The Association of GSK3β with E2F1 Facilitates Nerve Growth Factor-induced Neural Cell Differentiation*

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It is widely acknowledged that E2F1 and GSK3β are both involved in the process of cell differentiation. However, the relationship between E2F1 and GSK3β in cell differentiation has yet to be discovered. Here, we provide evidence that in the differentiation of PC12 cells induced by nerve growth factor (NGF), GSK3β was increased at both the mRNA and protein levels, whereas E2F1 at these two levels was decreased. Both wild-type GSK3β and its kinase-defective mutant GSK3β KM can inhibit E2F1 by promoting its ubiquitination through physical interaction. In addition, the colocalization of GSK3β and E2F1 and their subcellular distribution, regulated by NGF, were observed in the process of PC12 differentiation. At the tissue level, GSK3β colocalized and interacted with E2F1 in mouse hippocampus. Furthermore, GSK3β facilitated neurite outgrowth by rescuing the promoter activities of Cdk inhibitors p21 and p15 from the inhibition caused by E2F1. To summarize, our findings suggest that GSK3β can promote the ubiquitination of E2F1 via physical interaction and thus inhibit its transcription activity in a kinase activity independent manner, which plays an important role in the NGF-induced PC12 differentiation.

GSK3β, a multifunctional serine/threonine (Ser/Thr) kinase that was originally identified as a regulator of glycogen metabolism, is now known to be a key kinase in several important signaling pathways. The kinase activity of GSK3β is positively regulated by phosphorylation of its Tyr216 and negatively regulated by N-terminal phosphorylation of its Ser9 (1). GSK3β is involved in several signaling pathways important for early central nervous system patterning and neuronal differentiation. For example, GSK3β down-regulates β-catenin via phosphorylation through the formation of a multicomponent complex with adenomatous polyposis coli and Axin, and thus inhibits the transcription of downstream genes in Wnt signaling (2). In addition, GSK3β regulates cell cycle by phosphorylating cyclin D1 and p21 (3–5). Also, it activates MRp2, an actin-binding protein of the kelch-related protein family, and promotes neurite outgrowth in NGF-stimulated PC12 cells. Treatment of PC12 cells with GSK3β-interfering RNAs results in the inhibition of neurite outgrowth (6). Moreover, GSK3β promotes dendrite formation through the ILK–GSK3β pathway in sympathetic neurons (7), and it regulates the phosphorylation of CRMP-2, adenomatous polyposis coli, and MAP1B, which are separately related to the polymerization, stabilization, and dynamics of microtubules, principal cytoskeletal components of axons (8).

The E2F family has at least 10 members, classified as E2Fs (E2F1 to -8) and DPs (DP1 and -2). Physiological E2F exists as E2F/DP heterodimers in mammalian cells, binding and regulating E2F-targeted genes (9, 10). The E2F family plays a crucial role in regulating cell cycle progression at the G1–S transition mediated by pRB, a retinoblastoma tumor suppressor protein. pRB is phosphorylated in a cell cycle-dependent manner by cyclin-dependent kinases (Cdks). In quiescent cells, pRB binds to E2F heterodimers, leading to transcriptional inactivity and cell cycle arrest. Subsequently, the cell cycle-dependent kinase complexes Cdk4/Cdk6-cyclin D and Cdk2-cyclin E are activated and then phosphorylate pRB. Inactivation of pRB by phosphorylation leads to the dissociation and activation of E2F, which facilitates the activation of E2F-targeted genes, allowing the expression of genes required for cell cycle progression and S phase entry (9, 11). Although overexpression of E2F1 in tissue culture cells advances quiescent cells into the S phase and therefore stimulates cell proliferation and can be oncogenic, loss of E2F1 in mice results in tumorigenesis, demonstrating that E2F1 also functions as a tumor suppressor (12, 13). In addition, the E2F family is crucial in regulating cell differentiation. It has been reported that E2F1 is down-regulated in differentiating PC12 cells and developing quail neural retina (14, 15), whereas deregulated E2F1 blocks the terminal differentiation by overriding the p15/p16-pRB-E2F2 checkpoint in the G1 phase of the cell cycle (16).

On the whole, GSK3β and E2F are important to cell differentiation. However, their relationship in cell differentiation remains to be elucidated. Previous reports have found that

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§ The abbreviations used are: NGF, nerve growth factor; Cdk, cyclin-dependent kinase; RB, retinoblastoma; siRNA, small interfering RNA; WT, wild type; TRITC, tetramethylrhodamine isothiocyanate.
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GSK3β reduces the level of cyclin D1 by inhibiting its transcription and promoting its degradation and thus depresses the activity of the Cdk-cyclin D1 complex. This would decrease the phosphorylation of pRB and therefore the activation of E2F1 and its targeted genes, including itself (3, 9, 17). These together offered indirect evidence for the regulation of E2F1 by GSK3β.

