CSN5/JAB1 Interacts with the Centromeric Components CENP-T and CENP-W and Regulates Their Proteasome-mediated Degradation*

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Background: CSN5 (COP9 signalosome subunit 5), functions as a key modulator of cell cycle and cancer.
Results: CSN5 interacts with CENP-T and CENP-W and promotes their proteasome-mediated degradation.
Conclusion: CSN5 may regulate mitosis by affecting the stability of inner kinetochore components, CENP-T-CENP-W.
Significance: These findings demonstrate the first direct link of CSN5 and the mitotic apparatus highlighting the role of CSN5 as a key cell cycle regulator.

The CENP-T-CENP-W complex is a recently identified inner centromere component that plays crucial roles in the formation of a functional kinetochore involved in cell division during mitosis. Using yeast two-hybrid screening, we identified an interaction between CENP-T and CSN5, the fifth component of the COP9 signalosome and a key modulator of the cell cycle and cancer. Co-immunoprecipitation revealed that CSN5 directly interacts with both CENP-T and CENP-W. Ectopically expressed CSN5 promoted the ubiquitin- and proteasome-dependent degradation of CENP-T-CENP-W. The formation of a CENP-T-CENP-W complex greatly enhanced the stabilities of the respective proteins, possibly by blocking CSN5-mediated degradation. Furthermore, dysregulation of CSN5 induced severe defects in the recruitment of CENP-T-CENP-W to the kinetochore during the prophase stage of mitosis. Thus, our results indicate that CSN5 regulates the stability of the inner kinetochore components CENP-T and CENP-W, providing the first direct link between CSN5 and the mitotic apparatus, highlighting the role of CSN5 as a multifunctional cell cycle regulator.

The COP9 signalosome (CSN)2 is a conserved protein complex involved in protein degradation via the ubiquitin-proteasome pathway (1). The CSN complex typically consists of 8 subunits that show significant homology with the 19 S lid subcomplex of the 26 S proteosome and translation initiation factor eIF3 (2). The best characterized function of the CSN is its isopeptidase catalytic activity that cleaves the covalently bound Nedd8 protein of the Cullin family and thereby regulates the activity of the Cullin-ring ubiquitin ligases (3). CSN has been of considerable interest to tumor biologists, because is known to play a crucial role in the degradation of multiple key players involved in essential cellular activities of cancer progression (3). CSN5, the fifth element of the CSN complex, was originally discovered as a transcriptional co-activator of c-Jun, Jab1 (Jun activation domain-binding protein 1) (4). CSN5 is now of great interest not only as an important catalytic subunit for the deneddylation of Cullin-ring ubiquitin ligases but also as a multifunctional cell cycle regulator (5). Furthermore, accumulating evidence has revealed that CSN5 is an oncoprotein. CSN5 is overexpressed in tumors of varying origin, and CSN5 knockdown inhibits the proliferation of cancer cells (6). Moreover, CSN5, both as a part of the CSN holoenzyme and independently, actively participates in essential cellular functions, such as cell proliferation, cell cycle progression, apoptosis, and the maintenance of genome stability (2, 7). CSN5 also interacts with a number of important cellular regulators including Cyclin E, p53, and β-Catenin. Among these proteins, the expression of Myc, Cyclin A, Cdk2, and p27, were stabilized in CSN5 knockdown cells, whereas those of Skp2, c-Jun, and HIFI-α were destabilized in the same model system (2, 8).

CENP-W was initially identified as a putative oncogene, cancer up-regulated gene 2 (CUG2), which is commonly up-regulated in various types of human cancers and is highly oncogenic in a mouse fibroblast cell line (9). Recently, CENP-W was identified as a centromeric protein that interacts with another novel centromere member, CENP-T (10, 11). As a complex, CENP-T-CENP-W functions prior to other centromere components as a prerequisite factor for the recruitment of centromere components. Cellular depletion of CENP-T-CENP-W induces severe defects in the assembly of a functional kinetochore and aberrant chromosome segregation (10, 11). Furthermore, we recently localized CENP-W to the nucleolus during interphase, where it was found to interact with the nucleolar phosphoprotein, Nucleophosmin-1/B23 (12). We proposed that CENP-W mediates the interaction between the pre-kinetochore complex and nucleoli during interphase, and may therefore contribute to the maintenance of centromere protein stability and the recruitment of kinetochore components during mitosis (12).
In the present study, we identified an interaction between CSN5 and CENP-T-CENP-W and showed the ability of CSN5 to regulate the protein stability of CENP-T-CENP-W through the ubiquitin-mediated degradation pathway. Our findings suggest that a novel role exists for CSN5 that enables it to regulate the integrity of the functional kinetochore complex.

