Corrections

MEDICAL SCIENCES. For the article "Dynamic interplay between nitration and phosphorylation of tubulin cofactor B in the control of microtubule dynamics," by Suresh K. Rayala, Emil Martin, Iraida G. Sharina, Poonam R. Molli, Xiaoping Wang, Raymond Jacobson, Ferid Murad, and Rakesh Kumar, which appeared in issue 49, December 4, 2007, of *Proc Natl Acad Sci USA* (104:19470–19475; first published November 28, 2007; 10.1073/pnas.0705149104), the authors note that, due to a printer's error, Fig. 2 appeared incorrectly. This error does not affect the conclusions of the article. The corrected figure and its legend appear below.

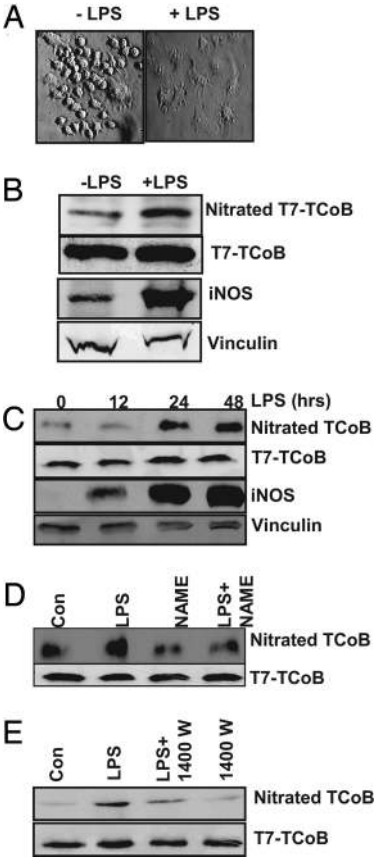

Fig. 2. Nitration of TCoB is iNOS-dependent. (*A*) Representative transmission images showing change in morphology of RAW 264.7 cells after LPS/γ-IFN treatment. (*B*) T7-TCoB was immunoprecipitated from transiently transfected RAW 264.7 cells that were treated with LPS (1 μg/ml)/γ-IFN (50 units/ml) for 16 h, separated by SDS/PAGE, and immunoblotted with the indicated antibodies. (*C*) T7-TCoB was immunoprecipitated from transiently transfected RAW 264.7 cells that were treated with LPS/γ-IFN at various time points, separated by SDS/PAGE, and immunoblotted with the indicated antibodies. (*D* and *E*) T7-TCoB was immunoprecipitated from transiently transfected RAW 264.7 cells that were pretreated with either 1400W (100 μM) or L-NAME (10 mM) for 4 h and/or LPS/γ-IFN, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies.

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GENETICS. For the article "Distinctive patterns of microRNA expression in primary muscular disorders," by Iris Eisenberg, Alal Eran, Ichizo Nishino, Maurizio Moggio, Costanza Lamperti, Anthony A. Amato, Hart G. Lidov, Peter B. Kang, Kathryn N. North, Stella Mitrani-Rosenbaum, Kevin M. Flanigan, Lori A. Neely, Duncan Whitney, Alan H. Beggs, Isaac S. Kohane, and Louis M. Kunkel, which appeared in issue 43, October 23, 2007, of *Proc Natl Acad Sci USA* (104:17016–17021; first published October 17, 2007; 10.1073/pnas.0708115104), the authors note that the affiliation information for authors Maurizio Moggio and Costanza Lamperti was incorrect in part. Their correct affiliation is "Unità Operativa di Neurologia, Centro Dino Ferrari, Università degli Studi di Milano, Istituto di Ricovero e Cura a Carattere Scientifico Fondazione Ospedale Maggiore, 20122 Milano, Italy." The corrected affiliation line appears below.

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Dynamic interplay between nitration and phosphorylation of tubulin cofactor B in the control of microtubule dynamics

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Tubulin cofactor B (TCoB) plays an important role in microtubule dynamics by facilitating the dimerization of α- and β-tubulin. Recent evidence suggests that p21-activated kinase 1 (Pak1), a major signaling nodule in eukaryotic cells, phosphorylates TCoB on Ser-65 and Ser-128 and plays an essential role in microtubule regrowth. However, to date, no upstream signaling molecules have been identified to antagonize the functions of TCoB, which might help in maintaining the equilibrium of microtubules. Here, we discovered that TCoB is efficiently nitrated, mainly on Tyr-64 and Tyr-98, and nitrated-TCoB attenuates the synthesis of new microtubules. In addition, we found that nitration of TCoB antagonizes signaling-dependent phosphorylation of TCoB, whereas optimal nitration of TCoB requires the presence of functional Pak1 phosphorylation sites, thus providing a feedback mechanism to regulate phosphorylation-dependent MT regrowth. Together these findings identified TCoB as the third cytoskeleton protein to be nitrated and suggest a previously undescribed mechanism, whereby growth factor signaling may coordinately integrate nitric oxide signaling in the regulation of microtubule dynamics.

