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Noncanonical Mode of ERK Action Controls Alternative αβ and γδ T Cell Lineage Fates

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SUMMARY

Gradations in extracellular regulated kinase (ERK) signaling have been implicated in essentially every developmental checkpoint or differentiation process encountered by lymphocytes. Yet, despite intensive effort, the molecular basis by which differences in ERK activation specify alternative cell fates remains poorly understood. We report here that differential ERK signaling controls lymphoid-fate specification through an alternative mode of action. While ERK phosphorylates most substrates, such as RSK, by targeting them through its D-domain, this well-studied mode of ERK action was dispensable for development of γδ T cells. Instead, development of γδ T cells was dependent upon an alternative mode of action mediated by the DEF-binding pocket (DBP) of ERK. This domain enabled ERK to bind a distinct and select set of proteins required for specification of the γδ fate. These data provide the first in vivo demonstration for the role of DBP-mediated interactions in orchestrating alternate ERK-dependent developmental outcomes.

INTRODUCTION

Differences in the extent of extracellular regulated kinase (ERK) signaling play a critical role in virtually every major developmental or differentiation process encountered by lymphocytes. Indeed, ERK activity is required for αβ T cell progenitors to traverse the β-selection checkpoint, which restricts developmental progression to progenitors with productively rearranged T cell receptor (TCR) β loci (Fischer et al., 2005). Differential activation of ERK has also been implicated in positive and negative selection in the thymus, which shapes the mature TCR repertoire of αβ T cells (Daniels et al., 2006; McNeil et al., 2005; Melichar et al., 2013). After positive selection, $CD4^+$ and $CD8^+$ T cell lineage commitment is determined by the duration of TCR signaling, as codified in the kinetic signaling model. This model proposes that adoption of the $CD4^+$ T cell fate requires prolonged TCR signals, while adoption of the $CD8^+$ T cell fate is dependent upon transient signals, and the longevity of ERK signaling might play an important role in this process (Singer et al., 2008). Finally, peripheral αβ T cell effector fates are also influenced by the extent of ERK signaling, because inhibition of ERK activity under T helper 17 (Th17) polarization conditions impairs Th17 differentiation while favoring production of regulatory T (Treg) cells (Liu et al., 2013). Differences in ERK activation have also been correlated with separation of the αβ and γδ T cell fates, although the importance of these differences in controlling the αβ versus γδ lineage separation process has never been investigated. Despite the central role that ERK signaling plays in essentially every lymphoid fate decision, the basis by which differences in ERK signaling promote the specification of alternative cell fates remains poorly understood (Raman et al., 2007).

Consequently, we have investigated the basis by which differences in ERK signaling specify alternate developmental fates, using separation of the αβ and γδ lineages as a model. Divergence of the αβ and γδ lineages is controlled by the strength of TCR signals, with weak and strong TCR signals favoring adoption of the αβ and γδ fates, respectively, irrespective of the TCR isotype from which they originate (Hayes and Love, 2006). Indeed, we and others showed that a single γδTCR that normally directs adoption of the γδ fate can divert progenitors to the αβ fate when its ability to transduce signals is attenuated (Haks et al., 2005; Hayes et al., 2005). The signaling cascades whose graded activation is responsible for alternate specification of the αβ and γδ fates remain poorly understood, but do involve differential activation of ERK (Lauritsen et al., 2009); however, the importance of the differences in ERK signaling for fate choice has never been directly evaluated.

Here, we report that adoption of the γδ fate is dependent upon ERK signals that are stronger and more prolonged than those associated with commitment to the αβ fate. Most surprisingly, the stronger and more prolonged ERK signals that promote adoption of the γδ fate do not depend upon the ability of ERK to phosphorylate conventional substrates through its D-domain, despite the fact that approximately 80% of ERK2 substrates are thought to be targeted through the D-domain (Carlson and White, 2012). Instead, adoption of the γδ fate depends upon an alternative mode of ERK action that utilizes a different docking mechanism mediated by its DEF binding pocket (DBP). Indeed, these data provide the first demonstration that the prolonged ERK signals that promote γδ T cell fate specification depend not on conventional substrate targeting through the D-domain, but instead depend on an alternate mode of ERK action mediated by its DBP, which posttranscriptionally induces the molecular effectors responsible for execution of ERK-mediated developmental outcomes.

RESULTS

Maturation of γδ Cells Is Dependent upon ERK Signaling

In addressing the molecular basis of ERK-mediated specification of lymphoid fates, we focused on αβ versus γδ lineage commitment, where graded induction of ERK activity had previously been noted, but its importance had not been evaluated (Hayes et al., 2005; Jensen et al., 2008). We have shown, using the KN6 γδTCR transgenic (Tg) model, that thymic progenitors adopt the γδ fate in the presence of the KN6 selecting ligand, H-2T10^{d} (Lig^{+}), but are diverted to the αβ fate if TCR signaling is attenuated by impairing ligand expression through β2M deficiency (Lig^{-}) (Haks et al., 2005). KN6 progenitors adopting the γδ fate in the presence of ligand (Lig^{+}) in vivo exhibit greater ERK phosphorylation as measured by intracellular staining (Figure 1A). Moreover, ERK phosphorylation was also more pronounced in fetal KN6 progenitors adopting the γδ fate in vitro on ligand-expressing (Lig^{+}) OP9-DL1 stromal cells, than in those adopting the αβ fate in the absence of ligand (Lig^{-}) (Figure 1A). ERK phosphorylation was greatest in immature CD24^{hi} cells and was progressively lost during maturation (see Figures S1A and S1B available online), consistent with a recent report indicating that signaling thresholds increase during maturation of γδ progenitors (Wencker et al., 2013). Collectively, these data demonstrate that adoption of the γδ fate is associated with markedly enhanced ERK signaling.

To determine whether γδ lineage commitment was dependent upon greater ERK activity, we investigated the effect of ERK1 and ERK2 deficiency on γδ T cell development. *Erk2* was conditionally ablated in T lineage progenitors using *Ptcra-Cre* (Luche et al., 2013), while *Erk1* was ablated in the germline (Fischer et al., 2005). *Ptcra-Cre*-mediated ablation of *Erk2* began in DN3 (CD4^{-}CD8^{-}CD44^{-}CD25^{+}) thymocytes and was complete in DN4 (CD4^{-}CD8^{-}CD44^{-}CD25^{-}) and γδTCR^{+} thymocytes (Figure S1C). Consistent with previous reports, ablation of both *Erk1* and *Erk2* (ERK deficiency) reduced thymic cellularity and impaired development of αβ T cell progenitors beyond the β-selection checkpoint at the DN3 stage, blocked the maturation of CD4^{+}CD8^{+} (double-positive; DP) thymocytes to the CD4^{+} and CD8^{+} stage (Figure S1D) (Fischer et al., 2005), and abrogated the development of CD1d-αGalCer reactive NKT cells (Figure S1E) (Hu et al., 2011). Moreover, ERK-deficiency reduced the absolute number of CD24^{lo} mature γδ lineage cells, suggesting that maturation of γδ T cell progenitors was dependent upon ERK signaling (Figure S2A). We further investigated the effect of ERK deficiency on particular Vγ subsets and found that ERK deficiency reduced the number of CD24^{lo} mature Vγ1 and Vγ2 progenitors in the thymus and the number of Vγ3^{+} dendritic epidermal T cell (DETC) in the skin (Figures S2A and S2B). Ablation of either *Erk1* or *Erk2* alone did not affect the numbers of γδ T cells in thymus, spleen, or skin (Figures S2C–S2E). These data demonstrate that ERK signaling is required for maturation of γδ T lineage cells in the thymus.

ERK Signaling Regulates αβ versus γδ T Cell Lineage Commitment

Because elevated ERK signaling is important for γδ T cell maturation, we wished to determine whether attenuation of ERK signaling resulted in a fate-switch to the αβ lineage. To determine whether ERK-deficiency diverted γδTCR^{+} progenitors to the αβ fate as evidenced by their development to the DP stage, we assessed the effect of ERK-deficiency on the development of TCRβ-deficient progenitors, which can express the γδTCR, but not the pre-TCR or αβTCR. ERK deficiency blocked the maturation (i.e., CD24 downmodulation) of TCRβ-deficient, γδTCR-expressing thymocytes and impaired the induction of CD73 among CD24^{hi} immature progenitors (Figure 1B). We recently demonstrated that CD73 induction marks γδTCR^{+} CD4^{-}CD8^{-} (double-negative; DN) thymocytes that have committed to the γδ T cell lineage (Coffey et al., 2014). Along with impairing γδ T cell lineage commitment and maturation, ERK deficiency also diverted TCRβ-deficient γδTCR^{+} progenitors to the αβ lineage and the DP stage of development (Figure 1B). The diversion of these γδTCR^{+} progenitors to the αβ T cell fate in ERK-deficient mice was also associated with substantial reductions in γδ T cells in the spleen (Figure 1C) and Vγ3^{+} DETC γδ in the skin (Figure 1D). Taken together, these data indicate that the increased ERK activity observed in cells adopting the γδ T cell fate is required for both adoption of the γδ T cell fate and for repression of the αβ T cell fate. These data also demonstrate that while ERK-deficiency abrogated the ability of the γδTCR to repress the αβ T cell lineage, ERK-deficiency did not block the ability of the γδTCR to promote development of progenitors beyond the β-selection checkpoint to the DP stage.

