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Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin

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Two seemingly unrelated hallmarks of memory CD8+ T cells are cytokine-driven proliferative renewal after pathogen clearance and a latent effector program in anticipation of rechallenge. Memory CD8+ T cells and natural killer cells share cytotoxic potential and dependence on the growth factor interleukin 15. We now show that mice with compound mutations of the genes encoding the transcription factors T-bet and eomesodermin were nearly devoid of several lineages dependent on interleukin 15, including memory CD8+ T cells and mature natural killer cells, and that their cells had defective cytotoxic effector programming. Moreover, T-bet and eomesodermin were responsible for inducing enhanced expression of CD122, the receptor specifying interleukin 15 responsiveness. Therefore, these key transcription factors link the long-term renewal of memory CD8+ T cells to their characteristic effector potency.

Successful eradication of intracellular pathogens and viruses depends on proper functioning of the cell-mediated immune system. The key effector cells of this immunity are type 1 CD4+ T cells, CD8+ T cells and natural killer (NK) cells. A characteristic common to each of these lineages is secretion of the cytokine interferon-γ (IFN-γ). In addition, cytotoxic CD8+ T cells and NK cells lyse target cells through the elaboration of perforin and granzymes. The T-box transcription factor T-bet, encoded by *Tbx21*, has been proposed to be a 'master regulator' of cell-mediated immunity capable of controlling the expression of genes encoding effector molecules (called 'effector genes' here) of CD4+ and CD8+ T cells, as well as NK cells[1–6].

In addition to regulating the effector genes of cell-mediated immunity, T-bet functions in the maturation and homeostasis of NK T cells (NKT cells) and NK cells[6] and may also contribute to the induction of CD8+ T cell memory[4,5,7]. T-bet seems to have a nonredundant function in T helper type 1 (T_H1) cell differentiation *in vitro* and *in vivo*[3]. Despite its possible involvement in the development and function of the cytotoxic lineages, there seems to be a substantial T-bet-independent component of CD8+ T cell and NK cell effector function and homeostasis[3–9].

Eomesodermin (Eomes) is a T-box transcription factor that is highly homologous to T-bet and is expressed in activated CD8+ T cells as well as resting and activated NK cells[6,8]. Overexpression studies and antagonism using dominant negative factors have suggested that Eomes and T-bet might have cooperative or redundant functions in regulating the genes encoding IFN-γ and cytolytic molecules in CD8+ T cells[8]. Whether Eomes is truly essential for T-bet-independent control of cell-mediated immunity has not been tested using mice with compound mutations. Homozygous deletion of *Eomes* results in early embryonic lethality[10]. We therefore tested whether cooperative or redundant regulation of effector function by these T-box factors could be inferred from the phenotype of compound mutant $Eomes^{+/-}Tbx21^{-/-}$ mice.

We report that mice with compound mutations in the genes encoding T-bet and Eomes had additive defects in effector gene expression by CD8+ T cells and NK cells. In addition, $Eomes^{+/-}Tbx21^{-/-}$ mice had considerable depletion of interleukin 15 (IL-15)–dependent lineages, including NK cells and memory phenotype CD8+ T cells. Moreover T-bet and Eomes were necessary and sufficient for enhanced expression of CD122, the receptor that confers cellular responsiveness to IL-15.

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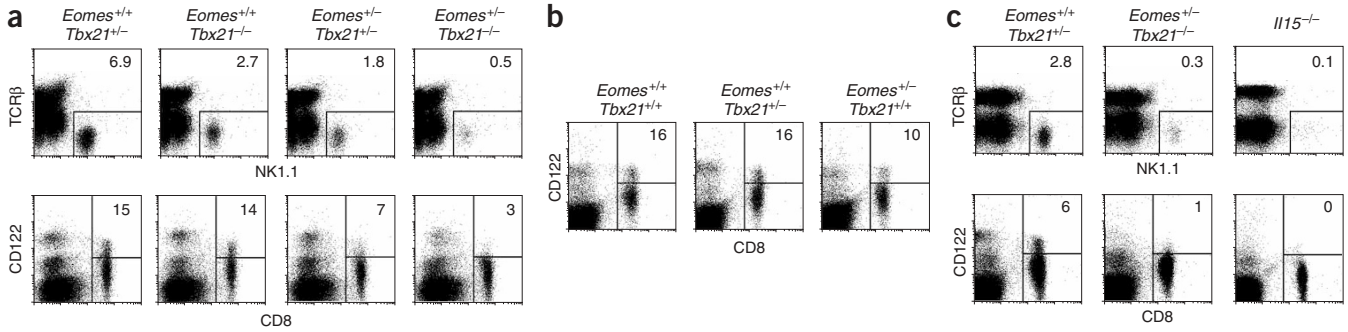

Figure 1 *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice have considerable depletion of IL-15-dependent lineages. (**a**) Flow cytometry of NK cells and memory CD8$^+$ T cells in freshly isolated peripheral blood (top row) and splenocyte samples (bottom row) from 16-week-old littermates (genotypes, above plots). Numbers in dot plots indicate percentage of NK cells among all events (top row) or percentage of CD8$^+$ cells that are CD122^{hi} (bottom row). Results are representative of more than ten similar experiments with comparable results using blood, spleen and lymph nodes. (**b**) Flow cytometry of memory CD8$^+$ T cells from peripheral blood of 16-week-old littermates (genotypes, above plots) derived from interbreeding of *Eomes*$^{+/-}$ and *Tbx21*$^{+/-}$ mice. Results are representative of five similar experiments. (**c**) Flow cytometry of NK cell (top row) and memory CD8$^+$ T cell (bottom row) populations in 6-week-old littermate *Eomes*$^{+/+}$*Tbx21*$^{+/-}$ and *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice and age-matched *Il15*$^{-/-}$ mice. Results are representative of three similar experiments. Numbers in dot plots in **b**,**c** indicate percentages as in **a**.

These results extend an emerging framework in which key transcription factors that specify lineage function can also specify the responsiveness to proprietary growth signals. They also provide a molecular link between the programming of effector and memory CD8$^+$ T cells.

RESULTS

Eomes$^{+/-}$*Tbx21*$^{-/-}$ mice are phenotypically similar to *Il15*$^{-/-}$ mice

To maximize the relevant littermate cohorts for an allelic series of *Eomes* and *Tbx21* compound mutants, we adopted the primary breeding strategy of mating *Tbx21*$^{-/-}$ mice with *Eomes*$^{+/-}$*Tbx21*$^{+/-}$ mice; this generated *Tbx21*$^{+/-}$, *Tbx21*$^{-/-}$, *Eomes*$^{+/-}$*Tbx21*$^{+/-}$ and *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ littermates. To complete the 'allelic series', we used separate matings to compare wild-type mice with simple heterozygous mutations of *Tbx21* and *Eomes*.