**EXPERIMENTAL PROCEDURES**

**Cells, Plasmids, and siRNAs—**Transient transfection of 293T cells was performed using either Effectene™ (Qiagen) or poly-ethyleneimine reagent (Sigma) according to the vendor’s instructions. HeLa-FLAG-CENP-W stable cells (11) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum under standard cell culture conditions. HeLa cells stably expressing CENP-T were also generated with pcDNA-3FLAG-CENP-T plasmid (11). To generate the GST-CSN5 construct, full-length CSN5 was amplified from the cDNA clone obtained from the 21C Frontier Human Gene Bank (Korea). Then, the PCR product was subcloned into the pBEG vector (11) using the BamHI-Ndel restriction sites. The ORF of CSN5 was also subcloned into EGFP-C1 vector (Clontech) using BglIII and XbaI sites. For siRNA treatment, cells were transfected with siRNAs synthesized from Bioneer (Korea) using Lipofectamine™ (Invitrogen). The target sequences were as follows: CSN5, GCCUCAGUAUCGAUGAAA; CENP-T, CAGUAGUGGCCAGGGCUUCA; and CENP-W, a mixture of CAGAUAAAGGCGGAAGGCUC and UACUGGCCGCAGCAAAGGU.

**Yeast Two-hybrid Screening, Protein Binding, and Antibodies—**By using EcoRI and Sall, the C terminus region (amino acids 455–546) was amplified and subcloned into the pGBKTK7 vector (Clontech) to generate a bait plasmid. Screening was performed with the Matchmaker™ two-hybrid system and the pre-transformed human fetal testis library (Clontech).

For co-immunoprecipitation, cells were first lysed in a lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% Triton X-100) supplemented with Protease Inhibitor Mixture (Sigma). The cleared lysate was then mixed with 1 μg of an appropriate antibody or preimmune mouse IgG (Santa Cruz) for 2 h at 4 °C, followed by protein A-agarose (Amersham Biosciences) treatment. For glutathione S-transferase (GST) pull-down, the cell lysate was incubated with 20 μl of a 50% slurry suspension of glutathione-agarose beads (Peptron, Korea) for 2 h at 4 °C with gentle rotation. For the in vitro protein-binding assay, CSN5, CENP-W, and CENP-T were first subcloned into the pET15b bacterial expression vector (Novagen). Next, the His-tagged recombinant proteins were expressed using the Escherichia coli S30 T7 protein expression system (Promega), and co-immunoprecipitation was carried out using an anti-CSN5 antibody (Santa Cruz). Anti-FLAG and anti-Myc antibodies were purchased from Sigma, and anti-His, anti-HA, anti-GFP, anti-CENP-A, anti-CENP-B, anti-Cyclin A, and anti-p27 antibodies from Santa Cruz Biotechnology.

**Half-life Determination and Ubiquitination Assay—**To measure the half-life of a protein, cyclohexamide (Sigma) was added at 100 μg/ml to cells. Then, cells were harvested at different time points and the half-life was quantified by densitometric measurement of the Western blot intensity. For the in vivo ubiquitination assay, 293T cells or HeLa stable cells were transfected with HA-ubiquitin plasmid (kindly provided by Dr. J. Ahn of Chungnam National University, Korea). After 24 h, cells were lysed and target proteins were immunoprecipitated with the appropriate antibody. Co-fractionated ubiquitinated proteins were then identified by Western blotting with an anti-HA antibody.

**Fluorescence Microscopy—**For CSN5 co-localization analysis, HeLa cells were incubated with either hydroxyurea (2 mM) or nocodazole (100 ng/ml) for 12 h prior to fixation in 4% freshly prepared paraformaldehyde in PBS for 15 min. Next, cells were permeabilized with 0.1% Triton X-100 in PBS, the coverslips were incubated with anti-FLAG and anti-CSN5 antibody, followed by FITC-labeled anti-mouse antibody (Vector Laboratories) and Cy3-linked anti-rabbit antibody (Jackson ImmunoResearch). Imaging was performed using an Olympus IX70 fluorescence microscope at ×200 magnification.

**Glycerol Gradient Centrifugation—**For cell fractionation, 10 ml of linear glycerol gradient (10–40%) containing 10 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol, was prepared. After loading the cell lysate onto the glycerol gradient, the sample was centrifuged at 40,000 × g in a Beckman SW 41 Ti rotor for 16 h at 4 °C. The fractions (0.7 ml) were collected from the bottom and labeled as fraction 1.

