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Review
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FOXP3 and Its Cofactors as Targets of Immunotherapies

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ABSTRACT

Forkhead box P3 (FOXP3) is a "master regulator" of regulatory T cells (Tregs), which are a subset of T cells that can suppress the antigen-specific immune reaction and that play important roles in host tolerance and immune homeostasis. It is well known that FOXP3 forms complexes with several proteins and can be regulated by various post-translational modifications (PTMs) such as acetylation, phosphorylation, ubiquitination, and methylation. As a consequence, the PTMs change the stability of FOXP3 and its capability to regulate gene expression, and eventually affect Treg activity. Although FOXP3 *per se* is not an ideal drug target, deacetylases, acetyltransferases, kinases, and other enzymes that regulate the PTMs of FOXP3 are potential targets to modulate FOXP3 and Treg activity. However, FOXP3 is not the only substrate for most of these enzymes; thus, unwanted "on target/off FOXP3" side effects must be considered when inhibitors to these enzymes are used. In this review, we summarize recent progress in the study of FOXP3 cofactors and PTM proteins, and potential clinical applications in autoimmunity and cancer immunity.

1. Forkhead box P3 as a "master regulator" of regulatory T cells

Regulatory T cells (Tregs) are the dominant subset of T cells that suppress host immunity. They were first described as a cluster of differentiation (CD)$4^{+}CD25^{high}$ T cell population [1], and were later found to dominantly express the forkhead box P3 (FOXP3) protein [2]. The history of FOXP3 has been well summarized in a review written by Ramsdell and Ziegler [3]. Since the identification of Tregs, an enormous number of studies have been conducted to reveal the role and function of this unique T cell subset.

FOXP3 is a transcription factor belonging to the forkhead box family of transcription factors. It contains three major domains: the N-terminal domain, which is called the "repressor domain" [4]; the zinc finger and leucine zipper domain (ZL domain), which is centrally located and facilitates the formation of FOXP3 homodimers, or heterodimers with other FOXP family members such as FOXP1 and FOXP4 [5]; and the highly conserved forkhead (FKH) domain at the C terminus, which is responsible for DNA binding.

The *Foxp3* gene has been cloned as a causal gene for scurfy mice [6] and for immunodysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome in humans [7,8]. Scurfy mice have 2 base pairs (bp) insertions in the exon 8 of *Foxp3*, which results in malfunction of the FOXP3 protein and the death of mice at approximately three weeks of age [6]. On the other hand, more than 70 *FOXP3* mutations have been found in IPEX patients thus far [9–11]. The majority of these mutations are located in the FKH domain, but they are also found throughout the entire protein, indicating that all of the domains in FOXP3 could be important for its immune-suppressing function.

FOXP3 is known to form large complexes involving many proteins. Li and Greene [12] were the first to discover that FOXP3 forms large complexes ranging in size from 300 kDa to more than 1200 kDa. Later, using biochemical and mass spectrometric approaches, Rudra et al. [13] found that FOXP3 is able to form complexes with more than 360 proteins. Interestingly, Kwon et al. [14] have shown that FOXP3 forms different sizes of complexes, and that each complex has distinct cofactors. For example, FOXP3 forms large complexes with RelA, lysine acetyltransferase (KAT)5 (also known as Tat-interactive protein-60 (Tip60)), and p300, and smaller complexes with Ikaros family zinc finger protein (IKZF)3, Yin Yang 1 (YY1), and enhancer of zeste homolog 2 (EZH2). These results clearly indicate that FOXP3 orchestrates many cofactors to regulate its targeted genes in order to render T cells with a regulatory function.

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2. Association of FOXP3 with transcription factors

Several transcription factors have been identified as FOXP3 cofactors:

Nuclear factor of activated T cells (NFAT): NFAT is important for peripherally induced Tregs (pTregs) or *in vitro* induced Tregs (iTregs), but not thymic-derived Tregs (tTregs) [15]. NFAT binds to FOXP3 and suppresses NFAT-targeted gene expression [16].

Runt-related transcription factor 1 (RUNX1): RUNX1 interacts with FOXP3 and induces FOXP3 signature genes [17]. In addition, RUNX1 forms a complex with core-binding factor subunit beta (CBFβ) and directly binds to the conserved non-coding sequencing region 2 (CNS2) in the *Foxp3* promoter region [18], which is highly de-methylated in Tregs and is important for maintaining FOXP3 expression [18] and for Treg cell lineage stability [19].

