

Letter to the Editor

Critical link between glycogen synthase kinase 3 β and forkhead box P3 in patients with chronic rhinosinusitis with nasal polyps

To the Editor:

Regardless of the distinct inflammatory profiles in patients with chronic rhinosinusitis with nasal polyps (CRSwNP) between western and eastern populations, the downregulation of forkhead box P3 (Foxp3; the master regulator for regulatory T [Treg] cells) has been uniquely observed.^{1,2} Previous studies on Foxp3 expression in patients with CRSwNP mainly focused on the transcriptional level, and the posttranslational regulation of Foxp3 expression in patients with CRSwNP has not been determined.

Glycogen synthase kinase 3 (GSK-3) is a serine-threonine protein kinase that exists in 2 isoforms in mammalian cells, namely GSK-3 α and GSK-3 β .³ GSK-3 β functions in diverse cellular processes that have been implicated in a range of human pathologies, including asthma and CRSwNP.^{4,5} On the basis of the bioinformatics analysis of molecular structure, we found Foxp3 contains the putative GSK-3 β phosphorylation motif as well. To address whether GSK-3 β promotes upper airway inflammation by regulating Foxp3 expression at the posttranslational

level, we examined expression of GSK-3 β and Foxp3 in polyp tissues of patients with CRSwNP and investigated the possible modulatory mechanism of GSK-3 β on Foxp3 expression *in vitro*.

We enrolled 52 patients with CRSwNP, 21 patients with chronic rhinosinusitis without nasal polyps (CRSsNP), and 27 healthy control subjects to address this issue. Clinical characteristics are listed in Table E1 in this article's Online Repository at www.jacionline.org (see the Methods section in this article's Online Repository at www.jacionline.org for further details). The study protocols were approved by ethics committee boards, and all subjects provided written informed consent. Sinonasal samples were collected, and expression of GSK-3 β and Foxp3 was examined by using immunohistochemical (IHC) staining, quantitative RT-PCR (qRT-PCR), flow cytometry, and Western blot analysis. The primer sequences used for qRT-PCR analysis are listed in Table E2 in this article's Online Repository at www.jacionline.org. Moreover, the regulatory effects of GSK-3 β on Foxp3 stability in cultured cell lines were examined (see the Methods section in this article's Online Repository).

Data were analyzed by using the Kruskal-Wallis *H* test followed by the unpaired Mann-Whitney *U* test or 1-way ANOVA followed by the Student *t* test. Correlations between the various parameters were assessed by using Spearman rank correlation analysis. A *P* value of less than .05 was defined as statistically significant.

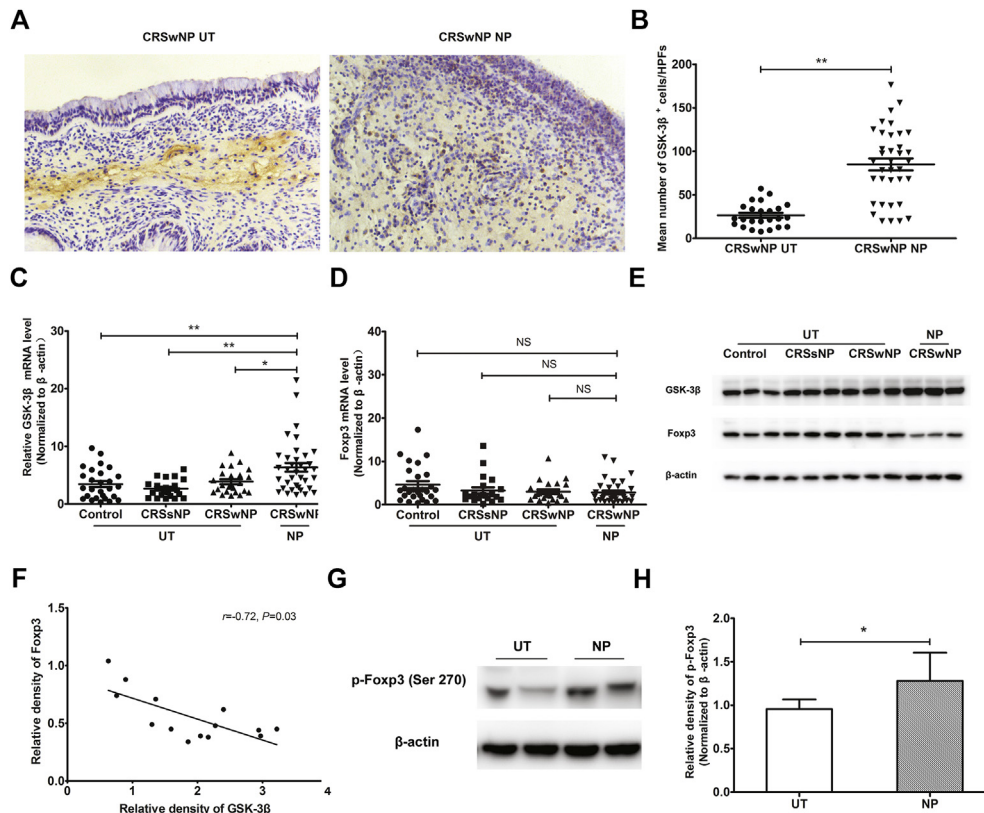


FIG 1. Expression of GSK-3 β and Foxp3 in sinonasal tissues. **A**, Immunoreactivity of GSK-3 β in sinonasal tissues (magnification $\times 200$). **B**, Mean number of GSK-3 β ⁺ cells in sinonasal tissues. **C** and **D**, mRNA level of GSK-3 β and Foxp3 in sinonasal tissues. **E**, Representative Western blot results of GSK-3 β and Foxp3 protein expression in sinonasal tissues. **F**, Relative protein levels of GSK-3 β and Foxp3 in NP tissues. **G**, Representative western blot results of p-Foxp3 (Ser 270) in NP tissues and UTs. **H**, Relative protein level of p-Foxp3 in NP tissues compared with UTs. NS, No significance. **P* < .05 and ***P* < .01.

