SCYL1BP1 modulates neurite outgrowth and regeneration by regulating the Mdm2/p53 pathway

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ABSTRACT SCY1-like 1–binding protein 1 (SCYL1BP1) is a newly identified transcriptional activator domain containing a protein with many unknown biological functions. Recently emerging evidence has revealed that it is a novel regulator of the p53 pathway, which is required for neurite outgrowth and regeneration. Here we present evidence that SCYL1BP1 inhibits nerve growth factor–mediated neurite outgrowth in PC12 cells and affects morphogenesis of primary cortical neurons by strongly decreasing the p53 protein level in vitro, all of which depends on SCYL1BP1’s transcriptional activator domain. Exogenous p53 rescues neurite outgrowth and neuronal morphogenesis defects caused by SCYL1BP1. Furthermore, SCYL1BP1 can directly induce Mdm2 transcription, whereas inhibiting the function of Mdm2 by specific small interfering RNAs results in partial rescue of neurite outgrowth and neuronal morphogenesis defects induced by SCYL1BP1. In vivo experiments show that SCYL1BP1 can also depress axonal regeneration, whereas inhibiting the function of SCYL1BP1 by specific short hairpin RNA enhances it. Taken together, these data strongly suggested that SCYL1BP1 is a novel transcriptional activator in neurite outgrowth by directly modulating the Mdm2/p53-dependent pathway, which might play an important role in CNS development and axonal regeneration after injury.

INTRODUCTION

Neurite outgrowth, sprouting, and regeneration require an orchestrated sequence of events. There is compelling evidence that the tumor suppressor protein p53 regulates neurite outgrowth and regeneration in vitro and in vivo. As a transcription factor, p53 is required for nerve growth factor (NGF)–mediated differentiation of PC12 cells via regulation of high-affinity NGF receptor TrkA levels (Zhang et al., 2006). p53 is also required for neurite outgrowth in cultured cells, including primary neurons and PC12 cells, as well as for axonal regeneration in vivo via increased expression of coronin 1b (a filamentous actin–binding protein), the small GTPase Rab13 (Di Giovanni et al., 2006), and the cGMP-dependent protein kinase type I (Tedeschi et al., 2009b). In addition, p53 plays a direct role in axon outgrowth by occupying the promoter of the proaxon growth gene GAP-43 (Tedeschi et al., 2009a). In addition to its transcriptional function, p53 plays a novel role in axonal growth cone behavior, acting as a suppressor of ROCK activity (Qin et al., 2009). These results suggest that p53 regulates neurite outgrowth and regeneration in both transactivation-dependent and transactivation-independent manners. Nonetheless, few endogenous cellular pathway molecules have been reported to be involved in the regulation of neurite outgrowth through a transcriptional activity or a nontranscriptional effect of p53, such as dysbindin-1 (Ma et al., 2011) and calpain (Qin et al., 2010), respectively.
SCYL1BP1 is a protein with a short half-life and is maintained at low or undetectable levels by continual proteolytic degradation in normal cells. Sustained degradation of p53 is mediated by the ubiquitin-proteasome system (Maki et al., 1996), mainly by the E3 ligase Mdm2 (Fuchs et al., 1998; Fang et al., 2000). By binding p53, Mdm2 also conceals the transactivation domain of p53 and therefore inhibits its transcriptional activity (Kubbutat and Vousden, 1998). In addition, Mdm2 is required to promote p53 nuclear export and subsequently decrease its transcriptional activity (Boyd et al., 2000; Geyer et al., 2000). Mdm2 itself is also a transcriptional target of p53 (Kubbutat and Vousden, 1998; Prives, 1998). These data reveal a feedback loop that regulates both p53 activity and the expression of Mdm2, which is critical for maintaining normal cellular physiology. Challenged with stress signals such as hypoxia or DNA damage, the feedback loop of Mdm2-p53 is disturbed, leading to apoptosis or malignancy (Marine et al., 2006). However, little is known about the role of the Mdm2-p53 feedback loop in neurite outgrowth and regeneration.

