Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs

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Activation of naive CD8$^+$ T cells with antigen induces their differentiation into effector cytolytic T lymphocytes (CTLs). CTLs lyse infected or aberrant target cells by exocytosis of lytic granules containing the pore–forming protein perforin and a family of proteases termed granzymes. We show that effector CTL differentiation occurs in two sequential phases in vitro, characterized by early induction of T-bet and late induction of Eomesodermin (Eomes), T-box transcription factors that regulate the early and late phases of interferon (IFN) γ expression, respectively. In addition, we demonstrate a critical role for the transcription factor Runx3 in CTL differentiation. Runx3 regulates Eomes expression as well as expression of three cardinal markers of the effector CTL program: IFN–γ, perforin, and granzyme B. Our data point to the existence of an elaborate transcriptional network in which Runx3 initially induces and then cooperates with T-box transcription factors to regulate gene transcription in differentiating CTLs.

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Naive CD8$^+$ T cells differentiate into effector CTLs with the ability to lyse antigen-bearing target cells by exocytosis of lytic granules containing perforin and granzymes, and to produce inflammatory cytokines such as IFN-γ and TNF upon restimulation through the TCR (1, 2). In vivo experiments have elucidated many critical parameters governing the development and evolution of primary CTL responses (3, 4). In this study, we have used in vitro systems such as those developed to study CD4$^+$ T cell differentiation to define the molecular basis of effector CTL differentiation (5, 6).

The T-box transcription factors Eomesodermin (Eomes) and T-bet are needed for important aspects of effector and memory CTL differentiation (7). In uninfected mice, compound deletion of the *Tbx21* (encoding T-bet) and *eomesodermin* genes is associated with a selective loss of CD8$^+$ T cells with an IL–2Rβ–high, memory phenotype (8). Mice deficient for both T-bet and Eomes in T cells have impaired ex-

pression of cytolytic mediators, manifest poor cytolytic activity, and fail to control acute lymphocytic choriomeningitis virus infection (9). Nevertheless, the specific roles of T-bet and Eomes in clonal expansion and CTL differentiation have not yet been resolved: in particular, it is not known whether these transcription factors function redundantly to control effector CD8$^+$ T cell differentiation, and whether they do so directly by targeting specific effector cytokine and cytolytic genes.

Runx proteins, a family of three DNA-binding transcription factors, control thymocyte differentiation and the CD4/CD8 lineage decision (10–13). Runx3 and perforin mRNA are expressed by double-positive (DP) thymocytes and CD8$^+$ single-positive (SP) thymocytes but not in CD4$^+$ SP cells (14). Although Runx3 is not expressed in naive CD4$^+$ T cells, its expression is up-regulated during Th1 cell

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differentiation, and Runx3 influences Th1 cell differentiation and function through direct regulation of the *Il4* and *Ifng* cytokine genes (15, 16). In contrast, all three Runx proteins are expressed in mature CD8^{+} T cells (10, 12), and Runx3-deficient CD8^{+} T cells show reduced cytolytic activity (12, 13). We therefore tested whether Runx3 influenced cytolytic T cell differentiation.

In this report, we show that Runx3 and T-box factors synergistically regulate CTL differentiation and function. T-bet is induced quickly upon TCR stimulation and is required for early programming of cytokine production (17), whereas Eomes is induced later during differentiation and sustains IFN-γ expression. Runx3 is required for Eomes and perforin expression, and both Eomes and Runx3 bind at the *Prf1* locus; in contrast, perforin expression is unaffected in T-bet–deficient cells. T cells lacking Runx3 show decreased expression of IFN-γ and granzyme B, and Runx3 also binds the promoter regions of the *Ifng* and *Gzmb* genes. Collectively, these results provide evidence for a complex transcriptional network in which Runx3 is a primary regulator of *Gzmb* expression but synergizes with T-bet and Eomes, respectively, to promote transcription of the *Ifng* and *Prf1* genes.

RESULTS AND DISCUSSION

A cell culture system to monitor effector CTL differentiation

We used a simple cell culture system to examine the kinetics of effector gene expression during CD8^{+} T cell differentiation. Naive CD8^{+} T cells from P14 TCR transgenic mice were activated for 2 d with anti-CD3 and anti-CD28 or with splenic APCs in the presence of Gp33 peptide, and were cultured in media containing 100 U/ml of recombinant human IL-2 (rhIL-2). We used TCR transgenic mice for these experiments because they provide a reliable source of CD8^{+} T cells that are truly naive; however, we chose not to stimulate cells with antigen in most experiments so as to avoid contamination with proteins and nucleic acids derived from APCs. There were only minor differences in gene expression during differentiation induced by antigen/APC versus anti-CD3/anti-CD28, and the major conclusions presented in this report are the same for both activating conditions.

Under our culture conditions, activated CD8^{+} T cells expanded exponentially and accumulated for >8 d. We limited our analysis to the first 6–8 d after activation, a period that coincides with clonal expansion of CD8^{+} T cells after activation in vivo.

Distinct expression kinetics of perforin and granzyme B during CTL development in culture

Our experiments revealed clear differences in the kinetics of perforin, granzyme B, and cytokine expression during CD8^{+} T cell activation (Fig. 1). Naive T cells showed detectable expression of perforin mRNA as well as perforin protein (Fig. 1, A–D). Relative to its expression in naive T cells, perforin (*Prf1*) mRNA expression did not increase appreciably at day 2 but showed a reproducible decrease at day 4, followed by robust reexpression between days 4 and 8 (Fig. 1, A–D).

In contrast, granzyme B (*Gzmb*) mRNA was low or undetectable in naive T cells but was strongly up-regulated by day 2 after stimulation and increased progressively until day 6 (Fig. 1, A and B); similarly, granzyme B protein was expressed by day 4 and remained high until day 6 (Fig. 1 E). As expected, a small fraction of naive T cells expressed the cytokines IFN-γ and TNF in response to stimulation, and this capacity increased significantly in differentiated cells (Fig. 1 E; see also Fig. 2 A).

