ARTICLE
Journal of Cellular Biochemistry 108:1325–1336 (2009)

Journal of Cellular Biochemistry

GSKIP, an Inhibitor of GSK3β, Mediates the N-Cadherin/β-Catenin Pool in the Differentiation of SH-SY5Y Cells

Ching-Chih Lin,[1] Chia-Hua Chou,[1,2] Shen-Long Howng,[3] Chia-Yi Hsu,[1] Chi-Ching Hwang,[1] Chihuei Wang,[4] Ching-Mei Hsu,[2] and Yi-Ren Hong[1,5*]

[1]*Department of Biochemistry, Faculty of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC*
[2]*Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan, ROC*
[3]*Department of Neurosurgery, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, ROC*
[4]*Department of Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC*
[5]*Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC*

ABSTRACT

Emerging evidence has shown that GSK3β plays a pivotal role in regulating the specification of axons and dendrites. Our previous study has shown a novel GSK3β interaction protein (GSKIP) able to negatively regulate GSK3β in Wnt signaling pathway. To further characterize how GSKIP functions in neurons, human neuroblastoma SH-SY5Y cells treated with retinoic acid (RA) to differentiate to neuron-like cells was used as a model. Overexpression of GSKIP prevents neurite outgrowth in SH-SY5Y cells. GSKIP may affect GSK3β activity on neurite outgrowth by inhibiting the specific phosphorylation of tau (ser396). GSKIP also increases β-catenin in the nucleus and raises the level of cyclin D1 to promote cell-cycle progression in SH-SY5Y cells. Additionally, overexpression of GSKIP downregulates N-cadherin expression, resulting in decreased recruitment of β-catenin. Moreover, depletion of β-catenin by small interfering RNA, neurite outgrowth is blocked in SH-SY5Y cells. Altogether, we propose a model to show that GSKIP regulates the functional interplay of the GSK3β/β-catenin, β-catenin/cyclin D1, and β-catenin/N-cadherin pool during RA signaling in SH-SY5Y cells. J. Cell. Biochem. 108: 1325–1336, 2009.

KEY WORDS: WNT SIGNALING PATHWAY; RETINOIC ACID; NEURITE OUTGROWTH; CYCLIN D1

Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase active in several signaling pathways, is involved in the regulation of cell fate, including Wnt signal transduction, protein synthesis, glycogen metabolism, mitosis, and apoptosis [Cohen and Frame, 2001; Jope and Johnson, 2004]. GSK3 has two structurally similar isoforms, GSK3α and GSK3β, in mammals. Earlier reports indicated that the developmental profiles of GSK3α and GSK3β expression are different and that the regulation and functions of these two proteins are not always identical [Liang and Chuang, 2007].

GSK3β plays an important role in neuron development. Emerging evidence has shown that GSK3β plays a pivotal role in regulating the specification of axons and dendrites [Jiang et al., 2005; Gärtner et al., 2006]. Shi et al. [2004] also indicate that global inhibition of GSK3β activity led to a defect in axon development in hippocampal neurons. Axon outgrowth is dependent on actin and microtubule cytoskeleton dynamics. Several studies show that GSK3β phosphorylates microtubule-associated proteins (MAPs) such as tau and appears to reduce their binding to microtubules, rendering the microtubules more dynamic, favoring axon growth [Goold and Gordon-Weeks, 2005; Zhou and Snider, 2005]. Therefore, MAPs phosphorylated by GSK3β is critical for axon development.

GSK3β also plays an essential role in the canonical Wnt signal transduction pathway [Grimes and Jope, 2001; Logan and Nusse, 2004]. It is well documented that in the absence of Wnt signaling, phosphorylation of β-catenin by GSK3β results in its ubiquitination

Abbreviations used: GSK3, glycogen synthase kinase 3; GSKIP, GSK3β interacting protein; RA, retinoic acid; MAP, microtubule-associated proteins; APC, adenomatous polyposis coli; NF, neurofilament; DAPI, 4′-6-diamidino-2-phenylindole.

Ching-Chih Lin and Chia-Hua Chou contributed equally to this work.

Grant sponsor: National Science Council (Taiwan, ROC); Grant numbers: NSC95-2314-B-037-050-MY1, NSC 95-2745-B-037-003-URD, NSC-96-2320-B-037-004; Grant sponsor: Ministry of Education (Taiwan, ROC); Grant numbers: MOE-97-U19609-1, 1ab-14, MOE 98-U19609-6-3; Grant sponsor: NHRI (Taiwan, ROC); Grant number: NHRI-EX98-9809SI.

*Correspondence to: Prof. Yi-Ren Hong, Faculty of Medicine, Department of Biochemistry, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 708, Taiwan, ROC. E-mail: m835016@cc.kmu.edu.tw

Received 30 April 2009; Accepted 31 August 2009 • DOI 10.1002/jcb.22362 • © 2009 Wiley-Liss, Inc.
Published online 14 October 2009 in Wiley InterScience (www.interscience.wiley.com).

and subsequent degradation by proteosomes [Shimizu et al., 1997]. Conversely, with the Wnt signal, GSK3β moves away and results in unphosphorylation of β-catenin [Li et al., 1999]. This causes β-catenin to accumulate in the nucleus, where it affects transcription of cell-cycle genes such as cyclin D1 and myc [Behrens et al., 1996]. Alternatively, β-catenin not only influences cellular events as a necessary transcriptional co-activator but also has an important role in cell adhesion complexes. The association between β-catenin and N-cadherin enhances cell-to-cell interactions necessary for neuronal differentiation [Yap et al., 1997]. Consequently, the distribution of β-catenin is critical in the neuron development. In our previous study, we found a novel GSK3β interaction protein (GSKIP) that binds to GSK3β and is able to negatively regulate GSK3β in the Wnt signaling pathway [Chou et al., 2006]. In light of these results, we began exploring the role of GSKIP in neuronal differentiation using overexpression in a human neuroblastoma cell line SH-SY5Y as a model system. Here, we sought to examine whether the interplay between GSK3β and GSKIP is involved in the neuron development. Interestingly, neurite extension following RA treatment in GSKIP-overexpressing cells was blocked. Furthermore, GSKIP expression promotes cell-cycle progression via increasing the accumulation of β-catenin in the nucleus, but downregulating the association of β-catenin and N-cadherin in the membrane. These results suggest that overexpression of GSKIP can affect the transcriptional state of the cell. Finally, we postulate that GSKIP mediates N-cadherin-bound β-catenin pool and it is important during neuron development.

MATERIALS AND METHODS

CELL CULTURE, DIFFERENTIATION, AND TREATMENT

Human neuroblastoma SH-SY5Y cell line from American Type Culture Collection (ATCC) was used for these experiments. SH-SY5Y cells were cultured in D-MEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum, 1% nonessential amino acids (Gibco), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C in a humidified 5% CO_2 incubator. Cells were cultured up to 70% confluence in 100-mm diameter dishes and fed once every 3 days. To induce neuronal differentiation, SH-SY5Y cells were seeded at 1×10^6 cells/cm^2 in 100-mm diameter culture dishes in D-MEM/F12 medium containing 10% FBS. When cells were 40–50% confluent, differentiation was initiated by addition of 10 μM all trans-retinoic acid (RA; Sigma). The cells were kept under these conditions for 5 days, changing the medium every 2 days. GSK3β inhibitors, such as 10 mM LiCl or 30 μM SB415286, were pretreated 1 h before RA treatment, and were maintained during RA-mediated differentiation.

CLONING AND DNA SEQUENCING

To construct the pEGFP-GSKIP plasmid from the yeast two-hybrid working assay, DNA fragments encoding GSKIP were amplified by PCR using Taq polymerase (TaKaRa). The PCR fragments were then inserted into the *Bam*HI and *Xho*I sites of the pEGFP (Clontech) vector. Site-directed mutagenesis experiments to create the GSKIP L130P mutants (leucine 130 to proline) were carried out according to the manufacturer's protocol (Stratagene). The nucleotide sequencing was performed with an ABI PRISMTM 3730 Genetic Analyzer (Perkin-Elmer).

ANTIBODY PRODUCTION

The full length of GSKIP was used to raise antibodies. To generate antibody against human GSKIP, His-tagged fusion proteins were constructed using the PET bacterial expression system (Novagene). Protein expression was carried out in the *Escherichia coli* strain BL21 (DE3) using Luria broth (10 g Bacto tryptone, 5 g yeast extract, and 5 g NaCl/L). Recombinant proteins were expressed for 3 h with 1 mM isopropyl-b-D-thiogalactoside (IPTG) and purified on nickel columns under denaturing conditions, as described by the manufacturer (Novagene). For immunization, purified proteins (250 mg of the His-tagged GSKIP fusion proteins) were injected into New Zealand white rabbits. Immune sera were obtained on days 90 and 120.

