Redundant Role for Early Growth Response Transcriptional Regulators in Thymocyte Differentiation and Survival[1]

John H. Carter,* Juliet M. Lefebvre,[§] David L. Wiest,[§] and Warren G. Tourtellotte[2]*[†‡]

The early growth response (Egr) family of transcriptional regulators consists of four proteins that share highly conserved DNA-binding domains. In many cell types, they are coexpressed and appear to have cooperative roles in regulating gene expression during growth and differentiation. Three Egr proteins, Egr1, Egr2, and Egr3, are induced during thymocyte differentiation in response to pre-TCR signaling, suggesting they may be critical for some aspects of pre-TCR-mediated differentiation. Indeed, enforced expression of Egr proteins in developing thymocytes can recapitulate some aspects of pre-TCR signaling, but the mechanisms by which they contribute to β-selection are still poorly understood. Egr3 stimulates proliferation of β-selected thymocytes, and Egr3-deficient mice have hypocellular thymuses, defects in proliferation, and impaired progression from double-negative 3 to double-negative 4. Surprisingly, Egr1-deficient mice exhibit normal β-selection, indicating that the functions of Egr1 during β-selection are likely compensated by other Egr proteins. In this study, we show that mice lacking both Egr1 and Egr3 exhibit a more severe thymic atrophy and impairment of thymocyte differentiation than mice lacking either Egr1 or Egr3. This is due to a proliferation defect and cell-autonomous increase in apoptosis, indicating that Egr1 and Egr3 cooperate to promote thymocyte survival. Microarray analysis of deregulated gene expression in immature thymocytes lacking both Egr1 and Egr3 revealed a previously unknown role for Egr proteins in the maintenance of cellular metabolism, providing new insight into the function of these molecules during T cell development. *The Journal of Immunology,* **2007, 178: 6796–6805.**

T cell development in the thymus progresses through well-defined stages characterized by the expression of the CD4 and CD8 cell surface markers. The earliest T cell precursors express neither CD4 nor CD8 (double-negative (DN)[3] thymocytes), which progress through a series of maturational steps to form double-positive (DP) thymocytes that express both CD4 and CD8, and then finally to mature single-positive (SP) thymocytes that express either CD4 or CD8. DN thymocytes can be further subdivided into several substages of maturation on the basis of CD25 and CD44 expression, namely DN1 ($CD44^+$ and $CD25^-$), DN2 ($CD44^+$ and $CD25^+$), DN3 ($CD44^-$ and $CD25^+$), and DN4 ($CD44^-$ and $CD25^-$) thymocytes (1). As part of this complex maturational process, rearrangement of the *TCRβ* gene occurs at the DN3 stage during which cells express the successfully rearranged *TCRβ* gene along with the pre-TCRα (pTα) chain to form the functional pre-TCR receptor complex. Signaling through the pre-TCR receptor involves a variety of cellular responses, including robust proliferation, enhanced cell survival, down-regulation of the *Rag1* and *Rag2* recombinase genes, reduced CD25 expression, decreased pTα expression, induction of the TCRα chain, and allelic exclusion of the nonrearranged *TCRβ* locus (2). Differentiation through DN3 to DN4 is known as β-selection because only cells with a functionally rearranged TCRβ chain survive this critical transition.

Thymocyte development depends upon normal pre-TCR signaling to engage a variety of molecules, including members of the early growth response (Egr), E protein, TCF/LEF, NF-κB, and NFAT transcriptional regulators (3–7). Although their precise function and the target genes regulated by them are largely unknown (8), it appears likely that at least some of the proliferative and antiapoptotic regulatory pathways engaged during thymocyte maturation converge on the promoters of the cyclin D3 and the Bcl-2-related A1 genes (9–11).

Egr proteins have a particularly important role as effectors of pre-TCR signaling, yet the precise stage of thymocyte development and mechanisms through which they act are still poorly understood. There are four Egr genes (*Egr1–4*), three of which (*Egr1–3*) are expressed during thymocyte differentiation. Egr proteins contain highly conserved zinc-finger DNA-binding domains that can bind a number of common target gene promoters and potentially cooperate in regulating their expression (12). In thymocytes, Egr1, Egr2, and Egr3 are induced by pre-TCR signaling and overexpression of these proteins in pre-TCR signaling-deficient thymocytes can partially facilitate progression through the β-selection checkpoint (13, 14). In addition, Egr protein overexpression down-regulates Rag1, Rag2, and pTα, and up-regulates TCR-Cα in vitro, mimicking some aspects of endogenous pre-TCR signaling. Conversely, dominant-negative Egr proteins inhibit progression from DN3 to DN4 in vitro (13). In vivo, Egr1-deficient thymocytes have a small but significant defect in positive selection but have no apparent defect in β-selection (15). In contrast, Egr3-deficient thymocytes have a partial block at the DN3

*Department of Pathology, [†]Division of Neuropathology, and [‡]Department of Neurology, Northwestern University, Chicago IL, 60611; and [§]Immunobiology Working Group, Division of Basic Sciences, Fox Chase Cancer Center, Philadelphia, PA 19111

Received for publication November 2, 2006. Accepted for publication March 8, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Institutes of Health (NS046468 and NS040748) and a Howard Hughes Faculty Scholar Award (to W.G.T.). J.H.C. was supported by a Predoctoral Fellowship from the National Institutes of Health (CA009560) and the National Institutes of Health Medical Scientist Training Program (GM008152).

[2] Address correspondence and reprint requests to Dr. Warren G. Tourtellotte, Department of Pathology, Northwestern University, W127, 330 East Chicago Avenue, Chicago, IL 60611. E-mail address: warren@northwestern.edu

[3] Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; pTα, pre-TCRα; Egr, early growth response; 1/3 DKO, Egr1 and Egr3 double knockout; WT, wild type; 7-AAD, 7-aminoactinomycin D; qPCR, quantitative real-time PCR; P, postnatal day; GO, Gene Ontology; TFBS, transcription factor-binding site; HSC, hemopoietic stem cell.

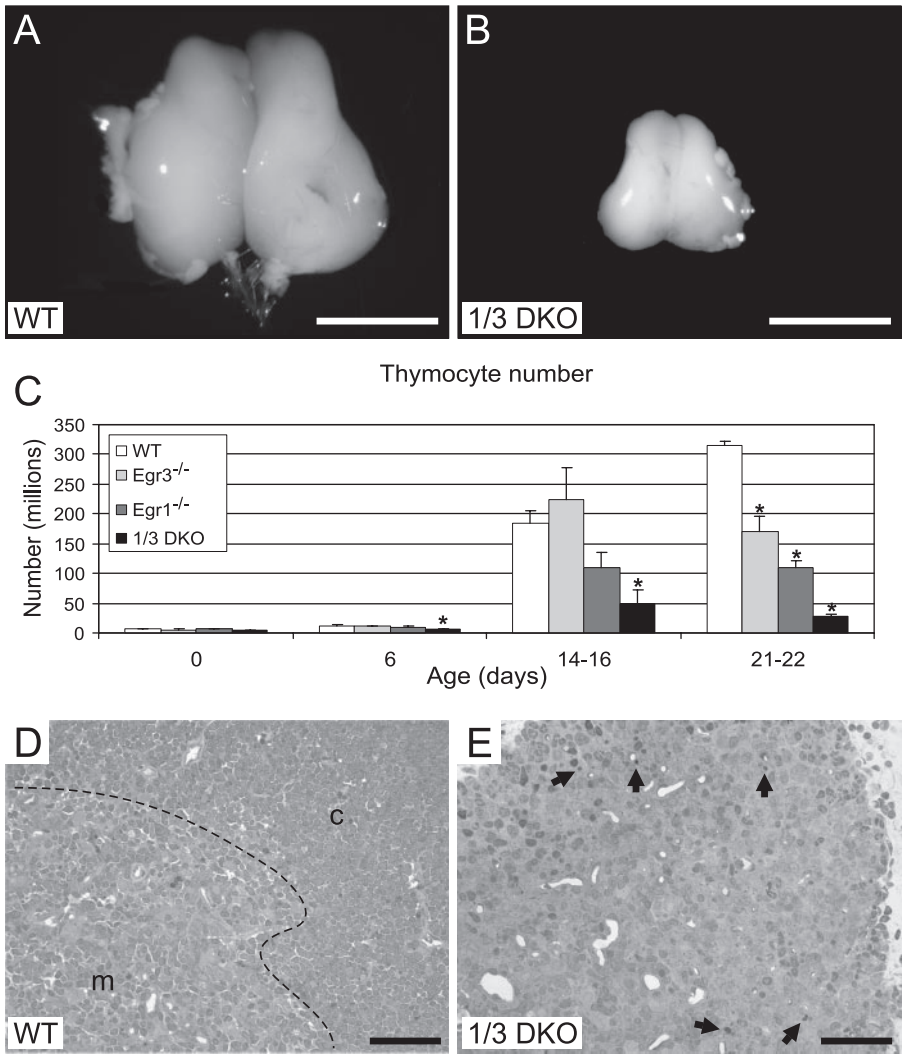

FIGURE 1. Thymic atrophy in 1/3 DKO mice. *A*, Gross appearance of a WT thymus and a (*B*) 1/3 DKO thymus at P21. *C*, Thymocyte counts during early development in WT, Egr1$^{-/-}$, Egr3$^{-/-}$, and 1/3 DKO mice. Significant decreases in cellularity were seen by P6 in 1/3 DKO mice which became more pronounced at P14 and P21. In Egr1$^{-/-}$ and Egr3$^{-/-}$ thymuses, significantly decreased cellularity was not observed until P21. *D*, Histological appearance of a WT thymus at P25 showing normal thymic architecture with obvious separation between cortex (c) and medulla (m). *E*, Histological appearance of a 1/3 DKO thymus at P25 showing effacement of the normal thymic architecture and numerous pyknotic nuclei (arrowheads). *, $p < 0.01$, Student's paired *t* test; scale bar, 5 mm for *A* and *B*, 50 μm for *D* and *E*).

