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<th>Germinal center dysregulation by histone methyltransferase EZH2 promotes lymphomagenesis( Main article )</th>
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Germinal center dysregulation by histone methyltransferase EZH2 promotes lymphomagenesis


Protection against deadly pathogens requires the production of high-affinity antibodies by B cells, which are generated in germinal centers (GCs). Alteration of the GC developmental program is common in many B cell malignancies. Identification of regulators of the GC response is crucial to develop targeted therapies for GC B cell dysfunctions, including lymphomas. The histone H3 lysine 27 methyltransferase enhancer of zeste homolog 2 (EZH2) is highly expressed in GC B cells and is often constitutively activated in GC-derived non-Hodgkin lymphomas (NHLs). The function of EZH2 in GC B cells remains largely unknown. Herein, we show that \( \text{Ezh2} \) inactivation in mouse GC B cells caused profound impairment of GC responses, memory B cell formation, and humoral immunity. EZH2 protected GC B cells against activation-induced cytidine deaminase (AID) mutagenesis, facilitated cell cycle progression, and silenced plasma cell determinant and tumor suppressor B-lymphocyte–induced maturation protein 1 (BLIMP1). EZH2 inhibition in NHL cells induced BLIMP1, which impaired tumor growth. In conclusion, EZH2 sustains AID function and prevents terminal differentiation of GC B cells, which allows antibody diversification and affinity maturation. Dysregulation of the GC reaction by constitutively active EZH2 facilitates lymphomagenesis and identifies EZH2 as a possible therapeutic target in NHL and other GC-derived B cell diseases.

Introduction

Protective immunity against pathogens relies on the production of high-affinity antibodies by long-lived plasma cells (PCs). Moreover, the ability to respond faster and with more potent antibodies to subsequent encounters with the same infectious agent depends on the generation of long-lived memory B cells. Both, high-affinity memory B cells and PCs differentiate from antigen-specific B cells that are recruited into the GC reaction during T cell–dependent immune responses (1). In GCs, B cells undergo clonal expansion, a process during which they accumulate mutations at high frequency within the Ig heavy and light chain variable (V) region genes. The highly dynamic nature of the GC reaction is characterized by repeated cycles of cell division, Ig somatic mutation, and strict selection based on the ability of B cells to capture and present antigen to T follicular helper cells (2). These processes occur within distinct areas of the GC reached by B cells through migratory paths regulated by chemokine gradients (1). The molecular determinants enabling cyclic reentry of B cells into the proliferating and mutating compartment of centroblasts, preventing terminal differentiation and the ensuing exit from the GC, remain poorly characterized.

Polycomb group (PcG) proteins act within 2 main polycomb repressive complexes (PRC1 and PRC2) to promote gene silencing. PRC1 and PRC2 catalyze posttranslational modifications of specific lysine residues in core histone tails, resulting in chromatin compaction (3). Changes in chromatin conformation regulated by PcG activity represent important molecular switches that control cell differentiation, proliferation, and survival in prenatal and postnatal life (4). Enhancer of zeste homolog 2 (EZH2) is the main catalytic subunit of PRC2. Through its SET domain, EZH2 catalyzes histone H3 lysine 27 trimethylation (H3K27me3), which is enriched at transcription start sites (TSSs) of repressed genes (5). Together with H3K4me3, H3K27me3 is found at promoters of regulators of lineage specification, where it acts to fine-tune their expression (6).

EZH2 is expressed at high levels in human GC B cells (7, 8). Moreover, whole-exome sequencing efforts have revealed that EZH2 gain-of-function mutations are among the most common genetic alterations identified in diffuse large B cell lymphoma (DLBCL) and follicular lymphoma originating from GC B cells (9, 10). Together, these results point to a critical role of EZH2 in GC B cell function and in the pathogenesis of GC-derived non-Hodgkin lymphoma (NHL).

Using GC B cell–specific gene targeting in mice, we show that EZH2 methyltransferase activity is required to protect GC B cells against genotoxic damage induced by activation-induced cytidine deaminase (AID). Moreover, we found that EZH2 is necessary to...
repress B-lymphocyte-induced maturation protein 1 (Blimp1) and Irf4 expression in GC B cells to limit terminal B cell differentiation induced by IL-21. Through these mechanisms, EZH2 ensures the persistence of B cells in the GC reaction, thus allowing the generation of high-affinity antibodies and memory B cells. We also found that constitutively active EZH2 is critical to stably repress tumor suppressor BLIMP1 expression in GC-type DLBCL cells, thereby possibly contributing to lymphomagenesis.

Results

Ezh2 is upregulated in mouse GC B cells. To investigate the expression of Ezh2 in mouse mature B cell subsets, we performed quantitative RT-PCR (qRT-PCR) analysis. Low Ezh2 mRNA levels were detected in follicular, marginal zone, and B-1 mature B cell subsets. A substantial increase in both Ezh2 transcript (Figure 1A) and protein (Figure 1B) levels was detected in B cells upon recruitment into the GC reaction during a T cell–dependent immune response. Within the GC, EZH2 was expressed predominantly in proliferating CXCR4+ centroblasts (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI70626DS1). The differentiation of GC B cells into long-lived IgG1+ memory B cells or PCs coincided with lower, yet detectable, Ezh2 transcripts (Figure 1A). These results pointed to a role of EZH2 in GC B cell physiology.

