

Foxp3 occupancy and regulation of key target genes during T-cell stimulation

Alexander Marson^{1,2*}, Karsten Kretschmer^{6,7*}, Garrett M. Frampton^{1,2}, Elizabeth S. Jacobsen¹, Julia K. Polansky⁶, Kenzie D. MacIsaac³, Stuart S. Levine¹, Ernest Fraenkel^{4,5}, Harald von Boehmer^{6,7} & Richard A. Young^{1,2}

Foxp3⁺CD4⁺CD25⁺ regulatory T (T_{reg}) cells are essential for the prevention of autoimmunity^{1,2}. T_{reg} cells have an attenuated cytokine response to T-cell receptor stimulation, and can suppress the proliferation and effector function of neighbouring T cells^{3,4}. The forkhead transcription factor Foxp3 (forkhead box P3) is selectively expressed in T_{reg} cells, is required for T_{reg} development and function, and is sufficient to induce a T_{reg} phenotype in conventional CD4⁺CD25⁻ T cells⁵⁻⁸. Mutations in *Foxp3* cause severe, multi-organ autoimmunity in both human and mouse⁹⁻¹¹. FOXP3 can cooperate in a DNA-binding complex with NFAT (nuclear factor of activated T cells) to regulate the transcription of several known target genes¹². However, the global set of genes regulated directly by Foxp3 is not known and consequently, how this transcription factor controls the gene expression programme for T_{reg} function is not understood. Here we identify Foxp3 target genes and report that many of these are key modulators of T-cell activation and function. Remarkably, the predominant, although not exclusive, effect of Foxp3 occupancy is to suppress the activation of target genes on T-cell stimulation. Foxp3 suppression of its targets appears to be crucial for the normal function of T_{reg} cells, because overactive variants of some target genes are known to be associated with autoimmune disease.

We developed a strategy to identify genes whose promoters are bound by Foxp3 and whose expression is dependent on that transcription factor (Fig. 1). To generate two cell lines that are genetically matched except for Foxp3, we transduced a Foxp3⁻CD4⁺ murine T-cell hybridoma with FLAG-tagged Foxp3. This approach was favoured over comparison of *ex vivo* cells, which are heterogeneous with regard to activation status. The lines provided sufficient numbers of homogeneous cells with appropriate controls to facilitate both location analysis and expression analysis. FACS (fluorescence-activated cell sorting) analysis confirmed that Foxp3 is expressed in the hybridoma at levels comparable to those in *ex vivo* CD4⁺CD25⁺ T_{reg} cells (Supplementary Fig. S1). Previous work has shown that conventional CD4⁺ T cells ectopically expressing Foxp3 do not upregulate interleukin 2 (Il2) secretion following T-cell receptor (TCR) dependent stimulation¹³. To confirm that the Foxp3⁺ hybridomas contain functional Foxp3, we assayed Foxp3⁻ and Foxp3⁺ cells for Il2 secretion. Indeed, FACS analysis revealed that Il2 secretion is strongly inhibited in phorbol myristate acetate (PMA)/ionomycin stimulated Foxp3⁺ hybridomas compared to stimulated Foxp3⁻ hybridomas (Supplementary Fig. S2).

To identify direct targets of Foxp3, DNA sequences occupied by the transcription factor were identified in a replicate set of experiments using chromatin-immunoprecipitation (ChIP) combined

with DNA microarrays. For this purpose, DNA microarrays were used that contain 60-mer oligonucleotide probes covering the region from -8 kilobases (kb) to +2 kb relative to the transcript start sites for approximately 16,000 annotated mouse genes¹⁴. The sites occupied by Foxp3 were identified as peaks of ChIP-enriched DNA that span closely neighbouring probes (Fig. 2). Foxp3 was found to occupy the promoters of 1,119 genes in PMA/ionomycin stimulated hybridomas (Supplementary Tables S1 and S2). The well-characterized Foxp3 target gene^{12,15}, *Il2*, was among the genes occupied by Foxp3 (Fig. 2a). Most of the promoters occupied by Foxp3 in stimulated T cells were also occupied in unstimulated cells (Supplementary Tables S1 and S2, and Supplementary Fig. S3). However, at some promoters Foxp3 binding increased considerably in cells stimulated with PMA/ionomycin (Fig. 2a and Supplementary Fig. S3). Control immunoprecipitation experiments in Foxp3⁻ cells, which produced few positive signals, confirmed the specificity of these results (Supplementary Fig. S4). Our confidence in the binding data was further strengthened by the discovery of a DNA sequence motif, which matches the consensus forkhead motif, at the genomic loci that were bound by Foxp3 (Fig. 2b and Supplementary Table S5). This motif distinguishes Foxp3 bound regions from unbound regions tiled on the promoter arrays with a high level of confidence ($P < 10^{-41}$). Instances of this motif are significantly more likely to be conserved in Foxp3 bound regions than in

