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ORIGINAL ARTICLE

Histone deacetylase 5 blocks neuroblastoma cell differentiation by interacting with N-Myc

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The N-Myc oncoprotein induces neuroblastoma, which arises from undifferentiated neuroblasts in the sympathetic nervous system, by modulating gene and protein expression and consequently causing cell differentiation block and cell proliferation. The class IIa histone deacetylase 5 (HDAC5) represses gene transcription, and blocks myoblast, osteoblast and leukemia cell differentiation. Here we showed that N-Myc upregulated HDAC5 expression in neuroblastoma cells. Conversely, HDAC5 repressed the ubiquitin–protein ligase NEDD4 gene expression, increased Aurora A gene expression and consequently upregulated N-Myc protein expression. Genome-wide gene expression analysis and protein co-immunoprecipitation assays revealed that HDAC5 and N-Myc repressed the expression of a common subset of genes by forming a protein complex, whereas HDAC5 and the class III HDAC SIRT2 independently repressed the expression of another common subset of genes without forming a protein complex. Moreover, HDAC5 blocked differentiation and induced proliferation in neuroblastoma cells. Taken together, our data identify HDAC5 as a novel co-factor in N-Myc oncogenesis, and provide the evidence for the potential application of HDAC5 inhibitors in the therapy of N-Myc-induced neuroblastoma and potentially other c-Myc-induced malignancies.

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INTRODUCTION

Neuroblastoma, which arises from undifferentiated neuroblasts of the sympathetic nervous system, is the most common extracranial solid tumor in early childhood. Amplification of the *MYCN* oncogene and consequent N-Myc oncoprotein overexpression in neuroblastoma tissues correlate with poor prognosis in patients.[1–3]

Like c-Myc, N-Myc oncoprotein exerts tumorigenic function by modulating gene transcription[4–7] and by upregulating protein expression,[8,9] leading to cell differentiation block and cell proliferation. N-Myc oncoprotein is degraded by the E3 ubiquitin–protein ligase Fbxw7[10,11] and NEDD4[12] and stabilized by Aurora A.[11]

Histone deacetylases (HDACs) have emerged as essential modulators in gene transcription.[13] To date, 18 HDACs have been identified in humans and grouped into four classes (classes I–IV). Based on their similarity to yeast HDACs, class II HDACs are subdivided into class IIa (HDAC4, 5, 7 and 9) and class IIb (HDAC6 and 10). The class IIa HDAC5 has a crucial role in cell differentiation and development in normal human tissues including myocardium, skeletal muscle and brain.[14–16] Multiple lines of evidence also suggest that HDAC5 contributes to tumorigenesis. For instance, upregulation of HDAC5 enhances medulloblastoma cell survival, and a high level of HDAC5 expression in tumor tissues is associated with poor prognosis in medulloblastoma patients.[17] In addition, HDAC5 blocks leukemia cell differentiation,[18] enhances cancer cell proliferation, and blocks cancer cell apoptosis and autophagy.[19]

Here, we investigated the role of HDAC5 in *MYCN* overexpressing neuroblastoma cells. We demonstrated that HDAC5 protein expression was increased by N-Myc, and that HDAC5 upregulated N-Myc oncoprotein expression in neuroblastoma cells. HDAC5 and N-Myc, and HDAC5 and SIRT2, repressed the expression of different common subsets of genes through different mechanisms, and HDAC5 blocked cell differentiation and induced cell proliferation.

RESULTS

N-Myc upregulates the expression of HDAC5 protein, which in turn upregulates the expression of N-Myc protein, in neuroblastoma cells

Myc oncoproteins modulate gene and protein expression by directly binding to target gene promoters,[5] leading to transcriptional activation, and by enhancing ribosome biogenesis and mRNA translation to protein.[8,9] As both N-Myc and HDAC5 are known to block cell differentiation, we examined whether N-Myc modulated HDAC5 gene and protein expression. As shown in Figures 1a and b, transfection of *MYCN*-amplified BE(2)-C and CHP134 human neuroblastoma cells with two different N-Myc siRNAs (N-Myc siRNA-1 and N-Myc siRNA-2) significantly reduced N-Myc mRNA and protein expression. Although showing no effect on HDAC5 mRNA expression (Figure 1a), N-Myc siRNA-1 and N-Myc siRNA-2 reduced HDAC5 protein expression in BE(2)-C and CHP134 cells (Figure 1b). These data demonstrate that N-Myc

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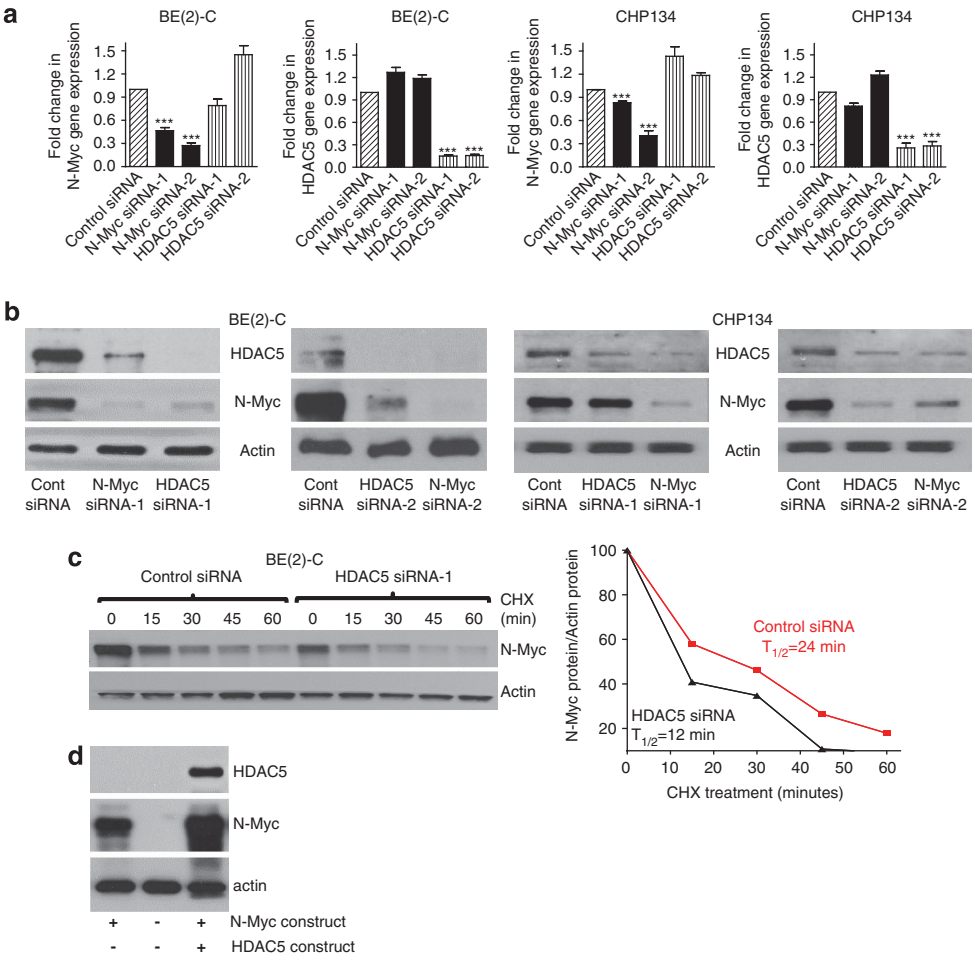

