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# Tet DNA demethylase is required for plasma cell differentiation by controlling expression levels of IRF4

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## Abstract

**Antibodies produced by plasma cells are critical for protection from infection. It has been demonstrated that global epigenetic modification, such as changes in DNA methylation, occurs during differentiation of plasma cells from B cells. However, the precise mechanisms by which DNA methylation controls plasma cell differentiation are not fully understood. We examined the effect of deficiency of DNA demethylases, Tet2 and Tet3, on B-cell activation and plasma cell differentiation, by generating conditional Tet2/3 double-KO (Tet dKO) B cells. We found that Tet dKO B cells failed to differentiate into plasma cells upon immunization with antigens. Tet dKO B cells proliferated normally and were capable of generating cells with IRF4$^{int}$, but not with IRF4$^{hi}$, the majority of which were CD138$^{+}$ plasma cells. IRF4 overexpression rescued the defect of Tet dKO B cells in plasma cell differentiation, suggesting that Tet2/3-dependent high IRF4 expression is required for plasma cell differentiation. We identified CpG sites in the *Irf4* locus that were demethylated specifically in plasma cells and in a Tet2/3-dependent manner. Our results suggest that Tet2/3-dependent demethylation of these CpG sites is dispensable for initial IRF4 expression but is essential for high IRF4 expression which is prerequisite for plasma cell differentiation.**

*Keywords:* B cells, development, epigenome, transcription factors

## Introduction

Antibodies are effector proteins that recognize and neutralize invading pathogens and are produced by terminally differentiated B cells, called plasma cells (1). Differentiation into plasma cells is a lineage switch from B cells and thus accompanied by dramatic changes in gene expression, which extinguish B-cell identity but provide the ability to secrete large amounts of antibodies constantly (2). The transcription factors that facilitate this lineage transition are Blimp-1, Xbp-1 and IRF4 (3). Blimp-1 is recognized as a master regulator for plasma cell development and function as a transcriptional repressor of B-cell factors, such as Pax5 or Bcl6 (4, 5). Xbp-1 plays a critical role in the unfolded protein response that is induced when a cell needs to handle large amounts of proteins, such as antibodies in the case of plasma cells (6). The function of IRF4 could differ depending on its expression level (7). Higher levels of IRF4 are essential not only for Blimp-1 induction (8) but for plasma cell survival (9).

It has been demonstrated that epigenetic modifications critically regulate the lineage transition from B cells to plasma cells. A recent study has revealed that genomic DNA isolated from plasma cells is hypomethylated, compared to that from naive B cells (10). DNA hypomethylation occurs after multiple rounds of cell divisions, which is coincident with the generation of CD138$^{+}$ plasma cells (10, 11). The role of DNA

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methylation for establishing the plasma cell program was confirmed by experiments with inhibitors of DNA methylation (10) or genetic deletion of *de novo* DNA methyltransferase (12), both of which resulted in a significant increase in plasma cell differentiation. However, the precise mechanisms by which DNA methylation regulate plasma cell differentiation are not fully understood.

Ten-eleven translocation proteins (Tet1, Tet2 and Tet3) have been identified as DNA demethylases which catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and other intermediates in the DNA demethylation pathway (13). Tet proteins play key roles during early development and also contribute to lineage development (14, 15). In B cells, Tet2 and Tet3, but not Tet1, are abundantly expressed (16–19) and have crucial roles in their development and activation (20). Loss of *Tet2* and *Tet3* at early stages of B-cell development impaired rearrangement of B-cell receptor and thus resulted in developmental arrest at the pro-B to pre-B transition (15, 21). More recently, two studies have demonstrated that Tet2 and Tet3 play important roles during peripheral B-cell activation. Lio *et al*. have reported that these Tet enzymes control class-switch recombination by augmenting activation-induced deaminase (AID) expression (22). Schoeler *et al*. have revealed that Tet 2 and Tet3 are also required for antibody production (23). In the absence of *Tet2* and *Tet3*, antigen-specific antibody production *in vivo* was significantly decreased, suggesting that Tet enzyme-mediated DNA demethylation is critically involved in plasma cell differentiation. Yet, the molecular mechanisms by which Tet enzymes regulate plasma cell differentiation have been totally unclear.

In this study, we investigated how Tet enzymes control antigen-induced plasma cell differentiation from naive B cells, in which *Tet2* and *Tet3* were acutely deleted using ERT2cre. Our results suggest that Tet-mediated demethylation of *Irf4* locus allows high IRF4 expression, which thus facilitates efficient plasma cell differentiation.

## Methods

### Mice

$Tet2^{f/f}$ mice (24), $Tet3^{f/f}$ mice (24) and B1-$8^{hi}$ mice (25) are described elsewhere. Rosa26-ERT2cre mice were obtained from Taconic Farms. C57BL/6 mice were purchased from CLEA Japan. All mice were bred and maintained under specific pathogen-free conditions and all animal experiments were performed under the institutional guidelines of Osaka University.

### *Immunization, treatments and adoptive transfer*

Mice were immunized with 100 μg of hapten 4-hydroxy-3-nitrophenylacetyl (NP) coupled to chicken γ globulin (NP-CGG) precipitated with Imject alum (Thermo Fisher Scientific), or 50 μg of NP-Ficoll by intra-peritoneal (i.p.) injection. The deletion of the loxP-flanked *Tet2* and *Tet3* alleles was induced by oral administration of 2 mg tamoxifen (Sigma-Aldrich) in sunflower oil (Sigma-Aldrich) once per day for 5 consecutive days. Adoptive transfer of B1-$8^{hi}$ B cells was performed as described previously (26). In brief, splenic B cells from B1-$8^{hi}$ mice were purified by magnetic depletion using anti-CD43 MicroBeads and the AutoMACS system (Miltenyi Biotec). Purified $CD45.1^{+}$B1-$8^{hi}$ B cells containing $1 \times 10^5$ NP-binders were transferred intravenously into recipient C57BL/6 mice. To track cellular division, the purified B1-$8^{hi}$ B cells were labeled with CellTrace™ Violet (CTV, Thermo Fisher Scientific) before adoptive transfer.

