Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras-ERK MAPK cascade

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Activation of mitogen-activated protein kinase (MAPK) pathways leads to cellular differentiation and/or proliferation in a wide variety of cell types, including developing thymocytes. The basic helix-loop-helix (bHLH) proteins E12 and E47 and an inhibitor HLH protein, Id3, play key roles in thymocyte differentiation. We show here that E2A DNA binding is lowered in primary immature thymocytes consequent to T cell receptor (TCR)-mediated ligation. Whereas expression of E2A mRNA and protein are unaltered, Id3 transcripts are rapidly induced upon signaling from the TCR. Activation of Id3 transcription is regulated in a dose-dependent manner by the extracellular signal-regulated kinase (ERK) MAPK module. These observations directly connect the ERK MAPK cascade and HLH proteins in a linear pathway.

The E proteins are members of the highly conserved basic helix-loop-helix (bHLH) family of transcriptional regulatory proteins. This subclass of HLH proteins includes the mammalian E2A, E2-2 and HeLa E-box–binding (HEB) proteins and the *Drosophila* gene product Daughterless. E proteins are closely related within the bHLH domain and two conserved NH_2-terminal-transactivation domains. They have the potential to bind canonical E-box elements either as homodimers or as heterodimers with tissue-specific bHLH proteins[1]. The E proteins perform numerous functions during vertebrate development. For example, the mammalian E proteins play key roles in cell-fate decisions by functioning as dimerization partners for lineage-specific HLH proteins such as the myogenic factors MyoD, myogenin and Myf5 and the neurogenic factors mammalian achaete-scute homolog 1 (MASH1), MASH2, neurogenin and NeuroD (also called Beta2)[2–4].

E proteins are also required for various aspects of lymphocyte development. B lymphopoiesis in E2A-deficient mice is arrested at an early stage that precedes the onset of immunoglobulin gene rearrangement[5,6]. Similarly, T-lineage differentiation is dependent on the activity of both E2A and HEB. Thymocytes in HEB-null mutant mice are blocked at the immature single-positive (SP) stage. In contrast, thymocyte development in E2A-deficient mice is disrupted at an early double-negative (DN) stage, before the rearrangement of the T cell receptor (TCR) β chain locus[7–9]. Additionally, proper thymocyte maturation, which is mediated by both major histocompatibility complex (MHC) class I– and class II–restricted TCRs, requires E2A activity[10].

E2A proteins have also been implicated in cell growth, apoptosis and lymphomagenesis. For example, mice carrying targeted mutations of the locus that encodes E2A rapidly develop malignant T cell lymphomas and these E2A-deficient lymphomas undergo apoptosis when activity of the gene is restored[7,11,12]. Additionally, ectopic expression of E2A inhibits cell cycle progression and promotes apoptosis in a human T-acute lymphoblastic leukemia (T-ALL)[13]. There is now considerable evidence linking the development of various T cell lymphomas with the inactivation of E2A activity, which suggests that E2A functions as a tumor suppressor[12,14].

Transcriptional activity of the E proteins can be regulated by the expression of another subclass of HLH proteins called Id proteins. The Id HLH inhibitor proteins lack a basic DNA-binding domain and consequently they function as dominant-negative inhibitors of the E proteins by forming non-DNA-binding heterodimers[15]. The family of genes that encode the Id proteins includes four mammalian members (*ID1*, *ID2*, *ID3* and *ID4*) and the *Drosophila* gene *extramachrochaete* (*emc*)[15]. Recent gene-targeting experiments have shown functional roles for the vertebrate Id proteins in several developmental processes. Although Id1 expression was shown to be dispensable for normal murine development, Id2-null mutant mice showed profound defects in peripheral lymphoid organ formation and development of natural killer (NK) cells and Id3-deficient mice displayed defects during the B cell secondary immune response[11,16,17].

Other data has recently revealed a key role for Id1 and Id3 in angiogenesis. Specifically, $Id1^{-/-}Id3^{-/-}$ double-deficient mice showed vascular abnormalities in the forebrain and a lack of sprouting and branching of blood vessels into the neuroectoderm[18]. In addition, tumors failed to grow in $Id1^{+/-}Id3^{-/-}$ mice as a result of poor vascularization and an inability to metastasize[18]. Id3 is also required for proper development of T-lineage cells[19]. Specifically, maturation of DP thymocytes into SP cells is severely defective in the absence of Id3[19]. In contrast, thymocyte maturation in $E2A^{-/-}Id3^{-/-}$ double-deficient mice is normal,

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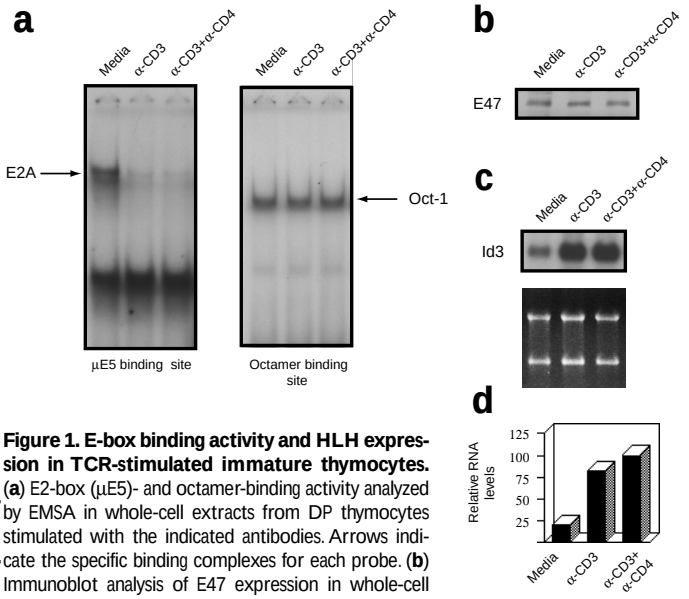

