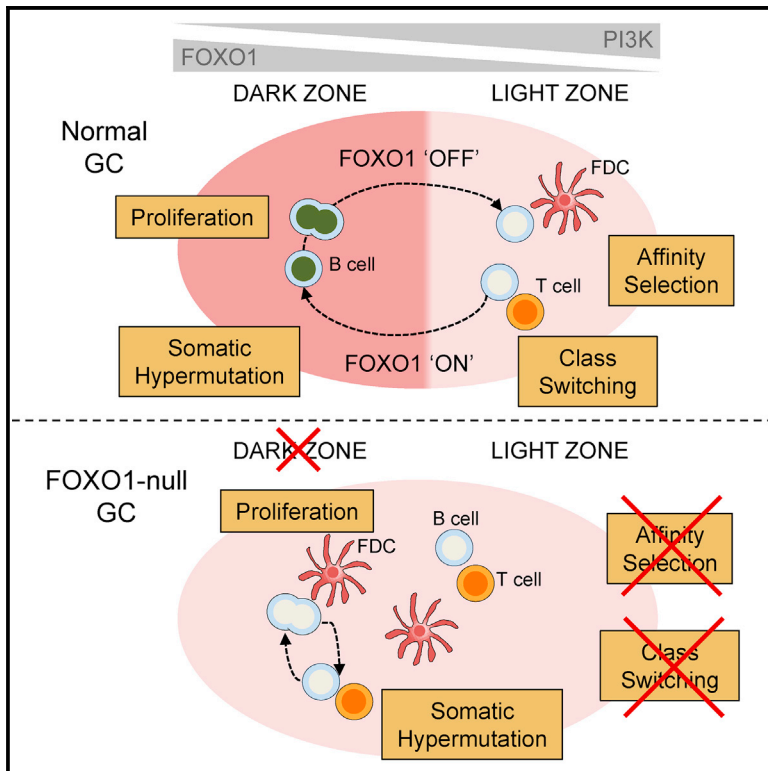# The FOXO1 Transcription Factor Instructs the Germinal Center Dark Zone Program

Graphical Abstract

Authors

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In Brief

The factors that control germinal center polarity and cyclic reentry are unknown. Dalla-Favera and colleagues demonstrate that the transcription factor FOXO1 instructs a gene program that is required for germinal center dark zone development. Mouse germinal centers devoid of FOXO1 expression fail to support affinity maturation and class switch recombination.

Accession Numbers

GSE69216
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Highlights

- FOXO1 expression in the germinal center is restricted to dark zone B cells

- FOXO1-null mouse germinal centers lack dark zones and lose architectural polarity

- FOXO1 deletion impairs affinity maturation and IgG1 class switch recombination

- FOXO1 instructs the dark zone gene program directly and by licensing BCL6 activity

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CellPress

The FOXO1 Transcription Factor Instructs the Germinal Center Dark Zone Program

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SUMMARY

The pathways regulating formation of the germinal center (GC) dark zone (DZ) and light zone (LZ) are unknown. In this study we show that FOXO1 transcription factor expression was restricted to the GC DZ and was required for DZ formation, since its absence in mice led to the loss of DZ gene programs and the formation of LZ-only GCs. FOXO1-negative GC B cells displayed normal somatic hypermutation but defective affinity maturation and class switch recombination. The function of FOXO1 in sustaining the DZ program involved the trans-activation of the chemokine receptor CXCR4, and cooperation with the BCL6 transcription factor in the trans-repression of genes involved in immune activation, DNA repair, and plasma cell differentiation. These results also have implications for the role of FOXO1 in lymphomagenesis because they suggest that constitutive FOXO1 activity might be required for the oncogenic activity of deregulated BCL6 expression.

INTRODUCTION

Germinal centers (GCs) are specialized environments where mature B lymphocytes (B cells) undergo repeated rounds of clonal expansion and secondary diversification of their immunoglobulin (Ig) genes. The purpose of the GC reaction is the generation of high-affinity B cells destined to differentiate into memory B cells and plasma cells and support immune adaptive responses (De Silva and Klein, 2015; Victora and Nussenzweig, 2012). GC B cells are also cells of origin for a majority of B cell non-Hodgkin lymphomas, which are caused by genetic alterations leading to the deregulation of pathways especially relevant to GC physiology (Basso and Dalla-Favera, 2015).

GCs are organized into two topologically and functionally distinct compartments: the dark zone (DZ), where proliferative expansion and Ig somatic hypermutation (SHM) occur; and the light zone (LZ), where B cells expressing high-affinity antibodies are selected in response to cues provided by follicular dendritic cells (FDCs) and T follicular helper lymphocytes and where they undergo class switch recombination (CSR). Recent studies have provided important insights into the complex dynamics of GC physiology by showing that GC B cells cyclically transit between LZ and DZ, thus replacing the classic definition of centrocytes (or LZ GC B cells) and centroblasts (or DZ GC B cells) as irreversible B cell developmental stages. The iterative transit of GC B cells between these two compartments sequentially links SHM-based Ig diversification to affinity selection events and drives the progressive increase in B cell receptor affinities (Allen et al., 2007; Oprea and Perelson, 1997; Victora et al., 2010). It has been proposed that transit between LZ and DZ is prompted by external cues found in each of the two GC compartments (Victora et al., 2012; Victora and Nussenzweig, 2012). This idea has been recently challenged to suggest that the DZ-to-LZ transition is driven by a cellular "timer" of yet unknown nature, which is cyclically reset by T-cell-dependent affinity-based selection events in the LZ (Bannard et al., 2013; Gitlin et al., 2014). Although several transcription factors have been identified for their involvement in the establishment or maintenance of the GC structure, little is known about those that drive DZ versus LZ polarity and GC B cell interzonal transit.

Because the pathogenesis of GC-derived B cell lymphomas often involves the deregulation of key pathways controlling GC development, our attention was captured by FOXO1, a member of the Fox-O family of Forkhead transcription factors (Eijkelenboom and Burgering, 2013; Hedrick, 2009) that is recurrently targeted by specific mutations in Burkitt lymphoma (BL) and diffuse large B cell lymphoma (DLBCL) (Morin et al., 2011, 2013; Pasqualucci et al., 2014; Schmitz et al., 2012; Trinh et al., 2013). FOXO1 has been shown to play essential activities at critical stage transitions during B cell development (Dengler et al., 2008; Srinivasan et al., 2009). Its role has been characterized in
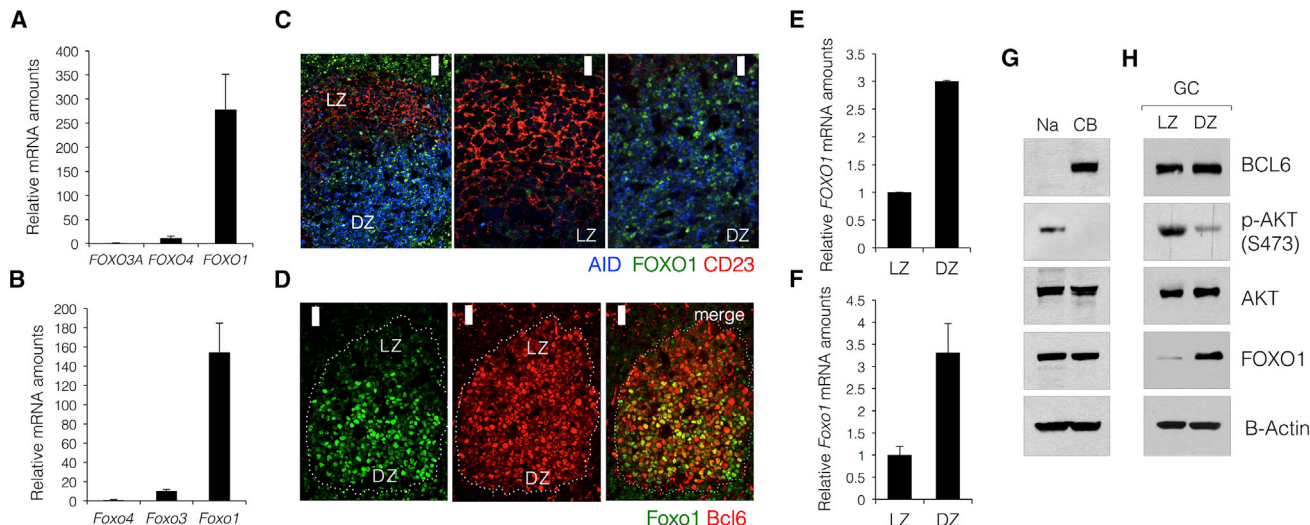
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Figure 1. FOXO1 Protein Expression Is Restricted to the GC Dark Zone

(A and B) Relative mRNA amounts of FOXO gene family members in human (A) and mouse (B) GC B cells, as measured by quantitative RT-PCR (average ± SD; n = 3). Data are shown as fold change relative to the gene with the lowest expression (arbitrarily set to 1), after normalization to the expression of a reference housekeeping gene (*Actb*, beta-actin).

