Rho-associated Coiled-coil Kinase (ROCK) Protein Controls Microtubule Dynamics in a Novel Signaling Pathway That Regulates Cell Migration

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Background: ROCK regulates microtubule acetylation.

Results: ROCK phosphorylation of TPPP1/p25 inhibits the interaction between TPPP1 and HDAC6, resulting in increased HDAC6 deacetylation of microtubules, leading to increased cell motility.

Conclusion: ROCK phosphorylation of TPPP1 is a novel signaling pathway that regulates cell migration via increased HDAC6 activity and reduced MT acetylation.

Significance: This newly discovered ROCK/TPPP/HDAC6/MT signaling pathway might have important implications for cell motility and invasion.

The two members of the Rho-associated coiled-coil kinase (ROCK1 and 2) family are established regulators of actin dynamics that are involved in the regulation of the cell cycle as well as cell motility and invasion. Here, we discovered a novel signaling pathway whereby ROCK regulates microtubule (MT) acetylation via phosphorylation of the tubulin polymerization promoting protein 1 (TPPP1/p25). We show that ROCK phosphorylation of TPPP1 inhibits the interaction between TPPP1 and histone deacetylase 6 (HDAC6), which in turn results in increased HDAC6 activity followed by a decrease in MT acetylation. As a consequence, we show that TPPP1 phosphorylation by ROCK increases cell migration and invasion via modulation of cellular acetyl MT levels. We establish here that the ROCK-TPPP1-HDAC6 signaling pathway is important for the regulation of cell migration and invasion.

The dynamic reorganization of the MT network during cell division and migration relies on a precise balance between MT growth, stabilization, and depolymerization. Several known mechanisms modulate these events, including MT-associated proteins that promote MT polymerization and depolymerization, as well as MT posttranslational modifications, including phosphorylation, acetylation, polyglutamation, and detyrosination, that regulate MT stability. Importantly, acetylation of MTs correlates with polymer stabilization (9, 10). MT-bound α-tubulin subunits are reversibly acetylated on lysine 40 (Lys-40) by αTAT1 (11), MEC-17 (12), and the Elongator complex (13) acetyl transferases, whereas its deacetylation is regulated by histone deacetylase 6 (HDAC6) (14) and Sir2-like 2 (SIRT2) (15, 16).

It was suggested that tubulin acetylation is regulated by Rho-GTPase signaling. Treatment of cells with the Rho inhibitor C3 exoenzyme (17) or the ROCK inhibitor Y-27632 increases acetyl-MT levels (18–21), whereas overexpression of the constitutively active Rho mutant, Rho-V14, decreases its levels (17). Although it has been described that Rho regulates HDAC6 activity in osteoclasts via Rho-mediated inhibition of the HDAC6/mDia2 interaction that reduces its deacetylase activity (17), the role of ROCK signaling in the regulation of MT acetylation is yet to be elucidated.

TPPP1/p25 is a ubiquitously expressed 25-kDa protein belonging to the tubulin polymerization promoting protein (TPPP) family that includes TPPP2 (p20) and TPPP3 (p18) (22, 23). Overexpression of TPPP1 in cells promotes MT polymerization and an increase in MT acetylation, whereas introduction of TPPP1 RNAi reduces MT acetylation (24). TPPPP1 regulates MT acetylation via binding to HDAC6 and inhibiting its activity (25). Recent reports suggest that in vitro TPPP1 activity is regulated by the ROCK substrates LIM kinase 1 and 2 (LIMK1 and 2) (24, 26).

We report here that ROCK-mediated phosphorylation of TPPP1 inhibits the TPPP1/HDAC6 interaction to drive a decrease in acetylated MT levels in cells, therefore resulting in increased cell migration and invasion.

