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# Functional roles for evolutionarily conserved Spt4p at centromeres and heterochromatin in *Saccharomyces cerevisiae*

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**The kinetochore (centromeric DNA and associated proteins) mediates the attachment of chromosomes to the mitotic spindle apparatus and is required for faithful chromosome transmission. We established that evolutionarily conserved *Saccharomyces cerevisiae SPT4*, previously identified in genetic screens for defects in chromosome transmission fidelity (*ctf*), encodes a new structural component of specialized chromatin at kinetochores and heterochromatic loci, with roles in kinetochore function and gene silencing. Using chromatin immunoprecipitation assays (ChIP), we determined that kinetochore proteins Ndc10p, Cac1p, and Hir1p are required for the association of Spt4p to centromeric (*CEN*) loci. Absence of functional Spt4p leads to altered chromatin structure at the *CEN* DNA and mislocalization of the mammalian CENP-A homolog Cse4p to noncentromeric loci. Spt4p associates with telomeres (*TEL*) and *HMRa* loci in a Sir3p-dependent manner and is required for transcriptional gene silencing. We show that a human homolog of *SPT4* (*HsSPT4*) complements *Scspt4*-silencing defects and associates with *ScCEN* DNA in an Ndc10p-dependent manner. Our results highlight the evolutionary conservation of pathways required for genome stability in yeast and humans.**

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## Introduction

In order to proliferate and develop, organisms depend on the accurate segregation of their chromosomes. Errors in this process lead to aneuploidy, birth defects, developmental disorders, and possibly cancer (Lengauer *et al*, 1998). *CEN* DNA is the *cis*-acting locus that specifies the binding of sequence-specific DNA-binding proteins and assembly of additional kinetochore proteins. Both the structural and regulatory components that define a functional kinetochore are essential for faithful chromosome transmission during mitosis and meiosis. The kinetochore maintains cohesion between sister chromatids, mediates the attachment of chromosomes to the mitotic spindle, and directs their subsequent movement to the spindle poles. The kinetochore is also the site through which completion of metaphase is sensed by the cell-cycle regulatory machinery, which coordinates the synchronous separation of chromosomes at the onset of anaphase (Kitagawa and Hieter, 2001).

In contrast to the complex centromeric structure of other eukaryotes, the *CEN* DNA sequence in *Saccharomyces cerevisiae* is relatively short (125 bp) with three conserved elements, *CDEI*, *CDEII*, and *CDEIII* (Fitzgerald-Hayes *et al*, 1982). The approximately 50 or more kinetochore proteins identified to date are classified based on whether they co-localize or interact with *CEN* DNA (inner kinetochore), spindle microtubules (outer kinetochore), or between the inner and outer kinetochore components (Kitagawa and Hieter, 2001; Cheeseman *et al*, 2002). Molecular analysis of the kinetochore complexes will aid in understanding of their functional roles. Binding of CBF3, a multiprotein complex containing Ctf13p, Ndc10p, Cep3p, and Skp1p to *CDEIII*, is critical for kinetochore assembly (Cheeseman *et al*, 2002).

In *S. cerevisiae*, the kinetochore chromatin domain is delimited on each side by strong nuclease-hypersensitive sites and is flanked by arrays of phased nucleosomes (Bloom and Carbon, 1982; Funk *et al*, 1989; Schulman and Bloom, 1991; Glowczewski *et al*, 2000). Modified and specialized histones are crucial for the assembly and function of the kinetochores and centromeric chromatin. The core centromeric chromatin contains the histone H3 variant Cse4p, the homolog of mammalian CENP-A (Stoler *et al*, 1995; Meluh *et al*, 1998). Genetic analyses have shown that increased histone levels impair mitotic chromosome segregation (Meeks-Wagner and Hartwell, 1986). Furthermore, mutations in genes encoding histones H2A, H2B, H4 and chromatin remodeling complex RSC proteins, (Sth1p, Snf5p), lead to chromosome missegregation, increased nuclease sensitivity in *CEN* DNA, and adjacent chromatin (Han *et al*, 1987; Saunders *et al*, 1990; Smith *et al*, 1996; Tsuchiya *et al*, 1998; Keith *et al*, 1999; Pinto and Winston, 2000; Hsu *et al*, 2003). These studies suggest a direct role for histones in centromere function and establish that nucleosome organization and function at the centromere is distinct from its function elsewhere in the genome.

In addition to kinetochore function, specialized chromatin is required for epigenetic functions such as the establishment and maintenance of gene silencing (Krude, 2002). Silent chromatin is present at the mating type loci (*HMLα* and *HMRa*) and at telomeric (*TEL*) DNA in *S. cerevisiae* (Grunstein, 1997). Using genetic analyses, we have previously shown that *SPT4* is required for faithful chromosome transmission and kinetochore function. We determined that *spt4* mutants show a 100-fold increase in the loss of a nonessential reporter chromosome, test positive in an *in vivo*

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assay for defects in kinetochore integrity, genetically interact with kinetochore mutants, and extracts exhibit defects in the binding of minichromosomes to microtubules (Basrai *et al*, 1996). The *spt* (suppressor of Ty) mutants were also identified in a genetic screen for suppression of transcriptional defects associated with Ty delta insertions in the 5′ regions of *HIS4* and *LYS2* (Winston *et al*, 1984). *SPT4* interacts genetically and biochemically with *SPT5* and *SPT6* (Swanson and Winston, 1992; Krogan *et al*, 2003; Lindstrom *et al*, 2003), and the Spt4p/Spt5p/Spt6p complex is required for assembly or stabilization of nucleosomes (Swanson and Winston, 1992; Bortvin and Winston, 1996), and transcription elongation (Hartzog *et al*, 2002; Rondon *et al*, 2003).

In this paper, we establish that Spt4p is a new structural and functional component of the centromeric and heterochromatic loci linking chromatin structure with kinetochore function and gene silencing. We used ChIP experiments and mutants to analyze the specific association of Spt4p with different chromosomal loci. Consistent with a role in kinetochore structure and function, we determined that Spt4p is required for the integrity of centromeric chromatin and restriction of the localization of Cse4p to kinetochores. Also, we established a novel role of Spt4p in gene silencing and complementation of the yeast mutant phenotypes by human *SPT4*. Interestingly, we provide the first example of an *in vivo* association of a human protein, namely HsSpt4p, with *CEN* DNA in budding yeast. Our results further demonstrate how yeast can be used as a model system to study the fundamental process of chromosome segregation in humans.

## Results

### *Spt4-GFP co-localizes with kinetochores in S. cerevisiae*

Previously, we used genetic analyses to show that Spt4p is required for chromosome segregation and kinetochore integrity (Basrai *et al*, 1996; Kerscher *et al*, 2001). The *spt4-138* and *spt4*Δ strains exhibit temperature-sensitive growth at 37°C and defects in chromosome transmission fidelity (*ctf*); so we decided to determine whether Spt4p co-localized with a kinetochore marker (Ndc10p). We tagged the C-terminus of Spt4p with green fluorescent protein (GFP) in a strain expressing Ndc10p tagged at the C-terminus with the hemagglutin epitope (HA). The Spt4-GFP fusion protein was functional, as the strain containing the tagged protein did not exhibit temperature-sensitive growth at 37°C (Figure 1A) or a *ctf* phenotype (data not shown). Subcellular localization showed that Ndc10p-HA was localized to the kinetochores as two or three distinct foci per nucleus (Figure 1B) (Sharp *et al*, 2002). In a majority of the cells (>90%), Spt4-GFP was localized to three to seven nuclear foci. One or two of these Spt4-GFP foci overlapped with kinetochore-containing Ndc10p-HA foci in greater than 90% of the cells (Figure 1B, merged). These results indicate that a subset of Spt4-GFP foci co-localize with the kinetochores. Spt4-GFP foci that do not overlap with Ndc10p-HA may represent an association with other chromosomal loci.

