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De novo DNA Methyltransferases Dnmt3a and Dnmt3b regulate the onset of *Igκ* light chain rearrangement during early B-cell development

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Immunoglobulin genes V(D)J rearrangement during early lymphopoiesis is a critical process involving sequential recombination of the heavy and light chain loci. A number of transcription factors act together with temporally activated recombinases and chromatin accessibility changes to regulate this complex process. Here, we deleted the de novo DNA methyltransferases Dnmt3a and Dnmt3b in early B cells of conditionally targeted mice, and monitored the process of V(D)J recombination. Dnmt3a and Dnmt3b deletion resulted in precocious recombination of the immunoglobulin κ light chain without impairing the differentiation of mature B cells or overall B-cell development. Ex vivo culture of IL-7 restricted early B-cell progenitors lacking Dnmt3a and Dnmt3b showed precocious Vκ-Jκ rearrangements that are limited to the proximal Vκ genes. Furthermore, B-cell progenitors deficient in Dnmt3a and Dnmt3b showed elevated levels of germline transcripts at the proximal Vκ genes, alterations in methylation patterns at *Igκ* enhancer sites and increased expression of the transcription factor E2A. Our data suggest that Dnmt3a and Dnmt3b are critical to regulate the onset of *Igκ* light chain rearrangement during early B-cell development.

Keywords: B-cell · DNA methylation · Immunoglobulin · V(D)J rearrangement

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Introduction

The development of lymphocytes (B or T cells) through differentiation of HSCs into several successive progenitor stages involves highly coordinated events orchestrated by many different genes, which often act at specific stages of cell differentiation. These genes encode lineage-specific transcription factors, growth factors, and chemokines and their receptors [1–4]. The primary function of B lymphocyte is to produce high level of antigen-specific antibodies. To generate a very large repertoire of antigen receptors (antibodies) early B cells undergo a somatic recombination process known as V(D)J recombination that assembles variable (V), diversity (D), and joining (J) gene segments of the immunoglobulin (*Ig*) locus to create a large diversity of antibodies [5, 6].

The mouse Ig heavy (*IgH*) and light (*IgL*) chain (κ or λ) loci become activated during B-cell development in a step-wise manner for V(D)J recombination. First, the *IgH* gene rearranges, involving sequential D_H-J_H at the pro-B cell stage and then V_H-DJ_H joining at the pre-B cell stage. Upon successful *IgH* rearrangement, the *IgL* locus is poised for rearrangement of V-J gene segments at

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the small pre-BII cell stage, thereby giving rise to functional *Ig* genes in successive stages of B-cell development [6, 7]. The V(D)J recombinase proteins Rag-1 and Rag-2 are responsible for the induction of cleavage and recombination at conserved flanking regions (recombination signal sequences, RSS) of the V, D, and J gene segments within the *Ig* loci. Accessibility of RSS sites at the *Ig* locus for Rag-1 and Rag-2 mediated synapsis and cleavage is dependent on chromatin structure and epigenetic marks [7–9].

DNA methylation is a covalent modification of the genomic DNA that involves addition of a methyl (CH_3) group to the 5′ cytosine residue at CpG dinucleotides (reviewed in [10]). This epigenetic mark is usually associated with transcriptional repression, and is essential for normal embryonic development, X-chromosome inactivation, and genomic stability. CpG methylation is mainly catalyzed by the DNA methyltransferases (Dnmt) Dnmt1, Dnmt3a, and Dnmt3b [11]. Dnmt1 acts as "maintenance" methyltransferase that methylates hemi-methylated CpGs, whereas Dnmt3a and Dnmt3b (Dnmt3a/3b) are "de novo"methyltransferases that modify unmethylated DNA. Targeted deletion of either the maintenance Dnmt or the de novo Dnmt genes in mouse demonstrated that Dnmts are essential for proper embryonic development [11–13]. Mutations in the *Dnmt3b* gene in human cause a rare autosomal disease, called ICF (immunodeficiency, centromere instability, facial abnormalities) syndrome, with some B-cell specific aspects that include defective B-cell-negative selection and terminal differentiation [14–16]. ICF missense mutations in the *Dnmt3b* gene in mice resulted in similar phenotypes as in human ICF patients [17]. Notably, deletion or mutation in *Dnmt3a/3b* gene cause defects in the development of hematopoietic cells. Previous studies have demonstrated impaired repopulation abilities of HSCs [18–20] and T-cell death by apoptosis [17] when any of the *Dnmt* genes is deleted. Together these studies demonstrated an important role for these enzymes in the hematopoietic system.

De novo DNA methylation of CpG dinucleotides is a known key epigenetic modification that influences tissue and context-specific gene expression [21]. Many genes show changes in expression during early lymphopoiesis and in the process of V(D)J recombination [4]. However, it is unclear whether de novo DNA methylation has an influence on this process [22]. In the present study, using B-cell specific conditional knockout mice for the de novo methyltransferases, *Dnmt3a* and *Dnmt3b*, we interrogated the role of these enzymes in early B-cell development and V(D)J rearrangement.

Results

Deletion of both Dnmt3a and Dnmt3b in B cells enhances Ig kappa rearrangement in vivo

To study the function of the de novo DNA methyltransferases Dnmt3a and Dnmt3b in B cells, we made use of conditionally targeted (floxed) mice in conjunction with *mb1-cre* mice, which express Cre recombinase throughout B-cell development [23]. Mice carrying floxed alleles for both *Dnmt*3a and *Dnmt3b* (*Dnmt3a*$^{F/F}$:*Dnmt3b*$^{F/F}$) were intercrossed with mice carrying the *mb1-cre* allele to generate *Dnmt3a/3b*$^{F/F}$+ *mb1-cre* mice (called hereafter *Dnmt3a/3b KO*) and *Dnmt3a*$^{F/F}$:*Dnmt3b*$^{F/F}$ mice that did not carry the *mb1-cre* allele (called hereafter control mice). Cre-mediated deletion completely ablated the expression of both *Dnmt3a* and *Dnmt3b* in B cells (Supporting Information Fig. 1A).

We used flow cytometry in conjunction with staining of single cells suspensions with combinations of B-cell specific surface markers in order to identify and isolate successive stages of B-cell development. Both *Dnmt3a* and *Dnmt3b* are expressed throughout B-cell development, albeit at varying levels: while *Dnmt3a* is expressed relatively equally in all stages examined, *Dnmt3b* shows the highest level in pre-BI cells and is expressed at a lower level in following stages (Supporting Information Fig. 1B). We tested whether B-cell specific ablation of Dnmt3a and Dnmt3b has an impact on B-cell development and for this examined a number of B-cell subsets in control and *Dnmt3a/3b KO* mice. The number of cells corresponding to the major B-cell populations did not differ between the two genotypes (Supporting Information Fig. 1C). Likewise, the number of splenic CD19$^+$ B cells was not affected by the deletion of these two enzymes (Supporting Information Fig. 1D). Thus, even though *Dnmt3a* and *Dnmt3b* are expressed throughout B-cell development, ablation of both enzymes does not appear to be detrimental.

The *IgH* and *IgL* loci undergo rearrangement of their V, D, and J gene segments in B cells during early developmental stages in the bone marrow (BM). To examine whether B-cell specific deletion of Dnmt3a and Dnmt3b has an impact on this process we analyzed V(D)J recombination in B cells lacking Dnmt3a and Dnmt3b. For this, genomic DNA from pre-BI cells was tested for V(D)J rearrangement by semiquantitative PCR. *Ig* rearrangement of the *IgH* locus normally starts first with DJ joining in pro-B cells followed by V-DJ joining in pre-BI cells. Subsequently, VJ rearrangements take place at the *IgL* loci in small pre-BII cells (reviewed in [6]). Therefore we first examined the DJ and V-DJ rearrangement at the *IgH* locus. Pre-BI cells from mice that lack both Dnmt3a and Dnmt3b did not show any altered DJ or V-DJ arrangements at either proximal or distal V regions of the *IgH* locus (Fig. 1A). In contrast, in these cells as well as in large pre-BII cells, premature *Igκ* (Vκ-Jκ) rearrangement was observed as compared with cells derived from control mice, which undergo robust Vκ-Jκ rearrangement as expected only at the small pre-BII cell stage (Fig. 1B). Interestingly, in splenic mature B cells no difference in *Igκ* rearrangement pattern was observed between the two genotypes.

