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CENP-B promotes the centromeric localization of ZFAT to control transcription of noncoding RNA

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The centromere is a chromosomal locus that is essential for the accurate segregation of chromosomes during cell division. Transcription of noncoding RNA (ncRNA) at the centromere plays a crucial role in centromere function. The zinc-finger transcriptional regulator ZFAT binds to a specific 8-bp DNA sequence at the centromere, named the ZFAT box, to control ncRNA transcription. However, the precise molecular mechanisms by which ZFAT localizes to the centromere remain elusive. Here we show that the centromeric protein CENP-B is required for the centromeric localization of ZFAT to regulate ncRNA transcription. The ectopic expression of CENP-B induces the accumulation of both endogenous and ectopically expressed ZFAT protein at the centromere in human cells, suggesting that the centromeric localization of ZFAT requires the presence of CENP-B. Coimmunoprecipitation analysis reveals that ZFAT interacts with the acidic domain of CENP-B, and depletion of endogenous CENP-B reduces the centromeric levels of ZFAT protein, further supporting that CENP-B is required for the centromeric localization of ZFAT. In addition, knockdown of CENP-B significantly decreased the expression levels of ncRNA at the centromere where ZFAT regulates the transcription, suggesting that CENP-B is involved in the ZFAT-regulated centromeric ncRNA transcription. Thus, we concluded that CENP-B contributes to the establishment of the centromeric localization of ZFAT to regulate ncRNA transcription.

The centromere is a chromosomal locus that is essential for the accurate segregation of chromosomes during cell division (1, 2). Dysregulation of the centromere function leads to genomic instability and aneuploidy, both of which are frequently observed in many human cancers. The human centromere is composed of highly repetitive AT-rich DNA sequences, known as α-satellite DNA (3, 4). The α-satellite DNA is defined by a 171-bp monomeric sequence unit. The α-satellite monomers show 50–70% identity to each other and are arranged tandemly in a head-to-tail fashion to form a higher-order repeat (HOR) unit. The HOR unit is repeated hundreds to thousands of times to produce a large homogeneous array at the centromere (3, 4). However, centromere functions are epigenetically specified by the histone H3 variant CENP-A rather than *via* the DNA sequence (5, 6). In addition, the transcription of noncoding RNA (ncRNA) at the centromere plays an important role in centromere function (7–11). Therefore, centromeric transcription is tightly regulated, and its dysregulation may lead to an impaired centromere function.

ZFAT (Zinc-Finger protein with AT-hook) is a nuclear protein that contains one AT-hook domain and 18 C2H2 zinc-finger domains (12, 13). The ZFAT genes have been identified in vertebrates with highly conserved amino acid sequences (14), suggesting their crucial role in physiological processes. Indeed, we have previously demonstrated that ZFAT is essential for embryonic development (15) and the homeostasis of T cells (16–18) and adipocytes (19). Recently, we reported that ZFAT plays an important role in the centromeric ncRNA transcription in human cells (20). The ectopic expression of ZFAT increased centromeric ncRNA levels at specific chromosomes, including chromosomes 17 and X (20). Furthermore, depletion of ZFAT reduced the centromeric ncRNA levels at chromosomes 17 and X (20). Therefore, ZFAT regulates the centromeric ncRNA transcription at specific chromosomes, including chromosomes 17 and X.

ZFAT binds to a specific 8-bp DNA sequence at the centromere, named the ZFAT box (20). The ZFAT box (GAA(T/A)(C/G)TGC) is highly conserved and widely distributed at whole α-satellite DNA regions of every chromosome (20). At the centromere, ZFAT interacts with histone acetyltransferase KAT2B to induce acetylation of lysine 8 in histone H4 (H4K8ac). Furthermore, the bromodomain-containing protein BRD4 accumulated at the centromere through its binding to the KAT2B-induced H4K8ac, which activates RNA polymerase II-dependent ncRNA transcription. These findings indicate that ZFAT binds to the centromere to regulate ncRNA transcription through histone modifications (20). However, the centromeric levels of ZFAT protein vary between chromosomes and also within the centromeric regions. Therefore, the precise mechanisms of

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centromeric localization of ZFAT at the molecular level remain elusive.

A variety of proteins have been identified as components of the centromere. In mammals, CENP-B was the only known centromeric protein with sequence-specific DNA-binding activity (21, 22); however, ZFAT was then recently identified as a centromeric DNA-binding protein (20). CENP-B binds to a 17-bp consensus motif at the centromere, named the CENP-B box, at the N-terminal region (22, 23). The role of CENP-B at the centromere had remained elusive for years until several recent studies have demonstrated that CENP-B plays an essential role in centromere functions (24–27). The binding of CENP-B at the centromere is required for the establishment and maintenance of the centromere through its interaction with CENP-A and CENP-C. However, it has also been reported that CENP-B interacts with many other centromeric and noncentromeric proteins (28–30), suggesting that there are unresolved roles identified for CENP-B.

In this study, we demonstrate a novel function for CENP-B in the centromeric localization of ZFAT. An increase in the centromeric CENP-B levels causes an accumulation of ZFAT at the centromere. ZFAT interacts with the acidic domain of CENP-B. Furthermore, knockdown of CENP-B in human cells decreases the centromeric levels of ZFAT, as well as those of ncRNA. These results suggest important roles for CENP-B in the establishment of centromeric localization of ZFAT to regulate ncRNA transcription.

Results

Ectopic expression of CENP-B stimulates the formation of ZFAT foci at the centromere

To elucidate the precise molecular mechanism for the centromeric localization of ZFAT, in particular its relationship with other centromeric proteins, we investigated the colocalization of human ZFAT (hZFAT) with CENP-A or CENP-B using immunofluorescence analysis. In HeLa cells, ectopically expressed ZFAT formed foci in the nucleus, most of which were colocalized with the foci of endogenous CENP-A or CENP-B (Fig. 1, *A* and *B*). While the foci of HA-hZFAT-EGFP were larger than those of CENP-A, the regions with endogenous CENP-B foci almost overlapped with those of HA-hZFAT-EGFP foci (Fig. 1*C*). These results demonstrate that CENP-B is associated with the centromeric localization of ZFAT.

To examine the contribution of CENP-B to the centromeric localization of ZFAT, in HeLa cells we coexpressed hZFAT-HA and EGFP-CENP-B. Of interest, the ectopic coexpression of CENP-B with ZFAT considerably stimulated the formation of ZFAT foci that colocalized with CENP-B foci, compared with cells coexpressing hZFAT-HA and EGFP (Fig. 1, *D* and *E*). Furthermore, the ectopic expression of Kusabira Orange 2 (KuOr)-CENP-B stimulated foci formation of endogenous ZFAT protein in HT1080 cells (Fig. 1, *F* and *G*). Together, these results indicate that CENP-B is involved in the centromeric localization of ZFAT.

Endogenous ZFAT protein interacts with endogenous CENP-B protein in human and mouse cells

As the immunofluorescence analysis demonstrated that ectopically expressed CENP-B was involved in the centromeric localization of ZFAT (Fig. 1), we next examined the interaction between ZFAT and CENP-B endogenous proteins using coimmunoprecipitation (Co-IP) analysis. The ZFAT protein was coimmunoprecipitated with the CENP-B protein using an anti-CENP-B antibody in HEK293 cells, and vice versa (Fig. 2*A*). Similarly, the interaction between mouse Zfat and Cenp-B endogenous proteins was also observed in NIH3T3 mouse fibroblast cells (Fig. 2*B*). These results demonstrate that endogenous ZFAT interacts with endogenous CENP-B and that their interactions are conserved in human and mouse cells.