We evaluated antigen-dependent cytolytic function in a short-term assay in which target cell death was measured within 2 h (Fig. 1 F). By limiting the duration of TCR stimulation, this strategy minimizes cytolysis secondary to new gene expression during the period of the assay. Naive T cells did not display significant cytolytic function in this short-term assay (unpublished data), most likely because they express immature (unprocessed) forms of perforin and lack the capacity to degranulate (18, 19). Even after activation for 2 or 4 d, the cells showed poor cytolytic activity (Fig. 1 F), in striking contrast to their capacity for efficient cytokine production (Fig. 1 E). Only cells cultured until day 6 displayed robust cytotoxicity, as judged by their ability to induce apoptosis in a large number of target cells (Fig. 1 F).

These results show that after a strong priming stimulus through TCRs and co-stimulatory receptors in vitro, granzyme B expression and the ability to produce effector cytokines are programmed early, whereas perforin expression and cytolytic function are induced later, during the phase of clonal expansion in IL-2. Therefore, the two major effector functions of CTL, cytokine production and cytolytic activity, are not intrinsically coregulated.

Distinct kinetics of T-bet and Eomes expression during CTL differentiation

The T-box transcription factors T-bet and Eomes have been linked to the regulation of genes encoding effector cytokines (e.g., *Ifng*) and genes important for cytolytic function (e.g., *Prf1* and *GzmB*) (20). We investigated the kinetics of expression of these transcription factors in our in vitro cultures (Fig. 1, A–D). T-bet mRNA and protein were not detectable in naive CD8^{+} T cells, but were strongly induced upon TCR priming (day 2) and remained expressed through day 6 of differentiation (Fig. 1, A and C; quantified in Fig. 1, B and D). In contrast, Eomes expression was low or undetectable at both the mRNA and protein levels in naive CD8^{+} T cells, and TCR priming in culture had only a modest effect on its expression at day 2 (Fig. 1, A and C). Strong induction of Eomes mRNA and protein was only observed at day 4 and later (Fig. 1, A and C). T-bet mRNA expression slightly preceded the expression of *GzmB* mRNA; similarly, Eomes mRNA and protein were expressed ~1 d ahead of the reexpression of perforin mRNA and protein, respectively (Fig. 1, B and D).

