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# Inhibition of Sirtuin 6 Induces Neuroblastoma Differentiation

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**Abstract.** *Background/Aim: Sirtuins (SIRTs) play crucial roles in various signaling pathways that modulate differentiation and proliferation. We sought to elucidate the role of SIRTs in differentiation and proliferation of human neuroblastoma (NB). Materials and Methods: NB cells were treated with nicotinamide (NAM), a non-specific SIRT inhibitor, SIRT-targeted short hairpin RNAs, and retinoic acid to assess cell growth and differentiation. Results: SIRTs are involved in proliferation and differentiation using NAM in BE(2)-C cells. Specifically, SIRT6 knockdown in BE(2)-C cells reduced cell proliferation, induced neurite extension, corresponding with induction of $p21^{CIP1}$ expression and $G_1$ cell-cycle arrest. These effects were rescued by forced re-overexpression of SIRT6. SIRT6 expression was reduced in differentiated human NB sections, and RA-induced differentiation in BE(2)-C cells. Conclusion: SIRTs have important oncogenic properties in NB beyond its established functions in aging and genome stability. SIRT6 may represent a novel target for developing future therapeutics for the treatment of aggressive NBs.*

Neuroblastoma (NB) is the most common extracranial pediatric solid tumor arising from neural crest precursors (1). Afflicted children have diverse clinical courses with some tumors spontaneously regressing while others metastasize and are refractory to our most aggressive treatment strategies (2). Tumors presumably arise due to molecular defects that promote proliferation and block terminal differentiation (3). Escalating chemotherapeutic strategies targeting aberrant proliferation are the mainstay treatment of children with advanced-stage disease, but are limited by their intolerable side-effect profiles (2). Far less effort has been devoted to developing pro-differentiating strategies. Retinoic acid (RA) derivatives are pro-differentiating agents that are utilized in the treatment of high-risk children with minimal residual disease following myeloablation with autologous stem cell transplantation (4).

Differentiation is regulated by broad transcriptional changes, suggesting that epigenetic aberrations may promote tumorigenesis by blocking differentiation (3). Targeting class I and II histone deacetylases has emerged as a pro-differentiating strategy in NB, but no reports to date have evaluated the role of class III histone deacetylases in NB tumorigenesis (5). Sirtuins (SIRTs) are a family of class III histone deacetylases comprising seven known members that share significant sequence homology and uniformly utilize NAD+ as a cofactor (6). Sequence-based phylogenetic analysis revealed that mammalian SIRTs can be divided into four classes: SIRT1–SIRT3 belong to class I, SIRT4 to class II, SIRT5 to class III, and SIRT6 and SIRT7 to class IV. Class I (SIRT1-SIRT3) and IV (SIRT6,7) have broad deacetylase activity, while SIRT4 functions predominately as an ADP-ribosyltransferase and the deacetylase activity of SIRT5 is limited to carbamoyl phosphate synthetase I, a mediator of the urea cycle (6). SIRTs are found in different subcellular compartments, such as in the cytoplasm (SIRT1 and SIRT2), nucleus (SIRT1, -6, and -7) and the mitochondria (SIRT3, -4 and -5) (7, 8). SIRTs regulate a diverse array of cellular processes including metabolism, cellular senescence, and genomic stability with an increasingly recognized role in cancer (6, 9).

In particular, SIRT6 is a nuclear localized protein that regulates metabolism, inflammation, cellular senescence, genomic stability, and stress response *via* its activity as a mono-ADP-ribosyltransferase and deacetylase for both histone and non-histone proteins (10-15). The role of SIRT6 in carcinogenesis has received considerable interest with seemingly organ-dependent oncogenic and tumor suppressor effects (16). Specifically, SIRT6 expression is decreased in



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colon, pancreatic, liver, and head and neck cancers (16). Mechanistically, these findings have been linked to decreased SIRT6 levels increasing the expression of MYC and HIF-1α, promoting proliferation and glycolysis (17). Conversely, SIRT6 expression is increased in prostate and breast cancers, where its tumor suppressor effects have been linked to regulation of FOXO3a and p53 (17).

The role of SIRT in regulating proliferation or differentiation in NB has not been reported previously. Given the demonstrated efficacy of class I and II histone deacetylases in promoting NB differentiation, we hypothesized that blocking SIRTs would promote NB differentiation.

