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Brief Reviews

The Journal of Immunology

Genomic Analysis of Foxp3 Function in Regulatory T Cells

Gabriel A. Dolsten*,† and Yuri Pritykin*,‡

Regulatory T (Treg) cells are critical for tolerance to self-antigens and for preventing autoimmunity. Foxp3 has been identified as a Treg cell lineage-defining transcription factor controlling Treg cell differentiation and function. In this article, we review the current mechanistic and systemic understanding of Foxp3 function enabled by experimental and computational advances in high-throughput genomics. ***The Journal of Immunology*****, 2023, 210: 880–887.**

Regulatory T (Treg) cells form a specialized immunosuppressive lineage of CD4 T cells and are essential for tolerance to self-antigens and for preventing autoimmunity and inflammation (1–3). Foxp3, encoded on X chromosome, has been identified as a Treg cell lineage-defining Forkhead family transcription factor (TF) controlling Treg cell differentiation and function (4–6). Mutations in the *Foxp3* gene are associated with the rare but severe human immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (7), and ectopic Foxp3 expression in activated conventional CD4 T cells is sufficient to confer suppressive function (8).

Despite the critical role of Foxp3 in Treg cell development and function, the underlying mechanisms remain unclear. It remains contested whether Foxp3 operates as a transcriptional activator or repressor (9–12). Deciphering the role of Foxp3 is difficult due to differential regulation of a mosaic of TFs during Treg cell development and function upstream and downstream of Foxp3 expression. Disentangling direct effects of Foxp3 from its indirect effects and the effects of other TFs remains a challenge.

Experimental and computational advances in high-throughput genomics combined with genetically engineered mouse models and other standard methods of immunology have offered a range of tools for probing critical questions of Treg cell biology. In this article, we review applications of experimental and computational approaches that have elucidated critical aspects of Treg cell biology and Foxp3 function.

Functional transcriptomic studies of Treg cells with normal and altered Foxp3 function

High-throughput genome-wide transcriptomic profiling of isolated bulk cell populations enables their functional characterization by comparing the resulting multidimensional vectors of gene expression across populations and enables identification of the underlying molecular regulators and pathways (13). The Treg-specific transcriptomic program has been studied by isolating Treg cells using $CD4^+$ and IL-2R α-chain (CD25) (14) or a Foxp3-GFP knock-in mouse model (6). These studies have identified Treg-specific genes such as Foxp3, Il2ra, Ikzf2 (Helios), CTLA-4, GITR, and Lrrc32 (9) ranging from being nearly unique to Treg cells (e.g., Foxp3) to being present in other cells, albeit at lower levels (e.g., Ikzf2). The Treg transcriptomic program includes as many as 3000 differentially expressed genes between Treg cells and naive conventional $CD4^+$ T (Tcon) cells, although only 600 of these genes may be shared among different studies (12, 15–18) (Fig. 1A). A range of results across studies could be attributed to varying quality of the data, different experimental designs, and variability in choices and parameters of computational methods, all improving over time.

Bulk RNA sequencing (RNA-seq) studies are well complemented by single-cell RNA-seq (scRNA-seq), which can provide comprehensive characterization of Treg cell heterogeneity and avoid biases present in bulk data (19–23). Early scRNA-seq analyses in Treg cells were consistent with bulk RNA-seq studies, recovering Treg-specific markers such as Foxp3 and Ikzf2. Despite limited resolution, one early study identified a considerable overlap of the transcriptomic states between some Treg and Tcon cells, particularly depending on the activation status and presumably on the TCR signaling strength (19); this intermixing of transcriptomes of Treg and Tcon cells has been observed in subsequent studies (24).

Although splenic or thymic Treg cells have been the primary focus of studies, Treg cells also reside in nonlymphoid tissues and possess distinct functions, mechanisms, and transcriptomes (25, 26). A recent scRNA-seq profiling of the nonlymphoid Treg cells revealed molecular signatures of Treg cell tissue adaptation and specificity, including a nonlymphoid trajectory that was shared between gut and skin and conserved in humans (27). Functional single-cell genomic analysis across multiple experimental systems and human diseases has enabled identification of relatively rare subpopulations of Treg cells (28–31).