### *Flow cytometry analysis*

Single cell suspensions of spleen cells were prepared in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA), stained with antibodies and analyzed on a FACSCanto II (BD Biosciences). Anti-B220 (RA3-6B2) and CD45.2 (104) were purchased from BD Biosciences. Anti-CD45.1 (A20) and CD138 (28102) were purchased from BioLegend. APC-conjugated NP was prepared as described previously (26). For intracellular staining with anti-IRF4 (3E4; Thermo Fisher Scientific), the cells were fixed and permeabilized using a Foxp3 staining kit (Thermo Fisher Scientific). Donor cell-derived activated B cells ($CD45.1^{+}NP^{+}B220^{+}CD138^{-}$) or plasma cells ($CD45.1^{+}NP^{+}CD138^{+}$) were sorted using FACSaria II (BD Biosciences).

### *Retroviral IRF4 expression*

Tamoxifen-treated B1-$8^{hi}$ $Tet2^{fl/fl}$ $Tet3^{fl/fl}$ ERT2cre mice or B1-$8^{hi}$ ERT2cre mice were immunized i.p. with 50 μg of NP-Ficoll in PBS. Six hours later, purified splenic B cells were cultured with 2 μg $ml^{-1}$ of anti-CD40 for 18 h *in vitro*. Cells were then infected with viral supernatants in the presence of 8 μg $ml^{-1}$ of polybrene by spin infection for 90 min at 800 × *g*. Retrovirus expressing IRF4 was generated by PCR cloning of IRF4 cDNA with the primers IRF4-EcoRI (5′-AGGAATTCCGCACGCGTCATGAACT-3′) and IRF4-XhoI (5′-TGCTCGAGTATTTCTTCTCACTCTT-3′). The PCR product was inserted into the pMCs-ires-GFP retroviral (RV) vector. The retroviral vectors were transfected into PLAT-E cells and viral supernatants were collected 2 days later.

### *ELISPOT assay*

Sorted cells were incubated overnight at 37°C on $NP_{29}$-BSA-coated plates. Antibody-secreting cell (ASC) spots were detected with goat anti-mouse immunoglobulin (Southern Biotech) and alkaline phosphatase-conjugated anti-goat IgG antibody (Santa Cruz), and were visualized by BCIP/NBT substrate (Promega).

### *Real-time quantitative PCR*

Total RNA was prepared with RNeasy micro kit (Qiagen) and was transcribed with ReverTra Ace qPCR Master Mix (TOYOBO). Real-time quantitative PCR (qPCR) was performed as described previously (27) with Power SYBR Green PCR Master Mix (Applied Biosystems). Expression of Actb mRNA was used for normalization. PCR primers are listed in Supplementary Table 1.

*CpG methylation analysis by bisulfite sequencing*

Genomic DNA was isolated from 1 × $10^5$ FACS-sorted cells using QIAamp DNA Micro Kit (Qiagen) and was subjected to bisulfite treatment using MethylEasy Xceed (Human Genetic Signatures). Modified DNA was amplified by PCR with the primers listed in Supplementary Table 1. The PCR products were sub-cloned into pGEM-T Easy vector (Promega) and sequenced.

*Tet2 chromatin immunoprecipitation assay*

Purified B cells were cultured with lipopolysaccharide (LPS) for 4 days. Cells were harvested and chromatin immunoprecipitation (ChIP) was performed using the ChIP-IT Express Enzymatic Kit (Active Motif) and anti-Tet2 antibody (Abcam), according to the manufacturer's protocol. Quantitative PCR was performed with the primers listed in Supplementary Table 1.

*Statistical analysis*

Statistical analysis was performed with unpaired Student's *t*-test or two-way analysis of variance (ANOVA) with Sidak's multiple comparisons test using GraphPad Prism software version 8. *P*-values <0.05 were considered significant. Error bars denote SD.

## Results and discussion

*Tet2/3 are required for plasma cell generation in vivo*

To examine the role of Tet2 and Tet3 for antigen-driven plasma cell differentiation, we crossed $Tet2^{fl/fl}$ and $Tet3^{fl/fl}$ mice with ERT2cre mice that express Knock-In B1-$8^{hi}$ BCR heavy chain (25), which is specific for the hapten 4-hydroxy-3-nitrophenylacetyl (NP) when combined with endogenous Igλ light chains. We administered tamoxifen to the B1-$8^{hi}$ $Tet2^{fl/fl}$ $Tet3^{fl/fl}$ ERT2cre mice to acutely delete Tet2 and Tet3, or to the B1-$8^{hi}$ ERT2cre mice as a control (Fig. 1A). Tet2 and Tet3 were both efficiently deleted in tamoxifen-treated mice (Fig. 1B). We purified 'Tet dKO' B cells or 'control' B cells from the tamoxifen-treated mice and transferred these B cells into congenically marked recipient mice, followed by immunization with antigens (Fig. 1A). This experimental setting allows us not only to examine B-cell intrinsic roles of Tet2/3 for antigen-dependent B-cell activation and differentiation, but to circumvent secondary effects caused by prolonged Tet2/3 deficiency during B-cell development.

