Human Zwint-1 Specifies Localization of Zeste White 10 to Kinetochores and Is Essential for Mitotic Checkpoint Signaling*

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Chromosome segregation in mitosis is orchestrated by dynamic interaction between spindle microtubules and the kinetochore, a multiprotein complex assembled onto centromeric DNA of the chromosome. Here we show that Zwint-1 is required and is sufficient for kinetochore localization of Zeste White 10 (ZW10) in HeLa cells. Zwint-1 specifies the kinetochore association of ZW10 by interacting with its N-terminal domain. Suppression of synthesis of Zwint-1 by small interfering RNA abolishes the localization of ZW10 to the kinetochore, demonstrating the requirement of Zwint-1 for ZW10 kinetochore localization. In addition, depletion of Zwint-1 affects no mitotic arrest but causes aberrant premature chromosome segregation. These Zwint-1-suppressed cells display chromosome bridge phenotype with sister chromatids inter-connected. Moreover, Zwint-1 is required for stable association of CENP-F and dynamitin but not BUB1 with the kinetochore. Finally, our studies show that Zwint-1 is a new component of the mitotic checkpoint, as cells lacking Zwint-1 fail to arrest in mitosis when exposed to microtubule inhibitors, yielding interphase cells with multinuclei. As ZW10 and Zwint-1 are absent from yeast, we reasoned that metazoans evolved an elaborate spindle checkpoint machinery to ensure faithful chromosome segregation in mitosis.

Chromosome movements during mitosis are governed by the interaction of spindle microtubules with a specialized chromosome domain located within the centromere. This specialized region, called the kinetochore (1, 2), is the site for spindle microtubule-centromere association. In addition to providing a physical link between chromosomes and spindle microtubules, the kinetochore has an active function in chromosomal segregation through microtubule motors and spindle checkpoint sensors located at or near it (3–5).

Eukaryotic organisms require extraordinary fidelity in chromosome segregation during meiosis and mitosis as aberrant chromosome segregation can be catastrophic to an organism or its progeny. One of the evolutionarily conserved multiprotein complexes essential for the fidelity of chromosome segregation contains several proteins, including ZW10 (Zeste White 10) and ROP ( Rough Deal) (6–8). Mutations in the Drosophila ZW10 or ROD genes cause similar defects, most noticeably in lagging chromatids that remain at the metaphase plate late in anaphase, leading to high levels of aneuploidy among daughter cells.

ZW10 and ROD proteins display remarkable dynamics in their intracellular location during cell division (7, 9, 10). Both proteins accumulate strongly at the outer kinetochore plates during prometaphase. At metaphase, ZW10 and ROD depart from the kinetochores and relocate onto spindle microtubules. During anaphase, the proteins are no longer found on kinetochore microtubules and instead localize exclusively to the kinetochores of the separating chromosomes.

Besides binding to ROD, ZW10 is responsible for localization of cytoplasmic dynein to kinetochores (10–12) via a direct contact with dynamitin, a component of the dynactin complex (11, 13). HZwint-1 (Human ZW10 interacting protein-1, referred to as Zwint-1 hereafter) was identified in a yeast two-hybrid screen for proteins interacting with ZW10. Zwint-1 targets to the kinetochore prior to ZW10 in prophase HeLa cells and remains at the kinetochore until late in anaphase. The spatial-temporal distribution of Zwint-1 relative to ZW10 raises the possibility that Zwint-1 may be responsible for ZW10 localization to the kinetochore. However, the inter-relationship between Zwint-1 and ZW10 is not known, nor whether Zwint-1 is essential and sufficient for ZW10 localization.

