Hip3 Interacts with the HIRA Proteins Hip1 and Slm9 and Is Required for Transcriptional Silencing and Accurate Chromosome Segregation*

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The fission yeast HIRA proteins Hip1 and Slm9 are members of an evolutionarily conserved family of histone chaperones that are implicated in nucleosome assembly. Here we have used single-step affinity purification and mass spectrometry to identify factors that interact with both Hip1 and Slm9. This analysis identified Hip3, a previously uncharacterized 187-kDa protein, with similarity to S. cerevisiae Hir3. Consistent with this, cells disrupted for hip3 exhibit a range of growth defects that are similar to those associated with loss of Hip1 and Slm9. These include temperature sensitivity, a cell cycle delay, and synthetic lethality with cdc25–22. Furthermore, genetic analysis also indicates that disruption of hip1+ is epistatic with mutation of hip1+ and slm9+. Mutation of hip3+ alleviates transcriptional silencing at several heterochromatic loci, including in the outer (otr) centromeric repeats, indicating that Hip3 is required for the integrity of pericentric heterochromatin. As a result, loss of Hip3 function leads to high levels of minichromosome loss and an increased frequency of lagging chromosomes during mitosis. Importantly, the function of Hip1, Slm9, and Hip3 is not restricted to constitutive heterochromatin loci, since these proteins also repress the expression of a number of genes, including the Tf2 retrotransposons.

Centromeres play a critical role in the precise segregation of chromosomes, and as a result, defects in centromere function lead to aneuploidy (1). The fission yeast Schizosaccharomyces pombe provides an excellent system for the study of centromeres (2). In contrast to the budding yeast Saccharomyces cerevisiae, which has simple “point” centromeres (3), S. pombe has large complex centromeres that occupy between 35 and 110 kb and are arranged as a central core (cnt) flanked by arrays of repeated (imr and otr) elements (2). In this respect, S. pombe centromeres are reminiscent of the complex regional centromeres of metazoans. Furthermore, ultrastructural studies have revealed that the overall architectural organization of fission yeast centromeres is conserved with their human counterparts (4). Fission yeast centromeres are organized into distinct chromatin domains (2, 5). An inner domain is assembled into specialized chromatin in which core histone H3 is replaced by Cnp1, the fission yeast homologue of CENP-A (6), whereas the outer regions are associated with chromatin that resembles the pericentric heterochromatin of higher cells (2). Marker genes inserted into these outer regions are subject to heritable inactivation (7, 8), which is dependent upon the RNA interference machinery, the methylation of histone H3 on lysine 9, and the association of a number of proteins, including Swi6, a homologue of mammalian HP1 (heterochromatin protein 1) (2, 5, 9–12). In addition, the integrity of pericentric heterochromatin in fission yeast is dependent upon the two HIRA proteins Hip1 and Slm9 (13, 14), since loss of either protein alleviates silencing in the otr centromeric repeats and results in increased levels of chromosome missegregation (13).

The S. pombe HIRA proteins Hip1 and Slm9 are members of a family of histone chaperones that are conserved in all eukaryotes (15). S. cerevisiae also has two HIRA proteins (Hir1 and Hir2) (16, 17), whereas higher eukaryotes have a single protein (15, 18–20). HIRA proteins were originally identified in S. cerevisiae as repressors of histone expression, since mutation of HIR1 or HIR2 was found to result in the constitutive transcription of six of the eight budding yeast histone genes (17, 21). Subsequently, HIRA proteins have been implicated in nucleosome assembly and the organization of repressive chromatin (13, 22–25). In higher eukaryotes, HIRA has been identified as a critical component of a replication-independent nucleosome deposition pathway (26, 27). Furthermore, in budding yeast, HIRA proteins functionally overlap with CAF-1 (chromatin assembly factor 1), since the mutation of HIR1 or HIR2 exacerbates the chromosome segregation and transcriptional silencing defects associated with loss of CAF-1 (23, 25, 28). The fission yeast HIRA proteins Hip1 and Slm9 are required for heterochromatic silencing at centromeres and also at the mating type (mat) region even in the presence of functional CAF-1 (13). Recently, human HIRA has been shown to be required for the formation of senescence-associated heterochromatin in human cells (24), indicating that HIRAs have roles in repressive chromatin in mammals.