Our results showed mRNA levels of T-box transcription factor, GATA-3, and retinoic acid-related orphan receptor C were significantly increased in polyp tissues than in healthy uncinate process tissues (UTs) of patients with CRSwNP, and mRNA levels of proinflammatory cytokines (TNF- α , IFN- γ , IL-1 β , IL-4, IL-5, IL-13, IL-6, IL-17A, and IL-22) were significantly increased in polyp tissues than those in healthy UTs of patients with CRSwNP (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org). As shown in Fig 1, A and B, nasal polyp (NP) tissues had significantly stronger expression of GSK-3 β than healthy UTs, as indicated by increased numbers of GSK-3 β ⁺ cells ($P < .01$), but had significantly weaker expression of Foxp3 than healthy UTs, as indicated by decreased numbers of Foxp3⁺ cells ($P < .01$, see Fig E3 in this article's Online Repository at www.jacionline.org). GSK-3 β mRNA and protein levels were significantly increased in NP tissues compared with those in 3 groups of healthy UTs ($P < .01$; Fig 1, C and E), whereas Foxp3 protein (but not mRNA) levels were significantly decreased in NP tissue compared with those in healthy UTs ($P < .01$; Fig 1, D and E, also see Fig E4 in this article's Online Repository at www.jacionline.org). In addition, a negative association of relative GSK-3 β and Foxp3 protein levels in patients with CRSwNP was observed ($r = -0.72$, $P < .05$; Fig 1, F), providing a clue that GSK-3 β might modulate Foxp3 production in NPs. In the following study we further observed that frequencies of Foxp3⁺CD4⁺ cells (Treg cells) were significantly lower in NP tissues than in healthy UTs ($P < .01$, see Fig E5 in this article's Online Repository at www.jacionline.org) and identified the colocalization of GSK-3 β and Foxp3 protein in Treg cells (Foxp3⁺CD4⁺ cells) in NP tissues, PBMCs, and healthy tonsils by using flow cytometric analysis (see Fig E6 in this article's Online Repository at www.jacionline.org).

Next, we examined whether and how GSK-3 β posttranslationally modulates Foxp3 production *in vitro*. After cotransfection of hemagglutinin-tagged GSK-3 β and FLAG-tagged Foxp3 into HEK293 T cells, we obtained the evidence that GSK-3 β physically interacts with Foxp3 by means of immunoprecipitation and Western blot analysis. Moreover, the Foxp3 protein level was significantly changed in response to GSK-3 β activator, inhibitor, or GSK-3 β small interfering RNA (siRNA) but not in the presence of the proteasome inhibitor MG132, suggesting GSK-3 β -mediated Foxp3 degradation is proteasome dependent (see Fig E7 in this article's Online Repository at www.jacionline.org). Consistently, significantly prolonged half-life of Foxp3 protein was found in HEK 293T cells transfected with FLAG-tagged Foxp3 with GSK-3 β siRNA. Using autoradiography and mass spectrometry, we obtained evidence that phosphorylated Foxp3 (p-Foxp3) was produced and that a motif in Foxp3 between residues 270 and 275 was phosphorylated in the presence of GSK-3 β . Moreover, by using an *in vitro* kinase assay, we identified Ser 270/274, but not Ser 271/275, as the phosphorylation site in Foxp3 protein in response to GSK-3 β . Conversely, when Ser 270/274 (A) or Ser 271/275 (B) was mutated, we only obtained p-Foxp3 in mutant B in the presence of GSK-3 β (see Fig E8 in this article's Online Repository at www.jacionline.org). Moreover, we found that the p-Foxp3 (Ser 270) level was increased in polyp tissues ($P < .01$; Fig 1, G and H) and observed a positive association of the relative protein levels of p-Foxp3 (Ser 270) and GSK-3 β (data not shown). Our findings thus demonstrated a critical link of GSK-3 β and Foxp3 in driving persistent inflammation in patients with CRSwNP.

Although increased GSK-3 β expression has been observed in NP tissues, its proinflammatory effect and the underlying mechanism have not been well documented. In this study we sought to investigate whether GSK-3 β promotes inflammatory response in NP tissues of patients with CRSwNP by targeting Foxp3 to subsequently affect the inhibitory function of Treg cells. It is well recognized that active GSK-3 β is able to phosphorylate a series of substrates with the consensus motif S/TX-X-X-Phospho-S/T and usually results in ubiquitin-mediated degradation.⁶ One of the most well-known substrates of GSK-3 β is β -catenin, which is an important regulator of the Wnt- β -catenin signaling pathway. Phosphorylation of β -catenin by GSK-3 β leads to the instability of β -catenin, reducing translocation of β -catenin into the nucleus, which can be associated with tumorigenesis.⁷ In this study we established the critical link between GSK-3 β and Foxp3 by means of tissue examination and *in vitro* analysis. Previously, Graham et al⁸ reported that GSK-3 β inhibition is able to potentiate the suppressive activity of Treg cells, but whether other forms of posttranslational modifications, such as phosphorylation, can affect Foxp3 stability and Treg cell function has been largely unknown.

In a recent study on rheumatoid arthritis, Nie et al⁹ investigated the relationship between Treg cells with pathogenic T cells at the site of chronic inflammation. They identified Ser 418 as a key phosphorylation site of Foxp3 that was responsible for the impaired Treg cell function in response to TNF- α . In this study we found a novel phosphorylation motif (Ser 270/274) of Foxp3, which can be recognized by proinflammatory GSK-3 β . Interestingly, when we examined the p-Foxp3 protein using specific antibody against Ser 270 in NP tissues, we found p-Foxp3 (Ser 270) and showed a positive association of GSK-3 β and p-Foxp3 (Ser 270) protein levels. Therefore these findings propose a molecular mechanism regulating inhibitory function of Treg cells, which might expand our knowledge on the pathogenesis of CRSwNP.

