Post-translational Regulation of Foxp3

-Identification of novel molecular targets for immune modulation-

Regulatie van het immuunsysteem door modificaties van Foxp3

(met samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General introduction

Maintenance of immune homeostasis is a complex process allowing the immune system to be both aggressive enough to eradicate cells that express foreign antigens, and yet provide sensitivity to tolerate cells expressing self antigens. Key modulators allowing tolerance of host antigens, thereby preventing autoimmunity are the regulatory T cells (Treg). These Treg are a subset of CD4+ lymphocytes, characterized by a high expression of the IL-2 receptor α-chain CD25, and comprises approximately 5% of the total CD4+ T cell population in both mice and men[1-4]. Although several subtypes of Treg have been described (Tr1, Th3), this thesis will only focus on CD4+CD25+ Treg. Treg (also described as suppressor T cells), are able to restrain immune activation, thereby mediating self-tolerance. For over four decades Treg have been linked to the protection of autoimmunity[5-7]. Co-culturing mouse or human CD4+ or CD8+ T cells with CD4+CD25+ cells *in vitro* dramatically impairs proliferation of the T cells[1, 2]. Indirect evidence for the importance of Treg in the suppression of autoimmunity arose from mice thymectomy studies early in life, at the time in which the thymus is still developing its T lymphocyte population. This was found to result in the onset early of auto-inflammatory like diseases including gastritis, thyroiditis, and type 1 diabetes. In thymectomy studies using rats, diabetic mice, or young chickens similar results were observed[8-11]. The dramatic autoinflammatory immune phenotype could be prevented by the administration of CD4+ cells, and in particular CD4+CD25+ cells[4, 12, 13]. Furthermore, depletion of CD4+CD25+ Treg in mice led to similar symptoms as observed in the thymectomised mice[14]. Taken together, these data were the first to demonstrate that a population of CD4+CD25+ Treg cells are crucial for maintaining immune homeostasis and provided the basis for studies aiming to more specifically study these cells.

Natural versus induced Treg

Over the last five years there has been increasing evidence that there are in fact two distinct subsets of CD4+CD25+ Treg in mice and humans, the natural Treg (nTreg) that are generated in the thymus, and the induced Treg (iTreg) that differentiate from naive T cells in

the periphery[15, 16]. CD4+CD25+ Treg with suppressive capacity are found in the thymus with the same frequency as observed in the periphery. Moreover, these cells do not appear to have homed to the thymus from the periphery, and this implies Treg can be produced by the thymus[17, 18]. During thymic selection in the thymic medulla, thymocytes with a high affinity for cognate self-peptide complexes are thought to acquire CD25 expression and develop into Treg[19]. Thymocytes with a low affinity for self peptides are apparently unable to differentiate into Treg. Since the population of Treg in mice deficient for the T cell co-stimulator CD28 is significantly reduced, CD28 co-stimulation was also reported to be critical in the selection process[20].

While there is clear evidence of CD4+CD25+ Treg development in the thymus, it has also been reported that these cells can differentiate directly from CD4+CD25- T cells in the periphery[21, 22]. Chen *et al.* demonstrated that stimulation of the T cells receptor (TCR) in combination with TGF-β was sufficient to induce development of CD4+CD25+ suppressive iTreg from CD4+CD25- T cells[23]. Similar results were also obtained using either alloantigen treatment or immunization with low doses of antigen. Low dose antigen delivery through implantation of osmotic pumps in mice also resulted in conversion of CD4+CD25- cells into CD4+CD25+ iTreg[24, 25]. Taken together, these data demonstrate that there are two subsets of Treg, those that develop in the thymus or in the periphery. Although both nTreg and iTreg can suppress activation and proliferation of Teff cells, iTreg appear to be less suppressive *in vitro*[26].

Mechanisms of CD4+CD25+ Treg mediated suppression

It has become clear that Treg can inhibit the proliferation and activation of effector T cells (Teff; also known as T helper cells) *in vitro* and *in vivo*. Although it was originally thought that these cells can only inhibit immune activation by the secretion of inhibitory cytokines, is has now become apparent that there are multiple potential mechanisms underlying Treg

mediated suppression. These can be grouped in at least four different modes of action which are discussed below (**Fig. 1**).

Suppression mediated by inhibitory cytokines

The immunosuppressive cytokine IL-10 can be produced by Treg and adaptive transfer of IL-10 producing Treg into mice with airway inflammation was reported to control the inflammatory process, and this effect was abrogated by administration of specific anti-IL-10 receptor antibodies[24, 27, 28]. Furthermore, in an induced colitis mouse model, in which colitis is induced by infusion of CD4+CD45^{high} T cells in immunodeficient mice, Treg isolated from IL-10 deficient mice were unable to protect from colitis, and the protective effect of wild-type Treg could again be abrogated by infusion with anti-IL-10 receptor antibodies[29].

TGF-β can also be produced by Treg, and Treg can also express membrane-tethered TGF-β on their cell-surface[30, 31]. Both *in vitro* and *in vivo* studies report that TGF-β is crucial for Treg mediated suppression[32, 33]. T cell specific deletion of *Tgfb1* or anti-TGF-β treatment was reported to result in airway allergic responses and colitis[34-36]. Furthermore, in a mouse inflammatory bowel disease (IBD) model, Treg cannot suppress Teff cells that express a dominant negative TGF-ß receptor that are therefor resistant to TGF-β mediated suppression[37]. *In vitro* experiments have demonstrated that Treg cannot suppress the proliferation of proliferating CD4+ cells when the two cell populations are physically separated[27, 38], demonstrating that Treg mediated suppression must contain a cell-cell contact dependent component. This observation also suggests that the dominant manner of TGF-β mediated suppression is through the membrane-tethered form. Another possible explanation is that cytokines are most effective when produced in very close proximity to the target cell, maintaining a high local concentration.

Another inhibitory cytokine that can be produced by Treg in mice is IL-35[39]. Ectopic expression of IL-35 in naïve CD4+ cells converted these cells into suppressive CD4+CD25+ Treg. *In vitro* proliferation of CD4+ T cells could be impaired by addition of recombinant IL-35[39]. In addition, IL-35 knockout Treg were unable to control disease in a mouse

inflammatory bowel disease model[39]. Since IL-35 secretion was not detected in a second study utilizing human Treg[40].

Suppression mediated by cytolysis

Human Treg have been shown to produce both perforin and granzyme A (or granzyme B in mice), which can induced apoptosis in Teff cells[41-43]. Mouse Treg deficient for granzyme B were found to be less suppressive compared to wild-type Treg[44]. In addition, granzyme B expressing Treg had an improved capacity to prevent rejection of skin grafts in a transplantation mouse model[45]. Since granzyme B and perforin production has previously only been assigned as a mechanism used by activated Teff cells, its role in Treg mediated suppression requires further investigation.

Suppression mediated by disruption of the metabolic state of the target cell

Treg express high levels of CD25, the IL-2 receptor α-chain, on their cell surface. Since IL-2 is a cytokine critical for activation and proliferation of T effector cells, it has been proposed that Treg could suppress Teff cells by consumption of locally available IL-2[38, 46]. Pandiyan *et al*. were able to show that in mice Treg-mediated deprivation of IL-2 resulted in apoptosis of Teff cells, which could be reversed by addition of exogenous IL-2[47]. In contrast, two recent studies have shown that while human Treg can consume IL-2, IL-2 deprivation does not per se result in apoptosis and therefore is likely not a mechanism used in general by Treg[48, 49].

Recently it has been proposed that Treg can suppress Teff cells through regulation of cyclic AMP (cAMP) levels. Treg have high intracellular levels of cAMP, and this cAMP can be directly transferred to Teff through membrane gap-junctions[50]. Treg mediated suppression of Teff cells was found to be severely impaired by cAMP antagonists or inhibitors of gap-junctions[50]. An additional mechanism by which Treg can increase the intracellular levels of cAMP in neighboring Teff cells is through expression of a CD73 and CD39 on their cell surface. These ectoenzymes hydrolyze ATP and ADP to AMP and adenosine. Adenosine

can then subsequently activate the adenosine receptor 2a ($A_{2a}R$) on Teff cells, resulting in increased intracellular cAMP and suppression of Teff cell activation and proliferation[51, 52].

Suppression mediated by targeting dendritic cells

The suppressive capabilities of Treg can also be indirectly mediated through interaction with dendritic cells (DC). The co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4) is highly expressed on Treg and can interact with CD80 and CD86 ligands on the surface of DC[27, 53, 54]. This interaction results in upregulation and secretion of indoleamine 2,3-dioxygenase (IDO) by DC, a potent suppressor of Teff cells. Treg-mediated suppression of CD4+ T cell proliferation in the presence of DC was found to be impaired in the presence of CTLA-4 blocking antibodies or when CTLA-4 deficient Treg were used, demonstrating that CTLA-4 is critical for Treg-mediated suppression[55, 56]. Treg can also modulate immune responses through direct inhibition of DC maturation, resulting in inefficient stimulation of Teff cells[57]. This is thought to occur by interaction of lymphocyte activation gene 3 (LAG3; CD223), a CD4 homologue on the Treg membrane with MHC class II molecules on the DC surface thereby abrogating DC maturation. Consistent with this, LAG3 deficient Treg were unable to abrogate DC maturation, demonstrating that LAG3 by Treg can indirectly result in impaired CD4+ T cell activation[57].

Taken together, recent studies have demonstrated that Treg are able to suppress immune responses by diverse mechanisms, utilizing the expression of a variety of surface molecules and cytokine production. It remains unclear if human Treg utilize all these mechanisms to maintain immune homeostasis, and if so, if some are more important than others. Further research will be necessary to better understand the dominant mechanism(s) of Treg mediated suppression in humans.

A role for Treg in autoimmunity?

Since the discovery of Treg, their significance in the pathogenesis of numerous, especially autoimmune related, diseases have been extensively studied. Possible defects resulting in autoimmunity include: insufficient Treg numbers, Treg with impaired suppressive capacity, or Teff cells that are resistant to Treg-mediated suppression[58].

Treg numbers in peripheral blood are significantly reduced in both systemic lupus erythematosus (SLE) and ulcerative colitis compared to healthy controls[59-64]. In addition, the number of Treg inversely correlates with disease, suggesting that reduced Treg numbers could be a critical component of disease pathogenesis[61, 62]. In contrast, the percentage of Treg in the inflamed lamina propria and mesenteric lymph nodes of ulcerative colitis patients was fund to be increased compared to controls[63, 64]. Increased numbers of Treg have been reported in peripheral blood and skin lesions of psoriasis patients and synovial fluid in the joints of arthritis patients[65-68]. However, increased Treg numbers at inflammatory loci is apparently insufficient to control the local inflammation, suggesting that their suppressive capacity is impaired. Indeed, analysis of the suppressive capacity of Treg isolated from rheumatoid arthritis patient revealed that these cells were inefficient in suppressing the production of TNF-α and IFN-γ by Teff cells[69]. Furthermore, Treg isolated from either lesional skin or peripheral blood from psoriasis patients, type 1 diabetes patients, multiple sclerosis (MS) patients, or SLE patients all demonstrate abrogated suppression of CD4+ T cell proliferation[70-76].

Although it is clear that suppression by Treg is impaired in these diseases, there may also be a role for Teff cells. Since some of these studies were performed utilizing autologous Teff cells, there is still a possibility that here Treg are suppressive, but the Teff are resistant to suppression. In studies to determine whether patient Teff cells are indeed more resistant to Treg mediated suppression, Treg from healthy controls were co-cultured with control or patient Teff cells, and suppression of Teff cell proliferation was assessed. Teff of patients with type 1 diabetes, SLE, and Crohn's disease were observed to be partly resistant to Treg mediated suppression[71, 74, 76-78].

These data suggest that strategies to increase Treg numbers/function would be a promising approach to resolve autoimmune diseases. In addition, it will be necessary to identify the specific factors mediating resistance of Teff for Treg mediated suppression, which could reveal novel therapeutic targets to modulate immune responses.

Foxp3: the master regulator of Treg ontogeny and function

Although suppressive Treg were first functionally identified in 1970, isolation of these cells and therefore research into their biology remained difficult since there were no available markers to obtain purified cell populations[5]. Over the last few years, multiple "Treg markers" have been identified, being defined as upregulated in Treg versus Teff cell, but none of these markers demonstrate total speciticity for Treg (**Table 1**). At the start of this millennium it was evidently shown that the transcription factor Foxp3, a member of the Forkhead box transcription factor family, was selectively expressed by CD4+CD25+ Treg[1, 2, 79]. Although all forkhead family members have a homologous DNA-binding domain their individual roles have been specifically implicated in a variety of biological processes. For example, Foxp1 is critical for brain and lung development, while Foxp2 is necessary for speech and language development (reviewed by Coffer *et al.*[80]). Structural analysis of the Foxp3 protein has revealed several functional domains; a proline-rich repressor domain which is necessary for transcriptional activity, zinc finger and leucine zipper domains which are important for protein-protein interactions and the forkhead DNA-binding domain (**Fig. 2**)[79, 81, 82]. In human but not mouse, Treg there are two Foxp3 isoforms expressed, a full-length form and one lacking exon 2 (**Fig. 2**)[83]. Although CD4+ T cells transduction with either isoform will generate cells with a Treg phenotype, there are some minor functional differences as the transcription factor RORγt associates with the exon 2 encoded region of Foxp3 (further discussed below)[84, 85].

Foxp3 is crucial for both Treg function and development, and both scurfy mice, which express a mutated form of Foxp3, as well as Foxp3 knockout mice die within 3 weeks after

birth by exuberant multi-organ infiltration of activated T cells, caused by a Treg deficiency[1, 2, 14, 86]. Mutations in the *Foxp3* gene are also found in humans resulting in a rare autoimmune disease termed immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX)[87]. Patients suffer from similar symptoms to scurfy mice including massive lymphoproliferation, early onset diabetes, thyroditis, eczema, food allergies, and chronic wasting[81, 87, 88]. Although treatment with immune suppressive drugs decreases the onset and severity of IPEX symptoms, only a bone marrow transplantation can result in total remission. Forced expression of Foxp3 in mouse or human CD4+CD25- cells was found to induce a Treg phenotype generating cells that *in vitro* were able to impair proliferation of co-cultured CD4+ and CD8+ cells[1, 2, 14]. Consistent with this, in a mouse colitis model injection of Foxp3 transduced CD4+ cells, but not control transduced cells, significantly reduced disease scores and colitis pathology in the gut. These pioneering studies could demonstrate that ectopic expression of Foxp3 in CD4+ T cells results in the generation of functional Treg both *in vitro* and *in vivo*[1]. In addition, ablation of Foxp3 in mature Treg in loxP-flanked Foxp3 mice resulted in increased production of IL-2 and the loss of their suppressive capacity, demonstrating that sustained Foxp3 expression is critical for maintenance of their suppressive phenotype[89].

In vitro TCR stimulation of CD4+CD25- T cells utilizing anti-CD3 and anti-CD28 antibodies results in a transient expression of Foxp3 mRNA and protein, possibly to prevent excessive activation. However the majority of these cells only temporarily upregulate Foxp3 protein levels, losing Foxp3 expression several days after stimulation. These “ex-Foxp3” T cells are non-suppressive in both *in vitro* and *in vivo* assays[26, 90]. In contrast, a small population retains Foxp3 protein expression and are suppressive, demonstrating that stable Foxp3 expression in needed for maintenance of the Treg phenotype[83, 91].

Foxp3 post-translational modifications

The function of many intracellular proteins, particularly transcription factors, can be altered by post-translational modifications. Here, one or more amino acids are covalently modified, often modulating subcellular localization, activation state, interaction with other proteins, or protein turnover. Although over 20 post-translational modifications (PTMs) have been characterized, the best studied PTMs include acetylation, phosphorylation and ubiquitination (**Fig. 3, 4**).

Acetylation

Protein acetylation is a tightly regulated process in which an acetyl group from acetyl-CoA is transferred to a specific substrate[92]. A majority of acetylated proteins appear to be directly involved in transcriptional regulation, either as histones or transcription factors[93]. Although the N-terminus of several proteins have been reported to be acetylated, the majority of acetylation occurs at the ξ-amino nitrogen specific lysine residue[92, 94]. The process of acetylation is reciprocally regulated by lysine acetyl transferases (KATs) and lysine deacetylases (KDACs).

Acetylation of Foxp3 is an important post-translational modification which has recently been shown to result in diverse biological outcomes[95-97]. TGF-ß stimulation of Treg was reported to increase Foxp3 acetylation, while treatment of CD4+CD25+ cells with TGF-ß also enhanced Foxp3 association to chromatin, and site-specific recruitment of Foxp3 to the human IL-2 promoter[96]. Acetylation was also shown to regulate Foxp3 protein stability, as proteasomal degradation was abrogated when Foxp3 was hyperacetylated[97]. Acetylation was subsequently shown to impair Foxp3 poly-ubiquitination, thereby resulting in increased Foxp3 protein levels. Since both ubiquitination and acetylation are restricted to lysine residues, it is likely that the molecular mechanism underlying these observations involves competition between ubiquitination and acetylation for free lysine residues. This theory is strengthened by the observation that a Foxp3 mutant in which all lysine residues are mutated to argenine is extremely stable, comparable to acetylated Foxp3[97].

Two distinct KATs have thus far been demonstrated to increase Foxp3 acetylation[95, 97]. The ubiquitously expressed KAT p300 can interact with Foxp3, and ectopic expression p300 increases Foxp3 acetylation. In an *in vitro* acetylation assay Foxp3 was found to be directly acetylated by p300, demonstrating that Foxp3 is indeed a direct substrate[97]. The KAT TIP60 has also has been shown to associate with Foxp3, and Foxp3 acetylation can be increased by ectopic expression of TIP60[95, 97].

There are four different classes of KDACs based upon their sequence identity and domain organization. Class I includes HDAC 1, 2,3 and 8, Class II: HDAC 4, 5, 6, 7, 9 and 10, Class III: SIRT1-7 and Class 4 only comprises of HDAC 11. The class II KDACs: HDAC7, HDAC9 and class III KDAC SIRT1 have all been reported to associate with Foxp3[95, 97]. Ectopic expression of SIRT1 decreases Foxp3 expression and conversely SIRT1 inhibition increases Foxp3 protein levels, however specific KDAC-mediated deacetylation still needs to be demonstrated[97]. HDAC7 was shown to both interact with Foxp3 and also decrease its transcriptional activity[95]. Since HDAC7 is catalytically inactive, its effect on Foxp3 transcriptional activity cannot be directly mediated by Foxp3 deacetylation.

Over the last few years the potential of KDAC inhibition to manipulate immune responses has been increasingly studied[98]. In several reports the effect of KDAC inhibitors on Treg mediated suppression has been analyzed. The broad range KDAC inhibitors: TSA, SAHA, tubacin, BML-210, MS-275, SB, bufexmac and SIRT specific inhibitor nicotinamide all increased Treg suppressive capacity *in vitro*[97, 99, 100]. HDAC9 or SIRT1 knock-down both increased Treg mediated suppression of proliferating Teff cells *in vitro*[101, 102]. While mice treated with the broad spectrum KDAC inhibitors TSA, SAHA, VPA, splitomycin, or a SIRT1 inhibitor all showed down-modulation of immune responses in colitis, arthritis, and transplantation models[99, 101-103]. Furthermore, mice with targeted deletions of Sirt1 in either CD4+ T cells or Foxp3+ Treg cells exhibited prolonged survival of mice receiving MHC-mismatched cardiac allografts[101]. Since acetylation of Foxp3 is important for its transcriptional activity and therefore Treg mediated suppression, it is interesting to speculate

that the effect of these diverse KDAC inhibitors on Treg function is mediated through regulation of Foxp3 activity.

Phosphorylation

Phosphorylation is perhaps the most well-studied post-translational modification, affecting about one third of all proteins in cells. Protein phosphorylation is regulated by over 500 kinases, whereas dephosphorylation is regulated by only a few dozen phosphatases[104]. Most proteins are phosphorylated at serine and threonine residues (98%), however a small proportion can be tyrosine phosphorylated[105].

Surprisingly little is in fact known concerning Foxp3 phosphorylation, and there is only a single report demonstrating that Foxp3 is phosphorylated[96]. Here, Foxp3 was immunoprecipitated from human CD4+CD25+ Treg and threonine phosphorylation was analyzed by Western blotting utilizing an anti-phospho-threonine antibody. Analysis revealed that both Foxp3 isoforms can be phosphorylated on threonine residues, however these observations need to be more rigorously investigated and the function of Foxp3 phosphorylation on transcriptional activity still remains unclear[96].

Ubiquitination

Protein ubiquitination is tightly regulated by a large family of E1, E2, and E3 ligases. E1 activates ubiquitin in an ATP-requiring reaction to generate a high-energy thiol ester intermediate and uses its active cysteine site to associate with ubiquitin. Subsequently, the E1 transfers ubiquitin to the ubiquitin conjugating enzyme E2. Next, one of the 40 E3 ubiquitin ligase family members associates ubiquitin to a lysine residue of the substrate. Since there are only a few E2 ligaeses, substrate specificity is achieved by the combination of E2 and E3 ligases. Together these enzymes catalyze addition of 8.5 kDa ubiquitin protein to lysine residues of the target protein. Here, the initial ubiquitin can serve as an acceptor for further cycles of ubiquitin modification, eventually resulting in elongation of a poly-ubiquitin chain[106]. Ubiquitin contains seven lysines that can potentially themselves be ubiquitinated to

form the poly-ubiquitin chain. Lysine-48 (48K) linked poly-ubiquitination is the best studied form of protein ubiquitnation and marks a protein for degradation by the 26S proteasome[107, 108]. Both mono- and non-K48 linked poly-ubiquitination (such as K11, K29, and K63) have been implicated in the regulation of protein function and localization and have been demonstrated to regulate in multiple cellular processes including DNA repair, apoptosis, antigen processing and transcription[109-112]. Deubiquitination is regulated by deubiquitinating enzymes (DUBs) which consist of over 60 distinct family members. Although there is specificity for substrates, DUB specific consensus sites have not yet been identified [113, 114].

Relatively little is known concerning Foxp3 ubiquitination, although both mono- and poly-ubiquitination of Foxp3 has been observed in cells ectopically expressing Foxp3[97]. Furthermore, poly-ubiquitination can be negatively regulated by increased Foxp3 acetylation resulting in increased protein stability[97].

Foxp3 binding partners

In general, the activity of transcription factors is mediated by the association with specific co-factors. Foxp3 has been shown to be part of a 600 kDa protein-complex (**Fig. 5**)[115]. As previously discussed, the KATs p300 and TIP60 have been reported to associate with Foxp3 increasing both its transcriptional activity and protein stability[95, 97]. In addition, the KDACs HDAC7/9 and SIRT1 also interact with Foxp3, impairing its transcriptional function[95, 97].

Foxp3 has been shown to homo-dimerize or dimerize with its close transcription family relative Foxp1, an association mediated by their leucine zipper domains[115]. Both interactions are abrogated by delK250 or delE251 mutations which have been described in IPEX patients. These mutations also dramatically impaired both association of Foxp3 with chromatin, as well as Foxp3 transcriptional activity[115]. These data demonstrate that dimerization is essential for association with the DNA and therefore transcriptional activity, difining the molecular mechanism responsible for the clinical observations in the delK250/delE251 IPEX patients.

Nuclear factor for activated T cells (NFAT), nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB), activator protein 1 (AP-1), and acute myeloid leukaemie (AML-1) are transcription factors essential in controlling adaptive immunity[116-119]. NFAT forms co-operative complexes with AP-1, and together modulate cytokine transcription after T cell activation[118]. Both NFAT and NF-κB are thought to have a common evolutionary origin, based on their related DNA-binding domains[117, 119]. These transcription factors can upregulate T cell cytokines including IL-2, IL-4 and IFN-γ, cytokines whose production can be suppressed by Foxp3[42, 120, 121]. NFAT physically associates with Foxp3 and the transcriptional activation of NFAT target genes is blocked by ectopic expression of Foxp3[120]. Based upon the crystal structure of NFAT bound to the DNA-binding domain of Foxp2, it was reported that NFAT is likely to associate with the DNA-binding domain of Foxp3. Mutating residues within the Foxp3 DNA binding domain predicted to associate with NFAT, interfered with Foxp3 transcriptional activity, and Treg mediated suppression in an autoimmune diabetes model[122]. Equally, Betelli *et al.* reported that in cells ectopically expressing both Foxp3 and NF-κB these proteins can associate, resulting in impaired transcription of NF-κB gene targets[120].

Foxp3 has also been described to associate with c-Jun, a member of the AP-1 transcription factor family. A c-Jun-Foxp3 complex was observed by immunoprecipitation utilizing an ectopically expressing cell line as well as primary mouse Tregs, and was increased by phosphorylation of c-Jun[119]. Association of these transcription factors alters c-Jun subnuclear localization as well as the affinity of AP-1/c-Jun for chromatin, resulting in abrogated transcriptional activity of c-Jun transcriptional targets[119].

The transcription factor AML1/Runt-related transcription factor 1 (Runx1) is involved in T cell development in the thymus and can regulate both IL-2 and IFN-γ transcription[123]. Foxp3 was shown to co-localize and physically interact with AML1 in human Treg. Increased production of both IL-2 and IFN-γ by AML1 was demonstrated to be inhibited by co-transduction of Foxp3 in CD4+ T cells. These data have been validated utilizing an IL-2 promoter reporter,

demonstrating that Foxp3 suppresses T cell activation by decreasing IL-2 and IFN-γ by inhibiting AML1 mediated transcription[124].

Retinoic acid-related orphan receptor γt (RORγt) is essential for development and function of Th17 T cells[85, 125]. Th17 cells are a subset of T helper cells that express IL-17, and are thought to play a key role in the onset of auto-immune diseases[85, 125]. Foxp3 and RORγt can antagonize each others activity by direct association, resulting in the development of either Th17 or Treg cells, although the precise molecular mechanism underlying this observation remains unclear[84, 85]. Interestingly, the Foxp3delExon2 isoform is unable to associate with RORγt, mapping the interaction site to exon2 in Foxp3, and also for the first time showing a functional difference between the two Foxp3 isoforms[84, 85].

A recent report has shown that Foxp3 can also associate with EOS, a zinc-finger transcription factor of the Ikaros family[126]. EOS appears to be necessary for Foxp3-mediated transcription since EOS knock-down resulted in impaired transcription of Foxp3 target genes in reporter assays. EOS was shown to recruit the transcriptional repressor C-terminal-binding protein 1 (CTBP1) to the Foxp3-EOS complex, resulting in transcriptional repression. Furthermore, EOS knock-down resulted in enhanced IL-2 and IFN-γ gene expression in mice Treg, without influencing Foxp3 protein expression. In addition, in a mouse colitis model, infusion of EOS deficient Treg was unable to reduce colitis scores, in contrast to wild-type cells, demonstrating that EOS is necessary for the suppressive capacity of Treg[126].

Interferon regulatory factor-4 (IRF4) is a transcription factor essential for Th2 effector cell differentiation, but is also expressed in Treg[127]. Zheng *et al.* reported that Foxp3 directly associates with IRF4 in mouse Treg, and that this interaction is necessary for Treg mediated suppression of T helper 2 (Th2) immune responses[127]. CD4+ T cells from mouse with IRF4 deficient Treg display an increased activation status, and upregulated cytokines including IL-4 and IL-5. These mice showed dysregulated Th2 responses, resulting in tissue lesions with pronounced plasma cell infiltration[127]. The precise mechanism how the interaction of these factors result in TH2 specific suppression needs to be further evaluated.

Thesis outline

Since Foxp3 is crucial for both the differentiation and function of Treg, the aim of the current study was to gain a better understanding of the molecular mechanisms regulating Foxp3 functionality. In this thesis we have focused on modulation by post-translational modifications as well as association with co-factors.

In **Chapter 2** we demonstrate that acetylation of Foxp3 can be reciprocally regulated by the KAT p300 and KDAC SIRT1. Increased acetylation prevents poly-ubiquitination and protein degradation, resulting in increased Foxp3 protein levels. Inhibition of KDAC activity in both primary human and mouse primary Treg increases Foxp3 protein expression and Treg mediated suppression of Teff cell proliferation. These data demonstrate that Foxp3 protein levels and therefore Treg mediated suppression can be regulated by acetylation of Foxp3.

In **Chapter 3** the role of SIRT KDACs in Foxp3 deacetylation was investigated further. SIRT1, but not SIRT2-7, co-localizes with and negatively influences Foxp3 protein levels. Ectopic expression of SIRT1 increased Foxp3 poly-ubiquitination, thereby increasing Foxp3 proteasomal degradation. Furthermore, SIRT1-mediated Foxp3 deacetylation abrogated Foxp3 transcriptional activity in human Treg, demonstrating that SIRT1 plays a central role in modulation of Treg mediated suppression.

Chapter 4 focuses on the regulation of Foxp3 poly-ubiquitination. We show that the ubiquitin specific protease 7 (USP7) can deubiquitinate Foxp3, thereby increasing Foxp3 protein levels. Inhibition of DUB activity or knock-down of USP7 in human Treg decreases Foxp3 protein levels and abrogates Treg mediated suppression *in vitro*. In contrast to control Treg, Treg in which DUB activity was inhibited are ineffective in reducing disease scores in a mouse colitis model. Work presented here demonstrates that modulation of Foxp3 poly-ubiquitination by USP7 is a critical mechanism for regulation of Treg mediated suppression.

Chapter 5 focusses on the regulation of Foxp3 transcriptional activity by associating co-factors. Here we show that the interaction of Foxp3 with the Wnt-transcriptional effectors TCF1 and β-catenin negatively affects Foxp3 transcriptional activity. Consistent with this,

inhibition of Wnt signaling in human Treg increases Treg mediated suppression, while activation of Wnt signaling abrogates Treg function *in vitro* and *in vivo* utilizing two autoimmune mouse models. Since Wnt3a production is increased by activated T cells, as observed in the joints of juvenile idiopathic arthritis patients, our data demonstrates that mononuclear cells can regulate Treg function by Wnt production.

The consequences of the findings in this thesis are discussed in **Chapter 6**.

Tables

Table 1. Treg markers. Markers found to be upregulated in Treg compared to Teff cells in mouse and human. IL, interleukin. TNFRSF, Tumor necrosis factor receptor super family.

Figure legends

Figure 1. Mechanisms of Treg mediated suppression. Treg can suppress the activation and proliferation of Teff cells by mechanisms involving: **a**, targeting DCs. **b**, inhibitory cytokines. **c**, cytolysis. **d**, disruption of the metabolic state of target cells. Treg, regulatory T cells. Teff, T effector cells. DC, dendritic cell. IDO, indoleamine 2,3-dioxygenase. CTLA4, cytotoxic T-lymphocyte antigen 4. MHC, major histocompatibility complex. LAG3, lymphocyte activation gene 3. TGF-β, transforming growth factor β. IL, interleukin. cAMP, cyclic adenosine monophosphate. $A_{2A}R$, adenosine receptor 2a.

Figure 2. Schematic representation of two Foxp3 isoforms. Mutations found in IPEX patients are indicated. RD, repressor domain. ZF, zinc-finger domain. LZ, leucine zipper domain, FKH, forkheadbox DNA-binding domain. The second isoform is Foxp3 lacking exon 2.

Figure 3. Allignment of mouse and human Foxp3. Mouse and human Foxp3 are aligned. Boxed amino acids are conserved. Conserved amino acids that can be post-translationally modified by acetylation, ubiquitination or phosphorylation are highlighted.

Figure 4. Foxp3 post-translational modifications. Schematic representation of the enzymes that regulate Foxp3 post-translational modifications. K, lysine residue. S, serine residue. T, threonine residue. Y, tyrosine phoshorylation. KAT, lysine acetyl transferase. KDAC, lysine deacetylase. DUB, deubiquitinase.

Figure 5. Foxp3 binding partners. Proteins that associate with Foxp3. RD, repressor domain. ZF, zinc-finger domain. LZ, leucine zipper domain, FKH, forkheadbox DNA-binding domain.

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Human	Mouse	Function
CD4	CD4	Co-receptor for the T cell receptor
Foxp3	Foxp3	Transcription factor
CTLA4	CTLA4	Immune inhibitory receptor
CD28	CD28	T cell co-stimulatory protein
GITR	GITR	Immune inhibitory receptor
TLR5	TLR5	Toll like receptor 5
TLR7	TLR7	Toll like receptor 7
TGF-ß	TGF-ß	Immune inhibitory cytokine
$CD25^{high}$	$CD25^{intermediate\text{-}high}$	IL-2 receptor α-chain
CD27		TNFRSF7
$CD127^{low}$		IL-7 receptor α-chain
HLA-DR		Major histocompatibility complex II
CCR4		Chemokine receptor 4
CCR8		Chemokine receptor 8
	CD30	TNFRSF8
	OX40	TNFRSF4
	4-1BB	TNFRSF9
	CCR2	Chemokine receptor 2
	CCR6	Chemokine receptor 6
	CCR7	Chemokine receptor 7
	CXCR3	CXC chemokine receptor 3
	LAG-3	Immune inhibitory receptor
	PD-1	Immune inhibitory receptor
	TLR4	Toll like receptor 5
	TGF-ßR1	TGF-ß receptor type 1

Table 1.

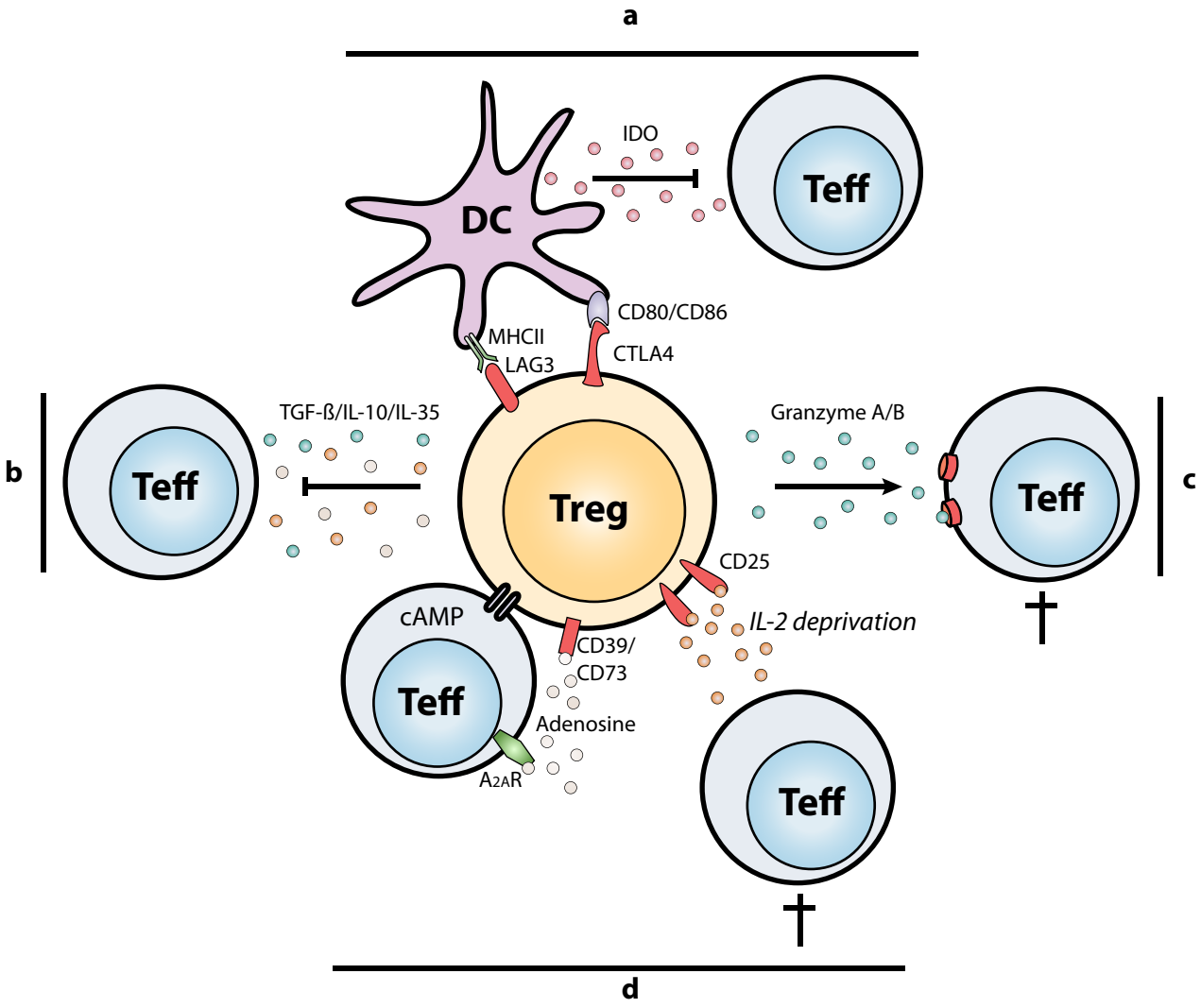
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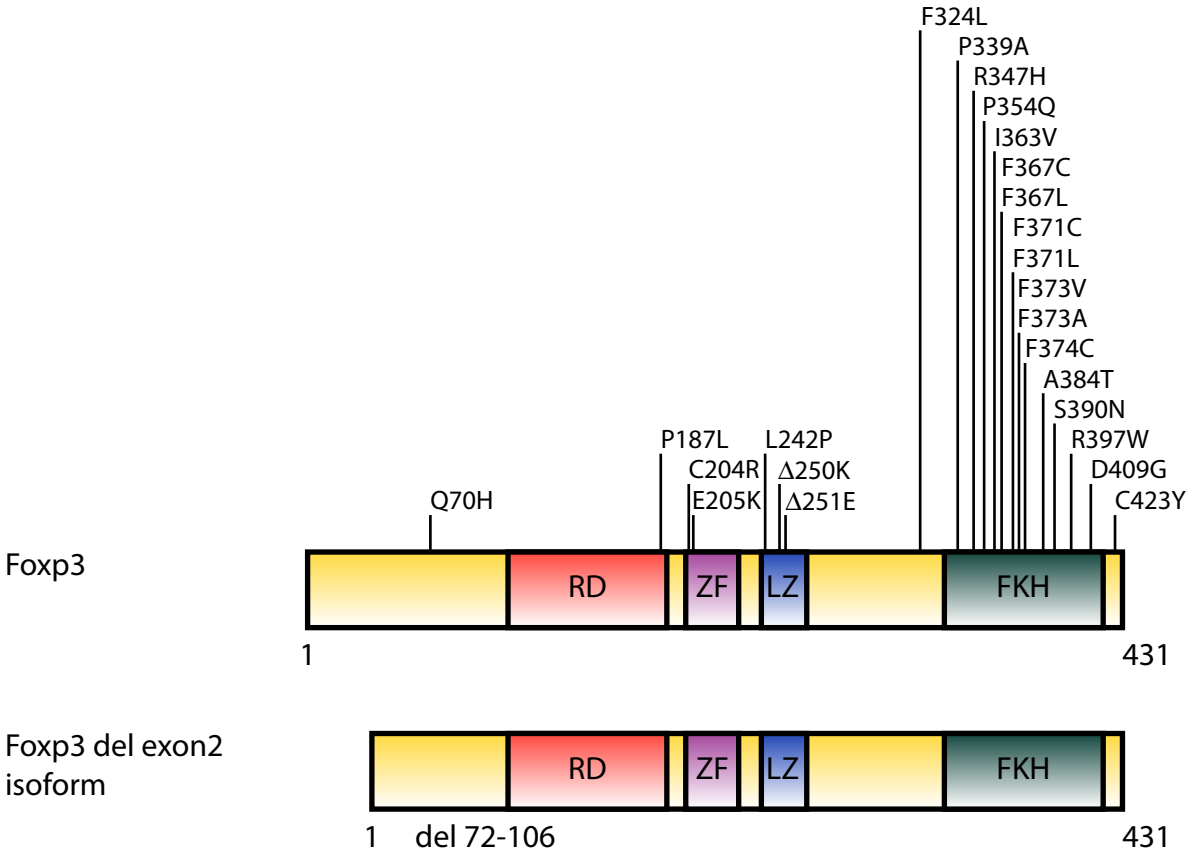
Figure 1.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L242P
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E205K
Δ251E
Foxp3
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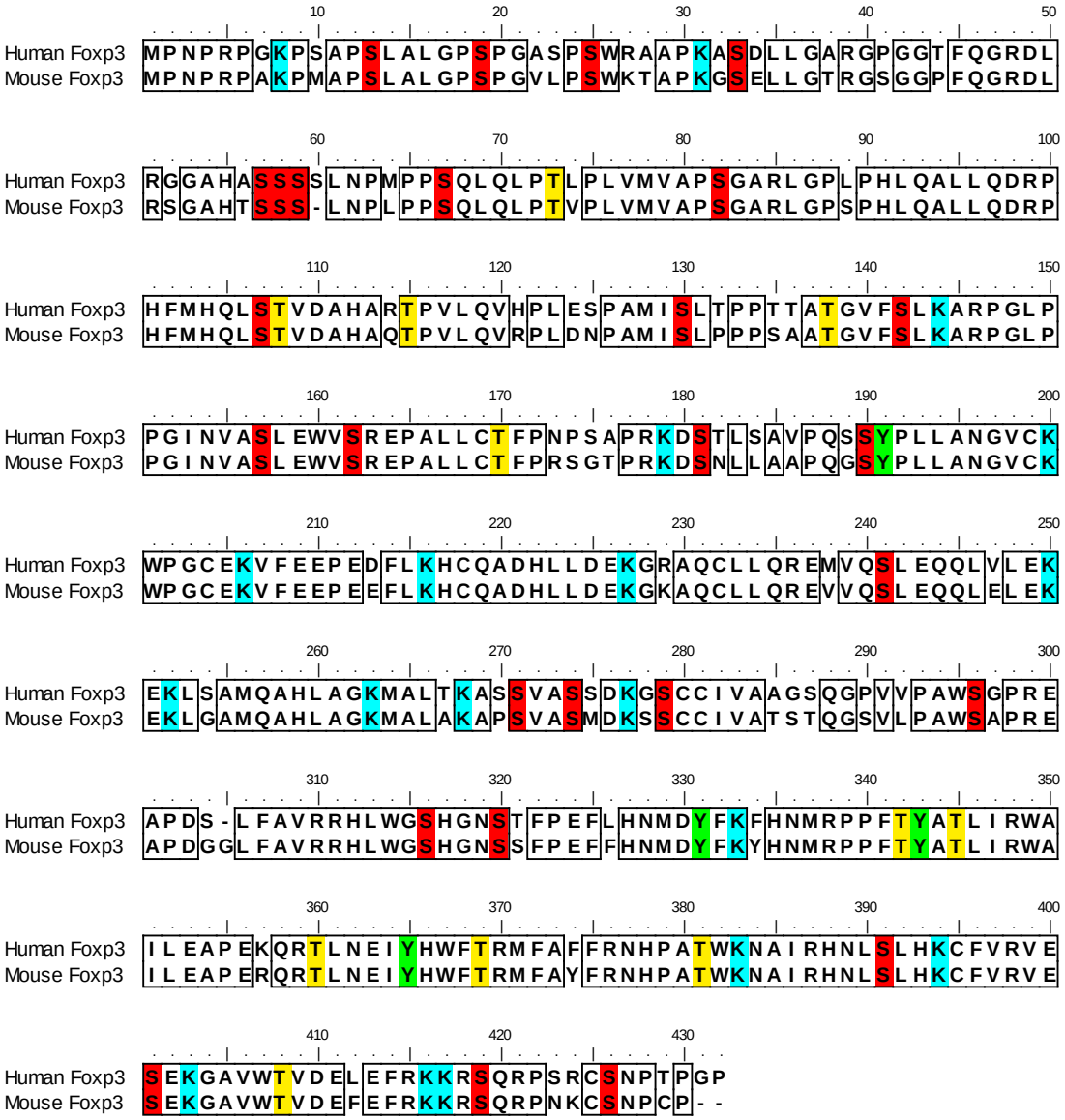
431

Figure 2.


