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Ternary Complex Factors SAP-1 and Elk-1, but Not Net, Are Functionally Equivalent in Thymocyte Development

Patrick Costello,*1 Robert Nicolas,*1 Jane Willoughby,*2 Bohdan Wasylyk, † Alfred Nordheim, ‡ and Richard Treisman*

The ternary complex factors (TCFs; SAP-1, Elk-1, and Net) are serum response factor cofactors that share many functional properties and are coexpressed in many tissues. SAP-1, the predominant thymus TCF, is required for thymocyte positive selection. In this study, we assessed whether the different TCFs are functionally equivalent. Elk-1 deletion, but not the hypomorphic Netβ mutation, exacerbated the SAP-1 positive selection phenotype, but triply deficient thymocytes were no more defective than SAP-1−/− Elk-1−/− cells. Inactivation of the other TCFs did not affect SAP-1-independent processes, including β-selection, regulatory T cell selection, and negative selection, although reduced marginal zone B cells were observed in SAP-1−/− Elk-1−/− animals. Ectopic expression of Elk-1, but not Net, rescued positive selection of SAP-1−/− thymocytes; thus, SAP-1 and Elk-1 are functionally equivalent in this system, and the SAP-1 null selection phenotype reflects only its high expression in the thymus. Array analysis of TCR-stimulated double-positive cells identified SAP-1–dependent inducible genes whose transcription was further impaired in SAP-1−/− Elk-1−/− cells; thus, these genes, which include Egr-1 and Egr-2, represent candidate mediators of positive selection. Chromatin immunoprecipitation revealed subtly different promoter targeting between the different TCFs. Ectopic expression of Egr-1 restored positive selection in SAP-1 null thymocytes, establishing it (and possibly other Egr family members) as the major effector for ERK–SAP-1 signaling in thymocyte positive selection. The Journal of Immunology, 2010, 185: 000–000.

The ternary complex factors (TCFs; SAP-1, Elk-1, and Net) are vertebrate Ets domain proteins that link transcription to MAPK signaling, in partnership with the serum response factor (SRF) transcription factor (1). Relatively little is known about their biological roles in vivo. SAP-1 plays an important role in thymocyte positive selection, the Ras-ERK–dependent process that ensures the survival of cells bearing a productively rearranged TCR (2) (see Refs. 3 and 4 for reviews). In contrast, SAP-1 is not required for negative selection, the ERK-independent process by which thymocytes bearing high self-avidity TCRs are deleted.

The online version of this article contains supplemental material.

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; DN, double-negative; DP, double positive; E, Elk-1−/−; Elk, Elk-1; ES, SAP-1−/− Elk-1−/−; ESN, SAP-1−/− Elk-1−/− Netβ; GEO, Gene Expression Omnibus; IRES, internal ribosome entry site; MSCV, murine stem cell virus; nc, no SRF binding site confirmed within 10 kb of the transcription start site; S, SAP-1−/−; Sap, SAP-1−/−; SIF, single-positive; SRF, serum response factor; TCF, ternary complex factor; Treg, regulatory T; W, wild-type; WT, wild-type.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10S16.00 from the T cell repertoire (2, 3). In contrast, Elk-1 inactivation causes no obvious phenotype, whereas the Netβ mutation leads to lymphangiectasia (5, 6).

Although SAP-1 and Net are highly expressed in the tissues affected by their inactivation (2, 7), it remains unclear whether the phenotypes result from this as opposed to TCF-specific functions. Although the TCF proteins share many biochemical and functional properties (8, 9), they differ in DNA-binding specificities (10), interaction with protein partners (11), transcriptional activation and repression (9, 12), and generation of repressive isoforms (1, 8). SRF target genes specifically recruit TCFs or the myocardin family SRF coactivators, but the basis of this specificity is not understood (13). Such differences may account for the observation that SAP-1 is not required for β-selection and regulatory T (Treg) cell development, which are also pre-TCR– or TCR-dependent. In contrast, TCF functional redundancy might explain why the SAP-1 positive-selection defect is mild compared with the strong effect of SRF deficiency (14) (P. Costello, R. Nicolas, and R. Treisman, unpublished observations). Interestingly, previous knockout studies showed that two downstream targets of SAP-1, the zinc finger proteins Egr-1 and Egr-2, are also implicated in positive selection (15–17).

In this study, we used the thymocyte system to investigate TCF functional specificity and target gene activation in detail. We show that Elk-1, but not Net, contributes to thymocyte development in SAP-1−/− cells and that ectopic expression of Elk-1, but not Net, can rescue the selection defect in SAP-1–deficient cells. Thus, SAP-1 and Elk-1, but not Net, are functionally equivalent in this system. We show that the three TCFs display subtly different target gene specificities in vivo and identify Egr family genes as major targets of TCF signaling in thymocyte development. The ability of ectopically expressed Egr-1 to rescue the thymocyte-selection defect in SAP-1−/− cells establishes Egr-1, and possibly other members of the Egr family, as the major effector of ERK signaling in thymocyte positive selection.
Materials and Methods

Mice

SAP-1−/−, Elk-1−/−, and NetΔ3 (2, 5, 6), kept under specific-pathogen-free conditions, were backcrossed to C57/BLJ mice (The Jackson Laboratory, Bar Harbor, ME) for a minimum of five generations and were subsequently crossed with OT-II and HY TCR transgenic mice (2). Elk-1 is on the X chromosome; for clarity we refer to Elk-1 nulls as Elk-1−/− regardless of gender and specify gender in the text and figures when appropriate. For reconstitutions, bone marrow from the femurs of 6-wk-old animals or fetal liver cell suspensions from 15-4-d embryos were injected into the tail vein (5 × 10^5 cells/mouse), and analysis was performed 6 wk later. Donor and host cell lines were distinguished using CD45 alloantigens (donor, CD45.2; host, CD45.1) as described (2). Animal experimentation, approved by the Cancer Research UK Animal Ethics Committee, was carried out under Home Office License PPL 80/2152.