Xingmei Wu, MD^{a,*}

Sihua Wang, PhD^{b,*}

Miaomiao Han, PhD^{c,d,*}

Bin Song, PhD^e

Ping Ye, MD^b

Shanshan Ma, MD^e

Jun Li, MD^b

Fenghong Chen, MD^a

Geng Xu, PhD^a

Qingqing Ding, PhD^f

Jiahong Xia, PhD^b

Huabin Li, PhD^{a,c,d}

From ^athe Allergy Center, Otorhinolaryngology Hospital, the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China; ^bthe Department of Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; ^cthe Allergy Division, Department of Otolaryngology, Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; ^dthe Shanghai Key Laboratory of Translational Medicine on Ear and Nose Diseases, Shanghai, China; ^ethe Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China; and ^fthe Department of Pathology, University of Missouri, Columbia, Mo. E-mail: qingqing_ding@hotmail.com Or: jiahong.xia@hust.edu.cn Or: allergyli@163.com.

*These authors contributed equally to this work.

Supported by grants from the National Natural Science Foundations of China (nos. 81172512, 81271054, 81271056, and 81470673) and the Shanghai Committee of Science and Technology (no. 14DZ2260300).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Van Bruaene N, Pérez-Novo CA, Basinski TM, Van Zele T, Holtappels G, De Ruyck N, et al. T-cell regulation in chronic paranasal sinus disease. *J Allergy Clin Immunol* 2008;121:1435-41, e1-3.
2. Cao PP, Li HB, Wang BF, Wang SB, You XJ, Cui YH, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. *J Allergy Clin Immunol* 2009;124:478-84, e1-2.
3. Luo J. Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. *Cancer Lett* 2009;273:194-200.
4. Linke R, Pries R, Konnecke M, Bruchhage KL, Böske R, Gebhard M, et al. Glycogen synthase kinase 3 in chronic rhinosinusitis: two faces of a single enzyme in one disease. *Ann Allergy Asthma Immunol* 2013;110:101-6.
5. Bao Z, Lim S, Liao W, Lin Y, Thiernemann C, Leung BP, et al. Glycogen synthase kinase-3beta inhibition attenuates asthma in mice. *Am J Respir Crit Care Med* 2007;176:431-8.
6. Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, et al. Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. *Mol Cell* 2005;19:159-70.
7. Ding Q, He X, Hsu JM, Xia W, Chen CT, Li LY, et al. Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. *Mol Cell Biol* 2007;27:4006-17.
8. Graham JA, Fray M, de Haseth S, Lee KM, Lian MM, Chase CM, et al. Suppressive regulatory T cell activity is potentiated by glycogen synthase kinase 3β inhibition. *J Biol Chem* 2010;285:32852-9.
9. Nie H, Zheng Y, Li R, Guo TB, He D, Fang L, et al. Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-α in rheumatoid arthritis. *Nat Med* 2013;19:322-8.

<http://dx.doi.org/10.1016/j.jaci.2015.08.014>

METHODS

Patients and tissue samples

This study was approved by local ethics committee boards, and written informed consent was obtained from each subject. Fifty-two patients with CRSwNP, 21 patients with CRSsNP, and 27 healthy control subjects were recruited from the First Affiliated Hospital of Sun Yat-sen University. The diagnosis of CRSwNP and CRSsNP was based on history, clinical examination, nasal endoscopy, and computed tomography, which was in accordance with the "European position paper on rhinosinusitis and nasal polyps 2012."^{E1} The presence or absence of NPs was recorded basis on the results of nasal endoscopy. Patients with an established immunodeficiency, pregnancy, coagulation disorder, diagnosis of classic allergic fungal sinusitis, or Samter triad did not participate in the study. Healthy control subjects were patients with traumatic neuropathy undergoing endoscopic optical decompression and did not have sinus disease. None of the subjects used oral or nasal glucocorticoids or other medications (eg, antibiotics or antileukotrienes) for 4 weeks before sample collection. Details of subjects' characteristics are included in Table E1.

NP tissues and healthy UTs were collected during transnasal endoscopic surgery and used for qRT-PCR, IHC staining, and Western blot analysis, for example. Human tonsils were collected as healthy controls from patients with obstructive sleep apnea during pharyngeal surgery. In addition, single-cell suspensions isolated from sinonasal tissues and PBMCs were collected for flow cytometric analysis.

IHC staining

Paraffin sections (4 μ m) were used, and IHC staining was performed by using the peroxidase-labeled streptavidin-biotin technique. Briefly, 4- μ m-thick sections were deparaffinized by means of serial treatment. After blocking endogenous peroxidase in 3% hydrogen peroxide and 1% BSA, the sections were incubated overnight at 4°C in the presence of the primary antibodies GSK-3 β (1:250; Epitomics, Cambridge, Ill) and Foxp3 (1:50; Thermo Fisher, Rockford, Ill). Thereafter, each section was incubated with a secondary antibody and then with horseradish peroxidase-labeled streptavidin complex (Zhongshanjinjiao, Beijing, China). Peroxidase distribution was revealed by incubating the sections in a solution containing 3% 3,3'-diaminobenzidine tetrahydrochloride before counterstaining with hematoxylin and placement under cover slips. Negative control studies were performed by replacing the primary antibodies with normal IgG in appropriate concentrations.

The sections were blindly examined and coded with no awareness of the clinical data by using an Olympus CX40 Microscope (Olympus Optical, Hamburg, Germany). The number of positive cells was counted in 10 high-powered fields ($\times 400$ magnification) and averaged.

qRT-PCR

The mRNA level was detected through real-time qPCR analysis, as we described elsewhere.^{E2} Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. Reverse transcription was performed, and cDNA was synthesized from 2 μ g of total RNA by using an oligo (dT) 18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan) for quantitative PCR. RNA integrity and the success of the reverse transcription reaction were monitored by means of PCR amplification of β -actin transcripts. mRNA expression was determined with the ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, Calif) and SYBR Premix Taq (TAKARA). The primer sequences are listed in Table E2. PRISM samples contained $1 \times$ SYBR Green Master Mix, 1.5 μ L of 5 μ mol/L primers, and 25 ng of synthesized cDNA in a 25- μ L volume.

