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SGTb regulates a surface localization of a guidance receptor BOC to promote neurite outgrowth

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ABSTRACT

Neuritogenesis is a critical event for neuronal differentiation and neuronal circuitry formation during neuronal development and regeneration. Our previous study revealed a critical role of a guidance receptor BOC in a neuronal differentiation and neurite outgrowth. However, regulatory mechanisms for BOC signaling pathway remain largely unexplored. In the current study, we have identified Small glutamine-rich tetratricopeptide repeat (TPR)-containing b (SGTb) as a BOC interacting protein through yeast two-hybrid screening. Like BOC, SGTb is highly expressed in brain and P19 embryonal carcinoma (EC) cells differentiated into neuronal cells. BOC and SGTb proteins co-precipitate in mouse brain and differentiated P19 EC cells. Furthermore, BOC and SGTb co-localize in neurites and especially are concentrated at the tip of neurites in various neuronal cells. SGTb depletion attenuates neuronal differentiation of P19 cells through reduction of the surface level of BOC. Additionally, SGTb depletion causes BOC localization at neurite tip, coinciding with decreased p-JNK levels critical for actin cytoskeleton remodeling. The overexpression of SGTb or BOC restores JNK activation in BOC or SGTb-depleted cells, respectively. Finally, SGTb elevates the level of surface-resident BOC in BOC-depleted cells, restoring JNK activation. Taken together, our data suggest that SGTb interacts with BOC and regulates its surface level and consequent JNK activation, thereby promoting neuronal differentiation and neurite outgrowth.

1. Introduction

Small glutamine-rich tetratricopeptide repeat (TPR)-containing b (SGTb) belongs to the SGT (small glutamine-rich TPR-containing protein) family and is initially found to bind to the non-structural protein of parvovirus H [1]. SGTb is a brain-specific isoform of broadly expressed SGTa. The TPR domain of the SGT family interacts with a variety of molecules including β-amyloid peptides [2], cysteine-string protein [3], Hsc70 [3,4], Hsp90 [4] and ubiquitin-dependent endocytosis motif. On the other hand, the C-terminal fragment (glutamine-rich domain) of SGT plays a role in the association of SGT with in vitro translated rat type I glucose transporter (GLUT1) [5]. SGT proteins have been implicated in diverse biological processes such as neuronal synaptic transmission [6], cell cycle [7], protein folding [5] and apoptosis [8]. SGTb shares similar characteristics with SGTa, which negatively regulates chaperone activity of Hsp70/Hsc70 as a co-chaperone [9] and plays a role in neuronal apoptosis triggered by oxidative stress [10]. However, the role of SGTb in neurogenesis or neuronal function is currently unknown.

Neurite outgrowth is a critical step for neurogenesis and neuronal circuitry formation during neuronal development and regeneration. Axon guidance molecules, such as netrin, slit or sonic hedgehog (Shh)

Abbreviations: BOC, Brother of CDO; DCX, doublecortin; EC, embryonal carcinoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT1, type I glucose transporter; GST, glutathione S transferase; ICD, intracellular domain; ITS, insulin-transferrin-selenite; JNK, Jun N-terminal kinase; MAP2, myelin-associated protein 2; PLL, poly-L-lysine; RA, retinoid acid; SGT, small glutamine-rich tetratricopeptide repeat-containing protein; SGTb, Small glutamine-rich tetratricopeptide repeat-containing b; Shh, sonic hedgehog; TRP, tetratricopeptide repeat

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play important roles in axonal growth and navigation of growth cones to innervate targets, which involve remodeling and reorganization of the cytoskeleton [11]. Previous studies have shown that an immunoglobulin superfamily member, BOC functions as Shh co-receptor in canonical and noncanonical Shh signaling in neuronal development and axon outgrowth/guidance [12]. BOC plays a critical role for proper guidance of commissural neurons guided by Shh in spinal cord development [13]. The depletion of BOC in the forebrain of mice and zebrafish leads to axon guidance defects [13,14]. Furthermore, BOC deficiency causes defects in the formation of synapses to generate the cortical microcircuitry [15]. Previously, we have shown that BOC promotes neuronal differentiation and neurite outgrowth in P19 embryonal carcinoma (EC) cells or C17.2 neuronal progenitor cells through JNK activation [12]. In addition, Shh treatment also enhances JNK activation, which is diminished by BOC depletion correlating with impaired neurite outgrowth. However, regulatory mechanisms for BOC signaling pathway in neuritogenesis remain largely unexplored.

In the current study, we have identified SGTb as a BOC interacting protein from a yeast two-hybrid screening. SGTb and BOC are expressed in brain with the highest level and they were increased during neuronal differentiation. SGTb and BOC proteins can be co-precipitated from brain homogenates and lysates of differentiated neuronal cells. The depletion of SGTb results in decreased neuronal differentiation of P19 EC cells and C17.2 neuronal progenitor cells. SGTb depletion decreases JNK activity while overexpression of BOC or SGTb augments JNK activation. Furthermore, SGTb overexpression restores JNK activation and neuronal differentiation of BOC-depleted cells. SGTb depletion leads to reduction of surface BOC level without change in the total BOC expression level. Taken together, these data suggest that SGTb promotes neuronal differentiation and neurite outgrowth through modulation of BOC's surface localization and consequent JNK activation.

2. Materials and methods

2.1. Yeast two-hybrid screening

Yeast two-hybrid screening was performed by using MATCHMAKER GAL4 Two-Hybrid System following manufacturer's instructions (Clontech, Mountain View, CA, USA). Briefly, intracellular region of human BOC was sub-cloned into pLexA containing DNA-binding domain (pLexA-iBOC). Yeast strain EGY48 (Clontech) cells were transformed with p8op-lacZ to create EGY48[p8op-lacZ] and then followed by the co-transformation with pLexA-iBOC and cDNA library plasmids (pB42AD), which were constructed with mRNAs from embryonic 13.5 mouse brain. To screen the interacting proteins, the transformed cells were selected on synthetic dropout (SD) plate lacking uracil, histidine, tryptophan, leucine in the presence of X-gal.

2.2. Expression vector constructions

The expression vectors for pSuper/shBOC, pcDNA3.1/Flag-BOC and GST-tagged human BOC-intracellular region were previously described [12,16,17]. For yeast two-hybrid screening study, intracellular region of human BOC was amplified by PCR and subcloned into pLexA. To generate GFP-BOC, full-length of human BOC was subcloned into pEGFP-N1. To obtain the full length of SGTb, SGTb-coding region was amplified by PCR using cDNA generated from 2-month-old C57BL/6J mouse brain. The PCR products were then ligated into the vector pcDNA 3.1-Myc, pDsRed-N1, pGEX-2 T, pVFT2S or pB42AD. For the domain interaction analysis, full length and mutant forms of SGTb were amplified by PCR and subcloned into pEBG-2 T. The primer sequences used in this study are listed in Table 1.

