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Research Article

TTDN1 is a Plk1-interacting protein involved in maintenance of cell cycle integrity

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Abstract. Polo-like kinase 1 (Plk1) is a highly conserved serine/threonine kinase that plays critical roles in many cell cycle events, especially in mitosis. In the present study, we identified TTDN1 as a potential interacting partner of Plk1 in yeast two-hybrid screens. Sequence analysis indicates that TTDN1 contains a consensus Plk1-binding motif at its C terminus. TTDN1 colocalizes with Plk1 at the centrosome in mitosis and the midbody during cytokinesis. TTDN1 is phosphorylated by Cdk1 in mitosis, and this is required for its interaction with Plk1. Site-directed mutagenesis indicates that TTDN1 is phosphorylated at multiple residues, including Ser93 and Ser104. Mutation of Thr120 of TTDN1 abolishes its interaction with Plk1, suggesting phosphorylation of Thr120 in the consensus Plk1-binding motif is required for its interaction with Plk1. Overexpression of TTDN1 or its knockdown by siRNA causes multi-polar spindles and multiple nuclei, suggesting that TTDN1 plays a role in regulating mitosis and cytokinesis.

Keywords. Plk1, Cdk1, TTDN1, cell cycle, phosphorylation.

Introduction

Phosphorylation by mitotic kinases, including Cdk1 and the Polo-like kinases (Plks), is instrumental in driving critical mitotic transitions. Plk1 is an evolutionarily highly conserved Ser/Thr kinase that plays critical roles in many cell cycle events, such as centrosome maturation, G_2/M transition, Cdk1 activation, anaphase-promoting complex activation, and cytokinesis [1–4]. Plk1 is overexpressed in a range of human tumors, including prostate tumors, ovarian carcinomas, heptoblastomas and elanomas, and Plk1 overexpression was shown to coincide with bad prognosis [5–8]. Therefore, Plk1 is useful as a prognostic marker for outcome of disease. In addition, inhibition of Plk1 activity by various approaches causes growth arrest and apoptosis of transformed cells, suggesting that Plk1 is a potential target for drug development against cancers [9].

Plk1 contains an N-terminal Ser/Thr kinase domain and a noncatalytic C-terminal region composed of two homologous ~70–80 residue segments termed Polo box domain (PBD). The PBD of Plk1 is responsible for binding to its substrates phosphorylated by "priming" kinase and essential in targeting its catalytic activity to specific subcellular structures critical for mitosis. For example, phosphorylation of cyclin B1, Cdc25 and Wee1 by Plk1 contributes to the activation of cyclin-dependent kinase 1 (Cdk1), which in turn promotes the entry into mitosis [10–12]. Plk1 regulates centrosome maturation by phosphorylating the

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centrosomal protein Nlp, and blocks its targeting to centrosomes [13]. In prophase, Plk1 is required for the removal of cohesion from chromosome arms by phosphorylating the SA2 subunit of cohesion [14, 15]. Plk1 also phosphorylates Emi1, an inhibitor of the anaphase-promoting complex or cyclosome, and mediates the degradation of Emi1 in early mitosis [16, 17]. During late mitosis, Plk1 interacts with and phosphorylates the central spindle proteins MKLP1/2, Nir2, Cep55 and NudC, which is required for the completion of cytokinesis [18–24].

In this report, we identified TTDN1 as a Plk1-interacting protein. TTDN1 has been reported as the first disease gene for non-photosensitive trichothiodystrophy (TTD) [25], but little is known for its physiological functions. Our findings suggest that TTDN1 is phosphorylated by Cdk1 and associated with Plk1 in mitosis, which plays a role in maintenance of cell cycle integrity.

Materials and methods

Reagents. Mouse monoclonal antibodies against FLAG (Sigma) and HA (Covance, Berkeley, CA) epitopes, rabbit polyclonal antibody against Plk1 (Zymed), human embryonic kidney 293 and HeLa cells (ATCC, Manassas, VA), the human 293 cDNA library (Clontech) were purchased from the indicated manufacturers.

Constructs. Mammalian expression plasmids for FLAG-tagged Plk1 and TTDN1 were constructed by PCR amplification of the corresponding cDNA fragments and subsequent cloning into a cytomegalovirus (CMV) promoter-based vector containing an N-terminal HA or FLAG tag. The plasmid of pTTDN1-EGFP was generated by inserting TTDN1 cDNA into multiple cloning sites of pEGFP-N1 (BD Biosciences Clontech). The TTDN1 point mutant plasmids were generated by site-directed mutagenesis and the mutations were verified by DNA sequencing.

Yeast two-hybrid screening. The cDNA encoding the PBD of Plk1 was inserted in-frame into the pGBT9 plasmid (Clontech). The human 293 cDNA library was screened and the isolation of positive clones was performed by following the manufacturer's protocols.