## Materials and Methods

*Antibodies and reagents*. Primary antibodies used were for SIRT6 (Sigma-Aldrich, St. Louis, MO, USA), p21, neuron-specific enolase (NSE) and neurofilament (NF-M) from Cell Signaling (Beverly, MA, USA). All secondary antibodies against mouse and rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). β-actin antibody and all other reagents were from Sigma-Aldrich.

*Cell culture and transfections*. Human NB cell line, BE(2)-C was purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere containing 5% $CO_2$ and 95% air. For transfections, $5\times10^5$ cells were plated per well in 6-well plates and transfected the next day with Lipofectamine™ 2000 (Invitrogen, Rockville, MD, USA) as per the manufacturer's protocol. All stably transfected cells were selected with puromycin (Cellgro Mediatech Inc., Herndon, VA, USA) at 2.5 μg/ml for 2 weeks. For *SIRT6* overexpression and silencing, pCDH-SIRT6 and pSR-shSIRT6 (Addgene, Cambridge, MA, USA), respectively, were used. *SIRT6* short hairpin RNA (shRNA) target sequence was 5'-GGTCTCACTTTGTTACTTGTT-3'.

*Cell proliferation*. Cells were seeded onto 96-well plates at a density of $1\times10^4$ cells per well in RPMI culture medium with 10% FBS. After allowing 24 h for attachment, the cells were treated with control media (RPMI culture media with 10% FBS) or medium with nicotinamide (NAM; 20 mM) in triplicates. The cell number was assessed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) for various culture times as previously described (18). For transfected cells, the cell proliferation assays were performed as described above without further treatment.

*Cell morphology and immunofluorescence*. The cells were plated and grown on coverslips. Cell morphological neurite formation was assessed and imaged daily with a Nikon inverted phase-contrast microscope (Nikon Eclipse TS100, Nikon Instruments Inc. Melville, NY, USA). For RA-induced cell morphological change, the cells were treated with RA at 5 μM for four days. For immunofluoroscent staining, cells were plated and grown on cover slips, then were fixed with 4% paraformaldehyde for 20 min at room temperature (RT), permeabilized with 0.1% Triton X-100 for 15 min and blocked with 1% bovine serum albumin/phosphate-buffered saline (PBS) for 30 min. Cell were incubated with primary antibodies (1:100) against NF-M for 1 h at RT, washed five times with PBS and then incubated with secondary antibodies for 30 min at RT. The nuclei were counterstained with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA). The immunofluorescence signal was observed and pictured under a fluorescence microscope (Nikon Ecliose E600).

*Western blot analysis*. Cell lysates were prepared by incubating cells in lysis buffer (125 mM Tris–HCl, pH 6.8, 2% SDS, 10% v/v glycerol) at 4˚C for 10 min. Protein concentrations were quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins were separated on a NuPAGE Novex 4-12% Bis-Tris gels and electrophoretically transferred to polyvinylidenedifluoride membranes (Bio-Rad Laboratories), which were blocked with 5% dry milk in PBS. Immunoreaction was performed with antibodies against NSE, p21, SIRT6, NF-M, and β-actin, and the bands were visualized by developing the blots using an enhanced chemiluminescence system according to the manufacturer's instructions (Perkin Elmer, Waltham, MA, USA).

*Cell-cycle analysis*. Cell-cycle distribution was analyzed by flow cytometry as described elsewhere (8). Briefly, $1\times10^6$ cells were trypsinized, washed once with PBS, and fixed in 70% ethanol. Fixed cells were again washed with PBS, incubated with RNase (100 μg/ml) for 30 min at 37˚C, stained with propidium iodide (50 μg/ml), and analyzed on a 3-laser BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). The percentage of cells in different cell cycle phases was analyzed using Cell-FIT software (Becton-Dickinson Instruments, San Jose, CA, USA). Flow Cytometry experiments were performed in the Vanderbilt University Medical Center, Flow Cytometry Shared Resource (Nashville, TN, USA).

*Immunohistochemical analysis*. Vanderbilt University Institutional Review Board reviewed IRB# 091331 and determined the study does not qualify as "human subject" research per §46.102(f) (2). Samples of discarded tissue without identifiable private information were obtained from Surgical Pathology at Vanderbilt University Medical Center. Tissues were fixed in formalin for 3 days and embedded in paraffin wax. Paraffin-embedded sections (4 μm) were deparaffinized in three xylene washes followed by a graded alcohol series, antigen retrieval performed with 10 mM sodium citrate buffer, and then blocked with solution for 1 h at RT. They were incubated with primary antibody against SIRT6 (1:400 dilutions) overnight at 4˚C, washed with PBS, incubated with secondary antibodies for 30 min at RT, and developed with 3,3'-diaminobenzidine reagent. All sections were counterstained with hematoxylin, and then dehydrated with ethanol and xylene. Coverslips were mounted and slides observed by light microscopy.