TRANSFECTION AND RNA INTERFERENCE

For transient transfection studies, SH-SY5Y cells were seeded onto glass coverslips at a density of 1×10^5 cells per 12-well plate. One microgram of pEGFP vector alone, pEGFP-GSKIP or pEGFP-GSKIP mutants (L130P) DNA was transfected into SH-SY5Y cells, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h transfection, the cells were cultured in fresh medium and then processed as described below for the different assays. In RNA interference, β-catenin siRNA and scrambled siRNA duplex were purchased from Invitrogen. The siRNA transfection protocol suggested by the manufacturer was optimized as follows: 1×10^5 or 1×10^6 cells were plated onto 12-well plates or 100 mm dishes and left to grow overnight, respectively. The following day, cells were transfected with the siRNA duplex (final concentration 50 nM) using Lipofectamine 2000. After 24 h transfection, the cells were induced by addition of 10 μM all trans-RA for 5 days. In all cases, the cells were doubly transfected with siRNA duplex after 72 h transfection during RA treatment.

IMMUNOFLUORESCENCE AND MICROSCOPY

For immunofluorescent labeling, cell cultures were rinsed several times with PBS and fixed in 4% paraformaldehyde for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min. Fixed cells were rinsed in PBS, and nonspecific binding was blocked with 5% normal goat serum (NGS)/1% bovine serum albumin (BSA) in PBS, pH 7.4, for at least 30 min at 37°C. All subsequent antibody incubations were carried out in the same blocking solution. After a brief wash, the cells were incubated for 45 min at 37°C with the primary antibodies. Primary antibodies were anti-NF-200 monoclonal antibodies (1:500 dilution, Sigma), anti-β-catenin polyclonal antibodies (1:100 dilution, Santa Cruz), or anti-cyclin D1 polyclonal antibodies (1:250 dilution, Abcam). After extensive washes with PBS, the cultures were then incubated with the appropriate secondary antibody conjugated to either Alexa 488 or Alexa 568 (1:500 dilution, Molecular Probes, USA) for 45 min at 37°C. Finally, the cells were incubated for 5 min with 4'-6-diamidino-2-phenylindole (DAPI; 1 μg/ml, Roche) prior to mounting (Molecular Probes). Confocal images were obtained using an Olympus IX71 microscope (100× UPlanFl objective 1.3 NA) at 0.2 μm z-steps,

controlled by FLUOVIEW software (Universal Imaging). All images were imported into Adobe Photoshop v7.0 for contrast manipulation.

WESTERN BLOTTING

Cellular lysate was prepared using RIPA buffer. The protein content was determined by a Bio-Rad Protein Assay system. Nuclear extracts were prepared using the Nuclear/Cytosol Fractionation kit (Biovision Research Products, Mountain View, CA) according to the manufacturer's instructions. Proteins were separated on 12% SDS–PAGE and transferred onto PVDF membrane. Then the membrane was incubated with primary antibodies: anti-GSKIP (our preparation), anti-phospho (Ser396)-Tau and anti-phospho (Thr205)-Tau (Biosourse), anti-GSK-3 (recognize both GSK-3α and GSK-3β), anti-phospho (Tyr216)-GSK-3β (BD), anti-phospho (Ser9)-GSK-3β (Cell Signaling), anti-GFP, anti-β-catenin, anti-β-actin (Santa Cruz), anti-N-cadherin (upstate), anti-E-cadherin (Zymed), or anti-cyclin D1 (Abcam). The secondary antibodies used were goat anti-mouse or anti-rabbit IgG conjugated to HRP (Zymed), and the ECL reagents (Amersham) were used for immunodetection.

SEAP AND LUCIFERASE ACTIVITY ASSAY

The construction of pTcf4RE-luc and pIRES2-hyg-β-catenin (T41A, S45A) was described previously [Hsu et al., 2006]. For luciferase activity assay, SH-SY5Y cells were seeded onto glass coverslips at a density of 1×10^5 cells per 24-well plate. Cells were co-transfected with 0.5 μg of pEGFP vector alone, pEGFP-GSKIP, or pIRES2-hyg-β-catenin (T41A, S45A) and 0.5 μg of pTcf4RE-luc and 0.2 μg of pSEAP2-control (Clontech), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Following transfection, the cells were washed and incubated with or without 10 μM all trans-RA treatment. After 48 h, 25 μl of the supernatant was collected for secreted alkaline phosphatase (SEAP) activity analysis. The supernatant was mixed with an equal volume of reaction buffer and chemiluminescent substrate (Tropix) and measured by TopCount NXT™ (Perkin-Elmer) in chemiluminescent mode. The remaining cells for luciferase activity assay were determined using a dual-luciferase reporter assay kit (Promega) following manufacturer's instructions and measured by TopCount NXT™ (Perkin-Elmer) in luciferase mode.

IMMUNOPRECIPITATION

SH-SY5Y cells (1×10^6) transfected with pEGFP vector alone or pEGFP-GSKIP were treated with or without RA for 5 days. Then cells were harvested and washed with phosphate-buffered saline. The lysate was prepared by adding 1 ml of immunoprecipitation assay buffer (50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% deoxycholate, and 10 μg/ml each of leupeptin, aprotinin, and 4-(2-aminoethyl)benzenesulfonyl fluoride) to the cells. Then the lysate was centrifuged using a microcentrifuge at 10,000*g* for 20 min. The supernatant was added to anti-N-cadherin antibody (Upstate) at 4°C for 1 h. Protein-A/G-agarose beads (30 μl) (Calbiochem) were added to the lysate, and the mixture was incubated with shaking for 1 h at 4°C. The beads were finally collected by centrifugation and washed three times with immunoprecipitation assay buffer. Proteins binding to the beads were eluted by adding 20 μl of 2× electrophoresis sample buffer and separated by SDS–polyacrylamide gel electrophoresis. Immunoblotting was analyzed by using anti-β-catenin antibody (Santa Cruz).

CELL GROWTH CURVE AND MTT ASSAY

For growth curve, cells were seeded on 50 mm culture dish at 2×10^4 cells. At the indicated time, the viability and total cell number were counted. For MTT assay, cells were seeded onto 96-well plates at 2×10^4cells/well in D-MEM/F12 medium, and cells were incubated for 24 h. MTT was added (final concentration 0.5 μg/ml per well) and incubated for 4 h (37°C at 5% carbon dioxide), and the reaction stopped by adding DMSO, and the formazan dye solubilized. Optical density was read at 595 nm using a microplate reader.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard derivation. Statistical significance was tested using Student's *t*-test, and statistical significance was achieved when the *P*-value was <0.05.

RESULTS

EXPRESSION OF GSKIP CONFERS RESISTANCE TO RA-MEDIATED DIFFERENTIATION

To gain insight into the involvement of GSK3β and GSKIP in neuron differentiation, we used human neuroblastoma SH-SY5Y cells as a model of neuronal cell differentiation. In the absence of RA, native SH-SY5Y cells had triangular phase-bright bodies with short neurites. Our previous study had shown that GSKIP interacts with GSK3β and acts as an inhibitor, but not GSKIP (L130P) mutant [Chou et al., 2006]. Therefore, we used GSKIP (L130P) mutant as a control. Expression of the wild-type GFP-GSKIP and GFP-GSKIP (L130P) mutant did not cause any change in their shape (Fig. 1A, left). After 5 days of RA treatment, neurite extension was 2–4 times the length of the cell body in native and GFP-GSKIP (L130P) mutant cells, but not in GFP-GSKIP-transfected cells (Fig. 1A, right). Neurofilament heavy chain (NfH) is a neuron-specific protein for the neuro-axonal compartment. Differentiation of the neuronal cells was further confirmed by immunostaining with an anti-neurofilament heavy chain antibody (NF-200) as a neuronal differentiation marker. Immunostaining using anti-NF-200 antibody showed enhancement of immunoreactivity in native and GFP-GSKIP mutant (L130P) transfected cells after RA treatment. The result was similar to using another differentiation marker, anti-GAP43 antibody (data not shown). Conversely, the immunoreactivity was not enhanced after RA treatment of GFP-GSKIP-transfected cells, indicating that neuronal differentiation was blocked (Fig. 1B). To further determine that GSKIP blocks neuronal differentiation by inhibiting GSK3β activity, SH-SY5Y cells were treated with LiCl or SB415286, an inhibitor of GSK3. In the presence of LiCl or SB415286, neurite extension was inhibited in RA-mediated differentiation cells (Fig. 1C). A similar result was shown in the immunostaining images (Fig. 1D). The effect of GSK3 inhibitors on neurite outgrowth in SH-SY5Y cells was summarized in Table I. These data collectively suggest that the activity of GSK3 is required