stage that is largely explained by a reduced proliferation of Egr3-deficient DN4 thymocytes (16). Transgenic overexpression of Egr3 in Rag1-deficient thymocytes (which are normally blocked at DN3) leads to increased thymocyte proliferation and partial rescue of the β-selection defect in these cells. The transient kinetics of Egr3 expression appear to be critical for proper development, as sustained overexpression of Egr3 inhibits the expression of RORγT through induction of the transcriptional inhibitor Id3 (17, 18). In turn, repression of RORγT leads to decreased Bcl-x_L expression and increased apoptosis in DP thymocytes.

Although Egr1 and Egr3 single knockout mice have helped to define their role in thymocyte development, other Egr-dependent processes may still be uncharacterized due to potential overlapping function of these proteins, because Egr1, Egr2, and Egr3 are all up-regulated at the DN3/DN4 transition and during positive selection, they may regulate a common set of target genes. In this study, we examined the impact on thymocyte development of simultaneously abrogating Egr1 and Egr3 function in developing thymocytes. Egr1 and Egr3 double knockout mice (referred to as 1/3 DKO) had consistently severe thymic atrophy not seen in mice lacking only Egr1 or Egr3. Although the relative proportions of DN, DP, and SP thymocytes were normal in most 1/3 DKO mice, there was a partial block at the DN3/DN4 transition and a reduction of thymocyte proliferation. Strikingly, there was massive apoptosis of developing thymocytes in 1/3 DKO mice not seen in mice lacking only Egr1 or Egr3. Chimeric transplant studies showed that thymocyte death was a consequence of a cell autonomous survival defect of 1/3 DKO thymocytes. Although the gene regulatory networks modulated by Egr1 and Egr3 during progression from the DN to DP stage are likely to be very complex, we identified a number of genes deregulated in DN thymocytes lacking both Egr1 and Egr3. These results may provide new insights into the molecular mechanisms through which Egr transcriptional regulators facilitate normal T cell development in the thymus.

Materials and Methods

Animals

Egr1-deficient and Egr3-deficient mice were generated and genotyped as previously described (19–22). Egr1-deficient mice were backcrossed 10 generations and Egr3-deficient mice were backcrossed 4 generations to the C57BL/6J strain. Heterozygous matings were established and littermate wild-type (WT) mice were used as controls for all experiments in which comparisons were made between WT and *Egr* gene-deficient mice. B6.SJL-Ptprca Pepcb/BoyJ mice were purchased from The Jackson Laboratory. All experimental procedures complied with protocols approved by the Northwestern University Institutional Animal Care and Use Committee.

Immunohistochemistry and TUNEL histochemistry

For immunohistochemical analysis, anesthetized mice (ketamine, 150 mg/kg; xylazine 10 mg/kg; i.p.) received intracardiac PBS (0.1 M (pH 7.4)), followed by aldehyde perfusion (4% paraformaldehyde, 0.1 M phosphate buffer (pH 7.4)). The thymuses and spleens were removed, cryoprotected in 30% sucrose-PBS overnight, frozen in OCT embedding medium (VWR), and stored at −80°C. Tissue sections were cut on a freezing microtome at

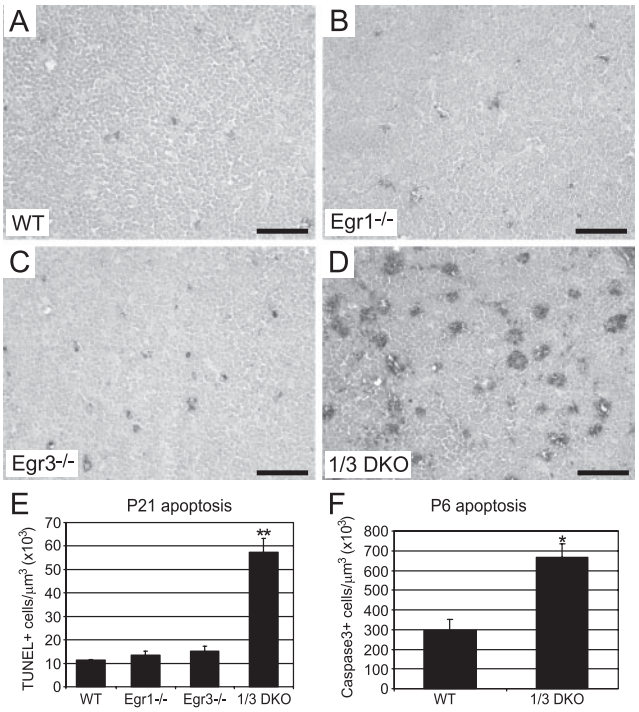

FIGURE 2. Increased apoptosis in 1/3 DKO thymus. *A–D*, TUNEL histochemistry from P21 thymuses showed low levels of endogenous apoptosis in (*A*) WT, (*B*) Egr1$^{-/-}$, (*C*) Egr3$^{-/-}$, and (*D*) greatly increased apoptosis in 1/3 DKO thymuses (results are representative of three independent animals for each genotype). *E* and *F*, Quantification of activated caspase 3^{+} cells in WT and 1/3 DKO thymus observed at (*E*) P6 and (*F*) P21. There was a highly significant increase in apoptosis in 1/3 DKO thymuses. Values represent mean ± SEM for three mice of each genotype. *, $p < 0.05$, **, $p < 0.0001$, Student's paired *t* test; scale bar, 50 μm.

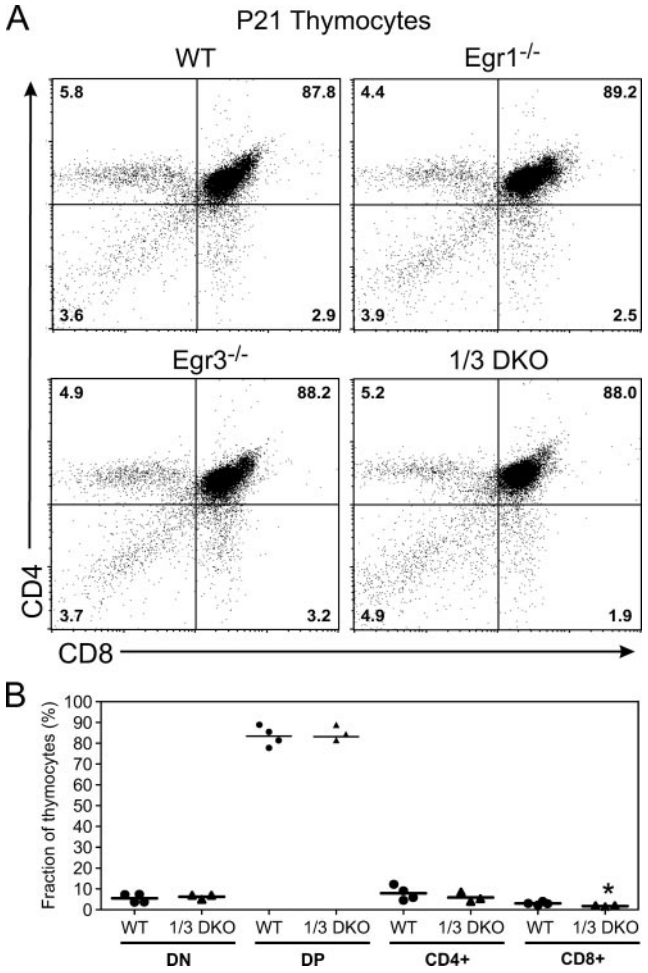

FIGURE 3. Thymocyte differentiation in *Egr* gene-deficient mice. *A*, Representative FACS analysis of CD4 and CD8 expression on WT, Egr1$^{-/-}$, Egr3$^{-/-}$, and 1/3 DKO thymocytes. *B*, Scatter plot showing pooled data from four WT and three 1/3 DKO mice. The CD8^{+} SP population was significantly reduced compared with WT (*, $p < 0.05$, Student's paired *t* test).

