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Insights into transcriptional regulation by FOXP3

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1. ABSTRACT

FoxP3 recently entered the spotlight as a critical component of regulatory T cell development and function. Several groups are presently engaged in an effort to uncover the mechanistic details of its contribution to this critical T cell subset. Despite this, the mechanism of FoxP3-mediated transcriptional repression and the affected target genes are still largely unknown. First, we discuss insights from work on other Fox family members with an emphasis on those with known roles in the immune system. Second, we review recent data concerning the molecular mechanism of FoxP3 function and its role in human disease. Finally, we consider what is known about FoxP3 target genes and their effect on T cell physiology.

2. THE FOX FAMILY OF TRANSCRIPTION FACTORS

Transcription factors are modular proteins with unique domains and motifs mediating DNA binding, protein-protein interactions, and transcriptional activation or repression. Forkhead box (Fox) proteins are a growing family of transcription factors classified by an approximately 110 amino acid monomeric DNA-binding domain, termed a forkhead (FKH) domain. Fox genes have been identified in species ranging from yeast to humans, with involvement in a broad range of developmental processes. First identified as the *fork head* (*fkh*) gene product in *Drosophila melanogaster*, Fkh has a high protein sequence similarity to the hepatic nuclear factor 3 (HNF3) family (now known as the FoxA family) (1, 2).

Table 1. Phenotypes of mice deficient in immunologically-relevant *Fox* family genes

Gene	Expression	Disease/Knockout	Immunological Function	References
Foxq1	abundant expression in the liver, moderate expression in the kidney, and low level expression in the lung, brain, and testis	satin (sa) mutation, where fur coat is silky due to a defect in differentiation of the hair shaft	suggested to play a role in natural killer NK cell activity, yet this defect appears to be attributed to genetic background differences, and/or the synergistic effect of the beige (bg) mutation	84, 85
Foxp1	expressed in developing nervous system, heart, lung and liver	N/A	regulates tissue macrophage differentiation in monocyte cell lines	86
Foxd2	expressed in T cell and monocytes	approximately half of the animals exhibit mild renal abnormalities	postulated to modulate T cell proliferation by adjusting sensitivity to cAMP levels	22, 23
Foxn1	found in the liver, lung, intestine, renal cortex and urinary tract of the developing embryo; expressed in the intestine, testis and thymus of adult tissue	nude (nu) phenotype; abnormal development of the epidermis, lack of hair, and lack of thymus	thymic epithelial cell development and T cell lymphopoiesis	26, 15
Foxj1	expressed by all structures containing ciliated cell; also found lymphocytes	deficient animals die during embryonic development or shortly after birth, with survivors displaying severe defects; fetal liver chimeras develop Th1 hyperproliferation and cytokine production with spontaneous NF-kappaB activity; decreased levels found in lymphocytes of SLE prone strains	significant role in modulating NF-kappaB activity and T cell activation	31, 32, 33, 34, 35, 36
Foxo3a	mainly expressed in peripheral lymphoid tissues; found in lymphocytes	Develop lymphocytic infiltrate disease, with NF-kappaB hyperactivation, Th cell hyperactivation and dysregulated cytokines; Foxo3a activity is decreased in SLE prone strains	NF-kappaB activity and lymphocyte proliferation	40

The Fox family is subdivided into at least seventeen classes (or subfamilies) based on structural similarity, each denoted by a letter (FoxA through FoxQ) (3). Individual genes of a subfamily are identified with a number following the subgroup classification (*i.e.* FoxA2).

In 1993, the first crystal structure of a FKH domain from FoxA3 bound to DNA was completed (4). This lepidopterous structure was termed a 'winged helix' for the three alpha helices arranged in a helix-turn-helix conformation flanked by two 'wings' comprised of loops and beta strands. While the majority of Fox family proteins bind to a DNA consensus sequence (XYZAAYA, X=A/G, Y=C/T, Z=A/C), more distant subfamilies (H, M, N, O, P, and Q) bind to sequences that only partially resemble this consensus motif (*i.e.* FoxO subfamily and insulin response elements) (5-9). Eight clusters of Fox genes have been identified in humans, with at least twenty additional Fox genes scattered throughout the genome (10). Fox proteins are involved in a diverse range of biological processes ranging from inner ear formation to autoimmunity, speech, and language development (11-13).

Mounting evidence indicates a significant role for Fox family genes, including FoxP3, in both development and regulation of the immune system (14-19). However, the mechanisms by which these genes are regulated and their specific targets are poorly understood. Fox family members of immunological relevance are discussed below, with a primary focus on the molecular mechanism and potential targets of Foxp3. Table 1 summarizes the immune related phenotypes of *Fox* family knockout mice.

3. FOX FAMILY MEMBERS IN THE IMMUNE SYSTEM

3.1. FoxD1/2

The stromal cells of the kidney express FoxD1, where it regulates the rate of differentiation of mesenchyme into tubular epithelium as well as growth and branching of the ureter and collecting system (20). *Foxd1* null mice die within 24 hours of birth due to renal failure secondary to malformation of the kidney. A highly related family member, FoxD2, is expressed in several tissues of the developing mouse embryo, including the kidney and central nervous system (21). Interestingly, *Foxd2* null animals are viable with roughly forty percent exhibiting renal abnormalities of hypoplasia and hydroureter (22). T cells from the *Foxd2* null animals show decreased sensitivity to cAMP-mediated inhibition of proliferation. *Foxd2* is expressed within the anterior presomitic mesoderm and nascent somites of the murine embryo (21). Later in development, expression is also detected in the developing tongue, nose, whiskers, kidney and limb joints (21). In human peripheral blood, *FOXD2* mRNA is detected in monocytes and T cells, but absent in B cells (23). While further investigation is necessary, Johansson and colleagues suggest that Foxd2 modulates T cell proliferation by adjusting sensitivity to cAMP levels. Interestingly, Gavin and colleagues reported that Foxp3 may also indirectly affect cAMP levels (24).

