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REPORT

Budding yeast CENP-A^{Cse4} interacts with the N-terminus of Sgo1 and regulates its association with centromeric chromatin

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ABSTRACT

Shugoshin is an evolutionarily conserved protein, which is involved in tension sensing on mitotic chromosomes, kinetochore biorientation, and protection of centromeric (*CEN*) cohesin for faithful chromosome segregation. Interaction of the C-terminus of Sgo1 with phosphorylated histone H2A regulates its association with *CEN* and pericentromeric (peri-*CEN*) chromatin, whereas mutations in histone H3 selectively compromise the association of Sgo1 with peri-*CEN* but not *CEN* chromatin. Given that histone H3 is absent from *CEN* and is replaced by a histone H3 variant CENP-A^{Cse4}, we investigated if CENP-A^{Cse4} interacts with Sgo1 and promotes its association with the *CEN* chromatin. In this study, we found that Sgo1 interacts with CENP-A^{Cse4} *in vivo* and *in vitro*. The N-terminus coiled-coil domain of Sgo1 without the C-terminus (*sgo1-NT*) is sufficient for its interaction with CENP-A^{Cse4}, association with *CEN* but not the peri-*CEN*, and this *CEN* association is cell cycle dependent with maximum enrichment in mitosis. In agreement with the role of CENP-A^{Cse4} in *CEN* maintenance of Sgo1, depletion of CENP-A^{Cse4} results in the loss of Sgo1 and *sgo1-NT* from the *CEN* chromatin. The N-terminus of Sgo1 is required for genome stability as a mutant lacking the N-terminus (*sgo1-CT*) exhibits increased chromosome missegregation when compared to a *sgo1-NT* mutant. In summary, our results define a novel role for the N-terminus of Sgo1 in CENP-A^{Cse4} mediated recruitment of Sgo1 to *CEN* chromatin for faithful chromosome segregation.

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Introduction

Erroneous chromosome segregation causes chromosomal instability (CIN), which contributes to aneuploidy observed in several cancers, developmental disorders, and birth defects.[1–3] Checkpoints such as the spindle assembly checkpoint (SAC) serve as a quality control mechanism to block the progression of the cell cycle from metaphase to anaphase and prevent CIN.[4,5] The SAC monitors kinetochore-spindle attachment and tension between sister chromatids.[5–7] The chromosome passenger complex (CPC) which contains the evolutionarily conserved aurora kinase Ipl1 (aurora B), Sli15, Bir1, and Nbl1[8] responds to erroneous kinetochore-spindle attachments.[8] The CPC is recruited to the kinetochore microtubule interface and phosphorylates its substrates thereby allowing for error correction and cell cycle progression.[8]

In addition to the CPC, evolutionarily conserved Shugoshin (Sgo1 in yeast) also plays a role in sensing kinetochore tension.[9,10] The molecular function of shugoshin during meiosis is well established and is evolutionarily conserved among eukaryotic organisms.[9,11] One of the functions of Sgo1 is to protect sister-chromatid cohesion duing meiosis I.[12–15] Protection of cohesion is achieved by recruitment of heterotrimeric protein phosphatase PP2A to the centromeric chromatin mediated by the N-terminal coiled-coil domain of Sgo1 during meiosis.[11,12,15–17] In addition to cohesin protection, shugoshins are also involved in biorientation of homologous chromosomes and spindle checkpoint silencing during meiosis.[9,11] In contrast to a well-defined role of Sgo1 in meiosis, there are fewer studies describing a role for Sgo1 in mitosis. In fission yeast, Sgo1 affects centromeric (*CEN*) localization of Ipl1 kinase during mitosis,[10,18] and *Xenopus* egg extracts depeleted of Sgo1 show altered aurora B kinase activity.[19] In budding yeast, Sgo1 has a limited role in cohesion protection in mitosis,[20–22] instead, it is involved in tension sensing on mitotic chromosomes, and regulation of kinetochore biorientation.[20,21,23,24]

Sgo1 associates with core *CEN* and peri-*CEN* (50 kb DNA flanking the *CEN)* regions in budding yeast as well as in other eukaryotic organisms.[9,21,23,25] Phosphorylation of histone H2A (S121) by the Bub1 kinase contributes to the recruitment of Sgo1 at the core *CEN* and peri-*CEN* regions.[24,26,27] The Bub1-H2A-shugoshin mitotic pathway is evolutionarily conserved from budding yeast to mammalian systems.[28–31] In addition, the mitotic kinase, Mps1 has been shown to act as an upstream regulator of Sgo1, and *CEN* recruitment of Sgo1 requires Mps1 kinase activity in budding yeast and human cells.[32,33]

In meiosis, deletions of kinetochore genes *IML3* and *CHL4* selectively compromise the association of Sgo1 with peri-*CEN* but not the core *CEN*.[21] Studies with mitotic cells have shown that an interaction of histone H3 with Sgo1 is required for the association of Sgo1 with peri-*CEN* but not the core *CEN*, and that the H3-Sgo1 interaction is essential for tension sensing,

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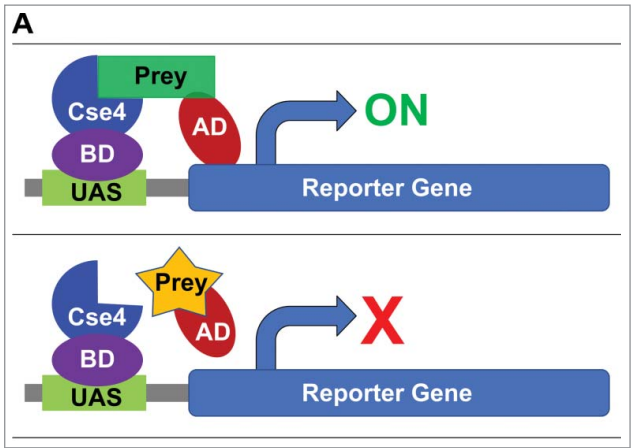
and kinetochore biorientation.[23] However, histone H3 is not present in budding yeast *CEN* nucleosomes,[34,35] hence additional recruitment mechanisms must regulate the localization of Sgo1 to the core *CEN*.

Budding yeast contains "point" *CENs* that are small in size with a well-defined conserved 125 bp DNA sequence in contrast to regional *CENs* in other eukaryotes, which are composed of alpha-satellite DNA repeats ranging in size from 0.1 to 5 Mb.[34,36,37] Despite the variations in *CEN* DNA, all eukaryotic centromeres contain a specific histone H3 variant (CENP-A in humans, Cse4 in budding yeast) that replaces histone H3 in the *CEN* nucleosomes.[38–40] Unlike regional *CENs* where CENP-A nucleosomes are interspersed with histone H3 nucleosomes,[38,39] point *CENs* contain only Cse4 nucleosomes, and are devoid of histone H3 nucleosomes making budding yeast an ideal model system to investigate the differences between Sgo1 at the peri-*CEN* and core *CEN*. Hence, we investigated the *in vivo* interaction of Sgo1 with CENP-A^{Cse4}, defined the domain of Sgo1 that interacts with CENP-A^{Cse4}, and identified the minimum domain of Sgo1 that regulates its association with the core *CEN* chromatin for faithful chromosome segregation.

In this study, we show that Sgo1 interacts with CENP-A^{Cse4} *in vivo* and *in vitro*. The N-terminus coiled-coil domain of Sgo1 (termed as *sgo1-NT*; amino acid residues 1–150) is sufficient for its interaction with CENP-A^{Cse4}. Full length Sgo1 and *sgo1-NT* associate with *CEN* chromatin and their *CEN* association is regulated by the cell cycle with maximum enrichment observed in mitotic cells. While Sgo1 associates with the core *CEN* and peri-*CEN* regions, *sgo1-NT* only associates with the core *CEN*. CENP-A^{Cse4} is required for the association of Sgo1 and *sgo1-NT* with core *CENs* as depletion of CENP-A^{Cse4} results in the loss of Sgo1 and *sgo1-NT* from the *CEN* chromatin. Moreover, we found that strains with deletion of the N-terminus of Sgo1 (*sgo1-CT*) exhibit errors in chromosome segregation. Our results have uncovered a novel interaction of the N-terminus of Sgo1 with CENP-A^{Cse4} that is required for faithful chromosome segregation.



