BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma

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Edited by Carlo M. Croce, Ohio State University, Columbus, OH, and approved May 13, 2009 (received for review April 8, 2009)

The BCL6 proto-oncogene encodes a transcriptional repressor that is required for germinal center (GC) formation and whose deregulation by genomic lesions is implicated in the pathogenesis of GC-derived diffuse large B cell lymphoma (DLBCL) and, less frequently, follicular lymphoma (FL). The biological function of BCL6 is only partially understood because no more than a few genes have been functionally characterized as direct targets of BCL6 transrepression activity. Here we report that the anti-apoptotic proto-oncogene BCL2 is a direct target of BCL6 in GC B cells. BCL6 binds to the BCL2 promoter region by interacting with the transcriptional activator Miz1 and suppresses Miz1-induced activation of BCL2 expression. BCL6-mediated suppression of BCL2 is lost in FL and DLBCL, where the 2 proteins are pathologically coexpressed, because of BCL2 chromosomal translocations and other mechanisms, including Miz1 deregulation and somatic mutations in the BCL2 promoter region. These results identify an important function for BCL6 in facilitating apoptosis of GC B cells via suppression of BCL2, and suggest that blocking this pathway is critical for lymphomagenesis.

apoptosis | DLBCL | germinal center

The BCL6 proto-oncogene encodes a transcriptional repressor of the POZ/BTB zinc-finger protein family (1, 2), which binds to specific DNA sequences and represses the transcription of its target genes via recruitment of corepressor complexes (1–4). In the B-cell lineage, BCL6 is expressed in germinal centers (GC) (5), the site in which B cells undergo somatic hypermutation (SHM) and class-switch recombination (CSR) of immunoglobulin (Ig) genes, and are selected on the basis of the production of antibodies with high affinity for the antigen (6). BCL6 is also an essential requirement for GC formation, because mice lacking BCL6 cannot form these structures (7–9). BCL6 expression is then turned off at the end of the GC reaction by a variety of signals, including CD40 receptor engagement (10–12), B-cell receptor (BCR) signaling (13), and genotoxic stress (14). Downregulation of BCL6 is necessary for GC B cells to mature toward plasma cells, because BCL6 is a repressor of *PRDM1*, a master regulator of plasma cell differentiation (15, 16).

In ≈30% of diffuse large B cell lymphoma (DLBCL) and 10% of follicular lymphoma (FL) cases, chromosomal translocations juxtapose heterologous partner chromosomes to the intact coding region of BCL6 (17–19), leading to its deregulated expression by promoter substitution (20). In addition, SHM-derived mutations in the BCL6 5′ regulatory region (21–23) deregulate BCL6 expression by disrupting its negative autoregulatory circuit in ≈10% of DLBCL (24, 25) and by preventing CD40-induced IRF4-mediated downregulation in a minority of cases (12). The role of BCL6 in lymphoma pathogenesis is underscored by the fact that mice expressing deregulated BCL6 alleles develop DLBCL (26).

A critical issue in the understanding of BCL6 function in GC development and lymphomagenesis is the identification of its regulatory program, i.e., the compendium of genes that are direct targets of its transrepressive activity. A mixture of direct and indirect BCL6 targets have been identified by gene expression profiling (15), and >400 promoters have been shown to be bound by BCL6 in transformed B cells (27). Nonetheless, only a few direct target genes have been functionally validated in normal GC B cells, including (*i*) the B7–1/CD80 coreceptor molecule, whose repression prevents premature activation of B cells within the GC (28); (*ii*) the p53, ATR, and CHEK1 genes, whose repression by BCL6 prevents the sensing and response to SHM/CSR-induced genotoxic stress in GC B cells (29–31); and (*iii*) the *PRDM1* gene (15, 16). In addition, BCL6 has been shown to suppress the transcription of genes whose promoters lack a BCL6 binding site by physically interacting with the transcriptional activator Miz1, which is recruited to promoters linked to an Inr element. By this mechanism, BCL6 suppresses the transcription of the cell-cycle arrest gene *CDKN1A* (p21) (32), thus facilitating the proliferation of GC B cells.

The results herein add a biologically important function to BCL6 by identifying the BCL2 gene, encoding a key anti-apoptotic molecule with oncogenic functions in FL and DLBCL (33–35), as a novel target of its transrepression activity in GC B cells. We show that BCL6 does not bind directly to the BCL2 promoter, but suppresses its activity via binding to Miz1. Finally, we demonstrate that BCL6-mediated suppression of BCL2 can be altered in DLBCL by different mechanisms, including chromosomal translocations of the BCL2 gene, somatic mutations in the BCL2 promoter region, and deregulated expression of Miz1. These results have implications for the understanding of GC biology and lymphomagenesis.

