A threshold level of NFATc1 activity facilitates thymocyte differentiation and opposes notch-driven leukaemia development

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NFATc1 plays a critical role in double-negative thymocyte survival and differentiation. However, the signals that regulate Nfatc1 expression are incompletely characterized. Here we show a developmental stage-specific differential expression pattern of Nfatc1 driven by the distal (P1) or proximal (P2) promoters in thymocytes. Whereas, preTCR-negative thymocytes exhibit only P2 promoter-derived Nfatc1β expression, preTCR-positive thymocytes express both Nfatc1β and P1 promoter-derived Nfatc1α transcripts. Inducing NFATc1α activity from P1 promoter in preTCR-negative thymocytes, in addition to the NFATc1β from P2 promoter impairs thymocyte development resulting in severe T-cell lymphopenia. In addition, we show that NFATc1 activity suppresses the B-lineage potential of immature thymocytes, and consolidates their differentiation to T cells. Further, in the pTCR-positive DN3 cells, a threshold level of NFATc1 activity is vital in facilitating T-cell differentiation and to prevent Notch3-induced T-acute lymphoblastic leukaemia. Altogether, our results show NFATc1 activity is crucial in determining the T-cell fate of thymocytes.

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Differentiation of CD4⁻CD8⁻ double-negative (DN) cells to the CD4⁺ or CD8⁺ single-positive (SP) T cells in the thymus is regulated by a complex network of signalling pathways involving multiple transcription factors at various stages of development. On the basis of their differentiation status, DN thymocytes consist of four distinct populations, CD4⁻CD8⁻DN1, CD4⁺CD8⁻DN2, CD4⁺CD8⁺DN3 and CD4⁺CD8⁻DN4 (ref. 1). DN3 thymocytes upon rearrangement of their T-cell receptor β (Tεb) locus express a functional TCRβ chain, which, in combination with the preTεα (∊εα) chain forms the preTCR (pTCR). PreTCR signalling is essential for the differentiation of DN3 cells to DN4 and later stages. We have shown recently that the transcription factor NFATc1 plays a critical role during early stages of thymocyte development. A haematopoietic lineage cell-specific ablation of NFATc1 activity blocked thymocyte development at DN1 stage, the earliest stage of thymic T-cell development. Mice deficient for NFATc2, NFATc3 or both, do not show any apparent defect in thymocyte development, suggesting a critical role played by NFATc1 during thymic T-cell development.

Ligation of TCR with cognate peptide-MHC complex in vivo, or stimulation of T cells with anti-CD3 plus anti-CD28 Abs in vitro increases intracellular Ca²⁺ levels, which in turn activate the serine/threonine phosphatase calcineurin. Active calcineurin dephosphorylates multiple serine/threonine residues in NFAT proteins and facilitates their nuclear translocation. We have previously elucidated a novel NFAT activation pathway in pTCR-negative thymocytes, which plays an indispensable role in their survival and differentiation. In contrast to the calcineurin-mediated dephosphorylation pathway, in pTCR-negative thymocytes IL-7-JAK3 signals activated NFATc1 via phosphorylation of tyrosine 371 in the regulatory domain. Both, the calcineurin-mediated ‘conventional’ and IL-7-JAK3-mediated ‘alternative’ pathways though explained the post-translational mechanisms of NFAT activation, the transcriptional regulation of Nfatc1 itself is poorly understood. A previous study showed Nfatc1 expression in T cells is autoregulated by NFATc1 (ref. 5). In this study, we have delineated the signalling pathways that regulate Nfatc1 expression with distinct promoter usage at pTCR-negative and -positive thymocytes. Further, we provide evidence in support of a critical role of NFATc1 in suppressing lineage plasticity of immature thymocytes towards non-T lineages, and the essentiality of a threshold level of NFATc1 activity at the pTCR-positive DN3 stage in facilitating the T-cell fate of thymocytes by preventing the development of T-Acute Lymphoblastic Leukaemia (T-ALL).

**Results**

**Differential usage of Nfatc1 promoters in thymocytes.** In T cells, two distinct promoters, a distal (P1) and a proximal (P2), initiate Nfatc1 expression. Due to alternative splicing and usage of two different polyadenylation (pA) sites, six Nfatc1 isoforms; three from P1 promoter (Nfatc1a, αβ and αc) and three from P2 promoter (Nfatc1bα, βB and βC), respectively, are synthesized (Supplementary Fig. 1a)⁶⁷. To distinguish whether any particular NFATc1 isoform is prevalent in pTCR-negative and -positive thymocytes we analysed wild-type (WT) DN1–DN4 cells for Nfatc1 expression. Interestingly, we observed an exclusive P2 promoter activity in the pTCR-negative DN3 cells, whereas pTCR-positive DN4 cells exhibited both P2 as well as P1 promoter activity (Fig. 1a; Supplementary Fig. 1b). Exclusive P2 activity in pTCR-negative thymocytes was supported by an active chromatin configuration, as indicated by histone modifications and a concomitant recruitment of RNA polymerase II (Pol II) at the Nfatc1 P2 promoter in Rag2⁻⁻⁻⁻⁻⁻ DN3 cells, whereas only little H3K4me3 was detectable at the P1 promoter (Fig. 1b). Analysis of small RNA-sequ data⁵, to score for bidirectional paused transcripts enriched at promoters further confirmed significant enrichment of transcriptional start site RNAs at P2 promoter over P1 (Fig. 1b). Using an additional set of primers to detect total α- or β-specific transcripts derived from the P1 or P2 promoters respectively, again we observed an exclusive P2 activity in DN3, and both P1 and P2 activity in DN4 cells (Supplementary Fig. 1c). Further, in agreement with our observation regarding induction of P1 promoter activity at the pTCR-positive stages, we detected NFATc1 proteins in DN4 cells but not in DN3 cells (Fig. 1c; Supplementary Fig. 1d). Keeping in view the findings of a previous report⁹, that in naive T cells, constitutive basal level TCR signalling maintains the P2 promoter activity, and TCR-antigen ligation induced signals are responsible for the P1 promoter activity, it was quite intriguing how thymocytes lacking even the pTCR exhibit such a robust Nfatc1 expression.