2.3. Cell cultures and immunocytochemistry

P19 EC cells, C17.2 neuronal progenitor cells and 293 T cells were cultured as previously described [12,18]. Briefly, P19 EC cells were cultured in α-MEM (alpha-minimum essential medium, Thermo Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Thermo Scientific). To induce differentiation, 3.5×10^5 EC cells were seeded and incubated in retinoic acid (RA) medium (α-MEM, 10% FBS and 0.5 μM all-trans-RA (Sigma-Aldrich, St. Louis, MO, USA)) in 100 mm bacterial grade dishes for 2 days. After induction with RA, the aggregated cells were seeded onto poly-L-lysine (PLL)-coated culture dishes containing insulin-transferrin-selenite (ITS) medium (DMEM/F12, 1× ITS (Sigma-Aldrich)) and incubated for 2 days. P19 EC cells were transiently transfected by using Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) and incubated for 36 h as previously described [17], followed by induction of differentiation for indicated times. For the induction of neuronal differentiation with C17.2, the proliferation medium, DMEM containing 10% FBS was switched to DMEM containing 2% horse serum (HS, Thermo Scientific) at 80–90% of cell confluence. 293 T cells were cultured in DMEM containing 10% FBS.

Immunostaining was carried out as previously described [12,18,19]. Briefly, fixed cells were permeabilized with 0.5% Triton X-100 at room temperature for 15 min, and incubated with primary antibodies such as anti-BOC, anti-SGTb or anti-β-Tubulin III antibodies at 4 °C for overnight. After incubation with secondary antibodies, images were obtained by confocal microscopy by using Zeiss LSM-510, LSM-710 Meta confocal microscopy at Sungkyunkwan University, school of medicine-microscopy Shared Resource Facility. The length of neurites was measured by using NeuronJ program [20]. Secondary fluorescent antibodies used in this study were Alexa Fluor 488 donkey anti-mouse antibody, Alexa Fluor 488 donkey anti-goat antibody and Alexa Fluor 594 donkey anti-rabbit antibody (Invitrogen). Fluorescence microscopy was performed with Nikon ECLIPS TE-2000 U and NIS-Elements F software (Nikon, Tokyo, Japan). The information of primary antibody used in this study are listed in Table 2.

2.4. siRNA transfection

Control siRNA and SGTb siRNA were purchased from Sanghai GenePharma Company (Shanghai, China). The sequences of control and

Table 1
The primers used in this study.

Name	Purpose	Sequence
BOC	RT-PCR	F: TGCTCTGGGTGTCTTCATCA
		R: ATGGCATGATCAGGTAGTTG
	Cloning pEGFP-N1/BOC	F: AATGCTCGAGATGCTGCGTGGGACGATGACGGC
		R: CATTGAATTCGAATTGTGAGAGGTGGTGTTTCAA
	Cloning pLexA-BOC	F: GCGGATCCATCTGATTGTCGGGGTT
		R: TAGCGGCCGCCTAAATTGTGGGAGG
SGTb	RT-PCR	F: TCATCTGTGAAGCCCCTAGTTTA
		R: ACGGAGTTTGTGAACATTTCTGT
	Cloning pEBG-2 T/SGTb	F:AATGACTAGTATGTCATCTGTGAAGCCCCTA
		R:CATTATCGATCTACTCGGGCACTGAGTTTGAAAG
	mapping domain	F: AATGACTAGTGATGTGGGAAAGGCGGACCAG
		R: CATTATCGATCTACTTATACGAATCATTCTCAGG
		F: AATGACTAGTTCAAATCTGAAAATAGCAGAA
		R: CATTATCGATTCAGGAGTGTTCGTCTGCACTGCT
	Cloning pcDNA-myc/SGTb	F:CTCGTAAGCTTCTCGTG GAGATATTTAAGCGTC
		R: TGGTCCTCGAGGGAGTGTTCGTCTGCACTGCTGC
	Cloning pDsRed-N1/SGTb	F: AATGCTCGAGATGTCATCTGTGAAGCCCCTAG
		R: CATTGAATTCGGGAGTGTTCGTCTGCACTGCTGC
β-Tubulin III	RT-PCR	F: CAACGTCAAGGTAGCCGTGT
		R: TCCGATTCCTCGTCATCATC
NeuroD	RT-PCR	F: TGACCTTTCCCATGCTGAAT
		R: AAGTGCTAAGGCAACGCAAT
GAPDH	RT-PCR	F: ACCACAGTCCATGCCATCAC
		R: TCCACCACCCTGTTGCTGTA

Table 2
The antibodies used in this study.

Antibodies	Source	Cat#	Host
SGTb	Abcam	ab202419	Rabbit
BOC	R&D systems	AF 2385	Goat
JNK	Santa cruz biotechnology	SC-571	Rabbit
phospho-JNK	Santa cruz biotechnology	SC-6254	Mouse
Doublecortin	Abcam	ab77450	Rabbit
β-Tubulin III	Sigma-aldrich	T8660	Mouse
NeuroD	Santa cruz biotechnology	SC-1086	Goat
Myelin-associated protein 2	Cell signaling technology	4542S	Rabbit
Neurofilament-M	Cell signaling technology	SC-20013	Mouse
N-cadherin	Abcam	Ab18203	Rabbit
β-Tubulin	Sigma-Aldrich	T5293	Mouse
N-Cadherin	Abcam	Ab18203	Rabbit
GAPDH	Abfrontier	LF-PA0018	Rabbit
HSP90	Santa cruz biotechnology	SC-7947	Rabbit
Flag	Sigma-aldrich	F1804	mouse
Myc	Santa cruz biotechnology	SC-40	Mouse
GST	Santa cruz biotechnology	SC-469	Rabbit

SGTb siRNA used in this study are as following: Control, 5'-UUC UCC GAA CGU GUC ACG UTT AGC UGA CAC GUU CGG AGA ATT-3′; siSgtb#1, GCU GCU CAA AGC AAA UUA ATT UUA AUU UGC UUU GAG CAG CTT; siSgtb#2 GCA GUA UCU CGU UAC AUU UTT AAA UGU AAC GAG AUA CUG CTT. To deplete SGTb, 50 nM of indicated siRNA and 10 μl of transfection reagent (Lipofectamin 2000) were used.