**RESULTS**

**CSN5 Interacts with CENP-T—**We previously identified CENP-T as the binding partner of CENP-W by yeast two-hybrid screening (9). In the present study, we used the same approach to identify new CENP-T-interacting proteins. Because full-length CENP-T was self-active when fused to the Gal4 DNA-binding domain, we used a truncated C-terminal region (amino acids 455–546) of CENP-T as bait. This region contains the Histone 2A (H2A) domain (amino acids 477–523), which we identified using the SMART (Simple Modular Architecture Research Tool) protein domain analysis program. Screening was performed against a human testis cDNA library. Among the selected clones, we isolated CENP-T, suggesting that CENP-T can exist as a dimer. We also identified a cDNA sequence of CSN5, the fifth component of CSN.

To confirm the interaction between CSN5 and CENP-T, we performed co-immunoprecipitation with ectopically expressed GST-CSN5 and FLAG-CENP-T in 293T cells. CSN5 was co-isolated with full-length CENP-T as well as with the C-terminal region of CENP-T (Fig. 1A). Likewise, GST pull-down experiments revealed that the C-terminal region (amino acids 455–546) of CENP-T was eluted with GST-CSN5 (Fig. 1B). The CSN5-CENP-T interaction was then examined endogenously by immunoprecipitation using an anti-CSN5 antibody and HeLa cell lysates, followed by Western blot analysis with an anti-CENP-T antibody (12). CENP-T was present in the CSN5 fraction, but not in the normal IgG fraction (Fig. 1C). Finally, to demonstrate direct binding between CSN5 and CENP-T, we performed an in vitro binding assay using recombinant proteins obtained from an E. coli lysate-based cell-free expression system. Co-immunoprecipitation with an anti-CSN5 antibody revealed that His-CSN5 expressed in vitro directly interacts with recombinant His-CENP-T (Fig. 1D).
To determine which CSN5 domain is required for the interaction with CENP-T, a series of truncated versions of CSN5 were generated and tested for CENP-T binding activity. The deneddylation activity of CSN5 was previously mapped to the JAB1/MPN/Mov34 metalloenzyme (JAMM) motifs located within the Pad1p N-terminal (MPN) domain (Fig. 1E). We found that the interaction between CSN5 and CENP-T also depended on the N-terminal region containing the MPN domain (amino acids 1–190). Interestingly, additional deletion analysis revealed that the N-terminal short fragment containing the JNK-binding domain (amino acids 1–53) (13) has greater CENP-T binding activity than the MPN domain (amino acids 54–190). Conversely, we mapped the CENP-T region required for binding to CSN5 using CENP-T deletion mutants. The H2A domain within CENP-T, previously used as bait for yeast two-hybrid screening, was sufficient for interaction with CSN5 (Fig. 1F).

CSN5 Promotes Degradation of CENP-T—Based on studies that indicate CSN5 is an important regulator of proteasome-dependent degradation of key proteins in the cell cycle (8), we evaluated the ability of CSN5 to modulate CENP-T protein stability. First, we determined if CENP-T degradation is proteasome-dependent using MG132, a potent inhibitor of proteasomes, in HeLa-FLAG-CENP-T stable cells. After blocking protein synthesis with cyclohexamide treatment, we determined that the half-life of CENP-T increased greatly following MG132 treatment, indicating that proteasomes are involved in the breakdown of CENP-T (Fig. 2A). Next, HeLa-CENP-T sta-

