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UXT is a novel regulatory factor of regulatory T cells associated with Foxp3

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Regulatory T (Treg) cells are a constitutively immunosuppressive subtype of T cells that contribute to the maintenance of immunological self-tolerance and immune homeostasis. However, the molecular mechanisms involved in the regulation of Treg cells remain unclear. In the present study, we identified ubiquitously expressed transcript (UXT) to be a novel regulator of human Treg-cell function. In cultured human Treg cells, UXT associates with Foxp3 in the nucleus by interacting with the proline-rich domain in the N-terminus of Foxp3. Knockdown of UXT expression in Treg cells results in a less-suppressive phenotype, demonstrating that UXT is an important regulator of the suppressive actions of Treg cells. Depletion of UXT affects the localization stability of Foxp3 protein in the nucleus and downregulates the expression of Foxp3-related genes. Overall, our results show that UXT is a cofactor of Foxp3 and an important player in Treg-cell function.

Keywords: Cofactor · Foxp3 · Immune suppression · Regulatory T (Treg) cell · UXT

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Introduction

Regulatory T (Treg) cells are a subset of $CD4^+$ T cells that express high levels of the IL-2R α-chain (CD25). In both mice and humans, Treg cells comprise only 5–10% of circulating $CD4^+$ cells [1]. Treg cells have the ability to actively suppress immune responses and represent a predominant tolerance-inducing modality [2].

Forkhead box P3 (Foxp3) is a 47 kDa member of the forkhead/winged-helix family of transcription factors and is involved in regulating immune system development and function [3]. The fox family members are both transcriptional repressors and activators. They all have a forkhead domain, which is crucial for their roles in DNA binding and nuclear localization [4]. In particular, Foxp3 has been shown to be the master control gene for Treg-cell development and function [5, 6]. It confers the suppressive phenotype of Treg cells by inhibiting the activation of target genes of T-cell stimulation [7]. It also acts as a transcriptional repressor at the promoter regions of many cytokines such as IL-2 and GM-CSF [8, 9]. Studies [10–14] indicate that

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Foxp3 may form a dynamic superamolecular complex with a variety of molecular partners, such as NFAT, NF-κB, AML1/Runx1, AP-1, RORγt and TIP60, to regulate transcription. However, further identification of Foxp3-interacting proteins is needed in order to elucidate the mechanisms in which Foxp3 orchestrates the cellular and molecular programs involved in Treg-cell function.

The ubiquitously expressed transcript (UXT) gene, located in Xp11.23-p11.22, is 7.35 kb in length and is composed of seven exons. It encodes a protein of 157 amino acid residues with a calculated molecular weight of 18 246 and a calculated isoelectric point of 7.02 [9]. UXT interacts with the *N*-terminus of the androgen receptor and regulates androgen responsive genes [15]. It has also been described as a suppressor of cell transformation and a co-regulator of NF-κB [16, 17].

UXT is expressed in many tissues, including the heart, skeletal muscle, kidney, liver, adrenal gland, lymph node, thymus, and peripheral blood leukocyte [18]. In the current study, we use several approaches to provide evidence that UXT is expressed in Treg cells and is a novel regulatory factor of Foxp3. We propose that UXT may contribute to the sustained presence of Foxp3 inside the nucleus and promote Foxp3 transcriptional activity as well as regulate Treg-stimulated gene expression, thus playing a role in the immunsuppressive functions of Treg cells.

Results

Ubiquitously expressed transcript (UXT) is expressed in Treg cells

UXT is ubiquitously expressed in human tissues and has multiple influences on cell functions. To investigate the expression of UXT in human lymphocytes, we isolated several subsets of lymphocytes and found UXT expressed in T cells including the Treg cells and conventional T (Tconv) cells, and B cells (Supporting Information Fig. 1). Furthermore, we isolated $CD4^+CD25^+$Treg cells from five healthy volunteers to examine the expression and regulation of UXT in human Treg cells. As shown in Figure 1A and B, UXT mRNA and protein were present in all samples (Fig. 1C and D).

Treg cells were immunostained with UXT and Foxp3 antibodies to determine the interactions between UXT and Foxp3. As shown in Figure 1E, UXT and Foxp3 colocalized in the nucleus and perinuclear regions. In addition, UXT co-immunoprecipitated with Foxp3 in Treg-cell extracts (Fig. 1F), thus confirming that UXT is indeed associated with Foxp3.

UXT mediates the immune suppression of Treg cells

Foxp3 serves as a specific lineage transcript factor in Treg cells in mediating suppression of lymphocyte proliferation. To examine whether UXT enhances the suppressive activity of Treg cells, purified $CD4^+C25^+$ Treg cells were transfected with UXT siRNA (siRNA428). Successful knockdown of UXT expression was confirmed by Western blot analysis (Fig. 2A and B). There were no differences in the viability of transfected Treg cells compared to that of non-specific (NS)-transfected (control) or UXT-overexpressed cells (Supporting Information Fig. 2).

Interestingly, the antiproliferative effect of Treg cells co-cultured with $CD4^+CD25^-$ cells was significantly decreased in the presence of UXT siRNA (Fig. 2C), as evidenced by an increase in BrdU incorporation, an indicator of cell proliferation. The effect of siRNA428 was dose dependent (Fig. 2D). To further assess proliferation, $CD4^+$ cells were cultured for 4 days and tested using the carboxyfluorescein succinimidyl ester dilution assay. UXT siRNA-transfected Treg cells showed decreased suppressive function on WT autologous $CD4^+CD25^-$ T cells (Fig. 2E), thus confirming the results observed using the BrdU assay. In addition, the production of IL-4 and IFN-γ was significantly increased in UXT siRNA-transfected Treg cells compared to that of control. Overexpression of UXT in Treg cells had minimal effects on the secretion of IL-4 and IFN-γ (Fig. 2F and G).

Next, we examined the effect of UXT knockdown in Treg cells on the cytotoxic activity of $CD8^+$ T cells toward MKN45 cells. As shown in Figure 3A, co-culture of activated $CD8^+$ T cells with autologous Treg cells attenuated the cytotoxic activity against MKN45 cells (25.1 ± 6.6% at E/T ratio 20:1, $n = 6$) compared with that of $CD8^+$ T cells cultured without Treg cells (73.2 ± 11.4% at E/T ratio 20:1, $p < 0.05$). Co-culture of activated $CD8^+$ T cells with Treg cells treated with control siRNA had minimal effect on CTL activity compared with co-cultures with UXT siRNA-transfected Treg cells (20.7 ± 6.7% versus 45.6 ± 14.2% at E/T ratio 20:1, $p < 0.05$). These results indicate that autologous Treg cells significantly suppress the cytotoxic function of $CD8^+$ T cells and knockdown of UXT can partly abolish the function of Treg cells.

Furthermore, perforin and IFN-γ levels were significantly reduced in the presence of Treg cells. Perforin yielded 415.7 ± 53.6 ng/L in the Treg-siRNA428 ($p < 0.05$) versus 278.3 ± 38.4 ng/L in the Treg-control and 236.7 ± 16.6 ng/L in the Treg-UXT group. Similarly, IFN-γ yielded 415.8 ± 53.6 ng/L in the Treg-siRNA428 ($p < 0.05$) versus 278.7 ± 38.4 ng/L in the Treg-control and 236.9 ± 16.6 ng/L in the Treg-UXT group (Fig. 3B and C).

