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Tubulin polymerization promoting protein 1 (TPPP1)

A DNA-damage induced microtubule regulatory gene

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The eukaryotic cell cycle relies heavily on the mechanical forces vested by the dynamic rearrangement of the microtubule (MT) network. Tubulin Polymerization promoting Protein 1 (TPPP1) alters MT dynamics by driving MT polymerization as well as stabilization, via increasing MT acetylation. It increases MT rigidity, which results in reduced cell proliferation through downregulation of G_1/S-phase and mitosis to G_1-phase cell cycle transitioning. In this communication, we provide further evidence that TPPP1 may be an important regulator of genomic homeostasis. Our preliminary data show that long-term TPPP1 overexpression reduces cell viability via induction of apoptotic cell death pathways. Moreover, induction of DNA-damage results in increased TPPP1 expression, which is inhibited in the absence of expression of the tumor suppressor p53.

Keywords: TPPP1, HDAC6, ROCK, Cdk, Cell cycle, p53, signal transduction

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In eukaryotic cells, microtubules (MT) form highly organized macro-molecular structures whose spatial arrangement define cellular morphology as well as organelle distribution in interphase cells. In addition to these global MT matrices, MT subsets form or contribute to specialized structures important for the eukaryotic cell life cycle including the transient mitotic spindle and astral microtubules as well as centrosomes and centrioles. To achieve these conformations, MT populations exhibit both static and dynamic characteristics, which are achieved through several mechanisms including specialized microtubule-associated proteins (MAPs) that modulate MT polymerization-depolymerization kinetics and MT post-translational modifications that affect MT stability. Herein, we discuss the MAP Tubulin Polymerization Promoting Protein 1 (TPPP1), a protein that we have recently identified as an inhibitor of the cell cycle,[1] and provide additional insight into its possible role as a tumor suppressor gene.

Tubulin Polymerization Promoting Protein 1 (TPPP1)

Tubulin Polymerization Promoting Protein 1 (TPPP1), also known as p25 (protein of MW 25 kDa), is a member of the TPPP family that in vertebrates also includes TPPP2 (p20) and TPPP3 (p18).[5,6] It is a ubiquitously expressed unique MAP that functions to increase MT polymerization and MT acetylation, a stabilizing modification.[3,5,7,8] At present, the precise mechanism by which TPPP1 increases MT polymerization is unclear. It binds soluble 'tubulin' purified from bovine brain,[5] however it is not known whether TPPP1 binds to α-tubulin or β-tubulin subunits or to the α-/ β-tubulin heterodimer. TPPP1 is a Mg^{2+} dependent GTP-binding protein;[5,9] therefore, it may act as a nucleotide exchange co-factor to enhance the polymerizing GTP-tubulin pool and increase the rate of MT polymerization or alternatively, it may act as a scaffold protein to bind and deliver tubulin heterodimers to nascent protofilaments. In contrast, TPPP1 modulates MT acetylation by binding

