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# Protein deacetylase SIRT1 in the cytoplasm promotes nerve growth factor-induced neurite outgrowth in PC12 cells

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ABSTRACT

**SIRT1, a NAD$^+$-dependent protein deacetylase, is known to have neural functions. However, despite its cytoplasmic expression in some neural cells, its cytoplasmic function, if any, is unknown. Here we found that PC12 (pheochromocytoma) cells expressed SIRT1 in the cytoplasm. Nerve growth factor (NGF)-induced neurite outgrowth of these cells was promoted by activators of SIRT1, while inhibitors of SIRT1 or SIRT1-siRNA significantly inhibited it. The overexpression of a mutant SIRT1 that localised to the cytoplasm but not the nucleus enhanced the NGF-dependent neurite outgrowth, and a cytoplasmic dominant-negative SIRT1 suppressed it. Thus, cytoplasmic SIRT1 increases the NGF-induced neurite outgrowth of PC12 cells.**


## 1. Introduction

SIRT1, an NAD$^+$-dependent class III histone deacetylase [1], deacetylates various nuclear proteins and participates in cell differentiation, cell survival, tumorigenesis, and cell metabolism [2,3]. SIRT1 is highly expressed in the foetal and adult brain [4,5]. It inhibits some neurological toxic insults in animal models of Alzheimer's disease and amyotrophic lateral sclerosis [6]. Mutant, Wallerian degeneration slow, mice have high NAD biosynthetic activity, and the NAD-induced increase in SIRT1 activity may help protect the axons [7]. In the postnatal period, SIRT1 binds to the transcription factor Hes1 and promotes gliogenesis [8]. In addition, we have shown that SIRT1 participates in embryonic neuronal differentiation [9].

SIRT1 is a nucleocytoplasmic shuttling protein [10]. It is predominantly expressed in the cytoplasm of normal cardiomyocytes, and translocates into the nucleus to protect cells from oxidative stress during chronic heart failure [11]. Thus, in the heart, cytoplasmic SIRT1 may serve as a reservoir for nuclear SIRT1. On the other hand, in neural precursor cells isolated from the embryonic brain, SIRT1 is transiently localised to the nucleus in response to differentiation stimuli and then re-translocates to the cytoplasm [9]. SIRT1 is also predominantly expressed in the cytoplasm of some NeuN$^+$ neurons in the gray matter [9], but the cytoplasmic function of SIRT1 in neurons, if any, is unknown.

PC12 cells, derived from a rat pheochromocytoma, are a well-established model for studying neurite outgrowth. PC12 cells treated with nerve growth factor (NGF) cease proliferation, exhibit somatic hypertrophy, extend neurites, and differentiate [12].

In this study, we used PC12 cells to examine the function of cytoplasmic SIRT1.

## 2. Materials and methods

### 2.1. Plasmids and siRNAs

Constructs for nucleus-directed (SIRT1-EGFP), cytoplasm-directed (mtNLS-EGFP), or nucleus-directed dominant-negative (H355Y-EGFP) SIRT1-fused EGFP were described previously [10]. Cytoplasm-directed dominant-negative SIRT1-fused EGFP (mtNLS-H355Y-EGFP) was constructed by inserting the *Bgl*II–*Eco*RV fragment from mtNLS-EGFP into H355Y-EGFP digested with *Bgl*II and *Eco*RV. The construct was confirmed by DNA sequencing. Three kinds of SIRT1-siRNAs (B-Bridge) were used [11]. The sense and antisense SIRT1-siRNAs were as follows: No. 1: 5′-GAACAAA-GUUGACGAUUUAtt-3′ (sense)/5′-UAAAUCGUCAACUUUGUUCtt-3′

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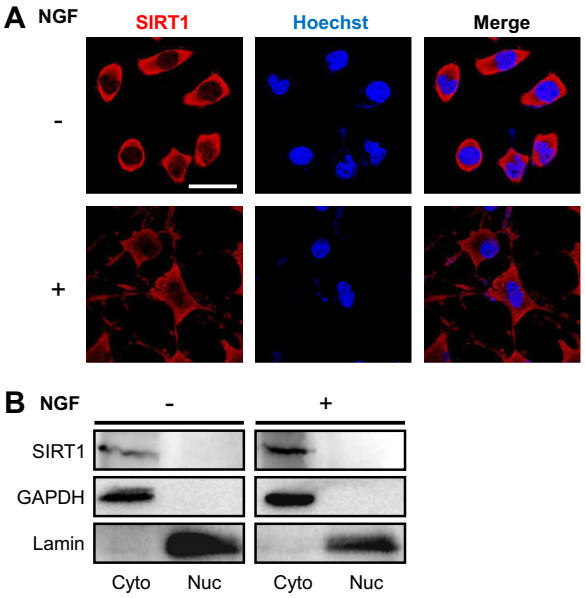