(C) Immunofluorescence on paraffin sections of human tonsil tissue. CD23 labels follicular dendritic cells (i.e., light zone; LZ) and AID the dark zone (DZ), respectively. Scale bars represent 100 μm.

(D) Immunofluorescence on paraffin section of murine spleen (10 days post-SRBC immunization). Bcl6 staining is used to identify GC B cells. Scale bars represent 50 μm.

(E and F) Relative *Foxo1* mRNA amounts in human (E) and mouse (F) DZ versus LZ GC B cells (average ± SD; n = 3). Data are shown as fold change relative to the expression in LZ GC B cells (arbitrarily set to 1), after normalization to *Actb* expression.

(G and H) Immunoblot analysis in purified human tonsillar B cell populations (abbreviations are as follows: Na, naive B cells; CB, CD77^{+} GC B cells) (G) and LZ and DZ GC B cells (H). Naive B cells were purified with anti-IgD antibodies, which can transiently induce modest activation of BCR signaling (Rickert, 2013). Shown are the results of one representative experiment out of three.

greater detail in immature B cells, where FOXO1 controls the expression of stage-specific genes either directly or through complex transcriptional interactions (Lin et al., 2010; Mansson et al., 2012). FOXO1 deletion during early B cell development results in loss of *Il7ra*, *Rag1*, and *Rag2* expression, defective V to (D)J rearrangement, and a severe developmental blockade at the pro-B cell stage due to compromised proliferation, survival, and differentiation (Amin and Schlissel, 2008; Dengler et al., 2008). Relatively less characterized is the role of FOXO1 in mature B cells and, in particular, GC development. Previous work shows that ablation of FOXO1 in mature B cells does not apparently interfere with GC development, but prevents CSR both in vivo and in vitro, a defect that has been attributed to reduced transcription of activation-induced cytidine deaminase (AID) (Dengler et al., 2008; Omori et al., 2006).

In order to analyze in greater detail the role of FOXO1 in GC development, we studied mice engineered to specifically delete FOXO1 in GC B cells. We show that, in contrast to the initial findings in mature B cells (Dengler et al., 2008), GC development in the absence of FOXO1 is only apparently normal. In fact, FOXO1 deletion prevented the formation of GC DZs, consistent with its restricted expression and the lack of PI3K signaling in this compartment. FOXO1 was also essential for antibody affinity maturation as well as CSR and functions in part by licensing the activity of BCL6, a GC master regulator (Basso and Dalla-Favera, 2012). These findings have implications for our understanding of the normal physiology of the GC reaction and the role of

FOXO1 mutations in the pathogenesis of GC-derived B cell lymphomas.

RESULTS

Polarity of the PI3K-FOXO1 Axis in GCs

In order to comprehensively determine the pattern of expression of FOXO family members in GC B cells, we examined mRNA expression of *FOXO1* (*Foxo1*), *FOXO3A* (*Foxo3a*), and *FOXO4* (*Foxo4*). The results show that *FOXO1* (*Foxo1*) was the only family member effectively expressed in human and murine GC B cells (Figures 1A and 1B). FOXO1 protein expression was confirmed by immunofluorescence and immunoblot analyses in GC B cells—where notably, expression was restricted to the DZ (Figures 1C, 1D, and 1H)—and in naive B cells (Figures 1C and 1G). DZ GC B cells showed almost ubiquitous nuclear expression of FOXO1 (Figures 1C and 1D). The polarity in FOXO1 protein expression correlated with about 3-fold higher expression of FOXO1 transcripts in DZ GC B cells (Figures 1E and 1F), suggesting transcriptional regulation. Because the activity of FOXO factors and their subcellular localization is also controlled by the phosphatidylinositol 3′ OH kinase (PI3K) pathway (Burgering, 2008), we examined the presence of Ser473-phosphorylated AKT kinase, a bona fide surrogate of PI3K pathway activity, in purified DZ and LZ GC B cells. We found that PI3K activity was virtually absent in DZ B cells and was restricted to LZ B cells (Figure 1H). Thus, DZ B cells were

characterized by FOXO1 nuclear expression and absence of PI3K signaling, whereas PI3K signaling was detected in LZ GC B cells, where FOXO1 expression was largely absent.

FOXO1 Is Essential for the Development and Maintenance of the GC Dark Zone

To understand the contributions of FOXO1 to the control of GC programs, we analyzed mice in which *Foxo1* was conditionally deleted in GC B cells by crossing mice carrying floxed *Foxo1* alleles (*Foxo1*^{fl/fl}) (Paik et al., 2007) with Cγ1-*cre* animals (Casola et al., 2006). The resulting *Foxo1*^{fl/fl}×Cγ1*cre* mice were able to generate normal numbers of GCs and Bcl6^{+} GC B cells upon immunization with sheep red blood cells (SRBCs), despite the complete lack of Foxo1 expression in these cells (Figures 2A, 2B, and S1A–S1C). However, *Foxo1*-null GCs were completely devoid of phenotypic DZs, which are typically identified by the surface expression of the Cxcr4 chemokine receptor, Cd86, and Cd83 (Cxcr4^{hi}Cd86^{lo}Cd83^{lo}) (Victora et al., 2010, 2012), and were entirely composed of B cells displaying the phenotype of LZ cells (Cxcr4^{lo}Cd86^{hi}) (Figures 2C and 2D). This specific defect was not seen in *Foxo1*^{+/fl} mice, which developed GCs with normal polarity (Figures 2C and 2D). The absence of DZs was already apparent in early GCs (day 4) and continued throughout the GC reaction (Figures S1D and S1E), although it did not prevent GCs from persisting until late stages (21 days) (Figure S1F and S1G). Post-GC differentiation was not affected, as shown by the normal number of plasma cells at 3 weeks post-immunization (Figures S1H and S1I). Although cell division is known to be largely restricted to the DZ in normal GCs (Allen et al., 2007; Gitlin et al., 2014; Victora et al., 2010), *Foxo1*-null, LZ-only GCs contained a fraction of proliferating B cells equivalent to that observed in *Foxo1*^{+/+} GCs (Figures 2E and 2F), suggesting that cell cycle and phenotypic polarity can be uncoupled in GC B cells (see Discussion).

