Global analysis of core histones reveals nucleosomal surfaces required for chromosome bi-orientation

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The attachment of sister kinetochores to microtubules from opposite spindle poles is essential for faithful chromosome segregation. Kinetochore assembly requires centromere-specific nucleosomes containing the histone H3 variant CenH3. However, the functional roles of the canonical histones (H2A, H2B, H3, and H4) in chromosome segregation remain elusive. Using a library of histone point mutants in *Saccharomyces cerevisiae*, 24 histone residues that conferred sensitivity to the microtubule-depolymerizing drugs thiabendazole (TBZ) and benomyl were identified. Twenty-three of these mutations were clustered at three spatially separated nucleosomal regions designated TBS-I, -II, and -III (TBZ/benomyl-sensitive regions I–III). Elevation of mono-polar attachment induced by prior nocodazole treatment was observed in H2A-I112A (TBS-I), H2A-E57A (TBS-II), and H4-L97A (TBS-III) cells. Severe impairment of the centromere localization of Sgo1, a key modulator of chromosome bi-orientation, occurred in H2A-I112A and H2A-E57A cells. In addition, the pericentromeric localization of Htz1, the histone H2A variant, was impaired in H4-L97A cells. These results suggest that the spatially separated nucleosomal regions, TBS-I and -II, are necessary for Sgo1-mediated chromosome bi-orientation and that TBS-III is required for Htz1 function.

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Introduction

Eukaryotic chromosomes consist of repeating structural units known as nucleosomes (Kornberg, 1974). The structure of the nucleosome is comprised of a canonical histone octamer containing two histone H2A–H2B dimers and a histone (H3–H4)₂ tetramer, which together are surrounded by ~146 base pairs of DNA (Luger *et al*, 1997). CenH3 and H2A.Z are evolutionarily conserved histone variants with specific cellular localization patterns. Centromere-specific nucleosomes containing CenH3 (Cse4 in budding yeast; CENP-A in humans) serve as the sites for kinetochore assembly (Black and Bassett, 2008; Torras-Llort *et al*, 2009). Nucleosomes containing H2A.Z (Htz1 in budding yeast) are enriched in the promoter regions of most genes as well as in the pericentromeric regions of chromosomes (Krogan *et al*, 2004; Albert *et al*, 2007; Greaves *et al*, 2007), suggesting that H2A.Z contributes to chromosome segregation.

There is much debate over the composition of centromere-specific nucleosomes. A recent report indicated that the centromere-specific octameric nucleosomes are composed of Cse4 and histones H2A, H2B, and H4 (Camahort *et al*, 2009). Other studies showed that centromere-specific nucleosomes contain Cse4, histone H4, and the non-histone protein Scm3, but not histone H2A or H2B (Mizuguchi *et al*, 2007). However, it is unclear whether Scm3 is a component of centromere-specific nucleosomes (Camahort *et al*, 2009; Pidoux *et al*, 2009; Shivaraju *et al*, 2011). Controversy also exists over the three-dimensional structure of centromere-specific nucleosomes. A recent report showed that CenH3-containing nucleosomes were suggested to induce positive DNA supercoils and wrap DNA in a right-handed manner, in contrast to canonical nucleosomes, which wrap DNA in a left-handed manner, with negative supercoiling (Furuyama and Henikoff, 2009). However, a recent study of the structure of (CENP-A–H4)₂ showed no evidence for positive DNA supercoils (Sekulic *et al*, 2010). Therefore, a variety of further experimental approaches are needed to establish the three-dimensional state of the centromere-specific nucleosomes.

In addition to the Cse4-containing centromere-specific nucleosomes, several studies have explored the roles of the canonical histones in chromosome segregation. The temperature sensitivity of a histone H4 double substitution mutant defective in mitotic chromosome transmission (H4-T82I/A89V) was reversed by Cse4 overexpression (Smith *et al*,

1996). Two single substitution mutations in histone H2A (H2A-S19F or H2A-G29D) caused an increase in ploidy (Pinto and Winston, 2000). Histone H2B was also implicated in centromere-kinetochore function (Maruyama *et al*, 2006). Furthermore, covalently modified core histones, together with the linker histone H1, may have an important role in converting nucleosomes into a highly compact state during chromosome segregation (Trojer and Reinberg, 2007; Xu *et al*, 2009). Thus, canonical histones could be involved in a variety of mitotic functions.

For faithful chromosome segregation, each of the sister kinetochores must attach to microtubules that extend from opposite spindle poles, a process called chromosome bi-orientation (also known as amphitelic attachment or bi-polar attachment) (Tanaka *et al*, 2005; Walczak *et al*, 2010). Chromosome bi-orientation results from the tension between sister kinetochores (Dewar *et al*, 2004) and is assured by the chromosomal passenger complex (CPC), which consists of Ipl1/Aurora B kinase, Slh1/INCENP, Bir1/Survivin, and Nbl1/Borealin (Biggins and Murray, 2001; Tanaka *et al*, 2002; Sandall *et al*, 2006; Ruchaud *et al*, 2007; Nakajima *et al*, 2009). The CPC destabilizes kinetochore-microtubule attachment in the absence of tension between sister kinetochores (Tanaka *et al*, 2002). Consequently, tensionless syntelic attachment (mono-orientation), in which both sister kinetochores attach to microtubules emanating from the same spindle pole, is corrected to bi-polar attachment. The localization of the CPC to the kinetochore is partially regulated by the interaction between Bir1 and shugoshin (Sgo1 in budding yeast; Sgo2 in fission yeast) (Kawashima *et al*, 2007). Consistent with this finding, in budding yeast, shugoshin (Sgo1) is necessary for the proper establishment of chromosome bi-orientation (Indjeian *et al*, 2005). In fission yeast, shugoshin (Sgo2) is recruited onto centromeres *via* phosphorylation of histone H2A at serine 121 by Bub1 kinase (Kawashima *et al*, 2010). However, it is not clear whether a single post-translational modification is necessary and sufficient to trigger chromosome bi-orientation (Hayashi *et al*, 2009). Indeed, phosphorylation of histone H3 at threonine 3 by the Haspin kinase recruits the CPC subunit Survivin to centromeres in human cells (Kelly *et al*, 2010; Wang *et al*, 2010; Yamagishi *et al*, 2010).

Although canonical histones are involved in a variety of mitotic functions, comprehensive analyses to understand their functional roles in chromosome segregation have not been performed. Thus, a global library of histone point mutants (the histone-GLibrary) (Matsubara *et al*, 2007; Sakamoto *et al*, 2009; Sato *et al*, 2010) was used to identify the amino-acid residues on the surfaces of canonical histones that are required for faithful chromosome segregation. The mitotic function of canonical histones was dissected using several representative histone point mutants that were identified by their sensitivity to microtubule-depolymerizing drugs. These mutants were used to analyse the roles of canonical histone residues in chromosome bi-orientation.

Results

Identification of histone residues that conferred sensitivity to thiabendazole and benomyl

Most chromosomal instability mutants in budding yeast show sensitivity to microtubule-depolymerizing drugs (Stearns

et al, 1990). To identify canonical histone residues required for faithful chromosome segregation, mutants from the histone-GLibrary (Matsubara *et al*, 2007; Sakamoto *et al*, 2009) were assessed for their sensitivity to the microtubule-depolymerizing drugs thiabendazole (TBZ) and benomyl. Of 423 viable mutants, 24 histone point mutants (H2A, 11; H2B, 2; H3, 8; H4, 3) were sensitive to both TBZ and benomyl (Figure 1A; Supplementary Figure S1). Interestingly, most of the mutations which were found to confer TBZ/benomyl sensitivity occurred within histones H2A and H3, for which histone variants have been identified (Htz1 and Cse4, respectively). In contrast, fewer TBZ/benomyl-sensitive strains were identified carrying mutations in histones H2B and H4, which have no variants in budding yeast.

