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ORIGINAL ARTICLE

Transcriptional upregulation of histone deacetylase 2 promotes Myc-induced oncogenic effects

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Myc oncoproteins and histone deacetylases (HDACs) modulate gene transcription and enhance cancer cell proliferation, and HDAC inhibitors are among the most promising new classes of anticancer drugs. Here, we show that N-Myc and c-Myc upregulated *HDAC2* gene expression in neuroblastoma and pancreatic cancer cells, respectively, which contributed to N-Myc- and c-Myc-induced cell proliferation. Cyclin G2 (CCNG2) was commonly repressed by N-Myc and HDAC2 in neuroblastoma cells and by c-Myc and HDAC2 in pancreatic cancer cells, and could be reactivated by HDAC inhibitors. 5-bromo-2′-deoxyuridine incorporation assays showed that transcriptional repression of CCNG2 was, in part, responsible for N-Myc-, c-Myc- and HDAC2-induced cell proliferation. Dual crosslinking chromatin immunoprecipitation assay demonstrated that N-Myc acted as a transrepressor by recruiting the HDAC2 protein to Sp1-binding sites at the *CCNG2* gene core promoter. Moreover, HDAC2 was upregulated, and CCNG2 downregulated, in pre-cancerous and neuroblastoma tissues from N-Myc transgenic mice, and c-Myc overexpression correlated with upregulation of HDAC2 and repression of CCNG2 in tumour tissues from pancreatic cancer patients. Taken together, our data indicate the critical roles of upregulation of HDAC2 and suppression of CCNG2 in Myc-induced oncogenesis, and have significant implications for the application of HDAC inhibitors in the prevention and treatment of Myc-driven cancers.

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Introduction

Myc family oncoproteins are commonly upregulated in human cancer. *MYCN* oncogene amplification and consequent N-Myc oncoprotein overexpression are seen as a clonal feature in 20–25% of tumours, and correlate with poor prognosis in patients with neuroblastoma (Maris and Matthay, 1999; Brodeur, 2003). *MYC* oncogene amplification and consequent c-Myc oncoprotein overexpression occur in 54% of pancreatic cancer cell lines (Mahlamaki *et al*., 2002) and 33% in human primary pancreatic tumours (Schleger *et al*., 2002).

Myc oncoproteins induce malignant transformation by binding to cognate DNA sequences and modulating gene transcription, leading to cell proliferation (Pelengaris *et al*., 2002; Fernandez *et al*., 2003; Patel *et al*., 2004). Myc oncoproteins are well known to induce gene transcription by direct binding to the Myc responsive element E-Box at target gene promoters. Recently, we have demonstrated that Myc oncoproteins repress transcription of the tissues transglutaminase gene by recruiting histone deacetylase (HDAC) 1 protein to the tissue transglutaminase gene promoter (Liu *et al*., 2007).

Recruitment of HDAC proteins to gene promoters induces histone hypo-acetylation and transcriptional repression, particularly of tumour suppressor genes (Marks *et al*., 2001). Multiple lines of evidence demonstrate that histone hypo-acetylation is a necessary step in tumour initiation and progression. For example, in a comprehensive panel of normal cells, cancer cell lines, normal tissues and primary tumours, a global loss of mono-acetylation of histone H4 is seen only in cancer cells and is associated with the early stage of tumourigenesis (Fraga *et al*., 2005). This histone hypo-acetylation is suggested to be a common hallmark of cancer, and termed a 'cancer signature'.

One of the HDAC proteins which are overexpressed in human tumour tissues is HDAC2. HDAC2 overexpression promotes tumour cell proliferation and blocks apoptosis (Huang *et al*., 2005; Hrzenjak *et al*., 2006). In patients with pancreatic (Fritsche *et al*., 2009)

or colon (Weichert *et al*., 2008) cancer, HDAC2 is overexpressed in tumour tissues and the overexpression correlates with an advanced disease stage and/or poor prognosis.

HDAC2 is transcriptionally upregulated by c-Myc in colon cancer cells deficient in the tumour suppressor gene, adenomatosis polyposis coli (Zhu *et al*., 2004), and is one of the genes most significantly upregulated in N-Myc overexpressing, compared with N-Myc low-expressing, neuroblastoma cell lines in a complementary DNA microarray study (Raetz *et al*., 2003). We have, therefore, examined the mechanism by which N-Myc upregulates *HDAC2* gene expression, and in turn, the mechanism by which HDAC2 modulates the oncogenic effects of N-Myc in neuroblastoma and c-Myc in pancreatic cancer.

Results

N-Myc oncoprotein upregulates HDAC2 *gene expression by directly binding to its promoter*

We first performed real-time reverse transcription PCR (RT–PCR) analysis of *HDAC2* gene expression in *MYCN*-amplified BE(2)-C and *MYCN* stable transfectant SHEP-S1 neuroblastoma cells after transfection with scrambled control small interfering RNA (siRNA) or N-Myc specific siRNAs. As shown in Figure 1a, both N-Myc siRNA no.1 (N-Myc siRNA-1) and no.2 (N-Myc siRNA-2) significantly reduced N-Myc and HDAC2 gene expression. To confirm that overexpression of N-Myc was sufficient to induce HDAC2 gene expression, we extracted primary bone marrow-derived B cells from normal mice, and transfected them with retroviral constructs overexpressing the full-length human N-Myc complementary DNA or empty vector. As shown in Figure 1b, N-Myc overexpression induced HDAC2 mRNA expression by about 2.8-fold in the primary bone marrow-derived B cells.

The N-Myc oncoprotein can induce gene transcription by directly binding to a Myc-responsive element E-Box at target gene promoters. Our promoter analysis confirmed a Myc-responsive element E-Box at –380 bp upstream of the *HDAC2* gene transcription start site (Figure 1c). Chromatin immunoprecipitation (ChIP) assay with an anti-N-Myc antibody and PCR primers targeting this E-Box confirmed that N-Myc protein directly bound to the *HDAC2* gene core promoter (Figure 1d).

Transcriptional upregulation of HDAC2 promotes neuroblastoma cell proliferation

HDAC2 has been shown to induce cancer cell proliferation and to block apoptosis (Huang *et al*., 2005; Hrzenjak *et al*., 2006). We therefore examined whether N-Myc-induced transcriptional upregulation of HDAC2 contributed to an N-Myc-induced cancer phenotype. As shown in Figure 2a, N-Myc siRNA-1 and N-Myc siRNA-2 reduced N-Myc and HDAC2 protein expression, wehreas HDAC2 siRNA-1 and HDAC2 siRNA-2

