

BCL11B functionally associates with the NuRD complex in T lymphocytes to repress targeted promoter

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BCL11 genes play crucial roles in lymphopoiesis and have been associated with hematopoietic malignancies. Specifically, disruption of the BCL11B (B-cell chronic lymphocytic leukemia/lymphoma 11B) locus is linked to T-cell acute lymphoblastic leukemia, and the loss of heterozygosity in mice results in T-cell lymphoma. BCL11 proteins are related C₂H₂ zinc-finger transcription factors that act as transcriptional repressors. Here, we demonstrate the association of the endogenous BCL11B with the nucleosome remodeling and histone deacetylase (NuRD) complex, one of the major transcriptional corepressor complexes in mammalian cells. BCL11B complexes from T lymphocytes possess trichostatin A-sensitive histone deacetylase activity, confirming the functionality of the complexes. Analysis of the BCL11B–NuRD association demonstrated that BCL11B directly interacted with the metastasis-associated proteins MTA1 and MTA2 through the amino-terminal region. We provide evidence for the functional requirement of MTA1 in transcriptional repression mediated by BCL11B through the following: (1) overexpression of MTA1 enhanced the transcriptional repression mediated by BCL11B, (2) knockdown of MTA1 expression by small interfering RNA inhibited BCL11B transcriptional repression activity and (3) MTA1 was specifically recruited to a BCL11B targeted promoter. Taken together, these data support the hypothesis that the NuRD complex mediates transcriptional repression function of BCL11B.

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Introduction

Development of lymphocytes from hematopoietic stem cells occurs through a series of regulated differentiation events controlled by transcription factors and signaling molecules (reviewed in Engel and Murre, 2001; Douagi *et al.*, 2002; Georgopoulos, 2002; Quong *et al.*, 2002;

Warren and Rothenberg, 2003; Medina and Singh, 2005). Alterations in the function of transcription factors can result in abnormal differentiation of progenitors and generation of continuously proliferating cells that progress to leukemia and lymphoma (Fischer and Malissen, 1998; Liberg and Sigvardsson, 1999). Recently, two novel transcriptional regulators, BCL11A and BCL11B (B-cell chronic lymphocytic leukemia/lymphoma 11A and 11B), were shown to play an important role in lymphopoiesis (Liu *et al.*, 2003; Wakabayashi *et al.*, 2003b; reviewed in Durum, 2003). Distinctively, BCL11A is required for B-cell development (Liu *et al.*, 2003), while BCL11B is necessary for T-cell development (Wakabayashi *et al.*, 2003b). Lately, BCL11B was also demonstrated to play an important role in the specification and development of corticospinal motor neurons (Arlotta *et al.*, 2005). In addition, both BCL11A and BCL11B have been implicated in lymphoproliferative disorders (Nakamura *et al.*, 2000; Satterwhite *et al.*, 2001; Miyazawa *et al.*, 2002; Wakabayashi *et al.*, 2003a; Przybylski *et al.*, 2005). Specifically, the translocation t(2;14)(p13;q32.3), involving the BCL11A locus, was associated with several cases of B-cell chronic lymphocytic leukemias (Satterwhite *et al.*, 2001), while the inversion inv(14)(q11.2q32.31) resulted in disruption of the BCL11B locus, absence of wild-type BCL11B transcripts and T-cell acute lymphoblastic leukemia (T-ALL) (Przybylski *et al.*, 2005). Likewise, loss of heterozygosity at the mouse *Bcl11b* locus resulted in thymic lymphomas and skin tumors, bringing additional support for the association of BCL11 genes with malignancy (Miyazawa *et al.*, 2002; Wakabayashi *et al.*, 2003a). Moreover, overexpression of BCL11B suppressed cell growth, suggesting that BCL11B may function as a tumor suppressor (Wakabayashi *et al.*, 2003a). Conversely, targeted overexpression in myeloid lineage of a splice isoform of BCL11A/Evi9, which lacks the carboxy-terminal zinc-fingers with a role in DNA binding, resulted in myeloid leukemia in BXH2 mice (Nakamura *et al.*, 2000). Thus, BCL11A and BCL11B are implicated in oncogenesis, although the mechanisms are, as yet, uncharacterized.

BCL11A and BCL11B are Kruppel-like C₂H₂ zinc-finger proteins that have been previously found to elicit

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transcriptional repression when either tethered to promoters through COUP-TF (chicken ovalbumin upstream promoter transcription factor) nuclear receptors or heterologous DNA binding domains (Avram *et al.*, 2000; Senawong *et al.*, 2003, 2005), or through direct binding to response elements (Avram *et al.*, 2002). The deacetylase SIRT1 (silent mating type information regulation 2 homolog 1) was implicated in transcriptional repression mediated by BCL11 proteins (Senawong *et al.*, 2003, 2005). However, the transcriptional repression function of endogenous BCL11 proteins still remains poorly characterized.

Using an unbiased approach involving immunoprecipitation and mass spectrometry analysis, we report here that endogenous BCL11B associates with the nucleosome remodeling and deacetylase (NuRD) complex in T lymphocytes. Moreover, we demonstrate that endogenous BCL11B complexes from CD4⁺ T lymphocytes harbor histone deacetylase (HDAC) activity sensitive to trichostatin A (TSA), an inhibitor of the NuRD HDACs. The NuRD components, metastasis-associated proteins MTA1 and MTA2, were found to directly interact with BCL11B and the interaction was mediated through the amino-terminal region of BCL11B (amino acids 1–141). Deletion of the first 45 amino acids of BCL11B abolished the interaction. In addition, we demonstrate that the transcriptional repression mediated by BCL11B was dependent on at least one of the MTA proteins, MTA1. MTA1 knockdown by small interfering RNA (siRNA) reduced the BCL11B-mediated transcriptional repression, while overexpression of MTA1 significantly potentiated this activity. Finally, chromatin immunoprecipitation (ChIP) experiments demonstrated that MTA1 was specifically recruited to a BCL11B targeted promoter.

Results

Endogenous BCL11B associates with the NuRD complex in CD4⁺ T cells

BCL11B was previously shown to be expressed and play an important role in T lymphocytes (Wakabayashi *et al.*, 2003b). We therefore employed immunoprecipitation and mass spectrometry analysis to identify the complexes associated with endogenous BCL11B in CD4⁺ T lymphocytes, in order to gain information about the cellular role of BCL11B. We first analysed the bands migrating around 116 kDa, which we predicted would correspond to BCL11B splice isoforms based on the Western blot analysis (Figure 1a) and on previous reports (Senawong *et al.*, 2003; Wakabayashi *et al.*, 2003b). Table 1 shows the results of the mass spectrometry analysis, confirming the validity of the procedure and clearly demonstrating that the anti-BCL11B (B26-44) antibodies immunoprecipitate BCL11B.

Eight other proteins immunoprecipitated with the anti-BCL11B antibodies were identified by mass spectrometry analysis as components of the NuRD complex. These include Mi2 β , MTA1, MTA2, HDAC1, HDAC2,

RbAp46, RbAp48 and MBD3 (Table 2). The presence of all the NuRD components in the immunoaffinity-purified BCL11B complexes, but not in the IgG complexes, was confirmed by Western blot analysis (Figure 1b, compare lanes 3 and 2).

To rule out the possibility that the NuRD components may be isolated through nonspecific interactions with the anti-BCL11B antibodies, reversed immunoprecipitations were conducted with antibodies against several components of the NuRD complex, including Mi2 β (Figure 1c, lane 3), MTA2 (Figure 1c, lane 5), MBD3 (Figure 1c, lane 7), HDAC2 (Figure 1c, lane 9) and MTA1 (Figure 1c, lane 11), and the presence of BCL11B was detected by Western blot analysis. The results showed that in all cases, BCL11B was immunoprecipitated with antibodies against components of the NuRD complex (Figure 1c, lanes 3, 5, 7, 9 and 11), but not with IgG (Figure 1c, lanes 2, 4, 6, 8 and 10). In addition, results recently published employing immunoprecipitation with anti-RbAp48 (p48, a component of the NuRD complex) antibodies and mass spectrometry analysis identified BCL11B in the RbAp48 (p48) complexes (Gururaja *et al.*, 2002), further supporting our hypothesis that BCL11B associates with the NuRD complex.