Analysis of the effect of ERK deficiency on αβ versus γδ lineage commitment using the KN6 γδTCR Tg model produced similar results. Indeed, Rag2-deficient progenitors expressing only the KN6 γδTCR adopt the γδ fate in the presence of T10^{d} ligand (KN6 Tg Lig^{+}), as evidenced by their retention of the DN phenotype and downregulation of the maturation marker, CD24 (Figure 1E, left panels) (Haks et al., 2005); however, ERK-deficiency not only blocked the maturation of KN6 γδTCR Tg progenitors developing in the presence of ligand, but it also robustly diverted those progenitors to the αβ T cell fate, as indicated by their development to the DP stage (Figure 1E, right panels). This involved striking increases in the absolute number of αβ lineage DP thymocytes, as well as reductions in the absolute number of mature CD24^{lo} γδ T cells that normally develop in

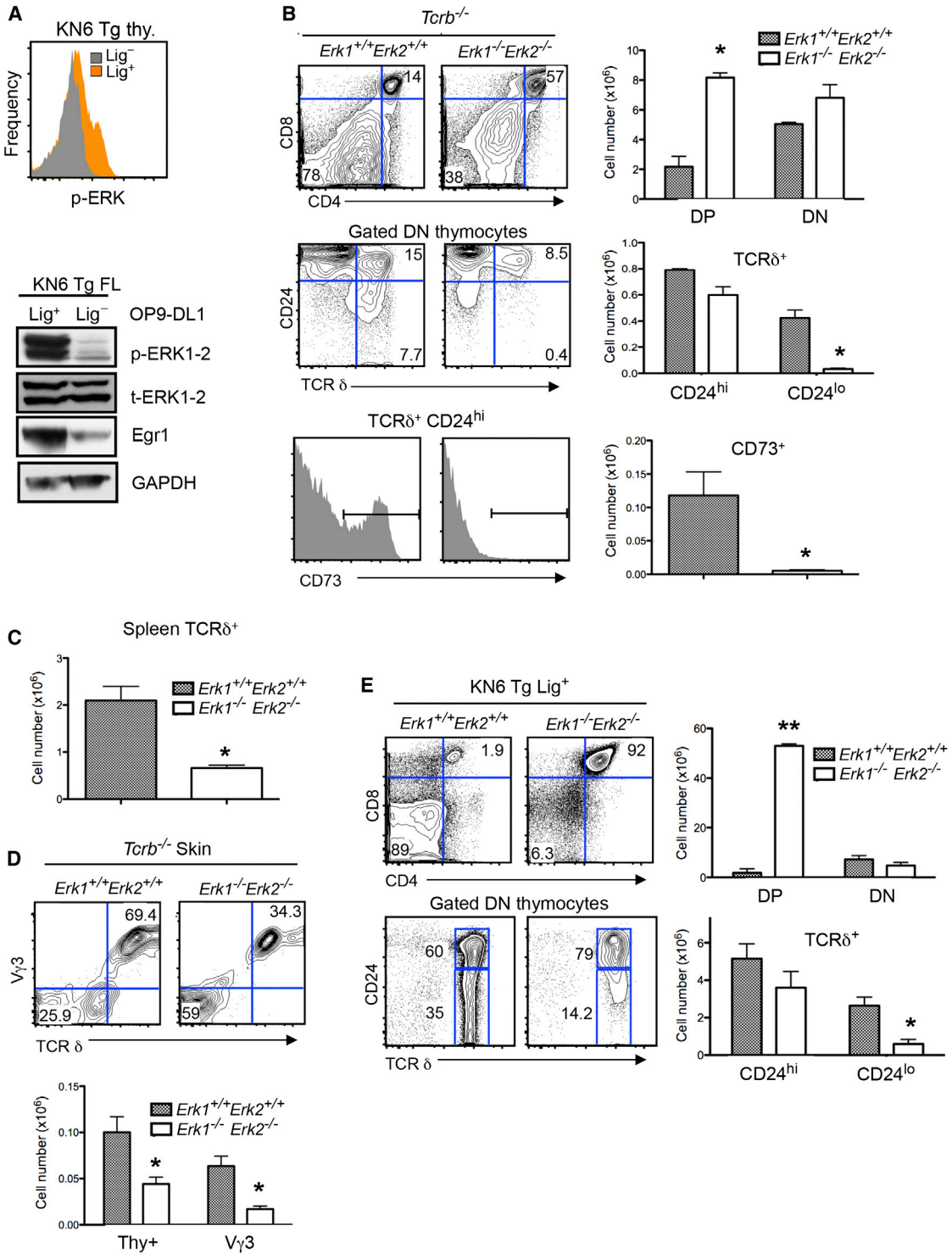

Figure 1. ERK-Deficiency Diverts γδ TCR-Expressing Progenitors to the αβ T Cell Fate

(A) Suspensions of explanted thymocytes from KN6 Tg ligand expressing (Lig$^+$) and ligand-deficient (Lig$^-$) mice were fixed and permeabilized, following which phospho-ERK staining was assessed on electronically gated TCRδ-positive progenitors. ERK phosphorylation and Egr1 protein expression were assessed by immunoblotting of detergent extracts from E14.5 KN6 γδ TCR Tg fetal liver progenitors cocultured for 7 days on OP9-DL1 cells expressing (Lig$^+$) or lacking ligand (Lig$^-$).

(B and C) Thymocytes and splenocytes from 6- to 7-week-old *Ptcra-Cre*$^-$*Erk1*$^{+/+}$*Erk2*$^{fl/fl}$ (*Erk1*$^{+/+}$*Erk2*$^{+/+}$) and *Ptcra-Cre*$^+$*Erk1*$^{-/-}$*Erk2*$^{fl/fl}$ (*Erk1*$^{-/-}$*Erk2*$^{-/-}$) *Tcrb*$^{-/-}$ mice were analyzed by flow cytometry with the indicated antibodies. Gate frequencies of the indicated populations were used to calculate the absolute number of thymocyte subsets, which are depicted graphically (right panels). Cumulative data shown are the means ± SEM from at three independent experiments. *$p < 0.05$

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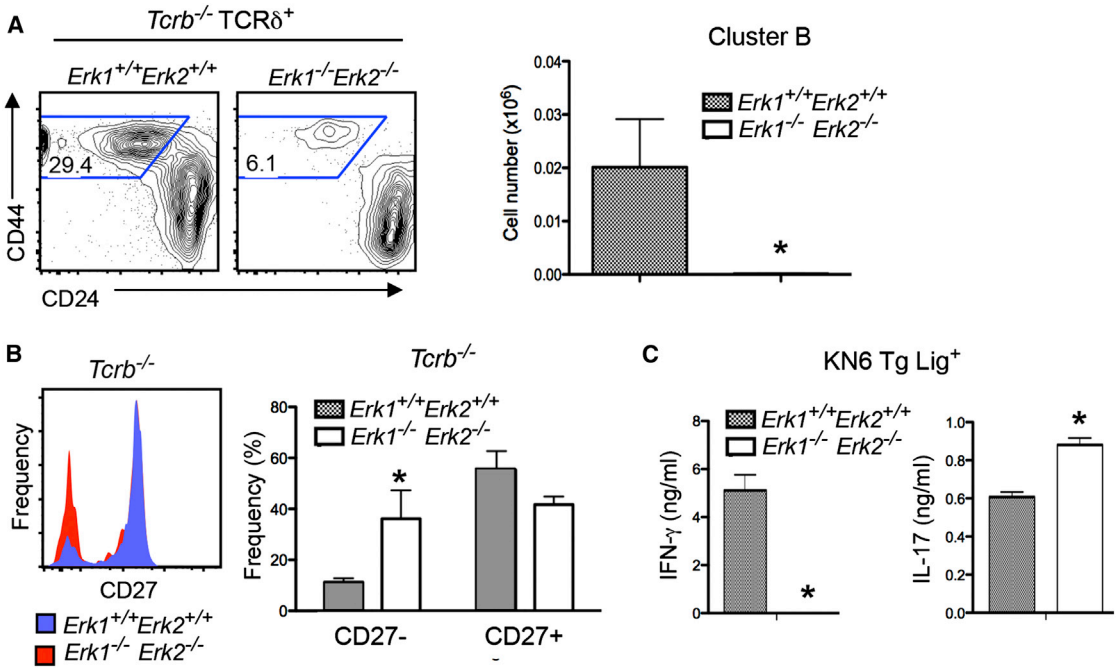

Figure 2. Intrathymic Acquisition of Effector Function Is Altered by ERK Deficiency

(A) Functional, cluster B ($CD44^+CD24^-$) γδ progenitors were analyzed by flow cytometry. The mean of $CD44^+CD24^-$ cluster B cells ± SEM is depicted graphically.