To explore the nature of the ZW10-Zwint-1 interaction at the kinetochore and their respective functions in spindle checkpoint signaling, we used a yeast two-hybrid assay to pinpoint an interface for such an interaction. Our biochemical studies confirmed that Zwint-1 interacts with the N-terminal 80 amino acids of ZW10. In addition, expression of the mutant ZW10 protein in HeLa cells indicated that this Zwint-1 interacting domain is sufficient for ZW10 distribution to the kinetochore. Suppression of Zwint-1 protein synthesis by small interfering RNA liberated kinetochore localization of ZW10. Most significantly, depletion of Zwint-1 abrogates the mitotic checkpoint induced by microtubule disruptors. In addition, Zwint-1 is required for stable kinetochore localization of CENP-F and dynamitin. Given the inter-relations—

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1 The abbreviations used are: ZW10, Zeste White 10; Zwint1, ZW10 interacting protein-1; GST, glutathione S-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; siRNA, small interfering RNA; PIPES, 1,4-piperazinediethanesulfonic acid; DAPI, 4,6-diamidino-2-phenylindole; RNAi, RNA interference; ACA, anti-centromere antibody.
ship of ZW10-Zwint-1 established here, we propose that Zwint-1 links ZW10/ROD/dynein-mediated checkpoint signaling to chromosomal segregation dynamics.

**MATERIALS AND METHODS**

**Cell Cultures**—HeLa and 293T cells (American Type Culture Collection, Manassas, VA) were cultivated as subconfluent monolayers in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (HyClone, UT) and 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C with 10% CO2.

**CDNA Construction**—To generate GFP-tagged full-length ZW10 and deletion mutants, ZW10 cdna was digested with BamHI and XhoI and then cloned into pEGFP C3 vector (Clontech). The bacterial expression constructs of ZW10 were cloned into pGEX-5X-3 (Amer sham Biosciences), whereas Zwint-1 cDNA was cloned into pGEX-2T (Amersham Biosciences). FLAG-tagged Zwint-1 cDNA was cloned by inserting the PCR product into the pcDNA3 vector (Invitrogen) with EcoRI and XhoI digestion.

**Recombinant Protein Production**—Purification of recombinant proteins was carried out as described previously (14). Briefly, 1 liter of LB media was inoculated with bacteria transformed with GST-ZW10 and GST-Zwint-1, respectively. The protein expression was induced by addition of isopropyl-D-thiogalactopyranoside for 3 h. Bacteria media was inoculated with bacteria transformed with GST-ZW10 and gen) with EcoRI and XhoI digestion.

**GST fusion protein-bound Sepharose beads were incubated with 293T cell lysates and its accessory proteins were then washed and then boiled in SDS-PAGE sample buffer followed by fractionation of bound proteins on a 6–12% gradient gel. Proteins were then transferred onto nitrocellulose membrane for Western blotting using FLAG antibody (Sigma). Slides were examined with a Zeiss Axiovert-200 fluorescence microscope, and images were collected and analyzed with Image-5 (Carl Zeiss, Germany).

In some experiments, aliquots of oligonucleotide-treated HeLa cells were exposed to 100 ng/ml nocodazole for a period of 18 h to determine whether elimination of Zwint-1 abrogates the mitotic spindle checkpoint. These cells were then fixed and stained with DAPI for scoring the cell fate profiling under microscopy.

**Antibody Production**—GST-tagged Zwint-1 and ZW10 proteins were immunized into three Balb/c mice, respectively, according to standard protocol. These mice were boosted every 3 weeks until antibody titer reached 1:1500 as judged by Western blotting. The splenocytes from immunized mice were fused with FO myeloma cells (American Type Culture Collection, Manassas, VA) according to standard protocol. Mono-specific antibodies against ZW10 and Zwint-1 were selected based on binding and immunocytochemistry.

**Full-down Assay**—GST fusion protein-bound Sepharose beads were used as an affinity matrix to isolate proteins interacting with ZW10 by using the soluble fraction from mitotic cell lysates as described previously (14). Briefly, the GST-ZW10 (full-length and its deletion mutants) fusion protein-bound Sepharose beads were incubated with 293T cell lysate containing FLAG-tagged Zwint-1 for 4 h at 4 °C. After the incubation, the beads were extensively washed with PBS and boiled in SDS-PAGE sample buffer followed by fractionation of bound proteins on a 6–12% gradient gel. Proteins were then transferred onto a nitrocellulose membrane for Western blotting with a monoclonal GST antibody and FLAG antibody, respectively.