B

Gene	Molecular Role
HHF1	Histone H4, component of nucleosomes
MCM10	Initiation of DNA replication, DNA fork protection
MIF2	Kinetochore assembly, chromosome segregation
PPH21	Regulation of mitosis, catalytic subunit of PP2A
SAK1	Serine/threonine kinase for Snf1 complex
SCM3	CENP-A^{Cse4} chaperone, kinetochore assembly
SGO1	Cohesin protection, spindle tension sensing, spindle biorientation, chromosome segregation
SNF1	Serine/threonine kinase, role in transcription
SNF4	Activating gamma subunit of Snf1 kinase complex
SPS1	Putative serine/threonine kinase

Figure 1. Genome-wide two-hybrid screen for CENP-A^{Cse4} identified Sgo1 as an interacting partner. (A) Schematic of yeast two-hybrid assay to identify interactors of CENP-A^{Cse4} used here as a bait. BD = binding domain, AD = activating domain, UAS = upstream activating sequence. *GAL4* gene, a transcription factor, produces BD and AD protein products, which are required for transcription of the reporter gene. *GAL4-BD*+CENP-A^{Cse4} and *GAL4-AD*+Prey (yeast genes) fusion constructs were used. These fusion proteins alone cannot activate reporter gene transcription; however, expression of both fusion proteins allows interaction between CENP-A^{Cse4} and prey protein leading to transcription of the reporter gene. (B) List of genes showing statistically significant two-hybrid interaction with CENP-A^{Cse4}.

Results

A genome-wide two-hybrid screen with CENP-A^{Cse4} identified Sgo1 as an interacting protein

We performed a genome-wide yeast two-hybrid screen using full length CENP-A^{Cse4} to identify possible interactors of CENP-A^{Cse4} (Fig. 1A). CENP-A^{Cse4} was fused to the *GAL4* DNA-binding domain (DBD) and this bait strain was screened against the genome-wide array of 6000 prey strains each of these expresses a gene fusion of the open reading frame to the *GAL4* activation domain (AD)[41] and a mini-array of kinetochore and spindle strains.[42] This genome-wide set of two-hybrid strains has successfully been used to identify novel kinetochore protein-protein interactions.[42,43] The screen identified ten proteins that interact with CENP-A^{Cse4} (Fig. 1B). The Gene Ontology (GO) enrichments for biological process and cellular components for these proteins are available in the supplementary information (Table S1). Included amongst these are two kinetochore proteins, Scm3, Mif2, and histone H4, a constituent of the CENP-A^{Cse4} nucleosomes.[44] The identification of pertinent positives that are involved in kinetochore function (p-value = 2E-04; Table S1) validates the two-hybrid screen. Phosphatase (Pph21), and several inter-related kinases including two members of the Snf1 kinase complex (Snf1 and Snf4), its upstream kinase Sak1, as well as Sgo1 were also identified. Even though phosphorylation of CENP-A^{Cse4} has been previously reported,[45] a role for Snf1 kinase complex and Sak1 in modification of CENP-A^{Cse4} has not been described so far. We decided to focus on the interaction of Sgo1 with CENP-A^{Cse4} because of the role of Sgo1 in chromosome segregation. We confirmed the two-hybrid interaction using CENP-A^{Cse4} as a DBD-fusion and full length Sgo1 as an AD-fusion. A positive interaction results in the activation of the reporter gene *HIS3* and the growth of the transformants on medium lacking histidine with 3mM AT. We observed an interaction of CENP-A^{Cse4} with Sgo1, whereas no growth was observed with the AD vector (pADC), confirming the results of the genome-wide two-hybrid screen (Fig. 2A).

CENP-A^{Cse4} interacts in vivo with Sgo1 and its N-terminus coiled-coil domain

The identification of Sgo1 as a CENP-A^{Cse4} interacting protein was intriguing and hence we pursued further studies to

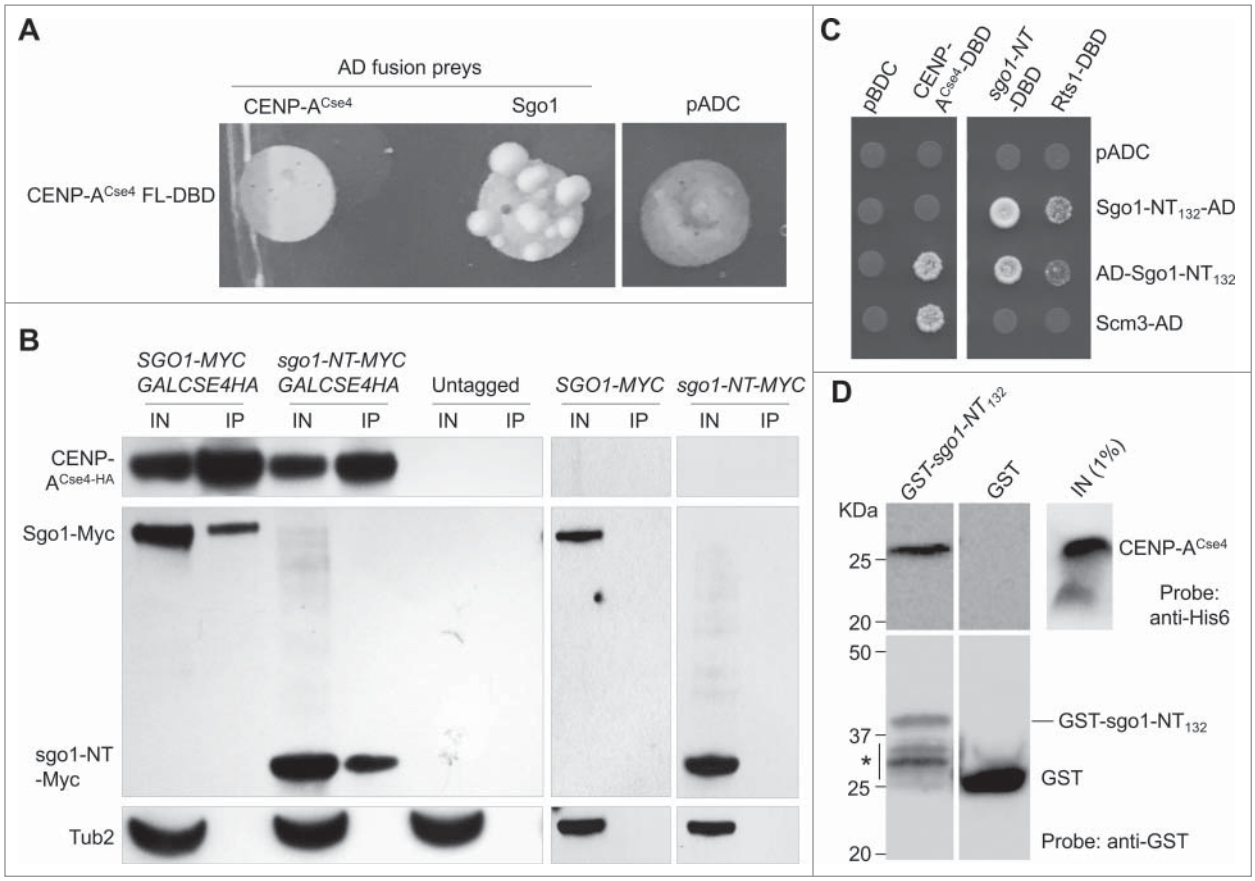


Figure 2. CENP-A^{Cse4} interacts with Sgo1 and *sgo1-NT in vivo* and *in vitro*. (A) Sgo1 prey constructs was tested against the CENP-A^{Cse4} bait construct. Sgo1 prey resulted in increased growth compared with vector (pADC) or CENP-A^{Cse4} prey when tested against the CENP-A^{Cse4} FL bait construct. (B) CENP-A^{Cse4} interacts *in vivo* with Sgo1 and *sgo1-NT*. Wild type strain (*SGO1-MYC*, YMB10165), *sgo1-NT-MYC* (YMB10160) expressing CENP-A^{Cse4} from galactose inducible promoter (*GAL1*) were grown in YEP with 2% galactose + 2% raffinose at 25°C. Untagged strain (OCF1533-4B), *SGO1-MYC* (AMY905), and *sgo1-NT-MYC* (YMB10076) were used as a control. Cell extracts were prepared for immunoprecipitation experiments using anti-HA agarose antibodies (A2095, Sigma-Aldrich). Eluted proteins were analyzed by Western blotting with anti-Myc (Sgo1-Myc or *sgo1-NT-Myc*; a-14, sc-789, Santa Cruz Biotechnology), anti-HA (CENP-A^{Cse4}; H6908, Sigma Aldrich), and anti-Tub2 (loading control) antibodies. IN = input, and IP = immunoprecipitated samples. (C) A minimal prey construct of AD-sgo1-NT$_{132}$ (amino acids 1–132) resulted in two-hybrid positive interactions with CENP-A^{Cse4}-DBD, *sgo1-NT* (amino acids 1–150) and Rts1-DBD. Scm3-AD was also positive with CENP-A^{Cse4}-DBD. Two-hybrid selection plates were SD–HLT and supplemented with 3-aminotriazole (1 or 3 mM). (D) Affinity pull-down of recombinant proteins shows that *GST-sgo1-NT$_{132}$* immobilized on glutathione beads can interact and pull-down His6-CENP-A^{Cse4} octasome from *E. coli* lysate. IN = input (1% of the input run on a separate gel). *Represents degraded or proteolyzed products. Pull-down experiments were performed three times with similar results.

investigate the biological relevance of this interaction. We performed immunoprecipitation (IP) experiments using agarose beads conjugated with anti-hemagglutinin (HA) antibodies as described in the materials and methods to examine if CENP-A^{Cse4} interacts with Sgo1 *in vivo* using a wild type strain that expresses Myc-tagged Sgo1 from its endogenous promoter, and HA-tagged CENP-A^{Cse4} from a *GAL1* promoter. IP results showed that Sgo1 interacts with CENP-A^{Cse4} *in vivo*. No signals were observed in control experiments performed using an untagged and Myc-tagged Sgo1 strains (Fig. 2B).

