The molecular regulation of Forkhead box P3

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Master program: Infection and Immunity

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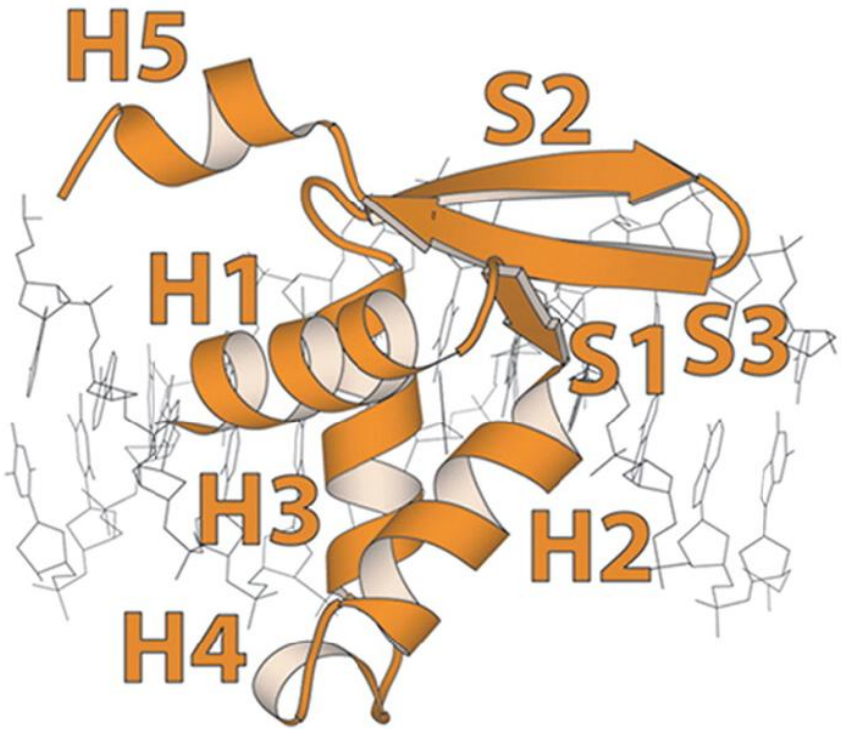

A cartoon representation of FOXP2, shown as ribbon drawings, bound to DNA, which is shown as a wire frame. H1 to H5 are alpha-helices and S1 to S3 form a three-stranded antiparallel β-sheet. Figure from Stroud et al., 2006 [1].

Outline

I. Introduction

The Forkhead Box transcription factor FoxP3 (Forkhead Box P3) is exclusively expressed in regulatory T cells [2, 3]. Clinical consequences of mutations that abrogate FoxP3 function are severe, reflecting both its pivotal role in regulatory T cells (Tregs) and the importance of these cells in providing peripheral immune tolerance [4, 5]. Although the amount of research on Treg and FoxP3 has greatly expanded in the last decade, several questions about these topics still remain unanswered. Relatively little is known about FoxP3 regulation at the molecular level and although a wide range of molecules that influence FoxP3 expression and activity have been identified and characterized, the contribution of the individual molecules to FoxP3 expression and activity *in vivo* is still under debate. This thesis will describe the role of FoxP3 in regulatory T cells, the features of the *FoxP3* gene and protein, the downstream targets of FoxP3 and finally the regulation of FoxP3. The focus will be strongly on the regulation of FoxP3; especially the different signal transduction pathways involved in regulation of FoxP3 expression and epigenetic regulation of FoxP3 expression as little is known about the post-transcriptional levels. Both murine FoxP3 and human FoxP3 will be denoted in this thesis as FoxP3, when specifically discussing one of the two species, the species will be mentioned.

Central tolerance is established in the thymus and bone marrow where progenitor lymphocytes are deleted upon reacting to self-antigens (Figure 1). However, some developing lymphocytes escape this selection and progress to become mature immune cells capable of mounting an autoimmune response (reviewed by Starr *et al.,* 2003) [6]. Several possible fates await these cells when encountering their specific antigens, depending on co-signalling from antigen presenting cells (APCs), the environment and the strength and duration of the T cell receptor (TCR). One possibility is that they become activated and initiate an immune response to their self-antigens. Another option is that they enter an anergic state and become functionally impaired or undergo apoptosis or cell-mediated death; the tolerance to auto-antigens established in this fashion is called peripheral tolerance (Figure 1) [6]. Regulatory T cells (Tregs), either directly derived from the thymus or induced from naive T cells, are capable of suppressing other immune cells and are therefore pivotal for establishing peripheral tolerance (reviewed by Piccirillo *et al.,* 2004) [7].

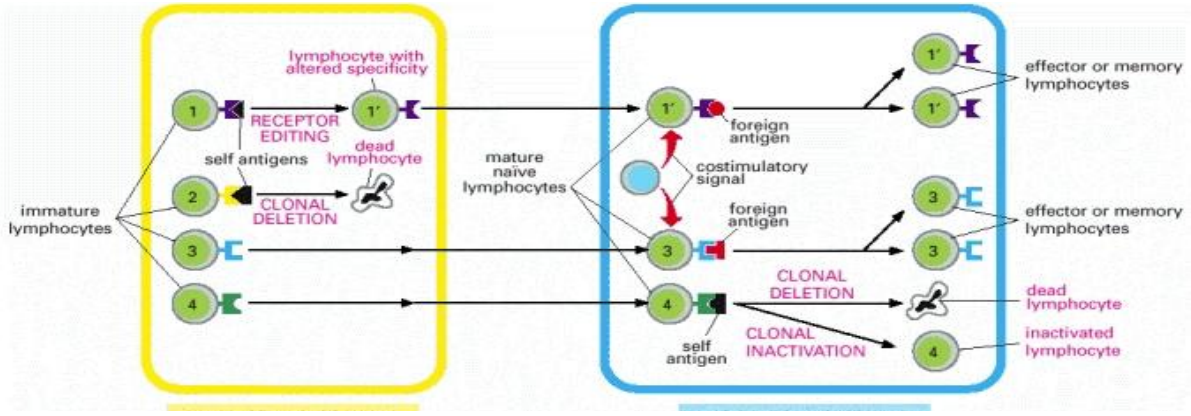

Figure 1. Mechanisms preventing auto-immune responses. Central tolerance is established in the thymus where immature lymphocytes recognizing self-antigens are either deleted or edited. Self-reactive lymphocytes escaping this mechanism are deleted or inactivated in the periphery in response to co-stimulatory signals provided by antigen presenting cells. Figure from Molecular Biology of the Cell. 4th edition. Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002.

The importance of correctly functioning FoxP3 for these Tregs and thus for the immune tolerance is illustrated by loss-of-function mutations in mice that lead to a phenotype called scurfy [8]. These mutations were first located to the *FoxP3* gene in 2001 by Brunkow *et al.* [4]. The *FoxP3* gene is X-linked and as the mutation is recessive it solely affects hemizygous males. Hallmarks of the scurfy mice are overproliferation of $CD4^+CD8^-$ T-cells, multi-organ infiltration and elevated cytokine levels, cumulating in death at 2-3 weeks of age. Lymphadenopathy, hypergammaglobulinemia and dermal inflammatory cell infiltration are common phenotypes in scurfy mice. All these features result from a failure to regulate $CD4^+CD8^-$ T-cells activity, due to a lack of regulatory T-cells (Tregs) [9-12]. Very similar to scurfy mice, FoxP3 mutations in humans cause the immune disorder IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) (Figure 2) [5]. This disorder was first described in 1982 and the precise incidence of this rare disease is still unknown [13]. The disease affects very young, male children and common symptoms are: dermatitis, cachexia, growth retardation, type 1 diabetes mellitus and enteropathy, the latter resulting in severe diarrhea. IPEX syndrome can lead to thyroiditis, hypothyroidism, haemolytic anemia, recurrent infections and is lethal in the first two years of life if left untreated; all symptoms caused by a Treg deficiency [14-16]. Therapy consists of bone marrow transplantation or potent immunosuppressive drugs; both have severe side effects and low efficacy, demonstrating the need for new therapeutic agents [13]. While FoxP3 is the master regulator of Tregs, continuous expression is required to confer suppressive phenotype to T cells (Figure 4). This is illustrated by the transient expression of FoxP3 in activated naive T cells (Tnaive) that do not gain suppressive capacities [2, 17, 18]. In addition, loss of FoxP3 expression abrogates the suppressive capacities of T cells [19-21]. These findings show FoxP3 mediated regulation dictates T cell phenotype and is therefore an interesting target for clinical intervention in T cell-associated diseases.

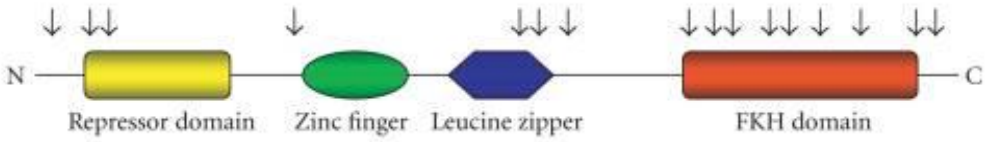

Figure 2. Location of the various mutations in the human FoxP3 gene causing IPEX in relation to the different conserved domains. Figure from Van der Vliet et al., 2007 [22].

In short, two mechanisms control *FoxP3* gene expression: epigenetic markers, modulating availability of the regulatory regions of the gene for the transcriptional machinery; and signal transduction pathways, ultimately affecting recruitment of the transcription machinery. In addition, FoxP3 mRNA (messenger RNA) stability and FoxP3 protein activity and stability are directly regulated [23, 24]. These mechanisms will be described in this thesis with the emphasis on the signal transduction pathways as these are studied most extensively. In addition to the different pathways, emphasis will be placed on the mediators of crosstalk between the different pathways as these could prove suitable targets for clinical intervention. The goal of this thesis is to provide an overview of what is known about FoxP3 regulation, and to describe which areas require further investigation. Ultimately, further research into the regulation of FoxP3 may lead to more insight in which molecules and mechanisms are critical in the regulation of FoxP3 and therefore in the function of regulatory T cells. Identifying those pivotal molecules will provide targets for intervention in cases where there is

dysfunctional FoxP3 regulation. These targets can subsequently be investigated for feasibility in treating diseases associated with dysfunctional Treg biology, such as IPEX, transplantation tolerance, cancer and viral infections. This might provide better therapeutic options than universally suppressing the immune system, which has severe side effects.

II. FoxP3 is the master regulator of Treg development and function

Peripheral tolerance is established by T cells outside the thymus and supplements the central tolerance to self-antigens created in the thymus (Figure 1). Tregs play a pivotal role in creating peripheral tolerance. This was first shown by performing neonatal thymectomy in mice or adult thymectomy followed by x-ray irradiation in rats, both leading to autoimmune disease. Transferring $CD4^+$ T cells from normal animals could prevent onset of autoimmune disease. These findings suggested that the thymus produces $CD4^+$ T cells that prevent autoimmunity [25]. Tregs have the ability to suppress the activation, proliferation and effector functions of several immune cells, including natural killer (NK) and NKT cells, B cells and antigen-presenting cells (APCs) [26] [27]. Numerous different ways in which Tregs suppress effector T cells (Teff) have been described, including cell-mediated cell death, cytokine signalling and induction of apoptosis (Figure 3), reviewed by Vignali *et al.,* 2008 [28]. How Tregs exert their effect and their role in different diseases is still under intense investigation. Tregs are also implied in transplantation tolerance [29], clearance of viral infections [30] and cancer (reviewed by Mougiakakos *et al.*) [31]. In cancer high levels of Tregs frequently accumulate in the tumour, disrupting Teff function and providing protection for the tumour [31]. In contrast, in several autoimmune diseases the functionality or number of Tregs is impaired, leading to an aberrant immune response to auto-antigens [29].

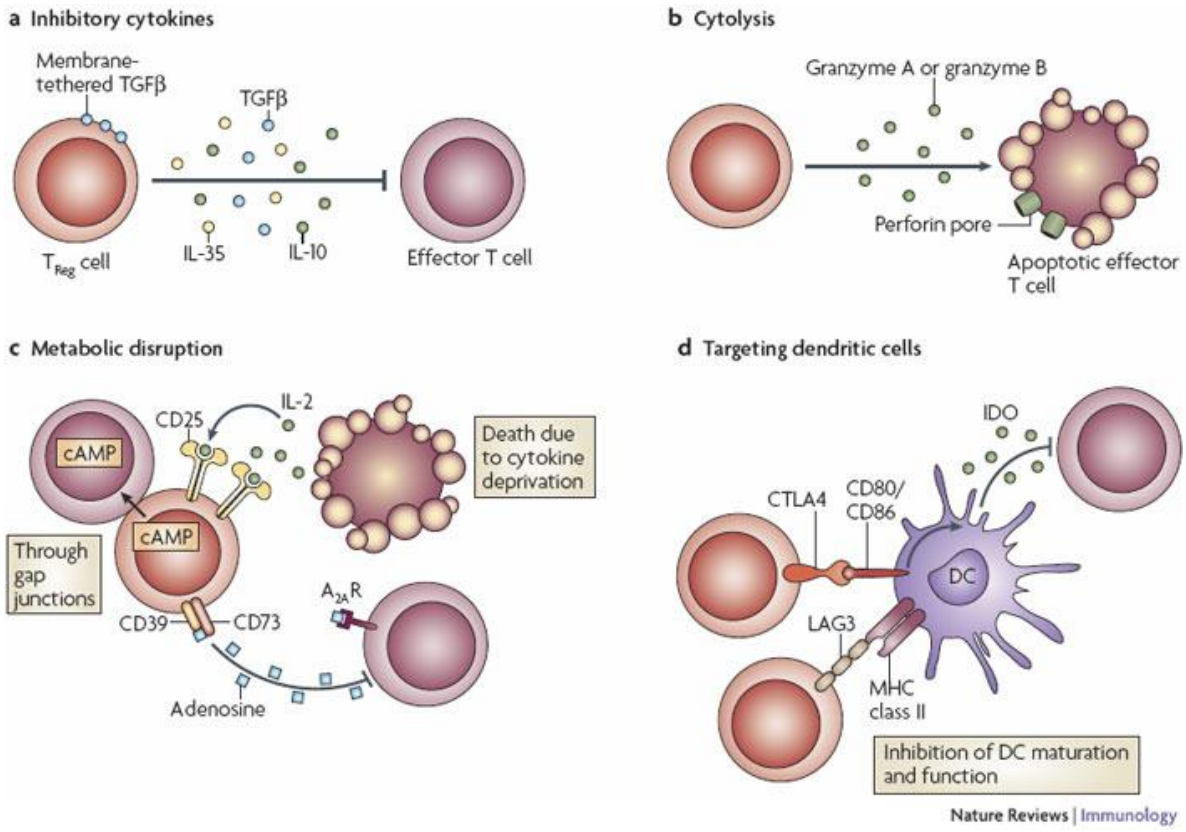

Figure 3. Regulatory T cells suppress effector T cells through a variety of mechanisms, including (A) signalling through inhibitory cytokines, (B) cytotoxic mechanisms, (C) competition for cytokines and (D) inhibiting APC function. Figure from Vignali et al., 2008 [28].

