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# Histone/protein deacetylases control Foxp3 expression and the heat shock response of T-regulatory cells

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## Abstract

Lysine ε-acetylation is a post-translational modification that alters the biochemical properties of many proteins. The reaction is catalyzed by histone/protein acetyltransferases (HATs), and is reversed by histone/protein deacetylases (HDACs). As a result, HATs and HDACs constitute an important, though little recognized, set of proteins that control the functions of T-regulatory (Treg) cells. Targeting certain HDACs, especially HDAC6, HDAC9, and Sirtuin-1 (Sirt1), can augment Treg suppressive potency by several distinct and potentially additive mechanisms. These involve promoting Forkhead box p3 (Foxp3) gene expression and preserving Foxp3 lysine ε-acetylation, which infers resistance to ubiquitination and proteasomal degradation, and increases DNA binding. Moreover, depleting certain HDAC can enhance the heat shock response, which increases the tenacity of Treg to survive under stress, and helps preserve a suppressive phenotype. As a result, HDAC inhibitor therapy can be used to enhance Treg functions in vivo and have beneficial effects on allograft survival and autoimmune diseases.

## Introduction

The ability to control the immune response is an important therapeutic goal in the management of many diseases. However, doing so requires finding a delicate balance between activation and attenuation. Unfortunately, most current therapeutic strategies targeting the immune system have relatively limited antigen specificity and therefore notoriously lack precision. For example, immune modulation after solid organ transplantation faces the challenge of achieving enough suppression to limit graft rejection without impairing the host's ability to protect against infections [1] and malignancy [2]. This comes in addition to numerous nonimmune toxicities [3]. Treatments for autoimmune conditions like inflammatory bowel disease face very similar problems [4]. Conversely, at the opposite end of the spectrum, cancer immunotherapy, while very promising and increasingly effective against a wide range of tumors, can predispose to autoimmunity [5].



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Throughout the search for more specific approaches, T-regulatory (Treg) cells have been recognized as an important T cell subset able to limit immune responses in an antigen-specific manner, and are crucial to maintaining self-tolerance [6,7•]. Treg-based therapies, such as ex-vivo expansion or efforts to enhance in vivo suppressive function offer a potential avenue towards more antigen directed immunosuppression [8•], and Tregs are now recognized as an obstacle and therapeutic target in anti-neoplastic treatments [9•]. The best established and most studied type of Treg cells are characterized by expression of the transcription factor forkheadbox-p3 (Foxp3), which plays a key role in their development and functions [10,11]. HDAC inhibitor (HDACi) use can augment Foxp3+ Treg production and induce various molecular changes that enhance their phenotype [12]. As a result, the suppressive capacity of murine [13], non-human primate [14] and human [15••] Tregs can be increased by treatment with histone/protein deacetylase inhibitors (HDACi) [16,17•], with therapeutic consequences in models of autoimmunity and transplantation [18,19••,20••, 21••]. At present, several histone/protein acetyltransferases (HATs) and histone/protein deacetylases (HDACs) have been implicated in Treg biology, and the relevant HDAC biology is summarized Figure 1. Aspects of this work are summarized in the following sections, with an emphasis on the lead HDAC and HAT molecular pathways that are known to influence Treg function in vivo as well as in vitro.

## Acetylation of Foxp3 prevents proteasomal degradation and increases Treg potency

Post-translational modifications expand the regulatory potential of proteins vastly beyond mere gene expression. Lysine (K) provides one of the most reactive residues that can engage in a myriad of biochemical alterations [22]. ε-amino acetylation can neutralize lysine's positive charge (Figure 2) and profoundly alter the biological functions of affected proteins [23,24••]. Historically, lysine acetylation was first appreciated in regard to post-translational modifications of histone tail residues that promoted chromatin accessibility and RNA synthesis [25]. Therefore, enzymes facilitating lysine acetylation were named histone acetyltransferases and those reversing it as histone deacetylases. Identification of an increasing number of biologically important non-histone targets prompted lysine acetyltransferase (KAT) and lysine deacetylase (KDAC) as an alternative nomenclature. In the current review, we use the terms histone/protein acetyltransferases (HATs) and histone/protein deacetylases (HDACs) so as to reflect these developments, and given a focus on the development of new therapeutic strategies for autoimmunity and transplantation, we emphasize consideration of HDACs and their inhibitors (HDACi).