The loss of the DZ compartment in *Foxo1*-null GCs was confirmed by the analysis of gene expression profiles, which showed a significant (p ≤ 10^{−3}) absence of gene signatures associated with the DZ program and the predominance of signatures characteristic of LZ GC B cells (Figure 2G). Only minor transcriptional changes were observed in *Foxo1*^{+/fl} GC B cells, which were consistent with their normal LZ and DZ distribution and suggestive of a negligible effect of *Foxo1* partial loss (Figure S2A). A considerable fraction of a gene signature that distinguishes DZ from LZ GC B cells in both human and mouse (Victora et al., 2012) was differentially expressed in *Foxo1*-null as compared to *Foxo1*^{+/+} GC B cells (Figure S2B, Table S1). The expression pattern of these genes in *Foxo1*-null cells largely resembled that of LZ GC B cells, and included *Cxcr4* and *Cxcr5*, which encode for chemokine receptors with a critical role in the maintenance of GC architectural polarity (Allen et al., 2004; Nie et al., 2004); *Cd86*, which encodes for the ligand of Cd28 and Ctla4 and is differentially enriched in the surface of LZ GC B cells (Victora et al., 2010); and *Prdm1* and *Irf4*, which encode for transcription factors required for plasma cell differentiation (Figure S2B, Table S1; Klein et al., 2006; Shapiro-Shelef et al., 2003). Notably, DZ-specific genes like *Aicda* and *Tcf3* and the LZ gene *Cd83* were not found to be differentially expressed (Figures S2C and S2D; Table S1). Quantitative analysis confirmed the altered expression patterns of these gene transcripts (Figure 2H). In

agreement with these results (Allen et al., 2004; Bannard et al., 2013), *Foxo1*-null GCs lacked proper stromal polarization and showed an even distribution of the follicular dendritic cell (FDC) network throughout this B cell compartment (Figure 2I). Overall, these results demonstrate that FOXO1 expression is an essential requirement in the establishment and maintenance of GC polarity and the DZ GC B cell phenotype.

Normal SHM but Loss of Affinity Maturation in *Foxo1*-Null GCs

Next, we investigated whether the loss of DZs in *Foxo1*-null GCs resulted in altered SHM, which is considered a typical DZ function (De Silva and Klein, 2015; Victora and Nussenzweig, 2012), as well as SHM-based affinity maturation, a process coupled to the cyclic transit of GC B cells between DZ and LZ (Allen et al., 2007; Gitlin et al., 2014; Victora et al., 2010). To this end, we immunized GC-specific *Foxo1*-null mice and *Foxo1*^{+/+} littermates with 4-hydroxy-3-nitrophenyl-acetyl (NP) hapten conjugated to keyhole limpet hemocyanin (KLH), which induces T-cell-dependent immune responses (Allen et al., 1988; Shih et al., 2002). The magnitude of the GC response and the defects in GC polarity observed upon SRBC immunization (Figures 2 and S1) were also reproduced in response to NP-KLH (Figure S3A). Because the identity of the mutations at IgV regions conferring high and low affinity for the NP-hapten are known (Weiss and Rajewsky, 1990), with this approach it is possible to (1) analyze SHM activity by examining the number of mutations in IgV regions and (2) to accurately assess affinity maturation by examining the appearance of NP-specific mutations in Lambda1-chain-bearing antibodies expressing the V186.2 VH gene, which dominate the anti-NP response (Allen et al., 1988; Shih et al., 2002). The results of this analysis showed that *Foxo1*-null GCs had a severely reduced number of B cells carrying CDR1 Trp33-Leu (W33L) high-affinity mutations (24.7% versus 60.5% in *Foxo1*^{+/+} GCs, p < 2 × 10^{−4}; Figure 3A) and consequently were defective in affinity maturation. This conclusion was further supported by the significant decrease in the numbers of NP-binding and Ig-Lambda-positive cells in *Foxo1*-null GCs (Figure S3B). However, the mutational load at day 14 post-immunization was not significantly lower in either the V186.2 regions (2.01 × 10^{−2} versus 1.53 × 10^{−2}, p = 0.17), which were subjected to NP-driven selection, or in the JH4 intron (2.80 versus 2.63, p = 0.83), which was not under selection (Figures 3B–3D). The evidence of normal SHM activity was consistent with normal *Aicda* mRNA and Aid protein expression (Figure S4). Thus, FOXO1 is not required for SHM, which can function even in a LZ context, but it is necessary for effective affinity maturation.

Defective CSR in *Foxo1*-Null GC

As previously observed in *Foxo1*^{fl/fl};*Cd21-cre* mice (Dengler et al., 2008), we found that the fraction of IgG1-switched B cells in *Foxo1*-null GCs was severely reduced, with the subsequent accumulation of IgM[+] GC B cells (Figures 4A and 4B). The defect in class-switch recombination was also observed in splenic B cells cultured ex-vivo and stimulated to switch to IgG1 (and IgG3) with lipopolysaccharide plus interleukin-4, as previously reported (Figure S5; Dengler et al., 2008). However, Aid expression in *Foxo1*-null GCs appeared to be unaltered in vivo
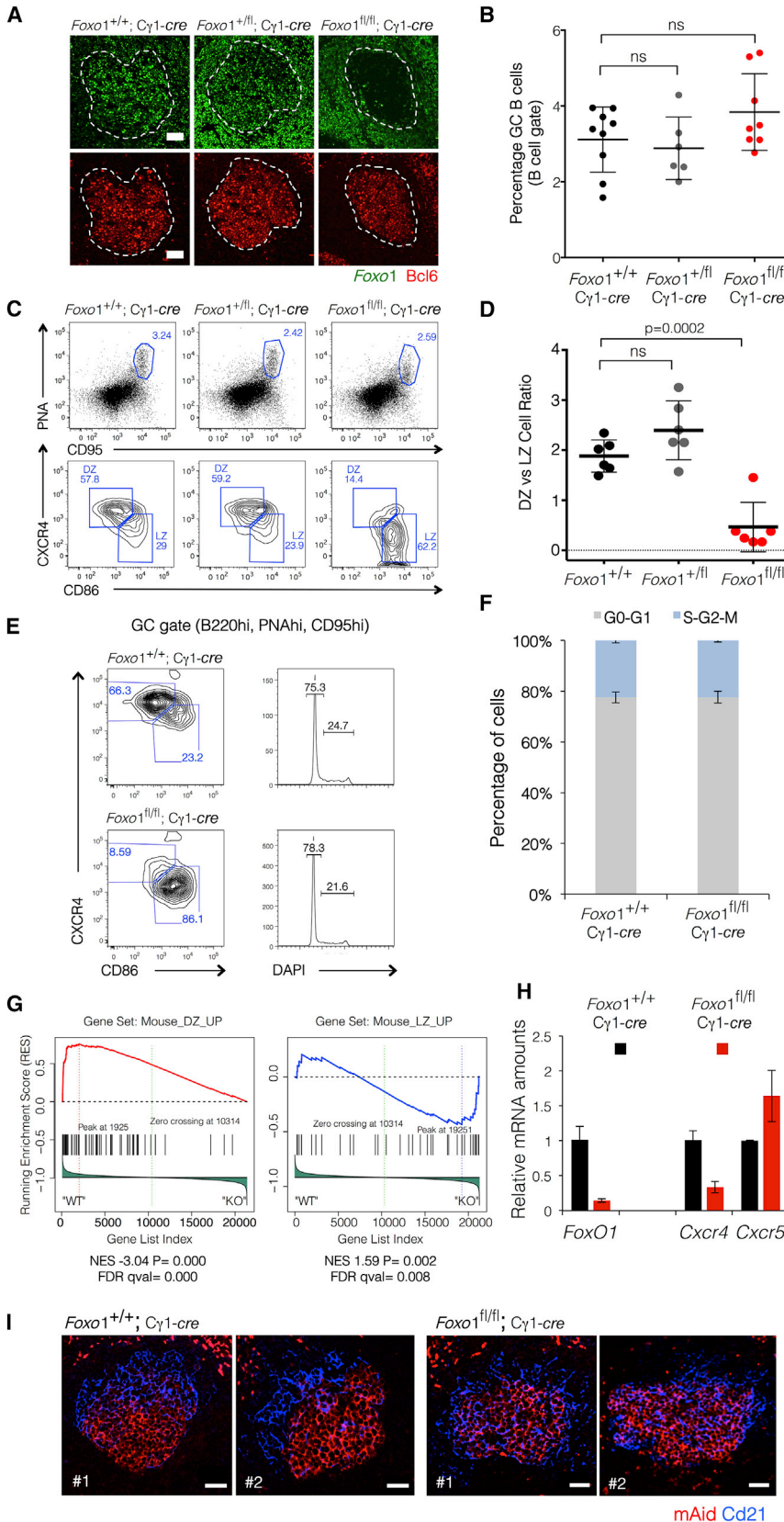
Figure 2. Loss of Dark Zone in *Foxo1*-Null GCs

(A) Immunofluorescence analysis of Foxo1 and Bcl6 protein expression on paraffin-embedded mouse spleen sections from *Foxo1*$^{fl/fl}$×Cγ1*cre*, *Foxo1*$^{+/fl}$×Cγ1*cre*, and *Foxo1*$^{+/+}$×Cγ1*cre* mice. Scale bars represent 100 μm.