Reduced number of GC B cells upon inducible Ezh2 inactivation. To study the specific role of EZH2 in GC B cells, conditional Ezh2

![Figure 1](image-url)
knockout (Ezh2−/−) mice carrying loxP sites flanking exons coding for the catalytic SET domain (11) were crossed with Cty1-cre knockout animals expressing Cre recombinase upon onset of Ig class switch recombination (CSR) (12). Ezh2−/− mice were also crossed with Cr2-cre transgenic mice to inactivate Ezh2 in all peripheral mature B cells (13). Ezh2fl/fl;Cty1-cre conditional mutants and Ezh2fl/+:Cty1-cre controls (hereafter referred to as controls) were immunized with alum-precipitated 4-hydroxy-3-nitrophenyl acetyl (NP) conjugated to chicken y globulin (CGG) to induce GC responses. Flow cytometry analysis revealed an average 3-fold reduction in percentages and absolute numbers of splenic CD19+CD38+CD95+ GC B cells in Ezh2fl/fl;Cty1-cre animals, 12 to 14 days after immunization, at the peak of the response (Figure 1, C and D, and Supplemental Figure 1C). Histological analysis of spleen sections of immunized mice revealed a significant reduction in peanut agglutinin (PNA)–positive GCs in immunized mice revealed a significant reduction in peanut agglutinin (PNA)–positive GCs in immunized mice revealed a 3-fold reduction in percentages and absolute numbers of splenic CD19+CD38+CD95+ GC B cells in Ezh2fl/fl;Cty1-cre animals, 12 to 14 days after immunization, at the peak of the response (Figure 1C). The counter selection of Ezh2−/− mutants (Supplemental Figure 1D) that showed normal distribution and number of peripheral B cell subsets (Supplemental Figure 1E and F).

To reveal the identity of the remaining GC B cells in Ezh2fl/fl;Cty1-cre animals, we determined the status of the Ezh2−/− by quantitative PCR (qPCR) analysis. Whereas Cre-mediated recombination occurred in the majority of GC B cells in control animals, GC B cells of Ezh2fl/fl;Cty1-cre mutants retained substantial levels of the unrecombined Ezh2−/− allele (Figure 1F), giving rise to full-length Ezh2 transcripts (Supplemental Figure 1G). The counter selection of Ezh2−/− mutant GC B cells was confirmed in Ezh2fl/fl;Cr2-cre mice (Supplemental Figure 1H) and in animals generated from crosses of Ezh2fl/fl;Cty1-cre mice to the R26-yfp Cre reporter strain. Flow cytometry analysis of splenic B cells isolated from immunized mice revealed a 4- to 6-fold reduction in the frequency of rearrangements carrying codon 33 TTG (W33L) as compared to controls (Table 1).

**Table 1**

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<th>Genotype</th>
<th>Mutations range</th>
<th>Mutation frequency</th>
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<tr>
<td>Ezh2+/−</td>
<td>1 to 14</td>
<td>2.05% (54/2,637)</td>
<td>77.8</td>
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<tr>
<td>Ezh2−/−</td>
<td>1 to 8</td>
<td>1.06% (28/2,637)</td>
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Sequence analysis of IgH rearrangements carrying V_{H}186.2 amplified by PCR from genomic DNA of Ezh2 control (Ezh2+/+:Cty1-cre/R26-yfp [Ezh2+/−]) and mutant (Ezh2fl/fl;Cty1-cre/R26-yfp [Ezh2−/−]) GC B cells sorted on the basis of YFP expression. Mutation frequency was calculated by dividing the number of point mutations by the number of sequenced nucleotides. Only PCR products containing 1 or more mutations were analyzed. Nine unique V_{H}186.2 sequences were analyzed for each genotype. Identical nucleotide substitutions present in different sequences were counted once if V_{H}186.2 rearrangements shared the same CDR3 region. The frequency of rearrangements carrying codon 33 TGG→TTG (W33L) was counted by dividing the number of unique V_{H}186.2 sequences bearing TTG substitutions by the total number of unique mutated V_{H}186.2 rearrangements.
For this purpose, mount an antigen-specific T cell–dependent antibody response. We next investigated the effects of GC specific inactivation of Ezh2 on the persistence of B cells within the GC reaction. These studies reveal that EZH2 is strictly required for the formation of IgG1 memory B cells (Table 1). Moreover, the frequency of replacement mutations, causing a cryptophan-to-leucine substitution at codon 33 that confers a 10-fold higher affinity for NP to the BCR, was reduced in mutant GC mice, as measured by flow cytometry after NP-CGG immunization. Values are relative to Riplpo expression. *P = 0.033 (t test). (D) Frequency of splenic GC B cells in Ezh2 control (Ezh2fl/fl:C1-cre) and mutant (Ezh2fl/fl:C1-cre; n = 12) mice after NP-CGG immunization. (E) Proportion of CaspGLOW–splenic GC B cells revealed by FACS analysis in Cdkn2a-deficient Ezh2 control (Ezh2fl/fl:C1-cre; n = 7) and mutant (Ezh2fl/fl:C1-cre; n = 5) mice after NP-CGG immunization. (F) Efficiency of Ezh2 inactivation in purified splenic GC B cells of NP-CGG–immunized Cdkn2a-deficient Ezh2 control (Ezh2fl/fl:C1-cre; n = 2) and mutant (Ezh2fl/fl:C1-cre; n = 2) mice. Data are representative of (A, B, and F) 2, (D and E) 4, and (C) 10 experiments, respectively. (C) Columns indicate mean ± SEM. (D and E) Columns indicate mean ± SD.

Ezh2 inactivation increases apoptosis of GC B cells. (A) Representative FACS analysis of CaspGLOW staining of GC B cells in the spleens of Ezh2 control (Ezh2fl/fl:C1-cre [Ezh2+/+]) and mutant (Ezh2fl/fl:C1-cre [Ezh2+-]) mice after NP-CGG immunization. Numbers indicate percentage of CaspGLOW– cells. (B) Mean frequency ± SD of CaspGLOW– GC B cells in Ezh2 control (Ezh2fl/fl:C1-cre; n = 6) and mutant (Ezh2fl/fl:C1-cre; n = 4) mice immunized with NP-CGG. **P = 0.002 (t test). (C) qRT-PCR analysis of Cdkn2a transcripts in purified GC B cells of Ezh2 control (Ezh2fl/fl:C1-cre; n = 12) and mutant (Ezh2fl/fl:C1-cre; n = 12) mice after NP-CGG immunization. Values are relative to Riplpo expression. *P = 0.018; **P = 0.0017 (t test). (E) Proportion of CaspGLOW–splenic GC B cells revealed by FACS analysis in Cdkn2a-deficient Ezh2 control (Ezh2fl/fl:C1-cre) and mutant (Ezh2fl/fl:C1-cre; n = 5) mice after NP-CGG immunization. (F) Efficiency of Ezh2 inactivation in purified splenic GC B cells of NP-CGG–immunized Cdkn2a-deficient Ezh2 control (Ezh2fl/fl:C1-cre; n = 2) and mutant (Ezh2fl/fl:C1-cre; n = 2) mice. Data are representative of (A, B, and F) 2, (D and E) 4, and (C) 10 experiments, respectively. (C) Columns indicate mean ± SEM. (D and E) Columns indicate mean ± SD.