After generating the mutant mice, we unexpectedly found that the lymphoid tissues of *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice had considerable depletion of NK cells (**Fig. 1a**). A previous line of *Tbx21*$^{-/-}$ mice is nearly devoid of NKT cells and has a substantial but incomplete defect in the maintenance of peripheral NK cells[6]. Our analyses of the allelic series of compound mutants showed that the *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice had a similar defect in NKT cell development as that of *Tbx21*$^{-/-}$ mice (**Fig. 1a**). The defect in the NK cell compartment in *Tbx21*$^{-/-}$ mice, however, was exacerbated considerably by the loss of one allele of *Eomes* (**Fig. 1a**). Mice with simple heterozygous mutations also had defects in NK and NKT cell composition. Loss of one allele of *Tbx21* eliminated most NKT cells, whereas loss of one allele of *Tbx21* or *Eomes* caused a partial NK cell defect (**Supplementary Fig. 1** online).

In addition to a considerable loss of NK cells, *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice had depletion of memory phenotype CD8$^+$ T cells with high expression of CD122, the β-chain of the IL-2 receptor (IL-2R) and IL-15R (**Fig. 1a**). This memory defect was not obvious from our analysis of simple *Tbx21*$^{-/-}$ mice, although it has been reported that the response of *Tbx21*$^{-/-}$ mice to viral infection is characterized by a moderate loss in the establishment of virus-specific memory cells[5] and impaired protection from vaccination[4,7]. By examining the littermates derived from an intercross of *Eomes*$^{+/-}$ and *Tbx21*$^{+/-}$ mice, we found that *Eomes*$^{+/-}$ mice had a small but detectable defect in memory phenotype CD8$^+$ T cells, whereas *Tbx21*$^{+/-}$ mice closely resembled wild-type littermates (**Fig. 1b**). *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice consistently had the most substantial defect, indicating that both T-box factors contributed to the homeostasis of memory CD8$^+$ T cells (**Fig. 1a**,**b**).

The considerable loss of NKT, NK and memory phenotype CD8$^+$ T cells resulting from the elimination of a single allele of *Eomes* on a *Tbx21*-null background was reminiscent of the phenotypes of mice

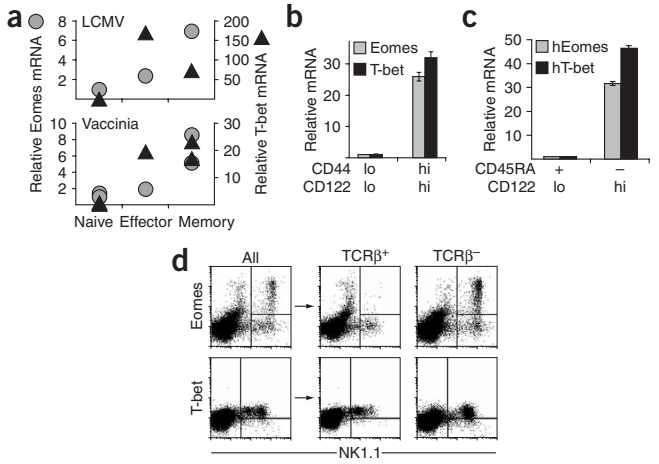

Figure 2 Eomes and T-bet are expressed in IL-15-dependent lineages. (**a**) Quantitative real-time RT-PCR of Eomes and T-bet mRNA. TCR-transgenic effector cells (days 8 and 6) and memory cells (days 100+ and 50+) were sorted from infected mice (LCMV and vaccinia, respectively). Data are one of three representative LCMV experiments. Two naive (T-bet values superimposed), one effector and two memory cohorts from vaccinia infection are presented. Data represent mean of triplicate determinations; s.e.m. values are all less than 12%. (**b**) Real-time RT-PCR of Eomes and T-bet mRNA of CD8$^+$ T cells from 16-week-old C57BL/6 mice sorted into CD44^{lo}CD122^{lo} (naive) and CD44^{hi}CD122^{hi} (memory) subsets. Results are representative of three identical experiments. Values for real-time PCR throughout represent mean ± s.e.m. of triplicate determinations, normalized to HPRT values. (**c**) Real-time RT-PCR of CD8$^+$ T cells from a healthy human donor sorted into CD45RA$^+$CD122^{lo} (naive) and CD45RA$^-$CD122^{hi} (memory) subsets. Similar results were obtained from two donors, hEomes, human Eomes; hT-bet, human T-bet. (**d**) *Eomes* reporter activity (enzymatic detection) and intranuclear T-bet (monoclonal antibody detection) in splenocytes from *Eomes*$^{+/-}$ mice. Results are representative of five experiments.

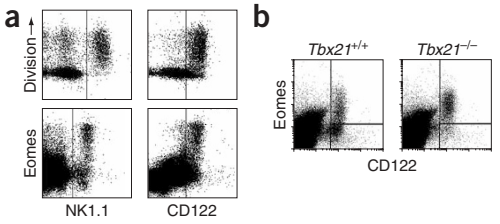

Figure 3 T-box factors coincide with CD122 and IL-15 responsiveness. (**a**) Top, analysis of cell division (vertical axis) and markers (horizontal axis) of splenocytes from *Eomes*$^{+/-}$ mice, cultured in recombinant IL-15 (without TCR ligation) for 6 d and labeled with bodipy red succinimidyl ester (fluorescence intensity inverted so that the direction of division parallels the intensity of Eomes expression below). Bottom, direct analysis of *Eomes* reporter activity and markers of freshly isolated splenocytes from *Eomes*$^{+/-}$ mice loaded with fluorescein digalactoside. (**b**) Direct analysis of *Eomes* reporter activity and CD122 expression of bone marrow cells isolated from *Eomes*$^{+/-}$*Tbx21*$^{+/+}$ or *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice. Results are representative of three experiments.

with alterations in the IL-15 signaling pathway, both IL-15 deficient[11] and IL-15Rα deficient[12], that have been characterized before. We therefore directly compared littermate *Tbx21*$^{+/-}$ mice and *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice with age-matched *Il15*$^{-/-}$ mice. Indeed, the phenotype of *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice approximated, albeit incompletely, the phenotype of *Il15*$^{-/-}$ mice in the depletion of NKT, NK and memory phenotype CD8$^+$ T cells (**Fig. 1c**). We obtained similar results comparing these mice with IL-15Rα-deficient mice (data not shown). The incomplete nature of the defect in *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice might be a consequence of the hypomorphic or 'partial knockdown' approach, which does not eliminate all the pertinent T-box factor alleles that could participate in the IL-15 pathway. In contrast, *Il15*$^{-/-}$ mice have a complete loss of IL-15 signaling. In conclusion, the allelic series of *Eomes* and *Tbx21* compound mutants showed that combining homozygous deletion of *Tbx21* with heterozygous deletion of *Eomes* approximated the features of IL-15 signaling mutants, with considerable loss of NKT cells, NK cells and memory phenotype CD8$^+$ T cells.

T-box factors in IL-15-dependent lineages

Eomes is expressed in effector CD8$^+$ T cells[8]. Consistent with the loss of circulating cytotoxic lineages in *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice, we found that T-bet and Eomes were expressed in both antigen-specific effector and memory T cells from mice infected with lymphocytic choriomeningitis virus (LCMV) and vaccinia virus (**Fig. 2a** and **Supplementary Fig. 2** online). In addition, circulating memory phenotype CD8$^+$ T cells had higher expression of both T-box factors in mice (**Fig. 2b**) and humans (**Fig. 2c**) than did naive CD8$^+$ T cells. Pathogen-specific or endogenous memory cells had higher expression of Eomes than did pathogen-specific effector cells. T-bet mRNA was highest, however, in effector cells and was either stabilized or decreased in memory cells, although it remained higher than in naive cells (**Fig. 2a**,**b** and **Supplementary Fig. 2** online).

