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The Key Regulator for Language and Speech Development, FOXP2, is a Novel Substrate for SUMOylation

Leslie J. Meredith,[1] Chiung-Min Wang,[1] Leticia Nascimento,[1] Runhua Liu,[2] Lizhong Wang,[2] and Wei-Hsiung Yang[1]*

[1]*Department of Biomedical Sciences, Mercer University School of Medicine, Savannah, Georgia 30404*

[2]*Department of Genetics and Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294*

ABSTRACT

Transcription factor forkhead box protein P2 (FOXP2) plays an essential role in the development of language and speech. However, the transcriptional activity of FOXP2 regulated by the post-translational modifications remains unknown. Here, we demonstrated that FOXP2 is clearly defined as a SUMO target protein at the cellular levels as FOXP2 is covalently modified by both SUMO1 and SUMO3. Furthermore, SUMOylation of FOXP2 was significantly decreased by SENP2 (a specific SUMOylation protease). We further showed that FOXP2 is selectively SUMOylated in vivo on a phylogenetically conserved lysine 674 but the SUMOylation does not alter subcellular localization and stability of FOXP2. Interestingly, we observed that human etiological FOXP2 R553H mutation robustly reduces its SUMOylation potential as compared to wild-type FOXP2. In addition, the acidic residues downstream the core SUMO motif on FOXP2 are required for its full SUMOylation capacity. Finally, our functional analysis using reporter gene assays showed that SUMOylation may modulate transcriptional activity of FOXP2 in regulating downstream target genes (*DISC1*,*SRPX2*, and *MiR200c*). Altogether, we provide the first evidence that FOXP2 is a substrate for SUMOylation and SUMOylation of FOXP2 plays a functional role in regulating its transcriptional activity. J. Cell. Biochem. 117: 426–438, 2016.

KEY WORDS: FOXP2; SUMOylation; TRANSCRIPTIONAL ACTIVITY

Forkhead box protein P2 (FOXP2), an 80-kDa (715 amino acid residues) protein, belongs to the forkhead-box/winged-helix transcription factor family. *FOXP2* gene (OMIM#605317) is the first gene implicated in a severe autosomal-dominant language and speech disorder, called developmental verbal dyspraxia (DVD, OMIM#602081), mainly from the series of studies of a multigenerational British family known as the KE family [Hurst et al., 1990; Lai et al., 2000, 2001]. FOXP2, is expressed in several tissues and many areas of the brain, especially in fetal brain during neuronal differentiation, suggesting that FOXP2 is important for the brain development and maturation [Ferland et al., 2003; Lai et al., 2003; Hisaoka et al., 2010; Reimers-Kipping et al., 2011]. Accumulated data suggest that two functional copies of FOXP2 are necessary for normal language and speech development, suggesting that haploinsufficiency is the likely etiology [Hurst et al., 1990]. Moreover, FOXP1, a close paralog of FOXP2, is necessary and sufficient for motor neuron diversity and coordinated actions [Dasen et al., 2008; Rousso et al., 2008], further suggesting that FOXP proteins of family are critical for brain and language development. FOXP2 can form homodimers and heterodimers with FOXP1 and FOXP4. Functional studies suggest that the leucine-zipper region of FOXP2 is required for dimerization and essential for transcriptional repression [Vernes et al., 2006]. As a transcription factor, FOXP2 can regulate a variety of genes including *DISC1* [Walker et al., 2012], *CNTNAP2* [Vernes et al., 2008], and *SRPX2/μPAR* [Roll et al., 2010], which most of them are associated with speech and language development. While the wild-type (WT) of FOXP2 is predominantly localized in the nucleus, significant amounts of human etiological FOXP2 R553H point mutation are found in the cytoplasm [Vernes et al., 2006]. Human etiological R553H point mutation in the forkhead domain of

Abbreviations: DISC1, disrupted in schizophrenia 1 protein; FOXP2, forkhead box protein P2; SRPX2, sushi repeat-containing protein SRPX2; SENP, sentrin-specific protease; SUMO, small ubiquitin-like modifier.

Leslie J. Meredith and Chiung-Min Wang have contributed equally to this work.

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*Correspondence to: Dr. Wei-Hsiung Yang, Department of Biomedical Sciences, Mercer University School of Medicine, Savannah, GA 31404. E-mail: yang_w@mercer.edu

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FOXP2 has been characterized in patients with speech-language disorder 1 (SPCH1, OMIM#602081) with a severe orofacial dyspraxia resulting in largely incomprehensible speech [Hurst et al., 1990; Lai et al., 2000, 2001]. FOXP2 also regulates neural development and outgrowth by down-regulating *CNTNAP2* gene, which is essential for cortical development and neuroblast migration [Vernes et al., 2011; Tsui et al., 2013]. Recent studies have demonstrated that FOXP2 also regulates lung development by targeting surfactant protein C gene [Zhou et al., 2008; Yang et al., 2010]. Although FOXP2 is a neuronal transcription factor, recent studies have demonstrated that FOXP2 is involved in cancer development and progression, such as breast [Cuiffo et al., 2014], prostate [Stumm et al., 2013], and ovarian [Ying et al., 2014] cancers. Overall, these results suggest that FOXP2 has a wide range of functional roles in neural coordination, synapse formation, brain maturation, language development, as well as cancer progression, but the underlying molecular mechanism is still poorly understood.