We then examined whether early recombination of the *Igκ* light chain in *Dnmt3a/3b KO* mice leads to an increase in the total number of cell surface Kappa expressing cells (κ$^+$) in the BM. However, flow cytometry analysis of BM cells stained for Ig Kappa protein did not reveal any difference in the proportion of cells expressing surface κ$^+$ cells when comparing control and *Dnmt3a/3b KO* mice (Supporting Information Fig. 2A). In contrast, intracellular kappa light chain expression was found to be increased in large pre-BII cells (Supporting Information Fig. 2B). Taken together, these data indicate that deletion of Dnmt3a and Dnmt3b in B cells

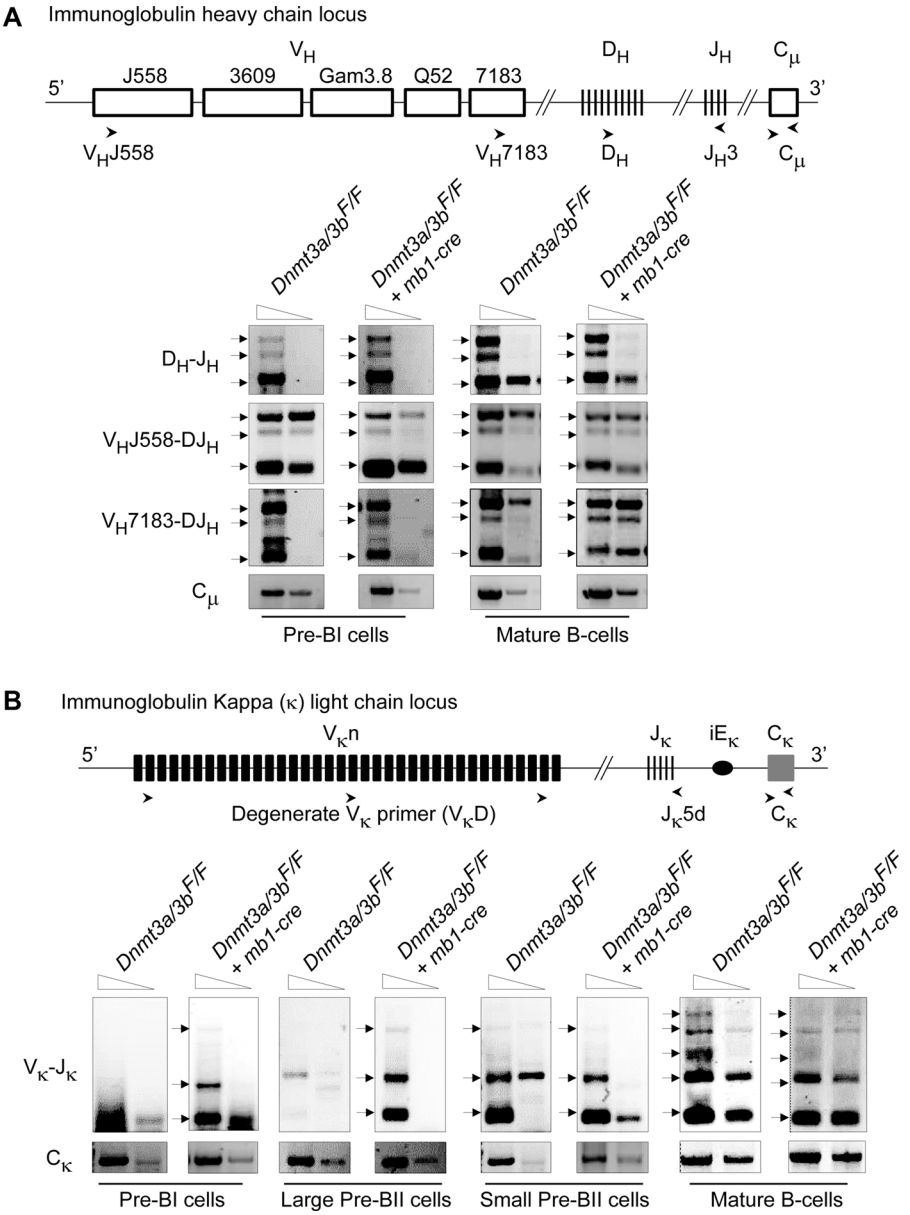

Figure 1. Dnmt3a and Dnmt3b deletion results in early rearrangement of Igκ light chain in pre-BI cells. (A) Top: Schematic representation of the immunoglobulin heavy chain locus (not to scale), indicating variable (V), diversity (D), joining (J), and constant (Cμ) regions and including the location of primers used for the PCR (arrow head). Bottom: Semiquantitative PCR analysis for *IgH* rearrangement. FACS sorted pre-BI cells from BM and MACS isolated mature B cells from spleen of control and *Dnmt3a/3bKO* mice were used. Genomic DNA isolated from indicated cell type was used for semiquantitative analysis for V-DJ rearrangement. PCR for Cμ served as control. Fourfold serially diluted DNA was used. Arrows indicate V or D gene recombined with three different J regions. (B) Top: Schematic representation of the Immunoglobulin Kappa (κ) light chain locus (not to scale), indicating the V, J, and C regions, as well as the intronic enhancer (iEκ). Primers used for the PCR (Vκ-Jκ recombination and Cκ) are indicated with an arrow head. VκD: degenerate primer for Vκ. Jκ5d: reverse primer specific for region downstream of Jκ5. Cκ: forward and reverse primers within the C region. *Bottom:* Semiquantitative PCR analysis for *Igκ* rearrangement using primers as indicated. FACS sorted pre-BI cells or pre-BII cells from BM and MACS isolated mature B cells from spleen of control and *Dnmt3a/3b KO* mice were used, as indicated. Genomic DNA isolated from indicated cell type was used for semiquantitative analysis for Vκ-Jκ rearrangement by PCR. Fourfold serially diluted DNA was used for each PCR reaction. Bands correspond to each of the four Jκ gene segments (marked with an arrow). PCR for Cκ served as control for the amount of genomic DNA used. (A and B) Data are representative of three independent experiments, using as samples pools of two mice per genotype.

leads to precocious Vκ-Jκ rearrangement and enhanced intracellular expression of Kappa protein.

Early recombination of κ light chain in ex vivo pre-BI cells lacking Dnmt3a and Dnmt3b

In order to further examine the mechanism of early *Igκ* rearrangement in pre-BI cells lacking Dnmt3a and Dnmt3b, we then isolated early progenitors with lymphoid and myeloid potential (EPLM) from the BM and expanded them ex vivo as described previously [24]. EPLM cells ($B220^+CD117^+CD93^+CD19^-NK1.1^-$) expanded *ex vivo* rapidly become positive for expression of CD19 and the cultures result in the accumulation of pre-BI cells that are $B220^+CD117^-CD93^+CD19^+$. These cells (hereafter called ex vivo pre-BI cells) are efficiently deleted for Dnmt3a and Dnmt3b when obtained from *Dnmt3a/3b KO* mice (Supporting Information Fig. 3A). V-DJ rearrangements at the *IgH* locus in these ex vivo pre-BI cells are similar to those in BM pre-BI cells, and are not influenced by deletion of Dnmt3a and Dnmt3b (data not shown).

Pre-BI cells are normally devoid of *Igκ* recombination; we therefore tested ex vivo pre-BI cells of both genotypes for Vκ-Jκ rearrangements by semiquantitative PCR. Indeed ex vivo *Dnmt3a/3b KO* pre-BI cells showed premature recombination of the *Igκ* locus, whereas the *Dnmt3a/3b$^{F/F}$* control cells were not rearranged at the *Igκ* locus. However, upon IL-7 withdrawal *Igκ* rearrangement was observed in control cells as well, as expected (Fig. 2A).