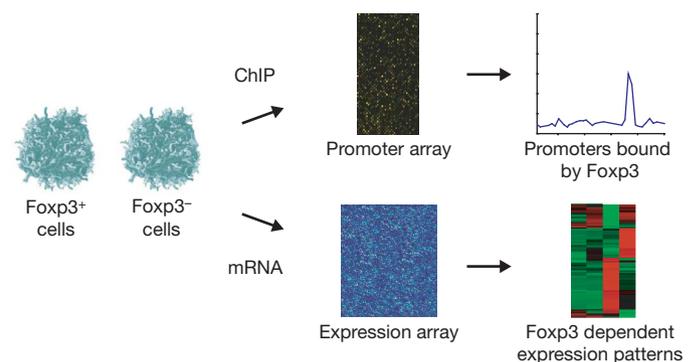


Figure 1 | Strategy to identify direct Foxp3 transcriptional effects. Genetically matched Foxp3⁺ and Foxp3⁻ cell populations were generated by transduction of FLAG-tagged Foxp3 into a Foxp3⁻ murine T-cell hybridoma. Foxp3 binding sites at promoters across the genome were identified by ChIP experiments with an anti-FLAG antibody. Foxp3 dependent transcriptional regulation was identified by gene expression profiling performed on each of these cell types.

¹Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA. ²Department of Biology, ³Department of Electrical Engineering and Computer Science, ⁴Biological Engineering Division, ⁵Computer Science and Artificial Intelligence Laboratory, Massachusetts Institute of Technology (MIT), Cambridge, Massachusetts 02139, USA. ⁶Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, USA. ⁷Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, USA.

*These authors contributed equally to this work

the genome scale experiments. Notably, in these experiments (Fig. 3d) all genes that were activated following stimulation in Foxp3^- cells and repressed in Foxp3^+ cells were activated in a calcineurin dependent manner, consistent with the notion that Nfat is involved in their activation. Finally, just as Foxp3 regulates the protein levels of secreted IL2 (Supplementary Fig. S2), cell surface

staining and FACS analysis show that Foxp3 regulates the level of Ly6a protein, demonstrating that Foxp3 transcriptional regulation of its targets modulates protein levels (Supplementary Fig. S6).

Taken together, the results from *ex vivo* T cells and the hybridoma system identify a core set of Foxp3 regulatory targets (Fig. 4a), most of which showed suppressed activation in stimulated Foxp3^+ cells. A

