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 I (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.
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 Duterque-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 (Barton et al., 1998; Eyquem et al., 2004), and Ets1 is essential for Th1 effector differentiation (Grenningloh et al., 1998). Ets1 participates in two important aspects of early thymocyte development, allelic exclusion during TCRβ 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 TCRβ+ CD24lo, 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 TCRβhi CD24lo DP thymocytes were present in the thymus of Ets1–/– 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 K1, thymocytes being positively selected express high levels of the transgenic Vα2 and Vβ8 TCR chains (Pircher et al., 1989). The frequency of Vβ8+ 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 TCRβ allelic exclusion (Eyquem et al., 2004). Nonetheless, the correlation between Vβ8 and Vα2 expression was excellent in both genotypes, and we used either marker to characterize P14 thymocytes undergoing selection. Postselection Vα2hi CD24lo 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 Vα2hi CD24lo 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–Ab (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 TCRβhi CD24lo thymocytes, notably to verify that the presence of this subset was not simply reflecting a requirement for Ets1 for CD24 expression. Normally, preselection TCRβhi 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<sup>−/−</sup> TCR<sup>β<sub>hi</sub></sup> DP thymocytes were uniformly CCR7<sup>hi</sup>, expressing CCR7 levels similar to those seen in Ets1<sup>+/+</sup> SP thymocytes and >10 times higher than those on TCR<sup>lo</sup> DP cells (either Ets1<sup>+/+</sup> or Ets1<sup>−/−</sup>; Fig. 3 A). To evaluate if these CCR7<sup>hi</sup> DP thymocytes migrated to the medulla, we compared thymus sections of Ets1<sup>−/−</sup> and Ets1<sup>+/+</sup> 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<sup>+</sup> medullary areas in P14 Ets1<sup>+/+</sup> 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<sup>−/−</sup> 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](image-url)
$Ets1^{+/+}$ 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.

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**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.
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 (TcRa2hi CD24lo) DP thymocytes from $Ets1^{-/-}$ P14 transgenic 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 TcRa2hi CD24hi 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 CD24hi counterparts, suggests that some maturelike DP cells may eventually silence $Cd4$ and $Cd8$ 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 (TcRa2hi CD24lo 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). Previous 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 (TcRa2hi CD24lo) DP thymocytes from $Ets1^{-/-}$ P14 transgenic 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 TcRa2hi CD24hi counterparts (Fig. 5 A, bottom and middle rows).

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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 TCRβhi (as gated in left plots). Expression of CCR7 on TCRβ SP 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). Overlaid 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, 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; Shiner 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 thymi, 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 (TCRhi) 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 5. Persistent CD4 expression in Ets1−/− maturelike DP thymocytes. (A) Thymocytes subsets from P14 TCR Ets1−/− mice were sorted as indicated in Fig. S3, stripped of their surface coreceptor molecules, and analyzed by flow cytometry for CD4 and CD8 surface expression after overnight single-cell suspension culture (right column). An aliquot of the pronase-treated cells was kept at 4°C and analyzed in parallel to verify the complete removal of CD4 and CD8 surface molecules after pronase digestion (third column). No change in surface coreceptor expression was seen in the absence of pronase treatment (two left columns). Data are representative of two separate experiments. Numbers in graphs indicate the mean fluorescence intensity of CD4 staining on CD8+ cells. (B) Splenocytes were prepared from 1-wk-old Ets1+/+ and Ets1−/− mice and analyzed as in Fig. 1 for expression of CD4, CD8, and TCRβ. CD4 versus CD8 two-parameter contour plots derived from TCRβ splenocytes show CD4+CD8+ splenocytes in Ets1−/− mice. Data are representative of six Ets1−/− and three Ets1+/+ neonates analyzed in two separate experiments.
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 +/- 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 experiments.
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+ (or Vα29+) CD24+ 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+ CD24+ 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 evolutionarily 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 AGGAAAGY sequence that matches the consensus for Ets1 DNA binding (Sharrocks, 2001; Verger and Duterque-Coguillaud, 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
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 V8. Two-parameter contour plots show Cd4 and Cd8 expression on gated V8 hi CD24 lo thymocytes. (B) Bar graph indicates the percentages (average ± SEM; n: number of mice of each genotype) of V8 hi or V8 lo 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.
assess Runx3 gene expression in the relevant CD24<sup>lo</sup> TCR<sup>hi</sup> 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 Cd4 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). If that is the case, the rescue of Cd4 silencing 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