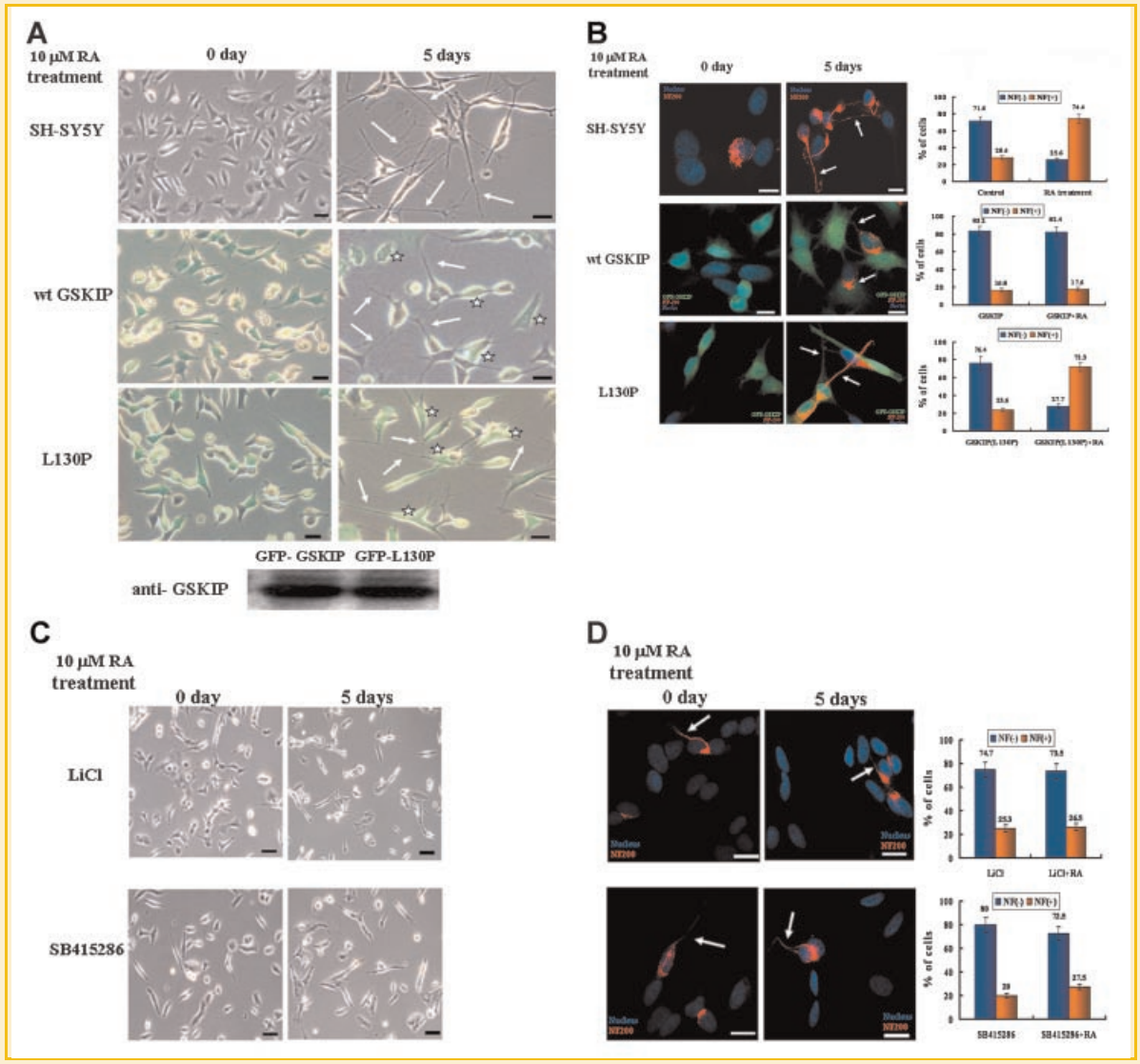

Fig. 1. Inhibition of GSK3β activity prevents SH-SY5Y cell differentiation in response to retinoic acid (RA) treatment. A: Phase-contrast image of each cell line before (left) and after (right) RA treatment. RA (10 μM) treatment induced elongation of neurites in native and GSKIP (L130P) mutant-transfected cells. GSKIP-transfected cells still remained triangular phase-bright bodies with short neurites and failed to develop elongated neurites even after 5 days of RA treatment. An immunoblot using anti-GSKIP antibody to detect GFP-GSKIP and GFP-GSKIP (L130P) mutant proteins in the extracts is shown in the bottom panel. B: The immunoreactivity of NF-200 was enhanced after RA treatment in native and GSKIP (L130P) mutant cells (upper and bottom panels), and remained unchanged in GSKIP cells (middle panels). Quantification of anti-NF-200-positive cells corresponding to the experiments is illustrated in immunofluorescence micrographs (left). C: Phase-contrast image of each cell treated with GSK3β inhibitors such as LiCl or SB415286 blocked RA-mediated neurite outgrowth in SH-SY5Y cells. D: The immunoreactivity of NF-200 remained unchanged in SH-SY5Y cells treated with LiCl or SB415286. Quantification of anti-NF-200-positive cells corresponding to the experiments is illustrated in immunofluorescence micrographs (left). Data are from three independent experiments and at least 300 cells are counted. Error bars represent SD. Stars indicate transfected cells. Arrows indicate elongated neurites. Scale bars represent 10 μm. The statistical test used was Student's *t*-test. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

for neurite outgrowth. Although we cannot exclude involvement of GSK3α in neuritogenesis, GSKIP might play a role in regulation of GSK3β during RA-mediated differentiation in SH-SY5Y cells.

GSKIP AFFECTS GSK3β ACTIVITY ON PHOSPHORYLATING A SPECIFIC SITE OF TAU DURING RA TREATMENT OF SH-SY5Y CELLS

To further investigate how GSKIP affects GSK3β and neurite outgrowth during RA treatment of SH-SY5Y cells, the activity of GSK3β was examined. It is well known that GSK3β is regulated by phosphorylation or protein–protein interactions. Our data showed that the amount of total GSK, GSK3β Y216 (the active form), and GSK3β Ser9 (the inactive form) showed no difference between GFP alone and GFP-GSKIP-transfected cells during RA treatment of SH-SY5Y cells (Fig. 2A), suggesting that GSK3β activity was not regulated through post-translational modulation by RA signaling. Indeed, tau is a major substrate of GSK3β and is hyperphosphorylated in the axon during neurite outgrowth. Therefore, we next examined how GSK3β mediated tau phosphorylation. As

TABLE I. Summary of anti-NF-200-positive SH-SY5Y, cells in different treatments

	Neurite outgrowth (%)
Control	28.4
Control + RA	74.4
GSK inhibitors	
LiCl	25.3
LiCl + RA	26.5
SB41	20.0
SB41 + RA	27.5
Nature occurred GSK inhibitor	
GSKIP	16.8
GSKIP + RA	17.6
Mutant GSKIP	
L130P	23.6
L130P + RA	72.3

shown in Figure 2B, the phosphorylated form (Ser396) of tau significantly increased in the presence of RA treatment (Fig. 2B, top panel, lane 2 compared to lane 1). Expression of the GSKIP-phosphorylated form (Ser396) of tau significantly decreased in the absence or presence of RA treatment, but not in GFP-alone cells (Fig. 2B, top panel, lane 3 compared to lane 1; lane 4 compared to lane 2). The data also showed that GSKIP had no influence on the phosphorylation of tau Thr205, the specific site phosphorylated by Cdk5 (Fig. 2B, middle panel). Altogether, these data indicate that GSK3β activity was not regulated by RA signaling and over-expression of GSKIP inhibited GSK3β activity, reducing the specific phosphorylated site (Ser396) of tau by GSK3β.

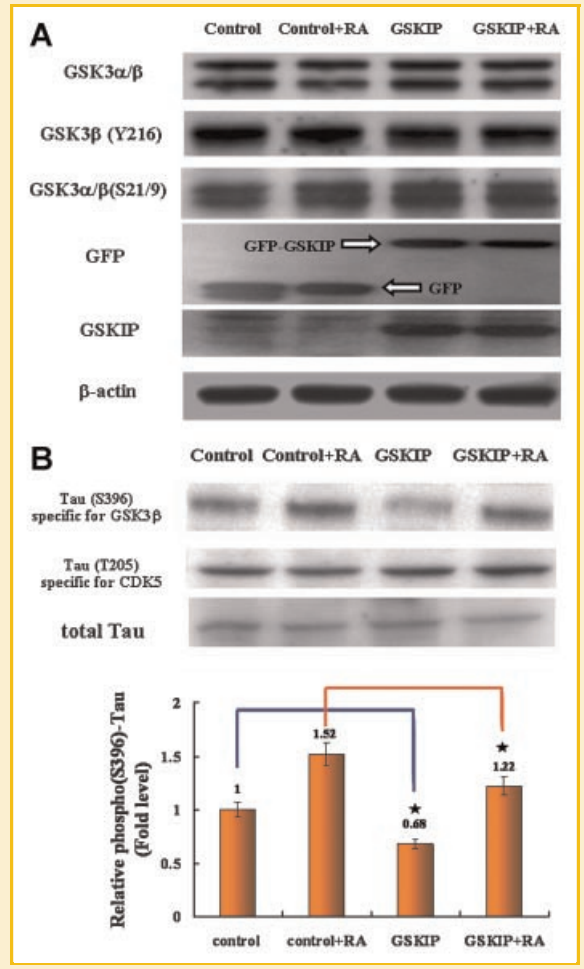

Fig. 2. GSKIP affects GSK3β activity phosphorylating a specific site of tau during RA treatment of SH-SY5Y cells. A: GSK3β activity was not regulated through post-translational modulation by RA. SH-SY5Y cells were transfected with GFP alone or GFP-GSKIP for 24 h, then incubated in the presence or absence of RA for 5 days. After 5 days treatment, cells were collected and total lysates were immunoblotted with the antibodies indicated in the left panel. Arrows indicate GFP-fusion proteins. Actin was used as a loading control. B: GSKIP affects GSK3β phosphorylation of a specific site of tau. The transfected cells were collected after 5 days of RA treatment, and total lysates were immunoblotted with the antibodies indicated in the left panel. The relative intensity of tau (Ser396) was quantified with a densitometer and normalized to the amount of total tau. Data are from three independent experiments. Error bars represent SD. The statistical test used was Student's *t*-test. Asterisks indicate $P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

