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Endogenous Transcription at the Centromere Facilitates Centromere Activity in Budding Yeast

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Summary

Background—The centromere (*CEN*) DNA-kinetochore complex is the specialized chromatin structure that mediates chromosome attachment to the spindle and is required for high-fidelity chromosome segregation. Although kinetochore function is conserved from budding yeast to humans, it was thought that transcription had no role in centromere function in budding yeast, in contrast to other eukaryotes including fission yeast.

Results—We report here that transcription at the centromere facilitates centromere activity in the budding yeast *Saccharomyces cerevisiae*. We identified transcripts at *CEN* DNA and found that Cbf1, which is a transcription factor that binds to *CEN* DNA, is required for transcription at *CEN* DNA. Chromosome instability of *cbf1*Δ cells is suppressed by transcription driven from an artificial promoter. Furthermore, we have identified Ste12, which is a transcription factor, and Dig1, a Ste12 inhibitor, as a novel *CEN*-associated protein complex by an *in vitro* kinetochore assembly system. Dig1 represses Ste12-dependent transcription at the centromere.

Conclusions—Our studies reveal that transcription at the centromere plays an important role in centromere function in budding yeast.

Introduction

In eukaryotic cells, all duplicated chromosomes are segregated equally to daughter cells during mitotic cell division. To make the event a success, a highly conserved architecture consisting of centromere (*CEN*) DNA, kinetochores, microtubules, and microtubule-organizing centers (MTOCs), must be properly assembled during each cell cycle [1].

The centromere of budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), which is known as a point centromere [2], has been well studied, and *S. cerevisiae* is a good model organism in which to investigate how kinetochore proteins assemble onto *CEN* DNA [3, 4]. Each *CEN* DNA of *S. cerevisiae* is an approximately 125-bp region that has three conserved centromere DNA elements CDEI, CDEII, and CDEIII [5]. CDEI includes the CACRTG region, where a homodimer of Cbf1 (helix-loop-helix family) associates [5, 6]. CDEII is a 78- to 86-bp sequence that is about 90% AT rich [5]. This element is thought to be important for histone and/or Ndc10 interaction [7–9]. The 25-bp CDEIII contains a conserved CCG motif that is crucial for forming the CBF3 complex (comprising Ndc10, Cep3, Ctf13, and

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Skp1) [10]. More than 70 kinetochore proteins have been identified in *S. cerevisiae* [3, 4]. The kinetochore comprises three layers that connect between *CEN* DNA and microtubules in a hierarchical manner: a DNA-binding layer, a linker layer, and a microtubule (MT)-binding layer. The *CEN* localization of most kinetochore proteins depends on the CBF3 complex in *S. cerevisiae*. Recent evidence suggests that kinetochores are disassembled during *CEN* DNA replication in early S phase but are reassembled in late S phase [11].

In diverse eukaryotic species such as the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), plants, and metazoans, regional *CEN* DNAs are much longer and more complex than point *CEN* DNA in budding yeast [2]. *S. pombe CEN* regions are large, repetitive structures that range from 35 kb to 110 kb in length [12]. The *CEN* regions in humans and *Drosophila melanogaste*r are composed of repetitive satellite elements, which are from 200 kb to 4 Mb long [13]. RNA interference (RNAi), a gene-silencing pathway triggered by double-stranded RNA, corresponds to the formation and the maintenance of centromeric heterochromatin in regional *CEN* DNA. These species use the ribonuclease III (RNase III) endonuclease Dicer and the effector protein Argonaute to generate small interfering RNAs (siRNAs) [14]. In contrast, the budding yeast *S. cerevisiae*, which has point *CEN* DNA, does not contain RNAi genes such as Dicer and presumably does not make use of the RNAi pathway [15].

Here, we have shown that transcription at the centromere makes a direct contribution to centromere function in budding yeast. The transcription factor Cbf1 contributes to *CEN* transcripts whereas Dig1 inhibits Ste12-dependent *CEN* transcripts. Although strong transcription by an artificial promoter deactivates centromere activity [16, 17], we found that a certain level of transcriptional activity is required for centromere function.

Results

Identification of *CEN3*-associated proteins

To identify novel proteins that associate with *CEN* DNA, an *in vitro* assembly system was used (Figure S1). Espelin *et al*. [18] found that Ndc10 binding with DNA extends beyond CDEIII. Thus, we prepared two *CEN3* constructs (Figure 1A). The short version of *CEN3* DNA, which is 134-bp long, consists of only CDEI, CDEII, and CDEIII. The long version, which is 184-bp long, contains the additional Ndc10 binding site. A mutant *CEN3* containing a point mutation in the CCG motif in CDEIII, which is deficient in centromere activity [10, 19], was used as a negative control. To increase the binding capacity, we used plasmids containing 8 tandem copies of *CEN3* (Figure S1A) [20]. To investigate whether *CEN* DNA and protein complexes are assembled properly, we first tested whether CBF3 components (Ndc10, Skp1, and Cep3) would bind to *CEN* DNA (Figures 1B and 1C). Indeed, Ndc10, Skp1, and Cep3 all bound to both the long and the short versions of wild-type *CEN* DNA. In contrast, these proteins did not bind to vector only or to the *CEN3* mutant beads, except that Ndc10 and Cep3 were loaded at a low level onto the long version of the mutant *CEN3* due to the extended CBF3. Sgt1 and Cdc28, which are not components of the kinetochore [21], were used as negative controls, and no signals were detected (Figure 1B). These results indicated that the core-kinetochore complex was assembled specifically in this *in vitro* system. Cse4 (the yeast homologue of human centromere-specific histone H3 variant CENP-A) and Scm3, which is required for Cse4 incorporation into the centromeric chromatin [22], bound to both the long and the short versions of *CEN3* DNA but not to the *CEN3* mutant DNA (Figures 1C and 1D; Figure S2). These results therefore suggest that localization of Cse4 and Scm3 with *CEN* DNA depends on the CBF3 complex, which is consistent with the previous finding that Cse4-*CEN3* association was decreased in *ndc10-1* mutants [23]. The association of Cbf1 with *CEN3* DNA was not altered by the mutation of the CCG motif in CDEIII (Figure 1E), which is also consistent with the previous finding that