To further substantiate our observation regarding selective promoter usage, and the role of pTCR signalling in inducing P1 activity, we investigated three different mouse models, where either there was no pTCR (Rag1⁻⁻⁻⁻⁻⁻), pTCR was absent but a downstream signalling molecule calcineurin was constitutively active (Calcineurin transgenic; ΔCam)⁹ or there was enhanced pTCR signalling (Notch3 transgenic; N3tg)¹⁰. In both Rag1⁻⁻⁻⁻⁻⁻ and ΔCam mice, T-cell development is blocked at the DN3 stage due to lack of pTCR signals⁹. In contrast, N3tg mice showed an enhanced DN3 to DN4 transition due to strong pTCR signals (Fig. 1d,e). Confirming our ChIP-Seq and RNA-Seq observations, analysis of Nfatc1 expression in Rag1⁻⁻⁻⁻⁻⁻ DN3 cells showed the presence of only Nfatc1β isoforms (P2 activity), similar to that in WT DN3 cells (Fig. 1f). Stimulating Rag1⁻⁻⁻⁻⁻⁻ DN3 cells with anti-CD3 Abs to mimic pTCR signalling, we readily detected Nfatc1α transcripts in addition to the Nfatc1β transcripts implying that pTCR signals can induce P1 promoter activity (Fig. 1f). Further, P1 promoter activity was autoregulated by NFATc1, as P1 activity disappeared without affecting P2 activity when anti-CD3 antibodies stimulated Rag1⁻⁻⁻⁻⁻⁻ DN3 cells were treated with cyclosporine A (CsA; Fig. 1f). In contrast to the WT and Rag1⁻⁻⁻⁻⁻⁻ mice, ΔCam DN3 cells showed a robust P1 activity in addition to the P2 promoter activity (Fig. 1g). The strong P1 activity in ΔCam DN3 cells was due to the autoregulatory loop maintained by constitutive calcineurin activity, as CsA treatment specifically extinguished Nfatc1 P1 activity without affecting the P2 activity (Fig. 1g).

Further, we observed a similar pattern of strong P1 and P2 promoter activity as that in ΔCam mice, in N3tg DN3 cells (Fig. 1h). Thus, it was evident that Nfatc1 is expressed from distinct promoters in pTCR-negative and -positive thymocytes, and pTCR signalling is necessary for the induction of Nfatc1 P1 promoter activity.

**NFATc1 activity is vital for DN thymocyte differentiation.** Due to exclusive P2 activity in pTCR-negative thymocytes, we investigated whether NFATc1β is solely critical for the differentiation of early DN thymocytes. To clarify this, we have generated a mutant mouse with floxed Nfatc1 P2 promoter element (P2floflo) to abolish NFATc1β activity in a tissue-specific manner (Supplementary Fig. 2a–d). We bred P2floflo mice with mice expressing cre-recombinase under Vav promoter (Vav-Cre)¹¹ to abolish Nfatc1 P2 activity during thymocyte development. Surprisingly, analysis of Vav-CreP2floflo mice showed completely normal T-cell development, which was indistinguishable from that of littermate controls (Fig. 2a–c; Supplementary Fig. 2e). The normal T-cell development in Vav-CreP2floflo mice was quite surprising, as loss of NFATc1 activity was expected to block thymocyte development at the DN1 stage². PCR with reverse
transcription (RT–PCR) analysis on pTCR-negative thymocytes showed a total lack of Nfatc1 P2-derived transcripts indicating ablation of Nfatc1 P2 promoter activity (Fig. 2d). However, Nfatc1 activity was not lost rather in absence of P2 activity we observed a robust P1 promoter activity in the Vav-CreP2fl/fl pTCR-negative thymocytes (Fig. 2d).

We have previously shown that Bcl-2 is a target of NFATc1 in thymocyte differentiation. Thus, in the Vav-CreP2fl/fl mice, Nfatc1 P1 promoter activity functionally compensated for the loss of P2 activity, underlining the indispensability of NFATc1 activity in thymocyte differentiation.