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Abbreviations used in this article: ATAC-seq, Assay for Transposase-Accessible Chromatin using sequencing; aTreg, activated regulatory T; ChIP-seq, chromatin immunoprecipitation sequencing; CUT&RUN, Cleavage Under Targets and Release Using Nuclease; DNase-seq, DNase I hypersensitive sites sequencing; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; KO, knockout; RNA-seq, RNA sequencing; rTreg, resting regulatory T; scRNA-seq, single-cell RNA sequencing; Tcon, conventional $CD4^+$ T; TF, transcription factor; Treg, regulatory T; WT, wild type.

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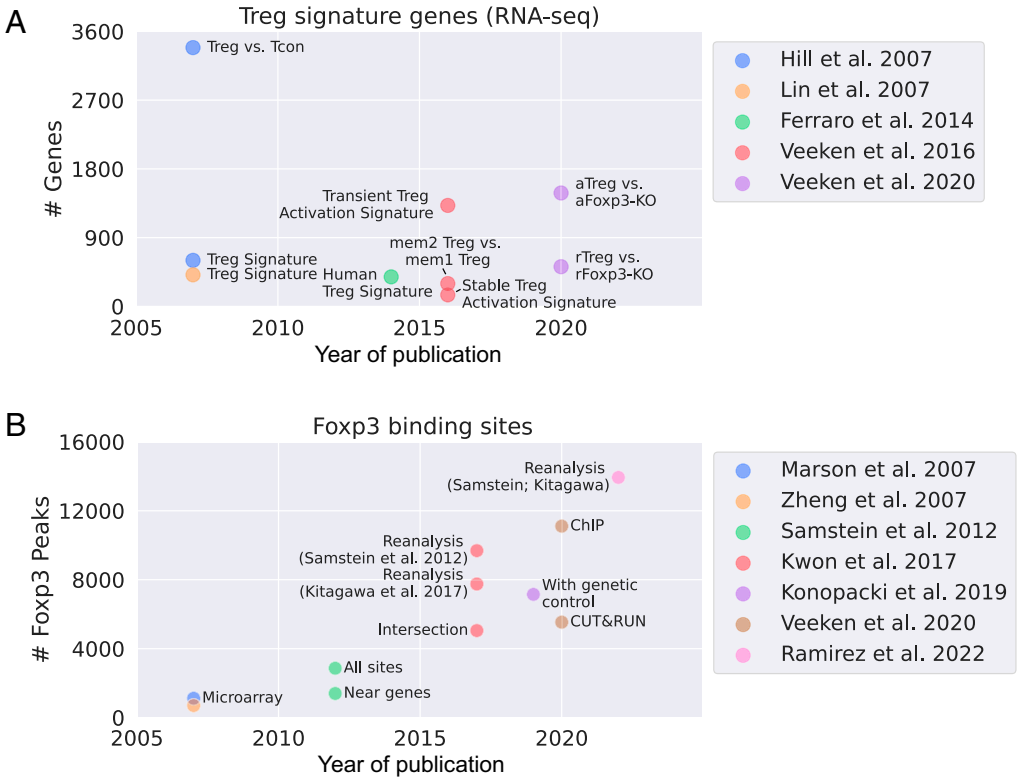

FIGURE 1. Published Treg cell gene signatures and Foxp3 binding sites. (**A**) Number of genes in published Treg cell–specific gene sets, along with the year of publication (only from publications where the list or the number of differentially expressed genes was provided) (12, 15–18). (**B**) Number of published Foxp3 binding sites, along with the year of publication (only from publications where the list or the number of peaks was provided) (10, 11, 18, 34, 53, 84, 85).

These studies collectively provide a robust characterization of physiological Treg cell states and transcriptomes.

The Treg transcriptomic program is characterized by differential expression of a number of TFs other than Foxp3, including Satb1, NFAT, Ikzf2, Stat5, Foxo1, Foxp1, and others (10, 32–34). To disentangle the role of Foxp3 in establishing the Treg cell identity, one study performed RNA-seq of primary $CD4^+$ T cells transduced with a mutant Foxp3 panel (35). Foxp3 mutants displayed perturbed transcriptomic programs with deficiencies in both gene activation and repression, suggesting that Foxp3 has diverse functions, perhaps by interacting with distinct molecular complexes. Importantly, similar perturbed transcriptomic programs were shared among mutants of disparate Foxp3 domains, suggesting that combinatorial and perhaps synergistic interactions between domains contribute to Foxp3 function.

