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Tubulin polymerization promoting protein 1 (TPPP1) increases β-catenin expression through inhibition of HDAC6 activity in U2OS osteosarcoma cells

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ABSTRACT

The Rho-associated coiled-coil kinase (ROCK) family of proteins, including ROCK1 and ROCK2, are key regulators of actin and intermediate filament morphology. The newly discovered ROCK substrate Tubulin polymerization promoting protein 1 (TPPP1) promotes microtubule polymerization and inhibits the activity of Histone deacetylase 6 (HDAC6). The effect of TPPP1 on HDAC6 activity is inhibited by ROCK signaling. Moreover, it was recently demonstrated that ROCK activity increases the cellular expression of the oncogene β-catenin, which is a HDAC6 substrate. In this study, we investigated the interplay between ROCK-TPPP1-HDAC6 signaling and β-catenin expression. We demonstrate that β-catenin expression is increased with ROCK signaling activation and is reduced with increased TPPP1 expression in U2OS cells. Further investigation revealed that ROCK-mediated TPPP1 phosphorylation, which prevents its binding to HDAC6, negates TPPP1-mediated reduction in β-catenin expression. We also show that increased HDAC6 activity resulting from ROCK signaling activation reduced β-catenin acetylation at Lys-49, which was also accompanied by its decreased phosphorylation by Caesin kinase 1 (CK1) and Glycogen synthase kinase 3β (GSK3β), thus preventing its proteasomal degradation. Overall, our results suggest that ROCK regulates β-catenin stability in cells via preventing TPPP1-mediated inhibition of HDAC6 activity, to reduce its acetylation and degradation via phosphorylation by CK1 and GSK3β.

1. Introduction

Rho-associated coiled-coil kinase (ROCK) signaling is a key regulator of several downstream targets, most notably a large array of actin regulatory and intermediate filament proteins. More recently, it was demonstrated that ROCK regulates cellular β-catenin expression, via an as yet undefined mechanism. Enhanced ROCK signaling increases β-catenin expression [1] and its transcriptional target cyclin D1 [2], whereas down-regulation of ROCK signaling reduces β-catenin and cyclin D1 levels [3]. These findings suggest that ROCK-mediated increases in cell proliferation may be partially due to increased β-catenin expression.

β-catenin is an oncogene that plays a crucial role in the canonical Wnt signaling pathway. Binding of Wnt ligands to the Frizzled (Fz)/LRP5/6 (Low Density Lipoprotein receptor protein 5 or 6) co-receptor complex activates the Wnt signaling cascade to increase β-catenin levels, through inhibition of its degradation by the "destruction complex", a multi-protein assembly consisting of the Adenomatous polyposis coli (APC), axin, Casein kinase 1 (CK1) and Glycogen synthase kinase 3β (GSK3β) proteins. Elevated β-catenin levels are accompanied by its cytoplasmic-nuclear translocation, where it binds to the Lymphoid enhancer factor/T-cell factor (LEF/TCF) family of DNA-binding proteins to displace their transcriptional repressor Groucho and promote the transcription of Wnt target proto-oncogenes including c-myc and cyclin D1 [4,5]. In the absence of Wnt signaling, β-catenin is tethered to the assembled "destruction complex", at which point it is fated for destruction via systematic phosphorylation at Ser45 by CK1 [6] and at Ser-31, Ser-37 and Thr-41 by GSK3β [7]. These modifications enable its binding to β-transducin repeat-containing protein (β-TrCP), a component of the ubiquitin E3 ligase, promoting β-catenin ubiquitination and proteasomal degradation [8–10]. In addition to these post-translational modifications, β-catenin is also acetylated on Lys-49, a site frequently mutated in tumors [11]. Acetylation is catalyzed by the CREB-binding protein (CBP)/p300 acetyltransferase, whereas its deacetylation is performed by Histone deacetylase 6 (HDAC6) [12]. Mutation of the Lys-49 to Arg,

Abbreviations: CK1, caesin kinase 1; GSK3β, glycogen synthase kinase 3β; HDAC6, histone deacetylase 6; ROCK, rho-associated coiled-coil kinase; TPPP1, tubulin polymerization promoting protein 1; β-TrCP, β-transducin repeat-containing protein.

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an amino acid that cannot be acetylated, increases β-catenin-mediated transcriptional activation of c-myc compared to its wild-type counterpart [11], suggesting that β-catenin acetylation regulates its stability, cytoplasmic-nuclear shuttling and/or gene promoter affinity.

Tubulin polymerization promoting protein 1 (TPPP1) is a Microtubule (MT) regulatory protein that drives MT polymerization and stabilization [13]. It stabilizes MTs through binding and inhibition of HDAC6 activity, resulting in increased levels of acetylated MT [14]. We recently demonstrated that the ROCK-TPPP1 signaling pathway prevents TPPP1-mediated inhibition of HDAC6 activity [15] resulting in decreased MT acetylation. Moreover, we established that TPPP1 overexpression decreases osteosarcoma (U2OS) cell proliferation while its knockdown increases it [16].