Xenopus and human HIRA proteins have been found to be components of large but poorly defined protein complexes (26, 29), and here we demonstrate that the S. pombe proteins Hip1 and Slm9 are also components of large protein complexes. Moreover, we have used affinity purifications coupled with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to identify potential components of these complexes. This analysis revealed that both Hip1 and Slm9 co-purify with a 187-kDa protein that we have called Hip3. Disruption of hip3 results in a range of phenotypes that are highly similar to those associated with mutation of hip1+ or slm9+. These include temperature sensitivity, a cell cycle delay, increased rates

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3 The abbreviations used are: MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; 5-FOA, 5-fluoroorotic acid; TBZ, thiabendazole.
of chromosome missegregation, and defective heterochromatin silencing. Furthermore, genetic analysis indicates that mutations in hip1<sup>+</sup>, slm9<sup>-</sup>, and hip3<sup>-</sup> are epistatic. Thus, the data suggest that Hip1, Slm9, and Hip3 function on a nucleosome assembly pathway that is required for the integrity of heterochromatin and accurate chromosome segregation.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Strains—**Routine culture of *S. pombe* and general genetic methods were performed as described previously (30). The strains used in this study are described in Table 1. The hip3<sup>-</sup> open reading frame was disrupted using one-step gene replacement. An 846-bp fragment from the 5′-end of the hip3<sup>-</sup> open reading frame was PCR-amplified using oligonucleotides Hip3KO<sub>A</sub> (5′-AGTTATTCTCCTTCTATGGC-3′) and Hip3KO<sub>B</sub> (5′-GCTAATTCGGGATACGAC-3′) and then cloned into pGEM-T (Promega) to yield pGEM-Hip3. The 1.8-kb *ura4<sup>+</sup>* cassette from pRep4 was then cloned into the HindIII site to give plasmid pGEM-Hip3::ura4<sup>-</sup>. The BGII fragment from pGEM-Hip3::ura4<sup>-</sup> was used to transform an NT4/NT5 diploid strain and integration at the correct segregation was confirmed by PCR. A locus was confirmed by PCR. A

**TABLE 1**

| Strain   | Genotype                        | Source         |
|----------|---------------------------------|----------------|
| SW5      | h<sup>-</sup> ade6-M126 leu1-32 ura4-D18 | Laboratory stock |
| SW4      | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 | Laboratory stock |
| SW307    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip1-2CTAP(ura4<sup>-</sup>) | This study |
| SW365    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 slm9-2CTAP(ura4<sup>-</sup>) | This study |
| pku1TAP  | h<sup>-</sup> ade6-704 leu1-32 ura4-D18 pku70-CTAP(KAN<sup>+</sup>) | A. Carr |
| SW387    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip1-Pk(ura4<sup>-</sup>) | Ref. 13 |
| SW390    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip1-Pk(ura4<sup>-</sup>) | Ref. 13 |
| SW401    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip1-Pk(ura4<sup>-</sup>) slm9-3XFLAG(ura4<sup>-</sup>) | This study |
| SW137    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip1::URA8 | Ref. 13 |
| SW138    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip3::URA8 | Ref. 13 |
| SW347    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip3::LEU2 | This study |
| SW375    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip3-3XFLAG(ura4<sup>-</sup>) | This study |
| SW376    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip3-3XFLAG(LEU2) | This study |
| SW382    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip3-3XFLAG(ura4<sup>-</sup>) slm9-2CTAP(ura4<sup>-</sup>) | This study |
| SW385    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip3-3XFLAG(LEU2) hip1-2CTAP(ura4<sup>-</sup>) | This study |
| SW248    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 slm9-3Pk(ura4<sup>-</sup>) | Ref. 13 |
| SW200    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 slm9-3Pk(ura4<sup>-</sup>) hip1::ura4<sup>-</sup> | This study |
| SW198    | h<sup>-</sup> leu1-32 ura4-D18 slm9-3Pk(ura4<sup>-</sup>) hip1-3Pk(ura4<sup>-</sup>) | This study |
| SW371    | h<sup>-</sup> leu1-32 ura4-D18 slm9::ura4<sup>-</sup> hip3::URA8 | This study |
| SW373    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip3::LEU2 hip1::ura4<sup>-</sup> | This study |
| JK2246   | h<sup>-</sup> leu1-32 ura4-D18 slm9::ura4<sup>-</sup> | Ref. 14 |
| SW152    | h<sup>-</sup> leu1-32 ura4-D18 slm9::ura4<sup>-</sup> hip1::ura4<sup>-</sup> | Ref. 13 |
| SW377    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 slm9::Ppk(ura4<sup>-</sup>) hip3::ura4<sup>-</sup> | This study |
| SW389    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip1::ura4<sup>-</sup> hip3-3XFLAG(LEU2) | This study |
| SW380    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip1::URA8 hip3-3XFLAG(ura4<sup>-</sup>) | This study |
| SW115    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 (Chl6-216-LEU2) | Ref. 13 |
| SW145    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 (Chl6-216-LEU2) hip3::URA8 | This study |
| FY412    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18/D/E CCl2(Shpk::ura4<sup>-</sup>) | Ref. 8 |
| SW391    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18/3XFLAG Hip3::URA8 | Ref. 14 |
| FY1182   | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 otRIR(Shpk::ade6) | Ref. 48 |
| SW139    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 slm9::Ppk(ura4<sup>-</sup>) otRIR(Shpk::ade6) | This study |
| SW151    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip1::URA8 otRIR(Shpk::ade6) | Ref. 13 |
| SW364    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 hip3::URA8 otRIR(Shpk::ade6) | This study |
| PG1672   | mat1-P17::LEU2 mat3-M(EcoRV)::ade6 ade6-M201 leu1-32 ura4-D18 | Ref. 40 |
| SW349    | mat1-P17::LEU2 mat3-M(EcoRV)::ade6 ade6-M201 leu1-32 ura4-D18 slm9::ura4<sup>-</sup> | Ref. 13 |
| SW150    | mat1-P17::LEU2 mat3-M(EcoRV)::ade6 ade6-M201 leu1-32 ura4-D18 hip1::ura4<sup>-</sup> | Ref. 13 |
| SW363    | mat1-P17::LEU2 mat3-M(EcoRV)::ade6 ade6-M201 leu1-32 ura4-D18 hip3::URA8 | This study |
| FY1862   | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 his3-D1 otRIR(Shpk::ade6) tel11::his3 tel22::ura4<sup>-</sup> | Ref. 49 |
| SW388    | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 tel22::ura4<sup>-</sup> hip3::LEU2 | This study |
| FY1004   | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18 clr4::LEU2 | R. Allshire |
| FY3019   | h<sup>-</sup> ade6-GN/NT leu1-32 ura4<sup>-</sup> hip1::URA8 | R. Allshire |
| FY2283   | h<sup>-</sup> ade6-M120 leu1-32 ura4-D18/D/E otRIR(Shpk::ade6<sup>-</sup>) rik1::LEU2 his1::URA8 | R. Allshire |
| sw64Δ    | h<sup>-</sup> ura4-D18 sw64-G4C | Ref. 50 |
GAATTTCCTCATGATATACTCG-3') and hip3PstI (5'-GGACATCTCG-CAGTAAATCCAGTCGACAAGTTTTCG-3'), digested with PstI and BamHI, and cloned into pRip42-3XFLAG. The resulting plasmid (pRip42-Hip3-FLAG3X) was digested with BclI before being transformed into the appropriate strain. A LEU2-based plasmid for FLAG tagging of the hip3+ open reading frame was constructed by digesting pRip42-Hip3-FLAG3X with HindIII and cloning the LEU2-containing HindIII fragment from pRep41 to give pRip41-Hip3-3XFLAG. Correct integration and expression of fusion proteins was confirmed by PCR and Western analysis.