Several factors have been described to affect the feedback loop of Mdm2-p53, such as retinoblastoma protein, transcriptional factor Yin Yang 1, promyelocytic leukemia (Bernardi et al., 2004), and SCY1-like 1-binding protein 1 (SCYL1BP1). SCYL1BP1 is a newly identified regulator of the Mdm2-p53 feedback loop, which directly binds to Mdm2 and promotes Mdm2 self-ubiquitination, subsequently stabilizing p53 (Yan et al., 2010a,b). SCYL1BP1, initially identified as an SCYL1-binding protein (Di et al., 2003), is an evolutionally conserved protein existing in different organisms ranging from plants to animals. SCYL1BP1 protein is observed in many normal tissues and is mainly localized in the cytoplasm, with a low level in the nucleus. Bioinformatics analysis reveals that SCYL1BP1 protein has a predicted bipartite nuclear localization signal, a breast carboxy-terminal domain, two coiled-coil domains, and a transcriptional activator domain (Di et al., 2003). All these results strongly suggest that SCYL1BP1 might play an important role in transcriptional activity. Therefore we hypothesized that SCYL1BP1 regulated the Mdm2-p53 feedback loop not only via proteins interactions, but also through unknown transcriptional mechanisms.

In the present study, we characterize the function of SCYL1BP1 in neurite outgrowth and regeneration. Overexpression of SCYL1BP1 inhibits NGF-mediated neurite outgrowth of PC12 cells and affects morphogenesis of cortical neurons via transcriptional regulating Mdm2 expression and consequently p53 inhibition. In addition, overexpression of SCYL1BP1 inhibits neuronal regeneration after facial nerve axotomy, whereas inhibiting the function of SCYL1BP1 overexpression of SCYL1BP1 inhibits neuronal regeneration after facial nerve axotomy, leading to apoptosis or malignancy (Marine et al., 2006). However, little is known about the role of the Mdm2-p53 feedback loop in neurite outgrowth and regeneration.

RESULTS

Exogenous SCYL1BP1 attenuates NGF-mediated neurite outgrowth of PC12 cells and affects the morphogenesis of cortical neurons

To investigate the role of SCYL1BP1 in axon outgrowth, we first examined expression patterns of SCYL1BP1 during NGF-mediated neurite outgrowth of PC12 cells and morphogenesis of primary cortical neurons cultured in vitro. Western blot showed that SCYL1BP1 expression was decreased at different times after 100 ng/ml NGF treatment in naive PC12 cells, which was similar to the dynamics we observed in primary neurons prepared from E18 rat cortex and cultured for different days in vitro (DIV, Figure 1, A and B). Alternatively, immunofluorescence staining was performed to identify the distribution of endogenous SCYL1BP1. In naive PC12 cells, SCYL1BP1 had a high level in the cytoplasm and a low level in the nuclei. After NGF treatment, SCYL1BP1 expression was largely decreased in both components, especially in nuclei (Figure 1C). Similar dynamics was observed in primary cortical neurons (Figure 1D). Furthermore, cell fractionation assays were performed and showed identical results (Figure 1, E and F). We then hypothesized that SCYL1BP1 could be important for neuronal morphogenesis.