3.2. FoxN1

Analysis of murine embryonic expression patterns show that Foxn1 is found in the mesenchymal and epithelial cells of the liver, lung, intestine, renal cortex and urinary tract. In adult tissue, expression is restricted to the intestine, testis, and thymus (25). Mutations in *Foxn1* give rise to the nude (nu) phenotype in mice, rats, and humans, which is characterized by the abnormal development of the epidermis, lack of hair, and absence of a thymus (15, 26). Expression of *Foxn1* is likely controlled by wingless (Wnt) glycoproteins and bone morphogenetic proteins (BMPs). BMPs are involved in cell fate determination and patterning of the embryo (27). Foxn1 upregulation was observed

upon treatment of intact thymic lobes with BMP4, suggesting that BMPs may act directly on the thymic stroma affecting thymopoiesis (28). Wnt proteins constitute a large group of secreted glycoproteins that have significant roles in cell fate, proliferation, migration, polarity, and death (29). Both thymocytes and thymic epithelial cells (TECs) secrete these glycoproteins, and blocking Wnt-mediated signaling inhibits Foxn1 expression. These data show that BMPs and Wnts play a significant role in Foxn1 expression, thymic epithelial development, and T cell lymphopoiesis (30).

3.3. FoxJ1

In mice and humans the expression pattern of FoxJ1 has been localized to the lung, spermatids, oviduct, choroid plexus, and fetal kidney – all structures containing ciliated cells (31-33). *Foxj1* null mice die during embryonic development or shortly after birth, with any surviving animals exhibiting hydrocephaly, defective ciliogenesis, and randomized left-right asymmetry (34, 35). In the immune system, *Foxj1* is expressed primarily by T cells and is rapidly down regulated following TCR or IL-2 stimulation. Using fetal liver chimeras, it was found that *Foxj1* deficient animals display systemic cellular autoimmunity, as well as T cell hyperproliferation and hyperreactivity. T cells in these animals are skewed toward a Th1 cytokine profile, can be activated solely by IL-2 (TCR stimulation is not required), and exhibit decreased levels of the I-kappaB-beta regulatory subunit associated with spontaneous NF-kappaB activity (36). These findings suggest a role for *Foxj1* in suppressing spontaneous NF-kappaB activation *in vivo*. Consistent with this idea, Foxj1 levels are significantly decreased in lymphocytes from systemic lupus erythematosus (SLE) prone strains of mice (BXSB and MRL/lpr mice) (36). This decrease is not observed in the corresponding non-autoimmune controls, suggesting a role for FoxJ1 in preventing autoimmunity. Thus it appears that *Foxj1* plays a significant role in modulating NF-kappaB activity and T cell activation. Interestingly, Foxp3 may participate in a closely related regulatory pathway. Bettelli and co-workers have recently suggested that Foxp3 inhibits NF-AT and NF-kappaB mediated transcription of the *IL-2*, *IL-4*, and *IFN*--gamma genes, as discussed later (37).

3.4. FoxO

Currently, the *Foxo* subfamily contains four members: *Foxo1*, *Foxo3a*, *Foxo4*, and *Foxo6*, which are mammalian homologues of the *Caenorhabditis elegans* (*C. elegans*) *dauer formation mutant 16* (*daf-16*) gene. In *C. elegans*, *daf-16* is involved in a wide variety of biological processes, including fertility, lifespan, and regulation of insulin signaling and metabolism (38-40). While the FoxO family of proteins displays a broad expression pattern in mice, it appears that *Foxo3a* is the predominant family member expressed in peripheral lymphoid tissues. In resting lymphocytes, Foxo3a remains transcriptionally active in the nucleus. Upon cellular activation, Foxo3a is phosphorylated, exported from the nucleus, and rendered transcriptionally inactive (40). *Foxo3a* null animals undergo overtly normal early development, but exhibit premature ovarian failure and T helper cell hyperactivity, which gives rise to a multi-organ lymphocytic infiltrate disease. The disease phenotype exhibited by *Foxo3a* null mice is similar to that of (*Foxp*- deficient) *scurfy* (*sf*) animals as discussed below. Although the *Foxo3a* phenotype is less severe than that of *Foxj1* deficient animals, it appears that Foxj1 and Foxo3a may perform similar roles within the immune system. Employing a reporter system in M12 and HEK 293T cell lines, Lin and colleagues demonstrate that Foxo3a modulates the activity of an NF-kappaB luciferase reporter. Analogous to Foxj1, Foxo3a suppressed spontaneous NF-kappaB reporter activity, while other Fox family members (Foxm1 and Foxq1) had little or no effect. Furthermore, Foxo3a activity is decreased in naïve T cells from lupus-prone stains of mice, as are Foxj1 levels in the same mouse strains (40). Given the overlap in function and diminished levels/activity of Foxj1 and Foxo3a observed in SLE animals, it is tempting to speculate that Foxj1 and Foxo3a may act cooperatively to modulate NF-kappaB activity.