There are two major types of FoxP3$^+$ Tregs; both are characterized by the expression of CD4, CD25 and FoxP3, while CD4$^+$CD25$^-$ T cells do not have a suppressive phenotype. The two FoxP3$^+$ Treg types are natural Tregs and induced Tregs. Thymus-derived Tregs are termed natural Tregs (nTregs) and have an antigenic specificity as broad as that of Tnaive [32]. In addition to these natural Tregs, naive T cells can also differentiate into antigen-specific Tregs upon chronic stimulation with an antigen, the so-called induced Tregs (iTregs). Differentiation into Tregs requires TGF-β and is facilitated by dendritic cell-delivered retinoic acid (RA) [33]. While these natural and induced Tregs differ in epigenetic markers (discussed later, Figure 7) and comprise two distinct cell populations, there is not yet a single marker distinguishing between them [34]. Several other Treg populations have been defined (Table 1); characterizing them is difficult due to heterogeneity in gene expression profile, phenotype and suppressive capacity. As research is still ongoing, the division made here is still subject to change [35-37]. Importantly, not all Treg populations express FoxP3; nonetheless, its pivotal role in inducing peripheral tolerance and preventing autoimmune disease is illustrated by IPEX patients and scurfy mice.

Treg population	**Express FoxP3**
CD4$^+$CD25$^+$ Foxp3$^+$ cells	**+**
Interleukin 10 (IL-10)–producing 'Tr1' cells [38]	-
Transforming growth factor-β (TGF-β)–producing T helper type 3 cells [39]	**-**

CD8+ T suppressor cells [40]	+ [41]
Natural killer T cells [42]	- [43]
CD4–CD8–T cells [44]	- [45]
γδ T cells [46]	- [47]

Table 1. The different Treg populations and expression of FoxP3 by these different populations.

FoxP3 is essential for development and functional maintenance of Tregs

Foxp3 was described as a master control gene for mouse Treg development and function with expression of FoxP3 being both necessary and sufficient for the development for regulatory T cells [3, 48, 49]. When naive mouse T cells are transduced with FoxP3 they start to upregulate the expression of other Treg markers, while the production of molecules associated with Teff is repressed. These downstream molecules of FoxP3 will be discussed in more detail later (Figure 5). In addition, FoxP3 inhibits the differentiation of naive T-cells to Th17-cells (T helper 17), a pro-inflammatory T cell closely linked to Tregs [50]. The fact that ectopic expression of FoxP3 induces Treg development and function, combined with the consequences of the loss-of-function mutations, implies FoxP3 expression in mouse $CD4^+$ T cells is sufficient to mark these T-cells as regulatory T cells in mice [51]. While in mice FoxP3 expression is restricted to Tregs, stimulation of the T cell receptor (TCR) induces FoxP3 expression in human $CD4^+CD25^-$ effector T cells. In several studies it was shown that this expression does not lead to anergy or suppressive properties [2, 17, 18]. Importantly, this expression is transient and FoxP3 is expressed at lower levels compared to natural Treg cells (Figure 4) [52, 53]. The biological reason for this transient upregulation is currently unknown but one hypothesis is that it may function as an intrinsic brake to regulate activation of Teff cells [54]. Prolonged FoxP3 expression can confer regulatory competence to activated T-cells [18, 55-57]. The precise regulation of transient versus constitutive expression is not yet known but epigenetic markers are likely involved [58]. The epigenetic regulation of FoxP3 will be discussed in more detail later. Loss of FoxP3 expression impairs the suppressive capacities of Tregs, showing constitutive expression of FoxP3 is required to maintain a regulatory T cell phenotype [19-21]. Thus, tight regulation of FoxP3 dictates T cell phenotype, making it an interesting subject for clinical interference in T cell-associated diseases. While FoxP3 regulation has been extensively studied, the contribution of the individual molecules to the regulation of FoxP3 is still unclear.

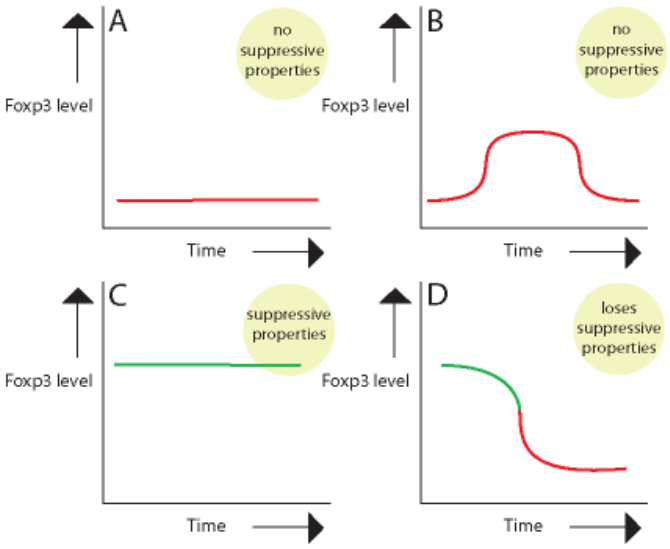

Figure 4. Continued high level of FoxP3 is required to confer suppressive properties to CD4$^+$ T cells. A. Low levels of FoxP3 expression does not lead to suppressive capacity in T cells. B. Transient expression of FoxP3 after TCR activation in low amounts compared to that observed in Tregs does not confer suppressive properties to these cells. C. Continued high level of FoxP3 leads to suppressive capacity. D. Loss of FoxP3 expression in a regulatory T cell leads to loss of suppressive potential

III. Treg program coordination by the transcription factor FoxP3

When naive mouse T cells are transduced with FoxP3 they upregulate the expression of Treg markers, including CD25, cytotoxic T-cell associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR). In contrast, production of pro-inflammatory cytokines such as IL-2, IFN-γ and IL-4 is repressed [49]. FoxP3 achieves this by forming transcriptional complexes with nuclear factor of activated T cells (NFAT), acute myeloid leukemia-1 /runt-related transcription factor 1 (AML1/Runx1) and HDACs [54]. From this we may conclude that FoxP3 is the transcription factor controlling the regulatory T cell program.

An experimental model in which green fluorescent protein (GFP) was inserted in the mouse *FoxP3* gene was created by Gavin *et al.*, 2007 [53] As this mutation was introduced in female mice, only half of the T cells contained this mutation due to random inactivation of the X-chromosome. This was required as full deletion of FoxP3 leads to a scurfy phenotype followed by death, seen in hemizygous males [4]. This model allowed dissection of the FoxP3 dependent from the FoxP3 independent features in Tregs. Their data suggests that FoxP3 both amplifies and stabilizes expression of several proteins that negatively regulate T-cell activation in conventional T cells upon TCR stimulation. These molecules include: Fibrinogen-like protein 2 (Fgl2), CD73, CD39 and CTLA-4. FoxP3 also represses pro-inflammatory cytokines including IL-4, IFN-γ, tumour necrosis factor α (TNF-α), IL-17 and IL-21. Moreover, FoxP3 modulates signalling and surface molecules in order to change the response to environmental cues and promote Treg stability. Downregulation of phosphodiesterase 3b, cGMP-inhibited (PDE3b) by binding of FoxP3 to a conserved intronic region in the *PDE3B* gene is crucial in this [53].

Three genome wide studies investigating FoxP3 have been conducted, all combining chromatin immunoprecipitation (ChIP) with expression profiling using microarrays. The study by Marson *et al*. in 2007 [59] used a mouse cell-line hybridoma, the second study used mouse Tregs [60] and the most recent study made use of human Tregs [61]. Of the 3836 genes associated with FoxP3 bound regions in humans and the 1977 genes associated with FoxP3 bound regions in mice, 888 genes are shared between the two species. Thus, 23% of the genes that are FoxP3 targets in humans are also FoxP3 targets in at least one of the mouse studies, with 107 genes that are common to all three data sets [59-61].

The study using hybridomas showed that most promoters bound by FoxP3 in stimulated cells were also occupied in unstimulated cells. Furthermore, unstimulated $FoxP3^+$ and $FoxP3^-$ T cells had only few differentially expressed genes. However, upon stimulation with PMA/ionomycin significant differences in expression in almost 1% of mouse genes was found between $FoxP3^+$ and $FoxP3^-$ cells, showing FoxP3 might exert most of its effects after stimulation [59]. Of the FoxP3-dependent genes approximately 10% is directly regulated by FoxP3. Importantly, FoxP3 transactivates genes of other transcription factors and miRNAs, indirectly influencing mRNA and protein levels (Figure 5). Among transcription factors upregulated by FoxP3 are PR domain zinc finger protein 1 (Prdm1), IRF-4, IRF-6, Helios, STAT5, Blimp1 and cAMP-responsive element modulator (Crem). These transcription factors target several FoxP3-dependent genes that are not directly occupied by FoxP3 and are Treg-associated molecules, including Fibrinogen-like protein 2 (Fgl2), IL-10 and Epstein-Barr virus induced gene 3 (Ebi3) [60]. Of the FoxP3-bound genes approximately 35% and 6% were upregulated in Tregs in the thymus and in the periphery respectively. A smaller proportion was downregulated in both the thymus and periphery [60]. The observation that only a small part of the FoxP3 bound genes are differentially regulated could be due to cofactors that are recruited by FoxP3 and that are required for expression. This is illustrated by the enrichment in the FoxP3 bound genes for the presence of a NFAT DNA sequence motif neighbouring the FoxP3 binding sites [59].

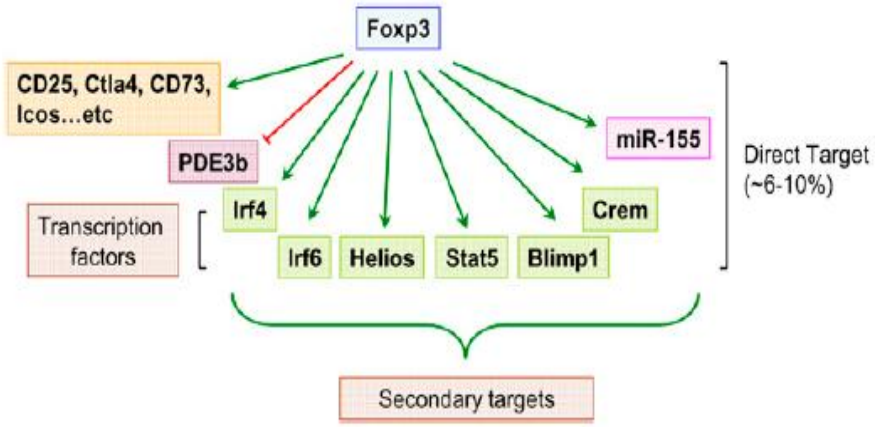

Figure 5. Representation of direct targets of FoxP3, consisting of transcription factors, microRNA, surface molecules and signalling pathways. 6-10% of the genes regulated by FoxP3 are regulated directly, while the majority is regulated indirectly. PDE3b = phosphodiesterase 3B, cGMP-inhibited. miR-155 = microRNA-155. Crem = cAMP-responsive element modulator Figure from Lu & Rudensky, 2008 [158]

Cluster analysis of the genes bound by FoxP3 shows these genes are enriched in genes that are implicated in TCR signalling, cell communication (CD25, Ctla4, neuropilin 1 (Nrp1) and Icos) and transcriptional regulation (Mitogen-activated protein kinase (MAPK) and PDE3b) [59-61]. Additionally, analysis of the differentially expressed genes in the thymus that were occupied by FoxP3 showed a group of genes involved in chromatin modifications and controlling gene expression [60]. Also, enrichment in genes involved in proliferation, activation and cell death in T cells in normal immune responses as well as inflammatory and autoimmune conditions was observed [61]. In the 888 FoxP3 target genes conserved between species a significant higher proportion of differentially expressed genes existed [61]. Among these conserved targets, genes encoding for the TCR signalling pathway (TCR and CD28), CTLA4 signalling and MAPK signalling were enriched [61]. As TCR signalling controls FoxP3 expression, this creates a feedback loop in which FoxP3 protein influences the expression of its own gene.

IV. Forkhead box protein P3 (FoxP3)

FoxP3 belongs to the family of Forkhead box transcription factors that contain an evolutionarily conserved 110 amino acid DNA binding domain called winged helix or forkhead domain [62]. FoxP3 belongs to the FoxP subfamily of this family of Fox proteins, characterized by a leucine zipper and classical zinc finger motif [63]. Other members of this subfamily are FoxP1, FoxP2 and FoxP4 [64]. While FoxP3 expression is restricted to the adaptive immune system [3], the other FoxP subfamily members are involved in a wide range of processes. FoxP1 is an important regulator of heart, brain, lung, kidney, testis, gut, macrophage and B-cell development in mice [65-69]. FoxP2 is involved in the development of the heart, lung and gut [68, 70], while FoxP4 regulates gut and pulmonary development [71].