(B) CD27 expression by γδTCR-expressing thymocytes from *Erk1*$^{+/+}$*Erk2*$^{+/+}$ (blue) and *Erk1*$^{-/-}$*Erk2*$^{-/-}$ (red) mice was analyzed by flow cytometry. The frequency of $CD27^-$ and $CD27^+$ cells ± SEM is depicted graphically.

(C) DN thymocytes from *Erk1*$^{+/+}$*Erk2*$^{+/+}$ and *Erk1*$^{-/-}$*Erk2*$^{-/-}$ KN6 γδTCR Tg mice were stimulated with plate-bound anti-CD3 Ab for 24 hr, and IFN-γ and IL-17 production was determined by antibody-capture ELISA. Data shown are the means ± SEM of at three independent experiments. *$p < 0.05$. Error bars represent SEM. See also Figure S3.

the presence of ligand (Figure 1E, right panels). The reduction in mature $CD24^{lo}$ γδ T cells in ERK-deficient mice was not associated with decreased proliferation but was accompanied by decreased survival (Figures S2F and S2G).

ERK Signaling Influences the Specification of γδ Effector Fate

Because ERK signaling plays a critical role in γδ lineage commitment, we sought to assess its role in the acquisition of effector function, which is largely specified in the thymus (Bonneville et al., 2010). Recent analysis indicates that thymic γδ T cells that have acquired the ability to produce the cytokines, interferon-γ (IFN-γ) or interleukin-17 (IL-17), are found in "cluster B," which is defined as $CD44^{hi}$ and $CD24^{lo}$ (Haas et al., 2009). As shown in Figure 2A, ERK deficiency markedly reduced the $CD44^{hi}$ $CD24^{lo}$ cluster B population in TCRβ-deficient mice, suggesting that ERK signaling is required for the acquisition of γδ T cell effector function. The choice of effector fate is purportedly influenced by TCR signal strength (Jensen et al., 2008), with adoption of the $CD27^+$ IFN-γ-producing fate requiring more intense signaling than the $CD27^-$ IL-17 producing effector fate (Ribot et al., 2009; Turchinovich and Hayday, 2011). To determine whether ERK deficiency altered effector fate choice, we analyzed CD27 expression and found that ERK-deficiency increased the frequency of $CD27^-$ cells in TCRβ-deficient mice (Figure 2B), consistent with the notion that weakening TCR signaling favored adoption of the IL-17-producing effector fate (Figure 2B). Thymocytes from KN6 γδTCR Tg mice predominantly produce IFN-γ when developing in the presence of ligands. ERK deficiency attenuated the ability of KN6 Tg thymocytes to produce IFN-γ, while augmenting the production of IL-17 (Figure 2C). The addition of IL-1 and IL-23, which is reported to enhance IL-17 production (Ribot et al., 2009), increased both IL-17 and IFN-γ production by ERK-deficient KN6 Tg thymocytes but did not eliminate the bias toward IL-17 production (Figure S3). Thus, enhanced ERK signaling is not only critical for γδ T cell lineage commitment, but also regulates maturation of cells into functional cluster B and influences selection of the IFN-γ or IL-17-producing fates.

(D) Dendritic epidermal γδ T cells were analyzed by flow cytometry on skin preps from *Erk1*$^{+/+}$*Erk2*$^{+/+}$ and *Erk1*$^{-/-}$*Erk2*$^{-/-}$ *Tcrb*$^{-/-}$ mice as above. Histograms depicting electronically gated Thy^+ cells and absolute numbers of the indicated populations are depicted graphically as above (bottom panel). *$p < 0.05$

(E) Development of *Erk1*$^{+/+}$*Erk2*$^{+/+}$ and *Erk1*$^{-/-}$*Erk2*$^{-/-}$ KN6 Tg thymocytes was assessed by flow cytometry on single cell thymic suspensions from 6- to 7-week-old mice (left panels). Gate frequencies of the indicated populations were used to calculate the absolute number of thymocytes subsets, which are depicted graphically (right panels). *$p < 0.05$

Error bars represent SEM. See also Figures S1 and S2.

The ERK Signals that Promote γδ Lineage Commitment Posttranscriptionally Induce Egr1

To determine whether the more robust ERK signaling that promotes γδ T cell lineage commitment and maturation is more intense, more prolonged, or both, we removed KN6 Tg thymocytes from the selecting environment in vivo where they were adopting the αβ (Lig^-) or γδ T cell fate (Lig^+) and cultured them in suspension in vitro to assess the kinetics of decay of ERK phosphorylation (Figure 3A). ERK phosphorylation in thymocytes adopting the γδ fate (Lig^+) persisted, and even increased, over the 60 min course of analysis (Figure 3A). Although the basis for this increase in ERK phosphorylation remains unclear, it might involve escape from negative regulation induced by chronic TCR signaling in vivo. In contrast to the prolonged ERK signaling observed in progenitors adopting the γδ T cell fate, ERK phosphorylation in thymocytes adopting the αβ fate (Lig^-) was both less intense and shorter in duration, as it began to diminish after 20 min of culture (Figure 3A). These data demonstrate that γδ T cell lineage commitment is associated with ERK signals of greater amplitude and duration, and raise the question of how these more intense and sustained ERK signals promote adoption of the γδ T cell fate.

ERK signals of greater amplitude would be expected to result in greater phosphorylation of conventional substrates such as ribosomal subunit kinase (RSK), which is mediated through the D-domain of ERK. Consistent with this expectation, RSK phosphorylation was more pronounced in adult KN6 γδTCR Tg thymocytes adopting the γδ T cell fate in the presence of $T10^d$ ligand and was ERK-dependent as it was diminished by ERK deficiency (Figure 3B).

ERK is also capable of interacting with binding partners through a distinct interface, the DBP-domain, although the relevance of this mode of action to development has never been assessed (Dimitri et al., 2005). The DBP-domain mediates interactions with DEF-domain-containing targets, many of which are encoded by IEG, and can increase their stability (Murphy and Blenis, 2006). This mode of action has been hypothesized to underlie the execution of ERK signals of increased duration (Murphy and Blenis, 2006). Accordingly, the prolongation of ERK signals associated with adoption of the γδ T cell fate would be expected to increase the stability of IEG protein products. To test this possibility, we focused on the IEG, Egr1, since we had previously shown that Egr proteins play an important role in γδ lineage commitment (Lauritsen et al., 2009). Quantitation of Egr1 mRNA and protein revealed that KN6 Tg thymocytes committing to the γδ T cell fate (Lig^+) expressed 2.5-fold more Egr1 mRNA than did cells adopting the αβ T cell fate, but more than 20 times more Egr1 protein, consistent with posttranscriptional control of Egr1 expression (Figure 3C) (Lee et al., 2010). This was not due to an intrinsic difference in Lig^- progenitors, because posttranscriptional accumulation of Egr1 protein also occurred in those progenitors upon antibody stimulation (Figure S4A). Moreover, the posttranscriptional accumulation of Egr1 protein in cells adopting the γδ T cell fate is dependent on ERK signaling, because Egr1 protein expression is markedly reduced in ERK-deficient KN6 Tg Lig^+ progenitors, despite expressing equivalent levels of Egr1 mRNA (Figure 3D). The dependence of Egr1 protein accumulation on ERK signaling raised the possibility that this involved physical interaction between Egr1 and active ERK. In fact, coimmunoprecipitation analysis revealed that Egr1 protein was associated with ERK in KN6 Tg thymocytes adopting the γδ T cell fate in the presence of ligand (Lig^+), but not in cells adopting the αβ fate in the absence of ligand (Lig^-) (Figure 3E). Association of ERK with Egr1 was observed in both anti-ERK and anti-Egr1 immunoprecipitates of Lig^+ thymocytes, but not in Lig^- thymocytes, even when Egr1 was transgenically overexpressed (Figure 3E). To determine whether the increased Egr1 protein observed in KN6 Tg Lig^+ thymocytes resulted from increased protein stability, we performed metabolic labeling and pulse-chase analysis. We found that Egr1 protein was far more stable in KN6 Tg Lig^+ thymocytes adopting the γδ T cell fate, than in KN6 Tg Lig^- Egr1 Tg thymocytes (Figure 3F). Global protein synthesis was unchanged in KN6 Tg Lig^+ thymocytes (Figure S4B). Taken together, these data demonstrate that adoption of the γδ T cell fate is associated with prolonged ERK signals that lead to the physical interaction of ERK with IEG such as Egr1, which increases Egr1 protein stability.

