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MTA3 and the Mi-2/NuRD Complex Regulate Cell Fate during B Lymphocyte Differentiation

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Summary

The transcriptional repressor BCL-6 regulates B lymphocyte cell fate during the germinal center reaction by preventing terminal differentiation of B lymphocytes into plasma cells until appropriate signals are received. Here, we report a cofactor, MTA3, a cell type-specific subunit of the corepressor complex Mi-2/NuRD, for BCL-6-dependent cell fate determination. MTA3 is expressed in the same pattern in germinal centers as BCL-6. BCL-6 physically interacts with Mi-2/NuRD and this interaction is sensitive to BCL-6 acetylation status. Depletion of MTA3 by RNAi impairs BCL-6-dependent repression and alters the cell-specific transcriptional pattern characteristic of the B lymphocyte. Remarkably, exogenous expression of BCL-6 in a plasma cell line leads, in an MTA3-dependent manner, to repression of plasma cell-specific transcripts, reactivation of the B cell transcriptional program, expression of B lymphocyte cell surface markers, and reprogramming of cell fate.

Introduction

In multicellular organisms, the essential biological processes of development and differentiation result in the creation of multiple cell types, each with its own unique pattern of gene expression. The portion of the genome active in any given cell type results from the balance of two opposing activities, transcriptional activation and transcriptional repression. For many differentiated cell types, the transcriptional program is under the control of master regulatory factors. A classic example of such a factor is MyoD, a basic helix-loop-helix transcription factor with the capacity to direct the muscle cell-specific transcriptional program (Weintraub et al., 1991). While master regulatory factors are frequently necessary for the establishment of cell type-specific transcription, it is clear that in most cases eukaryotic transcription factors utilize cofactors, termed coactivators and corepressors, to facilitate modulation of gene expression (Jaskelioff and Peterson, 2003).

Transcriptional regulation plays an integral role in the control of development and differentiation in the vertebrate immune system. The development of acquired immunity relies on the proper differentiation of B lymphocytes into antibody-secreting cells (Bishop et al., 2003). Central to this process is the germinal center reaction (McHeyzer-Williams et al., 2001). Germinal center B lymphocytes are engaged in a unique biological program, including isotype switching and somatic hypermutation. Following selection of high-affinity antibodies, these MHC class II-positive lymphocytes can differentiate into MHC class II-negative, immunoglobulin-secreting plasma cells (Calame et al., 2003). Key regulators of the germinal center B lymphocyte and plasma cell transcriptional programs are the transcriptional repressors BCL-6 and Blimp-1 (Calame et al., 2003). Considerable effort has been devoted to determination of the transcriptional programs downstream of these two repressors. In the germinal center B lymphocyte, BCL-6 activity is required for repression of markers, including Blimp-1, the cytokine CCL3, Syndecan 1, and the transcription factor XBP1, of the plasma cell transcriptional program (Shaffer et al., 2000). In contrast, Blimp-1 activity is required to stably repress markers, including CIITA, BLNK, Igβ, and SYK, of the germinal center B cell transcriptional program (Shaffer et al., 2002a). Recent work has demonstrated that Blimp-1 facilitates transcriptional repression through recruitment of the histone methyltransferase G9a (Gyory et al., 2004). BCL-6, in contrast, represses transcription through the recruitment of corepressors including NCoR (Huynh and Bardwell, 1998), SMRT (Wong and Privalsky, 1998), and BCoR (Huynh et al., 2000). While much progress has been made in the definition of an HDAC-dependent repression mechanism utilized by BCL-6, in the context of the germinal center B lymphocyte, the repression mechanism of this key regulator has not been fully defined.

The Mi-2/NuRD complex represents an abundant corepressor present in diverse cell types (Bowen et al., 2004). This complex is heterogenous in subunit composition, leading to the prediction that subunit heterogeneity produces functionally distinct corepressor complexes (Bowen et al., 2004). MTA3, a cell type-specific subunit of the Mi-2/NuRD complex, has been recently identified as a component of a genetic pathway regulating differentiation in mammary epithelial cells (Fujita et al., 2003). In this context, MTA3 is required for recruitment of Mi-2/NuRD to at least one promoter, resulting in HDAC-dependent transcriptional repression (Fujita et al., 2003). The role(s) of MTA3 or the Mi-2/NuRD complex in general as a regulator of cell fate determination in other contexts remain unknown. Whether specific subunits of Mi-2/NuRD, in addition to MTA3, act to provide unique functionality in cell type-specific transcriptional programs has not been determined.

In this work, we report a novel and intriguing cell type-specific function of MTA3 and the Mi-2/NuRD complex. MTA3 is expressed in a biologically distinct subset of lymphocytes: germinal center B cells. As the pattern of MTA3 expression mirrored the expression pattern of the master regulator, BCL-6, of B cell differentiation, the interaction between MTA3 and BCL-6 was examined.

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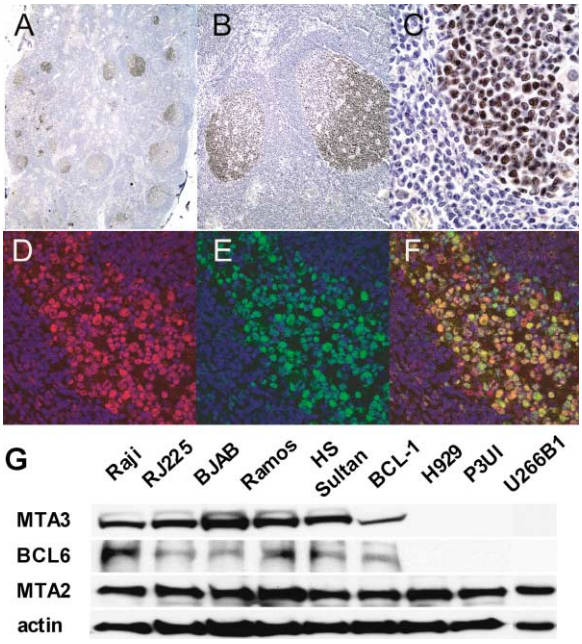

Figure 1. MTA3 and BCL-6 Are Expressed in Germinal Center B Lymphocytes

(A)–(C) display a section of lymph node with reactive follicular hyperplasia that was immunostained (23) with anti-MTA3 antibodies (magnifications: A, 40×; B, 100×; C, 400×). Following staining with secondary reagents, bound antibodies were detected by a standard DAB reaction. In a separate experiment, a section of chronically inflamed tonsil was double-stained with anti-MTA3 (D, red) and anti-Bcl-6 antibodies (E, green). The merged image is shown in (F). All nuclei were labeled with To-pro-3 iodide (D–F, blue).

(G) Immunoblot analysis of whole-cell extracts from the indicated human and mouse cell lines was performed with antibodies against the indicated proteins.

MTA3 physically associated with BCL-6 in extracts from B lymphocyte cell lines, and depletion of MTA3 from these cells by RNAi led to concomitant alterations in their cell type-specific transcriptional program. The interaction of BCL-6 and MTA3 was regulated by acetylation of BCL-6 itself, a modification known to affect the ability of BCL-6 to repress transcription (Bereshchenko et al., 2002). Moreover, exogenous expression of BCL-6 in a plasma cell line led to reprogramming of cell fate toward that of a B lymphocyte in an MTA3-dependent fashion. Together, these data suggest a prominent role for MTA3 in B cell fate determination.

