Foxp3 is a 47-kDa transcription factor central to regulatory T cell (Treg) function. The importance of Foxp3+ Tregs in controlling self-reactive T cells and preventing autoimmunity is well established. Our analysis of Foxp3 expression in natural Tregs led to identification of a shorter 41-kDa Foxp3 species in activated Tregs, indicating that Foxp3 may be processed by proteolytic cleavage upon cell activation. Searches of murine and human Foxp3 sequences for potential cleavage sites responsible for the generation of the short Foxp3 species revealed the presence of two RXXR proprotein convertase (PC) motifs, 48RLR51 and 414RKRR417, located near the N- and C-terminal ends, respectively. We show, using retroviral expression of Foxp3 in CD4+ T cells, that Foxp3 is cleaved at both the N- and C-terminal RXXR sites and that mutagenesis of the RXXR motif prevents cleavage. The cleaved forms of Foxp3 are found in the chromatin fraction but not in nuclear or cytoplasmic extracts. CD4+ T cells expressing Foxp3 species engineered to mimic N-terminally, C-terminally, or N- and C-terminally cleaved Foxp3 forms are functionally distinct, as indicated by differences in expression of key Treg genes, such as interleukin-10 and cytotoxic T-lymphocyte antigen 4 (CTLA-4). In addition, CD4+ cells expressing C-cleaved Foxp3 are superior to those that express WT Foxp3 in preventing experimental colitis. Coexpression of Foxp3 with PC1 or PC7 results in cleavage of the Foxp3 C terminus. The mechanism by which Foxp3 is processed likely extends to other members of the FoxP subfamily, because Foxp1 and Foxp2 also have N-terminal RXXR proteolytic cleavage motifs at similar locations to Foxp3. Our results indicate that the generation of fully functionally competent Tregs is complex and dependent on the generation of multiple forms of Foxp3 that have differing effects on Treg cytokine production and suppressive function.
Foxp3 Processing by Proprotein Convertases

Foxp3 is cleaved at both sites. CD4+ cells expressing the engineered forms of Foxp3 that mimic the N-cleaved, C-cleaved, or N&C-cleaved proteins are functionally distinct, as is evident by differences in the expression profiles of critical markers, their ability to suppress Teff cell proliferation, and differences in efficacy in an IBD model. Hence, the regulation of Treg activity is complex and includes proteolytic processing of Foxp3.

EXPERIMENTAL PROCEDURES

Mice—Female C57BL/6 mice (Jackson Laboratory) and female Rag1−/− (C57BL/6) mice (Taconic) were used in animal studies approved by the institutional animal care and use committee of the Children’s Hospital of Philadelphia.

Antibodies—Anti-mouse Foxp3 mAbs (FJK-16s, NRRF-30; eBioScience), and SP1 and Furin antibodies (catalog numbers sc-59 and sc-20801; Santa Cruz Biotechnology) were used for Western blots. Polyclonal anti-mouse Foxp3 antibody was generated by immunizing rabbits with a synthetic 11-amino acid (aa) peptide, QRPNKCSNPQP, corresponding to 419 – 429 aa of mFoxp3. Foxp3-specific polyclonal antibody was purified from high titer rabbit antiserum by affinity chromatography (Sigma-Genosys).

cDNA Cloning and Mutagenesis—mFoxp3 cDNA was amplified from thymus with Foxp3-specific forward and reverse primers (Integrated DNA Technologies). The mutations were introduced with a QuikChange site II-directed mutagenesis kit (Stratagene; catalog number 200523), and after sequence confirmation, fragments were recombined to Minr-1 vector for retroviral expression.

Retroviral Expression—Retroviruses were generated by cotransfection of Foxp3 mutants (in Minr-1 vector) with pCLeco (Invitrogen) helper plasmid, using Lipofectamine 2000 reagent (Invitrogen; catalog number 11668-027). Virus containing supernatant was collected and used to infect purified CD4+ or CD4+CD25+ T cells. Prior to infection with the retrovirus, CD4+ T cells were isolated by magnetic sorting, activated with PMA (3 ng/ml), ionomycin (1 mM), and IL-2 (5 units/ml) for 24 h, washed, and transduced using the 48- and 72-h viral supernatants obtained from transfected Phoenix cells (16). Transduced cells were expanded for 1–3 days, unless stated, and used in the suppression assays or for subcellular fractionation (16).