Budding yeast Sgo1 contains two distinct domains, which are evolutionarily conserved among eukaryotes: the N-terminus coiled-coil domain (*sgo1-NT*) that mediates homodimerization,[46] and interaction with proteins such as Rts1, a PP2A-B′ subunit,[47–49] and the C-terminus basic region, which is required for the interaction of Sgo1 with nucleosomes containing phosphorylated H2A.[26] Since the role of N-terminus of Sgo1 is not well defined, we investigated if the N-terminal domain of Sgo1 interacts with CENP-A^{Cse4} by performing IP experiments using a *sgo1-NT* strain carrying Myc-tagged N-terminus amino acid residues 1–150 expressed from its endogenous promoter. Western blot analysis showed an *in vivo* interaction between *sgo1-NT* and CENP-A^{Cse4} (Fig. 2B).

To further validate the *in vivo* interaction of the N-terminus of Sgo1 with CENP-A^{Cse4}, we analyzed additional N-terminal Sgo1 constructs using the two-hybrid system with different activation domain fusion contexts. A minimal construct of Sgo1 (amino acids 1–132) fused to the N-terminus of the activation domain (AD-sgo1-NT$_{132}$) was sufficient to interact with CENP-A^{Cse4} (Fig. 2C). Fusion of the Sgo1-NT$_{132}$ to the C-terminus of the activation domain (sgo1-NT$_{132}$-AD) did not result in an interaction indicating that this fusion context may hinder the interaction. Consistent with homodimerization of Sgo1 N-terminus,[46] a positive interaction was observed between Sgo1 bait (amino acids 1–150; *sgo1-NT-DBD*) and both the Sgo1 preys (AD-sgo1-NT$_{132}$ and sgo1-NT$_{132}$-AD). A similar result was observed for Rts1, which is known to interact with the Sgo1 N-terminal domain.[48,49] A positive interaction was observed between CENP-A^{Cse4} and Scm3 used as a control (Fig. 2C). Taken together, these results provide evidence for an *in vivo* interaction of Sgo1 with CENP-A^{Cse4} and show that *sgo1-NT* is sufficient for its interaction with CENP-A^{Cse4}.

CENP-A^{Cse4} interacts in vitro *with the N-terminus coiled-coil domain of Sgo1*

It is possible that a two-hybrid interaction between *sgo1-NT* and CENP-A^{Cse4} may be dependent on other proteins. Hence, we examined the interaction of CENP-A^{Cse4} and *sgo1-NT* using affinity purified CENP-A^{Cse4} and *sgo1-NT* constructs that were expressed in *Escherichia coli*. We expressed a GST-tagged N-terminus construct of Sgo1 encompassing amino acid residues 1–132 (*GST-sgo1-NT$_{132}$*; predicted molecular weight of 41.4 KDa) and immobilized the protein onto glutathione agarose beads. *GST-sgo1-NT$_{132}$* was tested against *E. coli* lysate containing RGSH6 tagged CENP-A^{Cse4} co-expressed with histones H2A, H2B and H4. Western blot analysis showed that *GST-sgo1-NT$_{132}$* pulled down CENP-A^{Cse4}, whereas the GST control failed to detect any CENP-A^{Cse4} (Fig. 2D). Taken together, these results show that the N-terminus coiled-coil domain of Sgo1 (*sgo1-NT*) is sufficient for its *in vitro* interaction with CENP-A^{Cse4} and does not require other yeast proteins to mediate the interaction.

N-terminus of Sgo1 is sufficient for association with the core CEN

The interaction of Sgo1 and *sgo1-NT* with CENP-A^{Cse4} *in vivo* and *in vitro* prompted us to examine if *sgo1-NT* can associate with *CEN* chromatin and if this was cell cycle regulated. Chromatin immunoprecipitation (ChIP) experiments were performed using wild type strains with Myc-tagged Sgo1 or *sgo1-NT* expressed from its endogenous promoter. Cells were synchronized in G1 (α-factor treatment), S-phase (hydroxyurea treatment), or in G2/M (nocodazole treatment) stages of the cell cycle. Western blot analysis confirmed the protein expression of Sgo1 and *sgo1-NT* in logarithmically grown cultures and a cell cycle-regulated protein expression of Sgo1 with detectable levels in S and G2/M phases and no detectable level in G1 cells (Fig. 3A) as reported previously.[47] The protein expression level of *sgo1-NT* was constant throughout the cell cycle, which is consistent with published data indicating that Sgo1 is degraded through a destruction box (amino acids 494–498) in the C-terminal region of the protein.[47] The cell cycle synchronization was confirmed by FACS analysis (Fig. 3B). Sgo1 enrichment was observed at core *CEN* (*CEN1, CEN3*, and *CEN5*) (Fig. 3, C, D and E) in logarthimically grown cells consistent with previous studies.[21,25] Remarkably, an association of *sgo1-NT* was also observed at core *CEN* in logarithmically grown cells (Fig. 3, C, D and E). No significant enrichment of either Sgo1 or *sgo1-NT* was detected at the negative control region *ACT1* or in an untagged control strain (Fig. 3, C, D, E and F). The enrichment of Sgo1 and *sgo1-NT* at *CEN* was significantly higher in mitotic (G2/M) cells, whereas no significant enrichment was observed in G1 and S-phase cells (Fig. 3C, D and E). Although statistically not significant (p-values = >0.05), the levels of *sgo1-NT* enrichment at core *CEN* in G2/M were slightly lower (~ 29.9 – 36.8%) than those observed for the full length Sgo1 (Fig. 3, C, D and E). These results show that the N-terminus of Sgo1 associates with the core *CEN* region in a cell cycle dependent manner independent of its constitutive expression throughout the cell cycle.

Sgo1 associates with peri-*CEN* regions, which include about 50 Kb DNA flanking the *CEN*.[9,21] In addition, peri-*CEN* regions in budding yeast are significantly enriched for cohesin components, Smc3 and Mcd1[50,51] consistent with the role of Sgo1 in protection of cohesins.[21,52] Hence, we examined whether *sgo1-NT* associates with peri-*CEN* regions in G2/M cells (Fig. 4A). As expected, Sgo1 associated at peri-*CEN* regions (Fig. 4B). In contrast to Sgo1, *sgo1-NT* was enriched significantly only at the core *CEN* but not at peri-*CEN* regions (Fig. 4B). No significant enrichment was detected in an untagged strain used as a negative control (Fig. 4B).