Cell synchronization. HeLa cells were synchronized by double-thymidine block and collected at the G_1/S border (0 h), S phase (4 h), G_2 phase (8 h), and mitosis (12 h). For mitotic synchronization of cells, cells were treated with 0.5 μg/ml nocodazole for 16 h. Mitotic arrested cells were collected by the "mitotic shake-off" method.

Antibody generation. Bacterially produced His-TTDN1 was used to raise polyclonal antibodies in mouse (Experimental animal center of Institute of Genetics and Developmental Biology, CAS, Beijing, China).

Immunofluorescence microscopy. Cells were fixed with ice-cold methanol for 10 min at –20°C, rehyrated three times with PBS, and blocked in 5% bovine serum albumin/PBS for 10 min. The cells were stained with primary antibody in blocking buffer for 1 h at 37°C, rinsed with PBS and stained again with FITC-conjugated Affinipure rabbit anti-mouse IgG or Texas Red-conjugated Affinipure goat anti-rabbit IgG (1:200 dilutions) for 1 h at 37°C. The cells were then rinsed with PBS containing DAPI and mounted in Prolong Antifade (Molecular Probes). The cells were observed with an OLYMPUS BX51 immunofluorescent microscope using a ×100 plan objective.

Co-immunoprecipitation and Western blot analysis. HeLa cells in interphase or synchronized in M phase were lysed in 1 ml lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The lysate was incubated with 0.5 μl of the indicated antibody and 25 μl of 1:1 slurry of Gamma Bind G Plus Sepharose (Amersham Biosciences) for at least 2 h. The Sepharose beads were washed three times with 1 ml lysis buffer containing 500 mM NaCl. The precipitates were fractionated on SDS-PAGE and Western blot analysis was performed.

RNAi experiments. Double-strand oligonucleotide corresponding to the target sequence was cloned into the pSuper.Retro RNAi plasmid (Oligoengine Inc.). The following sequence was targeted for human TTDN1 cDNA: CAGTATCTGTAGTGGATA.

Results

Identification of TTDN1 as a Plk1-interacting protein in yeast two-hybrid screens. Previous studies indicate that Plk1 regulates multiple stages of the cell cycle through interacting with different docking proteins and substrates. Structural analysis suggests that these interactions are mediated by the C-terminal PBD of Plk1 and a consensus motif in Plk1 targets [26–32]. To identify additional Plk1-interacting proteins, we used the yeast two-hybrid system to screen a human embryonic kidney 293 cell cDNA library with the PBD of Plk1 (amino acid 306 C terminal) as the bait. We screened a total of ~2×10^6 independent clones and obtained 31 β-gal-positive clones. Among the β-gal-positive clones, four encoded an uncharacterized protein designated as C7orf11 (chromosome 7 open reading frame 11) in the GenBank databases. A recent study indicated that mutation of C7orf11 was associated with non-photosensitive TTD and C7orf11 was renamed as TTDN1 [25]. However, the physiological functions of TTDN1 are unknown.

Human TTDN1 is a 179-amino acid protein (Fig. 1a). Blast searches of the GenBank databases indicate that TTDN1 is highly conserved in vertebrates, but not in lower eukaryotes (such as *Caenorhabditis elegans* and yeast) (data not shown). Structural analysis indicates that TTDN1 contains a consensus Plk1-binding motif between amino acids 116–121 (Fig. 1a), suggesting that TTDN1 is a candidate Plk1-interacting protein. Previous studies indicate that TTDN1 mRNA is ubiquitously expressed in examined tissues, including heart, placenta, liver, skeletal muscle, kidney and pancreas [33]. To determine whether TTDN1 is expressed in mammalian cells at the protein level, we raised a mouse polyclonal antibody against recombinant human full-length TTDN1. Western blot analysis with this antibody indicated that TTDN1 was expressed as a ~19-kDa protein in all examined cell lines, including HeLa, 293, HCT8 and U937 (Fig. 1b). This 19-kDa protein was slightly smaller than overexpressed HA-tagged TTDN1 and was not detected by the antibody pre-absorbed with the recombinant TTDN1, suggesting that the TTDN1 antibody we