16-μm thickness. The sections were blocked (3% normal serum-0.1% Triton X-100 in PBS) for 1 h at room temperature, incubated with affinity purified anti-activated caspase 3 Ab (Cell Signaling Technology) diluted in blocking buffer overnight at room temperature, and subsequently incubated with a biotinylated anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories). An avidin-biotin histochemistry reaction using diaminobenzidine as the chromagen was performed according to the manufacturer's specifications (Vector Laboratories). Quantification of caspase 3^{+} cells and measurement of tissue section volume was performed using unbiased stereology and optical fractionator procedures (StereoInvestigator; MicroBrightField). TUNEL histochemistry was performed as described (23) using biotin-labeled dUTP and visualized using avidin-biotin histochemistry with diaminobenzidine as the chromagen.

Resin histology

Thymus tissue was fixed in 2.5% glutaraldehyde/2% paraformaldehyde/0.1 M cacodylate buffer (pH 7.2), postfixed in 1% osmic acid in PBS, dehydrated through graded ethanol, and embedded in Epon. Sections were cut at 1 μm and stained with toluidine blue.

Flow cytometry

All Abs were from eBioscience. Thymus cell suspensions were stained for 20 min in PBS/2% FBS with the following fluorophore conjugated mAbs: anti-CD4-allophycocyanin or anti-CD8a-PE. For identification of DN thymocyte subsets, cells were first stained with biotin-labeled anti-CD4, anti-CD8, anti-CD11b, anti-NK1.1, and anti-$\gamma\delta$TCR, followed by streptavidin-allophycocyanin, anti-CD25-PE, and anti-CD44-FITC. For DNA content analysis, cells were first stained for surface markers and then resuspended in PBS/0.1% saponin (Sigma-Aldrich)/10 μg/ml 7-aminoactinomycin D (7-AAD) at room temperature for 30 min before FACS analysis. Intact cells were determined from forward and side scatter profiles.

OP9-DL1 cultures

OP9-DL1 monolayers were seeded with bone marrow hemopoietic stem cells (HSCs; cKit^{+}Sca-1^{high}Lin^{-}) isolated by flow cytometry from Egr-deficient mice and cultured as described, except that IL-7 levels were decreased to 0.2 ng/ml (24, 25). Lineage exclusion markers included CD19, Ter119, NK1.1, Gr1, CD11b, CD4, and CD8. Briefly, 10,000 HSCs were seeded on day 0, fed with cytokines on day 4 (5 ng/ml Flt3 ligand and 0.2 ng/ml IL-7), and then 300,000 cells were transferred to fresh subconfluent monolayers on day 7. Thereafter, 300,000 cells were transferred to fresh subconfluent monolayers every 4 days and analyzed by flow cytometry using the indicated Ab.

Bone marrow transplantation

Primary bone marrow cells were isolated from the tibias and femora of P21 WT and 1/3 DKO mice. B6.SJL-Ptprca Pepcb/BoyJ mice were used as recipients because they express a different CD45 isoform (CD45.1) than WT and 1/3 DKO (CD45.2) donor cells. Recipient mice were exposed to two sequential doses of gamma irradiation (550 rad/dose) 3–4 h apart. Immediately after the second irradiation dose, mice were injected with freshly isolated bone marrow cells (1×10^6 cells suspended in 200 μl of PBS) from WT and 1/3 DKO littermates by tail vein injection. The mice were administered antibiotic (polymyxin B sulfate and neomycin sulfate) containing water in a pathogen-free vivarium. To examine thymocytes derived from transplanted bone marrow cells (CD45.2^{+}), recipient CD45.1^{+} cells were excluded from the flow cytometric analysis.

Microarray analysis

DN thymocytes from three WT and three 1/3 DKO mice were sorted on a MoFlo cell sorter directly into TRIzol (Invitrogen Life Technologies). Total RNA (50 ng for each sample) was amplified using the MessageAmp II

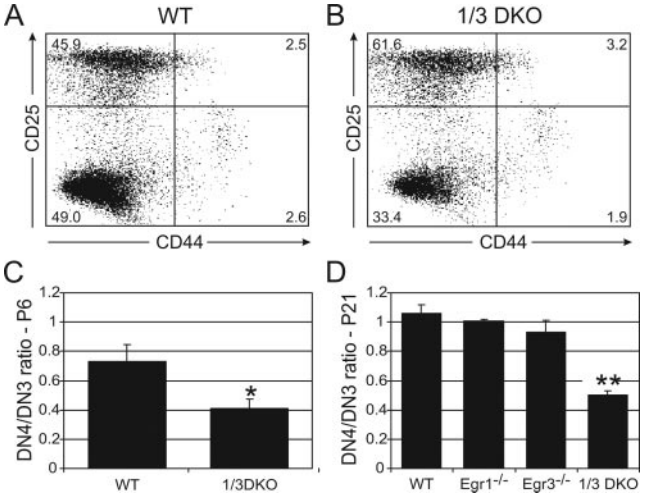

FIGURE 4. Altered populations of DN thymocytes in 1/3 DKO thymus. Representative FACS plot of CD25 and CD44 expression on DN WT (*A*) and 1/3 DKO thymocytes (*B*). DN thymocytes accumulate in the Lin^-; $CD44^-$; $CD25^+$ DN3 subset in 1/3 DKO mice. *C*, DN3/DN4 ratios are significantly decreased in 1/3 DKO thymocytes relative to WT at P6. *D*, DN3-DN4 ratios are significantly decreased in 1/3 DKO thymocytes relative to WT, $Egr1^{-/-}$, or $Egr3^{-/-}$ at P21 (values represent mean ± SEM for three to five mice of each genotype; *, $p < 0.05$ and **, $p < 0.01$, Student's unpaired *t* test).

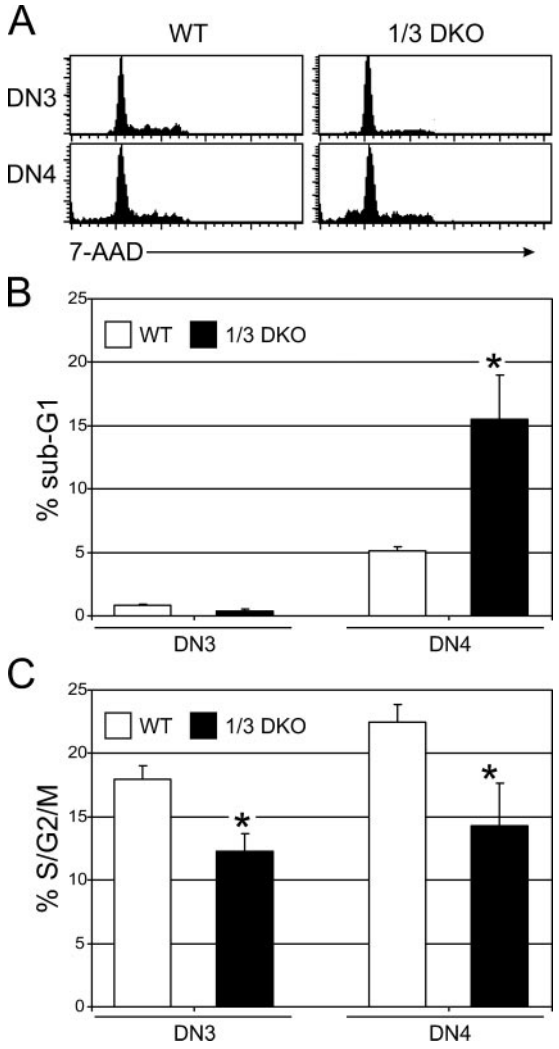

FIGURE 5. Abnormal cell cycle activity and apoptosis in 1/3 DKO DN3 and DN4 thymocytes. *A*, Representative histograms showing DNA content stained by 7-AAD in gated DN3 and DN4 thymocytes from WT and 1/3 DKO mice. *B*, An increased number of apoptotic cells as defined by the sub-G_1 population was observed in 1/3 DKO DN4 thymocytes. No differences were observed in the DN3 population. *C*, Quantification of proliferating cells as defined by the S/G_2/M population. Proliferation is significantly reduced in both DN3 and DN4 in 1/3 DKO thymocytes relative to WT (values represent mean ± SEM for three mice of each genotype; Student's *t* test; *, $p < 0.05$).

aRNA kit (Ambion). Microarray analysis was performed as described (26) using GeneChip mouse expression arrays (430A and 430B; Affymetrix). Briefly, 5 μg of antisense RNA was converted to cDNA using the Superscript Reverse Transcriptase (Invitrogen Life Technologies) and the T7-Oligo(dT) Promoter Primer kit (Affymetrix). The cDNA was purified using the GeneChip sample cleanup module (Affymetrix) and was then used for the in vitro synthesis of biotin-labeled cRNA using the GeneChip expression 3′-amplification reagents for in vitro transcription labeling (Affymetrix) at 37°C for 16 h. The cRNA was fragmented into 35- to 200-bp fragments using a magnesium acetate buffer (Affymetrix). A total of 10 μg of labeled cRNA were hybridized to the microarrays for 16 h at 45°C. The GeneChips were washed and stained according to the manufacturer's recommendations (Affymetrix) using the GeneChips fluidics station (model 450; Affymetrix). This procedure included washing the chips with PE-streptavidin, performing signal amplification by a second staining with biotinylated anti-streptavidin, and performing a third staining with PE-streptavidin. Each chip was scanned using the GeneChip scanner (model 3000; Affymetrix). Signal intensity and detection calls were generated using the GeneChip operating software (Affymetrix). The absolute intensity values of each chip were scaled to the same target intensity value of 150 to normalize the data for interarray comparisons. Six chip comparisons were generated using the samples from three WT and three 1/3 DKO DN thymocytes. Up-regulated and down-regulated genes with a >2-fold change in expression were identified using statistical measures performed on the nine interarray comparisons. The annotated MAGE-ML and image fields are available for all arrays at http://arrayconsortium.tgen.org.

