Sequential actions of EOMES and T-BET promote stepwise maturation of natural killer cells

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EOMES and T-BET are related T-box transcription factors that control natural killer (NK) cell development. Here we demonstrate that EOMES and T-BET regulate largely distinct gene sets during this process. EOMES is dominantly expressed in immature NK cells and drives early lineage specification by inducing hallmark receptors and functions. By contrast, T-BET is dominant in mature NK cells, where it induces responsiveness to IL-12 and represses the cell cycle, likely through transcriptional repressors. Regardless, many genes with distinct functions are co-regulated by the two transcription factors. By generating two gene-modified mice facilitating chromatin immunoprecipitation of endogenous EOMES and T-BET, we show a strong overlap in their DNA binding targets, as well as extensive epigenetic changes during NK cell differentiation. Our data thus suggest that EOMES and T-BET may distinctly govern, via differential expression and co-factors recruitment, NK cell maturation by inserting partially overlapping epigenetic regulations.
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K cells are group 1 innate lymphoid cells (ILCs) with an important role in antiviral and anti-tumor responses. NK cells share many features with tissue-resident ILC1s, such as the responsiveness to IL-15, IL-12 and IL-18, and the capacity to rapidly produce IFN-γ upon stimulation. However, they differ from ILC1s by their capacity to circulate in the blood, by their expression of multiple receptors of the Ly49 family, by their higher cytotoxic potential and by their expression of integrin subunits. In particular, CD49B and CD49A are expressed in a mutually exclusive manner by NK cells and expression of integrin subunits. Therefore, EOMES and T-BET balance each other during NK cell development. We show a dominant expression and a dominant role of EOMES in immature NK cells, and a more recent study suggested that T-BET stabilizes immature NK cells, somewhat contradicting previous findings.

Results

EOMES and T-BET balance each other during NK cell maturation and activation. To explore the roles of T-BET and EOMES in NK cells, we first measured the expression of both TFs during maturation. To unambiguously analyze NK cells and exclude ILC1s we defined NK cells as CD49B+/CD49A−/NK1.1+/CD3− and analyzed maturation based on the CD11B/CD27 classification (Supplementary Fig. 1A). Flow cytometry measurements indicated that EOMES expression in spleen NK cells was rather similar in stages 1 and 2 and then decreased in stage 3 NK cells, while T-BET had a reciprocal expression pattern (Fig. 1A). In the BM, EOMES levels showed the same pattern as in the spleen while T-BET levels were much lower compared to that of spleen NK cells (Supplementary Fig. 1B), as we previously observed.

We used Tbx21−/− mice and NCR1−iCre+/− Eomesfl/fl mice (referred to as NK-Eomes−/− thereafter) to study the consequences of T-BET or EOMES deficiency on NK cell development. We noted that in mice with one copy of Eomes, NK cells had near normal levels of EOMES protein, while in mice with one copy of Tbx21, the level of this TF was approximately half of that of WT mice (Fig. 1A). In T-BET deficient NK cells (i.e., from Tbx21−/− mice), a higher expression of EOMES was also noted in all subsets.
while EOMES-deficient NK cells (i.e., from NK-Eomes−/− mice) displayed higher T-BET expression (Fig. 1A). This suggested that EOMES and T-BET repress transcriptionally each other in NK cells or that the absence of one TF stabilizes the protein expression of the other one. Upon stimulation with cytokines ex vivo, IL-15 maintained T-BET and EOMES levels while a combination of IL-12 and IL-18 (together or not with IL-15) resulted in a strong up-regulation of T-BET and a decrease of EOMES, further supporting the concept that EOMES and T-BET balance each other during NK cell maturation or activation (Fig. 1B).

A previous article reported that T-BET and EOMES were localized both in the nucleus and in the cytoplasm of memory CD8+ T cells, which adds another layer of regulation for both TFs. To analyze the nuclear expression of EOMES and T-BET, we used Image cytometry (Image-Stream) and confocal microscopy. Image-Stream analysis allowed us to quantify the nuclear fraction of each TF in gated NK cell subsets.

**Fig. 1 EOMES and T-BET balance each other during NK cell maturation.**

**a** Flow cytometry measurement of T-BET and EOMES in gated NK cells from WT, Tbx21+/−, Ncr1Cre−/− (Eomes+/−), Ncr1Cre+/− X Eomes+/−, Tbx21+/− X Eomes+/− and Ncr1Cre−/− X Eomeslox/lox (Eomes−/−) mice, as indicated. Bar graphs show the mean ± SD fluorescence intensity (MFI) of EOMES and T-BET staining in gated NK cell subsets from spleen. Data are representative of 3 experiments and 3 mice are shown for each group. **b** WT spleen cells were stimulated O/N in the indicated conditions and T-BET and EOMES expression were measured by flow cytometry. Bar graphs show the mean ± SD fluorescence intensity (MFI) of EOMES and T-BET staining in gated NK cells. Data are representative of 2 experiments and 3 mice are shown for each group. **c** Image stream X (ISX) analysis of T-BET and EOMES expression in gated NK cells. **d** Quantification of nuclear T-BET and EOMES in gated NK cell subsets analyzed with IDEAS software. Bar graphs show nuclear MFI of T-BET and EOMES for the indicated subsets, normalized to the level of each TF in immature CD11B− NK cells. N = 1232, 5157 and 22403 cells analyzed for CD11B−, DP and CD27− subsets, respectively. **e** NK cells were sorted from WT mice in immature or mature subsets and subsequently stained for nucleus (DAPI), T-BET, EOMES and CD122 for confocal microscopy analysis. Shown are representative images of each staining and combinations for immature NK cells. Images are representative from three individual experiments. Unpaired t-tests (two-tailed) were used for statistical analysis of data presented in this figure.
of EOMES and T-BET, delimited by the DAPI staining (Fig. 1C). Using this method, we found that NK cell maturation was associated with a progressive shift in the nuclear ratio between EOMES and T-BET, from high EOMES/low T-BET to low EOMES/high T-BET (Fig. 1D). A confocal microscopy analysis then showed that EOMES and T-BET are predominantly nuclear in NK cells, and suggested that they localize in the same nuclear areas both in immature (Fig. 1E) and mature NK cells (Supplementary Fig. 1C, see also correlative analysis in Supplementary Fig. 1D).

EOMES is essential for early NK cell development and restrains T-BET-induced terminal maturation. A previous analysis of the role of T-BET and EOMES in NK cell development was hampered by a gating analysis that did not discriminate between NK cells and ILC122. We therefore revisited the role of both TFs in NK cell development and maturation using Tbx21−/−, NK-Eomes−/− and appropriate control mice by carefully gating NK cells (CD49B+CD49A−) and excluding ILC1 (CD49A+CD49B−) from the analysis (see Supplementary Fig. 1). Both T-BET and EOMES were important for NK cell homeostasis (Fig. 2A). However, EOMES had a more important role than T-BET in terms of NK cell numbers, i.e., the lack of EOMES caused the number of NK cells to drop by 5–10 fold both in BM and spleen, while the lack of T-BET rather increased the number of BM NK cells, and decreased that of spleen NK cells two to threefold (Fig. 2B). When examining maturation stages, EOMES was essential for the accumulation of all NK cell subsets, especially DP that were the only ones to decrease in percentage among total NK cells in both BM and spleen compared to controls (Fig. 2C). The strong decrease in the number of immature CD11B− NK cells in the BM in the absence of EOMES indicated an essential role of this TF in early NK cell development, unlike T-BET. Of note, the paucity of NK cells in NK-Eomes−/− mice was not compensated by an accumulation of CD49B+ILC1 either CD49B+ or CD49B− (Supplementary Fig. 2A).

