

Tubulin-tyrosine Ligase (TTL)-mediated Increase in Tyrosinated α -Tubulin in Injured Axons Is Required for Retrograde Injury Signaling and Axon Regeneration*[§]

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Background: Axon regeneration following nerve injury depends on retrograde injury signals.

Results: An increased tyrosinated α -tubulin level at the injury site is required for the retrograde transport of injury signals and timely activation of a pro-regenerative program.

Conclusion: An injury-induced increase in tyrosinated α -tubulin is important for axon regeneration.

Significance: Deciphering the mechanisms regulating the retrograde transport of injury signals is crucial for our understanding of regenerative mechanisms in peripheral neurons.

Injured peripheral neurons successfully activate a pro-regenerative program to enable axon regeneration and functional recovery. The microtubule-dependent retrograde transport of injury signals from the lesion site in the axon back to the cell soma stimulates the increased growth capacity of injured neurons. However, the mechanisms initiating this retrograde transport remain poorly understood. Here we show that tubulin-tyrosine ligase (TTL) is required to increase the levels of tyrosinated α -tubulin at the axon injury site and plays an important role in injury signaling. Preventing the injury-induced increase in tyrosinated α -tubulin by knocking down TTL impairs retrograde organelle transport and delays activation of the pro-regenerative transcription factor c-Jun. In the absence of TTL, axon regeneration is reduced severely. We propose a model in which TTL increases the levels of tyrosinated α -tubulin locally at the injury site to facilitate the retrograde transport of injury signals that are required to activate a pro-regenerative program.

Activation of a pro-regenerative transcriptional program is an important determinant of successful axon regeneration following injury or trauma to the nervous system. Peripheral neurons regenerate remarkably better than their counterparts in the central nervous system (1, 2). Defining how peripheral neurons respond to injury to increase their intrinsic regenerative capacity may suggest approaches to improve the recovery of neurological functions following central nervous system injury or in the case of extensive peripheral injury.

Successful axon regeneration depends on both extrinsic factors in the environment and the activation of intrinsic mechanisms that can stimulate axon regrowth. The retrograde transport of injury signals from the distantly located axonal lesion site to the nucleus represents an essential mechanism activating a pro-regenerative program (3, 4). Early studies in *Aplysia* have proposed that retrograde injury signaling occurs in two different temporal phases (5). The early phase involves transient calcium waves and other early ionic flux changes and may have a crucial role in priming retrograde injury signaling by eliciting epigenetic changes in the soma (6, 7). The second phase is mediated by molecular complexes activated locally at the injury site that are transported retrogradely by the molecular motor dynein toward the cell soma (4). Interfering with the retrograde transport of injury signals inhibits the activation of various transcription factors, such as c-Jun and STAT3, and prevents efficient regeneration and survival (8, 9).

Microtubules represent a fundamental structural element that supports the motor-mediated transport of vesicles and protein complexes along the axon. Tubulin tyrosination is a posttranslational modification believed to correlate with the dynamic properties of microtubules and to control the association of a pool of microtubule binding proteins (10, 11). The C-terminal tyrosine of newly synthesized α -tubulin is cyclically removed by an unknown carboxypeptidase and readded by tubulin-tyrosine ligase (TTL),³ the only known protein with this function (12–14). TTL binds to the $\alpha\beta$ tubulin dimer and adds a tyrosine specifically to α -tubulin (15). This cyclic process is essential because mice lacking TTL are perinatal lethal (16). Furthermore, mutations in α -tubulin residues that are engaged in the tubulin-TTL interface are linked to neurodevelopmental disorders (17, 18). Tyrosinated α -tubulin dimers are incorporated in polymerizing microtubules, therefore often marking

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³ The abbreviations used are: TTL, tubulin-tyrosine ligase; +TIP, plus end tracking protein; E, embryonic day; DIV, day(s) *in vitro*; DMSO, dimethyl sulfoxide; CHX, cycloheximide; DRG, dorsal root ganglion/ganglia; CAP-Gly, cytoskeleton-associated protein glycine-rich; P/CAF, p300/CBP associated factor; DLK, dual leucine zipper kinase.

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the dynamic plus end of microtubules, which are decorated by microtubule plus end tracking proteins (+TIPs) (10, 11, 19). Recent evidence suggests that +TIPs facilitate the initiation of retrograde axonal transport (20–23). For example, the association of the +TIP CLIP-170 specifically with tyrosinated α -tubulin contributes to the efficiency of minus end-directed transport of organelles (21, 24, 25). In addition, a component of the dynein-dynactin complex p150^{Glued} regulates the initiation of retrograde axonal transport in developing sensory neurons (22). Together with the observations of others and our observation that axon injury increases tyrosinated α -tubulin levels at the injury site (6, 26, 27), these findings suggest that tubulin tyrosination in response to injury may regulate retrograde injury signaling.

Here we reveal that axon injury rapidly increases tyrosinated α -tubulin levels in peripheral sensory neurons in a TTL-dependent manner. Injury-induced tyrosinated α -tubulin recruits +TIPs and is required for retrograde organelle transport following injury. Preventing the injury-induced increase in tyrosinated α -tubulin levels by knocking down TTL delays phosphorylation of the pro-regenerative transcription factor c-Jun and severely reduces axon regeneration. Taken together, our results suggest that a TTL-mediated increase in tyrosinated α -tubulin levels promotes axon regeneration by facilitating the retrograde transport of injury signals that are required for the activation of the pro-regenerative program.

Experimental Procedures

Animals and DRG Cultures—E12 pregnant mice were purchased from Charles River Laboratories, and 2-month-old C57 mice were used for surgery experiments. E13 embryonic DRG cultures were prepared as described previously (6). All procedures were approved by the Washington University in St. Louis School of Medicine Animal Studies Committee.

Antibodies and Lentiviruses—Antibodies used were as follows: tyrosinated tubulin (catalog no. T9028) and acetylated tubulin (catalog no. T7451) (Sigma); TTL (catalog no. 13618, Proteintech); α -tubulin (catalog no. Ab18251, Abcam); STAT3 (catalog no. 9132), CLIP-170 (catalog no. 8977), and p-c-Jun (catalog no. 9261) (Cell Signaling Technology); EB1 (catalog no. 610534) and p150^{Glued} (catalog no. 610473) (BD Biosciences), P/CAF (catalog no. AB9962, EMD Millipore); and anti- β III tubulin (catalog no. TUJ1, Covance, MMS-435P). To knock down TTL, shRNAs were used, and lentivirus was produced using pLKO.1 constructs from TRC (catalog nos. TRCN0000191515, TRCN0000191227, and TRCN0000278795) as described previously (6). LacZ shRNA was used as a control.

