Histone methyltransferase DOT1L controls state-specific identity during B cell differentiation

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Abstract

Differentiation of naive peripheral B cells into terminally differentiated plasma cells is characterized by epigenetic alterations, yet the epigenetic mechanisms that control B-cell fate remain unclear. Here, we identified a role for the histone H3K79 methyltransferase DOT1L in controlling B-cell differentiation. Mouse B cells lacking Dot1l failed to establish germinal centers (GC) and normal humoral immune responses in vivo. In vitro, activated B cells in which Dot1l was deleted showed aberrant differentiation and prematurely acquired plasma cell characteristics. Similar results were obtained when DOT1L was chemically inhibited in mature B cells in vitro. Mechanistically, combined epigenomics and transcriptomics analysis revealed that DOT1L promotes expression of a pro-proliferative, pro-GC program. In addition, DOT1L indirectly supports the repression of an anti-proliferative plasma cell differentiation program by maintaining the repression of Polycomb Repressor Complex 2 (PRC2) targets. Our findings show that DOT1L is a key modulator of the core transcriptional and epigenetic landscape in B cells, establishing an epigenetic barrier that warrants B-cell naïveté and GC B-cell differentiation.

Keywords B-cell differentiation; DOT1L; germinal center B cell; plasma cell; PRC2

Subject Categories Chromatin, Transcription & Genomics; Immunology; Post-translational Modifications & Proteolysis

Article

Introduction

B lymphocytes are key cellular components of the adaptive immune system, and their functional deregulation is associated with immune deficiencies and autoimmunity (Rajewsky, 1996; LeBien & Tedder, 2008). Several well-coordinated processes during B-cell differentiation coincide with specific adaptations of the epigenome (Parra, 2009; Busslinger & Tarakhovsky, 2014; Bao & Cao, 2016). Given the critical contribution of B cells to the immune system, it is important to understand the molecular mechanisms underlying the epigenetic programming during their differentiation.

We previously identified DOT1L as a conserved epigenetic writer that catalyzes mono-, di-, or tri-methylation of lysine 79 of histone H3 (H3K79me) (van Leeuwen et al, 2002; Frederiks et al, 2008). DOT1L-mediated H3K79me resides on the nucleosome core surface away from histone tails (Vlaming et al, 2014). H3K79me is associated with active transcription, but its function in gene regulation remains unclear (Steger et al, 2008; Vlaming & van Leeuwen, 2016; Wood et al, 2018). DOT1L has gained wide attention as a specific drug target in the treatment of Mixed Lineage Leukemia (PLL) characterized by rearrangements of the MLL gene. Oncogenic MLL-fusion proteins recruit DOT1L, leading to hypermethylation of H3K79 and increased expression of MLL-target genes, thereby introducing a druggable dependency on DOT1L activity (Okada et al, 2006; Bernt et al, 2011; Daigle et al, 2011; McLean et al, 2014; Chen et al, 2015; Wang et al, 2016; Stein et al, 2018). In addition, we recently observed a similar dependency in a mouse model of thymic lymphoma caused by loss of the histone deacetylase HDAC1 (Vlaming et al, 2019). While DOT1L is emerging as a...
drug target in leukemia and lymphoma, the role of DOT1L in gene regulation during normal B lymphocyte development and differentiation is not known. Analysis of publicly available RNA-sequencing data shows that Dot1L expression is regulated during B-cell development (see below).

An active humoral immune response is characterized by the activation of clonally selected, antigen-primed B cells within secondary lymphoid organs. This results in the formation of a specific microenvironment, known as the germinal center (GC) (MacLennan, 1994, 2005). Rapidly proliferating GC B cells pass through the process of somatic hypermutation that lays the molecular basis of antibody affinity maturation (Odegard & Schatz, 2006; Di Noia & Neuberger, 2007; Calado et al, 2012; Victora & Nussenzweig, 2012; Pilzecker & Jacobs, 2019). Ultimately, B cells selected on the basis of antibody affinity may differentiate either into memory B cells to establish long-term immunological memory or via a plasma-blast stage into terminally differentiated, antibody-secreting plasma cells. Development and functionality of B lymphocytes are associated with dynamic changes in the epigenetic landscape (Martin-Subero & Oakes, 2018). Recent studies indicate that specific alterations in B-cell function and identity are intimately tied with well-established histone modifications such as H3K4 tri-methylation (H3K4me3) related with active gene promoters (Li et al, 2007; Zhang et al, 2015) and H3K27me3 associated with gene depression (Beguelin et al, 2016). Furthermore, the H3K27 methyltransferase EZH2, the catalytic component of the Polycomb repressive complex 2 (PRC2), has been shown to have an essential role in establishing GC B cells (Beguelin et al, 2013).

Here, we determined the role of the H3K79 methyltransferase DOT1L in normal mouse B-cell development by deleting Dot1L early in the B-cell lineage and investigating specific dependencies of B lineage cells on DOT1L. Our findings, corroborated by in vitro DOT1L inhibition studies, show that DOT1L fine-tunes the core transcriptional and epigenetic landscape of B cells and in doing so establishes a critical epigenetic barrier coordinating the stepwise transitions toward terminally differentiated plasma cells.

**Results**

**Effective deletion of Dot1L in B-cell lineage cells**

Given the DOT1L dependencies in leukemia (Daigle et al, 2013; Shukla et al, 2016; Wang et al, 2016; Stein et al, 2018) and lymphoma (Vlamig et al, 2019), we quantified the expression of Dot1L during normal B-cell development using publicly available data (Shi et al, 2015). We observed that Dot1L is transcriptionally regulated in B-cell subsets and more highly expressed in GC B cells (Fig 1A). To determine the relevance of this regulation in controlling the development and differentiation of B lineage cells, we inactivated Dot1L during early B-cell development by crossing the Mb1-Cre knockin allele into a Dot1L/+/ background. DOT1L is the sole enzyme responsible for H3K79me; knockout of Dot1L has been shown to lead to complete loss of H3K79me (Jones et al, 2008; Feng et al, 2010; Vlamig & van Leeuwen, 2016; Vlamig et al, 2019). However, loss of H3K79 methylation requires dilution of modified histones by replication-dependent and replication-independent mechanisms of histone exchange (De Vos et al, 2011; Radman-Livaja et al, 2011; Chory et al, 2018). Mb1-Cre was chosen because it leads to deletion of Dot1L at an early stage in B-cell development that is followed by successive rounds of replication. This ensures complete loss of H3K79me in all subsequent B-cell subsets. Dot1L was specifically and efficiently deleted in B cells, as confirmed at the transcript level (Fig EV1A and Appendix Fig S1), as well as by intracellular staining for the DOT1L product H3K79me2 in proB, preB, immature B cells, and mature B cells. While some proB cells retained H3K79me2, preB cells and all stages beyond lacked detectable levels of H3K79me2 (Fig 1B). As a control, the methylation mark remained unchanged in mature T cells from Mb1-Cre/−; Dot1L+/+ and Mb1-Cre/−; Dot1LWt/wt mice (Fig 1B). We refer to Mb1-Cre/−; Dot1L/+, Dot1L−/− and Mb1-Cre/−; Dot1LWt/wt B cells as Dot1L KO and WT cells, respectively. To study the impact of Dot1L ablation on the development of B cells in the bone marrow, we determined the cellularity of specific developmental subsets in the DOT1L-proficient and DOT1L-deficient settings. Early ablation of Dot1L resulted in an overall 1.6-fold reduction of bone marrow B lineage cells. This reduction appeared to be caused primarily by an early differentiation block at the proB to preB cell stage; preB cell were reduced 1.8-fold in the Dot1L KO as compared to WT and proB cells were increased by 2.0-fold. In line with this partial developmental inhibition, the cellularity of all subsequent stages of development including immature B and mature B cells was significantly reduced in the bone marrow (Figs 1C and EV1B).