Poor antibody responses and fewer memory B cells upon GC B cell–specific inactivation of Ezh2. We next investigated the effects of GC B cell–specific inactivation of Ezh2 on the ability of animals to mount an antigen-specific T cell–dependent antibody response. For this purpose, Ezh2fl/fl:C1-cre mutants and controls were immunized with NP-CGG. On average, NP-specific IgG1 titers were over 10-fold reduced in Ezh2 mutant mice throughout the primary immune response (Figure 2A). The percentage of high-affinity NP-specific IgG1 memory B cells was also significantly reduced in the spleens of Ezh2fl/fl:C1-cre animals 2 months after primary immunization (Figure 2B and Supplemental Figure 2A). The reduction was already detected at the peak of the primary GC response, indicating that EZH2 is necessary for the formation of IgG1 memory B cells (Supplemental Figure 2B).

To study the role of EZH2 in antibody affinity maturation, GC B cells from the spleens of NP-CGG–immunized Ezh2fl/fl:C1-cre:R26-yfp mice were sorted on the basis of YFP expression to enrich for Ezh2 mutants. Mutation analysis of the V_{H}186.2 gene expressed by NP-specific B cells revealed a substantial reduction in the overall mutation frequency in Ezh2 mutant GC B cells (Table 1). Moreover, the frequency of replacement mutations, causing a cryptophan-to-leucine substitution at codon 33 that confers a 10-fold higher affinity for NP to the BCR, was reduced in mutant GC B cells (Table 1). In accordance with these results, Ezh2fl/fl:C1-cre mice showed a reduction in high-affinity NP-specific IgG1 serum titers in primary and recall immune responses (Figure 2C). To assess whether the reduction in IgG1 titers was due to a intrinsic defect in Ig CSR, primary B cells from Ezh2fl/fl:Cr2-cre mice were stimulated in vitro with LPS and IL-4. Flow cytometry analysis revealed a sizeable fraction of IgG1+ B cells in Ezh2 mutant cultures (Figure 2D), thus excluding major defects in Ig CSR. The inactivation of Ezh2 in B cells was confirmed by expression of a truncated, catalytic-inert (EZH2^{33LE}) form of the protein and by a reduction in global H3K27me3 levels (Supplemental Figure 2C). Increased apoptosis of Ezh2 mutant GC B cells is independent of p19^{ARF} and resistant to BCL2 overexpression. To understand the mechanisms responsible for the loss of Ezh2 mutant GC B cells, Ezh2fl/fl:C1-cre and control mice were immunized with NP-CGG and GC B cells were analyzed by flow cytometry for the expression of active caspases as a readout of apoptosis. Inactivation of Ezh2 led to a 3-fold increase in the percentage of CaspGLOW– apoptotic GC B cells (Figure 3, A and B). Similar results were observed analyzing GC responses in Ezh2fl/fl:Cr2-cre mice (Supplemental Figure 3A).
In agreement with the established role of EZH2 in the repression of the Cdkn2a tumor suppressor locus (14), Cdkn2a transcripts increased over 9 fold in Ezh2 mutant GC B cells (Figure 3C). To test whether the proapoptotic factor p19ARF, encoded by Cdkn2a, was responsible for the death of Ezh2 mutant GC B cells, we analyzed Ezh2/Cdkn2a double-mutant mice (Ezh2fl/+:Cdkn2a–/–:Cay1-cre mice). Flow cytometry analysis of NP-CGG–immunized mutants and controls (Ezh2fl/+:Cdkn2a–/–:Cay1-cre mice) revealed a significant reduction in the percentage of double-mutant GC B cells compared with that in controls (Figure 3D), which was associated with an increased percentage of apoptotic cells (Figure 3E). Moreover, genomic qPCR analysis indicated the persisting counter selection of GC B cells carrying Ezh2 recombined alleles in compound mutants (Figure 3F). Next, we attempted to rescue apoptosis of Ezh2 mutant GC B cells through enforced expression of the antiapoptotic protein BCL2, using the E+ BCL2 transgene (15). Flow cytometry analysis and quantitative assessment of the Ezh2 allele revealed lower frequency of GC B cells, increased percentage of apoptotic cells, and counter selection of Ezh2 mutant GC B cells in compound mutants (Supplemental Figure 3, B–D). In conclusion, EZH2 sustains GC B cell survival independently of its repression of p19ARF and without limiting mitochondrial apoptosis.