Next, we quantified the recombination level between Vκ and Jκ1 by real-time PCR. The result showed that ex vivo pre-BI cells lacking Dnmt3a and Dnmt3b have approximately a tenfold increase in rearrangement compared with the control cells (Fig. 2B). In addition, to exclude the possibility that this effect

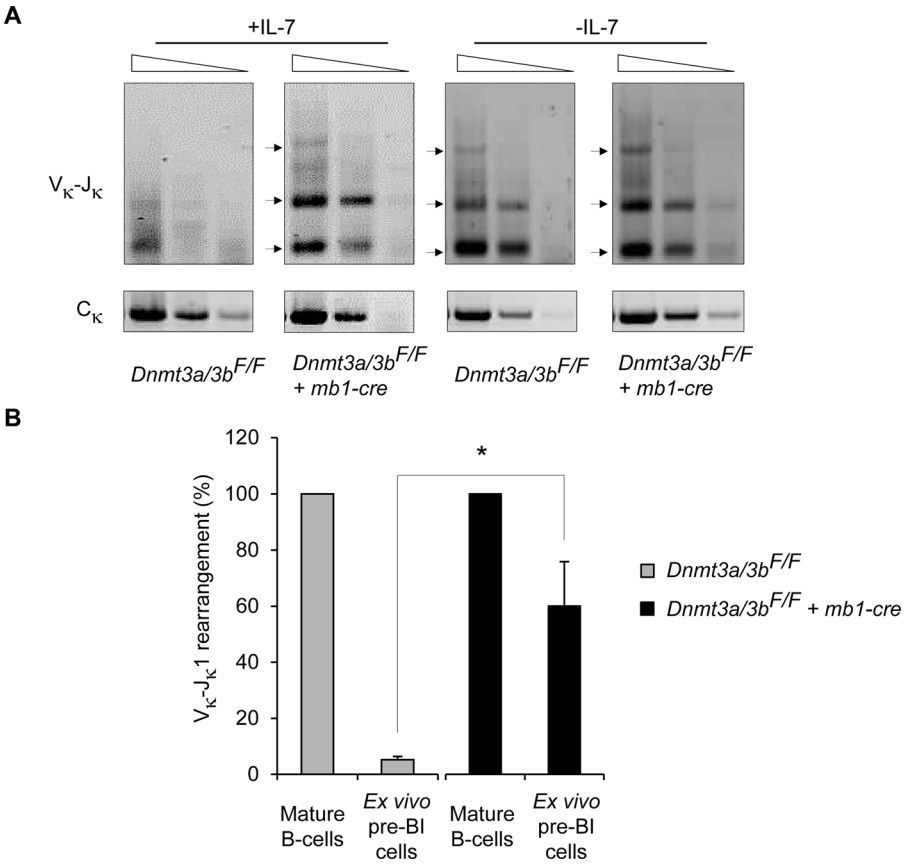

Figure 2. Premature recombination of Igκ light chain in ex vivo pre-BI cell cultures from Dnmt3a/3b KO mice. (A) Semiquantitative PCR analysis for *Igκ* Vκ-Jκ rearrangement in ex vivo pre-BI cells using degenerate VκD primer and a reverse primer downstream of Jκ5 (Jκ5d), as in Fig. 1A. Cells were maintained in presence or absence of IL-7, as indicated. Fourfold serially diluted genomic DNA prepared from ex vivo pre-BI cells from control and *Dnmt3a/3b KO* mice was used for the PCR amplification. Bands correspond to different Vκ-Jκ rearranged (marked with an arrow). PCR for Cκ served as control. Data shown are representative of four independent experiments, using as samples pools of two mice per genotype. (B) The amount of recombined Vκ-Jκ fragment in genomic DNA of ex vivo pre-BI cells were measured by quantitative PCR using degenerate VκD primer and a reverse primer specific for Jκ1. Levels of completely rearranged Vκ-Jκ1 product from corresponding mature B cells of each genotype was set to 100% after normalizing with internal control β-actin. Data represent four independent experiments, with DNA samples pooled from two mice per genotype for each experiment. $^*p < 0.05$, unpaired *t*-test.

might be due to a cre-mediated artifact, we also examined the rearrangement of *Igκ* in cells that are heterozygous for the floxed alleles (*Dnmt3a*$^{F/+}$;*Dnmt3b*$^{F/+}$) and carry *mb1-cre* (Supporting Information Fig. 3B). The results show that expression of mb1-cre by itself cannot induce Vκ-Jκ rearrangement in pre-BI cells when one allele of *Dnmt3a* and *Dnmt3b* are present (Supporting Information Fig. 3C). This therefore demonstrates that presence of Dnmt3a and Dnmt3b is important to delay the onset of *Igκ* rearrangement in early B-cell progenitors.

Lack of Dnmt3a and Dnmt3b allows germline transcription and proximal Vκ loci rearrangement

During early B-cell development antigen receptor diversity is achieved by the rearrangement of any of the 96 potentially functional Vκ genes with any of the four Jκ genes that results in either productive or unproductive rearrangement [7]. Given that Dnmt3a and Dnmt3b deleted pre-BI cells undergo premature *Igκ* rearrangement, we focused on the *IgL* locus to find where rearrangement occurs within the locus. We performed Vκ repertoire analysis on Vκ-Jκ1/2 recombined products by sequencing; to identify the recombination products, sequencing data were aligned to mouse *Ig* genes using the V-Quest online tool [25]. The Vκ usage data obtained from this analysis revealed that the *Dnmt3a/3b KO* ex vivo pre-BI cells preferentially recombine at proximal Vκ regions (Fig. 3A).

Since Dnmt3a and Dnmt3b deleted ex vivo pre-BI cells have preferential Vκ usage for the proximal V genes, we looked at the in vivo Vκ usage in BM pre-BI cells and in splenic mature B cells of control and *Dnmt3a/3b KO* mice. BM pre-BI cells from Dnmt3a and Dnmt3b deleted mice show proximal Vκ usage that is largely consistent with the results obtained with ex vivo pre-BI cells (Supporting Information Fig. 4A). In contrast, despite an increased proximal Vκ usage in either in vivo or ex vivo pre-BI cells, splenic resting B cells lacking Dnmt3a and Dnmt3b show a diverse Vκ usage that did not differ significantly from *Dnmt3a/3b*$^{F/F}$ control mice (Supporting Information Fig. 4B). Together, these results indicate that the absence of Dnmt3a and Dnmt3b has an impact on the Vκ-Jκ rearrangement process only at a very early stage of B-cell development; the difference in Vκ gene usage observed between BM pre-BI cells and splenic mature B cells could be erased by a normalization of the repertoire as a result of selection.

Premature recombination of the *Igκ* locus could reflect an open state of the chromatin. Assuming this, we expected that the transcription machinery might be active at the *Ig* locus in Dnmt3a and Dnmt3b deleted pre-BI cells. To test this, we examined the *Ig* locus germline transcripts (GLTs) and measured the expression of representative GLTs across the *Igκ* light chain locus genes (Fig. 3B). Ex vivo pre-BI cells deleted for Dnmt3a and Dnmt3b were found to have a nearly sixfold increase in the expression of GLTs at proximal Vκ genes (Fig. 3C) while the GLTs of more distal Vκ genes were not changed. Similarly, BM pre-BI cells from *Dnmt3a/3b KO* mice show an increase in the expression of proximal Vκ gene