Results

BCL6 Binds to the BCL2 Promoter via Miz1. To identify the full set of BCL6 direct target genes, we performed a genomewide integrated transcriptional (gene expression profiling), biochemical (ChIP-chip), and bioinformatic (ARACNe algorithm) (36) analysis in purified normal GC B cells. This approach identified BCL2 as a novel candidate target gene. As shown in Fig. 1*A*, BCL6 binds to BCL2 in a region between the P1 and P2 promoters (37). This region contains numerous canonical BCL6 binding sites (B6BS) (1) and several putative Inr elements that could mediate BCL6 binding to BCL2 via Miz1, as previously described for the *CDKN1A* promoter (32). Indeed, a luciferase reporter construct driven by BCL2 promoter sequences spanning this region was efficiently repressed by BCL6 in transient transfection assays (Fig. 1*B*, solid bars). However, BCL6 could still repress a reporter construct where all canonical BCL6 binding sites had been mutated (Fig. 1*B*, shaded

Author contributions: M.S., U.N., and R.D.-F. designed research; M.S., U.N., E.P., and Q.S. performed research; P.S., C.S., M.C., G.B., A. Califano, and A. Chadburn contributed new reagents/analytic tools; M.S., U.N., K.B., P.S., A. Chadburn, L.P., and R.D.-F. analyzed data; and L.P. and R.D.-F. wrote the paper.

Conflict of interest: The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0903854106/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.0903854106

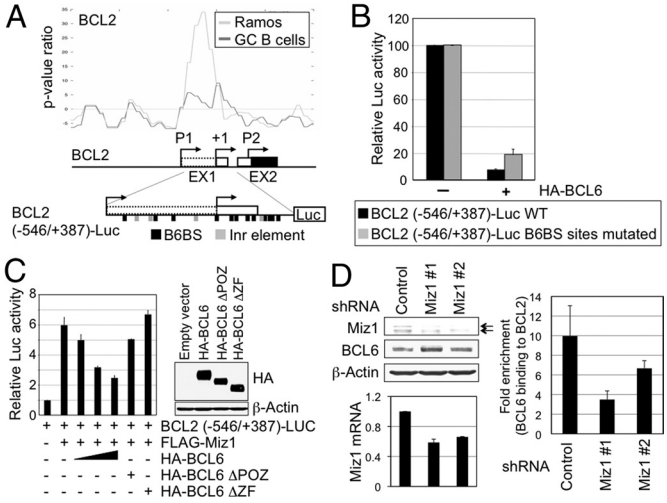

Fig. 1. BCL6 binds and represses BCL2 via Miz1. (*A*) ChIP-chip profile of BCL6 binding to the BCL2 promoter in centroblasts and Ramos cells; *P*-value ratio, logarithm of the ratio between the significance threshold of 0.001 and the *P*-value, identified by ChIP-on-chip significance analysis (CSA) (see *SI Methods*). The BCL2 genomic locus is aligned below. Position +1 is the first nucleotide of NM_000633.2. The bound region (−546/+387) includes 12 canonical BCL6 binding sites (B6BS) and 4 Inr elements, schematically shown in the luciferase reporter construct used in transient transfection assays. (*B*) Activity of the indicated BCL2 promoter-driven luciferase reporter constructs, transfected alone or with expression vectors encoding HA-BCL6 or pMT2T vectors (mean ± SD; $n = 2$). (*C*) Transient transfection assays were performed using the indicated constructs and luciferase activities were measured after 48 h (mean ± SD; $n = 2$). Western blot analysis using HA antibodies documents comparable expression levels of HA-BCL6 and its derivatives. (*D*) Western blot analysis of Miz1, BCL6, and β-actin expression in Ramos cells, transduced with lentiviral vectors expressing control shRNA or 2 different Miz1 shRNAs (*Left*). (*Upper*) Arrows point to specific Miz1 bands and (*Lower*) the corresponding mRNA levels are quantitated. (*Right*) qChIP analysis of the same cells shows significant loss of BCL2 promoter binding after silencing of Miz1 (mean fold enrichment ± SD from triplicates). Data are representative of 2 independent immunoprecipitations.