To eliminate the possibility that BCL11B may associate with the components of the NuRD complex through DNA, the immunoprecipitation was performed in the presence of ethidium bromide, a DNA-intercalating agent that can prevent proteins from binding to DNA (Lai and Herr, 1992). We demonstrated that the association of BCL11B with the components of the NuRD complex was not mediated through DNA, as it occurred even after treatment of the nuclear extracts with ethidium bromide (Figure 1d, lane 5).

We then addressed the question of whether the association of BCL11B with components of the NuRD complex occurs only in Jurkat cells or takes place in other T-cell lines as well. Indeed, BCL11B also associated with the NuRD complex in the nuclei of the human lymphoblastic TCR $\alpha\beta$, MOLT4 cell line (Figure 2a), demonstrating that the association is not limited to Jurkat cells. Similarly, the closely related BCL11A, expressed in B cells, associated with the NuRD complex in the B-cell lymphoma Raji cell line (Figure 2b), suggesting that BCL11A and BCL11B may have a common mechanism of function.

Taken together, these results demonstrate that endogenous BCL11B and BCL11A associate with the NuRD complex in T and B cells, respectively.

BCL11B-associated complexes possess HDAC activity sensitive to TSA

To confirm the functionality of the BCL11B complexes, we measured the HDAC activity of the BCL11B complexes. Jurkat nuclear extracts were immunoprecipitated with the anti-BCL11B (B26-44) antibodies or IgG and the immune complexes were tested for their ability to deacetylate ϵ -acetylated lysine residues with a histone-like sequence (Figure 3). HDAC activity of

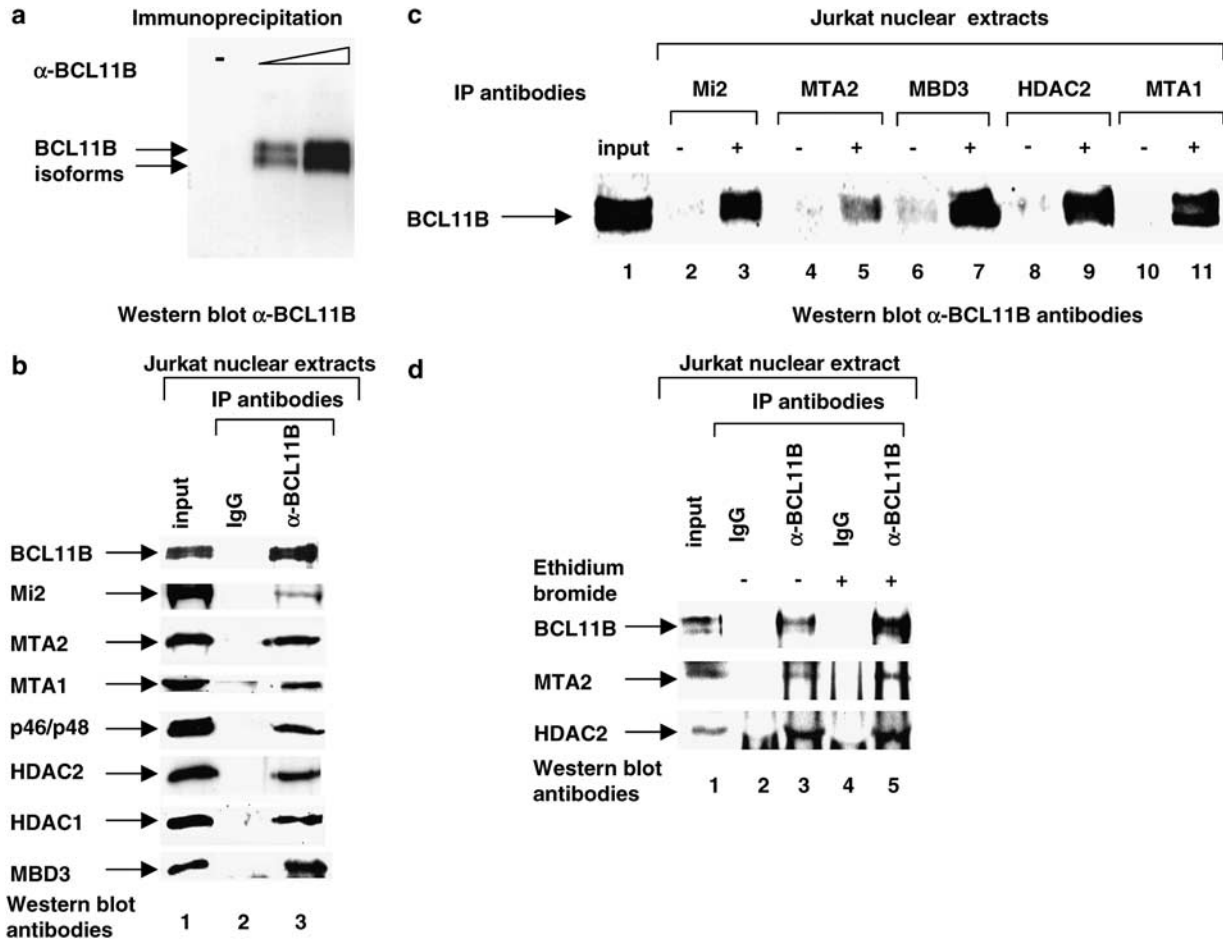


Figure 1 Association of BCL11B with the NuRD complex in Jurkat CD4⁺ T lymphocytes. (a) Immunoprecipitation and immunoblot analysis of nuclear extracts from Jurkat cells using the anti-BCL11B B26-44 antibodies. In all, 10 μg (lane 2) and 20 μg (lane 3) of affinity-purified anti-BCL11B B26-44 antibodies or 20 μg of IgG (lane 1) and protein G-agarose were used. The two isoforms of BCL11B are indicated by arrows. (b) The presence of the NuRD components in the BCL11B immune complexes was confirmed by immunoblot analysis using the indicated antibodies. Nuclear extracts from Jurkat cells were incubated with the anti-BCL11B B26-44 antibodies (lane 3) or IgG (lane 2) and the components of the NuRD complex were identified in Western blots with the NuRD antibodies specified on the left. Lane 1 shows 10% input. (c) Reverse immunoprecipitations with the indicated NuRD antibodies followed by SDS-PAGE and Western blot analysis with anti-BCL11B B26-44 antibodies. Lane 1 shows 10% input. (d) Immunoprecipitation of the Jurkat nuclear extract with anti-BCL11B B26-44 antibodies in the presence of ethidium bromide and Western blot analysis with antibodies against the NuRD components MTA2 and HDAC2. The nuclear extracts were incubated with 100 μg/ml ethidium bromide prior to immunoprecipitation, which was conducted as described in panel b

Jurkat extracts immunoprecipitated with anti-HDAC2 antibodies was used as a control in these experiments (Figure 3). Jurkat BCL11B immune complexes possessed HDAC activity significantly higher than background and in the same range as HDAC2 immune complexes (Figure 3, compare bar 1 with bars 3 and 2). In addition, BCL11B-specific HDAC activity was significantly inhibited by TSA (Figure 3, bar 4 *versus* 1), similar to the extent to which HDAC2-specific activity was inhibited (Figure 3, bar 2 *versus* 5), implicating TSA-sensitive HDACs. Similar results were obtained in MOLT4 cells (data not shown).

All these results demonstrate that BCL11B complexes from T lymphocytes possess active TSA-sensitive HDAC activity, which reflects the functional association of BCL11B with the NuRD complex.

