Kinetochore Components Required for Centromeric Chromatin Assembly Are Impacted by Msc1 in *Schizosaccharomyces pombe*

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ABSTRACT Eukaryotic chromosome segregation requires a protein complex known as the kinetochore that mediates attachment between mitotic spindle microtubules and centromere-specific nucleosomes composed of the widely conserved histone variant CENP-A. Mutations in kinetochore proteins of the fission yeast *Schizosaccharomyces pombe* lead to chromosome missegregation such that daughter cells emerge from mitosis with unequal DNA content. We find that multiple copies of Msc1—a fission yeast homolog of the KDM5 family of proteins—suppresses the temperature-sensitive growth defect of several kinetochore mutants, including *mis16* and *mis18*, as well as *mis6*, *mis15*, and *mis17*, components of the Constitutive Centromere Associated Network (CCAN). On the other hand, deletion of *msc1* exacerbates both the growth defect and chromosome missegregation phenotype of each of these mutants. The C-terminal PHD domains of Msc1, previously shown to associate with a histone deacetylase activity, are necessary for Msc1 function when kinetochore mutants are compromised. We also demonstrate that, in the absence of Msc1, the frequency of localization to the kinetochore of Mis16 and Mis15 is altered from wild-type cells. As we show here for *msc1*, others have shown that elevating *cnp1* levels acts similarly to promote survival of the CCAN mutants. The rescue of *mis15* and *mis17* by *cnp1* is, however, independent of *msc1*. Thus, Msc1 appears to contribute to the chromatin environment at the centromere: the absence of Msc1 sensitizes cells to perturbations in kinetochore function, while elevating Msc1 overcomes loss of function of critical components of the kinetochore and centromere.

KEYWORDS Msc1; CENP-A; Mis6; Mis15; Mis16/Mis18; Mis17; CCAN; lysine demethylase KDM5; RBP2; PLU-1; kinetochore; centromere

SEGREGATION of the genome at mitosis relies on the faithful association of spindle pole body-derived microtubules with regions of individual chromosomes that mediate chromosome attachment to the mitotic spindle. These chromosomal regions are epigenetically marked with a variant of canonical histone H3 known as CENP-A (Palmer *et al.* 1987; Allshire and Ekwall 2015). CENP-A-containing nucleosomes are found at centromeres in eukaryotic organisms ranging from unicellular budding and fission yeast, to human cells. CENP-A nucleosomes recruit complexes of proteins that form the kinetochore—the structure that mediates dynamic microtubule interactions with the mitotic spindle (Cheeseman 2014). Characteristics of the centromere vary between organisms: budding yeast chromosomes have point centromeres consisting of a single CENP-A-containing nucleosome, whereas fission yeast and human chromosomes have regional centromeres consisting of an array of CENP-A-containing nucleosomes (Westhorpe and Straight 2015). Regional centromeres of the three fission yeast chromosomes consist of distinct organizational structures: a central core (cnt) with flanking inverted repeats (imr), all of which have associated CENP-A nucleosomes, and large heterochromatic repeats to the outside of the imr region (otr) that contain histone H3 modified by methylation of lysine 9 in its N-terminal tail. Some report the presence of the histone H2A variant, H2A.Z in the otr region as well (Lawrence and Volpe 2009; Qiu *et al.* 2010).

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Kinetochores are protein structures that associate with CENP-A-containing nucleosomes to mediate the dynamic association between chromosomes and the growing and shrinking microtubules of the mitotic spindle (reviewed in Cheeseman 2014). Kinetochores of both fission yeast and mammalian cells include a complex of conserved proteins, collectively known as the Constitutive Centromere-Associated Network or CCAN, which consists of more than a dozen proteins that tend to remain with the centromere throughout the cell cycle (McAinsh and Meraldi 2011). As individual components of the CCAN are further investigated, evidence is emerging for dynamic association of some CCAN components as cells progress through the cell cycle (Hellwig *et al.* 2011; Fang *et al.* 2015; Nagpal and Fukagawa 2016).

In fission yeast, two components of the CCAN were identified in screens for mutants that exhibit a high frequency of mini-chromosome loss: Mis6 (CENP-I) (Takahashi *et al.* 1994) and Mal2 (CENP-O) (Fleig *et al.* 1996). Mis15 (CENP-N) and Mis17 (CENP-U) were identified in a direct visual screen of temperature-sensitive (ts) mutants that missegregate chromosomes at mitosis (Hayashi *et al.* 2004). Another CCAN protein, Sim4 (CENP-K), was identified in a screen for mutants that are defective for silencing in the middle of the centromere, a screen that gave rise as well to an allele of CENP-A (Pidoux *et al.* 2003). Several other components of the CCAN complex were identified by purification of proteins associated with Sim4 and Mal2 (Liu *et al.* 2005). Interestingly, only a subset of the CCAN proteins are conserved in budding yeast, suggesting that the association of the CCAN with an array of CENP-A containing nucleosomes requires a distinct set of proteins as compared to those needed to form a kinetochore on the single point centromere found in budding yeast (McAinsh and Meraldi 2011).

Direct screening for mutants defective in chromosome segregation also identified Mis16 and Mis18, proteins conserved between fission yeast and human cells (Hayashi *et al.* 2004). While a homolog of Mis16 is found in budding yeast (Hat2), a homolog of Mis18 is not, though Mis16 and Mis18 form a complex in both fission yeast and human cells (Fujita *et al.* 2007; Nardi *et al.* 2016; Subramanian *et al.* 2016). Two additional proteins, Mis19/Eic1 and Mis20/Eic2, which appear to be unique to fission yeast, associate with the Mis16/18 complex as well (Hayashi *et al.* 2014; Subramanian *et al.* 2014). The Mis16/18 complex is thought to facilitate loading of CENP-A to centromeres after DNA replication when CENP-A must be reincorporated following duplication of the chromosome and consequent dilution of the CENP-A containing nucleosomes between the daughter strands (Hayashi *et al.* 2004; Fujita *et al.* 2007). CENP-A protein levels at the central core are reduced in *mis16* and *mis18* strains (Hayashi *et al.* 2004). A histone chaperone, fission yeast Scm3, which may be analogous to human HJURP, has been described as a receptor for CENP-A loading, and proposed to act with the Mis18 complex, perhaps through histone deacetylation, to facilitate loading of CENP-A (Foltz *et al.* 2009; Pidoux *et al.* 2009; Barnhart *et al.* 2011).

Msc1 was identified in a fission yeast genetic screen for genes that in multiple copies suppressed the DNA damage-induced lethality of cells defective for the DNA damage checkpoint (Ahmed *et al.* 2004). Subsequent studies of cells disrupted for the *msc1* gene revealed a role for the protein in chromosome segregation (Ahmed *et al.* 2007), as cells lacking *msc1* exhibit a chromosome loss phenotype. Further genetic analysis (Ahmed *et al.* 2007) revealed synthetic lethal interactions with the CCAN component *mis6* (CENP-I), and with *mis12*, a component of the KMN protein network that is proposed to link microtubules to the kinetochore (Cheeseman *et al.* 2006). Double mutants of either *mis6* or *mis12* with a null allele of *msc1* result in inviability at temperatures typically permissive for the *mis* mutants (Ahmed *et al.* 2007). Curiously, increased expression of *msc1* suppresses the ts growth defect of cells with a mutation in the CENP-A encoding gene *cnp1-1* (Ahmed *et al.* 2007).

