

The transcription factor Ets1 is important for CD4 repression and *Runx3* up-regulation during CD8 T cell differentiation in the thymus

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The transcription factor Ets1 contributes to the differentiation of CD8 lineage cells in the thymus, but how it does so is not understood. In this study, we demonstrate that Ets1 is required for the proper termination of CD4 expression during the differentiation of major histocompatibility class 1 (MHC I)-restricted thymocytes, but not for other events associated with their positive selection, including the initiation of cytotoxic gene expression, corticomedullary migration, or thymus exit. We further show that Ets1 promotes expression of *Runx3*, a transcription factor important for CD8 T cell differentiation and the cessation of *Cd4* gene expression. Enforced *Runx3* expression in Ets1-deficient MHC I-restricted thymocytes largely rescued their impaired *Cd4* silencing, indicating that Ets1 is not required for *Runx3* function. Finally, we document that Ets1 binds at least two evolutionarily conserved regions within the *Runx3* gene in vivo, supporting the possibility that Ets1 directly contributes to *Runx3* transcription. These findings identify Ets1 as a key player during CD8 lineage differentiation and indicate that it acts, at least in part, by promoting *Runx3* expression.

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Abbreviations used: ChIP, chromatin immunoprecipitation; DP, double positive; SP, single positive; tRFP, tandem-dimer-tomato red fluorescent protein.

Thymocyte differentiation into the CD4 or CD8 lineages is a key event during the late steps of T cell development, in which precursors that have rearranged TCR β and TCR α genes and express both CD4 and CD8 (double positive [DP]) are selected into mature CD4 T cells if MHC II-restricted, or CD8 T cells if MHC I-restricted (Starr et al., 2003; Bosselut, 2004; Singer and Bosselut, 2004). Lineage differentiation is defined by the onset of new programs of gene expression, most prominently the changes in *Cd4* and *Cd8* transcription from

a DP to a single-positive (SP) CD4⁺CD8⁻ or CD4⁻CD8⁺ pattern. Several transcription factors selectively promote the differentiation of either CD4 or CD8 T cells. The zinc finger proteins Gata3 and Thpok (also called cKrox or Zbtb7b) are necessary for the generation of CD4 cells (Hernández-Hoyos et al., 2003; Pai et al., 2003; He et al., 2005; Sun et al., 2005), whereas the transcription factor *Runx3* is important for CD8 T cell development, notably by promoting the cessation of *Cd4* expression (Taniuchi et al., 2002a; Ehlers et al., 2003; Woolf et al., 2003; Egawa et al., 2007). This function of *Runx3* relies on the recruitment of *Runx3* molecules to a cis-regulatory silencer

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element located in the first intron of the *Cd4* gene (Taniuchi et al., 2002a, 2004). *Runx3* has been shown to be up-regulated during the differentiation of DP thymocytes into CD8 cells in the thymus (Sato et al., 2005; Egawa et al., 2007; Egawa and Littman, 2008), but little is known about the transcriptional circuitry that controls its transcription.

Ets1 is the prototype of a family of transcription factors that bind specific DNA sequences typically centered over a GGAA tetranucleotide motif (Sharrocks, 2001; Verger and Duterrque-Coquillaud, 2002). Multiple Ets factors are expressed in DP and SP thymocytes, including Ets1 and the related protein Ets2, both present throughout T cell development without marked preference for any T cell subset (Anderson et al., 1999). Despite this potential for functional redundancy, mice lacking Ets1 have impaired development of NK and T cells (Barton et al., 1998; Eyquem et al., 2004), and Ets1 is essential for Th1 effector differentiation (Grenningloh et al., 2005). Ets1 participates in two important aspects of early thymocyte development, allelic exclusion during TCR β gene rearrangement and the survival of early (pre-DP) thymocytes (Eyquem et al., 2004). Although *Ets1*^{-/-} mice have reduced thymocyte numbers as a result of these early effects, initial studies did not report major anomalies of late thymocyte development (Bories et al., 1995; Muthusamy et al., 1995; Barton et al., 1998). However, it was noticed that *Ets1*^{-/-} CD8 SP cells maintained low-level CD4 expression (Barton et al., 1998), a finding confirmed by a more recent study that showed that this defect is cell autonomous (Clements et al., 2006). How Ets1 affects CD8 lineage differentiation has remained poorly understood. Because Ets1 was reported not to affect expression of *Runx3*, it was proposed that *Ets1* disruption affected Runx3-mediated *Cd4* silencing (Clements et al., 2006).

In this study, we have examined how Ets1 contributes to CD8 T cell differentiation. We show that Ets1 promotes the proper cessation of CD4 expression during the differentiation of MHC I-restricted thymocytes. However, Ets1 is not required for Runx3-mediated *Cd4* silencing. Rather, Ets1 is important for *Runx3* expression in these cells and binds at least two regions of the *Runx3* gene. Our findings identify Ets1 as an important regulator of Runx3 expression and establish a novel connection in the network of transcription factors that control CD8 T cell differentiation in the thymus.

RESULTS

Ets1^{-/-} mice contain an MHC I-restricted "maturelike" DP thymocyte population

Consistent with previous studies (Barton et al., 1998; Eyquem et al., 2004; Clements et al., 2006), *Ets1*^{-/-} thymi were hypocellular (40–50% of wild-type littermates; Table S1). Flow cytometric analyses of CD4 and CD8 expression showed a reduced frequency of CD8 SP thymocytes contrasting with a normal or increased representation of CD4 SP cells (Fig. 1 A). Given the low cellularity of *Ets1*^{-/-} thymi, this resulted in substantially reduced numbers of CD8 SP thymocytes (25–30% of wild-type controls; Table S1). The most mature thymocyte subset, defined as TCR^{hi} CD24^{lo}, normally

comprises CD4 or CD8 SP cells that have successfully completed positive selection and escaped negative selection (Fowlkes and Pardoll, 1989; Kishimoto and Sprent, 1999). In *Ets1*^{-/-} mice, this subset included an unusual contingent of CD4⁺CD8⁺ thymocytes (Fig. 1, B and C). Such maturelike TCR^{hi} CD24^{lo} DP thymocytes were present in the thymus of Ets1-deficient newborn mice, indicating that this subset did not result from the accumulation over time of small numbers of long-lived thymocytes (Fig. S1). There was no maturelike DP subset in *Ets1*^{+/-} thymi, which were phenotypically similar to their *Ets1*^{+/+} counterparts (unpublished data) and were used as controls in some experiments.

We next evaluated if this unusual maturelike DP population resulted from MHC I- or MHC II-induced positive selection by generating *Ets1*^{-/-} mice carrying defined TCR specificities. In mice carrying the MHC I-restricted P14 TCR, which recognizes an LCMV-derived peptide presented by K^b, thymocytes being positively selected express high levels of the transgenic V α 2 and V β 8 TCR chains (Pircher et al., 1989). The frequency of V β 8⁺ thymocytes was lower in *Ets1*^{-/-} than in *Ets1*^{+/+} P14 mice (Fig. S2 A), presumably reflecting the role of Ets1 in early thymocyte development and notably its requirement for efficient TCR β allelic exclusion (Eyquem et al., 2004). Nonetheless, the correlation between V β 8 and V α 2 expression was excellent in both genotypes, and we used either marker to characterize P14 thymocytes undergoing selection. Postselection V α 2^{hi} CD24^{lo} thymocytes were present in *Ets1*^{+/+} and *Ets1*^{-/-} P14 mice in similar numbers (Fig. 2, A [middle] and B), and *Ets1* disruption did not prevent the up-regulation of CD69, a surface molecule normally expressed in response to TCR signaling (Swat et al., 1993; Fig. S2 B); thus, Ets1 was not required for thymocytes to respond to positively selecting TCR engagements. However, postselection V α 2^{hi} CD24^{lo} *Ets1*^{-/-} thymocytes were mostly CD4⁺CD8⁺, unlike their *Ets1*^{+/+} counterparts, which were predominantly CD4⁻CD8⁺ (Fig. 2, A [right] and B). Thus, MHC I-induced positive selection in the absence of Ets1 results in the generation of maturelike DP thymocytes. Correspondingly, the frequency and number of CD8 SP thymocytes were substantially reduced in *Ets1*^{-/-} P14 TCR mice relative to their *Ets1*^{+/+} counterparts (Fig. 2, A [left] and B).

In contrast with these findings, positive selection by the MHC II-restricted OT-II TCR, which recognizes an ovalbumin-derived peptide presented by I-A^b (Hogquist et al., 1994), did not result in the presence of a maturelike DP population on the *Ets1*^{-/-} background, and instead gave rise to a CD4-skewing of mature thymocytes similar to that on the *Ets1*^{+/+} background (Fig. S2 C).

