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Terf/TRIM17 stimulates degradation of kinetochore protein ZWINT and regulates cell proliferation

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Terf/TRIM17 is a tripartite motif protein that has been originally isolated from testis. Terf has been characterized to exhibit an E3 ubiquitin ligase activity and to undergo self-ubiquitination. The cellular function of terf and its substrates, however, remain elusive. In the present study, we performed a yeast two-hybrid screening assay using terf as bait and identified a positive clone coding for ZW10 interacting protein (ZWINT), a known component of the kinetochore complex required for the mitotic spindle checkpoint. Immunoprecipitation and western blot analyses showed that terf interacted with ZWINT and that overexpression of terf caused down-regulation of protein levels of ZWINT in mammalian cells. In addition, the coiled-coil domain of terf was required for the interaction with ZWINT. In a cell growth assay, stable transfection with terf decreased proliferation of MCF7 breast cancer cells. In contrast, the growth rate of MCF7 cells was increased by stable expression of ZWINT. Specific siRNAs targeting terf and ZWINT dampened these negative and positive effects of terf and ZWINT on cell proliferation, respectively. These results suggest that the E3 ubiquitin ligase terf causes protein degradation of ZWINT and negatively regulates cell proliferation.

Keywords: Terf/ZWINT/kinetochore/cell proliferation/protein degradation.

Abbreviations: CENP-H, centromere protein H; CGN, cerebellar granule neuron; RING, Really Interesting New Gene; siRNA, short interfering RNA; SNAP, soluble NSF attachment protein; SPRY, SPla and the RYanodine Receptor; terf, testis RING finger protein; TRIM, tripartite motif; ZW10, zeste-white 10; ZWINT, ZW10 interacting protein.

The tripartite motif (TRIM) family proteins, characterized by the conserved RING finger, B-box and coiled-coil domains, are involved in various cellular processes such as cell proliferation, oncogenesis and antiviral defense (*1–3*). Many lines of evidence point to the involvement of RING finger proteins in the ubiquitin-mediated protein modification (ubiquitination), accomplished by a complex process involving the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin ligase (E3). In this system, the TRIM proteins function as E3 ubiquitin ligases that bind both to an E2 conjugase through the RING finger domain and to the substrates via a portion associated with the RING finger domain. Ubiquitination alters the biophysical properties of the modified protein, the most common of which is the ubiquitin-mediated protein degradation through the 26S proteasome, where the target protein to be destroyed is tagged with ubiquitin.

Testis RING finger protein (terf)/TRIM17, a member of the TRIM family, has been identified using the polymerase chain reaction with degenerate primers for the RING finger domain (*4*). Recently, we found that terf undergoes ubiquitination *in vitro* in the presence of the E2 enzyme, UbcH6 (*5*). In addition, terf is conjugated with polyubiquitin chains and stabilized by the proteasome inhibitor MG132 in mammalian cells. Thus, terf functions as an E3 ubiquitin ligase and renders itself susceptible to proteasomal degradation through polyubiquitination. We also found that terf interacts with TRIM44, another member of the TRIM family. Since TRIM44 inhibits the ubiquitination of terf and stabilizes this protein, a new mechanism is proposed on the basis of regulation of E3 ubiquitin ligase activity for TRIM proteins. In addition, Lassot *et al.* (*6*) reported that the expression of mouse terf is induced in culture cells derived from cerebellar granule neurons (CGNs) under the apoptotic condition of serum-free medium containing 5 mM KCl. They also showed that the exogenous expression of terf was sufficient to induce CGN apoptosis even under survival conditions. In contrast, the RING finger mutants did not trigger significant apoptosis. This implies that the pro-apoptotic effect of terf depends on the E3 activity conferred by its RING domain. However, the biological functions, substrates and mechanism of action of terf E3 ubiquitin ligase remain elusive.

In the present study, using a yeast two-hybrid screening assay, we identified a kinetochore ZW10 interacting protein (ZWINT) (*7*) as a binding protein for terf. The results showed that the coiled-coil domain of terf is required for interaction with ZWINT and that

terf stimulates the degradation of ZWINT via the proteasomal pathway in mammalian cells. Moreover, stable expression of terf inhibits cell growth of breast cancer cells; in contrast, stable expression of ZWINT stimulates cell growth. We present a novel interactor and cellular function of the E3 ubiquitin ligase, terf.

