Development/Plasticity/Repair

A Novel Function for p53: Regulation of Growth Cone Motility through Interaction with Rho Kinase

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The transcription factor p53 suppresses tumorgenesis by regulating cell proliferation and migration. We investigated whether p53 could also control cell motility in postmitotic neurons. p53 isoforms recognized by phospho-p53-specific (at Ser-15) or "mutant" conformation-specific antibodies were highly and specifically expressed in axons and axonal growth cones in primary hippocampal neurons. Inhibition of p53 function by inhibitors, small interfering RNAs, or by dominant-negative forms, induced axonal growth cone collapse, whereas p53 overexpression led to larger growth cones. Furthermore, deletion of the p53 nuclear export signal blocked its axonal distribution and induced growth cone collapse. p53 inhibition-induced axonal growth cone collapse was significantly reduced by the Rho kinase (ROCK) inhibitor, Y27632 [(*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide]. Our results reveal a new function for p53 as a critical regulator of axonal growth cone behavior by suppressing ROCK activity.

Introduction

p53 is mostly known as a tumor suppressor, and p53 deficiency/mutation is found in >50% of human cancers (Hollstein et al., 1994; Levine et al., 1994; Vousden and Lu, 2002). The tumor-suppressing function of p53 is generally attributed to its activation at cell cycle checkpoints and to induction of DNA repair and apoptosis, among other regulatory processes. However, emerging evidence has extended these "classic" functions to regulation of cell migration as another mechanism to control tumor invasion (Roger et al., 2006). These findings have raised the possibility that p53 may regulate cell motility in many cell types, especially in developing neurons. The first hint that p53 might play a critical role in brain development originated from the observation that a fraction of p53-deficient embryos exhibited exencephaly and lethality (Armstrong et al., 1995). Subsequent research revealed that p53 expression is normally limited to the developing CNS of embryos and newborn mice (Komarova et al., 1997). Furthermore, high levels of p53 mRNA have been detected in the developing brain in areas showing little or no apoptosis (Gottlieb et al., 1997; Komarova et al., 1997), suggesting that p53 could participate in apoptosis-unrelated functions. Studies performed in rat PC12 cells showed that NGF-induced neuronal differentiation and neuritic growth were inhibited by the expression of a dominant-negative form of p53 (Fábián et al., 2006; Zhang et al., 2006). This effect of p53 on NGF-induced neuritic growth in PC12 cells and in cultured cortical neurons was associated with increased expression of coronin 1b [a filamentous actin (F-actin) binding protein] and the small GTPase, Rab13 (Di Giovanni et al., 2006). Furthermore, several genes belonging to the four axon guidance pathways (netrins, semaphorins, SLITs, and ephrins) are downregulated in cancer cells that have abnormal p53 function (Arakawa, 2005).

Growth cone motility depends on a dynamic actin network (Lin et al., 1996; Mallavarapu and Mitchison, 1999; Diefenbach et al., 2002) that is regulated by numerous proteins, including the Rho GTPase family (Kozma et al., 1997; Fournier et al., 2000; Dickson, 2001; Giniger, 2002). Mutations in proteins involved in Rho GTPase signaling are causative in some forms of mental retardation, which underlines the importance of Rho GTPase in brain development (Kutsche et al., 2000; Lower and Gecz, 2001; Newey et al., 2005; Benarroch, 2007). Rho GTPases activate two downstream kinases, p21-activated kinase (PAK) and Rho kinase (ROCK), with Rac activating the former and Rho the latter; both PAK and ROCK can phosphorylate and activate Lim domain kinase (LIMK). LIMK can then phosphorylate and inactivate cofilin, which favors actin polymerization (Ridley, 2006; Ishikawa and Kohama, 2007). p53 deficiency in mouse embryonic fibroblasts is associated with abnormal morphology and motility in a RhoA-ROCK-dependent manner (Gadea et al., 2007). Here, we report a novel and critical function for p53 in axonal growth cone motility regulation through suppression of ROCK activity. These findings reveal a novel mechanism that could potentially play critical roles in axonal wiring and in regeneration after neuronal injury.

Materials and Methods

Expression plasmid and transfection. The expression plasmids for green fluorescent protein (GFP) vector and GFP-p53 nuclear-export signal (NES) mutant were from AddGene (plasmid 12092). Enhanced GFP (EGFP)-p53 (originally from Invitrogen) and EGFP-p53-R175H mutant

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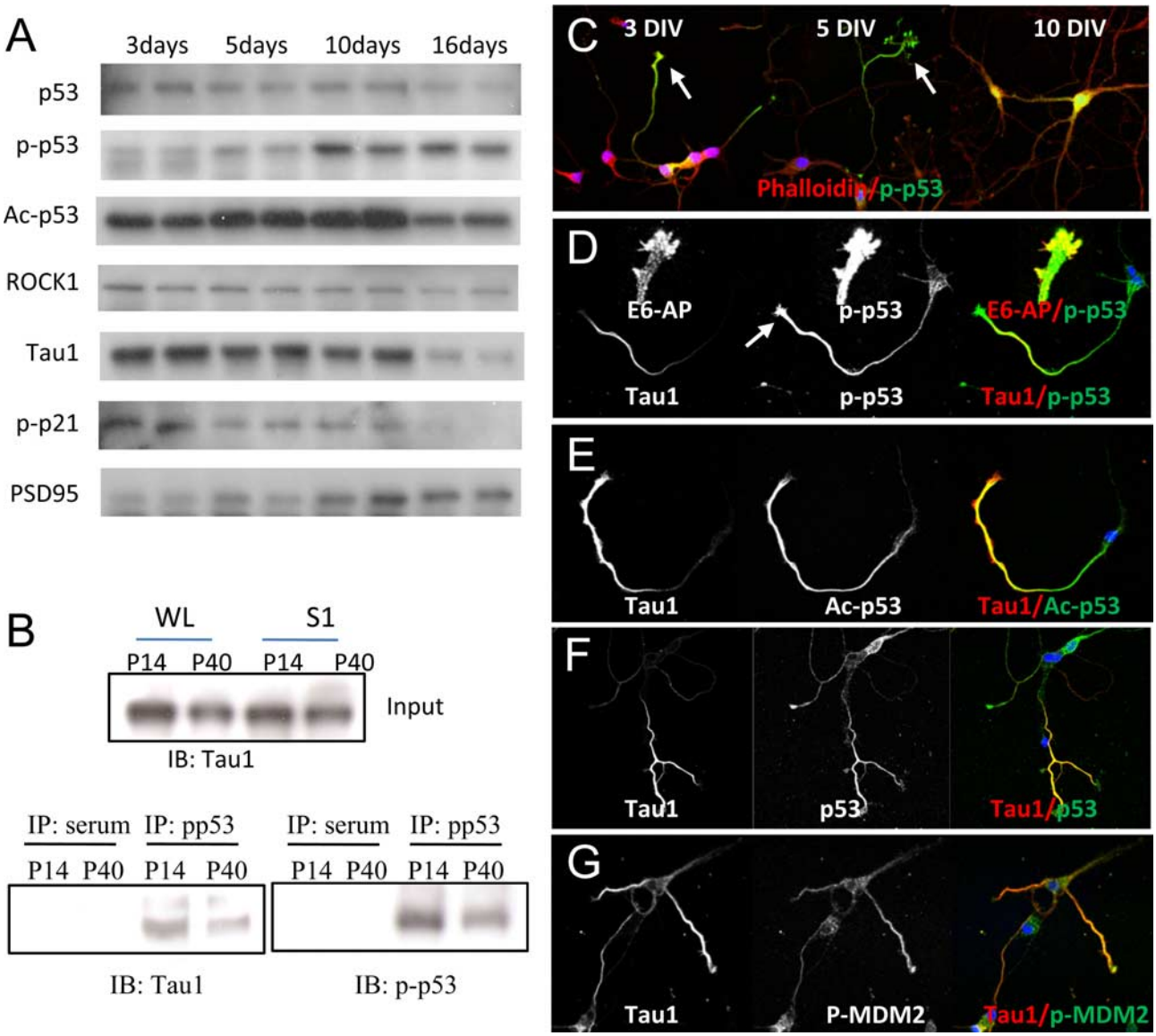