To test our hypothesis, we generated a naive PC12 cell line that stably expresses SCYL1BP1 and examined cell morphology by phase-contrast microscopy. We found that NGF treatment could induce PC12 cells to undergo differentiation, which was blocked by the overexpression of SCYL1BP1 (Figure 2B). To confirm this observation, we quantified neurite outgrowth. The percentage of cells with neurites longer than two body lengths was calculated, and at least 500 cells were assayed in each group. The percentage of cells with neurites longer than two body lengths was conspicuously decreased by SCYL1BP1, ranging from 15% to 4.5% (Figure 2C). To further investigate the role of SCYL1BP1 in axon outgrowth, we generated two mutants of SCYL1BP1: BP1-N, which lacks the transcriptional activator region, and BP1-T, which contains the C-terminal transcriptional activator region (Figure 2A). Like SCYL1BP1, BP1-T also attenuates NGF-mediated neurite outgrowth of PC12 cells (Figure 2, B and C). Because SCYL1BP1 impaired neurite outgrowth, MTT assays were performed to examine whether it affected cell viability; both SCYL1BP1 and BP1-T, but not BP1-N, increased PC12 cell viability (Supplemental Figure S1A). A
SCYL1BP1 knockdown experiment was also performed with lentivirus encoding green fluorescent protein (GFP) or together with SCYL1BP1 shRNA. SCYL1BP1 shRNA 1 resulted in ~80% reduction in SCYL1BP1 level when compared with control shRNA (Supplemental Figure S1B). SCYL1BP1 shRNA 1 did not affect naive PC12 cell neurite outgrowth without NGF, whereas after NGF treatment, SCYL1BP1 shRNA 1 enhanced neurite outgrowth of PC12 cells (Supplemental Figure S1, C and D).

In addition, we transfected cultured cortical neurons with lentivirus encoding GFP or together with SCYL1BP1, BP1-N, or BP1-T soon after plating and analyzed neuronal morphology at DIV 4. Infection of primary cortical neurons with lentiviral particles carrying the SCYL1BP1 or BP1-T plasmid resulted in a marked reduction in the total length of both dendrites and axons (Figure 2, D and E), which suggested that overexpression of SCYL1BP1 in cortical neurons could inhibit neuronal morphogenesis. Taken together, these data suggest that SCYL1BP1, with its transcriptional activator domain, serves as a potent suppressor of neuronal morphogenesis in vitro.

SCYL1BP1 directly, are the two major E3 ligases involved in p53 degradation. Mdm2 and Pirh2, both of which can bind to SCYL1BP1, with its transcriptional activator domain, may decrease p53 protein level by promoting its degradation in neuronal cells. Next we examined the mechanism by which SCYL1BP1 promotes p53 degradation. Mdm2 and Pirh2, both of which can bind to SCYL1BP1 directly, are the two major E3 ligases involved in p53 expression (Yan et al., 2010a). Down-regulation of p53 by SCYL1BP1 was attenuated when cells were treated with the 26S proteasome inhibitor MG132 (Supplemental Figure S2A), suggesting that the regulation of p53 by SCYL1BP1 was probably accomplished by accelerating protein turnover. To further verify this possibility, we analyzed the half-life of p53 protein. PC12 cells were treated with cycloheximide for 0.5–8 h to prevent further protein synthesis. As shown in Supplemental Figure S2B, the half-life of p53 protein was clearly reduced by ectopically expressed SCYL1BP1. All these results suggest that SCYL1BP1, with its transcriptional activator domain, may decrease p53 protein level by promoting its degradation in neuronal cells.

Next we examined the effect of p53 on SCYL1BP1-mediated inhibition of neurite outgrowth and neuronal morphogenesis. In NGF-treated PC12 cells, neurite outgrowth defect induced by SCYL1BP1 was largely prevented by cotransfecting p53 (Figure 3, C and D). p53 alone promoted neurite outgrowth in PC12 cells, as evidenced by extensive and longer neurites, which was consistent with a previous report (Zhang et al., 2006). Similar observations were made in primary cortical neurons (Figure 3, E and F). Thus we conclude that inhibition of neurite outgrowth and neuronal morphogenesis by SCYL1BP1 may result from destabilization of p53.