Both the *FoxP3* gene and protein have been well characterized and consist of several domains well conserved throughout evolution (Figure 6) [4, 72]. The gene encoding FoxP3 consists of eleven coding and three non-coding exons [4]. Two non-coding exons (-2a and -2b), which are separated by 640 base pairs (bps), are conserved at the extreme 5'-end. They splice to another non-coding exon (-1) 5000 bps further downstream [4]. These exons contain important regulatory elements that will be discussed in more detail later. N-terminally, a proline-rich repressor domain (PRR) exists that has an inhibitory effect on the NFAT (nuclear factor of activated T cells) mediated transcription of genes [26, 72]. The leucine zipper domain is essential for homodimerization of FoxP3, which in turn is essential for FoxP3 function [72]. This can be explained by the requirement of FoxP3 to form at least a homodimer in order for the forkhead domain to bind DNA with high affinity [73]. However, independently of the leucine zipper domain, FoxP3 can heterodimerize with the transcription factors NFAT, NF-kB (nuclear factor kappa B) and AML1/Runx1 (acute myeloid leukemia-1 /runt-related transcription factor 1) to regulate Treg functionality. The forkhead domain (FHD), encoded by exons 9, 10 and 11, has both a DNA binding function and a repressor function. This repressor function is dependent on interaction with other transcription factors such as NFAT [54]. Additionally, both ends of the FHD are involved in targeting of FoxP3 to the nucleus [72]. Zinc finger domains generally function as DNA binding domains, found in 3% of the human genes [4, 73, 74]. However no specific functions have been ascribed to the zinc finger domain in FoxP3 and although the domain is conserved in the FoxP subfamily, mutations in this domain are not associated with IPEX [63, 72, 73]. Of all IPEX patients, 60% have missense mutations

in the FKH domain, with other mutations being distributed throughout the gene, demonstrating the importance of the FKH domain for FoxP3 function (Figure 2) [75].

FoxP3 isoforms

While in mice only one FoxP3 isoform is expressed, three different FoxP3 splice variants exist in humans. In addition to the full length variant, one splice variant misses exon 2 [55], FoxP3Δ2, and the third variant misses both exons 2 and 7, FoxP3Δ2Δ7 [76]. Exon 2 encodes a protein interaction domain, which is required for binding to the retinoic acid receptor-related orphan receptors-α and γt (RORα and RORγt) (Figure 6) [77] [78]. This splice variant of FoxP3 also lacks N-terminal residues 67-198, which mediate the interaction with, and repression of, NFAT protein [72]. A histone deacetylator (HDAC) binding domain, which will be described in detail later, is also present in this region [26, 72, 79]. The splicing variant of FoxP3 lacking exon 7, which codes for part of the leucine zipper domain, shows this domain is required for FoxP3 suppressor function. The biological roles of both splice variants remain to be clarified, but the FoxP3Δ2Δ7 possibly exhibits a dominant negative effect over the full-length version [26, 80, 81]. The FoxP3Δ2 splice variant can induce a Treg phenotype and most of its effects are either redundant or additive to the FoxP3 full length splice variant effects [58, 80, 82, 83]. However, unlike the full length splice variant, the FoxP3Δ2 splice variant seems unable to repress Th17 differentiation through binding RORγt [82].

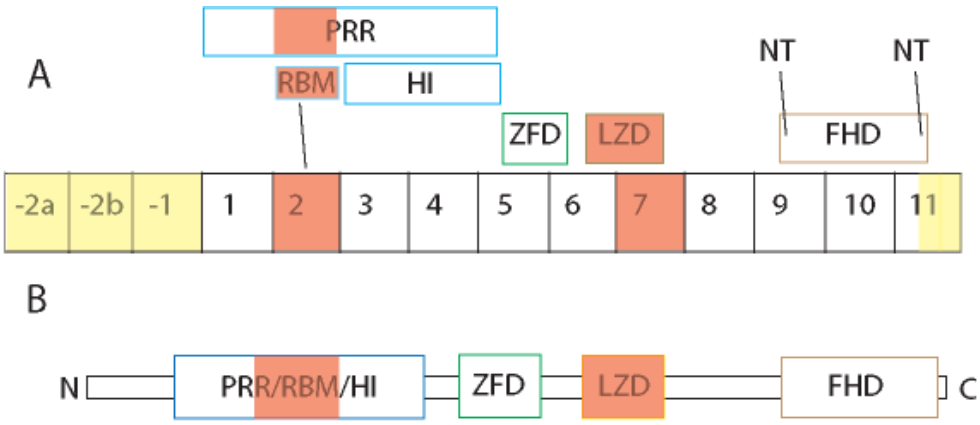

Figure 6. The conserved domains and regions of FoxP3. A. The exons of the FoxP3 gene showing the domains they code for as well as the non-translated regions (yellow) and splice variants (red). Only exons are depicted here. The two splice variants of FoxP3 miss exons 2, or 2 and 7 respectively, affecting the RBM and PRR or the RBM, PRR and LZD. B. FoxP3 protein with its conserved domains, the domains affected by the splice variants are again depicted in red. IE = intronic enhancer. RBM = ROR binding motif. PRR = proline-rich region. HI = HDAC interacting region. ZFD = zinc finger domain. LZD = leucine zipper domain. NT = region required for nuclear translocation. FHD = forkhead domain

IV. Mechanisms regulating FoxP3 expression levels

As described previously, tight regulation of FoxP3 expression levels and transcriptional activity is critical for correct development and functioning of Tregs. It is therefore not surprising that FoxP3 expression is regulated at different levels. An array of molecules influences transcription of the *FoxP3* gene. Furthermore, decay of FoxP3 mRNA is controlled and post translational modifications (PTMs) influence FoxP3 protein stability and protein activity [23, 24, 84]. Regulation of *FoxP3* gene transcription occurs through two main mechanisms. One mechanism consists of binding of transcription factors to the promoter or other regulatory elements of the gene, followed by recruitment of transcriptional complexes that either promote or inhibit gene transcription. Immediately upstream of the transcription start site is a FoxP3 region promoting transcription of the *FoxP3* gene, called the proximal promoter [85]. Several features characteristic of eukaryotic promoters, including TATA, GC, and CAAT boxes, consensus sequences that help recruit the transcriptional machinery, are found in this region. Additionally, several important transcription factor-binding sites (activator protein-1 (AP-1), NFAT) are located in this region [86]. Two well described regulatory elements are located in introns between -2b and -1, and upstream of the promoter called enhancer I and the upstream enhancer respectively [85]. In general, pro-inflammatory cytokines such as IL-4 and IL-6 have a negative effect on FoxP3 expression through mechanisms that will be explained in more detail later [87, 88]. IFN-γ (interferon-γ) however, exhibits complexity since it can both upregulate and downregulate FoxP3 expression through mechanisms discussed later [89]. Other molecules that have both a positive and a negative regulating effect in FoxP3 regulation are Notch and FoxP3 protein itself [90-93]. This mechanism and the most thoroughly studied molecules involved will be described in more detail. The second mechanism of regulation, discussed in the following section, is the generation of stable epigenetic markers on the *FoxP3* gene that change nucleosome structure to either allow or prevent the transcriptional complexes to access the *FoxP3* gene (Figure 7). A nucleosome consists of DNA wound around a complex of eight proteins, called histones.

Epigenetic: regulation of *FoxP3* gene transcription

Epigenetics describes the heritable regulation of gene expression without altering the underlying nucleotide sequence. Several epigenetic markers have been reported for the *FoxP3* gene, including histone modifications and methylation of the cytosine residue at CpG dinucleotides. Modifications on the tails of these histones influence chromatin structure and therefore transcription (Figure 7). The review by Lal & Bromberg, 2009 describes epigenetic mechanisms affecting FoxP3 transcription in more detail [85].

Approximately 60% of all human CpG dinucleotides are methylated and three DNA methyltransferases (DNMTs), DNMT1, DNMT3a, and DNMT3b, control DNA methylation of these CpG residues [94]. The first is involved in maintenance of methylation during cell division while the latter two are responsible for *de novo* methylation [94, 95]. Methylated CpG dinucleotides recruit histone deacetylases (HDACs) which remove the acetyl group from histone tails, increasing their positive charge. These positively charged histones then bind more strongly to the negatively charged nucleic acids, preventing transcription [96]. Conversely, acetylation of the histone tails, by histone acetyl transferases (HATs), introduces a negative charge on them, preventing histone binding to nucleic acids, making the DNA accessible to the transcription machinery (Figure 7). Other post-translation modifications (PTMs) that are common for histones are: phosphorylation, poly-ADP

(adenosine-di-phosphate) ribosylation, ubiquitination, and methylation affecting initiation, pausing and elongation of transcription [97]. Several studies have reported epigenetic markers on the *FoxP3* gene [85]. Addition of the methylation inhibitor 5-azacytidine (5-Aza) or knocking down Dnmt1 induces FoxP3 expression (Figure 5). HDAC inhibitors increase expression of FoxP3 in both $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells, indicating HDACs directly regulate FoxP3 expression and Treg development and function [98]

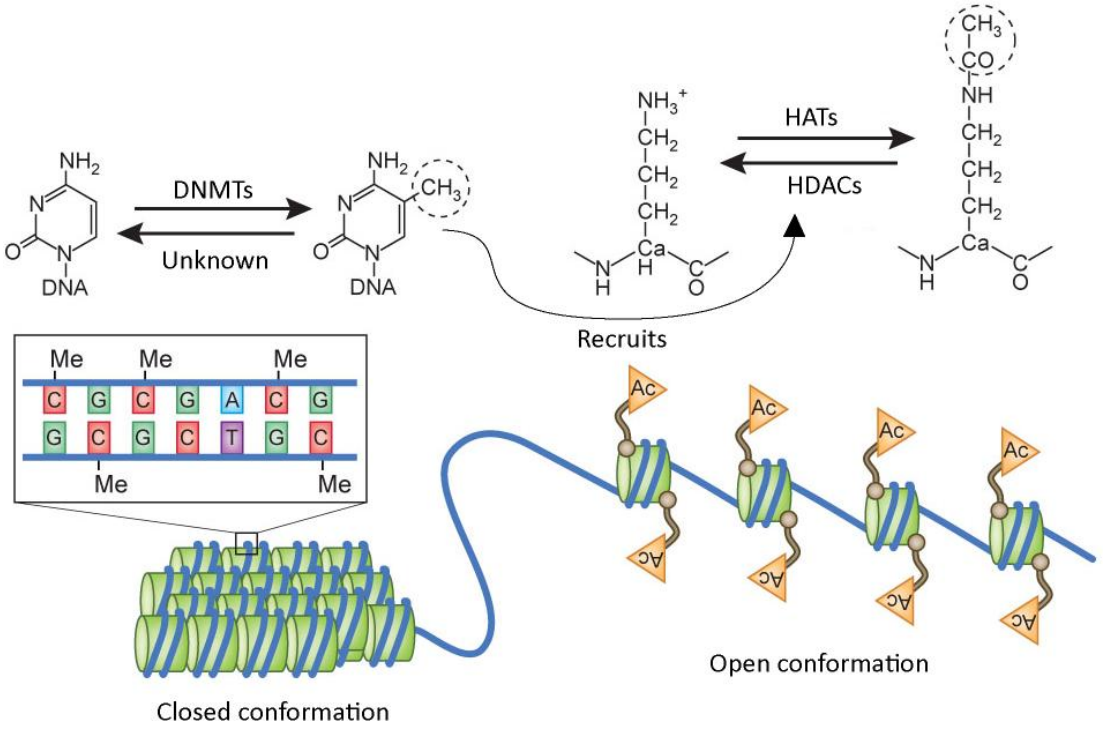

Figure 7. Epigenetic mechanisms regulating gene transcription. DNMTs methylate cytosine residues from CpG dinucleotides which leads to recruitment of HDACS that remove acetyl groups from histone tails. This leads to a "closed conformation" that prevents DNA transcription. In contrast, acetylated histone tails mediate an "open conformation" which allows transcription. DNMTs = DNA methyltransferases. Me = methyl-group. Ac = acetyl-group. HATs = histone acetyl transferases. HDACS = histone deacetylases. Adapted from Korzus et al., 2010 [99].

CpG methylation

Most CpG dinucleotides in the proximal promoter of the *FoxP3* gene are demethylated in natural Tregs while only partial demethylation is observed in naive or effector T cells [100]. TGF-β (transforming growth factor-β) signalling, which induces expression of FoxP3, was found to induce demethylation of the FoxP3 promoter and of the first CpG-containing conserved intronic region [101]. Demethylation of the latter region increases availability for cyclic-AMP response element–binding protein (CREB)/activating transcription factor (ATF). CREB/ATF acts downstream of the TCR and induces increased FoxP3 expression [101]. TGF-β signalling also leads to reduced DNMT expression through the inhibition of ERK (Extracellular signal-regulated kinases); this decreased DNMT expression leads to a lowered level of methylation of the FoxP3 promoter and thereby to upregulation of FoxP3 transcription [88, 101-103]. That inhibition of ERK is followed by decreased methylation has been previously observed in colon cancer cells [104]. IL-6 signalling induces methylation of the upstream

enhancer of FoxP3 by DNMT1, leading to repression of FoxP3 in natural Tregs. STAT3$^{-/-}$ mice showed that this repression of FoxP3 and induced methylation occurs in a STAT3 (Signal Transducer and Activator of Transcription 3)-dependent manner, as STAT3 induces transcription of DNMT1 [88].

Histone modifications

Di- and tri-methylation of histone H3 at lysine 4 (H3K4me2 and -3) are common modifications in regulatory regions of active genes. Indeed, in cells expressing FoxP3 and cells capable of turning on FoxP3 expression, these modifications are found near the transcription start site (TSS) and in the 5' untranslated region (UTR) of the *FoxP3* gene. Continued activation of the TCR induces demethylation of H3K4, providing a possible explanation for the observation that prolonged TCR signalling abrogates FoxP3 inducibility [105].

Another histone modification, H4 acetylation, is also associated with transcriptionally active genes. Retinoic acid (RA) is able to induce histone H4 acetylation, thereby inducing FoxP3 expression [106]. HDAC7 has been shown to deacetylate histones in the FoxP3 promoter can and in this way suppress transcription [107].

Signaling pathways regulating FoxP3 expression

Several distinct signal transduction pathways have been implied in the regulation of FoxP3 expression levels and the pathways that have been studied most extensively will be discussed here. The extensive crosstalk between the different pathways complicates the mechanisms by which they regulate FoxP3 expression. Additionally, environmental conditions, such as presence of certain pro-inflammatory interleukin, can alter the effect of a pathway on FoxP3 expression. Cis-regulatory feedback also exists as FoxP3 inhibits its own proximal promoter [93]. In general, signalling by other immune cells through ubiquitously expressed receptors or through binding of antigens to the TCR will initiate a cascade of intracellular events. Second messengers, such as cAMP (cyclic adenosine-mono-phosphate), will transduce the extracellular signal to an intracellular response, integrating and modulating extracellular signals. Intracellular proteins such as kinases and phosphatases transduce the signal, ultimately leading to the modulation of factors effecting gene expression, including transcription factors, HATs and HDACs.