Figure 1. Characterization of p53 and MDM2 expression in primary cultured neurons. ***A***, Levels of p53 and its associated proteins in cortical neurons cultured for different days *in vitro* (DIV). Neurons were cultured from E18 mouse embryos and incubated for 3, 5, 10, and 16 d and processed for immunoblotting with antibodies against p53 (total-p53), p53 phosphorylated at Ser-15 (p-p53) or acetylated at Lys-379 (Ac-p53), ROCK1, tau (Tau1), phosphorylated p21 (p-p21), or PSD95. ***B***, Association of p-p53 with tau proteins. Whole lysates (WL) or S1 fractions (supernatant obtained by lysate centrifugation at 16,000 × *g* for 30 min) were prepared from brains of P14 or P40 mice, and S1 fractions were used for immunoprecipitation (IP) with anti-p-p53 antibodies or control serum. Pull-down products were then probed with anti-p-p53 or Tau1 antibodies; p-p53 and Tau1 immunoreactive bands were only found in products precipitated by anti-p-p53 antibodies. IB, Immunoblot. ***C–G***, Immunofluorescent analysis of p53 and MDM2 distribution in cultured hippocampal neurons. Hippocampal neurons were prepared from E18 BALB/c mouse embryos and kept *in vitro* for 3–16 d before being processed for immunofluorescent staining. ***C***, p-p53 immunoreactivity (green) and F-actin (labeled by phalloidin; red) in hippocampal neurons of 3, 5, and 10 DIV. ***D***, Colocalization of p-p53 with tau (labeled by Tau1 antibody; red) in the axon and with E6-AP (inset; red) in a growth cone of a DIV4 neuron. The arrows in ***C*** and ***D*** indicate growth cones. ***E***, Colocalization of Ac-p53, acetylated p53, with tau in the axon of a DIV4 neuron. ***F***, Distribution of total p53 revealed by antibodies that label all isoforms of p53 in cell bodies, dendrites, and axons. ***G***, MDM2 is also colocalized with tau protein but is also present in cell bodies.

plasmids were gifts from Dr. Zhiqun Tan (University of California, Irvine, Irvine, CA); mutation of R to H was confirmed by sequencing analysis provided by City of Hope (Duarte, CA). Plasmid transfection was performed as previously described (Qin et al., 2006). Briefly, neurons were incubated with DMEM (HyClone) with the addition of (per milliliter) 1 μg of plasmid DNA, 40 μl of 0.25 M $CaCl_2$, and 41 μl of BES (*N*,*N*-bishydroxyethyl-2-aminoethane-sufonic acid), pH 7.1, for 3 h. Neurons were then changed to fresh culture medium and further cultured for 18–24 h before being processed for time-lapse experiments or immunostaining analysis.

Chemicals and antibodies. (*R*)-(+)-*trans*-*N*-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y27632), pifithrin-α, pifithrin-μ, and actinomycin-D were purchased from Calbiochem. Anti-E6-AP and anti-ROCK1 antibodies were from Sigma-Aldrich. Anti-phospho-p21 (Thr-145) antibody was from Santa Cruz Biotechnology. Tau1 antibody was from BioSource. Anti-tubulin, anti-phospho-p53 (Ser-15) and its blocking peptides, anti-acetyl-p53 (Lys-379), anti-GFP antibodies, and control rabbit serum as well as a p53 small interfering RNA (siRNA) kit (SignalSilence) were from Cell Signaling. Phalloidin conjugated with Alexa 594, mutant conformation-specific p53 (MU-p53) antibody, wild-type conformation p53 (WT-p53) antibody, Alexa 488-conjugated anti-rabbit, Alexa 594 anti-mouse, and cell survival/death detect kit were from Invitrogen.

Neuronal culture, immunofluorescence, and image analysis. Cortical and hippocampal neurons were dissociated from embryonic day 18 (E18) mouse embryos (BALB/c; Charles River) and cultured in Neuro-

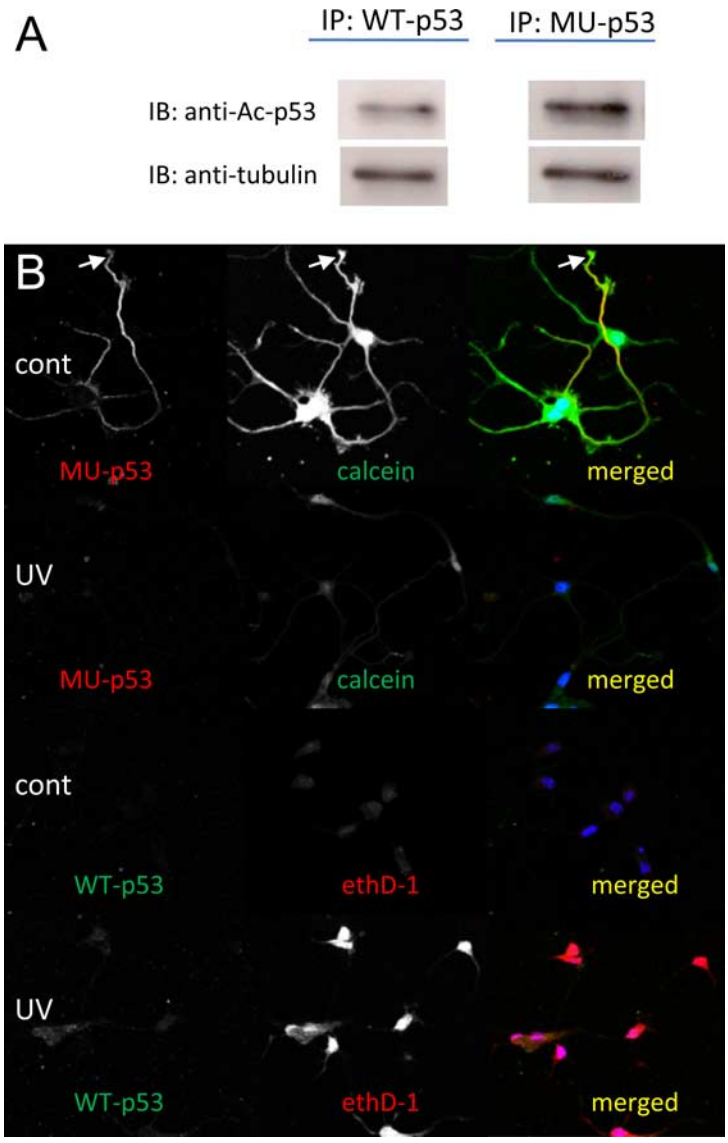

Figure 2. Axonally localized p53 proteins are labeled by a conformation mutant p53-specific antibody. ***A***, Primary cortical neurons at DIV4 were processed for immunoprecipitation (IP) with Pab-1620 and Pab-240 antibodies, which are specific for either wild-type or mutant conformation p53, respectively. Immunoprecipitated proteins were probed with antibodies against Ac-p53 or tubulin. IB, Immunoblot. ***B***, UV treatment reduced levels of conformation mutant p53 in axons and increased wild-type p53 levels in nuclei. Hippocampal neurons of DIV4 were treated with UV for 2 s and further incubated in culture medium for 8 h before being analyzed by a cell survival/death assay. Note that conformation mutant p53 (MU-p53) was mostly found in axons (arrows) in control live neurons (cont) labeled with calcein, whereas conformation wild-type p53 (WT-p53) was found in damaged nuclei double labeled with ethidium homodimer 1 (ethD-1) (red) in UV-treated neurons.