**TAP Purifications**—10-Liter cultures were grown at 30 °C in YEP medium (2 g/liter bactopeptone, 5 g/liter yeast extract, 30 g/liter glucose, 225 mg/liter adenine, histidine, leucine, uracil, and lysine) until they reached A<sub>695</sub> = 2. They were then diluted with an equal volume of fresh, prewarmed YEP and incubated for a further 2 h. Cells were harvested and washed twice with chilled water and twice with HB buffer (25 mM Tris-HCl, pH 7.5, 15 mM EGTA, pH 7.5, 15 mM MgCl<sub>2</sub>, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM NaF). Pellets were resuspended in an equal volume (v/v) of HB buffer containing 1 mg/ml phenylmethylsulfonyl fluoride and protease inhibitor mixture (Roche Applied Science) and disrupted into liquid nitrogen. Cells were crushed by grinding in a mechanical pestle and mortar as described previously (34). In order to purify the TAP-tagged proteins, powdered cell lysates were thawed at 4 °C and clarified by spinning at 15,000 rpm for 1 h. Proteins were immunoprecipitated by incubation at 4 °C for 2 h with 500 μl of a 50% slurry of IgG-coupled Dynal beads pre-equilibrated into HB buffer. Beads were recovered and washed three times with HB buffer, three times with IPP150 buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM NaF). Pellets were resuspended in 0.5 ml TEV cleavage buffer, and protein complexes were released by the addition of 300 units of TEV protease and incubating at 25 °C for 1 h. The recovered eluates were concentrated by spinning in Vivascin concentrator columns (10,000 molecular weight cut-off) and electrophoresed through Bio-Rad Criterion precast gels (4–20%). Gels were stained for 2 h with Simply Blue Safestain (Invitrogen), and bands were excised and subjected to MALDI-TOF mass spectrometry.

