Identification of Tension Sensing Motif of Histone H3 in *Saccharomyces cerevisiae* and Its Regulation by Histone Modifying Enzymes

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ABSTRACT To ensure genome stability during cell division, all chromosomes must attach to spindles emanating from the opposite spindle pole bodies before segregation. The tension between sister chromatids generated by the poleward pulling force is an integral part of chromosome biorientation. In budding yeast, the residue Gly44 of histone H3 is critical for retaining the conserved Shugoshin protein Sgo1p at the pericentromeres for monitoring the tension status during mitosis. Studies carried out in this work showed that Lys42, Gly44, and Thr45 of H3 form the core of a tension sensing motif (TSM). Similar to the previously reported G44S mutant, K42A, G44A, and T45A alleles all rendered cells unable to respond to erroneous spindle attachment, a phenotype suppressed by Sgo1p overexpression. TSM functions by physically recruiting or retaining Sgo1p at pericentromeres as evidenced by chromatin immunoprecipitation and by *in vitro* pulldown experiments. Intriguingly, the function of TSM is likely regulated by multiple histone modifying enzymes, including the histone acetyltransferase Gcn5p, and deacetylases Rpd3p and Hos2p. Defects caused by TSM mutations can be suppressed by the expression of a catalytically inactive mutant of Gcn5p. Conversely, G44S mutant cells exhibit prominent chromatin instability phenotype in the absence of *RPD3*. Importantly, the *gcn5*$^{-}$ suppressor restores the tension sensing function in *tsm*$^{-}$ background in a fashion that bypasses the need of stably associating Sgo1p with chromatin. These results demonstrate that the TSM of histone H3 is a key component of a mechanism that ensures faithful segregation, and that interaction with chromatin modifying enzymes may be an important part of the mitotic quality control process.

KEYWORDS histone H3; chromatin; mitosis; Shugoshin; *Saccharomyces cerevisiae*

FAITHFUL partitioning of the genome duplicates in mitosis requires that the sister chromatids be engaged in bipolar attachment to mitotic spindle. Once all chromosomes are appropriately captured by the spindles, anaphase starts with the action of separase that cleaves the cohesin complex, thus separating the two sister chromosomes (Nasmyth 2002). Premature anaphase onset causes aneuploidy, a common trait associated with spontaneous abortion, birth defects, and cancer. Chromosome biorientation is a result of sister chromatid cohesion by the cohesin complex, stable attachment of spindles to kinetochores, and that the two sister kinetochores each attach to spindles emanating from different spindle pole bodies (Kschonsak and Haering 2015). Erroneous attachment of spindles to kinetochore activates the spindle assembly checkpoint (SAC), which prevents metaphase-to-anaphase transition so that errors can be corrected (Akera and Watanabe 2016). The two essential elements of biorientation are the spindle–kinetochore interaction and the tension between sister chromatids (Goshima and Yanagida 2000; Pinsky and Biggins 2005; Wang *et al.* 2014). The latter results from the physical cohesion of the sister chromatids that resists the poleward pulling force from opposing mitotic spindles, a scenario also called "amphitelic attachment." If one of the two sister kinetochores is not attached (monotelic), or if both kinetochores are attached to spindles from the same spindle pole body (syntelic), tension will not be produced, and both copies of sisters will cosegregate, leading to aneuploidy (Pinsky and Biggins 2005). The physical form of tension

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detectable by cells remains a subject of investigation (Li and Nicklas 1995). Tension-dependent conformational changes of chromatin and cohesin near kinetochores are likely candidates (Chambers *et al.* 2012; Haase *et al.* 2012; Verdaasdonk *et al.* 2012). Besides the biorientation-induced separation of sister kinetochores within the confinement of cohesion (He *et al.* 2000), intrachromosomal extension of the distance between adjacent nucleosomes in the pericentric regions has also been suggested to be an outcome of bipolar attachment (Yeh *et al.* 2008). On the other hand, how cells interpret such structural changes induced by tension is unclear. One key player in tension sensing is the Shugoshin protein (Indjeian *et al.* 2005; Kitajima *et al.* 2006; Yamagishi *et al.* 2008; Yin *et al.* 2008). Homologs of Shugoshin are found in eukaryotes ranging from yeast to humans and are important for both meiotic and mitotic chromosome segregation (Kitajima *et al.* 2004; Watanabe 2005). Deleting *SGO1*, which encodes the sole copy of Shugoshin in the budding yeast, renders cells unable to detect or to respond to tensionless crises (Indjeian *et al.* 2005; Fernius and Hardwick 2007). During mitosis, Shugoshin is enriched at the centromeres and pericentromeres (Salic *et al.* 2004; Kiburz *et al.* 2005; Riedel *et al.* 2006), from which tension originates (Bloom *et al.* 2006). Centromeric recruitment of Shugoshin depends critically on the phosphorylation of Ser121 of H2A by the Bub1p kinase, as well as several heterochromatic marks at the pericentromeres (Kiburz *et al.* 2005; Fernius and Hardwick 2007; Yamagishi *et al.* 2008; Kawashima *et al.* 2010). Genetic and biochemical experiments revealed that Shugoshin interacts with Ipl1p, the kinase subunit of the chromosomal passenger complex (Campbell and Desai 2013; Ng *et al.* 2013), protein phosphatase 2A (PP2A) (Tang *et al.* 2006; Xu *et al.* 2009; Tanno *et al.* 2010; Liu *et al.* 2013a,b; Eshleman and Morgan 2014), and cohesion (Liu *et al.* 2013b). It is possible that Shugoshin proteins participate in the detection and/or correction of attachment error. Consistently, evidence has been presented for the biorientation-dependent removal of Shugoshin from pericentromeres (Eshleman and Morgan 2014; Nerusheva *et al.* 2014), suggesting that retaining this protein at centromeres and pericentromeres may be a crucial element that keeps the spindle assembly checkpoint at an "on" state before the establishment of biorientation. However, how Shugoshin interacts with SAC remains an open question.

Previously we reported that histone H3 plays a critical role in mitotic tension surveillance in budding yeast (Luo *et al.* 2010). Yeast cells harboring the Gly44-to-Ser (G44S) mutant allele of H3 exhibit phenotypes typical of those resulting from tension sensing defects, including chromosome instability, missegregation, and inability to activate the SAC when tension buildup is perturbed (Indjeian *et al.* 2005). This mutation apparently impairs the recruitment and retention of Sgo1p at pericentromeres, whereas the centromeric Sgo1p localization remains in large part normal (Luo *et al.* 2010). Moreover, scanning mutagenesis of H3 helped uncover multiple residues, including Gly44, required for faithful segregation of chromosomes (Kawashima *et al.* 2011; Ng *et al.* 2013). Together, these reports attest to the indispensable, yet frequently overlooked function of nucleosomes in the regulation of mitosis.

Nucleosomes are the basal components specifying both the structures and functions of chromatin. Dynamic changes in nucleosomes, including their post-translational modifications, critically affect nuclear activities, including transcription, replication, and recombination. Comparatively, how mitosis might be regulated by chromatin is only beginning to be understood. Here we present evidence that Gly44 of histone H3 is part of the TSM ^{42}KPGT that bridges the *N′* tail domain and the central histonefold domain of H3. Genetic assays also revealed that the function of H3 TSM is regulated by two opposing chromatin modifying activities, the histone acetyltransferase Gcn5p and deacetylases Rpd3p and Hos2p. Together, these results further demonstrate that chromatin, besides itself being the cargo of genome partitioning, plays an active role in ensuring faithful segregation.

Materials and Methods

Yeast strains and plasmid constructs

The yeast strains, plasmids, and primers used in this work are listed in Table 1, Table 2, and Table 3.

To assess the effects of histone H3 mutations from lysine 36 to lysine 56 on the benomyl sensitivity, pJL74 (a *LEU2* plasmid bearing histones H2A and H2B) was cotransformed with H3 mutant collection from the Boeke group (*TRP1* plasmids harboring H3 and H4 genes) that contains specific H3 mutations (Dai *et al.* 2008). *5-FOA* selection was conducted to select for yeast cells that had lost the plasmid pMK440 (a *URA3* plasmid bearing all four core histone genes). All other studies were carried out with histone mutations generated in pMK439 by two-step PCR site-directed mutagenesis (Luo *et al.* 2010).

To study the functions of *GCN5* on the H3-Sgo1p tension sensing mechanism, H3 mutations (on pMK439 *LEU2*$^+$) were introduced into yJL486 (*gcn5*Δ, pMK440 *URA3*$^+$) via plasmid shuffling and 5-FOA selection, resulting in strains yJL506 to yJL510 (see Table 1). yJL486 was constructed by transforming a 4.6-kb *gcn5::URA3* fragment, which is released from pMK147 by *Xho*I and *Xba*I, into yMK1141 (pMK440 *URA3*$^+$). Dominant negative HAT inactive mutants of *gcn5*, *gcn5E173H*, or *gcn5F221A* were carried on pMK144 and were directly transformed into cells bearing H3 mutations.

To delete *RPD3*, primers oXD37 and oXD38 were used to amplify the *Kluyveromyces lactis TRP1* selective marker from plasmid pBS1479. The PCR product was transformed into yMK1141 for tryptophan prototroph selection. The same strategy was used for knockout of *SIN3* (oXD33 and oXD34), *HDA1* (oXD25 and oXD26), *HOS1* (oXD79 and oXD80), and *HOS2* (oXD83 and oXD84). The single knockout strains were then subjected to plasmid shuffling and 5-FOA selection to obtain either WT or G44A H3.