basal (Invitrogen) with 10% bovine serum albumin (BSA), 2% B27, and 1% glutamine. For immunofluorescent analyses, cells were fixed with 4% paraformaldehyde in phosphate buffer, pH 7.4, for 15 min. After washing with 1× PBS, cells were permeabilized with 0.05% Triton X-100 in 1× PBS for 15 min, and incubated with preblock buffer (3% BSA, 0.02% Triton X-100 in 1× PBS) for 15 min before being probed with primary antibodies; the following primary antibodies were used: anti-E6AP (1:1000), anti-phospho-p53 (1:250), anti-acetyl-p53 (1:1000), mutant form-specific p53 antibody (1:250), wild-type form p53 antibody (1:250), and Tau1 (1:1500). All primary antibodies were diluted in preblock buffer and incubated at 4°C for 18 h. After six washes (six times for 10 min each time) with 1× PBS at room temperature, cells were incubated with secondary antibodies: Alexa 488-anti-rabbit (1:500), Alexa 594-anti-mouse (1:500); both antibodies were diluted in preblock buffer and incubated at room temperature for 1 h, washed with 1× PBS (six times for 10 min each time), and mounted with mounting medium containing DAPI (4′,6′-diamidino-2-phenylindole) (Vectashield) to stain nuclei. Immunofluorescent signal was detected by a Nikon confocal microscope.

Quantification of growth cone morphology and immunoreactivity was performed with confocal images taken with a 60× objective. Approximately 30–40 images were randomly selected from each culture dish (20 mm in diameter); at least six to eight dishes from three to six individual culture preparations were used for each experimental group. Quantification was done blindly by multiple researchers. Growth cones with <1 filopodium were considered collapsed. ImageJ software was used to quantify growth cone areas and intensity of p-p53 immunoreactivity. Results were expressed as means ± SEM, and p value was determined by one-way ANOVA followed by *post hoc* analysis; values of $p < 0.05$ were considered statistically significant.

Chemical treatment. Y27632 (10 μM), actinomycin-D (10 μM), pifithrin-α (0.5–1 μM), and pifithrin-μ (50 μM) were first dissolved in 10% DMSO and diluted in cultured medium; final DMSO concentration was <0.01%.

Time lapse imaging. Cells were washed once with 1× PBS and changed to prewarmed (37°C) imaging buffer (100 mM NaCl, 3 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 2% B27 dissolved in 1× PBS), and the culture dish was transferred to an imaging chamber with temperature kept at 37°C. Images were acquired with a Nikon confocal microscope; parameters for image acquisition were kept constant between different treatments.

UV treatment. Cells were washed once with 1× PBS, treated with UV (50 mJ/cm^2) for 2 s, and changed to fresh culture medium and fixed for immunostaining 8 h later.

Immunoprecipitation and immunoblotting procedures. For immunoprecipitation, mouse brains or neuronal cultures were lysed in lysis buffer [0.05 M Tris base, 0.9% NaCl, pH 7.6, and 0.5% Triton X-100 plus protease inhibitors mixture set III (1:100; EMD Biosciences) and phosphatase inhibitor mixtures 1 and 2 (1:500; Sigma-Aldrich)]. Lysate was centrifuged at 16,000 × g for 30 min at 4°C. The supernatant was then cleared with a mixture of protein A/G-agarose beads (each 50%) for 1 h at 4°C, and after a brief spin, the pellet was discarded. A small portion of the supernatant was used as input. The reminder of the supernatant was immunoprecipitated overnight with phospho-p53, control rabbit serum, or WT-p53 or MU-p53 antibodies. Immunoprecipitates were captured by incubation with protein A/G-agarose beads for 3 h at 4°C. After several washes, the beads are resuspended in 2× SDS sample buffer (4% SDS, 100 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 20% glycerol, and 0.2% bromophenol blue) and boiled for 10 min. The resulting proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes for immunoblotting using previously described protocols (Liao et al., 2007).

Results

Expression of p53 in axons and growth cones

We first evaluated whether p53 participated in the development and maturation of CNS neurons by determining levels and localization of native as well as phosphorylated and acetylated p53 in

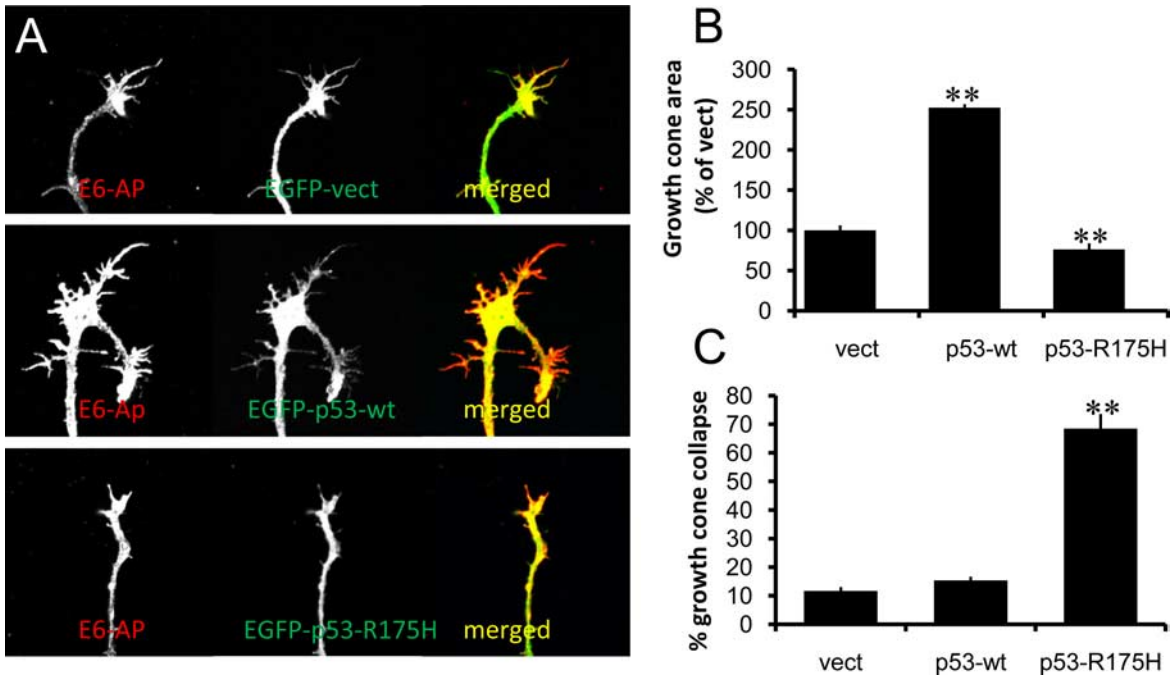

Figure 3. Overexpression of p53 enhances growth cone growth. ***A***, Effects of overexpressing p53 on growth cones. Hippocampal neurons were transfected on DIV3 and processed for immunofluorescence 18 h later. Compared with vectors (EGFP) alone (top panels), overexpression of wild-type p53 (EGFP-p53-wt) led to larger growth cones (middle panels), whereas overexpression of p53 with R175H mutation (EGFP-p53-R175H) resulted in growth cone collapse (bottom panels). ***B***, ***C***, Quantification of growth cone areas (***B***) and percentage of growth cone collapse (***C***) in hippocampal neurons transfected with vectors alone (vect), EGFP-wild-type-p53 (p53-wt), or EGFP-p53-R173H (p53-R175H). Data are means ± SEM; $n = 100$ neurons. $^{**}p < 0.01$ compared with values in vector-only-transfected neurons.