FIGURE 1. CSN5 interacts with CENP-T. A, co-immunoprecipitation between CSN5 and CENP-T. After 293T cells were transfected with GST-CSN5 and FLAG-CENP-T (either C-terminal H2A domain region (amino acids 455–546) or full-length), the cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody or normal mouse IgG. B, reciprocal interaction between GST-CSN5 and FLAG-CENP-T (H2A) using GST-pull-down. C, determination of binding activity at the endogenous level. Immunoprecipitation was performed with HeLa cell lysates using an anti-CSN5 antibody, and co-fractionated CENP-T was identified with anti-CENP-T antibody. D, in vitro binding assay. After recombinant His-CSN5 and His-CENP-T was obtained by using the E. coli S30 T7 protein expression system (Promega), co-immunoprecipitation was carried out with an anti-CSN5 antibody. E, identification of CSN5 region responsible for CENP-T binding. After a series of CSN5 deletion mutants were transfected into HeLa-CENP-T stable cells, co-isolated CENP-T was monitored after a GST pull-down experiment. F, domain mapping of CENP-T. After CENP-T deletion mutants were co-expressed with GST-CSN5 in 293T cells, GST pull-down was performed to identify the CENP-T domain co-isolated with CSN5. WB, Western blot; JBD, JNK-binding domain.
ble cells transfected with GST-CSN5 or GST were treated with cyclohexamide and used to examine CENP-T protein levels. The half-life of CENP-T was significantly decreased in GST-CSN5-transfected cells compared with GST expressing control cells (Fig. 2B). The N-terminal short fragment (amino acids 1–53), which was identified to be crucial in binding with CENP-T also promotes CENP-T degradation, suggesting that the effect of CSN5 to CENP-T is dependent on a protein-protein interaction. Moreover, we determined the effects of the CSN5 inhibitor, curcumin, on CENP-T protein stability. At 24 h post-transfection with CSN5, HeLa-CENP-T stable cells were treated with increasing concentrations of curcumin (0–20 μM) for 6 h prior to harvest. D, in vivo ubiquitination assay. After HA-ubiquitin was transfected to HeLa-CENP-T stable cells, immunoprecipitation (IP) was carried out with an anti-FLAG antibody. The ubiquitinated CENP-T fraction was identified with an anti-HA antibody. E, knock-down of CSN5 increases CENP-T stability. HeLa cells were incubated with CSN5 siRNA (100 nM) for 32 h before analysis by Western blotting. F, cellular distribution of CENP-T upon CSN5 co-expression. After overexpression of EGFP-CSN5 in HeLa-CENP-T stable cells, the localization of CENP-T was visualized using immunostaining with an anti-FLAG antibody, followed by Cy3-linked anti-mouse secondary antibody. Scale bar equals 10 μm.

CSN5 Regulates CENP-T-CENP-W Stability

CSN5 promotes the nuclear to cytoplasmic shuttling of target proteins, such as p27, has been observed in a number of other studies (5). To determine whether CSN5 can change the localization of CENP-T within cells, HeLa-FLAG-CENP-T stable cells were transfected with EGFP-CSN5. After immunostaining with an anti-FLAG antibody and Cy3-labeled secondary antibody, the EGFP-CSN5 and CENP-T signals were monitored. Although no cytoplasmic shuttling of CENP-T was observed, almost all CENP-T signals were lost in ∼30% of CSN5-transfected cells, suggesting that CSN5 co-expression facilitates CENP-T degradation (Fig. 2F). In contrast, CSN5 was found in both the cytoplasm and nucleus, and no significant differences were observed upon co-transfection of CENP-T.

CSN5 Interacts with CENP-W—Given that CENP-T functions as a complex with CENP-W (10, 14), we examined the ability of CSN5 to interact with CENP-W in vivo. 293T cells were transfected with FLAG-CENP-W and GST-CSN5 and
used in an in vivo binding assay. GST pull-down revealed that GST-CSN5 co-fractionated with FLAG-CENP-W (Fig. 3A), and reciprocal immunoprecipitation showed that CSN5 also bound to FLAG-CENP-W (Fig. 3B). To examine CSN5 and CENP-W interaction at the endogenous level, and because of the unavailability of specific antibodies for CENP-W, we used HeLa cells stably expressing FLAG-CENP-W (12). When immunoprecipitation was conducted using an anti-CSN5 anti-body, FLAG-CENP-W was clearly observed in the CSN5 complex (Fig. 3C), but not in the IgG control. We also examined the in vitro binding of recombinant CENP-W and CSN5 proteins using the E. coli cell-free expression system. His-CENP-W was co-fractionated with His-CSN5 when immunoprecipitated with an anti-CSN5 antibody in vitro (Fig. 3D), indicating a direct interaction between these 2 proteins. To evaluate the complex formation of these 3 proteins, we transfected 293T
cells with FLAG-CENP-T, Myc-CENP-W, and GST-CSN5 plasmids and performed immunoprecipitation with an anti-FLAG antibody. CSN5 and CENP-W were co-eluted with FLAG-CENP-T, whereas no specific bands were observed with normal mouse IgG (Fig. 3E), suggesting that these 3 proteins exist in the same complex. Taken together, these results demonstrate that CSN5 interacts with CENP-W as well as CENP-T, and these 3 proteins likely form a complex in vivo.

