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Msc1 Acts Through Histone H2A.Z to Promote Chromosome Stability in *Schizosaccharomyces pombe*

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ABSTRACT

As a central component of the DNA damage checkpoint pathway, the conserved protein kinase Chk1 mediates cell cycle progression when DNA damage is generated. Msc1 was identified as a multicopy suppressor capable of facilitating survival in response to DNA damage of cells mutant for *chk1*. We demonstrate that loss of *msc1* function results in an increased rate of chromosome loss and that an *msc1* null allele exhibits genetic interactions with mutants in key kinetochore components. Multicopy expression of *msc1* robustly suppresses a temperature-sensitive mutant (*cnp1-1*) in the centromere-specific histone H3 variant CENP-A, and localization of CENP-A to the centromere is compromised in *msc1* null cells. We present several lines of evidence to suggest that Msc1 carries out its function through the histone H2A variant H2A.Z, encoded by *pht1* in fission yeast. Like an *msc1* mutant, a *pht1* mutant also exhibits chromosome instability and genetic interactions with kinetochore mutants. Suppression of *cnp1-1* by multicopy *msc1* requires *pht1*. Likewise, suppression of the DNA damage sensitivity of a *chk1* mutant by multicopy *msc1* also requires *pht1*. We present the first genetic evidence that histone H2A.Z may participate in centromere function in fission yeast and propose that Msc1 acts through H2A.Z to promote chromosome stability and cell survival following DNA damage.

THE fission yeast *Schizosaccharomyces pombe* has proven to be a useful model system for studies of cell cycle events, including DNA replication, mitosis, and cytokinesis. These events must be executed with high fidelity to ensure chromosome integrity. Checkpoints monitor key events during the cell cycle and block subsequent events if earlier ones are incomplete, thereby increasing the fidelity of DNA replication and chromosome segregation (Hartwell and Weinert 1989; Murray 1992; Hartwell and Kastan 1994). The DNA damage checkpoint is activated in response to genotoxic stress such as irradiation or aberrant DNA replication (O'Connell *et al.* 2000) and delays cell cycle progression by inhibiting the activity of cyclin-dependent kinases (Cdk's), key regulators of cell cycle progression in all eukaryotic cells (Morgan 1997). In fission yeast, Cdc2 is the primary Cdk, which is dephosphorylated at tyrosine 15 to promote entry into mitosis (Gould and Nurse 1989). Tyrosine phosphorylation of Cdc2 is maintained by Wee1 and Mik1 (Lundgren *et al.* 1991), while the Cdc25 phosphatase dephosphorylates Cdc2 at the same site to initiate mitosis (Millar *et al.* 1991). In response to DNA damage, the protein kinase Chk1 is phosphorylated and inhibits mitotic entry by phosphorylating Wee1 and Cdc25 to prevent activation of Cdc2 (Rhind *et al.* 1997; Guo *et al.* 2000; Liu *et al.* 2000; Raleigh and O'Connell 2000; Lopez-Girona *et al.* 2001; Capasso *et al.* 2002). The phosphorylation of Chk1 is dependent on the protein kinase Rad3 (Walworth and Bernards 1996), which belongs to a subgroup of a phosphatidylinositol 3 kinase-like family that includes the human proteins ATM and ATR (Abraham 2001; Shiloh 2001; Melo and Toczyski 2002).

A fission yeast protein, Msc1, related to mammalian proteins RBP2 and PLU-1, was identified as a multicopy suppressor of cells defective for *chk1* function (Ahmed *et al.* 2004). RBP2, first isolated as a protein that interacts with the tumor suppressor Rb, is postulated to act as both a transcriptional activator and a repressor, depending on the context (Chan and Hong 2001; Benevolenskaya *et al.* 2005). PLU-1 was first isolated as a transcript upregulated in breast cancer cells that is normally expressed mainly in the testis and during development (Lu *et al.* 1999; Madsen *et al.* 2002). PLU-1 interacts with transcription factors to repress transcription in a reporter assay system (Tan *et al.* 2003). Like RBP2 and PLU-1, Msc1 has multiple domains suggestive of a role in modulating chromatin structure and/or

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function, including three plant homeodomain (PHD) motifs and Jumonji N (JmjN) and Jumonji C (JmjC) domains, although Msc1 lacks the AT-rich interacting domain (ARID) common to RBP2 and PLU-1 (Ahmed *et al.* 2004). While cells lacking *msc1* are relatively resistant to DNA-damaging agents, loss of *msc1* function exacerbates the DNA damage sensitivity resulting from loss of *chk1* function. Furthermore, *msc1* null cells are sensitive to the histone deacetylase inhibitor trichostatin A (TSA), as they lose viability relative to wild-type cells. Thus, it is possible that Msc1 plays a role in maintaining proper chromatin structure and function.

The faithful segregation of chromosomes at mitosis requires the coordinated action of multiple cell cycle checkpoints that monitor replication of the genome and the attachment of sister chromatids to the mitotic spindle apparatus (Amon 1999). Attachment of spindle microtubules to chromosomes occurs through the kinetochore, a specialized protein structure that associates with the centromeric region of the chromosome (Cleveland *et al.* 2003; Amor *et al.* 2004). Nucleosomes at the centromere contain CENP-A, a centromere-specific histone H3 variant. In fission yeast, loading of CENP-A at the centromere is dependent on the Mis6 complex and is promoted by the Ams2 protein (Takahashi *et al.* 2000; Chen *et al.* 2003; Hayashi *et al.* 2004). Mis6, a homolog of vertebrate CENP-I (Saitoh *et al.* 1997; Nishihashi *et al.* 2002), forms a complex with Mis15, Mis17, and Sim4 (Pidoux *et al.* 2003; Hayashi *et al.* 2004). Another evolutionarily conserved kinetochore protein, Mis12, forms a complex with Mis13 and Mis14 (Hayashi *et al.* 2004; Obuse *et al.* 2004), but Mis12 is dispensable for CENP-A loading in fission yeast (Goshima *et al.* 1999, 2003). In vertebrate cells, the Mis12 complex has been proposed to form the core binding site within the kinetochore for the attachment of spindle microtubules (Cheeseman *et al.* 2006). Fission yeast cells bearing mutations in kinetochore complex components exhibit unequal chromosome segregation (Saitoh *et al.* 1997; Goshima *et al.* 1999; Takahashi *et al.* 2000; Pidoux *et al.* 2003). In the event that microtubules do not attach or tension on the spindle is relieved, the mitotic spindle checkpoint is activated to prevent the onset of anaphase, exit from mitosis, and initiation of cytokinesis (Lew and Burke 2003; Pinsky and Biggins 2005). Mis6 is required in fission yeast for loading of the mitotic spindle checkpoint protein Mad2 at the kinetochore (Saitoh *et al.* 2005).