The middle domain of ZFAT interacts with the acidic domain of CENP-B

To elucidate the mechanism of the interaction between ZFAT and CENP-B, we next examined the interaction between ectopically expressed hZFAT-HA and FLAG-tagged CENP-B (FLAG-CENP-B) in HEK293 cells using Co-IP analysis. hZFAT-HA protein was coimmunoprecipitated with the FLAG-CENP-B protein using an anti-FLAG antibody, and vice versa (Fig. 3*A*). Furthermore, we examined the interaction between ZFAT and CENP-B after DNase treatment using Co-IP analysis (Fig. 3, *B* and *C*). Treatment with DNase resulted in the disappearance of DNA, indicating that DNA was properly degraded by DNase in the Co-IP buffer used (Fig. 3*C*). On the other hand, the DNase treatment did not affect the interaction between ZFAT and CENP-B (Fig. 3*B*), suggesting that ZFAT interacts with CENP-B independently of DNA.

To determine which ZFAT regions were involved in the interaction with CENP-B, we examined the interaction between FLAG-CENP-B and the deletion mutants of hZFAT-HA using Co-IP analysis (Fig. 3*D*). The ZFAT protein is composed of a highly conserved (among vertebrates) 18 zinc-finger domains (ZF, Fig. 3*E* (14)). Furthermore, the amino acid residues, other than ZF, in the C-terminal region of ZFAT are also highly conserved, whereas those in the N-terminal and middle regions of ZFAT have low conservation (14). While deletion of ZF1-8 of ZFAT did not affect the interaction with CENP-B (ZF-ΔN-2), a significant decrease in the interaction with CENP-B occurred with an additional deletion of the middle domain and ZF9-12 of ZFAT (ZF-ΔN-3, Fig. 3*D*). On the other hand, the deletion mutant of ZFAT, which does not have the C-terminal half but has the middle domain, retained the ability to interact with CENP-B (ZF-ΔC-2, Fig. 3*D*). These results demonstrate that the ZFAT middle domain is involved in its interaction with CENP-B.

To determine which CENP-B regions were involved in the interaction with ZFAT, we evaluated the interaction between hZFAT-HA and the deletion mutants of FLAG-CENP-B using Co-IP analysis. Human CENP-B is typically divided into four domains, which include DNA binding, integrase core, acidic, and dimerization domains (Fig. 4*A*). While the deletion mutant

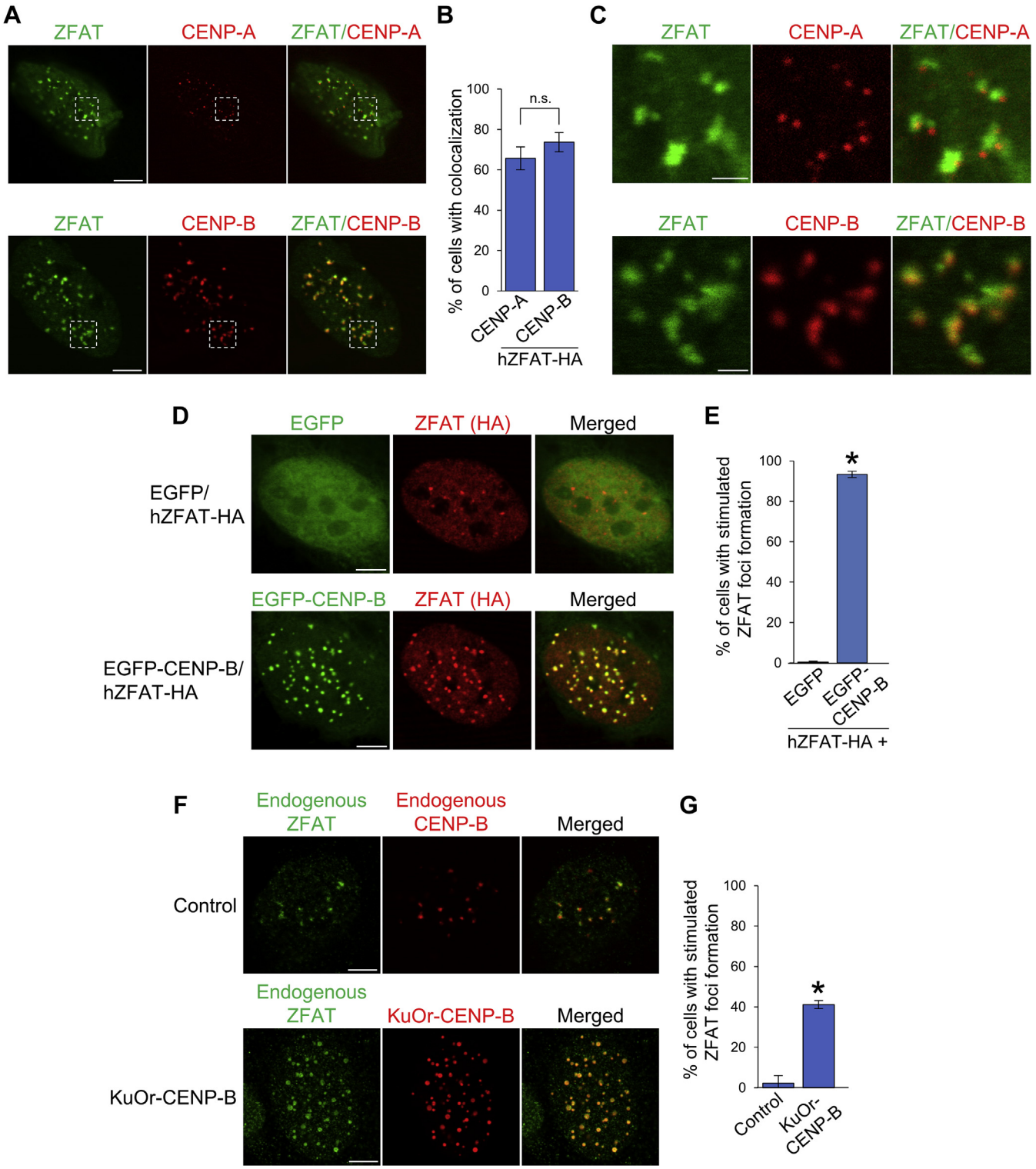

Figure 1. Ectopic expression of CENP-B stimulates the formation of ZFAT foci at the centromere. *A*, immunofluorescence images of ZFAT, and endogenous CENP-A or CENP-B in HeLa cells transiently expressing HA-hZFAT-EGFP. *B*, percentage of cells containing the ZFAT foci colocalizing with the foci of CENP-A or CENP-B, determined in (*A*). *C*, magnified images of the region enclosed by the white dotted line in (*A*). *D*, immunofluorescence images of HeLa cells transiently expressing hZFAT-HA, and EGFP (*upper panels*), or EGFP-CENP-B (*lower panels*). *E*, percentage of cells with stimulated ZFAT foci formation in cells expressing hZFAT-HA, determined in (*D*). *F*, immunofluorescence images of HT1080 cells transfected with a control vector, or the expression vector of Kusabira Orange 2 (KuOr)-CENP-B. Immunofluorescence images of endogenous ZFAT with endogenous CENP-B (*upper panels*), or KuOr-CENP-B (*lower panels*) are shown. *G*, percentage of cells with the stimulated foci formation of endogenous ZFAT in HT1080, determined in (*F*). *A*, *C*, *D*, and *F*, the data are representative of three independent experiments. *A*, *D*, and *F*, scale bar, 5 μm. *C*, scale bar, 1 μm. *B*, *E*, *G*, the data represent the mean ± SD of three independent experiments (N = 25–30 cells per one experiment). n.s., not significant. *$p < 0.05$, against cells transfected with the control vector.