T-BET deficiency resulted in an accumulation of DP both in terms of percentage and number in the BM and a near lack of CD27− cells in both organs analyzed (Fig. 2C), as previously shown,21 confirming the essential role of this TF in terminal NK cell maturation. We also observed an inverse pattern of KLRG1 expression, which is highly expressed in terminal mature NK cells, in the absence of EOMES and T-BET, i.e., an increased percentage of KLRG1+ NK cells and a decreased percentage of these cells among NK cell subsets when compared to controls respectively, suggesting that terminal maturation is promoted by T-BET and rather prevented by EOMES (Supplementary Fig. 2B). To further test this point we sorted NK cells at maturation stages 1 and 2 from Tbx21−/−, NK-Eomes−/− and control mice and adoptively transferred them into un-irradiated Ly5a X B6 (CD45.1/2) mice. Transferred NK cells were allowed to acclimate for two weeks before analysis of their maturation status in the spleen. The recovery rate was low for both EOMES and T-BET deficient NK cells compared to controls (Supplementary Fig. 2C). However, recovered NK-Eomes−/− NK cells from either stage 1 or stage 2 were still Eomes-deficient (Supplementary Fig. 2D), did not differentiate into ILC1s (Supplementary Fig. 2E) and had an accelerated maturation towards the CD27-CD11B+ stage 3 compared to controls, while Tbx21−/− NK cells had the opposite behavior (Fig. 2D), thus confirming the antagonistic effect of both TFs on NK cell maturation rate. Collectively, these results demonstrate the sequential roles of EOMES and T-BET in NK cell differentiation. Moreover, they show that in the absence of EOMES, the few developing NK cells can become hyper mature, possibly because of an excess of T-BET.

EOMES and T-BET promote NK cell survival at different maturation stages. To further document the role of EOMES and T-BET in NK cell development and homeostasis, we next assessed proliferation and survival of NK cell subsets developing in the absence of either factor. We evaluated NK cell proliferation using EdU incorporation or Ki67 staining. The main proliferative burst in NK cells normally occurs before the acquisition of the CD11B integrin, in the BM.35 Surprisingly, Eomes deletion did not change the level of proliferation of immature CD11B− NK cells in the BM, but rather increased that of DP in this compartment. In the spleen, all NK-Eomes−/− NK cell subsets had an increased proliferation rate, especially CD11B− and DP (Fig. 3A and Supplementary Fig. 3A). Tbx21 deletion increased the proliferation rate of mature CD27− NK cells and to a lesser extent that of DP, both in the BM and spleen, while CD11B− NK cells were hardly affected.

The increased proliferation of NK cells in the periphery of both knockout mouse models could be related to the increased availability of IL-15 due to the lack of its consumption by NK cells. To address this possibility, we transferred CTV-labeled Eomes−/− or Tbx21−/− NK cells with control NK cells into Ly5a congenic mice that have normal NK cell numbers. We then monitored the percentage of divided NK cells two weeks after transfer. As shown in Supplementary Fig. 3B, Tbx21−/− NK cells still divided more than control NK cells in these conditions. By contrast, Eomes−/− NK cells had a near-normal proliferation, indicating that EOMES does not intrinsically regulate proliferation in NK cells.

To explain the reduction in NK cells upon deletion of either factor, we hypothesized that both TFs could regulate their survival. Indeed, the percentage of apoptotic Annexin-V+ DP cells was increased in the absence of EOMES in both spleen and BM. Similarly, the percentage of apoptotic Annexin-V+ CD27− NK cells was increased in the absence of T-BET in both BM and spleen (Fig. 3B). As IL-15 is a major mediator of NK cell survival37, we next assessed how EOMES and T-BET regulated the response to this cytokine. Upon co-culture with IL-15 for 48 h, both EOMES and T-BET deficient NK cells showed a decreased viability compared to controls (Fig. 3C) suggesting that a balanced expression of EOMES and T-BET is necessary for optimal response to IL-15. At the subset level, the decreased viability was observed for T-BET deficient CD27− NK cells, and for EOMES-deficient DP NK cells (Supplementary Fig. 3C). CD122 surface levels were higher in T-BET deficient and lower in EOMES-deficient NK cells than in controls, respectively (Fig. 3D), showing that T-BET and EOMES regulate IL-15-mediated survival not simply through the control of CD122 expression. Altogether, these data demonstrate that EOMES and T-BET promote survival of maturing NK cells in a sequential manner, which could involve fine-tuning of IL-15 responsiveness.

Complementary roles of EOMES and T-BET in instructing the NK cell maturation program. Next, to gain molecular insight on the mechanisms of T-BET and EOMES action during NK cell development and maturation, we performed RNA-seq in immature CD11B− and mature CD27− NK cells from Tbx21−/−, NK-Eomes−/− and control mice. In accordance with the dominant expression of EOMES in immature cells (Fig. 1), we found that the loss of EOMES but not that of T-BET had a strong impact on gene expression in immature NK cells (Fig. 4A, B and Supplementary Data 1). Inversely, in mature NK cells, T-BET had a much more prominent role than EOMES on gene transcription (Fig. 4A, B). When examining differentially expressed genes (DEGs), EOMES was more often a transactivator of gene
expression (84% of DEGs in immature NK cells and 52% in mature ones) while T-BET was more often a repressor of gene expression (63% of DEG in mature NK cells, Fig. 4B). We queried the Immgen database to visualize the expression pattern of EOMES and T-BET-regulated genes across the whole immune system. EOMES-induced genes were found to be rather NK-specific while T-BET activated genes had a broader expression pattern (Supplementary Fig. 4A). The expression pattern of EOMES-repressed and T-BET-repressed genes was more comparable, but T-BET appeared more specialized in the repression of genes expressed in hematopoietic progenitors, or in B and T cell progenitors (Supplementary Fig. 4B). Of note, EOMES-dependent genes in immature NK cells include 23/91 of genes defining the NK cell signature as previously defined by the Immgen consortium, while only four genes were also regulated by T-BET in this list (Fig. 4C). Inversely, in mature NK cells, 25 and 6 of these genes were regulated by T-BET and EOMES, respectively (Fig. 4C), suggesting that both EOMES and T-BET contribute to define NK cell identity, but in a sequential manner.