UXT associates with Foxp3 inside the nucleus of Treg cells

UXT has been shown to contribute to the sustained presence of p65 inside the nucleus [17]. Thus, we hypothesized that UXT may also serve as a specific molecular chaperone for Foxp3 inside the nucleus. SGC-7901 and HEK293 cells were transfected with control (NS) or UXT (428) siRNAs and cultured with or without leptomycin B (LMB), a specific inhibitor of nuclear export signal dependent protein export from the nucleus. UXT siRNA-transfected cells had significantly lower amounts of Foxp3 and its localization appeared to shift from the nucleus to the

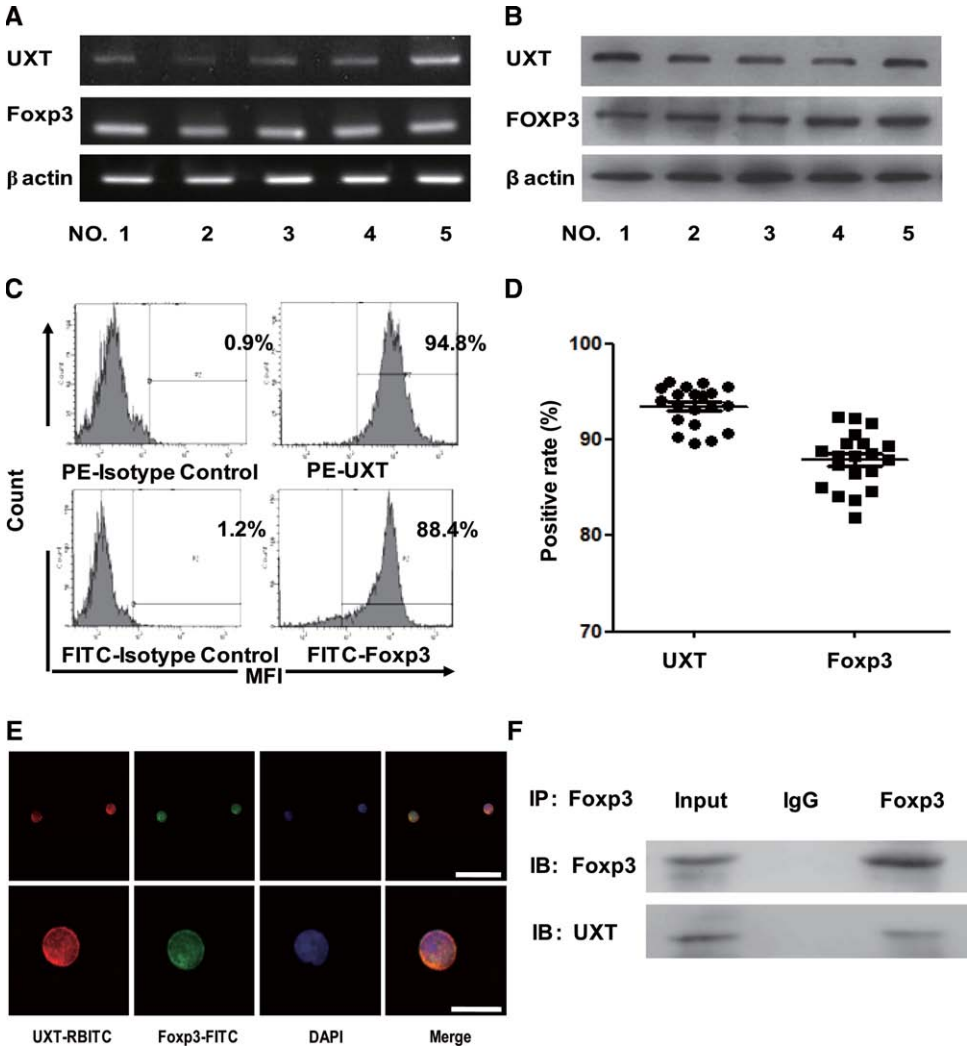

Figure 1. UXT is expressed in Treg cells and colocalizes with Foxp3. $CD4^+CD25^+$Treg cells were isolated from healthy volunteers. (A) Endogenous expression of UXT and Foxp3 in human Treg cells was determined by RT-PCR. (B) Western blot analysis of endogenously expressed UXT and Foxp3 in Treg cells. (A and B) Lanes 1–5 represent blood samples from five donors. (C) Treg cells were analyzed for UXT and Foxp3 expression by flow cytometry. (D) The percentage of Treg cells expressing UXT and Foxp3 was determined in the peripheral blood by flow cytometry (n = 20). Each symbol represents an individual sample. (E) Treg cells were fixed with paraformaldehyde and immunostained with polyclonal goat antibodies against Foxp3 and monoclonal mouse antibodies against UXT. The primary antibodies were detected by addition of FITC-coupled secondary antibody (green) and rabbit RBITC-coupled antibody (red), and the cells were evaluated by confocal microscopy. Cell nuclei were visualized by DAPI staining. Scale bars, 25 μm (top) and 10 μm (bottom). (F) Co-immunoprecipitation (IP) of UXT and Foxp3 from Treg-cell extracts. A representative image of three performed is shown.

cytoplasm. This phenomenon was dose dependent and transfection with control siRNA displayed no such effect. Immunofluorescence analysis suggested that cells cultured in the presence of LMB sustained nuclear localization of Foxp3, even when UXT expression was knocked down. This indicated that UXT positively regulates the nuclear localization and actions of Foxp3 (Fig. 4A). In addition, nuclear and cytoplasmic fractions were extracted and assayed by Western blot analysis. The results were similar to those observed with the immunofluorescence analysis (Fig. 4B). To assess the efficiency of extraction of nuclear and cytoplasmic fractions, we immunoblotted the cytoplasmic extracts with Lamin A and confirmed that they were pure and free of nuclear fractions (Fig. 4B).

UXT regulates Foxp3 transcriptional activity and its DNA-binding capacity

Foxp3 has the ability to occupy IL-2, CTLA-4, and CD25 promoters, both in T cells retrovirally transduced with Foxp3 and in "natural" $CD4^+CD25^+$ Treg cells that have been expanded with IL-2 [19,20]. Given that UXT was important in maintaining the presence of Foxp3 inside the nucleus, whether UXT regulates the transcriptional activity of Foxp3 in vivo to further influence the function of Treg cells remains unknown. To address this issue, transfected Treg cells were analyzed for Foxp3 occupancy at the promoters of IL-2, CTLA-4, and CD25 genes. ChIP analysis showed that Foxp3 binding to IL-2, CTLA-4, and CD25 was increased when UXT