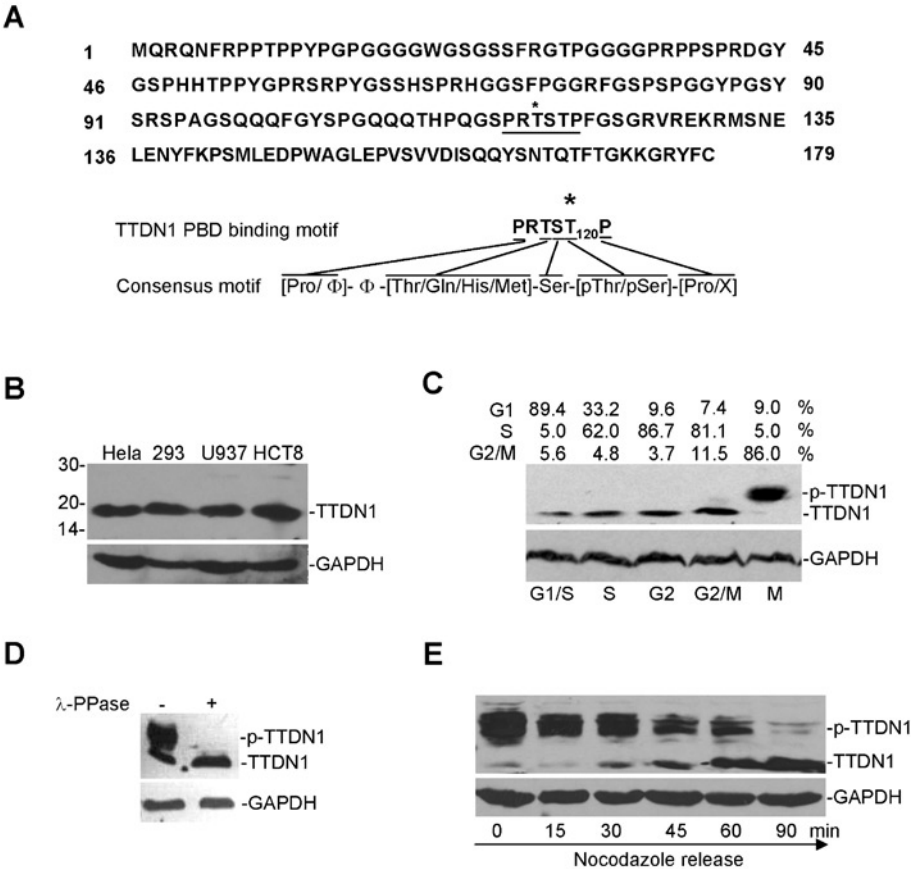

Figure 1. TTDN1 is phosphorylated in M phase. (*a*) Amino acid sequence of TTDN1. The potential Plk1-binding motif and its alignment with the consensus Plk1-binding motif are shown. (*b*) Expression of TTDN1. The indicated cell lysates were analyzed by Western blots with anti-TTDN1 (upper panel) or anti-GAPDH (lower panel) antibodies. (*c*) Western blot analysis of TTDN1 protein in different phases of the cell cycle. HeLa cells were synchronized at G_1/S by double-thymidine block. The synchronized cells were released from the block and then collected at various time points until the appearance of rounded-up morphology for most cells (an indication of cells in M phase). The collected cells were analyzed by flow cytometry and Western blot. (*d*) Effect of λ-PPase on M phase TTDN1. Mitotic HeLa cells were prepared by nocodazole arrest along with mitotic shake-off. The cells were lysed and the lysate was immunoprecipitated with anti-TTDN1 antibody. The immunoprecipitates were treated with λ-PPase or left untreated, and analyzed by Western blot with anti-TTDN1 antibody. (*e*) TTDN1 is dephosphorylated during upon exit from M phase. HeLa cells were synchronized in M phase by sequential thymidine and nocodazole treatment. The cells were then collected at the indicated times after nocodazole washout and lysed for Western blot analysis with anti-TTDN1 (upper panel) or anti-GAPDH (lower panel) antibody.

raised specifically recognized TTDN1 (data not shown).

TTDN1 is phosphorylated in M phase. To determine whether TTDN1 plays a role in cell cycle regulation, we determined whether its expression is modulated in the cell cycle. We synchronized HeLa cells at G_1, S, G_2, and M phase by double-thymidine block and release methods- and then performed Western blot analysis with TTDN1 antibody. The results indicated that expression levels of TTDN1 in each phase of the cell cycle were not significantly changed (Fig. 1c). However, TTDN1 in M phase cells has lower mobility compared to interphase cells (Fig. 1c). The simplest explanation for this observation is that TTDN1 is phosphorylated in M phase. To test this hypothesis, we immunoprecipitated TTDN1 from M phase cells. The immunoprecipitates were treated with λ-phosphatase (λ-PPase) or left untreated and analyzed by Western blot with anti-TTDN1 antibody. The results indicated that λ-PPase treatment caused TTDN1 to shift to the lower molecular weight form, suggesting that the higher molecular weight TTDN1 in M phase is a phosphorylated form of TTDN1 (Fig. 1d).

Regulation of M phase progression relies predominantly on protein phosphorylation and proteolysis. To determine whether TTDN1 is degraded or dephosphorylated following phosphorylation in M phase, we synchronized HeLa cells at M phase with nocodazole and then removed nocodazole for various times. As shown in Figure 1e, removal of nocodazole caused gradual decrease of phosphorylated TTDN1 and increase of unphosphorylated TTDN1 over a period of 90 min. Similar results were obtained in the

presence of cycloheximide, a protein synthesis inhibitor (data not shown). These data suggest that TTDN1 is dephosphorylated during cells' exit from M phase, excluding the possibility that phosphorylated TTDN1 is degraded while unphosphorylated TTDN1 is synthesized during cells' exit from M phase.