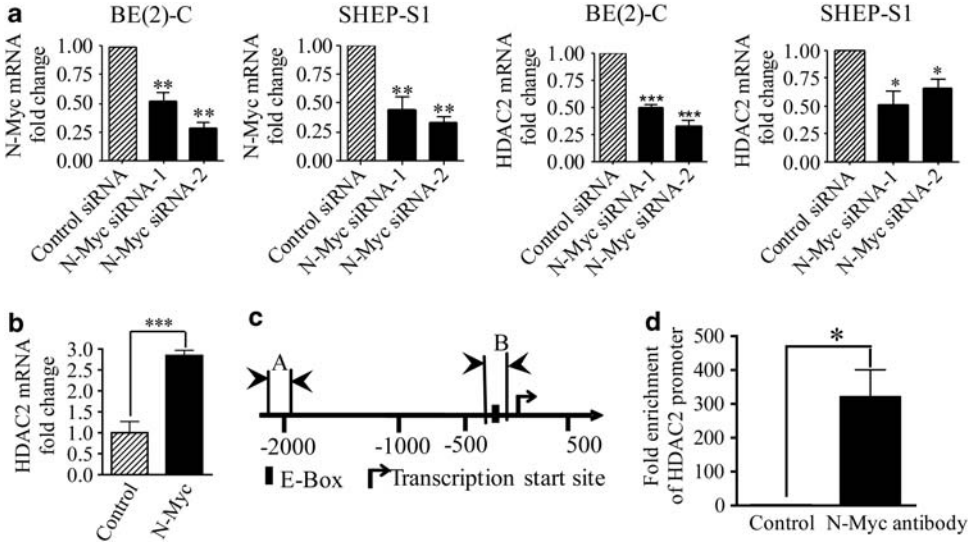


Figure 1 N-Myc oncoprotein upregulates HDAC2 gene expression. (**a**) BE(2)-C and SHEP-S1 neuroblastoma cells were transfected with scrambled control siRNA, N-Myc siRNA-1 or N-Myc siRNA-2 for 48 h, followed by RNA extraction and real-time RT–PCR analysis of N-Myc and HDAC2 gene expression. N-Myc and HDAC2 gene expression in control siRNA-transfected cells were artificially set as 1.0 (**b**). Bone marrow-derived B cells were extracted and purified from normal mice and transfected with constructs overexpressing N-Myc or empty vector, followed by RNA extraction and real-time RT–PCR analysis of HDAC2 gene expression. (**c**) Schematic representation of the human *HDAC2* gene promoter. The diagram includes: transcription start site (black arrow); N-Myc-responsive element E-Box (black box); target regions of PCR primers for quantitative ChIP assay (black arrow heads, amplicons A and B). (**d**) ChIP assay was performed with anti-N-Myc antibody and primers targeting amplicon A or B in BE(2)-C cells. Fold enrichment of HDAC2 gene promoter by anti-N-Myc antibody was calculated by dividing the PCR product from amplicon B by the PCR product from amplicon A. Symbols *($P<0.05$), **($P<0.01$) and ***($P<0.001$) indicated statistically significant differences. Error bars indicated standard error.

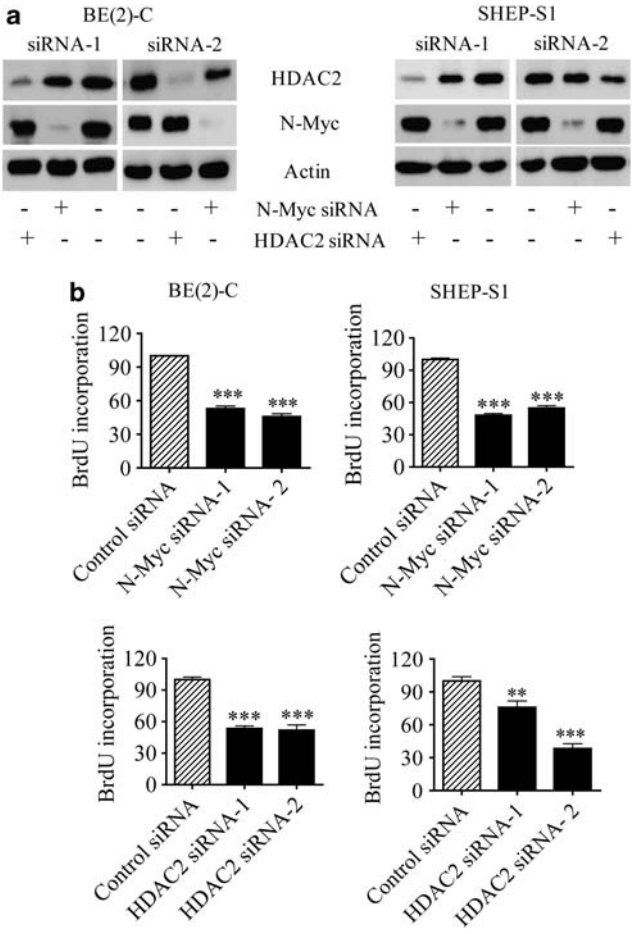


Figure 2 Transcriptional upregulation of HDAC2 promotes neuroblastoma cell proliferation. BE(2)-C and SHEP-S1 neuroblastoma cells were transfected with scrambled control siRNA, N-Myc siRNA-1, N-Myc siRNA-2, HDAC2 siRNA-1 or HDAC2 siRNA-2. (**a**) Cellular protein was extracted 72 h after transfection and subjected to immunoblot analysis with an anti-N-Myc antibody and an anti-HDAC2 antibody, followed by an anti-actin antibody as a loading control. (**b**) At 72 h after siRNA transfection, BE(2)-C and SHEP S1 cells were pulsed with BrdU for 2 h. Cell proliferation was examined by BrdU incorporation enzyme-linked immunosorbent assay, and expressed as fold change in optical density units of absorbance, comparing samples transfected with target gene siRNA and those transfected with control siRNA. Symbols $**(P<0.01)$ and $***(P<0.001)$ indicated statistically significant differences. Error bars indicated standard error.

reduced HDAC2 protein expression, in both BE(2)-C and SHEP-S1 neuroblastoma cells. Trypan blue cell viability assays showed that repression of HDAC2 or N-Myc with siRNAs in BE(2)-C and SHEP-S1 cells did not induce cell death (data not shown). 5-bromo-2′-deoxyuridine (BrdU) incorporation assays revealed that transfection with N-Myc siRNA-1 or N-Myc siRNA-2 for 3 days reduced cell proliferation by 50% in both BE(2)-C and SHEP-S1 cells (Figure 2b). Similarly, repression of HDAC2 gene expression with HDAC2 siRNA-1 or HDAC2 siRNA-2 reduced BE(2)-C cell proliferation by 50 and 50%, and SHEP-S1 cell proliferation by 30 and 65%, respectively. These data suggest that transcriptional upregulation of HDAC2 contributes to N-Myc-induced cell proliferation.

Cyclin G2 (CCNG2) is transcriptionally repressed by HDAC2 and N-Myc and reactivated by HDAC inhibitors

Both N-Myc and HDAC2 are well known to exert biological effects through transcriptional modulation. To identify transcriptional target genes responsible for HDAC2- and N-Myc-induced cell proliferation, we performed Affymetrix microarray analyses (Affymetrix, Santa Clara, CA, USA) comparing mRNA expression in BE(2)-C cells transfected with scrambled control siRNA, N-Myc siRNA-1 or HDAC2 siRNA-1 for 24 h. In summary (Table 1), 3.01% of genes were upregulated by N-Myc siRNA, comparing N-Myc siRNA to control siRNA microarray data (Supplementary Table T1 and T2), and 1.65% of genes were upregulated by HDAC2 siRNA, comparing HDAC2 siRNA to control siRNA microarray data (Supplementary Table T3 and T4). Among the genes upregulated by N-Myc siRNA, 12.93% were also upregulated by HDAC2 siRNA (Supplementary Table T5). Conversely, 4.89% of genes were downregulated by N-Myc siRNA (Supplementary Table T2), and none of the genes were upregulated by HDAC2 siRNA. Taken together, our data suggest that N-Myc siRNA upregulated genes and HDAC2 siRNA upregulated genes overlapped significantly more than would be expected by chance, and that HDAC2 and N-Myc commonly repress the expression of a subset of genes. CCNG2 stood out in this microarray analysis as the gene most dramatically upregulated by HDAC2 siRNA-1, and one of the genes most significantly activated by N-Myc siRNA-1.

