The Plant Homeodomain Fingers of Fission Yeast Msc1 Exhibit E3 Ubiquitin Ligase Activity*

Barbara E. Dul and Nancy C. Walworth

From the Department of Pharmacology, University of Medicine and Dentistry of New Jersey (UMDNJ)-Robert Wood Johnson Medical School and the Joint Graduate Program in Cellular and Molecular Pharmacology, UMDNJ-Graduate School of Biomedical Sciences and Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854

The DNA damage checkpoint pathway governs how cells regulate cell cycle progression in response to DNA damage (1). A screen for suppressors of a fission yeast chk1 mutant defective in the checkpoint pathway identified a novel Schizosaccharomyces pombe protein, Msc1. Msc1 contains 3 plant homeodomain (PHD) finger motifs, characteristically defined by a C4HC3 consensus similar to RING finger domains. PHD finger domains in viral proteins and in the cellular protein kinase MEKK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1) have been implicated as ubiquitin E3 protein ligases that affect protein stability. The close structural relationship of PHD fingers to RING fingers suggests that other PHD domain-containing proteins might share this activity. We show that each of the three PHD fingers of Msc1 can act as ubiquitin E3 ligases, reporting for the first time that PHD fingers from a nuclear protein exhibit E3 ubiquitin ligase activity. The function of the PHD fingers of Msc1 is needed to rescue the DNA damage sensitivity of a chk1Δ strain. Msc1 co-precipitates Rhp6, the S. pombe homologue of the human ubiquitin-conjugating enzyme Ubc2. Strikingly, deletion of msc1 confers complete suppression of the slow growth phenotype, UV and hydroxyurea sensitivities of an rhp6 deletion strain and restores deficient histone H3 methylation observed in the rhp6Δ mutant. We speculate that the target of the E3 ubiquitin ligase activity of Msc1 is likely to be a chromatin-associated protein.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Tel.: 732-235-5661; Fax: 732-235-4073; E-mail: walworna@umdnj.edu.
PHD fingers in Msc1 can act as E3 ligases and whether they can bind to histones. In this paper we show that the PHD fingers of Msc1 possess E3 ubiquitin ligase activity, for the first time demonstrating that a PHD domain from a nuclear protein can facilitate ubiquitin transfer.

**EXPERIMENTAL PROCEDURES**

**Yeast Plasmids, Strains, and Manipulation—**Standard *Schizosaccharomyces pombe* media and genetic manipulations were used as described by Moreno et al. (24). Yeast strains are listed in Table 1. For GST protein fusions each PHD finger was amplified by PCR and cloned in-frame with GST. Point mutations for the full-length protein were generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene) in a plasmid (pSPI) expressing *msc1* under its endogenous promoter and tagged with a triple HA tag. The same methodology was used to generate point mutations in the GST-PHD protein fusions. GST and GST-PHD proteins were purified from *Escherichia coli* on GSH-Sepharose using standard procedures (25). For yeast lysate preparation, all strains were grown to mid-log phase (0.5 × 10^7 cells/ml) at 30 °C. For experiments assaying drug sensitivity, strains were grown to 10^7 cells/ml and serially diluted 10-fold, and 5 μl of each dilution was spotted. To assay UV sensitivity, cells were grown to mid-log phase at 30 °C. 1000 cells were spread onto plates in triplicate and exposed to different doses of UV light using a Stratagene (Stratagene). Survival was determined by counting the number of colonies forming at each dose and dividing by the number of colonies that grew on the unirradiated control plates.

**Ubiquitylation Assays—**GST-PHD fusion proteins were incubated for 2 h at 30 °C in a final volume of 180 μl with 4 mM ATP, 10 mM MgCl₂, 86 mM ubiquitin (Sigma), 10 μM yeast E1 (Boston Biochem), 80 μM human E2 (Boston Biochem), and 200 μM histones extracted from *S. pombe* or purchased from Sigma. SDS/β-mercaptoethanol sample buffer was added and boiled, and the reaction was resolved by 10% SDS-PAGE, transferred to nitrocellulose, and probed with a rabbit antibody against ubiquitin (Sigma) or GST. To test the requirement for Zn^2+", the chelator tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN, Sigma) was added at 100 μM.

For ubiquitylation reactions performed with full-length Msc1 immunoprecipitated from *S. pombe* (see below), reactions were performed essentially the same way except that the reaction components were incubated in the presence of protein A-Sepharose beads (Amersham Biosciences) that had been used to capture the immunoprecipitated protein. After boiling, the reaction sample was spun down briefly at 15,000 × g, but only the supernatant was loaded onto the gel.