Quantitative real-time PCR (qPCR)

RNA isolation, reverse transcription, and qPCR analysis were performed as described (27). Quantification was performed by generating standard curves using control cDNA samples and all samples were normalized relative to expression of GAPDH. Primer sequences are available upon request.

Results

Egr1 is induced at multiple stages of thymocyte development, yet Egr1-deficient mice exhibit only subtle defects in positive selection and normal β-selection during thymocyte development (15). To test the hypothesis that multiple Egr proteins may perform redundant roles during thymocyte differentiation, mice with simultaneous loss of both Egr1 and Egr3 (1/3 DKO) were generated. Compared with WT littermates, thymuses from 1/3 DKO mice were markedly smaller in gross appearance (Fig. 1, *A* and *B*). Thymocyte cell counts were obtained from WT, $Egr1^{-/-}$, $Egr3^{-/-}$, and Egr1/3 DKO mice during the first 4 wk of life to more precisely define the timing and extent of the thymic atrophy. Thymocyte numbers were equivalent between all genotypes at birth, but as early as postnatal day 6 (P6) a statistically significant reduction in cell number was seen in 1/3 DKO thymuses compared with WT controls ($p = 0.0002$, Fig. 1*C*). This difference became more pronounced 2 and 3 wk after birth. By P21, thymocyte number in 1/3 DKO mice were reduced to 10% that of WT littermates. Although 1/3 DKO mice are ~50% smaller than normal littermates by P21, the differences in thymocyte cell number were significant even after correction for body weight (data not shown), indicating that the thymic atrophy was not simply the result of smaller body size. At P21, $Egr1^{-/-}$ and $Egr3^{-/-}$ mice also showed reduced cellularity. However, thymic atrophy was significantly more pronounced in 1/3 DKO mice relative to $Egr1^{-/-}$ and $Egr3^{-/-}$ ($p = 0.008$ and 0.004, respectively). In addition, relative to WT thymuses (Fig. 1*D*),

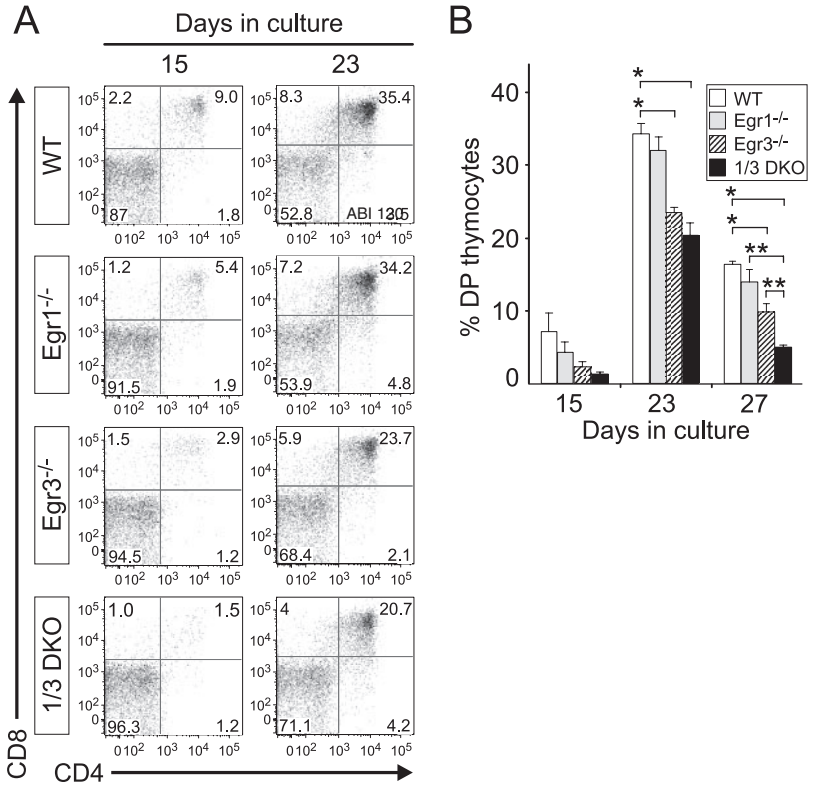

FIGURE 6. Impaired differentiation of Egr-deficient HSCs in vitro. *A*, Representative histograms of CD4 and CD8 expression on bone marrow HSCs cultured for 15 and 23 days on the OP9-DL1 stromal cells. Note intermediate decreases in DP cells from Egr1- and Egr3-deficient HSCs. DP cells are significantly further decreased in 1/3 DKO HSCs relative to WT. *B*, DP cells produced after 15, 23, and 27 days of culture on OP9-DL1 cells. At day 27, cultures of 1/3 DKO thymocytes showed significantly decreased differentiation to DP thymocytes compared with WT, Egr1$^{-/-}$, and Egr3$^{-/-}$ HSCs (values represent mean ± SEM for three experiments; Student's paired *t* test; ∗, $p < 0.01$; ∗∗, $p < 0.05$).

histological analysis of 1/3 DKO thymuses at P21 revealed effacement of normal thymic architecture and numerous pyknotic nuclei (Fig. 1*E*, arrows).

TUNEL histochemistry was performed on WT, Egr1$^{-/-}$, Egr3$^{-/-}$, and 1/3 DKO thymuses to evaluate the extent of apoptosis. In 1/3 DKO thymuses at P21, there was massive apoptosis not seen in WT, Egr1$^{-/-}$, or Egr3$^{-/-}$ littermates (Fig. 2, *A–D*). Quantification of TUNEL-positive cells demonstrated that 1/3 DKO mice, but not Egr1$^{-/-}$ or Egr3$^{-/-}$ mice, had significantly increased apoptosis at P21 (Fig. 2*E*, $p < 0.001$). Because TUNEL

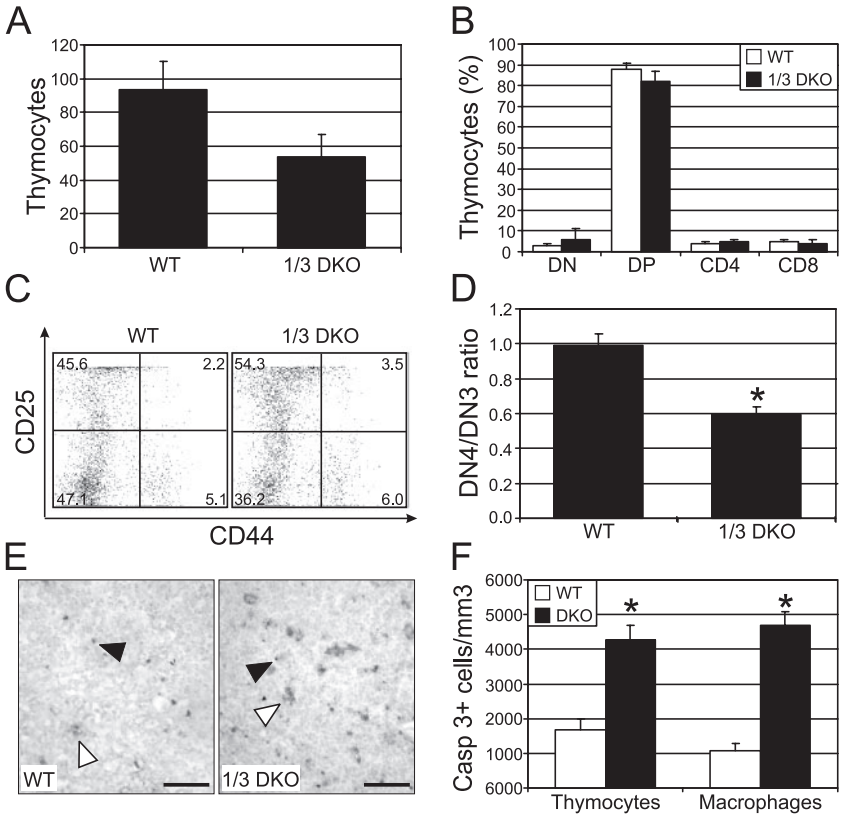

FIGURE 7. Thymocyte defects in 1/3 DKO mice are cell autonomous. *A*, The number of donor cells identified in transplanted thymuses was decreased when 1/3 DKO thymocytes were transplanted relative to WT (values represent total thymocytes multiplied by the percentage of CD45.1 donor cells for three mice of each genotype ± SEM). *B*, No significant differences in the relative percentages of DN, SP, CD4$^+$, and CD8$^+$ were identified between transplanted 1/3 DKO thymocytes compared with WT (values represent mean ± SEM for three mice of each genotype). *C*, DN thymocytes accumulate in the CD44$^-$, CD25$^+$ DN3 subset in 1/3 DKO mice relative to WT. *D*, The DN4/DN3 ratio in 1/3 DKO thymocytes is significantly reduced compared with WT (values represent DN4/DN3 ratio ± SEM for three mice of each genotype; Student's *t* test; ∗, $p < 0.01$). *E*, Immunohistochemistry for activated caspase 3 shows a marked increase in caspase 3$^+$ cells in mice transplanted with 1/3 DKO-derived bone marrow relative to WT. Caspase 3$^+$ thymocytes (black arrowheads) and macrophages (white arrowheads) can be distinguished (scale bar, 50 μm). *F*, Activated caspase 3$^+$ thymocytes and macrophages containing caspase 3$^+$ thymocytes are significantly increased in thymuses of mice receiving transplants with 1/3 DKO-derived bone marrow relative to WT (Student's paired *t* test; ∗, $p < 0.005$).