Table 1 Peptides of BCL11B identified by mass spectrometry analysis

Band	Tryptic peptides
116 kDa	QPFNSAWFLLQHAQNTHGFR IYLEPGPASSSLTPR LTIPPLGPEAVAQSPLMNFLGDSNPFNLLR LPGTPPLFSPPPR LSAEEMGLVAQHPSAFDR LLNPFQPSPK SDDGLSAASSPEPGTSELAGEGLK VMENVLGLALPQYGELLADK GGGFAPGTEPFPGFLFPR DLELPPAALIPSENVYSQWLVAASR DPFLGFTDAR FSTPPGDLLDGGLSGR

Table 2 Peptides of the components of the human NuRD complex identified by mass spectrometry analysis

Band	Identified NuRD component	Peptides
220 kDa	Mi2 β	LLEQALVIEEQLR GPFLSAPLSTIINWER
80 kDa	MTA1	DISSTLIALADK DFTDIQQDFLPWK LETQVWEAHNPLTDR VG DYVYFENSSSNPYLIR
70 kDa	MTA2	LCASCWIYWK CSVTLNETDILSQYLEK LNPADAPNPVVFVATK VKPTLIARPPVPLPAPSHPASTNEPIVLED
60 kDa	HDAC1	SFNLPLMLG GGGYTIR
60 kDa	HDAC2	TFNLPLLMLG GGGYTIR DGIDDES YGQIFKPIISK FNVGEDCPVFDGLEFCQLSTGGVAGAVK
46–48 kDa	RbAp48/46 ^a	IGEEQSPEDAEGPPELLFIHGGTAK TIFTGHTAVVEDVSWLLHESLFGSVADDQK LHSFESHKDEIFVQWSPHNETILASSGTDR EGYGLSWNP NLSGHELLSASDDHTICLWDISAVPK LVLGTHTSDEQNHLVIASVQLPNDDAQFDASHYDSEK
35 kDa	MBD3	LSGLNAFDIAEELVK

^aThe peptides identified for the 46–48 kDa correspond to both RbAp48 and RbAp46

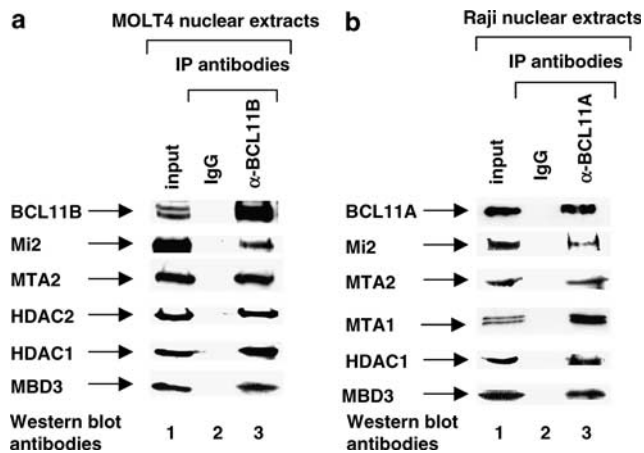


Figure 2 BCL11B associates with the NuRD complex in the T-lymphoblastic cell line MOLT4 and BCL11A associates with the NuRD complex in the B-lymphoblast-like Burkitt's lymphoma Raji cell line. (a) Immunoprecipitation and immunoblot analysis demonstrating the presence of the NuRD complex components in the immunoaffinity-purified BCL11B complexes in MOLT4 cells. Nuclear extracts from MOLT4 cells were immunoprecipitated with the anti-BCL11B B26-44 antibodies (lane 3) or IgG (lane 2) and the components of the NuRD complex were identified in Western blots by the specified NuRD antibodies. Lane 1 shows 10% input. (b) Immunoprecipitation and immunoblot analysis indicating the presence of the NuRD complex in the immunoaffinity-purified BCL11A complexes in Raji cells. Nuclear extracts from Raji cells were immunoprecipitated with anti-BCL11A antibodies (lane 3) or IgG (lane 2) and the components of the NuRD complex were identified in Western blots by the specified antibodies against components of the NuRD complex

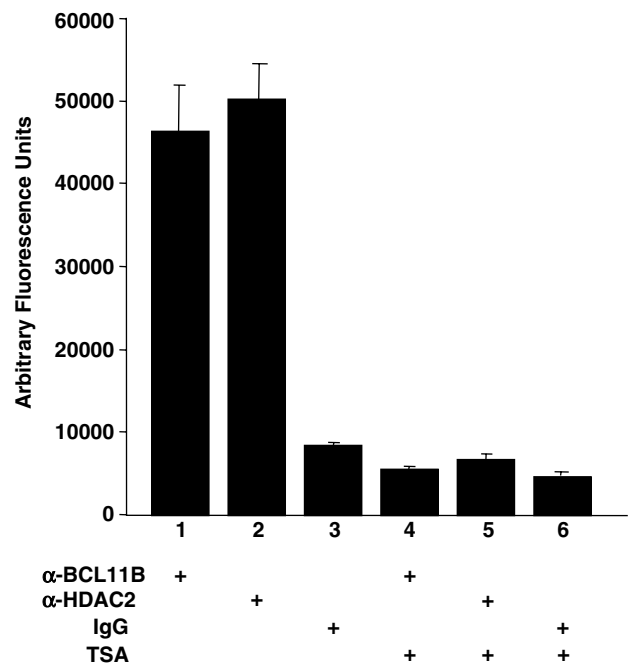


Figure 3 BCL11B complexes possess HDAC activity sensitive to inhibition by TSA. Jurkat nuclear extracts were immunoprecipitated with the anti-BCL11B B26-44 antibodies (lanes 1 and 4), HDAC2 (lanes 2 and 5) or IgG (lanes 3 and 6) in the presence (lanes 4–6) or absence (lanes 1–3) of 100 nM TSA and tested for deacetylase activity as described in Materials and methods using the HDAC fluorescent activity assay/drug discovery kit (BIOMOL). The fluorescence was measured on a fluorometric reader with excitation set at 360 nm and emission detection set at 460 nm. The deacetylase activity is expressed as arbitrary fluorescence units (AFU). The quantifications represent mean fluorescence activities \pm s.d. derived from three independent experiments

BCL11B directly interacts with MTA1 and MTA2 proteins, but not with other components of the NuRD complex

The immunoprecipitation experiments presented above indicated that endogenous BCL11B associates with the NuRD complex in T lymphocytes (Figure 1). However, immunoprecipitation cannot discriminate between direct binding and indirect association through a common intermediary protein or complex. To determine whether BCL11B interacts directly with components of the NuRD complex, protein–protein interaction assays were conducted with recombinant proteins. We purified bacterially expressed GST-BCL11B (glutathione *S*-transferase) on glutathione-Sepharose and tested for interaction with recombinant *in vitro*-translated components of the NuRD complex, or alternatively, we expressed GST fusions of the NuRD components and tested for interaction with recombinant *in vitro*-translated BCL11B. In these assays, BCL11B was found to interact strongly with MTA1 and MTA2 (Figure 4, upper panel), but not at significant levels above background with any other components of the NuRD complex, including Mi2 β (Figure 4, upper panel), MBD3, HDAC1, HDAC2, RbAp46 and RbAp48 (Figure 4, lower panel).

These results demonstrate that the association of BCL11B with the NuRD complex is direct and mediated primarily through the interaction with MTA1 and MTA2 proteins.

BCL11B–NuRD interaction is mediated through the amino-terminal region of BCL11B

In order to define the region(s) of BCL11B responsible for the interaction with MTA proteins, we performed *in vitro* GST pulldowns using bacterially expressed GST-BCL11B deletion mutants and recombinant *in vitro*-translated and labeled MTA1 and MTA2. The results showed that the region comprising amino acids 1–141, but not 141–412 or 407–813, interacted with both MTA1 and MTA2 (Figure 5a, compare lane 3 with lanes 5 and 6). In addition, deletion of the first 45 amino acids of BCL11B resulted in loss of the interaction, suggesting that this part of the amino-terminal region of BCL11B is required for the interaction with MTA proteins (Figure 5a, compare lane 4 with 3).