OVEREXPRESSION OF GSKIP INCREASES CYTOPLASMIC β-CATENIN AND PROMOTES THE TRANSLOCATION OF β-CATENIN INTO THE NUCLEUS

Chou et al. [2006] have indicated that GSKIP may also participate in the GSK3β–Axin–β-catenin complex as part of the Wnt signaling pathway. To elucidate whether GSKIP also affects the expression of β-catenin in neuronal differentiation, we investigated the protein level of β-catenin during RA-mediated differentiation in SH-SY5Y cells. When GSKIP was overexpressed in SH-SY5Y cells, the total level of β-catenin increased two times compared with control cells (Fig. 3A, lane 3 compared to lane 1). A similar result was also shown in the immunostaining images (Fig. 3B, bottom panel compared to top panel). However, it should also be noted that the amount of β-catenin was increased in control cells with RA treatment (Fig. 3A, lane 2 compared to lane 1) and it robustly increased almost four times in GSKIP overexpressed cells upon RA treatment (Fig. 3A, lane 4 compared to lane 1). Because the transcriptional activity of β-catenin requires its nuclear translocation [Yuan et al., 2005], we therefore examined β-catenin levels in cytosolic and nuclear fractions. To our surprise, the increase of β-catenin induced by RA did not translocate to the nucleus in control cells (Fig. 3C, top panel, lane 2 compared to lane 1). Alternatively, β-catenin increased significantly about 52% and 74% in the nuclear fractions of GSKIP-expressing cells regardless of RA treatment (Fig. 3C, top panel, lanes 4 and 3 compared to lane 1, respectively). Further, β-catenin modestly increased in the cytosolic fractions in control cells with RA treatment (Fig. 3D, top panel, lane 2 compared to lane 1), but there were no significant differences between GSKIP-expressing cells (Fig. 3D, top panel, lane 4 compared to lane 3). Moreover, our data showed that GFP-GSKIP-transfected cells could affect the transcriptional activity of β-catenin using a TCF/Lef luciferase reporter in the absence or presence of RA treatment, but the GFP-alone-transfected cells did not exhibit a significant difference (Fig. 3E). A typical β-catenin double mutant T41A/S45A, which stabilizes β-catenin, exhibited significantly higher activity as expected (Fig. 3E). These data indicate that overexpression of GSKIP stabilizes cytoplasmic β-catenin via inhibiting GSK3β activity and leads to an

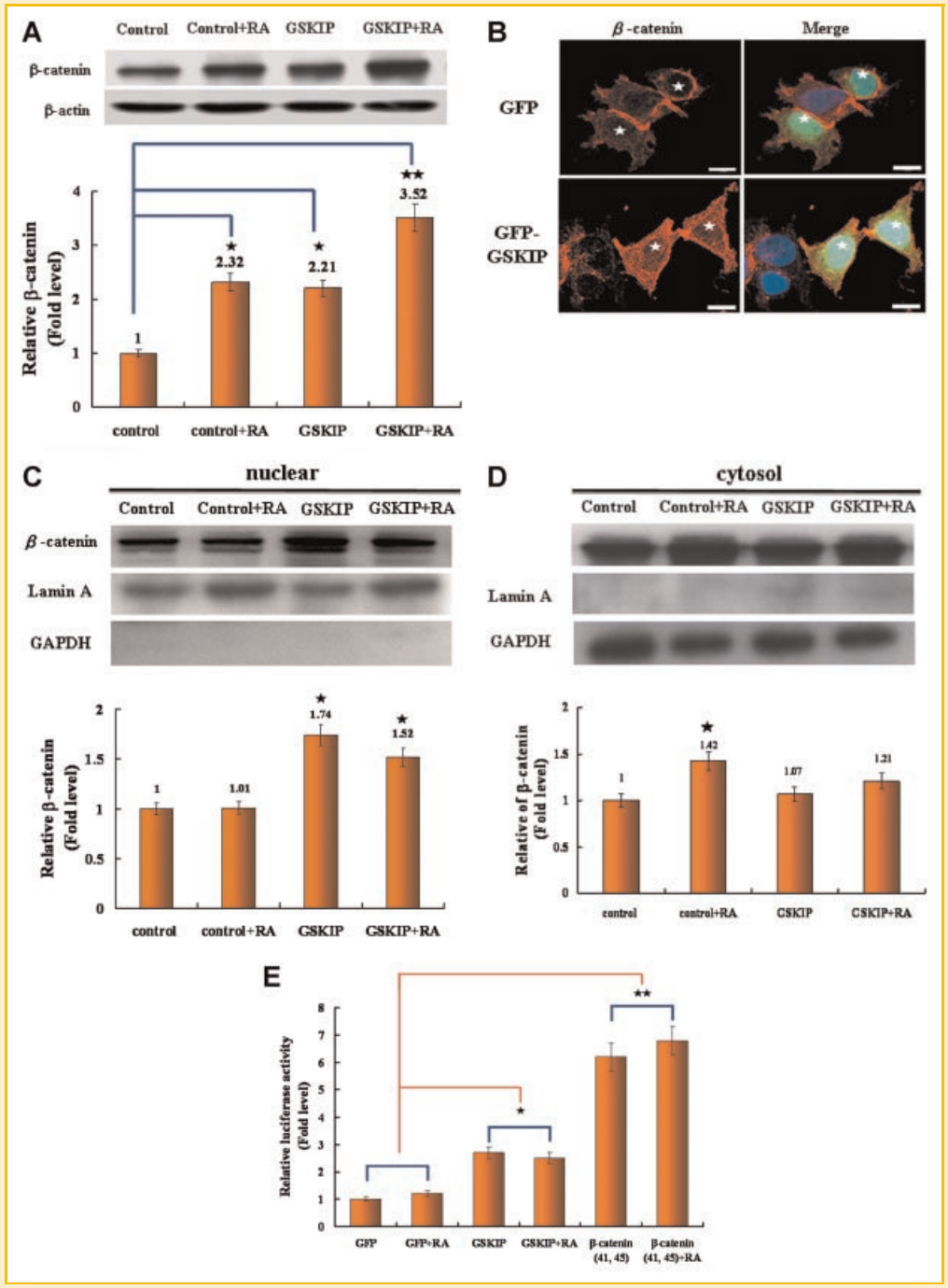

Fig. 3. GSKIP increases cytoplasmic β-catenin and promotes the translocation of β-catenin into the nucleus. A: Overexpression of GSKIP increases the amount of β-catenin in the absence or presence of RA treatment. The transfected cells were collected after 5 days RA treatment and total lysates were immunoblotted with anti-β-catenin antibodies. Actin was used as a loading control. The relative intensity of β-catenin was quantified with a densitometer and normalized to the amount of actin (bottom panel). B: The transfected cells were fixed and immunostained with anti-β-catenin antibody. Overexpression of GSKIP enhances the nuclear translocation of β-catenin. Immunoblotting of nuclear extracts (C) or cytosolic extracts (D) using anti-β-catenin antibody before and after 10 μM RA treatment. Purification of nuclear extracts and cytosolic extracts was verified using anti-lamin A and anti-GAPDH antibody, respectively. The relative intensity of β-catenin was quantified with a densitometer. E: The luciferase activities assay using pTcf4RE-luc was utilized to study the β-catenin TCF/LEF transcriptional activity and was normalized by the SEAP activity of pSEAP2-control and presented as a bar graph. Data are from three independent experiments. Error bars represent SD. Stars indicate transfected cells. Scale bars represent 10 μm. The statistical test used was Student's *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

increase in the amount of available free β-catenin translocation into nucleus.