bars), suggesting that these sites are not the critical mediators of BCL6 transsuppression. We therefore tested whether transcriptional repression of BCL2 by BCL6 is mediated by Miz1, a known activator of BCL2 (38). Fig. 1*C* shows that BCL6 was capable of suppressing Miz1-induced activation of BCL2 in a dose-dependent manner, and this activity requires the presence of both the BCL6 transrepression domain and the ZF domain, which mediates BCL6-Miz1 physical interaction (32), suggesting that transcriptional repression of BCL2 by BCL6 acts through Miz1. Note that mutation of the Inr elements, useful to demonstrate their direct involvement in the BCL6/Miz1 regulation, was not informative because it abrogates BCL2 transcription completely. Notably, shRNA-mediated silencing of Miz1 in B cells impairs the ability of BCL6 to bind the BCL2 promoter (Fig. 1*D*, *Right*), despite the presence of comparable BCL6 protein levels (Fig. 1*D*, *Left*). Taken together, these results suggest that BCL6 binds to the BCL2 promoter in vivo via Miz1.

BCL6 Suppresses BCL2 Transcription in GC B Cells. To demonstrate the physiologic significance of BCL2 suppression by BCL6, we analyzed the relative levels of BCL6 and BCL2 mRNA and protein in mature B cells by gene expression profile analysis of purified naive, GC, and memory B cells and double immunofluorescence analysis of normal lymphoid tissue sections. Consistent with previous data, BCL2 and BCL6 mRNA expression is mutually exclusive in B cells (Fig. 2*A*) (39). Accordingly, GC B cells, which uniformly express BCL6 at high levels, lack BCL2 protein expression, even when analyzed at the single-cell level (Fig. 2*B*). In addition, downregulation of BCL6 by CD40 and BCR activation in these cells leads to loss of BCL6 binding to the BCL2 promoter and upregulation of BCL2 expression (Fig. 2 *C* and *D*). This effect specifically depends on BCL6 because its shRNA-mediated silencing was sufficient to induce BCL2 expression in Ramos B cells (Fig. 2*E*). Overall, these results establish a physiologic and functionally relevant inverse relationship between BCL6 and BCL2 in GC B cells, supporting a direct mechanism for suppression of BCL2 by BCL6.

BCL6-Mediated Suppression of BCL2 Is Lost in Both BCL2 Translocation-Positive and BCL2 Translocation-Negative DLBCL. We then examined whether BCL6-mediated suppression of BCL2 is conserved in DLBCLs by characterizing the expression pattern (mRNA and protein) of these 2 factors in 148 samples representative of BCL2 translocated and nontranslocated cases. Immunohistochemical and Western blot analysis showed that BCL6 and BCL2 are coexpressed in a large fraction of DLBCLs, including 9/20 (45%) cell lines and 61/128 (48%) primary biopsies. Pathologic BCL2/BCL6 coexpression was found in the vast majority of cases carrying BCL2 translocations (7/9 lines and 16/21 biopsies) [supporting information (*SI*) Fig. S1 and Fig. 3], suggesting a role for the juxtaposed Ig enhancers in overriding BCL6-mediated repression (40). However, BCL6/BCL2 coexpression is also observed in 45/107 (42%) DLBCL cases lacking BCL2 translocations (Fig. 3*B*). These findings suggest that BCL6-mediated suppression of BCL2 is frequently disrupted in DLBCL, but this event can be explained by the juxtaposition of Ig regulatory elements only in a fraction of cases.

The Promoter Region of BCL2 Is Subjected to Aberrant SHM in DLBCL. To characterize the BCL2 locus in cases apparently resistant to BCL6-mediated suppression, we proceeded to a detailed structural analysis of the BCL2 genomic sequences by DNA amplification and direct sequencing of ≈3 kb spanning the BCL6-bound region and its coding exon 2. This analysis revealed that, with rare exceptions, including 1 sample in which BCL2 was not expressed and 1 sample carrying a non-IgH translocation, all translocated cases display a massive load of mutations, whose frequency (0.77%) exceeds by far the one (0.11%) previously reported for the BCL2 coding region in FL and transformed DLBCL (Fig. 4 and Fig. S2*A*) (41, 42). Mutations include mostly single base pair substitutions, but also deletions, duplications, and insertions, and are not detected in matched normal DNA from the same individuals, thus constituting somatic events clonally represented in the tumor (see Table S1 for a detailed list). The characteristics of the mutations, and in particular their distribution downstream of the promoter, the positive transition/transversion ratio, and the preferential hotspot (RGYW) targeting, strongly suggest that they are caused by the SHM mechanism (43) (Table S2). In 3 informative cases, RT-PCR amplification and sequencing using specific primers documented that the mutations were restricted to the translocated allele, while the nontranslocated allele was unmutated and not expressed (not shown), suggesting a role for the juxtaposed Ig enhancer in recruiting the SHM machinery to these alleles (40). Interestingly, a significant, although smaller number of mutations with analogous features were also detectable in cases lacking BCL2 translocations ($n = 19/94$; mutation frequency, 0.027%) (Fig. 4 and Fig. S2*A*). Because no variants were observed in normal GC B cells (consistent with their lack of BCL2 transcription) (Fig. S2*B*), these findings indicate that BCL2 mutations in nontranslocated cases represent a tumor-associated phenomenon, presumably due to the aberrant activity of the SHM mechanism, present in most DLBCL (44). Of note, in 20/121 (14.2%) samples sequenced, mutations extend to the BCL2 coding domain, leading to amino acid substitutions with potential functional consequences. Thus, one-third (41/121) of DLBCLs, including both BCL2 translocated and nontranslocated samples, display somatic mutations of the BCL2 regulatory region.