NF-κB molecules RelA and c-Rel: *c-Rel* knockout (KO) mice have a dramatically reduced Treg population, which occurs at the thymic development stage [20]. Rels are also important for pTreg development and FOXP3-regulated gene expression/suppression [21,22]. Interestingly, the *Foxp3*-specific deletion of RelA tends to result in more severe autoimmune symptoms than c-Rel deletion, which suggests that RelA is more important than c-Rel in Treg function [22].

Interferon regulatory factor 4 (IRF4): Treg-specific IRF4 deletion results in T helper 2 (Th2)-selective dysregulation and autoimmunity [23].

Together with the transcription factors listed above, many other transcription factors such as Eos (IKZF4) [24], Helios (IKZF2) [14,25], retinoic acid receptor-related orphan receptor (ROR)γ [26,27], RORα [28], hypoxia-inducible factor 1-alpha (HIF1α) [29], signal transducer and activator of transcription (STAT)3 [30], GATA3 [13], Kruppel's associated box-associated protein 1 (KAP1) [31,32], YY1 [13,33], and EZH2 [34] have also been identified as FOXP3 cofactors. Among these, HIF1α and YY1 seem to suppress FOXP3 function, while the others induce FOXP3 stability and/or assist FOXP3-targeted gene regulation in Tregs. It is notable that genetic deletion of these genes in Tregs using *Foxp3*$^{\text{YFP-Cre}}$ systems, which was developed by Rubtsov et al. [35], showed some kind of autoimmune phenotypes, but the result was not as severe as in scurfy mice (Table 1) [13,15,19,22,23,25,27,29,30,32,34]. These facts suggest that the association of those transcription factors with FOXP3 is important, but not critical, for overall FOXP3 function.

3. Association of FOXP3 with post-translational modulators

FOXP3 is regulated by post-translational modifications (PTMs) [36], which include phosphorylation, acetylation, ubiquitination, and methylation, among others. Compared with transcription factors discussed above, conditional deletion of PTM proteins in Tregs tends to result in more severe autoimmune phenotypes (Table 2) [37–49]. However, these PTM proteins also regulate cell functions by modifying histone and other proteins epigenetically. It remains to be elucidated whether PTM of FOXP3 alone is sufficient for Treg stability and function, or whether epigenetic modifications on any other proteins also contribute.

3.1. Acetylation and deacetylation of FOXP3

FOXP3 is known to be acetylated by KATs. KATs can add acetyl groups to the lysine of their target proteins. Among KATs, Tip60 (KAT5) and cyclic adenosine monophosphate response element-binding protein (CREB)-binding protein (CBP)/p300 (KAT3A/3B) directly bind to FOXP3 and acetylate it, which enhances DNA binding and stability [38,50,51].

Since acetylation sites in FOXP3 overlap with ubiquitination sites, acetylation by KATs prevents ubiquitin-dependent proteasomal degradation (as described below) and induces the stability of FOXP3 protein.

We have found that Tip60 and p300 acetylate each other and further induce FOXP3 acetylation [37]. A structure model suggests that the single acetylation of Tip60 at K327 by p300 would favor FOXP3 binding to the Tip60 cleft. Genetic deletion of Tip60 in Tregs demonstrated severe autoimmune problems similar to those of scurfy mice, suggesting that Tip60 is essential for Treg function.

We have also reported that the natural p300 inhibitor garcinol induces FOXP3 degradation via a lysosome-dependent pathway, which suggests that p300 plays a role in lysosome-dependent FOXP3 degradation [52]. However, single deletion of p300 [38] or CBP [39] only causes relatively mild autoimmunity. Since CBP and p300 have high homology and are functionally conserved [53], single deletion is not effective to limit p300 or CBP function. This has been confirmed by double deletion of p300 and CBP, which causes severe autoimmunity on a level similar to the deletion of Tip60 [39].

Lysine deacetylase, which is sometimes known as histone deacetylase (HDAC), also regulates FOXP3 functions. HDAC consists

Table 1
Phenotypes of genetic deletion of transcription factors in mice.

