Structural Integrity of Centromeric Chromatin and Faithful Chromosome Segregation Requires Pat1

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ABSTRACT The kinetochore (centromeric DNA and associated protein complex) is essential for faithful chromosome segregation and maintenance of genome stability. Here we report that an evolutionarily conserved protein Pat1 is a structural component of *Saccharomyces cerevisiae* kinetochore and associates with centromeres in a *NDC10*-dependent manner. Consistent with a role for Pat1 in kinetochore structure and function, a deletion of *PAT1* results in delay in sister chromatid separation, errors in chromosome segregation, and defects in structural integrity of centromeric chromatin. Pat1 is involved in topological regulation of minichromosomes as altered patterns of DNA supercoiling were observed in *pat1Δ* cells. Studies with *pat1* alleles uncovered an evolutionarily conserved region within the central domain of Pat1 that is required for its association with centromeres, sister chromatid separation, and faithful chromosome segregation. Taken together, our data have uncovered a novel role for Pat1 in maintaining the structural integrity of centromeric chromatin to facilitate faithful chromosome segregation and proper kinetochore function.

HIGH-FIDELITY chromosome segregation is important for normal cell growth and development. Errors in chromosome segregation result in aneuploidy, which has been associated with several human diseases and disorders, such as birth defects, developmental problems, and likely cancer (Lengauer *et al.* 1998). The kinetochore is essential for high-fidelity chromosome segregation in every eukaryotic organism (Burrack and Berman 2012; Maddox *et al.* 2012). The features of a kinetochore include centromeric (*CEN*) DNA, specialized protein complexes, and a unique chromatin structure (Bloom and Carbon 1982; Allshire and Karpen 2008; Burrack and Berman 2012; Choy *et al.* 2012; Maddox *et al.* 2012). The size and composition of *CEN* DNA varies among organisms ranging from the ~125 bp of defined DNA sequences in *Saccharomyces cerevisiae* (Clarke and Carbon 1980), to 4 kb–10 Mb of DNA composed of species-specific satellite DNA, microsatellite repeats, or retrotransposon-derived sequences in other eukaryotes (Allshire and Karpen 2008; Burrack and Berman 2012; Maddox *et al.* 2012). Despite the lack of *CEN* DNA sequence conservation, several

kinetochore proteins and multiprotein kinetochore subcomplexes are evolutionarily conserved (Kitagawa and Hieter 2001; Meraldi *et al.* 2006; Burrack and Berman 2012; Maddox *et al.* 2012). The structural and functional analysis of kinetochores has been an active area of research, and so far about 70 kinetochore proteins have been characterized using molecular genetics and biochemical approaches in *S. cerevisiae* (Cho *et al.* 2010). Many *S. cerevisiae* kinetochore proteins have orthologs in fission yeast, flies, *Caenorhabditis elegans*, and humans (Hartzog *et al.* 1996; Kitagawa and Hieter 2001; Meraldi *et al.* 2006; Przewłoka and Glover 2009; Lampert and Westermann 2011).

In addition to kinetochore proteins, structural integrity of *CEN* chromatin ensures high-fidelity chromosome segregation during the cell cycle (Newlon 1988; Verdaasdonk and Bloom 2011). *CEN* chromatin is organized into a unique, highly ordered, and topologically distinct structure in chromosomes (Bloom and Carbon 1982; Bloom *et al.* 1984; Saunders *et al.* 1990). Several factors such as chromatin-remodeling complexes (RSC complex) (Hsu *et al.* 2003), as well as post-translational modifications (Boeckmann *et al.* 2013), and altered dosage of histones regulate *CEN* chromatin structure for faithful chromosome segregation (Saunders *et al.* 1990; Pinto and Winston 2000; Au *et al.* 2008; Choy *et al.* 2011; Verdaasdonk and Bloom 2011; Yu *et al.* 2011; Choy *et al.* 2012; Au *et al.* 2013). Despite these studies, the molecular mechanisms that ensure the

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structural integrity of *CEN* chromatin for high-fidelity chromosome segregation are not fully understood.

In this study, we report that an evolutionarily conserved protein, *Pat1*, associates with *CEN* chromatin and regulates kinetochore structure and function to maintain faithful chromosome segregation. A deletion of *PAT1* results in altered structure of *CEN* chromatin, altered patterns of minichromosome supercoiling, defects in sister chromatid separation, and chromosome missegregation. *Pat1*, initially identified as a protein associated with topoisomerase II (Wang *et al.* 1996), is involved in the assembly of cytoplasmic foci containing non-translating mRNAs and decapping factors called P-bodies (Pilkington and Parker 2008), and a disruption of the *PAT1* gene causes errors in chromosome transmission fidelity (Wang *et al.* 1996). We determined that the role of *Pat1* in chromosome segregation is independent of its function in P-body assembly and translation repression. We have identified an evolutionarily conserved region (amino acid residues 254–422), which mediates the *CEN* association of *Pat1* and is required for sister chromatid separation and faithful chromosome segregation.

Materials and Methods

Strains, plasmids, and growth conditions

Yeast strains and plasmids used in this work are listed in Table 1. Strains were grown in YPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) or in synthetic medium containing 2% glucose and supplements to allow selection of plasmids.

Chromosome fragment loss assay

A colony color assay developed previously (Spencer *et al.* 1990) was used to measure the loss of a nonessential reporter chromosome fragment (CF). Strains were grown to logarithmic phase in synthetic media to maintain the CF; cultures were then diluted and plated on synthetic medium with limiting adenine at 30°. Loss of the CF results in red sectors in an otherwise white colony. The chromosome loss frequency was calculated by determining the percentage of colonies that were at least half red, indicating loss of the CF at the first cell division. At least 3000 colonies were counted from three individual transformants for each strain (Mishra *et al.* 2011).