ERK Promotes γδ T Cell Development through a Noncanonical, DBP-Mediated Mode of Action

Adoption of the γδ T cell fate is promoted by ERK signals that are more intense and prolonged, and lead to both greater phosphorylation of conventional substrates through the D-domain and stabilization of IEG such as Egr1, through the DBP-domain. To test the contribution of these two modes of ERK action to adoption of the γδ T cell fate, we mutated the ERK domains responsible. ERK2 interaction with DEF containing targets is mediated by the DBP domain and this can be disrupted by the Y261A mutation (Dimitri et al., 2005). Interactions with conventional substrates like RSK are mediated by the ERK2 D-domain and can be disrupted by the D319N mutation (Dimitri et al., 2005). To verify that these mutations had the intended effects, we expressed them in ERK-deficient fetal liver (FL) progenitors, where they were phosphorylated to similar extents upon PMA and ionomycin stimulation (Figure 4A). The D319N D-domain mutation attenuated RSK phosphorylation by ERK2 in KN6 $γδTCR^+$ progenitors cultured on ligand-expressing OP9 monolayers (Figure 4B); however, it did not disrupt the interaction of ERK2 with Egr1, as measured by coimmunoprecipitation (Figure 4C). The Y261A DBP-domain mutation, which disrupts interaction with DEF containing IEG targets, only minimally affected RSK phosphorylation, but completely blocked coprecipitation of ERK2 with Egr1 (Figures 4B and 4C). Consequently, the ERK2 D319N and Y261A mutations had the expected effects of blocking phosphorylation of conventional substrates and interaction with DEF domain-containing targets, respectively. To determine how disruption of substrate phosphorylation and interaction with DEF domain-containing targets affected γδ T cell development, we expressed the mutant ERK2 molecules in ERK-deficient KN6 γδTCR Tg FL progenitors and assessed the effect on maturation on OP9-DL1 monolayers expressing ligand (Figure 4D). As we observed in intact KN6 γδTCR Tg progenitors developing in vivo, ERK-deficiency impaired their maturation in vitro as measured by CD24 downmodulation (Figure 4D). Retroviral reconstitution of these cells with wild-type (WT) ERK2 restored maturation and this was not impaired by abrogation of the ability of ERK2

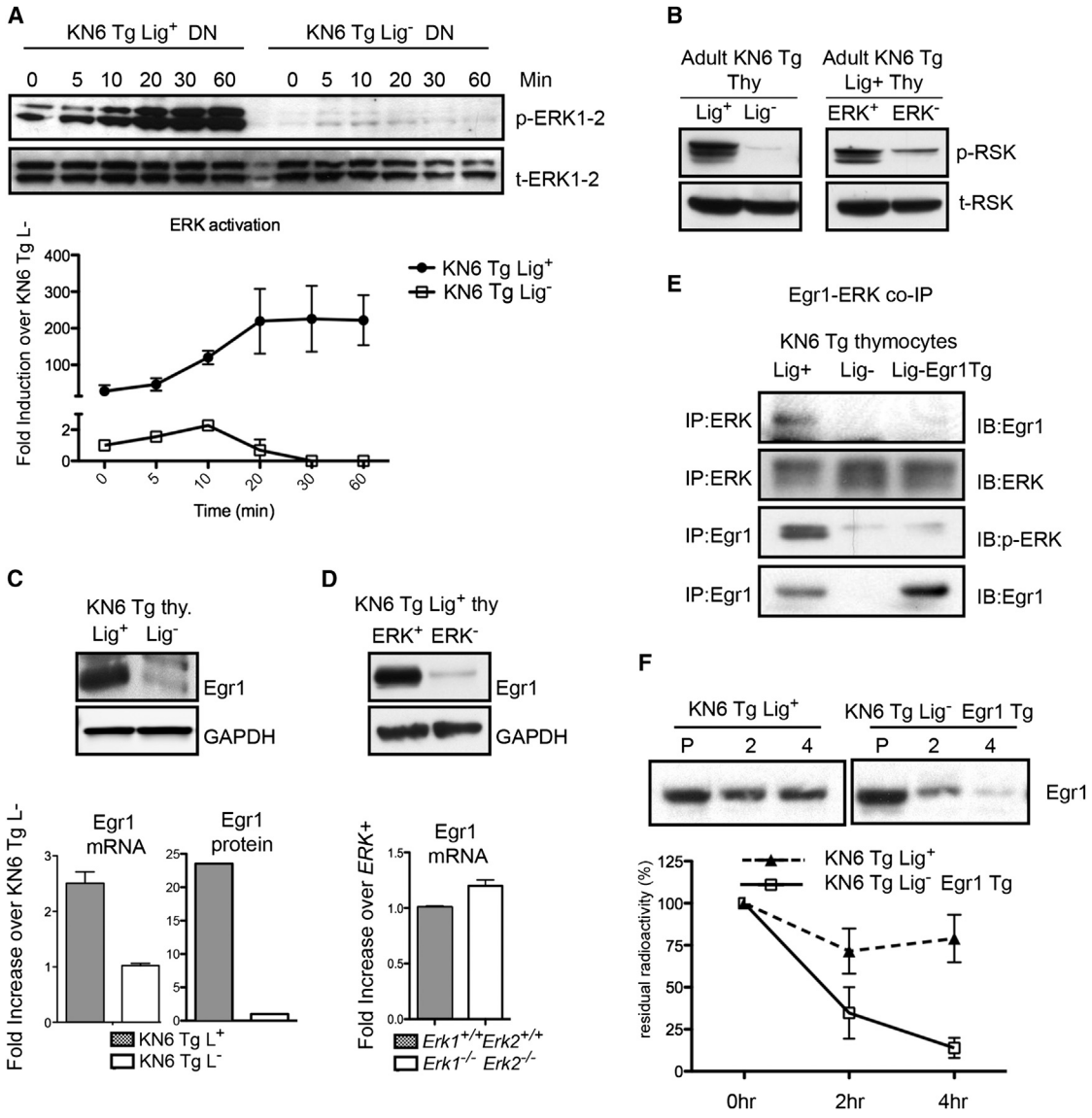

Figure 3. Prolonged and More Intense ERK Signals Associated with γδ T Cell Development Promote ERK-Egr1 Interactions and Increased Egr1 Protein Stability

(A) KN6 γδTCR Tg DN thymocytes adopting the γδ T cell fate (Lig$^+$) or αβ fate (Lig$^-$) were cultured in suspension and the persistence of ERK phosphorylation was assessed by anti-phospho ERK immunoblotting of detergent extracts.

(B) The role of ligand stimulation in ERK phosphorylation of RSK was examined in adult KN6 γδTCR Tg thymocytes developing in the presence (Lig$^+$) or absence of (Lig$^-$) T-10^d ligand (left panels) or lacking ERK (ERK$^+$ and ERK$^-$; right panels). RSK phosphorylation was measured by phosphoblotting.

(C and D) Egr1 protein and mRNA expression were measured by immunoblotting and real-time PCR, respectively, in KN6 Tg thymocytes adopting the γδ T cell fate (Lig$^+$) or the αβ T cell fate (Lig$^-$) in ERK-expressing (ERK$^+$) or deficient (ERK$^-$) mice. Protein levels were quantified by measuring the fluorescence emitted by bound anti-Egr1 antibody using the Li-COR.

(E) To assess the extent of ERK-Egr1 interaction, detergent extracts from DN of the indicated genotypes were immunoprecipitated with either anti-ERK or anti-Egr1 following which associated proteins were detected by immunoblotting.

(F) The stability of newly synthesized Egr1 protein was measured by performing metabolic labeling for 30 min with ^{35}S-methionine, chasing the labeled cells for 2 and 4 hr, and then immunoprecipitating Egr1 protein. The quantity of labeled Egr1 protein remaining during the chase was determined by phosphorimagery and normalized to incorporation during the pulse (bottom panel). The data are representative of at least two independent experiments. All error bars indicate mean ± SEM. See also Figure S4.

to phosphorylate conventional substrates (Figure 4D; ERK2 D319N). However, maturation was abrogated when the alterative mode of ERK action, enhancement of protein stability through DBP-domain interactions with IEG, was attenuated (Figure 4D; Y261A). These results indicate that the ERK signals that promote γδ T cell maturation do not require the ability of ERK to phosphorylate substrates like RSK, and instead depend on a noncanonical mode of ERK function that involves