The spatial positions of histone residues conferring TBZ/benomyl sensitivity were visualized using the yeast nucleosome core (White *et al*, 2001; Figure 1B–D). With the exception of H3-E97, these residues could be classified into three groups, TBZ/benomyl-sensitive regions (TBS)-I, -II, and -III, based on their spatial location within the nucleosome (Figure 1B–D; Table 1). TBS-I contains the nucleosome entry site, TBS-II contains the acidic patch, and TBS-III contains the interacting region between the histone H4 C-terminal tail and histone H2B $\alpha 2$ (Luger *et al*, 1997; White *et al*, 2001).

The nucleosome entry site (TBS-I) is further structurally divided into two sites: the nucleosomal DNA entry–exit site, which contains the N-terminal structured–unstructured border region of histone H3; and the docking site for histones H2A and H3, which contains the histone H2A C-terminal tail and the histone H3 αN region (Figure 1B–D). Based on their spatial positions, these two sites in TBS-I may be critical for facilitating structural changes within the nucleosome. Although histones are basic proteins, the acidic patch (TBS-II) contains a high concentration of acidic residues (Luger *et al*, 1997; Figure 1B) and may serve as a target site for chromatin-acting factor(s) (Barbera *et al*, 2006; Makde *et al*, 2010). The interacting region between the histone H4 C-terminal tail and histone H2B $\alpha 2$ (TBS-III) is adjacent to either a short β -strand of histone H2A or its variant Htz1 (Suto *et al*, 2000; White *et al*, 2001; Figure 1C; Supplementary Figure S2). Htz1 overexpression restored temperature sensitivity in H4-Y98H (*hhf1-39*) cells (Santisteban *et al*, 2000), suggesting that the TBS-III residues are likely to be important for the incorporation of Htz1.

Chromosomal instability in histone H2A C-terminal point mutants was not caused by a defect in the spindle assembly checkpoint

We first examined the mitotic phenotypes of the TBS-I mutations within the histone H2A C-terminal tail that resulted in TBZ/benomyl sensitivity (Figure 2A–C). The histone H2A C-terminal tail (aa 80–119 in *Xenopus laevis*, aa 81–120 in budding yeast; Luger *et al*, 1997) interacts with histones H3 and H4 and is essential for nucleosome formation. TBZ/benomyl-sensitive histone H2A C-terminal residues (H2A-I112, -H113, -N115, -L116, and -L117) interact with the histone H3 αN and $\alpha 2$ regions (White *et al*, 2001; Figure 2B and C, lower panels).

Since H2A-H113 and -N115 in budding yeast are not well conserved in higher eukaryotes (Figure 2D), these two residues were not further analysed. The evolutiona-

Figure 1 A genetic screen for TBZ- and benomyl-sensitive mutants with the histone-GLibrary (Matsubara *et al*, 2007; Sakamoto *et al*, 2009). (A) Sensitivity to microtubule-depolymerizing agents was determined by dropping three-fold serial dilutions of histone point mutants on the FY406 or MSY748 background onto SC agar plates containing 25 μ g/ml thiabendazole (TBZ) or 10 μ g/ml benomyl diluted in 0.125% dimethyl sulphoxide (DMSO). The plates were incubated at 30 °C for 3 days. (B) The position of histone residues that conferred TBZ/benomyl sensitivity on the electrostatic surface of the nucleosome core (PDB ID: 1ID3; White *et al*, 2001) looking down from the DNA superhelix axis. The positions of each residue, which is visible or not directly visible, are indicated by solid and dashed lines, respectively. Classification of TBS-I, -II, and -III residues is listed in Table I. (C, D) Side (C) and top (D) view of the same structure shown in (B).

Table I Classification of histone residues conferring TBZ and benomyl sensitivity

Group	Histone	Residues conferring TBZ and benomyl sensitivity
TBS-I	H2A	I112 H113 N115 L116 L117 S121
	H3	Y41 K42 G44 T45 R49 R52 I112
TBS-II	H2A	E57 L66 L86 E93 L94
	H2B	E116
TBS-III	H2B	D71
	H4	L97 Y98 G99

With the exception of H3-E97, histone residues conferring TBZ and benomyl sensitivity can be structurally classified into three groups.

Highly conserved hydrophobic residues of the histone H2A C-terminal tail (H2A-I112, -L116, and -L117) and a potentially phosphorylated residue (H2A-S121) were subjected to further analysis. H2A-S121A was identified as a TBZ/benomyl-sensitive mutation, which is consistent with the recent finding that Bub1-dependent phosphorylation at H2A-S121 is important for Sgo1 recruitment (Kawashima *et al*, 2010).

To define mitotic phenotypes in these histone H2A point mutants, cell-cycle progression was assessed after treatment

with the microtubule-depolymerizing agent nocodazole. Analysis by flow cytometry revealed a slight accumulation of the asynchronous histone H2A C-terminal point mutants (H2A-I112A, -L116A, -L117A, and -S121A) at the G2/M phase, as compared with H2A wild-type (WT) cells (Figure 2E). After release from nocodazole treatment, the H2A-I112A, -L116A, -L117A, and -S121A mutant cells each exhibited further defects in cell-cycle progression (Figure 2E). Therefore, the functionality of the spindle assembly checkpoint in the histone H2A point mutants was assessed using residues H2A-I112 and -L117 as representative histone H2A C-terminal conserved residues (Figure 2D).

In the presence of nocodazole, a spindle checkpoint mutant, *mad2* mutant cells escaped mitotic arrest, as previously reported (Li and Murray, 1991), while both H2A-I112A and -L117A cells remained in the G2/M phase (Figure 2F), suggesting that the spindle assembly checkpoint in both H2A-I112A and -L117A cells was functional. Pds1/securin was also evaluated in the histone point mutant cells by immunoblotting (Figure 2G and H), since Pds1/securin inhibits cell-cycle progression by binding to the separin Esp1; when the spindle

Figure 2 Histone H2A point mutants show chromosomal instability when released from mitotic arrest with nocodazole. (A) Nucleosome structure. Histones H2A, H2B, H3, H4, and DNA are yellow, red, blue, green, and white, respectively. (B, C) Enlarged views of the focused histone H2A C-terminal tail region (B) and the histone H3 residues located in proximity to H2A-I112 (C). Upper and lower panels represent the surface and cartoon models generated using the Pymol software application (see Materials and methods). (D) Multiple alignments of various histone H2A C-terminal sequences across species. Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Dm, *Drosophila melanogaster*; Xl, *Xenopus laevis*; Mm, *Mus musculus*; Hs, *Homo sapiens*. Red circles indicate the histone residues conferring TBZ/benomyl sensitivity. (E) Histone H2A point mutants in the C-terminal region show perturbation of cell-cycle progression when released from mitotic arrest with nocodazole (Noc). Samples were taken at the times indicated and analysed by flow cytometry as described in Materials and methods. Asyn, asynchronous cells. (F) The spindle assembly checkpoint in H2A-I112A and -L117A cells is functional. A deletion mutant of Mad2, a spindle assembly checkpoint protein, was used as a positive control. Samples were harvested at the indicated times and analysed by flow cytometry as described in Materials and methods. α -Factor was used to arrest cells in the G1 phase. (G) Experimental scheme for figure (H). Cells were arrested in G1 phase by the addition of α -factor for 3 h, and released from G1 arrest in the presence of nocodazole. (H) According to the scheme shown in (G), cells were lysed at the indicated times after release from G1 arrest and analysed by immunoblotting for Pds1-3HA and histone H3 (control).

assembly checkpoint is satisfied, Pds1 is degraded, liberating Esp1, and the cell progresses into anaphase (Ciosk *et al*, 1998). In the presence of nocodazole, Pds1/securin was

retained in H2A-I112A and -L117A cells to the same extent as in H2A-WT cells, but Pds1/securin was not detected in nocodazole-treated *mad2* cells (Figure 2G and H). These

results suggest that chromosomal instability in histone H2A C-terminal point mutants is not caused by a defect in the spindle assembly checkpoint.