The reduction in preB cell numbers and subsequent B cell subsets did not relate to a compromised cell viability (Fig EV1C–H). This suggests that impaired VDJ recombination in the absence of DOT1L-dependent pro-recombinogenic H3K79me marks (Xu et al, 2012; Deng et al, 2015) underlies the reduced B-cell cellularity in the Dot1L-KO setting. However, other causal factors cannot be excluded. Regardless of this partial developmental block, B cells could mature in the absence of DOT1L and H3K79 methylation, providing a system to study the role of this epigenetic mark in B-cell differentiation.

**Lack of DOT1L prohibits differentiation of germinal center B cells**

In the spleen of Mb1-Cre/−; Dot1L+/+ mice, B cellularity decreased 3.5-fold while T-cell numbers remained unaffected, resulting in a 2.0-fold decreased overall cellularity (Fig 2A and B). Similar to the results from the bone marrow B-cell subsets, the frequency of late apoptotic cells as measured by annexin V and DAPI staining of spleen cells (ex vivo) remained unaltered in the absence of DOT1L (Fig EV1I). This was further corroborated in situ by immunohistochemistry for the apoptosis marker cleaved caspase-3 (Fig EV1J). Among the various peripheral B-cell subsets, the strongest reduction was found in the number of marginal zone B cells and GC B cells to the extent that they were nearly absent (Figs 2C–H and EV2A). The reduction of GC B cells in Dot1L KO was of particular interest given the highest expression of Dot1L mRNA in this subset (Figs 1A and 2H), suggesting that the formation of germinal centers critically depends on DOT1L. Indeed, in situ histological analyses also revealed the absence of GCs in the spleen of Dot1L-KO mice (Fig 2I and J). Similarly, in Peyer’s patches, lack of DOT1L resulted in a marked reduction of GC B cells (Fig EV2B).
Figure 1. Expression levels, efficient deletion of Dot1L in B cells, and its effect on cellularity of B lineage subsets in the bone marrow.

A Expression of Dot1L in different B-cell populations (Shi et al., 2015). B1: B1 cells, MZB: marginal zone B, FOB: follicular B, GCB: germinal center B, SPLPB: spleen plasma blast, SPLPC: spleen plasma cells, and BMPC: bone marrow plasma cells. Expression is shown as transcript per million (TPM). Results represent the data from two biological replicates except SPLPB and BMPC which lack replicates (WT; n = 2). Bars indicate mean values.

B Intracellular flow cytometry staining for H3K79me2 in bone marrow B-cell subsets as well as splenic B and T cells from MB1Cre+/−; Dot1L−/− (WT) and MB1Cre+/−; Dot1L+/− (KO) mice. Results represent the data from two independent experiments.

C Statistical analysis of the absolute number of total nucleated cells from bone marrow B-cell subsets. Results represent the data pooled from three independent experiments, and numbers represent biological replicates for each group (WT; n = 5, KO; n = 7), except bone marrow cellularity (WT; n = 10, KO; n = 14). Bars and error bars indicate mean ± SD.

Data information: Statistical analyses were performed using the Student’s two-tailed unpaired t-test. Statistical significance was determined by calculating the P-value. A P-value of less than 0.05 was considered as significant.
Figure 2.

- A: Splenocytes
- B: B cells
- C: Marginal Zone Precursor cells
- D: Marginal Zone B cells
- E: Follicular B cells
- F: FOB-I cells
- G: T1 cells
- H: Gated on B220^+ 
  - WT: GCB 0.71
  - KO: GCB 0.08
- I: WT
- J: KO
- No of PNA GCs/section
Dot1L deletion impairs proliferation in response to T cell-dependent stimuli and reduces class switch recombination in response to T cell-independent stimuli in vitro

Following B-cell priming, B cells undergo class switch recombination (CSR) and form GCs. CSR is an important feature of humoral immunity and is linked to B-cell activation and initiation of proliferation. Given the critical role of Dot1L in GC B-cell differentiation, we have examined the CSR potential and proliferative capacity of naive splenic B cells stimulated in vitro. Upon the T cell-dependent stimulus mimic anti-CD40 + IL-4, the lack of Dot1L was found associated with a reduced frequency of class-switched cells (Figs 3A and EV3A). The reduction in CSR might relate to a defect in the CSR machinery or impaired proliferation. Upon anti-CD40 + IL-4 stimulation, the proliferative response of Dot1L-KO B cells was strongly impaired, as revealed by the dilution of a fluorescent label (Fig 3B). Further analysis based on frequency of switched cells (IgG1+) among different generations of cell divisions revealed that, overall, the reduction in frequency of switched cells in KO as compared to WT B cells was associated with impaired proliferation rather than a defect in CSR (Fig 3C). The reduced proliferation in response to T-dependent stimulation suggested that Dot1L-KO B cells are compromised in mounting T-cell-dependent immune responses, which is in line with the lack of GCs in Dot1L-KO mice.

Unlike stimulation with anti-CD40 + IL-4, mimicking T cell-dependent B-cell activation, the proliferative capacity of mature B cells was found indistinguishable in response to T cell-independent activation stimuli using the strong B-cell mitogen lipopolysaccharide (LPS) (Dziarski, 1982) or LPS + IL-4. These findings indicate that Dot1L-KO B cells are not intrinsically impaired in their proliferative potential (Fig EV3B and C). However, despite their proficiency in proliferation, the frequency of switched cells in Dot1L-KO B cells was found reduced in response to both LPS alone (inducing a switch to IgG3) or LPS + IL-4 (inducing a switch to IgG1) (Figs 3D and E, and EV3D–G). The underlying mechanism by which DOT1L might affect CSR under these conditions of strong activation warrants further investigation. RNA-Seq analysis of KO and WT B cells activated with LPS + IL-4 did not show defects in the mRNA expression of the known trans elements involved in CSR in Dot1L-KO B cells, (Appendix Table S1). However, under LPS conditions, DOT1L may affect these factors by unknown post-transcriptional mechanisms or by altering cis acting features associated with CSR, like switch region accessibility. Alternatively, the switching cells may be prone to die in the absence of DOT1L, as DOT1L has been associated with DNA damage responses and induction of apoptosis (Huyen et al., 2004; Nguyen & Zhang, 2011; Kari et al., 2019; Bian et al., 2020). In vitro activation of naive B cells with anti-CD40 + IL-4, LPS, and LPS + IL-4 led to a higher proportion of dead cells (Zombie NIR

**Figure 2.** Dot1L ablation affects the cellularity of mature peripheral B-cell populations with a strong reduction in Marginal Zone and Germinal Center B cells.