A dynamic cytoskeleton is critically important for numerous essential cellular processes like cell motility, phagocytosis, mitosis, and cytokinesis. The cytoskeleton undergoes major reorganization in response to a multitude of extracellular signals. Microtubules (MTs) constitute one of the major networks of dynamic cytoskeletal polymers (1). In general, the actin, intermediate filaments, and MT networks participate in the coordinated generation of a polarized cell response, such as cell migration (2). Recent studies have identified potential molecular mechanisms by which the MTs communicate with the actin cytoskeleton (3).

Cellular factors, including MT-associated proteins, influence MT stability and dynamics and are critical for the proper functioning of the MTs (4). MT synthesis requires a pool of α,β-tubulin dimers that are generated by tubulin-folding cofactors (TCoFs) through post-translational modification of α- and β-tubulins (5). Recent evidence suggests that growth factor-induced stimulation of p21-activated kinase 1 (Pak1) participates in the MT dynamics through the phosphorylation of tubulin cofactor B (TCoB) on Ser-65 and Ser-128 during the MT regrowth phase (6).

In addition to phosphorylation, nitration of tyrosine residues by nitrating agents is another mechanism to alter the functions of cytoskeletal proteins. Tyrosine nitration is a selective process, because not all tyrosine residues in a protein undergo nitration under physiological conditions (7, 8). It confers a negative charge, which can alter the local structure and hydrogen bonding of tyrosine with substrates and neighboring amino acids. Also, the bulky nitro group may induce steric perturbations in the structure of tyrosine and inhibit the binding of substrates to enzymes and/or disrupt protein–protein interactions (9, 10). It has been suggested that in neurofilaments tyrosine residues situated next to glutamic acid can be nitrated (11). In particular, several lines of investigation suggest that posttranslational nitrotyrosination compromises the function of the surface-exposed carboxyl-terminal domain of α-tubulin (12). Free nitro tyrosine (NO_2Tyr) is taken up by mammalian cells and irreversibly incorporated into α-tubulin, via a posttranslational mechanism catalyzed by the tubulin-tyrosine ligase (TTL) (12). Such modification can alter MT dynamics and function. The second example of nitrated cytoskeleton protein is Tau, a MT-binding protein (13). Tau undergoes nitration and oligomerization upon peroxynitrite treatment, and these modifications impair its MT-binding activity. In addition, Tau is regulated by serine/threonine phosphorylation in a signaling-dependent manner, which promotes tubulin polymerization and MT stability (14). It remains an open possibility that active control of the cytoskeleton proteins can be achieved by phosphorylation and nitration. However, the upstream modifiers that control nitration and phosphorylation have not been identified.

Here, we investigated the influence on the MT remodeling of tyrosine nitration of TCoB, a phosphoprotein that is required for the stabilization of native α-tubulin, MT regrowth, and completion of cytokinesis. We also delineated the influence of Pak1 phosphorylation of TCoB on tyrosine nitration and vice versa and provide evidence in support of a regulatory feedback inhibitory role of TCoB nitration on MT biogenesis in eukaryotic cells with stimulated Pak1 signaling.

Results

TCoB Is a Nitrated Protein. Our earlier studies showed that phosphorylation of TCoB facilitates MT assembly in mammalian cells (6). Because nitration of specific microtubule components is known to affect MT function (13), we investigated whether TCoB could be nitrated and whether its nitration affected Pak1-dependent activation of TCoB. Additionally, we evaluated the potential cross-talk between tyrosine nitration of TCoB and Pak1 signaling. A close examination of the amino acid sequence of TCoB revealed the presence of two potential nitration sites; tyrosine 64, exposed toward the surface directly adjacent to the Pak1 phosphorylation site (Serine 65), and tyrosine 98, which is localized between two negatively charged glutamate residues [supporting information (SI) Fig. 6].

To test the above hypothesis, we first determined whether TCoB could be nitrated. ZR-75 breast cancer cells, which express iNOS (15, 16), were transiently transfected with T7-

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The authors declare no conflict of interest.

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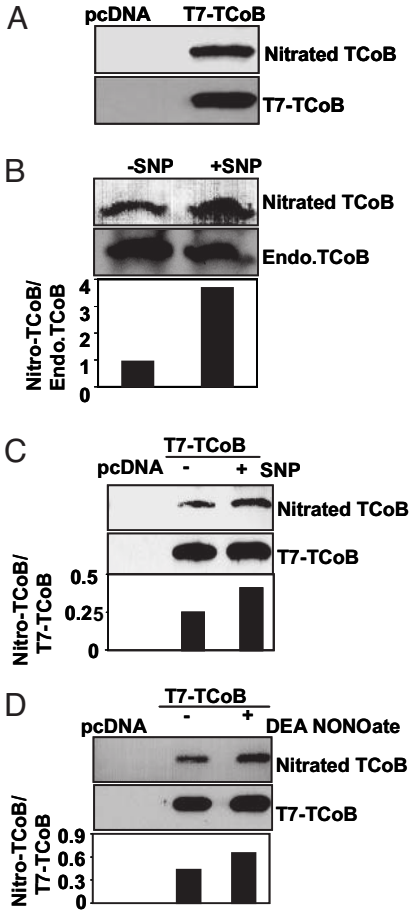