Figure 1. E-box binding activity and HLH expression in TCR-stimulated immature thymocytes. (**a**) E2-box (μE5)- and octamer-binding activity analyzed by EMSA in whole-cell extracts from DP thymocytes stimulated with the indicated antibodies. Arrows indicate the specific binding complexes for each probe. (**b**) Immunoblot analysis of E47 expression in whole-cell extracts from DP thymocytes stimulated with the indicated antibodies. (**c**) (Upper panel) Northern blot analysis of Id3 transcripts in DP thymocytes stimulated with the indicated antibodies. (Lower panel) An ethidium bromide picture of the gel is shown to demonstrate equivalent loading of each sample. (**d**) The relative level of Id3 RNA was quantified by phosphorimager and plotted. (**a–c**) Media alone served as a control.

which indicates a genetic interaction involving the E2A and Id3 proteins during thymocyte development[19].

During maturation in the thymus, interactions involving the α/β TCR chains and MHC molecules may promote cell survival and differentiation of developing thymocytes, a process called positive selection[20,21]. Thymocyte maturation requires the activation of Src family protein tyrosine kinases (PTKs) such as p56^{lck}, which signal through the activation of a mitogen-activated protein kinase (MAPK) cascade. For example, TCR and CD4- or CD8-mediated activation of p56^{lck} leads to intracellular calcium release and the activation of the Ras-Raf-extracellular-signal regulated kinase (ERK) pathway. Activation of both p56^{lck} and the Ras-Raf-ERK MAPK pathway is essential to promote thymocyte survival and maturation.

Here we provide evidence that E2A DNA binding is lowered in primary thymocytes upon TCR ligation. We show that p56^{lck}, Ras and ERK signaling regulate E2A DNA-binding activity. In addition, we show that activation of the ERK MAPK cascade in immature thymocytes leads to the induction of Id3 transcription. These observations provide a direct link between a distinct signaling module and a class of HLH proteins that is involved in tumor suppression and lymphocyte development.

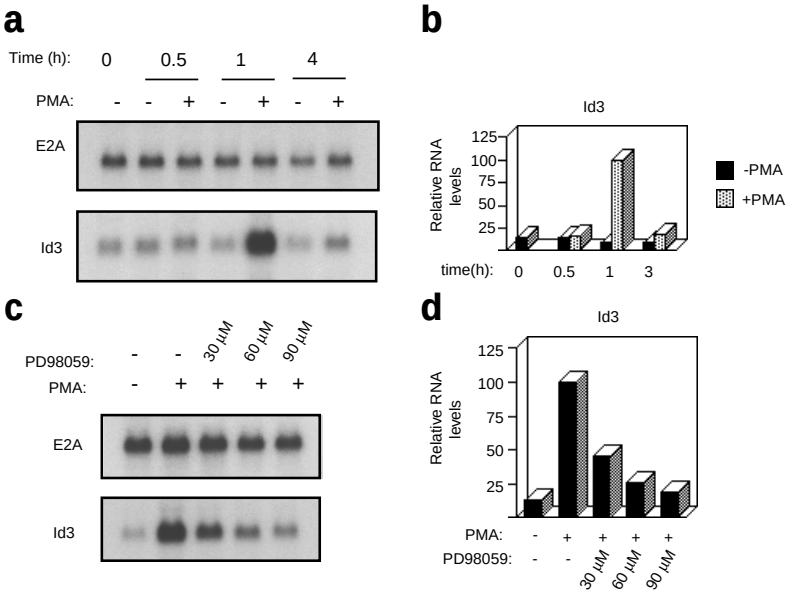

Figure 2. Id3 transcription is induced in immature thymocytes by PMA through the ERK MAPK pathway. (**a**) DP thymocytes were stimulated with PMA for the indicated times and RNA analyzed for E2A and Id3 mRNA by northern blotting. (**b**) The relative level of Id3 RNA was quantified by phosphorimager and plotted. (**c**) DP thymocytes were stimulated with PMA in the presence or absence of the MEK1 inhibitor, PD98059. E2A and Id3 RNA levels were analyzed by northern blotting. (**d**) The relative level of Id3 RNA was quantified and plotted.

Results

E47 DNA-binding activity upon TCR-mediated signaling

Thymocyte developmental progression is regulated at various checkpoints by signaling pathways that emanate from the TCR. At the DP to SP transition, this signaling cascade involves Ras and ERK. E2A bHLH proteins E12 and E47 and the HLH inhibitor protein Id3 are essential for proper maturation of DP thymocytes[10,19]. To establish a link between E2A activity and Id3 expression and signals emanating from the TCR, the DNA-binding activity of E2A and its heterodimeric partner, HEB, was analyzed in primary thymocytes in response to TCR signals. DP thymocytes derived from AND TCR recombination-activating gene 2–deficient (RAG2$^{-/-}$) H-2^{d} mice were incubated with antibodies to CD3 and CD4. Due to the inability to positively select the transgene or rearrange the endogenous genes that encode the TCR, thymi from these mice lack SP T cells and contain 90% immature DP cells[30]. Whole-cell extracts from stimulated and control cells were prepared and analyzed by electrophoretic mobility shift assay (EMSA) with an E2-box site or octamer-binding site as a probe. We found that E47-HEB DNA-binding activity in DP thymocytes was reduced by approximately 75% in response to CD3 ligation, whereas Oct-1 binding was unchanged (**Fig. 1a**). To determine whether TCR-mediated signaling modulates expression of the genes that encode E47 or HEB, protein expression was examined by immunoblotting (western blotting). Neither E47 nor HEB protein expression were altered upon CD3 ligation (**Fig. 1b** and data not shown).