EZH2 protects GC B cells against AID-dependent genotoxic damage. Both in vitro and in vivo studies have provided evidence that PcG proteins, including EZH2, can participate in DNA damage responses (16–19). We thus investigated whether EZH2 protected
B cells from genotoxic damage through H3K27 trimethylation. First, we measured the response of Ezh2 mutant B cells to acute DNA damage induced by ionizing radiation (IR). Primary resting B cells purified from mutant (Ezh2fl/fl:Cr2-cre) and control (Ezh2fl/+:Cr2-cre) mice were stimulated in vitro with LPS and, shortly after, were exposed to a single dose of 0.1 and 1 Gy of IR, respectively. The fraction of apoptotic B cells was determined 24 hours after irradiation by TUNEL staining. Whereas the majority of B cells succumbed to 1 Gy of IR (irrespective of Ezh2 genotype), the treatment with 0.1 Gy caused minor toxicity to Ezh2 proficient B cells. In contrast, the cultures of Ezh2 mutant B cells exposed to the same sublethal IR dose consisted of mostly TUNEL + cells (Figure 4A). In accordance with this result, we found a significantly higher number of DNA damage response foci marked by 53BP1 in Ezh2 mutant B cells after exposure to IR (Figure 4B). Since AID is a major determinant of genotoxic stress in GC B cells (20), we tested whether EZH2 inhibition increased the sensitivity of B cells to AID toxicity. Initially, we compared the behavior of Ezh2 mutant B cells after in vitro stimulation with either LPS/IL-4 or an antibody activating the Toll-like receptor homolog RP-105, as they differ in the ability to induce AID (Supplemental Figure 4A). Ezh2 mutant B cells responded to both mitogenic stimuli (Supplemental Figure 4B). However, only LPS/IL-4 stimulation, which induced robust AID expression, caused a significant increase in apoptosis of Ezh2 mutant B cells and lower numbers of viable cells after 4 days of in vitro culture (Figure 4C and Supplemental Figure 4C). This effect was particularly evident among IgG1 class-switched B cells (Supplemental Figure 4D). To directly assess the contribution of AID to the apoptosis of Ezh2 mutant GC B cells, Ezh2fl/fl:Cr2-cre mice were crossed with AID-deficient animals. Strikingly, the size of GCs and the absolute number and the percentage of GC B cells were largely rescued in Ezh2/Aicda double mutants (Figure 4, C–G). To determine whether the methylation status of H3K27me3 and H3K4me3 might be altered in primary GC B cells during AID-mediated DNA damage, we performed qRT-PCR analyses. Interestingly, among 67 candidate genes tested (Supplemental Table 2), Ezh2 mutant primary GC B cells showed a significant increase in expression of 21 genes (Figure 5D). In contrast, none of these genes were upregulated in Ezh2 deficient GC B cells. These findings provide direct evidence that EZH2 inhibition increased the sensitivity of B cells to AID toxicity.
EZH2 regulates GC B cell transcriptional program. Despite the consistent normalization of apoptosis, Ezh2fl/+;Aicda–/–;Cr2-cre compound mutants showed still a modest, yet reproducible, reduction in GC B cell numbers and, in few cases, Ezh2/Aicda double-mutant GC B cells remained counter selected (Supplemental Figure 4G). These results suggested additional function(s) of Ezh2 in GC B cells, genotoxicity linked to AID mutagenesis.

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To gain insights into the biological function of H3K27me3 targets in GC B cells, we performed a Gene Ontology analysis. Genes marked by H3K27me3 were significantly (P = 0.001) enriched for categories involved in developmental processes (Supplemental Figure 5A), whereas H3K4me3 preferentially marked genes involved in metabolism (Supplemental Figure 5B). To understand the influence of H3K27me3 on target gene expression, we compared the average transcript levels of genes marked in GC B cells, by H3K27me3, H3K4me3, or both, respectively. In accordance with previous observations (22), we found that genes marked by H3K27me3 were significantly (P < 2.2 × 10−16) less transcribed than those marked by H3K4me3 (Figure 5B). Transcripts encoded by bivalent genes showed intermediate levels, which were significantly lower (P < 2.2 × 10−16) than those derived from H3K4me3-marked genes (Figure 5B). These data pointed to a role for EZH2 in transcriptional silencing of target genes in GC B cells. To test this, we quantified the transcript levels of a representative set of genes in Ezh2 mutant and control GC B cells (n = 67) selected according to H3K27me3 status and relevance in B cell biology (Supplemental Table 2). We found that differentially expressed genes (P < 0.05) were all upregulated in Ezh2 mutant GC B cells (Figure 5C) and preferentially marked by H3K27me3 in Ezh2-proficient GC B cells (Figure 5D and Supplemental Figure 5C). These included key regulators of cell cycle progression (Cdkn1a, Cdkn1b, and Cdkn2a) and GC B cell differentiation (Id2, Blimp1, Irf4, and Xbp1; Figure 5C). For Irf4, we confirmed the upregulation at the protein level. Moreover, the fraction of cells expressing high Irf4 levels increased significantly.
in GCs of Ezh2 conditional mutants (Supplemental Figure 6, A and B). Instead, the expression of H3K4me3-only targets remained largely unaffected in response to Ezh2 inactivation (data not shown). Collectively, these data assign a critical control over cell cycle progression and differentiation of GC B cells to EZH2.

Ezh2 facilitates B cell proliferation independent of repression of CDK inhibitors. The identification of Cdkn1a, Cdkn1b, Cdkn2a as targets of H3K27me3 in GC B cells pointed to a role for EZH2 in the regulation of cell cycle progression, possibly through the repression of CDK inhibitors (8, 23). To test this hypothesis, given the difficulty in tracking the few Ezh2 mutant GC B cells in vivo, we performed cell cycle distribution analysis after in vitro activation of Ezh2 mutant B cells with membrane-bound CD40L and IL-4. Acute EZH2 inactivation in B cells led to an accumulation of cells in the G0/G1 phase of the cell cycle (Figure 6, A and B). Expression analysis revealed a substantial upregulation of the CDK inhibitor p16Ink4a in Ezh2 mutant GC B cells (Figure 6C). To determine whether induction of p16Ink4a was responsible for the defect in G1-to-S transition of Ezh2 mutant B cells, we analyzed the cell cycle profile of Ezh2/Cdkn2a double-mutant B cells after CD40L/IL-4 stimulation. In contrast to previous results (24, 25), Cdkn2a inactivation failed to normalize the cell cycle profile of Ezh2 mutant B cells (Figure 6D). Moreover, expression of other CDK inhibitors was largely unaffected in Ezh2 mutant primary B cells after mitogenic stimulation, excluding their possible involvement in the cell cycle defect (Figure 6C). All together these results unravel a novel mechanism that uncouples EZH2 control of G1-to-S transition from the repression of CDK inhibitors.