Materials and Methods

Yeast two-hybrid screen

The bait plasmid pGBKT7-terf was constructed by subcloning full-length terf, without the translation initiation codon, into the pGBKT7 vector (Clontech) in frame with the GAL4 DNA-binding domain. The AH109 yeast strain cells transforming the pGBKT7-terf bait were mated with the MATCHMAKER library Y187 cells that had been transformed with human testis cDNA library. After mating, diploids were plated on leucine–tryptophan–histidine–adenine-lacking SD medium (-LTHA-SD medium) plates. Colonies growing on the -LTHA-SD medium plates were picked and spread on -LTHA-SD medium plates containing X-α-Gal. Blue colonies were cultured for plasmid isolation. Isolated plasmids were subcloned into competent *Escherichia coli* cells (DH5α). Transformants were spread out on ampicillin-containing plates for the selection of clones bearing the pACT2 library vector. Inserts of the pACT2 vector were characterized by sequence analysis.

Mammalian expression plasmids

Flag- or RH-tagged human terf (Flag-terf or RH-terf) and ZWINT (Flag-ZWINT or RH-ZWINT) were amplified with specific primers by PCR using testis cDNA (Clontech), and cloned into the mammalian expression plasmid pcDNA3 (Invitrogen) (*5*). RH-tagged mutants of terf were constructed by subcloning the regions corresponding to amino acids 2–67 containing the RING (Really Interesting New Gene) domain (RH-terf R), 97–135 containing the B-box (RH-terf B), 143–177 containing the coiled-coil region (RH-terf CC), 348–477 containing the C-terminal region (RH-terf C) and 97–477 lacking the RING domain (RH-terf ΔR). RH-tagged ZWINT mutants were constructed by subcloning the regions corresponding to amino acids 2–114 containing the N-terminal region (RH-ZWINT N), 114–217 containing the two coiled-coil regions (RH-ZWINT CC), 217–277 containing the C-terminal region (RH-ZWINT C) and a fusion of 2–114 and 217–277 lacking the coiled-coil regions (RH-ZWINT ΔCC).

Cell culture and establishment of stable transformants expressing terf and ZWINT

Human embryonic kidney 293T cells and human breast cancer MCF7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 and a humidified atmosphere. For stable transfection, MCF7 cells were transfected with Flag-terf or Flag-ZWINT using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol and selected in DMEM containing 0.5 mg/ml G418. The stable transformants of MCF7 cells expressing Flag-terf (MCF7-terf #1 and #2), Flag-ZWINT (MCF7-ZWINT #1 and #2), and empty vector (MCF7-vec #1 and #2) were cloned.

Immunoprecipitation and immunoblotting

293 T cells plated in 100-mm culture dishes were transfected with 10 μg of expression vectors with a combination of terf- and ZWINT-expression plasmids for 36 h and further incubated with 10 μM MG132 or vehicle for 12 h. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM EDTA, 0.1% NP-40, 10 mM NaF and 10 μM MG132). The samples were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to polyvinylidene difluoride Immobilon transfer membranes (Millipore) and probed with rabbit antisera against human terf (*5*) or mouse monoclonal antibodies against Flag (SIGMA), RGS-His (RH; QIAGEN), and β-actin (SIGMA). Signals on the blots were detected with an ECL chemiluminescence detection kit (Santa Cruz). For immunoprecipitation, cell lysates were incubated with the indicated antibody for 1 h at 4°C. Then, protein G-sepharose beads (GE) or TALON beads (TAKARA) were added to collect the immunocomplexes for an additional 1 h. The pellets were washed three times with lysis buffer and subjected to western blot analysis.

Cell proliferation assay

MCF7-terf #1 and #2 cells and MCF7-ZWINT #1 and #2 cells were seeded in 96-well plates at a density of 5000 cells/well and transfected with 50 nM short interfering RNA (siRNA) targeting terf and ZWINT or control siRNA using Lipofectamine 2000 (Invitrogen). The siRNA target sequences were as follows: terf, 5′-GCGGAGAG AACGCATTGTGCTGG-3′ and ZWINT, 5′-CAGAGAATCTTCC AGATGATAAA-3′. A non-targeting control siRNA with no homology to the known gene targets in mammalian cells was used (*8*). Cell proliferation was assessed in terms of the cell viability by using a kit containing WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) according to the manufacturer's protocol (Nacalai Tesque, Kyoto, Japan). The reaction products were measured at an OD of 450 nm. The experiment was repeated in triplicates and the results were indicated as mean ± SD.