### *HA-Spt4p associates with chromosomal DNA*

We used a biochemical approach to assess the association of Spt4p with chromosomal loci using the ChIP technique. Strains containing an N-terminally tagged Spt4p with the HA epitope exhibit wild-type growth (Figure 1A). Chromatin

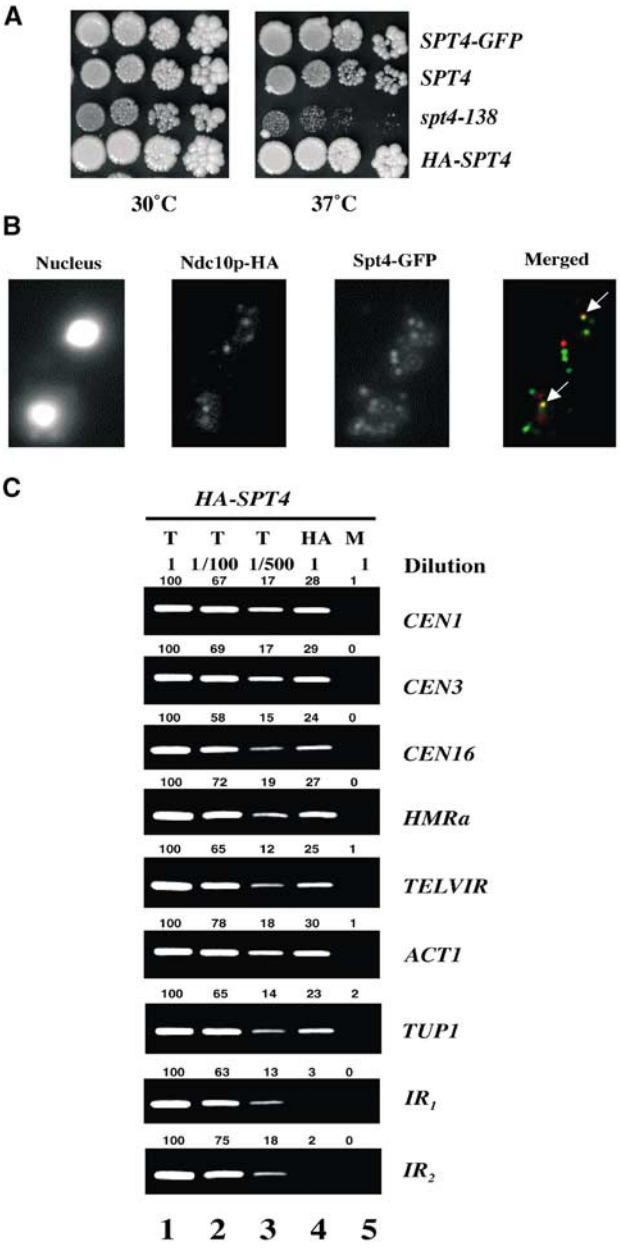


**Figure 1** Spt4p co-localizes with kinetochores and associates with chromosomal loci. (**A**) Epitope-tagged *SPT4* is functional. Growth of five-fold dilutions of wild-type (YPH499) and *spt4-138* (YMB54) strains was compared to *SPT4-GFP* (YMB1859) and *HA-SPT4* (GHY262) strains on YPD plates incubated at 30°C and 37°C for 3–4 days. The *spt4-138* strain exhibits a slower growth compared to the wild-type strain even at the permissive temperature of 30°C. (**B**) Subset of Spt4-GFP foci co-localizes with kinetochore. Chromosome spreads from strain (YMB1859) expressing Ndc10p-HA, and Spt4-GFP probed with anti-GFP or anti-HA antibodies. The arrows in the merged panel indicate co-localization of Ndc10p-HA (red), and Spt4-GFP (green) is shown in yellow. (**C**) HA-Spt4p associates with chromosomal loci. ChIP experiments were carried out using wild-type strain (GHY262) expressing chromosomally tagged *HASPT4* grown to logarithmic phase at 30°C. Different dilutions of chromatin samples from total (T) (undiluted, 1/100, 1/500), immunoprecipitated with anti-HA (HA), and mock (M) were analyzed using primers for core *CEN1*, core *CEN3*, core *CEN16*, *HMRa*, *TELVIR*, and *ACT1* or intergenic regions ($IR_1$ and $IR_2$) devoid of ORFs (chromosome IV, co-ordinates 1 157 000–1 157 200 and 1 523 000–1 523 180, respectively). Values for the quantitation of the data are shown above each of the lanes, with the value for undiluted total set to 100 for each row (lane 1). These data show that the PCR yield is proportional to the amount of starting material.

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extracted from wild-type cells expressing *HASPT4* was crosslinked with formaldehyde and HA-Spt4p/DNA complexes were immunoprecipitated using anti-HA antibody. PCR analyses of total (T), immunoprecipitated (HA), and mock (M) samples were carried out using different primer pairs. Different dilutions of the T DNA (undiluted, 1/100, 1/500) used in PCR reactions verified that the PCR yield was proportional to the amount of starting DNA (Figure 1C). No PCR product was obtained from mock samples, samples precipitated with anti-GFP antibody, samples from a control strain lacking epitope tagged *SPT4*, or samples without addition of crosslinker (data not shown). Our results showed that HA-Spt4p associated with *CEN* DNA, heterochromatic loci *HMRa*, *TELVIR* and actively transcribing genes *ACT1* and *TUP1*, but not with intergenic DNA regions ($IR_1$ and $IR_2$) devoid of ORFs. The enrichment of HA-Spt4p at various chromosomal loci was independent of the cell cycle stage (data not shown).

### *Kinetochore protein Ndc10p is required for the association of HA-Spt4p with core CEN loci*

The association of HA-Spt4p with *CEN* DNA and other chromosomal loci led us to hypothesize that the association of HA-Spt4p with a locus may be dependent on the presence of specific protein(s) at these loci. Hence, we evaluated the association of HA-Spt4p with different chromosomal loci in ChIP experiments using kinetochore, RNAPII, or silencing mutants. In the first of such experiments, we used a temperature-sensitive *ndc10-1* strain containing a mutation in the essential kinetochore gene *NDC10*, which fails to assemble a functional kinetochore at the nonpermissive temperature of 37°C (Goh and Kilmartin, 1993). The *ndc10-1* strain has been used to establish the specificity of the interaction of kinetochore proteins with *CEN* DNA (Goshima and Yanagida, 2000; He *et al*, 2001). ChIP experiments were performed using wild-type and *ndc10-1* strains expressing *HASPT4* grown at the permissive temperature (30 or 25°C) or after a shift to 37°C. Our data showed that HA-Spt4p associated with *CEN16*, *TELVIR*, and *ACT1* in the wild-type and *ndc10-1* strains grown at 30 or 25°C, respectively (Figure 2A and B). However, after the shift of the *ndc10-1* strain to 37°C, HA-Spt4p specifically failed to associate with the core *CEN16* DNA; the chromosomal association of HA-Spt4p to non-*CEN* loci such as *TELVIR* and *ACT1* was unaffected (Figure 2B). Similar results were obtained for association of HA-Spt4p with *CEN1* and *CEN3* (data not shown). Control experiments showed that the association of HA-Spt4p with *CEN* or non-*CEN* loci was unaffected when chromatin was prepared from wild-type cells shifted to 37°C (Figure 2A). These results show that Spt4p is a kinetochore protein and Ndc10p is required for the association of HA-Spt4p to *CEN* DNA.