Table I. *Genes in 1/3 DKO thymocytes*

Gene Symbol	Gene Name	Fold Change (microarray)	Fold Change (qPCR)
Genes down-regulated in 1/3 DKO thymocytes			
Khrdbs	KH domain containing, RNA binding, signal transduction-associated 1	10.27	ND
Srm	Spermidine synthase	7.38	ND
Sms	Spermine synthase	1.60	7.01
Runx1	Runt-related transcription factor 1	6.88	5.01
Hivep3	HIV type I enhancer-binding protein 3	5.66	ND
Myc	Myelocytomatosis oncogene	3.75	6.85
Cdk4	Cyclin-dependent kinase 4	2.30	4.83
E2F1	E2F transcription factor 1	1.90	2.55
Genes up-regulated in 1/3 DKO thymocytes			
Pdcd1	Programmed cell death 1	7.46	ND
Sla2	Src-like-adaptor 2	6.49	ND
Bim (Bcl2L11)	BCL2-like 11	4.39	2.7
Ctla4	CTL-associated protein 4	3.69	ND
Bik	Bcl2-interacting killer-like	3.40	4.3
Fasl	Fas ligand (TNF superfamily, member 6)	2.73	2.9

histochemistry labels cells in late stages of apoptosis when many thymocytes have been phagocytosed by macrophages (28), it is likely that the degree of apoptosis in the 1/3 DKO was underestimated. To determine whether increased apoptosis was present in very young mice, WT and 1/3 DKO littermates were analyzed at P6. To maximize detection of early apoptotic cells, immunohistochemistry for activated caspase 3, a sensitive and early marker of apoptosis, was performed. Consistent with the increased TUNEL reactivity observed in P21 mice, the number of activated caspase 3^+ thymocytes was significantly increased in 1/3 DKO thymuses at P6, indicating that the decreased thymic cellularity was at least in part a consequence of increased apoptosis (Fig. 2*F*).

To assess the effect of Egr1 and Egr3 deficiency on thymocyte differentiation, CD4 and CD8 expression were examined in single and double knockout thymocytes. Representative FACS analysis plots from P21 thymocytes are shown in Fig. 3*A*. The percentages of DN, DP, and CD4 SP thymocytes were statistically indistinguishable between WT and 1/3 DKO thymocytes (Fig. 3*B*). However, there was a small but significant decrease in the percentage of CD8 SP thymocytes in 1/3 DKO mice (2.2% in 1/3 DKO vs 4.0% in WT, $p = 0.002$). Thymocytes from 1/3 DKO thymuses at earlier ages, or in Egr1$^{-/-}$ or Egr3$^{-/-}$ mice at any age, were statistically similar to WT (data not shown). Because *Egr* genes have been shown to be essential for progression from the DN3 to the DN4 stage, substages of DN thymocyte development were examined. Consistent with previous data implicating Egr proteins in the progression through the β-selection checkpoint, there was a partial block at the DN3 stage in 1/3 DKO thymocytes (Fig. 4, *A* and *B*). This block was noted as early as P6, and it became more prominent by P21 (Fig. 4, *C* and *D*). By contrast, significant alteration in the DN4/DN3 ratio was not seen in Egr1$^{-/-}$ or Egr3$^{-/-}$ thymocytes (Fig. 4*D*). To simultaneously assess proliferation and apoptosis in substages of DN maturation, thymocytes were stained with the DNA-binding dye 7-AAD (Fig. 5*A*). Consistent with the increased apoptosis detected in situ in 1/3 DKO thymuses, 1/3 DKO DN4 cells exhibited increased numbers of cells with sub-G_0 DNA content compared with WT littermates (Fig. 5*B*). Proliferating DN3 and DN4 cells were also decreased in the 1/3 DKO thymus, and a similar decrease was seen with BrdU immunohistochemistry on WT and 1/3 DKO thymuses (Fig. 5*C* and data not shown). However, the magnitude of this decrease was not significantly different from previously reported for Egr3$^{-/-}$ mice (16). No reproducible differences in DN1 or DN2 proliferation or apoptosis could be detected in any genotype (data not shown).

Most 1/3 DKO thymuses exhibited cell loss, increased apoptosis, and relatively normal CD4 and CD8 expression. However,

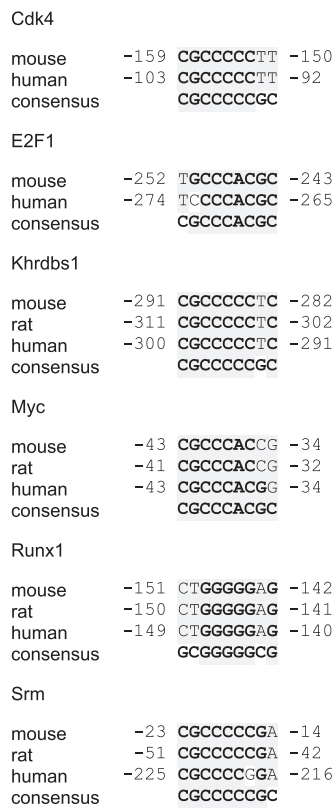

FIGURE 8. Evolutionarily conserved Egr-binding motifs in potential target genes. The 1000 bp upstream promoter regions of select genes in mouse, rat, and human were analyzed for potential Egr-binding sites. Conserved sequences were identified and compared with the defined Egr consensus-binding site GCG(T/G)GGGCG or its reverse complement. Bases highlighted in gray indicate interspecies conservation and bases in bold match the previously defined Egr consensus site. The numbering is relative to the transcriptional start sites as published in the respective genome databases.

Table II. *Gene expression changes*

	Arbitrary GAPDH Normalized Expression[a]				Ratios	
Gene	WT DN3	WT DN4	1/3 DKO DN3	1/3 DKO DN4	WT/DKO (DN3)	WT/DKO (DN4)
Myc	19,325	90,062	151	20,666	127.9	4.35
E2F1	3,387	3,726	1,766	1,576	1.9	2.1
Runx1	17,165	4,519	7,600	2,406	3.8	1.8
CD25[b]	71,123	522	73,329	125	0.96	4.2

[a] Values represent arbitrary units of expression normalized to GAPDH from qPCR.
[b] CD25 is included to confirm purity of the sorted populations.

three mice had severe cell loss (total thymic cellularity <10 million cells) and a significant decrease in the percentage of DP thymocytes (data not shown). These mice were suspected to have an intrinsic defect in thymocyte survival and differentiation, but also an extrinsic thymocyte killing, possibly mediated by glucocorticoids in response to stress or malnourishment. Such mice were excluded from the analysis shown above. However, to confirm that the phenotype observed was due to a thymocyte-intrinsic defect and not a secondary effect such as stress, bone marrow HSCs from WT, Egr1$^{-/-}$, Egr3$^{-/-}$, and 1/3 DKO mice were cultured on OP9-DL1 cells which are capable of inducing Notch signaling and T cell differentiation in vitro (25). After 15 days of coculture with OP9-DL1 cells, a relatively small percentage of marrow-derived cells expressed both CD4 and CD8 (DP) in all cultures (Fig. 6*A*). However, after 23 days in culture, Egr3$^{-/-}$ and 1/3 DKO cells had generated significantly fewer DP cells compared with WT (Fig. 6), a significant effect which was also seen after 27 days of culture (Fig. 6*B*). Consistent with a lack of defects in β selection in vivo, Egr1$^{-/-}$ cells generated DP cells with similar efficiencies compared with WT ($p > 0.05$ at all time points). These results support the hypothesis that impaired differentiation of the Egr3$^{-/-}$ and 1/3 DKO thymocytes is due to a cell-autonomous defect. Importantly, after 27 days of culture, 1/3 DKO cells showed significantly impaired differentiation compared with cells lacking only Egr1 or Egr3, indicating that defects seen in the 1/3 DKO mice cannot be attributed solely to loss of Egr3 ($p < 0.05$; Fig. 6*B*).