**Immunoprecipitations—**To immunoprecipitate Msc1, 200 ml of cultures were grown to mid-log phase (0.5 × 10^7 cells/ml), spun down by centrifugation, and resuspended in Tris-buffered saline at 10^7 cells/ml containing complete protease inhibitor (Roche Applied Science). Cells were lysed with acid-washed glass beads in a Fastprep vortexing machine (Bio101). The lysate was centrifuged at 2300 × g for 5 min at 4 °C. Protein concentration was determined using the method of Bradford with bovine serum albumin as a standard. One mg of protein was incubated with 25 μl of protein

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**TABLE 1**

**List of strains**

| Strain   | Genotype                  |
|----------|---------------------------|
| SP6      | h* leu1-32                |
| SP98     | h* leu1-32 ade6-210       |
| NW591    | h* leu1-32 ade6-216 ura4D18 chk1:ura4 pSPI/chk1-HA |
| NW1562   | h* leu1-32 msc1-HA- kan6  |
| NW1797   | h* leu1-32 msc1-HA- kan6  |
| NW1798   | h* leu1-32 msc1-HA- kan6  |
| NW1799   | h* leu1-32 msc1-HA- kan6  |
| NW1800   | h* leu1-32 msc1-HA- kan6  |
| NW1801   | h* leu1-32 msc1-HA- kan6  |
| NW1802   | h* leu1-32 msc1-HA- kan6  |
| NW1803   | h* leu1-32 msc1-HA- kan6  |
| NW1804   | h* leu1-32 msc1-HA- kan6  |
| NW1805   | h* leu1-32 msc1-HA- kan6  |
| NW1806   | h* leu1-32 msc1-HA- kan6  |
| NW1807   | h* leu1-32 msc1-HA- kan6  |
| NW1808   | h* leu1-32 msc1-HA- kan6  |
| NW1809   | h* leu1-32 msc1-HA- kan6  |
| NW1810   | h* leu1-32 msc1-HA- kan6  |
| NW1811   | h* leu1-32 msc1-HA- kan6  |
| NW1812   | h* leu1-32 msc1-HA- kan6  |
| NW1813   | h* leu1-32 msc1-HA- kan6  |
| NW1814   | h* leu1-32 msc1-HA- kan6  |
| NW1815   | h* leu1-32 msc1-HA- kan6  |
| NW1816   | h* leu1-32 msc1-HA- kan6  |
| NW1817   | h* leu1-32 msc1-HA- kan6  |
| NW1818   | h* leu1-32 msc1-HA- kan6  |
| NW1819   | h* leu1-32 msc1-HA- kan6  |
| NW1820   | h* leu1-32 msc1-HA- kan6  |
| NW1821   | h* leu1-32 msc1-HA- kan6  |
| NW1822   | h* leu1-32 msc1-HA- kan6  |
| NW1823   | h* leu1-32 msc1-HA- kan6  |
| NW1824   | h* leu1-32 msc1-HA- kan6  |
| NW1825   | h* leu1-32 msc1-HA- kan6  |
| NW1826   | h* leu1-32 msc1-HA- kan6  |
Histone Preparations—Histones were prepared according to Shi et al. (22) to test whether GST fusion proteins of the Msc1 PHD fingers were capable of binding to histones in vitro. No binding of histones to any of the Msc1 PHD fingers was detectable, although the control protein consisting of the PHD finger of ING2 fused to GST pulled down histones that have been shown to bind methylated lysine 4 of histone H3 (20–23, 27). To test whether the PHD fingers of Msc1 share the ability to bind histones, we performed the assay described by Shi et al. (22) to test whether GST fusion proteins of the Msc1 PHD fingers were capable of binding to histones in vitro. No binding of histones to any of the Msc1 PHD fingers was detectable, although the control protein consisting of the PHD finger of ING2 fused to GST pulled down histones that have been shown to bind methylated lysine 4 of histone H3 (20–23, 27). To test whether the PHD fingers of Msc1 share the ability to bind histones, we performed the assay described by Shi et al. (22) to test whether GST fusion proteins of the Msc1 PHD fingers were capable of binding to histones in vitro. No binding of histones to any of the Msc1 PHD fingers was detectable, although the control protein consisting of the PHD finger of ING2 fused to GST pulled down histones that have been shown to bind methylated lysine 4 of histone H3 (20–23, 27). To test whether the PHD fingers of Msc1 share the ability to bind histones, we performed the assay described by Shi et al. (22) to test whether GST fusion proteins of the Msc1 PHD fingers were capable of binding to histones in vitro. No binding of histones to any of the Msc1 PHD fingers was detectable, although the control protein consisting of the PHD finger of ING2 fused to GST pulled down histones that have been shown to bind methylated lysine 4 of histone H3 (20–23, 27). To test whether the PHD fingers of Msc1 share the ability to bind histones, we performed the assay described by Shi et al. (22) to test whether GST fusion proteins of the Msc1 PHD fingers were capable of binding to histones in vitro. No binding of histones to any of the Msc1 PHD fingers was detectable, although the control protein consisting of the PHD finger of ING2 fused to GST pulled down histones that have been shown to bind methylated lysine 4 of histone H3 (20–23, 27). To test whether the PHD fingers of Msc1 share the ability to bind histones, we performed the assay described by Shi et al. (22) to test whether GST fusion proteins of the Msc1 PHD fingers were capable of binding to histones in vitro. No binding of histones to any of the Msc1 PHD fingers was detectable, although the control protein consisting of the PHD finger of ING2 fused to GST pulled down histones that have been shown to bind methylated lysine 4 of histone H3 (20–23, 27). To test whether the PHD fingers of Msc1 share the ability to bind histones, we performed the assay described by Shi et al. (22) to test whether GST fusion proteins of the Msc1 PHD fingers were capable of binding to histones in vitro. No binding of histones to any of the Msc1 PHD fingers was detectable, although the control protein consisting of the PHD finger of ING2 fused to GST pulled down histones that have been shown to bind methylated lysine 4 of histone H3 (20–23, 27). To test whether the PHD fingers of Msc1 share the ability to bind histones, we performed the assay described by Shi et al. (22) to test whether GST fusion proteins of the Msc1 PHD fingers were capable of
Msc1 Exhibits Ubiquitin Ligase Activity