This detailed kinetic analysis suggested that, under our culture conditions, T-bet and Eomes contribute to distinct aspects of gene transcription during CTL differentiation. T-bet is required early for IFN-γ production, and our data suggested
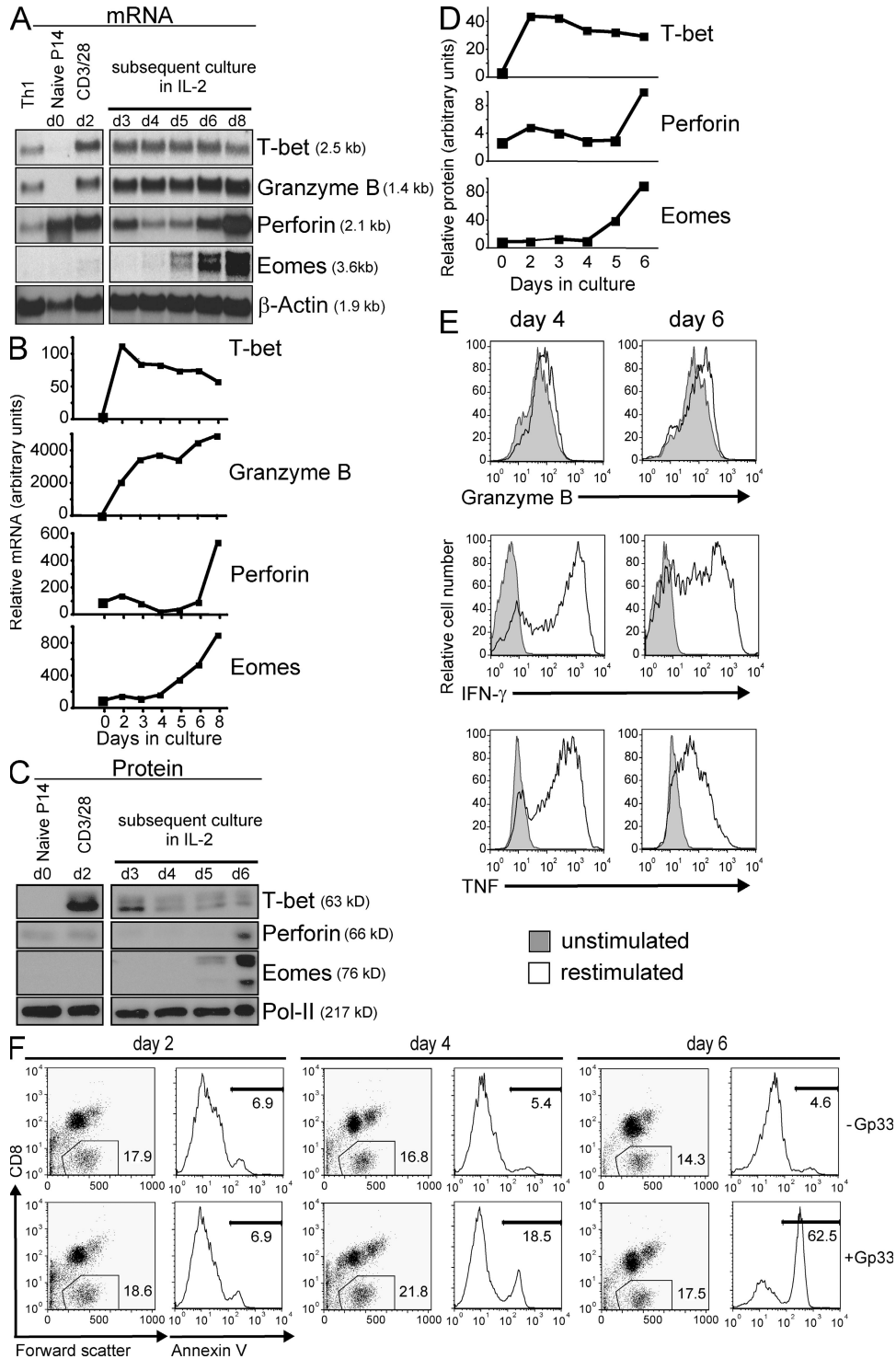
Figure 1. **Kinetics of gene expression during CD8+ T cell differentiation.** (A) Kinetics of *Prf1*, *Gzmb*, *Tbx21* (T-bet), and *Eomes* mRNA expression in differentiating P14 CD8+ T cells analyzed by Northern blotting. RNA from day 7 Th1 cells was used as a control. Sizes of mRNA transcripts are indicated. (B) Quantification of relative mRNA amounts by phosphorimager analysis. (C) Kinetics of protein expression in differentiating P14 CD8+ T cells analyzed by immunoblotting. Sizes of protein bands are indicated. (D) Relative protein amounts quantified from the Western blots. (E) Intracellular staining for granzyme B, IFN-γ, and TNF. Granzyme B staining was specific relative to an isotype control (not depicted). Cells were restimulated with PMA and ionomycin for 4 h. (F) FACS-based assay to measure cytolytic activity of P14 CD8+ T cells against EL4 targets loaded with 0 (−) or 1 (+) μM Gp33 peptide (effector-to-target ratio = 5:1). Percentage of Annexin V+ (apoptotic) target cells in the CD8-negative EL4 target population (dot plots) was determined (histograms). Cytolytic activity was blocked by incubation with 2 mM EGTA (not depicted), confirming involvement of the granule exocytosis (perforin–granzyme B) pathway. Data are representative of at least five (A–E) or three (F) independent experiments.
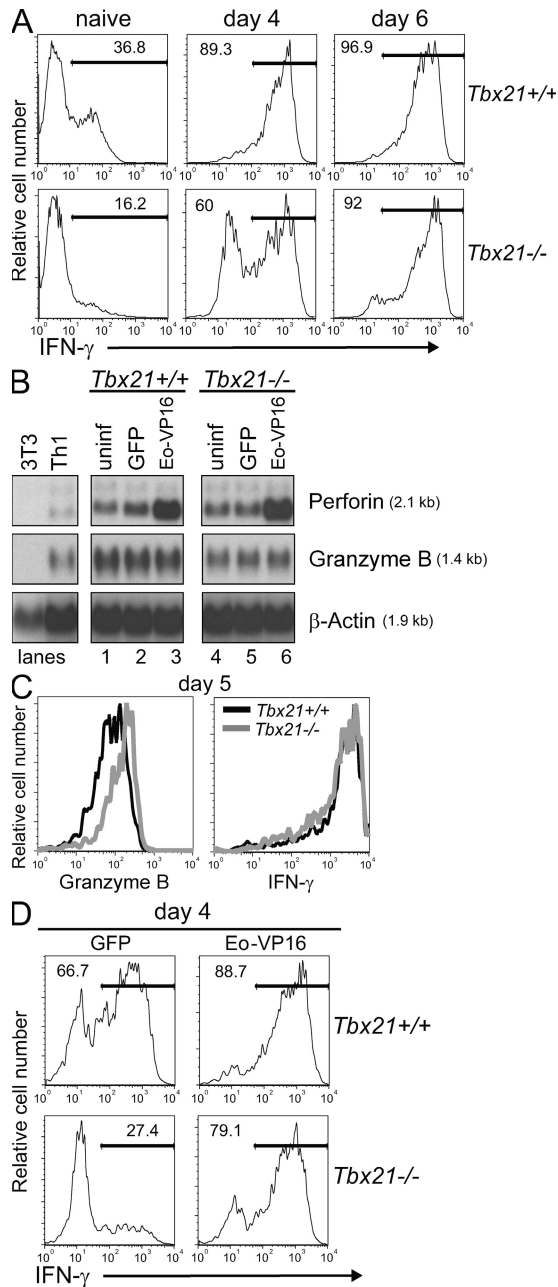
Figure 2. Regulation of perforin, granzyme B, and IFN–γ expression by T–bet and Eomes in differentiating CTLs. (A) IFN–γ expression by WT (*Tbx21*$^{+/+}$) and T-bet–deficient (*Tbx21*$^{−/−}$) T cells. Naive CD8$^+$ T cells, or cells activated and cultured for 4 or 6 d, were restimulated with PMA and ionomycin for 6 h, and IFN–γ expression was assessed by intracellular staining. Numbers show the percentage of IFN–γ$^+$ cells. (B) Northern blot analysis of *Prf1* and *GzmB* mRNA expression in WT or T-bet–deficient CD8$^+$ T cells activated and either left uninfected (uninf) or transduced with retroviruses expressing Eomes-VP16 (Eo-VP16) or an empty IRES-GFP cassette (GFP). Total cellular RNA was analyzed on day 6 of culture. The frequency of transduced cells in the cultures was equivalent for both constructs (∼65–70% GFP$^+$ cells; not depicted). (C) Granzyme B and IFN–γ expression by *Tbx21*$^{+/+}$ and *Tbx21*$^{−/−}$ T cells analyzed in restimulated cells that had been cultured for 5 d. (D) IFN–γ production by cells transduced with Eo-VP16 or control (GFP) retroviruses (RV) measured on day

that Eomes might not function during this early period but rather might contribute later to the control of perforin expression. Our data seemed most consistent with a model in which TCR signals induce T-bet, which in turn induces IFN–γ (17) and possibly granzyme B; subsequently, Eomes is induced during the period of clonal expansion in IL-2 and activates perforin expression.