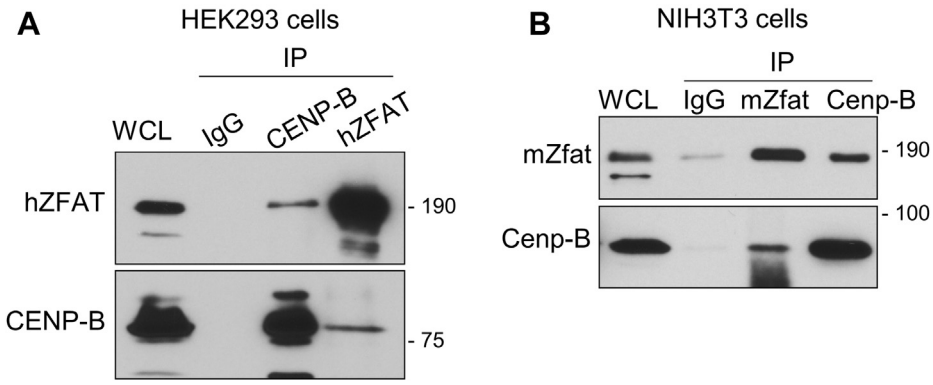

Figure 2. Endogenous ZFAT protein interacts with endogenous CENP-B protein. *A*, coimmunoprecipitation analysis of endogenous human ZFAT (hZFAT) and CENP-B in HEK293 cells using anti-ZFAT and anti-CENP-B antibodies or control IgG (IgG). *B*, coimmunoprecipitation analysis of endogenous mouse Zfat (mZfat) and Cenp-B in NIH3T3 cells using anti-mZfat and anti-CENP-B antibodies or control IgG (IgG). *A* and *B*, the molecular weight (in kDa) and position of the molecular weight markers are indicated on the *right* of the *panels*. The data are representative of three independent experiments. IP, immunoprecipitation; WCL, whole-cell lysates.

that lost the dimerization domain of CENP-B retained the ability to interact with ZFAT (ΔCB-4), deletion of the acidic domain of CENP-B markedly diminished the interaction with ZFAT (ΔCB-3, Fig. 4*B*). These results demonstrate that the acidic domain of CENP-B is involved in ZFAT interaction. Taken together, these results suggest that the middle domain of ZFAT interacts with the acidic domain of CENP-B.

CENP-B is required for the binding of ZFAT to centromeres at chromosomes, except the Y chromosome

To elucidate the roles of CENP-B in the centromeric localization of ZFAT, we evaluated the effects of depletion of CENP-B on ZFAT levels at centromeres. The centromeric ZFAT levels were determined by chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) analysis using primers for α-satellite DNA and other repetitive DNA sequences in HEK293 cells that transiently expressed HA-hZFAT-EGFP. The binding of HA-hZFAT-EGFP was observed at centromeric α-satellite DNA, but not at the genomic regions for other repetitive DNA sequences (Fig. 5*A*). We used siRNA to knockdown the CENP-B gene expression in HEK293 cells and confirmed the decreased expression of CENP-B protein through immunoblotting analysis using an anti-CENP-B antibody (Fig. 5*B*). Depletion of CENP-B significantly reduced the levels of ZFAT protein at centromeric α-satellite DNA, in comparison to cells transfected with the control siRNA (Fig. 5*A*), suggesting that CENP-B is required for the centromeric binding of ZFAT.

It is known that CENP-B binds directly to a 17-bp sequence found in the centromeric repetitive DNA sequences, termed CENP-B box, conserved within all human chromosomes, except the Y chromosome (31–33); therefore, CENP-B does not bind to the Y chromosome centromere. On the other hand, we have previously shown that ZFAT is bound to centromeres of every human chromosome, including the Y chromosome (20). Therefore, we examined the effects of CENP-B depletion on the centromeric ZFAT levels at the Y chromosome in HT1080 cells that transiently expressed HA-hZFAT-EGFP. The binding of HA-hZFAT-EGFP was observed in HT1080 cells at the centromeric α-satellite DNA of the Y chromosome, but not at the 5S ribosomal RNA region (Fig. 5*C*). The depletion of CENP-B did not significantly affect ZFAT protein levels at the centromeric α-satellite DNA of the Y chromosome, compared with cells transfected with control siRNA (Fig. 5*C*). These results demonstrate that CENP-B is not required for centromeric binding of ZFAT at the Y chromosome. Taken together, CENP-B is therefore essential for the binding of ZFAT to the centromere of chromosomes, except for the Y chromosome.

CENP-B is involved in ZFAT-regulated centromeric ncRNA transcription

To investigate the involvement of CENP-B in ZFAT-regulated centromeric ncRNA transcription, we first established quantitative reverse transcription-PCR (qRT-PCR) analysis for centromeric ncRNA using α-satellite DNA primers for chromosomes 17 and X in HT1080 cells (Fig. 6*A*). The ncRNA levels were determined in the presence or absence of reverse transcriptase (RTase), DNase, or RNase. The centromeric ncRNA levels in samples treated with RTase were significantly higher than those in samples without RTase. Furthermore, centromeric ncRNA was hardly detected in samples pretreated with RNase. These results indicate that the signals in qRT-PCR analysis are derived from RNA. On the other hand, treatment with DNase dramatically decreased the signal levels in qRT-PCR analysis, compared with those in samples untreated with DNase, indicating that contaminated genomic DNA is adequately removed by DNase treatment. Together, these results indicate that the levels of centromeric ncRNA are exactly measured by the qRT-PCR analysis.

We next evaluated the effects of CENP-B depletion on the centromeric ncRNA levels at chromosomes 17 and X, where ZFAT regulates the centromeric transcription. A siRNA-mediated knockdown of CENP-B did not have any effect on the expression levels of endogenous ZFAT protein in HT1080 cells (Fig. 6*B*). A depletion of CENP-B in HT1080 cells significantly decreased centromeric ncRNA levels at