Mdm2 knockdown rescues neurite outgrowth and neuronal morphogenesis defects caused by SCYL1BP1

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SCYL1BP1 modulates neurite outgrowth

To further investigate how SCYL1BP1 up-regulates Mdm2, we examined Mdm2 expression at both the mRNA and protein levels. Reverse transcription (RT)-PCR assays showed that overexpression of SCYL1BP1 or BP1-T significantly up-regulated the Mdm2 mRNA level in both PC12 cells and cultured cortical neurons (Figure 5, A and B). The half-life of Mdm2 was not notably altered with or without SCYL1BP1 overexpression (Figure 5C). These data suggest that SCYL1BP1 probably up-regulates Mdm2 expression mainly at the transcriptional level. To directly test whether SCYL1BP1 induces Mdm2 transcription rate, we amplified a fragment spanning Mdm2 degradation (Leng et al., 2003; Logan et al., 2004; Zhang et al., 2005; Duan et al., 2006; Yan et al., 2010a). We hypothesized that SCYL1BP1 might destabilize p53 protein by up-regulating Pirh2 and/or Mdm2. This hypothesis was first tested in both PC12 cells and cultured cortical neurons. Immunoblotting assays showed that overexpression of SCYL1BP1 or BP1-T up-regulated the expression of Mdm2 but not Pirh2 (Figure 4, A and B). Then we examined the role of Mdm2 knockdown on SCYL1BP1-induced neurite outgrowth and neuronal morphogenesis defects. siMdm2 resulted in ~70% reduction in Mdm2 level when compared with control siRNA (Figure 4, C and D). In NGF-treated PC12 cells, neurite outgrowth defects induced by SCYL1BP1 were partly prevented by Mdm2 knockdown (Figure 4, E and F). Similar results were obtained in primary cortical neurons (Figure 4, G and H). Taken together, these results demonstrate that SCYL1BP1, with its transcriptional activator domain, antagonizes p53-induced neurite outgrowth and neuronal morphogenesis by up-regulating the expression of Mdm2.
SCYL1BP1 induces Mdm2 transcription. (A, B) RT-PCR analysis of Mdm2 expressions in PC12 cells (A) and cultured cortical neurons (B) after overexpression of SCYL1BP1, BP1-N, or BP1-T. (C) SCYL1-BP1 did not change the half-life of Mdm2. PC12 cells were transfected with or without SCYL1-BP1. At 48 h posttransfection, the cells were treated with 50 μg/ml CHX for the indicated times. Lysates were prepared and analyzed by Western blotting for Mdm2 and the GAPDH loading control. (D) Schematic diagrams showing human and rat genomic DNA structures of Mdm2. In human, P1 is a constitutive promoter, and P2 is a p53-responsive promoter that is located between exons 1 and 2 designated as two distinct promoters, P1 and P2. P1 promoter, which is from nt −1513 to −513, contains exon 1. P2 promoter, which is from nt −512 to +287, contains exons 2 and 3. The total promoter from nt −1513 to +287 was designated as P1P2. Then all of them were cloned for luciferase assay (Figure 5D). We showed that P1P2 promoter led to a noticeable increase of luciferase activity by SCYL1BP1 or BP1-T, and P2 promoter led to a similar level, whereas P1 promoter led to a slight increase (Figure 5E). These results suggested that SCYL1BP1, with its transcriptional activator domain, could directly induce Mdm2 promoter, in which P2 promoter but not P1 promoter was required.

To demonstrate that SCYL1BP1 can directly bind to the Mdm2 promoter, we performed chromatin immunoprecipitation (ChIP) with primers to amplify fragments in both P1 and P2 promoters. Indeed, P2 promoter was immunoprecipitated by SCYL1BP1 in both PC12 cells and cultured cortical neurons, but P1 promoter was not (Figure 5, F and G). Subsequently we asked how SCYL1BP1 interacted with the P2 promoter of the Mdm2 gene during neurite outgrowth of both PC12 cells and primary cortical neurons. ChIP assays showed that their interaction decreased over time after NGF treatment in PC12 cells; an identical result was observed in cultured 1–11-DIV neurons (Supplemental Figure S3).