Statistical analyses

For comparison of cell proliferation between stable cell transformants of terf, ZWINT and control vector, the differences between the groups were first analysed by one-way ANOVA, and subsequent Dunnett's *post hoc* test for multiple comparisons was performed to determine the significance of differences between the groups. In siRNA experiments, results between the siRNA- and control siRNA-treated cells were compared by two-tailed Student's *t*-test. $P < 0.05$ were considered significant.

Results

Identification of ZWINT as an interacting protein for terf

We performed a yeast two-hybrid screening to identify proteins interacting with terf. For this screening, we used a construct encoding rat terf as bait (Fig. 1A; upper panel). During the screening of yeast clones that had been transformed with the human testis cDNA library, we identified a single clone coding for the human ZWINT (Fig. 1A; lower panel).

Interaction between terf and ZWINT

Next, we examined the protein–protein interaction between terf and ZWINT in mammalian cells. 293 T cells were transiently transfected with RH-terf and Flag-ZWINT expression plasmids for 48 h. Immunoprecipitation using anti-Flag antibody demonstrated that terf and ZWINT formed a complex in the cells (Fig. 1B).

Terf induces degradation of ZWINT

Since terf has been shown as an E3 ubiquitin ligase (*5*), we examined whether terf stimulates degradation of ZWINT protein through the proteasome, a major system of protein degradation. 293T cells were transfected with Flag-ZWINT together with an increasing amount of RH-terf in the absence or presence of the proteasome inhibitor MG132. As shown in Fig. 2, protein levels of ZWINT were dose-dependently decreased by the increasing amount of terf protein expression. However, MG132 treatment impaired the down-regulation of ZWINT protein by terf. These results indicate that terf decreased the expression level of ZWINT protein in a proteasome-dependent manner. Next, we characterized the protein–protein

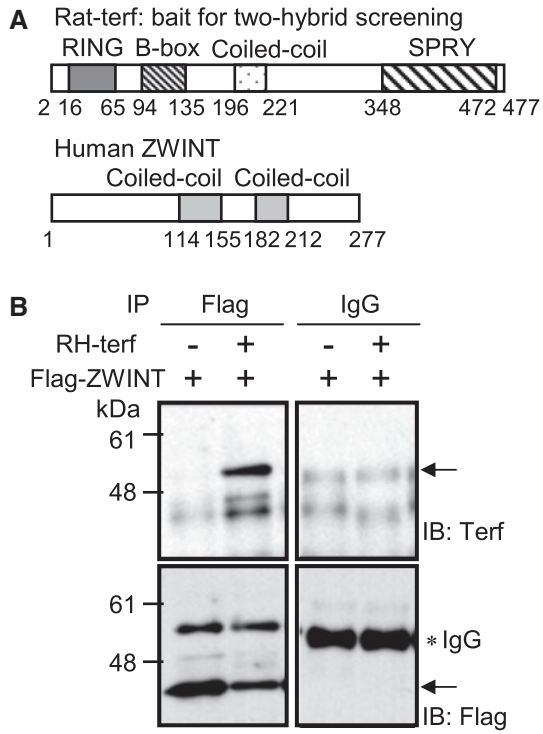

Fig. 1 Detection of the interaction between terf and ZWINT. (A) Schematic representation of rat terf used as bait for the yeast two-hybrid screening (upper panel), and full-length human ZWINT (lower panel). (B) Interaction between terf and ZWINT in mammalian cells. Lysate of 293T cells transfected with expression plasmids coding for RH-tagged terf and Flag-tagged ZWINT were immunoprecipitated with anti-Flag antibody or control IgG and then analysed by immunoblot using anti-terf or Flag antibody. Arrows show the positive signals for RH-terf and Flag-ZWINT proteins. Asterisk indicates non-specific signal for IgG.