*Statistical analysis*. The results are expressed as the means±SEM from at least three independent experiments. The values were evaluated *via* one-way ANOVA followed by Duncan's multiple range tests using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p<0.05$.

## Results

*NAM blocked NB cell growth*. We examined whether SIRTs regulate proliferation or differentiation in a *MYCN*-amplified NB cell line, BE(2)-C, using the non-specific SIRT inhibitor, NAM. NAM treatment (20 mM) markedly inhibited the

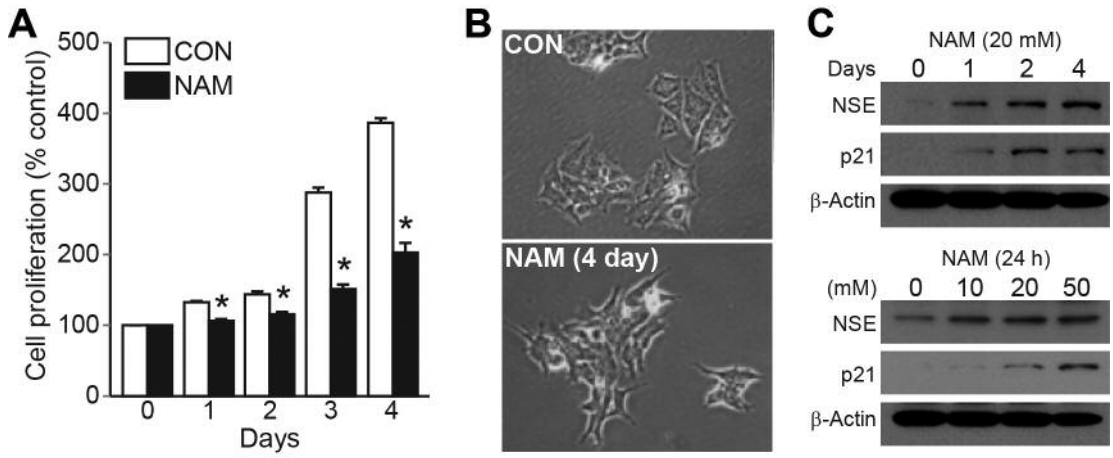


Figure 1. *Nicotinamide blocks neuroblastoma (NB) growth and promotes differentiation. A: Cells were treated with nicotinamide (NAM) (20 mM) over a time course and cell proliferation was determined using Cell Counting Kit-8. Data are the mean±SEM.* Significantly different at p<0.005 vs. CON). B: Neurite outgrowth consistent with neuronal differentiation was observed in BE(2)-C cells after treatment with NAM (20 mM) for 4 days. C: p21 and neuron specific enolase (NSE) protein expression is induced by NAM in both a time-and dose-dependent manner. β-Actin was used as an internal control.*

growth rate of BE(2)-C cells compared to the control cells, as analyzed *via* a tetrazolium-based proliferative assay (Figure 1A). Furthermore, NAM induced formation of neurite-like structures consistent with neuronal differentiation (Figure 1B). Next, we examined the effects of NAM in the expressions of proliferation and differentiation-related genes, p21 and NSE. NAM induced p21 and NSE expression in a time- and dose-dependent manner (Figure 1C). These findings suggested that SIRTs may play a critical role in NB proliferation and differentiation.

*Silencing SIRT6 promoted neuronal differentiation of NB*. We next sought to determine which SIRT family members were critical for NAM-mediated growth arrest. We performed stable knockdown of SIRT1, 2, 3, 6 and 7 in BE(2)-C cells using shRNA, and confirmed the silencing *via* western blotting. Among the five SIRTs examined, only silencing of *SIRT6* (with shSIRT6) significantly reduced proliferation compared to control-transfected cells (shCON), whose growth rate steadily increased at each time point (Figure 2A). These results suggested that SIRT6 regulates proliferation of human NB BE(2)-C cells.