**Fig. 1.** SIRT1 is predominantly expressed in the cytoplasm of PC12 cells. (A) SIRT1 immunostaining of undifferentiated and differentiated PC12 cells. For differentiation, PC12 cells were cultured in differentiation medium containing NGF for 3 days. SIRT1 was expressed in the cytoplasm and neurites. Bar, 10 μm. (B) Western blot analysis of SIRT1. Cytoplasmic (Cyto) and nuclear (Nuc) fractions were prepared from undifferentiated and differentiated PC12 cells. GAPDH and Lamin were cytoplasmic and nuclear markers, respectively.

(antisense), No. 2: 5′-GCAGAAACAGUGAGAAAAUtt-3′ (sense)/5′-AUUUUCUCACUGUUUCUGCtt-3′ (antisense) and No. 3: 5′-GCUCAGAGUUUGAGCAUAUtt-3′ (sense)/5′-AUAUGCUCAAACUCUGAGCtt-3′ (antisense). Control siRNAs (B-Bridge) were a cocktail of the following three siRNAs: 5′-AUCCGCGCGAUAGUACGUAtt-3′ (sense)/5′-UACGUACUAUCGCGCGGAUtt-3′, 5′-UUACGCGUAGCGUAAUACGtt-3′ (sense)/5′-CGUAUUACGCUACGCGUAAtt-3′ (antisense) and 5′-UAUUCGCGCGUAUAGCGGUtt-3′ (sense)/5′-CGUAUUACGCUACGCGUAAtt-3′ (antisense). Plasmids or siRNAs were electroporated into PC12 cells using a Nucleofector kit (Amaxa). To identify the cells containing siRNAs, pmaxGFP (Amaxa) was co-electroporated with the siRNAs.

### 2.2. Cell culture

PC12 cells were grown at 37 °C in 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% heat-inactivated horse serum and 5% foetal bovine serum. Cells were plated at 50–60% confluence. To induce neurite outgrowth, the PC12 cells were plated on poly-L-ornithine-coated coverslips, and the medium was changed to a differentiation medium (DMEM plus 2% horse serum and 1% foetal bovine serum) with 50 ng/ml NGF (Alomone Labs). Splitomicin was from BIOMOL and other chemicals were from Wako.

### 2.3. Immunocytochemistry

Immunostaining was carried out as described previously [10]. Anti-SIRT1 (1:500; [4]) and Alexa Fluor 594-conjugated goat

