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# Calcineurin sets the bandwidth for discrimination of signals during thymocyte development

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## Abstract

At critical times in development, cells are able to convert graded signals into discrete developmental outcomes; however, the mechanisms involved are poorly understood. During thymocyte development, cell fate is determined by signals originating from the α β T-cell receptor. Low-affinity/avidity interactions between the T-cell receptor and peptide–MHC complexes direct differentiation to the single-positive stage (positive selection), whereas high-affinity/avidity interactions induce death by apoptosis (negative selection)[1,2]. Here we show that mice deficient in both calcineurin and nuclear factor of activated T cells (NFAT)c2/c3 lack a population of preselection thymocytes with enhanced ability to activate the mitogen-activated protein kinase (Raf–MEK–ERK) pathway, and fail to undergo positive selection. This defect can be partially rescued with constitutively active Raf, indicating that calcineurin controls MAPK signalling. Analysis of mice deficient in both Bim (which is required for negative selection) and calcineurin revealed that calcineurin-induced ERK (extracellular signal-regulated kinase) sensitization is required for differentiation in response to 'weak' positive selecting signals but not



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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

**Author Contributions** E.M.G., M.M.W. and G.R.C. generated the hypotheses, designed the experiments and wrote the manuscript. E.M.G. performed the experiments and generated the figures. K.C.-B. generated the *NFATc3* conditional knockout mice, maintained this line in the *NFATc2*-null background and contributed to the experiments in Fig. 4. A.N.R. and L.H. contributed to pilot experiments and experiments shown in Fig. 2 and Supplementary Fig. 8. J.R.N. generated the *Cnb1* conditional knockout mice, conducted pilot experiments and contributed to experimental rationale. L.M. contributed to experiments shown in Supplementary Fig. 5. B.I. provided the *Raf-CAAX* transgenic mice.

in response to ‘strong’ negative selecting signals (which normally induce apoptosis). These results indicate that early calcineurin/NFAT signalling produces a developmental period of ERK hypersensitivity, allowing very weak signals to induce positive selection. This mechanism might be generally useful in the discrimination of graded signals that induce different cell fates.

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The calcineurin/NFAT[3,4] and the Raf–MEK–ERK[5–7] pathways have been shown to be required for positive selection of thymocytes but not for their negative selection. Calcineurin B1 (*Cnb1*)-deficient $CD4^+$ $CD8^+$ double-positive thymocytes lack calcineurin activity, fail to dephosphorylate NFATc transcription factors and are not positively selected (Fig. 1a and ref. 3). *Cnb1*-deficient thymocytes have normal phosphorylation of JNK (c-Jun N-terminal kinase), p38, protein kinase C-θ, protein kinase D and glycogen synthase kinase 3α after crosslinking of the T-cell receptor (TCR)(Fig.1band ref. 3). Actin polymerization and $Ca^{2+}$ influx were also normal in these cells (Supplementary Fig. 2a, b). Collectively, these results indicated that *Cnb1*-deficient thymocytes did not have widespread signalling defects downstream of the TCR. However, *Cnb1*-deficient thymocytes showed a specific and severe defect in ERK1/2 phosphorylation (Fig. 1c), resulting in almost undetectable induction of the ERK/Elk4 target gene Egr1 (ref. 6) after engagement of the TCR (Fig.1d). In addition, both MAP-kinase kinase (MEK)1/2and Raf activation were defective in *Cnb1*-deficient double-positive thymocytes (Fig. 1c, e, f) after stimulation by crosslinking of the TCR.

To test whether calcineurin activity was directly required for Raf activation, we stimulated thymocytes in the presence of the calcineurin inhibitor cyclosporin A (CsA) or the MEK1/2 inhibitor UO126. Acute *in vitro* inhibition of MEK1/2 with UO126 but not inhibition of calcineurin activity with CsA impaired ERK1/2 phosphorylation and Egr1 induction (Fig. 2a, b). In contrast, 10-day treatment of mice *in vivo* with CsA consistently recapitulated both the block in positive selection and defective ERK1/2 phosphorylation observed in *Cnb1*-deficient thymocytes (Fig. 2a, band Supplementary Fig. 3a). These data indicated that calcineurin activity was not directly required for ERK1/2 phosphorylation but was instead required during development to acquire the ability to activate ERK1/2 properly in response to subsequent TCR signalling.

When ERK1/2 phosphorylation was assayed by intracellular staining, two populations with different levels of ERK1/2 phosphorylation were observed in control double-positive CD69-negative thymocytes after crosslinking with anti-CD3ε antibody. The population of thymocytes with the higher level of ERK1/2 phosphorylation was absent in *Cnb1*-deficient and CsA-treated mice (Fig. 2c and Supplementary Fig. 3b). The specificity of the staining and uniformity of crosslinking was confirmed by pretreating the cells with UO126 and by counterstaining for anti-CD3ε, respectively (Supplementary Fig. 3c, d). We also examined the expression of TCR-β on *Cnb1*-deficient and control thymocytes and found no difference in the percentage of thymocytes with intermediate levels of TCR-β expression (double-positive TCR-$\beta^{int}$), a population that is absent from mice that fail to rearrange the TCR-α chain[8–10] (Supplementary Fig. 3e). We concluded that two thymocyte populations existed, within the double-positive CD69-negative population, with different abilities to phosphorylate ERK1/2 and that calcineurin activity was required for the presence of the

population with an increased ability to phosphorylate ERK1/2. We refer to these populations as ‘ERK low competence’ and ‘ERK high competence’ populations, respectively.

