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Myc represses transcription through recruitment of DNA methyltransferase corepressor

Carmen Brenner[1,7], Rachel Deplus[1,7], Céline Didelot[1,7], Axelle Loriot[2], Emmanuelle Viré[1], Charles De Smet[2], Arantxa Gutierrez[3], Davide Danovi[4], David Bernard[1], Thierry Boon[2], Pier Giuseppe Pelicci[4], Bruno Amati[4], Tony Kouzarides[5], Yvan de Launoit[1,6], Luciano Di Croce[3,4] and François Fuks[1,5,*]

[1]Free University of Brussels, Faculty of Medicine, Laboratory of Molecular Virology, Brussels, Belgium, [2]Ludwig Institute For Cancer Research, UCL, Brussels, Belgium, [3]ICREA and Center for Genomic Regulation, Barcelona, Spain, [4]Department of Experimental Oncology, European Institute of Oncology, Milan, Italy, [5]Wellcome/Cancer Research UK Institute and Department of Pathology, University of Cambridge, Cambridge, UK and [6]UMR 8117, CNRS Institut Pasteur de Lille, Université de Lille 1, Institut de Biologie de Lille, Lille, Cedex, France

The Myc transcription factor is an essential mediator of cell growth and proliferation through its ability to both positively and negatively regulate transcription. The mechanisms by which Myc silences gene expression are not well understood. The current model is that Myc represses transcription through functional interference with transcriptional activators. Here we show that Myc binds the corepressor Dnmt3a and associates with DNA methyltransferase activity *in vivo*. In cells with reduced Dnmt3a levels, we observe specific reactivation of the Myc-repressed *p21Cip1* gene, whereas the expression of Myc-activated E-boxes genes is unchanged. In addition, we find that Myc can target Dnmt3a selectively to the promoter of *p21Cip1*. Myc is known to be recruited to the *p21Cip1* promoter by the DNA-binding factor Miz-1. Consistent with this, we observe that Myc and Dnmt3a form a ternary complex with Miz-1 and that this complex can corepress the *p21Cip1* promoter. Finally, we show that DNA methylation is required for Myc-mediated repression of *p21Cip1*. Our data identify a new mechanism by which Myc can silence gene expression not only by passive functional interference but also by active recruitment of corepressor proteins. Furthermore, these findings suggest that targeting of DNA methyltransferases by transcription factors is a wide and general mechanism for the generation of specific DNA methylation patterns within a cell.

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*Corresponding author. Free University of Brussels, 808 route de Lennik, 1070 Brussels, Belgium. Tel.: +32 2 555 6243; Fax: +32 2 555 6257; E-mail: ffuks@ulb.ac.be
[7]These authors contributed equally to this work

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Introduction

The c-Myc (Myc) protein is an important regulator of many cellular processes, including growth, proliferation, differentiation and apoptosis (Pelengaris *et al*, 2002). These diverse cellular functions of Myc are closely tied to its ability to both activate and repress transcription (Pelengaris *et al*, 2002). Transcriptional activation by Myc occurs via dimerization with its partner Max and direct binding to specific DNA sequences, termed E-boxes. Myc stimulates gene expression in part at the level of chromatin, through its association with the cofactor TRRAP, thereby recruiting histone acetyltransferases such as GCN5 and Tip60 (Amati *et al*, 2001; Levens, 2003). Myc directly binds and stimulates the expression of a very large population of E-boxes containing genes (Levens, 2003).

In contrast to transcriptional activation by Myc, the mechanisms by which Myc silences gene expression are less well understood. An increasing number of target genes repressed by Myc have been identified, including the cyclin-dependent kinase inhibitors *p21Cip1*, *p15Ink4b*, *p27Kip1* as well as genes involved in cellular differentiation and metabolism (Eisenman, 2001; Wanzel *et al*, 2003). Genes repressed by Myc do not seem to involve its direct association to DNA, but rather Myc is recruited to core promoters through protein–protein interactions with positively acting transcription factors, such as TFII-I (Roy *et al*, 1993), NF-Y (Izumi *et al*, 2001), and Miz-1 (Peukert *et al*, 1997). The current model for Myc-mediated gene silencing is that Myc associates with these activators and passively interferes with their transactivation function. The most convincingly demonstrated mechanism of functional interference by Myc is through its interaction with the Miz-1 transcription factor. Several studies have shown that Miz-1 binds and activates promoters of several genes, including *p21Cip1* and *p15Ink4b*, and that transactivation by Miz-1 can be negatively regulated by its association with Myc (Seoane *et al*, 2001, 2002; Staller *et al*, 2001; Herold *et al*, 2002; van de Wetering *et al*, 2002). This is likely due, at least in part, because Myc competes with the coactivator p300 for binding to Miz-1 (Staller *et al*, 2001). In addition to functional interference with trancriptional activators, whether other mechanisms are involved in Myc-mediated gene silencing remains to be demonstrated.

DNA methylation at CpG dinucleotides is the major epigenetic modification in mammals and is known to be associated with transcriptional repression. This gene-silencing function can be related to the essential role played by CpG methylation for normal mammalian development (Jaenisch and Bird, 2003). The occurrence of DNA methylation within the genome is not random, but rather patterns of methylation are generated that are gene and tissue specific (Bird, 2002). How are DNA methylation patterns established is still poorly understood. Mechanistic insights into that question have begun to come from the characterization of the enzymes—the DNA methyltransferases—that generate methylation

patterns. Three active DNA CpG methyltransferases, Dnmt1, Dnmt3a, and Dnmt3b, have been identified in mammals (Bestor *et al*, 1988; Okano *et al*, 1998). Whereas Dnmt3a and Dnmt3b have been shown to be required for *de novo* methylation (Okano *et al*, 1998, 1999), Dnmt1 appears to function primarily as a maintenance methyltransferase, restoring methylated cytosines following DNA replication (Leonhardt *et al*, 1992). Several studies have shown that Dnmts can act as corepressors to silence gene expression, in part through their association with histone deacetylases (Fuks *et al*, 2000, 2001; Robertson *et al*, 2000; Bachman *et al*, 2001), that help maintain chromatin in a compacted and silent state. DNA methyltransferases have little intrinsic sequence specificity beyond CpG dinucleotide (Yoder *et al*, 1997), and therefore other parameters are likely to be required to target their enzymatic activities to preferred genomic loci. It has been proposed that Dnmts may be directed by alterations in the chromatin structure, whereby chromosomal regions would not be equally accessible to Dnmts (Bird, 2002; Burgers *et al*, 2002). Consistent with this notion, studies of two SNF2 family helicases, ATRX and Lsh2, have shown that mutants of these enzymes decrease CpG methylation (Gibbons *et al*, 2000; Dennis *et al*, 2001). In addition, findings in *Neurospora*, *Arabidopsis* and more recently in mammals have shown that histone methylation at Lys9 of H3, which is associated with gene silencing, facilitates DNA methylation (Tamaru and Selker, 2001; Jackson *et al*, 2002; Lehnertz *et al*, 2003). Thus, chromatin modification or remodelling proteins could be needed for recruitment of Dnmts to particular loci. Another explanation to account for the varying DNA methylation patterns could involve a CpG methylation-targeting mechanism steered by sequence-specific binding proteins. Evidence for this mechanism has come from recent work showing an association of Dnmts with the oncogenic transcription factor PML-RAR, which binds to the *RAR*β promoter and thereby recruits Dnmts to methylate and silence the targeted promoter (Di Croce *et al*, 2002).

In the present study, we report that Myc silences transcription by recruiting a DNA methyltransferase corepressor. We found that Myc associates with the Dnmt3a enzyme and targets its activity, through the DNA-binding protein Miz-1, to the *p21Cip1* promoter. Recruitment of Dnmt3a by Myc leads to methylation and silencing of the targeted *p21Cip1* gene. These data define a previously unrecognized pathway for Myc-mediated repression. In addition, our work sheds light on the poorly understood mechanisms by which specific CpG methylation patterns are established by DNA methyltransferases.

Results

Myc interacts with the corepressor Dnmt3a and associates with DNA methyltransferase activity

The mechanisms by which Myc silences gene expression remain unclear. Several studies indicate that Myc acts as a transcriptional repressor, at least in part, through its functional interference with transcriptional activators bound to different DNA sequences (Eisenman, 2001; Wanzel *et al*, 2003). In the present work, we considered whether Myc-mediated repression might in addition include an active mechanism involving the recruitment of corepressors. By means of an *in vitro* gluthatione S-transferase (GST) pull-down assay, we found that *in vitro* translated (IVT) and radiolabelled full-length Myc bound to the DNA methyltransferase Dnmt3a fused to GST (Figure 1A, left panel, lanes 4 and 5). Residues encompassing the conserved PHD-like motif of Dnmt3a were involved in the association with Myc (Figure 1A, left panel, lanes 4 and 5). In contrast, Myc failed to bind to the control GST alone or to the extreme N-terminal and C-terminal parts of Dnmt3a (Figure 1A, left panel, lanes 2, 3 and 6, respectively). We performed the reciprocal experiment using IVT full-length Dnmt3a and various GST fragments spanning the Myc protein. Figure 1A (right panel) shows that residues encompassing the conserved MBI and MBII domains of Myc contributed to its interaction with Dnmt3a.