Human Foxp3
Mouse Foxp3
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HFMHQLSTVDAHAQTPVLQVRPLDNPAMISLPPPSAATGVFSLKARPGLP
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PGINVASLEWVSREPALLCTFPRSGTPRKDSNLLAAPQGSYPLLANGVCK
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WPGCEKVFEEPEEFLKHCQADHLLDEKGKAQCLLQREVVQSLEQQLELEK
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EKLGAMQAHLAGKMALAKAPSVASMDKSSCCIVATSTQGSVLPAWSAPRE
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APDGGLFAVRRHLWGSHGNSSFPEFFHNMDYFKYHNMRPPFTYATLIRWA
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ILEAPERQRTLNEIYHWFTRMFAYFRNHPATWKNAIRHNLSLHKCFVRVE
SEKGAVWTVDELEFRKKRSQRPSRCSNPTPGP
SEKGAVWTVDEFEFRKKRSQRPNKCSNPCP--
K = lysine residue, acetyl and ubiquitin acceptor
S = serine residue, phospho acceptor
T = threonine residue, phospho acceptor
Y = tyrosine residue, phospho acceptor

Figure 3.

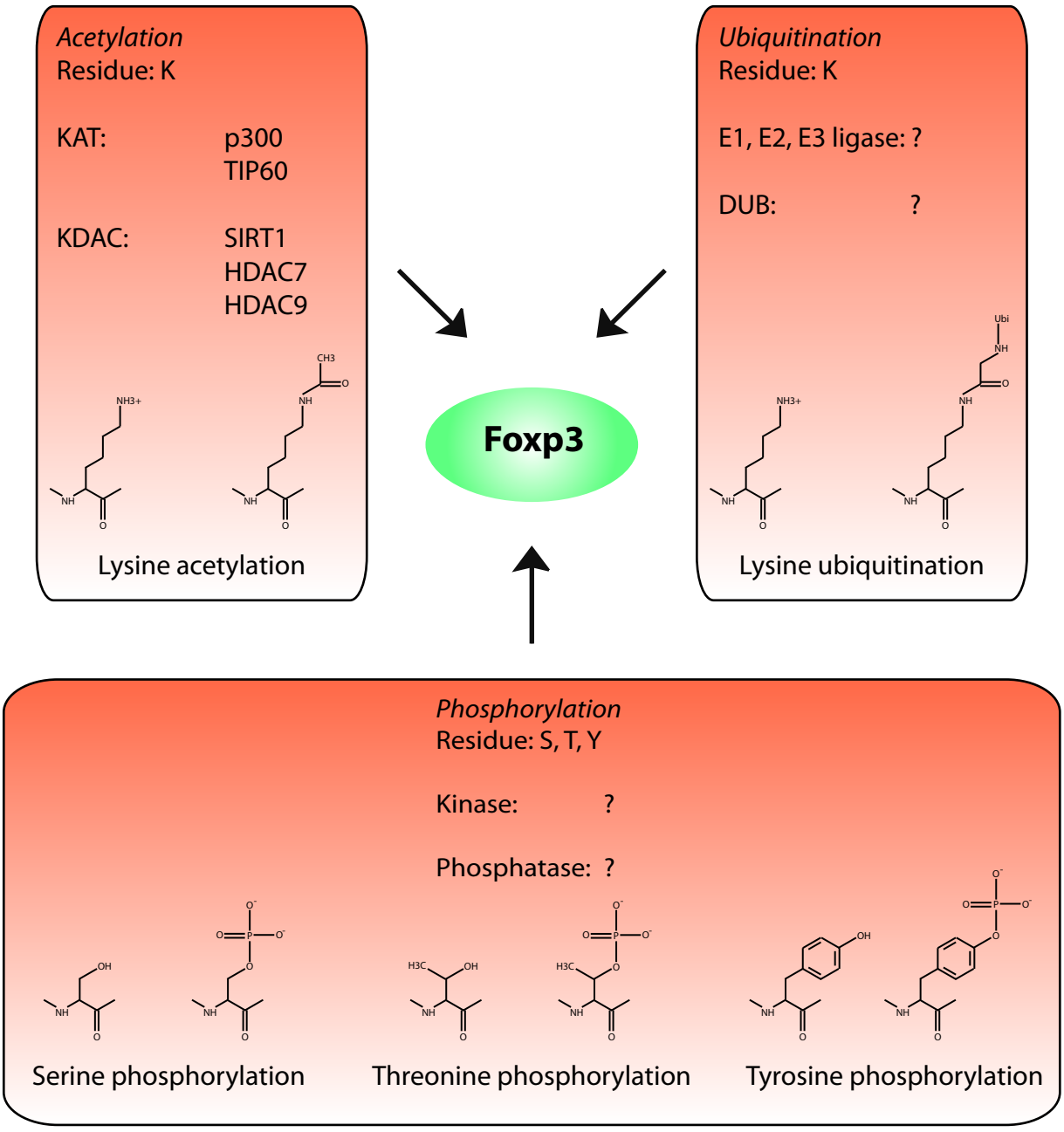
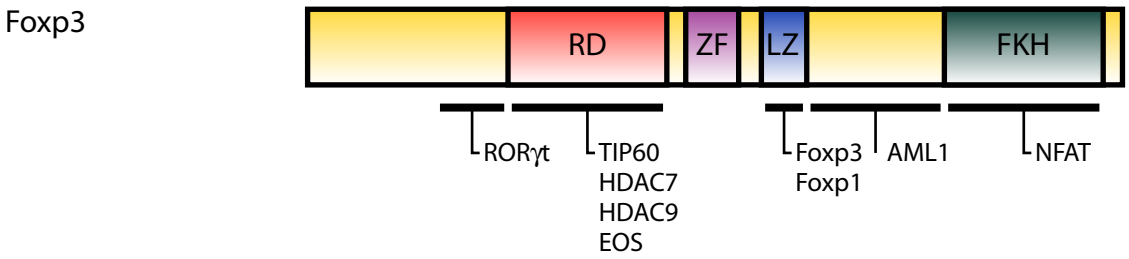

Acetylation
Residue: K
KAT: p300
TIP60
KDAC: SIRT1
HDAC7
HDAC9
Lysine acetylation
Ubiquitination
Residue: K
E1, E2, E3 ligase: ?
DUB: ?
Lysine ubiquitination
Foxp3
Phosphorylation
Residue: S, T, Y
Kinase: ?
Phosphatase: ?
Serine phosphorylation
Threonine phosphorylation
Tyrosine phosphorylation

Figure 4.



Binding site unknown: p300
SIRT1
NF-κB
AP-1
IRF-4

Figure 5.

Chapter 2

Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization.

Jorg van Loosdregt[1], Yvonne Vercoulen[2], Teun Guichelaar[3], Yoony Y.J. Gent[4], Jeffrey M. Beekman[1,2], Olivier van Beekum[5], Arjan B. Brenkman[5], Dirk-Jan Hijnen[4], Tuna Mutis[3], Eric Kalkhoven[2,5], Berent J. Prakken[2], Paul J. Coffer[1,2*]

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Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization

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Regulatory T cells (Tregs) are a specific subset of lymphocytes that are critical for the maintenance of self-tolerance. Expression levels of the transcription factor Foxp3 have been causally associated with Treg differentiation and function. Recent studies show that Foxp3 can also be transiently expressed in effector T cells; however, stable Foxp3 expression is required for development of a functional Treg suppressor phenotype. Here, we demonstrate that Foxp3 is acetylated, and this can be reciprocally regulated by the histone acetyltransferase p300 and the histone deacetylase SIRT1. Hyperacetylation of Foxp3 prevented polyubiquitination and proteasomal degradation, therefore dramatically increasing stable Foxp3 protein levels. Moreover, using mouse splenocytes, human peripheral blood mononuclear cells, T cell clones, and skin-derived T cells, we demonstrate that treatment with histone deacetylase inhibitors resulted in significantly increased numbers of functional Treg cells. Taken together, our data demonstrate that modulation of the acetylation state of Foxp3 provides a novel molecular mechanism for assuring rapid temporal control of Foxp3 levels in T cells, thereby regulating Treg numbers and functionality. Manipulating Foxp3 acetylation levels could therefore provide a new therapeutic strategy to control inappropriate (auto)immune responses. (Blood. 2010;115: 965-974)

Introduction

Regulatory T cells (Tregs) are a specific subset of lymphocytes that play a crucial role in the maintenance of self-tolerance.[1,2] These $CD4^+CD25^+$ cells can be distinguished from conventional T cells by the expression of a distinct subset of molecules, both on their cell surface as well as intracellularly.[3,4] The transcription factor Foxp3 is crucial for Treg differentiation and function, and various Foxp3 mutations, both in scurfy mice and IPEX (immune dysregulation polyendocrinopathy, enteropathy, X chromosome-linked syndrome) patients, result in the development of complex autoimmune disease, resulting from Treg deficiency.[5,6] Ectopic expression of Foxp3 in $CD4^+CD25^-$ T cells has also been shown to induce a suppressive phenotype, suggesting that stable Foxp3 expression is sufficient for development of functional Tregs.[2,3,7]

Foxp3 expression is not unique to lymphocytes; recent reports demonstrate that respiratory, thymic, prostate, and mammary epithelium cells also express Foxp3, although expression levels were low compared with Tregs.[8] Furthermore, it has been reported that in vitro T-cell receptor (TCR) stimulation of $CD4^+CD25^-$ effector T (Teff) cells can result in transient Foxp3 expression, which does not however generate T cells with a suppressive phenotype.[9,10] In contrast, TCR-stimulated $CD4^+CD25^-$ cells expressing high and stable Foxp3 levels develop suppressive capacity, illustrating that persistent Foxp3 expression is probably an essential step in the conversion of Teff cells into Tregs.[11,12]

Because Foxp3 is an essential transcription factor for maintenance of immune homeostasis, its activity must be tightly and specifically regulated. However, surprisingly little is currently understood concerning Foxp3 posttranslational regulation of this transcription factor. A recent study has demonstrated that Foxp3 can interact with histone deacetylase (HDAC) 7 and 9, and with the histone acetyltransferase (HAT) TIP60.[13] Although these data suggest that Foxp3 is able to form a multiprotein complex containing both HAT/HDAC molecules, the functional relevance of these observations remains to be further clarified. Furthermore, mice treated with HDAC inhibitor trichostatin A (TSA) have increased numbers of functionally improved Tregs correlating with reduced disease severity in an induced colitis model as well as an increased donor-specific allograft tolerance in a cardiac and islet transplantation model.[14] However, the molecular mechanism underlying improved Treg function by TSA treatment remains unclear, and it is not evident whether these are direct or indirect effects.

Here, we demonstrate that Foxp3 acetylation can be reciprocally regulated by the HAT p300 and the HDAC SIRT1. We show that Foxp3 protein has a short half-life and that acetylation prevents proteasomal degradation, dramatically increasing Foxp3 levels. Furthermore, modulating SIRT activity in mouse and human primary T cells regulates Foxp3 protein levels as well as the number and suppressive capacity of Tregs. Taken together, directly modulating the acetylation state of Foxp3 provides a novel molecular mechanism for assuring rapid temporal control of Foxp3 levels in T cells. Increasing Foxp3 acetylation levels may thus be a critical switch in the generation of induced Tregs from activated peripheral T cells.

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Methods

Antibodies, DNA constructs, and reagents

The following antibodies were used: mouse anti-Foxp3 clone PCH101 for fluorescence-activated cell sorter (FACS) analysis (eBioscience), rabbit anti-p300 (Santa Cruz Biotechnology), rabbit anti–acetyl-lysine (Cell Signaling Technology), mouse anti-Flag M2 from Sigma-Aldrich (Zwijndrecht), mouse antihemaglutinin (HA) clone 12CA5 from Santa Cruz Biotechnology, mouse antitubulin (Sigma-Aldrich), and anti-Myc monoclonal mice antibody were made using a hybridoma cell line. Foxp3 was cloned from MIGR1-Foxp3 (kindly provided by Dr S. Sakaguchi[7]) into pMT2 that already contained a Flag tag resulting in pMT2-Flag-Foxp3. Using side-directed mutagenesis, the pMT2-Flag-Foxp3ΔE250 mutant was constructed. pcDNA3 (Invitrogen), pcDNA3-HA-p300, pcDNA3-HA-TIP60 (kindly provided by Dr D. Trouche[15]), 6xHis-p300 (kindly provided by Dr W. L. Kraus[16]), pRSV-NFATc/A,[17] and Myc-SIRT1[18] (both kindly provided by Dr B. M. T. Burgering, University Medical Center, Utrecht, The Netherlands) have been described earlier. pCDNA3-p300-HA was generated by cloning a Not I-*Hin*dIII fragment from CMVβ-p300-HA (a gift from Dr R. Eckner, ESBATech AG, Zurich, Switzerland) into the respective cloning sites of pCDNA3. PEI (#23966) was purchased from Polysciences. TSA, nicotinamide (NAD), cycloheximide (CHX), epoxomycin, lactacystin, and MG132 were from Sigma-Aldrich.

Transfection of cells and luciferase assays

HEK293 cells were maintained in Dulbecco modified Eagle medium (Invitrogen) supplemented with 8% heat-inactivated fetal calf serum (FCS), penicillin, and streptomycin (Invitrogen) at 37°C and 5% CO_2. Cells were grown to 50% confluence in 6-well plates (Nunc) and transfected with a mixture of 1.5 μg DNA and 7.5 μL PEI overnight; the next day, cells were washed twice with phosphate-buffered saline (PBS) and cultured for 24 hours in medium. Cell lysates were prepared for Western blot analysis. For the luciferase assay, cells were transfected. Calcium-phosphate was used with 1 μg interleukin-2 (IL-2) promoter luciferase reporter from Panomics, 0.5 μg of pMT2-Foxp3, 0.5 μg pcDNA3-HA-p300, pcDNA3-NFATc/A, or 0.5 μg pcDNA3 empty vector and 7 μg pMT2 empty vector and 0.05 μg pRLTK Renilla (Promega) to normalize for transfection efficiency. Cells were transfected in a 6-well plate; 3 days after transfection, the cells were washed twice with PBS and lysed in 50 μL passive lysis buffer for 15 minutes, insoluble cell debris were spun down, and the supernatant fraction was assayed for luciferase activity using Dual-Luciferase Reporter Assay System (Promega).[19]

In vitro acetylation assay

Glutathione S-transferase (GST) fusion proteins were induced and purified as described previously.[20] The wild-type and catalytic AT2 mutant p300 proteins were synthesized in Sf9 cells using a baculovirus expression system and purified as previously described. A total of 1 μg GST-Foxp3, 0.5 μL (10 nCi) [14C]-acetylCoA (PerkinElmer), and 2 μg His(6x)-p300 or His(6x)-p300mutAT2 were incubated in AIPH buffer (20mM Tris-HCI pH 8.0, 60mM NaCI, 2mM EDTA, 0.2% NP-40, 40μM PMSF) for 40 minutes at 30°C. The reaction was stopped by 10 μL 5 × sample buffer. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Kodak XB films.

Confocal studies

HEK 293 cells were cultured on poly-L-lysine–coated (Sigma-Aldrich) microscope glasses, fixed in PBS containing 3% paraformaldehyde (Merck) for 15 minutes at 15°C, and subsequently 100% methanol (Merck) for 30 minutes at −20°C. Cells were preincubated with 10% normal goat serum (Jackson ImmunoResearch Laboratories) before mouse anti-Foxp3 (5 μg/mL; eBioscience) and of rabbit anti-p300 (5 μg/mL) was added for 1 hour, followed by PBS washes and incubation with 2 μg/mL goat anti–mouse Cy3 (Jackson ImmunoResearch Laboratories) or goat anti–rabbit Cy5 (Jackson ImmunoResearch Laboratories) conjugates (all antibody stainings were in PBS with 10% goat serum). Slides were then washed extensively, and cells were mounted in mowiol containing 3% 1,4-diazabiclo-(2,2,2)-octan followed by a glass cover as described.[21] Cells were examined with a Zeiss LSM 710 microscope (Carl Zeiss).

Western blots

Cells were lysed in Laemmli buffer (0.12M Tris-HCL, pH 6.8, 4% SDS, 20% glycerol, 0.05 μg/μL bromophenol blue, 35mM β-mercaptoethanol) and boiled for 5 minutes, and the protein concentration was determined. Equal amounts of sample were analyzed by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membrane (Millipore), and probed with the respective antibodies. Immunocomplexes were detected using enhanced chemiluminescence (GE Healthcare).

Isolated primary T cells

T cells from human PBMCs. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density-gradient centrifugation (Pharmacia) and $CD4^+$ cells were isolated by magnetic-activated cell sorting (MACS). Cells were cultured in for 7 days with RPMI 1640 supplemented with 2mM L-glutamine, 100 units/mL of penicillin/streptomycin (Invitrogen), and 10% (vol/vol) AB heat-inactivated (60 minutes at 56°C) human serum (Sanquin Blood Bank), and stimulated with plate-bound anti-CD3 (1.5 μg/μL), 300 IU/mL human IL-2, and 2 μg/mL anti–human CD28 in combination with 10mM NAM or 5μM resveratrol. Cells were cultured in triplicate in round-bottom 96-well plates (Nunc) at 37°C in an atmosphere of 5% CO_2 with 100% relative humidity.

T cells from human skin. Normal human skin was obtained from patients undergoing cosmetic surgery procedures. Three-dimensional matrices (Statamatrix) were obtained from Cell Sciences. Explant cultures were established as described.[22] Skin was cut into very small fragments and placed on the surface of a matrix. Each matrix was placed into 1 well of a 24-well plate in 2 mL/well of Iscove modified medium (Mediatech) with 20% heat-inactivated fetal bovine serum (Sigma-Aldrich), penicillin, and streptomycin, and 3.5 mL/L β-mercaptoethanol. Cultures were fed 3 times a week by careful aspiration of 1 ml of culture medium and replacement with fresh medium. Cells were harvested at 12 days. IL-15 (10 ng/mL) and IL-2 (5 IU/mL; R&D Systems) were added and refreshed with each feeding, in combination with 10mM NAM or 5μM resveratrol.

T cells from mouse splenocytes. Spleens were removed from healthy C57BL/6 mice after they were killed, and $CD4^+$ T cells were isolated from splenocytes using MACS separation. Cells were cultured for 5 days in flat-bottom plates coated with mu-aCD3 (1 μg/mL in pbs) in Iscove modified Dulbecco medium supplemented with 2 mM L-glutamine, 100 units/mL of penicillin/streptomycin (Invitrogen), and 10% (vol/vol) heat-inactivated (60 minutes at 56°C) FCS, 0.2 ng/mL murine IL-2, and 0.5 μg/mL anti–murine CD28 and 2 ng/mL transforming growth factor-β in combination with 10mM NAM or 5μM resveratrol. Cells were cultured in triplicate in round-bottom 96-well plates (Nunc) at 37°C in an atmosphere of 5% CO_2 with 100% relative humidity. The University Medical Center Utrecht approved this study.

T cell clone. The $CD4^+$ T-cell clone N3CA8 was generated as described elsewhere[23] and cultured for 6 days with RPMI 1640 supplemented with 2mM L-glutamine, 100 units/mL of penicillin/streptomycin (Invitrogen), and 10% (vol/vol) heat-inactivated pooled human serum (Sanquin Blood Banks) in 48-well tissue culture plates (BD Biosciences). The T-cell clone was stimulated with 40 IU/mL human IL-2 and anti-CD3/anti-CD28 Dynabeads (Invitrogen) at a bead/T-cell ratio of 1:10 in combination with 5mM NAM or 2.5μM resveratrol. Staining of surface markers and Foxp3 for flow cytometry was done according to the manufacturer's protocol (eBioscience).

Suppression assay

Mice CD4 T cells were cultured as described in "Methods" and stained by anti–mouse CD4 peridinin chlorophyll protein and anti–mouse CD25

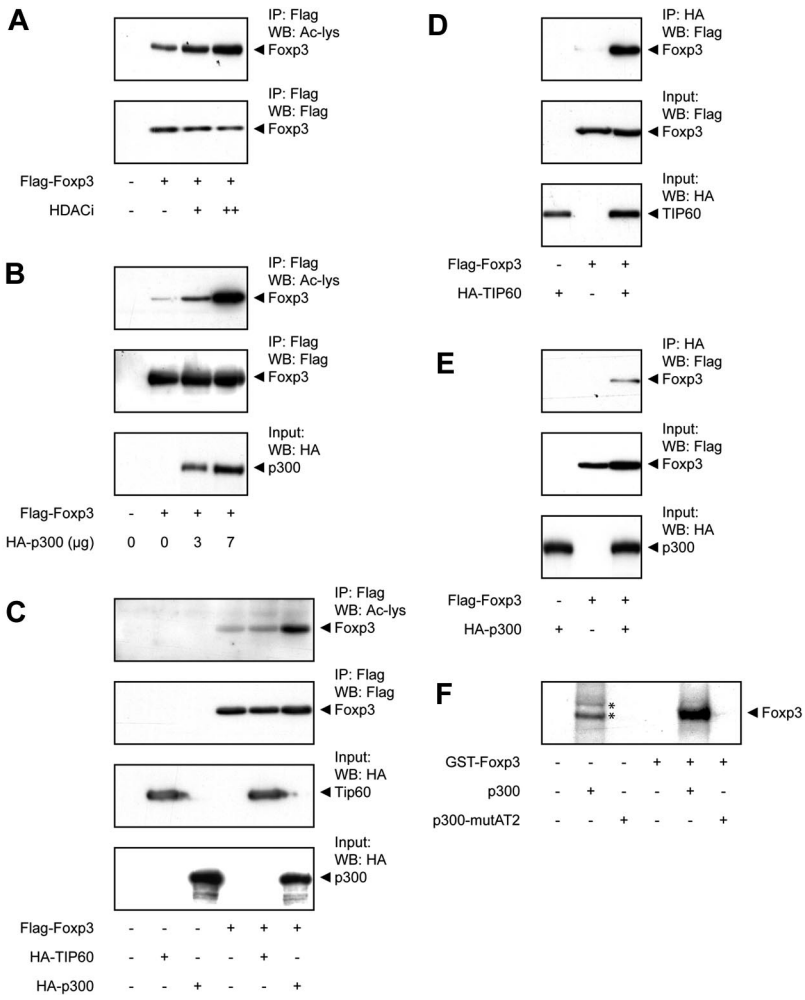

Figure 1. p300 promotes Foxp3 acetylation. (A) Flag immunoprecipitation from lysates from Flag-Foxp3–transfected cells. HEK 293 cells were treated with HDACi: + indicates 50 nm TSA and 1mM NAM; or ++, 250 nm TSA and 5mM NAM for 16 hours. Equal amounts of immunoprecipitated Foxp3 were separated by SDS-PAGE, and Western blots were probed for acetylated lysines (Ac-Lys) or Flag. (B) Cells were transfected with Flag-Foxp3 and/or HA-p300. Flag-Foxp3 was immunoprecipitated with anti-Flag beads and analyzed using acetyl lysines (Ac-Lys), Flag, or HA antibodies. (C) Cells were cotransfected with Flag-Foxp3 and HA-p300 or HA-TIP60 and immunoprecipitated using anti-Flag beads. Immunoblots were analyzed with acetyl-lysine (Ac-Lys) antibody, anti-Flag, or anti-HA. Cells were cotransfected with Flag-Foxp3, HA-Tip60 (D), or HA-p300 (E). Cell lysates were coimmunoprecipitated using anti-HA beads and analyzed using anti-Flag or anti-HA. (F) GST-Foxp3 fusion protein coupled to Sepharose beads was incubated with [14C]-labeled acetyl-CoA and p300 or the acetylase dead p300mutAT2; samples were separated on SDS-PAGE and analyzed using films sensitive for radioactivity. Results are representative of at least 3 independent experiments. *Aspecific band. IP indicates immunoprecipitation; and WB, Western blot.

antigen-presenting cells (APCs) (clone RM4-5 and PC61, respectively; both from BD Biosciences PharMingen). $CD4^+CD25^-$ cells Teff (10 000) were sorted by FACSAria directly into a 96-well plate, and $CD4^+CD25^+$ sorted cells were added at a ratio of 1:5. A CD4-depleted fraction of spleen cells (using anti–mouse CD4 microbeads and a single run on an LS column, MACS; Miltenyi Biotec) was irradiated at 35 Gy and served as a source for APCs. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were cultured in Iscove modified Dulbecco medium supplemented with 10% FCS, 2mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5×10^{-5}M 2-mercaptoethanol in the presence of 50 000 APCs and 1 μg/mL soluble anti-CD3 (clone 145-2C11; BD Biosciences PharMingen) without NAM for 120 hours. During the last 18 hours, 1 μCi (0.037 MBq) ^{3}H-thymidine (GE Healthcare) was added per well, and ^{3}H uptake was measured using a liquid scintillation β counter. Proliferative responses were calculated as the mean ^{3}H incorporation (cpm) of triplicate wells. To determine suppression of proliferation by measuring carboxyfluorescein succinimidyl ester (CFSE) dilution within the CD4 T effector cell population, we labeled freshly isolated splenocytes with 2μM CFSE for 7 minutes at 37°C. A total of 10 000 labeled splenocytes per well in a 96-well plate were stimulated with 1 μg/mL soluble anti-CD3, and 2000 $CD4^+CD25^+$ sorted cells were added. On day 4, cells were harvested, stained with anti-CD4, and CFSE dilution was measured on FACSCanto (BD Biosciences).

Quantitative PCR

mRNA was isolated using the Trizol according to the manufacturer's protocol (Invitrogen), and cDNA synthesis was performed using IScript cDNA synthesis kit (Bio-Rad). cDNA samples were amplified using SYBR Green supermix (Bio-Rad), in a MyiQ single-color real-time PCR detection system (Bio-Rad) according to the manufacturer's protocol. The following primers were used: Foxp3 forward: TCAAGCACTGCCAGGCG, Foxp3 reverse: CAGGAGCCCTTGTCGGAT, β_2-microglobulin (β_2M) forward: CCAGCAGAGAATGGAAAGTC, β_2M reverse: GATGCTGCTTACATGTCTCG. To quantify the data, the comparative threshold cycle method was used. Relative quantity was defined as $2^{-\Delta\Delta Ct}$. β_2M was used as reference gene.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test (Prism GraphPad Software). *P* less than .05 was considered statistically significant.

Results

Foxp3 interacts with and is acetylated by p300

To assess whether Foxp3 can be acetylated, cells were transfected with a Flag-tagged Foxp3 construct and incubated with the HDAC inhibitors (HDACi) TSA and NAM. Together these HDACi can inhibit a majority of HDACs. Foxp3 acetylation was analyzed by Flag immunoprecipitation and immunoblotting using an anti–acetyl lysine antibody. We observed a basal level of Foxp3 acetylation in the absence of HDACi, which was increased by addition of TSA/NAM in a dose-dependent manner (Figure 1A). To determine whether the ubiquitously expressed HAT p300 could

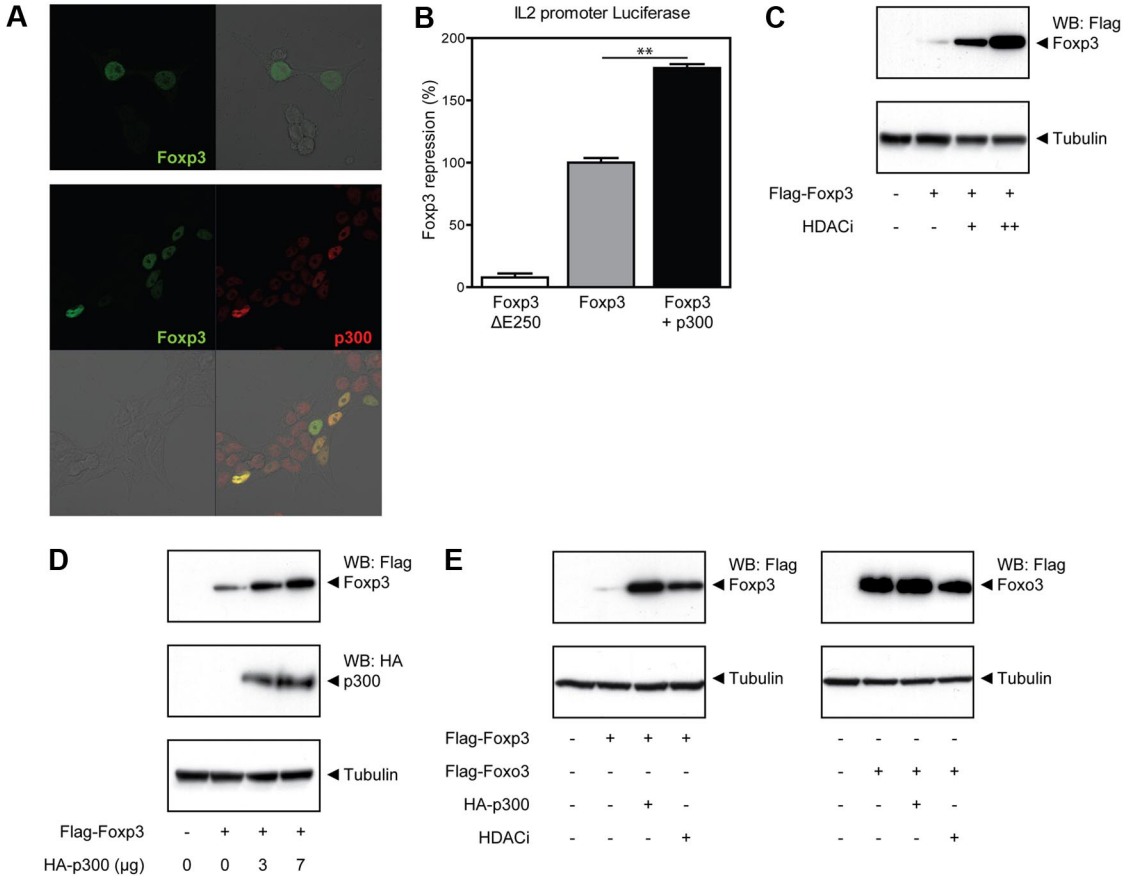

Figure 2. Acetylation modulates Foxp3 protein levels. (A) Representative examples of cells that were transfected with only Foxp3 (green; top panel). Subcellular distribution of cells that were cotransfected with Foxp3 (green) and p300 (red) are shown in the bottom panel. p300 was localized using an anti-p300 antibody that recognizes both endogenous and ectopically expressed p300. Colocalization of Foxp3 and p300 is indicated in yellow. (B) Foxp3 function was assessed by evaluating IL-2 promoter reporter activity. IL-2 promoter luciferase activity was analyzed in HEK 293 cells by cotransfecting NFAT with Foxp3 del E250 (▭), Foxp3 (▭), or Foxp3 with p300 (■). Repression of IL-2 luciferase activity by wild-type Foxp3 was set as 100%. Values were all normalized for cotransfected Renilla. $^{**}P < .01$. (C) HEK 293 cells were transfected with Flag-Foxp3 and treated with HDACi: + indicates 50 nm TSA and 1mM NAM; ++, 250 nm TSA and 5mM NAM. Immunoblots were probed for Flag expression or tubulin as loading control. (D) Cells were transfected with Flag-Foxp3 and increasing amounts of HA-p300. Western blots were incubated with antibodies against Flag, HA, or tubulin as indicated. (E) HEK 293 cells were transfected with Flag-Foxp3 or Flag-Foxo3 with or without HA-p300. Cells were treated with 100nM TSA and 2.5mM NAM for 16 hours (HDACi). Data are representative of at least 3 independent experiments.

acetylate Foxp3, cells were transfected with Foxp3 and increasing amounts of p300, and Foxp3 acetylation was again analyzed. Indeed, addition of p300 dose-dependently increased Foxp3 acetylation (Figure 1B). Because a recent study demonstrated interaction between Foxp3 and the HAT TIP60,[13] we wished to determine whether TIP60 could also acetylate Foxp3 in living cells. Lysates were prepared from HEK 293 cells cotransfected with Flag-Foxp3 and HA-TIP60 or HA-p300. Immunoblots reveal that, in contrast to p300, TIP60 cotransfection did not increase Foxp3 acetylation (Figure 1C). We next determined whether p300 and TIP60 could associate with Foxp3 by coimmunoprecipitation after cotransfection of cells with Flag-Foxp3 and HA-p300 or HA-TIP60. Both p300 and TIP60 were found to associate with immunoprecipitated Foxp3 (Figure 1D-E). Finally, we tested p300 for its ability to directly acetylate Foxp3 using an in vitro acetylation assay. GST-Foxp3 fusion protein coupled to Sepharose beads was incubated with [^{14}C]-labeled acetyl-CoA and p300, or an acetylase dead p300mutAT2, and samples were separated on SDS-PAGE and analyzed by autoradiography (Figure 1F). Incubation of Foxp3 with p300, but not the catalytically inactive variant, resulted in Foxp3 acetylation. These data demonstrate that, although both TIP60 and p300 can associate with Foxp3, Foxp3 acetylation is selectively mediated by p300.

Acetylation regulates Foxp3 protein levels

Because acetylation of the related Foxo Forkhead transcription factors has been reported to mediate their subcellular localization,[18,24] we analyzed Foxp3 subcellular localization under conditions of hyperacetylation. Using confocal microscopy, we found Foxp3 to be exclusively localized in the nucleus (Figure 2A top panel). In contrast to previous reports analyzing Foxo transcription factor localization, cotransfecting cells with p300 did not influence Foxp3 localization (Figure 2A bottom panel). However, ectopically expressed as well as endogenous p300 colocalized with Foxp3 in the nucleus, underscoring a role for p300 in nuclear Foxp3 acetylation.

To examine a possible role for p300 in Foxp3-mediated transcriptional activity, we performed transcription reporter assays in which cells were transfected with NFAT and an IL-2 promoter luciferase reporter together with Foxp3 in the absence or presence of p300[25] (Figure 2B). Foxp3 expression resulted in clear repression of IL-2 promoter activity, whereas the IPEX mutant Foxp3 ΔE250, which does not dimerize, was not transcriptionally functional as expected. Cotransfection of p300 significantly increased Foxp3 transcriptional repression ($P = .008$), indicating an increased functionality of acetylated Foxp3.

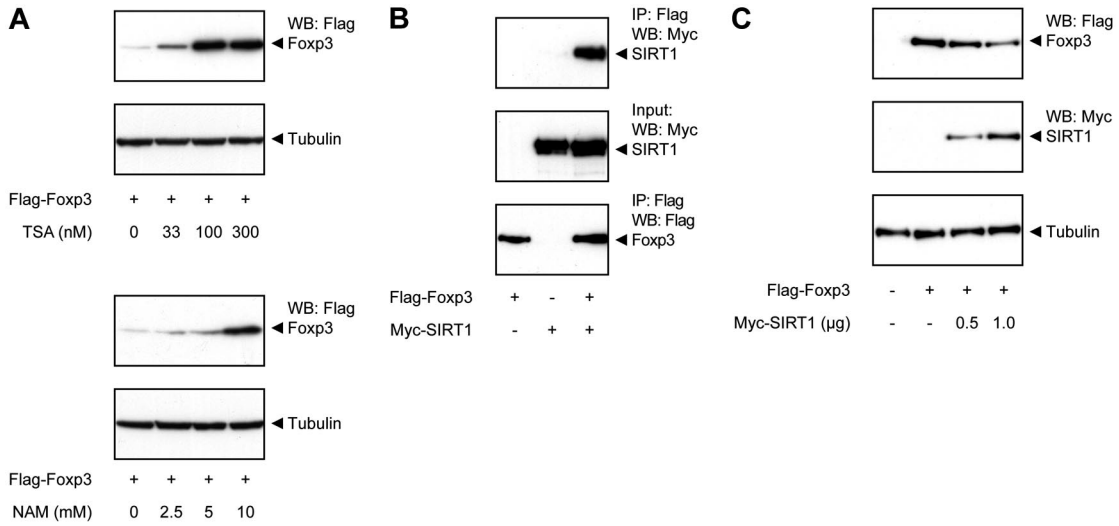

Figure 3. SIRT1 deacetylates Foxp3 and decreases its expression. (A) Flag-Foxp3–transfected HEK 293 cells were treated with increasing amounts of TSA (top panel) or NAM (bottom panel) for 16 hours. Foxp3 levels were determined using antibodies against Flag or tubulin as loading control. (B) Cells were cotransfected with Flag-Foxp3 and Myc-SIRT1. Cell lysates were immunoprecipitated using anti-Flag beads and analyzed using anti-Flag or anti-Myc antibodies. (C) Cells were cotransfected with Flag-Foxp3 and Myc-SIRT. Cell lysates were quantified and analyzed by Western blotting using anti-Flag antibody and tubulin as loading control.

Because p300-mediated acetylation resulted in increased Foxp3 activity, Foxp3 protein expression levels were analyzed. Cells were transfected with Foxp3, incubated with HDACi, and Foxp3 levels were determined by immunoblot analysis. We observed a dramatic, dose-dependent increase in Foxp3 protein levels in HDACi-treated cells (Figure 2C). Similarly, p300 cotransfection increased Foxp3 protein levels in a dose-dependent manner (Figure 2D). Expression levels of the related Foxo3 transcription factor, which has previously been described to be acetylated, was unaffected by HDACi treatment or p300 cotransfection[18] (Figure 2E). These results demonstrate that p300-dependent Foxp3 acetylation can regulate its protein expression level.

Because it has been described that Foxp3 function depends on homodimerization,[26] we also investigated whether dimerization is also necessary for p300-mediated acetylation. Flag-Foxp3 or Flag-Foxp3ΔE250 was immunoprecipitated, and acetylation levels were analyzed. Foxp3ΔE250 acetylation was comparable with that of wild-type Foxp3 in the presence or absence of p300 (supplemental Figure 1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). To determine whether acetylation of Flag-Foxp3ΔE250 also resulted in increased protein expression levels, cells transfected with Flag-Foxp3 or Flag-Foxp3ΔE250 were treated with HDACi or cotransfected with p300. Both Foxp3 and Foxp3ΔE250 protein levels were increased by HDACi and p300 to a similar extent (supplemental Figure 1B), indicating that acetylation and modulation of protein expression are not dependent on dimerization per se.

Because we observed that Foxp3 protein levels are modulated by p300-mediated acetylation, we also wished to examine the molecular mechanism regulating deacetylation. To further pinpoint which HDAC is responsible for the effects observed, cells were treated with either TSA or NAM, and Foxp3 expression levels were analyzed. TSA is an inhibitor of multiple HDAC families, whereas NAM selectively inhibits the SIRT HDAC family. Both TSA and NAM treatment increased Foxp3 protein levels in a dose-dependent manner (Figure 3A), indicating that Foxp3 can be deacetylated by members of at least 2 independent HDAC families.

To verify that SIRT is involved in modulation of Foxp3 expression, interaction between Foxp3 and SIRT was confirmed by coimmunoprecipitation after cotransfection of cells with Flag-Foxp3 and myc-tagged SIRT1 (Figure 3B). We next assessed whether SIRT1 could also modulate Foxp3 expression by cotransfecting cells with Flag-Foxp3 and myc-SIRT1. Immunoblots revealed that Foxp3 protein levels were clearly reduced in a dose-dependent manner in cells cotransfected with SIRT1 (Figure 3C). Taken together, these data support a role for acetylation/deacetylation in regulating Foxp3 protein expression levels, a process that can be reciprocally regulated by p300 and SIRT.

Acetylation impairs proteasome-mediated Foxp3 degradation.

To further evaluate the molecular mechanism underlying acetylation-dependent modulation of Foxp3 expression, proteasomal degradation was abrogated using the inhibitor MG132. Cells transfected with equal amounts of Flag-Foxp3 were incubated with HDACi with or without MG132, and Foxp3 protein levels were analyzed. Treatment with MG132 alone dramatically increased Foxp3 levels, indicating that Foxp3 is rapidly degraded by the proteasome. Importantly, treatment with HDACi did not further increase Foxp3 protein levels when cells were simultaneously incubated with MG132 (Figure 4A). To validate these observations, we repeated the experiments using additional specific proteasome inhibitors epoxomycin and lactacystin (Figure 4B). Similar results were obtained compared with MG132, indicating that Foxp3expression level is regulated in a proteasome-dependent manner.

These data suggest that Foxp3 proteasomal turnover is abrogated by HDACi. To confirm this, we made use of CHX, a widely used inhibitor of protein translation (Figure 4C). Cells were transfected with Flag-Foxp3 and then treated with CHX before analyzing Foxp3 expression levels. CHX treatment led to a decrease in Foxp3 protein levels within 6 hours. However, when the cells were also treated with HDACi, Foxp3 protein levels stabilized, confirming that Foxp3 protein stability is indeed increased through acetylation.