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**Figure 8.** Ets1 binds the Runx3 locus. (A) Ets1 promotes Runx3 expression in Th1-differentiating CD4 effectors. Bead-purified CD4<sup>+</sup>CD8<sup>-</sup> LN T cells from tRFP transgenic Ets1<sup>+/−</sup> or Ets1<sup>+/+</sup> mice were activated under Th1 conditions and analyzed for tRFP expression 5 d later. Overlaid histogram show tRFP expression in gated CD4<sup>+</sup>CD8<sup>-</sup> Ets1<sup>+/−</sup> (plain line) and Ets1<sup>+/−</sup> (dashed line) effector cells. The gray-shaded histogram shows background fluorescence in nontransgenic Ets1<sup>+/−</sup> 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 RORγ and TLR9 genes. The top and bottom graphs represent two distinct experiments, each from a separate chromatin preparation.
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–signalized 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 upregulate 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). “Praone 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 upregulation). It is also important to note that the pleiotropic effects of Ets1 disruption, including on TCRβ 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 (Picher et al., 1989; originally obtained from Taconic) or the OT-II TCR transgene (Barnelden et al., 1998; originally obtained from Jac), 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 rTFP cDNA (tdTomato, a gift from R. Tsien, University of California, San Diego, La Jolla, CA; Shaner et al., 2005), using recombinengineering technology (http://recombinengineering.ncifcrf.gov/), and microinjected into C57BL/6 fertilized oocytes. Resulting animals (IS-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 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 FACSVantage 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), (Invitrogen), reverse-transcribed from oligo-dT primers, and analyzed by quantitative real time PCR as previously described (Jenkinson et al., 2007), (Jenkinson et al., 2007). Gene expression values are normalized to β-actin in the same sample. Expression of Runx proteins was analyzed by immunohistology 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 staining, 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 and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. Nature. 377:635–638. doi:10.1038/377635a0

REFERENCES

Anderson, M.K., G. Hernandez-Hoyos, R.A. Diamond, and E.V. Rothenberg. 1999. Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. Development. 126:3131–3148.

Arman, M., J. Calvo, M.E. Trojanowska, P.N. Cockerill, M. Santana, M. Lopez-Cabrera, J. Vives, and F. Lozano. 2008. Transcriptional regulation of human CD5: important role of Ets transcription factors in CD5 expression in T cells. J. Immunol. 172:7519–7529.

Barnelden, M.J., J. Allison, W.R. Heath, and F.R. Carbone. 1998. Defective Ets-1 expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol. Cell Biol. 76:34–40. doi:10.1046/j.1440-1711.1998.00709.x

Barton, K., and G. Nucifora. 2000. AML1 haploinsufficiency, gene dosage, and the predisposition to acute leukemia. Bioessays. 22:214–218. doi:10.1002/(SICI)1521-1878(200003)22:3<214::AID-BIES2>3.0.CO;2-I

Bartos, K., N. Muthusamy, C. Fischer, C.N. Ting, T.L. Waluns, L.L. Lanier, and J.M. Leiden. 1998. The Ets-1 transcription factor is required for the development of natural killer cells in mice. Immunity. 9:555–563. doi:10.1016/S1074-7613(00)80638-X

Bories, J.C., D.M. Willeford, D. Grévin, L. Davidson, A. Camus, P. Martin, D. Stehelin, and F.W. Alt. 1995. Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. Nature. 377:635–638. doi:10.1038/377635a0