The abbreviations used are: ROCK, Rho-associated coiled-coil kinase; MLC, myosin light chain; MT, microtubule; Abs, antibodies; pMLC, phospho-myosin light chain; LIM, Lin-11, Isl-1, Mec-3.
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EXPERIMENTAL PROCEDURES

**Plasmid Constructs**—pBABE-FLAG-TPPP1, pGEX4T1-FLAG-ROCK1, pEF-BOS-FLAG-LIMK1, and pEF-BOS-FLAG-LIMK2 (24) were described previously. Constitutively active pcDNA3-FLAG-ROCK1Δ4 (amino acids 1–477) and pcDNA-FLAG-ROCK1Δ4-KD (amino acids 1–477, K105G) were a generous gift from Dr. S. Narumiya (Kyoto University, Japan). The TPPP1 alanine substitution mutants were synthesized by Geneart (Invitrogen) and cloned into the BamH1 and Xho1 sites of the pBabe-FLAG vector. The WT and alanine substitution mutants were cloned into the pEF-BOS-FLAG-LIMK1 vector as described previously. Constitutively active ROCK1Δ4 was purified from Sf9 insect cells by resuspension in buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Triton-X-100) and lysis in a French press at 10,000 p.s.i. followed by centrifugation at 20,000 × g for 30 min. ROCK1Δ4 was purified by incubation with anti-FLAG M2-agarose (Sigma) for 2 h at 4 °C, followed by washes with resuspension buffer and elution with 1 μg/ml FLAG peptide (Sigma) for 30 min at room temperature. GST-TPPP1 and GST-cofilin proteins were expressed and purified from bacteria by incubation with glutathione-Sepharose 4B (GE Life Sciences). GST-LIMK1 was purified from HEK293T cells as described above. All proteins were dialyzed in TBS.

**Immunoprecipitation**—U2OS cell extracts were precleared with ~2 μg of isotype control and 30 μl of protein A- or G-Sepharose (Amersham Biosciences) for 1 h at 4 °C. Cleared lysates were incubated with ~2 μg of rat IgG2a, rat anti-TPPP1 mAb (24), mouse IgG or mouse anti-HDAC6 mAb, and 50 μl of protein A/G-Sepharose and incubated at 4 °C overnight. FLAG immunoprecipitation was performed as described above.

**Immunoblotting**—Membranes were probed overnight in 5% BSA with the following antibodies: anti-α-tubulin (1:5000), anti-acetyl-α-tubulin (1:5000), anti-pMLC Thr-18/Ser-19 (1:1000) (Cell Signaling Technology), anti-FLAG 9H1 mAb clone (28) (1:1000), anti-GST (1:3000) (Millipore), and anti-TPPP1 mAb or polyclonal antibodies (24).

**In vitro Kinase Assays**—In vitro kinase assays were performed at a molar ratio of 1:40 kinase:substrate. Proteins were incubated with 5 μCi [γ-32P]ATP and 30 μM ATP in buffer containing 20 mM Hapes (pH 7.4), 10 mM MgCl2, 10 mM NaF, 1 mM Na3VO4, and complete protease inhibitor tablet (Roche) at 37 °C for 10 min. Reactions in the presence of 5 μM Y-27632 or 100 nM 22 were preincubated for 10 min prior to the addition of ATP. Assays were analyzed by SDS-PAGE and followed by autoradiography.

** Scratch-induced Migration and Invasion Assays**—Wound closure assays were performed by scratching confluent cell monolayers preincubated with 10 μg/ml mitomycin C (Sigma) for 2 h with a pipette tip coated in 100% FBS. At 0 time and 18 h post-scratch, phase-contrast images were acquired. Matrigel invasion assays were performed with cells treated with mitomycin C as described above using growth factor-reduced BD Matrigel™ Basement Membrane Matrix (BD Biosciences) according to the recommendations of the manufacturer.

**In vitro Tubulin Polymerization Assays**—In vitro tubulin polymerization assays were performed using the tubulin polymerization assay kit (catalog no. BK006P, Cytoskeleton). Briefly, 2 μM of GST or GST-p25 was phosphorylated in vitro with purified FLAG-ROCK1Δ4 and 30 μM ATP. Reactions were mixed with general tubulin buffer (80 mM PIPES (pH 6.9), 2 mM MgCl2, 0.5 mM EGTA), tubulin glycerol buffer (1 mM GTP with the addition of 5% glycerol), and 30 μg of purified tubulin. Tubulin turbidity was measured at 37 °C using a Polarstar optima plate reader configured to measure absorbance at A340 nm every minute for 40 min with the plate shaking every 10 s.