The mutant allele of *Eomes* we used here encoded β-galactosidase under control of *Eomes* regulatory elements[10], enabling discrimination of *Eomes* 'reporter gene' activity using flow cytometry. In addition, we used a technique of intranuclear monoclonal antibody staining to measure T-bet expression by flow cytometry[1]. We found both Eomes and T-bet were expressed in NK cells negative for T cell receptor-β (TCRβ$^-$; **Fig. 2d**). In contrast, T-bet but not Eomes was expressed in TCRβ$^+$ NKT cells (**Fig. 2d**), as suggested before[6]. These patterns of gene expression were therefore very consistent with the genetic data. T-bet alone was expressed in NKT cells, and T-bet seemed to be a nonredundant regulator of NKT cell development, given that deletion of an *Eomes* allele did not exacerbate the defect found in *Tbx21*$^{-/-}$ mice. This paralleled the function of T-bet in CD4$^+$ T_H1 differentiation. T_H1 cells expressed T-bet alone, and deletion of an *Eomes* allele did not exacerbate the T_H1 defect found in *Tbx21*$^{-/-}$ mice (data not shown). In contrast, T-bet and Eomes were both expressed in NK cells and memory CD8$^+$ T cells, and both factors seemed to conjointly regulate the homeostasis of the cytotoxic lineages, given that compound mutations exacerbated the defects of the simple mutants (**Fig. 1**).

T-box factor expression correlates with the 'CD122^{hi} state'

A common feature of cells with high expression of Eomes or T-bet (memory CD8$^+$ T, NK and NKT cells) was enhanced expression of CD122, the shared β-chain of IL-2R and IL-15R (**Fig. 2**). Both *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ and *Il15*$^{-/-}$ mice were deficient in these CD122^{hi} cell lineages (**Fig. 1**). Although basal CD122 expression is required for IL-2 signaling[13], enhanced CD122 expression (**Supplementary Fig. 3** online), called the 'CD122^{hi} state' here, confers IL-15 responsiveness on cells, accounting for the selective loss of CD122^{hi} cells in IL-15- and 15Rα-deficient mice[14–18]. In contrast to the function of CD122, which specifies cytokine responsiveness in *cis*, IL-15Rα is more critical in *trans*-presentation of IL-15 (refs. 16–21). With single-cell discrimination of *Eomes* reporter activity, the specificity of Eomes for the CD122^{hi} state was emphasized by the finding that the NK1.1

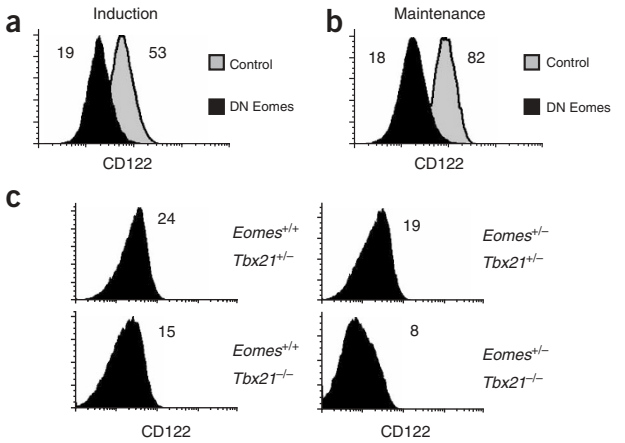

Figure 4 Eomes and T-bet are necessary for the induction and maintenance of the CD122^{hi} state. (**a**,**b**) Surface expression of CD122 of cells transduced with control retrovirus or dominant negative Eomes retrovirus (DN Eomes) containing the drosophila Engrailed repression domain in place of the endogenous *trans*-activation domain[8]. Numbers in plots indicate mean fluorescence intensity. (**a**) Naive P14 TCR-transgenic CD8$^+$ T cells were stimulated and then, 5 d after transduction, transduced cells were analyzed. (**b**) Memory phenotype (CD44^{hi}CD122^{hi}) CD8$^+$ T cells were sorted from older (over 6 months of age) wild-type mice and were stimulated and then, 4 d after transduction, transduced cells were analyzed. The reduction in CD122 expression caused by dominant negative Eomes approximates the basal expression in naive cells (**Supplementary Fig. 3** online). (**c**) CD122 induction in naive T cells (mouse genotypes, right margin) stimulated for 3 d before analysis. Numbers in plots indicate mean fluorescence intensity of CD122 staining on gated CD8$^+$ lymphocytes. Results are representative of two experiments.

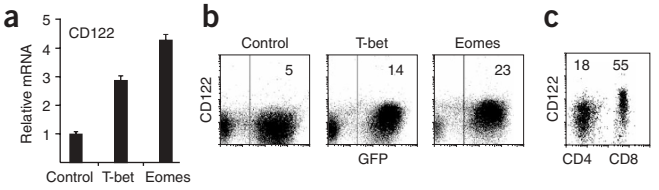

Figure 5 Eomes and T-bet are sufficient to induce the CD122^{hi} state. (**a**) Real-time RT-PCR of CD122 mRNA. CD4$^+$ T cells were stimulated in T_H2 conditions and were transduced with indicated bicistronic retroviruses; 6 d after stimulation, transduced cells were sorted for analysis. (**b**) Surface CD122 expression in developing T_H2 cells transduced for 6 d with retroviruses (above plots). Numbers in plots indicate mean fluorescence intensity of CD122 staining on GFP$^+$ cells. (**c**) CD122 staining of CD4$^+$ (left) or CD8$^+$ (right) effector and/or memory cells (CD44^{hi}). Multiparameter flow cytometry shows staining of CD8 (horizontal axis) and CD122 (vertical axis) of freshly isolated lymphocytes. Events are from a gate encompassing only CD44^{hi}CD4$^+$ cells plus CD44^{hi}CD8$^+$ cells. Numbers in plots indicate mean fluorescence intensity of CD122 staining. Results are representative of two experiments.

marker only partially delineated splenocytes capable of dividing in response to IL-15 and expressing Eomes (**Fig. 3a**). In contrast, the CD122^{hi} state marked almost all cells capable of dividing in response to IL-15 and expressing Eomes or T-bet (**Fig. 3a** and data not shown).

We also analyzed the extent to which T-bet and Eomes expression associated with the CD122^{hi} state throughout hematopoiesis by examining bone marrow from *Eomes* reporter mice on a T-bet-deficient background. The number of CD122^{hi} cells in the bone marrow of *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice was relatively normal, in contrast to the considerable peripheral defect (**Fig. 3b**; data not shown), suggesting that one allele of *Eomes* may be sufficient to develop but not sustain the IL-15-responsive lineages in a steady-state way. In the presence of T-bet, almost all Eomes-expressing cells in the bone marrow were CD122^{hi}, yet many CD122^{hi} cells did not have high expression of Eomes (**Fig. 3b**). In the absence of T-bet, however, most CD122^{hi} cells had high expression of Eomes (**Fig. 3b**). We found the same near-complete concordance between Eomes expression and the CD122^{hi} state in the peripheral lymphoid organs of *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice, although we recovered very few CD122^{hi} cells (data not shown). These results suggest that it may not be possible to separate the CD122^{hi} state (that is, IL-15 responsiveness) from the expression of Eomes or T-bet. In the absence of T-bet, CD122^{hi} lineages seemed to be lost (for example, NKT cells) or to be 'forced' to express Eomes.