Here we investigated the direct effect of GSK3β on E2F1 and found that in NGF-induced PC12 cells, E2F1 was down-regulated along with the up-regulated PC12 cells at both the mRNA and protein levels. Furthermore, we discovered that GSK3β, independent of its kinase activity, greatly suppressed E2F-luciferase activity by interacting with E2F1 and promoting its instability through a ubiquitin-proteasome pathway. Moreover, we observed neural cell-specific cytoplasmic colocalization of E2F1 and GSK3β and further consolidated our results in the mouse hippocampus. In addition, GSK3β could rescue the promoter activity of p15 and p21 inhibited by E2F1. Also, E2F1-suppressed neurite outgrowth was rescued by GSK3β, independent of its kinase activity. Altogether, our findings suggest that the regulation of E2F1 activity by GSK3β plays a role in NGF-induced neural cell differentiation.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—PC12 cells were obtained from Peking Union Medical College (China). NGF was purchased from Xiamen PKU Bioway Ltd. (China). Monoclonal GSK3β antibody (27C10) was purchased from Cell Signaling Technology, Inc. E2F1 antibody (sc-251), GSK3β small interference RNA (siRNA) (sc-35525), E2F1 siRNA (sc-35247), and control nonspecific siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. E2F1 plasmid was kindly provided by Dr. Michael D. Cole (Princeton University). GSK3β (WT/KM), E2F-luciferase, Wnt1, TopFlash-luciferase, LEF-luciferase, p15, and p21 promoters were applied as previously described (18, 19). Myc-tagged GSK3β deletion mutants were generated by restriction digestions and PCR and subcloned into pCMV-Myc. All of the mutants were verified by DNA sequencing.

Cell Culture—Hela, HEK293T, COS-7, and Hep3B cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone). Mouse neuroblastoma N2a cells were cultured in 50% Dulbecco’s modified Eagle’s medium and 50% Opti-MEM supplemented with 5% fetal bovine serum (HyClone). PC12 cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum and 10% horse serum. All of these cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1% fetal bovine serum and NGF at a final concentration of 50 ng/ml.

Luciferase Reporter Assay—HEK293T and PC12 cells were transfected with various plasmids as indicated in the figures.
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Two days after transfection, cells were harvested, and luciferase activities were measured by a luminometer (Berthold Technologies). Reporter activity was normalized to the control Renilla. Experiments were repeated in triplicate.

Transfection, Immunoprecipitation, and Immunoblotting—HEK293T cells were transiently transfected using calcium phosphate or Lipofectamine (Invitrogen). PC12 and N2a cells were transiently transfected using calcium phosphate (Promega). At 40 h post-transfection, the cells were lysed with 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Sigma) for 30 min at 4 °C. After centrifugation at 12,000 × g for 15 min, the lysates were immunoprecipitated with specific antibody and protein A-Sepharose (Zymed Laboratories Inc.) for 3 h at 4 °C. Thereafter, the precipitants were washed three times with washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and the immune complexes were eluted with sample buffer containing 1% SDS for 5 min at 95 °C and analyzed by SDS-PAGE. Immunoblotting was performed with specific antibody and secondary anti-mouse or anti-rabbit antibodies that were conjugated to horseradish peroxidase (Amersham Biosciences). Proteins were visualized by chemiluminescence.

Immunofluorescence—PC12 cells grown on collagen-coated glass coverslips were washed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 3% bovine serum albumin in phosphate-buffered saline for 60 min. The cells were then incubated with primary antibodies diluted in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 3 h and washed twice with phosphate-buffered saline and incubated with fluorescein isothiocyanate- or TRITC-conjugated anti-mouse or anti-rabbit antibodies for an additional 40 min. The nuclei were counterstained with 4’,6-diamidino-2-phenylindole (Sigma). Frozen sections of mouse brain were cut with a cryostat (Leica Microsystems) at a thickness of 10 μm and immunostained as above. Samples were examined under an Olympus Fluoview 500 microscope.