Flow cytometry

Cells were prepared by gentle disintegration of tissue through a 70-μm nylon filter using a syringe plunger and stained with saturating concentrations of Ab, as indicated in the figure legends. Analysis was performed on a FACS-Calibur (BD Biosciences, San Jose, CA) with CellQuest software. Events were collected and stored ungated in list mode. A MoFlo cell sorter (DakoCytometry, Fort Collins, CO) was used to isolate double-positive (DP) thymocytes, defined by cell surface marker expression, to ≥97% purity (FACS analysis). Abs (BD Biosciences CA) were conjugated to either FITC, PE, allophycocyanin, or biotin (revealed using streptavidin–TRICOLOR (Caltag Laboratories, Burlingame, CA) or streptavidin Alexa Fluor 750 allophycocyanin. Abs (Abs clones in parentheses): CD8 (53-5.8), CD4 (74-15C11), CD21/CD35 (7G6), and CD23 (B3B4). Progenitor analysis in the CD45.2 background was determined by real-time PCR with SYBR Green Invitrogen (11744-500) reagents or Roche Universal Probes and the TaqMan Universal PCR mix (4304437) with the ABI 7900HT PCR machine. Abs used were anti-SRF (sc-2567) and SRF (sc-13030), both from Santa Cruz Biotechnology, Santa Cruz, CA, anti-vp16 (V4388; Sigma-Aldrich), and mouse anti-human purified anti-mouse Elk-1 aa309–429, and anti-Net 1995 (20). Primer sequences for ChIP analysis were as follows: Egr1_p: 5′-CCGAGAAGCCATATAGGAAC-3′, 5′-ATATAAGGGCGCTCGCAAACAA-3′; Egr2_p: 5′-AATCGTTCCTCAGCTGATGTCA-3′, 5′-GCAGCTTTTGCTCGAAGCT-3′; Egr3_p: 5′-ATGCGTGGTGATGATGAGGTGATT-3′, 5′-CAGGTCTCTCCACCTTCCCAAGG-3′; Net_p: 5′-GGGCTTCTTGGCTCGATGTC-3′, 5′-GCCTTTTGGACCAAGAC-3′; Srf: 5′-TTGCGAAATTGGTGGTTTCT-3′, 5′-TAGTCTGAACCTAAGGCTT-3′; Per1: 5′-AATAAGGGAGAGACGTTGAC-3′, 5′-TCTTCTGTCACACATGGCAGC-3′; CD69: 5′-GGCTCTTGGACCAAGAC-3′, 5′-TTGCGAAATTGGTGGTTTCT-3′; Gpr171: 5′-TGGCCTACCTAAATGAGGACA-3′, 5′-AAGCTGCAAAGCTCTTAT-3′; Id3: 5′-TAAACCAGGCTCTTTCACCC-3′, 5′-GACAGTCAGGTGCTGTGGTGTA-3′; Ikaros: 5′-CCTCCTCTGTCTCTCA-3′, 5′-AAGCTGCAAAGCTCTTAT-3′; 5′-GACAGTCAGGTGCTGTGGTGTA-3′; Cre: 5′-GGGCTTCTTGGCTCGATGTC-3′, 5′-GCCTTTTGGACCAAGAC-3′; SAP-1, Elk-1, Net, and Egr-1 open reading frames were inserted into Retroviruses and reconstitution

Target genes were validated using SYBR Green-based real-time PCR (Invitrogen, Carlsbad, CA). Relative abundance of template cDNA was calculated according to the comparative C(T) method, normalizing to the abundance of Rps16 cDNA, whose level was invariant under all conditions. Primers for PCR validation of the array data and for TCF mRNA quantitation were as follows: CCACATGGACGA-3′, 5′-GGGCTTCTTGGCTCGATGTC-3′, 5′-TGCGTAAAGGCACTGGCTGTA-3′, 5′-CCCTGGTGAAGCTGATGTCA-3′, 5′-GTGCAGGCCATTCATGTGCAC-3′, 5′-GACAGTCAGGTGCTGTGGTGTA-3′, 5′-TGGCCTACCTAAATGAGGACA-3′, 5′-AAGCTGCAAAGCTCTTAT-3′; Id3: 5′-TAAACCAGGCTCTTTCACCC-3′, 5′-GACAGTCAGGTGCTGTGGTGTA-3′; Ikaros: 5′-CCTCCTCTGTCTCTCA-3′, 5′-AAGCTGCAAAGCTCTTAT-3′; 5′-GACAGTCAGGTGCTGTGGTGTA-3′; Cre: 5′-GGGCTTCTTGGCTCGATGTC-3′, 5′-GCCTTTTGGACCAAGAC-3′; SAP-1, Elk-1, Net, and Egr-1 open reading frames were inserted into Retroviruses and reconstitution

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described (13), with the following modifications: fixation was stopped by the addition of 250 mM glycerine, and sonication was with a Bioruptor UC 200 (full power, 4 min), with recovery using magnetic protein G beads (Invitrogen). The recovery of DNA quantitation was as follows: 5′-TGCGTAAAGGCACTGGCTGTA-3′, 5′-CCCTGGTGAAGCTGATGTCA-3′, 5′-GTGCAGGCCATTCATGTGCAC-3′, 5′-GACAGTCAGGTGCTGTGGTGTA-3′; Cre: 5′-GGGCTTCTTGGCTCGATGTC-3′, 5′-GCCTTTTGGACCAAGAC-3′; SAP-1, Elk-1, Net, and Egr-1 open reading frames were inserted into Retroviruses and reconstitution

Retrovectors and reconstitution

SAP-1, Elk-1, Net, and Egr-1 open reading frames were inserted into pMiG/murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-EGFP. Constructs were cotransfected with Vesicular stomatitis virus glycoprotein using Fugene into GP-293 cells under containment conditions. Forty-eight– and 72-h viral supernatants from the transfected GP-293 cells were subsequently used to infect Phox-2B cells by agglutinating with the expression of the relevant TCF or Egr-1 was monitored by immunoprecipitation with FLAG-M2 (Sigma-Aldrich) and Western blotting for SAP-1 (sc-13030; Santa Cruz Biotechnology), Elk-1 (sc-9182; Cell Signaling Technology, Beverly, MA), Net (sc-17860; Santa Cruz Biotechnology), or anti-FLAG-M2 HRP (Sigma-Aldrich). Coexpression of GFP was monitored by FACS.

Thymocytes were cultured for 2 h in RPMI 1640 with 10% FCS, 50 mM 2-ME. For stimulation, 107 cells were spun (1500 rpm, 5 min) onto anti-CD3-coated six-well plates, washed in PBS, and incubated for 30 min at 37°C, 10% CO2 before RNA preparation using GenElute Mammalian Total RNA miniprep columns (Sigma-Aldrich, St. Louis, MO).

Expression analysis

cDNA probes from triplicate total RNA preparations were generated by the Affymetrix GeneChip protocol. Microarray analysis used mouse MG-430 2.0 arrays (45.1 probe sets, representing unique reference mRNA records or 22,640 unique Unigene IDs). Data were preprocessed and quantile normalized using Thermo procedure within Bioconductor’s Affy package (18). The group-average expression values were calculated using the relevant (two-way) linear models, and differential genes were selected using an empirical Bayes method implemented in the Bioconductor limma package (18). A false-discovery rate of 0.05 was used as the cut-off. Data were deposited in the Gene Expression Omnibus (19) with the Bioconductor’s GEO database (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21546).

Statistical analysis

The statistical significance of flow cytometric experiments was assessed using the unpaired Student t test, except for the retroviral-reconstitution experiments; because it was unclear whether the data point distribution was Gaussian, we used the nonpaired Mann-Whitney U test.

Cell stimulation

Thymocytes were cultured for 2 h in RPMI 1640 with 10% FCS, 50 mM 2-ME. For stimulation, 107 cells were spun (1500 rpm, 5 min) onto anti-CD3-coated six-well plates, washed in PBS, and incubated for 30 min at 37°C, 10% CO2 before RNA preparation using GenElute Mammalian Total RNA miniprep columns (Sigma-Aldrich, St. Louis, MO).