GSKIP PROMOTES CELL-CYCLE PROGRESSION BY INDUCING CYCLIN D1 EXPRESSION

The nuclear translocation of β-catenin interacts with transcription factors of the LEF/TCF family to induce changes in cell-cycle gene expressions, such as cyclin D1 [Behrens et al., 1996]. Next we examined whether the downstream target gene of cyclin D1 could be turned on. Compared with control cells, the level of cyclin D1 was elevated significantly in cells expressing GSKIP even after RA treatment (Fig. 4A, lanes 3 and 4 compared to lane 1). Moreover, our data also showed that overexpression of GSKIP resulted in increasing accumulation of cyclin D1 in nucleus (Fig. 4B,C). A previous study showed that the nuclear localization of cyclin D1 is sufficient to induce differentiated neuroblastoma cells to reenter the cell cycle [Sumrejkanchanakij et al., 2006]. To further test whether GSKIP promotes cell-cycle progression during RA-mediated differentiation in SH-SY5Y cells, cell proliferation with cell growth and MTT assay were analyzed. Despite RA treatment, overexpression of GSKIP resulted in at least a 1–2 times increase in the total cell number compared with control cells (Fig. 4D). Cell number also increased at least 30% in GSKIP-expressing cells as shown by MTT assay (Fig. 4E). These data suggest that GSKIP expression elevates cyclin D1 accumulation and promotes cell-cycle progression during RA-mediated differentiation in SH-SY5Y cells.

GSKIP DOWNREGULATES N-CADHERIN EXPRESSION AND REDUCES RECRUITMENT OF CYTOPLASMIC β-CATENIN

It has been shown that exogenous expression of cadherins can recruit cytoplasmic β-catenin to the membrane pool [Sadot et al., 1998]. Previous studies have also reported that N-cadherin is essential for neurite outgrowth and is upregulated by RA treatment of neuronal differentiation P19 cells [Bixby and Zhang, 1990]. We next questioned whether the increased β-catenin induced by RA is recruited to the membrane pool with cadherin and whether GSKIP regulates the expression of N-cadherin resulting in inhibition of neurite outgrowth. As shown in Figure 5A, RA triggers the neuronal differentiation of SH-SY5Y cells by upregulating N-cadherin expression (Fig. 5A, lane 2 compared to lane 1; lane 4 compared to lane 3), but E-cadherin had no significant differences in each group (Fig. 5A, middle lane). To our surprise, overexpression of GSKIP downregulated N-cadherin expression in the absence of RA treatment (Fig. 5A, lane 3 compared to lane 1), but slightly increased back to a normal level with RA treatment (Fig. 5A, lane 4 compared to lanes 3 and 1, respectively). To further elucidate whether decrease in N-cadherin reduces the recruitment of cytoplasmic β-catenin, co-immunoprecipitation with N-cadherin and β-catenin was performed. As expected, the cytoplasmic β-catenin associated with N-cadherin increases upon RA treatment (Fig. 5B, lower panel compared to upper panel) but is decreasingly associated with N-cadherin in GSKIP-expressing cells (Fig. 5B, left panel compared to right panel). These data suggest that the increasing of N-cadherin-bound β-catenin pool was required for neuronal differentiation and GSKIP downregulates N-cadherin expression, resulting in a decrease in the recruitment of cytoplasmic β-catenin during RA treatment.

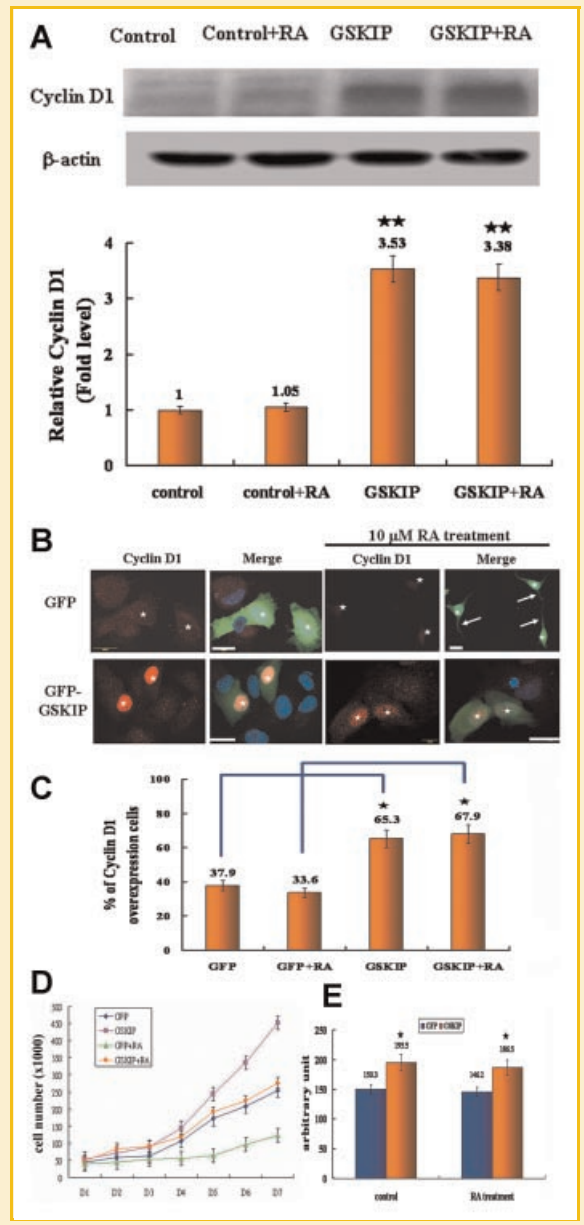

Fig. 4. GSKIP promotes cell-cycle progression by inducing cyclin D1 expression. A: Cyclin D1 was elevated significantly in cells expressing GSKIP even after RA treatment. The transfected cells were collected after 5 days RA treatment and total lysates were immunoblotted with anti-cyclin D1 antibodies. Actin was used as a loading control. The relative intensity of β-catenin was quantified with a densitometer and normalized to the amount of actin. B: SH-SY5Y cells were transfected with GFP alone or GFP-GSKIP without RA treatment (left) or with 5 days of RA treatment (right), respectively. Then cells were fixed and immunostained with anti-cyclin D1 antibody. C: Quantification of anti-cyclin-D1-positive cells corresponding to the experiments is illustrated in immunofluorescence micrographs (B). D: The transfected cells with or without RA treatment were collected at the indicated time points for cell number analysis. E: The transfected cells with or without RA treatment for 24 h were collected and MTT assay was performed. Data are from three independent experiments. Error bars represent SD. Stars indicate transfected cells. Arrows indicate elongated neurites. Scale bars represent 10 μm. The statistical test used was Student's *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

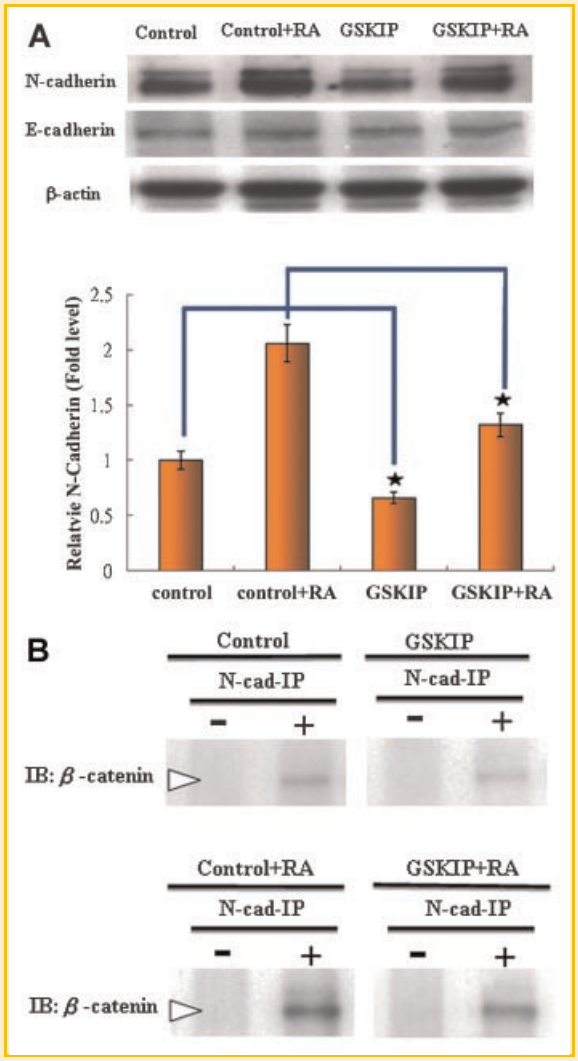

Fig. 5. GSKIP downregulates N-cadherin expression and reduces recruitment of cytoplasmic β-catenin. A: Overexpression of GSKIP downregulates N-cadherin expression with the RA treatment of SH-SY5Y cells. Cells were transfected with GFP alone or GFP-GSKIP for 24 h, then were collected after 5 days RA treatment and total lysates were immunoblotted with anti-N-cadherin and anti-E-cadherin antibodies. Actin was used as a loading control. The relative intensity of N-cadherin was quantified with a densitometer and normalized to the amount of actin. B: Overexpression of GSKIP reduces the formation of N-cadherin and β-catenin complex. The transfected cells were collected after 5 days RA treatment and total lysates were subjected to immunoprecipitation (IP) using anti-N-cadherin antibody and/or irrelevant IgG antibody. The resulting precipitates were then analyzed by Western blotting with the anti-β-catenin antibody. Data are from three independent experiments. Error bars represent SD. Arrowheads indicate β-catenin. Scale bars represent 10 μm. The statistical test used was Student's *t*-test. Asterisks indicate $P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

β-CATENIN IS REQUIRED FOR RA-MEDIATED NEURONAL DIFFERENTIATION IN SH-SY5Y CELLS

To further explore the effect of the β-catenin in RA-mediated differentiation, siRNA was transfected into the SH-SY5Y cells to inhibit the expression of β-catenin. As expected, the intensity of the β-catenin signal was remarkably reduced after treatment with siRNA–β-catenin, strongly suggesting that the siRNA treatment successfully suppressed β-catenin expression (Fig. 6A). After RA treatment, the differentiation of the neuronal cells was confirmed by using immunostaining with a neuronal differentiation marker NF-200. As shown in Figure 6B, neurite extension was blocked in β-catenin-depleted cells, but not in nonspecific siRNA cells after 5 days of RA treatment (data not shown). These results indicate that β-catenin plays an important role during RA-mediated neuronal differentiation in SH-SY5Y cells.