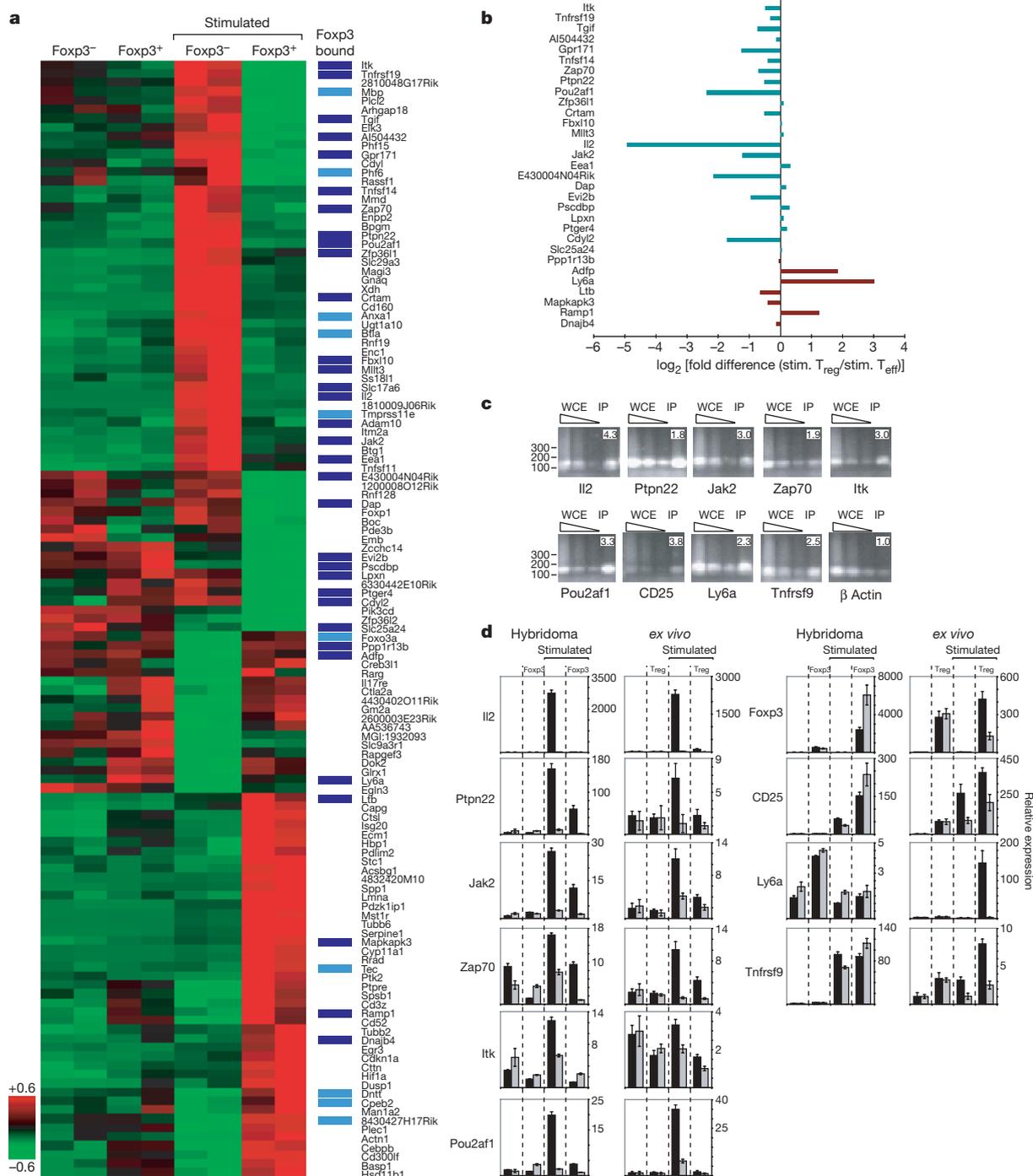


Figure 3 | Foxp3 directly suppresses the activation of target genes.

a, Replicate expression data for the 125 genes with Foxp3 dependent differential expression in stimulated hybridomas (false discovery rate, FDR < 0.05) were hierarchically clustered and displayed. The Z-score normalized induction (red) or repression (green) is shown for each gene. Direct targets of Foxp3 in stimulated hybridomas are indicated (dark blue for FDR < 0.05, light blue for FDR < 0.10). **b**, For repressed (green) and induced (red) Foxp3-bound targets in **a**, \log_2 [fold difference] in expression between stimulated *ex vivo* effector (T_{eff}) T cells and T_{reg} cells is displayed. *Slc17a6* and *Adam10* are not expressed in the *ex vivo* samples. **c**, Site-specific

PCR on 10 ng of ChIP DNA confirmed selected targets. Immunoprecipitated (IP) DNA was compared to serial dilutions (90, 30 and 10 ng of DNA) of unenriched whole cell extract (WCE) DNA. Enrichment ratios, shown at top right of each sub-panel, are normalized relative to the unenriched β actin control. DNA fragment size (bp) is indicated on the left of each row. **d**, The transcript levels of the panel of Foxp3 targets presented in **c** and of *Foxp3* were analysed by real-time RT-PCR in stimulated and unstimulated cells, with (grey) and without (black) cyclosporin A. Mean values \pm s.d. of relative expression, determined in triplicate, are shown for indicated genes.

