mutants are mostly temperature-sensitive or cold-sensitive and thus render compromised kinetochore function at restrictive temperatures. And subsequent characterization of these mutants greatly facilitated the identification of yeast kinetochore core subunits.

GFP-binding protein (GBP) is a 13-kDa soluble protein derived from a llama heavy chain antibody, which features with high binding affinity to GFP as well as to some GFP variants (Hamers-Casterman *et al.* 1993; Rothbauer *et al.* 2006, 2008; Kubala *et al.* 2010). Therefore, GBP has been rapidly applied in purification of protein complexes with GFP tags and protein targeting and various manipulations *in vivo* through GFP-tagged proteins in cultured mammalian cells and various model organisms in recent years (reviewed in Chen *et al.* 2017; Aguilar *et al.* 2019; Prole and Taylor 2019). Usually, forward genetic screenings for both systematic and individual gene-focused mutants in fission yeast are labor-intensive and time-consuming. Here, we describe the construction of a series of core structural kinetochore protein mutants in fission yeast by simply simultaneously expressing GFP-tagged kinetochore proteins and mCherry-tagged GBP.

Materials and methods

Fission yeast media, strains, and genetic analyses

Standard media [either YE (yeast extract) rich medium or EMM (Edinburgh minimal medium)] and culturing methods were used (Moreno *et al.* 1991; Forsburg and Rhind 2006). G418 disulfate (Sigma-Aldrich), hygromycin B (Sangon Biotech), or nourseothiricin (clonNAT; Werner BioAgents) was used at a final concentration of 100 μg/ml and thiabendazole (TBZ) (Sigma-Aldrich) at 5–15 μg/ml in YE media. For serial dilution spot assays, 10-fold dilutions of a mid-log-phase culture were plated on the indicated media and grown for 3–5 days at indicated temperatures. To examine the possible synthetic lethality (SL) of genetic combinations between alleles of GBP-mCherry, GFP-tagged kinetochore proteins, and spindle checkpoint mutants *mad2*Δ or *bub1*Δ, normal-looking 4-spore asci obtained after crosses between parental strains were dissected using a micromanipulator. At least 20 complete tetrads were dissected after each genetic cross, and the genotypes of colonies formed from germinated spores were deduced after being replicated on selective plates. The frequency of spores with expected genotypes failing to germinate was quantified, it was classified as SL, strong growth defect, or normal growth when the frequency was above 80%, between 20% and 80%, or below 20%, respectively. Yeast strains used and created in this study are listed in Supplementary Table S1.

Plasmid and yeast strain construction

To create strains expressing GBP-mCherry-2xNLS, 2xGBP-mCherry-2xNLS, or GBP-mCherry-9myc-TEV-2xNLS, two tandem SV40 nuclear-localization signal (NLS) (CCT AAG AAA AAA CGA AAA GTT GAG GAT CCT AAA AAG AAA CGA AAA GTT GAT) sequences were first introduced into the vector pUC119-P_{adh11}-GBP-mCherry-*hphMX6-lys1** by Quikgene method as previously described (Chen *et al.* 2017). The coding sequences of GBP-mCherry or 9myc-TEV were PCR amplified using vector pUC119-P_{adh11}-GBP-mCherry-*hphMX6-lys1** or pKANZA21-CFP-9myc-TEV (Sun *et al.* 2020) as template. Then, sequences corresponding to one extra copy of GBP-mCherry or 9myc-TEV were cloned into the vector pUC119-P_{adh11}-GBP-mCherry-2xNLS-*hphMX6-lys1** using the "T-type" enzyme-free cloning method as previously described (Chen *et al.* 2017). This resulted in pUC119-P_{adh11}-GBP-mCherry-GBP-mCherry-2xNLS-*hphMX6-lys1** and pUC119-P_{adh11}-GBP-mCherry-9myc-TEV-2xNLS-*hphMX6-lys1**. The promoter sequence in the latter plasmid was mutagenized to P_{adh1} by standard mutagenesis procedures to produce pUC119-P_{adh1}-GBP-mCherry-9myc-TEV-2xNLS-*hphMX6-lys1**. The resultant plasmids were linearized by *Apa*I and integrated into the *lys1* locus, generating the strains *lys1*Δ::P_{adh11}-GBP-*mCherry*::*hphMX6*, *lys1*Δ::P_{adh11}-GBP-*mCherry-2xNLS*::*hphMX6*, *lys1*Δ::P_{adh11}-GBP-*mCherry-GBP-mCherry-2xNLS*::*hphMX6*, *lys1*Δ::P_{adh11}-GBP-*mCherry*-9myc-TEV-2*xNLS*::*hphMX6* and *lys1*Δ::P_{adh1}-GBP-*mCherry*-9myc-TEV-2*xNLS*::*hphMX6*.

Western blot analyses

Western blot experiment was performed essentially as previously described (Chen *et al.* 2017). The primary antibodies used for immunoblot analysis of cell lysates were rabbit polyclonal anti-mCherry antibody (ab167453, Abcam; RRID: AB_2571870) and rabbit polyclonal anti-PSTAIRE (sc-53, Santa Cruz Biotechnology; RRID: AB_2074908). Secondary antibodies were anti-rabbit HRP conjugates (Cat# 88-1688-31, Thermo Fisher Scientific; RRID: AB_475768) and were read out using chemiluminescence.

