

# FOXP3 and its partners: structural and biochemical insights into the regulation of FOXP3 activity

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**Abstract** Forkhead box protein P3 (FOXP3) contributes to a unique transcriptional signature and serves as a functional marker of CD4<sup>+</sup>CD25<sup>+</sup> natural regulatory T cells. Dysfunction of FOXP3 in human is associated with fatal autoimmune disease known as immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) or X-linked autoimmunity–allergic dysregulation syndrome (XLAAD). FOXP3 also can act as a breast tumor suppressor of the v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (neuro/glioblastoma derived oncogene homolog (avian)) (Her2/neu) gene. While the suppressive functions of FOXP3 in maintaining the immune balance between tolerance and autoimmunity are obvious, the underlying molecular mechanism remains almost entirely undefined. Recent studies indicate that FOXP3 may form a dynamic superamolecular complex with a variety of molecular partners including transcription factors and enzymatic proteins to regulate transcription. How the FOXP3 ensemble changes in response to T-cell receptor signals and/or proinflammatory signal remains unclear although work from this laboratory has revealed its complexity. Structural information on FOXP3 complex may offer novel functional insights, as well as facilitate the development of rational means to modulate regulatory T-cell function in various human diseases.

**Keywords** Protein structure · Regulatory T cell · FOXP3 · Autoimmunity · Cancer

## Introduction

FOXP3 belongs to the forkhead/winged-helix family of transcription factors and plays a critical role by controlling immune homeostasis mediated by CD4<sup>+</sup>CD25<sup>+</sup> natural regulatory T cells (Treg) [1]. Mutations of *FOXP3* gene are responsible for the disease called

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immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [2, 3] or X-linked autoimmunity–allergic dysregulation syndrome (XLAAD) [4] in humans and the Scurfy phenotype [5] in mice.

Much effort has been focused on understanding the role of FOXP3 as a transcription repressor in Treg development and function. FOXP3 interacts with a range of partner proteins including transcription factors such as Forkhead box protein P1 (FOXP1) [6, 7], Nuclear factor of activated T cells (NFAT) [8, 9], nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- $\kappa$ B) [9], runt-related transcription factor 1/acute myeloid leukemia 1 (RUNX-1/AML1) [10] and Activator protein 1 (AP-1) [11], and post-translational modification enzymes such as histone acetyltransferase HIV-1 Tat interacting protein, 60 kDa (TIP60) [12] and histone deacetylase HDAC1, HDAC7, and HDAC9 [13]. FOXP3 may form differential dynamic multi-protein/DNA complexes to regulate gene transcription. Recently, FOXP3 has been identified as a breast cancer suppressor of the Her2/neu gene and FOXP3 expression is observed in epithelial cancer cells [14–18].

Despite the emerging role of FOXP3 in immunity, inflammation, and cancer, its mechanism of action currently remains unclear. FOXP3 may cooperate with multiple partners to modulate gene transcription and regulatory T-cell function. This review focuses on the structural aspects of the dynamic ensemble of FOXP3 associated protein/DNA complexes required for its physiologic activity.

## An overview of FOXP3 subdomains

The forkhead/winged-helix box-containing protein P subfamily (FOXP) transcription factor has four members including FOXP1, FOXP2, FOXP3, and FOXP4. Both FOXP3 and FOXP1 are expressed in regulatory T cells [6]. All the FOXP proteins contain conserved tandem zinc-finger-leucine-zipper oligomerization domain, and a highly conserved forkhead DNA-binding domain [6, 9, 19–21] (illustrated in Fig. 1). FOXP3 has a unique N-terminal proline-rich domain, which is distinct from the N-terminal Glutamine-rich domains of other FOXP subfamily members including FOXP1, FOXP2, and FOXP4. Two isoforms of FOXP3 proteins have been found in humans, namely FOXP3a and FOXP3b [12, 22, 23]. FOXP3b lacks exon 2 encoding region which is critical for its association with transcription factors ROR $\alpha$  [24] and ROR $\gamma$ t [25]. A range of FOXP3 mutations causing IPEX or involved in breast cancer have been extensively mapped over each functional domain encoding sequence [16, 23].