We used a single bromodeoxyuridine (BrdU) pulse to mark developing thymocytes[11] and establish the precursor/progeny relationship of these two populations. At early time points, most BrdU-positive, double-positive thymocytes were in the ‘ERK low competence’ state, with the percentage of BrdU-positive ‘ERK high competence’ double-positive thymocytes increasing over time (Fig. 2d, upper panel). The transition to the ‘ERK high competence’ state was not observed in *Cnb1*-deficient thymocytes (Fig. 2d, lower panel). These data indicated that the ‘ERK low competence’ state preceded the ‘ERK high competence’ state during normal thymocyte development and that calcineurin activity was required for this transition. The lag time for the development of the ‘ERK high competence’ population was consistent with a requirement for transcription.

Because the calcineurin phosphatase complex regulates the NFATc family of transcription factors[12,13], we analysed *NFATc2*/*NFATc3* double-knockout thymocytes, which also have impaired positive selection[4]. The development of the ‘ERK high competence’ population was impaired in *NFATc2*/*NFATc3* double-knockout thymocytes (Fig. 2e), the incomplete block probably reflecting a partly redundant function of NFATc1. These data indicated that calcineurin/NFAT signalling was required for the transition to the ‘ERK high competence’ state. The developmental requirement for calcineurin and NFAT activity for normal activation of the ERK pathway was specific to thymocytes because short-term or long-term treatment with cyclosporin had no effect on peripheral T and B lymphocytes (Supplementary Fig. 3f and data not shown). Analysis of MHCI/MHCII double-knockout mice revealed that the development of the ‘high ERK competence’ double-positive thymocyte population did not require TCR–MHC (major histocompatibility complex) interaction or positive selection (Supplementary Fig. 4a). Microarray analysis showed that 312 transcripts were differentially expressed in double-positive thymocytes from untreated and CsA-treated MHCI/MHCII double-knockout mice, indicating that calcineurin activity is required for preconditioning of double-positive thymocytes independently of positive selection (Supplementary Fig. 4b, c, and Supplementary Table 1). We speculate that the development of the ‘ERK high competence’ population depends on pre-TCR signalling and/or ligand-independent tonic TCR-αβ signalling and that the modulation of Raf activity could be due to a program of gene expression rather than a single modulator working at a single point in the pathway.

If a main mechanism by which calcineurin controlled positive selection were by modulating the sensitivity of the Raf–MEK–ERK pathway, restoring Raf signalling in the absence of calcineurin activity should lead to at least a partial rescue of positive selection. To test this, we obtained mice whose thymocytes express a constitutively active Raf-1 mutant protein (*Raf-CAAX* transgenic mice[14]), which does not induce positive selection in the absence of TCR signalling[14]. Mice were analysed between four and six weeks of age, before they developed any sign of thymic lymphoma. Long-term treatment with CsA completely blocked positive selection in control mice (Fig. 3a). However, positive selection is rescued in CsA-treated Raf-CAAX mice as assessed by the upregulation of CD69 and TCR-β and the development of mature $CD24^{low}Qa2^{high}$ single-positive CD4 and CD8 thymocytes (Fig. 3a–d). Moreover, CD4 single-positive and CD8 single-positive thymocytes that are rescued in

CsA-treated Raf-CAAX mice were able to respond functionally when stimulated with anti-CD3 and anti-CD28 antibodies (Supplementary Fig. 5). Analogous results were obtained when Raf-CAAX was expressed in *Cnb1*-deficient thymocytes (Supplementary Fig. 6). As expected, the decreased thymic cellularity that is observed in the absence of calcineurin activity as a consequence of impaired transition from double negative to double positive was not rescued by the *Raf-CAAX* transgene (Fig. 3e and Supplementary Fig. 6d). Raf-CAAX only partly restores ERK phosphorylation in thymocytes from CsA-treated mice (Supplementary Fig. 6e). The fact that the *Raf-CAAX* transgene does not rescue the double-negative to double-positive developmental block and does not restore appropriate timing or intensity of ERK signalling, together with additional ERK-independent Cnb1/NFAT targets (such as the transcription factor TOX) (ref. 15), might explain the incomplete rescue in *Cnb1*-deficient Raf-CAAX mice.