A. E1: + + + + - + + + E2: + + + + - + + + E3: P3 P3 P3 P3 P3 P3 P3 GST ATP: + + + + - + + + Substrate: H B L H H H -- H H α Ub α GST

B. E3: P1 P2 P3 GST α Ub 96 66 40 30 21 30 --- --- --- --- --- --- --- --- α GST

C. GST-PHD3 GST TPEN -- + + -- + + ZnCl2 -- -- + -- + + α Ub α GST

FIGURE 2. The PHD fingers of Msc1 possess E3 ubiquitin ligase activity. A, ubiquitylation depends on ATP, E1, E2, and a substrate. GST-PHD3 was incubated with ATP, E1, E2 (Ubc2), ubiquitin, and either S. pombe histones (H, lane 1), bovine serum albumin (B, lane 2), or lysozyme (L, lane 3). The remaining lanes show reactions containing GST-PHD3 leaving out ATP (lane 4), E1 (lane 5), E2 (lane 6), or histones (lane 7) or a complete reaction in the presence of a GST-PHD3 mutant (lane 8) or GST alone (lane 9). After incubation for 2 h at 30 °C, reaction mixtures were boiled in SDS sample buffer, divided in half, and resolved by 10% (upper panel) and 12% (lower panel) SDS-PAGE. After transfer to nitrocellulose, one blot was incubated with anti-ubiquitin antibody, and the other was with anti-GST antibody as indicated. B, mutations of cysteine residues compromise activity. The first or seventh cysteine in each GST-PHD domain was mutated to an alanine, and GST fusion proteins were produced. When assayed for ubiquitylation activity, the high molecular weight ubiquitin conjugates were either diminished or totally abolished when the mutant PHD fingers are used (Fig. 2, A, lane 8, and B). Thus, the activity of the PHD fingers is dependent on intact cysteine residues, suggesting that activity is likely to be zinc-dependent. To test this hypothesis, assays were done in the presence of a zinc chelator TPEN. As shown in Fig. 2C, TPEN abolishes high molecular weight ubiquitin conjugates, whereas the addition of ZnCl2 restores activity.

The high molecular weight material seen in the presence of the Msc1 PHD fingers could be due to polyubiquitylation (28) or to the attachment of multiple ubiquitin molecules on distinct lysine residues (mult ubiquitylation). To distinguish these possibilities, assays were performed in the presence of methylated ubiquitin (29). Methyl-ubiquitin forms the initial bond with a lysine residue through its C-terminal glycine residue. However, subsequent addition of methyl-ubiquitin to the initial one is blocked. As shown in Fig. 3A, high molecular weight ubiquitin conjugates are not formed when methyl-ubiquitin is used in place of unmodified ubiquitin.

Ubiquitin can form a number of chains, with the most well characterized occurring through lysine residues Lys-48 or Lys-63 (28). Chains formed through Lys-48 linkages usually target a protein for degradation. Chains formed through Lys-63 form an extended chain and are associated with events that, for example, transduce a signal or trigger a change in protein localization (30). Ubiquitin mutants with arginine substitutions at Lys-48 or Lys-63 were assayed for high molecular weight conjugate formation in the presence of Msc1 PHD fingers. As shown in Fig. 3B, wild-type ubiquitin or the K48R mutant each allows formation of high molecular weight ubiquitin conjugates. However, when the K63R mutant was used, the high molecular weight ubiquitin conjugates were abolished (Fig. 3B), consistent with polyubiquitin chains forming through Lys-63 linkages.