cultured cortical and hippocampal neurons with immunoblotting and immunofluorescence assays. Low levels of total p53 were consistently detected at 3–16 d *in vitro* (DIV), whereas levels of phosphorylated p53 (phosphorylated at residue Ser-15, hereafter referred to as p-p53) increased as a function of time in cultures (Fig. 1*A*). Immunoprecipitation assay indicated that anti-p-p53 pull-down products contained proteins labeled by Tau1 antibodies (Fig. 1*B*), whereas the precipitates from control serum did not, suggesting that p-p53 was associated with the axonal microtubule-associated protein tau. The specificity of the antibodies was also verified by preabsorbing experiments with p-p53-specific blocking peptides (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Confocal image analysis revealed that high levels of p-p53 were observed mainly in axons and axonal growth cones (Fig. 1*C*,*D*, arrows) in hippocampal neurons at DIV4; moderate levels of p-p53 were also present in cell bodies, whereas p-p53 was rarely present in dendrites. Phospho-p53 was colocalized with tau (Fig. 1*D*) but not with MAP2, a dendritic marker (data not shown), further confirming the axonal localization of p-p53. Expression of p53 acetylated at Lys-379 (equivalent to human Lys-382, referred to as Ac-p53) was detected with a specific antibody; Ac-p53 immunoreactivity was found mainly in axons but rarely in growth cones (Fig. 1*E*). In contrast, total p53 was distributed rather homogenously throughout neurons, albeit at a low level of expression (Fig. 1*F*). The same pattern of p-p53 expression was observed in neurons at DIV5; however, at DIV10 and DIV16, p-p53 was mainly found in cell bodies and neurites (Fig. 1*C*). At DIV4, p-p53 was also colocalized in axonal growth cones with E6-AP, an E3 ubiquitin ligase (Fig. 1*D*, insets). These results indicate that posttranslational modifications affect p53 distribution, with p-p53 being preferentially localized in axons as well as in growth cones and Ac-p53 in axons, especially at early developmental stages. In good agreement with these *in vitro* results, p-p53 immunoreactivity was also observed in mouse brains at E18 and postnatal day 1 (P1) (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

As MDM2 has been implicated in the regulation of p53 intracellular distribution (Boyd et al., 2000), we also examined its expression in cultured hippocampal neurons. Low to moderate levels of MDM2 phosphorylated at Ser-166 [which increases its activity (Mayo and Donner, 2001; Zhou et al., 2001)] were present in cell bodies and dendrites, whereas high levels were present in axons labeled with Tau1 antibodies (Fig. 1*G*).

p53 exists in two conformations: a wild-type form recognized by the antibody Pab-1620, and a "mutant" form recognized by the antibody Pab-240 (Gannon et al., 1990; Sasaki et al., 2007). These different conformations determine p53 activity and localization, with the mutant form exhibiting low or no transcriptional activity and mainly a cytoplasmic localization. Under certain conditions, these two conformations are exchangeable (Milner and Medcalf, 1991), a phenomenon that is actively pursued for developing cancer treatments (Selivanova and Wiman, 2007; Wang and El-Deiry, 2008). We used these two conformational specific antibodies to investigate whether p53 proteins localized in axons and growth cones were assuming a specific conformation. Immunoprecipitation experiments performed with the conformation-specific antibodies Pab-1620 and Pab-240 indicated that in primary cultured cortical neurons p53 existed in both wild-type and mutant conformations; both isoforms were acetylated, as immunoprecipitation pull-down products were recognized by anti-Ac-p53 antibodies (Fig. 2*A*). Furthermore, tubulin was found in immunoprecipitates prepared with both pAb-1620 and pAb-240 antibodies, suggesting that both forms of p53 are associated with this microtubule protein (Fig. 2*A*). However, confocal image analysis revealed that, whereas very low im-

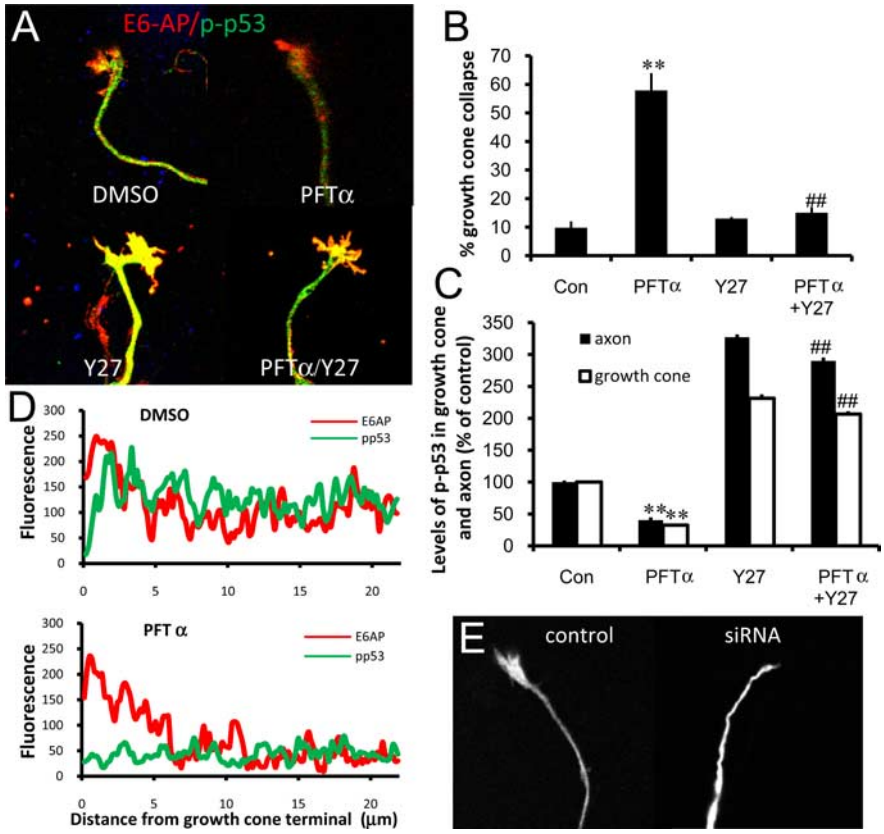

Figure 4. p53 inhibition-induced growth cone collapse is prevented by the ROCK inhibitor Y27632. ***A–D***, Pifithrin (PFT)-α-elicited p53 inhibition induces growth cone collapse, which is blocked by the ROCK inhibitor Y27632 (Y27). Hippocampal neurons were treated with DMSO (Con, vehicle), pifithrin-α (500 nM), pifithrin-α with ROCK inhibitor Y27632 (10 μM), or Y27632 alone on DIV3, and processed for immunofluorescence 20 h later. ***A***, Representative images of growth cones immunostained with anti-E6-AP (red) and anti-p-p53 (green) antibodies from different treatments. ***B***, ***C***, Quantification of percentage of growth cone collapse (***B***) and fluorescence intensity of anti-p-p53 immunostaining in axons and growth cones (***C***) ($n = 30$; $^{**}p < 0.01$ compared with DMSO treated; $^{\#\#}p < 0.01$ compared with pifithrin-α alone). Error bars indicate SEM. ***D***, Fluorescence profiles of anti-E6-AP (red) and anti-p-p53 (green) in axons and growth cones. Pifithrin-α treatment induced a marked decrease in p-p53 in axons and growth cones. ***E***, p53 siRNA treatment induces growth cone collapse. Hippocampal neurons were treated with a set of p53-specific siRNA (siRNA) or control siRNA (control) conjugated with GFP (20 nM) for 24 h and processed for image analysis.