We proposed that this developmental window of ERK hypersensitivity might transiently increase the dynamic range (or bandwidth) of TCR signals, thereby enabling thymocytes to respond to 'weak' positively selecting ligands. To test this, we used the OT-I TAP (transporter associated with antigen processing)-null mice mouse model[16,17] (OT-I TAP$^0$). We compared the ability of double-positive thymocytes from control and CsA-treated OT-I TAP$^0$ mice to respond to the negatively selecting peptide SIINFEKL and the positively selecting peptide RTYTYEKL (ref. 17). OT-I-positive double-positive thymocytes from control mice responded to both peptides by phosphorylating ERK1/2, inducing Egr1 and upregulating CD69 (Fig. 4a–c and Supplementary Fig. 7). The response to the positively selecting peptide is weaker and delayed, as has been previously reported[18,19]. In contrast, double-positive thymocytes from CsA-treated mice were able to respond partly to the negatively selecting peptide SIINFEKL but failed to respond to the positively selecting peptide RTYTYEKL (Fig. 4a–c and Supplementary Fig. 7). These data suggested that calcineurin-dependent ERK sensitization is required for a response to weaker positively selecting ligands, whereas stronger negative selecting signals are able to activate the ERK pathway to a certain extent even when the transition to the 'ERK high competence' state has not occurred.

If the role of calcineurin were to increase the 'signalling bandwidth' and allow effective discrimination of graded signals, one would predict that *Cnb1*-deficient thymocytes that were prevented from dying should be positively selected in response to stronger signals, which would normally induce cell death. To test this, we used mice deficient in *Bim*, which is necessary for negative selection[20]. In control mice, *in vivo* treatment with CsA completely blocked the development of single-positive thymocytes. However, differentiation occurred in *Bim*-deficient mice even in the absence of calcineurin activity as assessed by the development of TCR-β$^{high}$ CD4 single-positive and CD8 single-positive cells and upregulation of TCR-β, CD69 and CCR7 on double-positive cells (Fig. 4d and Supplementary Fig. 8a–c). The double-negative to double-positive developmental block that is observed in CsA-treated and *Cnb1*-deficient thymocytes[3], which results in an overall decrease in thymus cellularity in these mice, was not rescued by Bim deficiency (Supplementary Fig. 8c). Analogous results were obtained when *Bim*-deficient mice were crossed with conditional *Cnb1*-deficient mice (Supplementary Fig. 8d, e). Because Bim deficiency did not rescue development of the 'ERK high competence' population itself

(Supplementary Fig. 8f), we postulated that in *Bim*-deficient mice 'strong' negatively selecting ligands could trigger the activation of ERK in thymocytes that are in the 'ERK low competence' state. According to this hypothesis, in *Bim*-deficient mice negatively selecting ligands could circumvent the need for the calcineurin-dependent sensitization and could induce the differentiation of single-positive thymocytes that in normal circumstances are negatively selected. Indeed, analysis of *Bim*-deficient mice in a background (Balb/c) that expresses the MHC class II molecule I-E and allows the superantigen-mediated deletion of T cells[2] revealed that in CsA-treated *Bim*-deficient mice a higher percentage of CD4 single-positive thymocytes expressed TCR Vβ chains that are reactive to endogenous superantigens in comparison with controls, suggesting that single-positive thymocytes that develop in these mice are in fact those that received negatively selecting signals (Fig. 4e).

As a second approach to testing whether the role of ERK competence is to expand the signalling bandwidth, allowing the effective discrimination of weak signals, we examined thymocyte selection in the HYTCR transgenic mouse model. In this model most thymocytes express the HY TCR and are negatively selected by antigens expressed in male but not female mice[21]. As expected, the deletion of HY thymocytes in male mice (Supplementary Fig. 9a) was impaired by *Bim* deficiency[20]. Because CsA treatment resulted in the accumulation of immature CD8 single-positive cells (Supplementary Fig. 9a), we evaluated positive selection by monitoring CD69 upregulation and the development of $CD24^{low}$ and $Qa2^{high}$ CD8 single-positive cells. Treatment with CsA impaired CD69 upregulation and the development of mature CD8 single-positive thymocytes in control and *Bim*-deficient female mice, whereas it did not block the development of mature CD8 single-positive cells in male mice (Fig. 4f and Supplementary Fig. 9b, c). Similar results were obtained in the $HY^{CD4}$ model, in which TCR-α β expression is properly timed[22] (data not shown). These results suggested that the amount of ERK signalling provided by 'strong', negatively selecting signals in CsA-treated mice is able to induce differentiation and that negatively selecting signals do not require the development of 'ERK competence'.

Cell fate determination often occurs within morphogenic gradients that produce different cell fates at different points in the gradient, apparently as a result of signals of different intensities. A similar analogue-to-digital switch occurs in T-cell development (Supplementary Fig. 1), where signal intensity determines the outcome of TCR–MHC interactions. Our studies indicate that an early calcineurin–NFAT signal sensitizes the Raf–MEK–ERK pathway, allowing responses to weak TCR signals that would otherwise not be detected (Supplementary Fig. 1). In the absence of this calcineurin-dependent preconditioning, the signal intensity needed for positive selection overlaps with that needed for negative selection, and effective discrimination of graded developmental signals cannot occur.