Protein name	Mouse model	Phenotype	Ref.
NFAT	*Cd4*$^{\text{Cre}}$/(*NFAT1*$^{-/-}$*NFAT4*$^{-/-}$)/*NFAT2*$^{\text{fl/fl}}$	Impaired iTreg induction; functionally no difference	[15]
RUNX-CBFβ	*Foxp3*$^{\text{YFP-Cre}}$/*CBFβ*$^{\text{fl/fl}}$	Lymphadenopathy and splenomegaly at 5–8 weeks old; not lethal, no obvious sign of autoimmunity up to 8–10 months of age	[19]
c-Rel, RelA	*Cd4*$^{\text{Cre}}$/*c-Rel*$^{\text{fl/fl}}$	Impaired Treg maturation in thymus	[22]
	Cd4$^{\text{Cre}}$/*RelA*$^{\text{fl/fl}}$	Slight reduction in Treg population in thymus	
	Cd4$^{\text{Cre}}$/*c-Rel*$^{\text{fl/fl}}$/*RelA*$^{\text{fl/fl}}$	Dramatic reduction in Treg population in lymphoid tissues	
	Foxp3$^{\text{YFP-Cre}}$/*c-Rel*$^{\text{fl/fl}}$	No obvious symptoms; impaired Treg function in colitis model	
	Foxp3$^{\text{YFP-Cre}}$/*RelA*$^{\text{fl/fl}}$	Lethal by 15 weeks of age; no population change but impaired function of Tregs	
	Foxp3$^{\text{YFP-Cre}}$/*c-Rel*$^{\text{fl/fl}}$/*RelA*$^{\text{fl/fl}}$	Lethal by 4 weeks of age; dramatic reduction in Treg population and function	
IRF4	*Foxp3*$^{\text{YFP-Cre}}$/*IRF4*$^{\text{fl/fl}}$	Showing autoimmune phenotypes at 3–4 months of age	[23]
Helios	*Foxp3*$^{\text{YFP-Cre}}$/*Helios*$^{\text{fl/fl}}$	Not lethal; lymphadenopathy and splenomegaly at 6 months of age	[25]
RORγt	*Foxp3*$^{\text{YFP-Cree}}$/*RORC*$^{\text{fl/fl}}$	Ameliorate glomerulonephritis in mouse model	[27]
HIF1α	*Cd4*$^{\text{Cre}}$/*HIF1α*$^{\text{fl/fl}}$	Increased Treg and reduced Th17 population, resistant to EAE model	[29]
STAT3	*Foxp3*$^{\text{YFP-Cre}}$/*STAT3*$^{\text{fl/fl}}$	Inflammatory bowel disease symptoms by 12–14 weeks of age	[30]
GATA3	*Foxp3*$^{\text{YFP-Cre}}$/*GATA3*$^{\text{fl/fl}}$	Intestinal pathology and dermatitis develop after 6 months	[13]
KAP1	*Foxp3*$^{\text{YFP-Cre}}$/*KAP1*$^{\text{fl/fl}}$	Lymphadenopathy and lung inflammation develop at 8–12 weeks of age	[32]
EZH2	*Foxp3*$^{\text{YFP-Cre}}$/*EZH2*$^{\text{fl/fl}}$	50% of lethality by 175 d after birth; showing various autoimmune symptoms	[34]

EAE: experimental autoimmune encephalomyelitis; −/−: double KO; fl/fl: flox/flox, floxed; YFP: yellow fluorescent protein; Cre: Cre recombinase.

Table 2
Phenotypes of genetic deletion of post-translational modifications in mice.