Chromatin immunoprecipitation experiments

Chromatin immunoprecipitation (ChIP) experiments were performed with three independent biological replicates as described (Mishra *et al.* 2011). Antibodies used to capture protein–DNA complexes were α -FLAG (A2220, Sigma), α -HA (A2095, Sigma), α -Myc (A7470, Sigma), or α -GST (Z-5, sc-459, Santa Cruz Biotech). Quantitative real time ChIP–PCR was performed using Fast SYBR Green Master Mix in 7500 Fast Real Time PCR system (Applied Biosystems) as previously described (Mishra *et al.* 2011). The enrichment values were calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001) and are presented as percentage input. ChIP–PCR was performed using Hotstar-Taq polymerase Master Mix (Qiagen) (Mishra *et al.* 2011). Primer sequences are listed in Table 2.

Western blotting

Protein samples for Western blotting were prepared using the TCA procedure (Mishra *et al.* 2011) and quantified with the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein for each sample were size separated on polyacrylamide gels and transferred to nitrocellulose membrane. Primary antibodies used were α -HA (clone 12CA5, Roche), α -Flag (F-3165, Sigma), and α -Tub2 (Mishra *et al.* 2011). Secondary antibodies used were HRP-conjugated sheep α -mouse IgG (NA931V, Amersham), and HRP-conjugated sheep α -rabbit IgG (NA934V, Amersham).

Sister chromatid separation assay

Strains were grown in YPD or in selective medium to logarithmic phase at 30°, treated with 3 μ M α -factor (T-6901, Sigma) for 2 hr to synchronize cells in G1, washed, and released into pheromone-free YPD medium. α -factor was readded after the completion of mitosis (80 min after release) to arrest the cells in the subsequent G1 phase of the cell cycle. Samples were taken at 20-min time points after release from G1 for microscopic analysis. Cells were fixed as described (Warsi *et al.* 2008) and LacI–GFP foci were examined using a Zeiss Axioskop 2 microscope (Zeiss, Thornwood, NY).

Minichromosome supercoiling assay

Wild-type and *pat1 Δ* strains carrying a topologically closed circular minichromosome (pRS416) were grown in YPD at 25° to midlog phase, synchronized in G1 with α -factor, and released into pheromone free and nocodazole (20 μ g/mL) containing medium at 35° for 3 hr. Total DNA was purified as described (Warsi *et al.* 2008). DNA was fractionated by electrophoresis on 0.6% agarose gels containing 3 μ g/mL chloroquine in both the running buffer and the gel for supercoiling analysis. DNA was transferred to a nylon membrane and hybridized with a radiolabeled probe (1549-bp *ScaI*/*PvuII* fragment located adjacent to *CEN6* from pRS416). Cells for fluorescence-activated cell sorting (FACS) analysis were fixed in 70% ethanol, treated with RNase A, Proteinase K, washed with 0.2 M Tris-buffer, stained with propidium iodide, and assayed using a FACSort flow cytometer and Cell Quest software (BD Biosciences, Bedford, MA) (Mishra *et al.* 2011). Cells were examined using a Zeiss Axioskop 2 microscope (Zeiss) to determine the cell-cycle stages following the cell morphology and nuclear position as described previously (Calvert and Lannigan 2010).

Preparation of yeast nuclei and *DraI* accessibility assay

Yeast nuclei were prepared from wild-type and *pat1 Δ* strains grown in YPD at 30° to midlog phase as described previously (Saunders *et al.* 1990) and digested with *DraI* (0, 20, and 100 U/mL of nuclei) for 30 min at 37°. DNA was extracted from the chromatin as described previously (Saunders *et al.* 1990). For Southern hybridization, DNA was cleaved with *HindIII*, separated on 1.4% agarose gels, transferred to nylon membranes, and hybridized with a 900-bp radiolabeled DNA fragment flanking *CEN3* (Chr3: 114945–115844).

Table 1 *Saccharomyces cerevisiae* strains and plasmids

Strain	Genotype	Reference
A. <i>Saccharomyces cerevisiae</i> strains		
YPH1015	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 CEN3L.YPH278</i> <i>HIS3 SUP11</i>	Spencer <i>et al.</i> (1990)
JK421	<i>MATa ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 ndc10-1</i>	Goh and Kilmartin (1993)
JB577	<i>MATa leu2,3-112::LacO-LEU2 his3-11, 15::LacI-GFP-HIS3 trp1-1 ura3-1 ade2-1 can1-100</i> <i>CEN4-LacO-LEU2</i>	Warsi <i>et al.</i> (2008)
JG595	<i>MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 CSE4-12Myc::URA3</i> <i>SCM3-3Flag::kanMX</i>	(Camahort <i>et al.</i> 2007)
BY4741	<i>MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0</i>	Open Biosystems
YMB6398	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 CSE4-13Myc::LEU2</i>	Au <i>et al.</i> (2008)
YMB7963	<i>MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 CSE4-12Myc::URA3</i> <i>SCM3-3Flag::kanMX PAT1-3HA::TRP1</i>	This study
YMB8307	<i>MATa ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 ndc10-1 PAT1-3HA::TRP1</i>	This study
YMB8345	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 pat1Δ::kanMX CFIII</i> <i>(CEN3L.YPH278) HIS3 SUP11</i>	This study
YMB8402	<i>MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 pat1Δ::kanMX</i>	This study
YMB8421	<i>MATa leu2,3-112::LacO-LEU2 his3-11, 15::LacI-GFP-HIS3 trp1-1 ura3-1 ade2-1 can1-100</i> <i>CEN4-LacO-LEU2 pat1Δ::kanMX</i>	This study
YMB8422	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 CSE4-13Myc::LEU2</i> <i>pat1Δ::URA3</i>	This study
YMB8660	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 CEN-URA3 CFIII</i> <i>(CEN3L.YPH278) HIS3 SUP11</i>	This study
YMB8678	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 edc3Δ::kanMX CFIII</i> <i>(CEN3L.YPH278) HIS3 SUP11</i>	This study
YMB8706	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 pat1Δ::kanMX</i> <i>CEN-URA3 CFIII (CEN3L.YPH278) HIS3 SUP11</i>	This study
YMB8680	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 dhh1Δ::kanMX CFIII</i> <i>(CEN3L.YPH278) HIS3 SUP11</i>	This study
YMB8728	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 TOP2-18Myc-URA3 CFIII</i> <i>(CEN3L.YPH278) HIS3 SUP11</i>	This study
YMB8729	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 pat1Δ::kanMX</i> <i>TOP2-18Myc-URA3 CFIII (CEN3L.YPH278) HIS3 SUP11</i>	This study
YMB8900	<i>MATa leu2,3-112::LacO-LEU2 his3-11, 15::LacI-GFP-HIS3 trp1-1 ura3-1 ade2-1 can1-100</i> <i>CEN4-LacO-LEU2 mad2Δ::kanMX</i>	This study
YMB8901	<i>MATa leu2,3-112::LacO-LEU2 his3-11, 15::LacI-GFP-HIS3 trp1-1 ura3-1 ade2-1 can1-100</i> <i>CEN4-LacO-LEU2 pat1Δ::URA3 mad2Δ::kanMX</i>	This study
B. List of plasmids		
Plasmid	Description	Reference
pRS416	<i>CEN URA3</i>	Sikorski and Hieter (1989)
pRP1490 (<i>PAT1</i>)	<i>CEN URA3 PAT1-Flag</i>	Pilkington and Parker (2008)
pRP1491 (Δ254-422)	<i>CEN URA3 PAT1:Δ254-422-Flag</i>	Pilkington and Parker (2008)
pRP1492 (Δ422-697)	<i>CEN URA3 PAT1:Δ422-697-Flag</i>	Pilkington and Parker (2008)
pTW009	<i>CEN URA3 TOP2-18Myc</i>	Warsi <i>et al.</i> (2008)