Microscopy

GFP- and mCherry-fusion proteins were observed in cells after fixation with cold methanol. Cells were washed in PBS and resuspended in PBS plus 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) (Roche). Photomicrographs were obtained using a Nikon 80i fluorescence microscope coupled to a cooled CCD camera (ORCA-ER; Hamamatsu Photonics) or a Perkin Elmer spinning-disk confocal microscope (UltraVIEW® VoX) with a 100x NA 1.49 TIRF oil immersion objective (Nikon) coupled to a cooled CCD camera (9100-50 EMCCD; Hamamatsu Photonics) and spinning disk head (CSU-X1, Yokogawa). Image processing and analysis were carried out using Element software (Nikon), ImageJ software (National Institutes of Health), and Adobe Photoshop.

Statistical analysis

Experiments for quantification of protein levels of GBP-mCherry fusions and frequencies of chromosome missegregation were repeated three times. In order to determine statistical significance of our data, independent-samples *t*-tests were performed and *P*-values were calculated using SPSS19. $P < 0.05$ was considered statistically significant.

Results

Construction of strains expressing GBP-mCherry fusions with or without NLS

GBP or GFP-targeting nanobody is most commonly used in applications such as immunoprecipitation, relocation, or targeted proteasomal degradation of GFP-tagged proteins (reviewed in Chen *et al.* 2017; Aguilar *et al.* 2019; Prole and Taylor 2019). In the course of our use of GBP-fusion proteins as an intracellular tool for redirecting proteins of our interest to different subcellular loci in *S. pombe*, we noticed unintended perturbation of GFP-tagged protein function in a few cases (data not shown). To confirm those sporadic observations and examine whether GBP-mediated interference of protein function may offer a generic means for inactivation of certain set of proteins, we decided to construct yeast strains expressing GBP-mCherry fusion proteins which can be followed under fluorescent microscopy. To compare the potential effects of subcellular localization (nuclear *vs* cytoplasmic), tandem presence (1 *vs* 2 tandem GBP-mCherry cassettes), size [GBP-mCherry (39.72 kDa) *vs* 9myc-TEV protease (40.11 kDa)], and expression strength (driven by P_{adh11} *vs* P_{adh1} promoters) of GBP-mCherry fusions on functional perturbation of target proteins, we constructed a series of vectors carrying combined sequences of GBP-mCherry, two tandem SV40 NLSs or 9myc-TEV protease and obtained integrants at $lys1^+$ locus after transformation of linearized vectors (Figure 1A).

Under fluorescent microscopy, we could observe either cytoplasm- or nucleus-localized mCherry signals of GBP-mCherry fusions depending on the absence or presence of the SV40 NLSs (Figure 1B). Cells expressing P_{adh11}-2xGBP-mCherry-2xNLS gave the brightest nuclear mCherry signals among the tested constructs, which should be due to the presence of two copies of GBP-mCherry (Figure 1B). We also compared the protein levels of GBP-mCherry fusions by Western blotting, and found surprisingly that P_{adh11}-GBP-mCherry-9myc-TEV-2xNLS was least abundant (Figure 1C). However, expression of GBP-mCherry-9myc-TEV-2xNLS driven by the strongest constitutive *adh1* promoter variant (P_{adh1}) elevated its protein levels (Figure 1C), which was consistent with previous studies using this set of promoters (Tada *et al.* 2011; Chen *et al.* 2017).

GBP-mCherry is efficiently targeted to kinetochores in cells expressing GFP-tagged kinetochore proteins

We chose kinetochore proteins to test their possible functional interference by GBP, because almost all the core kinetochore subunits are essential for cell survival and therefore likely more sensitive to perturbation than nonessential proteins. Similar to higher eukaryotes, the *S. pombe* genome encodes approximately 30 core structural components of kinetochore, and these subunits mostly reside in three major complexes: Ndc80 complex, Mis12 complex, and Mis6-Sim4 complex (Fukagawa and De Wulf 2009; Figure 2A). Among them, seven kinetochore proteins were selected and included in our investigation, they represent inner kinetochore components (Cnp1, Mis6, and Dad1), outer kinetochore components (Mis12, Spc7, and Ndc80), and subunit linking the inner and outer kinetochore (Cnp3), respectively. In addition, we also included one cohesin complex subunit (Rad21). As the very first step, we examined whether all the GBP-mCherry fusions could be recruited to GFP-tagged kinetochore proteins (Figure 2B). As expected, we found that all GBP-mCherry fusions were efficiently redirected to kinetochores, as judged by the co-localization of dot-like GBP-mCherry and GFP signals (Figure 2C and Supplementary Figures S1A–S6A). Surprisingly, even the GBP-mCherry fusion without SV40 NLSs could be targeted to kinetochores inside nuclei, this was most likely because GBP-mCherry could bind to GFP-tagged kinetochore proteins soon after their respective translations and the bound complexes were co-transported from cytoplasm into nucleus. We noticed that all GBP-mCherry fusions, except the one without SV40 NLSs, could retain certain portions of GFP-tagged kinetochore proteins in nucleoplasm (Figure 2C and Supplementary Figures S1A–S6A). We also observed that when combined with Rad21-GFP, GBP-mCherry could be observed as concentrated dots at centromeres and dispersed signals in the nuclear chromatin, which is consistent with previous study (Tomonaga *et al.* 2000). These results demonstrated that the high affinity of the GFP for the GBP resulted in a steady-state relocation of GBP-mCherry fusion to the kinetochores or cohesin complexes.