Intriguingly, *SIRT6* knockdown induced morphological changes similar to those after NAM treatment. Specifically, *SIRT6* knockdown induced neurite-like outgrowth (Figure 2B, arrows), demonstrated by immunofluorescent staining of NF-M within neurite outgrowths. To further characterize the suppression of proliferation by SIRT6 knockdown, we analyzed the expression of proliferation and differentiation related molecules. As shown in Figure 2C, *SIRT6* knockdown increased the expression of p21, NSE, and NF-M when compared to shCON cells. Taken together, these findings demonstrate that *SIRT6* knockdown promotes neuronal protein expression patterns and morphology while suppressing NB growth.

*Re-overexpression of SIRT6 reversed the growth, morphological, and protein expression changes induced by SIRT6 knockdown*. To validate the role of SIRT6 in NB growth, we attempted to rescue knockdown-induced phenotype by forced overexpression of SIRT6. We analyzed the effects of *SIRT6* re-overexpression on the *SIRT6* knockdown cells. Forced re-overexpression rescued growth inhibition mediated by *SIRT6* knockdown and rescued neurite outgrowth (Figure 3A and B). Cell-cycle arrest initiated by *SIRT6* knockdown was successfully rescued with the overexpression of *SIRT6* as demonstrated by a return of $G_1$ and S phase cell populations to near control levels (Figure 3C). Consistent with a reduction in the S phase population, knockdown of *SIRT6* reduced the incorporation of BrdU compared to shCON cells (data not shown). Furthermore, increased expression of proliferation and differentiation-related genes, *p21* and NSE in shSIRT6 cells, were reduced by *SIRT6* re-overexpression (Figure 3D). Together, these findings demonstrate that features of growth arrest and differentiation induced by *SIRT6* knockdown can be reversed with forced *SIRT6* re-expression, confirming the critical role of SIRT6 signaling in modulating NB growth and differentiation.

*SIRT6 expression is decreased in differentiated NB and RA-induced cells*. The previous results provided evidence that SIRT6 is involved in differentiation and proliferation of NB cells. We attempted to correlate these results with SIRT6 expression in human NB tissues. We analyzed the SIRT6

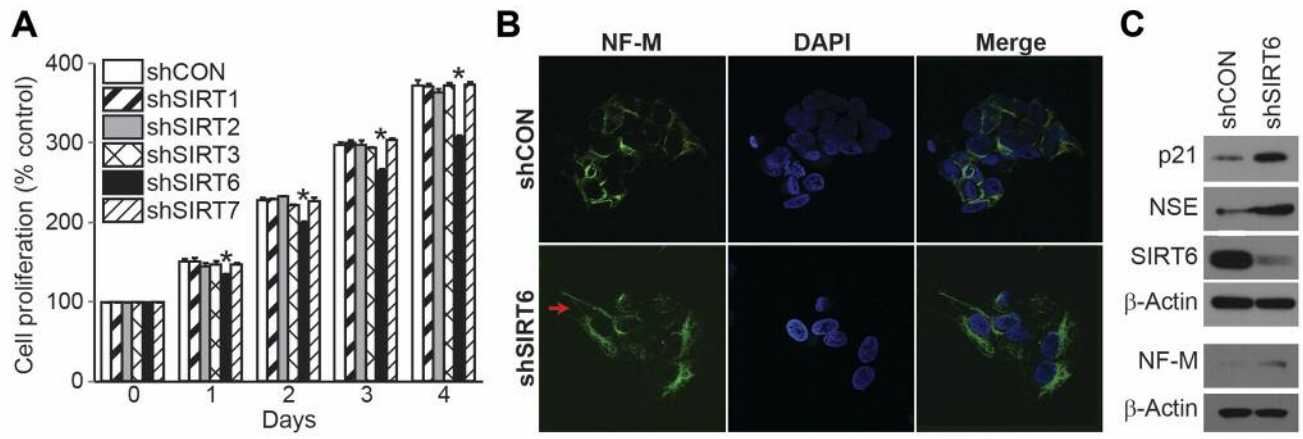


Figure 2. *Silencing sirtuin 6 (SIRT6) blocks proliferation and promotes neuronal differentiation in neuroblastoma (NB). A: Cell proliferation was determined using Cell Counting Kit-8 after silencing of different SIRTs. Data are the mean±SEM.* Significantly different at $p<0.05$ vs. control-transfected cells (shCON). B: Stable knockdown of SIRT6 enhances neurite outgrowth. Neurofilament (NF-M) staining of neurite-like structures after SIRT6 silencing was monitored by confocal microscopy (magnification, ×400) using an antibody recognizing NF-M. 4,6-Diamidino-2-phenylindole (DAPI) co-staining highlights nuclear staining. C: Western blot analysis was performed on proteins from SIRT6 knock-down BE(2)-C cells with the indicated antibodies. The silencing of SIRT6 was confirmed by western blotting. The expression of p21, neuron-specific enolase (NSE), NF-M was increased in SIRT6 silencing cells. β-Actin was used as a loading control.*