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Cbf1 binds to CDEI. We did not detect any *CEN* association with Spc105, Ndc80, or Mtw1 (the central kinetochore proteins); Dam1 or Dad1 (the outer kinetochore proteins); or α-tubulin (Figures 1E–1G). We found that Ipl1 (the yeast Aurora kinase) did associate with wild-type *CEN3* DNA but not with the mutant *CEN3* DNA (Figure 1F), implying that Ipl1 functions closely to the core-kinetochore *CEN* DNA complex.

Next we performed mass spectrometry to identify *CEN*-associating proteins. Dig1-Ste12, Sum1-Hst1, and Cdc14-Net1 complexes were identified in the *CEN* DNA magnetic beads by two independent mass spectrometry analyses as well as Cbf1 (Figure S3; data not shown). The specificity of these results was confirmed by performing immunoblotting of the elution from the *CEN* DNA magnetic beads. All of these proteins were associated with both wild-type and mutant *CEN3* DNA, except that Dig1 was loaded at a low level onto mutant *CEN3* DNA. (Figure 2A; Figure S4). Sum1-Hst1 is a part of the Sum1/Rfm1/Hst1 complex that represses meiotic genes during vegetative growth via histone deacetylation by Hst1 (Homologue of Sir Two) [24, 25]. Cdc14-Net1 is the core subunit of the RENT complex (Net1, Sir2, and Cdc14) involved in nucleolar silencing and telophase exit [26]. Ste12 is a transcription factor that is controlled by a mitogen-activated protein (MAP) kinase cascade in mating pheromone and invasive growth pathways [27]. Dig1 is an inhibitor of Ste12 [28, 29]. Ste12 and Dig1 were associated with the long version of *CEN3* but not the short version in the *in vitro* assembly system (Figure 2A; Figure S4), indicating that the binding region of these proteins exists outside of CDEIII. In fact, we found a Ste12-binding site, which is called a pheromone response element [PREs; TGAAAC(A/G)] [30], in the pericentromeric region of *CEN3* (Figure 3A). The chromatin immunoprecipitation (ChIP) assay showed that Cbf1, Ste12, and Dig1 coimmunoprecipitated specifically with *CEN* DNA (Figure 2B), indicating that these proteins associate with *CEN* DNA *in vivo*.

Identification of transcription at *CEN* DNA

These observations made us consider the possibility that transcription occurs from or through the centromere. Therefore, we tested whether transcripts occurred at the centromere in wild-type cells. Core centromeric transcripts of *CEN3*, *CEN9*, and *CEN13* were detected by reverse transcriptase PCR (RT-PCR) in the wild-type strain but not in *cbf1*Δ mutants (Figure 3B), indicating that Cbf1 is required for centromeric transcription. We also examined whether the core or pericentromeric transcripts of *CEN3* or *CEN9* were decreased in *ste12*Δ mutants or/and increased in *dig1*Δ mutants (Figures 3C and 3D). To detect the transcription, we designed primers upstream and downstream of the Ste12 binding site (Figure 3A). Interestingly, deletion of *DIG1* increased the core centromeric expression of *CEN3*, *CEN9*, and *CEN13* (Figures 3C and 3D). Similarly, one of the pericentromeric transcripts of *CEN9* (*CEN9* peri_L), which is derived from the left side of the putative Ste12 binding site, was high in *dig1*Δ mutants (Figures 3C and 3D). The core centromeric expression of *CEN3*, *CEN9*, and *CEN13* was slightly decreased in the *ste12*Δ mutants and the increased expression in the *dig1*Δ mutants was abolished by deletion of *STE12* (Figures 3C and 3D). This suggests that Ste12 has a positive role in basal *CEN* transcription but that full Ste12 transcription induction is repressed by Dig1. We also tested the transcription derived from the right side of the Ste12 binding site. The pericentromeric transcripts of *CEN3* (*CEN3* peri) were elevated in *dig1*Δ mutants and the elevated expression was abolished by deletion of *STE12*, as the case of the core centromeric transcripts (Figures 3C and 3D). In contrast, there were no significant differences in expression between wild-type and *dig1*Δ strain in the pericentromeric transcripts of *CEN9* (*CEN9* peri_R). Therefore, these results suggest that Dig1 inhibits core and pericentromeric transcription regulated by Ste12.

In *S. pombe*, RNA polymerase II is required for pericentromeric transcription mediated by the siRNA interference [31]. We thus examined whether the largest subunit (Rpb1) of RNA polymerase II is responsible for core and pericentromeric transcription using the

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temperature-sensitive mutant *rpb1-1* (Figure 3E). In wild-type cells, both core and pericentromeric *CEN3* transcripts were clearly detectable, as were *CDC28* and *ACT1* transcripts. As expected, *CDC28* and *ACT1* transcripts were greatly reduced in *rpb1-1* mutant cells (Figure 3E). In addition, *CEN3* core and pericentromeric transcripts were greatly reduced when the cells were shifted to the non-permissive temperature (37°C), which indicates that Rpb1 is required for both core and pericentromeric transcription of CEN3.