## METHODS

### Mice

*Cnb1* conditional knockout and Raf-CAAX mice have been previously characterized[3,14]. *Lck-cre* mice were a gift from C. Wilson. Because no consistent differences were observed among mice of the genotypes $Cnb1^{+/+}$-$lck^{Cre}$;$Cnb1^{fl/+}$-$lck^{Cre}$, $Cnb1^{\Delta/+}$-$lck^{Cre}$;$Cnb1^{f/f}$ or

*Cnb1*$^{fl/\Delta}$, they are collectively referred to as 'control mice' throughout this manuscript. In addition, no differences were observed between *Cnb1*$^{f/f}$-*lck*$^{Cre}$ and *Cnb1*$^{fl/\Delta}$-*lck*$^{Cre}$ mice, and these animals were used interchangeably for the experiments described in this study and compared with age-matched control mice. OT-I transgenic mice[16] on the TAP$^0$ background were a gift from K. A. Hogquist. C57/B6 *H2-A*$^b$/β*2m* double knockout mice, C57BL/6 and HY TCR transgenic mice were purchased from Taconic. *Bim*-deficient mice were purchased from the Jackson Laboratory. For *in vivo* treatment with CsA, mice were treated with daily intraperitoneal injections of CsA (30 mg kg$^{-1}$ d$^{-1}$) for ten or more days. Mice in which exon 3 of the *NFATc3* gene is flanked by loxP sites (*NFATc3*$^{f/f}$) were generated in our laboratory[4] and crossed to *Lck-cre* mice and to *NFATc2*-deficient mice[28] to obtain *NFATc2/NFATc3*-deficient thymocytes.

## Phospho-ERK intracellular staining

The staining for phospho-ERK was performed as indicated in the staining protocol provided by Cell Signalling. For co-staining with anti-BrdU and anti-phospho-ERK antibodies, the staining was performed in accordance with instructions from Cell Signalling for the anti-phospho-ERK antibody with the following modifications: first, DNase treatment was performed in accordance with the BD Pharmingen protocol after the blocking step, and second, anti-BrdU antibody was added at the same time as the anti-phospho-ERK antibody. For phospho-ERK staining in OT-I TAP$^0$ thymocytes, T2-H-2K$^b$ APCs were pulsed overnight at a concentration of $10^6$ ml$^{-1}$ with 2 μM SIINFEKL and 100 μM RTYTYEKL in RPMI medium. APCs were washed twice in PBS and then incubated with thymocytes at a 1:1 ratio at 37 °C for the indicated duration. Reaction was started with a 2-min centrifugation at 300*g*. Conjugates were disrupted by the addition of ice-cold PBS, 10mM EDTA, 2% paraformaldehyde and by vigorous pipetting. Cells were then left on ice for 5 min and then incubated at 25 °C for 15 min. Staining was performed in accordance with instructions from Cell Signalling, except that the incubation in ice-cold methanol was followed by an additional incubation overnight in methanol at −20 °C.

## Biochemical analysis

SDS–PAGE and immunoblotting were conducted with standard procedures. All phospho-specific antibodies and total protein antibody used for immunoblotting were purchased from Cell Signalling with the exception of anti-B-Raf and anti-Egr1 (Santa Cruz Biotechnology) and anti-ERK1/2 (Upstate Biotechnology).

## Microarray

The indicated thymocyte populations were sorted on ARIA and the purity was assessed to be at least 98% by reanalysis. Three independent RNA samples from thymocytes sorted from individual mice were analysed for each experimental group. Probe sets were first filtered with DMT software (Affymetrix) by eliminating those that did not have a 'present call' in all control samples (for increased calls) or all experimental samples (for decreased calls). Nine pairwise comparisons of the three experimental versus three control samples were performed with DMT software. To be considered significant, probe sets had to receive a 'change call' in 100% of comparisons, had to have an absolute log ratio of 1 or more, and had to be considered significantly changed by one-way analysis of variance (ANOVA) ($P \leq 0.01$). One-