The effect of Foxp3 dysfunction has also been studied extensively in vivo with the Foxp3-knockout (KO)-GFP mouse model (36). These mice possess a population of Foxp3-GPF^+ T cells known as "Treg cell wannabes" that have the activated *Foxp3* locus but do not have functional Foxp3 and cannot differentiate into functional Treg cells (36). Because Foxp3 is expressed on the X chromosome, female mice heterozygous for the Foxp3-KO allele are healthy, with a mosaic population of Treg and Treg wannabe cells allowing direct transcriptomic comparison.

Early transcriptomic analysis of Treg wannabes suggested that Foxp3 primarily reinforces TCR-dependent gene expression changes that occur before Foxp3 expression (15, 36). This observation was extended in a later study (18), in which the analysis was performed in resting ($CD44^{lo}CD62L^{hi}$) and activated ($CD44^{hi}CD62L^{lo}$) Treg, Foxp3-KO, and Tcon cells. Resting Foxp3-wild type (WT) Treg cells demonstrated modest changes in gene expression compared with resting Foxp3-KO Treg cells with ~500 differentially expressed genes. However, ~1500 differentially expressed genes were observed when comparing activated Foxp3-WT Treg cells with activated Foxp3-KO Treg cells. This suggested that Foxp3 not only regulates the establishment of Treg cell identity but is also involved in or interferes with Treg cell activation.

Studies of Foxp3 function in Treg cells have leveraged bulk RNA-seq and scRNA-seq in Foxp3-deficient mice and in humans with the IPEX syndrome (24, 36, 37). These analyses revealed a common Treg-like precursor with lower Foxp3 expression but high Ikzf2 and Il2ra expression shared by healthy donors and IPEX patients. Genes overexpressed in IPEX patients were expressed in both Treg and Tcon cells, suggesting that the IPEX signature may overlap with a broad T cell activation signature (24).

Differential Foxp3 exon usage and differential isoform expression may constitute an additional layer of complexity in characterizing Foxp3 function. Indeed, a recent analysis in a mouse model and in human patients with IPEX and other immunodeficiencies identified Foxp3 exon 2 as a critical regulator of Treg cell function (38).

A recent study introduced an engineered mouse model that enabled rapid restoration of Foxp3 expression in Treg wannabe cells in the context of established systemic inflammation and autoimmunity caused by lack of functional Treg cells (39). This resulted in a single pool of regenerated normal immunosuppressive Treg cells that resolved inflammation and provided long-term protection. scRNA-seq revealed that these persistent rescued Treg cells resembled a subset of the normal

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Treg cell pool, with a trajectory toward the activated Treg (aTreg) cell signature. A subcluster of these long-lived rescued Treg cells were enriched with an IL-2–Stat5 gene signature, a pathway associated with Treg cell self-renewal, and were less differentiated than most rescued Treg cells. This suggested that the corresponding cell subpopulation was likely contributing to local peripheral Treg cell maintenance.

Single-cell profiling in models with more intricate control of degraded or heightened Foxp3 expression in Treg cells and their counterparts can further reveal the mechanisms of Foxp3 function in Treg cell differentiation, maintenance, and activation across immunological contexts. We anticipate that future single-cell studies will focus both on characterizing heterogeneity within Treg cells and on better describing Treg cells within broader immune cell compartments, across tissues, immunological challenges, and developmental stages, in a range of mouse models and human conditions. Coupling this with single-cell TCR sequencing will enable refining such analyses with respect to TCR specificity and clonality. Emerging technologies will also enable functional transcriptomic studies of Treg cell interactions with other immune and nonimmune cells. This will further improve our understanding of Treg cell development and function, Treg cell trajectories of differentiation and response, and the role in these processes of Foxp3 and other factors. However, beyond gene expression analysis, more direct assays of TF function and activity are needed.