Table I. *Human tissue microarrays analyzed by immunohistochemistry for sirtuin 6 (SIRT6). The intensity of SIRT6 expression in tumors was defined as: −: negative; +: low expression; ++: moderate; +++: high. The table reports the number of the different tumors analyzed and their subdivision according to their immunohistochemistry score.*

| | Poorly differentiated | | | | Differentiated | | | |
|---|---|---|---|---|---|---|---|---|
| Intensity of SIRT6 expression | − | + | ++ | +++ | − | + | ++ | +++ |
| Number of samples | 0 | 0 | 3 | 5 | 4 | 0 | 2 | 3 |
| % Of samples | 0 | 0 | 37.5 | 62.5 | 44.44 | 0 | 22.22 | 33.33 |

expression in human NB sections. Overall, absence of SIRT6 was noted in 44% of differentiated NB, as compared to none of the undifferentiated NB, which demonstrated a trend towards significance ($p=0.08$; Figure 4A; Table I). To further understand the involvement of SIRT6 in differentiation/proliferation in NB, we analyzed the effect of SIRT6 on RA-induced neuronal differentiation. RA treatment successfully induced both neurite formation (Figure 4B) and enhanced expression of p21 and NSE (Figure 4C).

*Treatment with RA induced both a time and dosage-dependent inhibition of SIRT6 expression (data not shown).* Intriguingly, silencing *SIRT6* enhanced RA-induced neurite formation, while SIRT6 overexpression antagonized neurite formation (Figure 4B). RA-induced p21 and NSE expression patterns were similarly modulated by *SIRT6* silencing and overexpression. Specifically, *SIRT6* knockdown enhanced RA-mediated p21 and NSE expression, while overexpression of *SIRT6* antagonized p21 and NSE expression (Figure 4C). Overall, these findings suggest a novel inhibitory role of SIRT6 in the neuronal differentiation in NB.

## Discussion

The SIRT family has an increasingly recognized role in cancer initiation and progression (6, 19). The pleotropic effects of this family of proteins on metabolism, aging, cellular senescence, genomic stability appears to be largely homolog (1-7) and tissue-dependent (6). The role of SIRT in NB progression has not been previously reported. We have shown here that treatment with a non-specific SIRT inhibitor, NAM, induces differentiation and reduces proliferation in NB. shSIRT for different SIRT family members was utilized to demonstrate that SIRT6 is the primary member responsible for these effects, as