Overall, there was a limited overlap between T-BET and EOMES dependent genes both in immature and mature NK cells (Fig. 4D, Supplementary Data 1), with 114/166 (68%) EOMES dependent genes not regulated by T-BET and 691/744 (92%) T-BET dependent genes not regulated by EOMES. We also looked at the mutual regulation between EOMES and T-BET. There was a trend for more EOMES mRNA in the absence of T-BET and vice versa (Supplementary Fig. 4C) but this was not statistically significant, suggesting that posttranscriptional mechanisms may contribute to explain the results observed in Fig. 1 at the protein level.
Finally, 82/166 EOMES-dependent (52%) and 250/744 T-BET-dependent (35%) genes were regulated during NK cell maturation (Fig. 4E, F). Reciprocally, 294/699 genes normally regulated during NK cell maturation were dependent on either EOMES or T-BET (42%) (Fig. 4F), confirming that both factors are major drivers of NK cell maturation, acting in a complementary manner.

Specific and shared actions of T-BET and EOMES on NK cell transcriptome. To define which properties were commonly or specifically conferred to NK cells by EOMES and T-BET, we then performed functional annotations of the gene modules described in Fig. 4.

Only one gene was regulated together by T-BET and EOMES in both immature and mature NK cells: Cym, which encodes a mast cell enzyme of unknown function in NK cells (Fig. 4D). Fifty-two other genes were regulated both by T-BET and EOMES in at least one comparison (Fig. 4D). This gene module is presented in details in Fig. 5 and includes three subsets: genes induced by EOMES in immature NK cells and by T-BET in mature ones. This group comprises crucial mediators of granule-dependent cytotoxicity such as Prf1, Gzma and Serpinb9b, and “cell killing” was one of the significant terms in a functional annotation of this geneset (Fig. 5E) using Metascape. At the protein level, we confirmed that GZMA was strongly reduced in both T-BET and EOMES deficient NK cells (Fig. 5D). Cma1 encodes for another mast cell-protease present in granules that could also be involved in cytotoxicity. We assessed the cytotoxic potential of EOMES and T-BET deficient mature NK cells using a new technique adapted to low cell numbers. T-BET deficient NK cells had a normal cytotoxicity, while EOMES deficient NK cells were poorly cytotoxic compared to controls (Fig. 5F). The T-BET/EOMES co-induced gene set also included Slpr5 that is essential for NK cells egress from the BM. To examine how T-BET and EOMES contributed to this egress, we injected T-BET and EOMES deficient mice with anti-CD45.2 Ab for 5 min, thus brightly staining only cells that were in blood sinusoids. A reduced sinusoidal fraction was observed in BM and LN NK cells from T-BET deficient but not EOMES deficient mice (Supplementary Fig. 5A), showing that even though both TFs regulate Slpr5, T-BET is the major factor promoting blood circulation of NK cells. The frequency of blood NK cells was also quantified from T-BET deficient mice, NK cells were strongly reduced in the absence of both T-BET and EOMES. However, in T-BET deficient mice, NK cells were only reduced in the blood (Supplementary Fig. 5B, C) and not in the BM (Fig. 2A), which confirms the specific role of T-BET in NK cell blood circulation.

Several genes showed however opposite regulation by T-BET and EOMES (Fig. 5G), for example Cd27 and Cd69 were induced by EOMES but repressed by T-BET in NK cells, which we could also...
confirm at the protein level (Fig. 5H, I). These genes are normally expressed at high levels in immature NK cells and repressed in mature NK cells, suggesting that when T-BET is up-regulated upon terminal maturation, it substitutes EOMES on the promoter of these genes and represses their expression. Other genes may be regulated in a reciprocal manner, i.e., repressed by EOMES and induced by T-BET. This is the case of Ccl26 encoding for DNAM1, which expression was not statistically different between control and KOs at the mRNA level (Supplementary Data 1), but was clearly different at the protein level (Fig. 5I).

**EOMES-specific role in the induction of hallmark NK cell genes**. EOMES specific genes (i.e., regulated either in immature or in mature NK cells by EOMES and not T-BET) include mainly EOMES-inducible ones (Fig. 6A). A functional annotation of EOMES-activated genes suggested a link with NK cell activation, cytotoxicity and IFNγ production (Fig. 6B). Indeed, this module contains a large cluster of NK cell receptors (KlrA8, KlrE1, KlrA4, KlrC2, KlrG1) and the signaling adaptor SAP (encoded by Sh2d1a), suggesting that EOMES is specifically involved in the induction of NK cell receptors and associated signaling and functions. Indeed, when looking at the expression of a battery of NK cell receptors at the protein level, we observed a defect in the expression of several Ly49 or SLAM receptors in immature NK cells (encoded by Sell) (Fig. 6C), and genes involved in the cytotoxic function such as FastL and GzmK or in trafficking such as Cd62L (encoded by Sell, Fig. 6D) or the granule-contained CCL5 chemokine. Finally, EOMES induced the expression of many genes of unknown function such as Khdc1a and Khdc1b and several lncRNA (Mirt1, Gm12596 etc, see Supplementary Data 1).
To identify putative downstream mediators of EOMES transcriptional activity, we also examined TFs whose expression was dependent on EOMES. We found that *Kcnip3*, *Lzts1* and *Bhlhe40* were down regulated in the absence of EOMES, either in immature or in mature NK cells (Fig. 6F). *Bhlhe40* is known to promote mitochondrial metabolism in resident memory T cells and could therefore also regulate NK cell development. EOMES also behaved as a specific repressor of a subset of TFs including *Cebpg*, *Cbx2*, *Zfp689* and *Zfp683*. The latter, also known as Hobit, was previously shown to be essential for ILC1 development.

This suggested that *Eomes*−/− NK cells were closely related to ILC1s, even though they were gated as CD49B + CD49A−. To address this point, we performed a single cell RNA-seq analysis of CD11B− and CD11B + NK cells and of ILC1s from WT and NK−/− mice. Results presented in Fig. 6G, Supplementary Fig. 6 and Supplementary Data 2 show that *Eomes*−/− NK cells cluster with WT NK cells, apart from ILC1s, thus showing that the loss of EOMES is not sufficient to induce reprogramming into ILC1s in developing NK cells.

