Different molecular complexes that mediate transcriptional induction and repression by FoxP3

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FoxP3 conditions the transcriptional signature and functional facets of regulatory T cells (Treg cells). Its mechanism of action, whether as an activator or a repressor, has remained unclear. Here, chromatin analysis showed that FoxP3 bound active enhancer elements, not repressed chromatin, around loci over- or under-expressed in Treg cells. We evaluated the impact of a panel of FoxP3 mutants on its transcriptional activity and interactions with DNA, transcriptional cofactors and chromatin. Computational integration, confirmed by biochemical interaction and size analyses, showed that FoxP3 existed in distinct multimolecular complexes. It was active and primarily an activator when complexed with the transcriptional factors RELA, IKZF2 and KAT5. In contrast, FoxP3 was inactive when complexed with the histone methyltransferase EZH2 and transcription factors Y1 and IKZF3. The latter complex partitioned to a peripheral region of the nucleus, as shown by super-resolution microscopy. Thus, FoxP3 acts in multimodal fashion to directly activate or repress transcription, in a context- and partner-dependent manner, to govern Treg cell phenotypes.

Regulatory T cells (Treg cells) are of central importance in immunological tolerance and in the control of inflammatory processes. FoxP3, a transcription factor (TF) of the Forkhead family, is expressed specifically in Treg cells, is essential for their differentiation and function and is the defining factor of the lineage. Loss of FoxP3 function leads to Treg cell deficiency and to devastating multi-organ inflammation in scurfy mice and in human patients with the X-linked immunodeficiency syndrome IPEX.

Treg cells share a core transcriptional signature of genes that are over- or under-expressed in Treg cells versus their conventional CD4+ T cell counterparts (Tconv cells). This Treg signature encodes molecules that mediate Treg cell suppressor activity (such as IL-10, CTLA-4), but also includes transcripts typically induced (or repressed) upon T cell activation, in keeping with the self-reactive nature of the T cell antigen receptor in many Treg cells. Much of this signature is controlled by FoxP3. An important aspect of FoxP3’s function in maintaining Treg cell identity is the suppression of effector cytokines (such as IL-2, IL-4 or IL-17) that are produced by activated Tconv cells. Beyond that shared signature, Treg cell transcriptomes are further modified and adapted to their location and function. For instance, transcripts controlled by the nuclear receptor PPARγ promote metabolic adaptation to adipose tissue and are uniquely found in the Treg cells that reside there.

FoxP3 contains several structural modules: a short zinc finger of unknown function, a leucine-zipper domain for homo- or heterodimerization, and a C-terminal Forkhead domain (FKH), which is the primary DNA-binding domain but also interfaces with transcriptional co-regulators. The structure of the FKH domain is known, but the N-terminal region has characteristics of an Intrinsically Disordered Protein and has resisted structural determination. FoxP3 interacts physically with many other TFs, such as RUNX1, NFAT, Eos (IKZF4), IRF4, RORγ, RORα, HIF1α, STAT3, TCF1 and EZH2, and mass spectrometry analysis has further identified a large set of proteins that interact with FoxP3 within multiprotein complexes. Some of the interactions with these cofactors have been mapped to various regions of the FoxP3 protein, in the FKH or N-terminal domain, and several ‘tune’ Treg cell activity, modulating their ability to suppress particular T cell phenotypes or autoimmune diseases.

How these structural aspects are integrated, and how FoxP3 regulates its target genes, is incompletely understood. FoxP3’s transcriptional effects are thought to reflect sequence-specific binding to enhancers that affect its target genes. Accordingly, FoxP3 is detected on a large number of sites in the genome, some in close proximity to Treg signature genes, although the majority of FoxP3-binding sites are nowhere near any relevant genes, perhaps reflecting long-range interactions or a structural role in nuclear organization of the DNA. FoxP3-binding sites tend to be active enhancer elements but are not exclusive to FoxP3 and, in its absence, are occupied by other factors. Whether FoxP3 is a repressor or an activator, or both, has been interpreted diversely over time. FoxP3 was initially considered to be a repressor, in part because attention focused mainly on IL2 as a target, but broader analysis of the Treg signature and its effects on the chromatin of target genes led to a perspective of FoxP3 as a dual activator and repressor. More recently, a unifying model of FoxP3 action was proposed in which FoxP3 binds to enhancer elements, not repressed chromatin, around loci over- or under-expressed in Treg cells.
elements that are generally open in CD4+ T cells, recruiting EZH2 and the PRC2 repressor complex31. In this model, FoxP3 contributes to the upregulation of Treg signature genes only indirectly (by repressing repressors). However, it can be argued that this model is not easily compatible with transcriptional correlates of natural or engineered FoxP3 variants8,25.

FoxP3’s mechanism of action is clearly a key question to be resolved. We thus sought to determine how an array of sequence alterations spaced through the FoxP3 protein affected its binding to a set of cofactors, and how this impacted FoxP3’s transcriptional properties. Our results clearly identify two modes of operation for FoxP3 that correspond to distinct multimolecular complexes that segregate differentially within the nucleus.

RESULTS

FoxP3 binds active enhancers at both Treg-up and Treg-down loci

To begin to elucidate the mode of action of FoxP3 in specifying different components of the Treg signature, we re-analyzed several published chromatin immunoprecipitation (ChIP-seq) data sets that define the position of FoxP3 and chromatin marks of enhancer activity or repression in the mouse genome32,33. We defined ‘high-confidence’ FoxP3-binding sites in the genome as those replicated in two independent FoxP3 ChIP-seq data sets32,33, selected 5,000 sites with the highest signals and replicated in both studies, and parsed their distribution around loci encoding transcripts over- or under-expressed in Treg cells relative to Tconv cells (called ‘Treg-up’ and ‘Treg-down’, respectively, encompassing the 200 transcripts with most extreme differences). In keeping with previous conclusions24, FoxP3 was equally present in the vicinity of both Treg-up and Treg-down signature genes, predominantly within 50 kb of their transcriptional start site, evoking a direct action of FoxP3 (Fig. 1a; FoxP3 also bound some Treg-neutral loci (equally expressed in Treg and Tconv cells), albeit at significantly lower frequency). To determine which type of regulatory element bound FoxP3 in the vicinity of these signature genes, we evaluated the histone marks at these FoxP3 ChIP-seq peak regions in both Treg and Tconv cells. As expected, FoxP3-binding sites in the vicinity of Treg-up signature genes carried marks of active enhancers (H3K27ac and H3K4me1) but not a mark associated with repressed chromatin (H3K27me3) (Fig. 1b). Those H3K27ac signals were also present in Tconv cells but were greater in abundance in Treg cells than in Tconv cells (Fig. 1b), consistent with the conclusion that FoxP3 activates pre-existing enhancers26. Genome-wide, there was a positive correlation between binding of FoxP3 and H3K27ac signals, but a negative correlation to H3K27me3 (Fig. 1c). All these observations were compatible with the conclusion that FoxP3 increased enhancer activity (or the frequency of cells in which the enhancers were active) around Treg-up genes. Less expected, however, was the finding that FoxP3-binding sites around Treg-down signature loci also showed characteristics of active enhancers in both Treg cells and Tconv cells, with no detectable increase in H3K27me3 signals that might be expected from repression. Thus, FoxP3 generally appeared to bind to active enhancer elements.