**NFATc1 activity is essential for T-cell development.** To investigate the physiological significance of the distinct pattern of NFATc1 promoter activity in pTCR-negative and -positive thymocytes, we explored what impact NFATc1ζ will have on thymocyte development if it is co-expressed with NFATc1β in pTCR-negative thymocytes. To address this issue, we used mice in which a constitutively active version of Nfatc1ζA was knocked-in into Rosa-26 locus flanked by a floxed stop cassette (R26-caNfatc1ζA-Stopfl/fl; designated hereafter as Nfatc1ζAfl/fl) (Fig. 3e). To activate Nfatc1ζA expression in early thymocytes we bred Nfatc1ζAfl/fl mice with Vav-Cre mice. Surprisingly, analysis of Vav-CreNfatc1ζAfl/fl mice showed severely impaired thymocyte development as evident from a dose-dependent reduction in the size of the thymus, spleen and lymph nodes (Fig. 3a). Accordingly, the cellularity in these organs was drastically reduced in Vav-CreNfatc1ζAfl/fl mice leading to T-cell lymphopenia (Fig. 3a,b,c). Analysis of DN cells from Vav-creNfatc1ζAfl/fl mice to understand the reason behind the low thymic cellularity revealed a dose-dependent block in the transition of DN3 cells to the DN4 stage (Fig. 3d; Supplementary Fig. 3a). Confirming our earlier report regarding NFATc1-mediated regulation of Bcl-2 expression in developing DN thymocytes, enforced NFATc1ζ expression enhanced Bcl-2 levels in Vav-CreNfatc1ζAfl/fl DN3 cells compared with WT thymocytes.
NFATc1 suppresses lineage plasticity of immature thymocytes. T-cell development in the thymus follows a sequential process of T-lineage specification, and commitment. While DN1 cells retain the potential to differentiate into B cells, natural killer (NK) cells, dendritic cells (DCs) and macrophages, the lineage plasticity gets restricted to NK and DC lineages in DN2 cells, and finally is completely lost at the DN3 stage. As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). As in the WT mice (Fig. 4a). 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Figure 3 | *Nfatc1* P1 activity in addition to P2 activity in pTCR-negative cells impairs thymocyte differentiation. (a) Photograph showing the dosage-dependent effect of *Nfatc1* P1 activity on the size of the thymus, LNs and spleen in *Vav-CreNfatc1αAfl/+* and *Vav-CreNfatc1αAfl/fl* mice compared with *Nfatc1αAfl/fl* control mice. (b) Distribution of thymocyte subsets based on CD4 and CD8 expression in indicated mice. Number above each plot represents total thymic cellularity. (c) Cellularity in thymus, LNs and spleen from *Nfatc1αAfl/Afl*, *Vav-CreNfatc1αAfl/+* and *Vav-CreNfatc1αAfl/fl* mice (*n* = 10 per group). (d) Distribution of DN1–DN4 cells based on CD44 and CD25 expression among DN thymocytes from indicated mice. (e) Intracellular Bcl-2 expression in *Vav-CreNfatc1αAfl/fl* DN3 cells compared with that in *Nfatc1αAfl/Afl* control mice. MFI, mean fluorescence index. (f) Intracellular TCRβ expression in DN3 cells from indicated mice. Number inside each plot represents per cent TCRβ-positive cells. (g) Impaired differentiation of *Vav-CreNfatc1αAfl/fl* DN1–DN4 cells to DP stage on OP9-DL1 monolayer compared with WT cells *in vitro*. Numbers inside each plot represent per cent respective population. Data represent one of three independent experiments (*n* = 4 per group), and are shown as mean ± s.d., ***P*<0.0001, one-way analysis of variance.
were not affected (Fig. 4e). Corroborating this, *Ebf1* and *Pax5* expression were suppressed in Vav-Cre*Nfatc1*Δ^fl/fl^ DN1 cells, whereas gene expression necessary for NK and myeloid lineages was unaffected (Fig. 4f). However, the suppression of B-lineage potential of the Vav-Cre*Nfatc1*Δ^fl/fl^ DN1 cells was neither due to enhanced cell death nor due to any toxic effect of NFATc1 activity on the precursor cells. In a B-lineage permissible environment, FACs sorted DN1 thymocytes from Vav-Cre*Nfatc1*Δ^fl/fl^ mice developed comparable proportion of B220^+^ B cells as that in case of WT DN1 cells, when co-cultured on OP9 bone marrow stromal cell layer (Supplementary Fig. 3e). This observation ruled out that there was any inherent developmental restriction towards B-lineage differentiation in the Vav-Cre*Nfatc1*Δ^fl/fl^ DN1 cells.

**Integrin signalling induces *Nfatc1* P2 promoter activity.** The exclusive P2 activity in the WT pTCR-negative thymocytes led us to ask how the P2 promoter is regulated in these cells. Surprisingly, when we cultured DN4 thymocytes in vitro, not only all P1-directed transcripts disappeared but we also observed the absence of all P2-derived transcripts as well (Fig. 5a). However, annexin V analysis revealed that majority of cells in this culture condition were alive, ruling out cell death being the reason for the
Figure 5 | Integrin-cyclic-AMP signalling regulates Nfatc1 P2 activity. (a) RT-PCR analysis of Nfatc1 isoforms expression in WT DN4 cells cultured in medium only for 18 h compared with that in freshly isolated DN4 cells. (b) RT-PCR analysis of Nfatc1 isoforms expression in 8-CPT-cAMP treated WT DN3 and DN4 cells. (c) Analysis of Nfat1, Nfat1α, Nfat1β and other genes in WT CD4+ CD25+ Treg cells compared with CD4+ CD25− Teff cells. (d) Analysis of GFP expression levels in thymic Teff and Treg cells from various integrins in WT DN3 cells stimulated with CD48 Abs (5 μg) or PMA (100 ng) alone or in combination of both for 18 h. (j) Gene expression analysis for various integrins in WT DN3, DN4, CD4+ CD8− DP and CD4+ CD8+ SP cells from thymus as revealed by RT-PCR. (k) CD18 expression levels (MFI) in Treg and Teff cells from thymus and LNs of WT mice, (n = 4). (l) Analysis of Nfat1 expression pattern in fibronectin (Fibr.; 1 μg) or CD48Abs (0.5 μg) stimulated peripheral CD4+ T cells. Data are representative of three independent experiments and are shown as mean ± s.d., *P < 0.0001, paired t-test.

loss of Nfatc1 expression (Supplementary Fig. 4a). We have previously reported the responsiveness of Nfatc1 promoter activity to Forskolin (FSK) treatment in vitro. To investigate whether cyclic-AMP (cAMP) signalling is responsible for Nfatc1 P2 activity, we treated WT DN3 and DN4 cells with a cAMP analogue 8-CPT-cAMP. Interestingly, 8-CPT-cAMP treatment specifically induced the expression of Nfatc1β, both in DN3 and DN4 cells (Fig. 5b). To prove that P2 activity is cAMP signalling-dependent, we explored for a physiological context where the intracellular cAMP level is high. Several recent studies have reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Supplementary Fig. 4a). We have previously reported increased cAMP levels in CD4+ CD25+ Foxp3+ regulatory T (Treg) cells. The effect of cAMP in inducing Nfatc1 P2 promoter activity was confirmed as Treg cells exhibited stronger P2 promoter activity compared with CD4+ CD25− SP cells from thymus as revealed by RT-PCR (Su...
Foxp3$^+$ effector T (T$_{eff}$) cells (Fig. 5c). This observation was corroborated with higher level of green fluorescent protein (GFP) expression reflecting higher NFATc1 levels in T$_{eff}$ cells from Nfatc1-eGfp-Bac$^+$ reporter mice (Fig. 5d).

