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Plk1 phosphorylation of Topors is involved in its degradation

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Abstract

Topors is a DNA topoisomerase I- and p53-binding protein, and mainly functions as a p53 regulator. Accumulating evidence also supports the notion that Topors plays the role as a negative regulator of cell growth, and possibly as a tumor suppressor. Here, we demonstrated that Topors is also involved in normal mitotic progression, since Topors depletion delays mitotic entry and affects mitotic progression. Furthermore, Topors is degradated in response to the activation of the spindle checkpoint. Significantly, Polo-like kinase 1 (Plk1)-associated phosphorylation of Topors at S718 is essential for nocodazole-induced degradation of Topors.

Keywords

Topors; Protein degradation; Spindle checkpoint; Polo-like kinase 1; Phosphorylation

Introduction

Being identified as a protein that binds to both DNA topoisomerase I [1] and p53 [2], Topors (topoisomerase I-binding protein) is decreased or undetectable in colon, lung and brain adenocarcinomas, indicating that it might function as a tumor suppressor [3]. Consistent with this concept, the human Topors gene is located on chromosome 9p21 [4]; loss of heterozygosity in this region is frequently observed in several different malignancies, and Topors is also known to associate with promyelocytic leukemia (PML) nuclear bodies in the nuclei of exponentially growing cells. Forced expression of Topors in cancer cells inhibits cellular proliferation and colony formation, likely due to G0/G1 arrest and apoptosis [3, 5,

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6]. Containing an N-terminal C3HC4-type RING domain, Topors is the first example of a protein with both ubiquitin and SUMO-1 E3 ligase activity [7, 8]. Substrates of ubiquitin ligase activity of Topors include tumor suppressor p53, Hairy and NKX3.1 [8, 9]. Topors also functions as a SUMO-1 E3 ligase for DNA topoisomerase I, many chromatin-modifying proteins and p53 [7, 10]. While Topors-associated ubiquitination of its substrates lead to protein degradation, Topors-induced p53 sumoylation is accompanied by an increase in p53 protein levels [7]. Therefore, it is possible that the effects of Topors on a particular substrate may vary depending on the cellular context and the level of Topors [9].

Polo-like kinase 1 (Plk1) is emerging as a critical regulator in various mitosis-related events including centrosome maturation during G2/M transition, bipolar spindle formation during metaphase, sister chromatid segregation at the onset of anaphase and cytokinesis [11]. In addition, Plk1 is speculated as a negative regulator of p53, contributing to cell proliferation and transformation [12]. Although enough biochemical and genetic evidence from various systems has supported the essential role of Plk1 in mitosis and cell proliferation, its substrates identified so far are still very limited. In an effort to study Plk1 function as a potential p53 regulator, we recently reported that Topors is a Plk1 substrate [13]. We showed that Plk1 phosphorylation of Topors inhibits Topors-mediated sumoylation of p53, promotes p53 ubiquitination, thus leading to p53 degradation.

Considering the essential role of Plk1 in mitosis, we speculate that Topors, a Plk1 substrate, might also have additional mitosis-related function. Toward that end, we attempted to probe the potential function of Topors during mitotic progression. We found that Topors is degradated in response to tubulin poisons, and that Plk1-mediated phosphorylation of Topors is required for its degradation after the activation of the spindle checkpoint.

Materials and methods

Vector construction

Plasmids pLKO-puro.1-Topors-458 or -1781 (lentivirus vectors to deplete Topors by targeting two different positions) were constructed as described previously [14]. The targeting sequences of human Topors (GenBank accession no. NM_005802) are GGC AGAAGATGACTTCAAGG AA and GGGAAGATCAAGAAGTTCAGAA, corresponding to the coding regions of positions 458–478 and 1781–1801, respectively.

Cell culture, synchronization, and DNA transfections

HeLa, U2OS, H1299 and HEK293T cells were cultured in DMEM supplemented with fetal bovine serum. For synchronization, cells were treated with thymidine (2.5 mM) for 16 h, hydroxyurea (4 mM) for 24 h or nocodazole (100 ng/ml) for 12 h to arrest cells at G1, S or M phase, respectively. DNA transfection was performed using MegaTran 1.0 (ORIGENE). Plk1 siRNAs [15] were synthesized by Dharmacon, Inc. and transfected using Trans-messenger Transfection Reagent (QIAGEN).

