Point centromere activity requires an optimal level of centromeric noncoding RNA

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In budding yeast, which possesses simple point centromeres, we discovered that all of its centromeres express long noncoding RNAs (cenRNAs), especially in S phase. Induction of cenRNAs coincides with CENP-A^{Cse4} loading time and is dependent on DNA replication. Centromeric transcription is repressed by centromere-binding factor Cbf1 and histone H2A variant H2A.Z^{Htz1}. Deletion of *CBF1* and *H2A.Z^{HTZ1}* results in an up-regulation of cenRNAs; an increased loss of a minichromosome; elevated aneuploidy; a down-regulation of the protein levels of centromeric proteins CENP-A^{Cse4}, CENP-A chaperone HJURP^{Scm3}, CENP-C^{Mif2}, Survivin^{Bir1}, and INCENP^{Sli15}; and a reduced chromatin localization of CENP-A^{Cse4}, CENP-C^{Mif2}, and Aurora B^{Ipl1}. When the RNA interference system was introduced to knock down all cenRNAs from the endogenous chromosomes, but not the cenRNA from the circular minichromosome, an increase in minichromosome loss was still observed, suggesting that cenRNA functions *in trans* to regulate centromere activity. CenRNA knockdown partially alleviates minichromosome loss in *cbf1Δ*, *htz1Δ*, and *cbf1Δ htz1Δ* in a dose-dependent manner, demonstrating that cenRNA level is tightly regulated to epigenetically control point centromere function.

centromeric transcription | long noncoding RNA | centromere-binding factor Cbf1 | histone H2A variant Htz1 | chromosome instability

Eukaryotes, such as humans, mice, flies, and fission yeast, harbor regional centromeres consisting of tandemly repeated DNA that can be up to megabases long (1). The regional centromere is built on nucleosomes containing centromeric-specific histone H3 variant, CENP-A, that intersperse with histone H3-containing nucleosomes. The regional centromere is flanked by the pericentric heterochromatin, which can extend over several megabases. The formation and function of regional centromeres is regulated by epigenetics (2). Noncoding transcription in centromeric chromatin has been identified in many organisms harboring regional centromeres recently. A number of studies have proposed a role of centromeric transcription in regulating centromere activity (3). Inhibition of RNA polymerase II (RNAPII) by α-amanitin in human cells during mitosis resulted in a decrease in the level of centromeric protein CENP-C and an increase in chromosome missegregation (4). In addition, mutation of a subunit of the facilitates chromatin transcription (FACT) complex, which is a transcription elongation factor and a chromatin modifier, affects the deposition of CENP-A into the centromere (5). Some studies attributed the effect of centromeric transcription on centromere activity to the noncoding centromeric RNAs (cenRNAs). CenRNAs interact or associate with several important centromeric proteins, such as CENP-A (6, 7), CENP-C (8, 9), and components of the chromosomal passenger complex (CPC): Aurora-B, Survivin, and INCENP (9–11). Knockdown of cenRNAs resulted in abnormal nuclear morphology (11), mitotic defects (6), and misregulation of centromere proteins CENP-A (6), CENP-C (8, 9, 12), and Aurora-B (10, 13). On the other hand, ectopic expression of cenRNAs in mouse cells causes delocalization of Aurora-B from the centromere in mitotic chromosomes (14). Therefore, either too much or too little centromeric transcription can cause centromere malfunction, leading to chromosome instability (CIN) (15).

Budding yeast *Saccharomyces cerevisiae* contains point centromeres, which are ∼125 bp, consisting of three DNA elements: centromere DNA elements I–III (CDEI–III), built on a single CENP-A^{Cse4} nucleosome (16), and are flanked by a ∼2-kb array of uniformly spaced, phased nucleosomes (17, 18). Point centromeres are believed to be defined mainly by centromeric DNA sequences, which recruit specific centromeric DNA-binding proteins, including Cbf1 at CDEI, and Cbf3 complex at CDEIII. However, epigenetics has also been shown to be required for budding yeast de novo kinetochore assembly (19, 20). While it has long been known that forced, strong transcription across the point centromere in budding yeast inactivates the centromere (21), the positive impact of centromeric transcription in simple, point centromere function has only been shown recently (22). It is suggested that a certain level of centromeric transcription, regulated by transcription factors Cbf1 and Ste12, may provide a favorable epigenetic environment for normal centromere function (22). However, how centromeric transcription achieves a fine balance is unclear. Moreover, the timing of centromeric transcription and the identity and nature of the centromeric transcripts remain to be characterized.

Here, we show that centromeres in the budding yeast *S. cerevisiae* are transcribed and induced especially during S phase. Inner kinetochore protein Cbf1 and histone H2A variant H2A.Z^{Htz1} repress centromeric transcription. Up-regulation of centromeric transcription decreases the stability of a circular minichromosome

Significance

Budding yeast harbors a simple point centromere, which is originally believed to be sequence dependent without much epigenetic regulation and is transcription incompatible, as inserting a strong promoter upstream inactivates the centromere completely. Here, we demonstrate that an optimal level centromeric noncoding RNA is required for budding yeast centromere activity. Centromeric transcription is induced in S phase, coinciding with the assembly of new centromeric proteins. Too much or too little centromeric noncoding RNA leads to centromere malfunction. Overexpression of centromeric noncoding RNA reduces the protein levels and chromatin localization of inner centromere and kinetochore proteins, such as CENP-A, CENP-C, and the chromosome passenger complex. This work shows that point centromere is epigenetically regulated by noncoding RNA.

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The authors declare no conflict of interest.

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and endogenous chromosomes, down-regulates some centromeric and kinetochore protein levels, and reduces their chromatin association. To knock down cenRNAs from multiple chromosomes, we have constructed a transgenic strain in which the centromeric regions on all 16 chromosomes are converted to centromere 8 (*CEN8*), introduced the RNA interference (RNAi) system into yeast and expressed hairpin RNA targeting cenRNA8 sequence (23). Knockdown of the total pool of cenRNAs results in an increased loss rate of the circular minichromosome. In *cbf1Δ*, *htz1Δ*, or *cbf1Δ htz1Δ*, in which the cenRNA level is up-regulated, cenRNA knockdown partially recuses the minichromosome loss phenotype, indicating a finely balanced regulation of the cenRNA level is important for centromere function. Our results show that point centromeres are also controlled by epigenetics through cenRNAs, suggesting functional conservation of centromeric transcription among eukaryotic centromeres.

Results

Budding Yeast cenRNAs Are Transcribed in Both Strands and Contain Poly-A Tails. Reverse transcription PCR (RT-PCR) targeting haploid *S. cerevisiae* centromeric sequences, with expected product size of >200 bp, was performed to show that all centromeres can be transcribed to yield long noncoding RNAs (lncRNAs) (Fig. 1*A*).

cenRNA1, cenRNA3, and cenRNA8 were selected for further characterization as they gave strong RT-PCR signals (Fig. 1*A*). Strand-specific RT-PCR showed centromere 1, 3, and 8 are transcribed in both orientations (Fig. 1*B*). CenRNAs may contain poly-A tails because they can be reverse transcribed with the oligo(dT) primer (Fig. 1*C*). Detailed analysis of 5′ and 3′ ends of cenRNAs were performed by rapid amplification of cDNA ends (RACE). Sequencing of the RACE PCR products identified multiple 5′ and 3′ ends for each cenRNA analyzed, which are also referred to as the transcription start sites (TSSs) and 3′-end processing sites/transcription termination sites (TTSs) of cenRNAs, respectively (Fig. 1*D* and *SI Appendix*, Fig. S2). Sequencing of the 3′ RACE PCR products confirms the oligo(dT) primer was not binding to any A-rich sequences in the centromere-proximal region. As the 5′-RACE PCR products were designed to overlap with the corresponding 3′-RACE PCR products, and at least either the 5′- or 3′-RACE PCR product spans the centromeric region, we deduced the cenRNA sizes to be 462–1,754 nt (*SI Appendix*, Fig. S2). Adhering to the common threshold for defining ncRNAs (24–26), we confirmed that no open reading frame (ORF) encoding for more than 100 amino acids was found in the full-length cenRNA sequences. It was noted that sense cenRNA3 covers a short ORF, YCL001W-B, which encodes for hypothetical protein with unannotated function (*SI Appendix*, Fig. S2).