**siRNA Treatment and Assay for Knock-down Efficiency**—The siRNA sequence used for silencing of Zwint-1 corresponds to the coding region 163–183 (relative to the start codon). As a control, either a duplex targeting cyclophilin or scramble sequence was used (14). The 21-mer oligonucleotide RNA duplexes were synthesized by Dharmacon Research, Inc. (Boulder, CO). In the trial experiments, different concentrations of siRNA oligonucleotides were used for different time intervals of treatment as detailed previously, whereas transfection efficiency was judged based on the uptake of a fluorescein isothiocyanate-conjugated oligonucleotides (14). In brief, HeLa cells were synchronized and transfected with 21-mer siRNA oligonucleotides or control scramble oligonucleotides. The efficiency of the RNA-mediated protein suppression was judged by Western blotting analysis.

**Immunofluorescent Staining Analysis**—For immunofluorescence, cells were seeded onto sterile, acid-treated 18-mm coverslips in 6-well plates (Corning Glass). Double thymidine-blocked and released HeLa cells were transfected with 2 μg/ml Lipofectamine 2000 pre-mixed with various siRNA oligonucleotides as described above. In general, 36 h after transfection with siRNA or scrambled (control) oligonucleotides, HeLa cells were rinsed for 1 min with PHEM buffer (100 mM PIPES, 20 mM HEPES, pH 6.8, 5 mM EGTA, 2 mM MgCl2, and 4 mM glycerol) and were permeabilized for 1 min with PHEM plus 0.2% Triton X-100 as described previously (5, 15). Extracted cells were then firstly prepared 2% paraformaldehyde plus 0.05% glutaraldehyde in PHEM for 20 min and rinsed three times in PBS. Cells on the coverslips were blocked with 0.05% Tween 20 in PBS (TBPS) with 1% bovine serum albumin (Sigma). These fixed and permeabilized cells were incubated with various primary antibodies in a humidified chamber for 1 h followed by three washes to remove unbound antibody. FLAG epitope was visualized using Texas Red-conjugated goat anti-mouse IgG, whereas binding of anti-centromere antibody was visualized using Texas Red-conjugated goat anti-human IgG + IgM. DNA was stained with DAPI (Sigma). Slides were examined with a Zeiss Axiostar-200 fluorescence microscope, and images were collected and analyzed with Image-5 (Carl Zeiss, Germany).

**RESULTS**

**ZW10 Binds to Zwint1 via Its N-terminal Region**—Our early studies revealed that human Zwint-1, a 43-kDa ZW10-binding protein, interacts with ZW10 and co-localizes to the kinetochore of mitotic HeLa cells (12). To map the precise Zwint1 binding interface on ZW10, we employed a GAL4 yeast two-hybrid system in which a series of ZW10 deletion mutant cDNAs were co-transformed with Zwint1 cdna into yeast cells using β-galactosidase activity as a measure for protein-protein interaction. As summarized in Fig. 1A, the most N-terminal 80 amino acids are sufficient for Zwint-1 interaction. To validate the genetic interaction between ZW10 and Zwint-1 assessed in yeast and to test whether the N-terminal ZW10 is sufficient to bind Zwint1 in vivo, we carried out a pull-down assay in which FLAG-tagged Zwint-1 fusion protein from 293 cell lysates binds to bacterially recombinant GST fusion protein of ZW10 and its fragments as judged by FLAG antibody Western blotting (Fig. 1B). Western blot with GST antibody validated the level of GST fusion proteins used as affinity matrix. No FLAG-tagged Zwint-1 was absorbed by the GST protein, suggesting that the interaction between Zwint-1 and N-terminal ZW10 is specific and independent of the GST tag. To test whether the N-terminal ZW10 is sufficient to bind Zwint1 in vivo and to confirm the binding interface, we transfected FLAG-Zwint-1 cdna into 293T cells along with various GFP-ZW10 deletion constructs, and we isolated ZW10 proteins with GFP rabbit IgG-conjugated protein A-Sepharose beads. Western blot using monoclonal GST antibody, shown in Fig. 1C, validates that Zwint-1 binds to ZW10 via its N terminals (lanes 5 and 7). Further immunoprecipitation verified that the N-terminal 80 amino acids of ZW10 mediate ZW10-Zwint-1 interaction (data not shown). Thus, we conclude that the N terminus of ZW10 is sufficient for Zwint-1 binding.