**Immunofluorescence Microscopy**—Cells fixed in 100% methanol were blocked in 10% FBS prior to incubation with mouse anti-acetyl-α-tubulin (1:1000) and FITC-conjugated anti-α-tu-
bulin (1:200) (Sigma) antibodies. Cells were then incubated with Hoechst (1:10,000) and secondary Abs (anti-mouse Alexa Fluor 488 and anti-rat Alexa Fluor 594 (1:400)) (Invitrogen). Images were captured on a Nikon C1 confocal microscope.

Tandem MS/MS—GST-TPPP1 was digested from SDS-PAGE gel slices as described (29). Mass spectrometry was performed with an ABSciex tripleTOF equipped with a nano III source, running an information-dependent data acquisition program to fragment peptides, and the data were analyzed with Proteinpilot 4 software.

Data Analysis—Statistical analysis was performed with using two-tailed unpaired Student’s t-tests.

RESULTS

ROCK Signaling Modulates Tubulin Acetylation—Inhibition of ROCK activity results in increased MT acetylation in cells (18–21). We therefore endeavored to identify the mechanism by which ROCK controls acetyl tubulin levels. First, we treated the U2OS osteosarcoma cell line with the ROCK inhibitor Y-27632 (10 μM) or vehicle and analyzed acetyl-α-tubulin levels by immunoblotting (Fig. 1A) and immunofluorescence microscopy (Fig. 1B). In accordance with data published previously, inhibition of ROCK activity, as confirmed by decreased phospho-MLC (pMLC) levels, resulted in increased cellular MT
acetylation. To further investigate the role of ROCK signaling in the regulation of MT acetylation, we transiently overexpressed the constitutively active FLAG-ROCK1 or kinase dead ROCK1 (ROCK1-KD) truncated proteins in U2OS cells. Overexpression of ROCK1 but not of ROCK1-KD decreased acetyl-α-tubulin levels compared with the vector expressing cells, as shown by immunoblotting (Fig. 1C) and immunofluorescence microscopy (D). These results indicate that ROCK1 and/or its downstream effectors regulate MT acetylation in cells.

ROCK Phosphorylates TPPP1 in Vitro and in Cells—MT acetylation is regulated by two deacetylases, HDAC6 and SIRT2. HDAC6 activity is inhibited through its interaction with TPPP1, resulting in increased MT acetylation (25). We therefore hypothesized that ROCK phosphorylation of TPPP1 affects its interaction with HDAC6. To test this hypothesis, we first examined whether TPPP1 is a ROCK substrate. In vitro kinase assays were performed with GST-LIMK1 (1 nM) and GST-cofilin (40 nM) in the presence of 10% FBS and analyzed as described. TPPP1 is phosphorylated by ROCK in cells. Lysates from HEK293T cells transfected with F-TPPP1 alone or together with F-LIMK1 (left panels) or F-LIMK2 (right panels) were immunoprecipitated with GST beads and analyzed by autoradiography and immunoblotting. Lysates were analyzed for LIMK1, LIMK2, GAPDH (loading control), and p-cofilin as a measure of LIMK activity.