Histone H2A has a role in the establishment of chromosome bi-orientation

Among the histone H2A C-terminal residues conferring TBZ/benomyl sensitivity, H2A-I112 interacts with the largest number of histone H3 residues (L48, I51, and R52; see Supplementary Table S2 in Sakamoto *et al*, 2009) (Figure 2C). Each mutation of H3-L48 or -I51 conferred lethality (Matsubara *et al*, 2007; Dai *et al*, 2008; Nakanishi *et al*, 2008; Sakamoto *et al*, 2009), and the H3-R52A mutation showed sensitivity to TBZ and benomyl (Figure 1A), suggesting that H2A-I112 and its interacting histone residues in TBS-I are critical for faithful chromosome segregation.

To observe chromosome segregation in cells with histone point mutants, one centromere (*CEN5*) and one microtubule protein (Tub1) were labelled with GFP and CFP, respectively (Figure 3A). Continuous stretching of sister centromeres, a process known as ‘centromere breathing’, has been reported in cells after metaphase arrest induced by depletion of Cdc20, a mitotic anaphase-promoting complex/cyclosome (APC/C) activator (Tanaka *et al*, 2000). Since H2A-I112A cells exhibited nearly normal cell-cycle progression (see ‘Asyn’ in Figure 2E), it was postulated that the treatment of histone point mutants with a microtubule-depolymerizing drug would enhance chromosomal instability. To test this hypothesis, the rate of chromosome missegregation was investigated in both H2A-WT and -I112A cells released from mitotic arrest induced by Cdc20 depletion, with or without prior nocodazole treatment (Figure 3B and C). H2A-I112A cells with prior nocodazole treatment (+Noc) showed an increased rate of chromosome missegregation compared with the same cells without prior treatment (NT; Figure 3D). The stimulatory effect induced by nocodazole was confirmed using flow cytometry (Supplementary Figure S3). It was concluded that the microtubule-depolymerizing drug enhanced chromosomal instability in H2A-I112A cells.

Frequent chromosome missegregation in H2A-I112A cells suggests that the establishment of chromosome bi-orientation is defective in the mutant cells. Therefore, the establishment of chromosome bi-orientation was examined in H2A-I112A cells treated with or without nocodazole for 2 h, released from nocodazole, and then cultured for 2 h with Cdc20 depletion to reassemble spindles (see Figure 3E). H2A-I112A cells with prior nocodazole treatment (+Noc) showed an increased rate of mono-polar attachment compared with untreated cells (NT; Figure 3F and G). This observation suggests that histone H2A is required for chromosome bi-orientation.

Sgo1 function was impaired in H2A-I112A cells

It was suspected that the failure of chromosome bi-orientation in H2A-I112A cells was due to improper assembly of kinetochore or centromere proteins. The effect of the H2A-I112A point mutation on the centromeric localization of key modulators of chromosome bi-orientation, such as Sgo1, Bub1, and the CPC, was analysed using chromatin immunoprecipitation (ChIP). ChIP revealed that the CPC subunits (Ipl1 and Bir1) localized to the centromere of chromosome 3 (*CEN3*) less efficiently in H2A-I112A cells than in H2A-WT

cells (Figure 4A and B). A drastic reduction of Sgo1 localization to the centromere was observed in H2A-I112A cells, as compared with H2A-WT cells (Figure 4C). Unlike Sgo1, centromere localization of Bub1 kinase, a regulator of Sgo1 (Kawashima *et al*, 2010), was detectable in H2A-I112A cells (Figure 4D). These observations suggest that the H2A-I112A point mutation causes mono-polar attachment by inducing the mislocalization of proteins such as Sgo1 and the CPC.

Since chromatin structure is critical in the regulation of gene expression (reviewed in Li *et al*, 2007), aberrant transcriptional regulation of Sgo1 and the CPC in H2A-I112A cells may indirectly lead to reduced localization of these proteins to the centromere (Figure 4A–C). However, the mRNA levels of Sgo1 and the CPC subunits (Ipl1, Sli15, and Bir1) were not reduced in H2A-I112A cells compared with H2A-WT cells (Figure 4E). Therefore, it is unlikely that the reduced centromere localization of Sgo1 and the CPC is due to aberrant transcriptional regulation. On the other hand, the protein level of Sgo1, but not of the CPC subunits (Ipl1, Sli15, and Bir1), was unexpectedly lower in H2A-I112A cells than in H2A-WT cells (Figure 4F). The low overall levels of Sgo1 protein in H2A-I112A cells (Figure 4F) may explain the lower levels of the protein observed at the centromere (Figure 4C).

The localization of kinetochore proteins in addition to Sgo1, Bub1, and the CPC was also examined in H2A-I112A cells. To distribute sister chromatids to daughter cells, a variety of kinetochore protein complexes must be assembled at each sister centromere (Santaguida and Musacchio, 2009). The localization of the centromere-specific histone H3 variant Cse4, the Cse4-associated factor Scm3 (Mizuguchi *et al*, 2007), and Mif2 and Ctf3 (representative inner and outer kinetochore components, respectively) was not affected in H2A-I112A cells (Figure 4G–J), even when temperature-sensitive H2A-I112A cells (Supplementary Figure S4) were cultured at 37 °C. Furthermore, the H2A-I112A point mutation did not affect the localization of Sccl1, an essential subunit of the cohesin complex, at 37 °C (Figure 4K). Collectively, these observations suggest that the H2A-I112A point mutation specifically impairs the localization of Sgo1 and the CPC.

To understand functional relationships between H2A-I112 and Sgo1, the mitotic phenotypes of the H2A-I112A point mutant cells and *sgo1* deletion mutant cells were compared in the same genetic background. Like H2A-I112A cells, mitotic Ipl1 localization in *sgo1* cells was reduced to nearly half the level of that observed in WT cells (Figure 4L). Furthermore, prior nocodazole treatment induced missegregation and mono-polar attachment in *sgo1* cells (Figure 4M and N), as was found for H2A-I112A cells (Figure 3D and G) and shown in previous reports (Indjeian *et al*, 2005; Fernius and Hardwick, 2007). The similarity of the phenotypes of H2A-I112A and *sgo1* cells suggests that defective chromosome bi-orientation establishment in H2A-I112A cells is due to impaired Sgo1 function.

The establishment of chromosome bi-orientation was also examined in temperature-sensitive *ipl1-321* cells. High rates of mono-polar attachment were observed regardless of nocodazole treatment (Supplementary Figure S5), suggesting that the effect of prior nocodazole treatment is critical for mono-polar attachment in H2A-I112A cells but not in *ipl1-321* cells. The rate of mono-polar attachment was not increased further in *ipl1-321*/H2A-I112A double mutant cells, suggesting that the *ipl1-321* mutation is epistatic to the H2A-I112A mutation.

Figure 3 The H2A-I112A mutation causes mono-polar attachment, particularly after nocodazole treatment. **(A)** A schematic representation of the *tet* operator (*tetO*)/TetR-GFP system with kinetochore-microtubule bi-polar (left) and mono-polar (right) attachment in budding yeast. GFP-fused TetR binds *tetO* sequences integrated into the pericentromeric regions of chromosome 5 (*CEN5*-GFP). Centromere stretching in metaphase is observed when cells are arrested by depletion of Cdc20, a mitotic APC/C activator (Tanaka *et al*, 2000). **(B)** Time course for the observation of chromosome missegregation. Cells were released from the mitotic arrest caused by depletion of Cdc20 with (+ Noc) or without (NT, no treatment) additional nocodazole treatment for 2.5 h at 25 °C. Images of dividing cells were taken by time-lapse imaging. **(C)** Images shown are representative photographs merged with *CEN5*-GFP in cells in anaphase. The scale bar represents 1 μ m. **(D)** The rates of chromosome missegregation in H2A-WT and -I112A cells. Chromosome missegregation was evaluated by the behaviour of *CEN5*-GFP dots in dividing cells. The rates are represented as the mean $\pm$ standard deviation. **(E)** Time course for the observation of kinetochore-microtubule attachment in metaphase after nocodazole treatment. Cells were arrested by Cdc20 depletion with or without nocodazole (+ Noc or NT) for 2 h at 25 °C and released from the nocodazole block. After additional metaphase arrest by Cdc20 depletion for ~2 h, images of metaphase-arrested cells were taken by time-lapse imaging. **(F)** Images are representative photographs merged with *CEN5*-GFP and CFP-Tub1 in H2A-WT and -I112A cells in metaphase. The cyan signal of CFP-Tub1 has been converted to red. The scale bar represents 1 μ m. **(G)** The rate of mono-polar attachment in H2A-WT and -I112A cells. Kinetochore-microtubule attachment was evaluated by the movement of GFP-labelled *CEN5* in metaphase-arrested cells. The rates were represented as mean with standard deviation.