to nitrocellulose, one blot was incubated with anti-ubiquitin antibody, and the other was with anti-GST antibody as indicated. B, mutations of cysteine residues compromise activity. The first or seventh cysteine in each GST-PHD fusion was mutated to an alanine and incubated with ATP, E1, E2 (Ubc2), ubiquitin, and S. pombe histones. Reactions were processed as described in A. Lanes 1–3 are positive controls using wild-type PHD domains fused to GST. Lane 4 includes GST alone as a negative control. C, chelation of Zn⁺ compromises activity. GST-PHD3 or GST alone was incubated with ATP, E1, E2 (Ubc2), ubiquitin, and S. pombe histones (lanes 1 and 4) in the presence of 100 μM TPEN (lanes 2 and 5) or in the presence of 100 μM TPEN and 200 μM ZnCl₂ (lanes 3 and 6). Reactions were processed as in A but resolved by 8% (upper panel) and 12% (lower panel) SDS-PAGE.
Msc1 Associates with the S. pombe Homologue of Ubc2, Rhp6—In the ubiquitin conjugation enzymatic cascade, the E1 and E2 enzymes activate ubiquitin, whereas the E3 ligase generates the specificity of the reaction by bringing the substrate near the E2 carrying the ubiquitin (13). In initial assays of the PHD fingers of Msc1 for E3 ubiquitin ligase activity, nine different human E2 enzymes were tested, with the most robust activity seen with Ubc2. The fission yeast homologue of human Ubc2 is encoded by the gene encoding Msc1 from cells with an intact msc1 gene supports ubiquitylation in the in vivo assay. As shown in Fig. 4E, immunoprecipitated Msc1 from cells with an intact rhp6 gene supports ubiquitylation with or without the addition of added Ubc2 as an E2 (lanes 1 and 3, respectively). However, when Msc1 was immunoprecipitated from cells with a deletion of rhp6, no activity was detected (lane 4) unless Ubc2 was added (lane 2). Thus, co-precipitated Rhp6 supports Msc1 activity and may be the predominant E2 for Msc1 in cells.

The Function of the PHD Fingers Is Needed in Vivo—The gene encoding Msc1 was isolated in a screen for multicopy suppressors of a checkpoint-defective strain lacking chk1 in which DNA damage was generated by limiting the activity of DNA ligase (2). To test whether function of the PHD fingers is necessary for the ability of Msc1 to suppress this strain, a chk1Δ cdc17-K42 strain was transformed with plasmids expressing the single PHD domain mutants or the 6-Cys→Ala mutant. As shown in Fig. 5A, whereas a plasmid expressing wild-type Msc1 sustains growth at 32 °C, none of the mutants was capable of supporting colony formation. Thus, the E3 ligase activity associated with the PHD domains is important for the ability of Msc1 to allow survival when DNA ligase is limiting and the checkpoint pathway is compromised.

Multicopy Msc1 can also partially suppress the sensitivity of a chk1 mutant strain exposed to UV light or the topoisomerase I poison CPT (2). To test the functionality of the msc1 alleles for suppression of the chk1 mutant, plasmids were transformed into a chk1Δ strain. Surprisingly, in this strain with wild-type msc1 in the genome, the 6-Cys→Ala assay using the mutant with simultaneous mutation of all three PHD domains (Fig. 4C). We note that the lack of activity is despite the fact that the amount of Msc1–6-Cys→Ala protein is reproducibly elevated relative to the single mutants or the wild-type protein (Fig. 4C, lower panel).

The elevation in the amount of Msc1–6-Cys→Ala protein prompted us to consider the possibility that Msc1 self-ubiquitylates, leading to autoregulation of its own protein level. If this is the case and if Rhp6 is the appropriate E2, we anticipated that levels of Msc1 might be elevated in an rhp6Δ mutant. In the background of a strain lacking rhp6, however, the level of Msc1 is similar to that seen in a wild-type rhp6Δ background (Fig. 4D). Thus, although Msc1 may utilize Rhp6 to ubiquitylate other substrates in vivo, it is unlikely that Rhp6 targets Msc1 itself for degradation.

We also examined whether co-precipitating Rhp6 might be sufficient to support Msc1-mediated

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**Msc1 Exhibits Ubiquitin Ligase Activity**

![Figure 3](image_url)

**FIGURE 3.** The PHD fingers promote polyubiquitylation through Lys-63 linkages. A, methyl-ubiquitin blocks the formation of high molecular weight products. GST-PHD1, -2, and -3 or GST alone was incubated with ATP, E1, E2 (Ubc2), and *S. pombe* histones in the presence of wild-type ubiquitin (lanes 1–4) or a form of ubiquitin that is methylated (lanes 5–6). Reactions were processed as in Fig. 2A but resolved by 8 and 12% SDS-PAGE. The bottom panel is a longer exposure of the portion of the gel where ubiquitin migrates. B, formation of high molecular weight products requires Lys-63 of ubiquitin. GST-PHD1 (lanes 1–3) or GST alone (lanes 4–6) was incubated with wild-type (WT) ubiquitin (lanes 1 and 4), a ubiquitin mutant with an arginine substitution at lysine 48 (lanes 2 and 5), or a ubiquitin mutant with an arginine substitution at lysine 63 (lanes 3 and 6) along with ATP, E1, E2 (Ubc2), and *S. pombe* histones. Reactions were processed as in Fig. 2A.
Msc1 Exhibits Ubiquitin Ligase Activity

![Image](https://example.com/image)