We then investigated the mechanism of FoxP3’s action using retroviral transduction of FoxP3 into CD4+ Tconv cells, a good setting in which to assess the direct effects of a TF without the adaptations or redundancies that can occur in established cells in vivo. This approach has been used to assess FoxP3 in several studies and has been found to confer some, but not all, of the transcriptional and functional aspects of Treg cells6,7,34. We purified CD4+ Tconv cells from a FoxP3−/− mouse, activated them polyclonally, and transduced them with FoxP3. We then sorted the cells 72 h later, in a fixed window of expression of the Thy1.1 reporter encoded in the retroviral vector, pre-calibrated to ensure expression of FoxP3 equivalent to that of ex vivo Treg cells (thus avoiding overexpression artifacts by excluding cells with gross overexpression of FoxP3). Gene expression was profiled in these sorted cells by NanoString technology, with a custom code set (transcripts typical of the Treg signature and of tissue Treg cells, and some encoding major TFs and effector molecules of activated Tconv cells; Supplementary Table 1). We observed robust induction or repression of two gene sets relative to their expression in control cells transduced with empty vector (Fig. 1d). The induced transcripts substantially overlapped the set of genes overexpressed in the classic Treg signature (Fig. 1d). The overlap was not complete (Fig. 1d), as expected, because a sizeable segment of the Treg signature is independent of FoxP3 (refs. 6,7). Most of the induced transcripts corresponded to genes with enhancers more active in ex vivo Treg cells than in Tconv cells, as reflected by H3K27ac marks9 (Fig. 1e). In a reproduction of the paradox noted above, ~20% of the FoxP3-repressed transcripts also corresponded to Treg cell–specific enhancers (Fig. 1e). We also verified the relevance of FoxP3 transduction by ChIP-seq, immunoprecipitating the chromatin of cells transduced with 6x-HIS-tagged FoxP3. Distinct peaks were observed in cells transduced to express FoxP3 that were absent from the cells transduced with empty vector (Fig. 1f,g), which corresponded well to previously mapped FoxP3 binding in Treg cells26. A large proportion of the genes induced or repressed by FoxP3 transduction bound FoxP3 in transduced cells (88% or 89%, respectively; Fig. 1h). Thus, whether in ex vivo Treg cells or in transduced CD4+ T cells in which its action was more likely to be direct, FoxP3 seemed to activate a sizeable fraction of the Treg-up signature by binding to and increasing the activity of specific enhancers.

Determinants of FoxP3’s transcriptional functions

To gain further mechanistic insight on FoxP3’s interaction with transcriptional cofactors for the orchestration of transcriptional activation or repression, we engineered a set of 14 alanine-replacement mutants of FoxP3 that spanned various domains (Fig. 2a and Supplementary Table 2) and transduced them retrovirally as above. Flow cytometry of cells transduced to express those mutants, with standardization of FoxP3’s staining intensity to that of the co-transcribed Thy1.1 reporter, showed that all mutants had expression similar to that of wild-type FoxP3 (Supplementary Fig. 1a and Supplementary Table 3). Immunoblot analysis of extracts from transduced cells showed that all mutant proteins were full length (Supplementary Fig. 1b). Proper localization of the mutants to the nucleus was confirmed by immunofluorescence microscopy (Supplementary Fig. 1c). We then assessed how the alterations affected DNA binding, in a solution capture assay with biotinylated oligonucleotide with a dimer of the canonical 5’-AAACA motif (Supplementary Fig. 1d). All mutants in the set were able to bind DNA, albeit with a partial reduction in binding for a few mutants (Supplementary Fig. 1e).

We assessed how the substitutions impacted FoxP3’s ability to affect its transcriptional targets in CD4+ T cells with the retroviral transduction and signature profiling system. We compiled the results of two independent transductions for each mutant and compared the results with those of control cells transduced to express wild-type FoxP3 or empty vector (Fig. 2b and Supplementary Table 4). A range of results were obtained, with some mutants yielding a profile similar to that of wild-type FoxP3 and others showing severely affected activation and repression potential (Fig. 2b and Supplementary Table 4). Duplicates from independent experiments showed highly similar outcomes, as indicated by principal-component analysis (Fig. 2c). There was no segregation according to the position of the substitutions in the protein, although the most extreme effects were seen with substitutions in

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Figure 1 FoxP3 binds to active enhancers around both T\textsubscript{reg}-up loci and T\textsubscript{reg}-down loci. (a) FoxP3-binding peaks (replicated in independent published ChIP-seq data sets\cite{16,32}) in the vicinity of T\textsubscript{reg}-up or T\textsubscript{reg}-down genes or randomly selected neutral genes (left margin), plotted against distance from the transcription start site (TSS). (b) Density of histone modifications and of FoxP3 (key) in a 20-kb window around FoxP3-binding sites in chromatin of T\textsubscript{reg}-up or T\textsubscript{reg}-down genes in T\textsubscript{reg} cells and T\textsubscript{conv} cells. (c) Correlation of ChIP-seq signal intensity for FoxP3 versus several histone marks (vertical axis) in the vicinity of T\textsubscript{reg}-up or T\textsubscript{reg}-down genes (all in reads per million (rpm)); numbers in plots indicate Pearson correlation \(r\) and \(P\) value. (d) FoldChange in expression (NanoString quantification) of a selected gene set in activated CD4\(^+\) T cells at 48 h after transduction of retroviral vector encoding FoxP3 versus empty vector (horizontal axis), plotted against the significance of the difference in expression (\(t\)-test); transcripts of the classic T\textsubscript{reg} targets are highlighted in red or blue, respectively. (e) H3K27ac intensity (from published genome-wide data\cite{31}) at enhancers within 50 kb of FoxP3-induced genes (red) or FoxP3-repressed genes (blue) (as defined by FoxP3 transduction in d), presented as the ratio of the intensity in T\textsubscript{reg} cells to that in T\textsubscript{conv} cells, in resting cells (horizontal axis) plotted against that in activated cells (vertical axis). (f) Binding of FoxP3 around IL2ra and Pde3b in CD4\(^+\) T cells transduced with empty vector (EV) or FoxP3, with traces from ex vivo T\textsubscript{reg} cells\cite{31} for reference (bottom lines). (g) Genome-wide binding of FoxP3 in FoxP3-transduced CD4\(^+\) T cells plotted against that in ex vivo T\textsubscript{reg} cells\cite{31}. (h) Expression of transcripts in cells transduced with FoxP3 or empty vector (same plot as in d); green highlights denote genes that bind FoxP3 (by ChIP-seq analysis of transduced cells in g). Data are from published results (a,b,c,e) and/or are the average of two experiments (a,c–g) or four experiments (d,h).
Figure 2 Transcriptional activity of the FoxP3 mutants. (a) Position of the alanine-replacement mutants of FoxP3 (details, Supplementary Table 2), positioned relative to FoxP3’s zinc-finger (ZF), leucine-zipper (LZ) and FKH domains. Numbers above indicate amino acid position. (b) Transcription (NanoString analysis) of the selected gene set (as Fig. 1d) in activated CD4+ T cells transduced with retrovirus encoding wild-type (WT; green) or mutant FoxP3 or with empty vector (EV; yellow) and sorted at 72 h (matching expression of FoxP3 based on co-linear Thy1.1); results are presented as a ratio versus the mean value obtained for cells transduced with empty vector. Above plot, DNA-binding efficiency of the mutant or wild-type FoxP3 (as a ratio versus the mean value obtained for cells transduced with empty vector). Above plot, DNA-binding efficiency of the mutant or wild-type FoxP3 (as a ratio versus the mean value obtained for cells transduced with empty vector). (c) Principal-component analysis of the data in b; results are presented as a ratio versus the mean value obtained for cells transduced with empty vector. Above plot, DNA-binding efficiency of the mutant or wild-type FoxP3 (as a ratio versus the mean value obtained for cells transduced with empty vector). (d) General activation index for each mutant (average of the change in expression versus EV for all FoxP3-induced transcripts plotted against general repression index of each mutant (average of the change in expression versus EV for all FoxP3-repressed transcripts). (e) Suppressive activity of CD4+ T cells transduced with wild-type or mutant FoxP3 (top left corners), assessed by inhibition of the division of CFSE-labeled T cells supplemented with cells transduced with empty vector (black) or FoxP3 (green). (f) Suppression index from e plotted against the activation index from d; numbers in plots indicate Pearson correlation r and P value. Data are from two (all mutants) or four (EV and WT) experiments (b) or three experiments (e).
the FKH domain also found in some IPEX mutants (M371 and M338) (Fig. 2b). Although a general gradient of FoxP3 activity was observed, there was also a diversity of response patterns for individual targets (for example, Nrn1 compared with Gpr38). Not all targets were equally affected by the panel of mutants, including some unique effects (the ability of M354 to repress Zscan29, which was instead induced by M7) (Fig. 2b). There were some paradoxical effects, such as the induction by M342 and M338 of Il4 and Il5, which are normally repressed by wild-type FoxP3. Even the most severe mutants maintained some activity, such as the ability to repress Gama or Eomes (Fig. 2b).