Quantitative Real Time Reverse Transcription-PCR—Total RNA was isolated using Trizol (Roche Applied Science) reagent, and the genomic DNA was removed by DNase (Takara, Japan). Two micrograms of RNA was reverse-transcribed at 42 °C for 45 min in a 20-μl reaction mixture using the reverse transcription system (Promega). α-Actin was used as a control. Sequences for the forward primer and reverse primer were as follows: 5’-CCTCCATTC-AAGGCACATCCT-3’ (GSK3β forward), 5’-TGAGGCGTGT-CAGAAGCG-3’ (GSK3β reverse); 5’-GAAAGAAGACG-GTTGTCCACC-3’ (E2F1 forward), 5’-GAAATCAGGAGGTCAAGTC-3’ (GSK3β reverse); and 5’-AGTGCGAAGAGACTTG-3’ (α-actin forward), 5’-AGTGCGAAGAGACTTG-3’ (α-actin reverse). The reaction system and procedure were employed as previously described (20).

Isolation of Nuclear and Cytoplasmic Phase—The collected cells were incubated with lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 20 mM NaF, 10% glycerol, and 0.1% Nonidet P-40 on ice for 5 min. The samples were spun down at 4 °C for 15 min at full speed, and the supernatants were collected as cytoplasmic fractions. The pellets were washed with lysis buffer twice and sonicated in the lysis buffer to obtain the nuclear fractions.
**RESULTS**

**GSK3β Inhibits E2F1 in the Process of Neural Cell Differentiation**—Given that E2F1 and GSK3β have both been shown to relate with neural cell differentiation, their expressions were herein evaluated in the differentiation of PC12 cells induced by NGF. PC12 cells were incubated with NGF (50 ng/ml) for various time periods, from 1 to 8 days, and harvested for quantitative real time reverse transcription-PCR assay. Consistent with previous reports, the mRNA level of E2F1 was markedly down-regulated in a time-dependent manner. Interestingly, along with the down-regulation of E2F1, the mRNA level of GSK3β was significantly increased (Fig. 1A). Previous reports suggested that GSK3β could be an indirect regulator of E2F1 by influencing the activity of the Cdk-cyclin D complex and the phosphorylation level of RB protein (3). Thus, we examined the phosphorylation level of GSK3β in PC12 cells upon stimulation with NGF (50 ng/ml) with anti-phospho-GSK3β (Ser9) antibody. As shown in Fig. 1B, the Ser9 phosphorylation level of GSK3β was rapidly increased by NGF treatment (0.5 h) and began to decline 2 h later. Consistently, GSK3β was markedly increased, and E2F1 was decreased at the protein level. Moreover, we observed a dramatic decrease of E2F1 2 h after NGF stimulation, whereas the kinase activity of GSK3β at this time point was low, as indicated by its high Ser9 phosphorylation. This suggests that the kinase activity of GSK3β may not be the only contributing factor in the inhibition of E2F1 by GSK3β (Fig. 1B). To further substantiate the finding that the inhibition of E2F1 was caused by GSK3β, we overexpressed GSK3β in PC12 cells and analyzed the E2F1 expression level. As shown in Fig. 1C, the E2F1 mRNA level was down-regulated in GSK3β-overexpressed cells. Using siRNA, we also demonstrated that the E2F1 mRNA level was up-regulated when the endogenous GSK3β was knocked down by specific siRNA (Fig. 1C). Consistent results were obtained in the E2F-luciferase reporter assay. Knocking down of GSK3β obviously impaired the reduction of E2F1 by NGF in PC12 cells (Fig. 1D). Similar findings were observed in HEK293T cells that exogenous GSK3β dramatically inhibited E2F-luciferase activ-
GSK3β Down-regulates the Transcriptional Activity of E2F1 Independent of Its Kinase Activity—In order to verify whether the inhibition of E2F1 by GSK3β is kinase activity-dependent, we detected the effect of GSK3β wild type and its kinase-defective mutant on the transcriptional activity of E2F1 by a luciferase reporter assay in HEK293T cells. HEK293T cells were ideal for this purpose, because they synthesize E1A and SV40 T antigen, and thus the regulation of E2F1 by the pRB/Cdk pathway can be precluded (21). Therefore, the indirect effect of GSK3β on E2F1 through the regulation of cyclin D1 could be excluded. To identify whether the inhibitory effect of GSK3β on E2F1 is dependent on its kinase activity, TDZD-8, an effective pharmacological kinase inhibitor of GSK3β, was also applied. As shown in Fig. 2A, TDZD-8 had no effect on rescuing the inhibition of E2F1-luciferase induced by wild-type GSK3β and its kinase-defective mutant GSK3β KM. A similar result was observed in PC12 cells, and the efficiency of TDZD-8 was verified via Wnt-responsive LEF-luciferase reporter (Fig. 2, B and C). Taken together, these results indicate that GSK3β mainly inhibits the transcriptional activity of E2F1 in a kinase activity-independent manner. If GSK3β overexpression were to reduce E2F1 level in HEK293 cells, then the cell proliferation rate should be reduced. The following experiment carried out in HEK293 cells by Cell Counting Kit-8 confirmed that both wild-type GSK3β and GSK3β KM reduce cell proliferation (Fig. 2D).