Reactions were heated to 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing extension at 60°C for 60 seconds. Melting curve analysis was used to control for amplification specificity. The mean threshold cycle values were normalized to β -actin, and the relative mRNA levels of target genes were analyzed with $2^{-\Delta\Delta C_t}$. Experiments were performed in triplicate for each data point.

Flow cytometric analysis

Flow cytometric analysis was performed, as we described elsewhere.^{E3} Briefly, Fresh NP tissues and healthy UTs were cut into pieces of approximately 1 to 2 mm and digested in incomplete RPMI-1640 medium with endotoxin-free collagenase I (2 mg/mL; Sigma-Aldrich, St Louis, Mo) and DNase I (5 U/mL, TAKARA) for approximately 30 to 60 minutes at 37°C. The digested fragments were filtered through a 100-mm cell nylon mesh (BD Biosciences, San Jose, Calif) to disperse the cells. Mononuclear cells were then obtained from the dispersed cells by using Ficoll-Hypaque (Haoyang Bio, Tianjin, China) density gradient centrifugation at 2200 rpm for 20 minutes and then washed twice with Hank solution. The cells were suspended at a concentration of 1×10^6 /mL in complete RPMI-1640 medium containing 10% FCS (Sijiqing, Hangzhou, China), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L L-glutamine (all from Gibco, Grand Island, NY). PBMCs of 6 healthy control subjects were isolated by using Ficoll-Hypaque density gradient centrifugation.

For CD4 staining, cells were incubated with the CD4 mAb (eBioscience, San Diego, Calif) at 4°C in the dark for 30 minutes. After fixation and permeabilization with Permeabilization/Fixation buffer (BD Biosciences), these cells were stained with conjugated mAbs for Foxp3 (eBioscience), GSK-3 β (R&D Systems, Minneapolis, Minn), or both, according to the protocol of the Permeabilization/Fixation Kit. Stained cells were washed twice before analysis with a FACS Aria II cytometer (BD Biosciences).

Cell culture and stable gene transfection

HEK293 T and HeLa cell lines were cultured in Dulbecco modified Eagle medium plus GlutaMAX (Invitrogen, Carlsbad, Calif) and 5% FBS, penicillin (10 U/mL), and streptomycin (10 mg/mL, Invitrogen) in a humidified incubator at 37°C. Primary T cells were cultured in RPMI 1640 with L-glutamine (Lonza, Versviere, Belgium) and 10% FBS, penicillin (10 U/mL), and streptomycin (10 mg/mL). pCGN-GSK-3 β wild-type cells were kindly provided by Dr J. R. Woodgett. Foxp3 was cloned from pGEX6P-1 Foxp3 and pCMV5-MYC Foxp3 (kindly provided by Dr Shimon Sakaguchi) into pCDNA3.1(+) containing a Flag tag. After validation by means of DNA sequencing, it was named pCDNA3.1 (+)-Foxp3. The pGEX6P-1 Foxp3 and pCDNA3.1(+)-Flag-Foxp3 mutants were constructed by using a site-directed mutagenesis kit (Invitrogen). The GSK-3 β siRNA plasmid was purchased from Upstate Biotechnology (Lake Placid, NY). These plasmids were transfected into HEK293 T or HeLa cells or isolated Treg cells, respectively, by using Lipofectamine 2000 (Invitrogen), and the stable transfectants were selected with blasticidin (10 μ g/mL). Cycloheximide and MG132 (10 μ mol/L for 10 hours) were purchased from Sigma-Aldrich. GSK-3 β activator (staurosporine; 50 μ mol/L) or GSK-3 β inhibitors (5 μ mol/L SB216763 or GSK-3 β siRNA; Sigma-Aldrich) were added to the culture system.

Western blotting and immunoprecipitation

Protein levels were determined by means of Western blot analysis, as described elsewhere.^{E4} Briefly, tissues or cells were collected, and total cellular protein was extracted in 100 μ L of RIPA lysis buffer at 4°C for 30 minutes. The protein concentration was determined by using the Bradford method. Samples containing 10 μ g of protein were boiled, subjected to SDS-PAGE in 10% Tris-glycine gels, and transferred electrophoretically to polyvinylidene fluoride membranes. The membranes were incubated with 5% fat-free skim milk in Tris-buffered solution containing 0.05% Tween 20 (1 hour at room temperature) and then incubated with the following antibodies: mouse anti-Foxp3 (eBioscience); rat anti-Foxp3, mouse and rabbit anti-GSK-3 β , anti-phospho-(Ser9)-GSK-3 β , rabbit anti-phosphorylated protein, anti- β -actin, and anti-GAPDH (Cell Signaling, Danvers, Mass); and anti-Flag and anti-hemagglutinin (Santa Cruz Biotechnology, Santa Cruz, Calif). The Pierce Co-Immunoprecipitation (Co-IP) Kit and the Ubiquitin Enrichment Kit (Thermo Fisher) were used according to the manufacturer's instructions. Specific antibodies against the phosphorylation

site of Foxp3 protein (anti-Ser270 and anti-Ser274) were generated by Beijing Protein Innovation (Beijing, China). The membranes were then incubated with a horseradish peroxidase-linked secondary antibody and finally processed with an ECL chemiluminescence reaction kit (Cell Signaling), followed by exposure on medical film. The band density of the target protein relative to β -actin or glyceraldehyde-3-phosphate dehydrogenase was quantified by using Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, Calif).