munoreactivity was observed with anti-wild-type conformation p53 (Fig. 2*B*, bottom panels), moderate levels of immunofluorescence were found only in axons and growth cones of hippocampal neurons with the anti-conformational mutant antibody (Fig. 2*B*, top panels, arrows). Neurons immunopositive with mutant p53 antibodies were also labeled by calcein, an indicator of cell survival. Ultraviolet (UV) treatment was used to induce apoptosis, resulting in a marked increase in conformational wild-type p53 levels in nuclei of neurons labeled with ethidium (EthD-1) (a cell death marker) but in a decrease in conformational mutant p53 levels in axons and growth cones (Fig. 2*B*). Mutant p53 isoforms were mostly colocalized with p-p53 and Ac-p53 (data not shown). Together, these results indicate that in axons and growth cones p53 is not only modified by acetylation and phosphorylation, but also exists in a special three-dimensional conformation recognized by anti-mutant p53 antibodies. These special features may contribute to its axon/growth cone targeting and its promotion of axonal growth function.

p53 overexpression affects growth cones

To test whether genetic manipulations of p53 levels could affect axonal growth cones, hippocampal neurons were transfected at DIV3 with vectors containing EGFP linked to either wild-type p53 or p53 with the R175H mutation, a dominant-negative form of p53, and fixed 18 h later for microscopic analysis. In EGFP vector (used as a control)-transfected neurons, fluorescence was detected in cell bodies, dendrites, and axons. EGFP-wild-type-p53 (referred to as EGFP-p53-wt) was found preferentially in cell bodies, axons, and axonal growth cones (Fig. 3*A*, middle panels), suggesting that wild-type p53 was preferentially targeted to axons. However, EGFP-p53-R175H was mainly localized in cell bodies; only in a few neurons did EGFP-p53-R175H reach axons and axonal growth cones (Fig. 3*A*, bottom panels). Compared with neurons transfected with vector only (Fig. 3*A*, top panels), those transfected with EGFP-p53-wt exhibited larger axonal growth cones with larger lamellae and longer branched filopodia; growth cone area was 150% larger than in vector-transfected neurons (Fig. 3*B*). Immunoprecipitation experiments indicated that EGFP-p53-wt expressed in cortical neurons was pulled down by anti-MU-p53 antibodies, suggesting that the transfected wild-type p53 also assumed the mutant conformation (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). In contrast, neurons transfected with EGFP-p53-R175H had much smaller axonal growth cones with smaller lamellae and filopodia (Fig. 3*A*, *B*). Furthermore, expression of EGFP-p53-R175H led to a significant increase in growth cone collapse (Fig. 3*C*) and decreased expression of p-p53 and conformational mutant p53 in axons and growth cones (data not shown).

p53 suppression induces growth cone collapse

Potential functions of endogenous p53 in axonal development were first investigated by inhibiting p53 activity with pifithrin-α (Komarov et al., 1999) and by downregulating p53 expression with a specific set of siRNAs. Treatment of DIV3 hippocampal neurons with pifithrin-α (500 nM) for 24 h induced growth cone collapse (percentage of axons exhibiting growth cone collapse: 58 ± 6 vs 10 ± 2% in DMSO/vehicle treated; $p < 0.01$) (Fig. 4*A*, *B*). Pifithrin-α treatment also resulted in decreased p-p53 immunoreactivity in axons and growth cones (Fig. 4*C*,*D*). More recent experiments showed that treatment with 1 μM pifithrin for 2 min also induced growth cone collapse (72 vs 7% in DMSO/vehicle treated; average of two experiments). Pifithrin-α treatment did not induce significant changes in dendritic growth cone collapse (percentage of dendritic growth cone collapse: 19 ± 3 vs 19 ± 1% in DMSO/vehicle treated). Similarly, incubation with p53 siRNAs (20 nM) for 24 h induced growth cone collapse (percentage of axons exhibiting growth cone collapse: 50 ± 4 vs 19 ± 2% in control siRNA-treated) (Fig. 4*E*). Axons of p53 siRNA-treated neurons also exhibited lower levels of p-p53 compared with control siRNA-treated. These experiments demonstrated that blocking p53 function induces

growth cone collapse, whereas increasing p53 levels leads to larger growth cones.

ROCK inhibitor blocks p53 inhibition-induced growth cone collapse

Rho GTPases play important roles in neuronal polarization and axonal and dendritic growth (Bito et al., 2000). To test whether members of the Rho GTPase family were involved in p53-mediated regulation of axonal growth cone motility, an inhibitor of ROCK, Y27632, was included in various treatments (Kubo et al., 2007). When applied at 10 μM, Y27632 completely blocked pifithrin-α-induced growth cone collapse (Fig. 4*A*,*B*); growth cone collapse prevalence in pifithrin-α plus Y27632-treated neurons was 15 $\pm$ 2% (means $\pm$ SEM from three experiments and >100 neurons; $p < 0.01$ compared with pifithrin-α treated). However, treatment with 10 μM actinomycin-D did not block pifithrin-μ-induced growth cone collapse (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). In addition to inducing smaller growth cones, EGFP-p53-R175H was often found to form aggregates in cell bodies (Fig. 5). Y27632 also significantly decreased the number of EGFP-p53-R175H aggregates and promoted p-p53 and conformational mutant p53 axonal localization (Fig. 5, arrows) in EGFP-p53-R175H-transfected neurons. Quantitative analysis indicated that only 22% of EGFP-p53-R175H-transfected neurons treated with Y27632 contained aggregates, whereas >55% of neurons treated with vehicle showed aggregates ($n = 102$ neurons for DMSO-treated and 121 for Y27632-treated from three separate experiments; $p < 0.001$).