A–G Statistical analysis of absolute number of total nucleated splenocytes, splenic B- and T-cells, and indicated mature B-cell subsets in WT and KO mice. Results represent the data pooled from at least two independent experiments and numbers represent biological replicates for each group (A: (WT) n = 16, (KO) n = 17; B (B-cells); (WT) n = 16, (KO) n = 17; B (T-cells); (WT) n = 8, (KO) n = 9; C–E: (WT) n = 9, (KO) n = 9; F: (WT) n = 6, (KO) n = 6; G: (WT) n = 9, (KO) n = 9).

H Representative flow cytometry plots showing gating strategy (left panel) to identify germinal center B cells (PNA<sup>hi</sup>, CD95<sup>-</sup>) from the spleen of unchallenged WT and KO mice and statistical analysis of their absolute number (right panel). Results represent the data pooled from two independent experiments, and numbers represent biological replicates for each group (WT: n = 4, KO: n = 6).

I Identification of germinal centers by lectin histochemistry of Peanut agglutinin (PNA) in spleens from WT and KO mice. The scale bar: 20 μm.

J Quantification of germinal centers identified in spleen sections as shown in (I). Results represent the data from one experiment, and numbers represent biological replicates for each group (WT: n = 6, KO: n = 6).

Data information: Statistical analyses were performed using the Student’s two-tailed unpaired t-test. Statistical significance was determined by calculating the P-value. A P-value of less than 0.05 is considered as significant. Bars and error bars indicate mean ± SD.

**Figure 3.** Class switch recombination and proliferative potential of Dot1L-deficient B cells in response to T cell-dependent and T cell-independent stimuli in vitro.

A Statistical analysis of IgG1 switching of Dot1L-proficient (WT) and Dot1L-deficient (KO) B cells after 4 days of activation with anti-CD40 + IL-4. Results represent the data from one experiment, and numbers represent biological replicates for each group (WT: n = 4, KO: n = 4).

B Number of cell divisions traced by CTV dilution of B cells stimulated for 4 days with anti-CD40 + IL-4. Data represent three biological replicates for each genotype.

C Statistical analysis of the percentage of IgG1 switched cells per generation of proliferating WT and KO B cells after 4 days of stimulation with anti-CD40 + IL-4. Results represent the data from one experiment, and numbers represent biological replicates for each group (WT: n = 4, KO: n = 4).

D, E Statistical analysis of switching of WT and KO naive B cells activated for 4 days with LPS alone (IgG3 switching) (D) or with LPS + IL-4 (IgG1 switching) (E). Results represent the data pooled from at least four independent experiments, and numbers represent biological replicates for each group (D: (WT) n = 8, (KO) n = 9; E: (WT) n = 11, (KO) n = 13).

F Statistical analysis of IgG1 switching of naive B cells after 4 days of activation with anti-CD40 + IL-4 in the presence of the DOT1L inhibitor Pinometostat or DMSO as a control. Results represent the data from one experiment, and numbers represent biological replicates for each treatment (n = 4).

G Number of cell divisions traced by CTV dilution of B cells stimulated for 4 days with anti-CD40 + IL-4 either in the presence of DOT1L inhibitor Pinometostat or DMSO as a control. Data represents three biological replicates for each treatment.

H Statistical analysis of the percentage of IgG1 switched cells per generation of proliferating WT B cells after 4 days of stimulation with anti-CD40 + IL-4 either in the presence of DOT1L inhibitor Pinometostat or DMSO as a control. Results represent the data from one experiment, and numbers represent biological replicates for each treatment (n = 3).

I Statistical analysis of IgG3 switching of naive B cells after 4 days of activation with LPS alone in the presence of the DOT1L inhibitor Pinometostat or DMSO as a control. Results represent the data from one experiment, and numbers represent biological replicates for each treatment (n = 4).

Data information: Statistical analyses were performed using the Student’s two-tailed unpaired t-test. Statistical significance was determined by calculating the P-value. A P-value of less than 0.05 was considered as significant. Bars and error bars indicate mean ± SD.
positive) in Dot1L-KO compared to WT (Fig EV3H–K). In addition, the viable (Zombie NIR negative) subset in activated (LPS + IL-4 mediated) KO B cells consistently showed higher levels of cleaved caspase-3, a marker of apoptosis (Fig EV3L). Apparently, upon in vitro activation the viability of Dot1L-deficient B cells is compromised.

**In vitro inhibition of DOT1L activity in mature B-cell phenocopies early Dot1L deletion**

The pleiotropic changes associated with Dot1L ablation in developing B cells led us to investigate whether the effects observed in mature Dot1L-KO B cells in vitro could be recapitulated by inhibiting the methyltransferase activity of DOT1L. For this purpose, we employed the highly specific DOT1L inhibitor Pinometostat during T cell-dependent and T cell-independent activation of mature splenic B cells isolated from wild-type mice. Treatment with Pinometostat closely mimicked our observations regarding B-cell activation, CSR, proliferation, and viability in Dot1L-KO mice (Figs 3F–I and EV3M–T). These data highlight the relevance of DOT1L activity in controlling normal B-cell differentiation and proliferation induced by T-dependent stimuli in vitro. Importantly, these insights exclude potential indirect effects associated with the early ablation of Dot1L during B-cell ontogeny.
Figure 4.
Dot1L-deficient B cells fail to mount efficient immune responses in vivo and acquire plasma cell features in vitro

The virtual absence of GC B cells, the impaired proliferative response to T cell-dependent stimulation, and the decreased viability of Dot1L-deficient B cells upon activation implicated a severe defect of Mb1-Cre/-;Dot1Lfl/fl mice in establishing effective humoral immunity. In unchallenged Dot1L-KO mice, serum IgM titers appeared normal but IgG1 and IgG3 titers were decreased, while IgG2b and IgA titers were not significantly affected (Fig EV4A). To determine the immune responsiveness of Dot1L-deficient B cells, Mb1-Cre/-;Dot1Lfl/fl and Mb1-Cre/-;Dot1Lcr/wt mice were challenged with an acute lymphocytic choriomeningitis virus (LCMV) infection. During the LCMV infection, Dot1L-deficient B cells failed to establish GC B cells and generate plasma cells (Fig 4A and B). In accordance, these mice also failed to mount normal IgM serum titers against LCMV (Fig 4C). The inability of Dot1L-deficient B cells to establish GC B cells and generate plasma cells (Fig 4A and B). In accordance, these mice also failed to mount normal IgM serum titers against LCMV (Fig 4C). The failure to establish GCs in response to LCMV is also in line with the very low LCMV-specific IgG serum titers (Fig 4C). The inability of Mb1-Cre/-; Dot1Lcr/wt mice to mount efficient antibody responses to T cell-dependent antigen was confirmed using 4-hydroxy-3-nitrophenylacetyl conjugated to chicken gamma globulin (NP-CGG) in alum as immunogen (Fig 4D). Together, these data suggest that Dot1L is essential for establishing a normal humoral immune response. Upon in vitro activation of naïve Dot1L-KO B cells with LPS + IL-4, a significantly increased frequency of cells expressing the plasma cell marker CD138 (Sanderson et al, 1989; McCarron et al, 2017) was observed (Fig EV4F). This observation was supported by the increased proportion of cells expressing the pan-plasma cell transcription factor BLIMP1 (Tellier et al, 2016; Fig EV4G) in Dot1L-KO versus WT. However, these cells failed to downregulate B220, also known as CD45R (Fig EV4H). Downregulation of B220 is considered as a hallmark of post-mitotic plasma cells (Kallies et al, 2004). In addition, in vitro-activated Dot1L-KO B cells failed to differentiate into CD19 high-activated B-cell blasts or CD19-negative plasma cells, but instead they remained at a transitional state, expressing intermediate levels of CD19 on cell surface (Fig EV4I). Of note, these results were reproduced using the DOT1L inhibitor Pinometostat during in vitro stimulation of naïve WT B cells with LPS (Fig 5A–D). Together, these phenotypes observed upon in vitro activation suggest that in the absence of DOT1L-mediated H3K79 methylation, naïve B cells prematurely gain some plasma cell features which may form the basis of skipping the GC stage. However, these cells do not accomplish a complete plasma cell differentiation program.