Fig. 5 Shared transcriptional activities of T-BET and EOMES in NK cells. RNA-seq analysis of sorted Tbx21+/−, *Eomes*−/− and appropriate control CD11B− and CD27− NK cells (n = 3 per group with three sorts in total). DEGs were selected based on adjusted p < 0.05. a T-BET/EOMES co-repressed genes in immature or mature NK cells. Bar graphs show the log2 transformed fold change of genes between controls and T-BET or EOMES deficient mice, as indicated. b Bar graph showing the mean ± SD of *IL7R* expression in NK cells of the indicated genotype as measured by flow cytometry. The different controls for each genotype are pooled and annotated as “WT” in the graph which is same for the following graphs in (d, h, j). N = 3. c T-BET/EOMES co-induced genes in immature or mature NK cells, Bar graphs show the log2 transformed fold change of genes between controls and T-BET or EOMES deficient mice, as indicated. d Bar graph showing the mean ± SD of *GZMA* expression in NK cells of the indicated genotype as measured by flow cytometry. N = 3. e Functional annotation of the T-BET/EOMES induced gene set using Metascape. Bar graphs show selected terms among the most significant ones. f Cytoxicity assay using sorted Tbx21+/−, *Eomes*−/− and appropriate control CD27− NK cells as effectors and RNA-KR-Nano-luc cells as targets. Graphs show the mean cytotoxicity ± SD. Data are from 3 mice in each group, pooled from two independent experiments. g Bar graph showing the log2 transformed fold change of genes regulated in opposite ways by T-BET and EOMES, between controls and T-BET or EOMES deficient mice, as indicated. h–j Bar graphs showing the mean ± SD of *CD27* (h), *CD69* (i) and *DNAM-1* (j) expression in NK cells of the indicated genotype as measured by flow cytometry. N = 3. Unpaired t tests (two-tailed) were used for statistical analysis of data presented in this figure.
Fig. 6 EOMES-specific role in the induction of hallmark NK cell genes. RNA-seq analysis of sorted $Tbx21^{-/-}$, $Eomes^{-/-}$ and appropriate control CD11B− and CD27− NK cells ($N = 3$ per group with three sorts in total). DEGs were selected based on adjusted $p < 0.05$. a Genes specifically regulated by EOMES in immature or mature NK cells. Bar graph show the log2 transformed fold change between controls and EOMES deficient NK cells, as indicated. A few selected gene names are shown. b Functional annotation of the EOMES induced gene set using Metascape. Bar graphs show selected terms among the most significant ones. c–e Overlayed FACS histograms showing the expression of the indicated surface molecules in EOMES deficient vs control immature NK cells, as indicated. Grey histograms show control staining. f Heatmaps showing the log2 transformed fold change in the expression of the indicated TF between control and EOMES deficient NK cells. g The indicated subsets were subjected to scRNA-seq analysis as detailed in the methods. The heatmap shows the correlation between averaged profiles per sample, calculated on the top 100 most variable genes.
**T-BET-specific role in the repression of pluripotency and cell proliferation.** T-BET specific genes represent a large cluster of genes, with two thirds of them being repressed by T-BET and the other ones being induced by this TF (Fig. 7A for selected genes, and Supplementary Data 1 for the complete list). A functional annotation of T-BET repressed genes retrieved "cell cycle" and related terms as the most significant ones (Fig. 7B). "Adaptive immunity", "hematopoietic cell lineage", "T cell activation" and "myeloid cell differentiation" were other significant terms, which reflects the ability of T-BET to repress progenitor/pluripotency genes or genes expressed in other immune lineages. For example, T-BET strongly repressed the expression of Tcf7 or that of CD3 subunits. Gz2, encoding for GM-CSF was also specifically repressed by T-BET and not changed by EOMES.

Gene repression could be direct or indirect via other TFs induced by T-BET. Indeed, the most significant functional term associated with T-BET induced genes was "negative regulation of gene expression, epigenetic". Indeed, T-BET induced the expression of a large cluster of histone subunits (Hsit1h1c, Hsit1h1e, Hsit1h3a) or histone modification enzymes (Pufi, Hdac5, Ezh1, Epc1, Fig. 7C). Moreover, T-BET specifically induced the expression of many transcriptional repressors (Fig. 7C), some of which already known to regulate NK cell maturation such as Zeb2⁴⁶ and BLIMP (encoded by Pdml)⁴⁵, and some others like Pogk or Sertad1 with no described role in NK cells.

The functional analysis of T-BET-induced genes (Fig. 7B) also retrieved "IL-12 pathway" and "signaling by interleukins", which reflects the ability of T-BET to endow NK cells with specific response to cytokines. In particular, T-BET induced the expression of IL-12 and IL-21 receptors. As a consequence, Tbx21⁻/⁻ NK cells had a defective ability to respond to IL-12 as measured by STAT4 phosphorylation (Fig. 7D), and Tbx21⁻/⁻ but not Eomes⁻/⁻ had an impaired IFNγ secretion in response to IL-12/18 stimulation (Fig. 7E, F). "Macroautophagy" was also associated with T-BET-induced genes (Fig. 7B) while several terms related to anabolic pathways were associated with T-BET-repressed genes. This correlates with the known role of autophagy in NK cell development and survival⁴⁶, and with the increased apoptosis observed in the absence of T-BET¹⁹ (Fig. 3).

Importantly, even though T-BET is highly expressed in NK cells, we cannot exclude that some of the gene deregulations observed in Tbx21⁻/⁻ NK cells could be due to a role for T-BET in the NK cell environment. To address this possibility, we generated BM chimeric mice by transplanting a 1:1 mix of BM cells from Ly5a (CD45.1) and Tbx21⁻/⁻ mice (CD45.2) into sub-lethally irradiated Ly5aC57Bl/6 (CD45.1/2) mice. Eight weeks after transfer, we performed a 23-parameter flow cytometry characterization of Ly5a vs Tbx21⁻/⁻ NK cells in chimeric mice, and compared the results with those obtained when NK cells were characterized in individual donor mice (i.e., Ly5a vs Tbx21⁻/⁻). As shown in Supplementary Fig. 7, a strong correlation was observed between the expression ratios measured for each parameter in WT vs Tbx21⁻/⁻ mice and those in BM chimeric mice, suggesting that most of the effect of T-BET in NK cells is cell intrinsic.

**EOMES and T-BET share most of their DNA binding sites.** To complement the RNA-seq analysis, we sought to identify T-BET and EOMES binding sites in NK cells. In an effort to decrease experimental variability and avoid a bias linked to the use of different antibodies, we generated two mouse models allowing ChIP-seq analysis of T-BET and EOMES using the same antibody. In these models, an HA-V5 encoding tag was inserted at the 3’ end of Tbx21 and Eomes coding sequences (Fig. 8A, see "Methods"). Flow cytometry and WB analyses validated the expression of tagged T-BET and EOMES in NK cells even though HA-V5 tags decreased the expression of both EOMES and T-BET to some extent (Supplementary Fig. 8A, B). NK cells were present in normal frequency in both HA-V5-tagged mouse strains (Supplementary Fig. 8C), but the HA-V5 tag affected NK cell maturation in T-BET-HA-V5 mice (Supplementary Fig. 8D). The latter effect was, however, much less important than that observed in Tbx21⁻/⁻ mice, and we therefore proceeded with ChIP-seq of EOMES and T-BET using an HA antibody in freshly isolated NK cells from both models. We verified that EOMES and T-BET could be pulled down by the HA antibody using EOMES and T-BET western blotting (Supplementary Fig. 8E). T-BET were not co-immunoprecipitated upon EOMES pull down and vice versa (Supplementary Fig. 8E) suggesting that these TFs did not interact with each other, at least in resting NK cells.