Gene-expression analysis

cDNA probes from triplicate total RNA preparations were generated by the Affymetrix GeneChip protocol. Microarray analysis used mouse MG-430 2.0 arrays (45.1 probe sets, representing unique reference mRNA records or 22,640 unique Unigene IDs). Data were preprocessed and quantile normalized using Thermo procedure within Bioconductor’s Affy package (18). The group-average expression values were calculated using the relevant (two-way) linear models, and differential genes were selected using an empirical Bayes method implemented in the Bioconductor limma package (18). A false-discovery rate of 0.05 was used as the cut-off. Data were deposited in the Gene Expression Omnibus (19) with the Bioconductor’s GEO database (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21546).
Results

Elk-1 contributes to thymocyte positive selection in SAP-1 null mice

Although SAP-1+/− and Elk-1−/− mice are viable and fertile (2, 6), SAP-1+/− Elk-1−/− females appeared infertile, perhaps reflecting defective Egr family expression (21) (see Discussion). However, SAP-1+/− Elk-1−/− animals could be generated at low frequency by crossing SAP-1+/− Elk-1−/− males with SAP-1+/− Elk-1+/− or SAP-1+/− Elk-1+/− females. Thymocyte populations were analyzed in 6–8-wk-old mice in which Elk-1, SAP-1, or both TCFs were inactivated. We saw no alteration in SAP-1 or Net expression in Elk-1−/− thymocytes, nor was Net expression increased in SAP-1+/− Elk-1−/− cells (Supplemental Fig. 1) (2). Thymus cellularity was not affected by deletion of Elk-1, but it increased significantly upon deletion of SAP-1 and further increased by additional inactivation of Elk-1−/−, reflecting an increase in the absolute numbers of the major thymocyte populations (Fig. 1A). Total thymocyte numbers also increased upon inactivation of the TCF target gene Egr-1, and this has been attributed to Egr-1–dependent inhibition of progenitor cell entry into the thymus (22).

As previously reported, Elk-1 deletion alone had no effect on the proportion of mature TCRhi thymocytes or the generation of CD4 SP or CD8 SP thymocytes, but SAP-1 inactivation reduced the proportions of these cell types by ∼50% (Fig. 1B-D) (2, 6). However, in doubly deficient animals, the proportions of CD4 SP and CD8 SP thymocytes, as well as mature TCRhi cells, were reduced by an additional 50% (Fig. 1B-D). Although this indicates a positive-selection defect, the absolute numbers of SP thymocytes produced was comparable among the different strains, probably reflecting the increased cellularity of the TCF-deficient thymuses (Supplemental Fig. 2A, 2B). Expression of CD69, a positive-selection marker, was also reduced in SAP-1 null thymocytes and further impaired in SAP-1+/− Elk-1+/− cells (Supplemental Fig. 2C). When generated by bone marrow transplant into irradiated B6.SJL hosts, SAP-1+/− Elk-1+/− thymocytes also exhibited an ∼75% reduction in CD4 SP, CD8 SP, and TCRhi cells, indicating that the selection defect is cell intrinsic (Supplemental Fig. 3). The reduced proportions of SAP-1+/− Elk-1+/− SP thymocytes were retained in chimeric thymuses generated by mixed reconstitution with wild-type (WT) and SAP-1+/− Elk-1−/− cells; therefore, they do not reflect a reduction in the availability of positively selecting ligands (Fig. 1E).

To examine the selection of thymocytes bearing a defined TCR, we analyzed OT-II transgenic mice, in which thymocytes bearing TCRo Vα2 were selected into the CD4 lineage. Although SAP-1 deletion reduced CD4 SP thymocyte output by 60%, CD4 SP cells were reduced by 90% in two SAP-1+/− Elk-1−/− primary animals (Fig. 1F, Supplemental Fig. 4A). Comparable results were obtained in radiation chimeras (Supplemental Fig. 4B). In both systems, TCF inactivation decreased the proportion of CD4 SP thymocytes that were also Vα2+ (Supplemental Fig. 4C); this suggests that in these RAG2−/−, where non–OT-II thymocytes can also be selected, TCF inactivation somehow puts OT-II+ cells at a selective disadvantage compared with endogenous TCRs.

Mature NK1.1+ CD3+ NK T cells were significantly reduced in SAP-1 null animals and further reduced by simultaneous inactivation of Elk-1 (Fig. 1G). In contrast to SAP-1+/− animals, SAP-1+/− Elk-1+/− animals exhibited a slight reduction in CD4+ FoxP3+ cells, which was also seen in SAP-1+/− Elk-1−/− bone marrow chimeras, indicating that it results from an intrinsic thymocyte defect (Fig. 1H, Supplemental Fig. 3C) (23).

SAP-1−/− Elk-1−/− animals maintain negative selection

We used the HY-TCR transgene, which recognizes the male-specific HY Ag presented by H-2b class I D6, to investigate negative selection in SAP-1+/− Elk-1−/− animals. In female mice, thymocytes expressing the HY TCR are efficiently positively selected, developing into CD8 SP thymocytes, whereas in males, which express the HY Ag, HY TCR thymocytes are efficiently deleted early during ontogeny (24). WT, SAP-1+/−, and SAP-1−/− Elk-1−/− HY TCR males displayed greatly reduced thymic cellularity compared with females (Fig. 2B) (2). Deletion, as assessed by the absence of DP thymocytes, was as effective in SAP-1+/− Elk-1−/− HY TCR males as in WT animals (Fig. 2A, 2B). HY deletion was also unchanged in irradiation chimeras upon transfer of SAP-1+/− Elk-1−/− bone marrow into male hosts (data not shown). SRF-deficient thymocytes also exhibit male-specific deletion of HY TCR-transgenic thymocytes (P. Costello, R. Nicolas, and R. Treisman, unpublished observations). MMTV9 superantigen-mediated deletion of γδ thymocytes occurs efficiently in C57BL/6 × BALB/c mice but not in C57 BL6 animals, reducing the proportion of CD4 γδ thymocytes by 93% and 95%, respectively, while leaving γδ cells unaffected. In SAP-1−/− Elk-1−/− animals, deletion occurred to a similar extent (CD4 γδ, 87%; CD8 γδ, 92%). These data indicate that TCF–SRF signaling is not required for HY- or superantigen-mediated negative selection.

TCFs in early thymocyte development and other hematopoietic cell lineages

ERK signaling has been implicated in the DN3–DN4 (CD44− CD25+ /− / CD44+ CD25−) transition, which accompanies rearrangement of the TCR β-chain and assembly of a functional pre-TCR [reviewed in (3, 4)]; the Egr family of TCF target genes is also implicated in this process (25, 26). We previously observed no effect on early thymocyte development in SAP-1 null animals (2). SAP-1−/− Elk-1−/− deficient animals also did not display significant perturbation of CD44+ and CD25+ cell populations and showed normal progression through the β-selection checkpoint, as assessed by TCRβ expression in CD25+ cell populations (Fig. 2C–E).

B cell lineage-specific inactivation of SRF results in loss of marginal zone B cells and reductions in other B cell types (14). Unlike SAP-1−/− animals, bone marrow from SAP-1−/− Elk-1−/− animals contained increased numbers of B220+ B cells, with a reduced proportion of IgM+ IgD+ mature B cells (Supplemental Fig. 5A). Some small variations were observed between WT and SAP-1−/− Elk-1−/− hematopoietic stem cell populations (Supplemental Fig. 5B–D). Although splenic cellularity was comparable among all of the strains (Supplemental Fig. 6A), SAP-1−/− Elk-1−/− deficient animals exhibited a significant decrease in CD21/35high CD23−low marginal zone B cells, whereas the proportion of CD19+ splenocytes or CD21/35hi CD233hi follicular B cells was unaffected (Supplemental Fig. 6B, 6C).