While kinetochore protein structures are key elements in maintaining chromosome stability, the integrity of the centromeric chromatin, upon which kinetochores assemble, is critical as well. The function of mammalian centromeres relies not only on the histone variant CENP-A, but also on the histone H2A variant H2A.Z (Rangasamy *et al.* 2004). Recently, it has been shown that histone H2A.Z and CENP-A form distinct domains within the centromeric region (Greaves *et al.* 2007). While histone H2A.Z has not yet been localized in fission yeast, cells with a disruption in the *pht1* gene encoding histone H2A.Z exhibit chromosome loss (Carr *et al.* 1994). In budding yeast, histone H2A.Z is found throughout the genome, specifically within one or two nucleosomes that flank nucleosome-free promoters, perhaps establishing marks for the activation of transcription (Guillemette *et al.* 2005; Li *et al.* 2005; Raisner *et al.* 2005; Zhang *et al.* 2005; Millar *et al.* 2006). A chromatin-remodeling complex containing the Swr1 protein facilitates the exchange of histone H2A.Z for the core histone H2A (Mizuguchi *et al.* 2004).

In this study, we characterize the phenotype of cells lacking the fission yeast gene *msc1*. Msc1 is required for chromosome stability and exhibits synthetic genetic interactions with at least two critical kinetochore components, Mis6 and Mis12. Multicopy expression of Msc1 suppresses loss of function of the centromere-specific histone H3 variant CENP-A encoded by the temperature-sensitive allele *cnp1-1*. In addition, localization of wild-type CENP-A to the centromere is compromised in cells lacking *msc1*. As Msc1 coprecipitates components of the Swr1 chromatin-remodeling complexes, we also tested whether Msc1 might act through histone H2A.Z. Indeed, the ability of Msc1 to rescue a *chk1* mutant or a *cnp1* mutant requires the *pht1* gene. Given the role of the kinetochore in establishing proper spindle function, we tested whether suppression of *chk1* by Msc1 might require the spindle checkpoint protein Mad2 and found that it does. Thus, we suggest that Msc1 promotes chromosome stability in fission yeast by facilitating deposition or retention of the histone H3 variant CENP-A at the centromere. Furthermore, genetic evidence suggests that the ability of Msc1 to maintain chromosome stability and allow for appropriate responses to cell cycle checkpoints requires the presence of the histone H2A variant H2A.Z.

MATERIALS AND METHODS

Strains and growth condition: Strains are listed in Table 1. Standard genetic methods were utilized for strain construction (Moreno *et al.* 1991). Cells were grown at 30° unless otherwise indicated. Survival following UV treatment was determined as described previously (Walworth *et al.* 1993). For spotting assays, cells were grown to mid-log phase and 10-fold serial dilutions were made. Aliquots of 5 μl of each dilution were spotted on plates. For monitoring mitotic entry in the presence of a microtubule inhibitor, log-phase *cdc25-22* cells were shifted to 36° for 3 hr to synchronize them in G_2 and then shifted to 25° in the presence of 50 μg/ml thiobendazole (TBZ, Sigma, St. Louis). Samples were collected at 20-min intervals, fixed with 70% ethanol, and stained with DAPI and calcofluor before counting binucleate cells under the fluorescent microscope. Immunofluorescence studies were performed as described previously (Ahmed *et al.* 2004) with TAT-1 antibody to α-tubulin (a kind gift of Keith Gull) and DAPI staining to visualize nuclei. For determining the percentage of