Fig. 1. TCoB is a nitrated protein. (*A*) ZR-75 cells were transfected with pcDNA or T7-TCoB, and 2 mg of total protein lysates were immunoprecipitated with an anti-T7 agarose beads, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies. (*B*) Endogenous TCoB (endo-TCoB) was immunoprecipitated from ZR-75 cells treated with or without SNP (1 mM) for 4 h, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-TCoB antibodies. (*C*) T7-TCoB was immunoprecipitated from transiently transfected ZR-75 cells treated with SNP (1 mM) for 4 h, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies. (*D*) T7-TCoB was immunoprecipitated from transiently transfected ZR-75 cells that had been treated with or without DEA NONOate (100 μM) for 30 min, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies. All blots from *B–D* were quantitated with ImageQuant (version 5.1) software. The quantitation results were presented as ratio of the intensities of the anti-nitrotyrosine bands/intensities of the anti-T7-TCoB bands. This ratio was used to compare the relative levels of nitration in different lanes of the same gel but does not reflect the fraction of the protein that has been nitrated.

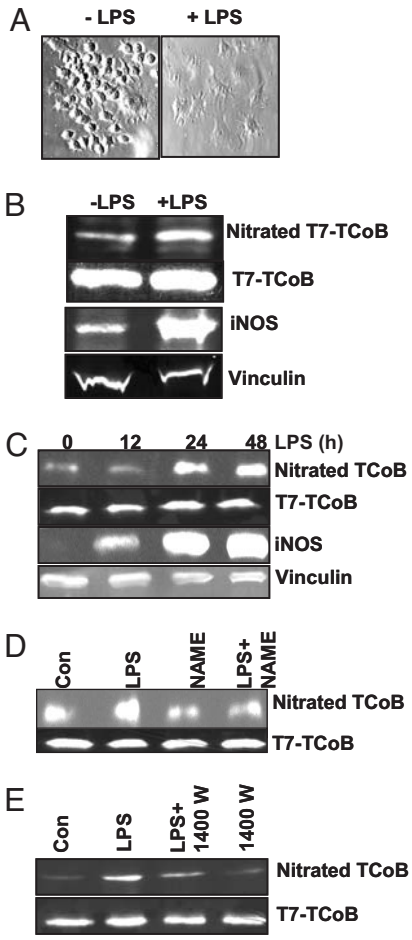

Fig. 2. Nitration of TCoB is iNOS-dependent. (*A*) Representative transmission images showing change in morphology of RAW 264.7 cells after LPS/γ-IFN treatment. (*B*) T7-TCoB was immunoprecipitated from transiently transfected RAW 264.7 cells that were treated with LPS (1 μg/ml)/γ-IFN (50 units/ml) for 16 h, separated by SDS/PAGE, and immunoblotted with the indicated antibodies. (*C*) T7-TCoB was immunoprecipitated from transiently transfected RAW 264.7 cells that were treated with LPS/γ-IFN at various time points, separated by SDS/PAGE, and immunoblotted with the indicated antibodies. (*D* and *E*) T7-TCoB was immunoprecipitated from transiently transfected RAW 264.7 cells that were pretreated with either 1400W (100 μM) or L-NAME (10 mM) for 4 h and/or LPS/γ-IFN, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies.

tagged TCoB or pcDNA vector. Immunoprecipitation of the cell lysates with anti-T7 agarose beads and subsequent Western blotting with a well characterized anti-nitrotyrosine antibody (17) revealed that, indeed, T7-TCoB could be nitrated (Fig. 1*A*). To validate these findings and to demonstrate that the endogenous TCoB is also nitrated under physiologic conditions, ZR-75 cells were treated with the nitric oxide (NO) donor sodium nitroprusside (SNP), and endogenous TCoB was immunoprecipitated by using a TCoB-specific antibody (6), followed by Western blotting with an anti-nitro-tyrosine antibody. Results showed that indeed, endogenous TCoB is efficiently nitrated (Fig. 1*B*). Further, we transfected ZR-75 cells with T7-TCoB and treated them with either SNP or DEA NONOate, two widely used NO donors. The levels of nitrated T7-TCoB increased upon nitric oxide donor treatment (Fig. 1 *C* and *D*). Together, these results suggest that TCoB, a cytoskeletal protein, is efficiently nitrated by NO donors under physiological conditions.