Structure-wise forkhead domain of FOXP3 has been best studied because of its high level of homology to that of FOXP2 which has been crystallized [26]. The forkhead domain of the FOXP subfamily is very divergent from those of the classical forkhead domains seen in other members of FOX family. A salient feature of the FOXP3 forkhead domain is that wing 1 may be partially truncated, and wing 2 appears to be converted from a loop to a helix.

The forkhead domain of FOXP2 exists in either a monomeric state or domain-swapped dimeric state [26]. However, the replacement of alanine 539 in FOXP2, a conserved residue in FOXP subfamily, to a proline that is found at the equivalent position of other FOX family members, renders the protein exclusively monomeric, suggesting that domain swapping is an evolutionary feature uniquely developed for the P subfamily of forkhead transcription factors. IPEX syndrome mutants F371C and F371L were predicted to disrupt the domain-swapped dimer. This is intriguing since FOXP3 has been thought to homo-dimerize [19, 20] and homo-oligomerize [6] through its leucine zipper motif. We have found that the FOXP3 N-terminal transcriptional repression domain can also self-associate

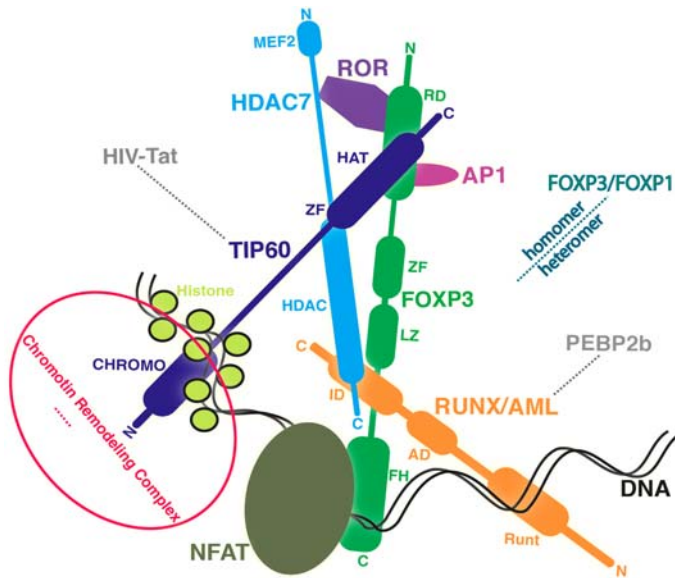


**Fig. 1** Schematic illustration of the FOXP3 associated multiprotein/DNA complex in regulatory T cells. FOXP3 may directly and indirectly recruit various transcription factors including FOXF1, NFAT, NF $\kappa$ B, AP-1, AML1/RUNX1, ROR $\alpha$  and ROR $\gamma$ t, and other enzymatic proteins such as TIP60 and HDAC7, to site-specific chromatin for transcriptional regulation in FOXP3<sup>+</sup> regulatory T cells. The dynamic ensemble of FOXP3 with its partners may be tightly regulated by T-cell receptor signals, co-stimulation signals, anti- or pro-inflammatory cytokines. Structural studies on FOXP3 complex ensemble will provide molecular basis for rational design of small molecular inhibitors to modulate FOXP3 activity and Treg function. Note that both FOXP3 and Runx1 can form homo-oligomers through the Leucine zipper domain and the C-terminal domain, respectively. For simplicity, however, only one copy of the related subunits was shown

to form oligomers (Song and Greene, unpublished). Thus, it is possible that multiple subdomains of FOXP3 may cooperate with each other to facilitate its homo-oligomerization.

So far the biological implications of domain swapping found in the forkhead domain of FOXP2 remain undetermined. Structural information of FOXP3 itself will help to understand the exact DNA-binding mode and the oligomerization mechanism.

### DNA-binding features of FOXP3

Members of the FOX protein family do not employ a single consensus sequence but rather follow a general pattern: 5'-RYMAAYA-3' (R = A or G; Y = C or T; M = A or C) [27]. Considering that the forkhead domain is highly conserved, the structural study of FOXP2 forkhead domain complexed with DNA has provided important insight regarding how FOXP3 may bind to DNA [26]. By wedging helix H3 into the DNA major groove, FOXP2 interacts with DNA predominantly through shape complementarity with only a few hydrogen bonds, predicting a relatively lower binding affinity. FOXP3 Mutants in human IPEX syndrome including I363V, A384T and R397W were shown to affect the protein–DNA binding through varied nonoverlapping mechanisms [8].