Ndc10p, a component of the inner kinetochore, associates with the core *CEN* DNA of about 250 bp (Meluh and Koshland, 1997). Hence, we carried out ChIP experiments to determine the extent to which perturbations in kinetochore structure due to the *ndc10-1* mutation affected the association of HA-Spt4p with *CEN* and flanking sequences. We determined that HA-Spt4p associates with core *CEN16* and the flanking DNA up to 3 kb on either side of *CEN16* in the *ndc10-1* mutant grown at 25°C (Figure 2C). However, HA-Spt4p specifically failed to associate with core *CEN16* DNA, with no effect on chromosomal association to flanking *CEN* DNA in

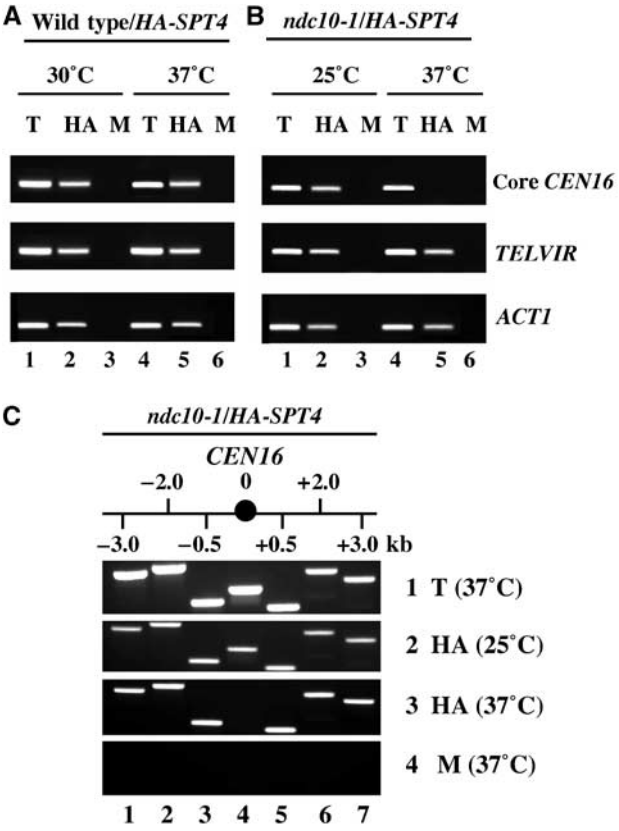


**Figure 2** Ndc10p is required for the association of HA-Spt4p to the core *CEN* loci. ChIP experiments were carried out using wild-type strain (GHY262) or *ndc10-1* strain expressing *HASPT4* (YMB1659) grown at 30 or 25°C and shifted to 37°C for 4 h. (**A, B**) Chromatin samples from T, HA, and M were analyzed using primers for core *CEN16*, *TELVIR*, or *ACT1*. (**C**) Chromatin samples from T (row 1), HA (rows 2 and 3) and M (row 3) were analyzed using primers for core *CEN16* (lane 4) and those that flank on either side (lanes 1–3 and 5–6).

the *ndc10-1* strain shifted to 37°C (Figure 2C). Based on these and earlier results (Figure 2B), we conclude that Ndc10p is required for the specific association of HA-Spt4p with core *CEN* DNA.

### *Centromeric chromatin structure is altered in spt4 mutants*

The specific association of HA-Spt4p with *CEN* DNA led us to examine as to whether Spt4p was required for the proper configuration of centromeric chromatin, by analyzing the endonuclease accessibility of chromatin structure at *CEN3* (Saunders *et al*, 1988, 1990). In wild-type cells, the kinetochore protects the three naturally occurring *Dra*I sites in *CDE*II from digestion (Figure 3A). Mutations in *CEN* DNA, kinetochore proteins, or depletion of histones H2B or H4 increase the accessibility of *CDE*II to *Dra*I (Saunders *et al*, 1988, 1990; Meluh *et al*, 1998). These experiments were carried out using Southern blot analysis of nuclear DNA digested with *Dra*I (different concentrations) and then with *Eco*RI, using a 0.9 kb *Hind*III–*Bam*HI fragment as a probe (Figure 3A). As can be seen in Figure 3B in a wild-type strain shifted to 37°C, the *Dra*I sites within *CDE*II of *CEN3* were minimally accessible (2.9 kb fragment). However, in the *spt4-138* mutant, we observed a marked increase in the

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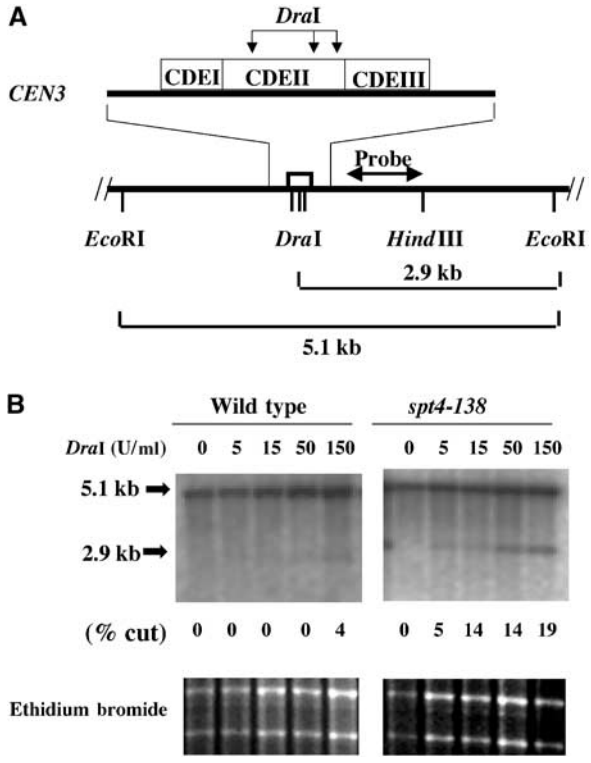


**Figure 3** Centromeric chromatin structure is altered in the *spt4-138* strain. (**A**) Representation of three *Dra*I sites within *CDEII* of the *CEN3*, the resulting *Dra*I–*Eco*RI or *Eco*RI–*Eco*RI fragments, and the probe used for Southern blot analysis. (**B**) *CEN3* chromatin structure of wild-type and *spt4-138* mutant. Nuclei prepared from logarithmic growing cultures of wild-type (PKY090) and *spt4-138* (YMB2192) strains grown at 37°C for 6 h were incubated with 0, 5, 15, 50, and 150 U/ml of *Dra*I at 37°C for 30 min. Southern blot analysis of the *Eco*RI digested DNA was performed and probed with a $^{32}$P-dCTP-labeled 0.9 kb *Hind*III–*Bam*HI fragment as shown in (A). The fraction of *CEN3* accessible to *Dra*I (% cut) was determined by quantification of the cut band (2.9 kb) divided by the sum of the uncut (5.1 kb) and cut bands (2.9 kb) using a Fuji Phosphorimager. The results were reproducible in multiple experiments, with values not differing by more than 0.01%.

accessibility of centromeric *Dra*I sites, with a five-fold increase in digestion as compared to the wild-type strain. We did not detect a significant difference in *Dra*I accessibility at the permissive temperature of growth (data not shown). These results showed that Spt4p is required for the integrity of centromeric chromatin.

### *Spt4p is required for restricting the association of Cse4p-HA with kinetochores*

The alteration of centromeric chromatin in the *spt4-138* strain led us to examine as to whether the compromised kinetochore structure was accompanied by changes in the localization of a kinetochore protein Cse4p. Cse4p, a homolog of mammalian CENP-A, is present at the kinetochores in almost all systems and is critically required for chromosome segregation (Smith, 2002). We compared the localization pattern of chromosomally tagged Cse4p-HA from wild-type and *spt4-138* strains using chromosome spreads and indirect immunofluorescence. In the majority of wild-type cells (>98%), Cse4p-HA was 'clustered' and localized to one or two foci (Figure 4A), consistent with previous observations (Meluh *et al*, 1998). In contrast, in the *spt4-138* strain, Cse4p-HA was localized to multiple foci within the nucleus (Figure 4A). Quantitative analysis showed that greater than 75% of the cells with *spt4-138* showed multiple Cse4p-HA foci compared to only 2% of the cells containing wild-type *SPT4* (Figure 4C). Similar results were obtained for the mislocalization of Cse4p-HA in the *spt4*Δ strain (data not shown). We next questioned whether Spt4p is required for localization of kinetochore protein Mtw1-GFP. Mtw1-GFP was localized to one or two kinetochore foci in greater than 95% of the wild-type cells (Figure 4B), as previously reported (Goshima and Yanagida, 2000; Iouk *et al*, 2002). An almost identical localization of Mtw1-GFP to one to two foci was also observed in the *spt4-138* strain (Figure 4B and C). We also determined that Spt4p was not required for the localization of Ndc10p (data not shown). These results suggest that Spt4p is required for the optimal localization of a subset of kinetochore proteins such as Cse4p.