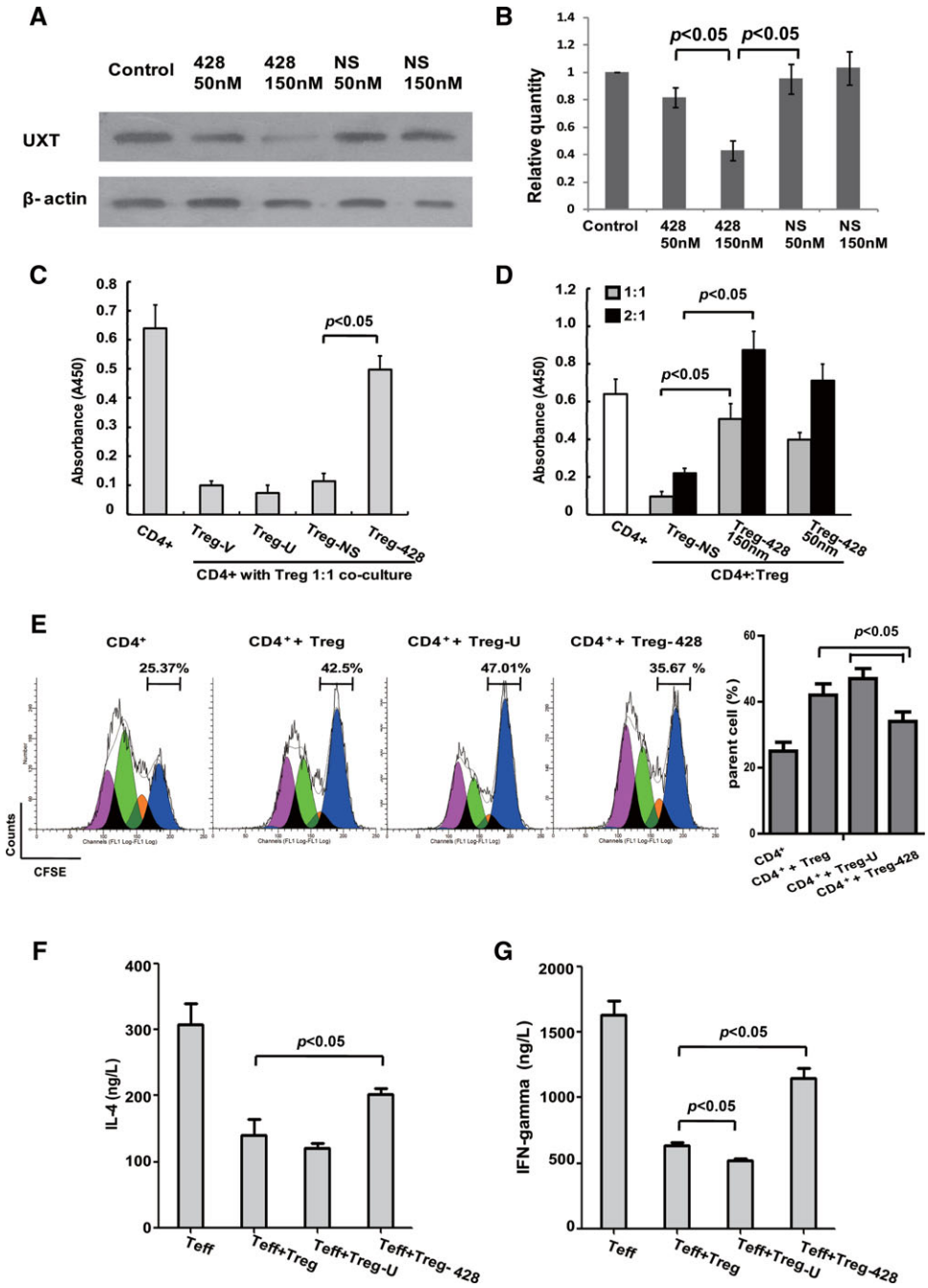

Figure 2. UXT mediates the immunosuppressive function of Treg cells. (A) Treg cells were transfected with the indicated siRNA. (A) Forty eight hours after transfection, cells were collected and subjected to Western blotting for determining the efficiency of siRNA transfection. β-actin was used as a loading control. (B) Data shown are mean ± SEM of the relative quantity from three replicate experiments. (C) $CD4^+C25^+$T cells were transfected with UXT (428) or control (NS) siRNA and cultured with autologous $CD4^+CD25^-$ responder T cells for 5 days, after which BrdU incorporation was assessed. Suppressive capacity of transfected Treg cells tested by co-culture of transfected Treg cells with $CD4^+$ responder cells at a ratio of 1:1. Treg-V: Cells transfected with control vector; Treg-U: Cells transfected to overexpress UXT; Treg-NS: Cells transfected with nonspecific control siRNA; Treg-428: Cells transfected with siRNA 428 that targets against UXT. (D) Treg cells were transfected with increasing doses of the indicated siRNA and then co-cultured with $CD4^+$ T responder cells for the suppressive capacity assay. (E) In the carboxyfluorescein succinimidyl ester assay, autologous $CD4^+CD25^-$ T cells were labeled with carboxyfluorescein succinimidyl ester and co-cultured with or without autologous $CD4^+CD25^+$ Treg cells for 6 days. $CD25^-$ cells were sorted for flow cytometry analysis, of which the culture supernatant was collected and applied for ELISA of (G) IL-4 (F) IFN-γ secretion. For all experiments, each group was evaluated in triplicate/quadruplicate. Data are shown as mean ± SD of replicates pooled from three experiments. The one-way ANOVA was used for statistical significance.

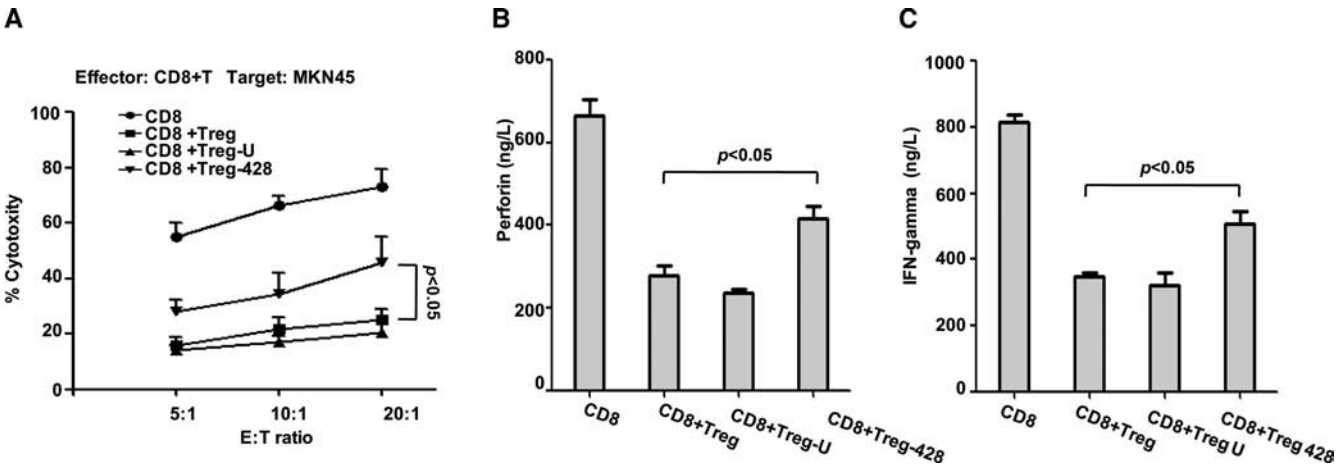

Figure 3. Knockdown of UXT in Treg cells significantly decreases their suppression of CTL cells. CTL cells primed with MKN45 lysate-loaded DCs were used as effectors, and the MKN45 cell line was used as target cells. (A) Cytotoxic activity of CTL cells co-cultured with Treg cells or siRNA transfected was measured by specific lysis of target cells. Also, the culture supernatant was collected and evaluated for lactate dehydrogenase activity released by the damaged cells, analyzed at the indicated E/T ratio. (B and C) The secretion of (B) perforin and (C) IFN-γ was measured by ELISA. Data are shown as mean ± SD of three replicates pooled from three experiments. The *t*-test was used for statistical significance.

