Isoform-Specific Inhibition of RORα-Mediated Transcriptional Activation by Human FOXP3

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FOXP3 is a forkhead family transcriptional repressor important for the development and function of CD4+CD25+ regulatory T cells. In humans, FOXP3 is expressed as two isoforms, a full-length form and a smaller form lacking exon 2. These two isoforms are expressed in approximately equal amounts in circulating regulatory T cells, and are induced equally in freshly activated CD4+CD25− T cells. Herein, we show that FOXP3 interacts with retinoic acid receptor-related orphan receptor (ROR)α, and that this interaction inhibits transcriptional activation mediated by RORα. Full-length FOXP3, but not the isoform lacking exon 2, interacts with RORα, and the region of FOXP3 involved in the interaction is encoded by exon 2. Mutation of the LxxLL motif in FOXP3, located in exon 2, abolished interaction and repression by FOXP3. Additionally, the inhibition of RORα by FOXP3 does not require an intact forkhead domain, demonstrating a mode of FOXP3 function that is independent of DNA binding. Interestingly, expression of RORα in T cells leads to the expression of genes that define Th17 cells, and the expression of each of these genes was inhibited by coexpression of full-length, but not ΔEx2, FOXP3. These data expand the possible targets of FOXP3-mediated repression and demonstrate functional differences between FOXP3 isoforms. The Journal of Immunology, 2008, 180: 4785–4792.

Immuneological tolerance consists of both a central component (selection and deletion in the thymus) and a peripheral component. A population of regulatory T (Treg) cells exemplified by the cell-surface expression of CD4 and CD25 have been identified and shown to actively suppress immune responses (1). These cells inhibit the activation of autoreactive T cells in an Ag-specific, cell contact-dependent manner (2–4). In rodents these cells have been shown to develop in the thymus, possibly as a consequence of escape from negative selection (2, 5, 6).

Although the molecular basis for the development and function of Treg cells remains unclear, the forkhead family transcription factor FOXP3 has been implicated in their development and function. Mice carrying the X-linked scurfy mutation develop a fatal lymphoproliferative disease exemplified by a lack of conventional CD4+CD25− Treg cells (7, 8). In both humans and mice, FOXP3 has been shown to be expressed predominantly in CD4+CD25− Treg cells. Additionally, in mice, FOXP3 has been shown to be capable of converting naïve CD4+CD25− to Treg cells when introduced via retrovirus or enforced transgene expression (8, 9). Thus, in mice, FOXP3 is both necessary and sufficient for the development and function of CD4+CD25+ Treg cells.

In humans, mutations in FOXP3 lead to the clinical syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which shares most of the phenotypic characteristics of scurfy (10–12). However, unlike mice, expression of FOXP3 is also induced upon stimulation of human CD4+CD25− T cells, suggesting additional roles for FOXP3 in human T cell function (13–15). Additionally, humans express two FOXP3 isoforms, one of which is encoded by a complete mRNA, and the other by a splice variant that lacks exon 2 (13, 15). Although the expression levels of these two isoforms appear to be equivalent in human Treg cells, the functional differences, if any, remain obscure.

The retinoic acid receptor-related orphan receptor (ROR) subfamily of nuclear receptors consists of three members, RORα, RORβ, and RORγ, which are termed NR1F1–NR1F3 by the Nuclear Receptor Nomenclature Committee. RORα is expressed in a variety of cell types and regulates many aspects of development, including neuronal cell development, bone metabolism, muscle differentiation, arteriosclerosis, immune response, and lymphocyte development (16). In vascular smooth-muscle cells, RORα negatively regulates inflammatory responses by preventing the expression of the inflammatory cytokines such as IL-6, IL-8, and cyclooxygenase-2 (17). Additionally, Dzhagalov et al. demonstrated that RORα indirectly regulates lymphocyte development by providing an appropriate microenvironment (18). RORβ is expressed in the tissues of CNS that are involved in processing the sensory information, and in anatomical components that are responsible for circadian rhythm (19). RORβ−/− mice showed postnatal retinal degeneration and abnormalities in circadian behavior. RORγ has been shown to play a crucial role in lymph node organogenesis and thymopoiesis. Mice deficient in RORγ lack lymph nodes and Pey er’s patches, and the thymocytes from these mice undergo accelerated apoptosis that leads to dramatic decrease of peripheral T cell numbers (20–22).
In this report we show that FOXP3 can interact with RORα and inhibit its ability to act as a transcriptional activator. This inhibition does not require DNA binding by FOXP3, and is only seen with the full-length protein, not the isoform lacking exon 2. This is the first report of a functional distinction between human FOXP3 isoforms, and it identifies a novel FOXP3-interacting protein.