Centromeric Transcription Is Induced in S Phase by DNA Replication. To determine if cenRNA expression was cell cycle dependent, cells were arrested in G1 phase by α-factor and then released into nocodazole-containing medium for G2/M phase arrest

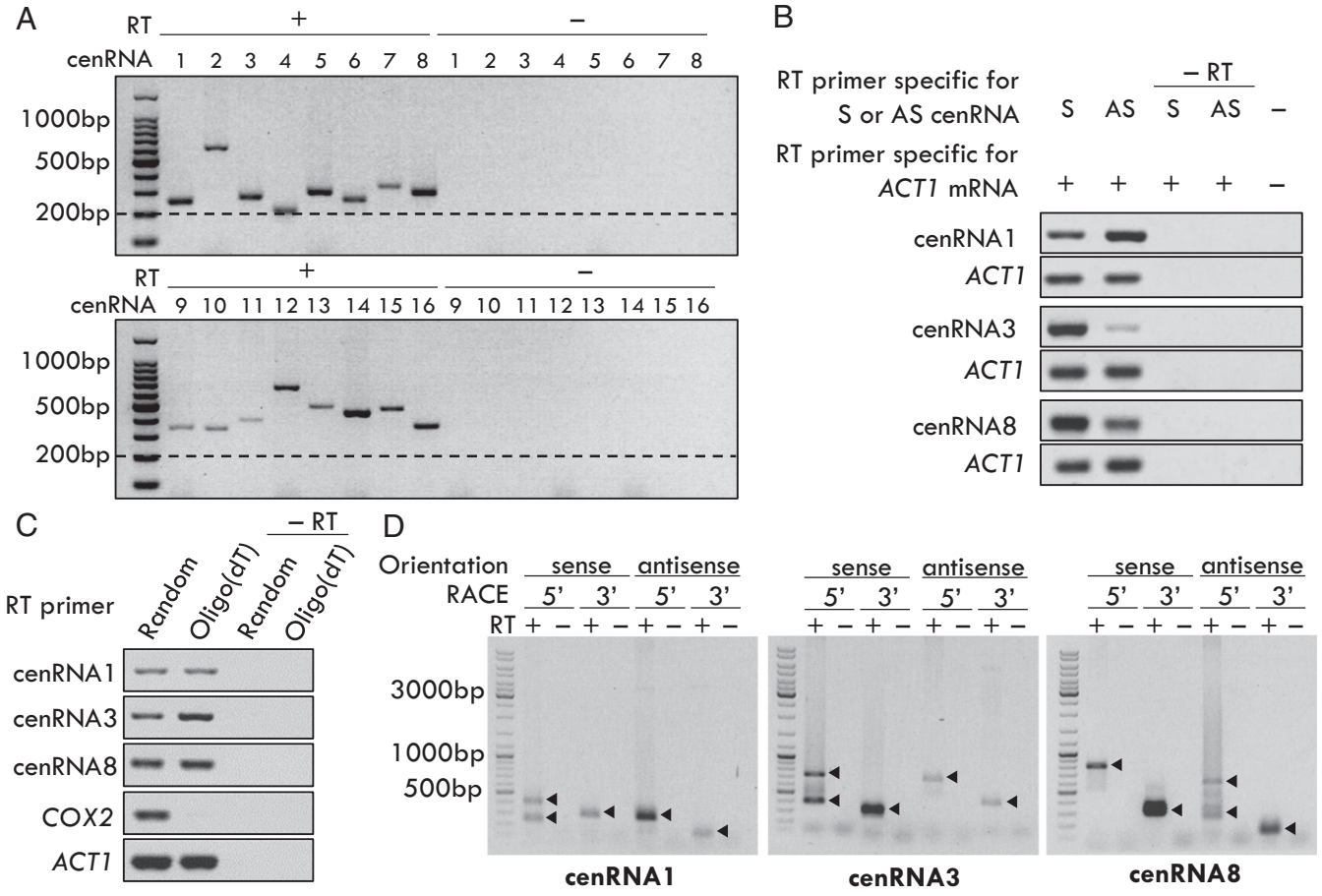

Fig. 1. Centromeres of the budding yeast *S. cerevisiae* express long noncoding RNAs in both sense and antisense orientations, containing poly-A tails, and with multiple start and end sites. (*A*) RT-PCR indicated that centromeres from all 16 chromosomes in *S. cerevisiae* are transcribed into RNAs with over 200 bp. (*B*) Strand-specific RT-PCR of cenRNA1, cenRNA3, and cenRNA8 indicated that centromeres are transcribed in both sense (S) and antisense (AS) orientations. RT primer specific for actin (*ACT1*) mRNA was added in the reaction as an internal control. (*C*) RT-PCR of cenRNA1, cenRNA3, and cenRNA8 using either random primer or oligo(dT)$_{12–18}$ primer. *COX2*, a mitochondrial mRNA without a poly-A tail, is used as the negative control. *ACT1* is used as the internal control. (*D*) RACE PCR of cenRNA1, cenRNA3, and cenRNA8.

(*SI Appendix*, Fig. S3*A*). We found that cenRNAs (indistinguishing sense, antisense, or transcription variants) were induced when cells entered G1/S phase (Fig. 2 *A* and *B*), which coincided with *CLN2* induction (at 10 min after release from α-factor arrest). CenRNA expression level peaked at S phase (20 min), similar to *HTA1* expression. When cells started to enter G2/M phase, as indicated by the expression of *CLB2* (50 min), cenRNA level dropped back to close to the baseline level at G1 phase (0 min). We also quantified the copy number of cenRNA. In asynchronous culture, cenRNA8 has a copy number of 0.002 molecules per cell (SEM = 0.0001). In S phase, cenRNA8 level increased 16-fold to 0.031 molecules per cell (SEM = 0.003) (*SI Appendix*, Text S1 and Fig. S1).

We next investigated the relationship between the S phase-induced centromeric transcription and DNA replication. We controlled the expression of Cdc6, a component of the pre-replication complex (27), by a galactose-inducible promoter. Yeast cells were first synchronized to G1 phase by α-factor arrest in galactose-containing (*CDC6* ON) medium, and then released into either galactose-containing (*CDC6* ON) or glucose-containing (*CDC6* OFF) medium. In the *CDC6* ON condition (Fig. 2 *C* and *D*), a 10-min delay in S phase entry was observed compared with the *CDC6* wild type (based on induction time of *CLN2* and *HTA1*, Fig. 2*A*), possibly due to a slower growth when galactose was used as the carbon source instead of glucose (28). Nonetheless, the cenRNA8 expression peak was always coherent with the time of *HTA1* induction (Fig. 2 *D* and *F*). DNA content analysis indicated that *CDC6* OFF cells remained in 1N DNA content (*SI Appendix*, Fig. S3*B*). *CDC6* ON cells showed a slightly slower emergence of bud compared with *CDC6* OFF cells (*SI Appendix*, Fig. S3*C*), again possibly because of a slower growth in galactose than in glucose. As for the expression of cell cycle marker genes, both conditions showed similar induction time of G1/S marker gene, *CLN2* (20 min, Fig. 2*C*). *CDC6* OFF condition impaired the induction of S phase marker gene *HTA1*, as its expression is dependent on DNA replication (29, 30). Expression of G2/M marker gene, *CLB2*, in the *CDC6* OFF condition was 20 min later than that in *CDC6* ON condition. Importantly, when DNA replication was inhibited in the *CDC6* OFF condition, cenRNA8 was no longer induced at S phase (Fig. 2*F*), similar to *HTA1*. CenRNA3 also showed reduced S phase induction in CDC6 OFF condition (*SI Appendix*, Fig. S3*D*). Thus, DNA replication is required for the S phase induction of centromeric transcription, at least in chromosomes III and VIII.