2.5. Protein analysis and surface biotinylation

Western blot analysis was carried out as previously described [21]. Briefly, cells were lysed in extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.2 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10 mM NaF and 1 mM Na_3VO_4) containing complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and then sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed followed by immunoblotting.

For co-immunoprecipitation assay (co-IP), 293 T cells were transfected by using Lipofectamin 2000 and 36 h later, cells were lysed followed by co-immunoprecipitation and immunoblotting. Brains from three male C57BL/6J mice at 2-months of age were harvested and extracted were prepared by cryo-pulverization with liquid nitrogen and homogenized by FastPrep^R-24 (MP Biomedicals, Santa Ana, CA, USA) with glass beads in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.5% Triton X-100) containing complete protease inhibitor cocktail, followed by centrifugation at 13,000 rpm for 30 min at 4 °C. Supernatant was collected and quantification with BSA kit. One mg of total cell lysates or whole mouse brain extracts were immunoprecipitated with one μg primary antibody overnight at 4 °C. For glutathione S transferase (GST)-pull down assay, lysates were incubated with 20 μl of the Glutathione Sepharose 4B agarose beads (GE Healthcare, little Chalfont, UK) overnight at 4 °C. To remove non-specific binding proteins, beads were washed with 500 μl of ice-cold lysis buffer for 5 min 3 times.

Cell surface biotinylation was performed as previously described [22]. Briefly, P19 EC cells were induced to differentiate for indicated time by switching to DM and incubating in phosphate-buffered saline (PBS) containing Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) with the final concentration of 1 mg/ml for 30 min on ice. After quenching the biotinylation, cells were lysed in extraction buffer containing protease inhibitor. Biotinylated proteins were recovered on streptavidin-agarose beads (Thermo Fisher Scientific), followed by SDS-PAGE.

2.6. RNA extraction and RT-PCR

Total RNA extraction from cells and mouse tissues were carried out as described previously [23]. Briefly, tissues from 2 male C57/BL/6J mice at 4 weeks of age were harvested and homogenized by FastPrepR-24 (MP Biomedicals, Santa Ana, CA, USA) followed by total RNA extraction with an easy-spin Total RNA Extract kit (iNtRON, Seongnam, Korea) according to the manufacturer's instructions. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the expression level of each gene. The primer sequences are shown in Table 1.

2.7. Statistical analysis

Statistical differences between groups were analyzed by Mann-Whitney *U* test using GraphPad Prism software (version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Statistically significant differences were determined at levels of $*P < .05$, $**P < .01$ and $***P < .001$.

3. Results

3.1. TPR region of SGTb interacted with the BOC's intracellular region

To identify the interacting protein of BOC, we performed the yeast two-hybrid screening using BOC's intracellular domain as a bait and found SGTb as an interacting protein with BOC's intracellular domain (ICD) (Fig. 1a). To verify such interaction, 293 T cells were transfected with Flag-tagged BOC (Flag-BOC) or Myc-tagged SGTb (Myc-SGTb) expression vectors and subjected to immunoprecipitation with anti-Myc or anti-Flag antibodies and immunoblotting. SGTb co-precipitated with BOC proteins (Fig. 1b, c). To define the interacting domain of BOC, GFP-SGTb-containing vector and GST-fusion vectors containing various regions of BOC's intracellular domain spanning amino acid (aa) 1 to aa237 (ICD/1–237) were co-transfected and subjected to GST-pulldown assay. The full-length ICD/1–237 and ICD/1–160 exhibited interaction with SGTb and ICD/1–160 interacted strongest (Fig. 1d). However, ICD/1–85, ICD86–161, ICD161–237 and ICD86–237 failed to interact with SGTb. Thus, it appears that ICD1–160 of BOC is primarily responsible for the interaction with SGTb. Next, we determined the domain of SGTb protein responsible for the interaction with BOC. 293 T cells were co-transfected with Flag-BOC and GST-fusion vectors containing various region of SGTb (Fig. 1e) and subjected to GST-pulldown assay. The full-length and TPR region spanning from aa81 to aa190 displayed the strongest interaction with BOC (Fig. 1e). Other regions such as aa1-aa80 and aa1-aa190 exhibited a moderate interaction with BOC, while aa191-aa304 and aa81-aa304 showed a weak interaction with BOC. These results suggest that BOC and SGTb interact via the TPR region of SGTb and the N-terminal intracellular region of BOC.

3.2. SGTb and BOC were induced in differentiating neurons and co-localized in neurites

Our previous study has shown that BOC plays a critical role in axon guidance as a Shh receptor and neurite outgrowth in neuronal differentiation of P19 EC cells and C17.2 neuronal progenitor cells [12]. To gain an insight into a role of SGTb in relation to BOC, the expression of SGTb and BOC was examined in various tissues. The quantitative RT-PCR analysis showed that the highest expression of SGTb and BOC was detected in brain while BOC was also expressed in other tissues such as kidney, testis, heart, lung, skeletal muscle and thymus unlike the exclusive expression of SGTb in brain (Fig. 2a). To address whether SGTb plays a role in neuronal differentiation, the expression of SGTb and BOC was examined in P19 cells. P19 cells were induced to differentiate into neurons by the treatment with RA for 2 days with a subsequent culturing of cells in ITS medium for additional 2 days (ITS2). The mRNA of SGTb and BOC were induced upon neuronal induction at one day or two days of RA treatment (RA1 or RA2), respectively. The strongest expression of these genes was observed at ITS2 (Fig. 2b). Two neuronal-specific genes, NeuroD and β-Tubulin III, were induced in cells at RA2