Control of Foxp3 expression in Treg is not limited to regulation at the level of Foxp3 gene transcription. Instead, the speed at which the Foxp3 protein is degraded is equally important. Recently, van Loosdregt et al. showed, in non-immune cells, that the class III HDAC Sirtuin-1 (Sirt1) directly co-localizes with Foxp3 and mediates its deacetylation and poly-ubiquitination [26•]. In related studies involving genetic deletion of Sirt1 or its pharmacologic inhibition, we showed that loss of Sirt1 activity in Tregs led to increased Foxp3 protein expression and increased Treg suppressive function, which translated into prolonged allograft survival [20••]. Additional HDACs are almost certainly involved in deacetylating Foxp3, including HDAC9 [13]. In contrast, hyperacetylation of Foxp3 through p300 makes it less susceptible to ubiquitination, which in turn increases the overall Foxp3 protein amount [27••]. Tip60 is another HAT known to acetylate Foxp3 [28]. Thus, leaving Foxp3 in a more acetylated state either through activation of relevant HATs, or deactivation and various HDACs, can render Foxp3 resistant to proteasomal degradation, and thereby control its expression (Figure 2). To date, only p300 and Sirt1 were shown to regulate Foxp3 acetylation and thereby prevent Foxp3 ubiquitination and turnover [26•,27••]. However, beyond resistance to ubiquitination, acetylation of Foxp3 acetylation markedly increases its

regulatory capability through improved DNA binding [13,29,30], e.g. at the interleukin-2 promoter when cooperating with nuclear factor of activated T-cells (NFAT) and competing with AP-1 for its DNA binding [31]. The biochemical details of Foxp3 acetylation were recently reviewed [32]. Therefore, acetylated Foxp3 persists longer and has higher function, depending on the lysine residues affected by acetylation.

## HDACs alter transcription factors of the Foxp3 gene

Since the discovery of Foxp3+ Tregs and appreciation of their significance, investigators have sought to understand the mechanisms of Foxp3 gene regulation. In 2006, Mantel et al. identified the Foxp3 promoter region and reported several binding sites for the transcription factors NFAT and AP-1 [33]. Subsequently, Zorn et al. reported IL-2 dependent STAT5 as another transcription factor relevant to Foxp3 gene expression [34]. Additional transcription factors were implicated based on insights from Treg cell biology. For example, since transforming growth factor (TGF)-β has long been reported as a factor inducing Treg from conventional T cells [35], its downstream targets were expected to be involved in Foxp3 gene regulation. Indeed, Tone et al. showed that Smad3, in conjunction with NFAT, can enhance Foxp3 gene expression [36]. Other transcription factors affecting Foxp3 gene expression include the NF-κB family members RelA (p65) and c-rel [37], NOTCH1 [38], Id3 [39], Runx [40], and others [41•]. Opposing these are negative regulators, notably GATA-3 [42] and STAT3 [43], which are involved in transitioning the differentiation from a Treg to Th2 or Th17 cell fate, respectively. Importantly, Ruan et al. reported that NFAT, SMAD, CREB, c-Rel and Rel-A form the so-called c-rel enhanceosome, which is now understood to be central to Treg induction and lineage commitment [44].