To compare the overall activating versus repressive activities of each mutant, we computed global activation and repression indices, averaging overall effects on induced or repressed targets, respectively. These proved to be highly correlated (Fig. 2d), which indicated that the trans-activation activities of FoxP3 and its trans-repression activities were generally governed by the same mechanisms, reminiscent of its binding to enhancer elements in the vicinity of both T_r++ up signature genes and T_re- down signature genes.

To search for functional correlates of the transcriptional effects reported above, we assessed the ability of cells transduced to express wild-type or mutant FoxP3 to inhibit the proliferation of activated T_conv cells34. Suppressive activity was observed after transduction of wild-type FoxP3 and of several mutants, but that was lost after transduction of M342 or M371 (Fig. 2e). The averaged suppression activity correlated well with the transcriptional activation index of the mutants (Fig. 2f); when assessed against the transactivation of specific FoxP3 targets, suppressive activity correlated strongly with the transactivation of genes encoding several effector molecules (such as Lrcc32 and Il2ra) but not with the transactivation of non-canonical FoxP3 targets (such as Il1rl1, Rorc and Il4) (Supplementary Fig. 1f). These results indicated a great degree of variegation in the mechanism through which FoxP3 activated or repressed its targets.

Mechanistic underpinning of transcriptional differences

One mechanistic interpretation of the variable effect of the FoxP3 mutants on its targets was that each substitution altered the range of enhancer elements that FoxP3 bound across the genome. To test this hypothesis, we selected three mutants with a range of transcriptional effects (one mild: M176; two harsher: M354 and M390) and performed ChIP-seq analysis of FoxP3 in transduced CD4+ T cells. No clear relationship to transcriptional effects was found: the mild mutant M176 bound chromatin much like wild-type FoxP3 did, as did the more severe mutant M390. But binding of M354 was clearly perturbed (with an average ratio of binding intensity, relative to that of wild-type FoxP3, of 0.54 across all FoxP3 binding sites; range 0.19–1.18 for individual peaks; Fig. 3a,b). We then correlated the ability of the mutants to bind naked DNA at the canonical FKRE dimer (values from Supplementary Fig. 1e) versus their ‘transcriptional output’ (activation and repression indices from Fig. 2d). Only limited correlation for either activation (r = 0.37, P = 0.16) or repression (r = 0.31, P = 0.27) was observed; several of the mutants with high DNA-binding potential had low activation or repression potential and vice versa (Fig. 3c). Together these data suggested that binding of DNA or chromatin by FoxP3 was important but was not
a primary determining factor in modulating its ability to activate or repress its target genes.

Given the lack of correlation between FoxP3 binding and transcriptional output, we hypothesized that altered transcriptional activity of the FoxP3 mutants might result from altered interactions with transcriptional cofactors. We thus tested the panel of FoxP3 mutants in co-immunoprecipitation assays together with 17 known FoxP3 cofactors (Supplementary Table 5). HEK293 human embryonic kidney cells were transfected to express FLAG-tagged FoxP3 together with each cofactor (also tagged), and proteins were immunoprecipitated from nuclear extracts with anti-FLAG (FoxP3). In agreement with the literature, the cofactors were specifically and efficiently co-immunoprecipitated with FoxP3 (Supplementary Fig. 2a), in a manner that did not artefactually result from parallel binding to DNA (not inhibited by treatment with DNase or intercalation of ethidium bromide; Supplementary Fig. 2b,c) or result from agglomeration after cell lysis (Supplementary Fig. 2d). When these cofactors were tested against the panel of FoxP3 mutants, no simple pattern emerged, and interactions with every cofactor proved unique (Fig. 4a,b and Supplementary Table 6). Even interactions with the related proteins IKZF1, IKZF2 and IKZF3 (three members of the IKAROS family) showed some similarities but were affected differentially by replacements in the N-terminal and FKH domains. In most cases, interactions with any one cofactor were influenced by substitutions in several domains of FoxP3, with perhaps the exception of STAT3 and the dominant effect of N-terminal substitutions. Many examples of enhanced binding were observed, most readily explained by displacement of a competing cofactor. The strongest of such contrasts were observed for the substitutions in the N-terminal region, where M1 and M7 dampened binding to IKZF3, NFAT1 and STAT3 while enhancing binding to KAT5 (TIP60), EP300 and IKZF1. Thus, we identified great complexity in the specification of FoxP3’s interactions with cofactors, in keeping with the flexibility conferred by the intrinsically disordered nature of much of this protein.

Connecting transcription and interaction with DNA or cofactors

To understand the relationship between FoxP3’s interactions with cofactors and its transcriptional activity, we correlated, for the panel of FoxP3 mutants, their ability to induce or repress individual target genes and their ability to bind individual cofactors. For induced transcripts, and with the exception of a few targets with more specific correlations (such as Rorc or Vipr1), a dominant pattern emerged:
Figure 5 Connecting co-factor interaction and transcriptional activity. (a) Example of the Pearson correlation (r, vertical axis) between the binding of FoxP3 mutants to RELA (top) or YY1 (bottom) and the ability of those mutants to affect expression of FoxP3-induced targets (Ctla4 and Lrc32) or a FoxP3-repressed target (Pde3b) (horizontal axes; normalized expression as in Fig. 2b); numbers in plots indicate Pearson correlation r and P value.

(b) Correlation between the ability of FoxP3 mutants to activate each FoxP3-induced (left) or FoxP3-repressed (right) target gene and the ability of those mutants to bind each co-factor (above plot), presented (key) as the coefficient from a linear model that included DNA binding as a covariate; hierarchically clustered, rows and columns. (c) ChiP-seq analysis of the binding of ELF1, ETS1 or FoxP3 (far left margin) to chromatin at FoxP3-induced loci (left) and FoxP3-repressed loci (right), in Tconv cells (as in Fig. 1f) or ex vivo Treg cells. (d) As in (c), compiled density of chromatin marks for ELF1 and ETS1 in a 10-kb window around FoxP3-binding sites for FoxP3-induced or FoxP3-repressed signature loci, in Tconv cells or Treg cells (key) (data from ref. 26). Data are from published results (c,d) and are an independent replicate (c) or average value (d) of two experiments.
activation of target genes correlated strongly with the mutant’s ability to interact with RELA or IKZF2 or, to a lesser extent, with KAT5, EP300 and GATA3. It correlated negatively with binding to IKZF3, YY1 or EZH2 (Fig. 5a, b). This dichotomy suggested that FoxP3 might engage in two main types of interactions that have activating or repressive properties. This interpretation is compatible with known biochemical activities: the histone acetyltransferases KAT5 and EP300 generally activate transcription by acetylating histones and other TFs and specifically FoxP3 (ref. 20); and the NF-κB pathway has a strong positive role in the differentiation and function of Treg cells.35,36 Conversely, IKZF3, EZH2 and YY1 are known repressors, in general and in the context of FoxP3 (refs. 31, 37–40), and NFAT1 is a central player in the repression of IL2 (ref. 15).

The transcripts repressed by FoxP3 again showed a paradoxical pattern. While one might have expected that FoxP3’s repressive association with negative cofactors would drive the downregulation, the exact opposite was observed: for most FoxP3-repressed transcripts, expression was positively correlated with binding to IKZF3, YY1 or EZH2 and was negatively correlated with binding to RELA (Pde3b, for example; Fig. 5). In other words, the positively acting FoxP3–RELA–IKZF2 complexes were the most effective at repression.