Next, we investigated which signalling pathway is involved in generating intracellular cAMP that drives NFATc1 expression in thymocytes and in T cells. Cell–cell interaction is mediated by various integrins, and thymocytes, as well as T cells express a number of integrins. CD2 (LFA-2) expressed on thymocytes and T cells has been shown to activate T cells$^{18,19}$. Murine CD2 binds to its ligand CD48 expressed on T cells as well as on the interacting cells and thereby can transduce signals both in cis and in trans$^{20–23}$. In addition, CD2 signalling in T cells have been reported to induce cAMP$^{24,25}$, and thereby the activation of cAMP response element binding (CREB) proteins$^{26}$. Mice lacking cAMP-CREB signalling show impaired thymocyte proliferation and altered fetal T-cell development$^{27,28}$. To check whether CD2-CD48 interactions regulate NFATc1 expression in DN thymocytes, we analysed CD2 and CD48 expression on various thymocyte subsets. We observed only a fraction of DN thymocytes expressed CD2 on their surface, which was increased in DP and SP thymocytes as well as in peripheral T cells (Fig. 5e). However, CD48 expression was highest on DN cells and lowest in DP and CD4$^+$ SP cells (Fig. 5f). Among DN thymocytes, DN1 cells expressed the highest level of CD2, whereas CD48 expression was high on all pTCR-negative DN populations (Fig. 5g,h). To prove whether CD2-CD48 signals could induce NFATc1 expression, we stimulated WT DN3 cells with CD48 antibodies and analysed for the generation of P1- and P2-directed transcripts. CD48 stimulation at a higher concentration induced the synthesis of both P1- and P2-directed NFATc1 transcripts in DN3 cells, while stimulation with PMA, reported to substitute CD2 co-stimulation$^{29}$, did not generate any NFATc1 RNA (Fig. 5i). However, CD48 at low concentration only induced NFATc1 P2

![Diagram](image_url)

**Figure 6 | Nfatc1 P1 activity is induced by a novel enhancer element.** (a) DNase I hypersensitive sites in the NFATc1 locus in human hematopoietic stem and CD3$^+$ T cells. Their corresponding position in the murine Nfatc1 gene is also shown. (b) Enhancer activity of the E2 element in inducing Nfatc1 P1 promoter activity in reporter assays. (c) NFATc1 expression levels as revealed by GFP expression in freshly isolated, or aCD3$^+$ aCD28 Abs stimulated CD4$^+$ T cells from Nfatc1-eGfp-Bac$^+$tg mice compared with that from Nfatc1-eGfp-Bac$^+$∆E2tg mice. (d) Immunoblot analysis for GFP or NFATc1α expression in unstimulated and 48 h aCD3$^+$ aCD28 Abs stimulated CD4$^+$ T cells from Nfatc1-eGfp-Bac$^+$∆E2tg mice compared with that from Nfatc1-eGfp-Bac$^+$ tg mice.
activity in the pTCR-negative thymocytes unraveling the effect of signal strength on the inducibility of P1 and P2 promoters (Supplementary Fig. 4b).

Although CD2-CD48 signalling induced Nfatc1 expression in vitro, these are unlikely to be the only integrins responsible for Nfatc1 expression in vivo. This was supported by the reported normal T-cell development in Cd2−/− mice30-32, as well as in mice injected with anti-CD2 antibodies33, and also in Cd48−/− mice34, indicating the involvement of additional integrins in regulating Nfat1 expression. Accordingly, analysis of thymocytes and T cells showed a differential expression pattern for various integrins (Supplementary Fig. 4c; Fig. 5j). CD4−CD25− Treg cells both in the thymus and in LNs expressed much higher levels of various integrins compared to CD4−CD25− T eff cells (Fig. 5k; Supplementary Fig. 4d), concurring with our observation regarding higher Nfat1 expression in Treg cells over T eff cells (Fig. 5c). Among the CD4+CD25+ population, CD25hi cells expressed highest level of various integrins, the most prominent being CD18, CD48, CD49d and CD62L (Supplementary Fig. 5a,b). This integrin expression pattern directly correlated to the expression in CD25hi cells as revealed from higher GFP levels in the Nfat1-eGfp-Bac transgenic reporter mice (Supplementary Fig. 5c). To further consolidate our observation, we stimulated WT CD4+ T cells with fibronectin, the ligand for CD18. Interestingly, fibronectin stimulation induced robust Nfat1 expression, which was mainly derived from the P2 promoter (Fig. 5l). In addition, similar to that in DN3 thymocytes (Fig. 5i), CD2-CD48 signalling also induced Nfat1 P2 activity in peripheral CD4+ T cells (Fig. 5l). Thus, the above observations established integrin-CAMP signalling as a critical component in regulating Nfat1 gene expression in thymocytes and in T cells.

An intronic cis-regulatory element controls P1 activity. To address the question why P1 promoter is inactive in the pTCR-negative thymocytes, we analysed DNA methylation status at the P1 promoter in these cells. However, methylation analysis did not reveal any significant difference between WT DN3 and DN4 cells (Supplementary Fig. 6a). To investigate whether epigenetic modifications on pTCR signalling at the DN3 to DN4 transition induce P1 activity we performed ChIP-Seq analysis of Rag2−/− DN3 cells stimulated with anti-CD3 Abs to mimic pTCR signals. However, no additional epigenetic changes were detected at the P1 promoter in the anti-CD3 stimulated Rag2−/− DN3 cells compared to the unstimulated cells (Supplementary Fig. 6b; Fig. 1b). These observations suggested that P1 promoter induction still requires involvement of additional cis-regulatory elements and/or trans-acting factors. To identify cis-regulatory elements essential for P1 promoter activity, we studied the DNase I hypersensitivity analysis of the human NFatc1 locus (Roadmap Epigenomics Project, www.roadmapepigenomics.org). Besides the P1 and P2 promoter elements, two very distinct DNase I hypersensitive sites designated as E1 and E2 were evident in the intron between exons 10 and 11 in the CD34+ human haematopoietic stem cells (Fig. 6a). Significantly, these elements were tissue-specific as compared with the haematopoietic stem cells, CD3+ human T cells showed only the presence of E2 site, which was conserved in murine T cells as well (Fig. 6a).