Analysis of FOXP2–DNA complex structure has indicated that the sequence of the forkhead domain from the FOXP subfamily is highly conserved with identical DNA-binding residues, but distinct from other members of the forkhead family. Sequence alignment

revealed that there are only several variable residues in this domain. Interestingly, these varied residues are located outside of the DNA-binding interface. Thus, it raises an immediate question: what distinguishes FOXP3 from other FOXP members in terms of DNA binding? Is it because of its interactions with NFAT1 or other DNA-binding partners that collectively control DNA binding?

Structural studies of other forkhead family members provided molecular insights into conformational changes upon DNA binding: a substantial DNA bend is induced while only minor structural changes occur in the forkhead domain [28, 29]. Since no individual structures is available for either the forkhead domain alone or the target DNA fragment, further study is required to verify whether this is the case for FOXP3.

So far the potential consequences of FOXP3 self-oligomerization and its influence on DNA binding have not yet been defined. Study of FOXP2 revealed a domain-swapped dimer that can bind two copies of DNA simultaneously [26]. The authors then suggested that under physiologic concentrations, forkhead domain swapping may be facilitated by the zinc finger/leucine zipper oligomerization domain in the full-length protein, and therefore play a functional role in DNA looping or chromosome modification.

### **FOXP3 cooperates with NFAT via its forkhead domain-mediated DNA binding**

NFAT is a key transcriptional regulator for both T-cell activation and T-cell tolerance [30–33]. In activated T cells, NFAT binds to DNA by forming cooperative complex with AP-1 [33].

Recent studies have shown that FOXP3 may also cooperate with NFAT through its forkhead domain to form a DNA-binding complex that is important for Treg function [8]. This complex is structurally analogous to the NFAT:AP-1:DNA regulatory element. The crystal structure of NFAT:FOXP2:DNA complex revealed a primarily polar interface with small hydrated pockets between the DNA-binding domains of NFAT and FOXP2, suggesting that FOXP3 may not form stable complex with NFAT in the absence of target DNA. According to the structural comparison between FOXP2:DNA and FOXP2:NFAT:DNA complexes, the FOXP3-binding site on the target DNA is shifted from the consensus site A to a less favorable nonconsensus site B upon NFAT cooperation. Notably, mutations on the predicted NFAT:FOXP3 interface disrupt FOXP3 function as a transcriptional repressor in Treg.

During the formation of the FOXP3:NFAT:DNA complex, FOXP3 might compete with AP-1 as they bind to the same region of the target DNA. However, competition alone is not sufficient for ensuring Treg suppressive function since deletion of the FOXP3 N-terminal region abrogates its modulation effects on gene expression. The FOXP3 N-terminal domain is required for recruiting other co-regulatory partners, which indicating that FOXP3-NFAT cooperation is only part of a bigger picture. The potential consequences of FOXP3 homo-association and association with other transcriptional co-repressors and co-activators on its DNA binding and interactions with NFAT need to be further investigated.

### **Phosphorylation dependent interaction of AP-1 with the N-terminus of FOXP3**

Fang and colleagues recently found that FOXP3 interacted with c-JUN/AP-1 both in an ectopically expressed system and at the endogenous level in Treg cells [11]. JNK-mediated c-JUN/AP-1 phosphorylation and activation is required for their interaction. FOXP3

altered the nuclear distribution pattern and DNA-binding activity of activated c-JUN/AP-1. Moreover, FOXP3 associates with JNK activated c-JUN/AP-1 via its N-terminal non-DNA-binding domain. Interestingly, the N-terminal region of FOXP3 is sufficient for FOXP3-mediated T-cell anergy, but not suppression, which is independent of its forkhead domain mediated DNA binding [11]. FOXP3 sequestered activated AP-1 somewhere in the nucleus, alternated the subnuclear distribution of activated AP-1 so that the activated AP-1 could not bind to chromatin [11]. It is unclear whether phosphorylated c-JUN could disrupt FOXP3:NFAT:DNA complex formation. Future structural studies will provide molecular insight into the stoichiometry of FOXP3-AP-1-NFAT ternary complex.