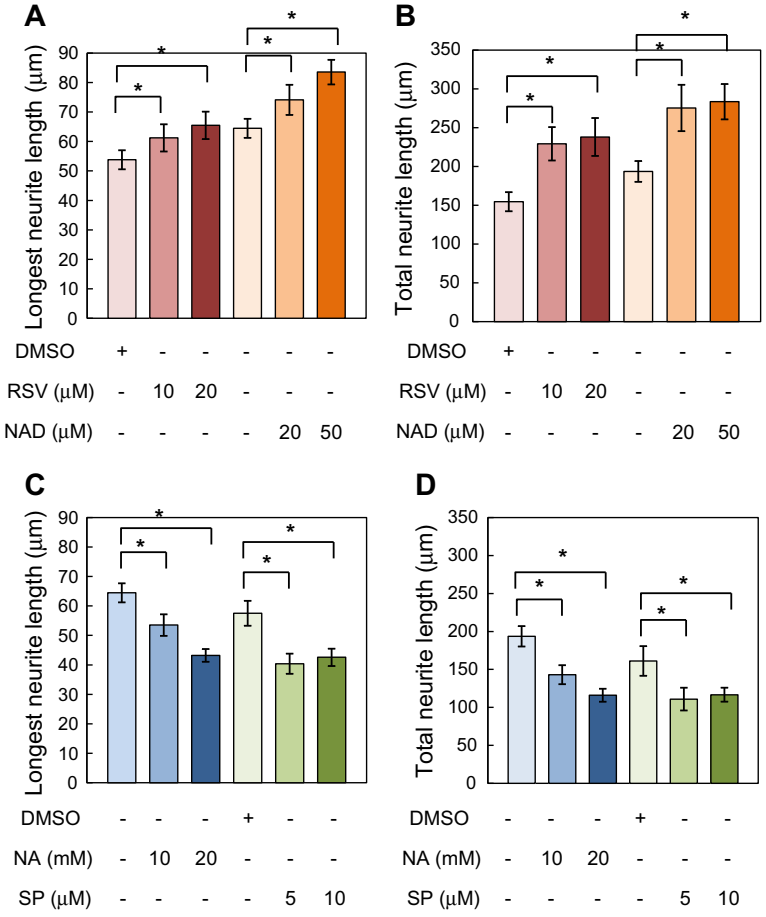


**Fig. 2.** Modulators of SIRT1 activity affect the NGF-induced neurite outgrowth. (A and B) PC12 cells were treated with an activator of SIRT1, either resveratrol (RSV) (10 or 20 μM) or NAD (20 or 50 μM) for 1 h, and then subjected to differentiation by NGF in the presence of the same activator for 3 days. (C and D) PC12 cells were treated with an inhibitor of SIRT1, either nicotinamide (NA) (10 or 20 mM) or splitomicin (SP) (5 or 10 μM) for 1 h, and then subjected to differentiation by NGF in the presence of the same inhibitor for 3 days. Neuron J was used to quantify the longest neurite length (A and C) and the total neurite length (B and D) for each cell. Differences were tested by a one-way ANOVA. $^{*}P < 0.05$ versus control cells treated with DMSO.

anti-rabbit antibodies (Molecular Probes) were used. Nuclei were stained with 1 μM Hoechst 33342 (Wako). For cell membrane staining, the cells were incubated with 2 μM cell tracker (Molecular Probes) for 5 min at 37 °C and then for 15 min at 4 °C before mounting. The samples were examined by confocal microscopy (Radiance 2100, Bio-Rad). Because confocal images cannot depict structures that extend out of the plane of focus, we merged two sections from different planes for each cell, to show the neurites. The length of neurites on a cell was measured with Neuron J (version 1.4.1), a program for neurite tracing and quantification developed by E. Meijering.

### 2.4. Immunoblotting

Immunoblotting was performed as described previously [9]. The Nuclear/Cytosol Fractionation kit (BioVision Research Products) was used to separate cellular fractions. Anti-GAPDH (Chemicon) and anti-Lamin A/C (Cell Signalling Technology) antibodies were used as cytoplasmic and nuclear markers, respectively. To strip bound antibodies, membrane filters were treated with ReBlot Plus (Chemicon).

### 2.5. Statistical analysis

More than 60 cells were examined in each experiment. Three independent experiments were carried out under each experimental condition. The results are presented as means ± S.E.M. Differences were tested by a one-way ANOVA. A *P*-value of less than 0.05 was considered significant.

## 3. Results

### 3.1. Predominant expression of SIRT1 in the cytoplasm of PC12 cells

We found that SIRT1 was predominantly expressed in the cytoplasm of undifferentiated PC12 cells (Fig. 1A). The treatment of PC12 cells with differentiation medium containing NGF-induced their differentiation and promoted neurite outgrowth. SIRT1 was still present in the cytoplasm and neurites of the PC12 cells 3 days after differentiation (Fig. 1A). Western blot analysis also showed that SIRT1 was predominantly detected in the cytoplasmic fractions from undifferentiated and differentiated cells (Fig. 1B).

### 3.2. Effects of SIRT1 modulators on NGF-induced neurite outgrowth

We next explored whether the neurite outgrowth of PC12 cells was affected by the SIRT1 activity. PC12 cells were treated with activators or inhibitors of SIRT1 1 h before being switched to differentiation medium, and they were then cultured under differentiation conditions with the SIRT1 modulators for 3 days. Most of the PC12 cells had several neurites after differentiation (see Fig. 4A).