(B) Percentage of splenic GC B cells (B220$^+$CD95^{hi}PNA^{hi}) in *Foxo1*$^{+/+}$ (n = 9), *Foxo1*$^{+/fl}$ (n = 6), and *Foxo1*$^{fl/fl}$ (n = 8) mice, as measured by cytofluorimetric analysis 10 days post-SRBC immunization.

(C) Representative contour plots of GC B cell DZ (Cxcr4^{hi}Cd86^{lo}) and LZ (Cxcr4^{lo}Cd86^{hi}) populations (n = 6 mice per genotype, 10 days post-SRBC immunization).

(D) Quantification of DZ versus LZ cell ratios (ratio of percentages of GC B cells in DZ or LZ gates), as displayed in (C) (average ± SD; n = 6).

(E) DZ versus LZ distribution (contour plots) and cell cycle profile histograms (DAPI = DNA content) of GC B cells from *Foxo1*$^{+/+}$ and *Foxo1*$^{fl/fl}$ mice (n = 3, one representative is displayed). Analysis was performed 8 days post-SRBC immunization.

(F) Percentage of GC B cells at different stages of the cell cycle (as shown in E; mean ± SEM; n = 3).

(G) Gene set enrichment analysis for DZ and LZ mouse gene signatures (Victora et al., 2012) in *Foxo1*$^{+/+}$ (WT) and *Foxo1*$^{fl/fl}$ (KO) GC B cell expression data (see also Figure S2, Table S1).

(H) Relative mRNA amounts (quantitative RT-PCR) of *Foxo1*, and *Cxcr4*, *Cxcr5* chemokine receptor genes in *Foxo1*$^{+/+}$ and *Foxo1*-null GC B cells (average ± SD; n = 3).

(I) Immunofluorescence analysis in *Foxo1*$^{+/+}$ and *Foxo1*-null GCs, 10 days post-SRBC immunization (spleen). Two representative GCs in two different animals are shown per genotype. Cd21 highlights follicular dendritic cells. mAid is normally polarized to the DZ in *Foxo1*$^{+/+}$ GCs (Victora et al., 2012). Scale bars represent 100 μm.
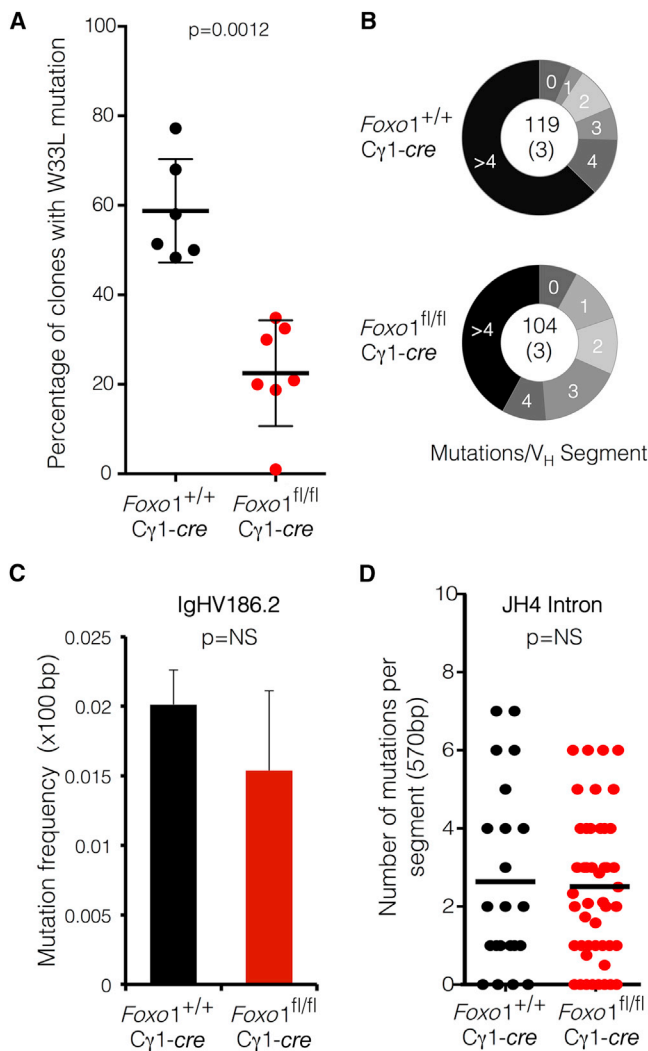
See also Figures S1 and S2.

Figure 3. Normal Somatic Hypermutation, but Loss of Affinity Maturation in *Foxo1*-Null GCs

(A) Percentage of NP high-affinity clones (carrying the W33L mutation in CDR1) in purified bulk GC B cells of *Foxo1*^+/+^ and *Foxo1*^fl/fl^ mice, 14 days post-immunization with NP-KLH (average ± SD). Each dot corresponds to a single animal (~30 unique clones/mouse; Mann-Whitney test).

(B) Distribution of the number of mutations per unique clones (VH186.2 segment). Numbers in the center of each pie refer to the number of individual sequences and (in brackets) number of analyzed animals.

(C) Mutation frequencies (number of mutations per 100 base pairs) in VH186.2 IgHV segments (average ± SD; n = 3; Mann-Whitney test).

(D) Mutation frequencies (number of mutations per segment) in JH4 intron (average ± SEM; n = 6) (p > 0.05, NS; Mann-Whitney test).

See also Figures S3 and S4.

(Figure S4). Therefore, we considered the possibility that absence of Foxo1 would preclude normal transcription at the immunoglobulin locus (i.e., *Ighg1*) and as a result, prevent class switch recombination (CSR). This notion was also suggested by the reduction in *Ighg1* transcripts observed in the gene expression profiles of these cells (Table S1).

We then used quantitative RT-PCR to conclusively assess the relative abundance of the diverse array of transcripts originating

from the IgH locus in GC B cell fractions and observed a severe reduction in germline (GLT) and post-switch (PST) transcripts arising from *Ighg1* in *Foxo1*-null B cells, in accordance with the lack of surface IgG1 expression (Figure 4C). *Ighg2b* also showed defective production of GLTs and PSTs in the splenic GC B cell population, whereas the abundance of *I-mu*, *IghA*, or *Ighg3* transcripts was not altered (Figures S6A and S6B). These observations were consistent with the quantification of switched GC B cells as measured by flow cytometry analysis (Figure S6C). These defects suggested a specific function for FOXO1 in regulating immunoglobulin GLTs, which was congruous with the presence of FOXO1 binding in three regions within the immunoglobulin heavy chain locus (IgH) in human GC B cells (Figure S6D and Table S2). These FOXO1-bound regions were specifically located at the I-mu and 3′ regulatory enhancers (Manis et al., 2002), as well as within a central super-enhancer region that lies centromeric to *IGHA1* (Figure S6D; Qian et al., 2014). This binding pattern was in agreement with the involvement of FOXO1 in the transcriptional regulation of the IgH locus. An equivalent spatial distribution of super-enhancers has been reported at the murine Ig locus (Kieffer-Kwon et al., 2013), implying a substantial conservation of local regulatory features across species. In addition, gene expression analysis of *Foxo1*-null GC B cells showed a severe reduction in Basic leucine zipper transcription factor ATF-like (*Batf*) transcripts (Figure S2B, Table S1). Batf is required for the expression of Ig germline transcripts and for effective CSR (Ise et al., 2011). Taken together, these results indicate that Foxo1 is required for CSR in vivo and suggest that impaired CSR in *Foxo1*-null GCs is at least in part due to defective transcriptional regulation at the IgH locus, specifically of germline transcripts and of *Batf*.