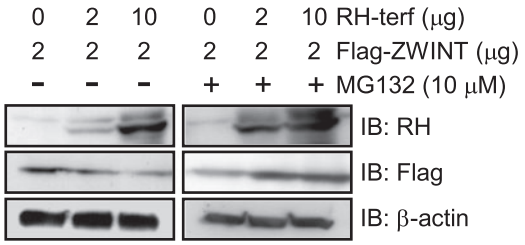

Fig. 2 Terf stimulates proteasomal degradation of ZWINT. 293T cells were transfected with 2 μg of Flag-ZWINT together with increasing amount of RH-terf for 48 h. After transfection, cells were treated with 10 μM MG132 or vehicle for 12 h. Cell lysates were subjected to immunoblot analysis using anti-RH, Flag and β-actin antibodies.

interaction between terf and ZWINT in mammalian cells. To identify the binding domain of terf, we constructed a series of deletion mutants of terf (Fig. 3A; left panel) and analysed their binding abilities to full-length ZWINT in 293T cells in the presence of MG132 by Talon bead precipitation (Fig. 3A; right panel). As shown in the figure, the almost same protein levels of Flag-ZWINT were verified among the lanes (input). RH-terf R consisting of the N-terminal RING domain, RH-terf B consisting of the B-box domain and RH-terf C consisting of the C-terminal region could not bind to ZWINT. However, full-length terf (RH-terf), RH-terf ΔR lacking the N-terminal RING domain and RH-terf CC consisting of the coiled-coil domain of terf could bind to ZWINT. These results indicate that the coiled-coil domain of terf is sufficient to sustain this protein–protein interaction. In order to identify the binding domains of ZWINT with terf, we constructed a series of deletion mutants of ZWINT (Fig. 3B; left panel) and analysed their binding abilities to full-length terf in the presence of MG132 by Talon bead precipitation (Fig. 3B; right panel). As shown in the figure, the almost same protein levels of Flag-terf were verified among the lanes (input). RH-ZWINT containing full-length ZWINT could bind to terf, whereas the deletion mutants RH-ZWINT N, RH-ZWINT CC, RH-ZWINT C and RH-ZWINT ΔCC could not. These results indicate that the whole region of ZWINT is necessary for interacting with terf.

Terf decreased cell proliferation in MCF7 cells

To analyse the cellular function of terf, we established stable transfectants of MCF7 cells with Flag-terf (MCF7-terf #1, #2), Flag-ZWINT (MCF7-ZWINT #1, #2) or empty vector (MCF7-vec #1, #2). Immunoblot analysis showed the stable expression of Flag-terf or Flag-ZWINT in these transfectants (Fig. 4A). Then, we evaluated cell proliferation of the stably transfected MCF7 cells (Fig. 4B). MCF7-terf #1 and #2 cells showed a reduction in cell proliferation compared to MCF7-vec #1 and #2. In contrast, MCF7-ZWINT #1 and #2 cells showed an acceleration of cell proliferation.

Next, we examined the effect of siRNAs targeting terf and ZWINT on cell proliferation of MCF7-terf and MCF7-ZWINT cells, respectively. The terf- and ZWINT-targeting siRNAs were confirmed to efficiently suppress the expression of exogenously introduced terf and ZWINT, respectively, in the stable transfectants of MCF7 cells (Fig. 5A). MCF7-terf #1 and #2 cells treated with terf siRNA increased cell proliferation when compared with those of control siRNA (Fig. 5B). Knockdown of ZWINT with siRNA decreased cell proliferation in MCF7-ZWINT #1 and #2 cells (Fig. 5B). These findings indicated that terf and ZWINT regulate cell proliferation in MCF7 cells.

Discussion

To explore substrates of the E3 ubiquitin ligase, terf, we screened a testis cDNA library by the yeast two-hybrid assay using terf as bait and identified a positive clone coding for ZWINT. We demonstrated that terf interacts with ZWINT in mammalian cells and stimulates degradation of ZWINT through the proteasomal pathway. MCF7 cells stably expressing terf showed decreased cell proliferation whereas those expressing ZWINT showed increased cell proliferation. These results suggest that the E3 ubiquitin ligase, terf, causes protein degradation of ZWINT and inhibits cell proliferation.