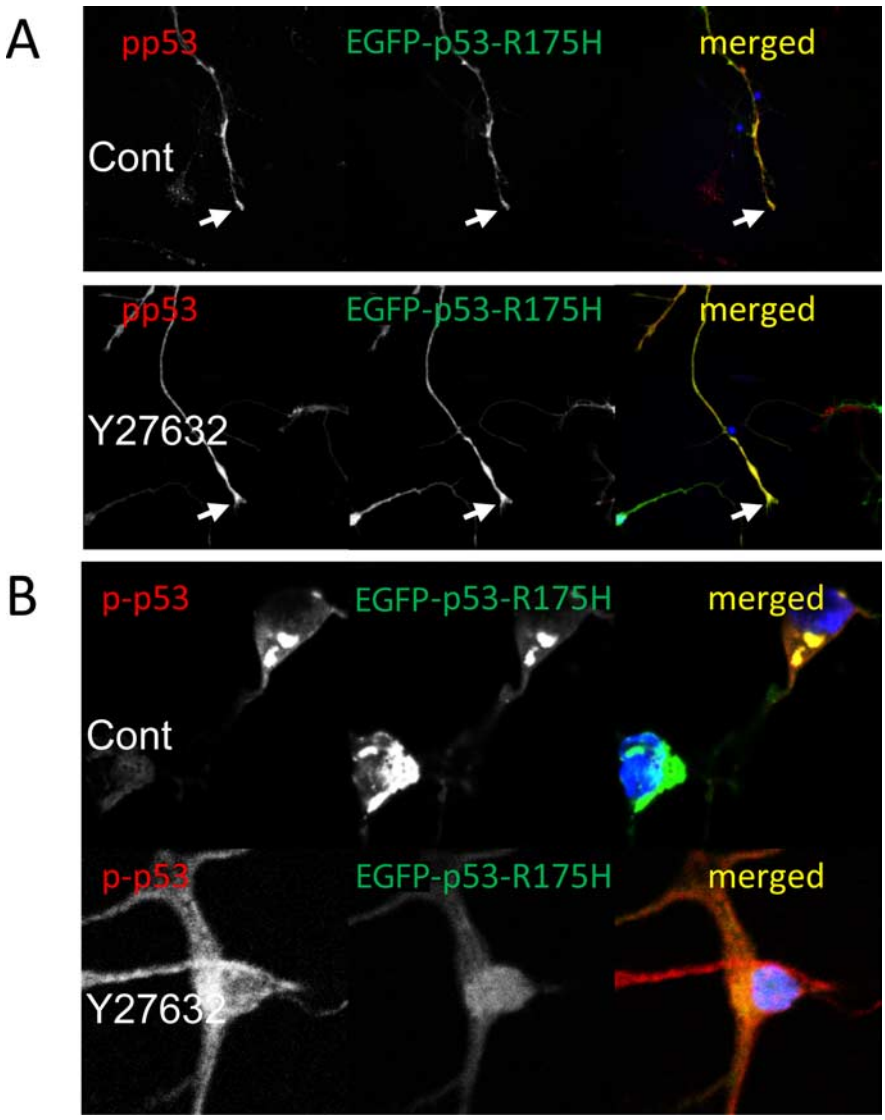

Figure 5. Expression of p53 with R175H mutation reduces p-p53 axonal distribution and induces its aggregation. Hippocampal neurons cultured for 3 d were transfected with EGFP-p53-R175H plasmids (green), treated with DSMO or Y27632 (10 μM), fixed on DIV4, and double stained with anti-p-p53 antibodies (red). ***A***, Images show that levels of p-p53 in axons and growth cones of EGFP-p53-R175H-transfected neurons are very low, and Y27632 treatment increases p-p53 intensity in axons and growth cones (arrows). ***B***, High-magnification images show that both EGFP-p53-R175H and p-p53 form clusters in cell bodies of DMSO-treated neurons but not of Y27632-treated neurons.

Pifithrin-μ-induced rapid growth cone collapse is also blocked by ROCK inhibition

Recently, another p53 inhibitor, pifithrin-μ, has been developed (Strom et al., 2006). Unlike pifithrin-α, this new p53 inhibitor suppresses p53-dependent apoptosis without affecting the transactivation function of p53. We therefore investigated the effects of pifithrin-μ to test whether p53 could promote growth cone development via transcription-independent pathways. Time lapse experiments showed that pifithrin-μ (50 μM) induced rapid growth cone collapse within 1 min after local application to DIV4 hippocampal neurons (Fig. 6*A*). Image analysis revealed a 73% growth cone collapse in inhibitor-treated neurons (50 μM for 2 min) compared with 14% in DMSO/vehicle treated ($n = 200$ growth cones from five experiments; $p < 0.001$) (Fig. 6*B*). The same treatment with pifithrin-μ also induced a rapid reduction in p-p53 levels in both axons and growth cones (Fig. 6*C*,*D*); a similar effect was observed for Ac-p53 (data not shown). Pifithrin-μ-induced rapid growth cone collapse and changes in levels of p-p53 were significantly reduced in the presence of Y27632 (Fig. 6*D*). Treatment with pifithrin-μ also significantly reduced levels of conformation mutant p53 in axons and growth cones, an effect that was also blocked by the ROCK inhibitor (data not shown).

Blocking p53 nuclear export results in growth cone collapse

Recent research has shown that p53 contains its own leucine-rich NES, and one study showed that the putative p53 NES is required for MDM2-independent shuttling of p53 from one nucleus to a second nucleus in heterokaryons (Stommel et al., 1999). A fusion construct of GFP and p53 with an NES mutation (L348A, L350A) [GFP–p53(NES–)] exhibited a predominant nuclear localization, which was not affected by cotransfection with HDM2 (Boyd et al., 2000). To test whether p53 function in growth cones required axonal localization, we transfected DIV3 hippocampal neurons