To further validate the interaction between Myc and Dnmt3a, we used a coimmunoprecipitation approach. We cotransfected mammalian U2OS cells with vectors expressing full-length Myc tagged with HA and full-length Dnmt3a tagged with GAL4, and analyzed the cell lysates by immunoprecipitation using an antibody against GAL4 (for Dnmt3a), followed by Western blotting with an antibody against HA (for Myc). Figure 1B (left panel) indicates that Myc interacts with Dnmt3a (lane 1), whereas no precipitate was detected after transfection of either HA-Myc or GAL4-Dnmt3a alone (lanes 2 and 3, respectively). The reverse experiment, that is immunoprecipitation of HA-Myc followed by Western blotting for GAL4-Dnmt3a, also allowed specific association between the proteins (Figure 1B, right panel). The interaction between Myc and Dnmt3a can also be demonstrated in untransfected cells. In this experiment, an immunoprecipitate obtained with Dnmt3a-specific antibody was shown to contain Myc (Figure 1C, upper left panel, lane 1). As controls, no precipitation of Myc was observed using an unrelated CREB1 antibody (Figure 1C, upper left panel, lane 2). The presence of Dnmt3a or CREB1 in immunoprecipitates was visualized by Western blotting using anti-Dnmt3a or anti-CREB1, respectively (Figure 1C, bottom left panel). The reverse endogenous coimmunoprecipitation of Dnmt3a with Myc was also observed (Figure 1C, right panel).

The binding of Myc to Dnmt3a led us to expect that Myc would be associated with DNA methyltransferase activity. To test this, we evaluated whether antibodies against Myc could immunoprecipitate DNA methyltransferase activity from untransfected cells. As shown in Figure 1D, immunoprecipitation of endogenous Myc from HeLa nuclear extracts with anti-Myc antibodies purified significant amount of DNA methyltransferase activity (lane 2), whereas control immunoprecipitation with antibodies against another nuclear protein (PLZF; lane 1) showed background activity. The Dnmt enzymatic activity bound to Myc is provided by Dnmt3a. Indeed, when Myc fused to GST was incubated with bacterially expressed and active Dnmt3a followed by Dnmt enzymatic assay, Myc associated with Dnmt3a methyltransferase activity (Figure 1E). The Myc-associated enzymatic activity could also be due to Dnmt3b but not to Dnmt1, since, using a coimmunoprecipitation approach, we found that Myc coimmunoprecipitated with Dnmt3b but not with Dnmt1 after cotransfection into mammalian cells (see Supplementary Figure 1). Taken together, these data indicate that endogenous Myc binds to the Dnmt3a enzyme and is associated with DNA methyltransferase activity *in vivo*.

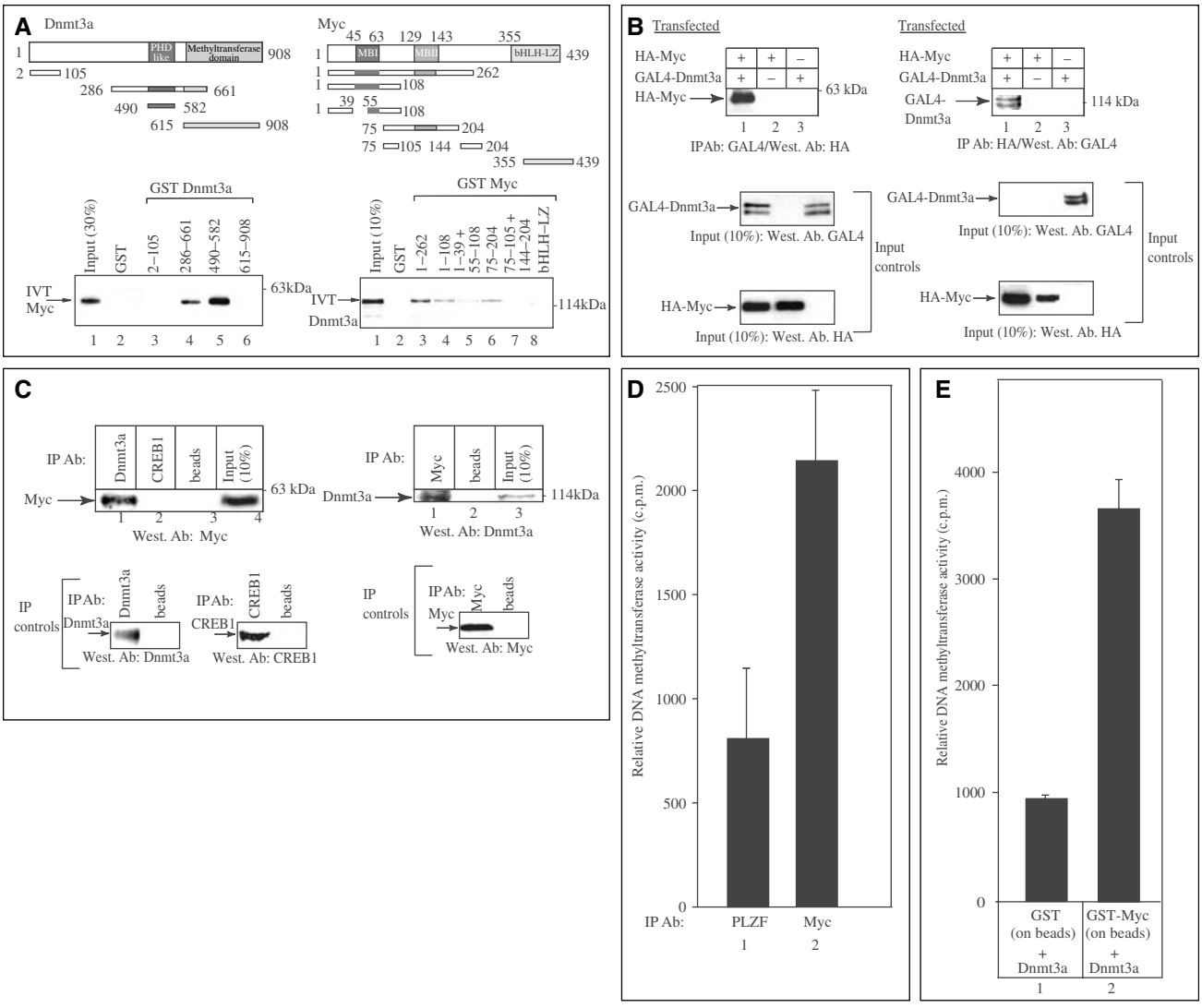
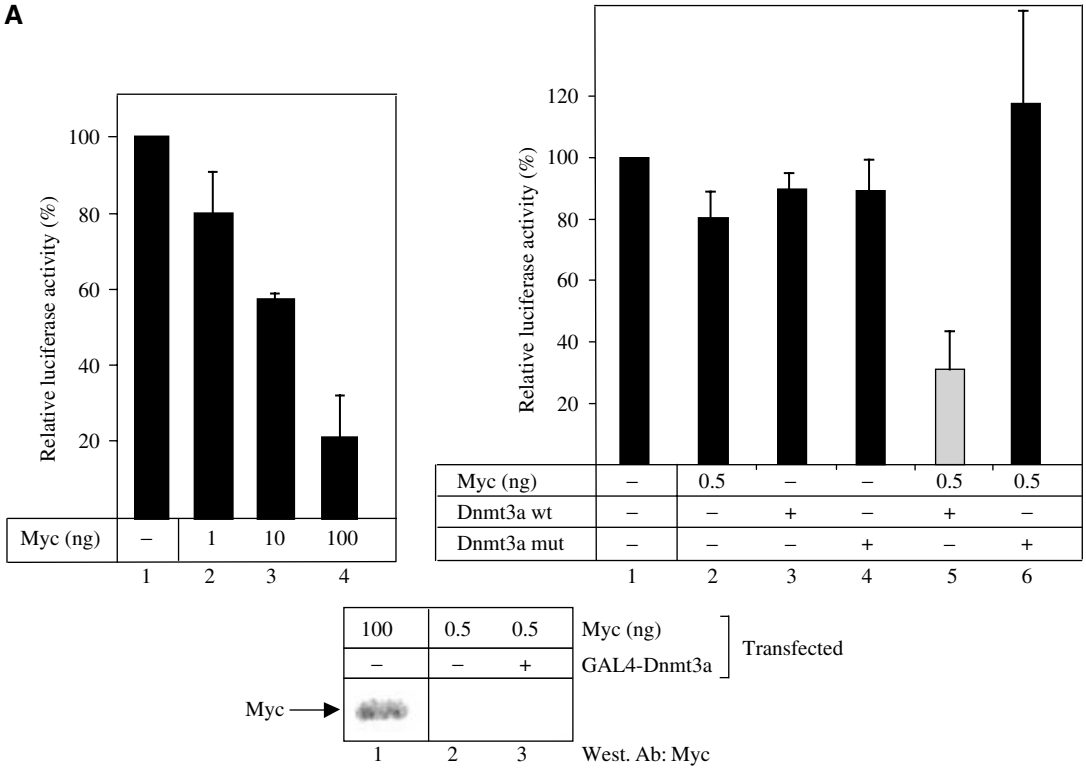