The ChIP-seq experiment was performed on two replicates of each genotype and on C57Bl/6 NK cells as a control, and the peak calling was performed relative to the control cells. We controlled the similarity of both replicates for each ChIP-seq (Supplementary Fig. 9A). Both T-BET and EOMES were found to be often bound to putative promoter or intron regions and less frequently to more distant sites relative to transcriptional start sites (Fig. 8B). We found comparable numbers of binding sites for EOMES and T-BET (5682 and 4774, respectively, Fig. 8C). About half of the peaks were shared between both TFs (Fig. 8C) according to the ChIP-seq peak calling algorithm. The same proportions were kept when assigning genes to each peak using the nearest TSS method (Fig. 8D). This suggested that T-BET and EOMES compete for many of their binding sites. This conclusion was even more evident when visualizing a heatmap of the union of all EOMES and T-BET peaks (Supplementary Fig. 9B) or the mean profile of these peaks (Supplementary Fig. 9C) suggesting that most binding sites were in fact shared between T-BET and EOMES. Importantly, the control ChIP-seq yielded only background values at the position of T-BET and EOMES binding sites (Supplementary Fig. 9B, C), which validated the specificity of our ChIP-seq analysis. Moreover, EOMES and T-BET peaks identified in our study were also identified in previous studies in NK or activated CD8 T cells that used anti-EOMES or anti-T-BET antibodies (Figs. 9D, E). Finally, we scanned EOMES and T-BET ChIP-seq peaks with the FIMO tool⁴⁷ for occurrences of the T-box motif and found it in 973 EOMES peaks and 786 T-BET peaks (about 20% in each case). To confirm this point in an unbiased manner, we then performed de novo detection of TF-binding motifs under T-BET and EOMES ChIP-seq peaks using the MEME-chip motif discovery suite⁴⁷. As expected, the canonical T-box motif was enriched under both EOMES and T-BET peaks, albeit more significantly for EOMES (Fig. 8E, F). Other motifs were also commonly enriched under EOMES and T-BET peaks, such as RREB1, RUNX1/3, SP1, and PBX3 motifs. However, there were also some EOMES-specific motifs, such as Fli1, previously identified to regulate NK cell development⁴⁴ and T-BET-specific motifs, such as STAT4, which operates downstream of IL-12 and is a known epigenetic regulator of NK cell activation⁵⁰.

We then combined the ChIP-seq analysis with our RNA-seq results in an attempt to identify direct T-BET and EOMES target genes. Genes associated with T-BET or EOMES binding were in general much more expressed than those without binding (Fig. 8G), and co-binding did not influence further this expression. Among genes for which we identified a nearby EOMES ChIP-seq peak, 79 were differentially expressed between WT and NK-Eomes⁻/⁻ NK cells (Fig. 8H). This group of functional EOMES targets was in most cases (58/79, or 73%) induced by EOMES (Supplementary Fig. 10A and full list in Supplementary Data 3), and notably contains many genes...
Fig. 7 T-BET-specific role in the repression of pluripotency and cell proliferation. RNA-seq analysis of sorted Tbx21−/−, Eomes−/− and appropriate control CD11B− and CD27− NK cells (n = 3 per group with three sorts in total). DEGs were selected based on adjusted p < 0.05. 

**a** Genes specifically regulated by T-BET in mature CD27− NK cells. Bar graphs show the log2 transformed fold change between controls and T-BET deficient mice, as indicated. A few selected gene names are shown. 

**b** Functional annotation of the T-BET-repressed and T-BET-induced genesets using Metascape. Bar graphs show selected terms among the most significant ones. 

**c** Transcription factors, histone subunits or histone modifying enzymes whose expression are dependent on T-BET. The heatmap shows the log2 transformed fold change in expression between control and T-BET deficient mature NK cells. 

**d** Flow cytometry analysis of STAT4 phosphorylation in WT vs T-BET deficient NK cells of the indicated subset in response to stimulation with IL-12 for 1 h. Graphs show the MFI ± SD. Data are from 3 mice representative of two experiments. 

**e, f** Flow cytometry analysis of intracellular IFNγ expression in gated spleen NK cells of the indicated genotype following culture in medium supplemented or not with IL-12 and IL-18. Bar graphs show the mean percentage of IFNγ positive NK cells ± SD. N = 6 mice in 2 experiments. Unpaired t tests (two-tailed) were used for statistical analysis of data presented in this figure.

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the number of genes analyzed is indicated in the expression (RNA-seq data) of genes bound by T-BET and/or EOMES (Chip-seq data). Expression of genes without any of them is shown as reference. The EOMES-regulated genes (RNA-seq).

Among genes for which we identified a nearby T-BET ChIP-seq peak, 225 were differentially expressed between WT and Tbx21+/− mature NK cells (Fig. S1, Supplementary Fig. 10C and Supplementary Data 3). A total of 97 of them corresponded to T-BET-induced genes and notably include TF (Zeb1, Blimp1, Id2, Irf8), many cytokines and cytokine receptors (Ifng, Il18r1, Il2r1, Il12r2b) but also genes involved in cytotoxicity (Fasl, Gzma, Gzmb, Prf1) or in trafficking (Slpr5). Examples of ChIP-seq tracks for direct T-BET targets are shown in Supplementary Fig. 10D.

Finally, we sought to determine if EOMES and T-BET binding was associated with chromatin remodeling. To address this question, we re-analyzed a set of published ATAC-seq data for NK cell precursors (NKp), BM immature NK cells (iNK) and spleen NK cells (mature or mNK) [13]. We then compared ATAC-seq profiles at EOMES or T-BET ChIP-seq peak positions between all three conditions. Results in Supplementary Fig. 11A, B show that ATAC profiles are on average much more intense in iNK or mNK compared to NKp at these positions, indicating opening of the chromatin during NK cell differentiation at sites of EOMES or T-BET occupancy. Next, we performed a footprinting analysis of the ATAC-seq data using the TOBIAS tool [51] to identify TF binding motifs in ATAC-seq “footprints”. Using TOBIAS, we performed differential TF binding analysis between NKp and iNK or mNK and mNK. For both comparisons, the most
significant differential binding was obtained for a cluster of T-box factors, that were enriched in iNK or mNK compared to NKp, EOMES and T-BET being part of this cluster (Supplementary Fig. 11C, D). When comparing iNK to mNK (Supplementary Fig. 11E), this cluster was not differential, suggesting that most of the chromatin remodeling induced by T-box factors occurs at the NKp to iNK transition, i.e., when EOMES is dominant over T-BET.

**Discussion**

Here, we provide molecular insight on how EOMES and T-BET control NK cell development and maturation. Using a variety of methods, we show that EOMES is dominantly expressed in immature NK cells and that deficiency in EOMES but not T-BET strongly affects the transcriptome of immature NK cells. In addition, we found that T-BET deficiency does not affect the number of immature NK cells, while EOMES deficiency resulted in a severe decrease of this subset and of other consecutive stages. Mechanistically, our data demonstrate that EOMES is essential to specify the NK cell lineage by inducing the expression of many hallmark genes important to provide survival signals (such as NK cell receptors) and by promoting optimal responsiveness to IL-15. In the absence of EOMES, immature NK cells were more apoptotic, confirming recent findings. However, they did not convert into ILC1s, as shown by the scRNA-seq analysis.