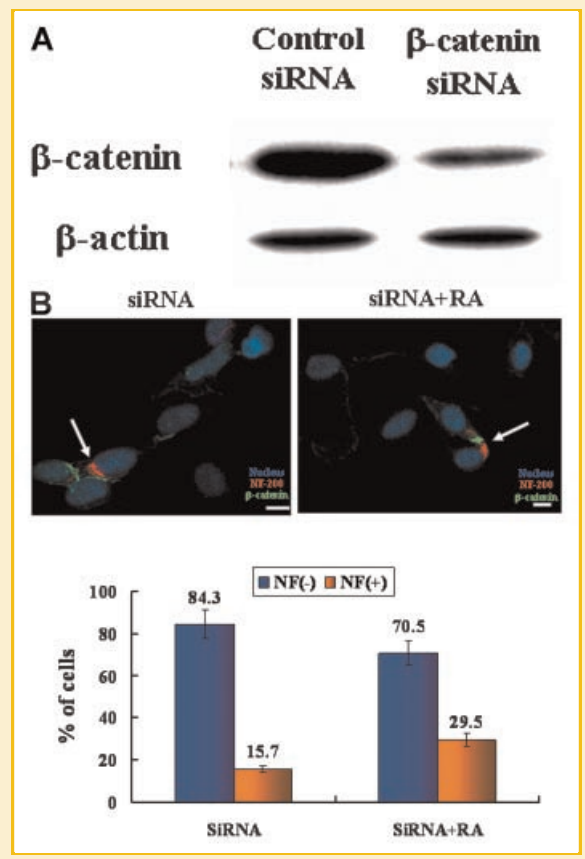

Fig. 6. β-Catenin is required for RA-mediated neuronal differentiation in SH-SY5Y cells. A: Western blot analysis of total extracts from SH-SY5Y cells treated for 72 h with a β-catenin-specific siRNA or a control siRNA. Actin was used as a loading control. B: The immunoreactivity of NF-200 remained unchanged in β-catenin-depleted SH-SY5Y cells. After 24 h β-catenin-specific siRNA or control siRNA transfection, 10 μM of trans-retinoic acid was added to the cells for 5 days. In all cases, the cells were doubly transfected with siRNA duplex after 72 h transfection during RA-mediated differentiation. Quantification of anti-NF-200-positive cells corresponding to the experiments is illustrated in immunofluorescence micrographs (top). Data are from three independent experiments and at least 300 cells are counted. Error bars represent SD. Arrows indicate elongated neurites. Scale bars represent 10 μm. The statistical test used was Student's *t*-test. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

GSKIP NEGATIVELY REGULATES GSK3β ACTIVITY IN RA-MEDIATED NEURITE OUTGROWTH

Overexpression of GSKIP inhibits the activity of GSK3β to prevent neurite outgrowth in RA-mediated differentiation, indicating that GSK3β activity is important for neurite outgrowth. These observations are consistent with previous reports using different model systems [Takehashi et al., 1999; Kishida et al., 2004; Shi et al., 2004; Goold and Gordon-Weeks, 2005; Kim et al., 2006]. Conversely, treatment of DRG neurons and Neuro-2a neuroblastoma cells with a GSK3β inhibitor like lithium or SB415286 has been reported to induce neurite outgrowth [Zhou et al., 2004]. Munoz-Montano et al. [1999] also demonstrated that high levels of Li+ inhibit neurite outgrowth in cerebellar granule cells, but lower levels (1–5 mM) promote it. The apparent discrepancy is explained by the need for an optimal level of GSK3β activity for neurite outgrowth, and the participation of other kinases such as ERK1/2, CDK5, PKA, and PI3K in different cell types during RA-mediated neuronal differentiation may be related [Lopez-Carballo et al., 2002; Canon et al., 2004]. GSK3β regulation mechanisms are not fully understood yet, and it is believed that it is regulated in multiple ways, including phosphorylation and protein–protein interactions with GSK3-binding proteins [Jope and Johnson, 2004]. In our case, GSK3β activity showed no changes with RA treatment of SH-SY5Y cells, suggesting that the activity of GSK3β is not regulated by RA signaling. Moreover, our data showed that GSKIP affects GSK3β activity to phosphorylate a specific site (Ser396) of tau in the absence or presence of RA treatment but has no effect on GSK3β Y216 (active form) and GSK3β Ser9 (inactive form) immunoreactivity. In our previous study, an interaction between GSKIP and GSK3β had been demonstrated by co-immunoprecipitation from HEK293 cell lysates co-expressing HA-tagged GSKIP and FLAG-tagged GSK3β [Chou et al., 2006]. Based on this evidence, we suggest that GSKIP negatively regulates GSK3β activity through protein–protein interactions. However, we have so far been unable to detect endogenous GSKIP in anti-GSK3β immunoprecipitates (unpublished data), suggesting that the interaction is transient and/or that only a small amount of GSKIP interacts with GSK3β.

GSKIP PLAYS A ROLE IN REGULATING THE PHOSPHORYLATION OF TAU DURING RA-MEDIATED DIFFERENTIATION IN SH-SY5Y CELLS

Tau plays a key role in regulating microtubule dynamics, axonal transport, and neurite outgrowth, and all of these functions of tau are modulated by site-specific phosphorylation [Johnson and Stoothoff, 2004]. Although many kinases can phosphorylate tau in vivo, emerging evidence suggests that GSK3β may play a major role in regulating tau phosphorylation both in physiological and pathological conditions [Cho and Johnson, 2004]. Lucas et al. [2001] have demonstrated that GSK3β can phosphorylate tau on Ser199, Thr231, Ser396, Ser400, Ser404, and Ser413 in vivo and in vitro. Moreover, previous studies have shown that phosphorylation of tau at the Ser396/Ser404 by GSK3β is increased in differentiated human neuroblastoma SH-SY5Y cells [Haque et al., 2004]. This indicates that the regulation of phosphorylated tau at the specific sites is critical for neuron to maintain normal cytoskeletal architecture and functions. Here our data demonstrate that GSKIP inhibits GSK3β activity to phosphorylate tau at the specific site (Ser396). However, there are no significant differences among the other phosphorylation sites (Fig. 2B, middle panel and unpublished data). It is possible that tau is phosphorylated by GSK3β, including both primed (S/T)XXX(S/T)-p and unprimed (S/T)P motifs [Jope and Johnson, 2004]. We suggest that GSKIP negatively regulates GSK3β activity to phosphorylate tau at the specific unprimed sites in differentiated SH-SY5Y cells. Indeed, the hyperphosphorylation of unprimed GSK3β sites in tau, such as the PHF-1 site (Ser396), may be pathological [Abraha et al., 2000]. It also suggests that GSKIP may be a regulator of tau pathological phosphorylation by GSK3β in Alzheimer's disease.

GSKIP INDUCES THE ACCUMULATION OF β-CATENIN IN NUCLEAR AND OVERCOMES THE EFFECT OF RA SIGNALING

As mentioned above, GSK3β also plays an essential role in the canonical Wnt signal transduction pathway [Grimes and Jope, 2001]. Wnt signals have also been implicated in promoting self-renewal during neural development. Overexpression of constitutively active β-catenin in neural stem cells increases neurogenesis primarily by decreasing cell-cycle exit of neural progenitors [Chenn and Walsh, 2002], and β-catenin expression in the developing spinal cord maintained neural progenitor cells in a proliferative state with decreased neuronal differentiation [Zechner et al., 2003]. Moreover, it has been suggested that stabilization of β-catenin results in maintenance of pluripotency in human embryonic stem cells [Sato et al., 2004] and inhibition of differentiation of murine ES cells [Aubert et al., 2002]. Furthermore, high β-catenin/TCF activity is able to drive cell proliferation during tumor formation by turning on the cell-cycle regulator cyclin D1 [Shtutman et al., 1999]. At the molecular level RA is known to inhibit G1–S progression and cyclin D1 expression. It has also been demonstrated that RA promotes ubiquitination and proteolysis of cyclin D1 during induced NT2/D1 cell differentiation [Spinella et al., 1999]. Moreover, NT2/D1 cells overexpressing cyclin D1 before and after RA treatment fail to exhibit a decline in cell proliferation, to differentiate, or G1 arrest. In our study, one notable finding was that overexpression of GSKIP markedly raises the levels of β-catenin in the nucleus resulting in an increase of cyclin D1 to promote cell-cycle progression even in the presence of RA treatment (Figs. 3 and 4). However, Diehl et al. [1998] show that GSK3β phosphorylates cyclin D1 at threonine 286, triggering rapid cyclin D1 turnover. Although we cannot definitively rule out whether the increase of cyclin D1 occurs through the activation of the β-catenin/TCF signaling pathway or the direct inhibition of GSK3β phosphorylation of cyclin D1. Our data suggest that GSKIP activates the β-catenin/TCF signaling pathway by inhibiting GSK3β and indeed participates in the regulation of cell-cycle progression during RA-mediated differentiation.