**FIGURE 4. Full-length Msc1 interacts with Rhp6.** A, Msc1 co-precipitates Rhp6. Strains having wild-type Rhp6 and either HA-tagged Msc1 or untagged Msc1 were used in immunoprecipitation (IP) analysis. Msc1 was immunoprecipitated with F7 antibody as described under “Experimental Procedures,” and the immune complexes were run out on 8 and 13% gels and probed using anti-HA (Y11) or anti-Rhp6 antibodies, respectively. Mock samples were treated in the same way without addition of antibody. B, Rhp6 coprecipitates Msc1. The same strains as in Fig. 4A were immunoprecipitated with antibody to Rhp6, then processed as above. HA-tagged Msc1 was detected with F7 antibody. C, full-length Msc1 promotes ubiquitylation. An msc1::kan<sup>Δ</sup> deletion strain was transformed with plasmids containing either HA-tagged full-length wild-type msc1 (WT), the indicated full-length mutants, or an empty vector. Msc1 was immunoprecipitated from these strains and used in the ubiquitylation assay as described under “Experimental Procedures.” Reactions were processed as described in Fig. 2A. D, the abundance of Msc1 is not altered in cells lacking Rhp6. Strains with the indicated genotypes containing either wild-type Msc1 (Msc1<sup>WT</sup>) or HA-tagged Msc1 (Msc1<sup>HA</sup>) were grown to mid-log phase, and protein was extracted as described under “Experimental Procedures,” separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-HA antibody or anti-Ded1 antibody, a cytoplasmic protein used as a loading control (46). E, immunoprecipitated Msc1 exhibits Rhp6-dependent ubiquitylation activity. HA-tagged Msc1 immunoprecipitated from cells with an intact (lanes 1 and 3) or a null (lanes 2 and 4) rhp6 gene was assayed for ubiquitylation activity with (lanes 1 and 2) or without (lanes 3 and 4) the inclusion of Ubc2 as an E2.

A mutant allele is as functional as the wild-type msc1 plasmid, suggesting that the E3 ligase activity of the mutants is not necessary for suppression under these conditions (Fig. 5B). The single mutants are somewhat less efficient than the 6-Cys → Ala mutant at promoting survival upon exposure of cells to either UV light (Fig. 5B) or CPT (Fig. 5C). It is possible that the increased stability of the 6-Cys → Ala mutant protein (Fig. 4C) may explain why it is a better suppressor than the single mutants in these assays. In any case, the result argues that when wild-type Msc1 is present in the cell, the E3 ligase activity of the mutants is not necessary to confer survival under these conditions as the presence of excess Msc1 protein, regardless of activity, is sufficient.

To test whether the mutant versions of Msc1 could support survival of a chk1Δ strain in the absence of any wild-type Msc1 protein, plasmids were transformed into the chk1Δ msc1Δ strain and assayed for sensitivity to UV light and CPT treatment. As shown in Fig. 5D, the single point mutants allowed the strain to survive exposure to UV light to approximately the same degree as when wild-type msc1 was expressed. However, in this assay, the 6-Cys → Ala mutant failed to confer survival. In the presence of CPT, neither the 6-Cys → Ala nor the alleles with mutations in the second or third PHD domain conferred survival, although mutation of the first PHD domain is tolerated (Fig. 5E).

Because the mutant forms of Msc1 are functional when there is wild-type Msc1 in the cell but are largely nonfunctional when there is no wild-type Msc1 present, we considered the possibility that Msc1 forms multimers with itself, which might account for the ability of excess mutant protein to function when wild-type protein is present. To test this possibility we performed IP experiments with strains in which genomic Msc1 was tagged with the FLAG tag, and HA-tagged Msc1 was introduced on a plasmid. However, we were unable to co-precipitate the proteins with either antibody to the HA tag or the FLAG tag (supplemental Fig. 2), suggesting that Msc1 does not form multimers.

Cells lacking msc1 exhibit a ~30-fold increase in the rate of chromosomal loss as compared with wild-type cells. The presence or loss of Chr16 can be followed by formation of white and red colonies, respectively (34). When grown in the presence of limiting adenine, the ade6-210 mutation causes a buildup of red pigment that is an intermediate in the adenine biosynthesis pathway. The artificial chromosome carries an ade6-216 mutation. When the ade6-210 and ade6-216 mutations are present simultaneously,

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<sup>3</sup> S. Ahmed, B. E. Dul, X. Qiu, and N. C. Walworth, in preparation.
they demonstrate interallelic complementation (34). A cell with both of these mutations will form white colonies at low adenine concentrations. Loss of the artificial chromosome can then be followed by the formation of red colonies.

Transformation of wild-type Msc1 restored chromosome stability to the $msc1^-$ strain, whereas transformation of empty vector alone did not (Table 2). Transformation of the single mutants essentially rescued the high rate of chromosome loss

### TABLE 2

| Strain                  | Loss/division | -Fold difference compared to wild type | -Fold difference compared to $msc1^-$ |
|-------------------------|---------------|----------------------------------------|--------------------------------------|
| $msc1^D$ + $pmsc1$      | 0.00024       | 1.00                                   | 0.03                                 |
| $msc1^D$ + $pSP1$       | 0.0078        | 32.55                                  | 1.00                                 |
| $msc1^D$ + $p6C -> A$   | 0.0041        | 17.12                                  | 0.52                                 |
| $msc1^D$ + $pPHD1-7$    | 0.00025       | 1.04                                   | 0.03                                 |
| $msc1^D$ + $pPHD2-7$    | 0.00094       | 3.90                                   | 0.12                                 |
| $msc1^D$ + $pPHD3-7$    | 0.00024       | 1.01                                   | 0.03                                 |
exhibited by \( msc1 \Delta \). The 6-Cys \( \rightarrow \) Ala mutant showed a chromosone loss rate intermediate between that conferred by wild-type Msc1 and that exhibited by the \( msc1 \Delta \) deletion. Thus, genome stability can be maintained as long as some PHD domains are functional.