Post-translational modifications (PTMs) of proteins are enzymatic steps in protein biosynthesis and are crucial for normal physiological functions in cells. Currently, there are more than 20 types of PTMs. Among them, the small ubiquitin-related modifier (SUMO) family, which is highly conserved from yeast to humans and a widely used reversible modification system, has emerged as an molecular switch for regulating various cellular pathways and biochemical processes, including cancer development and metastasis [Bellail et al., 2014], cell cycle regulation [Schimmel et al., 2014], nucleocytoplasmic translocalization [Sun et al., 2014], protein targeting and stability [Belaguli et al., 2012], signal transduction, and transcriptional regulation [Wang et al., 2014a]. In mammalian cells, four SUMO paralogs (SUMO1 to -4, approximately 11 kDa proteins in size) are encoded by four distinct *SUMO* genes. SUMO1 shares only approximately 46% identity to either the closely-related SUMO2 or SUMO3. In contrast to SUMO1, both SUMO2 and SUMO3 contain a conserved consensus SUMOylation site in their N-terminal regions, suggesting that both SUMO2 and SUMO3 are capable to form poly-SUMO chains [Tatham et al., 2001]. Currently, the biological role of SUMO4 has been associated with immune system and diabetes development [Song et al., 2012]; however, the biological significance of SUMO4 is still not well understood. Recent proteomic and developmental studies have demonstrated that modifications by SUMO1/2/3 may regulate both unique and redundant biological pathways and processes [Wang et al., 2014b].

Despite limited sequence identity (approximately 20% sequence identity), SUMO proteins share with ubiquitin a common three-dimensional (3D) structure and use a similar conjugation mechanism, a tightly enzyme-controlled cycle of conjugation and deconjugation. Newly translated SUMO proteins are processed by SUMO-specific proteases (SENPs) to remove C-terminal residues in SUMO and to expose a conserved di-glycine motif. After this initial cleavage step, SUMO is then activated at its C-terminus by the heterodimeric E1-activating enzyme SAE1/SAE2 in an ATP-dependent manner. The thioester-linked SUMO is then transferred to the SUMO-specific E2-conjugating enzyme UBE2I, which in turn recognizes specific substrates and catalyzes the formation of an isopeptide bond between the lysine residue of target protein and the glycine residue of SUMO. This conjugation step can be further mediated and facilitated by SUMO E3 ligases such as RanBP2 and the PIAS family of proteins, which determine substrate specificity and catalyze the transfer of SUMO from UBE2I [Pichler et al., 2002; Schmidt and Müller, 2002]. Covalent modification of proteins by SUMO is short-lived and reversible through action of the SENP family of isopeptidases. Even though the 3D-structure and enzymological cycle of conjugation of SUMO are very similar to those of ubiquitin, the biological functions of SUMOylation are much different from ubiquitination [Martin et al., 2007]. Moreover, unlike ubiquitination, SUMOylation of certain proteins does not target substrates to the proteasomal degradation [Martin et al., 2007; Wilkinson and Henley, 2010]. From the accumulated data, the most common group of SUMO substrates are transcription factors and co-factors, whose transcriptional activities are either down-regulated [Oshima et al., 2009; Wang et al., 2014a] or up-regulated [Kishi et al., 2003] by SUMO modification. Overall, the cellular SUMOylation system affects the function of numerous nuclear proteins, transcription factors, and cell-cycle regulators, and signal transduction mediators. Thus, understanding the regulation of protein SUMOylation is pivotal for various biological processes and human diseases such as transcriptional regulation and cancer development, and elucidating the significance of SUMOylation may identify useful strategies for developing therapeutic agents.

Since FOXP2 is a transcription factor essential for language and speech development and the majority of the transcription factors are the substrates of post-translational modifications, we hypothesized that FOXP2 is a substrate for modification by SUMO proteins, and SUMOylation of FOXP2 has significant impact on FOXP2 activity. In this report, we identify FOXP2 as a target for modification by the common SUMO machinery and provide evidence demonstrating that SUMOylation of FOXP2 plays a functional role in regulating its downstream target genes (*DISC1*, *SRPX2*, and *MiR200c*).

MATERIALS AND METHODS

REAGENTS

All cell culture reagents and protein A/G-agarose were purchased from Life Technologies/Thermo Scientific (Grand Island, NY). Antibodies against FOXP2, Tubulin, and Lamin A/C were purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). Antibodies against HA were purchased from Origene (Rockville, MD). Antibodies against β-Actin were purchased from Sigma (St. Louis, MO). Luciferase activity was measured using the Dual Luciferase Assay System (Promega, Madison, WI). Ni-NTA agarose was purchased from QIAGEN (Valencia, CA). Ginkgolic acid (15:1) was purchased from Nacalai USA (San Diego, CA).

DNA CONSTRUCTS

Human FOXP2 plasmid (pcDNA4.1-HIS-*FOXP2*) was kindly provided by Dr. Simon Fisher (University of Oxford, UK). Human pcDNA4.1-*FOXP2* (without HIS tag) plasmid was constructed by removal of the HIS tag sequence of pcDNA4.1-HIS-*FOXP2* plasmid in our laboratory. *DISC1* promoter luciferase plasmid (1Kb upstream the transcription start site in pGL4.1) was kindly provided by Dr. Kathryn Evans (University of Edinburgh, UK) and *SRPX2* promoter

luciferase plasmid (1.4Kb upstream the transcription start site in pGL3) was kindly provided by Dr. Pierre Szepetowski (Mediterranean Institute of Neurobiology (INMED) INSERM UMR_S901 Parc scientifique de Luminy, France). HA-*SUMO1*-pcDNA3, HA-*SUMO3*-pcDNA3, HIS-HA-*SUMO3*-pcDNA3, and FLAG-*SENP2*-pcDNA6 plasmids were previously established in our laboratory [Wang et al., 2014a]. Human pCMV6-*FOXP2-GFP* expression plasmid was purchased from Origene (Rockville, MD). pCMV6-K674R *FOXP2-GFP* and pCMV6-R553H *FOXP2-GFP* expression plasmids were generated by PCR-based mutagenesis from pCMV6-*FOXP2-GFP* plasmid. *MiR-200b/a/429* and *MiR-200c/141* promoter luciferase plasmids (both in pGL3) were kindly provided by Dr. Tewari (Fred Hutchison Cancer Research Center, Seattle). All constructs were verified by nucleotide sequencing.