To determine whether Tet2/3 is required for generation of plasma cells from antigen-activated B cells, we immunized mice with NP-CGG (Fig. 1C) or Ficoll (NP-Ficoll) (Fig. 1D) to elicit T-cell dependent or T-cell-independent immune responses, respectively. We found that control B cells robustly generated B220$^{lo}$CD138$^{hi}$ plasma cells, whereas Tet dKO B cells failed to differentiate into B220$^{lo}$CD138$^{hi}$ plasma cells upon immunization with NP-CGG or NP-Ficoll (Fig. 1C and D).

It has been demonstrated that plasma cells develop only after B cells divide multiple times (10). To examine whether Tet dKO B cells are defective in antigen-induced proliferation, purified Tet dKO B cells were labeled with CTV to track cellular division and were transferred into the recipients, followed by immunization with NP-CGG. As shown in Fig. 1(E), CTV dilution indicated that Tet dKO B cells were able to undergo multiple cellular division like control B cells. However, generation of CD138$^+$ cells, which were observed after over eight rounds of division by control B cells, was severely impaired in Tet dKO B cells even after such multiple rounds of division. These results suggest that the defect of Tet dKO B cells in plasma cell generation was not due to altered proliferation.

*Tet2/3 are required for high levels of IRF4 expression*

Previous studies have demonstrated that Tet2/3 regulate the expression of a transcription factor IRF4 during early B-cell development (15, 21). IRF4 is one of the key transcription factors that are essential for the development and function of plasma cells (3). Hence, we examined whether Tet dKO B cells are capable of expressing IRF4 upon antigen-stimulation *in vivo*. As shown in Fig. 2, Tet dKO B cells expressed IRF4 and maintained it at intermediate levels during day 3 to day 7 after activation. However, in sharp contrast to control B cells, Tet dKO B cells failed to up-regulate IRF4 expression to higher levels after day 5. High IRF4 expression in control B cells was coincident with generation of CD138$^+$ plasma cells. These results suggest that Tet2/3 are dispensable for initial induction of IRF4 expression in activated B cells, but are indispensable for higher levels of IRF4 expression, which is prerequisite for plasma cell generation (8).

*IRF4-RV restores plasma cell differentiation by Tet dKO B cells*

The aforementioned findings led us to hypothesize that the defect of Tet dKO B cells in plasma cell differentiation could be explained by their inability to express high levels of IRF4. To test this, we used a RV transduction of IRF4 to Tet dKO B cells. Pre-stimulated B1-$8^{hi}$ control or B1-$8^{hi}$ Tet dKO B cells were transduced with IRF4-RV and transferred into the recipient mice, followed by immunization with NP-CGG (Fig. 3A). As shown in Fig. 3(B), transduction of IRF4-RV into Tet dKO B cells significantly restored the generation of CD138$^+$ cells. The number of CD138$^+$ cells in IRF4-RV$^+$ Tet dKO cells was comparable to that of CD138$^+$ cells in empty-RV$^+$ control cells.

To examine whether IRF4-RV$^+$ CD138$^+$ Tet dKO cells are functional plasma cells, CD138$^-$ or CD138$^+$ cells in IRF4-RV$^+$ cells were sorted and their ability to secrete antigen-specific antibodies was tested by an enzyme-linked immunosorbent spot (ELISPOT) assay. NP-specific ASCs were detected only in CD138$^+$ cells (Fig. 3C). Importantly, the number of NP-specific ASCs in CD138$^+$ cells from IRF4-RV$^+$ Tet dKO cells was comparable with that from empty-RV$^+$ control cells, suggesting that CD138$^+$ cells derived from IRF4-RV$^+$ Tet dKO B cells function as plasma cells.

We next asked to what extent IRF4-RV established plasma cell identity in CD138$^+$ cells derived from Tet dKO B cells. We examined the expression of mRNA for Blimp-1 and Xbp-1, which are critical transcription factors, besides IRF4, for plasma cell differentiation, identity and function (1, 3). The levels of *Prdm1* mRNA were comparable, whereas the abundances of *Xbp1* mRNA were much lower in IRF4-RV$^+$CD138$^+$Tet dKO cells, compared to those in empty-RV$^+$