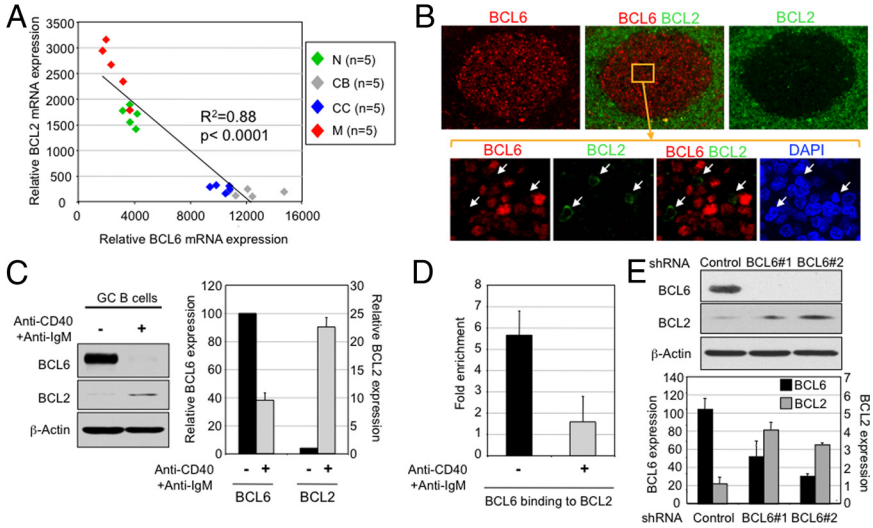

Fig. 2. Mutually exclusive BCL6 and BCL2 expression in normal GC B cells. (*A*) BCL2 and BCL6 mRNA expression levels in mature B cells subsets, as measured by gene expression profile analysis (N, naive; CB, centroblasts; CC, centrocytes; and M, memory B cells) (linear regression analysis). (*B*) Double immunofluorescence staining for BCL2 (green) and BCL6 (red) in a GC from a reactive tonsil. (*Lower*) Higher magnification of the selected area. DAPI (blue) was used for the detection of nuclei. (*C*) Western blot (*Left*) and qRT-PCR (*Right*) analysis of BCL2 and BCL6 expression in GC centroblasts treated with anti-CD40 and anti-IgM antibodies (mean ± SD of triplicates; data are representative of 2 independent experiments). (*D*) BCL6 binding to the BCL2 promoter, measured in the same cells by qChIP using anti-BCL6 antibodies (mean ± SD of triplicates). (*E*) (*Upper*) Western blot analysis of BCL6, BCL2, and β-actin expression in Ramos cells, transduced with lentiviral vectors expressing control shRNA or 2 different BCL6 shRNAs. (*Lower*) qRT-PCR analysis of BCL6 and BCL2 expression in the same cells (mean ± SD of triplicates from 2 independent experiments).

Suppression of BCL2 by BCL6 Can Be Impaired by Deregulation of Miz1 Expression. To dissect the mechanism leading to BCL6 and BCL2 coexpression in DLBCL, we first analyzed whether BCL6 can still bind to the BCL2 promoter in these cases. Quantitative ChIP (qChIP) assays performed in 5 BCL6+BCL2+ DLBCL cell lines revealed the absence of BCL6 binding in 3 samples, as opposed to 2 control cell lines carrying an intact BCL2 locus and lacking BCL2 expression (Fig. 5*A*, Fig. S1). Interestingly, no Miz1 protein expression was observed in these 3 cases (Fig. 5*A*, *Right*), indicating that the inability of BCL6 to bind the BCL2 promoter was conceivably because of the absence of its interacting partner. Cases expressing significantly lower amounts of Miz1 mRNA were found also among BCL6+BCL2+ DLBCL primary biopsies ($n = 5/61$) (Fig. 5*B*), thereby identifying a subset of DLBCLs in which loss of BCL2 repression by BCL6 may be caused by the lack of Miz1 expression, preventing BCL6 from reaching the BCL2 promoter.