We showed that T-BET was dominantly expressed in mature NK cells, and was critical for terminal NK cell maturation, which confirms previous observations. We also found that EOMES and T-BET balance each other’s expression at the protein level, which is important to mitigate the maturation rate. Indeed, in the absence of EOMES, residual NK cells tend to differentiate much more quickly than control NK cells. Reciprocally, the absence of T-BET resulted in the accumulation of DP NK cells. Thus, the EOMES to T-BET ratio is a crucial rheostat of NK cell maturation. Subtle changes in this equilibrium could have important consequences on the representation of maturation subsets.

T-BET acted more often as a repressor in NK cells, suppressing the transcriptional program of immature NK cells. This could be linked to its ability to induce the expression of known repressors such as Zeb2 and Blimp1, and of many enzymes, histones subunits and complexes involved in epigenetic reprogramming, as shown in the present study. We previously described that ZEB2-deficient and T-BET-deficient NK cells were phenocopies, and that ZEB2 overexpression partly compensated for T-BET deficiency in NK cells. Altogether, this suggests that T-BET induction of transcriptional repressors such as ZEB2 is essential to suppress the expression of genes expressed at previous developmental stages i.e., pluripotency, progenitors or immature NK cells. Our study points to many potentially novel transcriptional regulators downstream of T-BET such as POGK that contains a Krüppel-associated box domain (KRAB) repressor domain, or SERTAD1, a member of the Trip-Br family of TF, known to control cell proliferation. The indirect effect of T-BET via transcriptional repressors may also explain why there is overall a limited overlap between genes regulated by T-BET and EOMES, despite the fact that T-BET and EOMES DNA binding largely intersects. Interestingly, a previous analysis of EOMES and BRACHYURY actions in embryonic stem cells also showed that EOMES was rather involved in the induction of hallmark mesoderm genes, while BRACHYURY rather repressed neuroectoderm genes. Thus, division of labor between different T-box TFs may operate to favor stepwise differentiation in different tissues.

EOMES regulated the expression of very few TFs, with the notable exception of Bhlhe40 and Hobit. BHLHE40 could be especially important for NK cell metabolism downstream of EOMES, as suggested by a recent study in memory CD8+ T cells. In this study, Bhlhe40−/− memory T cells had a diminished expression of genes encoding the components of the mitochondrial membrane or genes involved in mitochondrial metabolism and/or OXPHOS, and a decreased oxygen consumption rate. Hobit has been previously reported to control tissue residency in lymphocytes, in cooperation with BLIMP1. As NK cells are the only ILCs capable of circulating in the blood vasculature and the only ILCs to express EOMES, we hypothesized that EOMES promoted blood circulation by repressing Hobit. However, we found little impact of EOMES deficiency on the ability of residual NK cells to exit the BM or LN, as opposed to the effect of T-BET that was much pronounced, especially in mature NK cells. This effect could be mediated by S1PR5, which inducibly contributes to NK cell trafficking, as we previously showed. Why ILC1s are tissue-resident and S1PR5 negative despite high levels of T-BET remains to be determined. Additional factors, such as ZEB2 that is induced at late stages of NK cell differentiation may be important to open the S1pr5 locus. T-BET was also necessary for the survival of mature CD27− NK cells, which correlated with the T-BET dependent induction of the “macropathoagy” pathway as captured by the functional annotation of the T-BET dependent geneset. Autophagy is known to be absolutely essential for NK cells and more generally for all ILC types. Glycolysis and oxidative phosphorylation are known to operate at very low intensity in NK cells and these pathways are even down regulated at the transcriptional level during NK cell differentiation. In this context, autophagy could be especially important to preserve NK cell integrity in quiescent mature NK cells. Autophagy is negatively regulated by the mTOR pathway, and we showed that activity of both mTORC1 and mTORC2 complexes was indeed progressively decreased during NK cell maturation. More recent studies have shown an interesting link between mTOR complexes and T-box factors. Indeed, deletion of Raptor, the essential subunit of mTORC1 resulted in a specific decrease of EOMES expression while deletion of Rictor had the reciprocal effect on T-BET. The latter effect could be through the posttranscriptional regulation of FOXO1 that is known to negatively regulate T-BET expression in NK cells. How mTORC1 specifically regulates EOMES and how the mTORC1/mTORC2 equilibrium is controlled during maturation remain open questions.

We generated novel T-BET and EOMES knock-in mice with the same HA-V5 insertion at the C-terminus of each factor. We anticipate that such tools will be very useful to study the role of both TFs in different contexts. The study of epigenetic regulation is indeed hampered by the limited availability of antibodies against TFs, and by the low expression of many of them. Using these tools, we identified a very strong overlap between EOMES and T-BET binding, which confirms a previous study in activated CD8+ T cells. However, the latter study used different antibodies for T-BET and EOMES, and in vivo activated CD8+ T cells vs in vitro activated CD8+ T cells as material for T-BET and EOMES ChIP-seq respectively, which could arguably lead to technical artifacts. Our study is thus the first comparison of genome-wide DNA binding by T-BET and EOMES using untouched primary lymphocytes.

Most T-BET and EOMES peaks were not associated with differential expression of the corresponding genes in T-BET or EOMES deficient NK cells, suggesting that other factors may compensate for the lack of T-BET or EOMES binding in most cases. This point is not specific of T-box TFs as similar
conclusions have been reached for many other TFs, as previously reviewed [46]. This binding is not necessarily equal to regulation, and it is probable that only a small fraction of all binding events have an important impact on gene expression.

As the EOMES/T-BET balance switches during maturation from dominance of EOMES in immature NK cells to dominance of T-BET in mature NK cells, we propose that in many occurrences and for direct T-box targets, EOMES comes first and binds T-box motifs to initiate transcription of NK cell genes. Then, when the level of T-BET increases and that of EOMES decreases, T-BET would come and replace EOMES. Even though this model requires formal demonstration, it would explain why many genes such as Pfy1 or Gzma are apparently co-induced by EOMES and T-BET. T-BET binding could either have the same effect as EOMES on gene expression (cases of Pfy1, Gzma, Sipr5, Il7r, etc.), or alternatively have an opposed effect (cases of Cd27, Cxcr3, Cd69, Dnai1 etc.). Opposed effects of T-BET and EOMES may involve secondary effectors as discussed above, or alternatively, different cofactors.

EOMES and T-BET were found to regulate mostly distinct gene sets, even though they bind essentially to the same genomic sites. This discrepancy could be linked to their different window of activity i.e., immature NK cells for EOMES versus mature NK cells for T-BET. Conceivably, transcriptional and epigenetic changes during maturation may indeed largely influence the outcome of TF binding. Another non-exclusive hypothesis could be that T-BET and EOMES-specific cofactors could influence how the binding of each TF influences gene transcription. The motif discovery analysis that we performed suggested that STAT4 and FLI1 could be preferential T-BET and EOMES cofactors respectively. More studies will be needed to formally address this point.