N-CADHERIN/β-CATENIN COMPLEX EXPRESSION AND FUNCTION IS NECESSARY TO MEDIATE THE EFFECTS OF RA ON SH-SY5Y CELLS

β-Catenin is present in two pools: a membrane pool required for cell–cell adhesion, and a cytoplasmic/nuclear pool responsible for β-catenin/TCF signaling. Membrane-bound β-catenin is associated

with cadherin/adherens junctions and functions to bridge cadherin to the cytoskeleton [Aberle et al., 1996]. In addition to being a key component of the Wnt signal transduction pathway, the transmembrane cadherin/catenin complex is expressed at high levels in both axons and dendrites from early neuronal development [Benson and Tanaka, 1998]. Several studies have shown that translocation of cytoplasmic β-catenin to the membrane can reduce β-catenin/TCF signaling [Gottardi et al., 2001; Stockinger et al., 2001]. In this study, our data show that β-catenin modestly increases after RA treatment in SH-SY5Y cells (Fig. 3A), but it has no effect on the regulation of cyclin D1 expression (Fig. 4A,B). It is possible that RA treatment increases β-catenin protein stability and enhances the affinity for adherins junctions in SH-SY5Y cells. N-cadherin as a cell adhesion molecule has been considered to play important roles in the development of the central nervous system (CNS) [Takeichi, 1995]. Further, cadherin inhibition of β-catenin signaling regulates the proliferation and differentiation of neural precursor cells [Noles and Chenn, 2007]. In the present study, we show that RA triggers neuronal differentiation of SH-SY5Y cells by upregulating N-cadherin expression and results in increasing the association of β-catenin with N-cadherin (Fig. 5). These observations are consistent with previous reports using different model systems [Shah et al., 2002]. Shah et al. also demonstrate that RA mediates the activation of the RAR/RXR pathway that directly or, more likely, indirectly regulates cadherin expression. However, overexpression of GSKIP downregulates N-cadherin expression regardless of RA treatment and this event reduces the ability to recruit cytoplasmic β-catenin (Fig. 5). It is not clear how GSKIP regulates N-cadherin expression in SH-SY5Y cells, but our data suggest that GSKIP increases the amount of β-catenin and downregulates N-cadherin expression resulting in promoting the translocation of β-catenin to the nucleus (Figs. 3 and 5). It strongly indicates that GSKIP reduces the expression of N-cadherin and this may be one of the factors that block neurite outgrowth during RA-mediated differentiation in SH-SY5Y cells.

THE FUNCTIONAL INTERPLAY OF GSK3β/β-CATENIN, β-CATENIN/CYCLIN D1, AND THE N-CADHERIN/β-CATENIN POOL DURING RA SIGNALING

Zechner et al. [2003] showed that Wnt signals, which are mediated by β-catenin and its downstream interaction partners, control proliferation and the balance between progenitor expansion and differentiation. We have demonstrated that when GSKIP affects β-catenin signals, SH-SY5Y cells exit the cell cycle less frequently, and instead continue to proliferate in RA treatment (Figs. 3 and 4). Moreover, by interfering with β-catenin signaling using siRNA, we observe that differentiation of SH-SY5Y cells was abolished but there was no effect on the cell survival (Fig. 6 and data not shown). It seems likely that β-catenin is not fully knocked down by siRNA and maintains the basal level for cell function. But the availability of

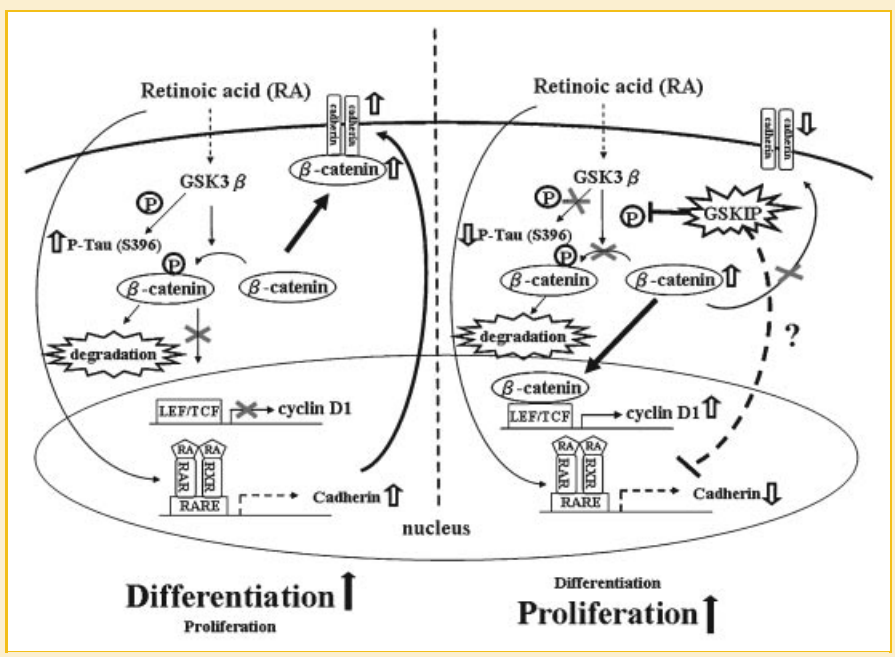

Fig. 7. A model of the functional interplay of GSK3β/β-catenin, N-cadherin/β-catenin, and β-catenin/cyclin D1 during RA-mediated SH-SY5Y neuronal differentiation. In the presence of RA signaling, RA and its receptors (RAR/RXR) form a complex to translocate to nucleus, and bind to RARE (RA response element) to initiate the transcription of cadherin. The increased cadherin translocates to membrane and also recruits and stabilizes cytoplasmic β-catenin. This recruitment prevents cytoplasmic β-catenin from translocating to the nucleus, which interacts with transcription factors of the LEF/TCF family to induce changes in cell-cycle gene expression, such as cyclin D1. Besides, tau (Ser396) is hyperphosphorylated by GSK3β. Cell proliferation terminates and differentiation initiates. Conversely, overexpression of GSKIP inhibits GSK3β phosphorylation of β-catenin and tau (Ser396) in RA-mediated SH-SY5Y neuronal differentiation. GSKIP also diminishes cadherin expression, which reduces recruitment of cytoplasmic β-catenin, although how GSKIP disturbs the RA signaling to downregulate cadherin expression is still unclear. The elevation in free β-catenin translocates to the nucleus and binds to LEF/TCF, promoting changes in the transcriptional machinery that lead to activation of several target genes, such as cyclin D1. The increase in cyclin D1 promotes cell-cycle progression and prevents cell-cycle exiting. As a consequence, cell proliferation restarts and differentiation is blocked.

β-catenin decreases and this event is sufficient to inhibit neurite outgrowth by RA signaling. In addition, RA-mediated β-catenin overexpression is involved in the cadherin/catenin cell adhesion complex rather than carrying out transcriptional activity. Moreover, downregulation of N-cadherin also reduces cytoplasmic β-catenin recruitment (Fig. 5B). Altogether, our data suggest that GSKIP may be involved in the regulation of N-cadherin and recruitment of β-catenin, which is critical for neuron differentiation.

In summary, our results suggest the model depicted in Figure 7 for RA-mediated differentiation of SH-SY5Y cells, showing that GSKIP regulates the functional interplay of GSK3β/β-catenin, β-catenin/cyclin D1, and N-cadherin/β-catenin pool during RA signaling. In SH-SY5Y cells, RA treatment increases the expression of a cadherin that mediates strong cell–cell adhesion and translocates β-catenin to the membrane, thereby mediating the effects of RA on cell morphology and differentiation. The decrease of β-catenin nucleus translocation leads to reduced cyclin D1 expression. This event evokes cell-cycle arrest and promotes cells to differentiate. On the other hand, overexpression of GSKIP has two significant effects on RA-mediated neuron differentiation. One is to inhibit GSK3β activity, leading to decreased phosphorylation of tau (S396) and protecting β-catenin from proteasome degradation. The other is to downregulate N-cadherin expression resulting in reducing the recruitment of β-catenin. Both effects elevate the amount of β-catenin nucleus translocation and cyclin D1 accumulation. Finally, GSKIP promotes cell-cycle progression and terminates cell differentiation in the SH-SY5Y cell model. Altogether, our results show that GSKIP is an important inhibitor of GSK3β and its effect on β-catenin at a convergence point of both the RA and β-catenin/TCF signaling pathways in SH-SY5Y cells. It would be interesting to further investigate the specific contribution of GSKIP actions to neuronal development.