Proteasomal degradation is mediated by polyubiquitination, which, similar to acetylation, occurs at lysine residues. This

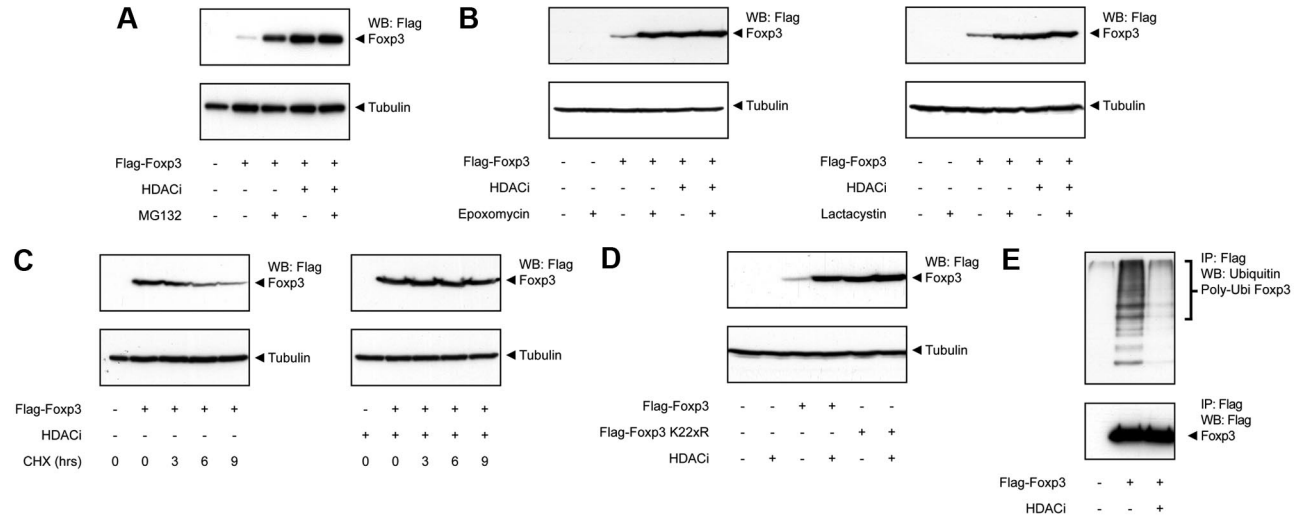

Figure 4. Foxp3 acetylation prevents proteasomal degradation. Flag-Foxp3–transfected HEK 293 cells were treated with 2μM MG132 (A), 100nM epoxomycin, or 10μM lactacystin (B) for 16 hours to inhibit proteasome function and/or HDACi TSA 100nM and NAM 2.5mM also for 16 hours. Foxp3 expression was analyzed using a Foxp3 antibody, and equal loading was verified by analyzing tubulin expression. (C) Cells were transfected with equal amounts of Flag-Foxp3. Half of the cells were treated with or without TSA 100nM and NAD 2.5mM for 16 hours (right panel) and 5 μg/mL CHX for the indicated time points. Foxp3 expression was analyzed using a Flag antibody, and tubulin expression was used as a loading control. Results are representative of at least 3 independent experiments. (D) Flag-Foxp3 or a Flag-tagged Foxp3 mutant in which all the lysines are mutated to arginines (Flag-Foxp3 K22xR) was transfected into HEK 293 cells. The cells were treated with the HDACi TSA (100nM) and NAM (2.5mM) for 16 hours. Cell lysates were made and immunoblotted for Flag and tubulin as control. (E) HEK 293 cells were transfected with Flag-Foxp3, treated with or without TSA 100nM and NAD 2.5mM for 16 hours, and cell lysates were immunoprecipitated using anti-Flag beads and analyzed using anti-ubiquitin and anti-Flag as transfection control.

suggests that acetylation of lysine residues may prevent ubiquitination and thereby inhibit Foxp3 degradation. To test this hypothesis, we generated a Foxp3 mutant in which all 22 lysines were mutated into arginines (Foxp3 K22xR). If direct Foxp3 ubiquitination is indeed responsible for increased protein turnover, then this mutant should exhibit enhanced stability; importantly, Foxp3 protein levels should no longer be modulated by HDACi. Cells were transfected with equal amounts of Flag-Foxp3 or Flag-Foxp3 K22xR and incubated with HDACi (Figure 4D). As previously demonstrated, hyperacetylation increased the levels of wild-type Foxp3. In contrast, Foxp3 K22xR protein levels were not altered by treatment with HDACi. Importantly, we observed that the levels of Foxp3 K22xR were considerably increased compared with wild-type Foxp3. To confirm our hypothesis that there is competition for the same lysines by acetylation and ubiquitination, we analyzed the effect of HDACi on the level of Foxp3 ubiquitination. Cells were transfected with Foxp3 and treated with HDACi. Subsequently, Foxp3 was immunoprecipitated, blotted, and evaluated using an antiubiquitin antibody. As shown in Figure 4E, there is a basal level of polyubiquitinated Foxp3. Treatment with HDACi, however, considerably reduced the amount of polyubiquitinated Foxp3.

Taken together, these data demonstrate that Foxp3 protein levels are rapidly turned over in a proteasome-dependent manner and that acetylation probably prevents entry of Foxp3 into this pathway by competing with polyubiquitination.

Acetylation increases Foxp3 levels in primary T cells

We next assessed the effect of acetylation on Foxp3 levels using primary T cells in 4 different models. T cells from 4 different sources were stimulated with IL-2, anti-CD3, and anti-CD28 to induce Foxp3, and Foxp3$^+$ cells were analyzed by flow cytometry. First, CD4$^+$ cells from human PBMCs were treated with either the SIRT activator resveratrol,[27] or the SIRT inhibitor NAM or control (Figure 5A; supplemental Figure 2). We observed significantly decreased numbers of Foxp3$^+$ cells in the resveratrol-treated cells, whereas the cells treated with NAM showed a significantly higher percentage of Foxp3$^+$ cells compared with control. Second, because it has recently been reported that Tregs play a crucial role in immune homeostasis of the skin,[28] T cells were isolated from human skin samples and treated with resveratrol or NAM for 14 days (Figure 5B). Analysis of Foxp3$^+$ cells again revealed that treatment with resveratrol decreased the percentage of Foxp3$^+$ cells, whereas NAM increased Foxp3$^+$ cell numbers. T cells were also isolated from healthy C57BL/6 mice spleens and cultured in the presence of resveratrol or NAM (Figure 5C). Activation of SIRT resulted in a diminished percentage of Foxp3$^+$ cells. Treatment with NAM, however, increased the number of Foxp3$^+$ cells by up to 10-fold. We also generated human T-cell clones, which were treated with resveratrol or NAM (Figure 5D). Again, resveratrol treatment decreased the numbers of Foxp3$^+$ cells, whereas inhibition of SIRT in these cells resulted in a significantly increased percentage of Foxp3$^+$ cells. Taken together, these data demonstrate that SIRT-mediated regulation of acetylation influences Foxp3 expression levels in both mouse and human primary T cells from a variety of origins.

Hyperacetylation of Foxp3 increases Treg function

Because we observed that acetylation impairs proteasomal degradation (Figure 4), we analyzed whether Foxp3 protein levels per cell were regulated by SIRT, as are the Foxp3$^+$ cell numbers. T cells from all 4 sources were treated with NAM or control and the mean fluorescence intensity of Foxp3$^+$ cells was analyzed by FACS (Figure 6A). The mean fluorescence intensity of the NAM-treated cells was significantly higher compared with the control-treated cells for all T-cell types, indicating that SIRT inhibition not only increased the number of Foxp3$^+$ cells but also the Foxp3 levels per cell compared with control. To elucidate whether the changes in Foxp3 expression are the result of increased protein stability, and not increased transcription, Foxp3 mRNA levels were analyzed. mRNA was isolated from control and NAM-treated T cells from the human T-cell clone that was previously used (Figure 5D). cDNA was prepared and analyzed using quantitative PCR for

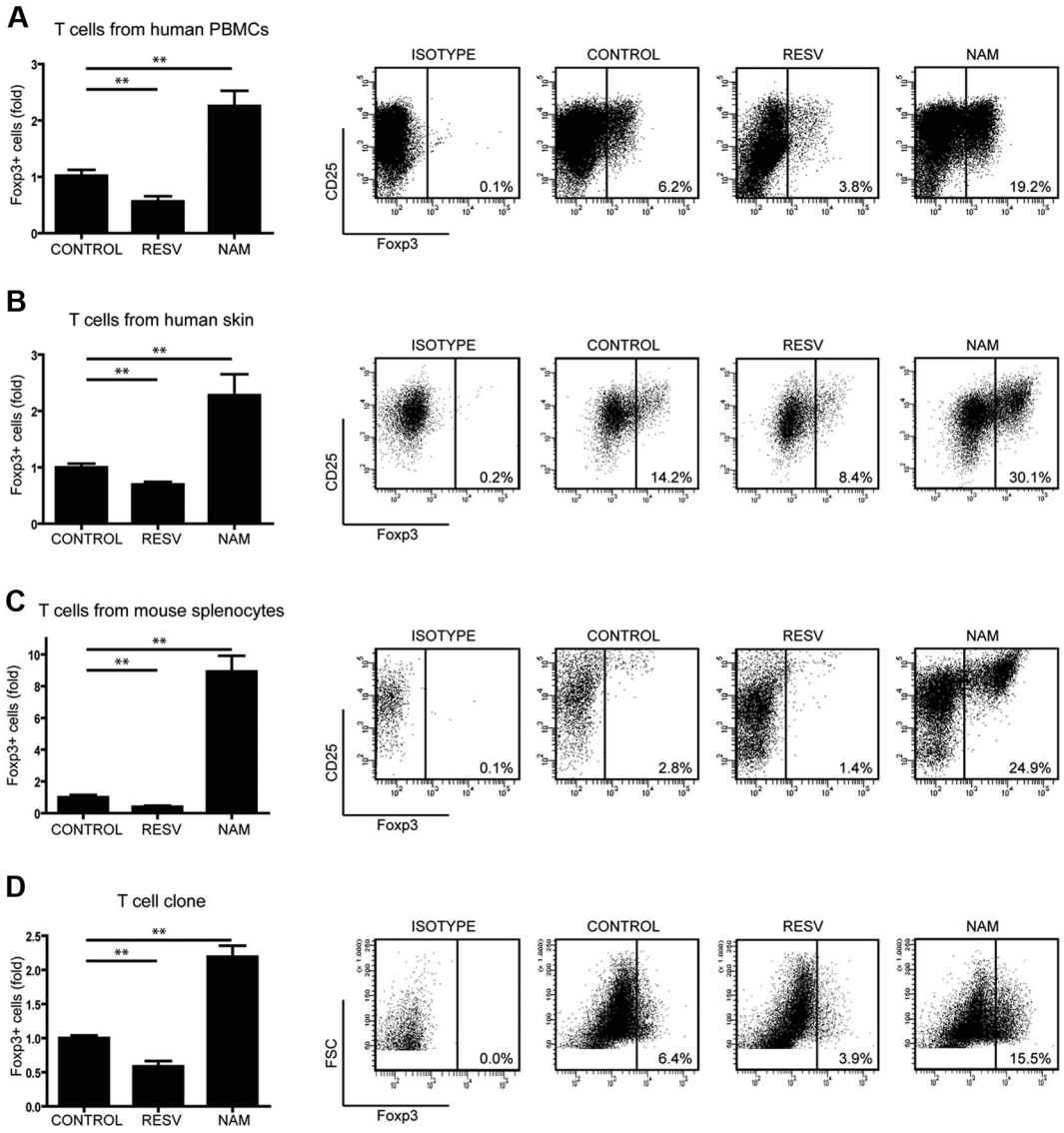

Figure 5. Treatment of primary T cells with a SIRT inhibitor results in increased Foxp3$^+$ cell numbers. Isolated primary T cells were cultured in the presence of IL-2, anti-CD3, and anti-CD28 (mouse splenocytes were also cultured in the presence of transforming growth factor-β). Cells were treated with the SIRT activator resveratrol, the SIRT inhibitor NAM, or carrier as control. The percentage of Foxp3$^+$ cells was determined using FACS technology. T cells originated from human PBMCs (A), human skin (B), mouse spleen (C), and a human T-cell clone (D). Results are the means of at least 4 independent experiments. $^{**}P < .01$.

Foxp3 and β_2M as reference. The ratio of Foxp3 versus β_2M per Foxp3$^+$ cell is shown in Figure 6B. No significant difference in Foxp3 mRNA levels per Foxp3$^+$ cell was observed, demonstrating that *Foxp3* transcription is not influenced by SIRT.

To confirm that acetylation not only regulates Foxp3$^+$ cell numbers and Foxp3 protein levels, but also Treg suppressor capacity, a suppression assay was performed. Primary T cells were stimulated and cultured with NAM for 5 days. Subsequently, the functionality of Foxp3$^+$ cells was analyzed in CFSE-based suppression assay (Figure 6C). NAM or control-treated CD4$^+$CD25$^+$ T cells were cocultured with effector T cells in a 1:5 ratio, and proliferation of Teff cells was measured. As expected, Tregs from the control-treated sample were able to suppress the proliferation of effector T cells. Tregs from the NAM-treated cultures suppressed T-cell proliferation compared with the control significantly better. These data clearly demonstrate that SIRT-mediated regulation of acetylation not only modulates Foxp3$^+$ cell numbers but also Foxp3 protein levels independently of increased *Foxp3* transcription. Importantly, inhibition of SIRT also results in functionally improved Treg cells.

Discussion

Foxp3 is a key transcription factor controlling immune homeostasis through regulating both the numbers and functionality of immunosuppressive Tregs. Although recent studies have focused on identifying Foxp3 transcriptional targets or the molecular mechanisms underlying transcriptional regulation of Foxp3 expression, analysis of specific posttranslational modifications regulating Foxp3 function have thus far not been critically addressed. Here we report, for the first time, that Foxp3 protein levels are directly controlled by acetylation, a process mediated by inhibition of proteasomal degradation. Moreover, we identified novel interaction partners that are key mediators of this of this process: the HAT p300 and the HDAC SIRT1. Furthermore, hyperacetylating Foxp3 in primary

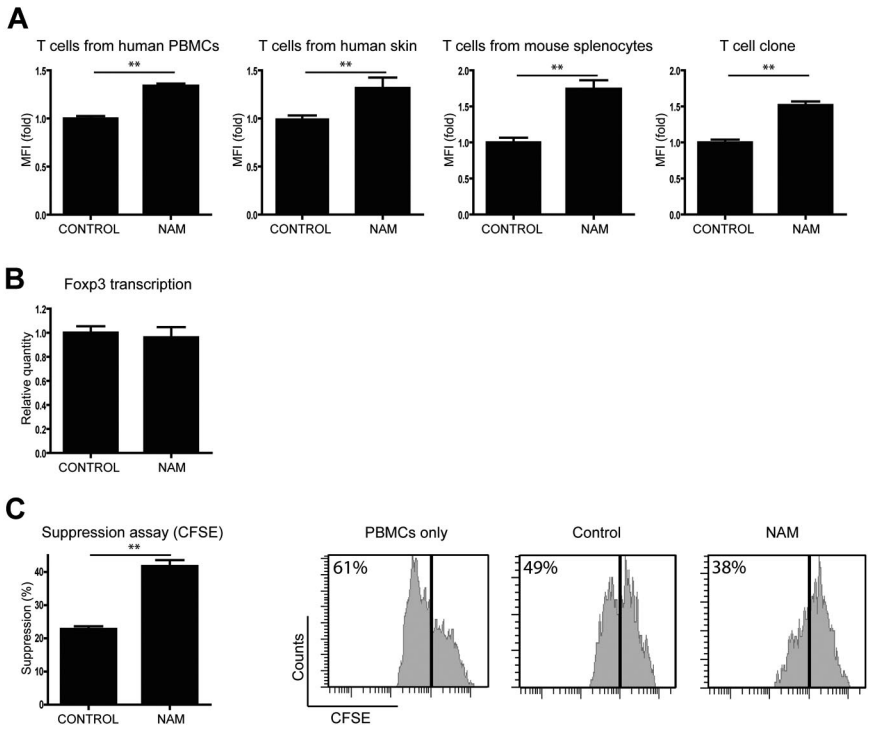

Figure 6. SIRT inhibition increases Treg functionality. Isolated primary T cells were cultured in the presence of IL-2, anti-CD3, and anti-CD28. Cells were treated with the SIRT inhibitor NAM or carrier as control. (A) Foxp3 levels per cells of different T-cell sources were analyzed by FACS, and data represent the mean fluorescence intensity of the Foxp3$^+$ population. (B) Cells from a human T-cell clone were stimulated and treated with NAM or control. Foxp3 transcription was determined with quantitative PCR, and results were corrected for the housekeeping gene β_2M and Foxp3$^+$ cell numbers. (C) CD4 T cells isolated from mouse splenocytes were stimulated using IL-2, anti-CD3, and anti-CD28 beads and treated with NAM or control. CD25$^+$ cells were sorted, and the function of these induced Tregs was analyzed using a standard suppression assay. A total of 10 000 effector T cells were cocultured with 2000 sorted CD25$^+$ cells for 4 days (without NAM). Proliferation of effector T cells on day 4 was measured by CFSE dilution within the CD4$^+$ effector T-cell population. Data shown are representative of at least 3 independent experiments. $^{**}P < .01$.

T cells resulted in more Foxp3$^+$ cells, higher Foxp3 protein levels per cell, and better suppressive capabilities. Our data provide novel insights into posttranslational mechanisms regulating Foxp3 function and provide a model by which Treg numbers and functionality can be rapidly modulated by the extracellular milieu.

A recent study by Tao et al has demonstrated that in vivo HDAC inhibition results in enhanced Treg-mediated suppression of homeostatic proliferation, decreased inflammatory bowel disease through Treg-dependent effects, and induction of permanent tolerance against islet and cardiac allografts.[14] Because we have demonstrated that HDACi increase Foxp3 expression through protein stabilization, this suggests a molecular mechanism explaining these in vivo observations. Importantly, we demonstrate that p300 increases Foxp3 expression levels through inhibition of proteasomal degradation. This is a novel and unique observation for members of the forkhead transcription factor family.[3] Degradation of other transcription factors is mediated by polyubiquitination of these proteins. Because lysine acetylation and ubiquitination are mutually exclusive, acetylation may thus prevent polyubiquitination through a competition-based mechanism. Indeed, in Figure 4, we demonstrate that, by mutating all Foxp3 lysines into arginines, we significantly increased Foxp3 protein levels, a process most probably mediated by inhibition of proteasomal degradation because Foxp3 K22xR cannot be polyubiquitinated. Inhibition of the proteasome-mediated degradation dramatically increased and stabilized Foxp3 protein levels, which could not be further increased by treatment with HDACi. Furthermore, treatment with HDACi did not further increase Foxp3 K22xR protein levels as observed in wild-type. In addition, we also show that treatment with HDACi dramatically decreased polyubiquitination of Foxp3. Taken together, we propose that polyubiquitination-mediated Foxp3 degradation can be impaired by acetylation of lysines in a competition-based mechanism. A similar mechanism has been previously described for RUNX3, Smad7, and p53 where polyubiquitination of these transcription factors was significantly impaired by hyperacetylation.[29-32]

Although p300 and TIP60 were both found to associate with Foxp3, we found that only p300 was able to acetylate Foxp3 (Figures 1, 2). Li et al recently demonstrated that Foxp3 is part of a transcriptional complex containing both HDAC and HAT.[13] This study reported that TIP60 overexpression promotes Foxp3-mediated transcriptional repression. However, as we did not observe TIP60-mediated Foxp3 acetylation, our data suggest that TIP60 predominantly acts indirectly on other (non) histone proteins in the transcription/repression complex, rather than directly modulating Foxp3 transcriptional function.

We also examined which HDAC is responsible for regulating Foxp3 deacetylation. Treatment with either TSA or NAM alone was sufficient to result in increased Foxp3 expression (Figure 3). TSA has a broad specificity, inhibiting HDAC families I, II, and IV. It has also recently been reported that both HDAC7 and HDAC9 associate with Foxp3 in a multimeric protein complex,[13] and it is possible that Foxp3 deacetylation can also be mediated by one of these HDACs. Because NAM is a specific inhibitor of the SIRT HDAC III family, we conclude that there is also a role for SIRT in regulating Foxp3 activity, which we could subsequently further substantiate by coimmunoprecipitation experiments (Figure 3). Furthermore, ex vivo treatment of CD4$^+$ T cells from 4 different sources with the SIRT activator resveratrol decreased the number of Foxp3$^+$ cells. Interestingly, multiple studies have reported that resveratrol has antitumor activity, although through an as yet undefined mechanism.[33,34] Because Tregs repress immune function,[35,36] it is interesting to speculate that resveratrol may act by reducing Foxp3 protein levels, thereby relieving Treg-mediated immune suppression and ultimately resulting in enhanced immune activity toward tumors.

Although there has been a general paradigm that Foxp3 is exclusively expressed by regulatory T cells, it was recently shown that respiratory, thymic, prostate, and mammary epithelial cells also express this transcription factor.[8] Foxp3 function has also been directly linked with tumor suppressor activity; it was demonstrated that Foxp3 binds and represses the promoter of the HER-2/erbB2

and SKP2 oncogenes.[37,38] In addition, somatic mutations and down-regulation of Foxp3 were found in human breast cancer samples and correlated significantly with HER-2/erbB2 and SKP2 expression. Because we have demonstrated that acetylation increases Foxp3 expression, it is plausible that treatment with HDACi may enhance the tumor suppressor activity of Foxp3 and therefore have beneficial effects on tumor formation in the breast epithelium.

It has now become clear that $CD4^+$ effector T cells can also express Foxp3 after TCR stimulation in vitro, and Foxp3 expression has been strongly correlated with hyporesponsiveness of activated T cells.[9-11] However, not all TCR-stimulated $Foxp3^+$ cells have suppressive capabilities. In nonsuppressive $Foxp3^+$ T-cell populations, Foxp3 expression was found to be transient, whereas stably expressing Foxp3 cells had true suppressive capabilities with a phenotype similar to that of naturally occurring Tregs.[9,10] We speculate that this critical difference in transient versus stable Foxp3 expression is a key switch in the generation of "true" suppressor Tregs and that this is directly regulated by acetylation. It is possible that normally antigen exposure activates T cells, resulting in transient Foxp3 expression levels, which may act as an "immune brake" preventing T-cell hyperactivation through repression of IL-2 and interferon-γ transcription.[39] However, when T cells are chronically stimulated, for example, by self-antigen, Foxp3 is acetylated stabilizing protein levels and allowing the initiation of a unique transcriptional program defining the Treg phenotype. Further work will be required to determine whether this is indeed the case.

Taken together, we have established that Foxp3 protein levels can be tightly regulated by acetylation. p300-mediated acetylation of Foxp3 plays a critical role in stabilizing Foxp3 protein levels. In contrast, we found that SIRT1-mediated deacetylation of Foxp3 results in reduced protein expression levels in cell lines as well as primary T cells. In addition, we demonstrate that the mechanism by which Foxp3 protein levels are stabilized by acetylation is by inhibition of proteasomal degradation. Our findings have important consequences for the development of novel molecular therapies regulating Treg numbers through pharmacologic stabilization of Foxp3 protein levels.

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Authorship

Contribution: J.v.L. wrote the paper, designed and performed the research, and analyzed data; Y.V., T.G., J.M.B., and O.v.B. designed and performed the research and analyzed data; Y.Y.J.G performed research and analyzed data; A.B.B., D.-J.H., T.M., E.K., and B.J.P. designed the research and analyzed data; and P.J.C. designed the research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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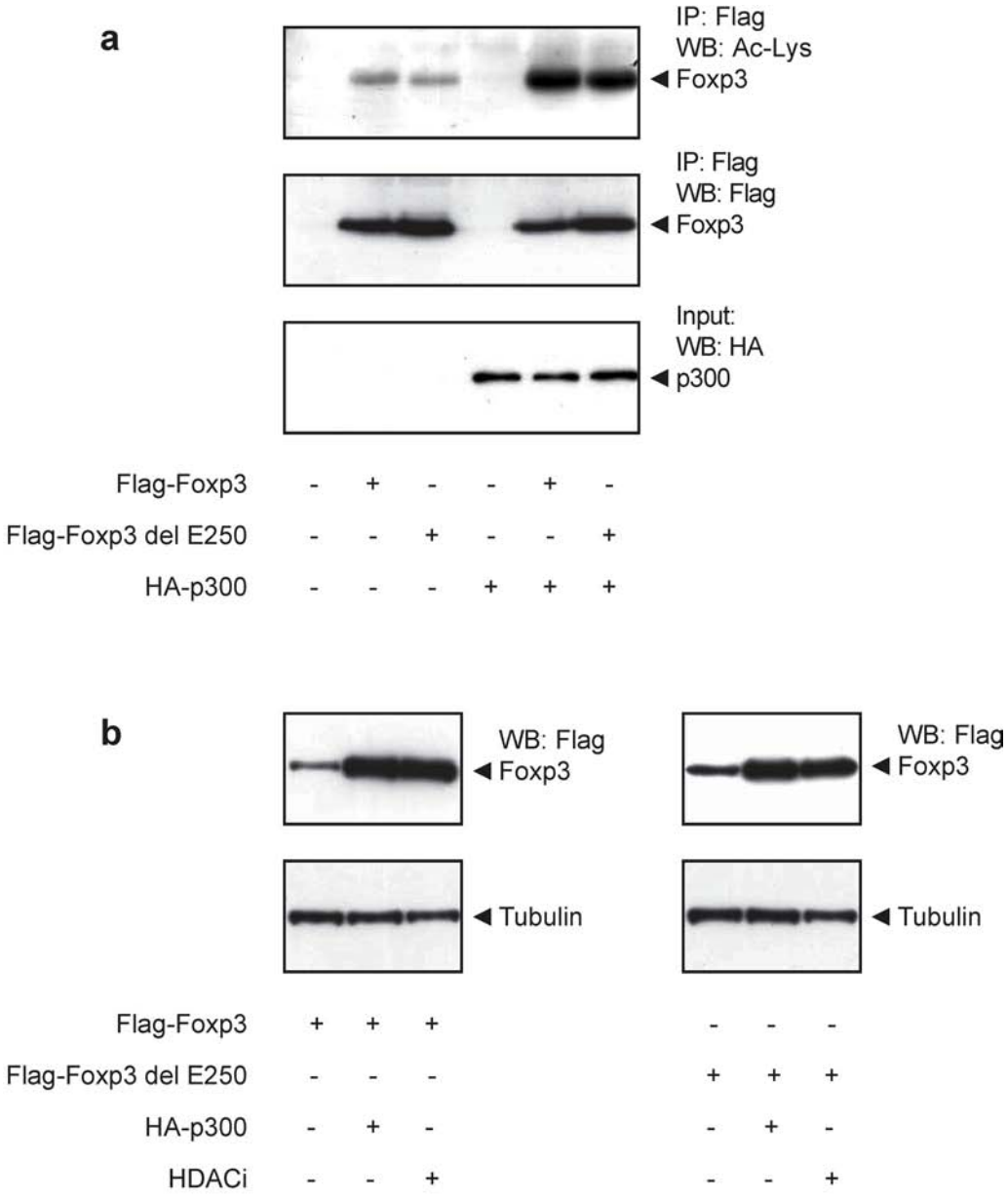
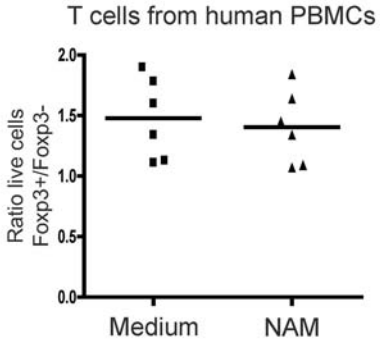


Figure S1. Foxp3 acetylation and increased expression does not depend on homo-dimerization

(A) Flag-Foxp3 or Flag-Foxp3ΔE250 was co-transfected with p300 in HEK 293 cells. Using equal amounts of input protein, Foxp3 acetylation was analyzed using antibodies specific for acetyl-lysine (Ac-Lys), Flag or HA. (B) Cells were co-transfected with Flag-Foxp3 or Flag-Foxp3 ΔE250 and/or p300 and treated with 100 nm TSA and 2.5 mM NAM for 16 hours. Protein lysates were quantified and subsequently western blots were probed utilizing an antibody recognizing Flag, HA, or tubulin as loading control. All western blots are representative of at least 3 independent experiments.

a



b

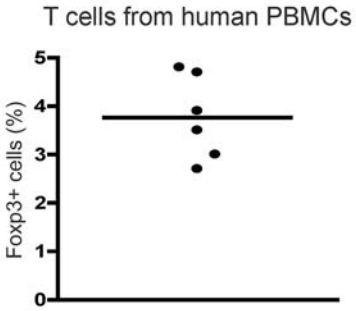


Figure S2. Increased Foxp3^{+} cell numbers after HDACi treatment is not due to preferential cell death

(A) Human CD4^{+} cells from six different donors were cultured in RPMI medium in the presence of IL2, anti CD3 and anti CD28 with or without NAM as described in figure 5 and 6. At day 7 cells were stained for FOXP3 to separate Foxp3^{-} from Foxp3^{+} cells and annexin V and 7-AAD to determine the percentage of apoptosis. The ratio of apoptotic cells in FOXP3^{+} vs FOXP3^{-} is depicted. (B) Analysis of Foxp3 positivity in CD4^{+} compartment of human PBMCs at day 0 as the cells go into culture.

Chapter 3

Rapid temporal control of Foxp3 protein degradation by Sirtuin-1

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Abstract

Maintenance of Foxp3 protein expression in regulatory T cells (Treg) is crucial for a balanced immune response. We have previously demonstrated that Foxp3 protein stability can be regulated through acetylation, however the specific mechanisms underlying this observation remain unclear. Here we demonstrate that SIRT1 a member of the lysine deacetylase Sirtuin (SIRT) family, but not the related SIRTs 2-7, co-localize with Foxp3 in the nucleus. Ectopic expression of SIRT1, but not SIRTs 2-7 results in decreased Foxp3 acetylation, while conversely inhibition of endogenous SIRT activity increased Foxp3 acetylation. We show that SIRT1 inhibition decreases Foxp3 poly-ubiquitination, thereby increasing Foxp3 protein levels. Co-transfection SIRT1 with Foxp3 results in increased Foxp3 proteasomal degradation, while SIRT inhibition increases Foxp3 transcriptional activity in human Treg. Taken together, these data support a central role for SIRT1 in the regulation of Foxp3 protein levels and thereby in regulation of Treg suppressive capacity. Pharmacological modulation of SIRT1 activity in Treg may therefore provide a novel therapeutic strategy for controlling immune responses.

Introduction

Protein acetylation is a tightly controlled process reciprocally regulated by lysine acetyl transferases (KATs) and lysine deacetylases (KDACs). The possibility of manipulating immune responses through specific modulation of protein acetylation is being increasingly examined[1]. *In vitro* and *in vivo* studies have demonstrated that treatment with a variety of KDAC inhibitors increases regulatory T cell (Treg) numbers, Treg mediated suppression and decreases disease scores in murine models or arthritis, allograft rejection and colitis[2-5]. Recently, several studies have reported Foxp3 acteylation, a transcription factor that is crucial for both Treg development and function[5-7]. Foxp3 has been reported to associate with the KATs TIP60 and p300[5;6], while TIP60 and p300 both promote Foxp3 acetylation resulting in both increased Foxp3 protein stability and chromatin binding [5-7]. The KDACs: HDAC1, HDAC7, HDAC9 and SIRT1 have all been reported to associate with Foxp3. However, although they have all been shown to impair Foxp3 transcriptional activity or decrease protein stability, none of these KDACs have been reported to directly reduce Foxp3 acetylation[5;6;8]. Very recently it has been shown that Foxp3 protein levels were increased in SIRT1 knock-out Treg resulting in increased suppressive capacity[9]. However, the molecular mechanisms underlying these observations or the specificity between SIRT family members have not yet been evaluated.

In humans, the class III lysine deacetylases also known as sirtuins (SIRTs) consists of seven members (SIRT1-7). This is a highly conserved gene family encoding nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases. All SIRTs contain a 250 amino acid core domain that shares 25-60% sequence identity[10]. Individual SIRTs are localized to specific subcellular compartments and have been reported to mediate a variety of cellular processes, including cell differentiation, chromatin remodelling, DNA repair, endocrine signalling, and apoptosis[11;12]. Currently little is understood concerning the role of SIRTs in immune responses, although it has been reported that SIRT1 deficient mice are more prone to develop autoimmunity and that Treg specific deletion of SIRT1 increases their

immunosuppressive capacity[9;13;14]. The role for specific SIRT family members in regulating Foxp3 acetylation levels still remains poorly understood.

Recently, we have reported that acetylation abrogates Foxp3 proteasomal degradation through a reduction in Foxp3 poly-ubiquitination levels[5]. As both ubiquitination and acetylation are restricted to lysine residues, a competition model for lysine modification could be envisaged. Here, we demonstrate that SIRT1, but not SIRTs 2-7, co-localizes with Foxp3 in the nucleus and negatively regulates Foxp3 protein levels. SIRT1 deacetylates Foxp3, resulting in increased Foxp3 poly-ubiquitination and proteasomal degradation. Finally, we show that SIRT1 inhibition results in increased FOXP3 transcriptional activity in human Treg. Taken together, these data suggest that specific modulation of SIRT1 activity may provide a therapeutic approach to pharmacologically control immune responses.

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The Study was approved by the Institutional Review Board of the UMC Utrecht. All participants provided written informed consent for the collection of samples and subsequent analysis

Antibodies, DNA constructs and reagents

The following antibodies were used: mouse anti-Foxp3 clone PCH101 for FACS analysis (eBioscience, San diego, CA), rabbit anti-acetyl-lysine (Cell signaling, Danvers, MA), mouse anti-Flag M2, and mouse anti-tubulin from Sigma (Zwijndrecht, The Netherlands), Mouse anti-hemaglutinin (HA) clone 12CA5, and rabbit anti-HSP90 from Santa Cruz Biotechnology (Santa Cruz, CA), (Sigma), HRP-conjugated anti-ubiquitin Enzo Life Sciences (Plymouth Meeting, PA, USA). pMT2 HA-Foxp3, pMT2 HA-Foxp3K22xR, pMT2 Flag-Foxp3 and pRSV-NFATC/A are previously described[5]. Flag-SIRT1-7 constructs have previously been described[15] although we have to re-subclone SIRT5. Cyclohexamide, nicotinamide, and MG132 were purchased from Sigma.

Cell culture and luciferase assays

HEK293 cells were maintained in DMEM (Invitrogen) supplemented with 8% heat-inactivated FCS, penicillin and streptomycin (Invitrogen) at 37°C and 5% CO_2. Cells were grown to 50% confluence in six wells-plates (Nunc, Roskilde, Denmark) and transfected with a mixture of 1.5 µg DNA and 7.5 µl PEI overnight, the following day cells were washed twice with PBS and cultured for 24 hours in medium. Cell lysates were separated by SDS-PAGE and analyzed by Western blot. For luciferase assay, cells were transfectied with 1 µg IL-2 promoter luciferase reporter from Panomics (Fremont, CA) 0.5 µg of pMT2-Foxp3, pcDNA3-NFATC/A or 0.5 µg pcDNA3 empty vector and 7 µg pMT2 empty vector and 0.05 µg pRLTK

renilla, (Promega, Leiden, the Netherlands) to normalize for transfection efficiency. Cells were transfected in a six-well plate, three days after transfection the cells were washed twice with PBS and lysed in 50 µl passive lysis buffer for 15 minutes, insoluble cell debris was spun down and the supernatant fraction was assayed for luciferase activity using Dual-Luciferase Reporter Assay System (Promega, Leiden, The Netherlands).

Confocal imaging

Localization studies

Transfected HEK293 cells seeded on poly-L-lysine-coated coverslips were washed with PBS before fixation using 3% paraformaldehyde (Merck, Nottingham, United Kingdom) for 10 minutes at 22°C. Cells were pre-incubated with PBS containing 10% normal donkey serum (Jackson Immunoresearch) and 0.5% saponin (Sigma-Aldrich) for 15 minutes. Next, cells were incubated for 60 minutes with mouse anti-Flag-FITC (2.5µg/ml) and rabbit anti-HA-TRITC 4µg/ml (Santa Cruz Biotechnology Inc.) in PBS containing 10% normal donkey serum and 0.5% saponin. Cells were washed three times with PBST (0.05% Tween), and mounted in Mowiol 4-88 (Sanofi-Aventis, Paris, France) containing DAPI. Cells were analyzed with a 63x objective on a Zeiss LSM 710 fluorescence microscope (Oberkochen, Germany).

Proximity ligation assay

PLA detection was performed according to the manufacturer's protocol. In short, cells grown on coverslips were fixed using 3% paraformaldehyde for 10 minutes at 22°C. Subsequently, cells were washed three times with PBS and blocked for 30 minutes at 22°C in PBS containing 10% normal donkey serum, 0.5% BSA, and 0.5% saponin. After blocking, cells were incubated for 60 minutes at 22°C with mouse anti-HA and rabbit anti-Flag antibodies in PBS containing 10% normal donkey serum, 0.5% BSA, and 0.5% saponin. Cells were washed three times with PBST (0.05% Tween) and incubated with the secondary mouse PLUS and rabbit MINUS antibodies for 1.5 hours at 37°C in the dark. Cells were washed three times in PBST before detection of the probe using the *in situ* PLA detection kit

(Abnova, Walnut, USA). Cells were analyzed with a 63x objective on a Zeiss LSM 710 fluorescence microscope.

Immunoprecipitation and Western blot analysis

Cells were lysed in a NP40 lysis buffer (0.05 M Tris-HCl pH7.5, 0.5% Nonidet P40, 0.15 M NaCl, 0.01 M EDTA), immunoprecipitation was performed utilizing anti-flag coupled beads (Sigma). Beads were washed 3x in lysis buffer, boiled and samples were separated by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and hybridized with antibodies as indicated. Immunocomplexes were detected using enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom).

Generation of iTreg

CD4+CD25- were isolated from human cord blood by magnetic-activated cell sorting (MACS) and cultured in RPMI 1640 supplemented 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 x 10^{-5} M 2-mercaptoethanol. Foxp3 expression was induced by culturing the cells for four days in combination with anti-CD3 anti-CD28 beads dynabeads, 300 IU IL-2 and 10 nM TGF-β.

Quantitative PCR

mRNA was isolated using the trizol according to the manufacturer's protocol (Invitrogen), cDNA synthesis was performed using IScript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA samples were amplified using SYBR green supermix (BIO-Rad), in a MyiQ single-color real time PCR detection system (Bio-Rad) according to the manufacturer's protocol. To quantify the data, the comparative Ct method was used[16]. Relative quantity was defined as $2^{-\Delta\Delta Ct}$ and β2-microglobulin was used as reference gene. Primers are listed in Table 1.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test (Prism GraphPad Software, San Diego, CA). $p<0.05$ was considered statistically significant.

Results

SIRT1 co-localizes with Foxp3 reducing Foxp3 protein expression levels

Recently, we reported that proteasome-mediated degradation of Foxp3 is inhibited by p300-mediated acetylation[5]. Increased acetylation prevented poly-ubiquitination and subsequently proteasomal degradation in a competition-based manner, as has been described for other transcription factors including RUNX3, SMAD7, and P53[17-19]. To further validate these observations we wished to determine whether there was specificity among sirtuin (SIRT) lysine deacetylase family members in their capacity to regulate both Foxp3 acteylation and ubiquitination.

Firstly, to analyze potential interactions between SIRT family members and Foxp3, the subcellular localization of both Foxp3 and SIRTs 1-7 was evaluated. Cells ectopically expressing HA-tagged Foxp3 and Flag-tagged SIRTs 1-7 were fixed and permeabilized. Subsequently, the localization of Foxp3 and SIRTs 1-7 were analyzed utilizing anti-HA and anti-Flag antibodies. Foxp3 was found specifically localized in the nucleus and this was unaffected by SIRT co-transfection (Figure 1A). Although all SIRTs were expressed to similar levels, we could only detect nuclear localization of SIRT1, 6 and 7. To investigate specifically which SIRTs could have the capacity to decrease Foxp3 expression levels, cells were transfected with both HA-Foxp3 and Flag-SIRTs 1-7, and Foxp3 protein levels were determined using anti-HA antibodies and blots quantified. Co-transfection of SIRT1 reproducibly decreased Foxp3 levels, while SIRTs 2-7 had no observable effect on Foxp3 expression (Figure 1B). Taken together, these data show that SIRT1 specifically co-localizes with Foxp3 and increased SIRT1 expression is sufficient to reduce Foxp3 protein levels.

SIRT1 directly associates with Foxp3

To determine whether SIRT1 associates with Foxp3 a co-immunoprecipitation assay was performed. Cells were transfected with both HA-Foxp3 and Flag-SIRT1, lysates were prepared and Foxp3 or SIRT1 were immunoprecipitated. SIRT1 was detected when Foxp3

was immunoprecipitated and *vice versa*, demonstrating their association (Figure 2A, B). To further verify these results an *in situ* proximity ligation assay (PLA) was performed (see Materials and Methods). Since a PLA signal can only be obtained when the proteins of interest are in extremely close proximity, this technique enables the detection of direct protein-protein interactions in cells. Association of transfected SIRT1 and Foxp3 in HEK293 cells was observed and interaction was localized specifically in the nucleus (Figure 2C).

SIRT1-mediated deacetylation increases Foxp3 poly-ubiquitination

To further evaluate the molecular mechanism underlying the effect of SIRT1 on Foxp3 protein levels, we first determined whether SIRT1 could decrease Foxp3 acetylation levels. Cells were co-transfected with HA-Foxp3 and Flag-SIRT1, lysates were prepared and HA-Foxp3 was immunoprecipitated, and acetylation was visualized by Western blotting using an anti-acetyl lysine antibody. Levels of Foxp3 acetylation were considerably reduced by SIRT1co-transfection (Figure 3A). To further validate these data we utilized nicotinamide (NAM), the widely used Sirtuin inhibitor[20;21]. NAM treatment increased acetylation of Foxp3 (Figure 3B), demonstrating that SIRTs can indeed modulate Foxp3 deacetylation. Since we previously demonstrated that acetylation of Foxp3 results in increased protein levels by preventing poly-ubiquitination[5], the effect of SIRT inhibition on Foxp3 ubiquitination was also analyzed. Cells ectopically expressing HA-Foxp3 were treated for three hours with the proteasome inhibitor MG132, lysed and Foxp3 was immunoprecipitated. Ubiquitinated Foxp3 was visualized by Western blotting utilizing anti-ubiquitin antibodies. SIRT inhibition dramatically reduced Foxp3 poly-ubiquitination (Figure 3C). Taken together, these data show that SIRT1-mediated deacetylation results in increased Foxp3 poly-ubiquitination.

SIRT1 regulates Foxp3 proteasomal degradation

Since we observed that SIRT1 can decrease Foxp3 deacetylation, thereby increasing its poly-ubiquitination, the effect of SIRT1 on Foxp3 protein stability was further investigated.