In Vitro Axotomy Assay—Mouse embryonic DRG spot cultures, *in vitro* axotomy, and regeneration assays were performed as described previously (6). Briefly, embryonic E13.5 DRGs were dissociated and plated in a defined region with 10^4 cells/ $2.5 \mu\text{l}$. Culture medium was added 10 min after plating. DRG were infected with shLacZ as a control or TTL shRNA at DIV2 or DIV3. At DIV6 or DIV7, DRG neurons were axotomized using a blade (FST, catalog no. 10035-10). At the time of axotomy, the DRG cultures were treated with DMSO or 10 mg/ml ($35 \mu\text{M}$) cycloheximide (CHX, Sigma) or left untreated. The cultures were fixed 2 h following axotomy, unless indicated

otherwise, and processed for immunostaining. Images were acquired using fluorescence microscopy (Nikon Eclipse TE2000-E, $\times 10/0.25$) and analyzed by ImageJ. To quantify tyrosinated α -tubulin levels, the fluorescence intensity of tyrosinated α -tubulin and total α -tubulin was measured using the average intensity of a one-pixel height line along the axons from DRG cell bodies to the axotomy site, as described previously (6). For c-Jun quantification, one to three fields of view from each spot were selected randomly. The number of p-c-Jun-positive nuclei, stained with DAPI, were counted. P/CAF was quantified by measuring the intensity of P/CAF-positive area surrounding the DAPI-positive nucleus and normalized to the total cellular area. All p-c-Jun and P/CAF images were acquired using the same exposure and intensity parameters. *In vitro* regeneration was assessed as described previously (6). Briefly, DRG neurons were fixed 0, 40, or 60 h after axotomy and stained for β III tubulin. Axons were visualized by fluorescence with a $\times 10$ objective (Nikon, TE2000E). A regeneration index was calculated from the images acquired post-axotomy. The fluorescence intensity of a square area ($2.7 \times 0.1 \text{ mm}$) at 0.1 mm distal to the axotomy line was measured using ImageJ and normalized to a similar area 0.1 mm proximal to the axotomy line.

Sciatic Nerve Ligation and Sample Preparation—Mouse sciatic nerve was unilaterally ligated and dissected at the indicated time after injury, as described previously (6). For Western blot analysis, the sciatic nerves were lysed in lysis buffer (Cell Signaling Technology) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 1.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mg/ml leupeptin, and protease inhibitor (Roche). For chemical treatments, sciatic nerve was additionally soaked with either DMSO or 1 mM CHX (Sigma) in Surgifoam (Johnson and Johnson) 20 min prior to sciatic nerve injury. For immunohistochemistry, the dissected sciatic nerve was fixed in 4% paraformaldehyde/PBS (Sigma) for 1 h, soaked in 20% sucrose overnight at 4 °C, and frozen in OCT (Tissue-Tek) with dry iced-cooled methanol.

Live Imaging of Mitochondria and Lysosome Transport—Mitochondrial movement was measured as reported previously (28). Briefly, embryonic spot-cultured DRG neurons were infected at DIV2 with control shRNA or TTL shRNA plus mitochondrially targeted DsRed fluorescence protein-expressing lentivirus. The cultured neurons were mounted on a heat-controlled stage on DIV6, and time-lapse images were acquired using a $\times 20$ Nikon objective at 37 °C. Images were acquired every 2 s for 5 min. The *x* and *y* axes scale bars of the kymographs indicate 10 μm and 10 s. For lysosome movement, embryonic spot-cultured DRG neurons were infected at DIV2 with control shRNA or TTL shRNA, cultured in phenol red-free neurobasal medium. At DIV6, 50 nM LysoTracker green DND-26 (Cell Signaling Technology) was added in cultured medium for 1 min, followed by live imaging using fluorescence microscopy (Nikon Eclipse TE2000-E, $\times 20/0.75$) in naïve uninjured neurons or 6 h after axotomy. Images were acquired every second with a 100–200 ms exposure time for a total of 1 min. Image sequences were analyzed using the ImageJ kymograph

