COMMENTARY

DNA bridging of FOXP3 ladder-like multimer: Unveiling a novel transcriptional regulation paradigm

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In the latest issue of *Nature*, Zhang et al. characterized a novel ladder-like structure of FOXP3-DNA interaction involving FOXP3 multimerization and remote DNA bridging through a combination of biochemistry, structural biology, cell biology, and bioinformatics analyses.[1] In this commentary, we highlight their key findings and provide our insights into the research paradigm for further exploration of a novel transcriptional regulation mode as well as a therapeutic strategy from the structural aspects of the FOXP3 complex (Figure 1).

Regulatory T (Treg) cells are a subset of T cells with suppressive function that are indispensable for both immune tolerance and immune homeostasis. FOXP3 serves as a lineage-specific transcription factor for Treg cells. It recognizes and binds to specific DNA sequences to orchestrate gene expression in the development and programming of Treg cells. FOXP3 mutations result in aggressive autoimmune disease in both scurfy mice and human patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, demonstrating the fate-determining function of FOXP3 in Treg cells.[2] Clarifying the distinct modes of FOXP3 binding to Treg signature genes can provide structural and mechanistic insights into Treg biology and facilitate the development of novel therapeutic strategies to modulate Treg cells in human diseases.

FOXP3 is a forkhead transcription factor family member containing a conserved forkhead motif binding domain at the C-terminal region. However, how these limited consensus modules achieve the functional diversity of Treg cells remains elusive. Several studies have been conducted to determine the different means of FOXP3 assembly to orchestrate the dynamic gene regulatory network of Treg cells. FOXP3 was found to interact with various binding partners and chromatin remodelers to modulate the expression of numerous target genes, such as to upregulate *Cd25*, *Ctla4*, and *Gitr* or to repress *Il2* and *Ptpn22*.[2] In addition, FOXP3 has been shown to form a domain-swapping structure of dimerization, which facilitates chromatin remodeling and long-distance DNA interactions.[3] In a study published in *Immunity* last year, Dr. Hur's team characterized a head-to-head (H-H) conformation of FOXP3 through the recognition of inverted-repeat forkhead motif in a single DNA molecule under physiological conditions.[4] Given the rarity of this motif in FOXP3 chromatin immunoprecipitation sequencing data, researchers are eager to explore novel FOXP3-DNA binding modes. Notably, FOXP3 was innovatively proposed to form homo-oligomers within a super molecular complex, with oligomerization playing a critical role in the binding affinity of FOXP3.[5] In this article, Zhang et al. demonstrated the precise structure and mechanism underlying FOXP3's multimerization. Unlike commonly held beliefs that the DNA-binding domain functions as a domain-swapped or H-H dimer, the cryoelectron microscopy (cryo-EM) structure elucidated that the FOXP3 multimer forms a unique ladder-like geometry. This structure is composed of two DNA molecules creating sidebars connected by five rungs. Each rung comprised two FOXP3 subunits that attach to the DNA fragments enriched with T_nG repeats. FOXP3 complex assembly provides novel insights into the high-order chromatin architecture and transcriptional modulation feature with high efficiency and flexibility. Targeting the interaction sites among FOXP3 proteins and modifying the FOXP3-DNA binding hold promise to coordinate the suppressive function of Treg cells and treat autoimmune diseases and cancer.

To determine the DNA-binding motifs of FOXP3, Zhang et al. conducted the FOXP3 pull-down assay coupled with next-generation sequencing and *de novo* motif analysis. They demonstrated that T_nG repeats are functional FOXP3 binding elements. In addition, they examined the affinity of T_nG repeats and revealed the preference of FOXP3 for T_3G binding. Subsequently, the authors examined the structure of FOXP3-T_3G repeats with single-particle cryo-EM and found that the two strands of DNA molecules with long spatial distances were pulled together by FOXP3 proteins, forming a ladder-like architecture. Meanwhile, they also observed that FOXP3 between ladders could interact with DNA in an alternating pattern of 8 and 12 bp intervals (inter-rung^{8bp} and inter-rung^{12bp}). These results demonstrated the crucial role of the interaction between 8 bp intervals in the formation of DNA-bridging filamentous aggregates. Although the mutations of the inter-rung^{12bp} exhibited only a small effect on the formation of the aggregates, the binding between FOXP3 and DNA returned to a moderate level when the number of interval sequences increased to about one helical periodicity. Such a periodic pattern revealed that the assembly of the FOXP3 multimer is not restricted by the precise position of FOXP3, indicating the structural flexibility of FOXP3.

The same study revealed that FOXP3 can bind to not only perfect T_3G repeats but also to an extended range of T_nG-repeat-like elements. Through further experiments, researchers have demonstrated that "imperfect T_3G repeats" can enhance the binding of FOXP3 to these suboptimal T_nG repeats by bridging with T_3G repeats. This discovery expands the range of DNA sequences that FOXP3 may specifically bind to as a transcription factor. Analysis of 3D genomic data indicated that the oligomeric structure of FOXP3 can facilitate long-range contact between T_nG repeat microsatellites. This enables the formation of enhancer-promoter loops and thereby regulates the expression of target genes and Treg functionality. Such a unique mechanism challenges our traditional understanding of the function of FOXP3 protein and offers a new perspective on how Treg cells regulate gene expression during immune responses.