Collectively, these data suggest that the reduced centromere localization of the CPC, which is due in turn to impaired localization of Sgo1, is the plausible mechanism for defective chromosome bi-orientation in H2A-I112A cells.

Multi-copy SGO1 restored the TBZ and benomyl sensitivity caused by point mutations of histones H2A and H3 in TBS-I and TBS-II

These data demonstrate that the H2A-I112 residue and a potentially modifiable H2A-S121 residue on the H2A C-terminal region of TBS-I seem to regulate Sgo1 function (Figures 1–4). It was, therefore, important to determine whether Sgo1 activity is also dependent on other histone H2A or H3 in TBS-I. To elucidate the functional relationships between the canonical histones and Sgo1, the rescue of TBZ/benomyl sensitivity in the histone point mutants by the

overexpression of Sgo1 was examined (Figure 5A). The overexpression of Sgo1 partially suppressed TBZ/benomyl sensitivity of H2A-I112A cells (Figure 5B). Of Bub1 and the components of the CPC (Ipl1, Sli15, and Bir1), overexpression of Ipl1 or Sli15 slightly rescued the TBZ/benomyl sensitivity of H2A-I112A cells (Supplementary Figure S6). With regard to the other histone residues positioned near H2A-I112, multi-copy SGO1 also reversed the TBZ/benomyl sensitivity of H2A-L116A, -L117A, and -S121A cells (Figure 5B). Furthermore, a suppressor effect of multi-copy SGO1 was observed in H2A-I112A/L116A/L117A triple mutant cells (Figure 5B), suggesting that these hydrophobic residues in TBS-I contribute to the functional interaction between histone H2A and Sgo1.

The effect of Sgo1 overexpression on TBZ/benomyl sensitivity in histone H3 point mutants in TBS-I (Figures 1B–D

Figure 4 The kinetochores localization of the CPC and Sgo1 is impaired in H2A-I112A cells. (A–D) H2A-WT and -I112A cells containing Ipl1-3HA (A), Bir1-13Myc (B), Sgo1-3HA (C), or Bub1-3HA (D) were grown in YPAD medium containing 15 μ g/ml nocodazole for 3 h at 25 °C. Cells were fixed with 1% formaldehyde for 15 min and subjected to ChIP. Input DNA and DNA co-immunoprecipitated with the anti-Myc or anti-HA antibody (IP) were amplified with a primer set corresponding to *CEN3* nucleotide sequences. The data are the average of two independent experiments. Error bars indicate the standard deviation. Dashed lines indicate the background level of the ChIP signal in an untaged strain. (E) The CPC (*IPL1*, *SLI15*, and *BIR1*) and *SGO1* mRNA levels in nocodazole-treated H2A-WT and -I112A cells. The data are the average of two independent experiments. Error bars indicate the standard deviation. (F) The CPC (Ipl1, Sli15, and Bir1) and Sgo1 protein levels in nocodazole-treated H2A-WT and -I112A cells. (G–K) The localization of major kinetochores components was unchanged in H2A-I112A cells. The centromere-specific histone H3 variant, Cse4 (G); representative proteins of the inner kinetochores, Scm3 (H) and Mif2 (I); a representative protein of the outer kinetochores, Ctf3 (J); and a cohesin component, Scc1 (K), were analysed. Cells were subjected to ChIP as described in (A–D) with the exception that the cells were incubated in the presence of nocodazole for 1 h at 37 °C rather than at 25 °C. Since H2A-I112A cells are temperature sensitive (Supplementary Figure S6), the effect of the H2A-I112A point mutation is expected to be detectable at 37 °C by ChIP. (L) The kinetochores localization of Ipl1, a subunit of the CPC, was analysed in *sgo1* cells. ChIP analysis was performed using WT and *sgo1* cells containing Ipl1-3HA as described in (A–D). (M, N) The rates of missegregation (M) and mono-polar attachment (N) were increased in *sgo1* cells. Under the same experimental conditions described in Figure 3B and E, the rates of missegregation and mono-polar attachment in WT and *sgo1* cells were evaluated with or without prior treatment with nocodazole.

and 5A; Table I) was next examined. The TBZ/benomyl sensitivity of representative histone H3 point mutants (H3-Y41A, -T45A, and -R52A; Figure 5C) was partially restored by the overexpression of Sgo1. Thus, the entire TBS-I region, including histone H2A and H3 residues, may be partially responsible for Sgo1 function.

Based on these results, the suppression of TBZ/benomyl sensitivity by the overexpression of Sgo1 was examined in

cells with point mutations in other residues within TBS-II and -III. Several representative histone point mutants were selected from each group (Figures 1B–D and 5A; Table I), and the suppressor effect of Sgo1 was tested in these mutants. The TBZ/benomyl sensitivity of the TBS-II residue point mutants (H2A-E57A and -E93A; Figure 5D) was partially reversed by the overexpression of Sgo1. However, the TBZ/benomyl sensitivity of TBS-III residue mutants (H4-L97A,

Figure 5 Sgo1 is a multi-copy suppressor of histones H2A and H3 point mutations in TBS-I and -II. (A) Illustration of an experimental approach to investigate the functional interaction between Sgo1 and TBS-I, -II, and -III residues (Figure 1B–D; Table I). (B) The sensitivity of histone H2A point mutants in the C-terminal region to 25 µg/ml TBZ and 10 µg/ml benomyl was determined using three-fold serial dilutions of cells. Each strain was transformed with a YEplac195 multi-copy vector (*URA3* marker, 2 µ) containing the *SGO1* gene. The plates were incubated for 3 days at 25 °C. (C–E) The sensitivity of representative TBS-I (C), TBS-II (D), and TBS-III (E) mutants to TBZ and benomyl was determined using three-fold serial dilutions of cells as described in (B).

-Y98A, and -G99A) was not rescued by the overexpression of Sgo1 (Figure 5E). These observations suggest that two groups of residues, TBS-I and -II, are important for the function of Sgo1.

Phenotypic and functional characteristics of the H2A-E57A (TBS-II) and H4-L97A (TBS-III) mutations

In addition to H2A-I112 (a TBS-I residue), the roles of the residues within TBS-II and -III in chromosome bi-orientation were also characterized. H2A-E57A and H4-L97A cells were selected for further analyses as representative mutants at TBS-II and -III, respectively (Figure 1; Table I). Flow cytometry suggested that both H2A-E57A and H4-L97A cells exhibited perturbation of cell-cycle progression induced by prior nocodazole treatment (Figure 6A). In the presence of nocodazole, *mad2* cells escaped from mitotic arrest, while both H2A-E57A and H4-L97A cells remained in the G2/M phase (Figure 6B). Furthermore, like H2A-I112A cells (Figure 2G and H), Pds1/securin was retained in both H2A-E57A and H4-L97A cells in the presence of nocodazole (Figure 6C), suggesting that the spindle assembly checkpoint in both H2A-E57A and H4-L97A cells was functional. Both H2A-E57A and H4-L97A cells showed an increased rate of mono-polar attachment following the nocodazole treatment as compared with untreated cells (Figure 6D), demonstrating that core histone residues including H2A-I112 (TBS-I), -E57 (TBS-II), and H4-L97 (TBS-III) are involved in the establishment of chromosome bi-orientation.

As in H2A-I112A cells (Figure 4C and F), the protein level of Sgo1 was lower in H2A-E57A cells than in H2A-WT cells (Figure 6E), resulting in severe mislocalization of Sgo1 at the centromere (Figure 6F). In contrast to H2A-I112A and -E57A cells, only mild centromere mislocalization of Sgo1 was observed in H4-L97A cells (Figure 6F), even though the protein level of Sgo1 was lower in H4-L97A cells than in H4-WT cells (Figure 6E). A reason why the overexpression of Sgo1 did not rescue the TBZ/benomyl sensitivity of H4-L97A cells in contrast to the case of H2A-I112A and -E57A cells (Figure 5) may be explained by the presence of sufficient level of functional Sgo1 at the centromere in H4-L97A cells.