Ezh2 is required for repression of a subset of BCL6 targets in GC B cells. We noticed that several genes upregulated in Ezh2 mutant GC B cells are bound with high significance and repressed by the transcriptional repressor BCL6 in GC B cells (26). Given the relevance of BCL6 in the regulation of the GC reaction (27), we investigated the influence of EZH2 on BCL6-dependent gene silencing. Specifically, we quantified the transcript levels of 49 highly significant (false discovery rate [FDR] ≤ 0.0001) BCL6 targets in Ezh2 mutant GC B cells (27). Of these, around one-third (n = 16) were marked by H3K27me3 in wild-type GC B cells (Supplemental Table 3). Whereas expression of BCL6 targets lacking H3K27me3 remained largely unaltered in Ezh2 mutant primary B cells after mitogenic stimulation, excluding their possible involvement in the cell cycle defect (Figure 6C). All together these results unravel a novel mechanism that uncouples EZH2 control of G1-to-S transition from the repression of CDK inhibitors.

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mals expressing BCL6 under the \( I^+ \) promoter that is constitutively active in B cells (28). Expression analysis performed on primary GC B cells of compound mutants revealed the failure of \( I^+ \)HABCL6 to normalize expression of a subset of H3K27me3/BCL6 shared targets (Figure 7B). These results indicate that EZH2 regulates BCL6 repressor function on a subset of common targets in GC B cells.

H3K27me3 marking of Blimp1 and Irf4 in GC B cells is erased upon induction of PC differentiation. Bioinformatic analysis of ChIP-seq data identified the PC determinant Blimp1 as a target of H3K27me3 in mouse GC B cells, in accordance with recent findings in the human counterparts (23). We confirmed this result by performing H3K27me3 ChIP-qPCR analyses on primary GC B cells (Figure 8A) and A20 and I.29\(+\) murine B lymphoma cells (Supplemental Figure 7A) expressing low levels of Blimp1 (Supplemental Figure 7F). Accordingly, the PRC2 core component Suz12 was recruited to the Blimp1 locus in BLIMP-negative B cells (Supplemental Figure 7B). In a similar fashion, screening by ChIP-qPCR identified H3K27me3 deposition in a region proximal to the Irf4 TSS in primary GC B cells and A20 and I.29\(+\) lymphoma cells (Figure 8A and Supplemental Figure 7A). These results establish that Blimp1 and Irf4 are direct targets of PRC2 in GC B cells.

To study the regulation of H3K27 trimethylation at the Blimp1 and Irf4 loci during terminal differentiation, we stimulated B cells in vitro with either LPS or IL-21. A substantial loss of H3K27me3 at the Blimp1 and Irf4 loci (Figure 8B) was detected within the first 24–48 hours after stimulation. In particular, for Irf4, this coincided with loss of Suz12 binding, increased H3K4 trimethylation, and recruitment of RNA polymerase II to the promoter, which correlated

Figure 8
H3K27me3 marks Blimp1 and Irf4 in GC B cells and restricts PC differentiation. (A) Blimp1 and Irf4 H3K27me3 ChIP-qPCR in primary GC B cells. Gapdh was assessed as negative control. Enrichment is relative to input and histone H3 density. Columns represent mean values. (B) Blimp1 and Irf4 H3K27me3 ChIP-qPCR in I.29\(+\) B cells before (-) and 48 hours after LPS stimulation and in A20 cells in response to 24 hours of IL-21 stimulation. (C) Immunoblot analysis of H3K27me3 and full-length (Ezh2\(^{\text{SET}}\)) EZH2 levels in representative Ezh2 control (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\); n = 3) and mutant (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\); n = 3) iGB cultures 4 days after CD40L/BAFF/IL-4 stimulation. Histone H3 was assessed as loading control. Quantification of H3K27me3 levels relative to controls is shown. (D) Blimp1 and Irf4 H3K27me3 ChIP-qPCR analysis in Ezh2 control (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\)) and mutant (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\)) iGB cells stimulated as in C. (E) qRT-PCR analysis of Irf4 and Blimp1 transcripts in Ezh2 control (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\); n = 3) and mutant (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\); n = 4) iGB cells stimulated as in C. Values are relative to Gapdh. Columns indicate mean ± SEM. \( * P = 0.0066 \) (t test). (F) Representative FACS analysis and (G) frequency of Irf4\(^{\text{hi}}\)CD138\(^{\text{+}}\) PCs in control (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\); n = 3) and mutant (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\); n = 3) iGB cultures after IL-21 stimulation. \( ** P = 0.0006 \) (t test). (H) IgG1 levels in the supernatant of Ezh2 control (Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\); n = 3) and mutant (Ezh2\(^{\text{fl}}\):Ezh2\(^{\text{fl}}\):C\(^{\text{a1-cre}}\); n = 3) iGB cultures after IL-21 stimulation. (G and H) Symbols represent individual samples; bars refer to mean values. (B and D) Fold enrichments are calculated as in A.
IL-21 stimulation, over one-third of differentiated into Irf4hiCD138+ PCs, significantly outnumbering those sis (Figure 8D). Importantly, upon triggered differentiation of a subset of CD40-activated B cells into Blimp1 that was confirmed at the transcribed with augmented Irf4 transcription (Supplemental Figure 7, C–E). Notably, induction of PC differentiation did not promote global H3K27 demethylation, as PRC2 targets Hoxa9 and Olig1 remained marked by H3K27me3 and bound by Suz12 in stimulated B cells (Supplemental Figure 7G). These results reveal that Blimp1 and Irf4 upregulation during onset of PC differentiation is accompanied by a substantial loss of H3K27me3 proximal to their TSS.

**Ezh2 restricts terminal B cell differentiation promoted by IL-21.** To assess the relevance of EZH2-dependent repression of Blimp1 and Irf4 in GC B cells, we determined the effects of acute Ezh2 inactivation on terminal differentiation. Primary Ezh2Cre expressing CD19+B cells were stimulated in vitro for 4 days with CD40L, BAFF, and IL-4 to generate proliferating GC-like B cells (also called iGB cells; ref. 29) that were ultimately differentiated into PC by the addition of IL-21 to the culture media (29). Efficient Ezh2 inactivation in iGB cells led to a substantial reduction in global H3K27me3 levels (Figure 8C) that was confirmed at the Blimp1 and Irf4 loci by ChIP-qPCR analysis (Figure 8D). Ezh2 mutant iGB cells expressed higher Irf4 and Blimp1 transcript levels (Figure 8E), which was alone sufficient to trigger differentiation of a subset of CD40-activated B cells into Irf4+CD138+ PCs (Supplemental Figure 8A). Importantly, upon IL-21 stimulation, over one-third of Ezh2 mutant iGB cells differentiated into Irf4+CD138+ PCs, significantly outnumbering those present in control cultures (Figure 8, F and G). In accordance with this result, conditioned media of Ezh2 mutant iGB cultures stimulated with IL-21 contained higher levels of soluble IgG1 antibodies (Figure 8H), which coincided with a greater number of IgG1+ antibody-secreting cells, when compared with controls (Supplemental Figure 8B). These results indicate that EZH2 repression of Blimp1 and Irf4 restricts IL-21-controlled PC differentiation.