CELL CULTURE AND TRANSFECTION

MCF7, HEK293, and SH-SY5Y cells were purchased from the American Type Culture Collection (Manassas, VA). MCF7 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal bovine serum and antibiotics (GIBCO) in humidified air containing 5% CO_2, at 37°C. SH-SY5Y cells were maintained in a 1:1 mixture of ATCC-formulated Eagle's minimum essential medium and F12 medium supplemented with 10% fetal bovine serum and antibiotics in humidified air containing 5% CO_2, at 37°C. After incubation, the cells were transfected with plasmids as indicated in each experiment using Fugene HD Transfection Reagent (Roche, Indianapolis, IN). Approximately 48 h after transfection, the cells were harvested. Luciferase activity was measured and normalized with Renilla activity. All experiments were performed three times in triplicate.

IMMUNOPRECIPITATION ASSAY AND Ni^{2+}–BEAD PULL-DOWN ASSAY

MCF7 or HEK293 cells (2×10^6) were seeded onto 10-cm plates. Approximately 24 h after transient transfection, cells were harvested and lysed in lysis buffer (40 mM HEPES, 120 mM sodium chloride, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM EDTA, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1% Triton X-100) containing protease inhibitor cocktail (Sigma), followed by rotation for 1 h at 4°C to solubilize proteins. Soluble protein was collected and immunoprecipitated with the indicated antibody overnight. Protein A/G agarose beads were added to protein lysates for 2 h at 4°C in the cold room. Beads were centrifuged and washed at least three times with lysis buffer. For Ni^{2+}-bead pull-down assays, Ni^{2+}-NTA agarose was used to precipitate HIS-tagged FOXP2 or HIS-tagged SUMO3 from cell lysates. Proteins were eluted by boiling in 50 μl of 2× Laemmli sample buffer, resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and processed for immunoblotting as described below.

IMMUNOBLOTTING

Protein lysates were allowed to rotate at 4°C for 30 min, and protein contents of the high-speed supernatant were determined using the BCA™ Protein Assay kit assay (Pierce/Thermo Scientific, Rockford, IL). Equivalent quantities of protein (20–50 μg) were resolved on SDS-PAGE gels, transferred to PolyScreen PVDF transfer membrane (PerkinElmer, Waltham, MA), and immunoblotted with specific antibodies. Results were visualized using the Supersignal West Dura Extended Duration Substrate kit (Pierce/Thermo Scientific). Band intensity was quantified by ImageJ program.

IN VIVO SUMOylation ASSAYS

The in vivo SUMOylation assay was carried out as previously described [Yang et al., 2009]. Briefly, MCF7 or HEK293 cells (2×10^6) were seeded in 10 cm plates and 24, Plate 24 h later were transfected with indicated HIS-*FOXP2* and HA-*SUMO1* or HA-*SUMO3* expression vectors. After 48 h, cells were harvested in 700 μl lysis buffer (500 mM NaCl, 10 mM imidazole, 45 mM Na_2HPO_4, 5 mM Na_2H2PO_4, 8 M urea, pH 8.0) containing complete protease inhibitors without EDTA (1 tablet/10 ml; Roche) and sonicated. Lysates were cleared and incubated with 100 μl of 50% Ni^{2+}-NTA agarose (QIAGEN) at room temperature for 60 min on a rotator. The resin was washed 3 times in wash buffer 1 (400 mM NaCl, 10 mM imidazole, 17.6 mM Na_2HPO_4, 32.4 mM $Na_2H_2PO_4$, 8 M urea, pH 6.75), washed 3 times in wash buffer 2 (150 mM NaCl, 10 mM imidazole, 17.6 mM Na_2HPO_4, 32.4 mM $Na_2H_2PO_4$, pH 6.75). Samples were resuspended in 2× EDTA SDS-PAGE sample buffer (with 150 mM imidazole). Samples (20 μl) were resolved by 8% SDS-PAGE and processed for immunoblotting using anti-FOXP2, anti-HA primary antibody. Images were captured in a Kodak Image Station 440 CF using Super Signal West Femto substrates (Pierce/Thermo Scientific).

CELLULAR LOCALIZATION STUDY

MCF7 cells cultured on 10-cm plates were transfected with WT, K674R, or R553H *FOXP2* expressing plasmids for 2 days. Nuclear and cytoplasmic fractions of cells were separately as previously described [Yang et al., 2009] and subjected to anti-FOXP2, anti-Lamin A/C, anti-Tubulin, and anti-β Actin immunoblotting. MCF7 cells cultured on 6-well plate were transfected with WT *FOXP2-GFP*, or K674R *FOXP2-GFP*, or R553H *FOXP2-GFP* expressing plasmids for 2 days. Cells were counterstained with DAPI and images were obtained with a fluorescence microscope (Olympus DP72) and camera.

CYCLOHEXIMIDE CHASE ASSAYS

MCF7 cells were treated with 50 μg/ml cycloheximide 30 h after transfections. Cells were harvested at indicated time points and cell lysates were subjected to western blotting.