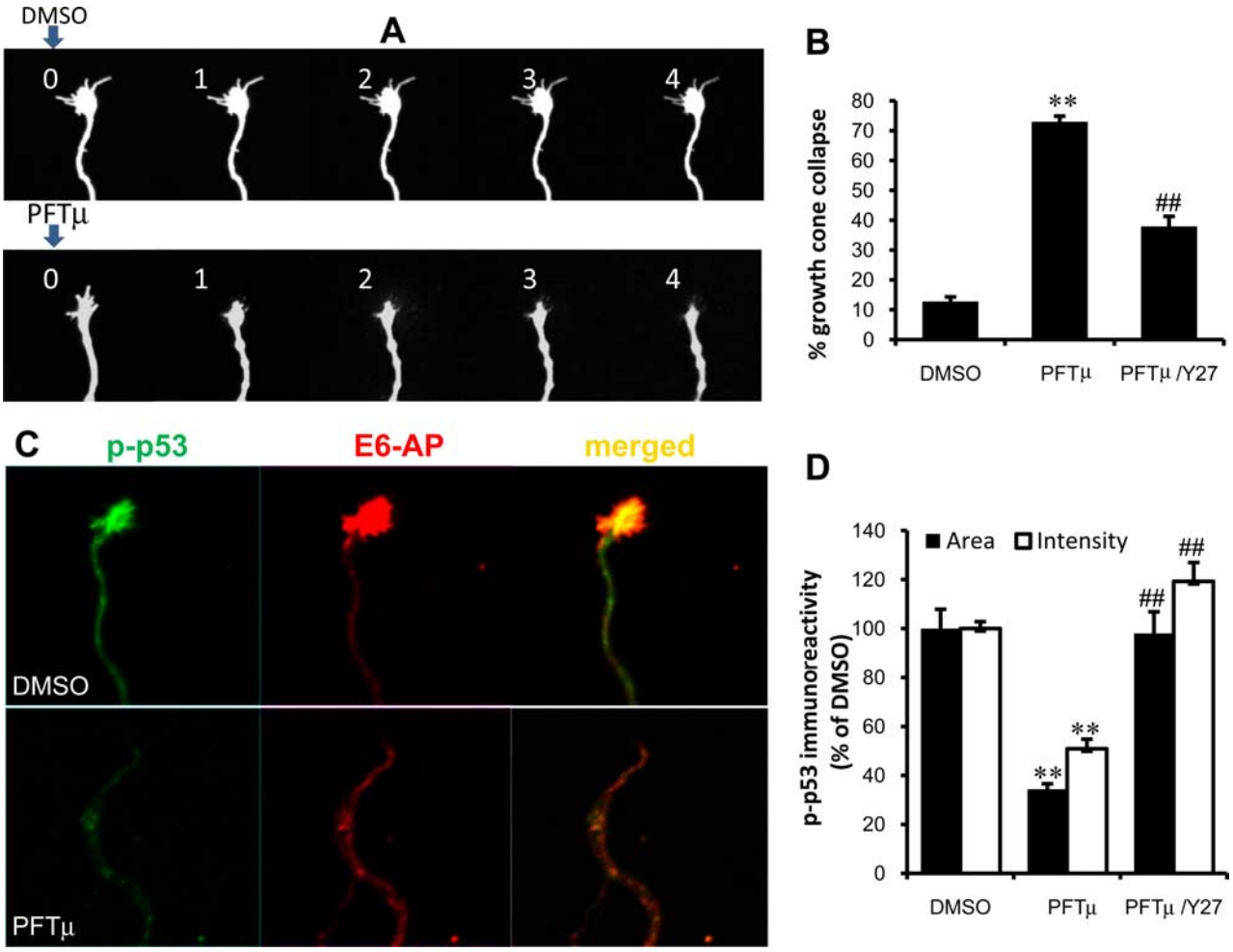

Figure 6. Effects of pifithrin-μ on p53 distribution and growth cones. ***A***, Rapid growth cone collapse is induced by pifithrin-μ. DIV4 hippocampal neurons were treated with 50 μM pifithrin-μ, a new p53 inhibitor that does not suppress p53-mediated transactivation, and confocal images were taken at a 1 min interval. Note that growth cone collapse was achieved by 1 min after inhibitor application. ***B***, Percentage of growth cone collapse in neurons treated for 2 min with DMSO/vehicle or pifithrin-μ. Error bars indicate SEM. ***C***, ***D***, Pifithrin-μ treatment for 2 min induces significant decreases in levels of p-p53 in axons and growth cones. ***C***, Representative images. ***D***, Quantification of p-p53 immunoreactive areas and intensity in growth cones ($n = 130$ growth cones; $^{**}p < 0.01$ compared with DMSO treated). Quantification indicates that pifithrin-μ induced growth cone collapse and decreases in p-p53 immunoreactivity were significantly reduced by ROCK inhibitor Y27632 (***B***, ***D***) ($n = 120$; $^{\#\#}p < 0.01$ compared with pifithrin-μ treated).

with the GFP–p53(NES–) constructs. In agreement with the previous report, GFP–p53(NES–) was predominantly expressed in nuclei of transfected hippocampal neurons (Fig. 7). Double immunofluorescence experiments showed that, in GFP–p53(NES–)-transfected neurons, both p-p53 (Fig. 7*A*) and the conformational mutant p53 (Fig. 7*B*) also exhibited a predominantly nuclear localization, and very low levels of both types of p53 were observed in axons and growth cones. Quantitative analysis showed that levels of p-p53 and MU-p53 in axons of GFP–p53(NES–)-transfected neurons were 18 and 6%, respectively, of those in axons of GFP-vector-transfected neurons ($n = 30$ axons; $p < 0.01$). Furthermore, overexpression of GFP–p53(NES–) also resulted in growth cone collapse (Fig. 7). These results suggest that a major portion of newly synthesized p53 (including endogenous) is preferentially transferred to the nuclei before being exported to cytoplasm and targeted to axons and growth cones and that transactivation-independent function of p53 is needed for axonal growth.

Discussion

In this study, we investigated the potential roles of the tumor suppressor p53 in the regulation of axonal growth cones. We first found that modified forms (posttranslationally and structurally) of p53 were highly expressed in axons and axonal growth cones in early developing neurons. Inhibition of p53 function by inhibitors or siRNAs resulted in growth cone collapse. In particular, both pifithrin-α and pifithrin-μ induced rapid growth cone collapse, within 2 min after application, a result that provided the first indication of a transcription-independent mechanism. Additional experiments showed that blockade of p53 nuclear export also produced growth cone collapse. Together, these data strongly support the existence of an unexpected and critical function of p53 in axonal growth cones, which could play important roles during CNS development and in axonal regeneration after brain injury.

Selective axonal and growth cone localization of modified p53

A surprising finding of our study was that, early in development, modified forms of p53 (phosphorylated, acetylated, and conformational mutant) were preferentially localized in axons and growth cones (phosphorylated and conformational mutant). During the initial polarization of neurons, a number of proteins are differentially transported to axons or dendrites (Samuels et al., 1996). As p53 was found in both dendritic and axonal com-

partments, the selective localization of the modified forms of p53 seems to be dictated by their modifications. This implies that the modified isoforms are preferentially targeted to axons or that p53 is selectively modified in axons. The latter possibility would require the selective localization of the appropriate enzymes, which has yet to be determined. Previous studies with cells derived from human neuroblastomas indicated that covalent modification of p53 may function as a common means to sequestrate p53 in the cytoplasm (Zaika et al., 1999), which further indicates that certain posttranslational modifications can in fact dictate p53 nuclear or cytosolic localization. Although this interpretation may explain the non-nuclear localization of covalently modified p53 in developing hippocampal neurons, it does not explain why modified p53 proteins are preferentially targeted to axons and not dendrites. A recent study has shown that p53 ubiquitination by HDM2 is required for p53 localization in the cytoplasm (Boyd et al., 2000); whether MDM2/HDM2 is involved in p53 axonal targeting remains to be determined. One possibility could be that p-p53 is more resistant to MDM2-mediated ubiquitination and degradation (Haupt et al., 1997). Intriguingly, overexpression of GFP–p53(NES–) also resulted in nuclear accumulation of endogenous p53. Based on these results, it is tempting to speculate that, in early developing hippocampal neurons, newly synthesized p53 proteins are first targeted to the nuclei in which they form oligomers and are perhaps phosphorylated before being exported together with MDM2 and targeted to axons and growth cones. This hypothesis also explains why overexpression of GFP–p53(NES–) not only induces growth cone collapse but also sequesters endogenous p53 in nuclei. Although the precise mechanism by which modified forms of p53 are preferentially localized in axons and growth cones in developing neurons remains to be determined, our results suggest that this selective localization of modified p53 forms is associated with p53 regulation of growth cone motility and possibly neuronal survival.