Results

MTA3 Expression in Lymphoid Tissue

In an immunohistochemical survey of MTA3 expression in breast cancer specimens (Fujita et al., 2003), a slide containing a lymph node was encountered. Closer examination of this and other slides containing secondary lymphoid tissues revealed high-level expression of MTA3 at germinal centers. Figure 1 (panels A–C) depicts a human lymph node stained for MTA3. High-level expression is largely restricted to the germinal center. Immunofluorescence analysis of frozen sections of human tonsil with antibodies to MTA3 and BCL-6, a repressor that functions during lymphoid development (Niu, 2002; Ye et al., 1993), revealed that these two transcriptional regulators were coexpressed in the same population of germinal center B lymphocytes (Figures 1D–1F).

The precise coexpression of MTA3 and BCL-6 suggested a functional role in the biology of the germinal center B lymphocyte. To investigate this further, immunoblot analysis of whole-cell extracts from human and mouse cell lines revealed high-level expression of both MTA3 and BCL-6 in cell lines representative of B lymphocytes; the cell lines include Raji (Epstein et al., 1966), RJ2.2.5 (Accolla, 1983), BJAB (Zech et al., 1976), Ramos (Klein et al., 1975), HS Sultan (Harris, 1974), and BCL-1 (Slavin and Strober, 1978) (Figure 1G). In contrast, the plasmacytoma lines H929 (Gazdar et al., 1986) and U266B1 (Nilsson et al., 1970) and the myeloma cell line P3U1 (Unkeless, 1979) exhibited little to no expression of these two proteins, suggesting that these cell lines could be utilized to investigate physical and functional interactions between the repressor BCL-6 and the corepressor Mi-2/NuRD.

MTA3 Associates with BCL-6

Exogenous expression of BCL-6 and MTA3 with epitope tags in 293T cells followed by immunoprecipitation revealed that the two proteins interacted, either directly or indirectly (Figure 2A). To authenticate this observation in a more physiologically relevant context, coprecipitation experiments were performed in whole-cell extracts of the B cell line RJ2.2.5. Immunoprecipitation of endogenous BCL-6 led to coprecipitation of MTA3 as well as three other subunits of Mi-2/NuRD: MBD3, HDAC1, and Mi-2 (Figure 2B). Converse experiments demonstrated that BCL-6 coprecipitated with four different subunits of the Mi-2/NuRD complex, including MTA3 (Figure 2C). We concluded from these experiments that BCL-6 forms a stable interaction with the MTA3-containing version of the Mi-2/NuRD corepressor complex. Interactions between BCL-6 and other corepressors have been reported (Dhordain et al., 1997; Huynh and Bardwell, 1998; Huynh et al., 2000; Wong and Privalsky, 1998). However, we failed to detect robust expression of two of these molecules, NCoR and SMRT, in extracts prepared from Raji and RJ2.2.5 (Figure 2D), indicating that these molecules are expressed at low levels in the immortalized B cell lines utilized in this work.

To dissect the interaction between MTA3 and BCL-6, protein-protein interaction assays were employed. Several regions of BCL-6 were produced in recombinant bacteria as GST fusions. The purified GST-BCL-6 derivatives were subsequently incubated with nuclear extracts from Raji cells. In this assay, robust interaction of BCL-6 with MTA3 and the Mi-2/NuRD complex was observed using the central region of the BCL-6 protein (Figure 3A). Other regions of BCL-6, including the BTB-POZ domain, failed to interact productively with MTA3 and the Mi-2/NuRD complex. This result was somewhat surprising as the interaction between BCL-6 and other corepressors, including NCoR (Huynh and Bardwell, 1998), SMRT (Huynh and Bardwell, 1998; Wong and Privalsky, 1998), and BCoR (Huynh et al., 2000), is mediated by the BTB-POZ domain. To determine whether the interaction between BCL-6 and the Mi-2/NuRD complex involved

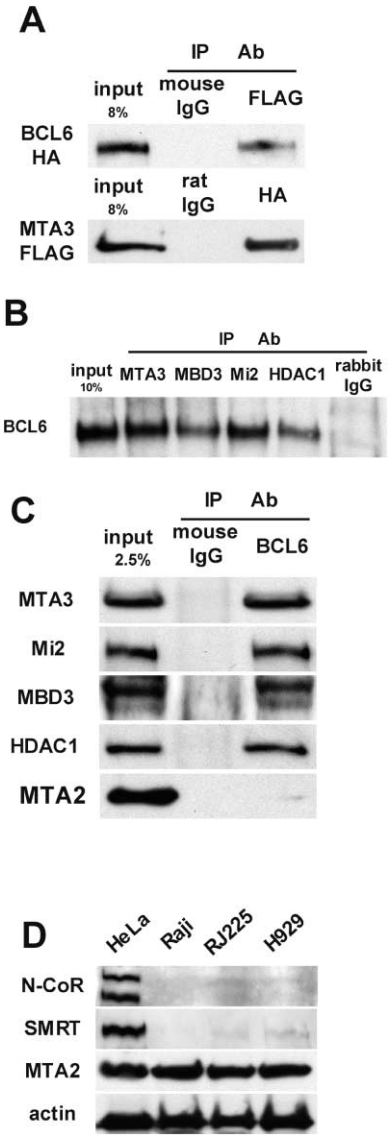

Figure 2. MTA3 Physically Associates with BCL-6 in B Cells

(A) Epitope-tagged versions of BCL-6 and MTA3 were expressed in 293T cells and immunoprecipitations were performed with the indicated epitope antibodies. Detection of BCL-6 and MTA3 by their respective epitope tags is indicated on the figure.

(B) The indicated antibodies to Mi-2/NuRD subunits were used in immunoprecipitations from RJ2.2.5 extracts. BCL-6 was detected in the precipitates as indicated in the figure.

(C) Immunoprecipitation of BCL-6 from RJ2.2.5 extracts was performed. Mi-2/NuRD subunits were detected in the precipitates using the indicated antibodies.

(D) Whole-cell extracts from the indicated cell lines were analyzed for NCoR, SMRT, MTA2, and actin by immunoblot as indicated in the figure.

a direct interaction between BCL-6 and MTA3, protein-protein interaction assays were performed with recombinant fragments of each protein. GST-MTA3 fusions were prepared in recombinant bacteria, purified, and tested for interaction with a purified recombinant fragment of BCL-6 containing the central region (Figure 3B). The carboxy-terminal 137 amino acids of MTA3 interacted specifically with the BCL-6 fragment in this assay, demonstrating a direct interaction between the C terminus of MTA3 and the central region of BCL-6.

The MTA3 Mi-2/NuRD Complex Directs Repression by the BCL-6 Central Repression Domain

The coordinate expression and physical interaction of BCL-6 with MTA3 suggested a functional interaction. To investigate the potential role of MTA3 in transcriptional repression by BCL-6, we employed a Gal4 tethering assay. BCL-6 fused in-frame to the Gal4 DNA binding domain was cotransfected into HeLa cells with a constitutively expressing luciferase reporter construct containing Gal4 binding sites (Fujita et al., 2003). HeLa cells were utilized because they are readily transfected and they express endogenous MTA3 at high levels (Fujita et al., 2003). Gal4-BCL-6 repressed transcription from the reporter in a dose-dependent manner (Figure 3C). As other corepressors have been demonstrated to interact with BCL-6 and mediate transcriptional repression by interaction with the BTB-POZ domain (Dhordain et al., 1997; Huynh and Bardwell, 1998; Huynh et al., 2000; Wong and Privalsky, 1998), specific regions of BCL-6 were tested in this assay for repressive activity. As expected, the BTB-POZ domain when fused to the Gal4 DNA binding domain repressed transcription from the reporter in a dose-dependent manner. However, a mutant version of BCL-6 lacking the BTB-POZ domain (construct Δ2, Figure 3C) also functioned as a transcriptional repressor in this assay. Further deletion mutations mapped the minimal region sufficient for transcriptional repression to the central 367 amino acids (construct Δ5, Figure 3C). These data suggested that BCL-6 has two independent transcriptional repression domains: the BTB-POZ domain and the central region.