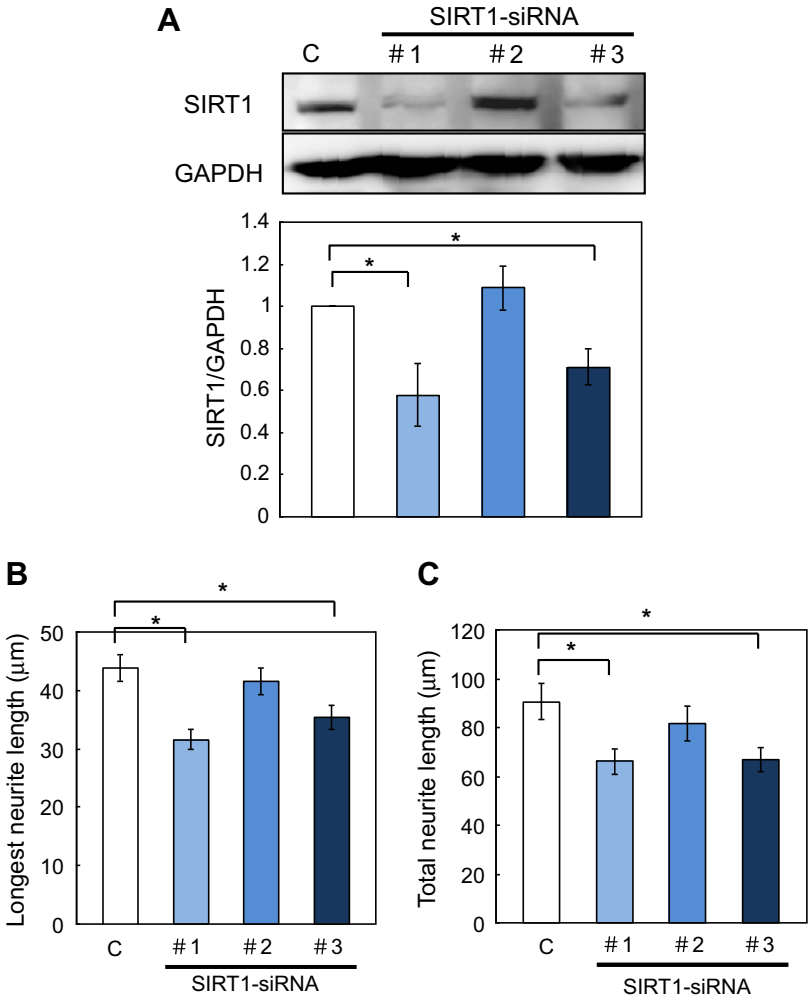


**Fig. 3.** SIRT1-siRNA represses NGF-induced neurite outgrowth. (A) Control siRNAs or each of SIRT1-siRNAs was electroporated into PC12 cells 24 h before differentiation, and then the cells were incubated in differentiation medium with NGF for 2 days. Representative immunoblots of SIRT1 and GAPDH are shown. #1 and #3 SIRT1-siRNAs but not #2 SIRT1-siRNA suppressed SIRT1 expression. The longest neurite length (B) and total neurite length (C) were quantified by Neuron J. Differences were tested by a one-way ANOVA. $^{*}P < 0.05$ versus cells expressing control siRNAs.

We evaluated the length of the longest neurite and the total neurite length for each cell. Resveratrol is a potent activator of SIRT1 [13]. The length of the longest neurite and the total neurite length were significantly longer for the cells treated with resveratrol than control cells (Fig. 2A and B). The administration of NAD, a SIRT1 cofactor, also increased the neurite outgrowth under differentiation conditions (Fig. 2A and B). In contrast, nicotinamide and splitomicin, inhibitors of SIRT1, significantly impaired the NGF-induced neurite outgrowth (Fig. 2C and D). None of these positive or negative modulators affected the number of neurites per cell (data not shown).

### 3.3. Inhibition of NGF-induced neurite outgrowth by SIRT1-siRNA

The sirtuin family contains seven members, SIRT1-7. SIRT1 modulators affect not only SIRT1 but also other sirtuins. To evaluate the function of SIRT1 specifically, three SIRT1-siRNAs or control siRNAs along with GFP were introduced into PC12 cells one day before differentiation, and the neurite outgrowth was examined after 2 days under differentiation conditions (Fig. 3A). The protein level of SIRT1 was significantly decreased by the #1 and #3 SIRT1-siRNAs but not by the #2 SIRT1-siRNA (Fig. 3A). The NGF-induced neurite outgrowth of the cells electroporated with the #1 or #3 SIRT1-siRNA significantly decreased compared with that of cells expressing control siRNAs (Fig. 3B and C). The neurite outgrowth was not affected by the #2 SIRT1-siRNA, that failed to decrease SIRT1 expression.