4. DISCOVERY OF THE FOXP SUBFAMILY

The Foxp subfamily originated with the discovery of two FKH domain-encoding genes expressed in embryonic and adult mouse lung, *Foxp1* and *Foxp2* (41). Both are also expressed in neural, intestinal, and cardiovascular cell types during embryogenesis, and repress transcription of the murine CC10 (Clara cell 10kDa protein) and human surfactant protein-C (SP-C) promoters *in vitro* (41). Foxp1 and Foxp2 contain a glutamine-rich amino-terminal region, a C2H2 zinc finger, a leucine zipper, and a FKH domain near the carboxy-terminal end (Figure 1). The presence of the glutamine-rich sequence, the C2H2 zinc finger, and the location of the FKH domain near the carboxy-terminus are unique features of the Foxp subfamily. Recently, two additional Foxp family members have been described. *Foxp4* was identified in an expressed sequence tag (EST) database based on homology to Foxp1 and Foxp2. It is expressed in the proximal and distal airway epithelium of the lung, as well as in the epithelial cells of the developing intestine (42). *Foxp3* contains at least three distinct structural domains: a FKH domain, a leucine zipper, and a C2H2 zinc finger. *Foxp3* was identified as the gene mutated in the *scurfy* mouse, a mutant that suffers from severe autoimmunity (12).

Foxp1, Foxp2, and *Foxp4* encode protein products of similar size. Interestingly, there are long regions of glutamine-rich sequences in the amino-terminal regions of Foxp1 and Foxp2, shorter regions in Foxp4, but none in Foxp3. Glutamine-rich sequences are found in the amino-terminal region of the androgen receptor (AR). By expressing mutant AR constructs containing various lengths of glutamine-rich sequence, Chamberlain *et al.* demonstrated that increasing the length of this domain negatively affected the transcriptional activity of the AR (43). These data prompted the authors to suggest that glutamine-rich domains may confer repressor function on transcriptional regulators. While the importance of this domain in transcriptional repression mediated by Foxp proteins has not been determined, the absence of this domain in Foxp3 may suggest a different mechanism of transcriptional repression.

Figure 1. Alignment of the four Foxp family members from mouse was performed using the ClustalW algorithm and blosum62mt2 matrix (VectorNTI AlignX program, Invitrogen, Carlsbad, CA). Poly-glutamine tracts of Foxp1 and Foxp2 Are shown in bold above and in gray at right (to scale). The C2H2 zinc finger domain is underscored in red, the coiled-coil/leucine zipper domain in green, and the FKH domain in blue. Note the uniqueness of Foxp3's amino-terminal end, lack of poly-glutamine, and much shorter carboxy-terminal extension beyond the FKH domain.

Similar to murine Foxp3, human FOXP3 contains a leucine zipper, a C2H2 zinc finger, a carboxy-terminal FKH domain, and lacks the glutamine-rich amino-terminal region. These proteins are highly homologous, sharing 86% amino acid (aa) sequence similarity overall, and 94% sequence similarity within the FKH domains. In contrast to

mice, a splice variant of FOXP3 has been identified in humans at both the mRNA and protein levels (44 and our unpublished observations). This variant lacks the sequence encoded by exon 2 (aa 70-104). The functional significance of this splice variant of FOXP3 has not been determined.

Foxp subfamily members share a highly conserved FKH domain, suggesting that they bind to similar target DNA sequences or bind to DNA in a similar manner. Indeed, Foxp1, Foxp2, and Foxp4 bind to DNA containing a FKH consensus site from the murine CC10 promoter *in vitro* (45). In addition, Foxp1 and Foxp2 bind to FKH consensus sites within the SV40 and *IL-2* promoters (46). Similarly, FOXP3 binds to DNA containing the FKH consensus site from the immunoglobulin heavy chain variable region (V_H) promoter (V1P) (47). Interestingly, the Foxp family may function cooperatively, as Foxp1, Foxp2, and Foxp4 homo- and heterodimerize (45). Since deletion of a conserved glutamic acid residue in the leucine zipper abolishes oligomerization (45), this oligomerization requires an intact leucine zipper. Finally, disruption of the leucine zipper inhibits both DNA binding and transcriptional repression of the murine CC10 promoter (45). Taken together, these results suggest that Foxp1, Foxp2 and Foxp4 bind to DNA as homodimeric or multimeric complexes, and that transcriptional repression requires DNA binding. Since all other characterized Fox proteins bind to DNA as monomers, this property of the Foxp family is unique (45).

Recently, a potential mechanism of transcriptional repression by Foxp1 and Foxp2 has been elucidated. Two domains in their amino-terminal regions were identified which when fused to the yeast Gal4 DNA-binding domain exhibit transcriptional repressor function. These domains lie within a 250 amino acid region in the middles of the proteins (~ amino acids 250 – 500) (Figure 1). One of these domains, originally termed domain 2, mediates protein-protein interaction with carboxy-terminal Binding Protein-1 (CtBP1), a transcriptional co-repressor known to interact with histone deacetylases (48). Co-expression of CtBP1 with full-length Foxp1 or Foxp2 results in a dose-dependent increase in their ability to repress the murine CC10 promoter. Furthermore, deletion of the CtBP1-binding region in domain 2 abrogates the ability of the Gal4-domain 2 fusion protein to repress transcription of the luciferase reporter containing Gal4 binding sites. These data suggest that Foxp1 and Foxp2 repress transcription by recruiting histone deacetylases, which then deacetylate histones associated with the promoters of their targets. Hypoacetylated histones are found in "closed" or "silent" chromatin, which correlates with transcriptional silence of the associated genes. However, another mechanism of transcriptional regulation by Foxp1, Foxp2, and Foxp4 is suggested by the following two findings. First, Foxp4 does not contain repressor domain 2 required for interaction with CtBP1, however, it still represses transcription of the murine CC10 promoter. Second, mutation of the CtBP1 consensus-interaction sequence in Foxp1 and Foxp2 does not affect the ability of full-length Foxp1 or Foxp2 to repress transcription of the murine CC10 promoter (45). Alternatively, these data may indicate that Foxp1, Foxp2, and Foxp4 employ multiple mechanisms for regulating transcription, and that different mechanisms may be employed for regulating different target genes.