Cells simultaneously expressing GBP-mCherry and GFP-tagged Cnp1 are sensitive to temperature and microtubule-depolymerizing drug

We next examined whether simultaneous expression of GBP-mCherry and GFP-tagged kinetochore proteins could cause any cell growth defects. Since mutations in fission yeast genes encoding components of the kinetochore are characteristically sensitive to microtubule-depolymerizing drug TBZ (Saitoh *et al.* 1997; Jin *et al.* 2002), we also tested the TBZ-sensitivity of all those strains. Our serial dilution spot assays showed that GBP-mCherry fusions exacerbated the growth of cells expressing Cnp1-GFP at 33°C and 37°C (Figure 3A) and also at 30°C in the presence of TBZ (Figure 3B). All the other GFP-tagged kinetochore proteins were not sensitive to expressing GBP-mCherry fusions at all temperatures tested or in the presence of TBZ (Figure 3 and Supplementary Figures S1–S3, S5, and S6), except three GBP-mCherry fusions without TEV only slightly caused the Mis12-GFP to be sensitive to 37°C (Supplementary Figure S4B). Quite surprisingly, a few GBP-mCherry fusions rescued the high temperature-sensitive phenotype of Ndc80-GFP and Spc7-GFP (Figure 3A and Supplementary Figure S5B). Together, these data suggested that kinetochore-targeted GBP-mCherry likely perturbs the integrity of centromeric chromatin, which is essential for kinetochore assembly.

Kinetochore-targeted GBP-mCherry causes chromosome segregation defects

It has been well established in previous studies that the chromosome missegregation phenotype that resulted in large and small daughter nuclei is the hallmark of mutations in authentic kinetochore components (Saitoh *et al.* 1997; Goshima *et al.* 1999; Takahashi *et al.* 2000; Jin *et al.* 2002; Hayashi *et al.* 2004). Interestingly, we found that all strains simultaneously expressing GBP-mCherry and GFP-tagged kinetochore proteins showed an elevated frequency of chromosome missegregation due to unequal segregation of chromosomes at 30°C, among which Cnp1-GFP was most sensitive to the presence of GBP-mCherry (Figure 4). It is noteworthy that it was the tandem presence of GBP-mCherry rather than the size of the fusion [comparing GBP-mCherry (39.72 kDa) with 9myc-TEV protease (40.11 kDa)] or the expression strength (comparing GBP-mCherry-9myc-TEV-2xNLS expressed under P_{adh11} with P_{adh1} promoters) contributed most significantly