Interaction between E2F1 and GSK3β in Cell Lines—Since the inhibitory effect of GSK3β on E2F1-luciferase was kinase activity-independent, GSK3β may regulate E2F1 through direct interaction. To confirm this, we performed a co-immunoprecipitation assay in different cell lines. In PC12 cells, E2F1 was detected in anti-GSK3β immunoprecipitation but not in the control

Figure 4. GSK3β destabilizes E2F1 by enhancing the efficiency of E2F1 ubiquitination. Overexpression of GSK3β destabilizes E2F1 in HEK293T (A) and PC12 cells (B). HEK293T or PC12 cells were seeded into two batches of 60-mm dishes. One batch was transfected with GSK3β plasmid (2.5 μg), and the other batch was transfected with pCMV vector plasmid (2.5 μg), and the total protein for loading was equal. The data shown are representative of three such experiments. The protein level at time 0 was set as 100%. C, overexpression of GSK3β in PC12 cells enhances the efficiency of endogenous E2F1 ubiquitination. PC12 cells were transiently transfected with pCMV-GSK3β-HA or pCMV empty vector (pCMV5) as indicated. At 8 h post-transfection, cells were treated with cycloheximide (CHX; 20 μg/ml) for 0–4 h, as indicated. Cell extracts were then harvested for immunoblotting (IB) by SDS-PAGE and blotted with anti-E2F1 antibody. The protein intensity was quantitated by BCA kit, and the total protein for loading was equal. The data shown are representative of three such experiments. The protein level at time 0 was set as 100%. C, overexpression of GSK3β in PC12 cells enhances the efficiency of E2F1 ubiquitination. PC12 cells were transiently transfected with pCMV-GSK3β-HA or pCMV empty vector (pCMV5) as indicated. At 8 h post-transfection, cells were treated with or without NGF (50 ng/ml) for 24 h. Thirty hours after transfection, proteasome inhibitor MG132 (10 μmol/liter) was added, as indicated. E2F1 was immunoprecipitated (IP) with anti-E2F1 antibody and analyzed by Western blot for anti-ubiquitin (Ub) antibody. Cell lysates were also subjected to direct Western blot analysis using anti-HA to detect GSK3β expression. D and E, overexpression of GSK3β (D) and GSK3β KM (E) enhances the efficiency of exogenous E2F1 ubiquitination. HEK293T cells were transiently transfected with pCMV-GSK3β-HA, pCMV-E2F1-FLAG, pCMV-Ubiquitin-6*His, or pCMV empty vector (pCMV5) as indicated. Thirty hours after transfection, proteasome inhibitor MG132 (10 μmol/liter) was added, as indicated. E2F1 was immunoprecipitated (IP) using anti-FLAG antibody (D) or nickel beads (E) and analyzed by Western blot for anti-His (D) or anti-FLAG (E) antibody. Cell lysates were also subjected to direct Western blot analysis with anti-HA antibody to detect exogenous GSK3β expression and anti-FLAG antibody to detect exogenous E2F1.

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immunoprecipitated with a nonspecific antibody (Fig. 3A). In mouse neuroblastoma N2a cells, GSK3β was detected in anti-E2F1 immunoprecipitation but not in the control (Fig. 3B). The endogenous interaction between E2F1 and GSK3β was also detected in different nonneural cell lines, including HEK293T, HeLa, COS-7, and Hep3B (Fig. 3C). GSK3β KM mutant could also interact with endogenous E2F1 (Fig. 3D). In addition, we found that the association between endogenous E2F1 and GSK3β in PC12 cells was enhanced by NGF treatment, which implies that the interaction between E2F1 and GSK3β is involved in cell differentiation (Fig. 3E). To identify the domain required for E2F1 binding, we constructed GSK3β deletion mutants (supplemental Fig. 1A). As shown in supplemental Fig. 1, B and C, GSK3β N terminus was required for its binding with E2F1. All of these results indicate that GSK3β and E2F1 interact specifically.