Materials and Methods

Yeast 2-hybrid screen

Human CD4⁺ CD25⁺ Treg cells were generated in vitro as described (13), and total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies). All human subjects research protocols were approved by the Benaroya Research Institute Institutional Review Board, and subjects provided written informed consent before participation in the study. Reverse transcription was performed with CDS III primer and SMART III Oligonucleotide of the Matchmaker Library Construction & Screening Kit (Clontech). The primers for PCR are: 5' PCR-Not (TAG CTC ACG CCG CCG CAA GCA GTG GTA TCA ACG CAG AGT GG) and 3' PCR-XhoI (GTA TCA GTG TCG AAT GAC GAG GGC GCC GAG GCC GCC GAC A). The prey vector pB42AD was modified as follows: BamHI and NotI sites were removed and a multiple cloning site containing NotI site was cloned between the BamHI sites. A cDNA library for the yeast 2-hybrid screen was generated by cloning the PCR product into this modified prey vector after the digestion with NotI and XhoI. A PCR product for the sequences encoding amino acids 1–198 of human FOXP3 was cloned in the bait vector pLexA. Yeast 2-hybrid screen and retest analysis were performed as described by the manufacturer (Clontech).

Cell culture and transfections

HEK 293 T cells were maintained in complete DMEM medium containing 5% FBS and 1% penicillin and streptomycin, and transfected using FuGENE 6 reagent (Roche). Jurkat cells were maintained and transfected according to the manuals of X-tremE GENE Q2 transfection reagent (Roche) and Cell Line Nucleofector Kit V (axama). Luciferase activity was measured using luciferase assay system (Promega) and the EG & G Berthold Lumat 9507 luminometer (PerkinElmer Life Sciences). All transfections included pSV-Biotin and pLexA-β-gal for HEK 293 T cells or pCMV-β-gal for Jurkat cells, and data were normalized to β-galactosidase activity.

RT-PCR for RORα

Human CD4⁺ CD25⁺ Treg cells were generated from PBMCs, and in vitro-generated CD4⁺ CD25⁺ regulatory T cells were produced as described (13). Total RNA was isolated using TRIzol reagent. Reverse transcription was performed with oligo(dT)₁₂–₁₈ and the SuperScript II RNase H⁻ reverse transcriptase (Invitrogen). The PCR primers and the annealing temperatures for the various are: RORα: forward (Eco RI sites) (forward: ACC CCG CTG AAC CAG GAA TC, reverse: GAA GTT CCG TCG CCC TCA ATG CAG); reverse (NotI sites) (forward: CCT GGA GGC AGA CAC TCA GGT, reverse: GAA GTT CCG TCG CCC TCA ATG CAG); reverse (NotI sites) (forward: CCT GGA GGC AGA CAC TCA GGT, reverse: GAA GTT CCG TCG CCC TCA ATG CAG); reverse (NotI sites) (forward: CCT GGA GGC AGA CAC TCA GGT, reverse: GAA GTT CCG TCG CCC TCA ATG CAG); reverse (NotI sites) (forward: CCT GGA GGC AGA CAC TCA GGT, reverse: GAA GTT CCG TCG CCC TCA ATG CAG). The PCR products were confirmed by DNA sequencing.

Retroviral transduction

Full-length human FOXP3 was cloned into retrovirus vector pLXSN-NGFR. Full-length human RORα transcript was PCR cloned into pLXSN-NGFR and sequenced. These plasmids, together with gag-pol and vsvg plasmids, were then transfected into 293 T cells to prepare retrovirus with Muris TransIT-LT1 transfection reagent (Mirus Bio). After 48 h incubation, the supernatant containing prepared retrovirus was harvested and stored at −80°C until use. Jurkat cells were plated in a 24-well plate, and retrovirus supernatant prepared above was added to the wells. Then the cells were spun at 2000 rpm for 90 min at 32°C and incubated for 3 days at 37°C. GFP-positive cells were sorted by FACS. These sorted cells were then stimulated with PMA (50 ng/ml) plus ionomycin (1 µg/ml) for 4 h. After stimulation, cells were harvested and total RNA was isolated as described above.