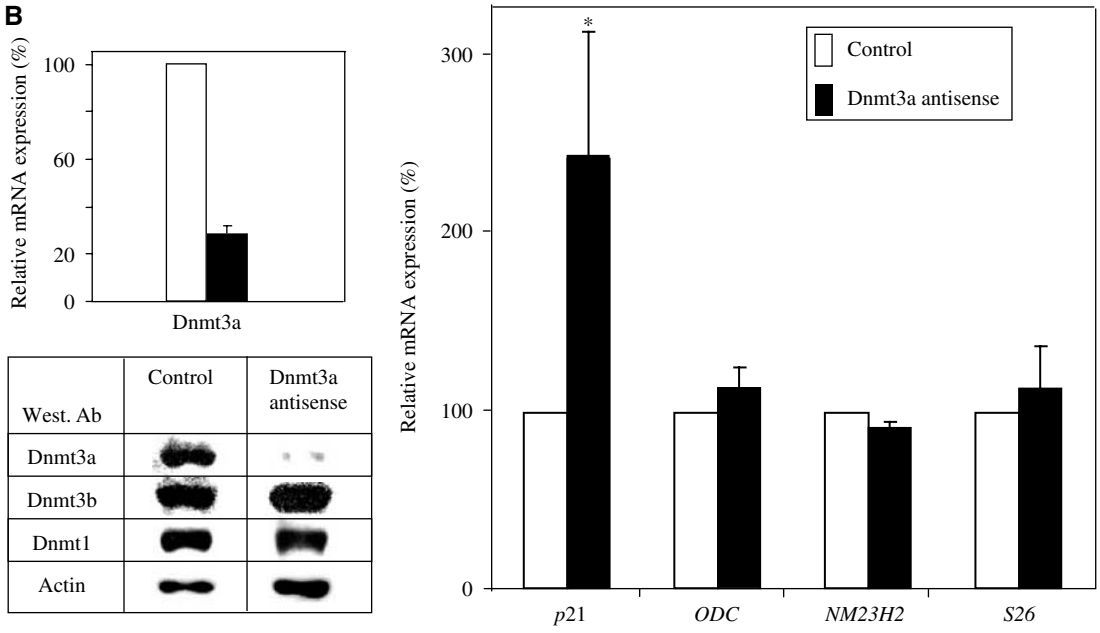
Figure 1 Myc binds the corepressor Dnmt3a and associates with DNA methyltransferase activity *in vivo*. (**A**) Representation of GST fusions of Dnmt3a (left panel) or Myc (right panel). The indicated GST fusions were tested in GST pull-down experiments using IVT full-length Dnmt3a (left) or Myc (right). (**B**) Myc coimmunoprecipitates with Dnmt3a. U2OS were transiently transfected as indicated (+) with expression vectors for HA-tagged Myc full-length and/or GAL4-tagged Dnmt3a full length. Left panel: cell extracts were precipitated with anti-GAL4 antibody and the presence of HA-Myc in the immunoprecipitates was visualized by Western blot analysis using anti-HA antibody. Right panel: anti-HA was used to immunoprecipitate HA-Myc and anti-GAL4 was used to immunoblot GAL4-Dnmt3a. Expression levels of the different proteins in the inputs were verified by Western blotting using anti-GAL4 or anti-HA antibodies (Input controls). (**C**) Myc coimmunoprecipitates with Dnmt3a from untransfected cells. Left panel: HeLa nuclear extracts were immunoprecipitated using anti-Dnmt3a, anti-CREB1 (used as negative control) or the beads only. Precipitated were blotted with anti-Myc antibody. Bottom: the presence of Dnmt3a or CREB1 in immunoprecipitates was visualized by Western blotting using anti-Dnmt3a or anti-CREB1, respectively. Right panel: reverse endogenous coimmunoprecipitation of Dnmt3a with Myc. (**D**) Endogenous Myc purifies DNA methyltransferase activity from nuclear extracts. HeLa nuclear extracts were immunoprecipitated with either a specific antibody against Myc (lane 2) or an irrelevant antibody (PLZF; lane 1). After washing, the immune complexes were tested for DNA methyltransferase activity. Activity is given as c.p.m. of radiolabelled methyl groups from *S*-adenosyl-L-[*methyl*-^{3}H]-methionine incorporated into an oligonucleotide substrate. (**E**) Myc-associated Dnmt activity is provided by Dnmt3a. DNA methyltransferase assay was performed as in (D) using immobilized-GST-Myc 1–204 and bacterially expressed and active Dnmt3a.

Figure 2 The Myc-repressed *p21Cip1* gene is silenced by Dnmt3a *in vivo*. (**A**) Left panel: U2OS cells were transfected with the reporter construct *p21Cip1* promoter-Luc together with increasing amounts of Myc full length (1–100 ng). The activity of the reporter in the absence of Myc is normalized to a value of 100. Right panel: Myc-mediated repression of *p21Cip1* promoter is enhanced by Dnmt3a wild type. U2OS cells were transfected with the reporter *p21Cip1* promoter-Luc together with combinations of limiting amount of expression vectors for Myc and Dnmt3a wild-type (Dnmt3a wt) or catalytic mutant (Dnmt3a mut). Bottom: Western blotting using anti-Myc shows that enhancement of Myc-mediated *p21Cip1* silencing by Dnmt3a (Figure 2A, right panel, lane 5) is not simply due to increased expression of Myc protein levels after cotransfection of Dnmt3a. (**B**) Depletion of Dnmt3a from cells reactivates endogenous *p21Cip1* expression. U2OS were treated either with Dnmt3a antisense inhibitor or with mismatch control. Left panel: Dnmt3a antisense specifically depletes Dnmt3a but not Dnmt1 or Dnmt3b. Quantitative real-time PCR analysis of Dnmt3a mRNA is shown, as well as Western blotting of the Dnmts. Actin serves as a loading control for Western blotting. Right panel: mRNA expression of the indicated genes was determined by quantitative real-time PCR and normalized against that of β-actin. Expression of each mRNA with mismatch control was set to a value of 100. Error bars represent standard deviations. Statistical significant reactivation of *p21Cip1* was observed with Dnmt3a antisense compared to control ($^*P<0.05$).

Dnmt3a specifically silences the Myc-repressed p21Cip1 gene

As Dnmt3a functions as a transcriptional corepressor (Bachman *et al*, 2001; Fuks *et al*, 2001), we next investigated whether Dnmt3a could act together with Myc to silence gene expression. We chose the *p21Cip1* gene as a Myc-inhibited gene because it is a *bona fide* Myc-repressed target (Coller *et al*, 2000; Herold *et al*, 2002; Seoane *et al*, 2002) and it is known that its expression can be downregulated by DNA methylation (Allan *et al*, 2000; Zhu *et al*, 2003). Figure 2A (left panel) shows that transient transfection of *p21Cip1* promoter together with increasing amounts of Myc led to





Myc-repressed gene	Yes	No	No	No
Myc-activated gene	No	Yes	Yes	No

a dose-dependent inhibition of its promoter activity. While expression of limiting amounts of Myc or Dnmt3a repressed *p21Cip1* activity only slightly (Figure 2A, right panel, lanes 2 and 3), cotransfection of Myc along with Dnmt3a provided a synergistic repressive effect on *p21Cip1* transcription (lane 5). High expression of Myc alone (Figure 2A, left panel, lane 4) leads to similar level of *p21Cip1* repression observed by cotransfection of Myc and Dnmt3a (Figure 2A, right panel, lane 5). Hence, it was possible that enhancement of Myc-mediated *p21Cip1* silencing by Dnmt3a (Figure 2A, right panel, lane 5) was simply due to increased expression of Myc protein levels after cotransfection of Dnmt3a. However, this is not the case, as shown by Western blotting of Myc protein after transfection of either high or low levels of Myc, in the presence or absence of Dnmt3a (Figure 2A, bottom panel). Together, these results suggest that Dnmt3a can act as a corepressor with Myc on the *p21Cip1* promoter.