Immunoprecipitation and western blotting

Total cell lysates were incubated with Topors (Novus Biologicals, catalog number H00010210-M01) antibodies overnight at 4°C, followed by a 2 h incubation with Protein A/G PLUS-Agarose beads (Santa Cruz Biotech). Immunocomplexs were resolved by SDS–PAGE and transfer to Immobilon-P membranes (Millipore). Immunoblot analyses were performed using antibodies against Topors, Plk1 (Santa Cruz Biotech, catalog number sc-17783), Mek1 (Santa Cruz Biotech, catalog number sc-6250), and myc (GeneTex, catalog number GTX29106), followed by anti-mouse or anti-rabbit horseradish peroxidase-linked secondary antibodies (Amersham) and detection using ECL reagent (Amersham).

Results and discussion

Topors is involved in mitotic progression

To analyze cellular functions of Topors, HeLa cells were transfected with pLKO-puro.1-Topors-458 or -1781 (lentivirus vectors to deplete Topors by targeting two different positions). After incubation with puromycin to select transfection-positive cells, cells were harvested and analyzed by western blotting. Topors was efficiently depleted upon transfection with both vectors (Fig. 1a, b). Transfection of a control vector did not affect cell cycle progression, whereas transfection of pLKO-puro.1-Topors led to an obvious accumulation of a cell population with 4N DNA content, as indicated in the FACS profiles of these cells (Fig. 1c). Moreover, Topors-depleted cells were subjected to a double thymidine block protocol (16 h thymidine treatment, release for 8 h, followed by a second thymidine block for 16 h) to be synchronized at the G1/S boundary, released into fresh medium for different times, and stained with phospho-histone H3 to follow mitotic progression. Whereas control cells reached a peak of mitotic index (20%) at 10 h post-release, Topors-depleted cells did not reach maximum phospho-H3 staining until 12 h post-release, with a much lower percentage of 8%. Therefore, Topors depletion seems to delay mitotic entry and affect mitotic progression (Fig. 1d).

Because Topors is a p53-binding protein and mainly acts as a p53 regulator in vivo [5], we then compared Topors depletion phenotypes of cells with different p53 backgrounds. For that purpose, three cancer cell lines were utilized: HeLa, whose p53 is significantly down-regulated due to overexpression of human papillomavirus E6 [16], p53-null H1299 cells, and U2OS cells, which are p53 wild type. These cell lines were transfected with pLKO-puro.1-Topors-1781, irradiated with 50 J/m^2 UV light, and harvested for FACS analysis. Serious apoptosis was observed upon Topors depletion in H1299 cells, indicating that p53-deficient cells are extremely sensitive to Topors depletion. Topors depletion followed by UV irradiation also led to cell death in HeLa cells, whereas no cell death was observed in U2OS cells after Topors depletion and UV irradiation (Fig. 1e). To further confirm our observation, we also generated p53-depleted U2OS cells using lentivirus-based RNAi approach. These isogenic U2OS cells were then depleted of Topors, followed by UV irradiation. Consistent with what we observed with H1299 cells, combination of Topors depletion and UV irradiation also led to serious cell death in p53-depleted U2OS cells (Fig. 1e, second panel). Altogether, these data suggest that p53 plays a critical role in Topors depletion-induced phenotypes in cancer cells.

Topors is destabilized in response to tubulin poisons

The Topors loss-of-function phenotypes described above prompted us to examine whether the protein level of Topors is regulated during cell cycle progression. Accordingly, HeLa cells were synchronized with the double thymidine block, released into fresh medium for different times, and harvested for western blotting. Topors expression was detected in different phases of the cell cycle and slightly increased in mitosis (Fig. 2a). Interestingly, in the presence of spindle poisons (both taxol and nocodazole), Topors was clearly destabilized (Fig. 2b, c, d, e), as indicated by a decrease of full-length protein and an increase of shorter fragments (labeled by stars on the right, Fig. 2c, d, e). Taxol prevents microtubule depolymerization, and nocodazole inhibits microtubule polymerization. The presence of both drugs leads to a mitotic block due to activation of the spindle checkpoint machinery [17]. MG132, a proteosome inhibitor, rescued nocodazole-induced Topors degradation, suggesting that the standard proteosome pathway is involved in Topors degradation in response to microtubule poisoning (Fig. 2b).