To create pXD32, a 2-μm URA3 RPD3 plasmid, primers oXD15 and oXD16, bearing 42 bp of homology to the vector

Table 1 Yeast strains used in this study

Strain	Relevant genotype	Source or reference
227a	*lys1*	Gift of E. Grayhack
70α	*thr3 met*$^-$	Gift of E. Grayhack
yJL171	*MAT***a** *ade2-1 bar1Δ can1-100 his3-11, 15::pGAL-MCD1::HIS3 leu2-3, 112 trp1-1::PDS1-Myc13::TRP1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pQQ18 [*ARS CEN LEU2 HTA1-HTB1 HHT2-HHF2*]	Luo *et al.* (2010)
yJL340	*MAT***a/α** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hht2-hhf2::Nat hta1-htb1::HPH hta2-htb2::Nat* pMK439G44S [*ARS CEN LEU2 HTA1-HTB1 hht2-G44S-HHF2*]	This study
yJL343	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1::SGO1-6HA::TRP1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pQQ18 [*ARS CEN LEU2 HTA1-HTB1 HHT2-HHF2*]	Luo *et al.* (2010)
yJL475	*MAT***a/α** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hht2-hhf2::Nat hta1-htb1::HPH hta2-htb2::Nat* pMK439K42A [*ARS CEN LEU2 HTA1-HTB1 hht2-K42A-HHF2*]	This study
yJL479	*MAT***a/α** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hht2-hhf2::Nat hta1-htb1::HPH hta2-htb2::Nat* pMK439T45A [*ARS CEN LEU2 HTA1-HTB1 hht2-T45A-HHF2*]	This study
yJL486	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1::gcn5 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pJH33 [*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study
yJL487	*MAT***a** *ade2-1 bar1Δ can1-100 his3-11, 15::pGAL-MCD1::HIS3 leu2-3, 112 trp1-1::PDS1-Myc13::TRP1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK439K42A [*ARS CEN LEU2 HTA1-HTB1 hht2-K42A-HHF2*]	This study
yJL492	*MAT***a** *ade2-1 bar1Δ can1-100 his3-11, 15::pGAL-MCD1::HIS3 leu2-3, 112 trp1-1::PDS1-Myc13::TRP1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK439T45A [*ARS CEN LEU2 HTA1-HTB1 hht2-T45A-HHF2*]	This study
yJL506	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1::gcn5 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pQQ18 [*ARS CEN LEU2 HTA1-HTB1 HHT2-HHF2*]	This study
yJL507	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1::gcn5 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK439G44S [*ARS CEN LEU2 HTA1-HTB1 hht2-G44S-HHF2*]	This study
yJL508	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1::gcn5 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK439G44A [*ARS CEN LEU2 HTA1-HTB1 hht2-G44A-HHF2*]	This study
yJL509	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1::gcn5 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK439K42A [*ARS CEN LEU2 HTA1-HTB1 hht2-K42A-HHF2*]	This study
yJL510	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1::gcn5 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK439T45A [*ARS CEN LEU2 HTA1-HTB1 hht2-T45A-HHF2*]	This study
yJL540	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1::SGO1-6HA::TRP1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK439K42A [*ARS CEN LEU2 HTA1-HTB1 hht2-K42A-HHF2*]	This study
yJL543	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1::SGO1-6HA::TRP1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK439T45A [*ARS CEN LEU2 HTA1-HTB1 hht2-T45A-HHF2*]	This study
yMK1141	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pMK440 [*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	Luo *et al.* (2010)
yMK1174	*MAT***a/α** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hht2-hhf2::Nat hta1-htb1::HPH hta2-htb2::Nat* pJH33 [*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study
yMK1243	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH* pQQ18 [*ARS CEN LEU2 HTA1-HTB1 HHT2-HHF2*]	Luo *et al.* (2010)
yXD24	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH hda1Δ::TRP1 /pMK440*[*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study
yXD26	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH sin3Δ::TRP1/pMK440*[*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study
yXD27	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH rpd3Δ::TRP1/pMK440*[*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study
yXD29	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH RPD3-3HA::TRP1/pMK440*[*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study
yXD87	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH hos1Δ::TRP1/pMK440*[*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study
yXD88	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH hos2Δ::TRP1/pMK440*[*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study
yXD100	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hht1-hhf1::KAN hht2-hhf2::KAN hta1-htb1::Nat hta2-htb2::HPH GCN5-13MYC::TRP1/pMK440*[*ARS CEN URA3 HTA1-HTB1 HHT2-HHF2*]	This study

pMK595 at the 5′ end, were used to amplify *RPD3* ORF from the yeast genome. The PCR product was cotransformed with *Not*I-digested pMK595 into yMK839. Ura$^+$ transformants were subjected to DNA extraction for bacterial transformation. Miniprep DNA was then verified by sequencing. To introduce the H150A catalytically dead mutation to Rpd3p, primers oXD59 and oXD60 were used for site-directed mutagenesis PCR with pXD32 as template, generating pXD33. The mutagenesis was confirmed by sequencing as well.

Myc-tagged Gcn5p and HA-tagged Rpd3p strains were created as previously described (Liu *et al.* 2010).

Table 2 Plasmid constructs used in this study

Plasmid	Main features	Source or reference
pJH33/pMK440	*pRS316-HTA1-HTB1 HHT2-HHF2*	Luo *et al.* (2010)
pJL51	*2 μm URA3 pADH1-3xHA-SGO1-tADH1*	This study
pJL52	*ARS1 CEN4 URA3 pADH1-3xHA-tADH1*	This study
pJL53	*ARS1 CEN4 URA3 pADH1-3xHA-SGO1-tADH1*	This study
pJL55	*pGEX-4T-1 3xHA-SGO1*	This study
pJL74	*pRS315-HTA1-HTB1*	This study
pMK120	*2 μm URA3* vector with *CUP1* promoter	Kuo *et al.* (1998)
pMK144	*2 μm URA3 pCUP1-GCN5*	Kuo *et al.* (1998)
pMK144E173H	*2 μm URA3 pCUP1-gcn5E173H*	Kuo *et al.* (1998)
pMK144F221A	*2 μm URA3 pCUP1-gcn5F221A*	Kuo *et al.* (1998)
pMK572	*2 μm URA3* vector with *ADH1* promoter and terminator	Luo *et al.*, (2010)
pMK573	*2 μm URA3 SGO1*	Luo *et al.*, (2010)
pQQ18/pMK439	*pRS315-HTA1-HTB1 HHT2-HHF2*	Luo *et al.*, (2010)
pXD32	*2 μm URA3* RPD3 with *ADH1* promoter and terminator	This study
pXD33	*2 μm URA3* rpd3H150A with *ADH1* promoter and terminator	This study

Yeast methods

Yeast growth media, conditions, and transformation were based on standard procedures (Sherman 1991). When appropriate, 5% casamino acids (CAAs) were used to substitute for synthetic amino acid mixtures as selective medium for uracil, tryptophan, or adenine prototroph. Yeast transformation was done with the lithium acetate method (Gietz *et al.* 1992).

Chromosome stability assays were conducted by measuring the mating behavior of diploid strains bearing WT or selective tension sensing mutants. Homozygous diploid cells created by the transformation of YCp50-HO (Herskowitz and Jensen 1991) were grown overnight in YPD, and then patched onto YPD plates and incubated at 30° for 2–3 days until saturation. Cell plates were replica plated to other fresh YPD plates covered with $\sim 5 \times 10^7$ *MAT***a** or *MAT*α tester cells and allowed to mate at 30° for 10 hr, followed by further replica plating to minimum medium plates. Mating between the tester and the subject strains resulted in complete complementation of nutrient requirement and were able to survive in the minimal medium. Genomic PCR that examined the status of the *MAT* loci on chromosome III was conducted by using the primers OJL100, OJL101, OJL102, and OJL103 targeting the *MAT* locus but not either of the two silent loci. The tension sensing test using the P_{GAL1}-*MCD1* strains was precisely according to Indjeian *et al.* (2005) using strains yJL171, yJL487, and yJL492. Western analyses of yeast proteins and benomyl washout assays were conducted as mentioned in Luo *et al.* (2010).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted as previously described (Kuo and Allis 1999; Luo *et al.* 2010) using primers listed in Table 3. To quantify the ChIP results, PCR products were purified and resolved by 9% polyacrylamide gel electrophoresis and stained by ethidium bromide. The captured gel images were then quantified by National Institutes of Health ImageJ. Intensities of each CEN/pericentric fragment were compared to a common internal control (*DED1*, *PGK1*, or *TEL*). The ratio was further normalized to 0.1% input DNA for PCR amplification carried out in parallel of all reactions. The ChIP data were obtained from at least three independent yeast cultures.

Sgo1p–H3 interaction

Histones were prepared according to Edmondson *et al.* (1996). Recombinant Sgo1p was prepared as previously described (Luo *et al.* 2010). For pulldown assays, ~5 μg of soluble recombinant Sgo1p was incubated with ~3 μg of yeast histones in 150 μl of HEMGT buffer at 4° for 1 hr. A total of 6 μl of glutathione beads along with 150 μl of the HEMGT buffer were added to the reactions and rocked gently at 4° for another hour. Beads were washed with 500 μl of the HEMGT buffer three times, 5 min each, followed by boiling in 2× SDS-PAGE loading buffer for 5 min. Eluate was resolved by 15% SDS-PAGE and blotted for anti-H3 Western analyses (Luo *et al.* 2010).

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Mutations of a cluster of amino acid residues of H3 cause mitotic chromosome instability

Our initial discovery that Gly44 of H3 is important for Sgo1p interaction and tension sensing (Luo *et al.* 2010) suggests that this residue is part of a motif directly responsible for Sgo1p recruitment. Several scanning mutagenesis studies of histone H3 indeed identified residues, some are close to Gly44, to be important for maintaining mitotic integrity and chromatin stability (Dai *et al.* 2008; Kawashima *et al.* 2011; Ng *et al.* 2013). To more specifically map the functional domain integrating Gly44 for tension sensing, we first compared benomyl hypersensitivity of strains bearing histone

Table 3 Oligos used in this study

Oligo	Sequence
CEN16 S	ATGCAAAGGTTGAAGCCGTTA
CEN16 AS	TTTGCCGATTTCGCTTTAGAAC
CEN16–0.3 kb S	GAAGCACTCCGACCTTTC
CEN16–0.3 kb AS	CTTGCCTTTTCTGGATCAG
CEN16–1.7	GATGAGCACATATGCATG
CEN16–1.7as	CTTAATCCATCAATTCTGG
CEN16+4.0	GCCCTGATAAAGTCGACC
CEN16+4.0as	GAACTCTTGCAAGTTGAAG
CEN16+6.5	CCGATGATGGTTGTTATG
CEN16+6.5as	CTCTAATAGTGGCAATGTTG
CEN16+9.1	AAACTCAATGATGACCTTG
CEN16+9.1as	TATGTTACTCTTACGATGTG
mk93	GGC AAG TGG TAT TCC GTA AG
mk93as	CTT GGT TTT CCT CTT AAG TG
OJL19	TGTCATCATGCGTATTAGAG
OJL20	CGTATAGGGAATTTAACGTC
OJL100	GACTAAAGTAGAGCAACA
OJL101	AGTGGAGTAATGCCACAT
OJL102	ACGTCTAGCTGAGCATGT
OJL103	GAACTGTCGAAACTGAGT
oXD15	ATCCATATGACGTTCCAGATTACGCTGCTCAGTGCGTATATGAAGCAACACCTTTTGATC
oXD16	TATCGGGGGGATCCACTAGTTCTAGCTAGAGCGGCCTCAATAGAATTCATTGTCATGCTC
oXD25	AGCATGGATTCTGTAATGGTTAAGAAAGAAGTACTGGAAAATTGAAGCTTGATATCGAAT
oXD26	TTCTTCATCACTCCATTCTTCAAACGAATCCAGTATAAAGTCTACGACTCACTATAGGGC
oXD33	AAAATGTCACAGGTTTGGCATAATTCGAATTCGCAATCAAACTGAAGCTTGATATCGAAT
oXD34	TTGAATCTTAGCCCCCTTGTCTGAAGATTCAGTATTCCCAGTTACGACTCACTATAGGGC
oXD37	CAGATGGTATATGAAGCAACACCTTTTGATCCGATCACGGTCTGAAGCTTGATATCGAAT
oXD38	ATAGAATTCATTGTCATGCTCAACATGTAGGTCCCTCGCATATACGACTCACTATAGGGC
oXD59	TCAACTATGCGGGTGGTTTGGCTCATGCAAAAAAATCGGAGGCTTCTGGGTTTTGTTATT
oXD60	AATAACAAAACCCAGAAGCCTCCGATTTTTTTTGCATGAGCCAAACCACCCGCATAGTTGA
oXD79	TAATATGAATTAATAAACACCTGTCCATTTTAGAAAAACGCTTGAAGCTTGATATCGAAT
oXD80	TCGCATTATTAATTTGTATTCAAACGACTAATTAAAACTATCTACGACTCACTATAGGGC
oXD83	AGTACGTTAAAATCAGGTATCAAGTGAATAACAACACGCAACTGAAGCTTGATATCGAAT
oXD84	AAAAAAAAAAACGGGAGATTAACCGAATAGCAAACTCTTAAATACGACTCACTATAGGGC