To establish whether endogenous Dnmt3a regulates the Myc-repressed *p21Cip1* gene *in vivo*, we treated U2OS cells with a previously characterized Dnmt3a antisense oligonucleotide inhibitor (Robert *et al*, 2003). Quantitative real-time PCR analysis indicated that messenger RNA (mRNA) *Dnmt3a* levels were markedly decreased in cells treated with Dnmt3a antisense compared to mismatch control (Figure 2B, left panel). Similarly, Western blot analysis after treatment with Dnmt3a antisense showed specific depletion of Dnmt3a protein levels, whereas Dnmt3b or Dnmt1 protein levels were not affected (Figure 2B, left panel). As shown in Figure 2B (right panel), *p21Cip1* mRNA levels were significantly elevated in the cells with reduced Dnmt3a levels. In contrast, the expression levels of *ODC* and *NM23-H2*, two E-box genes that are activated by Myc (Bello-Fernandez *et al*, 1993; Schuhmacher *et al*, 2001), were unchanged. The expression of *S26*, which is not regulated by Myc, was also not affected. Together, these data demonstrate that Dnmt3a is a specific repressor of the Myc-repressed *p21Cip1* gene *in vivo*.

Myc targets Dnmt3a to the p21Cip1 promoter

We next asked whether Dnmt3a could be recruited by Myc to *p21Cip1*. To test this, we performed chromatin immunoprecipitation experiments (ChIPs), first on cells transfected with either the *p21Cip1* promoter, and Dnmt3a alone, or in combination with an expression vector for HA-tagged Myc. We used primers located within the *p21Cip1* proximal promoter region as it is the region recognized by Myc (Herold *et al*, 2002; Seoane *et al*, 2002; van de Wetering *et al*, 2002). Figure 3A shows that, in the presence of overexpressed Myc, Dnmt3a can bind to *p21Cip1* (lane 2), whereas in the absence of exogenous Myc, Dnmt3a did not bind to the *p21Cip1* proximal promoter (lane 6).

The above-mentioned ChIP data were obtained in transfected cells and may be considered as an artificial system. Thus, we next determine whether recruitment of Dnmt3a by Myc could also be observed from untransfected cells. To this end, we tested in ChIP assays the well-characterized *c-myc* knockout rat fibroblasts (*c-myc−/−*) and their wild-type counterparts (*c-myc+/+*) (Mateyak *et al*, 1997). In *c-myc+/+* cells, the *p21Cip1* proximal promoter is bound by Dnmt3a (Figure 3B, lane 2). However, in *c-myc−/−* cells, Dnmt3a binding is significantly reduced (Figure 3B, lane 6). Similar ChIPs on the albumin promoter, to which Myc does not bind (Zeller *et al*, 2001), show no Dnmt3a binding

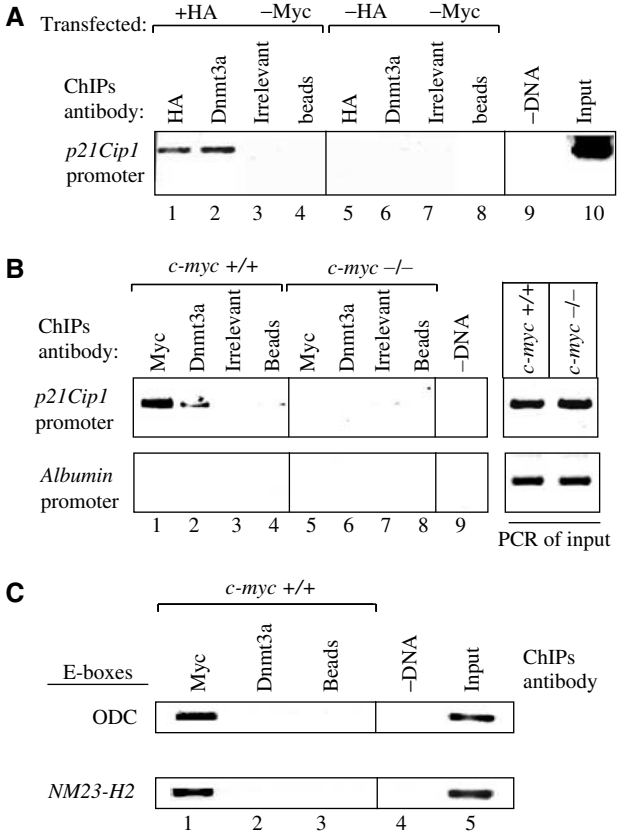


Figure 3 Myc recruits Dnmt3a to the *p21Cip1* promoter. (**A**) ChIPs from overexpressed cells show that binding of Dnmt3a to the *p21Cip1* promoter depends on Myc. 293 cells (one 6 cm dish) were transfected with *p21Cip1* promoter-Luc and Dnmt3a in the presence (lanes 1–4) or absence of HA-tagged Myc (lanes 5–8). The crosslinked chromatin was then immunoprecipitated as indicated. (The irrelevant antibody is against GFP.) The purified DNA was then amplified by PCR. Note that given the low amount of transfected 293 cells used, endogenous Myc is not detectable on *p21Cip1*. (**B**) ChIPs in untransfected *c-myc+/+* and *c-myc−/−* show Myc dependence for Dnmt3a recruitment to *p21Cip1* promoter *in vivo*. Crosslinked chromatin from one 15 cm dish of *c-myc+/+* (lanes 1–4) or *c-myc−/−* (lanes 5–8) were immunoprecipitated with the indicated antibodies (the irrelevant antibody was against HA) or the beads only. (**C**) ChIPs performed in *c-myc+/+* cells show that Dnmt3a does not bind the Myc-activated E-box genes *ODC* and *NM23-H2*.

(Figure 3B, lower panel). Consistent with data presented in Figure 2B, the Myc-activated E-box promoters, *ODC* and *NM23-H2*, associated with Myc in *c-myc+/+* cells but not with Dnmt3a (Figure 3C). Collectively, these data strongly suggest that Myc targets Dnmt3a selectively to the *p21Cip1* promoter.

Myc and Dnmt3a corepress p21Cip1 promoter through association of Myc with Miz-1

Recent data indicated that Myc does not bind directly to the *p21Cip1* proximal promoter but is recruited through its association with the DNA-binding protein Miz-1 (Herold *et al*, 2002; Seoane *et al*, 2002; van de Wetering *et al*, 2002). We therefore asked whether Miz-1 could be the factor that targets Myc and Dnmt3a to silence *p21Cip1* expression. To test this idea, we first determined whether Dnmt3a can associate with

Miz-1. As shown in Figure 4A, when Miz-1 and GAL4-tagged Dnmt3a expression vectors were transiently transfected in mammalian cells, we detected an interaction after immunoprecipitation with anti-GAL4 antibody, followed by Western blotting with an Miz-1-specific antibody. Dnmt3a also coimmunoprecipitated with Miz-1 in untransfected cells (Figure 4B). Further, immunoprecipitation of endogenous Miz-1 with anti-Miz-1 antibody purified significant DNA methyltransferase activity from HeLa nuclear extracts (Figure 4C, lane 2), whereas control immunoprecipitation using an irrelevant antibody (PLZF) gave background activity (Figure 4C, lane 1). These results indicate that Miz-1 can interact with the Dnmt3a DNA methyltransferase, consistent with its ability to associate