Perforin and granzyme B expression are not appreciably regulated by T–bet

To test the model outlined in the previous paragraph directly, we compared the expression of IFN–γ, perforin, and granzyme B in CD8$^+$ T cells from WT and *Tbx21* (T-bet)–deficient mice. As expected (17, 21), naive *Tbx21*$^{−/−}$ CD8$^+$ T cells produced IFN–γ poorly upon activation (Fig. 2 A). Notably, this deleterious effect of T-bet deficiency was only observed in differentiating CD8$^+$ T cells until day 4 of culture but was almost completely mitigated by day 6 (Fig. 2 A). This most likely reflected compensation by Eomes, which was strongly induced between days 4 and 6 (Fig. 1). In contrast, T-bet–deficient T cells cultured for 6 d showed no defect in perforin mRNA expression (Fig. 2 B, compare lanes 1 and 4). We consistently observed a modest reduction in *GzmB* mRNA in T-bet–deficient T cells (Fig. 2 B, compare lanes 1 and 4), which did not translate into a decrease in expression of granzyme B protein (Fig. 2 C).

To examine the role of Eomes, we transduced naive CD8$^+$ T cells from WT and *Tbx21*$^{−/−}$ mice with retroviruses containing internal ribosome entry site (IRES)–GFP that were either empty or encoded a strongly transactivating version of Eomes (Eo-VP16) (8), and expanded them for 6 d under our culture conditions. Eo-VP16, but not the empty GFP retrovirus, increased perforin expression in both WT and T-bet–deficient CD8$^+$ T cells (Fig. 2 B, lanes 2, 3, 5, and 6). As expected, Eo-VP16 also rescued the early defect in IFN–γ production observed in T-bet–deficient CD8$^+$ T cells (Fig. 2 D). However, Eo-VP16 did not induce *GzmB* mRNA expression in either WT or T-bet–deficient cells; thus, the partial T-bet dependence of *GzmB* mRNA expression cannot be compensated for by Eo-VP16.

Runx3 controls multiple aspects of the CTL differentiation program, in part through induction of Eomes

Because Runx3 is highly expressed in peripheral CD8$^+$ T cells, and because of the T-bet-Runx3 cooperation we observed earlier in CD4$^+$ T cells (15), we examined the role of Runx3 in effector CTL differentiation. We isolated CD8$^+$ T cells from *Runx3*$^{−/−}$ (KO) mice of the outbred ICR background and their WT *Runx3*$^{+/+}$ littermates by positive selection with anti-CD8 magnetic beads (Figs. S1 and S2, available at http://www.jem.org/cgi/content/full/

4 after 6 h of restimulation with PMA and ionomycin. Numbers show the percentage of GFP$^+$ IFN–γ$^+$ cells. Results are representative of three (A and C) or two (B and D) independent experiments.

jem.20081242/DC1). Strikingly, *Runx3*$^{-/-}$ CD8$^+$ T cells were strongly impaired in their ability to differentiate into effector CTLs, as judged by expression of perforin, granzyme B, and IFN-γ (Fig. 3). Compared with WT T cells, perforin mRNA and protein expression were essentially undetectable in *Runx3*$^{-/-}$ T cells at day 6 of culture (Fig. 3, A and B). *Runx3*$^{-/-}$ T cells also had no detectable Eomes expression; in contrast, T-bet expression was unimpaired (Fig. 3 A). Furthermore, Runx3 was required for maximal production of IFN-γ, but not TNF or IL-2, by CD8$^+$ T cells restimulated at day 6 (Fig. 3 C).

We previously reported that Th1 cell differentiation was regulated through a feed-forward loop in which T-bet is upregulated early and induces Runx3, after which T-bet and Runx3 cooperate to induce IFN-γ and silence IL-4, thus promoting stable differentiation toward the Th1 lineage (15, 22). Because (a) Runx3 appeared necessary for Eomes induction (Fig. 3 A), (b) the kinetics of Eomes expression paralleled those of perforin expression (Fig. 2), and (c) overexpression of Eo-VP16 in either WT or T-bet–deficient T cells led to an increase in both perforin and IFN-γ expression (Fig. 2, B and D), we asked whether CTL differentiation was also potentially regulated by a feed-forward loop involving these same two classes of Runx and T-box transcription factors. Specifically, we asked whether Runx3, which was necessary for Eomes induction, then cooperated with Eomes to regulate transcription of the effector CTL markers perforin, IFN-γ, and granzyme B.

To test this hypothesis, we used chromatin immunoprecipitation (ChIP) assays to ask whether Eomes and Runx3 bound regulatory regions of the *Prf1*, *Ifng*, and *Gzmb* genes (Fig. 3 D). Both proteins associated with gene regulatory regions in differentiated CTLs. Runx3 bound to the *Prf1* and *Gzmb* transcription start sites (TSS); to a known IL-2 responsive enhancer located near −1 kb of the *Prf1* gene (23); to the distal CTL-specific DNase I hypersensitive site 9 in the *Prf1* locus (24); to the *Ifng* promoter near the TSS, as previously reported for Th1 cells (10); and to several DNase I hypersensitive sites in the *Ifng* locus (Fig. 3 D and not depicted) (25). Eomes bound primarily to the *Prf1* TSS and the −1 kb enhancer; this binding was substantially greater than that observed at the promoter of the *Il2rb* gene, a known direct target of Eomes (8), and comparable to that observed at the *Ifng* TSS, a known target of T-box proteins in both Th1 and CD8$^+$ T cells (Fig. 3 D) (17).