Not surprisingly, HATs and HDACs are important to the functions of many of these transcription factors, e.g. through direct protein acetylation/deacetylation, or indirectly through modification of their own expression, DNA binding affinity, nuclear translocation or transcriptional activity, or by likewise affecting regulators of these transcription factors, providing many possibilities for complex biologic effects and for therapeutic intervention (Figure 3). For example, Sirt1 has been shown to deacetylate NF-κB, more specifically the RelA/p65 subunit at K310 [45]. Acetylation of p65 can augment gene transcription through several mechanisms; e.g. acetylation of K314/315 increases promoter selectivity, acetylation of K221 increases DNA binding, acetylation of K221/K218 decreases its binding to the IκBα inhibitor, and acetylation of K310 can increase the transcriptional activity of p65 [46]. Of note, loss of Sirt1 can augment RelA-dependent transcription in macrophages [47]. In our own studies, T cell or Treg-specific deletion of Sirt1 increased RelA K310 acetylation [20], which is relevant in regard to the concept of RelA being an important part of the c-rel enhanceosome [44], and could be one mechanism by which Sirt1 targeting can increase Foxp3 gene expression.

Another transcription factor of interest is the TGFβ-dependent SMAD3. Its transcriptional activity is, in part, regulated through proteasomal degradation, which is induced by estrogen receptor (ER)-α signaling [48•]. At the same time, activation of ERα by estrogen binding promotes the expression of HDAC6 [49]. We hypothesize, that HDAC6 may be involved in mediating ERα dependent SMAD3 degradation, and that thus, loss of HDAC6, could preserve SMAD3, and transcriptionally active phospho-SMAD3 (Figure 3). However, the role of HDAC6 in controlling TGFβ-dependent signaling in Treg cells currently remains poorly defined. From the epithelial-mesenchymal transition literature, Shan et al reported, that HDAC6 is actually required for phosphorylating SMAD3 [50]. However, such regulatory functions of HDAC6 could differ significantly between different cell types. We are currently investigating the effects of HDAC6 on SMAD3-regulated events at the TGFβ-dependent Foxp3 enhancer. It is noteworthy that Sirt1 can regulate ERα expression and Sirt1

inhibition can thus impair ERα signaling pathways [51]. Moreover, Sirt1 has also been shown to deacetylate SMAD3, which reduces its DNA binding capacity [52]. Disrupting the SMAD3/p300 interaction also diminished SMAD3 acetylation and led to a similar decline in DNA binding [53]. Thus, targeting Sirt1 may lead to (a) more preserved (phospho)-SMAD3 through diminished ERα signaling, and b) to a more transcriptionally active SMAD3 through acetylation.

A third transcription factor potentially subject to HDAC/HAT manipulation is IL-2-dependent STAT5. STAT5 is transported into the nucleus after tyrosine phosphorylation in the C-terminal region [54,55]. In addition, STAT5 is acetylated (K694, K701 and K359) by the HAT, CREB-binding protein (CBP), which promotes formation of a stable STAT5 dimer though acetylation at K696 and K701 [56]. Importantly, sustained phospho-STAT5 dimer binding to DNA favors histone acetylation and chromatin remodeling, which further augments transcription [57]. It is therefore possible that manipulation of either CBP, or a yet unidentified HDAC, could influence stability of the phospho-STAT5 dimer through acetylation, and thus influence Foxp3 gene transcription.

## Foxp3 gene methylation and histone acetylation

Over the past few years, increasing attention was dedicated to understanding methylation of the Foxp3 promoter and conserved non-coding sequences (CNS) within the Foxp3 gene [58,59]. Methylation of CpG islands and acetylation of histones determine accessibility of the DNA through chromatin remodeling. Of note, Zheng et al. found that three CNS within the Foxp3 gene convey lineage stability of the Treg phenotype through their methylation state and responsiveness to transcription Foxp3 factors [60••]. Therefore, the Foxp3 promoter and CNS regions are targets for epigenetic regulation of Foxp3 expression [61]. Liu et al. observed that the sumoylation ligase PIAS1 can recruit DNA methyltransferases and other factors promoting heterochromatin formation to the Foxp3 promoter and thus restrict mRNA transcription [62]. Since histone acetylation uncoils the DNA structure (euchromatin) making it more accessible to transcription factors, HATs and HDACs have become a subject of interest in epigenetic regulation of Foxp3 expression (Figure 3) [63•].