FOXO1 Transcriptional Program

To better understand the basis for FOXO1's requirement in the maintenance of the DZ program, we dissected its transcriptional network in GC B cells. By combining chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) with gene expression profiling, we could identify genomic loci bound by FOXO1, the expression of which changed in coordination with FOXO1 expression. ChIP-seq analysis performed in two distinct pools of GC B cells (CD77^+^) isolated from human tonsils identified ~4,500 genomic regions bound by FOXO1 in both biological replicates (Table S2). About half of these regions were found in close proximity (−5 kb/+4 kb) to transcription start sites (TSS; 21%) or at intragenic regions (29%), and the other half (50%) of FOXO1-bound regions were located within intergenic regions (Figure 5A). FOXO1 binding at gene promoters occurred mainly in close proximity (±1 kb) to TSS (Figure 5B), as commonly observed for other transcription factors (Koudritsky and Domany, 2008). However, FOXO1 was preferentially found at distal regions (>5 kb from the TSS), suggesting that it might have an important *cis*-regulatory role through direct participation at enhancer elements (Figure 5B). As expected, FOXO1-bound regions were significantly enriched for FOXO1 consensus binding sites (p ≤ 10^−10^; Figure 5C, Table S3).

Consistent with a role for FOXO1 in DZ formation, FOXO1 binding occurred preferentially at promoters of genes differentially expressed between DZ and LZ GC B cells (Figure 5D). Specifically, FOXO1 binding was detected at the promoter
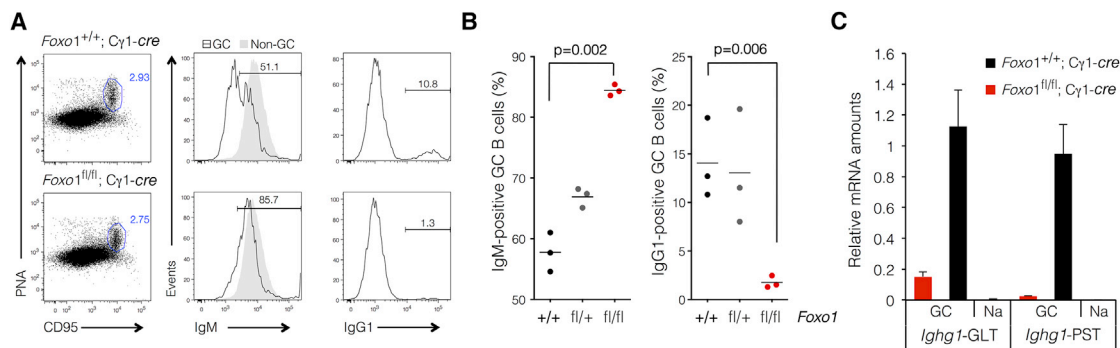
Figure 4. Defective IgG1 Class Switch Recombination in *Foxo1*-Null GCs

(A) Flow cytometry analysis of Ig class-switch events in *Foxo1*$^{+/+}$ and *Foxo1*$^{fl/fl}$ mouse GC B cells (spleen). Representative data for one out of three animals per genotype are displayed. The shadowed histograms correspond to surface IgM expression in non-GC B cells and are used here as a reference.

(B) Percentage of IgM$^+$ (left) and IgG1$^+$ (right) GC B cells in *Foxo1*$^{+/+}$, *Foxo1*$^{+/fl}$, and *Foxo1*$^{fl/fl}$ mice. Analysis was performed 14 days post-SRBC immunization (n = 3; Student's t test, two-tailed, unequal variance).

(C) Relative mRNA amounts measured by quantitative RT-PCR of germline (GLT) and post-switch (PST) IgG1 transcripts in FACS-sorted naive (Na) and in GC B cells of *Foxo1*$^{+/+}$ and *Foxo1*$^{fl/fl}$ mice (average ± SD; n = 3). GLT transcript amounts are shown for one out of three distinct primer sets.

See also Figures S5 and S6 and Table S6.

regions of genes that displayed significant downregulation (137 genes) or upregulation (235 genes) in DZ B cells (Table S2). This observation suggests a dual function for this factor both as a repressor and activator of transcription, and/or indicates the presence of indirect gene targets where FOXO1 might have no regulatory function despite binding to their promoters (see Discussion).

A majority of genes bound by FOXO1 and downregulated in DZ GC B cells were involved in the control of archetypal LZ programs, including B cell receptor (BCR) and CD40 signaling, T-cell-mediated activation, and cytokine and chemokine signaling (Table S2). In addition, we detected FOXO1 binding about 900 bp upstream of the *PRDM1-beta* isoform, and we showed that, as previously suggested (Vogel et al., 2014), its

binding contributes to *PRDM1* repression, consistent with the lack of PRDM1 expression in DZ B cells (see below and Figure 6).

The loci bound by FOXO1 and upregulated in DZ GC B cells were significantly enriched in positive modulators of proliferation (cell cycle and DNA replication) and negative modulators of DNA repair (e.g., *MDM2* and *MDM3*, which negatively control TP53) (Table S2). Of particular relevance was the upregulation of *CXCR4*, which encodes a chemokine receptor required for the organization of normal LZ and DZ compartments. Loss of CXCR4 expression has been previously demonstrated to prevent GC B cells from transiting to the DZ (Allen et al., 2004; Bannard et al., 2013; Nie et al., 2004). Collectively, these results suggest that FOXO1 might contribute to the LZ-to-DZ transition by silencing signaling pathways characteristic of the GC LZ
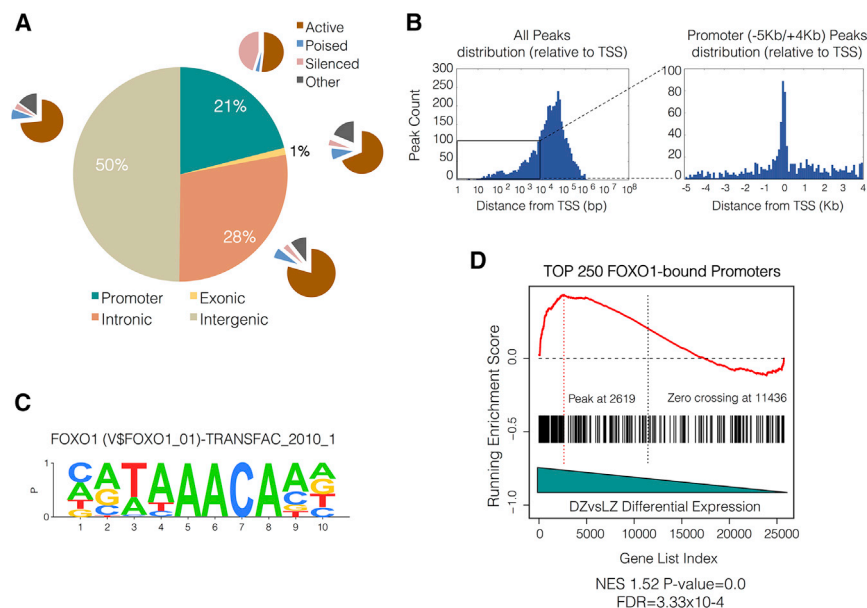

Figure 5. FOXO1 Transcriptional Network in GC B Cells

(A) Summary of the distribution of genomic bound regions (ChIP-seq) in human CD77$^+$ GC B cells. Regions are annotated as promoter (−5 kb/+4 kb around the transcription start site, TSS), intronic, exonic, or intergenic. The distribution of active, poised, silenced, and other regions based on the repertoire of histone modifications is shown per each type of bound loci (Ernst et al., 2011).