GSK3β Destabilizes E2F1 by Enhancing the Efficiency of E2F1 Ubiquitination—Next, we tested how this interaction inhibited the transcriitional activity of E2F1. The autoregulated effect of E2F1, which stimulates its own activity directly through the presence of binding sites in the promoters (22, 23), revealed that the repression of the protein level of E2F1 by GSK3β may also result in the repression of its own mRNA level. GSK3β has been shown to promote the degradation of several proteins via phosphorylation. Therefore, whether GSK3β could affect the E2F1 protein stability via direct interaction was analyzed. PC12 or HEK293T cells transfected with GSK3β or with pCMV vector (negative control) were treated with the protein synthesis inhibitor cycloheximide, and the protein level of the endogenous E2F1 proteins was examined by immunoblotting. As shown in Fig. 4, A and B, the E2F1 protein level decreased much more rapidly in GSK3β-overexpressed PC12 and HEK293T cells. Overexpression of GSK3β KM also caused rapid decrease of E2F1 protein in these cells (data not shown). These data support our results from the luciferase reporter assay in Fig. 2. Since previous studies demonstrated that E2F1 was degraded through the ubiquitin-proteasome pathway (24–26), the ubiquitination of E2F1 was examined in PC12 and HEK293T cells. As shown in Fig. 4C, ubiquitination of E2F1 was enhanced in PC12 cells overexpressing GSK3β or treated with NGF. In HEK293T, overexpression of both GSK3β and GSK3β KM led to enhanced E2F1 ubiquitination (Fig. 4, D and E). To confirm this, we examined ubiquitination of E2F1 by GSK3β C1 mutant, which cannot associate with E2F1. As shown in supplemental Fig. 1F, GSK3β C1 did not enhance the E2F1 ubiquitination. To summarize, we proved that the overexpression of GSK3β decreased the stability of E2F1 protein by promoting their ubiquitination.

NGF Regulates the Subcellular Distribution of GSK3β and E2F1—As important regulators in cell growth and differentiation, both GSK3β and E2F1 can shuttle between the cytoplasm and nucleus (27, 28). To consolidate the association between GSK3β and E2F1, we studied the subcellular localization of GSK3β and E2F1 in both neural and nonneural cell lines via immunofluorescence. Consistent with previous reports, in HEK293T and HeLa cells, E2F1 mainly localized in the nucleus as a transcription factor, whereas GSK3β localized in the cytoplasm (data not shown). However, in PC12 cells, E2F1 colocalized well with GSK3β. Before NGF stimulation, E2F1 predominantly colocalized with GSK3β in the cytoplasm (Fig. 5). After NGF stimulation, GSK3β expression increased markedly in both nucleus and cytoplasm. E2F1 obviously increased in the nucleus, leading to its enhanced nuclear colocalization with GSK3β (Fig. 5). To further confirm these results, we explored the interactions between E2F1 and GSK3β in different cellular compartments by separating the cytoplasm and nucleus in PC12 and HEK293T cells. Consistently, in untreated PC12 cells, E2F1 mainly localized and associated with GSK3β in cytoplasm, and their subsequent association in the nucleus was enhanced by NGF stimulation (Fig. 6A). In HEK293T cells, E2F1 mainly associated with GSK3β in the nucleus (Fig. 6B). We also detected the distribution of E2F1 and GSK3β following NGF time course stimulation and obtained consistent results (Fig. 6C). Furthermore, NGF-induced accumulation of E2F1 in

**FIGURE 5.** E2F1 colocalizes with GSK3β in PC12 cells. PC12 cells were seeded in poly-o-lysine-coated 35-mm dishes and on the following day were treated with or without NGF (50 ng/ml) for 3 days. After this, the cultures were fixed with paraformaldehyde and permeabilized with Triton X-100. Following this, cells were incubated with rabbit anti-GSK3β primary antibody and mouse anti-E2F1 primary antibodies and then rhodamine-conjugated anti-mouse and fluorescein isothiocyanate-conjugated anti-rabbit secondary antibodies. The nuclei were stained in blue with 4',6-diamidino-2-phenylindole (DAPI). Scale bar, 20 μm.
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A

NGF Treatment: IP antibody: Cyt/NU: NS - - Cyto/NU: - GSK3β E2F1 IB: GSK3β E2F1 IB: Tubulin IB: LaminB B