Gene expression analysis

For microarray experiment, total RNA was labeled with MessageAmp II-Biotin Enhanced Single Round aRNA Amplification Kit (Ambion), and microarray was performed using Affymetrix U133 Plus 2.0 Human GeneChips according to the manufacturer’s instructions. For quantitative PCR (qPCR) experiments, the first-strand cDNA was synthesized from total RNA as described above. qPCR experiments were then performed as described previously (23). Primer sets were as follows: human IL22, 5’-aa gaagctgtagcctcc-3’ and 5’-catgctggctagctgg-3’, human CXCR3, 5’-tgc ggctgtaggtgctc-3’ and 5’-atgctgtagctgggtggaa-3’, human GAPDH, 5’- gagaagctgtagctgggg-3’ and 5’-ggaaagttgtaggtggagtagt-3’. Data are presented as expression relative to GAPDH.

Results

FOXP3 interacts with RORα and suppresses its transcriptional activation

To begin an analysis of the mechanism of transcriptional repression by FOXP3, a yeast 2-hybrid screen was performed. The amino terminal 198 amino acids of human FOXP3 was used as bait

The supernatant was transferred to a fresh tube containing 400 µl of lysis buffer and precleared with protein G plus agarose beads (Santa Cruz Biotechnology) for 1 h before 4 µg of anti-FLAG-M2 (Sigma-Aldrich) or 2 µg of anti-GAL4-DBD (Santa Cruz Biotechnology) was added. Immunoprecipitates were collected with either protein-G (FLAG-Tag) or protein-A (GAL4-DBD). Western blot was conducted using rabbit anti-human FOXP3 (1/1000 dilution), anti-GAL4-DBD (1/500 dilution) or anti-FLAG-M2 (2 µg/ml).

Immunofluorescence

Five hours after transfection, HEK 293 T cells were seeded on poly-L-lysine-coated coverslips (BD Biosciences) and permitted to grow overnight. After being washed with PBS, cells were fixed in 4% formaldehyde for 15 min, washed 3 times for 5 min in PBS, and then permeabilized with PBT (0.1% Triton X-100 in PBS) for 10 min. After blocked with blocking buffer (10% normal donkey serum and 1% BSA in PBT) for 1 h, the coverslips were incubated with 5 µg/ml anti-FLAG Mab (Sigma) and rabbit anti-human FOXP3 Ab (1/1000 dilution) in 1% BSA/PBT for 1 h at room temperature. The coverslips were washed three times for 5 min in PBT and stained with 5 µg/ml Alexa Flouor 488 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes) for 1 h in 1% BSA/PBT. The samples were then counterstained with 0.2 µg/ml 4,6-diamidino-2-phenylindole (DAPI) in PBS for 1 min. After having been washed three times for 5 min in PBS, the coverslips were mounted in 50% glycerol/2.2% octane (DABCO) in 50% glycerol to prevent fading. The images were collected using Radiance 2000 confocal microscope (Bio-Rad).

Site-directed mutagenesis

Site-directed mutagenesis for RORα and FOXP3 was performed according to the instruction manual of QuickChange Site-Directed Mutagenesis Kit (Stratagene). The PCR for each mutagenesis was conducted with a pair of primers complementary to each other. The forward primers are: FLAG-RORα 5’GGAAGATGCTGATGGGATT-3’ and 5’CATTGGACGACTCTCAAACATACACAGGAGTGGATTGTAATGA-3’ and the reverse primer is: 5’GTAATGCTCCTCCTGCACGCCGCCGCTCACATGATGAACTACAAACTCCTGA-3’. The PCR products were sequenced. These plasmids, together with gag-pol and vsvg plasmids, were then transfected into 293 T cells to prepare retrovirus with Muris TransIT-LT1 transfection reagent (Mirus Bio). After 48 h incubation, the supernatant containing prepared retrovirus was harvested and stored at −80°C until use. Jurkat cells were plated in a 24-well plate, and retrovirus supernatant prepared above was added to the wells. Then the cells were spun at 2000 rpm for 90 min at 32°C and incubated for 3 days at 37°C. GFP-positive cells were sorted by FACS. These sorted cells were then stimulated with PMA (50 ng/ml) plus ionomycin (1 µg/ml) for 4 h. After stimulation, cells were harvested and total RNA was isolated as described above.

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to screen a library prepared from in vitro-generated CD4\(^+\)CD25\(^+\) Treg cells (13). This region of the FOXP3 was used, as it has been shown to be essential for the ability of FOXP3 to repress transcription (24). In the initial screen, three independent clones encoding ROR\(\alpha\) were isolated (Fig. 1A). The cDNA clones contain the putative ligand-binding domain and part of the hinge region. Human ROR\(\alpha\) gene encodes at least four isoforms, which share the same DNA-binding domain, hinge region, and the putative ligand-binding domain, but differ in their amino terminal domains (25).

