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

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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 Net2 mutation, exacerbated the SAP-1–positive selection phenotype, but triply deficient thymocytes were no more defective than SAP-12/2 Elk-12/2 Net2/2 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-12/2 Elk-12/2 animals. Ectopic expression of Elk-1, but not Net, rescued positive selection of SAP-12/2 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-12/2 Elk-12/2 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.

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Materials and Methods

Mice

SAP-1−/−, Elk-1−/−, and Neta6 (2, 5, 6), kept under specific-pathogen-free conditions, were backcrossed to C57/BL6j 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 14.5-d embryos were injected into the tail vein (5 × 10^6 cells/mouse), and analysis was performed 6 wk later. Donor and host cells were distinguished using CD45 alloantigens (donor, CD45.2; host, CD45.1) and are accessible through GEO Series accession number GSE21546.

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 FACSCalibur (BD Biosciences, San Jose, CA) for a minimum of five generations and were determined by real-time PCR with SYBR Green Invitrogen (11744-500) reagents or Roche Universal Probes and the TaqMan Universal PCR mix was determined by real-time PCR with SYBR Green Invitrogen (11744-500) reagents or Roche Universal Probes and the TaqMan Universal PCR mix.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChiP) was described. Primer sequences for ChiP analysis were as follows: Egr1_p: 5′-CGAGAAGCCGATTACCCACG-3′, 5′-ATATAGAGTTGCTCAGCATTACCA-3′; Ncoa7_in13: 5′-GAGCAAATGATGACCGCCAA-3′, 5′-CCAGCAGGAGGCTGTCGTTG-3′; and SRF_p: 5′-GGTGAAGACACTCAAGGCT-3′, 5′-TCATTCCCCACCCGACCTG-3′.

Retrovirology and reconstitution

SAP-1, Elk-1, Net, and Egr1 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 G418-selected cell lines used in the thymus. Expression of the relevant TCR or Egr1 was monitored by immunoprecipitation with FLAG-M2 HRP (Sigma-Aldrich), and in-house affinity-purified anti-mouse Elk-1 a309-429, and anti-Net 1995 (20). Primer sequences for ChiP analysis were as follows: Egr1_p: 5′-CGAGAAGCCGATTACCCACG-3′, 5′-ATATAGAGTTGCTCAGCATTACCA-3′; Ncoa7_in13: 5′-GAGCAAATGATGACCGCCAA-3′, 5′-CCAGCAGGAGGCTGTCGTTG-3′; and SRF_p: 5′-GGTGAAGACACTCAAGGCT-3′, 5′-TCATTCCCCACCCGACCTG-3′.

2 TCF FUNCTIONAL EQUIVALENCE IN THYMOCYTES

Target genes were validated using SYBR Green-based real-time PCR (Invitrogen, Carlsbad, CA). Relative abundance of template cDNA was calculated using the comparative C(XD/ΔC(X method, normalization 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: AY078069: 5′-TTAGGACCAAGACCGCAACTCT-3′, 5′-CATGAATCTGCGACATGACAF-3′; CD69: 5′-TGAGCTCTTCAAGACGACCTAT-3′, 5′-CTACAGCTTCGAGGATTTTCG-3′; Foxp3: 5′-CCAGAAGGCATGAGACGATC-3′, 5′-CAGGGTCTGTGAAGAGA-3′; SAP: 5′-TTCCCTCGGTCAGTTGAC-3′, 5′-TTCCCTCGGTCAGTTGAC-3′; Ncoa7: 5′-GAGCAAATGATGACCGCCAA-3′, 5′-CCAGCAGGAGGCTGTCGTTG-3′; and SRF: 5′-GGTGAAGACACTCAAGGCT-3′, 5′-TCATTCCCCACCCGACCTG-3′.

Statistical analysis

The statistical significance of flow cytometric experiments was assessed using the unpaired Student t test, except for the retrovirology-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, 105 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 GeneElute 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 was used mouse MGI:3501201 and 45,001 probe sets targeted on unique reference mRNA records or 22,640 unique Unigene IDs. Data were preprocessed and quantile normalized using Therma procedure within Bioconductor's limma package (18). A false-discovery rate of 0.05 was used as the cutoff for statistical testing. Microarray data were 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).
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), 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 TCRb 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 V6a2 are 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 V6a2+ (Supplemental Fig. 4C); this suggests that in these RAG2-/- cells, 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-2d class I Dd, 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 male mice 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 male 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 vß5+ thymocytes occurs efficiently in C57BL/6 × BALB/c mice but not in C57 BL6 animals, reducing the proportion of CD4 vß5+ and CD8 vß5+ thymocytes by 93% and 95%, respectively, while leaving vß6+ and vß8+ thymocytes unaffected. In SAP-1-/- Elk-1-/- animals, deletion occurred to a similar extent (CD4 vß5+, 87%; CD8 vß5+, 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 CD23low marginal zone B cells, whereas the proportion of CD19+ splenocytes or CD21/35mid CD23bright follicular B cells was unaffected (Supplemental Fig. 6B, 6C).

The Netb mutation does not affect positive selection

To investigate whether the third TCF, Net, also contributes to immune cell development, we studied mice carrying the Netb mutation, which encodes a truncated Net lacking the Ets domain (5).
SAP-1<sup>−/−</sup> Net<sup>b</sup> animals exhibited a chylothorax phenotype similar to that observed in Net<sup>b</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 Supplemental 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.