DOT1L supports a pro-proliferative, MYC-high GC stage and prohibits premature differentiation toward plasma cells in vitro

To unravel the underlying molecular mechanisms that prohibit Dot1L-KO GC B-cell differentiation and stimulate partial differentiation toward plasma cells, we performed RNA-Seq analyses of in vitro-activated B cells under Dot1L-proficient and Dot1L-deficient conditions (Fig 6A). To avoid confounding issues associated with impaired replication upon in vitro stimulation with anti-CD40 + IL-4, naïve B cells were stimulated in vitro with LPS + IL-4 as an
Figure 5.
activating condition that facilitates replication of both WT and KO B cells. Among the differentially expressed genes, the genes encoding the pro-GC transcription factor BACH2 and the pro-proliferative transcription factor MYC (Muto et al., 2004; Muto et al., 2010; Calado et al., 2012) were H3K79me2 methylated in WT cells (see below) and transcriptionally downregulated in activated DOT1L-deficient B cells (Fig EV5A, B and D). In agreement with this, Prdm1, repressed by BACH2 and encoding the pan-plasma cell transcription factor BLIMP1 (Martins & Calame, 2008; Tellier et al., 2016), was found upregulated (Figs 6B and EV5C). These transcriptional changes are

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**Figure 6. Transcriptome analysis of in vitro-activated B cells shows accelerated plasma cell differentiation and compromised activation of MYC-target genes in the absence of DOT1L.**

- **A** MA-Plot of normalized RNA-Seq data generated from three independent biological replicates for each genotype showing differential (false discovery rate (FDR) < 0.05) expression of genes between Dot1L-KO and WT B cells after 2 days of in vitro activation with LPS + IL-4.
- **B** Differential (FDR < 0.05) expression of Bach2, Prdm1 (encoding BLIMP-1), Cd138 (a plasma cell marker), and Myc transcripts as indicated by counts per million after TMM normalization from WT and KO. Data were generated from three independent biological replicates for each genotype. Bars and error bars indicate mean ± SD. Statistical significance is indicated by FDR after the Benjamini-Hochberg multiple testing correction performed by edgeR package using R language.
- **C, D** Enrichment of plasma cell signature genes in KO as compared to WT-activated B cells (C) and Enrichment of MYC-target genes in WT as compared to KO-activated B cells (D). Enrichment of gene sets is depicted by a BARCODE plot; P-value calculated via FRY test show the statistical significance of enrichment of each gene set. A P-value less than 0.05 was considered as significant.

Source data are available online for this figure.
DOT1L-mediated H3K79 methylation is associated with gene activity in B cells

To link the phenotypes of loss of DOT1L to its role as an epigenetic regulator in B cells, we generated genome-wide maps of DOT1L-mediated H3K79me2 in naïve and activated WT B cells by ChIP-Seq. H3K79me2 is known to mark the region downstream of the transcription start site of transcribed genes and positively correlate with gene activity (Steger et al, 2008; Huff et al, 2010; Vlaming & van Leeuwen, 2016; Godfrey et al, 2019). While H3K79me1 shows the same trends, it has a broader distribution, and H3K79me3 is detectable only at limited levels (Steger et al, 2008; Huff et al, 2010; Vlaming et al, 2019). Here, we analyzed how the gene expression changes in Dot1L-KO versus WT B cells related to genes being marked by H3K79me2 in WT cells. Both in naïve and activated B cells more than 83% of the differentially expressed genes was found upregulated in the absence of DOT1L; only a small subset was downregulated (Figs 7A and EV5I). The upregulation was biased toward more lowly expressed genes. The observed upregulation of genes in Dot1L-KO B cells was unexpected given the fact that H3K79me2 generally correlates with transcriptional activity (Schubeler et al, 2004; Steger et al, 2008; Wang et al, 2008; Bernt et al, 2011; Cano-Rodriguez et al, 2016; Yang et al, 2016; Wood et al, 2018; Godfrey et al, 2019). However, repressive functions of DOT1L have been proposed as well (Zhang et al, 2006a; Zhang et al, 2006b; Cecere et al, 2013; Xiao et al, 2016). Comparing H3K79me2 ChIP values in WT B cells with the gene expression changes caused by loss of DOT1L revealed that the genes upregulated in Dot1L-KO B cells were mostly hypomethylated in WT cells, indicating that they are likely indirectly affected by the loss of DOT1L (Fig 7A and B). In contrast, genes downregulated in Dot1L-KO B cells were generally highly expressed and H3K79 methylated in WT B cells, indicating that this gene set harbors the genes directly dependent on DOT1L. This set of candidate direct targets of DOT1L includes the previously mentioned pro-proliferative factor Myc, the pro-GC transcription factor Bach2, and Prdm1, a target of BACH2 (Fig EV5A–D). These findings show that DOT1L-mediated H3K79me2 is a mark of many active genes in B cells, but suggest that only a small fraction of these genes requires H3K79me2 for maintenance of gene expression, since only a subset of the active genes was downregulated in Dot1L-KO B cells. Similar observations in transcriptome changes were also made in CD5+ T cells lacking DOT1L as well as upon DOT1L inhibition in...
Figure 7.
The large number of genes found upregulated in DOT et al. Muhammad Assad Aslam et al. 

**DOT1L supports repression of PRC2 target genes**

The large number of genes found upregulated in Dot1L-KO B cells indicated that DOT1L positively regulates the expression of a tran-

scriptional repressor whose target genes are de-repressed in the absence of DOT1L. To identify such candidate repressors, we investig-
tigated the relatively small fraction of genes downregulated in naïve and activated Dot1L-KO B cells. Unbiased identification of upstream transcriptional regulators by Ingenuity Pathway analyses (IPA) pointed toward potential regulators that were differentially upregu-
lated in WT B cells (Tables 1 and 2). To further narrow down the

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*Genes with TMM normalized H3K79me2 score lesser than 10 were not considered.