To determine which isoforms are expressed in the human CD4\(^+\)CD25\(^+\) Treg cells, RT-PCR was conducted with the isoform-specific primers as indicated in Materials and Methods. RT-PCR followed by direct sequencing of the PCR bands revealed that isoforms 2 and 4 were expressed in freshly isolated CD4\(^+\)CD25\(^+\), CD4\(^+\)CD25\(^{low}\), and CD4\(^+\)CD25\(^{high}\) T cells, as well as in the in vitro-generated Treg cells (Fig. 1B). Because the yeast 2-hybrid cDNA clones showed that the FOXP3-interacting region is located C-terminal to the DNA-binding domain (Fig. 1A), full-length isoform 4 was used for studies presented here. To determine whether FOXP3 associates with ROR\(\alpha\), coimmunoprecipitation was conducted. FOXP3 and FLAG-ROR\(\alpha\) were transiently expressed, and immunoprecipitation was conducted using the mAb against FLAG-tag. FOXP3 coimmunoprecipitated with FLAG-ROR\(\alpha\), demonstrating a specific interaction between FOXP3 and ROR\(\alpha\) (Fig. 1C). Both FOXP3 and ROR\(\alpha\) are transcriptional factors, and they colocalize in the nucleus of transfected cells (data not shown). These data demonstrate that ROR\(\alpha\) is expressed in human CD4\(^+\)CD25\(^+\) Treg cells and interacts with FOXP3.

Upon binding to RORE, which is composed of a 6-bp AT-rich sequence 5'-a to a half-site core motif PuGATCA (\(Pu = A\) or \(G\)), ROR\(\alpha\) serves as a transcriptional activator (26). To examine the functional consequences of FOXP3-ROR\(\alpha\) interactions, a reporter containing four repeats of RORE was cotransfected with expression plasmids encoding either ROR\(\alpha\) or FOXP3 into Jurkat cells, and transcriptional activity was measured. As shown in Fig. 1D, cotransfection of the reporter and ROR\(\alpha\) resulted in a marked increase in transcriptional activity (29-fold). However, the introduction of FOXP3 suppressed the activation by ROR\(\alpha\) in a dose-dependent fashion. It has been shown that ROR\(\alpha\) is constitutively active and can either bind as a homodimer to a Rev/DR2 element or as a monomer to single RORE. We next examined whether FOXP3 suppresses ROR\(\alpha\) transactivation at single RORE site. Co-transfection assay was conducted as above except for using a reporter plasmid containing only single RORE. As expected, the transcriptional activation mediated by ROR\(\alpha\) was suppressed by FOXP3 (Fig. 1E). Taken together, these data show that FOXP3 can interact with ROR\(\alpha\), and that this interaction results in the suppression of ROR\(\alpha\)-mediated transcription.

Inhibition of ROR\(\alpha\) transcriptional activation by FOXP3 is not dependent on FKH domain

Previous work has shown that FOXP3 was capable of inhibiting NFAT-mediated transcription in a manner that was dependent on the FKH domain of FOXP3 (27). However, an inspection of the DNA sequence of the RORE used in our studies failed to detect a FKH binding site. To determine whether FOXP3 was inhibiting ROR\(\alpha\) transcriptional activation in a non-DNA-binding manner, a FOXP3 construct that lacked a functional FKH domain, but retained the bipartite nuclear localization motif (referred to as FOXP3\(^{ΔFKH}\)-NLS), was used for coimmunoprecipitation. When coexpressed, FOXP3\(^{ΔFKH}\)-NLS was capable of interacting with ROR\(\alpha\) (Fig. 2A), demonstrating that this interaction is not dependent on FOXP3-specific DNA binding.

We next sought to determine whether DNA binding was critical for the ability of FOXP3 to inhibit the ROR\(\alpha\)-mediated transcriptional activation. Cells were transfected with the RORE reporter, and expression plasmids encoding ROR\(\alpha\) and the ΔFKH-NLS version of FOXP3. FOXP3\(^{ΔFKH}\)-NLS was capable of inhibiting...
the ability of RORα4 to activate the expression of the RORE reporter gene (Fig. 2B). Thus, repression of RORα transcriptional activation by FOXP3 was independent of DNA binding, a novel finding for a member of the FOX family. The second FOXP3 isoform, FOXP3ΔE2, failed to bind to RORα and does not inhibit RORα-mediated transcriptional activation.