***In vitro* kinase assay, phosphorylation, and mass spectrum analysis**

For *in vitro* kinase assay and phosphorylation analysis, purified glutathione-S-transferase-Foxp3 fusion proteins were incubated with purified glutathione-S-transferase-GSK-3 β protein (Upstate Biotechnology, Lake Placid, NY) in the presence of 50 mmol/L ATP in a kinase buffer for 30 minutes at 30°C. The proteins were then separated by means of SDS-PAGE, followed by Western blot analysis with an anti-phosphorylated Ser/Thr antibody. Alternatively, proteins were visualized by means of

autoradiography. Mass spectrum analysis of *in vitro* GSK-3 β -p-Foxp3 protein was performed as described elsewhere.^{E5}

REFERENCES

- E1. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012;50:1-12.
- E2. Wen W, Liu W, Zhang L, Bai J, Fan Y, Xia W, et al. Increased neutrophilia in nasal polyps reduces the response to oral corticosteroid therapy. *J Allergy Clin Immunol* 2012;129:1522-8.e5.
- E3. Xiao L, Wei Y, Zhang YN, Luo X, Yang BY, Yu SF, et al. Increased IL-21 expression in chronic rhinosinusitis with nasal polyps. *Clin Exp Allergy* 2015; 45:404-13.
- E4. Ding Q, He X, Hsu JM, Xia W, Chen CT, Li LY, et al. Degradation of Mcl-1 by β -TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. *Mol Cell Biol* 2007;27:4006-17.
- E5. Kalkum M, Lyon GJ, Chait BT. Detection of secreted peptides by using hypothesis-driven multistage mass spectrometry. *Proc Natl Acad Sci U S A* 2003;100:2795-800.

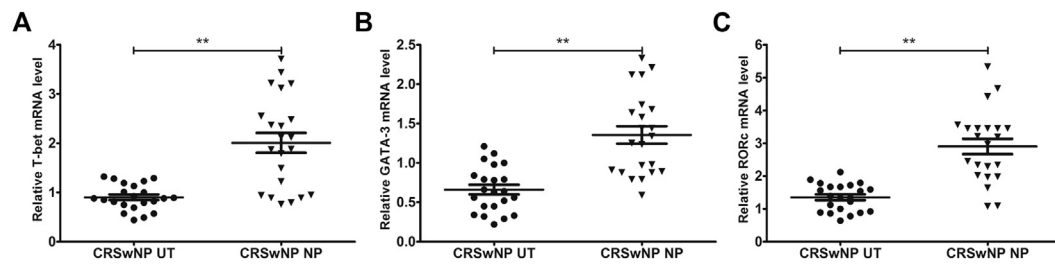


FIG E1. mRNA expression of T_H cell subsets in sinonasal tissues of patients with CRSwNP. mRNA levels of T-box transcription factor (*T-bet*; **A**), GATA-3 (**B**), and retinoic acid–related orphan receptor C (*RORc*; **C**) in NP tissues were significantly increased compared with those in UTs of patients with CRSwNP ($n = 22$). ** $P < .01$.

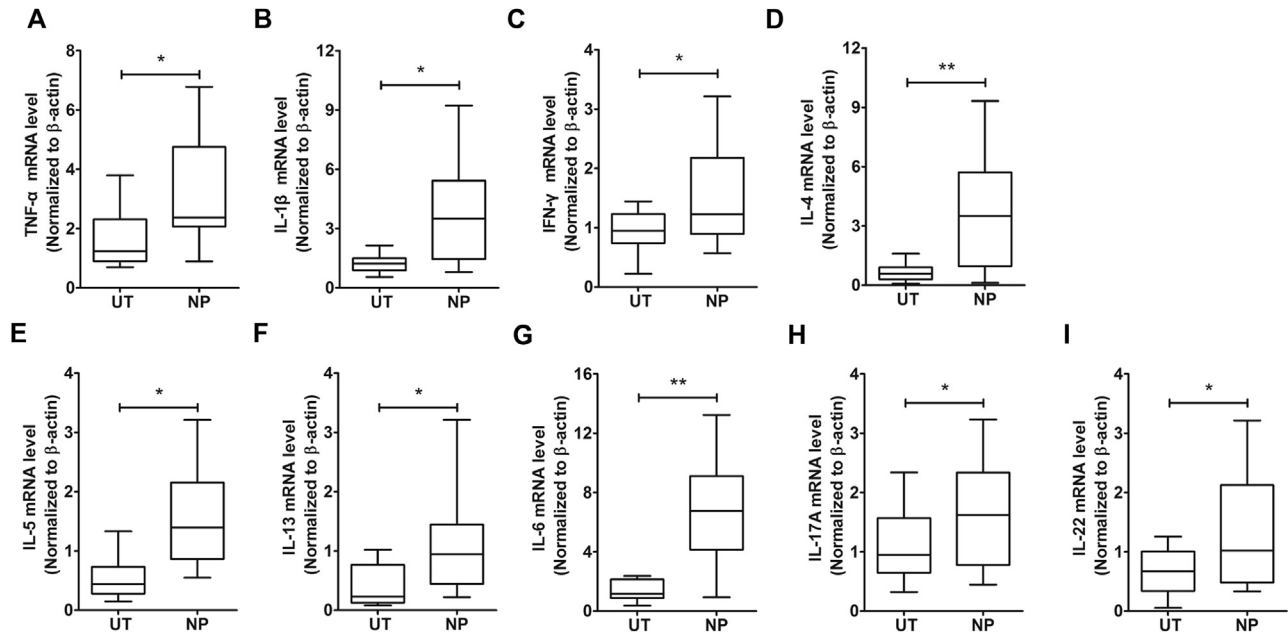


FIG E2. mRNA expression of inflammatory cytokines in sinonasal tissues of patients with CRSwNP. mRNA levels of TNF- α (A), IL-1 β (B), IFN- γ (C), IL-4 (D), IL-5 (E), IL-13 (F), IL-6 (G), IL-17A (H), and IL-22 (I) in NP tissues were significantly increased compared with those in UTs in patients with CRSwNP ($n = 22$) are shown. * $P < .05$ and ** $P < .01$.