Cse4p has been shown to associate with core *CEN* DNA using the ChIP technique (Meluh *et al*, 1998). Our data suggested that the absence of functional Spt4p may result in mistargeting of Cse4p to non-*CEN* DNA. We used the ChIP technique to compare the association of Cse4p-HA to chromosomal loci in wild-type and *spt4-138* strains. Consistent with previously published reports (Meluh *et al*, 1998), Cse4p-HA associated only with the core *CEN16* DNA in wild-type cells (Figure 4D). In contrast, we observed a striking difference in the association of Cse4p-HA in the *spt4-138* strain. In the *spt4-138* strain, Cse4p-HA associated not only with the core *CEN16* locus but also DNA flanking *CEN16* (−3.0 and +5.0 kb) and heterochromatic loci such as *HMRa* and *TELVIR*. Cse4p-HA was not enriched at the *ACT1* locus (Figure 4D). We determined that the mislocalization of Cse4p-HA in the *spt4-138* strain was not due to its altered expression, as Western blot analyses showed similar levels of expression of Cse4p-HA in wild-type and *spt4-138* strains (Figure 4E). Hence, based on subcellular localization and biochemical analyses, we conclude that in the absence of functional Spt4p, Cse4p-HA associates with additional centromeric and with noncentromeric loci such as *TELVIR* and *HMRa*.

### *Cac1Δ hir1Δ mutants show a defect in the association of HA-Spt4p with CEN loci*

Recent data have shown that Cac1p, a chromatin assembly factor, and Hir1p, a histone regulatory protein, associate with kinetochores and that *cac1*Δ *hir1*Δ strains exhibit chromosome missegregation and declustering of Cse4p foci similar to that observed in *spt4* mutants (Sharp *et al*, 2002). Hence, we determined whether Cac1p and Hir1p were required for the recruitment of HA-Spt4p to *CEN* DNA. ChIP experiments were carried out using wild-type, *cac1*Δ, *hir1*Δ, and *cac1*Δ *hir1*Δ strains expressing *HASPT4*. HA-Spt4p was enriched at core *CEN3*, *HMRa*, *TELVIR*, and *ACT1* in wild-type, *cac1*Δ, and *hir1*Δ strains (Figure 5). However, in the *cac1*Δ *hir1*Δ strains we failed to detect an enrichment of HA-Spt4p specifically with core *CEN3*; the association of HA-Spt4p with non-*CEN* loci was unaffected. Similar results were obtained for lack of enrichment of HA-Spt4p to core *CEN1* or *CEN16* in the *cac1*Δ *hir1*Δ strain (data not shown). Western blot analysis showed similar levels of expression of HA-Spt4p in the single and double deletion strains (data not shown). Based on our

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results, we conclude that the absence of both Cac1p and Hir1p leads to a defect in the association of HA-Spt4p with core *CEN* DNA.

### *RPB1 is not required for the association of HA-Spt4p with CEN DNA*

A complex containing Spt4p/Spt5p has been shown to be required for transcription elongation (Hartzog *et al*, 2002; Rondon *et al*, 2003) and Spt5p associates with the open reading frames of actively transcribing genes (Andrulis *et al*, 2000; Kaplan *et al*, 2000; Pokholok *et al*, 2002; Simic *et al*, 2003). Hence, we determined whether the association of Spt4p to *CEN* DNA required a major component of the transcriptional machinery. We constructed an *HASPT4* strain carrying a temperature-sensitive *rpb1-1* mutation in the gene encoding the largest subunit of RNAPII. When *rpb1-1* strains are shifted to the nonpermissive temperature, 37°C, transcription by RNAPII and growth of the strain are rapidly halted (Nonet *et al* 1987). ChIP experiments were carried out using *rpb1-1* and a wild-type strain expressing *HASPT4* grown at 25°C and after shift to 37°C for 1 h. Different dilutions of the T DNA (undiluted, 1/100, 1/500) used in PCR reactions verified that the PCR yield was proportional to the amount of starting DNA (Figure 6A). HA-Spt4p associated with core *CEN1* and to actively transcribed genes *ACT1* and *TUP1* in both the wild-type and the *rpb1-1* mutant grown at 25°C (Figure 6A, rows 1, 3, and 5). However, upon shift to 37°C, the association of HA-Spt4p with core *CEN1* DNA (Figure 6A, row 2) was unaffected, even though there was lack of enrichment of HA-Spt4p with *ACT1* or *TUP1* in the *rpb1-1* strain (Figure 6A, rows 4

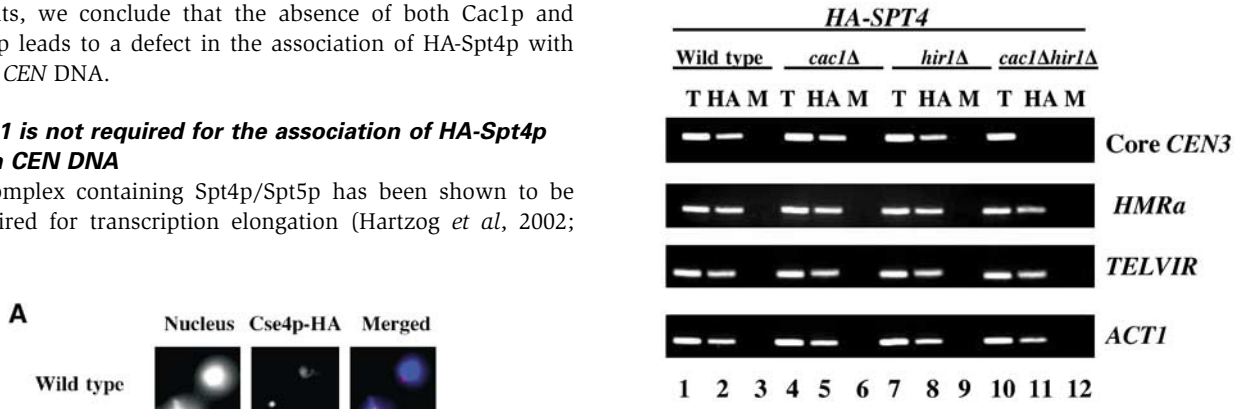


**Figure 5** HA-Spt4p fails to associate with *CEN* DNA in a *cac1*Δ *hir1*Δ strain. ChIP experiments were carried out using wild-type (PKY090), *cac1*Δ (PKY638), *hir1*Δ (PKY117), or *cac1*Δ *hir1*Δ (PKY632) strains expressing *HASPT4* (pMB237) grown at 30°C. Chromatin samples from T, HA, and M were analyzed using primers for core *CEN3*, *HMRa*, *TELVIR*, or *ACT1*.