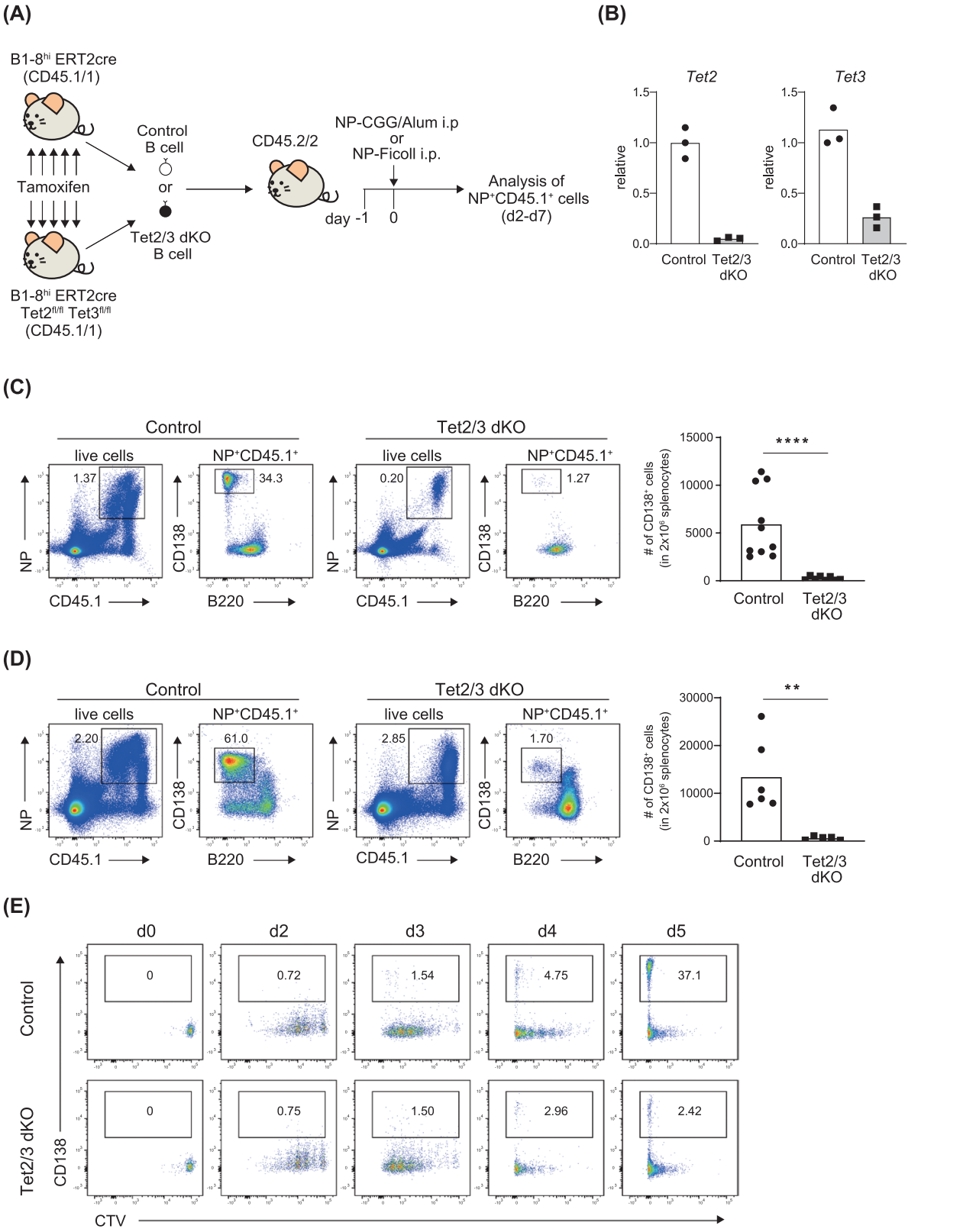


**Fig. 1.** Defect of Tet2/3 dKO B cells in plasma cell generation *in vivo*. (A) Schematic illustration of the experimental protocol for assessment of the effect of Tet2/3 deficiency in naive B cells on generation of CD138+ plasma cells. (B) Expression of *Tet2* or *Tet3* mRNA in purified control or Tet2/3 dKO B cells ($n = 3$ for each group). (C, D) Expression of B220 and CD138 in NP+CD45.1+ control or Tet2/3 dKO cells after immunization with NP-CGG (C) or NP-Ficoll (D). FACS dot plots are representative of three independent experiments. The number of donor-derived CD138+ cells in $2 \times 10^6$ splenocytes are summarized from three independent experiments (right graphs, $n = 6$–10). $^{**}P < 0.005$, $^{****}P < 0.0005$, unpaired Student *t*-test. (E) CD138 expression and CTV intensity of NP+CD45.1+ cells before (d0) or d2–d5 after immunization with NP-CGG. The frequencies of CD138+ cells are indicated. The data are from two independent experiments.