TCR/IL-2

A two-step model in which first a TCR signal results in the upregulation of CD25 (IL-2R α-chain) in a precursor Treg was suggested by Burchill *et al.* [108]. This upregulation of CD25, mediated by protein kinase C (PKC), primes the precursor cell to receive an IL-2 signal inducing FoxP3 expression via STAT5 [108, 109]. This model is used to artificially induce Treg differentiation from a CD4$^+$ population by, stimulating naïve CD4$^+$ cells with anti-CD3 (antibody against TCR), anti-CD28 (antibody against the coreceptor of TCR) and IL-2 [110]. However, these *in vitro* induced Tregs are unstable compared to natural Tregs originating from the thymus as they can convert to Teff upon stimulation with pro-inflammatory cytokines, such as IL-6 and IL-1β. This conversion is mediated by repressed FoxP3 levels and addition of all trans-RA to these cells provides resistance to this effect [111].

In addition to CD25 mediated upregulation of FoxP3 levels, downstream of the TCR several transcription factors can directly regulate transcription of the *FoxP3* gene. These factors, including NFAT, AP1, CREB/ATF and NF-κB, bind to the FoxP3 promoter region or an intronic element proposed to serve as an enhancer [86, 101]. NFAT-AP1 binding possibly enhances the binding of Smad3 to the intronic enhancer [112]. The strength of the Major histocompatibility complex (MHC) ligand interacting together with the density and duration of TCR interactions determine whether FoxP3 expression will be induced [113]. TCR-induced proliferation may recruit cell-cycle dependent maintenance of DNMT resulting in a silenced state of the Foxp3 locus, which could explain this phenomenon [114]. Alternatively, the TCR downstream transcription factors NFAT and NF-κB upregulate GATA (transcription factor that binds to the GATA sequence) expression, which represses FoxP3 transcription [115, 116]. A third explanation is that prolonged TCR signalling induces protein kinase B (PKB) signalling, discussed later, which in turn suppresses FoxP3 expression [105].

IL-2 was shown to be essential for the development of natural Tregs in the thymus and IL-2 signalling has also been associated with the survival, expansion and function of Tregs in the periphery [117-121]. Knock-out models have shown that IL-15 and IL-7 can compensate for IL-2 loss in Treg development [122]. It was therefore proposed that common γ-chain (γc) signalling rather than IL-2 signalling per se is crucial to Treg development since this receptor subunit is common to the receptors of all three interleukins. Indeed, mice lacking the γc show a complete lack of $FoxP3^+$ Tregs [123-125]. STAT5, a transcription factor phosphorylated and activated by Jak (Janus kinase), is a downstream target of γc cytokine signalling [126-128]. Two STAT5-binding sites are located in both the promoter and the conserved region inside the first intron of the *FoxP3* gene. The binding of phosphorylated STAT5 to these sites mediates upregulation of FoxP3 expression (Figure 8) [127, 129]. Furthermore, a constitutively active form of STAT5 rescues Treg generation in absence of IL-2 and leads to expansion of $CD4^+CD25^+$ Tregs [108]. Another member of the STAT family, STAT1, is phosphorylated after IL-27 and IFN-γ stimulation of Th1 cells and directly interacts with the FoxP3 proximal promoter, resulting in increased FoxP3 expression [130]. Two other STATs downregulate the expression of FoxP3 (Figure 8); STAT3 binds and inactivates an enhancer located in the second intron of the *FoxP3* gene after it is activated by IL-6, IL-21 and IL-27 signalling [112, 131-133]. This inhibits binding of Smad3 to the enhancer in the first intron and STAT3 thereby downregulates FoxP3 expression. The second STAT downregulating FoxP3 expression is STAT6, which binds the FoxP3 promoter and inhibits FoxP3 expression induced by TGF-β [134, 135]. STAT6 phosphorylation is mediated by phosphorylated JAK3, which is phosphorylated itself after IL-4 binds to its receptor. IL-4, expressed by Th2 cells, in this way inhibits FoxP3 expression [134, 135]. Retinoic acid in the presence of TGF-β can inhibit the binding of STAT6 to the Foxp3 promoter and enhance histone acetylation, counteracting the effect of IL-4 [135]. In complex with NFAT and NF-κB, STAT6 also induces the expression of GATA3, which binds directly to the FoxP3 promoter and represses FoxP3 expression (Figure 8) [115, 116].

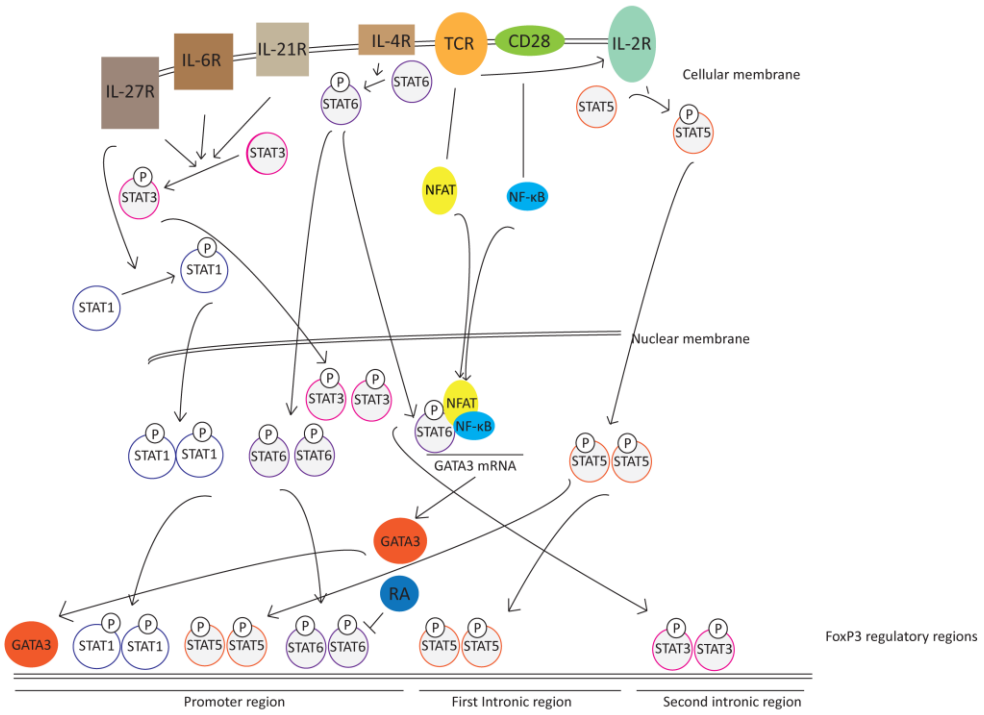

Figure 8. STAT signalling in the induction of FoxP3 expression. STAT5 can bind to the promoter region after phosphorylation and directly regulate FoxP3 transcription. STAT6 plays an important inhibitory role, both directly and through the induction of GATA3. Retinoic acid can partially revert the inhibitory effect of STAT6 by preventing its binding to the promoter region of FoxP3. STAT3, after activation by IL-6, IL-21 or IL-27 signalling can bind to the second intronic region of the FoxP3 gene which downregulates FoxP3 expression indirectly. STAT = Signal Transducer and Activator of Transcription. JAK = Janus kinase. NFAT = nuclear factor of activated T cells. NF-κB = nuclear factor κB. Figure is adapted from Shen et al., 2009 [123].

TGF-β/Smad

TGF-β regulates diverse cellular functions, playing an important role in cell fate, development and carcinogenesis. TGF-β signalling is primarily mediated by Smad proteins, although TGF-β can also activate Smad-independent pathways. After phosphorylation of the Smad proteins by the TGF-β receptor, they translocate to the nucleus where they regulate transcription after recruiting additional transcription factors [136]. TGF-β has been implicated in regulation of FoxP3 expression in both peripheral T cells and developing thymocytes [23, 110, 137, 138]. Membrane-bound TGF-β induces Smad3 phosphorylation which forms a complex with phosphorylated Smad4 and NFAT [139]. This complex binds to a conserved enhancer region in the intron between exon -2b and exon -1 [140] and this binding is facilitated by retinoic acid (Figure 9) [112]. Activity of this enhancer is required for the development of induced Tregs and Smad3 thus plays a pivotal role in the induction of FoxP3 expression in the thymus as well as in the periphery [140, 141]. The Smad3/Smad4 axis is inhibited by

Smad7, activated by IL-6R signalling, which in turn is downregulated by FoxP3. This leads to a positive feedback loop in which FoxP3 expression leads to more susceptibility to TGF-β signalling through the Smad3/Smad4 axis (Figure 9) [92]. Smad3 can also be directly recruited to a promoter site of FoxP3 in a so-called enhanceosome with c-reticuloendotheliosis (c-Rel), protein 65 (p65), NFAT and CREB that also induces FoxP3 expression (Figure 9) [142]. RA can bind to the nuclear receptors Retinoid A receptor (RAR) and Retinoid X receptor (RXR) which then bind to two sites in the enhancer in the first intronic region and in the FoxP3 promoter respectively [143]. This leads to increased acetylation of histones near the Smad3 binding site on the *FoxP3* gene, leading to an increased binding of Smad3 to the FoxP3 promoter and consequently to increased FoxP3 expression (Figure 9) [112, 140]. Deletion of the RAR binding site in the enhancer greatly reduces the upregulating activity of RA on FoxP3 expression and deletion of both sites leads to an almost complete loss of RA activity [112]

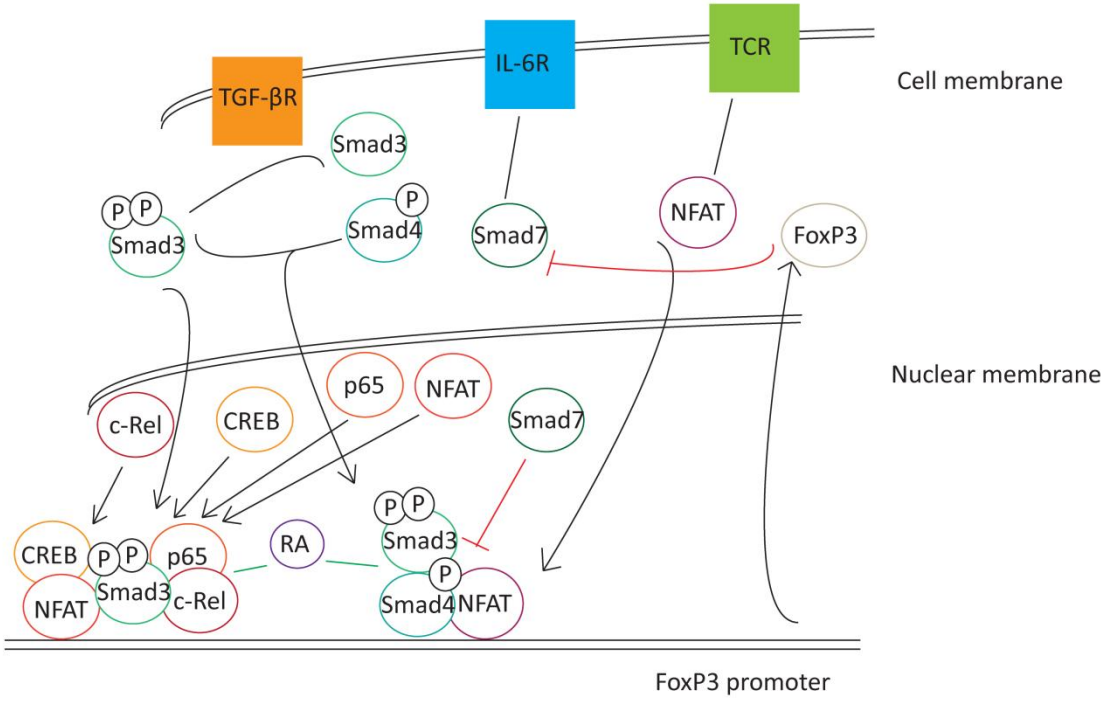

Figure 9. The TGF-β/Smad axis. TGF-β induces Smad3 that forms a complex with Smad4 and NFAT to induce FoxP3 transcription which in its turn releaves the inhibiting role of Smad7. The so-called enhanceosome complex consists of Smad3, c-Rel, NFAT, p65 and CREB. RA acetylates histones in the FoxP3 promoter and thereby induces Smad3 binding to the promoter. TGF-β = Transforming growth factor β, NFAT = nuclear factor of activated T cells. c-Rel = c-reticuloendotheliosis. NFAT = nuclear factor of activated T cells. p65 = protein 65. CREB = cAMP response element-binding. RA = retinoic acid

PKB/mTOR axis

The Phosphatidylinositol 3 kinase (PI-3K)/protein kinase B (PKB/c-Akt) signalling pathway plays an important role in cell growth and proliferation. In T cells the PI-3K/PKB/mTOR/FoxO axis is required for regulation of activation through the TCR [139]. Mammalian target of rapamycin (mTOR) is a kinase that regulates cell size, growth, proliferation, survival and metabolism; in the immune system mTOR

is involved in cell function in both the adaptive and innate immunity and regulates T cell proliferation and differentiation [144]. mTOR can form two different complexes with disparate functions: mTORC1 and mTORC2 respectively; while mTORC1 can be inhibited by rapamycin, mTORC2 is rapamycin-insensitive [145]. Four different FoxO transcription factor isoforms have been described, regulating a broad range of cellular processes including differentiation, survival, cell-cycle arrest, metabolism, stress resistance and tumour suppression [146].

PKB, under control of PI-3K, which is controlled by TCR signalling [105], as well as mTORC2, phosphorylates FoxO transcription factors, resulting in their exclusion from the nucleus. Since FoxO binding to regulatory elements in the FoxP3 promoter induces FoxP3 transcription, activation of this PKB pathway inhibits FoxP3 expression by preventing FoxO-mediated transcription (Figure 10) [147-149]. A FoxO-binding motif is located in the promoter and several consensus sequences are found in the FoxP3 locus [148, 149]. It was shown that proteins inhibiting PI3K, such as Casitas B-lineage lymphoma-b (Cbl-b), are required for efficient FoxP3 expression, while constitutively active PKB prevents FoxP3 induction [105, 150, 151]. Inhibition of mTORC2, which activates PKB, also leads to induction of FoxP3 expression [152]. The effect of inhibition of PI3K or mTOR on FoxP3 expression can be overcome by prolonged TCR signalling, possibly through epigenetic regulation [105, 153]. Knock-out experiments by Ouyang *et al.*, 2010 [149] showed two redundant FoxO transcription factors, FoxO1 and FoxO3a are important for this pathway. Deletion of both these transcription factors prevents induction, but probably not maintenance, of FoxP3 transcription [147, 149].