Deletion of Msc1 Suppresses Phenotypes Associated with the Absence of Rhp6—To evaluate genetic interactions between Msc1 and Rhp6, we obtained a strain lacking \( rhp6 \). As reported by others (35), a strain lacking \( rhp6 \) grows very slowly (Fig. 6A). Strikingly, combining \( msc1 \Delta \) with \( rhp6 \Delta \) fully restores growth (Fig. 6A). Deletion of \( rhp6 \) also confers sensitivity to DNA damaging agents, yet deletion of \( msc1 \) restores the ability to survive exposure to either hydroxyurea or UV light (Fig. 6, B and C). \( S.\ pomb\ e \ rhp6 \Delta \) cells are both temperature-sensitive (36) and cold-sensitive. Both of these phenotypes are also suppressed when \( msc1 \) is deleted in the \( rhp6 \Delta \) background (Fig. 6D).

We have previously reported that cells lacking Msc1 exhibit an increased level of histone H3 acetylation (2). In the course of examining other histone modifications in cells lacking \( msc1 \), we evaluated the methylation of histone H3 lysine 4. Deletion of \( msc1 \) does not in itself affect histone H3 K4 methylation when probed with antibody to mono-, di- or trimethylated forms of the protein (Fig. 6E and data not shown). However, as shown in Fig. 6E, deletion of \( rhp6 \) essentially abolishes H3 K4 dimethylation. Strikingly, methylation of histone H3 is completely restored in a \( msc1 \Delta rhp6 \Delta \) background. Although the explanation for this phenomena remains enigmatic, we speculate that Msc1 might inhibit the activity of a methyl transferase. Deletion of \( msc1 \) relieves that inhibition allowing for methylation to be restored when it is otherwise lost in the \( rhp6 \) mutant.

**DISCUSSION**

There has been disagreement in the literature over whether PHD fingers can act as E3 ubiquitin ligases (37–39). Although experimental evidence supports the notion that domains classified as PHD domains on the basis of sequence homology in both viral proteins and MEKK1 act as E3 ligases, it is possible that these PHD domains are in fact RING fingers that are sufficiently different from canonical RING fingers that they have been misclassified (39). However, since that proposal was initially made, two additional proteins with PHD fingers have been reported to act as E3 ubiquitin ligases, AIRE and mitochondrial ubiquitin ligase (MITOL) (17, 18). Bottomley et al. (40) performed structural analysis of the PHD finger in AIRE and argue that it is a PHD finger rather than a misclassified RING finger. Furthermore, in assays for E3 ligase activity using autoubiquitylation as the assay, these investigators were unable to detect activity. On the other hand, Uchida et al. (17) analyzed the ability of AIRE to ubiquitylate substrates in a rabbit reticulocyte lysate system with positive results. One possible expla-
nation for the discrepancy is that PHD fingers, unlike RING fingers, may be unable to produce autoubiquitination activity. Supporting this idea, the PHD fingers of Msc1 cannot autoubiquitylate. Formation of the high molecular weight ubiquitin conjugates by the Msc1 PHD fingers depends not only on E1, E2, and ATP but also on the presence of an exogenous substrate (see Fig. 2A, lane 7 for example).

Bottomly et al. (40) also suggest that the C-terminal helix that contains the last cysteine dyad coordinating the second metal binding site is not comparable with RING fingers in proteins shown to act as E3 ubiquitin ligases. In c-Cbl, this helix is part of a shallow hydrophobic groove suitable for van der Waals interactions with their E2 partners. The authors indicate that this groove is missing in AIRE and, therefore, might not be able to bind an E2, although this was not proven experimentally. We have shown that Msc1 can co-immunoprecipitate with the E2 Rhp6, the *S. pombe* homologue of Ubc2. Thus, although we do not have a structure for the PHD fingers of Msc1, our results suggest that they are more similar biochemically to the subset of RING fingers that may be misclassified as PHD fingers.

The physical interaction between Rhp6 and Msc1 suggests that they might function in the same cellular pathway. Unexpectedly, however, we demonstrate a genetic interaction between them that suggests a more complex relationship. Except for a mating deficit exhibited by the *rhp6* deletion, many of the known phenotypes of a strain lacking *rhp6* are suppressed rather than mimicked by deletion of *msc1*. We do not yet have a molecular explanation for this genetic interaction. However, it is clear from studies of Rad6, the Rhp6 homologue in budding yeast, that a single E2 associates with multiple E3 ubiquitin ligase partners (41–44). Thus, deletion of Rhp6, like Rad6, likely affects multiple pathways. In addition, in the absence of Rhp6, Msc1 may be free to perform other functions to the detriment of the cell. If this were the case, then deletion of *msc1* could restore such cells to health. Given the multiple functional domains of Msc1, we expect that it will have additional functional roles and may interact with many other cellular proteins. Additional genetic and biochemical approaches will be required to fully understand the relationship between Rhp6 and Msc1.

