Identification of Uhp1, a Ubiquitinated Histone-like Protein, as a Target/Mediator of Rhp6 in Mating-Type Silencing in Fission Yeast

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SUMMARY

Mating type silencing in *S. pombe* is brought about by cooperative interactions between cis-acting DNA sequences flanking *mat2P* and *mat3M* and the trans-acting factors namely, Swi6, Clr1-Clr4, Clr6 and Rik1. In addition, DNA repair gene *rhp6*, which plays a role in post-replication DNA repair and ubiquitination of proteins including histones, is also involved in silencing, albeit in a unique way: its effect on silencing and chromatin structure of the donor loci is dependent on their switching competence. Earlier, we hypothesized the existence of a mediator of Rhp6 that plays a role in reestablishment of the chromatin structure coincidentally with DNA replication associated with mating-type switching. Here, we report the identification of a 22kd protein as an *in vivo* target and mediator of Rhp6 in mating type silencing. Level of this protein is greatly elevated in *sng1-1/rhp6-* mutant and *rhp6Δ* as compared to wild type strain. Both the deletion and overexpression of the gene encoding this protein elicits switching-dependent loss of silencing. Furthermore, the 22kd protein undergoes Rhp6-dependent multiubiquitination and associates with *mat2* locus during S phase in wild type cells. Interestingly, it contains a histone-fold motif similar to that of histone H2A and like histone H2A, it interacts strongly with histone H2B *in vitro*. These results indicate that the 22kd protein, renamed as the ubiquitinated histone-like protein Uhp1, is an *in vivo* target/mediator of Rhp6 in silencing. Thus, regulation of association of Uhp1 with chromatin and ubiquitination followed by degradation may play a role in reestablishment of inactive chromatin structure at the silent mating type loci.

*Keywords:* silencing, *rhp6*, histone-fold protein, chromatin, remodelling, *Schizosaccharomyces pombe*
INTRODUCTION

Mating-type silencing in two distantly related yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* occurs by analogous mechanisms with the participation of cis-acting DNA sequences that flank the silent loci and trans-acting factors (1, 2). Early studies in *S. cerevisiae* showed that cis-acting sequences, which include an ACS (ARS1 Consensus Sequence) and binding sites for ABF1 and RAP1 are important for silencing (for review see Ref. 1). In addition, genes encoding MAR/SIR proteins (3-5) are also involved in silencing. Genetic and biochemical studies have shown a role of interaction between the N terminus of histone H4 and SIR3 in silencing (6-8). In parallel studies, mutations in ORC (Origin Recognition Complex) sub units were found to result in loss of silencing (9-12). These results, together with the earlier work of Nasmyth (13) and Rine (14) suggested a coupling between establishment of silent chromatin structure and DNA replication. In addition, mutations in yeast homologues of chromatin assembly factor (CAF1) also cause a defect in silencing (15, 16). However, recent studies have shown that DNA replication could be decoupled from silencing in strains where SIR1 protein could be recruited artificially (17, 18), rather than by ORC (19).

In *S. pombe*, mutations in *swi6*, *clr1- clr4*, *clr6* and *rik1* have been shown to abrogate silencing (20-24). Swi6p contains the conserved chromodomain and chromo-shadow domain (20). Likewise, Clr4p contains the SET domain (25), which is associated with methyltransferase activity directed towards Lysine 9 position in histone H3 (26, 27), while *clr3* and *clr6* encode histone deacetylases (24). An orthologue of SIR2 has also been found in *S. pombe* indicating the conservation of heterochromatin components in these two yeasts and higher eukaryotes (28, 29).

Among cis-acting sequences, K region spanning the *mat2-mat3* interval is required
for establishing a switching/silencing competent state (30). Strains deleted for this region, which includes a stretch of strong homology to the centromere repeats (31), exhibit two alternative states of switching and silencing of a ura4+ reporter gene, which are not only stably inherited mitotically but also segregate as "Mendelian" epialleles during meiosis (30). Furthermore, the dosage of Swi6 is critical for maintenance of these alternative epialleles (32). More importantly, the requirement of cis-acting sequences with ARS-like function, for silencing (33, 34), suggests an involvement of DNA replication in silencing in S. pombe. Very recently, DNA polymerase α has been shown to play a role in establishment of epigenetic chromosomal state, which is stably propagated during mitosis and meiosis (35), through interaction with the chromodomain protein Swi6. Thus, Polα may play a role in silencing presumably through recruitment of Swi6 (35, 36), indicating that DNA replication is directly coupled to the assembly of silent chromatin structure in S. pombe (36, 37).

It was shown recently that sng1-1, a mutation in DNA repair gene rhp6 also abrogates silencing (38). However, the effect of this mutation is unique: unlike the swi6 and clr1-4 mutations (20-23), it causes derepression of silent loci only if they are switching competent (38). It was proposed that Rhp6 plays a role during replication associated with switching in reestablishment of silent chromatin structure at the switching donor loci (38, 39). Likewise, a role of Rad6 in silencing at telomere and mating type loci was reported in S. cerevisiae (40). Rad6p can ubiquitinate histones in vitro (41) and other proteins and target them to degradation by proteasome according to the N-end rule (42). Recently, in vivo RAD6-dependent ubiquitination of histone H2B has been demonstrated in S. cerevisiae (43). However, this modification is essential for sporulation, not for silencing (43). In mice, disruption of one of the two copies of the RAD6 homologue (HHR6B) leads to male sterility, as HHR6B-dependent chromatin modification and nucleoprotein transition during
spermatogenesis fail to occur (44). Thus, an *in vivo* target/s for Rhp6/Rad6 that mediates its role in silencing remains to be discovered.