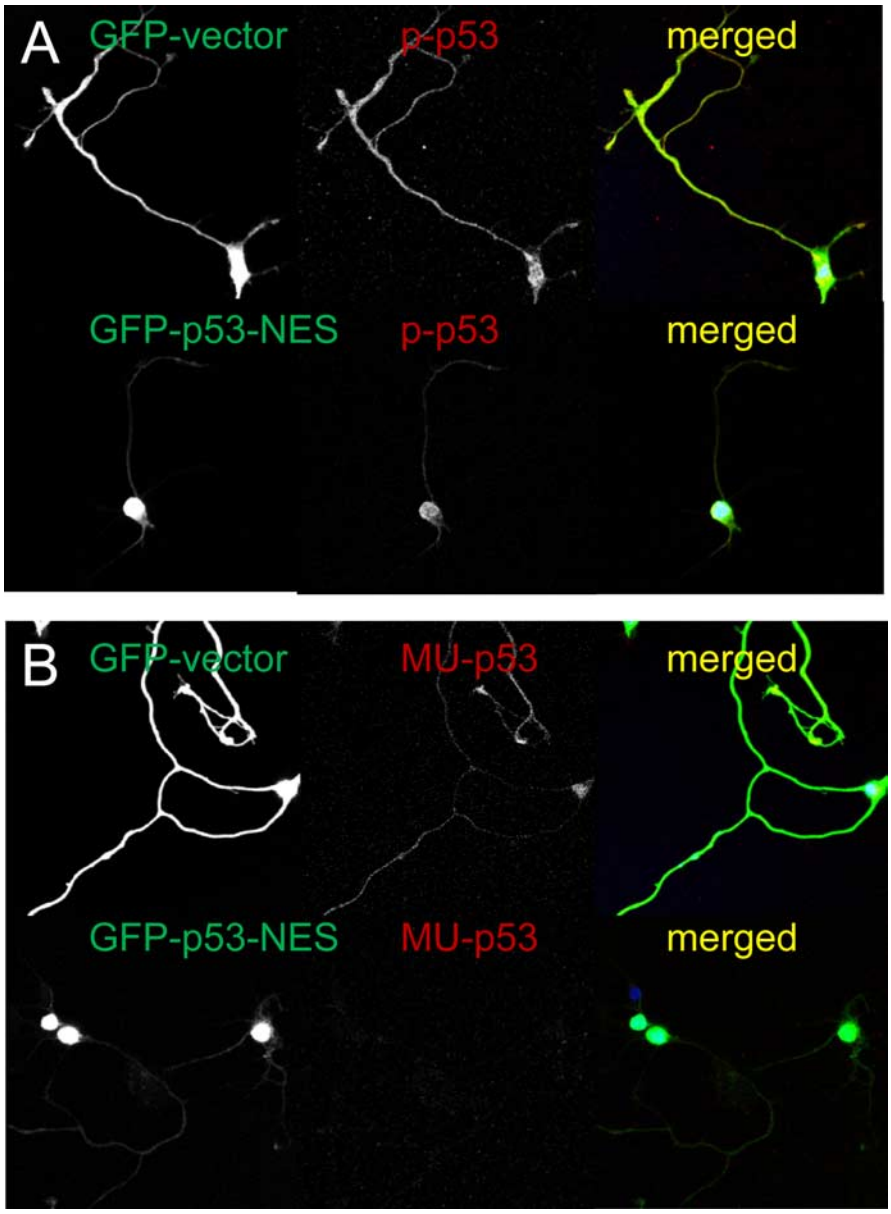

Figure 7. p53 nuclear export signal deletion induces p53 redistribution and growth cone collapse. Hippocampal neurons at DIV3 were transfected with a fusion construct of GFP and p53 with NES mutation (L348A, L350A) [GFP–p53(NES–)] and cultured for 18 h before being processed for immunofluorescence with anti-p-p53 (***A***) or anti-MU-p53 (***B***). Neurons transfected with GFP–p53(NES–) showed smaller growth cones compared with neurons transfected with only the GFP vector. Furthermore, levels of both p-p53 and MU-p53 in axons and growth cones were markedly decreased compared with levels in neurons without transfection. High levels of p-p53, but not of MU-p53, were observed in nuclei of transfected neurons.

In vitro development of cultured embryonic hippocampal neurons has been separated into five stages (for a recent review, see Arimura and Kaibuchi, 2007; Nikolić, 2008). Stage 1 is the "epithelial cell-like stage," characterized with a flat cell body with predominant lamellipodia and few very thin filopodia. Stage 2 is the "stage of minor processes" with morphologically indistinguishable short neurites. Stage 3 is characterized by the specification of one neurite into an axon. Stages 4 and 5 represent the continuation of axonal development and the growth of dendrites. Stages 1–3 generally occur during the first 2–5 d in culture, whereas the maturation of dendrites takes up to 21 d. A possible function for the high levels of modified p53 in axons during the early stages could be to contribute to the specification and maintenance of one process into an axon; similarly, decreased levels in axons (after DIV5) and finally complete disappearance after DIV10 could correspond to the switch from axonal development to dendritic and spine maturation.

Our results indicated that overexpression of p53-R175H led to growth cone collapse paralleled with decreases in p-p53 in axons and growth cones. R175H mutation is one of the most prevalent p53 mutations in human cancer mutations; the mutation occurs in the DNA binding domain and belongs to the family of conformational mutants (in contrast to contact site mutants) that inhibit p53 function by altering the structure of the protein (Cho et al., 1994). Classification of this family of mutants was mainly

attributable to the use of the Pab-240 antibody that recognizes residues exposed in the mutant but cryptic in the wild type (Milner and Medcalf, 1991). Previous *in vitro* experiments have shown that R175H p53 mutants have increased thermodynamic instability (Friedler et al., 2003); other studies revealed that co-translation of mutant p53 with wild type drives wild-type p53 proteins into mutant conformation (Milner and Medcalf, 1991). These results provide potential explanation for the formation of p53 aggregates in neurons expressing p53-R175H constructs. However, the R175H mutants expressed in hippocampal neurons also exhibited properties distinct from those found in cancer cells, as they were not recognized by Pab-240 antibodies. Application of the ROCK inhibitor Y27632 not only reversed mutant p53-induced growth cone collapse but also decreased p53 aggregates. Thus, the close association between the presence of mutant p53 aggregates, the lack of axonal localization of p-p53, and growth cone collapse further strengthens the notion that a special structure of p53 is needed for its presence and function in axons and growth cones.

A potential local function of p53 in growth cone regulation

Previous studies have shown that dominant-negative forms of p53 inhibited NGF-induced neuritic growth in rat PC12 cells (Fábián et al., 2006; Zhang et al., 2006) and cortical neurons (Di Giovanni et al., 2006). These effects were associated with p53-induced increases in transcription and expression of TrkA receptors (Zhang et al., 2006), coronin 1b (a F-actin binding protein), and the small GTPase, Rab13 (Di Giovanni et al., 2006). Our data provide three lines of evidence suggesting a transactivation-independent novel function for p53 in growth cone regulation. First, as mentioned in the previous section, axonal localization of modified p53 was closely associated with growth cone motility: growth cone collapse occurred in parallel with decreases in axonal levels of modified p53. Second, the p53 inhibitors pifithrin-α and pifithrin-μ induced rapid growth cone collapse (within minutes), an effect that was blocked by a ROCK inhibitor but not by the transcriptional inhibitor actinomycin D. Finally, blocking p53 nuclear export, which should not suppress p53 transaction, induced growth cone collapse and resulted in nuclear accumulation of modified p53 and downregulation of its expression in axons and growth cones. Together, these results indicate that p53 regulates growth cone motility via a local mechanism that involves the Rho kinase pathway.

p53 regulation of growth cone motility involves suppression of ROCK

Our study showed that growth cone collapse induced by p53 inhibitors or by p53-specific siRNA was significantly reduced in the presence of the ROCK inhibitor Y27632. These results suggest that p53 may function upstream of ROCK as a negative regulator of ROCK-induced growth cone collapse. However, application of the ROCK inhibitor Y27632 not only diminished growth cone collapse but also prevented the decreases in levels of modified p53 in axons and growth cones, which implies that ROCK activity plays some role in posttranslational modification of p53. Together, these data suggest that p53 and ROCK may interact in a reciprocal regulatory loop. The role of ROCK pathway in growth cone motility is well demonstrated, as ROCK regulates both the actin network and the microtubule system. Emerging evidence has also shown that p53 is associated with both actin filaments and microtubules, at least in several cancer cell lines (for a recent review, see O'Brate and Giannakakou, 2003). It is thus conceivable that p53 interacts with ROCK in the regulation of both the actin network and the microtubule system in growth cones. Recent studies have indicated that local protein synthesis, especially the synthesis of RhoA and β-actin, is critically involved in growth cone regulation (Wu et al., 2005; Leung et al., 2006). Emerging evidence also indicates that p53 tumor suppressing function is linked to its ability to inhibit protein translation (Fontoura et al., 1992; Marechal et al., 1994; Miller et al., 2000). Whether p53 suppresses Rho kinase pathway by inhibiting local protein translation is currently under investigation.

Although previous publications have indicated that p53 may promote neurite growth, our study provides the first evidence that p53 promotes axonal growth through local interaction with ROCK kinase. This function requires axonal targeting of p53 and is mostly transactivation independent, but is associated with p53 phosphorylation and acetylation. Finally, our results clearly indicate that p53 functions in the regulation of growth cone growth via inhibition of Rho kinase activity. The precise mechanism involved in this effect is not fully understood. Nevertheless, considering the number of pathological conditions associated with defects in axonal development and regeneration, our results clearly open new avenues for identifying new therapeutic targets and potentially new treatments for these conditions.

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