Ets1^{-/-} maturelike DP thymocytes are localized in the thymic medulla

It was important to assess other indicators of differentiation in *Ets1*^{-/-} DP TCR^{hi} CD24^{lo} thymocytes, notably to verify that the presence of this subset was not simply reflecting a requirement for Ets1 for CD24 expression. Normally, preselection TCR^{lo} DP thymocytes reside in the thymic cortex,

whereas positively selected thymocytes migrate to the medulla, as a result of their up-regulating the chemokine receptor CCR7 (Takahama, 2006). Flow cytometry analyses showed that *Ets1*^{-/-} TCRβ^{hi} DP thymocytes were uniformly CCR7^{hi}, expressing CCR7 levels similar to those seen in *Ets1*^{+/+} SP thymocytes and >10 times higher than those on TCR^{lo} DP cells (either *Ets1*^{+/+} or *Ets1*^{-/-}; Fig. 3 A). To evaluate if these CCR7^{hi} DP thymocytes migrated to the medulla, we compared thymus sections of *Ets1*^{-/-} and *Ets1*^{+/+} mice by immunohistological analysis of CD4 and CD8, and of the medullary cell marker cytokeratin 14 (K14;

Klug et al., 1998). These experiments were performed on mice carrying the P14 TCR transgene in which thymocyte differentiation is normally skewed toward the CD8 lineage. K14⁺ medullary areas in P14 *Ets1*^{+/+} thymi contained mostly CD8 SP and only a few CD4 SP cells, whereas the K14-negative cortical areas were occupied by DP thymocytes (Fig. 3 B, top). K14-positive medullary areas were clearly defined in P14 *Ets1*^{-/-} thymi; however, they were packed with DP thymocytes (Fig. 3 B, bottom) and could not be distinguished from the surrounding cortex on the basis of CD4 and CD8 expression alone, unlike the clear boundary seen in the P14

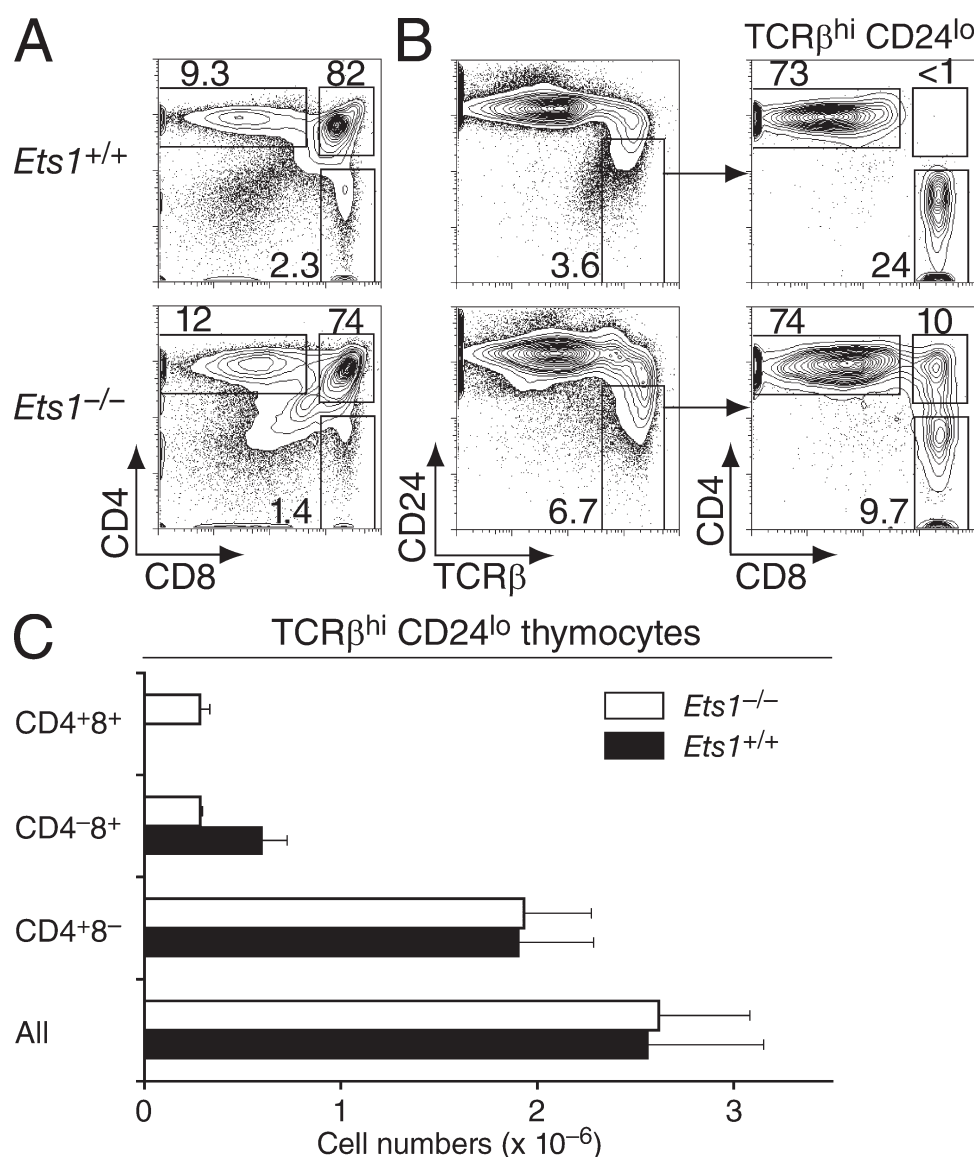


Figure 1. Mature thymocytes fail to resolve into CD4 and CD8 SP populations in *Ets1*-deficient mice. (A and B) Thymocytes from *Ets1*^{+/+} (top) or *Ets1*^{-/-} (bottom) mice were assessed by flow cytometry for surface expression of CD4, CD8, CD24, and TCRβ. Two-parameter contour plots are shown on all live cells for expression of CD4 and CD8 (A) or of CD24 and TCRβ (B, left); TCRβ^{hi} CD24^{lo} mature thymocytes are analyzed for CD4 and CD8 expression (B, right). Numbers next to boxes indicate the percentage of cells within that box. Note that the level of CD24 expression was not affected by *Ets1* disruption by itself. Data are from more than three experiments. (C) Bar graphs (mean ± SEM; *n* = 5 for each genotype) represent the absolute numbers of thymocytes within each mature (TCRβ^{hi} CD24^{lo}) subset.

Ets1^{+/+} thymus. We conclude from these analyses that *Ets1*^{-/-} maturelike DP thymocytes have the same medullary location as wild-type SP thymocytes.

Ets1^{-/-} maturelike DP thymocytes are CD8 lineage cells

Because MHC I-induced selection normally gives rise to CD8-lineage cells, we examined if *Ets1*^{-/-} maturelike MHC

I-restricted DP thymocytes were cells that undergo CD8 differentiation and fail to silence CD4, or cells that are redirected to the CD4 lineage and fail to silence CD8. We submitted maturelike *Ets1*^{-/-} P14 DP thymocytes to real-time RT-PCR analysis of mRNAs encoding the transcription factor Thpok and the cytotoxic marker perforin, normally expressed in CD4 and CD8 lineage thymocytes,