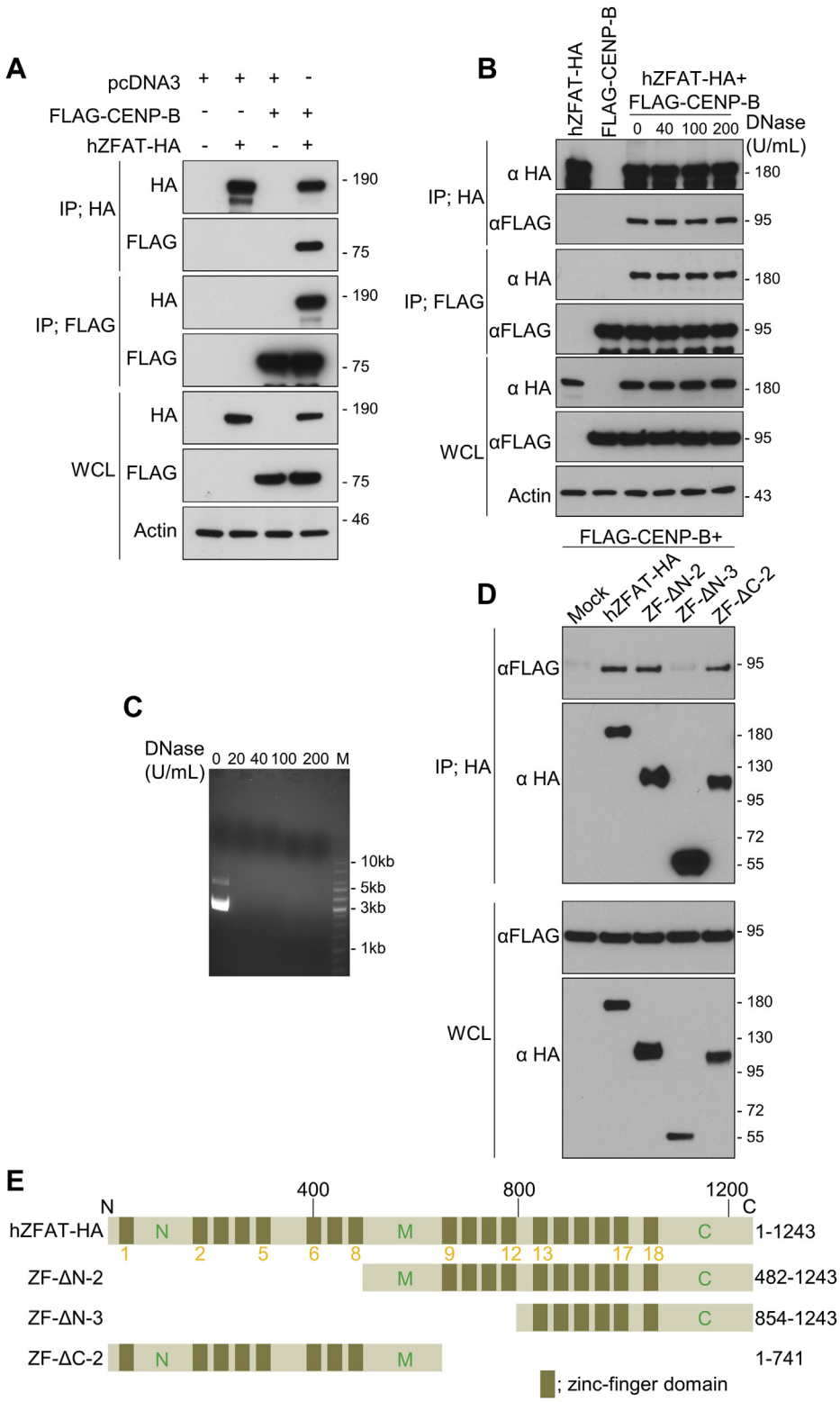

Figure 3. The middle domain of ZFAT is required for the interaction with CENP-B. *A*, coimmunoprecipitation analysis of hZFAT-HA and FLAG-CENP-B using anti-HA and anti-FLAG antibodies in HEK293 cells transfected with the indicated expression vectors. *B*, coimmunoprecipitation analysis of hZFAT-HA and FLAG-CENP-B after DNase treatment. *C*, Agarose gel electrophoresis of lysates treated with DNase at the indicated concentrations. M, DNA size marker. *D*, coimmunoprecipitation analysis of FLAG-CENP-B and the deletion mutants of hZFAT-HA using an anti-HA antibody in HEK293 cells that were transfected with the indicated expression vectors. *E*, schematic diagram of the deletion mutants of hZFAT-HA used in (*D*). N, N-terminal domain; M, middle domain; C, C-terminal domain. *A*, *B*, and *D*, the molecular weight (in kDa) and position of the molecular weight markers are indicated on the *right* of the *panels*. The data are representative of three independent experiments. IP, immunoprecipitation; WCL, whole-cell lysates.

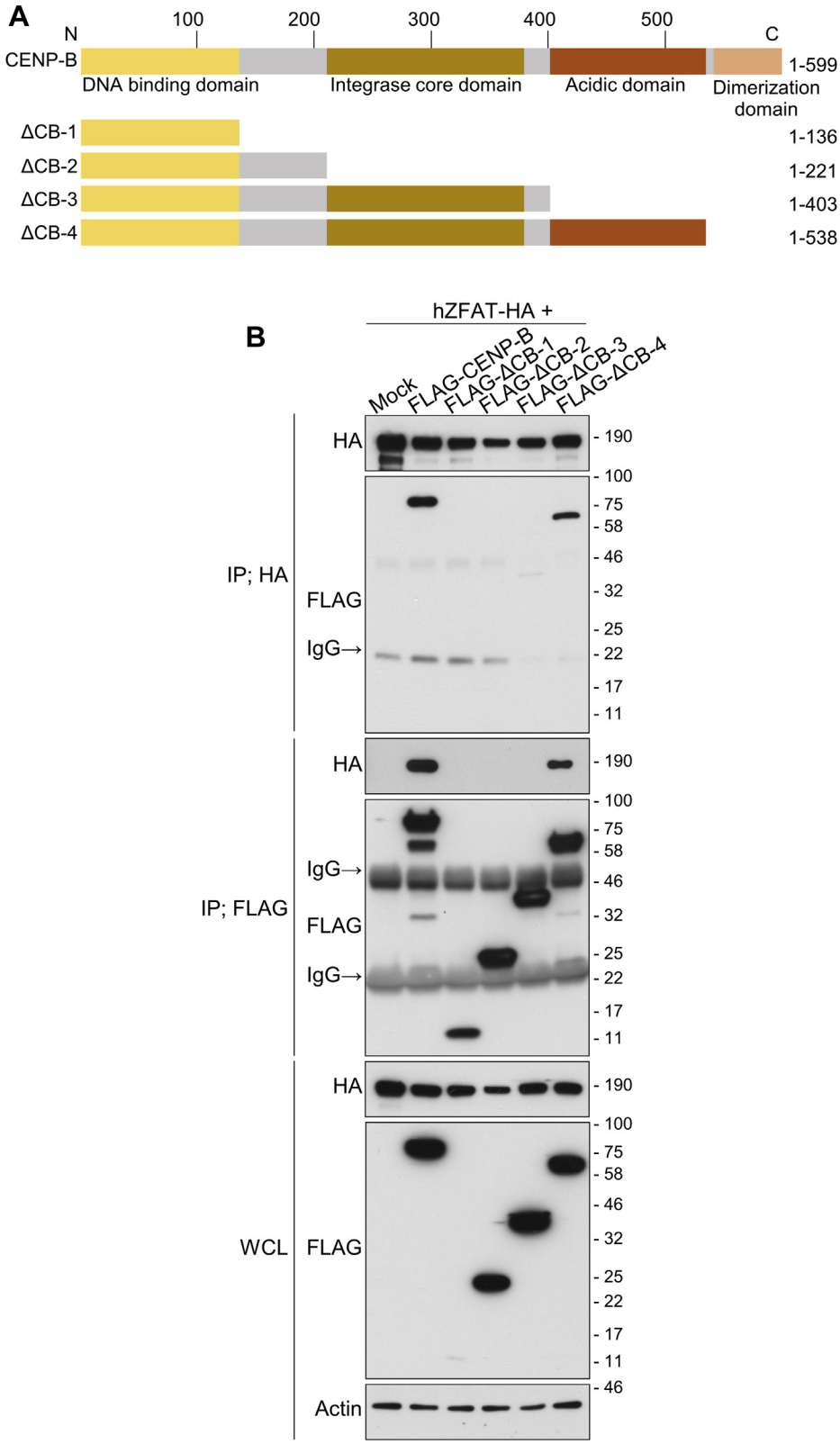

Figure 4. The acidic domain of CENP-B is required for the interaction with ZFAT. *A*, schematic diagram of the deletion mutants of FLAG-CENP-B used in (*B*). *B*, coimmunoprecipitation analysis of hZFAT-HA and the deletion mutants of FLAG-CENP-B using anti-HA and anti-FLAG antibodies in HEK293 cells that were transfected with the indicated expression vectors. The molecular weight (in kDa) and position of the molecular weight markers are indicated on the *right* of the *panels*. The data are representative of three independent experiments. IP, immunoprecipitation; WCL, whole-cell lysates.