We have demonstrated previously that Msc1 coprecipitates histone deacetylase (HDAC) activity (Ahmed *et al.* 2004). Given that increasing the level of Msc1 compensates for loss of *cnp1-1* function, and that the Mis16/18 complex helps to maintain deacetylation of histones to facilitate CENP-A loading, we hypothesized that Msc1 might influence activity of the Mis16/18 complex. To that end, we tested whether elevating Msc1 could rescue ts *mis16* or *mis18* mutants, or whether deletion of *msc1* exacerbates their phenotype. Indeed, at restrictive temperature, we find that extra copies of Msc1 can suppress, while deletion of *msc1* exacerbates, the growth defect and chromosome missegregation phenotype of *mis16* or *mis18* mutants. The C-terminal PHD domains, previously shown to be necessary to precipitate HDAC activity, are necessary for Msc1 to accomplish this function. Like Msc1, the Mis16/18 complex is conserved in mammalian cells, which, like fission yeast, have regional centromeres, but Mis18 is not conserved in budding yeast, which have point centromeres. CCAN components are conserved in organisms with regional and point centromeres, and we have also examined the relationship between Msc1 and components of the CCAN. Interestingly, elevating Msc1 levels rescues ts alleles of CCAN mutants *mis6*, *mis15*, and *mis17*, which have been shown by others to be rescued upon increased expression of *cnp1* (Hayashi *et al.* 2004). While elevating *cnp1* or *msc1* levels acts similarly to promote survival of the CCAN mutants, the rescue of *mis15* and *mis17* by *cnp1* does not require the presence of *msc1*. Finally, we examine the localization of the Mis16 component of the Mis16/18 complex as well as the CCAN component Mis15 in cells lacking Msc1. Consistent with exacerbation of the *mis16* phenotype by deletion of *msc1*, Mis16 protein is less frequently associated with the kinetochore in the absence of Msc1. In contrast, Mis15 protein is more frequently associated with the kinetochore when Msc1 is absent. We suggest that Msc1 contributes to the chromatin environment at the centromere such that in its absence, kinetochore function is compromised. On the other hand, in cells with diminished kinetochore function, elevating Msc1 can facilitate chromosome segregation and improve viability.

Materials and Methods

Strains and growth conditions

Standard *Schizosaccharomyces pombe* media and genetic techniques were used as described (Moreno *et al.* 1991). Transformation was done using the LiAC method. To construct double mutant strains, two strains with opposite mating types (h^+ and h^- in fission yeast) were crossed and mated on nitrogen-deficient SPA plates at 25° for at least 2 days. Asci from the crosses were incubated in 1× glusulase at 25° followed by plating on YES plates. Plasmids were transformed into strains as listed in Table 1. For spotting assays, all cells were grown at 25° in minimal media (PMA-leucine) unless otherwise noted. Cells were grown to midlog phase and fivefold serial dilutions prepared. Aliquots of each dilution were then transferred using a 48-pin Multi-Blot Replicator (VP Scientific), spotted and grown for 3–4 days at temperatures and on media as indicated in the figure legends. YES plates contain the following components per liter: 5 g yeast extract, 30 g glucose, 150 mg each of adenine, uracil, leucine, lysine and histidine, and 20 g of agar. PMA minus leucine plates contain the following components per liter: 32 g EMM (Sunrise Science Products), 150 mg each of adenine, uracil, lysine and histidine, and 20 g of agar.

Microscopy for mis phenotype

Cells were grown at 25° in media as indicated to midlog phase and diluted to 2.5×10^6 cells/ml. Subsequently, cells were transferred to fresh media at appropriate restrictive temperature for the period of time described in the figure legends. Methanol fixation was conducted by washing cells twice with ddH_2O, then incubating cells in 100% methanol for ≤ 20 min at −20°. Cells were pelleted and incubated in 30% methanol, 1× PBS at −20°. For glutaraldehyde fixation, cells were washed with ddH_2O then fixed in 0.5% glutaraldehyde for 10 min on ice. After three washes with cold ddH_2O, cells were resuspended in a small volume of ddH_2O. For DAPI staining, 0.4 μl of cells was mixed with 0.4 μl of 10 μg/ml DAPI solution. The DAPI-stained cell suspension was analyzed using a fluorescence microscope (Zeiss Axioplan 2) within 24 hr of fixation. Images were captured with a Zeiss AxioCam and analyzed with Openlab software.

Fluorescence microscopy

For imaging GFP signals, cells were grown at 25° to midlog phase, stained with DAPI as described above and imaged for GFP at 15–19 different focal depths through Z-stack scanning. By tracking dots throughout all individual images taken from each focal depth, we evaluated a minimum of 200 cells for each strain for the presence of discrete foci representing the centromere.

Western blot

For lysate preparation, strains were grown to midlog phase in indicated media at 25°. Cells were lysed in a buffer containing 10% 1× Protease Inhibitor (PI), 10% PMSF in 1× PBS with acid-washed glass beads in a Fastprep vortexing machine (Bio101). The following antibodies were used for Western Blot analyses: GFP B-2 (Santa Cruz), Goat anti-mouse IgG-HRP (Santa Cruz), α-tubulin T-5168 (Sigma), and polyclonal antibody to fission yeast Ded1 (Liu *et al.* 2002)

Table 1 Yeast strains

Strain	Genotype	Source
SP6	*h- leu1-32*	Laboratory stock
NW730	*h+ msc1::kan leu1-32*	Laboratory stock
NW1702	*h- leu1-32 ura4+ mis6-302*	Laboratory stock
NW2841	*h- leu1 mis15-68*	YGRC FY20041
NW2842	*h- leu1 mis16-53*	YGRC FY20042
NW2843	*h- leu1 mis17-362*	YGRC FY20043
NW2844	*h- leu1 mis18-262*	YGRC FY20044
NW2850	*h- leu1 ura4 mis16-GFP[ura4+]*	YGRC FY10474
NW2851	*h- leu1 ura4 mis15-GFP[ura4+]*	YGRC FY10468
NW2945	*h? leu1- mis15-68 msc1::kan^R*	This study
NW2946	*h? leu1- mis16-53 msc1::kan^R*	This study
NW2947	*h? leu1- mis17-362 msc1::kan^R*	This study
NW2948	*h? leu1- mis18-262 msc1::kanR his?*	This study
NW2949	*h- msc1::hygB mis16-GFP [ura+] leu1-*	This study
NW2950	*h- msc1::hygB mis15-GFP [ura+] leu1-*	This study

Data and reagent availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All strains constructed in the course of this study will be made available by request.