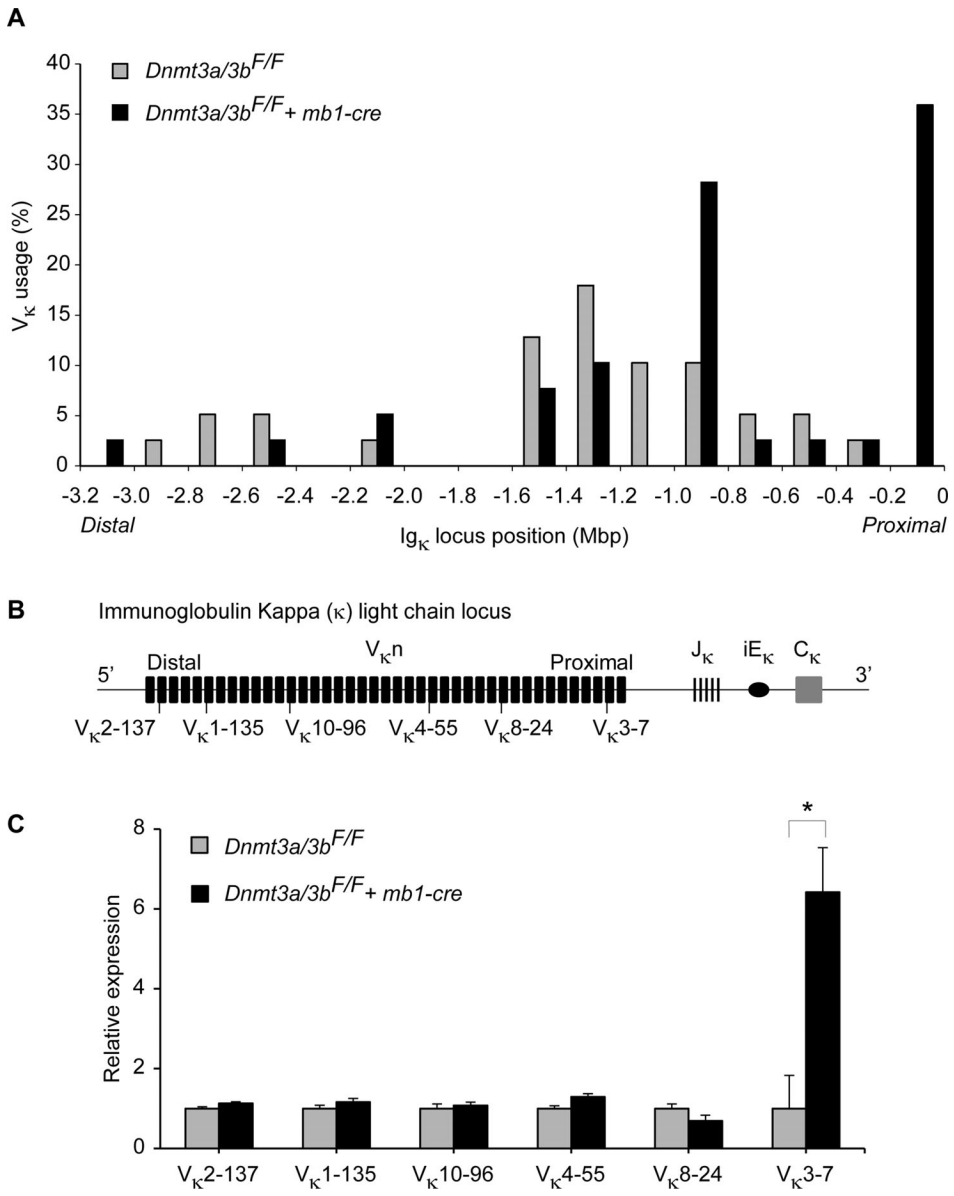

Figure 3. Preferential proximal Vκ rearrangements in Dnmt3a and Dnmt3b deleted ex vivo pre-BI cell cultures. (A) Productive and unproductive Vκ gene segment usage analysis in control and *Dnmt3a/3b KO* ex vivo pre-BI cells. Genomic DNA was isolated and used for PCR amplification of Vκ-Jκ2 recombination products; after cloning, positive clones were selected for DNA sequencing. Sequences were aligned with V-Quest database. Data represent the relative frequency of Vκ genes per 0.2 Mbp intervals in the *Igκ* locus that rearranged with Jκ1/2. Data are representative of four to six independent ex vivo pre-BI cell cultures (each from two mice) with a total of 40 and 39 sequences for control and *Dnmt3a/3bKO*, respectively. (B) Schematic representation of the *Igκ* locus (not to scale) indicating various regions along the locus (refer to Fig. 1B legends for details). The positions of specific Vκ genes along the locus are indicated. (C) Quantitative RT-PCR analysis for *Igκ* locus germline transcription in control and *Dnmt3a/3b KO* ex vivo pre-BI cells. Expression levels of different germline transcripts (GLTs) across the V regions of the κ locus as illustrated in (B) were normalized to the levels of *Gapdh*, whereby the values in control cells were set to one. Data are shown as mean +SEM and are representative of four independent ex vivo pre-BI cell cultures (each from two mice per genotype). $^*p < 0.05$, unpaired *t*-test.

GLTs (Supporting Information Fig. 5A). This is consistent with the data on Vκ usage in *Dnmt3a/3b KO* ex vivo pre-BI cells indicating that the proximal Vκ genes are accessible for rearrangement and transcription in the absence of Dnmt3a and Dnmt3b.

DNA methylation pattern at the Igκ locus is altered in Dnmt3a/3b KO pre-BI cells

To find whether preferential rearrangement and germline transcription are due to changes in methylation patterns, we examined the DNA methylation marks at the *Igκ* locus. For this, genomic DNA of ex vivo pre-BI cells from control and *Dnmt3a/3b KO* mice was subjected to sodium bisulfite treatment followed by PCR amplification (BS-PCR) at the Jκ region, at the promoter region of proximal Vκ genes and at the κ locus enhancer region [26–28] (Fig. 4A). While the methylation of CpG dinucleotides at Jκ1/2 region only showed a mild difference, we found clear changes in methylation at other Jκ regions in *Dnmt3a/3b KO* samples when compared with the control (Fig. 4B). Interestingly, *Dnmt3a/3b KO* cells exhibit a remarkable loss in methylation patterns at CpG dinucleotides at both downstream enhancer regions (3′Eκ and Edκ), whereas the intronic iEκ region is devoid of CpG methylation marks in both genotypes. Furthermore, the promoter regions of the proximal Vκ genes (*Vκ 3-7*) highly expressed in Dnmt3a- and Dnmt3b-deleted ex vivo pre-BI cells also show clear changes in DNA methylation (Fig. 4B). Next, we verified the methylation patterns of BM pre-BI cells of both control and *Dnmt3a/3b KO* mice. While we found less changes in methylation at the Jκ region as well as at the κ enhancer regions between the genotypes, we saw similar changes in methylation of promoter regions of the proximal Vκ genes in pre-BI cells lacking Dnmt3a and Dnmt3b (Supporting Information