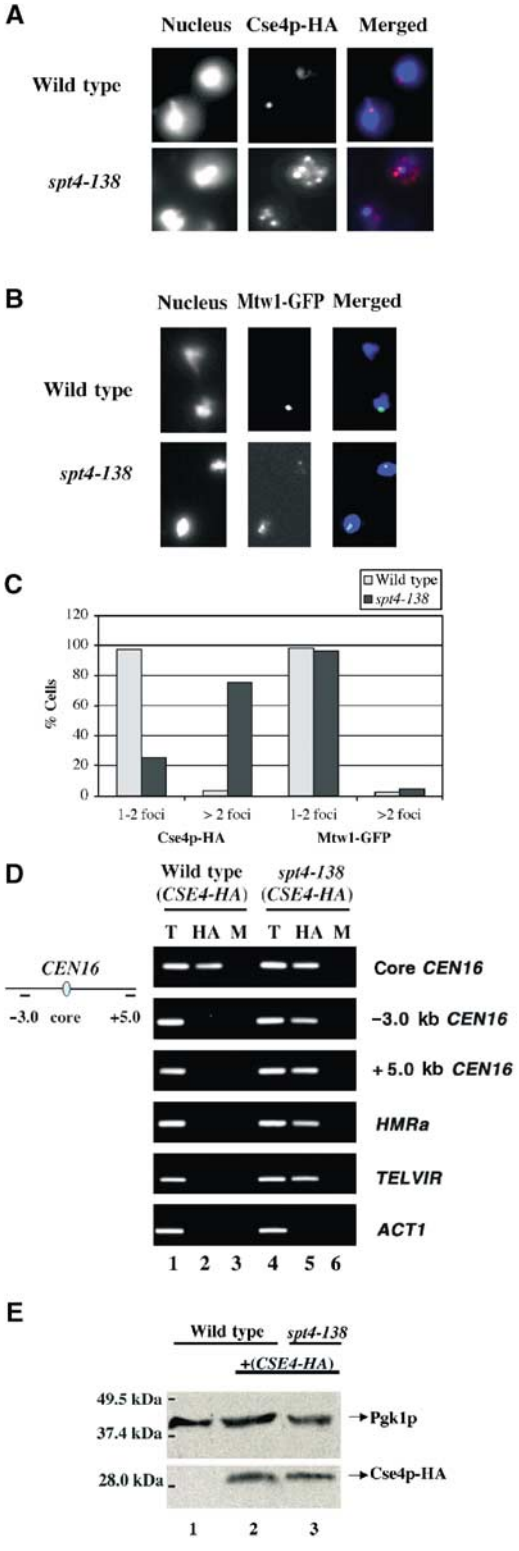


**Figure 4** Spt4p is required for restricting the association of Cse4p-HA with kinetochores. (**A, B**) Cse4p-HA, but not Mtw1-GFP, shows an altered localization pattern in the *spt4-138* strain. Chromosome spreads from wild-type (YPH98) and *spt4-138* (YMB54) strains expressing *CSE4-HA* (YMB2142 and YMB2140, respectively) or *MTW1* (YMB2231 and YMB2230, respectively) grown at 30°C were probed with anti-HA and anti-GFP antibodies. (**C**) Graphic representation of the percentage of wild-type and *spt4-138* cells showing Cse4p-HA and Mtw1-GFP foci corresponding to data in (A) and (B), respectively. At least 100 nuclei were counted in two independent experiments. Similar results were obtained with the *spt4*Δ strain (data not shown). (**D**) Chromosomal association of Cse4p-HA is altered in the *spt4-138* strain. ChIP experiments were carried out using wild-type (YMB2142) and *spt4-138* (YMB2140) strains expressing Cse4p-HA grown at 30°C. Chromatin samples from T, HA, and M were analyzed using primers to core *CEN16* and flanking DNA on either side at −3.0 or +5.0 kb, *HMRa*, *TELVIR*, or *ACT1*. (**E**) Expression of Cse4p-HA is not affected in the *spt4-138* strain. Western blot analysis was performed using the following strains: wild-type (lane 1), wild-type, and *spt4-138* strains expressing *CSE4-HA* (lanes 2 and 3). Blots were probed with anti-HA or anti-PGK (loading control) antibodies.


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and 6). HA-Spt4p was enriched at *CEN* DNA, *ACT1*, and *TUP1* in the wild-type strain at 37°C. These results show that *RPB1* is not required for the association of HA-Spt4p to core *CEN* DNA.

### *Sir3p is required for association of HA-Spt4p with TEL and HMRa*

Our data showed that kinetochore proteins, Ndc10p, Cac1p, and Hir1p, are required for the association of HA-Spt4p with *CEN* DNA, but not *TEL* and *HMRa*. We reasoned that the Spt4p complex at the centromere may be different from the one present at the *TEL* or *HMRa* loci. Hence, we questioned whether Sir3p, a component of *TEL* and *HMRa* chromatin, is required for the association of HA-Spt4p at these loci. ChIP experiments were carried out using wild-type and *sir3*Δ strains expressing *HASPT4*. Consistent with our previous observations, we determined that HA-Spt4p associates with *CEN1*, *HMRa*, *TELVIR*, and *ACT1* in the wild-type strain (Figure 6B). In contrast, the absence of *SIR3* (*sir3*Δ) resulted in a loss of enrichment of HA-Spt4p, specifically at *HMRa* and *TELVIR*. Sir3p was not required for the association of HA-Spt4p to *CEN1* or *ACT1*. Our results show that Sir3p is required for the enrichment of HA-Spt4p at *HMRa* and *TELVIR*. These results support our hypothesis for the presence of distinct HA-Spt4p complexes at *CEN*, *TEL*, and *HMRa*.