By reanalyzing a published set of ATAC-seq data we found large epigenetic changes that occur at sites of T-BET or EOMES binding during differentiation from progenitors to immature NK cells. As EOMES is dominant in immature NK cells, especially in the BM where T-BET is hardly expressed, this suggests that most epigenetic changes occurring during early NK cell specification are induced by EOMES. Moreover, we applied a novel computing tool (i.e., Tobias) that can analyse “footprints” in ATAC-seq data caused by TF binding. Using this tool, we confirmed the major impact of T-box TFs in NK cell differentiation from progenitors. Interestingly we did not uncover differential binding of T-box TFs between immature and mature NK cells, which likely reflects the strong impact of EOMES and T-BET on gene transcription in immature and mature NK cells, respectively.

In conclusion, our study reveals a major role for EOMES in NK cell lineage specification and induction of NK cell hallmark genes such as NK cell receptors and genes associated with cytotoxicity. Reciprocally, we found that T-BET promotes terminal NK cell maturation and survival via the repression of lineage and pluripotency genes indirectly via the induction of different repressors and the induction of specific properties such as responsiveness to IL-12. T-BET and EOMES binding is largely overlapping despite the specific effect of each TF on gene expression. This suggests that stage-specific epigenetic marks or specific co-factors influence the outcome of T-box binding on gene regulation. Our study also points to T-box TFs and especially EOMES as major drivers of epigenetic changes during NK cell differentiation.

**Methods**

**Mice.** Wild-type C57BL/6 J mice were purchased from Charles River Laboratories (L’Arbresle, Stock No: 009664, Ncr1-CreERT2, Eomes<cre/lox> and Tbx21<−/−> mice were previously described. This study was carried out in accordance with the French recommendations in the Guide for the ethical evaluation of experiments using laboratory animals and the European guidelines 86/609/CEE. The bioethic local committee CECAPP and the French Ministry of research approved all experimental studies involving live mice (protocol APAF1#297865)

T-BET and EOMES expression upon cytokine stimulation. 2 × 10⁵ splenocytes from C57BL/6 mice were prepared and then were cultured for 24 h with/without following cytokines IL-12 (25 ng/ml), IL-18 (5 ng/ml) and IL-15 (100 ng/ml). Cells were collected and were stained with anti-NK1.1, anti-CD3, followed by intracellular staining with anti-T-BET and anti-EOMES before analysis by flow cytometry.

**Immunostaining with confocal analysis.** Freshly sorted NK cells were seeded in a 96-Well Optical-Bottom Plate pre-coated with 100 μg/ml poly-L-lysine for 1 h incubation at 37 °C. After 4% PFA fixation for 15 min and three PBS washes, blocking step (PBS 3% BSA) for 30 min at RT was followed. Immunostainings were performed after a permeabilization step with 0.05% Triton X-100 for 7 min. Primary antibody antibodies were diluted in 3% BSA-PBS and added to the cell for one-hour incubation at room temperature. Primary antibodies used in this study include rat anti-CD122 (Bio X Cell, 4 μg/ml), rabbit anti-EOMES (Abcam, 4 μg/ml), FITC-conjugated mouse anti-T-BET (Biotrend, 1/50). After three washes with PBS, cells were incubated with the appropriate Alexa 555 conjugated anti-rat, AF647 conjugated anti-rabbit secondary antibody at 2 μg/ml in 3% BSA-PBS and add the cells to along with DAPI (4 μg/ml) for one-hour incubation at room temperature. After three gentle washes with PBS, cells were observed with a Zeiss LSM 800 laser scanning confocal microscope. The images and relative quantification were processed using Image J software.

**Imaging combined with flow cytometry analysis.** Sorted NK cells were prepared and stained with appropriate antibodies as described in “Flow cytometry”. The well-stained cells were then analyzed by Image Stream X technology (Amnis) at magnification x40 using IDEAS software. Mean fluorescence intensity (MFI) of T-BET and EOMES were analysed by applying masks (IDEAS software) to discriminate nuclear and cytoplasmic area based on the DAPI staining.

**Measurement of in vivo cell proliferation.** Mice were given two continuous intraperitoneal injection of 0.5 mg EdU (BD Biosciences) 12 h after the last EdU injection, mice were killed and organs harvested. Cells derived from BM and spleen were stained with mAb as described in “Flow cytometry”. After fixation and permeabilization, cells were stained with FITC anti-EdU antibody, according to manufacturer instructions. EdU incorporation for different cell populations was measured by flow cytometry. In some experiments Ly5a mice were injected with Cell Trace-Violet (CTV, Thermo-Fischer, used at 0.1 uM)-labelled spleen cells from C57BL6 mice or Tbx21<−/−> mice. Two weeks later, splen NK cells were purified and CTV dilution of transferred NK cells was monitored by flow cytometry.
IFN-γ production and degranulation upon cytokine stimulation. 1.5 × 10^6 splenocytes were cultured with Golgi-stop (BD Biosciences) in the presence of anti-CD107a (MPSH1, BD Biosciences) and phorbol myristate acetate (PMA, Sigma) and ionomycin (Ionomycin, Sigma) for 5 h with RMA-KR target cells (MHC class I deficient, 96-well plate with Rael1b positive) expressing the Nanoluciferase (lentivirus-mediated expression) at a concentration of 100 cells/well. Different ratio of NK to target cells were used: 30:1, 10:1, 3:1, 1:1, 0:1. After NK cell killing RMA-KR-derived NanoLuc was released in the culture supernatant. NanoLuc activity in the culture supernatant thus reflects target cell lysis. The total volume in culture wells was 200 μL, and plates were centrifuged briefly for 4 min at 500 x g. 50 μL of culture supernatants was collected, and NanoLuc activity was determined by adding 50 μL of NanoLuc reagent in black, flat-bottom, 96-well plates. Bioluminescence was measured for 0.1 s with a luminometer (TECAN).

**Cell cytotoxicity assay.** Sorted NK cell subsets were plated in 96-well, V-bottom plates and co-cultured for 4 h with RMA-KR target cells (MHC class I deficient, 96-well plate with Rael1b positive) expressing the Nanoluciferase (lentivirus-mediated expression) at a concentration of 100 cells/well. Different ratio of NK to target cells were used: 30:1, 10:1, 3:1, 1:1, 0:1. After NK cell killing RMA-KR-derived NanoLuc was released in the culture supernatant. NanoLuc activity in the culture supernatant thus reflects target cell lysis. The total volume in culture wells was 200 μL, and plates were centrifuged briefly for 4 min at 500 x g. 50 μL of culture supernatants was collected, and NanoLuc activity was determined by adding 50 μL of NanoLuc reagent in black, flat-bottom, 96-well plates. Bioluminescence was measured for 0.1 s with a luminometer (TECAN).