We have sought to identify the hypothetical target/mediator of Rhp6 in silencing. Earlier, we proposed such a mediator to function transiently during assembly of chromatin at the silent loci, following which it is ubiquitinated and degraded (38, 39). In this study, we have identified a 22kd protein as a mediator of Rhp6: this protein contains the histone-fold motif, interacts with histone H2B *in vitro* and is subject to ubiquitination *in vivo*. Accordingly, the protein has been renamed as ubiquitinated histone-like protein, Uhp1. Both over-expression and deletion of the uhp1 gene abrogate mating type silencing, thus demonstrating its role as a mediator of Rhp6 in assembly of heterochromatin and silencing.
EXPERIMENTAL PROCEDURES

Media and strains--Media were prepared according to Moreno et al. (45). To check sporulation levels, strains were grown at 30°C on PMA or PMA-leu plates (in case of selection for plasmid with LEU2 marker) for 4 days, colonies were stained with iodine for 2-3 minutes (45) and photographed. For quantitation of sporulation levels, cells were examined by light microscopy and the number of zygotic and haploid meiosis (hm) asci were counted. Strains used in this study and their genotypes are listed in Table 1.

Protein extraction from yeast cells--Cultures of appropriate strains were grown up to log phase, harvested and washed with buffer A (0.02M HEPES, 0.1M NaCl, 2mM EDTA, 0.625% glycerol and 1mM β-mercaptoethanol). Then, twice the volume of buffer B (Buffer A + 0.01mM PMSF) was added to the pellet. In addition, protease inhibitors (20µg each of pepstatin, aprotinin and leupeptin) and equal volume of glass beads (425-600 micron dia) were added. Cells were broken either by vortexing at 4°C or in a homogenizer. Each one min cycle was followed by a 30 sec incubation on ice till 80% lysis occurred (lysis of cells was checked under the microscope by adding 1% SDS). The lysed cells were centrifuged at 55,000rpm for 30 min at 4°C in TL-100-3 rotor of TL-100 Ultra centrifuge (BECKMAN) or SW-41 rotor of BECKMAN at 30,000 rpm for 45 minutes. Supernatant was collected, aliquoted and frozen at -70°C. Protein concentration was estimated according to Bradford (46) using BSA as standard. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially according to Laemmli (47).

Purification of the 22 kD protein--Since the level of 22 kD protein is elevated in rhp6- mutant, it was purified by conventional methods from the mutant strain. Total cell extract was prepared from a 4 liter culture of mutant strain grown in YEA medium at 30°C.
Fractional ammonium sulphate precipitation was done at 4°C and the 22kd protein, which was referred to as p22, was found to precipitate maximally at 40-60% ammonium sulphate concentration range. The precipitated fraction was redissolved, dialyzed and subjected to DEAE-Sepharose chromatography, where p22 appeared in the flow through fraction. Finally, the flow through fraction was subjected to preparative SDS-polyacrylamide gel electrophoresis, the p22 bands were excised and electroeluted. Upon SDS-PAGE and silver staining the protein was found to be purified to apparent homogeneity.

**V8 protease digestion and microsequencing** -- 20µg of the electroeluted Uhp1 was digested with V8 protease (which specifically cleaves peptide bonds at the C-group of acidic amino acid residue glutamic acid in Ammonium Carbonate buffer, pH 7.8 and glutamic acid and aspartic acid in Sodium or Potassium Phosphate buffer pH 7.8) in a reaction mix of 200 µl containing 50mM Sodium Phosphate buffer pH 7.8, 2mM EDTA and 1.2 µg of V8 protease at 37°C for 24 hours. The reaction mix was passed through a 5kd cutoff filter (Amicon). Samples were then subjected to SDS-PAGE and silver stained. The larger aliquot retained after V8-digestion was also subjected to SDS-PAGE in parallel lanes and electroblotted to PVDF membrane using 10mM CAPS buffer. Membrane was stained with 0.1% Coomassie blue stain in 50% methanol for 5 minutes. The required bands were excised, destained in 50% methanol and 10% acetic acid (10-20 minutes), washed with deionized water, dried and subjected to microsequencing using model 470 A Protein Sequencer (Applied Bio System) equipped with on-line PTH analyzer.

**Construction of GST-histone and HA-Uhp1 constructs** -- Genes encoding histones H2A, H2B and H3 were PCR amplified from genomic DNA with specific primers, as shown in Table 4, and cloned at BamHI and EcoRI sites of the vector pGEX-2T (Pharmacia). Similarly, *uhp1* gene was PCR amplified using specific primers shown in Table 4 and cloned
at NotI site of the vector pREP-1N-3HA (a kind gift of M. Yanagida), in which nmt1
promoter drives the expression of HA-tagged uhp1 gene. The resulting vector is called pHA-
Uhp1 (Table 1), in which there is an attachment of a C-terminal triple HA epitope tag to
Uhp1.

*Preparation of RNA, Northern blotting and Reverse Transcription Polymerase Chain
Reaction (RTPCR)*--RNA was prepared from the required strains as described by Schmitt et
al. (49). Conditions of RTPCR for detecting mat2Pc, mat3Mi and polα transcripts have been
described earlier (38). While RT-PCR products of mat2Pc and mat3Mi transcripts were
detected by Southern blotting that for Polα could be detected by ethidium staining, which
may reflect relative abundance of their respective transcripts. Northern blotting and
hybridization were performed according to Sambrook et al. (48) and the radioactive bands
were quantitated on Bio Rad Molecular Imager Fx using Quantity One Version 4.2.2
software.

*Protein extraction from E. coli strains expressing recombinant plasmids*--A freshly
grown single colony of E. coli strain JM109 expressing recombinant plasmid was inoculated
into 2-5 ml of LB-Amp medium and incubated overnight at 37°C. This culture was
reinoculated in to 25-100 ml of LB-Amp medium and grown at 37°C. IPTG was added to
final concentration of 1mM when OD600 reached 0.4-0.5. Cultures were incubated further at
37°C for 3-6 hr. Cells were collected by centrifugation and suspended in either PBS or TE
containing 1mM PMSF and sonicated 3-4 times at frequency of 15-20 for 20 sec each on
Branson Sonicator. After sonication 0.1% Triton X-100 was added and protein extract was
collected by centrifugation at 4°C for 15 min. Aliquots of protein extracts were frozen at -
20°C.

*Antibody production*--For raising polyclonal antibodies, rabbits were immunized
with Uhp1 bands excised from SDS gel, minced in PBS, mixed with Freund’s complete adjuvant (1:1 v/v) and injected subcutaneously at multiple sites on the rabbit’s back. Alternatively, affinity purified MBP-Uhp1 fusion protein was injected. Two booster injections were given with same amount of antigen mixed with Freund’s incomplete adjuvant at weekly intervals. A week after the last injection, blood samples were collected by puncturing the ear vein and specificity of the antibody was established by Western blotting.