**Protein Digestion**—Gel pieces containing the protein of interest were excised from the Coomassie Blue-stained gel, cut into small pieces, rehydrated with 50 μl of water for 5 min, and then destained with 50 μl of 25 mM Tris, pH 8, in 50% acetonitrile for 30 min. 50 μl of reduction buffer (10 mM Tris(2-carboxyethyl)phosphine in 25 mM Tris, pH 8) was added and incubated for 30 min at 56 °C. Next, 50 μl of alkylation buffer containing 100 μl iodoacetamide in water was added and incubated at room temperature in the dark for 30 min. The gel pieces were washed twice with 50 μl of water for 5 min and then dehydrated by washing twice with 100 μl of acetonitrile for 10 min at 30 °C. After drying under vacuum, the gel pieces were rehydrated on ice with 10 μl of digestion buffer containing 25 mM Tris, pH 8, 5 mM CaCl<sub>2</sub>, and 25 ng of trypsin (Promega, Madison, WI). After 10 min, a further 10–20 μl of the same buffer (without trypsin) was added to cover the gel slices, and the digests were then incubated for 16 h at 35 °C. The resulting tryptic peptides were then extracted twice using 10–20 μl of 0.1% trifluoroacetic acid in 60% acetonitrile at 56 °C for 30 min. Pooled extracts were dried using a SpeedVac and then redissolved in 10 μl of 0.1% trifluoroacetic acid and purified with Zip-Tip C<sub>18</sub> pipette tips (Millipore Corp., Billerica, MA), following the manufacturer’s recommended protocol. Peptides were eluted from the tip directly onto the MALDI plate with matrix solution of α-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile, 0.1% trifluoroacetic acid.

**MALDI Mass Spectrometry and Data Base Searching**—The MALDI-TOF mass spectrometer used was a Voyager DE-STR (Applied Biosystems Inc., Framingham, MA). The instrument was equipped with a delayed extraction ion source, used a nitrogen laser at 337 nm and was operated in reflector mode at accelerating voltages of 20–25 kV. Mass spectra were obtained over a mass range of 900–4000 Da, and monoisotopic peptide mass fingerprints were assigned and used for data base searches. Identifications were performed using the peptide mass fingerprint data and the Mascot search engine program (Matrix Science Ltd., London, UK), where the peptide mass tolerance was limited to 50 ppm, and searched against the NCBI nonredundant protein sequence data base.

**Co-immunoprecipitations**—Whole cell extracts were prepared as described previously (13) except that the standard lysis buffer was substituted with HB buffer. Immunoprecipitations were performed by adding 25 μl of IgG-coupled Dynal beads to 1 mg of whole protein extract and incubating for 1 h at 4 °C with gentle agitation. Beads were recovered and washed three times with 1 ml of HB buffer. Samples were electrophoresed through SDS-polyacrylamide gels and subjected to Western blotting using monoclonal anti-FLAG antibodies (Sigma) and/or a peroxidase anti-peroxidase-soluble complex produced in rabbit (Sigma).

**Immunofluorescence Microscopy**—Microscopy was performed as described previously (35). DAPI and fluorescein isothiocyanate fluorophores were captured by exciting cells with 450–490-nm and 365-nm wavelengths, respectively, using a Zeiss Axioscope microscope with a ×63 oil immersion objective and Axiovision imaging software.

**RNA Analysis**—RNA was purified and analyzed as described previously (13). Gene-specific probes were produced by PCR amplification from genomic DNA using the appropriate primers. All probes were labeled with [α-<sup>32</sup>P]dCTP by using a Prime-a-Gene labeling kit (Promega).

**RESULTS**

**Hip1 and Slm9 Are Components of Large Protein Complexes**—In higher cells, HIRA proteins have been reported to exist in large protein complexes (26, 29), but the composition of these complexes has yet to be defined. In order to determine whether the *S. pombe* HIRA proteins are also part of multisubunit complexes, a strain was constructed in which the chromosomal copy of *slm9*<sup>+</sup> was tagged with the 3XFLAG epitope and the chromosomal *hip1*<sup>+</sup> gene was tagged with the Pk epitope. A whole cell extract from the resulting strain was then subjected to gel filtration chromatography. Western blotting revealed that significant pools of Hip1 and Slm9 co-eluted within high molecular mass fractions (greater than 450 kDa) (Fig. 1). The presence of Hip1 and Slm9 in these fractions was not a consequence of these particular epitope tags, since similar results were obtained with strains expressing alternatively tagged versions of Hip1 and Slm9 (Fig. 2 and data not shown). Since we have previously demonstrated that Hip1 and Slm9 interact, these results...
suggest that a portion of these factors co-exist in a large complex or complexes.