Pericentromeric Htz1, the histone H2A variant, was reduced in H4-L97A cells

Most amino-acid residues in TBS-I and -II (Figure 1; Table I) are exposed on the nucleosome surface (White *et al*, 2001; Sakamoto *et al*, 2009). However, three residues in TBS-III, H2B-D71, H4-L97, and H4-Y98 (Figure 1) lie within the nucleosome structure (White *et al*, 2001; Sakamoto *et al*, 2009). Histone H2B α2 (H2B-D71) and the C-terminal tail of histone H4 (H4-Y98) interact with each other, and both regions are located adjacent to a short β-strand of the C-terminal tail region of histone H2A or its variant Htz1 (Suto *et al*, 2000; White *et al*, 2001; Figure 1C; Supplementary Figure S2). Htz1 overexpression has been reported to restore the temperature sensitivity of H4-Y98H (*hhf1-39*) cells (Santisteban *et al*, 2000). Thus, these buried residues (H2B-D71 and H4-L97, H4-Y98) could be important for the incorporation of Htz1. As expected, the TBZ/benomyl sensitivity of H4-L97A (TBS-III) but not H2A-I112A (TBS-I) or -E57A (TBS-II) cells was partially restored by the overexpression of Htz1 (Figure 6G). Furthermore, Htz1-containing nucleosomes were severely reduced in the pericentromeric regions of *CEN3* in H4-L97A (TBS-III) cells but not in H2A-I112A (TBS-I) or -E57A (TBS-II) cells (Figure 6H). The results suggested that H4-L97 (TBS-III), but not H2A-I112 (TBS-I) or -E57 (TBS-II), was responsible for Htz1 function during chromosome segregation.

Figure 6 The mitotic phenotypes of H2A-E57A (TBS-II mutant) and H4-L97A (TBS-III mutant) cells. **(A)** H2A-E57A and H4-L97A cells show perturbation of cell-cycle progression when released from mitotic arrest with nocodazole (+Noc). Samples were harvested at the times indicated and analysed by flow cytometry as described in Materials and methods. **(B)** The spindle assembly checkpoint in H2A-E57A and H4-L97A cells is functional. A deletion mutant of Mad2, a spindle assembly checkpoint protein, was used as a positive control (see Figure 2F). Samples were taken at the times indicated and analysed by flow cytometry as described in Materials and methods. **(C)** Immunoblotting of Pds1-3HA. Cells were subjected to immunoblotting as described in Figure 2H. **(D)** The rate of mono-polar attachment in H2A-E57A and H4-L97A cells. Kinetochore-microtubule attachment was evaluated by the behaviour of GFP-labelled *CEN5* in metaphase-arrested cells. **(E)** The protein level of Sgo1 is reduced in nocodazole-treated H2A-E57A and H4-L97A cells. **(F)** The localization of Sgo1 is impaired in H2A-E57A cells and moderately impaired in H4-L97A cells. Cells were subjected to ChIP as described in Figure 4A–D. **(G)** Htz1 is a multi-copy suppressor of TBZ/benomyl sensitivity in H4-L97A cells but not in H2A-E57A or -I112A cells. The sensitivity of the indicated histone point mutants to 25 µg/ml TBZ and 10 µg/ml benomyl was determined using three-fold serial dilutions of cells. Each strain was transformed with a YEplac195 multi-copy vector (*URA3* marker, 2 µ) containing the *HTZ1* gene. The plates were incubated for 3 days at 25 °C. **(H)** The localization of Htz1 is severely impaired in H4-L97A cells, but not in H2A-I112A or -E57A cells. Cells were subjected to ChIP as described in Figure 4A–D. Input DNA and DNA co-immunoprecipitated with the anti-FLAG antibody (IP) were amplified with primer sets targeting the left or the right side of *CEN3* nucleotide sequences.

Figure 7 Possible functions of TBS-I–III in the establishment of chromosome bi-orientation. The possible actions of TBS-I–III with Sgo1 or Htz1 in chromosome bi-orientation (bi-polar attachment) are described in Discussion. The nucleosome may be ‘the shugoshin (guardian) of shugoshin: Shugomaster’ to prevent its degradation by currently unknown proteases. The APC/C proteosomal complex is one candidate (Salic *et al*, 2004; Karamysheva *et al*, 2009).

Discussion

This study used the histone-GLibrary (Matsubara *et al*, 2007; Sakamoto *et al*, 2009; Sato *et al*, 2010) to clarify the roles of functional residues within canonical histones with respect to chromosome segregation. This strategy proved to be effective for chromosome segregation studies and led to the identification of 24 novel TBZ/benomyl-sensitive histone point mutations located within three newly identified nucleosomal regions (TBS-I, -II, and -III) required for faithful chromosome segregation (Figure 1). Among the TBS residues identified in this study, one residue in TBS-I (H3-G44; Luo *et al*, 2010) and three residues in TBS-III (H4-L97, -Y98, and -G99; Yu *et al*, 2011) have been very recently reported to be involved in mitotic function, though histone-GLibraries (Dai *et al*, 2008; Nakanishi *et al*, 2008) were partially utilized. The complete screening shown in the present study identified many novel residues within these surfaces that are involved in mitotic function (twelve additional residues in TBS-I, six new residues in TBS-II, and one additional residue in TBS-III). Notably, mutation of the H3-T3 and -S10 residues, which are known to be phosphorylated and involved in mitotic function in several eukaryotic cells (Wei *et al*, 1999; Kelly *et al*, 2010; Wang *et al*, 2010; Yamagishi *et al*, 2010), did not show TBZ/benomyl sensitivity. This result is likely explained by the histone ‘modification web theory’ (Hayashi *et al*, 2009), which explains why single mutation on a modifiable histone residue does not show any phenotypes (Matsubara *et al*, 2007). Among the TBS residues identified in this study, representative mutations of H2A-I112 (TBS-I), -E57 (TBS-II), and H4-L97 (TBS-III) caused defects in the establishment of chromosome bi-orientation (Figures 3G and 6D). Kinetochores localization of Sgo1 was impaired in H2A-I112A (TBS-I) and -E57A (TBS-II) cells (Figures 4C and 6F). Furthermore, two nucleosomal regions (TBS-I and -II) were shown to be critical for the suppressor effect of multi-copy *SGO1* (Figure 5B–D). H4-L97 (TBS-III) may be involved in Htz1 function (Figure 6G and H). Thus, the comprehensive mutational analyses presented here suggest that restricted and concentrated regions of canonical histones play important roles in mitotic chromosome segregation through the actions of Sgo1 and/or Htz1, and non-identified other chromatin-acting factors (Figure 7).

The roles of TBS-I and -II on the function of Sgo1 are shown schematically in Figure 7. Cell-cycle progression of

H2A-I112A (TBS-I) and -E57A (TBS-II) cells was inhibited by prior nocodazole treatment (Figures 2E and 6A). Elevation of mono-polar attachment was also observed in H2A-I112A and -E57A cells in response to prior nocodazole treatment (Figures 3G and 6D). Intriguingly, a similar phenotype was observed in both *sgo1* deletion mutant cells and in *bub1* mutant cells lacking the protein kinase domain, as previously reported (Figure 4L–N; Indjeian *et al*, 2005; Fernius and Hardwick, 2007), suggesting that both Sgo1 and Bub1 serve a similar role in the establishment of chromosome bi-orientation during mitosis. Indeed, it has been shown that Sgo1 is recruited to the centromere *via* phosphorylation of the TBS-I residue H2A-S121 by Bub1 kinase (Kawashima *et al*, 2010). Furthermore, an additional TBS-I residue, H3-G44, was also recently reported to functionally interact with Sgo1 (Luo *et al*, 2010). Given that all nine residues in TBS-I and -II tested here functionally interacted with Sgo1 (Figure 5), the entire TBS-I and -II nucleosome surface regions may be required for proper Sgo1 function (Figure 7). Since the Bub1-mediated phosphorylation of H2A-S121 is critical for the recruitment of Sgo1 to the centromere (Kawashima *et al*, 2010), some residues of TBS-I and -II, including H2A-I112, are expected to be required for the phosphorylation of H2A-S121. Since Bub1 was detectable at the centromere in H2A-I112A cells (Figure 4D), it is possible that the H2A-I112 is required for the activation of the Bub1 protein kinase. Alternatively, functional ability of unknown protein(s) regulating Bub1 kinase activity might be supported by the action of H2A-I112. These possibilities are not mutually exclusive. Determination of the phosphorylation state of H2A-S121 in each of the TBS-I and -II mutant cells, including the H2A-I112A mutants, will enable us to further understand the molecular mechanisms behind the regulation of Sgo1 activity by TBS-I and -II.