Results

Increased expression of msc1 improves viability of mis16/18 mutants and reduces mis16-53 chromosome missegregation

Fission yeast strains with hypomorphic alleles of kinetochore proteins encoded by *mis16* and *mis18* exhibit hyperacetylation of centromeric histones (Hayashi *et al.* 2004). Given the observed genetic interactions of *msc1* with mutants defective in kinetochore function (Ahmed *et al.* 2007) and the observation that Msc1 coprecipitates HDAC activity (Ahmed *et al.* 2007), we asked whether an increase in gene dosage of *msc1* can suppress the growth defect of strains with conditional, ts mutations in *mis16* and *mis18*. The *mis16-53* and *mis18-262* mutant strains were transformed with a multi-copy plasmid expressing full length Msc1 (p*msc1*), or with the empty vector pSP1 (Cottarel *et al.* 1993). Each transformed cell is expected to contain 5–10 copies of such plasmids, as the *S. pombe ars1* sequence allows replication (Forsburg 1993). To assess growth, spotting assays were conducted by growing cells to midlog phase at the permissive temperature of 25° followed by plating a series of fivefold dilutions at permissive (25°), semirestrictive (33°), and restrictive temperature (36°). As shown in Figure 1A, extra copies of the wild-type

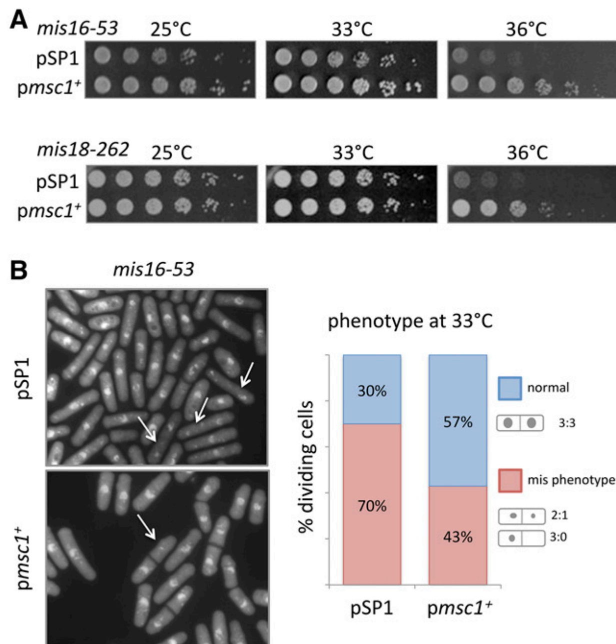

Figure 1 Extra copies of *msc1* restore viability to Mis16/18 complex mutants and decrease the frequency of the *mis* phenotype in *mis16-53*. (A) Strains with mutations in the Mis16/18 complex *mis16-53* and *mis18-262* were transformed with an empty vector pSP1 or plasmid carrying the *msc1* gene. The indicated strains were grown to midlog phase, serially diluted (fivefold), spotted on PMA-leucine plates and incubated at 25, 33, and 36°. (B) Cells of a *mis16-53* strain transformed with either empty vector (pSP1) or p*msc1*+ were grown to midlog phase then shifted to 33° for 8 hr. Cells were stained with DAPI and photographed by fluorescence microscopy. Arrows indicate the *mis* phenotype. Right panel: quantification of the percentage of dividing cells exhibiting the mis phenotype after shift to 33° for 8 hr ($n > 200$).

msc1 gene allowed both *mis16-53* and *mis18-262* mutant strains to grow at 36°.

At restrictive temperature, the *mis16* and *mis18* mutants exhibit chromosome missegregation such that most cells in the population arrest with an unequal distribution of chromosomes in the daughter cells (Takahashi *et al.* 1994; Hayashi *et al.* 2004). To test whether extra copies of *msc1* relieve the missegregation phenotype, we grew *mis16-53* and *mis18-262* mutant strains containing empty vector (pSP1) or full length *msc1* (p*msc1*) at 25° to midlog phase. Cells were shifted to 33° for 8 hr and stained with DAPI to visualize chromosomes (Figure 1B). In normally divided daughter cells, DNA should distribute evenly as observed in wild-type cells. In contrast, mutations in kinetochore components result in a chromosome missegregation phenotype characterized by daughter cells with unequal division of chromosomes (Takahashi *et al.* 1994; Saitoh *et al.* 1997; Hayashi *et al.* 2004): cells either contain large and small nuclei, with an apparent DNA ratio of 2:1, or all of the chromosomes segregate to one cell resulting in a DNA distribution of 3:0 (Figure 1B). Quantification of the fraction of the binucleate cell population with the *mis* phenotype reveals that 70% of *mis16-53* strains with the empty vector (pSP1) displayed the *mis* phenotype at 33° (Figure 1B). With extra copies of *msc1*, the fraction of cells with unequal chromosome segregation decreased to 43%. Thus, increased expression of *msc1* in the *mis16-53* strain improves viability and decreases the frequency of the *mis* phenotype.

Deletion of msc1 decreases viability of mis16/18 ts mutants and increases frequency of the mis phenotype associated with mis16-53

Given that elevated levels of Msc1 suppress the ts chromosome segregation and viability defect of the *mis16/18* ts mutants, we hypothesized that loss of function of *msc1* might further compromise the phenotype of *mis16/18* ts mutants. Strains with a null allele of *msc1* were crossed with *mis16-53* or *mis18-262* strains to generate double mutants. To test for the impact of loss of *msc1* function on growth of the *mis16* and *mis18* mutants, strains were grown at 25° overnight before preparing fivefold serial dilutions to plate at 25, 33, and 36°. As shown in Figure 2A, at permissive temperature (25°), cells of both single- and double-mutant strains are viable. At semipermissive temperature (33°), *mis16-53* grows well, while *mis16-53 msc1*Δ lost viability. At 36°, the *mis16-53* single mutant retains some viability, but the double mutant is completely inviable. While the *mis18*-262 mutant retains viability even at 36°, the *mis18-262 msc1*Δ strain is inviable at that temperature. Thus, loss of *msc1* lowers the restrictive temperature of *mis16/18* ts mutants suggesting that viability of the kinetochore mutants is compromised when Msc1 is absent.

To determine whether loss of Msc1 function alters the missegregation phenotype, we examined DAPI-stained *mis16-53* cells with and without *msc1* by fluorescence microscopy. The *mis16-53* and *mis16-53 msc1*Δ strains were grown to midlog phase at 25°, then shifted to 33° for 8 hr, fixed and examined by microscopy. As shown and quantified in Figure 2B, the frequency of cells with the *mis* phenotype at 33° is elevated in *mis16-53* cells lacking *msc1*. Whereas 55% of *mis16-53* cells exhibit the *mis* phenotype at 33°, 83% of *mis16-53 msc1*Δ show unequal chromosome segregation at this temperature. Without *msc1*, aberrant chromosome segregation caused by *mis16-53* and *mis18-262* mutants is exacerbated, consistent with the decreased viability of the double mutant cells as compared to the single mutants.

Msc1 downregulates Mis16 protein localization at the centromere without changing protein level

Other laboratories have reported that localization to the centromere of Mis16 and Mis18 proteins is codependent (Hayashi *et al.* 2004): when either Mis16 or Mis18 is mutated, localization to the centromere of the other is compromised. Given the observed genetic interactions of *msc1* with *mis16/18*, we sought to determine whether Msc1 might affect localization of the Mis16 component of the Mis16/18 complex.