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*Genes with TMM normalized H3K79me2 score lesser than 10 were not considered.
list, we filtered for genes that are (i) H3K9-dimethylated by DOT1L, (ii) encode transcriptional repressors, and (iii) play a role in B-cell differentiation/proliferation and GC formation. This led to the identification of the histone H3K79 methyltransferase EZH2 as a candidate factor (Tables 1 and 2). We verified that Ezh2 expression was reduced in Dot1L KO B cells (Fig 7C) and that the gene is H3K79-dimethylated in WT cells (Fig 7D), indicating that the expression of Ezh2 might be directly promoted by DOT1L activity (Fig 7C and D). Further supporting this notion, the expression of Ezh2 and Dot1L was found co-regulated in B-cell subsets (Fig 7E).

We next investigated the physiological relevance of the connection between DOT1L and EZH2. First, Ezh2-KO and Dot1L-KO B cells have overlapping phenotypes (Su et al, 2003; Béguelin et al, 2013; Caganova et al, 2013; Béguelin et al, 2017; Guo et al, 2018). Second, we took advantage of publicly available RNA-Seq data (Guo et al, 2018) from Ezh2-KO plasma cells to identify genes that require EZH2 for their repression (Fig EV5J). Using this as a signature of EZH2-dependent genes, we found that many of the genes de-repressed in Ezh2-KO cells were also found de-repressed in activated Dot1L-KO B cells (Fig EV5K). Many of these genes were found de-repressed also in naïve Dot1L-KO B cells (Fig EV5K). As an independent validation, we evaluated the expression of a known PRC2-target gene Cdkn1a (p21) (Fan et al, 2011; Sato et al, 2013; Béguelin et al, 2017) and found that it was upregulated in activated Dot1L-KO B cells (Fig 6C). Third, we analyzed the level of H3K27me3 and H3K79me2 in the set of genes that was de-repressed in Dot1L KO using our H3K79me2 ChIP-Seq data and publicly available H3K27me3 ChIP-Seq data from naïve B cells (Frangini et al, 2013). Further analysis revealed that this gene set was enriched for H3K27me3 in WT cells compared to expression-matched non-differentially expressed genes (Fig 7F). Lastly, chemical inhibition of both DOT1L (this study) and EZH2 (Scharer et al, 2018) upon in vitro stimulation led to enhanced dysfunctional plasma cell formation. Together, these findings suggest that in B cells, DOT1L supports the repression of PRC2 target genes, thus uncovering a previously unknown connection between two conserved histone methyltransferases associated with activation and repression, respectively. Together, our findings place H3K79 methylation by DOT1L at the heart of maintaining epigenetic identity of B cells, by orchestrating the activity of central transcriptional and epigenetic regulators such as BACH2, MYC, EZH2, and their target genes.

Discussion

Given the critical contribution of B cells to the immune system, it is important to understand the molecular mechanisms underlying the epigenetic programming during their differentiation. Taking advantage of a B cell-specific mouse knockout model, we observed that the H3K79 methyltransferase DOT1L has a central role in B-cell physiology. Among mature B cells, GC B cells express the highest levels of Dot1L and they were strongly reduced in Dot1L-KO mice. Indeed, GC B-cell differentiation was found to be critically dependent on DOT1L. Upon in vitro activation, Dot1L-KO B cells failed to proliferate and after an in vivo immune challenge these cells failed to differentiate into GC B cells and establish an effective immune response. In addition, we observed an accelerated but incomplete plasma cell differentiation in Dot1L-KO B cells in vitro. RNA-Seq data generated from naïve and in vitro-activated Dot1L-KO and WT B cells demonstrated a strong enrichment of genes associated with plasma cell differentiation among the genes upregulated in Dot1L KO. However, in vitro-activated Dot1L KO B cells failed to downregulate B220, indicating an incomplete differentiation into plasma cells (Kallies et al, 2004), which is in agreement with the failure of these cells to upregulate Irf4, a factor essential for plasma cell differentiation (Klein et al, 2006). Our results also revealed that DOT1L supports MYC and BACH2 activity, which B cells depend on to effectively differentiate into pro-proliferative GC B cells and maintain that state (Calado et al, 2012). Recent studies have shown that inhibition of DOT1L also leads to reduced Myc expression in multiple myeloma (Ishiguro et al, 2019), in MYC-driven B-cell lymphoma (Deshpande et al, 2018), and in androgen-dependent prostate cancers (Vatapalli et al, 2020) indicating that the connection between DOT1L and MYC has broad implications in B-cell physiology as well as other cell types. Furthermore, in neuroblastoma H3K79me2 methylation has been shown to be a strict prerequisite for MYC-induced transcriptional activation, indicating a mutual interaction between DOT1L and MYC (Wong et al, 2017). Interestingly, this novel interaction has also been reported recently in colorectal cancer (Yang et al, 2019). In addition to the crucial role in GC formation, we also identify DOT1L as a critical factor in maintaining MZ B cells. Further exploring the strong reduction of MZ B cells upon loss of Dot1L should provide additional insights regarding the contribution of DOT1L in orchestrating normal B-cell physiology.

In addition to supporting MYC and BACH2 activity, our findings also suggest that DOT1L supports the repression of target genes of PRC2. Regarding influence of EZH2 and DOT1L on GC B cells, it was found that in both spleen and Peyer’s patches, GC B cells were drastically reduced in the absence of EZH2 (Caganova et al, 2013) and DOT1L (this study). Phenotypically, this connection between DOT1L and PRC2 targets is further supported by overlap in transcriptome changes between Ezh2 KO and Dot1L KO B cells. The mechanism explaining the connection between DOT1L and PRC2 targets is unknown and needs further investigation. The observation that Ezh2 is normally H3K9-dimethylated and downregulated in Dot1L-KO B cells, and that Dot1L and Ezh2 are co-regulated in B cells indicates that DOT1L might promote repression of PRC2 targets by maintaining expression of Ezh2. Alternatively, DOT1L might affect a subset of PRC2 targets by altered activity of co-repressor complexes, as has been suggested for DOT1L in mouse ES cell differentiation (Ferrari et al, 2020). Finally, additional factors controlled by DOT1L may impact differentiation of B cells (e.g. see Tables 1 and 2). A direct stimulatory effect of H3K79me on H3K27me3 synthesis is not likely to be involved since these modifications occur at distinct locations in the genome and are associated with opposite transcriptional states. The link between DOT1L and multiple transcriptional regulators implies the existence of a complex regulatory network in B cells that warrants further investigation.

Understanding the critical role of DOT1L in B-cell physiology might uncover important implications in B-cell-related pathologies. Considering the requirement for DOT1L in supporting humoral immune responses that we show here, targeting DOT1L may offer an opportunity for immune suppression. Given the strong dependency of GC B cells on DOT1L and the role of DOT1L in controlling PRC2 targets and the oncogenic factor MYC, DOT1L inhibition may also offer a novel therapeutic angle in the treatment of diffuse large
B-cell lymphoma of the GCB type. In summary, in B cells, DOT1L has a central role in guiding dynamic epigenetic states controlling differentiation and ensuring functional immune responses, with a potential for clinical exploitation.