The role of centromeric transcription

Loss of Cbf1 causes chromosome loss and sensitivity to benomyl (a microtubule-depolymerizing drug) [32] (Figure 4B). To test whether deletion of *STE12* or *DIG1* influences centromere function, we investigated traits common to kinetochore mutants, such as benomyl sensitivity and infidelity of chromosome segregation (Figures 4A–4C). *ste12*Δ cells were mildly sensitive to benomyl and exhibited a substantial chromosome missegregation phenotype. Interestingly, *dig1*Δ cells were resistant to benomyl and had an increased fidelity of chromosome segregation than wild-type cells. Furthermore, *dig1*Δ *ste12*Δ cells were highly sensitive to benomyl and had a dramatic increase in chromosome missegregation, similar to the phenotype of *ste12*Δ cells. These observations indicate that both Ste12 and Dig1 affect the fidelity of chromosome segregation and revealed that the increased transcription at *CEN* DNA, at least more than wild-type level, is required for high fidelity of chromosome segregation. Next, we deleted the Ste12 binding site near *CEN3* on the chromosome fragment (Figure 4D), and found that the resulting Ste12 binding site deletion mutant exhibited a chromosome missegregation phenotype similar to those in *ste12*Δ or *dig1*Δ *ste12*Δ mutant cells (Figure 4E). Together, these results suggest that a certain level of transcriptional activity at *CEN* DNA is required for centromere function, whereas it was previously shown that a high level transcription through *CEN3* impairs centromere function [17].

To confirm this result, we constructed a strain in which the core *CEN3* transcripts on the chromosome fragment are controlled by the methionine-repressible *MET25* promoter (Figure 5A). When the cells are grown in medium containing methionine (2 mM), transcription from the *MET25* promoter is repressed. In contrast, when cells are grown in medium without or containing limited methionine (0.01 mM), strong transcription is induced from the *MET25* promoter, resulting in about 20-fold induction [33]. Wild-type cells, in the absence of methionine or in the presence of limited methionine, showed significant chromosome missegregation (Figure 5B; data not shown), indicating that a high level of transcription through *CEN3* impairs centromere function, as was previously shown [17].

If the hypothesis that *cbf1*Δ cells have reduced fidelity of chromosome segregation due to loss of core centromeric transcription is true, then a high level of transcription from the *MET25* promoter should be able to suppress the chromosome missegregation phenotype of *cbf1*Δ cells. Because *cbf1*Δ cells failed to grow in the methionine-free medium [32], the cells were grown in the medium containing 0.01 mM methionine to induce transcription from the *MET25* promoter. Indeed, the transcripts derived from the *MET25* promoter suppressed the chromosome missegregation phenotype of *cbf1*Δ cells (Figure 5C). We also constructed a *cbf1*Δ strain in which transcription through *CEN3* is induced by a *CUP1* promoter. Similarly, transcripts derived from the *CUP1* promoter partially suppressed the chromosome missegregation phenotype of *cbf1*Δ cells (Figure S5).

Next, we examined whether the same was true for *ste12*Δ. We constructed a *ste12*Δ strain in which the peri-*CEN3* transcripts are controlled by a *CUP1* promoter (Figure 5D). Transcription from the *CUP1* promoter suppressed chromosome instability of the *ste12*Δ mutants (Figure 5E). Together, these results strongly suggest that centromeric transcription contributes to centromere function.

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Discussion

In this study, we have identified transcripts at *CEN* DNA regulated by transcriptional factors both Cbf1 and Ste12 in an RNA polymerase II-dependent manner (Figure 6). Moreover, the silencing factors such as Hst1, Cdc14, and Sir1 are also part of the macromolecular complex of the kinetochore. These findings are reminiscent of transcriptional regulation at the regional centromere in higher eukaryotes.

Transcription at the centromere is important for centromere function

We identified novel *CEN*-associated proteins such as Dig1 and Ste12 by our *in vitro* kinetochore assembly system. The *CEN* DNA-protein complex contains only the inner kinetochore proteins (Figure 1H). Both Cbf1 and Ste12, which are known transcription factors [30, 32–34], associate with centromeric chromatin and help maintain mitotic chromosome stability. Our results strongly suggest that both Cbf1 and Ste12 regulate centromeric transcripts in an RNA polymerase II-dependent manner. Moreover, Dig1 inhibits centromeric transcripts. We have discovered a putative Ste12 binding site (TGAAACG) at peri-*CEN3* and another (TGTAACA) at peri-*CEN9* (Figure 3A). Using ChIP assays, we have shown that Ste12 associates with other *CEN* DNA (Figure 2B) and found putative Ste12 binding sites at pericentromeric regions in all 16 chromosomes (Table S1), suggesting that core or pericentromeric transcription by Ste12 is conserved in all chromosomes. Interestingly, there are multiple putative Ste12 binding site around some *CEN*s (Table S1). Ste12 inducible genes usually have more than one PRE in their promoter regions [35]. Pericentromeric regions regulated by Ste12 appear to be similar to the typical promoter region of Ste12 inducible genes. Moreover, genome-wide analyses by Harbison *et al*. [36] and Tachibana *et al*. [37] revealed that other transcriptional regulatory codes, which are specific DNA sequences that induce or repress gene expression, exist in some pericentromeric regions (Table S2). Thus, additional transcription factors might contribute to centromeric transcription.

The possible role of transcription at *CEN* DNA

CEN DNA might have evolutionarily originated from a promoter region. Hemmerich *et al*. [38] illustrated the analogy between the yeast centromere and the *MET* promoter. We also found a similarity between the yeast pericentromere and the promoter regulated by Ste12. It is tempting to speculate that the kinetochore assembly system on *CEN* DNA resembles the access of transcription or silencing factors on the promoter DNA.