The aim of this study was to investigate a mechanism that contributes to ROCK-mediated regulation of β-catenin levels. We show here that ROCK signaling regulates β-catenin levels via preventing the TPPP1/HDAC6 interaction to increase β-catenin deacetylation and expression.

2. Materials and methods

2.1. Plasmid constructs

pcDNA3-Flag-ROCK1Δ4 and pcDNA-Flag-ROCK1Δ4-KD constructs were a generous gift of Dr. S. Narumiya (Kyoto University, Japan) [17]. pBABE-Flag-TPPP1 [18], pBABE-Flag-TPPP1-S32A/S107A/S159A (TPPP1^{3Ala}) and pBABE-Flag-TPPP1-S32E/S107E/S159E (TPPP1^{3Glu}) plasmids were generated as previously described [15].

2.2. Mammalian cell culture

U2OS cells were cultured in DMEM supplemented with 10% FBS and maintained in a 5% CO_2 atmosphere at 37 °C. RNAi experiments were performed with hTPPP1 ON TARGETplus SMARTpool siRNA (Dharmacon). Cells were transfected with 10 nM of siRNA using the Lipofectamine™ 2000 (Life Technologies) transfection reagent in Opti-MEM® according to manufacturer's recommendations. Cells expressing ROCK constructs were transiently transfected with the appropriate constructs using the FuGene® 6 transfection reagent according manufacturer's instruction. U2OS cell lines stably expressing TPPP1 were generated by infection with amphotropic retroviruses as previously described [15].

2.3. Immunofluorescence microscopy

Cells grown on uncoated glass coverslips were fixed with ice-cold 100% methanol for 5 min followed by blocking in 10% FBS for 1 h at room temperature. Blocked cells were incubated with the following primary antibodies (Ab) overnight at 4 °C: Anti-Flag IgG (1:100) [19], Anti-β-Catenin IgG (1:1000) [Sigma; Cat #C7082] and Anti-phospho-MLC IgG S18/T19 [Cell Signalling; Cat #3674]. Incubation with anti-TPPP1 IgG (1:100) [18] was performed at room temperature for 1 h. Secondary Ab incubations were performed for 45 min at room temperature with the following Abs: Anti-Mouse IgG Alexa Fluor 488 (1:400) [Molecular Probes; Cat #A-11034], Anti-Rat IgG Alexa Fluor 594 (1:200) [Molecular Probes; Cat #A-21209] or Anti-Rabbit IgG Alexa Fluor 594 (1:400) [Molecular Probes; Cat #A-21207] together with Hoechst [1:10,000]. Coverslips were mounted on fluorescent mounting medium and stored at 4 °C. Images were captured on a Nikon C1 confocal microscope using the NIS software with a PLAPO VC 60X02PH NA1.4 objective lens. Confocal images were saved as grayscale TIFF files. Brightness adjustments and pseudo-coloring was performed with Adobe Photoshop v11.0.2 and applied to all images in a comparative group.

2.4. Immunoblotting

Immunoblotting was performed as previously described [15]. The following Abs were used: Anti-acetyl-α-Tubulin Lys40 (1:5000) [Sigma; Cat #T7451], Anti-acetyl-β-catenin Lys49 (1:500) [Cell Signalling; Cat #5934], Anti-c-myc (1:1000) [Life Technologies; Cat #AHO0062], Anti-Flag 9H1 clone (1:3000) [19], Anti-GAPDH HRP (1:3000) [Cell Signalling; Cat #3683], Anti-TPPP1 (1:1000) [18], Anti-α-tubulin (1:5000) [Sigma; Cat #T5168], Anti-β-catenin (1:3000) [Sigma; Cat #C7082], Anti-phospho-β-catenin Ser45 (1:1000) [Cell Signalling; Cat #5964], Anti-phospho-β-catenin Ser31/37/Thr41 (1:1000) [Cell Signalling; Cat #5961] and Anti-phospho-MLC Ser18/Thr19 (1:1000) [Cell Signalling; Cat #3647]. Protein band densitometry was performed using the ImageQuant 7 software (Molecular Dynamics (CA, USA)).

3. Results

3.1. ROCK regulates β-catenin levels in U2OS cells

Previous studies described that ROCK signaling increases β-catenin expression in various cell lines. To confirm that this is also the case in U2OS cells, we transiently expressed constitutively active Flag-ROCK1Δ4 (Flag-ROCK1), its K105G kinase dead mutant (Flag-ROCK1-KD) or vector control. Analysis of total β-catenin levels by immunoblotting (Fig. 1A) and immunofluorescence microscopy (Fig. 1B) showed that overexpression of Flag-ROCK1, but not that of Flag-ROCK1-KD, resulted in increased β-catenin levels as well as an increase in the level of its transcriptional target c-myc. Furthermore, inhibition of ROCK activity, by treatment of U2OS cells with the small-molecule ROCK inhibitor Y-27632, decreased β-catenin levels when analyzed by immunoblotting (Fig. 1C) and immunofluorescence microscopy (Fig. 1D). Therefore, we clearly show that ROCK signaling regulates β-catenin expression in U2OS cells.