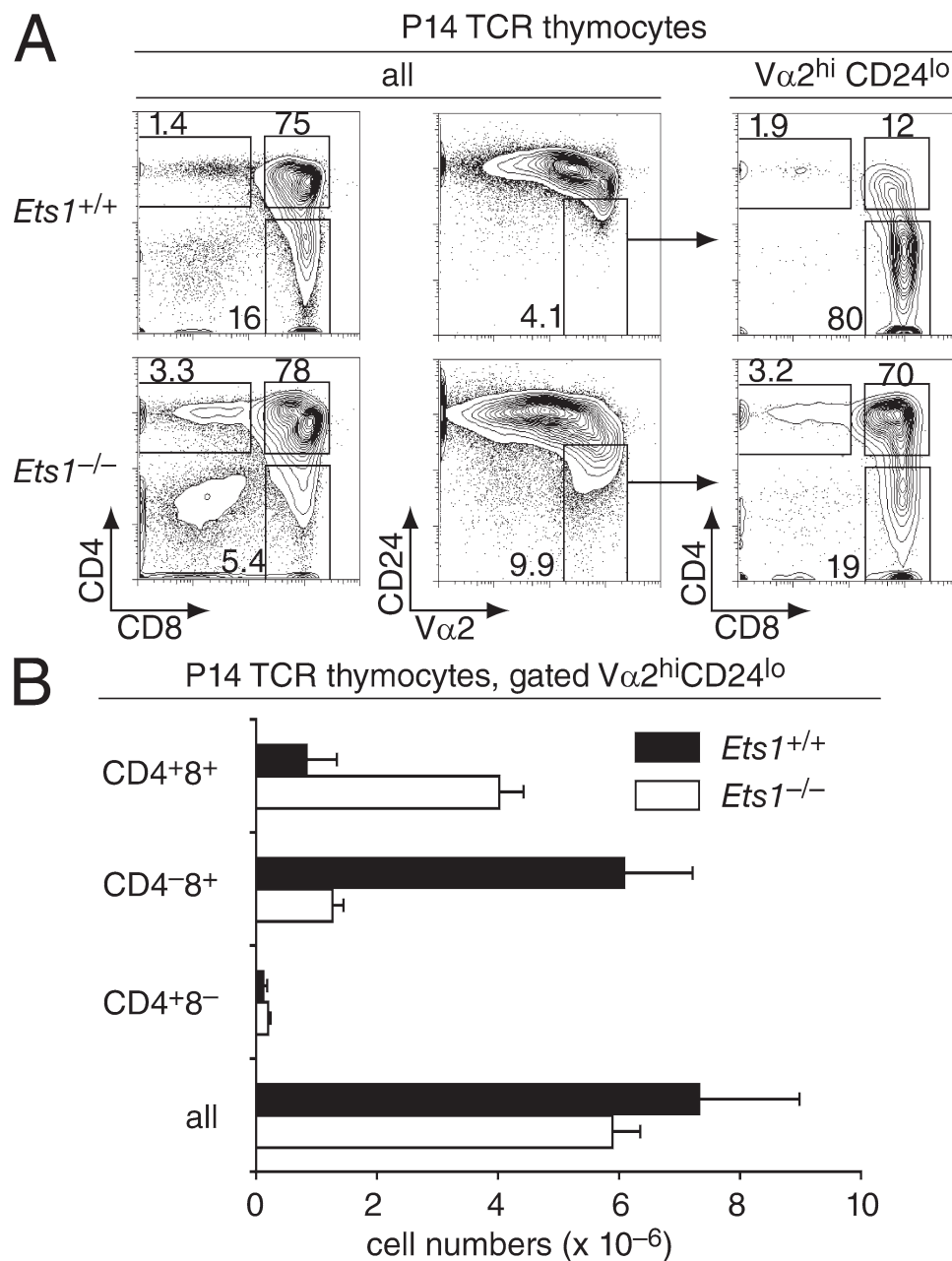


Figure 2. *Ets1*^{-/-} MHC I-restricted thymocytes fail to down-regulate CD4 during the late stages of positive selection. (A) Thymocytes from *Ets1*^{+/+} (top) or *Ets1*^{-/-} (bottom) mice carrying the P14 TCR transgene were assessed for expression of CD4, CD8, CD24, and the transgenic Vα2 TCRα chain. Two-parameter contour plots of CD4 and CD8 expression (left) show a reduced frequency of CD8 SP cells in *Ets1*^{-/-} thymi. The Vα2^{hi} CD24^{lo} subset is defined on two-parameter contour plots of CD24 and Vα2 expression (middle), and analyzed for CD4 and CD8 expression (right). Data are from more than three experiments. (B) Bar graphs (mean ± SEM) represent the absolute numbers of thymocytes within each mature (Vα2^{hi} CD24^{lo}) subset. Total thymocyte numbers (average ± SEM; × 10⁻⁶; n = 7) were 111 ± 23 and 43 ± 4.8 in *Ets1*^{+/+} and *Ets1*^{-/-} P14 mice, respectively.

respectively (Fig. 4, rightmost two columns). As expected, *Ets1*^{-/-} CD8 SP thymocytes expressed perforin but no Thpok (Fig. 4, second column). Importantly, the same was true of maturelike DP thymocytes (Vα2^{hi} CD24^{lo} DP cells from *Ets1*^{-/-} P14 transgenic mice; Fig. 4, left column), indicating that these cells had a gene expression pattern typical of CD8 lineage cells.

We considered the possibility that maturelike DP thymocytes might be in the process of silencing *Cd4*, so that their expression of surface CD4 molecules would not be indicative of active *Cd4* gene expression. To address this possibility, we measured CD4 and CD8 protein reexpression in sorted *Ets1*^{-/-} P14 thymocytes that had been “stripped” of their surface coreceptor proteins by mild pronase digestion (Suzuki et al., 1995). Previ-

ous studies had documented that surface reexpression of coreceptor molecules in this assay is indicative of *Cd4* and *Cd8* gene expression (Brugnera et al., 2000; Yu et al., 2003). Most maturelike (Vα2^{hi} CD24^{lo}) DP thymocytes from P14 transgenic *Ets1*^{-/-} mice reexpressed both CD4 and CD8 after pronase stripping, unlike their CD8 SP counterparts that only reexpressed CD8 (Fig. 5 A, bottom and top rows). However, CD4 reexpression levels were lower on maturelike DP than on their immature Vα2^{hi} CD24^{hi} counterparts (Fig. 5 A, bottom and middle rows). We draw two conclusions from these experiments. First, maturelike DP thymocytes actively express *Cd4*, indicating impaired silencing. Second, the lower CD4 reexpression by that population, compared with its CD24^{hi} counterparts, suggest that some maturelike DP cells may eventually silence *Cd4* and

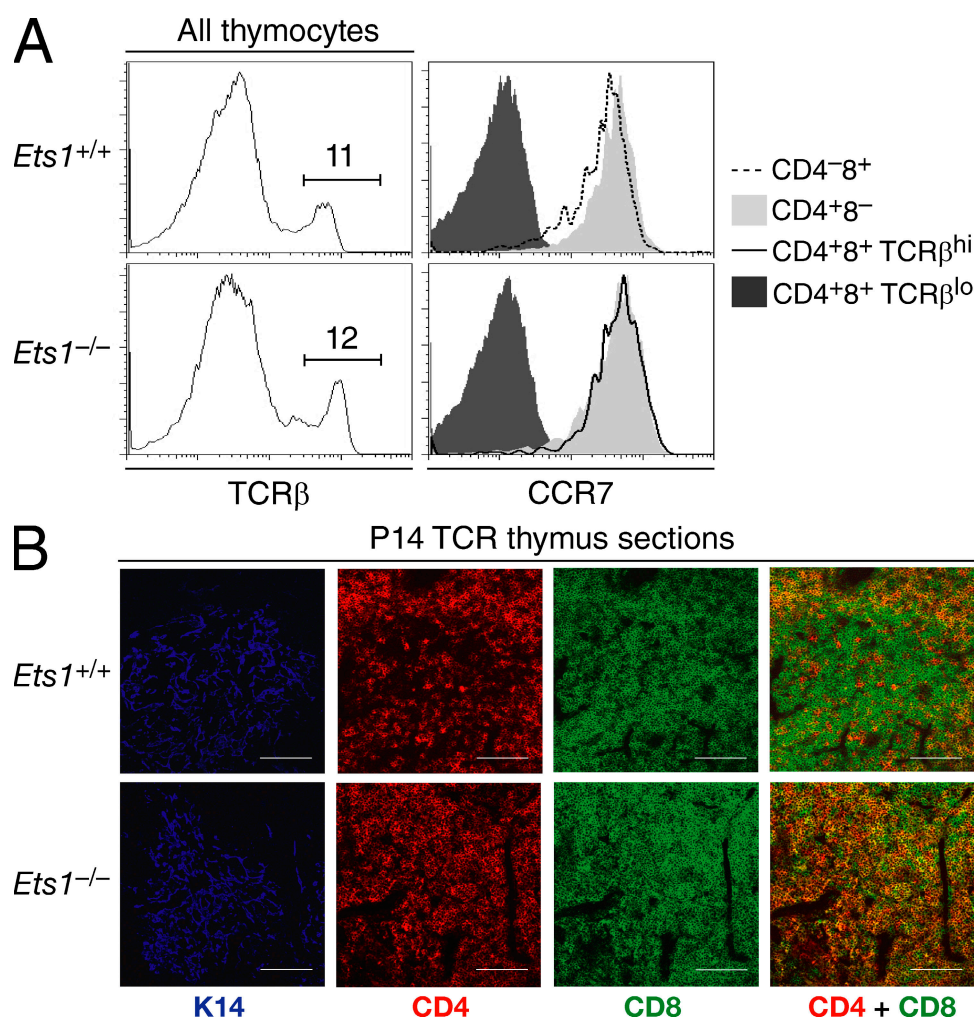


Figure 3. *Ets1*^{-/-} MHC I-restricted maturelike DP thymocytes migrate to the medulla. (A) Thymocytes from *Ets1*^{+/+} and *Ets1*^{-/-} mice were stained for surface expression of CD4, CD8, TCRβ and the chemokine receptor CCR7. Overlaid histograms (right plots) analyze expression of CCR7 on *Ets1*^{-/-} DP and CD4 SP thymocytes (bottom graph), and on *Ets1*^{+/+} CD8 and CD4 SP thymocytes (top graph), all TCRβ^{hi} (as gated in left plots). Expression of CCR7 on TCR^{lo} DP thymocytes is shown in both strains as a negative control (dark gray histograms). Data are representative of two experiments. (B) Frozen thymic sections were prepared from P14 transgenic *Ets1*^{+/+} or *Ets1*^{-/-} mice, stained for cytokeratin 14 (K14, pseudo-colored as blue, defining medullary areas), CD4 (red), and CD8 (green). Overlaying CD4 and CD8 staining (right) shows exclusion of DP cells from medullary areas in *Ets1*^{+/+} but not in *Ets1*^{-/-} mice. The red medullary staining in *Ets1*^{+/+} mice is contributed by the few CD4 SP cells that develop in these recombination-competent animals. Bars, 100 μm. Data are representative of three experiments.

convert to a CD8 SP phenotype. However, the presence of DP T cells in the spleen of adult and neonate *Ets1*^{-/-} mice (Fig. 5 B and not depicted) suggests that at least some maturelike DP thymocytes complete their intrathymic development before terminating *Cd4* expression.