T-box factors are required for the CD122^{hi} state

The near-invariant association of Eomes and T-bet with the CD122^{hi} state could be explained by several mechanisms: a common factor regulates Eomes, T-bet and CD122 in parallel; a CD122-mediated signal induces Eomes and T-bet; or Eomes and T-bet intrinsically induce the CD122^{hi} state. Our analysis yielded substantial support for the third possibility. We found that Eomes and T-bet were probably required for the establishment and maintenance of the CD122^{hi} state. We used two complementary approaches to test the requirement for the T-box factors in the generation of enhanced CD122 expression. First we introduced a dominant negative form of Eomes, containing a drosophila Engrailed repression domain in place of the endogenous *trans*-activation domain[8,22,23], via retroviral gene transfer into CD8$^+$ T cells. We found that dominant negative Eomes, which antagonizes the function of both Eomes and T-bet[8], blocked induction of the CD122^{hi} state in developing effector cells generated by *in vitro* stimulation of naive P14 transgenic CD8$^+$ T cells (**Fig. 4a**). In addition, dominant negative Eomes inhibited the maintenance of the CD122^{hi} state in memory phenotype CD8$^+$ T cells from older mice (**Fig. 4b**).

In the second approach, we assessed the induction of CD122 in newly activated naive CD8$^+$ T cells from compound mutant mice. Naive CD8$^+$ T cells from *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice activated *in vitro* showed defective induction of CD122 compared with that of cells from littermate control mice (**Fig. 4c**) or *Il15*$^{-/-}$ mice (**Supplementary Fig. 4** online). Despite the apparently critical function of Eomes and T-bet in controlling the CD122^{hi} state (**Fig. 4**), basal CD122 expression was probably regulated independently of Eomes and T-bet, as indicated by the uniform intensity of CD122 staining on naive CD8$^+$ T cells from the various mutants (**Fig. 1a**). Eomes and T-bet, therefore, seemed to be critical for inducing the CD122^{hi} state in naive cells that were differentiating into effector cells and also for maintaining the CD122^{hi} state in mature memory cells.

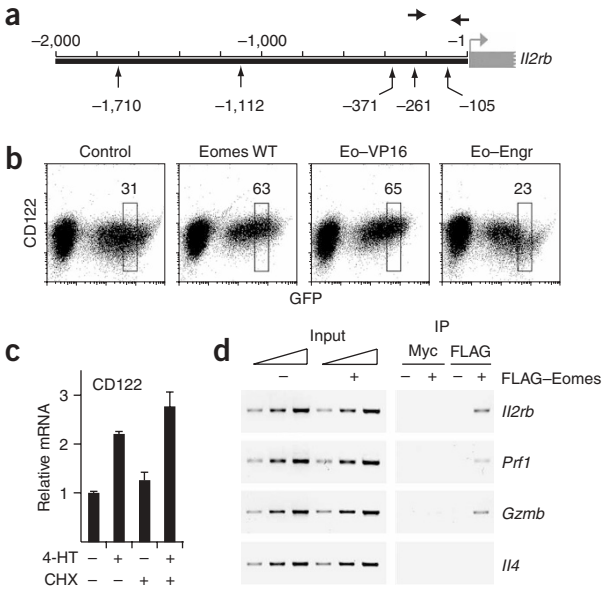

Figure 6 *Il2rb* is a direct 'target' of Eomes. (**a**) The upstream region of *Il2rb* contains T-box elements. Vertical arrows mark five potential motifs, with seven-eighths nucleotide identity to the core recognition element of xenopus Eomes[25]; horizontal arrows mark sites of PCR primers used in **d**. Numbers indicate nucleotide positions relative to the transcription start site. (**b**) Surface CD122 expression in developing T_H2 cells transduced for 4 d with retroviruses (above plots). The VP16 variant of Eomes (Eo–VP16) contains a strong activation domain in place of the endogenous *trans*-activation domain; the Engrailed variant of Eomes (Eo–Engr) is identical to the dominant negative Eomes (**Fig. 4a**). WT, wild-type. Numbers above boxed areas indicate mean fluorescence intensity of CD122 staining. (**c**) PCR of CD122 mRNA in developing T_H2 cells transduced with Eomes–ER and, 5 d later, treated for 6 h with various combinations (below graph) of 4-hydroxytamoxifen (4-HT) to induce Eomes and with cycloheximide (CHX) to inhibit protein synthesis. (**d**) Chromatin immunoprecipitation assay. Developing T_H2 cells were transduced with a FLAG-tagged Eomes fusion retrovirus and, 7 d later, transduced (+) and untransduced (–) cells were analyzed with anti-FLAG to detect enrichment of chromatin associated with tagged Eomes at the promoter (genes, right margin). *Prf1*, perforin; *Gzmb*, granzyme B. Right, immunoprecipitation (IP) with anti-Myc (negative control). PCR of immunoprecipitated samples is compared with threefold dilutions (wedges) of DNA before immunoprecipitation (input). Results are representative of two separate experiments.