Cells were co-transfected with HA-Foxp3 and Flag-SIRT1 and treated with the proteasome inhibitor MG132. After lysis, Foxp3 protein levels were analyzed by Western blotting utilizing anti-HA antibodies. Co-transfection of SIRT1 decreased Foxp3 protein levels, and this was reversed by MG132 treatment (Figure 4A). These data show that the SIRT1-mediated decrease in Foxp3 expression levels is proteasome-dependent**.** To further validate these observations, cells were transfected with HA-Foxp3 and treated with NAM for 16 hours. SIRT inhibition resulted in increased Foxp3 levels as expected (Figure 4B), while MG132 treatment had no additional effect on Foxp3 protein levels. To confirm that SIRT inhibition regulates Foxp3 expression post-translationally, cells were treated with cyclohexamide (CHX), an inhibitor of translation. CHX treatment of cells ectopically expressing HA-Foxp3 resulted in reduced Foxp3 protein levels (Figure 4C). However, there was no effect of CHX-treatment on Foxp3 protein levels when the cells were also treated with NAM, confirming that SIRT1 inhibition increases Foxp3 protein stability.

To confirm that deacetylation of Foxp3 is directly responsible for reduced proteasomal degradation we generated a Foxp3 mutant in which all lysines had been mutated (HA-Foxp3 K22xR), thereby preventing both acetylation and ubiquitination of Foxp3. Cells were transfected with wild-type Foxp3 or Foxp3 K22xR and subsequently treated with NAM. Foxp3 K22xR protein levels were increased compared to wild-type presumably since the Foxp3 K22xR mutant can no longer be poly-ubiquitinated (Figure 4D). Treatment with NAM again resulted in increased protein levels of wild-type Foxp3, however Foxp3 K22xR expression levels remained unaffected by SIRT inhibition.

To determine whether SIRTs can also regulate Foxp3 protein expression in human CD4+ Treg, CD4+ cells from human PBMC were cultured in the presence of IL-2, anti-CD3 and anti-CD28 to generate iTreg as previously described[5]. iTreg were treated with NAM and the percentage of CD4+Foxp3+ T cells, as well as Foxp3 protein levels per cell were determined by FACS. NAM treatment resulted in both increased CD4+Foxp3+ cell numbers as well as increased Foxp3 protein levels per cell (Figure 4E, F). Taken together, these data show that SIRT-mediated deacetylation increases proteasome mediated Foxp3 degradation in Treg.

SIRT1 activity inhibits Foxp3 transcriptional output

To determine whether SIRT1-mediated Foxp3 deacetylation also results in impaired Foxp3 transcriptional output an IL-2 promoter reporter assay was performed. Cells were transfected with IL-2 promoter luciferase, NFAT, Foxp3 and SIRT1 as previously described[5]. Transfection of Foxp3 resulted in suppression of IL-2 promoter activity (Figure 5A). Co-transfection with SIRT1 significantly impaired Foxp3 mediated repression of the IL-2 promoter. Since we have established that SIRT1 decreased Foxp3 protein levels through proteasomal degradation Foxp3, SIRT1 mRNA expression levels were analyzed during Treg induction. Treg were induced by stimulating CD4+CD25- cells from cord blood peripheral blood mononuclear cells (PBMC) using anti-CD3 anti-CD28 coupled dynabeads in combination with IL-2 and TGF-β for five days. Cells were isolated at day 0, 3, and 5, and SIRT1 and Foxp3 mRNA expression were analyzed by qRT-PCR (Figure 5B). Foxp3 mRNA levels were dramatically upregulated during iTreg differentiation, as expected. Furthermore, a significant decrease of SIRT1 mRNA at both day three and five was observed. We next analyzed whether increased Foxp3 protein levels by SIRT inhibition also results in increased Foxp3 transcriptional activity. Human CD4+ cells were isolated from PBMC and differentiated towards Foxp3+ iTreg in the presence or absence of NAM. mRNA expression of multiple Foxp3 transcriptional targets (IL-2, ITK, Zap70, c-Myc, Jak2) were analyzed[22]. Treatment with NAM reduced mRNA levels of all Foxp3 transcriptional targets in a dose-dependent manner (Figure 5C). Taken together, these data suggest that increased Foxp3 protein levels by SIRT1 inhibition also results in increased suppression of multiple Foxp3 transcriptional targets in iTreg.

Discussion

Stable expression of the transcription factor Foxp3 is essential for the development and function of Treg[23-26]. Although TCR activated T cells can transiently express Foxp3 protein, these cells lack suppressive capacities[27;28]. While many studies have focused on the regulation of *Foxp3* promoter activity, proteasome mediated regulation of Foxp3 expression levels has not yet been extensively investigated. Recently, we established that Foxp3 acetylation increases protein levels, and that deacetylation by KDACs decreases Foxp3 protein expression through increased proteasomal degradation[5]. Here we demonstrate that SIRT1, but not SIRTs 2-7, co-localizes with Foxp3 in the nucleus. SIRT1 can specifically regulate Foxp3 deacetylation, resulting in increased Foxp3 poly-ubiquitination and reduced Foxp3 protein levels. In addition, we show that SIRT inhibition results in both increased Foxp3 protein levels and transcriptional output in human iTreg. Taken together, these data provide novel insights into regulatory mechanisms modulating Foxp3 protein expression and thereby Treg numbers and suppressive capacity.

Several classes of KDAC inhibitors are currently being used as anti-inflammatory agents[1]. We and others have recently reported that inhibition of KDAC activity utilizing broad-spectrum KDAC inhibitors, including TSA, SAHA, Tubacin, BML-210, MS-275, SB, and Bufexmac can increase Treg-mediated suppression in *in vitro* suppression assays[4;5;29]. Treatment with TSA, SAHA, and VPA have also been shown to reduce colitis, and increase cardiac and island allograft survival *in vivo* in mouse models[2-4]. Importantly, Beier *et al*. recently reported that in both murine *sirt1* knock-out Treg, and Treg in which SIRT1 was specifically inhibited utilizing SIRT1 inhibitor EX-527**,** Treg mediated suppression was increased, resulting in increased cardiac allograft survival[9]. However, the specificity if SIRT family members for Foxp3 and the molecular mechanisms underlying these observations were not evaluated.

Here we demonstrate that treatment of human CD4+ T cells with SIRT inhibitor nicotinamide increases the number of CD4+Foxp3+ Treg as well as the amount of Foxp3 per cell by

impaired Foxp3 degradation, increasing Treg suppressive capacity. Nicotinamide, also known as vitamin B3, is a water-soluble vitamin and its use in the treatment of auto-immunity would therefore be cheaper and relatively safe compared to other commercially available KDAC inhibitors. In several human trials nicotinamide treatment has already shown to significantly reduce the development of type 1 diabetes[30;31]. In addition, nicotinamide treatment of patients with arthritis has been observed to result in disease remission[32;33]. IPEX patients have an enhanced susceptibility for diabetes type 1 and Treg adoptive transfer impairs diabetes in NOD mice[34], while depletion of Treg in collagen-induced arthritis mice hastens the onset of disease[35]. With this in mind, it is interesting to speculate that the positive effect of nicotinamide observed in current patient trials could be Treg mediated[36;37].

As previously mentioned, activated T cells can also transiently upregulate Foxp3 expression levels[27]. CD4 positive T cells stimulated with phorbol 12-myristate 13-acetate (PMA) and Ca^{2+} ionophore, or anti-CD3 and anti-CD28, transiently express both Foxp3 mRNA and protein[27;38;39]. In general, these Foxp3 positive cells do not develop a Treg phenotype and are unable to suppress proliferation or cytokine production of co-cultured T cells[27;38]. However, a small percentage of stimulated T cells express high and stable levels of Foxp3 and have also been demonstrated to be immune suppressive[27;28]. Murine Treg in which Foxp3 is conditionally deleted, lose their suppressive phenotype resulting in the production of Th1 cytokines[26]. These observations underscore the prevailing dogma that stable, high expression of Foxp3 is essential for maintaining the Treg phenotype, and stabilization of Foxp3 expression levels is a critical step for the development of induced Treg. It has recently been shown that SIRT1 is significantly upregulated in activated T effector cells, while in Treg SIRT1 is downregulated upon activation[9]. Since we show that SIRT1 mediated deacetylation of Foxp3 results in impaired protein stability, we propose a model in which Foxp3 acetylation may provide a post-translational “switch” allowing the development of iTreg. In this model, T cell activation results in expression of Foxp3 which will be stable and high when Foxp3 is acetylated, resulting in the development of suppressive iTreg. However,

in the presence of active SIRT1, as reported in activated T effector cells[9], T cells will not develop a Treg phenotype since Foxp3 will be deacetylated, subsequently poly-ubiquitinated and degraded by the proteasome. Therefore control of SIRT1 activity may be a defining factor for the initiation of a Foxp3-dependent transcriptional program and the development of Treg.

Taken together, we have demonstrated that SIRT1 but not SIRTs 2-7 directly deacetylates Foxp3, resulting in increased Foxp3 poly-ubiquitination and proteasomal degradation, thereby abrogating Treg functionality. Our findings suggest that manipulation of SIRT1 levels or activity may provide a novel therapeutic strategy to control (inappropriate) immune responses.

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Figure legends

Figure 1. SIRT1 co-localizes with Foxp3 and reduces its expression. (**A**) HEK293 cells that were transfected with HA-Foxp3 and Flag-tagged SIRT1-7. Cells were fixed, permiabilized and tagged proteins were visualized using anti-HA (red) or anti-Flag (green) antibodies. DAPI was used to visualize the nuclei (blue). Figure shows examples of three independent experiments. (**B**) HEK293 cells were transfected with HA-Foxp3 and Flag-SIRT1-7. After 48 hours cells were lysed, and Foxp3 levels were analyzed by Western blotting using anti-HA antibodies. Foxp3 protein levels were quantified and normalized for HSP90 expression. Data show a mean of five independent experiments + SEM. * $P<0.05$.

Figure 2. Nuclear association of Foxp3 and SIRT1. (**A**) HEK 293 cells were co-transfected with both HA-Foxp3 and Flag-SIRT1, lysed, and Foxp3 was immunoprecipitated and association of proteins was analyzed by Western blotting utilizing anti-Flag antibodies. (**B**) HA-Foxp3 and Flag-SIRT1 transfected cells were lysed, SIRT1 was immunoprecipitated and the association of Foxp3 was assessed as in (**A**). (**C**) SIRT1-Foxp3 interaction in transfected HEK293 cells was visualized using *in situ* proximity ligation assay (PLA). Cells were fixed and protein-protein interactions were visualized utilizing anti-SIRT1 and anti-Foxp3 antibodies as described in the Materials and Methods section. Punctate staining (red) indicates Foxp3-SIRT1 interaction as detected by the assay. Nuclei were stained using Hoechst. Representative images from at least three independent experiments are depicted.

Figure 3. SIRT1-mediated deacetylation increases Foxp3 poly-ubiquitination. (**A**) HEK293 cells ectopically expressing HA-Foxp3 were transfected with Flag-SIRT1. Cell lysates were prepared and HA-Foxp3 was immunoprecipitated. Acetylated Foxp3 was visualized by Western blotting using anti-acetylated lysine antibodies. (**B**) Analysis of Foxp3 acetylation after treatment with 20 mM nicotinamide (NAM) for 16 hours as in (**A**). (**C**) Cells were transfected with Flag-Foxp3 and treated with 20 mM NAM, Foxp3 was

immunoprecipitated from cell lysates and ubiquitination was analyzed by Western blotting utilizing anti-ubiquitin antibodies. * indicates aspecific band. Data shown are representative of three independent experiments.

Figure 4. SIRT1 regulates Foxp3 degradation. (**A**) HEK293 cells were co-transfected with HA-Foxp3 and Flag-SIRT1 and treated with 2 μM MG132 for 16 hours. Cells were lysed and Foxp3 protein levels were visualized by Western blotting using anti-HA antibodies. (**B**) Cells were transfected with HA-Foxp3 and treated with 20 mM NAM for 16 hours, Foxp3 protein levels were analyzed by Western blotting using anti-HA antibodies. (**C**) Cells ectopically expressing HA-Foxp3 were treated with both 20 mM NAM and 5 μg/ml cyclohexamide (CHX) for 16 hrs and Foxp3 levels were analyzed by Western botting. (**D**) HA-Foxp3 or a HA-tagged Foxp3 mutant in which all lysines were mutated to argenines (HA-Foxp3 K22xR) was transfected into HEK 293 cells. Cells were treated with 20 mM NAM for 16 hours. Cell lysates were prepared and Foxp3 levels were evaluated by immunoblotting for HA and HSP90 as control. (**E**) CD4+ T cells isolated from human PBMCs were cultured in the presence of 300 IU/ml IL-2, 2.0 μg/ml anti-CD28, and 1.5 μg/ml plate-bound anti-CD3 and treated NAM (1 or 9 mM). After 4 days, the percentage of Foxp3+CD25+ cells was analyzed by FACS. (**F**) Histogram of the data shown in (**E**). Data are representative of at least three independent experiments.

Figure 5. SIRT1 inhibition increases Foxp3 transcriptional output. (**A**) Utilizing an IL-2 promoter reporter, Foxp3 transcriptional function was analyzed. Cells were transfected with an IL-2 promoter luciferase reporter, NFAT, Foxp3 and SIRT1, cells were lysed and assayed as described in the Materials and Methods. Luciferase values were normalized for co-transfected renilla to correct for transfection efficiency. (**B**) iTregs were generated by culturing human CD4+CD25- human T cells with anti-CD3 and anti-CD28 coupled beads, in combination with 10 ng/ml TGF-β and 300 U/ml IL-2 for 5 days. At day 0, 3, 5 cells were harvested and mRNA expression of SIRT1 and Foxp3 was analyzed by qRT-PCR. (**C**)

Freshly isolated human CD4+ T cells were cultured in the presence of 300 U/ml IL-2, anti-CD3 and anti-CD28 and 0, 1 or 9 mM NAM for three days. mRNA expression of multiple transcriptional targets of Foxp3 was analyzed by qRT-PCR. Data are representative of at least three independent experiments, * $P<0.05$, ** $P<0.01$.

Tables

Table 1. Q-PCR primers

Gene	Forward primer	Reverse Primer
IL-2	5'-TCCTGTCTTGCATTGCACTAAG-3'	5'-CATCCTGGTGAGTTTGGGATTC-3'
ITK	5'-GTGTTTGCTCCAGATCGTGAG-3'	5'-GGTTGGATCATATTGGGCACAG-3'
cMyc	5'-TCCGGAAGGACTATCCTGCTG-3'	5'-GTGTGTTCGCCTCTTGACATT-3'
ZAP70	5'-TGCCCTTCTTCTACGGCAG-3'	5'-ACGAGCGACAGCACATAGC-3'
JAK2	5'-CAACAGAGCCTATCGGCATGG-3'	5'-GGGGTTTGATCGTTTTCTTTGG-3'
SIRT1	5'-TGCGGGAATCCAAAGGATAA-3'	5'-CAGGCAAGATGCTGTTGCA-3'
Foxp3	5'-TCAAGCACTGCCAGGCG-3'	5'-CAGGAGCCCTTGTCGGAT-3'
β_2M	5'-CCAGCAGAGAATGGAAAGTC-3'	5'-GATGCTGCTTACATGTCTCG-3'

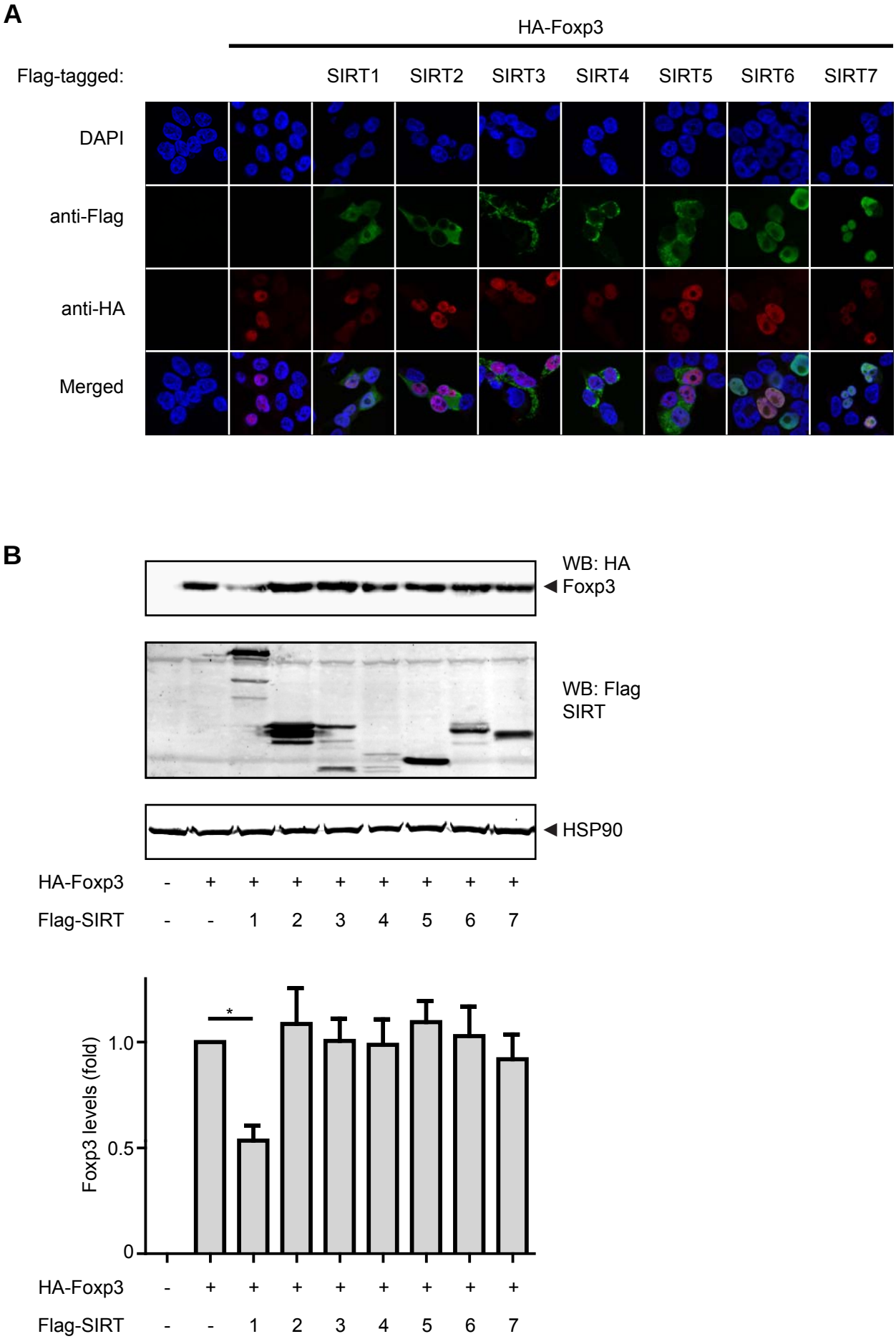
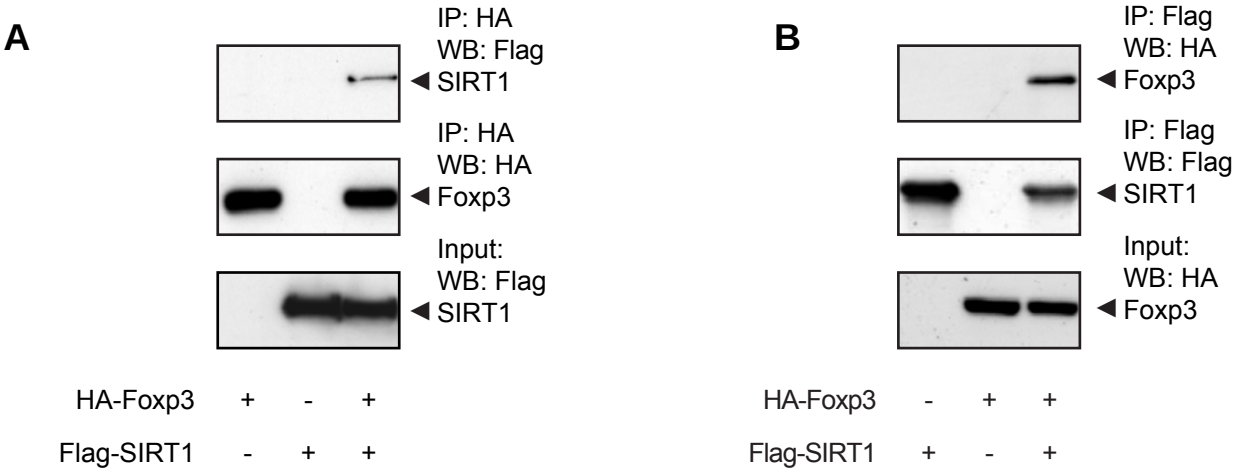


Figure 1. van Loosdregt et al.



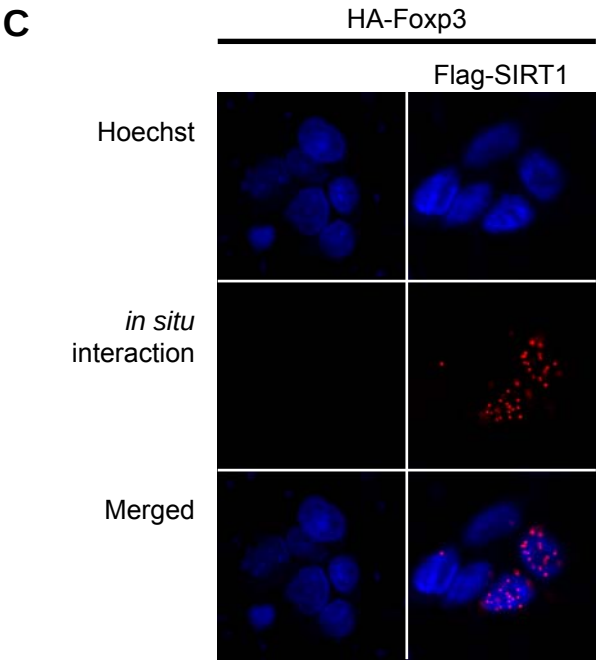


Figure 2. van Loosdregt et al.

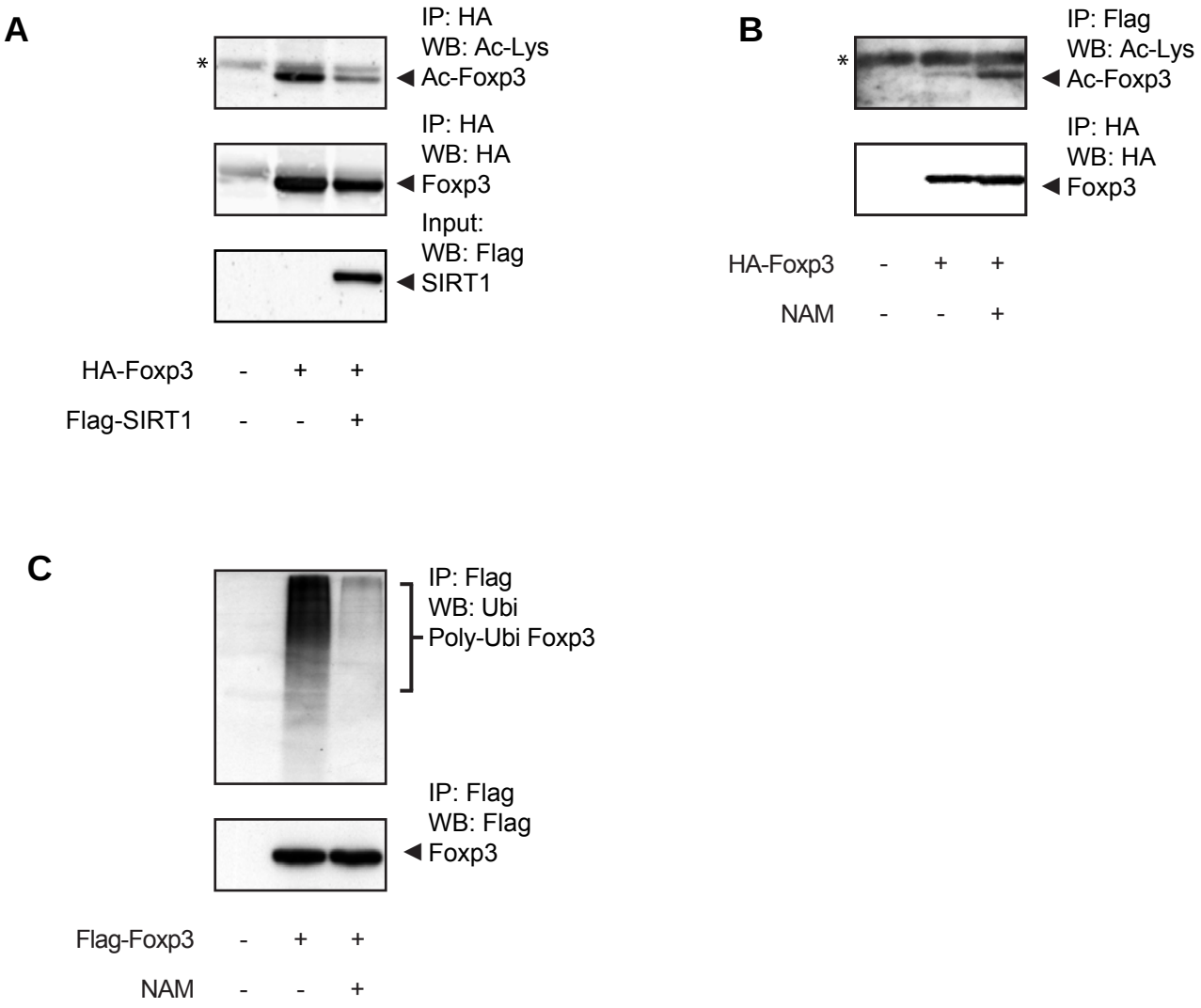


Figure 3. van Loosdregt et al.

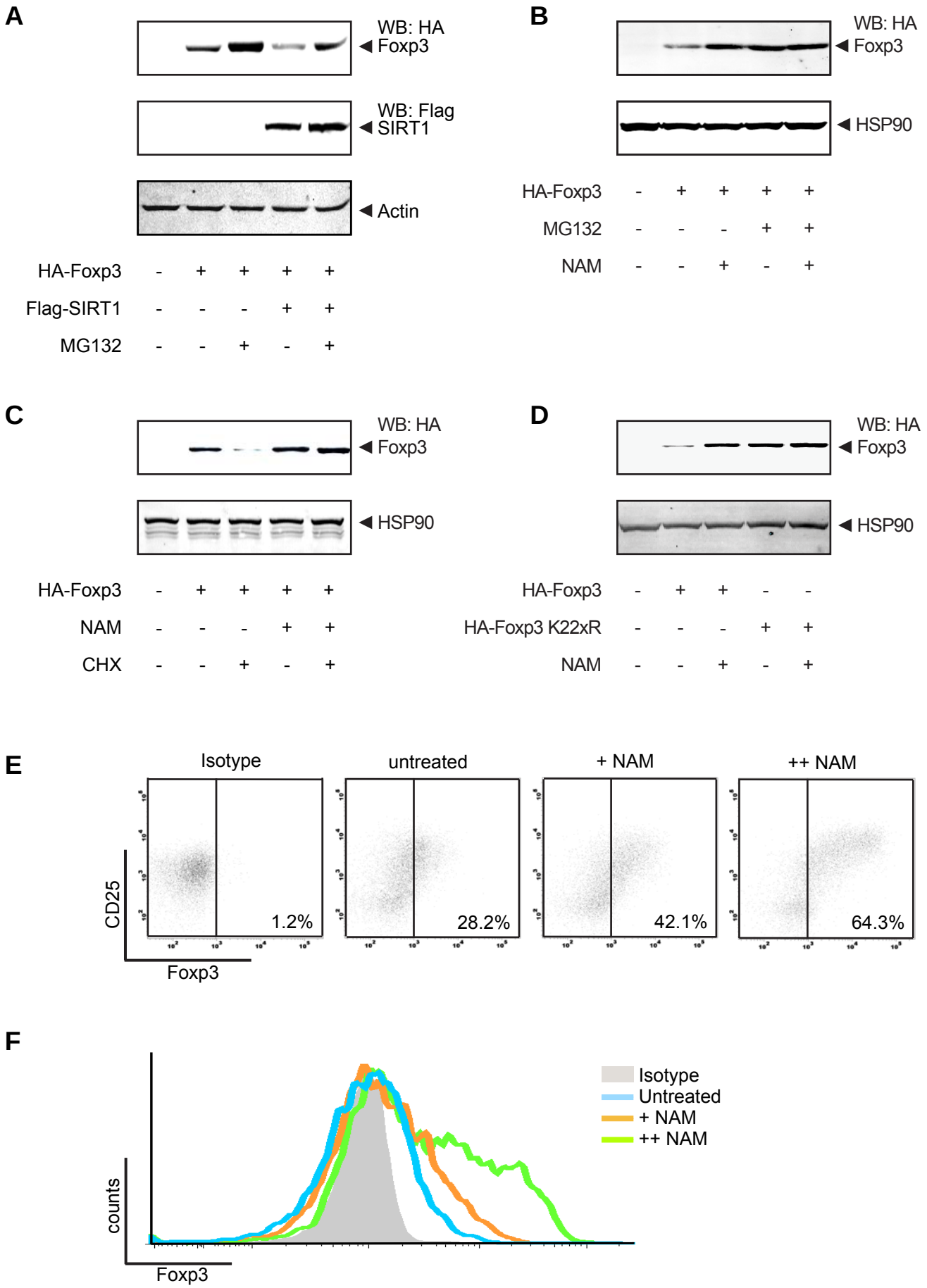


Figure 4. van Loosdregt et al.

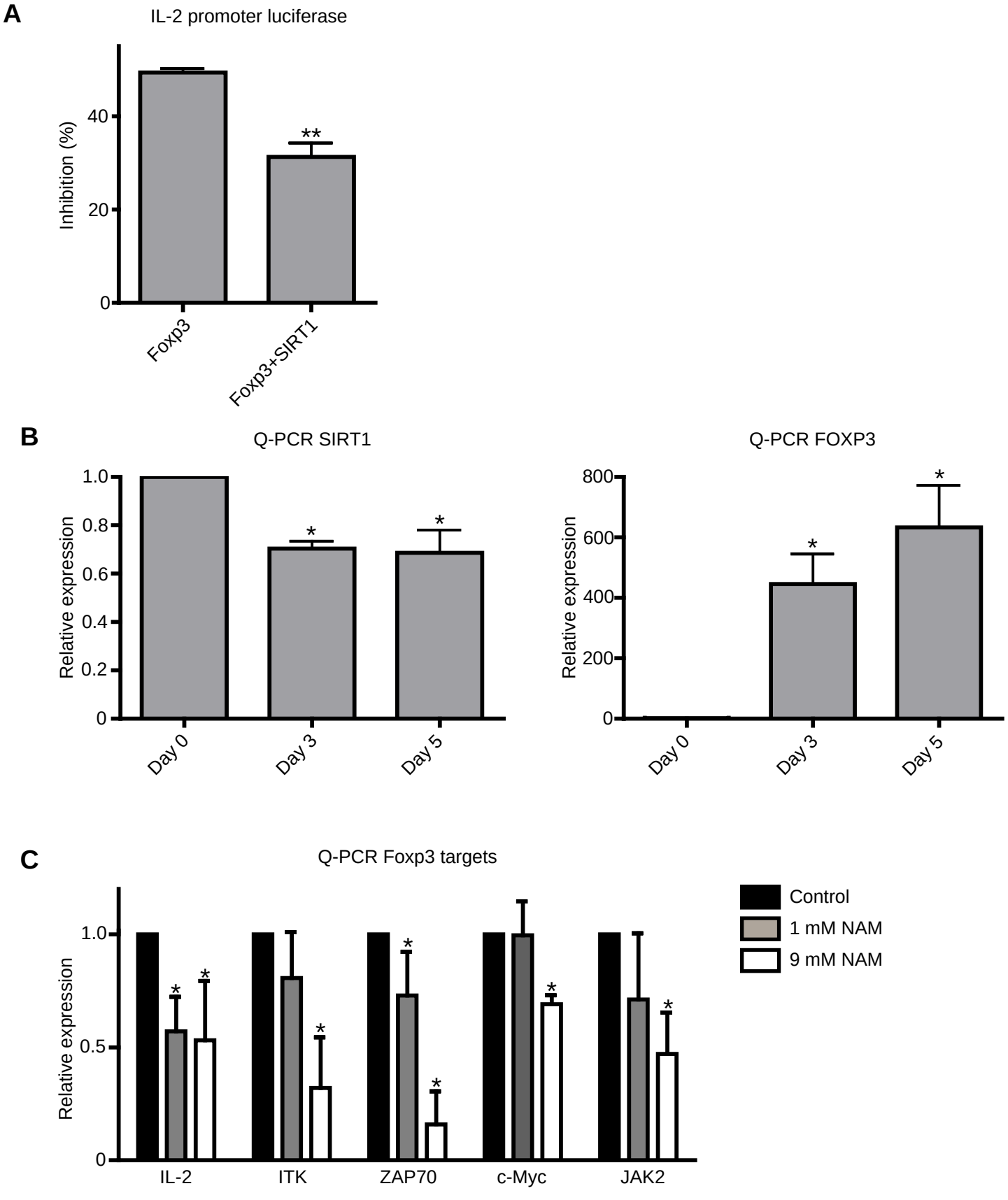


Figure 5. van Loosdregt et al.

Chapter 4

USP7/HAUSP mediated stabilization of Foxp3 increases Treg suppressive capacity

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Submitted

Abstract

Stable Foxp3 expression levels are required for the development of functional regulatory T cells (Treg). Here we demonstrate that Foxp3 expression is regulated through poly-ubiquitination on at least five independent lysine residues, resulting in proteasome-mediated degradation. The deubiquitinase (DUB) USP7 was found to be active in Treg and associated with Foxp3 in the nucleus. Ectopic expression of USP7 decreased Foxp3 poly-ubiquitination increasing both Foxp3 expression and transcriptional activity. Conversely, treatment with a pan DUB inhibitor, or USP7 knockdown decreased endogenous Foxp3 protein levels in Treg. Furthermore, USP7 knockdown significantly decreased Treg mediated suppression. In addition, in an induced colitis mouse model, inhibition of DUB activity in Treg abrogated their ability to resolve inflammation *in vivo*. Taken together, our data provides a novel molecular mechanism in which rapid temporal control of Foxp3 levels in T cells is regulated by USP7, thereby modulating Treg numbers and function.

Foxp3+ regulatory T cells (Treg) are a specific subset of CD4+ T cells that are crucial for the maintenance of self-tolerance[1, 2]. The X chromosome-encoded transcription factor Foxp3, a member of the Forkhead box family, is essential for both Treg development and function. Foxp3 mutations in mice as well as IPEX (immune dysregulation polyendocrinopathy, enteropathy, X chromosome-linked syndrome) patients result in the development of complex autoimmune diseases, due to Treg deficiency[1, 3]. Additionally, T cells manipulated to ectopically express Foxp3 acquire the Treg phenotype[1, 4].

Constitutive expression of Foxp3 has been demonstrated to be essential for the maintenance of Treg suppressor function[5]. Although the precise molecular mechanisms regulating expression of the *Foxp3* gene are incompletely understood, it has been reported that TGF-β, IL-2, or T cell receptor (TCR) stimulation of T cells can all result in increased *Foxp3* expression[6-8]. This is likely modulated by demethylation of the Foxp3 promoter or conserved non-coding regions in the *Foxp3* locus[6]. In addition, multiple transcription factors including NF-κB, SMAD3, NFAT, CREB/ATF and STAT5 have all been demonstrated to regulate the *Foxp3* transcription in. However the complex interactions between chromatin remodeling, enhancer elements and transcription factor activation still remain unclear.

Foxp3 expression in Treg is not unique since *in vitro* TCR stimulation of CD4+CD25- T cells results in transient expression of Foxp3 mRNA and protein. However, the vast majority of cells do not exhibit a suppressive phenotype, and it is possible that here Foxp3 acts to prevent T cell hyperactivation[9, 10]. In contrast, a small sub-population of these TCR stimulated CD4+CD25- cells does express both high and stable Foxp3 protein levels, acquiring suppressive capacity[11, 12]. These and other studies have led to the now widely accepted dogma that persistent expression of Foxp3 is essential for the maintenance of suppressor function. Since, stable Foxp3 protein levels are indispensable for the suppressive capacity of Treg, here we have focused on the molecular mechanism modulating this process.

Protein expression levels in cells can be regulated by both production and degradation rates. Much of the regulated proteolysis in eukaryotic cells is catalyzed by the ubiquitin-proteasome system[13]. Covalently attached ubiquitin chains of four or more ubiquitin proteins mark a

protein for degradation by the 26S proteasome[14, 15]. Protein ubiquitination is a tightly regulated process modulated by E1, E2, and E3 ligases which, in a complex, catalyze addition of ubiquitin to lysine residues of the target protein. Here, the initial ubiquitin serves as an acceptor for further cycles of ubiquitin modification, resulting in a developing poly-ubiquitin chain[16]. Protein deubiquitination is an equally well regulated process modulated by a large family of deubiquitinating enzymes (DUBs). DUBs catalyze removal of ubiquitin from specific protein substrates thereby preventing protein degradation, thereby increasing target protein levels[17].

Here we demonstrate that Foxp3 protein has a high turnover rate and that Foxp3 degradation is regulated by poly-ubiquitination. We establish that the DUB USP7 is active in primary Treg and associates with Foxp3. Ectopic expression of USP7 specifically decreased Foxp3 poly-ubiquitination resulting in increased Foxp3 protein levels. Conversely, knock-down of USP7 resulted in decreased Foxp3 protein levels. Furthermore, Treg function was noticeably decreased when USP7 was knocked-down or when DUB activity was inhibited *in vitro* and *in vivo*. Taken together, manipulation of Foxp3 ubiquitination provides a novel molecular mechanism for assuring rapid temporal control of Foxp3 levels in T cells, thereby regulating Treg numbers and function.

Results

Foxp3 protein expression is regulated by poly-ubiquitination

To investigate whether Foxp3 protein levels may be regulated post-transcriptionally, we utilized the protein translation inhibitor cyclohexamide (CHX). Foxp3 transfected cells or Treg were treated with CHX as indicated, cell lysates were prepared and Foxp3 protein levels were determined by Western blotting. In both transfected cells (**Fig. 1a**) and Treg (**Fig. 1b**) inhibition of translation led to a rapid decrease in Foxp3 protein levels, suggesting rapid Foxp3 protein degradation. Since protein degradation is commonly regulated by poly-ubiquitination, we analyzed the ubiquitination status of Foxp3. Cells were transfected with both His-tagged ubiquitin and HA-tagged Foxp3, lysates were prepared and ubiquitinated proteins were isolated using Ni-NTA beads. Foxp3 specific poly-ubiquitination was determined by Western blot analysis using anti-HA antibodies. A clear poly-ubiquitination pattern was observed with wild-type Foxp3 (**Fig. 1c**) and this was abrogated in a Foxp3 mutant in which all lysine residues were mutated (Foxp3 K22xR), indicating that Foxp3 is directly ubiquitinated. Short term treatment of cells with the pan deubiquitinase (DUB) inhibitor H1 dramatically increased Foxp3 poly-ubiquitination, suggesting that continuous deubiquitination of Foxp3 is required to maintain protein expression[18]. To confirm that Foxp3 can also be poly-ubiquitinated in human Treg, cells were treated with the proteasomal inhibitor MG132 for three hours to abrogate degradation of poly-ubiquitinated proteins. Cells were lysed and incubated with Tandem Ubiquitin Binding Entities (TUBE) coupled agarose beads to isolate ubiquitinated proteins. Again, Foxp3 specific poly-ubiquitination was visualized by Western blot analysis using anti-Foxp3 antibodies (**Fig. 1d**). In the presence of MG132 a clear poly-ubiquitin pattern was observed, demonstrating that also in human Treg Foxp3 is poly-ubiquitinated.

To determine whether Foxp3 poly-ubiquitination resulted in regulation of Foxp3 protein levels, HA-Foxp3 transfected cells (**Fig. 1e**) or Treg (**Fig. 1f**) were treated with DUB inhibitor H1 and Foxp3 protein levels were analyzed by Western blot. Treatment with H1 decreased

Foxp3 protein levels in both situations. Taken together, these data show that Foxp3 protein levels are regulated post-transcriptionally, a process that is poly-ubiquitination dependent and can be rescued by de-ubiquitination.

Identification of multiple sites of Foxp3 ubiquitination

Since we could demonstrate that Foxp3 poly-ubiquitination results in protein degradation, we further analyzed specifically which lysine residues could be ubiquitinated. Cells ectopically expressing Foxp3 were lysed, and Foxp3 was immunoprecipitated. Samples were trypsinized and subsequently analyzed by mass spectrometry as described. Five distinct lysine residues in Foxp3 were found to be ubiquitinated (K249, K251, K263, K267, K393; **Fig. 2a, b**). To further verify that these lysines can indeed be ubiquitinated, Foxp3 expression constructs were generated in which all lysines were mutated into arginines (Foxp3 K22xR) or modified to express combinations of the lysines identified. Foxp3 mutants were subsequently analyzed for ubiquitination as previously described. Addition of all five lysine residues identified by mass spectrometry rescued Foxp3 ubiquitination. Similarly addition of only lysines 263/267 or 249/251/393 restored Foxp3 ubiquitination to levels similar to wild-type Foxp3 (**Fig. 2c**). These data provide evidence for promiscuous ubiquitination of Foxp3 at multiple lysine residues, a phenomenon previously described for other proteins such as TRAF1, BRCA1, UCH-L1 and EGFR[19-22]. To evaluate whether the specific location of modification influences poly-ubiquitination, a Foxp3 mutant was generated in which ubiquitin was fused to the N-terminus. The Ubi-Foxp3 fusion protein was highly poly-ubiquitinated and rapidly degraded (**Fig. 2d**). To further verify that the ubiquitin fusion-protein was indeed poly-ubiquitinated, we generated an Ubi-K7xR-Foxp3 fusion construct in which all lysines in ubiquitin, but not in Foxp3, were replaced with arginine. Mutating the ubiquitin fusion protein markedly reduced Foxp3 poly-ubiquitination back to wild-type levels. Taken together, these data show that Foxp3 can be poly-ubiquitinated at multiple, independent lysine residues which result in rapid proteosome-mediated degradation.