STATISTICAL ANALYSES

All experiments were performed at least 3 times. Statistical analyses were performed using the Student's t test or a one-way ANOVA when more than two groups were compared. After the ANOVA analysis, the post-hoc multiple comparisons were performed by using the Tukey honestly significant difference (HSD) test to determine the statistical difference from each other among subgroups. For each test, P values of <0.05 was considered significant.

RESULTS

FOXP2 IS A SUBSTRATE FOR MODIFICATION BY SUMO

Human FOXP2 harbors one evolutionarily conserved motif at its C-terminal domain that conforms to the SUMOylation consensus

(Fig. 1A). To examine whether FOXP2 can be modified by SUMO proteins in mammalian cells, MCF7 cells were transiently transfected with *FOXP2* and HIS-tagged WT *SUMO3* or AA mutant *SUMO3* expression plasmids. The SUMOylated proteins were purified using Ni^{2+}-NTA resins under denaturing conditions. The SUMOylated FOXP2 was detected by using anti-FOXP2 antibody. Our data showed that the SUMOylated FOXP2 was clearly detected with a slower migrating band with molecular weight greater than 115 kDa in SDS-PAGE analysis when WT *SUMO3* was co-transfected (Fig. 1B). In contrast, AA mutant *SUMO3*, which the di-glycine residues of SUMO3 were mutated to di-alanine residues, completely lost the ability to enhance FOXP2 SUMOylation (Fig. 1B). These results clearly indicate that band shift of FOXP2 was indeed due to the covalent conjugation of SUMO3. A previous study has demonstrated that ginkgolic acid inhibits protein SUMOylation by blocking formation of the E1-SUMO intermediate [Fukuda et al., 2009]. To further confirm the data in Figure 1B, we next tested whether SUMOylation of FOXP2 can be inhibited by ginkgolic acid. We treated the cells with different concentrations of ginkgolic acid after transfection of SUMO3 into MCF7 cells. As shown in Figure 1C, SUMOylated FOXP2 was significantly reduced (~50%) after ginkgolic acid treatment (100 μM), suggesting that FOXP2 is indeed a substrate for SUMO3 modification.

Since SUMO1 shares only approximately 46% identity to either the closely-related SUMO2 or SUMO3, we next tested whether FOXP2 can be SUMOylated by SUMO1 in mammalian cells. First, we confirmed SUMOylated FOXP2 by SUMO3 using different tag system. MCF7 cells were transiently transfected with HIS-tagged *FOXP2* expression plasmids with or without HA-tagged *SUMO3* (WT or AA mutant) expression plasmids. Western blot analysis (Fig. 2A) of the FOXP2 preparations by Ni^{2+} chelate chromatography under denaturing condition revealed that a slowly migrating species (about 115 kDa) was detected in cells expressing WT FOXP2 alone (in long exposure image), suggesting HIS-tagged FOXP2 is able to be