The PKB/mTOR axis possibly employs other mechanisms to inhibit Treg induction as PKB plays a role in the IL-6R signalling pathway, which has been shown to block FoxP3 induction and drive differentiation of naive $CD4^+$ T cells towards Th17 cells [50, 151, 154, 155]. Moreover, TGF-β signalling downregulates mTORC1 through impairing Extracellular-signal-regulated kinases (ERK) [156]. mTORC1 has been found to inhibit TGF-β induced FoxP3 expression, showing the mTORC1 complex is not only regulated by TGF-β but is also involved in the downstream signalling of TGF-β [151]. This gives an indication of the complexity of the PKB/mTOR pathway that consists of several feedback loops and different mTOR-containing complexes [153].

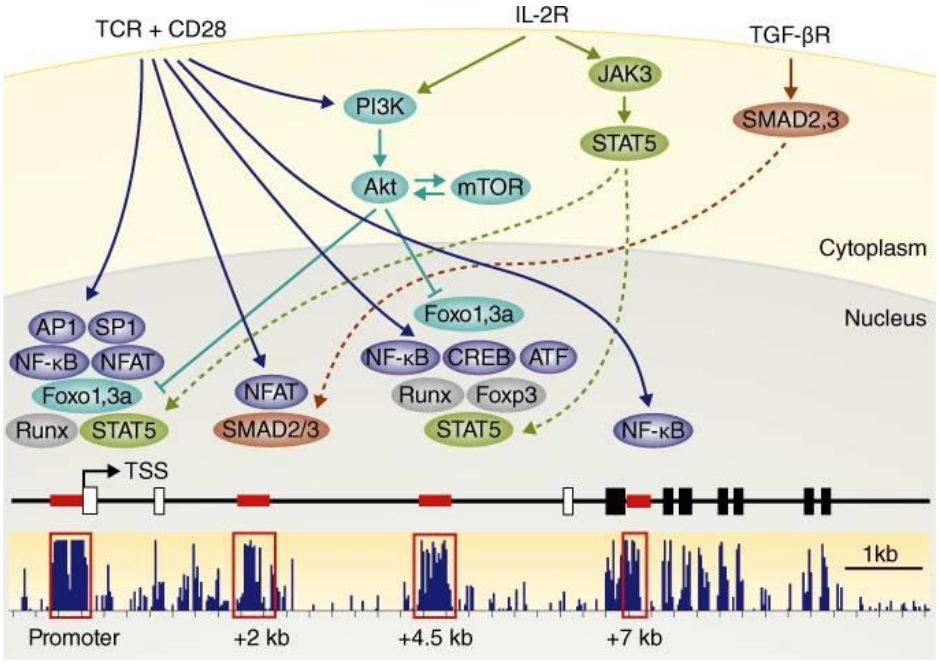

Figure 10. Signalling through mTOR/PKB axis. Upregulation of Akt (PKB) by PI-3K and mTOR inhibits Foxo-induced transcription of FoxP3. Foxo transcription factors are involved in inducing expression of FoxP3 at both the FoxP3 promoter and the second conserved intronic region. Both IL-2R and TCR signalling activate PI-3K and therefore prevent Foxo-induced FoxP3 expression. PI-3K = Phosphatidylinositol 3 kinase. PKB = protein kinase B. mTORC2 = mammalian target of rapamycin complex 2. FoxO = Forkhead box O. AP1 = Activator protein 1. SP1 = Specificity protein 1. Runx = Runt-related transcription factor. CREB = cAMP response element-binding. ATF = Activating transcription factor. TSS = transcription start site. Figure from Merkenschlager et al., 2010 [147]

Notch

Notch signalling is an evolutionary conserved mechanism for dictating cell fate which has also been implicated in regulating T cell maturation [157]**.** The membrane receptor family Notch consists of four receptors (Notch-1,-2,-3 and -4) and are receptors for Delta-like-1,-3 and -4 and Jagged-1 and -2 [158]. Membrane-bound TGF-β, which has been described to be involved in regulation of FoxP3 expression, can act through the Notch pathway [159, 160]. This was shown by blocking Notch cleavage, and therefore Notch intracellular domain (NICD) release, with γ-secretase inhibitor (GSI). Addition of GSI blocks TGF-β-induced FoxP3 expression, upregulation of genes downstream of FoxP3 and the suppressive capacities of Tregs [91]. Notch was later shown to have a dual role in the regulation of FoxP3 expression dependent on the level of Notch activity. Notch regulates FoxP3 via the NICD, cleaved after Notch activation. NICD exerts its effects on FoxP3 via the transcription factors CSL (CBF1/RBP-Jκ/Suppressor of Hairless/LAG-1) and Hairy and enhancer of split 1 (HES1) [91]. Binding of the NICD to CSL on a highly conserved CSL-binding site in the FoxP3 promoter induces recruitment of co-activators and the dislocation of repressors. HES1 can bind to a HES-binding site near the FoxP3 transcription start site. The first has a positive regulating effect, and dominates at low levels of Notch signalling, while the latter has a negative effect on transcription and is more important at higher levels. NICD alternatively interacts with Smad3 and CSL to activate the transcription of Hes1, showing TGF-β signalling can modulate Notch signalling [159 91]. Importantly, binding of NICD/RBP-J to

the FoxP3 promoter also occurs in $CD4^+CD25^-$ T cells showing Notch signalling might not be sufficient for Treg development [90].

IFN-γ/IRF

The cytokine Interferon-γ (IFN-γ) is a central player in regulation of immune responses. It is involved in anti-microbial responses, antigen processing, inflammation, growth suppression, cell death, tumour immunity and autoimmunity. IFN-γ can signal through the JAK/STAT pathway, leading to binding of STAT1 to Gamma Activating Sequences (GAS) in the promoters of target genes and regulation of various immune functions [161]. IFN-γ signalling also activates the Interferon regulatory factor 1 (IRF-1) which specifically represses FoxP3 transcription. It achieves this by binding a conserved IRF consensus element sequence (IRF-E) in the FoxP3 promoter, thereby downregulating the activity of the promoter [162]. Fragale *et al.*, 2008 have stated IRF-1 binding to the IRF-E might result in downregulation of FoxP3 by competition with c-Myb for an overlapping consensus sequence on the *FoxP3* promoter [162]. A study by Feng *et al.* in 2008 showed that addition of exogenous IFN-γ to Th2 cells resulted in suppression of IL-4, induction of Suppressor of cytokine signalling-1 (SOCS1) and upregulation of FoxP3 [89]. The former is mediated by IRF1 and IRF2, while the latter is mediated by the transcription factor SP2 [163, 164]. This shows IFN-γ signalling can play a dual role in the regulation of FoxP3. IFN-γ signalling, through IRF-1 and an IRF-E in the promoter of inducible nitric oxide synthase (iNOS), might lead to the upregulation of iNOS and thereby to the increased generation of nitric oxide (NO) [165]. NO can activate the STAT1 pathway, however the mechanism through which it achieves this has not yet been clarified [166]. Inhibition of NOS impairs FoxP3 transcription and supplying a NO donor in absence of IFN-γ partly mimics the positive effect of IFN-γ. Precisely how NO induces FoxP3 expression has not yet been clarified [166]. Another IRF, IRF-4, also represses FoxP3 expression; whether it directly binds to the IRF-E in the FoxP3 promoter is unclear [87, 139]. IRF-4 specifically enhances activation of the IL-4 promoter by interacting with STAT6 and this may be the mechanism by which IRF-4 represses FoxP3 expression [167, 168]. IRF-4 itself is also upregulated by IL-4 signalling, at least in B cells and upon costimulation with CD40, indicating a positive feedback loop between IRF-4 and IL-4 may exist [168]. IRF-4 is also an important factor in the differentiation towards a Th17 cell fate as it positively regulates the level of Retinoid orphan receptor γt (RORγt), which is a marker and regulator of Th17 cells [87].

cAMP/PKA

Prostaglandin E (PGE2) exposure induces FoxP3 expression in $CD4^+CD25^-$ T cells and upregulates FoxP3 in $CD4^+CD25^+$ Tregs [169]. In the absence of the E-prostanoid (EP) receptor or in the presence of cAMP inhibitors, this induction in FoxP3 expression is diminished [170, 171]. This indicates the cAMP/protein kinase A (PKA) inhibitory pathway is probably involved [172, 173]. cAMP is an ubiquitous second messenger involved in numerous signalling pathways, including TCR signalling in T cells. PKA can phosphorylate CREB, NFAT, NF-kB, Ras and ERK and thereby modulate their activity. The phosphorylation of the transcription factors CREB, NFAT and NF-κB occurs within their nuclear localization signal, increasing nuclear transport where they can exert their effect on transcription [173]. All substrates have been implicated in FoxP3 regulation through one of the pathways described earlier. Additionally, PKA phosphorylation of c-Src tyrosine kinase (Csk) increases its activity; Csk

directly inhibits TCR signalling by phosphorylating Lck and Fyn on their C-terminal end. This suppresses the activity Lck and Fyn, which are involved in TCR-signalling [173]. Whether this is detrimental or beneficial for FoxP3 expression is unclear as TCR signalling can both up- and downregulate FoxP3, depending on the intensity and duration of the signal. PGE2 mediated induction of FoxP3 expression has primarily been observed in tumours that secrete high levels of COX-2. COX-2 generates PGE2, leading to the differentiation of T cells into Tregs, providing immunotolerance for the tumour [169, 171]. Interestingly, LPS-activated monocytes can induce FoxP3 expression in resting $CD4^{+}CD25^{-}$ T cells via this pathway, modulating the immune response to pathogens [171].

To summarize, different signalling pathways regulate expression of FoxP3 using similar mechanisms. In general, a signal is received by a receptor, after which this signal is transduced intracellularly via second messengers. Ultimately, this leads to the recruitment of transcription factors or placement of epigenetic markers to the regulatory parts of the gene, influencing transcription activity. Important pathways including TCR, TGF-β, Notch, PKB/mTOR and cAMP/PKA signalling are involved, with extensive crosstalk going on between the different pathways and environmental cues affecting the activity of the different signalling cascades (Figure 11). Although these pathways have been extensively studied, especially the TCR and TGF-β signalling pathways, the understanding of how their combined signalling regulates FoxP3 expression remains incomplete. Additionally, other molecules that have not been studied so extensively with regard to this area are implicated in the regulation of FoxP3 expression; e.g. 17-β-estradiol (E2) [174] and N- and K-Ras [175].

Post transcriptional regulation of FoxP3

In contrast to the regulation of FoxP3 transcription, little is known concerning the regulation of FoxP3 mRNA. One regulator of FoxP3 mRNA has been described thus far: trichostatin A (TSA). This HDAC inhibitor can both enhance the activity of the FoxP3 promoter but at the same time increases the degradation of FoxP3 mRNA [23]. It is known however, that Dicer facilitates FoxP3 expression as well as the development of Tregs in the thymus. Dicer is involved in the generation of miRNAs which affect mRNA degradation and these miRNAs thus likely play a role, either directly on FoxP3 mRNA or indirectly, in the expression of FoxP3 [84]. The miRNAs miR-22 and miR-155 have been shown to influence the FoxP3-regulating PKB and IL-2 pathways respectively [176, 177]. miR-22 downregulates expression of PTEN, which results in an upregulation of PKB. Active PKB, as described earlier, suppresses the expression of FoxP3 [176]. miR-155 inhibits SOCS1 (suppressor of cytokine signalling 1) expression and this inhibition leads to increased IL-2R signalling, possibly through direct binding to the IL-2R [178]. In this way miR-155 increases the expression of FoxP3 [177]. Interestingly, miR-155, and likely miR-22 as well, is upregulated by FoxP3 itself, showing potential for creating feedback loops [61]. Thus, although regulation of FoxP3 mRNA probably contributes to FoxP3 expression levels, only one direct regulator is currently known and additional research is required to gain more insight in this field.

Post-translational modifications of the FoxP3 protein

Similar to mRNA regulation, little is known about the regulation of FoxP3 protein. FoxP3 can be acetylated and this modification is reciprocally regulated by the HAT p300 and the HDAC Silent information regulator two-like 1 (SIRT1) [24], as well as by other HDACs/HATs [98]. Adding HDAC inhibitors results in the acetylation of lysine residues in the forkhead domain of FoxP3 and upregulation of the Treg numbers [98]. This acetylation leads to increased binding to the promoters of FoxP3 target genes and substituting the lysine residues in the forkhead domain abrogates the ability of FoxP3 to bind these promoters [98]. TGF-β treatment increases the acetylation of FoxP3 and increases its association with chromatin [179]. TGF-β together with IL-6, which negatively regulates Treg numbers and function, leads to decreased association with chromatin. This decreased association can be abrogated by the addition of HDAC inhibitors [179]. Samanta *et al*., 2008 showed also that FoxP3 can be phosphorylated; however, the function of this phosphorylation has not been clarified yet [179]. The phosphorylation of FoxO can promote both FoxO nuclear localization and removal from the nucleus dependent on the residue which is phosphorylated [146]. This might possibly provide insight in the role of this phosphorylation for FoxP3. It was recently shown that hyperacetylation of the FoxP3 protein prevents proteasomal degradation by blocking sites of polyubiquitination, which leads to increased stability of FoxP3 protein [24]; showing another mechanism in which FoxP3 can be regulated by acetylation.