**Zwint-1 Binding Domain Is Required for Localization of ZW10 to Kinetochore**—To account for the molecular basis underlying the association of ZW10 with the kinetochore, we sought to identify its kinetochore-targeting domain. Different segments of GFP-ZW10 were co-transfected with FLAG-tagged Zwint-1 into HeLa cells and scored for the kinetochore localization by fluorescence microscopy. Western blot of transfected lysates showed that all the constructs, including full-length...
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Fig. 1. Genetic and biochemical characterization of ZW10-Zwint-1 interaction. A, schematic drawing of ZW10 and its fragments that encode various bait clones used for yeast 2-hybrid assay for Zwint-1 binding activity. B, reconstitution of ZW10-Zwint-1 association using recombinant fusion protein. Full-length (F) and fragments (N and C) of GST-ZW10 recombinant proteins purified on glutathione-agarose beads were used as affinity matrix for absorbing FLAG-tagged Zwint-1 as described under "Materials and Methods." GST protein-bound agarose beads (GST) were used as a control. An aliquot of cell lysates from 293T cells transfected with FLAG-Zwint-1 was loaded into an adjacent well as a positive control. After washing, proteins bound to agarose beads were fractionated on a SDS-polyacrylamide gel followed by transferring onto a nitrocellulose membrane. FLAG antibody reacts with a 34-kDa protein band of Zwint-1 fusion proteins from full-length and N-terminal ZW10 pull-down, but not GST and C-terminal ZW10 pull-downs, indicating that N-terminal ZW10 is sufficient to absorb Zwint-1 protein. Western blot with GST antibody validates the expression level of various recombinant GST fusion proteins. C, co-immunoprecipitation of N-terminal ZW10 and Zwint-1 from transfected 293T cells. 293T cells co-transfected with a FLAG-tagged Zwint-1 and various GFP-tagged ZW10 were extracted and subjected to immunoprecipitations using polyclonal antibody (pAb) to GFP. Control immunoprecipitation was performed using cell lysates from GFP-transfected 293T cells. Immunoprecipitates (IP) were analyzed by SDS-PAGE and immunoblotting (IB) using a FLAG antibody and a monoclonal GFP antibody (mAb), respectively. In the experiment shown in C, Western blotting verifies co-immunoprecipitation of Zwint-1 and full-length and N-terminal ZW10.

GFP-ZW10, expressed proteins of the predicted size (e.g. Fig. 1C). To verify that the GFP tag would not interfere with localization of ZW10, the full-length ZW10 was first tested. GFP-ZW10 was found to localize near the nuclear membrane in prophase cells (Fig. 2A), whereas FLAG-tagged Zwint-1 is located at the pre-kinetochore of newly condensed chromosomes exemplified by typical double-dot staining (c, arrowheads). This non-overlapping distribution profile becomes clear when these two images are merged with DNA staining (Fig. 2A, d, arrowheads).

However, in prometaphase cells, it was readily apparent that GFP-ZW10 is located at the kinetochores (Fig. 2A, e, arrowheads), which is co-localized with that of Zwint-1 staining. Thus, we concluded that Zwint-1 localizes to the kinetochore prior to ZW10, which is consistent with our early studies (12). Their distinct temporal orders of assembly to the kinetochore propelled us to examine the region(s) on ZW10 that mediate the Zwint-1 kinetochore association. Among the several fragments of ZW10 that were examined, all N-terminal fragments were clearly localized to kinetochores (e.g. Fig. 2B, a, arrowheads). In contrast, the C-terminal ZW10, for example amino acids 470–779, was found not to localize to kinetochores. Thus, the kinetochore-binding domain of ZW10 resides within the N-terminal 80 amino acids, which is sufficient for ZW10 localization to the kinetochore.