Induction of Id3 expression by ERK

E2A and Id3 interact genetically to regulate thymocyte developmental progression[19]. To determine whether inhibition of E47-HEB DNA-binding might be reflected by a change in Id3 concentration, mRNA was isolated and examined by northern blotting. We found that *in vitro* engagement of the TCR complex resulted in a three- to fourfold increase in Id3 transcripts (**Fig. 1c,d**).

The deficiency in thymocyte maturation in Id3-null mutant mice is similar to the defect observed in mice that lack Ras, MEK1 or ERK1 activity[19,31,32]. In addition, activation of the ERK MAPK cascade has been shown to be essential for receptor-mediated positive selection. Thus, we examined the potential role of the ERK pathway in regulating

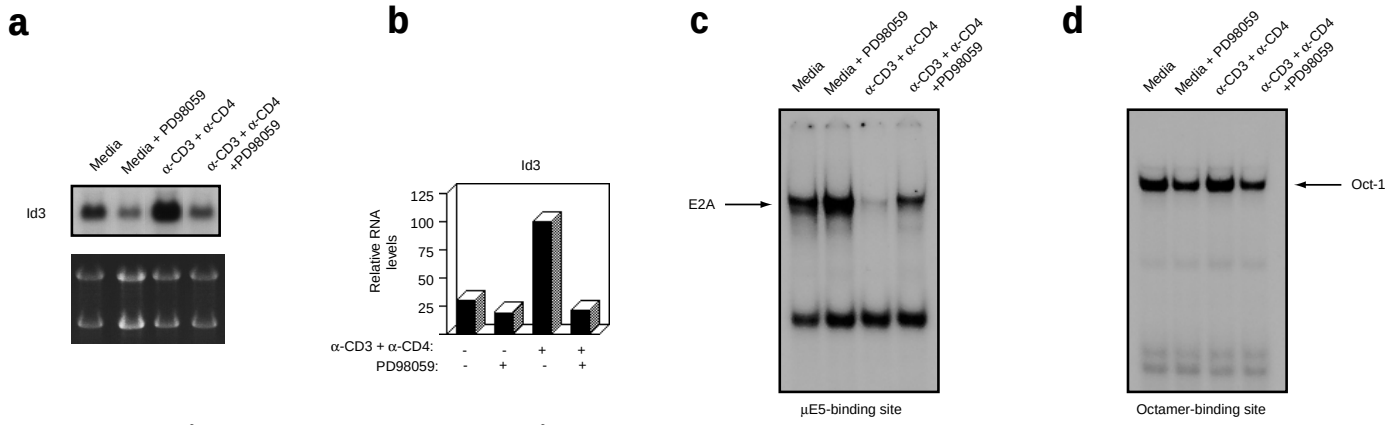

Figure 3. Id3 transcription is induced through TCR-mediated activation of the ERK MAPK pathway. (**a**) (Upper panel) Northern blot analysis of Id3 expression in DP thymocytes treated with anti-CD3 + anti-CD4 in the presence or absence of PD98059. (Lower panel) An ethidium bromide picture of the gel is shown to demonstrate equivalent loading of each sample. (**b**) The relative expression Id3 RNA was quantified by phosphorimager and plotted. EMSA analysis of (**c**) E2-box- and (**d**) octamer-binding activity in whole-cell extracts from DP thymocytes treated with the indicated antibodies in the presence or absence of PD98059. The arrows indicate the specific binding complexes for each probe. (**a,c,d**) Media alone served as a control.

expression of the gene that encodes Id3. Incubation of thymocytes with phorbol 12-myristate 13-acetate (PMA) results in the activation of protein kinase C (PKC), which has the ability to phosphorylate Raf and activate the ERK MAPK pathway[33–36]. DP thymocytes were incubated for varying amounts of time in the presence or absence of PMA and mRNA was isolated from the cells and analyzed by northern blotting (**Fig. 2a**). Id3 expression transiently increased tenfold 60 min after PMA treatment, whereas expression of E2A mRNA and protein remained unchanged (**Fig. 2a,b** and data not shown). To determine whether activation of Id3 expression was mediated through the ERK MAPK pathway, immature thymocytes were incubated with PMA in the absence or presence of PD98059, a pharmacological agent which specifically blocks phosphorylation of MEK1 and MEK2 by c-Raf[37,38]. The activation of Id3 transcription by PMA was inhibited by the addition of PD98059 in a dose-dependent manner, which indicated that activation of the ERK MAPK pathway in DP thymocytes induces Id3 expression (**Fig. 2c,d**).

To determine whether TCR-mediated activation of ERK also induces Id3 expression, DP thymocytes were incubated with anti-CD3 + anti-CD4 in the presence or absence of PD98059. Addition of PD98059 inhibited the induction of Id3 mRNA after CD3 ligation, which indicated that TCR-mediated activation of the ERK MAPK cascade modulates Id3 expression (**Fig. 3a,b**). Also, addition of PD98059 to anti-CD3 + anti-CD4–stimulated thymocytes resulted in increased E47-HEB DNA-binding activity (**Fig. 3c,d**). These data suggest that Id3 expression was activated in DP thymocytes by the ERK MAPK signaling module that originates from the TCR and that this increase in Id3 contributed to decreased E47-HEB DNA binding. We note, however, that E47-HEB DNA-binding activity was not restored to wild-type levels upon addition of PD98059, anti-CD4 + anti-CD3. This raises the possibility that additional signaling pathways may contribute to the overall decrease in DNA-binding activity.