Treg Suppression Assays—5 × 10^4 carboxyfluorescein succinimidyl ester-labeled CD4+CD25− (Teff) cells, isolated using magnetic beads (Miltenyi), were stimulated with CD3 mAb in the presence of irradiated syngeneic T-cell depleted splenocytes and varying ratios of activated CD4+ cells transduced with different Foxp3 constructs (17). Suppression of proliferation was determined from the CFSE profile of dividing Teff cells at 72 h.

Nuclear and Cytoplasmic Extraction—Subcellular fractions were prepared as described (19). For analysis of histones, cellular fractions extracted with 0.2 M H2SO4 were analyzed on acetic-acid urea gels (20).

Western Blots—The proteins were separated by 14 or 20% SDS-PAGE, blotted, and probed with the primary antibody followed by secondary antibody horseradish peroxidase and Luminol reagent (Santa Cruz Biotechnology; catalog number sc-2048).

DNA and Protein Estimation—DNA in the subcellular fractions was estimated using the diphenylamine-based color reaction assay described by Zaccharias Dische in 1930 and subsequently modified by Burton (21). Protein estimation was done using a Bio-Rad DC protein assay kit (catalog number 5000112).

Adoptive Transfer Model of IBD—For the adoptive transfer model of colitis, CD4+CD25− T cells were isolated from spleen and mesenteric lymph nodes were isolated using magnetic beads (Miltenyi Biotech) to >95% purity (by flow cytometry), and 1 × 10^6 cells were injected intraperitoneal into C57BL/6 background Rag1−/− mice together with Thy1.1+CD4+ cells (5 × 10^6) transfected with WT or mutated Foxp3 (22). The mice were monitored biweekly for clinical evidence of colitis, including weight loss, fecal blood, and stool consistency.

Quantitative PCR—Total RNA was prepared by lysing 1 × 10^6 cells with Qiashredder (Qiagen; catalog number 79654) and purification of RNA using RNeasy mini kit (Qiagen; catalog number 74104). cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems), primer/probe sets were obtained from Applied Biosystems, and quantitative PCR was performed using an ABI Prism 7000 Analyzer.

Pathology—Gut samples were paraffin-embedded and stained by hematoxylin and eosin, or snap frozen and stained by immunoperoxidase using anti-Foxp3 or an isotype control mAb (23).

RESULTS

Detection of the 41-kDa Foxp3 Species—Subcellular localization of Foxp3 is regulated by signal transduction (16). To determine the effect of Treg activation on Foxp3 expression and its cellular distribution, naturally occurring thymic-derived CD4+CD25+ Tregs from peripheral lymph nodes and spleen were isolated and activated with CD3 and CD28 mAbs, followed by subcellular fractionation and Western blot analysis. Treg cell activation resulted in increased levels of Foxp3, detected as a single 47-kDa species in nuclear and cytoplasmic extracts and especially in the chromatin fraction (Fig. 1, lane 6). In resting Tregs, the ratio of chromatin-bound Foxp3 to Foxp3 in nuclear extracts was very low, but with TCR stimulation almost equal amounts of Foxp3 were detected in these fractions (Fig. 1, lanes 1, 3, 4, and 6). Increased exposure revealed the presence of additional Foxp3 species, migrating both more quickly and more slowly than the major 47-kDa Foxp3 species (Fig. 1, lanes 7–10). Although most of these additional species were common to resting and activated Tregs, a 41-kDa Foxp3 species was found exclusively in the chromatin fraction of activated Tregs (Fig. 1, lane 10). The chromatin fraction used in this study had the characteristic histone profile of chromatin (24), indicating that subcellular fractionation was efficiently performed (Fig. 1, lanes 11–16). Activation of Tregs by PMA and ionomycin also showed the 41-kDa Foxp3 species to be exclusively in the chromatin fraction (Fig. 1, lane 19).