**Y641 mutant of EZH2 silences BLIMP1 expression through PRC2 to facilitate DLBCL growth.** BLIMP1 has been proposed tumor suppressor since its inactivation occurs frequently in human DLBCL and promotes B cell lymphomas in mouse models (30–32). In DLBCL, impaired BLIMP1 expression results from genetic alterations of the BLIMP1 locus or stable repression by BCL6 (32). BLIMP1 mutations have been almost exclusively associated with ABC-type DLBCL (30, 32). Since EZH2 gain-of-function mutations occur predominantly in GC-type DLBCL (9) and given that Blimp1 is a direct target of EZH2 repression in primary mouse GC B cells, we investigated whether EZH2 was required for stable BLIMP1 silencing in GC-type DLBCL. To this aim, we treated the DLBCL cells lines, SU-DHL-4 and SU-DHL-6, expressing constitutively active forms of EZH2 (9, 33) with the EZH2 small molecule inhibitor, GSK343 (34). Treatment of tumor cells with 2.5 μM GSK343 led to a reduction in global levels of H3K27me3, with interfering with EZH2 expression (Supplemental Figure 9, A and B). Importantly, exposure of DLBCL cells to GSK343 induced a substantial upregulation of BLIMP1 expression in both DLBCL lines (Figure 9A). This effect was associated with impaired in vitro lymphoma growth (Figure 9B) that was contributed by a partial arrest in cell cycle progression at the G1-to-S...
transition (Supplemental Figure 9C). To understand whether Y641 mutant of EZH2 exerted its catalytic activity within PRC2, we inhibited the expression of the essential PRC2 subunit EED in DLBCL cells. Downregulation of EED in SU-DHL-4 cells (Supplemental Figure 9, D and E) caused a global reduction in H3K27me3 levels (Supplemental Figure 9F) and, most importantly, a substantial upregulation of BLIMP1 expression (Figure 9C). Similar results were obtained in A20 lymphoma B cells expressing wild-type EZH2 (Supplemental Figure 10). Inhibition of EED interfered with in vitro DLBCL growth (Figure 9D) and impaired cell cycle progression (Supplemental Figure 9G), extending our data as well as that of others based on the use of EZH2 small molecule inhibitors (23, 35–37). In a subset of SU-DHL-4 cells, PRC2 inhibition alone was sufficient to cause the induction of surface CD138 expression and the downregulation of MHC class II molecules, which accompanies terminal differentiation (Supplemental Figure 9H). To test whether induction of BLIMP1 was alone sufficient to influence growth of B lymphoma cells expressing a constitutively active form of EZH2, we complemented WSU-DLCL-2 DLBCL cells with a BLIMP1 expression vector. Transient expression of BLIMP1 (Figure 9E) caused a severe impairment in in vitro growth of DLBCL, which ultimately led to fewer lymphoma cells retrieved 2 days after complementation (Figure 9F). All together, these results indicate that constitutively active EZH2 represses the tumor suppressor BLIMP1 via PRC2 to facilitate the growth of GC-type DLBCL.

**Discussion**

The strong induction of EZH2 in GC B cells, combined with the frequent occurrence of EZH2 gain-of-function mutations in GC-derived NHL, suggested a role for the PcG protein in the regulation of the GC reaction. Indeed, using cell type–specific gene targeting in mice, we show that Eb2 mutant myelotransferase activity is strictly required for GC B cell function. EZH2 inactivation resulted in fewer GCs, reduced numbers of high-affinity long-lived memory B cells, and impaired production of antigen-specific antibodies upon encounter with T cell–dependent antigens.

The failure to sustain the GC reaction was largely contributed by enhanced apoptosis of Eb2 mutant B cells, which was independent of p19ARF upregulation and resistant to constitutive BCL2 expression. Strikingly, instead, GC B cells lacking functional EZH2 became highly vulnerable to genotoxic stress associated with AID function. As a consequence of premature death, Eb2 mutant GC B cells carried fewer Ig V gene mutations and failed to be selected by antigen-driven selection. The enrichment for apoptotic cells among Ig class-switched Eb2 mutant B cells suggests a possible contribution of the PcG protein to the repair of DNA breaks generated during IgH isotype switching, extending previous findings (16, 18, 19, 38). In support of this, Eb2 mutant primary B cells displayed higher numbers of DNA damage foci marked by 53BP1 after exposure to a sublethal dose of IR. The failure of Eb2 mutant GC B cells to preserve genome integrity may trigger ultimately their death, possibly as a consequence of mitotic catastrophe. This scenario does not exclude that EZH2 may act to limit the genome-wide recruitment of AID via H3K27me3-dependent silencing of target genes (39, 40). Future studies will establish whether EZH2 inactivation increases the frequency of off-target mutations caused by AID, in particular at bivalent genes (40, 41).

Global identification of H3K27me3 targets combined with expression analyses revealed a critical contribution of EZH2 to the regulation of the GC B cell transcriptional program. H3K27me3 targets were preferentially repressed in GC B cells and strongly enriched for developmental regulators, supporting the notion that, in differentiated cells, PRC2 has a critical function to preserve silencing of transcriptional programs driving alternative cell fates. Importantly, EZH2 also exerted a direct control of the expression of a substantial number of genes that regulate the persistence and expansion of B cells within the GC reaction. EZH2 was required to repress Id2, the inhibitor of the E-box protein E2A, whose function is necessary for GC B cells (42). In GC B cells, EZH2 was also critical to prevent the expression of the cyclin-dependent kinase inhibitor p16INK4A encoded by the Cdkn2a locus, which regulates G1-to-S transition. In accordance with this, a higher proportion of Eb2 mutant cells was arrested at the Go/G1 stage after CD40 stimulation. Surprisingly, in contrast to previous reports, the cell cycle defect was neither rescued by Cdkn2a inactivation nor associated with substantial changes in the expression of other CDK inhibitors. These results unravel a novel mechanism through which EZH2 facilitates G1-to-S progression that is independent of CDK repression.