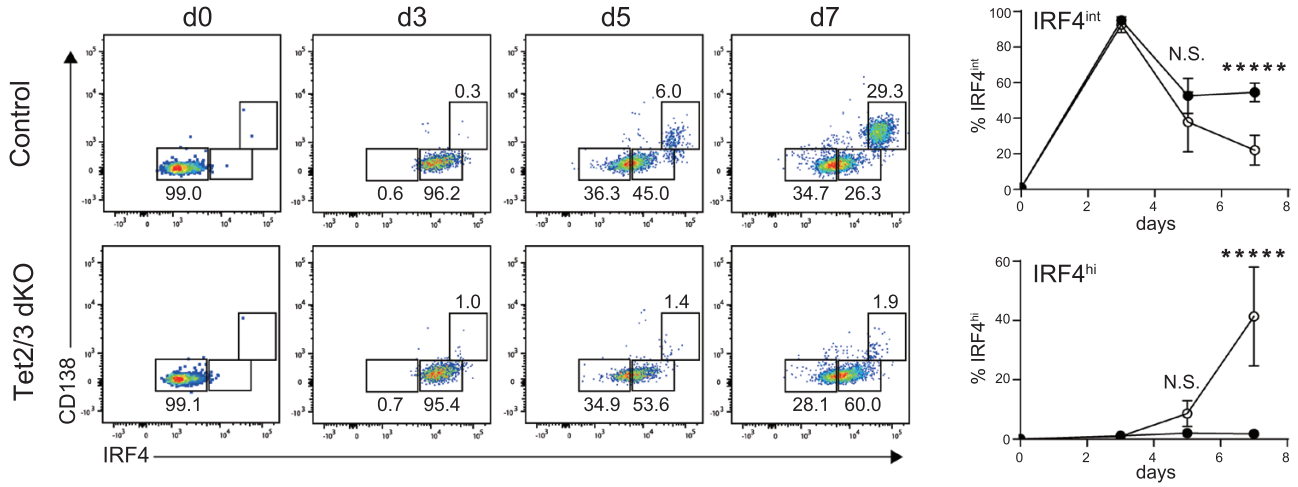


**Fig. 2.** IRF4 expression of Tet2/3 dKO B cells after antigenic stimulation *in vivo*. Expression of CD138 and intracellular IRF4 in control or Tet2/3 dKO B cells before (d0) or d3–d7 after immunization with NP-CGG. The frequency of IRF4$^{lo}$CD138$^{-}$, IRF4$^{int}$CD138$^{-}$ or IRF4$^{hi}$CD138$^{+}$ in CD45.1$^{+}$NP$^{+}$ control cells (open circles) or Tet2/3 dKO cells (closed circles) was indicated in FACS dot plots (left panel). The frequency of IRF4$^{int}$ or IRF4$^{hi}$ cells in CD45.1$^{+}$NP$^{+}$ cells at each time point was summarized (right panel, $n$ = 3–7 at each time point). *****$P$ < 0.0001, N.S., non-significant, two-way ANOVA with Sidak's multiple comparisons test. Bars indicate mean ± SD. All data are from two independent experiments.

CD138$^{+}$ control cells (Fig. 3D). These results suggest that IRF4-RV-induced CD138$^{+}$ Tet dKO cells established plasma cell identity partially.

Of note, our results indicate that expression of Blimp-1 and XBP-1 is differentially regulated during plasma cell differentiation. XBP-1 expression is dependent on Tet2/3, but not IRF4, whereas Blimp-1 expression requires IRF4 and can be induced in the absence of Tet2/3. It has previously been demonstrated that Xbp-1 is required for maximal antibody production, rather than plasma cell differentiation itself (28). This is consistent with our observation that IRF4-RV$^{+}$ Tet dKO CD138$^{+}$ cells gave rise to comparable number of ASC spots, but the size of the spots, which is correlated with amounts of antibody produced, was smaller in ELISPOT assay (Fig. 3C).

### *Tet2/3 are required for DNA demethylation in the Irf4 locus*

Given that Tet2/3-dependent high IRF4 expression is essential for plasma cell differentiation, we wished to understand how Tet2/3 control IRF4 expression. It has been demonstrated that CpG sites around *Irf4* are demethylated in CD138$^{+}$ plasma cells (10). However, it was unclear how the DNA methylation status at the *Irf4* locus is changed during plasma cell differentiation and how Tet2/3 regulate it. Hence, we extracted DNA from control or Tet dKO naive B cells (IRF4$^{lo}$ cells), activated CD138$^{-}$ B cells (IRF4$^{int}$ cells) or CD138$^{+}$plasma cells (IRF4$^{hi}$ cells) and examined the DNA methylation status of CpG sites at putative regulatory regions of the *Irf4* gene by bisulfite sequencing. The CpG sites at proximal promoter regions (position −191 to −57 relative to TSS) were completely demethylated both in control and Tet dKO naive B cells (Fig. 4A), suggesting that demethylation of these regions is presumably generated during early B-cell development and maintained until the stage of peripheral naive B cells.

On the other hand, CpG sites at distal, up-stream regions were demethylated in a Tet2/3-dependent manner. CpG sites at position −788 to −721 were demethylated both in activated B cells and plasma cells derived from control B cells, while those at position −1213 to −1020, −1468 to −1367 or −16458 to −16433 were demethylated only in plasma cells (Fig. 4A). Since IRF4 is expressed at comparable levels in control or Tet dKO activated B cells (Fig. 2), demethylation of CpGs at position −788 to −721 would not be required for initial induction of IRF4 expression. In contrast, demethylation of CpGs at up-stream regions (position −16458 to −1020) is likely to be critical for high IRF4 expression and thus plasma cell generation. DNA demethylation of these up-stream CpGs was highly dependent on Tet2/3, since IRF4-RV$^{+}$ CD138$^{+}$ Tet dKO plasma cells maintained a fully methylated status at these CpGs (Fig. 4B). Finally, we asked whether Tet enzymes bind to the *Irf4* locus. ChIP assay showed that Tet2 significantly bound to the regions including position −16470 to −16301 and −775 to −646 in the *Irf4* locus (Fig. 4C). We also observed weak binding of Tet2 to the other regions, including position −1442 to −1293 (data not shown). These results suggest that Tet enzymes bind to up-stream regions of the *Irf4* locus and promote DNA demethylation of CpGs in these regions, which facilitates high IRF4 expression and plasma cell differentiation. It is unclear whether demethylation of all of CpGs tested here is required for high IRF4 expression. Site-specific modulation of the DNA methylation status by CRISPR-dCas9-based epigenome editing (29, 30) will clarify the contribution of each CpG for IRF4 expression and plasma cell differentiation.

Our study demonstrated that Tet enzymes are required for up-regulation of IRF4, but not its induction, during B-cell activation. How does Tet-mediated DNA demethylation of the *Irf4* locus facilitate high expression of IRF4? Lio *et al.* have recently demonstrated that Tet enzymes augment AID expression in activated B cells (22). Mechanistically, Tet enzymes bind to distal enhancer elements in the *Aicda* locus, which then catalyze DNA demethylation and maintain chromatin accessibility at such regulatory elements, resulting in augmented *Aicda* expression. Thus, it is reasonable to speculate that Tet-binding regions in the *Irf4* locus, including −16 kb