To determine whether Runx3 controlled the expression of CTL effector genes through its induction of Eomes, we retrovirally expressed Runx3 and Eo-VP16 in CD8$^+$ T cells from *Runx3*$^{-/-}$ mice. Because of the limited number of CD8$^+$ T cells in these mice, and because we saw no difference between *Runx3*$^{-/-}$ CD8$^+$CD4$^-$ SP and CD8$^+$CD4$^+$ DP cells in our previous experiments, we used total *Runx3*$^{-/-}$ CD8$^+$ T cells without further fractionation as recipients for retroviral transduction. Reconstitution of *Runx3*$^{-/-}$ CD8$^+$ T cells with Runx3 restored expression of Eomes as well as perforin, granzyme B, and IFN-γ (Fig. 4, A and B). In addition, *Runx3*$^{-/-}$ T cells showed a
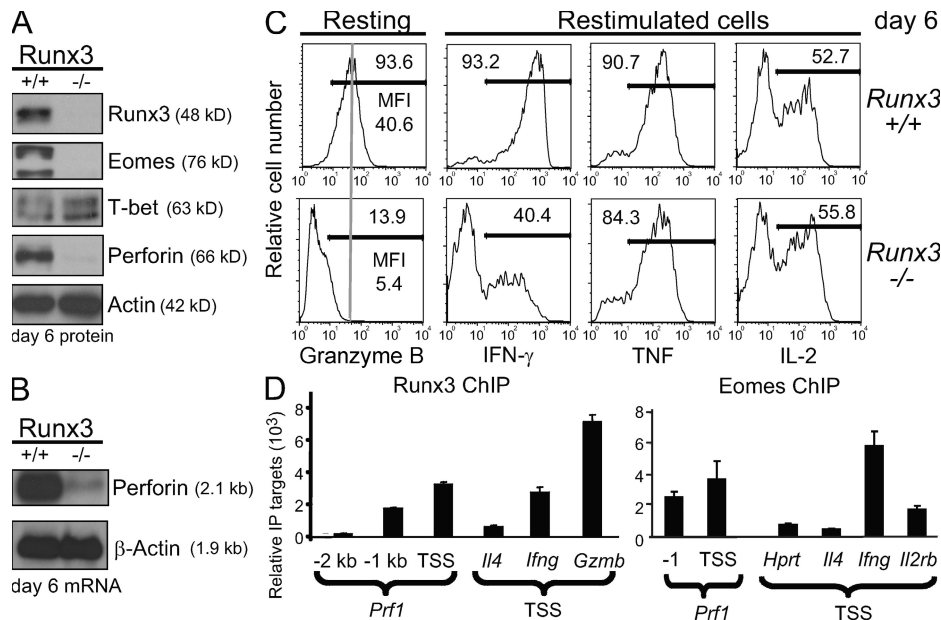

Figure 3. Key role for Runx3 in effector CTL differentiation. (A) Western analysis of Runx3, Eomes, T-bet, and perforin expression in *Runx3*$^{+/+}$ versus *Runx3*$^{-/-}$ CD8$^+$ SP T cells differentiated for 6 d. β-Actin was used as a loading control. (B) Northern blot analysis of *Prf1* mRNA expression in *Runx3*$^{+/+}$ versus *Runx3*$^{-/-}$ CD8$^+$ T cells differentiated for 6 d. β-Actin was used as a loading control. (C) Expression of granzyme B, IFN-γ, TNF, and IL-2 by resting or restimulated (6 h) *Runx3*$^{+/+}$ versus *Runx3*$^{-/-}$ CD8$^+$ SP T cells differentiated for 6 d. The vertical gray line indicates the granzyme B MFI for WT GFP$^+$ cells. Results in A–C are representative of two independent experiments. (D) ChIP analysis of binding of endogenous Runx3 and Eomes to the *Prf1* locus. Enrichment of the indicated genomic regions was evaluated by real-time PCR of DNA from immunoprecipitated and input chromatin. The data are the means of duplicate measurements from two chromatin preparations from two independent CD8$^+$ T cell differentiations. The efficiency of recovery of input for the −1-kb region of *Prf1* was 0.97% for the Runx3 ChIP and 0.5% for the Eomes ChIP.

compensatory up-regulation of Runx1, which was suppressed upon reconstitution with Runx3, indicating that Runx1 is a target of repression by Runx3. Notably, Eo-VP16 did not up-regulate perforin expression when expressed in $Runx3^{-/-}$ cells, even though it restored the capacity to induce IFN-γ expression upon TCR restimulation (Fig. 4, A and B). This result suggests strongly that perforin expression requires Runx3 and Eomes.

As expected from their defect in perforin and granzyme B expression, $Runx3^{-/-}$ CD8$^+$ T cells showed defective cytolytic activity in a mixed lymphocyte reaction (12). However, TCR-stimulated $Runx3^{-/-}$ CD8$^+$ cells were as effective as WT cells in killing tumor cells in a redirected CTL assay (12). Furthermore, CD8$^+$ cells from the peritoneal cavity of $Runx3^{-/-}$ mice immunized with certain tumor cells effectively killed these targets (13). Therefore, although activation of the perforin/granzyme B machinery is defective in $Runx3^{-/-}$ CD8$^+$ cells, these cells are not entirely devoid of cytolytic activity and could still effectively kill targets, possibly by alternative mechanisms such as the Fas–Fas ligand pathway.