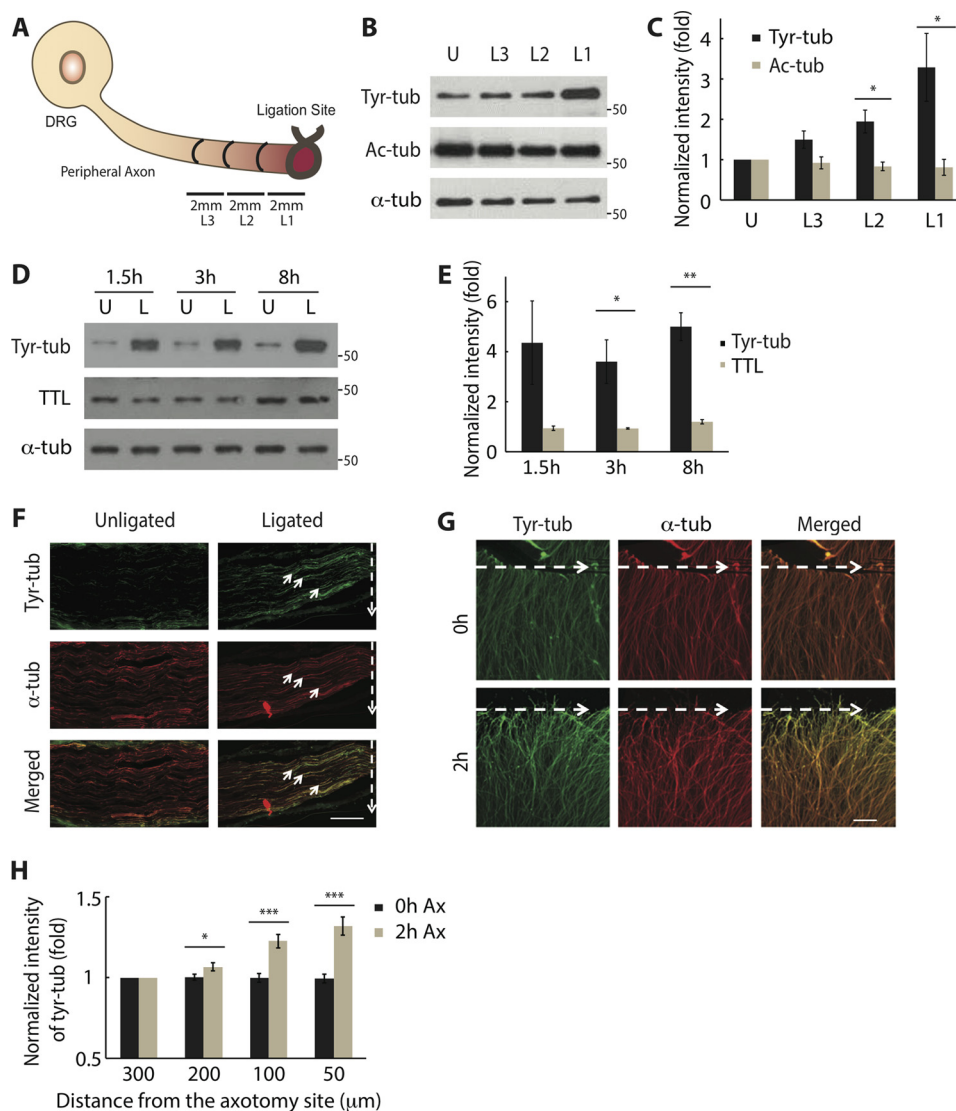


FIGURE 1. Axon injury increases tyrosinated α -tubulin levels. *A*, schematic of the analyzed proximal nerve segments. *B*, representative Western blot of unligated nerve (*U*) and proximal nerve segments L1, L2, and L3 1.5 h after ligation. *tub*, tubulin. *C*, quantification of *B*. Tyrosinated tubulin and acetylated tubulin levels were normalized to total α -tubulin (mean \pm S.E.; *, $p < 0.05$; Student's *t* test; $n = 4$). *D*, representative Western blot of unligated and ligated proximal sciatic nerve segments dissected at the indicated times. *E*, quantification of *D* (mean \pm S.E.; *, $p < 0.05$; **, $p < 0.01$; Student's *t* test; $n = 3$). *F*, representative fluorescence images of sciatic nerve segments unligated or ligated for 6 h and immunostained with the indicated antibodies. Scale bar = 100 μ m. *G*, spot-cultured DRGs were axotomized at DIV6 and immunostained with the indicated antibodies either immediately (0 h) or 2 h after axotomy. Scale bar = 100 μ m. The white dotted arrow indicates the axotomy line. *H*, the ratio of tyrosinated tubulin to α -tubulin was measured at the indicated distance away from the axotomy (*Ax*) site (mean \pm S.E.; *, $p < 0.05$; ***, $p < 0.001$; Student's *t* test, 0 h axotomy, $n = 21$; 2 h axotomy; $n = 19$).

plugin. Single axons were selected for the quantification of lysosome movement.

Statistical Analysis—Western blots were scanned and quantified by ImageJ, and Student's *t* test was used for statistical analysis. An ImageJ macro was used to measure fluorescence intensity in fluorescence images. Analysis of variance followed by Tukey test and Student's *t* test were used for statistical analysis between experimental sets.

Results

Axon Injury Increases the Tyrosinated α -Tubulin Level in Sensory Neurons—We have reported previously that nerve injury elicits changes in tubulin posttranslational modifications, with decreased acetylated α -tubulin and increased tyrosinated α -tubulin (6). We have shown that tubulin deacetylation

plays an essential role in growth cone dynamics and axon regeneration (6). Here we sought to understand the role of tyrosinated α -tubulin in the axonal response to injury. We first investigated the temporal and spatial changes in tyrosinated α -tubulin in injured sciatic nerves. To ensure detection of changes at early time points, we analyzed small samples (2 mm) of nerves proximal to the ligation site by SDS-PAGE and Western blot analysis (Fig. 1*A*). Increased tyrosinated α -tubulin levels occurred mostly in the 2-mm segment immediately proximal to the ligation site 1.5 h after injury (Fig. 1, *B* and *C*). At this early time point, we observed a trend toward decreased acetylated α -tubulin levels (Fig. 1, *B* and *C*), suggesting that injury-induced changes in tyrosinated α -tubulin levels precede changes in acetylated α -tubulin levels. These results are consistent with our previous findings (6), in which we showed a

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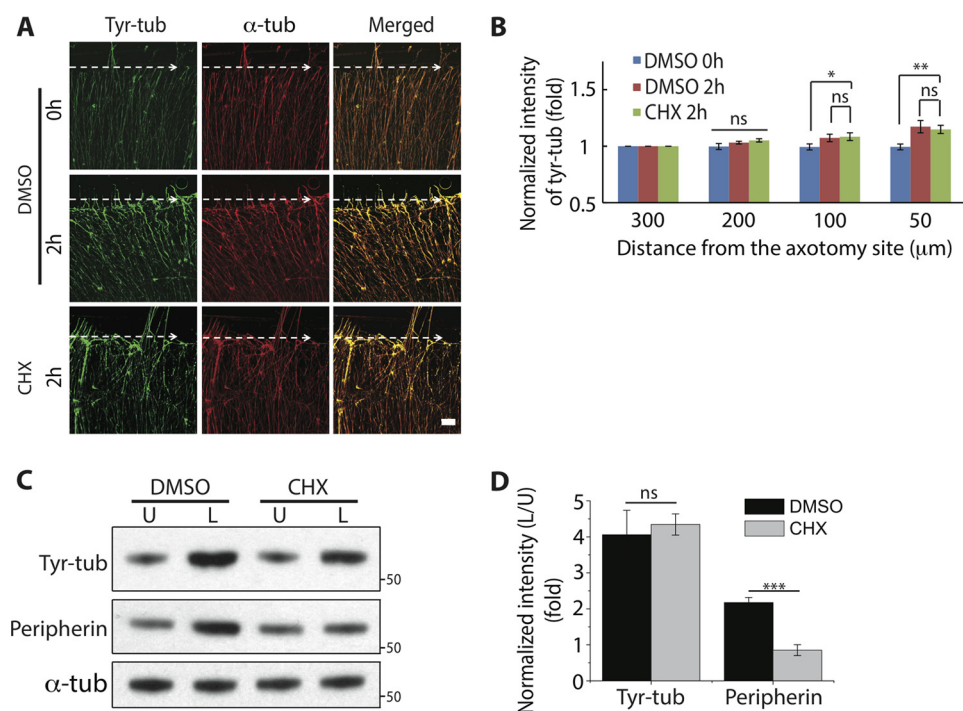


FIGURE 2. Injury-induced increase in tyrosinated α -tubulin does not require protein synthesis. *A*, DRG spot cultures were treated with either DMSO or CHX, axotomized at DIV6, and immunostained with the indicated antibodies. Scale bar = 100 μm . The white dotted arrows indicate the axotomy line. *tub*, tubulin. *B*, quantification of *A*. The intensity of tyrosinated α -tubulin was normalized to α -tubulin and measured at the indicated distance away from the axotomy site (mean \pm S.E.; *, $p < 0.05$; **, $p < 0.01$; ns, not significant; Student's *t* test; DMSO 0 h, $n = 13$; DMSO 2 h, $n = 10$; CHX 2 h, $n = 11$). *C*, Western blot of unligated (*U*) and 24-h-ligated (*L*) sciatic nerve treated with either DMSO or CHX. *D*, quantification of *C*. Tyrosinated α -tubulin and peripherin levels were normalized to total α -tubulin (mean \pm S.E.; ***, $p < 0.001$; Student's *t* test; $n = 3$; ns, not significant).

decrease in acetylated α -tubulin in a 3-mm segment 24 h after injury and in a 10-mm segment 6 h after injury. Increased tyrosinated α -tubulin levels in the 2-mm proximal nerve segment were maintained for up to 8 h (Fig. 1, *D* and *E*). We also observed an increase in tyrosinated α -tubulin levels near the injury site in sciatic nerve longitudinal sections (Fig. 1*F*). Because axons in peripheral nerves are surrounded by Schwann cells, we further tested whether tyrosinated α -tubulin levels increase in axons using embryonic dorsal root ganglion (DRG) spot cultures and *in vitro* axotomy, as described previously (6). In this culture system, DRG neurons are seeded within a defined area, allowing their axons to extend in a nearly parallel manner. DRG cultures were immunostained 2 h following axotomy for total α -tubulin and tyrosinated α -tubulin (Fig. 1*G*). The ratio of tyrosinated α -tubulin to α -tubulin was calculated over a 300- μm axonal section proximal to the axotomy line (Fig. 1*H*). Axotomy caused a significant increase in tyrosinated α -tubulin levels in proximity of the injury site (Fig. 1*H*). We note that the magnitude of the injury-induced increase in tyrosinated α -tubulin is greater *in vivo* (Fig. 1*E*) than *in vitro* (Fig. 1*H*) and could result from the dynamic rearrangement of microtubules in Schwann cells (29) present in the nerve *in vivo*. These results suggest that the increased tyrosinated α -tubulin level is an early and localized event triggered by axon injury.