We acquired a strain in which the endogenous *mis16*+ gene is tagged with GFP (Hayashi *et al.* 2004). By combining the tagged *mis16*:GFP strain with a deletion of *msc1*, we could analyze whether recruitment of Mis16 protein to the centromere

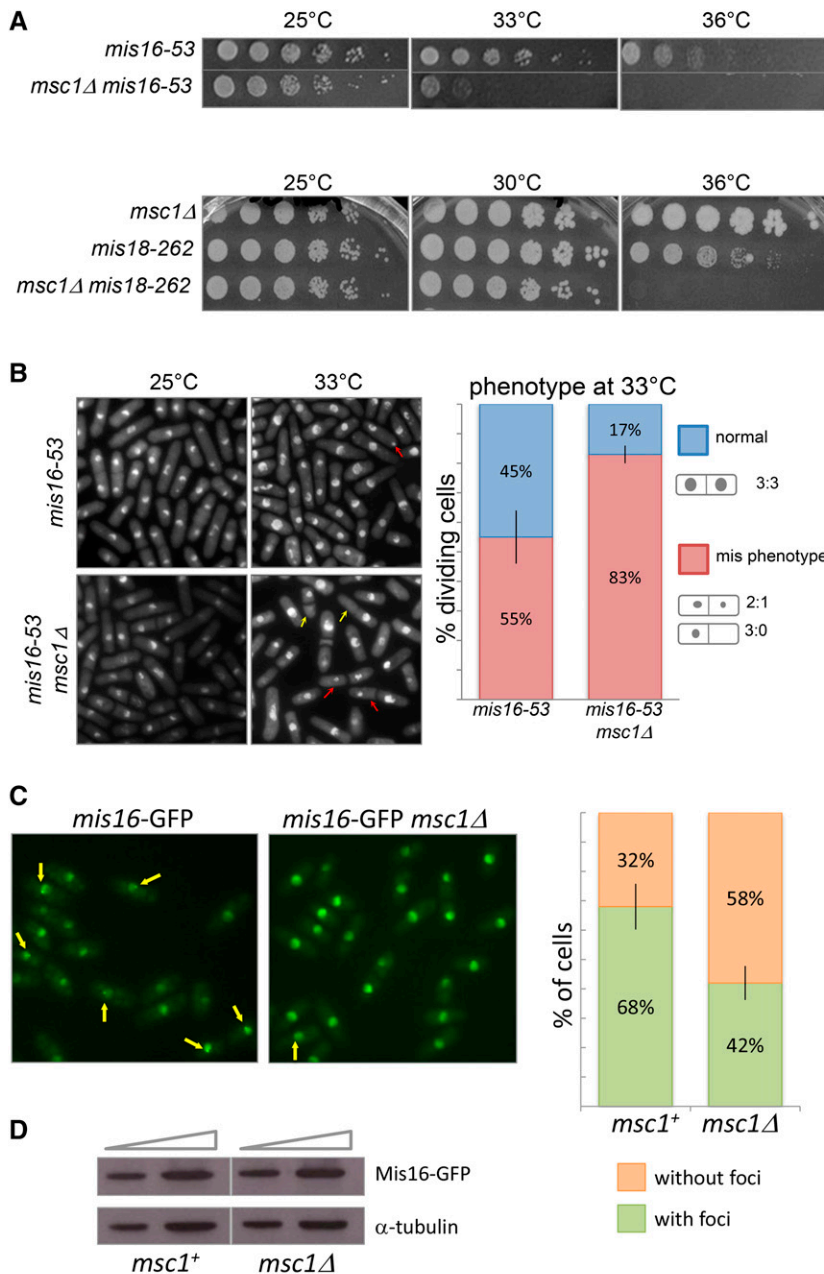

Figure 2 In the absence of *msc1*, the viability of mis16/18 mutants is compromised, the frequency of the *mis* phenotype associated with *mis16-53* is increased, and localization of Mis16 to the centromere is reduced. (A) The indicated strains were grown to midlog phase and fivefold serial dilutions were prepared, spotted on minimal media, and incubated for 3–4 days at the indicated temperatures. (B) Representative images of methanol-fixed cells grown to midlog phase at 25° (permissive temperature) and shifted to 33° (restrictive temperature) for 8 hr. Cells were stained with DAPI and photographed by fluorescence microscopy. Arrows indicate the *mis* phenotype: red (2:1), yellow (3:0). Right panel: quantification of the percentage of dividing cells exhibiting the *mis* phenotype after shift to 33° for 8 hr ($n > 200$). Data represent the mean of three counts of the number of cells exhibiting a normal *vs.* a *mis* phenotype; error bars indicate the SEM; the *P*-value for a two-tailed, unpaired *t*-test is 0.047. (C) The indicated strains with an integrated allele of *mis16* fused to GFP were grown at 25° to midlog phase. Localization of GFP-tagged Mis16 with or without *msc1* was observed by taking Z-stack pictures using a fluorescence microscope. Quantification of the frequency of cells with localization of Mis16-GFP to single foci is shown on the right. Data represent the mean of four determinations of protein localization with error bars indicating the SEM; the *P*-value for a two-tailed, unpaired *t*-test is 0.039. (D) GFP-tagged strains with wild-type *msc1* and with *msc1* deletion were grown to midlog phase. Two quantities of protein lysate from the indicated strains were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with antibody to GFP to detect Mis16-GFP. Detection of α-tubulin was utilized as a loading control.

would be altered. As reported (Hayashi *et al.* 2004), Mis16-GFP appears diffusely in the nucleus as well as in discrete foci by fluorescence microscopy, representing localization of Mis16-GFP to the centromere. GFP signals were observed at different focal depths by capturing individual images through Z-stack scanning. A single representative focal plane image is presented for Mis16-GFP cells in the left panel of Figure 2C. After collecting all image slices, the frequency of Mis16-GFP foci (indicated by yellow arrows) were scored and plotted. As shown in the right panel of Figure 2C, and, consistent with previously reported results (Hayashi *et al.* 2004), 68% of wild-type cells have foci. In cells lacking *msc1*, the percentage of cells with foci is reduced to 42%. Given that Mis16 undergoes a cycle of association and dissociation from the centromere during each cell cycle, this result suggests that Msc1 either facilitates Mis16 localization to the centromere or that the absence of *msc1* prolongs the period during which Mis16 is delocalized.

To determine whether the diminished localization of Mis16-GFP might be due to a change in protein abundance, we prepared cell lysates for Western blot analysis with anti-GFP antibody. Antibody to α-tubulin was used as a loading control. As shown in Figure 2D, the abundance of Mis16-GFP is equivalent in cells with and without *msc1*. Thus, deletion of *msc1* does not change the protein expression level of Mis16.

C-terminal PHDs of Msc1 are necessary for kinetochore mutant suppression

To gain insight into the domains of Msc1 necessary for supporting kinetochore function, we made use of previously