To map the region in FOXP3 that is responsible for the interaction with RORα, a yeast 2-hybrid test was used. A series of fusion constructs were made by cloning FOXP3 cDNAs for sequence between amino acids 1 and 198 into the bait vector pLexA based on the exon boundaries of the FOXP3 gene. The RORα cDNA for the hinge region and putative ligand-binding domain (amino acids 86 to C terminus of RORα4) was cloned into pB42AD as prey. As shown in Fig. 3A, only the bait fusion proteins containing the sequences between amino acids 71 and 105, encoded by exon 2, interacted with RORα. One of the RORα-interacting bait fusion proteins contains only exon 2. Consistent with this, a FOXP3 bait construct containing amino acids 1–198, but lacking exon 2, did not interact with RORα in the 2-hybrid test. This analysis shows that sequences encoded by exon 2 of FOXP3 are involved in the interaction with RORα.

Interestingly, there is a second isoform of FOXP3, lacking exon 2, that is coexpressed in human Treg cells (referred to as FOXP3ΔE2) (15). This isoform appears to be expressed at approximately equivalent levels with the full-length isoform. Additionally, both isoforms are induced upon activation of resting human CD4+CD25T cells (13). However, to date there have been few functional data distinguishing the two isoforms. Allan et al. showed that coexpression of the isoforms was required for optimal CD25 expression and acquisition of suppressor function by CD4+CD25 human T cells, suggesting a cooperative relationship between the isoforms (15).

To extend the results from the yeast 2-hybrid analysis, we assessed the ability of FOXP3ΔE2 to interact with RORα4. As shown in Fig. 3B, in contrast to full-length FOXP3, FOXP3ΔE2 failed to interact with RORα4. These data suggested the possibility that FOXP3 and FOXP3ΔE2 may differ in their ability to inhibit RORα-mediated function. We tested this directly by transfecting cells with the RORE reporter and RORα4, along with either FOXP3 or FOXP3ΔE2. As expected, coexpression of FOXP3 with RORα resulted in markedly reduced transcriptional activation of the RORE reporter (Fig. 3C). However, coexpression of FOXP3ΔE2 had no effect on the ability of RORα4 to activate the RORE reporter. The inability of FOXP3ΔE2 to repress RORα-mediated transcription was not due to an overall lack of function. When FOXP3ΔE2 was cotransfected with a reporter containing a consensus FKH binding site (27), it was as effective as full-length FOXP3 in repressing

![Figure 2](image-url)  
**FIGURE 2.** FOXP3-mediated inhibition of RORα transcriptional activation does not require DNA binding. A, HEK 293 T cells were transfected with the indicated (+ or −) expression plasmids, and lysates were immunoprecipitated with anti-FLAG tag mAb and immunoblotted with rabbit anti-hFOXP3 (top panel). Middle and bottom panels, Control immunoblots demonstrating expression of transfected expression plasmids. Identities of individual bands are indicated with arrows to the right of each panel. B, Jurkat cells were transfected with the 4xRORE-Luc reporter plasmid (67 ng/well), in the presence of absence of the indicated expression plasmids, and luciferase activity was measured 40 h later. The amounts of expression vectors used were: 33 ng/well pcDNA3-RORα4, and 132 ng/well pcDNA3-FOXP3ΔE2-NLS. Data are normalized to a cotransfected constitutive β-galactosidase reporter, and error bars are SD of triplicate wells.

![Figure 3](image-url)  
**FIGURE 3.** Exon 2 of FOXP3 binds to RORα, and FOXP3ΔE2 does not suppress the transcriptional activation by RORα4. A, Yeast 2-hybrid analysis of RORα-FOXP3 interaction, using RORα6–468 as prey, and the indicated regions of FOXP3 as bait. B, HEK 293 T cells were transfected with the indicated expression plasmids, and lysates were immunoprecipitated with anti-FLAG tag mAb and immunoblotted with rabbit anti-hFOXP3 (top panel). Middle and bottom panels, Control immunoblots demonstrating expression of transfected plasmids. Identities of individual bands are indicated with arrows to the right of each panel, and predicted position of FOXP3ΔE2 is indicated by arrowhead.
transcription from this reporter (Fig. 3D). Thus, the effect of deleting exon 2 on FOXP3 function is specific for RORα-mediated transcriptional repression, and it represents the first demonstration of a functional difference between FOXP3 and FOXP3<sup>AE2</sup>.