Materials and Methods

Mice

*Mb1-Cre*\(^{+/−}\)-*Dot1L^12107^ mice were derived by crossing the *Dot1Ltma^1a (KOMP)Wtsi* line—generated by the Wellcome Trust Sanger Institute (WTSl) and obtained from the KOMP Repository (www.komp.org)—with the *Mb1-Cre* strain kindly provided by M. Reth (Hobeika *et al*, 2006). Mice from this newly created *Mb1-Cre*\(^{+/−}\)-*Dot1L* strain were maintained under specific pathogen-free (SPF) conditions at the animal laboratory facility of the Netherlands Cancer Institute (NKI; Amsterdam, Netherlands). Mice used for experiments were between 6–8 weeks old and of both genders unless stated otherwise. All experiments were approved by the Animal Ethics Committee of the NKI and performed in accordance with the Dutch Experiments on Animals Act and the Council of Europe.

Genotyping PCR

Mice were genotyped for *Dot1L* using the forward primer (*Dot1L*: FWD, 5'-GCAAGGCTACAGCTTCATC-3') and reverse primer 1 (*Dot1L*: REV1, 5'-CACCAGGATGTCTCAATAATCTCA-3') to identify WT allele; *Dot1L*\(^{+/−}\) (517 bp) while the floxed allele; *Dot1L*\(^{−/−}\) (335 bp) was identified by using *Dot1L* FWD and reverse primer 2 (*Dot1L*: REV2, 5'-GAACCACAGGATGCTTCAG-3'). The region flanking floxed exon 2 of *Dot1L* was amplified by using My Taq DNA polymerase (meridian BIOSCIENCE, catalog number BIO-21107) and the following thermocycler conditions: Initial denaturation 95°C for 5 min, followed by 35 cycles of denaturation 95°C for 30 s, annealing 60°C for 30 s and extension 72°C for 1 min. Final extension was done at 72°C for 5 min. The WT allele (418 bp) for *Mb1* was detected by using forward primer (*Mb1*-FWD1: 5'-CTGCCGGGTATAAAGGGGGTC-3') and reverse primer (*Mb1*-REV1: 5'-CTTTGGCGGCTGTCGGACC-3') while *Cre* (219 bp) was detected by using forward primer (*Mb1*-FWD2: 5'-GTGCAAGCTGAACAAACAGGA-3') and reverse primer (*Mb1*-REV2: 5'-AAGGAGATGTGGATGCTGG-3'). PCR to amplify the region of the *Mb1* locus was performed by using My Taq DNA polymerase (meridian BIOSCIENCE, catalog number BIO-21107) and the thermocycler conditions used to amplify region of the *Mb1* locus consists of one cycle of Initial denaturation: 95°C for 3 min, annealing: 75°C for 5 min and extension: 72°C for 90 s followed by 25 cycles of denaturation: 94°C for 1 min, annealing: 63°C for 1 min, extension: 72°C for 90 s. Final extension was done at 72°C for 10 min.

Reverse transcriptase (RT)–PCR

Total RNA from FACS sorted mature B and T lymphocytes from the spleen of mice was isolated using RNeasy mini (Qiagen, cat. no. 74106) according to the manufacturer instruction. The cDNA libraries were prepared using Invitrogen Superscript III kit and Random hexamer primers (catalog number 18080051) according to the manufacturer instructions. Deletion of exon 2 (44 bp) from *Dot1L* transcript was detected by amplifying the portion of *Dot1L* cDNA with Taq polymerase (Invitrogen, catalog number 10342053) using forward primer (P1:FWD, 5'-CGAGAAGACTGAGCTGAG-3') and reverse primer (P2:REV, 5'-TGTTGGATGCTGCAGTGATG-3'). Thermocycler conditions used for amplification consists of Initial denaturation: 95°C for 3 min, followed by 15 cycles of denaturation: 95°C for 30 s, annealing: 65.7°C for 45 s and extension: 72°C for 1 min. This was followed by 25 cycles of denaturation: 95°C for 30 s, annealing: 60°C for 45 s, extension: 72°C for 1 min. Final extension was done at 72°C for 5 min. PCR fragments of 255 and 211 bp, for WT and KO, respectively, were extracted from 2% Precast Agarose E-gel (catalog number G401002) using E-gel Agarose electrophoresis system (Invitrogen) and subjected to sequencing using the same set of individual primers. Sequenced products were analyzed by SnapGene (version 4.1.6).

In silico analysis

The predicted translated product from the KO *Dot1L* transcript missing exon 2 was generated using SnapGene (version 4.1.6). Alignments of the open reading frame from the WT *Dot1L* transcript and the predicted translated product from the KO *Dot1L* transcript were performed by ClustalW under default settings.

Flow cytometry

Single cell suspensions were made from bone marrow, spleen, and Peyer’s patches. Bone marrow, spleen, and blood samples were subjected to erythrocyte lysis. Distinct cellular populations were identified using a combination of fluor-conjugated antibodies against surface markers (Appendix Table S3). Cells were stained with fluorescently labeled antibodies (Appendix Table S4). For intracellular staining, cells were fixed and permeabilized using the Transcription Factor Buffer set (Becton Dickinson, CA, catalog number 562574). Antibodies for intracellular staining were diluted in Perm/Wash buffer except for cleaved caspase-3. For H3K79me2 staining, cells were first stained with surface markers and fixed and permeabilized as described before. After fixation and permeabilization cells were washed with Perm/Wash containing 0.25% SDS. H3K79me2-specific antibody (Millipore, clone NL59, catalog number 04-835) was diluted 1:200 into Perm/Wash + 0.25% SDS and cells were incubated for 30 min. Cells were washed with Perm/Wash and incubated with the secondary antibodies Donkey anti-Rabbit AF555 (Thermo Scientific, catalog number A-31572) or Goat anti-Rabbit AF488 (Invitrogen, catalog number A-11034) 1:1,000 in Perm/Wash. For cleaved caspase-3 staining, the cells were fixed stained with surface markers and then fixed with 4% formaldehyde for 15 min at room temperature. After washing with 0.25% tween buffer, the cells were permeabilized with 0.25% tween buffer for 15 min at room temperature. Following permeabilization, the cells were stained with cleaved caspase-3 antibody as 1:50 diluted in 0.25% tween buffer for 45 min at room temperature under dark condition. For annexin V staining, following surface marker stainings, the cells were washed once with PBS and later with Binding Buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). Cells were stained with annexin V stain mix prepared as 1:20 in Binding Buffer and incubated for 20 min at room temperature in the dark.
Following annexin V (Biolegend, catalog number 640920) staining, the cells were washed once with Binding buffer and resuspended in Binding buffer containing DAPI (1:100). Flow cytometry was performed using the LSR Fortessa (BD Biosciences) and data were analyzed with FlowJo software (Tree Star Inc.). Histograms were smoothed.

Immunization

Adult mice were inoculated intravenously with sub-lethal dose 2×10^5 PFU (Plaque forming units) of lymphocytic choriomeningitis virus strain Armstrong. Serum was collected prior to immunization and 14 days post-immune challenge. For NP-CGG immunization, mice were injected intraperitoneally with 100 µg of alumprecipitated NP-CGG [(4-hydroxy-3-nitrophenyl) acetyl coupled to chicken CD40 (1 used at a concentration of 10 ng/ml. Cells were exposed to antigen 100 µg/ml, pen/strep, and 100 µg/ml of Lipopolysaccharide (LPS, 055:B5, Sigma) or LPS -mercaptoethanol. After washing, cells were cultured in complete IMDM medium (IMDM supplemented with 8% FCS, with pen/strep, and 100 µM β-mercaptoethanol). To determine the serum titers of NP-specific IgM and IgG1, mice were bled from the tail vein on days 0, 7, 14, 21, 28, and 35.