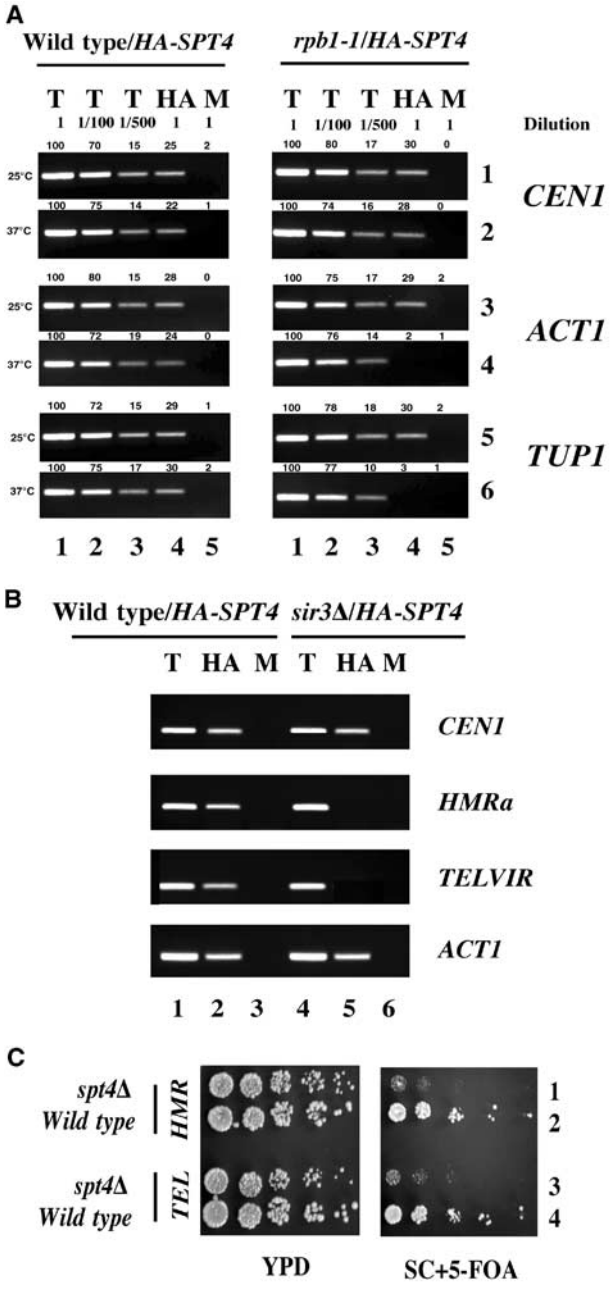


**Figure 6** RNAPII and Sir3p are not required for the association of HA-Spt4p to core *CEN* and *spt4* strains display a defect in silencing of reporter genes at heterochromatic loci. (**A**) HA-Spt4p associates with *CEN* DNA, but not with the actively transcribed genes *ACT1* and *TUP1* in a temperature-sensitive *rpb1-1* strain. ChIP experiments were carried out using wild-type (GHY501) and *rpb1-1* (GHY560) strains expressing *HASPT4* (pMB237) grown at 30°C and shifted to 37°C for 1 h. Different dilutions of chromatin samples from total (T) (undiluted, 1/100, 1/500), immunoprecipitated with anti-HA (HA), and mock (M) were analyzed using primers for core *CEN1*, *ACT1*, or *TUP1*. Values for the quantitation of the data are shown above each of the lanes, with the value for undiluted total set to 100 for each row (lane 1). These data show that the PCR yield is proportional to the amount of starting material. (**B**) HA-Spt4p associates with core *CEN* DNA, but not with *HMRa* and *TEL* in an *sir3*Δ strain. ChIP experiments were carried out using wild-type (BUY668) and *sir3*Δ (BUY671) strains expressing *HASPT4* (pMB237) grown at 30°C. Chromatin samples from T, HA, and M were analyzed using primers for core *CEN1*, *HMRa*, *TELVIR*, or *ACT1*. These results suggest that Spt4p may not be required for the transcription of *HMRa*. (**C**) Spt4p is required for silencing at *HMRa* and *TEL*. Silencing phenotypes were assayed by expression of *URA3* integrated adjacent to either *HMRa* in wild-type (BUY545) and *spt4*Δ (YMB1871) or *TELVIIL* (*TEL*) in wild-type (BUY668) and *spt4*Δ (YMB1849) strains. Five-fold serial dilutions of cells were plated on YPD, and SC + 5-FOA, and incubated for 3–4 days at 30°C. As a control, we determined that *spt4*Δ strains auxotrophic for *URA3* (*ura3*) do not exhibit growth defects on media containing 5-FOA.

### *Spt4p is required for heterochromatin gene silencing*

The association of HA-Spt4p with *TEL* and *HMRa* in an Sir3p-dependent manner suggested that Spt4p may have a role in heterochromatic gene silencing. In wild-type cells, silencing of a *URA3* reporter gene placed adjacent to *HMRa* or *TELVIIL* leads to the transcriptional inhibition of *URA3* (Kamakaka and Rine, 1998). The expression of *URA3* is assayed by comparing the growth of strains on rich media (YPD), and synthetic complete medium containing the metabolic poison 5-fluoro-orotic acid (SC + 5FOA), which inhibits the growth of *URA3* prototrophs (Boeke *et al*, 1984). Thus, growth on 5-FOA media reflects silencing, whereas growth inhibition is indicative of a defect in silencing of *URA3*. Our results showed that unlike wild-type cells in which the expression of *URA3* is silenced, the *spt4*Δ strain does not repress *URA3* expression at either the *HMRa* or *TEL* (Figure 6C), as evidenced by reduced growth on 5-FOA medium. Together, these data demonstrate that *spt4*Δ cells are defective in gene silencing at *HMRa* and *TEL*, and provide the first evidence for a functional role of Spt4p at heterochromatic loci.

### *Human SPT4 (HsSPT4) complements the silencing defects of S. cerevisiae spt4 (Scspt4) mutants and shows an Ndc10p-dependent association with S. cerevisiae CEN DNA*

We have previously shown that a human homolog of *ScSPT4* is able to complement functionally the *ctf*, *ts*, and *spt* pheno-

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types of *Scspt4* mutants (Hartzog *et al*, 1996). The results presented in this paper provide new evidence for a role for Spt4p in heterochromatic gene silencing. Hence, we determined whether *HsSPT4* functionally complements the silencing defects of *Scspt4* mutants. Consistent with our previous observations (Figure 6C), we determined that *spt4* transformants with vector alone showed growth inhibition on media containing 5FOA. Both *HsSPT4* and *ScSPT4* were able to complement the silencing defect of the *URA3* gene at *TEL* and *HMRa* in the *spt4*Δ strain (Figures 7A and B). Also, expression of *HsSPT4* does not alter the expression of the *URA3* gene at either *TEL* or *HMRa* in wild-type cells. These results show that *HsSPT4* is able to functionally complement the silencing phenotypes of *Scspt4* mutants.

The functional complementation of the *ctf* phenotype of *Scspt4* mutants by *HsSPT4* (Hartzog *et al* 1996) led us to examine whether *Hs*Spt4p associates specifically with *CEN* DNA. ChIP experiments were carried out using kinetochore mutant *ndc10-1* expressing *HsHA-SPT4* grown at 25°C and after shift to 37°C for 4 h. Similar to the results for *Sc*Spt4p-HA, we were able to observe an enrichment of HsHA-Spt4p with core *CEN16*, *HMRa*, *TELVIR*, and *ACT1* at 25°C (Figure 7C). However, *Hs*HA-Spt4p failed to associate specifically with only core *CEN16* upon shift to 37°C. Based on these results, we conclude that *Hs*HA-Spt4p associates with core *CEN* DNA of *S. cerevisiae* in an Ndc10p-dependent manner.

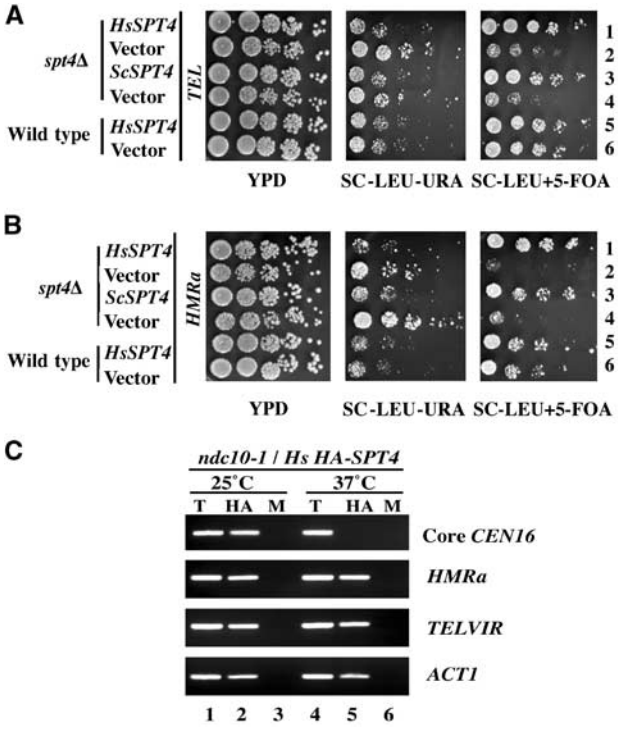


**Figure 7** Human *SPT4* complements the silencing phenotypes of an *Scspt4* mutant and associates with *S. cerevisiae* core *CEN* DNA in an Ndc10p-dependent manner. (**A**) and (**B**) Silencing phenotypes were assayed by determining the expression of *URA3* integrated adjacent to telomere VIIL (*TEL*) and *HMRa* in wild-type (BUY671 and BUY545, respectively) and *spt4*Δ strains (YMB1849 and YMB1871, respectively) expressing human *SPT4* (*HsSPT4*) (pMB299), *ScSPT4* (*ScSPT4*) (pMB237), or vector (vector). Serial dilutions of cells were plated on YPD, SC-LEU-URA, and SC-LEU + 5-FOA and incubated for 3–4 days at 30°C. (**C**) ChIP experiments were carried out using *ndc10-1* expressing p*HsSPT4/LEU2* (YMB1827) strain grown at 25°C and shifted to 37°C for 4 h. Chromatin samples from T, HA, and M were analyzed using primers for core *CEN16*, *HMRa*, *TELVIR*, or *ACT1*.