The present study demonstrated that TBS-I residues in the C-terminal tail region of histone H2A (H2A-I112, -L116, -L117, and -S121) were each required for the functional ability of Sgo1 and/or protein(s) functionally associated with Sgo1 (Figure 5B). In general, hydrophobic residues tend to serve as protein–protein interaction motifs (Neduvu and Russell, 2006). Thus, it is hypothesized that the putative conserved IXXLL motif within histone H2A in TBS-I regulates the functional association of Sgo1, protein(s) functionally associated with Sgo1, and/or other molecule(s). Likewise, histone H3 residues (from Y41 to R52) in TBS-I (Figures 1 and 5C), including H3-G44 (Luo *et al*, 2010), may serve as an

additional interaction motif or motifs to associate with Sgo1 and/or associated protein(s). Similar to TBS-I, as an exposed nucleosomal surface (Luger *et al*, 1997), the acidic patch in TBS-II may also be an important site in regulating the functional ability of Sgo1 and/or Sgo1-associated protein(s) (Figure 5D).

Since shugoshin (Sgo2 in fission yeast) is involved in the loading of the CPC onto kinetochores (Kawashima *et al*, 2007; Yu and Koshland, 2007), mutation of H2A-I112 was expected to induce Sgo1 and, subsequently, CPC mislocalization. Indeed, Ipl1, a CPC subunit, was moderately reduced at the centromere in H2A-I112A and *sgo1* cells (Figure 4A and L). Since the overexpression of the CPC component Ipl1 or Sli15 slightly rescued the sensitivity of H2A-I112A cells to TBZ and benomyl (Supplementary Figure S6), CPC dysregulation may occur in H2A-I112A cells. However, CPC localization was not completely abolished in H2A-I112A or *sgo1* cells (Figure 4A, B and L). Thus, regulatory factors other than Sgo1 may contribute to the localization of the CPC to kinetochores. In this context, it is interesting that the CPC is recruited to kinetochores by means of a histone modification at the unstructured N-terminal tail of histone H3 (phosphorylation of H3-T3) (Kelly *et al*, 2010; Wang *et al*, 2010; Yamagishi *et al*, 2010). Taking into account that both the structured-unstructured border site of the histone H2A C-terminal tail (H2A-S121; TBS-I) (Kawashima *et al*, 2010) and the structured core histones H2A and H3 (TBS-I and -II) functionally interact with Sgo1, both the tails and cores of histones within the nucleosome were required for Sgo1 and the CPC to establish chromosome bi-orientation (Figure 7).

Residues within TBS-III could genetically interact with histone variant Htz1 (Figure 6G) during chromosome segregation. Since Htz1-containing nucleosomes were decreased in the pericentromeric regions of H4-L97A (TBS-III) cells, but not of H2A-I112A (TBS-I) or -E57A (TBS-II) cells (Figure 6H), TBS-III may be important for the incorporation of Htz1 into nucleosomes. Since Htz1-containing nucleosomes could contribute to the unique structure of the simple 'point' centromeres in budding yeast as well as the large 'regional' centromeres in mammals (Krogan *et al*, 2004; Greaves *et al*, 2007), further analysis of the structural and functional relationships between Htz1 and the TBS-III residues in the establishment of chromosome bi-orientation (Figure 7) is required.

In addition to the histone variant Htz1, nucleosomes containing the histone H3 variant Cse4 are also known to play a central role in chromosome segregation (Black and Bassett, 2008; Torras-Llort *et al*, 2009). As described above (see Introduction), the components and structures of Cse4-containing nucleosomes are a topic of debate (Mizuguchi *et al*, 2007; Camahort *et al*, 2009; Pidoux *et al*, 2009; Shivaraju *et al*, 2011). Since ChIP analysis revealed no reduction of Cse4 at *CEN3* in H2A-I112A mutant cells (Figure 4G), the phenotypes caused by the H2A-I112A substitution are unlikely to be due to defects in Cse4 localization. Although this observation cannot discriminate which models for Cse4-containing nucleosomes in the 'Introduction' are correct, any present and future models on Cse4-containing nucleosomes should be compatible with the above result (Figure 4G).

Finally, we discuss about the reasons of the unexpected instability of Sgo1 in H2A-I112A (TBS-I), -E57A (TBS-II), and H4-L97A (TBS-III) mutant cells (Figures 4F and 6E). Since

TBS-I and -II genetically interacted with Sgo1 (Figure 5), disruption of the interaction between Sgo1 and the nucleosome in H2A-I112A (TBS-I) or -E57A (TBS-II) mutant cells might trigger Sgo1 degradation (Figure 7). Curiously, TBS-III did not genetically interact with Sgo1 (Figure 5); however, the instability of Sgo1 was also observed in H4-L97A (TBS-III) mutant cells (Figure 6E). This might be explained by poor deposition of nucleosomes at the pericentromeric regions in H4-L97A cells. Htz1 was drastically reduced at the pericentromeric regions in H4-L97A cells (Figure 6H) and furthermore, occupancy of the canonical core histones at the pericentromeric regions was very recently reported to be reduced in H4-L97A cells (Yu *et al*, 2011). Thus, the reduction of total amount of nucleosomes at the pericentromeric regions in H4-L97A cells might indirectly lead to the decrease of functional interaction between Sgo1 and the nucleosomes, and then might trigger Sgo1 degradation. Notably, vertebrate shugoshin is reportedly degraded by APC/C during the exit from mitosis (Salic *et al*, 2004; Karamysheva *et al*, 2009). It is, therefore, possible that APC/C also induces the degradation of Sgo1. Shugoshin (or Sgo1) was originally named after the Japanese word for 'guardian spirit' as it protects the centromeric protein cohesin from degradation (Watanabe, 2005). In this sense, the nucleosome, a fundamental unit of chromatin, might be considered 'the shugoshin (guardian) of shugoshin: Shugomaster' and might probably be 'the shugoshin (guardian) of chromatin-acting factors: Shugomaster' (Figure 7). It is of note that the role of histones, the nucleosomal constituents, as 'the shugoshin (guardian) of DNA' has been well known since 1974 (Kornberg, 1974).

In conclusion, the results from the analysis of a global library of histone point mutants (the histone-Glibrary; Matsubara *et al*, 2007; Sakamoto *et al*, 2009; Sato *et al*, 2010) provided several novel insights into the establishment of chromosome bi-orientation. The original concept of the comprehensive point mutant library has now been applied to highly conserved histone variants Htz1 (Kawano *et al*, 2011) and Cse4 (Camahort *et al*, 2009), which are essential for faithful chromosome segregation (Stoler *et al*, 1995; Meluh *et al*, 1998; Krogan *et al*, 2004). These comprehensive point mutant libraries of canonical histones and their variants will be powerful tools for elucidating the molecular mechanisms of chromatin remodelling during chromosome segregation.