Zwint-1 Is Necessary and Sufficient for Localization of ZW10 to the Kinetochore—Because immunoprecipitation experiments did not reveal an interaction between N-ZW10 and endogenous ZW10, the localization of ZW10 N-terminal fragments at kinetochores was not because of its interaction with endogenous ZW10 but likely via its association with Zwint-1 given their interaction and the spatio-temporal order of their targeting to the kinetochore. Therefore, we tested whether Zwint-1 might specify the kinetochore localization of ZW10. To this end, we introduced RNA interference (RNAi) oligonucleotides to Zwint-1 by transfection into HeLa cells. To determine the efficient timing for knocking down the Zwint-1 protein level by using the RNAi, we transfected HeLa cells with 100 nM oligonucleotides, and we collected cells at different intervals after the transfection. As shown in Fig. 3A, Western blotting with a Zwint-1 antibody revealed that the RNAi oligonucleotide caused remarkable suppression of the Zwint-1 protein level at 48 h, whereas control cells treated with irrelevant oligonucleotides (e.g. cyclophilin) express normal Zwint-1 levels (not shown). This suppression is relatively specific as it did not alter the levels of other proteins such as CENP-F, ZW10, Dynamitin,
and β-tubulin. Quantitative immunoblotting of Zwint-1 revealed that 100 nM Zwint-1 RNAi oligonucleotide caused a remarkable 7.3-fold suppression of Zwint-1 protein. Because Zwint-1 synthesis in the ~25% of untransfected cells with little or no oligonucleotide was unlikely to be markedly diminished, the observed ~7.3-fold inhibition at 100 nM must represent almost complete inhibition of Zwint-1 in 76 ± 3% of successfully transfected cells. Similarly, siRNA-mediated suppression of ZW10 caused a time-dependent reduction of ZW10 protein levels. Quantitative immunoblotting revealed that 100 nM RNAi oligonucleotide effected a remarkable 7.1-fold suppression of ZW10 protein, which represent almost complete inhibition of ZW10 in 75 ± 3% of successfully transfected cells.

We next examined whether elimination of the Zwint-1 protein altered localization of ZW10 to the kinetochore, so we treated HeLa cells with siRNA oligonucleotide. Forty eight hours after the transfection, cells were collected and stained for ZW10, Zwint-1, and DNA, respectively. As shown in Fig. 3C (a and c), ZW10 and Zwint-1 staining marks characteristic centromere localization, respectively. Most interestingly, ZW10 kinetochore localization was liberated from the kinetochore of the cells with depleted Zwint-1 (Fig. 3C, e and g), suggesting that Zwint-1 is required for targeting of ZW10 to the kinetochore. To test whether elimination of Zwint-1 has a global effect on kinetochore assembly, we then tested for the localization of ACA to the kinetochore. As shown in Fig. 3C, k, ACA labeling displays a typical kinetochore localization in Zwint-1-depleted cells, suggesting that ZW10 localization to kinetochore is likely dependent on Zwint-1.

Because Zwint-1 binds to ZW10, we then tested whether ZW10 is also required for the localization of Zwint-1 to the kinetochore. As shown in Fig. 3C, a, Zwint-1 labeling displays a typical kinetochore localization in ZW10-depleted cells, indicating that Zwint-1 localization to kinetochore is independent of ZW10. Thus, we conclude that Zwint-1 specifies the kinetochore localization of ZW10.

Zwint-1 Is Required for Kinetochore Localization of CENP-F and Dynamitin—Next, we examined the effect of Zwint-1-depletion on the distribution of other kinetochore proteins such as CENP-E and CENP-F. In scramble-transfected control cultures, ZW10, CENP-E, and CENP-F all co-localize to the kinetochores of prometaphase cells (Fig. 4, b–d, arrowheads). In cells in which Zwint-1 had been depleted, the levels of CENP-E present at kinetochores appeared largely unaltered (Fig. 4, g and o). In contrast, the levels of kinetochore-bound ZW10 and CENP-F appeared diminished (Fig. 4, f and h). Quantification of normalized pixel intensities shows that, when Zwint-1 was reduced to less than ~5% of its control value, kinetochore-bound ZW10 and CENP-F proteins were nearly undetectable, whereas kinetochore-bound CENP-E level was virtually unchanged. Elimination of ZW10 did not significantly diminish the localization of CENP-F to the kinetochore (data not shown). Thus, we conclude that localization of CENP-F to kinetochore is dependent on Zwint-1.