**FIGURE 2.** ROCK phosphorylates TPPP1 in vitro and in cells. A, TPPP1 is an in vitro ROCK1 substrate. ROCK1 (1 nM) and GST-TPPP1 (40 nM) were incubated in the presence of [γ-32P]ATP (lane 3). Reactions were preincubated with the ROCK inhibitor Y-27632 (5 μM) (lane 4) or with the LIMK inhibitor 22j (100 nM) (lane 5). TPPP1 phosphorylation was analyzed by autoradiography (AR) (top panel). IB, immunoblot. B, 22j inhibits LIMK1-mediated cofilin phosphorylation. In vitro kinase assays were performed with GST-LIMK1 (1 nM) and GST-cofilin (40 nM) in the presence of 10% FBS and analyzed as described. Lysates were blotted for pMLC as a control for ROCK activity. The numbers below the top panel represent the ratio between the values of lanes 1 and 2 (0.6), lanes 1 and 3 (3), and lanes 1 and 4 (1.3). D, U2OS cells expressing F-TPPP1 were treated with Y-27632 (10 μM) or vehicle and analyzed as described in C. D, LIMK1 or LIMK2 do not phosphorylate TPPP1 in cells. HEK293T cells were transfected with GST-TPPP1 alone or together with F-LIMK1 (left panels) or F-LIMK2 (right panels) or (E) with LIMK1 or LIMK2 siRNA and metabolically labeled with [32P]orthophosphate. GST-TPPP1 was pulled-down (PD) and analyzed by autoradiography and immunoblotting. Lysates were analyzed for LIMK1, LIMK2, GAPDH (loading control), and p-cofilin as a measure of LIMK activity.
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22j (30), as it was suggested that the LIMKs phosphorylate TPPP1 (24, 26). We showed that although inhibition of LIMK activity decreased the phosphorylation of its substrate cofilin (Fig. 2B), the level of TPPP1 phosphorylation by ROCK was unchanged (A, lane 5), therefore confirming that TPPP1 is phosphorylated by ROCK in vitro.

To confirm that TPPP1 is also an in vivo ROCK substrate, we analyzed the effect of ROCK inhibition on TPPP1 phosphorylation in cells. We metabolically labeled HEK293T or U2OS cells overexpressing FLAG-TPPP1 (F-TPPP1) with 32P-or-thophosphate in the presence or absence of 10 μM Y-27632. We demonstrated that inhibition of ROCK activity, demonstrated by decreased pMLC levels in cell lysates, significantly decreased TPPP1 phosphorylation compared with the controls in HEK293T (Fig. 2C Lanes 1 and 2) as well as in U2OS cells (Fig. 2D). Furthermore, although TPPP1 phosphorylation was greatly increased in cells stimulated with 10% FBS (3-fold) (Fig. 2C, lanes 1 and 3), inhibition of ROCK activity in FBS-stimulated cells reduced TPPP1 phosphorylation 2-fold in comparison to FBS only-treated cells (C, lanes 3 and 4). These results strongly suggest that TPPP1 is phosphorylated by ROCK, not only in vitro but also in cells.

To rule out the possibility that the ROCK-activated kinases LIMK1 and 2 are responsible for the phosphorylation of TPPP1 in vivo, which has been suggested previously (24, 26), we labeled HEK293T cells overexpressing FLAG-LIMK1 (F-LIMK1), FLAG-LIMK2 (F-LIMK2), or vector control with 32P-orthophosphate and analyzed the level of TPPP1 phosphorylation. Overexpression of LIMK1 or 2 did not alter the level of TPPP1 phosphorylation, despite increased phosphorylation of their established substrate cofilin (Fig. 2E). Furthermore, RNAi-mediated down-regulation of LIMK1 or LIMK2 levels had no effect on TPPP1 phosphorylation (Fig. 2F). Overall, these results indicate that ROCK phosphorylates TPPP1 directly in vitro and in cells.

ROCK Phosphorylates TPPP1 on Ser-32, Ser-159, and Ser-107—We next identified the TPPP1 phosphorylation sites. Initially, we characterized TPPP1 phosphorylation stoichiometry, which revealed that it was phosphorylated on three serine residues by ROCK (data not shown). Subsequently, we analyzed the in vivo TPPP1 phosphorylation sites by MS/MS and identified that TPPP1 is phosphorylated on three potential ROCK phosphorylation motifs (R/K XX S/T or R/K X S/T) (31, 32) in cells: Ser-32, Ser-107, and Ser-159 (data not shown).