H3 mutations from K36 (the end of the tail domain) through K56 (the end of the αN helix of the histone core). Benomyl depolymerizes mitotic spindles. Cellular hypersensitivity to benomyl is a trait shared by many mutants with defects in mitotic regulation. Figure 1A shows that K42A, G44A, and T45A mutations caused strong hypersensitivity to benomyl, and that a milder phenotype was caused by the adjacent H39A, R40A, Y41A, and R49A mutations. Intriguingly, P43A, flanked by K42 and G44, apparently is phenotypically neutral.

Crystal structures of nucleosomal particles show that the ^{42}KPGT region adapts a unique turn structure transitioning from the flexible tail domain to the well-structured histone-fold domain (White *et al.* 2001) (Supplemental Material, Figure S1). Curiously, both K42 and T45 have been shown to be modified post-translationally (Baker *et al.* 2010; Hyland *et al.* 2011) for the control of gene expression and replication, respectively. Their role in mitosis, if any, is unclear. To examine the potential involvement of K42 and T45 modifications in mitosis, we introduced additional mimetic mutations and tested the cellular response to benomyl treatment. Figure 1B shows that the K42R nonacetylatable mutant behaved similarly to the WT but K42Q cells were hypersensitive to benomyl. The T45E phosphomimetic mutation caused cell death, due possibly to dysregulation of replication (Baker *et al.* 2010). The differential effects of alanine, glutamine, and arginine substitutions at K42 suggest that maintaining a positive charge at this position is critical but the actual side chain, that is, lysine or arginine, is not as critical. On the other hand, the K42R mutation alone could not rescue the benomyl hypersensitivity phenotype of G44S (which shared comparable traits with G44A) (fourth row, Figure 1B), indicating that glycine at this position is essential and cannot be rescued by a constitutive, positive charge at position 42.

The original G44S mutation that impairs tension sensing can be suppressed by overexpressing Sgo1p, the key factor for this checkpoint function ensuring error-free segregation (Indjeian *et al.* 2005; Luo *et al.* 2010). If the newly identified mutant alleles also perturbed the function of tension sensing, we predicted that they would be suppressed by Sgo1p overexpression as well. Results confirm this hypothesis (Figure 1C). In the presence of a multicopy plasmid bearing a constitutively expressed *SGO1* gene, all mutant alleles tested showed apparent restoration of robust growth in the presence of benomyl. Neighboring residues replaced by alanine (*i.e.*, His39, Arg40, Tyr41, and Arg49) were all rescued by 2μ

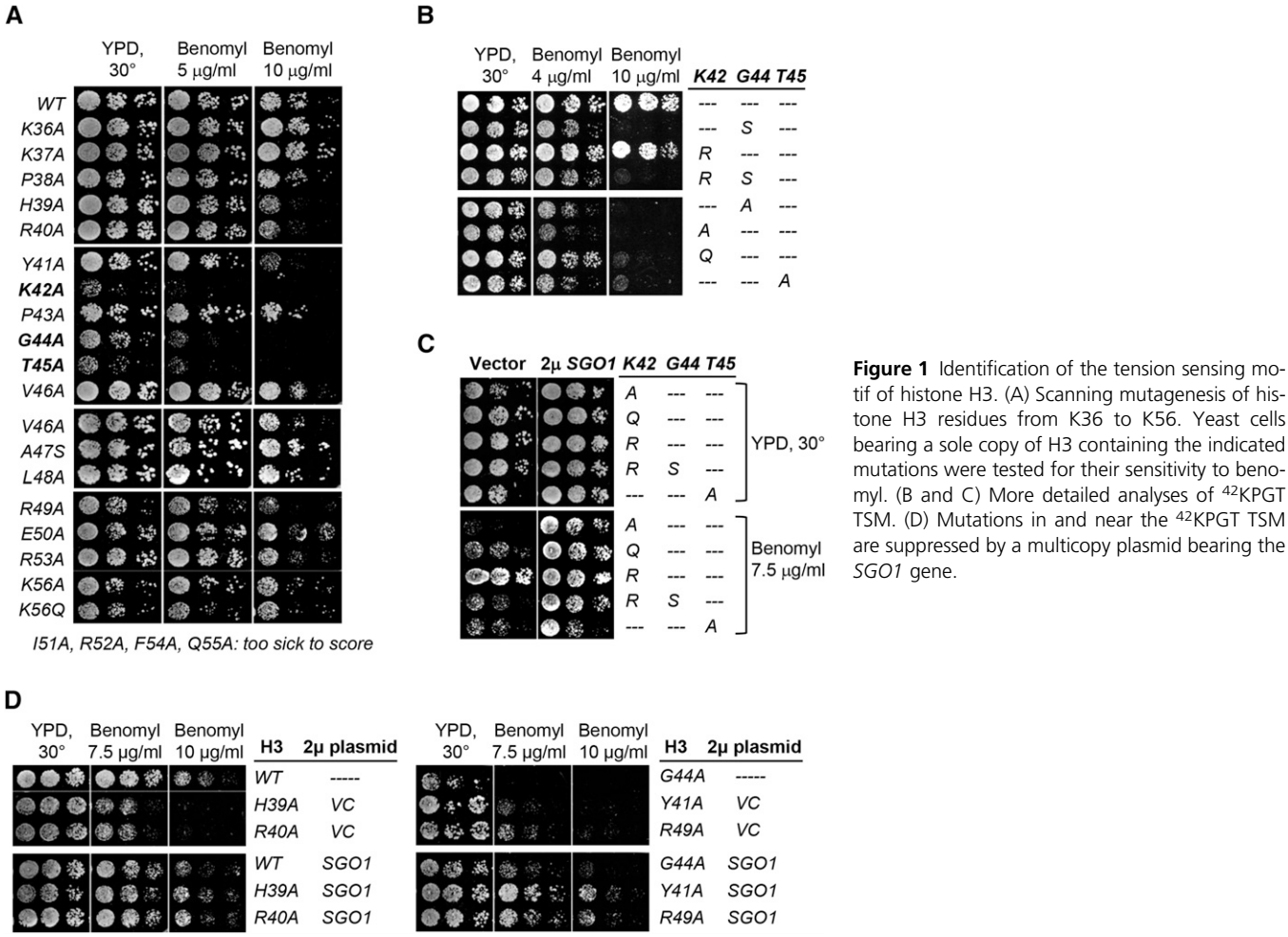

Figure 1 Identification of the tension sensing motif of histone H3. (A) Scanning mutagenesis of histone H3 residues from K36 to K56. Yeast cells bearing a sole copy of H3 containing the indicated mutations were tested for their sensitivity to benomyl. (B and C) More detailed analyses of ^{42}KPGT TSM. (D) Mutations in and near the ^{42}KPGT TSM are suppressed by a multicopy plasmid bearing the *SGO1* gene.

SGO1 (Figure 1D). These data suggest that K42, G44, and T45 form the core of a TSM that also requires several nearby residues for full function.

Chromosome instability conferred by K42, G44, and T45 mutants

Mitotic defects frequently trigger chromosome instability and aneuploidy, a phenotype that can be realized by the acquisition of the ability of diploid cells to mate. In budding yeast, only haploids can mate. Normal diploid cells inherit the transcriptionally active *MAT***a** and *MAT*α mating loci on chromosome III from the two haploid parents. Concomitant expression of these two loci in diploid cells represses genes essential for mating. The **a**/α diploid cells thus are nonmater. If there is an increase in chromosome instability, sporadic loss of one of the two chromosome III homologs from a population will enable the underlying strain to mate as both *MAT***a** and *MAT*α mating types, regardless of the ploidy for the rest of the genome. The emergence of this bimater phenotype thus manifests chromosome instability and aneuploidy (Yuen *et al.* 2007). To take advantage of this method to examine the effects of TSM mutations on chromosome stability, we first generated diploid strains bearing homozygous K42A, G44S, or T45A mutation. As an additional control, we included another novel histone mutant allele, H4 R35S, which was also hypersensitive to benomyl but was not suppressed by Sgo1p overexpression (J. Luo and M.-H. Kuo, unpublished data). Diploid cells from single colonies were grown on solid medium as patches before replica plating to haploid mating tester strains. Successful mating to either tester strain complemented the nutrient marker gene defects, hence allowing cells to grow on the synthetic defined (SD) minimal plate. Figure 2A shows that a substantial portion of diploid cells bearing any of these H3 mutant alleles were able to mate with both tester strains, while WT or H4 R35S mutant cells maintained their diploid nonmater character. Genomic PCR using primers differentiating *MAT***a** and *MAT*α loci demonstrated that the nonmater isolates maintained both copies of chromosome III, that is, the two PCR fragments were of roughly equal intensities. On the other hand, those capable of mating, WT haploid or mutant diploid, showed substantial bias toward one of the two copies. The mating type correlated well with the intensities of the two bands (Figure 2B), strongly suggesting that the bimater phenotype was due to imbalance of the two chromosome III homologs. To further rule out the possibility that these maters were a result of meiosis before or during experiments that would have generated mating-capable haploids, we did fluorescence activated