5. ROLE OF FOXP SUBFAMILY MEMBERS IN HUMAN DISEASE

The expression of Foxp1, Foxp2, and Foxp4 proteins in developing lung airway and intestinal epithelium suggests a role for Foxp family members in tissue differentiation. The functional importance of Foxp family members is emphasized by the prevalence of Foxp mutations in several mouse and human diseases. FOXP1 expression is dysregulated in a variety of human tumors such as diffuse large B-cell lymphomas, leading to speculation that FOXP1 may play a role in tumorigenesis (49). Conversely, increased expression of FOXP1 correlates with poor prognosis of diffuse large B-cell lymphoma patients (50, 51). These conflicting reports underscore the importance of identifying the transcriptional targets and mechanism of FOXP1 function. Mutations in FOXP2 have been linked to speech and language disorders in humans (13). As yet, no reports have described mutations in FOXP4 resulting in human disease. However, abnormalities in cardiogenesis have been discovered in Foxp4-deficient mice (52).

The identification of *foxp3* as the gene mutated in *scurfy* mice led to an investigation of its possible role in a human syndrome with clinical features similar to those seen in *scurfy* animals. FOXP3 mutation or loss of expression results in the human syndrome Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX). Affected males present with systemic autoimmune disease affecting bowel, skin, endocrine organs, and blood (53-56). In general, affected individuals develop symptoms very early in infancy and usually die in the first two years of life. Without ablative bone marrow transplant, this disease is fatal by one to two years of age. Accumulating data demonstrates the critical roles that Foxp subfamily members play in tissue differentiation, tumorigenesis, and immune system regulation. This underscores the importance of determining their mechanisms of function as well as their transcriptional targets.

6. FOXP3: THE BLACK SHEEP OF THE FOXP SUBFAMILY

6.1. Identification of FoxP3

Unlike other Fox gene subfamilies, all Foxp subfamily members function as transcriptional repressors and have carboxy-terminal rather than amino-terminal FKH domains. Thus it is tempting to speculate that the location of the FKH domain is a determining factor in whether a Fox family transcription factor induces or represses transcription of its targets. Foxp1, Foxp2, and Foxp4 are relatively large proteins (706 aa, 715 aa, and 686 aa, respectively,) in comparison to Foxp3 (430 aa) (Figure 1). The glutamine-rich sequences in the amino-terminal regions of Foxp1, Foxp2, and Foxp4, but not Foxp3,

account for most of the difference in size. In addition, there are longer sequences carboxy-terminal to the FKH domain in Foxp1, Foxp2, and Foxp4 relative to Foxp3. Collectively, these differences in structure indicate Foxp3 may function quite differently even from its own P subfamily members.

Foxp3 was first identified on the X chromosome by positional cloning as the gene mutated in the *scurfy* mouse, a spontaneously arising strain in which males bearing the mutant allele are characterized by wasting, exfoliative dermatitis, lymphadenopathy, hepatosplenomegaly, and the presence of autoantibodies (12, 57). The autoimmune syndrome observed in *scurfy* mice is due to the absence of naturally-arising CD4+CD25+ regulatory T cells (T_{reg}). In the absence of T_{reg}, autoreactive CD4+ T cell activation proceeds unchecked, resulting in dysregulated cytokine production, severe multi-organ lymphocytic infiltration, and other immunopathology (12, 57). Targeted deletion of *Foxp3* in mice results in development of *scurfy*-like symptoms and ultimately death at approximately 3 weeks of age, confirming that *Foxp3* deficiency is responsible for the *scurfy* phenotype. This autoimmune disease is prevented by neonatal adoptive transfer of T_{reg} from wild-type mice (58), or by breeding *scurfy* mice with Foxp3 transgenic mice (59). These Foxp3 transgenic mice express elevated levels of Foxp3, due to the integration of multiple copies of a genomic *Foxp3* cosmid clone (gene dosage effect). Due to the similarity of immunopathology between Foxp3-deficient mice and IPEX syndrome, mutation of FOXP3 in humans is suspected to result in a lack of T_{reg}, and subsequent autoimmunity. However, further study is required of IPEX patients in order to confirm this hypothesis.

6.2. Foxp3 Function

Murine studies have been particularly revealing with respect to the importance of Foxp3 in the regulation of peripheral auto-reactive T cells. However, relatively little is known about the molecular mechanism of FOXP3 function, particularly in humans. Our work, as well as that from other groups, has shown that FOXP is localized to the nucleus (17, 47). We have previously demonstrated that FOXP3 functions as a transcriptional repressor when expressed in either non-lymphoid cells or T cell lines using a reporter plasmid containing FKH binding sites as target (47). In addition, FKH consensus sites have been identified adjacent to NF-AT binding sites in the murine *IL-2*, *IL-4*, *GM-CSF*, and human *IL-2* promoters, suggesting that FOXP3 regulates expression of activation-induced cytokines. In support of this hypothesis, a short sequence from the murine *IL-2* promoter competes with the FKH consensus site for binding to FOXP3, suggesting that FOXP3 binds to the *IL-2* promoter (47). Furthermore, FOXP3 expression inhibits the activity of a reporter containing NF-AT binding sites from the murine *IL-2* promoter. Since nuclear localization, DNA binding, and transcriptional repressor function of FOXP3 all depend upon its presence, the FKH domain is pivotal for FOXP3 function. FOXP3-expressing T cells (both primary human CD4+T cells and FOXP3-transduced T cells) are hyporesponsive to TCR stimulation, making little or no IL-2, IL-4, IFN-gamma, or IL-10 (44, 47, 60, 61). The presence of FKH consensus sites in the promoters of cytokine genes coupled with the repressive effect of FOXP3 on activation-induced cytokine expression argue that FOXP3 inhibits transcription of these genes by binding to FKH consensus sites in their promoters.