Epigenomic and transcriptional regulation of Treg cells by Foxp3

Epigenomic profiling is crucial for studying mechanisms of gene expression regulation and is complementary to gene expression profiling. Chromatin immunoprecipitation sequencing (ChIP-seq) or Cleavage Under Targets and Release Using Nuclease (CUT&RUN) can be used for genome-wide identification of epigenomic features such as TF binding or histone marks (40–43). Assays such as DNase I hypersensitive sites sequencing (DNase-seq) or Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) provide an unbiased genome-wide view of chromatin accessibility; help characterize individual regulatory elements, such as promoters, enhancers, and repressors; and by employing publicly available TF binding sequence specificity information (e.g., in the form of positional weight matrices or motifs), can help characterize TF binding patterns (44–46). These assays are complemented by profiling of three-dimensional chromosomal organization using Hi-C or HiChIP (47–51). All these molecular modalities have been applied to Treg cells, particularly for further characterization of Foxp3 function.

Current estimates of Foxp3 genome-wide binding landscape range from 7000 to 11,000 peaks (Fig. 1B). Foxp3 belongs to a Forkhead family of TFs whose DNA sequence binding specificity is determined by the Forkhead motif. However, only a minority of Foxp3 peaks contain the Forkhead motif, suggesting that many Foxp3 peaks may be bound by Foxp3 indirectly and are indeed enriched with TCF, Ets, Runx, and other motifs (32, 33). This suggests that different Foxp3 binding sites may associate with distinct TF complexes, perhaps with distinct functions, e.g., activation or repression (9, 11) (Fig. 2). Indeed, analysis of differential expression between normal Treg and Foxp3-KO cells for genes at or near Foxp3 binding sites demonstrated that Foxp3 can both activate and suppress gene expression (36). A subset of Foxp3 peaks may also be associated with an alternative Foxp3 DNA-binding motif Forkhead-like that has been observed in vitro for other Forkhead family TFs (52).

Accessibility profiling in Treg and Tcon cells demonstrated a striking similarity in their epigenetic landscapes. A DNase-seq study found that 99% of regulatory elements are shared between Treg and Tcon cells (10). Of the 1% of Treg-specific enhancers, many occurred near canonical Treg genes, such as Ikzf2, Il2ra, and Lrrc32, suggesting that only a subset of active enhancers play a key role in determining Treg cell function. Subsequent analysis using H3K27ac HiChIP confirmed that activation of these canonical Treg genes is associated with significantly higher levels of Treg-specific three-dimensional chromatin looping (53). Genetic studies have confirmed that polymorphisms in Treg-specific enhancers are strongly associated with autoimmune diseases such as type 1 diabetes and colitis (54, 55). Therefore, despite broad similarity in the enhancer landscapes of Treg and Tcon cells, a key set of enhancers appears critical for establishing Treg cell identity.

Strikingly, despite its role as the Treg cell lineage-defining factor, Foxp3 appears to be only weakly associated with Treg-specific accessibility. One study found that as many as 98% of Foxp3 binding sites occur at enhancers shared between Tcon and Treg cells, with only 2% of Foxp3 binding sites occurring at Treg-specific enhancers (10).

A follow-up study leveraging DNase-seq in aTreg and resting Treg (rTreg) and $CD4^+$ T cells further explored the association between accessibility and Foxp3 binding (9). Most accessible sites in aTreg cells were also present in activated effector or naive $CD4^+$ T cells. However, Foxp3-bound sites showed lower accessibility in aTreg cells than in rTreg or effector T cells, and genes proximal to Foxp3-bound sites were repressed in aTreg cells. Immunoprecipitation experiments showed that Foxp3 interacted with the Polycomb repressive complex 2 in aTreg cells, but not rTreg cells, and Polycomb-mediated silencing was dependent on Foxp3, suggesting that Foxp3 complexes may poise accessible sites for repression during Treg cell activation.

An ATAC-seq analysis explored whether the enhancer landscape of Treg cells changes in progression from rTreg to aTreg to "inflammation-experienced" memory Treg cells isolated before, during, and after exposure to acute inflammation, respectively (17). Of ~34,000 accessible peaks in the genome, only 1159 peaks were differentially accessible in all pairwise comparisons, of which only a minority were stably maintained between aTreg and memory Treg cells. These results suggest that mature Treg cells resemble memory cells likely because of their developmental requirements for IL-2 and heightened TCR signaling. The majority of chromatin and transcriptional changes induced on Treg cell activation appeared to be transient, and "activation-experienced" Treg cells found in the secondary lymphoid organs returned to their basal state, with few long-lasting changes in their chromatin accessibility or gene expression related to cell migration to nonlymphoid tissues maintained after resolution of inflammation.