Ets1 promotes Runx3 expression

We concluded from the previous findings that *Ets1* disruption impaired the cessation of *Cd4* expression during CD8 differentiation. The lineage specificity of *Cd4* expression is determined by the *Cd4* silencer, a 434-bp element located in the first intron of the *Cd4* gene (Taniuchi et al., 2004). The silencer is activated in CD8-differentiating thymocytes, a process that normally requires the recruitment of the transcription factor Runx3, whose expression is up-regulated during CD8 differentiation (Taniuchi et al., 2002a; Woolf et al., 2003; Grueter et al., 2005). Thus, the impaired *Cd4* silencing in *Ets1*^{-/-} thymocytes indicated that *Ets1* is important for the expression of Runx3 molecules, for their ability to repress *Cd4* expression, or for both.

To distinguish between these possibilities, we first examined *Runx3* expression in *Ets1*^{-/-} thymocytes. In wild-type mice,

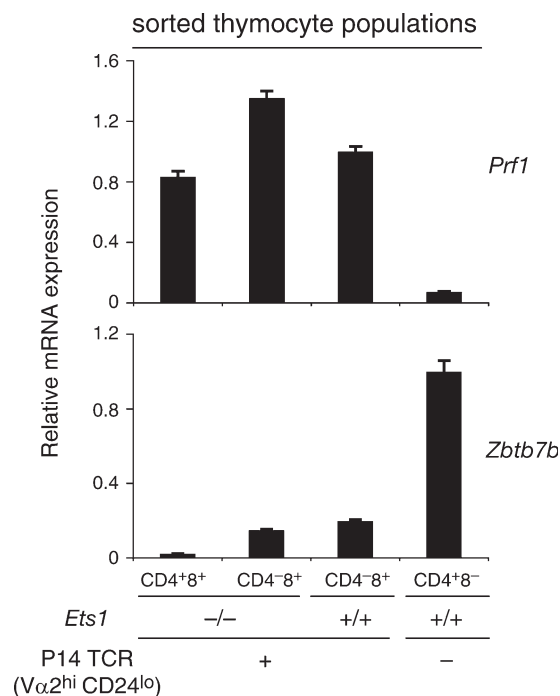


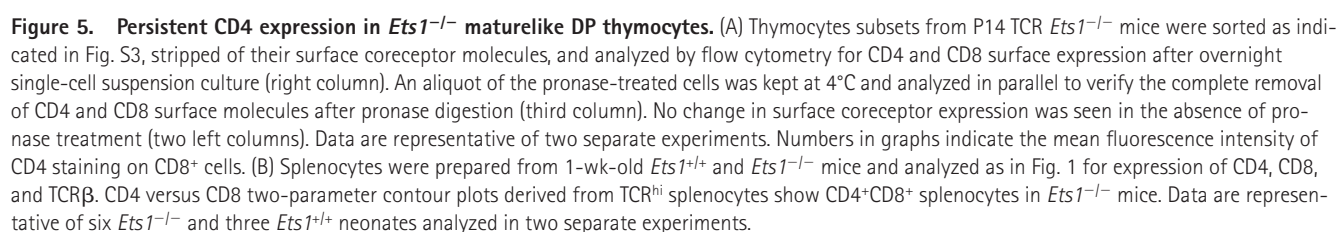
Figure 4. *Ets1*^{-/-} MHC I-restricted maturelike DP thymocytes are CD8 lineage cells. Expression of the genes encoding Thpok (*Zbtb7b*) and perforin (*Prf1*) was analyzed by real-time RT-PCR on sorted Vα2^{hi} CD24^{lo} CD4⁺CD8⁺ and CD4⁻CD8⁺ thymocyte populations from *Ets1*^{+/+} and *Ets1*^{-/-} mice carrying the P14 TCR transgene (left three columns) and on TCR^{hi} CD4⁺CD8⁻ thymocytes from wild-type mice (right column). mRNA levels, normalized on β-actin expression in the same sample, are shown relative to those in wild-type CD4⁺CD8⁻ cells (Thpok) or *Ets1*^{+/+} P14 CD4⁻CD8⁺ cells (*Prf1*). Bars indicate the mean values derived from triplicate determination from a single sorted population; error bars show standard deviations. Data are representative of three or more independent sorted samples for each population.

there is little or no *Runx3* gene expression in preselection DP thymocytes, and its preferential up-regulation during the DP to CD8 SP transition results in higher mRNA levels in CD8 than in CD4 SP thymocytes (Taniuchi et al., 2002a; Liu and Bosselut, 2004; Egawa et al., 2007). Analyses of *Runx3* expression in maturelike DP thymocytes selected by endogenously rearranged TCRs are hampered by the small numbers of these cells; an additional level of complexity comes from alternative promoter usage in the *Runx3* gene, resulting in mRNA species that appear to not equally contribute to Runx3 protein synthesis (Egawa et al., 2007; Egawa and Littman, 2008). To overcome these obstacles, we introduced into *Ets1*^{-/-} mice a transgenic BAC reporter in which the sequence coding for the tandem-dimer-tomato red fluorescent protein (tRFP; Shaner et al., 2005) had been inserted into the second exon of the *Runx3* gene (Fig. S4 A and unpublished data). As the tRFP cDNA insertion respects all *Runx3* noncoding sequences, and as tRFP translation is initiated from endogenous *Runx3* ATG codons, expression of tRFP in the thymus matched expression of endogenous Runx3 protein (Woolf et al., 2003; Egawa et al., 2007). In wild-type thymus, we readily detected tRFP in a subset of DN cells and in CD8 lineage thymocytes, whereas little or no expression was seen in DP and CD4 lineage cells (Fig. S4 B and unpublished data). Similarly, there was little tRFP fluorescence in *Ets1*^{-/-} CD4 lineage cells. However, fluorescence intensities in CD8 SP thymocytes were slightly lower in *Ets1*^{-/-} than in their wild-type counterparts and tRFP expression in maturelike DP thymocytes was half of that in wild-type CD8 SP thymocytes (Fig. 6 A). In fact, the fraction of positively selected (TCR^{hi}) thymocytes that expressed *Runx3*, as well as their level of expression, were lower in *Ets1*-deficient than *Ets1*-sufficient thymocytes, indicating that the low expression observed on maturelike DP cells did not result from a gating bias (Fig. 6 B). These experiments indicated that *Ets1* is important for appropriate *Runx3* expression.

We verified that reduced expression of the *Runx3* reporter was indicative of reduced endogenous Runx3 expression using mice carrying the P14 TCR transgene. *Runx3* mRNA expression was lower in *Ets1*^{-/-} maturelike DP than in CD8 SP cells, whether wild-type or *Ets1*^{-/-} (Fig. 6 C), and the same was true of Runx3 protein expression (Fig. 6 D), demonstrating that *Ets1* disruption results in defective Runx3 up-regulation during the positive selection of MHC I-restricted thymocytes. This defect was specific to Runx3, as expression of Runx1 remained unchanged in all three subsets in *Ets1*^{-/-} mice (Fig. 6 D). Consistent with analyses of Runx3 reporter mice, *Ets1* was not strictly required for Runx3 up-regulation; in *Ets1*^{-/-} mice Runx3 was detectable (although low) in maturelike DP thymocytes and present at subnormal levels in CD8 SP cells (Fig. 6, C and D). Thus, although *Ets1* is not required for Runx3 expression, it is necessary for its proper up-regulation during CD8 lineage differentiation.