To investigate the requirement for MTA3 function in transcriptional repression driven by BCL-6 in the Gal4 tethering assay, RNA interference was utilized. In the Gal4 tethering assay, transcriptional repression by full-length BCL-6 was reduced by MTA3 siRNA treatment (Figure 3D). Repression driven by the central region of BCL-6 was affected to a somewhat greater extent by MTA3 depletion. We concluded that MTA3 contributes to transcriptional repression by Gal4-BCL-6 through interaction with a transcriptional repression domain in the central region of BCL-6.

Acetylation of BCL-6 Alters Its Interaction with MTA3 and Its Ability to Repress Transcription

The central region of BCL-6 is of immense interest as Dalla-Favera and colleagues have defined in this region of the protein acetylation sites that are key regulators of transcriptional repression function (Bereshchenko et al., 2002). As the region defined in this work overlaps with the acetylation sites of BCL-6 (Figure 3A), we asked whether BCL-6 acetylation status impacted MTA3-dependent transcriptional repression. Mutant versions of BCL-6 were constructed that either prevent any acetylation (KKYK to KKYR mutation) or mimic constitutive acetylation (KKYK to QQYQ) in the context of the Gal4-BCL-6 fusion. Transcriptional repression assays were again performed in HeLa cells using the luciferase reporter described above (Figure 3E). Both wild-type BCL-6 and the KKYR mutant exhibited robust transcrip-

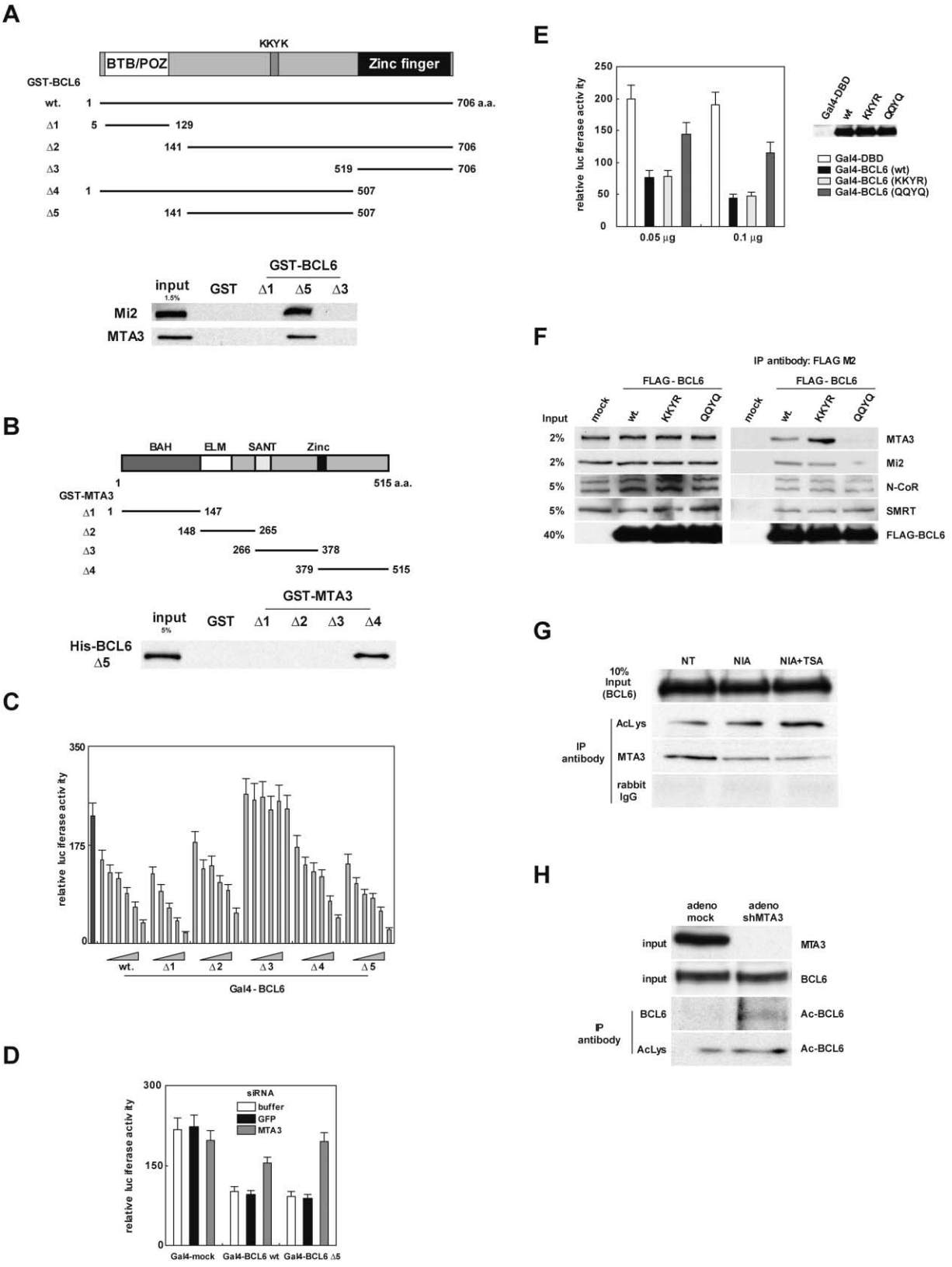

Figure 3. Direct Interaction between MTA3 and BCL-6 Is Regulated by Acetylation

(A) The indicated regions of BCL-6 were fused to GST and produced in recombinant bacteria. The BCL-6-GST fusions were incubated with Ramos nuclear extracts and interacting proteins were detected by immunoblot.

(B) The indicated regions of MTA3 were produced as GST fusion proteins in recombinant bacteria. After incubation with purified recombinant BCL-6 (the Δ5 mutant), interacting proteins were analyzed by immunoblot.

tional repression activity in the tethering assay. In contrast, the QQYQ mutant, which mimics constitutive acetylation, was unable to direct transcriptional repression of the reporter.

The above results suggest that the MTA3-dependent repression function of BCL-6 is sensitive to the acetylation status of BCL-6. As MTA3 interacts directly with BCL-6, in a region including the sites of acetylation, we asked whether modification status of BCL-6 affected its capacity to interact with MTA3. For these experiments, BCL-6 derivatives were expressed exogenously in HeLa cells that express high levels of NCoR, SMRT, and MTA3 (Figure 2D). Immunoprecipitation of BCL-6 was followed by immunoblot analysis to analyze coprecipitation of NCoR, SMRT, or the MTA3-containing Mi-2/NuRD complex. Overexpression of wild-type BCL-6 resulted in coprecipitation of all corepressor complexes analyzed (Figure 3F). The KKYR BCL-6 mutant that cannot be acetylated also coprecipitated all three corepressors (Figure 3F). In contrast, the QQYQ mutant that mimics constitutive acetylation coprecipitated NCoR and SMRT with comparable efficiency to wild-type but was impaired in its ability to coprecipitate MTA3 and the Mi-2/NuRD complex. These data support the conclusion that association of MTA3 with the central repression domain of BCL-6 was sensitive to posttranslational modification. In addition, these results also indicate that BCL-6 interacts productively with NCoR and SMRT and that this interaction is not altered by modification of BCL-6.