Because Polo-like kinase 1 (Plk1) is one critical mitotic kinase and is essential for the spindle checkpoint activation [18], we asked whether Plk1 is also involved in nocodazole-induced Topors degradation. BTO1, a specific Plk1 inhibitor [19], rescued nocodazole-induced Topors degradation, indicating that Plk1 is involved in this process (Fig. 2b, d, e). Moreover, Plk1 depletion by RNA interference also stabilized Topors in the presence of nocodazole (Fig. 2e). Thus, we concluded that Plk1-associated kinase activity is essential for Topors degradation in response to tubulin poisons.

Plk1-associated phosphorylation of Topors regulates its function in mitosis

Next, we asked whether Plk1 directly interacts with Topors. Accordingly, HeLa cells were treated with thymidine, hydroxyurea and nocodazole to arrest cells at G1, S and M phase, respectively, and harvested. Lysates were subjected to anti-Topors IP, followed by anti-Plk1 western blotting. As indicated in Fig. 3a, Plk1 binds to Topors in both S and M phase, but not in G1 phase. In a separate study, we recently reported that Plk1 phosphorylates Topors at S718 in vivo [13]. To analyze the functional significance of Plk1 phosphorylation of Topors, we generated different forms of myc-Topors (WT or S718A) and their expression in mammalian cells was confirmed (Fig. 3b, c). It has been well established that Topors localizes in punctuate nuclear regions associated with promyelocytic leukemia nuclear bodies [6]. As shown in Fig. 3c, both myc-Topors-WT and -S718A localized to nuclear bodies, suggesting that Plk1 phosphorylation of Topors does not affect the subcellular localization of the protein.

To further confirm the functional significance of Plk1-associated phosphorylation of Topors in its degradation, we transiently expressed GFP-Topors (WT or S718A) in HeLa cells. We consistently observed a much higher expression level of Topors-S718A than that of Topors-WT (Fig. 4a). Overexpressed WT-topors was destabilized in response to nocodazole treatment, but, in contrast, Topors-S718A remained stable in the presence of nocodazole (Fig. 4b), consistent with the data presented in Fig. 2d and e. We then tried to analyze half-lives of Topors with different phosphorylation states at S718. For that purpose, U2OS cells stably expressing myc-Topors (WT or S718A) [13] were treated with or without nocodazole

to activate or not the spindle checkpoint, followed by incubation with cycloheximide, an inhibitor of protein translation, for different times. In the presence of nocodazole, the half life of myc-Topors was less than 20 min, but the half life of myc-Topor-S718A was about 2 h (Fig. 4d). In striking contrast, half-lives of both Topors-WT and Topors-S718A are longer than 4 h in the absence of nocodazole (Fig. 4c). Finally, overexpression of Topors-S718A increased the cell population with positive phospho-H3 staining, indicating the significance of Plk1 phosphorylation of Topors in mitotic progression (Fig. 4e, f).

Regulation of p53 stability has been described as the major function of Topors [5, 7]. In this study, we showed that Topors is also involved in mitotic progression and that its stability is regulated by the spindle checkpoint. The protein level of Topors is increased in response to DNA damage [5], in agreement with the notion that Topors mainly functions as a tumor suppressor. The results presented here showed that the level of Topors is also regulated in response to tubulin poisoning, suggesting that stabilization/degradation of Topors might be a general mechanism to regulate its function. More significantly, Plk1-associated phosphorylation of Topors is essential for the degradation of Topors in response to the activation of the spindle checkpoint. BubR1, a classical spindle checkpoint protein, has been reported as a substrate of Plk1 [18]. The data presented here provide evidence that Plk1 might have multiple substrates in response to the activation of the spindle checkpoint. We might point out that we were not able to detect a significant reduction of Topors level in synchronized cells when Plk1 level was elevated (Fig. 2a). Under that experimental condition, after 10 h of release from the double thymidine block, cells are not as synchronized as in the early stages of release from the block. Therefore, in order to detect the decrease of Topors level as Plk1 increases, we need to add nocodazole to trap the cells at mitosis. Once we added nocodazole, Topors level was always significantly reduced (Fig. 2b, c, d, e).