We have showed previously that ZW10 links cytoplasmic dynein to the kinetochore by its association with dynamitin (11). To verify if Zwint-1 is required for ZW10-dependent assembly of dynamitin to the kinetochore, we then examined the
effect of Zwint-1 suppression on the kinetochore localization of dynamitin. In scramble oligonucleotide-transfected control cells, Zwint-1, CENP-E, and dynamitin all co-localized to prometaphase kinetochores (Fig. 4, j–l, arrowheads). However, kinetochore localization of dynamitin diminished (Fig. 4 n) as siRNA treatment suppressed the Zwint-1 protein expression (Fig. 4 p). Thus, our studies indicate that Zwint-1 specifies the kinetochore localization of CENP-F and ZW10 that is essential for dynamitin localization to the kinetochore.

Zwint-1 Is Required for ZW10 Kinetochore Localization

To explore whether depletion of Zwint-1 causes any cell cycle-related defects in HeLa cells, we collected control oligonucleotide-treated and siRNA-treated cells, stained with DAPI, ACA, and ZW10. Control anaphase cells treated with scramble oligonucleotide exhibited a total separation of two sets of sister chromatids (Fig. 5 A, b). However, cells treated with Zwint-1 siRNA display a typical chromosome bridge phenotype, in which the majority of sister chromatids is separated with one or more sister chromatids entangled (Fig. 5 A, f, arrow), a phenotype similar to that seen in Drosophila cells lacking either ROD or ZW10 (16, 17). Careful examination of these Zwint-1-suppressed cells also revealed that sister chromatids failed to segregate into two opposite poles (Fig. 5 A, h, arrowheads). The failure in separating equal sister chromatids prior to entry into anaphase resulted in unequal distribution of chromatids, which leads to aneuploidy. We surveyed 150 mitotic cells from three different experiments, in which both sets of sister chromatids were in the same focal plane, from Zwint-1 siRNA oligonucleotide-treated and control oligonucleotide-treated cells. We counted the number of cells displaying typical chromosome bridge phenotype and expressed it as the percentage of the total cell population that is in mitosis. As shown in Fig. 5 B, a summary from three different experiments shows that the depletion of Zwint-1 resulted in significant increases in cells bearing lagging chromosomes (39.7 ± 4.9%; p < 0.01 compared with that of control) and chromosome bridges (43.1 ± 5.4%; p < 0.01 compared with that of control). A survey of ZW10-depleted cells from three different experiments indicated that loss of ZW10 generated a large fraction of aberrant cells with chromosome bridges (41.3 ± 5.7%), which is similar to that seen Zwint-1-depleted cells. These data demonstrate that loss of Zwint-1 is responsible for
Chromatin bridges and lagging chromosomes are frequently seen in cells that lack the mitotic checkpoint because these cells divide in the presence of unaligned chromosomes (e.g. Ref. 14). We therefore assessed whether cells depleted of Zwint-1 could be arrested in mitosis in the presence of microtubule inhibitors such as nocodazole. 16 h after release from the G1/S boundary, HeLa cells transfected with scramble oligonucleotide were arrested in mitosis after nocodazole treatment (Fig. 5C, a/H11032–d/H11032). In contrast, cells depleted of Zwint-1 (Fig. 5C, a/H11032) or ZW10 (Fig. 5C, e/H11032) protein exited mitosis without nuclei dividing and separating, which formed highly aberrant nuclei that were probably polyploid (Fig. 5C, b/H11032 and f/H11032, respectively).

As shown in Fig. 5D, a summary from three different experiments showed that the depletion of Zwint-1 resulted in significant increases in cells bearing aberrant multinuclei (65.5 ± 7.9%; p < 0.01 compared with that of control). A survey of ZW10-depleted cells from three different experiments indicated that loss of ZW10 generated a large fraction of aberrant multinuclei (57.9 ± 5.9%; p < 0.01 compared with that of control), which is similar to that seen in Zwint-1-depleted cells. Conversely, there were dramatic decreases in the number of mitotic cells in Zwint-1-depleted cells, which was a hallmark of checkpoint failure.