Nitration of TCoB Depends on Inducible Nitric Oxide Synthase. Previous studies have established an important role for inducible nitric oxide synthase (iNOS, or NOS-2) in protein tyrosine nitration (18). To test whether TCoB nitration could be induced by iNOS, we used a well characterized mouse macrophage cell line RAW 264.7. It is known that upon stimulation with lipopolysaccharide (LPS), RAW 264.7 cells undergo distinct morphological changes, induce iNOS activity, and exhibit an increased oxidative stress (19), which provides a favorable environment for the nitration of endogenous proteins. Stimulation of T7-TCoB-transfected RAW 264.7 cells with LPS/γ-IFN resulted in distinct dynamic morphological changes as expected (Fig. 2*A*) and increased nitration of TCoB that correlated with iNOS protein expression (Fig. 2*B*), suggesting that NO derived from iNOS was responsible for the increased levels of TCoB

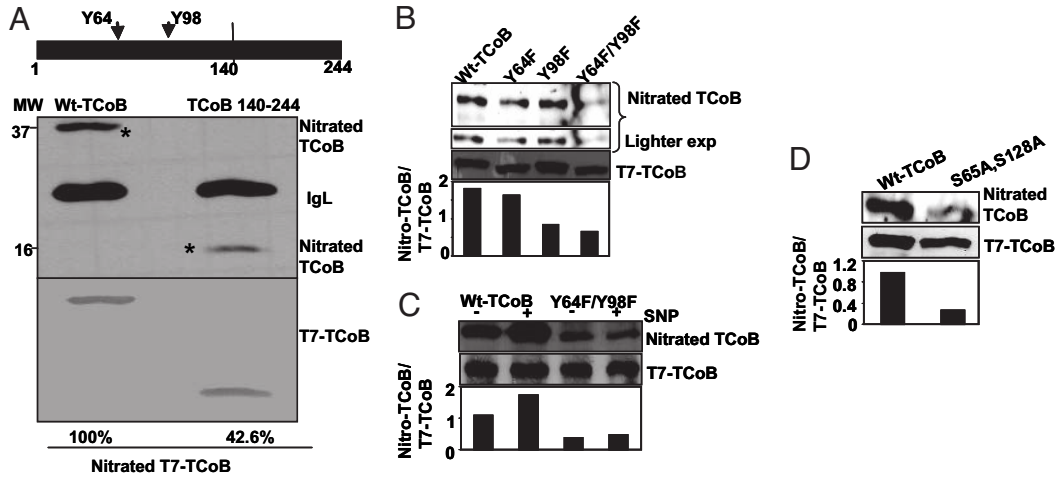

Fig. 3. Identification of the major nitrated tyrosine residues on TCoB. (*A*) Schematic representation of potentially nitrated tyrosine residues on TCoB. TCoB was immunoprecipitated from ZR-75 cells transfected with either full-length T7-TCoB or T7-TCoB fragment encompassing amino acids 140–244, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies. The altered mobility of the bands is expected because of the difference in protein sizes. The immunoblots were quantitated with ImageQuant (version 5.1) software. The quantitation results were expressed as percent nitrated. (*B*) TCoB was immunoprecipitated from ZR-75 cells transfected with full-length T7-TCoB, T7-TCoB-Y64F, T7-TCoB-Y98F, or T7-TCoB-Y64F, Y98F, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies. (*C*) TCoB was immunoprecipitated from ZR-75 cells transfected with full-length T7-TCoB or T7-TCoB-Y64F, Y98F that were either treated with or without SNP, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies. (*D*) TCoB was immunoprecipitated from ZR-75 cells transfected with full-length T7-TCoB or T7-TCoB-S65A, S128A and treated with SNP for 12 h, separated by SDS/PAGE, and immunoblotted with anti-nitrotyrosine and anti-T7 antibodies. For all blots from *B–D*, the immunoblots were quantitated with ImageQuant (version 5.1) software. The quantitation of results was calculated as ratios of the anti-nitrotyrosine protein band to that of the anti-T7-TCoB protein band. This ratio was used to compare the relative levels of nitration in different lanes of the same gel but does not reflect the fraction of the protein that has been nitrated.

nitration (Fig. 2*C*). As expected, we could detect some basal levels of nitrated TCoB, possibly resulting from residual levels of iNOS and/or nitrite accumulation in the culture medium (20). To confirm the role of iNOS in TCoB nitration, we next treated the RAW 264.7 cells with inhibitors of NOS, L-NAME (Nω-Nitro-L-arginine methyl ester) and 1400W, before stimulating with LPS/γ-IFN, and evaluated the status of nitrated TCoB. Both L-NAME and 1400W blocked the ability of LPS to increase TCoB nitration (Figs. 2 *D* and *E*), suggesting that endogenously synthesized NO was the primary source of TCoB nitration. Together, these findings suggested a role for iNOS in the nitration of TCoB.