NGF (mg/ml): 5 60 60 60 240 480 1d 3d 5d

IB: GSK3β E2F1 IB: E2F1 IB: Tubulin IB: LaminB

C

NGF treatment

D

FIGURE 6. Endogenous distribution of E2F1 and GSK3β. A, NGF enhances the association between GSK3β and E2F1 in the nucleus. PC12 cells were treated or untreated with NGF (50 ng/ml) in 100-mm dishes. Three days later, the cytoplasm and nucleus partition of cells were separated for immunoprecipitation (IP) with anti-E2F1 antibody or a nonspecific antibody (ns). E2F1-associated GSK3β was revealed by anti-GSK3β immunoblotting (IB) (top). The protein distribution was confirmed by anti-GSK3β or anti-E2F1 immunoblotting with the total cell lysates (middle two panels). Cytoplasmic protein tubulin and nuclear protein LaminB were immunoblotted as control (bottom two panels). B, GSK3β associates with E2F1 both in the cytoplasm and nucleus in nonneural cells at endogenous levels. Cytoplasm and nucleus partition of HEK293T cells were separated for immunoprecipitation with anti-E2F1 antibody or a nonspecific antibody. E2F1-associated GSK3β was revealed by anti-GSK3β immunoblotting (top two panels). The protein distribution was confirmed by anti-GSK3β or anti-E2F1 immunoblotting with the total cell lysates (middle two panels). Cytoplasmic protein tubulin and nuclear protein LaminB were immunoblotted as control (lower two panels). C, NGF induces translocation of E2F1 from cytoplasm to nucleus. PC12 cells were treated with NGF (50 ng/ml) for different times as indicated. Cytoplasm and nucleus partitions of cells were then separated for immunoblotting by anti-phospho-GSK3β antibody (top), anti-GSK3β antibody, anti-E2F1 antibody (middle two panels), and anti-tubulin or anti-LaminB antibody (bottom two panels). D, change of GSK3β and E2F1 distribution induced by NGF was quantitatively analyzed. Equal amounts of PC12 cells were seeded into 60-mm dishes and treated with or without NGF (50 ng/ml) for 3 days. Three days later, the cytoplasm and nucleus partitions of cells were then separated for immunoblotting by anti-GSK3β antibody and anti-E2F1 antibody (top). The histogram shows a statistical analysis of relative protein expression densities in the cytoplasm and nucleus from three independent experiments. E, statistical analysis of GSK3β and E2F1 distribution stimulated with or without NGF.

![Image](image_url)

the nucleus was notable, especially when the phosphorylation of GSK3β (Ser9) was rapidly diminished (Fig. 6C). A quantitative analysis of GSK3β and E2F1 distribution was shown in Fig. 6, D and E. Although total expression of E2F1 decreased, nucleus-localized E2F1 was enhanced with NGF stimulation, which implied that pRB-E2F1 complex formation in the nucleus was increased when GSK3β exhibited the high kinase activity.

E2F1 Colocalizes and Interacts with GSK3β in the Cytoplasm in Mouse Hippocampus—To further confirm the results above, we detected the interaction between GSK3β and E2F1 and examined their localization in the mouse brain. Samples from the hippocampal region of 3-month-old mice were homogenized to perform the immunoprecipitation assay, and obvious interaction between GSK3β and E2F1 was observed (Fig. 7A). The frozen sections of the hippocampus were also immunostained, and the observation supported the colocalization of GSK3β and E2F1 (Fig. 7, B and C). Interestingly, GSK3β and E2F1 displayed a cytosolic colocalization in neurons, which implies in terminally differentiated neurons that the association and staying of GSK3β and E2F1 in the cytoplasm may be important to keep neurons in a quiescent state and to prevent them from proliferation.