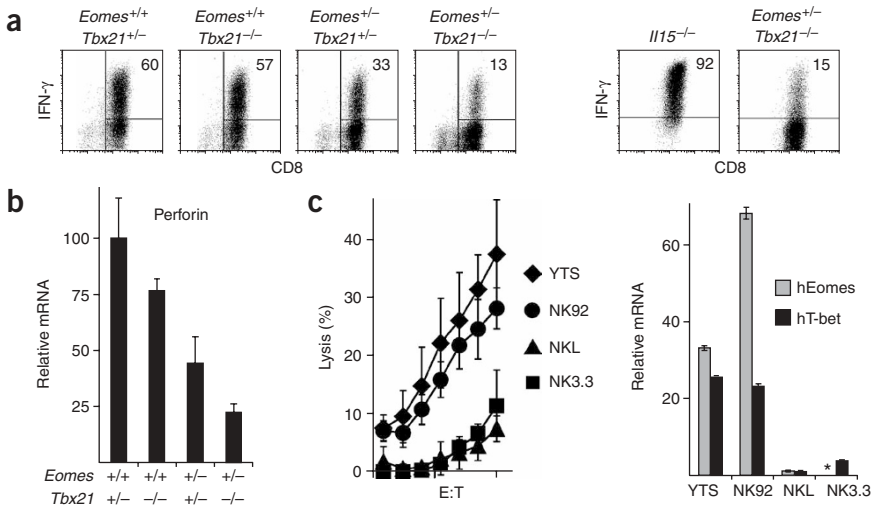

Figure 7 Eomes and T-bet coordinate effector function in parallel with their coordination of IL-15 responsiveness. (**a**) Expression of CD8 and IFN-γ in CD8$^+$ T cells from littermates, positively selected from splenocyte suspensions and stimulated with immobilized anti-CD3 plus anti-CD28 and recombinant IL-2 for 3 d before analysis (left), or in T cells from age-matched *Il15*$^{-/-}$ and *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice stimulated for 3 d with soluble anti-CD3 plus anti-CD28 and recombinant IL-2 (right). Numbers in dot plots indicate percentage of CD8$^+$ cells expressing IFN-γ. Identical patterns of IFN-γ induction were obtained with phorbol 12-myristate 13-acetate and ionomycin (shown here) or anti-CD3 (data not shown) for restimulation. (**b**) RT-PCR of perforin mRNA in sorted NK cells. NK1.1$^+$TCRβ$^-$ cells were sorted from bone marrow (genotypes, below graph). Bone marrow NK cells were used because of the scarcity of peripheral NK cells in *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice. Results are representative of two separate experiments. (**c**) Left, cytolytic activity of four human NK lines tested against ^{51}Cr-loaded target cells (E:T, effector/target). Right, RT-PCR of human Eomes (hEomes) and human T-bet (hT-bet) mRNA. *, undetectable. Results represent mean ± s.e.m. of three separate experiments.

T-box factors are sufficient for the CD122^{hi} state

In addition to their apparent requirement for enhanced CD122 expression, the two T-box factors might be sufficient for induction of the CD122^{hi} state in a nonpermissive cell type. Ectopic expression of T-bet or Eomes retrovirus in developing T$_H$2 cells, which do not normally express T-bet or Eomes, induced increased CD122 mRNA expression, as measured by real-time PCR (**Fig. 5a**). To determine whether the T-box factors could induce substantial surface protein expression, we also performed flow cytometry on transduced T$_H$2 cells (**Fig. 5b**). Both Eomes and T-bet enhanced CD122 expression relative to that of cells transduced with control retrovirus. The induction of CD122 as assessed by mean fluorescence intensity (2.8-fold by T-bet and 4.6-fold by Eomes; **Fig. 5b**) resembled the characteristic difference in CD122 expression that distinguished effector and/or memory (CD44^{hi}) CD8$^+$ T cells from CD4$^+$ T cells (3.1-fold; **Fig. 5c**). Although Eomes and T-bet may require cooperation from another factor expressed in T cells to achieve induction, the addition of either T-box factor alone was able to induce the CD122^{hi} state in a nonpermissive cell type.

Il2rb, a direct target of Eomes

Given the results of loss-of-function and gain-of-function approaches, our findings suggested that Eomes and T-bet might be necessary and sufficient for enhanced CD122 expression and IL-15 responsiveness throughout the immune system (**Figs. 3–5**). Those results were consistent with the published finding that *Tbx21*$^{-/-}$ NKT cells are defective in CD122 expression, which can be restored by reintroduction of T-bet via retroviral gene transfer[6]. In contrast, loss of T-bet did not perturb CD122 expression in NK cells[6] or CD8$^+$ T cells (**Figs. 1a** and **4c**), which could be attributable to the expression of Eomes in those lineages (**Fig. 2**). We therefore sought evidence for the ability of Eomes and T-bet to *trans*-activate *Il2rb*, the gene encoding CD122.

Sequence inspection of the promoter region[24] of *Il2rb* disclosed five potential Eomes recognition sites[25] within 2 kilobases of the transcriptional start site (**Fig. 6a**), indicating that the T-box factors could potentially act directly at the *Il2rb* locus. We also obtained evidence that Eomes could be functioning to directly activate *Il2rb* rather than indirectly repressing a negative regulator of *Il2rb*. We analyzed a set of Eomes variants in which the endogenous *trans*-activation domain of Eomes[23] was replaced by either a strong activation domain (from the herpes simplex virus protein VP16)[26] or a strong repression domain (from the drosophila Engrailed protein)[22]. When transduced into developing T$_H$2 cells, the dominant activating form resembled the wild-type Eomes construct, whereas the dominant repressing form acted in the opposite way (**Fig. 6b**). Eomes is therefore more likely to be functioning as an activator of *Il2rb* than an indirect repressor.

To obtain more direct biochemical evidence supporting the idea that Eomes activates *Il2rb*, we used two independent strategies. Although the VP16 variant of Eomes induced CD122, it could have acted indirectly by inducing another activator, which would have required new protein synthesis for translation of the intermediate gene product. If this were the case, induction of CD122 mRNA by Eomes would be inhibited by the drug cycloheximide, which impairs protein synthesis. Instead, we found that *trans*-activation of *Il2rb* by Eomes did not require new protein synthesis (**Fig. 6c**). We transduced developing T$_H$2 cells with a retrovirus encoding a fusion protein of Eomes and the estrogen receptor ligand-binding domain (Eomes–ER), which becomes activated after the addition of 4-hydroxytamoxifen. After allowing the transduced cells to 'rest', we treated them for 6 h with 4-hydroxytamoxifen in the presence or absence of cycloheximide to inhibit new protein synthesis. The dose of cycloheximide used inhibited induction of CD122 protein, assessed at later time points (data not shown). However, induction of CD122 mRNA by Eomes–ER was insensitive to cycloheximide, consistent with the potential of Eomes to act directly at the *Il2rb* locus without synthesizing an intermediate activator (**Fig. 6c**).

To determine whether Eomes could bind directly to the *Il2rb* promoter, we constructed an epitope-tagged Eomes retrovirus and introduced the construct into developing T$_H$2 cells. We fixed FLAG-tagged Eomes-expressing and untransduced cells and immunoprecipitated chromatin-associated proteins with antibody to the FLAG epitope or to an irrelevant Myc epitope. Immunoprecipitation of FLAG-tagged Eomes specifically enriched for chromatin at the *Il2rb* promoter, as judged by semiquantitative gene-specific PCR amplification. Binding of Eomes at *Il2rb* was comparable to its binding at two other putative targets of Eomes and T-bet, the genes encoding perforin and granzyme B[6,8] (**Fig. 6d**). In contrast, FLAG-tagged

Eomes did not specifically associate with chromatin at the *Il4* promoter (**Fig. 6d**). These findings suggest that Eomes may directly *trans*-activate *Il2rb*.

Eomes and T-bet jointly regulate cytotoxicity

Our initial reason for generating compound mutant mice was to determine whether Eomes is responsible for the T-bet-independent control of effector gene expression that is pertinent to cell-mediated immunity[3–5,9]. We found that $CD8^+$ T cells from *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice activated *in vitro* had defective IFN-γ induction, a phenotype distinct from that of *Tbx21*$^{-/-}$ mice (**Fig. 7a**) and reminiscent of the introduction of dominant negative T-bet or dominant negative Eomes into T-bet-deficient $CD8^+$ T cells[8]. That result suggested that the combined actions of Eomes and T-bet comprise a nonredundant pathway controlling effector differentiation of $CD8^+$ T cells, diminishing the concern that T-box factors other than Eomes and T-bet were being inhibited in prior studies using dominant negative retroviruses[8].

Although *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice were phenotypically similar to IL-15-deficient mice in loss of the IL-15-responsive lineages, we found that the defect in effector function in compound T-box factor mutants was not shared by IL-15 pathway mutants (**Fig. 7a**). This result suggests that effector molecule defects in *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice are not simply a secondary consequence of defective IL-15 signaling. Instead, the T-box factors may 'program' IL-15 responsiveness and effector function in parallel rather than in series.

