LETTERS

Foxp3-dependent programme of regulatory T-cell differentiation

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Regulatory CD4$^+$ T cells (T_R cells), the development of which is critically dependent on X-linked transcription factor Foxp3 (forkhead box P3), prevent self-destructive immune responses[1]. Despite its important role, molecular and functional features conferred by Foxp3 to T_R precursor cells remain unknown. It has been suggested that Foxp3 expression is required for both survival of T_R precursors as well as their inability to produce interleukin (IL)-2 and independently proliferate after T-cell-receptor engagement, raising the possibility that such 'anergy' and T_R suppressive capacity are intimately linked[2–4]. Here we show, by dissociating Foxp3-dependent features from those induced by the signals preceding and promoting its expression in mice, that the latter signals include several functional and transcriptional hallmarks of T_R cells. Although its function is required for T_R cell suppressor activity, Foxp3 to a large extent amplifies and fixes pre-established molecular features of T_R cells, including anergy and dependence on paracrine IL-2. Furthermore, Foxp3 solidifies T_R cell lineage stability through modification of cell surface and signalling molecules, resulting in adaptation to the signals required to induce and maintain T_R cells. This adaptation includes Foxp3-dependent repression of cyclic nucleotide phosphodiesterase 3B, affecting genes responsible for T_R cell homeostasis.

In males, Foxp3 deficiency results in fatal early-onset systemic autoimmune disease[5]. In heterozygote *Foxp3*$^{wt/null}$ females only one-half of T cells harbours the mutant *Foxp3* allele due to random X-chromosome inactivation, whereas autoimmunity is controlled by a normal T_R population expressing the *Foxp3* wild-type allele. Thus, we were able to genetically mark cells actively transcribing a *Foxp3*^{null} allele, yet lacking Foxp3 protein (hereafter called T_{FN} for *Foxp3*^{null}-expressing T cells), through an in-frame insertion of *GFP* into a stop-codon-disrupted *Foxp3* locus (*Foxp3*^{gfpko}) and investigate their features in mice (Fig. 1a; see also Supplementary Figs 1 and 2a). Female *Foxp3*$^{gfpko/wt}$ mice were healthy, whereas male *Foxp3*^{gfpko} mice developed the same severity of autoimmunity as *Foxp3* knockout (*Foxp3*^{null}) mice[6], resulting in death at ~4 weeks of age. Thymocyte and peripheral lymphoid organ cellularity did not differ between *Foxp3*$^{gfpko/wt}$ and *Foxp3*$^{gfp/gfp}$ mice, nor did the proportion of Foxp3$^+$ T_R cells and Foxp3$^-$ CD4$^+$ T cells (data not shown). As our main focus was to characterize T_{FN} cells in healthy *Foxp3*$^{gfpko/wt}$ mice, analysis of autoimmune male *Foxp3*^{gfpko} mice is included as Supplementary Fig. 2.

T_{FN} cells constituted ~1–3% of mature CD4$^+$ thymocytes and peripheral CD4$^+$ T cells, indicating that Foxp3 is not required to rescue T_R precursors from negative selection (Fig. 1b, c). This is consistent with a reported abundance of T-cell receptors (TCRs) characteristic of T_R cells in *Foxp3*^{null} mice[7]. As ectopic expression of Foxp3 has been shown to induce a state of hyporesponsiveness in CD4$^+$ T cells[3], we expected T_{FN} cells to appear highly activated, similar to CD25$^+$Foxp3$^-$CD4$^+$ T cells (Fig. 1b), which exhibit characteristics of activated, pro-inflammatory T cells[8]. T_{FN} cells, however, were small and CD62L^{high}, more similar to T_R cells and naive CD4$^+$ cells than to CD25$^+$Foxp3$^-$CD4$^+$ T cells (Fig. 1b, d). T_{FN} cells expressed intermediate levels of CD25, CD44, CTLA4, GITR and ICOS in comparison to T_R cells (Fig. 1b, d; see also Supplementary Fig. 3a). Notably, reduced IL-7R expression, which is considered to be a distinguishing feature of T_R cells, was lowest on T_{FN} cells in comparison to the other T-cell subsets (Fig. 1d). In contrast to T_R cells, T_{FN} cells exhibited negligible proliferative activity *in vivo* based on proliferation-associated antigen Ki67 expression (Fig. 1e).