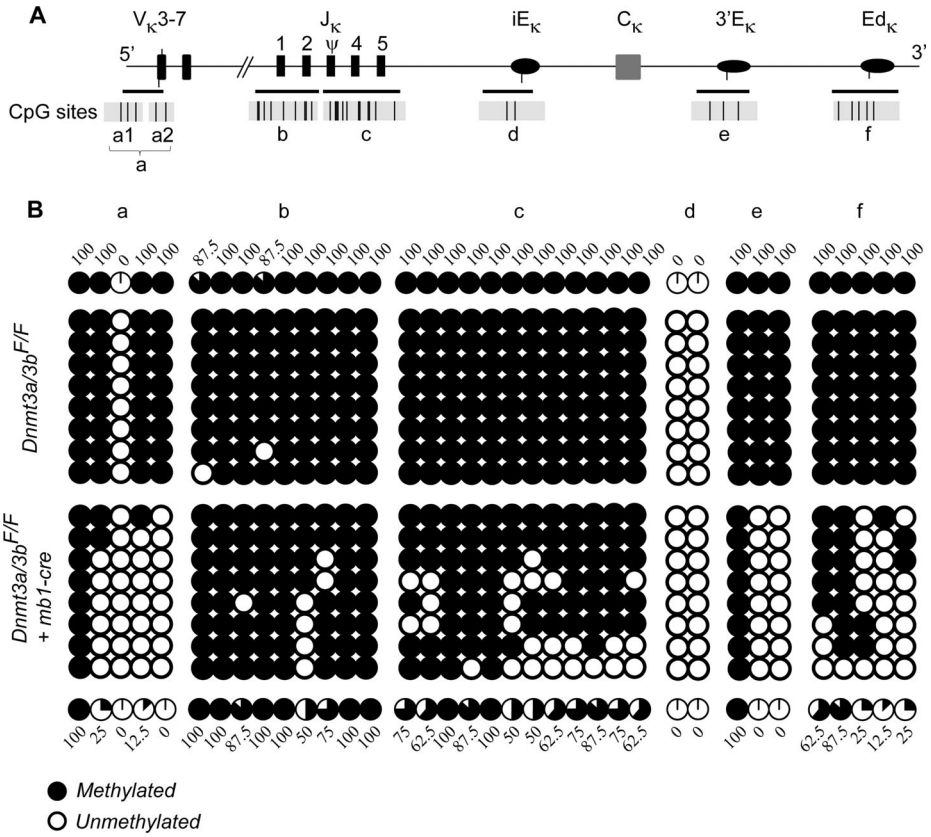

Figure 4. Altered methylation patterns at Igκ locus in absence of Dnmt3a and 3b. (A) Schematic representation of *Igκ* locus (not to scale), highlighting the regions analyzed by bisulfite PCR analysis (a–f). Region 'a' includes two amplicons a1 and a2 with 3 and 2 CpGs, respectively. (B) Bisulfite sequencing PCR analysis at indicated regions with ex vivo pre-BI cells (control and *Dnmt3a/3bKO*). The genomic DNA prepared from these cells were subjected to bisulfite conversion reaction followed by PCR using primers designed for specific target regions a, b, c, d, e, and f. The resulting PCR products were cloned in pGEM-T vector and positive clones (minimum of 10) were sequence verified for modified cytosine residue. Filled (dark) circles and unfilled open circles represent methylated and unmethylated CpGs, respectively. The percentage of methylation at each CpG is indicated numerically and in the form of a pie diagram. Data represent sequencing results from three independent ex vivo pre-BI cell cultures.

Fig. 5B). Collectively these results indicate that Dnmt3a and Dnmt3b have a direct regulatory activity in inducing changes in the methylation pattern of the *Igκ* locus at specific regions.

Increased expression of transcription factor E2A in cells lacking Dnmt3a and Dnmt3b

Next, we investigated the expression of critical B-cell specific factors known to be involved in early B-cell development. Among the various factors analyzed we found that ex vivo pre-BI cells lacking Dnmt3a and Dnmt3b show a significant increase in the expression of the precursor-B cell receptor (pre-BCR) components *Vpreb1* and *Igll1* (*λ5*) and of the critical B-cell transcription factor E2A *(Tcfe2a)* (Fig. 5A). In this analysis, we also observed a difference in the expression of the recombination activating genes (*Rag-1* and *Rag-2*), although not statistically significant. Increase in the expression of Rag-1 and Rag-2 is often associated with a cell-cycle arrest at specific developmental stages [29, 30]. Therefore, we examined the proliferation of control and *Dnmt3a/3b KO* ex vivo pre-BI cells. Interestingly, long-term culture of ex vivo pre-BI cells from mice lacking both Dnmt3a and Dnmt3b showed impaired proliferation and alteration in cell cycle with a partial block in the G1 phase (Supporting Information Fig. 6A and B). To rule out that cell-cycle-induced Rag expression might have led to premature Vκ rearrangements, we used cell cultures at earlier time points (at day 7), at which the cumulative cell numbers of both control and *Dnmt3a/3b KO* ex vivo pre-BI cells show no difference (Supporting Information Fig. 6A). These ex vivo pre-BI cells have equal levels of Rag-2 expression, irrespective of whether deleted for Dnmt3a and Dnmt3b or not (Supporting Information Fig. 7A). Interestingly, we also found an increase in the expression of proximal Vκ gene GLTs (Supporting Information Fig. 7B) and rearrangement of Vκ-Jκ (Supporting Information Fig. 7D) in cells lacking Dnmt3a and Dnmt3b. Thus, the activation of premature rearrangement of *Igκ* is due to the loss of Dnmt3a and Dnmt3b and not due to the loss of their proliferation potential.

E2A, which is encoded by the *Tcfe2a* gene, is indispensable for the proper initiation of B-lymphopoiesis and is known to bind at enhancer regions during V(D)J recombination at the pre-BI cell stage [31–33]. We examined the expression of E2A protein by Western blotting at an early time point in ex vivo pre-BI cell cultures and found that *Dnmt3a/3b KO* cells showed increased expression of E2A when compared with control cells (Fig. 5B).

Another possible mechanism allowing for an increase in E2A expression is the attenuation of IL-7R signaling in pre-BI cells. Normally, IL-7R signaling leads to phosphorylation of STAT5 (pSTAT5) and thereby controls the survival, expansion, and maintenance of pre-BI cells [34]. IL-7 withdrawal allows for dephosphorylation of STAT5, exit from the cell cycle and initiation of *Igκ* rearrangement in the subsequent developmental stage (pre-BII cells). E2A is known to play a critical role during this transition. We therefore examined whether IL-7 signaling might be defective in *Dnmt3a/3b KO* ex vivo pre-BI cells. As shown in Fig. 5B,

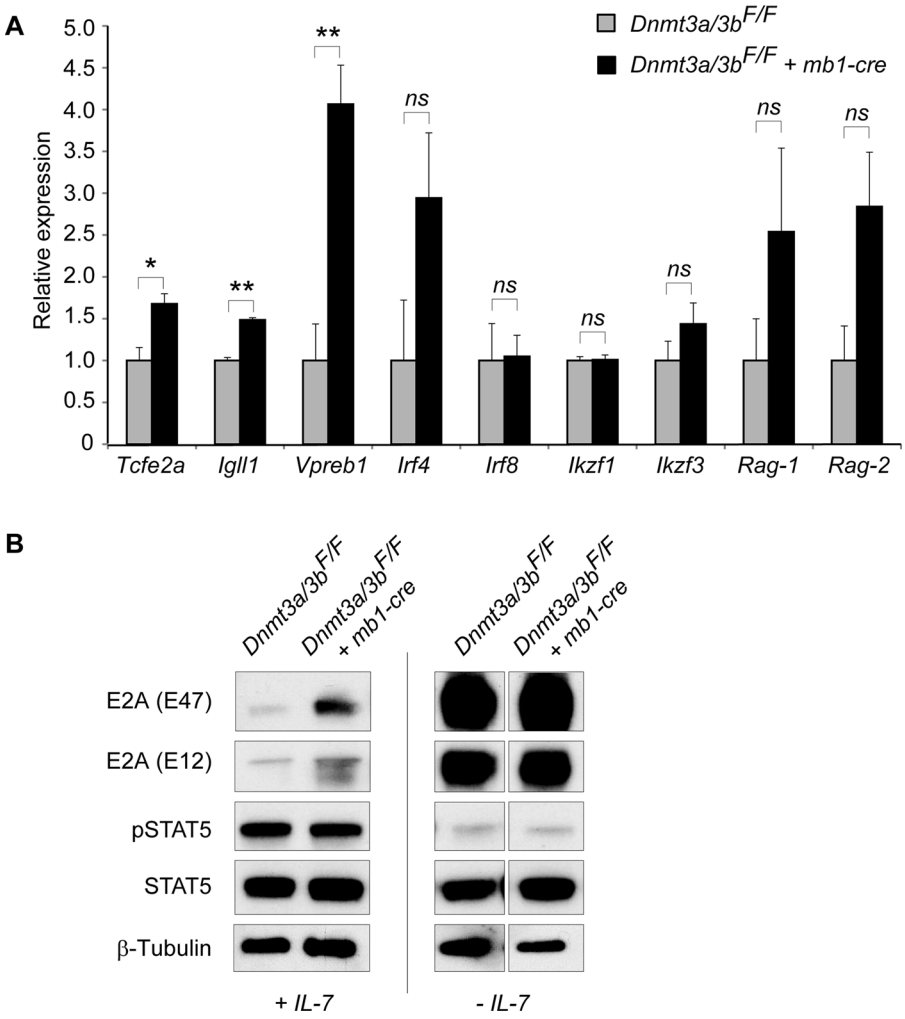

Figure 5. Altered gene expression patterns upon Dnmt3a and Dnmt3b deletion. (A) Difference in the expression of *Vpreb1*, *Igll1* (λ5), and *Tcfe2a* in *Dnmt3a/3b KO* ex vivo pre-BI cells. Total RNA from control and *Dnmt3a/3b KO* ex vivo pre-BI cells was reverse transcribed and the resulting cDNA was analyzed for the expression of indicated genes by quantitative RT-PCR. Expression levels are represented as relative to the expression levels of *Gapdh* with the expression level in control cells set to 1. Data are shown as mean +SEM and are representative of four independent ex vivo pre-BI cell cultures (each from two mice per genotype). $^{*}p < 0.05$, $^{**}p < 0.005$ and ns, not significant; unpaired *t*-test. (B) Western blotting analysis for indicated proteins. Lysates were prepared from ex vivo pre-BI cells of the indicated genotypes, cultured for 48 h either in the presence or absence of IL-7. Blots are representative of two independent experiments.