Figure 1. N-Myc upregulates HDAC5 protein expression, and HDAC5 upregulates N-Myc protein expression in neuroblastoma cells. (**a**, **b**) BE(2)-C and CHP134 cells were transfected with scrambled control (Cont) siRNA, N-Myc siRNA-1, N-Myc siRNA-2, HDAC5 siRNA-1 or HDAC5 siRNA-2 for 48 h, followed by RNA and protein extraction, real-time RT-PCR (**a**) and immunoblot (**b**) analyses of N-Myc and HDAC5 mRNA and protein expression. Error bars represent s.e. $***P<0.001$. (**c**) BE(2)-C cells were transfected with scrambled control siRNA or HDAC5 siRNA-1 for 48 h, and treated with 50 μM cycloheximide (CHX) for the last 0, 15, 30, 45 or 60 min. Protein was extracted from the cells and subjected to immunoblot analysis of N-Myc and actin. N-Myc protein level was normalized by actin, the ratio of N-Myc protein/actin protein was artificially set as 1.0 for samples un-treated with CHX, and half-life ($T_{1/2}$) of N-Myc protein was obtained from the line chart. (**d**) HEK293 cells were co-transfected with a pcDNA3.1-empty vector or pcDNA3.1-HDAC5 expression construct, together with a pShuttle-empty vector or a pShuttle-N-Myc expression construct for 48 h. Protein was extracted for immunoblot analysis of N-Myc, HDAC5 and actin protein expression.

upregulates HDAC5 protein expression through a post-transcriptional mechanism.

Figure 1 also showed that the expression of N-Myc protein (Figure 1b), but not N-Myc mRNA (Figure 1a), was reduced, when HDAC5 was knocked down by HDAC5 siRNA-1 or HDAC5 siRNA-2. We next treated BE(2)-C cells with 50 μM cycloheximide for different time points after transfection with control siRNA or HDAC5 siRNA-1 for 48 h. Immunoblot analysis showed that N-Myc protein half-life was reduced from approximately 24 min in BE(2)-C cells transfected with control siRNA to approximately 12 min in BE(2)-C cells transfected with HDAC5 siRNA-1 (Figure 1c). To further confirm that HDAC5 modulates N-Myc protein expression, we transfected human embryonic HEK293 cells, which do not express endogenous N-Myc protein, with a Flag-tagged N-Myc expression construct and/or a Flag-tagged HDAC5 expression construct for 48 h. Immunoblot analysis showed that overexpression of HDAC5 increased N-Myc protein expression in the embryonic HEK293 cells (Figure 1d). As N-Myc protein is stabilized when phosphorylated at serine 62 (S62) and destabilized when phosphorylated at threonine 58 (T58),[11] we next co-transfected HEK293 cells with a construct expressing empty vector or HDAC5, together with a construct expressing empty vector, S62 mutant (S62A) N-Myc or T58 mutant (T58A) N-Myc. Immunoblot analysis showed that overexpression of HDAC5 significantly upregulated the expression of total N-Myc protein in cells transfected with S62 mutant N-Myc, and slightly upregulated the expression of total N-Myc protein in cells transfected with T58 mutant N-Myc (Supplementary Figure S1). Taken together, these data indicate that HDAC5 upregulates N-Myc protein expression by increasing N-Myc protein stability.

HDAC5 represses the expression of the ubiquitin–protein ligase NEDD4 and upregulates the expression of Aurora A

HDACs exert biological effects by modulating gene transcription and protein stability. To understand how HDAC5 upregulates N-Myc protein expression, we performed differential gene expression studies with Affymetrix gene assay in BE(2)-C cells 30 h after transfection with control siRNA, HDAC5 siRNA-1 or N-Myc siRNA-1. Results from the microarray hybridization were loaded into R package (http://www.r-project.org/), followed by analysis with BioConductor software packages (http://www.bioconductor.org/). As shown in Supplementary Table S1, one of

the genes most significantly reactivated by HDAC5 siRNA-1 was the E3 ubiquitin–protein ligase NEDD4, which is known to target N-Myc protein for ubiquitination and degradation.[12] Importantly, NEDD4 was also upregulated by N-Myc siRNA-1, although below twofold and not listed in Supplementary Table 2, in BE(2)-C cells 30 h after siRNA transfection. To validate the Affymetrix microarray data, we performed chromatin immunoprecipitation (ChIP) assays with an anti-N-Myc antibody, an anti-HDAC5 antibody, an anti-acetylated histone H3 (acetyl-H3) antibody or a control antibody. The immunoprecipitated DNA was PCR amplified using primers targeting the negative control region or core promoter region of the NEDD4 gene promoter. The ChIP assays showed that the anti-N-Myc and the anti-HDAC5 antibodies efficiently immunoprecipitated the region of the NEDD4 gene core promoter, and that knocking down HDAC5 expression with HDAC5 siRNA-1 in BE(2)-C cells increased the presence of acetyl-H3 at the NEDD4 gene core promoter by approximately twofold (Figure 2a). Consistent with these results, real-time reverse transcriptase–PCR (RT–PCR) and immunoblot studies demonstrated that the expression of NEDD4 mRNA (Figure 2b) and protein (Figure 2c) was upregulated in BE(2)-C and CHP134 cells after transfection with N-Myc siRNA-1, N-Myc siRNA-2, HDAC5 siRNA-1 or HDAC5 siRNA-2.

The Affymetrix microarray data also showed that Aurora A, which is known to be upregulated by N-Myc and to stabilize N-Myc protein,[11] was slightly downregulated by HDAC5 siRNA-1. Consistent with this finding, our real-time RT–PCR and immunoblot studies showed that HDAC5 siRNA-1 and HDAC5 siRNA-2 reduced Aurora A mRNA (Figure 2d) and protein (Figure 2e) expression in BE(2)-C and CHP134 cells. Taken together, the results suggest that HDAC5 upregulates N-Myc protein expression by repressing NEDD4 and upregulating Aurora A gene expression.