VI. Discussion

$FoxP3^+$ Tregs are indispensible for normal immune function, which is illustrated by IPEX patients and scurfy mice, and maintenance of FoxP3 expression is required for the development and function of these cells [4, 5]. The transcription factor FoxP3 is part of the Forkhead box family of proteins and is the key controller of Treg differentiation and maintenance. The tight regulation of FoxP3 expression and activity is required as transient expression of FoxP3 upon TCR stimulation is not sufficient to initiate differentiation of $CD4^+$ $CD25^-$ T cells into the regulatory T cell lineage [52, 53]. In addition, loss of FoxP3 in Tregs leads to loss of regulatory capacities of these cells (Figure 4) [19, 21, 57]. Thus, insight in the regulation of FoxP3 can provide opportunities to affect regulatory T cell numbers and function in the clinic. As Tregs are implied not only in autoimmune disease but also in transplantation tolerance [29], clearance of viral infections [30] and cancer [31], this is an important subject of study. As discussed in this thesis, FoxP3 is regulated on many different levels; transcription of the *FoxP3* gene is regulated by epigenetic modulation as well as the recruitment or inhibition of the transcriptional machinery through different signalling pathways. In addition, FoxP3 mRNA regulation and post-translational modifications of the FoxP3 protein are very likely to affect FoxP3 levels and function [23, 24, 84].

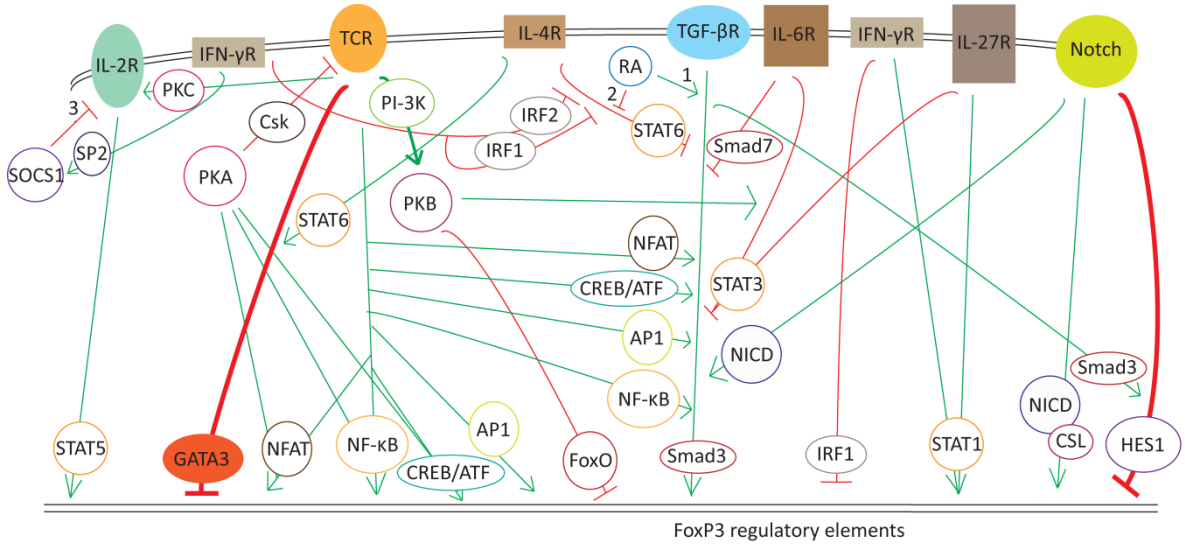

Figure 11. Interactions between the different signalling pathways regulating transcription of FoxP3. The molecules mediating the crosstalk between the different pathways are depicted here. Red arrows depict an inhibiting function, while green arrows indicate a stimulatory role. Thicker arrows are the result from longer, or higher concentrations of, signalling. The location of the arrows does not represent location of signalling. The intracellular domain of Notch, NICD, also binds Smad3 and this NICD/Smad3 complex interacts with the FoxP3 promoter in a transcriptional complex [91], thus Notch and TGF-β signalling interact to promote FoxP3 transcription. Membrane-bound TGF-β in its turn promotes Notch signalling [135, 139, 159]. TGF-β signalling also cooperates with the TCR pathway via several molecules to induce FoxP3 transcription. TCR, through PKC, upregulates CD25, which enables IL-2R signalling [109]. IFN-γ signalling suppresses IL-2R and TGF-β signalling by inducing SOCS1 and inhibiting IL-4 respectively [166]. The induction of SOS1 is mediated by SP2 and the inhibition of IL-4 signalling is mediated by IRF1 and IRF2 [163, 164]. Prolonged TCR signalling activates the PKB/mTOR axis through PI-3K which inhibits FoxP3 transcription [105]. PKA, downstream of PGE2, also interacts with TCR signalling through various molecules [173]. In addition to the interactions between these pathways several molecules affect these pathways without directly influencing FoxP3 expression. IL-6 signalling leads to STAT3 binding to, and inactivation of, the enhancer in the second intron of FoxP3; this prevents TGF-β signalling by inhibiting the binding of pSmad3 to the enhancer in the first intron [112, 134, 135]. STAT6, which is activated after IL-4 signalling inhibits TGF-β signalling and promotes the inhibitory signalling via the TCR pathway [115, 116]. This IL-4 signalling can be inhibited by RA, which also directly facilitates the transcription through the Smad pathway [112, 135]. 1. RA binding to the enhancer of the first intronic region of FoxP3 leads to acetylation of histones near the Smad3 binding site, followed by increased Smad3 binding to the FoxP3 promoter [112, 140]. 2. RA can, in the presence of TGF-β, prevent binding of STAT6 to the FoxP3 promoter, counteracting the effect of IL-4 on TCR signalling [135]. 3. SOCS1 can directly bind to, and inhibit, the IL-2R [178]. SOCS1 = suppressor of cytokine signalling 1. IL = interleukin. IFN-γ = interferon-γ. PKA = protein kinase A. TCR = T cell receptor. PKB = protein kinase B. RA = retinoic acid. TGF-β = Transforming growth factor-β. PKC = protein kinase C. CSk = C-src tyrosine kinase. NFAT = nuclear factor of activated T cells. NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells. CREB/ATF = cAMP response element-binding/ activating transcription factor-1. AP1 = activator protein 1. STAT = Signal Transducers and Activators of Transcription protein. PI-3K = Phosphatidylinositol 3-kinases. FoxO = Forkhead box protein O. NICD = Notch intracellular domain. HES1 = Hairy and enhancer of split 1. Csl = CBF1/RBP-Jκ/Suppressor of Hairless/LAG-1

Many different signalling pathways, to which a myriad of functions has been ascribed, have been demonstrated to directly regulate Foxp3 expression. Crosstalk between these different pathways is very common and some of the direct interactions between the different pathways are highlighted in

Figure 11. The interactions shown there are only the direct interactions existing between the major different pathways. Competition for binding sites, as well as for cofactors, and indirect interactions also link these different pathways. In addition, epigenetic markers placed by one molecule, affect the availability of binding sites and accessibility of the gene to the transcriptional machinery for all pathways. Moreover, FoxP3 also employs several feedback loops, both stimulatory and inhibitory, regulating its own expression. FoxP3 mRNA stability has been shown to be regulated and FoxP3 protein is subject to post-translational modifications altering protein stability and transcriptional activity [23, 24, 84]. However, compared to regulation of transcription, these fields are understudied. Post-translational modifications for the FoxP3 family member FoxO have been investigated and proven to be pivotal for its function [146]. Therefore, it is very likely that research into the PTMs, and mRNA regulation as well, will provide valuable information regarding FoxP3 regulation.

Pathways that have been studied thoroughly are: TCR/IL-2, Notch and TGF-β/Smad. Much is known about the role of Retinoic acid and IFN-γ/IRF also. In contrast, relatively little is known about PKB/mTOR and cAMP/PKA. Furthermore, the information about the involvement of the latter two pathways is fragmentated. A possible explanation for this lies in the specificity of the different pathways. While TCR signalling is implicitly limited to T cells; PI3K, PKB, mTOR, cAMP and PKA are ubiquitous and perform many different roles that extend the field of immunology. The last group of molecules involved in FoxP3 regulation are those about which almost nothing is known in this regard, including Ras and 17-β-estradiol [174, 175].

Concluding, although much is known about the regulation of FoxP3 already, much more is to be discovered. There are a few regions of the FoxP3 field which are especially open for further investigation. First, the role of the understudied pathways mTOR/PKB and cAMP/PKA has to be elucidated further. In addition, the crosstalk between the different pathways and which molecules exert this crosstalk also needs clarification; this could reveal which targets affect multiple ways of FoxP3 expression and are possibly good candidates for clinical intervention. Finally, the post-transcriptional levels, mRNA regulation as well as PTMs of the FoxP3 protein, of FoxP3 expression and activity regulation remain understudied. Increased insight in the regulation of FoxP3 may lead to new targets for intervention as the identification of critical processes, such as FoxP3 acetylation, and molecules pivotal for the regulation of FoxP3 expression and function, might be used as a target for intervention in diseases associated with dysfunctional Tregs. For example, as acetylation of FoxP3 enhances its function [23, 24, 179], leading to an increase in Treg function and numbers, inducing this acetylation could be a therapeutic strategy to obtain greater number of functional Tregs. These interventional strategies are potentially more specific for FoxP3 and Tregs than general immunosuppressive drugs which are currently widely used in the clinic. This could reduce side effects of treatment and lead to better clinical perspectives for people suffering from the many diseases associated with regulatory T cells, including: IPEX, multiple forms of cancer and transplantation tolerance [29, 31]. In cancer, Tregs are associated with poor prognosis as they provide tolerance for the tumour and impairing Treg functionality or numbers could be used as immunotherapy here [31]. As it is important to specifically lower Treg numbers while not affecting Teff cells, targeting FoxP3 is a promising strategy in cancer therapy. In several autoimmune diseases, including IPEX, an aberrant Treg response is observed. Improving this response by intervening in the FoxP3 pathway is an alternative to immunosuppressive drugs that make the patient more susceptible to infectious diseases and cancer [29]. After transplantation, true tolerance to the graft could potentially be achieved through inducing Tregs specific for the donor tissue [180]. This would

alleviate the need for continued administration of immunosuppressive drugs. These three situations: cancer, autoimmune diseases and transplantations, require treatment that specifically affects Tregs. Altering FoxP3 expression or functionality through targeting one of the key molecules regulating FoxP3, described in this thesis, would be an excellent strategy to achieve this given the essential role of FoxP3 in Treg biology

References

1. Stroud JC, Wu Y, Bates DL, Han A, Nowick K, Paabo S, Tong H, Chen L. Structure of the forkhead domain of FOXP2 bound to DNA. Structure 2006 Jan;14(1):159-66.

2. Morgan ME, van Bilsen JH, Bakker AM, Heemskerk B, Schilham MW, Hartgers FC, Elferink BG, van der Zanden L, de Vries RR, Huizinga TW, Ottenhoff TH, Toes RE. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. Hum Immunol 2005 Jan;66(1):13-20.

3. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003 Feb 14;299(5609):1057-61.

4. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 2001 Jan;27(1):68-73.

5. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001 Jan;27(1):20-1.

6. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. Annu Rev Immunol 2003;21:139-76.

7. Piccirillo CA, Shevach EM. Naturally-occurring CD4+CD25+ immunoregulatory T cells: Central players in the arena of peripheral tolerance. Semin Immunol 2004 Apr;16(2):81-8.

8. Godfrey VL, Wilkinson JE, Russell LB. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. Am J Pathol 1991 Jun;138(6):1379-87.

9. Lyon MF, Peters J, Glenister PH, Ball S, Wright E. The scurfy mouse mutant has previously unrecognized hematological abnormalities and resembles wiskott-aldrich syndrome. Proc Natl Acad Sci U S A 1990 Apr;87(7):2433-7.

10. Kanangat S, Blair P, Reddy R, Daheshia M, Godfrey V, Rouse BT, Wilkinson E. Disease in the scurfy (sf) mouse is associated with overexpression of cytokine genes. Eur J Immunol 1996 Jan;26(1):161-5.

11. Blair PJ, Bultman SJ, Haas JC, Rouse BT, Wilkinson JE, Godfrey VL. CD4+CD8- T cells are the effector cells in disease pathogenesis in the scurfy (sf) mouse. J Immunol 1994 Oct 15;153(8):3764-74.

12. Godfrey VL, Rouse BT, Wilkinson JE. Transplantation of T cell-mediated, lymphoreticular disease from the scurfy (sf) mouse. Am J Pathol 1994 Aug;145(2):281-6.

13. Gambineri E, Perroni L, Passerini L, Bianchi L, Doglioni C, Meschi F, Bonfanti R, Sznajer Y, Tommasini A, Lawitschka A, Junker A, Dunstheimer D, Heidemann PH, Cazzola G, Cipolli M, Friedrich W, Janic D, Azzi N, Richmond E, Vignola S, Barabino A, Chiumello G, Azzari C, Roncarolo MG, Bacchetta R. Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: Inconsistent correlation between forkhead box protein 3 expression and disease severity. J Allergy Clin Immunol 2008 Dec;122(6):1105,1112.e1.

14. Powell BR, Buist NR, Stenzel P. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. J Pediatr 1982 May;100(5):731-7.

15. Torgerson TR. Regulatory T cells in human autoimmune diseases. Springer Semin Immunopathol 2006 Aug;28(1):63-76.

16. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 2002 Aug;39(8):537-45.

17. Pillai V, Ortega SB, Wang CK, Karandikar NJ. Transient regulatory T-cells: A state attained by all activated human T-cells. Clin Immunol 2007 Apr;123(1):18-29.

18. Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, Roncarolo MG, Levings MK. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol 2007 Apr;19(4):345-54.

19. Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, Olek S, Dietmaier W, Andreesen R, Edinger M. Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation. Eur J Immunol 2009 Apr;39(4):1088-97.

20. Allan SE, Song-Zhao GX, Abraham T, McMurchy AN, Levings MK. Inducible reprogramming of human T cells into treg cells by a conditionally active form of FOXP3. Eur J Immunol 2008 Dec;38(12):3282-9.

21. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol 2007 Mar;8(3):277-84.

22. van der Vliet HJ, Nieuwenhuis EE. IPEX as a result of mutations in FOXP3. Clin Dev Immunol 2007;2007:89017.

23. Liu Z, Zhang C, Sun J. Deacetylase inhibitor trichostatin A down-regulates Foxp3 expression and reduces CD4+CD25+ regulatory T cells. Biochem Biophys Res Commun 2010 Sep 24;400(3):409-12.

24. van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, van Beekum O, Brenkman AB, Hijnen DJ, Mutis T, Kalkhoven E, Prakken BJ, Coffer PJ. Regulation of treg functionality by acetylation-mediated Foxp3 protein stabilization. Blood 2010 Feb 4;115(5):965-74.