3.2. ROCK-TPPP1 signaling regulates β-catenin levels

Our recent study demonstrated that TPPP1 knockdown increases cell proliferation [16]. Since β-catenin is a well known mitogen that is regulated by HDAC6 activity [20], we investigated the possibility that TPPP1, through inhibition of HDAC6 activity, may reduce β-catenin levels. We previously established that ROCK-TPPP1 signaling negates TPPP1-mediated inhibition of HDAC6 activity [15], therefore we analyzed β-catenin expression in stable U2OS cells expressing wild-type TPPP1, TPPP1^{3Ala} (ROCK phospho-inhibitory mutant), TPPP1^{3Glu} (ROCK phospho-mimetic mutant) or vector by immunoblotting (Fig. 2A) and immunofluorescence microscopy (Fig. 2B). Our analysis revealed that overexpression of wild-type TPPP1 or TPPP1^{3Ala} significantly decreased β-catenin levels, with TPPP1^{3Ala} exhibiting a greater reduction in β-catenin levels. In contrast, overexpression of TPPP1^{3Glu}, a mutant that mimics its ROCK phosphorylated form, had no affect on β-catenin expression. Moreover, down-regulation of endogenous TPPP1 levels with a TPPP1-specific siRNA significantly increased β-catenin levels and its transcriptional target c-myc compared to the non-targeting (NT) control as demonstrated by immunoblotting (Fig. 2C) and immunofluorescence microscopy (Fig. 2D). These results suggest that ROCK and TPPP1-mediated regulation of β-catenin expression is dependent on their modulation of HDAC6 activity. This is supported by the differences in β-catenin levels in the TPPP1 and TPPP1^{3Ala} expressing cells that is likely due to partial phosphorylation of the wild-type TPPP1.