To validate the microarray data, we performed real-time RT–PCR analyses of CCNG2 gene expression in BE(2)-C and SHEP-S1 cells after siRNA transfection and in normal bone marrow-derived B cells over-expressing N-Myc. As shown in Figure 3a, CCNG2 gene expression is upregulated by N-Myc siRNA-1, N-Myc siRNA-2, HDAC2 siRNA-1 and HDAC2 siRNA-2 in both BE(2)-C and SHEP-S1 cells. Consistently, forced overexpression of N-Myc in normal B cells reduced CCNG2 gene expression (Figure 3b). Immunoblot analyses demonstrated that N-Myc siRNA-1, N-Myc siRNA-2, HDAC2 siRNA-1 and HDAC2 siRNA-2 all upregulated CCNG2 protein expression in BE(2)-C and SHEP-S1 cells (Figure 3a).

Because pan-HDAC inhibitors repress the deacetylase activity of HDAC2, we examined whether the pan-HDAC inhibitor, trichostatin A (TSA), could activate the expression of genes repressed by N-Myc. Affymetrix microarray data showed that treatment with TSA for 24 h upregulated 2.26% (Supplementary Table T6), and downregulated 0.86% (Supplementary Table T7), of genes in BE(2)-C cells. Among the genes upregulated by N-Myc siRNA (Supplementary Table T1), 13.82%, including CCNG2, were also upregulated by TSA (Supplementary Table T8 and T9). By comparison, among the genes downregulated by N-Myc siRNA (Supplementary Table T2), only 0.77% was also

Table 1 Genes commonly modulated by N-Myc siRNA-1 and HDAC2 siRNA-1 by more than twofold, as identified by Affymetrix microarray analysis, in neuroblastoma BE(2)-C cells 24 h after siRNA transfection

Gene expression	*N-Myc siRNA/control siRNA microarray*	*Modulation of N-Myc siRNA target genes by HDAC2 siRNA*
Genes upregulated by N-Myc siRNA	↑ 3.01% (557/18507)	↑ 12.93% (72/557)
		↓ 4.13% (23/557)
Genes downregulated by N-Myc siRNA	↓ 4.89% (905/18507)	↑ 4.97% (45/905)
		↓ 0.00% (0/905)

Abbreviations: HDAC, histone deacetylases; siRNA, small interfering RNA.
↑ Indicated upregulation of N-Myc siRNA target genes by HDAC2 siRNA, ↓ indicated downregulation of N-Myc siRNA target genes by HDAC2 siRNA.

upregulated by TSA. Taken together, these data suggest that N-Myc siRNA and TSA commonly activate the expression of a subset of genes including CCNG2. Real-time RT–PCR analyses showed that TSA had no effect on N-Myc (Supplementary Figure S1), but significantly reactivated CCNG2, gene expression in BE(2)-C and SHEP-S1 cells (Figure 3c), and immunoblot analyses demonstrated that TSA upregulated CCNG2 protein expression (Figure 3c). Moreover, the class I HDAC specific inhibitor MS-275 (Bolden *et al*., 2006) also reactivated CCNG2 expression in the neuroblastoma cells (Supplementary Figure S2). These results suggest that both N-Myc and HDAC2 repress CCNG2 gene expression, and that HDAC inhibitors can be applied to reverse this effect.

Transcriptional repression of CCNG2 contributes to N-Myc and HDAC2-induced cell proliferation

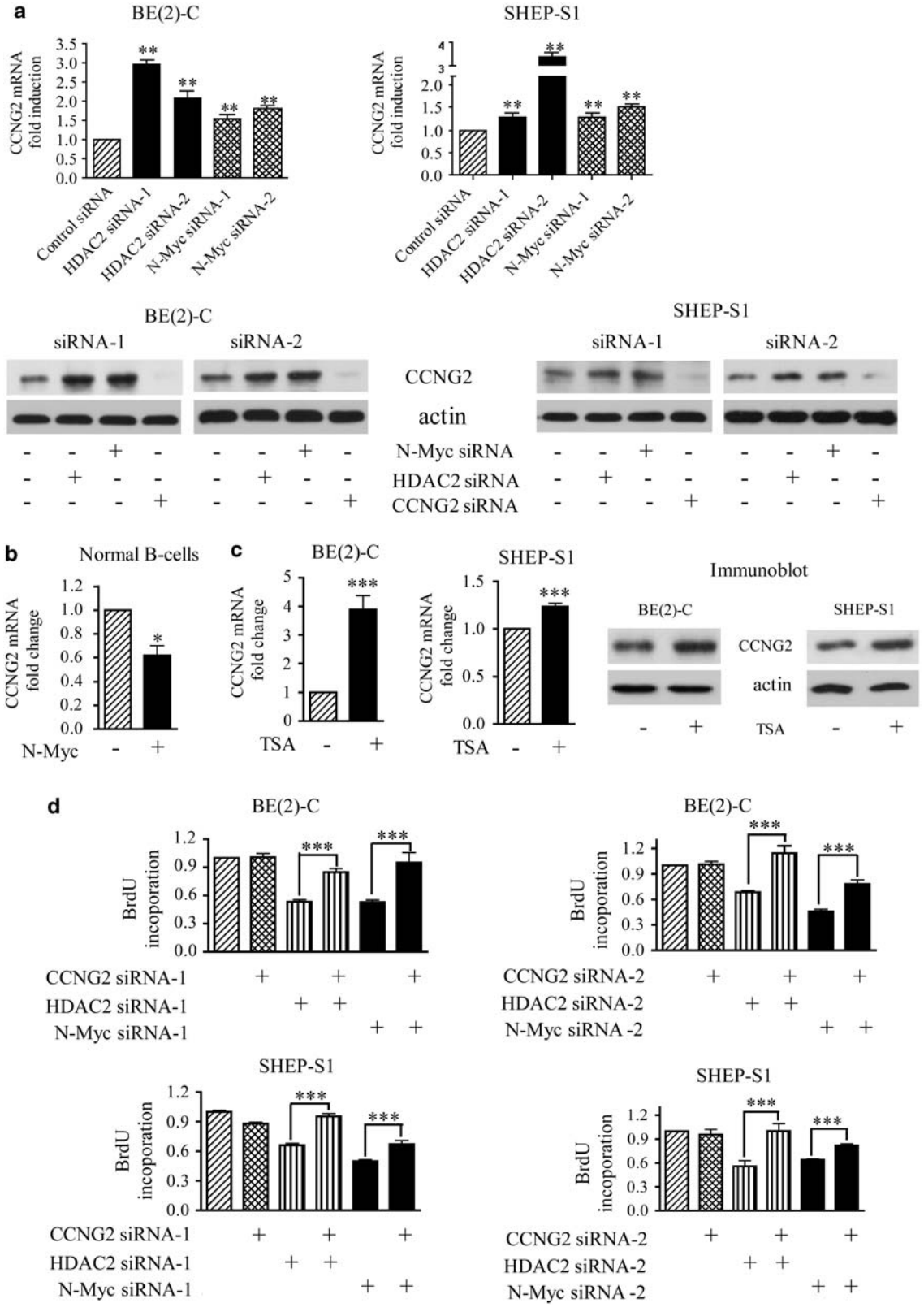
Unlike most other cyclins, CCNG2 blocks cell cycle progression and induces cell growth arrest (Bennin *et al*., 2002; Kim *et al*., 2004). We therefore examined whether transcriptional activation of CCNG2 contributed to cell growth arrest induced by N-Myc siRNAs and HDAC2 siRNAs. As shown in Figure 3d, Although repression of CCNG2 gene expression alone did not have an effect on cell proliferation, CCNG2 siRNA-1 and CCNG2 siRNA-2 partly blocked growth arrest because of the two sets of N-Myc siRNAs and the two sets of HDAC2 siRNAs in both BE(2)-C and SHEP-S1 cells. These data indicate that transcriptional repression of CCNG2 contributes to N-Myc- and HDAC2-induced cell proliferation in neuroblastoma cells.