(B) Distribution of FOXO1 peaks in relation to their distance from the closest TSS for all the regions (left) or only for the regions found in proximity (−5 kb/+4 kb) of the TSS (right).

(C) Position weight matrix of FOXO1 binding site motif.

(D) Pre-ranked gene set enrichment analysis of the most significantly (top 250) FOXO1-bound genes in the genes differentially expressed between DZ and LZ B cells.
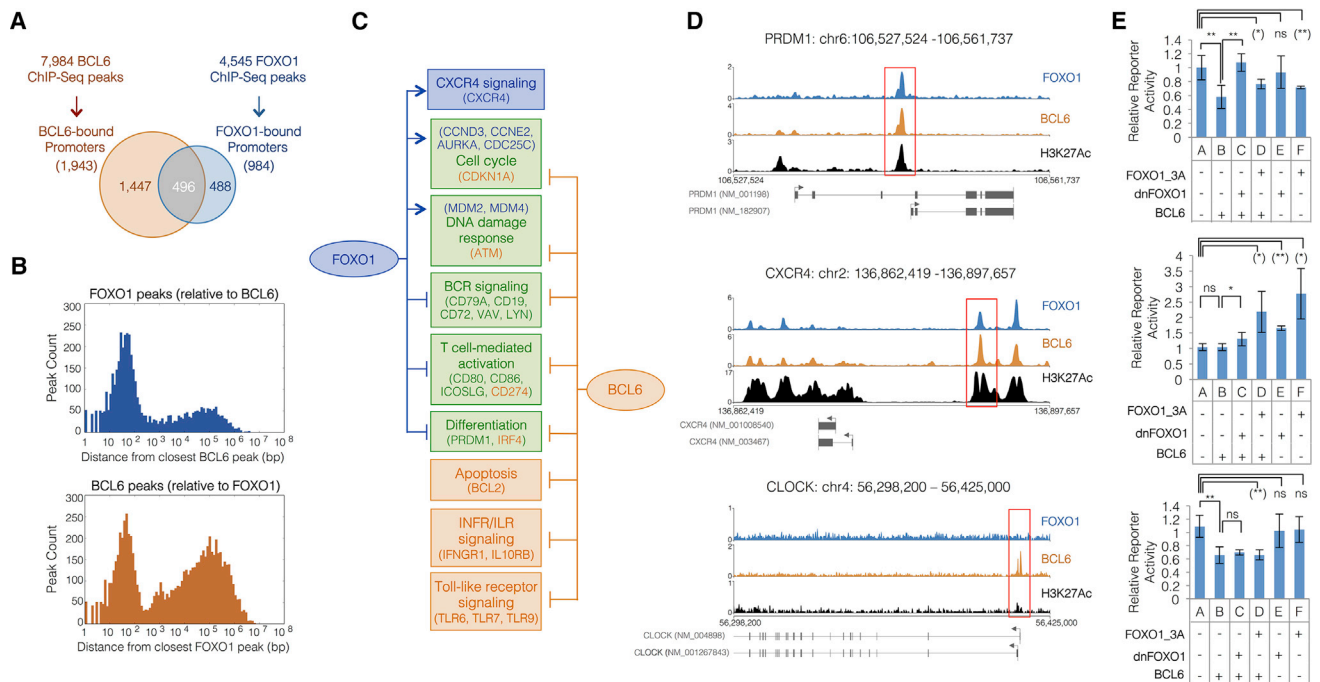
See also Tables S2 and S3.

A

7,984 BCL6
ChIP-Seq peaks

4,545 FOXO1
ChIP-Seq peaks

BCL6-bound
Promoters
(1,943)

FOXO1-bound
Promoters
(984)

1,447 | 496 | 488

B

FOXO1 peaks (relative to BCL6)

Peak Count
Distance from closest BCL6 peak (bp)

BCL6 peaks (relative to FOXO1)

Peak Count
Distance from closest FOXO1 peak (bp)

C

FOXO1

CXCR4 signaling
(CXCR4)

(CCND3, CCNE2,
AURKA, CDC25C)
Cell cycle
(CDKN1A)

(MDM2, MDM4)
DNA damage
response
(ATM)

BCR signaling
(CD79A, CD19,
CD72, VAV, LYN)

T cell-mediated
activation
(CD80, CD86,
ICOSLG, CD274)

Differentiation
(PRDM1, IRF4)

Apoptosis
(BCL2)

INFR/ILR
signaling
(IFNGR1, IL10RB)

Toll-like receptor
signaling
(TLR6, TLR7, TLR9)

BCL6

D

PRDM1: chr6:106,527,524 -106,561,737

FOXO1
BCL6
H3K27Ac

106,527,524   106,561,737
PRDM1 (NM_001198)
PRDM1 (NM_182907)

CXCR4: chr2: 136,862,419 -136,897,657

FOXO1
BCL6
H3K27Ac

136,862,419   136,897,657
CXCR4 (NM_001008540)
CXCR4 (NM_003467)

CLOCK: chr4: 56,298,200 – 56,425,000

FOXO1
BCL6
H3K27Ac

56,298,200   56,425,000
CLOCK (NM_004898)
CLOCK (NM_001267843)

E

Relative Reporter Activity

** ** (*) ns (**)

	A	B	C	D	E	F
FOXO1_3A	−	−	−	+	−	+
dnFOXO1	−	−	−	−	+	+
BCL6	−	+	+	+	−	−

Relative Reporter Activity

ns * (*) (**) (*)

	A	B	C	D	E	F
FOXO1_3A	−	−	−	+	−	+
dnFOXO1	−	−	+	−	+	+
BCL6	−	+	+	+	−	−

Relative Reporter Activity

** ns (**) ns ns

	A	B	C	D	E	F
FOXO1_3A	−	−	−	+	−	+
dnFOXO1	−	−	+	−	+	+
BCL6	−	+	+	+	−	−

Figure 6. Cooperative Transcriptional Control by FOXO1 and BCL6

(A) Overlap of FOXO1 and BCL6 bound promoters (−5 kb/+4 kb around TSS), as consistently assessed by ChIP-seq in two biological replicates of human (CD77^{+}) GC B cells.

(B) Distribution of FOXO1 (top) or BCL6 (bottom) bound peaks in relationship to their distance from each other's closest peak.

(C) Schematic representation of the pathways affected by BCL6 and FOXO1 coordinately (green) or in a specific fashion.

(D) ChIP-seq read tracks for *PRDM1*, *CXCR4*, and *CLOCK* loci. Red boxes highlight the bound regions subcloned in luciferase reporter vectors used in (E).

(E) Relative reporter activity measured in transient luciferase (nanoluc) reporter assays performed in Ramos B cell line, using vectors featuring the same regulatory regions highlighted in red in (D) (average ± SD; n = 6). Abbreviations are as follows: dnFOXO1, dominant-negative FOXO1; FOXO1-3A, constitutively active FOXO1 (Nakae et al., 2000).

See also Table S4.

program (activation and differentiation) while sustaining those functions typical of DZ GC B cells (cell proliferation and negative modulation of DNA repair).

Cooperative Transcriptional Control by FOXO1 and BCL6

Although a number of transcription factors have been shown to be required for GC development, little is known about the possible relationship among their activities. In order to explore this issue, and in particular to identify transcription factors important for FOXO1 function in GC B cells, we used the MINDy and MARINa algorithms, which predict modulators of transcriptional networks based on mutual information analysis of gene expression and on the co-regulation of transcriptional signatures, respectively (Lefebvre et al., 2010; Wang et al., 2009). This initial analysis identified FOXO1 as a modulator of the activity of BCL6, a GC-specific proto-oncogene and master regulator of GC formation.

To further explore this relationship, we first investigated the possible overlap in target gene binding by BCL6 and FOXO1 via paired ChIP-seq analysis in two independent biological replicates of human GC B cells. This analysis revealed a significant overlap between genomic regions bound by these two transcription factors (p = 2.2 × 10^{−16}). This overlap involved ∼500 loci

corresponding to 25% of all BCL6-bound promoters (496 out of 1,943) and 50% of FOXO1-bound promoters (496 out of 984) (Figure 6A; Table S4). Overall, a majority of FOXO1-bound regions were found to cluster in close proximity (<1,000 bp) to BCL6-bound regions, whereas BCL6 binding occurred with FOXO1 in a distinct group of targets, as well as in a large number of additional loci (Figure 6B).