N-Myc and HDAC5 repress a common subset of genes by forming an N-Myc-HDAC5 protein complex

As both N-Myc and HDAC5 repressed NEDD4 gene expression (Figures 2b and c), we examined whether N-Myc and HDAC5 repressed a common subset of genes by Affymetrix microarray analysis of differential gene expression in BE(2)-C cells 30 h after transfection with control siRNA, N-Myc siRNA or HDAC5 siRNA (Supplementary Table S1). Results showed that N-Myc siRNA upregulated 3.32% (614/18 507) and downregulated 5.39% (998/18 507) of the genes represented on the Affymetrix microarray (Supplementary Table S2). Importantly, N-Myc siRNA upregulated

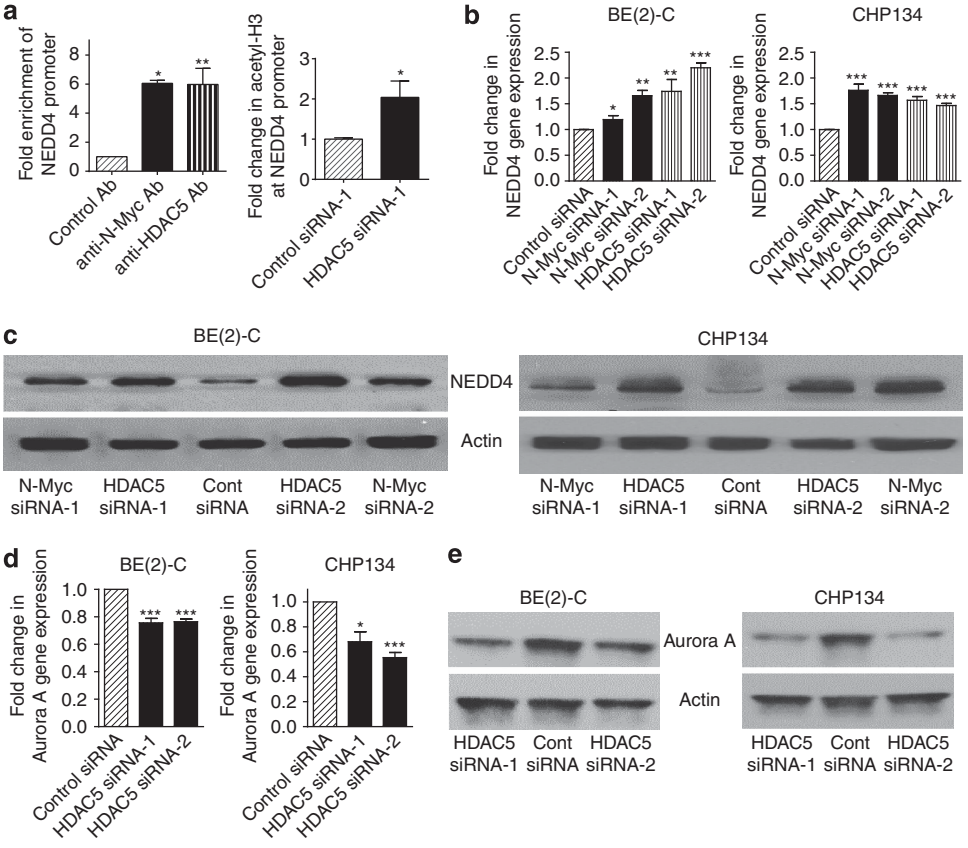

Figure 2. HDAC5 represses NEDD4 and upregulates Aurora A expression. (**a**) ChIP assays were performed in BE(2)-C cells with an anti-N-Myc antibody (Ab), anti-HDAC5 Ab, anti-acetylated histone H3 (acetyl-H3) Ab or negative control Ab, and real-time PCR with primers targeting control region (− 2500-bp upstream of the NEDD4 gene transcription start site) or primers targeting part of the NEDD4 gene core promoter region (− 394 to − 263-bp upstream of the NEDD4 gene transcription start site). Fold enrichment of NEDD4 gene core promoter by the control antibody, anti-N-Myc or anti-HDAC5 antibody was calculated by dividing PCR products from primers targeting the NEDD4 gene core promoter by PCR products from primers targeting control region, and fold enrichment by control antibody was artificially set as 1.0. Fold change in acetyl-H3 at NEDD4 gene core promoter was obtained after dividing fold enrichment of acetyl-H3 at NEDD4 gene core promoter in HDAC5 siRNA-1-transfected samples by fold enrichment of acetyl-H3 at NEDD4 gene core promoter in control siRNA-transfected samples, and fold enrichment by control siRNA was artificially set as 1.0. (**b**, **c**) BE(2)-C and CHP134 cells were transfected with control (Cont) siRNA, N-Myc siRNA-1, N-Myc siRNA-2, HDAC5 siRNA-1 or HDAC5 siRNA-2 for 48 h. NEDD4 mRNA and protein expression was analyzed by real-time RT-PCR (**b**) and immunoblot (**c**). (**d**, **e**), BE(2)-C and CHP134 cells were transfected with control (Cont) siRNA, HDAC5 siRNA-1 or HDAC5 siRNA-2 for 48 h, followed by real-time RT-PCR (**d**) and immunoblot (**e**) analyses of Aurora A mRNA and protein expression. Error bars represent s.e. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

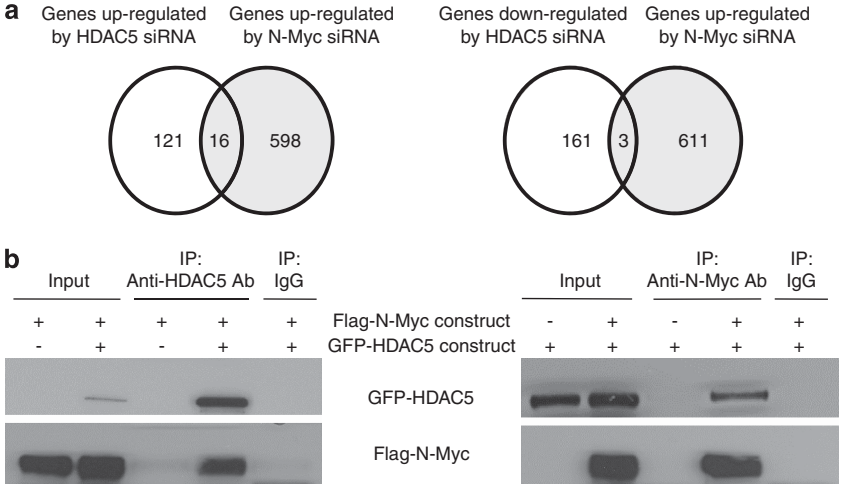

Figure 3. N-Myc and HDAC5 repress a common subset of genes by forming an N-Myc-HDAC5 protein complex. (**a**) Genes commonly modulated by N-Myc siRNA and HDAC5 siRNA by more than twofold, as identified by Affymetrix microarray analysis, in neuroblastoma BE(2)-C cells 30 h after siRNA transfections. (**b**) HEK293 cells were co-transfected with a pShuttle-Flag-N-Myc construct and a pcDNA3.1-GFP-empty vector or pcDNA3.1-GFP-HDAC5 construct (left panel), or co-transfected with a pcDNA3.1-GFP-HDAC5 construct and a pShuttle-Flag-empty vector or a pShuttle-Flag-N-Myc construct (right panel), followed by protein extraction and co-immunoprecipitation (IP) with a control immunoglobulin G (IgG), an anti-N-Myc or an anti-HDAC5 antibody (Ab). Eluted proteins were immunoblotted with an anti-Flag or an anti-GFP antibody.