Materials and methods

Yeast strains

The genetic backgrounds of the yeast strains used in this study are given in Table II. Yeast strains FY406 and MSY748 (S288C background) and the plasmids carrying histone genes used in a genetic screen for TBZ/benomyl-sensitive mutants (Figure 1A) have been previously described (Matsubara *et al*, 2007; Sakamoto *et al*, 2009). For other assays, new histone point mutants were constructed on a W303 background using transformation or tetrad dissection techniques, as described below. For the point mutant analysis of histones H2A and H2B, the two copies of genes encoding histones H2A and H2B (*HTA1-HTB1* and *HTA2-HTB2*) were deleted in the parental strain YKH2AB (W303 background). This deletion was rescued with a plasmid carrying the WT histone H2A and H2B (*HTA1-HTB1*) genes along with the selective marker *URA3* (pSAB6). For the point mutant analysis of histones H3 and H4, the two copies of genes encoding histones H3 and H4 (*HHT1-HHF1* and *HHT2-HHF2*) were deleted in the parental strain YKH34 (W303 background) and rescued with a plasmid carrying the WT histone H3 and H4 (*HHT1-HHF1*) genes along with the selective marker *URA3*.

Table II Yeast strains

Figure	Strain	Genotype	Source
1A	FY406	<i>MATa lys2-128 his3Δ200 leu2Δ1 trp1Δ63 ura3-52 hta1-htb1::LEU2 hta2-htb2::TRP1</i> [pSAB6 <i>HTA1-HTB1</i> (URA3)]	Matsubara <i>et al</i> (2007)
1A	MSY748	<i>MATα lys2-128δhis4-912δ leu2-3,112 ura3-52 Δ(HHT1-HHF1) Δ(HHT2-HHF2)</i> [pMS329 <i>HHT1-HHF1</i> (URA3)]	Matsubara <i>et al</i> (2007)
—	YK402	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 bar1::hisG</i>	Ogiwara <i>et al</i> (2007)
2E, 2F, 5B, 5D, 6A, 6B, 6G, S4, S6 5C, 5E, 6A, 6B, 6G	YKH2AB	YK402 <i>hta1-htb1::CgTRP1 hta2-htb2::hphMX4</i> [pSAB6 <i>HTA1-HTB1</i> (URA3)]	This study
2F	YKH34	YK402 <i>hht1-hhf1::CgHIS3 hht2-hhf2::hphMX4</i> [pMS329 <i>HHT1-HHF1</i> (URA3)]	This study
6B	YSK501	YKH2AB <i>mad2::kanMX6</i>	This study
2H, 6C	YSK502	YKH34 <i>mad2::kanMX6</i>	This study
2H	YSK503	YKH2AB <i>PDS1-6His-3HA::loxP-kan^r-loxP</i>	This study
6C	YSK504	YKH2AB <i>PDS1-6His-3HA::loxP-kan^r-loxP mad2::CgLEU2</i>	This study
4A, 4F, 4L, 6E	YSK505	YKH34 <i>PDS1-6His-3HA::loxP-kan^r-loxP</i>	This study
4B, 4F	YSK601	YKH2AB <i>IPL1-6His-3HA::loxP-kan^r-loxP</i>	This study
4C, 4F, 6E, 6F	YSK602	YKH2AB <i>BIR1-13Myc::kanMX6</i>	This study
4D	YSK603	YKH2AB <i>SGO1-6His-3HA::loxP-kan^r-loxP</i>	This study
4F	YSK604	YKH2AB <i>BUB1-6His-3HA::loxP-kan^r-loxP</i>	This study
4G	YSK605	YKH2AB <i>SLI15-6His-3HA::loxP-kan^r-loxP</i>	This study
4H	YSK606	YKH2AB <i>CSE4-6His-3HA::loxP-kan^r-loxP</i>	This study
4I	YSK607	YKH2AB <i>SCM3-6His-3HA::loxP-kan^r-loxP</i>	This study
4J	YSK608	YKH2AB <i>MIF2-6His-3HA::loxP-kan^r-loxP</i>	This study
4K	YSK609	YKH2AB <i>CTF3-6His-3HA::loxP-kan^r-loxP</i>	This study
4L	YSK610	YKH2AB <i>SCC1-6His-3HA::loxP-kan^r-loxP</i>	This study
6E, 6F	YSK611	YKH2AB <i>IPL1-6His-3HA::loxP-kan^r-loxP sgo1::CgLEU2</i>	This study
6E	YSK612	YKH34 <i>SGO1-6His-3HA::loxP-kan^r-loxP</i>	This study
6H	YSK613	YKH34 <i>IPL1-6His-3HA::loxP-kan^r-loxP</i>	This study
6H	YSK614	YKH2AB <i>HTZ1-6His-3FLAG::kanMX6</i>	This study
3C, 3D	YSK615	YKH34 <i>HTZ1-6His-3FLAG::kanMX6</i>	This study
3F, 3G, 4E, 4M, 4N, 6D, S3, S5	KT410	<i>MATa hta1-htb1::CgTRP1 hta2-htb2::hphMX4</i> [pSAB6 <i>HTA1-HTB1</i> (URA3)] <i>CEN5::tetO2x112::HIS3 leu2::TetR-GFP::LEU2 cdc20::P_{MET3}-CDC20::TRP1 BAR1 RAD5</i>	This study
4M, 4N 6D	KT437	<i>MATa hta1-htb1::CgTRP1 hta2-htb2::hphMX4</i> [pSAB6 <i>HTA1-HTB1</i> (URA3)] <i>CEN5::tetO2x112::HIS3 leu2::TetR-GFP::LEU2 trp1::CFP-TUB1::TRP1 cdc20::P_{MET3}-CDC20::TRP1 bar1 RAD5</i>	This study
S5	YSK701	KT437 <i>sgo1::kanMX6</i>	This study
	KT447	<i>MATa hht1-hhf1::CgHIS3 hht2-hhf2::hphMX4</i> [pMS329 <i>HHT1-HHF1</i> (URA3)] <i>CEN5::tetO2x112::HIS3 leu2::TetR-GFP::LEU2 trp1::CFP-TUB1::TRP1 cdc20::P_{MET3}-CDC20::TRP1 BAR1 RAD5</i>	This study
	KT449	<i>MATα hta1-htb1::CgTRP1 hta2-htb2::hphMX4</i> [pSAB6 <i>HTA1-HTB1</i> (URA3)] <i>CEN5::tetO2x112::HIS3 leu2::TetR-GFP::LEU2 trp1::CFP-TUB1::TRP1 cdc20::P_{MET3}-CDC20::TRP1 BAR1 RAD5 ip1-321</i>	This study

(pMS329). C-terminal 3HA, 13Myc, and 3FLAG tagging and deletion mutants were performed using standard PCR-based methods, as previously described (Ogiwara *et al*, 2007). Standard yeast culture medium and transformation techniques were used. The complete oligonucleotide sequences of primers used for disruption, tagging, and confirmation will be provided on request. The *CEN5::tetO2x112* (Tanaka *et al*, 2000), *TetR-GFP* (Michaelis *et al*, 1997), and *P_{MET3}-CDC20* (Uhlmann *et al*, 2000) constructs have been previously described. KT410–449 background strains were produced by crossing the above strains. To construct the histone point mutants, parental strains carrying either (i) *hta1-htb1*, *hta2-htb2*, and pSAB6 *HTA1-HTB1* (URA3) or (ii) *hht1-hhf1*, *hht2-hhf2*, and pMS329 *HHT1-HHF1* (URA3), as listed in Table II, were transformed with each plasmid carrying either the mutant gene or the corresponding WT gene, as a control (pRS313 *HTA1-HTB1* (HIS3), pRS412 *HTA1-HTB1* (ADE2), pRS315 *HHT1-HHF1* (LEU2), or pRS412 *HHT1-HHF1* (ADE2)). The resulting transformants were spread on plates containing 5-fluoroorotic acid to select cells that had lost pSAB6 or pMS329.

Construction of plasmids

Plasmids carrying histone genes with the selective marker ADE2 were constructed. The histone H2A and H2B (*HTA1-HTB1*) genes were isolated by PCR and inserted into a single-copy vector, pRS412 (ADE2) (Brachmann *et al*, 1998). Histone H2A point mutations were introduced into pRS412 *HTA1-HTB1* (ADE2) using the QuickChange site-directed mutagenesis kit (Stratagene). The *SGO1*, *HTZ1*, *IPL1*, *SLI15*, *BIR1*, and *HTA1/HTB1* genes were

isolated by PCR and inserted into a multi-copy vector, YEplac195 (URA3 selective marker, 2μ) (Gietz and Sugino, 1988). The complete oligonucleotide sequences of primers used for plasmid construction will be provided on request.