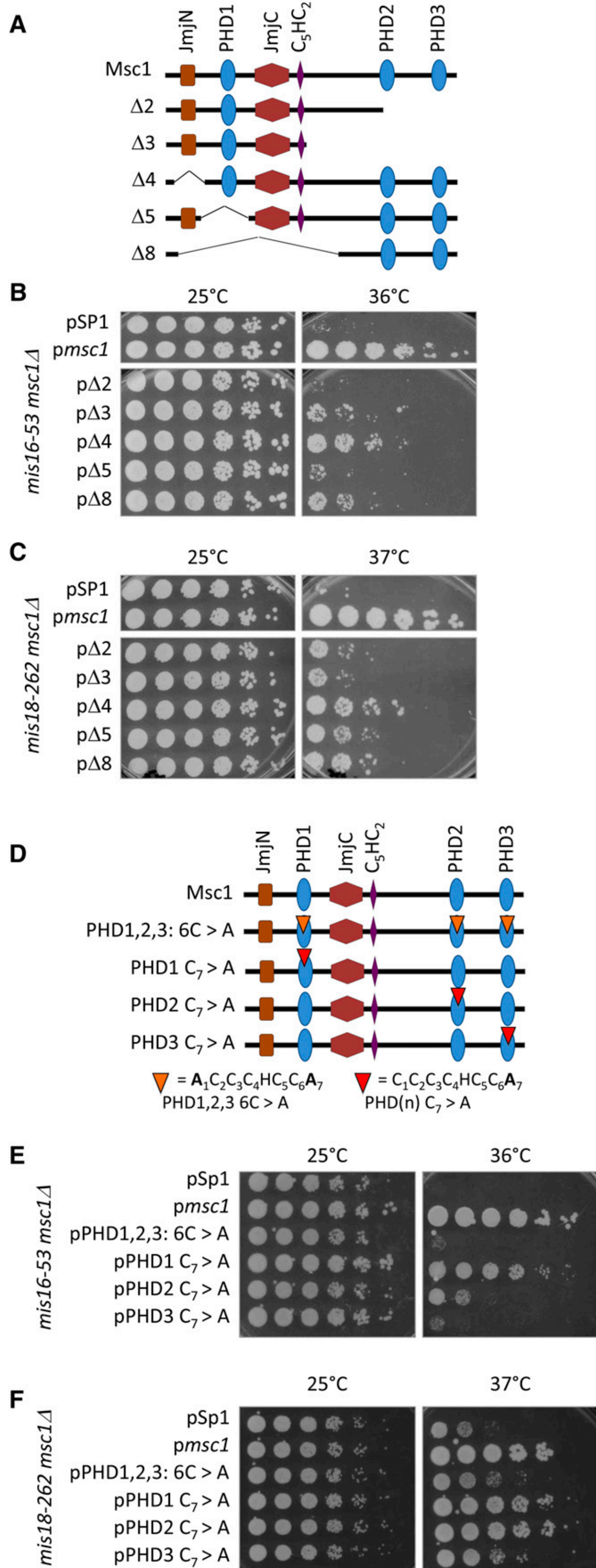

Figure 3 The C-terminal PHD domains of Msc1 are important for its function when *mis16* or *mis18* are compromised. (A) Schematic of Msc1 deletion mutations (Ahmed *et al.* 2004). (B, C) The indicated plasmids were transformed into the indicated strains. Cells were grown to midlog phase in PMA-leucine at 25°, serially diluted, then spotted on PMA-leucine plates (B), or YES plates (C), and incubated at the indicated temperatures. (D) Schematic of point mutations in Msc1 (Dul and Walworth 2007). (E, F) The indicated plasmids were transformed into the indicated strains. Cells were grown to midlog phase in PMA-leucine at 25°, serially diluted, then spotted on to PMA-leucine plates (E) or YES plates (F) and incubated at the indicated temperatures. YES plates were used for assaying *mis18* growth as the temperature sensitivity is more pronounced on YES than on minimal media.

constructed *msc1* plasmids (Ahmed *et al.* 2004; Qiu *et al.* 2010) with deletions of one or more conserved domains (Figure 3A). Plasmids containing deletion alleles were transformed into *mis16-53 msc1*Δ and *mis18-262 msc1*Δ, along with empty vector (pSP1) and a plasmid expressing full-length *msc1* as negative and positive controls, respectively. As shown in Figure 3B, when the wild-type *msc1* gene is introduced, cells are able to grow on plates at 36°, whereas the empty vector does not support any growth at this temperature. A plasmid missing the jmjN domain (Δ4) or N-terminal half of *msc1* (Δ8) allowed some growth. In contrast, deletion constructs lacking PHD domains (Δ2, Δ3, and Δ5) seem incapable of supporting viability of the *mis16-53 msc1*Δ mutant. Similar observations were found in the *mis18-262 msc1*Δ strain when the same plasmids were introduced (Figure 3C). Thus, we conclude that the PHD domains are necessary for Msc1 to confer viability on cells that have compromised kinetochore function due to mutations in *mis16* or *mis18*.

While the PHD domains seem to be critical for Msc1 function in the context of compromised kinetochore function, deletion mutants may affect protein folding. We have shown in previous studies that point mutations of conserved cysteine residues in the PHD domains of Msc1 affect their activity as E3 ubiquitin ligases (Dul and Walworth 2007). Therefore, we transformed plasmids containing point mutations within the PHD1, PHD2, and PHD3 domains into *mis16-53 msc1*Δ and *mis18-262 msc1*Δ strains to assess growth. As shown in Figure 3D, if the first and seventh cysteine in all three PHD domains of *msc1* are mutated (6C > A), cells are unable to grow at 36°. With a single point mutation in the N-terminal PHD1 domain, *mis16-53 msc1*Δ cells grow well at 36° (Figure 3E), which indicates that PHD1 may not be important for Msc1 function in the context of compromised kinetochore function due to mutation in *mis16*. However, point mutations in PHD2 and PHD3 dramatically reduce the ability of Msc1 to function in the context of compromised *mis16* function, as neither the PHD2C_7 > A nor the PHD3C_7 > A plasmids support robust growth at 36°. For the *msc1*Δ *mis18-262* mutant, PHD3 seems to be most critical as PHD3C_7 > A is most compromised for growth (Figure 3F). Previous results have shown that C-terminal PHD2 and PHD3 domains of Msc1 are important for precipitating HDAC activity (Ahmed *et al.* 2004). Given that *mis16-53* or *mis18-262* mutations increase centromeric histone acetylation levels at the centromere and

reduce cell viability (Hayashi *et al.* 2004), our results are consistent with the hypothesis that, through its C-terminal PHD domains, Msc1 may recruit HDAC to the centromere to suppress compromised kinetochore mutants.

Msc1 restores viability to CCAN mutants

We have shown previously that increased expression of *msc1* restores viability to cells with defective CENP-A due to a ts allele of *cnp1* (Ahmed *et al.* 2007). CENP-A loading at the centromere is facilitated through a largely conserved set of proteins that associate with CENP-A nucleosomes and constitute the constitutive centromere-associated network or CCAN (Cheeseman and Desai 2008). To test whether elevating *msc1* levels might rescue mutations in components of the CCAN, we transformed cells with either an empty vector or a plasmid expressing *msc1* into ts mutants of *mis6*, *mis15*, and *mis17*, which encode CENP-I, CENP-N, and CENP-U, respectively. As shown in Figure 4A, multicopy expression of *msc1* restores growth to each of these strains, though for *mis6*, suppression is achieved only at the restrictive temperature of 33°, and not at 36°. As shown in Figure 4B, extra copies of *msc1* also alleviates the *mis* phenotype, increasing the number of cells undergoing a normal mitosis in the case of *mis15-68*, by ~1.7-fold.

We showed previously that deletion of *msc1* compromises the viability of a *mis6* mutant (Ahmed *et al.* 2007). To test whether *msc1* is important for survival of mutants with defects in the CCAN components encoded by *mis15* and *mis17*, we generated double mutants of *mis15-68* and *mis17-362* with *msc1*Δ. As shown in Figure 5, A and B, deletion of *msc1* compromises the viability of both *mis15* and *mis17*, and exacerbates the *mis* phenotype in these cells at 33°: the frequency of the *mis* phenotype for *mis15-68* at 33° is increased from 39% when Msc1 is present in the cells to 85% when cells lack *msc1*.

To assess which domains of Msc1 are important for function when the CCAN is compromised, we introduced plasmids harboring deletions of various domains of Msc1 into double mutant cells with ts *mis15* or *mis17* alleles in combination with deletion of *msc1*. As shown in Figure 6A, truncation of the C-terminal domain of Msc1 eliminates Msc1 function in the context of *mis15-68* or *mis17-362*, consistent with our previous report on *mis6-305* (Ahmed *et al.* 2007). Similarly, as shown in Figure 6B, whereas a point mutation changing cysteine to alanine in position seven of PHD1 retains function in the CCAN mutants, mutating the analogous cysteine in either PHD2 or PHD3 abolishes function. Thus, as is the case for *mis6-305* (Ahmed *et al.* 2007), PHD2 and PHD3 are important for Msc1 function when *mis15* or *mis17* are compromised.