Protein name	Mouse model	Phenotype	Ref.
Tip60	*Foxp3*$^{YFP\text{-}Cre}$/*Tip60*$^{fl/fl}$	Lethal by 4 weeks of age; significant reduction in Treg population and function	[37]
CBP/p300	*Foxp3*$^{YFP\text{-}Cre}$/*EP300*$^{fl/fl}$	Mild autoimmunity develops at 10 weeks of age; less Treg-suppressive function	[38]
	Foxp3$^{YFP\text{-}Cre}$/*CREBBP*$^{fl/fl}$	50% of mice have enlarged peripheral lymphoid tissues at 8–10 weeks of age; less Treg function	[39]
	Foxp3$^{YFP\text{-}Cre}$/ (*CREBBP*$^{fl/fl}$*EP300*$^{fl/fl}$)	Lethal by 4 weeks of age; dramatic reduction in Treg population and function	[39]
HDAC3	*Foxp3*$^{YFP\text{-}Cre}$/*Hdac3*$^{fl/fl}$	Lethal by 6 weeks of age; impaired Treg function	[40]
HDAC6	KO	Increased Treg-suppressive function; regulated FOXP3 acetylation	[41]
HDAC9	KO	Increased Treg-suppressive function	[42]
HDAC11	*Foxp3*$^{YFP\text{-}Cre}$/*Hdac11*$^{fl/fl}$	Increased Treg-suppressive function; regulated FOXP3 acetylation	[43]
SIRT1	*Foxp3*$^{YFP\text{-}Cre}$, *Cd4*^{Cre}/*Sirt1*$^{fl/fl}$	Increased Treg-suppressive function; regulated FOXP3 acetylation	[44]
Pim2	KO	Increased FOXP3 expression and Treg-suppressive function	[45]
CDK	KO	Increased Treg-suppressive function	[46]
Cbl-b	KO	Increased Treg population	[47]
USP7	*Foxp3*$^{YFP\text{-}Cre}$/*USP7*$^{fl/fl}$	Lethal by 4 weeks of age; significant reduction in Treg population and function	[48]
USP21	*Foxp3*$^{YFP\text{-}Cre}$/*USP21*$^{fl/fl}$	Lymphadenopathy and splenomegaly develop at the age of 6–8 months	[49]
PRMT5	*Foxp3*$^{YFP\text{-}Cre}$/*PRMT5*$^{fl/fl}$	Lethal by 4 weeks of age; significant reduction in splenic Treg and overall Treg function	—[a]

CBP: cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)-binding protein; HDAC: histone deacetylase; SIRT1: sirtuin-1; CDK: cyclin-dependent kinase; Cbl-b: Casitas-B-lineage lymphoma protein-b; USP: ubiquitin-specific peptidase; PRMT: protein arginine methyltransferase.
[a] Unpublished data by Nagai et al.

of four different classes based on the cellular location, size, number of catalytic pockets, and homology to yeast prototypes [54]:

- Class I: HDAC1, 2, 3, and 8;
- Class II: HDAC4, 5, 6, 7, 9, and 10;
- Class III: SIRT1–7;
- Class IV: HDAC11.

Class I HDACs appear to enhance Treg functions. Conditional deletion of HDAC3 in Tregs results in decreased Treg function and severe scurfy-like phenotypes [40]. Other Class I HDACs, namely HDAC1 and HDAC2, also bind FOXP3 at the N terminal to regulate the expression of many genes [55]. These Class I HDACs do not seem to alter FOXP3 PTM; rather, they seem to be recruited by FOXP3, which in turn counteracts the hyper-acetylation of the promoter, and thereby switches off inflammatory genes.

Li et al. [55] have found that FOXP3 binds directly to Tip60, HDAC7, and HDAC9 at the N-terminal region to form a chromatin-remodeling complex. Although the association of HDAC7 with FOXP3 and Tip60 seems to be important in order for FOXP3 to repress the targeted gene [56], the precise mechanism is as yet unknown. Since HDAC7 has little enzymatic activity and needs to form a complex with other factors such as HDAC3 in order to function [40], HDAC7 may be important in recruiting other FOXP3 cofactors. However, deletion of HDAC6 [41,57], HDAC9 [42,57], sirtuin-1 (SIRT1) [44,57,58,59], or HDAC11 [43] increases the acetylation level and overall expression of FOXP3, in addition to enhancing Treg functions.

3.2. Interaction of FOXP3 with kinases

Phosphorylation of FOXP3 was first described by Samanta et al. [60]. Since then, several kinases, such as cyclin-dependent kinase (CDK)2, Pim1, Pim2, and lymphocyte-specific protein tyrosine kinase (LCK), have been reported to phosphorylate FOXP3 [18,36]. Phosphorylation of FOXP3 by CDK2 [46,61], Pim1 [62], and Pim2 [45] decreases FOXP3 function. CDK-deficient Tregs show higher suppressive functions than wild-type cells. Mutation of the CDK binding site on *FOXP3* increases FOXP3 expression and function.