Identification of Major Nitrated Tyrosine Residues on TCoB. We next attempted to identify the potential nitration sites on TCoB. Because TCoB contains multiple tyrosine residues that could be potentially nitrated, we examined the nitration of TCoB deletion constructs lacking the first 139 aa. This construct lacks two likely nitration sites, Tyr-64 and Tyr-98. Transfection of this construct into ZR-75 cells revealed that the deletion of amino acids 1–139, which contain the N-terminal functional region and part of the coiled-coil region, exhibited a reduction in the level of TCoB nitration by 57.4% as compared with the nitration level in the full-length control WT-TCoB (Fig. 3*A*). This finding suggests that Tyr-64 and Tyr-98 are two major nitration sites that account for >50% of the total TCoB nitration. To validate this finding, we substituted either or both Tyr-64 and Tyr-98 by phenylalanine and tested the ability of these mutant constructs to undergo nitration in ZR-75 cells. The mutation of both tyrosines to phenylalanine in the full-length TCoB reduced the overall nitration by >50% (Fig. 3*B*), confirming that Tyr-64 and Tyr-98 constitute the major sites of nitration of TCoB. This was further validated by the finding that this particular mutant (TCoB, Y64F,Y98F) failed to undergo NO-dependent nitration (Fig. 3*C*), whereas the WT-TCoB does.

The finding that TCoB can undergo both nitration and phosphorylation raised an important question about the effect of TCoB phosphorylation on its nitration. To investigate the potential regulatory cross-talk between nitration and phosphorylation of TCoB, we examined the ability of a TCoB double mutant lacking both Pak1 phosphorylation sites (TCoB-S65A, S128A) to undergo nitration. ZR-75 cells were transfected with wild-type T7-TCoB and the T7-TCoB-S65A,S128A mutant and treated with the NO donor SNP. Lysates were immunoprecipitated with anti-T7 agarose beads and blotted with an anti-nitrotyrosine antibody. We observed that the nitration of TCoB-S65A, S128A was substantially lower than of wild-type TCoB (Fig. 3*D*). Taken together, these results suggested that optimal TCoB nitration requires functional Pak1 phosphorylation sites and, thus, raised the possibility that the phosphorylation and nitration of TCoB are dynamically regulated.

Nitration Antagonizes Signaling-Dependent Phosphorylation of TCoB. Because TCoB phosphorylation by Pak1 is necessary for optimal ability of TCoB to stimulate MT regrowth (6), we examined whether nitration of TCoB has any effect on its phosphorylation in response to Pak1-activating signals. ZR-75 cells were transfected with T7-TCoB, metabolically labeled with [^{32}P]orthophosphoric acid, and treated with SNP or DEA NONOate. There was a decrease in the level of TCoB phosphorylation in cells exposed to the NO donors compared with control untreated cells (Fig. 4*A*). Consistent with this finding, we found a reduction in the phosphorylation of the endogenous TCoB in ZR-75 cells after treatment with the NO donor SNP (Fig. 4*B*). To rule out the possibility that the reduction in phosphorylation was caused by inhibition of Pak1 activity by the NO donor, we performed a Pak1 kinase assay, which did not show any changes in the activity of Pak1 after donor treatment (Fig. 4*C*), suggesting lack of any inhibitory effect of NO donor on the Pak1 activity. Furthermore, to identify the site where phosphorylation is decreased by nitration, ZR-75 cells were transfected with T7-TCoB, T7-TCoB-S65A, and T7-TCoB-S128A mutant, metabolically labeled with [^{32}P]orthophosphoric acid, and treated with SNP. We found that treatment with SNP

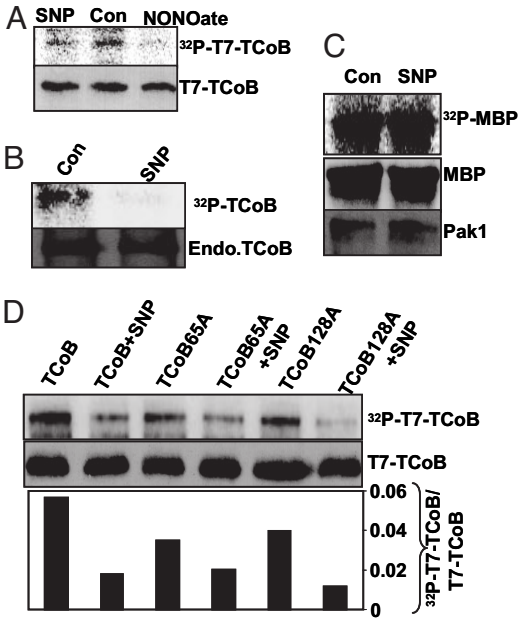

Fig. 4. Nitration antagonizes signaling-dependent TCoB phosphorylation. (*A*) ZR-75 cells were transfected with pcDNA or T7-TCoB and labeled with [^{32}P]orthophosphoric acid for 16 h. Cells were then treated with DEA NONOate for 30 min or SNP (1 mM) for 1 h. Cell lysates were immunoprecipitated with an anti-T7 agarose beads, and phosphorylation of TCoB was analyzed by autoradiography. After autoradiography, membranes were blotted with anti-T7 antibody. (*B*) ZR-75 cells were labeled with [^{32}P]orthophosphoric acid for 16 h and treated with SNP (1 mM) for 1 h. Cell lysates were immunoprecipitated with antibodies against endogenous TCoB, resolved by SDS/PAGE, and transferred to membranes. After autoradiography, the membranes were blotted with anti-TCoB antibody. (*C*) Lysates from Fig. 4B were assayed for Pak1 kinase activity by using MBP as the substrate. (*D*) ZR-75 cells were transfected with T7-TCoB, T7-TCoB-S65A, and T7-TCoB-S128A mutant, metabolically labeled with [^{32}P]orthophosphoric acid, and treated with or without SNP (1 mM) for 4 h. Cell lysates were immunoprecipitated with anti-T7 agarose beads, resolved by SDS/PAGE, and transferred to membranes. After autoradiography, the membranes were blotted with T7-antibody. The quantitation of results was calculated as ratios of the phosphorylated protein band to that of the anti-T7-TCoB protein band. This ratio was used to compare the relative levels of phosphorylation in different lanes of the same gel but does not reflect the fraction of the protein that has been phosphorylated. Con, control.