TTDN1 is phosphorylated by Cdk1 *in vitro* and *in vivo.* It has been shown that Plk1 docks to prephosphorylated targets in M phase. Since Cdk1 is a major M phase kinase critically involved in regulation of mitosis, we determined whether TTDN1 is phosphorylated by Cdk1. To determine whether TTDN1 is phosphorylated by Cdk1 *in vivo*, 293 cells were transfected with TTDN1-EGFP for 24 h. The transfected cells were synchronized at M phase with nocodazole and then treated with a specific Cdk inhibitor, roscovitine, or left untreated for 1 h and cell lysates were analyzed by Western blot with anti-TTDN1 antibody. The results indicated that roscovitine completely inhibited TTDN1 phosphorylation in M phase cells (Fig. 2a), suggesting that TTDN1 is phosphorylated by Cdk during M phase *in vivo.* Overexpression of cyclin B1 (Δ90), a non-degradable mutant of cyclin B1 and activator of Cdk1, increased the phosphorylation of TTDN1 in M phase (Fig. 2b). *In vitro* kinase assays indicated that recombinant TTDN1 was phosphorylated by purified Cdk1/cyclin B1 (Fig. 2c). Taken together, these data suggest that TTDN1 is phosphorylated by Cdk1 in mitosis.

Cell cycle-dependent localization of TTDN1. Since TTDN1 is phosphorylated in the M phase, we investigated the localization of TTDN1 at each phase of the cell cycle. Immunofluorescent staining experiments indicated that TTDN1 was mostly localized in the nucleus in interphase (Fig. 3a). During M phase, TTDN1 was enriched at the centrosome and dispersed in the cytoplasm (Fig. 3a). At telophase, TTDN1 was concentrated at the midbody (Fig. 3a). These results indicate that the location of TTDN1 is regulated during the cell cycle.

TTDN1 interacts with Plk1 in M phase in a phosphorylation-dependent manner. In our yeast two-hybrid screens, we identified TTDN1 as a protein interacting with the PBD of Plk1. To determine whether TTDN1 colocalizes with Plk1, we performed immunofluorescent staining experiments. The results indicated that TTDN1 colocalized with Plk1 at the centrosome in metaphase and at the midbody in telophase (Fig. 3b). These results suggest that TTDN1 is colocalized with Plk1 in M phase.

Our results indicated that TTDN1 was phosphorylated by Cdk1 in M phase (Fig. 2). These data suggest that phosphorylated TTDN1 may interact with Plk1 *in vivo.* To determine whether this is the case, we performed co-immunoprecipitation experiments with interphase and nocodazole-synchronized M phase HeLa cells. The results indicated that TTDN1 interacted with Plk1 in M phase but not interphase