In vitro, both T_{FN} and T_R cells showed an anergic phenotype, which was reversed in both T_{FN} and T_R cells by provision of IL-2; however, proliferation was more readily restored in T_{FN} cells by limited CD28 co-stimulation (Fig. 1f). Thus, anergy in T_{FN} cells is less stable in comparison to T_R cells. T_{FN} cells were also similar to T_R cells—and distinct from T_N (GFP$^-$CD25$^-$CD4$^+$ T cells from *Foxp3*$^{gfp/gfp}$ mice) and CD25$^+$Foxp3$^-$CD4$^+$ T cells—in their inability to generate IL-2 and T-helper 1 and 2 (T_H1 and T_H2) cytokines (Fig. 1g). Thus, IL-2 expression may be blocked transcriptionally during T_R cell differentiation in a Foxp3-independent manner. Lack of IL-2 production and decreased IL-7R make both T_{FN} and T_R cells reliant on exocrine IL-2, but T_{FN} cells are probably less competitive due to intermediate CD25 levels. Indeed, we found that T_{FN} cells were hyper-responsive to elevated IL-2 levels *in vivo* (Supplementary Fig. 7). Together, our analyses show that T_R cell development coincides with Foxp3-independent acquisition of several key T_R cell characteristics.

Unlike T_R cells, some T_{FN} cells produced either IL-10 or IL-17, resembling Tr1 (IL-10-producing Foxp3$^-$CD4$^+$ T cells[9]) and T_H17 cells, respectively (Fig. 1g; see also Supplementary Fig. 2g). Consistent with observed IL-17 production, both T_{FN} and CD25$^+$Foxp3$^-$CD4$^+$ T cells were found to express orphan nuclear receptor RORγt (*Rorc*), which has been shown to both promote IL-17 and block IL-2 production in T_H17 cells[10] (Supplementary Fig. 3b). Notably, *Rorc* transcription was reduced only fourfold in T_R relative to T_{FN} cells but *Il17* transcription was suppressed 7,500-fold, suggesting an ability of Foxp3 to over-ride RORγt activity.

The defining feature of T_R cells is their ability to suppress pro-inflammatory immune function. T_{FN} cells were incapable of suppressing CD4$^+$CD25$^-$ T-cell proliferation *in vitro* (Fig. 2a) and controlling effector T-cell expansion and ensuing splenomegaly and lymphadenopathy after adoptive co-transfer into T-cell-deficient recipient mice (Fig. 2b, c). Spleens and lymph nodes of mice that had received CD45RB^{high}CD25$^-$CD4$^+$ effector T cells with T_{FN} cells were similar in cellularity to those of mice that had received effector T-cells alone, whereas the co-transfer of T_R cells with effector T cells