The NetΔ mutation does not affect positive selection

To investigate whether the third TCF, Net, also contributes to immune cell development, we studied mice carrying the NetΔ mutation, which encodes a truncated Net lacking the Ets domain (5).
SAP-1<sup>−/−</sup> Net<sup>−/−</sup> animals exhibited a chylothorax phenotype similar to that observed in Net<sup>−/−</sup> animals and did not display any additional gross developmental perturbations. Because this prevented the analysis of thymocyte populations, we used 14.5-d fetal livers to reconstitute hematopoiesis in irradiated B6.SJL recipients. Net<sup>−/−</sup> thymocyte profiles were similar to WT, and the combination of Net<sup>−/−</sup> and SAP-1<sup>−/−</sup> mutations did not significantly increase the severity of the SAP-1<sup>−/−</sup> phenotype (Fig. 3A, compare Supplementary Fig. 3A,3B) (2). Thus, Net does not contribute to thymocyte selection in the absence of SAP-1, even though it is expressed at significant levels in thymocytes.

**FIGURE 1.** Elk-1 contributes to positive selection in SAP-1<sup>−/−</sup> mice. A, Thymus cellularity in WT (n = 9), Elk-1<sup>−/−</sup> (n = 9), SAP-1<sup>−/−</sup> (n = 9), and SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> (n = 8) animals. B, TCR<sup>β</sup> thymocytes in WT (n = 8), Elk-1<sup>−/−</sup> (n = 7), SAP<sup>−</sup> (n = 7), and SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> (n = 8) mice. C, Representative CD4/CD8 thymocyte profiles and TCR<sup>β</sup> staining. D, CD4 SP and CD8 SP thymocytes. E, Percentage of CD4 SP and CD8 SP thymocytes in mixed WT/SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> bone marrow chimeras (n = 4). F, CD4<sup>+</sup> Foxp<sup>3</sup> thymocytes in WT (n = 2), SAP<sup>−/−</sup> (n = 2), and SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> (n = 2) OT-II transgenic thymocytes. G, NK1.1<sup>+</sup> CD3<sup>+</sup> CD4<sup>+</sup> thymocytes in WT (n = 14), Elk-1<sup>−/−</sup> (n = 10), SAP<sup>−/−</sup> (n = 12), and SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> (n = 12) mice. H, Foxp3<sup>+</sup> CD4<sup>+</sup> thymocytes in WT (n = 14) and SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> (n = 12) mice. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001 (Student t test).
Elk-1⁻/⁻ Net⁻/⁻ and macroscopically indistinguishable from WT, demonstrating that the triple TCF mutation does not grossly impair embryonic viability to this stage. Surprisingly, the SAP-1⁻/⁻ Elk-1⁻/⁻ Net⁻/⁻ thymocyte profiles were similar to those seen in animals reconstituted with SAP-1⁻/⁻ Elk-1⁻/⁻ cells (Fig. 3B–E).

All of the genotypes reconstituted thymuses of comparable cellularity, with comparable DN thymocyte profiles and the SAP-1⁻/⁻ and SAP-1⁻/⁻ Elk-1⁻/⁻ thymocyte profiles (Supplemental Fig. 7A, 7B). In thymus, SAP-1⁻/⁻ Elk-1⁻/⁻ Net⁻/⁻ TCR⁺ and SP cells seemed to be slightly more abundant relative to SAP-1⁻/⁻ Elk-1⁻/⁻ cells (Fig. 3C, 3D); this effect was more marked in the periphery, suggesting that the Net⁻/⁻ mutation may relieve some aspects of the SAP-1⁻/⁻ Elk-1⁻/⁻ phenotype (Supplemental Fig. 7C–F; see Discussion). Although animals reconstituted with SAP-1⁻/⁻ Elk-1⁻/⁻ fetal liver cells showed a reduced level of FoxP3⁺ CD4 SP cells, similar to primary animals, triply defective FoxP3⁺ CD4 SP thymocytes, which also expressed the Treg cell-associated markers CTLA-4, glucocorticoid-induced tumor necrosis receptor, and CD103, were produced at WT levels (Fig. 3E). Together, these results showed that the intact Net protein is neither required for Treg cell selection nor responsible for the residual positive selection seen in SAP-1⁻/⁻ Elk-1⁻/⁻ animals.

Elk-1, but not Net, can functionally substitute for SAP-I

We next investigated whether ectopic expression of Elk-1 or Net was able to rescue the thymocyte-selection defect in the SAP-1⁻/⁻ OT-II transgenic TCR model discussed above. SAP-1⁻/⁻ OT-II TCR⁺ bone marrow was infected with retroviruses expressing SAP-1, Elk-1, or Net, together with GFP, and used to reconstitute RAG2⁻/⁻ recipient animals (Fig. 4A, Supplemental Fig. 9). In each case, expression of the different TCF mRNAs in the GFP + populations was comparable to that of endogenous SAP-1 mRNA in WT thymocytes; the low level of SAP-1 RNA present in the GFP⁻ thymocyte populations presumably reflects their contamination with WT stromal cells from the recipient (Fig. 4A).

Cells transduced with vector alone did not significantly affect CD4⁺ Vα2β⁺ cell counts in the GFP⁺ thymocyte populations, which were comparable to that observed in radiation chimeras.

**FIGURE 2.** Negative selection and early thymocyte development are unimpaired in SAP-1⁻/⁻ Elk-1⁻/⁻ mice. A, CD4 and CD8 expression in HY TCRβ (F-23.1)-gated thymocytes. B, Cellularity and DP thymocytes in female WT (n=5), male WT (n=4), male SAP-1⁻/⁻ (n=3), and male SAP-1⁻/⁻ Elk-1⁻/⁻ (n=2) mice. Error bars represent SEM (WT and SAP-1⁻/⁻) or half-range (SAP-1⁻/⁻ Elk-1⁻/⁻). Effect on cellularity was also seen in male B6.SJL radiation chimeras (WT, 10.8 ± 2.6 x 10⁶ [n=3]; SAP-1⁻/⁻ Elk-1⁻/⁻, 12.6 ± 1.2 x 10⁶ [n=4]). C, CD4⁺ CD8⁻ DN populations in lineage-negative WT and SAP-1⁻/⁻ Elk-1⁻/⁻ thymocytes. D, DN1, DN2/3, and DN4 thymocytes in WT (n=11) and SAP-1⁻/⁻ Elk-1⁻/⁻ animals (n=9). E, Intracellular TCRβ expression in DN cells.
with SAP-1⁻/⁻ OT-II TCR⁺ bone marrow (Fig. 4B, Supplemental Fig. 4). In contrast, the SAP-1 retrovirus effectively rescued generation of CD4⁺ Vα2KD cells in the GFP⁺ population (Fig. 4B, Supplemental Fig. 4B). A similar result was obtained with bone marrow transduced with the Elk-1 virus but not the Net virus (Fig. 4B). We conclude that Elk-1, but not Net, can rescue the positive-selection defect arising from SAP-1 inactivation, provided that it is expressed at an appropriate level.