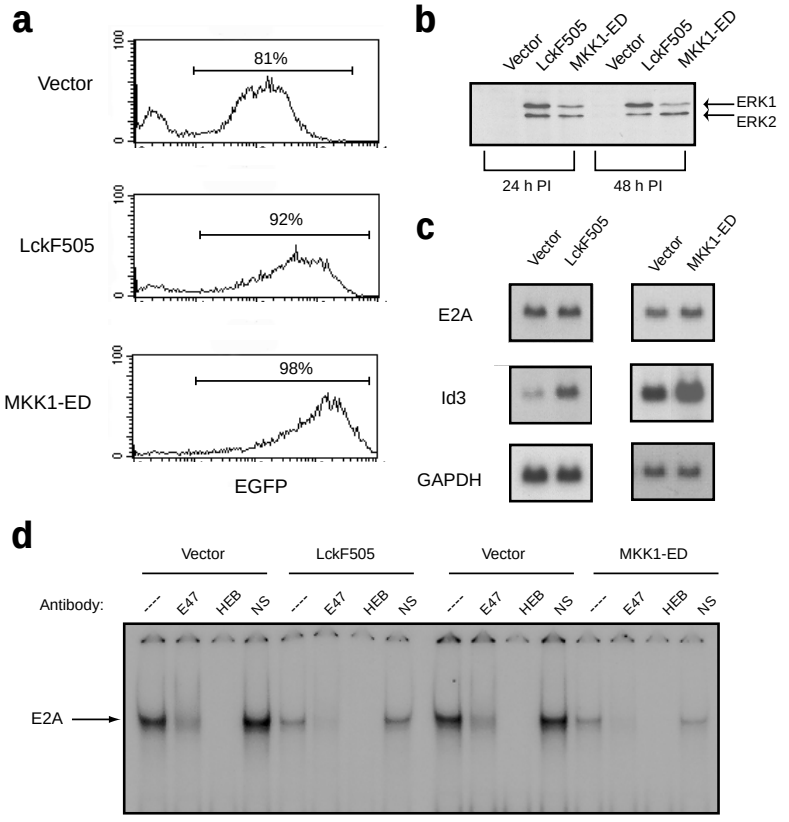

Figure 4. Constitutively active forms of p56^{lck} and MEK1 induce Id3 transcription in the immature DP T cell line 16610D9. (**a**) EGFP expression in 16610D9 cells 48 h after transduction with the indicated retroviruses. The percentage of EGFP$^+$ cells is indicated. (**b**) Immunoblot analysis of 16610D9 whole-cell extracts transduced with the indicated retroviruses. Whole-cell extracts were prepared either 24 h or 48 h after transduction (PI) and analyzed for phosphorylated ERK expression. (**c**) Northern blot analysis of Id3, E2A and GAPDH RNA expression in 16610D9 cells transduced with the indicated vectors. The blots were stripped and probed sequentially with the indicated probes. (GAPDH served as a control.) (**d**) EMSA analysis of E-box-binding activity in whole-cell extracts prepared from 16610D9 cells transduced with the indicated retroviruses (vector denotes the control vector was used). The extracts were preincubated with antibodies to either E47, HEB or a nonspecific antibody (NS) before the addition of the probe. The arrow indicates the E2A-containing complex. (**a–d**) Vector denotes that a control, empty, retrovirus was used.

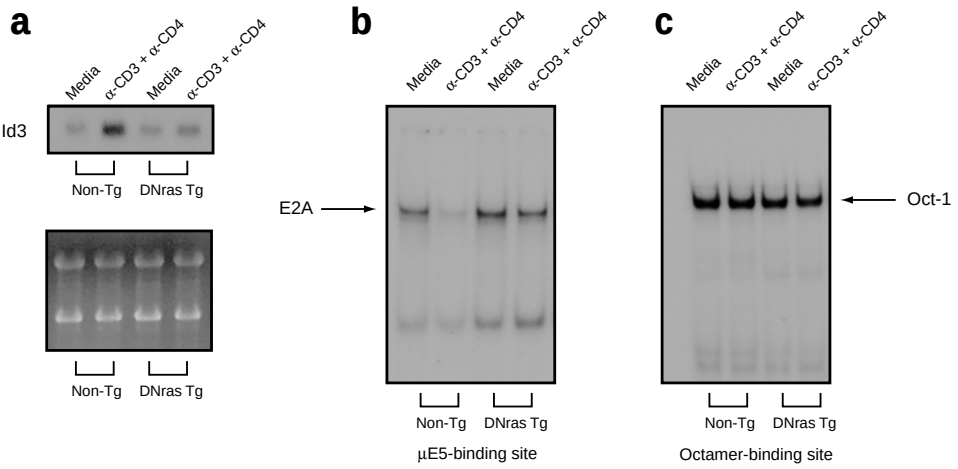

Figure 5. Inhibition of the Id3 induction in thymocytes expressing a DN-Ras transgene. (**a**) (Upper panel) Northern blot analysis of Id3 expression in thymocytes from nontransgenic (non-Tg) or dominant-negative Ras transgenic (DNras Tg) mice stimulated with the indicated antibodies. (Lower panel) An ethidium bromide picture of the gel is shown to demonstrate equivalent loading of the samples. Whole-cell extracts prepared from the stimulated cells were analyzed by EMSA for (**b**) E-box- and (**c**) octamer-binding activity. The arrows indicate the specific binding complex for each of the probes. (**a–c**) Media alone served as a control.