Quantitative real-time PCR (qPCR) was performed to determine the susceptibility of chromatin to *DraI* digestion (100 U/mL) using primers flanking *DraI* sites of *CEN3* and *ADP1*. Values were normalized to an untreated control (no *DraI*) to determine the fraction of DNA digested by the *DraI* (100 U/mL) enzyme.

Results

Pat1 regulates chromosome segregation and associates with CEN DNA

A previous study reported that a disruption of the *PAT1* gene by insertion of *LEU2* within the coding region results in errors in chromosome segregation in mitosis and meiosis

(Wang *et al.* 1996). We deleted the entire *PAT1* gene in a wild-type strain containing a reporter CF and examined the frequency of CF loss using a colony color assay in which the loss of CF results in red sectors in an otherwise white colony (Spencer *et al.* 1990). Colonies that were at least half red reflecting the loss of CF in the first cell division were counted (Figure 1A, see inset). The frequency of CF loss in *pat1Δ* strains is about 20-fold higher than that of the wild-type strain (Figure 1A). The CF loss phenotype of *pat1Δ* strains is complemented by a plasmid expressing *PAT1* from its native promoter (Figure 1A). The frequency of CF loss observed in *pat1Δ* strain is similar to that reported for deletion of kinetochore genes (Kastenmayer *et al.* 2005; Ma *et al.* 2012).

Table 2 List of PCR primers used in this study

Locus	Forward (5'–3')	Reverse (5'–3')	Reference
<i>CEN1</i>	CTCGATTTCGATAAGTGTGCC	GTGCTTAAGAGTTCTGTACCAC	Choy <i>et al.</i> (2011)
<i>CEN3</i>	GATCAGCGCCAAACAATATGG	AACCTCCACCAGTAAACGTTTC	Choy <i>et al.</i> (2011)
<i>CEN5</i>	AAGAACTATGAATCTGTAAATGACTGATTCAAT	CTTGCACTAAACAAGACTTTATACTACGTTTAG	Choy <i>et al.</i> (2011)
<i>HSC82</i>	AGGGTAAATTGGCTTATCTGATTATA	TCCAAATACACAATCCAGCATATC	Mizuguchi <i>et al.</i> (2007)
<i>HML</i>	CACAGCGGTTTCAAAAAAGCTG	GGATTTTATTTAAAAATCGAGAGG	Choy <i>et al.</i> (2011)
<i>CEN3–L1</i>	ATATTGTTTGGCGCTGATCGCC	TTGATGAACCTTTCAAAGATGAC	Choy <i>et al.</i> (2011)
<i>CEN3–L2</i>	TCATCTTTGAAAAGTTCATCAAGG	GATAACAAAGCATGGTATGGCG	Choy <i>et al.</i> (2011)
<i>CEN3–L3</i>	GCCATACCATGCTTTGTTATCGTC	TATTATGCTCCCCTGGATTTTATGCG	Choy <i>et al.</i> (2011)
<i>CEN3–L4</i>	AGCATTAGAGCCACTGTCATTTT	ATAATTAAGATACGAATGTGTTCTGTTG	This study
<i>CEN3–R1</i>	TTTACTGGTGAAGTTTTGCTCA	GTCAACGAGTCCTCTCTGGCTA	Choy <i>et al.</i> (2011)
<i>CEN3–R2</i>	GAGAGGACTCGTTGACGTAGAA	GAATATGATAATGGTTACACCAGTAGG	Choy <i>et al.</i> (2011)
<i>CEN3–R3</i>	TGTAACCATTATCATATTCATGAC	GATTTAATGCACGTTATGTTTCG	Choy <i>et al.</i> (2011)
<i>CEN3–R4</i>	ACTGACAGCACCATTAAATCAATCA	TATCCTCAGTAGAGGGCAAAGTT	This study
<i>CEN3–DraI</i>	TTGATGAACCTTTCAAAGATGAC	GTCAACGAGTCCTCTCTGGCTA	Choy <i>et al.</i> (2011)
<i>ADP1–DraI</i>	ATCCAAATGTGCTCAAGATAGTAGC	CACCAACAACATTTACTAGCAGTG	This study