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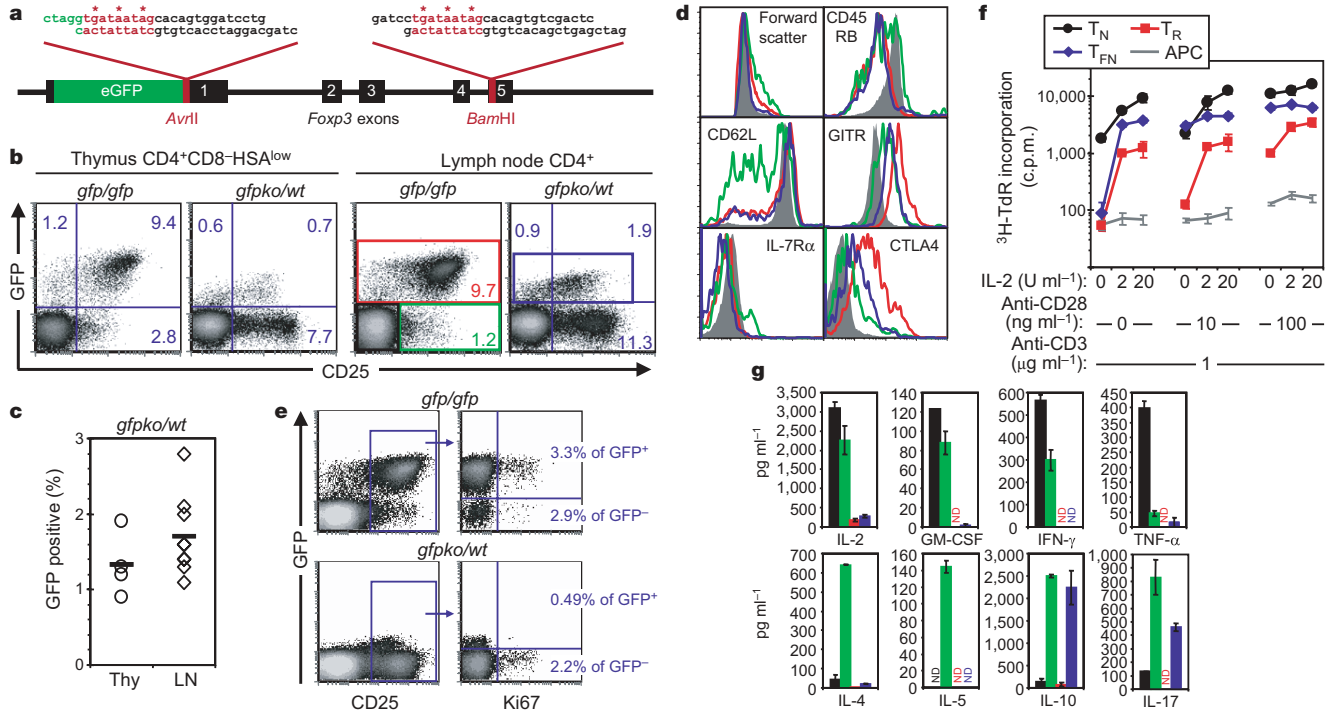

Figure 1 | Phenotype and frequency of cells transcribing the *Foxp3*^{gfpko} locus in *Foxp3*$^{gfpko/wt}$ mice. **a**, We modified our previously described GFP–Foxp3 fusion protein targeting construct[8] by inserting stop codon/frameshift cassettes downstream of GFP and also into *Foxp3* exon 5. In this manner, potentially important regulatory sequences were retained without the possibility that cryptic re-initiation of Foxp3 translation would generate full-length protein. **b**, Flow cytometric analysis of $CD4^+CD8^-HSA^{low}$ thymocytes or $CD4^+$ lymph node cells from female *Foxp3*$^{gfp/gfp}$ or *Foxp3*$^{gfpko/wt}$ mice. **c**, Frequency of GFP^+ cells among either $CD4^+CD8^-HSA^{low}$ thymocytes (Thy, circles) or $CD4^+$ lymph node cells (LN, diamonds) for individual mice. **d**, Flow cytometric analysis of $CD4^+$ lymph node cells for the indicated markers. The four cell subsets are demarcated in **b** as follows: GFP^-CD25^- T_N cells (grey), $GFP^-CD25^+Foxp3^-CD4^+$ T cells (green), GFP^+ T_R cells (red) from *Foxp3*$^{gfp/gfp}$ mice, and GFP^+ T_{FN} cells from *Foxp3*$^{gfpko/wt}$ mice (blue). **e**, Ki67 staining of lymph node cells from the indicated mice. **f**, Proliferation of sorted T cells in response to the indicated reagents. Results are representative of three independent experiments. Data represent the mean and standard deviation for triplicate wells. **g**, Cytokine production by sorted cells from *Foxp3*$^{gfp/gfp}$ (T_N, $CD25^+Foxp3^-CD4^+$, T_R cells) or *Foxp3*$^{gfpko/wt}$ (T_{FN} cells) mice in response to plate-bound anti-CD3 and anti-CD28 assayed by enzyme-linked immunosorbent assay. Results are representative of three separate experiments; data represent mean and maximum/minimum for duplicate wells and colours are as described in **d**.