25. Sakaguchi S, Wing K, Miyara M. Regulatory T cells - a brief history and perspective. Eur J Immunol 2007 Nov;37 Suppl 1:S116-23.

26. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol 2010 Jul;10(7):490-500.

27. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: A jack of all trades, master of regulation. Nat Immunol 2008 Mar;9(3):239-44.

28. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol 2008 Jul;8(7):523-32.

29. Fritzsching E, Kunz P, Maurer B, Poschl J, Fritzsching B. Regulatory T cells and tolerance induction. Clin Transplant 2009 Dec;23 Suppl 21:10-4.

30. Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. Science 2008 May 30;320(5880):1220-4.

31. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. Adv Cancer Res 2010;107:57-117.

32. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531-62.

33. Apostolou I, Verginis P, Kretschmer K, Polansky J, Huhn J, von Boehmer H. Peripherally induced treg: Mode, stability, and role in specific tolerance. J Clin Immunol 2008 Nov;28(6):619-24.

34. Chatenoud L. Natural and induced T CD4+CD25+FOXP3+ regulatory T cells. Methods Mol Biol 2011;677:3-13.

35. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochov G, Sakaguchi S. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 2009 Jun 19;30(6):899-911.

36. Baecher-Allan C, Wolf E, Hafler DA. MHC class II expression identifies functionally distinct human regulatory T cells. J Immunol 2006 Apr 15;176(8):4622-31.

37. Ito T, Hanabuchi S, Wang YH, Park WR, Arima K, Bover L, Qin FX, Gilliet M, Liu YJ. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. Immunity 2008 Jun;28(6):870-80.

38. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006 Aug;212:28-50.

39. Faria AM, Weiner HL. Oral tolerance. Immunol Rev 2005 Aug;206:232-59.

40. Chang CC, Ciubotariu R, Manavalan JS, Yuan J, Colovai AI, Piazza F, Lederman S, Colonna M, Cortesini R, Dalla-Favera R, Suciu-Foca N. Tolerization of dendritic cells by T(S) cells: The crucial role of inhibitory receptors ILT3 and ILT4. Nat Immunol 2002 Mar;3(3):237-43.

41. Wong M, La Cava A, Singh RP, Hahn BH. Blockade of programmed death-1 in young (new zealand black x new zealand white)F1 mice promotes the activity of suppressive CD8+ T cells that protect from lupus-like disease. J Immunol 2010 Nov 1.

42. Kronenberg M. Toward an understanding of NKT cell biology: Progress and paradoxes. Annu Rev Immunol 2005;23:877-900.

43. Ronet C, Darche S, Leite de Moraes M, Miyake S, Yamamura T, Louis JA, Kasper LH, Buzoni-Gatel D. NKT cells are critical for the initiation of an inflammatory bowel response against toxoplasma gondii. J Immunol 2005 Jul 15;175(2):899-908.

44. Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. Nat Med 2000 Jul;6(7):782-9.

45. Duncan B, Nazarov-Stoica C, Surls J, Kehl M, Bona C, Casares S, Brumeanu TD. Double negative (CD3+ 4- 8-) TCR alphabeta splenic cells from young NOD mice provide long-lasting protection against type 1 diabetes. PLoS One 2010 Jul 2;5(7):e11427.

46. Hayday A, Tigelaar R. Immunoregulation in the tissues by gammadelta T cells. Nat Rev Immunol 2003 Mar;3(3):233-42.

47. Kuhl AA, Pawlowski NN, Grollich K, Blessenohl M, Westermann J, Zeitz M, Loddenkemper C, Hoffmann JC. Human peripheral gammadelta T cells possess regulatory potential. Immunology 2009 Dec;128(4):580-8.

48. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for scurfin in CD4+CD25+ T regulatory cells. Nat Immunol 2003 Apr;4(4):337-42.

49. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003 Apr;4(4):330-6.

50. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006 May 11;441(7090):235-8.

51. Buckner JH, Ziegler SF. Functional analysis of FOXP3. Ann N Y Acad Sci 2008 Nov;1143:151-69.

52. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, Sakaguchi S. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol 2004 Nov;16(11):1643-56.

53. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, Rudensky AY. Foxp3-dependent programme of regulatory T-cell differentiation. Nature 2007 Feb 15;445(7129):771-5.

54. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008 May 30;133(5):775-87.

55. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 2003 Nov;112(9):1437-43.

56. Walker MR, Carson BD, Nepom GT, Ziegler SF, Buckner JH. De novo generation of antigen-specific CD4+CD25+ regulatory T cells from human CD4+CD25- cells. Proc Natl Acad Sci U S A 2005 Mar 15;102(11):4103-8.

57. Allan SE, Alstad AN, Merindol N, Crellin NK, Amendola M, Bacchetta R, Naldini L, Roncarolo MG, Soudeyns H, Levings MK. Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. Mol Ther 2008 Jan;16(1):194-202.

58. Zhou X, Bailey-Bucktrout S, Jeker LT, Bluestone JA. Plasticity of CD4(+) FoxP3(+) T cells. Curr Opin Immunol 2009 Jun;21(3):281-5.

59. Marson A, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, Levine SS, Fraenkel E, von Boehmer H, Young RA. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. Nature 2007 Feb 22;445(7130):931-5.

60. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature 2007 Feb 22;445(7130):936-40.

61. Sadlon TJ, Wilkinson BG, Pederson S, Brown CY, Bresatz S, Gargett T, Melville EL, Peng K, D'Andrea RJ, Glonek GG, Goodall GJ, Zola H, Shannon MF, Barry SC. Genome-wide identification of human FOXP3 target genes in natural regulatory T cells. J Immunol 2010 Jul 15;185(2):1071-81.

62. Weigel D, Jackle H. The fork head domain: A novel DNA binding motif of eukaryotic transcription factors? Cell 1990 Nov 2;63(3):455-6.

63. Li S, Weidenfeld J, Morrisey EE. Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. Mol Cell Biol 2004 Jan;24(2):809-22.

64. Kaufmann E, Muller D, Knochel W. DNA recognition site analysis of xenopus winged helix proteins. J Mol Biol 1995 Apr 28;248(2):239-54.

65. Tamura S, Morikawa Y, Iwanishi H, Hisaoka T, Senba E. Expression pattern of the winged-helix/forkhead transcription factor Foxp1 in the developing central nervous system. Gene Expr Patterns 2003 May;3(2):193-7.

66. Ferland RJ, Cherry TJ, Preware PO, Morrisey EE, Walsh CA. Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain. J Comp Neurol 2003 May 26;460(2):266-79.

67. Schon C, Wochnik A, Rossner A, Donow C, Knochel W. The FoxP subclass in xenopus laevis development. Dev Genes Evol 2006 Oct;216(10):641-6.

68. Shu W, Lu MM, Zhang Y, Tucker PW, Zhou D, Morrisey EE. Foxp2 and Foxp1 cooperatively regulate lung and esophagus development. Development 2007 May;134(10):1991-2000.

69. Hu H, Wang B, Borde M, Nardone J, Maika S, Allred L, Tucker PW, Rao A. Foxp1 is an essential transcriptional regulator of B cell development. Nat Immunol 2006 Aug;7(8):819-26.

70. Lai CS, Fisher SE, Hurst JA, Vargha-Khadem F, Monaco AP. A forkhead-domain gene is mutated in a severe speech and language disorder. Nature 2001 Oct 4;413(6855):519-23.

71. Lu MM, Li S, Yang H, Morrisey EE. Foxp4: A novel member of the foxp subfamily of winged-helix genes co-expressed with Foxp1 and Foxp2 in pulmonary and gut tissues. Mech Dev 2002 Dec;119 Suppl 1:S197-202.

72. Lopes JE, Torgerson TR, Schubert LA, Anover SD, Ocheltree EL, Ochs HD, Ziegler SF. Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor. J Immunol 2006 Sep 1;177(5):3133-42.

73. Koh KP, Sundrud MS, Rao A. Domain requirements and sequence specificity of DNA binding for the forkhead transcription factor FOXP3. PLoS One 2009 Dec 1;4(12):e8109.

74. Klug A. The discovery of zinc fingers and their development for practical applications in gene regulation and genome manipulation. Q Rev Biophys 2010 Feb;43(1):1-21.

75. Ziegler SF. FOXP3: Of mice and men. Annu Rev Immunol 2006;24:209-26.

76. Smith EL, Finney HM, Nesbitt AM, Ramsdell F, Robinson MK. Splice variants of human FOXP3 are functional inhibitors of human CD4+ T-cell activation. Immunology 2006 Oct;119(2):203-11.

77. Du J, Huang C, Zhou B, Ziegler SF. Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. J Immunol 2008 Apr 1;180(7):4785-92.

78. Ichiyama K, Yoshida H, Wakabayashi Y, Chinen T, Saeki K, Nakaya M, Takaesu G, Hori S, Yoshimura A, Kobayashi T. Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. J Biol Chem 2008 Jun 20;283(25):17003-8.

79. Bettelli E, Dastrange M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. Proc Natl Acad Sci U S A 2005 Apr 5;102(14):5138-43.

80. Mailer RK, Falk K, Rotzschke O. Absence of leucine zipper in the natural FOXP3Delta2Delta7 isoform does not affect dimerization but abrogates suppressive capacity. PLoS One 2009 Jul 1;4(7):e6104.

81. Kaur G, Goodall JC, Jarvis LB, Hill Gaston JS. Characterisation of Foxp3 splice variants in human CD4+ and CD8+ T cells-identification of Foxp3Delta7 in human regulatory T cells. Mol Immunol 2010 Aug 3.

82. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature 2008 May 8;453(7192):236-40.

83. Aarts-Riemens T, Emmelot ME, Verdonck LF, Mutis T. Forced overexpression of either of the two common human Foxp3 isoforms can induce regulatory T cells from CD4(+)CD25(-) cells. Eur J Immunol 2008 May;38(5):1381-90.

84. Cobb BS, Hertweck A, Smith J, O'Connor E, Graf D, Cook T, Smale ST, Sakaguchi S, Livesey FJ, Fisher AG, Merkenschlager M. A role for dicer in immune regulation. J Exp Med 2006 Oct 30;203(11):2519-27.

85. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. Blood 2009 Oct 29;114(18):3727-35.

86. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, Schmidt-Weber CB. Molecular mechanisms underlying FOXP3 induction in human T cells. J Immunol 2006 Mar 15;176(6):3593-602.

87. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, Arpaia E, Mak TW, Kamradt T, Lohoff M. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. Nat Immunol 2007 Sep;8(9):958-66.

88. Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. J Immunol 2009 Jan 1;182(1):259-73.

89. Feng G, Wood KJ, Bushell A. Interferon-gamma conditioning ex vivo generates CD25+CD62L+Foxp3+ regulatory T cells that prevent allograft rejection: Potential avenues for cellular therapy. Transplantation 2008 Aug 27;86(4):578-89.

90. Ou-Yang HF, Zhang HW, Wu CG, Zhang P, Zhang J, Li JC, Hou LH, He F, Ti XY, Song LQ, Zhang SZ, Feng L, Qi HW, Han H. Notch signaling regulates the FOXP3 promoter through RBP-J- and Hes1-dependent mechanisms. Mol Cell Biochem 2009 Jan;320(1-2):109-14.

91. Samon JB, Champhekar A, Minter LM, Telfer JC, Miele L, Fauq A, Das P, Golde TE, Osborne BA. Notch1 and TGFbeta1 cooperatively regulate Foxp3 expression and the maintenance of peripheral regulatory T cells. Blood 2008 Sep 1;112(5):1813-21.

92. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol 2004 May 1;172(9):5149-53.

93. Eckerstorfer P, Novy M, Burgstaller-Muehlbacher S, Paster W, Schiller HB, Mayer H, Stockinger H. Proximal human FOXP3 promoter transactivated by NF-kappaB and negatively controlled by feedback loop and SP3. Mol Immunol 2010 Jul;47(11-12):2094-102.

94. Bestor TH, Coxon A. Cytosine methylation: The pros and cons of DNA methylation. Curr Biol 1993 Jun 1;3(6):384-6.

95. Leonhardt H, Page AW, Weier HU, Bestor TH. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 1992 Nov 27;71(5):865-73.

96. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 1997 Sep 18;389(6648):251-60.

97. Spencer VA, Davie JR. Role of covalent modifications of histones in regulating gene expression. Gene 1999 Nov 15;240(1):1-12.

98. Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, Wells AD, Hancock WW. Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med 2007 Nov;13(11):1299-307.

99. Korzus E. Manipulating the brain with epigenetics. Nat Neurosci 2010 Apr;13(4):405-6.

100. Janson PC, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. FOXP3 promoter demethylation reveals the committed treg population in humans. PLoS One 2008 Feb 20;3(2):e1612.

101. Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: A role for DNA methylation. J Exp Med 2007 Jul 9;204(7):1543-51.

102. Venuprasad K, Huang H, Harada Y, Elly C, Subramaniam M, Spelsberg T, Su J, Liu YC. The E3 ubiquitin ligase itch regulates expression of transcription factor Foxp3 and airway inflammation by enhancing the function of transcription factor TIEG1. Nat Immunol 2008 Mar;9(3):245-53.

103. Luo X, Zhang Q, Liu V, Xia Z, Pothoven KL, Lee C. Cutting edge: TGF-beta-induced expression of Foxp3 in T cells is mediated through inactivation of ERK. J Immunol 2008 Mar 1;180(5):2757-61.

104. Lu R, Wang X, Chen ZF, Sun DF, Tian XQ, Fang JY. Inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway decreases DNA methylation in colon cancer cells. J Biol Chem 2007 Apr 20;282(16):12249-59.

105. Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, Knight ZA, Cobb BS, Cantrell D, O'Connor E, Shokat KM, Fisher AG, Merkenschlager M. T cell receptor signaling controls Foxp3 expression via PI3K, akt, and mTOR. Proc Natl Acad Sci U S A 2008 Jun 3;105(22):7797-802.

106. Kang SG, Lim HW, Andrisani OM, Broxmeyer HE, Kim CH. Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. J Immunol 2007 Sep 15;179(6):3724-33.

107. Li B, Samanta A, Song X, Iacono KT, Bembas K, Tao R, Basu S, Riley JL, Hancock WW, Shen Y, Saouaf SJ, Greene MI. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. Proc Natl Acad Sci U S A 2007 Mar 13;104(11):4571-6.