Pim1 phosphorylates FOXP3 at S418, which reduces the chromatin binding of FOXP3 to targeted genes, and prevents further phosphorylation at S422 of FOXP3, which is reported to be important for FOXP3 function [63]. *Pim2* is primarily identified as a FOXP3-dependent gene and is involved in Treg expansion *in vitro* [64]. We have found that Pim2 interacts with FOXP3 directly, and phosphorylates it at the N-terminal site [45]. *Pim2* KO mice have Tregs with higher FOXP3 expression and enhanced suppressive function, and are resistant to dextran sodium sulfate (DSS)-induced colitis; this suggests that phosphorylation by Pim2 also affects FOXP3 stability and function. LCK phosphorylates FOXP3 at Y342 in cancer cells and upregulates its function [65]. However, the mechanism by which LCK interacts with FOXP3 and affects subsequent phosphorylation is as yet unknown.

Phosphorylation of FOXP3 at S418 by an unknown kinase augments the DNA binding of FOXP3 to chromatin [63]. Dephosphorylation of this site by tumor necrosis factor (TNF)-induced protein phosphatase I (PP1) has been observed in arthritis patients, whose Tregs show markedly impaired suppressive function [63].

3.3. Ubiquitination and degradation of FOXP3

Protein ubiquitination is a multistep process that is regulated by the E1, E2, and E3 ligase families. Ubiquitination is caused by the binding of ubiquitin peptides on the lysine residue of the targeted protein. On its own, the ubiquitin peptide has seven lysine residues, including K48 and K63. At least one of these lysine residues on each ubiquitin peptide must be utilized in order to form a polyubiquitin chain [66]. Lysine-48-linked (K48) polyubiquitination is the dominant form that marks proteins for degradation by the 26S proteasome, while non-K48 polyubiquitinations are usually implicated in regulating the targeted protein's function, rather than in protein degradation [67]. The majority of FOXP3 ubiquitination is related to K48 ubiquitination and degradation [18,36].

Several pathways induce FOXP3 ubiquitination. HIF1α, which is induced under hypoxic conditions and directly binds to FOXP3, induces FOXP3 polyubiquitination and degradation [29]. Chemokine (C–C motif) ligand 3 (CCL3) signaling also induces polyubiquitination by activating protein kinase B alpha (PKBα) pathways in the psoriatic microenvironment; however, the precise mechanism is still unclear [68]. During T cell receptor (TCR) stimulation, selective inhibition of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-AKT signaling pathway was found to suppress the proliferation of Tregs, but not of conventional T cells to any significant degree; this suggests that Tregs are more dependent on PI3K-AKT pathways, and that AKT inhibitors can be used to limit Treg infiltration into tumors and to induce immunity against tumors, if they are able to be delivered specifically to Treg [69].

The enzymes that ubiquitinate FOXP3 for degradation are the C terminus of HSC70-Interacting Protein (CHIP) and Casitas-B-lineage lymphoma protein-b (Cbl-b). Chen et al. [70] have shown that STUB1 causes K48 type ubiquitination of FOXP3 at residues K227, K250, K263, and K268 in a heat shock protein (HSP)70-dependent manner. Specific knockdown of STUB1 or HSP70

increases FOXP3 expression, and STUB1 overexpression in Tregs decreases FOXP3 expression level and results in reduced suppressive Treg function in both *in vitro* studies and *in vivo* mouse models. Cbl-b also binds to FOXP3 upon TCR stimulation, and, in collaboration with STUB1, ubiquitinates and subsequently degrades FOXP3 [47].

As polyubiquitination reduces FOXP3 stability, it is expected that deubiquitinases will increase FOXP3 stability. Ubiquitin-specific peptidase (USP)7 [48,71] and USP21 [49] have been reported to directly interact with FOXP3 to induce stability by degrading ubiquitin. In a demonstration of the importance of USP7 and USP21, Treg-specific deletion of either of these peptidases was shown to cause autoimmune reaction in mice [48,49]. It is interesting to note that although both of these deubiquitinases cause autoimmunity, the deletion of USP7 resulted in a more severe phenotype. In those Tregs, Tip60 expression was also dramatically reduced, indicating that this severity is also partially due to Tip60 malfunction.