A recent study of murine Foxp3 supports this hypothesis. Retroviral transduction of *Foxp3* into primary CD4+ T cells inhibits TCR-induced expression of IL-2, IL-4, and IFN-gamma (37). Interestingly, this effect appears specific to Foxp3, as forced expression of neither Foxp1 nor Foxp2 inhibited IL-2, IL-4, or IFN-gamma production. Expression of a mutant *Foxp3* construct lacking the first 199 amino acids completely abrogated the ability of Foxp3 to inhibit IL-2 production (37), suggesting that the amino terminus was required for transcriptional repression. However, this experiment does not rule out the possibility that deletion of the amino terminus disrupts the proper folding of Foxp3 and is otherwise not involved in repressor activity.

Additional studies suggest that ectopic Foxp3 expression inhibits cytokine gene expression with NF-AT sites as likely targets (47). Recent work has also shown that Foxp3 inhibits NF-kappaB-mediated transcriptional activation, but not NF-kappaB DNA-binding (37). This suggests that Foxp3 does not compete with NF-kappaB for binding to DNA. Taken together, these data suggest that Foxp3 controls cytokine gene transcription through inhibition of the two transcription factors critical for their activation, NF-AT and NF-kappaB. In support of this conclusion, NF-AT and NF-kappaB activities are elevated in CD4+ T cells from *scurfy* mice, and introduction of Foxp3 into these cells lowers the activity of each factor (37, our unpublished observations).

The mechanism by which Foxp3 performs this function remains unclear. Recent work from Bettelli et al. suggests that Foxp3 directly interacts with both NF-AT and the p65 subunit of NF-kappaB (37). In addition, Foxp3 inhibits transcription of a Gal4 luciferase reporter initiated by Gal4-NF-AT and Gal4-p65 (NF-kappaB) fusion constructs (37). Since the reporter lacked FKH binding sites, these data are consistent with a direct interaction between Foxp3 and NF-AT/NF-kappaB. These data clearly demonstrate that Foxp3 blocks IL-2, IL-4, or IFN-gamma production in primary CD4+ T cells in response to TCR stimulation, most likely by inhibiting NF-AT and NF-kappaB activity.

6.3. Role of Transcriptional Co-repressors in FoxP Function

As described above, Foxp1 and Foxp2 associate with the transcriptional repressor CtBP1, suggesting a mechanism for inhibition of transcription. At this point, it remains unclear whether Foxp3 also associates with other co-factors that aid in its ability to repress transcription. Recent work showing that the chromatin structure of the *IL-2* promoter differs in CD4+CD25- and CD4+CD25+ T cells suggests a possible mechanism for Foxp3-mediated

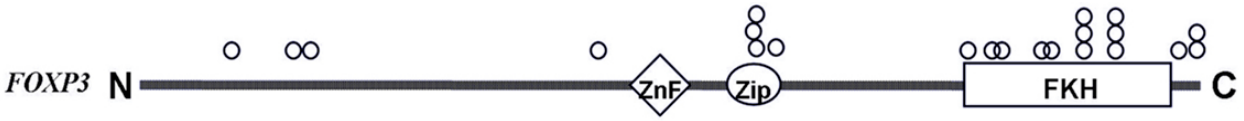

Figure 2. Mutations identified in IPEX patients cluster around identified structural domains. Linear schematic of FOXP3 demonstrates clustering of missense mutations (open circles) identified in patients with the phenotype of IPEX syndrome. Note that the mutations cluster within the FKH domain, leucine zipper, and a functionally undefined area of the amino-terminus. Each open circle represents a single missense mutation.

regulation of *IL-2* transcription (62). The chromatin containing the *IL-2* promoter in CD4+CD25- T cells is in an 'open' configuration, while the same region is 'closed' in CD4+CD25+ T cells (62). As *Foxp3* is primarily expressed in CD4+CD25+ T cells, it may be involved in remodeling the chromatin of genes whose transcription it regulates. The identification of FKH consensus sites in the human and mouse *IL-2* promoter, as well as in other cytokine genes (47) supports this conclusion. However, to date the precise mechanism by which Foxp3 regulates transcription is not known.

6.4. Structure-Function Analysis of FOXP3

Much effort is currently being expended to define functional domains within FOXP3. As described above, the FKH domain is critical for both DNA binding and nuclear localization. Confirmation of its role in nuclear localization comes from site-directed mutation of two lysine residues (K415 and K416) at the carboxy end of the FKH domain to glutamic acid. This mutant form of FOXP3, when expressed in cell lines, localizes to the cytoplasm (JEL, T. R. Torgerson, L.A. Schubert, S.D. Anover, H. D. Ochs, and SFZ, unpublished observations). The functional properties of the remainder of the protein are not well understood. However, studies on other members of the FOXP family may be relevant to the analysis of FOXP3 function. For example, the leucine zipper domain of Foxp1 and Foxp2 is critical for homo- and heterodimer formation (45). Additionally, deletion of the leucine zipper domain from Foxp1 and Foxp2 abrogates their ability to act as transcriptional repressors. This domain is also likely involved in dimerization of FOXP3 (see below).