way ANOVA was run with GENESIS software[27] on all probe sets, after filtering for absent calls. Probe sets were annotated by submitting them to the Affymetrix analysis website (www.affymetrix.com/analysis/index.affx).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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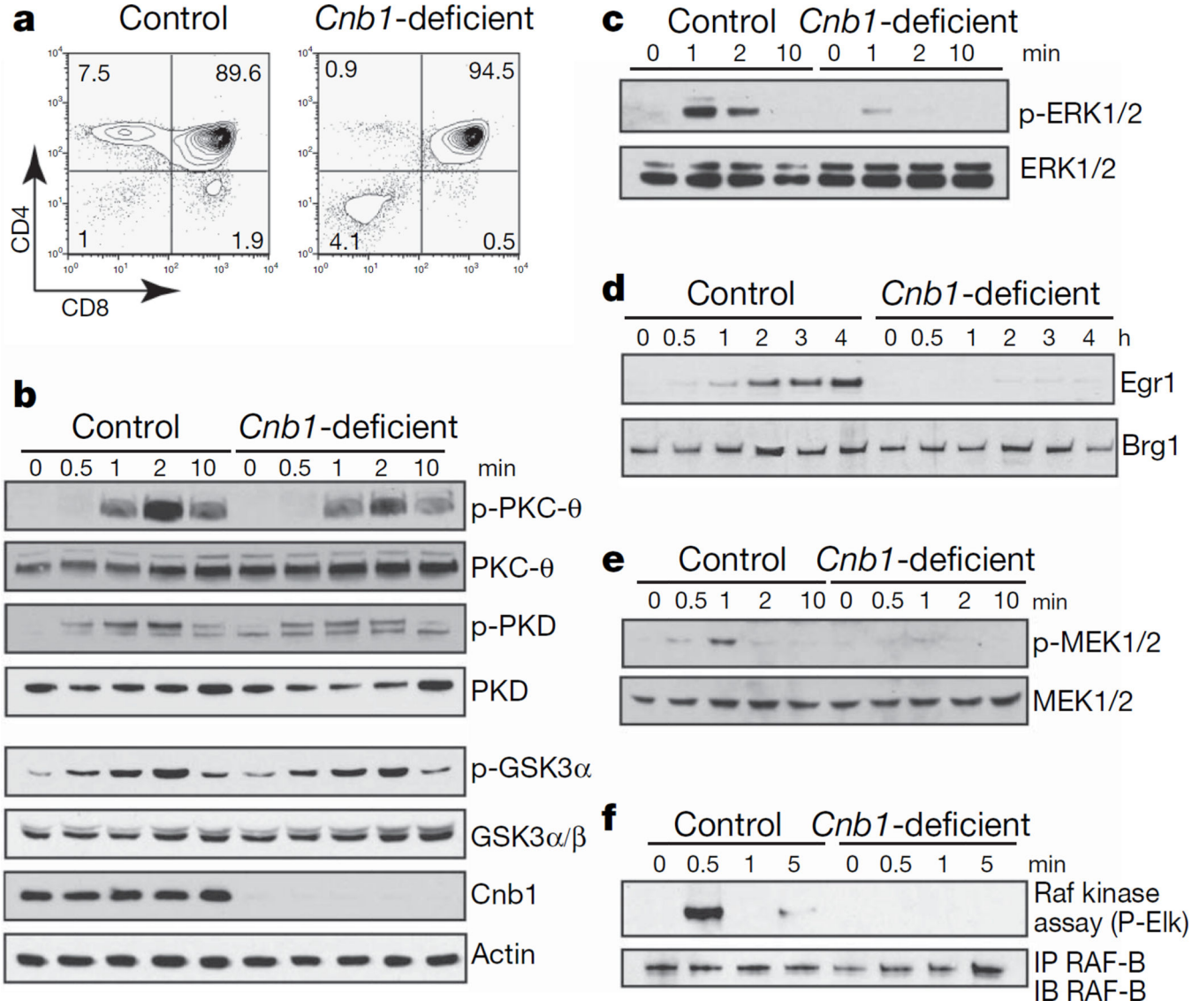


**Figure 1. Specific and severe defect in Raf–MEK–ERK activation in *Cnb1*-deficient thymocytes**

**a**, Expression of CD4 and CD8 on *Cnb1*-deficient and control thymocytes. The numbers in the corners of the panels represent the percentage of cells in each quadrant. **b**, Immunoblot analysis of phosphorylated and total proteins in *Cnb1*-deficient and control double-positive thymocytes after CD3ε crosslinking. GSK, glycogen synthase kinase; PKC, protein kinase C; PKD, protein kinase D. **c**, Immunoblot analysis of phosphorylated ERK1/2 in *Cnb1*-deficient and control double-positive thymocytes after CD3ε crosslinking. **d**, Immunoblot analysis of Egr1 induction in double-positive thymocytes from *Cnb1*-deficient and control littermates. Brg1 shows equal loading. **e**, Immunoblot analysis of phosphorylated MEK1/2 in *Cnb1*-deficient and control double-positive thymocytes after CD3ε crosslinking. **f**, Raf-B kinase activity in *Cnb1*-deficient and control double-positive thymocytes after CD3ε crosslinking. IP, immunoprecipitation; IB, immunoblotting.