Analysis of Miz1 mRNA expression in primary DLBCLs also revealed the existence of cases where pathologic BCL2 and BCL6 coexpression is associated with significantly higher levels of Miz1 mRNA (Fig. 5*B*) ($P < 0.001$ as compared to cells displaying a normal inverse correlation between BCL6 and BCL2 expression). This subset was largely restricted to translocation-negative, BCL2 unmutated cases ($n = 14/32$, 43.8%, corresponding to ≈23% of all BCL2+BCL6+ DLBCLs), suggesting that exceedingly high levels of Miz1 may stimulate BCL2 expression via out-titrating BCL6. Thus, loss of BCL2 suppression by BCL6 may be due to both Miz1 downregulation and overexpression, the cause of which is presently unknown.

BCL6-Mediated Suppression of BCL2 Can Be Impaired by BCL2 Mutations. To directly assess whether BCL2 mutations affect BCL6/Miz1-mediated suppression of BCL2, we analyzed the response of DLBCL-derived mutant alleles in transient cotransfection/reporter gene assays. Alleles were selected among BCL2/BCL6 double-positive cases showing normal Miz1 expression and preserved BCL2 binding. Although the relative ability of BCL6 to repress Miz1-induced activation was comparable in all 6 alleles tested, 3 of them (2062, 2106, and SUDHL6) displayed significantly enhanced responses to Miz1-mediated transcriptional activation (2- to 3-fold), resulting in the overall increased expression of the reporter, as further evidenced by using increasing doses of BCL6 (Fig. 5*C*). Because the only variable in the experiment is the presence of mutations in the tested alleles, these results indicate that a subset of BCL2 mutations leads to increased response to Miz1. Given the complexity of the mutation pattern in these alleles, some of which

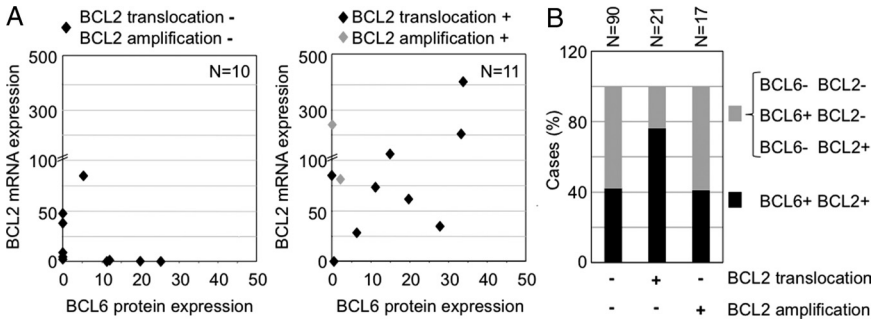

Fig. 3. Pathologic coexpression of BCL6 and BCL2 in DLBCL. (*A*) Relative expression levels of BCL2 mRNA (qPCR) and BCL6 protein (WB analysis) in DLBCL cell lines with or without t(14;18) and BCL2 amplification. (*B*) Distribution of cases displaying pathologic BCL2/BCL6 coexpression in primary DLBCL biopsies.