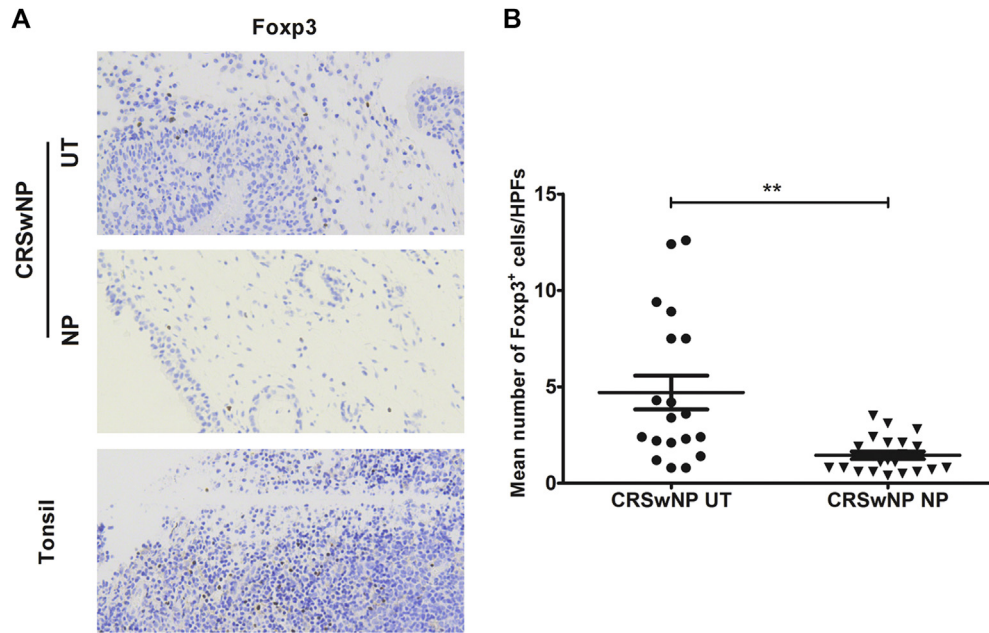


FIG E3. Expression of Foxp3 in sinonasal tissues of patients with CRSwNP. **A**, Immunoreactivity of Foxp3 in NP tissues and UTs of patients with CRSwNP and tonsils of healthy control subjects (as a positive control, magnification $\times 200$). **B**, Mean number of Foxp3⁺ cells was significantly decreased in NP tissues compared with UTs. ** $P < .01$.

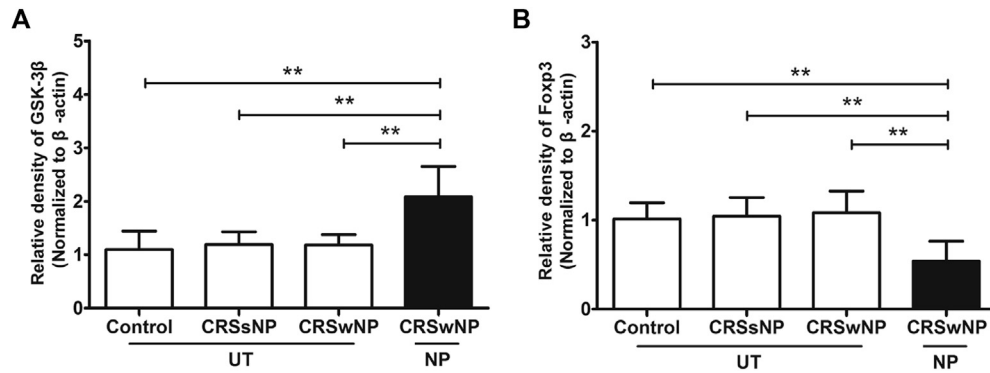


FIG E4. Relative protein levels of GSK-3 β and Foxp3 in sinonasal tissues of patients with CRSwNP. **A**, Relative protein levels of GSK-3 β were significantly increased in NP tissues compared with those in 3 other healthy UT groups. **B**, Relative protein levels of Foxp3 were significantly decreased in NP tissues compared with those in the other 3 healthy UT groups ($n = 14$). $**P < .01$.

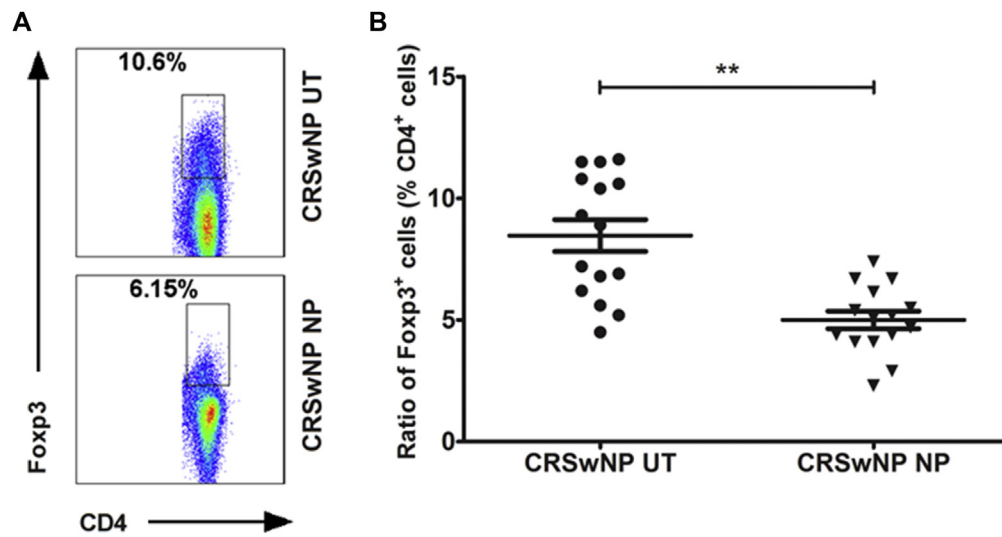


FIG E5. Ratio of Foxp3⁺CD4⁺ cells in sinonasal tissues. **A**, Representative flow cytometric results of Foxp3⁺CD4⁺ cells in NP tissues and UTs of patients with CRSwNP. **B**, Frequencies of Foxp3⁺CD4⁺ cells were significantly decreased in NP tissues compared with those in UTs. ** $P < .01$.