**Target gene induction in TCF knockout animals**

We next analyzed the effects of the different TCF mutations on the transcriptional program induced in DP thymocytes following TCR activation. We focused mainly on Elk-1 and SAP-1, because the functional analysis did not indicate a role for Net in thymocyte development. The induced levels of 54 genes were significantly reduced upon deletion of SAP-1, Elk-1, or both; 26 of the genes are known to be SRF targets by functional or ChIP approaches (Table I) (27). All SAP-1–sensitive genes and the majority of Elk-1–sensitive genes were equally or more severely affected in SAP-1⁻/⁻ Elk-1⁻/⁻ cells (Table I, Supplemental Table I). Transcript levels were also confirmed by RT-PCR (Fig. 5A, Supplemental Fig. 8). SAP-1 target genes exhibited differential sensitivity to Elk-1 inactivation (Fig. 5A, Table I). Egr-1, Egr-2, and Fos induction was substantially impaired in SAP-1⁻/⁻ Elk-1⁻/⁻ cells compared with SAP-1⁻/⁻ cells, whereas Egr-3 and Ncoa7 transcription was reduced maximally by SAP-1 inactivation alone, and Nr4a1 and JunB only showed significantly decreased induction in SAP-1⁻/⁻ Elk-1⁻/⁻ cells. Consistent with the observation that Net does not contribute to positive selection, induction of the Egr family and Nr4a1 was comparable in SAP-1⁻/⁻ Elk-1⁻/⁻ and SAP-1⁻/⁻ Elk-1⁻/⁻ Net⁺/⁺ thymocytes (Fig. 5B). Genes that are sensitive to deletion of SAP-1 but not Elk-1 and that are further impaired in SAP-1⁻/⁻ Elk-1⁻/⁻ cells, such as Egr-1, are strong candidates to mediate positive selection.

In the array analysis, SAP-1 inactivation significantly reduced basal transcript levels of only 26 genes, whereas inactivation of Elk-1 affected transcripts corresponding to ∼600 probe sets. Basal expression of these, together with ∼1000 additional genes, was further impaired in SAP-1⁻/⁻ Elk-1⁻/⁻ cells (Supplemental Table I). These results suggest that the Elk-1 TCF may preferentially contribute to basal gene expression in DP thymocytes.

**Elk-1 and SAP-1 share genomic targets in WT cells**

To assess whether TCF-dependent inducible genes are direct targets for the TCFs, we performed ChIP experiments on unstimulated total thymocytes. SRF was detectable at all of the promoters tested,
although the efficiency of recovery varied over a 30-fold range, presumably reflecting differences in cross-linking efficiency and/or binding stoichiometry (Fig. 5C; see Discussion). In general, deletion of SAP-1, the principal thymocyte TCF, did not affect SRF recovery, but it did result in a significant decrease in SRF on the Nr4a1 and Ncoa7 genes (see Discussion), whereas deletion of Elk-1 had no substantial effect on SRF recovery. We also carried out Net ChIP assays, although with this Ab recoveries were more variable. In WT cells, recovery of all promoters was comparable to the background level; SAP-1 and Elk-1 substantially increased recruitment of Net to the Egr-1, Egr-2, JunB, and Fos promoters was detected in WT, but not Elk-1 null cells, and it was increased substantially in SAP-1−/− cells, suggesting that the two proteins compete for these promoters in WT cells. In contrast, SAP-1 deletion had little or no effect on Elk-1 binding to the Egr-3, Nr4a1, SRF, or Ncoa7 promoters. We also carried out Net ChIP assays, although with this Ab recoveries were more variable. In WT cells, recovery of all promoters was comparable to the background level; SAP-1 and Elk-1 substantially increased recruitment of Net to the Egr-1,
JunB, and Fos promoters but not to the other promoters tested (Fig. 5C). Taken together, these results showed that the three different TCFs have subtly different promoter-targeting specificities in vivo (see Discussion).

Ectopic Egr-1 expression can rescue the SAP-1 selection defect

The gene-expression data strongly suggest that Egr family genes, particularly Egr-1 and Egr-2, play an important role in positive selection downstream of ERK-TCF signaling. Previous studies

Table I. Microarray analysis

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<th>Probe ID</th>
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<th>CD3WT/BSWt*</th>
<th>CD3Elk/CD3WTa</th>
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Triplicate RNA preparations from DP thymocytes unstimulated or stimulated for 30 min by plate-bound CD3, from the indicated TCF genotypes, were analyzed. Microarray data have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (19) and are accessible through GEO Series accession number GSE21546 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21546). SRF target genes are from previous functional analyses (27) (C. Esnault and R. Treisman, unpublished observations). The Ncoa7 transcripts probably represent the ERK-inducible Ncoa7B transcript (39), as SRF target (Supplemental Fig. 8B).

*CD3 inducibility in WT cells.

Ratio of the CD3-induced probe signal in TCF mutant cells to WT.

ppp, 0.0001; *pp, 0.001; pp, 0.01; p, 0.05. Elk, Elk-1<sup>2/2</sup>; ES, SAP-1<sup>2/2</sup>

Egr-1/2; Sap, SAP-1<sup>2/2</sup>. nc, no SRF binding site confirmed within 10 kb of the transcription start site; Sap, SAP-1<sup>−/−</sup>.

JunB, and Fos promoters but not to the other promoters tested (Fig. 5C). Taken together, these results showed that the three different TCFs have subtly different promoter-targeting specificities in vivo (see Discussion).

Ectopic Egr-1 expression can rescue the SAP-1 selection defect

The gene-expression data strongly suggest that Egr family genes, particularly Egr-1 and Egr-2, play an important role in positive selection downstream of ERK-TCF signaling. Previous studies...
showed that inactivation of Egr-1 or Egr-2 can impair positive selection (15–17). To test whether Egr family proteins are sufficient to promote positive selection, we tested whether ectopic expression of Egr-1 was able to rescue the thymocyte-selection defect in the SAP-1^{−/−} OT-II TCR+ bone marrow transduced with a retrovirus expressing Egr-1 and used to reconstitute RAG2^{−/−} recipient animals (Fig. 4A; Supplemental Fig. 9A). Cells transduced with the Egr-1 retrovirus effectively rescued generation of CD4⁺ Vα2⁺ cells in the GFP⁺ population (Fig. 6). Thus, Egr family proteins are the major effectors of ERK–SAP-1 signaling in thymocyte-positive selection (see Discussion).

Discussion

We investigated differential functionality in immune cell development between members of the TCF family of Ets domain SRF cofactors. The Elk-1 and Net TCFs do not contribute significantly to thymocyte development in the presence of SAP-1. Combination of the SAP-1 null mutation with the Elk-1 null further decreased output of SP thymocytes compared with SAP-1^{−/−} cells; however, it did not impair other processes in which ERK signaling has been previously implicated, such as progression through the TCRβ-rearrangement checkpoint, nor did it result in decreased negative selection in the HY transgenic model. Combination of the Net^{−/−} mutation with the SAP-1 null had no additional effect on thymocyte development; residual positive selection was observed, even in triply deficient SAP-1^{−/−} Elk-1^{−/−} Net^{−/−} cells. Consistent with these data, ectopic expression of Elk-1, but not Net, restored thymocyte selection in the SAP-1 null OT-II TCR transgenic model. The failure of ectopic Net expression to rescue SAP-1 null phenotypes may reflect differences in its ability to activate genes required for thymocyte development or its weaker potency as an activator (9). Our data suggest that only SAP-1 and Elk-1 are functionally equivalent in this system and that only the low relative expression level of Elk-1 prevents it and SAP-1 from exhibiting true functional redundancy.