Only the N-terminal RXXR Motif Is Present in Other FoxP Subfamily Members—The detection of a shorter Foxp3 species only in activated Tregs suggested Foxxp3 may be endoproteolytically processed upon activation to yield the 41-kDa species. Our search for proteolytic sites in both mouse and human Foxp3 revealed two RXXR motifs (14RDLR51 and 414RKKR417)
that represent potential cleavage sites for PC enzymes (Fig. 2). The presence of a serine residue immediately after the C-terminal RKKR (RKKRS) in mouse and human Foxp3 indicated Foxp3 may be cleaved at this site, because serine is most commonly found next to the RXXR motifs recognized by PCs (25, 26). The N-terminal RDLR is also followed by serine (RDLRS) in mouse and by glycine (RDLRG) in the human, another acceptable amino acid for cleavage by PCs (27). The search for RXXR motifs in other FoxP subfamily members showed that Foxp3 is unique in having the C-terminal RXXR motif (Fig. 2). However, N-terminal RXXR motifs are also found at similar locations in mouse and human Foxp1 and Foxp2. These N-terminal RXXR motifs in Foxp1–3, located within the first 51 aa, are in a region that does not include the zinc finger, leucine zipper, or forkhead domains common to FoxP subfamily members. Within the first 51 aa, Foxp1 and Foxp2 are only 6 and 14% identical to the N-terminal end of Foxp3. The presence of the N-terminal RXXR motifs in Foxp1–3, in a region of limited homology, suggests these members are processed by a common mechanism (Fig. 2).

**Foxp3 Is Cleaved at the N-terminal RXXR Motif**—To determine whether Foxp3 is cleaved at the RXXR sites, we mutated the N-terminal 48RDLR51 sequence to 48HDLH51, effectively abolishing the RXXR motif without altering the positive charge. This mutation was introduced to full-length Foxp3 (429 aa), as well as to a construct that encodes a short 417-aa Foxp3 mimicking Foxp3 cleaved at the C-terminal cleavage site immediately following the RKKR (C-cleaved Foxp3; RKKR followed by a stop codon, missing the last 12 aa). We also engineered a size control Foxp3 that is the same size (366 aa) as a Foxp3 cleaved at both the N and C termini. These constructs were retrovirally expressed in CD4+CD25− T cells, separated into subcellular fractions, and analyzed by Western blotting. A schematic diagram of these constructs is shown in Fig. 3a. Western blot of the chromatin fractions shows the 366-aa size control and the shorter Foxp3 species generated from C-cleaved Foxp3 species comigrate, in agreement with the possibility that the 41-kDa species may be the result of N-terminal cleavage of Foxp3. Moreover, the expression of the Foxp3 mutants that have lost their N-terminal RXXR motif did not yield any detectable 41-kDa species (Fig. 3b, lanes 4 and 5), indicating the N-terminal cleavage event in Foxp3 is dependent on the presence of an intact RXXR motif. We then analyzed the nuclear and cytoplasmic extracts, and similar to natural Tregs, the 41-kDa species was detected in the chromatin fraction but not in the nuclear or cytoplasmic extracts (Fig. 3c), suggesting a distinct function for this Foxp3 species.

**An N-terminal Specific Foxp3 mAb Does Not Recognize the 41-kDa Foxp3**—Analysis using mAbs also showed that the 41-kDa species is generated by N-terminal cleavage of Foxp3 because NRRF-30 mAb, specific to the Foxp3 N-terminal end, did not recognize the 41-kDa species, in contrast to the binding of another mAb, FJK-16s, specific to a more central Foxp3 epitope (Fig. 3d). Thus, a subpopulation of chromatin-bound Foxp3 in activated Tregs is N-cleaved. N-terminal

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**FIGURE 1.** 41-kDa Foxp3 species is detected in the chromatin fraction of activated natural Tregs. Foxp3 expression was analyzed by Western blotting in nuclear (N) and cytoplasmic extracts (C) and chromatin (Chr) fraction using FJK-16s mAb. Lanes 1–3, fractions from resting Tregs; lanes 4–6, fractions from CD3/CD28 mAb-activated Tregs; lanes 7 and 8, nuclear extracts, similar to lanes 1 and 4 but overexposed; lanes 9 and 10, chromatin fractions similar to lanes 3 and 6 but overexposed. These lanes (9 and 10) were from the same experiment with the same extraction and placed together using Adobe Photoshop without any other manipulation. Lanes 11–16, parallel samples to the ones shown in lanes 1–6 were analyzed for histone content by acetic-acid urea-PAGE. Lanes 17–19, N and C extracts and Chr fraction were prepared from Tregs after activation with PMA/ionomycin. The arrows next to lanes 10 and 19 mark the 41-kDa Foxp3 species.