GSK3β Facilitates Neurite Outgrowth by Rescuing the Cdk Inhibitors p21 and p15 from Suppression Caused by E2F1—Previous studies have reported that loss of E2F1 function resulted in an elevation of Cdk inhibitors, leading to a decrease in Cdk activity and pRB phosphorylation, and a further derepression of pRB-E2F-targeted genes that are required for cell differentiation (29). Therefore, NGF treatment might induce the expression of Cdk inhibitors by down-regulating E2F1 and further induce neurite outgrowth in PC12 cells. To verify this, we cotransfected p21 promoter, E2F1, and GSK3β constructs with or without NGF treatment in PC12 cells. As shown in Fig. 8A, NGF induced the transcriptional activity of p21, and GSK3β obviously rescued the luciferase activity of p21 promoter from the inhibition caused by E2F1. GSK3β KM also dramatically rescued the inhibition caused by E2F1. A similar result was observed in the promoter activity of p15, another Cdk inhibitor (30) (Fig. 8B). Based on this, we further examined the involvement of E2F1 in the neurite outgrowth of PC12 cells. As expected, overexpression of E2F1 suppressed neurite outgrowth, whereas GSK3β promoted it in NGF-induced PC12 cells. When the endogenous GSK3β was knocked down by specific siRNA, the neurite outgrowth was suppressed (Fig. 8D). Our results also showed that GSK3β KM, which had less effect than wild-type GSK3β in mediating neurite outgrowth, could still rescue neurite outgrowth from the suppression caused by E2F1 (Fig. 8C). In order to identify the specificity of neurite outgrowth regulated by the association of GSK3β and E2F1, we cotransfected GSK3β deletions and E2F1 in PC12 cells with or without NGF stimulation. As shown in Fig. 8C, a randomly selected deletion C1, which had no inhibitory effect on E2F1
and no interaction with E2F1, could not rescue the inhibited neurite outgrowth by E2F1. The transcriptional activities of E2F1 and Wnt signaling regulated by GSK3β deletions were detected by reporter assay via E2F-luciferase and TopFlash-luciferase reporters (supplemental Fig. 1, D and E). All of these data suggest that GSK3β facilitates neurite outgrowth by rescuing the neuronal differentiation from the inhibitory effect caused by E2F1.

**DISCUSSION**

The neuroendocrine cell line PC12, derived from rat pheochromocytoma, a tumor arising from chromaffin cells of the adrenal medulla, is a useful model system to study neuronal differentiation. NGF is an effective factor in inducing neuronal differentiation of PC12. Several studies showed that NGF promotes neurite growth rates partly by coupling the mitogen-activated protein kinase pathway to the activation of GSK3β and thereby the phosphorylation of MAP1B (8, 31). Furthermore, NGF can down-regulate cell cycle-related E2F1, E2F3, and E2F5, whereas it up-regulates E2F4 during the neuronal differentiation of PC12 cells, which may occur through the specific inhibition of Cdns and induction of cyclin D1 (14, 32).

Previous studies implicated that GSK3β and E2F1 could be related. However, the details are unclear. One possible way in which GSK3β regulates E2F1 is that GSK3β down-regulates cell cycle-dependent cyclin D1, therefore affecting the Cdk-cyclin D1 complex in cytoplasm, inhibiting the phosphorylation of pRB, and consequently repressing the release of E2F1 from the pRB complex (3). Here, we demonstrated the interaction between GSK3β and E2F1 and proposed another way by which GSK3β regulates the activity of E2F1 independent of its kinase activity. Interestingly, E2F1 was recently proved to be phosphorylated by GSK3β (33).

In this study, we found that the protein level of E2F1 decreased along with the increase of the total protein level of GSK3β but not with the level of its phosphorylated form in PC12 cells stimulated by NGF. We also discovered that the kinase-defective mutant GSK3β KM could also repress the transcriptional activity of E2F1. Additionally, the E2F1-inhibited activities of p15 and p21 promoters were rescued by both GSK3β and GSK3β KM. All of these data implied that the kinase-dependent regulation of E2F1 by GSK3β is not the only way that E2F1 is regulated under some conditions. It was reported that
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**FIGURE 8. GSK3β facilitates neurite outgrowth.** A and B, inhibition of p21 promoter-luciferase (p-p21-luciferase) activity (A) and p15 promoter-luciferase (p-p15-luciferase) activity (B) by E2F1 could be rescued by GSK3β overexpression in PC12 cells. PC12 cells were cotransfected with p21 promoter-luciferase reporter plasmid (0.2 μg) (A) or p15 promoter-luciferase reporter plasmid (0.2 μg) (B) and the constructs encoding E2F1 (0.1 μg), GSK3β (0.025 μg), and GSK3β KM mutant (0.025 μg) as indicated. Sixteen hours after transfection, NGF (50 ng/ml) was added to certain wells as indicated. All of the experiments were performed by cotransfection of Renilla (20 ng) as an internal control. The transfected cells were lysed for luciferase assay at 30 h post-transfection. Each experiment was performed in triplicate, and the data were represented as the mean ± S.D. of three independent experiments after being normalized to the Renilla activity. C and D, quantification of the effect of GSK3β WT/KM/C1, E2F1 (C), and GSK3β siRNA (D) on the neurite outgrowth. PC12 cells were transfected with GSK3β WT/KM/C1 (1 μg/each), E2F1 plasmids (1 μg), or GSK3β siRNA (50 nM) as indicated. At 20 h post-transfection, cells were treated with NGF (50 ng/ml). Three days later, cells were observed, and neurite outgrowth was measured. At least six randomly selected fields (containing 200 cells/field) were quantified to obtain the histogram. ns, nonspecific control siRNA. The bars in the graph represent the mean ± S.E. *p < 0.05.