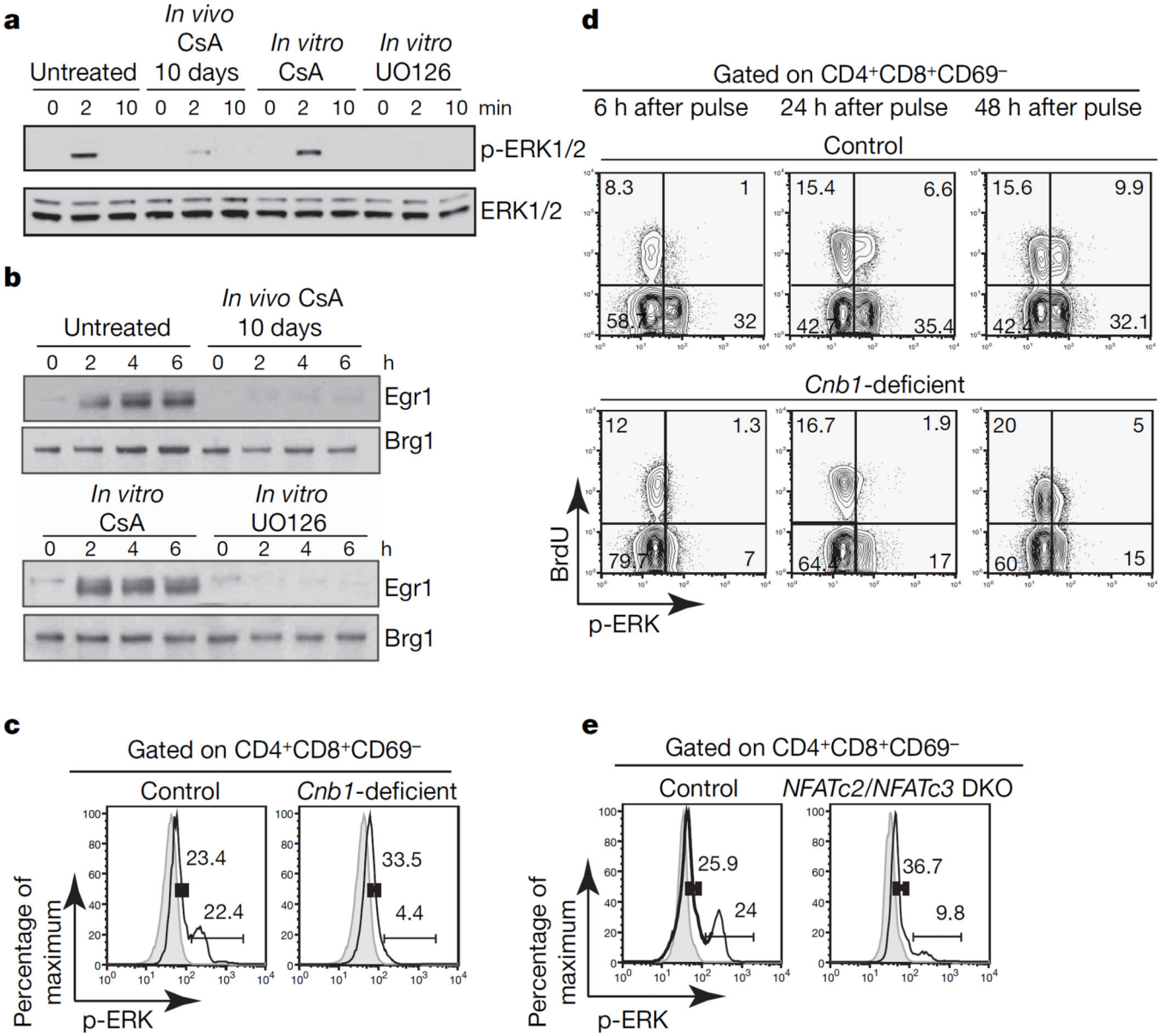


**Figure 2. Developmental but not direct requirement for calcineurin/NFAT activity for proper activation of ERK**

**a**, ERK1/2 phosphorylation in double-positive cells from untreated and CsA-treated mice in the presence of CsA (200 ng ml$^{-1}$) or UO126 (10 μM) after CD3ε crosslinking. **b**, Erg1 induction in double-positive cells from untreated or CsA-treated mice and in double-positive cells stimulated in the presence of CsA (200 ng ml$^{-1}$) or UO126 (10 μM) after CD3ε crosslinking. Brg1 shows equal loading. **c**, ERK1/2 phosphorylation in double-positive CD69-negative *Cnb1*-deficient and control thymocytes after CD3ε crosslinking for 2 min (solid lines). Grey areas, unstimulated. **d**, BrdU incorporation and ERK1/2 phosphorylation in double-positive CD69-negative thymocytes from *Cnb1*-deficient mice and control littermates injected once with BrdU after CD3ε crosslinking for 2 min. The numbers in the corners of the panels represent the percentage of cells in each quadrant. **e**, ERK1/2

phosphorylation in *NFATc2*/*NFATc3* double knockout (DKO) and control double-positive CD69-negative thymocytes after CD3ε crosslinking for 2 min (solid lines). Grey lines, unstimulated. The numbers in graphs **c** and **e** represent the percentage of cells in the indicated interval.