Thymocyte positive selection persists in animals lacking all TCFs Matings between SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> Net<sup>−/−</sup> males and SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> Net<sup>−/−</sup> females were used to generate triply TCF-deficient embryos. Four of 30 14.5-d embryos examined were SAP-1<sup>−/−</sup>

**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>hi</sup> thymocytes in WT (n = 8), Elk-1<sup>−/−</sup> (n = 7), SAP-1<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>B</sup> staining. D, CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes. E, Percentage of CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes in mixed WT/SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> bone marrow chimeras (n = 4). F, CD4<sup>+</sup> Vα2<sup>+</sup> thymocytes in WT (n = 2), SAP-1<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> thymocytes in WT (n = 14), Elk-1<sup>−/−</sup> (n = 10), SAP-1<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<sup>−/−</sup> Net<sup>−/−</sup> and macroscopically indistinguishable from WT, demonstrating that the triple TCF mutation does not grossly impair embryonic viability to this stage. Surprisingly, the SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> Net<sup>−/−</sup> thymocyte profiles were similar to those seen in animals reconstituted with SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> cells (Fig. 3B–E).

All of the genotypes reconstituted thymuses of comparable cellularity, with comparable DN thymocyte profiles and the SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> thymocyte profiles (Supplemental Fig. 7A, 7B). In thymus, SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> Net<sup>−/−</sup> TCR<sup>β</sup> and SP cells seemed to be slightly more abundant relative to SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> cells (Fig. 3C, 3D); this effect was more marked in the periphery, suggesting that the Net<sup>−/−</sup> mutation may relieve some aspects of the SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> phenotype (Supplemental Fig. 7C–F; see Discussion). Although animals reconstituted with SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> fetal liver cells showed a reduced level of FoxP3<sup>+</sup> CD4 SP cells, similar to primary animals, triply defective FoxP3<sup>+</sup> CD4 SP thymocytes, which also expressed the T<sub>reg</sub> cell-associated markers CTLA-4, glucocorticoid-induced tumor necrosis receptor, and CD103, were produced at WT levels (Fig. 3E).

Taken together, these results showed that the intact Net protein is neither required for T<sub>reg</sub> cell selection nor responsible for the residual positive selection seen in SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> animals.

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

We next investigated whether ectopic expression of Elk-1 or Net was able to rescue the thymocyte-selection defect in the SAP-1<sup>−/−</sup> OT-II transgenic TCR model discussed above. SAP-1<sup>−/−</sup> OT-II TCR<sup>β</sup> bone marrow was infected with retroviruses expressing SAP-1, Elk-1, or Net, together with GFP, and used to reconstitute RAG2<sup>−/−</sup> recipient animals (Fig. 4A, Supplemental Fig. 9). In each case, expression of the different TCF mRNAs in the GFP<sup>+</sup> populations was comparable to that of endogenous SAP-1 mRNA in WT thymocytes; the low level of SAP-1 RNA present in the GFP<sup>+</sup> 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<sup>+</sup> V<sub>β</sub>2<sup>+</sup> cell counts in the GFP<sup>+</sup> thymocyte populations, which were comparable to that observed in radiation chimeras.

FIGURE 2. Negative selection and early thymocyte development are unimpaired in SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> 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<sup>−/−</sup> (n = 3), and male SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> (n = 2) mice. Error bars represent SEM (WT and SAP-1<sup>−/−</sup>) or half-range (SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup>). Effect on cellularity was also seen in male B6.SJL radiation chimera (WT, 10.8 ± 2.6 × 10<sup>6</sup> [n = 3]; SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup>, 12.6 ± 1.2 × 10<sup>6</sup> [n = 4]). C, CD4<sup>+</sup> CD8<sup>−</sup> DN populations in lineage-negative WT and SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> thymocytes. D, DN1, DN2/3, and DN4 thymocytes in WT (n = 11) and SAP-1<sup>−/−</sup> Elk-1<sup>−/−</sup> 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\(^{+}\) V\(\alpha\)2\(^{hi}\) cells in the GFP\(^{+}\) population (Fig. 4B, Supplemental Fig. 9B). 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.

\section*{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.

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. 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.

\subsection*{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. SAP-1 was detectable at each of the promoters tested, and recoveries were reduced to background levels in SAP-1−/− cells (Fig. 5C). In general, SAP-1 recoveries were comparable to those seen with SRF, apart from at the Srf promoter, which responds to signaling predominantly via the myocardin-related transcription factors, which bind SRF competitively with SAP-1 (13). Elk-1 binding 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 show that Egr family genes are induced by ERK signaling and are essential for T-cell development.

### Table I. Microarray analysis

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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).
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-1/2 OT-II transgenic TCR model discussed above. SAP-1/2 TCR+ bone marrow was infected 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+Va2hi 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 Netb/d 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−/− Netb/d 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...
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-earlie 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 co-factor 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 other TCFs (9), and RNAs encoding inhibitory isoforms, such as SRF, albeit weakly (35), or the myocardin-related transcription factors proteins. We cannot exclude the possibility that the truncated polypeptide encoded by Netδ (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 transcription 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−/− 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.

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Disclosures

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References