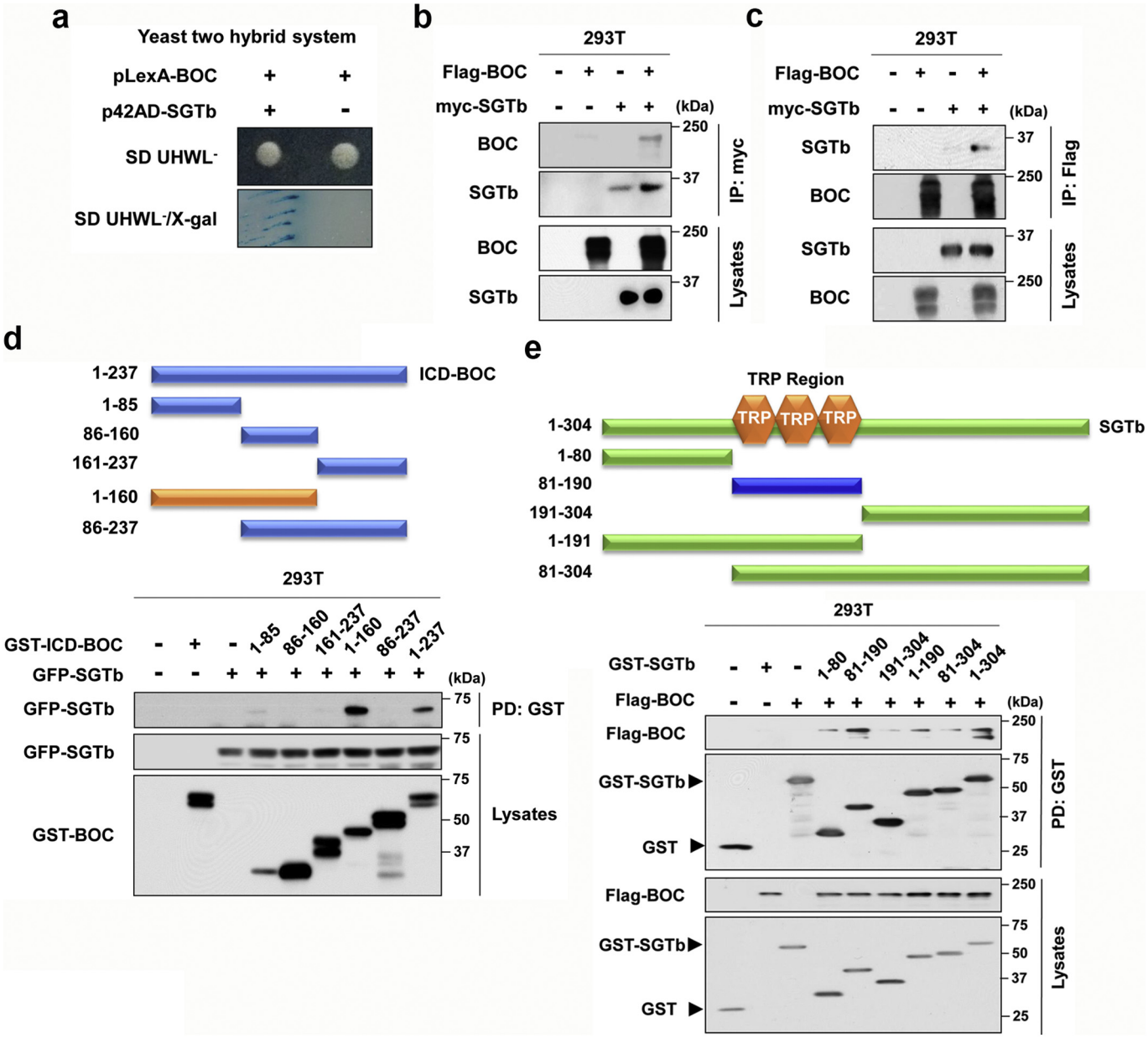

Fig. 1. TPR domain of SGTb mediated the interaction with the BOC's intracellular region. (a) SGTb was identified from Yeast two-hybrid screening using the intracellular domain of BOC as a bait. Yeasts were transformed with the indicated vectors for the intracellular regions of BOC fused with Gal4-DNA binding domain (pLexA-BOC) and SGTb fused with Gal4 activation domain (p42AD-SGTb). The transformants were cultured on selected media lacking Uracil, Histidine, Tryptophan and Leucine (SD UHWL$^-$), and the interaction between BOC and SGTb was indicated by β-galactosidase activity (blue labeling). (b and c) 293 T lysates, which were transfected with pcDNA-Flag-BOC, −myc-SGTb or control pcDNA expression vectors for 36 h, were immunoprecipitated with anti-myc or anti-Flag antibodies, followed by western blot analysis with anti-Flag or anti-myc antibodies. (d) For GST-pulldown analysis, 293 T cells were transfected with the indicated GST-fused intracellular domain of BOC and GFP-tagged full length of SGTb. PD, pulldown. (e) GST-pulldown analysis was performed with 293 T cells, which were transfected with various GST-fused SGTb constructs and Flag-BOC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and ITS1. Consistently, the protein level of BOC was strongly induced at ITS1 when three neuronal differentiation markers, DCX, β-Tubulin III and MAP2, and SGTb levels were also increased (Fig. 2c). These data suggest that BOC and SGTb are coexpressed in differentiating neurons.

Next, we asked whether BOC and SGTb proteins could interact endogenously. To do so, mouse brain extracts were subjected to immunoprecipitation with the control IgG or anti-BOC antibody and immunoblotting. SGTb was co-precipitated with BOC (Fig. 2d). In addition, lysates of P19 cells from differentiation time course were subjected to immunoprecipitation with anti-SGTb antibodies (Fig. 2e). Endogenous BOC proteins were co-immunoprecipitated with SGTb in P19 cells at ITS1, suggesting for a role of SGTb in neuronal differentiation. To further define the interaction between SGTb and BOC, differentiated P19 cells at ITS1 and ITS2 were subjected to immunostaining for BOC (green), SGTb (red) and β-Tubulin III (blue) (Fig. 2f). BOC and SGTb were expressed in developing neurites as shown in the inset of each panel. The co-localization of these proteins was more robust in neurites of cells at ITS2, especially at the tip of neurites. Similar pattern of co-localization of BOC and SGTb in neurites were observed in differentiated C17.2 cells, a pheochromocytoma PC12 cells, E13.5 embryonic cells and whole mouse brain (Supplementary Fig. S1). Taken together, BOC and SGTb are coexpressed in