To identify the region of CSN5 responsible for CENP-W binding, we performed GST pull-down using GST-CSN5 deletion mutants. CENP-W had a different binding specificity from CENP-T: a truncated CSN5 containing the MPN domain (amino acids 54–190) was sufficient to interact with CENP-W (Fig. 3F), whereas the presence of the short N terminus fragment (amino acids 1–53), important in CENP-T affinity, inhibited the interaction between CENP-W and CSN5. Both the large N-terminal region (amino acids 1–190) and the short N-terminal fragment (amino acids 1–53) did not bind with CENP-W. Furthermore, we examined the region of CSN5 necessary for the interaction with CENP-W. Our results indicate that the middle region of CENP-W (amino acids 31–60) most likely mediates its interaction with CSN5 (Fig. 3G).

CSN5 May Regulate CENP-W Stability—To determine whether CSN5 can also regulate CENP-W protein stability, we examined the degradation rate of CENP-W in the presence and absence of CSN5. 293T cells were transfected with FLAG-CENP-W and GST-CSN5 for 24 h, followed by cyclohexamide treatment. The degradation rate of CENP-W was slightly accelerated in CSN5 co-transfection (Fig. 4A). To confirm this observation, we transfected A549-FLAG-CENP-W stable cells with CSN5 siRNA (100 nM) for 32 h before cell harvest. 

FIGURE 4. CENP-W is destabilized by CSN5. A, CSN5 may promote CENP-W degradation. To measure the degradation rate of CENP-W, 293T cells were transfected with FLAG-CENP-W together with GST-CSN5 or GST control. Then, cells were harvested at various time points after the addition of cyclohexamide (100 μg/mL). B, CENP-W is destabilized by CSN5. After an increasing amount of CSN5 plasmid was transfected to A549-FLAG-CENP-W stable cells, the CENP-W protein level was analyzed by Western blot. C, effect of curcumin on CENP-W stability. At 24 h post-transfection of GST-CSN5 and FLAG-CENP-W, various concentrations of curcumin (0–40 μM) were treated to cells for 6 h before harvest. D, the effect of MG132 on CENP-W degradation. To measure the decay rate of CENP-W in the presence of MG132, MG132 (20 μM) was added to the 293T cells expressing CENP-W and CSN5, and incubated for 4 h prior to cyclohexamide treatment. E, in vivo ubiquitination assay. 293T cells were transfected with HA-ubiquitin and FLAG-CENP-W in the presence or absence of GST-CSN5, and immunoprecipitation was carried out with anti-FLAG antibody. F, cellular localization of CENP-W. HeLa cells were transfected with FLAG-CENP-W and GFP-CSN5 and immunostained with anti-FLAG antibody (red). Scale bars, 10 μm. G, depletion of CSN5 using siRNA. HeLa-FLAG-CENP-W stable cells were incubated with CSN5 siRNA (100 nM) for 32 h before cell harvest. H, CENP-W-binding activity of CSN5 mutants. I, effect of CSN5 mutant proteins on CENP-W degradation.

To confirm this observation, we transfected A549-FLAG-CENP-W stable cells with increasing concentrations of GST-CSN5 plasmid and found that CENP-W protein levels decreased in proportion to the concentrations of CSN5 in transfected cells (Fig. 4B). Moreover, the CSN5 inhibitor curcumin induced CSN5 destabiliza-
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...tion, which in turn resulted in CENP-W stabilization, indicating that CSN5 controls CENP-W protein levels (Fig. 4C). Next, we determined if the observed accelerated CENP-W degradation by CSN5 is a proteasome-mediated process. As shown in Fig. 4D, incubation with MG132 clearly abrogated CSN5-mediated CENP-W degradation. Finally, we performed an in vivo ubiquitination assay, which revealed that the ubiquitination of CENP-W significantly increases when CSN5 is co-transfected in cells (Fig. 4E). Taken together, these results show that CSN5 promotes the proteasomal degradation of CENP-W.

Again, no cytoplasmic shuttling of CENP-W was observed when CENP-W and CSN5 were co-expressed in HeLa cells (Fig. 4F); however, we did notice there was a change in the nuclear distribution of CENP-W. As previously reported (12), CENP-W is predominantly accumulated in nucleolar compartments when transiently expressed alone (second row of Fig. 4F). However, when CSN5 was co-expressed, CENP-W was visible as numerous dots in most cells (third row of Fig. 4F). We also performed a knockdown experiment in HeLa-CENP-W stable cells by using CSN5 siRNA. Prolonged treatment (~72 h) of cells with CSN5 siRNA resulted in decreased levels of both CENP-W and CENP-T, perhaps because of the disruption of global balance or to secondary effects from other CSN5 target molecules (data not shown). However, upon inhibition of CSN5 expression for a shorter period (~32 h), an increase in CENP-W, CENP-T, as well as previously identified substrates, such as Cyclin A1 and p27, was observed (Fig. 4G). Taken together, these findings indicate that CSN5 promotes the ubiquitin-dependent proteasomal degradation of the CENP-T-CENP-W complex. We also evaluated the effect of CSN5 on CENP-W using CSN5 mutant proteins. The D151N mutant, which does not bind with CENP-W resulted in attenuated CENP-W degradation activity (Fig. 4, H and I). In contrast, the E76A mutant accelerated CENP-W degradation, indicating that the protein-protein interaction is also important in the CSN5-mediated degradation of CENP-W.