TABLE 1

Strains used in this study

Strain	Genotype	Source
SP6	*h⁻ leu1-32*	Lab stock
NW309	*h⁺ leu1-32 nda3-KM311*	Lab stock
NW653	*h⁺ leu1-32 ura4D18 chk1::ura4 cdc17-K42 ade6-216*	Lab stock
NW731	*h⁺ leu1-32 msc1::kan^R cdc25-22*	Lab stock
NW1469	*h⁻ leu1-32 mis12-HA::LEU2*	Mitsuhiro Yanagida
NW1525	*h⁻ leu1-32 ura4D18 cnp1::ura4 lys1⁺::cnp1-1*	Mitsuhiro Yanagida
NW1526	*h⁻ leu1-32 lys1⁺::cnp1-GFP*	Kohta Takahashi
NW1564	*h⁻ leu1-32 msc1::kan^R*	This study
NW1580	*h⁻ leu1-32 mis12-375*	Mitsuhiro Yanagida
NW1601	*h⁻ leu1-32 msc1::kan^R nda3-KM311*	This study
NW1608	*h⁻ leu1-32 msc1-myc::kan^R*	This study
NW1617	*h⁺ leu1-32 mis12-375 msc1::kan^R*	This study
NW1698	*h⁻ leu1-32 ura4D18 cdc25-22*	This study
NW1699	*h⁺ leu1-32 ura4D18 cdc25-22 msc1::kan^R*	This study
NW1700	*h⁺ leu1-32 ura4D18 cdc25-22 mad2::ura4 ade6-210*	Matthew O'Connell
NW1702	*h⁻ leu1-32 mis6-302*	Mitsuhiro Yanagida
NW1703	*h⁺ leu1-32 mis6-302 msc1::kan^R ade6-210*	This study
NW1704	*h⁺ leu1-32 ura4D18 mad2::ura4 chk1::ura4 cdc17-K42 ade6-216*	This study
NW1713	*h⁻ leu1-32 ura4D18 mad2::ura4 chk1::ura4 ade6-216*	This study
NW1718	*h⁻ leu1-32 ura4D18 mad2::ura4 cdc17-K42 ade6-210*	This study
NW1736	*h⁺ leu1-32 ade6-210 ura4D18 msc1::kan^R cnp1::ura4 lys1⁺::cnp1-1*	This study
NW1737	*h⁻ leu1-32 lys1⁺::cnp1-GFP msc1::kan^R*	This study
NW1800	*h⁺ leu1-32 ade6-210 msc1::kan^R*	This study
NW1828	*h⁺ leu1-32 ade6-210 ura4D18 pht1::ura4*	Antony Carr
NW1829	*h⁺ leu1-32 ade6-210 ura4D18 pht1::ura4 msc1::kan^R*	This study
NW1830	*h⁻ leu1-32 ade6-210 ura4D18 mis6-302 pht1::ura4 msc1::kan^R*	This study
NW1831	*h⁻ leu1-32 ura4D18 mis12-375 pht1::ura4*	This study
NW1832	*h⁺ leu1-32 ura4D18 mis12-375 pht1::ura4 msc1::kan^R*	This study
NW1833	*h⁻ leu1-32 ade6-210 ura4D18 pht1::ura4 cdc17-K42*	This study
NW1834	*h⁺ leu1-32 ade6-210 ura4D18 arg3-D4 pht1::ura4 chk1::arg3 cdc17-K42*	This study
NW1835	*h⁺ leu1-32 ade6-210 arg3-D4 chk1::arg3 cdc17-K42*	This study
NW1836	*h⁻ leu1-32 ade6-210 ura4D18 mis6-302 cnp1::ura4 lys1⁺::cnp1-1*	This study
NW1837	*h⁺ leu1-32 ade6-210 ura4D18 pht1::ura4 cnp1::ura4 lys1⁺::cnp1-1*	This study
NW1839	*h⁺ leu1-32 ade6-210 ura4D18 pht1::ura4 msc1::kan^R cnp1::ura4 lys1⁺::cnp1-1*	This study
NW1854	*h⁺ ade6-210 (Ch16 ade6-216) leu1-32*	Chris Norbury
NW1855	*h⁺ ade6-210 (Ch16 ade6-216) msc1::kan^R leu1-32*	This study
NW1856	*h⁺ ade6-210 (Ch16 ade6-216) pht1::ura4 leu1-32 ura4D18*	This study
NW1857	*h⁺ ade6-210 (Ch16 ade6-216) msc1::kan^R pht1::ura4 leu1-32 ura4D18*	This study

cells with lagging chromosomes, cells were grown at 18° and cells with elongated anaphase spindles as visualized with TAT-1 antibody were counted (Ekwall *et al.* 1995). The number of cells in anaphase with more than two DAPI staining spots were counted as having lagging chromosomes.

Chromosome loss assay: The minichromosome Ch16 (Niwa *et al.* 1986) was introduced into *msc1::kan^R* and *msc1⁺* backgrounds using standard genetic techniques. Cultures were grown to mid-log phase in medium lacking adenine at 30°. An aliquot was removed to determine the percentage of Ade⁻ cells. Remaining cells were then grown for 24 hr at 30° in rich medium containing adenine, and samples were removed and plated on media with limiting adenine. The number of red colonies were counted and chromosome loss was calculated from the following formula: loss rate $= 1 - (F/I)^{1/N}$, where F is the final percentage of white colonies and I is the initial percentage of white colonies, while N is the number of generations between I and F.

Chromatin immunoprecipitations: Strains with GFP-tagged *cnp1* (Takahashi *et al.* 2000) were grown in 100-ml cultures to mid-log phase. Cells were pelleted and resuspended in 40 ml of YEA to which 1.4 ml of 37% formaldehyde was added. The samples were shaken gently at 25° for 15 min. Six milliliters of 1.25 M glycine was added, and the samples were transferred to 50-ml tubes and incubated overnight in the cold room. The following day the samples were washed twice with ice-cold TBS and resuspended in 400 μl of FALB (50 mM HEPES–potassium hydroxide, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% Na-deoxycholate) plus protease inhibitors (PI) (Ren *et al.* 2000). Cells were lysed with acid-washed glass beads in a Fastprep vortexing machine (Bio101). The lysate was centrifuged at 16,100 × *g* for 5 min at 4° and the supernatant was discarded. The pellets were resuspended in 800 μl of FALB+PI. These samples were then sonicated at a 30% duty cycle for 20 sec four times and for 10 sec once. The samples were pelleted in a microfuge at 16,100 × *g* for 10 min at 4°. The supernatants were harvested and brought to a final volume of 1 ml. A volume of 200 μl was set aside as the input fraction and the remaining material was precleared by incubating with washed sepharose beads in the cold room for 30 min. The

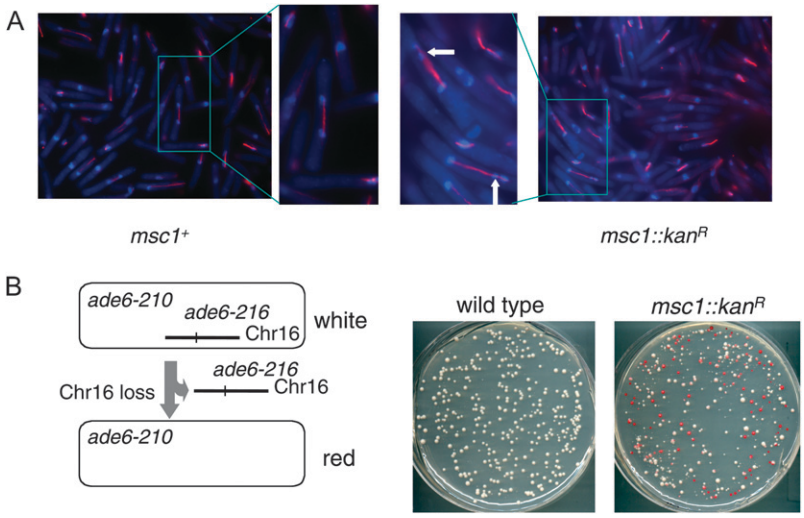

FIGURE 1.—Msc1 is required for chromosome stability. (A) Strains with a *cdc25-22* mutant allele were synchronized in G_2 by incubation at 36.5° and released to permissive temperature. After 90 min, cells were fixed with gluteraldehyde and processed for immunofluorescence with antitubulin antibody (red). Nuclei are stained with DAPI (blue). Portions of the micrographs in boxes are enlarged for better viewing of normal (left) and lagging (right) chromosomes (white arrows). (B) Schematic of chromosome loss assay. Ch16 is a minichromosome carrying the *ade6-216* allele that complements in *trans* the *ade6-210* allele (NIWA *et al.* 1986). Loss of Ch16 results in cells that solely express the *ade6-210* allele, which confers a red color to the colonies. From the parent strains that are either *msc1*$^+$ or *msc1::kan*^{R}, single white colonies were grown as described in MATERIALS AND METHODS. Equal numbers of cells of the indicated strains were plated on plates with a limiting concentration of adenine.