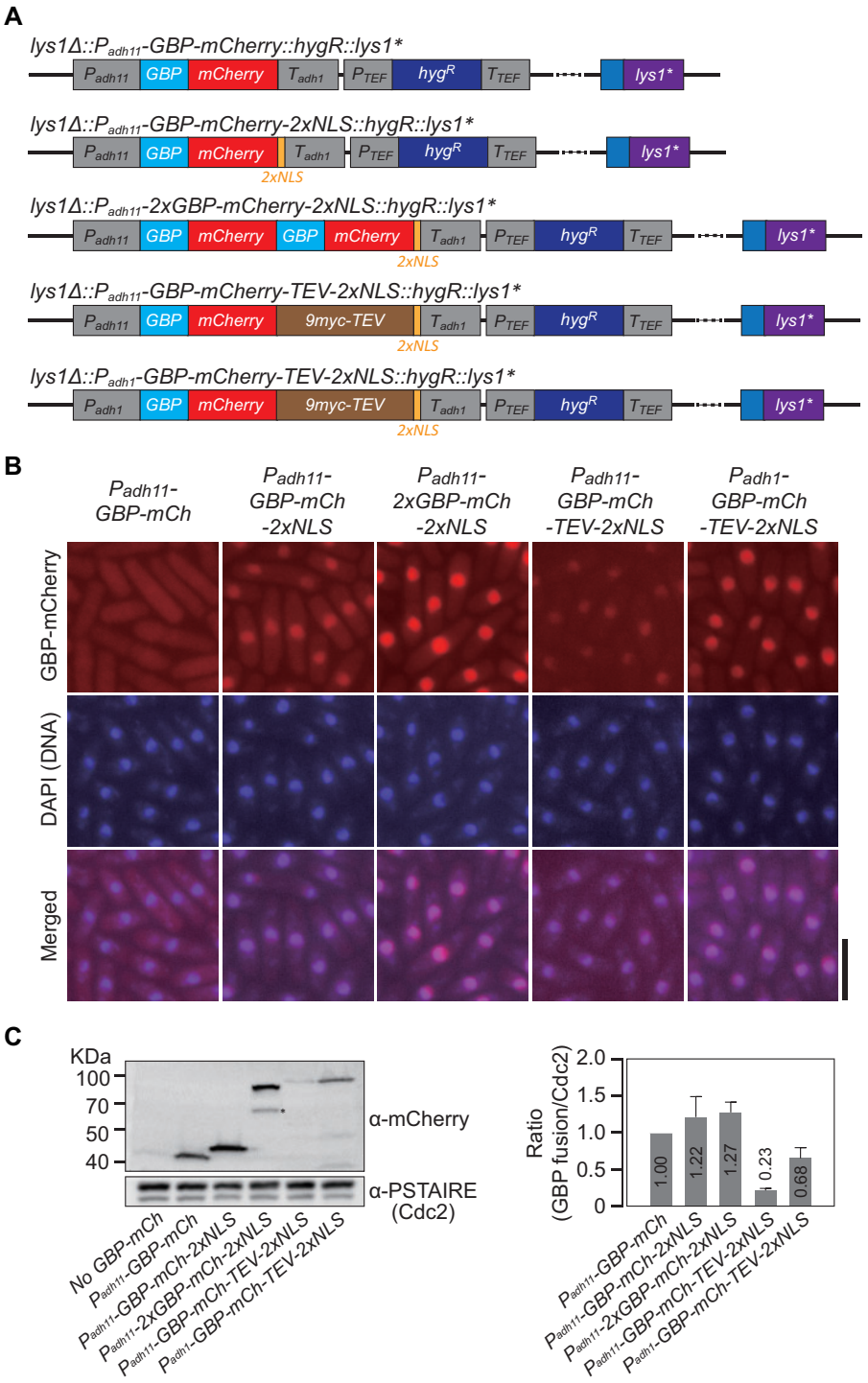

Figure 1 Construction of strains expressing nuclear-localized GBP-mCherry fusion. (A) Cartoon depicting the structure of the genomically integrated GBP-mCherry fusions at *lys1*$^{+}$ locus. (B) Representative images of cells expressing GBP-mCherry fusion proteins driven by promoters P_{adh11} or P_{adh1}. Cells were grown to early log phase in liquid YE, and then collected, fixed, DAPI-stained and visualized by using fluorescence microscopy. Scale bar, 10μm. (C) Western blot analyses of GBP-mCherry fusion protein levels. (Left) Samples were collected and prepared from early log phase cultures and subjected to immunoblotting analyses using anti-mCherry and anti-Cdc2 antibodies. (Right) GBP-mCherry levels were normalized to those of total Cdc2 for each sample, with the relative ratio between P_{adh21}-GBP-mCherry and Cdc2 set as 1.0. The experiment was repeated 4 times and the mean value and standard deviation (SD) for each sample was calculated. Asterisk indicates a likely degradation band.

to the disruptive effect on all tested kinetochore proteins (Figure 4). We also noticed that GFP tagging of Spc7 itself results in defective chromosome segregation to a certain degree, but GBP-mCherry fusions did not further exacerbate these defects (Figure 4). Taken together, our data further supported the idea that kinetochore-targeted GBP-mCherry efficiently compromises