Exogenous SCYL1BP1 attenuates physiological nerve regeneration

The in vitro data suggest that SCYL1BP1 inhibits NGF-induced, p53-dependent neurite outgrowth of PC12 cells and morphogenesis of cortical neurons. We next asked whether SCYL1BP1 could play a role in axonal regeneration in vivo. To this end, we compared the extent of nerve regeneration in rats injected with lentivirus encoding GFP or SCYL1BP1 or saline into facial nuclei after facial nerve axotomy, an established model for regeneration (Raivich et al., 2004). The extent of regeneration was assessed by counting fluorescent motor neurons in the facial motor nuclei with the retrograde nerve tracer FluoroGold at 21 d after facial nerve injury. It is striking that there was a significant decrease in the number of regenerating fibers in SCYL1BP1-injected animals as compared with saline or GFP animals (Figure 6A). The percentage of FluoroGold-positive neurons was 28.8 ± 4.5% and 26 ± 3.5%, respectively, in the saline and GFP animals versus 11.77 ± 1.6% in the SCYL1BP1-injected animals (Figure 6, B and C); this difference was highly significant (t test, p < 0.01). To examine the toxic effects of lentivirus encoding GFP or together with SCYL1BP1, we measured the survival and DNA integrity of facial nuclei. Both showed slight toxic effects, as a single terminal deoxynucleotidyl transferase dUTP nick end labeling–positive cell was observed (Supplemental Figure S4). To further investigate the role of SCYL1BP1 in nerve regeneration, we also used lentivirus encoding SCYL1BP1 shRNA. As expected, SCYL1BP1 knockdown enhanced axonal regeneration (Figure 6, D and E).

**DISCUSSION**

Complex neuronal morphology forms the basis of proper neuronal connectivity and brain function. Establishment of neuronal morphology during development is a highly regulated process, with sprouting and elongation of neurites, later developing into axons and dendrites. This multistep process is regulated by both intrinsic genetic programs and external signals (Whitford et al., 2002; Miller and Kaplan, 2003).

SCYL1BP1 was initially identified as an SCYL1-binding protein (Di et al., 2003), but little is known about its biological function. Recent work showed that loss-of-function mutations of SCYL1BP1 caused gerodermia osteodysplastica (Hennies et al., 2008), which
suggested that SCYL1BP1 is an important pathogenic candidate gene. More recently, SCYL1BP1 was identified as a new regulator of the p53 pathway (Yan et al., 2010a). So it is perhaps not surprising that SCYL1BP1 has broader roles in physiology and pathology, such as the regulation of stress, metabolism, autophagy, tumors, and development. Here our specific aim is to investigate the potential roles of SCYL1BP1 in the regulation of neurite outgrowth and regeneration.

We first found that SCYL1BP1 decreased during NGF-mediated neurite outgrowth of PC12 cells and morphogenesis of cortical neurons cultured in vitro. Concomitantly, p53 protein increased. SCYL1BP1 or BP1-T overexpression attenuates NGF-mediated neurite outgrowth of PC12 cells and morphogenesis of cortical neurons by p53 inhibition. Furthermore, neurite outgrowth and neuronal morphogenesis defects induced by SCYL1BP1 are largely prevented by cotransfecting p53. In addition, SCYL1BP1 or BP1-T can directly induce Mdm2 transcription, and inhibition of Mdm2 function by specific siRNAs results in partial rescue of neurite outgrowth and neuronal morphogenesis defects induced by SCYL1BP1. Additional experiments showed that SCYL1BP1 also decreases axonal regeneration in vivo, whereas inhibiting the function of SCYL1BP1 by specific shRNA enhances it. Taken together, these data strongly support the existence of an unexpected and critical function of SCYL1BP1 in neurite outgrowth, which could play important roles during CNS development and in axonal regeneration after injury.

Bioinformatics analysis reveals that SCYL1BP1 protein has a predicted bipartite nuclear localization signal and a transcriptional activator domain (Di et al., 2003). These data strongly suggest that SCYL1BP1 may play an important transcriptional activating role. In the present study, we found that SCYL1BP1 bound to Mdm2 promoter and enhanced its transcription. Subsequently ChiP–quantitative PCR assays showed that SCYL1BP1 also bound to the promoters of NF-κB, interleukin 6, and RhoA, besides Mdm2 (Li, 2010). These data imply that SCYL1BP1 did play a role as a novel transcriptional activator.