To investigate the influence of E1 and E2 elements on Nfat1 expression, we generated luciferase reporter constructs with the P1 or P2 promoter in combination with the E1 or E2 element (Fig. 6b). On stimulation with PMA + ionomycin (I), in EL-4 thymoma cells the E2, but not the E1 element induced a strong Nfat1 P1 promoter activity compared with a mild increase in P2 promoter activity (Fig. 6b). We also tested two additional DNase I hypersensitive sites, E3 positioned in the intron between exons 3 and 4, and E4 positioned at the extreme 3′ region of Nfatc1 locus for their potential regulatory effect on Nfat1 promoter activity. However, both these elements were ineffective in inducing Nfat1 P1 promoter activity (Supplementary Fig. 6c), suggesting the essentiality of the E2 element in the context of P1 promoter activity.

To extend our characterization of the E2 enhancer, we analysed for the trans-acting factors, which might play a role in enhancing the E2-mediated P1 promoter activity. We detected binding motifs for three prominent lymphoid-specific transcription factors; Nfatc1, Gata3 and Pu.1 in the E2 element (Supplementary Fig. 6d). To investigate whether any of these factors positively modulates the E2 enhancer activity, we mutated the binding sites for individual factors and checked their enhancer potential for Nfat1 P1 promoter activity. In reporter assays, only the Nfatc1 mutant showed loss of enhancer activity, whereas Gata3 or Pu.1 mutants exerted no influence on Nfatc1 P1 promoter activity (Supplementary Fig. 6d). This was in line with the previous reports from our laboratory that Nfatc1 antagonizes the P1 promoter activity5, however, the involvement of the E2 element was hitherto unknown.

Further, to study the effect of E2 element on Nfat1 promoter in vivo, we generated Nfat1-eGfp-Bac-AE2 tg reporter mice by deleting 1kb DNA from intron 10 harbouring the E2 element (Supplementary Fig. 6e)17. Loss of E2 enhancer activity in Nfat1-eGfp-Bac-AE2 tg reporter mice, showed a clear reduction in Nfat1 expression in CD4+ T cells as evident from reduced GFP expression both in unstimulated as well as in stimulated cells compared with that in the Nfat1-eGfp-Bac tg reporter mice (Fig. 6c). This reduction in NFATc1 levels was due to a specific loss of P1-derived NFATc1α, as we observed a strong decrease in NFATc1α proteins in immunoblot analysis with antibodies against GFP as well as against NFATc1α, whereas loss of E2 enhancer activity did not influence NFATc1β protein levels (Fig. 6d; Supplementary Fig. 7). Thus, we have identified a novel cis-regulatory element E2, acting as an enhancer, specifically for the P1 promoter activity in T cells.

NFATc1 activity prevents experimentally induced T-ALL. At the DN3 stage, though pTCR signalling is vital for proliferation and differentiation of thymocytes, failure to do so will lead to the development of T-ALL, an aggressive form of leukaemia observed in both mouse and humans with constitutive Notch signalling35-40.

We have previously shown that T-cell development in ΔCam mice is severely blocked at DN3 stage due to a defect in pTCR formation9. Further analysis revealed a strong downregulation in Ptcra (pTγ) expression (Fig. 7a), in ΔCam DN3 cells. Ptcra expression is regulated by Notch signalling41, and accordingly, in N3 tg T cells pTγ expression is not extinguished38. The negative effect of NFATc1α on Ptcra expression in ΔCam DN3 cells suggests that Nfatc1 P1 activity might be involved in downregulating pTγ expression once the cells have received pTCR signals. Inhibition of NFATc1 activity by CsA treatment restored Ptcra expression in ΔCam DN3 cells (Fig. 7b), further strengthening the possibility that NFATc1α suppresses Ptcra expression. This was confirmed by a dose-dependent suppression of Ptcra expression in DN4 cells in Vav-CreNfatc1αA1/2+ and Vav-CreNfatc1αA1/2+ mice compared with WT cells (Fig. 7c). Further, ChIP assays confirmed NFATc1α binding at the Ptcra promoter in vivo in WT DN and ΔCam DN3 cells (Fig. 7d). Also, in reporter assays NFATc1α failed to induce Ptcra promoter activity whereas, it efficiently activated the murine I2 promoter.
**Figure 7** | NFATc1x activity prevents T-ALL development. (a) RT-PCR analysis for Ptcra expression in DN3 cells from WT, ∆Cam and Rag1−/− mice. (b) Ptcra expression levels in CsA treated or untreated DN3 cells from ∆Cam mice. (c) RT-PCR analysis for Cd3e and Ptcra expression in DN4 cells from WT, Vav-Cre Nfatc1xΔ/C农田 and Vav-Cre Nfatc1xΔ/C农田 mice. (d) ChIP assays for *in vivo* NFATc1, NFATc1x, RBPJκ and IkBα binding at Ptcra promoter in WT DN cells or in Rag1−/− and ∆Cam DN3 cells. (e) Luciferase reporter assay depicting the influence of NFATc1x on Ptcra and il2 promoter activity in unstimulated or P + I stimulated EL-4 thymoma cells. (f) Effects of NFATc1x activity on Notch-induced Ptcra promoter transactivation in unstimulated or ionomycin (I) stimulated Jurkat T-ALL cells as revealed by luciferase reporter assays. (g) Flow cytometry profiles depicting the distribution of CD4+ / CD8+ T-cell population (left panel), and CD4+ / CD8+ / CD44+ / CD25+ DN3 cells (right panel) in the thymus, LNs and spleen from mice with Notch3-induced T-ALL compared with WT littermate controls (n = 7 per group). Histograms depict the cellularity in the thymus, LNs and in the spleen. (h) RT-PCR analysis of Nfatc1x isoforms expression in N3-induced T-ALL cells compared with DN3 cells from WT or normal N3 tg mice. (i) Cellularity in the thymus, LNs and spleen from WT, N3 tg, ∆Cam and ∆Cam x N3 double-tg mice (n = 6 per group). Data are representative of three independent experiments and are shown as mean ± s.d., ***P < 0.0001, one-way analysis of variance. *P = 0.0039 or 0.0049 and **P = 0.0317 or 0.0207, unpaired t-test.
Discussion