was overexpressed in Treg cells. Interestingly, this binding was markedly diminished when UXT was knocked down (Fig. 5A–C). UXT-overexpressed Treg cells expressed higher amounts of CTLA-4 and CD25 than the control cells. Conversely, lower amounts of these molecules were observed in UXT siRNA-transfected Treg cells. Flow cytometry results showed that the protein expression of CTLA-4 and CD25 was also decreased in UXT siRNA-transfected cells (Fig. 5D–F).

We further investigated the activity of Foxp3 UXT siRNA-treated Treg cells treated with or without LMB. Foxp3 transport was tested by measuring the levels of CTLA-4 and CD25. UXT siRNA transfected Treg cells had significantly decreased levels of CTLA-4 and CD25 mRNA. Treatment of these transfected cells with LMB partially restored levels of CTLA-4 and CD25 mRNA to that of control cells (Supporting Information Fig. 3). This suggests that the Foxp3/UXT interaction contributes to Foxp3 transcriptional activity.

These data indicate that the expression of a certain level of UXT is necessary for the induction of genes that are directly regulated by Foxp3 and suggest that UXT plays a unique role in Foxp3 transcription.

UXT interacts with Foxp3 in yeast and human Treg cells

To examine the mechanisms of UXT in regulating Treg cells, we assessed the putative interactions between UXT and Foxp3 by backcrossing yeast using the yeast strain, AH109. AH109 was transformed with the pGADT7-UXT vector that contains the GAL4-AD with or without pGBKT7-Foxp3. The co-transfected clones showed positive staining for β-galactosidase (Fig. 6A), suggesting that the growth of AH109 in the selective medium resulted from the specific interaction between UXT and Foxp3 in vivo. To confirm the interaction between these two proteins, immunoprecipitation studies were performed in human cells. In this experiment, the pCMV(Myc)-UXT and pFLAG-Foxp3 vectors were constructed and transfected in HEK293 cells. Flag-tagged Foxp3 was expressed, together or separately, with Myc-tagged UXT and subjected to immunoprecipitation using anti-Flag or anti-Myc antibodies. As shown in Figure 6B, both UXT co-immunoprecipitated with Foxp3. The interaction between UXT and Foxp3 was further confirmed by confocal fluorescence microscopy. The result indicated that UXT and Foxp3 were predominantly colocalized in the nucleus (Fig. 6C).

UXT interacts with proline-rich domain in the *N*-terminus of Foxp3

To explore the UXT-binding region within Foxp3, we constructed a series of Foxp3 deletion mutants (Fig. 6D). The pGBKT7-T1∼5 vectors were constructed and used for AH109 (Δ LEU2) transformation. The transformed competence cells were applied on an SD/-Trp flat plate and our results showed that all of the Foxp3 mutants (T1∼T5) were expressed in the system (Supporting Information Fig. 4). Furthermore, we transformed AH109 containing pGADT7-UXT with full-length Foxp3 or subdomains cloned in the pGBKT7 vector. Interestingly, the Foxp3 fragments lacking amino acids 1–152 at the *N*-terminus were unable to interact with UXT. Other Foxp3 deletion mutants lacking amino acids 1–152, 1–97, 72–152, or 72–39, fully retained their binding capability with UXT (Fig. 6E and F). The binding interactions consisted of one proline-rich domain, whereas no interaction was detected between UXT and T2 (153–396), which did not contain the proline-rich domain. To summarize, these data revealed that the proline-rich domain of Foxp3 is essential in mediating its interaction with UXT.