**FIGURE 2.** Schematic diagram showing the RXXR proprotein convertase recognition/cleavage sites in mouse Foxp1, Foxp2, and Foxp3. RXXR motifs are shown in gray boxes, and the numbers above the RXXRs indicate the precise locations of cleavage. Corresponding human residues are shown beneath the mouse residues, as marked. The percentages indicate the identical aa residues for the first 51 aa between Foxp1, Foxp2, and Foxp3 (highlighted). The second Foxp3 diagram beneath the full-length 429-aa Foxp3 diagram shows the sizes of the fragments that would be generated after processing of Foxp3 at the N- and C-terminal RXXR sites. QKRR sequences in Foxp1 and Foxp4 (corresponding to the RKKR sequence of Foxp3) are followed by proline residues (shown in red), an amino acid not favored by most PCs at this position. ZF, zinc finger; LZ, leucine zipper, FHD, forkhead domain.
cleavage of Foxp3 may explain the discrepancy in results from different groups and help resolve the controversy over the specificity of N-terminal specific human PCH101 mAb versus other Foxp3-specific mAbs (28).

**Foxp3 Is Cleaved at the C-terminal RXXR Motif**—To determine whether C-terminal cleavage takes place, we made additional constructs to resolve the uncleaved and C-cleaved (missing the last 12 aa) Foxp3s, because the size difference between the two forms was too small to resolve satisfactorily by SDS-PAGE. Two new constructs were made that encode C-terminally extended Foxp3, so that the C-cleaved and uncleaved forms could be resolved. These constructs were retrovirally expressed in CD4+ T cells, and one of them, with an additional 19 aa at its C-terminal end (sequences past RKKR extended from 12 to 31 aa) was found to be suppressive in the in vitro Treg assay (data not shown) and could be C-cleaved (Fig. 4). Thus, Foxp3 gets cleaved very close to its C-terminal end, in addition to being cleaved at the N-terminal 48RDLR↓S52 (Fig. 4a, arrowhead marks the C-cleaved Foxp3 and arrow marks the 41-kDa species resulting from N-terminal cleavage). In control experiments, the samples were analyzed for their DNA and protein content, and the protein/DNA ratio of the chromatin fraction was determined to be 2.8 ± 0.2, in agreement with the protein/DNA ratios reported for chromatin (29, 30) (Table 1). In addition, we confirmed that the histone profile of the chromatin fraction matches the histone profile of chromatin (24) (Fig. 4a, lane 7). To find out whether the C-terminal proteolytic cleavage is dependent on an intact RXXR motif, the RKKR in the C-terminally extended Foxp3 was mutated to QNKS, effectively abolishing the motif. The constructs were expressed in CD4+ T cells, and chromatin proteins were separated on large gels and analyzed by Western blotting. Analysis of C-terminally extended Foxp3 (RKKR...) expression showed the presence of uncleaved C-terminally extended Foxp3, endogenous Foxp3 of the CD4+ cells, and C-cleaved Foxp3 (comigrating with the 417-aa size control protein) (Fig. 4b, C-cleaved Foxp3 marked with arrowheads, lanes 3 and 5; and size control, lane 6). In contrast, the corresponding sample from the QNKS--- mutant showed the presence of uncleaved C-terminally extended mutant Foxp3, the endogenous Foxp3 of the CD4+ cells, but lacked any detectable C-cleaved (417 aa) species, indicating that the QNKS--- mutant does not get cleaved at the C terminus in the absence of an intact RXXR motif.

**Detection of the Cleaved 12-aa Foxp3 Peptide**—To determine the presence of the 12-aa peptide cleaved from the C-terminal end of Foxp3, an anti-peptide polyclonal antibody was generated in rabbits directed to the last 11 aa of the mouse Foxp3 sequence (Fig. 5, a and b). This polyclonal antibody was then used in the Western blot analysis of spleen and heart extracts and recognized a ~1.3-kDa peptide in the spleen extracts (theoretical molecular mass of cleaved C-terminal peptide, 1.34 kDa). Nonlymphoid tissues, such as heart, did not show any trace of this peptide (Fig. 5c).

**Differential Effects of Foxp3 Processing on CTLA-4 and IL-10 mRNA Levels**—We found that the shorter 41-kDa Foxp3 species is generated only upon the activation of Tregs, indicating that different Foxp3 forms may have distinct functions. To study this further, we used quantitative PCR to determine mRNA levels of the CTLA-4 inhibitory receptor and the anti-inflammatory and immunosuppressive cytokine, IL-10, in CD4+ cells retrovirally transduced to express the engineered Foxp3 forms (mimicking either N-cleaved, C-cleaved, or N&C-
cleaved (double-cleaved) Foxp3. We found that resting cells expressing WT Foxp3 had the highest level of CT2-A-mediated suppression, but rather as a mixture of uncleaved and cleaved (N-, C-, and double-cleaved) forms and therefore does not represent a pure population. We have precluded the use of C-terminal cleavage-resistant mutants in our experiments because the C-terminal RKKR residues are also involved in DNA binding. Thus, the use of such uncleavable Foxp3 mutants would make functional interpretation of cleavage resistance difficult. In agreement with this reasoning, we found an almost total loss of suppressive activity using a Foxp3 mutant in which the RKKR is replaced with PNNW, (PNNW, followed by a stop codon; mutant residues underlined), highlighting the importance of the basic four-amino acid sequence RKKR in the function of Foxp3.