**Generation of Tbet-HAV5/Knockin mice.** A homologous recombination plasmid was designed to target the mouse Tbet gene. Using Red/ET cloning (Gen Bridges), we first replaced Tbet stop codon in a bacterial artificial chromosome (BAC) containing Tbet genomic sequence (clone number RP24-161F21; CHORI) by a 2xHAV5-STOP-loxP-IACE-Cre-PKG-g2b-neo-loxP cassette. We then retrieved the cassette with flanking Tbet genomic sequence spanning 3.5 kb upstream and 3 kb downstream of original stop codon. Standard procedure was used to generate genetically modified MJamA3 ES cells and mice (refer to Zomey-HAV5 K1 mice procedure). The following primers were used for genotyping the animals: Ex5-F2 and Ex6-R2 (Supplementary Table 2). The knock-in fragment size is 509 bp and the WT fragment size is 356 bp.

**Chlp-seq.** For sample preparation, 10 million NK cells from pooled spleens of HAV5-tagged T-BET and EOMES mice were isolated and crosslinked with 1% formaldehyde for 10 min at 37 °C. Crosslinking was stopped with 0.125 M glycine, then washed twice with ice-cold Wash Buffer including detergent (Active Motif, 35042). Flash freeze the cell pellet by immersing tubes into dry ice for 10 min. Resuspend each pellet in 5 ml ice-cold Swelling Buffer (Active Motif, 35042) supplemented with protease inhibitor cocktail (PIC) and PMSF for 30 min on ice. Pellets were then suspended in 3 ml Swelling Buffer. An equal volume of Wash Buffer (50 mM Tris-HCl [pH 8) supplemented with PIC and PMSF and incubated on ice for 10 min. Chromatin was then sheared using a Probe Sonicator device (Active motif) to obtain a fragments size range between 200 and 1000 bp. After clearance by centrifugation at 4 °C, sheared chromatin was used for immunoprecipitation of HA (4 μg, CST, cat. no. 2383) or normal rabbit IgG control (4 μg, Diagenode, cat. number C15410260) incubated overnight at 4 °C. Protein G magnetic-activated beads (Active Motif, catalog number 53034) were added to each immunoprecipitation reaction and incubate for 3 h at 4 °C. Each IP was washed five times with Wash Buffer (Active Motif, 35042) and chromatin-antibody complexes were eluted with Elution Buffer AM4. Chromatin was then reverse crosslinked and DNA was purified according to the manufacturer’s instructions (Active motif). Before sequencing, we pooled three Chlp samples for the same mice. Paired-end sequencing 2 x 100 bp was performed on HiSeq 4000 (Illimina) at the GenomEast platform.

**Bio-informatic analysis.** RNA-seq. Reads were processed using an in-house RNA-seq pipeline of GenomEast facility. Briefly, raw data were preprocessed using cutadapt (v 1.9.4) in order to remove primers and low-quality reads and then aligned to Wash Buffer including detergent (Active Motif, 35042) supplemented with PIC and PMSF. For cell transfer, 2 × 10^4 to 4 × 10^5 sorted cells were plated in 96-well, V-bottom plates and co-cultured for 4 h with RMA-KR target cells (MHC class I deficient, 96-well plate with Rael1b positive) expressing the Nanoluciferase (lentivirus-mediated expression) at a concentration of 100 cells/well. Different ratio of NK to target cells were used: 30:1, 10:1, 3:1, 1:1, 0:1. After NK cell killing RMA-KR-derived NanoLuc was released in the culture supernatant. NanoLuc activity in the culture supernatant thus reflects target cell lysis. The total volume in culture wells was 200 μL, and plates were centrifuged briefly for 4 min at 500 x g. 50 μL of culture supernatants was collected, and NanoLuc activity was determined by adding 50 μL of NanoLuc reagent in black, flat-bottom, 96-well plates. Bioluminescence was measured for 0.1 s with a luminometer (TECAN).

**Single cell RNA-seq.** Single cell RNA-seq was performed using SingleCell Discoveries, Utrecht, Netherlands using a SORT-seq protocol. Sequencing data were then processed. Low quality genes were removed based on a transcript per cell cutoff and data normalization for sequencing depth per cell and log-transformation. The most variable genes in the dataset were identified and the top 2000 was used for dimensionality reduction and clustering. Principal component analysis was done on the 2000 variable genes to identify the dimensionality in the dataset and the most relevant principal components are select for downstream analysis (dimensionality reduction and clustering). The first 8 principal components were selected for analysis, based on the final distribution of the samples in TSNE and UMAP space. Correlations between samples were calculated on the profiles averaged per sample.

**Generation of EOMES-HAV5/Knockin mice.** A homologous recombination plasmid was designed to target the mouse Eomes gene. A targeting vector containing Homology sequences to the Eomes locus and an irs-GFP-loxP-IACE-Cre-PKG-g2b-neo-loxP cassette was made previously. Using Red/ET cloning (Gen Bridges), we first replaced Tbet stop codon in a bacterial artificial chromosome (BAC) containing Tbet genomic sequence (clone number RP24-161F21; CHORI) by a 2xHAV5-STOP-loxP-IACE-Cre-PKG-g2b-neo-loxP cassette. We then retrieved the cassette with flanking Tbet genomic sequence spanning 3.5 kb upstream and 3 kb downstream of original stop codon. Standard procedure was used to generate genetically modified MJamA3 ES cells and mice (refer to Zomey-HAV5 K1 mice procedure). The following primers were used for genotyping the animals: Ex5-F2 and Ex6-R2 (Supplementary Table 2). The knock-in fragment size is 509 bp and the WT fragment size is 356 bp.
8. Hayakawa, Y. & Smyth, M. J. CD27 dissects mature NK cells into two subsets included in this article as Supplementary data 2. The data for all graphical in the Geo repository with the accession code GSE168242. The raw scRNA-seq data is

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Reads were mapped to the Mus musculus genome (assembly mm10) using Bowtie v1.0.0 with default parameters except for “-m 1 –strata –best”.

ATAC-seq datasets. The following published datasets were downloaded from GEO or SRA in SRA format and converted to FASTQ format using the fastq-dump program in the sratoolkit (version 2.1.9).

GEO identifier Sample name Condition

These data were analyzed using the Encode ATAC-seq pipeline v1.5.1. Briefly, bowtie v2.3.4.3 was used to align reads to the reference mouse genome mm10/GRCh38 and MACS2 v2.2.4 was used to call OCs. Footprinting analysis of ATAC-seq data was performed using the suite of tools TOBIAS. Briefly, TOBIAS ATACCorrect corrects Tn5 insertion bias, TOBIAS ScoreBigwig calculates footprint scores within regulatory regions, TOBIAS BINDetect estimates bound/unbound transcription factor binding sites. Known motifs were from the JASPAR CORE (2020) for vertebrate non-redundant database.

Statistical analysis. Statistical analyses were performed using Prism 5 (Graph-Pad Software). Two tailed unpaired t test, paired t test, and ANOVA tests with Bonferroni correction were used as indicated in the figure legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability ChIP-seq and RNA-seq data that support the findings of this study have been deposited in the Geo repository with the accession code GSE168242. The raw scRNA-seq data is included in this article as Supplementary data 2. The data for all graphical representations in this article are included in the Source Data File. Additional relevant information can be obtained by contacting the authors. New reagents and mouse lines are available upon request. Source data are provided with this paper.

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Competing interests

The authors declare no competing interests.

Additional information

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