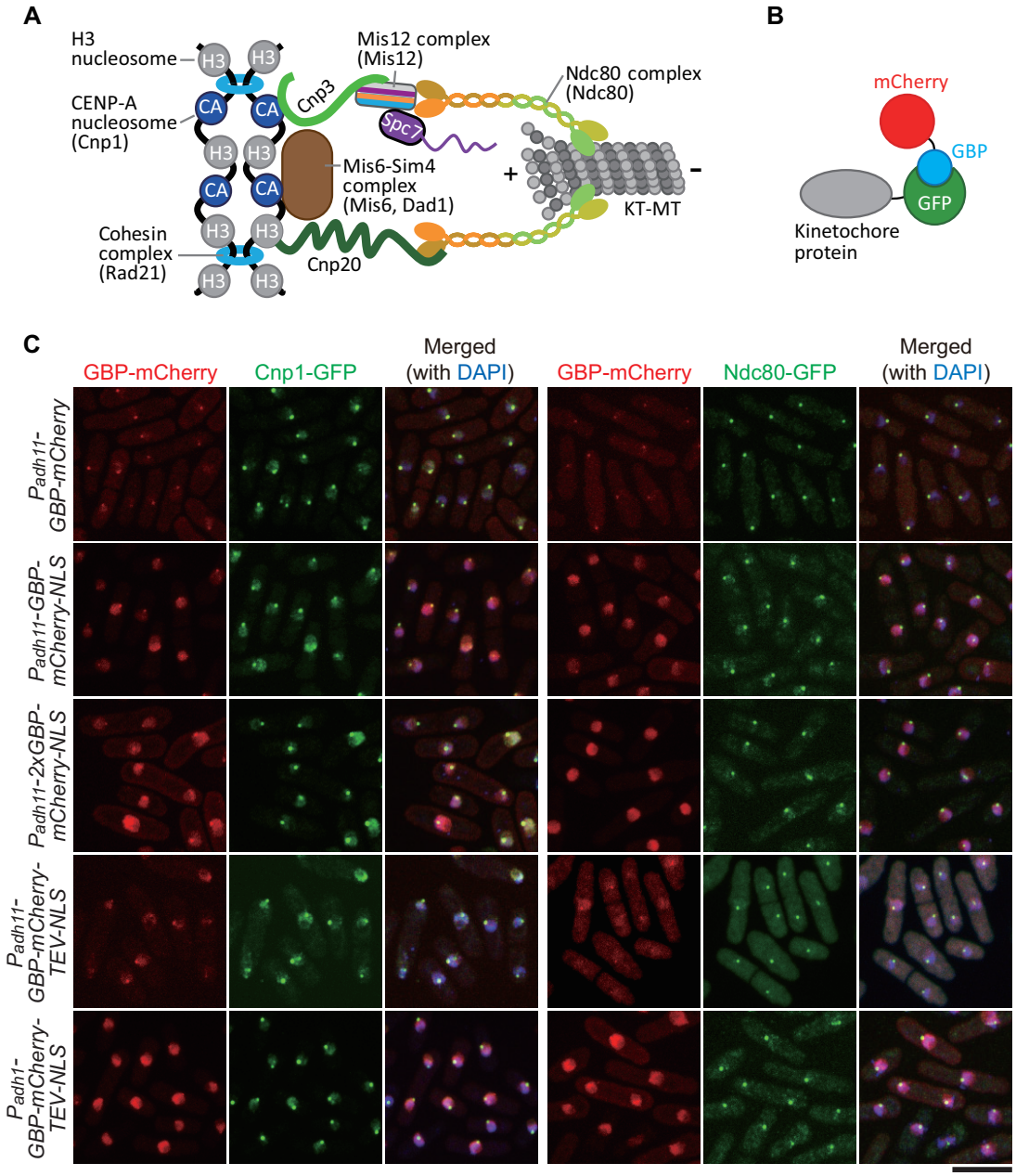

Figure 2 GBP-mCherry is efficiently targeted to kinetochores in cells expressing GFP-tagged kinetochore proteins. (A) Schematic of molecular organization model of fission yeast kinetochore. It may not necessarily represent the actual organization, as it is drawn mostly based on knowledge from vertebrate kinetochore. Proteins in parentheses are used for kinetochore targeting of GBP-mCherry fusions in this study. KT, kinetochore; MT, microtubule. (B) Schematic of strategy used for manipulated targeting of GBP-mCherry fusions to kinetochores by GFP-tagged kinetochore proteins. (C) Representative images of cells simultaneously expressing various GBP-mCherry fusion proteins and GFP-tagged kinetochore proteins Cnp1 and Ndc80. Cells were grown to early log phase in liquid YE, and then collected, fixed, DAPI-stained and visualized by fluorescence microscopy. Scale bar, 10μm.

or disrupts the integrity of the molecular architecture of kinetochores without the need for a temperature shift.

Strains carrying GBP-mCherry and GFP-tagged kinetochore proteins require the presence of spindle checkpoint for survival

The spindle checkpoint monitors kinetochore-microtubule interactions and generates a "wait anaphase" signal and delays anaphase onset upon any defective kinetochore-microtubule interactions (London and Biggins 2014b; Musacchio 2015). Among the major players of the checkpoint machinery, Bub1 together with Mad1 forms a platform at unattached kinetochores to recruit other components of the checkpoint (London and Biggins 2014a; Faesen *et al.* 2017; Ji *et al.* 2017). The Bub1-TPR domain is required for its interaction and recruitment of Mad3 (Leontiou *et al.* 2019), which further forms a diffusible mitotic checkpoint complex (MCC) with Mad2 and Cdc20 (Chao *et al.* 2012).

As the spindle checkpoint signals are initially generated at unattached kinetochores during mitosis, the compromised kinetochore architecture may compromise kinetochore-microtubule interaction and induce the activation of the checkpoint. We, therefore, tested the possible genetic interactions between the strains carrying kinetochore-targeted P_{adh11}-GBP-mCherry-2xNLS and the spindle checkpoint mutants *mad2*Δ or *bub1*Δ. Strikingly,