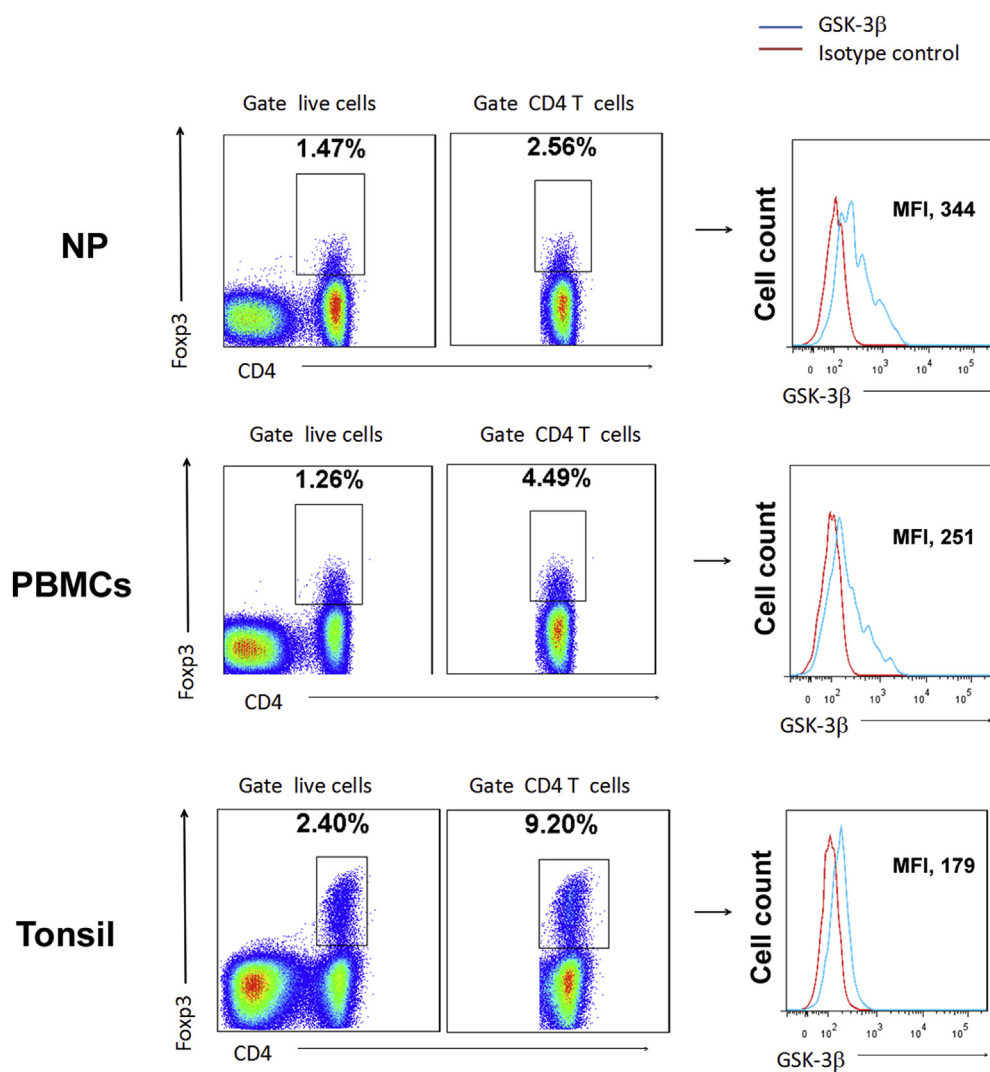


FIG E6. Colocalization of GSK-3 β and Foxp3 protein in Treg cells. Representative flow cytometric results of GSK-3 β ⁺ Treg cells (Foxp3⁺ CD4⁺) in NP tissues of patients with CRSwNP, as well as in PBMCs and tonsils of healthy control subjects, were shown. *MFI*, Mean fluorescence index.

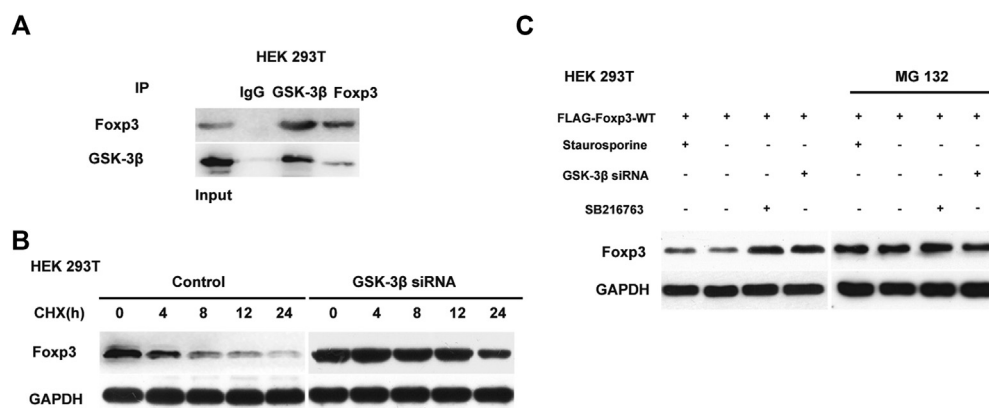


FIG E7. Interaction of GSK-3 β and Foxp3 protein *in vitro*. **A**, GSK-3 β physically interacts with Foxp3. **B**, GSK-3 β promotes the instability of the Foxp3 protein level. Inhibition of GSK-3 β with specific siRNA significantly prolonged the half-life of Foxp3 protein in HEK 293T cells. **C**, GSK-3 β modulates Foxp3 protein levels. *IP*, Immunoprecipitation.