What is the role of transcription at the centromere? Dynamic topological changes of DNA are known to occur during the transcription process [39]. A recent finding suggests that positive supercoiling is a general feature of centromeric nucleosomes in eukaryotic cells, although H3 nucleosomes induce negative supercoils [40]. Therefore, *CEN* transcription might be responsible for the proper topology of *CEN* DNA (Figure 6).

Comparison with RNAi machinery

In regional *CEN* DNA of higher eukaryotes, RNAi machinery is required for gene silencing in the assembly of pericentromeric heterochromatin. Budding yeast *S. cerevisiae* lacks the RNAi machinery, although other budding yeast species, such as *Saccharomyces castellii* and *Candida albicans*, has the machinery [15]. The level of transcription derived from the pericentromere is much higher than that from the core region of *CEN* DNA in *S. pombe* [41, 42]. Transfer RNA (tRNA) genes are thought to be the insulators that mark the distinct chromatin domains within the centromere. It has recently been reported that transcripts from the central core domain of fission yeast centromere are degraded by the exosome [42]. Increased level of transcription including *CEN3* region was also detected in exosome

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mutants in *S. cerevisiae* [43]. Considering these previous results, our findings raise the possibility that a certain level of transcription at *CEN* DNA is important for centromere function, which is evolutionally conserved, and thus, RNAi in higher eukaryotes might be needed only to control levels of transcripts at the centromere.

The RNA-silencing pathway in *S. pombe* contributes to heterochromatin formation and maintenance at centromeres [44]. In this process, chromodomain protein Swi6 recruits Clr3 histone deacetylase as a silencing factor and Epe1 transcriptional activator as an anti-silencing factor. The balance between the opposing activities of these proteins is essential for the determination of the transcriptional status at centromeres [44]. Sir1, which is the budding yeast silencing protein, is a functional component of centromeric chromatin [45]. We have found several silencing factors, such as Hst1-Sum1 and Cdc14-Net1, as *CEN*-associated proteins (Figure 2A). Therefore, transcriptional regulation, which is the balance of expression levels, is important to positively or negatively maintain the centromeric nucleosome (Figure 6). It will be interesting to decipher how centromeric transcription is regulated by these factors and how kinetochore assembly is regulated by centromeric transcription.

Experimental Procedures

Yeast strains

Table S3 presents the genotypes of yeast strains used for this study. Details of strain construction are provided in Supplemental Experimental Procedures.

Plasmids

Table S4 lists the plasmids used in the kinetochore assembly system *in vitro*. The construction of tandem *CEN3* plasmids is depicted in Figure S1. To construct the wild-type and mutant single copies of *CEN3* plasmids, 134-bp (short) or 184-bp (long) *CEN3* fragments were connected between the *Xho*I and *Sal*I sites of pBluescriptII SK(+).

Preparation of *CEN3* beads

Plasmid DNA was covalently modified with biotin with photoprobe long arm biotin reagents (SP-1020, Vector Laboratories, Burlingame, CA) by thermal coupling (95°C, 30 min). Next, 60 μg of biotinylated plasmid DNA was incubated with 3 mg of streptavidin-coated paramagnetic beads (Dynabeads M-280 Streptavidin, Invitrogen Dynal AS, Oslo, Norway) in a buffer containing 1 M NaCl, 10 mM HEPES-KOH (pH 7.6) and 1 mM EDTA using gentle rotation at room temperature for 8 h. The beads were washed and equilibrated in a buffer containing 300 μl of 10 mM HEPES-KOH (pH 7.6) and 1 mM EDTA.

Preparation of highly concentrated protein extracts

Highly concentrated protein extracts were prepared as previously described [20] with some minor modifications. In brief, yeast cells grown in YPD at 25°C were harvested and washed twice with cell wash buffer (20 mM HEPES-KOH (pH 7.8), 1 M Sorbitol) at 4°C and once with 10 volumes of lysis buffer (100 mM HEPES-KOH (pH 7.8), 0.8 M Sorbitol, 50 mM potassium glutamate, 10 mM MgOAc, and 2 mM EDTA). The cells were resuspended in 0.25 volume (per weight) of lysis buffer containing 4 mM DTT and 4× protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Yeast popcorn was prepared by dropping the cell suspension into liquid nitrogen. The frozen cells were disrupted in the electronic mortar grinder (RM100, Retsch, Germany) with liquid nitrogen for 1 h. After thawing the broken yeast powder, potassium glutamate was added to the homogenate to give a final concentration of 300 mM. The homogenate was incubated at 4°C for 30 min with gentle agitation and ultracentrifuged in a Beckman SW55 rotor at 33,000 rpm for 30 min at 4°C.

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The supernatant was also ultracentrifuged in a Beckman SW55 rotor at 55,000 rpm for 1 h at 4°C. The recovered supernatant was aliquotted, frozen in liquid nitrogen, and stored at −80°C. Typically, 10–15 g of the cell pellet was processed, and the protein concentration was from 50 to 100 mg/ml.