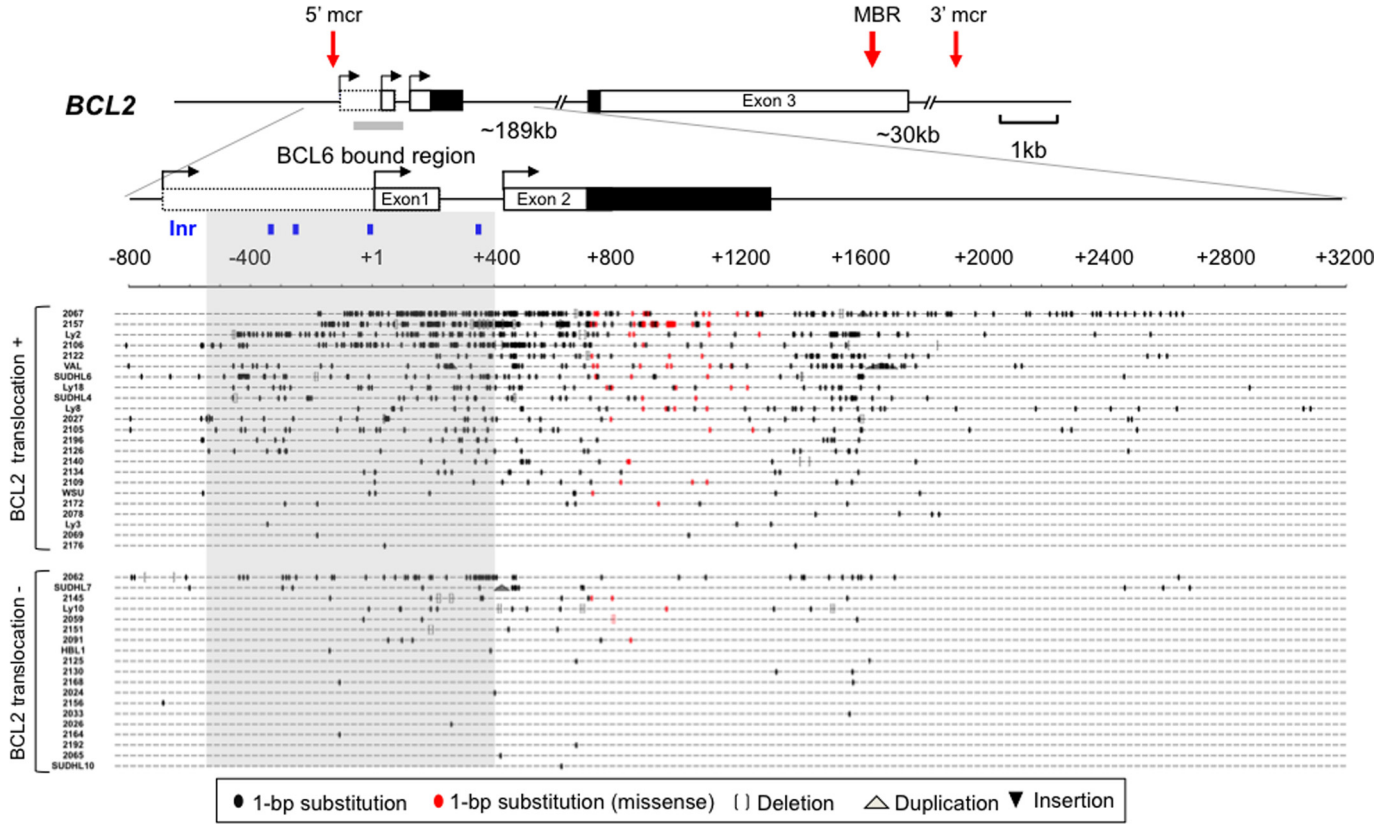

Fig. 4. Mutational analysis of the BCL2 locus. (*Upper*) Diagram of the BCL2 locus. Open and solid boxes: untranslated and translated sequences. Horizontal arrows indicate transcription initiation sites; vertical red arrows point to the 3 known translocation breakpoint clusters. (*Lower*) The region subjected to mutation analysis is expanded and aligned to individual DLBCL cases (1 line = 2 alleles), where each small segment represents a 25-bp interval and symbols depict distinct mutation events. The BCL6 bound region is highlighted in gray, and the 4 Inr elements are shown in blue below the map.

carry >50 mutational events, it is not possible at this stage to assess the contribution of each mutation to the observed abnormal response. However, this result indicates that mutations leading to an increased response to Miz1 transactivation can contribute to BCL2 deregulated expression in a subset of DLBCLs (Fig. S3).

Discussion

Mechanism of BCL2 Downregulation by BCL6. Previous reports have indicated that BCL6 can suppress target gene expression by directly binding to specific DNA sequences (1, 28) or by interacting with Miz1 (32) or AP1 factors (45), which in turn bind DNA directly. The results herein indicate that, in normal GC B cells, BCL6 occupies the BCL2 promoter and suppresses BCL2 transcription, and this function requires the presence of the Miz1 transcriptional activator. One important implication of this finding is that Miz1 levels can influence the ability of BCL6 to suppress BCL2, with low Miz1 levels limiting BCL6 function by preventing access to target promoters, and excessive Miz1 levels leading to the same net effect by out-titrating BCL6. Thus, the mechanism by which BCL6 suppresses BCL2 is analogous to the BCL6-mediated suppression of *CDKN1A* (32).