withdrawal of IL-7 from the culture medium led to a strong increase in E2A protein expression, irrespective of the presence or absence of Dnmt3a and Dnmt3b. Furthermore, IL-7R signaling and expression of pSTAT5 are preserved in these cells (Fig. 5B). Together, these observations indicate that the elevated E2A expression in pre-BI cells lacking Dnmt3a and Dnmt3b is not due to the loss of IL-7R signaling.

Altered DNA methylation at upstream regulatory regions of the Tcfe2a gene

To verify whether the increased expression of E2A was due to methylation changes at regulatory regions of the *Tcfe2a* gene, we examined their methylation status by BS-PCR. As the murine *Tcfe2a* gene consists of a CpG island promoter that is normally unmethylated, we extended our BS-PCR sequencing further upstream of the promoter region (Fig. 6A) and included regulatory regions that had been identified 6.5 kb upstream of the transcription start site [35]. Interestingly, ex vivo pre-BI cells lacking Dnmt3a and Dnmt3b showed marked differences in methylation pattern at specific regions along these upstream regulatory regions when compared with *Dnmt3a/3b*$^{F/F}$ control cells (Fig. 6B). These data indicate that Dnmt3a and Dnmt3b are involved in the methylation of the *Tcfe2a* gene in early B-cell progenitors.

Discussion

Immunoglobulin rearrangement is a tightly controlled process during early B-cell development [4]. Here, we show that the de novo methyltransferases Dnmt3a and Dnmt3b are involved in the process controlling the onset of *Igκ* light chain locus rearrangement. By using *mb1-cre* mice to conditionally delete these two enzymes in the B-cell lineage, we found that the rearrangement of *Igκ* is precociously activated in pre-BI cells (Fig. 1B). Yet, this does not impair B-cell development and the major B-cell subsets are not altered (Supporting Information Fig. 1). Gray et al. had used *CD19-cre* mice to delete these two enzymes [36] and had come to a similar conclusion regarding the normal presence of different B-cell populations. However, *mb1-cre* mice are known to induce more efficient B-cell specific deletion of floxed genes than the *CD19-cre* line, and allow complete deletion of floxed alleles already in the pre-BI compartment in the BM [23] unlike *CD19-cre*.

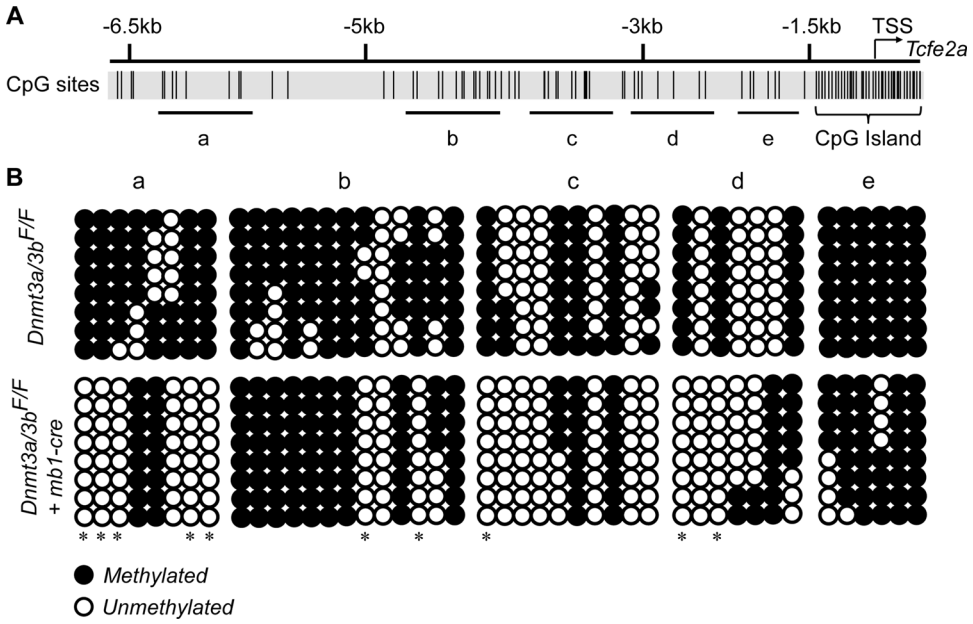

Figure 6. Methylation pattern at upstream regulatory region of the Tcfe2a gene. (A) Schematic representation of CpG distribution along the *Tcfe2a* gene locus between –6500 to +500 relative to the transcription start site (TSS; not to scale). The position of CpG dinucleotides is denoted by a black vertical line. The region around the TSS with above 50% of GCs within 100 bp window is denoted as CpG island. Underlined regions (a–e) indicate the Bisulfite PCR (BS-PCR) validated amplicons, which all span around 450–550 bp. (B) Results of BS-PCR performed for the indicated regions (a–e). Bisulfite converted genomic DNA of ex vivo pre-BI cells from control and *Dnmt3a/3b KO* were PCR amplified using specific primers and the resulting products were cloned in pGEM-T vector. Positive colonies were sequenced and methylation status of the CpG dinucleotides were verified that are indicated either with filled (dark) circles for methylated or unfilled open circles for unmethylated CpGs. Specific CpGs within the target region with 80–100% loss in methylation in the DKO are indicated with an asterisk (*). Data represent sequencing results for 10–12 colonies from each of two independent experiments with pools of cells from two mice per genotype.

The V(D)J rearrangement process starts with the rearrangement of the *IgH* locus in pro-B and pre-BI cells, followed by *IgL* rearrangement in small pre-BII cells [6]. Upon productive rearrangement of the *IgH* locus, Igμ is expressed at the cell surface together with the surrogate light chain components λ5 and VpreB to form the pre-BCR. Based on several studies it was proposed that presence of Igμ at the cell surface is required for *IgL* (κ or λ) rearrangement in normal or in transformed pre-B cells [37–39]. In contrast, other studies proposed that μ chain expression and pre-BCR signaling are not absolutely required for *Igκ* rearrangement [40, 41]. Following that, it was shown that *Igκ* rearrangement occurs independently of *IgH* rearrangement [42, 43]. Moreover, *Igκ* rearrangements can occur in pre-BI cells at the same time as *IgH* rearrangement but at low frequency or can precede *IgH* rearrangement [44]. Hence, it is evident from these studies that premature rearrangement can occur in pre-BI cells during normal B-cell development, albeit at low frequency. We have shown here that deletion of Dnmt3a and Dnmt3b induced early rearrangement of *Igκ* in pre-BI cells in vivo (Fig. 1B) or ex vivo (Fig. 2).

Given that the deletion of Dnmt3a and Dnmt3b leads to early *Igκ* rearrangement we analyzed in detail the mechanism underlying this phenotype. *Igκ* locus rearrangement is regulated by several critical factors and pathways; in particular, IL-7 and IL-7R signaling are crucial for the initiation of *Igκ* locus rearrangement in pre-BI cells. IL-7 stimulates continuous proliferation of pro-B and pre-BI cells and when IL-7 signaling is attenuated, cells exit the cell cycle and initiate *Igκ* rearrangement [45]. Downstream of IL-7 signaling, the activation of the transcription factor STAT5 opposes *Igκ* rearrangement by binding to the κ intronic enhancer (iEκ) and the 3'Eκ, restricting recombinase accessibility at the *Igκ* locus and also germline transcription [34, 46, 47]. We showed that in vitro expanded progenitor cells lacking Dnmt3a and Dnmt3b escape from the IL-7R barrier and undergo *Igκ* rearrangement (Fig. 2A).