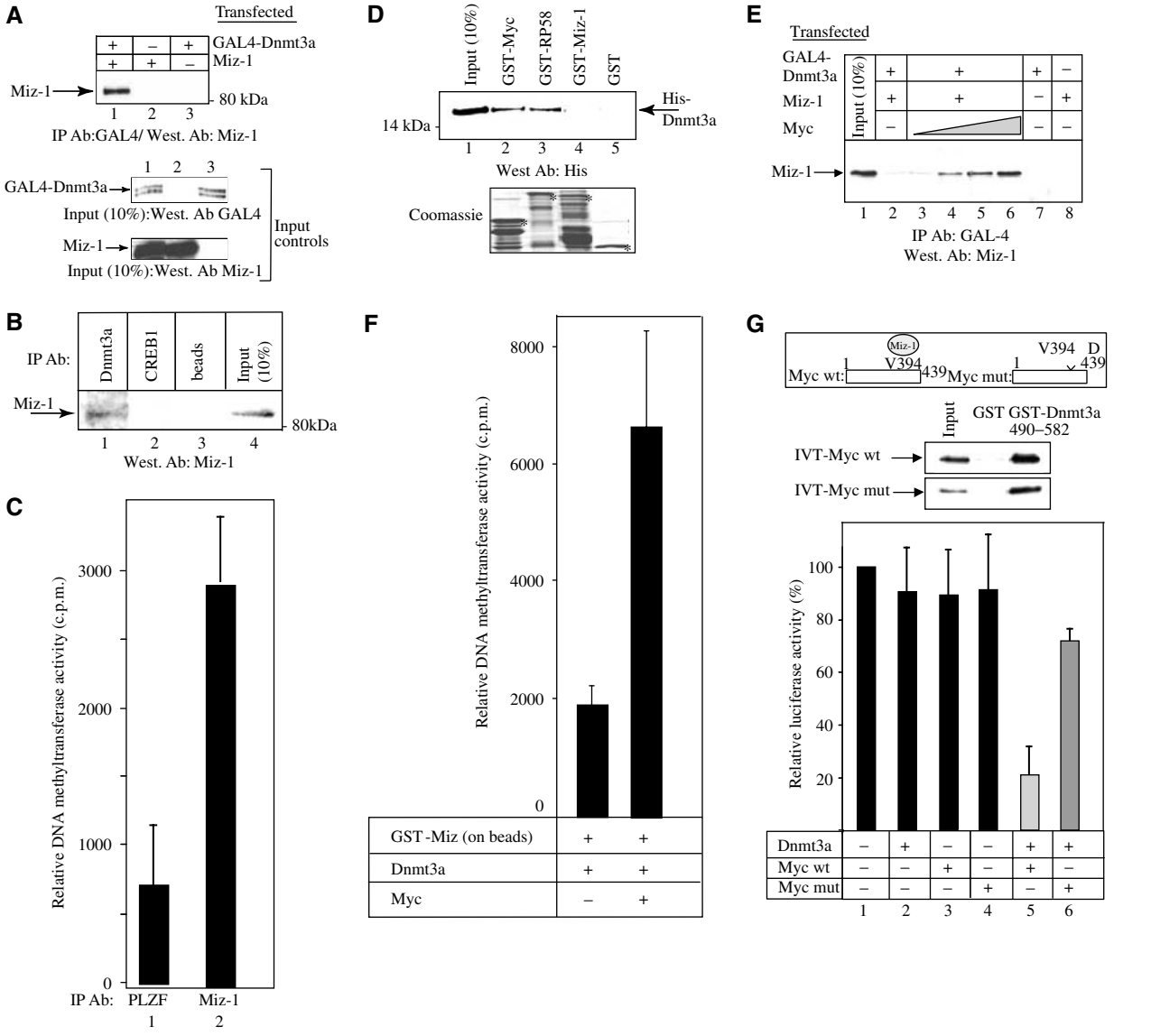


Figure 4 Myc corepresses *p21Cip1* promoter together with Dnmt3a through association with the DNA-binding protein Miz-1. (**A**) U2OS cells were transfected with GAL4-Dnmt3a and/or Miz-1. Cell extracts were then precipitated with anti-GAL4 antibody followed by Western blotting using a specific anti-Miz-1 antibody. Expression levels of the different proteins in the inputs were verified by Western blotting using anti-GAL4 or anti-HA antibodies (Input controls). (**B**) Miz-1 coimmunoprecipitates with Dnmt3a from untransfected cells. HeLa nuclear extracts were immunoprecipitated using anti-Dnmt3a, anti-CREB1 (used as negative control) or the beads only. Precipitated were blotted with a Miz-1 antibody. (**C**) Endogenous Miz-1 purifies DNA methyltransferase activity from HeLa nuclear extracts. (**D**) Myc binds directly to Dnmt3a, whereas Miz-1 only weakly binds directly to Dnmt3a. GST-Myc 1-204, GST-RP58, used as positive control (Fuks *et al*, 2001) or GST-Miz-1 were incubated with bacterially expressed His-Dnmt3a, followed by Western blotting using anti-His antibody. Coomassie stain gel show the input of GST fusion proteins used. (**E**) Myc mediates the interaction of Dnmt3a with Miz-1. U2OS were transfected with limited amount of GAL4-Dnmt3a and Miz-1 in the presence (lanes 3–6) or absence (lanes 2, 7 and 8) of increasing levels of Myc (1–4 μg). After immunoprecipitation of GAL4-Dnmt3a with anti-GAL4, Western blotting was performed using anti-Miz-1 antibody. (**F**) Myc increases the association of Miz-1 with Dnmt3a enzymatic activity. DNA methyltransferase assays were performed using immobilized-GST-Miz-1 and bacterially expressed and active Dnmt3a, in the presence or absence of bacterially expressed His-Myc. (**G**) Corepression of *p21Cip1* by Myc and Dnmt3a requires binding of Myc to Miz-1. Upper panel: Representation of Myc wt or the Myc mutant (MycV394D; unable to bind to Miz-1 and that lost Miz-1-dependent repression) (Herold *et al*, 2002). GST pull-downs using GST-Dnmt3a 490–592 and IVT Myc wt or Myc mut show that the Myc mutant still retains binding to Dnmt3a. Lower panel: U2OS were transfected with *p21Cip1*-Luc reporter and, as indicated, with limited amount of Dnmt3a and either Myc wt or the Myc point mutant.

with DNA methyltransferase activity *in vivo*. The Dnmt enzymatic activity bound to Miz-1 is provided by Dnmt3a (Figure 4F, see below) and also likely by Dnmt3b, but not Dnmt1. Indeed, coimmunoprecipitations after cotransfection into mammalian cells indicated that Miz-1 binds to Dnmt3b whereas Dnmt1 did not (see Supplementary Figure 2).

Several observations indicate that the interaction of Miz-1 with Dnmt3a is indirectly mediated by the binding of Dnmt3a to Myc. First, we performed a direct interaction assay by producing Dnmt3a, Myc, and Miz-1 as recombinant proteins in *Escherichia coli*. As shown in Figure 4D, GST-Myc bound histidine-tagged Dnmt3a (lane 2), whereas GST-Miz-1 (lane 4) did not or weakly. We used GST fused to the RP58 protein, a protein known to bind directly to Dnmt3a (Fuks *et al*, 2001), as a positive control (lane 3). Thus, Myc contacts Dnmt3a directly, while Miz-1 seems not. Second, similar coimmunoprecipitations as described in Figure 4A were performed, this time by transfecting limiting amounts of Miz-1 and GAL4-Dnmt3a, which resulted in only weak interaction between the two proteins (Figure 4E, lane 2). Overexpression of increasing amounts of Myc together with Miz-1 and GAL4-Dnmt3a strongly enhanced the association of Miz-1 with Dnmt3a (Figure 4E, lanes 3–6). As we cannot exclude that Miz-1 could weakly bind directly to Dnmt3a (Figure 4D, lane 4), it was possible that this enhanced Miz-1–Dnmt3a interaction was simply due to Myc expression increasing Dnmt3a levels. However, this possibility is unlikely since overexpression of Myc did not affect levels of Dnmt3a protein (Supplementary Figure 3). Following Dnmt3a-Miz-1 immunoprecipitation, we detected the presence of Myc by Western blotting using anti-Myc antibody (data not shown). Thus, these results suggest that Miz-1, Myc, and Dnmt3a can form a ternary complex. Next, and along the same line, DNA methyltransferase assays were performed using immobilized bacterially expressed GST-Miz-1 and recombinant Dnmt3a as a source of Dnmt enzyme, in the presence or absence of recombinant Myc. As shown in Figure 4F, addition of Myc significantly increased the association of Miz-1 with Dnmt3a enzymatic activity. This observation is consistent with coimmunoprecipitations presented in Figure 4E and suggests the formation of a trimeric Miz-1–Myc–Dnmt3a complex.

We then tested whether this ternary complex is involved in *p21Cip1* transcriptional silencing. For this, we performed corepression assays similar to those described in Figure 2B, using a point mutant of Myc (Myc mut) that is deficient in binding to Miz-1 and thereby unable to repress *p21Cip1* (Herold *et al*, 2002), while it still retains binding to Dnmt3a (Figure 4G, upper panel). Figure 4G (lower panel) shows that, while cotransfection of Dnmt3a together with Myc wt increased *p21Cip1* inhibition in a cooperative manner (lane 5), coexpression of Dnmt3a along with the Myc point mutant strongly impaired the repression of *p21Cip1* (lane 6). These results strongly suggest that corepression of *p21Cip1* promoter activity by Dnmt3a and Myc is dependent on interaction of Myc with Miz-1.

DNA methylation participates in Myc-mediated silencing of p21Cip1

Having shown that Myc binds the Dnmt3a DNA methyltransferase and that the latter silences *p21Cip1* expression, we next set out to establish whether DNA methylation was required for Myc to repress *p21Cip1*. To test this possibility, we first asked whether the repression mediated by Myc on *p21Cip1* promoter linked to the luciferase gene could be relieved by the addition of the DNA methylation inhibitor 5-azacytidine (5-AZC). As shown in Figure 5A, the repressive effect observed with Myc on reporter activity was substantially relieved in cells treated with 5-AZC. As 5-AZC is a broad-spectrum DNA methyltransferase inhibitor, we used a point mutant of Dnmt3a that abolishes its enzymatic activity (Hsieh, 1999) and found that, as compared to wild-type Dnmt3a, the mutant is unable to repress the *p21Cip1* promoter in cooperation with Myc (Figure 2A, right panel, lane 6).