Only a small number of genes exhibit strongly SAP-1–dependent transcription in DP cells, including the Egr-1, Egr-2, and Egr-3 zinc finger proteins; the AP-1 components c-Fos and JunB; and a putative transcriptional coactivator, Ncoa7. Of these, Egr-1 and Egr-2 represent the best candidates for genes required for positive selection, because their activity is most sensitive to SAP-1 deletion, is insensitive to Elk-1 deletion, and is further impaired in SAP-1^{−/−} Elk-1^{−/−} cells. Previous studies showed that inactivation of Egr-1 or Egr-2, but not Egr-3, results in impaired

FIGURE 5. Gene-expression analysis. A, RNA from DP cells of indicated genotypes (W, E, S, ES), unstimulated or stimulated with plate-bound αCD3 for 30 min, was analyzed by Affymetrix arrays or RT-PCR. Data were normalized to RPS16. Data are from three independent RNA preparations, and RT-PCR assays were performed in duplicate. For analysis of the inducible Ncoa7B precursor, see Supplemental Fig. 8B. B, RT-PCR of the target gene induction in W, ES, and ESN thymocytes. C, ChIP with SRF, SAP-1, Elk-1, and Net Abs, expressed as percentage input DNA. Horizontal lines represent background levels (recovery of GAPDH promoter). W, WT; E, Elk-1^{−/−}; ES, SAP-1^{−/−}/Elk-1^{−/−}; ESN, SAP-1^{−/−}/Elk-1^{−/−}/Net^{−/−}; S, SAP-1^{−/−}.

FIGURE 6. Ectopic expression of Egr-1 can rescue the selection defect in SAP-1^{−/−} OT-II TCR thymocytes. RAG2^{−/−} mice were reconstituted with SAP-1^{−/−} OT-II TCR transgenic bone marrow transduced with pMIG-IRES-GFP expressing Egr-1. GFP⁺ and GFP⁻ thymocyte populations were analyzed 6–8 wk later. Left panel, Proportion of CD4⁺Vα2⁺ thymocytes in GFP⁺ or GFP⁻ thymocyte populations. Compared with GFP⁻ control, the Egr-1 rescue was statistically significant (nonpaired Mann-Whitney U test; p = 0.0079). Right panels, Dot-plots of GFP⁻ and GFP⁺-gated CD4/CD8 profiles. ***p < 0.01.
positive selection (15–17). Our data show that ectopic expression of Egr-1 can suppress the positive-selection defect observed in the SAP-1−/− thymocytes; however, because functional redundancy exists among at least some Egr family members (28, 29), it is possible that Egr-2 also contributes to the selection process. Thus, Egr family proteins are the primary effectors of Erk–SAP-1 signaling in thymocyte positive selection. Immediately early genes are known to exhibit cross-regulation (30, 31), at least in fibroblasts; therefore, it remains possible that positive-selection defects are also associated with inactivation of c-Fos, JunB, Egr-3, and Ncoa7, although we note that the latter two do not seem to be Elk-1 targets. Because positive selection depends on TCR-induced ERK signaling, TCF target genes affected at the basal level are unlikely to be involved.

Neither SAP-1 nor the other TCFs is required for progress through the β-selection checkpoint. Previous work suggested that β-selection involves Egr family protein function (25, 26) and ERK signaling by the pre-TCR (3). It may be that β-selection requires only basal, TCF-independent levels of Egr proteins and that the ERK signaling requirement reflects induction of their cofactor Nab2 (32). Egr-1 overexpression in Rag-2−−/− animals, which cannot generate a pre-TCR or TCR signal, allows thymocyte development only to the intermediate single positive stage (25, 26), and transgenic expression of a constitutively active SAP-1 variant does so as well (P. Costello, R. Nicolas, and R. Treisman, unpublished observations). Thus, the non-Egr genes involved in β-selection transit are unlikely to be SAP-1 targets. We also observed that thymus size increased as SAP-1 and Elk-1 were progressively inactivated. We speculate that this reflects an increased ability of thymocyte progenitors to enter the thymus, because similar increases are seen upon inactivation of the TCF target gene Egr-1 (15, 22).

Our finding that SAP-1−/− Elk-1−/− Net−/− thymocytes remain competent for positive selection is surprising considering the severe impairment of positive selection in SRF-deficient animals (P. Costello, R. Nicolas, and R. Treisman, unpublished observations) (14). Moreover, some aspects of the SAP-1 Elk-1 null phenotype, such as Treg cell selection and reduced peripheral lymphocyte abundance, are reduced rather than enhanced in triply TCF-deficient animals. Net is a weaker transactivator than the other TCFs (9), and RNAs encoding inhibitory isoforms, such as Net-b (8), are expressed at similar levels to intact Net in thymocytes (Supplemental Fig. 1). We suggest that the ability of the Net mutation to rescue aspects of the SAP-1 Elk-1 null phenotype arises from the ability of other, more active factors to access SRF in the absence of Net. ChIP studies indicated that loss of SAP-1 allows the other TCFs to access at least some SAP-1 target promoters more effectively, and it is possible that in SAP-1 null cells other non-TCF transcription factors also can access TCF target genes required for positive selection. Such factors might be other Ets family proteins, of which many are expressed in any particular cell type (33, 34) and at least some of which can be recruited to DNA by SRF, albeit weakly (35), or the myocardin-related transcription factors proteins. We cannot exclude the possibility that the truncated polypeptide encoded by Netb (5) retains residual activity, although our functional studies suggest that this is unlikely (C. Esnault and R. Treisman, unpublished observations).

The ability of Elk-1 to rescue the SAP-1 positive-selection phenotype when expressed at an appropriate level suggests that these two TCFs must be able to act through shared targets to promote positive selection. Notwithstanding this, we found that the gene-targeting specificities of all of the TCFs are subtly different in vivo. SAP-1, but not Elk-1, is recruited to the Egr-3 and Ncoa7B promoters; therefore, SAP-1 deletion strongly impairs induction of these genes. Net is recruited to only a subset of SAP-1 targets, including Egr-1, c-Fos, and JunB, but not Egr-2 and Egr-3. SAP-1 has a less restricted DNA-binding specificity than Elk-1 (10, 36), which might partially explain these observations, but Net’s in vitro binding specificity has not been assessed. The array data indicated that SAP-1 deletion seems to affect induced transcript levels, whereas Elk-1 deletion predominantly affects basal transcription. Genome-wide analyses strongly implicated Ets family factors in the expression of housekeeping genes through interaction with strong consensus Ets motifs in their promoters (37, 38). Even if Elk-1 were to preferentially contribute to basal transcription in thymocytes, this is not essential for thymocyte selection, which is insensitive to Elk-1 inactivation.