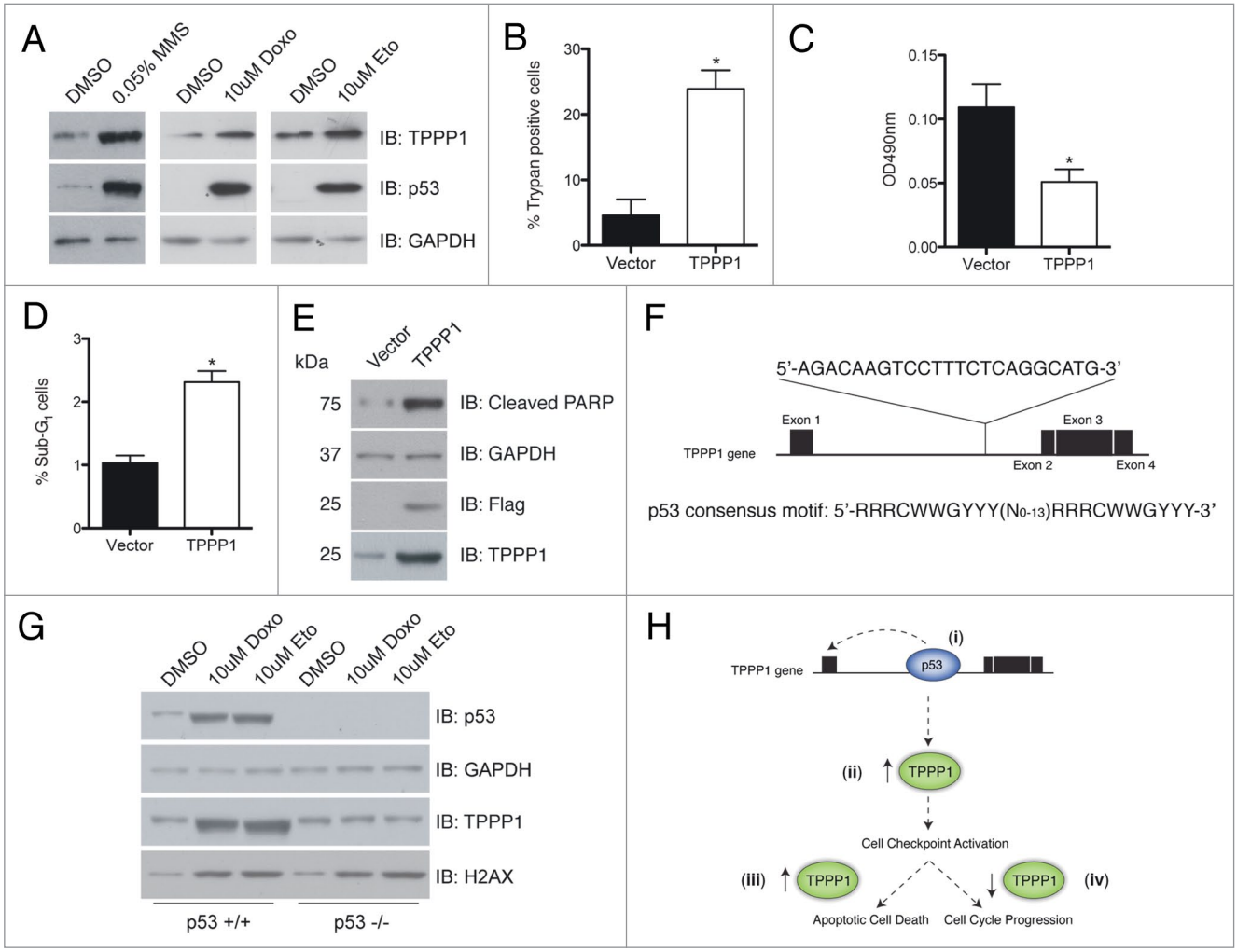

Figure 1. *TPPP1 expression increases in response to DNA-damage.* (**A**) Induction of DNA-damage increases TPPP1 expression. U2OS cells were incubated with MMS (0.05%), Doxorubicin (Doxo; 10 μM) or Etoposide (Eto; 10 μM) as described in the materials and methods. Cell lysates were analyzed by western blotting for TPPP1, p53 (experimental control) and GAPDH (loading control). (**B-D**) TPPP1 expression reduces cell viability. Cells stably expressing Flag-TPPP1 or vector were analyzed by cell counts and tyrpan blue counterstaining (**B**), MTS assays (**C**) or propium iodide staining and flow cytometry (**C**) (n = 3; *p < 0.05). (**E**) TPPP1 expression increases apoptotic cell death. Cells described in (**B-D**) were analyzed by western blotting for cleaved PARP (apoptosis marker), Flag, TPPP1 and GADPD (loading control). (**F**) The TPPP1 gene contains a putative p53-response element in intron 1. (**G**) DNA-damage induced increases in TPPP1 expression is inhibited in the absence of p53. HCT116 p53 +/+ and –/– cells were treated with Doxorubicin (Doxo) or Etoposide (Eto) as described in (**A**). Cell lysates were subject to western blotting for p53, H2AX (DNA-damage marker) and GAPDH (loading control). (**H**) Proposed model of TPPP1 regulation and function in cells. (i) After DNA damage the increased amount of p53 binds to the TPPP1 gene resulting in its increased transcription and protein expression. (ii) Increased TPPP1 contributes to cell cycle arrest, temporally enabling monitoring of DNA fidelity. (iii) In the case of high DNA-damage, TPPP1 expression and activity is prolonged to enable the induction of apoptotic cell death. (iv) However, if DNA is not compromised, TPPP1 activity is downregulated to enable cells to progress through the cell cycle.

to the α-tubulin deacetylase Histone deacetylase 6 (HDAC6) and inhibiting its activity, resulting in a reciprocal increase in MT acetylation.[3,10] Our recent work demonstrated that TPPP1 activity is inhibited by its phosphorylation by Rho-associated Coiled-coil kinase (ROCK) and cyclin dependent kinase 1 (Cdk1). Specifically, we show that ROCK-TPPP1 prevents TPPP1 binding and inhibition of HDAC6, whereas Cdk1-TPPP1 signaling inhibits its MT polymerizing activity.[1]

Expression of TPPP1 in cells enhances MT network rigidity through inhibition of HDAC6 activity and the consequential increase in MT acetylation. Early studies suggested that TPPP1 expression prevents the assembly of the mitotic spindle[5] and reduces cell division,[8] whereas ectopic expression of microRNA-206 (miRNA-206), a miRNA shown to reduce TPPP1 levels, increased the number of dividing cells.[11] Our recent study confirmed that TPPP1 overexpression and knockdown increased and decreased cell proliferation, respectively.[1] Interestingly, our analysis revealed that TPPP1 achieves this via reduction of both the G_1/S-phase and the mitosis to G_1-phase transitions. More specifically, we show that TPPP1-mediated HDAC6 inhibition delays the G_1/S-phase transition, whereas its regulation of the G_2/M-phase transition is dependent on this function and its promotion of MT polymerization. These results provide a compelling argument that TPPP1 is a

significant broad-spectrum enforcer of the cell cycle checkpoints.