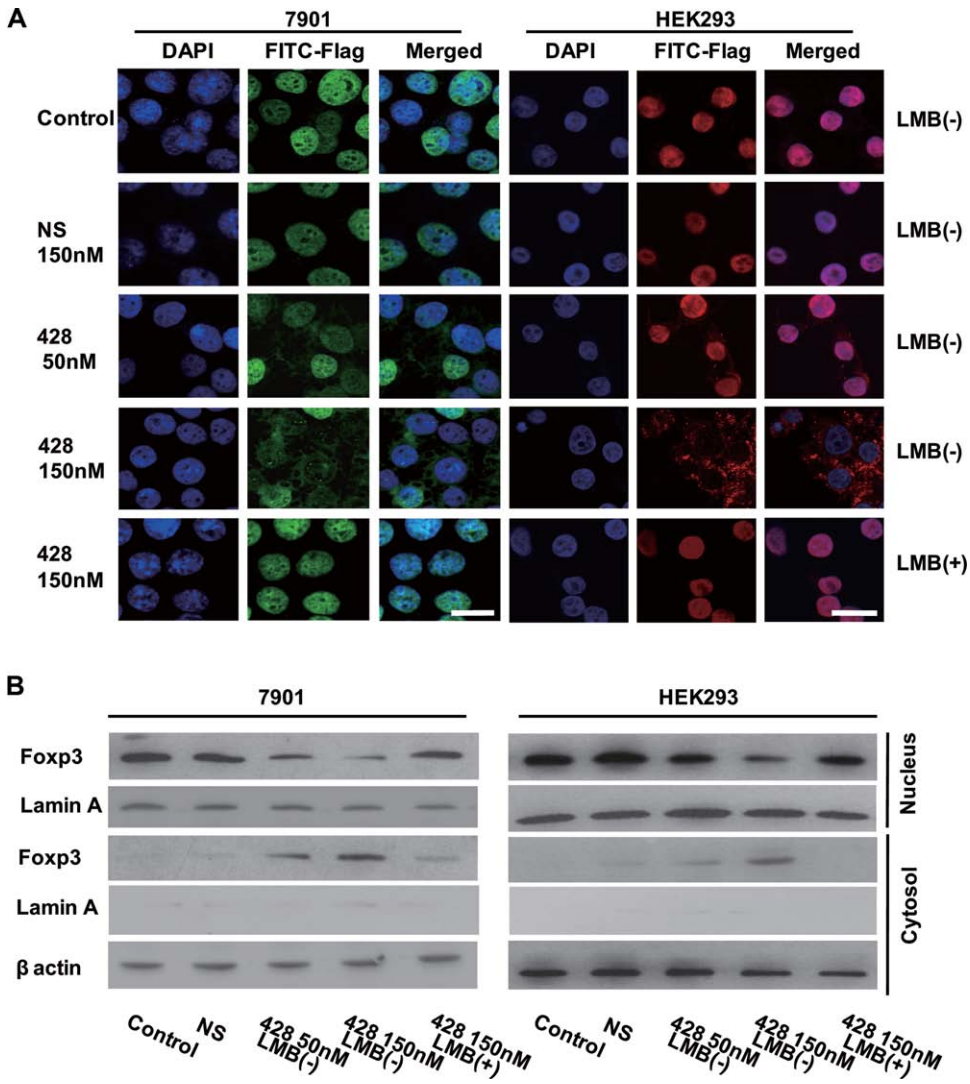

Figure 4. UXT retains Foxp3 inside the nucleus in SGC-7901 and HEK293 cells. Cells were infected with lentivirus to establish the stable cell lines that overexpress Flag-tagged Foxp3. (A) SGC-7901/Flag-Foxp3 (left) or HEK293/Flag-Foxp3 (right) cells were transfected with the indicated UXT siRNAs. After 48 h, cells were treated with or without 20 ng/mL LMB for 24 h and then stained with anti-Foxp3 primary antibody and FITC-conjugated secondary antibody. The nucleus was counterstained with DAPI. Control: cells treated with lipofectamine 2000; NS: cells transfected with nonspecific control siRNA; 428: cells transfected with siRNA 428. Scale bar, 20 μm. (B) Cytoplasmic and nuclear fractions were prepared and immunoblotted with the indicated antibodies, respectively. Cytosol extract fractions were immunoblotted with Lamin A to certify there was no nuclear fraction pollution in the cytosol extract fraction. Data shown are from one experiment representative of three performed.

Discussion

$CD4^+CD25^+$Treg cells play a critical role in the maintenance of peripheral immunological tolerance by acting as suppressors of diverse immune responses and important gatekeepers of immune homeostasis. Previous studies have reported that intratumoral Treg cells can suppress the functional capabilities, proliferation, production, and secretion of IFN-γ and interleukin (IL)-4 of autologous infiltrating $CD4^+CD25^-$ T cells [21]. Additionally, accumulating data indicated that Treg cells are capable of inhibiting $CD8^+$ T cells under different pathologic conditions such as infections [22–24]. An α-class PFD family protein named UXT was demonstrated to be widely expressed in human tissues, including the LN, thymus, and peripheral blood leukocyte. However, whether UXT regulates the function of Treg cells was unknown. Here, we showed that $CD4^+CD25^+$ Treg cells express UXT mRNA and protein. Both UXT and Foxp3 were predominantly colocalized in the nucleus and perinuclear regions. Furthermore, knockdown of UXT expression in Treg cells resulted in a less suppressive phenotype as evidenced by BrdU incorporation and CTL activity assays. Our study demonstrates that UXT is an important regulator of the suppressive actions of Treg cells.

As an α-class PFD family protein, UXT has the potential to function as a molecular chaperone in protein folding and stability [20] and to participate in transcriptional regulation [25]. UXT has been reported to contribute to the sustained presence of p65 inside the

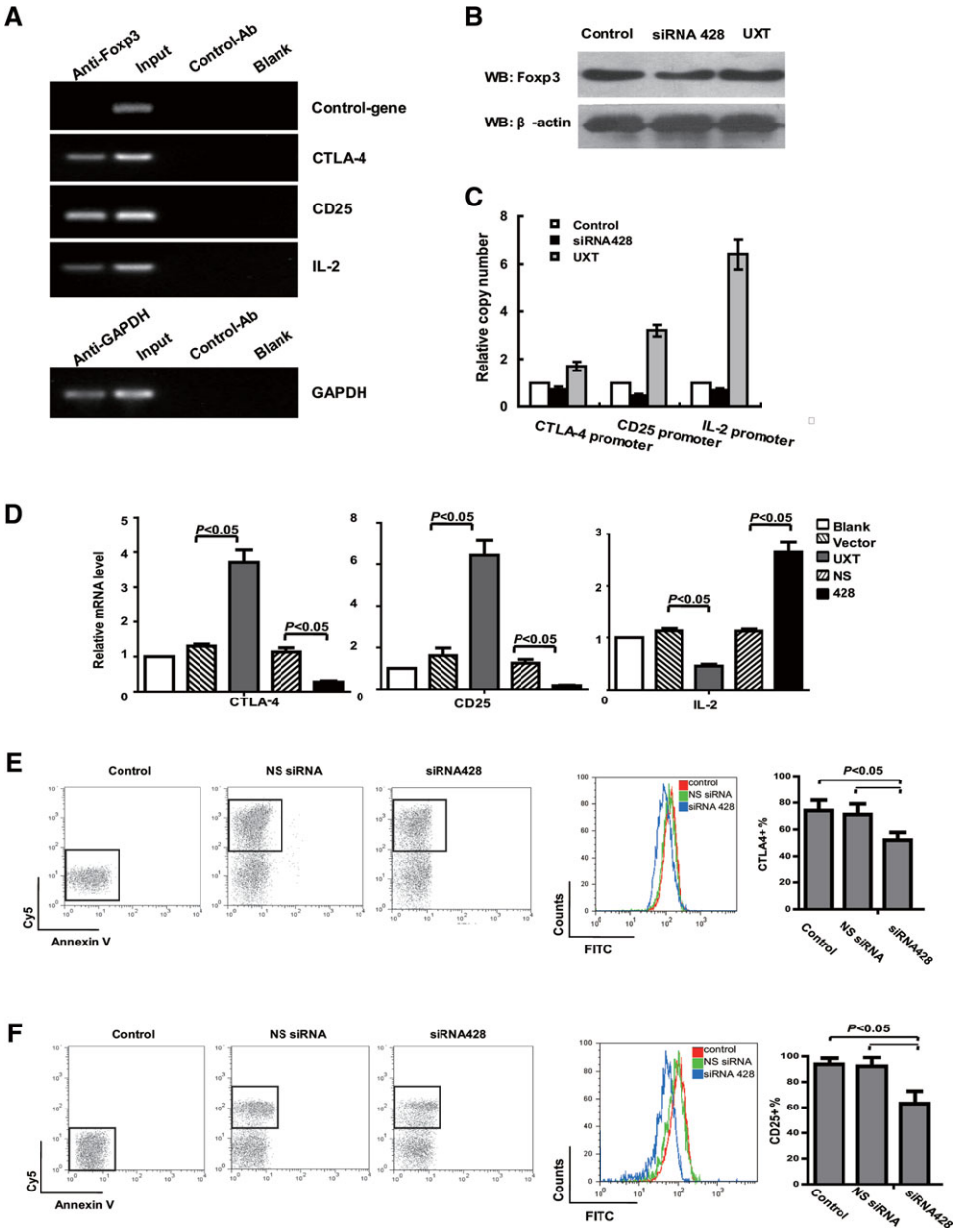

Figure 5. UXT influences Foxp3 transcriptional activity and its DNA-binding capacity. ChIP assay was performed to assess the binding of Foxp3 to the CD25, CTLA-4, and IL-2 promoters in Treg cells transfected with UXT, UXT siRNA (428) or control. The transfected Treg cells were cultured for four days and were restimulated for 30 min with PMA and ionomycin. Binding of endogenous Foxp3 to the promoters was determined by ChIP and real-time PCR. (A) Normal mouse IgG was used as a negative control antibody, anti-RNA Polymerase II as a positive control antibody, and GAPDH was used as a negative control gene. (B) Western blot analysis of Foxp3 expression in each group. (C) The numbers of copies of genomic DNA detected in each immunoprecipitation relative to a standard dilution of input. (D) The transfected Treg cells were collected and the relative mRNA expression of CTLA-4, CD25, and IL-2 was analyzed by RT-PCR. Blank: cells treated with nucleofector reagent; Vector: cells transfected with control vector; UXT: cells transfected with UXT; NS: cells transfected with nonspecific control siRNA; 428: cells transduced with UXT siRNA 428. (E and F) The surface expression of (E) CTLA-4 and (F) CD25 on Treg cells were analyzed by flow cytometry. Cy5-labeled siRNA was used to select transfected cell while annexin V was used for gating viable cells. (C–F) Data are shown as mean ± SD of three replicates pooled from three independent experiments. The *t*-test was used for statistical significance.

