A non-canonical function of Ezh2 preserves immune homeostasis

Ajithkumar Vasanthakumar^{1,2,†}, Dakang Xu^{3,4,5,†}, Aaron TL Lun^{1,2}, Andrew J Kueh^{1,2}, Klaas PJM van Gisbergen^{1,2}, Nadia Iannarella¹, Xiaofang Li^{3,4,5}, Liang Yu^{4,5}, Die Wang^{4,5}, Bryan RG Williams^{4,5} , Stanley CW Lee^{1,2}, Ian J Majewski^{1,2}, Dale I Godfrey^{6,7}, Gordon K Smyth^{1,8}, Warren S Alexander^{1,2}, Marco J Herold^{1,2}, Axel Kallies^{1,2}, Stephen L Nutt^{1,2,‡,*} & Rhys S Allan^{1,2,‡,**}

Abstract

Enhancer of zeste 2 (Ezh2) mainly methylates lysine 27 of histone-H3 (H3K27me3) as part of the polycomb repressive complex 2 (PRC2) together with Suz12 and Eed. However, Ezh2 can also modify non-histone substrates, although it is unclear whether this mechanism has a role during development. Here, we present evidence for a chromatin-independent role of Ezh2 during T-cell development and immune homeostasis. T-cell-specific depletion of Ezh2 induces a pronounced expansion of natural killer T (NKT) cells, although Ezh2-deficient T cells maintain normal levels of H3K27me3. In contrast, removal of Suz12 or Eed destabilizes canonical PRC2 function and ablates NKT cell development completely. We further show that Ezh2 directly methylates the NKT cell lineage defining transcription factor PLZF, leading to its ubiquitination and subsequent degradation. Sustained PLZF expression in Ezh2-deficient mice is associated with the expansion of a subset of NKT cells that cause immune perturbation. Taken together, we have identified a chromatin-independent function of Ezh2 that impacts on the development of the immune system.

Keywords chromatin; Ezh2; lymphocyte; methylation; polycomb

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Immunology; Post-translational Modifications, Proteolysis & Proteomics

DOI 10.15252/embr.201643237 | Received 18 August 2016 | Revised 18 January 2017 | Accepted 22 January 2017 | Published online 21 February 2017

EMBO Reports (2017) 18: 619–631

Introduction

Post-translational modifications to chromatin in the form of histone marks are known to be important for gene regulation. However, the enzymes that are responsible for laying down these marks can also modify non-histone proteins [1,2], making it important to understand whether such alternative mechanisms play a role in the regulation of cell fate. One of the key epigenetic silencing pathways involves the polycomb repressor complex 2 (PRC2) which consists of non-redundant components *Suppressor of zeste 12 homolog* (Suz12) and *Embryonic ectoderm development* (Eed) and the histone methyltransferase *Enhancer of zeste homolog 2* (Ezh2), which is responsible for the tri-methylation of lysine 27 of histone-H3 (H3K27me3) [3]. Recently, it has been found that a homolog of Ezh2, Ezh1 can also impart H3K27me3 and compensate for the loss of Ezh2 in some circumstances [4–6].

Ezh2 has also been shown to methylate non-histone proteins such as transcription factors resulting in outcomes such as functional repression [7] and degradation [8]. Ezh2 methylation of Vav1 or Talin has a role in actin polymerization [9] and cell migration [10]; however, the extent to which these events contribute to the differentiation of immune cells is unknown.

Here, we examined whether there was a role for chromatin-independent functions of Ezh2 in T-cell development. The process of T-cell development occurs in the thymus where hematopoietic progenitors (known as thymocytes) develop into mature T-cell lineages. Deletion of Ezh2 at this early point arrests T-cell development [9,11]. The major T-cell populations that arise are defined by the expression of either CD4 or CD8 molecules (known as co-receptors). These cells possess an $\alpha\beta$ T-cell

1 The Walter and Eliza Hall Institute of Medical Research, Parkville, Vic., Australia

2 Department of Medical Biology, The University of Melbourne, Parkville, Vic., Australia

3 Institute of Ageing Research, Hangzhou Normal University School of Medicine, Hangzhou, China

4 Hudson Institute of Medical Research, Monash University, Clayton, Vic., Australia

5 Department of Molecular and Translational Science, Monash University, Clayton, Vic., Australia

6 Department of Microbiology and Immunology, The University of Melbourne, Parkville, Vic., Australia

7 ARC Centre of Excellence for Advanced Molecular Imaging, The University of Melbourne, Parkville, Vic., Australia

8 Department of Mathematics and Statistics, The University of Melbourne, Parkville, Vic., Australia

*Corresponding author. Tel: +61 3 9345 2483; Fax: +61 3 9347 0852; E-mail: nutt@wehi.edu.au

**Corresponding author. Tel: +61 3 9345 2322; Fax: +61 3 9347 0852; E-mail: rallan@wehi.edu.au

†These authors contributed equally to this work as first authors

‡These authors contributed equally to this work as senior authors

receptor (TCR) that recognizes peptides bound to class I or class II major histocompatibility complex (MHC) molecules, respectively. The commitment to the CD4 or CD8 T-cell lineage occurs quite late in thymic development. Initially, precursors committed to the $\alpha\beta$ lineage, which have rearranged genes encoding their TCR chains (TCR β and TCR α), transition through a stage known as double-positive (DP) where they express both the CD4 and CD8 co-receptors. They then undergo a selection phase where only cells with appropriate avidity for self-ligands survive and differentiate into mature T cells. It is at this stage that the choice of the CD4 or CD8 lineage is made. There are also “non-conventional” T cells that develop in the thymus such as $\gamma\delta$ T cells and natural killer T (NKT) cells that are very important for innate responses. NKT cells develop during the DP stage and are a heterogeneous population of CD1d-restricted innate-like T cells that recognize glycolipid antigens [12,13]. Due to their potency in producing a range of different cytokines, NKT cell numbers must be kept in check as their aberrant expansion results in the activation of the adaptive immune system [14,15]. Thus, NKT cells have been implicated in a number of autoimmune diseases, such as asthma [16] and inflammatory bowel disease [17], as well as being targets for cancer immunotherapy [18]. NKT cells have their own distinct transcriptional profile [19] that depends on the master transcription factor promyelocytic leukemia zinc finger (PLZF, encoded by *Zbtb16*) [20,21].

Here, we identify a chromatin-independent role for Ezh2 in regulating T-cell development and immune homeostasis. We observed Ezh2-deficiency results in the expansion of NKT cells that maintain normal levels of H3K27me3. In contrast, removal of the PRC2-core components Suz12 and Eed led to H3K27me3 depletion and a dramatic loss of NKT cells. We identified a specific lysine residue of PLZF (K430) that is a target of Ezh2-induced methylation leading to the ubiquitination and degradation of the protein. Overall, we have demonstrated that Ezh2 can act in a chromatin-independent manner to control NKT cell development and preserve immune homeostasis potentially by fine-tuning levels of PLZF, via methylation-dependent ubiquitinylation.