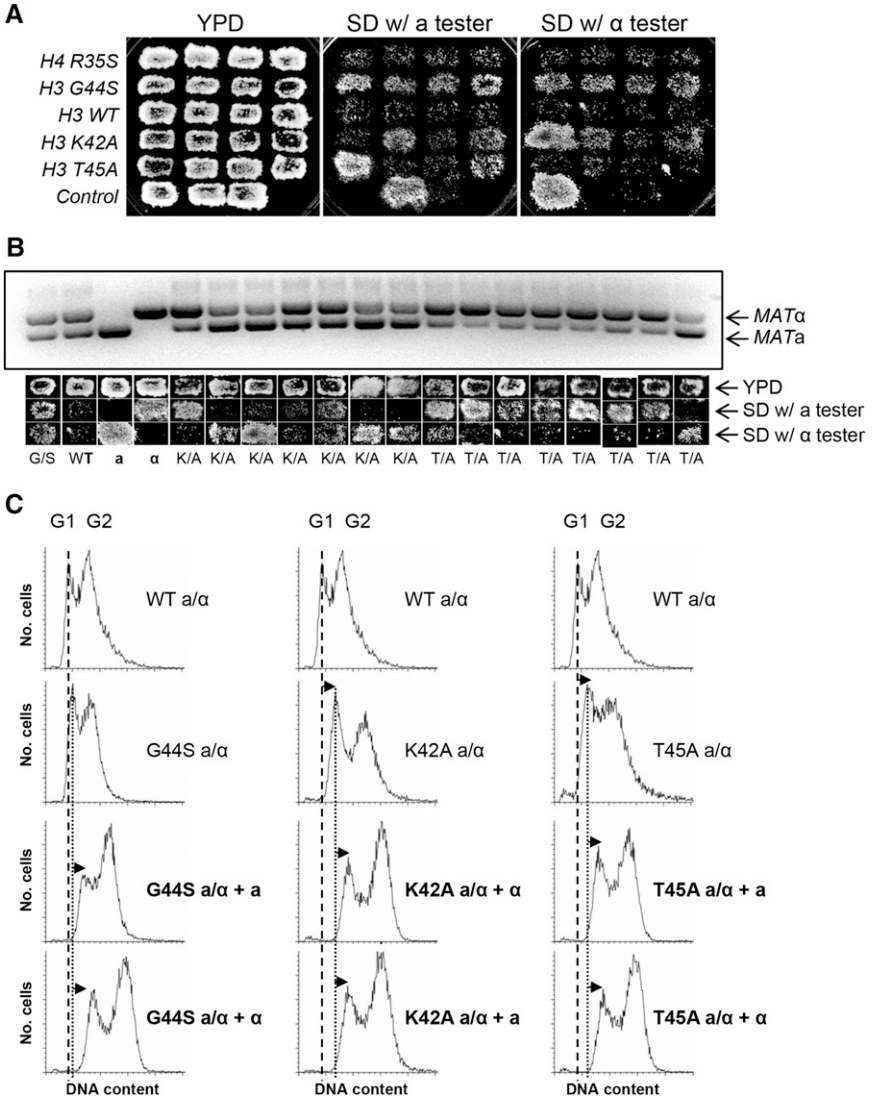

Figure 2 Mutations introduced to the TSM cause chromosome instability. (A) Diploid cells bearing homozygous mutant alleles of histone H3 or H4 were tested for their mating behavior. Cells mate with a tester strain (227a or 70α, see Table 1) acquire the ability to grow in SD minimal medium. Cells containing the R35S mutation in histone H4 are hypersensitive to benomyl and do not affect the tension sensing function. The three WT control strains on the bottom roll are, from left to right, *MAT*α, *MAT***a**, and *MAT***a**/α. (B) Genomic PCR reveals the stochastic loss of one of the two copies of chromosome III. Cells scraped from the YPD (before mating) patches were subjected to DNA isolation and PCR, using primers amplifying both silent mating loci concomitantly. The corresponding mating behaviors of each patch on YPD and SD plate are shown below each lane of the DNA gel. The relative intensity of the two mating loci PCR products correlates with the mating ability. (C) FACS analysis of the DNA content of asynchronous diploid strains before and after mating. Successful mating increases the DNA content. The G1 peak of the WT and each mutant diploid is marked by a broken and a solid vertical line, respectively. The right shift of the G1 peak after mating is indicated by arrows.

cell sorting (FACS) analysis to examine the overall DNA content of cells from the patches on YPD (before mating) and SD minimal plates (after mating). Figure 2C shows that prior to mating with the tester, all starting diploid cells contained 2N or slightly higher DNA content. After mating with the haploid tester cells, the DNA content further increased (marked by the arrows of the G_1 peaks), confirming the fusion of two sets of genome that resulted in triploidy.

In addition to high frequencies of aneuploidy (*i.e.*, bimater phenotype), the K42A, G44A, G44S, and T45A homozygous diploid cells had either a very low sporulation efficiency, or, when tetrads were formed, low germination rate (data not shown). Finally, in contrast to the histone H3 tension sensing mutants, the H4 R35S mutant behaved like the WT cells in mating tests (Figure 2A), arguing against the possibility that all benomyl hypersensitive mutants suffer from chromosome instability. We surmise that K42, G44, and T45 are important for cells to maintain mitotic chromosome stability.

K42A and T45A mutations compromise tension sensing function and Sgo1p recruitment

To more firmly link ^{42}KPGT to the tension sensing function, we tested the spindle assembly checkpoint activation in response to a tensionless crisis. Benomyl treatment depolymerizes microtubules, eliminating both attachment and tension, and consequently activates the SAC. Both K42A and T45A mutant cells were able to stabilize the securing protein Pds1p in response to benomyl insult (Figure S2). We then tested the spindle checkpoint activation induced specifically by the lack of tension. To this end, we placed the *MCD1* gene under the control of the galactose-repressible *GAL1–10* promoter (Indjeian *et al.* 2005). *MCD1* (also known as *SCC1*) encodes an integral component of the cohesin complex that holds sister chromatids together. WT and mutant strains were synchronized at, and released from G1 phase in a galactose-containing medium. Normal mitotic progression is manifested by the fluctuation of the securin protein Pds1p (Figure 3, solid lines). If cells were released from G1 arrest into a

glucose-containing medium that repressed *MCD1* transcription, Mcd1p deficiency disrupted cohesion and prevented tension buildup. Such a tensionless crisis activated the SAC by stabilizing Pds1p (broken line, top row, Figure 3). On the other hand, K42A and T45A mutant cells showed Pds1p fluctuation in both galactose- and glucose-containing media (middle and bottom rows, Figure 3). The inability of cells to respond to the lack of tension is in excellent agreement with the documented tension sensing defects caused by the G44S mutation (Luo *et al.* 2010). We therefore conclude that K42, G44, and T45 together form a TSM.

Yeast and other eukaryotic cells monitor the tension status via the Shugoshin family proteins (Marston 2015). Recruitment of the yeast Sgo1p to the pericentromeres is essential for the tension sensing function (Fernius and Hardwick 2007; Luo *et al.* 2010). Given that overexpressing *SGO1* suppresses the benomyl hypersensitivity phenotype of both K42A and T45A mutants (Figure 1C), we suspected that these two alleles also crippled the ability of H3 to retain Sgo1p at the pericentric region, hence compromising the tension sensing activity. To test this hypothesis, we conducted ChIP experiments to check the localization of Sgo1p in K42A or T45A mutant strains. Indeed, Sgo1p was recruited efficiently to CEN16 in all strains tested (Figure 4, A and B). However, the pericentric Sgo1p was significantly reduced in both mutants, despite the fact that the total levels of Sgo1p were comparable in all three strains tested (Figure 4C). The abrupt descent of the Sgo1p signal at as close as 0.3 kb to CEN16 in these two mutants strongly suggested that the recruitment of Sgo1p to the centromere, in which the canonical H3 is replaced by a centromere-specific H3 variant Cse4p, is mediated by a mechanism different from that for pericentric Sgo1p enrichment. This notion was further confirmed by *in vitro* pulldown assays that examined the physical interaction between H3 and Sgo1p. Figure 4D shows that the wild-type H3 purified from yeast was able to interact with a GST-tagged Sgo1p. K42A and T45A mutations weakened, but did not eliminate, the affinity for Sgo1p (Figure S4). Consistent with the observation that the P43A mutation did not cause benomyl hypersensitivity (Figure 1A), histone H3 bearing the P43A mutation bound Sgo1p similarly to WT. We thus conclude that both K42A and T45A mutations compromise severely the ability of H3 to interact with Sgo1p *in vitro* and *in vivo*, and that the TSM of H3 functions through physically recruiting or retaining Sgo1p at the pericentromeres.

The acetyltransferase Gcn5p is a negative regulator of the TSM

While it is highly likely that Sgo1p was first recruited to the centromeres and then spread to pericentromeres (Figure 4) (Fernius and Hardwick 2007; Kawashima *et al.* 2010; Luo *et al.* 2010), it is enigmatic as to how Sgo1p is confined at such loci when H3 is nearly ubiquitous in chromosomes. One possibility is that the H3–Sgo1p interaction is subjected to direct or indirect regulation at or surrounding centromeres such that only the pericentric H3 is amenable to Sgo1p

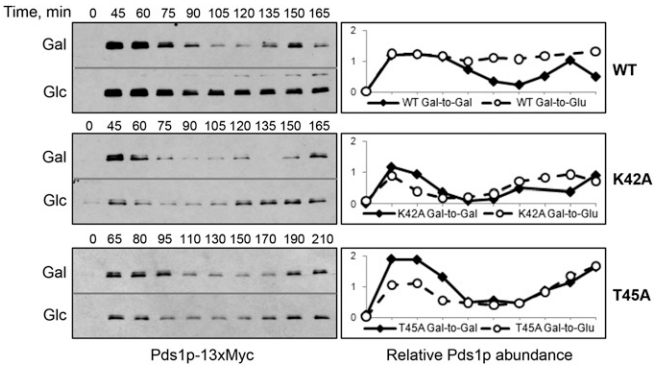

Figure 3 K42A and T45A mutations diminish the ability to activate the SAC when tension is absent. A cohesin complex component Mcd1p is under the control of the *GAL* promoter. Yeast cells were released from G1 arrest into galactose (Gal) or glucose (Glc) medium. The abundance of Pds1p securin was examined by immunoblotting and quantified by comparing with an internal control glucose-6-phosphate dehydrogenase (G-6-PDH) (see Figure S3).