samples were centrifuged at 4000 × *g* and the supernatant was transferred to a new tube and incubated with antibody overnight. The next day, 40 μl of sepharose bead volume was added to capture immune complexes and incubated in the cold room for 1 hr. The samples were pelleted and the beads were washed while rotating with the following buffers, each for 10 min in the cold room: FALB, FALB–500 mM NaCl, wash buffer (10 mM Tris–Cl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na-deoxycholate), and TE. After the TE wash, the immunoprecipitate (IP) beads were resuspended in 400 μl of TE. The input samples were brought up to 400 μl as well. Five microliters of 10 mg/ml RNase A were added and incubated at 37° for 30 min. To reverse the crosslinks, 20 μl of 10% SDS was added and the samples were incubated at 65° for 20 hr. The next day, the samples were incubated with 10 μl of 10 mg/ml proteinase K for 6 hr at 55°. The samples were then purified using a DNA QIAGEN (Valencia, CA) column or by phenol–chloroform extraction and precipitated. One microliter of each IP sample and 1 μl of each input sample (diluted fivefold) was used in the PCR reaction. Samples were amplified for 28 cycles, which results in reaction products that are linear with respect to the amount of template DNA used in the reactions. PCR products were separated on an agarose gel and photographed with a digital camera. Photographs were scanned and analyzed with National Institutes of Health ImageJ for quantitation of signals, which were in the linear range of the detection program. Enrichments were calculated as the ratio of signal at *cnt* or *imr* relative to *otr* [where CENP-A is not expected to bind (TAKAHASHI *et al.* 2000)] for the immunoprecipitates, normalized to the ratio of signals for the input DNA. Normalized enrichments, reported in Figure 4, were calculated as the ratio of E for the tagged strain *vs.* E for the untagged strain for the given PCR reaction.

RESULTS

Msc1 is important for chromosome stability: Previously we isolated Msc1 as a multicopy suppressor of a mutant defective for the checkpoint kinase Chk1. We showed that Msc1 associates with chromatin and coprecipitates a histone deacetylase activity (AHMED *et al.* 2004). In the course of examining the *msc1* null strain, we observed an unusual DAPI-staining pattern with individual cells often containing an extra DAPI-staining spot suggestive of lagging chromosomes. Therefore, we compared chromosome segregation in *msc1::kan*^{R} and *msc1*$^+$ cells synchronized in G_2 and released into mitosis. As shown in Figure 1A, cells lacking *msc1* exhibit multiple DAPI spots along the mitotic spindle (white arrows), whereas this phenotype is not observed in the wild-type cells. To quantitate this phenotype, cells were grown at low temperature and the frequency of lagging chromosomes on late anaphase spindles was determined as described (EKWALL *et al.* 1995). While wild-type cells exhibit lagging chromosomes at a frequency of 2.0 ± 0.3% (SD), cells lacking *msc1* show an elevated frequency of 12.6 ± 4.3% (SD).

Strains that exhibit the lagging-chromosome phenotype often exhibit an elevated rate of chromosome loss as well. To determine whether Msc1 is required for chromosome stability, we incorporated Ch16, a minichromosome derived from chromosome III (NIWA *et al.* 1986), in an *msc1* knockout strain by genetic manipulation. This strain carries the *ade6-210* mutation in the genome and the *ade6-216* mutation on the minichromosome, making the strain phenotypically Ade$^+$ and white in color because of *trans*-complementation of *ade6-210* and *ade6-216*. We examined the loss of the minichromosome by growing cells under nonselective conditions for several generations and then scoring the number of Ade$^-$ red colonies produced after plating on media with limiting adenine. As shown in Figure 1B and quantified in Table 2, the rate of chromosome loss in the *msc1::kan*^{R} deletion strain is elevated ~30-fold compared to an isogenic wild-type strain.

Chromatin structure at the core centromere region shows a unique pattern after micrococcal nuclease (MNase) digestion (POLIZZI and CLARKE 1991; TAKAHASHI *et al.* 1992). Since Msc1 has been implicated

TABLE 2

Frequency of chromosome loss

Strain	Chromosome loss (rate/generation)	Fold difference
Wild type	0.00024	1
*msc1*Δ	0.0083	35
*pht1*Δ	0.0036	15
*msc1*Δ *pht1*Δ	0.0078	33

in modification of histones (Ahmed *et al.* 2004), and a dramatic change in chromatin structure at the centromere could influence attachment of chromosomes to the mitotic spindle, we examined the nucleosome pattern following MNase digestion of chromatin isolated from wild-type and from *msc1* null cells. No differences were detected in the digestion pattern of centromeric chromatin when wild-type and *msc1::kan*^{R} cells were examined (supplemental data at http://www.genetics.org/supplemental/), indicating that no gross change in nucleosome organization results from the absence of *msc1*.

Msc1 exhibits genetic interactions with Mis12 and Mis6: The lagging-chromosome phenotype is common in fission yeast mutants that affect chromosome segregation and kinetochore function. To evaluate whether Msc1 influences kinetochore function, double mutants with strains harboring temperature-sensitive mutations in the genes encoding Mis12 and Mis6 (Saitoh *et al.* 1997; Goshima *et al.* 1999) were constructed. Deletion of *msc1* in the temperature-sensitive *mis12-537* background results in a lower restrictive temperature as compared to *mis12-537* alone (Figure 2A). While the *mis12-537* mutant readily forms colonies at 34°, the double-mutant *mis12-537 msc1::kan*^{R} is compromised for growth at this temperature. Likewise, a *mis6-302 msc1::kan*^{R} mutant is compromised for growth at 32° whereas the *mis6-302* strain is viable at this temperature (Figure 2B).