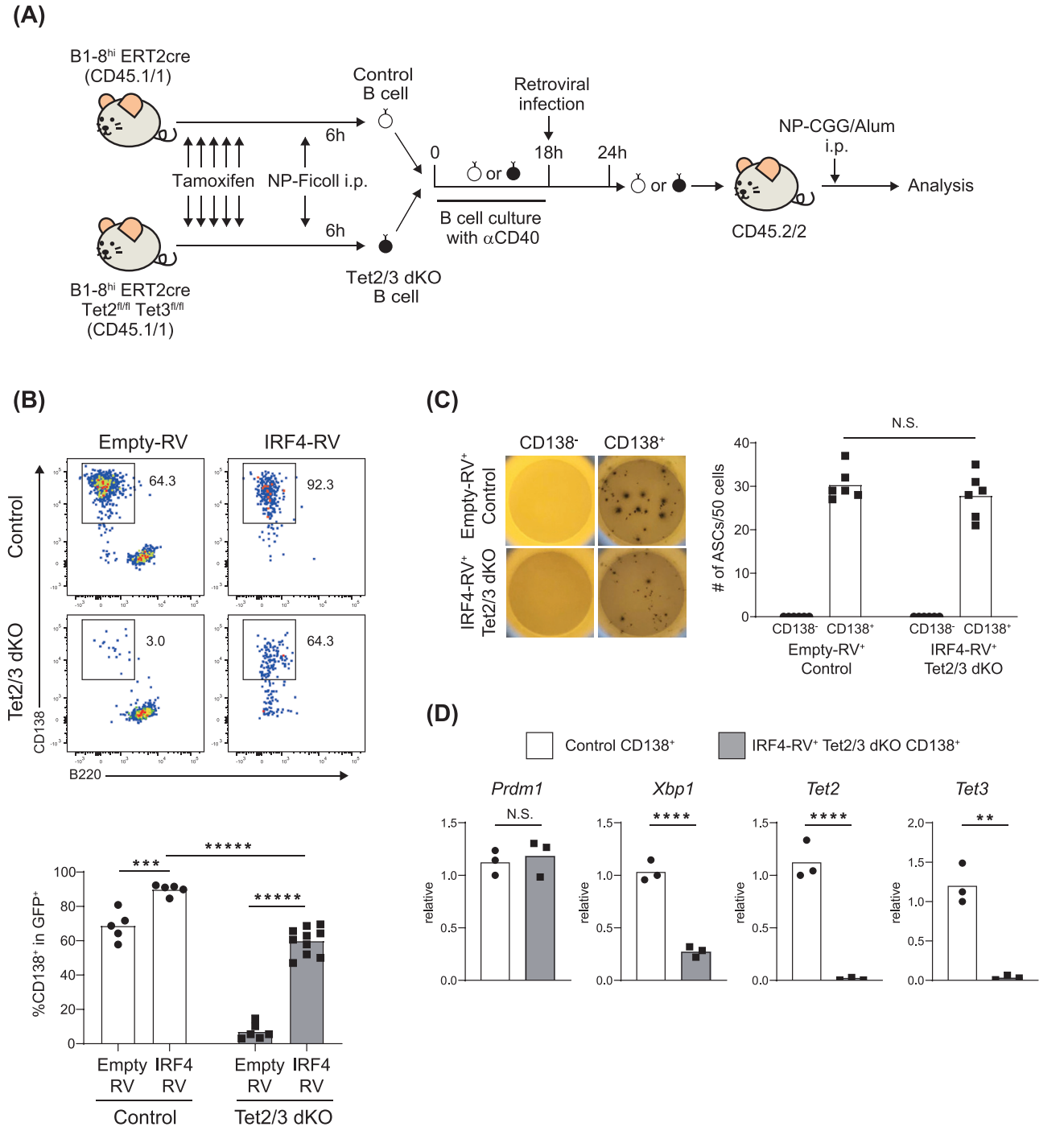


**Fig. 3.** The effect of IRF4-RV on plasma cell generation by Tet dKO B cells. (A) Schematic illustration of the experimental protocol for assessment of the effect of IRF4-RV expression in Tet2/3 dKO B cells on plasma cell generation. (B) CD138 expression in Empty-RV$^+$ (GFP$^+$) or IRF4-RV$^+$ (GFP$^+$) NP$^+$CD45.1$^+$ control or Tet2/3 dKO cells 5 days after immunization. FACS dot plots (upper panel) or bar graphs (lower panel, $n$ = 5–10) are from two independent experiments. ***$P < 0.001$, ****$P < 0.0005$, *****$P < 0.0001$, unpaired Student $t$-test. (C) ELISPOT for the presence of NP-specific ASCs in 50 sorted CD138$^-$ or CD138$^+$ control or Tet2/3 dKO cells infected with empty-RV or IRF4-RV. ASC spots (left panel) or bar graphs showing number of ASCs in sorted cells (right graphs, $n$ = 6) are from two independent experiments. N.S., non-significant, unpaired Student $t$-test. (D) Expression of *Prdm1*, *Xbp1*, *Tet2* or *Tet3* mRNA in sorted control CD138$^+$ cells or IRF4-RV$^+$Tet2/3 dKO CD138$^+$ cells. **$P < 0.005$, ****$P < 0.0005$, N.S., non-significant, unpaired Student $t$-test. All data are from two independent experiments ($n$ = 3).

relative to TSS, function as enhancer elements. Tet-mediated DNA demethylation of these regions might sustain chromatin accessibility and allow the recruitment of transcription factors or chromatin modifiers, which results in up-regulation of IRF4 expression. Future study will examine the enhancer activity of Tet-binding regions in the *Irf4* locus, and clarify more detailed mechanisms by which Tet-mediated epigenetic modification controls plasma cell differentiation.