CEN *association of Sgo1 and* sgo1-NT *is dependent on CENP-A^{Cse4}*

The enrichment of Sgo1 and *sgo1-NT* at *CEN* occurs in mitotic cells (Fig. 3), whereas budding yeast CENP-A^{Cse4} is recruited to the *CEN* in early S-phase and remains associated with *CEN* chromatin throughout the cell cycle.[53,54] Given our results for the association of Sgo1 and *sgo1-NT* with CENP-A^{Cse4} *in vivo* (Fig. 2B), we posited that CENP-A^{Cse4} may contribute to the maintenance of Sgo1 and *sgo1-NT* at the *CEN*. Hence, we constructed strains carrying endogenously expressed Myc-tagged Sgo1 or *sgo1-NT* in which CENP-A^{Cse4} expression was under the control of galactose inducible promoter (*GAL1*). CENP-A^{Cse4} is expressed only when these strains are grown in media containing galactose (CENP-A^{Cse4}-ON), whereas expression is shut-off when switched to media containing glucose (CENP-A^{Cse4}-OFF). ChIP experiments were performed using these strains grown at 25°C in galactose (CENP-A^{Cse4}-ON), and after shifting to glucose medium for three hours (CENP-A^{Cse4}-OFF). The galactose-induced expression of CENP-A^{Cse4} and glucose mediated shut-off was confirmed by Western blotting in logarithmically growing cultures (Fig. 5A) and DNA content was determined by FACS analysis (Fig. 5B). As expected the enrichment of CENP-A^{Cse4} at core *CEN* is significantly higher in CENP-A^{Cse4}-ON cells, whereas core *CEN*-associated CENP-A^{Cse4} levels are reduced significantly in CENP-A^{Cse4}-OFF conditions (Fig. 5C). Sgo1 and *sgo1-NT* both were significantly enriched at the core *CEN* under CENP-A^{Cse4}-ON conditions. However, the levels of Sgo1 and *sgo1-NT* were reduced significantly at core *CEN* in CENP-A^{Cse4}-OFF cells (~10-fold reduction of Sgo1; ~5-fold reduction of *sgo1-NT*) (Fig. 5D). No significant enrichment of CENP-A^{Cse4}, Sgo1, and *sgo1-NT* was detected at *ACT1*, or at core *CEN* and *ACT1* regions in an untagged control strain (Fig. 5C and D). These results show that CENP-A^{Cse4} is required for the maintenance of Sgo1 and *sgo1-NT* at the core *CEN* chromatin.

*Deletion of N-terminus of Sgo1 (*sgo1-CT*) causes errors in chromosome segregation*

The association of *sgo1-NT* with core *CENs* prompted us to examine if the N-terminus of Sgo1 regulates faithful chromosome segregation independent of its C-terminus basic region. A colony color assay was performed to measure the loss of a non-essential reporter chromosome fragment (CF)[55] in wild-type, *sgo1Δ* and *sgo1* mutant strains. *sgo1-NT* expresses Myc-tagged N-terminus amino acid residues 1–150; and *sgo1-CT*

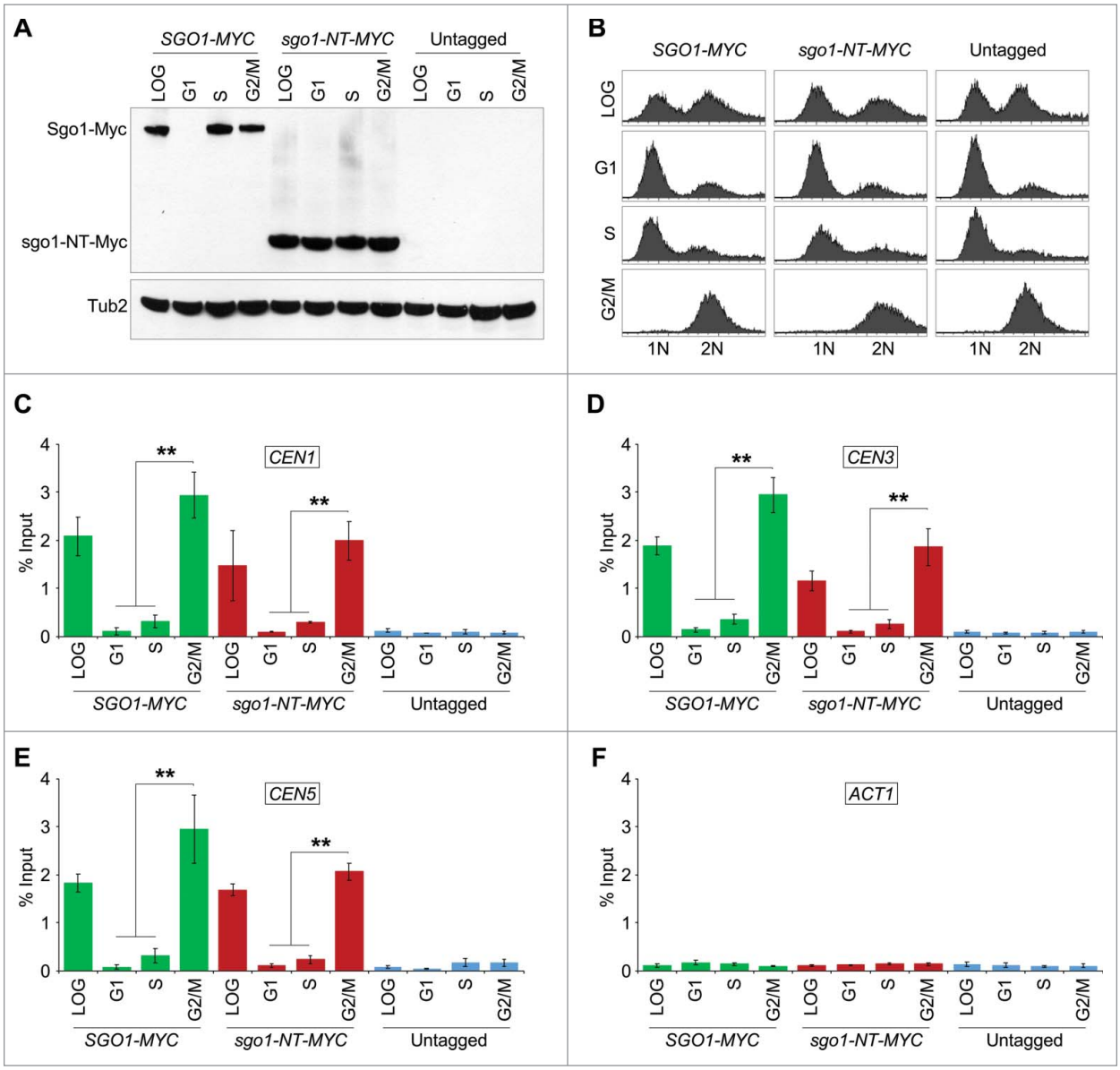


Figure 3. Sgo1 and *sgo1-NT* associate with core *CEN* in a cell cycle dependent manner. Wild type (*SGO1-MYC*, AMY905), *sgo1-NT-MYC* (YMB10076), and untagged control (OCF1533-4B) were grown in YPD to logarithmic phase (LOG) at 25°C, and synchronized in G1 with alpha factor (3 μM), S-phase with HU (0.2 M), and in G2/M with nocodazole (20 μg/mL) at 25°C for 2 hours. ChIP was performed using anti-Myc agarose beads (A7470, Sigma-Aldrich). Enrichment at *CEN* and at a negative control region *ACT1* was determined by qPCR and is shown as % input. Average from three biological replicates ± standard error is shown. **p value <0.01, Student's t test. (A) Western blotting showing expression of Sgo1-Myc and *sgo1-NT-Myc* in LOG or various stages of the cell cycle. Antibodies used were: anti-Myc (Sgo1 or *sgo1-NT*; a-14, sc-789, Santa Cruz Biotechnology), and anti-Tub2 (loading control) antibodies. (B) FACS profiles show DNA content representing various stages of the cell cycle. (C) ChIP-qPCR showing enrichment levels of Sgo1-Myc and *sgo1-NT-Myc* at *CEN1* in LOG phase or at various stages of the cell cycle. (D) ChIP-qPCR showing enrichment levels of Sgo1-Myc and *sgo1-NT-Myc* at *CEN3* in LOG phase or at various stages of the cell cycle. (E) ChIP-qPCR showing enrichment levels of Sgo1-Myc and *sgo1-NT-Myc* at *CEN5* in LOG phase or at various stages of the cell cycle. (F) ChIP-qPCR showing enrichment levels of Sgo1-Myc and *sgo1-NT-Myc* at a negative control region *ACT1* in LOG phase or at various stages of the cell cycle.

expresses Myc-tagged C-terminus amino acid residues 151–590 (Fig. 6A). The frequency of CF loss was measured by counting colonies that were at least half red indicating the loss of CF in the first cell division. The deletion of *SGO1* gene results in significantly higher chromosome segregation errors compared to the wild type strain (~ 6-fold, p-value = 0.008) (Fig. 6B) as previously reported.[27] The frequency of CF loss in *sgo1-CT* strains is not significantly different than that of *sgo1Δ* (p-value = 0.153), whereas the frequency of CF loss in *sgo1-NT* strain is significantly lower than *sgo1Δ* (p-value = 0.027), and *sgo1-CT* (p-value = 0.036) strains. The frequency of CF loss in *sgo1-NT* strain is significantly higher than the wild type strain (~ 2-fold, p-value = 0.025). Taken together, these results support a role for the N-terminus of Sgo1 in faithful chromosome segregation.