Multicopy expression of *msc1* suppresses loss of function of the histone H3 variant *cnp1-1*: Chromosome stability relies not only on proper function of kinetochore proteins, but also on properly assembled centromeric chromatin. The histone H3 variant CENP-A, encoded by *cnp1* in *S. pombe*, localizes to the *cnt* and *imr* regions of *S. pombe* centromeric chromatin (Kniola *et al.* 2001). Deletion of *msc1* in a temperature-sensitive mutant *cnp1-1* background does not exacerbate *cnp1-1* temperature sensitivity; indeed, it seems to improve growth somewhat at 30° (Figure 3A), although this behavior is not apparent when single colonies are streaked on plates (data not shown). On the contrary, multicopy expression of *msc1* suppresses the temperature sensitivity of *cnp1-1* (Figure 3B), as does overexpression of *ams2*, a gene identified by virtue of this property. Ams2 is implicated in loading of CENP-A at the centromere (Chen *et al.* 2003). Mis6 is also required for CENP-A to localize to centromeres (Takahashi *et al.* 2000), and as shown in Figure 3C, the ability of multicopy *msc1* to suppress *cnp1-1* requires *mis6* function.

To determine whether Msc1 affects localization of fission yeast CENP-A to the centromere, we performed chromatin immunoprecipitation experiments using GFP-tagged Cnp1. As shown in Figure 4, Cnp1-GFP localizes to the inner regions of the centromere (*cnt* and *imr*), but not to the outer region (*otr*) (Takahashi *et al.* 2000). In a mutant null for *msc1*, association of Cnp1-GFP to either *cnt* or *imr* is dramatically reduced.

Histone H2A.Z exhibits genetic interactions with *mis6* and *mis12* and is required for p*msc1* to suppress *cnp1-1*: Msc1 associates with Swr1 and other components of the Swr1 chromatin-remodeling complex (X. Qiu, S. Ahmed and N. C. Walworth, unpublished results). Since Swr1 exchanges histone H2A.Z for histone H2A (Mizuguchi *et al.* 2004), we hypothesized that Msc1 might carry out its functions through histone H2A.Z. Consistent with this hypothesis, deletion of the H2A.Z-encoding gene in *S. pombe*, *pht1*, has been reported to result in elevated chromosome loss rates (Carr *et al.* 1994). In our hands, the loss rate for a *pht1* null strain is elevated 15-fold above wild-type levels, whereas deletion of *msc1* or simultaneous deletion of both *pht1* and *msc1*

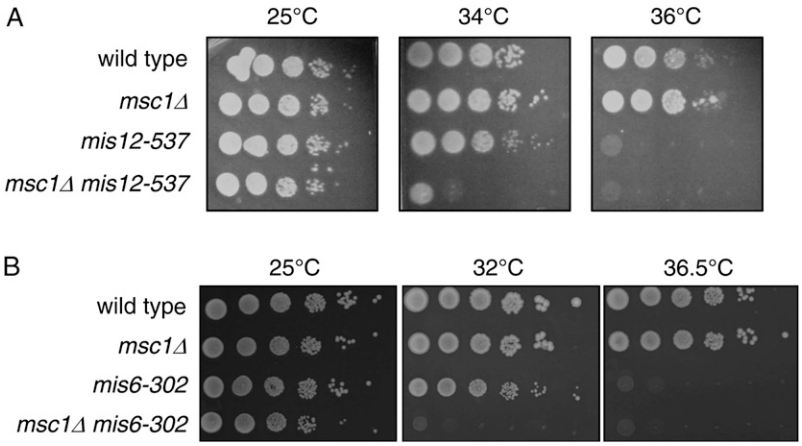

Figure 2.—Msc1 exhibits genetic interactions with Mis12 and Mis6. (A and B) The indicated strains were grown at 25° to mid-log phase. Tenfold serial dilutions were spotted on rich media plates and incubated at the indicated temperatures for 3–4 days.

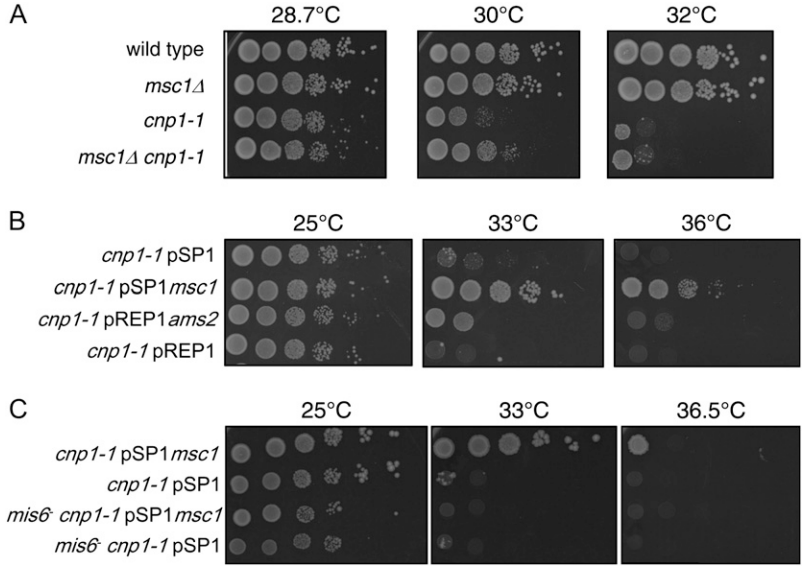

FIGURE 3.—An *msc1* null is not synthetically sick with *cnp1-1*, but multicopy *msc1* suppresses loss of function of CENP-A. (A) The indicated strains were grown at 25° to mid-log phase. Tenfold serial dilutions were spotted on rich media plates and incubated at the indicated temperatures for 3–4 days. (B and C) The *cnp1-1* (B) or *cnp1-1 mis6* (C) strain was transformed with the indicated plasmids. Cells were grown at 25° in minimal media to mid-log phase. Tenfold serial dilutions were spotted on minimal media plates and incubated at the indicated temperatures for 3–4 days.

results in an ~30-fold increase relative to wild-type cells (Table 2).