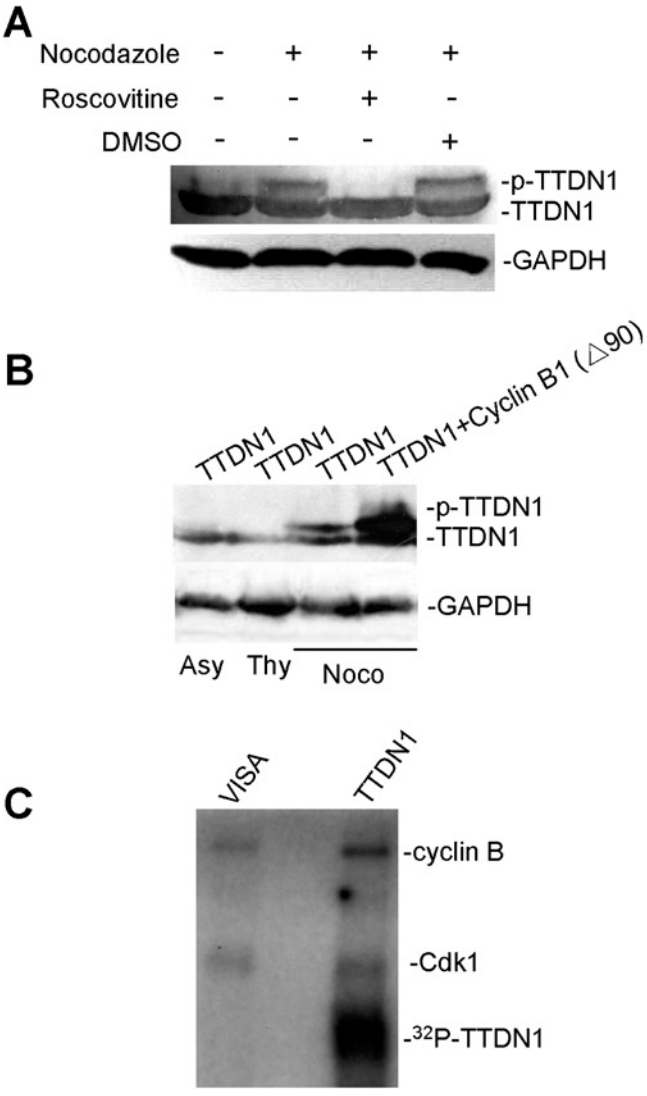

Figure 2. TTDN1 is phosphorylated by Cdk1 *in vitro* and *in vivo.* (*a*) TTDN1 is phosphorylated by Cdk *in vivo*. 293 cells transfected with TTDN1-EGFP were treated with nocodazole for 16 h, and then with or without the Cdk-specific inhibitor roscovitine (50 μM) for 1 h. Cell lysates were analyzed by Western blot with anti-TTDN1 antibody. (*b*) Cyclin B1 (Δ90) increases TTDN1 phosphorylation in M phase. 293 cells were co-transfected with cyclin B1 (Δ90) or control vector, together with TTDN1-EGFP. The transfected cells were synchronized in G_1/S or M phase or left unsynchronized. The cell lysates were analyzed by Western blots with anti-TTDN1 (upper panel) or anti-GAPDH (lower panel) antibody. (*c*) TTDN1 is phosphorylated by Cdk1 *in vitro.* Recombinant TTDN1 or VISA was incubated with recombinant Cdk1/cyclin B and *in vitro* kinase assay was performed with [^{32}P]ATP. The proteins were fractionated by SDS-PAGE and autoradiography was performed.

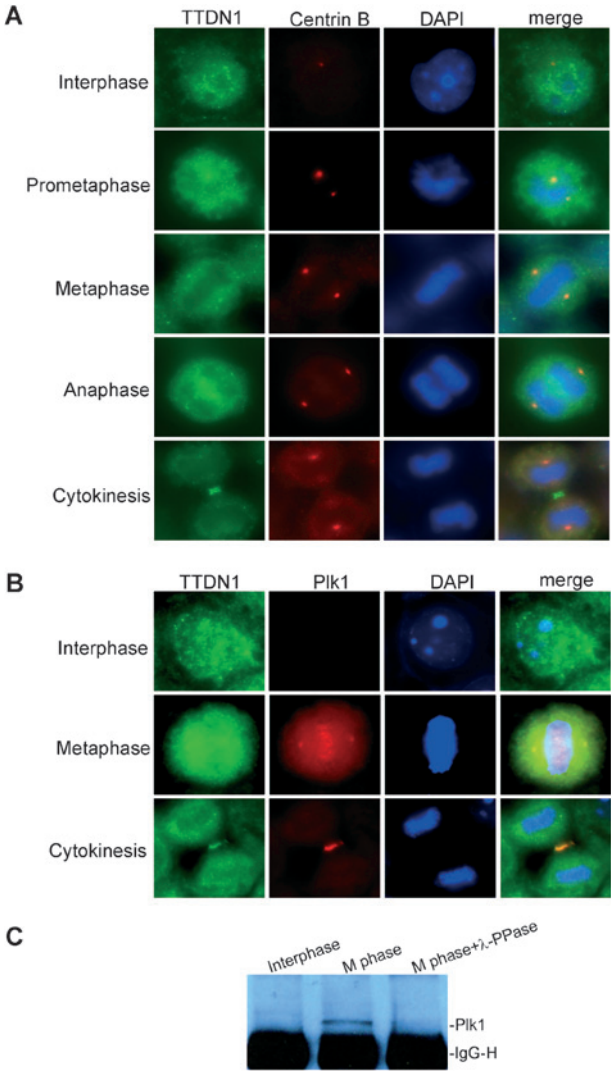

Figure 3. TTDN1 colocalizes with Plk1 and interacts with phosphorylated Plk1 in mitosis. (*a*) Cell cycle-dependent localization of TTDN1. HeLa cells were immunostained with anti-TTDN1 (green), anti-Centrin B (red) antibody and DAPI (blue). (*b*) Colocalization between TTDN1 and Plk1 in the centrosomes and the midbody. HeLa cells were immunostained with anti-TTDN1 (green), anti-Plk1 (red) antibody and DAPI (blue). (*c*) TTDN1 interacts with phosphorylated Plk1 in M phase. Lysates from HeLa cells of M phase were immunoprecipitated with anti-TTDN1 antibody. The immunoprecipitates were treated with λ-PPase or left untreated and then washed with lysis buffer. The immunoprecipitates were analyzed by Western blot with anti-Plk1 antibody.

(Fig. 3c). Furthermore, treatment of the immunoprecipitate with λ-PPase disrupted the interaction (Fig. 3c), suggesting that the interaction between TTDN1 and Plk1 is phosphorylation dependent.