Induction of Id3 expression by Lck and MEK1

To further examine the potential role of the ERK MAPK pathway in regulating Id3 transcription, activated forms of p56^{lck} (LckF505) and MEK1 (MKK1-ED) were retrovirally transduced into a murine DP T cell line, 16610D9. This cell line shows many characteristics of primary DP thymocytes, including high expression of heat-stable antigen (HSA), intermediate expression of TCR and low expression of CD5 and CD44[10]. In addition, lowering E2A DNA-binding activity by ectopic expression of Id3 in this cell line promotes the acquisition of markers that are characteristic of cells undergoing maturation[10]. Flow cytometric analysis of EGFP expression showed that 80–100% of the cells had been transduced by the retrovirus (**Fig. 4a**). Whole-cell extracts from transduced cells were analyzed for the presence of activated ERK and expression of Id3. Although phosphorylated ERK was virtually undetectable in control-infected cells, substantial amounts of phosphorylated ERK1 and ERK2 were present in cells expressing LckF505 or MKK1-ED (**Fig. 4b**). In addition, whereas E2A mRNA levels remained unchanged in transduced cells, expression of either activated p56^{lck} or MEK1 increased Id3 expression three- to fourfold (**Fig. 4c**). These data show that activated p56^{lck} and MEK1 have the ability to induce Id3 transcription in immature T cells, which is consistent with the data obtained from primary thymocytes.

To determine whether the changes in Id3 expression that were observed in the LckF505- and MKK1-ED–infected cells could affect E47-HEB DNA-binding activity, whole-cell extracts derived from transduced cells were analyzed by EMSA. Overall, E47-HEB DNA-binding activity was decreased 75–80% in cells expressing activated Lck or MEK1 (**Fig. 4d**). Preincubation of the cell lysates with E47- or HEB-specific antibodies directed against the HLH domain indicated that the complexes in the control and Lck- or MEK1-expressing cells were qualitatively similar (**Fig. 4d**).

Id3 induction requires Ras-mediated signaling

To provide further evidence that activation of the Ras-ERK pathway in primary thymocytes modulates Id3 mRNA levels and E2A DNA-binding activity, thymocytes derived from transgenic mice expressing a dominant-negative form of Ras (DN-Ras) were analyzed. These mice have a thymic phenotype that is similar to that of Id3-deficient mice[19,39]. Thymocytes were incubated with anti-CD3 + anti-CD4 and examined for the presence of Id3 mRNA and E2A DNA binding. Consistent with the previous data, Id3 mRNA was increased approximately three-to fourfold upon TCR ligation in nontransgenic control thymocytes (**Fig. 5a**). In contrast, Id3 mRNA levels were virtually unchanged in stimulated cells derived from mice that were expressing a DN-Ras transgene. Additionally, in thymocytes that were expressing DN-Ras, E2A-HEB DNA-binding activity was unaltered by treatment with anti-CD3 + anti-CD4. In contrast, binding activity was decreased by approximately 75% in the TCR-stimulated nontransgenic cell extracts (**Fig. 5b**). We obtained similar results using purified DP thymocytes from wild-type and DN-Ras transgenics (data not shown). Taken together, these observations indicate that Id3 RNA levels and E47 DNA-binding activity are regulated by the proto-oncogene, Ras, in primary immature thymocytes.

EGR1 ectopic overexpression activates Id3 transcription

To examine whether the ERK-activated induction of Id3 requires *de novo* protein synthesis, thymocytes were preincubated with cycloheximide. Cycloheximide treatment completely inhibited PMA-activated induction of Id3, which suggested that the ERK-mediated induction of Id3, albeit rapid, is indirect and requires *de novo* protein synthesis (**Fig. 6a**). Expression of the protein encoded by the immediate early growth response gene 1 (EGR1), is rapidly induced upon TCR ligation[40]. Additionally, EGR1 mRNA expression is modulated by the ERK MAPK pathway and ectopic expression of EGR1 in DP thymocytes promotes positive selection, which is similar to that observed in E47-deficient mice[10,40–42]. Both E47$^{-/-}$ HY TCR and EGR1 HY TCR double-transgenic mice showed enhanced positive selection, even in a β2-microglobulin–deficient background[10,42]. Similarly, EGR1 AND TCR double-transgenic mice and E47$^{-/-}$ AND TCR mice showed increased numbers of CD4 SP thymocytes[10,42]. These observations raised the possibility that EGR1 and E2A function in a linear pathway.

To determine whether EGR1 has the ability to activate Id3 expression, *Egr1* was ectopically expressed in 16610D9 cells. To analyze *Egr1* activity, whole-cell extracts were prepared from the transduced cells and analyzed by immunoblotting and EMSA. We found that EGR1 expression and DNA-binding activity were readily detectable only in cells transduced with the retrovirus carrying the EGR1 cDNA (**Fig. 6b,c**). To assess the ability of *Egr1* to activate transcription of Id3, RNA was isolated and examined by northern blotting. We found that Id3 expression increased approximately seven- to tenfold by the ectopic expression of *Egr1* (**Fig. 6d**). In contrast, E2A mRNA levels remained unchanged. Additionally, activation of EGR1 reduced E2A DNA-binding activity, which was consistent with the increase in Id3 mRNA. Oct-1 DNA-binding activity was unchanged in the presence of