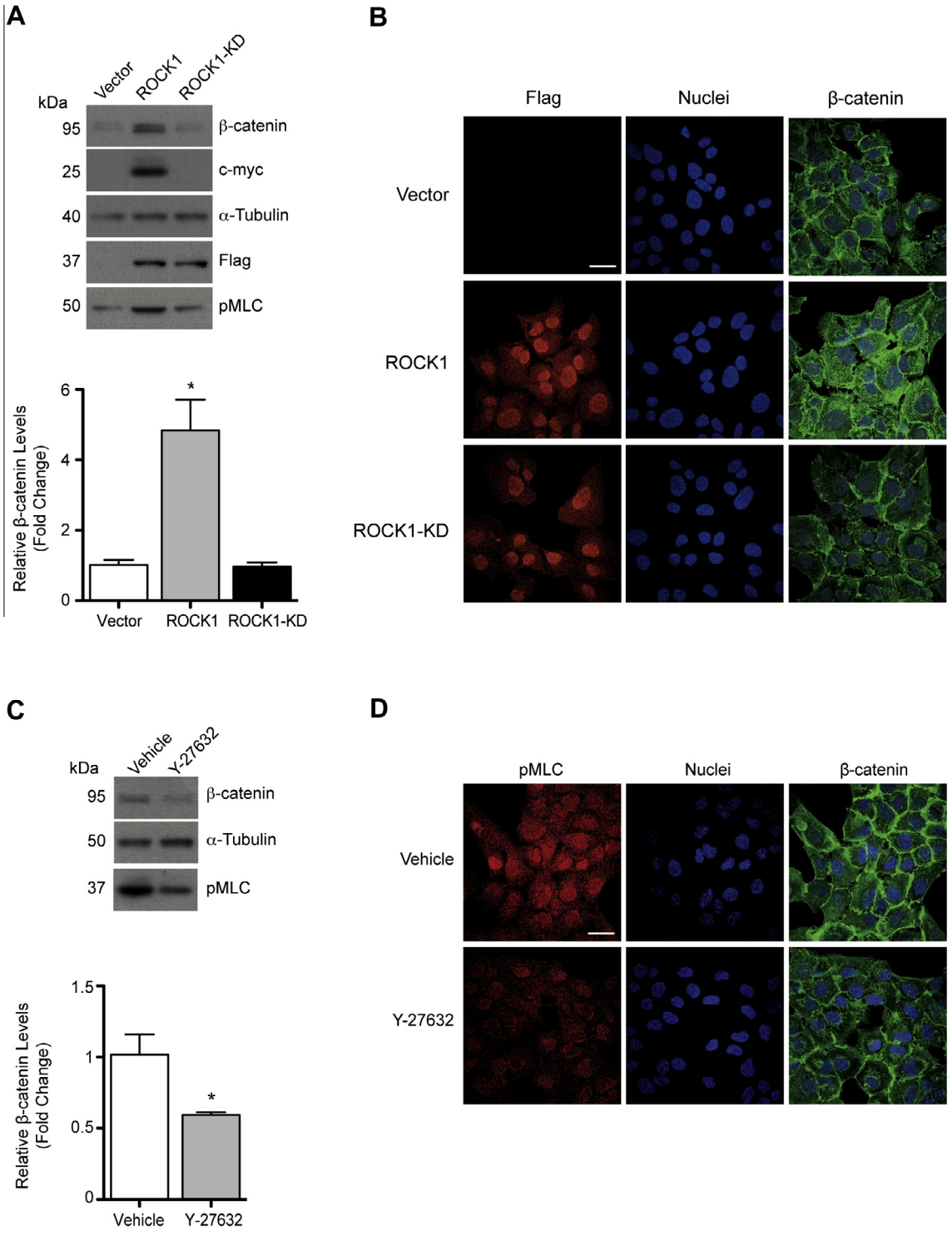

Fig. 1. ROCK activation increases β-catenin expression. U2OS cells were transiently transfected with constitutively active Flag-ROCK1Δ4 (F-ROCK1), its kinase dead (KD) mutant (F-ROCK1-KD) or vector. (A) Immunoblot analysis of the cell extracts probed for β-catenin, c-Myc, Flag, phospho-Myosin Light Chain (pMLC) (ROCK activity control) and α-tubulin (loading control) revealed a significant increase in β-catenin expression with ROCK1 activation as compared to vector control. (B) Immunofluorescence microscopy of cells stained for Flag (red), β-catenin (green) and nuclei (blue). (C and D) Inhibition of ROCK activity decreased β-catenin levels. U2OS cells were treated with the ROCK inhibitor Y-27632 (10 μM) or vehicle for 16 h. (C) Immunoblot analysis of the cell extracts probed for β-catenin, pMLC and α-tubulin (loading control) shows a significant decrease in β-catenin expression with ROCK inhibition. (D) Immunofluorescence microscopy of the cells stained for pMLC (red), β-catenin (green) and nuclei (blue). Scale bar is 50 μm. $N = 3$, $^{*}P < 0.05$.

3.3. ROCK signaling regulates β-catenin acetylation and phosphorylation

To test the possibility that ROCK-TPPP1 signaling increases β-catenin levels via affecting its post-translational modification, we analyzed its acetylation (Lys-49) and phosphorylation by CK1 (Ser-45) and GSK3β (Ser-33, Ser-37, and Thr-41) [21]. Western blot analysis of U2OS cell extracts expressing Flag-ROCK1, Flag-ROCK1-KD or vector that were normalized for total β-catenin levels revealed that expression of Flag-ROCK1, but not that of Flag-ROCK1-KD, reduced the level of β-catenin acetylation and phosphorylation compared to the vector control (Fig. 3A).