Results

A chromatin-independent role for Ezh2 in the development of NKT but not conventional $\alpha\beta$ T cells

To study the contribution of the PRC2 to T-cell development, we bred mice in which the exons of *Ezh2* [22], *Suz12* [23], or *Eed*

[6] are flanked by *loxP* sequences to transgenic mice expressing Cre recombinase under the control of the *Cd4* promoter [24], hereby referred to as conditional knockout (*CKO*) mice. We first confirmed deletion of the floxed alleles by PCR (Fig EV1A–C). We then examined the frequency of conventional $\alpha\beta$ CD4⁺ and CD8⁺ T cells (Fig 1A) and $\gamma\delta$ T cells (Fig 1B) in the thymus and spleen (not shown) of adult mice from all strains and found that they were similar to the wild-type counterparts suggesting that removal of these components at the DP stage did not affect the subsequent development of these populations. However, using CD1d tetramers loaded with PBS57, a derivative of the glycolipid antigen α -galactosylceramide (denoted NKT-tetramer), we observed a large population of TCR β ⁺ NKT cells in the thymus and spleen of the *Ezh2*^{CKO} mice (Figs 1C and EV2A and data not shown). This was in contrast to what was observed in the *Suz12*^{CKO} and *Eed*^{CKO} mice, which had a substantial loss of thymic NKT cells (Figs 1C and EV2A). Thus, we have revealed an unexpected difference in NKT cell development between mice deficient in Ezh2 vs. those deficient in the non-redundant components Suz12 and Eed.

Ezh2 is a histone methyltransferase that, when in complex with its PRC2 cofactors Suz12 and Eed, imparts the H3K27me3 mark that is associated with gene silencing [25]. Surprisingly, we did not observe a reduction in H3K27me3 levels in *Ezh2*^{CKO} NKT cells when compared to wild-type NKT cells by both flow cytometry and Western blot (Fig 1D and E). The maintenance of H3K27me3 was most likely due to compensation by the closely related Ezh1, as has been shown in some other circumstances [4–6,26]. Consistent with this notion, we found that Ezh1 was expressed in wild-type NKT cells and that its expression increased in the *Ezh2*^{CKO} cells (Fig 1D). This indicated that the canonical PRC2 function of laying down H3K27me3 occurred relatively normally and suggested that Ezh2 may control NKT cell development in a manner that is independent of its role as a chromatin-modifying enzyme. In support of this hypothesis, the removal of Suz12 and Eed which is known to compromise the ability of both Ezh2 and Ezh1 to methylate H3K27 [5,6] and lead to a loss of NKT cells resulted in a strong reduction in the levels of H3K27me3 (Fig 1F). Further evidence of PRC2 disruption comes from the substantially reduced Ezh2 levels in both the *Suz12* and *Eed*^{CKO} thymocytes (Fig EV2B), whereas Suz12 levels were unaltered in the *Ezh2*^{CKO} (Fig EV2C). In combination, these data indicate that the complete loss of the PRC2 function impairs NKT cell development, while the expansion of NKT cells in the absence of Ezh2 derives from the loss of a chromatin-independent function of this factor during NKT cell development.

Figure 1. Contrasting outcomes on NKT cell development upon deletion of individual PRC2 components.

- A–C Flow cytometric analysis of 6-week-old wild-type (WT) and *Ezh2*^{CKO}, *Suz12*^{CKO}, or *Eed*^{CKO} thymii showing proportion of (A) TCR β ⁺CD4 and CD8-expressing T cells, (B) TCR β ⁺TCR $\gamma\delta$ ⁺ T cells, or (C) TCR β ⁺PBS-57⁺ NKT cells. Numbers are the mean percentage in the indicated gate. Data are representative of two independent experiments.
- D Histogram overlay shows flow cytometric analysis of H3K27me3 (left panel) and Ezh1 (right panel) levels in wild type (WT) and indicated genotypes in thymic NKT cells. Gray shaded histogram represents isotype control.
- E Lysates of TCR β ⁺PBS-57⁺ NKT cells derived from WT or from five individual *Ezh2*^{CKO} mice were immunoblotted with antibodies specific for H3K27me3 or total histone-H3 as a loading control.
- F Histogram overlay shows flow cytometric analysis of H3K27me3 (left panel) and Ezh1 (right panel) levels in wild-type (WT) and indicated genotypes in thymic NKT cells. Gray shaded histogram represents isotype control.

Data information: Data in (A–F) are representative of at least two independent experiments.

Figure 1.

Ezh2 deficiency leads to the expansion of PLZF^{high} NKT cells

NKT cells arise from DP thymocytes and are thought to progress in a linear fashion from a CD24⁺CD44⁺NK1.1⁺ population (known as stage 0) to stage 1 (CD24⁺CD44⁺NK1.1⁺), stage 2 (CD24⁺CD44⁺NK1.1⁺), and stage 3 (CD24⁺CD44⁺NK1.1⁺) [13]. Recently, an alternative model has been proposed, in which some stage 2 and 3 NKT cells diverge from a common stage 0 or 1 progenitor to take on different transcriptional and functional properties [14,27]. This has led to the demarcation of some cells in stages 1 and 2 as “NKT2” as these cells have more T helper 2 like properties (such as high IL-4 production) and express high levels of the transcription factor PLZF and stage 3 as PLZF^{low}Tbet^{high} NKT1 for their functional similarity to T helper 1 cells [14,27]. To understand how Ezh2 regulates NKT cell development, we sought to determine which stage of differentiation is affected by the loss of Ezh2. Fractionation of the wild-type and Ezh2-deficient thymi showed a large proportion of the NKT cells from the *Ezh2*^{CKO} accumulated at stage 2 (Fig 2A) and expressed high levels of PLZF (Fig 2B) and IL-4 (Fig EV3A). Moreover, NKT cells from the *Ezh2*^{CKO} expressed low levels of Tbet and RORγt (Fig EV3B) and thus could be considered akin to NKT2 cells.

To investigate the transcriptional changes that occurred in NKT cells lacking Ezh2, we employed RNAseq. We isolated stage 1, 2, and 3 NKT cell populations from wild-type and *Ezh2*^{CKO} thymocytes (sort profile shown in Fig EV3C). A comparison of wild-type stage 1 vs. stage 3 NKT cells yielded over 1,500 differentially expressed genes (Figs 2C and EV3D, and Dataset EV1) illustrating the dramatic transcriptional changes that parallel PLZF downregulation during NKT cell maturation. Surprisingly, we found only 297 upregulated and 62 downregulated genes in *Ezh2*^{CKO} stage 2 NKT cells in comparison with their wild-type counterparts (Figs 2C and EV3D) and even fewer changes between the genotypes at stages 1 and 3 (Fig EV3E and Dataset EV2). The relatively minor changes in gene upregulation in the absence of Ezh2 correlated with the unaffected levels of H3K27me3 (Fig 1D and E). Interestingly, we found that a number of genes that were downregulated in stage 2 *Ezh2*^{CKO} NKT cells were Th1-type genes including *Tbx21* (encoding Tbet), *Il18rap*, and *Il2rb* (Fig 2C), genes which are highly expressed in the stage 3 population indicating that the loss of Ezh2 results in a block of cells at stage 2 or a skewing toward the NKT2 subset. Overall, this suggests that the loss of Ezh2 results in accumulation of stage 2/NKT2 type cells and that development of these cells appears to be independent of conventional pathway of Ezh2-PRC2 gene silencing.