## HDAC influence on the heat shock response

Pan-HDACi, but also HDAC6 and HDAC9 specific targeting, can augment activation of heat shock response (HSR) gene transcription in Treg cells [20,21]. The HSR enables the expression of chaperone proteins that alleviate and counter the sequelae of cellular stress, and is primarily induced through DNA binding of heat shock transcription factor-1 (HSF1) to heat shock elements in the regulatory regions of many genes. Under resting conditions, the majority of HSF1 monomer is inactive and bound to a complex of HSP90, HSP70, p23, and FK506 binding protein [64]. This complex can be disrupted by the emergence of reactive oxygen species and/or misfolded proteins, which leads to release of the HSF1 monomer. Furthermore, HSP90 hyperacetylation due to loss of HDAC6 can prompt dissociation from other proteins [65], including HSF1, which leads to activation of the HSR (Figure 4) [21]. The HSR is induced by nuclear translocation of HSF1, phosphorylation of serine residues, and formation of a competent HSF1 trimer capable of binding to the DNA [66]. Remarkably, unlike many other transcription factors, the DNA binding of HSF1 can be disrupted through acetylation, and stabilized by deacetylation through Sirt1 [67••]. Indeed, we found that Sirt1 deletion in Treg in vivo led to diminished induction of key HSR genes such as HSP70 and HSP27 [20••]. However, we also noted in preliminary studies, that Sirt1-deficient Treg show improved apoptosis free survival in response to heat shock challenge. We are currently investigating if Sirt1 might directly affect HSP70 acetylation and thereby influence the cellular HSR on a post-translational level. Of relevance, HSP70 acetylation has

been shown to increase its chaperone activity [68]. While this may help undo protein misfolding and aid Treg surviving in acidotic and hypoxic inflammatory tissues, it may also be important for Treg function. We have previously shown, that HSP70 can co-localize with Foxp3 (19••). We therefore hypothesize, that HSP70 can aid in the correct folding of newly synthesized cytoplasmic Foxp3 and aid in its transport to the nucleus (Figure 4).

## Additional considerations and conclusions

We are currently assembling evidence showing that several additional HDACs, beyond HDAC6, HDAC9 and Sirt1, have biologically relevant effects on Foxp3-dependent Treg biology. There are also, of course, numerous additional post-translational and epigenetic mechanisms beyond protein acetylation and DNA methylation that are relevant to modulation of Foxp3+ Treg function. We have recently reported evidence of the key role of proteolytic cleavage so as to generate a form of Foxp3 that appears most potent in terms of chromatin binding and control of Treg function [69], and identification of the mechanisms responsible for the nuclear translocation of Foxp3 [70]. Likewise, given limitations of space, the current review has not discussed Foxp3 phosphorylation, sumoylation or regulation via effects on mRNA stability. While these have intrinsic interest, we believe that the potential clinical application of our data most clearly relates to the use of selective HDACi so as to promote Foxp3+ Treg suppression in autoimmunity and post-transplantation. To that end, the availability of selective inhibitors of HDAC6 and Sirt1 has particular significance. Their testing in disease models, alone and in various combinations, is currently underway in our laboratory.

Highlights

- Histone/protein acetyltransferases (HATs) and histone/protein deacetylases (HDACs) control lysine acetylation, an important post-translational modification in histone and non-histone proteins.
- Acetylation of Foxp3, the master transcription factor of T-regulatory (Treg) cells, promotes increased DNA binding and resistance to proteasomal degradation.
- Several Foxp3 transcription factors can be regulated by acetylation (p65, STAT5), or indirectly though HDAC/HAT dependent mediators (SMAD3).
- Targeting HDAC6 or HDAC9 increases heat shock response (HSR) gene expression, which protects Treg from stress and preserves a suppressive phenotype.