USP7 associates with and deubiquitinates Foxp3

Since our data suggests that Foxp3 protein expression levels are continuously regulated by DUBs, a candidate approach was used to identify the relevant deubiquitinase. USP7 is an ubiquitinously expressed DUB that deubiquitinates the Forkhead box transcription factor Foxo4[23]. To determine whether USP7 could also deubiquitinate Foxp3, cells were transfected with Foxp3, USP7 and ubiquitin, lysed and Foxp3 poly-ubiquitination was analyzed as previously described. As shown in **Figure 3a**, Foxp3 poly-ubiquitination was reduced after ectopic expression of USP7, suggesting that USP7 can directly deubiquitinate Foxp3. To validate that Foxp3 is a direct USP7 substrate, association of the two proteins was analyzed by co-immunoprecipitation. Cells were co-transfected with Myc-USP7 and HA-Foxp3, Myc-USP7 was immunoprecipitated from cell lysates and analyzed for HA-Foxp3 association or *vice versa* (**Fig. 3b**). In both experiments a clear association was observed, indicating that Foxp3 can associate with USP7. To confirm that both proteins interact in human Treg, cells were fixed and permeabilized, and both endogenous Foxp3 and USP7 were visualized utilizing specific antibodies. Co-localization of both proteins was observed in the nucleus (**Fig. 3c**). Similar results were obtained in cells ectopically expressing GFP-tagged USP7 and mKate-tagged Foxp3 (**Supplementary Fig. 1**). To confirm that endogenous USP7 and Foxp3 can also directly associate in human Treg, an *in situ* proximity ligation assay (PLA) was performed (see Methods). Since a PLA signal can only be obtained when the proteins of interest are in extremely close proximity, this technique enables the detection of direct protein-protein interactions in cells. Association of USP7 and Foxp3 was observed and interaction was localized specifically to the nucleus (**Fig. 3d**). To further verify that USP7 is both expressed and active in regulatory T cells we used an HA-tagged probe that covalently binds active DUBs allowing isolation and identification[24, 25]. Cell lysates from both natural and induced Treg were incubated with the DUB probe, subsequently active DUBs were immunoprecipitated using anti-HA coupled beads and samples were separated by SDS-PAGE. Gels were stained for protein and bands were isolated and analyzed by mass spectrometry. Mass spectrometric analysis identified the most intense protein band as USP7

(coverage 26%), indicating that USP7 is an active DUB in Treg (**Fig. 3e**). Taken together, these data show that USP7, a DUB that is expressed in the same cellular compartment as Foxp3 and is active in both induced and natural Treg, can associate with and deubiquitinate Foxp3.

USP7 mediated Foxp3 deubiquitination results in increased protein expression

Next, we wished to determine whether, as would be predicted, USP7-mediated deubiquitination results in increased Foxp3 protein levels. Cells were co-transfected with Foxp3 and USP7, cell lysates were prepared and Foxp3 levels were determined by Western blot analysis. Ectopic expression of USP7 with Foxp3 noticeably increased Foxp3 protein levels (**Fig. 4a**). Since the Foxp3 K22xR cannot be poly-ubiquitinated, USP7 would not be expected to have an effect on protein levels. Indeed, Foxp3 K22xR protein levels were not increased by co-transfection of USP7, indicating that USP7 directly stabilizes Foxp3 protein levels by deubiquitination. To investigate whether these observations were specific for USP7, the effect of additional DUBs was analyzed. USP14 and UCH-L3 were both found to be active in Treg utilizing global DUB probe analysis (data not shown). As previously observed, USP7 increased Foxp3 expression (**Fig. 4b**). In contrast, neither USP14 nor UCH-L3 influenced Foxp3 protein levels, supporting a specific role for USP7 in Foxp3 deubiquitination.

To further verify the role of USP7 in stabilizing Foxp3 protein expression levels, USP7 knock down was performed. Utilizing a smart pool of four separate siRNAs, USP7 was knocked down in cells ectopically expressing Foxp3 (**Supplementary Fig. 1**). USP7 knockdown resulted in markedly reduced Foxp3 protein levels compared to the controls (**Fig. 4c**). To verify these data in primary human Treg, USP7 knockdown was performed utilizing an USP7 shRNA lentivirus and Foxp3 protein levels were subsequently determined by flow cytometry. USP7 knockdown was successful and resulted in dramatically decreased Foxp3 protein levels (**Fig. 4d**). These data demonstrate that USP7 activity in Treg stabilizes Foxp3 protein expression levels through inhibition of proteasome mediated degradation.

USP7-mediated Foxp3 deubiquitination improves Treg functionality

Since we observed a dramatic effect of USP7 on Foxp3 protein stability, the effect of USP7 on Foxp3 transcriptional activity was analyzed utilizing an IL-2 promoter luciferase reporter. Cells were transfected with NFAT, IL-2 promoter reporter, and Foxp3 with or without USP7. Foxp3 resulted in a clear repression of IL-2 promoter activity, whereas the well-described IPEX mutant Foxp3 Del250 was transcriptionally inactive (**Fig. 5a**). While co-transfection of Foxp3 Del250 with USP7 did not influence IL-2 reporter activity, USP7 significantly increased suppression mediated by wild-type Foxp3. To determine whether this also results in altered Treg-mediated suppression of T cell proliferation, an *in vitro* suppression assay was performed. CFSE labeled PBMC were co-cultured with sorted human CD4+CD25highCD127low Treg which were pre-treated with DUB inhibitor H1 (**Fig. 5b**). Proliferation of the CFSE labeled CD4+ T cells was significantly less suppressed by Tregs that had been pre-incubated with the DUB inhibitor, indicating that DUBs can regulate the suppressive capacity of Treg. To determine whether USP7 can specifically modulate Treg-mediated suppression, an USP7 knockdown was performed utilizing two distinct shRNAs and Treg functionality was addressed in an *in vitro* suppression assay. Both USP7 shRNAs significantly abrogated Treg mediated suppression compared to the scrambled shRNA control. Taken together, these data show that regulation of Foxp3 protein levels by USP7-mediated deubiquitination results in increased Treg-mediated suppression.

Inhibition of DUB activity abrogates Treg mediated suppression *in vivo*

To determine whether DUB activity could also modulate Treg mediated suppression *in vivo*, the well established mouse colitis model was utilized. Here, immune deficient mice are infused with CD4+CD45RBhigh T cells to induce colitis, and disease severity can be reduced by adoptive transfer of Treg[26, 27]. Sorted Treg isolated from Foxp3-GFP mice were pretreated with pan DUB inhibitor H1, and three weeks after Treg infusion, mice were sacrificed and colitis severity was scored by analyzing colon histology (**Fig. 6a**). Treatment with Treg

noticeably reduced disease scores compared to mice that did not receive Treg (**Fig. 6b,c**). Disease scores of mice receiving Treg pretreated with H1 were significantly increased compared to control mice. These data indicate that inhibition of DUB activity can significantly abrogate Treg-mediated suppression *in vivo*. To verify that all mice received the same number of Tregs, the percentage of GFP positive cells in the spleen was analyzed. In mice obtaining H1-treated Treg or untreated Treg the percentage of GFP positive CD4+ T cells was equal (**Fig. 6d**). To analyze if the impaired suppressive capacity of H1-treated Treg was the result of decreased Foxp3 expression, Foxp3 levels in T cells from the spleens were determined. Foxp3 protein levels were significantly reduced in H1-treated Treg compared to untreated Treg (**Fig. 6e**). These data show that inhibition of DUB activity reduces Foxp3 expression levels and thereby impairs Treg mediated suppression *in vivo*.

Discussion

The transcription factor Foxp3 is crucial for regulatory T cell development and function, and is therefore critical in controlling immune responses[1-5]. Although many studies have focused on transcriptional regulation of Foxp3 expression, the modulation of Foxp3 protein levels has not been investigated[6, 28]. Here we are the first to report that Foxp3 protein levels can be directly regulated by poly-ubiquitination. We demonstrate that Foxp3 is poly-ubiquitinated on at least five lysine residues. The de-ubiquitinase USP7 was found to interact with and deubiquitnate Foxp3, thereby increasing Foxp3 protein levels. Both, inhibition of DUB activity or USP7 knockdown in Treg resulted in decreased Foxp3 protein levels and impaired Treg mediated suppression *in vitro* and *in vivo*. These data provide novel insights into the molecular mechanisms regulating Foxp3 protein levels and therefore Treg functionality.

Utilizing mass-spectrometry we identified five distinct lysines residues that were ubiquitinated (K249, K251, K263, K267, K393). While a mutant of Foxp3 lacking all lysines (Foxp3 K22xR) was not ubiquitinated, addition of the five identified lysines in the Foxp3 K22xR background restored Foxp3 ubiquitination. Addition of K249/K251/K393 or K263/K267 was also sufficient to restore Foxp3 ubiquitination to wild-type levels, suggesting promiscuity of the lysine residues that are ubiquitinated. Furthermore, generating an N-terminal ubiquitin-Foxp3 fusion protein dramatically increased Foxp3 poly-ubiquitination, demonstrating that the specific location of Foxp3 poly-ubiquitinated lysine residues is apparently not critical. Promiscuity of ubiquitinated lysine residues is a commonly observed phenomenon and has been described for a variety of proteins including TRAF1, BRCA1, UCH-L1 and EGFR[19-22]. In addition, mutation of single ubiquitinated lysine residues may result in ubiquitination of secondary lysine residues that are not normally ubiquitinated, as has been observed in TCRξ chain and Gpa1[29, 30].

USP7 polymorphisms have been associated with multiple autoimmune diseases. In a genome-wide association study 14,000 cases of seven common diseases were compared to 3,000 controls[31]. Numerous single nucleotide polymorphisms in the USP7 gene were highly

significantly correlated with the autoimmune diseases including Crohn's disease, insulin-dependent diabetes mellitus, and rheumatoid arthritis. Since we demonstrate that USP7 increases Treg mediated suppression in multiple models, we propose that both activating or inhibiting USP7 polymorphisms could modulate Treg function and contributing to immune dysregulation.

While it was initially thought that Foxp3 expression was unique for Treg, several studies have recently reported that Foxp3 can also be expressed in activated T cells. Stimulation of human CD4 cells *in vitro* utilizing anti-CD3 and anti-CD28 or phorbol 12-meristate 13-acetate (PMA) and CA^{2+} ionophore results in transient expression of Foxp3, although protein levels are lower compared to nTregs[10, 11, 32]. Analysis of these T cells transiently expressing Foxp3 revealed that these cells could not suppress cytokine production or proliferation of co-cultured T cells[10, 11]. In contrast, TCR stimulated CD4+CD25- cells expressing high and stable Foxp3 levels develop suppressive capacity[9, 10]. In addition, ablation of Foxp3 in mature Tregs in mice with a loxP-flanked *Foxp3* allele resulted in loss of Tregs mediated suppression and the production of IL-2 and TH1 cytokines[5]. These data demonstrate that stable Foxp3 expression is a necessity for the suppressive phenotype of Treg, suggesting that the stabilization of Foxp3 may provide an essential step in the conversion of activated T cells into iTreg.

Treg are found in increased numbers both in, and in close proximity to, solid tumors[33]. They have been shown to inhibit tumor specific T cell immunity and contribute to growth of human tumors *in vivo*[34, 35]. Depletion of CD4+CD25+ cells *in vivo* augments the generation of tumor specific T cells and promotes the rejection of tumors derived from myeloma, sarcoma and melanoma[36-39]. These data suggest that inhibition of Treg numbers and function could provide a potent anti-tumor therapy. Recently, using high throughput screening, a small molecule compound HBX 41.108 was identified which inhibits USP7 activity with an IC_{50} value in the submicromolar range[40]. We propose compounds inhibiting USP7 could potentially be clinically relevant when used to treat diseases, since we have demonstrated

that inhibition of USP7 results in decreased Foxp3 protein levels and prevents Treg mediated suppression.

Taken together, here we propose a novel molecular mechanism regulating the efficiency of Treg mediated immune modulation. We demonstrate that Foxp3 protein levels can be tightly regulated by poly-ubiquitination mediated proteasomal degradation. Modulation of USP7 activity may provide a novel molecular therapy aimed to control (inappropriate) immune responses.

Methods

Antibodies, DNA constructs and reagents

The following antibodies were used: mouse anti-Foxp3 clone PCH101 for FACS analysis (eBioscience, San diego, CA), mouse anti-Flag M2 from Sigma (Zwijndrecht, The Netherlands), Mouse anti-hemaglutinin (HA) clone 12CA5 from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-tubulin (Sigma) and anti-Myc monoclonal mice Ab were made using a hybridoma cell line. Foxp3 was cloned from MIGR1-Foxp3 (kindly provided by Dr. S. Sakaguchi[4]) into pMT2 containing a HA tag generating pMT2-HA-Foxp3. pMT2-Flag-Foxp3, pMT2-Flag-Foxp3ΔE250, and pRSV-NFATC/A have been described previously[41]. pcDNA3 (Invitrogen Carlsbad,CA), Cyclohexamide and MG132 were purchased from Sigma. The HA-DUB probe and H1 have been described previously[18, 24].

Cell culture and luciferase assays

HEK293 cells were maintained in DMEM (Invitrogen) supplemented with 8% heat-inactivated FCS, penicillin and streptomycin (Invitrogen) at 37°C and 5% CO_2. Cells were grown to 50% confluence in six wells-plates (Nunc, Roskilde, Denmark) and transfected with a mixture of 1.5µg DNA and 7.5 µl PEI overnight, the following day cells were washed twice with PBS and cultured for 24 hours in medium. Cell lysates were separated by SDS-PAGE and analyzed by Western blot. For luciferase assay, cells were transfectied with 1µg IL-2 promoter luciferase reporter from Panomics (Fremont, CA) 0.5µg of pMT2-Foxp3, pcDNA3-NFATC/A or 0.5µg pcDNA3 empty vector and 7µg pMT2 empty vector and 0.05µg pRLTK renilla, (Promega, Leiden, the Netherlands) to normalize for transfection efficiency. Cells were transfected in a six-well plate, three days after transfection the cells were washed twice with PBS and lysed in 50µl passive lysis buffer for 15 minutes, insoluble cell debris was spun down and the supernatant fraction was assayed for luciferase activity using Dual-Luciferase Reporter Assay System (Promega, Leiden, The Netherlands).

Confocal imaging

Localization studies

Induced regulatory T cells in PBS containing 2 mM EDTA and 2% FSC were enabled to adhere to poly-L-lysine-coated coverslips and subsequently incubated with Foxp3 Fixation/Permeabilization solution (eBioscience) for 45 minutes at 4°C. Samples were washed with 1X Permeabilization buffer (eBioscience) and then pre-incubated with 1X Permeabilization buffer containing 10% normal human serum (Jackson Immunoresearch West Grove PA) for 30 minutes at room temperature. Next, cells were incubated for 60 minutes with mouse anti-Foxp3 antibody (eBio7979, eBioscience) and rabbit anti-USP7 antibody (A300-033A, Bethyl Laboratories, Montgomery TX) in 1X Permeabilization buffer containing 10% normal human serum. Cells were washed three times with 1X Permeabilization buffer and incubated for 60 minutes with donkey anti-mouse-Dylight 488 (Jackson Immunoresearch) and donkey anti-Rabbit-Dylight 549 (Jackson Immunoresearch) in 1X Permeabilization buffer containing 10% normal human serum. Cells were washed three times with 1X Permeabilization buffer and mounted in Mowiol 4-88 (Sanofi-Aventis, Paris, France) containing DAPI as previously described[42]. Cells were analyzed with a 63x objective on a Zeiss LSM 710 fluorescence microscope (Oberkochen, Germany).

Proximity ligation assay

PLA detection was performed using the Duolink II kit (Olink bioscience) according to the manufacturer's protocol. In short, induced regulatory T cells in PBS containing 2 mM EDTA and 2% FSC were enabled to adhere to poly-L-lysine-coated coverslips and subsequently incubated with Foxp3 Fixation/Permeabilization solution (eBioscience) for 45 minutes at 4°C. Samples were washed with 1X Permeabilization buffer (eBioscience) and then pre-incubated with 1X Permeabilization buffer containing 1% BSA and 10% normal human serum (Jackson Immunoresearch) for 30 minutes at room temperature. After blocking, cells were incubated for 60 minutes at room temperature with mouse anti-Foxp3 antibody (eBio7979, eBioscience) and rabbit anti-USP7 antibodyl (A300-033A, Bethyl Laboratories) in 1X Permeabilization

buffer containing 10% normal human serum. Cells were washed three times with 1X Permeabilization buffer, followed by PLA according to manufacturer's protocol. Cells were mounted in Mowiol 4-88 (Sanofi-Aventis,) containing DAPI, and subsequently analyzed with a 63x objective on a Zeiss LSM 710 fluorescence microscope within 24 hours.

TUBEs Ubiquitination assay

Ubiquitation of endogenously expressed protein was determined by use of agarose TUBE 2 (Lifesensors, Malvern PA). In short, induced regulatory T cells were treated with MG132 (20 mM) for 3 hours. Cells were then washed and lysed in TUBE cell lysis buffer (50 mM Tris-HCL pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 10% Glycerol). Lysates were sonicated (Bioruptor, Diagenode) for 3 x 30 seconds, sheered and clarified by high speed centrifugation. Supernatant was pre-cleared by incubation with prot-A-agarose for 30 minutes at 4°C. 5% total cell lysate was taken as input sample. Subsequently lysates were incubated with 15 ul TUBEs for 1 hour at 4°C while tumbling. Beads were collected by slow speed centrifugation and wash 3 times with TBS-T (20 mM Tris-HCL pH8.0, 0.15 M NaCL, 0.1% Tween-20). Polyubiquitinated proteins were eluted by resuspension in 1x SDS reducing sample buffer and analyzed by SDS-PAGE/western blotting for FOXP3 (eBio7979, eBioscience).

Isolation of active DUBs utilizing the HA-DUB probe

HA-immunoprecipitation of HA-DUB probe was performed as previously described[24]. In short, $2*10^6$ Treg were lysed by sonication in a buffer containing 1mM DTT, 250mM sucrose and 50mM Tris-HCl PH 7.4. 20 µg of protein extract was used for labeling with the probe at 37^0C for one hour. Subsequently, anti-HA coupled beads (Sigma) were added for two hours at 4°C. Beads were washed 3 times with ice-sample buffer and subjected to gel electrophoresis.

Immunoprecipitation and Western blot analysis

Transfected cells used for (co-)immunoprecipitations were lysed for 10 minutes at 4°C with Nonidet P-40 (NP-40) lysis buffer (0.5% NP-40, 50mM Tris-HCL pH 7.5, 150mM NaCl, 10mM EDTA) containing Halt protease inhibitor cocktail (1:100) Cell lysates were centrifuged for 10 minutes at 13.000 rpm at 4°C, 50µl of supernatant was used as an input control and the remaining lysate was incubated with antibody coupled beads for 2 hours at 4°C. Beads were washed 3 times with ice-cold NP-40 buffer, boiled in 1x sample buffer (60mM Tris pH 6.8, 2% SDS, 10%, glycerol, 2% β-mercaptoethanol and bromophenol blue) and subjected to gel electrophoresis

Cells were lysed in Laemmli buffer [0.12 mol/L Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 0.05µg/µl bromphenol blue, 35mmol/L ß-mercaptoethanol] and boiled for 5 minutes. Equal amounts of sample were separated by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with antibodies as detailed. Immunocomplexes were detected using enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom).

Flow cytometry

Cells were washed twice in FACS buffer (PBS containing 2% FBS) and blocked with rat serum for 15 minutes at 4°C. Subsequently, cells were incubated with antibodies for extra-cellular proteins for 30 minutes at 4°C. Cells were washed twice in FACS buffer, fixated in Cytofix/Cytoperm buffer (BD Biosciences) for 60 minutes at 4°C, and incubated with antibodies directed against intracellular proteins for 30 minutes at 4°C. Cells were analyzed utilizing FACS (BD Biosciences). FACSDiva (BD Biosciences) software was used for analysis.

***In vitro* suppression assay**

CD4 positive cells were isolated from human PBMC and CD4+CD25highCD127low nTreg were sorted and co-cultured PBMC labeled with 2µM CFSE (ratio 1:5) in anti-CD3 (clone OKT3) coated 96-wells. Cells were cultured for four days in RPMI medium supplemented

with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and $5x10^{-5}$M 2-mercaptoethanol. Proliferation of CD4 and CD8 positive cells was determined by measuring CFSE dilution using the FACS CANTO (BD Biosciences).

Generation of iTreg

CD4+CD25- were isolated from cord blood by magnetic-activated cell sorting (MACS) and cultured in RPMI 1640 supplemented 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and $5x10^{-5}$M 2-mercaptoethanol. Foxp3 expression was induced by culturing the cells for four days in combination with anti-CD3 anti-CD28 beads dynabeads, 300IU IL-2 and 10nM TGF-β.

Ubiquitin pulldown assay.

HEK293 cells were transfected with both His-tagged ubiquitin and HA-tagged Foxp3, after 48hrs the cells were treated with 20µM MG132 and 10µM H1 for 3 hours. Cells were lysed in a PH 8 urea buffer (8M Urea, 100mM NA2HPO4, 10mM TRIS PH 8.0, 0.2% TX-100, 10mM immidazole, 1 mM N-ethylmaleimide) and incubated with Ni-NTA beads for 2 hours at room temperature. Subsequently, the beads were washed twice in PH 8 urea buffer, twice in PH 6.3 urea buffer (8M Urea, 100mM NA2HPO4, 10mM TRIS PH 6.3, 0.2% TX-100, 10mM immidazole), and once in a wash buffer (20mM TRIS PH 8.0, 100mM NaCl, 20% glycerol, 1mM DTT, 10mM immidazole). The beads were boiled in sample buffer for 5 minutes, separated on SDS-PAGE and Foxp3 specific poly-ubiquitination was determined by Western blot analysis using anti-HA antibodies.

Mass spectrometric analysis

Proteins were separated utilizing 4-12% polyacrylamide gels and SDS-PAGE and stained with Biosafe. Protein bands were excised from gel, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (Roche, Almere, The Netherlands) as described[43]. Samples were subjected to nanoflow LC using C_{18} reverse phase trap columns

(Phenomenex, Utrecht, The Netherlands; column dimensions 2cm x 100µm, packed in-house) and subsequently separated on C_{18} analytical columns (column dimensions, 20cm x 50µm; packed in-house) using a linear gradient from 0 to 40% B (A = 0.1 M acetic acid; B = 95% (v/v) acetonitrile, 0.1M acetic acid) in 60min and at a constant flow rate of 150nl/min. Column eluate was directly coupled to a LTQ-Orbitrap-XL mass spectrometer (Thermo Fisher Scientific, New York, NY) operating in positive mode, using Lock spray internal calibration. Data were processed and subjected to database searches using MASCOT software (Matrixscience, London, UK) against Swiss Prot and non-redundant NCBI database, with a 10ppm mass tolerance of precursor and 0.8Da for the fragment ion.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test (Prism GraphPad Software, San Diego, CA). $p<0.05$ was considered statistically significant.

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Figure legends

Figure 1. Rapid turnover of the Foxp3 protein is mediated by poly-ubiquitination. (**a**) HEK 293 cells were transfected with HA-Foxp3 and treated with 5µg/ml Cyclohexamide (CHX) for 0, 4, 8hours (hrs). Cell lysates were blotted and analyzed using anti-HA or anti-HSP90 antibodies. (**b**) Treg were cultured in the presence of 5µg/ml CHX for 0, 2, 4, 8, 16 or 24 hrs. FOXP3 levels were determined by Western blotting using antibodies directed against Foxp3 and tubulin. (**c**) Cells were transfected with HA-Foxp3 or HA-Foxp3 K22xR in combination with HIS-Ubiquitin. Three hours prior lysis, cells were treated with 20µM MG132 and treated with 10µM of DUB inhibitor H1. Ubiquitinated proteins were pulled down using Ni-beads, immunoblotted and Foxp3 was visualized using anti-HA antibodies. (**d**) Tregs were cultured with 20µM MG132 for 3hrs, cells were lysed and ubiquitinated proteins were isolated utilizing TUBEs coupled beads. Ubiquitinated Foxp3 was visualized by Western blot using anti-Foxp3 antibodies. (**e, f**) HA-Foxp3 transfected HEK 293 cells or Treg were cultured with 5µM DUB inhibitor H1 for 8 hrs. Western blots were incubated with antibodies against, HA, Foxp3 or tubulin as indicated. Data shown are representative of at least three independent experiments, IP, immunoprecipitation. WB, Western blot.

Figure 2. Identification of multiple ubiquitination sites of Foxp3. (**a**) Immunoprecipitated HA-Foxp3 was analyzed for ubiquitinated lysines by mass-spectrometry. Peptides contining ubiquitinated lysine residues are depicted. Ubiquitinated lysine residues are underlined (**b**) Schematic representation of Foxp3 and the lysine residues that are ubiquitinated. ZF: zink finger motive, LZ: leucine zipper motive, DBD: DNA binding motive. (**c**) Cells were co-transfected with HIS-Ubiquitin and HA-Foxp3, HA-Foxp3 K22xR or HA-Foxp3 K22xR in which the arginines 249/251/263/267/393 were mutated back to lysines. Subsequently, the cells were treated with 20µM MG132 3hrs. Lysates were prepared and ubiquitinated proteins were isolated using Ni-beads, immunoblotted and analyzed using anti-HA antibodies. (**d**) Flag-Ubiquitin-Foxp3 or Flag-Ubiquitin-K7xR-Foxp3 fusion constructs (in which all seven

lysines in ubiquitin are mutated to arginines) were analyzed for ubiquitination (as in (**c**)). Data shown are representative of at least three independent experiments. WB, Western blot.

Figure 3. USP7 de-ubiquitinates Foxp3. (**a**) HEK 293 cells were transfected with HA-Foxp3, Myc-USP7, HIS-Ubiquitin and treated with 20μM MG132 for 3hrs. Ubiquitinated proteins were isolated from the cell lysate using Ni-beads, Foxp3 was visualized by Western blotting using antibodies specific for HA. (**b**) Cell lysates of HA-Foxp3 and Myc-USP7 transfected cells were immunoprecipitated using anti-HA or anti-Myc coupled beads. Immunoblots were analyzed using anti-HA or anti-Myc antibodies. (**c**) Representative confocal microscopy images of human Treg. Endogenous USP7 (red) and Foxp3 (green) was visualized utilizing specific antibodies, DAPI was used to visualize the nuclei (blue). (**d**) Foxp3-USP7 was visualized in human Treg using an *in situ* proximity ligation assay (PLA). Cells were fixed and protein-protein interactions were visualized utilizing anti-Foxp3 and anti-USP7 antibodies as described in the Methods section. Punctate staining (green) indicates a Foxp3-USP7 interactionas detected by the assay, DAPI was used to visualize the nuclei (blue). (**e**) iTreg and nTreg were lysed and incubated with a HA-DUB probe for 30 minutes. Utilizing anti-HA coupled beads active DUBs were immunoprecipitated, proteins were separated on gel and analyzed by mass spec (M.S.). Underlined amino acids represent the peptides that were identified. Data shown are representative of at least three independent experiments. IP, immunoprecipitation. WB, Western blot.

Figure 4. USP7 de-ubiquitinates Foxp3 in regulatory T cells. (**a**) HEK 293 cells were transfected with HA-Foxp3 or HA-Foxp3 K22xR and Myc-USP7. Cell lysates were quantified and analyzed by Western blotting using anti-HA antibody, and anti-HSP90. (**b**) Cells were co-transfected with HA-Foxp3 and Myc-USP7, Myc-USP14, or Flag-UCH-L3. Cell lysates were analyzed for Foxp3 expression by Western blotting utilizing anti-HA antibodies. (**c**) Cells were transfected with USP7 siRNA's as well as HA-Foxp3. Equalized protein lysates were immunoblotted and analyzed for HA. (**d**) USP7 was knocked down in iTregs using shRNA

lentivirus. Endogenous USP7 and Foxp3 levels were analyzed utilizing flow cytometry. All results are representative for at least three independent experiments. SC, Scrambled. SP, Smart Pool. IP, immunoprecipitation. WB, Western blot.

Figure 5. USP7 improves Treg function. (**a**) IL-2 promoter luciferase activity was analyzed in HEK 293 cells by co-transfecting NFAT with Foxp3 Del250 or wild-type Foxp3 with USP7. Repression of IL-2 reporter activity is depicted, all values were normalized for co-transfected renilla. (**b**) Sorted human CD4+CD25highCD127low Treg were pretreated with 10 μM DUB inhibitor H1 for 1 hour, washed three times and co-cultured with CFSE labeled PBMC in anti-CD3 coated wells for 4 days. CFSE dilution of CD4+ cells was analyzed by flow cytometry. (**c**) Analysis of Treg mediated suppression as in (**b**), USP7 knockdown in Treg was performed utilizing two different USP7 shRNA's (1), (2). A scrambled (SC) shRNA was used as control. Data shown are representative of at least three independent experiments, *p<0.05.

Figure 6. Inhibition of DUB activity abrogates Treg mediated suppression *in vivo*. (**a**) Colitis was induced by infusion of CD4+CD45RBhigh cells into immune deficient mice. Colitis severity can be abrogated by the administration of Treg. (**b**) Treg were pre-incubated with 10 μM H1 for 1 hour. Three weeks after Treg administration mice were sacrificed. Sections of the colon were analyzed and scored (five mice per group). (**c**) Representative hematoxylin and eosin stained tissue slides of the colon transcendence. (**d**) Analysis of GFP+CD4+ cells in the spleen. (**e**) Percentage of Foxp3 positive CD4+ T cells in the spleen. Mean + SEM, *p<0.05.

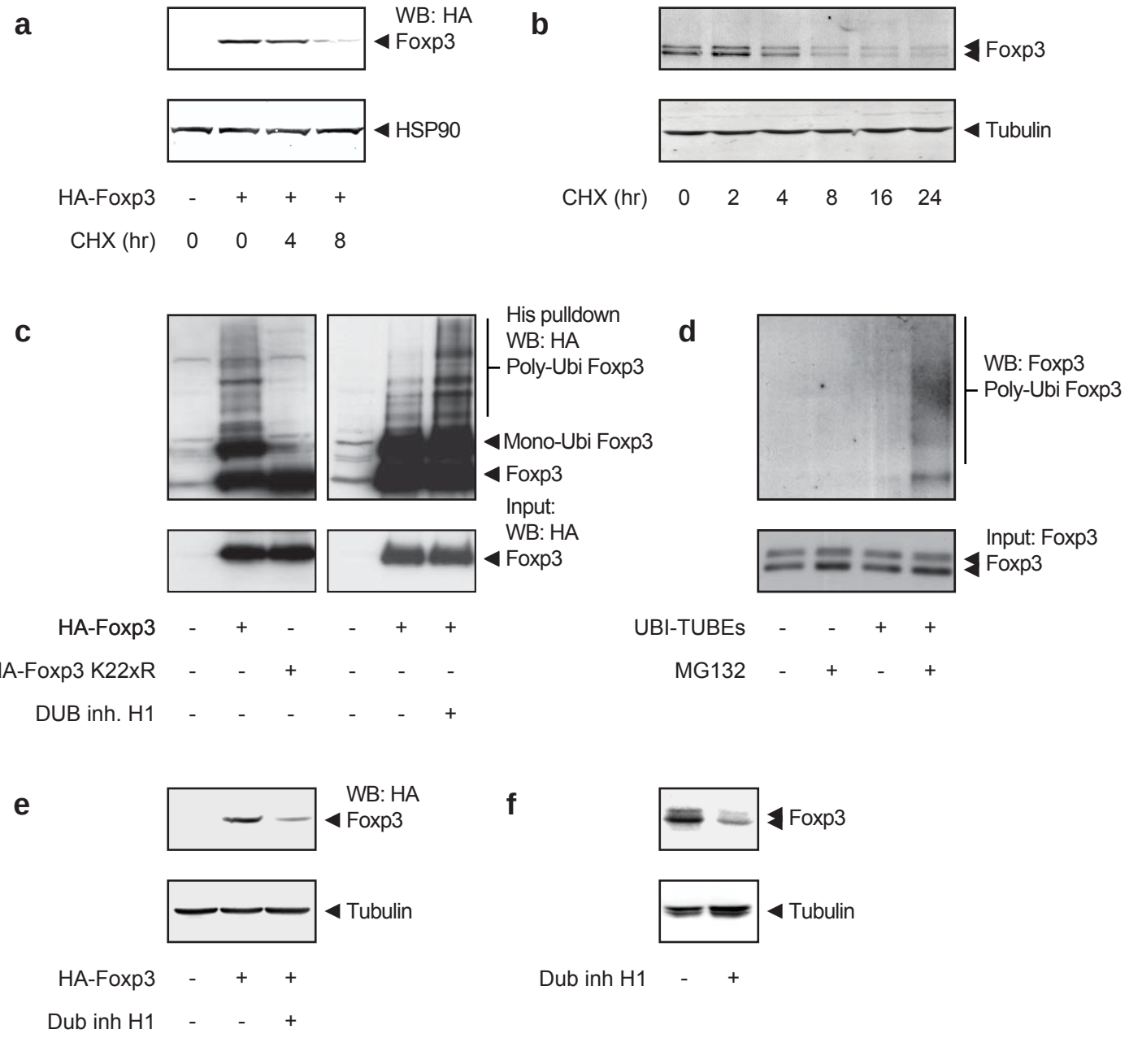
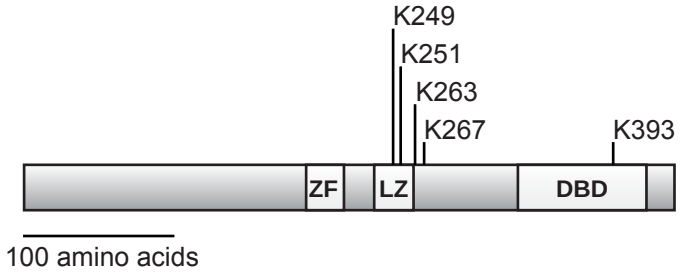


Figure 1. van Loosdregt et al.

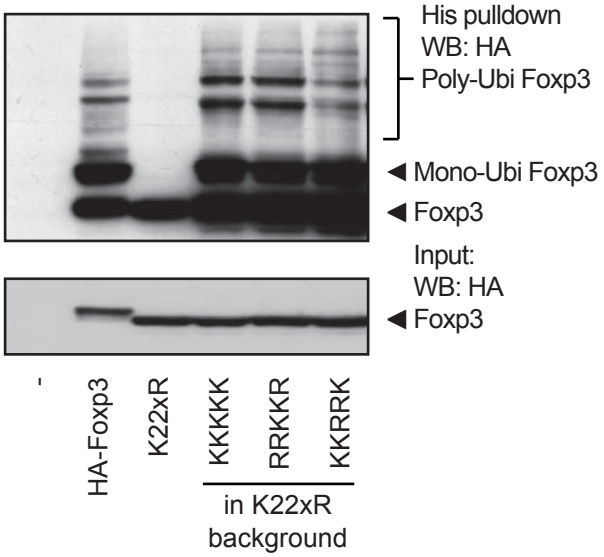
a

Peptide		Ubiquitinated Lysine
236-251	EVVQSLEQQLELE**K**EK	K249
244-262	QLELE**K**EKLGAMQAHLAG**K**	K249 K262
236-256	EVVQSLEQQLELEKE**K**LGAMQ	K251
263-276	MALA**K**APSVASMDK	K267
387-395	HNLSLH**K**CF	K393

b



c



d

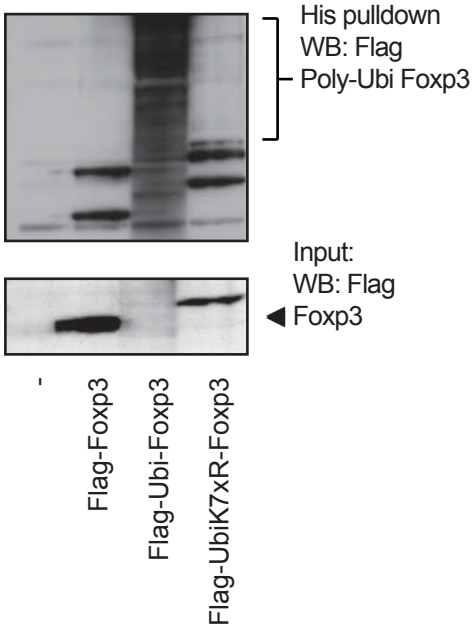
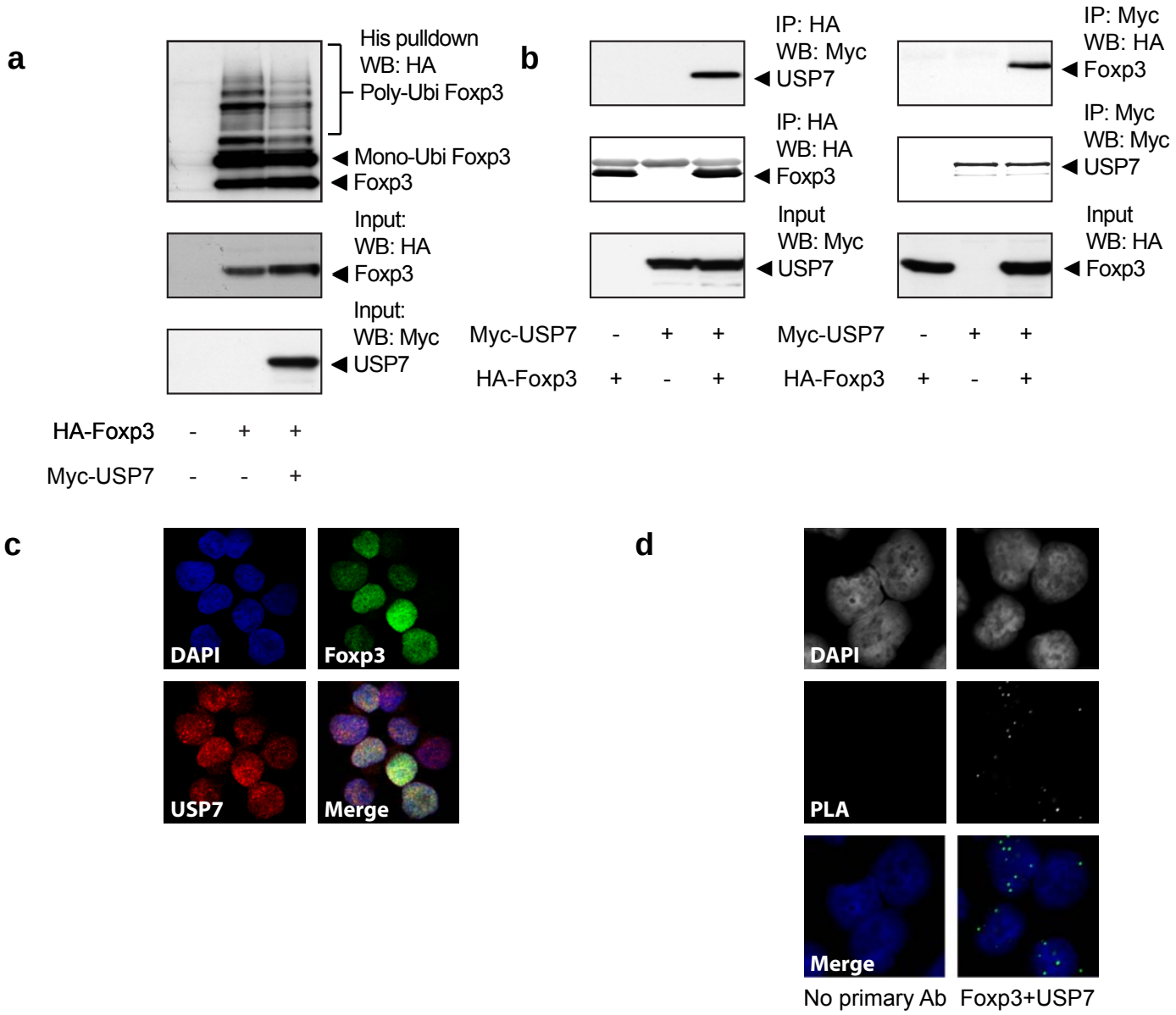


Figure 2. van Loosdregt et al.