To confirm that these sites are phosphorylated by ROCK, we generated mutants bearing single alanine substitutions and performed in vitro kinase assays with F-ROCK1. Fig. 3A demonstrates that a single mutation of Ser-32, Ser-107, or Ser-159 results in decreased TPPP1 phosphorylation compared with their wild-type counterpart. We therefore generated a triple TPPP1 S32A/S107A/S159A mutant construct, designated here as TPPP13Ala. In vitro kinase assays with this tri-
alanine TPPP1 mutant completely abolished TPPP1 phosphorylation by ROCK1 (Fig. 3B, top panel), thereby confirming that ROCK1 phosphorylates TPPP1 on Ser-32, Ser-107, and Ser-159.

ROCK Phosphorylation of TPPP1 Does Not Alter Its MT Polymerizing Activity—TPPP1 has two established cellular functions. It promotes MT polymerization and regulates HDAC6 activity (25, 33). To evaluate the impact of TPPP1 phosphorylation by ROCK on its MT polymerization activity, we performed in vitro MT polymerization assays with bacterially purified GST-TPPP1 that was phosphorylated in vitro with F-ROCK1. Both the wild-type and the ROCK1 phosphorylated GST-TPPP1 increased the rate and level of MT polymerization compared with spontaneous MT polymerization (Fig. 3C), therefore suggesting that TPPP1 phosphorylation by ROCK does not alter its MT polymerizing activity.

To confirm this in cells, we established stable U2OS cell lines expressing F-TPPP1, F-TPPP13Ala, F-TPPP13Glu (phospho-mimetic mutant), or vector alone and analyzed their MT network by staining with anti-α-tubulin Abs, followed by immunofluorescence microscopy. Overexpression of all three TPPP1 proteins resulted in increased MT levels compared with the vector-expressing cells (Fig. 3D), confirming that TPPP1 phosphorylation by ROCK does not alter its MT polymerizing activity.

ROCK Phosphorylation of TPPP1 Inhibits its HDAC6 Regulatory Activity—We next evaluated the impact of TPPP1 phosphorylation by ROCK on its HDAC6 regulatory activity. TPPP1 modulates HDAC6 activity via an inhibitory binding mechanism (25). Therefore, we initially analyzed the impact of Y-27632 treatment of U2OS cells on the interaction between endogenous HDAC6 and TPPP1 proteins. We first immunoprecipitated TPPP1 from these lysates and analyzed its ability to coprecipitate HDAC6. Inhibition of ROCK activity increased the interaction between TPPP1 and HDAC6 compared with vehicle-treated cells (Fig. 4A). Similarly, immunoprecipitation of endogenous HDAC6 from these lysates demonstrated that inhibition of ROCK activity increased its ability to interact with TPPP1 (Fig. 4B).

ROCK Phosphorylation of TPPP1 Results in Increased MT Acetylation—We have demonstrated that ROCK-mediated phosphorylation of TPPP1 inhibits its HDAC6 binding resulting in increased HDAC6 activity. To confirm these results, we analyzed the interaction between endogenous HDAC6 and wild-type F-TPPP1, F-TPPP13Ala, or F-TPPP13Glu overexpressed in U2OS cells. F-TPPP1 immunoprecipitation demonstrated that TPPP1 and TPPP13Ala interacted with HDAC6, with TPPP13Ala exhibiting a higher binding affinity to HDAC6 (Fig. 4C). In contrast, F-TPPP13Glu did not interact with HDAC6. Similarly, immunoprecipitation of HDAC6 revealed that it interacts with F-TPPP1 and F-TPPP13Ala but not with F-TPPP13Glu (Fig. 4D). The interaction between HDAC6 and TPPP1 is less efficient than the interaction between HDAC6 and F-TPPP13Ala, suggesting that wild-type TPPP1 is partially phosphorylated in cells. These results indicate that phosphorylation of TPPP1 by ROCK inhibits its binding to HDAC6.