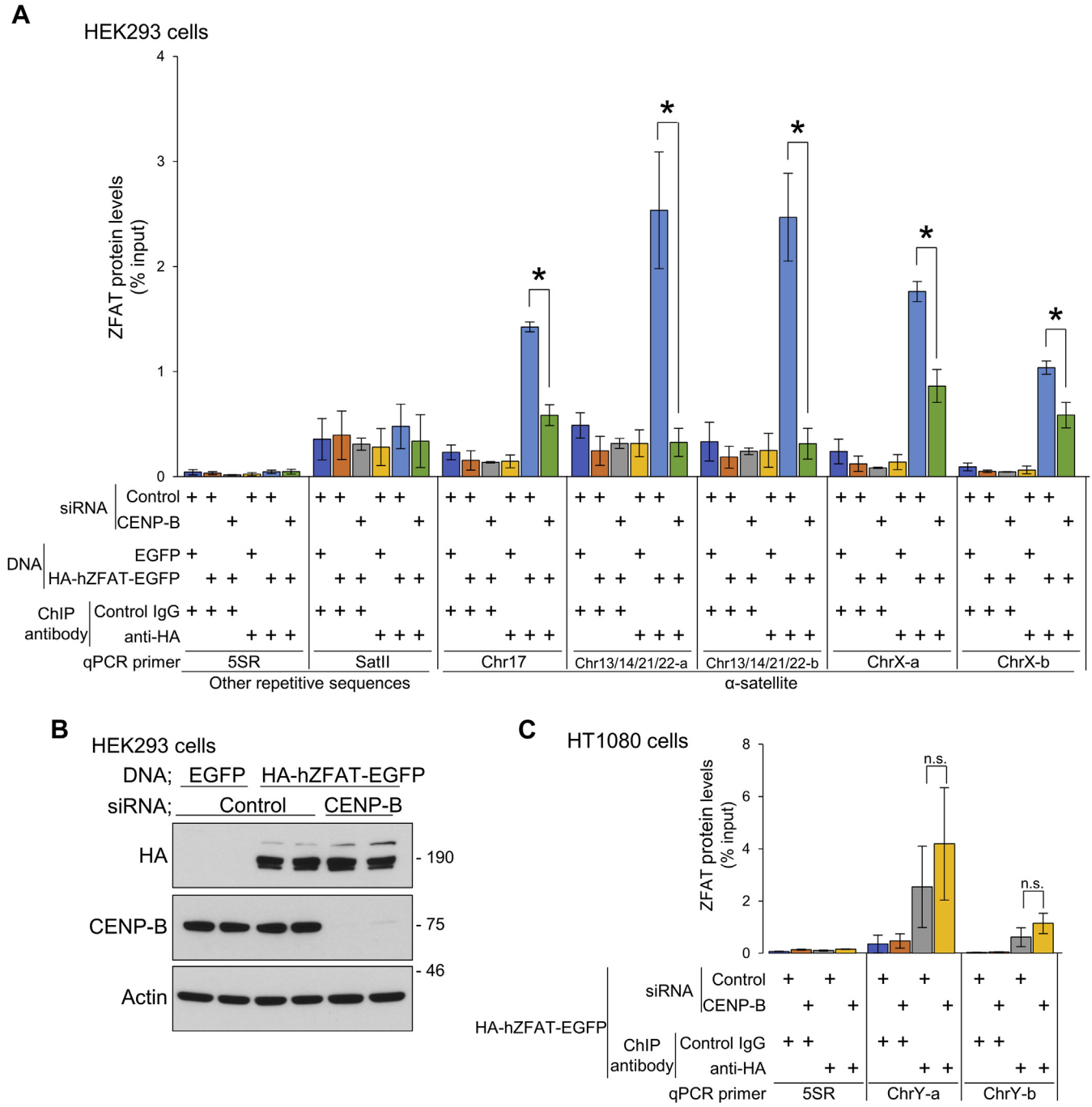

Figure 5. Depletion of CENP-B decreases the centromeric levels of ZFAT at the chromosomes, except the Y chromosome. *A*, ChIP-qPCR analysis of ZFAT at the human repetitive DNA sequences using an anti-HA antibody or control IgG in HEK293 cells that were transfected with the indicated expression vectors and siRNAs. *B*, immunoblotting analysis of ZFAT (HA) and CENP-B in HEK293 cells transfected with the indicated expression vectors and siRNAs. Each sample was analyzed in two adjacent lanes. The molecular weight (in kDa) and position of the molecular weight markers are indicated on the right of the panels. The data are representative of three independent experiments. *C*, ChIP-qPCR analysis of ZFAT at the centromeric α-satellite DNA of the Y chromosome and 5S ribosomal RNA using an anti-HA antibody, or control IgG in HT1080 cells that were transfected with the indicated siRNAs, and the expression vector for HA-hZFAT-EGFP. *A* and *C*, the data represent the mean ± SD of three independent experiments. *$p < 0.05$. n.s., not significant.

chromosomes 17 and X, in comparison to cells transfected with control siRNA (Fig. 6*C*). The centromeric ncRNA levels in CENP-B-depleted cells were comparable to those in ZFAT-depleted cells. Furthermore, a decrease in the centromeric ncRNA levels by CENP-B depletion was observed also in HeLa cells (Fig. 6*D*). On the other hand, codepletion of ZFAT and CENP-B showed no additive effects on the centromeric ncRNA levels in both HT1080 and HeLa cells (Fig. 6, *C* and *D*). These results suggest that CENP-B is involved in ZFAT-regulated centromeric ncRNA transcription. Furthermore, ZFAT and CENP-B may play roles in the same pathway of centromeric ncRNA transcription.