**Western blot analysis and Immunoprecipitation**--Western blotting was performed by using semi-dry blotter as instructed by the manufacturer (Sigma). After transfer membrane was blocked with 5% skim milk in 1X PBS at 4°C for 2-12 h. Primary antibodies were used at the following dilutions: 1:250 for anti-Uhp1 and 1: 2000 for anti-HA. Secondary antibody (Alkaline phosphatase-conjugated anti-rabbit IgG) was used at 1:2000 dilution. Incubations with antibodies and substrates were performed as per manufacturer’s instructions (Promega; 20µl of 50mg/ml NBT, 10µl of 50mg/ml BCIP, 40µl 1M MgCl2, 1.5ml of 1M Tris.HCl, pH 9.0 in total volume of 10ml). Alternatively, horse radish peroxidase-conjugated anti-mouse/anti-rabbit IgG (1:2000) was used in combination with ECL-Plus (Amersham) detection reagent as per manufacturer’s instructions.

**GST pull-down assay**--Protein extracts prepared from *E. coli* cells expressing GST-histone fusion proteins were allowed to bind to Glutathione-Sepharose beads in MTPBS buffer (100mM Na2HPO4, 16mM NaH2PO4 and 150mM NaCl) in a reaction mix of 100µl containing 1mg/ml BSA, 50µl of beads (50% v/v) and three increasing concentrations of GST-histone fusion protein for half an hour with shaking. The mixture was washed twice with MTPBS. To above reaction mix, 150µg of protein extract prepared from *h90 p22Δ rhp6* strain expressing pHA-Uhp1 construct, 1mg/ml BSA and 1X binding buffer (5X Binding
Buffer 750mM NaCl, 100mM Tris-Cl (pH 8.0), 5mM EDTA, 0.5% Triton-X and 5mM DTT) were added. The reaction mixture was incubated at room temperature for 1h and washed four times with washing buffer (100mM NaCl, 20mM Tris-Cl, 1mM EDTA, 0.1% Triton-X, 1mM EDTA). After adding 1X SDS loading dye, samples were boiled for 10 minutes and immunoblotted with anti-HA antibody.

Glutaraldehyde crosslinking experiment--Protein extract was prepared from \(h^{go}\) \(uhp1\Delta\) strain expressing the plasmid pHA-Uhp1 and subjected to cross-linking at room temperature for 30 minutes in a reaction mixture (50 µl) containing 20mM Tris-HCl (pH 7.5), 1mM ZnCl\(_2\), 35 µg of HA-Uhp1 extract and 0.005% and 0.01% glutaraldehyde. Reaction was stopped by addition of SDS loading buffer. Samples were boiled and immunoblotted with anti-HA antibody.

Ni-NTA chromatography--Ni-NTA chromatography was performed as per manufacturer’s instructions (Qiagen). pHis-Ub vector was transformed into appropriate strains and expressed in absence of thiamin as described earlier (50). 40µg protein was loaded in the input lanes while 1mg of extract was used for binding to Ni-NTA resin.

Chromatin immunoprecipitation (ChIP) assay--Localization of Uhp1 at \(mat2\) locus was quantitated by ChIP analysis, which was performed according to Ekwall and Partridge (51), using primers 12799 and 12754 for \(mat2\), and act1For and act1Rev for act1 gene. PCR conditions were: 94°C, 5 min; 35 cycles: 94°C-40sec, 51°C-30sec, 72°C-1min; 72°C, 5 min.
RESULTS

Elevated level of a 22kd protein in sng1-1/rhp6\(^{-}\) mutant and rhp6\(^{\Delta}\) strains--Earlier, we hypothesized that a protein target of Rhp6 may participate transiently in chromatin remodeling at the silent loci during switching, whereafter it is ubiquitinated, channelled to proteasome pathway and degraded (38, 39). Such a protein may be present at a low level in steady state population of wild type cells. However, in sng1-1/rhp6\(^{-}\) mutant it may accumulate because of lack of ubiquitination and degradation, and its continued association may perturb chromatin structure leading to derepression of silent cassettes (38, 39). To test this idea, we compared electrophoretic patterns of proteins from wild type and rhp6\(^{-}\)/sng1-1 mutant strains grown at 30\(^{\circ}\)C and 36\(^{\circ}\)C. Level of a 22kd protein was found to be greatly elevated at 30\(^{\circ}\)C in the sng1-1/rhp6\(^{-}\) mutant as compared to wild type strain (Fig. 1a, compare lanes 1 and 3). An elevated level of the 22kd protein was also observed in the rhp6\(^{\Delta}\) strain\(^{2}\). However, surprisingly, the level of elevation was reduced at 36\(^{\circ}\)C as compared to 30\(^{\circ}\)C in rhp6\(^{-}\) mutant (Fig. 1a, compare lanes 3 and 4). The reason is not clear. However, it is possible that some other ubiquitination pathway, like Hus5 (52), may be activated at 36\(^{\circ}\)C, which partially reduces p22 level.

The 22kd protein was purified by conventional methods of fractional ammonium sulfate precipitation, DEAE cellulose chromatography and electroelution. Purified protein was subjected to microsequencing but its N-terminus was found to be blocked. Therefore, it was subjected to digestion with V8 protease and the major proteolytic fragments were subjected to microsequencing. Two fragments of 17.5 and 13kd were also found to have their N-termini blocked (Fig. 1b). However, the 7.5kd band yielded the sequence...
KMHAAPKPNYPVVTL, which, upon data base search was found to match completely with an internal sequence of Obr1 (Fig. 1c; 53). This protein was earlier found to be elevated in brefeldin-resistant mutants of S. pombe (53), though it did not confer brefeldin resistance by itself. This protein was also reported as a “25kd” protein, which was elevated in crm1 mutant. The crm1 gene was shown to be involved in the maintenance of higher order chromosomal structure (54). However, p25 protein did not mediate crm1 function (54). Expression of p25 gene was found to be down regulated by Crm1 indirectly through down regulation of transcription factor Pap1 (55). Based on the new functions of p22/p25/Obr1, as described in Results section, it has been renamed as Uhp1.