The effect of 5-AZC on Myc-mediated silencing of *p21Cip1* was verified *in vivo* by monitoring the expression of *p21Cip1* in wild-type and knockout Myc rat fibroblasts that were treated or not with 5-AZC. RNA isolated from each cell type was reverse transcribed and amplified by polymerase chain reaction (RT–PCR). Figure 5B shows that, in the *c-myc*+/+ cells, *p21Cip1* mRNA levels were elevated when cells were treated with 5-AZC, whereas the drug had no effect on the expression of an unrelated housekeeping gene, *GAPDH*. In contrast, in the *c-myc*−/− cells, the level of *p21Cip1* mRNA was not affected by the addition of 5-AZC (Figure 5B). Reintroduction of Myc into knockout Myc cells (*c-myc*−/− +Myc) decreased *p21Cip*1 expression to a similar low level as observed in the parental *c-myc*+/+ cells, while 5-AZC treatment caused its re-expression (Figure 5B). These data strongly suggest that silencing of *p21Cip1* by DNA methylation requires the presence of Myc. To confirm these observations and since 5-AZC can have pleiotropic effects (Christman *et al*, 1985), we next carried out bisulphite genomic sequencing to compare the methylation status of *p21Cip1* proximal promoter between *c-myc*+/+ and *c-myc*−/− cells. Figure 5C indicates that several CpGs located within the *p21Cip1* proximal promoter showed differential methylation between *c-myc*+/+ and *c-myc*−/− cells (CpGs situated outside this cluster region did not show significant difference in methylation status; data not shown). Further, *c-myc*−/− cells in which Myc expression had been restored (*c-myc*−/− +Myc) gained methylation of *p21Cip1*, thus confirming that the presence of Myc is needed, at least in part, for DNA methylation of *p21Cip1* proximal promoter. These observations do not seem to be restricted to Rat1 fibroblasts as U2OS cells, which express Myc (data not shown), also show methylation of *p21Cip1* promoter (Figure 5D).

Discussion

Myc silences transcription by active recruitment of DNA methyltransferase corepressor

Recent studies indicate that transcriptional silencing by Myc significantly contributes to most of its biological functions; yet, the details underlying this process are still unclear. To date, Myc-mediated gene repression is known to involve a passive mechanism through interference with transcriptional activators (Eisenman, 2001; Wanzel *et al*, 2003). Here, we report a new mechanism by which Myc silences gene expression through recruitment of the Dnmt3a corepressor to a promoter, thereby leading to subsequent DNA methylation and silencing of the targeted promoter.

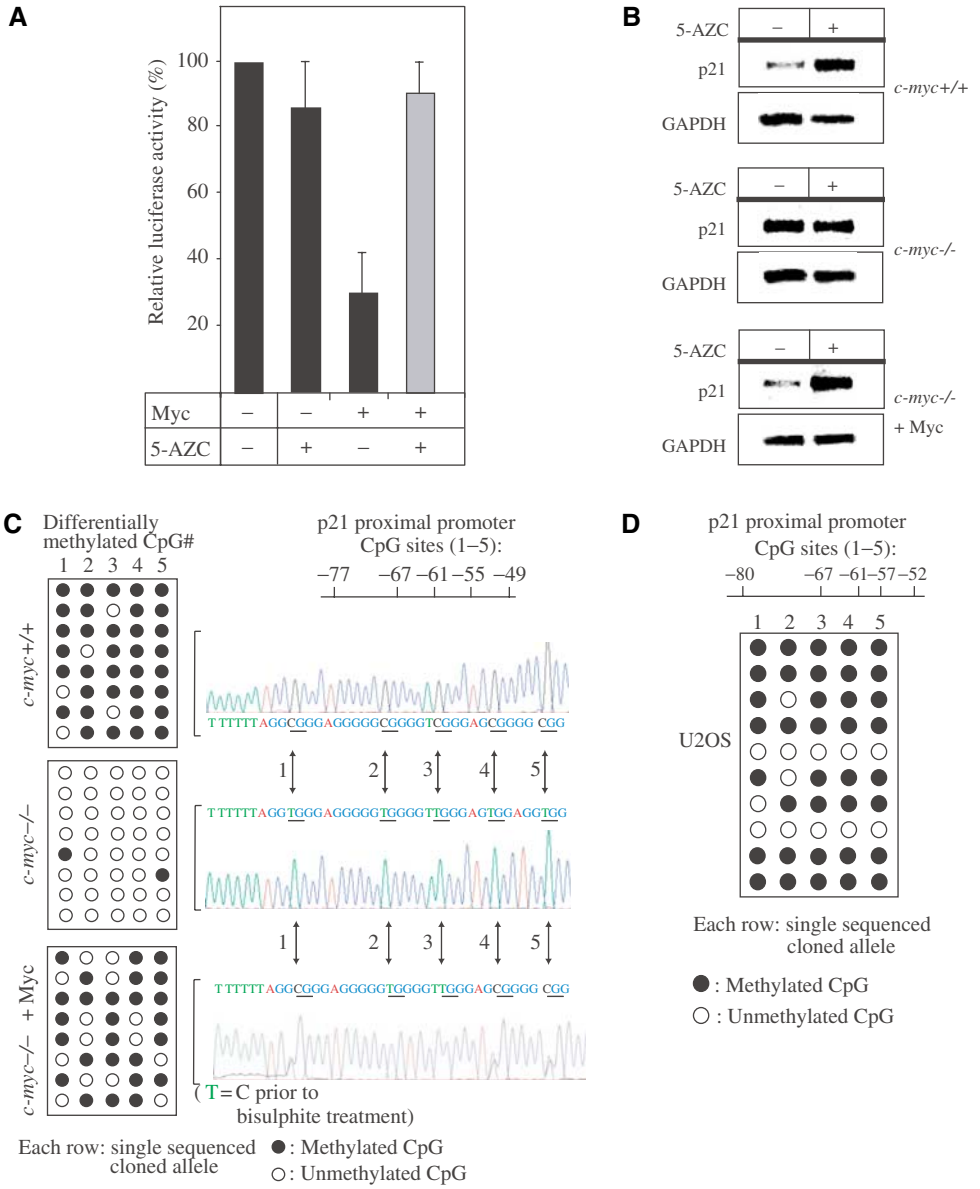


Figure 5 DNA methylation is required for Myc-mediated repression of *p21Cip1*. (**A**) Inhibition of DNA methyltransferase activity relieves repression by Myc. U2OS were transfected with *p21Cip1*-Luc reporter and Myc as indicated and treated or not with the demethylating agent 5-AZC (2 μM). (**B**) Inhibition of p21Cip1 by DNA methylation depends on the presence of Myc. *c-myc*+/+, *c-myc*−/− and '*c-myc*−/− +Myc' cells were treated or not with the inhibitor 5-AZC (5 μM). RNA was then isolated and equal amounts were analysed by RT–PCR for the expression of p21Cip1 and GAPDH (used as a negative control). (**C**) Bisulphite genomic sequencing shows that the *p21Cip1* proximal promoter is differentially methylated at specific CpGs in *c-myc*+/+ versus *c-myc*−/− cells. Left: several CpGs (numbered 1–5) showed consistent differential methylation in *c-myc*+/+ versus *c-myc*−/− cells (CpGs situated outside this cluster region did not show significant difference in methylation status; data not shown). Reintroduction of Myc into *c-myc*−/− cells led to a gain in *p21Cip1* methylation. Each row of circles represents a single sequenced cloned and each circle represents a single CpG. Right: Chromatogram of one sequenced clone derived from bisulphite-treated *c-myc*+/+, *c-myc*−/− or '*c-myc*−/− +Myc' cells. Underlined sequences show differential methylation between the three cell types; unmethylated C converted to T (italic) upon bisulphite treatment. (**D**) U2OS cells show methylation of the p21Cip1 proximal promoter after bisulphite sequencing. Top: representation of the methylated CpG sites in human p21 that are homologous in rat p21 promoter.

On Myc-repressed genes, Myc does not seem to bind directly to DNA but rather is recruited through protein–protein interaction with other transcription factors. As mentioned above, in the case of the *p21Cip1* gene, recent studies demonstrated that Myc binds to *p21Cip1* promoter through its association with the DNA-binding factor Miz-1 (Herold *et al*, 2002; Seoane *et al*, 2002; van de Wetering *et al*, 2002). The binding of Myc with Miz-1 switches Miz-1 from a transcriptional activator to a repressor of *p21Cip1* (Herold *et al*, 2002; Seoane *et al*, 2002; van de Wetering *et al*, 2002), likely by preventing the interaction of Miz-1 with its own coactivator (Seoane *et al*, 2001; Staller *et al*, 2001). Our studies suggest that silencing of *p21Cip1* by Myc and Dnmt3a involves the association of Myc with Miz-1. A model could be envisaged in which Myc switches Miz-1 from a transcriptional activator to a repressor by a dual mechanism: (i) as reported by others (Herold *et al*, 2002; Seoane *et al*, 2002), Myc prevents recruitment of a coactivator to Miz-1, and (ii) Myc brings

the corepressor Dnmt3a to Miz-1. These two mechanisms could be particularly important to keep tight control over *p21Cip1* regulation. The ability of Myc/Miz-1 to deliver methyltransferase activity may be relevant for any gene silenced by the Myc/Miz-1 complex. Thus, repression of the *p15ink4b* gene, a known Myc/Miz-1 target (Seoane *et al*, 2001; Staller *et al*, 2001) which is regulated by DNA methylation (Herman *et al*, 1996), may also involve the recruitment of DNA methyltransferase corepressor.