SAP-1 and Elk-1 are functionally equivalent, at least in the thymus. However, the ChIP data and previous studies (5) showed that Net can also access at least some SAP-1 target genes, suggesting that, in certain contexts, it might also function redundantly with the other TCFs. Given these considerations and the ubiquitous nature of TCF expression, it is surprising that the combination of SAP-1 with other TCF mutations did not significantly broaden the spectrum of phenotypes observed and that even SAP-1−/− Elk-1−/− Net−/− embryos remained viable into late gestation. True functional redundancy of SAP-1 and Elk-1 is likely to underlie the deficient generation of mature B cells in bone marrow and marginal zone B cells in spleen in SAP-1−/− Elk-1−/− Net−/− animals, a phenotype also seen upon deletion of SRF in B cells (14), and the infertility of SAP-1−/− Elk-1−/− females, a phenotype that probably reflects defective Egr family expression (21). Our data showed that at least some TCF tissue- or cell-specific phenotypes are a function of a “generic” TCF activity, reflecting the relative expression levels of the functionally equivalent proteins, rather than functional specificity. It will be interesting to probe TCF functional redundancy further by testing whether a particular TCF can fully substitute for another when expressed in situ.

Acknowledgments

We thank Diane Maurice, Victoria Lawson, Kathleen Weston, Cyril Enault, Facundo Batista, Caetano Reis e Sousa, Dominique Bonnet, and Fernando Alfonso for helpful discussions, advice, and communication of data prior to publication; Gavin Kelly and Philip East of the London Research Institute Bioinformatics and Biostatistics Facility for array data processing; Derek Davies and the London Research Institute Flow Cytometry staff for FACS support; and Stuart Pepper from the Cancer Research UK Affymetrix Facility, Paterson Institute for Cancer Research, for the array hybridizations.

Disclosures

The authors have no financial conflicts of interest.

References

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. TCF expression in thymic subsets. (a) Relative TCF mRNA expression in DP thymocytes. Standard Errors are indicated. (b) Levels of TCF mRNA expression in thymocyte subsets and regulatory T cells. The distribution of the three TCF mRNAs observed in DP thymocytes is also reflected in the double negative, single positive, and regulatory T cell populations, indicating that the regulation of TCF expression is similar throughout thymocyte development. Standard Errors are indicated. (c) Relative levels of Net, Net-b and Net-c in thymocytes. Primers detect Net plus Net-b plus Net-c; Net plus Net-b, and Net isoforms; for sequences see Supplementary Information. Expression levels were normalised to Rps16, and Net plus Net-b plus Net-c set to 100. Approximately half the Net RNA encodes Net-b.

Figure S2. SAP-1 / Elk-1 deficiency increases thymus cellularity and decreases the percentage of CD69+ thymocytes. (a) Absolute numbers of DP thymocytes in WT, Elk-1+/−; SAP-1+/−; SAP-1+/− Elk-1−/− animals. Data are mean ± SEM. (b) Absolute numbers of CD4 SP and CD8 SP thymocytes in WT(n=9), Elk-1−/− (n=9); SAP-1−/− (n=9); SAP-1−/− Elk-1−/− (n=8) animals. Data are mean ± SEM. (c) Proportion of CD69+ thymocytes in WT (n=9); Elk-1−/− (n=7); SAP-1−/− (n=7); SAP-1−/− Elk-1−/− (n=9) animals. Error bars, SEM; significance, by Student's t-test *, p < 0.05; ** p<0.01; ***, p<0.001.
Figure S3. The SAP-1<sup>-/-</sup> Elk-1<sup>-/-</sup> selection defect is cell autonomous. (a) Proportion of TCR<sup>hi</sup> thymocytes in radiation chimeras generated with WT (n=4); Elk-1<sup>-/-</sup> (n=4); SAP<sup>-/-</sup> (n=4); SAP-1<sup>-/-</sup> Elk-1<sup>-/-</sup> (n=4) bone marrow. (b) Proportion of CD4 SP thymocytes (filled columns) and CD8 SP thymocytes (open columns) in animals as in (a). (c) Percentage Foxp3<sup>+</sup> CD4+ thymocytes; animals as in (a). Error bars, SEM; significance, by Student's t-test *, p < 0.05; ** p<0.01; ***, p<0.001.

Figure S4. Increased selection defect in SAP-1<sup>-/-</sup>/Elk-1<sup>-/-</sup> deficient OT-II TCR transgenic animals. (a) Reduced proportion of CD4 SP thymocytes in OT-II transgenic animals. CD4 /CD8 and TCRβ profiles; numbers in dot plots indicate percentage of cells in each subset. (b) CD4+V<sub>α</sub>2+ thymocytes in irradiated B6.SJL mice reconstituted with bone marrow from WT, SAP<sup>-/-</sup> and SAP-1<sup>-/-</sup> Elk-1<sup>-/-</sup> OT-II TCR bone marrow. WT (n=3), SAP<sup>-/-</sup> (n=3) and SAP-1<sup>-/-</sup> Elk-1<sup>-/-</sup> (n=4). (c) TCF inactivation progressively reduces the proportion of total CD4+ thymocytes that are also V<sub>α</sub>2 positive; animals as in (b). Error bars, SEM; significance, by Student's t-test *, p < 0.05; ** p<0.01; ***, p<0.001.

Figure S5. B cell development in the bone marrow and hemaepoietic progenitor subsets. (a) Left, B220<sup>+</sup> cells in RBC depleted bone marrow in WT (n=10), Elk-1<sup>-/-</sup> (n=5), SAP-1<sup>-/-</sup> (n=10) and SAP-1<sup>-/-</sup> Elk-1<sup>-/-</sup> (n=9 ). Right, pro-B / pre-B (B220+IgM-IgD-), immature B (B220+IgM+IgD-) and mature B (B220+IgM+IgD+) cells in B220+ gated WT, Elk-1<sup>-/-</sup>, SAP-1<sup>-/-</sup> and SAP-1<sup>-/-</sup> Elk-1<sup>-/-</sup> RBC-depleted bone marrow from left. Standard Errors are indicated. (b) Lymphoid cell analysis. Top, Lineage-/lo; Lin-/lo c-Kit+ Sca-I-; Lin -/lo c-Kit+ Sca-I+ (LSK); Lin -/lo Kit+ Sca-I+ CD34+ Flt3-
(ST-HSC); Lin -/-lo Kit+Sca-I+ CD34+ Flt3+ (MPP); Lin -/-lo Kit+Sca-I+ CD34- Flt3- CD150+ (LT-HSC). 4 mice per genotype. Standard errors are indicated. (c) Myeloid stem cell analysis. Lineage -/-lo; Lin -/-lo c-Kit+ Sca-I-; Lin -/-lo c-Kit+ Sca-I- CD34+ CD16/32- (CMP), Lin-/lo c-Kit+ Sca-I- CD34+ CD16/32+ (GMP), Lin-/lo c-Kit+ Sca-I- CD34- CD16/32- (MPP), Lin-/lo Kit int Sca-I int IL7-R+ (CLP). 4 mice per genotype. Standard errors are indicated. (d) Representative dots plots for lymphoid and myeloid analysis in WT and SAP-1-/- Elk-1-/- bone marrow.