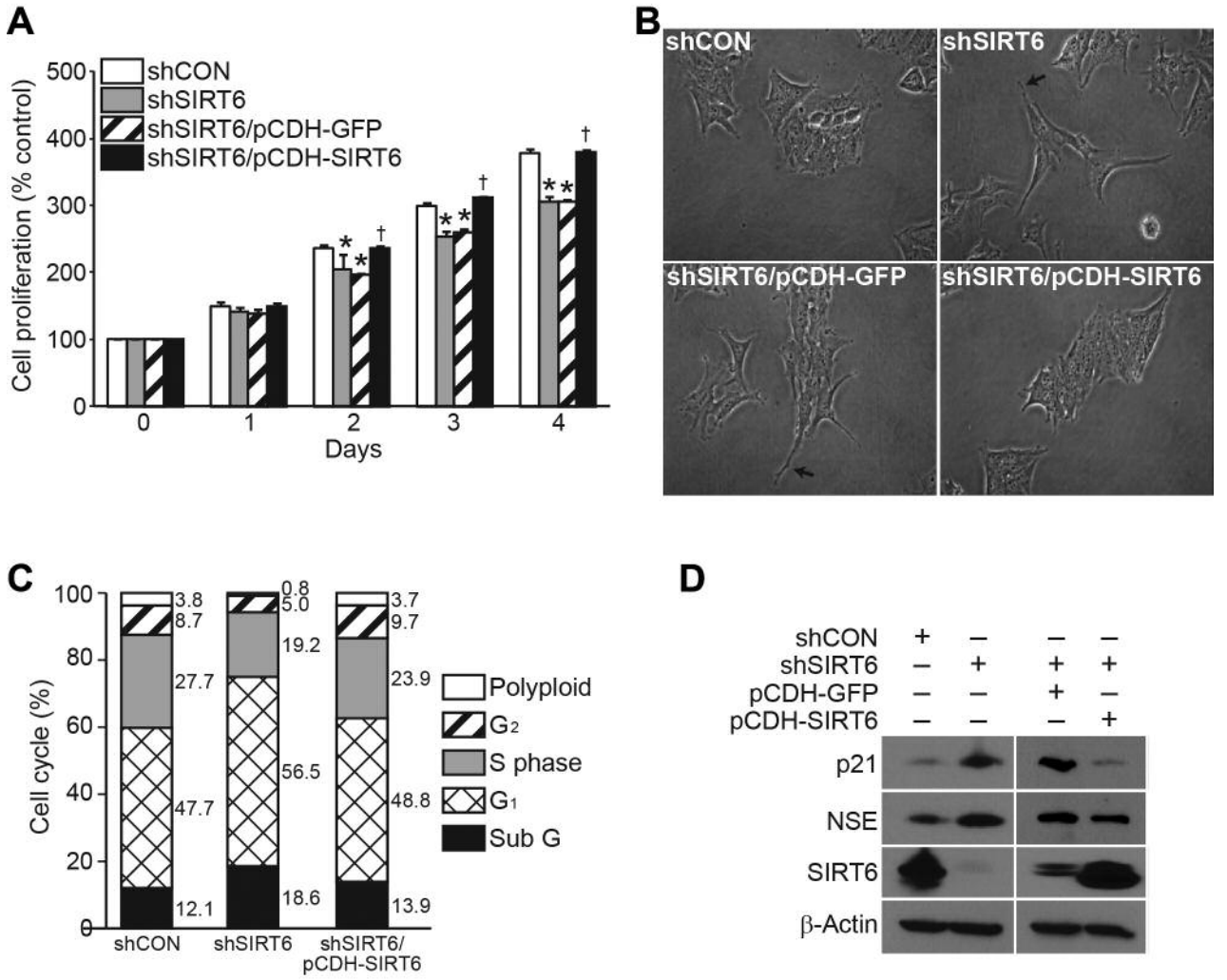


Figure 3. *Re-overexpression of sirtuin 6 (SIRT6) in BE(2)-C cells restored proliferation, and redound morphological change and expression of p21 and neuron-specific enolase (NSE). A, B: Growth curve and morphological change of each stable cell line [control-transfected cells (shCON), SIRT6 knockdown (shSIRT6), shSIRT6/pCDH-GFP and shSIRT6/pCDH-SIRT6] were analyzed using a Cell Counting Kit-8 and light microscopy. Data are the mean±SEM. Significantly different at p<0.05 vs. *shCON and †shSIRT6). C: A total of 1×10$^6$ cells were analyzed for cell-cycle distribution using flow cytometry. D: Western blot analysis was performed with the indicated antibodies in BE(2)-C cells after each stable transfection. β-Actin protein was used as an internal control.*

reduced proliferation and differentiation in NAM-treated BE(2)-C cells was recapitulated with stable knockdown of *SIRT6*.

Intriguingly, SIRT6 has been described as a regulator of both HIF1α and MYC transcription (17). Specifically, low levels of SIRT 6 have been linked to high levels of MYC and HIF1α in other cancer types, which are both transcription factors associated with a poor prognosis in NB (20, 21). Our findings in *MYCN*-amplified BE(2)-C cells suggest that this pattern is not conserved in NBs. Specifically, SIRT6 appears to regulate NB differentiation, with reduction in SIRT6 expression inducing cell growth arrest and promoting differentiation. These findings were further supported by the demonstrated increased expression of p21, NSE, and NF-M proteins and observed neurite formation under confocal microscopy. As such, these findings highlight that SIRT6 represses differentiation and lowering SIRT6 promotes differentiation in NB.