PLZF is a target for Ezh2 methylation and degradation

Recently, it has been shown that Ezh2 can methylate transcription factors in a process that could alter their function or lead to their degradation [7,8,28]. As PLZF (encoded by the *Zbtb16* gene) is a master regulator of NKT cell development [20,21] and was highly expressed in the expanded population of *Ezh2*^{CKO} NKT cells (Fig 2B), we investigated the possibility that it was the direct target of Ezh2. We confirmed previous reports that PLZF protein levels are dynamically regulated throughout normal NKT cell development, being high at stage 1 and reduced thereafter (Fig 3A) [20,29]. Strikingly, the down modulation of PLZF protein concentration during

progression from stage 1 to 2 did not correlate with *Zbtb16* transcript abundance, which remained high at stage 2 (Fig 3B), suggesting that post-translational modifications regulate PLZF protein stability. In *Ezh2*^{CKO} NKT cells, high PLZF amounts were sustained in stage 2 (Fig 3C), although the transcript levels were equivalent in wild-type and *Ezh2*^{CKO} NKT cells (Fig 3D and E). We also found that the amount of H3K27me3 was very low at the *Zbtb16* promoter of both the WT and *Ezh2*^{CKO} stage 2 NKT cells in comparison with that of *Hoxa11*, a gene known to be silenced in hematopoietic cells [30], providing more evidence for a chromatin-independent effect by Ezh2 on PLZF protein (Fig 3F).

PLZF stability is thought to be controlled by ubiquitin-mediated degradation [31], and recently, PLZF has been shown to be associated with the E3 ligase cullin 3 in NKT cells [32], raising the possibility that Ezh2 methylase activity may directly control PLZF ubiquitination and degradation. Such a mechanism is not without precedence as Ezh2 establishes a “methyl-degron” on the transcription factor RORα [8] (which was not differentially expressed in *Ezh2*^{CKO} NKT cells; Fig EV4A). We first confirmed that PLZF was indeed methylated in NKT cells and that this methylation was reduced in the absence of Ezh2 (Fig EV4B). As NKT cells are a very rare population, we characterized this mechanism further using the 293T cell line. We found that indeed Ezh2 and PLZF could interact (Fig 4A and B). In addition, the presence of Ezh2 led to increased PLZF methylation (Fig 4C). Moreover, inhibition of Ezh2 methyltransferase activity using the small molecule inhibitor GSK126 [33] reduced PLZF methylation suggesting that the enzymatic activity of Ezh2 is critical in this process (Fig EV4C). Interestingly, the presence of Suz12 was not required for this process as the knockdown of Suz12 expression (Fig EV4D) did not affect the levels of PLZF methylation in this system (Fig EV4E).

We then used a lysine methylation predicting tool [34] which highlighted K430 as a likely site for PLZF methylation (bioinfo.ncu.edu.cn/inquiries_PMeS.aspx). Interestingly, this site is highly conserved across species (Fig EV4F). Substitution of K430 to arginine (R) resulted in almost complete loss of Ezh2-induced PLZF methylation (Fig 4C). When Ezh2 was introduced into cells that co-expressed PLZF and ubiquitin-HA, we observed strong ubiquitinylation of PLZF, which was lost in the K430R mutant (Fig 4D). This correlated with increased stability of K430R PLZF after blocking protein synthesis with cyclohexamide (Fig 4E). Moreover, knockdown of endogenous Ezh2 with siRNA (Fig EV4G) resulted in increased stability of WT PLZF (Fig 4F), which did not alter *Zbtb16* transcription (Fig EV4H). In combination, these data demonstrate that PLZF is a novel target of the Ezh2-methyl-degron.

We next examined the effect of mutating of the Ezh2 methylation site in PLZF on primary T cells. To this end, we produced retroviruses encoding WT PLZF or K430R PLZF and observed a statistically significant increase in the levels of PLZF in primary CD4⁺ T cells carrying the K430R mutant (Fig EV5A). To determine whether NKT cell differentiation was altered, we transduced fetal liver stem cells with these vectors with the objective to reconstitute the immune system of lethally irradiated recipient mice. Unfortunately, we found that while we could obtain GFP⁺ T cells infected with the empty vector, we could not recover T cells that overexpressed either WT PLZF or PLZF K430R (data not shown). This suggests that PLZF introduction in stem cells restricts their ability to develop into T cells. Alternatively, we introduced these vectors into thymic

Figure 2. Ezh2 restricts the development of stage 2 NKT cells.

A Flow cytometric analysis showing expansion of stage 2 NKT cells in *Ezh2^{cKO}* mice. Left panel shows expression of NK1.1 and CD44 in WT (left) and *Ezh2^{cKO}* (right) NKT cells from the thymus. Numbers are the percentage of cells in the indicated quadrant. NKT cells corresponding to stages (S) 1, 2, and 3 are indicated on the plots. Bar graph represents the mean percentage of NKT cells in each stage $\pm$ SEM.

B Contour plots show the expression of NK1.1 and PLZF. Graph shows the percentage of PLZF⁺ NKT cells in both genotypes. Each dot represents an individual mouse; horizontal lines show the mean $\pm$ SEM. Student's *t*-test was performed to test for statistical significance.

C RNAseq analysis of differential gene expression in wild-type (WT) and *Ezh2^{cKO}* NKT cells (False discovery rate ≤ 0.1 relative to 1.2-fold threshold). Left panel shows comparison of gene expression profiles from WT and *Ezh2^{cKO}* stage 2 NKT cells. Right panel shows comparison of gene expression profiles from WT stage 1 and stage 3 NKT cells. In the left panel, the blue and red dots represent genes that were significantly up- or downregulated, respectively, in the *Ezh2^{cKO}* compared to WT with specific examples highlighted. In the right panel, blue and red dots represent genes significantly up- or downregulated in stage 3 compared to stage 1 WT NKT cells, respectively. In both graphs, the mRNA corresponding to the NKT cell-specific transcription factor PLZF (*Zbtb16*) is marked to illustrate its stage-specific expression pattern.

Data information: NKT cells in the plots in (A, B) were identified by binding to TCR β and the NKT-tetramer and are representative of at least two independent experiments.

Figure 3. Sustained expression of PLZF in *Ezh2*-deficient NKT cells is not associated with gene de-repression.

A WT thymic NKT cells were analyzed for stage-specific expression of PLZF protein by flow cytometry.
B Quantitative real-time PCR analysis of *Zbtb16* mRNA (encoding PLZF) in different stages of WT thymic NKT cells. Data are the mean $\pm$ SEM from two experiments.
C Flow cytometric analysis of PLZF expression in WT and *Ezh2^{cKO}* stage 1 (left), stage 2 (middle), and stage 3 (right) NKT cells.
D Bar graph shows expression of *Zbtb16* in WT and *Ezh2^{cKO}* stage 2 NKT cells. Error bars represent $\pm$ SEM from three independent experiments. Student's *t*-test was performed to test for statistical significance.
E RNAseq track showing reads mapping to the *Zbtb16* gene in WT and *Ezh2^{cKO}* from stage 1–3 NKT cells.
F ChIP enrichment of H3K27me3 at the transcriptional start site of *Zbtb16* and *HoxA11* from stage 2 NKT cells of the indicated genotypes. NKT cells stages were defined as shown in Fig EV3C. Data are the mean $\pm$ SEM from two experiments. NS, not significant ($P > 0.05$; Student's *t*-test).