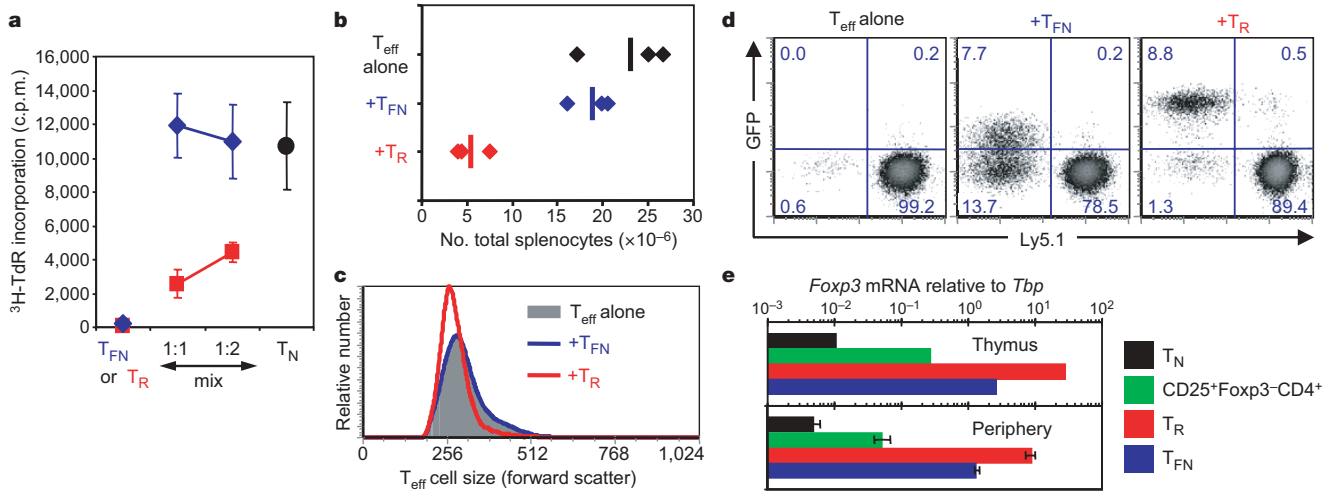

Figure 2 | Foxp3-dependent suppression and lineage stability. **a**, FACS purified T_R or T_{FN} cells were compared for their ability to suppress $CD25^-CD4^+$ T-cell proliferation in the presence of concavalin A and T-cell-depleted splenic antigen presenting cells. Data represent mean and standard deviation for triplicate wells; results are representative of four separate experiments. **b–d**, To examine suppressor activity *in vivo*, T_R or T_{FN} cells were co-transferred with allelically marked effector T cells into lymphopenic animals. Sorted $Ly5.1^+CD45RB^{high}CD25^-CD4^+$ effector T cells (T_{eff}, 3×10^5) were transferred into B6.SCID mice alone or with sorted T_R or T_{FN} cells (10^5). Five weeks after transfer, splenocytes were enumerated (**b**) and lymph node and spleen cells were analysed by flow cytometry. Cell size of the effector T-cell population (**c**) and abundance and GFP expression of the donor T_R or T_{FN} cell populations (**d**) are shown. Data are representative of two independent experiments. **e**, Quantification of *Foxp3* cDNA prepared from the indicated cell populations. For peripheral cells, error bars represent mean and maximum/minimum for biological duplicates.

significantly limited effector T-cell expansion. Similarly, effector T cells were increased in size in the presence of T_{FN} cells, but not T_R cells, likely reflecting increased proliferation (Fig. 2c). Lack of suppression was not due to loss of T_{FN} cells because donor T_{FN} cells competed efficiently with effector T cells during homeostatic expansion, manifested by an increase in their proportion and number (Fig. 2d).

T_{FN} cells also differed from T_R cells in their ability to retain *Foxp3* transcription. Whereas ~90% of donor T_R cells from *Foxp3*$^{gfp/gfp}$ mice retained expression of GFP-tagged Foxp3 protein, two-thirds of the expanded T_{FN} population lost GFP expression (Fig. 2d). Thus, in the course of homeostatic proliferation, *Foxp3* transcription in the absence of Foxp3 function was not efficiently retained. Furthermore, quantification of *Foxp3* messenger RNA in both thymic and peripheral T_{FN} and T_R cells supported the model that Foxp3 promotes its own transcription (Fig. 2e). Although we cannot rule out the possibility that the premature stop codons destabilize *Foxp3*^{gfpko} mRNA, we have found that conditional deletion of *Foxp3* exons 1–5 in mature T_R cells also results in an identical reduction of *Foxp3* mRNA levels[11]. Together, these findings demonstrate that stability of *Foxp3* expression and suppressor activity is dependent upon Foxp3 function rather than coinciding with its expression.