Effect of rhp6^-/-sng1-1 mutation on uhp1 mRNA and protein--We obtained ’p25’ gene described earlier (55) and the strain carrying disruption of ’p25’ gene from Dr. Toda. Western blotting using antibody raised against p22/Uhp1 protein showed absence of p22/Uhp1 band in p25 deletion strain, and enhanced levels in a strain carrying p25/uhp1 gene on a high copy plasmid, thus confirming that p22/uhp1 was identical to the product of obr1/p25 genes described earlier (53, 54).

To check at what level does sng1-1/rhp6^- mutation affect Uhp1 expression, we carried out Northern and Western blot analysis for wild type and sng1-1/rhp6^- mutant strains. Northern blots were probed with uhp1 and cdc2 genes (Fig. 2a, lanes 1 and 2). Phosphoimager analysis showed that sng1-1/rhp6^- mutation causes a reproducible two-fold elevation of the uhp1 mRNA, while Western blotting results showed an overall 16-fold higher Uhp1 level in sng1-1/rhp6^- mutant as compared to wild type strain (Fig. 2b, compare lanes 2 and 1; the faint band in the wild type lane 2 is indicated by an arrowhead). Thus,
sng1-1/rhp6− mutation affects the expression of uhp1 gene modestly at transcriptional level but more strongly at post-transcriptional level (about ~8-fold).

*Overexpression and deletion of uhp1 gene mimic the rhp6− mutant’s silencing defect.*—To check the involvement of Uhp1 in silencing, uhp1 gene cloned on a high copy vector was expressed in both homothallic (h90) and heterothallic, nonswitching strain Msmto (this strain carries a deletion of cis-acting sequences flanking mat1 locus that are required for generation of double strand break and hence switching; ref. 56). Likewise, disruption strains were generated in both switching and nonswitching background and their phenotype was compared with vector controls. Interestingly, we find that both overexpression and deletion of Uhp1 elicit haploid meiosis phenotype, indicative of silencing defect (Table 2). However, this effect was observed only in the switching (h90) strain, not in the non-switching (Msmto) strain (Table 2). Thus, manifestation of defects caused by overexpression and deletion of uhp1 show a dependence on switching competence of strains, which is similar to the phenotype displayed by sng1-1/rhp6− mutant (38).

To directly assess the effect of overexpression and deletion of uhp1 on silencing, RTPCR analysis was carried out to monitor the level of expression of silent copy transcripts in h90 and Msmto strains, as described earlier (38). Both overexpression and deletion of uhp1 caused an elevation of mat2Pc transcript only in a switching, h90 strain (Fig. 3, top panel, lanes 1-3) but not in non-switching, Msmto strain (Fig. 3, top panel, lanes 4-6). [It may be noted that a low level of mat2Pc transcript is detected even in the vector control of the switching h90 strain (Fig. 3, top panel, lane 1), but not in non-switching Msmto strain (Fig. 3, top panel, lane 4). This is in agreement with earlier results (38) where mat2Pc shows a low,
switching-dependent leakiness of expression.] A similar effect on expression of mat3Mi transcript was observed in h90 strain, with overexpression of uhp1 having a somewhat modest effect (Fig. 3, middle panel, lane 2) but a stronger effect caused by deletion of uhp1 gene (Fig. 3, middle panel, lane 3). However, no effect was observed in non-switching strain Msmto (Fig. 3, middle panel, lanes 4-6). Thus, overexpression of uhp1 elicits silencing defect similar to that exhibited by sng1-1/rhp6− mutant (38), that is, the effect is switching-dependent. These results show that Uhp1 does function as an in vivo mediator of Rhp6.

Interestingly, overexpression and deletion of uhp1 gene have opposite effects on the level of sporulation in h90 strain: while overexpression causes a reduction, deletion of uhp1 gene caused an elevation of sporulation level (Table 2). These results suggest that apart from its effect on silencing, Uhp1 also exerts an inhibitory effect on the level of switching and/or sporulation. Thus, Uhp1 may also mediate the role of Rhp6 in switching and/or sporulation. This is consistent with the fact that rad6/rhp6− mutants have reduced levels of sporulation (57, 58).

To assess the effect of overexpression and deletion of uhp1 on switching, we used the iodine staining assay. Iodine stains a starchy compound present in spore cell wall. Switching strains produce cells of both mating types, which mate and sporulate and give dark staining with iodine, while non-switching strains, such as Msmto, produce no spores and, therefore, give no iodine staining (45). It is particularly interesting that overexpression of uhp1 in h90 strain produces two types of colonies: dark staining and light staining (Table 2 and Fig. 4). Since mating type region is prone to rearrangements at a low rate (~ 10⁻⁴/generation; 59), some of which may possibly affect silencing, it is possible that cells of the light colony may have undergone a rearrangement. Such rearrangements are known to occur more frequently in
the $h^{90}$ as compared to the Msmto strains (59). However, Southern analysis showed no such rearrangement in the light staining colonies$^3$. Interestingly, light staining colonies again produced colonies that give light staining and switched to dark staining at a low rate of $\sim 10^{-4}$/generation$^3$. Thus, dark and light staining colonies may represent alternative metastable epigenetic states of switching and/or silencing, like those exhibited by the strain carrying deletion of $K$ region, as reported earlier (30).

Microscopic examination showed that the light-staining colony has a higher level of haploid meiosis as compared to the dark staining colony (Table 2). This level was also higher than that observed in $h^{90}$ strain carrying $uhp1$ deletion (Table 2), which is surprising, in view of equally high levels of $mat2Pc$ transcript in strains expressing $uhp1$ on a high copy vector or having $uhp1$ deletion (Fig. 3, lanes 2 and 3). This may be explained by the fact that RTPCR data for overexpression of $uhp1$ represent predominantly dark colonies (which contain very low, $\sim 1\%$, of light colonies), having a low level of haploid meiosis, which is similar to that observed for $uhp1\Delta$ strain (Table 2).