Given that Foxp3 appears to be weakly associated with differential accessibility, it is important to separate the direct and indirect effects of Foxp3 on chromatin accessibility and gene expression. For this purpose, a recent study implemented a powerful idea of functional genomic profiling in a hybrid F1

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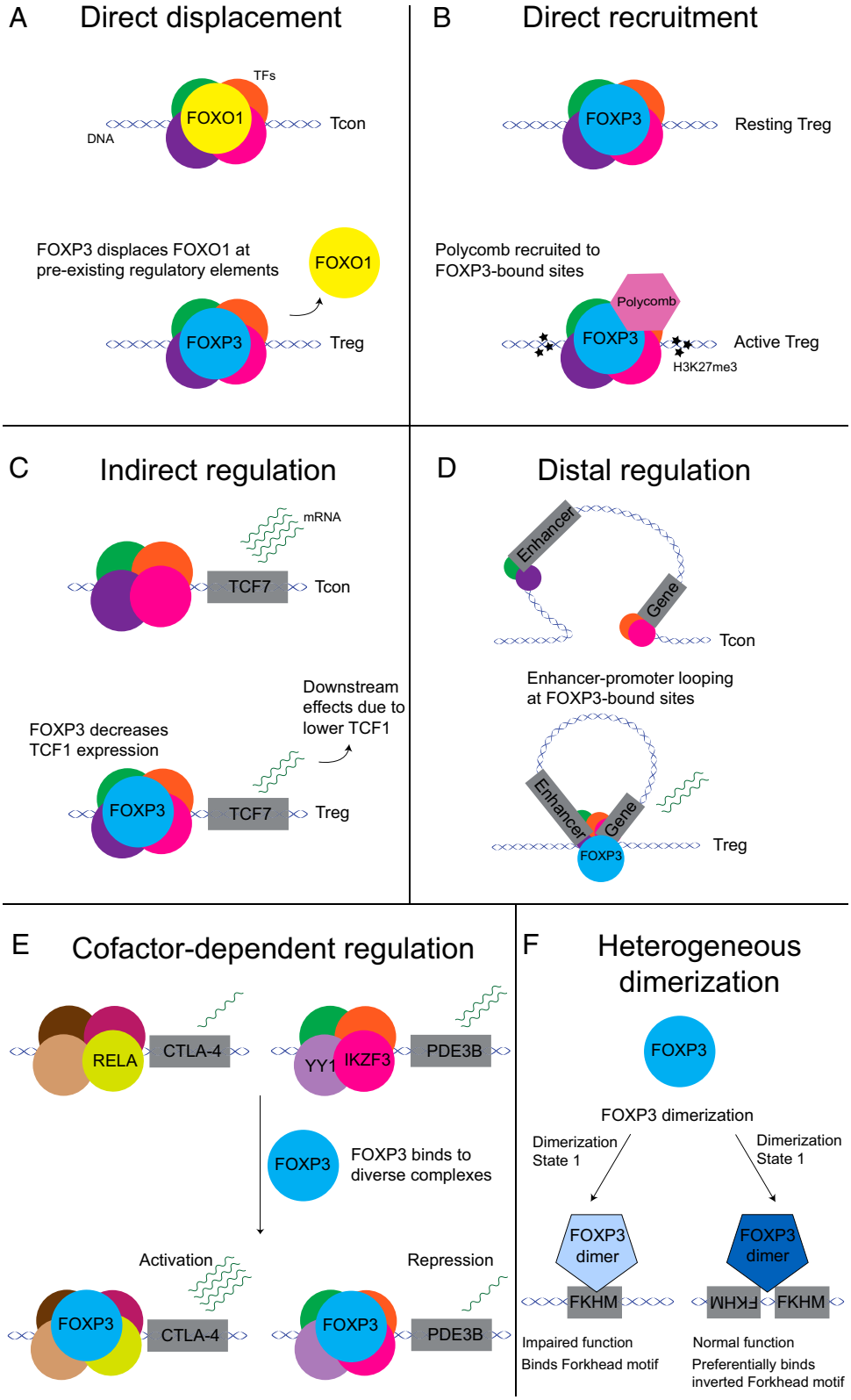