Enforced Runx3 expression restores CD4 silencing in *Ets1*^{-/-} thymocytes

Having shown that *Ets1* promotes *Runx3* expression during CD8 cell differentiation, we next investigated if *Ets1* was



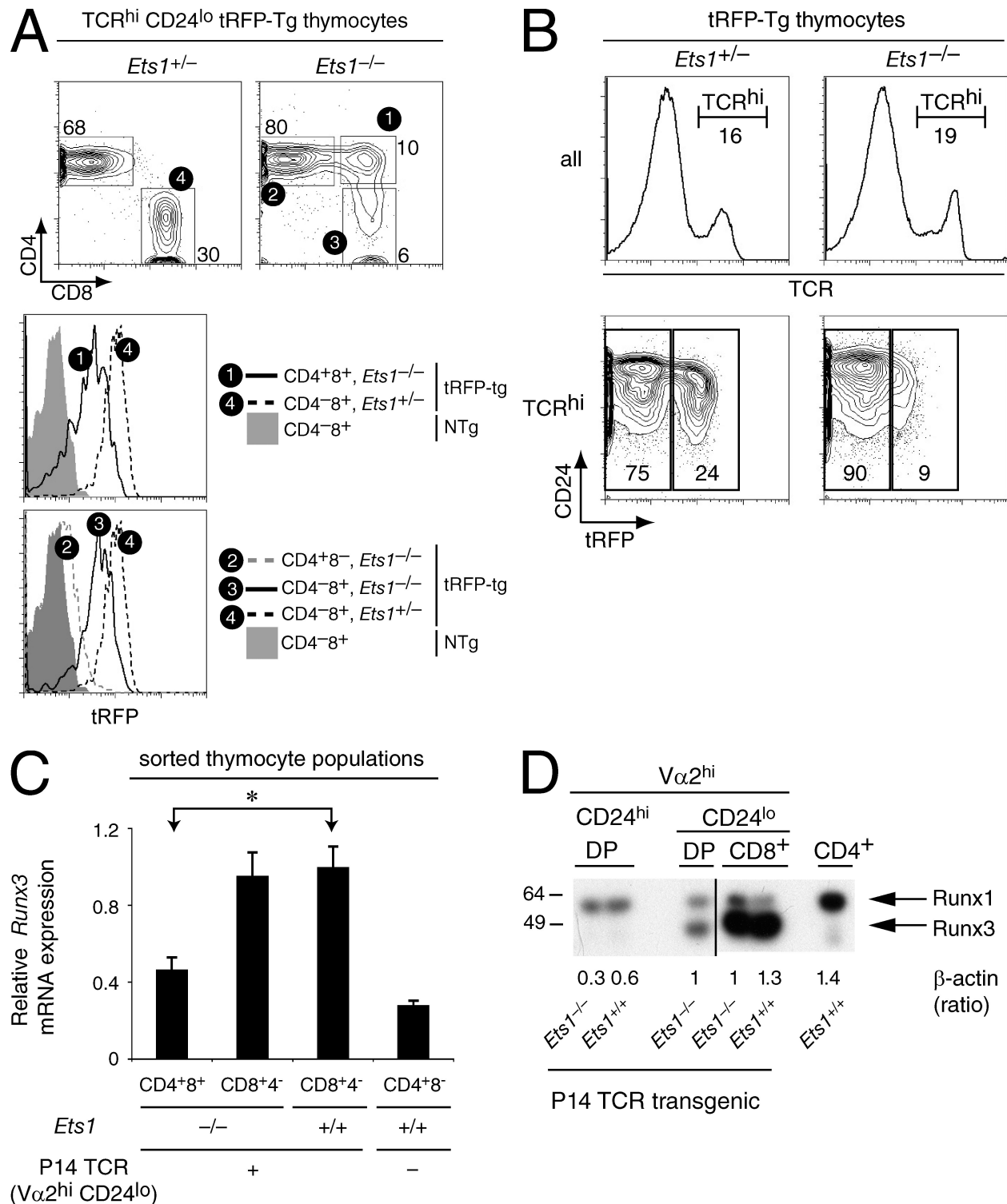


Figure 6. *Ets1* promotes *Runx3* expression. (A and B) Expression of *Runx3* was evaluated in mice carrying a BAC transgene in which a tRFP cDNA has been inserted within the second exon of *Runx3*. (A) Two-parameter contour plots of CD4 and CD8 expression (top) are gated on TCR^{hi} CD24^{lo} thymocytes from *Ets1*^{+/-} and *Ets1*^{-/-} mice. Subsets defined by boxes are numbered and analyzed for tRFP expression. Overlaid histograms (bottom) depict tRFP fluorescence in indicated subsets of tRFP-transgenic *Ets1*^{+/-} and *Ets1*^{-/-} mice. Gray-shaded histogram show background fluorescence in CD8 SP thymocytes from control *Ets1*^{+/-} nontransgenic mice. The mean intensity of tRFP fluorescence in subset 1 (maturelike DP cells from *Ets1*^{-/-} mice) was 49% of that in subset 4 (CD8 SP cells from tRFP-transgenic *Ets1*^{+/-} controls; mean on all three experiments). (B) Two parameter plots of tRFP and CD24 expression (bottom) are shown on TCR^{hi} gated cells (histograms, top). Data (A and B) is representative of three mice of each genotype analyzed in three separate

required for Runx3 to repress *Cd4*. To evaluate this possibility, we introduced into *Ets1*^{-/-} mice a *Runx3* transgene expressed throughout T cell development, starting at or before the preselection DP stage (Grueter et al., 2005). We reasoned that this transgene would fail to restore CD4 silencing in *Ets1*^{-/-} mice if silencer activation by Runx3 molecules required Ets1 expression. Unlike in wild-type mice, and as previously shown (Grueter et al., 2005), Runx3 was expressed at all post-DN stages in the transgenic mice, including preselection DP thymocytes (CD69⁻ cells) and CD4 lineage cells (Fig. S5 A). On a per-cell basis, expression of Runx3 molecules in transgenic CD69⁻ cells was not greater than that of endogenous Runx3 in wild-type CD8 lineage cells, indicating that the transgene did not result in Runx3 overexpression (Grueter et al., 2005; Fig. S5 A). Furthermore, expression of the transgene was not affected by *Ets1* disruption (Fig. S6).

We introduced the *Runx3* transgene into Ets1-sufficient and -deficient P14 TCR transgenic mice, and assessed the Vβ8^{hi} (or Vα2^{hi}) CD24^{lo} subset for expression of CD4 and CD8. Expression of the *Runx3* transgene on the P14 *Ets1*^{-/-} background resulted in a substantial reduction in the size of the maturelike DP subset (Fig. 7 A, right), with ~80% of the Vβ8^{hi} CD24^{lo} cells having down-regulated CD4 (Fig. 7 B). Transgenic Runx3 expression had previously been shown to cause CD4 down-regulation in preselection thymocytes (Telfer et al., 2004; Grueter et al., 2005; Kohu et al., 2005; Wildt et al., 2007), and analyses gated on all live cells showed that this was the case in Ets1-deficient thymocytes as well (Fig. S7). Because expression of transgenic Runx3 on a per-cell basis did not exceed that of endogenous Runx3 (Fig. S5 A), we interpret the early repression of *Cd4* as reflecting the premature expression of transgenic compared with endogenous Runx3 rather than being caused by Runx3 overexpression. We conclude from these experiments that Runx3-mediated *Cd4* silencing does not require Ets1, and that the impaired *Cd4* silencing observed in *Ets1*^{-/-} CD8 lineage cells is caused at least in part by their impaired Runx3 expression.

In vivo recruitment of Ets1 to the Runx3 gene

These findings prompted us to examine if Ets1 could directly promote *Runx3* transcription by binding to specific sites within the *Runx3* gene. To evaluate this possibility, we performed chromatin immunoprecipitation (ChIP) assays with an anti-Ets1 antibody that detects Ets1 binding to the IFN-γ promoter in Th1-differentiating effector T cells (Grenningloh

et al., 2005). Anti-Ets1 ChIP did not detect any specific Ets1 binding when performed on P14 TCR transgenic thymocytes (unpublished data), possibly because of the low representation of CD8-differentiating thymocytes, even in P14 transgenic mice, or of an insufficient Ets1 protein contents in thymocytes. To overcome these limitations, we considered that, because both Ets1 and Runx3 are expressed in Th1-differentiating effectors (Grenningloh et al., 2005; Djuretic et al., 2007; Naoe et al., 2007), it was possible that Ets1 was also important for *Runx3* expression in these cells. To evaluate this possibility, we assessed Th1 effectors carrying the *Runx3*-tRFP reporter. As expected, tRFP was expressed at greater levels in Th1 than in Th2 effectors (unpublished data); tRFP expression was lower in Ets1-deficient than in Ets1-sufficient Th1 effectors, indicating that Ets1 activation of *Runx3* expression is not limited to CD8-differentiating thymocytes (Fig. 8 A).