Significant efforts have been made to identify defects in proliferation that drive tumorigenesis in NB in the hope of developing targeted therapies, but relatively little effort has been focused on identifying targets in order to promote tumor differentiation. 13-Cis RA is administered in children with NB who have minimal residual disease following myeloablative chemotherapy and autologous stem cell

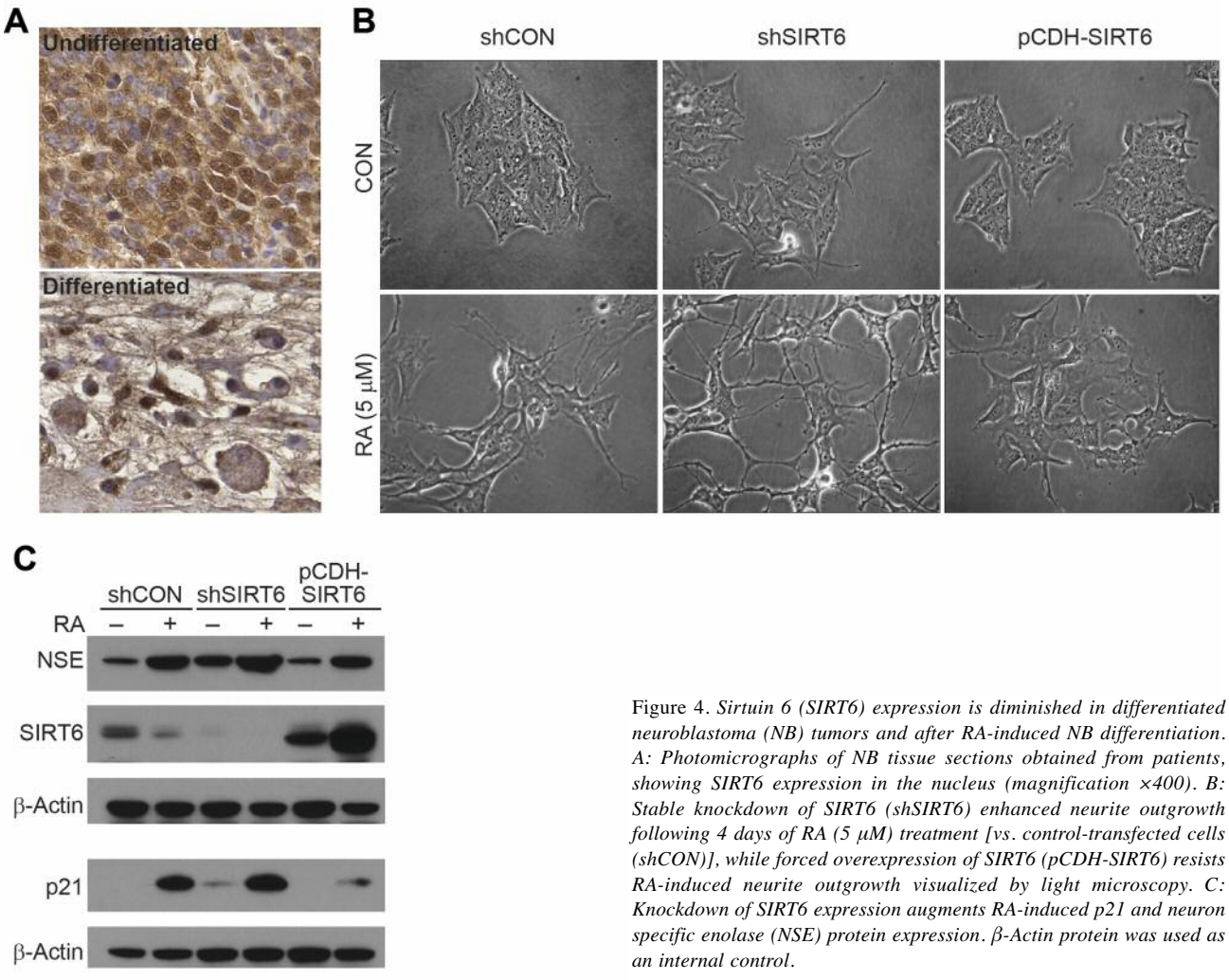


Figure 4. *Sirtuin 6 (SIRT6) expression is diminished in differentiated neuroblastoma (NB) tumors and after RA-induced NB differentiation. A: Photomicrographs of NB tissue sections obtained from patients, showing SIRT6 expression in the nucleus (magnification ×400). B: Stable knockdown of SIRT6 (shSIRT6) enhanced neurite outgrowth following 4 days of RA (5 μM) treatment [vs. control-transfected cells (shCON)], while forced overexpression of SIRT6 (pCDH-SIRT6) resists RA-induced neurite outgrowth visualized by light microscopy. C: Knockdown of SIRT6 expression augments RA-induced p21 and neuron specific enolase (NSE) protein expression. β-Actin protein was used as an internal control.*

transplantation (4). Histone deacetalyase inhibitors have also shown preclinical efficacy in NB models (22, 23). In fact, histone deacetalyase inhibitors have been shown to have synergistic effects with RA in eliciting NB differentiation (23, 24). These findings have prompted an ongoing phase I clinical trial evaluating the role of vorinostat with or without 13-cis-RA (National Cancer Institute, NCT01208454. ClinicalTrials.gov).