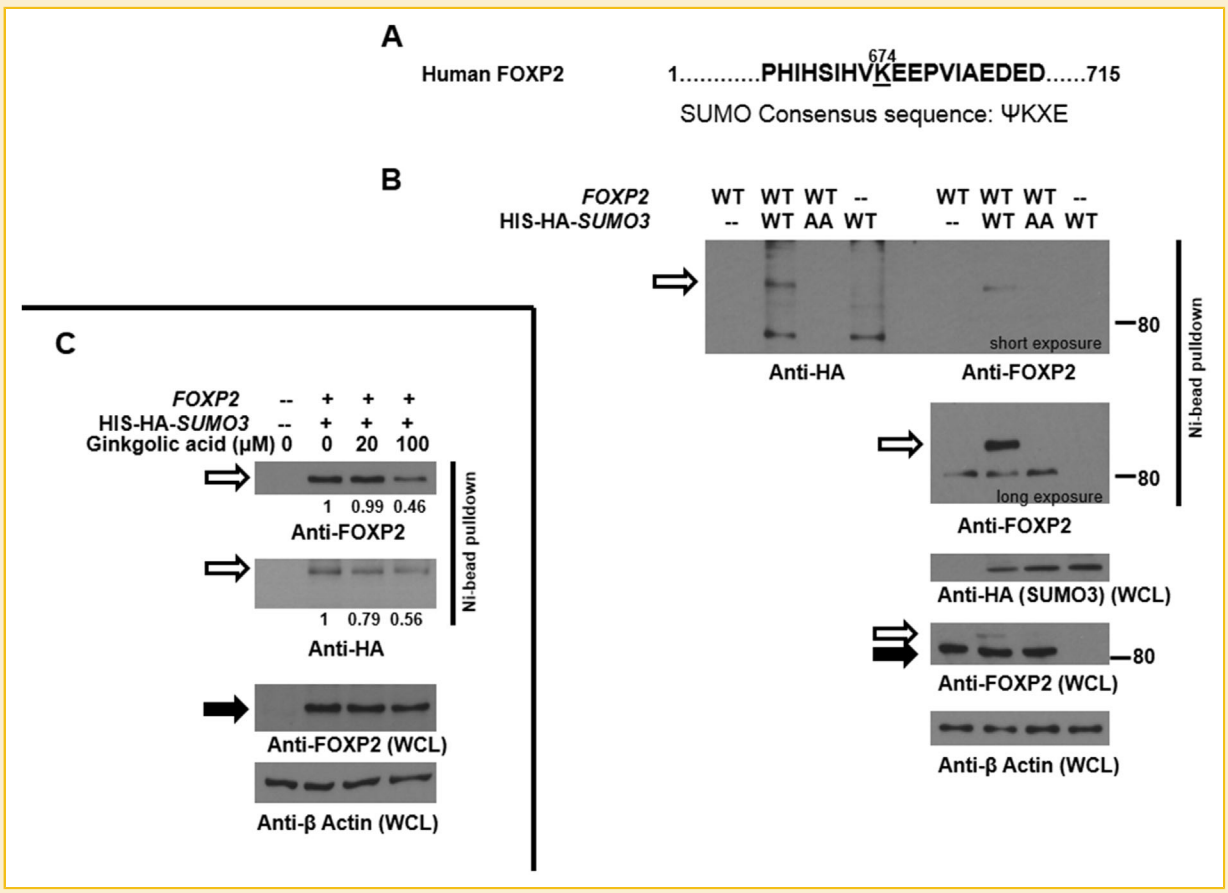

Fig. 1. FOXP2 can be SUMOylated. A: Sequence alignment of the human FOXP2 protein showing the region that contains the potential SUMO site (K674). B: SUMOylation of FOXP2 in mammalian cells. MCF7 cells were transiently transfected with 2 μg WT FOXP2 and 2 μg HIS-HA-SUMO3 (WT or AA mutant) expression vectors as indicated. After 48 h, cells were harvested and the cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-HA and anti-FOXP2 immunoblotting. Whole cell lysates (WCL) were subjected to anti-HA, anti-FOXP2, or anti-β-Actin immunoblotting for SUMO3, FOXP2, or β-Actin expression, respectively. The empty arrows indicate SUMOylated FOXP2. The solid arrows indicate non-SUMOylated FOXP2. C: Ginkgolic acid reduced FOXP2 SUMOylation. MCF7 cells were transiently transfected with 2 μg WT FOXP2 and 2 μg HIS-HA-SUMO3 expression vectors. After 42 h, cells were treated with various concentrations of ginkgolic acid (0, 20, 100 μM) for 6 h. Cells were harvested and the cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-HA and anti-FOXP2 immunoblotting. Whole cell lysates (WCL) were subjected to anti-FOXP2 or anti-β-Actin immunoblotting for FOXP2 or β-Actin expression, respectively. The empty arrows indicate SUMOylated FOXP2. The solid arrows indicate non-SUMOylated FOXP2. Experiments were performed three times with similar results.