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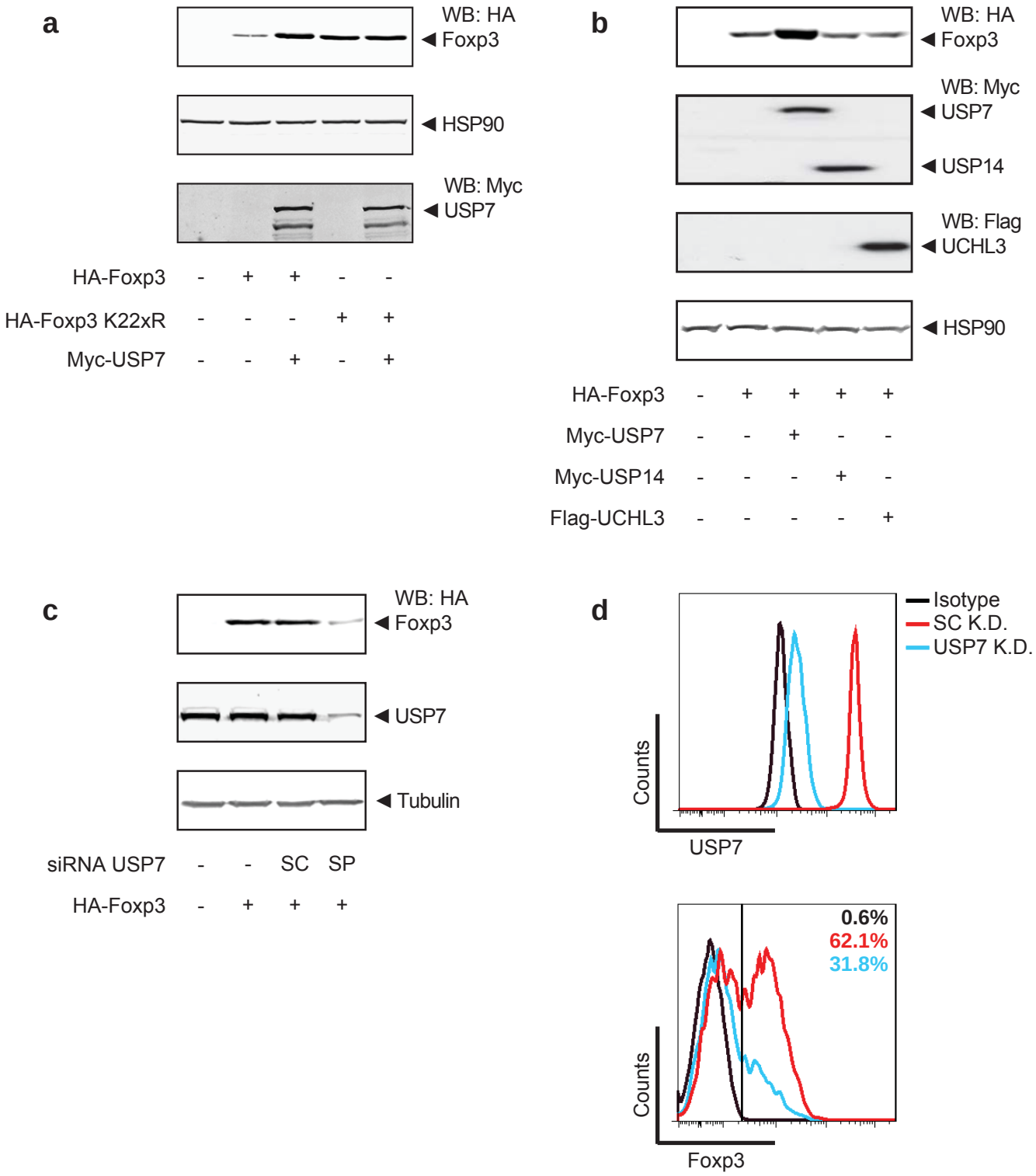

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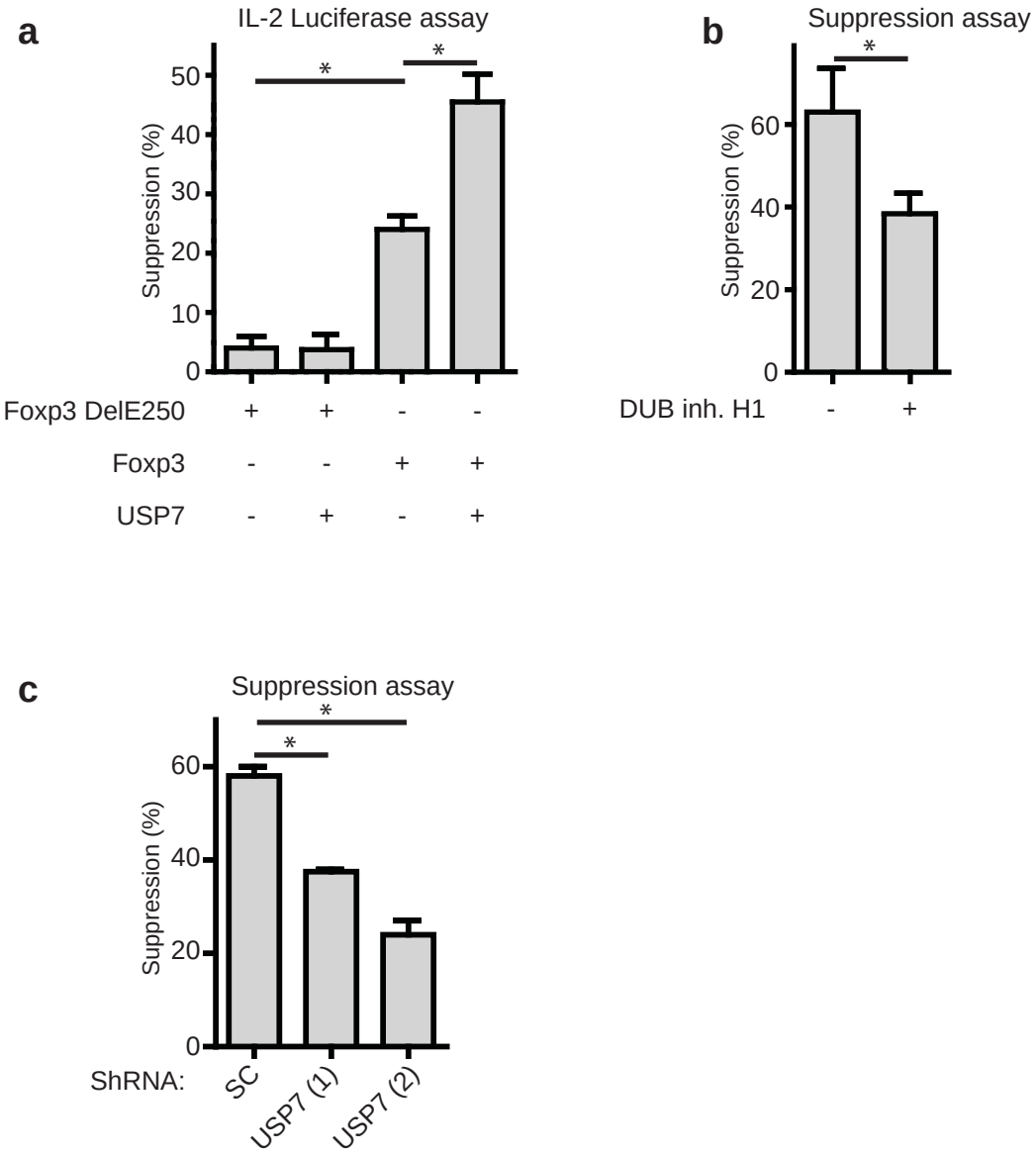

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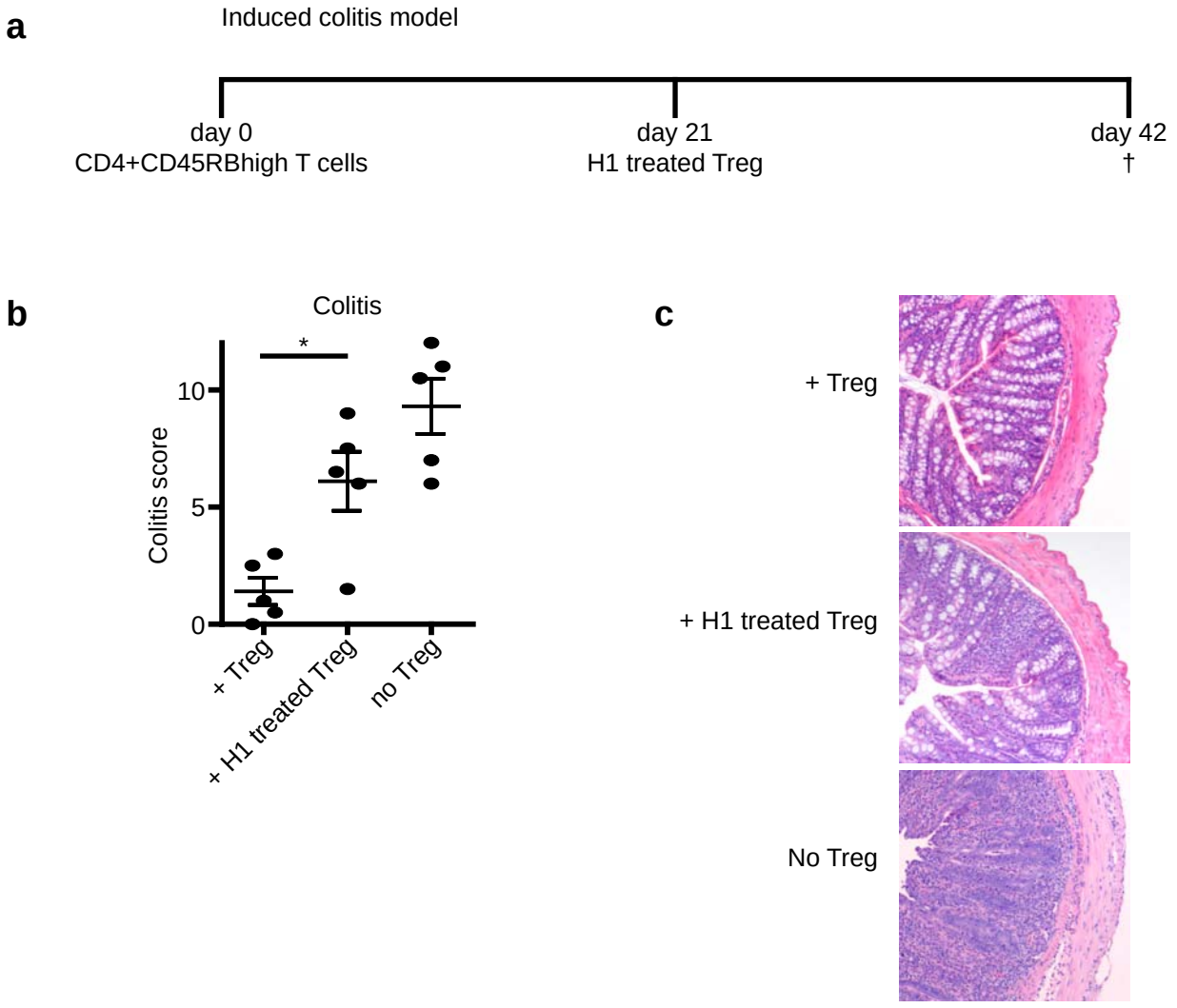

Figure 6. van Loosdregt et al.

Supplementary Figure 1. Colocalization of Foxp3 and USP7 in HEK293 cells. Representative examples of HEK293 cells that were co-transfected with only Foxp3-Mkate (red), USP7-GFP (green) and nuclei were visualized using DAPI (blue).

Supplementary Figure 2. USP7 knockdown in HEK293 cells using siRNA's. HEK293 cells were transfected with different siRNA's for USP7. Endogenous USP7 levels were analyzed by Western blotting utilizing anti-USP7 antibodies, tubulin was used as control. Scrambled (SC), Smart Pool (SP).

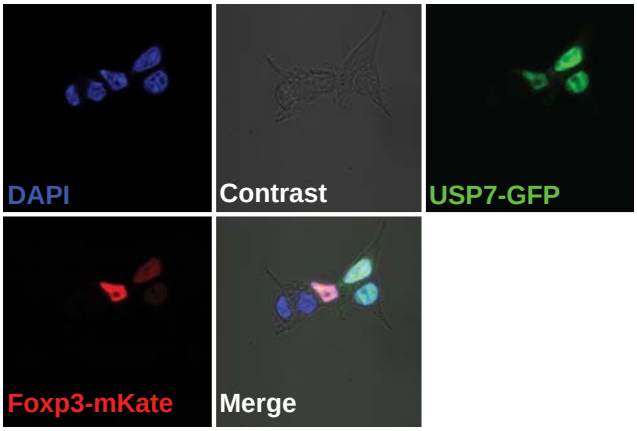

Figure S1. van Loosdregt et al.

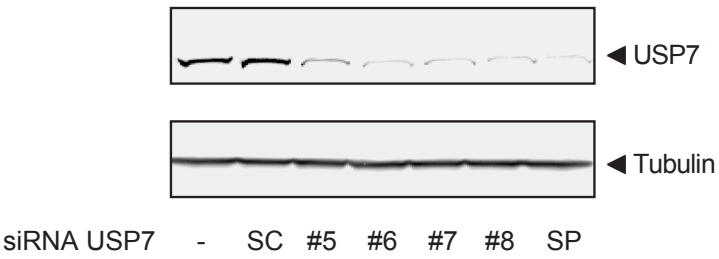

Figure S2. van Loosdregt et al.

Chapter 5

Canonical Wnt signaling negatively modulates T regulatory cell function by inhibiting Foxp3

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Submitted

Abstract

Foxp3 is crucial for both the development and function of regulatory T cells (Treg), however the post-translational mechanisms regulating Foxp3 transcriptional output remain poorly defined. Here, we demonstrate that Foxp3 associates with the Wnt-transcriptional effectors TCF1 and β-catenin. Active Wnt-signaling disrupts Foxp3 transcriptional activity, significantly reducing both human and mouse Treg-mediated suppression *in vitro*. Equally, disruption of Wnt signaling in Treg enhances their suppressive capacity. CD4+ activation increased Wnt3a production, and Wnt levels were dramatically increased in mononuclear cells isolated from synovial fluid versus peripheral blood from juvenile idiopathic arthritis patients. *In vivo* activation of Wnt signaling in murine colitis and arthritis models significantly abrogated the ability of Treg to resolve inflammation. Collectively our data demonstrate a novel post-translational mechanism whereby Wnt signaling directly regulates Treg-mediated suppression through control of Foxp3 transcriptional activity. We propose a model in which Wnt produced by activated mononuclear cells can repress Treg function allowing a productive immune response but if uncontrolled could lead to the development of autoimmunity.

Regulatory T cells (Treg) are a specific CD4+CD25+Foxp3+ T cell lineage that are crucial for self-tolerance[1, 2]. These cells suppress T cell mediated immune responses through multiple mechanisms including the production of inhibitory cytokines, direct cytolysis, metabolic disruption, and by modulation of dendritic cell (DC) and monocyte/macrophage function[3-7]. The Forkhead box transcription factor Foxp3 has been demonstrated to be uniquely required for Treg differentiation and function[1, 2]. Somatic mutations in Foxp3 result in the development of IPEX (immune dysregulation polyendocrinopathy, enteropathy, X chromosome-linked syndrome), a complex auto-immune disease caused by a lack of Treg[8, 9]. Although Foxp3+ Treg are potent suppressors of immune responses, large numbers of Foxp3+ Treg are often found at inflammatory sites in a variety of diseases including inflammatory bowel disease, diabetes type 1, multiple sclerosis, systemic lupus,and juvenile idiopathic arthritis[10-13]. These findings indicate that in many autoimmune diseases Treg function may be suppressed by the local inflammatory environment, potentially due to deregulated Foxp3 transcriptional activity.

Genome wide analysis of Foxp3-binding sites coupled to gene-expression profiling suggest that Foxp3 acts as both a transcriptional activator and repressor. It has been proposed that association of Foxp3 with additional transcription factors including NFAT, RUNX1 and AP-1 can post-transcriptionally regulate Foxp3 and therefore Treg suppressive function[14, 15]. In an unbiased screen to identify novel modulators of Foxp3 transcriptional activity we identified the transcription factor T Cell Factor 1 (TCF1) as a Foxp3 interaction-partner. TCF1 is the key transcription factor responding to canonical Wnt signaling and acts as a transcriptional activator in the presence of β-catenin[16, 17]. In the absence of β-catenin, nuclear TCF1 is associated with Groucho/TLE compressor proteins hence acting as a transcriptional repressor. Increased nuclear β-catenin levels result in competition for TCF with Groucho/TLE proteins, thereby activating transcription of Wnt target genes. β-catenin protein levels are regulated by a multimolecular destruction complex containing: axis inhibition protein (AXIN), adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β). In the absence of Wnt signaling, β-catenin is phosphorylated on multiple serine and threonine residues by CK1 and GSK3β subsequently resulting in polyubiquitination of β-

catenin, marking it for degradation by the 26S proteasome. Interaction of Wnt with FZD receptor results in inhibition of GSK3 activity, inactivating the destruction complex and rapidly increasing β-catenin protein levels[16, 18-20].

Within the immune system Wnt signaling has traditionally been studied during lymphocyte development or in the context of hematopoetic stem cell biology[16, 17, 20]. Here, we are the first to demonstrate that the Wnt-β-catenin-TCF1 module is a critical regulator of Treg function. We provide a molecular mechanism for the functional effects of Wnt signaling in Treg by demonstrating that Foxp3 directly associates with TCF1 and β-catenin, and Wnt signaling can inhibit Foxp3 transcriptional functionality. Activation of TCF1 utilizing GSK3 inhibitors or by the addition of Wnt3a significantly abrogated Treg suppressive capacity *in vitro*. In addition, Treg-mediated suppression was increased in Treg cultured in the presence of a specific Wnt production inhibitor or in $TCF1^{-/-}$ Treg. Furthermore, in two different mouse models of autoimmune disease, activation of Wnt signaling in Treg abrogated their suppressive function *in vivo*. Taken together, we demonstrate that Wnt signaling negatively regulates Treg function by modulation of Foxp3 transcriptional activity. These findings suggest that manipulation of Wnt signaling may provide a novel therapeutic strategy to control (inappropriate) immune responses.

Results

Association of Foxp3 with TCF1 and β-catenin

In an unbiased approach to globally identify transcription factors associating with Foxp3, a TF-TF array was performed utilizing an epitope-tagged Foxp3 expressing cell line according to the manufacturer's specifications (**Fig. 1a;** see Methods). Array analysis revealed that a member of the Wnt-associated transcription factor TCF/LEF family associates with Foxp3 (**Fig. 1b**). To validate this, a co-immunoprecipitation analysis was performed utilizing lysates of cells ectopically expressing TCF1 and epitope-tagged Foxp3. Foxp3 was immunoprecipitated, samples immunoblotted, and subsequently analyzed for the presence of TCF1. Foxp3 was found to associate with TCF1 (**Fig. 1c; lane 3**). To verify that this association was a protein-protein interaction and not DNA-mediated, lysates were treated with DNAse or EtBr to disrupt the protein-DNA interactions[21]. TCF1 was found to interact with Foxp3 irrespectively of DNAse/EtBr treatment. TCF transcriptional activity is regulated by association with β-catenin. To determine whether Foxp3 similarly associates with β-catenin, β-catenin was immunoprecipitated from cells ectopically expressing HA-β-catenin and Flag-Foxp3. Foxp3 was found to also associate with β-catenin and once again in a DNA-independent manner (**Fig. 1d**). To determine whether Foxp3 may influence the ability of TCF1 and β-catenin to associate, cells were transfected with β-catenin and TCF1 with or without Foxp3. β-catenin was subsequently immunoprecipitatied and association with TCF1 in the presence or absence of Foxp3 was analyzed. The β-catenin-TCF1 association was unaffected by the presence of Foxp3, indicating that Foxp3 does not disrupt this interaction (**Fig. 1e**). Taken together, these data show that Foxp3 can associate with both TCF1 and its transcriptional co-activator β-catenin.

Repression of TCF-mediated transcription by Foxp3

Since we observed that Foxp3 associates with TCF1 and β-catenin, it suggested that Foxp3 would likely modulate TCF transcriptional activity. To analyze the effect of Foxp3 on TCF transcriptional function, a TCF reporter construct was utilized containing six optimal TCF

binding sites fused to a luciferase cassette[16]. Cells were transfected with this reporter construct in combination with Foxp3, an inactive IPEX mutant (Foxp3 delE250), or the closely related transcription factors Foxp1 or Foxo3. A basal level of reporter activity was observed since HEK293 cells express endogenous TCF and β-catenin (**Fig. 2a**). Co-transfection with Foxp3 significantly decreased relative luciferase levels, while Foxp3delE250 and the other Forkhead transcription factors did not. As shown in **Figure 2b** a dose-dependent repression of TCF activity by Foxp3 was also observed. To determine whether Foxp3 could also inhibit TCF mediated transcription after β-catenin-induced activation, cells were co-transfected with the TCF luciferase reporter, β-catenin and Foxp3. Overexpression of β-catenin significantly increased reporter activity as expected (**Fig. 2c**), however Foxp3 was still capable of repressing β-catenin mediated TCF transcriptional activity. To asses if Foxp3 could also repress *bona fide* TCF targets genes, *c-myc* and *cyclin D1* promoter luciferase reporters were utilized. Cells were transfected with luciferase reporters, β-catenin and increasing amounts of Foxp3. Both the c-myc (**Fig. 2d**) and cyclin D1 (**Fig. 2e**) promoters were significantly inhibited by Foxp3 expression in a dose-dependent manner.

These data demonstrate that TCF transcriptional activity can be repressed by Foxp3 suggesting that in general TCF activity would be specifically decreased in Treg compared to CD4+ T cells. To investigate this, we analyzed mRNA expression of *AXIN2*, a TCF transcriptional target, in human CD4+ T cells. *AXIN2* expression was significantly decreased in CD4+CD25high+CD127low Treg compared to CD4+CD25- cells (**Fig. 2f**). To further validate these observations, AXIN2-LacZ reporter mice were analyzed in which β-galactosidase activity can be used as a measure of Wnt-activity. β-Galactosidase activity was determined in CD4+CD25- cells using FDG substrate, and compared to CD4+CD25high Treg. Wnt reporter activity, as measured by the percentage of Wnt responsive cells, was observed to be lower in Treg cells compared to CD4+CD25- cells (**Fig. 2g**), suggesting that Foxp3 inhibits TCF transcriptional activity in both human and mouse CD4+CD25high Treg.

Wnt signaling increases IL-2 production in T cells

To determine whether there was also a reciprocal effect of TCF on Foxp3 transcriptional activity, *IL-2* promoter activity was first analyzed since Foxp3 can directly inhibit *IL-2* transcription through promoter binding[14, 22, 23]. To investigate whether TCF could modulate IL-2 promoter activity, cells were transfected with an IL-2 promoter luciferase construct, TCF1 and β-catenin. TCF1 was found to significantly increase IL-2 promoter activity, and this was further increased by co-expressing β-catenin (**Fig. 3a**). To further study the effect of TCF activation on *IL-2* transcription, three chemically distinct GSK3 inhibitors were used to increase β-catenin levels, thereby activating endogenous TCF. All three inhibitors significantly increased IL-2 promoter activity to a similar degree, while the MeBIO negative control, had no effect (**Fig. 3b**). To determine whether Foxp3 was also able to suppress IL-2 promoter activity under conditions where TCF is activated by β-catenin, cells were co-transfected with IL-2 promoter reporter, β-catenin and Foxp3. Again, Foxp3 was also able to suppress IL-2 promoter activity after activation by β-catenin (**Fig. 3c**). To verify that TCF activation could increase IL-2 production in human T cells, freshly isolated peripheral blood mononuclear cells (PBMC) were cultured in the presence of LiCl to activate Wnt signaling, and IL-2 production was measured using multiplex technology. Treatment with LiCl significantly increased IL-2 levels in the supernatant (**Fig. 3d**). Taken together, these data show that Wnt signaling can induce *IL-2* transcription, increasing IL-2 production in PBMC.

Active Wnt signaling abrogates the suppressive capacity of Treg

Since we observed that TCF1 and Foxp3 can reciprocally regulate multiple transcriptional targets, we further assessed the role of Wnt signaling on Treg function. We first confirmed that components of the Wnt signaling cascade (TCF1, β-catenin, FZD receptors) were expressed in primary human Treg (**Supplementary Fig. 2**). To validate these data on the protein level, human Treg were lysed and TCF1, β-catenin and Foxp3 levels were determined by Western blotting using specific antibodies. TCF1, β-catenin and Foxp3 were all expressed in Treg and treatment with BIO stabilized β-catenin protein levels as previously described (**Fig. 4a**)[16, 18, 19]. To analyze Treg function, *in vitro* suppression assays were

performed by culturing sorted human Treg together with CFSE labeled PBMC in anti-CD3 coated wells for four days. Wnt signaling was again induced by incubation with three chemically distinct GSK3 inhibitors. All GSK3 inhibitors significantly impaired Treg suppressive function (**Fig. 4b**), suggesting that Wnt signaling in Treg indeed abrogates their suppressive capacity. Importantly, there was no direct effect of these inhibitors on PBMC proliferation (**Supplementary Fig. 3a**). To confirm that the observed results were indeed Treg specific the experiment was repeated, but pre-incubating Treg with GSK3 inhibitors for one hour. After pre-incubation, Treg were washed and co-cultured with the PBMC in absence of the inhibitors. Pre-treatment with GSK3 inhibitors, similarly abrogated Treg mediated suppression, confirming our previous observations (**Fig. 4c**). To further investigate the role of the Wnt signaling pathway in Treg mediated suppression, TCF1 null mutant mice were utilized. As expected, the number of peripheral CD4+ T cells in $TCF1^{-/-}$ mice was significantly reduced (**Supplementary Fig. 4a**)[16, 17]. However, the percentage of Treg was similar in $TCF1^{-/-}$ mice compared to control mice (**Fig. 4d; Supplementary Fig. 4b**). To investigate whether TCF1 may modulate Treg mediated suppression, Treg from wild-type and $TCF1^{-/-}$ mice were co-cultured with CFSE labeled wild-type CD4+ T cells and proliferation of the CFSE labeled cells was analyzed. Treg mediated suppression was reproducibly increased in $TCF1^{-/-}$ Treg compared to wild-type (**Fig. 4e**), indicating that the absence of TCF1 positively influences Treg mediated suppression but not Treg numbers. Taken together, these data indicate that active Wnt signaling can impair Treg mediated suppression.

Wnt proteins inhibit Treg function and are produced by activated PBMC

While previous experiments addressed the effect of directly activating TCF through modulation of β-catenin levels, we wished to determine the effect of direct addition of Wnt protein to Treg. To study the effect of Wnt proteins on Treg function purified Wnt3a or IWP-2, a specific inhibitor of Wnt production, were added during culture[24]. Addition of Wnt3a resulted in significantly impaired Treg mediated suppression of T cell proliferation (**Fig. 5a**). In contrast, IWP-2 mediated inhibition of Wnt production resulted in significantly increased Treg

suppressive capacity. Importantly, treatment with Wnt3a or IWP-2 did not directly affect PBMC proliferation (**Supplementary Fig. 3b**). To again confirm that these results were a direct effect on Treg, cells were pretreated with Wnt3a or IWP-2 for one hour, and after washing, Treg were cultured with the CFSE labeled PBMC for four days. Wnt3a pre-treatment inhibited Treg mediated suppression of PBMC proliferation, validating our previous observations (**Fig. 5b**). However, IWP-2 pre-treatment did not increase Treg mediated suppression, as was observed when present during the entirety of the suppression assay. Since IWP-2 inhibits Wnt production, this result implies that the source of Wnt proteins are PBMC, thereby resulting in inhibition of Treg mediated suppression. Since Wnt3a significantly abrogated Treg function, Wnt3a mRNA levels were analyzed in human CD4 T cells by qRT-PCR. We observed expression of Wnt3a in CD4+ T cells, and T cell activation utilizing anti-CD3 and anti-CD28 resulted in a significantly increased expression of Wnt3a mRNA (**Fig. 5c**). Treg are known to be present in high numbers in the joints of arthritis patients, however, they are unable to control local inflammation[10-13, 25]. We hypothesized that, in agreement with our *in vitro* data, PBMC at the inflammatory site may produce Wnt3a or related Wnt proteins, thereby inhibiting Treg function. PBMC as well as synovial fluid mononuclear cells (SFMC) were isolated from five juvenile idiopathic arthritis patients and Wnt3a mRNA was analyzed. We observed a dramatic increase of Wnt3a mRNA in SFMC compared to patient-matched PBMC (**Fig. 5d**). In conclusion, these data show that Wnt can directly modulate Treg function *in vitro* and that Wnt3a production is increased after activation of mononuclear cells both *in vitro* and *in vivo*.

Activation of Wnt signaling abrogates Treg mediated suppression *in vivo*

The data presented above demonstrate that Wnt signaling can directly modulate Treg mediated suppression. To assess the role of the canonical Wnt signaling in modulating Treg functionality *in vivo*, a proteoglycan induced arthritis (PGIA) model was first utilized[26, 27]. This autoimmune model of chronic arthritis is initiated by two injections of human PG in the adjuvant dioctadecyl ammonium bromide (DDA) in BALB/c mice. Importantly, this model is

Treg dependent since depletion of Treg results in significantly increased arthritis scores [27]. Arthritis was induced at day 0 and 21 and arthritis scores were analyzed three times per week by a visual scoring system of redness, swelling, and deformities of the paws[26]. To mimic Wnt signaling and activate TCF, mice were subjected to intra-peritoneal (I.P.) injections with low BIO (2mg/kg 3 times/week), high BIO (6mg/kg 2 times/week) or MeBIO (6mg/kg 2 times/week) as control from day 21 (**Fig. 6a**). In these experiments relatively young (16 week) mice were used which do normally not develop arthritis after two PG injections. As expected no mice developed arthritis in the control group (MeBIO). However, arthritis was observed in five out of six mice that received high BIO (**Fig. 6b, c**). Treatment with low BIO resulted in arthritis in three out of six mice, demonstrating a dose-dependent effect of BIO on disease scores. Mice that did not receive PG, but were treated with high BIO did not develop arthritis, demonstrating that arthritis was not induced by BIO-treatment itself (data not shown). 15 days after the second PG injection mice were sacrificed and the percentage of Foxp3 positive CD4+ cells was determined in both spleen and mesenteric lymph nodes (MLN). No significant differences were observed in Foxp3+ percentages or MFI, demonstrating that BIO treatment does not modulate Foxp3 expression levels (**Supplementary Fig. 5a, b**). To confirm that the increased arthritis scores in BIO treated mice are the result of impaired Treg mediated suppression an ex-*vivo* suppression assay was performed. Mice were sacrificed eighteen hours after I.P. injection with BIO or MeBIO. Equal numbers of Treg were isolated from the spleen and Treg mediated suppression of untreated CD4+ cells was analyzed. The suppressive capacity of Treg from BIO treated mice was significantly impaired compared to control treated mice, indicating that systemic activation of Wnt signaling abrogates Treg function (**Fig. 6d**).

To further investigate the relevance of Wnt signaling in modulating Treg functionality *in vivo*, an established mouse colitis model was next utilized[28, 29]. In this model, colitis is induced by the infusion of CD4+CD45RB+ T cells into immune deficient Rag1 $^{-/-}$ mice, and disease severity is diminished by adoptive transfer of Treg. Here, sorted Treg from Foxp3-GFP mice that were pre-treated with BIO (to activate TCF) or MeBIO were used. Three weeks after

Treg infusion the mice were sacrificed and colitis severity was scored by analyzing colon histology (**Fig. 6e**). Substantial colitis scores, indicated by severe distension of the colonic mucosa, were observed in mice that did not receive Treg, and infusion with control (MeBIO) treated Treg dramatically decreased colitis scores (**Fig. 6f, g)**. Importantly Treg pretreated with BIO, did not exhibit reduced colitis scores indicating that TCF activation abrogated Treg function *in vivo*. The number of GFP positive T cells in the spleen of mice with collitis were similar in all groups (**Supplementary Fig. 6**), indicating that treatment with BIO affected Treg function but not their numbers. Taken together, these data demonstrate that increased Wnt signaling abrogates Treg function *in vivo*.

Discussion

While Wnt signaling has traditionally been studied in the context of thymocyte development and stem cell biology[16, 17, 20], here we demonstrate an important functional role for canonical Wnt signaling in the regulation of Treg mediated suppression. We show that Foxp3 directly interacts with TCF1 and that their transcriptional targets can be reciprocally regulated (**Fig. 2, 3**). Activation of Wnt signaling in regulatory T cells, by addition of three chemically distinct GSK3 inhibitors, or treatment with Wnt3a, resulted in significantly impaired Treg function, while culturing Treg in the presence of a Wnt production inhibitor increased Treg function (**Fig. 4, 5**). In two different autoimmune mice models, TCF activation in Treg significantly increased disease scores, indicating that Wnt signaling can control Treg function both *in vitro* and *in vivo* (**Fig. 6**). Our data provide novel insights into the post-translational mechanisms regulating Foxp3 activity and provide a model by which Treg suppressor function can be rapidly and temporally modulated.

The role of Wnt proteins in regulating peripheral T cell function remains poorly understood and has not been extensively investigated. Several recent studies have demonstrated that the Wnt pathway can modulate CD8+ T cell responses. While TCF1 deficiency was shown to limit proliferation of CD8+ effector T cells and impair differentiation toward a central memory phenotype[30-32]. Ablation of β-catenin in mouse intestinal dendritic cells impaired their ability to induce Treg[33]. Furthermore, it has been reported that Treg over-expressing a stabilized β-catenin mutant through retroviral transduction resulted in increased survival[34]. However, continuous high levels of β-catenin are not favorable, as it has been shown that expression of stabilized β-catenin in thymocytes can result in the development of thymic lymphoma's[35]. Interpretation of experiments utilizing over-expression of stabilized β-catenin mutants are therefore difficult to interpret. Here, we are the first to show that the Wnt signaling directly modulates Foxp3 activity and thereby Treg function.

The role of Treg in autoimmunity has been extensively studied and while IPEX is the result of severely reduced number of Treg[8, 9], the observation that in most autoimmune diseases the number of Treg is normal or even increased, suggests that here these Treg are in some way

dysfunctional[36-38]. Treg suppressive capacity is severely impaired in a variety of autoimmune diseases including psoriasis, multiple sclerosis, diabetes type 1, systemic lupus, and rheumatoid arthritis[10-13, 25]. While there are potentially multiple mechanisms by which Treg suppressive capacity could be impaired, here we demonstrate that Wnt3a production could provide a possible explanation. The suppressive capacity of Treg was increased when PBMC were cultured in the presence of the Wnt production inhibitor IWP-2 (**Fig. 5a**) suggesting that PMBC can produce Wnt proteins thereby inhibiting Treg function. In support of this, we established that CD4+ T cells indeed produce Wnt3a, which was significantly increased after CD3/CD28-mediated activation (**Fig. 5c**). We also demonstrate that mononuclear cells in the synovial fluid of arthritis patients produce almost ten times more Wnt3a than PBMC from the same donor (**Fig. 5d**). Taken together, we propose that dysregulated Wnt production in the synovium inhibits Treg function, leading to an uncontrolled immune response and inflammation, as observed in juvenile idiopathic arthritis.

We demonstrate that activation of Wnt signaling in Treg utilizing LiCl significantly abrogates their suppressive capacity (**Fig. 4**). Several studies have reported that lithium intake is strongly correlated with an increased predisposition for autoimmunity. Lithium salts are used for the treatment of manic depression, and case studies reporting the onset of psoriasis after treatment with lithium go as far back as 1972. The incidence of psoriasis secondary to lithium treatment has been reported to be from 1.8% to 6%[39, 40]. In addition to psoriasis, there are multiple reports of onset of autoimmune thyroid disease as a result of lithium treatment[41, 42]. Furthermore, rats immunized with rat thyroglobulin and treated with lithium showed significantly increased anti-thyroglobulin levels compared to untreated littermates[43]. Since we have demonstrated that LiCl treatment inhibits Treg mediated suppression in multiple models, it is possible that increased autoimmunity phenotype observed in lithium-treated patients are caused by abrogated Treg function.

T cell activation and proliferation is necessary to clear infections, however, at the site of inflammation activated T cells have been reported to directly interact with Treg[44-46]. Since extensive or prolonged Treg activity would be detrimental for the resolution of infection, we

propose a model in which Wnt production by mononuclear cells could modulate the strength of the immune response by two means. Firstly, Wnt-mediated activation of FZD receptors expressed on Treg could dampen Treg mediated immune suppression through inhibition of Foxp3 transcriptional output. Secondly, increased Wnt production by mononuclear cells could result in increased TCF-mediated IL-2 production in T cells, resulting in both an autocrine and paracrine stimulation of T cell differentiation, growth, proliferation and survival[46, 47]. Taken together, we have established that canonical Wnt signaling regulates T cell mediated immune activation by both regulating IL-2 transcription and Treg function. Our findings support the notion that modulation of Wnt signaling could provide an important novel therapeutic target for manipulation of (inappropriate) immune responses.

Methods

Antibodies, DNA constructs and reagents

The following anti-human antibodies were used: mouse anti-Foxp3 clone PCH101 for FACS analysis (eBioscience, San Diego, CA), mouse anti-hemaglutinin (HA) clone 12CA5 from Santa Cruz, mouse anti-Flag (Sigma Aldrich, Zwijndrecht, The Netherlands) mouse anti-β-catenin (BD bioscienses, Breda, the Netherlands), rabbit anti-β-catenin (Cell signaling technology, Boston, MA), rabbit anti-TCF1 (Cell signaling), anti-CD4 (BIO-legend, San Diego, CA), anti-CD25, anti-CD127, anti-CD8 (all BD biosciences) Antibodies raised against mouse: anti-TCR, anti-CD4, anti-CD25, anti-CD8 (all BD bioscienses). HA-Foxp3 was cloned from MIGR1-Foxp3 (kindly provided by S. Sakaguchi[48]) into pMT2 containing a HA-tag generating pMT2-HA-Foxp3. pMT2-HA-Foxp3ΔE250 mutant was generated using site-directed mutagenesis, Flag-Foxp3 was described earlier[49], pcDNA3 (Invitrogen Carlsbad,CA), pRSV-NFATC/A, Foxo3 (both kindly provided by B. Burgering[50, 51], 6xTCF luc and TCF1 (kindly provided by M. van de Wetering[16, 51]). IL-2 promoter luciferase (Panomics, Fremont, CA). Foxp1 (kindly provided by A. Banham[52] has been previously described. LiCl (Sigma), SB 216763 (Sigma), BIO (Calbiochem), MeBIO (Calbiochem), IWP-2 (Sigma). Recombinant Wnt3a protein was produced in Drosophila S2 cells and purified using blue sepharose affinity and gel filtration chromatography as described[24].

TF-TF interaction array

HEK293 cells were transfected with HA-Foxp3 or empty vector. Forty-eight hours after transfection nuclear extract were prepared and the array was screened according to the manufacturer's protocol (Panomics). Immunoprecipitation was performed using anti-HA coupled beads (Sigma).

Cell culture and luciferase assays

HEK293 cells were maintained in DMEM (Invitrogen) supplemented with 8% heat-inactivated FCS, penicillin and streptomycin (Invitrogen) at 37°C and 5% CO_2. Cells were grown to 50%

confluence in six wells-plates (Nunc, Roskilde, Denmark) and transfected with a mixture of 1.5 µg DNA and 7.5 µl PEI overnight. Cell lysates were prepared for Western blot analysis. For the luciferase assay cells were transfected with 1.0 µg IL-2 promoter luciferase reporter with 0.5 µg of pMT2-Foxp3, pcDNA3-NFATC/A or pcDNA3 and 7 µg pMT2 empty vector and 0.05 µg pRLTK renilla, (Promega, Leiden, the Netherlands) to normalize for transfection efficiency. Cells were transfected in a six-well plate, three days after transfection the cells were washed twice with PBS and lysed in 50 µl passive lysis buffer for 15 minutes, insoluble cell debris was spun down and the supernatant fraction was assayed for luciferase activity using Dual-Luciferase Reporter Assay System (Promega, Leiden, The Netherlands).

Immunoprecipitation and Western blot analysis

Cells were lysed in a NP40 lysis buffer (0.05 M Tris-HCl pH7.5, 0.5% Nonidet P40, 0.15 M NaCl, 0.01 M EDTA), immunoprecipitation was performed utilizing anti-Flag coupled beads (Sigma). Beads were washed 3x in lysis buffer, boiled and samples were separated by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and hybridized with antibodies as indicated. Immunocomplexes were detected using enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom).

***In vitro* suppression assays**

Human

CD4+CD25highCD127low Treg were sorted from human PBMC and co-cultured with PBMC labeled with 2 µM CFSE (ratio 1:5) in anti-CD3 (clone OKT3) coated 96-wells. Cells were cultured for four days in RPMI medium supplemented with 10% FCS 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 x 10^{-5} M 2-β mercaptoethanol. Proliferation of CD4 positive cells was determined by measuring CFSE dilution using the FACS CANTO (BD Biosciences).

Mouse

CD4 T cells were isolated by MACS from mice spleen, $CD4^+CD25^+$ sorted cells were added to CFSE labeled splenocytes at a ratio of 1:5. Cells were cultured in IMDM supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 x 10^{-5} M 2-mercaptoethanol in the presence of 1 µg/ml soluble anti-CD3 (clone 145-2C11; BD Pharmingen) for four days. To determine suppression of proliferation the CFSE dilution within the CD4 T cell population was analyzed using by FACS

Quantitative PCR

mRNA was isolated using the trizol according to the manufacturer's protocol (Invitrogen), cDNA synthesis was performed using IScript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA samples were amplified using SYBR green supermix (BIO-Rad), in a MyiQ single-color real time PCR detection system (Bio-Rad) according to the manufacturer's protocol. Primers are described in **Supplementary Figure 1.** To quantify the data, the comparative Ct method was used[53]. Relative quantity was defined as $2^{-\Delta\Delta Ct}$ and β2-microglobulin was used as reference gene.

Transgenic mice

Conductin(AXIN2)-LacZ mice were generated and kindly provided by B. Jerchow and W. Birchmeier[54]. C57Bl/6 Tcf1-/- mice were originally described by Verbeek[17]. All mice were kept in the specified pathogen-free (SPF) breeding section of the Leiden University Medical Center, Leiden, The Netherlands.

Quantification of β-galactosidase activity

Intracellular β-galactosidase activity was measured by incubating cells with 2mM Fluorescein Di-β-D-galactopyranoside (FDG) substrate (Molecular Probes). FDG was loaded into the cells by hypotonic shock at 37°C for 1 minute, prior to cell surface antibody staining[55]. β-galactosidase reaction was stopped with 1mM phenylehtyl β-D-thiogalactopyranoside (PETG, from Molecular Probes).

Proteoglycan induced arthritis mouse model

Proteoglycan (PG) was purified from human articular cartilage and removed during knee joint replacement surgery by 4M guanidinium chloride extraction, and the human GAG side chains were depleted by digestion with chondroitinase ABC. Arthritis was induced in BALB/c mice by two intraperitoneal injections of 0.4 mg human deglycosylated PG in 2 mg of the synthetic adjuvant dimethyl dioctadecyl ammonium bromide (DDA; Sigma Aldrich, Zwijndrecht, The Netherlands) on days 0 and 21. Mice were intra-peritoneum injected with low BIO (2 mg/kg 3 times/week), high BIO (75 mg/kg 2 times/week) or MeBIO (6 mg/kg 2 times/week I.P.) as control from day 21. The onset and severity of arthritis were assessed three times a week in a blinded fashion by a visual scoring system as described previously[27]. In brief, the degree of joint swelling, redness, and deformation of each paw (scored from 0-4) was determined to express a total arthritis score. The mice were kept in the animal facility of the Utrecht University under regular conditions. The experiments were approved by the Animal Experiment Committee of the Faculty of Veterinary Medicine (Utrecht University).

Induced colitis mouse model

Rag1-/- mice were kept in the animal facility of the Utrecht University under specific pathogen free conditions. The experiments were approved by the Animal Experiment Committee of the Faculty of Veterinary Medicine (Utrecht University). Immunodeficient mice were injected with $3x10^5$ CD4+CD45high cells to induce colitis. After 21 days, $3x10^5$ Treg isolated from Foxp3-GFP mice were pretreated with 10 µM BIO or MeBIO for 1 hour, washed, and intra-vascular injected. Three weeks later the mice were sacrificed and colons were fixed in formalin for 24 hrs, embedded in paraffin, cut and stained by Hematoxylin and Eosin. Scoring was performed according to Berg et al.[56], in short: Grade 0: no infiltration of mononucleated cells. Grade 1: few foci of mononucleated cells, only slight depletion of goblet cells. Grade 2: many foci of mononucleated cells, infiltration in the lamina propria, however, not yet in the submucosa; diminished numbers of goblet cells. Grade 3: strong infiltration, also in the

submucosa; epithelial hyperplasia; number of goblet cells strongly diminished. Grade 4: transmural infiltration of mononucleated cells; strong epithelial hyperplasia, strong goblet cell depletion. Each segment of the colon (Ascending, Transverse, Descending) was given an individual score and the sum of these scores gave the overall histological score for each mouse. The percentage of GFP positive cells in the spleen and Mesenteric lymph nodes (MLN) were analyzed by FACS. The mice were kept in the animal facility of the Utrecht University under regular conditions. The experiments were approved by the Animal Experiment Committee of the Faculty of Veterinary Medicine (Utrecht University).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test (Prism GraphPad Software, San Diego, CA). $p<0.05$ was considered statistically significant.