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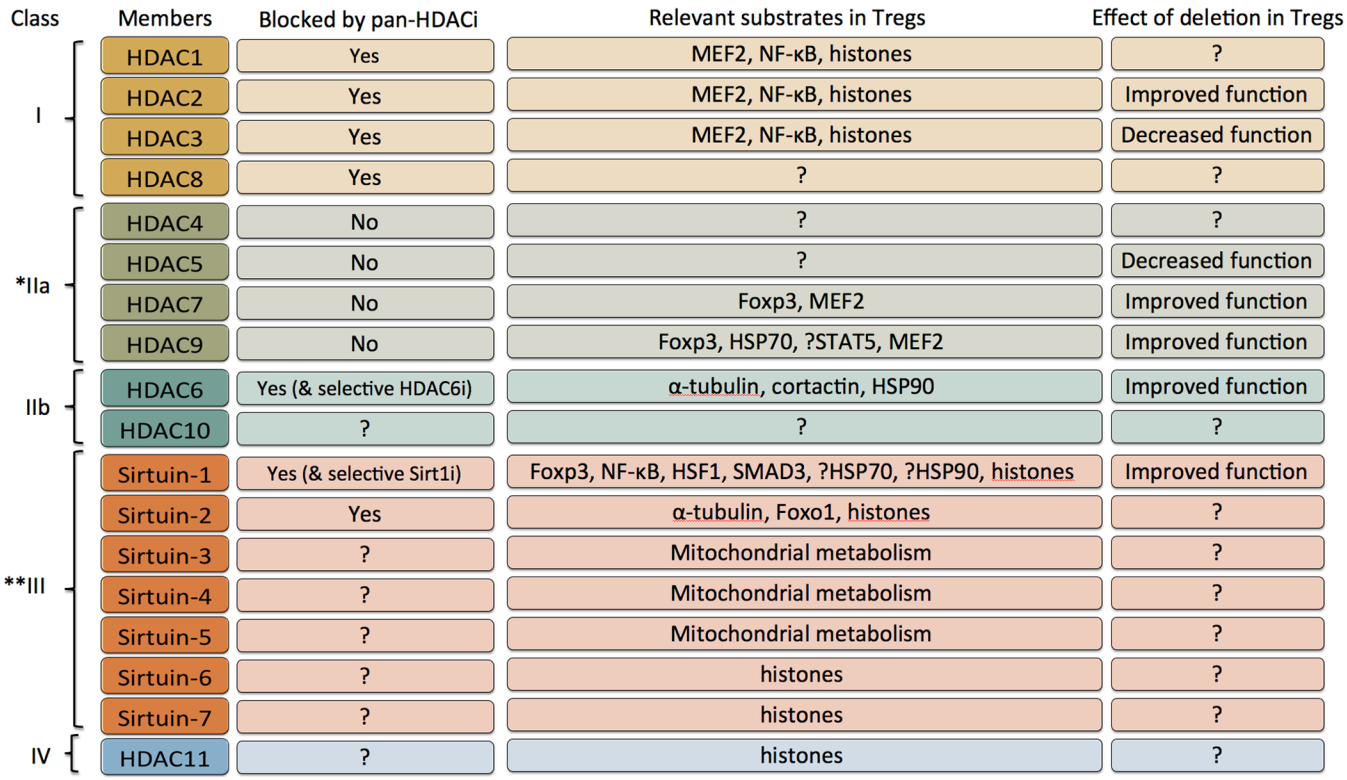