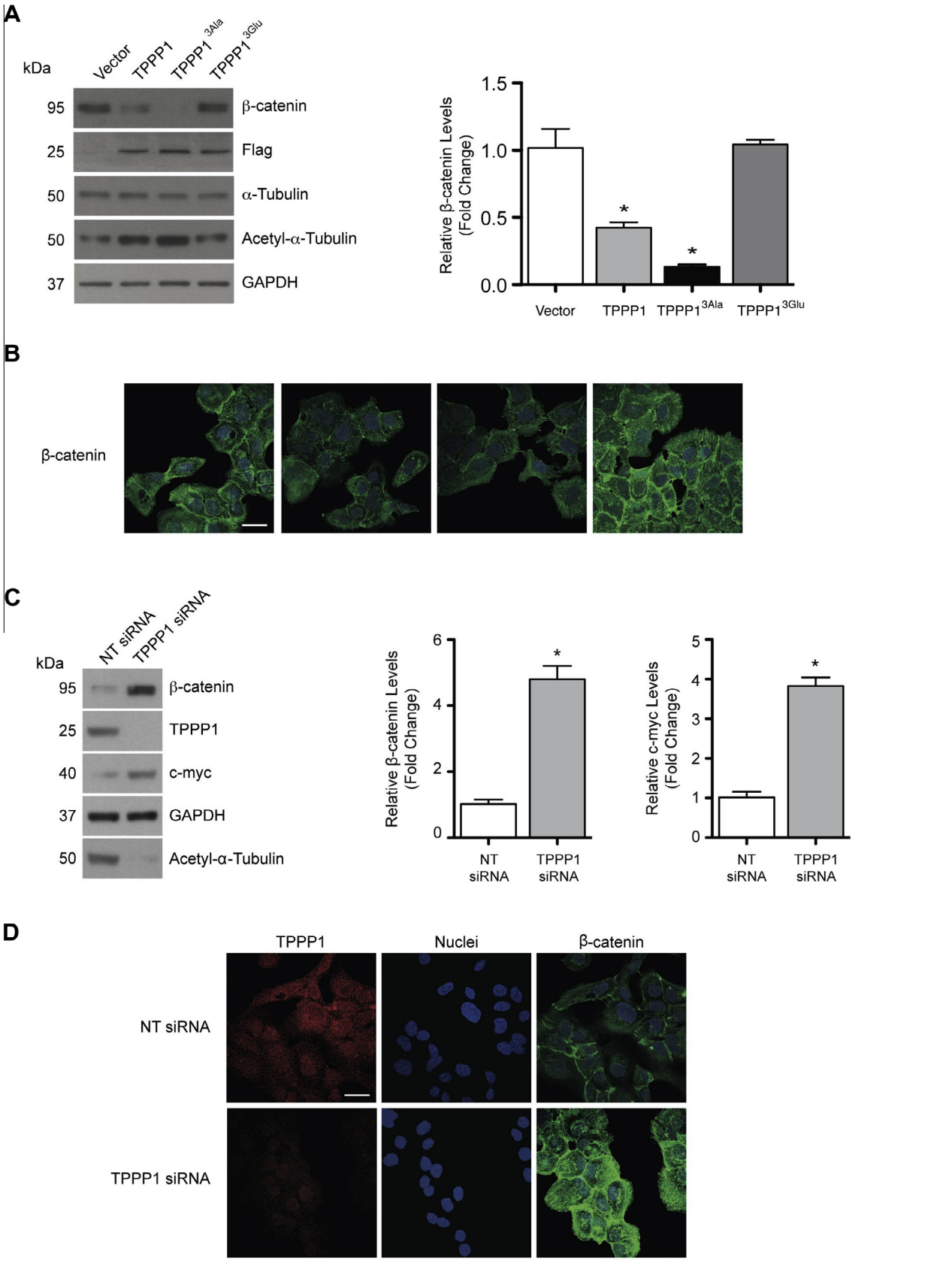

Fig. 2. TPPP1 expression results in reduced β-catenin levels. (A and B) TPPP1 overexpression decreases β-catenin levels in cells, which is inhibited by its phosphorylation by ROCK. (A) U2OS cells stably expressing wild-type TPPP1, TPPP1^{3Ala}, TPPP1^{3Glu} or vector were analyzed by immunoblotting and probed for β-catenin, F-TPPP1, α-tubulin, acetyl-α-tubulin and GAPDH (loading control). (B) Immunofluorescence microscopy of cells stained for β-catenin (green) and nuclei (blue). (C and D) Knockdown of TPPP1 increased β-catenin levels in cells. (C) U2OS cells were transiently transfected with TPPP1 or non-targeting (NT) siRNA. Cellular levels of β-catenin, TPPP1, c-Myc, acetyl-α-tubulin and GAPDH (loading control) levels were analyzed by immunoblotting and (D) immunofluorescence microscopy by staining them for TPPP1 (red), β-catenin (green) and nuclei (blue). Scale bar is 50 μm. $N = 3$, $^{*}P < 0.05$.

Conversely, treatment of U2OS cells with the ROCK inhibitor Y-27632 increased β-catenin acetylation and phosphorylation (Fig. 3B). These results suggest that ROCK signaling may regulate β-catenin levels through modulation of its acetylation and phosphorylation, which in turn affects its proteasomal degradation.

We next investigated a role for TPPP1 and ROCK-TPPP1 signaling in the regulation of β-catenin acetylation and phosphorylation by western blot analysis of U2OS cells expressing wild-type TPPP1 or its ROCK phospho-site mutants. Our results revealed that β-catenin acetylation and CK1/GSK3β-mediated phosphorylation were increased with wild-type TPPP1 or TPPP1^{3Ala} expression compared to vector control, with higher levels observed in the TPPP1^{3Ala} cell extracts which is likely due to partial wild-type TPPP1 phosphorylation (Fig. 3C). In contrast, expression of TPPP1^{3Glu} did not alter the level of β-catenin acetylation or phosphorylation. Subsequent evaluation of the affect of endogenous TPPP1 down-regulation revealed that knockdown of TPPP1 reduced the level of β-catenin acetylation and phosphorylation compared to the non-targeting (NT) control (Fig. 3D). Therefore, we establish that ROCK signaling increases β-catenin stability in cells via reduction of its post-translational modifications, as a consequence of its suppression of TPPP1-mediated HDAC6 inhibition. Moreover, these results suggest that acetylation of β-catenin reduces its stability.