While, the amino-terminal half of FOXP3 contains no obvious functional domains, it does contain a moderately high proportion of proline residues (Figure 1). We found that a fusion protein containing the DNA-binding domain of the yeast transcription factor GAL4 and the amino-terminal half of human FOXP3 (amino acids 1-198) is functional as a transcriptional repressor, while a fusion of the zinc finger and leucine zipper domains are nonfunctional. Although these constructs lack the FOXP3 nuclear localization signal sequence and leucine zipper domain, the GAL4 DNA-binding domain contains both nuclear localization and dimerization sequences, subserving these functions for the fusion proteins. Using this system our laboratory has defined two functional domains within the amino terminus of FOXP3. One domain, within amino acids 67-132, is involved in general transcriptional repression by FOXP3. The second domain is specifically required for repression of NF-AT-mediated transcription. Bettelli *et al*. also showed that Foxp3 directly interacts with NF-AT and the NF-kappaB subunit p65 (37). As for murine Foxp3, the region of interaction in human FOXP3 with NF-AT has not yet been identified. We demonstrated using differentially-tagged FOXP3 proteins that FOXP3 homodimerizes. This dimerization requires the leucine zipper domain, since deletion of a conserved glutamic acid residue (E251) within it inhibits dimerization. We have also demonstrated that both putative NLS sequences located on either side of the FKH domain are required for efficient nuclear localization when expressed in a cell line, clearly demonstrating that these sequences are indeed functional motifs. In summary, all FoxP subfamily members identified to date, including FoxP3, bind to DNA as dimers and utilize unique domains near their amino termini to repress transcription.

In an effort to further define important functional domains of FOXP3 *in vivo*, the *FOXP3* gene was sequenced in a large cohort of patients with the phenotypic features of IPEX syndrome. Overall, more than 30 mutations of FOXP3 have been reported (55, 63-65, T. R. Torgerson and H. D. Ochs, unpublished observations). Interestingly, the 16 missense mutations identified in FOXP3 cluster in 3 regions of the FOXP3 protein: the FKH DNA-binding domain, the leucine zipper, and a portion of the amino-terminal region with unknown function (Figure 2). Little, however, is known about how these mutations affect FOXP3 function in afflicted individuals. We demonstrated that mutations in FOXP3's leucine zipper and FKH domains analogous to mutations identified in IPEX patients result in a loss of transcriptional repression. The prevalence of mutations within the FKH domain argues that the ability of FOXP3 to bind to DNA is critical to its repressor function. Interestingly, mutation of the aforementioned conserved glutamic acid residue within the leucine zipper, which inhibits homodimerization of FOXP3, has also been identified in IPEX patients. This result suggests that homodimerization of FOXP3 is also required for FOXP3 function. These observations demonstrate a functional consequence for mutations of FOXP3 analogous to those found in IPEX and corroborates the assertion that mutations in FOXP3 are responsible for the development of IPEX in humans. It also suggests that dysregulated expression of FOXP3 transcriptional targets underpin the abnormal development and/or function of T_{reg}, thus leading to the severe autoimmunity observed in IPEX humans and *scurfy* mice. The identification of these transcriptional targets will be pivotal to understanding the ontogeny and function of naturally arising regulatory T cells.

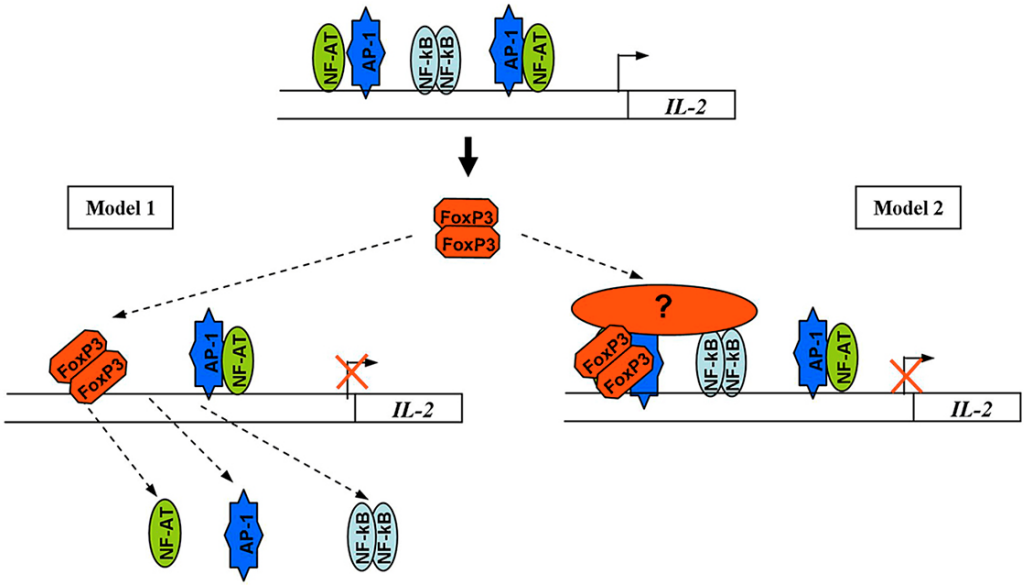

Figure 3. Molecular models representing mechanism by which FoxP3 represses transcription of the IL-2 promoter. Model 1 (left side): FoxP3 binds as a dimer to the FKH consensus site near the –280 NF-AT site, outcompeting NF-AT, AP-1, and NF-kappaB in binding to the IL-2 promoter, resulting in little or no transcription of the IL-2 gene. Model 2 (right side): FoxP3 recruits a transcriptional co-repressor to the IL-2 promoter, binds as a dimer, and directly interacts with NF-AT and NF-kappaB. The corepressor then silences transcription of IL-2 by an unknown mechanism, possibly involving recruitment of histone-modifying enzymes.