**Ubiquitination of Uhp1 in vivo and its dependence on Rhp6**-- Temperature sensitive mutants of proteasome subunit Mts3 have been shown to accumulate ubiquitinated derivatives of proteins at non-permissive temperature of 36$^\circ$C because of lack of degradation of multiubiquitinated proteins by the proteasome (60). To directly test whether Uhp1 is ubiquitinated in vivo, a construct expressing (His)$_6$-tagged ubiquitin (50) was transformed in to wild type, $uhp1\Delta$ and $mts3^{-1}$ strains. Cultures of transformed strains were first grown at 25$^\circ$C and then shifted to 36$^\circ$C for 4 hrs. Extracts prepared from these strains were subjected to Ni-NTA chromatography followed by immunoblotting with anti-Uhp1 antibody. Presence
of bands with reduced mobility in mts3-1 mutant (lane 1, Fig. 5) and their absence in uhp1Δ strain (lane 2, Fig. 5) directly demonstrates that Uhp1 undergoes multiple ubiquitination in vivo. Fainter bands are also observed in wild type cells (lane 3, Fig. 5), which may have reduced levels of ubiquitinated Uhp1, as compared to mts3-1 mutant, because of a functional proteasome.

To check whether ubiquitination of Uhp1 is cell cycle- and Rhp6-dependent, we subjected wild type and rhp6Δ mutant strains expressing His-Ub vector to cell cycle arrest in S-phase with hydroxyurea. Cells were harvested at hourly intervals after release of HU-arrest and the level of ubiquitination of Uhp1 monitored after Ni-NTA chromatography, followed by immunoblotting with anti-Uhp1 antibody. Results shown in Fig. 6 indicate appearance of one major ubiquitinated band of Uhp1 in wild type cells at 0 hr (Fig. 6a, lower panel, lane 5), whose level shows a slight (~ 2-fold) increase along with appearance of additional fainter bands with reduced mobility at 2hrs (corresponding to S phase) and 4hrs (Fig. 6a, lower panel, lanes 6-8). Importantly, no such bands were observed in rhp6Δ mutant (Fig. 6b, lower panel, lanes 5-8). These results clearly demonstrate that Rhp6 is essential for ubiquitination of Uhp1.

**Uhp1 is associated transiently with silent locus mat2P during S phase** -- The association of Uhp1 with silent locus mat2P was checked by ChIP assay at 0, 2 and 4 hrs after release of HU-arrest in a wild type strain expressing pHA-Uhp1. We find that Uhp1 is maximally associated with mat2P at 2 hrs, which coincides with S phase but not at 0 and 4 hrs (Fig. 7). Thus, Uhp1 appears to be associated with silent locus mat2P only during S phase.

**Uhp1 contains the histone-fold motif similar to histone H2A and interacts with**
**histone H2B--** Motif search did not reveal any structural motif or nuclear localization sequence in Uhp1 and only one short sequence with sub-optimum similarity to the PEST motif for ubiquitination was found (61, 62). However, a direct comparison with histone-fold motifs found in core histones by ClustalW analysis revealed a close similarity of the residues 13-76 in Uhp1 to the histone-fold motif in histone H2A, with about 20% identity and 40% similarity (Fig. 8a). This motif has been identified not only in core histones (63-65) but also in some TBP-associated factors (66). Maximum similarity was found in the middle helix $\alpha_2$ and loop regions L1 and L2 (Fig. 8a; Ref. 65). However, key basic residues that are required for interaction with DNA in H2A are not present in Uhp1 (Fig. 8a; 65). (Slightly less similarity was detected towards histone H2B and none with histones H3 and H4.)

Intriguingly, BLAST search (67) also revealed a strong homology with trp repressor binding protein (trpRBP) in *E. coli* and *B. subtilis* (E=1e-28; Fig. 8c). It has been shown that trpRBP does not interact with DNA but interacts with and modulates the activity of trp repressor (68). Thus, like histone H2A, Uhp1 could interact with either histone H2B and/or with other proteins that regulate mating type silencing. Interestingly, BLAST search also showed a high level of homology towards a hypothetical protein of *S. cerevisiae* (YDR032c, accession no. S61585; E=3e-49) with 60% identity and 77% similarity (Fig. 8b). It is possible that this protein may be the *in vivo* target of Rad6p in *S. cerevisiae*. Interestingly, both these proteins are similar in size to Uhp1 and share homology with it all along its length including the histone-fold.

To directly check whether, like histone H2A, Uhp1 can interact with its cognate partner H2B, we performed GST-pull down assay by incubating the extracts prepared from wild type strains expressing the HA-tagged *uhp1* gene with GST histone-fusion proteins,
where GST was fused with histones H2A, H2B and H3. Western blotting with anti-GST antibody confirmed the expression of fusion proteins from three GST-histone recombinant clones (Fig. 9a, lanes 1-3, arrowheads). Results indicate that histone H2B exhibits a strong interaction with Uhp1, while histone H2A does not interact at all and H3 interacts relatively weakly (Fig. 9b, compare lanes 7-9 with lanes 4-6 and 10-12). Thus, Uhp1 does interact with the cognate histone partner of H2A. Interaction with histone H4 could not be checked because GST-H4 fusion construct could not be expressed in *E. coli*, presumably because of unsuitable codon bias.

We also checked whether Uhp1 can interact with itself and form homodimers or multimers. Results of glutaraldehyde cross-linking show that Uhp1 does from homodimers *in vitro* (Fig. 9c). However, no multimers could be detected.