CENP-W and CENP-T Reciprocally Regulate Their Stability—In our double-transfection experiment with CENP-W and CENP-T, we observed that the level of 1 protein was affected by co-expression of another. To address this phenomenon, we monitored CENP-T protein levels after transfection of 293T cells with increasing concentrations of CENP-W (Fig. 5A). CENP-T protein levels were proportional to CENP-W levels. We also examined CENP-T protein stability in HeLa-CENP-T stable cells transfected with CENP-W. As shown in Fig. 5B, CENP-T decay was remarkably protected with the addition of CENP-W in HeLa-CENP-T stable cells. To determine whether the ubiquitination process was involved in this interaction, we carried out an in vivo ubiquitination assay examining CENP-T in the presence of CSN5 and CENP-W. The smeared bands of high molecular weight CENP-T were intensified by co-expression of CSN5 (Fig. 5C). However, the CSN5-mediated CENP-T ubiquitination was significantly inhibited by CENP-W co-expression, which suggests that CENP-T ubiquitination and degradation was inhibited when CENP-T was associated with CENP-W.

Conversely, to examine the effects of CENP-T on CENP-W stability, we transfected A549-CENP-W stable cells with increasing concentrations of CENP-T. CENP-W protein stability increased with CENP-T co-expression (Fig. 5D). Moreover, using an in vivo ubiquitination assay, we identified that co-expression of CENP-T significantly decreased CSN5-mediated ubiquitination of CENP-W (Fig. 5E), suggesting that CENP-T enhances CENP-W integrity by blocking CSN5-promoted ubiquitination. To confirm the observed protective relationship between CENP-T and CENP-W, we tested whether depletion of 1 protein would alter the stability of the other protein using siRNA treatment. Although suppression of CENP-T affected the stability of other kinetochore components, the most dramatic decrease resulting from reduction of CENP-T was that of CENP-W (Fig. 5F). Similarly, knockdown of CENP-W caused significant destabilization of CENP-T proteins, indicating that the interaction between these 2 centromere proteins greatly influences their stability.

To determine which domains are important for the binding of CENP-T to CENP-W, we performed domain mapping. GST pull-down revealed that only CENP-T proteins containing the H2A domain were co-isolated with GST-CENP-W (Fig. 5G). The same constructs interacted with CSN5 (Fig. 1F), indicating that both CSN5 and CENP-W interact with CENP-T through the H2A domain. For CENP-W, an N-terminal region (amino acids 1–60) that overlaps with the region previously identified to be involved in CSN5 binding (amino acids 31–60) (Fig. 3G), mediated the interaction with CENP-T (Fig. 5H). These results demonstrate that the region responsible for the interaction between CENP-T and CENP-W was also important for CSN5 binding, suggesting that the association between CENP-T and CENP-W may enhance their stability by blocking access of CSN5 to its binding sites, and consequently, CSN5-mediated degradation.

CSN5 Depletion Induces Defects in the Recruitment of CENP-T-CENP-W during Prophase—Although we determined that CSN5 directly interacts with kinetochore proteins, CENP-T and CENP-W, we also found that localization of these proteins is different in asynchronized cells (Figs. 2F and 4F): CSN5 was mostly observed to be dispersed both in the nucleus and cytoplasm, whereas CENP-T-CENP-W was scattered within the nucleus. To determine whether CENP-T and CENP-W exist as a complex with the CSN5 complex, we performed cell fractionation using the glycerol gradient (10–40%) technique. Previously, 2 different CSN5-containing protein complexes (large COP9 holoenzyme and small complex) were identified in cell fractionation analysis (15). CENP-T and CENP-W were co-sedimented with the small CSN5 complex, suggesting that these centromeric components may co-exist with a small CSN5 complex (Fig. 6A). Furthermore, CENP-T and CENP-W did not interact with CSN1 (Fig. 6B), which is known to only be present in the holoenzyme (16), suggesting that association of CSN5 with these centromere components may be independent of the function of COP9 holoenzyme. Next, the localization of CSN5 and CENP-T-CENP-W proteins was examined at different stages of the cell cycle by using HeLa stable cells. When cells were double-immunostained with anti-CSN5 and anti-FLAG antibodies, the general localization of the CSN5 did not match that of CENP-T or CENP-W during the cell cycle (data not shown). However, interestingly, we noticed CSN5 was localized...
either in the nucleolus or in the perinucleolar chromatin structure specifically during the late S phase (Fig. 6, C and D). Moreover, despite the scattered appearance in the late S phase cells, many CENP-T and CENP-W spots were present in the area surrounding the CSN5 proteins. To confirm the interaction in nucleoli, we performed subnuclear fractionation (12) using cells synchronized in late S phase. The following immunoprecipitation results indicate that CSN5 interacts with CENP-T and CENP-W in the nucleolar compartment (Fig. 6E).

