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Germinal center dysregulation by histone methyltransferase EZH2 promotes lymphomagenesis

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Introduction

Protective immunity against 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 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.

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EZH2 is upregulated in mouse GC B cells and required for GC formation. (A) Ezh2 transcript levels in B cell subsets relative to Rplp0. Columns represent mean ± SD of triplicates. Experiments were performed on pools of B cells sorted from 3 mice. (B) FACS analysis of EZH2 protein levels in follicular (FO) and GC B cells. Numbers indicate mean expression. (C) Representative FACS analysis of splenic B cells in NP-CGG–immunized Ezh2 control (Ezh2fl/fl:Cry1-cre) and mutant (Ezh2fl/fl:Cry1-cre) mice. Numbers indicate percentage of boxed GC B cells. (D) Frequency of splenic GC B cells in NP-CGG–immunized Ezh2 control (Ezh2fl/fl:Cry1-cre; n = 17) and mutant (Ezh2fl/fl:Cry1-cre; n = 22) mice. Symbols represent individual mice; bars refer to mean values. *P = 0.013 (t test). (E) Representative histological analysis of PNA-positive (brown) GCs (arrowheads) in the spleens of NP-CGG–immunized Ezh2 control (Ezh2fl/fl:Cry1-cre; n = 6) and mutant (Ezh2fl/fl:Cry1-cre; n = 6) mice. Scale bar: 200 μm. (F) Frequency of Ezh2 inactivation in GC B cells of Ezh2 control (Ezh2fl/fl:Cry1-cre; n = 3) and mutant (Ezh2fl/fl:Cry1-cre; n = 4) animals, quantified by genomic qPCR. (G) Frequency of YFP+ GC B cells in R26-yfp;Ezh2 control (Ezh2fl/fl) and mutant (Ezh2fl/fl) mice. (H) Summary of data on frequencies of YFP+ GC B cells in R26-yfp;Cry1-cre;Ezh2 control (n = 4) and mutant (n = 6) mice. (F and H) Columns indicate mean ± SD. **P = 0.002 (t test). Data represent (C and D) 10, (G) 5, (F, and H) 3, and (B, E, and F) 2 experiments, respectively.

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
knockout (Ezh2−/−) mice carrying loxP sites flanking exons coding for the catalytic SET domain (11) were crossed with Cγ1-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:Cr2-cre conditional mutants and Ezh2fl/fl:Cγ1-cre controls (hereafter referred to as controls) were immunized with alum-precipitated 4-hydroxy-3-nitrophenyl acetate (NP) conjugated to chicken immunoglobulin (CGG) to induce GC responses in immunized mice revealed a significant reduction in peanut agglutinin (PNA)–positive GCs in Ezh2−/− mice at the peak of the response (Figure 1, C and D, and Supplemental Figure 1C). Similar results were observed analyzing GC responses in Ezh2fl/fl:Cr2-cre mutants (Supplemental Figure 1D) that showed normal distribution and number of peripheral B cell subsets (Supplemental Figure 1, E and F).