Injury-induced Increase in Tyrosinated α -Tubulin Does Not Require Protein Synthesis—Newly synthesized α -tubulin is tyrosinated at its C terminus. The C-terminal tyrosine is removed cyclically by an unknown carboxypeptidase and readded by TTL (12–14). Given that α -tubulin mRNA is present in axons (30) and that local axonal protein synthesis plays impor-

tant roles in injury response (31), we first determined whether the observed increase in tyrosinated α -tubulin levels in injured axons results from local protein synthesis. We performed *in vitro* axotomy in DRG spot cultures in the presence of CHX, a well characterized inhibitor of protein synthesis that has been used in similar cultures (32), or DMSO as a vehicle control. We observed increased tyrosinated α -tubulin levels 2 h after axotomy in the presence of either DMSO or CHX (Fig. 2, *A* and *B*), suggesting that inhibition of protein synthesis does not affect tyrosinated α -tubulin levels following axon injury. We also observed that inhibition of protein synthesis *in vivo* in the sciatic nerve did not prevent the injury-induced increase in tyrosinated α -tubulin levels (Fig. 2, *C* and *D*). Peripherin, a protein known to be synthesized in injured axons (33), served as a control for the effectiveness of the CHX treatment. These results indicate that injury-induced increases in tyrosinated α -tubulin levels do not result from local protein synthesis.

Injury-induced Increase in Tyrosinated α -Tubulin Requires TTL—Previous studies have revealed that TTL knockdown reduces the level of tyrosinated α -tubulin in neurons (16). To determine whether TTL is responsible for the injury-induced increase in tyrosinated α -tubulin levels, we used an shRNA approach to knock down TTL in cultured DRG neurons. Knockdown of TTL at DIV1 lead to axon degeneration and decreased cell viability (data not shown), consistent with the essential role of TTL for neuronal organization (16). We then performed knockdown at DIV2 and tested the efficiency of the knockdown at DIV6. We found that a reduction in TTL levels correlated with a decreased level of tyrosinated α -tubulin without affecting the total levels of α -tubulin (Fig. 3*A*) but did not

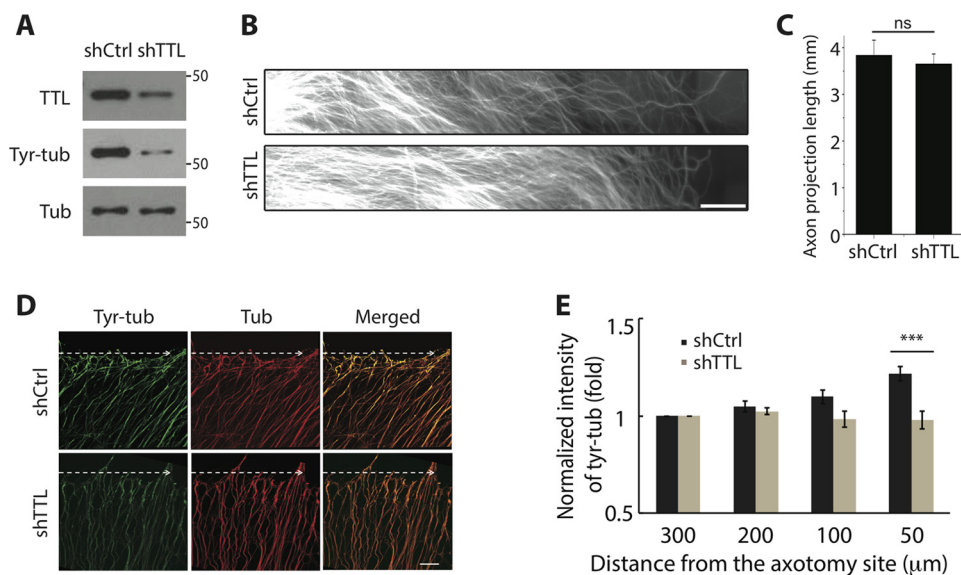


FIGURE 3. TTL mediates the injury-induced increase in tyrosinated tubulin levels. *A*, Western blot of DRG cultures infected with control shRNA (*shCtrl*) or TTL shRNA (*shTTL*) lentivirus at DIV2 and probed with the indicated antibodies at DIV6. *Tub*, tubulin. *B*, DRG spot cultures were infected at DIV2 with *shCtrl* or *shTTL*, fixed, and stained for β III-tubulin at DIV6. Axon radial length was calculated. *Scale bar* = 500 μm . *C*, quantification of *B* ($n = 8$, 10 for *shCtrl* and *shTTL*; mean \pm S.E.; *ns*, not significant). *D*, DRG spot cultures were infected with *shCtrl* or *shTTL* lentivirus at DIV2, axotomized at DIV6, and immunostained with the indicated antibodies 2 h following axotomy. The *white dotted arrows* indicate the axotomy line. *Scale bar* = 100 μm . *E*, quantification of *D*. The ratio of tyrosinated α -tubulin to α -tubulin was measured at the indicated distance away from the axotomy site (mean \pm S.E.; *, $p < 0.05$; ***, $p < 0.001$; *shCtrl*, $n = 10$; *shTTL*, $n = 15$).

affect axon growth (Fig. 3, *B* and *C*). Most importantly, knock-down of TTL prevented the axotomy-induced increase in tyrosinated α -tubulin levels (Fig. 3, *D* and *E*). These results suggest that TTL is required to increase the levels of tyrosinated α -tubulin following axon injury.

TTL-mediated Increase in Tyrosinated α -tubulin Recruits Plus End Tracking Proteins—The last three amino acids of tyrosinated α -tubulin (EEY) represent a motif recognized by the cytoskeleton-associated protein glycine-rich (CAP-Gly) domain, which is abundant in +TIPs such as the dynactin subunit p150^{Glu} and CLIP-170 (25, 34, 35). Therefore, the increase in tyrosinated α -tubulin following axon injury may recruit +TIPs. To test this hypothesis, we analyzed a 1.5-mm segment of ligated or unligated sciatic nerve for the presence of +TIPs. We observed increased levels of the +TIPs CLIP-170, p150^{Glu}, and EB1 in ligated nerve segments (Fig. 4, *A* and *B*). Given that p150^{Glu} facilitates the initiation of retrograde axonal transport (20, 22) and that the retrograde transport of injury signals is critical for axon regeneration (4, 9), we focused on p150^{Glu} in subsequent experiments. To determine whether p150^{Glu} accumulates at the injured axon tips, DRG cultures were immunostained 2 h following axotomy for total α -tubulin and p150^{Glu}, and the fluorescence intensity of p150^{Glu} was measured at 20 and 3 μm from the axon tip (Fig. 4, *C* and *D*). We observed a clear accumulation of p150^{Glu} at a distance of 3 μm from the injured axon tip. Interfering with tyrosinated α -tubulin levels by knocking down TTL prevented p150^{Glu} accumulation at the injured axon tip (Fig. 4, *E* and *F*). These results indicate that, following injury, the increased levels of tyrosinated α -tubulin recruit p150^{Glu} at the tip of injured axons.