To determine if the *pht1* deletion strain affects kinetochore function, crosses were carried out with *mis12-537* and *mis6-302*. As shown in Figure 5, deletion of *pht1* lowers the restrictive temperatures of *mis12-537* even more so than does deletion of *msc1* (Figure 5A). Like deletion of *msc1*, deletion of *pht1* also reduces the restrictive temperature of *mis6-302* (Figure 5B). A triple mutant of *mis6 msc1*Δ *pht1*Δ is as temperature sensitive as

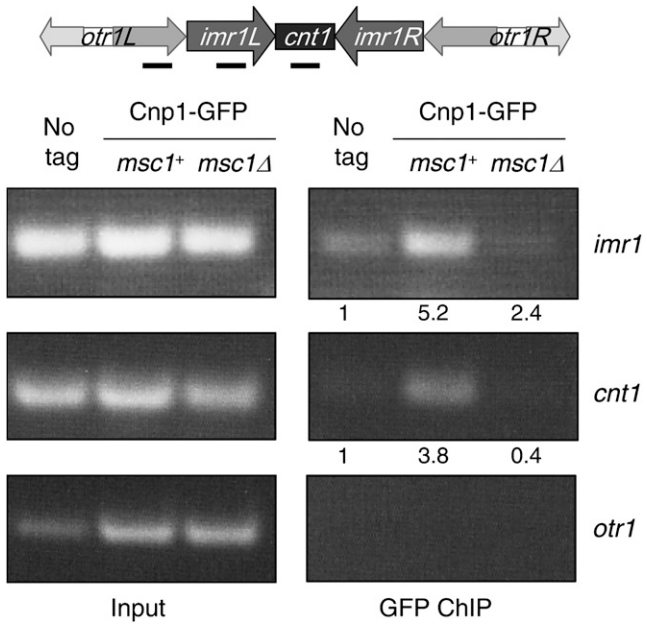

FIGURE 4.—CENP-A localization to the inner centromeric region is reduced in the absence of Msc1. Cells with GFP-tagged Cnp1 (CENP-A) with wild-type or a null allele of *msc1*, or an untagged control, were subjected to chromatin immunoprecipitation with antibody to GFP as described in MATERIALS AND METHODS. DNA from the lysates (input) or the immunoprecipitations were subject to PCR with primers specific to the central (*cnt*), inner (*imr*), and outer (*otr*) regions of the fission yeast centromere on chromosome I. Enrichment of *imr1* and *cnt1* was assessed compared to *otr1* in immunoprecipitates relative to input DNA. This experiment was performed three times with similar results.

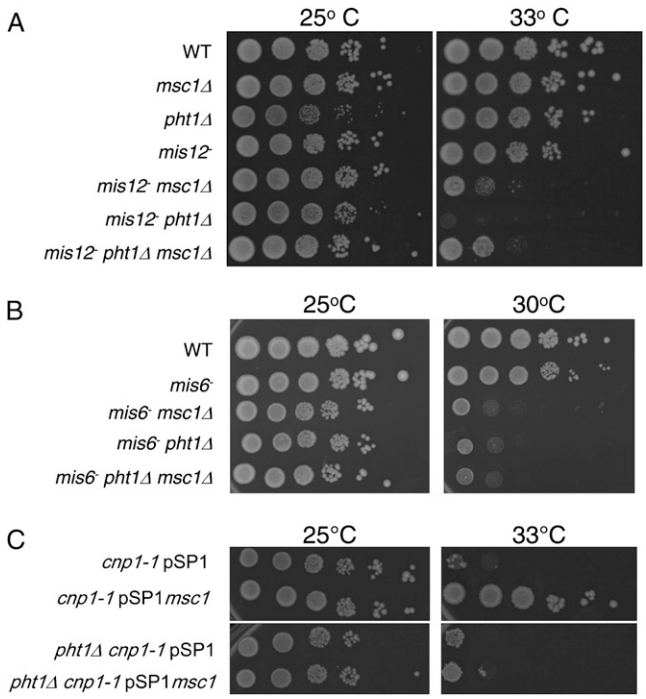

FIGURE 5.—A *pht1* null mutant exhibits genetic interactions similar to *msc1*. (A and B) The indicated strains were grown at 25° to mid-log phase. Tenfold serial dilutions were spotted on rich media plates and incubated at the indicated temperatures for 3–4 days. (C) The *cnp1-1* or *cnp1-1 pht1*Δ strain was transformed with the indicated plasmids. Cells were grown at 25° in minimal media to mid-log phase. Tenfold serial dilutions were spotted on minimal media plates and incubated at the indicated temperatures for 3–4 days.

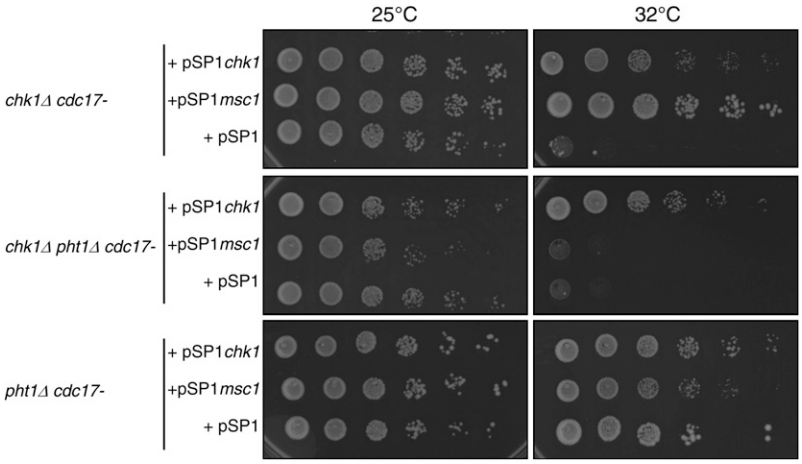

FIGURE 6.—The ability of multicopy Msc1 to restore survival of a *chk1*Δ strain requires histone H2A.Z. The indicated strains transformed with the indicated plasmids were grown in minimal media to mid-log phase at 25°. Serial dilutions (10-fold) were spotted on yeast nitrogen media plates lacking leucine and incubated at the indicated temperatures.

either *mis6-302 msc1*Δ or *mis6-302 pht1*Δ (Figure 5B). Paradoxically, the *mis12 pht1*Δ *msc1*Δ strain exhibits improved growth as compared to *mis12 pht1*Δ, equivalent to that seen for *mis12 msc1*Δ (Figure 5A).