We next examined whether the loss of ZW10 and Zwint-1 from kinetochores affected the ability of other checkpoint proteins to bind to kinetochores. Loss of Zwint-1 and ZW10 from kinetochores also did not affect the ability of Bub1 to bind to kinetochores in mitotic cells that were not treated with nocodazole (Fig. 4, r). Kinetochores depleted in Zwint-1 and ZW10 retained Bub1 in mitotic cells that were exposed to nocodazole.
**FIG. 5. Zwint-1 is essential for faithful chromosome segregation.** A, depletion of Zwint-1-impaired spindle checkpoint by promoting a premature anaphase (a–i). HeLa cells were transfected with Zwint-1 siRNA oligonucleotide and control oligonucleotide for 48 h followed by fixation and indirect immunofluorescence staining. This set of optical images was collected from an anaphase HeLa cell triply stained for human ACA (red), DAPI (DNA, blue), ZW10 (ZW10, green), and their merged images. As shown in f, Zwint-1-depleted cell entered in anaphase with a chromatin bridge (f and h, arrow). Most interestingly, ACA staining (g, arrowhead) verifies that this pair of chromatids failed to separate as the cell entered anaphase, leading to occurrence of aneuploidy. Bars, 10 μm. B, depletion of Zwint-1 and ZW10 by siRNA effected mitotic defects in chromosome segregation. HeLa cells were transfected with Zwint-1 siRNA oligonucleotide and control oligonucleotide for 48 h followed by fixation and DNA staining. Cells were then examined under a fluorescence microscopy to sort out the phenotypes. We categorized the phenotypes as normal mitosis (prometaphase/metaphase, anaphase/telophase) and those that carried lagging chromosomes and that prematurely exited from mitosis with unseparated sister chromatids and chromatin bridges. They were quantified and expressed as percentage of total mitotic cells. An average of 150 cells from three separate experiments was counted for HeLa cells treated with Zwint-1 and ZW10 siRNA oligonucleotides, respectively. The scramble oligonucleotides were transfected as control. Error bars represent S.E.; n = 3 preparations. *, p < 0.01 compared with the controls. C, Zwint-1 is required for mitotic spindle checkpoint (a–h). HeLa cells were transfected with siRNA oligonucleotides for Zwint-1 at the time of release from the double thymidine block. The transfected cells were treated with 100 ng/ml nocodazole at 8 h after the transfection. These nocodazole-treated transfected cells were harvested 24 h later followed by fixation and indirect immunofluorescence staining. This montage represents optical images collected from an anaphase HeLa cell triply stained for human ACA (red), DAPI (DNA, blue), ZW10 (ZW10, green), and their merged images. As shown in f, Zwint-1-depleted cells escaped from the mitotic checkpoint activated by nocodazole treatment and formed multinuclei (b and d). ACA staining (g, arrowhead) verifies that pre-kinetochore structure remains unchanged. Another example of cells displayed lagging chromosome in nocodazole-treated Zwint-1 depleted cells (arroew, f). Representative images from a control cell transfected with scrambled oligonucleotide were shown (a–d). Bars, 10 μm. D, elimination of Zwint-1 promotes mitotic exit without nuclei dividing and separation. HeLa cells were transfected with siRNA oligonucleotides for Zwint-1 and ZW10 at the time of release from the double thymidine block. The transfected cells were treated with 100 ng/ml nocodazole at 8 h after the transfection. These nocodazole-treated transfected cells were harvested 24 h later followed by fixation and indirect immunofluorescence staining. Cells were then examined under a microscope to determine their fates. They were quantified and expressed as percentage of transfected cells. An average of 150 cells from three separate experiments were counted for HeLa cells treated with Zwint-1 and ZW10 siRNA oligonucleotides, respectively. The scramble oligonucleotides were transfected as control. Error bars represent S.E.; n = 3 preparations. *, p < 0.01 compared with the controls.
These data demonstrate that loss of Zwint-1 is responsible for mitotic checkpoint signaling.

**DISCUSSION**

We have identified and characterized Zwint-1 as an important ZW10-binding partner that links ZW10 to the kinetochore and mitotic checkpoint. The temporal order of Zwint-1 prior to that of ZW10, together with the fact that elimination Zwint-1 liberates ZW10 from the kinetochore, indicates that Zwint-1 is required for ZW10 association with the kinetochore. We have examined the *in vivo* inter-relationship of the kinetochore proteins Zwint-1, ZW10, and CENP-F, and we found that ZW10 and CENP-F depend on Zwint-1 for stable association with the kinetochore. In addition, ZW10 and Zwint-1 proteins are required for the assembly of the dynein-dynactin complex onto kinetochores. These results are consistent with those reported for *Drosophila* ZW10 and ROD (16) and for human ZW10 (17). In addition, we elucidate the molecular basis of how ZW10 targets to the kinetochore. However, the low protein expression level of these ZW10 deletion mutants observed did not give any obvious mitotic phenotypes.