*Uhp1 derivatives lacking histone-fold and α2 helix exert a dominant negative effect on switching*--To check whether the histone-fold motif and the central α2 helix play a critical role in the function of Uhp1, we constructed clones of Uhp1 lacking histone-fold motif and α2 helix, and expressed them in *h90* strain. Interestingly, all the transformants carrying the constructs lacking histone fold or α2 helix gave light staining with iodine (Fig. 10). Southern analysis showed that these transformants had normal mating type organization and the level of double strand break. Microscopic examination of light staining transformants showed greatly reduced level of sporulation (Table 3). This defect may either be due to reduced mating efficiency caused indirectly by misregulation of *mat1* transcription or due to defects in switching and/or sporulation. Thus, constructs lacking histone-fold or α2-helix exert a dominant negative effect either on mating, switching or sporulation.
A novel mechanism for propagation of chromatin state--The main objective of this study was to identify the proposed mediator of Rhp6 in silencing. In an earlier study, Rhp6p was hypothesized to play a role in coupling the assembly of chromatin to DNA replication associated with switching (38, 39) Accordingly, existence of a mediator of Rhp6, that participates transiently in chromatin assembly at switching mating type loci, was proposed. In addressing this issue, we have made the following main findings. First, we find that a protein, renamed here as Uhp1, which contains the histone-fold motif and acts like H2A in binding to its cognate partner H2B, is elevated in the sng1-1/rhp6- mutant. Second, Uhp1 is regulated predominantly at post-translational level by Rhp6-dependent ubiquitination. Third, Uhp1 associates with chromatin in a cell cycle-dependent manner. Finally, both overexpression and absence of Uhp1 elicit silencing defect in wild type strains. These findings suggest a unique mechanism of propagation of chromatin state: by transient association of Uhp1 with chromatin, which is regulated by ubiquitin-mediated turnover. The modified as well as unmodified forms of Uhp1 may associate with chromatin during S phase, whereafter Uhp1 is degraded. Their association with silent mat2P donor locus during S phase coincides with switching, which occurs by a replication-coupled recombination mechanism (69). Thus, Uhp1 may participate in chromatin assembly during replication of switching mating type donor loci. Such a role is consistent with the proposed function of Rhp6 in reestablishment of chromatin structure of switching donor loci (38).

It remains to be checked how Uhp1 may be involved in chromatin assembly. It may be speculated that either one or both forms (ubiquitinated and unubiquitinated) of Uhp1 may associate with nascent nucleosomes, where they may substitute for H2A and/or H2B, generating a unique metastable nucleosome structure at the replication fork. This structure
may facilitate proper assembly of inactive chromatin after proteasome pathway helps to channel the ubiquitinated Uhp1 for degradation concomitantly with recruitment of histone H2A. In contrast, continuous presence of Uhp1, as happens in *sng1-1/rhp6* mutant or when *uhp1* is present on a high copy vector, might lead to a persistent alteration in chromatin structure, which may cause derepression of the silent loci. It is possible that in *rhp6* mutant, an excess amount of Uhp1 protein may result in an altered nucleosome structure with Uhp1 in place of H2A, which may not be able to form heterochromatin structure at the switching donor loci. Alternatively, chromatin lacking in ubiquitinated Uhp1 may not be recognized by other heterochromatin factors like Clr4 (see below). A similar ubiquitin-mediated regulatory mechanism operates in cell cycle control where cyclin degradation by ubiquitin-mediated proteolysis is critical for G1-S transition (70). Thus, a unique feature of this study is to demonstrate that a similar regulatory mechanism may operate in facilitating the propagation of chromatin structure.

**Mechanism governing regulation of Uhp1**—Surprisingly, *rhp6* mutant shows a 2-fold increase in *uhp1* mRNA, as a role of Rhp6 in transcription is quite unexpected. It is possible that Rhp6 may affect the stability of a specific regulator of expression of Uhp1, like Pap1 or Crm1 (55). However, as expected, our results suggest that Uhp1 level is regulated predominantly at post-translational level. In addition, our results indicate that Rhp6 is the E2 activity responsible for the ubiquitination of Uhp1, since no ubiquitination is detected in *rhp6* mutant, while multiply ubiquitinated moieties are detected in wild type cells, which accumulate further in *mts3-1* mutant.

The transient localization of Uhp1 at *mat2P* raises the question of how it may be transported to the nucleus and what regulates the association. Nuclear localization is
surprising in view of absence of the nuclear localization sequence. It is possible that nuclear entry may be regulated either by ubiquitination itself, or by an unknown protein that facilitates its nuclear entry. The S-phase-specific association with mat2 locus may be dictated by a hitherto undetectable modification, like phosphorylation. Another intriguing observation is lack of any ubiquitination motif in Uhp1. Although, not all proteins that are ubiquitinated possess such motifs, it is possible that ubiquitination of Uhp1 may be facilitated by an E3 activity, which remains to be discovered (61).

**Epigenetic function of Uhp1 in switching and silencing--**Recently, histone-code hypothesis has been proposed to explain the epigenetic phenomena wherein heterochromatin and euchromatin regions are associated with differently modified histones (71). According to this hypothesis, specifically modified histones may present a code for recognition by specific proteins, either transcription co-factors or repressors, which lead to either gene expression or gene silencing, respectively. Our study, showing the role of ubiquitination of a histone-like protein in chromatin assembly and in propagation of heterochromatin structure, helps to extend the histone-code hypothesis to non-histone chromosomal proteins. An interesting scenario may be where ubiquitinated Uhp1 is recognized by one or more of heterochromatin associated proteins, like Clr4 (see below). In this context, an interesting effect observed when Uhp1 was overexpressed in h90 strain, was manifestation of two alternative iodine staining colonies, the dark staining and the light staining. The light staining colonies exhibit a persistence of the light staining even after the plasmid was lost, suggesting a role of Uhp1 in establishing a stable epigenetic state2. Thus, Uhp1 may be important for initiating assembly of a structure that can propagate itself. This finding is reminiscent of the results of Grewal and Klar (30), where deletion of K region spanning the mat2-mat3 interval generated two
alternative epigenetic states showing different extents of switching/silencing. These states were metastable and switched to the other state at a low rate (~10^{-4}/generation) during mitotic growth. Interestingly, they also behaved as Mendelian alleles during meiosis, indicating that they had an imprinted memory of the chromatin structure, which could be propagated during mitosis and meiosis (30).

Interaction of Uhp1 with other factors involved in switching and silencing-- To check the genetic interactions with other silencing factors, we transformed Uhp1 into swi6 and clr1-clr4 mutant strains. Interestingly, we observe effect of overexpression of Uhp1 only in the h90, clr4 mutant, which yielded a light staining phenotype with reduced sporulation and switching but increase in the level of haploid meiosis. (The mating type organization and the level of DSB are not affected in these transformants.) Interestingly, unlike h90 strain, where less than 1% transformants gave light staining, almost 25% of transformants of the chromodomain mutant W31G and 2% in case of SET domain mutant G486D of Clr4 (25) gave light staining, suggesting that wild type Clr4 may function in conjunction with Uhp1. Recently, SET domain of Clr4 has been shown to be associated with histone methyltransferase activity specific for Lys9 position in histone H3 (25, 26). Furthermore, Lys9-methylated histone H3 is bound to Swi6 in heterochromatin regions (26, 27). It may be interesting to check whether Uhp1 physically interacts with Clr4 in vivo and helps to recruit it to heterochromatin regions.