To reveal the identity of the remaining GC B cells in Ezh2fl/fl:Cr2-cre animals, we determined the status of the Ezh2−/− gene 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:Cr2-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:Cγ1-cre mice to the R26-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) was counted once if identical nucleotide substitutions present in different sequences were counted once if each genotype. Nine unique V186.2 sequences were amplified by PCR from genomic DNA of Ezh2−/− (Ezh2fl/fl:R26-cre/R26-cre/Ezh2−/−) and mutant (Ezh2fl/fl:Cr2-cre/R26-cre/Ezh2−/−) 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 V186.2 sequences were analyzed for each genotype. Identical nucleotide substitutions present in different sequences were counted once if V186.2 rearrangements shared the same CDR3 region. The frequency of rearrangements carrying codon 33 TGG→TTG (W33L) was counted by dividing the number of unique V186.2 sequences bearing TTG substitutions by the total number of unique mutated V186.2 rearrangements.
B cell–specific inactivation of EZH2 is strictly required for the persistence of B cells within the GC reaction. These studies reveal that EZH2 is necessary for the formation of IgG1 memory response (Figure 2B and Supplemental Figure 2A). The reduction in global H3K27me3 levels (Supplemental Figure 2C). Thus excluding major defects in Ig CSR. The mutagenesis (Table 1). Moreover, the frequency of replacement mutations, causing a cryptophan-to-leucine substitution at codon 33 that confers a 10-fold increase in the percentage of CaspGLOW+ apoptotic GC B cells in the spleens of Ezh2 control (Ezh2+/+;C1-cre) and mutant (Ezh2−/−;C1-cre [Ezh2−−]) mice after NP-CGG immunization. Values are relative to Rplp0 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 (Ezh2+/+;C1-cre) and mutant (Ezh2−/−;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 (Ezh2+/+;C1-cre; n = 7) and mutant (Ezh2−/−;C1-cre; n = 5) mice after NP-CGG immunization. Data are representative of (A, B, and F) 2, (D and E) 4, and (C) 10 experiments, respectively. (C) Columns indicate mean ± SD.
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/fl:Cdkn2a–/–;C1a1-cre mice). Flow cytometry analysis of NP-CGG–immunized mutants and controls (Ezh2fl/+;Cdkn2a–/–;C1a1-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 \(\text{Ezh2}^\text{mutant}\) B cells to acute DNA damage induced by ionizing radiation (IR). Primary resting B cells purified from mutant (\(\text{Ezh2}^{\text{fl/fl}}:\text{Cr2-cre}\)) and control (\(\text{Ezh2}^{\text{fl/+}}:\text{Cr2-cre}\)) 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 \(\text{Ezh2}\) genotype), the treatment with 0.1 Gy caused minor toxicity to \(\text{Ezh2}\) proficient B cells. In contrast, the cultures of \(\text{Ezh2}\) 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 \(\text{Ezh2}\) 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 \(\text{Ezh2}\) 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). \(\text{Ezh2}\) 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 \(\text{Ezh2}\) 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 GC B cells. The sizes of GCs and the absolute number and the percentage of GC B cells were largely rescued in \(\text{Ezh2}/\text{Aicda}\) double mutants (Figure 4D).
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D and E, and Supplemental Figure 4, E and F). Importantly, AID inactivation led to the normalization of apoptosis of Ezh2 mutant GC B cells (Figure 4, F and G), allowing for the first time efficient recovery of cells that had recombined both Ezh2 alleles (Figure 4H). These results show that EZH2 protects against DNA damage-induced apoptosis, in particular, in GC B cells, genotoxicity linked to AID mutagenesis.

**Ezh2 regulates the GC B cell transcriptional program.** Despite the consistent normalization of apoptosis, Ezh2fl/fl:Aicda−/−;Cre2-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.

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 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 Ezh2fl/fl, H3K27me3, H3K4me3, or by both, respectively. In accordance with previous observations (22), we found that genes marked by H3K27me3 were significantly (P < 0.01) enriched for categories involved in developmental processes (Supplemental Figure 5A), whereas H3K4me3 preferentially marked genes involved in metabolism (Supplemental Figure 5B).

**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 1).** 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 IκB promoter that is constitutively active in B cells (28). Expression analysis performed on primary GC B cells of compound mutants revealed the failure of IκB-driven BCL6 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 (Supplementary 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
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 Ezh2fl/fl:Cyt1-cre 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 PCs 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 Irf4hiCD138+ PCs (Supplemental Figure 8A). Importantly, upon IL-21 stimulation, over one-third of Ezh2 mutant iGb cells differentiated into Irf4hiCD138+ PCs, significantly outnumbering those Irf4hiCD138+ PCs (Supplemental Figure 8A). Importantly, upon exposure to Blimp1-transfected control cells after normalization for GAPDH expression. Columns indicate mean of 2 independent experiments. Values are presented relative to control after normalization for HPRT expression. (F) Comparison of SU-DHL-2 cell numbers at the indicated days after transfection with control or Blimp1 expression vector, respectively. Data are representative of 2 experiments.