the expression of 11.68% (16/137) of the genes upregulated by HDAC5 siRNA (Supplementary Table S3), but only 2.19% (3/164) of the genes downregulated by HDAC5 siRNA (Supplementary Table S4 and Figure 3a). The data indicate that N-Myc and HDAC5 repress the expression of a common subset of genes.

We next investigated whether HDAC5 protein directly bound to N-Myc protein and formed a protein complex. Human embryonic HEK293 cells were transfected with an empty vector, an N-Myc expression construct and/or an HDAC5 expression construct, followed by protein extraction and co-immunoprecipitation with either an anti-N-Myc or an anti-HDAC5 antibody. The results showed that N-Myc antibody efficiently co-immunoprecipitated HDAC5 protein, and the anti-HDAC5 antibody efficiently co-immunoprecipitated N-Myc protein (Figure 3b), which strongly suggests that these two proteins form a protein complex.

The class III HDAC SIRT2 and the class IIa HDAC5 independently repress the expression of a common subset of genes without forming a protein complex

The class III HDAC SIRT2 has also been shown to repress NEDD4 gene transcription.[12] We therefore examined whether SIRT2 and HDAC5 repress the expression of a common subset of genes. Affymetrix microarray analysis was performed to identify genes that were differentially expressed in BE(2)-C cells 30 h after transfection with control siRNA, SIRT2 siRNA or HDAC5 siRNA (Supplementary Table S1). The results showed that SIRT2 siRNA upregulated 0.68% (126/18 507) and downregulated 0.96% (178/18 507) of the genes represented on the Affymetrix microarray (Supplementary Table S5). Importantly, SIRT2 siRNA upregulated 29.20% (40/137) of the genes upregulated by HDAC5 siRNA (Supplementary Table S6), but 0% (0/164) of the genes downregulated by HDAC5 siRNA (Figure 4a). The data indicate that SIRT2 and HDAC5 repress the expression of a common subset of genes.

We next investigated whether HDAC5 protein directly bound to SIRT2 protein. Human embryonic HEK293 cells were transfected with an empty vector, a SIRT2 expression construct and/or an HDAC5 expression construct, followed by protein extraction and co-immunoprecipitation with either an anti-SIRT2 or an anti-HDAC5 antibody. Results showed that the anti-SIRT2 antibody could not co-immunoprecipitate HDAC5 protein, and the anti-HDAC5 antibody could not co-immunoprecipitate SIRT2 protein (Figure 4b). Together, the data indicate that SIRT2 and HDAC5 do not form a protein complex, but repress the expression of a common subset of genes.

HDAC5 blocks neuroblastoma cell differentiation

HDAC5 has been shown to block myoblast and leukemia cell differentiation.[14,18] We next examined whether HDAC5 contributed to the block in differentiation induced by N-Myc-expression in neuroblastoma cells. BE(2)-C and CHP134 cells were transfected with control siRNA, N-Myc siRNA-1, N-Myc siRNA-2, HDAC5 siRNA-1 or HDAC5 siRNA-2. Ninety-six hours post-transfection, cells were subjected to immunocytochemistry study with an antibody against β-III tubulin, a well-known marker for neuronal differentiation.[20] As shown in Figure 5 and Supplementary Figure S2, compared with control siRNA, N-Myc siRNA-1, N-Myc siRNA-2, HDAC5 siRNA-1 and HDAC5 siRNA-2 all induced neurite outgrowth in BE(2)-C (Figure 5a) and CHP134 (Figure 5b) cells. Moreover, RT–PCR analysis revealed that HDAC5 siRNAs increased the expression of growth associated protein 43, tyrosine hydroxylase, maternally expressed 3 and β-III tubulin, which are markers for neuronal differentiation,[20–23] in the two neuroblastoma cell lines (Figures 5c and d). The data suggest that HDAC5 blocks neuroblastoma cell differentiation.

HDAC5 promotes neuroblastoma cell proliferation

To understand whether upregulation of HDAC5 leads to neuroblastoma cell proliferation and/or survival, we performed Alamar blue assays in neuroblastoma cells after transfection with control siRNA, N-Myc siRNAs or HDAC5 siRNAs. We demonstrated that knocking down N-Myc gene expression with N-Myc siRNA-1 or N-Myc siRNA-2 for 72 h reduced the numbers of viable BE(2)-C and CHP134 cells by approximately 30–40% (Figure 6a). Similarly, knocking down HDAC5 gene expression with HDAC5 siRNA-1 or HDAC5 siRNA-2 for 72 h reduced the numbers of viable BE(2)-C and CHP134 cells by approximately 40–70% (Figure 6a). To understand whether HDAC5 siRNAs induced cell death, we performed cell cycle analysis in BE(2)-C and CHP134 cells 72 h after transfection with control siRNA, HDAC5 siRNA-1 or