Runx3 and T-box factors control a complex program of transcriptional regulation during CTL differentiation

Collectively, these data provide evidence that Runx3, together with T-box factors, orchestrates a complex program of transcriptional regulation in differentiating CTL (Fig. 4 C). Runx3 is present in naive CD8$^+$ T cells before activation (12). It represses Runx1 and has a positive role in the induction of Eomes, granzyme B, perforin, and IFN-γ. Runx3 binds to promoters and putative regulatory regions of the latter three genes, suggesting a direct effect on gene expression. Additional experiments are needed to determine whether $Eomes$ and $Runx1$ are also direct target genes of Runx3.
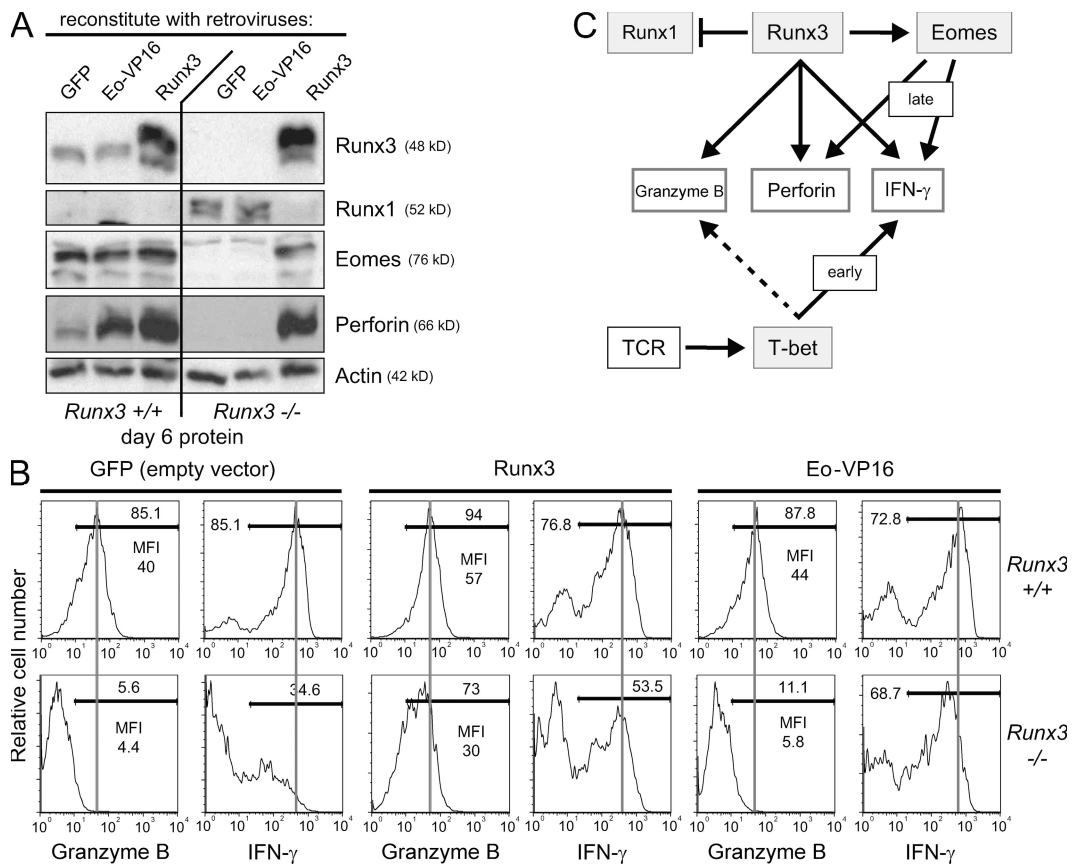

Figure 4.   Runx3 controls Eomes, perforin, granzyme B, and IFN-γ expression in effector CTLs. $Runx3^{+/+}$ or $Runx3^{-/-}$ CD8$^+$ T cells were activated and transduced with retroviruses bearing an empty IRES-GFP cassette (GFP) or also encoding Eomes-VP16 (Eo-VP16) or Myc-Runx3 (Runx3). The frequency of transduced cells in the cultures was equivalent for all constructs ($\sim$75–90% GFP$^+$ cells; not depicted). (A) Protein expression in whole-cell extracts (day 6) was analyzed by immunoblotting. Overexpression of Eomes-VP16 cannot be detected with the Eomes antibody, as the C-terminal epitope is within the region that has been replaced with the VP16 transactivation domain. (B) Expression of granzyme B and IFN-γ after culture for 6 d and restimulation for 4 h with PMA and ionomycin was determined by intracellular staining. The percentage of positively stained cells is shown above the gate; the mean fluorescence intensity (MFI) of granzyme B staining for the total population is shown below the gate. The vertical gray lines indicate the MFI for WT GFP$^+$ cells. Results are representative of at least two independent experiments. (C) Schematic diagram of the transcriptional network involving Runx3 and T-box factors. T-bet is induced by TCR signals and is essential for early IFN-γ expression. Runx3 is present in naive CD8$^+$ T cells and represses Runx1 and induces Eomes, perforin, granzyme B, and IFN-γ expression. Eomes may participate in sustaining late IFN-γ expression, whereas Runx3 and Eomes (but not T-bet) may cooperate to activate perforin expression. The dotted line indicates the partial effect of T-bet deficiency on $Gzmb$ mRNA but not granzyme B protein expression.

Surprisingly, Runx3 contributed to the optimal expression of TNF, IL-2, and IFN-γ at day 4 (Fig. S2). For TNF and IL-2, the requirement for Runx3 subsides by day 6 (Fig. 3 C), possibly because of compensation by Runx1, which is derepressed in *Runx3^−/−* cells (Fig. 4 A). Runx3 continues to be required for IFN-γ expression even at day 6, perhaps because of its role in the induction of Eomes expression (Fig. 4 A).

An unexpected finding was that the two T-box transcription factors, T-bet and Eomes, are up-regulated with very different kinetics in CD8^+ T cells under our culture conditions and have nonredundant roles in the subsequent expression of key effector proteins (Fig. 4 C). T-bet is needed early to confer on activated CD8^+ T cells the competence to produce IFN-γ upon restimulation, but its function is less important at later times. Eomes, which is induced late and functions downstream of Runx3, may substitute for T-bet in promoting the acute expression of IFN-γ in restimulated CTLs (8). Indeed, T-bet and Eomes both contribute to perforin expression in NK cells (8, 26), and Eomes induces granzyme B as effectively as T-bet in developing Th2 cells (7); thus, the relative roles of these T-box transcription factors vary depending on cell type.