Discussion

Evolutionarily conserved Sgo1 and its homologs play an important role in cohesin protection, spindle biorientation, and chromosome segregation in eukaryotes.[9,13,56–58] Multiple mechanisms regulate the interaction of Sgo1 with nucleosomes, microtubules, and chromatin associated proteins such as cohesin components.[9] Phosphorylation of histone H2A by Bub1 kinase regulates the association of Sgo1 to *CEN* and peri-*CEN*

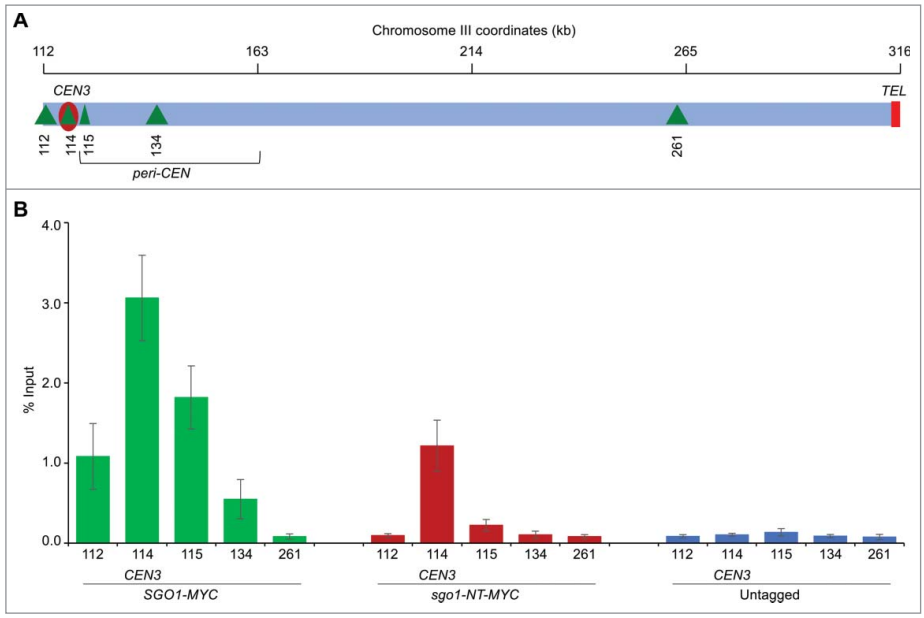


Figure 4. *sgo1-NT* associates with core *CEN* but not with peri-*CEN* chromatin. (A) Schematic of *CEN* and peri-*CEN* regions on chromosome III. Centromere (*CEN3*, violet oblong), cohesin associated regions (*CARs*; green triangle with vertical lines) are shown. *CEN* is located at 114 kb; peri-*CEN* regions 115 (1 kb from *CEN*), 112 (2 kb from *CEN*), 134 (20 kb from *CEN*), and chromosome arm *CAR* 261 (147 kb from *CEN*) were examined. (B) Association of Sgo1-Myc and *sgo1-NT-Myc* at *CEN* and peri-*CEN* regions. ChIP samples from cells synchronized in G2/M as described in **Figure 3** were used. Enrichment at *CEN3* and peri-*CEN* regions was determined by qPCR and is shown as % input. Average from three biological replicates ± standard error is shown.

chromatin.[26] Histone H3 interacts with Sgo1 for localization of Sgo1 to peri-*CEN* in mitosis.[23] However, mechanisms that regulate the localization of Sgo1 to the core *CEN* in mitosis have not been defined. In this study, we have shown that Sgo1 interacts with CENP-A^{Cse4} and the N-terminus of Sgo1 is sufficient for its interaction with CENP-A^{Cse4}, and association with core *CEN* chromatin for faithful chromosome segregation. This conclusion is derived from our observations showing: a) an *in vivo* and *in vitro* interaction between Sgo1 and CENP-A^{Cse4} mediated by the N-terminus of Sgo1 (*sgo1-NT*), b) a cell cycle dependent enrichment of Sgo1 and *sgo1-NT* to core *CENs*, c) Sgo1 and *sgo1-NT* fail to remain associated with core *CEN* chromatin upon depletion of CENP-A^{Cse4}, and d) deletion of N-terminus of Sgo1 (*sgo1-CT*) causes errors of chromosome segregation independent of its C-terminus basic region.

Our yeast two-hybrid screen for CENP-A^{Cse4} identified genes associated with kinetochore structure and function including Scm3 and Mif2, which have been previously shown to interact with CENP-A^{Cse4} and associate with *CEN* chromatin.[42,59–62] Scm3 is a CENP-A^{Cse4}-specific *CEN* assembly factor, which also protects CENP-A^{Cse4} from ubiquitin mediated proteolysis.[60,63–66] Histone H4, a part of the CENP-A^{Cse4} nucleosomes[36,44] was also enriched. These results are in agreement with previous *in vitro* study where CENP-A^{Cse4}, Scm3, and histone H4 have been shown to bind each other and form a stoichiometric complex.[54,64] In addition to kinetochore genes, we identified new and novel interactors of CENP-A^{Cse4}, which are linked with DNA replication and fork protection, Snf1 family kinases, mitotic regulation, and shugoshin.

Our results provide the first evidence of a molecular interaction between CENP-A^{Cse4} and Sgo1 in budding yeast. Sgo1 interacts with CENP-A^{Cse4} *in vivo* and *in vitro* and the interaction of CENP-A^{Cse4} with Sgo1 is mediated by the N-terminus coiled coil domain (*sgo1-NT*), a region of Sgo1 that regulates tension sensing and kinetochore biorientation.[47,49,56] A previous study on spatial distribution of budding yeast GFP-tagged proteins at the metaphase kinetochore revealed that CENP-A^{Cse4} and Sgo1 reside in close proximity, where the Sgo1-GFP foci are located 61 nm from CENP-A^{Cse4} toward the inner centromere.[28] The proximal fluorescent signals of tagged CENP-A^{Cse4} and Sgo1 suggest potential for an interaction. Moreover, Sgo1 localizes close to the CENP-A signals at metaphase chromosomes in human HeLa cells.[26] Furthermore, budding yeast strains with mutations and misregulation of CENP-A^{Cse4} exhibit phenotypes similar to the *sgo1Δ* strain, for example defects in kinetochore geometry, biorientation and errors in chromosome segregation.[28,54,67,68]

Sgo1 contains two evolutionarily conserved domains: the N-terminus coiled coil domain, and the C-terminus basic region.[9] A recent study has shown that mutations either in the N-terminus coiled coil region or the C-terminus phosphorylated H2A interacting motif of *SGO1* exhibit defects in sister chromatid separation and premature SAC silencing.[49] We determined that deletion of either N-terminus (*sgo1-CT*) or C-terminus (*sgo1-NT*) domains causes increased errors in chromosome segregation indicating that both domains are required and independently regulate chromosome segregation. Our results define a novel role for the N-terminus of Sgo1 in chromosome segregation as the presence of the N-terminus domain (*sgo1-NT*) reduced the frequency of chromosome loss when compared to strains lacking the N-terminus (*sgo1-CT*). The higher proficiency of chromosome segregation in the *sgo1-NT* strain is surprising considering that it only contains a short 150 amino acid N-terminal fragment of Sgo1 that forms a coiled coil based on the crystal structure.[46] The N-terminus of Sgo1 mediates homodimerization of Sgo1, which is required for kinetochore biorientation.[22,47] Our results suggest that the N-terminus homodimerization domain of Sgo1 alone is sufficient to interact with CENP-A^{Cse4} and has a role in regulating chromosome segregation.