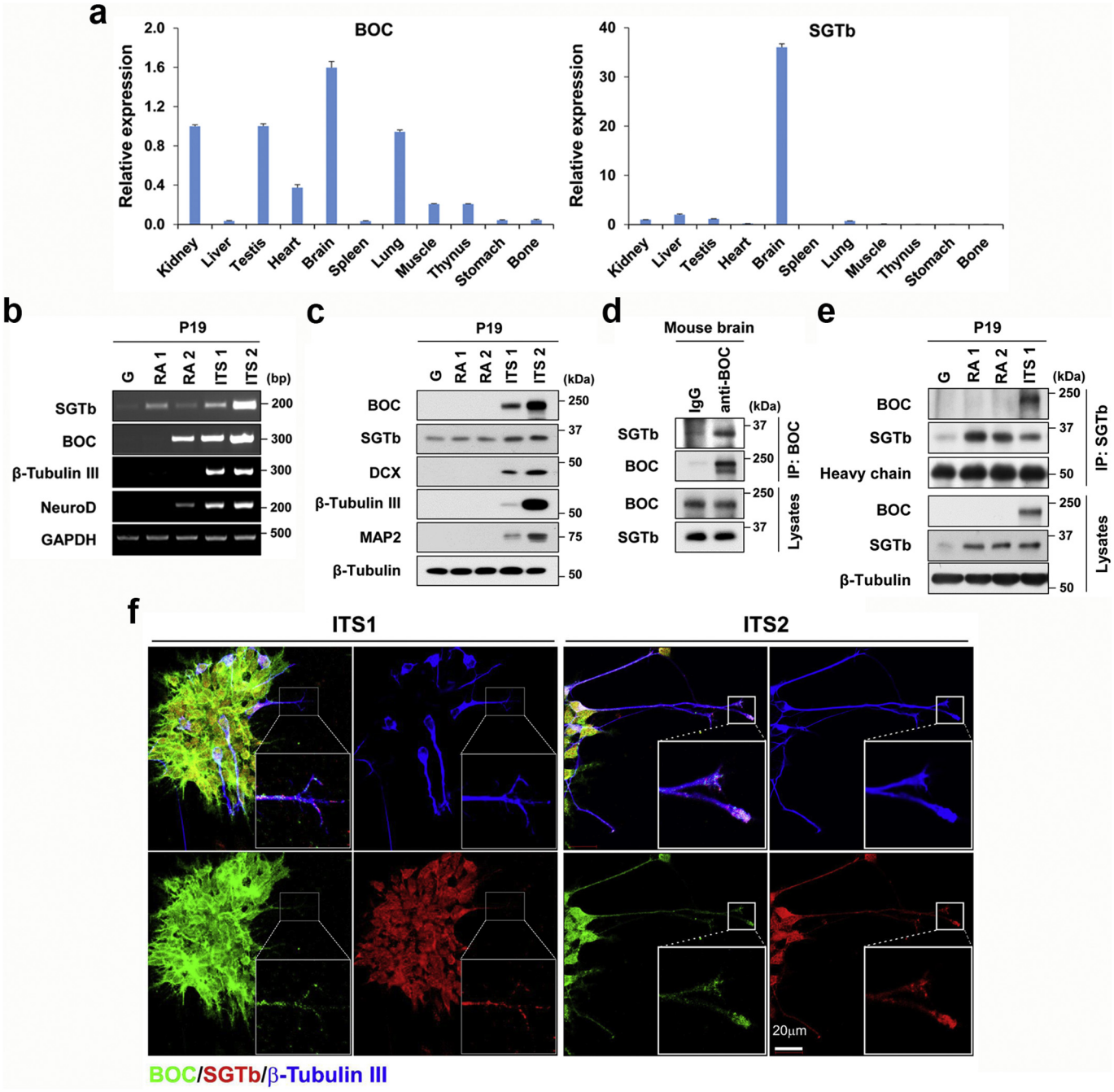

Fig. 2. SGTb and BOC were induced in differentiating neurons and co-localized in neurites. (a) qRT-PCR analysis was performed to determine the expression of BOC and SGTb using total RNAs isolated from tissues of two 4-week-old male mice. Values are means of triplicate. (b) Total RNAs isolated from P19 cells cultured in growth or differentiation medium were subjected to RT-PCR for the expression of SGTb, BOC, β-Tubulin III, NeuroD, and GAPDH as an internal control. (c) Lysates of P19 cells cultured in growth or differentiation medium were subjected to Western blot analysis with indicated antibodies. (d) Whole mouse brain lysates from a 2-month-old male mouse were immunoprecipitated with anti-BOC antibody and then blotted with indicated antibodies. (e) Lysates from P19 cells induced to differentiate were immunoprecipitated with anti-SGTb antibody followed by immunoblotting with the indicated antibodies. (f) Immunofluorescence staining for BOC (green), SGTb (red) and β-tubulin III (blue) in P19 cells at ITS1 and ITS2. The inset displays the tip of neurite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

differentiating neuronal cells and colocalized in the growing neurites.

3.3. SGTb was required for neuronal differentiation

To examine the role of SGTb in neuronal differentiation, P19 cells were transfected with siControl or two different siRNAs of SGTb (siSGTb) and induced to differentiate. Two different siSGTb constructs resulted in a significant knockdown of SGTb, compared to scrambled siRNA (Fig. 3a). At ITS2, BOC protein level did not alter greatly, while the neuronal differentiation markers, such as β-Tubulin III, NeuroD and MAP2 were reduced in SGTb-depleted cells, compared to the siControl cells. The quantification of β-Tubulin III levels of three different experiments demonstrated that SGTb depletion leads to reduced expression of β-Tubulin III (Fig. 3b). In addition, cells at ITS2 were immunostained for β-Tubulin III expression (Fig. 3c, d). The siControl cells showed strong β-Tubulin III stained neurites, while SGTb-depleted cells