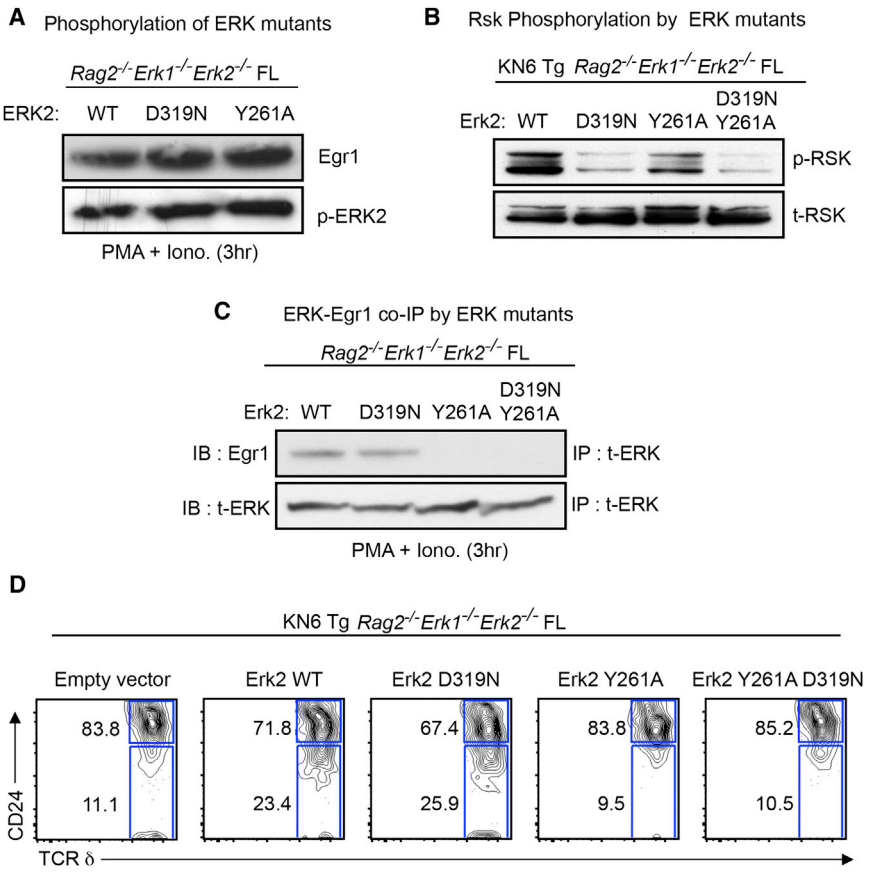

Figure 4. ERK Interaction with DEF Domain-Containing Targets through Its DBP Domain Is Required for ERK to Promote γδ T Cell Development

(A–C) To determine whether mutations abrogating function of the D-domain (D319N) or DBP domain (Y261A) of ERK2 had the intended effects, we transduced these mutant constructs along with WT ERK2 into progenitors of the indicated genotypes, cultured for 7 days on OP9-DL1 cells expressing ligand, and analyzed them without further treatment (B) or following stimulation with PMA and ionomycin (A and C). The effect of mutations on RSK phosphorylation was evaluated by phosphoblotting and the effect on ERK2-Egr1 association was assessed by immunoprecipitating with anti-ERK antibody and immunoblotting with anti-Egr1 or anti-ERK antibodies (A–C).

(D) The effect on γδ T cell development of abrogating ERK interaction with substrates (ERK2 D319N) or DEF domain-containing targets (ERK2 Y261A) was assessed by reconstituting ERK-deficient KN6 γδTCR Tg progenitors with WT or mutant ERK2 molecules and culturing them on ligand expressing OP9-DL1 monolayers. Following 6 days of culture, development was monitored by flow cytometry on electronically gated GFP$^+$ cells. Contour plots showing TCRδ and CD24 expression are depicted. The data are representative of at least two independent experiments.

DBP-domain mediated interactions with DEF domain-containing molecules like Egr1.

To determine whether development of γδ T cells in vivo was also dependent on DBP-domain based interactions with DEF domain containing targets, we constructed bone-marrow chimeras transduced with the *Erk2* mutants above. KN6 Tg *Rag2*$^{-/-}$*Erk1*$^{-/-}$*Erk2*$^{-/-}$ bone-marrow progenitors were retrovirally transduced with WT and mutant *Erk2* molecules and transferred into lethally irradiated *Rag2*$^{-/-}$ recipients (Figure 5). Repopulation of early hematopoietic progenitors was equivalent for all constructs (Figure S5). Consistent with the results of our in vitro analysis with FL progenitors (Figure 4D), bone-marrow chimeras reconstituted with ERK-deficient progenitors transduced with empty vector failed to commit to the γδ T cell fate as indicated by induction of CD73 (Figure 5A, top panel) (Coffey et al., 2014). γδ T cell lineage commitment (i.e., CD73 induction) was rescued by retroviral transduction with WT and D-domain mutant ERK2 (D319N); however, the DBP-domain mutant (Y261A) ERK2, which is unable to interact with DEF-domain containing targets, failed to rescue lineage commitment (Figure 5A; top panels). The DBP-domain (Y261A) ERK2 mutant also failed to restore maturation as indicated by CD24 downmodulation (Figure 5A; middle panels) or the acquisition of function as measured by the accumulation of the cluster B (CD44$^+$ CD24^{lo}) γδ T cell subset (Figure 5A; bottom panels). Conversely, the D-domain (D319N) ERK2 mutant restored both maturation and acquisition of effector fate to the same extent as WT ERK2. Taken together, these results support the surprising conclusion that γδ T cell lineage commitment and maturation do not depend on the ability of ERK to phosphorylate conventional substrates such as RSK, through its D-domain, despite the fact that most (80%) ERK targets are selected in this manner. Instead γδ T cell lineage commitment and maturation are critically dependent upon an alternative mode of ERK function, physical interaction with DEF-domain-containing targets mediated by its DBP domain. This represents the first demonstration of the importance of this noncanonical mode of ERK action in lymphoid development.

Prolonged ERK Signals Facilitate Transactivation of Targets by Egr1

Our data support a model where ERK promotes γδ T cell development through DBP-domain-mediated association with substrates that increases their expression by increasing their stability; however, it remained unclear how increasing protein stability might promote adoption of the γδ fate. One possibility is that these interactions could stabilize IEG that function as transcription factors, thereby enabling them to transactivate targets more effectively than is possible following transient ERK signals. To address this possibility, we focused on Egr1, because Egr1 is an IEG that we have previously shown to be an important effector of the TCR signals that promote γδ T cell development (Lauritsen et al., 2009). Egr1 modulated genes were identified by microarray analysis of a thymic lymphoma in which Egr1 was ectopically

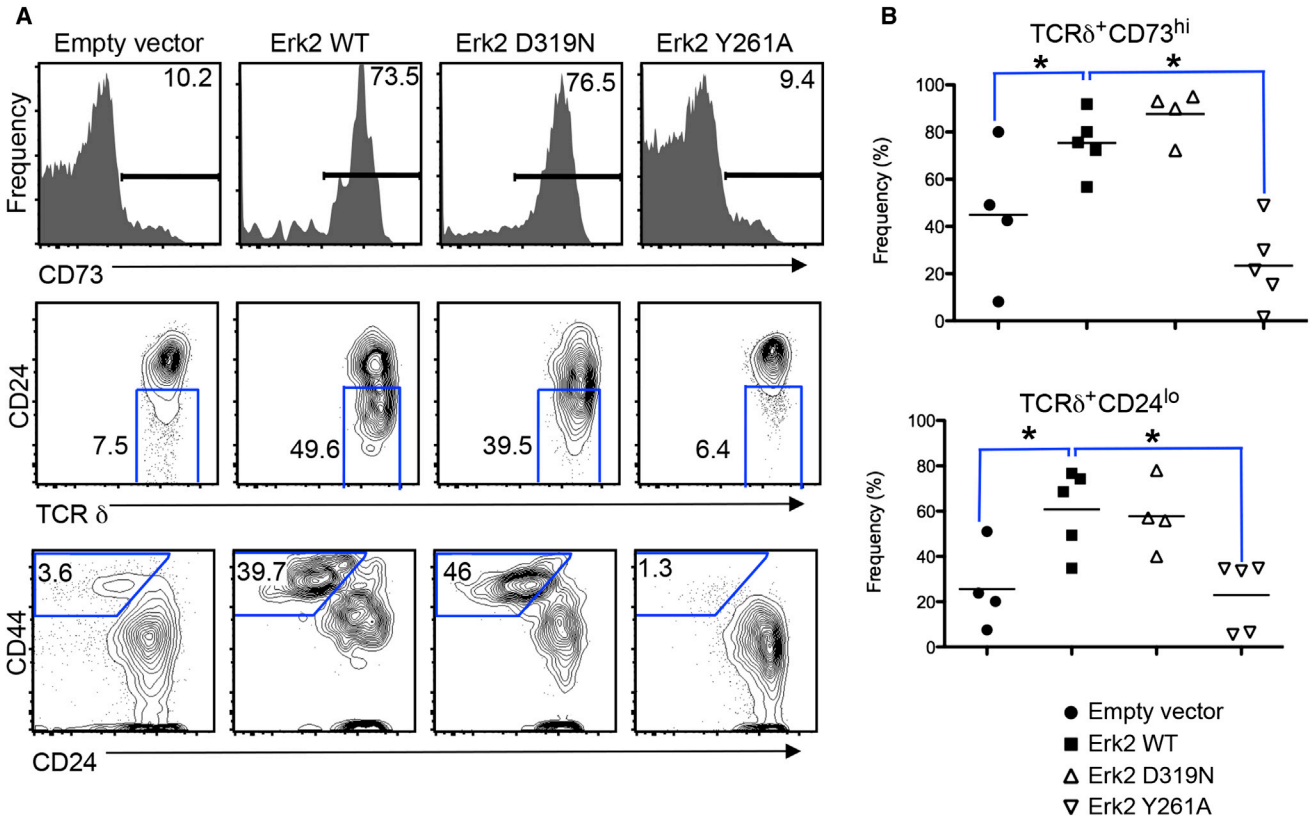

Figure 5. ERK Signals Require DBP-Mediated Interactions to Promote γδ T Cell Development In Vivo

(A) *Erk1*$^{-/-}$*Erk2*$^{-/-}$ KN6 γδTCR Tg *Rag2*$^{-/-}$ bone-marrow progenitors were retrovirally reconstituted with WT or the indicated ERK2 mutants and transferred to *Rag2*$^{-/-}$ hosts. After 7 weeks, the ability of the transduced ERK2 mutant to restore γδ T cell development was assessed by flow cytometry on electronically gated GFP^{+} thymocytes.