*Hip1 and Slm9 Co-purify with Hip3*—In order to identify proteins that interact with both Hip1 and Slm9, a single-step affinity purification approach was employed (36). Strains were constructed in which the *hip1*+ or *slm9*+ gene was tagged with the TAP epitope. Hip1-TAP and Slm9-TAP fusion proteins were purified from whole extracts using IgG-conjugated magnetic beads. Protein complexes were then eluted by treatment with TEV protease and analyzed by SDS-PAGE. In order to provide a control for these experiments, whole extracts derived from a strain expressing TAP-tagged Pku70 were also subjected to affinity purification. Staining of SDS-polyacrylamide gels revealed several protein bands that were present in both the Hip1-TAP and Slm9-TAP purifications but absent from the Pku70-TAP purification (Fig. 2A), so these proteins were identified by peptide mass fingerprint analysis using MALDI-TOF mass spectrometry. In agreement with our previous findings, Hip1 co-purified with Slm9 and vice versa. In addition, both Hip1 and Slm9 also co-purified with a large protein that was identified as SPBC31F10.14c, a 187-kDa protein that we named Hip3 (Hir in *pombe*). Furthermore, fractionation of a whole cell extract derived from slm9-TAP hip3–3XFLAG cells was fractionated over a Superose 6 column and analyzed by SDS-PAGE and Western blotting. D, whole cell extracts derived from the indicated strains were analyzed by SDS-PAGE and Western blotting with TAT-1 anti-tubulin antibodies (which serves as loading control) and either anti-Pk antibodies (Serotec) or anti-FLAG antibodies (Sigma).

**FIGURE 2. Hip3 co-purifies with Hip1 and Slm9.** A, affinity-purified TAP fusion proteins were analyzed by SDS-PAGE and stained with Simply Blue Safestain (Invitrogen). The strains used are indicated above the lanes and were Pku-TAP (pku70-TAP), Hip1-TAP (SW307), and Slm9-TAP (SW365). B, Hip3 co-immunoprecipitates with Hip1 and Slm9. Whole cell extracts derived from the appropriate strains were immunoprecipitated with IgG-coupled magnetic beads and subjected to SDS-PAGE and Western blotting with peroxidase anti-peroxidase (α-PAP) and anti-FLAG antibodies. The strains used were SW375 (lane 1), SW365 (lane 2), SW382 (lane 3), SW376 (lane 4), SW307 (lane 5), and SW385 (lane 6). C, whole cell extract from slm9-TAP hip3–3XFLAG cells was fractionated over a Superose 6 column and analyzed by SDS-PAGE and Western blotting. D, whole cell extracts derived from the indicated strains analyzed by SDS-PAGE and Western blotting with TAT-1 anti-tubulin antibodies (which serves as loading control) and either anti-Pk antibodies (Serotec) or anti-FLAG antibodies (Sigma).

Hip1 or Slm9 (Fig. 2D). Furthermore, disruption of *hip3*+ in a *slm9*Δ-Pk background also revealed that Slm9 protein levels were reduced by the absence of Hip3 (Fig. 2D). Therefore, the levels of Slm9 and Hip3 are interdependent, and also the level of both of these proteins is dependent upon Hip1. Taken together, these data are consistent with Hip1, Slm9, and Hip3 forming a complex.

Since loss of Slm9 does not result in reduced levels of Hip1, we examined whether Slm9 influences the subcellular localization of Hip1. Using immunofluorescence microscopy, we have previously demonstrated that Hip1 is predominantly localized to the nucleus (13) (see also Fig. 3). However, in an *slm9*Δ background, Hip1 was not enriched in the nucleus, and staining was observed throughout the cell (Fig. 3). Thus, Slm9 is required for the correct subcellular distribution of Hip1.

**Disruption of *hip3*+ Results in Growth Defects**—Further characterization of *hip3*Δ cells revealed that loss of Hip3 resulted in a number of readily detectable growth defects. In common with *hip1Δ* and *slm9Δ* cells, *hip3*Δ cells were temperature-sensitive, having a very limited ability to grow at elevated temperatures (>35 °C) (Fig. 4A). These mutants were also cold-sensitive, since they were unable to grow at 15 °C (Fig. 4A). Microscopic examination of *hip3*Δ cells also revealed an elongated morphology (Fig. 4B), a phenotype that is reminiscent of *hip1Δ* and
Fission Yeast HIRA Complexes

FIGURE 4. Deletion of hip3Δ results in temperature sensitivity and a cell cycle delay. A, the indicated strains were grown to exponential phase, subjected to 5-fold serial dilutions, and spotted onto YEP agar plates and YEP plates supplemented with TBZ at 7.5 or 10 μg/ml. Plates were incubated at 30 °C for 3 days (YEP) or for 5 days (YEP + TBZ).

FIGURE 5. hip3Δ cells are hypersensitive to spindle damage. The indicated strains were grown to exponential phase, subjected to 5-fold serial dilutions, and spotted onto YES5 agar plates and YES5 plates supplemented with TBZ at 7.5 or 10 μg/ml. Plates were incubated at 30 °C for 3 days (YES5) or for 5 days (YES5 + TBZ).