TTL-mediated Increase in Tyrosinated α -Tubulin Is Required for Retrograde Injury Signaling—Because p150^{Glu} accumulation at the plus end of microtubules contributes to the

efficiency of minus end-directed transport (20, 22), we hypothesized that the injury-induced increase in tyrosinated α -tubulin levels facilitates retrograde injury signaling. To test this hypothesis, we examined vesicular transport in spot-cultured DRG neurons, which have a uniform microtubule polarity with plus ends oriented distally (22), allowing for the quantitative analysis of organelle motility. We first analyzed the transport of mitochondria to test whether TTL knockdown affects bidirectional microtubule-dependent transport. Knockdown of TTL did not affect the proportion of motile mitochondria compared with control shRNA-treated neurons (Fig. 5, *A* and *B*, and expanded view, *Movies S1* and *S2*). We also observed no effects of TTL knockdown on the proportion or the velocity of anterogradely and retrogradely moving mitochondria (Fig. 5, *C* and *D*).

Next we analyzed lysosome retrograde transport because lysosomes have been shown to require p150^{Glu} enrichment in distal neurites for their transport out of the axon tip in cultured DRG neurons (22). In addition, the scaffolding protein JIP3, which is associated with retrograde injury signaling (9, 36), transports several classes of organelles (37), including lysosomes (38). Knockdown of TTL did not affect the proportion of motile lysosomes in uninjured neurons (Fig. 5, *E* and *F*, and expanded view, *Movies S3* and *S4*). The proportion of stationary to motile lysosomes was comparable with what has been measured by others (22). The velocities of lysosomes we measured (Fig. 5*G*) are also consistent with what has been seen by others (20, 22, 39) and were largely unaffected by TTL knockdown, although we note a small reduction in the velocity of anterogradely moving lysosomes. These results indicate that microtubule-dependent transport of organelles in uninjured neurons is not affected by a reduction of TTL levels.

We next measured lysosome transport following axotomy. We observed that TTL knockdown caused a reduction in the fraction of retrogradely moving lysosomes with a correspond-

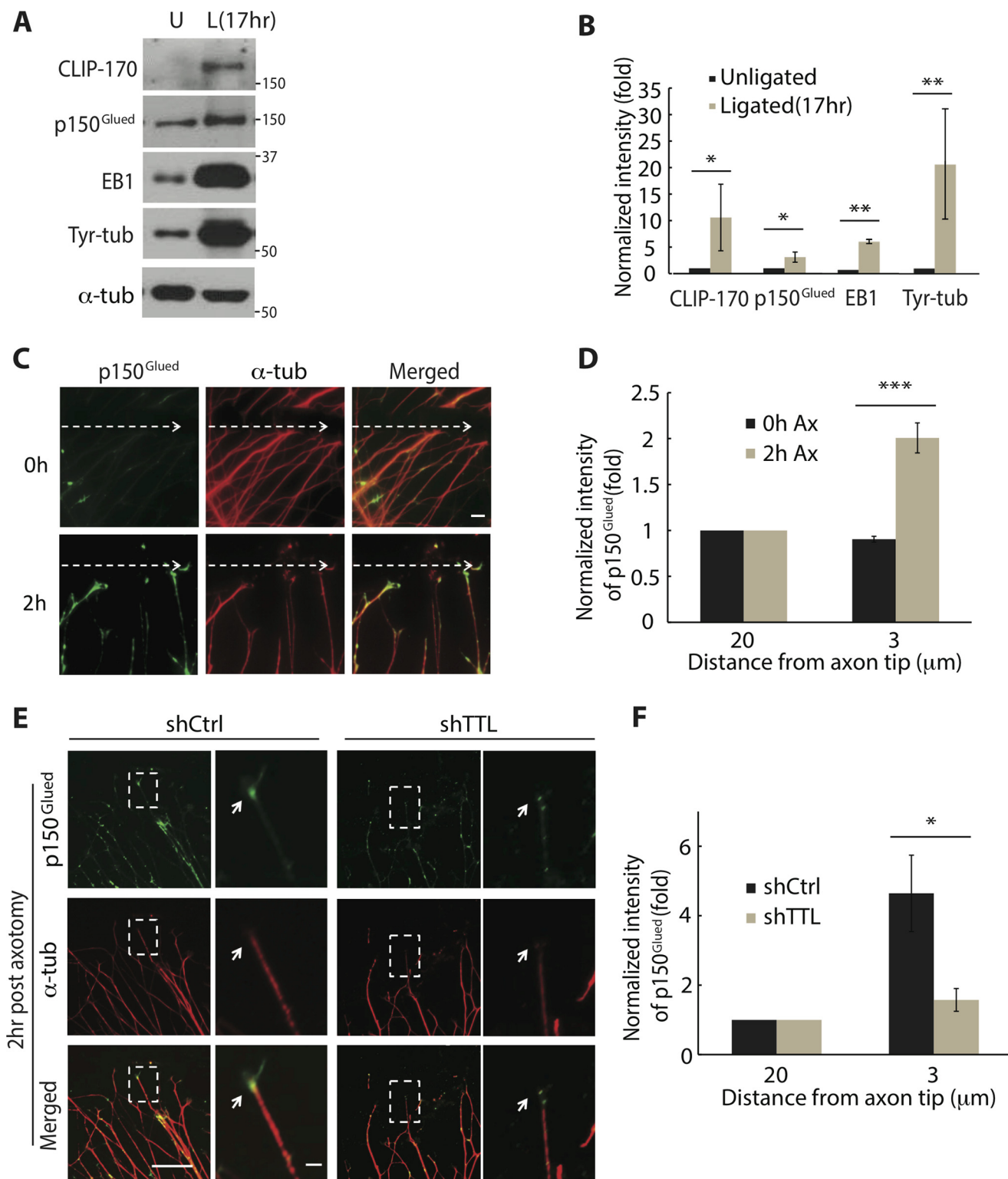


FIGURE 4. TTL-mediated increase in tyrosinated α -tubulin recruits +TIPs. *A*, representative Western blot of unligated (*U*) and ligated (*L*, 17 h) sciatic nerve analyzed with the indicated antibodies. *B*, quantification of *A* (mean \pm S.E.; *, $p < 0.05$; **, $p < 0.01$; Student's *t* test; $n = 5$). The intensity of the indicated protein was normalized to α -tubulin. *C*, DRG cultures were axotomized at DIV6 and immunostained with the indicated antibodies 2 h after axotomy. The white dotted arrows indicate the axotomy line. Scale bar = 10 μ m. *D*, quantification of *C*. The intensity of p150^{Glued} was normalized to α -tubulin and measured at the indicated distance away from the injury site (mean \pm S.E.; ***, $p < 0.001$; Student's *t* test, $n = 30$). *E*, DRG cultures were infected with shCtrl or shTTL lentivirus at DIV2, axotomized at DIV6, and immunostained with the indicated antibodies 2 h following axotomy. The white dotted areas are magnified in the right columns. Scale bars = 100 μ m (left columns) and 10 μ m (right columns). *F*, quantification of *E*. The fluorescence intensity of p150^{Glued} was normalized to α -tubulin and measured at the indicated distance away from the injury site (mean \pm S.E.; *, $p < 0.05$; Student's *t* test; $n = 13$).