(B) The frequency of TCRδ^{+} CD24^{lo} and TCRδ^{+} CD73^{hi} cells was depicted graphically, with each symbol representing an individual mouse and the horizontal line indicating the mean. *$p < 0.05$. See also Figure S5.

expressed. Egr1 expression resulted in the differential expression of 624 probes (Table S1). To determine whether these targets were enriched among those modulated during γδ T cell lineage commitment, we also performed microarray analysis of KN6 progenitors committing to the γδ T cell fate in the presence of ligand (Lig^{+}) or to the αβ fate in its absence (Lig^{-}) (Table S1). Indeed, hypergeometric analysis revealed that more than 40% of the Egr1 targets were found among those genes differentially expressed during commitment to the γδ T cell fate, representing substantial enrichment (Figure 6A; $p < 0.001$). Moreover, these Egr1 targets were more significantly modulated by the prolonged and more intense ERK signals associated with γδ T cell lineage commitment (Figure 6B; Lig^{+}) than by the shorter and less intense signals associated with adoption of the αβ fate (Figure 6B; Lig^{-}). Thus, the promotion of γδ development by ERK signals is accompanied by the stabilization of the IEG transcription factor Egr1, which enables it to more effectively transactivate its gene targets (Figures 6 and S6). ERK-IEG interactions might also promote alternative cell fates through actions on proteins in other gene ontology classes, because adoption of the γδ fate is also linked to modulation of DEF-domain containing genes involved in signal transduction and metabolism, as well as other functions (Figure 6C).

The Alternative Mode of ERK Function Is Required for Maturation of αβ Lineage DP Thymocytes

Because graded induction of ERK activity has been implicated in fate specification at a number of developmental checkpoints and lineage branch points encountered by lymphocytes, we sought to determine whether the DBP-mediated mode of ERK function might play an important role in other developmental processes. To do so, we employed zinc-finger nuclease (ZFN) mutagenesis to introduce the ERK2 Y261A mutation into the endogenous murine *Erk2* locus (Figure 7A). The *Erk2*^{Y261A} allele was then crossed to a floxed *Erk2* allele that was conditionally ablated in T lineage progenitors using *Ptcra-Cre*. Relative to ERK2 WT mice, ERK2 Y261A mice had equivalent thymic cellularity, but exhibited a defect in γδ T cell lineage commitment and maturation, as indicated by impaired CD73 induction and CD24 down-modulation, respectively (Figures 7B and 7C), consistent with our observations in the bone-marrow chimera model (Figure 5). Moreover, upon removal from the selecting milieu, immature CD73^{-}γδTCR^{+} progenitors from ERK2 Y261A mice exhibit more extensive diversion to the αβ fate, as indicated by their development to the DP stage when cultured on OP9-DL1 monolayers (Figure S7). Differentiation of αβ lineage thymocytes beyond the β-selection checkpoint to the DN4 stage and

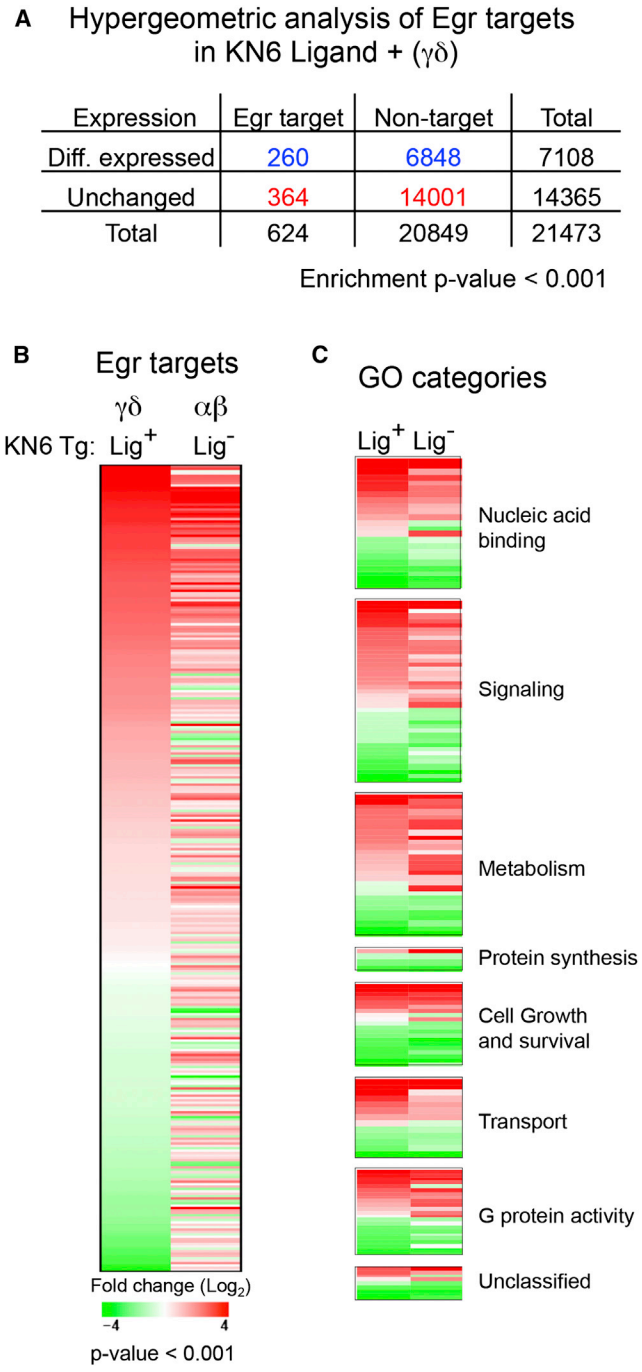

Figure 6. Egr1 Target Genes Are Preferentially Modulated during γδ T Cell Development

(A) Hypergeometric analysis was performed on Egr1-modulated genes to determine the extent of their enrichment among the genes whose expression was modulated during adoption of the γδ fate by KN6 Tg progenitors.

(B) Microarray analysis was performed on KN6 γδTCR Tg thymocytes adopting the γδ fate in the presence of ligand (Lig^+) and the αβ fate in the absence of ligand (Lig^-). Egr1 target genes were identified among the differentially expressed genes and displayed as a heatmap illustrating the relative expression of those Egr1 targets in Lig^+ or Lig^- thymocytes.

(C) DEF domain-containing genes that were differentially expressed in Lig^+ versus Lig^- thymocytes were grouped based on gene ontology and displayed in a heatmap illustrating the differences in expression in Lig^+ versus Lig^- thymocytes. The genes in the heat maps are significantly differentially expressed ($p < 0.0001$). See also Figure S6 and Tables S1 and S2.

ultimately to the DP stage was not impaired by the ERK2 Y261A mutation (Figures 7D and 7E); however, abrogation of ERK DBP-mediated processes by the ERK2 Y261A mutation dramatically impaired the differentiation of αβ lineage thymocytes to the $CD4^+$ and $CD8^+$ single-positive (SP) stages (Figure 7E). The impaired differentiation to the $CD4^+$ and $CD8^+$ SP stages was accompanied by a block in the generation of both $TCR\beta^{hi}$ $CD69^+$ and $TCR\beta^{hi}$ $CD69^-$ thymocytes (Figure 7E), which are positive selection intermediates (Yamashita et al., 1993).

Taken together, our data support a model whereby prolonged ERK signals are required to promote γδ T cell lineage commitment and maturation, by supporting ERK association with and stabilization of DEF domain-containing proteins, which modulates the cellular proteome independently of changes in mRNA levels. This alternative mode of ERK function is dispensable for the initial commitment of pre-TCR-expressing progenitors to the αβ fate, but is required for $\gamma\delta TCR^+$ progenitors to both adopt γδ T cell fate and suppress adoption of the αβ fate. Moreover, it also plays a critical role in the more distal developmental processes encountered by αβ lineage progenitors, including their positive selection.