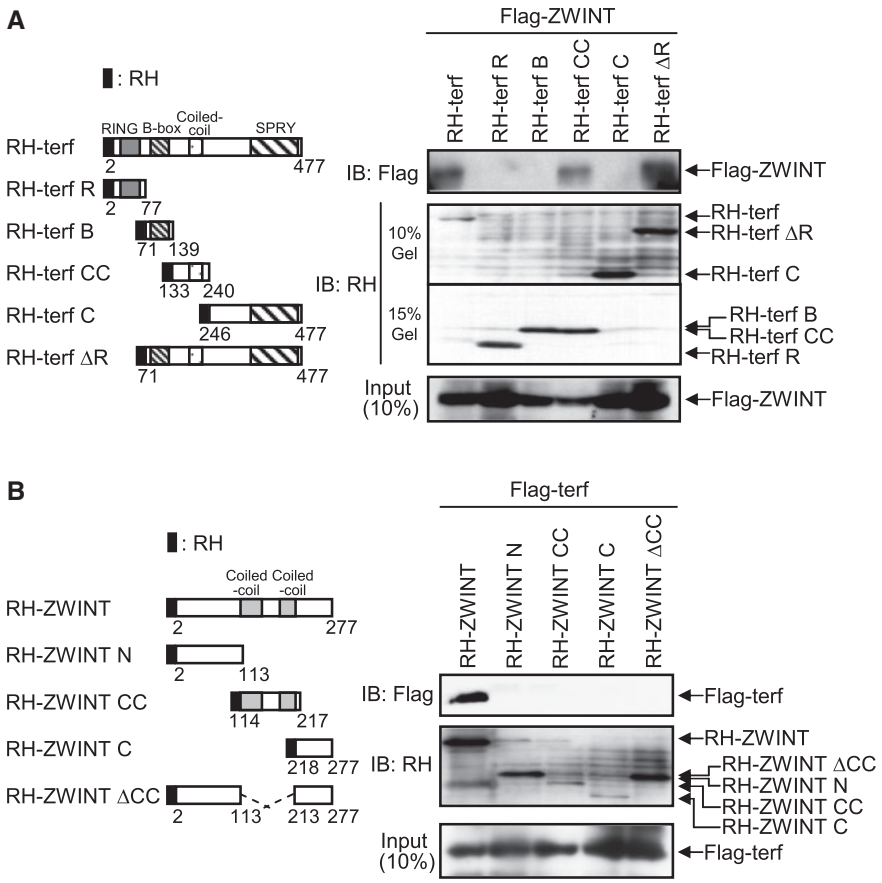

Fig. 3 Characterization of specific interaction between terf and ZWINT proteins. (A) Schematic diagram of RH-tagged full-length terf and its truncated mutants (left panel). 293T cells were transfected with Flag-ZWINT and the indicated expression vectors encoding terf and its truncated mutants. The RH-tagged proteins were precipitated from total cell lysates with cobalt-coated beads (TALON). The precipitants were analysed by western blot using anti-Flag or anti-RH antibody (right panels). For fine separation of terf-deletion mutants, 10% and 15% of polyacrylamide gels were used in SDS–PAGE. Right bottom: Immunoblotting of Flag-ZWINT in input proteins. (B) Schematic diagram of RH-tagged full-length ZWINT and its truncated mutants (left panel). 293T cells were transfected with Flag-terf and the indicated expression vectors encoding ZWINT and its truncated mutants. Cell lysates were subjected to TALON-bead precipitation, and the precipitants were analysed by western blot using anti-Flag or anti-RH antibody (right panels). For fine separation of ZWINT deletion mutants, 15% of polyacrylamide gel was used in SDS–PAGE. Right bottom: Immunoblotting of Flag-terf in input proteins.

ZWINT has been identified as a protein interacting with ZW10 that is required for chromosome motility and spindle checkpoint control. ZWINT co-localizes with ZW10 at the kinetochore, which specifies the attachments between the chromosomes and microtubules of the spindle, and is essential for accurate chromosome segregation (*9*, *10*). In prophase, ZWINT locates to the kinetochore before ZW10, and ZWINT remains at the kinetochore until late in anaphase. siRNA specific for ZWINT abolishes the localization of ZW10 to the kinetochore, indicating that ZWINT is required for ZW10 kinetochore localization (*11*). The ZWINT siRNA also causes a chromosome bridge phenotype with inter-connected sister chromatids, abrogates the microtubule inhibitor nocodazole-induced mitotic arrest and eventually triggers cell death (*12*). Collectively, these results and our data suggest that terf regulates cell growth through the proteasomal degradation of ZWINT, which is considered to play a critical role in the mitotic checkpoint.

Mitotic checkpoint proteins play a crucial role in cell-cycle regulation. For instance, the reduction in mitotic checkpoint proteins such as ZW10, BubR1 and Mad2 in human cancer cells provokes apoptotic cell death (*12*, *13*). Stable expression of the centromere protein (CENP)-A increases cell proliferation and stimulates tumorigenicity in nude mice (*14*). Recently, it has been reported that TRIM36, a member of the TRIM family, interacts with CENP-H, which has been identified as a constitutive component of the kinetochore (*15*, *16*). Moreover, TRIM36 decelerates the cell cycle and attenuates cell growth, suggesting that TRIM36 is potentially associated with chromosome segregation and instability (*16*).