3.4. ROCK-TPPP1 regulates β-catenin levels via HDAC6 regulation

The observation that β-catenin acetylation correlates with its phosphorylation by CK1 and GSK3β, events that occur in proteins fated for destruction, suggests that this modification also contributes to its degradation. To investigate this possibility we treated

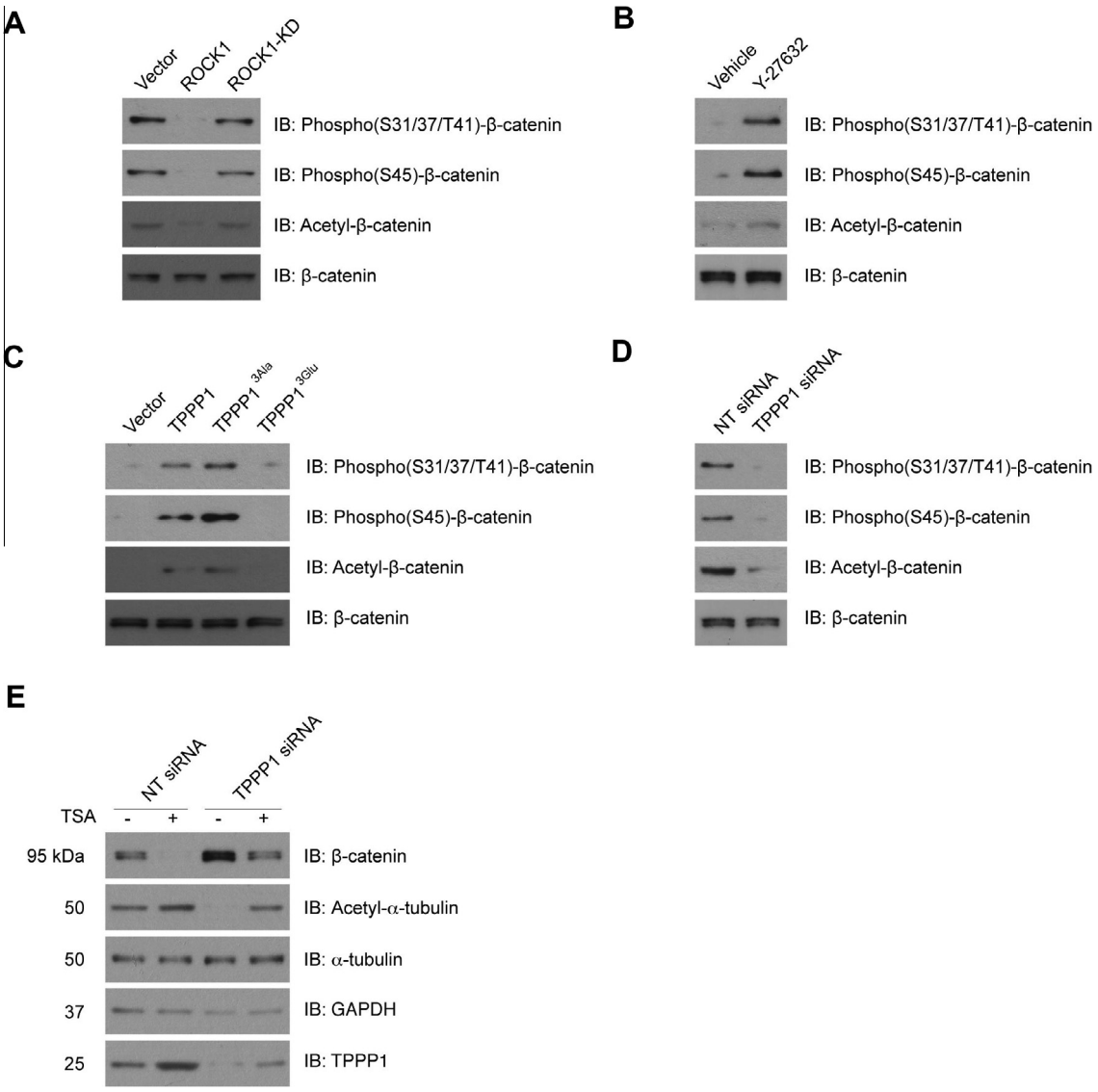

Fig. 3. ROCK-TPPP1 signaling regulates β-catenin levels via increasing its acetylation and phosphorylation. (A) ROCK signaling significantly reduces β-catenin acetylation and phosphorylation by Casein kinase 1 (CK1) and Glycogen synthase kinase-3β (GSK3β). U2OS cells transiently transfected with Flag-ROCK1, Flag-ROCK1-KD or vector and normalized for β-catenin levels were analyzed by immunobloting. The blots were probed with anti-acetyl-β-catenin (Lys-49), anti-phospho-(Ser-45)-β-catenin (CK1-mediated) and anti-phospho-(Ser-31/S37/Thr-41)-β-catenin (GSK3β-mediated) antibodies. (B) Inhibition of ROCK activity increases β-catenin acetylation and phosphorylation. Cell lysates treated with the ROCK inhibitor Y-27632 (10 μM) or vehicle were normalized and immunoblotted as described in (A). (C) TPPP1 expression increases β-catenin acetylation and CK1/GSK3β-mediated phosphorylation. Extracts from U2OS cells expressing wild-type TPPP1, TPPP1^{3Ala}, TPPP1^{3Glu} and vector normalized for β-catenin levels were analyzed by immunoblotting. Membranes were probed for acetyl-β-catenin (Lys-49), phospho-(Ser-45)-β-catenin (CK1-mediated, phospho-(Ser-31/S37/Thr-41)-β-catenin (GSK3β-mediated) and total β-catenin. (D) TPPP1 knockdown decreases β-catenin acetylation and CK1/GSK3 β-mediated phosphorylation. Cell extracts of U2OS cells transiently transfected with TPPP1 or non-targeting (NT) siRNA were analyzed as described in (C). (E) Inhibition of HDAC activity negates TPPP1 knockdown induced increases in β-catenin levels. U2OS cells transiently transfected with TPPP1 or NT siRNA for 48 h were treated with 100 nM Trichostatin A (TSA) or vehicle (DMSO) for 16 h and analyzed by immunoblotting. Membranes were probed for β-catenin, acetyl-α-tubulin, α-tubulin, TPPP1 and GAPDH (loading control).