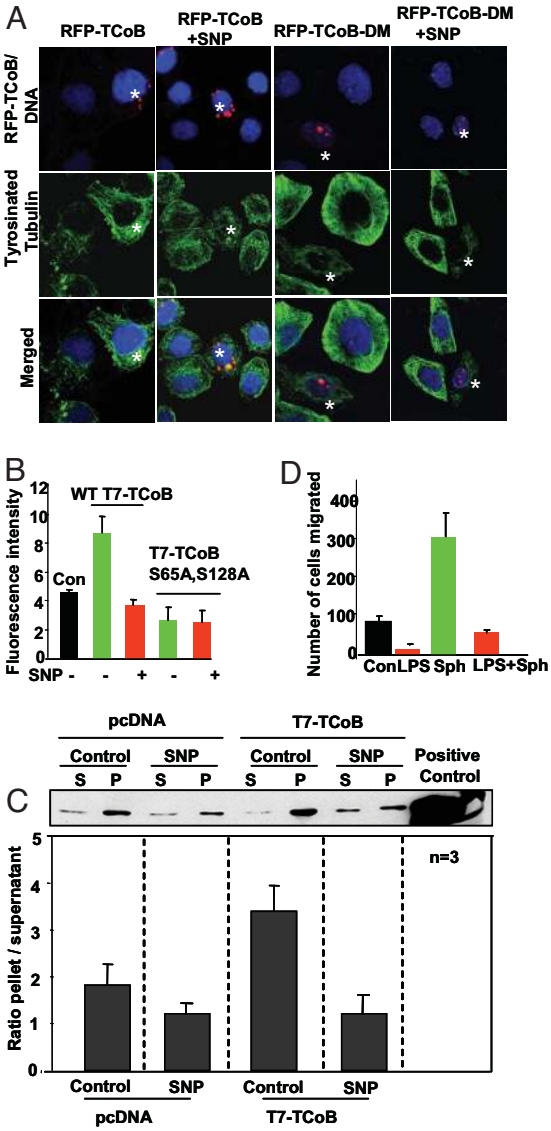

Fig. 5. TCoB nitration inhibits MT regrowth. (*A*) Representative immunofluorescent images showing effect of SNP (1 mM for 1 h) on tyrosination of tubulin in HeLa cells transfected with RFP-tagged wild-type TCoB or mutant TCoB-S65A,S128A. RFP-TCoB (red), tyrosinated tubulin (green), and DNA (blue). Star indicated cells transfected with RFP. DM, double phosphorylation mutant TCoB-S65A,S128A. (*B*) Bar chart showing quantitation for fluorescent intensity of HeLa cells transfected with TCoB in presence or absence of SNP as shown in *A*. Con, control. (*C*) ZR-75 cells transfected with either pcDNA or T7-TCoB were treated with SNP (1 mM) for 4 h and polymerized, and free-tubulin content was analyzed with the Cytoskeleton Microtubule assay kit. The bar chart shows the average ± SD calculated from three independent experiments. S, 100,000 × *g* supernatant; *P*, 100,000 × *g* pellet fractions. (*D*) Bar chart showing migration of RAW 264.7 cells in presence or absence of LPS (1 μg/ml) and sphingosine (Sph) (100 μM), as assayed by Boyden chamber. Cells (20,000 per well) were used in triplicate. Con, control.

reduced the phosphorylation status of both S128A and S65A mutants. However, the extent of reduction is significantly more with the S128A mutant, which retains a functional Ser-65, indicating that Ser-65 is the major site affected by nitration of TCoB (Fig. 4*D*).

Previous studies have shown that protein nitration can have an inhibitory effect on enzymatic activity (21). To test whether the decrease in TCoB phosphorylation in NO donor-treated cells was not caused by nitration of Pak1 itself, we tested the nitration status of Pak1. Immunoprecipitation of Pak1 from ZR-75 cells treated with NO donor, followed by immunoblotting with an anti-nitrotyrosine antibody, revealed no evidence of Pak1 nitration (data not shown). Taken together, these results suggest that nitration antagonizes signaling-dependent phosphorylation of TCoB, possibly through nitration of Tyr-64 and Tyr-98. Collectively, the above findings suggested that the electrostatic effect of phosphorylation on Ser-65 may inhibit nitration of Tyr-64 and Tyr-98 and vice versa (SI Fig. 6).