To characterize the Foxp3-dependent transcriptional programme, global gene expression profiling was performed. Automated gene clustering based on similarity in fold-change values between T_N cells and CD25$^+$Foxp3$^-$CD4$^+$ T cells, T_{FN} cells or T_R cells revealed that a distinct set of genes with moderate fold-change values in T_{FN} cells was further amplified in T_R cells. These genes encoded many of the cell surface markers currently used to identify T_R cells, such as CD25, GITR and CTLA4. To incorporate this expression pattern, gene clusters were then hand-curated to generate eight clusters with 'Foxp3-amplified' genes residing in clusters T4 and P4 (Fig. 3a, b; see also Supplementary Fig. 5). For both thymocytes and peripheral cells, Foxp3-dependent genes constituted the largest cluster, reflecting a dominant role for Foxp3 in broadly effecting cellular physiology and phenotype (Fig. 3a, b; clusters T3, P3). Notably, 'Foxp3-dependent' (T3/P3) and 'Foxp3-amplified' genes (T4/P4) most faithfully retained the same expression pattern in thymic and peripheral cells, with Foxp3-amplified genes containing the highest proportion of shared genes (Supplementary Fig. 6a). These findings corroborate our functional studies in elucidating a novel facet of T_R cell differentiation whereby transcriptional and functional characteristics induced concurrently or before *Foxp3* transcription are enforced by Foxp3 to become principal features of T_R cell biology.

To ascribe functional meaning to the defined gene clusters, comparisons with gene annotation databases were performed (Supplementary Fig. 6b, c), revealing that the Foxp3-dependent genes shared between thymic and peripheral data sets were significantly enriched for genes encoding cell surface and extracellular proteins. In contrast, the most enriched functional category for peripheral T_R cells was cell-cycle-associated genes (P6), demonstrating that Foxp3 rescues the inferior proliferative activity of T_{FN} cells in agreement with the analyses of cell size and Ki67 expression (Fig. 1d, e). Also for peripheral cells, the gene cluster specifically regulated by Foxp3 (P3) was enriched for genes involved in intercellular communication.