Centromeric Transcription Is Repressed by Kinetochore Protein Cbf1 and Histone H2A Variant H2A.Z^{Htz1}. To identify the regulators of centromeric transcription, 17 candidate genes, which are transcription regulators with predicted binding sites around the centromeres, were selected (*SI Appendix*, Datasets S1 and S2, and Fig. S4 *A* and *B*). Three deletion mutants were found to have perturbed cenRNA expression levels. *cbf1Δ* and *htz1Δ* up-regulated and *fkh2Δ* down-regulated the expression cenRNAs (Fig. 3*A*). Cbf1 is a helix–loop–helix transcription factor found in gene promoters involved in methionine biosynthesis (*SI Appendix*, Fig. S4*C*) (31, 32). It is also a centromeric protein that binds to the CDEI domain (33). H2A.Z^{Htz1} is a histone H2A variant that localizes to the pericentric chromatin, regulating centromere silencing and chromosome segregation in budding and fission yeast (34, 35). H2A.Z^{Htz1} also localizes to promoter regions

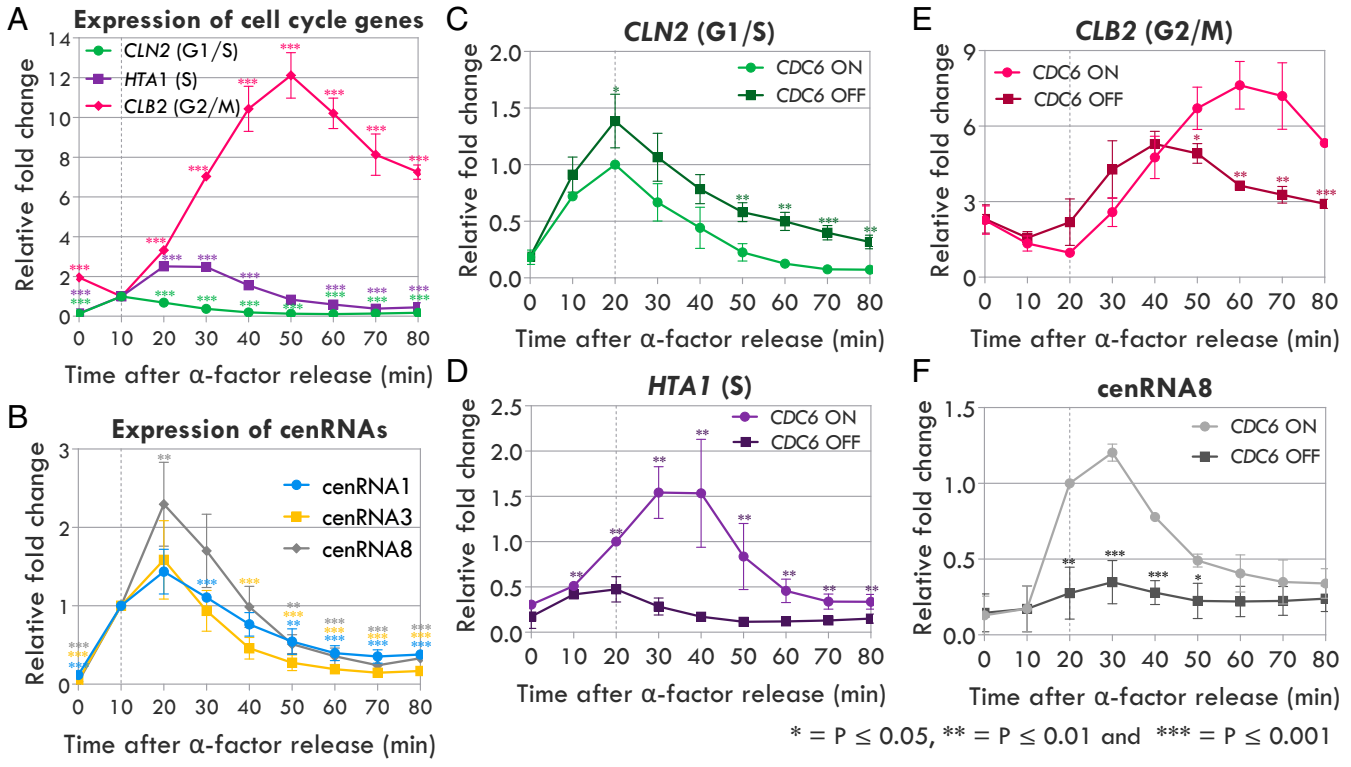

Fig. 2. Centromeric transcription is induced in S phase and is dependent on DNA replication. (*A* and *B*) RT-qPCR analysis of the mRNA expression levels of (*A*) cell cycle marker genes and (*B*) cenRNA1, cenRNA3, and cenRNA8 throughout cell cycle progression. Expression levels were quantified relative to that at 10 min after α-factor release (peak of *CLN2* expression). Statistical significances of the expression level (mean ± SD, $n = 3$) between the reference time point (10 min) and other time points were analyzed by multiple *t* test. (*C–F*) RT-qPCR analysis of the expression of (*C–E*) cell cycle marker genes and (*F*) cenRNA8 in *CDC6* ON or *CDC6* OFF condition. All data were quantified relative to the *CDC6* ON cells, at 20 min after α-factor release (peak of *CLN2* expression) (mean ± SD, $n = 3$). Statistical significances between the *CDC6* ON and *CDC6* OFF condition at each time point were analyzed by multiple *t* test.

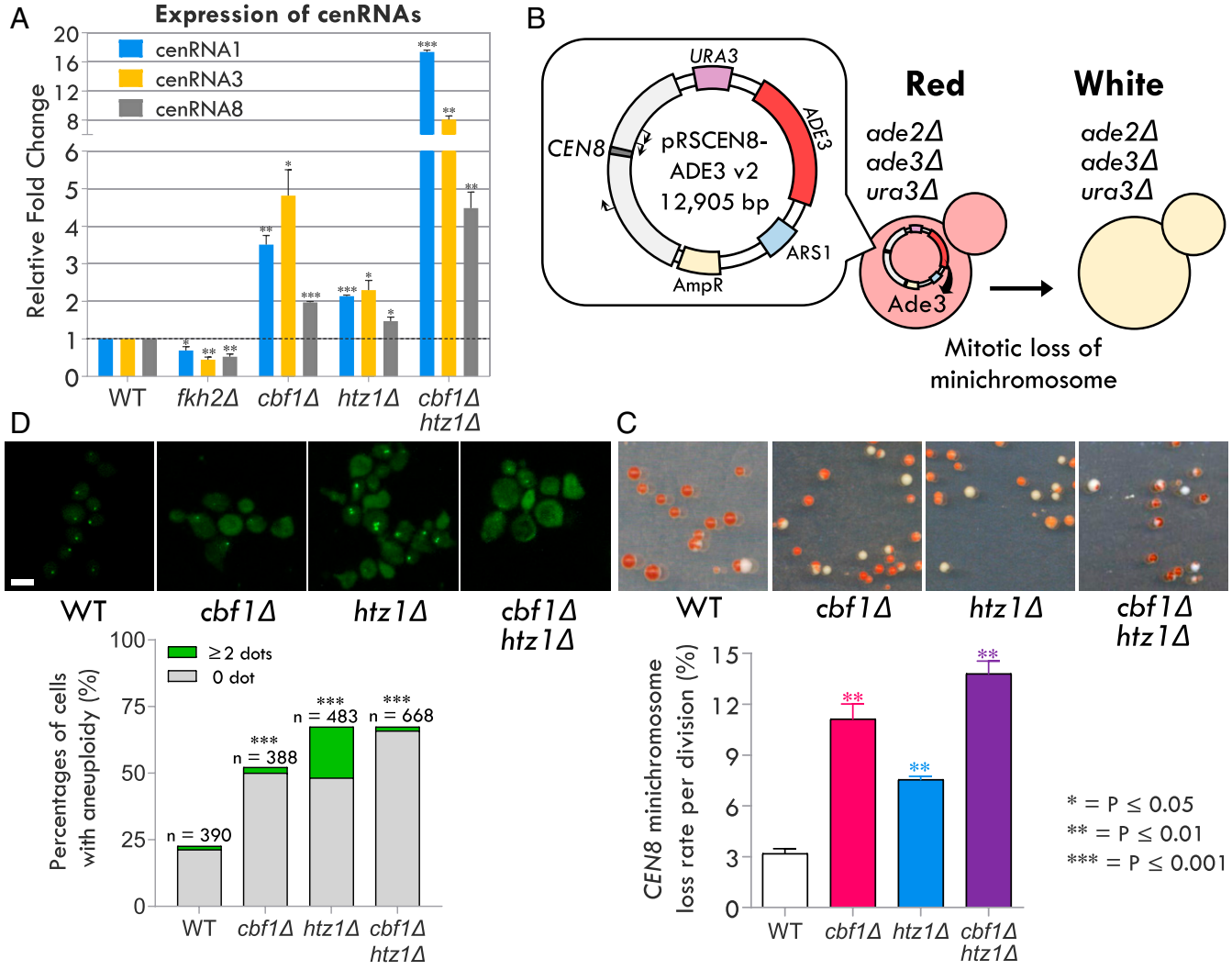

Fig. 3. Cbf1 and H2A.Z^{Htz1} repress centromeric transcription. (*A*) RT-qPCR analysis of cenRNA1, cenRNA3, and cenRNA8 expression in the deletion mutants, relative to the WT. Statistical significances of the expression level (means ± SD, $n = 3$) were analyzed with paired *t* test. (*B*) Illustration of the minichromosome loss assay. (*C*) Mitotic loss rates of the *CEN8* minichromosome per division in deletion mutants ($n = 3$). Statistical significances were analyzed with paired *t* test. (*D*) Aneuploidy frequency in deletion mutants. An array of 256 lacO repeats was integrated on chromosome III and visualized by lacI-GFP. (Scale bar, 5 μm.) Unbudded, G1 cells were counted and scored. Normal cells contained one GFP dot, while cells with aneuploidy contained none or two or more GFP dots. Statistical significances were analyzed by χ^2 test.