TCoB Nitration Inhibits Microtubule Regrowth. Because phosphorylation of TCoB by Pak1 is important for TCoB functions in the polymerization of MTs during the regrowth phase, we next examined whether inhibition of TCoB phosphorylation by nitration affects MT dynamics. We first confirmed that TCoB induces MT biogenesis in the present system, using an established MT regrowth assay and confocal scanning microscopy (22). Because newly polymerized MTs contain tyrosinated α-tubulin, we used an antibody specific for tyrosinated tubulin (green) as a marker of these newly polymerized MTs. Enhanced MT biogenesis was observed in most of the WT-RFP-TCoB-expressing cells compared with their neighboring untransfected cells, whereas WT-RFP-TCoB-expressing cells treated with ni-

tric oxide donor SNP showed attenuated MT biogenesis (Fig. 5 *A* and *B*). This implied that nitration of TCoB inhibited the regrowth of MTs. As expected from previous study (6), by using a Pak1 phosphorylation double mutant of TCoB (RFP-TCoB S65A, S128A), we showed a significant inhibition of MT regrowth as compared with the cells expressing the wild-type RFP-TCoB (Fig. 5 *A* and *B*). However, SNP treatment has no additional effect on regrowth of MT, further confirming that optimal TCoB nitration requires functional Pak1 phosphorylation sites. Comparable expression of the transfected RFP-tagged constructs was verified by Western blotting (SI Fig. 7). These findings validated that TCoB functions are inhibited by nitration, which prevents the phosphorylation and activation of TCoB.

To validate, in a more quantitative manner, the above findings that nitration inhibits the function of TCoB, we next used a MT/tubulin *in vivo* assay kit (23), which allows us to measure the ratio of tubulin incorporated into the MTs versus free-tubulin, as a direct measure of MT growth. As expected, with this assay, we observed an increase in the levels of polymerized tubulin in the ZR-75/TCoB cells compared with the ZR-75/pcDNA control cells (Fig. 5*C*). Comparable expression of the transfected T7-TCoB was verified by Western blotting (SI Fig. 8). Treatment with the nitric oxide donor resulted in a substantial decrease in the levels of polymerized tubulin in both ZR-75/pcDNA and ZR-75/TCoB cells; a situation similar to that observed in the above confocal experiment. Overall, these effects were more pronounced in TCoB-overexpressing cells. Together, results from these experiments indicate that nitration of TCoB inhibits its function in the regrowth of MTs and that such inhibition by nitration might serve as a feedback mechanism of signaling-dependent MT regrowth. Because Pak1 activation and subsequent phosphorylation of its substrate TCoB is important for cell-motility functions, we next wished to provide indirect functional evidence to support the finding that nitration inhibits phosphorylation. The mouse macrophage cell line RAW 264.7 cells were treated with Pak1-activating signals (sphingosine) or iNOS-inducing signal (LPS/γ-IFN) or both, and a Boyden chamber-based migration assay was performed. As expected, sphingosine promoted migration of the RAW 264.7 cells, whereas LPS, which activates NOS, inhibited basal as well as sphingosine-induced migration (Fig. 5*D*). This was further supported by our findings that ZR-75 cells treated with sphingosine showed enhanced MT regrowth as compared with control cells, whereas those treated with SNP or sphingosine and SNP showed a reduction in the polymerization of new MTs as evidenced by an established MT regrowth assay and confocal scanning microscopy (SI Fig. 9).

Discussion

The purpose of the present study was to gain deeper insights into the regulatory interplay between nitration and signaling-dependent phosphorylation of TCoB, one of the five tubulin-folding cofactors that coordinately control functional heterodimerization of α/β-tubulin to form the MT network. MTs exhibit dynamic instability because of stochastic transitions at MT ends between phases of growth and shrinkage (24), and reversible phosphorylation plays an important role in MT dynamics (25). Apart from the canonical principle that reversible serine and/or threonine phosphorylation plays an important role in the regulation of MT synthesis (26), recent evidence suggests a role for posttranslational nitrotyrosination of MT-interacting proteins in MT dynamics (13). More recently, Pak1 phosphorylation of TCoB was found to be essential for the rapid polymerization of new MTs (6). In the present study, we demonstrate that TCoB is nitrated and provide evidence for a dynamic interplay between the phosphorylation and nitration of TCoB in regulating the assembly and growth of new MTs.

A large body of evidence suggests a potential role of nitration in altering the MT structure and function via posttranslational incorporation into cytoskeletal proteins, such as Tau (13) and α-tubulin (27). However, it is currently unclear how the dynamic equilibrium between assembled and disassembled MTs is maintained. In this context, we examined the nitration of TCoB to strengthen the notion that tyrosine nitration of cytoskeletal proteins is a widely used regulatory mechanism to control MT dynamics in mammalian cells.