Mechanisms by which DNA methylation is targeted to preferred genomic sequences

How DNA methyltransferases establish DNA methylation patterns within a cell remains unclear. DNA methyltransferases have little sequence specificity beyond CpG dinucleotide (Yoder *et al*, 1997) and several mechanisms could be envisaged to explain the regional specificity they exhibit. One possibility could be that chromatin modifications or remodelling proteins are required for targeting Dnmts to particular loci. Studies in *Neurospora*, *Arabidopsis* and more recently in mammals revealed that histone methylation at Lys9 of H3 can direct methylation of DNA (Tamaru and Selker, 2001; Jackson *et al*, 2002; Lehnertz *et al*, 2003). In *Arabidopsis*, the adaptor protein LHP1, which binds with high affinity to histone H3 when methylated at Lys9, was found to interact with the CMT3 DNA methyltransferase (Jackson *et al*, 2002). Similarly, in mammals, Dnmts were found to physically associate with HP1 (Fuks *et al*, 2003a; Lehnertz *et al*, 2003). In addition, HP1 was recently reported to be essential for DNA methylation in *Neurospora* (Freitag *et al*, 2004). Hence, it was proposed that histone methylation would influence DNA methylation through the adaptor HP1, which would recruit DNA methyltransferases to CpG that has to be methylated (Jackson *et al*, 2002; Fuks *et al*, 2003a; Lehnertz *et al*, 2003).

Besides the proposed recruitment of Dnmts by chromatin-based mechanisms, the present work provides evidence that DNA methylation patterns can be regulated by the targeting of Dnmts to particular loci through their association with specific transcription factors. Our finding supports and extends our observations that the PML-RAR transcription factor associates with Dnmts, thereby allowing CpG methylation of its target gene (Di Croce *et al*, 2002). The targeting of DNA methyltransferases to specific loci by transcriptional regulators is reminiscent of mechanisms by which chromatin-modifying enzymes establish local changes in chromatin structure to regulate gene expression. Indeed, recruitment to promoters of histone acetyltransferases and deacetylases as well as histone methyltransferases by transcription factors seems to be a common and general strategy to bring their enzymatic activities to targeted genes (Kouzarides, 2002; Kurdistani and Grunstein, 2003). It is therefore likely that targeting of DNA methyltransferases to precise genes through their interaction with specific transcription factors may be a wide and general mechanism by which DNA methylation is generated at preferred loci.

Materials and methods

Plasmid constructions

We cloned the following sequences by PCR using appropriate sets of primers: full-length Dnmt3a wild-type or catalytic mutant (Hsieh, 1999), full-length Dnmt1 or Dnmt3b into pcDNA3.1-GAL4, full-length Dnmt3a wild-type or full-length Myc into pET-30 (Novagen) and Dnmt3a fragments into the vector pGEX (Pharmacia). The following plasmids have been described previously: pGEX-Dnmt3a 286–661, pGEX-Dnmt3a 490–582 and pGEX-RP58 (Fuks *et al*, 2001), pGEX-Miz-1 269-803, pGEX-Myc deletion constructs (Hateboer *et al*, 1993), pcDNA3-Myc wt and pcDNA3-Myc mut (Myc V394D point mutant deficient in Miz-1 binding) (Herold *et al*, 2002), pcDNA3 Miz-1 (Peukert *et al*, 1997), pcDNA3-HA-Myc (Frank *et al*, 2001), pBJ Myc (Frank *et al*, 2001) and *p21Cip1*-Luc reporter (2.4 kb).

GST fusion and histine-tagged proteins, in vitro translation, pull-down and direct interaction assays

Recombinant proteins were expressed in and purified from *E. coli* Top10 or Bl21 as described (Fuks *et al*, 2000). *In vitro* translation reactions and GST pull-down experiments have been described previously (Fuks *et al*, 2000). Dnmt3a, Myc wt or Myc mut were IVT from pcDNA3-GAL4-Dnmt3a, pcDNA3-Myc wt or pcDNA3-Myc mut, respectively. Direct interaction assays were performed as described (Fuks *et al*, 2001), using anti-His antibody (H1029, Sigma).

Cell culture, transfections and luciferase assays

Cell lines were maintained in DMEM supplemented with 8% fetal calf serum and grown at 37°C, 5% CO_2. The *c-myc+/+* (TGR1) and *c-myc−/−* (HO15.19) Rat1 fibroblasts (Mateyak *et al*, 1997) were a generous gift of J Sedivy. The '*c-myc−/−* +Myc' cells are *c-myc−/−* cells infected, as described (Frank *et al*, 2001), with a pBabe retrovirus expressing Myc. Transfections were performed using polyethylene imine (PEI) (Euromedex) as described previously (Deplus *et al*, 2002). Luciferase assays were performed with the Promega luciferase Assay System. Transfection efficiencies were normalized using a cotransfected plasmid encoding for β-galactosidase, which is measured in a β-galactosidase assay kit (Tropix). When used, 5-azacytidine was added 4 h following transfection (2 μM final; Sigma). The results shown are the average of at least three independent experiments with error bars displaying standard deviations.

Antisense treatment and quantitative real-time PCR

U2OS were treated for 3 h with 50 nM of the already described 2′-O-methylphosphorothioate Dnmt3a antisense or mismatch control oligonucleotides (Robert *et al*, 2003), in OptiMEM (Gibco) supplemented with 8.5 μg/ml LIPOFECTAMINE Reagent (Invitrogen). Cells were then incubated for 24 h in complete medium. RNA was extracted by Tripure reagent, reverse-transcribed and analysed by real-time PCR as described (Loriot *et al*, 2003). Specific primers and probes for the amplification are available on request. The expression levels of the genes of interest were normalized to the expression level of β-actin. The results shown are the average of three independent experiments with error bars displaying standard deviations. Statistical analyses were carried out using the Wilcoxon–Mann–Whitney test and significance was assigned at $P<0.05$. For control Western blotting, we used antibodies against Dnmt1 (a gift from S Pradhan), Dnmt3a or Dnmt3b (a gift from E Li) and actin (A5316, Sigma).

Immunoprecipitations and Western blot analysis

293 or U2OS cells were transiently transfected in culture dishes (10 cm diameter) with a total of 6 μg of plasmids as described (Deplus *et al*, 2002). Standard procedures were used for coimmunoprecipitations and Western blotting (Deplus *et al*, 2002). For endogenous immunoprecipitations, antibodies were incubated with HeLa nuclear extracts (4C Biotech) in IPH buffer (Fuks *et al*, 2000) at 4°C overnight. Antibodies used were against HA (12C5A, Roche), GAL4 (5C1, Santa Cruz), Myc (C33, Santa Cruz), Miz-1 (N17, Santa Cruz), CREB1 (24H4B, Santa Cruz) and Dnmt3a (IMG268; Imgenex).

DNA methyltransferase assay

DNA methyltransferase assays were carried out as described (Fuks *et al*, 2000). For immunoprecipitations preceding the DNA methyltransferase assay, we used antibodies from Santa Cruz against: Myc (C33), PLZF (F15) and Miz-1 (N17). For assays using recombinant and active Dnmt3a, we used bacterially expressed His-tagged Dnmt3a and immobilized GST-Myc 1–204 or GST-Miz-1 269–803. Where appropriated, eluted His-tagged Myc was also used.

RNA purification and RT–PCR analysis

Rat1 fibroblasts (Mateyak *et al*, 1997) were treated or not with 5-azacytidine as described before (Allan *et al*, 2000). Extraction of total RNA was carried out using Tripure reagent (Roche) according to the manufacturer's instructions. In all, 2 μg of RNA was reversed transcribed using random hexamers (Amersham/Pharmacia Biotech) and SuperscriptII reverse transcriptase (Life Technologies Inc.). PCR was achieved with Taq DNA polymerase (Promega) for 30–35 cycles of amplification.

Chromatin immunoprecipitation (ChIP)

ChIP was performed from either 293 transfected cells (one 6 cm diameter dish) or untransfected rat1 fibroblasts (one 15 cm diameter dish). Cells were crosslinked with formaldehyde (0.75%; Sigma) at room temperature for 10 min. Cells were rinsed twice with ice-cold PBS (pH 7.4) and collected in PBS. ChIPs were then performed essentially as described (Fuks *et al*, 2003b). We used anti-HA antibodies (12C5A, Roche), anti-GAL4 (5C1, Santa Cruz) or an unrelated antibody (anti-GFP, ab290, Abcam) in 293 cells, and anti-Myc (C33 or N262, Santa Cruz), anti-Dnmt3a (IMG268, Imgenex) or anti-HA (12C5A, Roche) in Rat1 cells. Immunoprecipitated DNA was analysed by PCR for the presence of human *p21Cip1* proximal promoter (−427 bp versus start site to +16). For rat *p21Cip1* proximal promoter, primers from −250 bp versus start site to +142 were used. Primers for *albumin* promoter (Zeller *et al*, 2001), and the E-box promoters *ODC* (Frank *et al*, 2001) and *NM23-H2* (Frank *et al*, 2001), were described previously. The cycle number and the amount of template were varied to ensure that results were within the PCR linear range.