nucleus [17]. Foxp3 is the master gene for differentiation and function of Treg cells and plays an important role in the negative immune regulation. Thus, we hypothesized that UXT may act as a specific molecular chaperone that regulates the nuclear localization of Foxp3. Our data determined that UXT can indeed keep Foxp3 inside the nucleus and that the loss of UXT affects the subcellular localization pattern of Foxp3. The change in the intracellular location of Foxp3 shifted from a cytoplasmic/perinuclear to a more nuclear expression, possibly due to posttranslational modifications [26]. Foxp family proteins are known to be regulated by

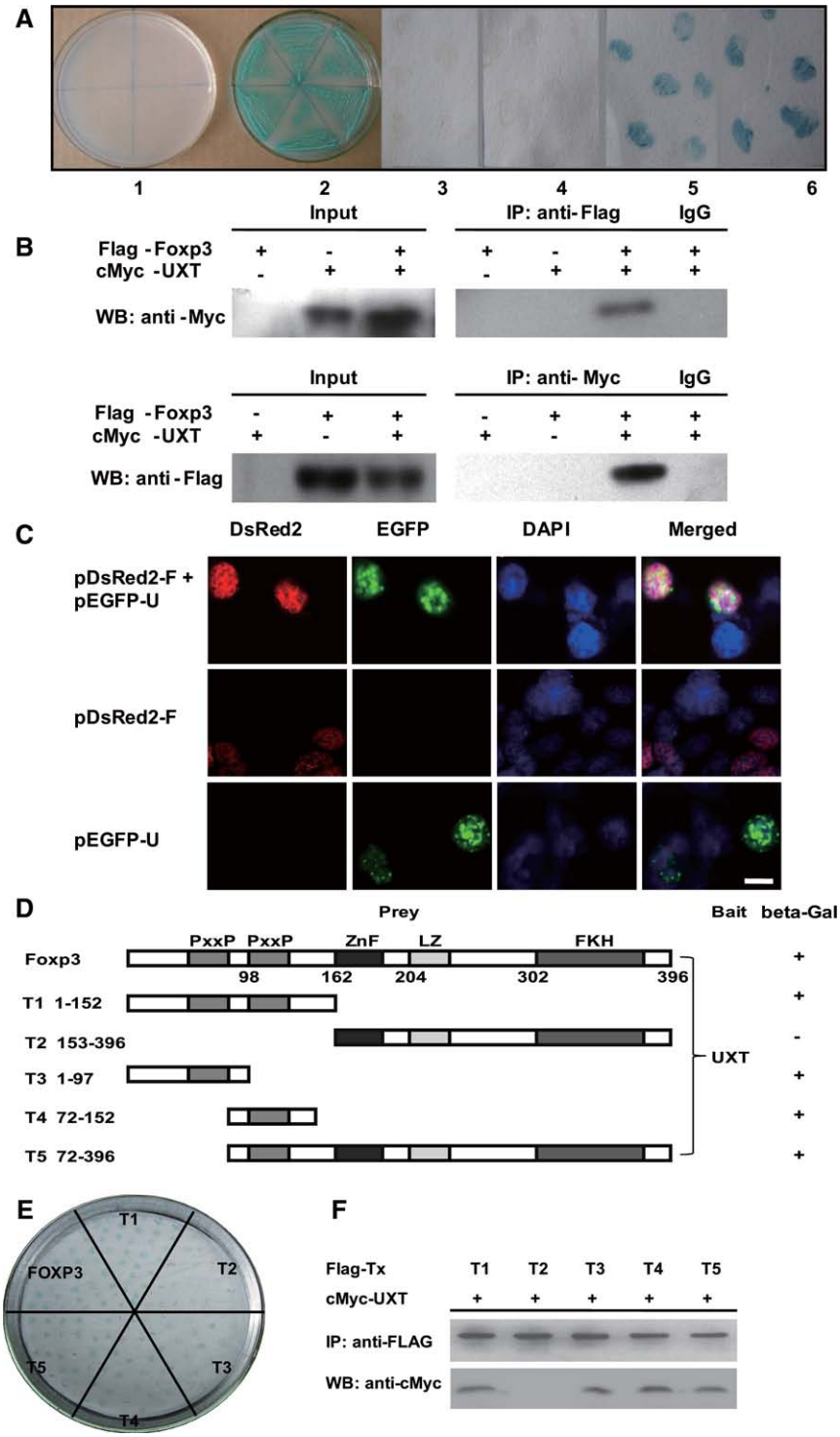

Figure 6. Foxp3 interacts with UXT. (A) α-galactosidase assay of the yeast strain AH109 after transformation with the pGADT7-UXT vector and with (2) or without (1) pGBKT7-Foxp3. Yeast cells were co-transfected with the indicated plasmids the β-galactosidase assay was performed. (3, 4: pGADT7-UXT) (5, 6: pGADT7-UXT and pGBKT7-Foxp3). (B) HEK293 cells were transfected with pCMV(Myc)-UXT and pFLAG-Foxp3 expression plasmids and harvested 60 h later. Co-IP of UXT and Foxp3 from 293T cell extracts using anti-Flag or anti-Myc antibodies is shown. (C) DsRed2-tagged Foxp3 was expressed, together or separately, with EGFP-tagged UXT in HEK293 cells for 48 h. The nuclei were visualized by DAPI staining. The cells were evaluated by confocal microscopy. Scale bar, 10 μm. (D) Schematic illustration of Foxp3 and its mutants. PxxP, Proline-rich domain; ZnF, zinc finger motif; LZ, leucine zipper; FKH, Forkhead domain. (E) The interaction of UXT with the Foxp3 *N*-terminal subdomains 1–152, 153–396, 1–97, 72–152 and 72–396 were assessed using the β-galactosidase assay. +, strong interactions (blue colonies); –, no interactions (white colony). (F) Myc-tagged full-length UXT was transfected into 293T cells along with Flag-tagged Foxp3's deletion mutants as indicated. 60 h later, whole cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. All data shown are from single experiments representative of three experiments performed.

site-specific phosphorylation, and the TCR stimulation activates unidentified kinases that phosphorylate Foxp3 and accelerate its rapid nuclear translocation. Foxp3 then undergoes acetylation by TIP60 and binds to the promoter regions of target genes to carry out its function as a transcription factor [27].