If the interaction of BCL-6 and MTA3 were modulated by acetylation, one would predict that drug treatments resulting in accumulation of the acetylated form of endogenous BCL-6 might impair its interaction with endogenous MTA3. Accordingly, Raji cells were cultured in the presence of niacinamide, an inhibitor of the NAD-dependent Sir2 class of deacetylases (Landry et al., 2000; Luo et al., 2001), with or without Trichostatin-A, an inhibitor of class I and class II histone deacetylases (Yoshida et al., 1990). Immunoprecipitations were subsequently performed with antibodies specific for acetyl-lysine, MTA3, or rabbit IgG as a control. Precipitates were analyzed by immunoblot for BCL-6. Consistent with previous reports (Bereshchenko et al., 2002), inhibition of NAD-dependent deacetylation resulted in accumulation of acetylated forms of BCL-6 (Figure 3G), an effect that was modestly enhanced by Trichostatin-A. Importantly, the amount of BCL-6 coprecipitating with MTA3 was reduced by deacetylase inhibition (Figure 3G).

As MTA3 is physically associated with HDAC1 and HDAC2 in the context of the Mi-2/NuRD complex (Fujita et al., 2003), we asked whether depletion of endogenous MTA3 from B lymphocyte cell lines might alter the steady-state levels of acetylated BCL-6. We were unable to achieve sufficient transfection of lymphocytes with siRNA, therefore we resorted to an alternative strategy. An adenovirus was constructed to deliver a DNA construct encoding a short hairpin RNA (shRNA) to deplete exogenous MTA3 in lymphocyte cell lines. While lymphocytes and plasma cells do not express high-affinity receptors for adenovirus, they can nonetheless be infected with high efficiency (Leon et al., 1998). Infection with the shRNA MTA3 adenovirus but not with a control shRNA adenovirus resulted in loss of MTA3 protein with no apparent effect on BCL-6 abundance (Figure 3H). Immunoprecipitations were performed with antibodies specific for acetyl-lysine and for BCL-6, and precipitates were analyzed by immunoblot for either acetyl-lysine or BCL-6 (Figure 3H). Depletion of MTA3 led to an accumulation of acetylated forms of BCL-6. These results suggest that either the HDAC activity associated with MTA3 may play an integral role in dynamic acetylation of BCL-6 or that association of the central repression domain of BCL-6 with MTA3 physically interferes with access of this region to the relevant acetyltransferase, believed to be p300 (Bereshchenko et al., 2002).

MTA3 Is Necessary for BCL-6-Dependent Transcriptional Repression In Vivo

While the above results indicated the potential importance of MTA3 for transcriptional repression function of BCL-6, issues relating to cellular context and overexpression needed to be addressed. Adenoviral shRNA delivery led to depletion of MTA3 protein in Raji and BJAB B cells (Figure 4A). As shown, analysis of relevant transcripts from the MTA3-depleted and control cell populations revealed that three plasma cell-specific transcripts, Blimp-1, CCL3, and Syndecan-1, were derepressed following MTA3 knockdown in both cell lines (Figure 4B). Thus, MTA3 function is required for full tran-

(C) A luciferase reporter plasmid containing the thymidine kinase promoter with upstream Gal4 binding sites was cotransfected into HeLa cells along with expression plasmids for the indicated regions of BCL-6 fused to the Gal4 DNA binding domain. Luciferase activity (normalized to Renilla luciferase transfection control) is depicted as a function of amount of Gal4-BCL-6 fusion transfected.

(D) Luciferase reporter assays were performed in HeLa cells as described in (C). Cells were treated with buffer, siRNA specific for GFP, or siRNA specific for MTA3. Luciferase activity (normalized to Renilla luciferase transfection control) is depicted for each condition.

(E) A luciferase reporter plasmid containing the thymidine kinase promoter with upstream Gal4 binding sites was cotransfected into HeLa cells along with expression plasmids for the indicated BCL-6 derivatives fused to the Gal4 DNA binding domain. Luciferase activity (normalized to Renilla luciferase transfection control) is depicted for each BCL-6 expression plasmid. The immunoblot at the left depicts expression of the different BCL-6 derivatives.

(F) The indicated BCL-6 derivatives were expressed in HeLa cells and immunoprecipitation was performed with the FLAG antibody. The left panel depicts input for each condition and the right panel depicts proteins precipitating with the FLAG BCL-6 derivative. Individual antibodies used to analyze the precipitates are indicated to the right of the figure.

(G) Raji B cells were grown with either no treatment, treatment with niacinamide, or treatment with niacinamide plus Trichostatin-A as indicated. Nuclear extracts prepared from each culture condition were immunoprecipitated with the indicated antibodies. BCL-6 abundance in the precipitates was analyzed with the anti-BCL-6 antibody.

(H) Raji B cells were infected with either a control adenovirus expressing a control shRNA or with an adenovirus expressing an MTA3-specific shRNA. Protein lysates from cells infected as indicated were analyzed with antibodies specific for MTA3 and BCL-6. Immunoprecipitations were performed with either anti-acetyl-lysine or anti-BCL-6 antibodies and analyzed using BCL-6 or acetyl-lysine antibodies.

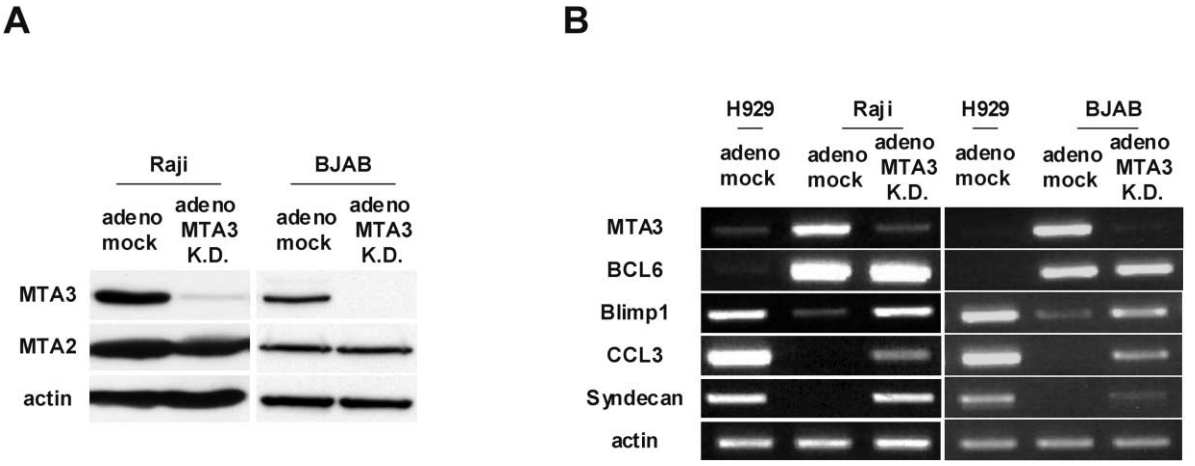

Figure 4. Endogenous MTA3 Is Required for the Transcriptional Repression Function of Endogenous BCL-6 in B Cells

(A) Raji and BJAB cells were infected with the adenovirus expressing shRNA-MTA3 or the control shRNA adenovirus. The immunoblot depicts MTA3, MTA2, and actin levels following infection.

(B) Transcript levels for the indicated gene products were determined by RT-PCR from uninfected, mock shRNA, and shRNA-MTA3 adenovirus-infected cells.

scriptional repression activity of endogenous BCL-6 at these three loci in multiple cell lines.

To examine in more detail the functional interaction of MTA3 and BCL-6 in lymphoid cells, we constructed adenovirus vectors for transient expression of these factors. Infection of H929 and U266B1 plasma cells with adenoviruses expressing BCL-6 and/or MTA3 led to infection of the majority of the cells in the population (data not shown) and high-level expression of the relevant proteins (Figure 5A and data not shown). To ascertain the role of BCL-6 and MTA3 on specific genes, the levels of transcripts of multiple loci differentially regulated in plasma cells and B lymphocytes were analyzed. By itself, MTA3 expression had no effect on any transcript examined. Expression of BCL-6 alone led to small changes in transcript abundance from these loci. However, coexpression of both BCL-6 and MTA3 led to robust repression of the set of transcripts associated with the plasma cell-specific transcriptional program, such as Blimp-1, CCL3, Syndecan-1, and XBP1 (Figure 5B). In contrast, multiple transcripts diagnostic of the B lymphocyte transcriptional program were significantly upregulated; these transcripts include CIITA, BLNK, Igβ, and SYK (Figure 5B).