CCNG2 gene expression is repressed by N-Myc and re-activated by HDAC inhibitors in pre-cancerous cells in MYCN transgenic mice

We have previously shown that 2-week-old homozygous N-Myc transgenic mice develop pre-cancerous neuroblast cell hyperplasia in coeliac and superior cervical ganglia, which develops into neuroblastoma by 3 weeks of age in 100% of the mice (Hansford *et al*., 2004). We next examined, whether N-Myc modulated HDAC2 and CCNG2 gene expression in these pre-cancerous cells of the ganglia from 2-week-old *MYCN* transgenic mice. As shown in Figure 4a, HDAC2 mRNA expression was increased by threefold, and CCNG2 gene expression reduced by more than 60%, in pre-cancerous cells from *MYCN* transgenic mice, compared with counterpart cells from wild-type mice. To test whether inhibition of HDAC activity could reactivate CCNG2 gene expression in the pre-cancerous cells, we extracted and purified coeliac and superior cervical ganglia cells from 2-week-

Figure 3 Transcriptional repression of CCNG2 is partly responsible for N-Myc and HDAC2-modulated cell proliferation. (**a**) BE(2)-C and SHEP-S1 cells were transfected with scrambled control siRNA, N-Myc siRNA-1, N-Myc siRNA-2, HDAC2 siRNA-1 or HDAC2 siRNA-2. The effect of N-Myc and HDAC2 on CCNG2 gene expression was analysed by real time RT–PCR and immunoblot. For RT–PCR studies, CCNG2 gene expression in cells transfected with scrambled control siRNA was artificially set as 1.0. For immunoblot studies, protein samples were lastly probed with anti-actin antibody as loading controls. (**b**) Normal bone marrow-derived B cells were transfected with a construct overexpressing empty vector or N-Myc. The effect of N-Myc on CCNG2 gene expression was analysed by real-time RT–PCR. (**c**) BE(2)-C and SHEP-S1 cells were treated with control or 0.3 μM TSA for 24 h. The effect of TSA on CCNG2 gene expression was analysed by real-time RT–PCR and immunoblot. (**d**) BE(2)-C and SHEP-S1 cells were transfected with scrambled control siRNA, N-Myc siRNA, HDAC2 siRNA, CCNG2 siRNA, combination of CCNG2 siRNA and N-Myc siRNA, or combination of CCNG2 siRNA and HDAC2 siRNA. At 72 h after transfection, cell proliferation was examined by BrdU incorporation enzyme-linked immunosorbent assay and expressed as fold changes. Symbols *($P<0.05$), **($P<0.01$) and ***($P<0.001$) indicated statistically significant differences. Error bars indicated standard error.

old homozygous N-Myc transgenic mice, and treated the cells with vehicle control or TSA for 24 h. As shown in Figure 4b, inhibition of HDAC activity reactivated CCNG2 gene expression by more than 200%. These results suggest that N-Myc upregulates HDAC2 and represses CCNG2 gene expression in pre-cancerous neuroblast cells of the ganglia during the first stages of tumour initiation.



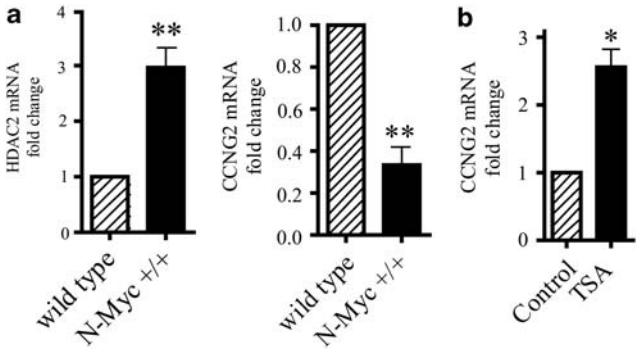


Figure 4 CCNG2 gene expression is repressed by N-Myc and re-activated by HDAC inhibitors in pre-cancerous cells in N-Myc transgenic mice. Coeliac and superior cervical ganglia were dissected from wild-type mice and homozygous N-Myc transgenic mice at 2-week-old, and ganglia cells purified. (**a**). RNA was extracted from the ganglia cells and subject to real-time RT–PCR analysis of HDAC2 and CCNG2 gene expression. Target gene expression in ganglia cells from normal mice was artificially set as 1.0. (**b**). Ganglia cells from N-Myc transgenic mice were treated with vehicle control or 0.1 μM TSA for 24 h, followed by RNA extraction and real-time RT–PCR analysis of CCNG2 gene expression. CCNG2 gene expression in ganglia cells treated with vehicle control was artificially set as 1.0. Symbols *($P<0.05$) and **($P<0.01$) indicated statistically significant differences.

N-Myc represses CCNG2 gene transcription by directly binding to HDAC2 at the Sp1 consensus site of CCNG2 gene core promoter

Both HDAC2 (Won *et al*., 2002) and N-Myc (Liu *et al*., 2007) are known to repress gene transcription by binding to Sp1-binding sites at target gene promoter. As both N-Myc siRNA and HDAC2 siRNA activated CCNG2 gene expression, we tested the hypothesis that N-Myc might repress CCNG2 gene transcription by recruiting HDAC2 to the CCNG2 gene promoter. Bioinformatic analysis of the CCNG2 gene promoter (−3000/+3000 from transcription start site), identified two regions enriched for Sp1 binding sites: one proximal to the transcription start site and a second one located about 1 kb downstream from the transcription start site (Figure 5a).

The CCNG2 promoter region was scanned for the presence of N-Myc, Sp1 and HDAC2 binding using the dual crosslinking ChIP assay (Nowak *et al*., 2005). A pre-immune serum was used as a negative control to determine the baseline of the non-specific background. As shown in Figure 5b, antibodies against N-Myc, HDAC2 and Sp1 all efficiently immunoprecipitated the two regions of the CCNG2 promoter carrying Sp1 binding sites. To confirm that transcriptional suppression of CCNG2 was mediated by N-Myc, we performed experiments using luciferase reporter constructs carrying different portions of the CCNG2 promoter: F3(−416/+1188), F2 (+146/+1188), F1(+700/+1188), F2Δ (+146/+825) and F1Δ (+700/+825). Reporters were transiently transfected into the TET21/N cells, a human neuroblastoma cell line carrying a *MYCN* transgene under the control of a TET-OFF promoter. Luciferase activity was monitored as a function of N-Myc expression. Results showed that deletion of the Sp1 region downstream of the transcription start site abolished N-Myc-mediated repression of the promoter, suggesting that this region was the target of N-Myc and HDAC2 (Figure 5c).

To determine whether N-Myc, HDAC2 and Sp1 are part of the same repressive protein complex, we performed co-immunoprecipitation assays using BE(2)-C cell nuclear extracts (Figure 5d). The extracts were incubated with a specific anti-HDAC2 antibody or with pre-immune immunoglobulinG as a negative control. The IP complexes were subsequently analyzed by immunoblot, using antibodies against Sp1, HDAC2 and N-Myc. Results showed that HDAC2 could co-immunoprecipitate both Sp1 and N-Myc. A reverse experiment, in which an anti-N-Myc antibody was employed as the immunoprecipitating agent, was also performed. Results showed that N-Myc could efficiently co-immunoprecipitate both HDAC2 and Sp1. Taken together, these findings suggest that N-Myc forms a protein complex along with HDAC2 and Sp1, and that the protein complex represses *CCGN2* gene transcription by binding the Sp1 containing region downstream of the transcription start site.