Bosselut, R. 2004. CD4/CD8 lineage differentiation in the thymus: from nuclear effectors to membrane signals. Nat. Rev. Immunol. 4:529–540. doi:10.1038/nri1392

Brugnera, E., A. Bhandoola, R. Cibotti, Q. Yu, T.I. Guinter, Y. Yamashita, S.O. Sharrow, and A. Singer. 2000. Correceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD4+ T cells. Immunity. 13:59–71. doi:10.1016/S1074-7613(00)80008-X

Clements, J.L., S.A. John, and L.A. Garrett-Sinha. 2006. Impaired generation of CD8+ thymocytes in Ets-1-deficient mice. J. Immunol. 177:905–912.

Cruz-Guilloty, F., M.E. Pipkin, I.M. Djuretic, D. Levinson, J. Lotem, M.G. Lichtenheld, Y. Groner, and A. Rao. 2009. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. J. Exp. Med. 206:51–59. doi:10.1084/jem.20081242

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 coexpressor reexpression assay (Fig. 5 A). Fig. S4 shows the schematic structure and expression pattern of the Runx3 rTFP 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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Grenningloh, R., B.Y. Kang, and I.C. Ho. 2005. Ets-1, a functional cofactor of T-bet and Runx3 cooperate to activate Il2 and slc1a2 in TH1 helper type 1 cells. Nat. Immunol. 8:143–153. doi:10.1038/ni1424

Egawa, T., and D.R. Littman. 2008. ThPOK acts late in specification of the T-bet, is essential for Th1 inflammatory responses. J. Exp. Med. 204:267–272. doi:10.1084/jem.20061982

Kim, W.Y., M. Sieweke, E. Ogawa, H.J. Wei, U. Engelmeier, T. Graf, and Y. Ito. 1999. Mutual activation of Ets-1 and AML1 DNA binding by direct interaction of their auto regulatory domains. EMBO J. 18:1609–1620. doi:10.1093/embobio/18.6.1609

Kioussis, D., and W. Ellmeier. 2002. Chromatin and CD4, CD8A and CD8B gene expression during thymic differentiation. Nat. Rev. Immunol. 2:909–919. doi:10.1038/nri852

Kishimoto, H., and J. Sreent. 1999. Several different cell surface molecules control negative selection of medullary thymocytes. J. Exp. Med. 190:65–73. doi:10.1084/jem.190.1.65

Klug, D.B., C. Carter, E. Crouch, D. Roop, C.J. Conti, and E.R. Richie. 1998. Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. Proc. Natl. Acad. Sci. USA. 95:11822–11827. doi:10.1073/pnas.95.20.11822

Kohu, K., T. Sato, S. Ohno, K. Hayashi, U. Uchino, N. Abe, M. Nakazato, N. Yoshida, T. Kikuchi, Y. Iwakura, et al. 2005. Overexpression of the Runx3 transcription factor increases the proportion of mature thymocytes of the CD8 single-positive lineage. J. Immunol. 174:2627–2636.

Liu, X., and R. Bosselut. 2004. Duration of TCR signaling controls CD4-CD8 lineage differentiation in vivo. Nat. Immunol. 5:280–288. doi:10.1038/nri1040

Liu, X., A. Adams, K.F. Wildt, B. Aronow, L. Feigenbaum, and R. Bosselut. 2003. Restricting Zap70 expression to CD4+CD8+ thymocytes reveals a T cell receptor-dependent proofreading mechanism controlling the completion of positive selection. J. Exp. Med. 197:363–373. doi:10.1084/jem.20021698

Muroi, S., Y. Naoe, C. Miyamoto, K. Akiyama, T. Ikawa, K. Masuda, H. Kawamoto, and I. Taniuchi. 2008. Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. Nat. Immunol. 9:1113–1121. doi:10.1038/nm1630