TABLE 2
Ch16 minichromosome-containing colonies were plated onto adenine-limiting agar, and the frequency of half-sectored colonies was determined.

| Genotype | Ch16 loss at 30 °C |
|----------|-----------------|
| Wild type | 0.026 (3845) |
| hir3Δ | 0.308 (4221) |

slm9Δ cells and is indicative of a cell cycle delay (13, 14). Mutations in both hip1Δ and slm9Δ result in a G2 delay, and, consistent with this, are synthetically lethal with cdc25Δ-22, a temperature-sensitive allele of cdc25 (13, 14). The Cdc25 phosphatase is a key regulator of progression from G2 into M-phase because it activates the cyclin-dependent kinase Cdc2 (37). In the similarities in the morphologies of hip1Δ, slm9Δ, and hip3Δ cells suggested that Hip3 may also be essential in a cdc25Δ-22 background. Indeed, subsequent genetic crosses revealed that hip3Δ cdc25Δ-22 cells were not viable. Microscopic examination of the double mutant revealed that it was able to germinate but failed to progress through more than seven cell divisions. Thus, like Hip1 and Slm9, Hip3 is required for normal cell cycle progression.

In order to investigate the genetic interactions between hip1Δ, slm9Δ, and hip3Δ, we constructed a series of double mutant strains. We have previously shown that deletion of hip1Δ and slm9Δ is epistatic (13). Similarly, examination of hip1Δ hip3Δ and slm9Δ hip3Δ strains indicated that they had growth defects and cell length phenotypes that were not detectably more severe than those of the parental strains (Figs. 4 and 5). Taken together, these data indicate that mutations in hip1Δ, slm9Δ, and hip3Δ are epistatic, suggesting that Hip3 functions in conjunction with Hip1 and Slm9. Nonetheless, it is worth noting that hip1Δ cells do have some phenotypes that are not shared with slm9Δ and hip3Δ cells; for instance, hip1Δ cells are severely impaired in their ability to undergo mating (13), whereas both hip3Δ and slm9Δ cells mate with similarly efficiency to wild type cells. This indicates that Hip1 has roles in sexual development that are independent of Hip3 and Slm9.

Hip3 Is Required for Accurate Chromosome Segregation—We have previously demonstrated that both Hip1 and Slm9 are required for accurate chromosome segregation, and this prompted us to examine the role of Hip3 in this process. Mutants that are defective in chromosome segregation are often sensitive to the drug thiabendazole (TBZ), which depolymerizes microtubules and abrogates formation of the mitotic spindle. Therefore, the ability of hip3Δ cells to grow on rich agar plates containing TBZ was examined. This revealed that, like hip1Δ and slm9Δ cells, hip3Δ cells are also sensitive to this spindle poison (Fig. 5). Next, we compared Ch16 minichromosome loss rates in wild type and hip3Δ backgrounds. The nonessential Ch16 minichromosome contains the ade6–216 allele that complements the ade6–210 allele (38). Therefore, in an ade6–210 background, cells that contain Ch16 are Ade+ and form white colonies on adenine-limiting media, whereas loss of Ch16 results in a Ade– phenotype and red colonies due to the build up of a biosynthetic intermediate. In the wild type background, the Ch16 minichromosome is faithfully segregated, and consistent with previous reports, loss rate were only 0.026% per division (Table 2). In contrast, in a hip3Δ background, Ch16 loss rates were 0.31%, indicating that loss of Hip3 increases chromosome missegregation by an order of magnitude. This is also consistent with Hip3 functioning in concert with Hip1 and Slm9, since loss of these proteins leads to similar rates of minichromosome loss (0.38 and 0.21%, respectively) (13).

That cells lacking Hip1, Slm9, or Hip3 exhibit hypersensitivity to TBZ and significantly increased rates of minichromosome loss suggested that they may have defects in mitosis. Therefore, this was investigated using immunofluorescence microscopy; fixed cells were incubated with TAT-1 α-tubulin antibodies and DAPI to visualize spindle microtubules and DNA, respectively (Fig. 6A). Examination of more than 200 late anaphase cells with long spindles in a wild type background revealed that only 0.4% of cells displayed lagging chromosomes (Fig. 6B). In comparison, 2% of anaphase cells had lagging chromosomes in the hip3Δ strain. Moreover, the hip1Δ and slm9Δ mutants also exhibited high levels of lagging chromosomes (8.9 and 8.3%, respectively). These findings confirm that, like Hip1 and Slm9, Hip3 is required for accurate chromosome segregation.