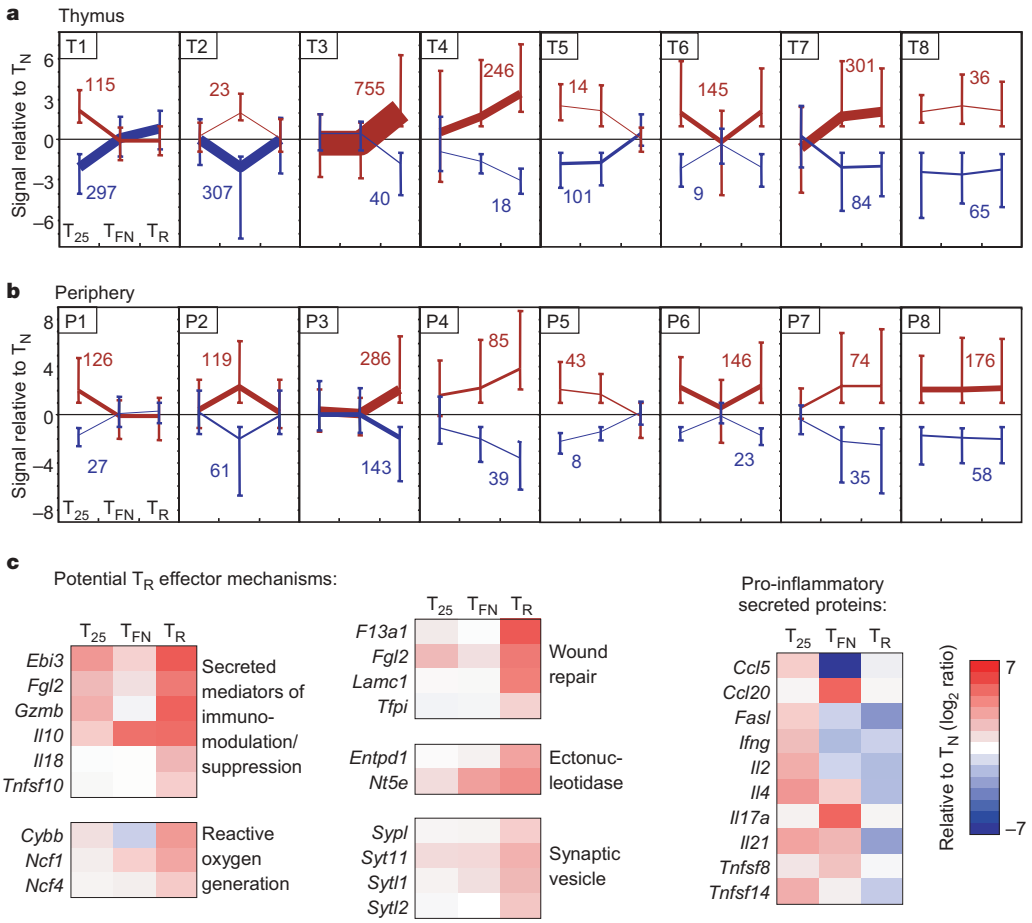

Figure 3 | Foxp3-amplified gene expression constitutes a defining characteristic of thymic and peripheral T_R cells. **a**, **b**, Genes differentially expressed in CD25$^+$Foxp3$^-$CD4$^+$ (T_{25}), T_R and T_{FN} cells relative to T_N cells were identified for both thymic (**a**) and peripheral (**b**) T-cell subsets. For each cluster, values and line thicknesses represent the number of upregulated or downregulated genes, and error bars represent the full range of log_2 expression ratios. **c**, Expression patterns of genes that may have a role, or have been demonstrated to have a role, in T_R cell effector function. Foxp3-mediated repression of genes encoding pro-inflammatory cytokines and chemokines is also shown.

Together, these data suggest that a second major role for Foxp3 is to adapt developing T_R cells by altering how environmental cues are integrated into cellular processes.

Because our experiments showed that suppressor function was Foxp3-dependent, we examined clusters T3 and P3 for genes defining potential T_R effector mechanisms (Fig. 3c). This analysis suggested several such mechanisms, including suppressive soluble factors[12–14], generation of extracellular adenosine[15–18], and release of reactive oxygen[19], and a possible, previously unanticipated role for T_R cells in regulating wound repair.

Among Foxp3-dependent genes, *Pde3b* (cyclic nucleotide phosphodiesterase 3B, cGMP-inhibited) was the most repressed (Fig. 4a, b). As cyclic AMP–protein kinase A pathways are well characterized attenuators of T-cell function[20], reduced PDE3B levels implied cAMP-mediated adaptation in T_R cells to chronic TCR and IL-2 signalling. Notably, naive T cells fail to downregulate PDE3B upon TCR engagement (Fig. 4b), and we found that Foxp3 binds a highly conserved region in the first intron of *Pde3b* (ref. 21). Thus, reduced PDE3B expression represents the first unique marker of T_R cells and may be considered more definitive then Foxp3 itself as it reports Foxp3 function. Hypothesizing that increased expression of PDE3B in T_R cells would be deleterious to T_R cell homeostasis, we introduced PDE3B or a catalytically inactive mutant, PDE3B(H801A)[22], into T_R cells by retroviral gene transfer (Supplementary Fig. 8a, f, g). After transfer into T-cell-deficient recipients, PDE3B-expressing T_R cells were reduced in number by ~60% in comparison to empty vector controls, whereas ectopic PDE3B expression in Foxp3$^-$CD4$^+$ T cells (T_H) had no effect on their homeostasis (Fig. 4c). T_R cells expressing PDE3B(H801A) also exhibited reduced numbers, although to a lesser degree. Loss of PDE3B-transduced T_R cells was due to cell death or inefficient expansion rather than loss of T_R phenotype, because enumeration of expanded donor T_R and T_H cells based on differing Ly5 congenic markers rather than T_R phenotype revealed the same reduction of T_R cell numbers (Fig. 4c).