DISCUSSION

ERK signaling plays a central role in many fate decisions; however, despite intensive efforts, the basis by which differences in ERK signaling contribute to the specification of alternative cell fates remains poorly understood (Raman et al., 2007). Most models postulate a prominent role for differential ERK substrate phosphorylation through its D domain. Here, we provide the first evidence that the differences in ERK activity that underlie separation of the αβ and γδ T cell lineages do so through a non-canonical, alternative mode of ERK function, that involves interaction with DEF domain-containing substrates through its DBP domain. In contrast, the ability of ERK to phosphorylate substrates through its D-domain is dispensable for this process, despite recent evidence indicating that most ERK substrates are targeted in this manner (Carlson and White, 2012). DBP domain-mediated interactions appear to impact fate-specification posttranscriptionally by increasing the stability of DEF domain-containing proteins. Among these are transcription factors such as Egr1, which play an important role in αβ versus γδ lineage commitment. The stronger and more prolonged ERK signals that promote adoption of the γδ T cell fate increase Egr1 protein stability, thereby enabling Egr1 and likely other IEG transcription factors to more effectively transactivate their targets than is possible in response to the weaker and more transient ERK signals associated with adoption of the αβ fate.

The involvement of stronger and more prolonged ERK signals in promoting adoption of the γδ T cell fate raises the question of how such signals are generated. Many regulators of ERK signal intensity and duration have been identified (Andreadi et al., 2012), such as the linkage of B-Raf to prolonged ERK activation (Tsukamoto et al., 2004). B-Raf is thought to prolong ERK

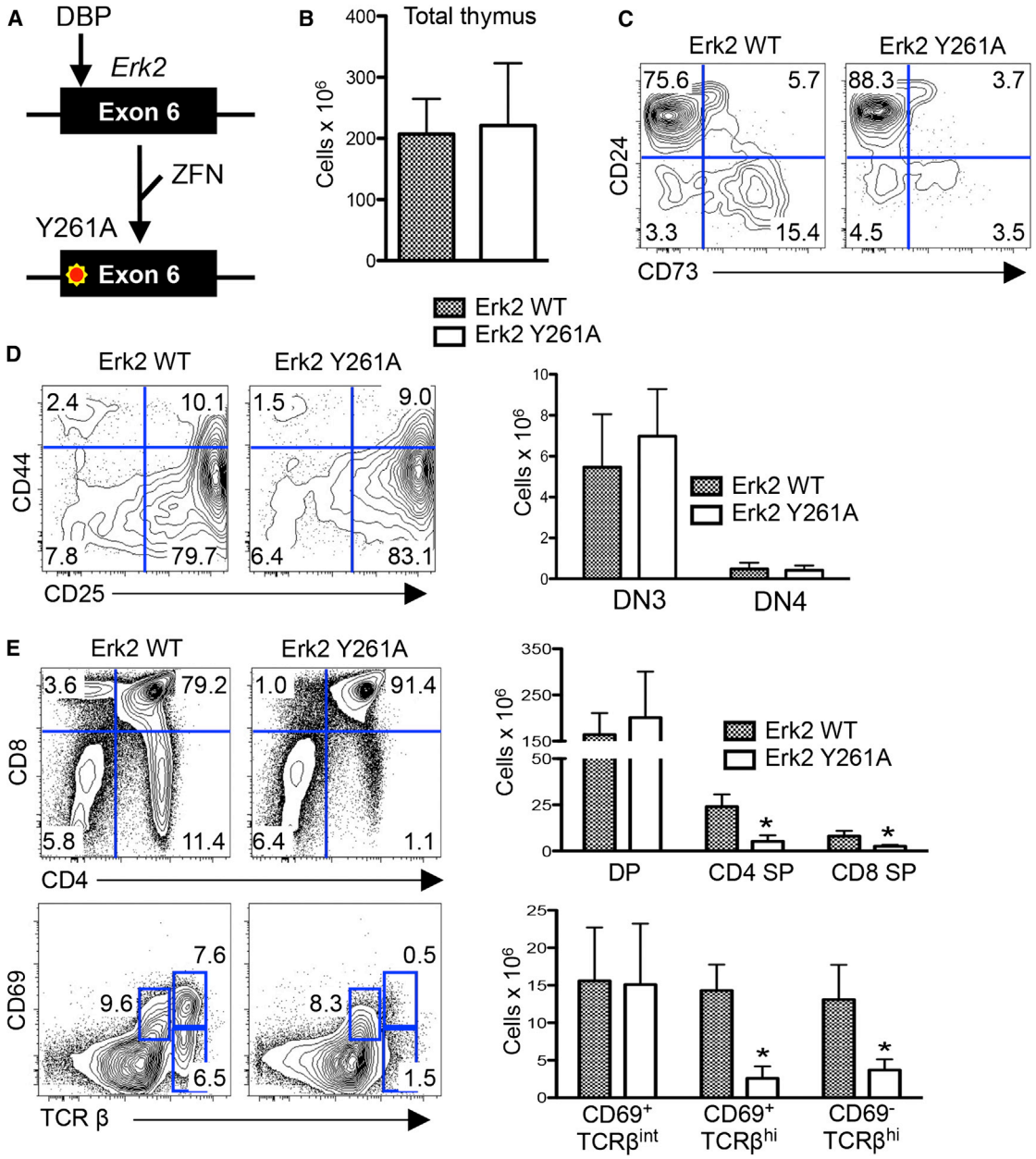

Figure 7. Role of ERK-DBP Interactions in Promoting Positive Selection of αβ T Cells

(A) Schematic of the strategy employed to mutagenize the DBP domain of ERK2 encoded by exon 6 of the murine *Erk2* locus.

(B–E) The effect of disabling the DBP domain of ERK2 on T cell development was assessed by performing flow cytometry on single cell suspensions of thymocytes from *Erk1*$^{+/-}$*Erk2*$^{fl/fl}$ *Ptcra-Cre*$^{-}$ and *Erk1*$^{+/-}$*Erk2*$^{Y261A/fl}$ *Ptcra-Cre*$^{+}$ mice. Gate frequencies of the indicated populations were used to calculate the absolute number of thymocyte subsets, which are depicted graphically (right panels). Data shown are the means ± SEM. *p < 0.05. See also Figure S7.

signaling through a newly described adaptor molecule, Kidins220, which is expressed in T lineage progenitors and associates with both the pre-TCR and γδTCR (Deswal et al., 2013). The duration of ERK signals has also been linked to the extent of heterodimerization of upstream signaling molecules, MEK1 and MEK2 (Catalanotti et al., 2009). Finally, pharmacologic inhibition of P2X7 (ATP-gated nonselective cationic receptor) has been reported to impair ERK activation, blunt Egr1 induction, and divert γδ T cell progenitors to the αβ T lineage fate (Frascoli et al., 2012).

How these regulators of ERK signaling might modulate ERK signals in the context of αβ versus γδ lineage commitment process remains unclear. One possibility is that these regulators of ERK signaling are differentially expressed in distinct progenitor pools, and could predispose certain progenitors to adopt the γδ fate. Nevertheless, accumulating evidence suggests that the pre-TCR and γδTCR complexes are acting instructionally to direct uncommitted progenitors to adopt a lineage fate, rendering this possibility less likely (Kreslavsky et al., 2008). An alternative

explanation is that these regulators selectively associate with either the pre-TCR or γδTCR and confer upon those receptors the tendency to induce weak or transient and strong or sustained ERK activation, respectively; however, none of the molecules identified to date have been selectively linked to either TCR isotype. Moreover, we demonstrate here that the KN6 γδTCR is capable of promoting either weak/transient or strong/sustained ERK signals (Figure 3). This observation raises another possibility, that the transduction of stronger and more sustained ERK signals by the γδTCR is dependent, at least in some cases, on TCR ligand-engagement. Consistent with this possibility, we recently identified a TCR-ligand inducible surface marker of γδ lineage commitment, CD73 (Coffey et al., 2014). CD73 is expressed by ~25% of γδTCR$^+$ cells in the thymus and by 90% of peripheral γδ T cells, suggesting that ligand-engagement is extensively involved in specification of the γδ T cell fate, even among the IL-17 producing γδ T cell effector subset, which has been suggested to develop in a ligand-naive manner (Coffey et al., 2014; Jensen et al., 2008). This is consistent with a recent report indicating that TCR agonist stimulation plays a role in the development of at least some subsets of IL-17 producing γδ T cell cells (Wencker et al., 2013). These authors also reported that agonist stimulation in the thymus serves to blunt γδTCR signaling capacity, consistent with our observation that CD24^{lo} mature KN6 γδTCR Tg γδ cells exhibit less ERK phosphorylation than CD24^{hi} immature γδ T cells (Figure S1) (Wencker et al., 2013).