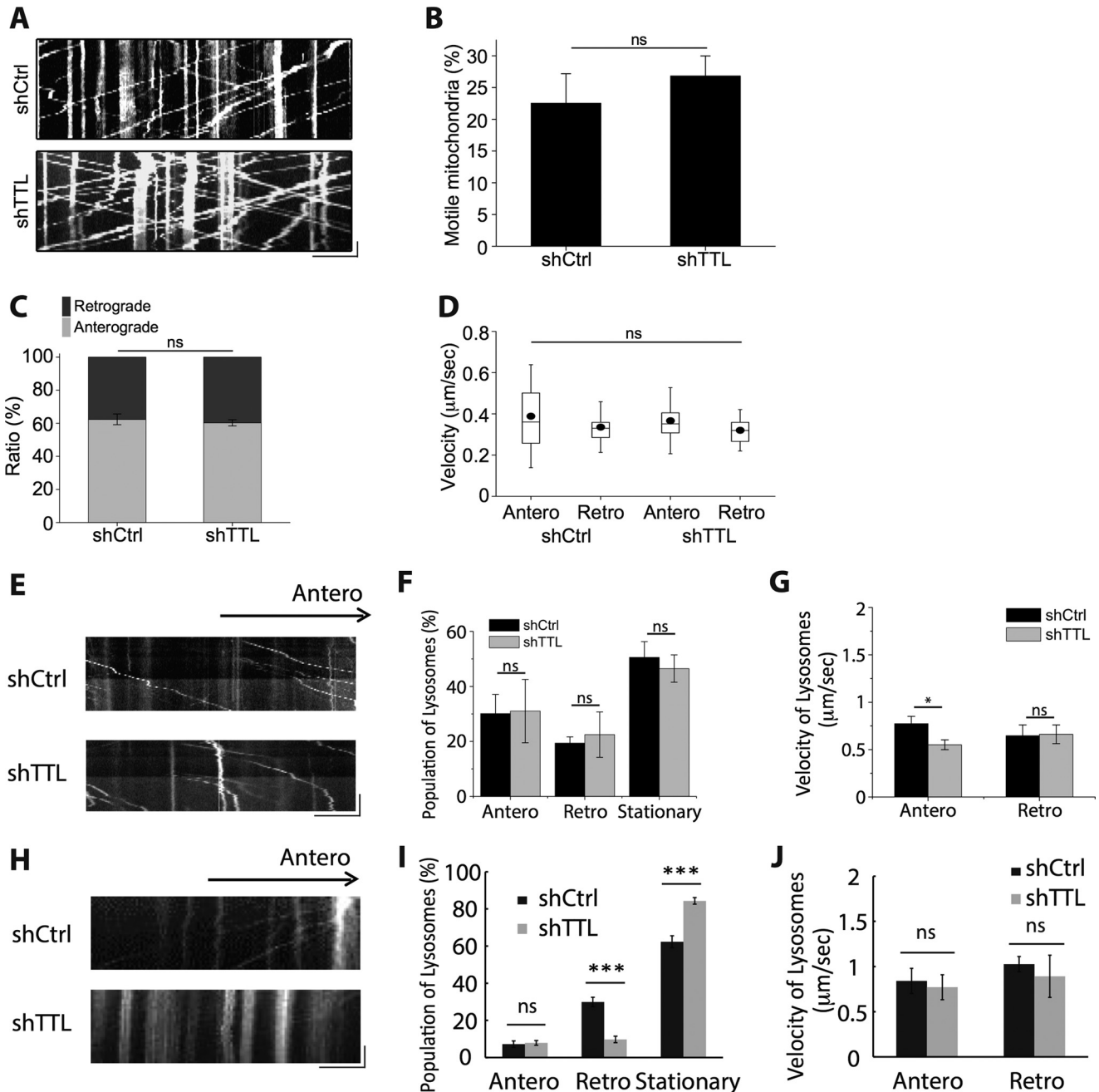


FIGURE 5. TTL-mediated increase in tyrosinated α -tubulin is required for retrograde injury signaling. *A*, DRG cultures were infected with shCtrl or shTTL and with mitochondrially targeted DsRed at DIV2 and imaged at DIV6. Kymographs were generated from movies of DsRed-labeled mitochondria. The x and y axes scale bar indicate 10 μm and 10 s, respectively. *B*, the percentage of motile mitochondria ($n = 11$ for shCtrl from four independent movies and $n = 16$ from five independent movies for shTTL (mean \pm S.E.; ns, not significant; Student's *t* test). *C*, the percentage of anterograde or retrograde moving mitochondria ($n = 43$ for shCtrl and $n = 52$ for shTTL; mean \pm S.E.; Student's *t* test). *D*, the velocity of motile mitochondria was calculated from the kymographs generated by ImageJ (box, 25% and 75%; dot, mean; whisker, S.D.; $n = 24, 15, 26$, and 26 for each condition by analysis of variance followed by Tukey test). *E*, kymographs of LysoTracker-labeled lysosomes from shCtrl or shTTL lentivirus-infected DRG spot cultures. Lentiviruses were infected at DIV2 and imaged at DIV6. ImageJ was used to generate kymographs. The x and y axes scale bar indicate 10 μm and 10 s, respectively. *F*, the percentage of anterograde or retrograde moving lysosomes (mean \pm S.E.; $n = 93$ for shCtrl from six independent movies and $n = 83$ for shTTL from seven independent movies; Student's *t* test). *G*, the velocity of lysosomes calculated from kymographs (mean \pm S.E.; *, $p < 0.05$; Student's *t* test; $n = 28, 18, 26$ and 19 for each condition). *H*, DRG cultures were infected with shCtrl or shTTL at DIV2, axotomized at DIV6, stained with LysoTracker, and imaged 6 h following axotomy. Kymographs were generated from movies of LysoTracker-labeled lysosomes. The x and y axes scale bar indicate 10 μm and 10 s, respectively. *I*, quantification of *H*. The percentage of lysosomes moving in either the anterograde or retrograde direction or remaining stationary was quantified (mean \pm S.E.; ***, $p < 0.001$; Student's *t* test; $n = 503$ for shCtrl and $n = 478$ for shTTL from 12 independent movies). *J*, quantification of *H*. The velocity of lysosomes was measured using ImageJ (mean \pm S.E.; $n = 503$ for shCtrl and $n = 478$ for shTTL).

ing increase in the stationary fraction compared with control shRNA-treated neurons (Fig. 5, *H* and *I*, and expanded view, [Movies S5 and S6](#)). The velocity of lysosomes was not affected

by TTL knockdown and was similar to the velocities measured in uninjured neurons (Fig. 5*G*). These results indicate that a TTL-mediated increase in tyrosinated α -tubulin is