## Discussion

The results presented in this paper provide direct evidence that evolutionarily conserved Spt4p is a new component of specialized chromatin at the centromere and heterochromatic regions in *S. cerevisiae*, with key roles in kinetochore structure and function as well as gene silencing. Our results show that Spt4p is required for: (a) integrity of centromeric chromatin and kinetochore structure and function, potentially mediated by restricting the localization of Cse4p, the homolog of mammalian CENP-A, to centromeric chromatin and (b) heterochromatic gene silencing. We determined that kinetochore proteins Ndc10p, Cac1p, Hir1p, the silencing protein, Sir3p, and RNA polymerase II subunit Rpb1p are required for the *in vivo* association of Spt4p to core *CEN* DNA, heterochromatic loci *TEL* and *HMRa*, and actively transcribing genes, respectively. These results show the presence of distinct HA-Spt4p complexes at *CEN*, *TEL*, *HMRa*, and actively transcribed loci. Furthermore, the human homolog of *SPT4* (*HsSPT4*) functionally complements the yeast mutant phenotypes and associates with *S. cerevisiae* core *CEN* DNA in an Ndc10p-dependent manner. These results underscore the evolutionary conservation of pathways required for genome stability and illustrate how the yeast model system can be used to understand the complex molecular architecture of the kinetochore and its role in the fundamental process of chromosome segregation in humans.

### *SPT4 is a component of centromeric chromatin*

Our previous analysis of *SPT4* demonstrated that *spt4* mutants show a 100-fold increase in the loss of a non-essential reporter chromosome, test positive in *in vivo* assays for defects in kinetochore integrity, and genetically interact with kinetochore mutants. Also, extracts from *spt4* mutants exhibit defects in the binding of minichromosomes to microtubules (Basrai *et al*, 1996). Combined, these data support the hypothesis that kinetochore structure and/or function are altered in the *spt4* mutants. The co-localization of a subset of Spt4-GFP foci with the kinetochore protein Ndc10p-HA and the *in vivo* association of HA-Spt4p with centromeric DNA provided further evidence for the role for Spt4p in kinetochore structure/function. We determined that components that constitute the inner kinetochore (Ndc10p) and chromatin (Cac1p, Hir1p) are required for the specific association of HA-Spt4p to core *CEN* DNA, but not to other loci. Ndc10p has been shown to be required for the association of inner, central, and outer kinetochore components to *CEN* DNA (Goshima and Yanagida, 2000; Cheeseman *et al*, 2002).

To gain direct evidence for the role of Spt4p in kinetochore structure, we determined whether the chromosome segregation and kinetochore integrity defects observed in the *spt4* mutants (Basrai *et al*, 1996) may be due to an alteration of centromeric chromatin structure. We determined that in an *spt4* mutant the *CEN3* chromatin is at least five times more accessible to *Dra*I digestion than *CEN3* chromatin from a wild-type strain. Similar increases in *Dra*I accessibility have been previously used to establish a structural role for

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histones (H2A, H2B, and H4), Cse4p, Cac1p, Hir1p, and chromatin remodeling proteins, Sth1p and Snf5p, in centromere structure (Han *et al*, 1987; Saunders *et al*, 1990; Smith *et al*, 1996; Tsuchiya *et al*, 1998; Pinto and Winston, 2000; Hsu *et al*, 2003).

Having established that Spt4p is a component of kinetochores, we addressed the impact of loss of functional Spt4p on kinetochore structure by examining whether Spt4p was required for the optimal localization of the kinetochore protein Cse4p. It has been suggested that the *CEN* DNA is wrapped around a Cse4p variant nucleosome (Basrai and Hieter, 1995; Stoler *et al*, 1995; Meluh *et al*, 1998; Keith and Fitzgerald-Hayes, 2000; Bjerling and Ekwall, 2002; Smith, 2002). We determined that Spt4p restricts the localization of Cse4p-HA exclusively to the kinetochores. ChIP experiments supported this conclusion, as the absence of functional Spt4p led to the association of Cse4p-HA to centromeric and noncentromeric loci such as *TELVIR* and *HMRa*. Overexpression of *CSE4* from a *GAL1* inducible promoter does not result in increased chromosome loss in wild-type strains (Sakelaris and Basrai, unpublished data). We have previously shown that there is a significant increase in chromosome loss in double mutants that combined mutations in *spt4* with those of either *CDEI* or *CDEII*, but not with those of *CDEIII* (Basrai *et al*, 1996). These results suggest that Spt4p interacts with *CDEI* and *CDEII*, but not *CDEIII*. A similar model has been proposed for the interaction of Cse4p based on the increased chromosome loss observed for *cse4* mutants when combined with mutations in *CDEI* and *CDEII*, but not *CDEIII* (Keith and Fitzgerald-Hayes, 2000; Smith, 2002). So far, we have failed to detect a genetic interaction between *SPT4* and *CSE4* alleles (data not shown). We speculate that the association of Cse4p-HA with noncentromeric DNA in an *spt4* mutant may: (a) lead to the recruitment of one or more kinetochore proteins to the noncentromeric sites affecting the steady-state levels of these proteins including Cse4p at the kinetochore or (b) affect the chromatin structure at the noncentromeric loci.

Cac1p and Hir1p have been identified as proteins that associate with centromeric chromatin. Strains lacking both *CAC1* and *HIR1* exhibit chromosome missegregation phenotypes and show a declustering of Cse4p foci similar to that observed in the *spt4* mutants (Sharp *et al*, 2002). *spt4* and *hir1* mutants are synthetically lethal and Spt4p and Hir1p interact biochemically (DeSilva *et al*, 1998; Formosa *et al*, 2002; G Hartzog and J Speer, personal communication). Furthermore, mutations in *SPT4*, *SPT5*, and *SPT6* confer phenotypes similar to those due to mutations in the *HIR* genes (Compagnone-Post and Osley, 1996). Our data on lack of association of Spt4p is the first example of a kinetochore protein that requires both Cac1p and Hir1p for centromeric association. It is possible that mislocalization of Cse4p in *cac1*Δ *hir1*Δ strains may be due to lack of enrichment of Spt4p at the kinetochores. Based on genetic and biochemical interactions as well as phenotypes for single- and double-mutant combinations between *cac1*Δ, *hir1*Δ, and *spt4*Δ, we propose that *SPT4* may be downstream of *CAC1* and *HIR1* in chromatin assembly at *CEN* loci.

Hence, based on genetic analyses, subcellular localization, *in vivo* association with *CEN* DNA, and centromeric chromatin studies, we propose that Spt4p is a key component of centromeric chromatin that is required for kinetochore function. Further studies with proteins such as Spt4p and associated proteins such as Spt5p and Spt6p may help us understand the molecular complexity of centromeric chromatin and understand the role of these and other proteins in kinetochore function.

### *Novel role of Spt4p in heterochromatic gene silencing*

Several proteins including the Sir proteins (Sir1p, Sir2p, Sir3p, and Sir4p) are required for the formation of repressive chromatin structure at heterochromatic loci such as *TEL* and *HMRa* (Hoppe *et al*, 2002; Rusche *et al*, 2002). Absence of Sir3p or other silencing protein(s) leads to defects in transcriptional silencing of reporter genes at these sites. Our results suggest that Sir3p is required for the association of HA-Spt4p with *TEL* and *HMRa*. Coupled with the fact that s*pt4* mutants show defects in silencing at *TEL* and *HMRa*, these data show that Spt4p has a role in heterochromatic gene silencing. Studies with *S. pombe* have shown that several kinetochore components co-localize to other silenced regions (Allshire and Pidoux, 2001). Cac1p, Hir1p, and our data on Spt4p represent examples of *S. cerevisiae* proteins with roles in gene silencing and kinetochore function in addition to other functions (Enomoto *et al*, 1997; Game and Kaufman, 1999; Sharp *et al*, 2002).