to regulate gene expression (36). Interestingly, *cbf1Δ htz1Δ* double mutant resulted in an additive up-regulation of cenRNA (Fig. 3*A*), suggesting that Cbf1 and H2A.Z^{Htz1} may act in different pathways to repress centromeric transcription. Chromatin immunoprecipitation (ChIP) also indicated that *cbf1Δ htz1Δ* has an elevated level of total RNAPII (subunit Rpb3) and active RNAPII mark (Ser2 phosphorylation on subunit Rpb1) around the centromere, consistent with its increase of cenRNA expression (*SI Appendix*, Text S2 and Fig. S5).

Fkh2 is a forkhead family transcription factor involved in regulating RNAPII transcriptional elongation (37). The down-regulation of cenRNAs in *fkh2Δ* supported that cenRNAs are transcribed by RNAPII (*SI Appendix*, Fig. S5) (22). However, Fkh2 was not chosen for further analysis because *fkh2Δ* also affected some cell cycle gene expression (38), which complicates the cell cycle expression analysis of cenRNAs.

We examined cenRNA expression in *cbf1Δ* and *htz1Δ* throughout the cell cycle after synchronization as above. Both mutants progressed the cell cycle similar to the WT, as demonstrated by the DNA content (*SI Appendix*, Fig. S6*A*) and the expression of cell cycle marker genes (*SI Appendix*, Fig. S6*B*). In all cell cycle stages, cenRNAs were up-regulated in both mutants. The S phase induction peak remained, except for cenRNA1 in *cbf1Δ*. CenRNAs up-regulation in all cell cycle phases was stronger in *cbf1Δ* than in *htz1Δ* (*SI Appendix*, Fig. S6*C*).

Up-Regulation of Centromeric Transcription Reduces Chromosome Stability, Centromere Protein Levels, and Chromatin Association. To examine centromere function in *cbf1Δ* and *htz1Δ*, the mitotic loss rate of a circular minichromosome was monitored based on the colors of the colony sectors (39). White-colored *ade2 ade3 ura3* yeast cells were transformed with a 13-kb circular minichromosome containing the 118-bp *CEN8* region together with an extended flanking pericentric region (2,746 bp upstream of CDEI and 2,002 bp downstream of CDEIII) and *URA3* as a selective marker (Fig. 3*B*). Maintenance of *ADE3*-expressing minichromosome in the *ade2 ade3 ura3* strain results in red cells. The basal loss rate of circular minichromosome is about 10^{-2} per cell division in the WT (40). Both *cbf1Δ and htz1Δ*

resulted in higher minichromosome loss rates (Fig. 3*C*). The minichromosome loss rate of *cbf1Δ* was higher than that in *htz1Δ*, consistent with their corresponding cenRNA overexpression levels (Fig. 3*A*), and the loss rate of *cbf1Δ htz1Δ* double mutant was the highest (Fig. 3*C*).

The frequency of aneuploidy in deletion mutants was also evaluated. An array of 256 copies of lac operon (lacO) repeats was integrated 22 kb to the left of *CEN3* (41). Lac repressor (lacI)-GFP binds on the lacO array and forms a single fluorescent dot that allows the tracking of the fate of chromosome III. The number of lacI-GFP dots was counted in unbudded, G1 haploid cells. Cells may have lost the lacI-GFP dot due to a loss of the whole chromosome III or intrachromosomal mitotic recombination of the long lacO repetitive array (42), whereas cells with two lacI-GFP dots could result from a gain of the whole chromosome III. We scored cells with either zero or two lacI-GFP dots as aneuploidy, which may slightly overestimate the aneuploidy rate. Nonetheless, consistent with the minichromosome loss assay, *cbf1Δ*, *htz1Δ*, and *cbf1Δ htz1Δ* showed increased rates of aneuploidy (Fig. 3*D*).

The levels of several centromere proteins were examined in the deletion mutants (Fig. 4*A*). A moderate reduction of CENP-A^{Cse4} was found in *htz1Δ* cells. A prominent, >50% reduction of CENP-A^{Cse4}, HJURP^{Scm3} (CENP-A chaperone), CENP-C^{Mif2}, and CPC components Survivin^{Bir1} and INCENP^{Sli15} protein levels were observed in *cbf1Δ htz1Δ*, while the level of two other CPC subunits, Borealin^{Nbl1} and Aurora-B^{Ipl1}, remained unchanged (Fig. 4*A* and *SI Appendix*, Fig. S7 *A* and *B* for quantification). This suggests that the inner kinetochore and CPC activity may be disrupted in *cbf1Δ htz1Δ*, when centromeric transcription is highly up-regulated. However, the mRNA levels of these centromeric proteins remain unchanged in the deletion mutants (*SI Appendix*, Fig. S7*C*).

We further analyzed the association of CENP-A^{Cse4}, CENP-C^{Mif2}, and Aurora-B^{Ipl1} on the chromatin by chromosome spreading (Fig. 4*B*). We found that the loss of CENP-A^{Cse4} and CENP-C^{Mif2} proteins on chromosomes was most striking in *cbf1Δ htz1Δ*, moderate in *cbf1Δ*, but not significant in *htz1Δ*. A significant loss of Aurora-B^{Ipl1} from the chromatin was found in both *cbf1Δ* and *htz1Δ*, and even more prominent in *cbf1Δ htz1Δ* (Fig. 4*B*), suggesting a disruption of centromere structure in these mutants. Although Aurora-B^{Ipl1} total protein level was unchanged in *cbf1Δ htz1Δ*, its prominent loss on chromosomes is possibly due to the decrease of the other regulatory components of the CPC, Survivin^{Bir1} and INCENP^{Sli15}.

Knockdown of Total cenRNAs Reduces Mitotic Stability of Minichromosome. We blocked centromeric transcription on a *CEN8* minichromosome with lacOs flanking *CEN8* by expression of lacI. It resulted in minichromosome loss (*SI Appendix*, Text S3 and Fig. S8), suggesting a crucial role of transcription in governing centromere activity. It is possible that the minichromosome loss phenotype resulted from a loss of centromeric transcription activity, a loss of cenRNA, or both. To distinguish these factors, we attempted to manipulate cenRNA levels without modulating centromeric transcription.