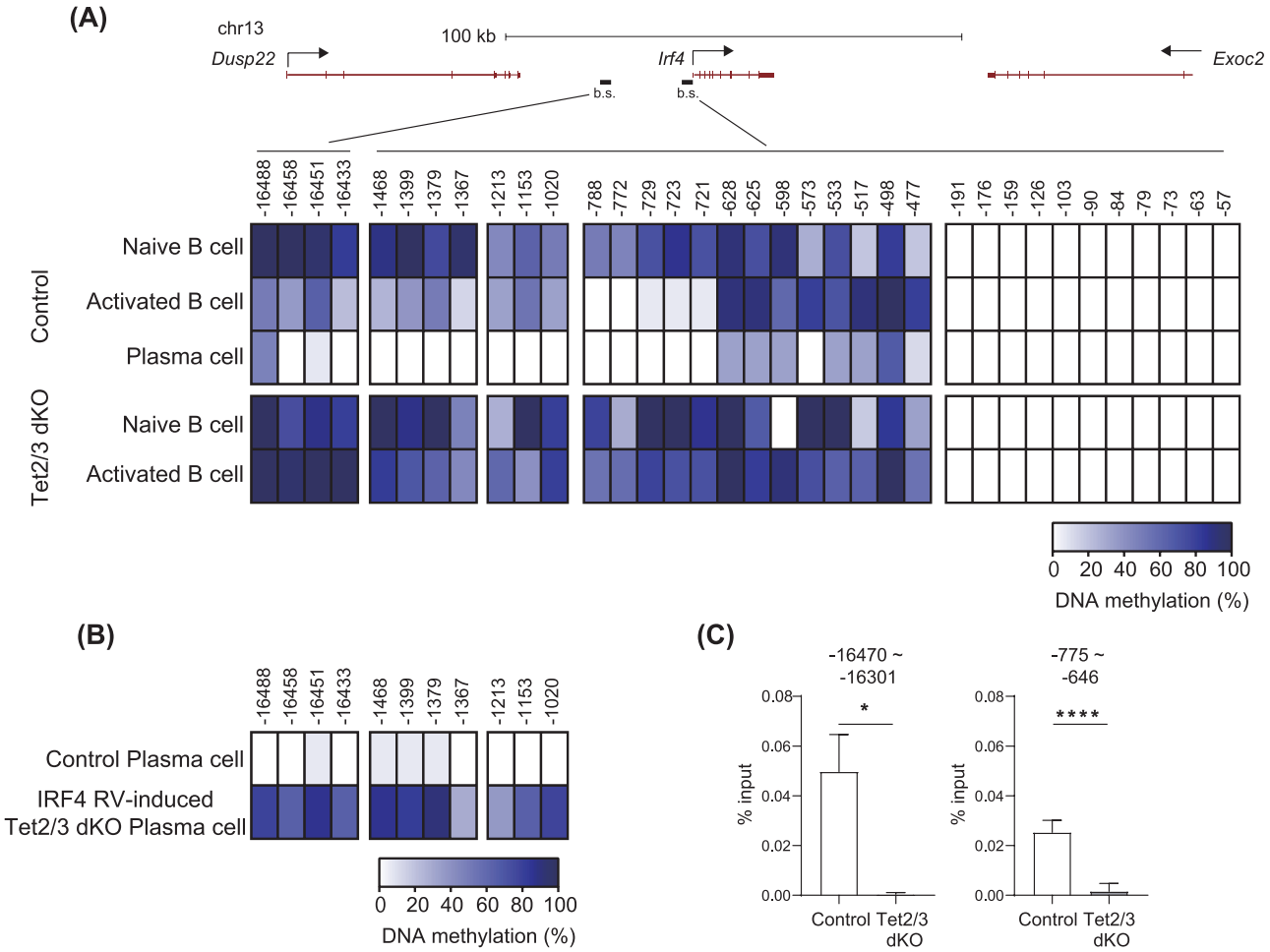


**Fig. 4.** DNA methylation status at the *Irf4* locus in Tet dKO cells. (A) CpG DNA methylation status at the *Irf4* locus in naive B cells, CD138⁻ activated B cells or CD138⁺ plasma cells derived from control or Tet2/3 dKO cells. The levels of DNA methylation at CpG sites within the regions indicated as horizontal bars below schematic diagram of the *Irf4* locus were examined by bisulfite sequencing (b.s.) and are shown as heat maps. The numbers indicated above the heat maps are the position of CpG relative to TSS of the *Irf4* gene; 16–32 clones for each CpG site were analyzed. The data are from two independent experiments. (B) DNA methylation status of CpG sites in up-stream regions of the *Irf4* locus in plasma cells derived from control B cells or IRF4-RV⁺ Tet2/3 dKO B cells. The data are from two independent experiments. (C) Tet2 binding to the *Irf4* locus. Genomic DNA was isolated from LPS-stimulated control or Tet2/3 dKO B cells and ChIP was performed with anti-Tet2 antibody. Binding of Tet2 to the indicated regions of the *Irf4* locus was examined by qPCR. The data are from two independent experiments.


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## References


1 Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M. and Corcoran, L. M. 2015. The generation of antibody-secreting plasma cells. *Nat. Rev. Immunol.* 15:160.

2 Shi, W., Liao, Y., Willis, S. N. *et al.* 2015. Transcriptional profiling of mouse B cell terminal differentiation defines a signature for antibody-secreting plasma cells. *Nat. Immunol.* 16:663.

3 Nutt, S. L., Taubenheim, N., Hasbold, J., Corcoran, L. M. and Hodgkin, P. D. 2011. The genetic network controlling plasma cell differentiation. *Semin. Immunol.* 23:341.

4 Lin, K. I., Angelin-Duclos, C., Kuo, T. C. and Calame, K. 2002. Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol. Cell. Biol.* 22:4771.