The requirement for MTA3 for efficient transcriptional repression at *CCL3*, *Syndecan-1*, *XBP1*, and *Blimp-1* could result from recruitment of MTA3 and the Mi-2/NuRD complex to these loci, or from an indirect effect. To differentiate between these two possibilities, chromatin immunoprecipitation (ChIP) assays were performed on the *CCL3* locus. MTA3 was recruited in a BCL-6 dependent manner to the human *CCL3* promoter following expression of these factors in the H929 plasma cell line (Figure 5C). Moreover, MTA3 was present naturally at the *CCL3* locus in the Raji B cell line (Figure 5D). These experiments suggested that BCL-6-dependent transcriptional repression in lymphoid cells requires the physical presence of the MTA3-containing Mi-2/NuRD complex in the vicinity of the promoter.

Coexpression of MTA3 and BCL-6 Reprograms Plasma Cell Fate

The above observation, that coexpression of BCL-6 and MTA3 led to repression of Blimp-1 and re-expression of CIITA (Figure 5B), raised the intriguing possibility that these events might lead to reprogramming of the plasma cell lines H929 and U266B1 to a B lymphocyte-specific transcriptional program. Blimp-1 is necessary, but not sufficient, for the differentiation of B lymphocytes into plasma cells (Shapiro-Shelef et al., 2003) and has been shown to repress expression of the class II transactivator, CIITA (Piskurich et al., 2000). CIITA is a coactivator required, along with several well-characterized transcription factors, for the expression of MHC class II proteins, such as HLA-DR, characteristic of B lymphocytes (Boss and Jensen, 2003; Ting and Trowsdale, 2002). Because four different transcriptional start sites direct CIITA expression in a cell type-specific manner (Muhlethaler-Mottet et al., 1997), RT-PCR was performed with primers differentiating between the cell type-specific transcription start sites to determine whether the B cell-specific promoter was used. In fact, the transcripts accumulating in H929 cells following coexpression of BCL-6 and MTA3 originate at promoter III, the primary promoter used in B lymphocytes (Figure 5E).

If coexpression of BCL-6 and MTA3 leads to reprogramming of the plasma cell transcriptional program to a B lymphocyte pattern, the expression of cell surface markers should reflect the change in cell fate. Analysis of the H929 plasma cell line for common markers reflective of B lymphocyte cell identity following infection with BCL-6 and/or MTA3 adenovirus revealed striking changes (Figure 6A). Cell surface expression of CD19 and HLA-DR was detected following transient expression of BCL-6, consistent with a shift from a plasma cell pattern to a B lymphocyte pattern. The percentage of cells converting was dramatically increased by coexpression of MTA3 (Figure 6B). CD19 expression, unlike HLA-DR, is CIITA independent. Thus, the pattern of pro-

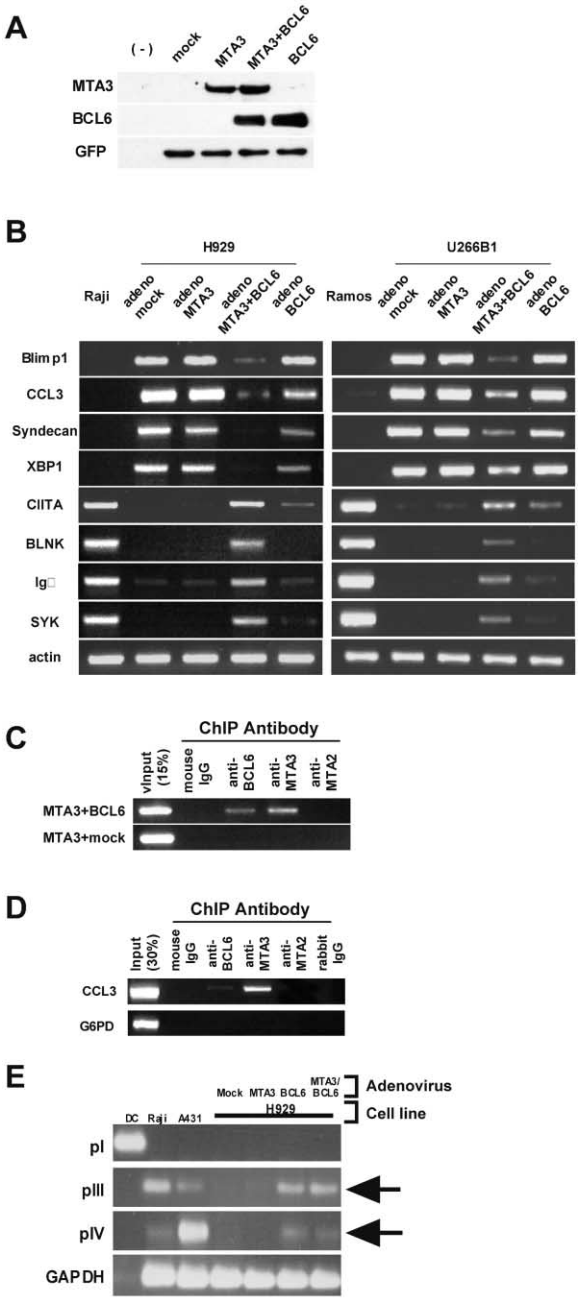

Figure 5. MTA3 and BCL-6 Expression in Plasma Cells Alters Their Transcriptional Program

(A) Adenoviruses expressing MTA3 and/or BCL-6 were used to infect H929 cells. The immunoblot depicts expression of MTA3 and/or BCL-6 following adenovirus infection.

(B) The panel depicts RT-PCR products derived from cells treated as indicated in the figure. RT-PCR from Raji and Ramos cells is shown as a control.

(C) Chromatin immunoprecipitation was performed on H929 cells following infection with either MTA3 adenovirus or MTA3 and BCL-6 adenoviruses. The indicated primary antibodies were used to precipitate chromatin following crosslinking. PCR to detect the CCL3 promoter region was performed on the precipitated DNA.

(D) Chromatin immunoprecipitation was performed on Raji cells with the indicated primary antibodies. PCR to detect the CCL3 promoter region was performed on the precipitated DNA.

(E) H929 cells were infected with control or adenoviral constructs expressing BCL-6 or MTA3. RNA was extracted 5 days post-infection and used for RT-PCR with CIITA isotype-specific primers. Plasmid expressing dendritic cell CIITA promoter 1 DNA and cDNA from Raji B cells and A431 epithelial cells treated with interferon γ were used as sources of templates to demonstrate CIITA promoter specificity of the assay. RT-PCR of the glyceraldehyde-3-phosphate dehydrogenase gene is shown as a loading control.