FIGURE 2. Putative mechanisms of Foxp3 function. (**A**) Direct displacement of TFs. Foxp3 competes with and displaces other TFs (e.g., Foxo1) at pre-existing regulatory elements (promoters, enhancers, or repressors) (10). (**B**) Direct recruitment of TFs. Foxp3 is poised at pre-existing regulatory elements in rTreg cells. During Treg cell activation, Foxp3 recruits the Polycomb complex to modify histones and repress gene expression (9). (**C**) Indirect regulation in *trans*. Foxp3 directly decreases expression of TCF1, resulting in indirect changes in gene expression (18). (**D**) Distal regulation at enhancer-promoter loops. Foxp3-bound sites engage in Treg cell–specific distal enhancer-promoter looping (53). (**E**) Cofactor-dependent regulation. Foxp3 is a member of different activating and repressive protein complexes (11). (**F**) Heterogeneous dimerization. Foxp3 can take different dimerization states with distinct motif binding preferences and functions (69).

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offspring of two well-characterized evolutionarily distant mouse strains (56, 57). This strategy provides a unique opportunity to leverage naturally occurring genetic polymorphisms between the strains for allele-specific analysis (18). Assignment of sequencing reads (e.g., from ATAC-seq, RNA-seq, ChIP-seq, or CUT&RUN) to one of the alleles (which is possible for a considerable fraction of reads) enables allele-specific quantification of functional signal. By statistically associating genome-wide allele-specific TF binding (as estimated from motif analysis or measured by ChIP-seq or CUT&RUN) with allele-specific accessibility and allele-specific gene expression, one can uncover *cis*-regulatory causal effects of TF binding on chromatin accessibility and gene expression while controlling for *trans* effects.

The study (18) used the F1 cross between the C57BL/6 strain and the wild-derived inbred mouse strain Cast/EiJ (Cast), which has roughly 20 million annotated genetic variants as compared with C57BL/6. Allele-specific analysis of ATAC-seq in rTreg and resting Tcon cells in these mice helped identify positive and, rarely, negative sequence-specific regulators of accessibility. Ets motifs appeared consistently associated with increased accessibility; other motifs were associated with differential accessibility only in a subset of peaks or cell-type comparisons. Despite determining Foxp3 binding to DNA, Forkhead motifs had a poor association with chromatin accessibility in rTreg cells. Instead, Sox motifs were significant positive regulators of accessibility in rTreg cells and were associated with stronger activity in rTreg "wannabe" as compared with rTreg cells. TCF1, a TF from the Sox family, had higher protein and transcript expression in rTreg wannabe than in rTreg cells (18). TCF1-encoding gene Tcf7 had higher accessibility in rTreg wannabes as compared with rTreg cells, except at the intronic site directly bound by Foxp3 in Treg cells. TCF1 CUT&RUN confirmed that sites with decreased TCF1 binding in rTreg cells demonstrated decreased accessibility as compared with Treg wannabe cells. This suggests that a substantial portion of Foxp3 activity may arise from regulation of TCF1 in *trans* (18).

Consolidating these findings with the increasing understanding of importance and molecular mechanisms of TCF1 function in other T cell subsets (58–63) can help to better understand the mechanisms of the Foxp3-TCF1 regulatory axis in Treg cells. Further exploration of the role of Foxp3 may require systematic Foxp3-dependent deletion of downstream Foxp3 targets to identify which of them are critical for Treg cell function and development. Functional genomic profiling in F1 hybrid mice may enable high-resolution analysis for disentangling multifactor regulatory mechanisms.

A separate axis of Foxp3 function is in its interactions, possibly both competitive and cooperative, with other Forkhead TFs. Analysis of DNase footprinting at Forkhead motifs revealed that Forkhead motifs were occupied both in Treg and Tcon cells, suggesting that other Forkhead factors may compete with and/or be replaced by Foxp3 in Treg cells (36). Strikingly, Foxp3 binding sites are bound by a number of different proteins, both in Treg cells (Runx1, Tcf1, Foxp1, Foxo1) and in other T lymphocytes (RORγt in Th17 cells) (64, 65).