Given that the CENP-T/CENP-W complex plays a key role in chromosome segregation during mitosis (10, 11), we determined if CSN5 depletion can affect the cell division process. Following synchronization of CSN5-depleted cells with nocodazole, mitotic cells were stained with an anti- α-Tubulin

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**FIGURE 5.** The complex formation between CENP-T and CENP-W increases their stability. A, co-transfection of CENP-W enhanced the CENP-T stability. 293T cells were transfected with FLAG-CENP-T together with an increasing amount of FLAG-CENP-W plasmid, and then, Western blotting (WB) was performed to monitor each protein. B, CENP-W transfection increased CENP-T stability. After 24 h post-transfection of CENP-W or vector control, cyclohexamide (CHX) was added to HeLa-FLAG-CENP-T stable cells. C, in vivo ubiquitination of CENP-T in the presence of CENP-W. After HeLa-CENP-T stable cells were transfected with the indicated plasmids, immunoprecipitation was performed with an anti-FLAG antibody. D, CENP-T increases CENP-W stability. A549-CENP-W stable cells were transfected with increasing amounts of FLAG-CENP-T plasmids. E, co-transfection of CENP-T suppressed CSN5-mediated CENP-W ubiquitination. F, knockdown of CENP-T or CENP-W using siRNA treatment. G, determination of CENP-T domains crucial for CENP-W binding. After FLAG-tagged CENP-T deletion constructs were transfected to 293T cells with GST-CENP-W, GST pull-down was carried out. H, domain mapping of CENP-W for CENP-T binding. After CENP-W domain mutants were co-transfected with EGFP-CENP-T to 293T cells, immunoprecipitation was performed with an anti-GFP antibody.
antibody for mitotic spindles and an anti-centromere antibody (ACA) for kinetochores. CSN5 knockdown induced significant abnormalities during mitosis: misaligned chromosome and multipolar spindles were frequently found in CSN5-depleted cells (Fig. 7A). Furthermore, CSN5 suppression significantly prolonged the duration of mitosis, as reflected by examination of different stages of mitosis (Fig. 7B).

The nucleolus, a protein-enriched subnuclear organelle, was recently proposed to play a role in the recruitment and organization of the prekinetochore complex during the interphase (17). Based on our finding that the CSN5/kinetochore interaction is most active near the nucleoli during the late S period of the cell cycle, we determined that CSN5 affects recruitment of the CENP-T/CENP-W complex during mitosis. For this purpose, we monitored the distribution of CENP-T/CENP-W at the prophase stage of mitosis in CSN5-depleted cells as well as in CSN5-overexpressing cells. CENP-T/CENP-W dots were clearly co-localized with the centromere markers recognized by ACA in control cells (Fig. 8B, left three columns). However, CENP-T/CENP-W was significantly dispersed throughout ~60% of CSN5-knockdown cells (Fig. 8, B, following three columns, and C). Similarly, CENP-T/CENP-W was highly dispersed when CSN5 was overproduced (Fig. 8B, right three columns). No significant changes were observed in the distribution of the core centromeric members, CENP-A and CENP-B, upon deregulation of CSN5 during mitotic prophase. These data suggest that CSN5 plays a role in the regulation of CENP-T/CENP-W stability during interphase, which is required for proper formation of the kinetochore complex during cell division.