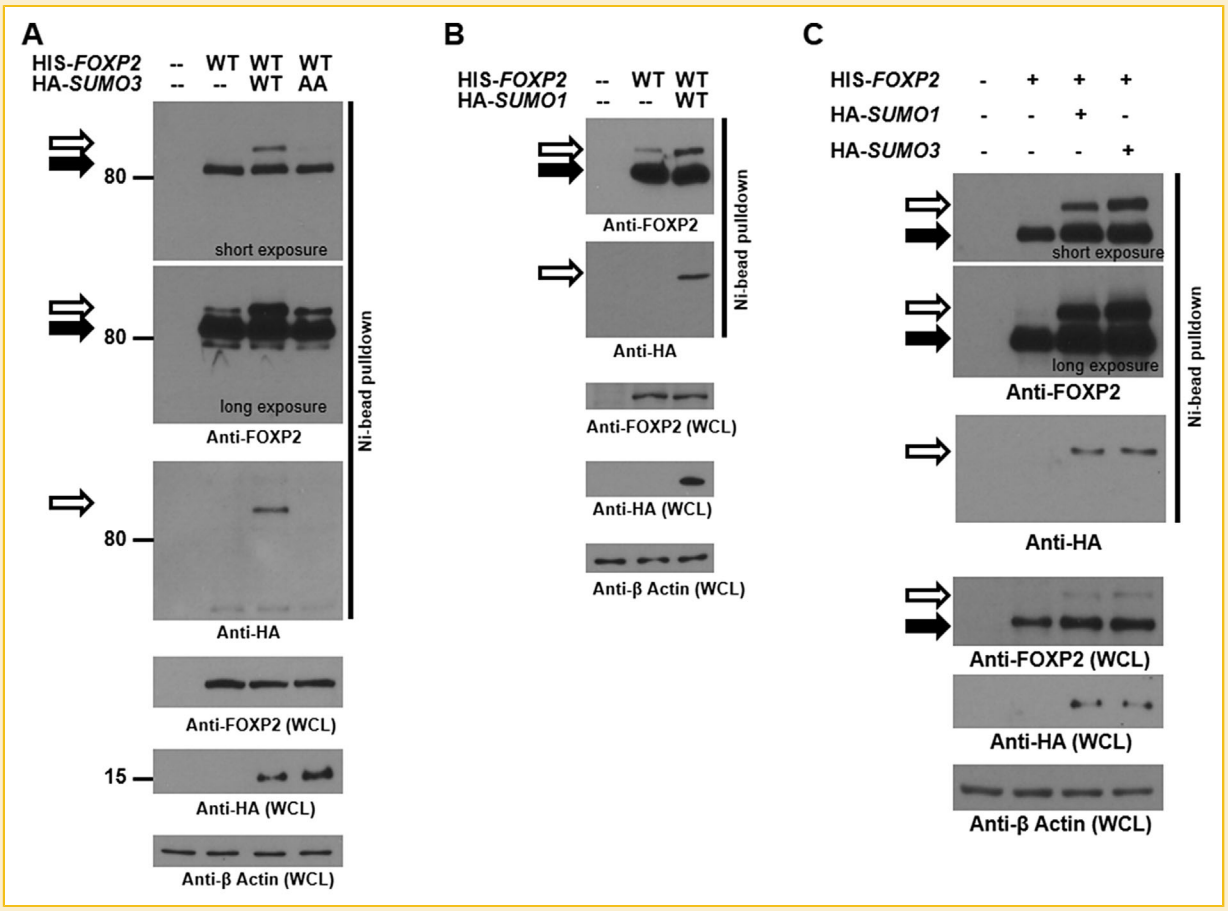

Fig. 2. FOXP2 can be modified by both SUMO1 and SUMO3. A: MCF7 cells were transiently transfected with 3 μg HIS-tagged WT *FOXP2* and 2μg HA-*SUMO3* (WT or AA mutant) expression vectors as indicated. After 48 h, cells were harvested and the cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-HA and anti-FOXP2 immunoblotting. Whole cell lysates (WCL) were subjected to anti-HA, anti-FOXP2, or anti-β-Actin immunoblotting for SUMO3, FOXP2, or β-Actin expression, respectively. B: MCF7 cells were transiently transfected with 3 μg HIS-tagged WT *FOXP2* and 2μg HA-tagged WT *SUMO1* expression vectors as indicated. After 48 h, cells were harvested and the cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-HA and anti-FOXP2 immunoblotting. WCL were subjected to anti-HA, anti-FOXP2, or anti-β-Actin immunoblotting for SUMO1, FOXP2, or β-Actin expression, respectively. C: MCF7 cells were transiently transfected with 3 μg HIS-tagged WT *FOXP2* and 2μg HA-tagged WT *SUMO1* or *SUMO3* expression vectors as indicated. After 48 h, cells were harvested and the cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-HA and anti-FOXP2 immunoblotting. WCL were subjected to anti-HA, anti-FOXP2, or anti-β-Actin immunoblotting for SUMO1/3, FOXP2, or β-Actin expression, respectively.

modified by endogenous SUMO proteins. Overexpression of WT SUMO3 significantly enhanced FOXP2 SUMOylation, further supporting the data on Figure 1B. However, AA mutant SUMO3 could not increase FOXP2 SUMOylation. Together, these results (both Figs. 1B and 2A) indicate that FOXP2 can be SUMOylated in mammalian cells by SUMO3 (closely-related to SUMO2). Similarly, using the same HIS-tagged FOXP2 system, we observed that FOXP2 is also able to be conjugated by SUMO1 (Fig. 2B). Next, we directly compared the efficiency of FOXP2 SUMOylation by SUMO1 and SUMO3. As shown in Figure 2C, both SUMO1 and SUMO3 are able to conjugate to FOXP2 with similar efficiency. Overall, our results provide direct evidence that FOXP2 can be modified by both SUMO1 and SUMO3 in mammalian cells.

Generally, SENP proteins are essential for de-conjugating SUMO proteins from target proteins as well as for activating SUMO proteins by removing the short C-terminal extension from immature SUMOs. In particular, SENP1 and SENP2 mainly involve in de-conjugation in mammalian cells. Therefore, we next tested whether SENP2 de-SUMOylates FOXP2. We expressed HIS-tagged *FOXP2* and HA-tagged *SUMO1* (Fig. 3A) or *SUMO3* (Fig. 3B) with or without FLAG-tagged *SENP2* in MCF7 cells. As shown in Fig. 3, a SUMOylated FOXP2 band was observed in cells expressing FOXP2 and SUMO1 (Fig. 3A) or SUMO3 (Fig. 3B). However, when SENP2 was co-expressed with FOXP2 and SUMO1 or SUMO3 in cells, the SUMOylated band was completely disappear, suggesting that SENP2 is involved in the process of de-SUMOylation of FOXP2.

LYSINE 674 IS THE MAIN SUMO-ACCEPTOR SITE ON FOXP2

To facilitate the analysis of FOXP2 SUMOylation, we next created HIS-tagged mutant forms of FOXP2 in which the acceptor lysine within the major SUMOylation motif was replaced with arginine (Fig. 4A). Importantly, these mutant forms of FOXP2 can be readily isolated and distinguished by virtue of the associated HIS tag. To determine whether K674 in FOXP2 is the major conjugation site for SUMO modification, we probed FOXP2 preparations isolated from MCF7 cells by Ni^{2+} chelate chromatography under denaturing