Biological Significance of BCL2 Repression by BCL6. The ability of BCL6 to suppress the anti-apoptotic function of BCL2 is consistent with the complex biology of the GC, where cells must be ready to undergo apoptosis if not rescued based on their affinity for the antigen. Consistent with this scenario, the transcriptional profile of GC centroblasts is characterized by downregulation of multiple anti-apoptotic genes and expression of pro-apoptotic genes (39, 46). While other genes with anti-apoptotic function may also be targeted by BCL6 directly or indirectly, its specific activity on BCL2 represents a critical function in ensuring that GC cells die if not rescued by the antigen and by other cellular signals. At the end of the GC reaction, engagement of the BCR by the antigen, signals from T cells (e.g., CD40 activation), and the accumulation of genotoxic stress lead to BCL6 downregulation and consequent release of BCL2 expression, which is typical for memory and plasma cells (46).

Heterogeneous Mechanisms Lead to BCL2 Ectopic Expression in DLBCL. Previous reports have indicated that the translocation t(14;18) leads to ectopic activation of BCL2 in most FL and up to one-third of DLBCL cases (34) and implied this event as the main mechanism leading to BCL2 expression in these lymphoma types (37, 40). The results herein modify these notions in several ways. First, a fraction of DLBCLs was identified in which BCL2 is coexpressed with BCL6 in the absence of BCL2 gross structural alterations, suggesting that additional mechanisms exist for BCL2 induction in DLBCL. Second, these results show that chromosomal translocations and amplifications are not the only genetic lesions affecting the BCL2 locus in DLBCL, because a significant load of mutations was found in both translocation-positive and -negative DLBCL cases. While the former likely derive from the spreading of SHM from adjacent Ig enhancers, the mechanism causing mutations in non-translocated alleles is unknown and may be part of the aberrant SHM activity that targets multiple genes in DLBCL (44).

Although the complexity and case-to-case variability of the mutational pattern prevent a simple interpretation or analysis of their role in each case, our results suggest at least 3 mechanisms by which BCL2 may escape BCL6-mediated suppression in DLBCL, beside or together with BCL2 chromosomal translocations. In a minority of cases, insufficient Miz1

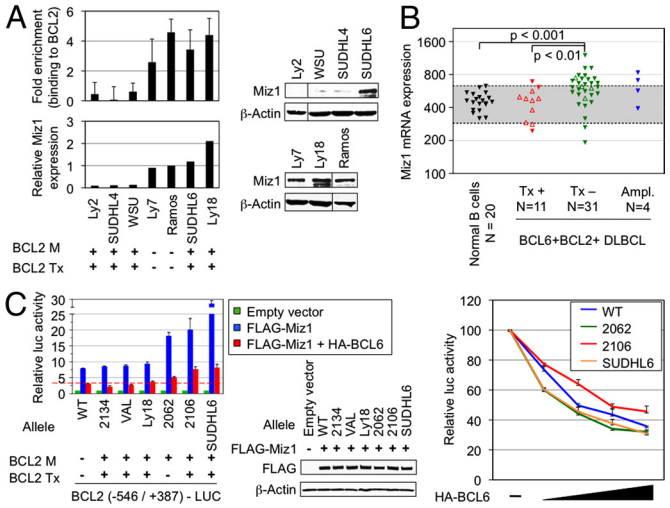

Fig. 5. Multiple mechanisms activate BCL2 expression in DLBCL. (*A*) qChIP analysis of BCL6 binding to the BCL2 promoter in 5 BCL2+BCL6+ DLBCL cell lines. (*Upper*) Ramos and Ly7, which do not express BCL2, served as controls (mean ± SD of triplicate PCR reactions from 2 independent ChIP experiments). Enrichment of ≥2 was considered as evidence for binding. (*Right*) Miz1 protein levels, measured in the same cell lines by Western blotting, were quantitated by densitometric analysis using ImageQuant 5.2 after β-actin normalization, with Ramos set as 1 (*Lower*). Tx, translocated; M, mutated. (*B*) Miz1 mRNA levels in normal B cells and DLBCL biopsies. Gray area: mean expression level of Miz1 in normal B cells, ±2 SD as assessed by the sigma approach. Solid and open triangles, BCL2 unmutated and mutated cases, respectively. (*C*) (*Left*) Luciferase activity of 6 DLBCL-derived mutant alleles in response to Miz1-induced activation and BCL6-mediated repression, vs. wild type (mean ± SD; $n = 2$). (*Middle*) Western blot analysis of Miz1 and β-actin on the same extracts controls for equal Miz1 amounts. (*Right*) The 3 mutant alleles with enhanced Miz1-induced activation were cotransfected with equal amounts of FLAG-Miz1 and increasing doses of HA-BCL6; Miz1-mediated activation of each reporter is set to 100%.