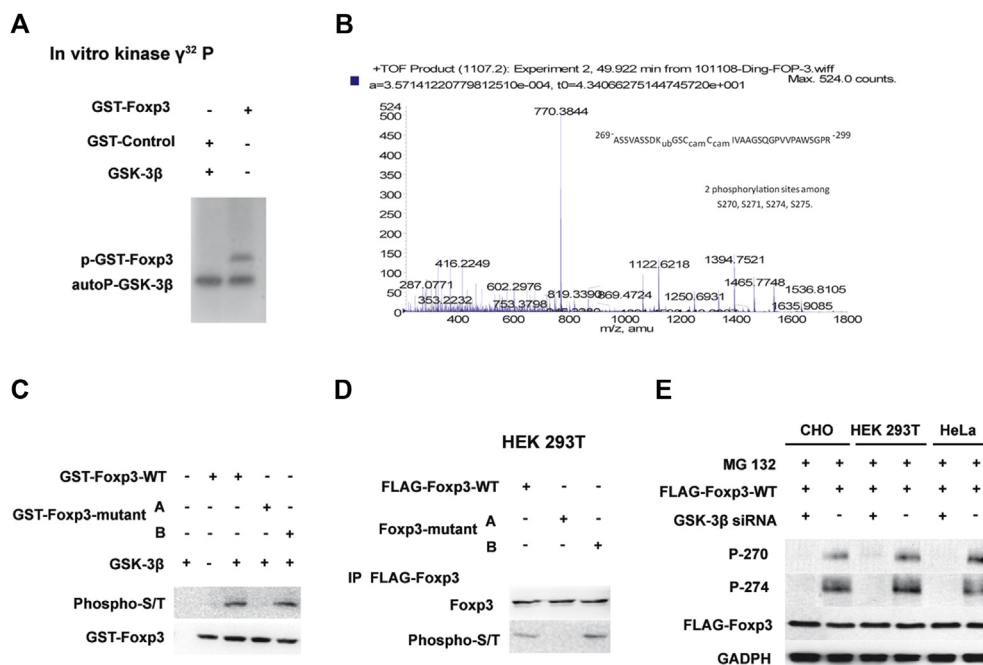


FIG E8. GSK-3 β phosphorylates Foxp3 by targeting a motif of Ser 270/274. **A**, GSK-3 β phosphorylates Foxp3. **B**, Mass spectrometric analysis revealed that a motif in Foxp3 between residues 270 and 275 can be phosphorylated by GSK-3 β . **C**, GSK-3 β phosphorylated Ser 270/274 in Foxp3 protein *in vitro*. **D**, Mutated Ser 270/274 cannot be phosphorylated *in vivo*. FLAG-Foxp3 wild-type or mutant A (270/274) or B (271/275) was transfected in HEK 293T cells. **E**, GSK-3 β phosphorylated Ser 270/274 in Foxp3 protein *in vivo*.

TABLE E1. Subjects' characteristics

Characteristics	Healthy (UT)	CRSsNP (UT)	CRSwNP (UT)	CRSwNP (NP tissue)
Subjects (no.)	27	21	52	
Sex (M/F)	14/13	12/9	32/20	
Age (y), range	20-42	23-55	22-65	
Atopy	NA	n = 7	n = 22	
Asthma history	NA	n = 9	n = 15	
AERD history	NA	NA	NA	
Sinus surgery history	NA	NA	n = 6	
Methodologies used				
IHC	n = 27 (14 M)	n = 21 (12 M)	n = 24 (14 M)	n = 36 (19 M)
qRT-PCR	n = 27 (14 M)	n = 21 (12 M)	n = 24 (14 M)	n = 36 (19 M)
Western blot	n = 12 (8 M)	n = 12 (7 M)	n = 14 (9 M)	n = 14 (9 M)
Flow cytometry	NA	NA	n = 21 (15 M)	n = 21 (15 M)
PBMC assay	n = 10 (6 M)	NA	NA	NA

AERD, Aspirin-exacerbated respiratory disease; *F*, female; *M*, male; *NA*, not applicable.

TABLE E2. Primer sequences used for qPCR analysis

Target genes	Forward 5'-3'	Reverse 5'-3'
GSK-3 β	CGT CAG GAA CAG GAC ATT TCA	ACA AGC TTC CAG TGG TGT TAG
T-bet	CGG CTG CAT ATC GTT GAG GT	GCA GTC ACG GCA ATG AAC TG
GATA-3	TCA TTA AGC CCA AGC GAA GG	GTC CCC ATT GGC ATT CCT C
RORc	CAT GTC CCG AGA TGC TGT CA	GAC CAC TGG TTC CTG TTG CTG
Foxp3	GAA ACA GCA CAT TCC CAG AGT TC	ATG GCC CAG CGG ATG AG
TNF- α	TCT TCT CGA ACC CCG AGT GA	GGA GCT GCC CCT CAG CTT
IL-1 β	CGA ATC TCC GAC CAC CAC TAC	TCC ATG GCC ACA ACA ACT GA
IFN- γ	TGC AGG TCA TTC AGA TGT AGC GGA	TGT CTT CCT TGA TGG TCT CCA CAC TC
IL-4	TTG CTG CCT CCA AGA ACA CAA CTG	TTC CTG TCG AGC CGT TTC AGG AAT
IL-5	TGC TGA TAG CCA ATG AGA CTC TG	TTT CCA CAG TAC CCC CTT GC
IL-6	TGT AGC CGC CCC ACA CA	GGA TGT ACC GAA TTT GTT TGT CAA
IL-13	TGG TCA ACA TCA CCC AGA ACC AGA	AGC CTG ACA CGT TGA TCA GGG ATT
IL-17A	CAA CCG ATC CAC CTC ACC	AGC CCA CGG ACA CCA GTA
IL-22	CCA GCT GCC TCC TTC TCT TG	TGC GGT TGG TGA TAT AGG GC
β -Actin	AAG ATG ACC CAG ATC ATG TTT GAG ACC	AGC CAG GTC CAG ACG CAG GAT

RORc, Retinoic acid-related orphan receptor C; *T-bet*, T-box transcription factor.