There has been considerable effort directed toward understanding how differences in ERK signaling lead to alternate developmental outcomes. One view is that ERK signals of differing intensity or duration might enable ERK to phosphorylate a distinct spectrum of substrates due to trafficking of ERK to distinct subcellular locations, such as cytosol versus nucleus. von Kriegsheim et al. recently reported that stimulation of PC12 cells with EGF or NGF leads to alternate fate outcomes, and these outcomes (proliferation versus neuronal differentiation, respectively) are associated with the targeting of ERK to distinct subcellular locations (von Kriegsheim et al., 2009). The differences in ERK localization were, in turn, linked to differential association with neurofibromin 1 (NF1) and PEA-15 (von Kriegsheim et al., 2009). NF1 is a Ras GTPase activating protein whose loss in T cells impaired both development and peripheral function (Ingram et al., 2002). PEA-15 association with ERK prevents its translocation to the nucleus, but its loss does not impair T cell development (Formstecher et al., 2001; Pastorino et al., 2010). Other mechanisms, such as differences in the dimerization of MEK1 and MEK2, have also been reported to influence nuclear localization (Catalanotti et al., 2009). Irrespective of the mechanism of altering the subcellular localization of ERK, this remains an attractive mechanism by which differences in the duration of ERK activity can lead to alternate cell fates. Nevertheless, our results suggest that at least in the context of αβ versus γδ lineage commitment and in positive selection of αβ lineage thymocytes, these potential differences in subcellular localization do not specify fate by phosphorylation of substrates through the D-domain of ERK, and instead, depend on an alternative mode of ERK action, mediated by its interaction with DEF domain-containing targets through its DBP domain.

How might interactions between ERK and DEF domain-containing proteins promote the alternate fates specified by differences in the intensity or longevity of ERK signals? The IEG (immediate early gene) sensor hypothesis posits that prolonged ERK signaling alters developmental outcomes by modulating the stability of IEG protein products (Murphy and Blenis, 2006). Specifically, irrespective of their intensity, transient ERK signals (less than 30 min) decay prior to synthesis of IEG protein products, resulting in their rapid degradation. Conversely, prolonged ERK signals (60 min or longer) are proposed to persist until IEG protein products are produced, enabling ERK to bind IEG containing DEF-domains through its DBP domain and increase their stability. Here, we provide the first evidence of the importance of this mechanism in development. We propose that there are several ways by which increasing protein stability might lead to a distinct developmental outcome. Stabilization of IEG encoding transcription factors enables those transcription factors to transactivate targets to a greater extent than would be possible under conditions where ERK signaling is transient, and we have observed this for the targets of the zinc-finger transcription factor Egr1 (Figure 6). The prolonged ERK signals also result in more profound repression of Egr targets than is observed in cells adopting the αβ T cell fate (Figure 6); however, the factors that determine whether a target is induced or repressed remain to be determined. While the prolonged ERK signals that promote γδ T cell lineage commitment clearly increased the stability and expression of Egr1 protein, this was not the case for all DEF-domain containing transcription factors expressed in cells adopting the γδ T cell fate. Specifically, while c-Jun protein levels were increased disproportionately relative to its mRNA, this was not observed for other DEF domain containing IEG, including c-Myc, Fra-2, and c-Fos (Figure S6). The factors that determine whether prolonged ERK signals will stabilize a particular DEF domain-containing target protein remain poorly understood. Thus, in order to understand the basis by which this mode of ERK action promotes fate specification in distinct developmental contexts, proteomic analysis must be performed to identify the subset of ERK targets that is stabilized. Nevertheless, it is clear that along with transcription factors, there are DEF domain-containing proteins that belong to other ontologic classes (signaling, metabolism, protein synthesis, etc.), raising the possibility that DBP-domain-mediated interactions with DEF-domain-containing targets can influence fate by affecting essentially any cellular process.

Our data support the surprising finding that the ability of ERK to phosphorylate conventional substrates such as RSK through its D-domain is dispensable for adoption of the γδ T cell fate. Instead, they reveal an unexpected dependence of this process on a noncanonical mode of ERK action, i.e., physical interaction with DEF domain-containing proteins mediated by the DBP motif, which acts to markedly increase expression and stability of target proteins. This mode of action, which had not previously been investigated in the context of normal developmental processes in vivo, is clearly critical for both specification of the γδ T cell fate and for positive selection of αβ T cell lineage thymocytes. Differential activation of ERK has also been implicated in essentially every developmental checkpoint encountered by T lymphocytes, (Bosselut, 2004; Hernández-Hoyos et al., 2000). These include ligand-mediated selection events that shape the

TCR repertoire of αβ lineage T cells, CD4$^+$ versus CD8$^+$ T cell lineage commitment, and peripheral effector fate specification (Daniels et al., 2006; Liu et al., 2013; McNeil et al., 2005; Singer et al., 2008). Accordingly, it will be important to investigate the mode of ERK action in these differentiation processes to assess their dependence on the alternative mode of ERK function, which involves its interaction with substrates through its DBP-domain.

EXPERIMENTAL PROCEDURES

Mice

All mice were maintained in Fox Chase Cancer Center's AALAC-accredited animal colony. KN6 γδ TCR Tg, (Haks et al., 2005), *Erk1*$^{-/-}$*Erk2*$^{fl/fl}$ (Fischer et al., 2005), and *Ptcra-Cre* mice (Luche et al., 2013) were described previously. TCRβ-deficient mice were purchased from Jackson Laboratory. *Erk1*$^{-/-}$*Erk2*$^{fl/fl}$ mice were crossed to *Ptcra-Cre* mice, and then to both KN6 γδ TCR Tg and TCRβ-deficient mice. All experiments were approved by the Institutional Animal Care and Use Committee.

Flow Cytometry

Flow cytometry was performed on single cell suspensions as described in Supplemental Experimental Procedures.

Coimmunoprecipitatation and Immunoblot Analysis

DN were isolated by negative selection using magnetic beads or by flow cytometry and lysed with NP40 lysis buffer at 4°C as described (Lauritsen et al., 2009). Equal quantities of protein were resolved by SDS-PAGE and immunoblotted with the following antibodies as described (Lauritsen et al., 2009): anti-pERK (Cell Signaling; 9106S), anti-total ERK (Cell Signaling; 9102), anti-pRSK (Cell Signaling; 12032), anti-Egr1 (Cell Signaling; 4153) or anti-total RSK (Cell Signaling; 8408), anti-c-Fos (Cell Signaling; 2250), anti-c-Jun (Cell Signaling; 9165), anti-c-Myc (Cell Signaling; 9402), anti-Fra2 (Santa Cruz; sc-604) and GAPDH (Millipore; MAB374). Fold-change values of p-ERK were calculated using the Odyssey Imaging System (Li-Cor). For coimmunoprecipitation analysis, NP40 extracts were immunoprecipitated with either anti-Egr1 or anti-ERK and subjected to immunoblotting with the indicated antibodies.

Pulse-Chase Analysis

Thymocytes were subjected to pulse-chase analysis, by labeling them for 30 min with ^{35}S-methionine/cysteine at 1 mCi/ml in cysteine- and methionine-free medium, following which the labeled cells were cultured as indicated in medium containing a 10-fold excess of unlabeled methionine. Egr1 was isolated from NP-40 extracts (anti-Egr1, Cell Signaling), resolved by SDS-PAGE, visualized by fluorography, and quantified by Image Gauge Software.

Bone-Marrow Chimeras

Bone-marrow chimeras were constructed as described in Supplemental Experimental Procedures.

Real-Time PCR

Expression of specific RNAs was quantified by real-time PCR as described in Supplemental Experimental Procedures.

Cytokine Production Analysis

Single cell suspensions of thymocytes were stimulated with 5 μg/ml plate bound anti-CD3 antibody with or without IL-1 and IL-23. Supernatants were added to duplicate wells on the ELISA plate and were assayed for IL-17 and IFN-γ using standard sandwich ELISA protocols.

Retroviral Transduction and OP9 Culture

Development of KN6 γδTCR expressing progenitors on OP9-DL1 monolayers was assessed as described in Supplemental Experimental Procedures.

ZFN-Mediated Mutagenesis of the *Erk2* Locus

Candidate site-specific ZFN were designed for 150 bp target sequences on each side of *Erk2* exon 6 (Sigma Life Sciences). Plasmids encoding 8 candidate ZFN (4 for each site) were transfected in all possible pairwise combinations into mouse Neuro2A cells to identify the most active ZFN, following which semiquantitative PCR was used to assess relative ability of paired ZFN to introduce DNA-breaks at *Erk2* exon 6. mRNAs encoding validated ZFN were generated by in vitro transcription and injected into fertilized mouse oocytes together with a 100 bp single-strand oligonucleotide spanning the cut site and encoding the single-codon change required to create the Y261A mutation in exon 6. Founder animals were screened by specific PCR assay and Cel1 digestion to identify animals carrying the Y261A mutation, which was confirmed by DNA sequencing.

Statistical Analysis

p values were analyzed from two-tailed Student's t test. The extent of enrichment of Egr targets among the genes modulated during commitment of KN6 progenitors to the γδ fate was assessed using hypergeometric analysis.

ACCESSION NUMBERS

Microarray data have been deposited with the Gene Expression Omnibus (GEO) database under GSE63730.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.10.021.

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