In vitro kinetochore assembly system

The *in vitro* kinetochore assembly system is based on the loading assay described elsewhere [20, 46]. Briefly, 35 μl of reaction buffer (57 mM HEPES-KOH [pH 7.6], 714 mM Sorbitol, 23 mM MgOAc, 5.7 mM EGTA, 2.3 mM DTT, 6.9 mM ATP, 46 mM creatine phosphate, 1.6 units of creatine phosphokinase, 1.1× protease inhibitor cocktail tablet, 5.1 μg/μl poly(dI-dC)•poly(dI-dC)) (Sigma-Aldrich, St. Louis, MO) was mixed with 40 μl of highly concentrated protein extracts and preincubated on ice for 10 min before addition of the Dynabeads. Next, 20 μl of 10 μg/μl bead suspension was placed on a magnetic separator, and 15 μl of supernatant was removed, leaving the beads in the tube. The preincubated mixture was added to the beads and mixed well by pipetting. The total volume of 80 μl of mixture was incubated using gentle rotation for 4 min at room temperature. The reaction was stopped by the addition of 9 vol (720 μl) of ice-cold wash buffer (50 mM HEPES-KOH (pH 7.6), 75 mM potassium glutamate, 1 mM EGTA, 5 mM MgOAc, 10% glycerol, 0.2% Triton X-100, 1 mM DTT, and 1× protease inhibitor cocktail tablet). After magnetic separation, the beads were washed 5 times with 800 μl of ice-cold wash buffer. The first wash also contained 0.1 μg/μl poly(dI-dC)•poly(dI-dC). Thereafter, the beads were washed once in 25 μl of 1% SDS, and the eluate was isolated by a magnetic separator. Finally, a 5-μl portion of Dynabeads (BEADS) or the supernatant (SUP) was loaded onto an SDS-polyacrylamide gel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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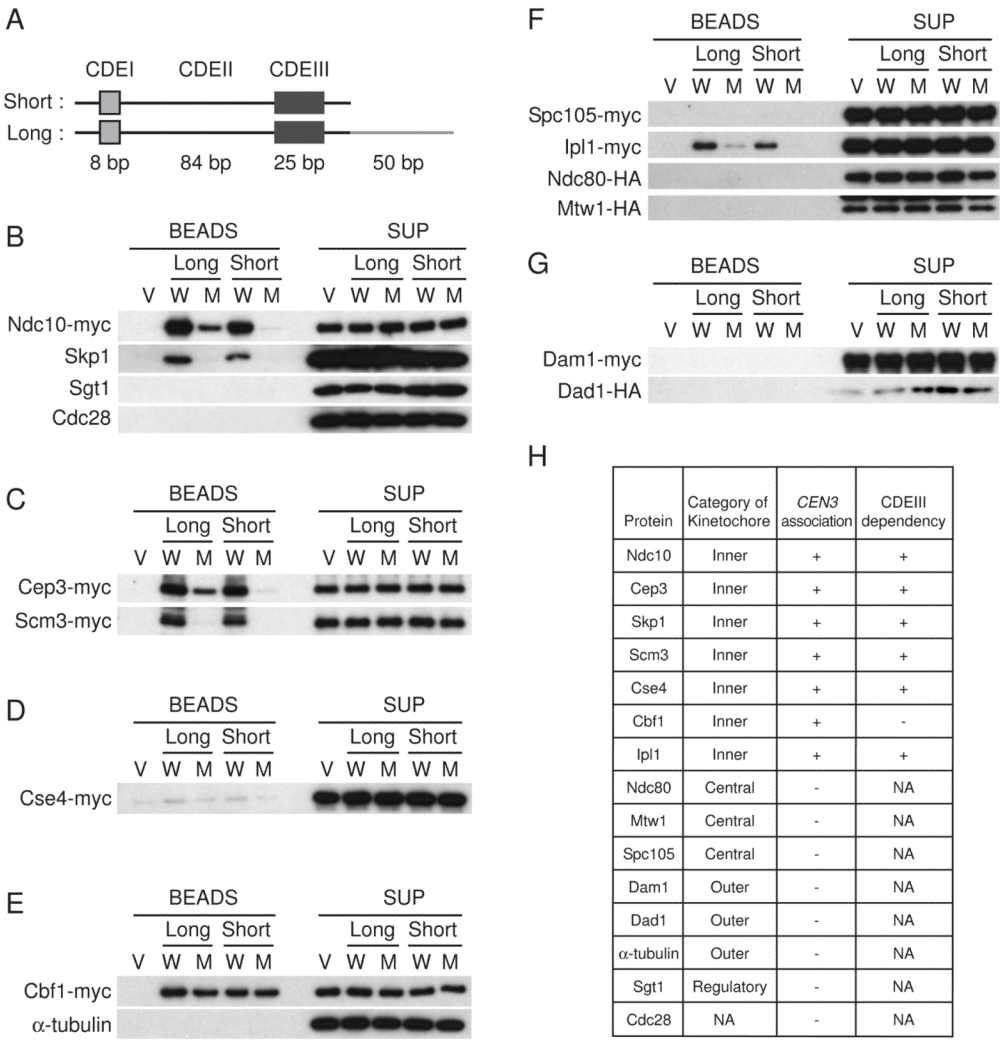

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Protein	Category of Kinetochore	*CEN3* association	CDEIII dependency
Ndc10	Inner	+	+
Cep3	Inner	+	+
Skp1	Inner	+	+
Scm3	Inner	+	+
Cse4	Inner	+	+
Cbf1	Inner	+	-
Ipl1	Inner	+	+
Ndc80	Central	-	NA
Mtw1	Central	-	NA
Spc105	Central	-	NA
Dam1	Outer	-	NA
Dad1	Outer	-	NA
α-tubulin	Outer	-	NA
Sgt1	Regulatory	-	NA
Cdc28	NA	-	NA