The centromeric ncRNA transcription is important for centromere functions. Previous reports showed that

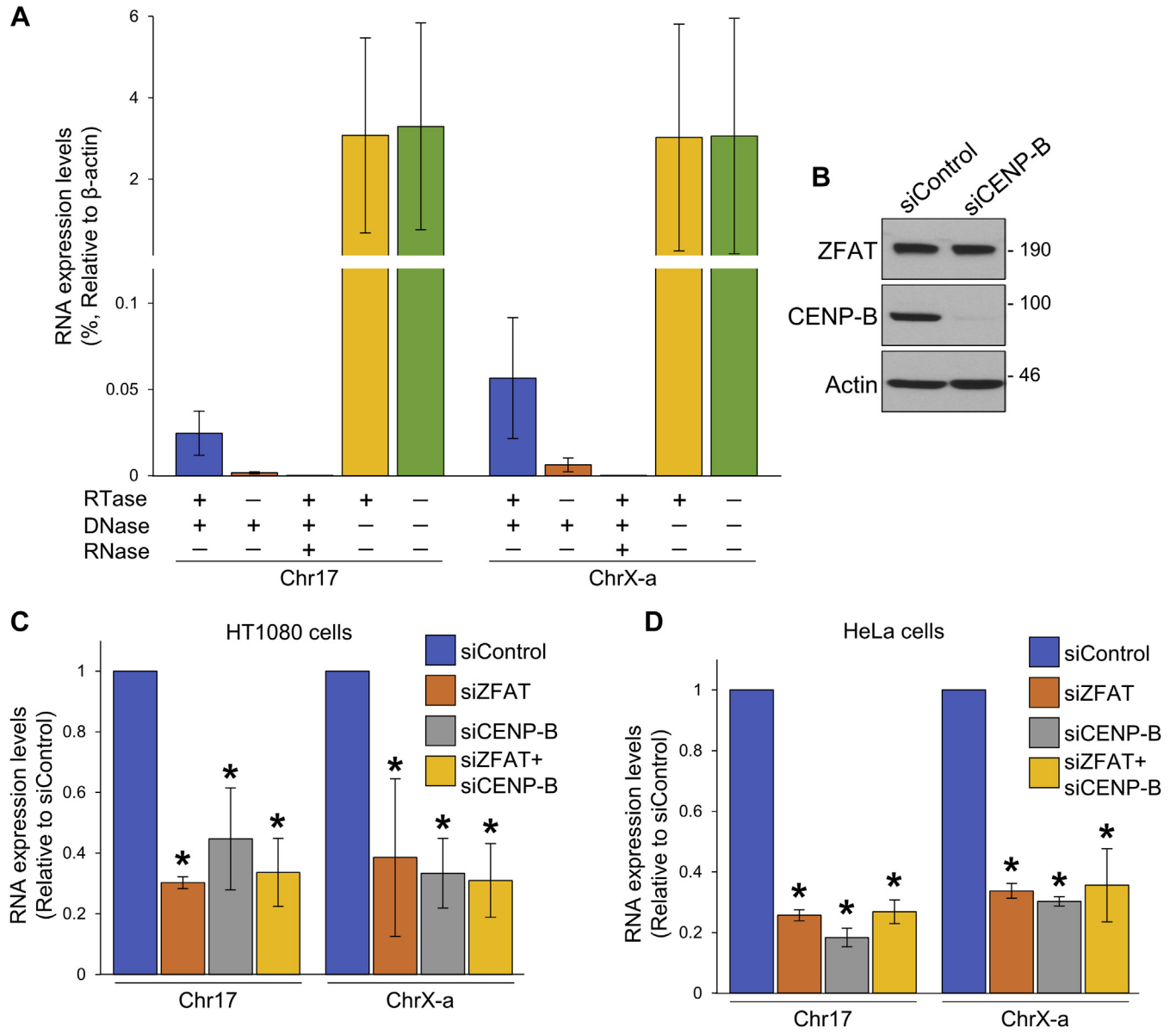

Figure 6. Depletion of CENP-B decreases the expression levels of centromeric ncRNA. *A*, qRT-PCR analysis of centromeric ncRNA in HT1080 cells. The RNA expression levels were determined in the presence or absence of reverse transcriptase (RTase), DNase, or RNase A and are shown as the relative values to those of β-actin. *B*, immunoblotting analysis of ZFAT and CENP-B in HT1080 cells that were transfected with siRNAs for either the control or CENP-B. The molecular weight (in kDa) and position of the molecular weight markers are indicated on the right of the panels. The data are representative of three independent experiments. *C*, qRT-PCR analysis of centromeric ncRNA in HT1080 cells transfected with the indicated siRNAs. *D*, qRT-PCR analysis of centromeric ncRNA in HeLa cells transfected with the indicated siRNAs. *A*, *C*, and *D*, the data represent the mean ± SD of three independent experiments. *$p < 0.05$, against siControl-transfected cells.

overexpression or knockdown of centromeric ncRNA results in chromosome segregation error (34–36). Indeed, we have previously shown that an abnormal spindle morphology was observed in ZFAT-depleted cells (20). To further elucidate the relationship between ZFAT and CENP-B, we examined the effects in HT1080 cells of CENP-B depletion on chromosome segregation using immunofluorescence analysis of α-tubulin. Depletion of CENP-B significantly increased the proportion of mitotic cells that had abnormal spindle morphology, compared with cells transfected with control siRNA (Fig. 7, *A* and *B*). The proportion of mitotic cells with abnormal spindle morphology in CENP-B-depleted cells was lower than that in ZFAT-depleted cells (Fig. 7, *A* and *B*). On the other hand, simultaneous depletion of ZFAT and CENP-B showed similar effects to single depletion of ZFAT, and no additive effects, on the formation of abnormal spindle morphology (Fig. 7, *A* and *B*). These results suggest a possibility that ZFAT may also play different roles from the centromeric ncRNA transcription in accurate spindle formation.

Discussion

We have recently reported that the zinc-finger transcriptional regulator ZFAT binds to the centromere to regulate