association. Because bacterially expressed Sgo1p and H3 interact well (Luo *et al.* 2010), it is possible that the H3–Sgo1p interaction might be negatively regulated *in vivo* by a reversible modification. Of the many chromatin modifying activities, we were particularly interested in the potential involvement of Gcn5p, a prototypical histone acetyltransferase well known for its role in transcriptional regulation. Additionally, Gcn5p controls the chromatin structure at the centromeric region, an activity shown to be important for ensuring timely mitotic progression (Zhang *et al.* 1998; Vernarecci *et al.* 2008). We suspected that Gcn5p might also be involved in the tension sensing function of H3 and therefore examined the effect of deleting *GCN5* from strains bearing mutations within the TSM. Figure 5A shows that deleting *GCN5* effectively suppressed the benomyl hypersensitivity of G44S, G44A, and K42A strains. Intriguingly, the T45A mutant was much less susceptible to this suppression, arguing against the idea that deleting *GCN5* resulted in a global change in benomyl flux or metabolism. To further delineate whether the suppression was related to the HAT (or lysine acetyltransferase) (KAT) activity of Gcn5p, we overexpressed two dominant negative alleles of *GCN5*, *gcn5 F221A* and *gcn5 E173H* (Kuo *et al.* 1998; Liu *et al.* 2005), that were devoid of the catalytic activity. Essentially identical results as those from *GCN5* ORF deletion were obtained (Figure 5B). The HAT activity of Gcn5p was thus concluded to be elemental to the mitotic function of Gcn5p. In addition to benomyl hypersensitivity, mutations at K42, G44, and T45 also caused cellular hypersensitivity to a nucleoside analog, hydroxyurea (HU) (Figure 5B). However, overexpression of the *GCN5* HAT inactive mutants had no effect on HU hypersensitivity, indicating that the genetic interaction between Gcn5p and H3 is specific to mitotic functions. The notion that Gcn5p acted as a negative regulator for the H3 mitotic function was further supported by the enhanced benomyl sensitivity when the WT Gcn5p was overexpressed in the K42A, G44A, and G44S mutants

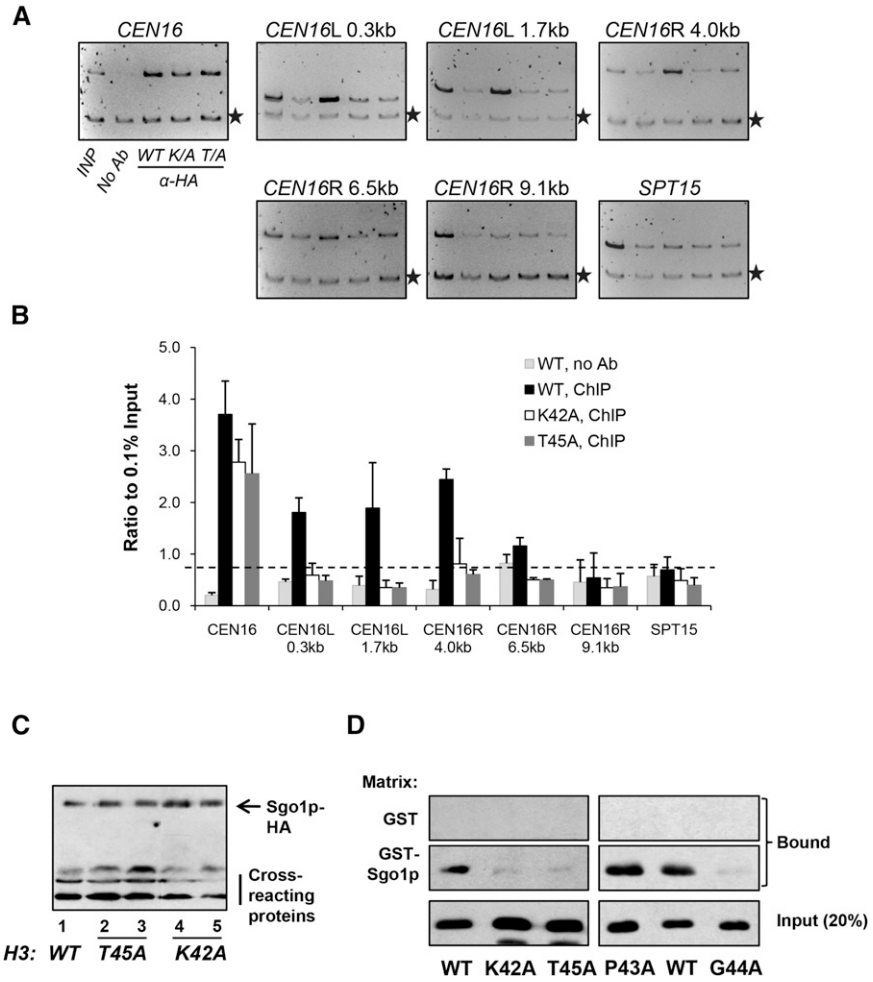

Figure 4 Defective TSM fails to retain Sgo1p in pericentromeres. (A and B) Chromatin immunoprecipitation of Sgo1p epitope-tagged with HA. Cells were arrested at G2/M by benomyl before ChIP. Each PCR reaction contained a pair of locus-specific primers and another common internal control corresponding to the PGK1 gene. Quantification results of PCR reactions are shown in B and C. The steady-state abundance of Sgo1p is not affected by mutations of the TSM. Yeast whole cell lysates were probed with anti-HA antibodies to detect the HA-tagged Sgo1p. (D) Sgo1p interaction with H3 is weakened by K42A, G44A, and T45A, but not by P43A mutation. Bacterially expressed GST–Sgo1p was incubated with core histones purified from yeast. H3 retained by GST–Sgo1p on the glutathione beads was eluted and resolved by SDS-PAGE. Results of Western blotting with and anti-H3 antibodies are shown. Error bars were calculated from at least three biological replica.

(Figure 5B). Moreover, the suppression brought about by *gcn5 F221A* and *gcn5 E173H* alleles was reverted upon *SGO1* deletion (Figure 5C), suggesting that Gcn5p regulates H3 function in the same pathway as Sgo1p.

The E173H *gcn5*$^{-}$ suppressor for mitotic defects caused by G44S was further examined by two additional methods (Figure 6). First, the cellular viability decline caused by progressively longer incubation with benomyl was assessed. Benomyl treatment depolymerizes microtubules and arrests cells at the G2/M phase. After returning to a benomyl-free medium, cells reestablish spindle–kinetochore attachment for metaphase-to-anaphase transition. Cell cycle progression with uncorrected attachment mistakes leads to aneuploidy and cell death. Defects in tension sensing thus cause elevated death rates (Luo *et al.* 2010) (Figure 6A). Overexpressing the dominant negative mutant Gcn5p restored viability of the G44S mutant to nearly the WT level (black broken line, Figure 6A). Second, the chromosome missegregation rate of the G44S mutant with or without the E173H allele of Gcn5p was further quantified by examining haploid cells bearing a GFP-marked *TRP1* locus 12 kb from *CEN4* (Straight *et al.* 1996). Haploid G1 phase cells with two GFP dots indicated missegregation from the previous round of mitosis. Figure 6B shows that the percentage of two-dotted G44S cells was significantly reduced by the *gcn5*$^{-}$ *E173H* allele. From Figure 5 and Figure 6, we concluded that the histone acetyltransferase Gcn5p functions as a negative regulator for the mitotic regulatory function of the histone H3 TSM.

Histone deacetylases Rpd3p and Hos2 interact genetically with the TSM of H3

If the HAT activity of Gcn5p plays a negative role in tension sensing, a lysine deacetylase(s) (KDAC) would likely be involved as well. If true, deleting such a deacetylase gene (equivalent to upregulating the HAT activity of Gcn5p) was predicted to cause a phenotype opposite of deleting *GCN5*, *e.g.*, elevated benomyl intolerance. Indeed, when *RPD3* or *HOS2* was deleted, the G44A cells exhibited benomyl hypersensitivity more severe than G44A, *rpd3*Δ, and *hos2*Δ single mutants (Figure 7A). Knocking out *SIN3*, which encodes a partner of Rpd3p for protein deacetylation (Kasten *et al.* 1997), caused the same phenotype as that of G44A *rpd3*Δ (Figure 7A), strongly suggesting that the Rpd3p/Sin3p deacetylase complex was involved. In contrast, the null alleles of two other KDAC genes *HDA1* and *HOS1* failed to cause a similar synthetic phenotype, indicating functional differentiation among these enzymes in mitosis. Besides benomyl hypersensitivity, the combination of *rpd3*Δ and the H3

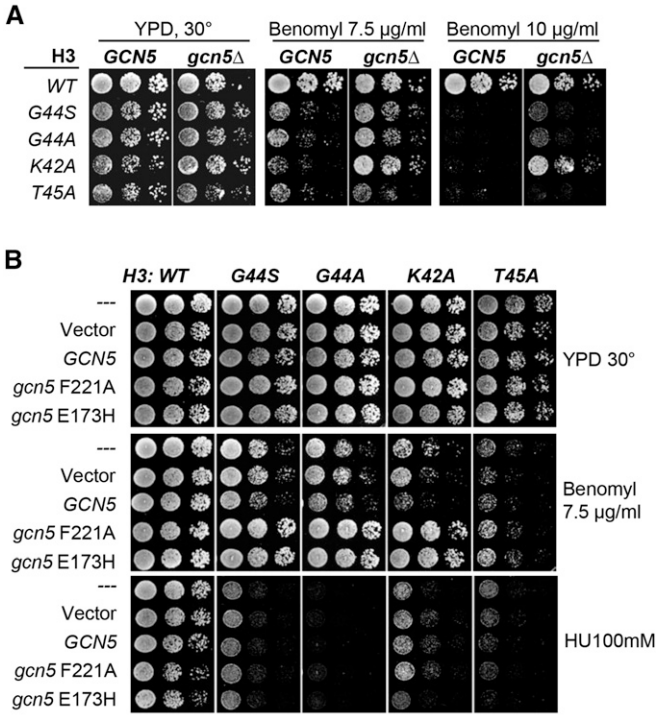

Figure 5 Gcn5p histone acetyltransferase is a negative regulator of the histone H3 TSM. (A) Deleting *GCN5* rescued the benomyl hypersensitivity phenotype of mutations at K42 and G44, but to a lesser extent for T45A allele. (B) The HAT activity of Gcn5p is linked specifically to the mitotic defect of TSM mutations. Dominant negative, catalytically inactive mutant Gcn5p bearing the E173H or F221A mutation (Kuo *et al.* 1998) was overexpressed in different TSM mutant strains. The cellular sensitivity to benomyl and HU was tested. Only benomyl hypersensitivity was rescued. (C) The E173H and F221A Gcn5p suppressors for TSM mutations require Sgo1p. *SGO1* was deleted in WT and different TSM mutants expressing different *GCN5* alleles. The cellular tolerance to benomyl was assessed.