Does Uhp1 function like a TAF?--Studies on TBP-associated factors (TAF’s) in Drosophila and human have led to the identification of TAF’s that are homologous in their histone-fold regions to H2B, H3 and H4. However, no TAF showing homology to histone H2A has been identified (65, 66). Because of its similarity to histone H2A in histone-fold
region and its interaction with H2B, the cognate partner of histone H2A, it is possible that Uhp1 may be the missing TAF corresponding to histone H2A. Here, it is pertinent to note the effect of overexpression of Uhp1 derivatives lacking the histone-fold and $\alpha_2$ helix: most of the transformants exhibit a much reduced iodine staining with lower level of switching and defect in silencing with high level of haploid meiosis. On the other hand, overexpression of intact Uhp1 produces light staining colonies at a rate of $\sim$1%. Thus, the histone-fold region may play an important role in the assembly of the functional complex involved in switching and silencing.

Possible Conservation of Uhp1 Function-- Finding of a high level of homology to trpRBP in several bacterial species is surprising, throwing up the possibility of a horizontal transmission between bacteria and yeast. It is tempting to speculate that certain class of transcriptional repressors may have crossed the species barrier. Furthermore, existence of a protein of unknown function but similar size from \textit{S. cerevisiae} with a high level of homology to Uhp1 is quite intriguing. It would be interesting to check whether it performs a similar function, that is, whether it serves as a target of Rad6 and plays a role in silencing in the budding yeast. These questions will be addressed in future studies.
REFERENCES


3091-3092


FOOTNOTES

1: The abbreviations used are: Amp, ampicillin; His, histidine; ura, uracil; ARS, autonomous replication sequence; MAR, mating regulator; SIR, silent information regulator; ORC, origin recognition complex; CAF1, chromatin assembly factor 1; Clr, cryptic loci regulator; Swi, switching; Rhp6, Rad6 homologue in pombe; sng, silencing not governed; PMA, pombe minimal medium with adenine; DEAE, diethyl aminoethyl; GST, glutathione-S-transferase; HU, hydroxyurea; HA, haemagglutinin; Ub, ubiquitin; Polα, DNA polymerase α; DSB, double strand break; TAF, TBP-associated factor; ChIP, chromatin immunoprecipitation.

2-4: Saini, Naresh and Singh, unpublished data
FIGURE LEGENDS

Fig. 1. **A protein of 22 kD, elevated in sng1-1/rhp6\textsuperscript{-} mutant as compared to wild type cells, is identical to the previously reported proteins p25 and Obr1.**  

* a. Protein extracts were prepared from wild type and *sng1-1/rhp6*\textsuperscript{-} mutant cells, which were grown at 30°C and 36°C. 30µg of each sample was subjected to SDS-PAGE (12.5%) and gel stained with Coomassie blue. M indicates molecular weight marker.  

* b. V8 protease digestion pattern of p22. 20µg of purified p22 was subjected to V8 protease digestion and a small aliquot subjected to SDS PAGE (12.5%) followed by silver staining. M, molecular weight marker.  

* c. Amino acid sequence of p22/p25/Obr1. Upon microsequencing, 7.5 kDa internal polypeptide shown in *b* yielded the 15 amino acid sequence (underlined), while the intact p22/Uhp1 as well as the 17.5 and 13kDa bands were found to have their N-termini blocked. BLAST search revealed the protein to be identical to the previously reported p25 protein (53) and obr1 (51).

Fig. 2. **Effect of sng1-1/rhp6\textsuperscript{-} mutation on the level of *uhp1* mRNA and protein.**  

* a. RNA was prepared from wild type (lane 2) and *sng1-1/rhp6*\textsuperscript{-} mutant (lane 1) strain. 20µg of total RNA was subjected to Northern blotting and hybridization with radiolabelled *uhp1* (upper panel) or *cdc2* (lower panel) probes. Signal was recorded and quantitated using Bio-Rad Phosphoimager.  

* b. Protein extracts
prepared from wild type (lane 2) and sng1-1/rhp6- strain (lane 1) were subjected to immunoblotting with anti-Uhp1 (upper panel) and TAT1 (lower panel) antibodies. Signals were quantitated on Gel Doc system of Pharmacia. The faint band corresponding to Uhp1 in wild type extract is indicated by an arrowhead.

Fig. 3. Both overexpression and deletion of \textit{uhp1} gene cause a loss of silencing, which is switching dependent. RT-PCR was carried out for \textit{mat2Pc} (top panel), \textit{mat3Mi} (middle panel) and \textit{pol\alpha} (bottom panel) transcripts using RNA prepared from wild type \textit{h}^{90} (lanes 1-3) or \textit{Msmto} strain (lanes 4-6), either alone (lanes 1 and 4) or containing \textit{uhp1} gene on a high copy vector pYA292 (lanes 2 and 5; a kind gift of T. Toda) or carrying \textit{uhp1} deletion (lanes 3 and 6).

Fig. 4. Overexpression reduces while deletion of \textit{uhp1} enhances the iodine staining of \textit{h}^{90} strain but not \textit{Msmto} strain. \textit{h}^{90} (upper panel) and \textit{Msmto} (lower panel) strains, transformed with vector alone (left panels) or \textit{uhp1} gene on a high copy vector pYA292 (middle panel) or carrying \textit{uhp1} deletion (right panel), were streaked on PMA plates lacking leucine. Colonies were stained with iodine after growth for 4 days and photographed.

Fig. 5. \textbf{Uhp1 is ubiquitinated \textit{in vivo}.} Accumulation of ubiquitinated forms of Uhp1 in proteasome mutant \textit{mts3-1} under non-permissive conditions. Wild type (lane 3), \textit{uhp1\Delta} (lane 2) and \textit{mts3-1} mutant (lane 1) strains carrying the His-Ub vector were grown initially at 25°C under conditions that allow expression of (His)6-tagged ubiquitin. Cultures at an OD\textsubscript{600} of 0.2-0.5 were shifted to 36°C and grown for 4 hours. Protein extracts were prepared and 1 mg of each protein
sample subjected to Ni-NTA chromatography. The Ni-NTA-bound fractions were immunoblotted with anti-Uhp1 antibody.