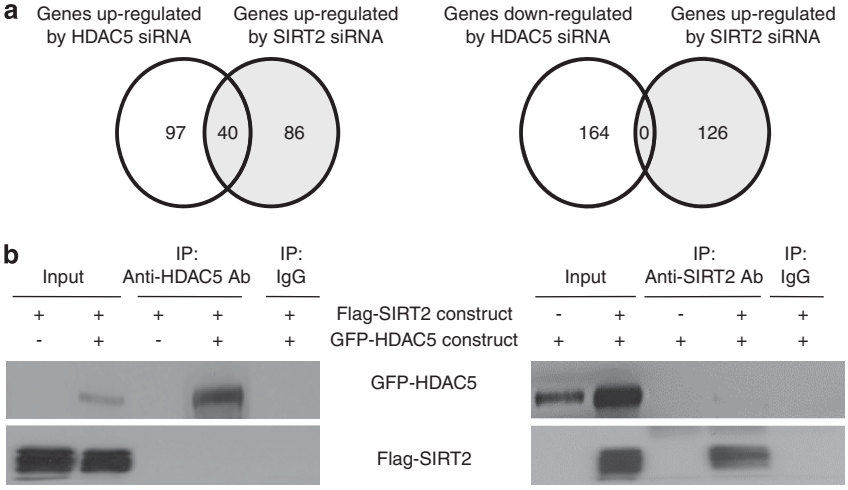

Figure 4. SIRT2 and HDAC5 independently repress the expression of a common subset of genes without forming a protein complex. (**a**) Genes commonly modulated by SIRT2 siRNA and HDAC5 siRNA by more than twofold, as identified by Affymetrix microarray analysis, in neuroblastoma BE(2)-C cells 30 h after siRNA transfections. (**b**) HEK293 cells were co-transfected with a pcDNA3.1-Flag-SIRT2 construct and a pcDNA3.1-GFP-empty vector or pcDNA3.1-GFP-HDAC5 construct (left panel), or co-transfected with pcDNA3.1-GFP-HDAC5 construct and a pcDNA3.1-Flag-empty vector or pcDNA3.1-Flag-SIRT2 construct (right panel), followed by protein extraction and co-immunoprecipitation (IP) with a control immunoglobulin G (IgG), an anti-SIRT2 or anti-HDAC5 antibody (Ab). Eluted proteins were immunoblotted with an anti-Flag or an anti-GFP antibody.

HDAC5 siRNA-2. Results showed that HDAC5 siRNAs did not consistently increase the percentage of cells at pre-G1 phase (Figure 6b). The data demonstrate that downregulation of HDAC5 impedes cell proliferation, but does not result in cell death.

DISCUSSION

Myc oncoproteins including c-Myc and N-Myc are well known to modulate gene transcription by directly binding to target gene promoters[5] and to upregulate protein expression by enhancing ribosome biogenesis, the formation of 7-methylguanosine caps on mRNAs and mRNA translation to protein.[8,9] In addition, Myc has recently been found to amplify the output of the existing gene expression program.[24,25] In this study, we have shown that N-Myc upregulates the expression of HDAC5 protein, but not HDAC5 mRNA, in *MYCN* overexpressing neuroblastoma cells. The data indicate that N-Myc upregulates the expression of HDAC5 via a post-transcriptional mechanism, possibly by enhancing HDAC5 protein synthesis.

Myc oncoproteins are well known to be degraded through the ubiquitin–protein ligase Fbxw7.[10,11] Recently, the HectH9/Huwe1 ubiquitin–protein ligase and the E3 ubiquitin–protein ligase NEDD4 have been shown to degrade,[12,26] whereas Aurora A has been shown to stabilize,[11] N-Myc oncoprotein. This study demonstrates that knocking down HDAC5 gene expression with siRNAs reduces the expression of N-Myc protein, but not N-Myc mRNA, and reduces N-Myc protein half-life by approximately 50% in human neuroblastoma cells. Consistently, forced overexpression of HDAC5 increases N-Myc protein expression in human embryonic cells transfected with N-Myc expression construct. RT–PCR and immunoblot studies reveal that repression of HDAC5 increases the expression of NEDD4 and decreases the expression of Aurora A. Taken together, our data suggest that HDAC5 upregulates N-Myc protein expression by repressing NEDD4 and enhancing Aurora A gene expression.

N-Myc represses the transcription of the tissue transglutaminase gene by recruiting HDAC1 protein to the tissue transglutaminase gene promoter, and represses the transcription of the cyclin G2 gene by recruiting HDAC2 to the cyclin G2 gene promoter.[27,28] As a HDAC, HDAC5 is well known to deacetylate histone H3 and histone H4, to partly block the histone acetylating effects of the HDAC inhibitor trichostatin A, and to repress gene transcription by forming transcriptional repressor complexes with transcriptional co-repressors at gene promoters.[29–33] Our previous Affymetrix microarray studies have identified NEDD4 as one of the genes most significantly upregulated by SIRT2 siRNA. This study has shown that NEDD4 is upregulated by N-Myc siRNA and is one of the genes most significantly reactivated by HDAC5 siRNA, that both N-Myc protein and HDAC5 protein bind to the NEDD4 gene core promoter, and that knocking down HDAC5 expression results in histone H3 acetylation at NEDD4 gene promoter. Genome-wide gene expression analysis reveal that N-Myc siRNA and HDAC5 siRNA upregulate the expression of a common subset of genes, whereas SIRT2 siRNA and HDAC5 siRNA upregulate the expression of another common subset of genes. Importantly, protein co-immunoprecipitation assays reveal that HDAC5 protein forms a complex with N-Myc protein, but not SIRT2 protein. Taken together, our data provide the first evidence that N-Myc oncoprotein can bind to the class IIa HDAC5, that Myc oncoproteins may possess a more widespread capacity for transcriptional repression by recruitment of class IIa HDAC proteins to target gene promoters and that Myc-mediated transcriptional repression could be reversed by inhibiting HDACs. By contrast, the class IIa HDAC5 and class III HDAC SIRT2 repress gene transcription independent of each other, although they repress the expression of a common subset of genes, possibly through similar mechanisms.

Unlike class I, IIb and III HDACs, class IIa HDACs including HDAC5 are mainly involved in cell differentiation block and cell proliferation (reviewed in Martin *et al.*[34]). HDAC5 blocks myoblast cell differentiation by binding to and inhibiting the activity of myocyte enhancer factor 2.[14] In osteoblast cells, HDAC5 interacts with Smad3 and Runx2 at the Runx2-binding DNA sequences, leading to histone deacetylation, repression of Runx2 target gene expression and osteoblast cell differentiation block.[35] Moreover, HDAC5 suppresses the transcriptional activity of the transcription factor GATA-1, and blocks murine erythroleukemia cell differentiation.[18] In this study, we demonstrate for the first time that knocking down HDAC5 gene expression results in neuroblastoma cell differentiation, which is characterized by neurite outgrowth. As N-Myc is known to block neuroblastoma cell differentiation, we hypothesize that HDAC5 blocks neuroblastoma