Additionally, we performed GST pulldowns with bacterially expressed and purified GST-BCL11B mutants and nuclear extracts from HeLa cells, which express the NuRD complex (Figure 5b), but not BCL11B (data not shown), to investigate whether the endogenous NuRD complex would associate with the amino-terminal domain of BCL11B as well. Similarly to what we observed in the *in vitro* pulldowns, BCL11B 1–141, but not 141–412 or 407–813, associated with the NuRD complex, and deletion of the BCL11B first 45 amino acids abrogated the association with the complex (Figure 5b). The same results were obtained when Jurkat nuclear extracts were used (data not shown).

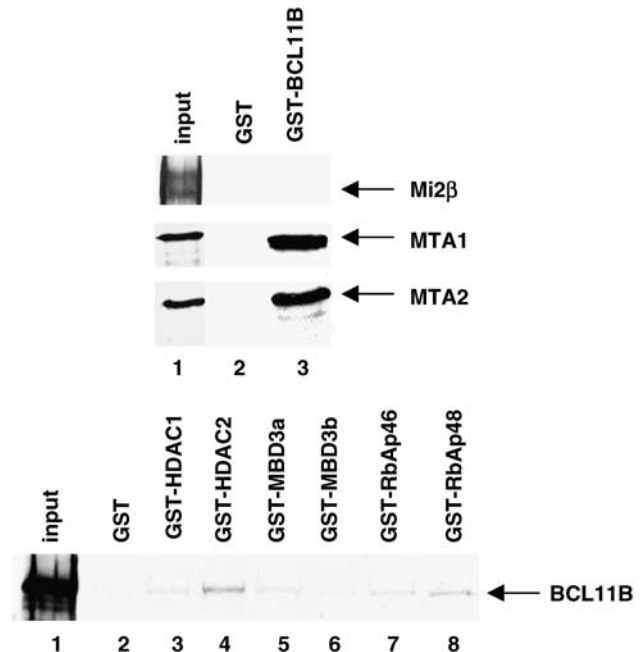


Figure 4 BCL11B interacts directly with MTA1 and MTA2, but not with other components of the NuRD complex. GST pull-down experiments employing bacterially expressed and glutathione-Sepharose-purified GST fusion proteins and *in vitro*-translated and [³⁵S]methionine-labeled proteins to test *in vitro* interactions. Upper panel: Mi2 β , MTA1 and MTA2 were *in vitro* translated and labeled with [³⁵S]methionine and incubated with 1 μ g of purified GST (lane 2) or GST-BCL11B (lane 3). Lower panel: BCL11B was *in vitro* translated and labeled with [³⁵S]methionine and incubated with 1 μ g of bacterially expressed and purified GST (lane 2) or GST-HDAC1 (lane 3), GST-HDAC2 (lane 4), GST-MBD3a (lane 5), GST-MBD3b (lane 6), GST-RbAp46 (lane 7) and GST-RbAp48 (lane 8). Lane 1 represents 10% of the [³⁵S]methionine-labeled input proteins in both upper and lower panels

These results together demonstrate that BCL11B interacts with MTA proteins through the amino-terminal region and the first 45 amino acids are required for the interaction.

BCL11B-mediated transcriptional repression is MTA1 dependent

As the natural target genes for BCL11B are yet unknown, we investigated the function of the BCL11B–NuRD complex using a reporter system in which BCL11B was expressed as a fusion with the GAL4 DNA binding domain (GAL4DBD) and co-expressed with reporters controlled by GAL4 response elements (17-mer-Luciferase). Both BCL11B and BCL11A were previously demonstrated to function as transcriptional repressors in similar systems (Avram *et al.*, 2000; Senawong *et al.*, 2003). Our results demonstrated that GAL4DBD-BCL11B specifically repressed two promoters controlled by GAL4 binding sites ((17-mer)₄-TK-Luc and pG5SV40-Luc) in HeLa (Figure 6a and data not shown), COS7 (Figure 6b) and Jurkat cells (Figure 8).

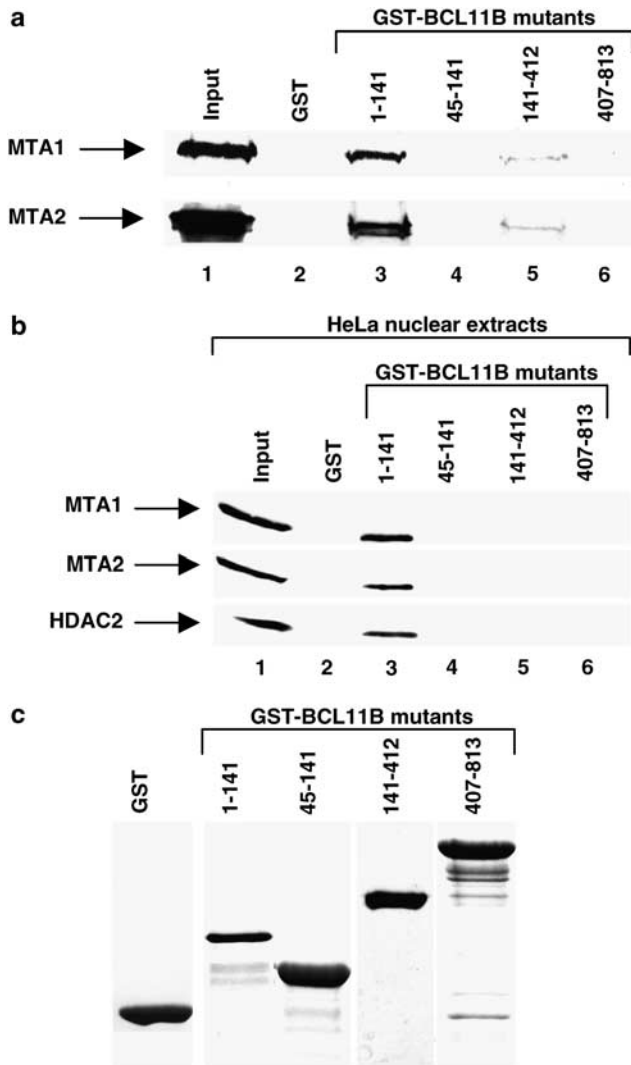


Figure 5 The amino-terminal region of BCL11B interacts directly with MTA1 and MTA2 and associates with the NuRD complex. **(a)** *In vitro* GST pulldown experiments employing bacterially expressed and glutathione-Sepharose-purified GST-BCL11B deletion mutants (1–141, lane 3; 45–141, lane 4; 141–412, lane 5; 407–813, lane 6) and *in vitro*-translated and [³⁵S]methionine-labeled MTA1 and MTA2. **(b)** Pull-down experiments of HeLa NuRD components with the GST-BCL11B deletion mutants (1–141, lane 3; 45–141, lane 4; 141–412, lane 5; 407–813, lane 6). The presence of NuRD complex components was analysed by immunoblotting with antibodies against MTA1, MTA2 and HDAC2, after precipitation of the nuclear extracts with GST-BCL11B mutants. **(c)** Coomassie-stained gel containing GST and GST-BCL11B fusion proteins used in panels a and b

To investigate whether the NuRD complex is involved in transcriptional repression mediated by BCL11B, we conducted reporter assays in which cells were transfected with GAL4DBD-BCL11B together with MTA1 or MTA2, and the (17-mer)₄-TK-Luc (Figure 6). In the presence of exogenously expressed MTA1 or MTA2, the repression mediated by BCL11B was enhanced both in HeLa (Figure 6a, compare bars 5 and 6 with bar 4) and COS7 cells (Figure 6b, compare bars 5 and 6 with bar 4). However, the enhancement of repression was only