To show that increased HDAC6 activity leads to increased tubulin deacetylation in U2OS cells overexpressing the TPPP1 proteins described above, we performed a Western blot analysis for acetyl-α-tubulin. Expression of TPPP1 and TPPP13Ala increased acetyl-α-tubulin levels (Fig. 4E), whereas acetyl-α-tubulin levels in cells expressing TPPP13Glu, which mimics phospho-TPPP1, remained similar to that of vector-expressing cells. Immunofluorescence staining of acetylated MTs confirmed that both TPPP1 and TPPP13Ala expression increased acetylated MT levels compared with control cells, whereas expression of TPPP13Glu had no effect (Fig. 4F).

Taken together, these results clearly demonstrate that phosphorylation of TPPP1 by ROCK inhibits its interaction with HDAC6, thereby resulting in increased HDAC6 activity in cells, without altering TPPP1-mediated regulation of MT polymerization.

TPPP1 Regulates Cell Migration and Invasion—It is well established that MT acetylation is involved in the regulation of cell migration (34). Therefore, we next characterized the role of the ROCK-TPPP1-HDAC6 signaling pathway in cell migration and invasion. We first analyzed cell migration by performing scratch-induced wound healing migration assays. Overexpression of TPPP1 significantly reduced wound-closure 18 h post-wounding compared with the control cells (Fig. 5, A and B). Similarly, overexpression of TPPP13Ala significantly reduced cell migration compared with both control and wild-type TPPP1 expressing cells, which is likely due to partial wild-type TPPP1 phosphorylation. In contrast, expression of TPPP13Glu increased wound healing compared with control cells (Fig. 5, A and B), suggesting that TPPP1 MT polymerizing activity contributes to cell migration. Finally, we compared the migration of U2OS cells transfected with TPPP1 or control siRNAs. Knockdown of TPPP1 significantly increased wound closure compared with controls (Fig. 5, C and D). These results clearly suggest that TPPP1-mediated regulation of HDAC6 activity leads to the inhibition of cell migration. Further analysis of the role of ROCK signaling in U2OS cell migration revealed that overexpression of ROCK increased wound-closure, whereas inhibition of its activity reduced wound-closure rates (supplemental Fig. 1, A–D). Therefore, we confirm that ROCK-mediated modulation of the TPPP1/HDAC6 interaction is important for the regulation of cell migration.

To study the role of TPPP1 in cell invasion, we performed Matrigel invasion assays and analyzed the migration of stable U2OS cells through Basement Membrane. Overexpression of TPPP1 and TPPP13Ala significantly reduced the invasiveness of these cells compared with the control (Fig. 5E), whereas overexpression of TPPP13Glu significantly increased their invasiveness. Furthermore, knockdown of TPPP1 significantly increased the invasiveness of cells (Fig. 5F). Similarly, ROCK overexpression and inhibition increase and decrease cell invasion, respectively (supplemental Fig. 1, E and F). These results demonstrate that TPPP1, via ROCK phosphorylation, regulates cell migration and invasion through its dual regulation of MT polymerization and HDAC6 activity.

DISCUSSION

We describe here a novel signaling pathway initiated by ROCK phosphorylation of TPPP1. This pathway, which includes ROCK-TPPP1-HDAC6, leads to increased HDAC6...
activity, resulting in decreased MT acetylation. Overall, the activation of this novel pathway by ROCK results in enhanced cell motility and invasion via decreased tubulin acetylation (Fig. 6).

ROCK1 and 2 have a large number of substrates and are important regulators of the actin cytoskeleton through phosphorylation of the LIMKs, MLC, and the myosin-binding subunit of the MLC phosphatase. Here we identified a novel MT regulatory ROCK substrate, TPPP1. TPPP1 is phosphorylated in vitro and in cells on multiple sites. These sites are phosphorylated in vitro by CDK5, ERK2, and protein kinase A, but only TPPP1 phosphorylation by ERK2 inhibited its tubulin polymerization activity (35). We show that TPPP1 phosphorylation by ROCK has no effect on its tubulin polymerizing activity, suggesting that the conformational changes induced by ROCK phosphorylation do not alter its interaction with MTs. We also clearly demonstrate that ROCK1, and not the LIMKs, is responsible for TPPP1 phosphorylation, as claimed previously (24, 26). As the ROCKs strongly interact and copurify with the LIMKs, the previously published findings are likely to reflect TPPP1 phosphorylation by ROCK but not by LIMKs, as demonstrated in this study.