Figure 4. An Ezh2 methyl-degron regulates PLZF stability.

- A Interaction between Ezh2 and PLZF. 293T cells were co-transfected with Ezh2-mCherry and PLZF-Flag-expressing plasmids. Whole-cell lysates were immunoprecipitated (IP) with anti-Flag or control IgG antibodies and then immunoblotted (IB) with Ezh2 and PLZF-specific antibodies. In total, 10% of total lysate was loaded as input.
- B 293T cells were co-transfected with Ezh2-Flag and PLZF-CFP-expressing plasmids. Whole-cell lysates were immunoprecipitated (IP) with anti-Flag or control IgG antibodies and then immunoblotted (IB) with PLZF and Ezh2-specific antibodies. In total, 10% of total lysate was loaded as input.
- C Detection of Ezh2-dependent methylation of PLZF by immunoblot of lysates from 293T cells co-transfected with control (mCherry) or Ezh2-mCherry and PLZF-Flag-expressing (WT or K430R) plasmids, whole-cell lysates were immunoprecipitated with an anti-Flag antibody and probed with methylated lysine and PLZF-specific antibodies.
- D Ezh2 induces the ubiquitinylation of PLZF. Plasmids encoding PLZF-Flag or PLZF K430R-Flag, and ubiquitin-HA were transfected with mCherry or Ezh2-mCherry into 293T cells for 48 h followed by IP of Flag-tagged PLZF. Cells were treated with 20 μ M MG132 for 4 h prior to lysis. Western blotting was performed with antibodies specific for HA (to examine ubiquitinylation status of PLZF) and anti-PLZF to determine input.
- E The stability of WT or K430 PLZF was assessed in 293T cells at the indicated times after blocking protein synthesis with cyclohexamide (CHX).
- F The stability of WT PLZF was assessed in 293T cells after knockdown of endogenous *Ezh2* by two independent shRNAs and culture in the presence of CHX for the indicated timepoints. Data are representative of at least two independent experiments.

progenitors and studied NKT cell development *in vitro*. Introduction of K430R PLZF led to an increased proportion of cells at stage 1 and a corresponding decrease in stage 3 (Fig EV5B). Overall, these data show that Ezh2 can target PLZF for degradation and that mutating this target site stabilizes PLZF and alters NKT cell differentiation.

PLZF^{high} NKT cells are the cause of immune perturbation in *Ezh2*^{CKO} mice

Finally, we studied how the loss of the Ezh2 affected immune homeostasis. Perturbations of CD8⁺ T-cell homeostasis by extrinsic factors, namely IL-4 [15], have been observed in mice lacking the tyrosine kinase Itk [35] and the transcription factors Klf2 [15] and Id3 [36] and have been attributed to the expansion of innate T-cell populations such as NKT or $\gamma\delta$ T cells [15]. A more detailed examination of the phenotypic characteristics of the T cells which developed in the absence of Ezh2 revealed a dramatic alteration specific to the activation status of the CD8⁺, but not the CD4⁺ T-cell population (Figs 5A and EV5C). *Ezh2*-deficient CD8⁺ T cells adopted memory-like characteristics including the upregulation of CD44, expression of the transcription factor Eomes, and the production of IFN γ , yet they maintained expression of CD62L, indicative of a central memory phenotype (Fig 5A and B). In line with this, we also found a dramatic increase in immunoglobulin E (IgE) in the serum of the *Ezh2*^{CKO} mice (Fig 5D) as has been noted previously [37], suggesting that B-cell homeostasis was also perturbed.

To investigate whether NKT cells were indeed the driver of the immune perturbation, we specifically removed them by generating *Ezh2*^{CKO} mice that also lacked CD1d, which is required for NKT development [38]. Loss of NKT cells also resulted in restoration in the proportion of CD8⁺ T cells with a memory phenotype to near wild-type levels (Fig 5C). Furthermore, the increased serum IgE in *Ezh2*^{CKO} mice was also dependent on the presence of NKT cells (Fig 5D). Thus, genetic ablation of Ezh2 in the T-cell lineage resulted in expansion of PLZF^{high} NKT cells that adversely impacted on adaptive immune cell homeostasis.

Discussion

While chromatin-independent functions of Ezh2 have been reported previously, they are not known to impact on the differentiation of immune cells. By using a combination of PRC2-deficient mice, we have identified that Ezh2 plays such an alternative role in the regulation of NKT cell development. The retention of H3K27me3 and the few de-repressed genes in *Ezh2*-deficient NKT cells, while surprising, has been also observed in PRC2-target genes in stem cells [5,6] and correlates with the evidence showing that Ezh1 can compensate for the role of Ezh2 to methylate H3K27 [4,5]. In support of an unconventional role for Ezh2 in the regulation of immune homeostasis, we showed that removal of the non-redundant components of PRC2, Suz12, and Eed ablated NKT cell development and did not phenocopy *Ezh2* deletion. Conversely, due to the profound defects in H3K27me3 and NKT cell survival without Suz12 or Eed, we cannot exclude the possibility that these factors are also involved in the methylation of PLZF as they have been shown to be required for non-histone methylation by Ezh2 [7,9], although our data suggest that Suz12 was not

required for this process. Future work will investigate the composition of the complex required to induce PLZF methylation.

Non-histone methylation by Ezh2 can result in a range of outcomes for the target protein, such as repression [7] or activation of transcriptional activity [28]. The results we describe here the result of PLZF methylation are in line with the previously described Ezh2-dependent methyl-degron [8]. In this case, methylation of ROR α by Ezh2 was recognized DDB1-CUL4-associated factor 1 (DCAF1), which acted as an adaptor for the recruitment of a cullin 4-containing ubiquitin ligase complex. Interestingly, cullin 3 is highly expressed in immune cells and has recently been shown to interact with PLZF in NKT cells [32].

In the *Ezh2*-deficient NKT cells, we observed high levels of PLZF that were not due to increased transcription and we identified PLZF as a novel substrate for the Ezh2 methyl-degron. Our data suggest that the sustained high-concentration of PLZF observed in the absence of Ezh2 may guide cells toward an NKT2 fate, either by diverting cells from stage 0/1 NKT progenitors or by stalling NKT2 from becoming NKT1. In line with this, *Tbx21* and its target genes *IL2rb* and *IL18ra* [39] were downregulated in stage 1 and 2 NKT cells lacking Ezh2. Interestingly, T-bet knockout mice have an accumulation of NKT2 cells [14,40] suggesting that a mutual antagonism may exist between PLZF and T-bet. We propose a working model, in which Ezh2-mediated degradation of PLZF guides thymic NKT development and specifically enables stage 3/NKT1 development. In the absence of Ezh2, PLZF levels are maintained, *Tbx21* is repressed, and stage 2/NKT2 cells accumulate, which subsequently perturbs the adaptive cells of immune system. This is supported by a recent study that showed that high levels of PLZF indeed push cells toward the NKT2 pathway [41]. This highlights the potential role of the Ezh2 methyl-degron in the rapid degradation of transcription factor levels in allowing stage 2 cells to pass to the next stage of development where *Zbtb16* transcription is subsequently repressed. This mechanism adds an additional layer of regulation in cell fate determination that may be at play in the differentiation of many cell types.