**CD4 Cells Expressing C-cleaved Foxp3 Prevent IBD**—Our hypothesis that one form of Foxp3 may be more efficacious in a particular in vivo model than other forms was tested in vivo using an adoptive transfer model of colitis (31). Rag1−/−(C57BL/6) mice were injected with a limiting number of 5 × 10^5 transduced Thy1.1^+ CD4^+ cells expressing either WT Foxp3 or C-cleaved Foxp3 (RKKR^+), or empty vector (control), plus 1 × 10^6 CD4^+CD25^− Teff cells. Additional mice received CD4^+CD25^− Teff cells alone. 5 × 10^5 transduced CD4^+ cells were transferred to mice based on prior experiments demonstrating that this number of cells resulted in only partial response. The weights of the animals in each group were monitored weekly for 45 days. Mice that received WT Foxp3 at the ratio of one Treg to two T effector cells (Fig. 7). It should be noted that WT Foxp3 expressed in the suppression assays is not present in the cells as a homogenous population, but rather as a mixture of uncleaved and cleaved (N-, C-, and double-cleaved) forms and therefore does not represent a pure population. We have precluded the use of C-terminal cleavage-resistant mutants in our experiments because the C-terminal RKKR residues are also involved in DNA binding. Thus, the use of such uncleavable Foxp3 mutants would make functional interpretation of cleavage resistance difficult. In agreement with this reasoning, we found an almost total loss of suppressive activity using a Foxp3 mutant in which the RKKR is replaced with PNNW, (PNNW, followed by a stop codon; mutant residues underlined), highlighting the importance of the basic four-amino acid sequence RKKR in the function of Foxp3.

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empty vector or no Tregs progressively lost weight and succumbed to disease, whereas mice receiving cells expressing WT Foxp3 showed minor weight loss but survived (p < 0.01 versus Minr-1 or no Treg groups) (Fig. 8a). In contrast to the use of WT Foxp3, mice receiving C-cleaved Foxp3 (RKKR) continued to gain weight (p < 0.05 versus WT Foxp3) (Fig. 8a). Histologic analysis showed mild mononuclear cell infiltration and edema in gut sections from mice receiving WT Foxp3, whereas mice receiving C-cleaved Foxp3 had essentially normal histology (Fig. 8b). Both groups showed considerable numbers of Foxp3+ mononuclear cells by immunoperoxidase staining, suggesting differences in weight loss were not caused by differences in cell recruitment

| 图 8. Percent weight loss after adoptive transfer of colitis in mice and treatment of disease by CD4+ cells expressing different Foxp3 forms. a, serial analysis of weight loss showing the benefit of therapy with C-cleaved Foxp3, b, comparison of duodenal samples collected at day 45. Injection of cells expressing WT Foxp3 was associated with mild mononuclear cell recruitment and villous edema, whereas animals receiving cells expressing C-cleaved Foxp3 had essentially normal histology (hematoxylin and eosin-stained paraffin sections; original magnifications, ×200). Immunoperoxidase staining showed infiltration by Foxp3+ mononuclear cells in both cases (hematoxylin-counterstained cryostat sections; original magnifications, ×400). The inset shows a lack of staining using isotype-matched control mAb. |
CD4+ CD25− T cells. C-terminally extended Foxp3 was used to enable detection of C-terminal cleavage, and CD4+ CD25− cells were used to minimize background from endogenous PC activity, because endogenous PC levels were found to drop in these cells upon activation (Fig. 9a). Analysis of subcellular fractions obtained from transduced cells showed that none of the three PCs cleaved the N-terminal end of Foxp3, whereas the C-terminal end was cleaved by both PC1 and PC7, and the cleaved form was detectable only in the chromatin fraction (Fig. 9b).