108. Burchill MA, Yang J, Vang KB, Moon JJ, Chu HH, Lio CW, Vegoe AL, Hsieh CS, Jenkins MK, Farrar MA. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. Immunity 2008 Jan;28(1):112-21.

109. Szamel M, Appel A, Schwinzer R, Resch K. Different protein kinase C isoenzymes regulate IL-2 receptor expression or IL-2 synthesis in human lymphocytes stimulated via the TCR. J Immunol 1998 Mar 1;160(5):2207-14.

110. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003 Dec 15;198(12):1875-86.

111. Lu L, Zhou X, Wang J, Zheng SG, Horwitz DA. Characterization of protective human CD4CD25 FOXP3 regulatory T cells generated with IL-2, TGF-beta and retinoic acid. PLoS One 2010 Dec 17;5(12):e15150.

112. Xu L, Kitani A, Stuelten C, McGrady G, Fuss I, Strober W. Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer i. Immunity 2010 Sep 24;33(3):313-25.

113. Gottschalk RA, Corse E, Allison JP. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. J Exp Med 2010 Aug 2;207(8):1701-11.

114. Josefowicz SZ, Wilson CB, Rudensky AY. Cutting edge: TCR stimulation is sufficient for induction of Foxp3 expression in the absence of DNA methyltransferase 1. J Immunol 2009 Jun 1;182(11):6648-52.

115. Wei J, Duramad O, Perng OA, Reiner SL, Liu YJ, Qin FX. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. Proc Natl Acad Sci U S A 2007 Nov 13;104(46):18169-74.

116. Mantel PY, Kuipers H, Boyman O, Rhyner C, Ouaked N, Ruckert B, Karagiannidis C, Lambrecht BN, Hendriks RW, Crameri R, Akdis CA, Blaser K, Schmidt-Weber CB. GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. PLoS Biol 2007 Dec;5(12):e329.

117. Malek TR, Yu A, Vincek V, Scibelli P, Kong L. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. implications for the nonredundant function of IL-2. Immunity 2002 Aug;17(2):167-78.

118. Bayer AL, Yu A, Adeegbe D, Malek TR. Essential role for interleukin-2 for CD4(+)CD25(+) T regulatory cell development during the neonatal period. J Exp Med 2005 Mar 7;201(5):769-77.

119. Almeida AR, Legrand N, Papiernik M, Freitas AA. Homeostasis of peripheral CD4+ T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4+ T cell numbers. J Immunol 2002 Nov 1;169(9):4850-60.

120. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. J Exp Med 2002 Sep 16;196(6):851-7.

121. de la Rosa M, Rutz S, Dorninger H, Scheffold A. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. Eur J Immunol 2004 Sep;34(9):2480-8.

122. Vang KB, Yang J, Mahmud SA, Burchill MA, Vegoe AL, Farrar MA. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development. J Immunol 2008 Sep 1;181(5):3285-90.

123. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol 2005 Nov;6(11):1142-51.

124. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. J Immunol 2007 Jan 1;178(1):280-90.

125. Malek TR, Yu A, Zhu L, Matsutani T, Adeegbe D, Bayer AL. IL-2 family of cytokines in T regulatory cell development and homeostasis. J Clin Immunol 2008 Nov;28(6):635-9.

126. Pfeifer AC, Timmer J, Klingmuller U. Systems biology of JAK/STAT signalling. Essays Biochem 2008;45:109-20.

127. Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, Watford WT, Laurence A, Robinson GW, Shevach EM, Moriggl R, Hennighausen L, Wu C, O'Shea JJ. Nonredundant roles for Stat5a/b in directly regulating Foxp3. Blood 2007 May 15;109(10):4368-75.

128. Lischke A, Moriggl R, Brandlein S, Berchtold S, Kammer W, Sebald W, Groner B, Liu X, Hennighausen L, Friedrich K. The interleukin-4 receptor activates STAT5 by a mechanism that relies upon common gamma-chain. J Biol Chem 1998 Nov 20;273(47):31222-9.

129. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, Bellucci R, Raderschall E, Canning C, Soiffer RJ, Frank DA, Ritz J. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. Blood 2006 Sep 1;108(5):1571-9.

130. Ouaked N, Mantel PY, Bassin C, Burgler S, Siegmund K, Akdis CA, Schmidt-Weber CB. Regulation of the foxp3 gene by the Th1 cytokines: The role of IL-27-induced STAT1. J Immunol 2009 Jan 15;182(2):1041-9.

131. Neufert C, Becker C, Wirtz S, Fantini MC, Weigmann B, Galle PR, Neurath MF. IL-27 controls the development of inducible regulatory T cells and Th17 cells via differential effects on STAT1. Eur J Immunol 2007 Jul;37(7):1809-16.

132. Huber M, Steinwald V, Guralnik A, Brustle A, Kleemann P, Rosenplanter C, Decker T, Lohoff M. IL-27 inhibits the development of regulatory T cells via STAT3. Int Immunol 2008 Feb;20(2):223-34.

133. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature 2007 Jul 26;448(7152):484-7.

134. Fu S, Zhang N, Yopp AC, Chen D, Mao M, Chen D, Zhang H, Ding Y, Bromberg JS. TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. Am J Transplant 2004 Oct;4(10):1614-27.

135. Takaki H, Ichiyama K, Koga K, Chinen T, Takaesu G, Sugiyama Y, Kato S, Yoshimura A, Kobayashi T. STAT6 inhibits TGF-beta1-mediated Foxp3 induction through direct binding to the Foxp3 promoter, which is reverted by retinoic acid receptor. J Biol Chem 2008 May 30;283(22):14955-62.

136. Derynck R, Zhang YE. Smad-dependent and smad-independent pathways in TGF-beta family signalling. Nature 2003 Oct 9;425(6958):577-84.

137. Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: The role of IL-2, TGF-beta, and IL-10. J Immunol 2004 May 1;172(9):5213-21.

138. Horwitz DA, Zheng SG, Gray JD. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. Trends Immunol 2008 Sep;29(9):429-35.

139. Shen Z, Chen L, Hao F, Wu J. Transcriptional regulation of Foxp3 gene: Multiple signal pathways on the road. Med Res Rev 2009 Sep;29(5):742-66.

140. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nat Immunol 2008 Feb;9(2):194-202.

141. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature 2010 Feb 11;463(7282):808-12.

142. Ruan Q, Kameswaran V, Tone Y, Li L, Liou HC, Greene MI, Tone M, Chen YH. Development of Foxp3(+) regulatory t cells is driven by the c-rel enhanceosome. Immunity 2009 Dec 18;31(6):932-40.

143. Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M, Lovey A, Kastner P, Grippo JF, Chambon P. Retinoic acid receptors and retinoid X receptors: Interactions with endogenous retinoic acids. Proc Natl Acad Sci U S A 1993 Jan 1;90(1):30-4.

144. Powell JD, Delgoffe GM. The mammalian target of rapamycin: Linking T cell differentiation, function, and metabolism. Immunity 2010 Sep 24;33(3):301-11.

145. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 2006 May 25;441(7092):424-30.

146. Hedrick SM. The cunning little vixen: Foxo and the cycle of life and death. Nat Immunol 2009 Oct;10(10):1057-63.

147. Merkenschlager M, von Boehmer H. PI3 kinase signalling blocks Foxp3 expression by sequestering foxo factors. J Exp Med 2010 Jul 5;207(7):1347-50.

148. Harada Y, Harada Y, Elly C, Ying G, Paik JH, DePinho RA, Liu YC. Transcription factors Foxo3a and Foxo1 couple the E3 ligase cbl-b to the induction of Foxp3 expression in induced regulatory T cells. J Exp Med 2010 Jul 5;207(7):1381-91.

149. Ouyang W, Beckett O, Ma Q, Paik JH, DePinho RA, Li MO. Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. Nat Immunol 2010 Jul;11(7):618-27.

150. Wohlfert EA, Gorelik L, Mittler R, Flavell RA, Clark RB. Cutting edge: Deficiency in the E3 ubiquitin ligase cbl-b results in a multifunctional defect in T cell TGF-beta sensitivity in vitro and in vivo. J Immunol 2006 Feb 1;176(3):1316-20.

151. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. J Exp Med 2008 Mar 17;205(3):565-74.

152. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, Worley PF, Kozma SC, Powell JD. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 2009 Jun 19;30(6):832-44.

153. Bruno L, Merkenschlager M. Directing T cell differentiation and function with small molecule inhibitors. Cell Cycle 2008 Aug;7(15):2296-8.

154. Hideshima T, Nakamura N, Chauhan D, Anderson KC. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene 2001 Sep 20;20(42):5991-6000.

155. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 2003 Aug 15;374(Pt 1):1-20.

156. Winter JN, Fox TE, Kester M, Jefferson LS, Kimball SR. Phosphatidic acid mediates activation of mTORC1 through the ERK signaling pathway. Am J Physiol Cell Physiol 2010 Aug;299(2):C335-44.

157. von Boehmer H. Notch in lymphopoiesis and T cell polarization. Nat Immunol 2005 Jul;6(7):641-2.

158. Curry CL, Reed LL, Golde TE, Miele L, Nickoloff BJ, Foreman KE. Gamma secretase inhibitor blocks notch activation and induces apoptosis in kaposi's sarcoma tumor cells. Oncogene 2005 Sep 22;24(42):6333-44.

159. Ostroukhova M, Qi Z, Oriss TB, Dixon-McCarthy B, Ray P, Ray A. Treg-mediated immunosuppression involves activation of the notch-HES1 axis by membrane-bound TGF-beta. J Clin Invest 2006 Apr;116(4):996-1004.

160. Blokzijl A, Dahlqvist C, Reissmann E, Falk A, Moliner A, Lendahl U, Ibanez CF. Cross-talk between the notch and TGF-beta signaling pathways mediated by interaction of the notch intracellular domain with Smad3. J Cell Biol 2003 Nov 24;163(4):723-8.

161. Saha B, Jyothi Prasanna S, Chandrasekar B, Nandi D. Gene modulation and immunoregulatory roles of interferon gamma. Cytokine 2010 Apr;50(1):1-14.

162. Fragale A, Gabriele L, Stellacci E, Borghi P, Perrotti E, Ilari R, Lanciotti A, Remoli AL, Venditti M, Belardelli F, Battistini A. IFN regulatory factor-1 negatively regulates CD4+ CD25+ regulatory T cell differentiation by repressing Foxp3 expression. J Immunol 2008 Aug 1;181(3):1673-82.

163. Elser B, Lohoff M, Kock S, Giaisi M, Kirchhoff S, Krammer PH, Li-Weber M. IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. Immunity 2002 Dec;17(6):703-12.

164. Letourneur M, Valentino L, Travagli-Gross J, Bertoglio J, Pierre J. Sp2 regulates interferon-gamma-mediated socs1 gene expression. Mol Immunol 2009 Jul;46(11-12):2151-60.

165. Coccia EM, Stellacci E, Marziali G, Weiss G, Battistini A. IFN-gamma and IL-4 differently regulate inducible NO synthase gene expression through IRF-1 modulation. Int Immunol 2000 Jul;12(7):977-85.

166. Feng G, Gao W, Strom TB, Oukka M, Francis RS, Wood KJ, Bushell A. Exogenous IFN-gamma ex vivo shapes the alloreactive T-cell repertoire by inhibition of Th17 responses and generation of functional Foxp3+ regulatory T cells. Eur J Immunol 2008 Sep;38(9):2512-27.

167. Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. J Exp Med 2002 Apr 15;195(8):1003-12.

168. Gupta S, Jiang M, Anthony A, Pernis AB. Lineage-specific modulation of interleukin 4 signaling by interferon regulatory factor 4. J Exp Med 1999 Dec 20;190(12):1837-48.

169. Baratelli F, Lin Y, Zhu L, Yang SC, Heuze-Vourc'h N, Zeng G, Reckamp K, Dohadwala M, Sharma S, Dubinett SM. Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. J Immunol 2005 Aug 1;175(3):1483-90.

170. Sharma S, Yang SC, Zhu L, Reckamp K, Gardner B, Baratelli F, Huang M, Batra RK, Dubinett SM. Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. Cancer Res 2005 Jun 15;65(12):5211-20.

171. Bryn T, Yaqub S, Mahic M, Henjum K, Aandahl EM, Tasken K. LPS-activated monocytes suppress T-cell immune responses and induce FOXP3+ T cells through a COX-2-PGE2-dependent mechanism. Int Immunol 2008 Feb;20(2):235-45.

172. Torgerson TR, Genin A, Chen C, Zhang M, Zhou B, Anover-Sombke S, Frank MB, Dozmorov I, Ocheltree E, Kulmala P, Centola M, Ochs HD, Wells AD, Cron RQ. FOXP3 inhibits activation-induced NFAT2 expression in T cells thereby limiting effector cytokine expression. J Immunol 2009 Jul 15;183(2):907-15.

173. Torgersen KM, Vang T, Abrahamsen H, Yaqub S, Tasken K. Molecular mechanisms for protein kinase A-mediated modulation of immune function. Cell Signal 2002 Jan;14(1):1-9.

174. Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). Int Immunol 2007 Mar;19(3):337-43.

175. Mor A, Keren G, Kloog Y, George J. N-ras or K-ras inhibition increases the number and enhances the function of Foxp3 regulatory T cells. Eur J Immunol 2008 Jun;38(6):1493-502.

176. Bar N, Dikstein R. miR-22 forms a regulatory loop in PTEN/AKT pathway and modulates signaling kinetics. PLoS One 2010 May 27;5(5):e10859.

177. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, Loeb GB, Lee H, Yoshimura A, Rajewsky K, Rudensky AY. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. Immunity 2009 Jan 16;30(1):80-91.

178. Sporri B, Kovanen PE, Sasaki A, Yoshimura A, Leonard WJ. JAB/SOCS1/SSI-1 is an interleukin-2-induced inhibitor of IL-2 signaling. Blood 2001 Jan 1;97(1):221-6.

179. Samanta A, Li B, Song X, Bembas K, Zhang G, Katsumata M, Saouaf SJ, Wang Q, Hancock WW, Shen Y, Greene MI. TGF-beta and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. Proc Natl Acad Sci U S A 2008 Sep 16;105(37):14023-7.

180. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: Take a billion or so and call me in the morning. Immunity 2009 May;30(5):656-65.

Molecular Biology of the Cell. 4th edition. Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002.