In vitro stimulation: class switch recombination, proliferation, and plasma cell formation

Single cells suspensions were prepared from the spleen of 6- to 8-week-old mice. Following erythrocyte lysis, naïve splenic B cells were enriched by the depletion of CD43-expressing cell using biotinylated anti-CD43 antibody (Clone S7, BD Biosciences), BD IMag Streptavidin Particles Plus, and the IMag® system (BD Biosciences), as described by the manufacturer. To measure their proliferative capacity, naïve B cells (CD43+) were labeled for 10 min at 37°C with 5 µM Cell Trace Violet (CTV, Life Technologies, Invitrogen™) in IMDM medium containing 2% FCS, with pen/strep, and 100 µM β-mercaptopethanol. After washing, cells were cultured in complete IMDM medium (IMDM supplemented with 8% FCS, with pen/strep, and 100 µM β-mercaptopethanol) at a density of 2 × 10^5 cells/well in 24-well plates. CSR to IgG3 and IgG1 was induced in T cell-independent manner by exposure to 5 µg/ml of Lipo polysaccharide (Escherichia coli LPS, 055:B5, Sigma) or LPS + IL-4. IL-4 was used at a concentration of 10 ng/ml. Cells were exposed to anti-CD40 (1 µg/ml, BD Clone HM40-3) and IL-4 (10 ng/ml) to induce IgG1 switching in a T cell-dependent manner. Four days later, the cells were harvested and either stained to determine CSR frequency or plasma cells formation. CTV dilution as an indicator of cell multiplication. In order to determine the effect of chemical inhibition of DOT1L on CSR and plasma cell formation under in vitro stimulation (LPS or anti-CD40 + IL-4), naïve B cells isolated from WT spleen were labeled with CTV and stimulated (LPS or anti-CD40 + IL-4) under the above-mentioned conditions either in the presence of DOT1L inhibitor Pinometostat (EPZ-5676, catalog number S7062, Selleckchem) at a concentration of 10 µM or 0.1% dimethyl sulfoxide (DMSO) as a control.

Enzyme-linked Immunosorbent Assay (ELISA)

LCMV-specific serum IgM and IgG levels were measured by ELISA. In short, Nunc-Immuno Maxisorp plates (Fisher Scientific) were coated overnight at 4°C with virus in bicarbonate buffer. Plates were subsequently incubated for 1h with blocking buffer (PBS/5% milk powder (Fluka Biochemika)). Sera from mice were diluted in PBS/1% milk powder and incubated for 1h at 37°C. Next, HRP-conjugated IgG and IgM antibodies (Southern Biotech) were diluted 1:4,000 in PBS/1% milk powder and incubated 1 h at 37°C. Plates were developed with TMB substrate (Sigma Aldrich), and the color reaction was stopped by the addition of 1 M H2SO4. Optical density was read at 450 nm (OD450) using a Microplate reader (Model 680, Bio-Rad).

To quantify NP-specific serum antibodies, plates were coated with 2 µg/ml NP30-BSA. Serum was added at a starting dilution of 1:100 followed by threefold serial dilutions and incubated for 2 h at room temperature. Bound serum antibodies were detected with polyclonal biotinylated goat anti-mouse IgM or anti-IgG1 (Southern Biotech, catalog numbers 1020-08 and 1071-08, respectively), streptavidin-alkaline phosphatase conjugate (Roche, catalog number 11089161001) and chromogenic substrate 4-nitrophenyl phosphate (Sigma, catalog number N2765-100TAB) in diethanolamine buffer. Purified monoclonal antibodies (B1-8g and 18-1-16y1, home-made) were used as standards for quantification.

To quantifying serum Ig titers, plates were coated with 1 µg/ml of goat anti-mouse Kappa and Lambda prepared in BBS (Borate Buffered Saline). Serum was diluted with 0.05% Tween in PBS and added at a starting dilution of 1:100, followed by fourfold serial dilutions. The plates were incubated overnight at 4°C. Bound serum antibodies were detected with biotinylated goat anti-mouse IgM, IgG1, IgG2b, IgG3, and IgA (Southern Biotech, catalog numbers 1020-08, 1071-08, 1090-08, 1100-08, and 1040-08, respectively), streptavidin-alkaline phosphatase conjugate (Roche, catalog number 11089161001), and chromogenic substrate 4-nitrophenyl phosphate (Sigma, catalog number N2765-100TAB) in diethanolamine buffer. Mouse IgM, IgG1, IgG2b, IgG3, and IgA antibodies (Southern Biotech, catalog numbers 0101-01, 0102-01, 0104-01, 0105-01, and 0106-01, respectively) were used as standards for quantification.

Lectin histochemistry

Lymphoid tissues such as spleen were fixed in EAF (ethanol, acetic acid, formaldehyde, saline) for 24 h and subsequently embedded in paraffin. 4-µm-thick sections were stained with the lectin Peanut Agglutinin (PNA, Vector Laboratories) at 1:1,500 dilution to reveal germinal centers. The sections were counterstained with hematoxyline.

Immunohistochemistry

Spleens were collected and fixed in EAF fixative (ethanol/acetate acid/formaldehyde/saline at 40:5:10:45 v/v/v). Fixed spleens were embedded in paraffin and sections of 4 µm thickness were made. Immunohistochemistry (IHC) was conducted for CD3 (Thermo Fischer Scientific, RM-9107-S1, at a dilution of 1:600), cleaved caspase-3 (Cell Signaling, catalog number 9661, at a dilution of 1:400), and Ki67 (Abcam, catalog number ab15580, at a dilution of 1:3000). The sections were reviewed with a Zeiss Axioskop2 Plus microscope (Carl Zeiss Microscopy, Jena, Germany), and images were captured with a Zeiss Axiocam HRc digital camera and processed with AxiomVision 4 software (both from Carl Zeiss Vision, München, Germany). The scale bars were set at 50 µm.
Sorting and in vitro activation for RNA and ChIP-Seq

For RNA sequencing, cells were first depleted for CD43- cells and either subjected to MACS sorting for CD19+ cells as naïve B cells or activated for 2 days with LPS + IL-4. Following activation, the cells were enriched for CD19+ by MACS according to the manufacturer instructions. For ChIP-Seq, CD43- cells were either FACS sorted for CD19+ as naïve B-cell pool or activated for 3 days with LPS + IL-4 and subjected to FACS sorting for CD19+ expression.

RNA-Seq sample preparation

MACS sorted CD19+ cells were resuspended in TRizol (Ambion Life Technologies), and total RNA was extracted according to the manufacturer’s protocol. Quality and quantity of the total RNA were assessed by the 2100 Bioanalyzer using a Nano chip (Agilent). Only RNA samples having an RNA Integrity Number (RIN) > 8 were subjected to library generation.