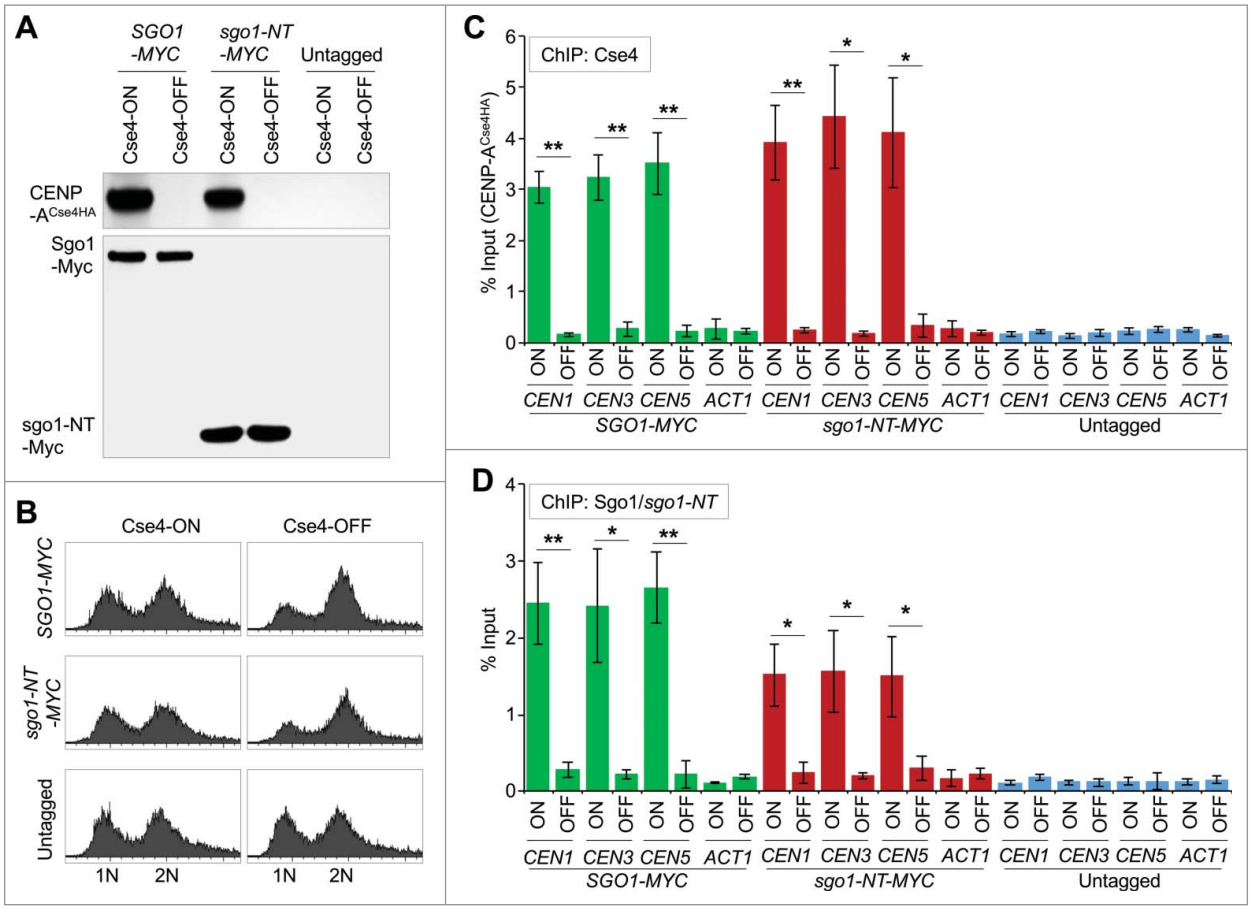


Figure 5. Association of Sgo1 and *sgo1-NT* with core *CEN* requires CENP-A^{Cse4}. Wild type strain (*SGO1-MYC*, YMB10165), and *sgo1-NT-MYC* (YMB10160) expressing HA-tagged CENP-A^{Cse4} from galactose inducible promoter (*GAL1*) were grown in YEP with 2% galactose + 2% raffinose at 25°C (Cse4-ON). Untagged strain (OCF1544-4B) was used as a control. Cells were collected, washed in dH_2O and grown at 25°C in YEP with 2% glucose for 3 hours to Shut-off the expression of CENP-A^{Cse4} (Cse4-OFF). Samples were collected for DNA content, protein extraction, and ChIP analyses. (A) CENP-A^{Cse4} protein levels are not detectable upon its depletion. Western blot analysis was carried out on whole cell protein extracts prepared from cultures grown under Cse4-ON and Cse4-OFF conditions as described above. Blots were probed with anti-HA (CENP-A^{Cse4}; clone 12CA5, Roche Molecular Systems), and anti-Myc (Sgo1-Myc or *sgo1-NT-Myc;* a-14, sc-789, Santa Cruz Biotechnology) antibodies. (B) Depletion of CENP-A^{Cse4} causes synchronization of cells in G2/M phase of the cell cycle as revealed by FACS analysis. (C) *CEN* levels of CENP-A^{Cse4} are reduced upon its depletion. ChIP for CENP-A^{Cse4} was carried out using anti-HA agarose beads (A2095, Sigma-Aldrich). Enrichment at *CEN1, CEN3, CEN5*, and *ACT1* (negative control) was determined by qPCR and is shown as % input. Average from three biological replicates ± standard error is shown. **p value <0.01, *p value <0.05, Student's t test. (D) Sgo1 and *sgo1-NT* fail to remain associated with *CEN* chromatin upon depletion of CENP-A^{Cse4}. ChIP for Sgo1-Myc and *sgo1-NT-Myc* was performed using anti-Myc agarose beads (A7470, Sigma-Aldrich). Enrichment at *CEN1, CEN3, CEN5*, and *ACT1* (negative control) was determined by qPCR and is shown as % input. Average from three biological replicates ± standard error is shown. **p value <0.01, *p value <0.05, Student's t test.

Our results showing expression of *sgo1-NT* in all phases of the cell cycle is consistent with a stabilized *sgo1* mutant (sgo1-Δdb-GFP) that is resistant to APC/C mediated degradation.[47] The sgo1-Δdb has the same levels of protein throughout the cell cycle yet exhibits appearance and delocalization timing (sgo1-Δdb-GFP foci) similar to the wild type Sgo1-GFP[47] consistent with our results showing lack of *CEN* localization for *sgo1-NT* in G1 and S-phases of the cell cycle. It is likely that molecular mechanisms other than Sgo1 protein expression or its destruction through APC/C may regulate its association with the *CEN* chromatin. Future studies will help us identify molecular factors involved in cell cycle regulated *CEN* association of Sgo1 in budding yeast.

We determined that the interaction of *sgo1-NT* with CENP-A^{Cse4} is sufficient for the enrichment of Sgo1 to *CEN* chromatin. The lower levels of *sgo1-NT* at core *CENs* compared to that observed for full length Sgo1 indicates that the C-terminus is also involved in recruitment of Sgo1 to the *CEN* consistent with studies in budding and fission yeast[26] and there is evidence for direct binding to phosphorylated H2A at inner centromeres in human cells.[69] The lack of enrichment of *sgo1-NT* from peri-*CEN* is supported by the absence of CENP-A^{Cse4} from the peri-*CEN* chromatin.[34,54,70,71] CENP-A^{Cse4} plays a critical role in enrichment and maintenance of Sgo1 and *sgo1-NT* at the core *CEN* chromatin. This conclusion is derived from results showing that Sgo1 and *sgo1-NT* fail to remain associated with the core *CEN* chromatin upon depletion of CENP-A^{Cse4}. We propose that CENP-A^{Cse4}, which is recruited to the core *CENs* in early S-phase and remains there throughout the cell cycle[53,54] facilitates the recruitment of Sgo1 specifically to the core *CEN* only during G2/M. Our results suggest that CENP-A^{Cse4} provides spatial specificity for maintenance of Sgo1 at the core *CEN* but additional mechanisms promote the dynamic recruitment and dissociation of Sgo1 through the cell cycle. The role of histone H3 in maintenance of Sgo1 at peri-*CEN* has been described previously,[23] and the Bub1-kinase mediated phosphorylation of H2A recruits Sgo1 to peri-*CEN* and *CEN*.[24,26,27,69]

In summary, our results show that CENP-A^{Cse4} interacts with Sgo1, and that this interaction is mediated by the