Mis15 associates with the kinetochore more frequently in cells lacking msc1

Some proteins of the CCAN, including CENP-N, the mammalian homolog of Mis15, have been shown to exhibit dynamic association with the centromere during cell cycle progression

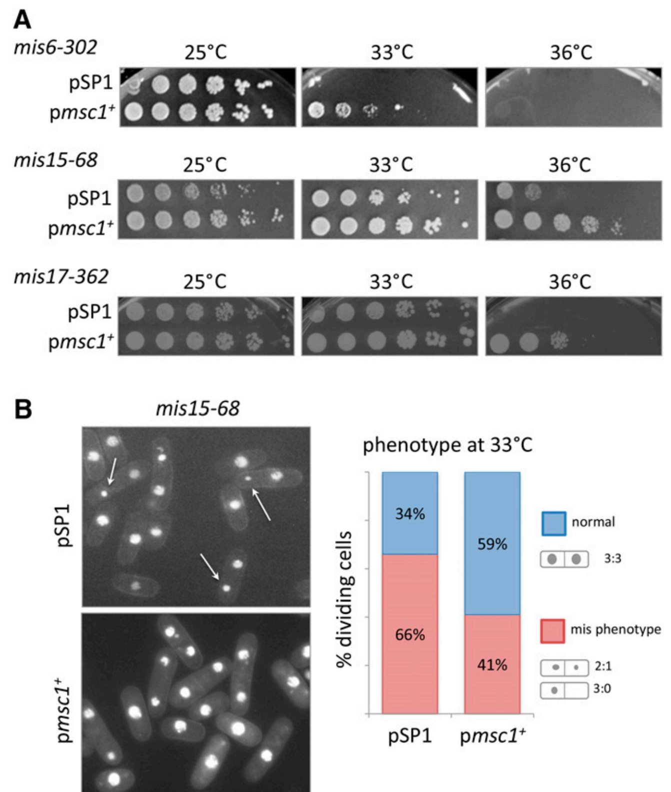

Figure 4 Extra copies of *msc1* restore viability to CCAN mutants, and decrease the frequency of the *mis* phenotype. (A) Strains with mutations in CCAN kinetochore *mis* mutants, *mis6-302*, *mis15-68*, and *mis17-362* were transformed with an empty vector pSP1 or plasmid carrying the *msc1* gene. The indicated strains were grown to midlog phase, serially diluted (fivefold), spotted on PMA-leucine plates, and incubated at 25, 33, and 36°. (B) Cells of a *mis15-68* strain transformed with either empty vector (pSP1) or p*msc1*+ were grown to midlog phase then shifted to 33° for 8 hr. Cells were stained with DAPI and photographed by fluorescence microscopy. Arrows indicate the *mis* phenotype. Right panel: quantification of the percentage of dividing cells exhibiting the *mis* phenotype after shift to 33° for 8 hr ($n > 200$).

(Hellwig *et al.* 2011; Fang *et al.* 2015). To examine kinetochore association of Mis15 in cells with and without Msc1, we utilized a strain with a chromosomal copy of *mis15* tagged with GFP. As shown in Figure 7A, and consistent with previously reported results (Hayashi *et al.* 2004), ~70% of wild-type cells expressing Mis15-GFP show characteristic punctate foci indicative of association with the centromere. Curiously, in cells lacking *msc1*, >80% of cells show the presence of foci, suggesting that centromeric association of Mis15 persists when *msc1* is absent. As was the case for Mis16, the abundance of Mis15-GFP as determined by Western blot analysis is unchanged in cells with and without *msc1* (Figure 7B).

Extra copies of cnp1 or msc1 act independently to improve viability of CCAN mutants

As previously reported by others, extra copies of CENP-A expressed from a plasmid encoding *cnp1* can rescue the ts growth defect of several *mis* mutants (Hayashi *et al.* 2004). Similarly, extra copies of *msc1* rescue *mis16/18* (Figure 1) as

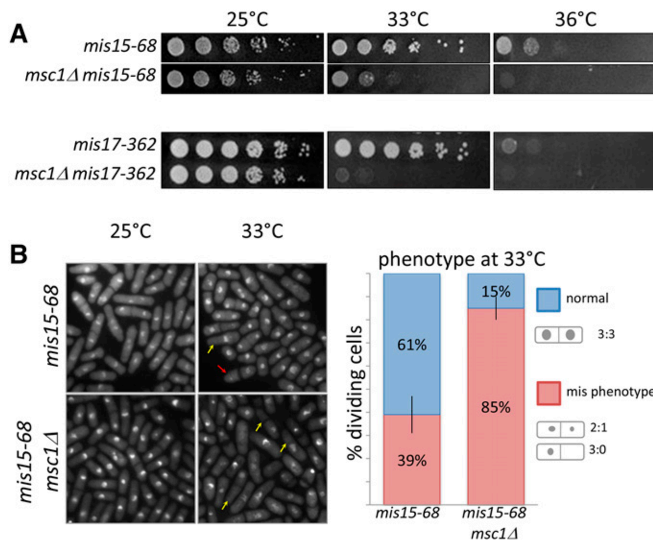

Figure 5 In the absence of *msc1*, viability of CCAN mutants is compromised and the frequency of the *mis* phenotype is elevated. (A) The indicated strains were grown to midlog phase and fivefold serial dilutions were prepared, spotted on minimal media, and incubated for 3–4 days at the indicated temperatures. (B) Representative images of methanol-fixed cells grown to midlog phase at 25° (permissive temperature) and shifted to 33° (restrictive temperature) for 8 hr. Cells were stained with DAPI and photographed by fluorescence microscopy. Arrows indicate the *mis* phenotype: red (2:1 segregation), yellow (3:0 segregation). Right panel: Quantification of the percentage of dividing cells exhibiting the *mis* phenotype after shift to 33° for 8 h ($n > 200$). Data represent the mean of three counts of the number of cells exhibiting a normal *vs.* a *mis* phenotype; error bars indicate the SEM; the *P*-value for a two-tailed, unpaired *t*-test is 0.009.