**Figure 1. HDACs and potential target proteins in Treg cells**

Summary of HDAC biology and Tregs, using a schematic adapted from Bush and McKinsey [71]. Note that * class IIa HDACs are currently thought to act primarily through protein/protein interactions and their deacetylase activity is mediated via recruitment of a class I HDAC, such as HDAC3; and **HDACi acting on Zn-dependent HDACs (class I, IIa and IV) are distinct from HDACi that act on NAD-dependent sirtuins (Class III). There are HDAC specific inhibitors for HDAC6 and Sirt1 (20••, 21••). Abbreviations are HDAC, histone/protein deacetylase; HSF, heat shock factor; HSP, heat shock protein; MEF-2, myocyte enhancer factor-2; NAD, nicotinamide adenine dinucleotide; and. Sirt1, Sirtuin-1.

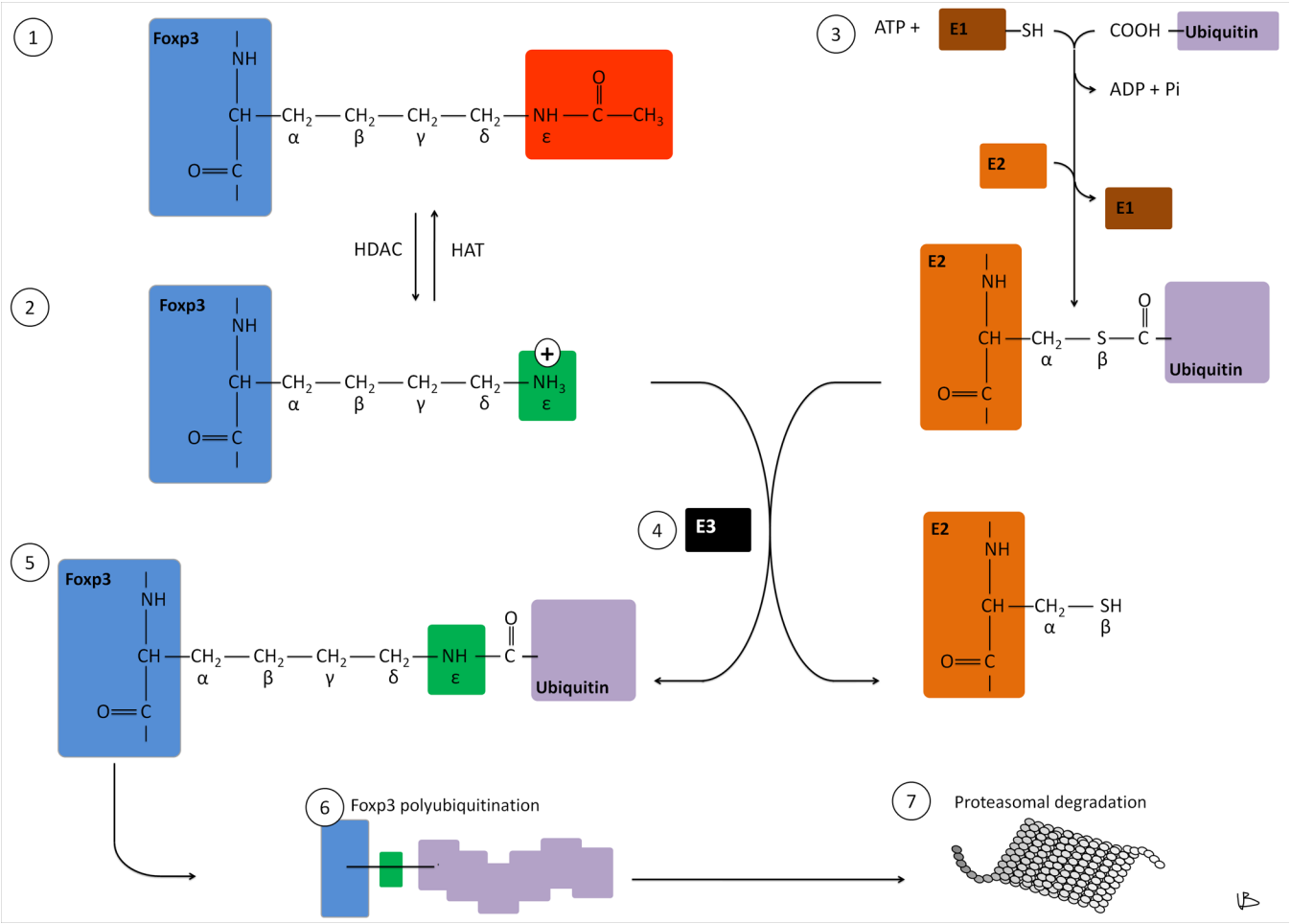


**Figure 2. Lysine ε-amino deacetylation promotes protein turnover and shortens Foxp3 lifespan**

(1) HATs can acetylate lysine residues in Foxp3 at the ε-$NH_2$ group. In the acetylated form, the lysine residue cannot enter the ubiquitination reaction. (2) HDACs can remove the acetyl-group from Foxp3, and make it susceptible to the ubiquitination reaction. (3) Activated ubiquitin is formed by binding of its c-terminus to a cysteine residue on ubiquitin-activating enzyme (E1) via a thiol bond. Next, E1 is replaced by ubiquitin-conjugating enzyme (E2). (4) The E2-ubiquitin complex can be linked to deacetylated lysine residues on Foxp3 via an ubiquitin-protein ligase (E3) forming an isopeptide bond (5). (6) Subsequently, other ubiquitins can bind to the ε-amino groups of lysine residues (K29 and K48) of ubiquitin already bound to Foxp3. (7) A chain of four or more ubiquitins is sufficient to indicate proteins for degradation in the proteasome.