We reconstituted the lost RNAi machinery in *S. cerevisiae* (43) by introducing *Saccharomyces castellii*'s Dicer (Dcr1) and Argonaute (Ago1) (23) (*SI Appendix*, Fig. S10*A*, *Left*). Although RACE analysis showed expression of cenRNAs from both orientations with complementary sequences (*SI Appendix*, Fig. S2), introduction of the RNAi machinery per se did not affect the level of cenRNA1,

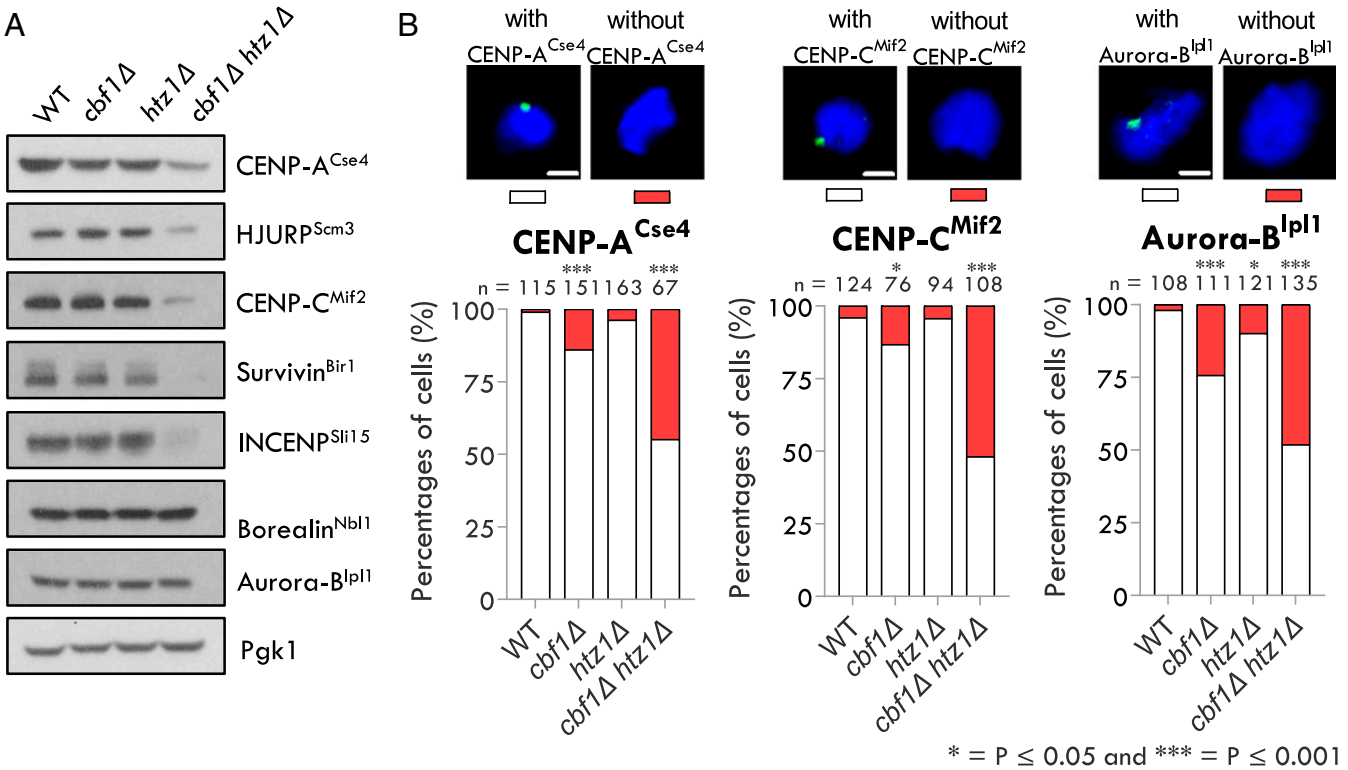

Fig. 4. *cbf1Δ* and *htz1Δ* exhibit compromised centromere function. (*A*) Expression of endogenously tagged centromeric proteins in the deletion mutants. Pgk1 expression levels from the experiment using CENP-A^{Cse4}–tagged strains were shown as representatives. Quantification of the protein expression level is shown in *SI Appendix*, Fig. S7*B*. (*B*) Deposition of CENP-A^{Cse4}, CENP-C^{Mif2}, and Aurora-B^{Ipl1} proteins (green) on the chromatin was examined by chromosome spread. DNA was stained with DAPI (blue). Representative images of DAPI spots with and without the protein signal are shown. (Scale bar, 2 μm.) Statistical significances were analyzed by χ^2 test.

cenRNA3, and cenRNA8 (*SI Appendix*, Fig. S10*B*), suggesting that cenRNAs are not in the form of double-stranded RNA (dsRNA).

To knock down specific RNA, hairpin RNA (hpRNA) under the control of the *TEF1* constitutively active promoter was integrated into the genome in a yeast strain expressing RNAi components and GFP (Fig. 5*A* and *SI Appendix*, Fig. S10*C*). *GFP* mRNA, as a knockdown control, was down-regulated by hpGFP (Fig. 5*B*). To knock down cenRNA, a hpRNA against cenRNA8 (hpcenRNA8) was integrated. In a strain carrying *CEN8* minichromosome (*SI Appendix*, Fig. S10*C*), with the endogenous *CEN8* sequence converted to *CEN3* sequence (*CEN8::CEN3*) (*SI Appendix*, Fig. S9*A*), hpcenRNA8 targeted the cenRNA8 from the *CEN8* minichromosome, but not the cenRNA expressed from chromosome VIII. HpcenRNA8 reduced cenRNA8 level to 39% (Fig. 5*B*). Both hpcenRNA8 and hpGFP specifically knocked down their targets without affecting the endogenously expressed cenRNA1 and *URA3*, the internal control gene on the minichromosome (Fig. 5*B*), or the growth rate of the cells (*SI Appendix*, Fig. S10*A*, *Right*). Surprisingly, no change in the *CEN8* minichromosome loss rate was observed in the cenRNA8 knockdown compared with the *GFP* knockdown (Fig. 5*C*). It is possible that merely knocking down the specific cenRNA from the minichromosome does not affect its stability. Potentially, cenRNAs from other chromosomes *in trans* can complement the function of the specific cenRNA from the minichromosome, cenRNA8, that has been knocked down.

To test this hypothesis, the total pool of cenRNAs needs to be depleted. *CEN8 × 16* strain was constructed in which all centromeres