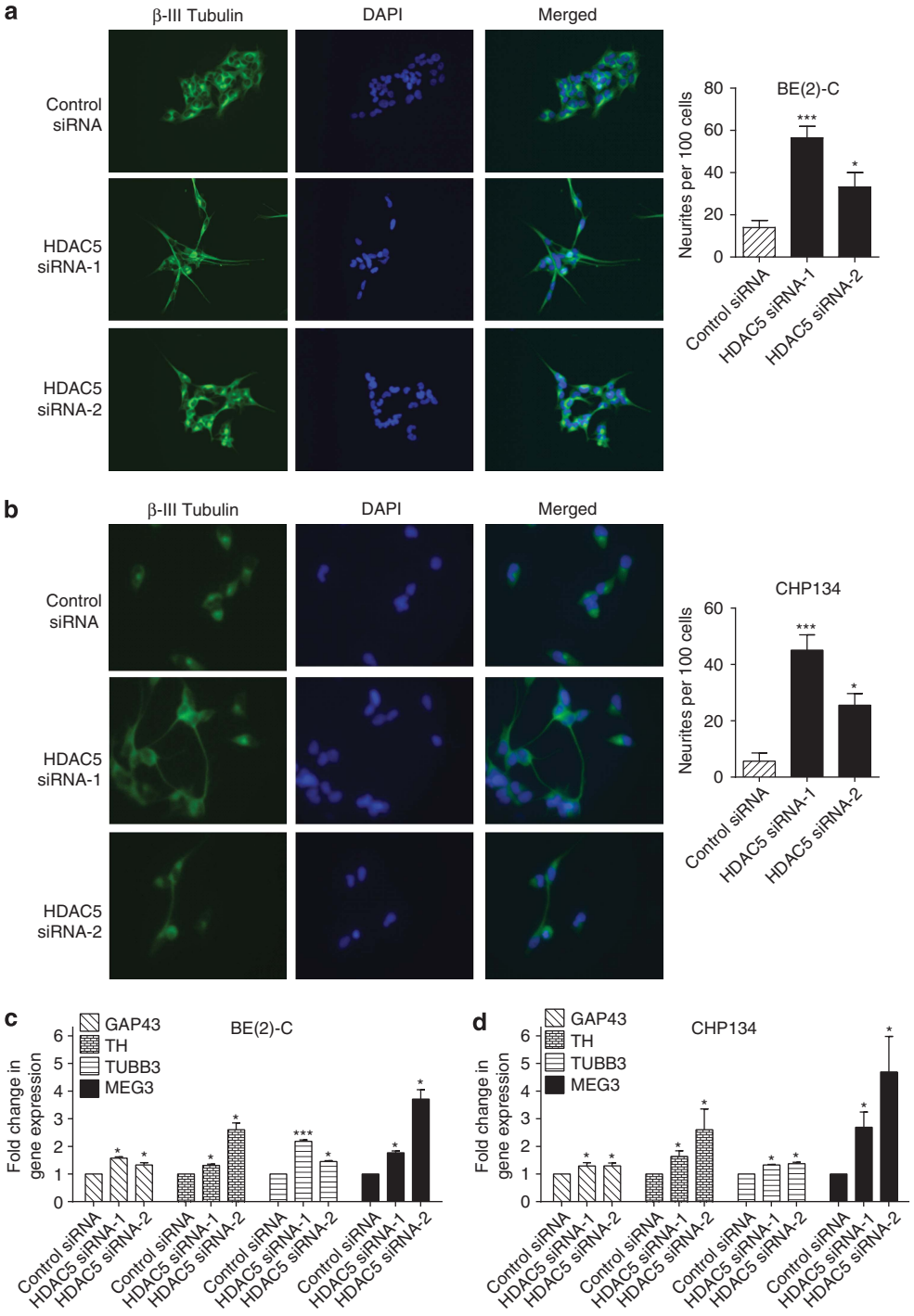

Figure 5. HDAC5 blocks neuroblastoma cell differentiation. (**a**, **b**) Neuroblastoma BE(2)-C (**a**) and CHP134 (**b**) cells were transfected with scrambled control siRNA, HDAC5 siRNA-1 or HDAC5 siRNA-2. After 96 h, cells were fixed and incubated with a rabbit anti-β-III tubulin antibody, followed a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody and 4,6-diamidino-2-phenylindole (DAPI). Cell differentiation was assessed by analyzing neurite outgrowth and expressed as the number of neurites per 100 cells. (**c**, **d**) Neuroblastoma BE(2)-C (**c**) and CHP134 (**d**) cells were transfected with scrambled control siRNA, HDAC5 siRNA-1 or HDAC5 siRNA-2 for 96 h, followed by RNA extraction and real-time RT-PCR analyses of the cell differentiation markers growth associated protein 43 (GAP43), tyrosine hydroxylase (TH), maternally expressed 3 (MEG3) and β-III tubulin (TUBB3). Error bars represent s.e. $^{*}P<0.05$ and $^{***}P<0.001$.

cell differentiation by upregulating N-Myc protein expression and/or by forming a transcriptional repressor complex with N-Myc protein, leading to transcriptional regulation of target genes involved in neuroblastoma cell differentiation.

HDAC5 is associated with actively replicating pericentric heterochromatin during late S phase, and has an important role in heterochromatin maintenance and assembly, cell cycle progression and survival.[19] Furthermore, HDAC5 protein is overexpressed in human medulloblastoma tissues, and knocking down HDAC5 expression leads to medulloblastoma cell growth inhibition and cell death.[17] This study reveals that repression of HDAC5 gene expression with siRNAs leads to neuroblastoma cell

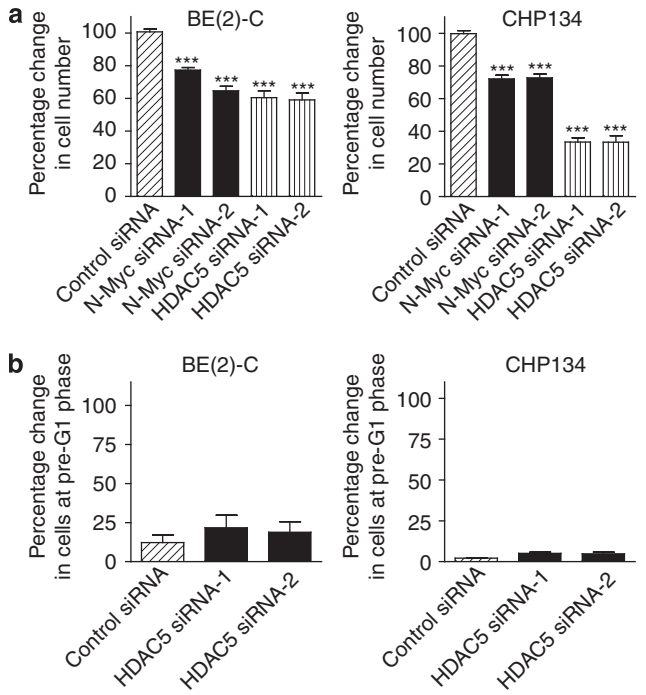

Figure 6. HDAC5 promotes neuroblastoma cell proliferation. (**a**, **b**) BE(2)-C and CHP134 cells were transfected with scrambled control siRNA, N-Myc siRNA-1, N-Myc siRNA-2, HDAC5 siRNA-1 or HDAC5 siRNA-2 for 72 h. (**a**) The number of viable neuroblastoma cells was examined by Alamar blue assays, and expressed as percentage change in cell numbers. (**b**) Cells were stained with propodium iodide, and subjected to flow cytometry analyses of cell cycle. The percentage of cells at pre-G1 phase was calculated with CellQuest cell cycle analysis program. Error bars represent s.e. $^{***}P < 0.001$.