Intriguingly, silencing the class III histone deacetalyase *SIRT6* sensitized BE(2)-C to RA treatment, demonstrating synergistic neurite outgrowth and p21 induction. In addition to the involvement of SIRT6 in proliferation and differentiation of NB cells, we found a trend towards decreased SIRT6 expression in differentiated human NB specimens (Figure 4A and Table I). Our failure to demonstrate statistical significance likely reflects the low number of tissue samples available in our microarray. In addition, we discovered that SIRT6 is decreased in RA-induced differentiation and that low SIRT6 levels are necessary for optimal RA-induced neuronal differentiation. Surprisingly, the expression level of SIRT6 was increased in RA-treated *SIRT6*-overexpressing cells. Specifically, RA suppressed SIRT6 expression in control cells, while we observed a paradoxic increase in SIRT6 expression in the presence of RA when BE(2)-C cells were transfected with our pCDH-driven overexpression vector. We attribute this discrepancy to the effects of RA on the autologous CDH promoter utilized in our overexpression model. Interestingly, the paradoxic RA-induced SIRT6 expression appeared to attenuate the level of RA-induced p21 expression as compared to RA-treated controls. Overall, these findings suggest that SIRT6 is an independent regulator of NB differentiation and low levels are a necessary component for optimal induction of differentiation using RA derivatives.

Regulation of the cell cycle plays a pivotal role in proliferation and differentiation. Mammalian cell-cycle progression is controlled by interplay between positive and negative regulatory factors. Cyclin-dependent kinases (CDKs), composed of regulatory cyclin and catalytic CDK subunits, are activated in a periodic manner to promote cell-cycle transitions while another family of proteins, the CDK inhibitors, act in a negative fashion by quenching the activity of CDKs (25). We analyzed the effects of *SIRT6* knockdown on cell-cycle progression, noting initiation of G1 arrest and reduced S phase population compared to shCON cells. Cell-cycle arrest was successfully abolished by re-overexpression of *SIRT6*. Induction of *p21*, a CDK inhibitor, blocks the formation of cyclin-CDK2, -CDK1, and -CDK4,6 complexes that regulate cell-cycle progression with increased $G_1$ and decreased S phase populations. Our demonstration of $G_1$ arrest accompanied with *p21* induction suggests that this CDK inhibitor is a critical mediator of neuronal differentiation by *SIRT6* knockdown.

The exact mechanism by which *SIRT6* knockdown induces increased p21 and NSE expression are not clear. p53 is the most well-known upstream molecules of p21. Intriguingly, BE(2)-C cells have been previously shown to have defective p53 signaling due to loss of chromosome 17 and a nonfunctional missense mutation in the remaining gene copy (26). These findings suggest that the induction of *p21* is in fact independent of p53 activation. Future studies will focus on the exact mechanisms of induction of *p21* and *NSE* hypothesizing that *SIRT6* silencing mediates the epigenetic regulation of these critical regulators of proliferation and differentiation.

In summary, our results demonstrate that silencing of *SIRT6* suppresses NB tumorigenesis through regulation of differentiation and proliferation-related genes. Our findings identified a novel histone deacetalyase family member that is critical for neuronal differentiation in NB. No specific small molecule inhibitors of SIRT6 had been identified at the time of this study. However, one SIRT6 inhibitor was recently identified by Sociali *et al*.; this SIRT6 inhibitor improves glucose tolerance in a mouse model of type 2 diabetes (27). Another report showed that the SIRT3-specific inhibitor, LC-0296, inhibits cell survival and proliferation, and promotes apoptosis of head and neck cancer cells. Additionally, LC-0296 works synergistically to increase sensitivity to radiation and cisplatin treatment (28). It is necessary to evaluate the effects of these specific SIRT inhibitors in NB. In conclusion, our findings suggest that SIRT6 may represent a novel target for future drug development strategies in NB.

## Conflicts of Interest

The Authors declare no potential conflicts of interest in regard to this study.

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