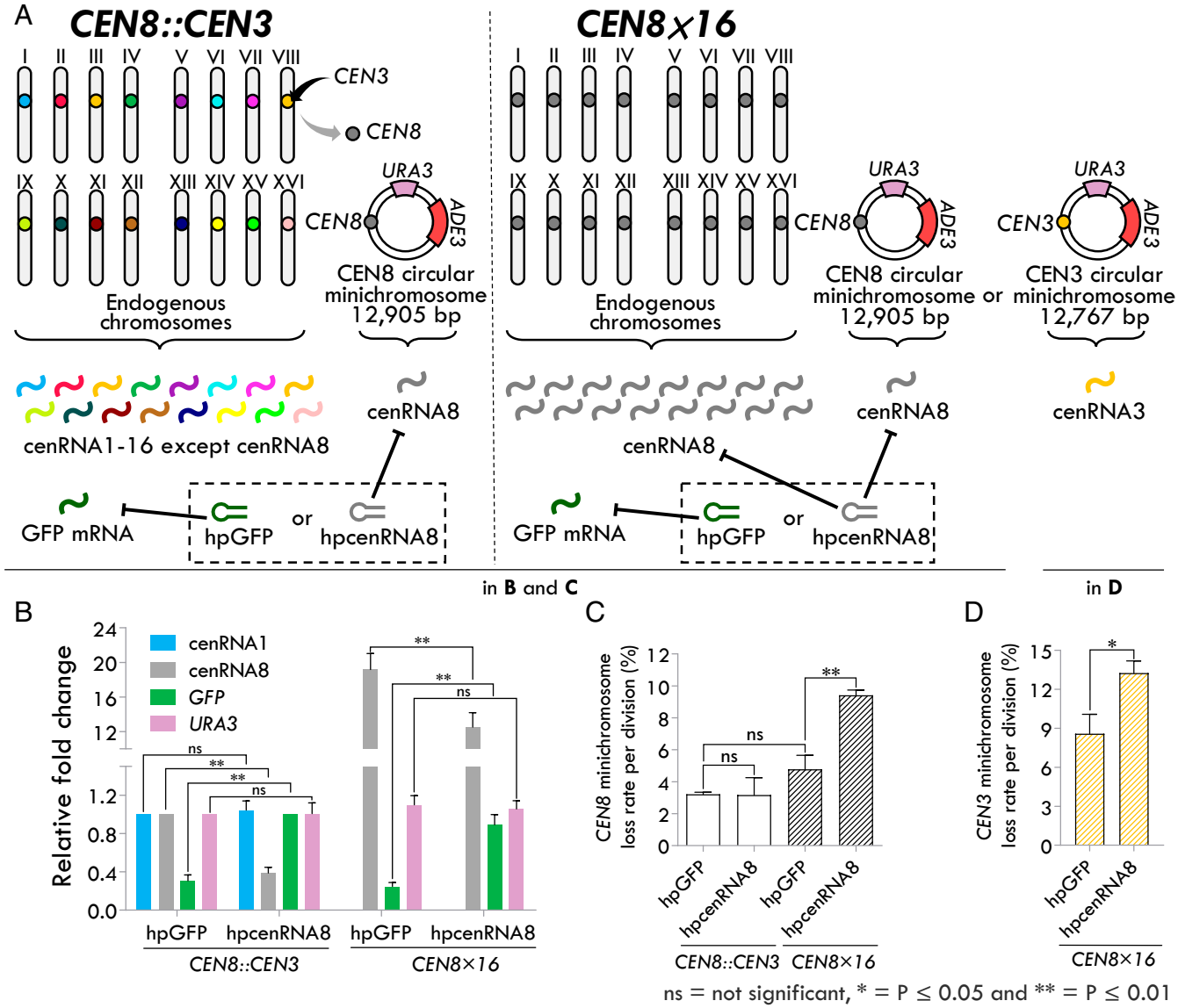

Fig. 5. Knockdown of total cenRNAs reduces mitotic stability of minichromosome. (*A*) Schematic diagram of the cenRNA knockdown experiments. In *CEN8::CEN3*, hpcenRNA8 knocked down cenRNA from the minichromosome only. In *CEN8 × 16*, hpcenRNA8 also targeted the cenRNAs from endogenous chromosomes. In *B* and *C*, strains in *CEN8::CEN3* and *CEN8 × 16* background were transformed with the *CEN8* minichromosome. In *D*, a strain in *CEN8 × 16* background was transformed with the *CEN3* minichromosome. (*B*) RT-qPCR analysis of the expression of cenRNA1, cenRNA8, *GFP*, and *URA3* in the RNAi-competent strains. For expression of cenRNA1, cenRNA8, and *URA3*, data were quantified relative to the hpGFP-expressing cells in *CEN8::CEN3*. For expression of *GFP*, data were quantified relative to the hpcenRNA8-expressing cells in *CEN8::CEN3*. Statistical significances of the expression level (mean ± SD, $n = 3$) were analyzed with paired *t* test. (*C*) *CEN8* and (*D*) *CEN3* minichromosome loss rates per division in the RNAi strains (means ± SD, $n = 3$). Statistical significances were analyzed with paired *t* test.

are converted to *CEN8* (*SI Appendix*, Fig. S9*B*). *CEN8* × *16* strain has a higher cenRNA8 expression level than the *CEN8::CEN3* strain (19.2-fold up-regulation, Fig. 5*B*). HpcenRNA8 targets all cenRNAs in the cells, resulting in a down-regulation of the total cenRNA pool. The absolute down-regulated level of cenRNA8 by hpcenRNA8 in the *CEN8* × *16* is still higher than that in the *CEN8::CEN3*; however, the total pool of cenRNAs in the *CEN8* × *16* strain is likely to be much lower (65% of total cenRNA) than that in the *CEN8::CEN3* strain [with 39% of cenRNA8, unchanged level of cenRNA1 (Fig. 5*B*) and probably unchanged cenRNAs from the other 14 endogenous chromosomes].

The mitotic loss rate of the *CEN8* circular minichromosome for hpGFP-expressing control strain were similar in *CEN8* × *16* and *CEN8::CEN3* background (Fig. 5*C* and *SI Appendix*, Fig. S10*D*). HpcenRNA8, hpGFP, or no hairpin strains have similar growth rates and cell cycle distribution (*SI Appendix*, Fig. S10*A*, *Right* and *SI Appendix*, Fig. S10*E*). Importantly, cenRNA8 knockdown in the *CEN8* × *16* resulted in an increased loss rate of *CEN8* circular minichromosome (Fig. 5*C*), which confirms that down-regulation of the total pool of cenRNAs, but not solely the specific cenRNA from one minichromosome, is detrimental to the centromere stability. These results imply that cenRNAs can regulate the centromere *in trans*. In addition, down-regulation of cenRNAs from other chromosomes can affect the stability of a minichromosome containing different CEN sequences (*CEN3* minichromosome, Fig. 5*D*), which further supports that cenRNAs are *trans* acting.

Knocking Down cenRNAs Can Partially Rescue Minichromosome Loss Caused by Up-Regulation of Centromeric Transcription. If the disruption of the centromere function in *cbf1Δ*, *htz1Δ*, and *cbf1Δ htz1Δ* is due to the up-regulation of cenRNAs, knockdown of cenRNA8 in these deletion mutants in the *CEN8* × *16* background should alleviate the mitotic loss of the minichromosome. With cenRNA8 knockdown, both single deletion mutants exhibited a reduced level of cenRNA8 (Fig. 6*A*) and a partial rescue of the *CEN8* minichromosome loss rate (Fig. 6*B* and *SI Appendix*, Fig. S11). In the *cbf1Δ htz1Δ* double deletion, the ampicillin gene (*AMP*) on the minichromosome was used as the internal control instead (as the expression of *URA3* was not comparable between hpGFP- and hpcenRNA8-expressing strains). Surprisingly, hpcenRNA8 reduced cenRNA8 to a relatively low level in *cbf1Δ htz1Δ* and rescued the minichromosome loss phenotype substantially (Fig. 6*B* and *SI Appendix*, Fig. S11). Importantly, this rescue experiment reflects a dose-dependent effect of the total cenRNA level on minichromosome loss rate, suggesting that cenRNA level has to be tightly regulated to epigenetically maintain proper centromere function (Fig. 6*B*).

Discussion

It is a common belief that point centromere in *S. cerevisiae* is governed mainly by the centromeric DNA sequence, and the role of epigenetics in the simple budding yeast centromere may have been overlooked. Unlike regional centromeres, in which epigenetics,