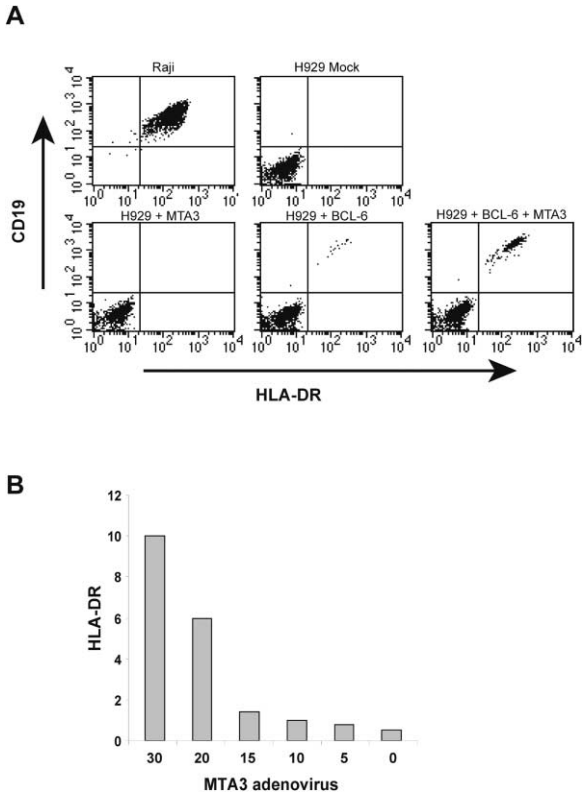

Figure 6. Coexpression of MTA3 and BCL-6 in H929 Cells Leads to Reversion to a B Cell-like Transcriptional Program

(A) Flow cytometric immunophenotyping was performed after 7 days infection with adenoviral constructs carrying BCL-6, MTA3, or control; all adenoviral constructs also carried GFP. Cells were surface labeled with Per-CP anti-HLA-DR and APC anti-CD19 or isotype control. Dot plots are shown for the following conditions: mock-infected control Raji cells; mock-infected H929 cells; MTA3 adenoviral-infected H929 cells; BCL-6 adenoviral-infected H929 cells; BCL-6 and MTA3 adenoviral-infected H929 cells. Gates were set based on isotype control staining levels; at least 10,000 total events were obtained. Analysis gates in FL-1 were placed on cells that displayed intermediate to high GFP expression (not shown). For these cells, fluorescence intensities in FL-3 (abscissa) and FL-4 (ordinate) are displayed on a four-decade logarithmic scale.

(B) The percentage of HLA-DR-positive cells is plotted as a function of the relative quantity of MTA3 adenovirus used to infect the cells with a constant quantity of coinfected BCL-6 adenovirus. The mean fluorescent intensities of the HLA-DR-positive populations were not statistically different (not shown). The gating strategy employed was identical to that employed in (A).

tein expression induced in this experiment includes not only CIITA-responsive genes but other B cell-specific proteins as well. Moreover, HLA-DR and CD19 expression were reactivated in the same population of cells and expression levels of these markers were indistin-

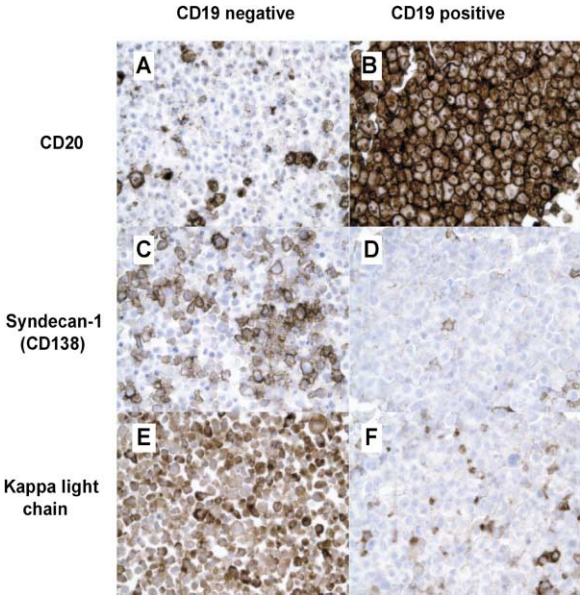

Figure 7. Coexpression of MTA3 and Bcl-6 in Plasmacytoma Cells Induces a Switch to a B Cell Immunophenotype

Immunocytochemical stains were performed on H929 cells 7 days after coinfection with MTA3 and Bcl-6 adenoviruses. Results are displayed for MACS-selected CD19-negative cells and CD19-positive cells at magnification 400× for all panels. After labeling with primary antibody and detection with secondary reagents, bound antibodies were identified by a standard DAB reaction.

guishable from those in Raji. This finding suggests that once a critical level of BCL-6/MTA3 is attained, resetting of the transcriptional program proceeds.

To examine whether additional alterations in protein expression were evident in H929 cells following transient expression of MTA3 and BCL-6, the CD19-positive and -negative populations were separated (Abts et al., 1989). Immunohistochemical methods were then used to analyze expression of markers of B cell and plasma cell differentiation. The mature B cell marker CD20 was robustly expressed in the CD19-positive population (Figures 7A and 7B). The plasma cell-selective marker Syndecan-1 (CD138) was expressed to a significantly higher extent in the CD19-negative population (Figures 7C and 7D). In addition, the CD19-negative cells displayed robust cytoplasmic staining for the Kappa immunoglobulin light chain, whereas the CD19-positive cells exhibited scant cytoplasmic staining with occasional cell surface expression, as expected (Figures 7E and 7F). Thus, transient expression of BCL-6 and MTA3 in a plasma cell line was sufficient to not only alter its transcriptional program toward that of a B lymphocyte but also to alter the pattern of protein synthesis reflective of a reversion in cell fate.

Discussion

Our results identify a molecule involved in transcriptional regulation during the B lymphocyte developmental program. MTA3 expression mirrors that of BCL-6 in cells of the B lymphocyte lineage and molecular analysis reveals a functional role in B cell-specific transcription. Elegant functional (Dhordain et al., 1997; Huynh and Bardwell, 1998; Wong and Privalsky, 1998) and structural data (Ahmad et al., 2003) link BCL-6 transcriptional repression function and the BTB-POZ domain to NCoR and SMRT. The results in this work suggest that MTA3 and the Mi-2/NuRD complex function to facilitate transcriptional repression by BCL-6 through interaction with the central region of the protein. This region of BCL-6 has been identified as an independent repression domain by other investigators (Dhordain et al., 1998; Zhang et al., 2001). Importantly, the repression function of this domain is modulated by protein modification (Bereshchenko et al., 2002), providing an acute means of regulating the transcriptional repression function of BCL-6 in response to biological cues. In the lymphoid cell lines utilized in this work, we failed to detect significant expression of either NCoR or SMRT (Figure 2D). We propose that utilization of MTA3 and the Mi-2/NuRD complex as a corepressor for BCL-6 extends its capacity to direct transcriptional repression. Multiple possible scenarios could result. In cells that express both NCoR/SMRT and MTA3, BCL-6-dependent repression could utilize both types of corepressor complex. The possibility of locus specificity for corepressors exists as does the possibility of combinatorial action. NCoR and SMRT can utilize HDAC3 to repress transcription (Li et al., 2000; Underhill et al., 2000; Urnov et al., 2000; Wang and Hiebert, 2001; Wen et al., 2000), whereas the MTA3-containing Mi-2/NuRD complex contains HDAC1 and HDAC2 (Fujita et al., 2003). This capacity to recruit unique histone deacetylases with potential differences in substrate specificity might expand the capacity of a transcriptional repressor such as BCL-6 to effectively silence transcription.

The involvement of MTA3 in the B lymphocyte transcriptional program supports the model (Bowen et al., 2004) that MTA3 represents a cell type-specific component of the well-described transcriptional corepressor, Mi-2/NuRD. In the context of mammary epithelial cells, MTA3 acts to repress genes implicated in dramatic cell fate alterations. The finding that MTA3 and the Mi-2/NuRD complex regulate cell fate in two biologically diverse models suggests that an important conserved function of this corepressor complex is repression of master regulatory genes integral to cell fate changes during development and differentiation. This prediction highlights the importance of cell type specificity of coactivators and corepressors, suggesting that alterations in the subunit composition of these transcriptional regulatory complexes may be integral to the establishment of cell type-specific transcriptional programs.