To assess the functional relevance of the Ezh2-PLZF methyl-degron on NKT cell development, we retrovirally introduced WT PLZF or PLZF K430R into developing thymic NKT cell cultures. These are technically challenging experiments with a range of variables. Nevertheless, we observed a skewing of the cells transduced with the K430R mutant toward stage 1 and a corresponding drop in the proportion of stage 3 NKT cells. This suggests that stabilizing PLZF does indeed alter NKT cell development; however, the modest effects observed also imply that this mechanism may not be the only one operating in the expanded NKT cells from the *Ezh2*^{CKO} mice. We speculate the Ezh2-PLZF methyl-degron is required at the transition between stages 1 and 2 to downregulate PLZF protein prior to transcriptional repression of *Zbtb16* at stage 3 and that introducing this stabilized version of PLZF into early NKT cell progenitors (stage 0) may lead to the early block at stage 1.

Recently, the groups of Tarakhovsky and Bosselut also observed NKT cell expansion in *Ezh2*^{CKO} mice, which they concluded, was caused by the loss of the canonical function of H3K27me3-associated repression of *Zbtb16* transcription [29]. Our data suggest that this is unlikely for a number of reasons. Firstly, we showed that the complete loss of the PRC2 function in *Suz12* or *Eed*^{CKO} mice impairs NKT cell development, suggesting that the expansion of NKT cells in

Figure 5. Loss of Ezh2 results in immune perturbation due to expansion of NKT cells.

A Wild-type (WT) and *Ezh2^{cko}* splenic CD8⁺ T cells were analyzed for central memory (CM) phenotype by flow cytometry. Top panel shows CD44 and CD62L expression by wild-type (WT, left) and *Ezh2^{cko}* (right) CD8⁺ T cells. Eomes expression by WT (left) and *Ezh2^{cko}* (right) CD8⁺ T cells is shown in the bottom panel. Graph shows percentage of CD44⁺CD62L⁺ of the total CD8⁺ T cells from mice of the indicated genotypes. Data are from two independent experiments.

B Representative profile and bar graph of the percentage of WT and *Ezh2^{cko}* CD8⁺ T cells that were positive for intracellular IFN γ after PMA/ionomycin stimulation for 3 h. Data are pooled from two independent experiments, and error bars represent $\pm$ SEM from four individual mice.

C Loss of NKT cells restores *Ezh2^{cko}* CD8⁺ T-cell phenotype to near wild-type levels. Left panels show expression of CD44 and CD62L on CD8⁺ T cells of the indicated genotypes. Graph shows percentage of CD44⁺CD62L⁺ of the total CD8⁺ T cells from mice of the indicated genotypes. Symbols in graphs indicate data points for individual mice from three independent experiments, and horizontal lines indicate $\pm$ SEM.

D Bar graph showing ELISA measurement of serum IgE levels in indicated genotypes. Data are representative of two independent experiments $\pm$ SEM from four individual mice.

Data information: Numbers in the plots in (A, C) are the percent cells in the indicated quadrant or gate. Student's *t*-test was performed to test for statistical significance.

the absence of Ezh2 derives from the loss of a chromatin-independent function of this factor during NKT cell development. Moreover, we found that the *Zbtb16* promoter in both wild-type and *Ezh2^{cko}* stage 2 NKT cells contains low levels of H3K27me3 and unaltered gene transcription, making it highly unlikely that Ezh2 deficiency would result in de-repression of an already active gene. Although transcription is unaltered, Ezh2-deficient NKT cells have sustained higher levels of PLZF implicating post-translational regulation of this protein.

In summary, we propose that through its control of PLZF concentration, Ezh2 maintains NKT cell numbers at appropriate levels and facilitates immune homeostasis. We believe that our study

establishes a paradigm for alternative roles of Ezh2 and non-histone methylation in the regulation of cell fate determination.

Materials and Methods

Mice

Ezh2^{fl/fl} mice [22], *Eed^{fl/fl}* mice [6], and *Cd1d^{-/-}* mice [42] were described previously. *Suz12^{fl/fl}* mice were generated at the Walter and Eliza Hall Institute [23]. The floxed strains were crossed to

Cd4Cre mice [24]. All mice lines have been maintained on a C57BL/6 (Ly5.2) background and were used between 4 and 8 weeks of age and were age- and sex-matched. Animal experiments were in accordance with the guidelines of the Walter and Eliza Hall Institute Animal Ethics Committee.

Plasmids

The plasmid for expression of CFP-tagged PLZF and Flag-tagged PLZF derivatives was generated by subcloning the human PLZF cDNA into pECFP-C1 or pCMV-Flag (Clontech Laboratories Inc.) as described previously [43]. The Lenti ORF clone of Human EZH2 (Myc-Flag-tagged) cDNA from OriGene Technologies. mCherry-tagged EZH2 was generated by subcloning into pmCherry-C1 (Clontech Laboratories Inc.). All cDNA constructs were verified by DNA sequencing.

Antibodies and flow cytometry

Fluorochrome-conjugated antibodies against the following mouse antigens were used for analysis by flow cytometry: brilliant violet- or phycoerythrin-conjugated tetramers of CD1d containing the α -GalCer derivative PBS57 were obtained from the Tetramer Core Facility of the US National Institutes of Health. CD4 (RM 4-5), CD62L (MEL-14), Ly5.2 (104), CD44 (IM7), IFN γ (XMG1.2), T-bet (04-46), EZH2 (clone 11), Ror γ t (Q31-378) from BD Pharmingen; CD8 (53-6.7), TCR β (H57-597), Ly5.1 (A20), Eomes (Dan11mag), IL-4 (BVD6-24G2), $\gamma\delta$ TCR (B 1.1), NK1.1 (PK136), Tbet (4B10) were from eBioscience. Anti-Ror α antibodies were from Abcam. Anti-PLZF polyclonal antibody (sc-11146) was from Santa Cruz; anti-PLZF mAb (2A9), anti-Ezh1 and anti-H3K27me3 were from EMD Millipore. Surface staining was carried out at 4°C for 30 min. Intracellular staining was performed using eBioscience Foxp3 staining kit as per the manufacturer's protocol. Antibody-stained cells were analyzed using BD FACS Canto II or BD Fortessa1.

Enrichment of NKT cells

Anti-CD8 (clone 53-6.7, rat IgG, produced in house) antibody was used to deplete CD8⁺ T cells and DP thymocytes from the thymi of 6- to 8-week-old C57BL/6 mice. Briefly, single-cell suspension of thymocytes was incubated with anti-CD8 antibody for 30 min at 4°C. Unbound antibody was washed, and the antibody-coupled cells were incubated with BioMag Goat Anti-Rat IgG beads (Qiagen) to deplete CD8⁺ T cells and DP thymocytes. The CD4⁺ T-cell-enriched fraction was further incubated with the phycoerythrin (PE)-conjugated NKT cell tetramer for 30 min at room temperature. Anti-PE microbeads (Miltenyi) were used to positively select the tetramer-coupled NKT cells.