Upregulation of HDAC2 and repression of CCNG2 gene expression contribute to c-Myc-induced pancreatic cancer cell proliferation

c-Myc and N-Myc are known to modulate gene transcription through common mechanisms (Pelengaris *et al*., 2002; Patel *et al*., 2004). We therefore tested the hypothesis that c-Myc might also induce tumour cell proliferation, in part, by modulating HDAC2 and CCNG2 gene expression in pancreatic cancer cells overexpressing c-Myc. As shown in Figure 6a, repression of c-Myc gene expression with c-Myc siRNA-1 or c-Myc siRNA-2 reduced *HDAC2* gene and protein expression in c-Myc overexpressing MiaPaca-2 pancreatic cancer cells. Repression of c-Myc, HDAC2 gene expression with siRNAs (Figure 6b) or inhibition of HDAC activity with the pan-HDAC inhibitor TSA (Figure 6c) significantly upregulated CCNG2 gene and protein expression in MiaPaca-2 cells, while TSA showed no effect on c-Myc expression (Supplementary Figure S1). Moreover, treatment with the class I HDAC specific inhibitor MS-275 also upregulated CCNG2 expression in the pancreatic cancer cells (Supplementary Figure S2). BrdU incorporation assays demonstrated that c-Myc siRNA-1, c-Myc siRNA-2, HDAC2 siRNA-1 and HDAC2 siRNA-2 all reduced MiaPaca-2 cell proliferation (Figure 6d). Although CCNG2 siRNA-1 and CCNG2 siRNA-2 alone had no effect, the CCNG2 siRNAs partly blocked MiaPaca-2 cell-growth arrest induced by the c-Myc siRNAs and the HDAC2 siRNAs. These results demonstrate that c-Myc transcriptionally activates HDAC2 and represses CCNG2 gene expression in pancreatic cancer cells, and that upregulation of HDAC2 and repression of CCNG2 contribute to c-Myc-induced pancreatic cancer cell proliferation.

HDAC2 is upregulated and CCNG2 downregulated in neuroblastoma tissues from N-Myc transgenic mice and c-Myc overexpressing tumour tissues from pancreatic cancer patients

We lastly examined whether N-Myc and c-Myc modulated HDAC2 and CCNG2 expression in tumour

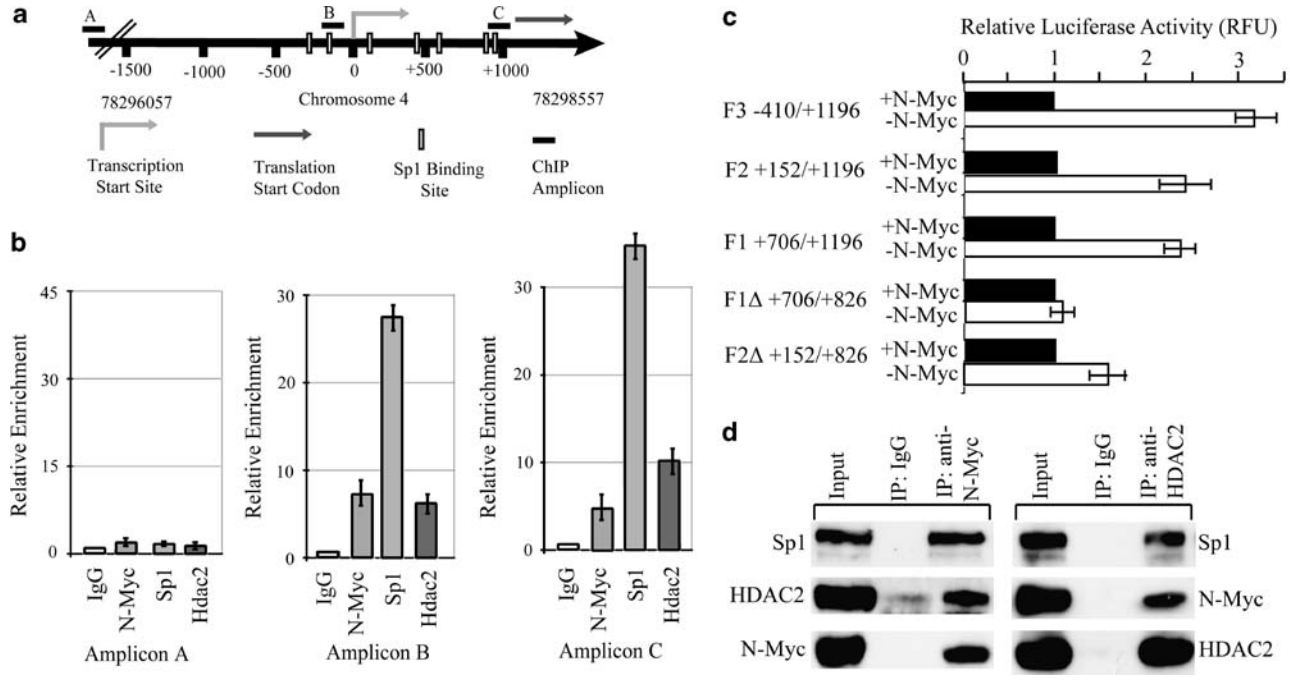


Figure 5 N-Myc and HDAC2 represses CCNG2 expression by direct binding to Sp1 protein at the CCNG2 promoter. (**a**) A schematic representation of the CCNG2 promoter containing the Sp1 binding sites. (**b**) Dual crosslinking ChIP and quantitative PCR were applied in BE(2)-C cells. Fold enrichment of CCNG2 promoter regions immunoprecipitated by anti-Sp1, N-Myc, HDAC2 antibodies and pre-immune serum was calculated as the logarithm of the difference between the cycle-threshold obtained with pre-immune serum and the cycle-threshold obtained with the specific antibody. Quantitative PCR targeting amplicon A and Sp1 binding sites (amplicon B and C) were performed in triplicate. Results were the average of three independent experiments. (**c**) Luciferase activity of the reporter constructs of CCNG2 promoter transfected in TET-21/N cells was determined in the presence (-tetracycline) and absence (+tetracycline) of N-Myc expression and normalized to that of Renilla. (**d**) Protein co-immunoprecipitation (IP) of N-Myc (*left*) and HDAC2 (*right*) in BE(2)-C nuclear protein extracts. Products obtained by immunoprecipitating with either anti-N-Myc or anti-HDAC2 antibody were probed with anti-Sp1, anti-HDAC2 and anti-N-Myc antibodies in immunoblot. IgG, immunoglobulin.

tissues *in vivo*. Because neuroblastoma tissues from 5-week-old N-Myc transgenic mice overexpressed N-Myc protein (Liu *et al*., 2007), we carried out immunohistochemistry analysis of HDAC2 and CCNG2 in the tumour tissues. As shown in Figure 7a, the expression of HDAC2 was very low in normal mature neurons and dramatically upregulated in adjoining tumour cells. In contrast, CCNG2 was highly expressed in the normal mature neurons and dramatically reduced in tumour cells. Moreover, immunohistochemistry study with control antibodies did not show positive staining in neuronal and neuroblastoma cells (Supplementary Figure S3).

c-Myc is frequently overexpressed in tumour tissues from pancreatic cancer patients (Schleger *et al*., 2002). We therefore tested whether c-Myc was associated with differential HDAC2 and CCNG2 expression in samples of human pancreatic cancer. In all, 4 normal and 63 pancreatic cancer tissue sections were stained with control, anti-c-Myc, HDAC2 and CCNG2 antibodies. Immunohistochemistry analysis showed that control antibodies did not generate positive staining in normal pancreas or pancreatic tumour cells (Supplementary Figure S3), and that 45 out of 63 (71%) tumour tissues had positive staining for c-Myc and 29 (46%) of these had significant proportion ($>2/3$) of tumour cells stained positive, whereas normal pancreas did not express c-Myc protein (Figure 7b). Importantly, expression of c-Myc in tumour tissues correlated with upregulation of HDAC2 ($P=0.0068$), and downregulation of CCNG2 ($P=0.0351$).