Interestingly, cells lacking *rhp6* exhibit a reduced level of histone H3 methylation on lysine 4, suggesting that Rhp6 normally acts in some way to promote methylation. When *msc1* is simultaneously disrupted in an *rhp6Δ* strain, methylation is restored, suggesting that Msc1 might normally act to limit methylation. Whether Rhp6 and Msc1 act in concert to control methylation or whether they impact on differing mechanisms to contribute to methylation remains to be determined. Given that the proteins physically interact and contribute to ubiquitylation activity, it is tempting to speculate that a target protein important for governing the level of histone methylation might be a target of the Rhp6/Msc1 E2/E3 activity. Clearly the relationship between Rhp6, Msc1, and the methylation status of histones remains to be dissected. It is interesting to note that a mammalian homologue of Msc1, Rbp2, has recently been reported to have histone demethylase activity, encoded by the JmjC domain (45). Although the JmjC domain of Msc1 does not share all conserved residues thought to be important for demethylase activity, the fact that deletion of Msc1 restores methylation would be consistent with the elimination of a demethylase activity.

The activity of the PHD fingers is important for Msc1 function *in vivo*. All three PHD fingers need to be intact to rescue *chk1* when damage is generated due to limiting amounts of DNA ligase. Our results also show that when all three PHD fingers have cysteine to alanine mutations, Msc1 can no longer promote survival of a *chk1* strain in response to UV- or CPT-induced DNA damage, although single mutations in any one finger still allow for survival. Although the single mutants still have E3 ligase activity when tested *in vitro*, this activity is significantly less than the wild-type activity. The ability of the single mutants to rescue *chk1* in the presence of UV and CPT, but not in the presence of unligated DNA, suggests that the demand for the function of the PHD fingers may be greater in the latter situation. Our analysis does not yet pinpoint the role of Msc1-mediated ubiquitylation, although identification of a physiological substrate will undoubtedly offer further insight.

Future studies will help elucidate the cellular targets of Msc1 and may explain why a single protein would possess three E3 ubiquitin ligase domains. We speculate that perhaps Msc1 could target several substrates simultaneously, such as multiple components of a complex. Msc1 is a nuclear protein and was previously shown to fractionate with chromatin (2). Thus, it is possible that the target of Msc1 might be a chromatin-associated protein. The relationships, if any, between the role of Msc1 in ubiquitylation, the methylation status of histones, and the ability of Msc1 to restore survival of a *chk1* deficient strain, remain to be examined.

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REFERENCES

1. Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002) *Annu. Rev. Genet.* 36, 617–656

2. Ahmed, S., Palermo, C., Wan, S., and Walworth, N. C. (2004) *Mol. Cell. Biol.* 24, 3660–3669

3. Defeo-Jones, D., Huang, P. S., Jones, R. E., Haskell, K. M., Vuocolo, G. A., Hanobik, M. G., Huber, H. E., and Oliff, A. (1991) *Nature* 352, 251–254

4. Lu, F. I., Sundquist, K., Backstrom, D., Poulsom, R., Hanby, A., Meier-Ewert, S., Jones, T., Mitchell, M., Pitha-Rowe, P., Freemont, P., and Taylor-Papadimitriou, J. (1999) *J. Biol. Chem.* 274, 15633–15645

5. Benevolenskaya, E. V., Murray, H. L., Branton, P., Young, R. A., and Kaelin, W. G., Jr. (2005) *Mol. Cell* 18, 623–635

6. Catteau, A., Rosewell, L., Solomon, E., and Taylor-Papadimitriou, J. (2004) *Int. J. Oncol.* 25, 5–16

7. Tan, K., Shaw, A. L., Madsen, B., Jensen, K., Taylor-Papadimitriou, J., and Freemont, P. S. (2003) *J. Biol. Chem.* 278, 20507–20513

8. Fattaey, A. R., Helin, K., Dembski, M. S., Dyson, N., Harlow, E., Vuocolo, G. A., Hanobik, M. G., Haskell, K. M., Oliff, A., Defeo-Jones, D., and Jones, R. E. (1993) *Oncogene* 8, 3149–3156