### *Function of Spt4p is evolutionarily conserved*

*SPT4* is conserved evolutionarily with homologs in *Schizosaccharomyces pombe*, *Mus musculus*, and humans (Chiang *et al*, 1996; Hartzog *et al*, 1996; Wood *et al*, 2002). We have shown previously that *Hs*Spt4p, which is 42% identical to *Sc*Spt4p, functionally complements the *ctf*, *ts*, and *spt* phenotypes of *S. cerevisiae* mutants (Hartzog *et al*, 1996). Here we show that *HsSPT4* complements the defects in the silencing of reporter genes at heterochromatic loci *TEL* and *HMRa* in *Scspt4* mutants. Our data on the association of *Hs*Spt4p with *S. cerevisiae CEN* DNA in an Ndc10p-dependent manner are consistent with the complementation of *ctf* phenotypes. These studies represent the first example of the *in vivo* association of a human protein to the kinetochores of budding yeast. Human homologs for key kinetochore proteins such as CENP-A, CENP-C, Skp1p, Sgt1p, and others have been described; however, only *HsSGT1* has been shown to complement the chromosome loss phenotype of *S. cerevisiae sgt1* mutants (Kitagawa and Hieter, 2001). Our results with Spt4p provide an exciting opportunity to study the association and order of assembly of human kinetochore proteins in budding yeast. The relatively simple *S. cerevisiae* centromere may provide clues to the fundamental order of assembly of the kinetochore of mammalian cells.

The significance of our studies with *SPT4* is further strengthened by the observations that most human cancers are associated with abnormal karyotypes and increased rates of chromosome missegregation (Lengauer *et al*, 1998). Mutations that cause genome instability in model organisms such as *S. cerevisiae* may help us identify genes such as *SPT4* that represent 'cross species candidate genes' for cancer predisposition. It is possible that *HsSPT4* is a prime target for mutation, leading to chromosome missegregation in human cancer. Future studies with *SPT4* in yeast and humans will help us determine the role of this gene in genome stability.

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**Table I** Yeast strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| YMB54 | *MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 spt4-138* | Basrai *et al* (1996) |
| YMB1849 | *MATα ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 URA3-TELVIIL spt4Δ::KAN* | This study |
| YMB1859 | *MATa ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1-1 LYS2 NDC10:HA3:URA3:HA3 SPT4GFP/HIS* | This study |
| YMB1871 | *MATa ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 HMRI::URA3 spt4Δ::KAN* | This study |
| YMB2140 | *MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 spt4-138 + [pCSE4-HA/URA3]* | This study |
| YMB2142 | *MATa ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1 + [pCSE4-HA/URA3]* | This study |
| YMB2230 | *MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 spt4-138* CFVII *(RAD2d.YPH277) URA3 SUP11 + [pMTW1-GFP/LEU2]* | This study |
| YMB2231 | *MATa ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1 + [pMTW1-GFP/LEU2]* | This study |
| YPH98 | MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1* | Spencer *et al* (1990) |
| YPH499 | MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1trp1Δ63* | Sikorski and Hieter (1989) |
| BUY545 | *MATa ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 HMRΔI::URA3* | R Kamakaka |
| BUY668 | *MATα ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 URA3-TELVIIL* | Dhillon and Kamakaka (2000) |
| BUY671 | *MATa/α ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 URA3-TELVIIL sir3Δ::TRP1* | R Kamakaka |
| GHY262 | *MATα his4-912δ lys2-128δ ura3-52Δ1 pep4::LEU2* HA-*SPT4 SPT5*-MYC | G Hartzog |
| GHY501 | *MATα his3-200δ his4-912δ lys2-128Δ ura3-52Δ1 leu2Δ1 rpb1Δ187::HIS3 + [pRP112 = RPB1CEN/URA3]* | G Hartzog |
| GHY560 | *MATα his3-200Δ his4-912δ lys2-128δ ura3-52Δ1 leu2Δ1 rpb1Δ187::HIS3 + [pRP1-1 = rpb1-1CEN/URA3]* | G Hartzog |
| JK421 | *MATa ade2-1 ura3-1 his3-11,1 trp1-1 leu2-3,112 can1-100 ndc10-1* | Goh and Kilmartin (1993) |
| PKY090 | *MATa leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 adh4::URA3-TEL VIIL* | P Kaufman |
| PKY117 | *MATa leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 hir1::HIS3 adh4::URA3-TEL VIIL* | P Kaufman |
| PKY632 | *MATa leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 cac1Δ::hisG hir1Δ::HIS3 URA3- ΔVIIL* | P Kaufman |
| PKY638 | *MATa leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100 cac1Δ::hisG URA3- VIIL* | P Kaufman |

## Materials and methods

### *Strains, plasmids, and growth conditions*

Yeast growth media and protocols are as described (Rose *et al*, 1990). The list of strains is given in Table I. The SPT4 ORF was tagged in frame with GFP or HA as described (Longtine *et al*, 1998). The plasmids used were pMB237 (HA-tagged SPT4) (Basrai *et al*, 1996), pMB299 (HA-tagged human SPT4) (Hartzog *et al*, 1996), and BMB 1058 (MTW1-GFP) (Iouk *et al*, 2002). CSE4 was tagged at its chromosomal locus using pCSE4HA/URA3 (pPM204)) (a gift from Pam Meluh, Memorial Sloan-Kettering Cancer CTR, NY, USA). Tagged strains were confirmed for in-frame fusions by PCR and Western blot analysis.

### *Chromatin immunoprecipitation*

'*In vivo*' crosslinking and chromatin immunoprecipitation (ChiP) were performed as described previously (Meluh and Broach, 1999), with the following modifications. Yeast strains were grown in YPD or selective media to an $OD_{600}$ of 1.0–1.2, then treated '*in situ*' with 1% paraformaldehyde for 30 min. Immunoprecipitation was performed on the diluted chromatin solution with anti-HA (Roche) or anti-GFP (Roche) or no antibody addition (mock). PCR analyses of total (T), immunoprecipitated (HA), and mock (M) samples were performed using primer pairs (sequences are available upon request). All ChIP experiments were carried out at least three times and often four times. Different dilutions of the template DNA were used in PCR reactions to verify that the PCR yield was proportional to the amount of starting DNA. Quantitation of the data in Figures 1C and 6A was performed using ImageQuant software.

### *Chromosome spreads and fluorescence microscopy*

Chromosome spreads were performed as described (Loidl *et al*, 1991). HA-tagged proteins were detected using antibody 16B12 (Covance) with secondary antibody Cy3-conjugated Affinipure Goat Anti-mouse IgG (Jackson ImmunoResearch). GFP-tagged proteins were detected using antibody Rabbit Alexa Fluor 488 (Molecular Probes). Nuclear morphology was examined by DAPI (4′,6-diamidino-2-phenylindole) staining. Fluorescence microscopy utilized a Zeiss Axioscope 2 microscope (Carl Zeiss Inc.) with a Cooke Sensicam (Cooke), a Chroma GFP filter set (CZ909, Chroma Technology Corp.), and a Uniblitz Shutter assembly (Uniblitz).

### *Western blot analysis*

Western blot analysis was as described previously (Iouk *et al*, 2002). The primary antibody used was anti-HA (clone 12CA5—Roche) or anti-Pgk1p (A-6457—Molecular Probes). The secondary antibody was HRP-conjugated sheep anti-mouse IgG (NA931V—Amersham).

### *Centromeric chromatin structure accessibility assay*

The *Dra*I assay was performed as described previously (Saunders *et al*, 1990; Meluh *et al*, 1998). Logarithmically growing cultures at 30°C were shifted to 37°C for 6 h. Nuclei were isolated and incubated with different concentrations of *Dra*I at 30°C for 30 min, followed by digestion with *Eco*RI and Southern blotting using a $^{32}$P-dCTP-labeled 0.9 kb *Hind*III–*Bam*HI fragment as a probe.

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