To understand this paradoxical correlation, we considered how FoxP3 might be exerting its repressive effects. In general, transcriptional repressors can be active or passive, as follows:41,42 they are active by recruiting dominant inhibitors (Polycomb or NuRD) or are passive by competing against more effective transactivators. We hypothesized that the FoxP3–RELA complex might be displacing more-effective transactivators at enhancers surrounding FoxP3-repressed loci. To investigate this notion, we analyzed published ChIP-seq data sets for differential binding in Treg cells versus Tconv in the immediate vicinity of FoxP3-binding sites (as for Fig. 1b). We found that binding of both ELF1 and ETS1 were lower in Treg cells than in Tconv cells at

**Figure 6** FoxP3’s active and inactive cofactors form different complexes. (a–e) Immunoblot analysis of cofactors co-immunoprecipitated (as in Fig. 1a) from lysates of cells transfected to express FLAG-tagged wild-type FoxP3 together with tagged EZH2 and KAT5 (TIP60) (a), RELA and EZH2 (b), IKZF3 and KAT5 (c), EZH2 and IKZF3 (d) or RELA and KAT5 (e); before the co-immunoprecipitation, lysates were pre-cleared with nonspecific IgG (−) or with antibodies to the cofactors (above lanes); blots were probed with antibodies to each tagged protein (right margins); below, quantification of band intensity (presented as percent remaining relative to amount in lysates precleared with control IgG) revealed by probing with antibodies (below graph) after preclearing of lysates as above (key). *P < 0.05, **P < 0.005 and ***P < 0.001, versus IgG (Student's t-test). (f) Immunoblot analysis (bottom) of FoxP3 in gel-filtration FPLC fractions of total lysates from FoxP3-transduced CD4+ T cells, and a Coomassie-stained gel (top). (g) Immunoblot analysis after gel filtration as in f, after immunoprecipitation of each fraction with anti-RELA or anti-EZH2 (left margin). (h) Immunoblot analysis of FoxP3 in gel-filtration fractions of total lysates from CD4+ T cells transduced with M7, M176 and M342 mutant FoxP3 (left margin). Data are representative of (top) or pooled from (below) three independent experiments (a–e) or are representative of two independent experiments (f), two independent experiments (g) or two independent experiments (h).
FoxP3-binding sites associated with FoxP3-repressed loci, but not at FoxP3-induced loci (Fig. 5c,d). Such patterns were not observed with other factors, in particular FOXO1, whose binding was higher in T_{reg} cells than in T_{conv} cells at both types of loci. These observations collectively suggested that both induction and repression by FoxP3 are determined by the same molecular complexes, but that the outcome varies with the target genes and the other complexes that can bind to the corresponding enhancers in its absence.

**FoxP3 in differentially active multimolecular complexes**

The results reported above suggested that FoxP3 forms complexes that have diametrically opposite transcriptional activities. We tested this hypothesis in several ways. First, to determine which cofactors belonged to the same FoxP3-containing complexes, or not, we performed FoxP3 co-immunoprecipitation experiments in which we preclared cell lysates with antibodies to one cofactor before probing for interaction between FoxP3 and a second cofactor. Preclaring the lysates with anti-EZH2 did not significantly reduce the total amount of FoxP3; it depleted the lysates, as expected, of complexes containing EZH2 but did not affect KAT5–FoxP3 complexes (Fig. 6a). The converse was true after pre-clearing of lysates with anti-KAT5 (Fig. 6a) or with anti-RELA (Fig. 6b). Preclearing with anti-IKZF3 also failed to remove KAT5–FoxP3 complexes (Fig. 6c). These observations suggested that EZH2 and IKZF3 might belong to one complex, while RELA and KAT5 belong to another. That interpretation was confirmed by the finding that anti-RELA and anti-KAT5 reciprocally depleted lysates of both RELA–FoxP3 and KAT5–FoxP3 complexes (Fig. 6d), as did EZH2 and IKZF3 (Fig. 6e). Thus, the associations suggested by the transcriptional correlations of Figure 5 were reproduced here as biochemical entities.

We then applied FPLC gel filtration to resolve FoxP3 molecular complexes in Foxp3-transduced CD4^+ T cells. In accordance with a previous study, FoxP3 was detected in several locations between 200 kDa and 2,000 kDa (Fig. 6f). Aliquots of each gel-filtration fractions were precipitated with anti-EZH2 or anti-RELA before immunoblot analysis of FoxP3. The FoxP3–RELA complexes were found in the larger size range (1,000–2,000 kDa), while FoxP3–EZH2 complexes were of intermediate size (~400–800 kDa) (Fig. 6g). We assessed the effect of some of the FoxP3 substitutions on these complexes. The M7 and M176 FoxP3 mutants, which had only mild effects on the effect of some of the FoxP3 substitutions on these complexes. The M7 and M176 FoxP3 mutants, which had only mild effects on the effect of some of the FoxP3 substitutions on these complexes. The M7 and M176 FoxP3 mutants, which had only mild effects on

**Different FoxP3 molecular complexes in different nuclear regions**

To further delineate the duality of the FoxP3-containing multimolecular complexes, we sought to visualize them within the nucleus. We used three-dimensional structured illumination microscopy, to visualize FoxP3 molecular complexes in transduced CD4^+ T cells transduced to express tagged FoxP3. FoxP3, RELA, and EZH2 were detected in discrete microclusters with an apparent size of 100–200 nm (Fig. 7a and Supplementary Video 1). FoxP3 and RELA tended to predominate at the center and EZH2 at the periphery of the nucleus (Fig. 7a and Supplementary Video 1). IKZF3, the other cofactor associated with repression, also localized to the periphery of the nucleus (Supplementary Fig. 3a). We then searched for co-localization in the three-dimensional images. In 48 nuclei from the transduced CD4^+ T cells (analyzed in seven independent experiments), we identified 24,380 FoxP3 microclusters, of which 6% or 4.8% colocalized with RELA or EZH2, respectively (as a reference for detection of colocalization, 75% of FoxP3 that was stained with two secondary antibodies labeled with different fluorochromes appeared to be colocalized under our detection criteria) (Fig. 7a). Fox3–RELA clusters dominated in the center of the nucleus, and Fox3–EZH2 did so at the periphery of the nucleus (Fig. 7a). Indeed, colocalization of FoxP3 with RELA or EZH2 tended to be mutually exclusive, as only 0.06% of the FoxP3 microclusters colocalized with both RELA and EZH2, versus the colocalization frequency 0.28% expected by chance (Fig. 7b). Essentially identical images and conclusions were obtained for ex vivo T_{reg} cells (Fig. 7c). Thus, the differential engagement of FoxP3 with an activating cofactor (RELA) or repressive cofactors (EZH2, IKZF3) detected biochemically corresponded to partitioning into different nuclear zones.

**DISCUSSION**

This study tackled the enigmatic question of how FoxP3 operates as a transcription factor, by associating dense mutagenesis with a systematic exploration of the determinism and functional impact of FoxP3’s interactions with its known cofactors. The results showed that FoxP3’s operation was keyed by binding to active enhancers, with either a repressive or activating outcome, depending on the target locus. This duality corresponded to FoxP3’s integration into different multimolecular complexes, which also controlled localization to different regions of the nucleus. FoxP3’s transcriptional activities were highly variegated, fitting for an interaction hub with the flexibility to adjust to the broad span of physiological roles of T_{reg} cells.
Many TFs are thought to have dual roles as transcriptional activators and repressors, in a gene- or context-dependent manner, but how they switch and balance the two functions has never been well established. In fact, the activator-versus-repressor debate is one of the oldest in molecular biology, going back to Englesberg’s ‘positive control’ versus Jacob and Monod’s ‘repressor models’ of transcriptional regulation in bacteria. The question has remained open for eukaryotic TFs, even for some as extensively studied as p53 (ref. 44). Transcriptional repressors can be passive or active: they can be passive by out-competing activators for binding to DNA sequence motifs or to co-activators, by forming inert heterodimers with activators; they can be active by recruiting inhibitory elements such as histone deacetylases, histone or DNA methylases, displacing target loci into inactive chromatin configurations or nuclear localizations. Transcriptional activation can result from the formation of scaffolds between enhancers and promoters into active transcriptional hubs, the decompaction of chromatin or the recruitment of elongation factors to release stalled polymerases. How does FoxP3’s function relate to such schemes for transcriptional regulation? Clearly, many of the present observations are not compatible with an interpretation in which FoxP3 would be primarily an active repressor via recruitment of EZH2, with transcription being largely indirect: both induction by FoxP3 and repression by FoxP3 appeared quickly after transduction, both induced loci and repressed loci showed enrichment for FoxP3-binding sites, and the finely variegated effects of FoxP3 substitutions were not readily compatible with indirect effects. The association of FoxP3 with active enhancers around both activated loci and repressed loci suggests that FoxP3 locates enhancers and modulates their activity, consistent with published conclusions.