Figure 1. Initial kinetochore assembly *in vitro*

(A) The 117-bp *CEN3* DNA consists of three conserved DNA elements, designated CDEI, CDEII, and CDEIII. CDEI consists of a conserved 8-bp sequence incorporating a 6-bp palindrome, which is important for Cbf1 association. Approximately 93% of the CDEII sequence is made up of A:T base pairs. CDEIII has a size of 25 bp and includes the completely conserved sequence CCG. As a negative control in the *in vitro* kinetochore assembly system, the CCG sequence of CDEIII was replaced to CCC. The short version of *CEN3* DNA has a length of 134 bp, containing only CDEI, CDEII, and CDEIII. The long version of *CEN3* DNA has a length of 184 bp, containing the same sequences plus an Ndc10-binding site (see Espelin *et al.*, 1997 [18]). (B–G) Protein extracts from cyclic cells were incubated with magnetic beads coupled to plasmid DNA. Vector (V) or plasmid containing 8 tandem copies of wild-type (W) or mutant (M) *CEN3* was used. After 4 min at room temperature with highly concentrated protein extract, the beads were collected and washed (Figure S1B). Proteins bound to the beads were analyzed by 4–15% SDS-PAGE and immunoblotting. The protein extracts derived from the tagged strains were as follows: (B) Ndc10-myc Ndc80-HA Mtw1-HA (Y2046); (C) Cep3-myc Scm3-myc Mif2-myc (Y2049); (D) Cse4-myc (Y2044); (E) Cbf1-myc Isw1-HA (Y2083); (F) Spc105-myc Ipl1-myc

Ndc80-HA Mtw1-HA (Y2047); and (G) Dam1-myc Stu2-myc Dad1-HA (Y2048). (H) Summary of kinetochore assembly in 4 min. NA: Not applicable.

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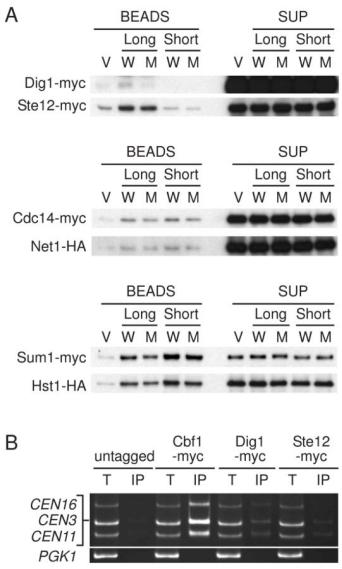

Figure 2. Dig1 and Ste12 are bound to centromeric chromatin

(A) Dig1-Ste12, Cdc14-Net1 and Sum1-Hst1 are bound to centromeric chromatin *in vitro*. *In vitro* kinetochore assembly system was executed as described in Figure 1. The protein extracts derived from the tagged strains were as follows: Dig1-myc Ste12-myc (Y2052); Cdc14-myc Net1-HA (Y2050); and Sum1-myc Hst1-myc (Y2051). (B) Anti-myc chromatin immunoprecipitation (ChIP) assays were performed from log-phase cells. Total lysate (T) and coimmunoprecipitated DNA (IP) were analyzed by PCR with primers specific to centromeric regions of chromosomes III, XI, and XVI and to a noncentromeric region (PGK1). The yeast strains used were as follows: untagged (YPH499), Cbf1-myc (Y2053), Dig1-myc (Y2054), and Ste12-myc (Y2055).

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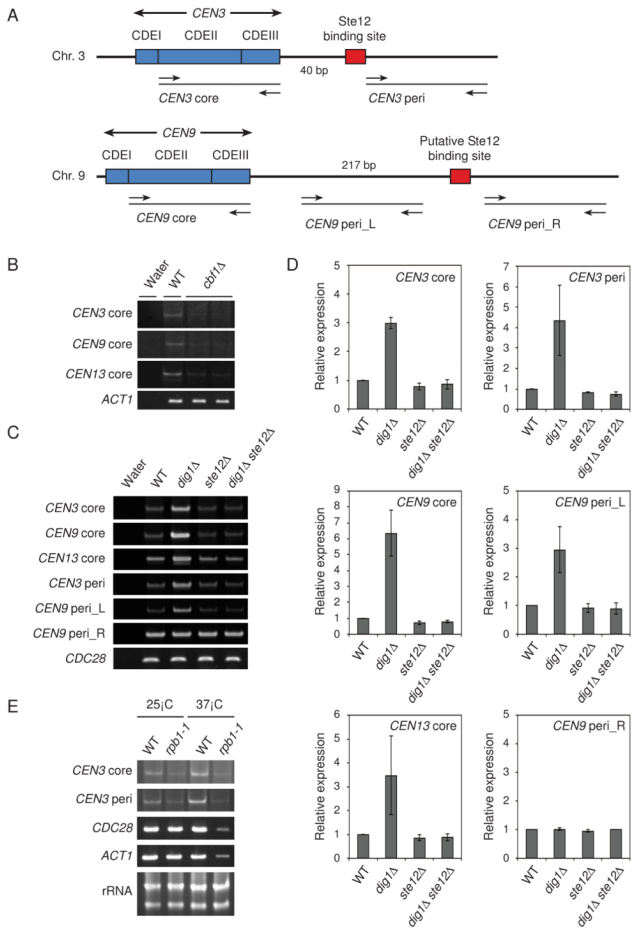

Figure 3. Centromeric transcripts in *Saccharomyces cerevisiae*

(A) Schematic representations of the *CEN3* and *CEN9* regions. Arrows mark the location of primers. The Ste12 binding site of *CEN3* (TGAAACG) is located 41–47 bp from CDEIII. The putative Ste12 binding site of *CEN9* (TGTAACA) is located 218–224 bp from CDEIII. (B) Transcription derived from *CEN3*, *CEN9*, and *CEN13* was detected in the wild-type but not in the *cbf1*Δ mutants. RT-PCR analyses were performed from log phase total RNA in wild-type (YPH499) and *cbf1*Δ mutants (Y1987 and Y1988). *ACT1* was used as a loading control. (C) Accumulation of transcripts derived from the core or pericentromeric regions in the *dig1*Δ mutant was determined with RT-PCR. *CDC28* was used as a loading control. The yeast strains used were as follows: wild-type (YPH499), *dig1*Δ (Y1979), *ste12*Δ (Y1985), and *dig1*Δ *ste12*Δ (Y2056). (D) Quantification of transcripts in (C). Relative expression levels were calculated by dividing *CDC28* expression level. (E) RNA polymerase II is required for the *CEN3* transcripts. RT-PCR analyses were performed from log phase total RNA in wild-type (YF7) and the largest subunit mutant of RNA polymerase II (*rpb1-1*) (YF38). *CDC28* and *ACT1* were used as positive controls. Ribosomal RNA was used as a loading control.