We demonstrated that Zwint-1 is an important component of the spindle checkpoint. Cells depleted of Zwint-1 failed to block mitosis in the presence of the spindle damage treated by the microtubule inhibitor nocodazole. In addition, Zwint-1 is critical for normal mitotic progression as cells lacking Zwint-1 exited mitosis with entangled chromosomes and unseparated sister chromatids, which led to formation of cells containing multinuclei and the genesis of aneuploidy phenotypes. This is a hallmark of the perturbation of the mitotic checkpoint that forces cells to exit mitosis prematurely, before all the chromosomes are aligned correctly. This phenotype resembles those observed when the functions of spindle checkpoint proteins Mad2, Bub1, BubR1, ZW10, and MPS1 were disrupted (19–21). Our findings are also consistent with loss of ROD and ZW10 function (17, 18), which demonstrates the roles of ROD and ZW10 in mitotic checkpoint surveillance. In fact, ZW10 and
ROD mutant flies were found to degrade cyclin B prematurely and to separate their sister chromatids precociously, which can potentially explain the high incidence of aneuploidy.

Previous studies show that neither ZW10 nor ROD is required by kinetochores to bind MAD1 and BUBR1 or to release these proteins in response to microtubule interactions (18). Recent studies show that BUB1 first localizes to the kinetochore followed by CENP-F and BUBR1, raising the possibility that CENP-F may be essential for association of BUBR1 to the kinetochore (22). In fact, a preliminary measure of pixel intensities revealed that suppression of Zwint-1, but not ZW10, by siRNA reduced kinetochore-bound BUBR1 levels by 61%. The fact that depletion of Zwint-1 attenuates the association of BUBR1 to the kinetochore provides a novel molecular basis to account for BUBR1 association with kinetochore. Given the differential requirement for assembly of CENP-E, CENP-F and BUBR1 to the kinetochore, our studies support the notion in which the kinetochore assembly involves several distinct but interactive pathways.

Cancer cells are often defective in maintaining a mitotic block in response to microtubule disruptors such as nocodazole and taxol, which leads to mis-segregation of chromosomes and biogenesis of aneuploidy (23). It has been demonstrated that haplo-insufficiency (loss of an allele) of the BUBR1 protein resulted in rapidly developing lung as well as intestinal adenocarcinomas in response to challenge with a carcinogen (24). In fact, we have shown recently (25) that suppression of BUB1 mitotic checkpoint signaling by either reduction of BUB1 protein level or inhibiting BUB1 kinase activity in human cancer cells is invariably lethal as the consequence of massive chromosome loss. Insufficiency to assemble BUB1 onto kinetochores in Zwint-1-depleted cells and the subsequent occurrence of aneuploidy in the presence of nocodazole mirrored the phenotype seen in the BUB1 haplo-insufficiency in genetically manipulated mouse cells (24). Thus, aberrant function of Zwint-1 pathway could lead to aberrant assembly of BUB1 to kinetochores, which provides another mechanism to compromise the mitotic spindle checkpoint in addition to BUB1 mutations found in colorectal cancer patients (26, 27). It would be of great interest to explore whether any cancer cells exhibit disrupted Zwint-1 activity. Our experimentation of manipulation of Zwint-1 expression and activity shown here may provide a useful system to dissect signaling cascade underlying Zwint-1-ZW10-mediated mitotic signaling cascade, which will aid in elucidating the pathogenesis of chromosome instability and identification of the other components that are involved in mitotic checkpoint signaling.

Taken together, our findings demonstrate a critical role of Zwint-1 in kinetochore assembly dynamics. The fact that elimination of Zwint-1 disrupts assembly of ZW10, CENP-F, and dynamitin to kinetochores, abrogates nocodazole-induced mitotic arrest, and induces chromosome cross-bridges in HeLa cells prematurely exited from anaphase demonstrates the importance of Zwint-1 in faithful chromosome segregation.

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**REFERENCES**


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