We have shown previously the indispensability of NFATc1 activity in early thymocyte development. Here we have delineated the signals that control NFATc1 expression initiated from two distinct promoters in a thymocyte developmental stage-specific manner and, how NFATc1 activity simultaneously facilitates the T-cell fate of the thymocytes, and prevents the pathogenesis of T-ALL. The finding that NFATc1 expression is directed from only P2 promoter at the pTCR-negative stages and from both P1 and P2 promoters at the pTCR-positive stages establish the differential threshold of NFATc1 activity required for thymocyte differentiation. Once the DN3 cells receive pTCR signals, they are irreversibly committed towards T-lineage only. Hence, the NFATc1 activity at the pTCR-positive stages is the key to enhance the threshold of total NFATc1 activity through which T-lineage commitment is established on pTCR signals. Thus, the switch from ‘NFATc1β’ only to both ‘NFATc1α and β’ at the pTCR-positive DN3 cells is absolutely necessary for T-lineage commitment in the thymus. In this regard our observation that NFATc1 activity specifically suppresses the B-lineage plasticity of the immature thymocytes provides an important clue as to how activities of various transcription factors stabilize T-cell fate during development. Importantly, the characterization of a novel enhancer element specifically regulating the P1 promoter activities holds immense significance in the context of T-lineage commitment. From our observations, it appears that epigenetically E2 element is in an open conformation at the DN3 stage, but it is the specific transcription factor occupancy, which is critical in inducing P1 promoter activity at the pTCR-positive stage. As we have shown earlier that NFATc1 nuclear levels increases from DN1 to DN3 stage thus, most likely making it possible to bind to the E2 element and autoregulates its own expression by inducing P1 promoter activity. The elucidation of the signalling pathways regulating NFATc1 gene regulation will help to better understand T-cell development and will provide insight for therapeutic mani-
Generation of Nfatc1 P2Δfl mice. To generate the Nfatc1 P2 promoter floxed allele, the 5’ end of the murine Nfatc1 gene was used as a target to isolate genomic genomic clones of the Nfatc1 gene. The 8.5 kb XbaI fragment containing the Nfatc1 P2 promoter was identified in DNase I hypersensitive site analysis. The blunt ended left (XbaI to XhoI) and right (XhoI to XbaI) arms were subcloned into the SmaI site of pGL3 basic vector. To incorporate the loxP sites into the left or right arm, loxP sites cut out from the pBS112 SX vector were inserted into the NdeI (448 bp), EcoT21 (5,020 bp) and NdeI (7536 bp) sites, respectively. The pFNNeoLoxP plasmid containing the neo gene for negative selection was used as basic vector in constructing the targeting vector. Enriched DN thymocytes CD8+ (TiB105), NK1.1 (1D4), CD19 (1D3) and MHC class II (2G9) followed by treatment of total thymocytes with biotinylated Abs against CD4 (GK1.5), were sorted and were used to generate the chimeric gene for positive selection and thymidine kinase for negative selection was used as basic vector in constructing the targeting vector. Embryonic stem cell clones positive for the targeted P2 floxed allele were selected and were used to generate the chimeric Nfatc1 P2Δfl mice by blastocyst injection method. Genomic tail DNA from the chimeric mice was analysed by long-distance PCR to confirm the genomic integration of the floxed Nfatc1 allele, and mice were bred with C57BL/6 mice for several generations. WT, Nfatc1 P2Δfl/+ and P2Δfl/fl mice were genotyped using the following primers: 5’-CTCCACGTTAATTCGTTCCG-3’ (forward) and 5’-TCTCCCTCAAAATGGCTCTC-3’ (reverse).

Generation of Nfatc1-eGfp-Bac-ΔE2 tg2 mice. Nfatc1-eGfp-Bac-ΔE2 construct was generated by deletion of a 1-kb fragment of the E2 element from the original Nfatc1-eGfp-Bac transgene cassette. The RCAP3-361H16 containing the murine Nfatc1 gene (mm9 chr.18, 80.779.051-80.993617, 214 kb) was further modified using bacterial homologous recombination to delete 1,000 bp from the intron 10 (mm9 chr.18, 80.809.298-80.808.298, 298 bp). Two homology arms flanking the region of interest were generated by PCR using the primers 5’-targ-box-E2-for/5’-targ-box-E2-rev. Cells were incubated with anti-NFATc1 (ImmunoGlobe, Ig-457), followed by Alexa Fluor 555-conjugated donkey anti-rabbit IgG (A-31572, Molecular Probes) and DAPI (4,6-diamidino-2-phenylindole; Molecular Probes). Image acquisition and analysis were done with a TCS SP2 Leica confocal microscope and software.

In vitro TNFα, or Rag1-/- and Acam DN3 cells with anti-Nfatc1 (Santracruz; sc-7294), anti-Nfatc1α, antiRBPJα (Cell Signaling; 5313) and anti-IκBα (Santa Cruz; sc-781) were done as described previously.26 Briefly, 1 x 10^6 freshly isolated DN thymocytes from WT, or DN3 cells from Rag1-/- and Acam tg mice were cross-linked using 1% formaldehyde. Isolated nuclei were digested so that genome length became 500–750 bp. Chromatin solution was pre-cleared with Sepharose G, and chromatin was immunoprecipitated by incubating with 6–8 μg of each antibody mentioned above overnight at 4°C. Immune complexes were collected on protein G-Sepharose beads and immunoprecipitates were eluted with 1% SDS, 50 mM NaHCO3. After reversal of cross-links and DNA precipitation, probes were used for amplification of the Pcrea promoter region bound to Nfatc1, Nfatc1α, and RBPjκ with the following primers: 5’-AGGAGGAAGATCAAGGCTG-3’ (forward) and 5’-ATCTTTGCTGCTAAATCTCC-3’ (reverse).