In light of these results, we examined if we could detect Ets1 binding to the *Runx3* promoter in Th1 effector cells. Indeed, ChIP assays demonstrated specific binding within a region surrounding the distal promoter, that is specifically active in CD8 lineage cells (Egawa et al., 2007; Egawa and Littman, 2008; amplified segments D and E; Fig. 8 B and Fig. S8 A). This evolutionary conserved region includes GGAA motifs compatible with Ets binding (Fig. S8 B). The enrichment of these segments in anti-Ets1 immunoprecipitates was two to three times greater than that of two irrelevant segments from the RORγ and TLR9 genes that we used as specificity controls (Fig. 8 B). Further supporting the conclusion that the binding around segments D and E was specific, little or no binding was detected to a region upstream of the promoter (amplified segment C; Fig. 8 B and Fig. S8, A and B). A search for additional conserved Ets motifs revealed potential binding sites in a region within the second *Runx3* intron. Indeed, ChIP assays found strong Ets1 binding to a region (amplified segment G; Fig. 8 B and Fig. S8, A and C) containing a highly conserved AGGAAGY sequence that matches the consensus for Ets1 DNA binding (Sharrocks, 2001; Verger and Duterque-Coquillaud, 2002). We conclude from these experiments that Ets1 is recruited to multiple sites within the *Runx3* locus in Th1 effectors; these findings support the possibility that Ets1 acts as a direct activator of *Runx3* expression.

In summary, the present study demonstrates ChIP binding of Ets1 on *Runx3*, Ets1-dependence of *Runx3* expression in two distinct cell types, and developmental rescue of Ets1 deficiency by Runx3 expression, and leads us to conclude

experiments. (C) Expression of *Runx3* was assessed as in Fig. 4 on the same mRNA preparations and is shown relative to that in *Ets1*^{+/+} P14 CD4-CD8⁺ cells. The difference between *Ets1*^{-/-} Vα2^{hi} CD24^{lo} DP and *Ets1*^{+/+} CD8 SP thymocytes for *Runx3* expression was statistically significant (*, $P < 10^{-4}$, two tailed Student's *t* test). Data are from more than three experiments. (D) Expression of Runx proteins was assessed in sorted thymocyte subsets by immunoblotting with an antibody directed against the Runt domain and recognizing both Runx1 and Runx3. CD4 SP thymocytes were sorted from wild-type mice and used as positive and negative controls for Runx1 and Runx3 expression, respectively. MW marker sizes are indicated on the left. Numbers underneath indicate expression of β-actin in each samples, quantified on the same membrane and expressed relative to that of wild-type CD8 SP thymocyte. The β-actin signal was consistently lower in DP thymocytes than in other cell subsets, but was not reproducibly affected by *Ets1* disruption. The figure is a composite of two parts of a single blot (separated as indicated by the vertical black bar). Data are from three determinations performed from two distinct sets of sorted cells.

that *Ets1* promotes *Cd4* silencing during CD8 lineage differentiation at least in part by promoting *Runx3* expression.

DISCUSSION

The activation of *Runx3* expression is a critical event during the differentiation of CD8 T cells from DP thymocytes, and signals the onset of *Cd4* down-regulation (Egawa and Littman, 2008). However, the control of *Runx3* expression in the thymus has remained largely mysterious. Although the CD4-differentiating factor Thpok represses *Runx3* (Egawa and Littman, 2008; Muroi et al., 2008; Wang et al., 2008b), it is not yet known whether this effect is direct, and no factor has been shown to promote *Runx3* expression in CD8-differentiating thymocytes. This study addressed this issue starting

from the observation that the transcription factor *Ets1* is required for the proper repression of CD4 during CD8 lineage differentiation. We show that *Ets1* disruption impairs expression of *Runx3* and we provide evidence that *Ets1* directly contributes to *Runx3* transcription. These findings identify *Ets1* as a new node in the transcriptional circuitry that orchestrates CD4-CD8 differentiation (Wang and Bosselut, 2009). Although these findings contrast with the opposite conclusion reached by a previous work (Clements et al., 2006), that study evaluated *Runx3* expression on unfractionated DP thymocytes expressing a diverse TCR repertoire. Both in wild-type and *Ets1*^{-/-} mice, unfractionated DP thymocytes mostly comprise preselection cells that express little or no *Runx3*, presumably making that approach not sensitive enough to

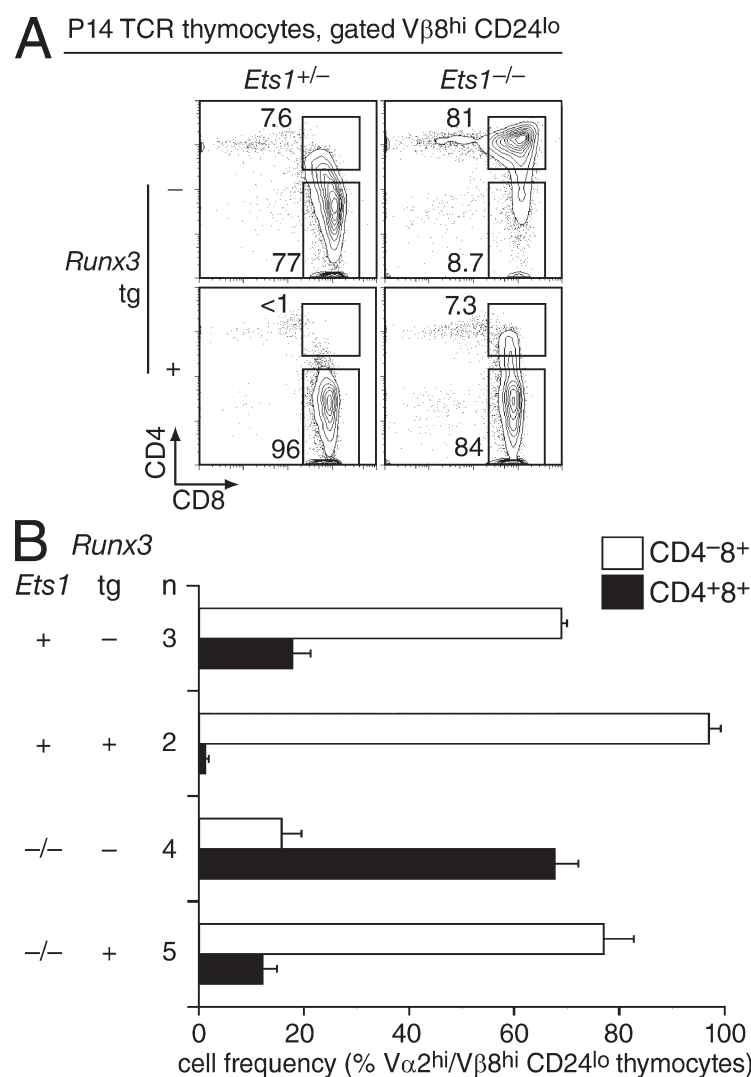


Figure 7. Enforced *Runx3* expression restores CD4 down-regulation in *Ets1*^{-/-} MHC I-restricted thymocytes. (A) Thymocytes were prepared from *Ets1*^{+/-} and *Ets1*^{-/-} mice, carrying either the P14 TCR transgene only or both the P14 and *Runx3* transgenes, and stained for CD4, CD8, CD24 and Vβ8. Two-parameter contour plots show CD4 and CD8 expression on gated Vβ8^{hi} CD24^{lo} thymocytes. (B) Bar graph indicates the percentages (average ± SEM; n: number of mice of each genotype) of Vα2^{hi} or Vβ8^{hi} CD24^{lo} DP and CD8 SP thymocytes in each strain (all carrying the P14 TCR transgene). In these experiments, *Ets1*-competent control mice (*Ets1*⁺) were either *Ets1*^{+/-} or *Ets1*^{+/+}; both genotypes resulted in similar *Ets1*-sufficient phenotypes. Data in (A) and (B) is from more than three experiments.