To determine whether suppression of the temperature sensitivity of *cnp1-1* by multicopy p*msc1* requires histone H2A.Z, a double mutant of *pht1*Δ and *cnp1-1* was constructed and transformed with the p*msc1* plasmid. As shown in Figure 5C, p*msc1* is unable to rescue *cnp1-1* in the absence of histone H2A.Z.

Histone H2A.Z and Mad2 are required for p*msc1* to suppress *chk1*: Msc1 was originally identified in fission yeast because of its ability to rescue a *chk1*-defective strain in which DNA ligase, encoded by *cdc17*, was mutated (AHMED *et al.* 2004). We tested whether the ability of Msc1 to rescue a *chk1*Δ *cdc17-K42* strain is also dependent on histone H2A.Z. As shown in Figure 6, while multicopy p*msc1* supports growth of a *chk1*Δ *cdc17-K42* strain at 32°, it cannot do so when *pht1* is deleted (middle). Deletion of *pht1* alone in the *cdc17-K42* strain does not compromise viability at 32° (bottom). Thus, the ability of p*msc1* to rescue loss of *chk1* function requires the presence of H2A.Z.

Recent results from others suggest that Chk1 may play a role not only in preventing the onset of mitosis when DNA is damaged, but also in slowing events within mitosis, particularly the metaphase-to-anaphase transition (COLLURA *et al.* 2005). Indeed, cells lacking the mitotic spindle checkpoint protein Mad2 are slightly sensitive to DNA-damaging agents, consistent with a possible back-up role for this checkpoint in promoting survival following DNA damage (COLLURA *et al.* 2005). Taking these observations into account, we tested the possibility that suppression of the *chk1* defect by multicopy *msc1* might require the function of Mad2. We constructed a strain with deletions of both *chk1* and *mad2* in the *cdc17-K42* background. As shown in Figure 7A, the triple mutant cannot be suppressed by expression of

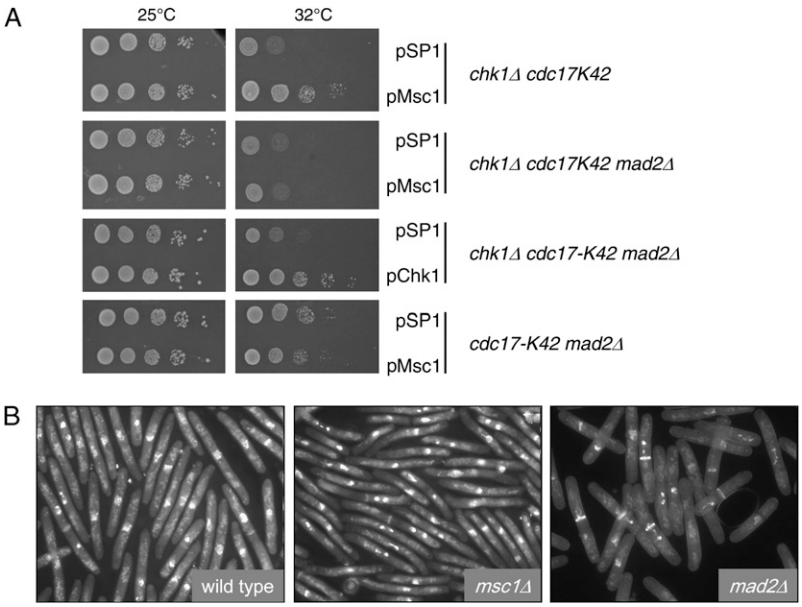

FIGURE 7.—The ability of multicopy Msc1 to restore survival of a *chk1*Δ strain requires Mad2 function, although Msc1 is not required directly for the spindle checkpoint. (A) The indicated strains transformed with the indicated plasmids were grown in minimal media to mid-log phase at 25°. Serial dilutions (10-fold) were spotted on yeast nitrogen media plates lacking leucine and incubated at the indicated temperatures. (B) Cells were blocked in G_2 by incubation at 36.5° and then released at permissive temperature in the presence of TBZ (50 μg/ml). At 20-min intervals cells were fixed with gluteraldehyde and processed for immunofluorescence with antitubulin antibody and stained with DAPI. For wild-type and *msc1*Δ cells, the 100-min time point is shown. For *mad2*Δ cells, the 60-min time point is shown.

p*msc1* (second panel), whereas a strain with the *mad2* gene intact can be suppressed (top panel). Thus, suppression of the *chk1* defect by multicopy *msc1* when DNA damage is present requires an intact spindle checkpoint. As expected, a p*chk1* plasmid was able to suppress the growth defect in the triple-mutant background (Figure 7A, third panel) by restoring DNA damage checkpoint function. Furthermore, the *cdc17-K42 mad2*Δ strain transformed with either the control vector plasmid or the p*msc1* plasmid is equally viable at 32° (Figure 7A, bottom panel), suggesting that Mad2 does not play a major role in promoting survival when DNA ligase function is compromised as long as Chk1 function is intact.

Msc1 does not play a direct role in the spindle checkpoint: To determine whether Msc1 might play a direct role in the mitotic spindle checkpoint, we compared the behavior of an *msc1*Δ strain to that of a *mad2* null. The null mutations of *msc1* and *mad2* were constructed in a *cdc25-22* background to allow synchronization of the cell population in G_2. Cells arrested in G_2 by incubation at 36.5° were released to 25° in the presence of the microtubule-destabilizing drug TBZ. Samples were collected at 20-min intervals and examined for progression through mitosis by visualizing nuclei with DAPI. As shown in Figure 7B, wild-type and *msc1*Δ cells remain arrested with condensed chromosomes in the presence of TBZ, even at 100 min. In contrast, even at 60 min, *mad2*Δ cells have already attempted to segregate their chromosomes exhibiting the cut phenotype and chromosome decondensation, indicating loss of spindle checkpoint function and progression through mitosis (He *et al.* 1997).