Chlp-Seq and RNA-Seq analysis. Chlp-seq and Small RNA-seq were performed as described previously using same standards and quality controls.

Chlp-Seq. Briefly, DN cells were isolated from Bl6 Rag2-/- mice and cross-linked with 1% formaldehyde. Histone ChlpS were performed by using cross-linked nuclear extracts from 5 million cells with 2 μg specific antibodies and 20 μl Dynabeads Protein G suspension (Life Technologies, USA), whereas 50 million cells were used for Pol II ChlpS. Antibodies used are described in Koch et al., with the exception of H3K27ac (ab4729; Abcam). DNA was extracted from immunoprecipitated chromatin and was used to prepare sequencing library using TrueSeq Chlp-Seq Library Preparation Kit (Illumina Inc., USA) according to manufacturer’s instructions, and sequenced on a Genome Analyzer II sequencing platform (Illumina Inc., USA). Data processing, including input subtraction and wig files generation was performed as previously described.

Small RNA-Seq. Total RNA was extracted from DN cells isolated from Rag2-/- cells using Trizol Reagent (Life Technologies, USA). Total RNA of 10 μg was fractionated by TBE-Urea PAGE to isolate RNA fragments ranging from 15 nucleotides to 70 nucleotides length. These size-selected small RNAs were used for preparing sequencing library using Small RNA-Seq Library Preparation Kit (Illumina Inc., USA) according to manufacturer’s instructions. Libraries were then sequenced on a Genome Analyzer II sequencing platform (Illumina Inc., USA). The RNA Seq data used is accessible in gene omnibus under the accession number GSE44578.

OP9-DL1 co-culture assays and in vitro cultures. 4 x 10^5 sorted WT or Var-CreNfatc1Δfl mice were cultured on monolayers of OP9-DL1 cells expressing the Notch ligand delta-like1 (OP9-DL1) in x-vivo 20 medium supplemented with rhLT3 ligand (5 ng ml^-1) and rhIFN-γ (1 ng ml^-1) for 4 days. Subsequently, thymocytes were analysed for differentiation into DP cells by flow cytometry. In vitro cultures of sorted DN1-DN4 cell populations or CD4+ T cells were performed either in medium (RPMI-1640, 10% FCS) or with CsA (100 ng ml^-1), B-CPT-cAMP (50 μM), Forskolin (10 μM), CD48 (0.5 μg ml^-1), PMA (100 ng ml^-1), Fibroactin (Fibr; 1 μg ml^-1) supplemented with rhIFN-γ (1 ng ml^-1).
2. Patra, A. K. et al. An alternative NFAT-activation pathway mediated by IL-7 is critical for early thymocyte development. Nat. Immunol. 14, 127–135 (2013).

3. Xanthoudakis, S. et al. An enhanced immune response in mice lacking the transcription factor NFAT1. Science 272, 892–895 (1996).

4. Ranger, A. M., Oukka, M., Reganin, J. & Cilmnich, L. H. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. Immunity 9, 627–635 (1998).

5. Chuvpilo, S. et al. Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. Immunity 16, 881–895 (2002).

6. Chuvpilo, S. et al. Multiple NF-ATc isoforms with individual transcriptional properties are synthesized in T lymphocytes. J. Immunol. 166, 7294–7301 (2001).

7. Serling, E., Chuvpilo, S., Liu, J., Hofer, T. & Palmetshofer, A. NFATc1 autoregulation: a crucial step for cell-fate determinations. Trends. Immunol. 27, 461–469 (2006).

8. Lepovic, C. et al. Divergent transcription is associated with promoters of transcriptional regulators. BMC Genomics 14, 914 (2013).

9. Patra, A. K. et al. PKB induces calcineurin/NFAT-induced arrest of Rag expression and pre-T cell differentiation. J. Immunol. 177, 4567–4576 (2001).

10. Bellavia, D. et al. Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. EMBO J. 19, 3337–3348 (2000).

11. de Boer, J. et al. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur. J. Immunol. 33, 314–325 (2003).

12. Baumgart, S. et al. Inflammation-induced NFATc1-STAT3 transcription complex promotes pancreatic cancer initiation by KrasG12D. Cancer Discov. 4, 688–701 (2014).

13. Porritt, H. E. et al. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. Immunity 20, 735–745 (2004).

14. Rupp, T. et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. J. Exp. Med. 204, 1303–1310 (2007).

15. Gavin, M. A. et al. Foxp3-dependent programme of regulatory T-cell differentiation. Nature 445, 771–775 (2007).

16. Conche, C., Boulla, G., Trautmann, A. & Randriamampita, C. T cell adhesion primes antigen receptor-induced calcium transients through a rise in adenosine 3',5'-cyclic monophosphate. Immunity 30, 33–43 (2009).

17. Hock, M. et al. NFATc1 induction in peripheral T and B lymphocytes. J. Immunol. 190, 2345–2353 (2013).

18. Meuer, S. C. et al. An alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep erythrocyte receptor protein. Cell 36, 897–906 (1984).

19. Yang, S. Y., Chouss, S. & Cresswell, P. A conserved pathway for T lymphocyte activation involving both the CD3-Ti complex and CD2 sheep erythrocyte receptor determinants. J. Immunol. 137, 1097–1100 (1986).

20. Kato, K. et al. CD48 is a counter-receptor for mouse CD2 and is involved in T cell activation. J. Exp. Med. 176, 1241–1249 (1992).