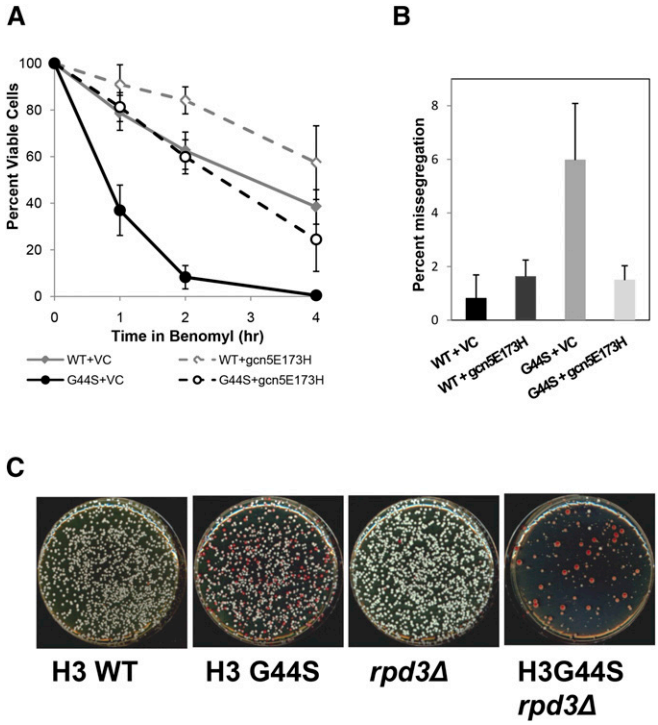

Figure 6 Gcn5p and Rpd3p affect chromosome stability via genetic interaction with the TSM. (A) WT and G44S strains bearing a *gcn5 E173H* overexpressing plasmid were treated with benomyl for the indicated time before plating to a benomyl-free medium to assess cellular viability. G44S cells lost viability fast but could be rescued by the dominant negative allele of Gcn5p. (B) Segregation of a GFP-marked chromosome was assessed by fluorescent microscopy. G1 phase cells bearing two GFP dots, indicating cosegregation of the indicator chromosome, were scored and expressed as percent of missegregation. (C) Deleting *RPD3* augments chromosome instability caused by a tension sensing mutation. The indicated yeast strains bearing an artificial chromosome that rescues the *ade2*$^-$ red colony phenotype were plated to YPD medium after overnight growth. Retention of the artificial chromosome gives rise to white colonies, the loss of which renders colonies exhibiting red color. The relative size of the red sector is dictated by the time of the chromosome loss during colonization. Total red colonies result from chromosome loss before inoculation to the plate. G44S *rpd3Δ* double mutant cells produce practically only all-red colonies. Error bars from A and B were calculated from at least three biological replica.

G44S allele caused significant frequency of chromosome loss, as revealed by the sectoring assays using a nonessential artificial chromosome (Figure 6C) (Spencer *et al.* 1990). Deleting *RPD3* alone did not cause a discernible effect on chromosome stability. Moreover, similar to the observation that a single catalytic dead mutation of Gcn5p was sufficient to elicit the suppression phenotype (see Figure 5 above), an H150A point mutation introduced to the active center of Rpd3p KDAC (Kadosh and Struhl 1998) was sufficient to cause synthetic benomyl hypersensitivity in the H3 G44A background (Figure 7B), indicating that the KDAC activity of Rpd3p was responsible for the observed mitotic defects. Western blotting of whole cell lysates demonstrated comparable abundance of the WT and the H150A species of Rpd3p (Figure S5). Moreover, all TSM mutants are suppressed by Sgo1p overexpression (Luo *et al.* 2010; Ng *et al.* 2013) (Figure 1D above). This suppression was ablated by deleting *RPD3* (black *vs.* white arrows, Figure 7C), indicating that Rpd3p is essential for Sgo1p to function in the surveillance of biorientation when the TSM is crippled (Figure S6).

A role of Rpd3p and Gcn5p in tension sensing was further supported by ChIP that showed enrichment of both enzymes at centromeres and pericentromeres (Figure 8). When these two proteins were epitope tagged (13×Myc for Gcn5p and 3×HA for Rpd3p) and ChIP'ed from benomyl-arrested G2/M phase cells, both CEN and pericentromeres harbored significant

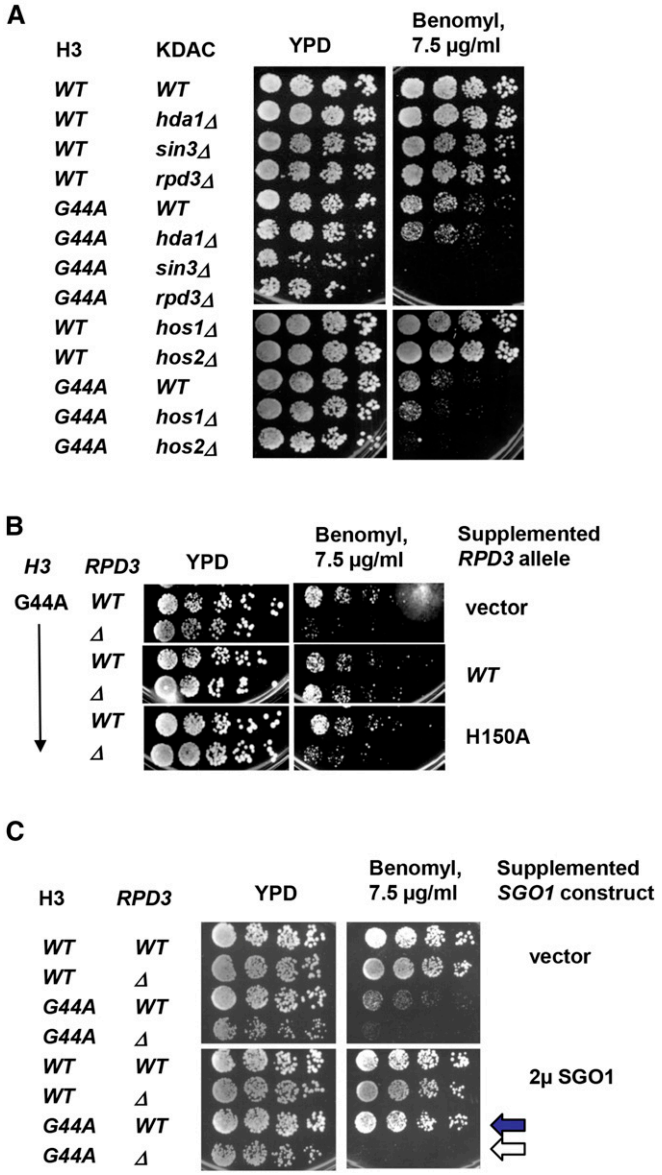

Figure 7 Histone deacetylases Rpd3p and Hos2p functionally interact with the H3 TSM. (A) Different HDAC genes were deleted in the G44A background to assess the effect on the benomyl hypersensitivity of the G44A cells. (B) The HDAC activity of Rpd3 underlies the genetic interaction between *RPD3* and H3 G44A allele. WT and G44A cells with an *rpd3Δ* null allele were transformed with an empty vector, WT *RPD3*, or *rpd3* bearing an inactivating H150A mutation. The cellular sensitivity to benomyl was examined. (C) Sgo1p multicopy suppressor for the G44A mutant allele of the TSM requires Rpd3p.

levels of Gcn5p and Rpd3p when compared with a transcriptionally silenced subtelomeric region. The enrichment of Gcn5p and Rpd3p at the centromeres and nearby regions is consistent with the notion that the mitotic function of Sgo1p and TSM are subjected to the local regulation by these two enzymes. To further delineate the molecular underpinning of how Gcn5p may control the Sgo1p–TSM interaction, ChIP was conducted to examine the recruitment of Sgo1p in the *gcn5 E173H* suppressor background. In contrast to the common notion that Sgo1p monitors the biorientation status only at centromeres and pericentromeres, the *gcn5 E173H* suppressor apparently rescues the G44S TSM mutant in a manner that does not require persistent presence of Sgo1p at the centromeres or the pericentromeric region (Figure 9). Conversely, overexpressing Sgo1p in the same mutant background successfully reestablished the pericentric Sgo1p recruitment (Figure 9B, white bars). These results suggest that Sgo1p may also have an activity that does not require stable association with centromeres or pericentromeres, and that this activity, liberated in *gcn5*$^-$ cells, complements the defects of the TSM of H3.

Discussion

This work characterizes a TSM in histone H3 that is important for mitotic checkpoint control. The core residues of this motif, Lys42, Gly44, and Thr45, are important for interactions with Sgo1p both *in vitro* and *in vivo*. Alanine substitutions of these three residues share similar mitotic phenotypes, and are all susceptible to the overexpression of *SGO1*, suggesting that they function in the same pathway for monitoring tension between sister chromatids. These results are consistent with the scanning mutagenesis experiments reported by others (Kawashima *et al.* 2011; Ng *et al.* 2013). ChIP assays showed that mutating residues of the TSM selectively diminished the pericentric, but not the centromeric recruitment of Sgo1p. We suggest that Sgo1p is first recruited to the centromeres by factors such as the kinase Bub1p and phosphorylated histone H2A (Fernius and Hardwick 2007; Kawashima *et al.* 2010; Williams *et al.* 2016), and then relocates to pericentromeres where histone H3 is part of the canonical nucleosomes (Luo *et al.* 2010). Retention of pericentric Sgo1p is mediated through the TSM of H3. The loss of pericentric domains of Sgo1p results in malfunction in tension sensing and chromosome missegregation. Intriguingly, the mitotic function of TSM appears to be regulated by the histone acetyltransferase Gcn5p, and at least two deacetylases, Rpd3p and Hos2p. That changing *GCN5*, *RPD3*, or *HOS2* status alone does not cause a significant mitotic phenotype in the WT H3 background suggests that these enzymes function within the TSM–Sgo1p tension sensing pathway, and likely play auxiliary roles that become indispensable when TSM is crippled.