To demonstrate whether there was a cell-autonomous defect in vivo, bone marrow cells from 1/3 DKO and WT mice were transplanted into normal recipients. Donor cells carry the *CD45.2* allele and recipient cells carry the *CD45.1* alleles, which are distinguishable by flow cytometry. Therefore, recipient cells were excluded from analysis by gating on CD45.2$^+$ cells derived from donor bone marrow cells. Total thymic cellularity was decreased ~2-fold in mice transplanted with 1/3 DKO bone marrow, although due to high animal-to-animal variability this difference did not reach statistical significance (Fig. 7*A*, $p = 0.06$). In addition, there was no significant difference between the number of DN, DP, and SP thymocytes between WT and 1/3 DKO-transplanted mice (Fig. 7*B*). However, analysis of DN thymocyte subpopulations in transplanted mice receiving 1/3 DKO bone marrow-derived cells again revealed a partial block at the DN3 population relative to mice transplanted with cells from WT mice (Fig. 7, *C* and *D*, $p = 0.004$). An increased number of apoptotic cells was noted in thymuses reconstituted with 1/3 DKO relative to WT-derived bone marrow cells (Fig. 7*E*). Moreover, caspase 3 reactivity was observed in both individual thymocytes (Fig. 7*E*, black arrowheads) and in macrophages (Fig. 7*E*, white arrowheads) which phagocytosed the apoptotic cells. In addition, there was a quantitative increase in the number of caspase 3$^+$ cells and macrophages in thymuses from mice transplanted with 1/3 DKO-relative WT bone marrow-derived cells (Fig. 7*F*, $p < 0.005$ for both comparisons).

The increased apoptosis observed in 1/3 DKO thymocytes suggests that Egr proteins may cooperate to regulate thymocyte survival factors. Because Bcl-2 has been proposed to be a downstream target of Egr1 (15), Bcl-2 was measured in DN, DP, and SP thymocytes using intracellular flow cytometry. However, levels of Bcl-2 protein in 1/3 DKO thymocytes were equal to or greater than the levels found in WT littermates (data not shown). Likewise, the mRNA expression of other antiapoptotic genes—including *A1*, *Mcl-1*, and *Bcl-x$_L$*—was equivalent in WT and 1/3 DKO thymocytes (data not shown).

To identify novel target genes induced by Egr proteins as thymocytes progress from DN3 to DN4, genome-wide expression analysis was performed on WT and 1/3 DKO DN thymocytes using Affymetrix microarray analysis. The signal values for all genes analyzed are listed in supplementary table I.[4] Six hundred thirty-nine genes were significantly down-regulated ≥2-fold in 1/3 DKO thymocytes relative to WT. The fold-changes in levels of expression, as well as confirmatory qPCR analysis for a select population of potential target genes, are shown in Table I. In addition, analysis of promoter regions of several of these genes revealed evolutionarily conserved Egr-binding motifs, suggesting that they may be subject to direct Egr-mediated transcriptional regulation (Fig. 8). Notably, the transcriptional regulators Myc and Runx1, both of which are involved in β-selection, were down-regulated in 1/3 DKO thymocytes, as was the proliferation regulator CDK4. The apoptosis that occurs in 1/3 DKO thymocytes skewed the DN3 and DN4 populations by 1.3- and 1.5-fold, respectively, relative to WT. To confirm that this had no significant effect on the gene expression results, we isolated DN3 and DN4 cells from WT and 1/3 DKO thymuses and examined the expression of a few deregulated genes by qPCR. The results indicated that gene expression changes were far more dramatic than could be explained by alterations in DN3/DN4 cell populations due to apoptosis (Table II). Moreover, expression of CD25 which is expressed at high levels in DN3 cells and low levels in DN4 cells demonstrates the purity of the cells tested and no significant alteration in expression between WT and 1/3 DKO mice. Interestingly, another 858 genes were up-regulated ≥2-fold in 1/3 DKO thymocytes, including the pro-apoptotic genes *Bcl2L11* (*Bim*), *Bik*, and *FasL*.

To identify functional classes of genes which were misregulated in thymocytes that lack both Egr1 and Egr3, significantly deregulated genes were clustered using Gene Ontology (GO) annotations with the EXPANDER software suite (29) (www.cs.tau.ac.il/~rshamir/expander). Surprisingly, several classes of genes involved in basic metabolic processes were significantly overrepresented among the genes down-regulated in 1/3 DKO DN thymocytes (Fig. 9*A*). Over 100 genes annotated as involved in cellular metabolism, comprising 13.1% of all genes in this class, were down-regulated

[4] The online version of this article contains supplemental material.

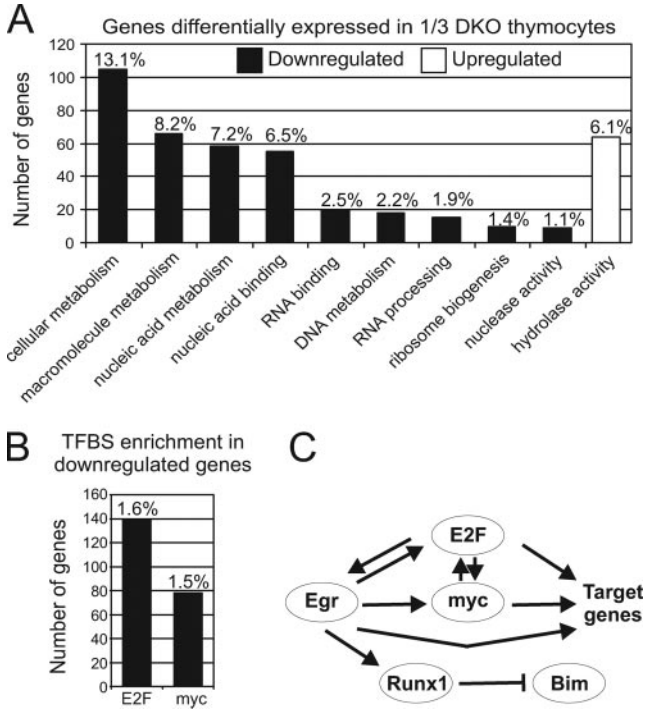

FIGURE 9. GO annotation and promoter analysis of genes deregulated in 1/3 DKO DN thymocytes. *A*, Genes that were significantly down-regulated (■) and significantly up-regulated (□) in 1/3 DKO DN thymocytes fell into several GO categories. The values depicted on the *y*-axis represent the total number of genes in each class with the percentages indicating the relative number compared with the total number of genes in the particular GO class represented on the microarray. *B*, Many genes that were down-regulated in 1/3 DKO thymocytes contained either E2F or Myc transcription factor-binding sites. *C*, Potential transcriptional network involving Egr, E2F, Runx1, Myc, and Bim proteins in DN thymocytes (see text).

in thymocytes lacking Egr1 and Egr3. These results suggest that Egr proteins, either directly or indirectly, regulate genes involved in maintenance of cellular metabolic activity. Among the genes up-regulated in 1/3 DKO thymocytes, only genes annotated as possessing hydrolase activity were enriched (Fig. 9*A*).

The EXPANDER program was also used to identify transcription factor-binding sites (TFBS) in potential *Egr* target genes which were down-regulated in 1/3 DKO thymocytes. Binding sites for two proteins, E2F and Myc, were found to be significantly enriched in the set of down-regulated genes ($p < 0.001$ for both factors). E2F sites were identified in the promoters of 140 genes (or 1.6% of E2F promoter-containing genes represented on the array) and Myc sites were identified in nearly 80 genes (or 1.5% of Myc promoter-containing gene represented on the array; Fig. 9*B*).

Discussion

Although Egr proteins have been proposed to be critical mediators of thymocyte differentiation, defining their essential role in vivo has been difficult due to some functional overlap between Egr1, Egr2, and Egr3. For example, in genetic backgrounds where thymocytes are unable to generate a pre-TCR signal, overexpression of Egr1 can partially drive differentiation past the DN4 stage in vitro and in vivo (13, 30). However, Egr1$^{-/-}$ mice show no defect in β-selection, indicating that the function of Egr1 is compensated by other Egr family members at this stage (15). Egr3-deficient mice exhibit thymic atrophy due to impaired proliferation after β-selection. In this study, we characterized thymocyte differentiation in mice lacking both Egr1 and Egr3, and found that these mice exhibit severe thymocyte loss due to impaired proliferation and increased apoptosis. The apoptotic thymocyte death was at least partially cell autonomous and occurred at the DN4 stage where *Egr* genes are first expressed. Although thymocyte cultures on OP9-DL1 cells confirmed a cell autonomous role for Egr1 and Egr3 in thymocyte survival, the overall thymic cellularity was not decreased to the same extent in the bone marrow chimeras as it was in 1/3 DKO mice. These results may suggest that Egr-dependent environmental or non-thymocyte-intrinsic factors also contribute to thymocyte survival in vivo. One possibility is that thymocyte conditioning by thymic stromal interactions may also depend upon Egr-mediated transcription. The extent to which Egr-dependent gene regulation in thymic stromal cells may influence thymocyte development is currently unknown.