Cells lacking Dnmt3a and Dnmt3b show preferential recombination at the proximal Vκ region accompanied by proximal germline transcripts (Fig. 3A and C). This suggests that the local chromatin may be open and allows access to the recombination and transcription machinery. Changes in DNA methylation pattern at the proximal Vκ genes, as well as at the κ enhancer regions in Dnmt3a and Dnmt3b deleted cells provide evidence for a possible direct role of these enzymes in altering the accessibility at the *Igκ* locus. These changes were found to be more marked in ex vivo pre-BI cells than in cells directly isolated from the BM. We do not know the reason for this difference but several options can be considered. It is possible that exposure to IL-7 might have been different between the two samples: while pre-BI cells in culture are uniformly exposed to IL-7 in the culture medium, the pre-BI cells isolated from the mice might have been exposed to different concentrations of IL-7 in the BM niche, resulting in differences in the activation of the IL-7/STAT5 pathway in individual cells. Alternatively, and not mutually exclusive, it is also possible that Dnmt3a and Dnmt3b control the timing and integrity of Vκ repertoire

establishment by methylation dependent as well as methylation-independent mechanisms. The rearrangement and elevated transcription of proximal Vκ genes observed in pre-BI cells lacking Dnmt3a and Dnmt3b is clearly dependent on the presence of these enzymes, but may be DNA methylation-independent, as only few changes in DNA methylation are visible in cells directly isolated from mice. The changes in methylation over these regions may be secondary effects, only apparent in cell culture. The DNA-binding zinc finger protein CTCF was recently reported to control the proximal Vκ germline transcription and *Igκ* locus rearrangement [48]. Strikingly, deletion of CTCF in B cells leads to an increased level of the proximal Vκ transcripts together with increase in proximal Vκ usage similar to what we observe in the absence of Dnmt3a and Dnmt3b. Furthermore, CTCF occupancy is related to demethylation signatures at genomic regions [49]. From our data it is thus conceivable that Dnmt3a and Dnmt3b represent an alternate regulatory pathway for keeping the Vκ germline transcription and locus rearrangement in check.

Igκ recombination at the pre-BII stage is activated by the binding of transcription factors such as IRF4, IRF8, E2A, and PU.1 at the iEκ and 3'Eκ enhancers [33, 50, 51]. We found here that E2A is upregulated in IL-7 restricted ex vivo pre-BI cultures lacking Dnmt3a and Dnmt3b (Fig. 5), and thus possibly can bind to the enhancers to initiate *Igκ* rearrangement. We observed that the upstream regulatory regions of *Tcfe2a* have lost DNA methylation marks in these cells (Fig. 6), suggesting that these two enzymes contribute to the regulation of this important transcription factor. Recent reports have identified DNA methylation changes at distal regulatory regions during cellular differentiation [52, 53].

The catalytic activity of Dnmt3a and Dnmt3b is known to be stimulated by another member of the Dnmt3 family, Dnmt3L [54, 55]. By itself Dnmt3L does not have methyltransferase activity but can recruit Dnmt3a/3b at sites of transcriptional activity and thereby allow them to modulate DNA methylation [56, 57]. In addition, isoforms of Dnmt3b including catalytically inactive forms like those that are seen in ICF syndrome can modulate the activity of Dnmt3a-Dnmt3L complexes and alter genomic methylation patterns [58]. It has been reported that Dnmt3a and Dnmt3b can interact with histone deacetylases (HDACs), methyl-binding proteins and through their association with such protein complexes act as corepressors to silence gene expression in a methylation-independent manner [59–61]. For example, Dnmt3a and Dnmt3b association with HDACs is considered to help maintain chromatin in a compact and silent state [62, 63]. While deletion or inhibition of HDAC1 and HDAC2 blocked B-cell development at the pre-BII cell stage [64, 65], deletion of Dnmt3a and Dnmt3b does not impair B-cell development ([36] and this study), although *Igκ* recombination is impacted. Furthermore, Dnmt3a was recently shown to be required for differentiation of hematopoietic stem cells [66]. In addition to their de novo methyltransferase activity, Dnmt3a/Dnmt3b also can as function as maintenance methyltransferases to preserve DNA methylation after DNA replication, similarly to Dnmt1 (reviewed in [67]). This was shown to occur especially in densely methylated or repetitive sequences regions [68, 69] and different Dnmts cooperate to maintain methylation patterns [70].

Recent reports have underscored an active DNA methylation turnover and demethylation signature upon binding of transcription factors to the regulatory regions [71, 72]. In the V(D)J rearrangement paradigm it is widely accepted that demethylation of the *Ig* locus has profound importance for the recombination process [22]. Localized demethylation at the recombination intermediates is known to be critical in B cells during Ig rearrangement [73]. However, it is not clearly understood if and how de novo DNA methylation could influence *Ig* recombination. An in vitro study showed that CpG methylation of heptamers of RSS sites can inhibit Rag-1/2 mediated cleavage of the V(D)J segments [74] suggesting a direct control of the recombination mechanism by DNA methylation. Another study has linked localized DNA methylation and Ig locus silencing [75, 76]. Our work shows that de novo Dnmt3a and Dnmt3b can influence methylation at the *Igκ* locus enhancer region and also the upstream regulatory regions of genes important for initiation of *Igκ* rearrangement, such as *Tcfe2a*. Thus, Dnmt3a and Dnmt3b actively take part in the repression of specific regions by DNA methylation and thereby prevent the onset of *Igκ* rearrangement at the very early stage of B-cell lineage.

Materials and methods

Mice

Conditional Dnmt3a ($Dnmt3a^{F/F}$) and Dnmt3b ($Dnmt3b^{F/F}$) mice of 129SvJ background were obtained from Dr. En Li (CNIBR, Shanghai). We crossed $Dnmt3a^{F/F}$ mice with $Dnmt3b^{F/F}$ mice to produce *Dnmt3a* and *Dnmt3b* floxed homozygous offspring ($Dnmt3a/3b^{F/F}$). For the generation of B-cell specific deletion of *Dnmt3a* and *Dnmt3b* we used *mb1-cre/+* transgenic mice (C57BL/6 background). The *mb1-cre/+* mice were bred to $Dnmt3a/3b^{F/F}$ mice to obtain $Dnmt3a/3b^{F/F}$ with *mb1-cre* ($Dnmt3a/3b^{F/F}$+ *mb1-cre*) or $Dnmt3a/3b^{F/F}$ without *mb1-cre* (control) mice. Animals were housed in a controlled environment and experiments were conducted in accordance with the ordinance provided by Cantonal Veterinary Office, Basel-Stadt, Switzerland and were approved by the Animal Committee of the Friedrich Miescher Institute for Biomedical Research.

Flow cytometry, Intracellular staining, and B-cell sorting

Erythrocyte depleted BM cells from 3–6 weeks old female mice were resuspended in staining buffer (2% fetal bovine serum in PBS) containing either FITC-, PE-, APC-, APC/Cy7-, PE/Cy5.5, Brilliant Violet or biotin conjugated anti-CD45R/B220, anti-CD19 (1D3), anti-CD117 (2B8), anti-CD43, anti-CD2, anti-CD25, anti-Ig Kappa, and anti-IgM (all from BD Biosciences) followed by

secondary labeling with Streptavidin (SA) conjugated PE/Cy5.5 (Life technologies) or APC/Cy7 (BD Biosciences). Cell sorting was performed with a MoFlo (DakoCytomation) or FACS Aria II (BD Biosciences). B-cell subsets were isolated as follows; Pre-BI cells: $B220^+CD117^+CD19^+CD43^{high}CD25^-CD2^-$, large pre-BII cells: $B220^+CD117^-CD19^+CD43^{high}CD25^+CD2^-$ and small pre-BII cells: $B220^+CD117^-CD19^+CD43^-CD25^+CD2^+$. Splenic mature resting B cells were isolated by incubating single cell suspensions of splenocytes with anti-CD43 microbeads (Miltenyi Biotec) in MACS buffer (0.5% BSA, 2 mM EDTA in PBS) followed by purification over a MACS column (Miltenyi Biotec). Intracellular staining of Ig Kappa was done using Cytofix/Cytoperm Buffer kit (BD Biosciences) following the manufacturer's instructions. Flow cytometry was performed with a FACS Calibur (BD Biosciences) and analysis was done with FlowJo software (Tree Star).