Hip3 Is Required for Transcriptional Silencing—Next, we determined whether Hip3 is required for the integrity of centromeric chromatin, since disruption of this chromatin is known to result in chromosome missegregation (2). S. pombe has large complex centromeres that are organized into two distinct transcriptionally silent domains: a central region, which is the kinetochore assembly site, and the heterochromatic outer domains (10, 11). In order to determine whether Hip3 is required for transcriptional silencing in the outer domain, we utilized a strain in which an ade6– marker gene is inserted in the otr repeats of chromosome 1 (Fig. 7A). In a wild-type background, this marker gene is subjected to strong transcriptional silencing, and cells form red colonies in adenine-limited conditions. However, deletion of hip3Δ in this background resulted in the formation of light pink colonies, demonstrating...
that transcriptional silencing in the \( \text{otr} \) centromeric repeats is reduced, indicating that Hip3 is required for the integrity of pericentric heterochromatin (Fig. 7A). In order to establish whether this effect was confined to the outer region, we utilized a second strain harboring the \( \text{ura4}^+ \) marker gene in the central core of centromere 2 (Fig. 7B). Silencing of the \( \text{ura4}^+ \) allows cells to grow in the presence of 5-FOA, but expression of \( \text{ura4}^+ \) renders this compound toxic. We observed that deletion of \( \text{hip3}^- \) in this background results in cells being no more sensitive to 5-FOA than wild-type cells, indicating that silencing of the reporter is maintained (Fig. 7B). This suggests that, like \( \text{sim9}^- \) and \( \text{hip1}^- \), \( \text{hip3}^- \) is not required for function of the central core.

In addition to centromeres, heterochromatic transcriptional silencing is also observed at the mating (\( \text{mat} \)) locus and also at telomeres, although there is known to be specialization in the factors required at these different loci (39). Examination of a strain harboring a \( \text{ura4}^+ \) marker gene in telomere 2 indicated that deletion of \( \text{hip3}^- \) resulted in a slight increase in sensitivity to 5-FOA, but these cells were unable to grow on medium lacking uracil, indicating that silencing of the telomeric \( \text{ura4}^+ \) marker gene is largely maintained (Fig. 7C). To determine the influence of Hip3 upon \( \text{mat} \) silencing, we employed a strain in which the \( \text{ade6}^- \) marker gene was inserted next to the \( \text{mat3}^- \) locus (40). As previously reported, in a wild type background this strain formed red colonies on adenine-limited medium, indicating that the marker gene is silenced (Fig. 7D). However, a strain lacking Hip3 formed light pink colonies, indicating that silencing of the marker gene was reduced (Fig. 7D). Taken together, these data suggest that Hip3 contributes to the function of heterochromatin in the outer repeat (\( \text{otr} \)) sequences of centromeres and at the \( \text{mat} \) locus.
Fission Yeast HIRA Complexes

![Image](VOLUME 281 • NUMBER 13 •)

FIGURE 8. Hip1, Slm9, and Hip3 repress the expression of TF2 retrotransposons. Total RNA was prepared from the indicated strains and subjected to Northern blotting with the indicated probes.

Hip1, Slm9, and Hip3 Repress the Expression of Specific Genes, Including TF2 Retrotransposons—We next sought to determine whether Hip1, Slm9, and Hip3 were required for repressive chromatin at other loci. Microarray analysis has previously suggested that a number of fission yeast genes are regulated by heterochromatin (41, 42). For instance, mutations in the RNA interference machinery (ago1Δ, rdp1Δ, and dcr1Δ) and in a histone methyl transferase (clr4Δ) result in elevated levels of hsp16” and SPBC19C7.04c transcripts. Consistent with this, Northern analysis revealed that transcript levels were also elevated in strains carrying mutations in other key heterochromatin silencing factors (swi6Δ, rik1Δ, and chp1Δ) (Fig. 8). Furthermore, when compared with a wild type background, hsp16” and SPBC19C7.04c mRNA levels were also increased in hip1Δ, slm9Δ, and hip3Δ mutants, and in agreement with our previous results, transcript levels were not further increased by combining mutations in hip1Δ, slm9Δ, and hip3Δ (Fig. 8). These results suggest that, in addition to regions of constitutive heterochromatin, HIRA proteins regulate the chromatin structures that repress the expression of these genes.

Transposable elements in a number of cell types are known to be packaged into repressive chromatin structures (43). Therefore, we next examined whether HIRA proteins regulate the expression of long terminal repeat retrotransposons. The S. pombe genome contains 13 integrated full-length copies of the TF2 long terminal repeat retrotransposon (44), and Northern blotting revealed that only low levels of TF2 RNA were detectable in wild type cells (Fig. 8). However, deletion of hip1Δ, slm9Δ, or hip3Δ resulted in a large increase in TF2 transcript levels, demonstrating that HIRA complexes are required to silence the expression of fission yeast retrotransposons. Interestingly, the level of TF2 RNA was not increased by loss of silencing factors, such as Swi6,Clr4,Rik1, and Chp1 (Fig. 8), indicating that these elements are repressed by a mechanism that is different from centromeric repeats and also the hsp16” and SPBC19C7.04c genes. Thus, our results suggest that HIRA complexes have important roles in several distinct modes of transcriptional silencing in fission yeast.