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Figure legends

Figure 1. Foxp3 associates with TCF1 and β-catenin. (**a**) Panomics TF-TF interaction array was used to identify new Foxp3 interacting transcription factors. (**b**) HA-Foxp3 was immunoprecipitated from HA-Foxp3 transfected HEK293 cell lysate. Specific biotin-labeled transcription factor binding sites were added, washed, boiled and hybridized on the array. Bound oligo's were visualized using streptavidin antibodies. (**c**) HEK 293 cells were co-transfected with Flag-Foxp3 and TCF1. Lysates were treated with 100 U/ml DNase or 25 µg/ml EtBr for 20 min, Foxp3 was immunoprecipitated and Western-blots were analyzed using anti-TCF1 or anti-HA antibodies. (**d**) Association of Flag-Foxp3 and HA-β-catenin was analyzed as in (**c**). (**e**) HEK 293 cells were co-transfected with HA-β-catenin, TCF1 and Flag-Foxp3. Cells were lysed and an immunoprecipitation using HA-beads was performed, TCF1 was visualized using anti-TCF1 antibodies. Results depicted are representative of at least 3 independent experiments. IP, immunoprecipitation. WB, Western blot.

Figure 2. Foxp3 inhibits TCF transcriptional activity. (**a**) HEK 293 cells were co-transfected with TCF-luciferase reporter, renilla and Forkhead transcription factors and luciferase-reporter activity analyzed. (**b**) TCF-reporter activity was analyzed with increasing amounts of Foxp3. (**c**) TCF-reporter activity was analyzed in HEK293 cells transfected with Foxp3 in combination with β-catenin. (**d**) Increasing amounts of Foxp3 were co-transfected with β-catenin, renilla and a c-myc promoter luciferase reporter construct. (**e**) CyclinD1 promoter reporter activity was analyzed with increasing amounts of Foxp3. In all experiments luciferase and renilla activity was analyzed and values were normalized for co-transfected renilla. (**f**) CD4+ or CD4+CD25highCD127low (Treg) cells were sorted from human PBMC. qRT-PCR was used to determine *AXIN2* mRNA levels, results were corrected for β2M. (**g**) Splenocytes of AXIN2-LacZ mice were stained for CD4, CD25 and Wnt-reporter activity was determined by β-galactosidase. Splenocytes were gated on their CD4 expression and high expression of CD25 (Treg) or absence of CD25 positivity. Results depicted are the mean + SEM of at least three independent experiments.

Figure 3. TCF activation inhibits IL-2 promoter activity. (**a**) HEK 293 cells were transfected with a IL-2 promoter luciferase reporter construct, NFAT, and renilla. Cells were co-tranfected with β-catenin and TCF1 and luciferase activity measured. (**b**) IL-2 promoter activity was analyzed as in (a), cells were treated with GSK3 inhibitors to activate TCF; 10mM LiCl, 10μM SB 216763, 200nM BIO and 200nM MeBIO as control. (**c**) IL-2 promoter activity was analyzed in cells co-transfected with β-catenin and Foxp3. (**d**) Freshly isolated PBMC were cultured for 2 days in the presence of 1.5μg/ml plate bound anti-CD3 and 0.01μg/ml soluble anti-CD28 with or without 10mM LiCl. Supernatants were analyzed for IL-2 levels using multiplex technology. All results depicted are the means + SEM of at least three independent experiments.

Figure 4. Wnt signaling regulates regulatory T cell function *in vitro*. (**a**) Human Treg were treated with 2μM BIO or MeBIO for 4 hours, and subsequently lysed. TCF1, β-catenin and Foxp3 levels were visualized by Western-blotting utilizing specific antibodies. (**b**,**c**) Human T cell suppression assays were performed to analyze Treg mediated suppression. Sorted CD4+CD25highCD127low Treg were co-cultured with CFSE-labeled PBMC in anti-CD3 coated wells for four days. CFSE dilution of CD4+ cells was analyzed using FACS. (**b**) Suppression by Treg in the presence of TCF activators LiCl (5mM), SB216763 (10μM), BIO (100nM) or control MeBIO (100nM) was analyzed. (**c**) Sorted Treg were pre-incubated with 10mM LiCl, 40μM SB216763, 10μM BIO or 10μM MeBIO for one hour, washed and functionality analyzed by suppression assay. (**d**) CD4 positive splenocytes from both wild-type and $TCF1^{-/-}$ mice were analyzed for CD25 expression. (**e**) *In vitro* T cell suppression assays were performed to analyze Treg functionality. Sorted CD4+CD25high Treg from wild-type or $TCF1^{-/-}$ mice were co-cultured with CFSE-labeled wild-type splenocytes in the presence of soluble anti-CD3 wells for four days. CFSE dilution of CD4+ cells was determined by FACS analysis. All results depicted are the means + SEM of at least three independent experiments.

Figure 5. Increased Wnt production by activated PBMC. Human T cell suppression assays were performed to analyze Treg-mediated suppression. Sorted CD4+CD25highCD127low Treg were co-cultured with CFSE-labeled PBMC in anti-CD3 coated wells for four days. CFSE dilution of CD4+ cells was analyzed using FACS. (**a**) Suppression of Treg in the presence of Wnt3a (400ng/m) or Wnt production inhibitor IWP-2 (2μM). (**b**) Treg were pre-treated with Wnt3a (800ng/ml) or IWP-2 (4 μM) for one hour. Cells were washed and used for suppression assay. (**c**) Wnt3a mRNA levels were analyzed by qRT-PCR from CD4+ T cells that were sorted from human PBMC and cultured overnight in the absence or presence of anti-CD3 and anti-CD28 coated beads. (**d**) Wnt3a mRNA levels were determined in PBMC or SFMC from five different juvenile ideopathic arthritis (JIA) patients. Mean + SEM, all experiments were performed in triplicate and for at least three independent donors.

Figure 6. Activating TCF *in vivo* abrogates Treg function. (**a**) Arthritis was induced by two injections of PG in DDA on day 0 and day 21. Mice were treated with 6mg/kg BIO or MeBIO twice a week or 2mg/kg BIO three times a week and disease was scored (six mice/group) as described in the Methods section. (**b**) Mice with an arthritis score of at least 2 are depicted in time. (**c**) Mean arthritis scores are shown. (**d**) Mice I.P. injected with 6mg/kg BIO or MeBIO were sacrificed 18 hours after injection. Treg isolated from the spleen were co-cultured with CFSE labelled CD4+ cells in the presence of anti-CD3 for four days and Treg mediated suppression was determined by CFSE dilution. (**e**) Colitis was induced by infusion of CD4+CD45RBhigh cells into immune deficient mice. Colitis can be abrogated by the administration of Treg at day 21. (**f**) Treg were pre-incubated with 10μM BIO or MeBIO for one hour. Sections of the colon were analyzed and scored (five mice per group) as described in Materials & Methods. (**g**) Representative hematoxylin and eosin stained tissue slides of the colon transcendence are shown.

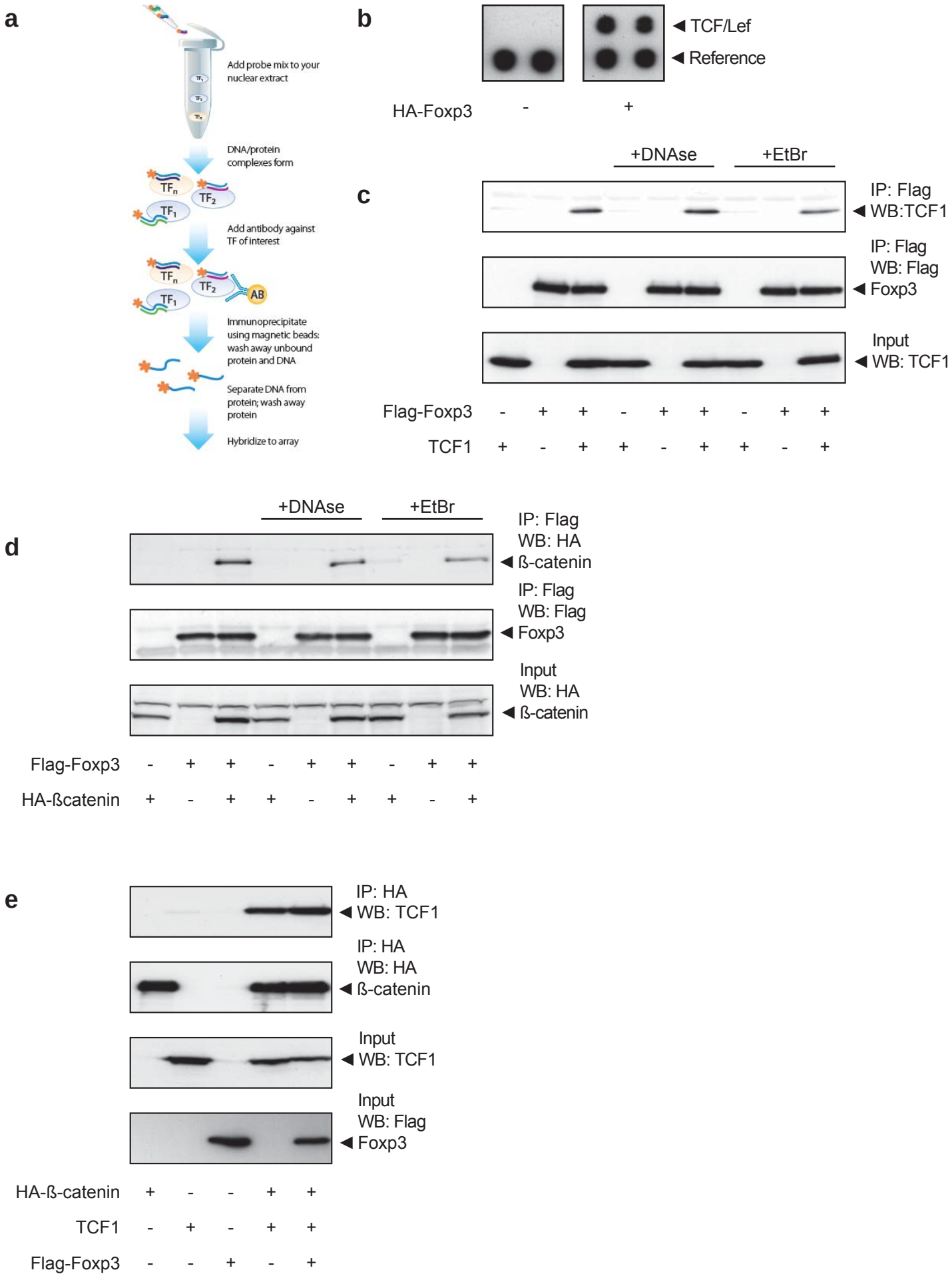
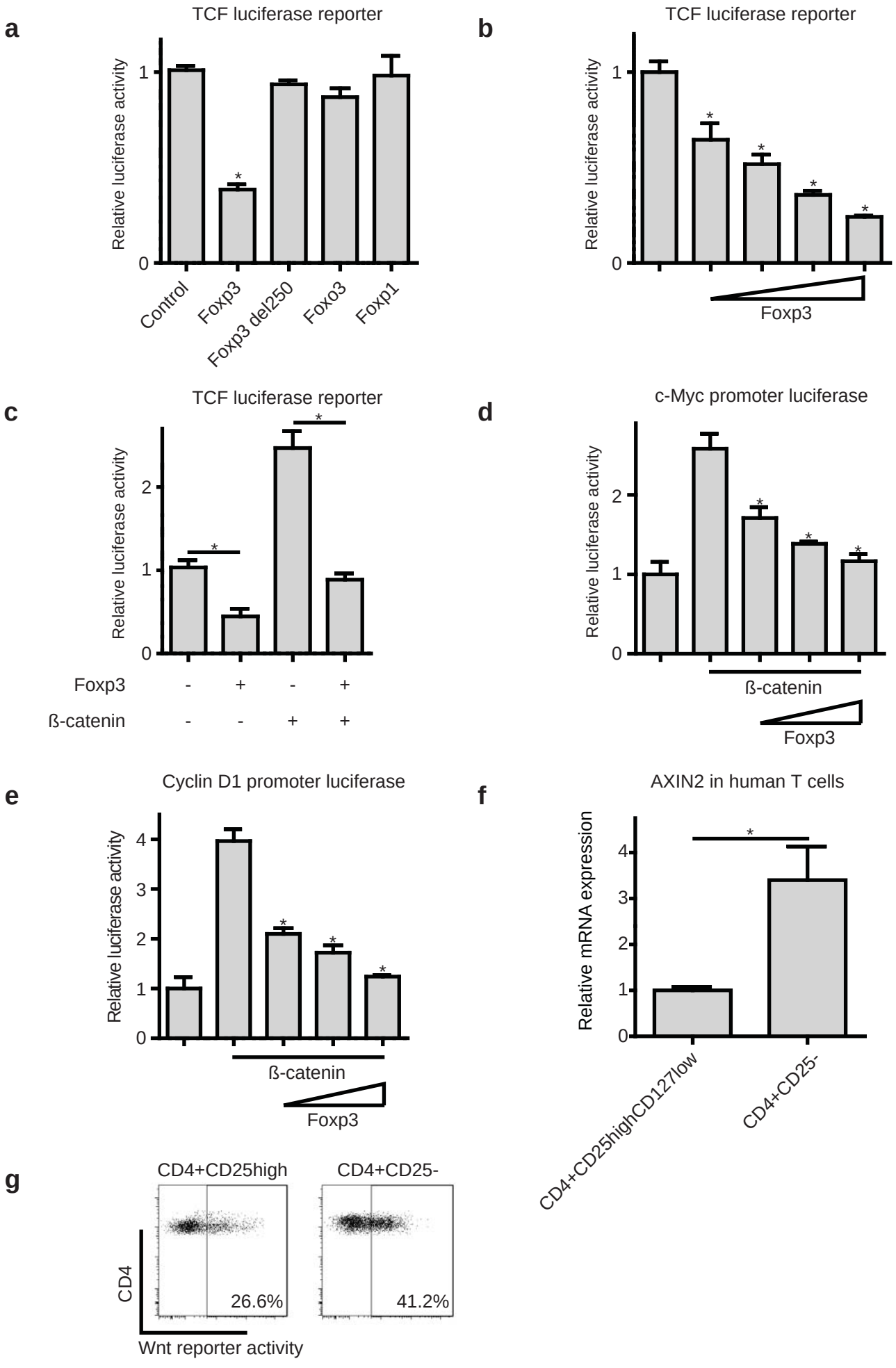


Figure 1. van Loosdregt et al.



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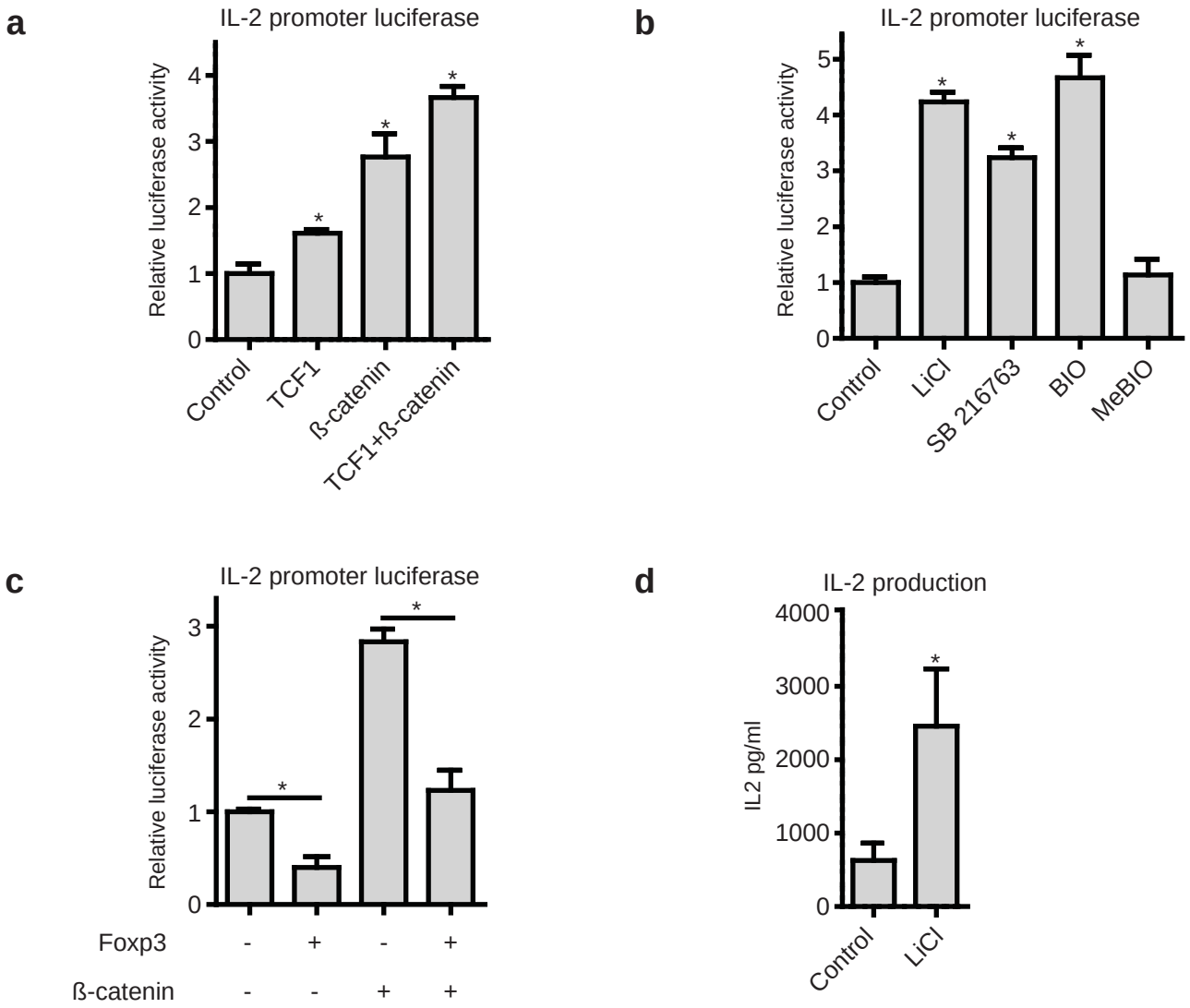

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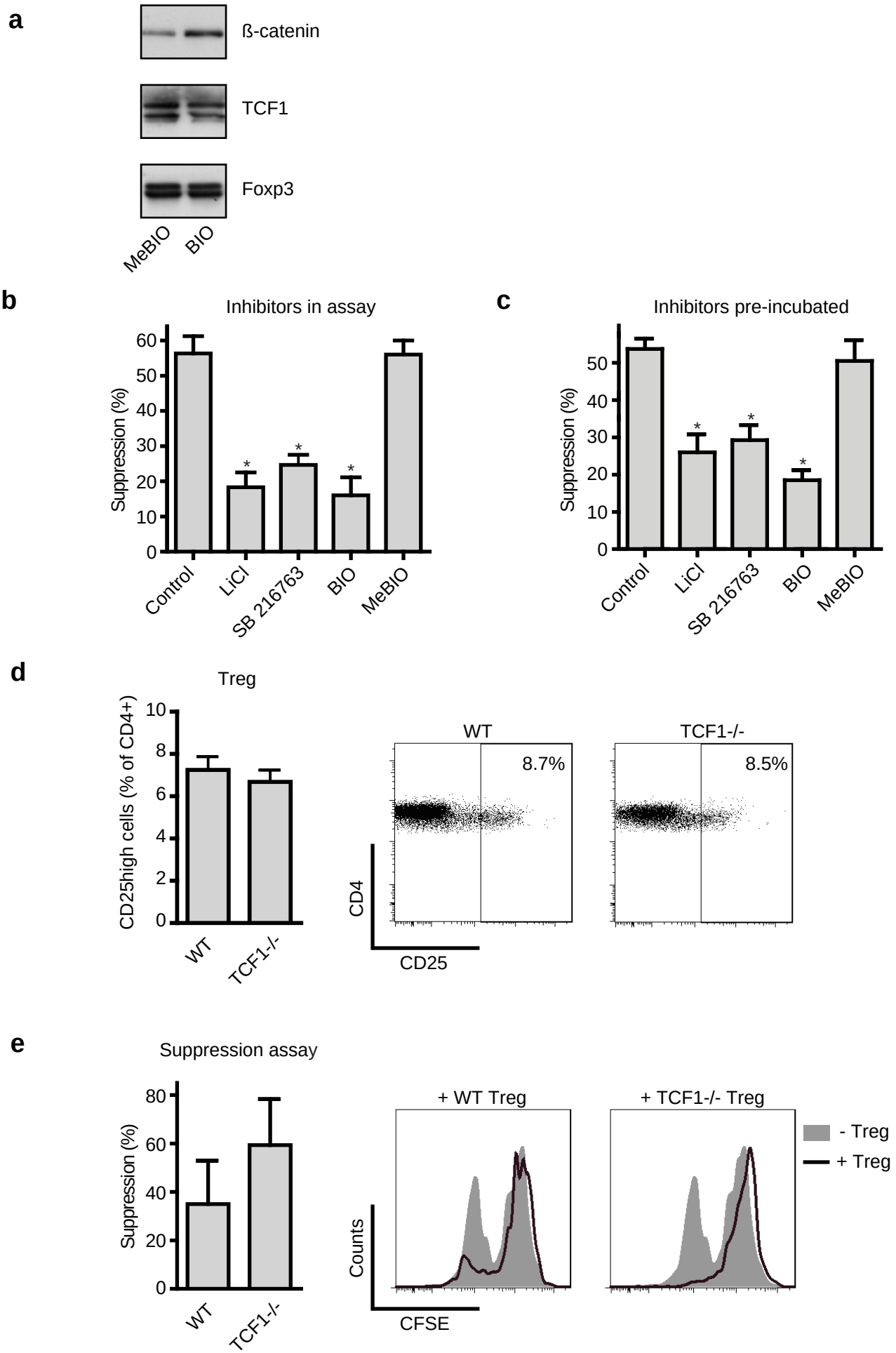

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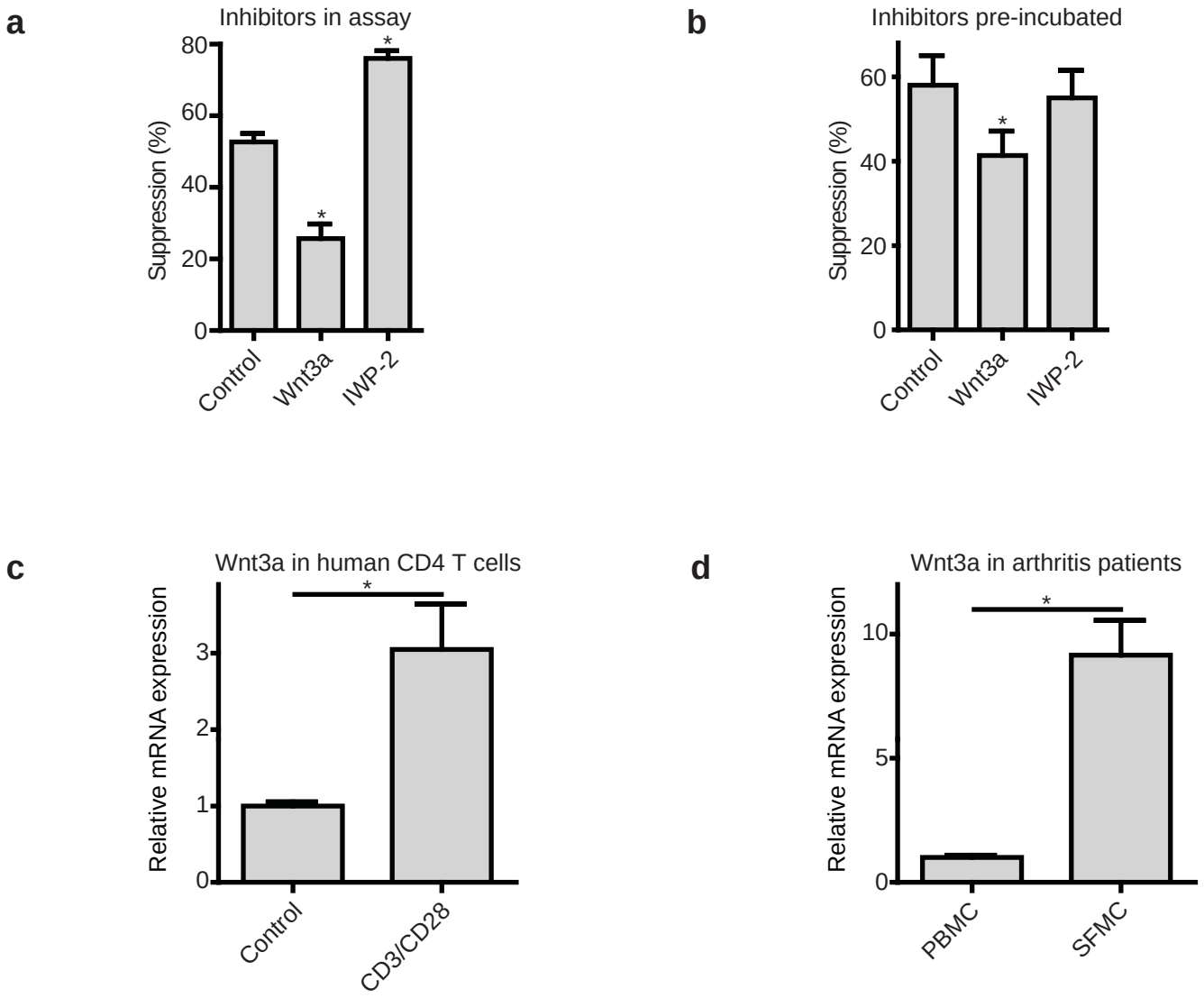

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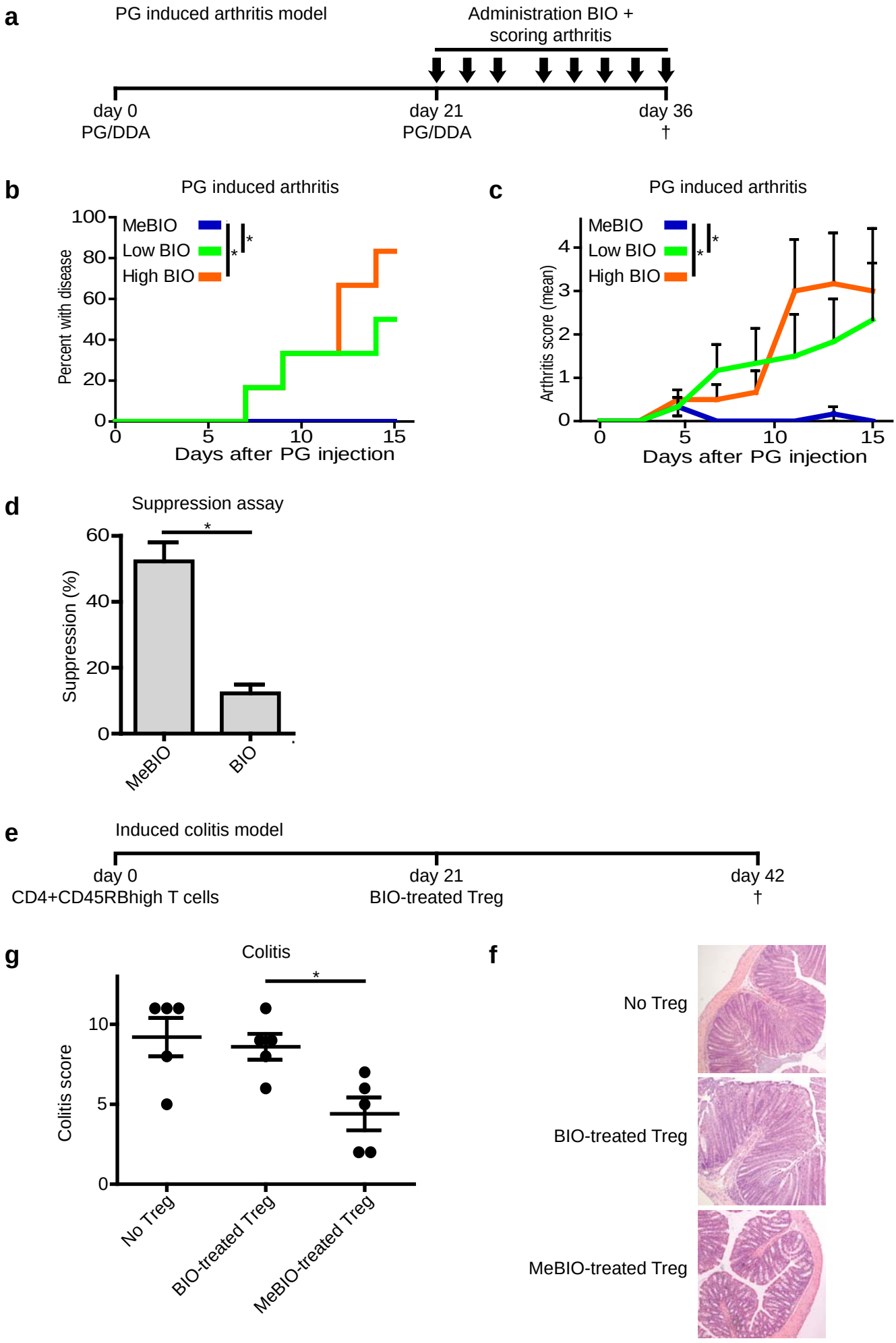


Figure 6. van Loosdregt et al

Supplementary Figure 1. Primers utilized for quantitative PCR.

Supplementary Figure 2. TCF1, TCF4, β-catenin and FZD expression in regulatory T cells. CD4+CD25highCD127low Tregs and CD4+ T cells were sorted from human PBMC, mRNA expression was analyzed for (**a**) TCF1, (**b**) TCF4, (**c**) β-catenin and (**d**) FZD receptors. β2-microglobulin (β2-M) was used as a reference gene. Mean CT-values normalized for β2-M are depicted + SEM. FZD1 was undetectable. Values depicted are representative for three independent donors.

Supplementary Figure 3. CD4+ T cell proliferation is unaffected by modulators of Wnt signaling. Human PBMC were CFSE labeled and cultured in the presence of (**a**) 5mM LiCl, 10μM SB216763, 100nM BIO, 100nM MeBIO or (**b**) 400ng/ml Wnt3a, 2μM IWP-2 for four days in anti-CD3 coated wells. Proliferation of CD4 positive cells was analyzed by CFSE dilution. Values depicted are representative for three independent donors.

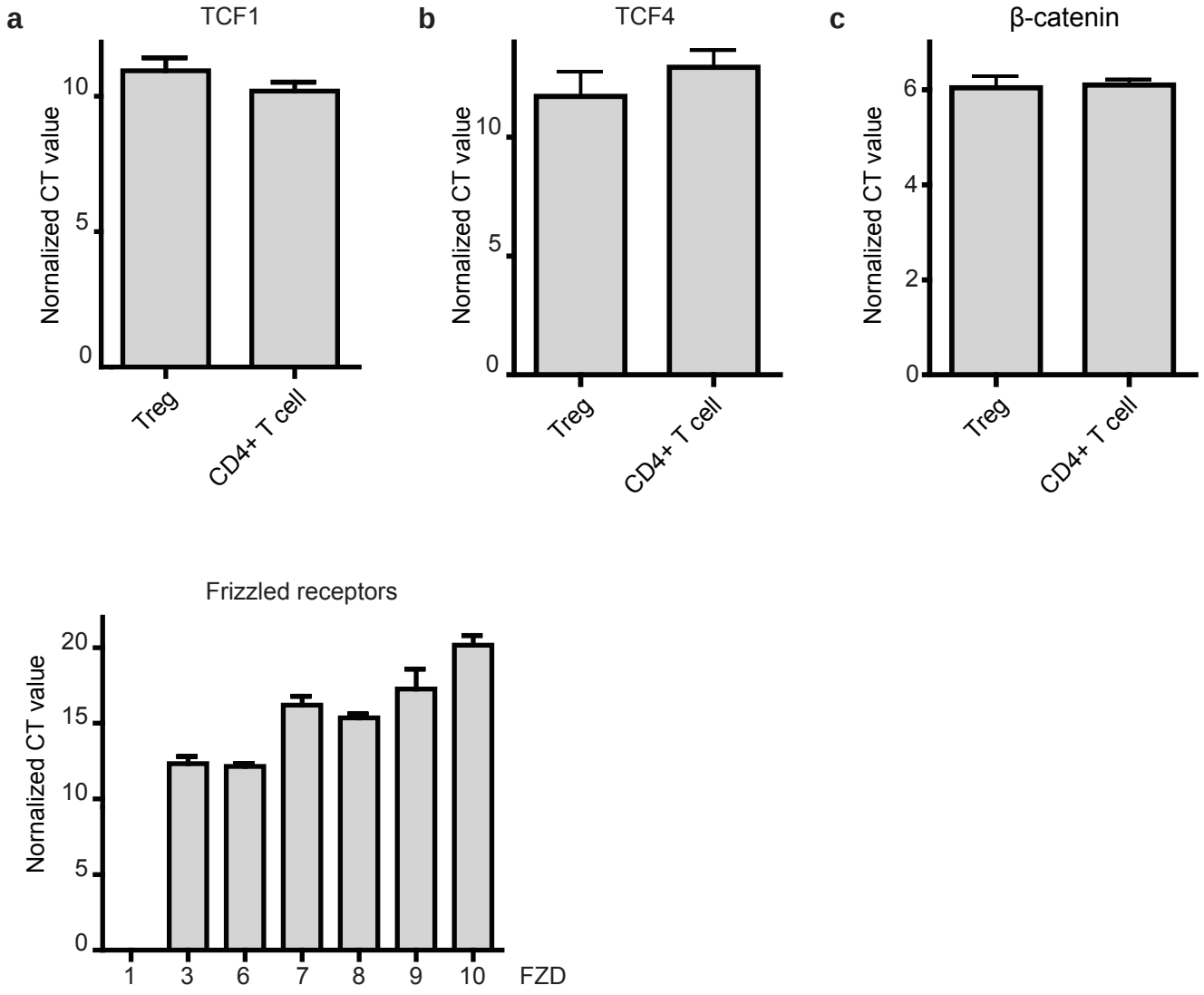
Supplementary Figure 4. Analysis of CD4+ T cell numbers in TCF-/- mice compared to wild-type. (**a**) PBMC were isolated from wild-type and TCF1-/- mice and analyzed by FACS for CD4 expression. (**b**) FACS sorted CD4+CD25+ splenocytes from TCF1-/- or wild-type mice were analyzed for Foxp3 expression. N=3

Supplementary Figure 5. Foxp3+ expression is unaffected in mice treated with BIO. Arthritis was induced by two injections of PG on day 0 and 21. From day 21 mice were injected with 6mg/kg MeBio or BIO twice a week (BIO high) or three times a week with 2mg/kg (BIO low). After 15 days of treatment mice were sacrificed. The percentage (**a**) or mean fluorescence intensity (MFI; **b**) of Foxp3 positive cells in the spleen was analyzed by FACS. Six mice per group.

Supplementary Figure 6. Numbers of Foxp3+ cells are unaffected by treatment with BIO. Immune deficient mice were injected with $3x10^5$ CD4+CD45high cells to induce arthritis. After 21 days, $3x10^5$ Tregs from Foxp3-GFP mice were pretreated with 10μM BIO or MeBIO for 1 hour, washed, and I.V. injected. 21 days later the mice were sacrificed and the percentage of GFP positive cells in the spleen and Mesenteric lymph nodes (MLN) were analyzed by FACS. Five mice per group.

β2M	fw: CCAGCAGAGAATGGAAAGTC
	rv: GATGCTGCTTACATGTCTCG
Wnt3a	fw: TCACGCTCTCGGGGCGGACTC
	rv: ACAGCCAGCGACCACCAGATCGG
AXIN2	fw: AGCCAAAGCGATCTACAAAAGG
	rv: GGTAGGCATTTTCCTCCATCAC
FZD1	fw: GCTGCGGAGAGTTGCGCTCTCTA
	rv: CGAGCCCAAGCCGCTTTCG
FZD3	fw: CGCCGGGGTCTGAGGATAGCA
	rv: ACATGGGGCACATTTGGCCAACA
FZD6	fw: TCAGCGGCTTGTATCTTGTGCCA
	rv: TGGTCGAGCTTTTGCTTTTGCCTGA
FZD7	fw: CTCTCCCAACCGCCTCGTCGCA
	rv: ACAGAGGCCCAGGGACGAAAGC
FZD8	fw: GCATGGAGTGGGGTTACCTGTTG
	rv: ATGCCCTTACACAGCGGCAC
FZD9	fw: CACCGTGGCGGTCTTCATGCTCAA
	rv: CGGCCAGCTGCTATCTTGCGGT
FZD10	fw: CACCTGGGCGCTCCAAGAAGAGG
	rv: ATACCGGGAAGCGAGGGAAGC
TCF1	fw: GTGACAAAAGGCCCCTTTCCGAC
	rv: CACAGGCCTGGCTGATTCCTTGT
TCF4	fw: TACAGAGGCATGCCACCAGGACT
	rv: TGGTGTCAGGTCCTCATCGTCA
β-catenin	fw: GAAGGTGTGGCGACATATGCAGCT
	rv: ATCCAAGGGGTTCTCCCTGGGC

Supplementary Figure 1.