Figure 9

Y641 mutant of Ezh2 silences BLIMP1 expression through PRC2 to facilitate DLBCL growth. (A) BLIMP1 transcripts in SU-DHL-4 and SU-DHL-6 DLBCL cells 12 days after treatment with GSK343 or vehicle, as revealed by qRT-PCR analysis. Columns indicate mean ± SD of triplicates. Expression is relative to that of vehicle-treated control cells after normalization for B2M expression. (B) Growth curves of SU-DHL-4 (black line) and SU-DHL-6 (gray line) DLBCL cells treated with either GSK343 (solid line) or vehicle (dotted line). Average cell numbers at the indicated time points ± SEM of 4 replicates is shown. ***P < 0.001 (2-way ANOVA). (C) BLIMP1 transcript levels in SU-DHL-4 DLBCL cells after infection with lentiviruses expressing control (ctrl) or anti-EED (EED) shRNAs, respectively. Results are presented relative to control after normalization for GAPDH expression. Columns indicate mean ± SD of 2 independent experiments. Values are presented relative to control after normalization for HPRT expression. (F) Comparison of SU-DHL-2 cell numbers at the indicated days after transfection with control or Blimp1 expression vector, respectively. Data are representative of 2 experiments.
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 Ezh2 methyltransferase 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 Ezh2 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, Ezh2 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 Ezh2 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, Ezh2 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 Ezh2 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 Ezh2 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 Ezh2 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 Ezh2 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 Ezh2 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 Ezh2 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 PcG 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 Ezh2 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 Ezh2 mutant B cells to exit the GC as antibody-secreting cells. Moreover, whereas EZH2 inactivation interferes...
neither with Blimp1-controlled *Xbp1* expression nor with the establishment of the transcriptional program that supports the unfolded protein response (short-lived *Ezh2* mutant PCs secrete normal amounts of antibodies in vitro), additional experiments will be required to determine whether *EZH2* is required for long-term PC persistence (54).

The identification of *Blimp1* as a direct target of *EZH2* is relevant for the understanding of the mechanisms through which the PcG protein may contribute to the pathogenesis of DLBCL. *BLIMP1* loss-of-function mutations occur frequently in ABC-type DLBCL and accelerate lymphomagenesis in mouse models, supporting its 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 in the treatment of GC-derived DLBCL and follicular lymphoma.

**Methods**

*Mice.* *Ezh2*<sup>−/−</sup>, *R26-3rfp*, *Cre2-cre*, *Cry1-cre*, *Ckln2a−/−*, *Eμ-BCL2*, *Aicda−/−*, and *Ij/HablC6* mice were previously described (11–13, 28, 57–60). Compound mutants were backcrossed to *C57BL/6* mice for 6 or more generations and kept under specific pathogen-free conditions. Eight- to sixteen-week-old mice were immunized by intraperitoneal injection of alum-precipitated NP-CGG<sub>25,56</sub> (100 µg per mouse; Biosearch Technologies) or NP-CGG<sub>25</sub>/PBS (50 µg per mouse) for secondary responses. Sheep red blood cells (1 x 10<sup>6</sup> cells per mouse; Oxoid) were resuspended in PBS and injected ipronetropically.

*Antibodies.* Flow cytometry was performed using fluorescent- or biotin-labeled antibodies reactive to mouse CD19 (1D3; Bioscience), CD21 (8D9; ebioscience), CD23 (B384; ebioscience), CD38 (90; ebioscience), IgD (11.26; ebioscience), CD184 (2811; ebioscience), CD95 (5d2; BD Biosciences), CD138 (281-2; 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 (R33-18-10; K. Rajewsky). Antibodies against human MHC class II and CD138 were provided by S. Cenci (San Raffaele Scientific Institute). NP binding was assessed using NP-BSA conjugated to allophycocyanin (provided by M. Hikida, Kyoto University, Kyoto, Japan) and stained with Cytofix/Cytoperm (BD Biosciences), and stained with IRF4-specific (3E4; BioLegend), H3K27me3-specific (C36B11; Cell Signaling), or *EZH2*-specific (AE25.13; K. Helin, University of Copenhagen, Copenhagen, Denmark) mAb conjugated in house with Alexa Fluor dyes according to manufacturer’s instructions (Molecular Probes, Invitrogen). Samples were acquired on a FACScalibur (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