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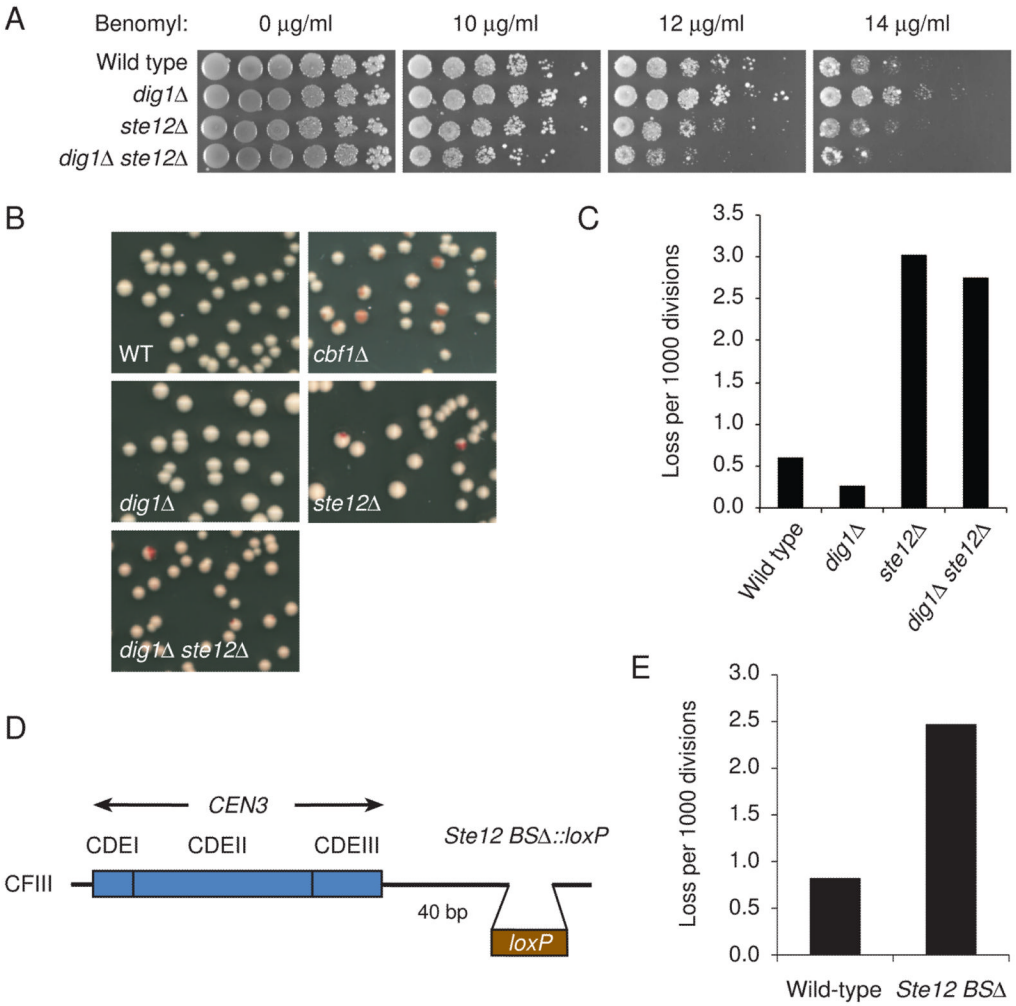

Figure 4. Centromeric transcripts contribute to centromere function

(A) Benomyl resistance of *dig1*Δ cells and benomyl sensitivity of *ste12*Δ and *dig1*Δ *ste12*Δ cells. Yeast cells were spotted in 5-fold dilutions from 5×10^4 cells per spot on YPD plates containing benomyl. The plates were incubated at 23°C for 5 days and photographed. The yeast strains used were as follows: wild-type (YPH499), *dig1*Δ (Y1978), *ste12*Δ (Y1985), and *dig1*Δ *ste12*Δ (Y2056). (B) *cbf1*Δ, *ste12*Δ, and *cbf1*Δ *ste12*Δ mutants sectoring phenotypes. Each strain includes a single *SUP11*-marked chromosome III fragment containing *CEN3*. (C) Chromosome loss rate in null mutants was determined by half-sector analysis. Wild type: 18 half-sectored colonies/30,214 total colonies; *dig1*Δ: 6/22,972; *ste12*Δ: 44/14,567; *dig1*Δ *ste12*Δ: 40/14,575. The yeast strains used in B and C were as follows: wild-type (Y14), *cbf1*Δ (Y2011), *dig1*Δ (Y2016), *ste12*Δ (Y2060 and Y2061), and *dig1*Δ *ste12*Δ (Y2062 and Y2063). (D) Schematic diagram showing deletion of the Ste12 binding site (*Ste12 BS*Δ) on the chromosome fragment. (E) Increased chromosome missegregation in *Ste12 BS*Δ. Chromosome loss rate was determined by half-sector analysis. Wild-type (Y14): 7 half-sectored colonies/8,550 total colonies; *Ste12 BS*Δ (Y2082): 20/8,085.