Bisulphite genomic sequencing

Methylation status of the *p21Cip1* promoter in Rat1 fibroblasts or U2OS was assessed by bisulphite genomic sequencing. *Bam*HI- or *Eco*RI-digested genomic DNA (5 μg) from these cells was subjected to sodium bisulphite modification and the *p21Cip1* promoter was amplified as described (Di Croce *et al*, 2002). The amplified product was subcloned into the pCR2.1 vector by TA cloning (Invitrogen) and sequenced via automated sequencing. Primer sequences for rat *p21Cip1* amplification were as follows: sense strand 4149 5′-TGGTTTTTATTTGGGTAGTAGTTG-3′, antisense strand 4766 5′-CCTACCTCCAATTCCCCTTAACTC-3′; sense strand 4163 5′-GTAGTA GTTGTTAAAAGGATTTTG-3′, antisense strand 4741 5′-AACACTAT AACAACTCACACCTCT-3′. Primer sequences for human *p21Cip1* amplification were as follows: sense strand 5′-AAAAGTTAGATT TGTGGTTTATTT-3′, antisense strand 5′-TCTCACCTCCTCTAAATA CCTC-3′; sense strand 5′-GGGAGGAGGGAAGTGTTTTT -3′, antisense strand 5′-CAACTACTCACACCTCAACTAAC-3′.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

Allan LA, Duhig T, Read M, Fried M (2000) The p21(WAF1/CIP1) promoter is methylated in Rat-1 cells: stable restoration of p53-dependent p21(WAF1/CIP1) expression after transfection of a genomic clone containing the p21(WAF1/CIP1) gene. *Mol Cell Biol* **20:** 1291–1298

Amati B, Frank SR, Donjerkovic D, Taubert S (2001) Function of the c-Myc oncoprotein in chromatin remodeling and transcription. *Biochim Biophys Acta* **1471:** M135–M145

Bachman KE, Rountree MR, Baylin SB (2001) Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. *J Biol Chem* **276:** 32282–32287

Bello-Fernandez C, Packham G, Cleveland JL (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc Natl Acad Sci USA* **90:** 7804–7808

Bestor T, Laudano A, Mattaliano R, Ingram V (1988) Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J Mol Biol* **203:** 971–983

Bird A (2002) DNA methylation patterns and epigenetic memory. *Genes Dev* **16:** 6–21

Burgers WA, Fuks F, Kouzarides T (2002) DNA methyltransferases get connected to chromatin. *Trends Genet* **18:** 275–277

Christman JK, Schneiderman N, Acs G (1985) Formation of highly stable complexes between 5-azacytosine-substituted DNA and specific non-histone nuclear proteins. Implications for 5-azacytidine-mediated effects on DNA methylation and gene expression. *J Biol Chem* **260:** 4059–4068

Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, Golub TR (2000) Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc Natl Acad Sci USA* **97:** 3260–3265

Dennis K, Fan T, Geiman T, Yan Q, Muegge K (2001) Lsh, a member of the SNF2 family, is required for genome-wide methylation. *Genes Dev* **15:** 2940–2944

Deplus R, Brenner C, Burgers WA, Putmans P, Kouzarides T, de Launoit Y, Fuks F (2002) Dnmt3L is a transcriptional repressor that recruits histone deacetylase. *Nucleic Acids Res* **30:** 3831–3838

Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M, Fuks F, Lo Coco F, Kouzarides T, Nervi C, Minucci S, Pelicci PG (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* **295:** 1079–1082

Eisenman RN (2001) Deconstructing myc. *Genes Dev* **15:** 2023–2030

Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B (2001) Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes Dev* **15:** 2069–2082

Freitag M, Hickey PC, Khlafallah TK, Read ND, Selker EU (2004) HP1 is essential for DNA methylation in neurospora. *Mol Cell* **13:** 427–434

Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T (2000) DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat Genet* **24:** 88–91

Fuks F, Burgers WA, Godin N, Kasai M, Kouzarides T (2001) Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J* **20:** 2536–2544

Fuks F, Hurd PJ, Deplus R, Kouzarides T (2003a) The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res* **31:** 2305–2312

Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T (2003b) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem* **278:** 4035–4040

Gibbons RJ, McDowell TL, Raman S, O'Rourke DM, Garrick D, Ayyub H, Higgs DR (2000) Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nat Genet* **24:** 368–371

Hateboer G, Timmers HT, Rustgi AK, Billaud M, van't Veer LJ, Bernards R (1993) TATA-binding protein and the retinoblastoma gene product bind to overlapping epitopes on c-Myc and adenovirus E1A protein. *Proc Natl Acad Sci USA* **90:** 8489–8493

Herman JG, Jen J, Merlo A, Baylin SB (1996) Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. *Cancer Res* **56:** 722–727

Herold S, Wanzel M, Beuger V, Frohme C, Beul D, Hillukkala T, Syvaoja J, Saluz HP, Haenel F, Eilers M (2002) Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol Cell* **10:** 509–521

Hsieh CL (1999) *In vivo* activity of murine *de novo* methyltransferases, Dnmt3a and Dnmt3b. *Mol Cell Biol* **19:** 8211–8218

Izumi H, Molander C, Penn LZ, Ishisaki A, Kohno K, Funa K (2001) Mechanism for the transcriptional repression by c-Myc on PDGF beta-receptor. *J Cell Sci* **114:** 1533–1544

Jackson JP, Lindroth AM, Cao X, Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* **416:** 556–560

Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* **33** (Suppl)**:** 245–254

Kouzarides T (2002) Histone methylation in transcriptional control. *Curr Opin Genet Dev* **12:** 198–209

Kurdistani SK, Grunstein M (2003) Histone acetylation and deacetylation in yeast. *Nat Rev Mol Cell Biol* **4:** 276–284

Lehnertz B, Ueda Y, Derijck AA, Braunschweig U, Perez-Burgos L, Kubicek S, Chen T, Li E, Jenuwein T, Peters AH (2003) Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr Biol* **13:** 1192–1200

Leonhardt H, Page AW, Weier HU, Bestor TH (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* **71:** 865–873

Levens DL (2003) Reconstructing MYC. *Genes Dev* **17:** 1071–1077

Loriot A, Boon T, De Smet C (2003) Five new human cancer-germline genes identified among 12 genes expressed in spermatogonia. *Int J Cancer* **105:** 371–376

Mateyak MK, Obaya AJ, Adachi S, Sedivy JM (1997) Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. *Cell Growth Differ* **8:** 1039–1048

Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* **99:** 247–257

Okano M, Xie S, Li E (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* **19:** 219–220

Pelengaris S, Khan M, Evan G (2002) c-MYC: more than just a matter of life and death. *Nat Rev Cancer* **2:** 764–776

Peukert K, Staller P, Schneider A, Carmichael G, Hanel F, Eilers M (1997) An alternative pathway for gene regulation by Myc. *EMBO J* **16:** 5672–5686

Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, MacLeod AR (2003) DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat Genet* **33:** 61–65

Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP (2000) DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet* **25:** 338–342

Roy AL, Carruthers C, Gutjahr T, Roeder RG (1993) Direct role for Myc in transcription initiation mediated by interactions with TFII-I. *Nature* **365:** 359–361

Schuhmacher M, Kohlhuber F, Holzel M, Kaiser C, Burtscher H, Jarsch M, Bornkamm GW, Laux G, Polack A, Weidle UH, Eick D (2001) The transcriptional program of a human B cell line in response to Myc. *Nucleic Acids Res* **29:** 397–406

Seoane J, Le HV, Massague J (2002) Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* **419:** 729–734

Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, Massague J (2001) TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol* **3:** 400–408

Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Moroy T, Bartek J, Massague J, Hanel F, Eilers M (2001) Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol* **3:** 392–399

Tamaru H, Selker EU (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* **414:** 277–283

van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H (2002) The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111:** 241–250

Wanzel M, Herold S, Eilers M (2003) Transcriptional repression by Myc. *Trends Cell Biol* **13:** 146–150

Yoder JA, Soman NS, Verdine GL, Bestor TH (1997) DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. *J Mol Biol* **270:** 385–395

Zeller KI, Haggerty TJ, Barrett JF, Guo Q, Wonsey DR, Dang CV (2001) Characterization of nucleophosmin (B23) as a Myc target by scanning chromatin immunoprecipitation. *J Biol Chem* **276:** 48285–48291

Zhu WG, Srinivasan K, Dai Z, Duan W, Druhan LJ, Ding H, Yee L, Villalona-Calero MA, Plass C, Otterson GA (2003) Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter. *Mol Cell Biol* **23:** 4056–4065