levels may prevent BCL6 from reaching the BCL2 promoter and repressing its transcription (7/18 translocation-positive and 2/32 translocation-negative cases) (Fig. 5 *A* and *B* and Fig. S3). This mechanism may complement the deregulating activity of the Ig enhancers in cases with structurally altered BCL2 genes. Alternatively, abnormally high levels of Miz1, as observed in over one third of translocation-negative BCL2+BCL6+ DLBCLs, may out-titrate the ability of BCL6 to suppress BCL2 by generating BCL6-free Miz1 molecules. This scenario seems largely restricted to BCL2 unmutated cases, suggesting that abnormally high levels of Miz1, unchecked by BCL6, may substitute for Ig enhancer elements in transactivating BCL2. Finally, a subset of mutations occurring in both translocation-positive and -negative DLBCLs may cause abnormally high Miz1 responses. Together, these 3 mechanisms may account for at least 50% of cases displaying pathologic coexpression of BCL6 and BCL2, independent of chromosomal translocations (Fig. S3). It is important to note that not all mutations may have functional significance, because the SHM mechanism displays a semirandom distribution, in which functionally relevant and irrelevant sequence changes may be coselected during tumorigenesis. Mutations were also found within the BCL2 coding domain, as previously reported in t(14;18)+ FL and transformed DLBCL cases. These mutations did not affect the ability of BCL2 to synergize with BCL6 and induce cell transformation in soft agar/colony formation assays (Fig. S4). Overall, these results underscore the complexity of the mechanisms responsible for BCL2 activation in DLBCL and the need for further studies, including testing on the role of mutations and the mechanisms leading to Miz1 under- and overexpression.

Implications for Lymphomagenesis. The pathologic coexpression of BCL6 and BCL2 in DLBCL was previously reported and may identify patients with unfavorable prognosis, although this issue is controversial (47). Abnormal BCL2 expression may allow GC B cells to survive in the presence of death signals (e.g., Fas) and in the absence of survival signals (e.g., BCR, CD40), thus contributing to lymphomagenesis by increasing the pool of cells that can be targeted by additional genetic alterations in the GC environment. Preliminary evidence for the pathologic effect of BCL6 and BCL2 expression was obtained in Rat1 cells, where cotransfection of both genes led to a synergistic transformation effect (Fig. S4). This result will have to be corroborated by directing the coexpression of BCL6 and BCL2 to GC B cells in transgenic mice. The results herein suggest that combination modalities targeting the multiple oncogenic activities of BCL6 and the anti-apoptotic function of BCL2 may represent a rational approach for the treatment of a subset of DLBCLs (48, 49).

Materials and Methods

Full details of the methods used are presented in *SI Text*.

Transient Transfection/Reporter Assays. Transient transfection/reporter assays were performed on 293T cells as described, using the indicated constructs (1, 12). All experiments were performed in duplicate, and luciferase activities were measured 48 h posttransfection using the dual-luciferase reporter assay kit (Promega) according to the manufacturer's protocol.

BCL2 Mutation Analysis. Genomic DNA was extracted by standard methods, and 6 overlapping PCR products were used to amplify the BCL2 genomic sequences spanning position ≈−800 to ≈+2700 from the first exon of the BCL2 transcript variant alpha (NM_000633.2) (see Table S3 for PCR primers and conditions). Purified amplicons were sequenced directly from both strands (Genewiz) and analyzed as described (44). The somatic origin of the mutations was confirmed by analysis of matched normal DNA where available. Mutation analysis of BCL2 in normal B-cell subpopulations (naive and CB) and in the IMR91 fibroblast cell line was done as reported (44).

ACKNOWLEDGMENTS. We thank S. V. Nandula and V. V. Murty for the BCL2 FISH and M. Brahmachary for help in statistical analysis. U.N. was supported by grants from Oncosuisse, the Swiss National Science Foundation, the Bernese Scholarship for Cancer Research, and a grant from Zuppinger-Stiftung (Bern, Switzerland). C.S. was supported by the Mildred Scheel Stiftung für Krebsforschung. This work was supported by grants from the National Institutes of Health (PO1 CA9265-07, to R.D.-F.) and the Leukemia and Lymphoma Society.

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