### 3.4. Modulation of NGF-induced neurite outgrowth by cytoplasmic SIRT1

The differentiated PC12 cells expressed SIRT1 in the cytoplasm and neurites (Fig. 1A). We next explored whether the overexpression of cytoplasmic SIRT1 would promote the NGF-induced neurite outgrowth of PC12 cells. mtNLS-EGFP (cytoplasmic SIRT1), which has mutations in two of SIRT1's nuclear localisation signals [10], was expressed in the cytoplasm of PC12 cells (Fig. 4A middle panel, inset). The length of the longest neurite on the cells overexpressing cytoplasmic SIRT1 was significantly longer than that of control cells overexpressing EGFP alone (Fig. 4A and B).

When mtNLS-H355Y-EGFP (cytoplasmic dominant-negative SIRT1) was expressed in PC12 cells, it was detected in the cytoplasm as expected (Fig. 4A right panel, inset). The cytoplasmic dominant-negative SIRT1 failed to increase the NGF-induced neurite extension under differentiation conditions (Fig. 4A and B), demonstrating that the promotion of neurite outgrowth by cytoplasmic SIRT1 required SIRT1's enzymatic activity. Furthermore, the total length of the neurites on cells expressing the cytoplasmic dominant-negative SIRT1 was significantly shorter than that on control cells under differentiation conditions (Fig. 4C).