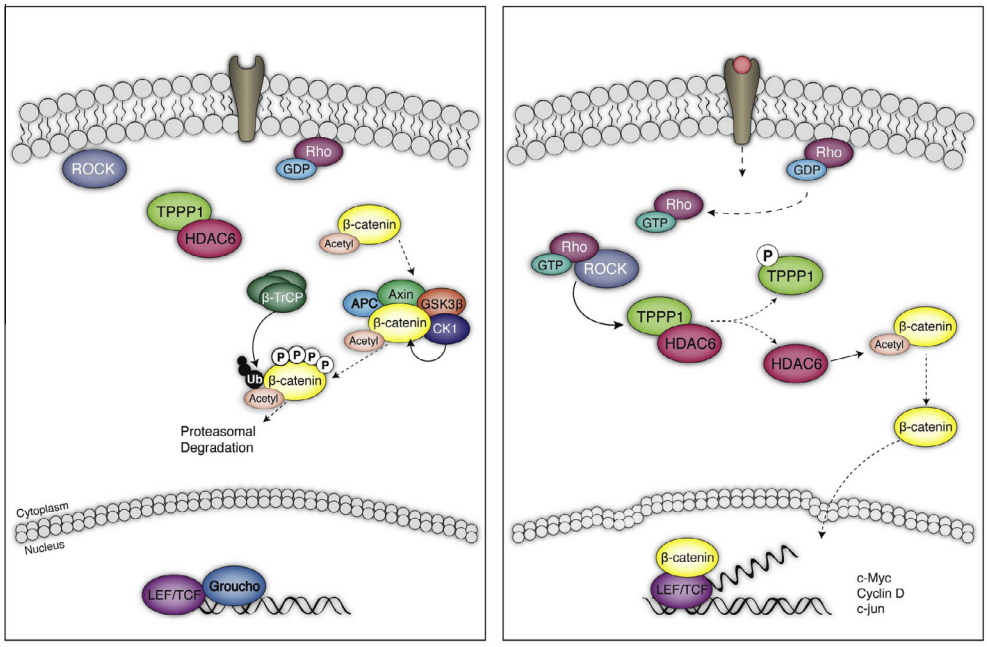

Fig. 4. ROCK-TPPP1 signaling regulates β-catenin levels. Under basal unstimulated conditions TPPP1 binds to and inhibits HDAC6 activity to increase β-catenin acetylation. Acetylated β-catenin binds to the 'destruction complex", which is composed of Adenomatous polyposis coli (APC), axin, Casein kinase 1 (CK1), and Glycogen synthase kinase 3β (GSK3β), and is phosphorylated (P) by both kinases. Thereby promoting its ubiquitination and proteasomal degradation, leading to decreased total β-catenin levels in cells (left panel). Activation of the Rho-ROCK signaling pathway results in TPPP1 phosphorylation. This negates the HDAC inhibitory TPPP1/HDAC6 interaction leading to decreased β-catenin acetylation and phosphorylation. As a consequence, β-catenin does not bind to the ubiquitin E3 ligase β-transducin repeat-containing protein (β-TrCP), thereby preventing its ubiquitination (Ub) and proteasomal degradation, which results in increased total β-catenin levels in cells (right panel).

U2OS cells, transiently transfected with TPPP1 or NT siRNA, with 100 nM of the broad-spectrum HDAC inhibitor Trichostatin A (TSA) or vehicle (DMSO) and analyzed total β-catenin levels. The results demonstrate that inhibition of HDAC activity decreased β-catenin levels in control NT siRNA transfected cells compared to the DMSO control (Fig. 3E). However, inhibition of HDAC activity in TPPP1 siRNA treated cells resulted in the inhibition of TPPP1 knockdown-mediated increases in β-catenin expression, therefore further supporting a role for β-catenin acetylation in the regulation of its stability. Interestingly, treatment of cells with TSA resulted in an additional unanticipated increase in endogenous TPPP1 levels, suggesting that TPPP1 acetylation may increase its stability.