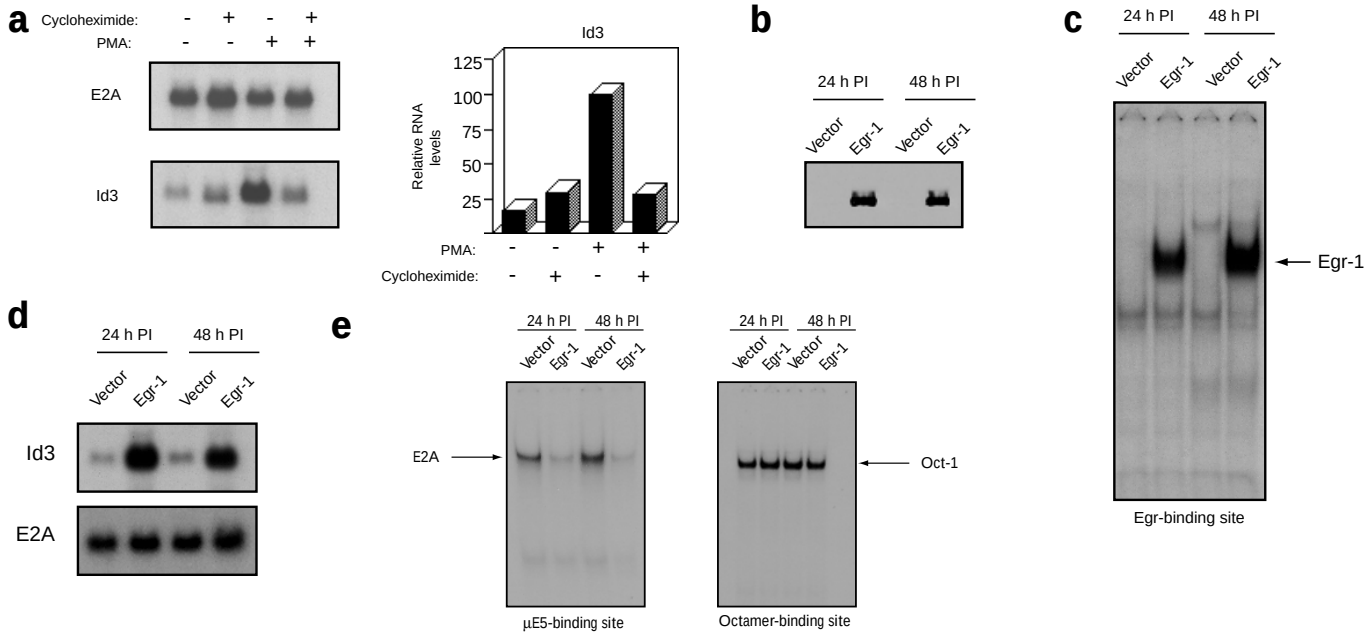

Figure 6. Transcriptional activation of Id3 is indirect and can be mediated by EGR1. (**a**) (Left panels) Northern blot analysis of E2A and Id3 expression in DP thymocytes stimulated with PMA in the presence or absence of cycloheximide. (Right panel) The relative amounts of Id3. (**b**) Immunoblot analysis of EGR1 (Egr-1) expression in 16610D9 cells transduced with retrovirus expressing *Egr1* either 24 h or 48 h after infection. (**c**) EMSA analysis of *Egr1*-binding activity in *Egr1*-transduced 16610D9 cells 24 h or 48 h after infection. (**d**) Northern blot analysis of Id3 and E2A expression in 16610D9 cells infected with the *Egr1*-expressing retrovirus 24 h or 48 h after infection. (**e**) E-box- and octamer-binding activity was analyzed by EMSA in whole-cell extracts prepared from 16610D9 cells infected with *Egr1*-containing vector 24 h or 48 h after infection. (**b–e**) Vector denotes that a control, empty, retrovirus was used.

EGR1 (**Fig. 6e**). Thus, these data show that EGR1 has the ability to increase Id3 mRNA levels and decrease E2A DNA-binding activity.

The data described here show that EGR1 has the ability to activate Id3 expression. We next determined whether activation of EGR1 expression by the ERK MAPK pathway precedes that of Id3. Thus, mRNA was isolated from DP thymocytes stimulated with PMA or antibodies to CD3 and CD4 in the presence or absence of PD98059 and analyzed for the presence of Id3 and EGR1 (**Fig. 7a,b**). As expected, EGR1 mRNA levels were rapidly increased by the ERK MAPK pathway and preceded that of Id3. In addition, induction of EGR1 was blocked by the presence of DN-Ras in a similar manner to that shown for Id3 (**Fig. 7c**). To determine whether the ERK-mediated induction of Id3 requires *de novo* mRNA synthesis, thymoctyes were incubated with actinomycin D 30 min after treatment with PMA. EGR1 transcription was not affected by the presence of actinomycin D, whereas the amount of Id3 decreased to an amount that was comparable to those seen in untreated cells (**Fig. 7d**). These data indicate that the induction of Id3 expression requires transcriptional initiation and is consistent with a direct linear relationship between the ERK MAPK pathway and Id3.

Discussion

The data we present here position the ERK MAPK signaling cascade and HLH proteins, both involved in thymocyte maturation, in a common pathway. Although E2A mRNA and protein expression are not modulated by TCR signaling *in vitro*, Id3 expression is induced and E2A DNA-binding activity is inhibited. We show that the TCR-mediated activation of Id3 expression is mediated by the Lck-Ras-ERK MAPK module. We propose that decreased E2A DNA-binding activity upon TCR signaling promotes thymocyte maturation, which is consistent with the phenotypes of the E2A- and Id3-deficient mice.