Immunoblotting analysis. Immunoblotting analyses were performed with antibodies specific for *EZH2* (AE25-13; K. Helin), H3K27me3 (07-449; Upstate), histone H3 (07-690; Millipore), and EED (AA19-30; K. Helin), respectively. Immunoblot images were acquired using the ChemiDoc system (Bio-Rad).

*Analysis of apoptosis and DNA damage.* GC B cell apoptosis was measured with the CaspGLOW Staining Kit (BioVision). Irradiation was performed using a Faxitron (X-Ray Corporation) on MACS-purified primary B cells stimulated in vitro for 48 hours with 20 µg/ml LPS. TUNEL staining was performed using the In situ Cell Death Detection Kit (Roche) following the manufacturer’s instructions. Cells were acquired on a FACScalibur (BD Biosciences), and data were analyzed with FlowJo software (Tree Star). S3BP1- DNA damage foci were quantified in LPS-activated primary B cells 2 hours after exposure to 0.2 Gy of IR. To detect S3BP1+ foci, cells were fixed in PBS/4% paraformaldehyde, permeabilized, and stained with S3BP1-specific antibody (Novus Biologicals, NB100-304), and foci were visualized with a fluorescent-labeled secondary antibody in combination with DAPI staining. Photographs were acquired using an upright fluorescence microscope (Olympus AX-70; x40 magnification).

Cell cycle analysis. iGB cells were pulsed with EdU (44 µM; Invitrogen) for 1 hour followed by EdU measurement using the Click-iTEdU Alexa Fluor 647 Flow Cytometry Assay kit according to the manufacturer’s instructions (Invitrogen). DLBCL were labeled with BrDU (33 µM; Sigma-Aldrich) for 1 hour followed by determination of BrDU according to manufacturer’s instructions (BD Pharmingen). Samples were stained with propidium iodide, acquired on a FACScalibur (BD Biosciences), and analyzed using FlowJo software (Tree Star).

**ELISA.** To quantify NP-specific serum antibodies, plates were coated with 2 µg/ml NP<sub>23</sub>-BSA or NP<sub>4</sub>-BSA, respectively (Biosearch Technologies), and bound were antibodies detected with biotinylated anti-IgM (R33.24.12) or anti-igG1 (S50331; 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 AB85-1 [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 (ABS-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<sup>-ΔΔCt</sup>. *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 V<sub>H</sub> gene mutation analysis. Ig rearrangements bearing the V<sub>H</sub>186.2 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 multisport glass slides (Hendley)
were stained with rat anti-mouse IgD (BD Biosciences) and biotin-conjugated PNA (Vector Laboratories), followed by streptavidin peroxidase. Sections were washed, and streptavidin complex (Vector Laboratories) was added. Stains were developed with 3,3-diaminobenzidine tetrahydrochloride to reveal peroxidase conjugate and fast blue for biotinylated PNA. Slides were mounted with Immunumount (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 mixture (Sigma-Aldrich). 40 ng sonicated chromatin was immunoprecipitated with 3.5 to 5 μg of antibodies to H3 (ab1791; Abcam), H3K27me3 (C6361B; Cell Signaling), and H3K4Me3 (04-745; Millipore) or an IgG control (ab57415; Abcam). Antibodies (3.5–5 μg) specific for SUZ12 (D394F; Cell Signaling) or RNA polymerase-II (ab5131; 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 Supplementary 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 (C6361B; 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 Supplementary 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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