Surprisingly, however, Eomes and T-bet appeared nonredundant in their ability to induce two other markers of CTL function, *Prf1* and *Gzmb* (Fig. 4 C). Rather, T-bet and Eomes were involved in regulating granzyme B and perforin expression, respectively: up-regulation of T-bet and Eomes mRNA and protein closely preceded up-regulation of *Gzmb* and *Prf1* mRNA and protein, respectively. T-bet had no role in perforin expression under our culture conditions, and Eo-VP16 did not affect granzyme B expression when expressed in *T-bet^−/−* or *Runx3^−/−* cells. Because conventional Eomes-deficient mice die before precursor cells can be isolated for bone marrow transfers (27) and because T cells conditionally deficient in Eomes have only recently been described (9), we were unable to introduce Runx3 into Eomes-deficient CD8^+ T cells to test formally whether Eomes cooperated with Runx3 to induce perforin expression.

Collectively, our data are consistent with a transcriptional network in which preexisting Runx3 cooperates with the induced T-box factors T-bet and Eomes and IL-2Rβ signals (unpublished data) to orchestrate CTL differentiation (Fig. 4 C). Our data recall the "feed-forward" interaction between T-bet and Runx3 that we previously described in CD4^+ (Th1) T cells (15) but are distinct in two respects: in differentiating Th1 cells, T-bet is induced by TCR signals and IFN-γ, and in turn induces Runx3 (15), whereas in differentiating CD8^+ T cells, preexisting Runx3 is required to induce the T-box transcription factor Eomes. Whole-genome experiments in these and other systems will be required to establish whether cooperation between T-box and Runx family transcription factors is a general feature of cellular differentiation programs.

MATERIALS AND METHODS

Antibodies and reagents. The following antibodies used for intracellular or surface stains were obtained from eBioscience: anti–IL-2, anti–IFN-γ, anti-TNF, anti–granzyme B, anti-CD8, anti-CD25, and anti-CD44. Anti-CD69 was purchased from BD. For ChIP experiments, the anti-Eomes antibody was obtained from Abcam and the anti-Runx3 antibody was produced by the Groner laboratory. The following antibodies were used for immunoblotting: antiperforin (Abcam), anti-Eomes (Abcam), and anti–Pol-II (Santa Cruz Biotechnology, Inc.). The T-bet antibody was provided by L. Glimcher (Harvard School of Public Health, Boston, MA).

The following reagents were used for the experiments presented in this report: Annexin V–FITC Apoptosis Detection Kit (BD), CD8 Negative Isolation Kit (Invitrogen), CD8 MicroBeads (Miltenyi Biotec), and SYBR Green PCR Core Reagents (Applied Biosystems). The Gp33 peptide (KAVYNFATC) was synthesized by the Tufts University Core Facility, and 10 mM of stock solutions was prepared in DMSO.

Isolation and culture of primary CD8^+ T cells. CD8^+ T cells from 4–8-wk-old *Tcra^−/−* × P14 TCR transgenic (Taconic), C57BL/6J WT, or *Tbx21^−/−* (The Jackson Laboratory) mice were purified (>95% purity) by negative selection (Invitrogen) from pooled spleen and lymph node cells. CD8^+ T cells from *Runx3^−/−* mice on the ICR background were purified by positive selection (Miltenyi Biotec). All mice were maintained in specific pathogen-free barrier facilities and used according to protocols approved by the Immune Disease Institute and the Harvard Medical School Animal Care and Use Committees. For stimulation, purified CD8^+ T cells were cultured at 10^6 cells/ml (10 ml) in T25 flasks coated with 1 µg/ml each of anti-CD3 (clone 2C11) and anti-CD28 (clone 37.51) by pretreatment with 300 µg/ml goat anti–hamster IgG. After 48 h, cells were removed from the TCR stimulation and recultured at a concentration of 5 × 10^5 cells/ml in media supplemented with 100 U/ml rhIL-2. Every 24 h, viable cells were counted and readjusted to 5 × 10^5 cells/ml with fresh media containing the corresponding amount of rhIL-2.

Isolation of CD8^+ T cells from Runx3^−/− mice. Runx3-deficient T cells fail to silence CD4 expression normally (Fig. S1) (12, 13). We therefore further fractionated the positively selected CD8^+ T cells from Runx3 KO mice into CD8^+CD4^− SP or CD8^+CD4^+ DP cells by separation using anti-CD4 magnetic beads. This yielded a Runx3 KO SP "enriched" population that contained 75% CD8^+CD4^− cells and a KO DP enriched population that contained 85% CD8^+CD4^+ cells (Fig. S1). The cells were stimulated with anti-CD3^+ anti-CD28 for 2 d before removing them from the TCR stimulus and culturing them in media containing 100 U/ml IL-2. As previously reported, TCR–induced proliferation of *Runx3^−/−* CD8^+ T cells was severely impaired, irrespective of CD4 expression (Fig. S1) (12, 13). However, the *Runx3^−/−* cells showed cell-surface expression patterns indicative of activated cells, including up-regulation of CD25 and CD69 (Fig. S1). As expected from their ability to up-regulate CD25, *Runx3^−/−* CD8^+ T cells responded to IL-2 supplementation after day 2 and efficiently expanded until day 6 of the culture period, albeit at slower rates compared with WT cells (Fig. S1). Although a fraction of the KO DP cells silenced CD4 expression after activation, the ratio of SP/DP cells in each enriched population remained constant thereafter, and we did not observe any major differences between these two populations throughout the culture period, indicating that in terms of effector CTL differentiation and under our culture conditions, *Runx3^−/−* CD8^+ T cells that also coexpress CD4 are indistinguishable from those that do not. The data presented in Fig. S2 are from Runx3 KO SP cells, whereas those shown in Figs. 3 and 4 are from total Runx3 KO CD8 cells.