Muthusamy, N., K. Barton, and J.M. Leiden. 1995. Defective activation and survival of T cells lacking the Ets-1 transcription factor. Nature. 377:639–642. doi:10.1038/377639a0

Naoe, Y., R. Setoguchi, K. Akiyama, S. Muroi, M. Kuroda, F. Hatam, D.R. Littman, and I. Taniuchi. 2007. Repression of interleukin-4 in T helper type 1 cells by Runx/CBF binding to the Il4 silencer. J. Exp. Med. 204:1749–1755. doi:10.1084/jem.20062456

Par, S.Y., M.L. Truitt, C.N. Ting, J.M. Leiden, L.H. Glimcher, and I.C. Ho. 2003. Critical roles for transcription factor GATA-3 in thymocyte development. Immunity. 19:863–875. doi:10.1016/S1074-7613(03)00328-5

Pincher, H., K. Bürki, R. Lang, H. Hengartner, and R.M. Zinkernagel. 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature. 342:559–561. doi:10.1038/342559a0

Pognonec, P., K.E. Bouloakis, R. Bosselut, C. Boyer, A.M. Schmitt-Verhult, and J. Ghysdael. 1990. Identification of a Ets1 variant protein unaffected in its chromatin and in vitro DNA binding capacities by T cell antigen receptor triggering and intracellular calcium rises. Oncogene. 5:603–610.

Prosser, H.M., D. Wotton, A. Geggone, J. Ghysdael, S. Wang, N.A. Speck, and M.J. Owen. 1992. A phorbol ester response element within the human T-cell receptor beta-chain enhancer. Proc. Natl. Acad. Sci. USA. 89:9934–9938. doi:10.1073/pnas.89.20.9934

Pufall, M.A., G.M. Lee, M.L. Nelson, H.S. Kang, A. Velyvis, L.E. Kay, L.P. McIntosh, and B.J. Graves. 2005. Variable control of Ets-1 DNA binding by multiple phosphates in an unstructured region. Science. 309:142–145. doi:10.1126/science.1111915

Sato, T., S. Ohno, T. Hayashi, C. Sato, K. Kohu, M. Satake, and S. Habu. 2005. Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocyte differentiation. Immunity. 22:317–328. doi:10.1016/j.immuni.2005.01.017

Sawada, S., J.D. Scarborough, N. Killeen, and D.R. Littman. 1994. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell. 77:917–929. doi:10.1016/0092-8674(94)01040-6
Setoguchi, R., M. Tachibana, Y. Naoe, S. Muroi, K. Akiyama, C. Tezuka, T. Okuda, and I. Taniuchi. 2008. Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development. Science. 319:822–825. doi:10.1126/science.1151844

Shaner, N.C., P.A. Steinbach, and R.Y. Tsien. 2005. A guide to choosing fluorescent proteins. Nat. Methods. 2:905–909. doi:10.1038/nmeth819

Sharrocks, A.D. 2001. The ETS-domain transcription factor family. Nat. Rev. Mol. Cell Biol. 2:827–837. doi:10.1038/sj.nrm2810

Singer, A., and R. Bosselut. 2004. CD4/CD8 coreceptors in thymocyte development, selection, and lineage commitment: analysis of the CD4/CD8 lineage decision. Adv. Immunol. 83:91–131. doi:10.1016/S0065-2776(04)83003-7

Singer, A., S. Adoro, and J.H. Park. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4+ versus CD8-lineage choice. Nat. Rev. Immunol. 8:788–801. doi:10.1038/nri2416

Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. Annu. Rev. Immunol. 21:139–176. doi:10.1146/annurev.immunol.21.120601.141107

Sun, G., X. Liu, P. Mercado, S.R. Jenkinson, M. Kypriotou, L. Feigenbaum, P. Galéra, and R. Bosselut. 2005. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. Nat. Immunol. 6:373–381. doi:10.1038/ni183