NKT cell cultures and retroviral infection

Anti-CD8 (clone 53-6.7, rat IgG, produced in house) antibody was used to deplete CD8⁺ T cells and DP thymocytes from the thymi of 6- to 8-week-old C57BL/6 mice. Briefly, single-cell suspension of thymocytes was incubated with anti-CD8 antibody for 30 min at 4°C. Unbound antibody was washed, and the antibody-coupled cells were incubated with BioMag Goat Anti-Rat IgG beads (Qiagen) to deplete CD8⁺ T cells and DP thymocytes. NK1.1⁺CD8⁺ cells were FACsorted

and cultured on OP9-DL1 in the presence of 5 ng/ml of recombinant IL-7 (Peprotech). After 2 days, these cells were spin-infected at 37°C in the presence of polybrene with either empty GFP retrovirus, WT PLZF GFP, or K430R PLZF GFP retrovirus. Five days later, NKT-tetramer⁺ cells were examined for the expression of CD44 and NK1.1 by flow cytometry.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed following an adapted protocol by Upstate/Millipore. In brief, stage 2 NKT cells were isolated from the thymus and cross-linking was performed by addition of 1% formaldehyde at room temperature for 10 min, followed by sonication and immunoprecipitation with 10 μ g of anti-H3K27me3 (Millipore). qPCR was performed using the following primers: *Zbtb16* (forward: 5'-AGCCCTTGCCTGTACAAAGA-3', reverse: 5'-TGCCTCACCAACCTTTCTTC-3'), *Hoxa11* (forward: 5'-AGGAGAAGGGGTTTCCTCAA-3', reverse: 5'-CTCCGCGGTTTGTCAATAAT-3').

Real-time PCR analysis

Total RNA was prepared using RNeasy kit (Qiagen) from WT or *Ezh2^{CKO}* NKT cells that were purified by flow cytometry. cDNA was made using iScript reverse transcription kit (Bio-Rad) as per the manufacturer's protocol. Real-time PCR was performed with the SYBR Green PCR kit (Bioline). Analyses were performed in triplicate, and mean normalized expression was calculated with the Q-Gene application with *Gapdh* as the reference gene. The following primers were used: *Gapdh* (forward: 5'-ACGGCCGATCTTCTTGTGCA-3', reverse: 5'-AATGGCAGCCCTGGTGACCA-3'), *Zbtb16* (forward: 5'-GACGCAC TACAGGGTTTACA-3', reverse: 5'-CGTTGTGTGTTCTCAGGTGC-3').

Immunoprecipitation and immunoblotting from NKT cells

NKT-tetramer-enriched thymocytes (1×10^7) were lysed with 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% (v/v) Nonidet P-40, 1 mM dithiothreitol, and 1 $\times$ protease inhibitor mix (Roche) for 30 min on ice. Lysates were cleared by centrifugation, pre-cleared with protein G Dynabeads (Invitrogen) for 1 h at 4°C, and incubated with anti-PLZF bound protein G Dynabeads for 12 h at 4°C. Beads were washed three times with cold lysis buffer followed by three washes with PBS and one wash with PBS with 0.05% Triton X-100. Bound proteins were eluted by boiling the beads with 50 μ l of 2 $\times$ Laemmli sample buffer. For immunoblotting, whole-cell extracts were prepared by lysing flow cytometrically sorted NKT cells in RIPA buffer (Millipore). Proteins were resolved in denaturing conditions in 4–12% gradient SDS-PAGE (Life technologies) and were transferred onto nitrocellulose membrane (Bio-Rad). Membrane was probed with following antibodies: mouse anti-PLZF (Santa Cruz), rabbit anti-methyl-lysine (Abcam), rabbit anti-Ezh2 (Cell Signaling), rabbit anti-Suz12 (Cell Signaling), rabbit anti-histone H3 (Cell Signaling), and goat anti-lamin B1 (Santa Cruz).

Immunoprecipitation and Immunoblotting from 293T cells

For the detection of PLZF ubiquitylation, cells were transfected with plasmids encoding PLZF-Flag and HA-Ub with either mCherry

or Ezh2-mCherry. After incubation for 48 h, cells were treated with 20 μ M MG132 for 4 h. Cells were then lysed with an NP40-containing lysis buffer and incubated with anti-Flag M2 antibodies. Antibody complexes were isolated with protein Affinity beads (Sigma-Aldrich), and immunocomplexes were run on an SDS-PAGE gel. Ubiquitinated PLZF was visualized by immunoblotting with anti-HA antibody (Life Technologies).

For detection of PLZF lysine methylation, 293T cells were transfected with plasmids encoding PLZF-flag with either mCherry or Ezh2-mCherry. After 48 h, cells were lysed with an NP40-containing lysis buffer and incubated with anti-Flag M2 antibodies. Antibody complexes were isolated with protein Affinity beads (Sigma-Aldrich), and immunocomplexes were analyzed by SDS-PAGE and immunoblotting with anti-methylated lysine (Abcam) and anti-PLZF (Calbiochem). Inhibition of Ezh2 methyltransferase activity was performed by treating the cells with 10 μ M of GSK126 (Selleck, S7061) for 48 h.

For co-immunoprecipitation, 293T cells were transfected with plasmids encoding PLZF-Flag and Ezh2-mCherry or Ezh2-Flag and PLZF-CFP. After 48 h, cells were lysed with an NP40-containing lysis buffer and incubated with anti-Flag M2 antibodies. Antibody complexes were isolated with protein Affinity beads (Sigma-Aldrich), and immunocomplexes were analyzed by SDS-PAGE and immunoblotting with anti-Ezh2 and anti-PLZF. Protein bands were detected and quantified on a Li-Cor Odyssey infrared imaging system. To analyze protein stability, the PLZF and PLZF K430R plasmids were transfected into 293T cells which and after 24 h were then treated with cycloheximide (20 μ g/ml) and analyzed at the indicated times after treatment.

Knockdown of *Suz12*

The MIT CRISPR design software was used for the design of sgRNAs (<http://crispr.mit.edu>). sgRNA sequence is as follows: *Suz12* exon 1: 5'-ACGGCTTCGGCGGCAAATC-3'. Lentiviral particles were produced by transient transfection of 293T cells grown in 10-cm Petri dishes with 10 μ g of vector DNA (containing pFgh1UTG vector with target sgRNA inserted and the pFUCas9mCherry vector) along with the packaging constructs pMDL (5 μ g), pRSV-rev (2.5 μ g), and pVSV-G (3 μ g) using standard calcium phosphate precipitation. Virus-containing supernatants were collected at 48–72 h after transfection and passed through a 0.45- μ m filter. To establish *Suz12* knockdown 293T cells treated with 8 ng/ml polybrene in the viral supernatant, incubated for 30 min at 37°C, and then centrifuged at 887 g for 2.5 h at 32°C. Doxycycline hyclate (Sigma-Aldrich D9891) was used for treatment of cell lines to induce expression of the sgRNA for 3 days.