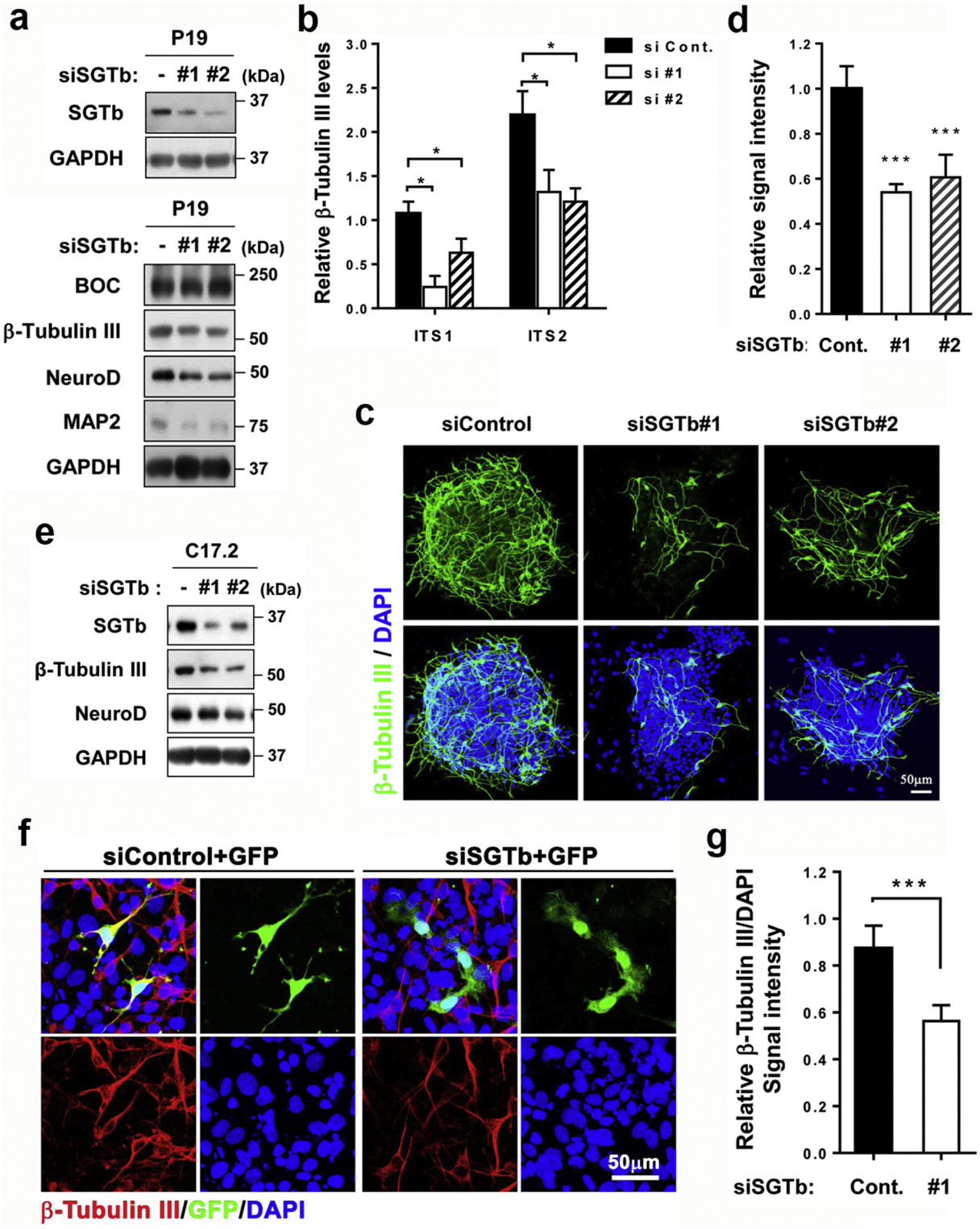

Fig. 3. SGTb depletion caused impaired neuronal differentiation. (a) Lysates from P19 cells, which were transfected with siControl or siSGTb for 36 h and differentiated until ITS2 were immunoblotted with indicated antibodies. For SGTb depletion, two different types of siRNA were utilized. (b) Quantification for expression of β-tubulin III from three individual experiments shown in panel a. Band intensity was quantified by using the ImageJ software. $N = 3$, $^{*}p < .05$. (c) Immunofluorescence staining was performed with siControl- or siSGTb-transfected P19 cells at ITS2 for β-tubulin III (green). Nuclei are visualized by DAPI staining. (d) Quantification of relative β-Tubulin III signal intensities per clusters shown in panel c. $N > 10$. $^{***}p < .001$. (e) C17.2 cells, which were transfected with siControl or siSGTb for 36 h and subsequently differentiated for 3 days (D3), were subjected to immunoblot analysis with anti-SGTb, anti- β-Tubulin III, and anti-NeuroD antibodies. Anti-GAPDH antibody was used for loading control. (f) Immunostaining with anti-β-Tubulin III antibodies in C17.2 cells transfected with siControl or siSGTb plus GFP-expressing plasmid at D3. DAPI staining was performed to visualize nuclei. (g) Quantification of relative β-Tubulin III signal intensity shown in panel f. Values are represented as percentile. $N = 7$. $^{***}p < .001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

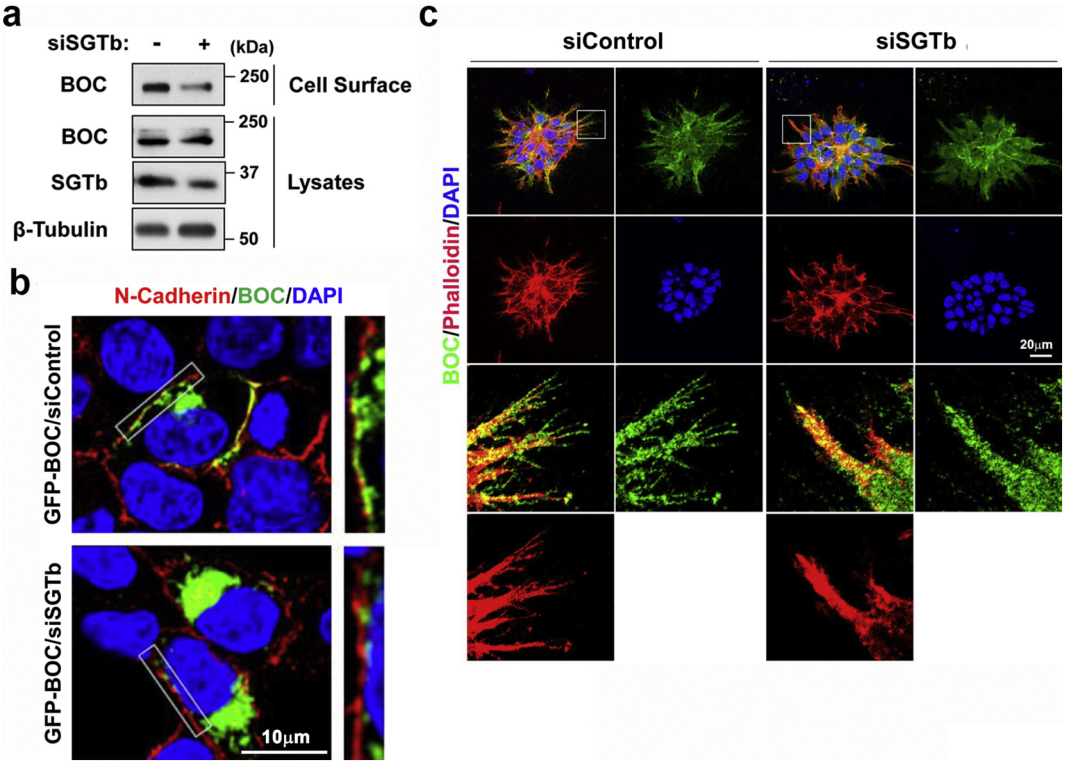

Fig. 4. SGTb depletion resulted in decreased surface resident BOC levels and its localization in the tip of neurite. (a) P19 cells transfected with siControl or siSGTb for 36 h and differentiated for 1 day in RA medium were subjected to the surface biotin labeling, followed by pulldown with streptavidin and immunoblotting with anti-BOC antibody. (b) 293 T cells co-transfected with GFP-BOC plus siControl or siSGTb for 36 h were subjected to immunofluorescence staining with anti-N-Cadherin antibody, and DAPI staining was performed to visualize nuclei. (c) Immunostaining for BOC (green) and Phalloidin (red) in P19 cells transfected with siControl or siSGTb for 36 h and differentiated in RA medium for 2 days. Nuclei were visualized by DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