ChIP-seq data analysis has confirmed the interplay between Foxp3 and other Forkhead TFs, including Foxo1 and Foxp1, which share many binding sites with Foxp3 (9, 34, 66). Foxo1 is upregulated in mature thymocytes and especially Treg cells. Foxo1 ChIP-seq in Treg cells suggested that Foxo1 binding was lower in Treg as compared with Tcon cells, possibly because of competition with Foxp3 (10). More broadly, the Foxo family of TFs may have synergistic effects for Treg cell development, perhaps through coordination with Foxp3 (67). Foxp1 is another Forkhead family factor expressed in Treg cells whose DNA-binding Forkhead motif is indistinguishable from the Foxp3 Forkhead motif. Foxp1 function in Treg cells was explored using ChIP-seq with appropriate genetic controls (34). Quantitative comparison of TF binding across genotypes was achieved via data normalization using background genomic regions. Unlike Foxo1, for which a subset of binding sites was replaced by Foxp3 in Treg cells, Foxp1 binding sites were largely shared between Treg and Tcon cells. In Treg cells, the majority of Foxp1 and Foxp3 binding sites were shared. Moreover, Foxp1 deletion led to statistically significant reduction of Foxp3 ChIP-seq signal genome-wide. A possible explanation is that the loss of Foxp1-Foxp3 heterodimers led to a redistribution of Foxp3 homodimers. Overall, Foxp1 has an essential nonredundant function in Treg cells by enforcing Foxp3-mediated regulation of gene expression (34, 68). An aggregated analysis of ChIP-seq and CUT&RUN data for Foxp3 and other Forkhead TFs across contexts could provide a more comprehensive picture of reproducible genome-wide Foxp3 and cofactor binding and its association with chromatin features in Treg cells.

Foxp1 and Foxp3 can form homodimers and heterodimers that enhance their binding (69–71). Preference of the Foxp3 dimers to bind to two Forkhead motifs in an inverted repeat separated by a 4-nt-long gap was observed in vitro and was associated with the head-to-head dimers, whereas swapped dimers had no such DNA-binding pattern (69). However, ChIP-seq data analysis showed no statistically significant enrichment for closely adjacent Forkhead motifs at Foxp1/Foxp3 peaks (18, 27, 29, 30). Therefore, the exact nature of sequence specificity of Foxp3 dimers and their interacting TF complexes in vivo remains incompletely understood. For example, Foxp1 and/or Foxp3 factors could mediate long-range chromatin interactions by bridging distant DNA elements with Forkhead motifs (71, 72).

Foxp3 HiChIP in Treg cells revealed 13,000 enhancer-promoter loops, but whether these are directly mediated by Foxp3 or Foxp3 exploits pre-existing looping organization remains unclear. Indeed, loops identified in Foxp3 HiChIP in Treg cells had high H3K27ac HiChIP intensity in Tcon cells, consistent with the notion that Foxp3 operates primarily at pre-established enhancers (53). This also suggests that properly normalizing HiChIP signal and disentangling multiple potential confounding effects, including from histone acetylation or TF binding levels, and using appropriate controls is critical for statistically accurate HiChIP data analysis, much like it is known to be critical for ChIP-seq data analysis. More unbiased methods for assaying chromatin architecture, such as Hi-C or Micro-C, would be useful for corroborating the results of HiChIP analysis. Furthermore, it will be important to reach a better understanding of what constitutes a three-dimensional chromatin interaction event of interest, e.g., whether multiple bin-to-bin interactions for proximal genomic bins should be considered multiple loops or a single looping event, and what other higher-order structural features are functional. Further integrating such analyses with other epigenomic data modalities, e.g., ChIP-seq, CUT&RUN, and CUT&Tag, currently available at much higher resolution than HiChIP or Hi-C, will help better understand the underlying molecular mechanisms of chromatin organization and the role of Foxp3 and other factors.

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Regulation of Foxp3 expression and function

Although the mechanisms of activation and regulation of Foxp3 in Treg cells remain incompletely understood, low- and high-throughput genomic and genetic analyses have led to multiple fundamental observations.