For protein tyrosine nitration, several structural determinants are important (*i*) surface accessibility; (*ii*) preference for tyrosine residues in loop structures; (*iii*) proximity to negatively charged residues; (*iv*) absence of steric hindrances; and (*v*) paucity of reactive cysteine/methionine residues in the vicinity of tyrosine residues (13). Protein sequence analysis of TCoB raised the possibility that TCoB may also undergo nitration, because we found evidence of positioning of the predicted tyrosines toward the surface of the molecule in the loop structure and the presence of negatively charged moieties near the sites of nitration. This was combined with the interesting fact that there is a Pak1 phosphorylation site, Ser-65, a site required for optimal MT regrowth, next to the Tyr-64. It is possible that nitration of Tyr-64 and Tyr-98 may affect the ability of Pak1 to phosphorylate Ser-65 because of induced conformational changes in the connecting loop between strands 3 and 4, resulting from the introduction of the negative charge. Because Pak1 promotes MT regrowth, these observations suggest that tyrosine nitration and Pak1 phosphorylation might have opposing effects on MT regrowth.

Our finding that endogenous TCoB could be nitrated by iNOS activity is important, because it reveals the effectiveness of signal-dependent modification of TCoB. In this study, we obtained several lines of evidence reinforcing the notion that TCoB tyrosine nitration occurs with biological selectivity and affects protein structure and function. We showed that selective nitration of the N terminus (Tyr-64 and Tyr-98) of TCoB constitutes >50% of the total nitration of TCoB. We also found that tyrosine nitration of TCoB inhibits its ability to undergo phosphorylation, thus destabilizing MT regrowth. These results are consistent with the hypothesis that nitrotyrosination invokes conformational changes, either directly or via allosteric interactions, that lead to changes in MT dynamics (12). However, our present finding that phosphorylation-inactive mutants of TCoB cannot be nitrated introduces a new role of TCoB nitration as a feedback mechanism to antagonize phosphorylation-dependent MT synthesis. Phosphorylation and nitration of TCoB may thus play a role in maintaining a constant balance between growth and regrowth of MTs (SI Fig. 10).

In conclusion, the findings presented here show that TCoB is a cytoskeletal protein that is nitrated, in addition to Tau and α-tubulin. In addition, this study provides evidence that nitration and signaling-dependent phosphorylation are dynamically controlled and target TCoB, which has an essential role in MT dynamics.

Materials and Methods

Cell Culture and Reagents. HeLa and ZR-75 cells were purchased from the American Type Culture Collection, and murine macrophage-like RAW 264.7 cells have been described (19). All cells were cultured in RPMI medium 1640 supplemented with 10% FBS. For transient-transfection studies, RAW or ZR-75 cells cultured in 60-mm dishes were transfected with 5 μg of either pcDNA3.1A or T7-TCoB by using the FuGENE-6 transfection reagent following standard suggested protocols. NOS2 was induced in RAW cells by using a mixture of γ-IFN (50 units/ml) and LPS (1 μg/ml) for the indicated times. Anti-nitrotyrosine (clone 1A6) from Upstate Biotechnology. Goat anti-mouse secondary antibodies conjugated with Alexa Fluor 488 (green) and the DNA dye DAPI, Topro-3 (blue) were purchased from

Molecular Probes. SNP was purchased from Calbiochem; L-NAME was from Sigma; 1400W and DEA NONOate were from Alexis Biochemicals. Antibody against endogenous TCoB has been described (6). The source of various specific antibodies is detailed in *SI Text*.

Cell Extracts, Immunoblotting, Immunoprecipitation, Pak1 Kinase Assay, *in Vivo* Phosphorylation, Confocal Microscopy, and Microtubule Regrowth Assays. These assays were performed as described (6) and as outlined in *SI Text*.

Plasmid Construction. The red fluorescent protein RFP-tagged TCoB, T7-TCoB-S65AS128A, and T7-TCoB-140–244 constructs were generated as described (6). TCoB-Y64F, TCoB-Y98F, and TCoB-Y64FY98F double mutations were generated by site-directed mutagenesis by using a QuikChange mutagenesis kit from Stratagene.

Tubulin *in Vivo* Assay. MT/tubulin *in vivo* assay was performed by using a kit (cat. no. BK038) from Cytoskeleton per the manufacturer's instructions and is detailed in *SI Text*.

Migration Assay. Briefly, 60% confluent RAW 264.7 cells were serum-starved for 24 h and treated with γ-IFN (50 units/ml) and LPS (1 μg/ml) for 16 h, as detailed in *SI Text*.

Quantitation and Reproducibility. Quantitation of the individual cell fluorescence intensity was calculated from 30 cells per condition by using NIH Image J software. The immunoblots were quantitated with ImageQuant (version 5.1) software. The quantitation results were expressed as ratios of the intensity score of the anti-nitrotyrosine bands/intensity score of the anti-T7-TCoB band. This ratio was used to compare the relative levels of nitration and phosphorylation in different lanes of the same gel but does not reflect the fraction of the protein that has been nitrated or phosphorylated. The results presented are representative of at least three replicate experiments.

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