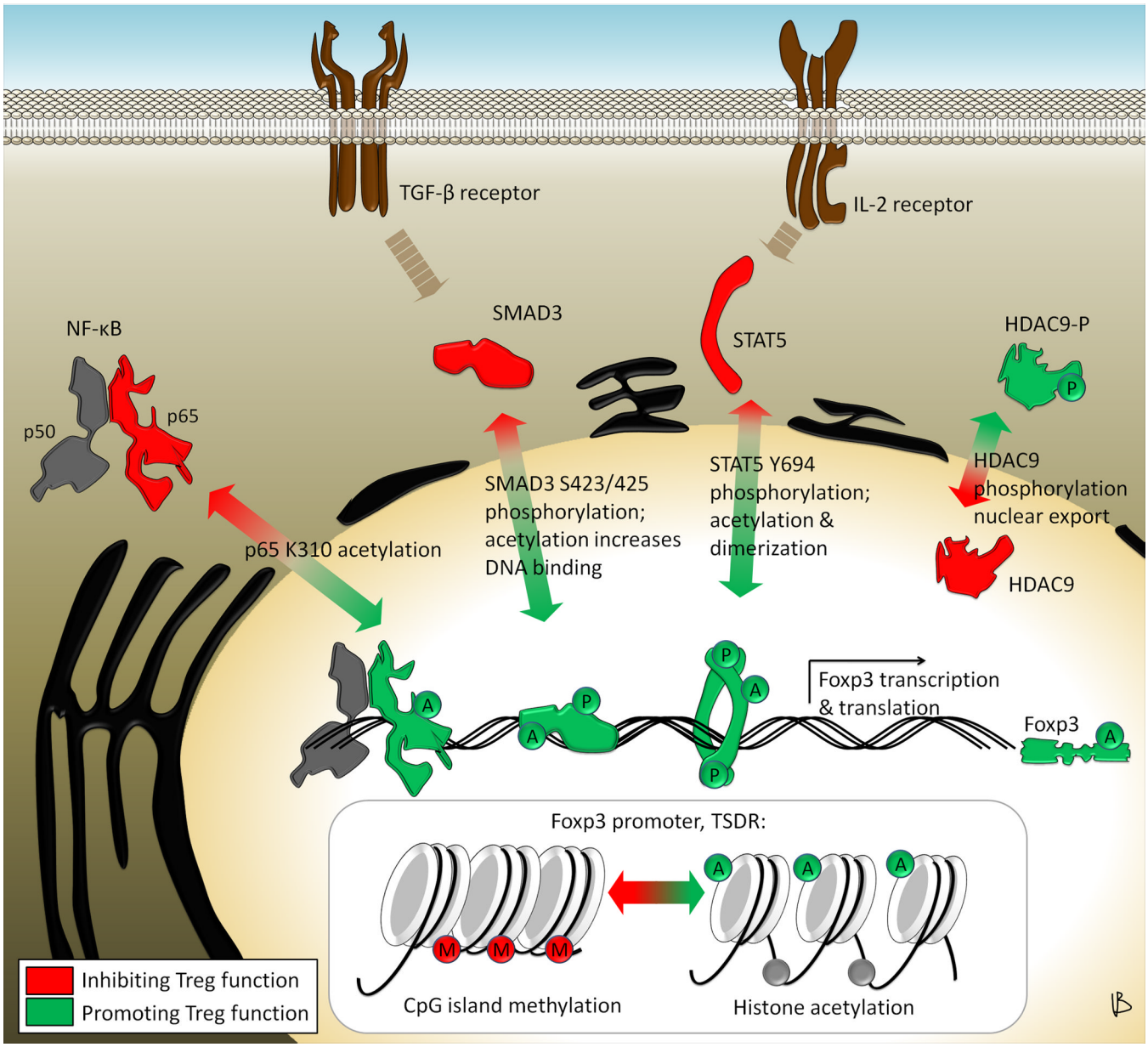


**Figure 3. HDACs control Foxp3 gene expression**

Targeting certain HDACs can increase Foxp3 gene expression by aiding nuclear translocation of transcription factors and promoting histone acetylation at the Foxp3 promoter and the Treg-specific-demethylated region (TSDR). The p65 subunit of NF-κB can be deacetylated at K310 through Sirtuin-1. Acetylation of p65 at K310 is important for NF-κB nuclear translocation, and p65 is an integral part of the c-rel enhanceosome promoting Foxp3 expression. Likewise, SMAD3 and STAT5, both effectors of TGF-β and IL-2 receptor signaling, respectively, depend upon nuclear translocation as well, which is facilitated by phosphorylation. Proteasomal degradation of nuclear phosphorylated SMAD3 can be favored by HDAC6 activity (see text), whereas