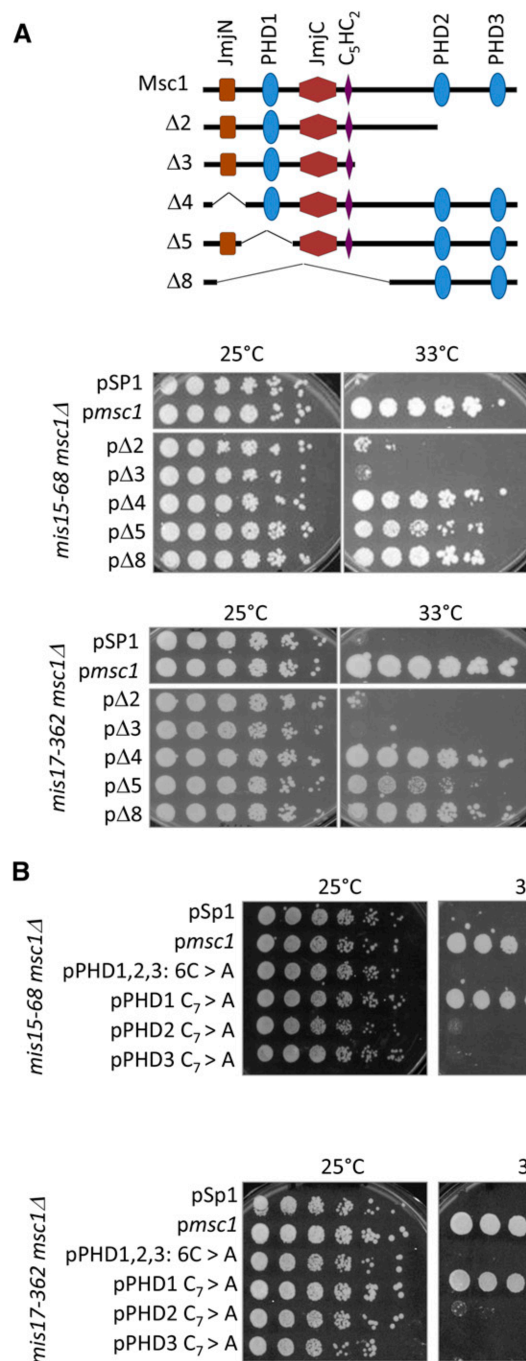

Figure 6 The C-terminal PHD domains are important for Msc1 function when CCAN function is compromised. (A) Schematic of plasmids expressing deletion mutants of Msc1 as described in Figure 3. Plasmids were transformed into the indicated strains. Cells were grown to midlog phase at 25°, serially diluted, then spotted on to PMA-leucine plates and incubated at the indicated temperatures. (B) The indicated plasmids (shown schematically in Figure 3D) were transformed into the indicated CCAN mutant strains. Cells were grown to midlog phase at 25°, serially diluted, then spotted on PMA-leucine plates and incubated at the indicated temperatures.

well as mutants in CCAN components (Figure 4). We entertained the possibility that *msc1* might rescue *mis* mutants by elevating expression of *cnp1*. To test this possibility, we expressed *msc1* in cells with a GFP-tagged allele of *cnp1* incorporated in the genome. Cells transformed with an empty vector served as a control for Cnp1-GFP protein expression. As shown in Figure 8A, there is no apparent difference in the amount of Cnp1-GFP protein expressed in cells at either 25 or 33° when cells were transformed with the empty vector (pSP1) or with p*msc1*. Thus, it seems unlikely that *msc1* rescues *mis* mutants by elevating Cnp1. To determine whether *cnp1* and *msc1* might be acting through different mechanisms, we asked whether *cnp1* on a plasmid could rescue CCAN mutants *mis15* and *mis17* when *msc1* is absent from cells. As shown in Figure 8, B and C, p$cnp1^+$ rescues both the *mis15-68* and *mis17-362* mutants in the absence of *msc1*. Thus, we conclude that p$cnp1^+$ rescue of the CCAN mutants does not require *msc1*.

Discussion

Kinetochore proteins Mis16 and Mis18 form the Mis16/18 complex required for localization of newly synthesized histone H3 variant CENP-A^{Cnp1} to the centromere (Hayashi *et al.* 2004; Fujita *et al.* 2007). By mutating either of these kinetochore proteins, unequal chromosome segregation resulting in the *mis* phenotype occurs (Hayashi *et al.* 2004). Furthermore, kinetochore mutations decrease the frequency of CENP-A^{Cnp1} localization, and elegant mechanisms have

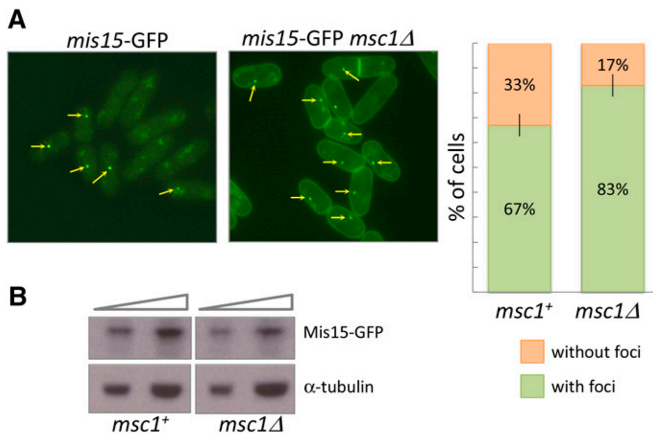

Figure 7 In the absence of Msc1, the frequency at which Mis15 localizes to the centromere is elevated. (A) The indicated strains with an integrated allele of *mis15* fused to GFP were grown at 25° to midlog phase. Localization of GFP-tagged Mis15 with or without *msc1* was observed by taking Z-stack pictures using a fluorescence microscope. Quantification of the frequency of cells with localization of Mis15-GFP to single foci is shown on the right. Data represent the mean of four determinations of protein localization, with error bars indicating the SEM; the *P*-value for a two-tailed, unpaired *t*-test is 0.045. (B) GFP-tagged strains with wild-type *msc1* and with *msc1* deletion were grown to midlog phase. Two quantities of protein lysate from the indicated strains were transferred to nitrocellulose membrane, and incubated with antibody to GFP to detect Mis15-GFP. Detection of α-tubulin was utilized as a loading control.

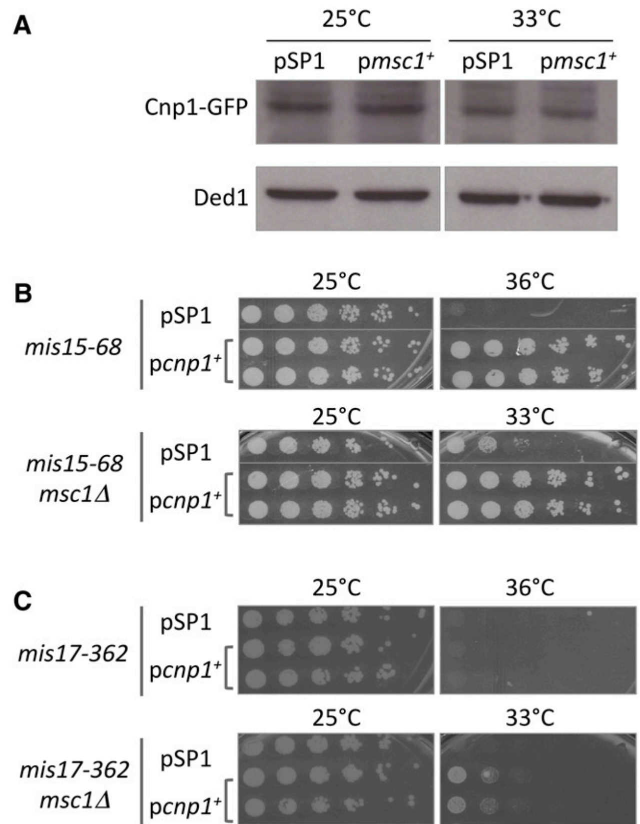

Figure 8 Extra copies of *cnp1*+ rescue viability of CCAN mutants even in the absence of *msc1*. (A) Elevated expression of *msc1* does not alter the expression of Cnp1-GFP. A *mis6-302* mutant with a chromosomally integrated Cnp1-GFP allele strain was transformed with empty vector pSP1 or p*msc1*. Cells were grown to midlog phase at 25°, and then shifted to 33° for 5 hr. Cell lysates were prepared and aliquots separated on SDS-PAGE then transferred to nitrocellulose. Western blot assays were conducted by incubating with antibody to Cnp1-GFP (anti-GFP) or Ded1 (anti-Ded1) as a loading control. (B, C) CCAN *mis* mutants and *mis msc1*Δ double mutants were transformed with pSP1 or p*cnp1* encoding CENP-A. Cells were grown to midlog phase at 25°, serially diluted, then spotted on PMA-leucine plates and incubated at the indicated temperatures for 3–4 days.