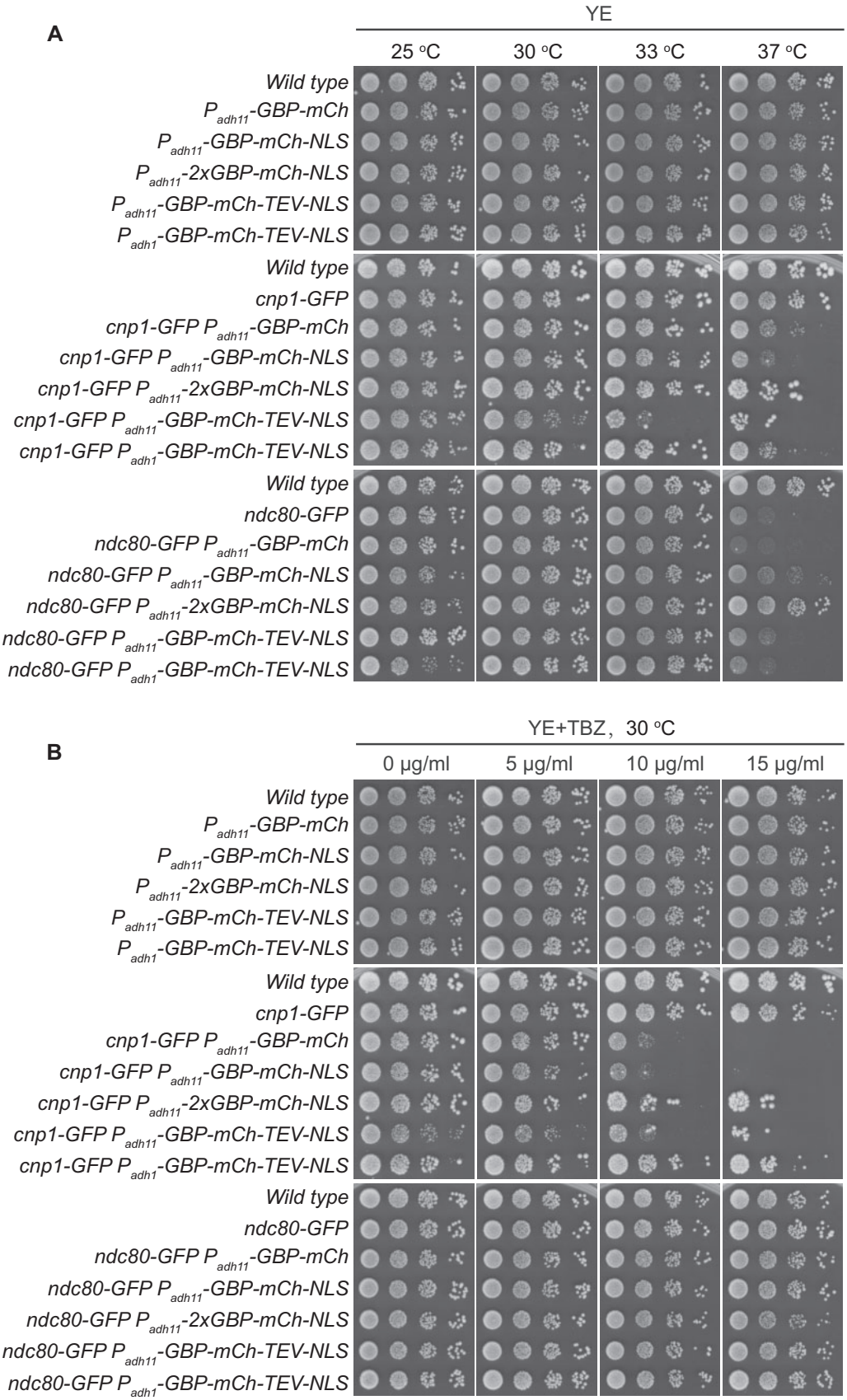

Figure 3 Cells simultaneously expressing GBP-mCherry and Cnp1-GFP are sensitive to high temperatures and TBZ. Serial dilutions (10-fold) of the indicated strains were either spotted on YE plates and incubated at the indicated temperatures (A), or on YE plates with different concentrations of TBZ at 30°C (B). Plates of samples grown at 30°C in (A) is shown as the "0 μg/ml" TBZ control in (B). Note that P_{adh21}-GBP-mCherry-NLS and P_{adh21}-2xGBP-mCherry-NLS rescued the growth defects of Ndc80-GFP strain at 37°C (A).