**DISCUSSION**

In this study we demonstrate that activation of Tregs leads to proteolytic cleavage of Foxp3 and that the RXXR motifs located close to the N- and C-terminal ends, 48RDLR21 and 414RKKR417, are sites for proteolytic cleavage. We show Foxp3 can be cleaved at either the N- or C-terminal site, or at both sites, and the processed protein is exclusively found in the chromatin fraction. We find CD4+ cells expressing the different engineered forms of Foxp3 show differences in their expression of the CTLA-4 receptor and IL-10. CTLA-4 expression is highest in resting cells that express WT Foxp3. In contrast, cells expressing the C-cleaved or the double-cleaved forms of Foxp3 have a high level of IL-10 expression. Samples corresponding to WT Foxp3 were found to have very low levels of IL-10, similar to samples corresponding to empty vector. The induction of IL-10 expression in cells expressing C-cleaved Foxp3 is particularly meaningful because IL-10 is reported to be important in preventing IBD in the adoptive transfer model of colitis (32), and mice with CD4+ cells deficient in IL-10 expression develop spontaneous disease (33). Tregs that express IL-10 are found to be localized in large and small intestines (34). Exogenous addition of IL-10 to CD4+ cell cultures during their activation differentiates them into a phenotype that produces high levels of this cytokine, and these IL-10-producing cells are found to be capable of preventing colitis in SCID mice when coadministered with pathogenic splenic T cells (32). It is likely that expression of C-cleaved Foxp3 in transduced CD4+ T cells converts them into a Tr1-like (contact-independent type 1-like) regulatory T cell phenotype (35). Tr1 cells are a subset of CD4+ T cells that have only been partially characterized and are defined by their expression of high levels of IL-10. Tr-1-like subsets of Tregs have also recently been reported (36).

The finding that Foxp3, a nuclear, DNA-binding protein, is processed by enzymes of the PC family is surprising because PCs are known to process secreted proteins and peptide hormones (25, 37, 38). Our data indicate that both PC1 and PC7 are capable of processing Foxp3 at its C-terminal end when coexpressed. PC7 mRNA levels in active Tregs are ~15-fold more than PC1, and in agreement with our observations, high level PC7 expression is reported in lymphoid organs (39, 40). Hence, PC7 may be the principal enzyme to process the C-terminal end of Foxp3 in natural Tregs, but additional work is needed to prove this point. The N-terminal end of Foxp3 is not processed by Furin, PC1, or PC7, indicating involvement of another PC in N-terminal processing. Substrate specificities of PCs are affected by amino acid residues at positions P2–P4 (R^X^A^X^P^4). In addition, the surrounding residues at both sides of the RXXR motif (extending to P8 and P4) contribute to recognition specificity (26, 41, 42). Cleavage preference of individual PCs is rather complex, and the lack of information makes the design of specific inhibitors very difficult (42). Foxp3 C-terminal RXRX motif is totally comprised of basic amino acids. In contrast, the N-terminal RDLR has an aspartic acid at P3 and leucine at P2, an indication that N-terminal processing of Foxp3 likely requires a PC with a different substrate specificity.

The fact that only a minor portion of Foxp3 is found processed may reflect the level of cellular demand for the processed form. The rate of Foxp3 processing may also depend on the availability of active PCs (members of the PC family of enzymes themselves require processing to become active) or depend on additional factors for proper presentation of Foxp3 to the PC. More 41-kDa Foxp3 species is generated in natural Tregs following activation with PMA plus ionomycin, compared with activation with CD3/CD28 stimulation, indicating that the type and duration of activation contribute to how much 41-kDa species is detected. PMA plus ionomycin treatment also results in the appearance of several additional Foxp3 species ranging between 41 and 47 kDa, and these species remain to be identified.
Foxp3 Processing by Proprotein Convertases

Furin processes many substrates, including pro-transforming growth factor-β, to their active form (43). Mice with furin-deficient Tregs have recently been shown to be unable to keep T cell expansion and activation under control, and these mice develop IBD at approximately 6 months, highlighting the importance of PCs in the maintenance of peripheral tolerance (44). Our studies here suggest that PCs may control Treg function via additional mechanisms, namely by direct cleavage and regulation of Foxp3 activity.

The identification of the molecules that interact with the N- and C-terminal ends of Foxp3 will likely help explain how Foxp3 achieves diverse effects in altering the expression of different genes. It is possible that N- or C-terminal cleavages of Foxp3 lead to major topological changes and alter its DNA binding properties. In support of such conformational changes, we consistently find that the N-terminal cleavage in an inaccessible for processing by the PC(s) (Fig. 3b, lane 3 versus lane lane 3), indicating the release of the C-terminal tail results in a structural change, making the N-terminal site more accessible for processing by the PC(s) (Fig. 3

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