Once FoxP3 is bound to an enhancer, the potential for interaction with various cofactors seems to be the dominant driver of its functional outcome, judging from the correlation between cofactor binding and target gene transcriptional activation. Transactivation by FoxP3 was for the most part positively correlated with the ability of mutant FoxP3 to form a complex with RELA, IKZF2, EP300 or KAT5, and was negatively correlated with its ability to form a complex with EZH2, YY1, IKZF3, NFAT1 or STAT3. As discussed above, these results make biochemical and functional sense. This genetic evidence for distinct FoxP3 complexes with differential transcriptional potential was directly confirmed by biochemical experiments. We thus propose a model in which FoxP3 can be alternatively assembled in different complexes. It usually potentiates an enhancer when together with RELA–KAT5–EP300, possibly through acetylation-mediated activation via BRD4 and P-TEFb. It is inactive when complexed with IKZF3–YY1–EZH2, which leads to repression by recruitment of the NuRD and Polycomb assemblies and displacement away from active regions of the nucleus. The outcome of FoxP3’s activity would thus result from the balance between these two complexes, varying with genomic location (with different enhancers being more or less favored by each complex) and also influenced more generally by changes in the cell state, organismal location or environmental cues, possibly via post-translational modifications.

One might have expected that FoxP3 repressor complexes would operate dominantly on repressed loci: better binding to EZH2–YY1–IKZF3 leading to better repression. However, the exact opposite was observed: binding of FoxP3 mutants to EZH2, YY1 or IKZF3 correlated positively with the expression of FoxP3-repressed targets (and binding to RELA or IKZF2 correlated negatively to expression of these target genes). In other words, FoxP3–RELAKAT5 complexes were here the better inhibitors of FoxP3-repressed targets. One possible explanation for this paradox would be that repression by FoxP3 is mostly indirect, via the induction of repressive feedback factors. This interpretation is consistent with the close correlation between induction and repression indices but does not fit with the rapid effect after transduction or the binding of FoxP3 in the vicinity of repressed loci. Rather, we propose that the FoxP3–RELAKAT5 complex, can also behave as a passive repressor, interfering with stronger activating complexes. These loci appear to be repressed, but simply because they are less efficiently activated. There is precedent for the CREL–FoxP3 complex being reported as repressive. In support of that notion, the high ELF1 and ETS1 signals in Treg cells were lower in Treg cells, but only at FoxP3-repressed loci in Treg cells (with no difference at FoxP3 induced loci). This behavior might not be exclusive to ELK1 and ETS1 (displacement of AP1 is also possible), but these repress plausible candidates for the displacement that would accompany passive repression.

Beyond this ‘dominant theme’ of the RELA- and EZH2-containing complexes, however, several of FoxP3’s targets obeyed different modes of transactivation, with some even appearing to be independent of DNA binding since activated by FKH mutant devoid of DNA-binding activity (we cannot rule out the possibility that these results reflected ‘squelching’, or de-repression by dominant negative variants). Some of the ‘atypical’ responses to mutations make sense, such as the coordinated de-repression of H4 and H5, which are co-regulated in the Treg subset of helper T cells. Interestingly, atypically regulated targets included Il1r1 and Rorc, whose expression in Treg cells is largely restricted to tissue Treg cells. That observation suggests that FoxP3 has an inherent ability to activate these transcripts, which is revealed, or ‘de-inhibited’, by cofactors induced in Treg cells by tissue-localization cues.

In conclusion, this study has uncovered a multimodal operation of FoxP3 and the complexes it belongs to: it acted most frequently as an activator, acted on other loci as a passive repressor by ‘tuning down’ enhancer activity, and acted as an active repressor by associating with the major repressor complexes. Importantly, we do not know how rapidly tunable these complexes are in individual Treg cells, and it will be important to determine whether the population averages observed here reflect frequencies of binary states or whether they fluctuate rapidly in every cell. This view of FoxP3 as a multimodal interaction hub is consistent with the fine-tuning of Treg cell transcriptional programs needed to adjust to the broad span of Treg cell physiology.}

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

*Note:* Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

H.-K.K. and H.-M.C. performed the experiments; all authors designed the study and analyzed and interpreted the data, and H.-K.K., C.B. and D.M. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.
Foxp3-dependent and -independent molecules specific for FOXP3 is a homo-oligomer and a component of a supramolecular network. Analysis of FOXP3 reveals multiple domains required for its function. FOXP3 controls regulatory T cell function through cooperation with Treg function.

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ONLINE METHODS

Mice and cells. C57/BL/6J and Foxp3<sup>GFP</sup> mice and mutants were bred in an SPF facility at Harvard Medical School, under Institutional Animal Care and Use Committee protocol 02954. Plat-E cells were obtained from Cell Biolab and tested negative for mycoplasma contamination.

Cloning and mutagenesis. To construct the FoxP3 alanine-scanning library, Foxp3 cDNA was amplified from Tqg cell cDNA and was inserted into the MSCV-IRES-THY1.1 retroviral vector<sup>7</sup> with a terminal FLAG-tag motif (FLAG-Foxp3<sup>361</sup>). From that template, alanine replacement mutations were generated by substitution of groups of six amino acids for M1 (M,PNPRA), M7 (A,KPMAPS<sup>14</sup>), M14 (I<sub>L</sub>ALGPS<sub>5</sub>PS), M151 (G153<sub>V</sub>INVAS156), M176 (P<sub>2</sub>TKBDNS181), M215 (K<sub>2</sub>HCQADH232) and M235 (R<sub>25</sub>EVVQS<sub>26</sub>Qsa) in each individual amino acid within the FKH domain, including M328 (P<sub>388</sub>), M342 (Y<sub>392</sub>) M354 (P<sub>358</sub>), M359 (T<sub>359</sub>), M371 (F<sub>372</sub>), M390 (S<sub>390</sub>) and M409 (D<sub>409</sub>). Directed mutagenesis used the QuikChange XLII SiteDirected Mutagenesis Kit (Agilent, Santa Clara, CA). All coding sequences in the mutant plasmids were verified by Sanger sequencing. To generate six-histidine (6xHIS)-tagged Foxp3, mouse Foxp3 cDNA was cloned into pENTR-Topo (ThermoFisher) and was transferred into pDEST40 (Invitrogen) by Gateway cloning. To express Foxp3 cofactors, cDNA encoding human IKZF1, IKZF3, IKZF2, YY1, HDAC1, HDAC3, STAT4, IRF4, RUNX1, EZH2, KAT5, RELA, RORC or GATA3 was obtained from the DF/HCC DNA Resource Core and was cloned into pDest vectors (GFP tag, pDest-N1 (Addgene); 6xHIS tag, pDest40 (Invitrogen); V5-6xHIS, MSCV-Att1-cdbb-Att2-V5-6xHIS-IRES-GFP) by Gateway cloning. Mouse cDNA encoding Foxp1 or Not1 was obtained from Addgene, and was expressed similarly. For Foxp3 ChIP-seq experiment with anti-V5 and anti-6xHIS, cDNA from wild-type, M7, M354 and M390 plasmids was re-cloned into MSCV-Att1-cdbb-Att2-V5-6xHIS-IRES-GFP vector, which generated by insertion of Att1-cdbb-Att2-V5-6xHIS into MSCV-IRES-GFP vector.