**DISCUSSION**

Affinity purifications of Hip1 and Slm9 have identified Hip3 as a protein that interacts with S. pombe HIRA proteins. Consistent with this, loss of Hip3 function leads to growth defects that are highly similar to those associated with loss of Hip1 or Slm9. Genetic analysis also indicates that mutations in hip1”, slm9”, and hip3” are epistatic, and furthermore Hip3 protein levels are dependent upon Hip1 and Slm9. Thus, all of the available evidence indicates that Hip3 functions in a complex with Hip1 and Slm9. Nonetheless, Slm9 does not precisely co-fractionate with Hip3 by gel filtration chromatography, and furthermore, Hip1 has roles in sexual development that are independent of Hip3 and Slm9. Thus, fission yeast cells may contain several distinct HIRA complexes.

Hip3 is related (29% similar) to the budding yeast protein Hir3. Mutations in S. cerevisiae Hir3 lead to phenotypes that are closely related to those associated with mutations of Hir1 and Hir2 (17, 23, 45), the counterparts of Hip1 and Slm9. Furthermore, during the final preparation of this manuscript, Prochasson et al. (46) demonstrated that S. cerevisiae Hir3 forms a stable complex with Hir1 and Hir2. Thus, there appears to be a level of conservation in the composition of HIRA complexes in budding and fission yeast. Prochasson et al. (46) also demonstrated that S. cerevisiae HIRA complexes contain a fourth protein called Hpc2. Blast analysis of the S. pombe genome has revealed the presence of an uncharacterized protein (SPBC947.08c), which is a putative homologue of Hpc2. Although this protein was not identified by our affinity purifications, it will be important to determine whether it interacts with Hip1, Slm9, and Hip3.

Hip1, Hip3, and Slm9 are necessary for accurate chromosome segregation, because they are required for centromeric chromatin. Fission yeast centromeres are composed of distinct chromatin domains, a central domain that is flanked by domains of heterochromatin associated with the outer (otr) repeats. It is known that deposition of Cnp1, the CENP-A histone variant, in the central domain is critical for kinetochore assembly and thus centromere function (6). However, Hip1 and Slm9 are not required for Cnp1 deposition, and mutations in hip1”, slm9”, and hip3” do not alleviate central core silencing. Instead, all three proteins are required for full function of the heterochromatin domains that flank the central domain. It has been proposed that these domains of heterochromatin interact to form a loop (47) that presents central domain and thus the kinetochore in the correct orientation for microtubule attachment (2). Disruption of heterochromatin is believed to be one cause of merotely, where microtubules from both spindle poles attach to the same kinetochore. Importantly, merotely is thought to result in lagging chromosomes during mitosis, and therefore it is significant that loss of Hip1, Slm9, and Hip3 all lead to increased levels of lagging chromosomes. Other data are also consistent with aberrant heterochromatin in cells lacking HIRA proteins. We have found that in common with other mutations that disrupt pericentric heterochromatin (10, 11), levels of the transcripts derived from the centromeric otr repeats are elevated in hip1Δ and slm9Δ mutants (data not shown).

S. pombe HIRA proteins repress the expression of both hsp16” and SPBC19C7.04c. Expression of these genes is also elevated by other mutations that disrupt heterochromatin (clr4Δ, swi6Δ, chp1Δ). Although the chromatin associated with these genes has not yet been shown to bear the classic hallmarks of heterochromatin (such as histone H3 lysine 9 methylation and Swi6/Hir1 binding), it is tempting to suggest that their expression is repressed by packaging into heterochromatic structures.

We have also demonstrated that Hip1, Slm9, and Hip3 are required for silencing of TF2 retrotransposons. Transposable elements are known to be repressed in a range of cell types, and indeed, in a number of cases transposons are packaged into heterochromatic structures (43). It is therefore interesting that repression of TF2 elements does not require Clr4 and therefore the methylation of histone H3 on lysine 9; nor does it require other heterochromatin factors, such as Swi6, Rik1, or Chp1. Thus, although TF2 expression is regulated by HIRA proteins, it seems that retrotransposons are silenced by a mechanism that is distinct from centromeres and the mat locus. As such, our data suggest that HIRA proteins are required for the assembly and/or maintenance of several distinct forms of repressive chromatin. Furthermore, it will be important to determine the role of HIRA proteins in retrotransposon silencing in higher eukaryotes.
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