Fig. 6. **Rhp6-dependence of ubiquitination of Uhp1.** Cells of wild type (a) and *rhp6*/*sng1-1* mutant (b) strain expressing His-Ub vector were first subjected to HU-arrest for 4 hrs. Cells were washed free of HU and grown further for 0, 1, 2, 3 and 4 hours. Septation index was monitored and plotted (a and b, top panels). (a and b, lower panels), 1.0 mg of protein extract prepared from samples harvested at 0hr (lane 5), 1hr (lane 6), 2hr (lane 7) and 4hr (lane 8) was subjected to Ni-NTA chromatography and the Ni-NTA bound fractions were electrophoresed along with 40µg of each protein fraction at 0hr (lane 1), 1hr (lane 2), 2hr (lane 3) and 4hr (lane 4) and immunoblotted with anti-Uhp1 antibody.

Fig. 7. **Uhp1 is localized to mat2 locus during S phase.** ChIP analysis of a wild type *h90* strain expressing an integrated copy of HA-tagged Uhp1. Cells were grown to early log phase (OD600 of 0.2-0.3) and subjected to HU arrest. Samples were collected at 0hr (lane 1), 2hr (lane 2) and 4 hrs (lane 3) after release of HU arrest and subjected to ChIP analysis using oligos to amplify *mat2P* and *act1* genes. The *mat2*-to-*act1* signal ratio was determined for each NIP and IP sample and the enrichment ratio determined using the IP/NIP ratio for 0hr sample as 1.

Fig. 8 **Uhp1 contains histone-fold motif similar to that of histone H2A and presence of homologous proteins in *E. coli* and *S. cerevisiae.* a, ClustalW alignment of Uhp1 with histone-fold motif of histone H2A. Maximum homology is seen in
central helix α2. Residues in H2A that have been shown to interact with DNA are also indicated (65), which are lacking in Uhp1. b and c, BLAST search also shows a high degree of homology of Uhp1 with a hypothetical protein of unknown function (YDR032c) from *S. cerevisiae* (*b*) and with trp repressor binding protein (trpRBP) from *E. coli* (*c*).

**Fig. 9. Uhp1 exhibits specific interaction with histone H2B in vitro.** *a*, Extracts prepared from *E. coli* strains expressing GST-H2A (lane 1), GST-H2B (lane 2), GST-H3 (lane 3) and GST alone (lane 4) were immunoblotted with anti-GST antibody. Arrowheads indicate the positions of GST-histone fusion proteins. *b*, GST-pull down assay was performed by adding nothing (lane 1), beads alone (lane 2), GST extract (lane 3) and three increasing amounts of extracts of cells expressing GST-H2A (lanes 4-6), GST-H2B (lanes 7-9) and GST-H3 (lanes 10-12) to the extract prepared from *S. pombe* strain lacking *uhp1* gene but containing the *pHA-uhp1* construct. Proteins remaining bound to glutathione-agarose beads were immunoblotted with anti-HA antibody. *c*, Uhp1 can form homodimers in vitro. Extract prepared from a wild type strain expressing pHA-Uhp1 was subjected to cross-linking with 0.005 and 0.01% glutaraldehyde and subjected to immunoblotting with anti-HA antibody. 0 denotes no glutaraldehyde.

**Fig. 10. Histone-fold motif is important for Uhp1 function.** *h90* strain carrying a *mat3-linked ura4* marker was transformed with vector alone, *uhp1* gene on high copy vector (pYA292; Table 1), and *uhp1* derivative clones lacking histone-fold motif or α2 helix. Transformants were streaked on PMA plates lacking leucine. After 4 days of growth colonies were stained with iodine and photographed.
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<th>Genotype</th>
<th>Source</th>
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<td>SPJ25</td>
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<td>This study</td>
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<td>SPJ107</td>
<td>h&lt;sup&gt;90&lt;/sup&gt;, leu1-32, ura4D18, ade6-216, sng1-1/rhp6&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>pREP1N/3HA</td>
<td>Vector containing a triple HA tag and NotI site for protein fusion and expression in <em>S. pombe</em></td>
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<td>His-Ub</td>
<td>A pombe vector in which (His)&lt;sub&gt;6&lt;/sub&gt;-tagged Ubiquitin is expressed under control of the <em>nmt</em> vector</td>
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Table 2. Changes in the level of zygotic asci and 'hm' asci due to overexpression and deletion of the *uhp1* gene.

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<td>2</td>
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<td><em>h</em>₉₀, <em>mat3::ura4/uhp1↑</em> (Dark staining)</td>
<td>50</td>
</tr>
<tr>
<td><em>h</em>₉₀, <em>mat3::ura4/uhp1Δ</em></td>
<td>85</td>
</tr>
<tr>
<td><em>Msmt0/vector</em></td>
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<tr>
<td><em>Msmt0/uhp1↑</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Msmt0/uhp1Δ</em></td>
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↑ denotes overexpression; Δ denotes deletion. For each measurement 500 cells were counted.
Table 3  **Level of zygotic asci and 'hm' asci on overexpression of Uhp1 derivatives lacking histone-fold and α2 helix**

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<td>'hm' asci</td>
<td>Zygotic asci</td>
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<td>-</td>
<td>75</td>
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- denotes Nil

For each measurement 500 cells were counted.
Table 4  Primers used in this study

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<td>GCA TGA ATT CTG AGC GTT CGC CAC CGA G</td>
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<tr>
<td>Uhp1-α2Δ</td>
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Fig. 2
Fig. 3
Fig. 4
Fig. 6
Fig. 7
Identity = 20%  Similarity = 40%

0  Residues making main chain contacts with DNA
0  Residues making side chain contacts with DNA
* Lysine residue spanning the major groove

Fig. 8
Fig. 9
Fig. 10
Identification of Uhp1, a ubiquitinated histone-like protein, as a target/mediator of Rhp6 in mating-type silencing in fission yeast
Alpana Naresh, Sharanjot Saini and Jagmohan Singh

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