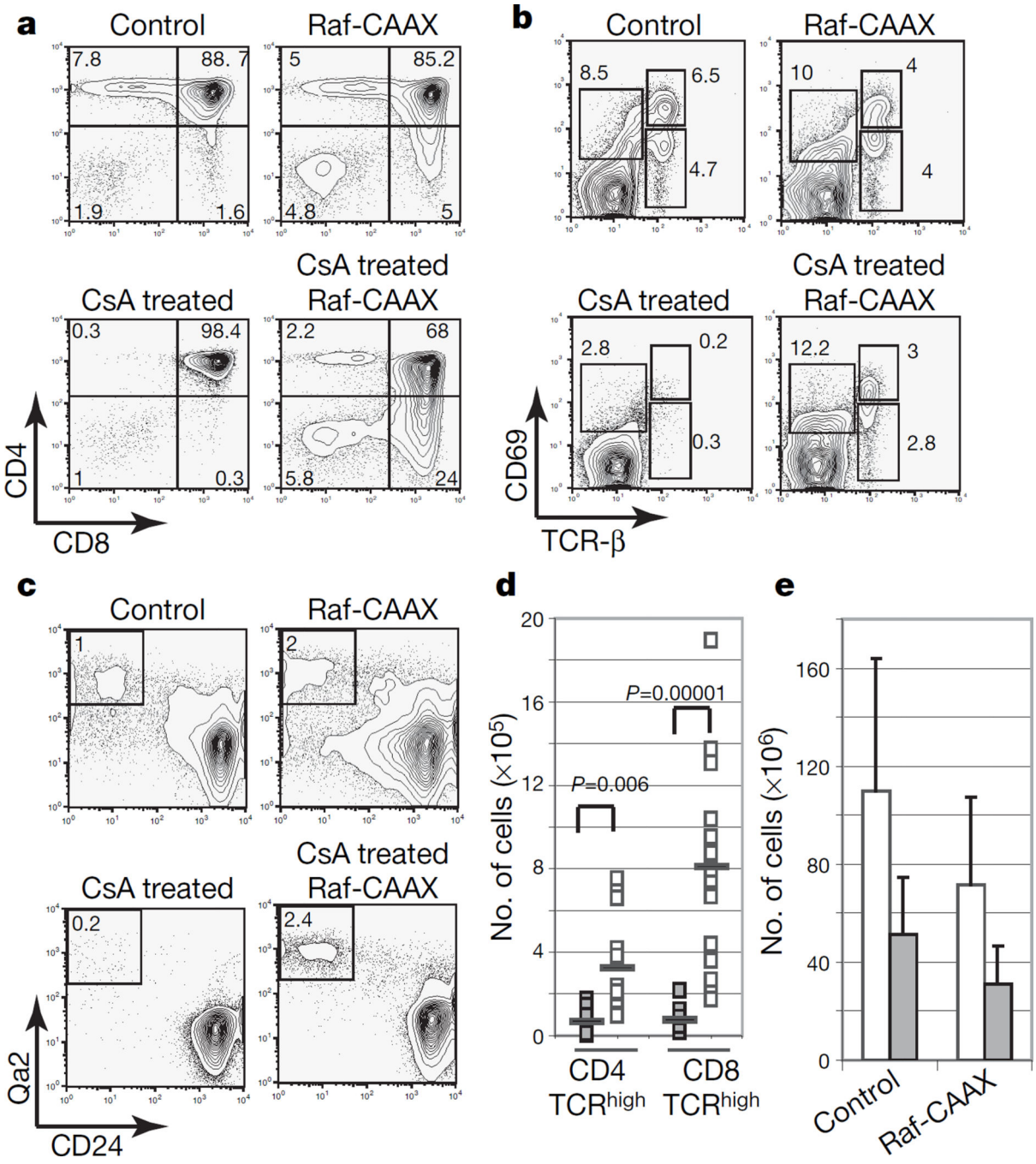


**Figure 3. Reconstitution of Raf–MEK–ERK signalling partly rescues positive selection in the absence of calcineurin activity**

**a**, Expression of CD4 and CD8 in Raf-CAAX transgenic and control mice treated with CsA or left untreated. **b**, Analysis of CD69 and TCR-β expression on thymocytes from Raf-CAAX transgenic and control mice treated with CsA or left untreated. **c**, Analysis of CD24 and Qa2 expression on thymocytes from Raf-CAAX transgenic and control mice treated or not with CsA. **d**, Absolute numbers of $TCR^{high}$ CD4 and CD8 single-positive cells ($n \geq 8$, each square represents an individual mouse; bar equals mean value). *P* values refer to a one-

tailed *t*-test. Open symbols, CsA, Raf-CAAX; filled symbols, CsA. **e**, Absolute numbers of thymocytes for mice of indicated genotype ($n \geq 8$; error bars show s.d.). Open bars, control; filled bars, CsA-treated. The numbers in the corners of the panels represent the percentage of cells in each quadrant.