Retroviral production and titration. For the production of retroviral particles, Plat-E packaging cells<sup>49</sup>, were transfected with TransIT-T293 (Mirus Bio) per the manufacturer’s protocol. In brief, complete culture medium was changed to DMEM with 10% FCS 6 h before transfection, and cells were transfected with 6 µg of each retroviral plasmid and 4 µg of Gag, Pol and Env packaging plasmid (pCL-Eco, Addgene) together with 40 ng of each retroviral plasmid and 4 µl of complete RPMI medium supplemented with 10% FCS, 3 mM t-glutamine, 10 mM sodium pyruvate, 10 mM non-essential amino acids, 100 µM penicillin-streptomycin, 50 µM β-ME and 50 µM human IL-2 (Peprotech). For viral infection, culture medium was

ChIP-seq. For Foxp3 ChIP-seq, we used a previously published procedure, with minor modification<sup>50</sup>. Activated CD4<sup>+</sup> T cells transduced with wild-type or mutant Foxp3 were sorted according to THY1.1 reporter expression to select cells with normal levels of Foxp3 expression (matching ex vivo T<sub>reg</sub> cells) as above. These cells (7 × 10<sup>6</sup> cells per sample) were cross-linked with 1% of formaldehyde for 10 min and were lysed on ice in RIPA buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 140 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 0.1% sodium deoxycholate) supplemented with complete protease inhibitor cocktail (Roche). Chromatin was sheared using an AFA Focused-ultrasonicator (Covaris) for 9 min (duty cycle 2%, intensity 3, cycle-burst 200) and the sheared material was cleared by a 10 min centrifugation at 13,000 r.p.m. at 4 °C. The cleared material was immunoprecipitated overnight at 4°C with the antibody combination to maximize immunoprecipitation efficiency by targeting different parts of Foxp3 protein (5 µg of anti-V5 (R960-25; Invitrogen) and 5 µg of anti-6xHIS (ab9108; Abcam)) conjugated with magnetic Protein G beads. Beads were sequentially washed with 1 ml of washing buffer (five times for each buffer; 10 min for each washing) including ice-cold RIPA buffer, high-salt RIPA buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate), LiCl buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 250 mM LiCl, 0.5% NP-40 and 0.5% sodium deoxycholate) and TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)). Chromatin was eluted from the beads, treated with 1 µg DNase-free RNase (Roche) for 30 min at 37°C and with Proteinase K (Roche) for 2 h at 37°C, followed by reversing crosslinks by incubation at 65°C for 16 h. Reverse-crosslinked DNA was purified with SPRI beads (Agencourt AMPure XP beads, Beckman Coulter) and sequential steps of end repair, A-base addition, adaptor-ligation and PCR amplification (15 cycles) were performed to prepare the ChIP-seq library for each sample. ChIP-seq libraries were size-selected for 200- to 500-bp fragments with SPRI beads. Equivalent amounts of barcoded libraries were pooled and sequenced on a NextSeq 500 (Illumina) instrument. To control for background noise, we prepared samples immunoprecipitated with anti-V5 and anti-6xHIS (identified above) using chromatin from empty-vector-transduced T cells.

Short reads (50 bp, single end) were aligned to the mouse reference genome (mm10) using the bowtie aligner<sup>51</sup>. Reads with multiple alignments were removed with samtools (v1.1) and de-duplicated with picard (v1.130). To identify peaks from ChIP-seq reads, we used the HOMER package maketagDirectory followed by the findPeaks command with the 'histone' parameter. To visualize individual ChIP-seq data on Integrative Genomics Viewer (IGV)<sup>52</sup>, we converted bam output files from picard into normalized bigwig format using the bamCoverage function in deepTools (v1.6) with options -fragmentLength 200 – normalizeUsingRPKM<sup>53</sup>. HOMER-generated peak files for H3K27ac, H3K4me1, H3K27me3 and Foxp3 were used to analyze with the ROSE algorithm described previously<sup>54</sup>, wherein Foxp3 peaks are stitched together if they are located within 10 kb of each other. Line plots as in Figure 1b were generated using ngs.plot<sup>55</sup>.

To derive a robust list of Foxp3 chromatin binding sites from previous ChIP-seq data, raw fastq files from<sup>26,52</sup> were mapped to the mm10 reference genome using bowtie<sup>51</sup>. Peaks from both studies were called using HOMER findPeaks function with an FDR of 1% using the parameter -style factor and respective input controls, recovering 9,685 (ref. 26) and 7,751 (ref. 32) Foxp3 peaks. Intersection of peaks were derived from both data sets using the BEDTools intersect function with a 50% reciprocal overlap requirement, yielding 5,047 robust Foxp3 peaks.

Evaluation of Foxp3 expression. For immunohistochemistry, HEK cells were seeded on glass coverslips and transfected with empty vector (EV), plasmid encoding wild-type or each mutant Foxp3, by TransIT-T293 (Mirus). After 72 h, cells were fixed with cold 100% methyl alcohol for 20 min at −20°C, then washed three times with PBS. Cells were blocked with 5% BSA in PBS for 1 h and incubated with anti-FLAG (M2, Sigma) (1:200 dilution) overnight at 4°C,
followed by staining with donkey-anti-mouse-IgG-Cy5 (715-175-130; Jackson ImmunoResearch Laboratory) (1:1,000 dilution) for 1 h at room temperature. The DNA-binding dye DAPI (100 nM, Invitrogen) was used for nuclear counterstaining. Images were acquired on a Zeiss Axios M1 fluorescence microscope. For flow-cytometry analysis of FoxP3 and THY1.1 expression in transduced primary T cells after infection to express EV or wild-type or mutant FoxP3, cells were stained with anti-THY1.1 (OX-7), fixed and permeabilized with 1× Fixation/Permeabilization buffer (eBioscience) overnight at 4 °C, stained with anti-FoxP3 (eBioscience, #14-5773) in 1× Permeabilization buffer for 1 h at 4 °C and analyzed on an LSRII (Becton Dickinson). For immunoblot analysis of FoxP3, cells were sorted by THY1.1 expression and nuclear proteins were extracted with a nuclear extraction kit (Active motif: 40010). These lysates were resolved by SDS-PAGE and subjected to immunoblot analysis with anti-FLAG (M2; Sigma) and HRP-conjugated anti-mouse-IgG (115-035-003; Jackson ImmunoResearch Laboratory).

DNA binding assay. Activated CD4+ T cells (2 × 10⁶) were transduced to express FLAG-tagged wild-type or mutant FoxP3, then were harvested, stained with anti-THY1.1 (OX-7; BioLegend) and sorted for a window of colinear THY1.1 reporter expression determined to ensure a match with FoxP3 expression in splenic Treg cells. After sorting, nuclear extracts were prepared (Active motif: 40010), and total protein in each sample was quantified by a BCA protein assay (ThermoFisher, 23225). A 25-µg double-stranded biotinylated oligonucleotide encompassing two copies of the canonical FoxP3 motif (underlined) (5′-CAAGGTTAAACAGGGATTTTGCGGT-3′) or a control oligonucleotide with a scrambled motif (5′-CAAGGCGCAGGCGTCTAGGCCGTC-3′) was used as a probe. Protein–DNA binding was analyzed with Episeeker DNA-protein binding assay kit (Abcam, ab17139) according to the manufacturer's protocol. In brief, 40 ng of probe and 50 µg of each extract were incubated on streptavidin-coated plate, which was washed with 1× washing buffer, and FLAG-tagged FoxP3 was detected with anti-FLAG (Sigma; F1804) and HRP-conjugated anti-mouse-IgG (115-035-003; Jackson ImmunoResearch Laboratory) with TMB as substrate. Binding was assessed by absorbance on a micro-plate reader at 450 nm.