Supplementary Figure 2. van Loosdregt et al

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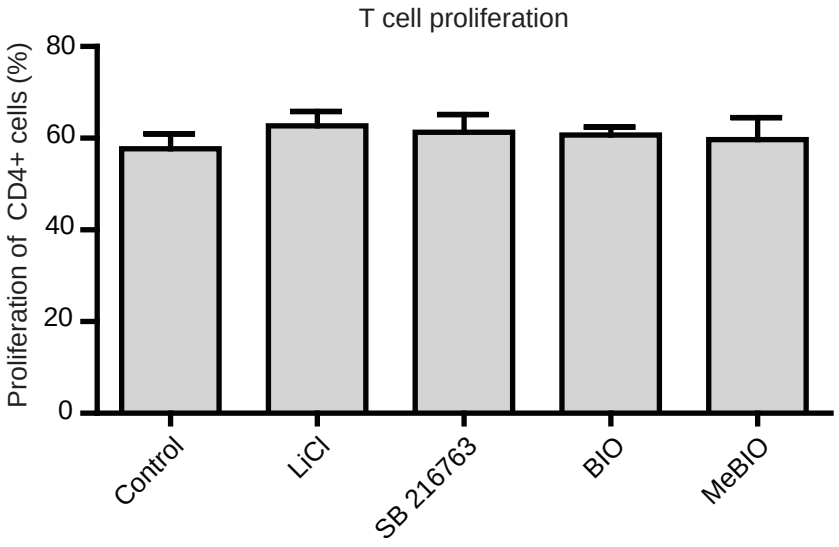

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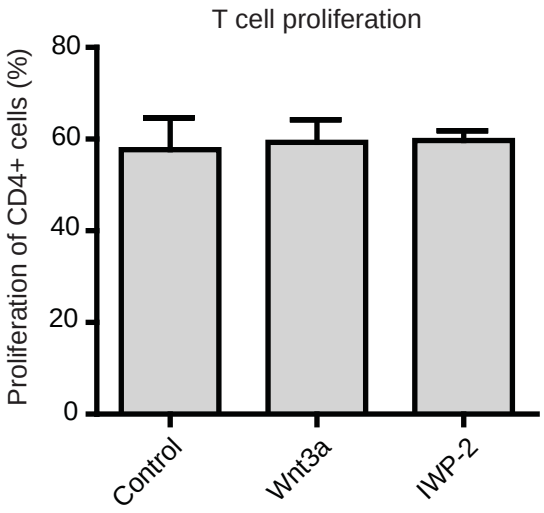
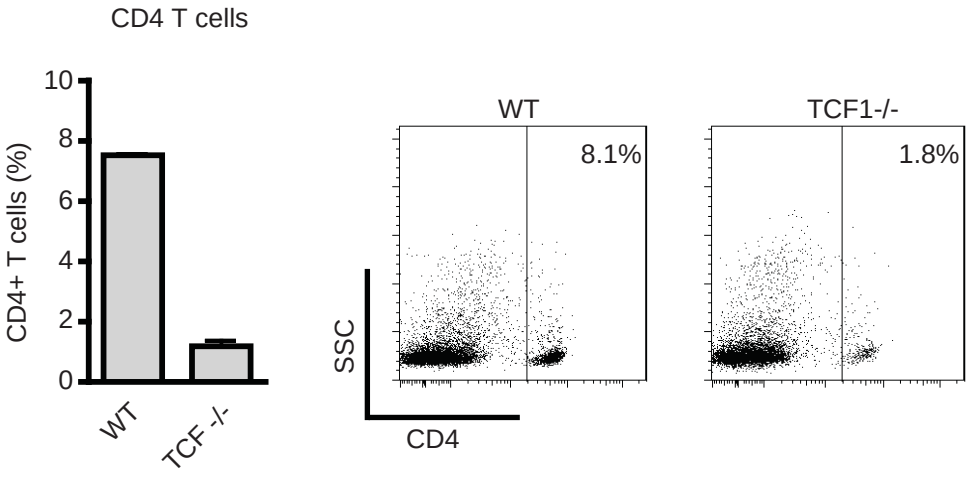


Supplementary Figure 3. van Loosdregt et al

a



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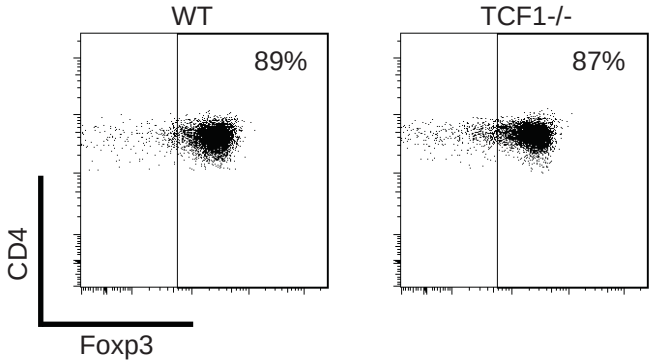

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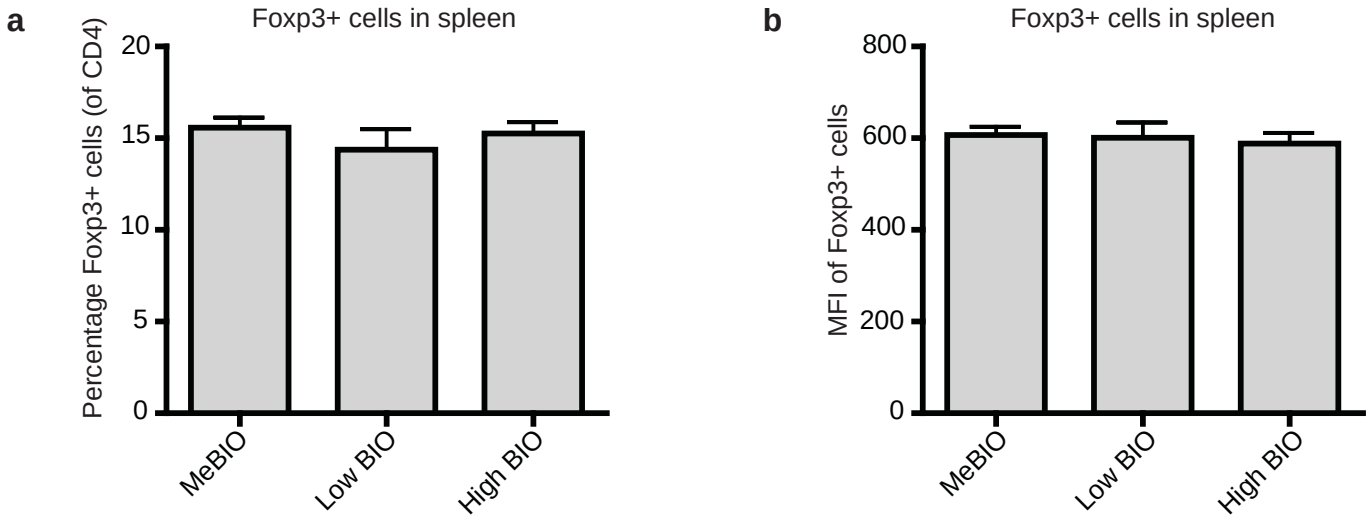
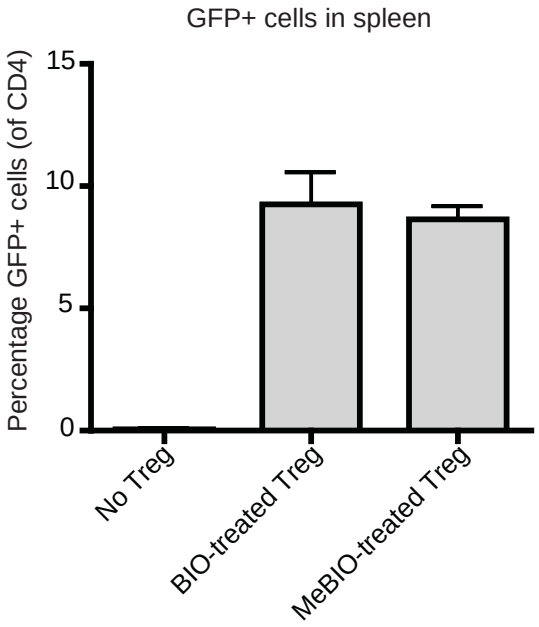
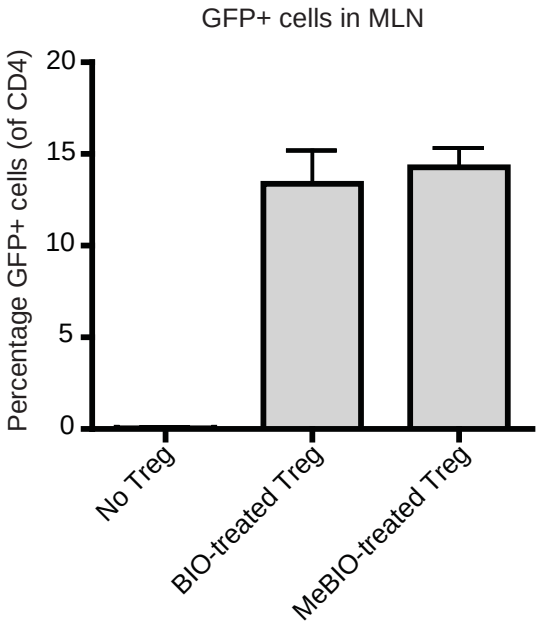


Supplementary Figure 5. van Loosdregt et al





Supplementary Figure 6. van Loosdregt et al

Chapter 6

General discussion

The transcription factor Foxp3 is of critical importance for both regulatory T cell (Treg) development and function, and thereby the maintenance of immune homeostasis[1-3]. Although the transcriptional regulation of *Foxp3* has been well investigated, mechanisms of post-translational regulation remain poorly understood[4-6]. Here we sought to investigate the molecular mechanisms regulating Foxp3 protein expression and the potential for modulation of transcriptional activity by co-factor association. We report, several novel findings including: a) regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization, (b) rapid temporal control of Foxp3 protein degradation by Sirtuin-1, (c) Increased Treg mediated suppression through USP7/HAUSP-mediated Foxp3, (d) inhibition of Treg function through inhibition of Foxp3 by canonical Wnt signaling. The implications of these observations are discussed below.

Regulation of Foxp3 protein levels

Since Foxp3 is a critical regulator of Treg mediated suppression, manipulation of Foxp3 protein levels would potentially therapeutic modulation of immune responses. In **Chapters 2, 3 and 4** of this thesis, we show that the Foxp3 protein has a high turnover rate, and proteasome inhibition dramatically increases Foxp3 protein levels. Moreover, we demonstrate that Foxp3 proteasomal degradation is regulated by reversible Foxp3 poly-ubiquitination. Inhibition of deubibiquitinases (DUBs) was found to increase poly-ubiquination and reduce Foxp3 expression levels. Foxp3 can associate with and is deubiquitinated by ubiquitin specific protease-7 (USP7), resulting in increased Foxp3 protein levels and improved Treg mediated suppression *in vitro*. In an *in vivo* mouse colitis model inhibition of DUB activity impeded the ability of Treg to reduce disease severity.

An acetylation/ubiquitination competition model regulating Foxp3 protein stability.

Poly-ubiquitination can be impaired by Foxp3 acetylation, and Foxp3 can be reciprocally acetylated by the KAT p300 and deacetlyated by the KDAC SIRT1. Treatment of cells

ectopically expressing Foxp3 with KDAC inhibitors decreased Foxp3 poly-ubiquitination. In addition, hyperacetylation of Foxp3 either by ectopic expression of p300 or by inhibition of SIRT1 dramatically reduced Foxp3 turnover, and resulted in increased Foxp3 protein levels. Lysine ubiquitination and acetylation are mutually exclusive, suggesting that acetylation can prevent Foxp3 poly-ubiquitination through a competition based mechanism (**Fig. 1**). A similar mechanism has been described several proteins. For example, Smad7 is acetylated by p300 on two N-terminal lysine residues and mutation of either of these lysines to alanines, or acetylation of these lysines by ectopic expression of p300, resulted in reduced Smad7 degradation and increased protein levels[7]. Both lysine residues were also found to be poly-ubiquitinated, a process that was impaired by p300-mediated acetylation. Jin *et al.* have demonstrated that the transcription factor RUNX3 is subjected to ubiquitin-dependent degradation mediated by the E3 ligase Smurf1[8]. Similar to Smad7, acetylation of RUNX3 by p300 reduced RUNX3 poly-ubiquitination and stabilized the protein. Mutation of three RUNX3 lysine residues greatly reduced RUNX3 acetylation and resulted in severely impaired Smurf1 mediated degradation. Protein expression of tumor suppressor p53 can also be regulated by both acetylation and ubiquitination. The C-terminus of p53 can be acetylated at several distinct lysine residues as the result of DNA-damage[9-11]. The same C-terminal region was shown to be ubiquitinated by the E3 ligase MDM2, resulting in p53 degradation[12]. While mutation of six C-terminal lysine residues did not interfere with the MDM2-p53 association, it prevented ubiquitination and degradation, resulting in increased p53 protein levels [13]. Furthermore, treatment with the HDAC inhibitor TSA dramatically increased acetylation and stabilization of p53[10]. We propose that, similar to Smad7, RUNX3 and p53, Foxp3 protein expression is regulated by an acetylation/ubiquitination competition based mechanism.

Utilizing mass spectrometry, five distinct lysine residues in Foxp3 were found to be ubiquitinated (K249, K251, K263, K267, K393). Restoring these five lysine residues in a lysine-deficient Foxp3 mutant re-established Foxp3 poly-ubiquitination to wild-type levels. However, equal Foxp3 ubiquitination levels were observed with either the combination K249/K251/K393 or K263/K267. In contrast, mutation of these lysines to argenines in wild-

type Foxp3 did not reduce Foxp3 ubiquitin levels (data not shown). Taken together, these data support the conclusion that there is considerable promiscuity in target lysine ubiquitination. Furthermore, a fusion protein in which ubiquitin was fused to the N-terminus of Foxp3 resulted in dramatically increased Foxp3 poly-ubiquitination and degradation, suggesting that poly-ubiquitination occurs irrespective of the site of initial ubiquitination. Foxp3 is not the first protein for which promiscuity of lysine ubiquitination has been described. Utilizing mass spectrometry, TNF-R associating factor 1 (TRAF1) was found to be ubiquitinated on two lysine residues, although always independently[14]. However, mutation of both lysine residues, was required to abrogate ubiquitination. In accordance, utilizing mass spectrometry the DUB UCH-L1 was also found to be ubiquitinated on at least four lysine residues[15]. Similarly, mutation of all four lysine residues did not result in reduced ubiquitination levels. Only a UCH-L1 protein in which all 16 lysine residues were mutated showed severely reduced ubiquitination levels.

Mass spectrometric analysis of Foxp3 revealed that lysine residues K249, K251, K263, and K267 that were found to be ubiquitinated can be also acetylated (data not shown). Since these lysine residues can both be acetylated and poly-ubiquitinated, this strongly support a model in which Foxp3 acetylation and poly-ubiquitination compete for free lysine residues, thereby regulating Foxp3 degradation and protein expression levels.

These data demonstrate that p300, SIRT1 and USP7 can all regulate Foxp3 protein expression levels. Although very little is known concerning the role of growth factors or cytokines in regulating these enzymes, it is interesting to speculate as to how the activity of these factors may be regulated during immune responses. When a active immune response is desirable, immune modulatory cytokines such as TNF-α, IL1-ß, IL-6 and IFN-γ are produced. One could imagine that these cytokines could activate SIRT1, or deactivate p300 or USP7, resulting in a rapid and temporal decrease in Foxp3 protein levels and reduced Treg function. Conversely, when an immune response needs to be terminated, anti-inflammatory cytokines including IL-10 and TGF-ß are produced. These cytokines could potentially activate p300 or USP7 thereby stabilizing Foxp3 protein and increasing Treg

mediated suppression (the potential effect of TGF-ß on Foxp3 protein levels will be further discussed, see below). Further research regarding activation of these enzymes is required to test this hypothesis.

Manipulation of Foxp3 protein expression as a novel therapeutic target

Several autoimmune diseases have been linked with impaired Foxp3+ Treg numbers (reviewed by JH Buckner[16]). In both systemic lupus erythematosus (SLE) and ulcerative colitis Treg numbers are reduced compared to healthy controls, and inversely correlate with disease severity[17-20]. Since we show that Foxp3 degradation can be regulated by p300, SIRT1 and USP7 it is interesting to speculate how manipulation of these enzymes could be used as a therapeutic approach to control (auto)immune diseases. There is already some evidence that manipulation of immune responses can be achieved through modulation of the global acetylation state of Treg (reviewed by Wang *et al.*[21]). Incubation of Treg with KDAC inhibitors including: trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), tubacin, BML-210, MS-275, sodium butarate (SB), bufexmac and nicotinamide (NAM) all increased Treg mediated suppression of CD4+ T cell proliferation *in vitro*[22-24]. Consistent with this, mice treated with the KDAC inhibitors TSA, SAHA, valproic acid (VPA), or splitomycin all showed decreased immune responses in colitis, arthritis and cardiac allograft transplantation models [23, 25, 26]. HDAC inhibitors are already in clinical use for treatment of a variety of neurologic and psychiatric disorders. For example, the HDAC inhibitor VPA is the most commonly used treatment for epilepsy[27]. Sodium butyrate and SAHA treatment have been found to enhance the formation of memory in mice[28]. In addition, HDAC inhibitors are currently being utilized for cancer treatment (as reviewed by Marks pa *et al.* and Richon *et al.*[29, 30]). Although the molecular mechanisms by which these HDAC inhibitors facilitate their effects often remain unclear, it is often proposed that epigenetic histone modifications are responsible. Based upon our own studies and others, another possibility is that HDAC inhibitor treatment modifies the acetylated state of critical transcription factors, thereby manipulating their activity and expression levels, similar to our observations with Foxp3.

Most recently it has been reported that specific inhibition of SIRT1 activity in Treg abrogates their suppressive capacity in both *in vitro* suppression assays, and in a MHC-mismatched cardiac allograft transplantation mouse model. Furthermore, SIRT1 mRNA expression was shown to be significantly increased in Teff 24 hours after activation with anti-CD3 and irradiated antigen presenting cells[31]. In contrast, activation of Treg resulted in reduced SIRT1 expression. Since we show that SIRT1 mediated deacteylation of Foxp3 increases protein stability resulting in increased Treg number and function, activation of Treg would result in increased Treg mediated suppression due to decreased SIRT1 levels. In addition, mice with a targeted deletion of SIRT specifically in CD4+ T cells or Foxp3+ Treg both exhibited prolonged survival of MHC-mismatched cardiac allograft[31]. Since we have identified SIRT1 as a specific KDAC for Foxp3 deacetylation, the SIRT1 inhibitor nicotinamide, also known as vitamin B3, is a potential candidate for therapeutic use. Nicotinamide is a water-soluble vitamin and is therefore safer and cheaper compared to other KDAC inhibitors. Indeed, in several human trials, nicotinamide treatment resulted in remission of autoimmune diseases including rheumatoid arthritis and reduced development of diabetes type 1[32-35]. In addition, numerous studies have reported that oral nicotinamide treatment of acne vulgaris succesfully reduced associated inflammatory lesions (reviewed by Nirem *et al*.[36]).

Modulation of Foxp3 poly-ubiquitination could also be a potential target for the regulation of immune responses. In this thesis we demonstrate that USP7 deubiquitinates Foxp3, and therefore increases Foxp3 protein levels. Treatment of Treg with a pan-DUB inhibitor decreased Foxp3 poly-ubiquitination, thereby reducing Foxp3 protein expression, resulting in impaired Treg mediated suppression *in vitro* and *in vivo*. Since the DUB inhibitor we used can potentially inhibit all DUBs and thereby influence a variety of cellular processes including DNA repair, apoptosis, antigen processing and transcription of multiple transcription factors, a more specific approach is desirable[37-39]. Recently the first USP7, HBX 41.108, was published[40]. This small-molecule compound inhibits USP7 activity with an IC_{50} value of submicromolar range, in contrast it did not decrease the activity of several proteases including renin, plasmin, calpain-1 and UCH-L1, demonstrating specificity. Treg are found in

increased numbers both in, and in close proximity to, solid tumors and have been shown to inhibit tumor specific T cell immunity and contribute to growth of human tumors *in vivo*[41-43]. Depletion of CD4+CD25+ cells *in vivo* augments the generation of tumor specific T cells and promotes the rejection of tumors derived from myeloma, sarcoma and melanoma[44-48]. Since we demonstrated that USP7 inhibition resulted in decreased Foxp3 expression levels and impaired Treg mediated suppression a specific USP7 inhibitor, such as HBX 41.105 could potentially be used to increase tumor directed immune responses in cancer patients. Interestingly, MDM2 the major E3 ligase of the tumor suppressor p53 has been identified as a substrate of USP7[49-51]. It was demonstrated that specifically MDM2, and not p53 itself, can be deubiquitinated by USP7 resulting in increased p53 degradation[51, 52]. Inhibiting USP7 may therefore act as a double edged-sword, increasing both p53 tumor-suppressor protein levels as well as triggering an anti-tumor immune response.

Regulation of Foxp3 transcriptional activity

In **Chapter 5** we demonstrate that Wnt signaling is an important negative regulator of Treg mediated suppression. Here we show that Foxp3 can associate with the Wnt-transcriptional effectors TCF1 and ß-catenin. TCF1 can inhibit the transcriptional activity of Foxp3. Equally, disruption Wnt signaling in Treg enhanced their suppressive capacity, while Treg mediated suppression was abrogated upon activation of Wnt signaling, both *in vitro* and in two auto-immune mouse models.

Foxp3 associates with TCF1/ß-catenin

Although we show that Foxp3 can associate with TCF1 and ß-catenin, the precise composition of this complex remains unclear, particularly since these experiments were performed in cells ectopically expressing both TCF and ß-catenin. Assuming that both TCF1 and Foxp3 associate with the DNA, several models are possible. One possibility is that al three proteins associate with each other, here all factors interact with both the other proteins (**Fig. 2a**). Another possibility is that ß-catenin acts as a scaffold bridging association of TCF1

with Foxp3 (**Fig. 2b**). A third option is that ß-catenin and Foxp3 can only associate with TCF1 (**Fig. 2c**). Additional experiments utilizing cells that not endogenously express these factors will provide further insight into the precise composition of this complex.

Utilizing promoter reporter assays, we demonstrate that Foxp3 could abrogate transcriptional activation of TCF1 targets c-myc and cyclin D1. In addition, TCF1 activation resulted in increased IL-2 promoter activity, which has been reported to be a direct target of Foxp3[53, 54].

The exact molecular mechanism by which TCF1 activation results in impaired Treg mediated suppression remains unclear. Since both transcription factors are expressed in Treg and can opposingly regulate their transcriptional targets, the activation state of TCF1 may determine the transcriptional activity (**Fig. 3**). In the absence of Wnt, ß-catenin is rapidly degraded and TCF1 will be inactive, here Foxp3 will be dominant resulting in suppressive Treg. In the presence of Wnt, ß-catenin can activate TCF1 shifting the balance toward TCF1, thereby overruling Foxp3 and resulting in impaired Treg mediated suppression. Other transcription factors that have been reported to associate with Foxp3 include AML1, NFAT and NF-κB, which can all increase transcription of the T cell cytokines IL-2, IL-4 and IFN-γ, which can be downregulated by Foxp3[55-60]. We propose that the Foxp3-TCF1 association is comparable to its interaction with AML1, NFAT or NF-κB. Additional experiments in which the association of Foxp3 and or TCF1 with their DNA binding sites can be studied, such as EMSA or CHIP, methodologies could be used to gain information regarding the underlying mechanism behind these observations.

It has been reported that ß-catenin can modulate Treg survival[61] . Here a constitutively stable form of ß-catenin was transduced into CD4+CD25+ mouse Treg, resulting in increased survival when cultured *in vitro*. Ten days after co-injection of ß-catenin and control trancduced cells into mice significantly more ß-catenin transduced cells could be retrieved in the peripheral blood of these mice. In an induced colitis mouse model, CD4+CD45RB^{high} cells were co-injected with ß-catenin transduced Treg into TCRa-/-TCRb-/- mice. ß-catenin transduced Treg demonstrated improved disease scores compared to control Treg. Although these data indeed show that mouse Treg transduced with this artificial form of ß-catenin

show increased survival, this study lacks any supporting evidence utilizing additional methodologies to manipulate Wnt signaling which would further strengthen the conclusions. For example additional experiments utilizing GSK-3 inhibitors, TCF or ß-catenin knockout Treg would help to validate this work. We demonstrated that Wnt production is increased upon T cell activation, as observed at inflammatory loci. Furthermore, we show that Wnt signaling, and thus ß-catenin activation, results in reduced Treg mediated suppression. Together these data would suggest that at sites of inflamation Treg activity is suppressed to allow rapid immune activation resulting in clearance of the infection. While Treg should be inactive during the onset of a productive immune response they must remain present in order to terminate the response when cleared. Possibly Wnt production could increase the survival of these cells assuring that they remain present to initiate termination of the response. Additional research at the site of inflammation is required to determine if these cells indeed show increased survival.

Wnt signaling as a target for immune modulation

The role of Wnt signaling regulating mature T cell function remains poorly understood and has not been extensively investigated. Recently is has been demonstrated that TCF1 can modulate CD8 responses[62]. TCF1-/- mice were crossed with OT-1 transgenic mice in which TCR can recognize ovalbumin. CD8 cells from these or control mice were transferred into mice that were infected with listeria monocytogenes expressing Ova, and proliferation of transferred cells was analyzed. Significantly less proliferation was observed for the TCF1-/- CD8 cells compaired to wild-type. OT-1 cells isolated from the spleen of TCF-/- mice showed reduced IL-2 production compared to controls, demonstrating that TCF1 activation increases CD8+ T cell proliferation and activation. Since we show that Wnt signaling abrogates Treg mediated suppression, this indicates that Wnt signaling can activate immune responses both by directly inducing CD8 T cell proliferation and activation, as well as suppressing Treg suppressive capacity.

Increased numbers of Treg are observed in patients with psoriasis and arthritis[63-66]. Since this increased number of Treg is apparently insufficient to control the local inflammation, this suggests that their suppressive capacity is abrogated. Indeed, Treg mediated suppression was reported to be impaired in patients with rheumatoid arthritis, psoriasis, type 1 diabetes, multiple sclerosis, or SLE[67-73]. We demonstrate that isolated CD4+ T cells express Wnt3a, and this is increased upon T cell receptor activation. In addition we show that synovial fluid mononuclear cells from juvenile arthritis patients express up to ten fold more Wnt3a compared to paired PBMC. Furthermore, treatment of Treg-PBMC co-cultures with a Wnt production inhibitor significantly increased Treg mediated suppression. Based upon these data we propose a model in which immune responses can be rapidly and reversibly modulated through regulation of Wnt levels. Treg can maintain immune homeostasis by constant moderate suppression of T cell activation and proliferation (**Fig. 4a**). Upon infection, when Treg mediated suppression is unwanted, T cell receptors are activated, resulting in local production of Wnt resulting in both upregulation of the T cell stimulatory cytokine IL-2 and impairment of Treg mediated suppression through Foxp3 transcriptional inhibition (**Fig. 4b**). When the infection is resolved, Wnt levels drop and Treg regain their functionality, restoring immune homeostasis (**Fig. 4c**). Furthermore, we propose that dysregulated Wnt production, as observed in the synovium of juvenile arthritis patients, inhibits Treg function, resulting in an uncontrolled immune response and inflammation (**Fig. 4d**).

We demonstrate that systemic modulation of Wnt signaling *in vivo* by intraperitoneal injection with the GSK-3 inhibitor BIO abrogates Treg mediated suppression resulting in more aggressive immune responses. Manipulation of Wnt signaling may therefore be a potent therapeutic target for manipulation of immune responses. Suppression of Wnt signaling or Wnt production, thereby suppressing immune responses, could potentially be used to treat patients with auto-immunity. In our experiments Wnt production was inhibited utilizing IWP-2, an inhibitor that is directed against Porcn. Since secretion of Wnt first requires palmitoylation of Wnt by Porcn, this inhibitor inhibits Wnt production and not Wnt activity. Although this compound significantly increase Treg mediated suppression of Teff cell proliferation *in vitro,*

we did not perform *in vivo* experiments utilizing this inhibitor. Additional experiment in autoimmune mouse models are necessary to further investigate the potential of this compound to treat autoimmunity. Another possibility is to inhibit Wnt signaling utilizing Wnt antagonists of which there are two classes (reviewed by Kawano *et al*.[74]). The first class of antagonists can directly interact with soluble Wnt, thereby preventing association with its Frizzled receptor. This class include Wnt inhibitory factor 1 (WIF-1), Cerberus, and secreted Frizzled–related protein (sFRP). There are at least seven different sFRPs, which all share a cysteine rich domain including ten conserved cysteine residues that are shared by Frizzled receptors, suggesting that this domain may be the Wnt-interaction site. Potentially, small molecule inhibitors directed to this cystine rich domain would block Wnt interaction with its receptor, thereby preventing activation of the Wnt signaling pathway. The second class of Wnt antagonists comprises certain members of the Dickkopf (Dkk) family (Dkk1-4), which can associate with LRP5/6 of the Wnt receptor complex. Dkk-1 was reported to inhibit Wnt-mediated stabilization of ß-catenin, and TCF mediated transcription in an artificial reporter assay, demonstrating its antagonistic function[75, 76]. Blocking LGR5/6 could therefore be a potential therapeutic target to inhibit Wnt signaling and therefore increase Treg mediated suppression.

In addition, activation of Wnt signaling could be a strategy to increase anti-tumor responses in cancer patients. We demonstrated that *in vitro* treatment of Treg with LiCl, a GSK-3 inhibitor, and therefore TCF1 activator, dramatically reduced Treg suppressive capacity. LiCl is already widely used to treat patients with manic depression, and several case studies have reported that LiCl treatment is correlated with increased auto-immune diseases including psoriasis and autoimmune thyroid disease[77-80]. This suggests that indeed systemic treatment with LiCl will result in increased immune activation and can potentially be used to increase an anti-tumor immune response. Wnt signaling can also be activated utilizing Wnt agonists. Very recently, it was shown that the Wnt agonist R-spondin can be used to activate Wnt signaling *in vivo*[81]. R-spondin can relieve Dkk-1 inhibition, thereby activating the Wnt pathway [82]. Wnt signaling fulfils a critical role in intestinal epithelial cell proliferation, maturation and

regeneration[83]. Intravenous injection with R-spondin resulted in increased mRAN expression of the TCF target genes *Axin2*, *Ascl2* and *Lgr5* in the small intestine[81]. In addition, radiation-induced colitis was reduced by injection with R-spondin, as the proliferation of intestinal stem cells was increased in these mice, demonstrating that treatment with this Wnt agonist is a potential method to modulate Wnt signaling *in vivo*.

SIRT1: regulator of Treg mediated suppression

SIRT1 has recently been shown to be able to increase Wnt signaling. Protein levels of disheveled (Dvl), an effector of Wnt receptor activation, were reduced as a result of SIRT1-knock-down[84]. SIRT1 and Dvl can form a complex and inhibition of SIRT1 significantly inhibited transcription of TCF target genes (**Fig. 1**). We have shown that inhibition of SIRT1 increases Treg mediated suppression and that Wnt signaling negatively influences Treg suppressive capacity. The modulation Dvl protein levels by SIRT1 could therefore potentially be part of the molecular mechanism regulating Treg functionality.

Foxp3 can be to be acetylated by both TIP60 and p300, however we have found that only the KDAC SIRT1 can specifically deacetylate Foxp3[24, 85]. SIRT1 has been also reported to impair both TIP60 and p300 activity[86-88]. SIRT1 can directly interact with TIP60, and ectopic expression of SIRT1 but not SIRT2-7 dose-dependently reduced acetylation of TIP60, while an inactive SIRT1 mutant did not. Furthermore, SIRT1 was able to repress TIP60 mediated acetylation of its target histone 2A (H2A), while SIRT1 knock-down resulted in excessive acetylation of H2A[86, 87]. These data demonstrate that SIRT1 can deacetylate TIP60, thereby abrogating its activity. SIRT1 was also reported to directly associate with the C-terminus of p300 in cells ectopically expressing both proteins[88]. SIRT1 was found to deacetylate p300, while treatment with the SIRT1 inhibitor nicotinamide increased p300 acetylation. SIRT1 but not an inactive SIRT1 mutant could repress p300 KAT activity, demonstrating that SIRT1 mediated deacetylation of p300 can regulate its activity.

Since SIRT1 modulates the activity of the Treg modulators: Foxp3, TIP60, p300 and Dvl, SIRT1 may impair Treg mediated suppression on multiple molecular levels. Manipulation of SIRT1 activity or protein levels could therefore be a potent novel therapeutic target.

TGF-ß regulates Foxp3 functionality

Transforming growth factor-ß is critical for both Foxp3 induction and maintenance, and Treg function[89-91]. Stimulation of human or mouse CD4+CD25- cells with TGF-ß induces Foxp3 expression, and in TGF-ß1 knockout mice, Treg numbers in the periphery but not in the thymus are reduced compared to control mice[92-95]. In addition, TGF-ß receptor deficient Treg develop normally in the thymus but are poorly maintained in the periphery[96]. Blocking TGF-ß signaling in T cells resulted in the onset of colitis in mice[97], while the transfer of wild-type Treg into colitis mice, but not Treg in which TGF-ß signaling was blocked, into colitis mice abrogated disease severity[98]. Samanta *et al*. reported that TGF-ß stimulation of Foxp3 transfected Jurkat T cells increased Foxp3 acetylation, by immunopreciption of Foxp3 and visualizing acetylation utilizing an anti-acetylated lysine antibody[99]. It is interesting to speculate that TGF-ß may increase p300 activity or inhibit SIRT1 and could therefore influence Foxp3 acetylation. More research is needed to study this effect in more detail.

TGF-ß can also activate TGF-ß activated kinase 1 (TAK1) which in turn can activate NEMO-like kinase (NLK)[100]. Utilizing TCF reporter assays, it has been shown that ectopic expression of NLK resulted impaired ß-catenin/TCF mediated transcription[100]. Since we demonstrated that suppression of TCF1 increases Treg suppressive capacity, the TGF-ß-TAK1-NLK pathway may contribute in the increased effect on Treg mediated suppression by TGF-ß stimulation (**Fig. 1**).

In this thesis we show that Foxp3 can be subjected to post-translational regulation, resulting in altered Treg mediated suppression. We demonstrate that regulation of both Foxp3 acetylation and ubiquitination, can dramatically modulate Foxp3 protein expression and therefore Treg mediated suppression. In addition, we report that Wnt signaling can negatively

regulate Foxp3 transcriptional activity and Treg suppressive capacity. Taken together, we have identified several novel therapeutic targets, which could potentially be used to treat autoimmunity, infections and cancer.

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Figure legends

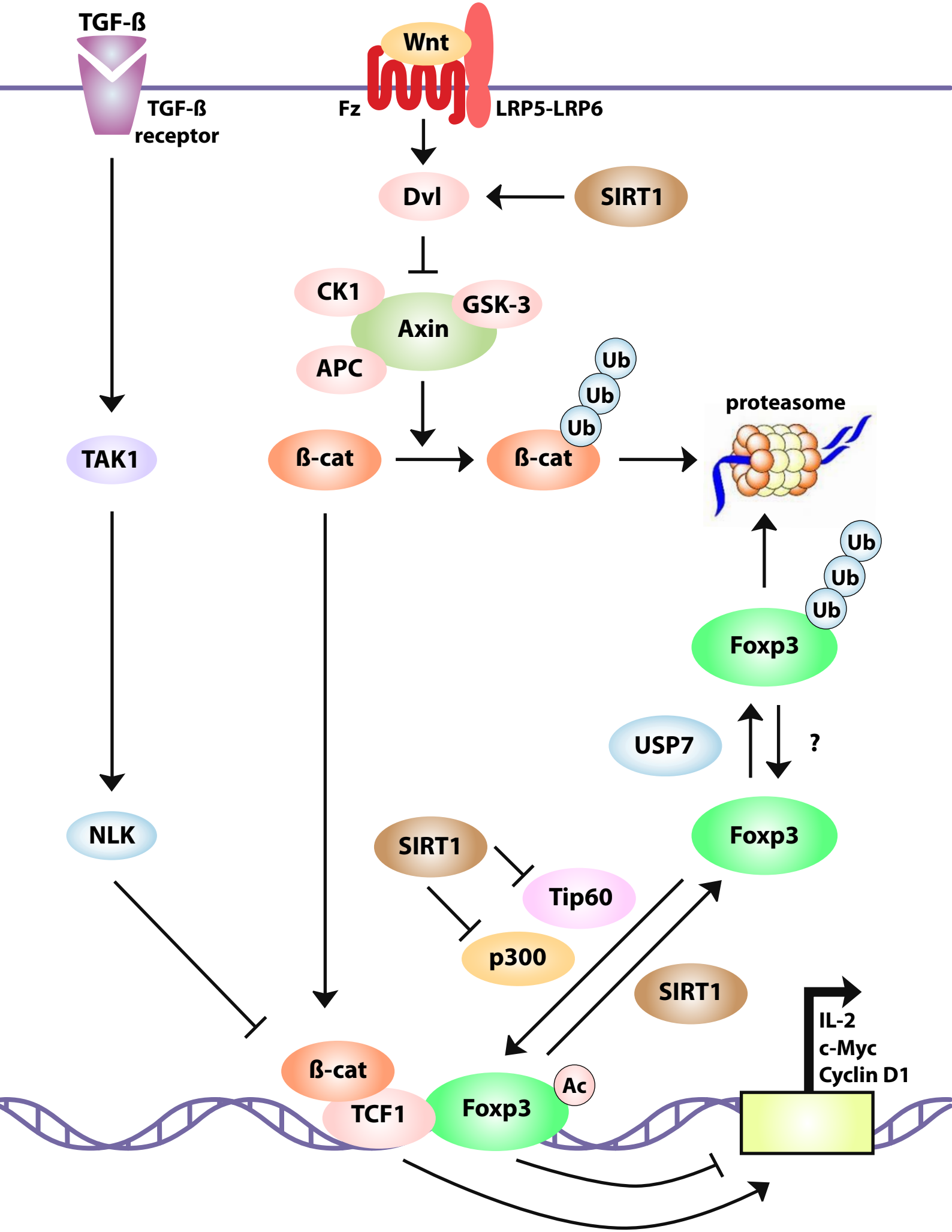
Figure 1. Molecular mechanisms regulating Foxp3 activity. Foxp3 can repress transcription of numerous genes including IL-2, c-myc, and cyclin D1. Reciprocally, ß-catenin/TCF1 activates transcription of the same genes. TCF1 is activated by canonical Wnt signaling. Here, Wnt binding to its cognate Frizzled receptor activates Dvl, which can abrogate a ß-catenin destruction complex. In the presence of Wnt, ß-catenin protein levels increase, ß-catenin translocates to the nucleus, associating with and activating TCF1. TGF-ß stimulation activates TAK1 and subsequently NLK, NLK can impair transcriptional activity of TCF1. Foxp3 protein levels are regulated by both acetylation and ubiquitination in a competition based fashion. Acetylated Foxp3 is has a higher affinity to associate to DNA, Foxp3 can be acetylated by Tip60 or p300 and can be deacetylated by SIRT1. Deacetylated Foxp3 can be poly-ubiquitinated which results in proteasomal degradation. Foxp3 de-ubiquitination is mediated by USP7.

Figure 2. Possible ß-catenin-TCF1-Foxp3 complex formations. **a**, All three proteins associate with each other. **b**, ß-catenin acts as a scaffold associating TCF1 with Foxp3. **c**, ß-catenin or Foxp3 associate independently with TCF1.

Figure 3. Potential regulatory mechanism of transcritpional activation/repression. In the absence of Wnt, TCF1 will be inactive, here Foxp3 will be the dominant treanscription factor resulting in suppressive Treg. In the presence of Wnt, ß-catenin can activate TCF1 shifting the balance toward TCF1, thereby overruling Foxp3 and resulting in non-suppressive Treg.

Figure 4. Model of Wnt mediated regulation of immune responses. Immune homeostasis is maintained by Treg which suppress Teff cell activation and proliferation. Upon infection or

immune activation T cells are activated, resulting in Wnt3a production. Wnt3a activates its receptor, resulting in TCF1 activation increasing IL-2 transcription. In addition, Wnt signaling in Treg abrogates Foxp3 transcriptional activity, thereby reducing Treg suppressive capacity. During termination of immune activation, Wnt3a levels are reduced, restoring Treg mediated suppression and immune homeostasis. Furthermore, we propose that dysregulated Wnt3a production, as observed in the synovium of juvenile arthritis patients, inhibits Treg function, resulting in an uncontrolled immune response and inflammation.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TGF-ß receptor
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proteasome
TAK1
ß-cat
ß-cat
Foxp3
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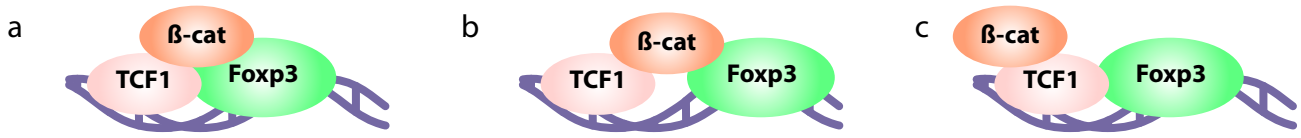
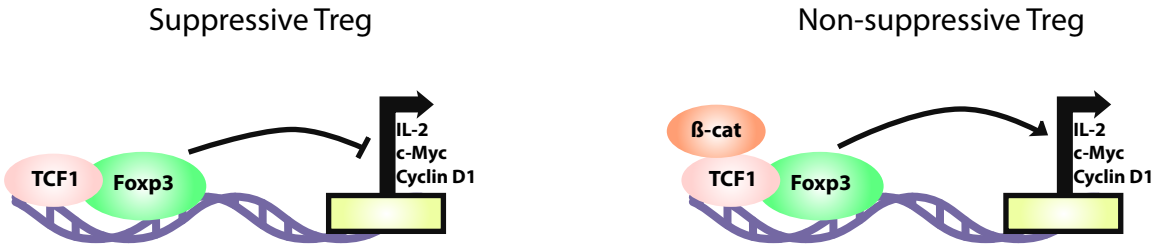


Figure 2


Suppressive Treg
TCF1
Foxp3
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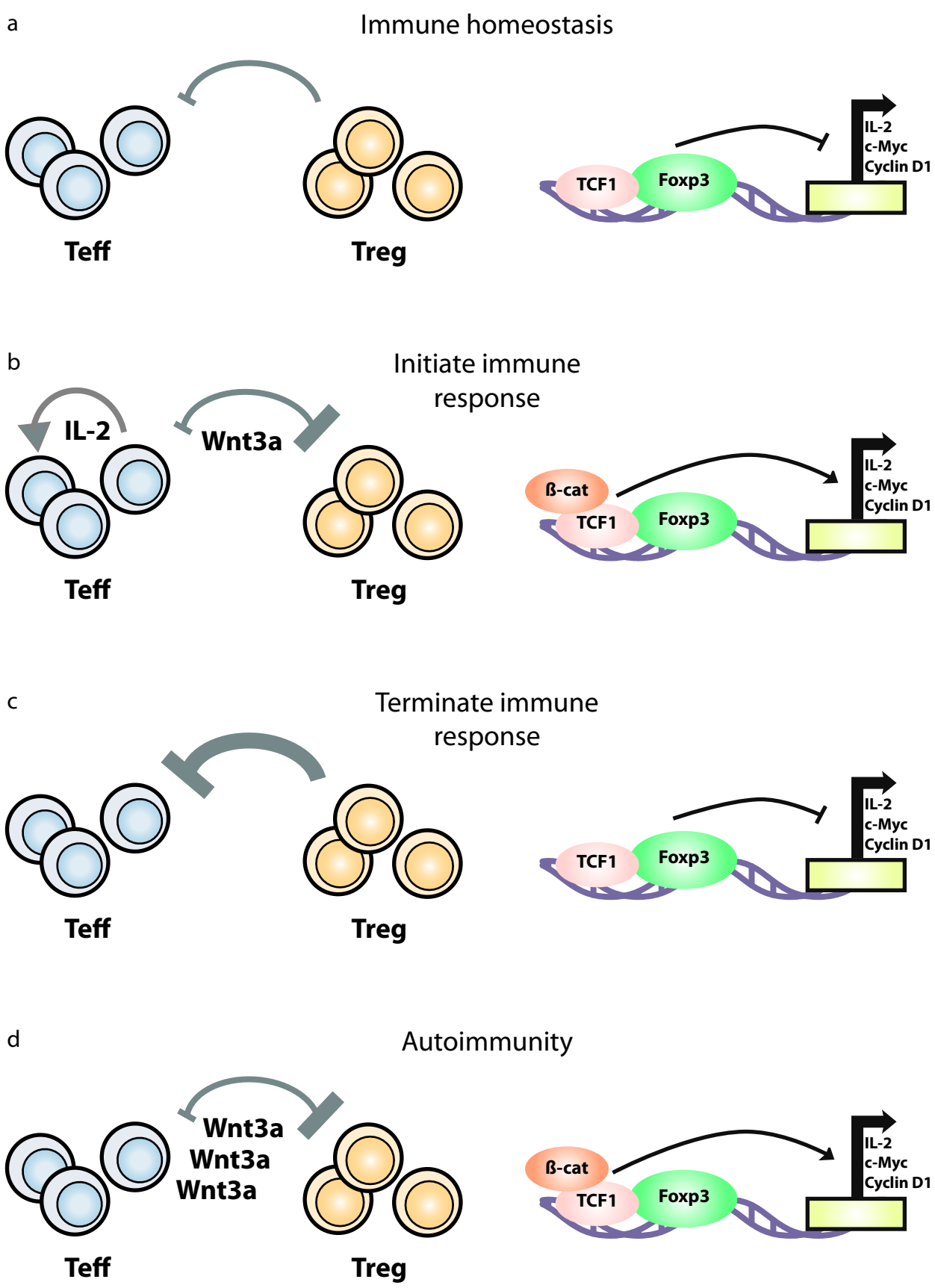
Cyclin D1
Non-suppressive Treg
ß-cat
TCF1
Foxp3
IL-2
c-Myc
Cyclin D1

Figure 3


a
Immune homeostasis
Teff
Treg
TCF1
Foxp3
IL-2
c-Myc
Cyclin D1
b
Initiate immune response
IL-2
Wnt3a
Teff
Treg
ß-cat
TCF1
Foxp3
IL-2
c-Myc
Cyclin D1
c
Terminate immune response
Teff
Treg
TCF1
Foxp3
IL-2
c-Myc
Cyclin D1
d
Autoimmunity
Wnt3a
Wnt3a
Wnt3a
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Treg
ß-cat
TCF1
Foxp3
IL-2
c-Myc
Cyclin D1

Figure 4