Previous data showed that SCYL1BP1 could directly bind to Mdm2 and promote Mdm2 self-ubiquitination (Yan et al., 2010b). Therefore we hypothesize that there is a new regulating loop between SCYL1BP1 and Mdm2, in which SCYL1BP1 up-regulates Mdm2 at the transcriptional level and down-regulates it at posttranscriptional level (Figure 7). In addition to p53, NF-xB and

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**FIGURE 6:** SCYL1BP1 modulates physiological nerve regeneration. (A) Lentivirus encoding GFP or together with SCYL1BP1 were injected into facial nuclei after facial nerve axotomy, an established model for regeneration. Scale bar, 500 μm. (B) The extent of regeneration was assessed by counting fluorescent motor neurons in the facial motor nuclei with the retrograde nerve tracer FluoroGold 21 d after injury. Scale bar, 20 μm. (C) Bar graphs show quantitation of regenerating facial motor neurons 21 d after facial transection. The percentage of FluoroGold-positive neurons was 28.8 ± 4.5% and 26 ± 3.5%, respectively, in the saline and GFP animals vs. 11.77 ± 1.6% in the SCYL1BP1-injected animals (five animals per group, unpaired two-tailed t test with p < 0.01). (D) The extent of regeneration was assessed by counting fluorescent motor neurons in the facial motor nuclei with the retrograde nerve tracer FluoroGold 21 d after injury. Scale bar, 20 μm. (E) Bar graphs show quantitation of regenerating facial motor neurons 21 d after facial transection. The percentage of FluoroGold-positive neurons was 27.3 ± 3.5% in control animals vs. 49.77 ± 4.2% in SCYL1BP1 shRNA-injected animals (five animals per group, unpaired two-tailed t test p < 0.01).

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**FIGURE 7:** Model of SCYL1BP1 regulation and action in neurite outgrowth. SCYL1BP1 binds to Mdm2 promoter and enhances its transcription. Mdm2 inhibits p53 activity. p53 is also required for neurite outgrowth. Whether the role of SCYL1BP1 in neurite outgrowth suppression depends on transcriptional regulation of the NF-κB and/or RhoA pathway needs further investigation. Solid lines, the data from our present work. Dotted lines, the data from previous reports or our hypothesis.
RhoA were also reported to play important roles in neural process growth, elaboration, and structural plasticity (Koh, 2006–2007; Boersma et al., 2011; Gutierrez and Davies, 2011). Accordingly, we conclude that SCYL1BP1 binds to Mdm2 promoter and enhances its transcription, ultimately inhibiting p53 activity and suppressing neurite outgrowth. Whether the role of SCYL1BP1 in neurite outgrowth suppression depends on transcriptional regulation of the NF-κB and/or RhoA pathway needs further investigation (Figure 7).

In conclusion, our work provides strong evidence for a novel role of SCYL1BP1 in neurite outgrowth and nerve regeneration and provides the molecular framework for such a function. Modulation of this SCYL1BP1/Mdm2/p53-dependent pathway may provide a novel therapeutic target for facilitating neuroplasticity and neuroregeneration after injuries to the central or peripheral nervous system.

MATERIALS AND METHODS
Expression plasmid preparation
To obtain full-length rat SCYL1BP1, 5′-end primer (5′-CGC TCG AGG TAT GCC TCA GGG TTG GGC TG-3′) and 3′-end primer (5′-AGG GTA CCC GTG GCC ACA GCG GCT GGA AAG-3′) were designed to amplify a cDNA library of rat cortex by PCR. The PCR products were cloned into pEGFP-N3 at Xhol and KpnI sites. An identical method was used for BP1-N, BP1-T, and hemagglutinin (HA)-tagged rat p53. To generate the luciferase reporter under the control of the Mdm2 promoter (P1P2), a 1800-base pair DNA fragment containing the Mdm2 promoter was amplified from genomic DNA purified from PC12 cells using an upstream primer, 5′-TTG AGC TCT TAG GTC CCC TGG AAT GAC AG-3′, and a downstream primer, 5′-ATC TCG AGT GCC TTT CTA AAG CCA GCG TC-3′. The DNA fragment was then cloned into pGL3-basic vector at SacI and XhoI sites. To generate the luciferase reporters P1 and P2, a SacI and XhoI fragment obtained from the P1P2 was cloned into pGL3-Basic (Promega, Madison, WI). All constructs were verified by sequencing.