growth inhibition, but not cell death. This is consistent with our previous observation that N-Myc induces proliferation, but shows no effect on survival, in neuroblastoma cells.[27,28]

In summary, this study demonstrates that N-Myc upregulates HDAC5 protein expression, and that HDAC5 represses NEDD4 gene expression, increases Aurora A gene expression and consequently upregulates N-Myc protein expression in neuroblastoma cells. HDAC5 and N-Myc repress the expression of a common subset of genes by forming a protein complex, whereas HDAC5 and SIRT2 independently repress the expression of another common subset of genes. Moreover, HDAC5 blocks cell differentiation and induces cell proliferation. Taken together, our data identify HDAC5 as a crucial co-factor in N-Myc oncogenesis, and provide the evidence for the potential application of HDAC5 inhibitors for the therapy of N-Myc-induced neuroblastoma and potentially other c-Myc-induced malignancies.

MATERIALS AND METHODS

Cell culture

BE(2)-C human neuroblastoma and HEK293 primary embryonic cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. CHP134 human neuroblastoma cells were cultured in RPMI 1640 medium supplemented with L-glutamine and 10% fetal calf serum.

siRNA and plasmid transfection

Cells were transfected with siRNAs from Qiagen (Hamburg, Germany) or Ambion (Austin, TX, USA) or plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) with the protocol we described previously.[28,36,37]

Real-time RT–PCR

Gene expression in tumor cells was examined by quantitative real-time PR–PCR as described previously, with the expression of β-actin mRNA as a loading control.[28,36]

Immunoblot analysis

For the analysis of protein expression by immunoblot, cells were lysed, protein extracted and separated by gel electrophoresis. After western transfer, membranes were probed with mouse anti-N-Myc antibody (1:1000; Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit anti-NEDD4 (1:500), rabbit anti-Aurora A (1:500; Cell Signaling, Danvers, MA, USA), rabbit anti-HDAC5 (1:500), rabbit anti-GFP (1:10 000; both from Abcam, Cambridge, MA, USA) and mouse anti-Flag (1:2000; Sigma, St Louis, MO, USA). Protein bands were visualized with SuperSignal (Pierce, Rockford, IL, USA). The membranes were lastly re-probed with an anti-actin antibody (Sigma) as loading controls.

Alamar blue assays

Alamar blue assays were carried out as we described previously.[38] Briefly, cells were transfected with various siRNAs in 96-well plates. After 72 h, cells were incubated with Alamar blue (Invitrogen) for 5 h, then plates were read on a microplate reader at 570/595 nm. Results were calculated according to the optical density absorbance units and expressed as percentage change in cell number.

Cell cycle analysis

Seventy-two hours after siRNA transfection, cells were harvested and then re-suspended at a concentration of 2×10^6 cells/ml in solution containing 2 μg/ml RNase (Sigma) and 50 μg/ml propidium iodide (Sigma). Cells were then run on FACScan (Becton Dickinson, Bedford, MA, USA), and cell cycle was analyzed with CellQuest software (Becton Dickinson).

Cell differentiation assay

Ninety-six hours after transfections, cells were fixed and incubated with a rabbit antibody against the neuronal differentiation marker β-III tubulin (Covance, Princeton, NJ, USA), followed by a fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G and 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Cell images were captured under a fluorescence microscope and stored. Cell differentiation was assessed by analyzing neurite outgrowth as we described and expressed as neurites per 100 cells.[27]

Affymetrix microarray study

BE(2)-C neuroblastoma cells were transfected with scrambled control siRNA, HDAC5 siRNA-1 or N-Myc siRNA-1. Thirty hours after transfection, RNA was extracted from the cells with RNeasy mini kit (Qiagen). Differential gene expression was examined with Affymetrix GeneChip HG-U133 Plus 2 Arrays (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. Results from the microarray hybridization were loaded into R package, followed by analysis with BioConductor software packages.

Protein co-immunoprecipitation assays

HEK293 human embryonic cells were transiently co-transfected with a pcDNA3.1-green fluorescent protein (GFP)-empty vector or pcDNA3.1-GFP-HDAC5 construct,[14] together with pShuttle Flag-empty vector or pShuttle-Flag-N-Myc construct[12] with Lipofectamine 2000 (Invitrogen). In separate experiments, the cells were co-transfected with a pcDNA3.1-GFP-empty vector or pcDNA3.1-GFP-HDAC5 construct, together with a pcDNA3.1-Flag-empty vector or pcDNA3.1-Flag-SIRT2 construct.[12] Thirty-six hours after transfections, cellular protein was extracted and incubated overnight with 2 μg of a control, anti-N-Myc, anti-HDAC5 or anti-SIRT2 antibody. Eluted proteins were immunoblotted with an anti-Flag or anti-GFP antibody.

ChIP assays

ChIP assays were performed with an anti-N-Myc, anti-HDAC5 or control mouse antibody and PCR with primers targeting negative control region or core promoter region of NEDD4 gene promoter with the protocol we have described.[27] Fold enrichment of NEDD4 gene core promoter by

the anti-N-Myc or anti-HDAC5 antibody was calculated by dividing the PCR product from NEDD4 gene core promoter region by the PCR product from the negative control region.