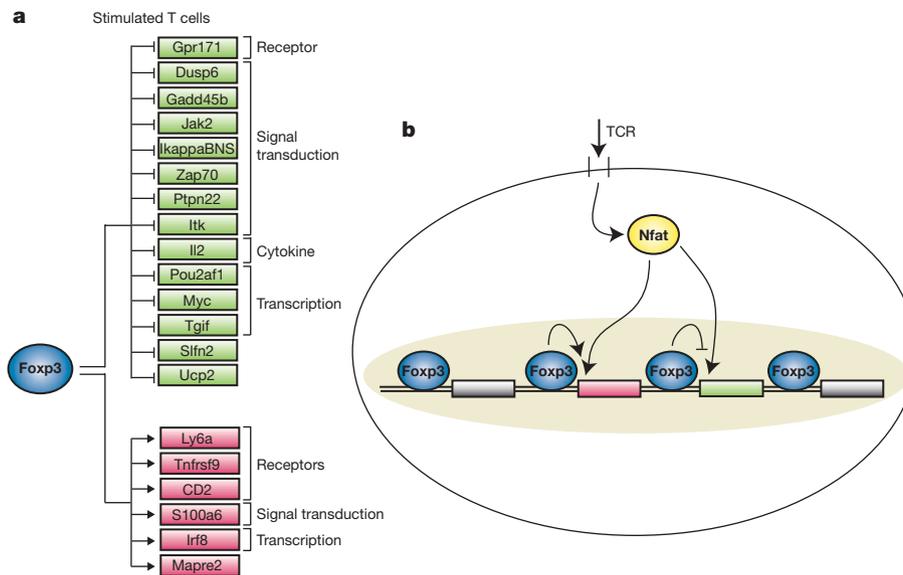


Figure 4 | Core direct regulatory effects of Foxp3. **a**, Shown here are a subset of direct Foxp3 targets that exhibit consistent transcriptional behaviour in hybridomas and in *ex vivo* T cells (Supplementary Fig. S5). **b**, Foxp3 binds to a large set of promoters both in unstimulated and stimulated T cells, but Foxp3 transcriptional regulation is more

smaller number of Foxp3 target genes was upregulated in stimulated Foxp3⁺ cells, including some encoding cell surface molecules with known roles in immunoregulation, such as Ly6a (ref. 25) and Tnfrsf9 (4-1BB) (ref. 26). The results from the hybridoma system indicate that Foxp3 occupies regions of most of its target promoters in both unstimulated and stimulated conditions, but increased binding at some promoters and regulation of most targets is observed after stimulation. Furthermore, in hybridomas the major function of Foxp3 at these genes is to suppress the level of gene activation that would occur if this transcription factor were not expressed (Fig. 4b). Conceivably, the Foxp3 dependent downregulation of T-cell activation and cytokine genes, and upregulation of immunosuppressive cell surface molecules, contribute to both the hypo-responsive and suppressive T_{reg} phenotype.

Mutations in some Foxp3 target genes are already known to be associated with autoimmune disease. The protein tyrosine phosphatase Ptpn22 is a notable example. In our experiments, *Ptpn22* is one of the highest confidence direct targets of Foxp3, it is upregulated on stimulation in Foxp3⁻ cells, and this upregulation is inhibited in Foxp3⁺ hybridomas (Fig. 3a) and *ex vivo* T_{reg} cells (Fig. 3b, d). Ptpn22 modulates the signal cascade downstream of the TCR, and mutations in the human *PTPN22* have been associated with type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus and Graves' disease, as well as other autoimmune diseases^{27–29}. A recent report suggests that one *PTPN22* single-nucleotide polymorphism associated with autoimmunity is a gain-of-function mutation³⁰. Our findings are compatible with the hypothesis that the gain-of-function mutation might be pathogenic if mutant *PTPN22* is overactive in T_{reg} cells³⁰.