Mapping of phosphorylation and Plk1-binding sites of TTDN1. To get insight into the molecular mechanism of interaction between TTDN1 and Plk1, we attempted to determine the phosphorylation sites of TTDN1. We constructed mammalian expression plasmid for TTDN1-GFP fusion protein. When overexpressed in 293 cells, TTDN1-GFP could be phosphorylated in M phase (Fig. 4a). Based on sequence analysis, we identified ten candidate phosphorylation sites (Ser/Thr followed by Pro) in TTDN1. To determine whether these candidate sites were actually phosphorylated, we constructed a series of TTDN1 mutants in which each of the ten sites (Ser40, Ser47, T51, Ser66, Ser80, Ser82, Ser93, Ser104, Ser115 and T120) was individually replaced with alanine. 293 cells were transfected with each of these proteins and treated with nocodazole for synchronizing the cells in M phase. Western blot analysis indicated that mutations of two sites, including Ser93 and Ser104, significantly affect the phosphorylation of TTDN1 in M phase (Fig. 4a). However, when Ser93 and Ser104 were double mutated, we could still detect a lower molecular weight band representing phosphorylated TTDN1, suggesting that additional site(s) are also phosphorylated. Recent studies of high-throughput protein phosphorylation analysis suggest that Thr120 is also phosphorylated in M phase [34]. In our experiments, T120 mutation had minimal effect on the migration of native and phosphorylated TTDN1 (Fig. 4a). It is possible that the phosphorylation of T120 has minimal effect on its migration and this was not detected in our Western blot analysis.

Thr120 is localized in the consensus Plk1-binding motif. Based on previous studies, phosphorylation of this site in TTDN1 should be a prerequisite for its interaction with Plk1. To test this, we determined whether Thr120 mutation could interact with Plk1 in co-immunoprecipitation experiments. 293 cells transfected with TTDN1-GFP or its T120A mutant were synchronized in M phase with nocodazole. Cell lysates were immunoprecipitated with anti-TTDN1 antibody and the immunoprecipitates were analyzed by Western blot with anti-Plk1 antibody. The results indicated that mutation of Thr120 to alanine in TTDN1 abolished its interaction with Plk1 in M phase, while mutation of Ser104 had no effect (Fig. 4b). These results suggest that TTDN1 interacts with Plk1 in phase through its consensus Plk1-binding motif.

TTDN1 plays a role in maintenance of cell cycle integrity. Since TTDN1 interacts with Plk1 in M phase, we analyzed the role of TTDN1 in cell cycle regulation. We transfected TTDN1-EGFP into HeLa cells and examined the phenotypic consequences. We found that overexpression of TTDN1 caused fragmentation of the nucleus in 8% of transfected cell 3 days after transfection, comparing to less than 2% in EGFP-transfected cells (Fig. 5a), suggesting that TTDN1 plays a role in cell cycle regulation.