These results identified pathways co-regulated by BCL6 and FOXO1 through binding to the same regulatory regions, pathways independently regulated by each gene, as well as those co-regulated through distinct targeting (Figure 6C; Table S4). The set of FOXO1 and BCL6 co-bound genes downregulated in DZ B cells was significantly enriched for genes involved in the modulation of BCR signaling, T-cell-mediated B cell activation, and plasma cell differentiation (*PRDM1*). Conversely, other BCL6-dependent GC cellular programs, including apoptosis, as well as the interferon, interleukin, and Toll-like receptor signaling pathways (Basso et al., 2010; Ci et al., 2009), were not included among the FOXO1 target gene repertoire (Figure 6C; Table S4). Of interest was the dual and complementary action of FOXO1 and BCL6 on modulating DNA repair—by BCL6-mediated suppression of *TP53* (Phan and Dalla-Favera, 2004)—and activation of its negative regulator *MDM2* by FOXO1, and enforcing or sustaining proliferation—through suppression of the cell-cycle

arrest gene *CDKN1A* (Phan et al., 2005) by BCL6 and activation of positive cell cycle modulators by FOXO1.

To further address the functional relationship between FOXO1 and BCL6 in the modulation of specific gene targets, we performed reporter assays in a GC-derived BL cell line (Ramos), which expresses both BCL6 and nuclear FOXO1. We specifically analyzed the effects of FOXO1 and BCL6 on a group of target promoter and regulatory regions (*PRDM1*, *CXCR4*, and *CLOCK*) that display differential binding by either one or both of these two factors at their regulatory regions. In these assays, FOXO1 activity was manipulated by using a dominant-negative form (FOXO1Δ256; dnFOXO1) that acts by displacing endogenous FOXO1 from its targets, or by using a constitutively active, AKT-insensitive form of FOXO1 (FOXO1-3A) (Nakae et al., 2000).

As a representative example of BCL6 and FOXO1 shared targets that are downregulated in DZ B cells, we tested the regulatory region located just upstream of the PRDM1-beta isoform (Figure 6D, top). Whereas co-expression of FOXO1 and BCL6 decreased *PRDM1* reporter activity significantly, expression of dnFOXO1 was able to override BCL6 repression. Conversely, the presence of FOXO1-3A restored BCL6 repression, suggesting that at this *PRDM1* regulatory region, BCL6-mediated repression is dependent on FOXO1 (Figure 6E, top). Modulation of the *CXCR4* locus was tested as an example of a BCL6 and FOXO1 co-bound target that is upregulated in DZ B cells. The analyzed *CXCR4* regulatory region, located in a putative enhancer 5′ to *CXCR4* (Figure 6D, middle), was unresponsive to BCL6, whereas it was activated by FOXO1-3A expression. These data suggest that FOXO1, but not BCL6, contributes to *CXCR4* transcriptional activation, as genomic binding does not always imply transcriptional regulation (Figure 6E, middle). Finally, we tested the BCL6-bound promoter region of a canonical BCL6 target (*CLOCK*), which is specifically downregulated in GC B cells (Figure 6D, bottom; Basso et al., 2010). Consistent with the ChIP-seq profile at this locus (bound only by BCL6), transcription of the *CLOCK* reporter construct was efficiently repressed by BCL6 but unresponsive to FOXO1 (Figure 6E, bottom).

Overall, the data described above suggest that FOXO1 and BCL6 cooperate in the transcriptional modulation of a subset of targets including the plasma cell master regulator PRDM1 and point to a licensing role for FOXO1 in a fraction of the BCL6 transcriptional repertoire in GC B cells.

DISCUSSION

The first finding of our study is that the GC DZ zone is characterized by the expression of FOXO1 with the consequent absence of PI3K signaling, which is detectable only in the LZ. Although we cannot exclude undetectable low activity of PI3K signaling that might be required to support "tonic" BCR signaling in DZ B cells (Srinivasan et al., 2009), the clear inverse relationship between FOXO1 expression and AKT phosphorylation, as well as the observation that the early experimental activation of PI3K in GC B cells causes the disappearance of the DZ (see the accompanying paper by Sander et al. [2015]), is consistent with a major polarized role of PI3K restricted to the LZ. This idea, together with previous results showing that NF-κB, a major transcriptional hub for several pathways including BCR, TNF receptor, and Toll-like receptor signaling, is not active in DZ B cells

(Basso et al., 2004; Shaffer et al., 2001), further supports the conclusion that the DZ is essentially a proliferative compartment, mainly dedicated to clonal expansion and sheltered from any immune activation signals. The absence or active repression of critical B cell pathways would make DZ B cells immune to premature activation and differentiation prior to proper clonal expansion and affinity selection.

The phenotype of *Foxo1*-null GCs, i.e., a fully formed but LZ-only GC, is notable in that it suggests a re-evaluation of a number of functions that are considered to typically characterize the DZ phenotype. First, the presence of actively proliferating cells displaying the phenotype of LZ B cells indicate that rapid proliferative expansion is not a specific program of DZ cells, but rather that it can be instructed at the initiation of the GC reaction and after each round of DZ cyclic reentry by signals that can be independent of a full DZ phenotype. Second, SHM, a typical function of DZ centroblasts (De Silva and Klein, 2015; Victora and Nussenzweig, 2012), can apparently also occur in LZ B cells, suggesting that AID induction and the entire machinery of SHM can be activated independent of the DZ program. Conversely, the selection of B cells expressing high-affinity antibodies to T-cell-dependent antigens, a function normally executed in the LZ of the GCs (Victora and Nussenzweig, 2012), is impaired in *Foxo1*-null LZ-only GCs. We suggest that absence of DZ programs and the premature initiation of signals promoting the activation and survival of GC B cells in *Foxo1*-null GCs might prevent effective affinity maturation by overriding the affinity selection checkpoints controlling cyclic re-entry of GC B cells from LZ to DZ. This notion is consistent with the constitutive expression of immune activation and BCR signaling programs observed in *Foxo1*-null GCs. Thus, a function of FOXO1 in GC B cells is to actively enforce the suppression of these programs as LZ cells transit into the DZ during cyclic reentry. The requirement for FOXO1 in this process might also explain why the simple repositioning of GC B cells, as it occurs in *Cxcr4*-deficient GCs (Allen et al., 2004; Bannard et al., 2013), is not sufficient to change their phenotype.

Our results confirm previous reports indicating that FOXO1 is required for CSR, a LZ-typical function that is surprisingly lost in the absence of DZ B cells. However, in contrast with previous reports (Dengler et al., 2008; Omori et al., 2006), we conclude that the CSR defect in these cells is apparently not due to a defect in AID expression, which we found normally active. This finding is further supported by the normal activity of SHM and the selective CSR defect involving only some Ig isotypes. We attribute the discrepancy with the previous report to the fact that our results show that in vivo, AID is normally present in *Foxo1*-null GC B cells, whereas previous conclusions were based on the lack of CSR and low AID expression in non-GC B cells activated in vitro (Dengler et al., 2008; Omori et al., 2006), a less physiological context where we have in fact confirmed the suboptimal induction of AID. Conversely, our results indicate that defective CSR might be at least in part due to faulty activation of Ig sterile transcripts in the absence of FOXO1, as suggested by the decreased expression of these transcripts in *Foxo1*-null GC B cells, as well as by the binding of FOXO1 to critical enhancer elements within the Ig locus. Nonetheless, we cannot fully exclude that indirect effects caused by reduced expression of additional factors (i.e., *Batf*)

in *Foxo1*-null GC B cells could also contribute to the defects in CSR (Ise et al., 2011).