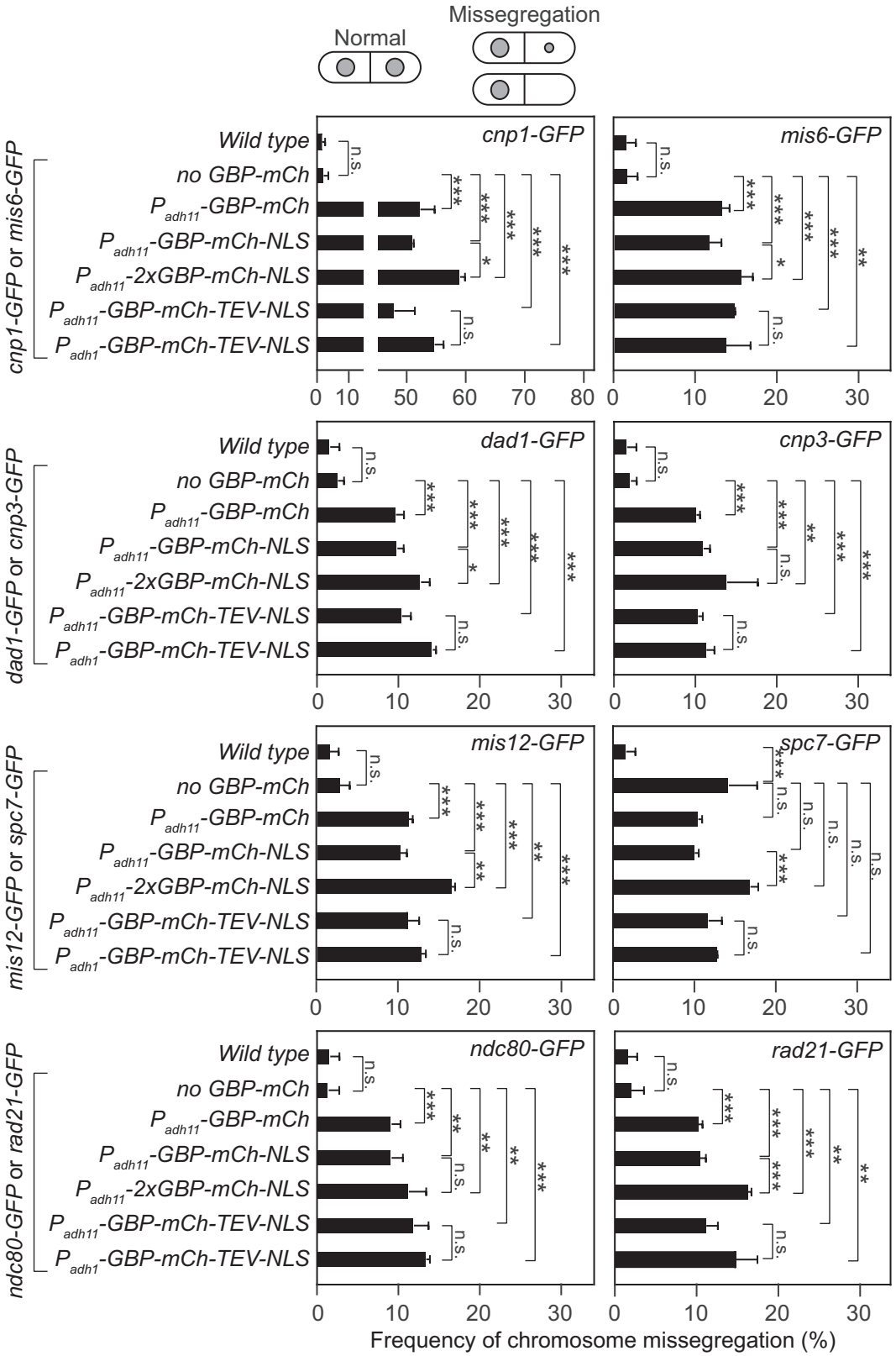

Figure 4 Kinetochore-targeted GBP-mCherry causes chromosome segregation defects. Cells were grown at 30°C to early log phase and collected, fixed and stained with DAPI. Anaphase and telophase cells were examined by fluorescence microscopy and chromosome missegregation was quantified for each strain. The experiment was repeated 3 times and the mean value and standard deviation (SD) for each sample was calculated. 100–200 cells were analyzed for each strain. n.s., no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 1 Summary of the genetic interactions between the strains carrying kinetochore-targeted P_{adh11}-GBP-mCherry-2xNLS and the spindle checkpoint mutations *mad2Δ* or *bub1Δ*

Combinations or mutations	*mad2Δ*	*bub1Δ*
cnp1-GFP	+++	+++
cnp1-GFP P_{adh11}*-GBP-mCherry-2xNLS*	±	SL
mis6-GFP	+++	+++
mis6-GFP P_{adh11}*-GBP-mCherry-2xNLS*	SL	SL
dad1-GFP	+++	+++
dad1-GFP P_{adh11}*-GBP-mCherry-2xNLS*	SL	SL
cnp3-GFP	+++	+++
cnp3-GFP P_{adh11}*-GBP-mCherry-2xNLS*	+++	SL
mis12-GFP	+++	+++
mis12-GFP P_{adh11}*-GBP-mCherry-2xNLS*	+++	+++
spc7-GFP	+++	+++
spc7-GFP P_{adh11}*-GBP-mCherry-2xNLS*	+++	SL
ndc80-GFP	+++	+++
ndc80-GFP P_{adh11}*-GBP-mCherry-2xNLS*	SL	SL
rad21-GFP	+++	+++
rad21-GFP P_{adh11}*-GBP-mCherry-2xNLS*	SL	SL

Note: Genetic interactions are shown as synthetic lethality (SL), strong growth defect (±), and normal growth (+++).

most combinations were synthetic lethal (see Table 1), but not when P_{adh11}-GBP-mCherry-2xNLS was absent (Supplementary Figure S7). These data are consistent with the notion that, in most cases, cells simultaneously expressing GBP-mCherry and GFP-tagged kinetochore proteins likely have defects in the attachment of microtubules to kinetochores that trigger spindle checkpoint-dependent delays. Thus, the presence of checkpoint provides time for cells to correct chromosome attachment errors, ensure faithful chromosome partition into daughter cells and cell survival.

Discussion

The single-domain camelid nanobodies that specifically bind GFP or RFP have been very useful as tools for not only affinity purification, but also for a broader range of applications, such as ectopic recruitment or targeting to or from proteins with GFP or RFP tags, recoloring, targeted degradation and inactivation, calcium sensing and more (Rothbauer *et al.* 2006, 2008; Fridy *et al.* 2014; Chen *et al.* 2017; Aguilar *et al.* 2019; Prole and Taylor 2019). In this study, we described the use of the GBP fusion proteins as a tool to inactivate some kinetochore proteins in fission yeast. This strategy turned out to be most efficient to perturb the function of inner kinetochore, although we do not quite understand the mechanistic details yet. It is possible that binding of this small polypeptide with around 120 amino acids to our tested kinetochore proteins causes altered dynamics or interaction capability of these proteins, which eventually collapse the massive multiprotein assembly. Very interestingly, our observations that the function of GFP-tagged proteins can be inactivated by GBP are not unique, because a very recent study also reported that GBP binding to dynamin-2-GFP and GFP-Tumor Protein D54 (TPD54/TPD52L2) in HeLa cells inhibits or perturbs the function of these two human proteins, which are involved in endocytosis and anterograde traffic respectively (Kuey *et al.* 2019).