**FOX3P interacts with the activation function 2 (AF2) motif of RORα via an LxxLL motif in exon 2**

Using the approach described above, we mapped the regions of RORα4 involved in the interaction. The original FOXP3 bait (amino acids 1–198) was used for this yeast 2-hybrid analysis. A series of deletion mutants of RORα4 were generated, beginning at amino acid 86, and cloned into the PB42AD vector (Fig. 4A). This start site for the deletions was chosen so as to include all RORα sequences from the longest cDNA clone obtained from the original yeast 2-hybrid (Fig. 1A). This yeast 2-hybrid assay showed that a construct that contained the carboxyl-terminal 42 amino acids of RORα4 was capable of binding to FOXP3, while all other constructs lacking this sequence failed to bind (Fig. 4B). To determine whether the carboxyl-terminal 42 amino acids can physically interact with FOXP3, we fused this region to the GAL4 DNA-binding domain (GAL4-DBD). HEK 293 T cells were transiently transfected with expression vectors for FOXP3 and the RORα4-GAL4-DBD fusion protein, and possible interaction was determined by coimmunoprecipitation. As shown in Fig. 4C, FOXP3 was capable of direct association with the carboxyl-terminal region of RORα. This 42 amino acid region contains the AF2 motif (Fig. 4D), which is essential for the activity of RORα through interactions with coactivators or corepressors (28, 29). The importance of this region to the interaction with FOXP3 was tested by generating a RORα construct with a stop codon inserted at position 448, resulting in a version of RORα4 that is truncated just before the AF2 motif (Δ448–468) (Fig. 4D). This construct failed to interact with FOXP3, confirming the role of the AF2 helix in the interaction with FOXP3 (Fig. 4E). The AF2 helix of RORα1 interacts with the coactivator GRIP-1, and leucine and phenylalanine residues at positions 510 and 511, respectively, are critical for this interaction (30). To determine whether these two residues are also critical for the interaction of RORα4 and FOXP3, the equivalent residues in RORα4 (L455 and F456) were mutated to alanine, and the resulting construct was tested for its ability to interact with FOXP3. FLAG-RORα<sup>F455,F456AA</sup> failed to interact with FOXP3, demonstrating the importance of the AF2 helix for interaction with FOXP3 (Fig. 4E).

The AF2 motif of nuclear receptors is the binding site for both corepressors and coactivators. The coactivators bind to AF2 domain of the ligand-bond receptors via their motif LxxLL (31, 32). The AF2 helix of the RORα is in a constitutively active conformation, and it is required for interaction with coactivator proteins (16, 26, 28). Thus, binding to AF2 would be predicted to be via an LxxLL motif in FOXP3. Inspection of the amino acid sequence of FOXP3 revealed a single LxxLL motif (LQALL), located in the sequence encoded in exon 2. To determine whether this sequence was involved in RORα-FOX3P interactions, the last two leucine residues were changed to alanine (amino acids 95 and 96). As a control, leucine residues at positions 74 and 76 were also mutated to alanine. Constructs expressing these two mutant forms of FOXP3 (L<sup>95</sup>Q, L<sup>96</sup>A and L<sup>74</sup>L, L<sup>76</sup>A) were cotransfected into HEK 293 T cells with FLAG-RORα4 and a communoprecipitation assay was performed. FOXP3<sup>L<sup>95</sup>Q,L<sup>96</sup>A</sup> failed to communoprecipitate with RORα, whereas FOXP3<sup>L<sup>74</sup>L,L<sup>76</sup>A</sup> communoprecipitated as well as full-length FOXP3 (Fig. 5A). To test the functional consequences of this loss of interaction, the ability of these mutant forms of FOXP3 were assessed for their ability to repress the RORα-mediated activation of the RORE-Luc reporter construct. Jurkat cells were transfected with constructs encoding FOXP3 (full-length, ΔE2, and LL mutations) and RORα4, and then either the RORE-Luc or 3xFKH-Luc reporters. Full length
FOXP3 and FOXP3\textsuperscript{LL74,76AA} completely inhibited activation of the RORE and 3xFKH reporters (Fig. 5, B and C). However, FOXP3\textsuperscript{LL74,76AA}, similar to FOXP3\textsuperscript{ΔEF}, failed to inhibit activation of the RORE-Luc reporter, but it did inhibit the 3xFKH-Luc reporter. These data show that full-length FOXP3 interacts with the AF2 motif of ROR\(\alpha\) via a LxxLL motif located in sequences encoded by exon 2, and that this interaction inhibits ROR\(\alpha\)-mediated transcriptional activation.