Compared to the previous findings, both the intra- and inter-rungs between FOXP3 in adjacent ladders differ from the H-H dimer conformation. Furthermore, the authors investigated whether intra-rung interactions affect T_nG recognition, and the biochemical analysis indicated that the intra-rung interaction of FOXP3 in the ladder is essential for the formation of DNA-bridging filamentous aggregates. Cellular experiments further demonstrated that these intra-rung mutations could disrupt FOXP3-induced gene expression and the suppressive function of Treg cells. Extending their findings to different species and transcription factors, Zhang et al. showed that ladder-like multimerization and T_nG repeat recognition are evolutionarily conserved and shared among four FOXP transcription factors.

Overall, these discoveries unveil a novel mechanism of DNA recognition that encompasses the homomultimerization of transcription factors and DNA bridging, underscoring the involvement of microsatellites in transcriptional regulation and diseases. To be noted, previous studies commonly overlooked the biological function and implication of microsatellite sequences, while Zhang et al. suggested that microsatellites are indispensable for the binding affinity and accuracy of transcription factors. Meanwhile, microsatellite variations may be associated with gene expression alterations in homeostasis and disease.

The research team highlighted the enormous potential that these findings hold in biology and clinical applications while also acknowledging new challenges and areas of inquiry. Questions such as how to precisely regulate the FOXP3 activity under physiological and pathological contexts, as well as the impact of this

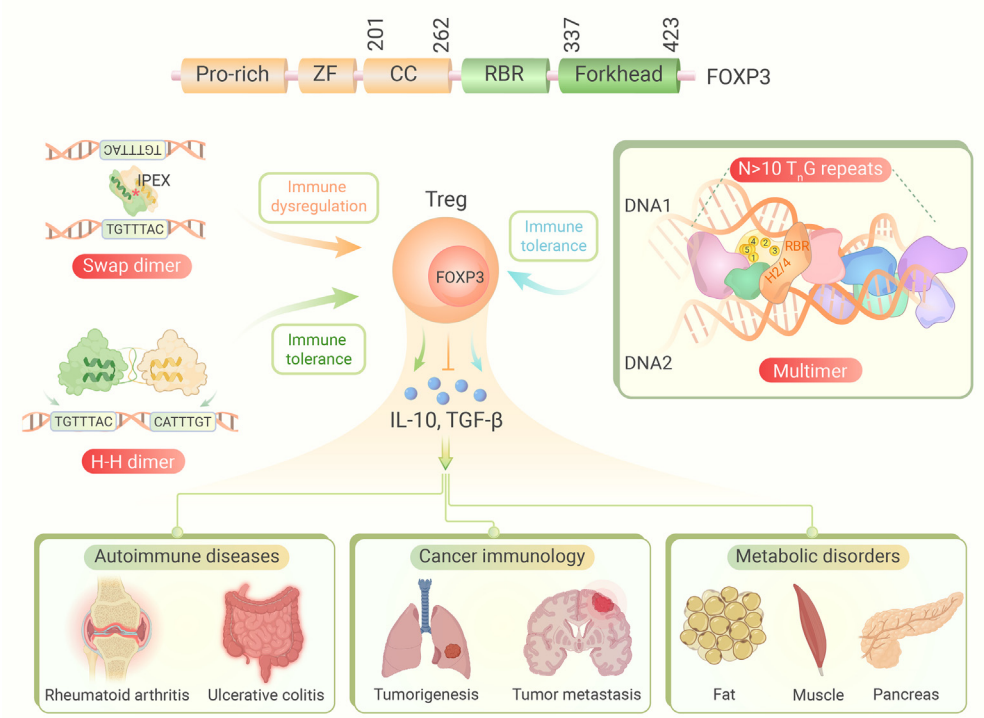

Figure 1. Different conformations of FOXP3 in Treg cells and potential prospect on immune diseases treatment (Top) The schematic diagram of mouse FOXP3 subdomains. ZF, zinc finger; CC, coiled coil; RBR, Runx1-binding region. (Middle left) The schematic model of FOXP3-swapped dimer conformation, along with mutations indicated by a red asterisk, triggering Treg dysfunction in immune dysregulation, polyendocrinopathy, enteropathy, X-linked disease. Under physiological conditions, FOXP3 can fold into a head-to-head (H-H) dimer conformation and maintain immune homeostasis. (Middle right) Zhang et al. clarified that the FOXP3 multimer binds to two DNA molecules enriched with T_nG repeats and forms a ladder-like structure critical for enhancer-promoter loop formation and transcriptional regulation. Functional residues in the intra-rung interface are shown as yellow spheres (1, Arg356; 2, Val396; 3, Val398; 4, Val408; 5, Asp409/Glu410/Phe411). Mutations of these residues will disrupt T_nG repeat binding and DNA bridging of FOXP3. (Bottom) Based on insights into FOXP3 structure, the therapeutic implications of immune diseases, including autoimmune diseases, cancer immunology, and metabolic disorders, are prospected.

regulation on other components of the immune system, require further exploration. Additionally, this study underscores the significance of inter-disciplinary research in the biomedical field, particularly the integrated application of biochemistry, structural biology, cell biology, and bioinformatics. Such an interdisciplinary approach can achieve a more comprehensive understanding of the functionalities of proteins or other biomolecules, laying a solid foundation for unraveling their biological mysteries and developing innovative therapeutic strategies.

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DECLARATION OF INTERESTS

The authors declare no competing interests.