If this were the case, then pharmacologically induced DNA-damage might elicit an increase in TPPP1 expression or activity to enforce cell cycle checkpoints enabling the repair of DNA or to induce apoptotic cell death programs. Interestingly, our preliminary data demonstrate that treatment of cells with the intercalator Doxorubicin, the topoisomerase II targeting Etoposide or the alkylating agent Methyl methanesulfonate (MMS) increase TPPP1 expression (**Fig. 1A**). These data suggest that induction of TPPP1 expression is a general mechanism in response to DNA damage as a means of activating the cell cycle checkpoints. Moreover, long-term expression of TPPP1 reduces cell viability (**Fig. 1B-D**) via induction of apoptotic cell death (**Fig. 1E**), thereby adding more credence to the idea that TPPP1 is an important monitor of genomic integrity.

Finally, p53 is an important tumor suppressor gene that acts as a transcriptional regulator of several genes essential for the precise regulation of cell division. Interestingly, during our investigation, we observed that the TPPP1 gene contains a putative p53 response element [5'-RRRCWWGYYY-(N_{0-13})-RRRCWWGYYY-3'],[12] located in intron 1 (**Fig. 1F**). Given the presence of a p53 binding motif and that TPPP1 expression is increased in response to DNA damage, also an inducer of p53 expression, TPPP1 may be a p53 transcriptionally upregulated gene. Our preliminary analysis of wild-type HCT116 colorectal cells (p53+/+) or HCT116 cells lacking p53 (p53-/-) revealed that in the absence of p53, the increase in TPPP1 expression after DNA-damage was inhibited (**Fig. 1G**).

Materials and Methods

Plasmids, tissue culture and pharmacological treatments

All plasmid constructs were generated as previously described.[1] U2OS osteosarcoma and HCT116 colorectal carcinoma cells were cultured and maintained in DMEM supplemented with 10% FBS. U2OS cells stably expressing TPPP1 were generated as previously described.[1] Drugs used in this study were dissolved in solvents recommended by the manufacturer. Methyl methanesulfonate (MMS; Sigma) treatment was performed with 0.05% MMS 2 h prior to washout and recovery for two hours. Doxorubucin (10 μM; Sigma) and etoposide (10 μM; Sigma) incubations were performed for 16 h.

Immunoblotting

Immunoblotting was performed as previously described.[2] The following antibodies (Abs) were obtained from Cell Signaling: anti-cleaved PARP and GAPDH-HRP. The anti-TPPP1 Ab was previously described.[3] Anti-p53 and anti-H2AX Abs were generous gifts of A/Prof Ygal Haupt (Peter MacCallum Cancer Centre, Melbourne) and Dr Boris Sarcevic (St. Vincent's Institute, Melbourne), respectively.

Cell viability assays

Sub-G_0 cell populations were determined by flow cytometry of Propidium Iodide labeled cells performed as previously described[1] and analyzed using the FlowJo (v8.8.6) software. Cell metabolism, as a measure of cell viability, was performed using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega) by analysis of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] metabolism at 24 h post-plating. Cell viability counts were conducted by seeding cells at a density of $2x10^5$ cells/well in 6-well plates 24 h prior to trypsinization and resuspension in PBS. Equal volumes of cell suspension and 0.4% (w/v) trypan blue solution were mixed and incubated at room temperature for 5 min. Total cell and dead cell, trypan blue positive, counts were performed with a hemocytometer using a light microscope.

Bioinformatics and data analysis

TPPP1 genomic sequences were obtained from NCBI (AC_000137) and analyzed for the presence of a p53-response element [5'-RRRCWWGYYY-(N_{0-13})-RRRCWWGYYY-3'][4] using the ApE software (M. Wayne Davis, University of Utah, http://biologylabs.utah.edu/jorgensen/wayned/ape/). Data are expressed as mean ± SEM of three independent experiments and analyzed by two-tailed unpaired t-tests.

Conclusion

In conclusion, we hypothesize that TPPP1 is an important enforcer of the cell cycle checkpoints and that correct regulation of its activity may be important for maintaining tissue homeostasis. Moreover, given its potential role as a tumor suppressor gene, we propose that aberrant inactivation of TPPP1 may initiate the hyper-proliferative stage of cancer development. Specifically, we propose a model whereby TPPP1 transcription is promoted by p53-mediated binding to a response element located in Intron 1 of the TPPP1 gene. As a result of p53-mediated cis-activation of the TPPP1 gene, TPPP1 expression is increased to enforce cell cycle checkpoints. In the case that DNA fidelity is compromised, prolonged p53 and consequential TPPP1 expression contributes to the initiation of apoptotic cell death programs (**Fig. 1H**).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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