The other main effector arm of cell-mediated immunity is cytoxicity, which seems to be most characteristic of cells that express both Eomes and T-bet (NK and $CD8^+$ T cells). Consistent with a causal function in specifying the effector program, we noted progressive loss of perforin mRNA in bone marrow NK cells from compound mutant *Eomes* and *Tbx21* mice (**Fig. 7b**). We also found ample correlative support for this model from our analysis of human cells. Expression of Eomes and T-bet in human $CD8^+$ T cell subsets mirrored the expression of perforin and granzyme B as well as CD122 (**Supplementary Fig. 5** online). Likewise, we found that the cytolytic capacity of four human NK lines positively correlated with expression of Eomes and T-bet (**Fig. 7c**). Thus, Eomes and T-bet seem to cooperate in specifying IL-15 responsiveness and the spectrum of effector functions for cell-mediated immunity in mice and humans.

DISCUSSION

Several studies have established the importance of T-bet in regulating the differentiation and effector function of the cell-mediated immune system[1–6]. T-bet has a critical and nonredundant function in the differentiation and function of T_H1 cells, at least in part through regulating the expression of IFN-γ[1–6] and IL-12Rβ2 (refs. 2,27,28). T-bet also contributes to the expression of IFN-γ, perforin and granzyme B in cytotoxic $CD8^+$ T and NK cells[4,6,8]. A substantial portion of the effector function of $CD8^+$ T cells and NK cells, however, is preserved in the absence of T-bet[3–9].

The paralogous T-box factor eomesodermin has been suggested to regulate the T-bet-independent control of cytotoxic effector function[8]. We therefore generated mice with compound mutations of *Tbx21* and *Eomes* to investigate their relative contributions to cytotoxic effector function. Our results have indicated that $CD8^+$ T cells and NK cells from mice with compound mutations of *Eomes* and *Tbx21* had defects in effector gene expression, which became progressively more substantial as more alleles of the T-box genes were inactivated. Those findings provide solid genetic evidence that T-bet and Eomes function in a cooperative and partially redundant way to regulate effector gene expression of the cytotoxic lineages.

Our analysis of *Eomes*$^{+/-}$*Tbx21*$^{-/-}$ mice has also demonstrated an unanticipated phenotype distinct from their defective cytotoxic 'programming': the loss of almost all IL-15-dependent cell lineages from their peripheral lymphoid organs. In this way, the compound T-box mutants were phenotypically similar to IL-15 mutants, lacking NKT cells, NK cells and memory $CD8^+$ T cells. We noted a tight association between expression of Eomes or T-bet and high expression of CD122, the receptor dictating the ability of NKT cells, NK cells and memory $CD8^+$ T cells to respond to IL-15. The unexpected phenotype was explained by the observations that the T-box factors seemed to be necessary and sufficient for enhanced expression of CD122 and that they were capable of directly *trans*-activating *Il2rb*, which encodes CD122.

These results extend the finding that the terminal maturation of NKT and NK cells is regulated by T-bet[6]. T-bet-deficient mice have a near-complete absence of NKT cells, whereas NK cell homeostasis is only partially defective. These findings are consistent with the distinct and overlapping expression patterns of Eomes and T-bet. Eomes is not expressed in NKT cells, rendering T-bet nonredundant in the regulation of NKT cell homeostasis[6]. Likewise, Eomes is not expressed in T_H1 cells and T-bet is indispensable in T_H1 lineage commitment[3]. In contrast, T-bet and Eomes have overlapping expression in NK cells and memory $CD8^+$ T cells and act in a partially redundant way in those lineages.

Cytokines frequently seem to act as critical regulators of both homeostasis and differentiation of immune cell lineages. Whether and how these seemingly distinct effects are coupled has remained enigmatic. Our data have shown that the development and maintenance of a cell lineage can indeed be coupled to its specialized functions by a cell-intrinsic mechanism. Our results suggest that this could be accomplished by a key transcription factor (or factors) that can simultaneously specify restricted responsiveness to a cytokine and specify a program of gene expression that mediates the 'signature' functions of the cell. Although deficiency in IL-15 is associated with loss of mature cytotoxic lineages, IL-15 might not be chiefly responsible for specifying the cytoxic program. Instead, Eomes and T-bet may regulate cytotoxic programming and, in parallel, make cytotoxic cells able to respond to IL-15 by inducing CD122.

This function for IL-15 is reminiscent of the function of IL-12 in driving T_H1 cell maturation. Although deficiency in IL-12 is associated with loss of T_H1 cells, IL-12 might not be chiefly responsible for specifying T_H1 cell lineage commitment; instead, T-bet seems to enact a program of T_H1 cell gene expression that includes induction of IFN-γ[1–6] and, in parallel, enhanced expression of IL-12Rβ2 (refs. 2,27,28), making developing T_H1 cells more responsive to further proliferation and differentiation mediated by IL-12 (refs. 2,27,29).

Likewise, in $CD8^+$ T cells and NK cells cytokines could still regulate cell function by positive feedback at genes encoding the T-box factors or through direct action at a cytotoxicity gene. Indeed, IL-15 seems to enhance T-bet expression in NK cells[6] and enhance effector function in $CD8^+$ T cells[30]. Future efforts should be directed toward determining what factors initiate the activation of Eomes and T-bet in NK cells and $CD8^+$ T cells and what function IL-15 or other cytokines might have in triggering or converging with the Eomes and T-bet pathways.

The induction of long-lived protective immunity against intracellular microbes and the eradication of chronic viral infection have remained elusive goals. The factors controlling the ontogeny, function and durability of memory $CD8^+$ T cells, as well as their precise lineage relationship, have remained controversial[31–41]. Two hallmarks of memory $CD8^+$ T cells are their capacity for rapid recall of effector function when re-encountering a pathogen and their ability to

undergo IL-15-driven proliferative renewal in the absence of pathogen attack[14,15,39,42–44]. The identification of T-bet and Eomes as key regulators of both effector genes and IL-15 responsiveness may thus serve as an initial step in understanding the intrinsic aspects of memory programming. Likewise, it will be important to determine whether 'pathological' programming of memory[33–35] is associated with dysregulated expression of Eomes and T-bet.

We do not yet know whether other aspects of the memory cell differentiation program, besides maintenance of the $CD122^{hi}$ state and effector potency, are regulated by Eomes and T-bet. The memory program also encompasses changes in a broad set of cellular pathways, including signal transduction, survival and apoptosis, cell cycle regulation, metabolism, nuclear function, and cytoskeletal and membrane organization[31,37,45–47]. Future efforts should be aimed at determining whether the broad set of memory gene expression changes are directly 'downstream' of Eomes and T-bet. Alternatively, Eomes and T-bet may simply control a relatively narrow set of memory genes, whereas the remainder of the gene expression could be the indirect effect of changes in cytokine responsiveness. An example of the latter scenario would be the induction of enhanced CD122 expression by Eomes and T-bet, with subsequent IL-15 signaling allowing the memory cell to escape replicative senescence via induction of telomerase[47].