Essential genes cannot be deleted from the genome. Therefore, to date, genetic and functional studies of the essential proteins in fission and budding yeasts have relied mostly on the use of mutant strains carrying conditional temperature-sensitive (*ts*) or cold-sensitive (*cs*) alleles. To isolate this type of mutants, error-prone mutagenesis (or random mutagenesis) coupled with *in vitro* selection has been commonly used. The procedures to obtain desired mutants can be tedious, however, these alleles are often leaky and require analysis under nonphysiological conditions, and their inactivation requires a relatively long period of time.

In recent years, several approaches aiming at conditional depletion of a protein of interest have been developed in yeasts and mammalian systems, such as the auxin-inducible degron (AID) and the Trim-Away technologies, in which protein knockdown is achieved by recruiting a target protein to an E3 ubiquitin ligase and followed by rapid degradation of target proteins by the ubiquitin-proteasome pathway (Nishimura *et al.* 2009; Clift *et al.* 2017; Yesbolatova *et al.* 2020). In addition, strategies called "anchor-away" or "knocksideways" involving protein sequestering or rerouting to cytoplasm or mitochondria have also been developed. These methods are based on the rapamycin-induced heterodimerization between the FKBP domain from FKBP12 (homolog of budding yeast FPR1 and fission yeast Fkh1) and rapamycin-binding (FRB) domain from mTOR (homolog of budding yeast TOR1 and fission yeast Tor1) (Geda *et al.* 2008; Haruki *et al.* 2008; Robinson *et al.* 2010). For techniques of AID, "anchor-away" and "knocksideways," they all require simultaneously the tagging of target proteins with "aid" degron (for AID system) or FRB domain (for "anchor-away" or "knocksideways" system), introducing modified plant F-box protein TIR1 (for AID system) or FKBP-fusions (for "anchor-away" or "knocksideways" system) into cells, and the presence of inducing drugs (auxin for AID system or rapamycin for "anchor-away" or "knocksideways" system) (Geda *et al.* 2008; Haruki *et al.* 2008; Nishimura *et al.* 2009; Robinson *et al.* 2010; Yesbolatova *et al.* 2020). For applications in yeasts, "anchor-away" technique also requires a *tor* mutant background and deletion of FKBP12 homolog to eliminate competition from the endogenous TOR system (Haruki *et al.* 2008; Ding *et al.* 2014). To employ the Trim-Away technique, a specific antibody must be developed for each protein of interest, which might be cost-inefficient and challenged by antibody quality and specificity. Another limitation for the Trim-Away is that the major component of the system, the cytosolic antibody receptor TRIM21, is only present in mammals, thus it excludes its applications in nonmammalian systems.

As we demonstrated here, the strategy of using the GBP fusion proteins as a rapid, straight-forward and generic means to generate yeast mutants could potentially provide a powerful method, which can be applied in both systematic and individual gene-focused studies, and can be used to investigate both essential genes (*e.g.*, *cnp1*$^+$, *cnp3*$^+$, *mis6*$^+$, *mis12*$^+$, *spc7*$^+$, *ndc80*$^+$, and *rad21*$^+$ in this study) and nonessential genes (*e.g.*, *dad1*$^+$ in this study). Compared to other presently available protein-knockdown systems, this strategy requires the least effort to set up the test. Practically, GBP fusion proteins can be introduced into any yeast strains carrying GFP-tagged target proteins simply by genetic crosses. In *S. pombe*, a huge variety of proteins have been individually or systematically tagged with GFP or GFP variants for purposes of investigation on localization and function. Therefore, nanobody-mediated inactivation of protein function may be used as a direct or alternative method for perturbing functions of these off-the-shelf GFP-tagged proteins. In principle, this method can be extended to mCherry- or RFP-tagged proteins by using RFP nanobody. Furthermore, it should be possible to extend this method to other nonyeast systems, such as mammalian system, for the purpose of manipulating intracellular signaling.

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Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Supplementary material is available at G3 online.

Author contribution

Conceptualization: Q.-W.J. and Y.W.; Methodology: Q.-W.J., Y.W., and L.-L.D.; Validation: Y.W.; Formal analysis: D.-J.D., Q.-C.X., G.-S.J., F.S., L.-L.D., Q.-W.J., and Y.W.; Investigation: D.-J.D., Q.-C.X., G.-S.J., F.S., J.-L.C., L.S., J.-Q.W., and S.-M.W.; Data curation: D.-J.D., Q.-C.X., L.-L.D., Q.-W.J., and Y.W.; Paper writing: Q.-W.J. and Y.W.; Supervision: L.-L.D. and Y.W.; Project administration: Q.-W.J. and Y.W.; Funding acquisition: Q.-W.J.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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