Discussion

More than a dozen HDAC inhibitors are currently in clinical trials for the treatment of malignancies of various organ origins, and the HDAC inhibitor SAHA is already in clinical use for the treatment of cutaneous T cell lymphoma. HDAC2 promotes cancer cell proliferation (Hrzenjak *et al*., 2006), and mutation of HDAC2 in mice reduces tumour incidence (Zimmermann *et al*., 2007). In this study, we have shown that N-Myc oncoprotein upregulates HDAC2 gene transcription by directly binding to its gene promoter in neuroblastoma cells, that forced overexpression of N-Myc in normal non-malignant cells induces HDAC2 gene expression, and that c-Myc upregulates HDAC2 gene transcription in pancreatic cancer cells. Suppression of HDAC2, like suppression of Myc oncoproteins, results in cell growth arrest in both N-Myc overexpressing neuroblastoma and c-Myc overexpressing pancreatic cancer cells. Moreover, HDAC2 gene expression is upregulated in pre-cancerous cells and neuroblastoma cells from N-Myc transgenic mice and in cancer cells from human pancreatic tumour tissues, compared with counterpart

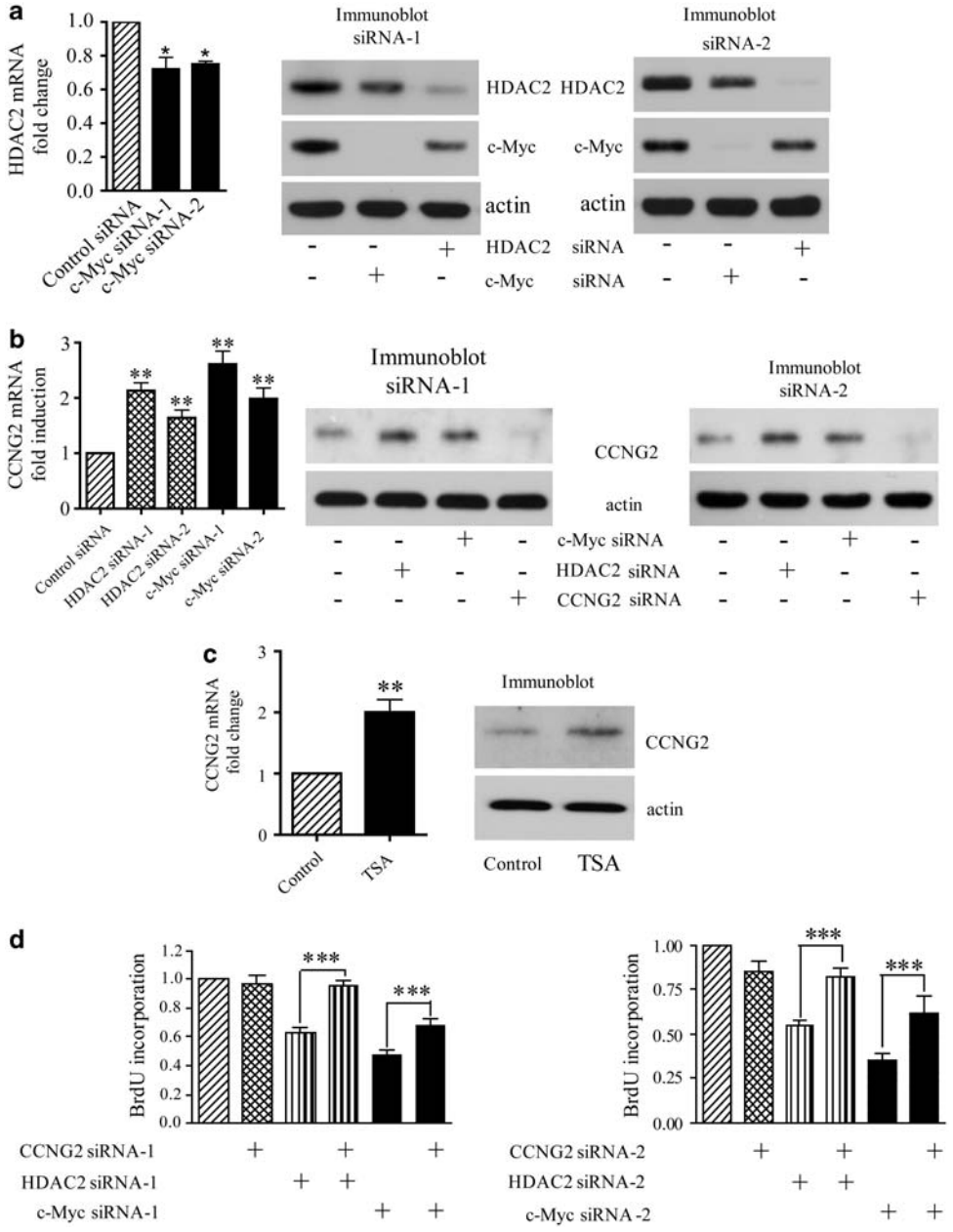


Figure 6 c-Myc represses pancreatic cancer cell proliferation partly through upregulating HDAC2 and repressing CCNG2 gene expression. (**a**) MiaPaca-2 pancreatic cancer cells were transfected with scrambled control siRNA, c-Myc siRNA-1 or c-Myc siRNA-2. The effect of c-Myc on HDAC2 gene and protein expression was analysed by real time RT–PCR and immunoblot. HDAC2 gene expression in cells transfected with scrambled control siRNA was artificially set as 1.0. For immunoblot, protein samples were lastly probed by anti-actin antibody as loading controls. (**b**) and (**d**) MiaPaca-2 cells were transfected with scrambled control siRNA, c-Myc siRNAs, HDAC2 siRNAs, CCNG2 siRNAs, combination of CCNG2 siRNAs and c-Myc siRNAs, or combination of CCNG2 siRNAs and HDAC2 siRNAs. (**b**) The effect of the siRNAs on CCNG2 expression was analysed by real-time RT–PCR and immunoblot. (**d**) Cell proliferation was examined by BrdU incorporation enzyme-linked immunosorbent assay and expressed as fold changes. (**c**) MiaPaca-2 cells were treated with 0.3 μM TSA. The effects of TSA on CCNG2 gene and protein expression were analysed by real-time RT–PCR and immunoblot. Symbols *($P<0.05$), **($P<0.01$) and ***($P<0.001$) indicated statistically significant differences. Error bars indicated standard error.

normal cells. Taken together, our data demonstrate that Myc oncoproteins are capable of upregulating HDAC2 gene expression, that Myc-induced HDAC2 overexpression contributes to Myc-induced cancer cell proliferation, and that HDAC2 overexpression is likely to be one of the key factors responsible for Myc-induced malignant transformation, tumour initiation and progression *in vivo*.