E2F1 is degraded in proteasome. We, herein, verified the ubiquitination of E2F1 and demonstrated that GSK3β promotes the ubiquitination of E2F1, offering a possible mechanism through which GSK3β suppresses the protein stability of E2F1. Moreover, as an autoregulated factor, the repression of the E2F1 protein level by GSK3β may also accelerate the repression of its own mRNA level in turn, which explains why GSK3β inhibited transcriptional activity of E2F1 simultaneously.

Inactivation of GSK3β results in CRMP-2 dephosphorylation, which leads to the enhanced microtubule polymerization and axon growth; activated GSK3β thus impairs neuronal polarization (6, 34, 35). It was also observed that the neureutrate outgrowth of NGF-stimulated PC12 cells can be repressed by GSK3β RNA interference (6). Additionally, PC12 cells overexpressing Wnt1, an activator of Wnt signaling that can inactivate GSK3β and advance cell proliferation, fail to extend neurite after treatment with NGF, despite the presence and activation of high affinity NGF receptors encoded by the trk gene as well as the induction of early response genes (36–38). Here in our study, GSK3β was inactivated in the first several h and reverted to the activated form in the following days. Only the total GSK3β level was increasing along with the prolonged time stimulated by NGF. In addition, the following results of GSK3β overexpression and knocking down in neurite outgrowth in PC12 cells partially demonstrated the promoting effect of GSK3β on neurite outgrowth in PC12 cells. Simultaneously, E2F1 decreased during differentiation. Enhanced interaction between GSK3β and E2F1 as well as accelerated ubiquitination of E2F1 were also observed. E2F1, whose expression is down-regulated in cell differentiation and whose overexpression blocks the terminal differentiation by overriding the p15/p16-pRB-E2F checkpoint in the G1 phase of the cell cycle, has been demonstrated to be important to cell differentiation in previous reports (15, 16). Here, we also identified the inhibitory effect of E2F1 on neurite outgrowth in PC12 cells induced by NGF and discovered the rescuing effect of GSK3β on neurite outgrowth inhibited by E2F1 through cell cycle regulation. All of these data suggest that repression of E2F1 by GSK3β accelerates neuronal differentiation in PC12 cells. To substantiate this, we generated several deletions of GSK3β and mapped GSK3β for its interaction domain with E2F1. We found that the deleted form, which lacks the E2F1 interaction domain, also lacks the ability to inhibit E2F1 transcriptional activity, induce E2F1 ubiquitination, or rescue E2F1-inhibited neurite outgrowth. This result indicates that the association between GSK3β and E2F1 is required for cell differentiation.

The functions of the E2F family are largely dependent on their subcellular distribution. E2F-1, -2, and -3, which have a nuclear localization signal sequence, are localized in nuclei, whereas E2F-4 and -5, which lack a nuclear localization signal sequence, are localized in nuclei, whereas E2F-4 and -5, which lack a nuclear localization signal sequence, can shuttle between the cytoplasm and nuclei (14, 28, 39–42). Although E2F1 has a nuclear localization signal sequence and is normally localized in the nuclei, it has been reported to be localized in the cytoplasm of terminally differentiated myotubes, both in vitro and in vivo (28). Our data in immunofluorescence and isolation of the nuclear and cytoplasmic phase showed that in untreated PC12 cells, E2F1 localizes mainly in the cytoplasm and subsequently translocates partially into the nucleus when stimulated by NGF. Also, in mouse hippocampal regions composed of mature neurons, E2F1 turned cytoplasm-localized again. Based on these results, cytoplasm-
localized E2F1 could be a signal for cell quiescence. As for the accumulation of E2F1 in the nucleus of PC12 cells stimulated by NGF, another protein pRB, which is pivotal in the regulation of E2F1, would play a key role. For example, increased GSK3β would phosphorylate RB by its kinase activity-dependent pathway and keep the inhibitory effect of pRB on E2F1 via their association. However, much work is needed to further elucidate the detailed mechanism.

In conclusion, we have demonstrated a kinase activity-independent manner by which GSK3β associates with E2F1, inhibits its protein level via promotion of its ubiquitination, and further suppresses its transcriptional activity. Combined with the GSK3β kinase activity-dependent manner, the inhibitory effect of GSK3β on E2F1 plays an important role in PC12 differentiation.

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