Small interfering RNAs and transfection
Mdm2 small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA; sc-63266). Briefly, the siRNA product generally consists of pools of three to five target-specific 19- to 25-nt siRNAs designed to knock down Mdm2 expression. Transfection was performed according to manufacturer's instructions.

Recombinant lentiviral vector packaging and infection
To get SCYL1BP1-overexpressing lentivirus, full-length SCYL1BP1 was cloned into the pLenti6/entre plasmid (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. A 3-μg amount of pLenti6/entre-SCYL1BP1-EGFP vector and 9 μg of ViraPower Pack (Life Technologies) according to manufacturer's instructions. A 3-μg amount of recombinant lentiviral vector packaging and infection (10 min × 14,000 relative centrifugal force [rcf], 4°C). The supernatant was saved as the cytosolic fraction, and the nuclear pellets were centrifuged (10 min × 14,000 relative centrifugal force [rcf], 4°C). The supernatant was collected and the remaining tissue pellet re triturated. The combined supernatants were centrifuged through a 4% bovine serum albumin (BSA) layer and the cell pellet resuspended in neuronal seeding medium, which consisted of B27-Neurobasal Medium (Life Technologies, Carlsbad, CA) supplemented with 1% 100x antibiotic-antimycotic solution, 25 μM Na glutamate, 0.5 mM l-glutamine, and 2% B27 Supplement (Life Technologies).

Cell fractionation
Subcellular fractionation was performed as described (Lenferink et al., 2001). Briefly, cell pellets were incubated in a hypotonic buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM ethylene glycol tetraacetic acid, 20 mM NaF, 100 μM Na3VO4, and protease inhibitor mixture) for 30 min at 4°C on a rocking platform. Then cells were homogenized (Dounce, 30 strokes), and their nuclei were pelleted by centrifugation (10 min × 14,000 relative centrifugal force [rcf], 4°C). The supernatant was saved as the cytosolic fraction, and the nuclear pellets were incubated in a nuclear lysin buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) for 1 h at 4°C on a rocking platform. The nuclear fraction was collected by centrifugation (10 min × 14,000 rcf, 4°C).

Western blot analysis
Cells were washed and collected from plates in phosphate-buffered saline (PBS) solution, resuspended with 2x sample buffer, and boiled for 5 min. Proteins were then resolved in 10% SDS–PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore).

Immunofluorescence analyses
For immunofluorescence analyses, cells were fixed with 4% paraformaldehyde in phosphate buffer, pH 7.4, for 15 min. After washing with 1x PBS, cells were permeabilized with 0.05% Triton X-100 in 1x PBS for 15 min and incubated with preblock buffer (3% BSA, 0.02% Triton X-100 in 1x PBS) for 15 min before being probed with primary antibodies; the following primary antibodies were used: anti-Tuj1 (1:500), anti-SCYL1BP1 (1:250), anti-Mdm2 (1:200), and HA (1:500). 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 305–anti-goat (1:500), Alexa 488–anti-rabbit (1:500), or Alexa 568–anti-mouse (1:500); all 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 Hoechst 33258 (Sigma-Aldrich) to stain nuclei. The immunofluorescence signal was detected by a Nikon confocal microscope (Nikon, Melville, NY).