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Abbreviations

Plk1	Polo-like kinase 1
Topors	DNA topoisomerase I-binding protein

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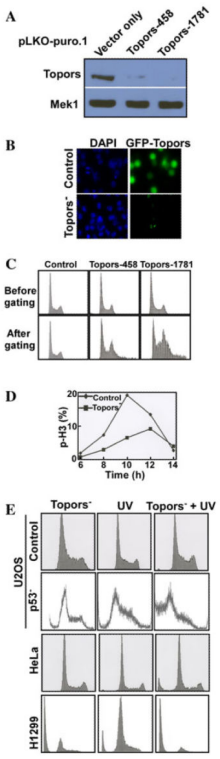

Fig. 1.

Topors is involved in mitotic progression of HeLa cells. **a** Western blotting to indicate an efficient Topors depletion using RNAi. Cells were transfected with pLKO-puro.1-Topors-458 or -1781 (lentivirus vectors to deplete Topors by targeting two different positions), incubated overnight, and selected with puromycin for two days. After floating cells were removed, the remaining attached cells were harvested and analyzed by western blotting. **b** IF to show Topors depletion using RNAi. Cells growing on coverslips were co-transfected with pLKO-puro.1-Topors-1781 and GFP-Topors at a ratio of 3:1, incubated for 2 days, and stained with DAPI. **c** Topors-depleted cells have increased 4N DNA content. Cells were co-transfected with pLKO-puro.1-Topors and GFP at a ratio of 3:1, incubated for 2 days, and stained with propidium iodide for FACS analysis. FACS profiles are shown either before or after GFP-positive cells were gated. **d** Topors-depleted cells show defects in mitotic progression. Cells growing on coverslips were transfected with pLKO-puro.1-Topors-1781 and subjected to thymidine block for 16 h. After release into fresh medium containing puromycin for 8 h, cells were blocked with thymidine for a second time for 16 h in the presence of puromycin. Cells were released into fresh medium for the times indicated, and stained with phospho-histone H3 antibodies. **e** Cancer cells with different p53 backgrounds were depleted of Topors, irradiated with 50 J/m^2 UV light, and harvested for FACS analysis

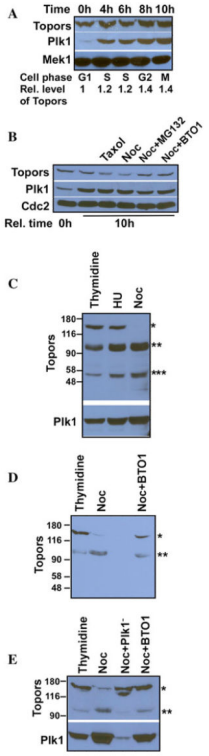

Fig. 2.

Topors is destabilized in response to tubulin poisons in HeLa cells. **a** Topors levels in different phases of cell cycle. Cells were synchronized by the double thymidine block, released for different times as indicated, and harvested for western blotting. **b** Topors is destabilized in response to spindle poisons. Cells were synchronized by the double thymidine block, released for 10 h in the presence of various drugs as indicated, and harvested for western blotting. Both taxol (10 μM) and nocodazole (100 ng/ml) were added upon release from the block. MG132 (10 μM) and BTO1 (100 μM) were added for 3 h prior to the harvest. **c** Stability of Topors in response to three different drugs. Cells were treated with 2.5 mM thymidine for 16 h, 4 mM hydroxyurea (HU) for 24 h or 100 ng/ml nocodazole (Noc) for 12 h, and harvested for western blotting. The position of full-length Topors is indicated by one star on the right, whereas the positions of Topors degradation products are labeled by two or three stars. **d** A Plk1 inhibitor prevents nocodazole-induced destabilization of Topors. Cells were treated with thymidine, nocodazole or nocodazole in the presence of 100 μM BTO1. **e** Plk1 depletion stabilizes Topors in the presence of nocodazole. Cells were depleted of Plk1 using RNA interference (lane 3), treated with nocodazole (lanes 2, 3, 4) or thymidine (lane 1), and harvested for western blotting