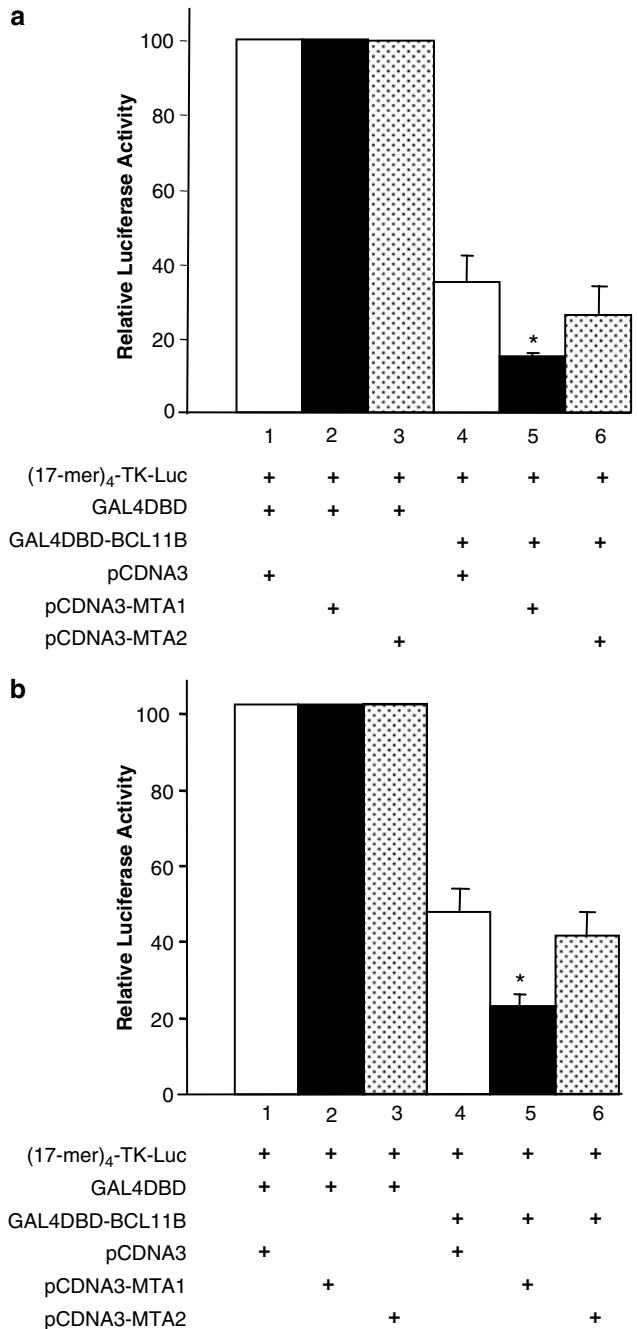


Figure 6 Transcriptional repression by BCL11B is significantly augmented by MTA1. HeLa **(a)** and COS7 cells **(b)** were transiently transfected with (1) the (17-mer)₄-TK-Luc reporter (lanes 1–6), (2) GAL4DBD-BCL11B (lanes 4–6) or GAL4DBD (lanes 1–3), and (3) pCDNA3-MTA1 (lanes 2 and 5), pCDNA3-MTA2 (lanes 3 and 6) or pCDNA3 (lanes 1 and 4). Efficiency of transfection was normalized to *Renilla* luciferase, as described in Materials and methods. For quantification, the luciferase activity in cells transfected with GAL4DBD was set at 100% (lanes 1–3), and the luciferase activities in cells transfected with GAL4DBD-BCL11B were expressed as average percentages of the GAL4DBD values (lanes 4–6). The quantifications represent means of three independent experiments ± s.d. The value in lane 5 is statistically different from the value in lane 4, as determined by Student's *t*-test (*P* < 0.05)

statistically significant in the case of MTA1 (Figure 6a and b, bar 5). The levels of expression of MTA1 and MTA2 were comparable (data not shown).

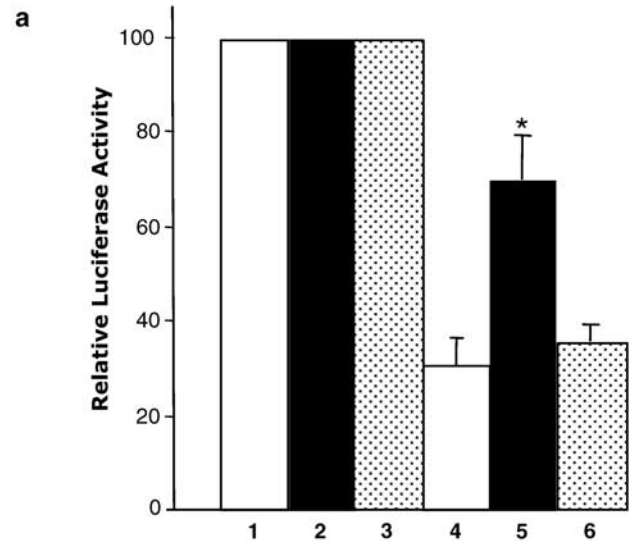
In addition, we employed siRNA technology to decrease the level of endogenous MTA1 and MTA2, predicting that this would inhibit the transcriptional repression mediated by BCL11B. Indeed, the decrease in MTA1 by siRNA (Figure 7b) significantly reduced the BCL11B-mediated transcriptional repression (Figure 7a, compare lanes 5 and 4), demonstrating that MTA1 is required for this function. However, the decrease in MTA2 levels by siRNA (Figure 7c) did not significantly affect the transcriptional repression mediated by BCL11B (Figure 7a, compare lanes 6 and 4).

These results taken together suggest that MTA1 is required for the transcriptional repression mediated by BCL11B in the reporter system employed.

BCL11B recruits MTA1 to a targeted promoter

Similar to what we observed in HeLa and COS7 cells (Figure 6), GAL4DBD-BCL11B repressed expression of pG5SV40-Luc reporter in Jurkat cells (Figure 8a, upper panel). If the NuRD complex is involved in the transcriptional repression function of GAL4DBD-BCL11B and MTA1 mediates the association of the complex with BCL11B, then MTA1 should be associated with this promoter specifically in the presence of GAL4DBD-BCL11B. To test this, Jurkat cells were transfected with pG5SV40-Luc reporter and GAL4DBD or GAL4DBD-BCL11B and the association of MTA1 and MTA2 proteins with the promoters was analysed by ChIP. Our results showed that MTA1 was associated with the promoter only in the presence of GAL4DBD-BCL11B, but not in the presence of GAL4DBD alone (Figure 8, lower panel, lanes 1 and 2), while MTA2 was not enriched on the promoter targeted by BCL11B (Figure 8, lower panel, lanes 3 and 4). In addition, this occurred only on the promoter that contained GAL4 binding sites, as MTA1 was not enriched on SV40-Luc promoter control (data not shown). Together, these data indicate that MTA1, but not MTA2, is specifically recruited by BCL11B to a

targeted promoter, and therefore supports the hypothesis that the NuRD complex is implicated in BCL11B-mediated transcriptional repression.