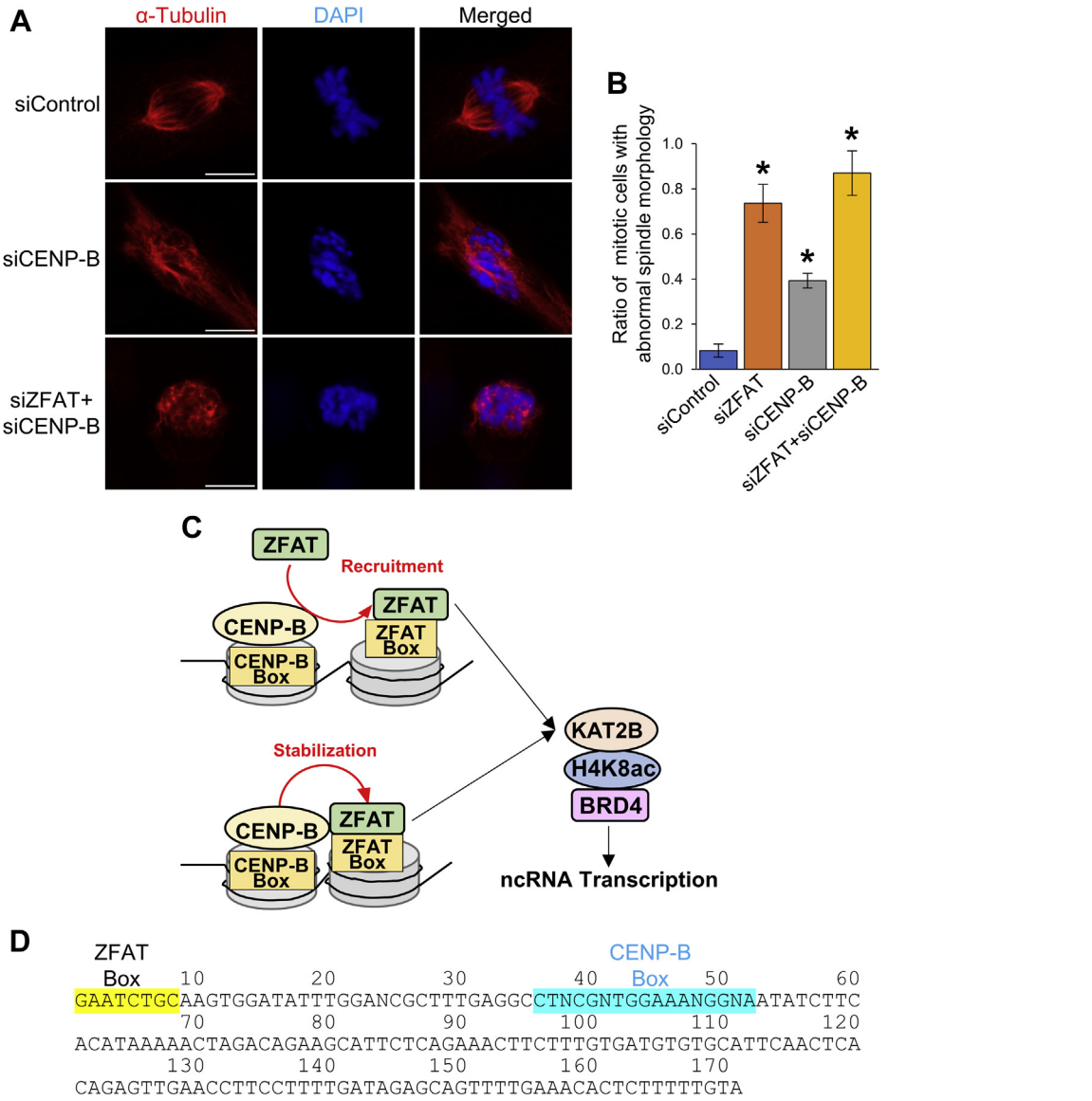

Figure 7. Depletion of CENP-B results in chromosome segregation error. *A*, immunofluorescence images of α-tubulin and DAPI in HT1080 cells transfected with the indicated siRNAs. Scale bar, 5 μm. The data are representative of three independent experiments. *B*, percentage of cells with an abnormal spindle morphology in mitotic cells transfected with the indicated siRNAs, determined in (*A*). The data represent the mean ± SD of three independent experiments. N = 25–30 cells per one experiment. *$p < 0.05$, against siControl-transfected cells. *C*, the model of the role of CENP-B in the centromeric localization of ZFAT. *D*, position of the ZFAT box and CENP-B box in the consensus α-satellite DNA sequence. The consensus nucleotide sequence for α-satellite DNA monomer is shown with reference Romanova *et al.* (39). Sequences for the ZFAT box and CENP-B box are colored in *yellow* and *blue*, respectively.

ncRNA transcription (20). ZFAT binds to a specific 8-bp DNA sequence called the ZFAT box, which is widely distributed at centromeres of every chromosome (20). However, the levels of centromeric ZFAT vary between chromosomes and within centromeric regions. Therefore, the precise molecular mechanisms of the centromeric localization of ZFAT needed to be identified. In this study, we demonstrate that CENP-B plays an important role in the centromeric localization of ZFAT. The centromeric localization of ZFAT relied on the presence of CENP-B protein. Furthermore, ZFAT interacts with the acidic domain of CENP-B. Importantly, depletion of CENP-B reduced the centromeric levels of ZFAT protein and those of ncRNA. These findings suggest that CENP-B contributes to the establishment of centromeric localization of ZFAT to regulate ncRNA transcription.

A role of CENP-B in the establishment of centromeric localization of ZFAT

In this study, we identified that the centromeric localization of ZFAT relied on the presence of CENP-B (Figs. 1 and 5). Furthermore, the evident interaction between ZFAT and CENP-B was observed in both human and mouse cells (Fig. 2). However, we have previously shown that ZFAT binds directly

to the centromeric DNA at specific nucleotide sequences, named the ZFAT box (20, 37), suggesting that ZFAT does not bind to centromeres through the CENP-B protein, nor the CENP-B box. Therefore, we propose two possible models of CENP-B roles in the centromeric localization of ZFAT. One is that CENP-B recruits ZFAT to the centromere, and then ZFAT binds to the ZFAT box; the other is that CENP-B stabilizes the binding of ZFAT to the ZFAT box at the centromere (Fig. 7*C*). The interactions between both the middle domain of ZFAT and the acidic domain of CENP-B are important for the establishment of centromeric localization of ZFAT by recruiting to or by stabilizing at the ZFAT box.

Based on sequence analysis of hundreds of α-satellite DNA monomers, 171-bp consensus nucleotide sequence has been identified (38–40). In the consensus sequence for α-satellite DNA monomer, the ZFAT box is only 27-bp away from the CENP-B box (Fig. 7*D*), suggesting that these binding sequences exist close together for the interaction between ZFAT and CENP-B in most of the α-satellite DNA.

In this study, we showed that the middle domain of ZFAT interacts with the acidic domain of CENP-B. On the other hand, the ZFAT domains, which bind to the ZFAT box, remain elusive. Our preliminary study using a luciferase reporter assay with ZFAT deletion mutants showed that clusters of zinc-finger domains (ZF) of ZFAT (ZF2-5 and ZF6-8) are involved in the binding of ZFAT to the ZFAT box. Further elucidation of molecular mechanisms by which ZFAT recognizes the ZFAT box should be addressed in future studies.

We previously showed that depletion of ZFAT resulted in the decreased centromeric ncRNA levels and abnormal spindle morphology using two different siRNAs against the *ZFAT* gene, demonstrating that ZFAT is required for centromeric ncRNA transcription and accurate chromosome segregation (20). Here, we showed that depletion of CENP-B resulted in decreases in the centromeric levels of ZFAT (Fig. 5) and ncRNA (Fig. 6), and caused chromosome segregation error (Fig. 7) using one siRNA against the *CENP-B* gene. These results suggest that CENP-B is required for the centromeric binding of ZFAT and the ZFAT-regulated centromeric ncRNA transcription. However, the possibility that the off-target effects by the CENP-B siRNA cause these results is not completely ruled out.

In our previous study, ChIP-seq analysis of ZFAT showed that the centromeric ZFAT levels differed between chromosomes in human cells (20). Similarly, it has also been reported that the centromeric CENP-B levels vary between human chromosomes (32). As shown in Figure 1, an increase in the centromeric CENP-B levels through the ectopic expression of CENP-B elevated the ZFAT levels at the centromere (Fig. 1, *D* and *F*). Furthermore, immunofluorescence analysis of endogenous ZFAT in HT1080 cells showed that the ZFAT foci were detected only at centromeres with high CENP-B signals (Fig. 1*F*). These results suggest that there is a correlation in the centromeric levels between ZFAT and CENP-B and CENP-B may play an important role in the determination of the centromeric ZFAT levels.