The chromosome-loss phenotype observed in *pat1Δ* strain (Figure 1A) prompted us to examine whether *Pat1* associates with *CEN* DNA. Previous studies have shown that *Pat1* localizes to the nucleus (Teixeira and Parker 2007) and associates with the chromatin fraction in *S. cerevisiae* (Wyers *et al.* 2000). However, the genomic regions of *Pat1* association are yet to be identified. We constructed a strain that expressed HA-tagged *PAT1* from its native promoter and determined that the epitope tagging does not affect the function of *PAT1* as this strain does not exhibit a growth defect (Supporting Information, Figure S1). ChIP experiments revealed that *Pat1* associates with *CEN1*, *CEN3*, and *CEN5* in wild-type cells (Figure 1, B, C, and E). ChIP–qPCR results showed that *Pat1* associates with about a 1.5-kb DNA region around *CEN3*, exhibiting relatively higher enrichment at the central core region of *CEN3* (Figure 1B). As expected, no significant enrichment was detected at the non-*CEN* *HML* locus (Figure 1B). In a control experiment using an untagged strain, no significant enrichment was observed by ChIP–qPCR at either the *CEN* or non-*CEN* *HML* regions (Figure S2).

Previous studies have shown that the inner kinetochore protein *Ndc10* is required for the assembly of functional kinetochores in budding yeast (Goh and Kilmartin 1993). Several kinetochore proteins fail to associate with centromeres in the *ndc10-1* mutant at the nonpermissive temperature (37°) (Goh and Kilmartin 1993; Janke *et al.* 2001; Hajra *et al.* 2006; Camahort *et al.* 2007; Winey and Bloom 2012). Hence, we examined whether *CEN* association of *Pat1* is dependent on *NDC10*. ChIP experiments were done to examine the *CEN* association of *Pat1* in wild-type and *ndc10-1* strains grown at the permissive temperature (25°) and after shift to the nonpermissive temperature (37°). Western blot analysis showed that the expression of *Pat1* is similar in wild-type and *ndc10-1* strains grown at 25° and 37° (Figure 1D). ChIP–qPCR showed that *Pat1* levels at *CEN1*, *CEN3*, and *CEN5* are reduced (~6–10 fold), specifically in *ndc10-1* mutants at the nonpermissive temperature (Figure 1, C and E). There was no significant enrichment of

Pat1 at non-*CEN* regions (*HML* and *HSC82*) relative to that observed at the *CENs* (Figure 1E). Based on these results we conclude that *Pat1* associates with centromeres and this requires *NDC10* gene.

***Pat1* is required for the structural integrity of centromeric chromatin**

To determine the role of *Pat1* in kinetochore structure, we examined the susceptibility of *CEN* chromatin (*CEN3*) to digestion by the restriction enzyme *DraI* in wild-type and *pat1Δ* strains. Three closely spaced *DraI* recognition sequences are within the *CDE-II* region of *CEN3* (see schematic, Figure 2A), which are protected from nuclease digestion due to the kinetochore protein complex (Saunders *et al.* 1990; Meluh *et al.* 1998; Crotti and Basrai 2004). Altered accessibility to *DraI* has been observed in kinetochore mutants and this approach has been used extensively to examine the structural integrity of *CEN* chromatin (Saunders *et al.* 1990; Meluh *et al.* 1998; Myhre and Bloom 2003; Yu *et al.* 2011). Yeast nuclei treated with different concentrations of *DraI* were analyzed by Southern hybridization using a 900-bp DNA probe flanking *CEN3* (see schematic Figure 2A). We observed increased *DraI* accessibility of *CEN3* chromatin as evident from the higher signal intensity of the smaller (cut) *DraI*–*HindIII* fragments in the *pat1Δ* compared to the wild-type strain (Figure 2B). FACS and nuclear morphology analyses showed no major differences in the cell-cycle distribution between wild-type (WT) and *pat1Δ* strains (Figure S3A). We validated and quantified the increased *DraI* sensitivity by quantitative PCR using primers flanking *CEN3* or a non-*CEN* region (*ADP1*). The *CEN* chromatin in *pat1Δ* strain was more susceptible to *DraI* digestion (approximately ninefold) than that observed for chromatin from a WT strain (Figure 2C). To determine if the increased nuclease sensitivity in *pat1Δ* strain was centromere specific, we examined the *DraI* sensitivity of the *ADP1* locus, which also contains three closely spaced *DraI* sites (see schematic, Figure 2C). The *ADP1*–chromatin (histone H3–chromatin) in *pat1Δ* and wild-type strains exhibited low (~1.0–1.5%)

Figure 1 Pat1 regulates chromosome segregation and associates with CEN DNA. (A) Deletion of *PAT1* results in a chromosome-loss phenotype. Frequency of chromosome loss in wild type (WT; YPH1015), *pat1Δ* (YMB8345), and *pat1Δ* strain transformed with *CEN-PAT1* (pRP1490) was determined as described in *Materials and Methods*. Representative image (see inset) showing the loss of the nonessential chromosome fragment in the first cell division ($\geq$ half red sector) is shown. At least 3000 colonies were counted and average from three independent transformants $\pm$ SE is shown. (*) P -value < 0.05 , Student's t -test. (B) Pat1 is enriched at the core *CEN3*. ChIP was performed for Pat1-3HA in wild-type strain (YMB7963) grown in YPD at 30°. Enrichment of Pat1 to the region surrounding *CEN3* was determined by qPCR and is shown as % input. Average from three independent biological replicates $\pm$ SE is shown. The non-*CEN* locus *HML* was used as a control. (C) Pat1 associates with *CEN* DNA in a *NDC10*-dependent manner. ChIP experiments were performed for Pat1-3HA in wild-type (WT, YMB7963) and *ndc10-1* (YMB8307) strains grown in YPD at permissive temperature (25°) and after a 6-hr shift to nonpermissive temperature (37°). Association of Pat1 at *CEN1* is examined by PCR. IN (input), IP (DNA from ChIP with α -HA antibodies), and M (DNA from ChIP with α -GST antibodies). (D) Pat1 protein levels are not altered in *ndc10-1* strain as revealed by Western blotting with α -HA antibodies. Tub2 used a loading control. (E) Enrichment of Pat1 to *CEN* (*CEN1*, *CEN3*, *CEN5*), and non-*CEN* (*HML*, *HSC82*) DNA in wild-type (WT, YMB7963) and *ndc10-1* (YMB8307) strains was determined by qPCR and is shown as % input. Average from three independent biological replicates $\pm$ SE is shown. There was no significant enrichment of Pat1 at non-*CEN* regions (*HML*, *HSC82*) relative to that observed at the *CENs*. (*) P -value < 0.05 , (**) P -value < 0.01 , ns, significantly not different at 5% level (P -value > 0.05 , Student's t -test).

and similar levels of accessibility by *DraI* (Figure 2C). These results suggest that *Pat1* is required for the structural integrity of *CEN* chromatin.