5 Shaffer, A. L., Lin, K. I., Kuo, T. C. *et al.* 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 17:51.

6 Shaffer, A. L., Shapiro-Shelef, M., Iwakoshi, N. N. *et al.* 2004. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* 21:81.

7 Sciammas, R., Shaffer, A. L., Schatz, J. H., Zhao, H., Staudt, L. M. and Singh, H. 2006. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* 25:225.

8 Ochiai, K., Maienschein-Cline, M., Simonetti, G. *et al.* 2013. Transcriptional regulation of germinal center B and plasma cell fates by dynamical control of IRF4. *Immunity* 38:918.

9 Tellier, J., Shi, W., Minnich, M. *et al.* 2016. Blimp-1 controls plasma cell function through the regulation of immunoglobulin secretion and the unfolded protein response. *Nat. Immunol.* 17:323.
10 Barwick, B. G., Scharer, C. D., Bally, A. P. R. and Boss, J. M. 2016. Plasma cell differentiation is coupled to division-dependent DNA hypomethylation and gene regulation. *Nat. Immunol.* 17:1216.
11 Scharer, C. D., Barwick, B. G., Guo, M., Bally, A. P. R. and Boss, J. M. 2018. Plasma cell differentiation is controlled by multiple cell division-coupled epigenetic programs. *Nat. Commun.* 9:1698.
12 Barwick, B. G., Scharer, C. D., Martinez, R. J. *et al.* 2018. B cell activation and plasma cell differentiation are inhibited by *de novo* DNA methylation. *Nat. Commun.* 9:1900.
13 Pastor, W. A., Aravind, L. and Rao, A. 2013. TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. *Nat. Rev. Mol. Cell Biol.* 14:341.
14 Dawlaty, M. M., Breiling, A., Le, T. *et al.* 2014. Loss of Tet enzymes compromises proper differentiation of embryonic stem cells. *Dev. Cell* 29:102.
15 Lio, C. W., Zhang, J., Gonzalez-Avalos, E., Hogan, P. G., Chang, X. and Rao, A. 2016. Tet2 and Tet3 cooperate with B-lineage transcription factors to regulate DNA modification and chromatin accessibility. *Elife* 5:e18290.
16 Jiao, J., Jin, Y., Zheng, M. *et al.* 2019. AID and TET2 co-operation modulates FANCA expression by active demethylation in diffuse large B cell lymphoma. *Clin. Exp. Immunol.* 195:190.
17 Lio, C. J. and Rao, A. 2019. TET enzymes and 5hmC in adaptive and innate immune systems. *Front. Immunol.* 10:210.
18 Mouly, E., Ghamlouch, H., Della-Valle, V. *et al.* 2018. B-cell tumor development in Tet2-deficient mice. *Blood Adv.* 2:703.
19 Wille, C. K., Li, Y., Rui, L., Johannsen, E. C., and Kenney, S. C. 2017. Restricted TET2 expression in germinal center type B cells promotes stringent Epstein-Barr virus latency. *J. Virol.* 91:e01987.
20 Tsagaratou, A., Lio, C. J., Yue, X. and Rao, A. 2017. TET methylcytosine oxidases in T cell and B cell development and function. *Front. Immunol.* 8:220.
21 Orlanski, S., Labi, V., Reizel, Y. *et al.* 2016. Tissue-specific DNA demethylation is required for proper B-cell differentiation and function. *Proc. Natl Acad. Sci. USA* 113:5018.
22 Lio, C. J., Shukla, V., Samaniego-Castruita, D. *et al.* 2019. TET enzymes augment activation-induced deaminase (AID) expression via 5-hydroxymethylcytosine modifications at the *Aicda* superenhancer. *Sci Immunol* 4:eaau7523.
23 Schoeler, K., Aufschnaiter, A., Messner, S. *et al.* 2019. TET enzymes control antibody production and shape the mutational landscape in germinal centre B cells. *FEBS J.* 286:3566.
24 Tanaka, S., Ise, W., Inoue, T. *et al.* Tet2 and Tet3 in B cells are required to repress CD86 and prevent autoimmunity. *Nat. Immunol.,* in press. doi:10.1038/s41590-020-0700-y
25 Shih, T. A., Roederer, M. and Nussenzweig, M. C. 2002. Role of antigen receptor affinity in T cell-independent antibody responses *in vivo*. *Nat. Immunol.* 3:399.
26 Shinnakasu, R., Inoue, T., Kometani, K. *et al.* 2016. Regulated selection of germinal-center cells into the memory B cell compartment. *Nat. Immunol.* 17:861.
27 Ise, W., Inoue, T., McLachlan, J. B. *et al.* 2014. Memory B cells contribute to rapid Bcl6 expression by memory follicular helper T cells. *Proc. Natl Acad. Sci. USA* 111:11792.
28 Taubenheim, N., Tarlinton, D. M., Crawford, S., Corcoran, L. M., Hodgkin, P. D. and Nutt, S. L. 2012. High rate of antibody secretion is not integral to plasma cell differentiation as revealed by XBP-1 deficiency. *J. Immunol.* 189:3328.
29 Choudhury, S. R., Cui, Y., Lubecka, K., Stefanska, B. and Irudayaraj, J. 2016. CRISPR-dCas9 mediated TET1 targeting for selective DNA demethylation at BRCA1 promoter. *Oncotarget* 7:46545.
30 Vojta, A., Dobrinić, P., Tadić, V. *et al.* 2016. Repurposing the CRISPR-Cas9 system for targeted DNA methylation. *Nucleic Acids Res.* 44:5615.