FIGURE 4. ROCK phosphorylation of TPPP1 inhibits HDAC6 binding and tubulin acetylation. A, inhibition of ROCK activity increases the interaction between TPPP1 and HDAC6. U2OS cell extracts treated with Y-27632 (lane 4) or vehicle (lane 3) were incubated with IgG (lane 2) or rabbit anti-TPPP1 polyclonal Abs (lanes 3 and 4). The immunoprecipitated proteins (IP) and total cell extracts were analyzed by immunoblotting (IB) for TPPP1 and HDAC6. B, immunoblots of extracts from A immunoprecipitated with mouse anti-HDAC6 mAb and probed as in A. C, ROCK phosphorylation of TPPP1 inhibits its binding to HDAC6. Extracts from U2OS cells expressing F-TPPP1 and its mutants were purified with anti-FLAG M2-agarose, and total lysates and IP were immunoblotted and probed with anti-HDAC6 and anti-FLAG mAbs. D, cell extracts from C were immunoprecipitated with anti-HDAC6 or IgG Abs. Total lysates and IPs were analyzed by immunoblotting as in C. E, immunoblot of lysates from U2OS cells stably expressing F-TPPP1 and its mutants were analyzed for acetyl tubulin, α-tubulin, GAPDH (loading control), and F-TPPP1. F, the U2OS cell lines described in E were stained for nuclei (blue) and acetylated MTs (green) and analyzed by immunofluorescence microscopy. Scale bar = 50 mm. The numbers below the top panels in A, B, D, and E represent the fold changes in the indicated protein levels.
The interaction between TPPP1 and HDAC6 results in the inhibition of HDAC deacetylase activity and, therefore, increased MT acetylation. We show here that phosphorylation of TPPP1 by ROCK prevents its interaction with HDAC6, resulting in an increase in its deacetylase activity. Our findings establish, for the first time, that ROCK signaling regulates tubulin acetylation through its phosphorylation of TPPP1. These findings reveal a mechanism by which ROCK regulates MT acetylation and confirm previous studies demonstrating that Rho-ROCK inhibition increases acetyl-α-tubulin levels (18–21), whereas Rho activation decreases it (17).

Tubulin acetylation is very important for many cellular processes, including cell migration and invasion. Previous studies linked increased tubulin acetylation with reduced cell motility.
and invasion (36, 37), whereas overexpression of HDAC6, which reduced tubulin acetylation, increased their invasiveness (38), thereby suggesting that aberrant tubulin acetylation may contribute to malignancies. Our data establish that overexpression of TPPP1 reduces cell migration and invasion, whereas its down-regulation increases cell migration and invasion. Similarly, ROCK-mediated TPPP1 phosphorylation, which inhibits its interaction of HDAC6, thus resulting in reduced MT acetylation, increases cell migration and invasion. Although it is likely that TPPP1-mediated regulation of tubulin acetylation is primarily responsible for its regulation of cell migration, it cannot be ruled out that dual regulation of HDAC6 and tubulin polymerization by TPPP1 contribute to decreased cell migration. Our studies and those of others show that activation of ROCK contributes to increased cell migration and invasion (39) and that inhibition of ROCK signaling inhibits cell migration (40), therefore strengthening our observation that ROCK-TPPP1 signaling increases cell migration.

In conclusion, our work has identified TPPP1 as a novel ROCK substrate. When TPPP1 is phosphorylated by ROCK, it is unable to bind to HDAC6 and inhibit its activity, resulting in a decreased cellular level of acetylated tubulin and increased cell migration (Fig. 6).

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