***pat1Δ* strain exhibits defects in sister chromatid separation and alteration in superhelicity of minichromosomes**

The chromosome-loss phenotype observed in *pat1Δ* strains may be due to defective kinetochore function and thus, we examined if *pat1Δ* strains show defects in sister chromatid separation by measuring the separation of sister chromatids labeled with a GFP reporter (LacI-GFP) inserted 13 kb from *CEN4* (Warsi *et al.* 2008). Wild-type and *pat1Δ* strains were synchronized in G1 with α -factor, and budding index and sister chromatid separation was examined at 20-min intervals after release from the G1. In the wild-type strain a maximum number of large budded cells was observed at 100 min post G1 release, which correlates with the frequency of sister chromatid separation ($\sim 85\%$; Figure 3). However, in the *pat1Δ* strain, the highest numbers of large budded cells and sister chromatid separation were observed at 140 min post G1 release (Figure 3). As expected, only 2–5% of G1 synchronized cells exhibited separated sister chromatids

(Figure 3). To examine whether delay in sister chromatid separation of *pat1Δ* strain is due to activation of the spindle assembly checkpoint (SAC), we deleted *MAD2*, a component of the SAC, in the *pat1Δ* strain, and measured the budding index and frequency of sister chromatid separation as described above. The delay in sister chromatid separation observed in *pat1Δ* was suppressed by deletion of *MAD2* (Figure 3). Similar to the wild-type strain, the highest numbers of large budded cells and sister chromatid separation were observed at 100 min post-G1 release in *pat1Δmad2Δ* strain (Figure 3). These results indicate that *PAT1* is required for sister chromatid separation and that *MAD2* contributes to the delay in sister chromatid separation in *pat1Δ* strains.

The interaction of *Pat1* with topoisomerase II (Wang *et al.* 1996), together with the observation showing altered structure of *CEN* chromatin in a *pat1Δ* strain, prompted us to examine whether *Pat1* affects the topological structure of minichromosomes. The electrophoretic mobility of DNA topoisomers on agarose gels is mainly based on their writhe; however, separation of topoisomers on chloroquine containing agarose gels reduces the twist of DNA. As the linking number in a circular minichromosome is fixed, the reduction

Figure 2 Pat1 is required for the structural integrity of CEN chromatin. (A) Schematic of restriction enzyme sites in the three elements (CDEI, II, and III) within CEN3. Indicated are the three Dral recognition sites within CDEII of CEN3. The 900-bp probe used for Southern blotting is indicated by a shaded bar. HindIII restriction sites and resulting HindIII–HindIII and Dral–HindIII are shown. (B) CEN chromatin shows increased nuclease sensitivity in *pat1Δ* strains. Southern blot analysis of nuclei prepared from logarithmic growing cultures of wild-type (WT, YMB6398) and *pat1Δ* (YMB8422) strains in YPD at 30° were incubated with 0, 20, and 100 U/mL Dral at 37° for 30 min. DNA prepared from Dral-treated samples were digested with HindIII and Southern blots were hybridized with the probe as indicated in A. Signal intensities were quantified using Image J (Schneider *et al.* 2012), and the percentage of CEN3 chromatin cleaved by Dral (% cut) represents the ratio of signal intensity value of cleaved DNA (cut) over signal intensities from Dral-resistant DNA (uncut). (C) qPCR analysis revealed increased nuclease sensitivity of CEN chromatin in *pat1Δ* strains. Dral (100 U/mL) treated samples were assayed by qPCR using primers flanking the Dral sites in CEN3 and a non-CEN region encoding ADP1 (marked as arrows on schematic). Graph shows the susceptibility of CEN3 and ADP1 chromatin to the Dral digestion as measured by fraction of DNA cut by 100 U/mL Dral (% cut) over the untreated control (no Dral). Average from three independent biological replicates $\pm$ SE is shown. (*) *P*-value < 0.05, and ns, significantly not different at 5% level (*P*-value > 0.05, Student's *t*-test).

in twist is compensated for by an increase in writhe (Prunell 1998; Furuyama and Henikoff 2009; Shivaraju *et al.* 2012). Negatively supercoiled DNA is relaxed by chloroquine due to addition of positive writhe; hence it migrates more slowly on an agarose gel, whereas positively supercoiled DNA is tightened by chloroquine and runs more quickly on a gel (Prunell 1998; Furuyama and Henikoff 2009; Shivaraju *et al.* 2012). We assayed the topological behavior of a minichromosome containing CEN6 from wild-type and *pat1Δ* strains using chloroquine gels. Deletion of PAT1 results in alteration in topological structure of minichromosomes as topoisomers from the minichromosome in *pat1Δ* strain migrate more slowly on chloroquine containing agarose gel compared to those observed in the wild-type strain (Figure 4A). We next examined the superhelicity of minichromosome in strains treated with nocodazole, which depolymerizes microtubules and releases spindle tension. The supercoiling pattern of minichromosomes in wild-type and *pat1Δ* strains treated with nocodazole was similar to that observed for untreated cells (Figure 4A). These results suggest that the altered pattern of minichromosome supercoiling in *pat1Δ* strain is not

Figure 3 Pat1 is required for sister chromatid separation. Wild-type (WT, JBY577), *pat1Δ* (YMB8421), *mad2Δ* (YMB8900), and *pat1Δmad2Δ* (YMB8901) strains marked with LacI–GFP (CEN4–proximal) synchronized in G1 with α -factor, released into pheromone-free medium, and sampled at 20-min time intervals were examined for cell morphology (top) and sister chromatid separation in cells as indicated schematically (bottom). At least 100 cells were examined for every time point. Average $\pm$ SE derived from three independent biological replicates is shown.

due to tension imposed by the mitotic spindle. The differences in topological distribution of minichromosomes observed between wild-type and *pat1Δ* strains is not a consequence of biased cell-cycle distribution (Figure S3B).