Taken together, we propose two models for the mechanism by which FoxP3 represses transcription of its targets, using IL-2 as a model target gene (Figure 3). In the first model, FoxP3 dimerizes and binds to the FKH consensus site within the distal composite NF-AT/AP-1 site of the *IL-2* promoter. Binding of FoxP3 thus blocks or displaces NF-AT and AP-1 from binding DNA (or from interacting with each other), resulting in repression of *IL-2* transcription in response to TCR stimulation. In the second model, FoxP3 dimerizes and associates with a transcriptional co-repressor, such as a histone deacetylase or another modifier of chromatin. FoxP3 binds to the distal NF-AT/AP-1 site in the *IL-2* promoter, recruiting the transcriptional co-repressor to the *IL-2* promoter. Recruitment of the co-repressor results in modification of the associated histones, and subsequent silencing of the *IL-2* promoter. The finding that Foxp3 expression does not affect the ability of NF-AT, NF-kappaB, or AP-1 to bind to their respective sites in the *IL-2* promoter *in vitro*, contradicts the first model (37, 62). Furthermore, the finding that chromatin containing the *IL-2* promoter remains "closed" in primary murine T_{reg} in response to strong stimuli supports the second model (62). The identity of the transcriptional co-repressor that interacts with FoxP3 and the subsequent modifications to the *IL-2* promoter are not known, and are the subject of intense study.

7. THE TRANSCRIPTIONAL PROGRAM OF FOXP3 – EVIDENCE FOR TARGET GENES AND EFFECTS ON REGULATORY T CELL PHYSIOLOGY

7.1. The FoxP3 Transcriptome

Mounting evidence suggests that FoxP3 is a critical regulator and lineage-determining factor for the CD4+CD25+ cell contact-dependent T_{reg} compartment. However, the specific target genes and biochemical pathways governed by this transcription factor remain largely elusive. At least four studies have been published comparing the transcriptome of regulatory and effector CD4+ T cells (T_{eff}) by microarray, though there is little concordance between their results (17, 24, 66, 67).

In addition to analyzing non-identical sources of T_{reg} and T_{eff} , each of the four aforementioned publications reports clearly non-identical subsets of their data. Nevertheless, in all three studies that included the activated T_{eff} reference sample (24, 66, 67), three genes were similarly upregulated in T_{reg} and not activated T_{eff} (Table 2). Eleven more genes were similarly upregulated in T_{reg} in at least two of the three studies. None of the genes common to at least two of the three published lists were downregulated in T_{reg}, which is perhaps surprising since FoxP3 is thought to be exclusively a transcriptional repressor. Thus, genes upregulated in response to FoxP3 expression are likely targets of other transcriptional repressors which are themselves repressed by FoxP3. In our own unpublished observations, however, there are approximately as many genes downregulated as there are upregulated in response to FoxP3 expression. In addition, under some circumstances, truncated FOXP3 constructs are capable of enhancing transcription in reporter assays.

Considering these results, one can speculate that FoxP3 induces deficits in T cell receptor and cytokine signaling, though no data have been published showing such a functional connection. In addition, none of the differentially regulated genes reported in these studies appear sufficient to explain the T_{reg} bias toward apoptosis

Table 2. Genes upregulated in T_{reg} relative to T_{eff}

Gene Name	Category	Description
SOCS2	Signaling	Phosphatase, growth factor signaling
TIAM-1	Signaling	Guanine nucleotide exchange factor
GBP-3	Signaling	G-protein
IL-2R alpha, CD25	Cell Surface	IL-2 receptor alpha chain
IL-2R beta, CD122	Cell Surface	IL-2 receptor beta chain
CTLA-4	Cell Surface	T cell inhibitory receptor
GITR, TNFRSF18	Cell Surface	T cell costimulatory receptor
OX40, TNFRSF4	Cell Surface	T cell costimulatory receptor
NRP-1, Neuropilin-1	Cell Surface	Unknown function in T cells
CD81, TAPA-1	Cell Surface	T and B cell costimulator, tetraspannin
Lymphotoxin	Secreted	Inflammation, lymphoid organ development
ECM-1	Secreted	Unknown function in T cells
OCTN-2, SLC22A5	Metabolism	High affinity carnitine transporter
beta-enolase	Metabolism	Distal glycolysis

Genes reported by all three publications analyzed are shown in bold. Other genes are reported by at least two of the three.

observed by some groups (68, 69, D. J. Kasprowicz unpublished observations) (discussed further below). Induction and maintenance of *FoxP3* expression is likely only one (albeit major) component determining the transcriptional profile of regulatory T cells, as they bear surface markers consistent with previous activation and likely experience a unique set of developmental signals from their effector counterparts. Thus, an experimental system in which absence or presence of *FoxP3* expression is the only variable will be required to definitively identify FoxP3 direct target genes. This might be accomplished in part by limiting expression analyses to genes differentially regulated within hours of *FoxP3* induction or direct protein transduction. It will also be crucial to conduct these experiments in primary T cells, since much of the apoptotic and proliferative machinery apparently regulated by FoxP3 is likely dysregulated in cell lines.