9. Schindler, U., Beckmann, H., and Cashmore, A. R. (1993) *Plant J.* 4, 137–150

10. Jacobson, S., and Pillus, L. (1999) *Curr. Opin. Genet. Dev.* 9, 175–184

11. Capili, A. D., Schultz, D. C., Rauhser, F. J., III, and Borden, K. L. B. (2001) *EMBO J.* 20, 165–177
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12. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67**, 425–479
13. Pickart, C. M. (2001) *Annu. Rev. Biochem.* **70**, 503–533
14. Boname, J. M., and Stevenson, P. G. (2001) *Immunity* **15**, 627–636
15. Coscoy, L., Sanchez, D. J., and Ganem, D. (2001) *J. Cell Biol.* **155**, 1265–1273
16. Lu, Z., Xu, S., Joazeiro, C., Cobb, M. H., and Hunter, T. (2002) *Mol. Cell* **9**, 945–956
17. Uchida, D., Hatakeyama, S., Matsushima, A., Han, H., Ishido, S., Hotta, H., Kudoh, J., Shimizu, N., Doucas, V., Nakayama, K. I., Kuroda, N., and Matsumoto, M. (2004) *J. Exp. Med.* **199**, 167–172
18. Yonashiro, R., Ishido, S., Kyo, S., Fukuda, T., Goto, E., Matsuki, Y., Ohmura-Hoshino, M., Sada, K., Hotta, H., Yamamura, H., Inatome, R., and Yanagi, S. (2006) *EMBO J.* **25**, 3618–3626
19. Goto, E., Ishido, S., Sato, Y., Ohgimoto, S., Ohgimoto, K., Nagano-Fujii, M., and Hotta, H. (2003) *J. Biol. Chem.* **278**, 14657–14668
20. Pena, P. V., Davrazou, F., Shi, X., Walter, K. L., Verkhusha, V. V., Gozani, O., Zhao, R., and Kutateladze, T. G. (2006) *Nature* **442**, 100–103
21. Li, H., Ilin, S., Wang, W., Duncan, E. M., Wysocka, J., Allis, C. D., and Patel, D. I. (2003) *Nature* **442**, 91–95
22. Shi, X., Hong, T., Walter, K. L., Ewalt, M., Michishita, E., Hung, T., Carney, D., Pena, P., Lan, F., Kaadige, M. R., Lacoste, N., Cayrou, C., Davrazou, F., Saha, A., Cairns, B. R., Ayer, D. E., Kutateladze, T. G., Shi, Y., Cote, J., Chua, K. F., and Gozani, O. (2006) *Nature* **442**, 96–99
23. Wysocka, J., Swigut, T., Xiao, H., Milne, T. A., Kwon, S. Y., Landry, J., Kauer, M., Tackett, A. J., Chait, B. T., Badenhorst, P., Wu, C., and Allis, C. D. (2006) *Nature* **442**, 86–90
24. Moreno, S., Klar, A., and Nurse, P. (1991) *Methods Enzymol.* **194**, 795–823
25. Smith, D. B., and Johnson, K. S. (1988) *Gene (Amst.)* **67**, 31–40
26. Edmondson, D. G., and Roth, S. Y. (1998) *Methods* **15**, 355–364
27. Martin, D. G., Baetz, K., Shi, X., Walter, K. L., Macdonald, V. E., Wlodarski, M. J., Gozani, O., Hieter, P., and Howe, L. (2006) *Mol. Cell. Biol.* **26**, 7871–7879
28. Pickart, C. M., and Fushman, D. (2004) *Curr. Opin. Chem. Biol.* **8**, 610–616
29. Hershko, A., and Heller, H. (1985) *Biochem. Biophys. Res. Commun.* **128**, 1079–1086
30. Haglund, K., and Dikic, I. (2005) *EMBO J.* **24**, 3353–3359
31. Leggett, D. S., Jones, D., and Candido, E. P. (1995) *DNA Cell Biol.* **14**, 883–891
32. Jentsch, S., McGrath, J. P., and Varshavsky, A. (1987) *Nature* **329**, 131–134
33. Xie, Y., and Varshavsky, A. (1999) *EMBO J.* **18**, 6832–6844
34. Wang, S. W., Read, R. L., and Norbury, C. I. (2002) *J. Cell Sci.* **115**, 587–598
35. Reynolds, P., Koken, M. H., Hoeijmakers, J. H., Prakash, S., and Prakash, L. (1990) *EMBO J.* **9**, 1423–1430
36. Singh, J., Goel, V., and Klar, A. J. (1998) *Mol. Cell. Biol.* **18**, 5511–5522
37. Aravind, L., Iyer, L. M., and Koonin, E. V. (2003) *Cell Cycle* **2**, 123–126
38. Coscoy, L., and Ganem, D. (2003) *Trends Cell Biol.* **13**, 7–12
39. Scheel, H., and Hofmann, K. (2003) *Trends Cell Biol.* **13**, 285–288
40. Bottomley, M. J., Stier, G., Pennacchini, D., Legube, G., Simon, B., Akhtar, A., Sattler, M., and Musco, G. (2005) *J. Biol. Chem.* **280**, 11505–11512
41. Wood, A., Krogan, N. J., Dover, J., Schneider, J., Heidt, J., Boateng, M. A., Dean, K., Gelshani, A., Zhang, Y., Greenblatt, J. F., Johnston, M., and Shilatifard, A. (2003) *Mol. Cell* **11**, 267–274
42. Dohmen, R. J., Madura, K., Bartel, B., and Varshavsky, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7351–7355
43. Bailly, V., Lauder, S., Prakash, S., and Prakash, L. (1997) *J. Biol. Chem.* **272**, 23360–23365
44. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) *Nature* **419**, 135–141
45. Klose, R. J., Yan, Q., Tothova, Z., Yamane, K., Erdjument-Bromage, H., Tempst, P., Gilliland, D. G., Zhang, Y., and Kaelin, W. G., Jr. (2007) *Cell* **128**, 889–900
46. Liu, H.-Y., Nefsky, B. S., and Walworth, N. C. (2002) *J. Biol. Chem.* **277**, 2637–2643