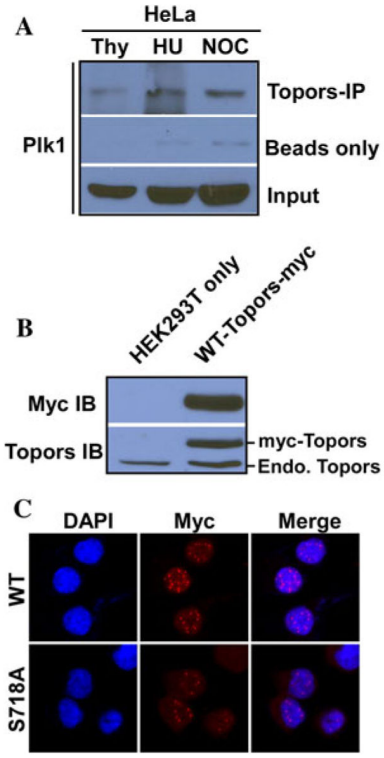

Fig. 3.

Plk1 interacts with Topors. **a** Plk1 co-immunoprecipitates with Topors. HeLa cells were treated with 2.5 mM thymidine for 16 h, 4 mM hydroxyurea (HU) for 24 h or 100 ng/ml nocodazole (Noc) for 12 h and harvested. Lysates were subjected to anti-Topors IP, followed by anti-Plk1 western blotting. **b** Western blotting to indicate expression of myc-Topors. **c** HeLa cells were transfected with myc-Topors (WT or S718A) and subjected to anti-myc IF

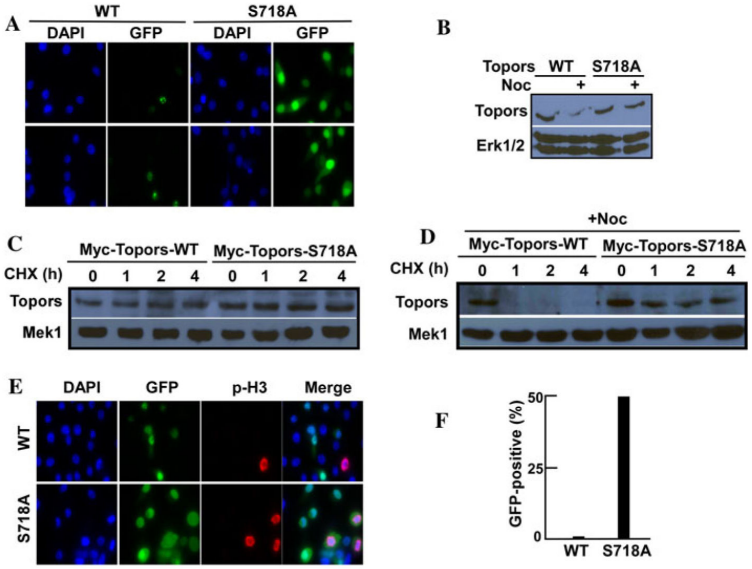

Fig. 4.

Plk1 phosphorylation might regulate Topors function in mitosis. **a** The S718A mutation stabilizes Topors. HeLa cells growing on coverslips were transfected with GFP-Topors (WT or S718A), incubated overnight, and harvested for DAPI staining. **b** The S718A mutant is less sensitive to nocodazole treatment. HeLa cells were transfected with GFP-Topors (WT or S718A), treated with nocodazole, and harvested for western blotting. **c, d** U2OS cells stably expressing myc-Topors (WT or S718A) were treated with (**d**) or without (**c**) nocodazole for 10 h, incubated with cycloheximide (CHX) for different times, and harvested for anti-myc western blot. **e, f** Overexpression of the S718A mutant leads to a partial mitotic block. HeLa cells growing on coverslips were transfected with GFP-Topors (WT or S718A), incubated overnight, and harvested for phospho-histone H3 staining. Panel C shows representative images. Panel D indicates percentages of GFP-positive cells in M phase (phospho-H3 positive) cells