Topoisomerase II (Top2) associates with CEN chromatin in budding yeast and its activity is required to maintain genome stability (Cline and Hanawalt 2006; Warsi *et al.* 2008; Baldwin *et al.* 2009). Based on the physical interaction between Pat1 and Top2 (Wang *et al.* 1996), we examined if alteration in minichromosome topology and defects in kinetochore function observed in *pat1Δ* strain are due to alteration in levels of centromere-associated Top2 in these strains. ChIP results showed that the level of CEN-associated

Figure 4 Pat1 affects the topological state of minichromosomes. (A) Deletion of *PAT1* causes alteration in topological structure of minichromosomes. Southern blot analysis of total DNA from wild-type (BY4741) and *pat1Δ* (YMB8402) strains with topologically closed circular minichromosome (pRS416). Total DNA was resolved on agarose gels with and without chloroquine (3 μ g/mL), blotted to a nylon membrane, and hybridized with radiolabeled unique DNA fragment derived from minichromosome. Plasmid DNA from *Escherichia coli* was used as control. Migration pattern of topoisomers are marked with a bracket. (B) *CEN* association of Top2 is not affected in *pat1Δ* strains. ChIP of Top2-Myc at *CEN* (*CEN1*, *CEN3*) in wild-type (WT, YMB8728), and *pat1Δ* (YMB8729) strains grown at 30°. Untagged strains (YMB8660 and YMB8706) were used as a control. Enrichment was determined by qPCR and is shown as % input. Average from three independent biological replicates $\pm$ SE is shown. ns, significantly not different at 5% level (P -value >0.05 , Student's t -test).

Top2 was not affected in *pat1Δ* (15.75% of input at *CEN1*, and 11.84% at *CEN3*) compared with the levels observed in a wild-type strain (12.29% at *CEN1* and 9.59% at *CEN3*) (P -values = >0.05) (Figure 4B). No significant enrichment was detected by ChIP-qPCR at *CEN1* and *CEN3* using an untagged control strain (Figure 4B).

Role of Pat1 in chromosome segregation is independent of its function in P-body assembly and translation repression

Previous studies have defined domains of *Pat1* involved in P-body function in budding yeast (Pilkington and Parker 2008). To define a domain required for the chromosome

segregation function of *Pat1*, we assayed two different alleles of *pat1* (Pilkington and Parker 2008) (Figure 5A) for a CF loss phenotype. Western blotting confirmed the expression of the *pat1* alleles (Figure 5B). Chromosome loss assays showed that *pat1-Δ254–422*, which does not show any defects in P-body assembly and translation repression (Pilkington and Parker 2008), exhibits a CF loss phenotype (CF loss rate = 1.92 ± 0.27 , mean $\pm$ SE) similar to that observed for *pat1Δ* (1.57 ± 0.22) strains (Figure 5C), whereas *pat1-Δ422–697* that exhibits defective P-body assembly and translation repression (Pilkington and Parker 2008) did not show a significant CF loss phenotype (Figure 5C). Wild-type strain expressing *pat1* alleles does not result in increased CF loss (Figure S4). Based on these results, we denote the *Pat1* domain from amino acids 254–422 as the chromosome transmission fidelity (CTF) region.

We next examined whether deletion of other components of P-body assembly and mRNA decapping lead to a chromosome-loss phenotype. For example, *Pat1* interacts with *Ecd3* and *Dhh1* (Coller and Parker 2005; Swisher and Parker 2011), and these are activators of the P-body assembly, translation repression, and mRNA decapping (Coller and Parker 2005; Pilkington and Parker 2008; Swisher and Parker 2011). Our results showed that the frequency of CF loss in *ecd3Δ* and *dhh1Δ* strains is statistically similar to that of a wild-type strain (Figure 5D). The *pat1Δ* strain exhibited a CF loss frequency about 20-fold higher than the wild-type strain (Figure 5D). Together, these data show that the role of *PAT1* in chromosome segregation is independent of its function in P-body assembly and translation repression.

The CTF region mediates association of Pat1 with centromeres and is required for sister chromatid separation

To determine if the CTF region is necessary for the *CEN* association of *Pat1*, ChIP experiments were performed using strain expressing *PAT1* or *pat1* alleles ($\Delta254–422$, $\Delta422–697$) (Figure 5A). Full-length *Pat1* and *Pat1-Δ422–697* were shown to associate with *CEN* DNA (Figure 6A). Consistent with the increased CF loss (Figure 5C), a deletion of the CTF region in *pat1-Δ254–422* results in a failure of *Pat1* to associate with *CEN* DNA (Figure 6A). In a control experiment, no significant ChIP-PCR signal was detected at *CEN* using an untagged strain (Figure 6A). These results show that the CTF region of *Pat1* mediates its *CEN* association and regulates chromosome segregation.

We next examined whether a deletion of the CTF region of *Pat1* is sufficient for the defects in sister chromatid separation observed in *pat1Δ* strains. Similar to the results of a *pat1Δ* strain (vector), a deletion of the CTF region (*pat1-Δ254–422*) showed defects in sister chromatid separation compared to wild-type or *pat1Δ* strain with *pat1-Δ422–697* or full-length *PAT1* (Figure 6B). As expected, only 2–7% of cells had separated sister chromatids in G1 (Figure 6B).