The simplest example of this prediction has been observed if one follows the role of CIITA. CIITA is the limiting factor governing whether a cell expresses MHC class II genes as well as the antigen presentation accessory genes, invariant chain, HLA-DM, and HLA-DO (Boss and Jensen, 2003). During B cell development, MHC class II expression occurs in the pro-B cell stage, increases during B cell maturation, but is extinguished in terminally differentiated plasma cells. Blimp-1 has been shown to mediate the silencing of MHC class II genes through the repression of CIITA expression (Piskurich et al., 2000). The interaction of Blimp-1 with promoter III of the CIITA gene is consistent with the findings reported

here and supports the model that MTA3 and BCL-6 regulate MHC class II expression by repressing the expression of Blimp-1. Preliminary studies suggest that specific histone modifications associated with gene expression are lost during the B cell, plasma cell transition and it is likely that Blimp-1 mediates these events.

The differentiation program of B lymphocytes is regulated, in part, by a negative feedback mechanism involving the transcriptional repressors BCL-6 and Blimp-1 (Lin et al., 2003; Shaffer et al., 2002b; Shapiro-Shelef et al., 2003). These transcription factors exhibit mutually exclusive patterns of expression and definition of the transcriptional programs downstream of their function supports the notion that these repressors are master regulators of B lymphocyte and plasma cell identity (Shaffer et al., 2000, 2002a). In fact, targeted disruption of Blimp-1 in the lymphocyte lineage leads to a failure to produce plasma cells (Shapiro-Shelef et al., 2003). Likewise, inappropriate expression of BCL-6 following translocation is believed to contribute to lymphomagenesis, at least in part by maintaining repression of Blimp-1 (Shaffer et al., 2002b). Feedback between transcriptional repressors is an evolutionarily ancient strategy to produce stable patterns of gene expression (Monod and Jacob, 1961). The molecular details of transcriptional repression utilized in such systems differ, but the fundamental strategy remains the same.

The molecular mechanisms utilized by Blimp-1 and BCL-6 provide interesting insights into cell type-specific patterns of transcription during eukaryotic development and differentiation. Recent evidence has shown that Blimp-1 (PRD-BF1) interacts with the histone methyltransferase G9a (Gyory et al., 2004), which has been shown to mediate methylation of lysine 9 of histone H3 (H3K9), leading to a repressive state (Tachibana et al., 2001). The utilization of lysine methylation as an epigenetic mark by a master regulator of the plasma cell transcriptional program raises an interesting concept. During differentiation, cell type changes progress from a state of pluripotency to one of restricted developmental potential. Concurrent with these alterations in cell physiology are epigenetic alterations that contribute to cell type-specific transcriptional patterns. The example of B lymphocyte development and ultimate terminal differentiation into plasma cells highlights this phenomenon. In the germinal center B lymphocyte, a cell that must rapidly respond to environmental cues, a master transcriptional regulator, BCL-6, utilizes an epigenetic modification, lysine acetylation, that is freely and rapidly reversible. In contrast, a terminally differentiated version of the germinal center B lymphocyte, the plasma cell, is less responsive to external stimuli and exhibits restricted capacity to alter its transcriptional program. The primary epigenetic mark deposited by a master regulator of the plasma cell program, Blimp-1, is lysine methylation, a mark with considerably greater chemical and biological stability than lysine acetylation. Thus, in this example, a terminally differentiated cell utilizes a chemically more stable epigenetic mark in establishment of a cell type-specific transcriptional program than does an intermediate cell type in the differentiation pathway. We propose that one strategy to restrict plasticity of cell type-specific transcriptional programs involves utilization of chemically and biologically stable epigenetic marks in terminally differentiated cells and more labile modifications in intermediate cell types.

Experimental Procedures

Cell Culture, Transfection, and Adenovirus Infection

Lymphocyte and plasma cell lines were cultured in RPMI (Cellgro), and HeLa and 293T cells were cultured in DMEM with 10% (v/v) fetal bovine serum (Gibco). Cells were transfected with Lipofectamine (Invitrogen) or Fugene 6 (Roche). Adenovirus carrying human MTA3 and human BCL6 was prepared as described (He et al., 1998). The virus was purified by CsCl banding with 10^{11} to 10^{12} plaque-forming units and introduced into cells. Luciferase activities were determined as described (Fujita et al., 2003). Values are the means and standard deviations of the results from three independent experiments.

Plasmids

Expression vectors were constructed by subcloning *BCL6* cDNA (kind gift of Ricardo Dalla-Favera) into pCDNA3 (Invitrogen) and pCMV-Gal4 (pcDNA3-BCL6 and pCMV-Gal4-BCL6). pcDNA3-MTA3 was described (Fujita et al., 2003). The fragment containing five Gal4 binding sites was inserted into the *TK*-promoter-containing pGL2-Basic vector (Promega). The promoter regions of human *CCL3* were amplified from human genomic DNA and cloned into pGL2-Basic. Primers for *CCL3* promoter amplification are as follows:

5′-GGTACCTAGAGTTATAAACGATGCTG-3′

5′-AAGCTTGGGCTCGAGTGTCAGGAGAG-3′

The Δ1 (5–129 aa), Δ2 (141–706 aa), Δ3 (141–706 aa), Δ4 (1–507 aa), and Δ4 (141–507 aa) of *BCL6* cDNA were subcloned into pCMV-Gal4. The mutants of BCL6 (KKYR, QQYQ) were generated by mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene).

Antibodies and Western Blotting

Antibodies against MTA3, MTA2, Mi2, and MBD3 were previously described (Fujita et al., 2003). Anti-NCoR and -SMRT polyclonal antibodies were the kind gift of Jiemin Wong. Other antibodies utilized were anti-HDAC1 and anti-HDAC2 (Santa Cruz), anti-BCL6 (DAKO), anti-GFP (ZYMED), anti-β-actin (Chemicon), anti-HA (Roche), anti-FLAG (M2), and anti-FLAG (M5) (Sigma).

Construction of Recombinant Protein

The Δ1 (1–147 aa), Δ2 (148–265 aa), Δ3 (266–378 aa), and Δ4 (379–515 aa) of *MTA3* cDNA and the Δ1 (5–129 aa), Δ3 (519–706 aa), and Δ5 (141–507 aa) of *BCL6* cDNA were subcloned into pGEX-4T-1 (Amersham Pharmacia). The Δ5 (141–507 aa) of *BCL6* cDNA was subcloned into pRSET (Invitrogen). GST or His fusion proteins were expressed in *E. coli* BL21(DE3) and were purified by glutathione-agarose (sigma) or Probond resin (Promega).

GST Pull-Down Assay

Bacterially expressed GST and GST-fused proteins (2 μg) were immobilized on glutathione-agarose beads and incubated with either whole lysates from Raji cells or His-tagged proteins (1 μg) in a buffer (250 μl) containing 0.1% TritonX-100, 50 mM HEPES, pH 7.4, 100 or 400 mM NaCl, 5% glycerol, 2 mM DTT, 0.1 mg/ml BSA and protease inhibitors for 1 hr at 4°C.

Immunoprecipitation

Cells were lysed with hypotonic buffer: 0.5% Triton X-100, 10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.2 mM PMSF, and 1 mM dithiothreitol (DTT). After centrifugation at 4000 rpm for 2 min, nuclear pellets were extracted with 10 mM Tris (pH 7.5), 500 mM NaCl, 3 mM $MgCl_2$, 10% glycerol, 1% CHAPS, 0.2 mM PMSF, and 1 mM DTT. After centrifugation, the supernatants were dialyzed versus 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 20% glycerol, 0.2 mM PMSF, and 1 mM DTT. Aliquots of nuclear extract (200–400 μg) were incubated with specific antibodies or with control IgG (Santa Cruz) for 1 hr at 4°C followed by incubation for 1 hr with 25 μl of protein G/A agarose (Oncogene).