Defining the precise function of these two T-box factors in hematopoiesis and the various phases of host defense will require complex conditional and temporal gene deletion. Homozygous deficiency of T-bet is already known to cripple T_H1 differentiation and the development of the NKT cell lineage. Eliminating one allele of *Eomes* on a T-bet-deficient background compounded the effector defects of NK cells and $CD8^+$ T cells and resulted in a considerable depletion of the mature cytotoxic lineages, NK cells and memory $CD8^+$ T cells. The unexpectedly deleterious phenotype resulting from the simple elimination of three of four alleles suggests that much of the development and persistence of an 'armed' cell-mediated immune system might be controlled by the combined actions of Eomes and T-bet.

METHODS

Mice. All animal work was done in accordance with Institutional Animal Care and Use Guidelines of the University of Pennsylvania, Emory University and Harvard University. C57BL/6 mice, OT-I TCR-transgenic mice (Jackson Laboratories), DO11.10 TCR-transgenic mice and P14 TCR-transgenic mice (Taconic Farms) were housed in specific pathogen–free conditions before use. *Eomes*$^{+/-}$ embryonic stem cells were generated as described[10] and mice with germline mutation were 're-derived' by injection of stem cells into C57BL/6 blastocysts. *Tbx21*$^{-/-}$ mice were generated by targeted deletion of exons 2–6 and were phenotypically identical to published *Tbx21*$^{-/-}$ mice[3] (data not shown). *Tbx21*$^{-/-}$ and *Eomes*$^{+/-}$ mice were backcrossed to C57BL/6 mice for four generations and were intercrossed to produce compound heterozygous mice. For most experiments, littermates from the mating of *Tbx21*$^{-/-}$ and *Eomes*$^{+/-}$ *Tbx21*$^{+/-}$ mice were used between 6 and 16 weeks of age. Seventh-generation backcross C57BL/6 *Il15*$^{-/-}$ mice were bred as homozygous knockouts and were used with age-matched *Eomes* and *Tbx21* compound mutant mice.

Viral infections. LCMV- and vaccinia-specific effector and memory $CD8^+$ T cells were generated as described[31,36,37]. $CD8^+$ T cells (2×10^5) from naive P14 TCR-transgenic mice (Thy-1.1^+) or OT-I TCR-transgenic mice (CD45.1^+) were transferred intravenously into nonirradiated C57BL/6 (Thy-1.2^+ CD45.2^+) recipients. Then, 1 d later, recipients were infected with 2×10^5 plaque-forming units (PFU) LCMV Armstrong strain by intraperitoneal injection or with 5×10^6 PFU recombinant vaccinia virus expressing chicken ovalbumin (VV-OVA). LCMV-specific effector (day 8) and memory (day 100+) P14 $CD8^+$ T cells were sorted from pooled spleens on the basis of Thy-1.1 expression. VV-OVA-specific effector (day 6) and memory (day 50+) OT-I $CD8^+$ T cells were sorted from pooled spleens on the basis of CD45.1 expression. For analysis of endogenous memory cells, C57BL/6 mice were infected with either LCMV or VV-OVA as described above. H-$2D^b$ NP396 and H-$2D^b$ GP33 tetramers were used to sort LCMV-specific memory $CD8^+$ T cells (day 45+). H-$2K^b$ SIINFEKL tetramer was used to sort VV-OVA-specific memory $CD8^+$ T cells.

Mouse cell culture and stimulation. Polyclonal T cell stimulation was done as described[2,8,28]. For $CD8^+$ T cell cultures, CD4-depleted splenocyte samples (3×10^6 cells/ml) from naive mice were stimulated with soluble monoclonal antibody to CD3 (monoclonal anti-CD3; 1 μg/ml; BD Biosciences), monoclonal anti-CD28 (0.5 μg/ml; BD Biosciences) and human recombinant IL-2 (100 U/ml). In some cases, $CD8^+$ T cells from naive mice were purified with microbeads coated with anti-CD8α (Miltenyi Biotec), followed by positive selection with an autoMACS Separator (Miltenyi Biotec). Purified $CD8^+$ T cells were stimulated with immobilized anti-CD3 (5 μg/ml), soluble monoclonal anti-CD28 (2 μg/ml) and human recombinant IL-2 (200 U/ml). Splenocytes from DO11.10 or P14 TCR-transgenic mice were stimulated with peptide (0.2 μg/ml; ovalbumin amino acids 323–339 or LCMV glycoprotein amino acids 33–41, respectively) and human recombinant IL-2 (40 U/ml for DO11.10 and 100 U/ml for P14), using resident splenocytes as antigen-presenting cells. For T_H2-polarizing conditions, recombinant IL-4 (5 U/ml), anti-IL-12 (5 μg/ml; BD Biosciences) and anti-IFN-γ (5 μg/ml; BD Biosciences) were added to cultures. For IL-15-induced proliferation experiments, freshly isolated splenocytes were labeled for 6 d with the fluorescent dye bodipy red succinimidyl ester (Molecular Probes) and then were cultured with IL-15 (100 ng/ml; Peprotech).

Retroviral transduction and constructs. Bicistronic retroviruses expressing T-bet, Eomes or dominant negative Eomes upstream of an internal ribosomal entry sequence and green fluorescent protein were constructed and prepared as described[2,8,28]. The VP16 variant of Eomes was constructed like dominant negative Eomes: the N-terminal region, including the T-box domain, of Eomes (amino acids 1–522) was fused to the *trans*-activation domain of herpes simplex virus protein VP16 (amino acids 410–490)[26]. The Eomes–ER construct was also generated by PCR-based mutagenesis. The nucleotide sequence encoding a modified hormone-binding domain (amino acids 281–599) of mouse estrogen receptor was fused to the 3′ end of the *Eomes* codons. For activation of Eomes–*ER*, 200 nM 4-hydroxytamoxifen (Sigma-Aldrich) was used. Preliminary confirmation of the activity of this construct that could be regulated by the drug was demonstrated by its ability to induce IFN-γ expression in developing T_H2 cells only in a 4-hydroxytamoxifen-dependent way (data not shown). Cycloheximide (10 μg/ml; Sigma-Aldrich) was used to inhibit protein synthesis in the Eomes–ER experiment. The FLAG epitope–tagged Eomes construct was generated by PCR-based mutagenesis for insertion of two sequential FLAG epitopes upstream of the *Eomes* codons. Retroviral transduction was done as described[2]. At 24 h after stimulation, T cells were resuspended in retroviral supernatant containing polybrene (8 μg/ml; Sigma-Aldrich) and were centrifuged at 6,000*g* for 90 min at 25 °C. After spin infection, cells were cultured in fresh media containing the same cytokine milieu used in the initial stimulation.

Flow cytometry. Surface staining, intracellular cytokine staining and flow cytometry were done as described[2]. Antibodies used for flow cytometry were purchased from BD Biosciences. For flow cytometry of *Eomes* reporter activity, *Eomes*$^{+/-}$ cells were loaded by hypotonic shock with fluorescein digalactoside according to the manufacturer's instructions (Molecular Probes). For intranuclear detection of T-bet, fixed cells were made permeable with 0.15% Triton-X and 1% FBS in PBS and were stained with fluorescein isothiocyanate–conjugated monoclonal anti-T-bet (Santa Cruz Biotechnology).