In the present study, we found that terf binds with ZWINT and stimulates the degradation of ZWINT in a proteasome-dependent manner. Based on our findings, we consider a couple of possible mechanisms for the terf-mediated ZWINT degradation: (i) terf will directly ubiquitinate ZWINT and promote the degradation of ZWINT in a proteasome-dependent manner; (ii) the interaction of terf with ZWINT will cause the dissociation of ZWINT from a undefined protein that functions as a stabilizer for ZWINT; or (iii) the interaction of terf with ZWINT will accelerate

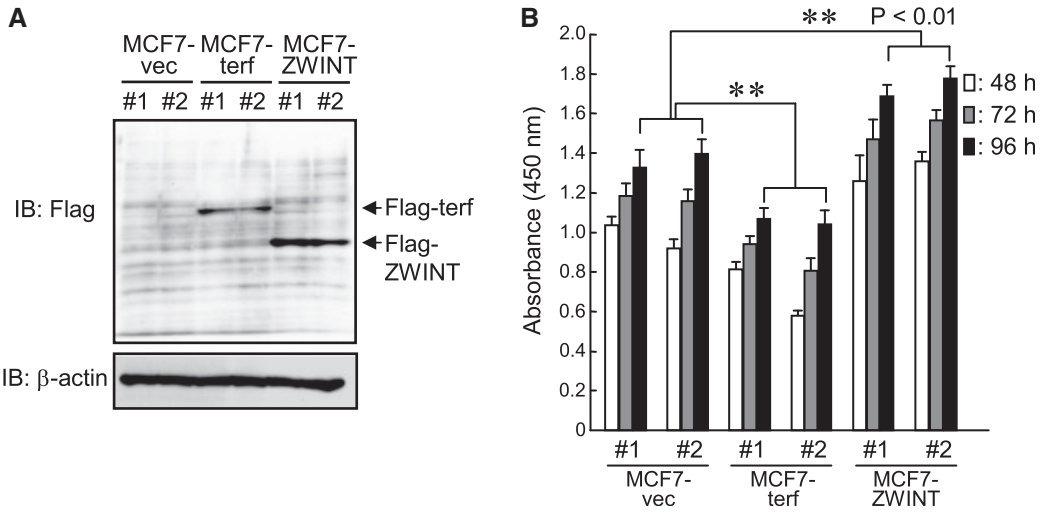

Fig. 4 Terf and ZWINT regulate growth of MCF7 cells. (A) MCF7 cells stably expressing Flag-terf (MCF7-terf #1 and #2), Flag-ZWINT (MCF7-ZWINT #1, #2) and empty vector (MCF7-vec #1, #2) were established. Expression levels of Flag-terf and Flag-ZWINT were examined by western blot analysis of cell lysates. (B) MCF7-terf #1 and #2, MCF7-ZWINT #1 and #2, and MCF7-vec #1 and #2 cells were seeded in 96-well plates at a density of 5000 cells/well. Proliferation of those cells was examined using a tetrazolium salt (WST-8) assay where the formation of formazan was determined photometrically at 450 nm. The results are shown as mean ± SD from triplicate experiments. $^{**}P < 0.01$ (by one-way ANOVA and Dunnett's *post hoc* test).