Knockdown of *Ezh2* and assessment of PLZF stability

Short hairpin RNA (shRNA)-mediated silencing was performed by transfecting 293T cells with GIPZ lentiviral human shRNA vector for three different expressing 19 nucleotide shRNAs against EZH2 (EZH2 target sequence 5'-TTAAGATTTCCGTTCTTTC-3', 5'-TATTGGTGTGACACCGA-3', 5'-TTATCATACACTTCCCTC-3'). A non-targeting shRNA (5'-ATCTCGCTTGGCGAGAGTAAG-3') was used as negative control (Dharmacon, Thermo Fisher Scientific, Lafayette, CO, USA). Samples were collected after cycloheximide (20 μ g/ml)

treatment at the indicated time to determine the protein levels of PLZF. EZH2 silencing in 293T cells was assessed by both Western blotting and qRT-PCR after 72 h post-transfection.

RNA sequencing and bioinformatic analysis

Thymocytes were isolated from wild-type or *Ezh2^{CKO}* mice and depleted of CD8⁺ cells using magnetic beads coupled to an anti-CD8 antibody. NKT cells identified by CD1d-tetramer and TCR β were sorted into different stages using a BD Influx cell sorter to purity of typically > 97% using CD44, NK1.1, and CD24 antibodies. RNA purification was performed following the manufacturer's protocol using the RNeasy Plus Mini Kit (Qiagen). RNA samples were sequenced at the Australian Genome Research Facility using the Illumina HiSeq sequencing system. An average of 30 million single-end 100-bp reads was obtained for each sample, split evenly over four technical replicates. Reads were aligned to the *mm10* build of the mouse genome using *subread* [44] with default parameters. Over 97% of reads were successfully aligned in each sample. Technical replicates for each sample were pooled into a single library. For each library, mapped reads with a mapping quality score greater than or equal to 30 were assigned to mouse genes in the NCBI RefSeq mouse annotation build 38 using *featureCounts* [45]. An average of 74% of mapped reads was counted into genes for each library.

Read counts for each gene were then used in a differential expression (DE) analysis using the *limma* and *edgeR* packages [46,47]. Lowly expressed genes were first filtered out if the average log-count per million (as computed by the *aveLogCPM* function) was < 1. *Xist* and any genes on the Y chromosome were also removed to eliminate sex effects. Normalization was performed using the TMM method [48] to remove composition bias between libraries. Counts were then log-transformed using the *voom* function [49]. A linear model was fitted to the log-counts using the computed precision weights. Sample variances for each gene were computed and shrunk toward a mean-variance trend using a robust empirical Bayes strategy [50]. For each contrast, a *P*-value was computed for each gene using the *treat* method [51] relative to a fold change threshold of 1.2. The Benjamini–Hochberg method was then applied to control the false discovery rate (FDR). DE genes were defined as those that were detected at a *treat* FDR of 10%.

MA plots were generated for each contrast by plotting the average log-count per million against the shrunken log-fold change for each gene. Briefly, each log-fold change was computed using the GLM framework in *edgeR* with a prior count of 3 [52].

Statistics

If not stated otherwise, a Student *t*-test was performed to test for statistical significance; error bars denote mean $\pm$ SEM.

Expanded View for this article is available online.

Acknowledgements

We thank David Tarlinton, Amanda Light, Simon Willis, Jane Visvader, Marnie Blewitt, and Sarah Kinkel from the Walter and Eliza Hall Institute and Nicholas Williamson (Bio21 University of Melbourne) for technical assistance, advice,

and discussions. We also thank Alexander Tarakhovsky (Rockefeller University) for the *Ezh2^{fl/fl}* mice and Stuart Orkin (DFCI, Harvard) for the *Eed^{fl/fl}* mice. This work was supported by grants and fellowships from the National Health and Medical Research Council of Australia (RSA, SLN, WSA, GKS, AK, BRGW, DX, DIG), the Australian Research Council (RSA, SLN, AK), the Sylvia and Charles Viertel Foundation (AK), an American Asthma Foundation Grant (SLN, RSA), the National Natural Science Foundation of China (DX) (81273247, 81472655, and 31670905), the National Basic Research Program of China (2012CB911204), and the Netherlands Organization for Scientific Research (KJPvG). This study was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC Independent Research Institute Infrastructure Support scheme and the Australian Cancer Research Fund.