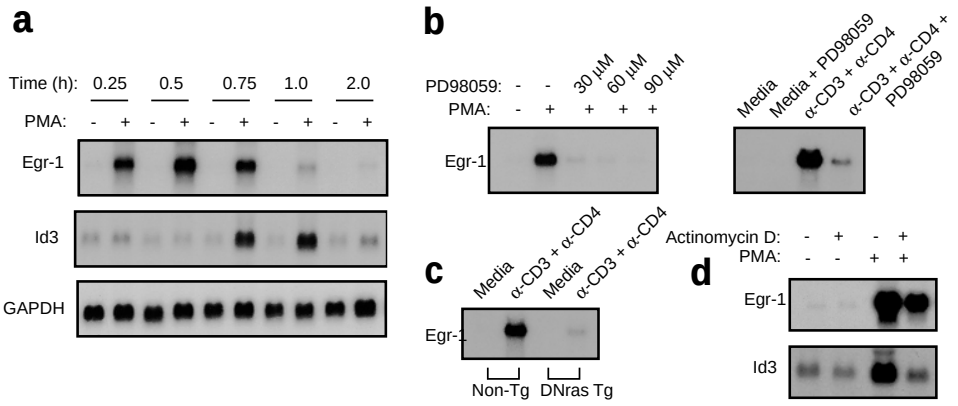

Figure 7. EGR1 expression is activated by the ERK MAPK pathway before Id3 and activates Id3 transcription. (**a**) Northern blot analysis of EGR1 and Id3 expression in DP thymocytes stimulated with PMA for the indicated times. (**b**) Northern blot analysis of EGR1 expression in DP thymocytes stimulated with PMA or anti-CD3 + anti-CD4 in the presence or absence of PD98059. (**c**) Northern blot analysis of EGR1 expression in thymocytes from non-Tg or DN-Ras Tg mice stimulated anti-CD3 + anti-CD4. (**d**) Northern blot analysis of Id3 and EGR1 expression in DP thymocytes stimulated with PMA in the presence or absence of actinomycin D. (**b,c**) Media alone served as a control.

E protein activity is required during three distinct stages of thymocyte development. The E2A proteins function during T-lineage commitment, whereas HEB is required during the transition from the DN to DP stage[7,8]. In addition, E2A and Id3 are required during maturation of DP thymocytes[10,19]. At this latter checkpoint, decreased activity of E2A promotes thymocyte maturation, whereas the absence of Id3 affects cell survival and/or differentiation[10,19]. Despite these advances, little progress has been made in understanding how E protein activity is regulated during thymocyte development.

Our data place Ras signaling and HLH proteins, both involved in thymocyte positive-selection, in a common pathway. Upon TCR ligation, the activated ERK MAPK module up-regulates the expression of the immediate-early gene *Egr1*, which has the ability to activate (either directly or indirectly) Id3 expression. We propose that the increase in Id3 expression contributes to the inhibition of E47-HEB DNA-binding activity. Recent data indicate that, indeed, E2A and Id3 interact in T cells to form stable heterodimers. This is consistent with the genetic interactions observed in E2A-Id3 compound-mutant mice[19]. However, it is conceivable that activation of the ERK MAPK pathway also results in modifications of the Id or E2A proteins that contribute to the observed decrease in DNA-binding activity.

Transgenic mice that overexpress EGR1 show disrupted thymocyte maturation[42]. The thymic phenotype of these mice is similar to that of E2A-deficient mice[10,42]. On the other hand, EGR1–deficient mice do not show a defect in thymocyte selection[43]. This raises the question of whether induction of Id3 requires the presence of EGR1. We would suggest that EGR2 and EGR3, both closely related to EGR1 and similarly expressed, have the ability to replace the activity of EGR1[43]. In addition, these data do not prove that EGR1 regulates Id3 expression *in vivo*. Double-or triple-null mutant mice that contain targeted deletions in the genes that encode EGR should resolve this question.

We found that the extent of Id3 induction is responsive to the dose of MEK1 inhibitor. These data suggest that Id3 mRNA can be differentially regulated by the amplitude of the ERK MAPK signal. ERK MAPK signal strength has been implicated in the CD4- *versus* CD8-lineage commitment and we observed alterations in the proportion of $CD4^+$ *versus* $CD8^+$ cells in E2A-deficient mice[7,10]. These observations raise the possibility that differential signaling of the ERK MAPK pathway modulates Id3 expression and E2A activity to influence lineage commitment as well.

The observations described here also have implications for the role of E2A as a tumor suppressor. Mice carrying targeted mutations at the E2A locus rapidly develop highly malignant T cell lymphomas[7,11]. Additionally, there is considerable evidence linking the inactivation of E2A with the progression of human T-ALL[13]. Both the $p56^{lck}$ and ERK MAPK modules have been implicated in the development of T cell lymphomas. Ectopic expression of $p56^{lck}$ and MEK1 in transgenic mice leads to rapid development of T cell lymphomas with similar kinetics of appearance and phenotype as described for mice that are deficient in E2A activity[7,11,44–46]. It is conceivable that the oncogenic effects of $p56^{lck}$ and ERK activity are due to inappropriate inhibition of E2A activity. We also note that the serine kinase TPL2, which is also known as COT, has the ability to activate MEK1 and has also been linked with the ERK MAPK module[47–49]. TPL2 was originally identified as an oncogene associated with the development of Moloney murine leukemia virus–induced lymphomas[50,51]. It is possible that TPL2 activates expression of the genes that encode the Id proteins in thymocytes, thus leading to the aberrant inactivation of E2A and the development of thymic lymphomas.

The observations we describe here show that the ERK MAPK pathway regulates E47 and HEB DNA-binding activity, at least in part, by modulating Id3 expression. Further studies will be required to determine whether the MAPK module also functions in other stages of thymocyte development to regulate E2A activity. In addition, because mutations within the genes that encode Ras are among the most frequent mutations in human cancers, it will be important to determine whether E2A activity and expression of the genes that encode Id are modulated in such neoplasms.