In summary, our data indicate that Foxp3 binds to the promoters of well-characterized regulators of T-cell activation and function. In the T-cell hybridomas studied here, the major role of this transcription factor is to dampen the induction of key genes when T_{reg} cells are stimulated. In *ex vivo* T_{reg} cells, Foxp3 could also activate the expression of a greater number of genes, perhaps owing to the greater abundance of certain transcriptional cofactors in these cells. Some of the identified Foxp3 target genes have been previously implicated in autoimmune diseases, implying that a therapeutic strategy to recapitulate the function of this transcription factor may have clinical utility for these diseases.

extensive in stimulated T cells. The genomic regions where Foxp3 binds are enriched for an Nfat binding site DNA motif. In the hybridomas, Foxp3 predominantly acts to directly suppress the activation of its target genes.

METHODS

A detailed description of all materials and methods used can be found in Supplementary Information.

Growth of murine CD4⁺ T-cell hybridomas and *ex vivo* T cells. CD4⁺ 5B6-2 hybridoma cells expressing a PLP_{139–151}-specific TCR, which was kindly provided by V. Kuchroo, were cultured in Dulbecco's modified Eagle medium (Invitrogen). Primary murine CD4⁺ T cells were cultured in RPMI-1640 medium (Invitrogen). For gene expression profiling, real-time RT-PCR, and location analysis, cells were cultured in the absence or presence of 50 ng ml⁻¹ phorbol 12-myristate 13-acetate (PMA) and 200 ng ml⁻¹ ionomycin at 37 °C and harvested after 6 h. Where indicated, cells were preincubated for 1 h with 2 μM cyclosporin A. Details of cell generation and isolation are provided in Supplementary Information.

Antibodies and ChIP assays. Detailed descriptions of antibodies, antibody specificity and ChIP methods used in this study are provided in Supplementary Information.

Promoter array design and data extraction. The design of the oligonucleotide-based promoter array set and data extraction methods are described in Supplementary Information. The microarrays used for location analysis in this study were manufactured by Agilent Technologies (<http://www.agilent.com>).

Motif analysis. Discovery of the Foxp3 sequence motif from the ChIP-chip binding data was performed using the THEME algorithm. The Foxp3 motif learned by THEME and the Nfat motif from the TRANSFAC database (version 8.3) were used to scan all arrayed sequences to identify matches to the motifs.

Functional classification of bound genes. Comparison of Foxp3 target genes to annotated KEGG biological pathways was performed using the online DAVID tool (<http://niaid.abcc.ncifcrf.gov/>).

Gene expression profiling. For each hybridoma culture condition, total RNA was prepared from 1 × 10⁷ cells using Trizol (Gibco) followed by additional purification using the RNeasy Mini Kit (Qiagen). Biotinylated antisense cRNA was then prepared according to the Affymetrix standard labelling protocol (one amplification round). For each primary T-cell culture condition, total RNA was isolated from 5 × 10⁵ cells with RNeasy. Biotinylated antisense cRNA was prepared by two rounds of *in vitro* amplification using the BioArray RNA Amplification and Labelling System (Enzo Life Sciences) according to the protocol for 10–1,000 ng of input RNA provided by the manufacturer. Biotinylated cRNAs of hybridomas and primary T cells were fragmented and hybridized to Affymetrix GeneChip Mouse Expression Set 430 2.0 arrays at the Microarray Core Facility (Dana-Farber Cancer Institute).

Quantitative RT-PCR. To determine transcript levels in T-cell hybridomas and *ex vivo* T cells, RNA was isolated, reverse-transcribed and subjected to real-time PCR performed on an ABI PRISM thermal cycler using SYBR Green PCR core reagents (Applied Biosystems). Detailed information is provided in Supplementary Information.

Received 13 October; accepted 27 November 2006.

Published online 21 January 2007.

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

Acknowledgements We thank members of the Young, von Boehmer and Fraenkel laboratories, as well as R. Jaenisch and D. K. Gifford, for discussions and critical review of the manuscript, especially T. I. Lee, J. Zeitlinger and D. T. Odom. We also thank Biology and Research Computing (BaRC), especially T. Dicesare for graphic assistance, as well as E. Herbolzheimer for computational and technical support. K.K. was supported in part by a fellowship grant from the German Research Foundation. This work was supported in part by a donation from E. Radutzky, and by the Whitaker Foundation (E.F.) and the NIH (H.v.B. and R.A.Y.).

Author Contributions R.A.Y. and H.v.B. contributed both as senior and corresponding authors.

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