**Figure S6. Peripheral B cell development in TCF deficient animals** (a) Absolute numbers of splenocytes in WT (n=15), Elk-1-/- (n=6), SAP-1-/- (n=12) and SAP-1-/- Elk-1-/- (n=9) animals. Mean ± SEM are indicated. (b) CD21/35 and CD23 expression on CD19+ gated splenocytes. Numbers indicate percentage of cells in the MZB gate (CD21+/35+ CD23-) and follicular B cells (CD21/35^int CD23^+). (c) MZB cells, follicular B cells and CD19+ cell numbers in WT (n=16); Elk-1-/- (n=7); SAP-1-/- (n=14); SAP-1-/- Elk-1-/- (n=13) animals. Error bars, SEM; significance, by Student’s t-test *, p < 0.05; ** p<0.01; ***, p<0.001.

**Figure S7. Thymus cellularity, early thymocyte development and Peripheral T cells in SAP-1-/- Elk-1-/- Netδδ reconstituted animals.** (a) Thymic cellularity in WT (n=12); SAP-/- (n=12); SAP-1-/- Elk-1-/- (n=8) and SAP-1-/- Elk-1-/- Netδδ (n=12) B6.SJL radiation chimeras reconstituted with fetal liver cells. (b) DN1, DN2/3 and DN4 populations in WT (n=8), SAP-/- (n=8), SAP-1-/- Elk-1-/- (n=8) and SAP-1-/- Elk-1-/- Netδδ (n=8) lineage-negative thymocytes. The differences between genotypes were not statistically significant. (c) TCRhi cells in the lymph nodes of WT (n=12);
SAP\(^{-/-}\) (n=11); SAP-1\(^{-/-}\) Elk-1\(^{-/-}\) (n=8) and SAP-1\(^{-/-}\) Elk-1\(^{-/-}\) Net\(^{\delta\delta}\) (n=12). Standard Errors are indicated. (d) CD4+ (filled columns) and CD8+ lymph node T cells (open columns) in samples from (c) above. Standard Errors are indicated. (e) TCR\(^{hi}\) cells in the spleens of all four genotypes, mice numbers as above. (f) splenic CD4 (filled columns) and CD8 T cells (open columns). Error bars, SEM; significance, by Student's t-test *, p < 0.05; ** p<0.01; ***, p<0.001.

**Figure S8. TCF target genes.** (a) qRT-PCR validation of microarray data. RNA from DP thymocytes of different genotypes (W, wildtype; E, Elk-1\(^{-/-}\); S, SAP-1\(^{-/-}\); ES, SAP-1\(^{-/-}\) Elk-1\(^{-/-}\)) either unstimulated or stimulated with plate-bound \(\alpha\)CD3 for 30 min was analysed by Affymetrix arrays (open bars) or by qRT-PCR (solid bars). Data were normalised to RPS16, and wildtype CD3-induced levels set to 100%. (b) Structure and expression of the Ncoa7 gene. Left, Ncoa7A and Ncoa7B exons are shown as black boxes and the SRE as a red box; transcription start sites indicated. Red lines, location of the primer pairs used for RNA analysis. Right, analysis of Ncoa7 transcription using the intronic primer pair within the Ncoa7B transcript, displayed as in (a).

**Figure S9. Retroviral expression of SAP-1 and Elk-1 and rescue of SAP-1\(^{-/-}\) OT-ll TCR selection defect.** Upper panel, \(\alpha\)FLAG immunoprecipitation from 2 x 10\(^6\) Phoenix cells infected with pMIG-IRES-GFP or pMIG-IRES-GFP encoding SAP-1, Elk-1 or Net, immunoblotted with FLAG, SAP-1, Elk-1 or Net antibodies. Lower panel, histograms showing GFP expression profiles of the infected Phoenix cell lines (uninfected cells, solid grey).
SUPPLEMENTARY TABLE LEGEND

Table S1. Microarray analysis of basal and CD3-stimulated transcripts in DP thymocytes from wildtype and TCF mutant mice.

<table>
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<tr>
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<th>Wildtype</th>
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<th>Sap-1^-/-</th>
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<td><strong>SAP-1^-/- Elk-1^-/- (ES)</strong></td>
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<td><strong>were used for</strong></td>
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<td><strong>cDNA synthesis.</strong></td>
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<td><strong>Following hybridisation</strong></td>
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<td><strong>to Affymetrix MOE430 2.0 GeneChips, 2572 probes were identified where the normalised intensity values changed by at least 1.5-fold in any genotype or condition at p&lt;0.05. Included in the tables are Actb and Gapdh controls, as well as probes for Rps16, the gene that exhibited the least variation with respect to genotype or CD3 stimulation.</strong></td>
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**Column C:** CD3-induced transcript levels in wildtype cells expressed as fold induction over basal. Highlighting: 1.5 to 2.0-fold increase, orange; >2.0-fold increase, red; 1.5 to 2.0-fold decrease, light green; >2.0-fold decrease, dark green.

**Columns D, E, F:** CD3-induced transcript levels in TCF mutant cells expressed relative to the CD3-induced transcript level in wildtype cells. Highlighting: 1.5 to 2.0-fold decrease, orange; >2.0-fold decrease, red; 1.5 to 2.0-fold increase, light green; >2.0-fold increase, dark green.

**Columns G-J:** Statistical significance of results in columns C-F. Each value is colour-coded as follows: p<0.0001, red; p<0.001, green; p<0.05, blue; not significant, black.

**Columns K-M:** Basal transcript levels in TCF mutant cells expressed relative to the basal transcript level in wildtype cells. Highlighting as for columns D-F.

**Columns N-P:** Statistical significance of results in columns K-M, highlighted as for columns G-J.

**Columns Q-T:** CD3-induced transcript intensity values for each probe set.

**Columns U-X:** Basal transcript intensity values for
each probe set. Microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE21546 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21546).

**Tab "ALL".** Whole dataset

**Tab "CD3 p<0.05".** All probes where CD3 crosslinking induces a change in signal significant at p<0.05.
Costello et al, Figure S1
WT SAP-1-/- Elk-1-/-

A B

CD4 SP CD8 SP

Costello et al, Figure S2
Costello et al, Figure S3
Costello et al, Figure S4

A

WT OT-II Tg

SAP-1−/− OT-II Tg

SAP-1−/− Elk-1−/− OT-II Tg

% CD4+ Vα2+ thymocytes

% CD4 thymocytes that are Vα2+

B

C

**

***

***

WT SAP-1−/− SAP-1−/−Elk-1−/−
Costello et al. Figure S5
A. Bar graph showing splenic cellularity X 10^6 for WT, Elk-1^-/-, SAP-1^-/-, and SAP-1^-/- Elk-1^-/-.

B. Flow cytometry plots showing CD19+ gated cells for WT, Elk-1^-/-, SAP-1^-/-, and SAP-1^-/- Elk-1^-/- with CD21/35 and CD23 axes.

C. Graphs showing % MZB, % Follicular B cells, and % CD19+ Splenocytes for WT, Elk-1^-/-, SAP-1^-/-, and SAP-1^-/- Elk-1^-/-.

Costello et al, Figure S6
Figure S7

A: Bar graph showing cellularity x10^6.

B: Bar graph showing % DN lineage negative thymocytes.

C: Bar graph showing % TCRhi lymph node.

D: Bar graph showing % TCRhi spleen.

E: Bar graph showing % splenocytes.

Costello et al, Figure S7
Gene structure of Ncoa7

B

Gene structure of Ncoa7

Costello et al, Figure S8
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Costello et al, Figure S9