showed much less staining as shown in the relative signal intensity compared to control (β-Tubulin III signal intensity normalized to the DAPI signal). Similarly, SGTb-depleted C17.2 cells also exhibited reduced expression of β-Tubulin III (Fig. 3e). Furthermore, C17.2 cells were co-transfected with siControl or siSGTb, and GFP expression vector to label the transfected cells and induced to differentiate for 3 days. SGTb depleted cells labelled with GFP were devoid of β-Tubulin III staining, compared to the siControl cells (Fig. 3f, g). These data suggest that SGTb is required for neuronal differentiation.

3.4. SGTb depletion caused a decrease in the surface level of BOC and its localization in neurite tips

To gain insight in the interaction between SGTb and BOC, P19 cells were transfected with siControl or siSGTb and induced to differentiate, followed by surface biotinylation (Fig. 4a). SGTb-depleted cells had decreased levels of BOC protein at the cell surface, while total BOC levels in lysates did not alter. To further examine a role of SGTb in the cell surface localization of BOC, P19/siControl or P19/siSGTb cells were transfected with a BOC-GFP vector and subjected to immunostaining with an N-Cadherin antibody to label the membrane, followed by confocal microscopy. BOC-GFP proteins were found at the cell membrane at the cell-to-cell contact site and intracellular compartments in both cell types. The signals of BOC-GFP and N-Cadherin were colocalized at the membrane in siControl cells, whereas SGTb-depleted cells had diminished signals for their colocalization (Fig. 4b). To further localize the expression of BOC, siControl and SGTb-depleted P19 cells were immunostained for BOC and labelled for actin with phalloidin (Fig. 4c). BOC proteins were localized in the growing neurites as well as at the tip of neurites that were devoid of phalloidin staining. Similar to siControl cells, SGTb-depleted cells also showed BOC staining in neurites however the localization of BOC at the tip of neurites was almost entirely abolished. Thus, SGTb is critical for the surface localization of BOC.

3.5. SGTb regulated neuronal differentiation through JNK activation

Previously, we have shown that BOC promotes neuronal differentiation and neurite outgrowth through JNK activation [12]. To examine the regulatory mechanism of SGTb-mediated neuronal differentiation, siControl and siSGTb-transfected P19 and C17.2 cells were induced to differentiate, followed by immunoblotting analysis for JNK activation by using antibodies against an active, phosphorylated form of JNK (p-JNK). SGTb-depleted P19 cells showed reduced levels of p-JNK, while the total JNK level did not alter, compared to the control cells (Fig. 5a, b). Consistently, the depletion of SGTb in C17.2 showed decreased p-JNK levels (Supplementary Fig. S2). To address this further, P19 cells were transfected with BOC or SGTb and induced to differentiate. As previously shown in P19 and C17.2 cells [12], BOC overexpression greatly enhanced the expression of neuronal markers, β-Tubulin III in P19 cells at ITS1 (Fig. 5c). Furthermore, the level of p-JNK was elevated, likely contributing to promotion of neurite outgrowth. Similar to BOC overexpression, SGTb overexpression also elevated the expression of β-Tubulin III and p-JNK without alteration in total JNK levels, compared to the control cells. These data suggest that BOC and SGTb promote neuronal differentiation and neurite outgrowth through activation of JNK.

Next, we analyzed the effect of SGTb overexpression on JNK activation of BOC-depleted cells. SGTb overexpression in BOC-depleted cells restored the level of p-JNK, compared to the control BOC-depleted cells (Fig. 5d). In a converse experiment, BOC overexpression also restored p-JNK levels in SGTb-depleted cells (Fig. 5e). To examine the