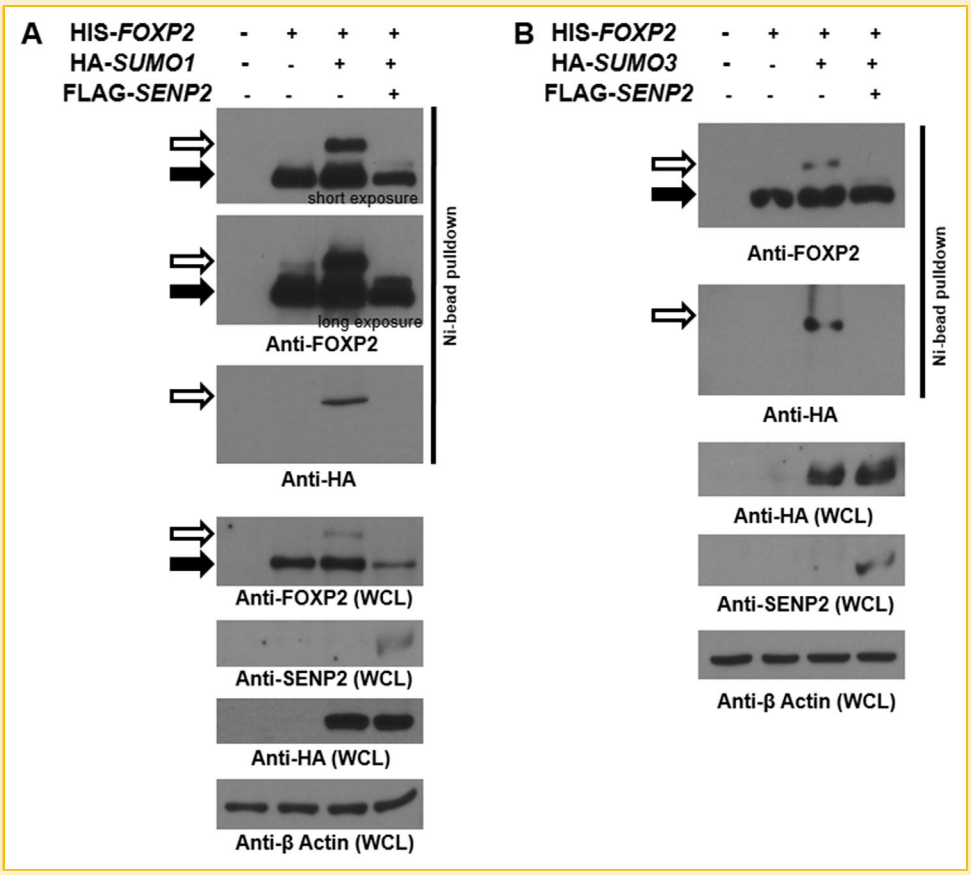

Fig. 3. SENP2 reduces FOXP2 SUMOylation. MCF7 cells were co-transfected with or without FLAG-tagged *SENP2*, HA-tagged SUMO1 (A) or SUMO3 (B), and HIS-tagged *FOXP2* expression plasmids. The cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-FOXP2 and anti-HA immunoblotting. WCL were subjected to anti-HA, anti-FOXP2, anti-SENP2, or anti-β-Actin immunoblotting for SUMO1/3, FOXP2, SENP2, or β-Actin expression, respectively. Experiments were performed three times with similar results.

condition. As shown in Figure 4B, a slowly migrating species (about 115 kDa) was detected in cells expressing WT FOXP2 alone (long exposure image). We interpret this form as being FOXP2 modified by endogenous SUMO. Moreover, the level of SUMOylated FOXP2 is dramatically increased when exogenous SUMO1 was co-transfected with FOXP2. Interestingly, disruption of the SUMO conjugation motif (K674R) led to a complete loss of detectable SUMOylation of FOXP2, suggesting that K674 is the major SUMO site on FOXP2. We observed similar result when exogenous SUMO3 was co-transfected with FOXP2 in MCF7 cells (Fig. 4C). Taken together, these results indicate that FOXP2 can be SUMOylated in mammalian cells and K674 is the major SUMO conjugation site on FOXP2.

SUMOylation IS DECREASED IN HUMAN ETIOLOGICAL FOXP2 R553H POINT MUTATION

Since FOXP2 R553H is an etiological point mutation found in all 15 affected members of the KE family with speech-language disorder [Lai et al., 2001], we next investigated whether this mutation affects SUMOylation of FOXP2. HIS-tagged *FOXP2* (WT, K674R, or R553H) expression plasmid was co-transfected with or without HA-tagged *SUMO3* plasmid in HEK293 cells for 48 h, and the HIS-tagged FOXP2 was precipitated by Ni^{2+} bead. As shown in Figure 5A, over-expression of SUMO3 dramatically increased SUMOylation of WT FOXP2 but not K674R FOXP2. Interestingly, we discovered that SUMOylation of R553H FOXP2 was significantly reduced compared to WT FOXP2 when SUMO3 was co-expressed. The similar result was observed in MCF7 cells (Fig. 5B). The quantitative analysis showed that approximately 50% of SUMOylation was lost in R553H mutation (Fig. 5C) in both HEK293 and MCF7 cells, likely suggesting that R553 residue in DNA-binding region may affect the full SUMOylation capacity of FOXP2.

EXTENDED CONSENSUS NEGATIVELY CHARGED AMINO ACID-DEPENDENT SUMOylation (NDSM) MOTIF IS ESSENTIAL FOR FOXP2 SUMOylation

Previous studies including ours on SUMO substrates demonstrate that SUMOylation is regulated by the acidic residues located downstream from the core consensus SUMO site of the target proteins [Yang et al., 2006; Wang et al., 2013]. From FOXP2 amino acid sequence, we found that four acidic residues (E681-D684) are located downstream from the core consensus SUMO site, K674, of FOXP2 (Fig. 1A). Therefore, to facilitate the analysis of negatively charged amino acid-dependent SUMOylation (NDSM) on FOXP2 SUMOylation, we next created HIS-tagged mutant forms of FOXP2 in which the acidic residues (EDED)