The transcriptional target genes of HDAC2 have not been investigated by screening methodologies such as microarray, and the mechanism through which HDAC2 promotes cancer cell proliferation has not been

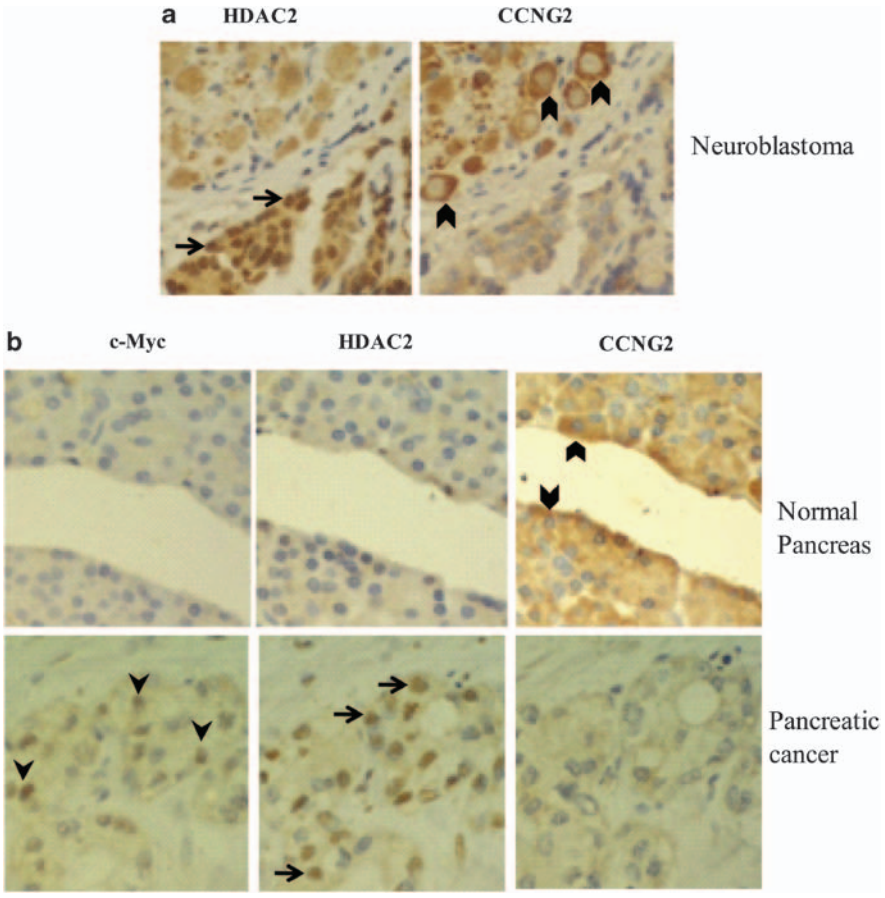


Figure 7 HDAC2 is upregulated and CCNG2 downregulated in neuroblastoma tissues from N-Myc transgenic mice and c-Myc overexpressing tumour tissues from pancreatic cancer patients. (**a**) Neuroblastoma tissue sections from 5-week-old N-Myc transgenic mice were stained by immunohistochemistry with an anti-HDAC2 or an anti-CCNG2 antibody and visualized with diaminobenzidine (DAB; brown). The nucleus was counter-stained with haematoxylin (blue). Residual normal mature neurons were located at the upper left hand corner. (**b**) Normal and cancerous human pancreatic tissues were stained by immunohistochemistry with anti-c-Myc, HDAC2 or CCNG2 antibodies and visualized with DAB. The nucleus was counter-stained with haematoxylin. Arrowheads, arrows and chevrons indicated positive nuclear, nuclear and cytoplasmic staining of c-Myc, HDAC2 and CCNG2, respectively.

reported. This study has identified a subset of genes, including CCNG2, as commonly repressed by N-Myc and HDAC2 in neuroblastoma cells and by c-Myc and HDAC2 in pancreatic cancer cells. We have further shown that Myc oncoproteins repress CCNG2 gene expression in primary normal cells, in pre-cancerous cells and neuroblastoma cells from N-Myc transgenic mice, and in tumour cells from human pancreatic cancer patients. Importantly, treatment with HDAC inhibitors re-activates CCNG2 gene expression in Myc over-expressing primary normal cells, pre-cancerous cells form N-Myc transgenic mice, and neuroblastoma and pancreatic cancer cells. Moreover, consistent with previous reports that CCNG2 blocks cell-cycle progression from G1 to S phase and induces cell growth arrest (Bennin *et al*., 2002; Kim *et al*., 2004; Xu *et al*., 2008), we have demonstrated that repression of CCNG2 gene expression with siRNAs significantly reverses neuroblastoma and pancreatic cancer cell growth arrest due to repression of Myc and HDAC2. These results suggest that N-Myc, c-Myc and HDAC2 promotes cell proliferation partly by blocking gene transcription of CCNG2, and that repression of CCNG2 gene expression by Myc and HDAC2 may be an important step in Myc-induced oncogenesis *in vivo*. Nevertheless, this does not exclude the importance of other Myc target genes in Myc-induced cell proliferation. It is likely that repression of CCNG2 is one of the critical steps in Myc-induced cell-cycle progression from G1 phase to S phase, that modulation of other Myc target genes are critical for Myc-induced cell cycle progression at other cell cycle phases and/or checkpoints, and that perturbing the effect of Myc on one of the critical cell cycle genes can significantly attenuate Myc-induced cell cycle progression and cell proliferation. Moreover, our data highlight the potential application of HDAC inhibitors for the prevention and treatment of Myc-overexpressing cancers.

Myc family proteins are well known to activate gene transcription by binding to a Myc-responsive element E-box, and to inhibit gene transcription by binding to a repressive initiator element at the target gene promoter (Li *et al*., 1994). Recent studies have revealed that Myc oncoproteins are capable of suppressing gene transcription of tissue transglutaminase by recruiting HDAC1 (Liu *et al*., 2007), and suppressing gene transcription of

ID2 by recruiting HDAC3 (Kurland and Tansey, 2008), to the target gene promoter regions. Our current study has shown that N-Myc can recruit the HDAC2 protein to the *CCNG2* gene core promoter at the Sp1-binding site, that N-Myc represses CCNG2 promoter activity, and that transcriptional repression of CCNG2 is reversed by suppression of HDAC2 with siRNAs or HDAC inhibitors. Moreover, our co-immunoprecipitation assay has revealed that N-Myc, HDAC2 and Sp1 proteins form a protein complex. These data suggest that N-Myc and HDAC2 are contemporaneously bound to Sp1 in a repressor complex, which is bound to DNA at its consensus binding site, and that the recruitment of HDAC2 is essential for N-Myc-induced transcriptional suppression of CCNG2. Although HDAC2 has been previously shown to be recruited to Sp1-binding sites at the target gene promoter (Won *et al*., 2002), our data provide the first evidence that a Myc oncoprotein can suppress transcription by recruiting HDAC2 to a target gene promoter, and that Myc-mediated transcriptional repression could be reversed by HDAC inhibitors in both pre-cancerous and cancer cells. Importantly, as Affymetrix microarray analysis has shown that a subset of genes are commonly repressed by N-Myc and HDAC2 by more than twofold in neuroblastoma cells, Myc oncoproteins may possess a more widespread capacity for transcriptional suppression of tumour suppressor genes by recruiting HDAC2 protein to the target gene promoter.

In summary, this study demonstrates transcriptional upregulation of HDAC2, the interaction between HDAC2 and Myc oncoproteins at the CCNG2 gene promoter, and transcriptional repression of CCNG2 as novel mechanisms responsible for Myc oncoprotein-induced cell proliferation. As Myc oncoproteins increase HDAC2 and repress CCNG2, and HDAC inhibitors reactivate CCNG2, gene expression in both tumour cells and pre-cancerous cells, our findings provide further support for the application of HDAC inhibitors in the prevention and therapy of cancer types characterized by Myc oncoprotein overexpression.