ACKNOWLEDGMENTS

This work was supported by NSC95-2314-B-037-050-MY1 (Taiwan, ROC) to S.L.H. and NSC 95-2745-B-037-003-URD, NSC-96-2320-B-037-004, and MOE-97-U19609-1, 1ab-14, MOE 98-U19609-6-3 and NHRI-EX98-9809SI to Y.R.H.

REFERENCES

Aberle H, Schwartz H, Kemler R. 1996. Cadherin–catenin complex: Protein interactions and their implications for cadherin function. J Cell Biochem 61:514–523.

Abraha A, Ghoshal N, Gamblin TC, Cryns V, Berry RW, Kuret J, Binder LI. 2000. C-terminal inhibition of tau assembly in vitro and in Alzheimer's disease. J Cell Sci 113:3737–3745.

Aubert J, Dunstan H, Chambers I, Smith A. 2002. Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. Nat Biotechnol 20:1240–1245.

Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W. 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382:638–642.

Benson DL, Tanaka H. 1998. N-cadherin redistribution during synaptogenesis in hippocampal neurons. J Neurosci 18:6892–6904.

Bixby JL, Zhang R. 1990. Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. J Cell Biol 110:1253–1260.

Canon E, Cosgaya JM, Scsucova S, Aranda A. 2004. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell 15:5583–5592.

Chenn A, Walsh CA. 2002. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science 297:365–369.

Cho JH, Johnson GV. 2004. Primed phosphorylation of tau at Thr231 by glycogen synthase kinase 3beta (GSK3beta) plays a critical role in regulating tau's ability to bind and stabilize microtubules. J Neurochem 88:349–358.

Chou HY, Howng SL, Cheng TS, Hsiao YL, Lieu AS, Loh JK, Hwang SL, Lin CC, Hsu CM, Wang CH, Lee CI, Lu PJ, Chou CK, Huang CY, Hong YR. 2006. GSKIP is homologous to the Axin GSK3β interaction domain and functions as a negative regulator of GSK3β. Biochemistry 45:11379–11389.

Cohen P, Frame S. 2001. The renaissance of GSK3. Nat Rev Mol Cell Biol 2:769–776.

Diehl JA, Cheng M, Roussel MF, Sherr CJ. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 12:3499–3511.

Gärtner A, Huang X, Hall A. 2006. Neuronal polarity is regulated by glycogen synthase kinase-3 (GSK-3beta) independently of Akt/PKB serine phosphorylation. J Cell Sci 119:3927–3934.

Goold RG, Gordon-Weeks PR. 2005. The MAP kinase pathway is upstream of the activation of GSK3beta that enables it to phosphorylate MAP1B and contributes to the stimulation of axon growth. Mol Cell Neurosci 28:524–534.

Gottardi CJ, Wong E, Gumbiner BM. 2001. E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. J Cell Biol 153:1049–1060.

Grimes CA, Jope RS. 2001. The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65:391–426.

Haque N, Gong CX, Sengupta A, Iqbal K, Grundke-Iqbal I. 2004. Regulation of microtubule-associated proteins, protein kinases and protein phosphatases during differentiation of SY5Y cells. Brain Res Mol Brain Res 129:163–170.

Hsu HT, Liu PC, Ku SY, Jung KC, Hong YR, Kao C, Wang C. 2006. Beta-catenin control of T-cell transcription factor 4 (Tcf4) importation from the cytoplasm to the nucleus contributes to Tcf4-mediated transcription in 293 cells. Biochem Biophys Res Commun 343:893–898.

Jiang H, Guo W, Liang X, Rao Y. 2005. Both the establishment and the maintenance of neuronal polarity require active mechanisms: Critical roles of GSK-3beta and its upstream regulators. Cell 120:123–135.

Johnson GV, Stoothoff WH. 2004. Tau phosphorylation in neuronal cell function and dysfunction. J Cell Sci 117:5721–5729.

Jope RS, Johnson GV. 2004. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 29:95–102.

Kim WY, Zhou FQ, Zhou J, Yokota Y, Wang YM, Yoshimura T, Kaibuchi K, Woodgett JR, Anton ES, Snider WD. 2006. Essential roles for GSK-3s and GSK-3-primed substrates in neurotrophin-induced and hippocampal axon growth. Neuron 52:981–996.

Kishida S, Yamamoto H, Kikuchi A. 2004. Wnt-3a and Dvl induce neurite retraction by activating Rho-associated kinase. Mol Cell Biol 24:4487–4501.

Li L, Yuan H, Weaver CD, Mao J, Farr GH III, Sussman DJ, Jonkers J, Kimelman D, Wu D. 1999. Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. EMBO J 18: 4233–4240.

Liang MH, Chuang DM. 2007. Regulation and function of glycogen synthase kinase-3 isoforms in neuronal survival. J Biol Chem 282:3904–3917.

Logan CY, Nusse R. 2004. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20:781–810.

Lopez-Carballo G, Moreno L, Masia S, Perez P, Barettino D. 2002. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid

is required for neural differentiation of SH-SY5Y human neuroblastoma cells. J Biol Chem 28:25297–25304.

Lucas JJ, Hernandez F, Gomez-Ramos P, Moran MA, Hen R, Avila J. 2001. Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. EMBO J 20:27–39.

Munoz-Montano JR, Lim F, Moreno FJ, Avila J, Diaz-Nido J. 1999. Glycogen synthase kinase-3 modulates neurite outgrowth in cultured neurons: Possible implications for neurite pathology in Alzheimer's disease. J Alzheimers Dis 1:361–378.

Noles SR, Chenn A. 2007. Cadherin inhibition of beta-catenin signaling regulates the proliferation and differentiation of neural precursor cells. Mol Cell Neurosci 35:549–558.

Sadot E, Simcha I, Shtutman M, Ben Z, Geiger B. 1998. Inhibition of β-catenin-mediated transactivation by cadherin derivatives. Proc Natl Acad Sci USA 95:15339–15344.

Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med 10:55–63.

Shah S, Pishvaian MJ, Easwaran V, Brown PH, Byers SW. 2002. The role of cadherin, beta-catenin, and AP-1 in retinoid-regulated carcinoma cell differentiation and proliferation. J Biol Chem 277:25313–25322.

Shi SH, Cheng T, Jan LY, Jan YN. 2004. APC and GSK-3β are involved in mPar3 targeting to the nascent axon and establishment of neuronal polarity. Curr Biol 14:2025–2032.

Shimizu H, Julius MA, Giarré M, Zheng Z, Brown AM, Kitajewski J. 1997. Transformation by Wnt family proteins correlates with regulation of beta-catenin. Cell Growth Differ 8:1349–1358.

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 96:5522–5527.

Spinella MJ, Freemantle SJ, Sekula D, Chang JH, Christie AJ, Dmitrovsky E. 1999. Retinoic acid promotes ubiquitination and proteolysis of cyclin D1 during induced tumor cell differentiation. J Biol Chem 274:22013–22018.

Stockinger A, Eger A, Wolf J, Beug H, Foisner R. 2001. E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. J Cell Biol 154:1185–1196.

Sumrejkanchanakij P, Eto K, Ikeda MA. 2006. Cytoplasmic sequestration of cyclin D1 associated with cell cycle withdrawal of neuroblastoma cells. Biochem Biophys Res Commun 340:302–308.

Takehashi M, Yasutake K, Tomizawa K. 1999. Lithium inhibits neurite growth and tau protein kinase I/glycogen synthase kinase-3beta-dependent phosphorylation of juvenile tau in cultured hippocampal neurons. J Neurochem 73:2073–2083.

Takeichi M. 1995. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 7:619–627.

Yap AS, Brieher WM, Pruschy M, Gumbiner BM. 1997. Lateral clustering of the adhesive ectodomain: A fundamental determinant of cadherin function. Curr Biol 1:308–315.

Yuan J, Zhang J, Wong BW, Si X, Wong J, Yang D, Luo H. 2005. Inhibition of glycogen synthase kinase 3beta suppresses coxsackievirus-induced cytopathic effect and apoptosis via stabilization of beta-catenin. Cell Death Differ 12:1097–1106.

Zechner D, Fujita Y, Hülsken J, Müller T, Walther I, Taketo MM, Crenshaw EB, Birchmeier W, Birchmeier C. 2003. Beta-catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. Dev Biol 15:406–418.

Zhou FQ, Snider WD. 2005. Cell biology. GSK-3beta and microtubule assembly in axons. Science 308:211–214.

Zhou FQ, Zhou J, Dedhar S, Wu YH, Snider WD. 2004. NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. Neuron 42:897–912.