Statistical analysis

All data for statistical analysis were calculated as mean ± s.e. Differences were analyzed for significance using analysis of variance among groups or unpaired *t*-test for two groups. A probability value of 0.05 or less was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003; **3**: 203–216.
2 Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet* 2007; **369**: 2106–2120.
3 Gustafson WC, Weiss WA. Myc proteins as therapeutic targets. *Oncogene* 2010; **29**: 1249–1259.
4 Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, Greene J *et al.* Genomic targets of the human c-myc protein. *Genes Dev* 2003; **17**: 1115–1129.
5 Patel JH, Loboda AP, Showe MK, Showe LC, McMahon SB. Analysis of genomic targets reveals complex functions of myc. *Nat Rev Cancer* 2004; **4**: 562–568.
6 Pelengaris S, Khan M, Evan G. C-myc: more than just a matter of life and death. *Nat Rev Cancer* 2002; **2**: 764–776.
7 Eilers M, Eisenman RN. Myc's broad reach. *Genes Dev* 2008; **22**: 2755–2766.
8 van Riggelen J, Yetil A, Felsher DW. Myc as a regulator of ribosome biogenesis and protein synthesis. *Nat Rev Cancer* 2010; **10**: 301–309.
9 Cole MD, Cowling VH. Transcription-independent functions of myc: regulation of translation and DNA replication. *Nat Rev Mol Cell Biol* 2008; **9**: 810–815.
10 Welcker M, Orian A, Jin J, Grim JE, Harper JW, Eisenman RN *et al.* The fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-myc protein degradation. *Proc Natl Acad Sci USA* 2004; **101**: 9085–9090.
11 Otto T, Horn S, Brockmann M, Eilers U, Schuttrumpf L, Popov N *et al.* Stabilization of n-myc is a critical function of aurora a in human neuroblastoma. *Cancer Cell* 2009; **15**: 67–78.
12 Liu PY, Xu N, Malyukova A, Scarlett CJ, Sun YT, Zhang XD *et al.* The histone deacetylase sirt2 stabilizes myc oncoproteins. *Cell Death Differ* 2013; **20**: 503–514.
13 Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* 2006; **5**: 769–784.
14 McKinsey TA, Zhang CL, Lu J, Olson EN. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature* 2000; **408**: 106–111.
15 Renthal W, Maze I, Krishnan V, Covington 3rd HE, Xiao G, Kumar A *et al.* Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron* 2007; **56**: 517–529.
16 Long X, Creemers EE, Wang DZ, Olson EN, Miano JM. Myocardin is a bifunctional switch for smooth versus skeletal muscle differentiation. *Proc Natl Acad Sci USA* 2007; **104**: 16570–16575.
17 Milde T, Oehme I, Korshunov A, Kopp-Schneider A, Remke M, Northcott P *et al.* Hdac5 and hdac9 in medulloblastoma: novel markers for risk stratification and role in tumor cell growth. *Clin Cancer Res* 2010; **16**: 3240–3252.
18 Watamoto K, Towatari M, Ozawa Y, Miyata Y, Okamoto M, Abe A *et al.* Altered interaction of hdac5 with gata-1 during mel cell differentiation. *Oncogene* 2003; **22**: 9176–9184.
19 Peixoto P, Castronovo V, Matheus N, Polese C, Peulen O, Gonzalez A *et al.* Hdac5 is required for maintenance of pericentric heterochromatin, and controls cell-cycle progression and survival of human cancer cells. *Cell Death Differ* 2012; **19**: 1239–1252.
20 Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M *et al.* Differentiation of mouse embryonic stem cells into a defined neuronal lineage. *Nat Neurosci* 2004; **7**: 1003–1009.
21 Manji SS, Sorensen BS, Klockars T, Lam T, Hutchison W, Dahl HH. Molecular characterization and expression of maternally expressed gene 3 (meg3/gtl2) rna in the mouse inner ear. *J Neurosci Res* 2006; **83**: 181–190.
22 Silvagno F, Guarnieri V, Capizzi A, Pescarmona GP. Synergistic effect of retinoic acid and dehydroepiandrosterone on differentiation of human neuroblastoma cells. *FEBS Lett* 2002; **532**: 153–158.
23 Summerhill EM, Wood K, Fishman MC. Regulation of tyrosine hydroxylase gene expression during differentiation of neuroblastoma cells. *Brain Res* 1987; **388**: 99–103.
24 Lin CY, Loven J, Rahl PB, Paranal RM, Burge CB, Bradner JE *et al.* Transcriptional amplification in tumor cells with elevated c-myc. *Cell* 2012; **151**: 56–67.
25 Nie Z, Hu G, Wei G, Cui K, Yamane A, Resch W *et al.* C-myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell* 2012; **151**: 68–79.
26 Zhao X, Heng JI, Guardavaccaro D, Jiang R, Pagano M, Guillemot F *et al.* The hect-domain ubiquitin ligase huwe1 controls neural differentiation and proliferation by destabilizing the n-myc oncoprotein. *Nat Cell Biol* 2008; **10**: 643–653.
27 Liu T, Tee AEL, Porro A, Smith SA, Dwarte T, Liu PY *et al.* Activation of tissue transglutaminase transcription by histone deacetylase inhibition as a therapeutic approach for myc oncogenesis. *Proc Natl Acad Sci* 2007; **104**: 18682–18687.
28 Marshall GM, Gherardi S, Xu N, Neiron Z, Trahair T, Scarlett CJ *et al.* Transcriptional upregulation of histone deacetylase 2 promotes myc-induced oncogenic effects. *Oncogene* 2010; **29**: 5957–5968.
29 Grozinger CM, Hassig CA, Schreiber SL. Three proteins define a class of human histone deacetylases related to yeast hda1p. *Proc Natl Acad Sci USA* 1999; **96**: 4868–4873.
30 Huang EY, Zhang J, Miska EA, Guenther MG, Kouzarides T, Lazar MA. Nuclear receptor corepressors partner with class ii histone deacetylases in a sin3-independent repression pathway. *Genes Dev* 2000; **14**: 45–54.
31 Kao HY, Downes M, Ordentlich P, Evans RM. Isolation of a novel histone deacetylase reveals that class i and class ii deacetylases promote smrt-mediated repression. *Genes Dev* 2000; **14**: 55–66.
32 Zhou X, Richon VM, Rifkind RA, Marks PA. Identification of a transcriptional repressor related to the noncatalytic domain of histone deacetylases 4 and 5. *Proc Natl Acad Sci USA* 2000; **97**: 1056–1061.
33 Lemercier C, Verdel A, Galloo B, Curtet S, Brocard MP, Khochbin S. Mhda1/hdac5 histone deacetylase interacts with and represses mef2a transcriptional activity. *J Biol Chem* 2000; **275**: 15594–15599.
34 Martin M, Kettmann R, Dequiedt F. Class iia histone deacetylases: regulating the regulators. *Oncogene* 2007; **26**: 5450–5467.
35 Kang JS, Alliston T, Delston R, Derynck R. Repression of runx2 function by tgf-beta through recruitment of class ii histone deacetylases by smad3. *EMBO J* 2005; **24**: 2543–2555.
36 Marshall GM, Liu PY, Gherardi S, Scarlett CJ, Bedalov A, Xu N *et al.* Sirt1 promotes n-myc oncogenesis through a positive feedback loop involving the effects of mkp3 and erk on n-myc protein stability. *PLoS Genet* 2011; **7**: e1002135.
37 Tee AEL, Marshall GM, Liu PY, Xu N, Haber M, Norris MD *et al.* Opposing effects of two tissue transglutaminase protein isoforms in neuroblastoma cell differentiation. *J Biol Chem* 2010; **285**: 3561–3567.
38 Liu T, Liu PY, Tee AEL, Haber M, Norris MD, Gleave ME *et al.* Over-expression of clusterin is a resistance factor to the anti-cancer effect of histone deacetylase inhibitors. *Eur J Cancer* 2009; **45**: 1846–1854.

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