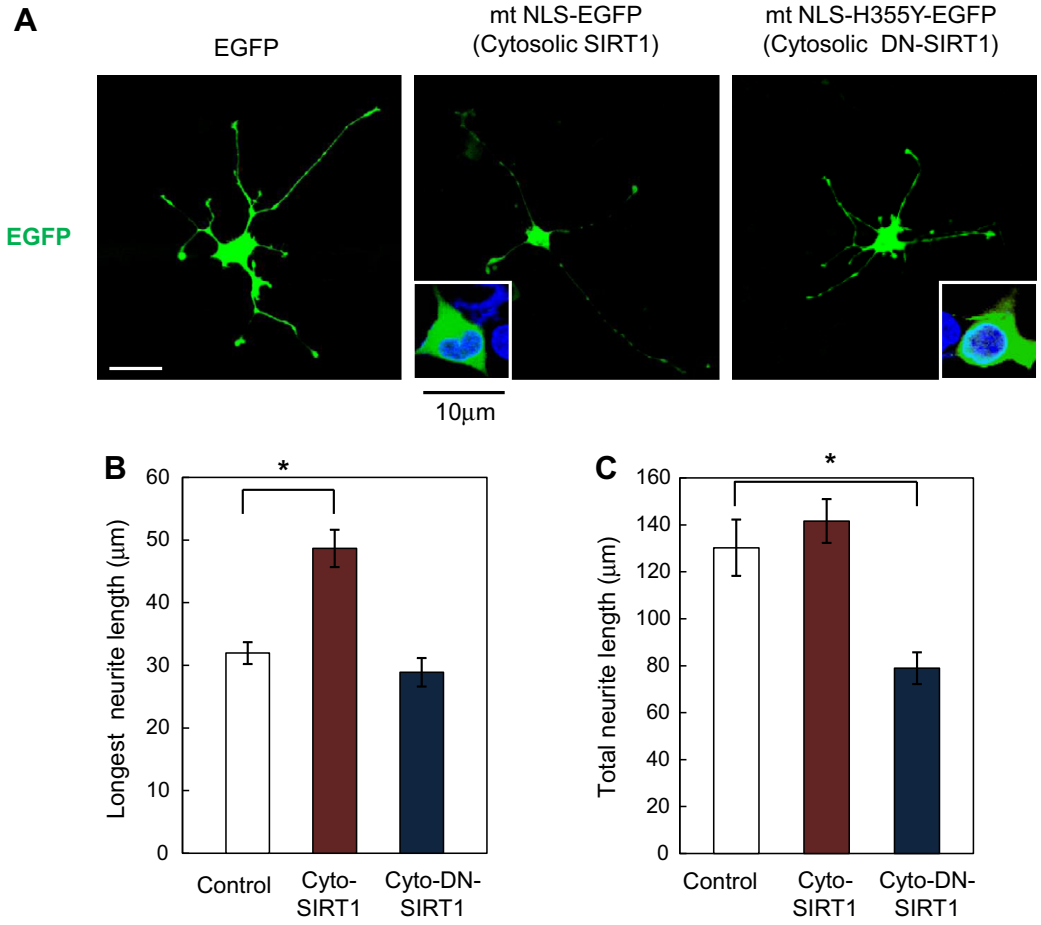


**Fig. 4.** Cytoplasmic SIRT1 promotes NGF-induced neurite outgrowth. (A) EGFP, mtNLS-EGFP (cytoplasmic SIRT1), or mtNLS-H355Y-EGFP (cytoplasmic dominant-negative SIRT1) was expressed in PC12 cells 24 h before differentiation, and then the cells were differentiated with NGF for 2 days. Representative images of EGFP (green) are shown. The focus was adjusted to identify neurites. Insets are confocal images with nuclear staining (blue). Bars: 10 μm. The longest neurite length (B) and the total neurite length (C) were quantified by Neuron J. Differences were tested by a one-way ANOVA. $^{*}P < 0.05$ versus control cells expressing EGFP.

Finally, we examined SIRT1-EGFP (nuclear SIRT1) and H355Y-EGFP (nuclear dominant-negative SIRT1; DN-SIRT1), which were exclusively localised to the nucleus of PC12 cells (Fig. 5A). The overexpression of neither nuclear SIRT1 nor DN-SIRT1 promoted the NGF-induced neurite outgrowth (Fig. 5B and C); rather, these molecules reduced the total neurite length.

## 4. Discussion

In this study, we demonstrated that SIRT1 was expressed in the cytoplasm of PC12 cells and modulated their NGF-dependent neurite outgrowth. We further showed that cytoplasmic SIRT1 but not nuclear SIRT1 promoted the neurite outgrowth. Neural precursor cells express SIRT1 in the cytoplasm [9]. The in utero electroporation of SIRT1-siRNA into neural precursor cells of the E14 brain abolishes the normal bipolar formation of the cells examined at E17; instead, these cells are round and have few or short processes [9]. Taken together, these data indicate that SIRT1 may participate in the formation of axons or dendrites by neurons during brain development. A neurite differentiates into an axon or a dendrite in the brain. At present, it is not known if SIRT1 affects axonal or dendritic formation. To determine whether SIRT1 affects axon-dendrite specification, studies using primary cultures of hippocampal pyramidal neurons or cortical neurons should be performed.

SIRT1 deacetylates nuclear proteins such as histones, transcription factors, and cofactors. Because cytoplasmic SIRT1 and cytoplasmic dominant-negative SIRT1 affected neurite outgrowth (Fig. 4), SIRT1 may directly deacetylate and modulate a cytoplasmic protein that participates in the formation and extension of neurites. Accordingly, SIRT1 is expressed in neurites (Fig. 1A). A recent study using GST-fusion SIRT1 and mass spectrometry showed that SIRT1 interacts with several cytoplasmic and membrane-bound proteins, including molecules of the Ras superfamily signalling pathway [14]. Because the Ras GTPase family is involved in