The detection of protein-protein interactions in Ramos or Raji cells was described (Bereshchenko et al., 2002). Briefly, we treated

Ramos cells with 5 mM NIA and/or 1 μM Trichostatin A (TSA) for 5 hr. Cells were lysed with hypotonic buffer with 5 mM NIA and/or 1 μM TSA (sigma). After centrifugation, nuclear pellets were extracted with 20 mM HEPES (pH 7.9), 500 mM KCl, 0.2 mM EDTA (pH 8.0), 2 mM EGTA (pH 8.0), 0.75 mM spermidine, 0.15 mM spermine, 25% glycerol, 0.2 mM PMSF, 2 mM DTT, 10 mM NIA, and/or 50 μM TSA (sigma). After centrifugation, the supernatants were dialyzed versus 20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA (pH 8.0), 0.2 mM EGTA (pH 8.0), 20% glycerol, 0.2 mM PMSF, 2 mM DTT, 10 mM NIA and/or 10 μM TSA (sigma), and/or 5 mM sodium n-Butyrate. Aliquots of nuclear extracts (200–400 μg) were incubated with specific antibodies for 1 hr at 4°C followed by incubation for 1 hr with 25 μl of protein G/A agarose.

RNA Extraction and RT-PCR

Total RNA was extracted by a modified guanidium thiocyanate-phenol-chloroform extraction as described (Fujita et al., 2003). cDNA was synthesized with M-MLV reverse transcriptase (Life Technologies) using random hexamers as described (Fujita et al., 2003). The cDNA was amplified using the following primers:

Blimp1: 5′-ACTTGCAACAAGGGCTTTAc-3′; 5′-ACCTTGCATTGGTATGGTTT-3′

CCL3: 5′-GCAACCAGTTCTCTGCATCA-3′; 5′-ACTCCTCACTGGGGTCAGC-3′

β-actin: 5′-CTCTTCCAGCCTTCCTTCCT-3′; 5′-AGCACTGTGTTGGCGTACAG-3′

CIITA (total): 5′-CTGAAGGATGGTGGAAGACCTGGGAAAGC-3′; 5′-GTGCCCGATCTTGTTCTCACTC-3′

CIITA (pI): 5′-GGAGACCTGGATTTGGCCCT-3′; 5′-GAAGCTCCAGGTAGCCACCTTCTA-3′

CIITA (pIII): 5′-GGGGAAGCTGAGGGCACG-3′; 5′-GAAGCTCCAGGTAGCCACCTTCTA-3′

CIITA (pIV): 5′-GCGGCCCCAGAGCTGG-3′; 5′-GAAGCTCCAGGTAGCCACCTTCTA-3′

GAPDH: 5′-CCATGGGAGGTGAAGGTCGGAGTC-3′; 5′-GGTGGTGCAGGAGGCATTGCTGATG-3′

XBP-1: 5′-TCTGGCGGTATTGACTCTTC-3′; 5′-GAGAAAGGGAGGCTGGTAAG-3′

Syndecan-1: 5′-GAGCAGGACTTCACCTTTGA-3′; 5′-TTCGTCCTTCTTCTTCATGC-3′

SYK: 5′-AGATCTGGCTGCAAGAAATG-3′; 5′-ATGCTTCCCACATCAACACT-3′

BLNK: 5′-CCTGTGATCGAAAGTCTGCT-3′; 5′-TTGCTTCAATAAATCGCACA-3′

Ig-β: 5′-TTCTGTCAGCAGAAGTGCAA-3′; 5′-GGAAGATAGGCACGATGATG-3′

Amplification of CIITA followed previously described conditions (Nagarajan et al., 2002).

Chromatin Immunoprecipitation Assay (ChIP)

Cells (5×10^5) were crosslinked (in PBS) with 1% formaldehyde. Crude cell lysates were sonicated. Immunoprecipitation was performed with specific and control antibodies (Upstate). The *CCL3* promoter region was detected by PCR amplification with the following primers: 5′-tgggttgtttccacagaac-3′ and 5′-ctgaatgaaagctggaggaa-3′. G6PD amplification followed the conditions of Coffee et al. (1999).

siRNA and Adenoviral shRNA Knockdown Experiments

siRNA duplexes designed in the open reading frames of *MTA3* and *GFP* were as described (Fujita et al., 2003). siRNAs were transfected using Oligofectamine following the manufacturer's instructions (Invitrogen). Knockdown of MTA3 using adenoviral delivery was accomplished by annealing the following primers for generation of shRNA-MTA3:

Forward 5′-GATCCCGGAGGATACCTTCTTCTACTCAGAAGCTTGTGAGTAGAAGAAGGTATCCTCTTTTTT-3′

Reverse 5′-CTAGAAAAAAGAGGATACCTTCTTCTACTCACAAGCTTCTGAGTAGAAGAAGGTATCCTCCGG-3′

Annealed primers were subcloned predigested pGE-1 (Stratagene). shRNA-MTA3 adenovirus was constructed by excising the shRNA-MTA3 fragment along with the U6 promoter from shRNA-MTA3/pGE-1 and ligating the fragment into pAdTrack, from which the CMV promoter used for protein expression had been deleted. All other adenovirus manipulations were performed as described for overexpression.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections were obtained from the archives of the Emory University Division of Anatomic Pathology. FFPE sections of tissue culture cell pellets were prepared after selection with CD19-microbeads on a MACS column (Millenyi Biotech). Sections of 4 μm thickness on glass slides underwent standard deparaffinization and rehydration (Jaye et al., 2003). Antigen unmasking and staining of tissue sections with polyclonal anti-MTA3 antibody was performed as described previously (Fujita et al., 2003). Antigen unmasking and staining of cell pellets with antibodies to CD20, CD138, and kappa and lambda immunoglobulin light chains (DAKO) were performed according to manufacturer's instructions. Detection with the LSAB2 System (DAKO) for immunoperoxidase staining followed the manufacturer's recommendations. Following diaminobenzidine (DAB) exposure, sections were counterstained with hematoxylin before mounting for light microscopy and photography.

Immunofluorescence Microscopy

Cryostat sections were cut to 8 μm thickness, placed on glass slides, and fixed and permeablized for 10 min at room temperature with ice-cold PBS, 3.7% paraformaldehyde, 0.5% Triton X-100. After three washes with PBS, sections were blocked for 30 min at room temperature with PBS, 3% BSA. Sections were stained for 1 hr at room temperature with primary antibody cocktail (mouse anti-Bcl-6 [Dako, Carpinteria, California] at 1:10 and rabbit anti-MTA3 at 1:200) and washed with PBS. Bound primary was detected with secondary reagent cocktail composed of Alexa-568-goat-anti-rabbit IgG (H & L) and Alexa-488 donkey anti-mouse IgG (H & L; Molecular Probes, Eugene, Oregon), each at 1:1000. After washing with PBS, nuclei were stained with To-pro-3-iodide (Molecular Probes) at 1:1000 in PBS for 2 min. After washing, sections were mounted and cover slipped for confocal microscopy as described (Ivanov et al., 2004).

Flow Cytometry

Cells were harvested on day 6 or day 7 following adenoviral infection and brought to 1×10^6 to 5×10^6 cells in 100 μl in PBS, 0.5% BSA. Cell suspensions were incubated for 20 min with 5 μl of peridinin chlorophyll protein (Per-CP)-anti-HLA-DR and allophycocyanin (APC)-conjugated anti-CD19 monoclonal antibodies or isotype controls (BD Biosciences, San Jose, California). After washing, cells were fixed with 1.8% paraformaldehyde for 10 min and washed once with PBS, 0.5% BSA. Cells were analyzed on a FACSCaliber cytometer (Becton Dickinson, San Jose, California), and data were acquired and analyzed as described (Jaye et al., 2001).

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