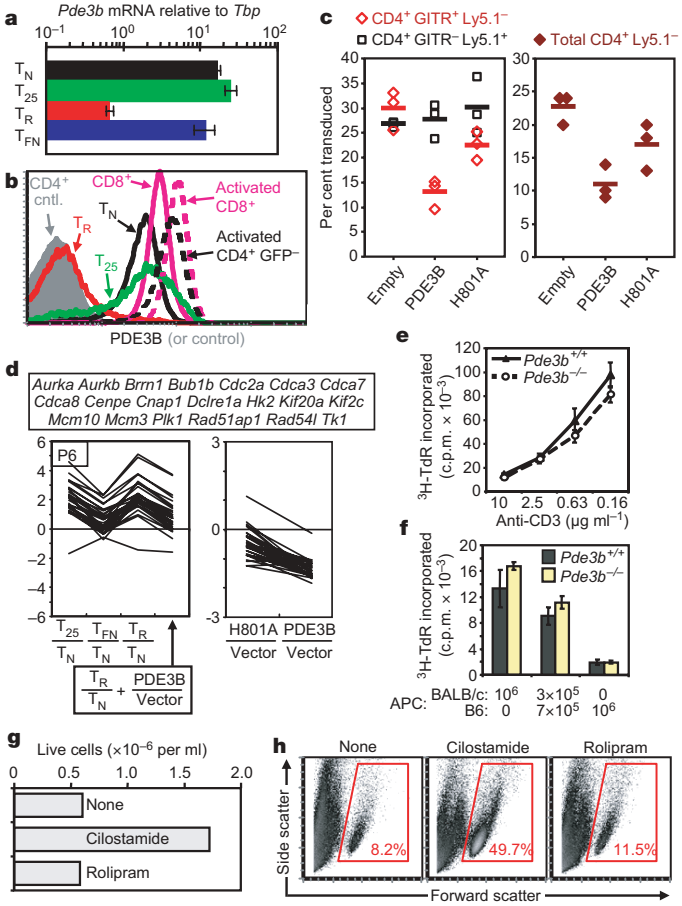

Figure 4 | Reduced PDE3B expression permits normal T_R cell homoeostasis and T_R-specific gene expression. **a**, **b**, *Pde3b* gene expression was analysed by real-time polymerase chain reaction (**a**) and by flow cytometry (**b**). Error bars represent mean and maximum/minimum for biological duplicates. Expression in GFP$^-$CD4$^+$ and CD8$^+$ gated cells after 3 days stimulation of total *Foxp3*$^{gfp/gfp}$ splenocytes with ConA is also shown (dashed lines). T_{25}, CD25$^+$Foxp3$^-$CD4$^+$ T cells. **c**, Frequency in recipient mice of transduced (GFP$^+$) T_R cells (Ly5.1$^-$CD4$^+$GITR$^+$) or T_N cells (Ly5.1$^+$CD4$^+$GITR$^-$), as well as the frequency of transduced Ly5.1$^-$CD4$^+$ cells (see Supplementary Fig. 8a). Results are representative of five separate experiments. **d**, The effect of ectopic PDE3B (PDE3B/Vector) on expression of the listed cell-cycle-associated genes in cluster P6 was added to T_R/T_N values for the same genes (Fig. 3b) and plotted as described (Supplementary Fig. 8c). The effect of PDE3B(H801A) and PDE3B on the same genes is also shown. **e**, **f**, Proliferative responses of *Pde3b*$^{+/+}$ or *Pde3b*$^{-/-}$ CD4$^+$ T cells to anti-CD3 in the presence of irradiated APC (**e**) and to allogeneic (BALB/c) stimulator cells (**f**). Results are representative of two separate experiments; data represent mean and standard deviation for triplicate wells. **g**, **h**, Pre-activated CD4$^+$CD25$^-$ T cells were cultured with IL-2 alone or with the PDE3 inhibitor cilostamide or the PDE4 inhibitor rolipram as described in Supplementary Methods. Viable cell counts (**g**) and percentage live cells (**h**) are shown. Results are representative of three separate experiments.