Of note, the majority of H3K27me3 targets that we found upregulated in Eb2 mutant GC B cells are bound and repressed by BCL6 (26), which is essential for GC B cell function (27). This result underscores a possible cooperation between PRC2 and BCL6 in the repression of common targets, which include important negative regulators of GC B cell function, such as Id2 and Blimp1. Our data support a scenario in which EZH2 primarily regulates BCL6 repressor activity, as enforced BCL6 expression was not sufficient in Eb2 mutant GC B cells to restore repression of a representative subset of shared targets.

One of the main findings of this study is the identification of PC determinants Blimp1 and Irf4 (43–45) as direct targets of EZH2 repression in GC B cells. Blimp1 and Irf4 genes were marked by H3K27me3 and bound by PRC2 in B cells prior to onset of PC differentiation. This regulation was critical to repress Blimp1 and Irf4, since transcripts for both PC determinants were significantly upregulated in Eb2 mutant GC B cells. The induction of the PC program in GC B cells following EZH2 inhibition was completed by the upregulation of Xbp1, which is required to sustain high rates of Ig synthesis (46, 47). Acute Eb2 inactivation was sufficient to trigger terminal differentiation of a subset of CD40-activated B cells. More strikingly, the addition of IL-21 substantially boosted differentiation of PC mutant B cells into antibody-secreting cells in response to CD40 stimulation. This result indicates that EZH2 limits the amplitude of the PC response induced by IL-21 (48), thereby possibly extending the persistence of B cells in the GC (49, 50). We propose that, in GC B cells, EZH2 modulates IL-21–dependent Blimp1 expression via H3K27 trimethylation (51, 52). Therefore, signals regulating PRC2 recruitment at the Blimp1 locus are predicted to influence the balance between IL-21–dependent Bcl6 and Blimp1 expression and, thereby, the decision of B cells to persist rather than exit from the GC reaction. The control of Irf4 expression exerted through H3K27me3 is likely to contribute to the regulation by EZH2 of the fate of GC B cells (53).

The increased susceptibility of Eb2 mutant B cells to PC differentiation is seemingly in contrast with reduced serum Ig titers measured in PcG mutant animals after immunization with T cell–dependent antigens. The increased apoptosis may represent a major obstacle for Eb2 mutant B cells to exit the GC as antibody-secreting cells. Moreover, whereas EZH2 inactivation interefes...
Methods

In the treatment of GC-derived DLBCL and follicular lymphoma. EZH2 inhibitors (35–37) in combination with inducers of terminal lymphomagenesis. These results provide a rationale for use of mechanism through which constitutively active EZH2 contributes to the establishment of a protective role as tumor suppressor (30–32). The rare occurrence of BLIMP1 mutations in GC-type DLBCL suggests the existence of additional mechanisms that could prevent its expression in this form of NHL. Using pharmacological inhibition of EZH2 and functional inactivation of PRC2, we provide evidence that constitutively active EZH2 enforces BLIMP1 silencing in GC-type DLBCL cells. While our manuscript was under review an independent study reached similar conclusions (23). Importantly, we provide evidence that BLIMP1 induction alone is sufficient to impair in vitro growth of GC-type DLBCL expressing mutant EZH2. Thus, epigenetic silencing of BLIMP1 may represent an important mechanism through which EZH2 supports lymphoma growth.

In conclusion, our data indicate that EZH2 methyltransferase activity is strictly required for the establishment of a protective long-term B cell adaptive immune response. EZH2 accomplishes this function through the coordinated regulation of GC B cell differentiation, proliferation, and response to genotoxic damage imposed by AID. Conversely, enforced BLIMP1 repression, coupled to protection against AID mutagenesis, acceleration of S-phase entry, and support for BCL6 function, may represent the mechanism through which constitutively active EZH2 contributes to lymphomagenesis. These results provide a rationale for use of EZH2 inhibitors (35–37) in combination with inducers of terminal differentiation, such as IL-21 (55, 56) and genotoxic agents, in the treatment of GC-derived DLBCL and follicular lymphoma.

Mice. Ezh2flnull, R26-εyp, Cr2-cre, C57BL/6J mice were previously described (11–13, 28, 57–60). Compound mutants were backcrossed to C57BL/6J mice for 6 or more generations and kept under specific pathogen-free conditions. Eight- to sixteen-week-old mice were infected with intraperitoneal injection of Alum-precipitated NPs (Biosearch Technologies) or NPs (30 µg per mouse) for secondary responses. Sheep red blood cells (1 x 10^6 per mouse; Oxo) were reinfused in PBS and injected intraperitoneally.

Antibodies. Flow cytometry was performed using fluorescent- or biotin-labeled antibodies reactive to mouse CD19 (1D3, eBioscience), CD21 (BD Biosciences), CD23 (B3B4, eBioscience), CD38 (90, eBioscience), IgD (11.26, eBioscience), CD184 (2B11, eBioscience), CD95 (J502; BD Biosciences), CD138 (2B1-2, BD Biosciences), IgG1 (A85-1; BD Biosciences), IgM (R33.24.12; K. Rajewsky), Max Delbrück Center for Molecular Medicine, Berlin, Germany), CD45R/B220 (RA3-6B2; K. Rajewsky), and IgG (R33-18; K. Rajewsky). Antibodies against human MHC class II and CD138 were from BD Biosciences, and bound were antibodies detected with biotin-conjugated anti-mouse IgG1 (MCA1287 [BD Pharmingen]).