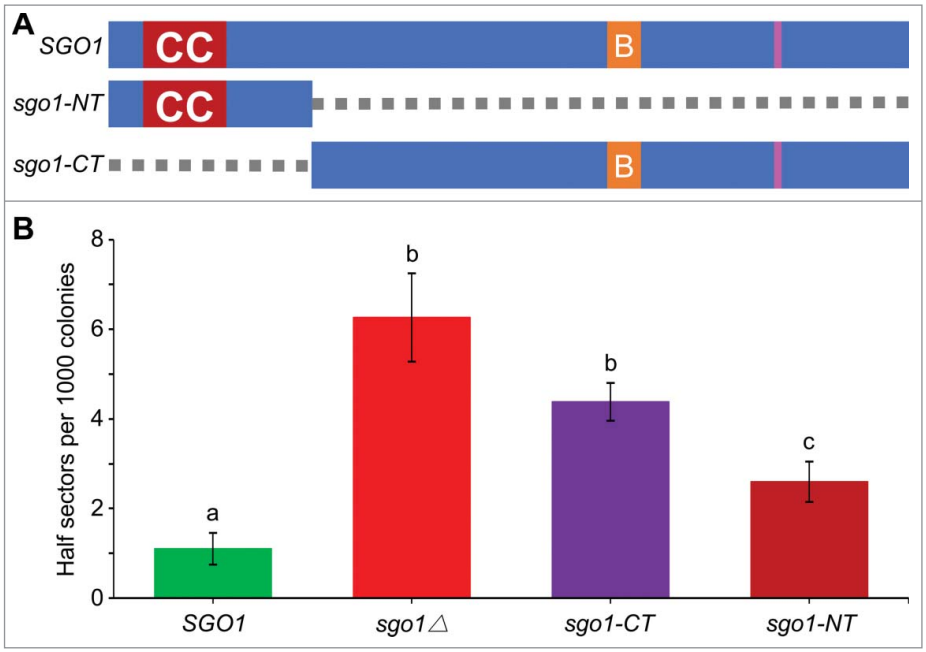


Figure 6. The N-terminus of Sgo1 (*sgo1-NT*) is required for faithful chromosome segregation. (A) Schematic of full-length *SGO1* and its mutant alleles. *sgo1-NT* includes amino acids residues 1–150 (deletion of amino acids 151–591), whereas, *sgo1-CT* includes amino acids residues 151–591 (deletion of amino acids 2–150). Symbols: CC denotes the N-terminus coiled coil domain (amino acids 43–87); B denotes the C-terminus basic SGO1 motif (amino acids 366–390), and pink vertical line represents destruction box (amino acids 494–498). (B) *sgo1-NT* is required for faithful chromosome segregation independently of C-terminus basic domain. Frequency of CF loss in wild type (*SGO1*; YPH1018), *sgo1Δ* (YMB10224), *sgo1-CT* (YMB10221), and *sgo1-NT* (YMB10225) strains was determined as described in Materials and Methods. At least 1000 colonies from three independent transformants were counted. Average from three biological experiments ± standard error. Values sharing the same letter are not significantly different at a 5% level based on the analysis of variance ($p > 0.05$).

N-terminus coiled coil domain of Sgo1, which regulates association with the core *CEN* chromatin. We also define a functional role of the N-terminus of Sgo1 in chromosome segregation. Misregulation of CENP-A^{Cse4}[54,72–74] as well as human homologs of Sgo1 (*SGOL1* and *SGOL2*) have been observed in several human cancers.[75,76] Hence, future studies aimed at defining how the spatial distribution of Sgo1 at *CENs* affects chromosome segregation may allow us to determine if misregulation of CENP-A^{Cse4} and Sgo1 contribute to aneuploidy in human cancers.

Materials and methods

Strains, and culture conditions

Budding yeast strains and plasmids are shown in Table 1. Strains were grown in yeast peptone dextrose (YPD; 1% yeast extract, 2% Bacto-peptone, 2% glucose), yeast extract peptone (YEP) with 2% galactose + 2% raffinose, or in yeast synthetic medium with 2% glucose.

Chromosome transmission fidelity assay

We used a colony color assay[55] to determine the frequency of non-essential chromosome fragment (CF) loss in wild type and *sgo1* mutant strains. The loss of CF results in red sectored growth in an otherwise a white colored colony. Strains were grown to logarithmic phase in synthetic glucose medium without histidine to maintain the CF. Cells were collected by centrifugation, dilutions were prepared, and plated on complete synthetic glucose medium with limiting adenine at 25°C. The frequency of CF loss was determined by counting the number of colonies exhibiting sectors which were at least half red, indicative of CF loss in the first cell division. At least 1000 colonies of three individual transformants were examined for each strain.

ChIP and quantitative PCR (qPCR) analysis

ChIP experiments were performed with three biological replicates using a previously described protocol.[54,77] Protein-DNA complexes were captured using anti-Myc agarose (A7470, Sigma-Aldrich, http://www.sigmaaldrich.com/catalog/product/sigma/a7470?lang = en&region = US) antibodies. ChIP-qPCR was performed using Fast SYBR Green Master Mix (https://www.fishersci.com/shop/products/applied-biosystems-sybr-fast-green-master-mix-6/p-4926995) in 7500 Fast Real Time PCR System (Applied Biosystems) using conditions: 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec, 60°C for 30 sec. The enrichment values were calculated using the $_{dd}C_T$ method [78] and are shown as % input. Primer sequences are listed in Table 1.

IP experiments and Western blotting

IP experiments were performed as described previously.[51,63] Briefly, strains were grown to logarithmic phase, and cells were collected by centrifugation. Cell pellets were dissolved in lysis buffer (100 mM Tris [pH 7.5], 0.1 mM EDTA, 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 10% glycerol, and protease inhibitors[63]), and whole-cell extracts were prepared by beadbeating using FastPrep-24 5G bead beater (MP Biomedicals, http://www.mpbio.com/product.php?pid = 116005500). Immunoprecipitations were captured using anti-HA agarose antibodies (A2095, Sigma-Aldrich, http://www.sigmaaldrich.com/catalog/

Table 1. List of yeast strains, plasmids, and primers used in this study.

Strain	Genotype	Reference
(A) Saccharomyces cerevisiae strains:		
OCF1533-4B	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2,3–112 can1-100*	Orna Cohen-Fix
YMB10160	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2,3–112 can1-100 sgo1Δ::sgo1-NT-9Myc::TRP1 GALCSE4-3HA::NAT*	This study
YMB10165	*MATa ade2-1, leu2-3, ura3, his3-11, can1-100, GAL, psi+, SGO1-9MYC::TRP1 GALCSE4-3HA::NAT*	This study
AMY905	*MATa ade2-1, leu2-3, ura3, his3-11, can1-100, GAL, psi+, SGO1-9MYC::TRP1*	Adele Marston
YMB10076	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2,3-112 can1-100 sgo1Δ::sgo1-NT-9Myc::TRP1*	This study
PJ69-4A MATa	*MATa trp1-901 leu2,3-112 ura3-52 his3Δ200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	Stan Fields
PJ69-4A MATalpha	*MATalpha trp1-901 leu2,3-112 ura3-52 his3Δ200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	Stan Fields
YPH1018	*MATalpha ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIII (CEN3L.YPH278) HIS3 SUP11*	Phil Hieter
YMB10221	*MATalpha ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIII (CEN3L.YPH278) HIS3 SUP11 sgo1-CT (amino acids 151–591)-9Myc::TRP1*	This study
YMB10224	*MATalpha ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIII (CEN3L.YPH278) HIS3 SUP11 sgo1Δ::KAN*	This study
YMB10225	*MATalpha ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIII (CEN3L.YPH278) HIS3 SUP11 sgo1-NT (amino acids 1–150)-9Myc::TRP1*	This study

(B) List of plasmids: Plasmid	Description	Reference
pGEX Sgo1-1-132	pGEX-6P-1 Sgo1 1–132 cloned into *BamH-I* and *Xho-I* sites	This study
pJL55	pGEX-4T-1 3xHA-SGO1 cloned into the *BamH-I* and *Xho-I* sites	Min-Hao Kuo
pET28 Cse4 Octamer	pET28 with Cse4 octamer genes with individual ribosome binding sites (RBS): *Nco*-I-6xH-3c-H2A-*EcoR-I*-RBS-H2B-*Sal-I*-RBS-H4-*Not-I*-RBS-RGS6H-CSE4-*Xho-I*	Peter Kaiser
pBDC	*CEN-TRP1 ADH*-GAL4 DBD	Stefan Milson
pADC	*CEN-LEU2 ADH*-GAL4 DBD	Stefan Milson
pKT0301	pBDC Cse4-DBD recombinational cloning into the *Nru-I* site	This study
pVB0264	pBDC Sgo1 1-150-DBD	This study
pKT0307	pADC Sgo1 1-132-AD	This study
pVB0268	pADC AD Sgo1 1–132	This study
pKTY2353	PJ69-4A MATalpha pBDC-Rts1-DBD	This study
pKTY2371	pJ69-4a MATa pADC Rts1-AD	This study
pGEX Sgo1-1-132	pGEX-6P-1 Sgo1 1–132 cloned into *BamH-I* and *Xho-I* sites	This study
pAG414-sgo1-NT-EGFP	P_{GAL1} –*sgo1-NT*-GAL4-DBD(1-74)-EGFP	This Study