Figure 5 Role of Pat1 in chromosome segregation is independent of its function in P-body assembly and translation repression. (A) Schematic of full-length *PAT1* and its alleles. *pat1Δ* strain transformed with *PAT1* or its mutant alleles as described in Table 2 were used for experiments shown in B and C. Results from chromosome loss (C) are summarized in the table. (#) Results of P-body assembly and translation repression for these alleles were derived from a previous study (Pilkington and Parker 2008). (B) Western blot analysis with α -Flag antibodies showing expression of Pat1-Flag and its alleles (Pat1-Δ254-422-Flag, Pat1-Δ422-697-Flag). (C) The CTF region of Pat1 (amino acid residues 254-422) regulates chromosome segregation. Wild-type (WT, YPH1015) and *pat1Δ* strain (YMB8345) transformed with vector, *PAT1* and its alleles were assayed for chromosome-loss phenotype as described in *Materials and Methods*. Values sharing the same letter are not significantly different at a 5% level based on analysis of variance (P -value >0.05) (D) Deletion of P-body encoding genes *EDC3* and *DHH1* does not exhibit an increased chromosome-loss phenotype. Wild-type (WT, YPH1015), *edc3Δ* (YMB8678), *dhh1Δ* (YMB8680), and *pat1Δ* (YMB8345) strains were assayed for the chromosome-loss phenotype as described in *Materials and Methods*. Average from three independent transformants $\pm$ SE is shown. (**) P -value <0.01 , Student's t -test.

To determine if the CTF region of *Pat1* is evolutionarily conserved, we compared *Pat1* protein sequences from budding yeast, other fungal species, and humans using BLAST alignment program (<http://www.ncbi.nlm.nih.gov>). Sequence alignment revealed a unique 30-amino acid sequence motif within the CTF region of *Pat1*, which is highly conserved (40% identity, 73% similarity) between budding yeast, other fungi, and humans (Figure 6, C and D) despite the differences in the centromere structure in these species (Mishra *et al.* 2007; Ketel *et al.* 2009; Verdaasdonk and Bloom 2011; Burrack and Berman 2012; Choy *et al.* 2012).

Discussion

The structural dissection of the yeast kinetochore has been an area of active research since the discovery of centromeres in 1980 (Clarke and Carbon 1980), and about 70 proteins have been identified so far (Cho *et al.* 2010). In this study, we have shown that the evolutionarily conserved protein *Pat1* is a component of the budding yeast kinetochore and is required for the structural integrity of *CEN* chromatin. These conclusions are derived from data which demonstrate that *Pat1* (a) associates with centromeres in a *ND10*-dependent manner, (b) affects the structural integrity of *CEN* chromatin, (c) modulates the superhelicity of minichromosomes, and (d) mediates chromosome segregation and sister chromatid separation.

The association of *Pat1* with centromeres suggests that *Pat1* is a structural component of *CEN* chromatin. Specificity of association is derived from experiments in which we observed that *CEN* enrichment of *Pat1* is reduced significantly in the kinetochore mutant *ndc10-1* at the nonpermissive temperature (37°). Similar results for a requirement of *Ndc10* have been reported for *CEN* association of other kinetochore proteins (Janke *et al.* 2001; Crotti and Basrai 2004; Hajra *et al.* 2006; Camahort *et al.* 2007). *Ndc10* is a prerequisite for kinetochore formation in budding yeast and is essential for the proper assembly of *CEN* chromatin (Goh and Kilmartin 1993; Camahort *et al.* 2007). The depletion of *CEN*-bound *Pat1* in an *ndc10-1* mutant further suggests that a functional kinetochore is required for *CEN* association of *Pat1*.

CEN chromatin is composed of a highly ordered and topologically unique structure in chromosomes (Bloom and Carbon 1982; Saunders *et al.* 1990). Our data reveal that *Pat1* is critical for the structural integrity of *CEN* chromatin. Increased *DraI* accessibility of *CEN* chromatin to the levels observed for *pat1Δ* strains has been used to define a structural role for *Cse4*, *Chl4*, *Spt4*, and core histones (H2A, H2B, and H4) in kinetochore function (Saunders *et al.* 1990; Meluh *et al.* 1998; Pinto and Winston 2000; Myhre and Bloom 2003; Crotti and Basrai 2004; Yu *et al.* 2011). Consistent with a role for *Pat1* in *CEN* chromatin structure, the *pat1Δ* strains exhibit phenotypes characteristic of kinetochore mutants such as defects in sister chromatid separation and chromosome-loss phenotypes (Strunnikov *et al.* 1995; Meluh *et al.* 1998; Ortiz *et al.* 1999; Ma *et al.* 2012). The chromosome-loss phenotype in *pat1Δ* strain is further supported by a previous study in which a partial deletion of *PAT1* coding region showed errors in chromosome transmission fidelity (Wang *et al.* 1996).

The altered superhelicity of minichromosomes in a *pat1Δ* strain suggests that *Pat1* regulates the topological state of minichromosomes. Although *Pat1* physically interacts with *Top2* (Wang *et al.* 1996), the defects in minichromosome topology in a *pat1Δ* strain are not likely due to the altered levels of centromere-associated *Top2*. Unlike *pat1Δ* strains, catalytically inactive *top2-4* mutants exhibit no defects in superhelicity