	1	2	3	4	5	6
GAL4DBD	+	+	+			
GAL4DBD-BCL11B				+	+	+
CTR siRNA	+			+		
MTA1 siRNA		+			+	
MTA2 siRNA			+			+

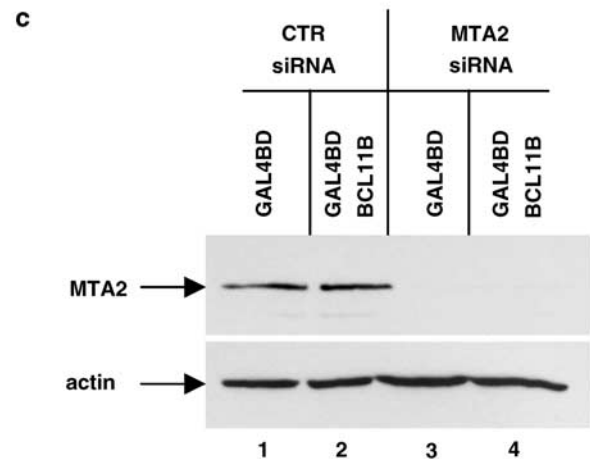
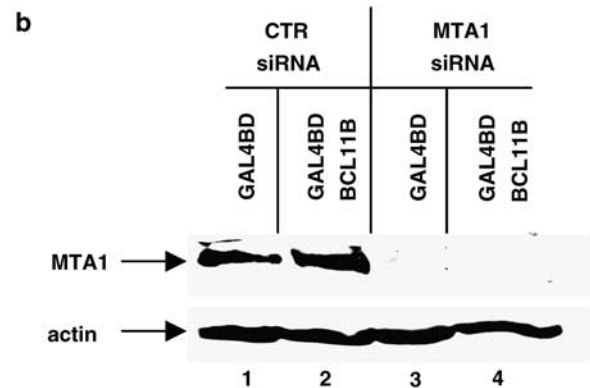


Figure 7 Repression mediated by BCL11B is diminished as a result of knockdown of MTA1 by siRNA. **(a)** Luciferase reporter assays from HeLa cells transfected with the following: (1) (17-mer)₄-TK-Luc reporter (lanes 1–6); (2) GAL4DBD (lanes 1–3) or GAL4DBD-BCL11B (lanes 4–6), and (3) nontargeting siRNA (lanes 1 and 4), MTA1 siRNA (lanes 2 and 5) or MTA2 siRNA (lanes 3 and 6). For quantification, the luciferase activity in cells transfected with GAL4DBD was set at 100% (lanes 1–3), and the luciferase activities in cells transfected with GAL4DBD-BCL11B were expressed as average percentages of the GAL4DBD values (lanes 4–6). The quantifications represent means of three independent experiments ± s.d. The value in lane 5 is statistically different from the value in lane 4, as determined by Student's *t*-test ($P < 0.05$). **(b, c)** Immunoblots for MTA1 **(b)** and MTA2 **(c)** after transfection of HeLa cells with nontargeting (lanes 1 and 2) or MTA-specific siRNAs (lanes 3 and 4) and with the following plasmids: (17-mer)₄-TK-Luc reporter (lanes 1–4), GAL4DBD (lanes 1 and 3) or GAL4DBD-BCL11B (lanes 2 and 4). Actin was used as loading control

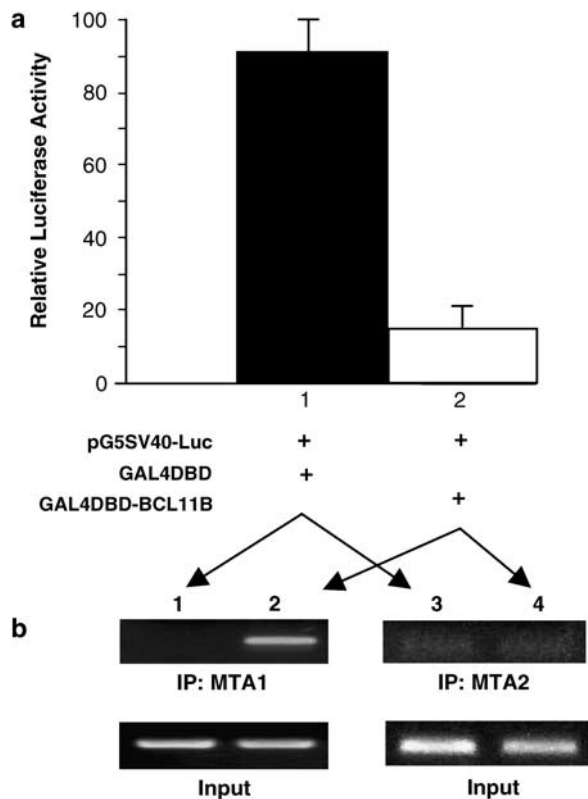


Figure 8 BCL11B specifically recruits MTA1 to a targeted promoter. The upper panel (**a**) represents the luciferase activity of Jurkat cells transfected with (1) pG5SV40-Luc reporter, which contains five tandem repeats of the GAL4 binding sites in front of the SV40 minimal promoter, and (2) GAL4DBD (black bar) or GAL4DBD-BCL11B (white bar). The value in lane 2 is statistically different from the value in lane 1, as determined by Student's *t*-test ($P < 0.05$). The lower panel (**b**) represents ChIP analysis of the samples used in the upper panel. Chromatin was immunoprecipitated with antibodies against MTA1 or MTA2 proteins after crosslinking and amplified with primers specific for the $5 \times$ GAL4-SV40 promoter (pG5SV40-Luc). Results are representative of three independent experiments

Discussion

BCL11A and BCL11B were previously shown to function as transcriptional repressors when tethered to promoters through COUP-TF nuclear receptors or heterologous DNA binding domains (Avram *et al.*, 2000; Senawong *et al.*, 2003, 2005) or through direct binding to their conserved response elements (Avram *et al.*, 2002). However, there is limited information with regard to the repression complexes associated with the endogenous BCL11B in T lymphocytes. Using immunoprecipitation and mass spectrometry analysis, we identified the NuRD complex as the major corepressor complex associated with the endogenous BCL11B in CD4⁺ T cells. We also demonstrate that BCL11B-containing complexes from the CD4⁺ T Jurkat and MOLT4 cells possess HDAC activity and this activity is sensitive to inhibition by TSA, an inhibitor of the NuRD HDACs. In addition, our results show that the related BCL11A is associated with the NuRD complex

in B lymphocytes, suggesting a common mechanism of action for both BCL11 proteins.

Metastasis-associated proteins MTA1 and MTA2 (Xue *et al.*, 1998; Zhang *et al.*, 1999; Mazumdar *et al.*, 2001) are the components of the NuRD complex and were found to interact directly with BCL11B. In addition to MTA1 and MTA2, recently, a third MTA protein, MTA3, was identified as being associated with the NuRD complex in breast epithelial cells, connecting estrogen receptor signaling to the cell adhesion molecule E-cadherin (Fujita *et al.*, 2003). MTA3/NuRD complex was also found to play a critical role in B-cell differentiation in the germinal center in relation with the transcriptional repressor BCL6 (Fujita *et al.*, 2004). However, in our study, none of the peptides identified by immunoprecipitation and mass spectrometry analysis corresponded to MTA3.

Here, we provide several lines of evidence that the NuRD complex component MTA1 plays an essential role in the transcriptional repression function of BCL11B: (1) overexpression of MTA1 significantly enhanced the transcriptional repression mediated by BCL11B, (2) knockdown of MTA1 expression by siRNA diminished the transcriptional repression activity of BCL11B and (3) MTA1 was specifically recruited to a BCL11B targeted promoter in Jurkat cells. Interestingly, we identified both MTA1 and MTA2 as being present in the BCL11B-associated complexes and, as shown above, both proteins directly interacted with BCL11B. However, our data implicated predominantly MTA1 in transcriptional repression function of BCL11B in the reporter system employed, and in the recruitment of the NuRD complex to the BCL11B targeted promoter. It is possible that BCL11B–MTA2 association may be significant for another function of BCL11B yet unknown, or MTA2 may preferentially recruit the NuRD complex to specific BCL11B targeted promoters. Other transcription factors, such as YY1, were shown previously to interact preferentially with MTA2, BCL6 with MTA3 (Fujita *et al.*, 2004), while p53 was shown to be associated with both MTA1 and MTA2 proteins (Luo *et al.*, 2000; Yao and Yang, 2003).