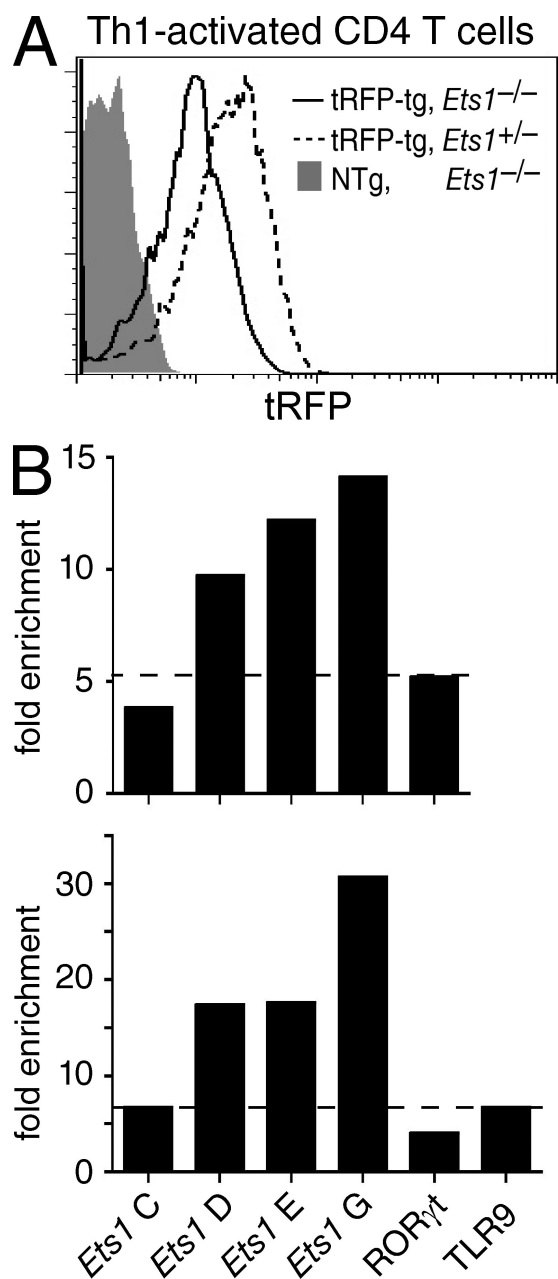


Figure 8. Ets1 binds the *Runx3* locus. (A) Ets1 promotes *Runx3* expression in Th1-differentiating CD4 effectors. Bead-purified CD4⁺CD8⁻ LN T cells from tRFP transgenic *Ets1*^{-/-} or *Ets1*^{+/-} mice were activated under Th1 conditions and analyzed for tRFP expression 5 d later. Overlaid histogram show tRFP expression in gated CD4⁺CD8⁻ *Ets1*^{-/-} (plain line) and *Ets1*^{+/-} (dashed line) effector cells. The gray-shaded histogram shows background fluorescence in nontransgenic *Ets1*^{-/-} Th1 effectors activated in parallel. Data are from two mice of each genotype analyzed in two separate experiments. (B) Recruitment of Ets1 protein to the *Runx3* gene in vivo was assessed by ChIP assays on Th1 effector T cells. Bar graphs represent fold-enrichment of segments C, D, E, and G from the *Runx3* gene (Fig. S8 for location) in anti-Ets1 immunoprecipitates, expressed as indicated in Materials and methods. Horizontal bars depict background enrichment as detected in the RORγt and TLR9 genes. The top and bottom graphs represent two distinct experiments, each from a separate chromatin preparation.

assess *Runx3* gene expression in the relevant CD24^{lo} TCR^{hi} thymocyte population.

Deciphering the roles of Runx proteins in positive selection and lineage differentiation has been complicated by the functional redundancy between the two *Runx* genes expressed in developing T cells, *Runx1* and *Runx3* (Taniuchi et al., 2002a; Woolf et al., 2003; Egawa et al., 2007). Disruption of both genes in DP thymocytes prevents CD8 cell differentiation (Egawa et al., 2007). In post-DN thymocytes, expression of *Runx1* is somewhat promiscuous, whereas expression of *Runx3* is largely restricted to CD8-differentiating cells, suggesting that Runx3 is the main component of the Runx activity that promotes CD8 differentiation. Indeed, *Runx1* inactivation does not affect CD4 expression during CD8 cell differentiation (Taniuchi et al., 2002a; Woolf et al., 2003; Egawa et al., 2007). However, the sole disruption of *Runx3* impairs CD8 lineage differentiation only partially, and notably results in incomplete *Cd4* derepression in CD8 lineage thymocytes (Taniuchi et al., 2002a; Woolf et al., 2003; Egawa et al., 2007). Disruption of *Runx3* also results in increased Runx1 protein expression in CD8 lineage cells (Egawa and Littman, 2008), which is instrumental in attenuating the consequences of *Runx3* disruption. Indeed, hemizygous inactivation of *Runx1* in *Runx3*-deficient thymocytes completely abrogates *Cd4* silencing by CD8 cells (Woolf et al., 2003), a result that is in line with other observations underscoring the sensitivity of Runx function to gene dosage (Barton and Nucifora, 2000).

Indirect comparisons with published studies (Taniuchi et al., 2002a; Woolf et al., 2003; Egawa et al., 2007) suggest that the loss of *Cd4* silencing is greater in *Ets1*^{-/-} than in *Runx3*-deficient CD8 lineage thymocytes, despite the residual Runx3 expression in the former. We see three potential explanations to this apparent paradox. First, unlike disruption of *Runx3*, disruption of *Ets1* did not result in compensatory Runx1 up-regulation, consistent with a more pronounced effect on *Cd4* silencing. Second, it is possible that Ets1 affects the expression of additional factors involved in *Cd4* silencer function. Notably, two silencer DNA motifs, presumably recruiting thus far unknown factors, are required for *Cd4* repression during CD8 lineage differentiation (Taniuchi et al., 2002b). It is conceivable that Ets1 is important for the expression of such additional silencer-binding proteins, thereby controlling multiple key players of CD8 lineage differentiation. If that is the case, the rescue of *Cd4* repression in *Ets1*^{-/-} cells by Runx3 could indicate partial redundancy between such factors and Runx3. Alternatively, it is possible that the expression of these unknown silencer-binding factors is itself under the control of Runx3. In line with this possibility, Runx3 uses such a “feed-forward” loop to promote cytotoxic gene expression in effector CD8 T cells (Cruz-Guilloty et al., 2009).

Third, it has been proposed that Ets1 could bind the silencer and directly cooperate with Runx3 to repress *Cd4* expression (Clements et al., 2006), in line with the in vitro synergy between Ets1 and Runx1 for TCR and BCR enhancer activation (Kim et al., 1999; Erman et al., 1998; Goetz

et al., 2000; Gu et al., 2000). Specifically, a putative Ets1 motif exists between the two Runx binding sites of the silencer (Sawada et al., 1994; Taniuchi et al., 2002a) raising the possibility that Ets1 would bind the silencer and cooperate with Runx3 to promote *Cd4* silencing. Although we did not observe any cooperative effect of Ets1 and Runx3 on silencer activity in cotransfection experiments (unpublished data), the present study does not directly evaluate this hypothesis. However, the fact that enforced Runx3 expression in *Ets1*^{-/-} thymocytes results in efficient *Cd4* silencing indicates that this function of Runx3 is not strictly Ets1-dependent.

Potential targets of Ets1, including CD5 or TCR genes, have been identified in vitro (Ho et al., 1990; Prosser et al., 1992; Tung et al., 2001; Arman et al., 2004) and in a large-scale ChIP study of Ets1 binding in the Jurkat human T cell line (Hollenhorst et al., 2007). However, only a few genes, including IFN- γ (Grenningloh et al., 2005) and *Runx3* (this study) have been shown to both recruit and require Ets1 for their expression in vivo. We detected direct binding of Ets1 to the *Runx3* locus in Th1 effectors, that, similar to CD8-differentiating thymocytes, express both Ets1 and Runx3 (Grenningloh et al., 2005; Djuretic et al., 2007; Naoe et al., 2007) and in which Ets1 similarly promotes *Runx3* expression. These observations support the possibility that Ets1 directly promotes *Runx3* expression by binding the *Runx3* gene. While the two areas of Ets1 binding we identified on *Runx3* are more than 30 kb apart on the sequence, it is possible that they are in close vicinity in the three-dimensional structure of the nucleus. That such an architecture is important for Ets1-mediated activation of *Runx3* expression would be consistent with our observation that neither the distal promoter region nor the intronic Ets1 motif, when analyzed in isolation, respond to Ets1 in cotransfection experiments in T cell lines (unpublished data).

That Ets1 promotes *Runx3* expression is consistent with the expression pattern of these two genes. *Ets1* and *Runx3* are coexpressed at multiple stages of T cell differentiation, including in early DN thymocytes, in CD8 lineage cells, in Th1 effectors, and in NK T cells (Anderson et al., 1999; Taniuchi et al., 2002a; Woolf et al., 2003). However, although expression of *Runx3* in the T cell lineage is stage specific, expression of *Ets1* is more promiscuous and is not restricted to CD8 lineage cells during positive selection. This brings two possibilities as to the function of Ets1 in *Runx3* expression. First, it is possible that Ets1 serves as a “permissive” or priming factor, that makes thymocytes competent to express *Runx3* but would not serve to convert extra-cellular clues into *Runx3* expression. Second, intrathymic signals could trigger post-translational modifications that constrain Ets1 activity, thereby making Ets1 a “signal-sensor” that contributes to adjust *Runx3* levels in response to environmental cues. Ets1 DNA binding in vitro is inhibited by the calcium-induced phosphorylation of serines encoded within its exon 7 (Pognonec et al., 1990; Pufall et al., 2005). Although these modifications do not appear essential for Ets1 functions during Th1 effector differentiation (Grenningloh et al., 2008), there is genetic evidence that they affect Ets1 activity in thymocytes (Higuchi et al., 2007).