Figure 6 The CTF region of Pat1 (amino acid residues 254–422) is required for the *CEN* association and sister chromatid separation. (A) The CTF region mediates the *CEN* association of Pat1. ChIP experiments showing association of Pat1–Flag or its alleles to *CEN1*. Lanes: IN (input), IP (DNA from ChIP using α -Flag antibodies), and M (DNA from ChIP using α -GST antibodies). Untagged strain was used as a control. (B) Deletion of the CTF region of Pat1 contributes to delay in sister chromatid separation. Wild-type (WT, JBY577) with vector (pRS416), and *pat1Δ* (YMB8421) with vector (pRS416), or *pat1* alleles: *pat1Δ*–254–422 (pRP1491), *pat1Δ*–422–697 (pRP1492), and full-length *PAT1* (pRP1490) marked with LacI–GFP (*CEN4*–proximal) were grown in 1× SC–URA glucose (2%) at 30°, synchronized in G1 with α -factor, and released into pheromone-free medium. Samples were taken at time points (min) after release from G1. Readback α -factor 80 min after release to block the cells in next G1. Cells were fixed and were examined for cell morphology (top) and sister chromatid separation as indicated schematically (bottom). At least 100 cells were examined for every time point. Average $\pm$ SE derived from three independent biological replicates is shown. (C) The CTF region of Pat1 contains an evolutionarily conserved sequence motif. The protein sequences of Pat1 CTF region from different fungal species: *S. cerevisiae* (*S. cer*), *S. kudriavzevii* (*S. kud*), *S. arboricola* (*S. arb*), *Zygosaccharomyces rouxii* (*Z. rou*), *Ashbya gossypii* (*A. gos*), *Kluyveromyces lactis* (*K. lac*), *Candida glabrata* (*C. gla*), *C. albicans* (*C. alb*), *C. dubliniensis* (*C. dub*), and human were analyzed using the motif-based sequence analysis software MEME (<http://meme.nbcr.net>). Sequence alignment of the evolutionarily conserved motif is shown. Identical amino acid residues are shown with blue shading, and similar amino acid residues are marked with an asterisk. (D) The consensus sequence of the evolutionarily conserved motif is shown in the Logo format drawn with WebLogo software (Crooks *et al.* 2004).

of minichromosomes such that no differences in the mobility of topoisomers were observed between *top2-4* and wild-type strains (Warsi *et al.* 2008). In addition, no alteration in *CEN* superhelicity was observed in topoisomerase mutants (*top1*, *top2*, and *top3* mutants) in budding yeast (Saunders *et al.* 1990). Although it is unclear how Pat1 affects the topological state of minichromosomes, data presented in this study begin to provide a functional link between topological structure and chromosome segregation. Chromosome topology is important for kinetochore function because *pat1Δ* strains, which show altered supercoiling of minichromosome exhibit defects in chromosome segregation. In a recent study, addition of a kinetochore protein *Scm3* into *in vitro* supercoiling assays containing

CEN nucleosomes resulted in some changes in the superhelicity of minichromosomes (Shivaraju *et al.* 2012). Despite these studies, the molecular mechanisms by which a kinetochore protein affects chromosome topology are yet to be determined.

The kinetochore function of Pat1 is separable from its previously described cytoplasmic role in P-body assembly and translational repression (Pilkington and Parker 2008; Swisher and Parker 2011). This conclusion is based on three independent experimental observations. First, deletion of the CTF regions causes a chromosome loss phenotype, but exhibits no observable defects in P-body assembly and translation repression (Pilkington and Parker 2008). Second, the

lack of a chromosome-loss phenotype with a deletion of the *PAT1* region containing codons 422–697 that mediates P-body assembly and translation repression (Pilkington and Parker 2008). Third, strains deleted for P-body assembly and decapping components (*edc3Δ* and *dhh1Δ*) do not exhibit chromosome segregation defects.

Structure–function analysis uncovered a distinct central CTF region (amino acid residues 254–422) of *Pat1* for chromosome segregation. A deletion of the CTF region abolishes association of *Pat1* with centromeres, leads to chromosome-loss phenotype, and defects in sister chromatid separation. These results could mean that the CTF region directly mediates *CEN* binding of *Pat1*. Alternatively, deletion of CTF region may likely alter the protein structure of *Pat1* in a way that prevents its *CEN* association. Our analysis showed that the CTF region of *Pat1* is highly conserved and contains a sequence motif that is found in budding yeast, other fungal species, and humans. We interpret these observations to imply that the role of *Pat1* in kinetochore function may be evolutionarily conserved. Although the role of the human ortholog of *Pat1* (*Pat1b*) in kinetochore function has not been defined, nuclear localization of *Pat1b* has been observed in humans (Marnef *et al.* 2012). Furthermore, mutations and misregulation of *Pat1b* have been observed in several human cancers (Araujo *et al.* 2012; Liu *et al.* 2012; Dulak *et al.* 2013). Hence, future studies aimed at understanding the role of the human homolog of *Pat1* in the structure and function of the kinetochore may help us identify potential therapeutic targets for cancer therapy.

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Supporting Information

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Structural Integrity of Centromeric Chromatin and Faithful Chromosome Segregation Requires Pat1

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Figure S1 Epitope tagging of Pat1 or Scm3 does not affect the function of these proteins. Serial dilutions (5-fold) of untagged (JG595) and tagged (Pat1-3HA and Scm3-3Flag) (YMB7963) strains were plated on YPD plates and grown at 25°C and 37°C.

Figure S2 Control ChIP-qPCR data using an untagged strain. ChIP experiments were performed with an untagged strain (JG595) using α -HA antibodies as described in Figure 1. Enrichment to the region surrounding *CEN3* was determined by qPCR and is shown as % input. Average from three independent biological replicates $\pm$ standard error is shown. The non-*CEN* locus *HML* was used as a control.

Figure S3 Defects in CEN chromatin structure and minichromosome supercoiling in *pat1Δ* strains are not a consequence of biased cell cycle distribution. FACS was used to analyze the DNA content. Cell cycle stages were determined based on cell morphology and nuclear position by microscopic examination of 100 cells for each strain as described in the Materials and Methods. (A) Increased *DraI* sensitivity in *pat1Δ* strains is not due to the differences in the cell cycle distribution. FACS profile and the distribution of cell cycle stages for wild-type (WT) and *pat1Δ* strains used in Figure 2B and C. (B) Altered topological structure of minichromosomes in *pat1Δ* strains is not due to biased cell cycle distribution. FACS profile and the distribution of cell cycle stages for wild-type (WT) and *pat1Δ* strains used in Figure 4A.

Figure S4 Expression of *pat1* alleles in a wild type strain does not exhibit increased chromosome loss phenotype. Wild type strain (YPH1015) with chromosome fragment transformed with vector (pRS416), *PAT1* (pRP1490) and its alleles (pRP1491, and pRP1492) were assayed for chromosome loss phenotype. Values sharing the same letter are not significantly different at a 5% level based on analysis of variance (p -value = >0.05).