Transcriptional analysis. For NanoString profiling, the NanoString nCounter system (NanoString Technologies, Seattle, WA) was used to digitally count transcripts in multiplex reaction. In brief, 5 µl of lysate (1,000 cells per µl) in RLT buffer was hybridized for 18 h with a custom code set (Supplementary Table 1), then was washed and quantitated using the nCounter Analysis System at maximum counting (555 images per sample). For processing, each result was normalized relative to that of positive and negative spike-in probes from the External RNA Control Consortium (ERCC), the background from negative-control probes was subtracted, and the values were normalized relative to average expression of control genes (Actb, Gapdh, Hprt, Mita1, Rpl119 and Tbp).

In vitro suppression assay. CD4⁺FoxP3+ Tcon cells from Foxp3GFP mice were isolated and labeled with 10 µmol/L CFSE (Invitrogen) in RPMI 1640 at 1 × 10⁶ cells per ml at 37°C for 20 min. CD4+ T cells infected with retroviral vector encoding wild-type or mutant FoxP3 or EV were sorted after 72 h for equivalent levels of the colinear THY1.1 retroviral reporter and were co-cultured with CFSE labeled Tcon cells at a ratio of 1:1 in the presence of beads coated with anti-CD3 and anti-CD28. Proliferation was measured as CFSE dilution after 72 h of co-culture. The suppression index was calculated as (PnEV/PnWt) / PnEV, where PI is the proliferation index (determined simply as the frequency of Tcon cells that proliferated, with all proliferated peaks included), of cells transduced with EV (PnEV) or of cells transduced with ‘x’ (Pnx).

Computational analysis. To identify robust FoxP3 binding in chromatin (Fig. 1), we combined by rank intensity two available FoxP3 ChIP-seq data sets, retaining peak regions that were called in the top 5,000 of peak intensity (integrated read count per million) in both data sets. The log₂IPF or log₂IPreg-down genes in Figure 1a-c were the 200 genes most overexpressed in Treg cells or Tcon cells, respectively, and ‘neutral’ genes were 200 randomly selected genes (150 iterations averaged in Fig. 1a) among transcripts with change in expression in Treg cells relative to their expression in Tcon cells of >0.9-fold and <1.1-fold.

To analyze the significance of transactivation or trans-repression by FoxP3 in transduced cells, a simple value for change in expression (fold value) was computed as the mean for all cells transduced with wild-type FoxP3 versus all cells transduced with EV (4 or 5 biological replicates) and a point-wise Pvalue derived from a t-test of the log of these values. Treg cell signature genes were those identified by published microarray analyses⁵,7. To focus the analysis of mutation effects on a robust set of trans-activated or repressed transcripts, genes upregulated by FoxP3 (Fig. 1d) were selected on the basis of the volcano plot of as those with a change in expression of >1.8-fold (or <0.55-fold) and a P value (−log₁₀p) of >1.3 after transduction with wild-type FoxP3. We also included in this analysis a few gene transcripts changed by threefold or more in at least one mutant (average of two determinations). Principal-component analysis (Fig. 2c) was performed on expression values for those selected genes in cells transduced by all mutants, the princomp function in R, with the first two components plotted.

From that selection, a general ‘repression index’ (Fig. 2d) was computed for each mutant by averaging the change in expression (fold values) for all FoxP3-repressed transcripts (equivalently weighting each transcript by first normalizing to the mean change in expression (fold value) for the transcript), and the ‘activation index’ was derived similarly from the FoxP3-upregulated gene set.

To relate suppressive activity of cells transduced with wild-type or mutant FoxP3 with their transcriptional programs, a Pearson correlation coefficient was calculated between in vitro suppression index and the activation and repression indices (Fig. 2f), or against expression of each FoxP3-induced or FoxP3-repressed transcript (as change in expression (fold value) vs EV control; Supplementary Fig. 1f).

To relate DNA-binding activity of the mutants FoxP3 to their ability to affect transcription (Fig. 3c), a Pearson correlation coefficient was calculated between DNA-binding activity (normalized to wild-type activity) and the activation or repression indices derived from NanoString profiling of transduced cells.

To relate the transactivation or trans-repression activity of the FoxP3 mutants to their ability to bind cofactors, a linear model was fit between target genes expression in the transduced cells (as change in expression (fold value) relative to EV control) and the factor-binding results (normalized to wild-type FoxP3 values), both log-transformed, adding the DNA-binding ability as an additional covariate to test whether the patterns might indirectly reflect DNA-binding activity (in practice, omitting the DNA binding term yielded very similar coefficients).

Gel-filtration chromatography. Activated CD4+ T cells transduced with wild-type or mutant FoxP3 (1 × 10⁶ cells per sample) were lysed in NP-40 lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1% NP-40 10% glycerol, 150 mM NaCl, 1 mM PMSF and 1× Proteasine inhibitor cocktail; Roche, #0469316001) for 2 h with gentle vortexing at 4°C. The lysates were sedimented at 15,000 r.p.m. for 30 min at 4 °C to remove aggregates, and the supernatant was injected into an FPLC Superose 6 column (10 mm × 300 mm, GE Healthcare Life Science) at a flow rate of 400 µl/min in PBS. 27 fractions of 400 µl were collected and were immediately concentrated by filter centrifugation (Centricon 10,000-kDa cutoff, Millipore) down to 50 µl resolved by SDS-PAGE and probed by sequential immunoblot analysis of FoxP3 (eBioscience, #14-5777). FoxP3-containing fractions were incubated with anti-REL(A) (Abcam, #ab7970) or anti-EZH2 (Active motif, #AC22), revolved by SDS-PAGE and immunoblotted with anti-FoxP3 (eBioscience, #14-5773).

Co-immunoprecipitation and immunoblot analysis. HEK293 cells (1 × 10⁶) were co-transfected as described above with plasmids encoding FLAG-tagged wild-type or mutant FoxP3 and another TF (tagged with HA, 6xHis or GFP). About 48 h after the transfection, cells were harvested and lysed in NP-40 lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1% NP-40 10% glycerol, 150 mM NaCl, 1 mM PMSF and 1× proteasine inhibitor cocktail) for 20 min. Cell lysates were centrifuged at 13,000 r.p.m. and 4 °C for 30 min, and protein concentration was determined by Bradford protein assay (Bio-Rad). Approximately 1 µg of...
protein lysate were incubated with slow rotation for 4 h at 4 °C with a complex of anti-FLAG (identified above) pre-bound to Protein G–coupled magnetic beads (Life Technologies) (per sample, 10 µg of anti-FLAG and 30 µl of Protein G suspension complex prepared by overnight incubation at 4 °C, washed with lysis buffer twice just before use).

After washing with lysis buffer, proteins were eluted from the beads in 30 µl of 1x Laemmli Sample Buffer for 10 min at 100 °C, resolved by 7–10% SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad). Membranes were blocked with 5% skim milk in 1x TBS buffer (50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) for 1 h at room temperature and probed with each primary antibody overnight at 4 °C (anti-FLAG (Sigma, #F1804), anti-Foxp3 (Abcam, #ab2290), anti-Foxp1 (Abcam, #ab16645) and anti-6XHIS (Abcam, #ab9108)). After five washes with 1x TBS supplemented with 0.05% Tween-20, membranes were incubated with HRP-conjugated secondary antibody (110-035-003; Jackson Immunoresearch Laboratory) and were developed with ECL prime Western Blotting Detection Reagent (GE Healthcare). Chemiluminescent images were acquired with ChemiDoc XR+ System (Bio-Rad) and were analyzed using AlphaView Software (version 3.2.2; ProteinSimple).

To exclude the possibility of DNA-contamination-mediated nonspecific interaction, 1 µg of protein lysate were incubated with anti-FLAG (Sigma, #F1804)–bead complex in the presence of 10 µg/ml ethidium bromide, or the immunoprecipitates bound to the beads were treated with 1 µg/ml of RNA free DNaseI (Roche) for 30 min at 37 °C.