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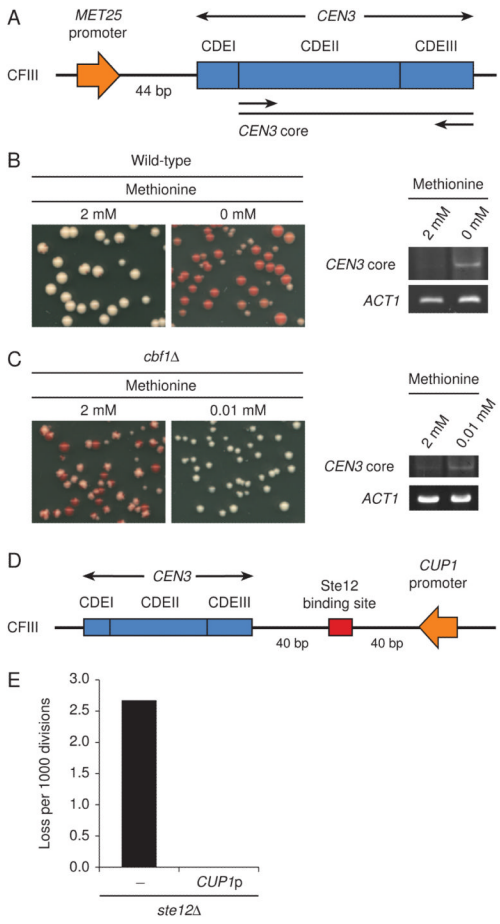

Figure 5. Centromeric transcripts induced by an artificial promoter suppress the chromosome missegregation phenotype of *cbf1*Δ and *ste12*Δ

(A) A schematic diagram showing the integration of the *MET25* promoter in front of CDEI on the chromosome fragment. (B) Overexpressed transcripts impair centromere function. P_{MET25}-*CEN3* (Y392) cells were precultured in minimal (SD) medium plus 2 mM methionine (to repress the *MET25* promoter) and lacking uracil (to keep the chromosome fragments). Colony color assay was performed after plating the cells onto SD plates, limiting the amount of adenine and adding the indicated concentration of methionine. RT-PCR analysis was performed from the total RNA taken after 3 h of induction. (C) Centromeric transcripts from a *MET25* promoter suppress the sectoring phenotype of *cbf1*Δ mutants. *cbf1*Δ P_{MET25}-*CEN3* (Y1990) cells were examined as described in (B). The medium containing 0.01 mM methionine was used as a derepressing condition. (D) Schematic diagram showing the integration of the *CUP1* promoter in front of the Ste12 binding site on the chromosome fragment. (E) Yeast cells were precultured in SD medium lacking tryptophan (to maintain the chromosome fragments). Colony color assay was performed after plating the cells onto SD plates, limiting the amount of adenine. *ste12*Δ (Y2060): 15 half-sectored colonies/5,615 total colonies; *ste12*Δ P_{CUP1}-*CEN3* (Y2080); 0/12,622.

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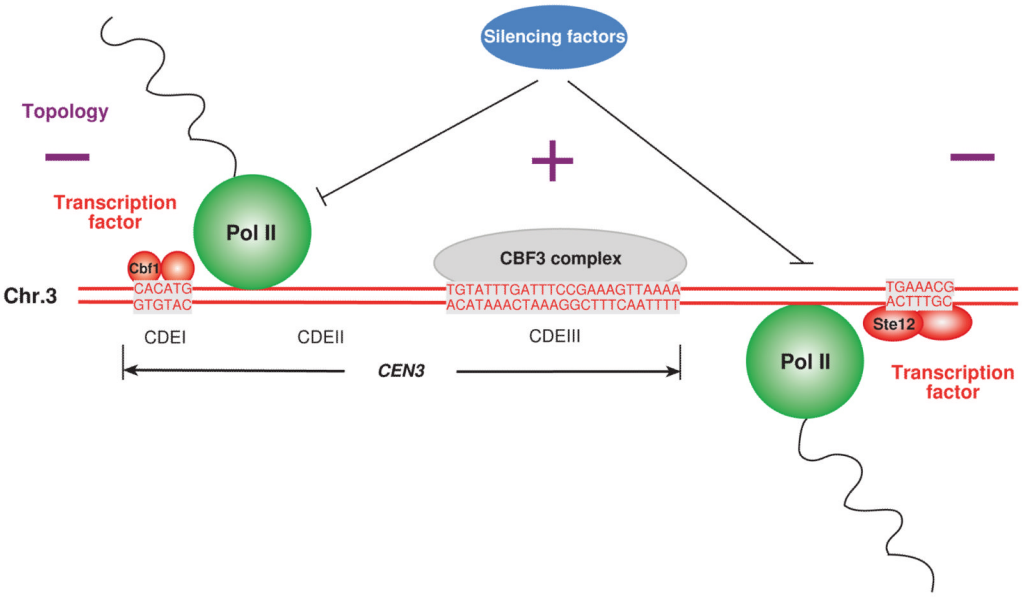

Figure 6. Transcription at the centromere plays an important role in centromere function

CEN3, which is 117 bp, is composed of three regions, CDEI, CDEII, and CDEIII. The sequence of CDEIII, which binds to CBF3 (the core kinetochore complex), and Cbf1 and Ste12 (transcription factors) binding sites are shown in grey boxes. Cbf1 and Ste12 contribute to transcription at the centromere. On the other hand, silencing factors, such as Sir1, Hst1, or Cdc14, may inhibit the transcription. RNA polymerase II is required for core- and peri-centromeric transcription. Predicted DNA topology is shown as plus (positive DNA supercoils) or minus (negative DNA supercoils). Intriguingly, topological analysis of yeast minichromosomes revealed that functional centromeres induce positive DNA supercoils [40]. Transcription at the centromere may generate proper topology of *CEN* DNA.