Quantitative RT-PCR. Total RNA was extracted with Trizol Reagent (Invitrogen). RNA (1 μg) was reverse-transcribed with random hexamer primers. An ABI PRISM 7900HT Sequence Detection System was used for quantitative real-time PCR according to the manufacturer's instructions (Applied Biosystems). The following primer and probe sets were used for sequence-specific detection (with the dyes 6-carboxyrhodamine (VIC), N,N,N′,N′-tetramethyl-6-carboxyrhodamine (TAMRA) and 6-carboxyfluorescein (6FAM)): hypox-

anthine guanine phosphoribosyl transferase (*HPRT*) sense, 5′-CTCCTCAGA CCGCTTTTTGC-3′, antisense, 5′-TAACCTGGTTCATCATCGCTAATC-3′, and probe VIC-5′-CCGTCATGCCGACCCGCAG-3′-TAMRA; mouse *Eomes* sense, 5′-TGAATGAACCTTCCAAGACTCAGA-3′, antisense, 5′-GGCTTGAGGCAAA GTGTTGACA-3′, and probe, 6FAM-5′-AGAAGTTTTGAACGCCGTACCGA CCTCCA-3′-TAMRA; mouse *Tbx21* sense, 5′-CCTGCAGTGCTTCTAACACAC AC-3′, antisense, 5′-CTCCGCTTCATAACTGTGTTC-3′, and probe, 6FAM-5′-TGTCCCCAGCCAGTAAGGCTGTGAG-3′-TAMRA; mouse (perforin) *Prf1* sense, 5′-GCAGCTGAGAAGACCTATCAGGAC-3′, antisense, 5′-TCTGAGCGC CTTTTTGAAGTC-3′, and probe 6FAM-5′-GTACCAGGCGAAAACTGTACAT GCGACACTCT-3′-TAMRA. Presynthesized Taqman Gene Expression Assays (Applied Biosystems) were used to amplify the following sequences (gene symbols and Applied Biosystems primer set numbers in parentheses): mouse CD122 (*Il2rb*; Mm00434264_m1), mouse granzyme B (*Gzmb*; Mm00442834_m1), human eomesodermin (*EOMES*; Hs00172872_m1), human T-bet (*TBX21*; Hs00203436_m1) and human HPRT (*HPRT1*; Hs99999909_m1). 'Test' gene values are expressed relative to that of HPRT, with the lowest experimental value standardized at 1.

Chromatin immunoprecipitation. Cells were fixed for 10 min at 25 °C with 1% paraformaldehyde. After incubation, glycine was added to a final concentration of 0.125 M to 'quench' the paraformaldehyde. After 5 min of incubation at 4 °C, cells were washed and lysed with lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin and 1 μg/ml of leupeptin). Lysates were sonicated (Branson Sonifier 450) to reduce DNA length to between 200 to 1,000 base pairs. The soluble fraction was diluted with dilution buffer (16.7 mM Tris, pH 8.1, 150 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100 and protease inhibitors), was precleared with salmon sperm DNA and protein A and protein G agarose beads and was then incubated with 1 μg of antibodies specific for FLAG (Sigma-Aldrich) or Myc (Upstate Biotechnology). Immune complexes were precipitated with protein A and protein G agarose beads. Immunoprecipitates were washed successively with low-salt buffer (20 mM Tris, pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and protease inhibitors), high-salt buffer (20 mM Tris, pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and protease inhibitors) and LiCl wash buffer (1 mM Tris, pH 8.1, 1 mM EDTA, 1% deoxycholic acid, 0.25 M LiCl, 1% NP-40 and protease inhibitors) and were eluted for 15 min twice at 25 °C with an elution buffer (0.1 M $NaHCO_3$, 1% SDS and 10 mM dithiothreitol). The eluent was incubated at 65 °C for 6 h to reverse crosslinks and DNA was extracted with phenol and chloroform. Ethanol-precipitated DNA was resuspended in Tris-EDTA buffer and was analyzed by PCR. The oligonucleotides used as primers for the analysis of promoter binding were as follows: *Il2rb* sense, 5′-GTGCTCATGCGTGAGCAGAAG-3′, and antisense, GCCCACTTCCTGTATAGAGGATG-3′; *Prf1* sense, 5′-CTCAGAAGCAGGGAG CAGTC-3′, and antisense, 5′-TGCGATCTATCCCCAGGCAG-3′; *Gzmb* sense, 5′-ACTAGATGGTCATGCTTGGTCCTG-3′, and antisense, 5′-TATGAAAACTC CTGCCCTACTGCC-3′; *Il4* sense, 5′-TCTGGGCCAATCAGCACCTCTCTT-3′, and antisense, 5′-GCACCCTCGGACACCTGTG-3′. 'Titrations' of input DNA (threefold) were used to control for sample loading and to ensure semiquantitative linear amplification.

Human cells and cell lines. Human peripheral blood lymphocytes were obtained in accordance with Emory University institutional review board approval and were sorted into various subsets with monoclonal antibodies to human CD8α, CD45RA and CD122. Four immortalized human NK cell lines, YTS, NK-92, NKL and NK3.3, were grown in media containing human recombinant IL-2 (100 U/ml).

Cytotoxicity assay. Target cells were of the major histocompatibility complex class I–negative Epstein-Barr virus–transformed B lymphoblastoid cell line 721.221. The 721.221 cells were labeled for 1 h at 37 °C with 100 μCi ^{51}Cr (as $NaCrO_4$; PerkinElmer) per 1×10^6 cells, followed by four washes in RPMI medium. Washed target cells (1×10^4) were then mixed with serially diluted NK cell samples in 96-well plates to achieve NK/target ratios ranging from 10:1 to 0.3:1. Mixtures were incubated for 4 h at 37 °C in a total volume of 200 μl RPMI with 10% FCS. After incubation, plates were centrifuged at 300*g* for 10 min and 100 μl of the supernatant was transferred into a Lumiplate (PerkinElmer). The amount of ^{51}Cr present was measured by light intensity with a TopCount XL (experimental c.p.m.). The total amount of ^{51}Cr present in labeled target cells was determined by lysis with 1% Nonidet-P40 in double-distilled H_20 (total c.p.m.). The background release of ^{51}Cr from target cells was determined by evaluation of supernatant of target cells incubated in media alone (spontaneously released c.p.m.). Percent target cell lysis was calculated as 100 × [(experimental c.p.m. – spontaneously released c.p.m.) / (total c.p.m. – spontaneously released c.p.m.)].

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin

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In the version of this article initially published online, the third sentence of the abstract was incorrect. The correct sentence is as follows: "We now show that mice with compound mutations of the genes encoding the transcription factors T-bet and eomesodermin were nearly devoid of several lineages dependent on interleukin 15, including memory CD8+ T cells and mature natural killer cells, and that their cells had defective cytotoxic effector programming." The error has been corrected for the HTML and print versions of the article.

Corrigendum: Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin

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In the print version of this article and the version initially published online, some labels for *Tbx21* in **Figure 7b** are incorrect. They should read as presented here. The error has been corrected in the HTML and PDF versions of the article. This correction has been appended to the PDF version.