(C) List of primers: Locus/plasmids	Forward (5′ – 3′)	Reverse (5′ – 3′)	Reference
CEN1	CTCGATTTGCATAAGTGTGCC	GTGCTTAAGAGTTCTGTACCAC	[82]
CEN3	GATCAGCGCCAAACAATATGG	AACTTCCACCAGTAAACGTTTC	[82]
CEN5	AAGAACTATGAATCTGTAAATGACTGATTCAAT	CTTGCACTAAACAAGACTTTATACTACGTTTAG	[82]
ACT1	AATGGCGTGAGGTAGAGAGAAACC	ACAACGAATTGAGAGTTGCCCCAG	[67]
112	AGCATTAGAGCCACTGTCATTTC	ATAATTAAGATACGAATGTGTTCGTTG	[51,83]
115	TGTAACCATTATCATATTCATGAC	GAT TTA ATG CAC GTT ATG TTT CG	[82]
134	CCGATGGTTAGGATTTCCAACG	GGTTTTCAGAACAGAATGGGGC	[84,85]
261	TTGCCACAGCCACAGATATAACTG	GATGGACAAAGCGTTGTATCCG	[85,84]
pADC AD Sgo1 1–132	CAAGCTATACCAAGCATACAATCCAAGATGCCGAAGAGAAAAATTGCTCC	GGAATTAATTCCGCTTTATCCATCTTTGCAAAGGCGCTCGAACTGGGCAAATTTTC	This study
pBDC Cse4-DBD	CAACTCCAAGCTTGAAGCAAGCCTCGATGTCAAGTAAACAACAATG	CGATAGAAGACAGTAGCTTCATCTTTCGAATAAACTGTCCCCTGATTCTTC	This study
pBDC Sgo1 1-150-DBD	GCTTGAAGCAAGCCTCGATGTCAAGTAAACAACAATGGGTT	CAGTAGCTTCATCTTTCGTCATGACAATGAGCATGATCTTGA	This study
pADC Sgo1 1-132-AD	AGCATACAATCCAAGATGCCGAAGAGAAAAATTGCTCCT	CGCTTTATCCATCTTTGCAAAGGCTCAGCTCGAACTGGGCAAATTTTC	This study
pGEX Sgo1-1-132	GGCCCCTGGGATCCATGCCGAAGAGAAAAATTGCT	TGCGGCCGCTCGAGTCAGCTCGAACTGGGCA	This study
pBDC-Rts1-DBD	CAACTCCAAGCTTGAAGCAAGCCTCGATGATGCGTGGTTTCAAGCAAAG	CGATAGAAGACAGTAGCTTCATCTTTCGCTGTATCTCGCTGTCACAATCAT	This study
pADC Scm3-AD	CAACTCCAAGCTTGAAGCAAGCCTCGATGAAAACCAATAAGAAAATTTC	CGATAGAAGACAGTAGCTTCATCTTTCGATCTCGTAATATCCTTTTCGAG	This study

product/sigma/a2095?lang = en&region = US). Beads were washed in the lysis buffer for 5 min at room temperature three times, and eluted in SDS buffer (1% SDS, 1 x TE pH 8.0). Total protein extracts were prepared with TCA procedure and quantified using Bio-Rad DC protein assay (Bio-Rad Laboratories, http://www.bio-rad.com/en-ch/product/dc-protein-assay). Protein samples were analyzed using Western blotting by size fractionation on SDS polyacrylamide gels, followed by transfer to the nitrocellulose membranes. Primary antibodies used were anti-HA (H6908, Sigma-Aldrich, https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Datasheet/2/h6908dat.pdf), anti-HA (clone 12CA5, Roche Molecular Systems, http://www.sigmaaldrich.com/catalog/product/roche/roaha?lang = en&region = US), anti-Myc (a-14, sc-789, Santa Cruz Biotechnology, https://www.scbt.com/scbt/product/c-myc-antibody-a-14), and anti-Tub2[54]. Secondary antibodies used were HRP-conjugated sheep anti-mouse IgG (NA931V, Amersham Biosciences, http://www.gelifesciences.com/webapp/wcs/stores/servlet/productById/en/GELifeSciences-us/25005173), and HRP-conjugated donkey anti-rabbit IgG (NA934V, Amersham Biosciences, http://www.gelifesciences.com/webapp/wcs/stores/servlet/productById/en/GELifeSciences-us/25005179).

Purification of recombinant proteins from Escherichia coli

Recombinant proteins were purified from *E. coli*. Expression of GST, GST-Sgo1 and GST-Sgo1$_{1\text{-}132}$ were performed in BL21DE3 cells using 100 mL of LB media at 30°C with 3 h induction with 1 mM IPTG (I0328, TCI America, http://www.tcichemicals.com/eshop/en/us/commodity/I0328/). Cells were pelleted, resuspended and lysed with BPER (78248, Thermo Scientific, https://www.thermofisher.com/order/catalog/product/78248) in the presence of protease inhibitor cocktail (88666, Pierce Chemicals, https://www.thermofisher.com/order/catalog/product/88666) and nuclease (Universal nuclease, 88700, Pierce Chemicals, https://www.thermofisher.com/order/catalog/product/88700) as per manufacturer instructions. The CENP-A^{Cse4} octamer was expressed in BL21DE3 cells and kept on ice for 30 min before induction to enhance soluble expression of the CENP-A^{Cse4} octamer. CENP-A^{Cse4} octamer was induced with 0.4 mM IPTG for 3 h. Lysis of the CENP-A^{Cse4} octamer expression strain was perfomed with BPER, protease inhibitor cocktail, nuclease and was supplemented with 2 M NaCl.

In vitro *protein-protein interaction assay*

The GST and GST-tagged Sgo1 lysates were incubated with 120 μL of glutathione-agarose bead slurry (16101, Pierce Chemicals, https://www.thermofisher.com/order/catalog/product/16101) for 1 h at 4°C and washed five times with equilibration buffer (50 mM Tris-HCl (pH 7.5),150 mM NaCl, 0.1% NP40) and 1 x protease inhibitor (88666, Pierce Chemicals, https://www.thermofisher.com/order/catalog/product/88666). The GST only expression strain lysate was diluted 10 x to ensure a similar level of protein was immobilized on all beads. The GST and GST-Sgo1 protein immobilized beads were incubated for 2 h at 4°C with 1 mL of *E. coli* CENP-A^{Cse4} octamer expression strain lysate prepared from a 100 mL culture. Beads were washed with 1 mL of equilibration buffer five times. Washed beads were resuspended and boiled at 95°C with 40 μL of SDS-PAGE loading dye. Samples were electrophoresed on 4–20% TGX Stain-Free Gels (Bio-Rad, http://www.bio-rad.com/en-ch/sku/4568095-4-20-mini-protean-tgx-stain-free-protein-gels-12-well-20-ul) and immunblotted with anti-His6 antibody (https://www.thermofisher.com/antibody/product/6x-His-Tag-Antibody-clone-HIS-H8-Monoclonal/MA1-21315) or anti-GST antibody (CGAB-GST, GeneCopoeia, http://www.genecopoeia.com/product/anti-gst-antibody/).

Yeast two-hybrid assay

To screen for CENP-A^{Cse4} protein-protein interactions using yeast two-hybrid, a bait vector was constructed using pBDC and transformed into the haploid strain PJ69-4A MATa.[79] This bait strain was then screened against a genome-wide array of prey strains[41] (PJ69-4A MATα) in an automated manner using previously described methods.[42,80] To confirm positives, new yeast two-hybrid vectors were constructed and strains were manually retested for positive yeast two-hybrid interactions. Various Cse4, Sgo1 and controls were used as fusion proteins with Gal4 DNA-binding domain (DBD) or Gal4 activation domain (AD) and cloned into the pBDC (*TRP1* marked) and pADC (*LEU2* marked) vectors.[81] The pBDC and pADC vectors were linearized using *Nru-I* (fusion to the N-terminus of DBD or AD) or *Pvu-II* (fusion to the C-terminus of DBD or AD) and the gene of interest was PCR amplified and cloned into these vectors using homologous recombination (primers are listed in Table 1). Plasmids were rescued from yeast and confirmed by sequencing. Bait (pBDC) plasmids were transformed into the PJ69-4A MATa strain and prey (pADC) plasmids were transformed into the PJ69-4A MATα haploid strain. Bait and prey strains were mated to form diploid strain by growing the haploid strains in YPAD (Yeast Extract-Peptone-Adenine-Dextrose) medium overnight at 30°C and 50 μL of each strain was mixed in a 96 well plate and diploids were spotted on YPAD plates using a 96 pin spotting tool (VP Scientific, http://www.vp-scientific.com/96_solid_pin_replicator.htm) and incubated overnight at 30°C. To select for the DBD and AD plasmids in diploids, yeast patches were spotted on synthetic dextrose (SD) without tryptophan and leucine (SD-TL) using the 96 pin replicating tool and incubated for 2–3 days at 30°C. Cells from the SD-TL plate were transferred and resuspended into 96 well plates containing 50 μL of SD-TL medium. An aliquot of the yeast diploids were spotted on SD-TL and SD without tryptophan, leucine or histidine (SD-TLH) with 1 mM or 3 mM 3-amino-1,2,4-triazoloe (3-AT, 09540, Fluka, St. Louis, MO) plates and incubated at 30°C for 5–7 days. Positive interactors showed growth on SD-TLH with 3 mM 3-AT.

Disclosure statement

The authors declare no competing financial interests.

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