4. Discussion

Our results reveal a novel inverse correlation between TPPP1 and β-catenin protein levels and its transcriptional target c-Myc, which is inhibited by ROCK-mediated TPPP1 phosphorylation. These findings describe a novel biochemical pathway, whereby ROCK signaling increases β-catenin expression through prevention of TPPP1-mediated HDAC6 inhibition. Specifically, our data show that enhanced ROCK activity is accompanied by reduced β-catenin acetylation and phosphorylation by the 'destruction complex' kinases CK1 and GSK3β, which is likely to increase total β-catenin levels through reducing its proteasomal degradation (Fig. 4).

We recently established that ROCK activation increased U2OS cell proliferation [22]. Additionally, our study showed that TPPP1 overexpression decreased cell proliferation, via delaying the G_1/S-phase and Mitosis to G_1-phase transitions, and that ROCK-mediated TPPP1 phosphorylation inhibits its regulation of cell growth [22]. These results suggest that ROCK signaling and TPPP1 regulate cell proliferation through modulation of β-catenin expression and its transcriptional targets. This is supported by previous studies demonstrating that β-catenin levels are increased during S-phase [23] and that activation of ROCK is necessary for cells to transit into S-phase [2]. Moreover, we demonstrated that inhibition of TPPP1-mediated HDAC6 regulation promotes S-phase entry [22].

The present study also demonstrates that ROCK activation and TPPP1 knockdown result in increased β-catenin expression, which correlates with its reduced phosphorylation by the "destruction complex" kinases CK1 and GSK3β as well as acetylation on Lys-49. Conversely, we show that reduced ROCK activity and TPPP1 overexpression result in decreased β-catenin levels that are paralleled by increases in its phosphorylation and acetylation. Although phosphorylation of β-catenin is an established pathway that ultimately results in its proteasomal degradation, the role of β-catenin acetylation is not yet established. Given that β-catenin acetylation correlates with its phosphorylation, this is suggestive of a role for acetylation in the regulation of its stability, which is supported by data demonstrating that expression of the β-catenin K49R mutant increases c-Myc expression compared to wild-type [11].

Similarly, overexpression of HDAC6 increased c-Myc levels while inhibition of its activity decreased c-Myc [20] and cyclin D1 expression [24]. Although TPPP1 modulation of β-catenin acetylation is likely to be its primary biochemical mechanism for the regulation of total β-catenin levels, ROCK signaling is likely to additionally contribute to other facets of the Wnt signaling pathway. For example, mouse bone marrow macrophages isolated from heterozygous ROCK1 knockout mice exhibit enhanced phosphorylation of GSK3β by cGMP-dependent protein kinase II (cGKII) [25] and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [26] which inhibits GSK3β activity [27], suggesting that ROCK1 may regulate the "destruction complex" assembly and activity. In addition, phosphorylation of β-catenin by the Rac1 effector p21-activated kinase 1 (PAK1) promotes its stabilization [28], whereas knockdown or inhibition of PAK1 activity decreases β-catenin and c-Myc expression [29,30]. As ROCK reduces Rac1-PAK1 pathway activation through inhibition of the Rac1 Guanine-exchange factor (GEF) STEF [31], ROCK signaling potentially modulates β-catenin stability via inhibition of Rac1 signaling. Furthermore, the RhoA-ROCK pathway is activated in response to the binding

of Wnt-3 [32], Wnt-3a [33], Wnt-5 and Wnt-11 [34] ligands to the Frizzled/LDLR5/6 co-receptor complex through the promotion of a complex between dishevelled (Dvl) and disheveled-associated activator of morphogenesis 1 (Daam1) that binds RhoA and promotes its activity [35]. Therefore, these results suggest that the ROCK-TPPP1 pathway is not the only mechanism by which ROCK signaling regulates β-catenin levels.

In conclusion, our study shows that β-catenin expression correlates with increased ROCK signaling and with reduced TPPP1 levels in U2OS cells. ROCK-mediated TPPP1 phosphorylation, which prevents its binding to HDAC6, negates TPPP1-mediated reduction of β-catenin expression. Furthermore, increased HDAC6 activity resulting from ROCK signaling activation reduced β-catenin acetylation at Lys-49, which was also accompanied by its decreased phosphorylation by Caesin kinase 1 (CK1) and Glycogen synthase kinase 3β (GSK3β), thus preventing its proteasomal degradation. Overall, our results suggest that ROCK regulates β-catenin stability in cells via preventing TPPP1-mediated inhibition of HDAC6 activity, to reduce its acetylation and degradation via phosphorylation by CK1 and GSK3β (Fig. 4).

Acknowledgments

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