The benomyl hypersensitivity phenotype caused by K42A, G44A, and G44S alleles is suppressed by deleting *GCN5* or inactivating its HAT activity and is augmented by the loss of *RPD3*, *SIN3*, or *HOS2* deacetylase genes. Chromosome instability is also rescued by the E173H catalytically inactive mutant of Gcn5p (Figure 6, A and B), but drastically augmented by the deletion of *RPD3* (Figure 6C). Importantly, the suppressor activity of *gcn5*$^-$ alleles depends critically on Sgo1p (Figure 5C). The *SGO1* high-copy suppressor for G44A is practically eliminated in the absence of *RPD3* (Figure 5C), whereas the chromosome loss rate of G44S cells rises greatly (Figure 6C). Together, these results strongly suggest that protein acetylation is a critical component of tension sensing

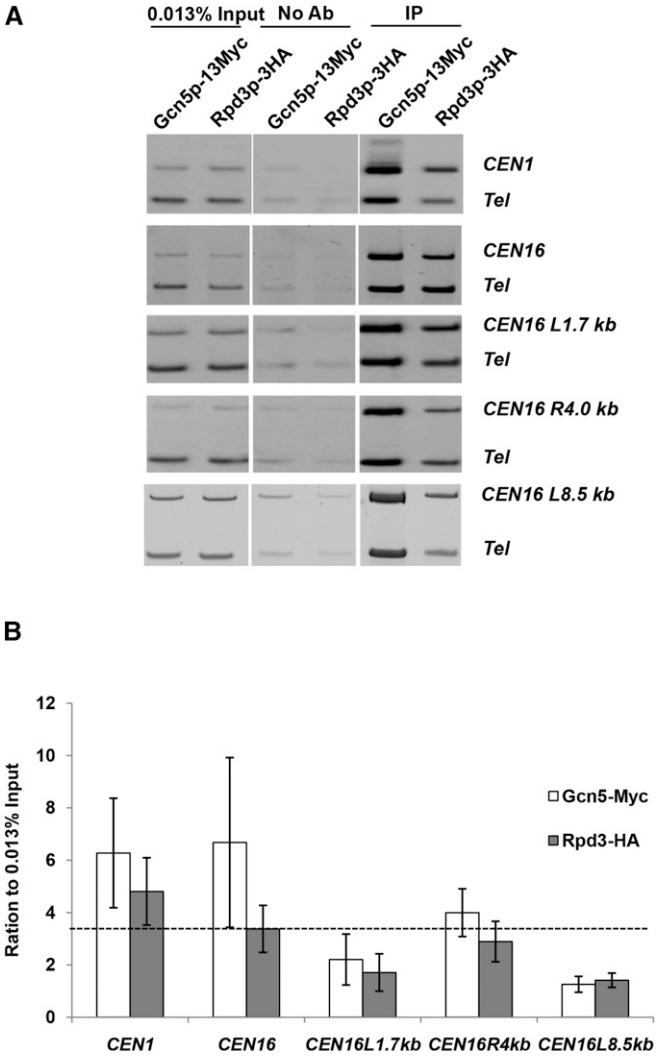

Figure 8 Both Gcn5p and Rpd3p are present in centromeres and pericentromeres. Myc-tagged Gcn5p and HA-tagged Rpd3p were ChIP'ed with the cognate antibodies. Quantitative PCR was conducted with primers corresponding to a selective chromosomal locus and a common telomeric region (*Tel*) as the internal control. The ratio of selective *vs.* the telomere control were calculated and normalized to that of input. The dotted line in B represents the experiment-to-input baseline. Error bars are from three biological replica.

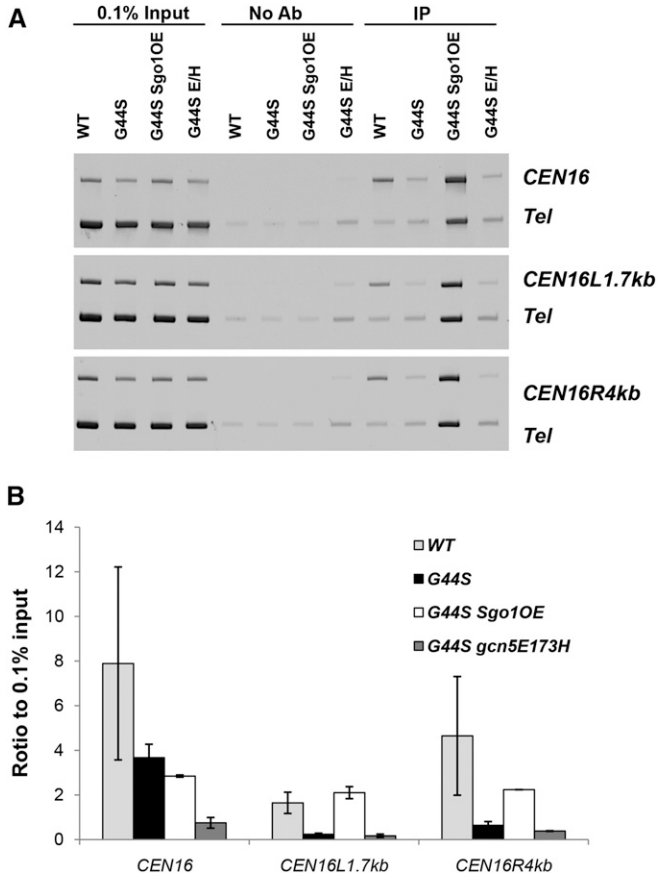

Figure 9 *gcn5 E173H* suppressor rescues tension sensing motif mutation independently of pericentric Sgo1p recruitment. Association of Sgo1p with *CEN16* and the nearby pericentric regions was examined by ChIP. See Figure 8 legends for assay details. OE, overexpression. Errors were calculated from two biological replica.

executed by H3 and Sgo1p. Indeed, both Gcn5p and Rpd3p are found to be enriched at centromeres and pericentromeres during mitosis (Figure 8). It is tempting to speculate that one or more centromeric and pericentric proteins are acetylated by Gcn5p in its antagonizing the H3–Sgo1p mitotic function. While the TSM includes a potentially acetylatable lysine residue, it is unlikely that Lys42 is a functional acetylation target herein because the K42R mutation, which mimics a constitutively unacetylated state, does not suppress the concomitant G44S mutant (Figure 1B). Instead, one can envision that acetylation of Sgo1p by Gcn5p diminishes the affinity for the TSM. The biochemical test of this hypothesis is currently being pursued. In addition to Sgo1p, cohesin may also be targeted by Gcn5p and Rpd3p. Cohesin plays an important role in localizing Sgo1p to the pericentromeres (Hou *et al.* 2007; Gutierrez-Caballero *et al.* 2012; Liu *et al.* 2013a). The recruitment of mammalian cohesion complex to centromeres and pericentromeres requires the acetyltransferase activity of San (Hou *et al.* 2007) and Esco1 (Whelan *et al.* 2012). However, our genetic data indicate that the acetylation is a negative regulator of tension sensing. If one of the cohesin subunits is acetylated by Gcn5p, this action may inhibit either the pericentric loading of cohesin or its interaction with Sgo1p.

It is also intriguing that the suppression brought about by eliminating the activity of Gcn5p does not require stable association of Sgo1p with centromeres and pericentromeres (Figure 9), which is in stark contrast to the observation that overexpressing Sgo1p restores the pericentric Sgo1p enrichment. One explanation is that the association between Sgo1p and chromatin, though restored, is more dynamic in the *gcn5*$^{-}$ background due to, *e.g.*, changes in centromeric and pericentric chromatin structure, so that the standard ChIP procedures fail to efficiently detect pericentric Sgo1p. Alternatively, it is possible that Sgo1p's tension sensing function includes a phase that is separable from chromatin

binding. This function is normally kept dormant by Gcn5p and is awakened upon deleting *GCN5*. For example, Jin and Wang (2013) suggested that Sgo1p functions in the SAC silencing network. However, the physical form or location of Sgo1p in this network remains to be delineated. The pericentromere and centromere binding-independent function of Sgo1p as revealed by the *gcn5*$^{-}$ suppressor may provide a direction for more work.

Compared with other mutations in the TSM, the T45A allele responds poorly to the suppression by *gcn5* and less well to multicopy *SGO1* overexpression (Figure 1C and Figure 5B, respectively). Because K42A, G44A, G44S, and T45A alleles all show weakened interactions with Sgo1p (Figure 4D) (Luo *et al.* 2010), it seems likely that Thr45 plays a qualitatively distinct role in mediating H3–Sgo1p interaction. One possibility is that the H3–Sgo1p interaction requires the side chain of Thr45, whereas Lys42 and Gly44 together provide an environment (*e.g.*, the sharp-turn structure, Figure 1E) that facilitates the Thr45–Sgo1p contact. If true, this hypothesis suggests that a post-translational modification at this residue would impose a significant impact on Sgo1p interaction. Indeed, Thr45 phosphorylation has been reported in both yeast and humans. The yeast Dbf4p–Cdc7p complex catalyzes Thr45 phosphorylation for DNA replication (Baker *et al.* 2010), whereas the mammalian Thr45 phosphorylation event is linked to apoptosis (Hurd *et al.* 2009) and to DNA damage (Lee *et al.* 2015). The phosphomimetic T45E mutation causes cell death (Baker *et al.* 2010 and data not shown), preventing detailed genetic experiments for the possible involvement of Thr45 phosphorylation in mitosis. However, our preliminary biochemical experiments showed that phosphorylation at Thr45 blocked *Sgo1p* interaction *in vitro* (data not shown), suggesting that Dbf4p–Cdc7p or another kinase may control the H3–Sgo1p interaction via Thr45 phosphorylation. Additionally, given the potential contribution of acetylation in the regulation of the TSM, it is worth noting that serine and threonine can also be acetylated (Mukherjee *et al.* 2006, 2007; Tweedie-Cullen *et al.* 2012). Whether Gcn5p could use Thr45 as an acetylation target for mitotic control awaits to be examined.

Sgo1p undergoes biorientation-dependent removal from chromatin (Nerusheva *et al.* 2014), suggesting that Sgo1p and, in particular, its interaction with chromatin is tightly linked to the status of tension. Pericentric chromatin structural changes seem to be an obligatory outcome of bipolar attachment (Haase *et al.* 2012; Verdaasdonk *et al.* 2012; Stephens *et al.* 2013). In tension defects, *sgo1Δ* cells either cannot activate the spindle checkpoint (Indjeian *et al.* 2005) or cannot prevent the silencing of SAC (Jin and Wang 2013). The latter model was supported by a recent report that Sgo1p, along with Ipl1p, Dam1p, and protein phosphatase 1 (PP1), is required to keep SAC on in the absence of tension (Jin and Wang 2013). The data presented above demonstrate a causal role of the loss of pericentric Sgo1p in crippling the tension sensing function. We suggest that the TSM of histone H3 in the pericentromeres functions initially as a docking site for Sgo1p, which is recruited to the centromeres by *Bub1p* and phosphorylated H2A, while cells establish bipolar attachment. Biorientation generates tension, which in turn alters chromatin structure in the pericentromeres (Haase *et al.* 2012; Verdaasdonk *et al.* 2012; Stephens *et al.* 2013), resulting in conformational changes of the TSM that disrupt Sgo1p–H3 interaction. The eviction of Sgo1p molecules from the pericentromere of the last pair of chromosomes under tension silences the checkpoint, hence allowing cells to progress from metaphase to anaphase. Whether the mere absence of Sgo1p or the exiting Sgo1p proactively shuts off SAC awaits to be delineated.