Mice lacking both Egr1 and Egr3 also exhibited fewer CD8$^+$ SP cells, although this could not be replicated in normal recipients transplanted with 1/3 DKO bone marrow. It may be that this represents a non-cell-autonomous defect. Conversely, because there was only a small reduction in CD8$^+$ SP cells in the 1/3 DKO mice compared with WT mice, it is possible that the bone marrow reconstitution experiments lacked sufficient power to detect this difference.

Although these results confirm a role for Egr proteins in the progression through the β-selection checkpoint, we note some discrepancies with previously reported literature. Notably, we were unable to identify a significant block at the DN3 stage in Egr3$^{-/-}$ thymocytes, although consistent with published reports we find that the Egr3$^{-/-}$ thymus is smaller than WT littermates (16). This difference may be explained by differences in genetic background (C56Bl6/129SV vs B6.AKR). However, taken together, the combined results suggest that Egr3 is required for normal thymocyte differentiation and simultaneous loss of Egr1 and Egr3 leads to more profound defects.

Cell death in 1/3 DKO thymocytes was not accompanied by the loss of known survival molecules. To understand which genes depend upon the expression of Egr1, Egr3, or both proteins, Affymetrix microarray analysis was performed to identify novel target genes. The transcription factors Runx1 and Myc, both known to play important roles during β-selection, were down-regulated in 1/3 DKO DN thymocytes. Impairment of Runx1 function in DP thymocytes leads to an up-regulation of Bim and increased sensitivity to apoptosis (31). As Runx1 expression in the thymus is highest in DN thymocytes (32), it is possible that reduction of Runx1 in DN thymocytes similarly leads to increased apoptosis. Consistent with this possibility, proapoptotic Bim was up-regulated in 1/3 DKO DN thymocytes (Table I).

To identify functional classes of genes deregulated in 1/3 DKO DN thymocytes, GO annotations were assigned to genes with significant changes in expression compared with WT. Surprisingly, several classes of genes involved in basic metabolism were significantly enriched in the set of genes down-regulated in 1/3 DKO cells. These results suggest that Egr-mediated pre-TCR signaling may influence thymocyte survival by maintaining metabolic processes in a manner analogous to that proposed for signals downstream of Notch1 and TCR stimulation (33, 34). For example, spermidine, and the related polyamine spermine, are known to be critical regulators of cell survival (35). By qPCR analysis, both Srm and Sms, which encode spermine synthase, were significantly down-regulated in 1/3 DKO thymocytes. Several studies have indicated that intracellular spermine is protective against apoptosis in thymocytes, raising the possibility that reduction in polyamines may sensitize 1/3 DKO thymocytes to apoptosis (36–38). Alternatively, it is possible that down-regulation of metabolic genes represents a nonspecific consequence of apoptosis initiated

through other mechanisms. However, because the expression of over 1200 genes annotated in the "cellular metabolism" GO category did not vary between WT and 1/3 DKO (expression ratios 0.9–1.1) the results do not appear to represent a global down-regulation of metabolism that would likely be associated with apoptosis.

To further characterize transcriptional networks affected by the loss of both Egr1 and Egr3, TFBS were analyzed in the promoters of some deregulated genes. Egr-binding sites were identified in the promoters of 68 genes, including evolutionarily conserved sites in the promoters of Runx1, Myc, E2F1, and cdk4. However, after applying the Bonferroni correction for multiple tests, the enrichment of Egr-binding sites was not considered significant compared with the total set of TFBS in the TRANSFAC database (data not shown). This may indicate that Egr proteins bind to promoter sites which differ from the canonical consensus site used in the TRANSFAC database. In support of this possibility, similar promoter analysis on the set of genes induced by overexpression of Egr3 in myotubes also did not yield significant enrichment of Egr-binding sites (Ref. 26 and data not shown), yet several directly regulated target genes have been identified and well-characterized from the array data set (26, 27). Alternatively, this may indicate that a large number of down-regulated genes are indirectly regulated by Egr1 and/or Egr3.

Because a number of transcriptional activators were down-regulated in 1/3 DKO thymocytes, the promoters of down-regulated genes were scanned for significant enrichment of binding sites for these proteins. For example, Myc- and E2F-binding sites were significantly overrepresented in promoter regions of the down-regulated genes. This was noteworthy as both Myc and E2F1 were down-regulated in cells lacking Egr1 and Egr3. Given that Myc is known to transactivate a large number of genes involved in metabolism and ribosome synthesis (39), it is possible that the down-regulation of metabolism-related genes may reflect an indirect effect through a decrease in Myc expression. Furthermore, E2F4/p130 complexes repress a large number of metabolic genes during growth arrest, making it possible that transcriptionally active E2F proteins contribute to the expression of these genes during periods of growth such as seen after β-selection (40). This raises the possibility that reductions in E2F and Myc contribute to further deregulation of gene expression, leading to functional impairments of 1/3 DKO thymocytes. It is also possible that Myc and E2F1 modulate each other's expression or that of Egr1 based on previous overexpression studies and/or chromatin immunoprecipitation experiments (41–44). A potential regulatory network involving Egr, E2F, Myc, Runx, and Bim is shown in Fig. 9*C*.

Some genes previously proposed to be targets of Egr proteins appeared normal in 1/3 DKO thymocytes. For example, Bcl-2 was shown to be modestly reduced in 3A9Tg, Egr1$^{-/-}$CD4^{low}/CD8^{low} thymocytes after stimulation with anti-CD3 (15). Here, we report that Bcl-2 expression was equivalent or slightly increased in 1/3 DKO DN thymocytes (see supplementary table I) and this was confirmed at the protein level in DN, DP, CD4$^+$, and CD8$^+$ cells by intracellular flow cytometry (data not shown). Although it is possible that Egr proteins affect the kinetics of Bcl-2 induction during positive selection, our data suggest that neither Egr1 nor Egr3 are essential for normal expression of Bcl-2 in vivo. Similarly, Id3 was initially proposed to be a potential Egr target by Bain and colleagues (45) who showed that Egr1 and Id3 were both induced by MAPK signaling with kinetics showing Id3 expression followed Egr1. Further studies demonstrated that overexpression of Egr3 can induce Id3 and lack of Egr3 results in a partial block at DN3, providing a model where Egr3 serves to regulate proliferation after β-selection (16, 18). However, in this study, the level of Id3 was not reduced in 1/3 DKO thymocytes compared with WT littermates. This raises two possible explanations. First, although overexpression of Egr3 may be sufficient to induce Id3, Egr function may not be required for normal Id3 expression in vivo. Alternatively, remaining Egr2 protein may be able to compensate for a loss of Egr1 and Egr3. Further studies will be required to distinguish these two possibilities.

In summary, we demonstrate that Egr1 and Egr3 serve redundant roles in the maintenance of cell survival during thymocyte differentiation in vivo. The mechanism by which these transcription factors contribute to cell survival remains to be precisely defined, but it does not appear to depend upon the up-regulation of known antiapoptotic molecules. One possibility is that failure to up-regulate essential metabolic enzymes and subsequent depletion of macromolecule precursors may increase sensitivity to apoptotic stimuli. Given the number of proapoptotic molecules which are up-regulated in 1/3 DKO thymocytes, it is also possible that Egr proteins serve to actively repress genes which normally would kill cells which do not receive a pre-TCR signal. Additional studies will be needed to further define the role of Egr proteins, including Egr2, in mediating some of the diverse phenotypic changes which follow successful passage through T cell developmental checkpoints.

Acknowledgments

We thank members of the National Institute for Neurological Diseases microarray consortium (TGen) for performing and analyzing the Affymetrix microarrays. We thank members of the Tourtellotte laboratory for advice on the manuscript, and Dr. L. Li for technical assistance with bone marrow transplants.

Disclosures

The authors have no financial conflict of interest.