In vitro cell culture

The OP9 mouse stromal cell line was maintained in complete IMDM (Life Technologies) supplemented with 50 μM β-mercaptoethanol, 1 mM glutamine, 0.03% w/v primatone (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2% heat inactivated fetal bovine serum (Sigma). Isolation and ex vivo culture of BM-derived EPLM cells was done as described previously [24, 77]. Briefly, BM single cell suspension was stained with FITC-B220, APC-CD117 (2B8), PE-CD19 (1D3), PE-NK1.1 (PK136), and biotin conjugated early B-lineage marker CD93 (493) (all from BD Biosciences) followed by secondary labeling with SA-PE/Cy5.5 (Life technologies). EPLM cells ($B220^+CD117^+CD93^+CD19^-NK1.1^-$) were isolated by FACS sorting. cells were collected in complete IMDM medium containing 5 ng/mL IL-7 (Peprotech) and cocultured with γ-irradiated OP9 stromal cells. Pre-BI cells derived from EPLM cultures (ex vivo pre-BI cells) were harvested by MACS for $CD19^+$ cells between 7 and 21 days for use in various experiments. For IL-7 withdrawal experiments, 48 h prior to harvest cells were cultured in complete medium containing 0.1 ng/mL IL-7.

Vκ-Jκ rearrangement and Vκ gene usage analysis

Genomic DNA was extracted using the Wizard SV Genomic DNA extraction kit (Promega). PCR for Vκ-Jκ rearrangement was performed semiquantitatively on fourfold serially diluted genomic DNA using degenerate VκD primer [41] and a primer downstream of Jκ5 [78]. For the control of DNA amounts we amplified non-recombining genomic region (Cκ) [79]. PCR products were separated on agarose gels and visualized by labeling with SYBR safe DNA stain (Life technologies). For Vκ gene repertoire analysis, genomic DNA was amplified using degenerate VκD primer and a primer near Jκ2 (Jκ2-1°) [80]. PCR products were separated on agarose gels, extracted using Wizard SV PCR and Gel clean-up system (Promega) and ligated into pGEM-T easy vector system (Promega). Positive bacterial colonies were subjected to sequencing in both directions by using SP6 and T7 primers on ABI3730 × l DNA Analyzer (Life Technologies). Sequences were analyzed by V-Quest sequence alignment online tool provided by the IMGT consortium [25].

Table 1. Primer sequences

RT-PCR primers	
Gene	Primer sequence (forward and reverse)
Tcfe2a	5′-CATGCTAGGTGACGGCTCTT-3′
	5′-AGCGAGCCATTAACCTCAGA-3′
Bisulfite sequencing PCR primers	
Target region	Primer sequence (forward and reverse)
Vκ 3-7 (a1)	5′-TGTTAGGAATTTATTTTTTTGATTAATT-3′
	5′-AAAAATTCTCTTATTTCCTTTCATCTAT-3′
Vκ 3-7 (a2)	5′-TTGGTTTATAGTGTGAGTTATAGGATAA-3′
	5′-AAAAAAATAAAAAATTCAAAAAACTAAA-3′
Jκ 1-2(b)	5′-TAGTGAGGAGGGTTTTTGTATAGTTAG-3′
	5′-AACAAAAAAACAACTTAACAAAATTAAACT-3′
Jκ ψ-5 (c)	5′-TAGTTTTAGGTAGGTTTTTGTAAAGG-3′
	5′-AAACAAAAAATCTAACACTATATACCAC-3′
iEκ (d)	5′-TTAAGGATTTTTTAAAATTTTTGAAATT-3′
	5′-ACTAACTTTCATTTTCTCCTATCTCTTC-3′
3′Eκ (e)	5′-TGTTAAGATTTGGTTATTAAGAGGTAGA-3′
	5′-AAAATATAAAATACACCACCCAAACTAT-3′
Edκ (f)	5′-AAATTGAATTATTTTTAATTTTTTGTGA-3′
	5′-TCTTTTACCAAATCCATCTACACTAC-3′
Tcfe2a (a)	5′-TTAGTATAAGGAGGTGGTTATTAGTGAT-3′
	5′-TTAATTAAAACAAATTTAAAAAAAATCC-3′
Tcfe2a (b)	5′-TTTGTTATTTTAGTTTTTTGAAAGTGAG-3′
	5′-ACATTAAACTTTTCCACCTTAAACTTAT-3′
Tcfe2a (c)	5′-TAATATTTATTTTTGGTGATGGAGTTTA-3′
	5′-CCCTCCTAACTATACCTTTTATTCTACT-3′
Tcfe2a (d)	5′-AGTAGAATAAAAGGTATAGTTAGGAGGG-3′
	5′-ACCCACCATATAAAAATACAACATATAA-3′
Tcfe2a (e)	5′-GGGTAAATATTTAATTTTATGGGTTTAG-3′
	5′-CAAACTAACCTCCAACTTCCTATATAA-3′

Reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was purified from cells with the RNeasy kit (Qiagen). First strand cDNA synthesis was performed by reverse transcription using oligo (dT) primer and Improm Kit (Promega). RT-qPCR was performed in triplicates on a StepOne Plus Real Time PCR system (Applied Biosystems) using FastStart Universal SYBR Green Master (Roche) followed by melting curve analysis. PCR results were analyzed using StepOne software (Applied Biosystems) and relative expression (to *Gapdh*) was calculated using the ΔCt method. Primers sequences used for qRT-PCR other than those that were published [48] are listed in Table 1.

The amount of Vκ-Jκ rearrangement in genomic DNA of ex vivo pre-BI cells was quantitatively measured by qPCR using a VκD degenerate primer and a reverse primer specific for Jκ1, as described previously [81]. Germline levels were normalized to the levels of a β-actin genomic region and the levels of completely rearranged Vκ-Jκ1 product from corresponding splenic mature B cells of each genotype were set to 100%.

Bisulfite treatment and DNA methylation analysis

For sodium bisulfite conversion 500 ng of genomic DNA was used as starting material and then purified using an EpiTect Bisulfite Kit (Qiagen). BS-PCR-specific primers were designed using MethPrimer online tool [82] and are listed in Table 1. Converted DNA was amplified in AmpliTaq Gold Polymerase plus Buffer Kit (Roche) under the following PCR program: 15 min of initial incubation at 95°C followed by 20 cycles of 95°C for 30 s 60°C (0.5°C decreasing per cycle) for 30 s, and 72°C for 1 min. This was followed by 36 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 1 min and a final extension for 15 min at 72°C. PCR products were separated on agarose gel, extracted using Wizard SV PCR and Gel clean-up system (Promega) and ligated into pGEM-T easy vector system (Promega). Positive bacterial colonies were picked and DNA was sequenced using SP6 primer on ABI3730xl DNA Analyzer (Life Technologies). For each condition, 10–15 separate clones were analyzed using BiQ Analyzer software [83].

Western blot

Protein extracts were prepared in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% w/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS) and the amount of protein was estimated using BCA protein assay kit (Pierce). Fifty micrograms of protein lysates were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Protein blots were probed with antibodies to TCF-3/E2A (MAb clone 826927; R&D systems) or phospho-STAT5 (Tyr694) (Cell Signaling) or STAT5 (sc-835; Santa Cruz).

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Abbreviations: **BM**: bone marrow · **Dnmt**: DNA methyltransferase · **EPLM**: early progenitors with lymphoid and myeloid potential · **GLT**: locus germline transcript · **HDAC**: histone deacetylase

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