When the first 45 amino acids were deleted from BCL11B, the interaction with MTA proteins and, consequently, the association with the NuRD complex were abolished. In a BLAST search, we found that this region of BCL11 proteins has similarity to the corresponding region of the transcriptional repressors belonging to Sall family (data not shown). Recently, it has been reported that the amino-terminal region of Sall1 is required for the interaction with a corepressor complex containing HDAC1/2, RbAp46/48 and MTA1/2 (Kiefer *et al.*, 2002), which may be the NuRD complex core. However, the component(s) of the corepressor complex that directly interacted with Sall has not been identified (Kiefer *et al.*, 2002). Based on the sequence similarity between the amino-terminal regions of BCL11 and Sall proteins, and the fact that BCL11B interacts with MTA proteins through this region, it is possible that Sall proteins also associate with the MTA proteins and NuRD complex through the same amino-terminal

region, and both BCL11 and Sall transcriptional repressors function through a similar mechanism. Interestingly, within the N-terminal region, the six extreme amino acids of BCL11, Sall and FOG proteins were found to be identical (data not shown). In two recent reports, the extreme N-terminus of FOG-1 was defined as a new repressor domain (Lin *et al.*, 2004; Hong *et al.*, 2005), that mediates the association with the NuRD complex through MTA and RbAp48/46 (Hong *et al.*, 2005). It is therefore possible that the same domain in the BCL11 amino-terminal region is involved in the interaction with MTA proteins. However, BCL11, Sall and FOG proteins also contain a CCHC-type zinc-finger motif in the amino-terminal region. In FOG proteins, such domains were previously demonstrated to associate with GATA CCCC-type zinc-fingers (Kowalski *et al.*, 2002). Interestingly, both MTA1 and MTA2 proteins contain CCCC-type zinc-finger domains (Yao and Yang, 2003). Therefore, we propose that the interaction of BCL11, Sall and FOG with MTA proteins may also involve the CCHC zinc-finger, in addition to the extreme N-terminus.

The MTA members are widely expressed in normal tissues and highly expressed in metastatic tumors (Kumar *et al.*, 2003). Two independent studies suggest that MTA proteins do not colocalize in the same NuRD complex and do not functionally substitute each other (Fujita *et al.*, 2003; Yao and Yang, 2003). Based on these studies and on phylogenetic analysis, it was proposed that MTA members are molecular markers for distinct forms of the NuRD complexes, being responsible for the functional specialization of the complex (Bowen *et al.*, 2004). However, the function of MTA proteins in the NuRD complex is yet unknown. Our data suggest that one potential role for MTA proteins is to mediate the association of the NuRD complex with distinct transcriptional regulators, potentially functioning as adaptors.

The NuRD complex was found to mediate the function of several other transcriptional repressors. These include the mammalian Ikaros proteins (Kim *et al.*, 1999), the Kruppel transcriptional repressors functioning through the corepressor KAP1 (Schultz *et al.*, 2001) and the *Drosophila* Tramtrack (Murawsky *et al.*, 2001) and hunchback (Kehle *et al.*, 1998). A common characteristic of these transcriptional repressors is that, different from BCL11B, all associate with the NuRD complex through the interaction with Mi2 protein, which possesses ATP-dependent nucleosome remodeling activity (Xue *et al.*, 1998; Zhang *et al.*, 1999).

The NuRD complex possesses both ATP-dependent nucleosome remodeling and HDAC activities (Xue *et al.*, 1998; Zhang *et al.*, 1999). These activities allow the generation of a transcriptionally nonpermissive environment by deacetylating histones on targeted promoters (Xue *et al.*, 1998; Zhang *et al.*, 1999; Liu and Bagchi, 2004). The NuRD complex HDACs HDAC1 and HDAC2 belong to class I, which together with class II HDACs are inhibited by TSA treatment (Xue *et al.*, 1998; Zhang *et al.*, 1999; Thiagalingam *et al.*, 2003). In addition, a third class of deacetylases, resistant

to inhibition by TSA, was identified, with the main representative Sir2 (silent information regulator 2). The yeast Sir2 deacetylase is implicated in transcriptional repression of the mating type locus (Imai *et al.*, 2000; Li *et al.*, 2001; reviewed in Guarente, 1999, 2000; Gartenberg, 2000). Interestingly, the human homolog of the yeast Sir2, SIRT1, was recently shown to associate with BCL11B and BCL11A and enhance the transcriptional repression function of BCL11 proteins in transient reporter assays (Senawong *et al.*, 2003, 2005). However, SIRT1, although possessing deacetylase activity, seems to prefer in mammalian cells other substrates than histones, including p53 (Vaziri *et al.*, 2001; Langley *et al.*, 2002; Cheng *et al.*, 2003) and FOXO (Brunet *et al.*, 2004). In addition, experimental evidence from knockout mice, as well as stem cell studies, failed to prove that the mouse Sir2 homolog is involved in gene silencing and uses acetylated histones as substrates (McBurney *et al.*, 2003a,b). Therefore, it is possible that the enhancement of the transcriptional repression function of BCL11 proteins by SIRT1 may occur through their deacetylation or deacetylation of BCL11-associated proteins, rather than of the histones on BCL11 targeted promoters. Further studies are necessary to investigate these possibilities. In unbiased experiments however, we failed to detect any peptides corresponding to SIRT1 by mass spectrometry analysis in complexes immunoprecipitated with anti-BCL11B antibodies, although we detected peptides corresponding to all the components of the NuRD complex, including the HDACs HDAC1 and HDAC2. One possibility is that the amount of SIRT1 present in the BCL11B complexes was under the level detectable by mass spectrometry analysis or the BCL11B antibodies that we used could not efficiently immunoprecipitate SIRT1-containing BCL11B complexes. Another possibility is that under the extraction conditions that we employed, SIRT1 may have dissociated from the BCL11B complexes.

One can envision that BCL11B would specifically recruit the NuRD complex to targeted promoters and consequently repress their expression. Any event impairing the association of BCL11B with the NuRD complex or the association of BCL11B with the targeted promoter and consequently the recruitment of the NuRD complex to the promoter may affect the control of the target genes. Disruption of the BCL11B locus, as a result of *inv(14)(q11.2q32.31)* observed in some cases of T-ALL, resulted in loss of wild type and expression of the fusion transcripts containing exons 1–3 of BCL11B in-frame with the first exon of TCRD locus (Przybylski *et al.*, 2005). Similarly, loss of heterozygosity in mice resulted in T-cell lymphoma (Wakabayashi *et al.*, 2003a). What is common in both situations is lack of wild-type BCL11B expression, which may consequently result in deregulation of target genes. It is tempting therefore to speculate that BCL11B may control negatively, through recruitment of the NuRD complex, expression of genes involved in cellular transformation and growth control. In addition, in the case of T-ALL associated with the *inv(14)(q11.2q32.31)*, it is also

possible that the fusion of BCL11B exons 1–3 in-frame with the first exon of TRDC is leukemogenic. The BCL11B exons 1–3 contain amino acids 1–213. We show here that the amino-terminal region of BCL11B interacts with MTA proteins and NuRD complex. Therefore, the fusion BCL11B exons 1–3–TRDC exon 1 will likely associate with the NuRD complex, assuming that it localizes to the nucleus. However, the fusion protein lacks any DNA binding zinc-fingers. Therefore, more likely, BCL11B target genes will not be appropriately regulated in the leukemic cells. Another possibility is that the fusion protein does not localize to the nucleus and consequently target genes cannot be silenced through recruitment of the NuRD complex. Lack of nuclear localization of an amino-terminal BCL11B fusion is suggested by the natural splice isoform of the related BCL11A, BCL11AS, which contains the first 243 amino acids and which does not localize to the nucleus (Nakamura *et al.*, 2000). Identification of BCL11B target genes will provide more insights into the mechanisms through which BCL11B controls leukemic transformation.

Materials and methods

Cell culture

Jurkat, MOLT4, Raji, COS7 and HeLa cells were obtained from ATCC. Jurkat, MOLT4 and Raji cells were grown in RPMI 1640 media containing 2 mM L-glutamine and 10% fetal bovine serum (FBS) and HeLa and COS7 cells were grown in DMEM supplemented with 10% FBS.