We further determined the role of TTDN1 in cell cycle

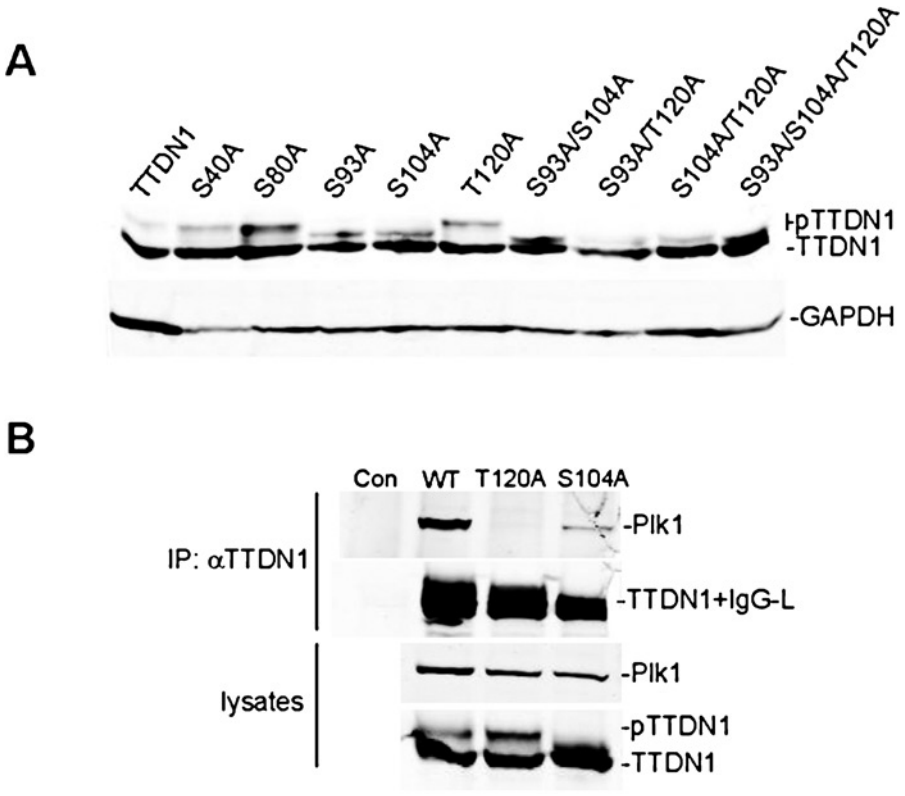

Figure 4. Mapping of TTDN1 phosphorylation sites and Plk1-binding motif. (*a*) HeLa cells were transfected with wild-type or the indicated TTDN1 mutants. The transfected cells were synchronized in M phase by nocodazole treatment and analyzed by Western blots with anti-TTDN1 (upper panel) or anti-GAPDH antibody (lower panel). (*b*) Mutation of T120 abolishes TTDN1's interaction with Plk1 in M phase. HeLa cells were transfected with wild-type or the indicated mutant TTDN1. The transfected cells were synchronized in M phase by nocodazole and the lysates were immunoprecipitated by anti-TTDN1 antibody. The immunoprecipitates (upper two panels) and the lysates (lower two panels) were analyzed by Western blot with anti-Plk1 and anti-TTDN1 antibodies.

regulation by inhibiting TTDN1 expression in HeLa cells. We constructed three TTDN1 RNAi plasmids and found that one of them could significantly inhibit the expression of TTDN1 in HeLa cells as suggested by Western blot analysis (Fig. 5b). We transfected the TTDN1 RNAi plasmid into HeLa cells and selected with puromycin. One week after selection, we performed immunofluorescent staining experiments. The results indicated that approximately 20% of the TTDN1-RNAi transfected cells showed phenotypes of multiple nuclei, collapse or multiple-polar mitotic spindles (Fig. 5c). To further determine whether TTDN1 plays a role in M phase progression, we synchronized the TTDN1-RNAi cells by thymidine blocks. Following the release of thymidine, the TTDN1-RNAi cells could progress into M phase. However, the DNA content of a significant fraction of the cells is larger than 4N, indicating that these cells had normal DNA replication but failed to divide (Fig. 5d). The stable cell line C7 has the same phenotype with C4 (data not shown). These data suggest that TTDN1 plays a role in maintenance of cell cycle integrity by regulating mitosis or cytokinesis.

Discussion

Human Plk1 plays several different roles during mitotic progression and these functions require precise targeting of Plk1 to particular subcellular structures such as centrosomes and kinetochores. The recent demonstration that the C-terminal PBD of Plk1 serves as a phosphopeptide-binding domain has led to an attractive model according to which the temporal and spatial control of Plk1 action involves PBD-mediated targeting of the kinase to pre-phosphorylated docking proteins [27, 35, 36].

In the present study, we identified TTDN1 as a substrate of Cdk1 and an interacting partner of Plk1 in M phase. Previous reports indicated that TTDN1 was mutated in patients with Amish brittle hair syndrome (ABSH) and in other non-photosensitive TTD cases with mental retardation and decreased fertility [25, 37]. However, the physiological functions of TTDN1 were unknown. Our data indicate that overexpression or depletion of TTDN1 causes significant deregulation of the cell cycle, such as disrupted mitotic spindles and formation of multiple nuclei with DNA content larger than 4N, suggesting that TTDN1 plays a role in maintenance of cell cycle integrity.

GenBank database searches identified predicted proteins with sequence similarity to human TTDN1 in other mammalian species, but not in lower eukary-