For the preclerking experiments, HEK293 cells (1 × 10^6) were triply transfected to express FLAG-tagged Foxp3 together with two other effectors (either of RELA-GFP, KAT5–6XHIS, IKZF3-GFP or 6XHIS and EZH2-HA) together with Foxp3. 48 h after transfection, the protein lysate was first preclerated with specific antibody (control IgG (BioLegend, #410404), anti-RELA, anti-EZH2, anti-6XHIS for KAT5 or anti-GFP for IKZF3; 30 µg per sample) for 12 h at 4 °C, and precleared proteins were removed by induction with Protein G bead conjugated with magnetic bead (200 µl per sample) for 2 h at 4 °C. Subsequently, precleared lysate was used for immunoprecipitation with anti-FLAG (Sigma, #F1804) for precipitation of Foxp3 complexes. Immunoprecipitated protein was analyzed immunoblot with specific antibodies (anti-Foxp3, 6XHIS, anti-GFP or anti-HA; all identified above) as described above.

Super-resolution microscopy (three-dimensional structured illumination microscopy). Activated CD4^+ T cells transduced with wild-type or mutant Foxp3 were immobilized on coverslips (1.5 µm) coated with anti-CD3 (#100313, BioLegend) (1 µg/ml), fixed with 4% formaldehyde for 10 min at room temperature and washed five times with PBS. Fixed cells were permeabilized with ice-cold 100% methyl alcohol at 4 °C for 20 min and were washed ten times with PBS. Cells were stained with purified rat anti-Foxp3 (1:200 dilution), (eBioscience, #14–5773), rabbit-anti-EZH2 (1:200), mouse anti-RELA (1:100) and rabbit anti-IKZF3 (1:200) for 1 h at room temperature and washed, followed by staining with donkey anti-mouse-IgG-alexa488 (ThermoFisher, #A-21202), donkey anti-rat-IgG-alexa594 (ThermoFisher, #R37119) and donkey anti-rabbit-IgG-alexa647 (ThermoFisher, #A31573) (all secondary antibodies; 1:2000 dilution) for 1 h at room temperature. Three-dimensional structured illumination microscopy (3D-SIM) data were collected on a DeltaVision OMX V4 Blaze system (GE Healthcare) equipped with a 60x/1.42 N.A. Plan Apo oil immersion objective lens (Olympus), and three Edge 5.5 SCMOS cameras (PCO). Alexa488 was exited with a 488-nm laser and a 528/48 emission filter, Alexa594 was exited with a 571-nm laser and 609/37 emission filter and Alexa647 was exited with a 645-nm laser with 683/40 emission filter. z-stacks of –2 µm were acquired with a z-step of 125 nm and with 15 raw images per plane (five phases, three angles). Spherical aberration was minimized using immersion oil matching. Super-resolution images were computationally reconstructed from the raw data sets with a channel-specific measured optical transfer function (OTF) and a Wiener filter constant of 0.001 using CUDA-accelerated 3D-SIM reconstruction code based on a published article. 3D-SIM images were analyzed with the Imaris Bitplane program. The spot function in Imaris automatically located Foxp3, EZH2 and RELA protein on the basis of size and intensity thresholds. Each spot was represented by a sphere of arbitrary size (X, Y and Z) determined by Imaris (default setting, automatic mode). To determine the colocalization from SIM data, we first verified channel alignment and the absence of chromatic aberration at 0.2-µm diameter TetaSpeck microsphere beads (Invitrogen) stained throughout with multiple fluorescent dyes. Clusters were called as colocalized when their centers were less than 100 nm apart (there is uncertainty in the actual position of an object in SIM, owing to the length of the molecules and of the antibody pair used for detection). As positive control, we imaged Treg cells with primary antibody to Foxp3 (Fk1-16s, eBioscience), then simultaneously counterstained with Alexa 488- and Alexa 568- conjugated secondary antibodies (all identified above) (~75% of Foxp3 spots were called in both wavelengths). To calculate spot intensity of co-localized spots, RELA or EZH2 spots that overlapped with Foxp3, voxels were further determined with the centered fluorescence intensity of each Foxp3 spot based on Imaris default settings. As a control, the same number of Foxp3 or RELA spots was randomly selected to calculate their EZH2–RELA or Foxp3–EZH2 intensity.

Data availability. The source data that support the findings of this study are available from the corresponding author upon request. Foxp3 ChIP-seq data sets have been deposited in the GEO database with accession code GSE102281. A Life Sciences Reporting Summary for this paper is available.
Experimental design

1. Sample size
Describe how sample size was determined.

Sample size was chosen according to standard practices in the field. ChIP-seq samples were prepared in duplicate. Nanostring experiments contained 2~4 replicate per sample. All biochemical experiments were performed 2~5 as described in figure legends.

2. Data exclusions
Describe any data exclusions.

No data were excluded.

3. Replication
Describe whether the experimental findings were reliably reproduced.

All of data were successfully reproduced by each attempt.

4. Randomization
Describe how samples/organisms/participants were allocated into experimental groups.

No randomization in this study.

5. Blinding
Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

No blinding test in this study.

Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Describe the software used to analyze the data in this study. Flowjo, S-PLUS, R and Prism were used.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company. No unique materials were used in this study.

Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species). All antibodies in this study were described in Method section and their validation data are available on the manufacturer’s website.

Eukaryotic cell lines

a. State the source of each eukaryotic cell line used. Hek293 cells from ATCC and Plat-E cell from Cell Biolabs, Inc.
b. Describe the method of cell line authentication used. None of the cell lines used have been authenticated.
c. Report whether the cell lines were tested for mycoplasma contamination. All cell lines were tested negative for mycoplasma contamination.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No commonly misidentified cell lines were used.

Animals and human research participants

Provide details on animals and/or animal-derived materials used in the study. Primary Tconv and Treg cells were isolated from 8 weeks old female C57Bl/6 mice.

This study did not involve human research participants.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation. Splenic Tconv(CD4+Foxp3-) were infected with EV, WT or mutant FoxP3 and stained with DAPI, CD4, Thy1.1 and FoxP3.
6. Identify the instrument used for data collection. LSR II
7. Describe the software used to collect and analyze the flow cytometry data. Flowjo
8. Describe the abundance of the relevant cell populations within post-sort fractions. Around 99% by double sorting.
9. Describe the gating strategy used. Starting cells were gated by FSC/SSC gates and then DAPI to select healthy/live population. These cells were further gated by CD4 to check the expression of Thy1.1 and FoxP3.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. □
ChIP-seq Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data deposition

1. For all ChIP-seq data:
   a. Confirm that both raw and final processed data have been deposited in a public database such as GEO.
   b. Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

2. Provide all necessary reviewer access links. The entry may remain private before publication.

   - Publicly available ChIPseq data from: for Tconv; H3K27ac (DRR061059, DRR061060), H3K4me1 (DRR061078), H3K27me3 (DRR061088) and for Treg; H3K27ac (DRR061061, DRR061062), H3K4me1 (DRR061079), H3K27me3 (DRR061089), FoxP3 (DRR061112, GSE40686). New data currently being deposited into GEO.

3. Provide a list of all files available in the database submission.

4. If available, provide a link to an anonymized genome browser session (e.g. UCSC).

Methodological details

5. Describe the experimental replicates.

6. Describe the sequencing depth for each experiment.

   - 50 million of raw reads, 5 million of uniquely mapped reads, 85 bps single-end

7. Describe the antibodies used for the ChIP-seq experiments.

   - anti-V5; Invitrogen, and anti-6XHIS; Abcam

8. Describe the peak calling parameters.

   - HOMER (findPeaks using Histone mode).

9. Describe the methods used to ensure data quality.

   - Fold over local region required = 4.00 and Poisson p-value over local region required = 1.00e-04

10. Describe the software used to collect and analyze the ChIP-seq data.

    - Short reads were aligned to the mouse reference genome (mm10) using the bowtie aligner. Reads with multiple alignments were removed with samtools (v1.1) and de-duplicated with picard (v1.130). To identify peaks from ChIP-seq reads, we used the HOMER package makeTagDirectory followed by the findPeaks command with the ‘histone’ parameter.