Author contributions

AV designed the research, performed experiments, analyzed data, and wrote the manuscript. DX designed the research, performed experiments, and analyzed data. KPJMvG, NI, LY, XL, and DW performed experiments and analyzed data. ATLL and GKS performed bioinformatics analysis. BRGW, DIG, and AK helped to design experiments and provided critical reagents. MJH and AJK generated critical reagents. SCWL, IJM, and WSA developed and characterized the *Suz12^{fl/fl}* mice. SLN and RSA designed and supervised the research and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Zhang X, Wen H, Shi X (2012) Lysine methylation: beyond histones. *Acta Biochim Biophys Sin* 44: 14–27
- Roth SY, Denu JM, Allis CD (2001) Histone acetyltransferases. *Annu Rev Biochem* 70: 81–120
- Margueron R, Reinberg D (2011) The Polycomb complex PRC2 and its mark in life. *Nature* 469: 343–349
- Margueron R, Li G, Sarma K, Blais A, Zavadil J, Woodcock CL, Dynlacht BD, Reinberg D (2008) Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. *Mol Cell* 32: 503–518
- Shen X, Liu Y, Hsu YJ, Fujiwara Y, Kim J, Mao X, Yuan GC, Orkin SH (2008) EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. *Mol Cell* 32: 491–502
- Xie H, Xu J, Hsu JH, Nguyen M, Fujiwara Y, Peng C, Orkin SH (2014) Polycomb repressive complex 2 regulates normal hematopoietic stem cell function in a developmental-stage-specific manner. *Cell Stem Cell* 14: 68–80
- He A, Shen X, Ma Q, Cao J, von Gise A, Zhou P, Wang G, Marquez VE, Orkin SH, Pu WT (2012) PRC2 directly methylates GATA4 and represses its transcriptional activity. *Genes Dev* 26: 37–42
- Lee JM, Lee JS, Kim H, Kim K, Park H, Kim JY, Lee SH, Kim IS, Kim J, Lee M et al (2012) EZH2 generates a methyl degron that is recognized by the DCAF1/DBB1/CUL4 E3 ubiquitin ligase complex. *Mol Cell* 48: 572–586
- Su IH, Dobenecker MW, Dickinson E, Oser M, Basavaraj A, Marqueron R, Viale A, Reinberg D, Wulfeing C, Tarakhovsky A (2005) Polycomb group protein ezh2 controls actin polymerization and cell signaling. *Cell* 121: 425–436
- Gunawan M, Venkatesan N, Loh JT, Wong JF, Berger H, Neo WH, Li LY, La Win MK, Yau YH, Guo T et al (2015) The methyltransferase Ezh2 controls cell adhesion and migration through direct methylation of the extranuclear regulatory protein talin. *Nat Immunol* 16: 505–516
- Simon C, Chagraoui J, Kros J, Gendron P, Wilhelm B, Lemieux S, Boucher G, Chagnon P, Drouin S, Lambert R et al (2012) A key role for EZH2 and associated genes in mouse and human adult T-cell acute leukemia. *Genes Dev* 26: 651–656
- Godfrey DI, Stankovic S, Baxter AG (2010) Raising the NKT cell family. *Nat Immunol* 11: 197–206
- Bendelac A, Savage PB, Teyton L (2007) The biology of NKT cells. *Annu Rev Immunol* 25: 297–336
- Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA (2013) Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol* 14: 1146–1154
- Weinreich MA, Odumade OA, Jameson SC, Hogquist KA (2010) T cells expressing the transcription factor PLZF regulate the development of memory-like CD8+ T cells. *Nat Immunol* 11: 709–716
- Umetsu DT, DeKruyff RH (2006) A role for natural killer T cells in asthma. *Nat Rev Immunol* 6: 953–958
- Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, Glickman JN, Siebert R, Baron RM, Kasper DL et al (2012) Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 336: 489–493
- Mattarollo SR, Smyth MJ (2013) NKT cell adjuvants in therapeutic vaccines against hematological cancers. *Oncoimmunology* 2: e22615
- Cohen NR, Brennan PJ, Shay T, Watts GF, Brigl M, Kang J, Brenner MB, ImmGen Project C (2013) Shared and distinct transcriptional programs underlie the hybrid nature of iNKT cells. *Nat Immunol* 14: 90–99
- Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, Chua K, Eidson M, Kim HJ, Im JS et al (2008) The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol* 9: 1055–1064
- Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, Lantz O, Bendelac A (2008) The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29: 391–403
- Su IH, Basavaraj A, Krutchinsky AN, Hobert O, Ullrich A, Chait BT, Tarakhovsky A (2003) Ezh2 controls B cell development through histone H3 methylation and IgH rearrangement. *Nat Immunol* 4: 124–131
- Lee SC, Miller S, Hyland C, Kauppi M, Lebois M, Di Rago L, Metcalf D, Kinkel SA, Josefsson EC, Blewitt ME et al (2015) Polycomb repressive complex 2 component Suz12 is required for hematopoietic stem cell function and lymphopoiesis. *Blood* 126: 167–175
- Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Nguyen S et al (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15: 763–774
- Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298: 1039–1043
- Ezhkova E, Lien WH, Stokes N, Pasolli HA, Silva JM, Fuchs E (2011) EZH1 and EZH2 cogovern histone H3K27 trimethylation and are essential for hair follicle homeostasis and wound repair. *Genes Dev* 25: 485–498
- Constantinides MG, Bendelac A (2013) Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol* 25: 161–167
- Kim E, Kim M, Woo DH, Shin Y, Shin J, Chang N, Oh YT, Kim H, Rhee J, Nakano I et al (2013) Phosphorylation of EZH2 activates STAT3 signaling

- via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. *Cancer Cell* 23: 839–852
29. Dobenecker MW, Kim JK, Marcello J, Fang TC, Prinjha R, Bosselut R, Tarakhovsky A (2015) Coupling of T cell receptor specificity to natural killer T cell development by bivalent histone H3 methylation. *J Exp Med* 212: 297–306
30. Heng TS, Painter MW, Immunological Genome Project C (2008) The immunological genome project: networks of gene expression in immune cells. *Nat Immunol* 9: 1091–1094
31. Sobieszczuk DF, Poliakov A, Xu Q, Wilkinson DG (2010) A feedback loop mediated by degradation of an inhibitor is required to initiate neuronal differentiation. *Genes Dev* 24: 206–218
32. Mathew R, Seiler MP, Scanlon ST, Mao AP, Constantinides MG, Bertozzi-Villa C, Singer JD, Bendelac A (2012) BTB-ZF factors recruit the E3 ligase cullin 3 to regulate lymphoid effector programs. *Nature* 491: 618–621
33. McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, Liu Y, Graves AP, Della Pietra III A, Diaz E et al (2012) EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature* 492: 108–112
34. Shi SP, Qiu JD, Sun XY, Suo SB, Huang SY, Liang RP (2012) PMeS: prediction of methylation sites based on enhanced feature encoding scheme. *PLoS ONE* 7: e38772
35. Atherly LO, Lucas JA, Felices M, Yin CC, Reiner SL, Berg LJ (2006) The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells. *Immunity* 25: 79–91
36. Vervokakis M, Boos MD, Bendelac A, Kee BL (2010) SAP protein-dependent natural killer T-like cells regulate the development of CD8(+) T cells with innate lymphocyte characteristics. *Immunity* 33: 203–215
37. Tumes DJ, Onodera A, Suzuki A, Shinoda K, Endo Y, Iwamura C, Hosokawa H, Koseki H, Tokoyoda K, Suzuki Y et al (2013) The polycomb protein Ezh2 regulates differentiation and plasticity of CD4(+) T helper type 1 and type 2 cells. *Immunity* 39: 819–832
38. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR (1997) Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. *Immunity* 6: 459–467
39. Jenner RG, Townsend MJ, Jackson I, Sun K, Bouwman RD, Young RA, Glimcher LH, Lord GM (2009) The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. *Proc Natl Acad Sci USA* 106: 17876–17881
40. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, Gapin L, Glimcher LH (2004) T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 20: 477–494
41. Pobezinsky LA, Etzensperger R, Jeurling S, Alag A, Kadakia T, McCaughy TM, Kimura MY, Sharrow SO, Guinter TI, Feigenbaum L, et al (2015) Let-7 microRNAs target the lineage-specific transcription factor PLZF to regulate terminal NKT cell differentiation and effector function. *Nat Immunol* 16: 517–524
42. Sonoda KH, Exley M, Snapper S, Balk SP, Stein-Streilein J (1999) CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site. *J Exp Med* 190: 1215–1226
43. Xu D, Holko M, Sadler AJ, Scott B, Higashiyama S, Berkofsky-Fessler W, McConnell MJ, Pandolfi PP, Licht JD, Williams BR (2009) Promyelocytic leukemia zinc finger protein regulates interferon-mediated innate immunity. *Immunity* 30: 802–816
44. Liao Y, Smyth GK, Shi W (2013) The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* 41: e108
45. Liao Y, Smyth GK, Shi W (2014) FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30: 923–930
46. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43: e47
47. Robinson MD, McCarthy DJ, Smyth GK (2010) EdgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139–140
48. Robinson MD, Oshlack A (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* 11: R25
49. Law CW, Chen Y, Shi W, Smyth GK (2014) Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol* 15: R29
50. Phipson B, Lee S, Majewski IJ, Alexander WS, Smyth GK (2016) Robust hyperparameter estimation protects against hypervariable genes and improves power to detect differential expression. *Ann Appl Stat* 10: 946–963
51. McCarthy DJ, Smyth GK (2009) Testing significance relative to a fold-change threshold is a TREAT. *Bioinformatics* 25: 765–771
52. McCarthy DJ, Chen Y, Smyth GK (2012) Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res* 40: 4288–4297