Drug sensitivity assays

Agar plates for drug sensitivity assays were prepared with synthetic complete (SC) medium supplemented with 25 μg/ml TBZ (Sigma) or 10 μg/ml benomyl (Aldrich). TBZ and benomyl were diluted in 0.125% dimethyl sulphoxide, which alone had no effect on the growth of histone point mutants at that concentration. Three-fold serial dilutions of strains with the indicated genotypes were spotted onto SC agar plates with or without each drug, starting with 1×10^5 cells. For the comprehensive screening of TBZ/benomyl-sensitive histone point mutants (Figure 1A), the plates were incubated for 3 days at 30 °C. For suppressor analysis by overexpression of Sgo1 or Htz1 (Figures 5 and 6), the plates were incubated for 3 days at 25 °C. Experiments were performed in duplicate and repeated several times.

Flow cytometry

In Figures 2E and 6A, WT cells and the indicated histone point mutants were grown in YPAD medium containing 15 μg/ml nocodazole (Noc) for 3 h at 25 °C. Cells were released from the G2/M block by washing in YPAD medium and were then incubated in fresh YPAD medium. In Figures 2F and 6B, WT cells, the indicated histone point mutants and *mad2* cells were grown in YPAD medium containing 100 ng/ml α-factor for 3 h at 25 °C. Cells were released

Table III Primer sets for ChIP and RT-PCR assays

Target locus	Primer name	Primer sequence
CEN3 (ChIP)	CEN3-FW3	GATCAGCGCCAAACAATATGG
	CEN3-RV3	AACTTCCACCAGTAAACGTTTC
CEN3 left (ChIP)	CEN3-FW1	GCATGGTATGGCGCGGAAGAAACCG
	CEN3-RV1	GGATGGTATATTTTAAGACACAGCC
CEN3 right (ChIP)	CEN3-FW5	TTGCGTATAATCCGTTTTCATCACC
	CEN3-RV5	GAGTTGAATTTCGTGTGATAATGCAAC
IPL1 (RT-PCR)	IPL1-CT-FW1	GTCTTGGCGTTTGAAGTACTGACC
	IPL1-RT-RV1	CAAGGCGCATTTCTATCTTTGGGGTC
SLI15 (RT-PCR)	SLI15-CT-FW1	GATGAGGCTAGCGTAACCTTTAGCG
	SLI15-RT-RV1	CGGTTTCAACCTGTTTAGCCTAGG
BIR1 (RT-PCR)	BIR1-CT-FW1	CAAATGCCTGCAGAAGAGTTGGAC
	BIR1-RT-RV1	GCCCATTTTCTTAGCAATATCGATC
SGO1 (RT-PCR)	SGO1-CT-FW1	GAAAATTTCTACTACGCGACCCCTCC
	SGO1-RT-RV1	GAGTTTCACTGCGCTTACCATTTCC
TDH1 (RT-PCR)	TDH1-RT-FW2	GGTAAGTTGACCGGTATGGCTTTC
	TDH1-RT-RV2	GGCGGAGTAACCGTATTCGTTATC

from the G1 block by washing in YPAD medium and then incubated at 25 °C in fresh YPAD medium containing 15 µg/ml nocodazole. Cells were collected at the indicated times and fixed with 80% ethanol at 4 °C. The fixed cells were resuspended in 400 µl of 50 mM sodium citrate, sonicated briefly, and treated with 0.3 mg/ml ribonuclease A for 1 h at 37 °C. Cells were treated with 16 µg/ml propidium iodide and incubated for 30 min. The DNA content of cells was analysed using a Becton-Dickinson FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Immunoblotting

In Figures 4F and 6E, yeast cells were grown in YPAD medium containing 15 µg/ml nocodazole for 3 h at 25 °C. In Figures 2H and 6C, cells were grown in YPAD medium containing 100 ng/ml α -factor for 3 h at 25 °C. Cells were released from the G1 block by washing in YPAD medium and incubated at 25 °C in fresh YPAD medium containing 15 µg/ml nocodazole. Cells were collected at the indicated times following the nocodazole treatment. The samples were resuspended in 150 µl of 0.1 M NaOH, incubated at room temperature for 10 min, and centrifuged at 12 000 r.p.m. for ~10 s. The cell pellets were resuspended in 2 × SDS sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2 M DTT, 20% Glycerol) and incubated at 95 °C for 5 min. The protein extracts were resolved on 10–15% SDS-polyacrylamide gels and transferred onto PVDF membranes. The proteins were detected with a monoclonal anti-HA antibody (3F10; Roche), a monoclonal anti-Myc antibody (9E10; Santa Cruz Biotechnology), or a polyclonal anti-histone H3 antibody (Abcam).

Microscopy

The *tetO*/TetR-GFP system was previously described (Michaelis *et al*, 1997; Tanaka *et al*, 2002; Dewar *et al*, 2004). Images were taken using a Personal DV microscope (Applied Precision) with a ×100 1.4 numerical aperture optical lens, a cooled CCD camera (Photometrics CoolSNAP HQ), and softWoRx software (Applied Precision). Time-lapse images were collected every 15–30 s for 10–60 min with 3–5 µm z sections (separated by 0.7 µm) at 25 or 37 °C. The JP4 filter set (Chroma) was used to distinguish GFP and CFP signals. Exposure time to excitation light was 0.1 s for both the YFP/JP4 and CFP/JP4 channels with the appropriate neutral density filters. Images were subsequently deconvoluted and analysed with softWoRx software. For figures, z stacks were projected into two-dimensional images.

ChIP and quantitative PCR

ChIP assays were carried out using a monoclonal anti-HA antibody (3F10 or 12CA5; Roche), a monoclonal anti-Myc antibody (9E10; Santa Cruz Biotechnology), or a monoclonal anti-FLAG antibody (M2; Sigma), as previously described (Ogiwara *et al*, 2007). A multi-beads shocker (Yasui Kikai) was used to prepare cell extracts in Figures 4D, L, 6F, and H. Quantitative expression data were obtained using the Thermal Cycler Dice Real-Time System (TaKaRa). The primers used for ChIP assays are described in Table III.

Yeast RNA extraction and reverse transcription

Yeast total RNA was prepared by hot phenol extraction. Cells were grown in YPAD medium containing 15 µg/ml nocodazole at 25 °C for 3 h. Then, 2×10^7 cells were harvested and resuspended in 150 µl of TES solution (10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% SDS) and the same volume of acid phenol. The samples were incubated at 65 °C for 1 h and chilled on ice for 5 min. The mixtures were centrifuged at 14 000 r.p.m. at 4 °C for 5 min and the aqueous phase was recovered. The aqueous phase was mixed with the same volume of acid phenol and then centrifuged at 14 000 r.p.m. for 5 min at 4 °C. The total RNA in the aqueous phase was collected by ethanol precipitation. The RNA pellet was air dried for 30 min and resuspended in 50 µl of DEPC (diethylpyrocarbonate)-treated water. The total RNA samples were treated with RNase-free bovine pancreatic DNase I (Sigma) at room temperature for 30 min to eliminate DNA contamination. The reaction was stopped by the addition of 5.0 mM EDTA pH 8.0, followed by inactivation at 70 °C for 10 min. The RNA samples were mixed with the same volume of phenol:chloroform (5:1) and centrifuged at 6000 r.p.m. for 5 min at 4 °C. The total RNA of the aqueous phase was purified by ethanol precipitation. The RNA pellet was air dried for 30 min and resuspended in 20 µl of DEPC-treated water. cDNA was prepared with ReverTra Ace (TOYOBO) using 1.0 µg of total RNA. Quantitative expression analysis was performed with the Thermal Cycler Dice Real-Time System (TaKaRa). The primers used for quantitative expression analysis are described in Table III.

Molecular graphics

The molecular graphics were prepared using the PyMOL program (<http://www.pymol.org/>).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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