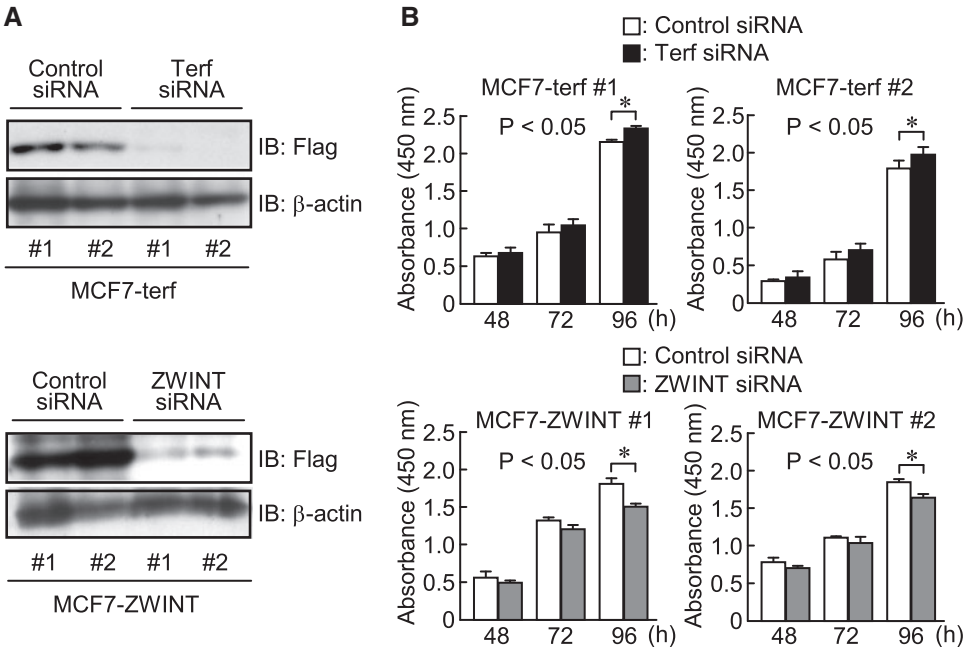

Fig. 5 Specific siRNAs targeting terf and ZWINT restore the effects of terf and ZWINT, respectively, on cell proliferation. (A) MCF7 cells stably expressing Flag-terf (MCF7-terf #1 and #2) or Flag-ZWINT (MCF7-ZWINT #1 and #2) were transfected with 50 μM siRNA targeting terf or ZWINT, respectively, for 72 h. For negative control, control siRNA was used in the transfection. After transfection, cell lysates were prepared and subjected to western blot analysis using anti-terf and anti-β-actin antibodies. (B) Proliferation of these cells was evaluated using the WST-8 assay up to 96 h. The results were shown as mean ± SD from triplicate experiments. $^{*}P < 0.05$ (by Student's *t*-test).

the complex formation with other factor(s), which leads to the proteasomal degradation of the whole complex. As far as we performed ubiquitination experiments for several times with various different conditions, we consider that terf does not directly ubiquitinate and degrade ZWINT. Although the terf-dependent ZWINT ubiquitination is the most plausible explanation for the ZWINT degradation, we assume that other mechanisms will rather contribute to the phenomenon. Similar to our findings, it has been also shown that a kinetochore CENP-H is an interactor of TRIM36, although CENP-H itself is not a direct ubiquitinated substrate for TRIM36 (*16*). Thus, we assume that TRIM proteins potentially interact with kinetochore proteins regardless of their E3 ligase activities and play a role in chromosome segregation and cell-cycle regulation.

Lassot *et al.* (*6*) reported that terf induces apoptosis in primary culture cells derived from CGNs depending on the E3 ubiquitin ligase activity conferred by

its RING domain. It may be possible that terf down-regulates the protein level of ZWINT and causes apoptosis in the brain. In addition, ZWINT has been shown to interact with Rab3c and SNAP25 proteins (*17*). Rab3c, one of the Rab3 proteins, is a small GTP-binding protein of the Ras superfamily. The Rab3c isoform is mainly expressed in the brain and is associated with synaptic vesicles and secretory granules in brain and neuroendocrine cells (*18*). Synaptosomal-associated protein of 25 kDa (SNAP25) is a SNARE (an acronym derived from SNAP receptor) protein that is highly expressed in the brain, where it performs essential functions in pre-synaptic neurotransmitter release. Since Rab3 and SNAP25 play essential roles in neuronal exocytosis pathways, ZWINT is suggested to be involved in pre-synaptic events in addition to its function as a kinetochore protein. In neuronal cells, terf may play another role in pre-synaptic events by regulating the turnover of ZWINT protein; however, these roles will be elucidated by future studies.

Previously, we demonstrated that abundant expression of terf is found in cells from the testis (*4*). The testis contains actively dividing cells to produce sperm, and spermatogonia show mitotic proliferation to undergo self-renewal division. Moreover, spermatogonia also divide and differentiate into spermatocytes, which undergo meiosis to originate spermatids. Kinetochore proteins play an essential role in centromere organization during meiotic cell division as well as mitosis (*19*). Studies on terf and ZWINT in testis cells will resolve their physiological function *in vivo*.

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Conflict of interest

None declared.

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