been proposed to account for the role of Mis18 orthologs in this process (Nardi *et al.* 2016). Whereas fission yeast Mis18 forms a homotetramer via YIPPEE domains in the N-terminus, the conserved and related human Mis18α and Mis18β proteins form a heterotetramer (Nardi *et al.* 2016; Subramanian *et al.* 2016). In both organisms, the Mis18 oligomers serve to target Mis18BP1 and the histone chaperone HJURP to the centromere to facilitate loading of CENP-A. Notably, Mis18 is not conserved in budding yeast, which, rather than having a regional centromere composed of multiple CENP-A nucleosomes interspersed in an array of histone-H3-containing nucleosomes, has single CENP-A nucleosomes on each chromosome that form point centromeres (Sullivan *et al.* 2001).

Like Mis18, Msc1 is not conserved in budding yeast, though it plays a critical role in maintaining genomic stability in fission yeast (Ahmed *et al.* 2004). While nonessential for viability, strains harboring a deletion of *msc1* exhibit elevated levels of chromosome loss, the lagging chromosome phenotype, and compromised viability when combined with conditional mutants in a number of genes encoding proteins involved in chromosome segregation. Furthermore, multi-copy expression of *msc1* robustly suppresses a ts CENP-A^{Cnp1} mutant (*cnp1-1*), while localization of CENP-A^{Cnp1} to the centromere is compromised in *msc1* null cells (Ahmed *et al.* 2007). Homologs of Msc1, which comprise the four members of the KDM5 family of proteins, are conserved in mammalian cells (Ahmed *et al.* 2004), and implicated in a range of nuclear functions including transcriptional repression and histone modifications (Barrett *et al.* 2002; Benevolenskaya *et al.* 2005; Liefke *et al.* 2010; Nishibuchi *et al.* 2014).

In the present study, the results of phenotypic and genetic analysis of Msc1 lead us to hypothesize that Msc1 affects chromosome stability by influencing kinetochore protein function. Extra copies of *msc1* suppress loss of function of the kinetochore mutant proteins in the Mis16/18 complex, relieving the ts growth defect of mutants in either subunit, and the chromosome missegregation (mis) phenotype associated with *mis16-53*. Given that Mis16/18 complexes are required for CENP-A^{Cnp1} deposition and normal chromosome segregation, we conclude that Msc1 may affect Mis16 and Mis18 protein function by either changing the environment of the centromere to facilitate Mis16/18 complex recruitment to the centromere, or supporting or compensating for functions of the Mis16/18 complex to influence CENP-A^{Cnp1} recruitment.

Msc1 associates with the Swr1 complex, which exchanges histone H2A.Z for histone H2A (Qiu *et al.* 2010) at discrete

locations in the genome. H2A.Z has been reported to localize to the fission yeast centromeric outer repeats (Lawrence and Volpe 2009; Qiu *et al.* 2010). Curiously, deletion of *msc1* or *swr1* relieves the mitotic arrest phenotype of cells with a cold-sensitive mutation in *dis1* (Qiu *et al.* 2010). Dis1 encodes a protein that facilitates microtubule polymerization (Ohkura *et al.* 1988, 2001). Analysis of the suppression of *dis1* mutants by *msc1*Δ suggests that deletion of *msc1* alters the chromatin landscape at the centromere in such a way as to permit productive microtubule/kinetochore interactions that silence the spindle assembly checkpoint, and relieve the mitotic arrest imposed by inactivation of *dis1* (George and Walworth 2015). The present study suggests that effects of Msc1 on the chromatin landscape at the centromere also influence the function of the Mis16/18 complex and the inner kinetochore CCAN complex. Increased expression of Msc1 improves viability and alleviates chromosome missegregation in ts mutants in subunits of both Mis16/18 and the discrete complexes that comprise the CCAN. Furthermore, in cells deficient for Msc1, localization is altered for representative subunits of these complexes, namely Mis16 and Mis15.

The early description of the CCAN as a stable platform upon which dynamic components of the kinetochore associate during cell cycle progression has been revised in recent years as it has become apparent that some components display cell-cycle-dependent association with the centromere. For example, CENP-N (Mis15 in fission yeast), which is thought to bind directly to the CATD domain of CENP-A, binds to kinetochores during S phase and G2 phase in mammalian cells, but is dissociated during mitosis and G1 phase (Hellwig *et al.* 2011). A particular region of CENP-A that becomes blocked when chromatin is compacted as cells progress through mitosis into G1 has been proposed to act as the switch for CENP-N binding (Fang *et al.* 2015). We observe that, in cells lacking Msc1, Mis15 exhibits increased frequency of association with the centromere (Figure 7), suggesting that dissociation may be reduced when the function of Msc1 is absent. One model to explain this observation is that, in the absence of *msc1*, the CATD of CENP-A may be altered in such a way that CENP-N (Mis15) has a higher propensity to remain associated with it, thereby prolonging its association with the centromere. Further studies to analyze in detail the timing of Mis15 association with the centromere in the presence and absence of Msc1 may reveal possible mechanisms to explain the observed data.

Rapid advances in identifying the protein subcomplexes that assemble to form the kinetochore, and of the mechanisms that lead to its formation, have led to elegant models for kinetochore structure and function (Cheeseman 2014; Dimitrova *et al.* 2016; Nardi *et al.* 2016; Petrovic *et al.* 2016; Subramanian *et al.* 2016). Unlike the proteins that comprise the core components of the kinetochore, Msc1 is nonessential, though it appears to modulate the ability of kinetochore proteins to execute their essential functions. As a member of the KDM5 family of proteins, Msc1 and its mammalian counterparts possesses conserved functional domains that may contribute to their cellular roles, and could serve as relevant drug targets to modulate function. The PHD domains have been shown to facilitate ubiquitylation of substrate proteins (Dul and Walworth 2007) and the JmjC domain of the mammalian protein KDM5A exhibits demethylase activity (Christensen *et al.* 2007; Klose *et al.* 2007). KDM5 family proteins are found in various protein complexes with other proteins that mediate chromatin modification. As such, they may serve regulatory roles and modulate local chromatin structure to affect a variety of nuclear functions: transcription, chromosome compaction, or chromosome segregation. While the absence of Msc1 is not lethal to the single celled organism *S. pombe*, genome integrity is compromised as cells exhibit an elevated rate of chromosome loss, evidenced by the appearance of lagging chromosomes indicative of merotelic chromosome attachments to the mitotic spindle. Such attachments may lead to aneuploidy. Significantly, under circumstances in which microtubule polymerization is compromised by mutation of the *dis1* gene leading to mitotic arrest, the elevated frequency of merotelic attachments conferred by loss of function of Msc1 or particular HDACs, allows cells to escape from mitotic arrest (George and Walworth 2015). Thus, deciphering ways in which Msc1 and the KDM5 proteins might be modulated has implications for discovering new ways to interfere with mitosis and to appreciate how cells might overcome mitotic blocks.

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