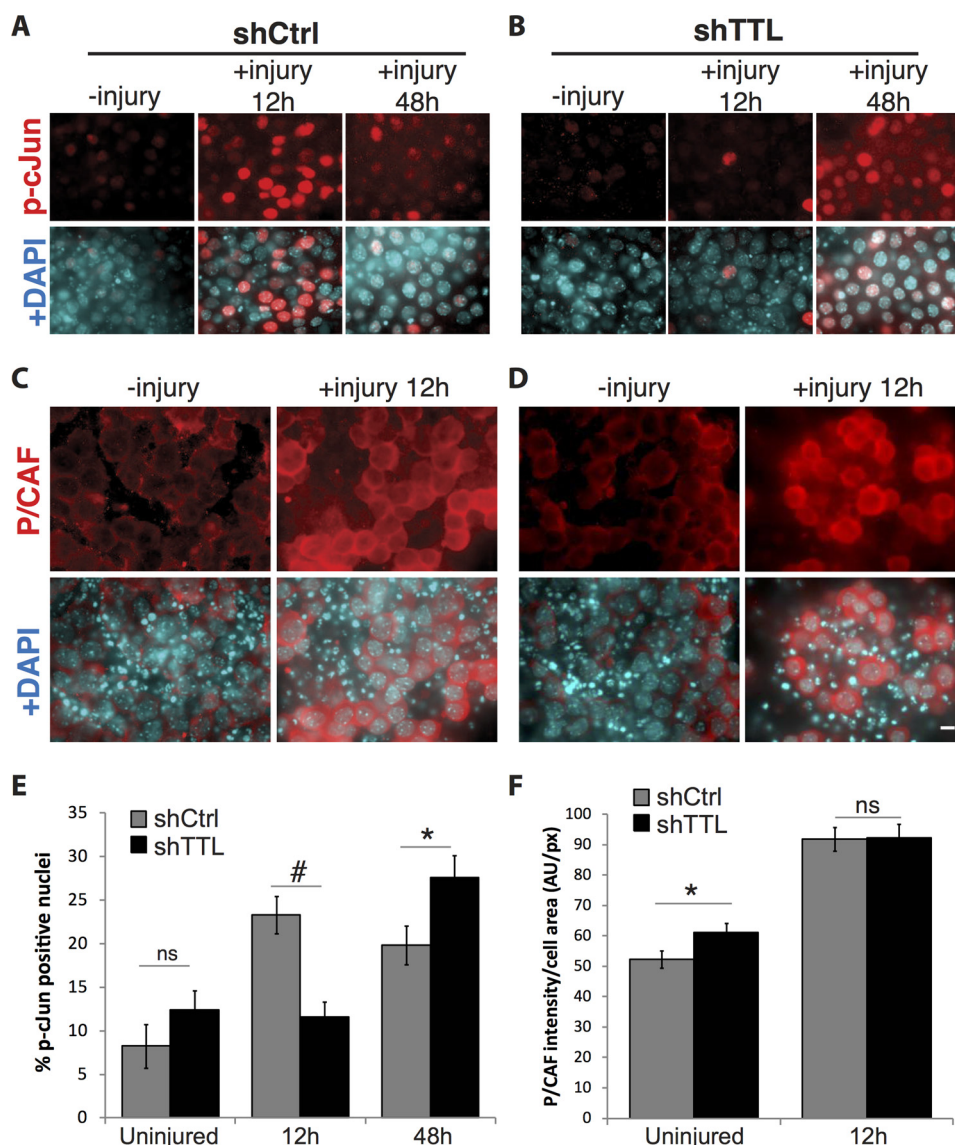


FIGURE 6. Knockdown of TTL delays the activation of c-jun in injured neurons. *A* and *B*, representative images of DRG cultures infected with either shCtrl (*A*) or shTTL (*B*) at DIV3, axotomized at DIV7, and immunostained 12 or 48 h after axotomy with DAPI (nuclei) and phosphorylated c-Jun (p-c-Jun). *C* and *D*, as in *A* and *B*, but DRG were stained for DAPI and P/CAF. Scale bar = 10 μ m. *E*, quantification of *A* and *B*. p-c-Jun activation is delayed in TTL knockdown neurons compared with control neurons. $n = 20$ (shCtrl uninjured), 29 (shCtrl 12 h), 19 (shCtrl 48 h), 17 (shTTL uninjured), 30 (shTTL 12 h), and 18 (shTTL 48 h) images analyzed from three replicates and 4/spots/replicate. *, $p < 0.05$; #, $p \ll 0.001$; ns, not significant. ANOVA followed by Tukey. *F*, quantification of *C* and *D*. P/CAF induction is not altered by TTL knockdown. $n = 40$ (shCtrl uninjured), 35 (shCtrl 12 h), 30 (shTTL uninjured), and 35 (shTTL 12 h) neurons analyzed from two replicates and 4 spots/replicate.

required for the efficient retrograde transport of lysosomes in injured axons.

Knockdown of TTL Delays the Activation of c-jun in Injured Neurons—If tyrosinated α -tubulin contributes to retrograde injury signaling, then activation (phosphorylation) of the regeneration-associated gene c-Jun (40) could be impaired or delayed in neurons lacking TTL. Indeed, we observed that knockdown of TTL delayed the appearance of phosphorylated c-Jun in DRG nuclei in response to axotomy (Fig. 6, *A* and *B*). Although, in control neurons, c-Jun phosphorylation was detected 12 h post-axotomy and persisted up to 48 h, c-Jun phosphorylation was only detectable 48 h post-axotomy in TTL knockdown neurons (Fig. 6, *A*, *B*, and *E*). Note that only one side of the spot culture is axotomized, so not all cell somas had their axon severed. We also examined the levels and localization of

the histone acetyl transferase P/CAF, which has been shown to accumulate in DRG soma in response to sciatic nerve injury in an ERK signaling-dependent manner (41). We observed increased P/CAF expression after injury in both control and TTL knockdown neurons (Fig. 6, *C*, *D*, and *F*). These results suggest that TTL depletion delays retrograde transport back to the cell body of a subset of injury signals.

TTL-mediated Increase in Tyrosinated α -Tubulin Is Required for Axon Regeneration—Because failure to activate c-Jun in response to injury correlates with a failure to regenerate axons (40), TTL depletion should limit axon regeneration. To test this possibility, we performed an *in vitro* regeneration assay, as described previously (6). Control DRG axons displayed a regenerative index of $\sim 40\%$ 40 h post-axotomy and up to $\sim 55\%$ 60 h post-axotomy. In contrast, a reduction in TTL levels