DISCUSSION

At the attachment site of spindle microtubules, a number of proteins and subcomplexes that reside in the kinetochore play key structural and regulatory roles during mitosis and meiosis (17). Although the timely regulation of the kinetochore destructive pathway is crucial for proper progression of the cell cycle, little has been described concerning the mechanism of degradation for the inner components of the kinetochore complex. Only recently, small ubiquitin-like modifier protein-targeted ubiquitin ligase was found to control the degradation of the inner kinetochore components, CENP-I and CENP-H (18), and the ICP0 protein of herpes simplex virus type 1 was reported to induce the proteasome-dependent degradation of CENP-A (19) and CENP-B (20).

The gene encoding CENP-W was originally described as a putative oncogene that is highly up-regulated in various human tumors. Although oncogenes are often up-regulated in cancer tissues, many previous studies have also revealed that various well known oncogenes, such as ras and myc, can drive cell apo-
FIGURE 7. Analysis of mitotic activities after CSN5 knockdown. A, aberrant mitosis was frequently observed in CSN5-depleted cells. After HeLa cells were incubated with CSN5 siRNA (100 nM) for 32 h, nocodazole (100 ng/ml) was added to the culture medium for another 12 h. After thorough washing, cells were harvested at 10–15-min intervals and used for immunostaining. Anti-α-Tubulin antibody and FITC-labeled anti-mouse IgG antibody was used for visualizing the distribution of mitotic spindles, and ACA (Cortex Biochem) and Alexa 594-linked anti-human IgG antibody (Invitrogen) for the centromere. B, CSN5 suppression prolonged the duration of mitosis. Representative images of each time point in mitotic cells were presented from CSN5-depleted cells and control siRNA-treated cells. At various time points, the number of cells in each stage of mitosis was counted, and the percentage of each stage was calculated.
ptosis or senescence upon their overexpression (21, 22). We previously showed that the abrupt expression of CENP-W also induces massive cell death in a SKOV-3 ovarian cancer cell line (23) as well as in zebrafish embryos (24). It is therefore likely that abnormal activation of CENP-W may provoke the unnecessary activation of a cell death program, although as an essential component of kinetochore, CENP-W functions as a mitotic activator. Collectively, previous results suggest that the protein level of CENP-W needs to be tightly regulated to maintain proper cell cycle function.

The nucleolus is a specialized subnuclear compartment and its primary role is in rDNA transcription and ribosome biogenesis. Recent studies revealed that the nucleolus is not simply an RNA factory, but rather a complex, interesting multifunctional organelle implicated in many cellular processes (25). Furthermore, because of the discovery of a complex composed of the typical nucleolar protein, B23, and the centromere inner complex, it has been proposed that the nucleolus plays a sequestering role in the formation of the pre-kinetochore complex during interphase (17). Our recent study demonstrated that CENP-W associates with nucleoli and interacts with B23 (12). Moreover, the interaction between CENP-W and B23 during interphase is required to maintain CENP-W stability and the proper recruitment of CENP-W during mitosis (12). In this study, although the protein levels of CSN5, CENP-T, and CENP-W remained relatively constant throughout the cell cycle (data not shown), we repeatedly observed that CSN5 is localized around the nucleolar compartment during the late S period. Based on these findings, we suggest that CSN5 most likely interacts with CENP-T-CENP-W during interphase, which correlates with the proposed role of nucleoli in the regulation of the integrity of the pre-kinetochore complex.

As a member of CSN complexes and in the monomeric form, CSN5 has been reported to directly interact with various proto-oncogenes and tumor suppressors to regulate their protein stability, thereby functioning as a key modulator of cell cycle progression and cell proliferation (3). In MEF cells lacking CSN5, loss of CSN5 inhibited progression of the cell cycle at multiple points and markedly increased the population of cells with higher ploidy (8). Moreover, CSN5 knockdown caused mitotic defects, G2/M arrest, and cell apoptosis in the human myeloid progenitor cell line K562 (26). Our present study in HeLa cells also revealed that depletion of CSN5 prevented mitotic progression and resulted in structural defects in the mitotic apparatus. Given that the kinetochore plays pivotal roles in chromosome segregation and mitotic progression, our findings that CSN5 directly interacts with CENP-T-CENP-W to promote their degradation may provide a direct functional link between CSN5 and the abnormalities observed during mitosis. Furthermore, based on the previous findings that the CENP-T-CENP-W complex may function upstream of the formation of the inner kinetochore complex (10), we speculate that CSN5 regulates the cell division process by controlling the stability of CENP-T-CENP-W, and consequently the whole integrity of the kinetochore inner complex. Our present study showed that CSN5 regulates the stability of the inner kinetochore proteins and contributes to the maintenance of their homeostatic balance, which elevates the status of CSN5 as a key cell cycle modulator.
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