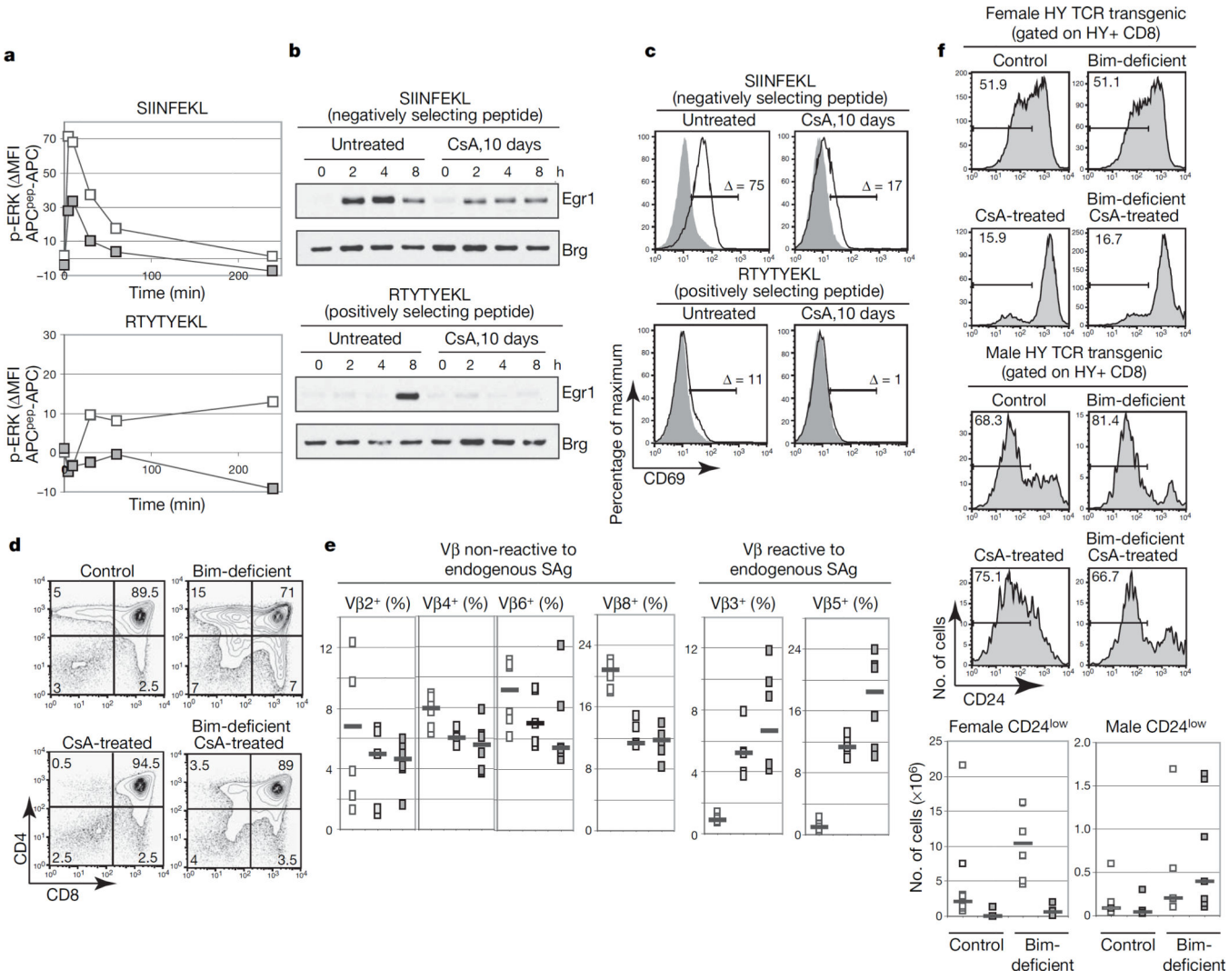


**Figure 4. Transition to the 'high ERK competence' state is required to respond functionally to positively selecting ligands**

**a**, Phospho-ERK1/2 in OT-I double-positive thymocytes from mice stimulated with SIINFEKL or RTYTYEKL. White symbols, untreated; grey symbols, CsA-treated. **b**, Egr1 upregulation in OT-I double-positive thymocytes stimulated with SIINFEKL or RTYTYEKL. **c**, CD69 upregulation in OT-I double-positive thymocytes after 4 h of stimulation. Δ indicates the percentage difference in CD69 thymocytes stimulated by APCs only or by peptide-pulsed APCs. Solid lines, APCs plus peptide; grey areas, APCs only. **d**, CD4 and CD8 expression in thymocytes from mice of the indicated genotypes. **e**, Percentage of CD4 single-positive expressing Vβ chains reactive or not to endogenous superantigens (squares show results for individual mice; bars show medians; $n \geq 3$). White symbols, untreated control; light grey symbols, *Bim*-deficient; dark grey symbols, *Bim*-deficient and CsA-treated. **f**, Percentages (numbers shown in each panel refer to the percentage of cells in the indicated interval) and absolute numbers of $HY^+$ CD8 single-positive $CD24^{low}$ cells in

mice of the indicated genotypes (squares show results for individual mice, bars show means; $n \geq 4$). White symbols, untreated; grey symbols, CsA-treated.