RNA-Seq library preparation

Strand-specific cDNA libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina) according to the manufacturer’s protocol. The libraries were analyzed for size and quantity of cDNAs on a 2100 Bioanalyzer using a DNA 7500 chip (Agilent), diluted, and pooled in multiplex sequencing pools. The libraries were sequenced as 65 base single reads on a HiSeq2500 (Illumina).

RNA-Seq preprocessing

Strand-specific RNA reads (11–33 million reads per sample), 65 bp single-end, were aligned against the mouse reference genome (Ensembl build 38) using Tophat (version 2.1, bowtie version 1.1). Tophat was supplied with a Gene Transfer File (GTF, Ensembl version 77) and was supplied with the following parameters: “--pre-filter-multithreads –no-coverage-search –bowtie1 –library-type fr-first-strand”. In order to count the number of reads per gene, a custom script which is based on the same ideas as HTSeq-count has been used. A list of the total number of uniquely mapped reads for each gene that is present in the provided Gene Transfer Format (GTF) file was generated. Genes that have no expression across all samples within the dataset were removed. Analysis was restricted to genes that have least two counts per million (CPM) value in all samples in specific contrasts, to exclude very low abundance genes. Differential expression analysis was performed in R language (version 3.5.1) on only relevant samples using edgeR package and default arguments. For analyses where we performed expression matching, we chose genes with an absolute log2 fold changes less than 0.1 and false discovery rate corrected P-values above 0.05 that were closest in mean expression to each of the genes being matched without replacement. The RNA-Seq datasets reported in this article have been deposited at the National Center for Biotechnology Information under the accession number GSE138909.

Ingenuity pathway analysis

Lists of differentially expressed genes (FDR < 0.05) between WT and KO B cells both from naïve and activated conditions were submitted to IPA using default settings to identify potential upstream regulators.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was carried out after differential expression analysis and was shown as barcode plot where the genes were ranked according to the log2 fold change between the compared conditions. Statistical significance for the enrichment of gene set was determined by Fast approximation to mroast (FRY) gene set test (Wu et al, 2010) from limma package (version 3.44.3) (Ritchie et al, 2015), and two-sided directional P-value less than 0.05 was considered significant. For Fig EV5H, GSEA was performed by Fast Gene Set Enrichment Analysis (fgsea) Bioconductor package (version 1.14.0) (preprint: Korotkevich et al, 2019) using ranked, shrunken fold changes from the differential expression analysis as input.

ChIP-Seq sample preparation

Sorted cells were centrifuged at 500 rcf. The pellet was resuspended in IMDM containing 2% FCS and formaldehyde (Sigma) was added to a final concentration of 1%. After 10-min incubation at RT, glycin (final concentration 125 mM) was added and incubated for 5 min. Cells were washed twice with ice-cold PBS containing Complete, EDTA-free, protein inhibitor cocktail (PIC) (Roche). Cross-linked cell pellets were stored at −80°C. Pellets were resuspended in cold Nuclei lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) + PIC and incubated for at least 10 min. Cells were sonicated with a PICO sonicator to an average length of 200–500 bp using 30 s on/30 s off for 3 min. After centrifugation at high speed, debris was removed and 9× volume of ChIP dilution buffer (50 mM Tris–HCl pH 8, 0.167 M NaCl, 1.1% Triton X-100, 0.1% sodium deoxycholate) + PIC and 5× volume of RIPA-150 (50 mM Tris–HCl pH 8, 0.15 M NaCl, 1 mM EDTA pH 8, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate) + PIC was added. Shearing efficiency was confirmed by reverse crosslinking the chromatin and checking the size on agarose gel. Chromatin was pre-cleared by adding ProteinG Dynabeads (Life Technologies) and rotation for 1 h at 4°C. After the beads were removed 2 µl H3K79me1, 2 µl H3K79me2 (NL59, Merck Millipore, catalog number 04-835) and 1 µl H3K4me3 (Abcam, catalog number ab8580) were added and incubated overnight at 4°C. ProteinG Dynabeads were added to the IP and incubated for 3 h at 4°C. Beads with bound immune complexes were subsequently washed with RIPA-150, two times
RIPA-500 (50 mM Tris–HCl pH 8, 0.5 M NaCl, 1 mM EDTA pH 8, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate), two times RIPA-LiCl (50 mM Tris–HCl pH 8, 1 mM EDTA pH 8, 1% Nonidet P-40, 0.7% sodium deoxycholate, 0.5 M LiCl) and TE. Beads were resuspended in 150 µl Direct elution buffer (10 mM Tris–HCl pH 8, 0.3 M NaCl, 5 mM EDTA pH 8, 0.5% SDS) and incubated overnight at 65°C, and input samples were included. Supernatant was transferred to a new tube, and 1 µl RNase A (Sigma) and 3 µl ProtK (Sigma) were added per sample and incubated at 55°C for 1 h. DNA was purified using Qiagen purification columns.

**ChIP-Seq library preparation**

Library preparation was done using KAPA LTP Library preparation kit using the manufacturer’s protocol with slight modifications. Briefly, after end-repair and A-tailing adaptor were ligated followed by Solid Phase Reversible Immobilization (SPRI) clean-up. Libraries were amplified by PCR and fragments between 250 and 450 bp were selected using AMPure XP beads (Beckman Coulter, catalog number A63881). The libraries were analyzed for size and quantity of DNAs on a 2100 Bioanalyzer using a High Sensitivity DNA kit (Agilent), diluted and pooled in multiplex sequencing pools. The libraries were sequenced as 65 base single reads on a HiSeq2500 (Illumina).

**ChIP-Seq preprocessing**

ChIP-Seq samples were mapped to mm10 (Ensembl GRCm38) using BWA-MEM with the option “-M”. Duplicate reads were removed using MarkDuplicates from the Picard toolset with “VALIDATION_STRINGENCY = LENIENT” and “REMOVE_DUPLICATES = true” as arguments. Bigwig tracks were generated from these bam files by using bamCoverage from deepTools using the following arguments: “-of bigwig –binSize 25 –normalizeUsing RPGC –ignoreForNormalization chrM –effectiveGenomeSize 2652783500”. Bigwig files were loaded into R using the “import.bw” function from the rtracklayer R package.

Statistical analyses

Statistical analyses were performed using Prism 7 (GraphPad). Data are presented as mean ± SD except for Fig 4D where it is presented as mean ± SEM. Unless stated otherwise, the unpaired Student’s t-test with two-tailed distributions was used to calculate the P-value. A P-value < 0.05 was considered statistically significant.

**Data availability**

The RNA-Seq and ChIP-Seq datasets reported in this article have been deposited at the National Center for Biotechnology Information under the accession number GSE138909 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138909) and GSE138906 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138906), respectively.

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**Author contributions**

Conception and design, MAA, MFA, EMK-M, Fvl, and HJ. Acquisition of data, MAA, MFA, EMK-M, FIM, MC, INP, TVW, and J-YS. Analysis and interpretation of data, MAA, MFA, EMK-M, RA, Edw, KR, Fvl, and HJ. Bioinformatics Analysis, MAA, TvdB, MFA, Edw, and idR. Writing of the manuscript, MAA, MFA, EMK-M, Fvl, and HJ.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


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