DISCUSSION

This study demonstrates a role for fission yeast Msc1 in chromosome stability. Genetic interactions with core components of the kinetochore and with the centromere-specific histone H3 variant CENP-A suggest that Msc1 may influence either directly or indirectly the structure or function of the site at which the mitotic spindle associates with the chromosome. We postulate that Msc1 acts through the histone H2A variant H2A.Z because the ability of multicopy Msc1 to elicit phenotypes is dependent on the presence of the H2A.Z gene, *pht1*. Furthermore, a mutant deficient in H2A.Z (*pht1*Δ) shares some phenotypes with a mutant deficient in Msc1 (*msc1*Δ). CENP-A and H2A.Z have been shown to bind within distinct centromeric domains in mammalian chromosomes (Okada *et al.* 2006; Greaves *et al.* 2007). CENP-A is a critical functional component of the fission yeast centromere (Takahashi *et al.* 2000) and the work described here provides genetic evidence supporting the possibility that histone H2A.Z could function within the fission yeast centromere as well. Detailed analysis of H2A.Z localization in fission yeast has not as yet been reported.

Msc1 was first identified in fission yeast as a protein that in multiple copies restores survival to a strain lacking Chk1 function (Ahmed *et al.* 2004). Two homologs of Msc1 exist in mammalian cells, one of which (PLU-1) is upregulated in breast cancer cells (Lu *et al.* 1999) and both of which (PLU-1 and RBP2) have been implicated in the control of gene expression by virtue of their associations with other proteins (Fattaey *et al.* 1993; Mao *et al.* 1997; Lu *et al.* 1999; Tan *et al.* 2003; Catteau *et al.* 2004). In fission yeast, cells lacking Msc1 are viable, but exhibit sensitivity to the histone deacetylase inhibitor TSA (Ahmed *et al.* 2004). Msc1, like its mammalian counterparts, possesses several motifs consistent with roles in chromatin modification and/or transcriptional control. These include three PHD fingers, JmjN, and JmjC domains (Ahmed *et al.* 2004). However, RBP2 and PLU-1 possess an ARID motif that does not appear to be conserved in Msc1. Published data for RBP2 and PLU-1 currently favor roles in transcriptional regulation. Whether fission yeast Msc1 influences transcriptional control is under investigation.

PHD domains have been suggested to have two disparate activities. The structural similarity of PHD domains to RING domains (Pascual *et al.* 2000; Capili *et al.* 2001) suggested that they might possess E3 ubiquitin ligase activity, which has been demonstrated for several PHD motifs found in non-nuclear proteins (Coscoy *et al.* 2001; Lu *et al.* 2002; Goto *et al.* 2003; Yonashiro *et al.* 2006). More recently, the PHD domains of at least two sets of proteins have been shown to behave as histone H3 di- or trimethyl-binding domains (Li *et al.* 2006; Martin *et al.* 2006; Pena *et al.* 2006; Shi *et al.* 2006; Wysocka *et al.* 2006). The PHD fingers of Msc1 do not appear to bind to histones, but do possess ubiquitin E3 ligase activity (Dul and Walworth 2007). Whether the corresponding PHD domains of RBP2 or PLU-1 possess either function remains to be determined.

JmjC domains have garnered attention recently as a subset of such domains have been shown to possess histone demethylase activity (Tsukada *et al.* 2006). Indeed, the JmjC domains of RBP2 and PLU-1 are reported to demethylate histone H3-K4 (Christensen *et al.* 2007; Klose *et al.* 2007; Yamane *et al.* 2007). However, many of the amino acid residues implicated in iron and α-ketoglutarate binding, which are thought to be essential for enzymatic activity of this domain, are not conserved in Msc1 (Klose *et al.* 2006). Thus, elucidation of the functional activity of the JmjC domain in Msc1 will require further investigation.

The results of our phenotypic and genetic analysis of Msc1 lead us to hypothesize that the protein affects chromosome stability by directly or indirectly promoting kinetochore attachment to the mitotic spindle. The fact that multicopy Msc1 robustly suppresses a *cnp1-1* mutant suggests that Msc1 might facilitate CENP-A incorporation or retention at the centromere, and indeed, CENP-A localization to the centromere is reduced

in an *msc1* null strain. However, the fact that *cnp1* is an essential gene whereas *msc1* is not indicates that a minimal level of CENP-A may be present at the centromere in the *msc1* null strain. Indeed, the fact that centromeres in the *msc1* null strain show a digestion pattern when exposed to nuclease (see supplemental Figure 2 at http://www.genetics.org/supplemental/) similar to that seen for wild-type cells suggests that CENP-A is present in sufficient amounts at the inner core to retain the chromatin structure characteristic of this region (Takahashi *et al.* 2000).

Histone H2A.Z must be present for multicopy p*msc1* to suppress the *cnp1-1* mutant or to suppress loss of *chk1* function. A molecular explanation for these requirements awaits characterization of the fission yeast Swr1 protein complex and determination of whether Swr1 promotes H2A.Z deposition as it does in budding yeast, as well as determination of the relationship of Msc1 to that complex. Notably, a clear homolog of Msc1 is absent from the budding yeast genome. It is possible that the mechanism by which Msc1 promotes cell survival in the absence of Chk1 may be through increasing the stability or segregation of damaged chromosomes. In support of this speculation, we note that suppression by p*msc1* of a *chk1* mutant also requires an intact mitotic spindle checkpoint since suppression is lost in cells in which both *chk1* and *mad2* are compromised.

A relationship between various checkpoint pathways in fission yeast has begun to emerge. Sugimoto *et al.* (2004) reported that a hydroxyurea-induced delay to mitotic entry in cells lacking the protein kinase Cds1 requires the function of Mad2, just as it requires the function of Chk1 (Lindsay *et al.* 1998). In addition, Collura *et al.* (2005) have reported that Crb2, in conjunction with Chk1, is required to delay the metaphase-to-anaphase transition in cells treated with the topoisomerase I poison camptothecin in a Mad2-dependent fashion. The mechanism by which Chk1 influences the Mad2-dependent checkpoint pathway in fission yeast and the role that Msc1 plays in promoting checkpoint function remain to be determined.

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