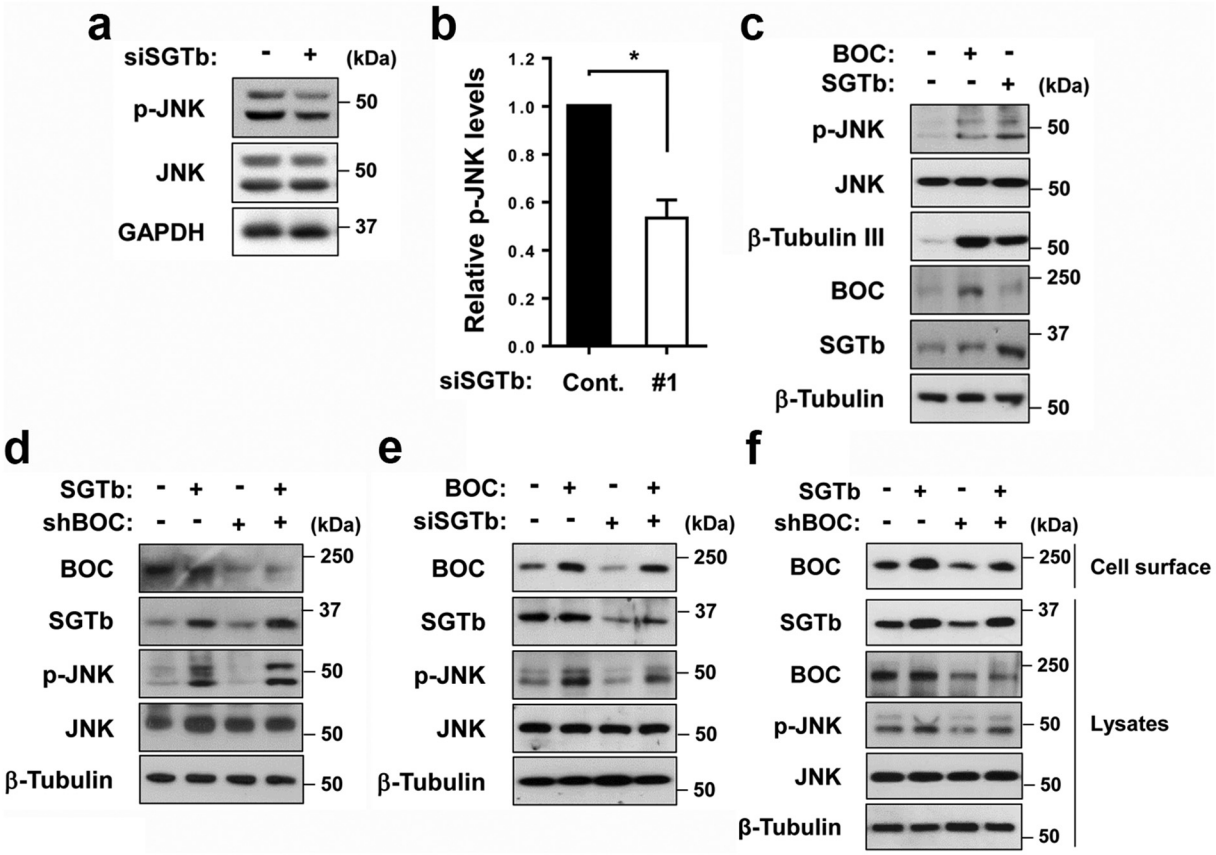

Fig. 5. SGTb regulated neuronal differentiation through JNK activation. (a) Immunoblot analysis for p-JNK and JNK levels in P19 cells, which were siControl- or siSGTb-transfected for 36 h and induced to differentiate until ITS1. GAPDH was used as a loading control. (b) Quantification of the relative level of p-JNK to JNK level shown in panel a. Band intensity was quantified by using the ImageJ software. $N = 3$. $^{***}p < .001$. (c) P19 cells, which were transfected with BOC or SGTb cells for 36 h and subsequently differentiated cells at ITS1, were subjected to immunoblotting analysis with the indicated antibodies. (d) P19/pSuper and P19/shBOC cells were transfected with control or SGTb-expressing vectors for 36 h and induced to differentiate at ITS1, followed by immunoblotting analysis with the indicated antibodies. (e) P19/siControl and P19/siSGTb cells were transfected with control or BOC-expressing vectors for 36 h and induced to differentiate until ITS1, followed by immunoblotting analysis with the indicated antibodies. (f) P19 cells, which were transfected with SGTb or shBOC for 36 h and induced until RA2, were subjected to the surface biotin labeling, followed by pulldown with streptavidin and immunoblotting with anti-BOC antibodies. Total cell lysates were also analyzed as a control.

mechanism of the rescuing effects of SGTb on JNK activation in BOC-depleted cells, BOC-depleted cells were transfected with SGTb expression vectors and cells were subjected to surface biotinylation. SGTb overexpression restored the surface level of BOC in the BOC-depleted cells, likely contributing the transduction of signaling from cell surface to activate JNK (Fig. 5f). Taken together, these data suggest that SGTb overexpression can rescue impaired neuronal differentiation of BOC-depleted cells. Thus, SGTb might regulate BOC's localization at the tip of neurite and JNK activation thereby regulating neurite outgrowth.

4. Discussion

Previous studies have shown that BOC acts as a receptor for an axon guidance cue Shh and also promotes neuronal differentiation and neurite outgrowth through JNK [11]. In this study, we have identified SGTb as an interacting protein of BOC, and it is required for neuronal differentiation and neurite outgrowth by regulation of BOC's surface localization and consequent JNK activation. The expression patterns of SGTb and BOC in differentiating neurons suggest that these genes are involved in neuronal differentiation. In addition, SGTb and BOC exhibit the highest expression in adult brain, suggesting for an additional role of these genes in brain function. This is consistent with a previous study showing an exclusive expression of SGTb in brain, while its isoform SGTa exhibits a broad expression pattern in various tissues [24]. However, its role in brain function is currently unclear. The interaction of SGTb with BOC appears to be primarily mediated through the TPR domain. The TPR domain has been shown to mediate interactions of SGTb with a variety of proteins thereby regulating diverse biological processes such as neuronal synaptic transmission, the cell cycle, protein folding, growth hormone receptor signaling and apoptosis [25–28]. One of the interacting protein is Hsp70 that is known to participate in a number of physiological and pathophysiological processes [29]. Considering that SGTb interacts with chaperone proteins, like HSP70, it might be involved in the protein quality control [9]. Thus, it is conceivable that SGTb might regulate the surface localization of BOC partly through the protein quality control that is critical for protein maturation. Interestingly, SGTb depletion in P19 EC cells resulted in perturbed growth cone formation and the absence of BOC in the growth cone (Fig. 4). The deletion of BOC function in mice led to impaired axon guidance of spinal commissural neurons in response to Shh [13]. Although the physiological function of SGTb is currently unclear, SGTb could play a role in Shh/BOC-mediated axon guidance.

A number of studies have suggested the important role of JNKs in neuronal development, neurite out growth and axonal regeneration [30–32]. JNKs have been shown to regulate cytoskeleton reorganization through phosphorylation of diverse proteins regulating microtubule stability, including DCX and microtubule-associated proteins, MAP2 and MAP1B [33–35]. These proteins are also induced during neuronal differentiation, and the time point of BOC induction is coinciding with induction of DCX and MAP2 (Fig. 2). Thus, BOC-mediated

JNK activation might be important for neurite extension through microtubule stability control. Our previous study has shown that BOC depletion resulted in reduced expression of DCX and MAP2, accompanied by reduction in JNK activation in P19 neuronal differentiation [31]. Similar to BOC depletion, SGTb depletion also caused decrease in MAP2 expression and JNK activation, and SGTb overexpression restored neuronal differentiation and JNK activation caused by BOC depletion. Considering that SGTb depleted cells had decreased levels of BOC at the cell surface observed by surface biotinylation, it can be suggested that SGTb regulates BOC's surface localization and in turn it recruits the intracellular components regulating JNK activity and microtubule stability that are critical for neuritogenesis. Further study will be required for the detailed analysis of the crosstalk between these proteins in regulation of brain function. In summary, SGTb regulates surface level of BOC and its downstream signal, JNK activity in neurite outgrowth and neuronal differentiation.

5. Conclusions

Taken together, our data demonstrate a positive role of SGTb in neuronal differentiation of P19 EC cells, and SGTb interacts with BOC, leading to JNK activation and enhancing BOC localization at neurite tip. Therefore, our current study provides new insight how SGTb regulates BOC-mediated JNK activity in control of neurite outgrowth and neuronal differentiation.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

TAV, SJL, GUB and JSK conceptualized the project, designed the experiments and wrote the manuscript. TAV, SJL, YEL and JRL performed the experiments, interpreted results and performed the statistical analysis. GUB and JSK supervised the project.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.01.003.

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