3.4. Methylation of FOXP3

A recent mass spectrometric approach has shown that FOXP3 can be di-methylated. Using isomethionine and specific arginine methylation antibody, Geoghegan et al. [72] identified 2502 arginine methylation sites from 1257 tissue-specific and housekeeping proteins in human T cells. From these peptides, they identified a FOXP3 peptide that is methylated at the R51 position. Working separately, we identified protein arginine methyltransferase (PRMT)5, which symmetrically di-methylates arginines [73], as a novel cofactor of FOXP3[†]. Conditional deletion of PRMT5 in Tregs resulted in a severe scurfy-like phenotype, suggesting that FOXP3 methylation is also critical for FOXP3 functions.

4. Therapeutic applications targeting FOXP3 and its cofactors

Because Tregs are the dominant T cells that suppress host immunity, they are considered to be an important target for clinical applications in autoimmunity [74] and cancer immunotherapy [75]. For this purpose, many inhibitors and modulators have been tested in attempts to directly regulate FOXP3 cofactors or the expression of the FOXP3 protein (Fig. 1). However, most of these studies have been performed in animal models, as proofs of concept. Only a few have been tested in clinical settings.

4.1. HDAC inhibitors

Since HDAC inhibitors are capable of enhancing FOXP3 expression and boosting the number and functions of $FOXP3^{+}CD4^{+}CD25^{+}$ Tregs, these inhibitors have been used as therapeutics to enhance Treg activity in disease models for autoimmune and organ transplantation. In alignment with enhanced Treg functions, induced acetylation of FOXP3 in Tregs and upregulation of several Treg-associated markers with functional activity (FOXP3, glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), cytotoxic T-lymphocyte-associated protein (CTLA)4, programmed cell death protein (PD)-1, and interleukin (IL)-10) have been observed in the presence of HDAC inhibitors [76].

In a BALB/c to C57BL/6 cardiac transplantation model, the HDAC inhibitor trichostatin in combination with low-dose rapamycin achieved permanent allograft survival [77]. The role of Tregs in this procedure was confirmed by a pre-transplantation thymectomy and anti-CD25 depleting monoclonal antibodies (mAb) (PC-61), which led to the depletion of central and peripheral $CD4^{+}CD25^{+}$ Tregs and accelerated the rejection of allografts.

Pan-HDAC inhibitors such as trichostatin A (TSA) and valproic acid have been tested in disease models to treat autoimmune diseases such as colitis [77] and collagen-induced arthritis [78]. By regulating Treg functions, valproic acid has also been used to enhance adenovirus vector-mediated airway gene expression in a cystic fibrosis mouse model [79].

In experimental autoimmune prostatitis (EAP), cellular and humoral prostate-specific autoimmune response is easily detectable in the male sex accessory gland of rats. This autoimmune

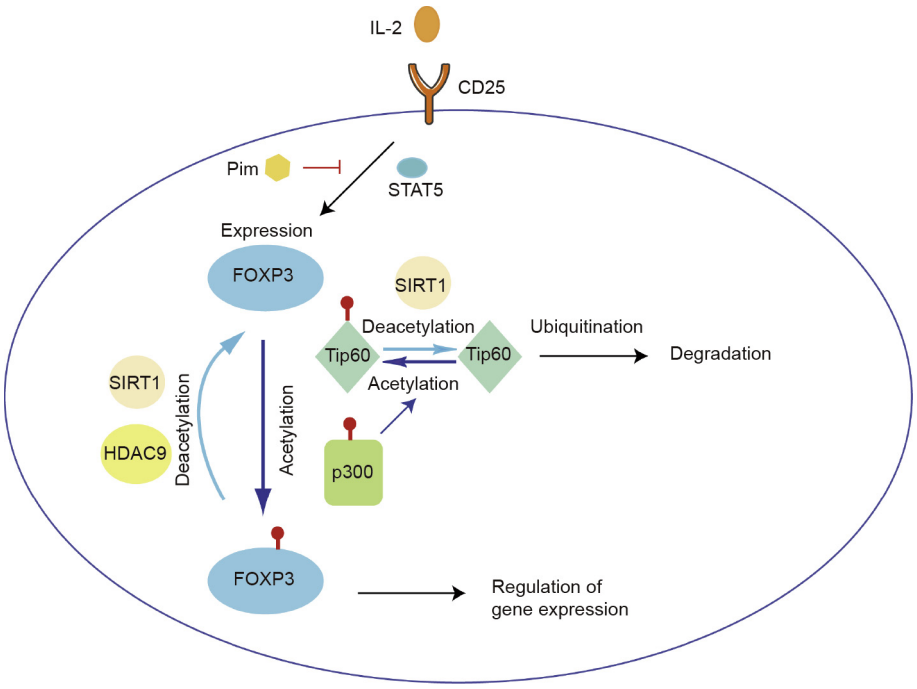

Fig. 1. A roadmap for targets to regulate FOXP3 and Treg functions. IL: interleukin.