Antibodies

Anti-BCL11B (B26-44) antibodies are polyclonal affinity-purified antibodies developed against a unique peptide covering amino acids 26–44, contained within both isoforms of BCL11B. Anti-BCL11A (A3-171) antibodies are polyclonal antibodies developed against BCL11A amino acids 1–171. Anti-Mi2 β , -MTA2 and -MBD3 antibodies were kind gifts from Dr Paul Wade. Anti-RbAp46/p48 antibodies were purchased from Upstate and anti-HDAC1, -HDAC2 and -MTA1 were from Santa Cruz Biotechnology.

Plasmid constructs

BCL11B was subcloned in pM (BD Biosciences) and pGEX-2T (Amersham) to generate the GAL4DBD-BCL11B and GST-BCL11B fusions, respectively. MTA2 was subcloned into pCDNA3 from pCMV-Flag-MTA2 (Zhang *et al.*, 1999), which, together with BSK-Mi2 β (Zhang *et al.*, 1999), was a kind gift from Dr Danny Reinberg. pCDNA3::Flag-MTA1 (Fujita *et al.*, 2003) was a kind gift from Dr Paul Wade. GST-RbAp46 and GST-RbAp48 (Verreault *et al.*, 1998) were kindly provided by Dr Bruce Stillman, GST-HDAC1 and -HDAC2 (Yang *et al.*, 1996) were kind gifts from Dr Edward Seto and GST-MBD3 (Saito and Ishikawa, 2002) was a kind gift from Dr Fuiuki Ishikawa. The firefly luciferase reporter vectors were generous gifts from Dr Ming-Jer Tsai ((17-mer)₄-TK-Luc) and Dr Michael Rauchman (pSV40-Luc and pG5SV40-Luc) (Klaver and Berkhout, 1994; Kiefer *et al.*, 2002). (17-mer)₄-TK-Luc and pG5SV40-Luc vectors contain four and five, respectively, tandem repeats of GAL4 response elements

upstream of the minimal herpes simplex virus thymidine kinase (TK) and SV40 promoters respectively.

Cellular fractionation and immunoprecipitations

Cells were lysed by incubation in BN buffer (15 mM Tris, pH 7.5, 60 mM KCl, 5 mM MgCl₂, 15 mM NaCl, 250 mM sucrose, 0.3% NP-40 and protease inhibitors). The nuclei were pelleted by centrifugation at 2000 r.p.m. for 5 min at 4°C and then lysed by resuspension in NEB buffer (25 mM Tris, pH 8, 250 mM NaCl, 10% glycerol, 0.2% NP-40 and protease inhibitors) for 30 min at 4°C. The extract was sonicated three times for 10 s and then the extract was cleared by centrifugation. The supernatant was incubated with the specified antibodies overnight and then with protein A–Sepharose CL-4B (Pharmacia) or protein G-agarose (Sigma).

Mass spectrometry analysis

For mass spectrometry analysis, nuclear extracts from 1×10^8 Jurkat cells, obtained as described above, were incubated with 60 μ g of B26-44 BCL11B antibodies or IgG crosslinked to protein G-agarose overnight. The BCL11B-associated complexes were separated in 4–20% gradient gels. The gels were stained with Biosafe Coomassie (Bio-Rad), and specific bands that appeared in three independent experiments in relation to the presence of anti-BCL11B antibodies, but not to IgG, were excised from the gel, trypsinized (Shevchenko *et al.*, 1996) and then the tryptic peptides were analysed using Micromass Q-ToF 2 mass spectrometry at the Proteomics Core Facility of the Center for Functional Genomics (CFG) of SUNY at Albany. The resulting tandem mass spectra were compared directly with amino-acid sequence databases using a Mascot computer algorithm.

HDAC activity

Nuclear extracts from Jurkat or MOLT4 cells prepared and immunoprecipitated as described above were assayed for HDAC activity using the HDAC fluorescent activity assay/drug discovery kit (BIOMOL Research Laboratories). This assay system allows detection of a fluorescent signal, upon deacetylation of substrates with a histone-like sequence containing ϵ -acetylated lysine residues. In short, the beads or the extracts were incubated with 100 μ M acetylated substrate in 100 μ l assay buffer. The reactions were incubated at 37°C for 30 min and then stopped by the addition of the developer. The fluorescence was measured on a fluorometric reader with excitation set at 360 nm and emission detection set at 460 nm. HDAC activity was expressed as AFU. For inhibitor assays, the reactions were carried out in the presence of 100 nM TSA (Sigma).

GST pulldown experiments were conducted as described previously (Avram *et al.*, 2000, 2002).

Transient transfections and reporter assays

HeLa cells were transfected using Lipofectamine plus (Invitrogen) with the following plasmids: (1) (17-mer)₄-TK-Luc or pG5SV40-Luc (1 μ g), (2) pM (GAL4DBD) or pM::BCL11B (GAL4DBD-BCL11B fusion) (0.125 μ g), (3) expression vectors (pCDNA3) encoding MTA1 or MTA2 proteins (0.075 μ g) and (4) *Renilla* reporter vector pRL-Luciferase (BD Bioscience) (0.005 μ g), as normalization control for transfection efficiency. The cells were harvested 48 h after transfection and luciferase activity was measured using the luciferase assay system (Promega). Jurkat cells were transfected by electroporation using a Gene Pulser II (Bio-Rad). Briefly, 10^7 cells

were incubated with firefly luciferase reporter plasmids SV40-Luc or pG5SV40-Luc (10 µg), pM or pM::BCL11B (20 µg), and pRL-Luciferase plasmid (0.6 µg), as normalization control for transfection efficiency, in 300 µl RPMI media and electroporated at 300 V and 975 µF. At 2 days after transfection, cells were harvested for ChIP or luciferase assays.

Gene knockdown by siRNA

MTA1- and MTA2-specific siRNAs (SMART pool) and control nontargeting siRNA were purchased from Dharmacon. HeLa cells were transfected with 400 pmol siRNA and 10 µl Lipofectamine 2000. A second transfection was conducted after 48 h with 400 pmol siRNA, 2 µg firefly luciferase reporter, 0.01 µg pRL-Luciferase and 0.5 µg GAL4DBD or GAL4DBD-BCL11B plasmids. Cells were harvested after 72 h for luciferase assays and immunoblot analysis.

ChIP assays

Jurkat cells were crosslinked with 1% formaldehyde for 10 min at room temperature. ChIP was performed according to Upstate Biotechnology protocol with minor modifications. Briefly, the chromatin was sheared to DNA fragments with an average size of 500 bp and then incubated overnight with 2 µg of anti-MTA1, -MTA2 or appropriate isotype controls and protein A-Sepharose CL-4B (Amersham Biosciences) for 2 more hours. The beads were sequentially washed with low-salt, high-salt and LiCl containing buffers. The crosslinking was reversed by treatment with 5 M NaCl followed by RNase A and proteinase K digestion and then the DNA fragments were eluted in 50 µl Tris-EDTA pH 8 buffer. A 2 µl portion of the eluted DNA was used for PCR amplification. In all cases, the input was 2%. The following primers crossing the luciferase

promoter were used for amplification: forward: 5'-tgtatctatgg-tactgtaactg-3' and reverse: 5'-ctttatgtttttggcgtcttcc-3'.

Abbreviations

BCL11B, B-cell chronic lymphocytic leukemia/lymphoma 11B; ChIP, chromatin immunoprecipitation; COUP-TF, chicken ovalbumin upstream promoter transcription factor; GST, glutathione S-transferase; HDAC, histone deacetylase; NuRD, nucleosome remodeling and deacetylase; MTA1, metastasis-associated protein 1; MTA2, metastasis-associated protein 2; SIRT1, silent mating type information regulation 2 homolog 1; Sir2, silent information regulator 2; TSA, trichostatin A.

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