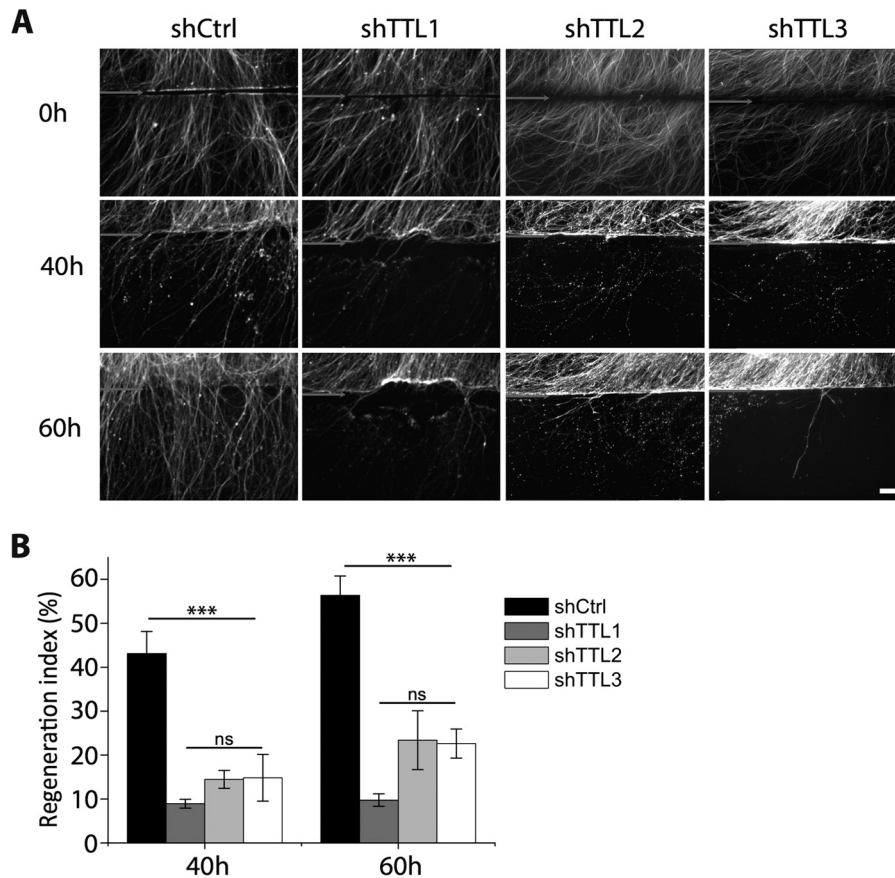


FIGURE 7. **TTL is required for axon regeneration.** *A*, DRGs were infected at DIV3 with control shRNA or three different TTL shRNAs, and *in vitro* axotomy was performed at DIV7. DRG were fixed and stained for β III tubulin 40 or 60 h after axotomy. *Red arrows* indicate the axotomy line. *Scale bar* = 100 μ m. *B*, a regeneration index was calculated (mean \pm S.E.; ***, $p < 0.001$; ns, not significant; $n = 9$).

dramatically decreased the regenerative ability, with a regenerative index of $\sim 10\%$ 40 h and $\sim 20\%$ 60 h post-axotomy (Fig. 7, *A* and *B*). Note that, for this experiment, we used three different TTL shRNAs to test for the specificity of the observed effects. Despite the appearance of c-Jun phosphorylation 48 h post-axotomy (Fig. 6, *A* and *C*), TTL knockdown impaired regeneration at the two time points tested. These results suggest that reducing the levels of TTL and, therefore, the levels of tyrosinated α -tubulin may also affect the reorganization of the microtubule cytoskeleton, a process that is needed to transform a damaged axon tip into a new growth cone (42). Taken together, these experiments indicate that a TTL-mediated increase in tyrosinated α -tubulin contributes to retrograde injury signaling and activation of a pro-regenerative program that enhances axon regeneration.

Discussion

Injury signaling is an important mechanism by which neurons initiate a regenerative response (4), but the mechanisms that initiate retrograde injury signaling remain unclear. Here we show that a TTL-dependent increase in tyrosinated α -tubulin following axon injury is required for the retrograde transport of a subset of injury signals and for axon regeneration.

Tubulin tyrosination occurs on α -tubulin dimers that are then incorporated into polymerizing microtubule plus ends (15). Therefore, tyrosinated α -tubulin often marks the dynamic

plus end of microtubules, which controls the association of a subset of microtubule binding proteins (10, 11). A diverse class of +TIPs decorates the growing ends of microtubule. A CAP-Gly domain, present in several +TIPs, including CLIP-170 and p150^{Glued}, is believed to favor interaction with tyrosinated α -tubulin (25). p150^{Glued}, a major subunit of the dynactin complex that functions with dynein, regulates the initiation of retrograde axonal transport (20, 22). Our data indicate that, although TTL knockdown does not affect axonal transport in uninjured conditions, TTL is required following axon injury to enhance the capture of p150^{Glued} at injured axon tips containing a high level of tyrosinated α -tubulin and to enhance the retrograde transport of lysosomes. Our results are consistent with prior observations revealing that the CAP-Gly domain of p150^{Glued} is required to enrich dynactin at distal neurite tips and enhances the flux of lysosomes from distal neurites but that the CAP-Gly domain is not required for the axonal transport of lysosomes (22).

Other CAP-Gly proteins, such as CLIP-170, which we found enriched in injured axons, may also participate in injury signaling. Indeed, CLIP-170 decoration of dynamic microtubules is required for the efficient retrograde transport of organelles (21). Furthermore, CLIP-170 recognizes a composite site consisting of tyrosinated α -tubulin and EB1 (24), and an EB1/CLIP-170/dynactin-dependent mechanism is required for the effi-

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cient initiation of transport of distinct types of cargos, including mitochondria, early endosomes, and late endosomes/lysosomes (43, 44).

Injury-induced increase in tyrosinated α -tubulin may also play a role in the reorganization of the microtubule cytoskeleton, a process that is needed to transform a damaged axon tip into a new growth cone (42). In mice lacking TTL, neurons have reduced tyrosinated α -tubulin, resulting in impaired growth cone organization and pathfinding (45) as well as an erratic neurite growth rate (16). Furthermore, tyrosinated α -tubulin recruits the kinesin family member KIF3C to destabilize microtubules (46). Loss of KIF3C in adult sensory neurons results in increased stable, overgrown, and looped microtubules and alters axon growth and regeneration (46). These studies suggest that KIF3C may function downstream of TTL-mediated tubulin tyrosination to control microtubule dynamic properties in the growth cone of regenerating axons. Therefore, a TTL-dependent increase in tyrosinated α -tubulin may contribute to axon regeneration through its functions on retrograde injury signaling as well as its action on growth cone dynamics.

TTL activity is likely to be precisely controlled in time and space to properly orchestrate axon regeneration. Because we observed that the levels of TTL did not change at the injury site, injury-induced signaling pathways are likely to regulate TTL activity. Local activation of TTL may strongly increase the probability of the α -tubulin dimer to be tyrosinated during the dynamic process of de- and repolymerization of microtubules at the injury site. Phosphorylation of TTL has been proposed as a potential mechanism for the regulation of tubulin tyrosination (47), and several kinases are activated at the injury site, including DLK/JNK (9, 36, 48), ERK (33), and PKC (6). However, decreased JNK activity in neurons within the central nervous system increases tyrosinated α -tubulin levels (49, 50), and loss of DLK-1 function in *Caenorhabditis elegans* results in increased tyrosinated α -tubulin levels (51). Blocking calcium-mediated PKC activation does not prevent the increase in injury-induced tyrosinated α -tubulin (6). Further studies will determine whether specific signaling pathways in injured axons contribute to the regulation of TTL activity. Because TTL is uniquely able to modify the tubulin dimer (15), its role in injured axons points toward a microtubule-specific mechanism.

The tubulin detyrosination and retyrosination cycle involves a still unidentified tubulin carboxyl peptidase (10). In contrast to TTL, which specifically binds tubulin dimers (15), detyrosination occurs on polymerized microtubules (52). It will be interesting to determine whether the detyrosination process is also regulated in injured axons and contributes to axon regeneration.

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