**Full-length FOXP3 inhibits in vivo targets of ROR\(\alpha\)**

The data presented above demonstrate that FOXP3 and ROR\(\alpha\) interact via sequences in exon 2 of FOXP3 and the AF2 domain of ROR\(\alpha\). These studies also showed, using a ROR\(\alpha\)-based reporter, that FOXP3 was capable of inhibiting ROR\(\alpha\)-mediated transcriptional activation. We next extended these studies to examine whether FOXP3 could regulate endogenous ROR\(\alpha\) target genes. Jurkat T cells were infected with a retrovirus encoding ROR\(\alpha\), in the presence or absence of FOXP3, and transcript array analysis was performed. Jurkat T cells were used because they do not express FOXP3 following stimulation, allowing for an analysis of direct ROR\(\alpha\)-FOXP3 interaction. Several genes were found to be regulated by ROR\(\alpha\) expression, including IL-17, IL-22, CXCR3, and FasL, were up-regulated in cells expressing ROR\(\alpha\), but not in cells expressing both ROR\(\alpha\) and FOXP3. B. Expression of IL-22 and CXCR3 mRNA relative to control GAPDH (in triplicate wells ± SD) in these three groups was determined by real-time PCR as described in Materials and Methods. Transcript level in control group (MIGR1) was considered as 100%.

**Discussion**

Herein, we show that FOXP3 can physically interact with ROR\(\alpha\). RORs are nuclear orphan receptors that activate transcription through specific binding to ROR response elements in the promoter of target genes (26). This interaction with FOXP3 resulted in the inhibition of ROR-mediated transcriptional activation. Interestingly, our data have two additional novel findings concerning the function of FOXP3. First, the forkhead domain of FOXP3 was
not required for the inhibition, demonstrating a DNA binding-independent function for FOXP3 function. Second, only the full-length isoform of FOXP3 was capable of interaction and inhibition, not the Δexon 2 isoform. This is the first functional distinction shown between the two isoforms.

In both humans and mice, FOXP3 is predominantly expressed in the CD4+CD25+ Treg cell population. Studies from both species have shown that it is necessary for proper Treg cell development. Mice that lack FOXP3, either through spontaneous or targeted mutation, fail to develop Treg cells and succumb to fatal autoimmune lymphoproliferative disease (7, 33). Mutations in the human FOXP3 gene result in IPEX syndrome, where affected individuals display symptoms consistent with a deficit in Treg cells (11, 12, 34). In contrast, ectopic expression of FOXP3 converts mouse CD4+CD25− T cells to regulatory phenotype (7, 8). The ability of ectopic expression of FOXP3 to convert human CD4 T cells to a regulatory phenotype is less clear (15, 35).

However, despite the obvious importance of FOXP3 in the development and function of Treg cells, its mechanism of action remains obscure. Structural analysis of FOXP3 has revealed several functional domains, including those required for dimerization (24, 36) and for transcriptional repression (24, 37). Additionally, Bettelli et al. also showed that FOXP3 can directly interact with NF-AT and the NF-κB subunit p65 (37). Although the region of interaction with FOXP3 was not precisely identified, deletion analysis did show that the amino terminal half of FOXP3 was involved in the association. Recently, FOXP3 was shown to bind cooperatively with NF-AT on the IL-2 gene promoter (38). Coocrystallization of the forkhead domain of FOXP2 and the Ret homology region of NFAT1 showed that several residues in the FOXP2 forkhead domain interacted with the NFAT1 Ret homology region. Using structure-guided mutagenesis of the equivalent residues in the forkhead domain of FOXP3, it was shown that these interactions were critical for FOXP3-mediated inhibition of IL-2 gene expression (38).

We have now defined another functional domain within FOXP3, the region encoded by exon 2 (amino acids 71–105). In contrast to the mouse, there are two isoforms of human FOXP3, differing by the inclusion or exclusion of exon 2 (13, 15). It appears that the two isoforms are equally expressed in both naturally occurring and in vitro-derived human Treg cells. However, no functional differences were reported for the FOXP3 isoforms (15). We have now shown that sequences in exon 2 are involved in the interaction between FOXP3 and RORγt, and that FOXP3ΔE2 does not interact with RORγt or inhibit its ability to serve as a transcriptional activator. It is possible that the two isoforms are expressed in distinct Treg cell populations, as single-cell analysis has not been performed and serological reagents that distinguish between the isoforms have not been developed. Interestingly, an examination of an IPEX patient with a mutation in exon 2 suggests that expression of the ΔE2 isoform is not sufficient to rescue Treg cell function. This individual had a single nucleotide deletion at position 227 (227delT), resulting in a frameshift and a severely truncated protein (39). Because this nucleotide is in exon 2, the deletion would be expected to be spliced out of the ΔE2 mRNA, and thus a normal FOXP3ΔE2 protein should be generated. The patient presented with several symptoms of IPEX, including autoimmune enteropathy and autoimmune thyroiditis, by 5 mo of age (39). Thus, expression of the ΔE2 isoform is not sufficient for proper Treg cell development and/or function, consistent with our data demonstrating functional differences between the isoforms.