### **FOXP3 interacts with transcription factor AML1/Runx1**

The Runx transcription factors are also known as AML, CBF $\alpha$ , or PEBP2 $\alpha$ . The domain organization of Runx proteins includes a N-terminal Runt domain that is critical for DNA binding, a C-terminal activation domain (AD) as well as an inhibition domain (ID). Structural studies of Runx1 Runt domain and its complexes with CBF $\beta$  and DNA revealed a closed-to-open conformational transition that is crucial for DNA binding [34–37]. Runx1 may also form homodimers through its C-terminal region, which affects its activity and function [38].

FOXP3 has been shown to interact with AML1/Runx1, and probably also with AML2/Runx3 and AML3/Runx2 [10]. The conserved ID shared by all three members of the Runx protein family is essential for the interactions between AML1 and FOXP3. The loop region between the leucine zipper and forkhead domains of FOXP3 is essential for RUNX binding.

We suggest that FOXP3 and Runx1 may bind to DNA at the same time with their DNA-binding domains disposed in a similar manner as seen in the Foxp2-NFAT-DNA complex. Other regions of the two proteins may interact to facilitate this cooperated DNA binding for transcription regulation. Meanwhile, DNA binding of both proteins could, in turn, stabilize the interactions between FOXP3 and Runx1. However, it remains unclear how the homooligomerization of FOXP3 and the dimerization of Runx1, as well as the conformational change of the Runt domain influence FOXP3-Runx1 interactions and therefore their cooperation in DNA binding.

It is also possible that other factors such as HDAC7 and NFAT may affect the interactions between FOXP3 and Runx through modifying the conformation of FOXP3. Notably in this context, the C-terminal region of HDAC7 can directly interact with the inhibition domain (ID) of Runx2 [39]. Since the ID domain is also involved in the FOXP3-Runx interactions, thus the association of Runx proteins with HDAC7 and FOXP3 could be competitive. On the other hand, HDAC7 inhibits the transcriptional activity of Runx2 through its N-terminal domain in a deacetylation-independent manner [39]. It would be interesting to test whether this kind of inhibition will affect the suppressor function of FOXP3-centered complex.

### **FOXP3 interacts with ROR $\gamma$ t through exon 2 region**

FOXP3 may also physically interact with retinoic acid receptor (RAR)-related orphan receptor (ROR) family transcription factors including ROR $\gamma$ t and ROR $\alpha$  [24, 25, 40]. As a subfamily member of nuclear receptors, the thymus-specific expressing isoform, ROR $\gamma$ t, is critical for the development and function of Th17 cells [41, 42]. Moreover, Yang et al. have

found that another subfamily member of ROR proteins, ROR $\alpha$ 4, also expressed in Th17 cells may cooperate with ROR $\gamma$ t in the regulation of Th17 differentiation [43]. Full length FOXP3, but not the short isoform lacking exon 2 interacts with ROR $\alpha$  and inhibits ROR $\alpha$ -mediated transcriptional activation [24]. This interaction is dependent on the LxxLL motif within the exon 2 encoding region of FOXP3, but not on the forkhead domain.

The FOXP3 binding region of ROR $\alpha$  has been mapped to the c-terminal AF2 motif. FOXP3 also interacts with ROR $\gamma$ t and inhibits ROR $\gamma$ t-directed IL-17 expression. The balance between FOXP3 and ROR $\gamma$ t expression and function may regulate the lineage differentiation of either Th17 or Treg [25, 40]. Detailed biochemical and structural studies of the FOXP3-ROR interaction will contribute to understanding the mechanism by which FOXP3 antagonizes ROR $\gamma$ t function in Th17 cell differentiation.

### **FOXP3 associates with enzymatic protein TIP60 directly through its proline-rich domain**

HIV-tat interacting protein TIP60 is a multifunctional acetyltransferase that plays important role in divergent biological processes including cellular signaling, DNA damage repair, cell cycle, and apoptosis [44, 45]. As a transcriptional co-regulator, TIP60 is extensively involved in many transcription-regulating complexes for enzymatic modification of associated protein factors and/or chromatin [44, 45]. A recent study of oncogene-induced DNA damage response reveals that TIP60 suppresses tumorigenesis in a haplo-insufficient and p53-independent manner [46].