Materials and methods

Cell culture
SHEP-S1, BE(2)-C, TET-21N and MiaPaca-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Mouse bone marrow-derived B cells were extracted from mouse bone marrow as described previously (Rolink *et al*., 1991), and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol and 10ng/ml recombinant mouse interleukin-7. All animal work was approved by the Animal Care and Ethics Committee of the University of New South Wales, Sydney, Australia.

siRNA transient transfection
Tumour cells were transfected with 20 nM siRNA using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Validated scrambled control siRNA, or siRNAs specifically targeting N-Myc, c-Myc, HDAC2 or CCNG2 were purchased from Qiagen (Qiagen, Hamburg, Germany) and/or Ambion (Ambion, Austin, TX, USA).

Quantitative real-time RT–PCR analysis
N-Myc, c-Myc, HDAC2 and CCNG2 mRNA expression was examined by quantitative real-time RT–PCR technique as described previously (Liu *et al*., 2007), with the mRNA expression of β-actin used as a loading control.

Immunoblot analysis
Cells were lysed, protein extracted and separated by gel electrophoresis. After western transfer, membranes were probed with mouse anti-N-Myc antibody (diluted 1:1000), mouse anti-c-Myc antibody (1:1000) or goat anti-HDAC2 antibody (1:200; all from Santa Cruz Biotech, Santa Cruz, CA, USA), followed by horseradish peroxidase-conjugated anti-mouse (1:8000; Santa Cruz Biotech) or anti-goat (1:20 000) antiserum (Pierce, Rockford, IL, USA). Protein bands were visualized with SuperSignal (Pierce). The membranes were lastly re-probed with anti-β-actin antibody (Sigma, St Louis, MO, USA) as loading controls.

Affymetrix microarray
Neuroblastoma BE(2)-C cells were transfected with scrambled control siRNA, N-Myc siRNA-1 or HDAC2 siRNA-1. At 30 h after transfection, RNA was extracted from the cells with RNeasy mini kit (Qiagen). Differential gene expression was examined with Affymetrix GeneChip Gene 1.0 ST Arrays (Affymetrix), according to the manufacturer's instruction. Results from the microarray hybridization were analysed with GeneSpring software (GeneSpring, Santa Clara, CA, USA).

Cell-proliferation assay
The cell-proliferation assay was carried out with the BrdU Colorimetric Cell Proliferation Kit (Roche) as we described previously (Liu *et al*., 2007). Briefly, three days after siRNA transfection, cells were pulsed with 10 μM BrdU for 2 h, fixed and incubated with an anti-BrdU antibody and a substrate solution consecutively. Absorbance was finally read with a plate reader. Modulation of cell proliferation was expressed as a fold change in optical density units of absorbance, comparing samples transfected with target gene siRNA and those transfected with control siRNA.

Dual crosslinking ChIP
Dual crosslinking ChIP was performed as previously described (Liu *et al*., 2007), with 5 μg immunoglobulinG as a control, anti-N-Myc, anti-Sp1 and anti-HDAC2 antibodies. Promoter region was detected with quantitative PCR with specific primer pair: distal forward 5′-AGAGAGCACTGGATAACAAAG-3′, distal reverse 5′-TGAATGACTACAGCAAGAGG-3′; transcription start site forward 5′-CTGGGAGGAAGGGTCG GATG-3′, transcription start site reverse 5′-GGGCTTCCAA ACCTGTCTCG-3′; +1000 site forward 5′-GTCCCTTCA CCCGCTCCTTGTC-3′, +1000 site reverse 5′-CATGACC TGCCAAGTGCTCTGC-3′.

Co-immunoprecipitation assay
A total of 1 mg of nuclear extract from BE(2)-C cells were incubated with 1 μg of mouse pre-immune serum or anti-N-Myc monoclonal antibody or anti-HDAC2 monoclonal antibody. N-Myc co-immunoprecipitated proteins were immunoblotted with anti-Sp1 polyclonal antibody and anti-HDAC2 monoclonal antibody; reversely HDAC2 co-immunoprecipitated

proteins were immunoblotted with anti-Sp1 polyclonal antibody and anti-N-Myc monoclonal antibody.

Luciferase assay
CCNG2 promoter constructs F1, F2, F3 were obtained as previously described (Stossi *et al*., 2006). F1Δ and F2 Δ was obtained from F1 and F2, respectively, constructs by Whole Around PCR using specific primer pair CCNG2 Δ F 5′-TGGGGATTGCCCTGGGGCTCTG-3′ and CCNG2 BglII Δ R 5′-AACAGATCTCTTCTGCCCAGGGGACTGCAGTGGC-3′. TET-21/N were transiently transfected using Lipofectamine2000 (Invitrogen) according to manufacturer's instructions. At 6 h after transfection, medium was replaced and cells were treated with tetracycline 1 μg/ml for 48 h before luciferase Assay. Firefly and Renilla activity was measured with a Dual Luciferase Assay (Promega, Madison, WI, USA).

N-Myc transgenic mouse studies
A 2-week-old homozygous N-Myc transgenic mice and matched 2-week-old wild-type mice from the same hemizygous N-Myc transgenic mothers were killed. After superior cervical and coeliac ganglia were dissected, ganglia cells were purified and cultured as we have described previously (Hansford *et al*., 2004). Ganglia cells from N-Myc transgenic mice were treated with vehicle control or 0.1 μM TSA for 24 h. Moreover, six N-Myc transgenic mice at the age of 5 weeks were killed and neuroblastoma tissues removed, formalin fixed and paraffin embedded.

Tissue microarrays
Formalin-fixed and paraffin-embedded tissue blocks for each patient were obtained from anatomical pathology departments. Tissue microarrays were constructed with each resected specimen represented by a minimum of 3×1 mm tissue cores. Immunohistochemistry was performed on 4 μm serial sections of paraffin-embedded, formalin-fixed tissue mounted on SuperFrost slides (Menzel-Glaser, Braunschweig, Germany). Studies involving human tissue blocks were approved by Human Ethics Committee of Garvan Institute.

Immunohistochemistry
Mouse tissue sections and human tissue microarrays were de-paraffinised, rehydrated, blocked with 3% hydrogen peroxide and serum, and incubated with rabbit anti-HDAC2 (1:400), goat anti-CCNG2 (1:300) or mouse anti-c-Myc antibody (1:300; all from Santa Cruz Biotech) overnight. The tissue sections were then incubated with biotinylated swine anti-rabbit (1:500), swine anti-goat (1:500) or rabbit anti-mouse (1:500) antibody (DakoCytomation, Glostrup, Denmark), followed by incubation with streptavidin-horseradish peroxidase (DakoCytomation) and final development with diaminobenzidine (DakoCytomation). Analyses of the immunohistochemistry staining were performed using the scoring system which we previously described (Biankin *et al*., 2009). Briefly, high level of expression of c-Myc, HDAC2 and CCNG2 was defined as positive staining with intensity 3+ in >33% of cells; moderate–high staining was defined as intensity 2+ in >33% of positive staining, up to intensity 3+ in 33% of cells; and low expression was defined as any staining with 1+ intensity, up to intensity 2+ in 33% of cells.

Statistical analysis
All data for statistical analysis were calculated as mean ± standard error. Differences were analyzed for significance using ANOVA among groups or unpaired *t*-test for two groups. The analysis on the correlation between the distribution of the expression of c-Myc, HDAC2 and CCNG2 was performed using χ^2-test. 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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