RNA isolation and RT-PCR analysis
Total RNA was isolated using TRIzol reagent (Invitrogen) and used for RT-PCR analysis. The pS3 and Mdm2 probe was made from a cDNA fragment amplified by RT-PCR with an upstream primer (5′-GCC ATC TAC AAG AAG TCA CAA-3′) and a downstream primer (5′-CAG GCA CAA ACA CGA ACC-3′). The Mdm2 probe was made from a cDNA fragment amplified by RT-PCR with an upstream primer (5′-GCC ATC TAC AAG AAG TCA CAA-3′) and a downstream primer (5′-TCT CAC GAA GGG TCC AAC-3′).

Luciferase assay
In all, a luciferase reporter (300 ng) along with pEGFP-N3, SCYL1BP1, BP1-N, or BP1-T (200 ng) and 25 ng of Renilla luciferase assay vector pRL-CMV (Promega, Madison, WI) were transfected into PC12 cells plated in a 24-well plate. All experiments were performed in triplicate according to the manufacturer’s instructions (Promega).

ChIP assay
ChIP assay was performed according to the manufacturer’s instructions (Pierce, Rockford, IL). Briefly, after 2 × 10^6 PC12 cells or cortical neurons were planted into 100-mm-diameter dishes, chromatin was cross-linked in 1% formaldehyde in PBS, and nuclei were extracted. Chromatin was digested with micrococcal nuclease to yield 500–1000-base pair DNA fragments and immunoprecipitated with SCYL1BP1 antibody. After reverse cross-linking and phenol-chloroform extraction, DNA fragments bound by SCYL1BP1 were purified over a column. PCR was performed to visualize the enriched DNA fragments. Primers designed to amplify the Mdm2 promoter were as follows: P1, the 5′-end primer nt −1086 base pairs (CCC CAC TTC CTC CTC GCT CAA) and the 3′-end primer nt −693 base pairs (AGA ACT CGC CAC CGG CCA); and P2, the 5′-end primer nt −4 base pairs (GCC AAT GTG CAA TAC CAA CAT GTC) and the 3′-end primer nt +287 base pairs (TGG CTT TCT AAA GCC AGC GTC AG).

Facial nerve transection
Facial nerve transection was performed as described earlier with slight modification (Di Giovanni et al., 2006). Briefly, the animals were anesthetized by an intramuscular injection of a combination of xylazine (13 mg/kg) and ketamine HCl (85 mg/kg). A skin incision was made behind the right ear and the facial nerve exposed; transection was made 2 mm posterior to the foramen stylomastoideum. The bregma bar was set at 0.0. The injection rate was 0.25 μl/min, and after the infusion the glass pipette was slowly retracted 1 mm and left in place for an additional 3 min before being withdrawn.

Regeneration in facial nuclei in the brain stem after facial nerve transection was evaluated at day 21 by retrograde axonal tracing with FluoroGold. Three days before being killed, animals from each group were anesthetized, and 1.5 ml of 3% FluoroGold was injected through a micropipette with a tip diameter of 25 mm into the whisker pads bilaterally. After 3 d, the animals were deeply anesthetized and perfused with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain stem was removed and placed in 30% sucrose for 24 h. The entire facial nuclei were sectioned and reconstructed, and all sections of both facial nuclei per each animal were evaluated (169 for the control and 157 for the transected side). Transverse serial frozen sections, 20 μm thick, were cut and mounted on slides and photographed under a fluorescence microscope. Only labeled neurons with visible nuclei were counted and taken to represent the number of regenerated motor neurons. Thus the overall ratio of FluoroGold-positive neurons was compared in the saline, Lenti-EGFP, and Lenti-EGFP-SCYL1BP1 injection groups (five animals per group). The Abercrombie formula for cell counting was included in our analysis to correct for tissue volume.

Image acquisition and quantitation
Images were obtained using either a Nikon TE2000-S or a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) confocal microscope with 40×/numerical aperture (NA) 1.3 or 63×/NA 1.4 objective. Neuronal morphology was traced and analyzed by Neurolucida software (MBF Bioscience, Williston, VT).

Statistical analysis
Values were expressed as mean ± SEM. Student’s t test was used to measure significance of differences between two groups. Statistical significance was defined as p < 0.05.

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