It has been shown that TIP60 associates with HDAC7 through its conserved N-terminal zinc finger-containing region and HDAC7 activity is required for the repressive effect of TIP60 [47]. Crystal structure of the TIP60 acetyltransferase HAT domain showed that the zinc finger motif is positioned aside from the wall of the substrate binding cleft (PDB code: 2OU2, Wu H et al. unpublished), suggesting that this region is involved in the coordination between acetylation and deacetylation. At this point, however, further work is required to elucidate how TIP60 and HDAC7 cooperate or compete with each other in the FOXP3-mediated suppressor complex to regulate gene transcription.

We have reported that TIP60 promotes FOXP3 acetylation and knockdown of endogenous TIP60 relieves FOXP3-mediated repression in vivo [12]. Recently, we have also discovered that the N-terminal proline-rich domain of FOXP3 directly interacts with the C-terminal HAT domain of TIP60 (Li and Greene, unpublished data). Structural studies of FOXP3-TIP60 complex will provide molecular bases for rational design of small molecules to modulate FOXP3 function.

### **The role of TIP60 in FOXP3–HDAC7 interaction**

Histone deacetylases (HDACs) catalyze protein deacetylation, a reverse process of protein acetylation that is carried out by histone acetyltransferases (HATs) such as TIP60. It is thought that multiprotein regulatory complexes may recruit HATs and HDACs to regulate the acetylation of lysine residues in the N-terminal tails of core histones, and therefore modify the local structure of chromatin, resulting in either activation or repression of gene transcription [48].

Dysfunctions/abnormalities of HATs and HDACs have been found in a variety of diseases such as leukemia and diabetes [49, 50]. Human HDACs are divided into four classes

according to sequence similarity. As a class II member, HDAC7 is expressed in a tissue-specific manner and its subcellular localization is differentially regulated depending on cell type and signaling [48, 51, 52]. HDAC7 may regulate the expression of various genes which play important roles during cell differentiation and development [53].

Recently, the crystal structure of the catalytic domain of human HDAC7 has been determined, offering new mechanistic insights into class II HDACs function in health and disease [54]. Intriguingly, the structure of HDAC7 shows a novel zinc-binding motif (CCHC-motif) that could be involved in association with additional factors and/or regulatory proteins. This is consistent with the notion that only class II HDACs becomes enzymatically active upon association with appropriate factors in a multiprotein complex [53, 55, 56].

In this regard, TIP60 is an interesting candidate that may regulate acetylation/deacetylation activity through interactions with HDAC7 under the context of FOXP3-mediated suppressor complex. Moreover, Xiao et al. showed that the region containing amino acid 241–533 in HDAC7, located N-terminal to the catalytic domain, is essential for association with the zinc finger region (aa 261–366) in TIP60 [47].

We found FOXP3 associated with HDAC7 in an oligomerization-independent manner [12]. Deletion of the N-terminal proline-rich repression domain (amino acid 107–190) of FOXP3 abolished its association with both HDAC7 and TIP60. Furthermore, knockdown of TIP60 increased the amount of HDAC7 associated with FOXP3 (Li and Greene, unpublished data). Thus, it appears that the binding of TIP60 and HDAC7 to FOXP3 is dynamic and competitive. Moreover, this process may involve the interactions between TIP60 and HDAC7. TIP60 itself is also an acetylated protein [57] (Li and Greene, unpublished data). It is unclear whether HDAC7 directly deacetylates either TIP60 or FOXP3 *in vivo*. Future structural and biochemical studies are required to elucidate how TIP60 and HDAC7 may interact with each other to affect their association with FOXP3 and therefore modify the function of regulatory T cell.

## Conclusion

Recent biochemical studies have firmly indicated that FOXP3 does not function alone, but rather coordinates with multiple transcriptional regulators in a dynamic assembled supermolecular complex (illustrated in Fig. 1). In addition to the partners mentioned above, many other factors including histones, chromatin remodeling enzymes, RNA binding proteins, molecular chaperones and transcription factors may directly or indirectly interact with FOXP3 in a dynamic manner in response to extracellular stimuli [58].

Depending on the physiologic status of expression, activation, and subcellular localization of the binding partners as well as FOXP3 itself, FOXP3 may form differential transcription-regulating complexes through combinatorial interactions with these partners in response to physiologic stimuli, such as T-cell receptor stimulation, cell surface receptor costimulation, and anti- or pro-inflammatory signaling. Structural and biochemical insights into these complex ensembles will deepen our understanding of FOXP3 function and facilitate the development of potential applications under disease conditions.

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