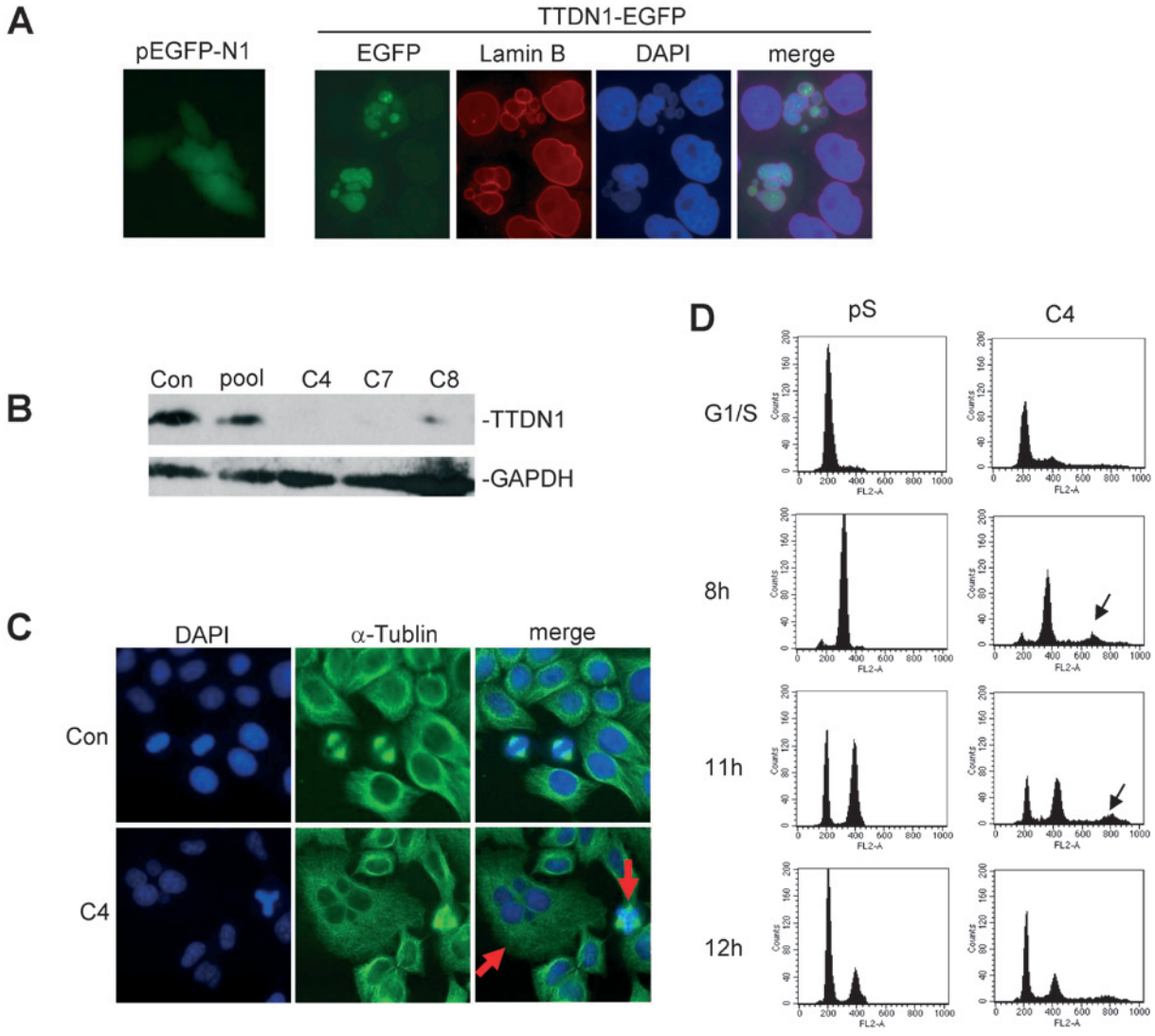

Figure 5. TTDN1 plays a role in maintenance of cell cycle integrity. (*a*) Overexpression of TTDN1 causes fragmentation of the nucleus. HeLa cells were transfected with pTTDN1-EGFP or pEGFP. The cells were stained with anti-TTDN1 (green), anti-Lamin B (red) and DAPI (blue) 3 days after transfection. (*b*) Down-regulation of TTDN1 by RNAi. HeLa cells were transfected with control TTDN1 RNAi plasmid. The cells were selected with puromycin for 7 days and analyzed for TTDN1 expression by Western blot and immunostaining. (*c*) Immunostaining of control or TTDN1 RNAi-transfected stable cell lives. The cells were stained with DAPI or anti-α-tubulin antibody. The red arrows point to a multiple-nuclei interphase cell or a multi-polar mitotic cell. (*d*) Flow cytometry analysis of TTDN1 RNAi-transfected cells. Black arrows point to cell populations with DNA content larger than 4N.

otic species such as *C. elegans* and yeast. It is possible that TTDN1 is not required for cell cycle progression but is involved in maintenance of cell cycle integrity in mammalian cells. In this context, overexpression or depletion of TTDN1 did not affect the cell cycle in most mammalian cells, but caused disruption of mitotic spindles and formation of multiple nuclei in a small fraction of cells.

Our studies clearly indicate that TTDN1 is phosphorylated by Cdk1 in M phase. Based on our and others' studies, TTDN1 was phosphorylated at several residues, including Ser93, Ser104, Thr120, and other unidentified sites. Thr120 is located in the consensus Plk1-binding motif and mutation of this residue abolished TTDN1's interaction with Plk1, suggesting that this motif is important for TTDN1's interaction with Plk1.

The different mitotic location of endogenous TTDN1 is very interesting. The movement of TTDN1 from the nucleus to the centrosome coincides with its phosphorylation. Based on our data, we propose a model whereby the Cdk1 acts as a regulatory molecule to phosphorylate TTDN1 upon mitotic entry. The phosphorylated TTDN1 is then able to interact with Plk1 located at the centrosome in the M phase and at the midbody in the telophase. Through the interaction

between TTDN1 and Plk1, TTDN1 plays an accessory role in regulating the functions of Plk1 and proper completion of the cell cycle progression.

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