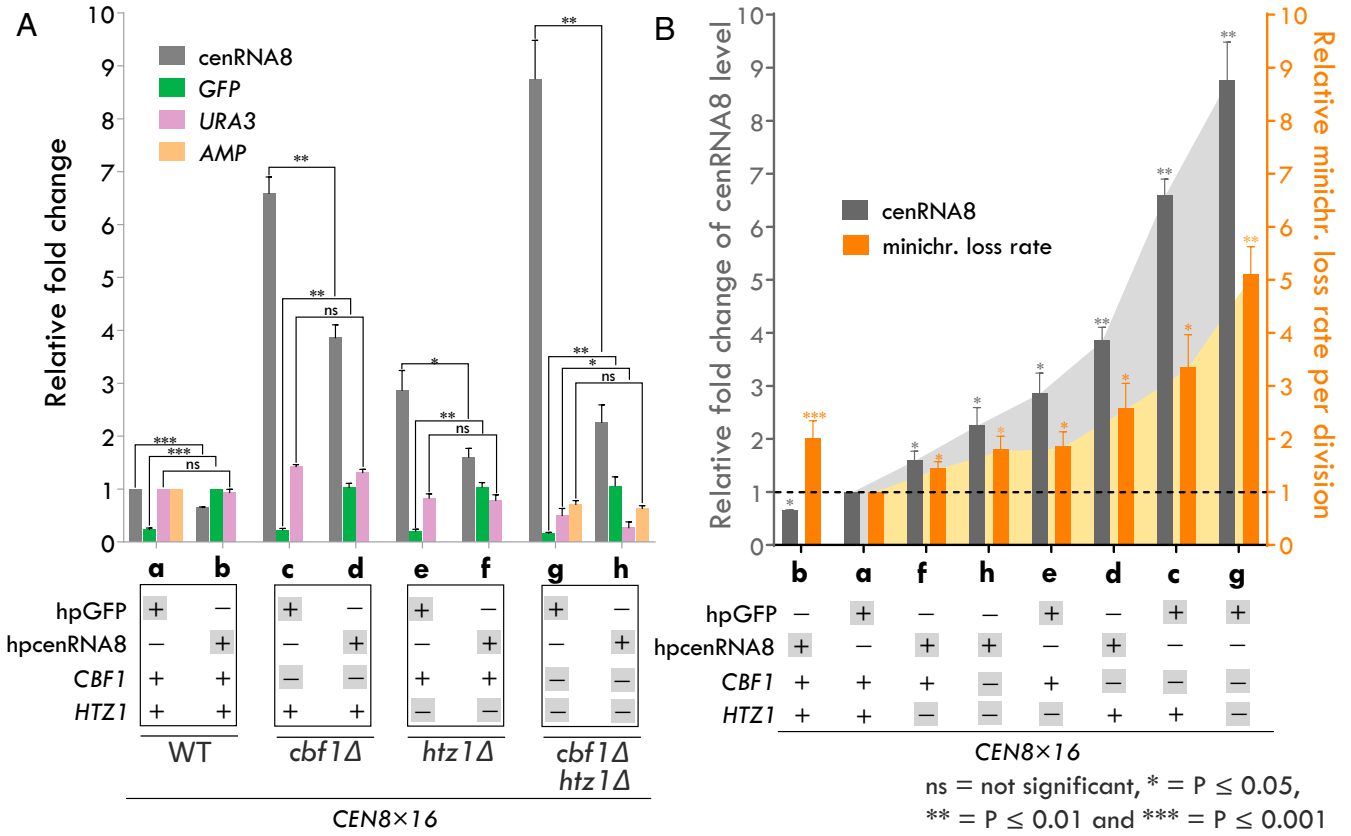

Fig. 6. Knockdown of total cenRNA level rescues minichromosome loss in *cbf1Δ*, *htz1Δ*, and *cbf1Δ htz1Δ*, suggesting a dose-dependent effect of cenRNA on centromere activity. (*A*) RT-qPCR analysis of the expression of cenRNA8, *GFP*, *URA3*, and *AMP* in the RNAi-competent strains in *CEN8* × *16* background. For expression of cenRNA8, *URA3*, and *AMP*, data were quantified relative to the hpGFP-expressing WT cells. For expression of *GFP*, data were quantified relative to the hpcenRNA8-expressing WT cells. Relative fold changes of expression were shown as means ± SD ($n = 3$). Statistical significances were analyzed with paired *t* test. (*B*) The *CEN8* minichromosome loss rates relative to that of the hpGFP-expressing WT cells were plotted, together with and ordered by the relative expression levels of cenRNA8 from *A*. The strains are also labeled "a–h" for easy referencing in *A* and *B*. The relative cenRNA8 expression levels and the relative *CEN8* minichromosome loss rates were expressed as means ± SD ($n = 3$). Statistical significances between hpGFP-expressing WT cells and other yeast strains were analyzed with paired *t* test.

including the histone H3 variant CENP-A and proximal histone modifications, plays an important role in initiating kinetochore formation, kinetochore assembly in point centromere is initiated by the binding of the CBF3 complex to the CDEIII domain. Mutations in the CDEIII domain completely inactivate the function of the point centromere (44–46). Nonetheless, it does not imply that epigenetics is not involved in regulating the function of budding yeast centromeres (19, 20). Budding yeast centromeres also contain CENP-A^{Cse4}, and have transcriptional activity, which is important in maintaining normal centromere activity (22). In this study, we demonstrated that budding yeast centromeres require an optimal level of centromeric transcripts (cenRNAs) to achieve centromere function.

We found that budding yeast cenRNAs were expressed in low copy number (*SI Appendix*, Text S1 and Fig. S1). To characterize these low abundant transcripts, we utilized PCR-based techniques. The 3′ RACE shows the presence of poly-A tails on cenRNAs (Fig. 1 *C* and *D*), consistent with the finding that budding yeast cenRNAs are RNAPII transcribed (*SI Appendix*, Fig. S5) (47). CenRNAs are expressed in both sense and antisense orientations (Fig. 1*B*). However, even though the cenRNA transcripts from the two orientations have some complementary sequences, they likely do not form dsRNA (*SI Appendix*, Fig. S10*B*). Similarly, in mice, in which the minor satellite transcripts are present in both orientations simultaneously, the sense and antisense cenRNAs are not processed by the RNAi (14). The exact reason of not forming dsRNA is unknown, but it is possible that the single-stranded cenRNAs are binding with interacting proteins or DNA (such as the R loops formed by telomeric repeat-containing RNA and its template DNA) (48), which hinders dsRNA formation. In fact, treatments with different RNases have demonstrated that single-stranded RNA, but not dsRNA, was required for the association of CENP-C at the human centromere (9).

We identified that budding yeast centromeres from at least three chromosomes are transcribed during S phase, and the induction of cenRNA8 is dependent on DNA replication (Fig. 2*F* and *SI Appendix*, Fig. S3 *B* and *C*). During centromeric DNA replication in S phase, kinetochores are transiently disassembled by the DNA replication machinery, and centromeres are detached from the microtubules and moved away from the spindle pole body (SPB) in a short period of time (49). Soon afterward, centromeres are recaptured by the microtubule after the reassembly of kinetochores (49) and reloading of CENP-A^{Cse4} (50). In humans, centromeric transcription and CENP-A loading are coupled and occur in late mitosis to early G1 phase (6). Interestingly, we found that these events also occur coincidently in budding yeast, but in S phase. We suggested that point centromere may be transcribed only when centromeric DNA is replicated and kinetochore is transiently disassembled, which only last for 1–2 min (49), resulting in low copy numbers of cenRNA (*SI Appendix*, Fig. S1).

Deletion mutant screening has discovered that the level of centromeric transcription is controlled by two proteins that bind to the centromeres or in proximity: the inner kinetochore protein Cbf1 and histone H2A variant H2A.Z^{Htz1}. The deletion of *CBF1* and *H2A.Z^{Htz1}* does not remove the S phase cenRNA induction (*SI Appendix*, Fig. S6 *B* and *C*); instead, centromeric transcription activity is up-regulated throughout the cell cycle, indicating that Cbf1 and H2A.Z^{Htz1} are centromeric transcription repressors, and there may be S phase-specific transcription activator yet to be identified.

Cbf1 forms a homodimer and binds to the E-box consensus sequence CACGTG present at a number of gene promoters and CDEI (51, 52). Cbf1 may control transcription by chromatin remodeling, possibly through the interaction with the chromatin-remodeling ATPase Isw1 (53). Intriguingly, Cbf1 is an activator for methionine genes, but a repressor for *LAC1* gene in the ceramide biosynthetic pathway (54). In contrast to the much clearer role of Cbf1 in transcriptional control, its role in centromere is vague. By binding to the centromeric CDEI domain, Cbf1 induces a bend on the CDEI DNA, but the significance of this bending is unclear (55). Cbf1 is not essential, but deletion of it renders centromeric regions more accessible to DNaseI digestion (52, 56), consistent with its role in centromeric transcription repression. Possibly, Cbf1 modulates the centromere chromatin structure similar to its activity at promoter regions (52). On the contrary, Ohkuni and Kitagawa (22) suggested that Cbf1 promotes centromeric transcription. The reason for this discrepancy is unknown, but we noticed that they used the RNeasy Extraction kit, instead of hot phenol for cenRNA extraction. The kit may cause incomplete lysis, resulting in a skew toward small RNA fraction (57, 58). It is possible that Ohkuni and Kitagawa were detecting a subfraction of small-sized cenRNAs or degraded products, which were down-regulated upon *CBF1* deletion.

Histone H2A variant H2A.Z is distributed throughout the genome, but is enriched in promoters to control transcriptional activation and repression (36, 59). In regional centromeres, H2A.Z localizes to centromeric chromatin, where H2A.Z/H3 nucleosomal domains are interspersed between H2A/CENP-A nucleosomal domains and also to pericentric heterochromatin nonuniformly (60). H2A.Z in regional centromeres is involved in establishing pericentric heterochromatin (61), the 3D organization of the centromere (60), and sister chromatid cohesion (62). Disruption of H2A.Z resulted in chromosome missegregation in fission yeast, mouse, and monkey kidney cells Cos-7 (35, 63). In addition, H2A.Z^{Pht1} is involved in silencing centromeric chromatin in fission yeast (35). In budding yeast, H2A.Z^{Htz1} is also present at the *HMR* locus. Deletion of H2A.Z^{Htz1} or mutation of heterochromatin protein Sir1 derepressed the *HMR* locus, and overexpression of H2A.Z^{Htz1} restored the silencing (64). In mouse cells, H2A.Z nucleosomes interact with HP1α, a conserved heterochromatin protein. H2A.Z-containing nucleosomal arrays are more compacted than H2A arrays, which favor the binding of HP1α. Upon binding with HP1α, the chromatin compacts further to create a specialized conformation in the heterochromatin (65). In budding yeast point centromeres, H2A.Z^{Htz1} nucleosomal domains are found in the flanking "pericentric" chromatin, which usually starts 100–200 bp away from the centromere and spans ∼600 bp (66). Consistent with its silencing function above, we found that H2A.Z^{Htz1} is a repressor of centromeric transcription in budding yeast.