Our results suggest that depletion of UXT affects the localization stability of Foxp3 protein in the nucleus. One possible mechanism for the phenomenon mentioned above is that the loss of UXT releases Foxp3 from the promoter region of downstream genes, which consequently causes Foxp3 to be exported from nucleus into the cytoplasm. Collectively, our study reveals that UXT is essential for the function of Foxp3 in the nucleus, which uncovers a new mechanism of Foxp3 regulation. However, further investigation is needed to determine whether or not the interaction between

UXT and Foxp3 is required for its function in Treg cells. This study does show that UXT can directly regulate the expression of Foxp3. Overexpression of UXT in Treg cells resulted in the upregulating of Foxp3 in both mRNA and protein levels (Supporting Information Fig. 5). This finding may potentially contribute to the mechanism by which UXT controls Treg-cell function. Currently, the pathways and molecular mediators involved in this function are unknown and present an important area of interest for future studies.

Foxp3 solidifies Treg-cell lineage stability through modification of cell surface and signaling molecules, resulting in adaptation to the signals required to induce and maintain Treg cells [28]. Treg cells constitutively express CD25 and CTLA-4 [29–31] and the network of Treg-cell suppression mechanisms includes these surface molecules that play a crucial role in the generation of $CD4^+CD25^+$ Treg cells. Recent studies suggested that Foxp3 acts as both transcriptional activators and repressors and that the direct targets of Foxp3 make up approximately 10–20% of Foxp3-dependent genes [24, 26]. Foxp3 has the ability to occupy IL-2, CTLA-4, and CD25 promoters, both in T cells retrovirally transduced with Foxp3 and in "natural" $CD4^+CD25^+$ Treg cells that have been expanded with IL-2 [19]. We further investigated how expression of the Foxp3-dependent genes (CD25 and CTLA-4) was influenced by knocking down endogenous UXT through siRNA transfections. As we expected, CD25 and CTLA-4 inductions were significantly attenuated when endogenous UXT expression was suppressed. These findings provide further evidence of how changes in UXT levels affect the expression of Foxp3-related genes.

Although multiple co-interactor and signal transduction pathways have been demonstrated to regulate Foxp3 function, the regulators of Foxp3 remain incompletely understood and a more comprehensive study of Foxp3 is needed. In this study, we initially identified UXT as a Foxp3-binding partner. Although the functional relationship between UXT and Foxp3 warrants further investigation, we have made several observations concerning UXT. Confocal fluorescence microscopy results indicated that both UXT and Foxp3 were predominantly colocalized in the nucleus and perinuclear regions, thus raising the possibility that these proteins interact with each other to regulate gene expression. The interaction of UXT and Foxp3 was further confirmed by immunoprecipitation and the yeast two-hybrid assay. To explore the UXT-binding region within Foxp3, a series of Foxp3 deletion mutants were constructed and we found that UXT interacts with the proline-rich domain in the *N* terminal of Foxp3. At least one proline-rich domain was essential and sufficient to mediate its interaction with UXT. The role of these proline-rich domains in the repressor activity of Foxp3 is still unknown [27].

Although the importance of Foxp3 in Treg-cell development and function has been demonstrated, the mechanisms of these specific biochemical functions remain unclear. The functions of Treg cells change according to the amount of UXT, as observed in our experiments, thus strongly suggesting that UXT is a crucial Foxp3 interacting protein that may be required for the suppressive function of Foxp3. Taken together, our data provide the first evidence linking UXT proteins to Foxp3, a protein involved in the immunosuppressive function of Treg cells. We propose that UXT may contribute to the sustained presence of Foxp3 inside the nucleus and to promote the transcription activity of Foxp3 as well as Treg-mediated gene expression. The elucidation of the mechanisms involved in the in vivo interactions and regulation of Foxp3 will help in developing drugs that target Treg cells, which may hold great clinical therapeutic potential.

Materials and methods

Materials

Treg cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories). Anti-human CD3 and anti-human CD28 were purchased from BD Biosciences. Phorbolmyristate acetate (PMA) and ionomycin were from Sigma-Aldrich. Jurkat nucleofector kits were purchased from Amaxa. UXT siRNA duplexes were chemically synthesized by Gene Pharma. The UXT siRNA sequences were as follows: 428, CCAAGGACUCCAUGAAUAUTT; and the control siRNA sequence was UUCUCCGAACGUGUCACGUTT. The primers were chemically synthesized by GenScript. The following primer pairs were used: CTLA-4 forward 5′- GAGTATGCATCTCCAGGCAA -3′, reverse 5′-CGCACAGACTTCAGTCACCT-3′; CD25 forward 5′-AGTGAGACTTCCTGCCTCGT-3′, reverse 5′-GGCCACTGCTACCT GGTACT-3′; Fopx3 forward 5′-TTCGAAGAGCCAGAGGACTT-3′, reverse 5′-ATGGCACTCAGCTTCTCCTT-3′; UXT forward 5′-TGGACCATCGAGACAAGGTA-3′, reverse 5′-CTCCGAGTGCTTA GCTTCCT-3′; β-actin forward 5′-CTGTCCACCTTCCAGCAGATGT-3′, reverse 5′-CGCAACTAAGTCATAGTCCGCC-3′. The following antibodies were used: anti-c-Myc antibody (Sigma), monoclonal anti-FLAG M2 antibody (Sigma), polyclonal anti-UXT antibody (Abnova), rabbit polyclonal anti-β-actin (Santa Cruz Biotechnology). T-cell isolation kit was from Miltenyi Biotec. The chromatin immunoprecipitation kit, BD matchmaker pretransformed libraries, and BrdU cell proliferation kit was purchased from Upstate Biotechnologies, BD Biosciences, Clontech, and Roche, respectively.

Construction of plasmids

pGADT7-UXT was generated by PCR amplification with 5′- and 3′-oligonucleotides, CGGAATTCATGGTCTTCCCCCTCCCCACTC, and GGGGATCCTCAATGGTGAGGCTTCTCTGGG, respectively, and the insertion of UXT fragments into *Eco*RI/*Bam*HI of pGADT7. For co-immunoprecipitation, pCMV(Myc)-UXT was created by inserting an *Eco*R/*Kpn*I UXT PCR fragment amplified from pGADT7-UXT with oligonucleotides: 5′-CG GGATCCGTATGGTCTTCCCCCTCCCCAC-3′ and 5′-GGGGTACC TCAATGGTGAGGCTTCTCTGGG-3′. pFLAG-Foxp3 was created

by PCR amplification of pGBKT7-Foxp3 with primers, 5′-CG AAGCTTATGCCCAACCCCAGGCCTGG-3′ and 5′-GGGGTACCTCA GGGGCCAGGTGTAGGG-3′, the product of which was digested with *Hind* III and *Kpn*I and inserted into the corresponding site of pFLAG. The Foxp3 deletion mutants were created by inserting *Eco*RI/*Bam*HI-digested PCR fragments generated using the following primers: sense for T1(1–52) 5′-CG GAATTC ATGCCCAACCCCAGGCCTG-3′, antisense for T1 5′-CG GGATCC TCAGGGCACAGCCGAAAGGGTG-3′; sense for T2(153–396) 5′-CGGAATTC ATGCAGAGCTCCTACCCACTGC-3′, antisense for T2 5′-CGGGATCC TCAGGGGCCAGGTGTAGGGTTG-3′; sense for T3(1–97) 5′-CGGAATTC ATGCCCAACCCC AGGCCTG-3′, antisense for T3 5′-CGGGATCCTCATGTGAG GCTGATCATGGCTGGG-3′; sense for T4(72–152) 5′-CG GAATTCATGTCAACGGTGGATGCCCACG-3′, antisense for T4 5′-CGGGATCC TCAGGGCACAGCCGAAAGGGTG-3′; sense for T5(72–396) 5′ -CGGAATTCATGTCAACGGTGGATGCCCACG-3′, antisense for T5 5′-CGGGATCC TCAGGGGCCAGGTGTAGGGTTG-3′.