21. Kaplan, A. J. et al. Production and characterization of soluble and transmembrane murine CD2. Demonstration that CD48 is a ligand for CD2 and that CD48 adhesion is regulated by CD2. J. Immunol. 151, 4022–4032 (1993).

22. Zhu, B., Davies, E. A., van der Merwe, P. A., Calvert, T. & Leckband, D. E. Demonstration that CD48 is a ligand for mouse CD2 and that CD48 adhesion is regulated by CD2. J. Immunol. 151, 4022–4032 (1993).

23. Hahn, W. C., Rosenberg, Y., Burakoff, S. J. & Bierer, B. E. Interaction of CD2 with its ligand lymphocyte function-associated antigen-3 induces adenosine 3',5'-cyclic monophosphate production. Immunity 30, 33–43 (2009).

24. Carrera, A. C., Rincon, M., De Landazuri, M. O. & Lopez-Botet, M. CD2 is involved in regulating cyclic AMP levels in T cells. Eur. J. Immunol. 18, 961–964 (1988).

25. Hwang, W. C., Rosenberg, Y., Burakoff, S. J. & Bierer, B. E. Interaction of CD2 with its ligand lymphocyte function-associated antigen-3 induces adenosine 3',5'-cyclic monophosphate production in T lymphocytes. J. Immunol. 147, 1439–1450 (1993).

26. Carrera, A. C., Rincon, M., De Landazuri, M. O. & Lopez-Botet, M. CD2 is involved in regulating cyclic AMP levels in T cells. Eur. J. Immunol. 18, 961–964 (1988).

27. Rudolph, D. et al. Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein. Proc. Natl Acad. Sci. USA 95, 4481–4486 (1998).

28. Burgart, K. et al. Defective thymocyte proliferation and IL-2 production in transgenic mice expressing a dominant-negative form of CREB. Nature 379, 81–85 (1996).

29. Rudolph, D. et al. Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein. Proc. Natl Acad. Sci. USA 95, 4481–4486 (1998).

30. Huling, T., Tiefenthaler, G., Meyer zum Buschenfelde, K. H. & Meuer, S. C. Alternative pathway activation of T cells by binding of CD2 to its cell-surface ligand. Nature 326, 298–301 (1987).

31. Killeen, N., Stuart, S. G. & Littman, D. R. Development and function of T cells affected with a disruption in CD24. J. Immunol. 148, 4226–4236 (1992).

32. Teh, S. J., Killeen, N., Tarakhovsky, A., Littman, D. R. & Teh, H. S. CD2 regulates the positive selection and function of antigen-specific CD4+ CD8+ T cells. Blood 89, 1318–1318 (1997).
32. Evans, C. F., Rall, G. F., Killeen, N., Littman, D. & Oldstone, M. B. CD2-deficient mice generate virus-specific cytotoxic T lymphocytes upon infection with lymphocytic choriomeningitis virus. J. Immunol. 151, 6239–6264 (1993).

33. Kyewski, B. A. et al. The effects of anti-CD2 antibodies on the differentiation of mouse thymocytes. Eur. J. Immunol. 19, 951–954 (1989).

34. Gonzalez-Cabrero, J. et al. CD48-deficient mice have a pronounced defect in CD4(+) T cell activation. Proc. Natl Acad. Sci. USA 96, 1019–1023 (1999).

35. Paganin, M. & Ferrando, A. Molecular pathogenesis and targeted therapies for NOTCH1-induced T cell acute lymphoblastic leukemia. Blood Rev. 25, 83–90 (2011).

36. Weng, A. & Ferrando, A. A. The role of NOTCH1 signaling in T-ALL. Eur. J. Immunol. 19, 951–954 (1989).

37. Bellavia, D. et al. Combined expression of pTalpha and Notch3 in T cell leukemia identifies the requirement of preTCR for leukemogenesis. Proc. Natl Acad. Sci. USA 99, 3788–3793 (2002).

38. O’Neil, J. et al. Activating Notch1 mutations in mouse models of T-ALL. Blood 107, 781–785 (2006).

39. Talora, C. et al. Cross talk among Notch3, pre-TCR, and Tal1 in T-cell development and leukemogenesis. Blood 107, 3313–3320 (2006).

40. Reizis, B. & Leder, P. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. Genes Dev. 16, 295–300 (2002).

41. Reizis, B. & Leder, P. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. Genes Dev. 16, 295–300 (2002).

42. Afantis, I., Gounari, F., Scorrano, L., Borowski, C. & von Boehmer, H. Constitutive pre-TCR signaling promotes differentiation through Ca2+ mobilization and activation of NF-kappaB and NFAT. Nat. Immunol. 2, 403–409 (2001).

43. Koch, F. et al. Transcription initiation platforms and GTF recruitment at tissue-specific enhancers and promoters. Nat. Struct. Mol. Biol. 18, 956–963 (2011).

44. Capobianco, A. J., Zagouras, P., Blaumueller, C. M., Artavanis-Tsakonas, S. & Bishop, J. M. Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. Mol. Cell. Biol. 17, 6265–6273 (1997).

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Author contributions
S.K.H. generated the Nfatc1-eGfp-Bac-AE2 tg mice and performed all E2 enhancer related studies; R.R. did the methylation analysis and analysed the Nfatc1-eGfp-Bac-AE2 transgenic mice; K.M. performed the ChIP assays; K.P.K. contributed in generating the Nfatc1P2ΔD mice; M.A.M, P.C. and J.C.A. performed the ChIP-Seq and RNA-Seq analysis; A.A. assisted in OP9 and OP9-DL1 co-culture experiments, and establishing the Nfatc1P2ΔD mice line; V.E. generated the ROSA26-caNfatc1-A-StopΔD mice and, C.T. and I.S. contributed with N3 tg mice and Jurkat cell line related experiments. E.S. contributed to several aspects of this study and wrote the manuscript. A.K.P. conceived the project, designed and performed majority experiments and wrote the manuscript.

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