7.2. FoxP3, Apoptosis, and Proliferation

In addition to uncertainty regarding target genes, the cell physiologic effects of *FoxP3* expression are controversial. CD4+CD25+ T_{reg} have been described both as biased toward (68, 69, D. J. Kasprowicz unpublished observations) and against (70, 71) apoptosis. The balance of evidence, however, suggests that *FoxP3*-expressing CD4+CD25+ cells are generally more sensitive to various apoptotic triggers than are their non-*FoxP3*-expressing CD4+CD25- counterparts *in vitro*. This does not seem to be the case *in vivo* (71), most likely due to the availability of pro-survival cytokines such as IL-2 and IFN-beta (68).

Although it is now clear that T_{reg} require a signal through the IL-2 receptor for development in the thymus and/or survival in the periphery (72-74), the mechanism by which *FoxP3* expression imposes this requirement is not known. In other settings of extreme dependence on a cytokine for survival, dramatically decreased expression of anti-apoptotic Bcl-2 family members and glycolytic pathway components (75) has been reported. Indeed, T_{reg} have been reported to express lower levels of Bcl-2 than T_{eff} (68), though only slightly so. Published microarray analyses, as well as our own data, also do not appear to support a specific or global decrease in glycolytic rate potential at the mRNA level. Thus, the molecular mechanism of FoxP3-induced apoptotic bias does not closely resemble growth factor withdrawal as might be expected given the strict dependence of T_{reg} on IL-2 (72-74).

Identification of this mechanism will be useful not only in understanding T_{reg} physiology, but also in maintenance of T_{reg} following adoptive transfer into human patients for the treatment of autoimmune disease. Experience from adoptive tumor immunotherapy has shown that poor survival of *in vitro*-expanded T cells post-infusion is a major barrier to positive clinical outcome (76). This may also be true for T_{reg}, and the systemic toxicity of high-dose exogenous IL-2 in humans obviates its use to maintain the transferred cells. Determining the FoxP3 target genes responsible for apoptotic bias and proliferative anergy might identify pharmaceutical targets for preferentially preserving and expanding both natural and expanded T_{reg}.

In addition to their suicidal tendencies, *FoxP3*-expressing cells have long been described as anergic. Although both effector and regulatory CD4+ T cells are capable of homeostatic and antigen-driven proliferation *in vivo* (24), T_{reg} are relatively anergic to proliferative stimuli *in vitro* (77, D. J. Kasprowicz unpublished observations). This phenotype may be closely related to the aforementioned apoptotic bias, or it may reflect a specific block to T cell receptor-driven cell cycle progression. In support of this, expression microarray analyses have shown elevated levels of inhibitory phosphatases (24, 66, 67), but no functional connection to these has yet been made. It has been shown that this *in vitro* anergy is reversible by addition of strong costimulation and an extremely high IL-2 concentration (78). The implications of this finding for the mechanism of T_{reg} anergy are unclear, however, as activated effector T cells expressing CD25 respond maximally to more than twenty-fold lower IL-2 concentrations (79). This may indicate that these superphysiological stimuli indirectly bypass rather than reverse T_{reg} anergy.

7.3. Molecular Mediators of Suppression and FOXP3

Ectopic expression of Foxp3 has been shown sufficient to confer on CD4+CD25- T cells the ability to suppress proliferation of non-Foxp3-expressing CD4+CD25- T cells. (18, 58). It is therefore reasonable to speculate that the molecular mediators of suppression are FOXP3 target genes. Although there is evidence implicating CTLA-4 (80), GITR (66), LAG-3 (81), and TGF-beta (82) as potential FoxP3 targets involved in this phenotype, the importance of these for T_{reg} function is disputed and none have been proven direct or indirect FoxP3 target genes. As the former three are induced upon activation in normal effector (FoxP3-) CD4+ , they are certainly not exclusive targets of FoxP3. Aside from these four, no other potentially suppressive cell surface proteins have arisen from published expression microarray analyses.

However, the conclusion that natural CD4+CD25+FoxP3+ T_{reg} suppression is contact-dependent has been based almost entirely on the ability of a transwell membrane to block suppression. It is also possible that not T_{reg}:T_{eff} contact but extreme proximity is required. Short range effects such as might be mediated enzymatically by indoleamine 2,3 dioxygenase (IDO) via tryptophan catabolism and consequent immunosuppressive metabolite production have been suggested (83). Thus, while the search for the molecular mediator of suppression may be limited to direct and indirect FOXP3 target genes, it cannot be confined to cell surface proteins. Further, as there is no direct evidence that FOXP3 can act as a transcriptional activator, the mechanism by which it regulates these putative suppression mediators is likely indirect.

8. PERSPECTIVE

While FoxP3 is capable of conferring and maintaining the regulatory T cell phenotype, the mechanism by which this occurs is still unclear. It appears that this process requires the transcriptional repressor function of FoxP3, however, the specific genes it targets (besides those of activation-inducible cytokines) remain to be identified. Lastly, is FoxP3 always a repressor, or can it directly induce transcription of some genes similar to other Fox family members? Much of our understanding of FoxP3 has come from studies in mice. However, there are minor and at least one potentially major difference between mouse and human FoxP3 – the coexpression of a truncated isoform of the protein lacking the second coding exon in human T_{reg}. Although the functional properties of this splice variant are unknown, it may give rise to discrepancies between mouse and human studies regarding T_{reg}. A better understanding of FoxP3 function at the mechanistic and target gene level will no doubt aid in transforming T_{reg} into a therapeutic tool for the treatment of human autoimmunity and potentially other types of disease.

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