STAT5 dimerization is enhanced by acetylation. Abbreviations are: A, acetylated; P, phosphorylated; M, methylated CpG island.

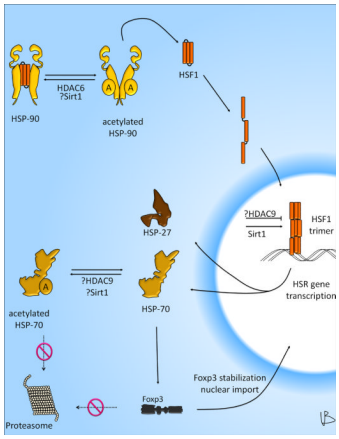


**Figure 4. HDACs and the Treg heat shock response**

At rest, HSP-90 is bound to multiple client proteins, including HSF1. Stress or HSP90 acetylation (reversed by HDAC6) cause displacement of HSF1, which unfolds, translocates into the nucleus and forms transcriptionally active trimer. The HSF1 trimer is stabilized in its deacetylated state through Sirt1, and turns on heat shock response genes, such as HSP-27 and HSP-70. HSP-70 is deacetylated by HDAC9, and perhaps Sirt1, which confers resistance to proteasomal degradation. HSP-70 can also act as a chaperone for newly translated Foxp3 and aid in its nuclear import. Abbreviation used: A, acetylated.