Suzuki, H., J.A. Punt, L.G. Granger, and A. Singer. 1995. Asymmetric signaling requirements for thymocyte commitment to the CD4+ versus CD8+ T cell lineages: a new perspective on thymic commitment and selection. Immunity. 2:413–425. doi:10.1016/1074-7613(95)90149-3

Swat, W., M. Desing, H. von Boehmmer, and P. Kuselkov. 1993. CD69 expression during selection and maturation of CD4+8+ thymocytes. Eur. J. Immunol. 23:739–746. doi:10.1002/eji.1830230326

Takahama, Y. 2006. Journey through the thymus: stromal guides for T-cell development and selection. Nat. Rev. Immunol. 6:127–135. doi:10.1038/nri1781

Taniuchi, I., M. Osato, T. Egawa, M.J. Sunshine, S.C. Bae, T. Komori, Y. Ito, and D.R. Littman. 2002a. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing in T lymphocyte development. Cell. 111:621–633. doi:10.1016/S0092-8674(02)01111-X

Taniuchi, I., M.J. Sunshine, R. Festenstein, and D.R. Littman. 2002b. Evidence for distinct CD4 silencer functions at different stages of thymocyte differentiation. Mol. Cell. 10:1083–1096.

Taniuchi, I., W. Ellmeier, and D.R. Littman. 2004. The CD4/CD8 lineage choice: new insights into epigenetic regulation during T cell development. Adv. Immunol. 83:55–89. doi:10.1016/S0065-2776(04)83002-5

Telfer, J.C., E.E. Heidblom, M.K. Anderson, M.N. Laurent, and E.V. Rothenberg. 2004. Localization of the domains in Runx transcription factors required for the repression of CD4 in thymocytes. J. Immunol. 172:4339–4370.

Tung, J.W., S.S. Kunnawatana, L.A. Herzenberg, and L.A. Herzenberg. 2001. The regulation of CD5 expression in murine T cells. BMC Mol. Biol. 2:5. doi:10.1186/1471-2199-2-5

Verger, A., and M. Duterque-Coquillaud. 2002. When Ets transcription factors meet their partners. Bioessays. 24:362–370. doi:10.1002/bies.10068

Wang, L., and R. Bosselut. 2009. CD4-CD8 lineage differentiation: Thpok into the nucleus. J. Immunol. 183:2903–2910. doi:10.4049/jimmunol.0901041

Wang, L., K.F. Wildt, J. Zhu, X. Zhang, L. Feigenbaum, L. Tesarollo, W.E. Paul, B.J. Fowlkes, and R. Bosselut. 2008a. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. J. Immunol. 183:1122–1130. doi:10.1089/jimmunol.0801167

Wang, L., K.F. Wildt, E. Castro, Y. Xiong, L. Feigenbaum, L. Tesarollo, and R. Bosselut. 2008b. The zinc finger transcription factor Zbtb17b represses CD8-lineage gene expression in peripheral CD4+ T cells. Immunity. 29:876–887. doi:10.1016/j.immuni.2008.09.019

Wildt, K.F., G. Sun, B. Grueter, M. Fischer, M. Zamisch, M. Ehlers, and R. Bosselut. 2007. The transcription factor Zbtb17b promotes CD4 expression by antagonizing Runx-mediated activation of the CD4 silencer. J. Immunol. 179:4405–4414.

Woof, E., C. Xiao, O. Fanaru, J. Lotem, D. Rosen, V. Negeau, Y. Bernstein, D. Goldenberg, O. Brenner, G. Berke, et al. 2003. Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. Proc. Natl. Acad. Sci. USA. 100:7731–7736. doi:10.1073/pnas.1232420100

Yu, Q., B. Erman, A. Bhardwaj, S.O. Sharrow, and A. Singer. 2003. In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature CD8+ T cells. J. Exp. Med. 197:475–487. doi:10.1084/jem.20021765