An additive effect in the up-regulation of cenRNA level (Fig. 3*A*), the minichromosome loss rate and aneuploidy (Fig. 3 *C* and *D*), is observed in the double deletion of *CBF1* and H2A.Z^{Htz1}, suggesting that multiple pathways are involved in centromeric transcription repression for optimal centromere activity. Besides the up-regulated level of cenRNAs, an increased enrichment and up-regulated activity of RNAPII in double deletion of *CBF1* and H2A.Z^{Htz1} may have an impact in centromere function (*SI Appendix*, Text S2 and Fig. S5). RNAPII was found to be accumulating on both ends of the budding yeast centromeres (67). In fission yeast, stalling of RNAPII promotes the deposition of CENP-A^{Cnp1} (68). It is possible that both Cbf1 and H2A.Z^{Htz1} are required to maintain a suitable chromatin environment around the centromere to keep centromeric transcription activity in a fine balanced control.

The effect of strong transcription on the budding yeast centromere was studied 30 y ago by inserting a galactose-inducible promoter upstream of the centromere on an endogenous chromosome (*GAL-CEN*) (21). The strong centromeric transcription inactivates the centromere completely, leading to growth arrest. However, in double deletion of *CBF1* and *H2A.Z^{Htz1}*, the cells are still dividing with comprised centromere activity (Figs. 3 and 4). A number of centromeric proteins, including CENP-A^{Cse4}, HJURP^{Scm3}, CENP-C^{Mif2}, Survivin^{Bir1}, and INCENP^{Sli15}, are down-regulated, possibly due to destabilized centromeres.

Chromosome spreading also indicated that there is a loss of chromatin-associated CENP-A^{Cse4}, CENP-C^{Mif2}, and Aurora-B^{Ipl1} in *cbf1Δ* and *htz1Δ*. Intriguingly, the homologs of these proteins in other higher eukaryotes are known to be interacting with cenRNAs (6, 8–10, 12).

In *cbf1Δ htz1Δ* double deletion, the down-regulation of CENP-A^{Cse4} and HJURP^{Scm3} protein levels likely causes a defect in reloading CENP-A^{Cse4} at S phase. The decrease of CENP-C^{Mif2} level suggests a disruption in inner kinetochore. In maize, the DNA binding activity of CENP-C is stabilized by interacting with cenRNA (8), and inhibition of RNAPII during mitosis by α-amanitin decreases the level of CENP-C at the kinetochore (4). CPC is a master regulator of mitosis consisting of four subunits: the enzymatic component Aurora-B^{Ipl1}, and the regulatory components Survivin^{Bir1}, Borealin^{Nbl1}, and INCENP^{Sli15}. CPC undergoes a dynamic change of localization throughout the cell cycle. CPC localizes to the inner centromere from G1 until anaphase. During anaphase, CPC moves to the anaphase spindle and spindle midzone (69). The regulatory components govern the dynamic localization of CPC, which allows Aurora-B to phosphorylate different substrates spatially to regulate mitotic activities such as kinetochore–microtubule attachments, spindle assembly checkpoint, and cytokinesis (70). In budding yeast, kinetochore transiently disassembles from the centromere and also detaches from the microtubule in S phase (49), and soon afterward, CPC relocalizes to the inner centromere to promote sister kinetochore biorientation (71). The main function of Survivin is to direct CPC to the inner centromere (72). INCENP acts as a scaffold in CPC by interacting with Aurora-B (73) and Survivin (74). The mislocalization of Aurora B^{Ipl1} in *cbf1Δ htz1Δ* is probably due to the down-regulation of regulatory components Survivin^{Bir1} and INCENP^{Sli15}. In mouse cells, overexpression of cenRNAs impaired centromere function with mislocalization of Aurora-B (14). In *cbf1Δ* and *htz1Δ* single mutants, although the total level of CPC proteins were not affected, the localization of Aurora-B could be disrupted by overexpression of cenRNAs (10, 13). Alternatively, the loss of Aurora B^{Ipl1} signal from the chromatin in these mutants may be caused by declustering of individual centromeres, which occurs when the interaction between the inner and outer kinetochore is disrupted (75). The signal of Aurora B^{Ipl1} from individual declustered centromeres may become too weak to be detected. We postulate that the function of centromeric transcription is tightly linked to the S phase-specific events that happen at the centromere, in particular CENP-A^{Cse4} loading, kinetochore reassembly, and CPC dynamic localization, as shown in our model regarding the regulation of centromeric transcription by Cbf1 and H2A.Z^{Htz1}, and the function of centromeric transcription and cenRNAs (*SI Appendix*, Fig. S12).

Using the introduced RNAi system, our study knocks down ncRNA in *S. cerevisiae* for functional study. In *CEN8 × 16* background, knockdown of cenRNA8 caused an elevated loss of both *CEN8*- and *CEN3*-containing minichromosomes, suggesting a potential *trans* action of cenRNAs (Fig. 5 *C* and *D*). By comparing the cenRNA sequences from different centromeres, no significant homology can be found. It is intriguing that cenRNAs could function *in trans* without significant sequence similarity. It is tempting to overexpress exogenous *trans* cenRNA for functional analysis; however, it is not clear whether cenRNAs will be functional if they are not expressed from a functional centromere. In the dicentric chromosome experiment (21), one of the centromeres (*GAL-CEN*) is inactivated by strong transcription, producing a large amount of cenRNAs. If these cenRNAs are functional, the other centromere on the same chromosome should be disrupted, but indeed, it remains functional. In fact, exogenous cenRNA overexpressed from a plasmid fails to introduce any abnormalities in HeLa cells (11). We reason that only cenRNA expressed from a functional centromere is in close proximity to interact with kinetochore proteins with promiscuous RNA-binding activity, such as Aurora-B (13) and CENP-C (8), making the cenRNA functional. The *trans* action of cenRNA may be potentiated by centromere clustering in budding yeast, in which the microtubules tether all centromeres to a confined region near the spindle pole body during most of the cell cycle (49, 76). There may be a local "cenRNA cloud" at the centromere cluster during S phase, allowing the interaction of cenRNAs to all centromeres/kinetochores.

Our rescue experiment by knocking down cenRNA in *cbf1Δ*, *htz1Δ*, and *cbf1Δ htz1Δ* provided a strong support that cenRNA overexpression in these mutants disrupts centromere activity (Fig. 6). In *cbf1Δ htz1Δ*, hpcenRNA8 knockdown was more efficient compared with the single deletions (Fig. 6*A*), probably because the increased amount of cenRNA8 provided more templates for subsequent siRNA generation and amplification. The fine balance of centromeric transcription in budding yeast was first suggested by Ohkuni and Kitagawa (22). Our current work described the importance of this fine balance quantitatively. While we cannot exclude the possibility that Cbf1 and H2A.Z^{Htz1} may have multiple mechanisms to affect centromeric function, our results indicate that cenRNA up-regulation is at least one of the reasons for the centromere malfunction in *cbf1Δ*, *htz1Δ*, and *cbf1Δ htz1Δ*. Whether misregulating centromeric transcription activity per se (without affecting cenRNA level) contributes to centromere malfunction is a question that remains to be determined. Nonetheless, this study provides evidence that either too much or too little cenRNA is detrimental to centromere activity, suggesting a tight regulation of cenRNA level is important for normal centromere function.

Methods

The genotypes of yeast strains used in this study are shown in *SI Appendix*, Table S1. Plasmids used in this study are listed in *SI Appendix*, Table S2. Additional information on methods used in this study, including strain and plasmid construction, cloning, media, culture conditions, growth analysis, RNA extraction, RT-PCR, RT-qPCR, absolute quantification of centromeric RNA, RACE, cell cycle synchronization, flow cytometry, budding index examination, deletion mutant screening, circular minichromosome loss assay, aneuploidy assay, protein extraction and Western blotting, chromosome spreading, microscopy imaging and analysis, chromatin immunoprecipitation, and statistical analysis, can be found in *SI Appendix, Supplementary Methods*.

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