ATAC-seq analysis and conditional and constitutive enhancer targeting studies were leveraged to examine the activity of individual enhancers in the Foxp3 locus (73–75). Four Foxp3 enhancers, CNS0, CNS1, CNS2, and CNS3, appear to regulate different aspects of Foxp3 expression and maintenance. During Treg cell development, CNS0 and CNS3 enhancers are poised and are active in precursor cells. Binding of STAT5 to CNS0 and c-Rel to CNS3 facilitates induction of Foxp3 in response to IL-2 and TCR signaling, respectively. CNS1, although dispensable for Treg cell differentiation in the thymus, appears to facilitate extrathymic differentiation of Treg cells (1, 76). Finally, CNS2 was shown to serve as "maintenance enhancer" enabling continuous expression of Foxp3 in dividing Treg cells in the presence of limiting amounts of IL-2, an inducing cue promoting Treg cell differentiation (77). H3K27ac HiChIP in immature thymic CD4 single-positive T cells, thymic Treg precursors, and thymic Treg cells revealed activation and coordination of these Foxp3 enhancers during Treg cell development through gain or loss of physical contact with the Foxp3 promoter (73). Thus, proper activation of Foxp3 requires coordinated regulation of multiple enhancers, and uncovering the role of these enhancers in different cell contexts (such as aTreg and rTreg cells) may shed light on Foxp3 regulation. An unresolved question is the degree to which Foxp3 expression is reversible, i.e., whether Treg cells can undergo reprogramming into conventional Th cells. An understanding of which factors contribute to activation of the Foxp3 enhancers, as well as how they are maintained in various states of inflammation or immune dysfunction, will be critical to resolving these questions of Treg stability (78).

Foxp3 undergoes significant transcriptional and posttranscriptional regulation (79, 80). Although CRISPR genome editing in primary T cells faces unique challenges (81), several groups have leveraged high-throughput CRISPR screens to characterize regulators of Foxp3 in Treg cells (82, 83). These screens identified several novel complexes involved in modulation of Foxp3 expression, including the SAGA and SWI/SNF complexes. Specific hits included Usp22, Rnf20, and Brd9. Usp22, a member of the SAGA complex, is involved in deubiquitination of H2BK120, and knockout of Usp22 led to an increase of H2BK120ub ChIP-seq signal at the Foxp3 locus and many other sites in the genome, including at Foxp3-bound sites (82). In addition, Usp22 appears to deubiquitinate Foxp3 itself, suggesting that regulation may occur both on transcriptomic and proteomic levels (82). Brd9, a member of the ncBAF SWI/SNF complex, was also found to play an important role in Foxp3 regulation (83). Brd9 KO diminished Foxp3 binding across the genome, either because of diminished levels of Foxp3 itself or because of loss of specific interactions between Brd9 and the Foxp3 protein.

Together, these results demonstrate the value of high-throughput screens and suggest that regulation of Foxp3 involves diverse factors. Genetic screens with additional more informative readouts, such as expression of Foxp3 target genes, rather than just Foxp3 itself, or screens probing differential exon and isoform usage or posttranscriptional regulation, may be useful for further uncovering complex regulatory mechanisms of Foxp3 expression and function.

Conclusions

It has been more than two decades since Foxp3 was first discovered as the critical protein responsible for differentiation and function of Treg cells. However, the molecular mechanisms by which this singular TF defines the Treg cell identity remain incompletely understood. The case study of Foxp3 therefore exemplifies the myriad challenges involved in attaining a complete explanation for how a TF confers its biological effects. "Solving" the function of Foxp3 would represent an advance in basic T cell biology with therapeutic implications and provide a blueprint for how to solve the function of other lineage-defining TFs.

High-throughput genomics has been an essential tool for understanding the function of Foxp3 and related fundamental problems in Treg cell biology. The tools described earlier were used to describe gene expression profiles of diverse cell types at a bulk and single-cell level; to assay the binding sites of a range of TFs; to examine genome-wide chromatin accessibility and epigenetic state and define regulatory elements; and to determine physical contacts between regulatory regions. These and yet unseen next-generation genomics technologies applied in sophisticated mouse models, combined with rigorous computational analyses, will continue to yield insights into Foxp3 and Treg cell function. We anticipate that next-generation technologies will help us more accurately define the regulatory mechanisms of Foxp3 expression during Treg cell development, differentiation, activation, and function, as well as during interaction with other immune and nonimmune cell types across tissues; will enable studying the relationship between Foxp3 function and TCR specificity and activation at a single-cell level; will provide comprehensive characterization of the dynamics of direct and indirect context-specific interactions of Foxp3 protein with TF cofactors and the chromatin; and will help better connect these characterizations with the downstream effects on chromatin features and gene expression associated with Treg cell phenotypes.

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