[†] Unpublished data by Nagai et al.

inflammation is similar to human chronic prostatitis and chronic pelvic pain syndrome. Treatment with an HDAC inhibitor, MS-275, significantly reduced the local accumulation of immune cells and messenger RNA (mRNA) levels of representative pro-inflammatory molecules in prostate tissue. Most importantly, MS-275 treatment increased the fraction of $FOXP3^+CD4^+$ Tregs in both lymph nodes and peripheral blood. This study suggests that MS-275 can be used as a potential candidate for the treatment of inflammatory prostatitis [80].

Most of the HDAC inhibitors that are used to treat autoimmune diseases exhibit broad inhibitory activity. However, their targets are believed to be Class II HDACs [81]. Since Class I HDACs are positively associated with Treg activity, selective inhibitors for Class I HDACs may have better application for cancer therapies.

As HDAC inhibitors also affect other proteins, especially histones, they can be used to target cancer cells. In fact, several HDACs have been approved to treat several types of cancer; these include romidepsin, vorinostat, and belinostat for T cell lymphoma, and chidamide for relapsed or refractory peripheral T cell lymphoma in China.

However, these HDAC inhibitors may have activity beyond attacking cancer cells *per se*. In clinical studies, declines in Tregs were observed in patients treated with belinostat in addition to chemotherapy, and the magnitude of the decline was associated with response to treatment ($P = 0.0041$) and progression-free survival ($P = 0.021$) [82]. Declines in T-cell immunoglobulin and mucin-domain containing (TIM)-3^+CD8^+ T cells were more substantial in responders than in non-responders ($P = 0.049$).

In a triple-negative breast cancer (TNBC) model, the HDAC inhibitor vorinostat was shown to up-regulate PD-L1 mRNA and protein expression in TNBC cells in a time-dependent manner, and down-regulate co-cultured $CD4^+FOXP3^+$ Tregs *in vitro*. In an *in vivo* triple-negative 4T1 breast cancer mouse model, vorinostat significantly enhanced the *in vivo* response to PD-1/CTLA4 blockade [83]. Of course, clinical studies are needed to confirm whether this HDAC inhibitor, in combination with a checkpoint inhibitor strategy, can be evolved into an effective clinical treatment for human TNBC.

4.2. Histone acetyltransferase inhibitors

Small-molecule allosteric compounds that modify the association of cofactors with FOXP3 are may be good candidates for regulating FOXP3 functions. Using a cavity-induced allosteric modification (CIAM) approach [84], we have developed an allosteric modifier for Tip60 [85]. The allosteric modifiers B7A and SGF003 had less of an impact on histone acetylation by Tip60, but were found to induce association between Tip60 and its target protein, including FOXP3. Treatment with these compounds in mouse colitis and collagen-induced arthritis (CIA) models significantly improved the autoimmune symptoms [85].

A384T, a natural IPEX mutation in *FOXP3*, disables the binding of FOXP3 to Tip60 but retains FOXP3's normal DNA binding capability and protein stability. Nevertheless, the normal Treg function is impaired. Interestingly, treatment with CIAM compounds was found to rescue the expression profiles of FOXP3 regulating genes and the suppressive function of T cells [85].

Tip60 and p300 inhibitors are also good candidates for tumor therapy. We found that the Tip60 inhibitor NU9056, which is reported to inhibit prostate cancer growth by inducing apoptosis, specifically reduces Treg suppression without affecting effector T cell proliferation at low concentrations (data not shown). p300 inhibitors have also been shown to promote tumor immunity by suppressing Treg functions [38,39].

4.3. Targeting FOXP3 expression

In addition to targeting the PTM of FOXP3 to change the protein stability, transcription of *Foxp3* in Tregs can be targeted in order to modulate Treg functions. EZH2 is a histone–lysine N-methyltransferase enzyme that shows increased activity in tumor-infiltrating Tregs, in both human and mouse models [86]. Using specific EZH2 inhibitors, Wang et al. [86] were able to destabilize FOXP3 expression and reprogram tumor-infiltrating Tregs to exhibit pro-inflammatory activity. In a murine colorectal tumor model (MC38), oral treatment with an EZH2 inhibitor decreased the growth of tumors in immune-competent mice, but not in T cell-deficient recombination activating gene 1 $(Rag\text{-}1)^{-/-}$ mice; this finding indicates critical involvement of tumor-infiltrating Treg activity.