References

1. Godfrey, D. I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol. Today* 14: 547–553.
2. Kruisbeek, A. M., M. C. Haks, M. Carleton, A. M. Michie, J. C. Zuniga-Pflucker, and D. L. Wiest. 2000. Branching out to gain control: how the pre-TCR is linked to multiple functions. *Immunol. Today* 21: 637–644.
3. Aifantis, I., F. Gounari, L. Scorrano, C. Borowski, and H. von Boehmer. 2001. Constitutive pre-TCR signaling promotes differentiation through Ca^{2+} mobilization and activation of NF-κB and NFAT. *Nat. Immunol.* 2: 403–409.
4. Engel, I., C. Johns, G. Bain, R. R. Rivera, and C. Murre. 2001. Early thymocyte development is regulated by modulation of E2A protein activity. *J. Exp. Med.* 194: 733–745.
5. Engel, I., and C. Murre. 2004. E2A proteins enforce a proliferation checkpoint in developing thymocytes. *EMBO J.* 23: 202–211.
6. Goux, D., J. D. Coudert, D. Maurice, L. Scarpellino, G. Jeannet, S. Piccolo, K. Weston, J. Huelsken, and W. Held. 2005. Cooperating pre-T-cell receptor and TCF-1-dependent signals ensure thymocyte survival. *Blood* 106: 1726–1733.
7. Voll, R. E., E. Jimi, R. J. Phillips, D. F. Barber, M. Rincon, A. C. Hayday, R. A. Flavell, and S. Ghosh. 2000. NF-κB activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. *Immunity* 13: 677–689.
8. Michie, A. M., and J. C. Zuniga-Pflucker. 2002. Regulation of thymocyte differentiation: pre-TCR signals and β-selection. *Semin. Immunol.* 14: 311–323.
9. Aifantis, I., M. Mandal, K. Sawai, A. Ferrando, and T. Vilimas. 2006. Regulation of T-cell progenitor survival and cell-cycle entry by the pre-T-cell receptor. *Immunol. Rev.* 209: 159–169.
10. Mandal, M., C. Borowski, T. Palomero, A. A. Ferrando, P. Oberdoerffer, F. Meng, A. Ruiz-Vela, M. Ciofani, J. C. Zuniga-Pflucker, I. Screpanti, et al. 2005. The *BCL2A1* gene as a pre-T cell receptor-induced regulator of thymocyte survival. *J. Exp. Med.* 201: 603–614.
11. Sicinska, E., I. Aifantis, L. Le Cam, W. Swat, C. Borowski, Q. Yu, A. A. Ferrando, S. D. Levin, Y. Geng, H. von Boehmer, and P. Sicinski. 2003. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* 4: 451–461.
12. Swirnoff, A. H., and J. Milbrandt. 1995. DNA-binding specificity of NGFI-A and related zinc finger transcription factors. *Mol. Cell. Biol.* 15: 2275–2287.
13. Carleton, M., M. C. Haks, S. A. Smeele, A. Jones, S. M. Belkowski, M. A. Berger, P. Linsley, A. M. Kruisbeek, and D. L. Wiest. 2002. Early growth response transcription factors are required for development of CD4$^-$CD8$^-$ thymocytes to the CD4$^+$CD8$^+$ stage. *J. Immunol.* 168: 1649–1658.

14. Shao, H., D. H. Kono, L. Y. Chen, E. M. Rubin, and J. Kaye. 1997. Induction of the early growth response (Egr) family of transcription factors during thymic selection. *J. Exp. Med.* 185: 731–744.
15. Bettini, M., H. Xi, J. Milbrandt, and G. J. Kersh. 2002. Thymocyte development in early growth response gene 1-deficient mice. *J. Immunol.* 169: 1713–1720.
16. Xi, H., and G. J. Kersh. 2004. Early growth response gene 3 regulates thymocyte proliferation during the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$. *J. Immunol.* 172: 964–971.
17. Xi, H., and G. J. Kersh. 2004. Sustained early growth response gene 3 expression inhibits the survival of CD4/CD8 double-positive thymocytes. *J. Immunol.* 173: 340–348.
18. Xi, H., R. Schwartz, I. Engel, C. Murre, and G. J. Kersh. 2006. Interplay between RORγt, Egr3, and E proteins controls proliferation in response to pre-TCR signals. *Immunity* 24: 813–826.
19. Lee, S. L., L. C. Tourtellotte, R. L. Wesselschmidt, and J. Milbrandt. 1995. Growth and differentiation proceeds normally in cells deficient in the immediate early gene *NGFI-A*. *J. Biol. Chem.* 270: 9971–9977.
20. Tourtellotte, W. G., and J. Milbrandt. 1998. Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor Egr3. *Nat. Genet.* 20: 87–91.
21. Tourtellotte, W. G., R. Nagarajan, A. Bartke, and J. Milbrandt. 2000. Functional compensation by Egr4 in Egr1-dependent luteinizing hormone regulation and Leydig cell steroidogenesis. *Mol. Cell. Biol.* 20: 5261–5268.
22. Whitehead, J., C. Keller-Peck, J. Kucera, and W. G. Tourtellotte. 2005. Glial cell-line derived neurotrophic factor-dependent fusimotor neuron survival during development. *Mech. Dev.* 122: 27–41.
23. Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119: 493–501.
24. Allman, D., A. Sambandam, S. Kim, J. P. Miller, A. Pagan, D. Well, A. Meraz, and A. Bhandoola. 2003. Thymopoiesis independent of common lymphoid progenitors. *Nat. Immunol.* 4: 168–174.
25. Schmitt, T. M., R. F. de Pooter, M. A. Gronski, S. K. Cho, P. S. Ohashi, and J. C. Zuniga-Pflucker. 2004. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat. Immunol.* 5: 410–417.
26. Albert, Y., J. Whitehead, L. Eldredge, J. Carter, X. Gao, and W. G. Tourtellotte. 2005. Transcriptional regulation of myotube fate specification and intrafusal muscle fiber morphogenesis. *J. Cell Biol.* 169: 257–268.
27. Li, L., J. Carter, X. Gao, J. Whitehead, and W. G. Tourtellotte. 2005. The neuroplasticity-associated *arc* gene is a direct transcriptional target of early growth response (Egr) transcription factors. *Mol. Cell. Biol.* 25: 10286–10300.
28. Surh, C. D., and J. Sprent. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* 372: 100–103.
29. Shamir, R., A. Maron-Katz, A. Tanay, C. Linhart, I. Steinfeld, R. Sharan, Y. Shiloh, and R. Elkon. 2005. EXPANDER—an integrative program suite for microarray data analysis. *BMC Bioinformatics* 6: 232.
30. Miyazaki, T. 1997. Two distinct steps during thymocyte maturation from $CD4^-CD8^-$ to $CD4^+CD8^+$ distinguished in the early growth response (Egr)-1 transgenic mice with a recombinase-activating gene-deficient background. *J. Exp. Med.* 186: 877–885.
31. Abe, N., K. Kohu, H. Ohmori, K. Hayashi, T. Watanabe, K. Hozumi, T. Sato, S. Habu, and M. Satake. 2005. Reduction of Runx1 transcription factor activity up-regulates Fas and Bim expression and enhances the apoptotic sensitivity of double positive thymocytes. *J. Immunol.* 175: 4475–4482.
32. Lorsbach, R. B., J. Moore, S. O. Ang, W. Sun, N. Lenny, and J. R. Downing. 2004. Role of RUNX1 in adult hematopoiesis: analysis of RUNX1-IRES-GFP knock-in mice reveals differential lineage expression. *Blood* 103: 2522–2529.
33. Ciofani, M., and J. C. Zuniga-Pflucker. 2005. Notch promotes survival of pre-T cells at the β-selection checkpoint by regulating cellular metabolism. *Nat. Immunol.* 6: 881–888.
34. Rathmell, J. C., M. G. Vander Heiden, M. H. Harris, K. A. Frauwirth, and C. B. Thompson. 2000. In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. *Mol. Cell* 6: 683–692.
35. Schipper, R. G., L. C. Penning, and A. A. Verhofstad. 2000. Involvement of polyamines in apoptosis: facts and controversies: effectors or protectors? *Semin. Cancer Biol.* 10: 55–68.
36. Brune, B., P. Hartzell, P. Nicotera, and S. Orrenius. 1991. Spermine prevents endonuclease activation and apoptosis in thymocytes. *Exp. Cell Res.* 195: 323–329.
37. Hegardt, C., G. Andersson, and S. M. Oredsson. 2001. Different roles of spermine in glucocorticoid- and Fas-induced apoptosis. *Exp. Cell Res.* 266: 333–341.
38. Hegardt, C., G. Andersson, and S. M. Oredsson. 2003. Spermine prevents cytochrome c release in glucocorticoid-induced apoptosis in mouse thymocytes. *Cell Biol. Int.* 27: 115–121.
39. Dang, C. V., K. A. O'Donnell, K., K. I. Zeller, T. Nguyen, R. C. Osthus, and F. Li. 2006. The c-Myc target gene network. *Semin. Cancer Biol.* 16: 253–264.
40. Cam, H., E. Balciunaite, A. Blais, A. Spektor, R. C. Scarpulla, R. Young, Y. Kluger, and B. D. Dynlacht. 2004. A common set of gene regulatory networks links metabolism and growth inhibition. *Mol. Cell* 16: 399–411.
41. Fernandez, P. C., S. R. Frank, L. Wang, M. Schroeder, S. Liu, J. Greene, A. Cocito, and B. Amati. 2003. Genomic targets of the human c-Myc protein. *Genes Dev.* 17: 1115–1129.
42. Leone, G., J. DeGregori, R. Sears, L. Jakoi, and J. R. Nevins. 1997. Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. *Nature* 387: 422–426.
43. Polager, S., Y. Kalma, E. Berkovich, and D. Ginsberg. 2002. E2Fs up-regulate expression of genes involved in DNA replication, DNA repair and mitosis. *Oncogene* 21: 437–446.
44. Ren, B., H. Cam, Y. Takahashi, T. Volkert, J. Terragni, R. A. Young, and B. D. Dynlacht. 2002. E2F integrates cell cycle progression with DNA repair, replication, and G_2-M checkpoints. *Genes Dev.* 16: 245–256.
45. Bain, G., C. B. Cravatt, C. Loomans, J. Alberola-Ila, S. M. Hedrick, and C. Murre. 2001. Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras- ERK MAPK cascade. *Nat. Immunol.* 2: 165–171.