Transcriptional profiling of recovered cells suggested mechanistic underpinnings for reduced homeostatic fitness of PDE3B-transduced T_R cells, including reduced expression of genes encoding mitochondrial and biosynthetic proteins (Supplementary Fig. 8b). Remarkably, cell cycle genes shared by CD25$^+$Foxp3$^-$CD4$^+$ T cells and T_R cells (cluster P6) reverted to T_{FN}-like expression levels in PDE3B-transduced T_R cells in a manner that was largely dependent on PDE3B catalytic activity (Fig. 4d). This reversion to T_{FN}-like gene expression was also observed for many other Foxp3-dependent genes (Supplementary Fig. 8c). In apparent contrast to the deleterious effect of ectopic PDE3B on T_R cell homeostasis, we found no difference in the proliferative responses of PDE3B-deficient and -sufficient CD4$^+$ T cells[23], and proliferation of wild-type T cells was not altered by PDE3 inhibitors (Fig. 4e, f and data not shown). However, we observed a substantial increase in viability and numbers of pre-activated T_N cells after IL-2-driven expansion in the presence of a PDE3, but not PDE4, inhibitor, suggesting that apoptosis associated with chronic IL-2-induced proliferation may be facilitated by PDE3B activity (Fig. 4g, h). Thus, we have identified *Pde3b* repression as a central component of Foxp3-dependent T_R cell maintenance, as its re-expression resulted in a reduction of biosynthetic processes, the attenuation of Foxp3-dependent proliferative fitness and the loss of some Foxp3-dependent gene expression.

Our study suggests that rather than initiating a *de novo* developmental programme in self-reactive T cells, Foxp3 takes advantage of preceding and coincidental features of T_R precursor cells probably facilitated by TCR signalling. This is accomplished through consolidation of the state of anergy and paracrine IL-2 dependence. Furthermore, Foxp3 amplifies and stabilizes expression of genes encoding cell surface or secreted molecules—like Fgl2, CD73, CD39, TRAIL or CTLA4—normally elaborated by conventional T cells upon TCR stimulation and capable of negative feedback regulation of T-cell activation '*in trans*'. At the same time, Foxp3 enforces repression of TCR-activation-dependent immune response effector cytokines including IL-4, interferon-γ, tumour-necrosis factor-α, IL-17 and IL-21. These observations might explain failure to identify a single non-redundant mechanism of suppression mediated by T_R cells. Finally, Foxp3 alters how T_R cells respond to environmental cues by modulating cell surface and signalling molecules to promote both T_R homeostasis and lineage stability. Essential adaptation to these signals results from Foxp3-dependent downregulation of PDE3B to support normal homeostasis and metabolic function and maintain a part of the T_R cell transcriptional programme. Our results suggest that, in resemblance of evolutionary processes, a lineage commitment factor may act in both an opportunistic and

adaptive fashion during cellular differentiation by amplifying beneficial and correcting disabling features of precursor cells.

METHODS

Mice. B6.SCID, TCR$\alpha^{-/-}$ and TCR$\beta\delta^{-/-}$ mice were obtained from The Jackson Laboratory, and C57BL/6 mice were from the Charles River Breeding Laboratories. All mice were maintained at the University of Washington specific pathogen-free facility. Generation of *Foxp3*^{gfpko} is described in Supplementary Methods.

Flow cytometry and cell sorting. Staining and sorting of cells and intracellular staining for Ki67 and PDE3B are described in Supplementary Methods.

***In vitro* assays.** T-cell stimulation, suppression and cytokine release assays were performed according to conventional methods as described in Supplementary Methods. To examine cell survival in the presence of PDE inhibitors, $CD25^{-}CD4^{+}$ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies. On day 2 cells were removed from antibody-coated plates and on day 4 cells were washed and plated at 2×10^5 cells ml^{-1} with IL-2 (100 U ml^{-1}) or IL-2 plus cilostamide or rolipram (each at 5 mM). On day 7 cells were split 1:4 into the same conditions and on day 13 cells were counted and analysed by flow cytometry.

Gene expression analysis. Gene expression was determined with Affymetrix mouse 430 2.0 microarrays and clusters were generated by Pearson squared *K*-means clustering with MultiExperiment Viewer (TIGR) and by hand, as described (Supplementary Methods).

PDE3B expression. Mixtures of transduced and non-transduced $CD25^{+}CD4^{+}$ cells from B6 mice and $CD25^{-}CD4^{+}$ cells from B6.SJL mice ($Ly5.1^{+}$) were transferred into TCR$\alpha^{-/-}$ recipients. Three weeks after transfer cells were analysed by flow cytometry and isolated by fluorescence-activated cell sorting (FACS) for gene expression analysis (Supplementary Methods and Supplementary Fig. 8).

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

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