Mechanisms of the centromeric localization of ZFAT at the Y chromosome, independently of CENP-B

We previously demonstrated that ZFAT is bound to the centromere of every chromosome, including the Y chromosome (20). In contrast, it is understood that CENP-B does not bind to the Y chromosome centromere as it does not contain the CENP-B box (31–33). Indeed, depletion of CENP-B did not result in a decrease in the centromeric ZFAT levels at the Y chromosome, unlike other chromosomes that were evaluated in this study (Fig. 5). These results suggest that CENP-B is not involved in the centromeric binding of ZFAT at the Y chromosome. Kasinathan *et al.* (41) have previously reported that the binding of CENP-B to the centromere induces the formation of a particular DNA structure called non-B DNA, which differs from the common B form of DNA. The non-B DNA structure is believed to play an important role in CENP-A deposition and centromeric transcription (41). Interestingly, the centromere at the Y chromosome is enriched in such non-B DNA structures, compared with the other chromosome centromeres (41). These DNA structures that are characteristics of the Y chromosome centromere, such as non-B DNA, may facilitate the centromeric binding of ZFAT without CENP-B. Furthermore, at the Y chromosome, alternative proteins to CENP-B may support the centromeric binding of ZFAT. Elucidating the molecular mechanisms of centromeric localization of ZFAT at the Y chromosome will require further studies.

A novel function of CENP-B in centromeric ncRNA transcription through interaction with ZFAT

Several recent studies have indicated that CENP-B plays an important role in the establishment and maintenance of the centromere. It has been reported that CENP-B is required for the deposition of CENP-A to the centromere to establish a functional human artificial chromosome (27). Furthermore, the binding of CENP-B to the centromere maintains the fidelity of chromosome segregation by stabilizing the CENP-C protein at the centromere (24). Recently, Hoffmann *et al.* (26) reported that in T cells, CENP-B plays physiologically important roles in the maintenance of centromere position during cell proliferation from the resting to activating states, through mainly recruiting CENP-A to the centromere. In addition to these known CENP-B roles, a novel role for CENP-B in the centromeric localization of ZFAT, identified in this study, is important in centromere functions. Indeed, depletion of CENP-B resulted in a decrease in the expression levels of ncRNA at the centromere where ZFAT regulates transcription (Fig. 6). Therefore, CENP-B contributes to the establishment of centromeric localization of ZFAT to regulate ncRNA transcription.

Experimental procedures

Cell culture

The cell lines HeLa, HEK293, HT1080, and NIH3T3 were cultured at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle

Medium (DMEM, Wako Pure Chemical Industries), supplemented with 10% fetal calf serum and penicillin/streptomycin.

Constructs

The constructs and primers used in this study are detailed in Tables S1 and S2 respectively. The expression vectors for human ZFAT have been previously described (20). cDNA for human CENP-B was amplified from reverse transcription products obtained from Jurkat cells and cloned into plasmid DNA for expression in cultured mammalian cells. The expression vectors were verified by DNA sequencing.

Immunofluorescence microscopy

Cells were seeded onto a 12-mm diameter glass coverslip coated with 0.1 mg/ml poly-L-lysine (Sigma-Aldrich, P5899) and placed into 24-well plates and subsequently transfected with plasmid DNA using Lipofectamine 3000 (Invitrogen). After 24 h, the transfected cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, subsequently washed thrice with PBS, permeabilized, and blocked with 5% FBS or 1% nonfat dry milk in PBS containing 0.3% Triton X-100 for 30 min at room temperature and subsequently incubated with the primary antibodies. Antibodies used for immunostaining are detailed in Table S3. Following incubation, the cells were washed thrice with PBS and subsequently incubated with secondary antibodies conjugated with fluorescent dyes, for 1 h at room temperature. Cells were then washed thrice with PBS, stained with DAPI, mounted using Fluorescence Mounting Medium (Dako), and viewed using a TCS SP5 laser-scanning confocal microscope (Leica Microsystems).

Immunoblotting and coimmunoprecipitation

The immunoblotting procedure was performed as previously described (19, 37, 42) using antibodies detailed in the Table S3. Co-IP was performed as previously described (43). To treat lysates with DNase before Co-IP, cells were lysed in Co-IP buffer for DNase (50 mM Tris-HCl, pH 8; 150 mM NaCl; 60 mM $MgCl_2$; 10 mM $CaCl_2$; 10% glycerol; and 1% NP-40) by incubation for 30 min at 4 °C. Cell pellets were removed by centrifugation, and supernatants were treated with DNase (Roche, #04716728001) for 15 min at 37 °C. Then, Co-IP was performed as previously described (43). To confirm DNA degradation, an aliquot of lysate was treated with DNase after addition of 500 ng plasmid DNA and then analyzed by agarose gel electrophoresis.

siRNA transfection

The siRNAs against CENP-B and ZFAT were purchased from Thermo Scientific (for CENP-B, CENPBHSS101784, 5′-AGUCGUACCAUAGACUGGUCUCGGU-3′; for ZFAT, NM_020863.4_stealth_3360, 5′-UGGCUUGAAGGUGGUGGAAAUUGAU-3′). The cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's reverse transfection protocol. Briefly, cells were seeded with siRNA (20 pmol)–Lipofectamine RNAiMAX (5 μl) complexes in six-well plates at a density of 2×10^5 cells per well. After 48 h, the cells were transfected with plasmid DNA using Lipofectamine 3000 and incubated for a further 24 h. Following incubation, the cells were utilized for downstream analyses.

Chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR)

The antibodies and primers used in ChIP and qPCR are detailed in Tables S2 and S3. For ChIP-qPCR analysis of ZFAT using an anti-HA antibody, HEK293 cells were transfected with the expression vector for EGFP or HA-hZFAT-EGFP using Lipofectamine 3000. After 24 h, the cells were cross-linked with 1% formaldehyde, and $EGFP^+$ cells were sorted using FACSAria II (BD Biosciences) as previously described (17, 18). The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and sonicated using a Bioruptor (Cosmo Bio) for 15 cycles at 1 min with 30 s on/off. After the ChIP procedure, the beads were serially washed with RIPA, RIPA containing 500 mM NaCl, and RIPA containing 250 mM LiCl and TE buffers (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). qPCR was performed using TB Green Premix Ex Taq GC (Perfect Real Time) (Takara Bio) with ABI PRISM 7900HT (Applied Biosystems).

Quantitative RT-PCR (qRT-PCR)

For those cells that were transfected with siRNA against CENP-B using RNAiMAX for 48 h, the total RNA was extracted using TRIZol reagent (Life Technologies). cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). Control samples without reverse transcriptase were performed to identify DNA contamination. qPCR was performed using the Thunderbird SYBR qPCR Mix (Toyobo) with ABI PRISM 7900HT (Applied Biosystems) according to the manufacturer's instructions. The primers used for qPCR are detailed in Table S2.

Statistical analysis

The data were expressed as the mean ± standard deviation. The statistical analyses were performed using an unpaired two-tailed Student's t test. A $p < 0.05$ denoted a statistically significant difference.

Data availability

All data are contained in the article or available on request by contacting the corresponding author: sshirasa@fukuoka-u.ac.jp

Supporting information—This article contains supporting information (43, 44).

Author contributions—S. I. and S. S. conceptualization; S. I., K. Y., S. H., and K. N. data curation; S. I. formal analysis; S. I. and S. S. funding acquisition; S. I. investigation; S. I. methodology; S. I. and

S. S. project administration; S. S. resources; S. S. supervision; S. I. and K. Y. writing–original draft; S. I., K. Y., S. H., K. N., T. T., and S. S. writing–review and editing.

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Abbreviations—The abbreviations used are: ChIP, chromatin immunoprecipitation; Co-IP, coimmunoprecipitation; HOR, higher-order repeat; ncRNA, noncoding RNA; ZFAT, zinc-finger protein with AT-hook.

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