ELISA. To quantify NP-specific serum antibodies, plates were coated with 2 µg/ml NP23-BSA or NP4-BSA, respectively (Biosearch Technologies), and bound were antibodies detected with biotin-conjugated anti-IgM (R33.24.12) or anti-IgG1 (S0331; BD Pharmingen) antibodies. IgG1 levels in conditioned media of iGB cultures were measured using a combination of rat anti-mouse IgG1 antibodies (MCA1289 [ABD Serotec] and A85-1 [BD Pharmingen]).

ELISPOT. MultiScreen HTS-HA filter plates (MHSAS4510; Millipore) were coated with 1 µg rat anti-mouse IgG1 (LO.MG1-13; ABD Serotec). Cells were seeded for 6 hours at 37°C, and bound IgG1 antibodies were detected with biotinylated rat anti-mouse IgG1 (A85-1; BD Pharmingen) followed by streptavidin coupled to alkaline phosphatase (ELISPOT Kit; R&D Systems). Images of plates were acquired, and the number of spots was scored using ImageJ software.

qPCR analysis. qRT-PCR was performed in triplicate with SYBR Green I Master Mix using primer combinations listed in Supplemental Table 4 or using custom-made TaqMan Array Micro Fluidic Cards (Applied Biosystems). Probe sets are listed in Supplemental Table 4. Expression values were calculated as 2^-ΔΔct. Ezh2 gene copy number was quantified by qPCR using primers annealing to genomic DNA lying within the loxP-flanked segment (Supplemental Table 4). Values were calculated using the comparative CT method. To normalize for DNA input, a segment of the Gapdh gene was amplified using primers listed in Supplemental Table 4.

Ig Vδ2 gene mutation analysis. Ig rearrangements bearing the Vδ2I86.2 gene were analyzed as described previously (61). PCR primers are listed in Supplemental Table 4.

Histology. Snap-frozen spleens were embedded in Cryo-M-Bed (Bright Instruments). Sections mounted onto multispot glass slides (Hendley)
were stained with rat anti-mouse IgD (BD Biosciences) and biotin-conjugated PNA (Vector Laboratories), followed by rabbit anti-rat Ig coupled to horseradish peroxidase. Sections were washed, and streptavidin complex (Vector Laboratories) was added. Stains were developed with 3,3-diaminobenzidine tetrahydrochloride to reveal horseradish peroxidase–conjugated antibodies and fast blue for biotinylated PNA. Slides were mounted with Immunomount (Thermo) and analyzed with a Leitz Wetzlar Dialux microscope. Photographs were taken with a Leica DM6000 microscope.

ChiP. B cells were fixed in 1% (vol/vol) PBS/formaldehyde, and the reaction was stopped by adding 0.125 M glycine. Fixed cells were harvested by centrifugation, washed in PBS, and resuspended in lysis buffer (0.5% SDS, 50 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, and 0.02% NaN3) containing 1 mM PMSF and 0.1% (vol/vol) protease inhibitor cocktail (Sigma–Aldrich). 40 μg sonicated chromatin was immunoprecipitated with 3.5 to 5 μg of antibodies specific for H3 (ab1791; Abcam), H3K27me3 (C36B11; Cell Signaling), and H3K4Me3 (ab37415; Abcam). Antibodies (3.5–5 μg) specific for SUZ12 (D39F6; Cell Signaling) or RNA polymerase-II (ab3131; Abcam) and an IgG control (ab37415; Abcam) were used to immunoprecipitate 500 μg chromatin. Antibody-bound DNA was reverse cross-linked, purified, and assayed in triplicate by real-time PCR using primers listed in Supplemental Table 3. ChIP-seq analysis was performed in duplicate on GC B cells sorted from pools of C57BL/6 mice immunized with SRBC. 10 ng sonicated chromatin before (input) and after immunoprecipitation with antibodies specific for H3K27me3 (C36B11; Cell Signaling) and H3K4Me3 (MC315; Millipore) was processed with the Illumina ChIP-Seq Sample Prep and Multiplexing Oligonucleotide Kits. DNA libraries were quantified and used for cluster generation and sequencing on a HiSeq 2000 instrument following the manufacturer’s protocol. ChIP-seq raw data have been deposited in the Gene Expression Omnibus database (GSE50912). Bioinformatic analyses of ChIP-seq data are described in the Supplemental Methods.

Statistics. Statistical analyses were done by 2-tailed unpaired Student’s t test, except for gene expression data using TagMan Array Cards, for which a non-parametric Wilcoxon rank-sum test was applied. Fisher’s exact test was used to assess the enrichment of H3K27me3 targets within specific gene lists. P values equal to or lower than 0.05 were considered indicative of significance. These P values were FDR corrected for multiple comparisons throughout the analysis, with the exception of the identification of enriched chromatin domains (for which much stricter thresholds were applied).

Study approval. Animal experimentation was approved by the IFOM Animal Care Committee (IACUC) and the Italian Ministry of Health.

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4. Aldiri I, Vetter ML. PRC2 during vertebrate development. *Cell Signaling) or RNA polymerase-II (ab5131; Abcam) or an IgG control (ab37415; Abcam). Antibodies (3.5–5 μg) specific for SUZ12 (D39F6; Cell Signaling) or RNA polymerase-II (ab3131; Abcam) and an IgG control (ab37415; Abcam) were used to immunoprecipitate 500 μg chromatin. Antibody-bound DNA was reverse cross-linked, purified, and assayed in triplicate by real-time PCR using primers listed in Supplemental Table 3. ChIP-seq analysis was performed in duplicate on GC B cells sorted from pools of C57BL/6 mice immunized with SRBC. 10 ng sonicated chromatin before (input) and after immunoprecipitation with antibodies specific for H3K27me3 (C36B11; Cell Signaling) and H3K4Me3 (MC315; Millipore) was processed with the Illumina ChIP-Seq Sample Prep and Multiplexing Oligonucleotide Kits. DNA libraries were quantified and used for cluster generation and sequencing on a HiSeq 2000 instrument following the manufacturer’s protocol. ChIP-seq raw data have been deposited in the Gene Expression Omnibus database (GSE50912). Bioinformatic analyses of ChIP-seq data are described in the Supplemental Methods.

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