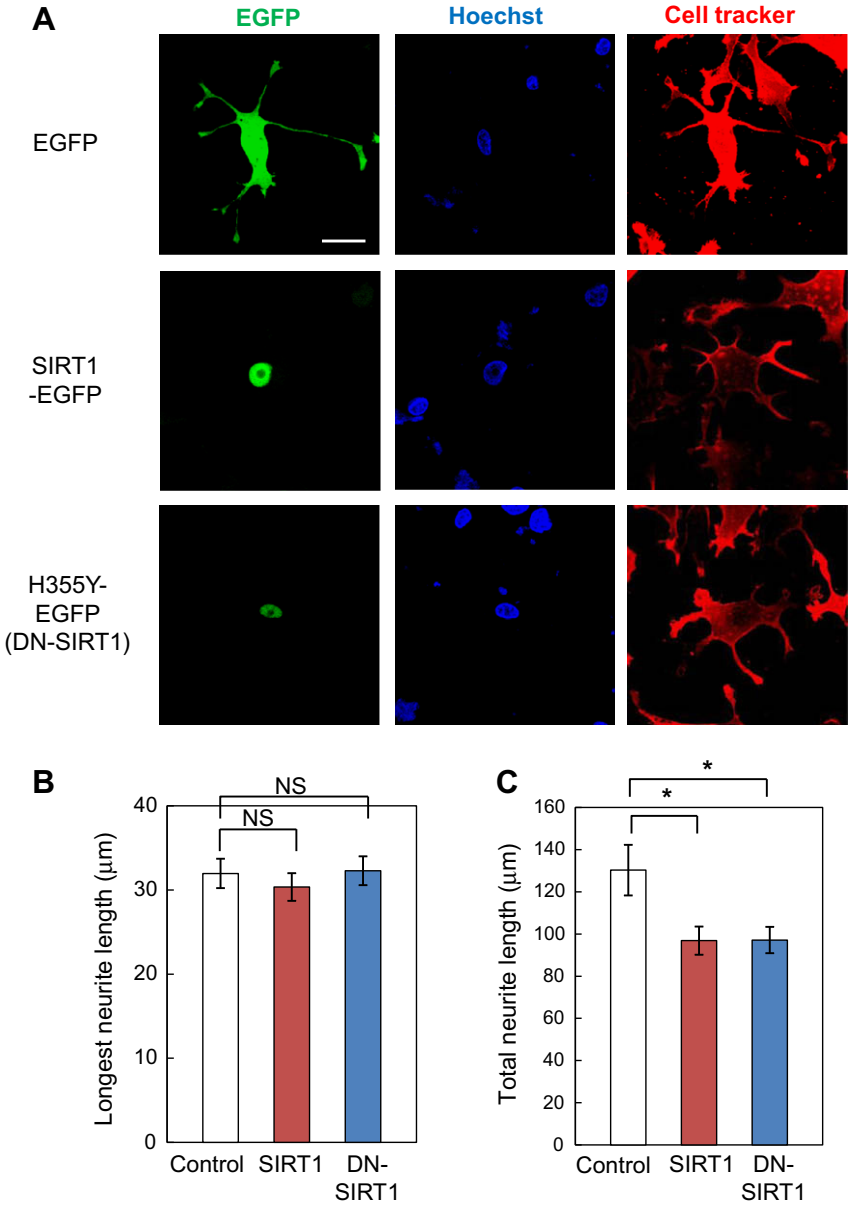


**Fig. 5.** Nuclear SIRT1 fails to promote NGF-induced neurite outgrowth. (A) EGFP, SIRT1-EGFP (nuclear SIRT1), or H355Y-EGFP (nuclear dominant-negative SIRT1) was expressed in PC12 cells, and then the cells were differentiated with NGF for 2 days. Representative images of EGFP (green), nuclear staining (blue), and cell tracker (red) are shown. Bars: 10 μm. The longest neurite length (B) and total neurite length (C) were quantified by Neuron J. Differences were tested by a one-way ANOVA. NS, no significant difference. $^{*}P < 0.05$ versus control cells expressing EGFP.

neurite outgrowth [15], SIRT1 may affect small GTPase cascades. The inhibition of HDAC6, a class II cytoplasmic histone deacetylase, has been shown to affect neurite growth [16]. Because HDAC6 is also predominantly expressed in the cytoplasm, a cytoplasmic protein that affects neurite outgrowth may be modulated by acetylation and deacetylation. SIRT2, another member of the sirtuin family, is a tubulin deacetylase. SIRT2 inhibits the neurite outgrowth of postmitotic hippocampal neurons [17]. Our preliminary study failed to show the deacetylation of tubulin by SIRT1 (data not shown). Thus, SIRT1 and SIRT2 may play different roles in the cytoplasm. Nuclear SIRT1 and dominant-negative SIRT1 inhibited the outgrowth of the total neurites per cell (Fig. 5C). At present, the mechanism by which nuclear overexpression of SIRT1 inhibits neurite outgrowth is unknown.

In summary, in PC12 cells, cytoplasmic but not nuclear SIRT1 promotes NGF-induced neurite outgrowth in a manner that depends on SIRT1's deacetylation activity. The modulation of SIRT1 localisation and activity may be useful as a novel therapeutic tool against neuronal diseases.

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