Cell purification

Enriched buffy coat samples were obtained from healthy volunteers and peripheral blood samples were collected from colon carcinoma patients. All studies were approved by the local institutional review board and written informed consent was obtained from all donors. PBMCs were isolated by Ficoll-Plaque density gradient centrifugation. After two washes with PBS, human $CD4^+C25^+$Treg cells and $CD4^+CD25^-$T cells were purified from PBMCs using the Treg cell isolation kit (Miltenyi Biotec Auburn, CA, USA). Cells were >95% pure, as determined by FACS analysis.

Immunofluorescence analysis

Cells (either grown on coverslips or suspended in solution) were fixed for 10 min with 4% PFA and washed twice with cold PBS and permeabilized with PBS containing 0.2% Triton X-100 for 10 min. After three more washes with cold PBS, the cells were blocked for 30 min with 1% BSA at room temperature. Cells were then stained with the indicated primary antibodies followed by fluorescein-conjugated secondary antibodies. Nuclei were counterstained with DAPI. Images were captured at room temperature using a confocal microscope (FluoView™ FV1000, Olympus).

T-cell transfections

Cells (5×10^6) were centrifuged at $200 \times g$ for 10 min at room temperature. The supernatants were discarded and the cell pellets were carefully resuspended in 100 μL Nucleofector® Solution containing plasmids or siRNA. Cell suspensions were transferred into cuvettes and transfections were carried out with the appropriate Nucleofector® Program. Afterward, ~500 μL of the preequilibrated culture media were added to each cuvette and the cells were transferred onto 12-well plates, which had been preincubated/equilibrated in a humidified 37°C/5% CO_2 incubator for at least 30 min.

T-cell proliferation assay

T-cell proliferation was determined using the 5-bromo-2-deoxyuridin (BrdU) based Cell Proliferation ELISA kit (Roche, Heidelberg, Germany). Five hours post electrotransformation treatments, both $CD4^+CD25^-$ and $CD4^+C25^+$Treg cells were cultured at different ratios with or without autologous $CD4^+CD25^-$ responder T cells (4×10^5) in anti-CD3 (10 μg/mL) plate-bound 96-well plates. IL-2 (300 U/mL) and anti-CD28 (2 μg/mL) were added to the cells. After 5 days in culture, the cells were pulsed with BrdU and assessed for BrdU incorporation 4 h later. The one-way ANOVA was used for statistical significance and a p value of less than 0.05 was considered significant.

CTL assay

MKN45 lysate-loaded dendritic cell (DC) stimulated CTLs were used as effectors, and the MKN45 cell line was used as target cells. MKN45 cells were seeded at a density of 5×10^4 cells/well in 96-well plates. Effector cells were incubated with MKN45 at effector and target (E/T) ratios (5:1, 10:1, and 20:1) at 37°C under 5% CO_2. Following electrotransformation treatments, $CD4^+C25^+$T cells were added to the plate, with 2×10^5 cells/well. In parallel, target cells were incubated alone to measure basal apoptosis. The CTL activity was measured by lactate dehydrogenase release. Percent cytotoxicity was calculated as follows: ((Experimental release–Effector spontaneous release–Target spontaneous release)/(Target maximum release–Target spontaneous release)) × 100%. Perforin and IFN-γ secretion were measured by commercially available ELISA kits.

Nuclear protein extractions

Nuclear extractions of cells were prepared using the Nuclear-Cytosol extraction kit (KEYGEN, KGP1100), according to the manufacturer's instructions.

Chromatin immunoprecipitation

Electrotransformation-treated Treg cells were stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) and the CHIP assay was performed using an immunoprecipitation assay kit (Millipore Corporation, MA, USA), according to the manufacturer's instructions. The presence of target DNA fragments in both the input and recovered DNA immunocomplexes was detected by PCR. The following promoter-specific primers were used: human

IL-2 promoter sense (5′-CAGGATGGTTTTGACAAAGAGAACA-3′) and antisense (5′-TTCATACAATGAACGCCTTCTGTATG-3′); CTLA-4 promoter sense (5′-TATACTCTCCAAGACTCCACGT-3′) and antisense (5′-GGTTTAGCTGTTACGTCGTAAAGA-3′); CD25 promoter sense (5′-GAGATCACAGAACAGAGTAGGC-3′) and antisense (5′-CTCTCAGTCTGTCATCTTGGC-3′); GAPDH promoter sense (5′-TACTAGCGGTTTTACGGGCG-3′) and antisense (5′- TCGAACAGGAGGAGCAGAGAGCGA-3′).

Yeast two-hybrid assay

Full-length Foxp3 or subdomains cloned in the pGBKT7 vector were transformed in the yeast strain AH109 (Δ LEU2) containing pGADT7-UXT. Transformants were mated overnight in 200 μL nonselective YPDA rich medium, of which 50–100 μL of suspended yeast were plated onto dual-selective media. Interactions were probed by growth on SD/-Leu/-Trp/-His medium. These clones were grown at 30°C for 8 to 10 days and then streaked in SD/-Leu/-Trp/-His/-Ade/X-α-GAL medium to verify the activation of all the reporter genes (ADE2, HIS3, and LacZ). Segregation was confirmed by the observation of white and blue colonies. Then the β-galactosidase assays were performed as described by Clontech.

Immunoprecipitation

HEK293 cells were transfected with appropriate expression plasmids. Sixty hours after transfections, the cells were harvested and whole-cell lysates were subjected to either immunoblot analysis (10% of lysates) or immunoprecipitation (90% of lysates). For immunoprecipitation (IP), the samples were precleared by addition of protein A/G-Sepharose slurry followed by 30 min incubation at room temperature. The supernatant was subsequently incubated with the appropriate antibody for 2 h at 4°C, followed by precipitation of the immune complexes with protein A/G-sepharose. The complexes were washed three times with ice-cold precipitation buffer, and then subjected to SDS-PAGE followed by Western blotting with specific antibodies as indicated in each figure.

Statistics

Data were analyzed for statistical significance using SPSS software. A p value of less than 0.05 was considered significant.

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Abbreviation: **UXT**: ubiquitously expressed transcript

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