Our analyses identify a large number of genes that are bound and differentially expressed in correlation with FOXO1 expression. However, none of these genes can be conclusively defined as a direct transcriptional FOXO1 target based only on combined ChIP-seq and GEP analysis. A second and related complication in the interpretation of our results is that both upregulated and downregulated genes appear in the set of candidate FOXO1 targets. This result might again reflect the indirect nature of some targets and/or the possible dual activity of FOXO1 as a trans-repressor or trans-activator and is in line with previous conflicting conclusions on its transcriptional function (Glauser and Schlegel, 2007; Greer and Brunet, 2005).

The set of direct and/or indirect target genes displays a significant enrichment in pathways that are consistent with a DZ function of FOXO1. We consider the overlap of the FOXO1 program with the BCL6 program to be of particular relevance, given the central regulatory role of BCL6 in GC physiology and GC-derived malignancies (Basso and Dalla-Favera, 2012; Hatzi and Melnick, 2014). Notably, a significant fraction of loci bound and apparently regulated by FOXO1 coincide with those bound and repressed by BCL6, including genes involved in the modulation of B cell receptor (BCR) signaling, T-cell-mediated B cell activation, and plasma cell differentiation (*PRDM1*). In the case of *PRDM1* we have shown that the trans-repressive function of BCL6 was dependent on the activity of FOXO1 on the same promoter region, an observation that suggests a strong cooperative action between these two transcription factors in establishing functions critical for DZ development. In addition to common programs, each gene appeared to have a separate set of targets, which, in the case of FOXO1, involved mostly a positive correlation with gene programs supporting cell proliferation. It is worth noting, however, that among those genes separately controlled by either transcription factor, we find some coherently regulating the same functions, but via opposite control of negative and positive regulators. Notable examples of these cooperative mechanisms are represented by the negative regulation of DNA repair, via upregulation of the negative regulator of TP53 *MDM2* by FOXO1 and direct downregulation of *TP53* by BCL6, and the positive regulation of cell proliferation via upregulation of cell cycle and DNA regulation genes by FOXO1 and trans-repression of the cell cycle arrest gene *CDKN1A* by BCL6.

Finally, the results herein provide some clues on the role of FOXO1 mutations in lymphomagenesis. Missense mutations, predominantly clustering on residues involved in PI3K-mediated phosphorylation and inactivation of FOXO1, are detectable in ~12% of BL and ~9% of DLBCL (Morin et al., 2011, 2013; Pasqualucci et al., 2014; Schmitz et al., 2012; Trinh et al., 2013). The recurrent and clustered nature of these mutations strongly supports their pathogenetic role in these malignancies. Initial functional analysis of such mutations has suggested that they might induce resistance to PI3K-mediated phosphorylation and inactivation in the cytoplasm (Trinh et al., 2013). The results of the present study suggest that constitutively active FOXO1 might contribute to malignant transformation by pathologically maintaining part of the DZ GC B cell program, including constitutive cell proliferation, blocking differentiation, and impairing DNA repair. The requirement of FOXO1 for BCL6-dependent repression of *PRDM1* suggests that constitutive FOXO1 activity might also be required for the oncogenic activity of deregulated BCL6 expression.

EXPERIMENTAL PROCEDURES

Mouse Strains and Immunization

Foxo1^{fl/fl} mice (FVB.129S6(Cg)-Foxo1tm1Rdp/J [Paik et al., 2007] back-crossed for >10 generations into the C57BL/6J background) and Cγ1-*cre* (B6.129P2(Cg)-Ighg1tm1(IRES-cre)Cgn/J) mice (Casola et al., 2006) were crossed to obtain GC-specific *Foxo1*-null animals. Mice were housed in specific-pathogen-free environments, and experiments conformed to the ethical principles and guidelines of our Institutional Animal Care and Use Committee.

~10- to 12-week-old mice were immunized with SRBCs (Cocalico Biologicals) or NP (4-hydroxy-3-nitrophenyl-acetyl) conjugated to KLH (BioSearch), mixed 1:1 in complete Freund's adjuvant (CFA; Sigma), by intraperitoneal injection (Victora et al., 2012).

B Cell Isolation and Flow Cytometry Analysis

Mononuclear cells were isolated from spleens of immunized mice with the Mouse B cell Isolation Kit (Miltenyi), as previously described (Victora et al., 2012). Cell pools were stained with specific antibodies (Table S5) and analyzed or sorted with a BD LSRII (or BD Fortessa) or a BD FACSAria device, respectively. GC B cell fractions were isolated as previously described (Victora et al., 2012). Further details on staining and flow cytometry procedures are provided in the Supplemental Experimental Procedures. FlowJo software (TreeStar; RRID: nif-0000-30575) was used for data analyses and plot rendering.

Immunofluorescence Analysis

All immunofluorescence analyses were performed on formalin-fixed, paraffin-embedded sections from mouse and human secondary lymphoid tissues, as previously described (Cattoretti, 2013; Cattoretti et al., 2006). The procedures are detailed in the Supplemental Experimental Procedures, and the antibody list is provided in Table S5.

Ex Vivo Class Switch Recombination and Affinity Maturation Analyses

For ex vivo class switch recombination assays, murine B cells were activated in vitro by co-stimulation with lypopolysaccharide (LPS) and mouse interleukin-4 (IL-4) for 3–4 days. Conditional deletion of *Foxo1* in *Foxo1*^{fl/fl} B cells was achieved with 45 min incubation with TAT-Cre (Excellgen). Cells were harvested at the end point and processed for gene expression and phenotypic analysis.

For the analysis of somatic hypermutation events and affinity maturation, we isolated genomic DNA from sorted murine GC B cells, 14 days after NP-KLH immunization. The IgH VH186.2-JH2 segment was amplified via high-fidelity PCR, as described (Schwickert et al., 2009). PCR products were subcloned and single colonies selected for Sanger sequencing. Sequence analysis and alignments were done with the HighV-Quest tool (The International Immunogenetics Information System, IMGT) (Lefranc, 2011). Only clones corresponding to the V186.2 gene (IGHV 72*01, IMGT nomenclature) were considered in the analysis.

Additional technical details are provided in the Supplemental Experimental Procedures.

Luciferase Reporter Assays

The Ramos Burkitt lymphoma cell line (ATCC CRL-1596) was electroporated (Neon system, Life Technologies) in multiple (6–10) replicates with reporter constructs encoding for Nanoluc luciferase (Promega) under the control of selected human regulatory regions (Supplemental Experimental Procedures). At 3 days post-electroporation, cells were harvested and reporter activity was measured with the Nano-Glo Luciferase Assay System (Promega).

Gene Expression Analyses via Quantitative RT-PCR and Microarray Profiling

cDNA was obtained from total RNA using a linear amplification method (Ovation RNA Amplification System; NuGEN), and it was subsequently used for SYBR green-based quantitative RT-PCR analysis or 3′ IVT expression

analysis (GeneChip Mouse Genome 430.2 arrays, Affymetrix). Primer sequences are detailed in Table S6. A detailed account of the experimental procedures for gene expression experiments and bioinformatics analysis of these data is included in the Supplemental Experimental Procedures.

ChIP-Seq Analyses

ChIP-seq experiments were performed on two independent samples of human tonsilar GC B cells after formaldehyde crosslinking and ultrasonication, via standard techniques. A detailed protocol of experimental procedures, with details on antibodies, chromatin immunoprecipitation steps, library preparation, and bioinformatics pipeline for data analysis can be found in the Supplemental Experimental Procedures.

Statistic Analyses

GraphPad Prism v.6.0 software (GraphPad, RRID: rid_000081) was used for all statistical analyses. The specific use of parametric (t tests) and non-parametric tests (Fisher's exact test and Mann-Whitney test) is reported in each figure legend.

ACCESSION NUMBERS

The gene expression profiling data have been deposited in the GEO database under accession number GSE69216. The ChIP-seq data have been deposited in the GEO database under accession number GSE68349.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.10.015.

AUTHOR CONTRIBUTIONS

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