FACS-based cytotoxicity assay. To measure cytotoxicity, EL4 thymoma target cells were loaded with 0 or 1 µM Gp33 peptide for 2 h before a 2-h coincubation with P14 CD8^+ T cells at the effector-to-target ratios indicated in the figures in 96-well round-bottom plates. After the coincubation period, cells were stained with Annexin V–FITC and anti-CD8–allophycocyanin. Data analysis was performed with FlowJo software (Tree Star, Inc.); EL4 target cells (CD8-negative events) were gated, and the percentage of Annexin V^+ target cells was determined.

Cytokine and surface marker staining. To assess cytokine production, cells were restimulated with 10 nM PMA + 1 µM ionomycin for 6 h (unless indicated otherwise in the figures), and intracellular cytokine stains were performed as previously described (28). To detect expression of surface molecules,

cells were washed in PBS, resuspended in FACS wash buffer (3% FBS, 0.1% sodium azide, 30 mM Hepes, 1× PBS) containing the antibodies indicated in the figures at previously optimized concentrations, incubated for 15 min at room temperature (RT), washed, and resuspended in 2% formaldehyde fixative solution before acquisition on a FACSCalibur (BD).

Retroviral transduction of primary CD8⁺ T cells. For transduction experiments, viral supernatants were generated by calcium phosphate transfection of Phoenix cells and concentration by overnight centrifugation at 6,000 *g*. At ∼42 h after the initial TCR activation of 10^6 CD8⁺ T cells per well in 12-well plates, the culture media was removed and replaced with complete media supplemented with 8 µg/ml polybrene containing fresh plus concentrated virus. The plates were centrifuged at 700 *g* for 1 h at RT before returning to 37°C for an additional 5 h. Retroviral constructs for Eomes-VP16 and the MIG control empty vector were a gift from S.L. Reiner (University of Pennsylvania, Philadelphia, PA) (8).

ChIP and real-time PCR analysis. 20×10^6 CD8⁺ T cells per immunoprecipitation were fixed by adding a 1/10th volume of fixation solution (11.1% formaldehyde, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Hepes) to 1 volume of culture media and were incubated for 10 or 30 min at RT. Fixation was stopped with 120 mM glycine on ice for 5 min. Fixed cells were washed 2× with cold PBS, 1× with cold solution I (10 mM Tris [pH 7.5], 10 mM EDTA, 0.5 mM EGTA, 1% Triton X-100), and 1× with cold solution II (10 mM Tris [pH 7.5], 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). After washes, cell pellets were resuspended at 40×10^6 cells/ml in ChIP lysis buffer (150 mM NaCl, 25 mM Tris [pH 7.5], 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate plus protease and phosphatase inhibitors), and chromatin was sheared with a sonicator to yield 0.5–1-kb DNA fragments. After preclearing the sheared chromatin with protein A–sepharose beads and removing 5% as input chromatin, immunoprecipitation was performed by adding optimized antibody amounts (per 20×10^6 cell equivalents: 2.5 µg anti-Eomes, 1:100 dilution anti-Runx3), followed by overnight incubation at 4°C; protein A–sepharose beads were added for the last 3 h of the incubation period. Beads were washed 2× with RIPA buffer (50 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% deoxycholate), 1× with high salt buffer (50 mM Tris [pH 8], 500 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS), and 1× with TE buffer. After the last wash, DNA was eluted by resuspending the beads in elution buffer (1% SDS, 100 mM NaHCO₃). Both input and ChIP chromatin were then treated with RNase A (5 µg total) for 1 h at 37°C, followed by the addition of proteinase K (100 µg total) and overnight incubation at 65°C to reverse cross-linking. DNA was then purified with QIAquick columns (Gel Extraction Kit; QIAGEN) according to the manufacturer's instructions and resuspended in a 50-µl volume. For real-time PCR detection of immunoprecipitated targets using the SYBR Green PCR Kit, a standard curve was obtained with serial dilutions of input DNA for each sample, and 1 µl ChIP DNA was used per PCR reaction (performed in duplicates). Melt curves and agarose gels were analyzed to ensure amplification of specific target sequences. Refer to Table S1 (available at http://www.jem.org/cgi/content/full/jem.20081242/DC1) for a list of primer sets. The data are presented as the number of immunoprecipitated target sequences relative to input chromatin, assuming two copies of target sequence per cell equivalent used for the ChIP.

Northern and Western blot analyses. RNA isolation and Northern blot analysis was performed as previously described (29). In brief, 10 µg of total RNA was loaded per lane and transferred to positively charged nylon membranes (Hybond-N⁺; GE Healthcare), which was confirmed by ethidium bromide staining of ribosomal RNA species on the membrane. Membranes were hybridized with 1 ng/ml α-[³²P]dCTP–labeled trichloroacetic acid precipitable probe in ExpressHyb hybridization buffer (Clontech Laboratories, Inc.). All cDNA probes were confirmed to have the appropriate single-copy specificity under these conditions using genomic Southern blot analysis. Band intensities were acquired by phosphorimaging analysis.

For Western analysis, whole-cell protein lysates were obtained from CD8⁺ T cells at the time points indicated in the figures during clonal expansion in 100 U/ml IL-2 with lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1% NP-40) by resuspending samples in 10 µl per 10^6 cells and incubating on ice for 30 min in the presence of protease inhibitors. Immunoblot analysis was performed with the antibodies indicated in the figures after SDS-PAGE (10–30 µg of total protein was loaded per well). Quantification of detected protein was performed with an Intelligent Dark Box unit (LAS-3000; Fujifilm) and normalized for loading with the amount of RNA Pol-II detected in each lane.

Online supplemental material. Fig. S1 shows the characterization of peripheral CD8⁺ T cells from *Runx3⁻/⁻* mice. Fig. S2 shows effector protein expression by Runx3 WT and KO cells at day 4 of in vitro culture. Primer sequences used for ChIP experiments are shown in Table S1. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081242/DC1.

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