Acknowledgments

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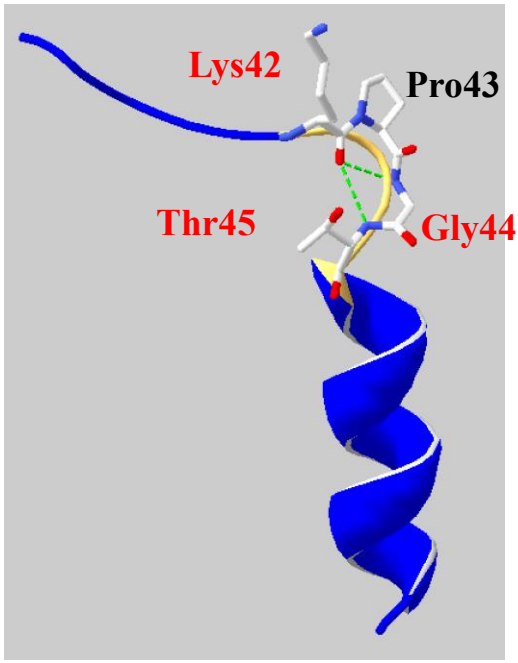
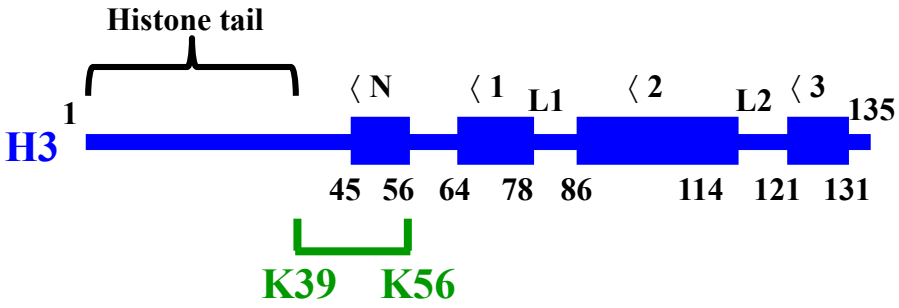
Supplemental Figure S1





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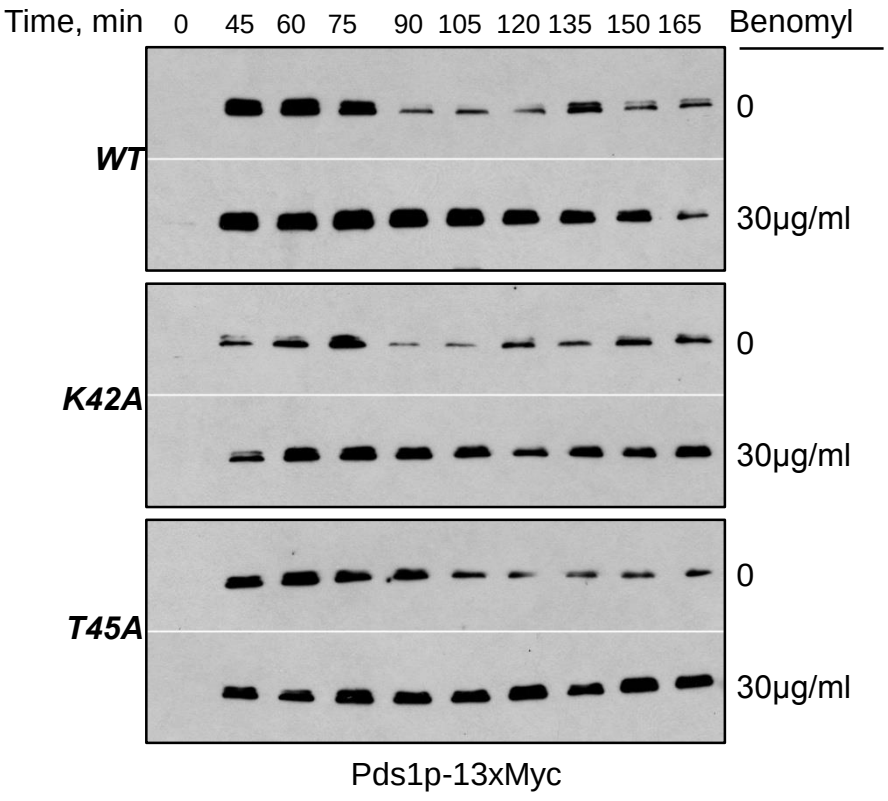

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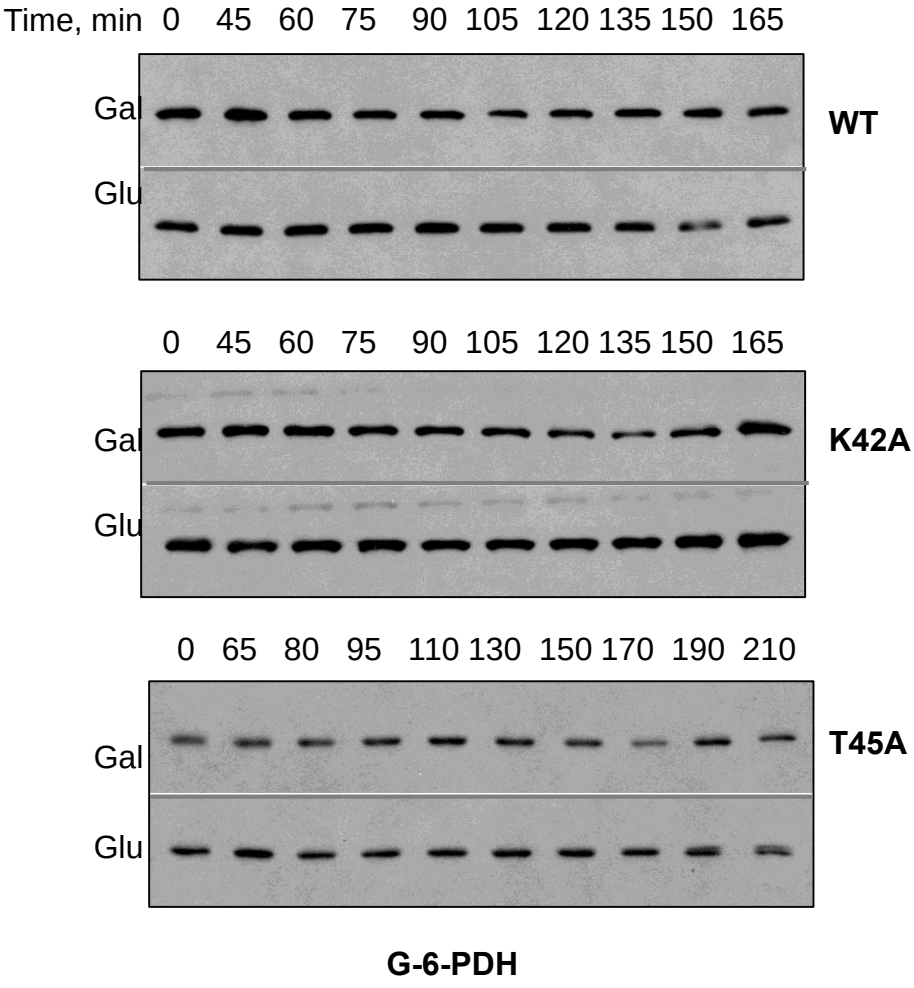

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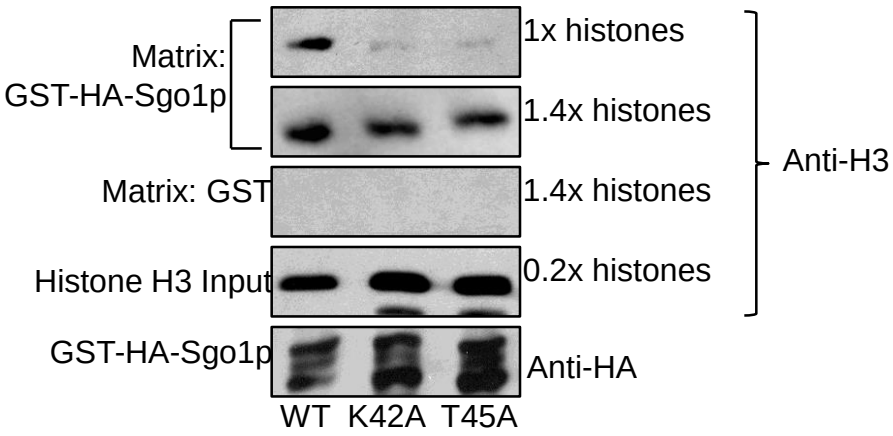

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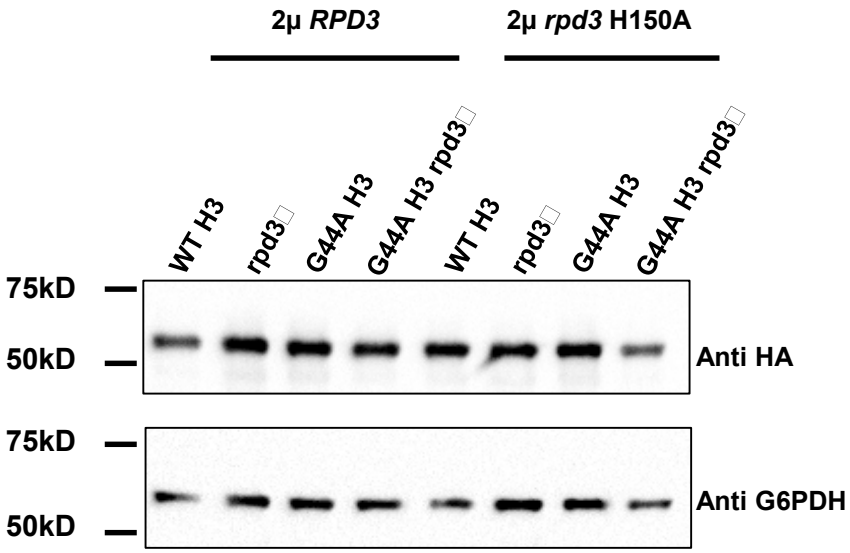

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