The possibility that the intracellular calcium concentration, and thereby TCR signals, affects Ets1 activation of *Runx3* expression raises a provocative correlate with the biology of CD4–CD8 lineage choice. Current models propose that TCR signals are of longer duration in MHC II– than in MHC I–signaled thymocytes, and thereby promote CD4 over CD8 lineage choice (Singer and Bosselut, 2004). In this perspective, it is conceivable, although at present speculative, that persistent TCR signals in MHC II–restricted thymocytes would result in sustained Ets1 phosphorylation, which in turn would minimize Ets1 recruitment to the *Runx3* gene and contribute to limit its expression. It is likely that multiple mechanisms contribute to match lineage choice to MHC specificity in the thymus and affect *Runx3* expression (Singer et al., 2008), and further work will be needed to evaluate the potential role of Ets1 phosphorylation in this process. However, mice genetically engineered to express only Ets1 molecules lacking exon 7–encoded sequences (and therefore not subject to phosphorylation-induced inhibition of DNA binding) have a slightly increased frequency of CD8 SP thymocytes (Higuchi et al., 2007), consistent with the possibility that increased Ets1 DNA binding would favor *Runx3* expression and CD8 cell differentiation.

Because Runx activity also contributes to repress the CD4–committing factor Thpok (Setoguchi et al., 2008), it could be envisioned that impaired *Runx3* expression as a result of *Ets1* disruption would cause MHC I–restricted thymocytes to up-regulate Thpok and therefore to fail CD8 differentiation or to be redirected into the CD4 lineage. However, we did not detect Thpok expression in *Ets1*^{-/-} MHC I–restricted thymocytes, possibly because Runx1 expression in these cells was sufficient to prevent their up-regulation of Thpok.

The heterogeneity of CD4 and *Runx3* expression in *Ets1*^{-/-} MHC I–restricted thymocytes is reminiscent of the variegated CD4 expression by *Runx3*–deficient CD8 lineage cells, which include maturelike DP thymocytes similar to those found in *Ets1*^{-/-} mice (Taniuchi et al., 2002a; Woolf et al., 2003). Expression of *Cd4* and *Cd8* genes is also subject to variegation as a result of mutations of the *Cd4* silencer and of *Cd8* enhancers, respectively (Kioussis and Ellmeier, 2002; Taniuchi et al., 2004). “Pronase stripping” analyses suggest a second source for the heterogeneity of *Cd4* expression by *Ets1*^{-/-} CD8 lineage cells, namely that *Ets1* disruption delays, rather than prevents, *Cd4* silencing (and presumably *Runx3* up-regulation). It is also important to note that the pleiotropic effects of *Ets1* disruption, including on TCR β allelic exclusion (Eyquem et al., 2004), may indirectly affect the sequence of developmental events that normally characterize positive selection. It is possible that such changes feedback on lineage-specific gene expression programs, and, combined with environmental constraints unique to each cell, contribute to the heterogeneous *Runx3* and *Cd4* expression of *Ets1*^{-/-} thymocytes.

In summary, the present study demonstrates the transcription factor Ets1 is required for the proper cessation of *Cd4* expression during the intrathymic development of MHC I–restricted CD8 lineage cells, and that it acts at least in part by promoting the expression of the *Cd4* repressor *Runx3*.

MATERIALS AND METHODS

Mice. Wild-type C57BL/6 (B6) mice were obtained from the National Cancer Institute (NCI) animal production facility. Mice carrying a disrupted *Ets1* locus (Barton et al., 1998) were maintained heterozygous and intercrossed to obtain *Ets1*^{-/-} mice. Mice carrying the P14 TCR transgene (Pircher et al., 1989; originally obtained from Taconic) or the OT-II TCR transgene (Barnden et al., 1998; originally obtained from Jax), and mice carrying a *Runx3* transgene (Grueter et al., 2005) were intercrossed with *Ets1*^{+/-} animals to generate *Ets1*^{-/-} mice with the desired transgene combination. Mice were housed in a specific pathogen-free facility and were analyzed between 6 and 12 wk of age, unless otherwise indicated. The BAC reporter transgene for *Runx3* expression was prepared as previously described (Wang et al., 2008a) by inserting a tRFP cDNA (tdTomato, a gift from R. Tsien, University of California, San Diego, La Jolla, CA; Shaner et al., 2005), using recombinase technology (<http://recombineering.ncicrf.gov/>), and microinjected into C57BL/6 fertilized oocytes. Resulting animals (I5-founder-derived line) were backcrossed to *Ets1*^{-/-} mice. Animal procedures were approved by the NCI Animal Care and Use Committee.

Cell preparation, staining, and analyses of gene expression. Single-cell suspensions of thymocytes and splenocytes were prepared and stained as described previously (Liu et al., 2003). Flow cytometry data were acquired either on a two-laser FACSCalibur or on an LSR-II cytometer (both from BD) using the software and configuration provided by the manufacturer. Data were analyzed with FlowJo Software (Tree Star, Inc.). Dead cells were excluded from analyses on the basis of Forward Light Scatter and either propidium iodide, DAPI, or 7-AAD gating. Cell sorting was performed on a FACS Vantage SE (BD). RNA was extracted from sorted cells with TRIzol (Invitrogen), reverse-transcribed from oligo-dT primers, and analyzed by quantitative real time PCR as previously described (Jenkinson et al., 2007), using an ABI PRISM 7900HT sequence detection system (Applied Biosystems) and previously published primer and probe sets (Jenkinson et al., 2007). Gene expression values are normalized to β -actin in the same sample. Expression of Runx proteins was analyzed by immunoblotting of whole-cell lysates with an antibody that recognizes Runx1 and Runx3, a generous gift of M. Satake, Tohoku University, Sendai, Japan (Sato et al., 2005); expression of β -actin on the same membrane was assessed as a loading control, and quantified using the Odyssey system (Li-Cor) where indicated. Analyses of CD4 and CD8 reexpression after pronase stripping were conducted as previously described (Brugnera et al., 2000).

Immunohistology. OCT-embedded frozen tissue sections were air-dried 15 min before acetone fixation. For costaining, sections were incubated simultaneously with optimal dilutions of polyclonal anti-mouse keratin 14 (Covance Research), FITC anti-mouse CD8 (clone 53-6.7), and anti-mouse CD4 (clone H129-19; BD). Immunoreactivity to CD4 was enhanced by tyramide amplification (PerkinElmer). Controls included slides incubated with normal rabbit IgG or isotype-matched rat IgG. Microscopic analysis was performed with a LSM 510 confocal microscope (Carl Zeiss, Inc.).

ChIP assays. ChIP was performed from *Ets1*^{+/-} Th1 effector CD4⁺ cells as previously described (Grenningloh et al., 2005). A detailed protocol is available upon request. The following antibodies were used: rabbit anti-Ets1 (C-20) and control rabbit IgG (both from Santa Cruz Biotechnology, Inc.). Isolated DNA fragments were amplified by quantitative PCR (Mx300P, Stratagene) using the following primers: Segment C (F, 5'-GTTGACTGGT-GGGAATAAAG-3'; R, 5'-AGGGTTTGGCACATACTG-3'), segment D (F, 5'-AACACCCTAAGAGCATCAAA-3'; R, 5'-TTTATGGGAGTT-GGGATTTA-3'), segments E (F, 5'-ATCCACAAACAGAAAGCCTA-3'; R, 5'-TGTCAACCCAATCTCACAT-3'), and segment G (F, 5'-TAACCGGTAACCTGGGATG-3'; R, 5'-CGCTGAGGTTGAGAGTGT-3'). For each target segment, fold enrichment was defined as the ratio of the target in the anti-Ets1 immunoprecipitates relative to the control IgG immunoprecipitate, calculated as $2^{[\text{anti-Ets1 cycle number}] - [\text{control cycle number}]}$.

Online supplemental material. Fig. S1 shows thymocyte subsets in *Ets1*^{-/-} newborn mice. Fig. S2 analyzes T cell selection in TCR transgenic *Ets1*^{-/-} thymi. Fig. S3 displays the sorting strategy used in the coreceptor reexpression assay (Fig. 5 A). Fig. S4 shows the schematic structure and expression pattern of the *Runx3* tRFP BAC reporter. Fig. S5 shows expression of the *Runx3* transgene in thymocyte subsets. Fig. S6 documents that *Ets1* disruption does not affect expression of the *Runx3* transgene. Fig. S7 shows that *Ets1* is not required for *Runx3*-mediated CD4 repression. Fig. S8 displays the location and sequence of Ets1 binding regions detected by ChIP analyses on the *Runx3* gene. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20092024/DC1>.

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