We have mapped the FOXP3-binding region in RORγt to the AF2 motif at its C terminus. This AF2 domain (helix 12) has been shown to be essential for the trans-activation of a wide variety of nuclear receptors (28, 29, 40). In most nuclear receptors it can adopt either active or repressive conformation, depending on whether the ligand is bound. In the ligand unbound state, the AF2 domain is bound by corepressors that inhibit transcriptional activation. This binding is dependent on an LxxIxxxL/I motif encoded by the corepressor (41–43). Upon ligand binding, a conformation change takes place in the AF2 domain, allowing the binding of transcriptional coactivators to a pocket consisting of the AF2 domain, and helices 3, 3′, 4, and 5 of the ligand binding domain (28). The binding to the AF2 helix is mediated through a specific amino acid sequence on the coactivator (LxxLL) (44). RORγt is an orphan receptor, and the AF2 helix is in a constitutively active conformation. The AF2 helix is required for RORγt to interact with coactivators, and removal or disruption of AF2 motif abolished its transcriptional activation (30, 31). We have shown that both the deletion of AF2 helix and the introduction of point mutations inside the AF2 motif abolish the capability of RORγt to interact with FOXP3. Additionally, mutation of motif LQALL (FOXP3L105,96AA) inside the sequence encoded by exon 2 abolishes both FOXP3-RORγt interaction and FOXP3-mediated inhibition of RORγt-specific transcriptional activation. Thus, FOXP3 uses a binding motif used by coactivators to bind to and inhibit RORγt.

The role of the ROR-FOXP3 interactions in Treg cells is still unclear. RORγt has been reported as a negative regulator for inflammatory response using the primary smooth-muscle cells as a model (17). Both FOXP3 and RORγt inhibit the expression of multiple cytokines, but by different mechanisms. FOXP3 represses the transcription activity of NFAT at the promoters of cytokine genes, including IL-2 (27). However, RORγt interferes with the NF-κB signaling pathway by inducing the production of IκBα, a major inhibitory protein of the NF-κB pathway, and thus inhibits the expression of inflammatory cytokines (17). Therefore, to some extent RORγt and FOXP3 have similar functions in the regulation of cytokine genes.

Our data also suggest that RORγt and RORγt may play overlapping and complementary roles in driving Th17 differentiation. As shown in Fig. 6, many of the genes that distinguish the Th17 lineage, including IL-17 and IL-22, are up-regulated following RORγt expression in Jurkat cells. Furthermore, as we have seen with RORγt (L. Zhou, J. E. Lopes, D. Littman, and S. F. Ziegler, manuscript in preparation), this up-regulation is inhibited by coexpression of FOXP3. Consistent with this possibility, IL-17 expression in CD4+ T cells from the guts of RORγt-null mice is not completely abrogated (45), suggesting a potential role for RORγt in IL-17 expression. As RORγt is expressed in CD4+ T cells and RORγt expression is inducible, one potential role would be in maintaining baseline expression, while RORγt is involved in inducible expression following exposure of the cell to inflammatory cytokines.

RORγt is expressed in both Treg cells and effector T cells, as shown in the present studies. To uncover its role in the Treg cells, the CD4+CD25+ T cells were isolated from staggerer mice for in vitro regulatory function assay. Although the number of CD4 T cells is much lower than that of littermate control, the in vitro function of Treg cells from staggerer mice appears to be normal (data not shown). Perhaps this finding is not surprising in that the mutation in the staggerer mouse results in a dominant-negative form of RORγt (46), which would be predicted to behave in a manner similar to wild-type RORγt bound to FOXP3.

In conclusion, we showed that RORγt is expressed in both effector and Treg cells and FOXP3 associates with RORγt and suppresses its transcription response, suggesting the role of FOXP3-RORγt interaction in the function of Treg cells. Additionally, the
isoform FOXP3isoform is functionally different from the full-length isoform in interaction and suppression, suggesting the distinct roles of the isoforms in the Treg cells.

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