Based on the current understanding of the roles played by Pim1 and Pim2 kinases in the depression of FOXP3 expression and Treg functions, inhibitors of these kinases can be used as therapeutics for natural or experimental autoimmune diseases. Pim1/2 inhibitors have been shown to enhance Treg function in both human [62] and mouse [45] models.

High-dose IL-2 has been explored as a cancer immunotherapy to boost T cell functions and tumor immunity; however, it somehow leads to toxicities. It has also been observed that high-dose IL-2 leads to defective suppressive capacity for Tregs. It was reported that the toxicity of high-dose IL-2 was ameliorated in a mouse model for the human immune system (HIS) through the use of a Pim1 kinase inhibitor, Kaempferol [87].

Tregs have abundant CD25 (IL-2Rα), which is the receptor for IL-2. The induction of FOXP3 and the differentiation of Tregs in the thymus are highly dependent on IL-2 and on STAT5, a key IL-2R downstream target. Although IL-2 has been used to amplify Tregs *in vitro*, it is not an ideal Treg-specific therapeutic agent due to its activity on other T cells. Recently, a fully human anti-IL-2 antibody, F5111.2, which belongs to the group of antibodies that inhibit IL-2 binding to IL-2Rβ and reduce binding to IL-2Rα, was shown to stabilize IL-2 in a conformation that results in preferential STAT5 phosphorylation *in vitro* and selective Treg expansion *in vivo* [88]. The therapeutic potential of F5111.2 for autoimmune diseases was demonstrated in three types of disease model: the induction of remission of type 1 diabetes in a non-obese diabetic (NOD) mouse model; the reduction in disease severity in a model of EAE; and the protection of mice against xenogeneic graft-versus-host disease [88].

4.4. Other inhibitors

Interestingly, some of FOXP3's epigenetic or post-translational modifiers are common therapeutic targets in cancer cells; this suggests that therapeutics can be developed to inhibit both tumor growth and Treg suppression function [89,90]. PRMT5 is well known to be overexpressed in many types of tumors [73,91]. Our recent studies have shown that *S*-adenosylmethionine-competitive inhibitors—but not substrate-competitive inhibitors—efficiently reduce FOXP3 methylation, and specifically reduce Treg-suppressive functions in both human and mouse Tregs. An *S*-adenosylmethionine-competitive PRMT5 inhibitor has been used to enhance anti-erbB2/neu targeted therapy by inducing tumor immunity.[†]

Regulation the association of FOXP3 with other cofactors may be a new strategy for regulating Tregs. Lozano et al. have developed cell-permeable peptides to bind FOXP3 and inhibit its interaction with NFAT [92] or acute myeloid leukemia (AML)/RUNX1 [93].

[†] Unpublished data by Nagai et al.

Both studies showed tumor-inhibiting activities in mouse tumor models. However, therapeutic peptides tend to have low storage stability and a short half-life *in vivo*. This disadvantage prevents the clinical adoption of this approach and must be overcome with better strategies in future [94,95].

5. Conclusion

FOXP3, a master regulator of Tregs, has a variety of cofactors and thus regulates its targeted gene through very complicated mechanisms. Although FOXP3 is reported to express in other cell types, including activated T cells, several types of immune cells, and tumor cells, Tregs remain as the dominant cell type that expresses FOXP3 at very high levels [18]. Thus, targeting FOXP3 is an interesting idea for future clinical applications addressing both autoimmune diseases and cancers. Targeting FOXP3 PTM proteins and tumor growth simultaneously may be a good strategy for cancer treatment. Allosteric modification of FOXP3 cofactors may be a new strategy for regulating FOXP3 function. By revealing FOXP3 ensembles and its precise regulation, it may be possible to fine-tune Treg functions for many types of diseases.

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Compliance with ethics guidelines

Yasuhiro Nagai, Lian Lam, Mark I. Greene, and Hongtao Zhang declare that they have no conflict of interest or financial conflicts to disclose.

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