Methods

***In vitro* thymocyte stimulation.** Plates (10 cm) were coated for 2 h at 37 °C with mouse anti–rat IgG (10 μg/ml, Jackson ImmunoResearch Labs, West Grove, PA) and goat anti–hamster IgG (10 μg/ml, Southern Biotechnology, Birmingham, AL) in carbonate buffer (0.1 M at pH 9.5) before the addition of 10 ml of hybridoma supernatant from the 145-2C11 line (anti-CD3) and the GK1.5 line (anti-CD4) or RPMI 1640 media alone and incubated overnight at 4 °C. Immature DP thymocytes were obtained by dissecting thymocytes from AND TCR transgenic RAG2-deficient mice in an $H\text{-}2^d$ background. DN-Ras transgenic mice were as described[39]. Thymocytes were dissected into RPMI 1640 media that contained 10% fetal bovine serum, glutamine, penicillin, streptomycin and 2-mercaptoethanol (50 μM). The coated plates were washed with PBS, 5×10^7 thymocytes in RPMI media (15 ml) were added to each plate and plates were incubated at 37 °C for 4–8 h. Thymocytes were then washed once in PBS and the cell pellet either resuspended in Trizol reagent (1 ml) for RNA preparation (Gibco-BRL, Gaithersburg, MD) or frozen on dry ice for preparation of whole-cell extract as described below. For the PMA time-course analysis, thymocytes were resuspended in RPMI at a concentration of 2.5×10^6 cells/ml and 4 ml of mixture was plated in a 6 cm tissue culture dish for each time point. PMA or DMSO (10 μl of 4 μg/ml) was added to each plate. Cells were then cultured at 37 °C for the indicated times. Experiments involving addition of the MEK inhibitor PD98059 (New England Biolabs, Beverly, MA) or cycloheximide were done as described above except that after 90 min in media alone, 60 μM of PD98059 (in DMSO) or 10 μg/ml of cycloheximide was added and the cells cultured for an additional 90 min at 37 °C before the addition of PMA (or DMSO). Cells were then cultured for an additional 1 h in the presence of PMA. Experiments involving addition of actinomycin D were done as described above except that 15 min after PMA treatment, 5 μg/ml of actinomycin D was added and the cells cultured for an additional 45 min before preparation of RNA.

Whole-cell extract preparation, EMSA and immunoblotting. The frozen cell pellet was thawed, resuspended at a concentration of 10 μl/10^7 cells in cold buffer C—which contained Hepes (20 mM at pH 7.9), NaCl (0.4 M), EDTA (1 mM), EGTA (1 mM), DTT (1 mM), PMSF + protease inhibitors (1 mM) and 1% NP-40—and vortexed vigorously for 2 min at 4 °C. Debris was pelleted and the supernatant removed as the whole-cell extract. Double-stranded DNA probes were end-labeled using T4 polynucleotide kinase and purified over a G25 sepharose column. Whole-cell extract (7–15 μg) was used in a gel-shift assay as described[52]. The sequence of the μE5 and EGR1 oligoprobes is as follows: μE5: 5′–TCGAAGAACAC-CTGCAGCAGCT–3′ and EGR1 5′–CCCGGCGCGGGGGCGATTTCGAGTCA–3′. The sequence of the Oct oligoprobe was as described[53]. For antibody supershifts, the whole-cell extracts were preincubated with monoclonal anti-E47 (1 μg, G127-32, PharMingen, San Diego, CA), anti-HEB (G127-382, PharMingen) or anti–Bcl-2 (1 μg, for a nonspecific control, 65111A, PharMingen) before the addition of the labeled probe. For immunoblotting, whole-cell extract (10–20 μg) was run on a 10% SDS-polyacrylamide gel and transferred to immobilon (Millipore, Bedford, MA). Blots were incubated with anti-E47 (1 μg/ml, G127-32, PharMingen) or anti-EGR1 (1 μg/ml, EGR1 588, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature or with an anti-phospho-ERK (New England Biolabs) overnight at 4°C. Detection of the HRP-conjugated secondary antibodies was done with ECL or ECL + reagents (Amersham, Little Chalfont, UK).

Northern blot analysis. Total RNA was prepared from cells by resuspension in Trizol (Gibco-BRL) as per the manufacturers instruction. Total RNA (5 μg) was separated on a 1% formaldehyde gel and then blotted to Nytran Plus (Schleicher and Schuell, Keene, NH). A 350-bp E2A probe was generated from an E2A cDNA by PCR using the following primers: sense: 5′–CATCCATGTCCTGCGAAGCCA–3′ and antisense: 5′–TTCTTGTCCTCTTCG-GCGTC–3′. A 200-bp Id3 probe was generated from an Id3 cDNA by PCR using the following primers: sense: 5′–CGCACTGTTTGCTGCTTTAGG–3′ and antisense: 5′–GTAGCA GTGGTTCATGTCGTC–3′. All PCR-generated probes were run on a 2% agarose gel and purified by Qiaex (Qiagen, Valcencia, CA).

Retroviral supernatant production and T lymphoma transduction. The 16610D9 murine cell line was as described[10]. The retroviral vector LZRSpBMN-linker-IRES-enhanced green fluorescent protein (S-003) was from H. Spits (The Netherlands Cancer Institute, Amsterdam, The Netherlands). A constitutively active form of mouse Lck (Y505F) was cloned as an *Eco*RI fragment into the *Eco*RI site of S-003. A constitutively active fragment of MEK1 (MKK1-ED) was obtained by PCR of MKK1-ED with the following primers: sense: 5′–CGGGATCCGGTCCAAAATGCCCAAGAAGAA–3′ and antisense: 5′–GCGGCCGCGGGACTCGCTCTTTGTTGCTTCCCA–3′. The PCR product was cloned into the pGem-T vector (Promega, Madison, WI), sequenced and cloned as a *Bam*HI-*Not*I fragment into the *Bam*HI-*Not*I sites of S-003. The cDNA for mouse EGR1 was

cloned as an *Eco*RI fragment into the *Eco*RI site of S-003. Transfection of the ϕNX-eco packaging line, preparation of the retrovirus and transduction of the cells was a described[10,12]. Transduced cells were collected 24–48 h after infection and analyzed by flow cytometry and RNA and whole-cell extract prepared as described above.

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