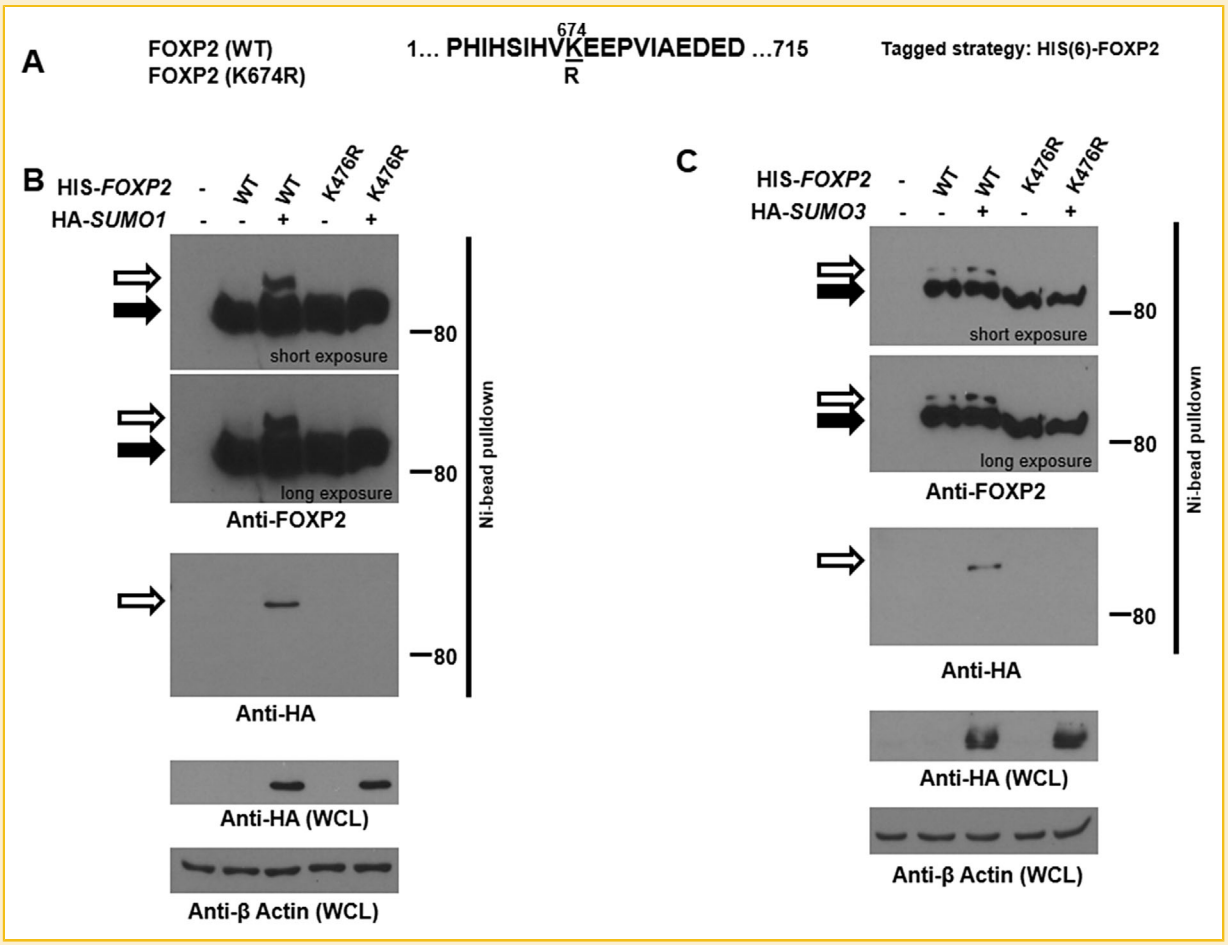

Fig. 4. Lysine 674 is the major SUMO site in FOXP2. A: Schematic representation of the human FOXP2 protein with the lysine-to-arginine FOXP2 mutant generated in this study to determine the potential SUMOylation site on FOXP2. MCF7 cells were transiently transfected with 3 μg HIS-tagged WT *FOXP2* or *FOXP2* in which lysine 674 (K674R) was mutated to arginine and 2 μg HA-tagged WT *SUMO1* (A) or *SUMO3* (B) expression vectors were subjected to Ni^{2+} bead pulldown, followed by anti-FOXP2 and anti-HA immunoblotting. WCL were subjected to anti-HA or anti-β-Actin immunoblotting for SUMO1/3 or β-Actin expression, respectively. Experiments were performed three times with similar results.

downstream from the core consensus SUMO site (K674) were replaced with alanines (Fig. 6A). HIS-tagged *FOXP2* (WT, EDAA, or K674R) expression plasmid was co-transfected with or without HA-tagged *SUMO3* plasmid in MCF7 cells for 48 h, and the HIS-tagged FOXP2 was precipitated by Ni^{2+} bead under denaturing condition. As shown in Figure 6B, similar to previous results, over-expression of SUMO3 significantly enhanced SUMOylation of WT FOXP2 but not K674R FOXP2. Interestingly, we found that SUMOylation of EDAA FOXP2 was dramatically reduced compared to WT FOXP2 when SUMO3 was co-expressed. The quantitative analysis showed that approximately 70% of SUMOylation was reduceed in EDAA FOXP2 protein (Fig. 6C), strongly suggesting that acidic residues (E681-D684) downstream from the core consensus SUMO site (K674) is essential for full SUMOylation capacity of FOXP2.

IMPAIRED SUMOylation DOES NOT AFFECT FOXP2 CELLULAR LOCALIZATION AND STABILITY

A previous report has demonstrated that while the WT FOXP2 is predominantly localized in the nucleus, significant amounts (approximately 50%) of human etiological R553H FOXP2 are found in the cytoplasm [Vernes et al., 2006]. We next examined whether SUMO conjugation to FOXP2 is associated with modulation of its subcellular localization. As can be seen in Figure 7A, subcellular fractionation revealed that WT FOXP2 is observable predominantly in the nuclear fractions of MCF7 cells. Consistent with the previous report, we observed significant amounts of R553H FOXP2 were localized in both the nucleus and cytoplasm (Fig. 7A). Interestingly, the nuclear distribution was not visibly altered in cells expressing the K674R SUMOylation-deficient FOXP2 (Fig. 7A left). We next transfected MCF7 cells with expression vectors bearing WT *FOXP2-GFP*, K674R *FOXP2-GFP*, or R553H *FOXP2-GFP*, and used fluorescence to localize the POXP2 proteins. As expected and consistent with previous reports, WT FOXP2-GFP, K674R FOXP2-GFP were localized in the nucleus only (Fig. 7A right). However, R553H FOXP2-GFP was localized in both nucleus and cytoplasm (Fig. 7A right). Our data suggest that SUMOylation does not affect FOXP2 cellular localization.

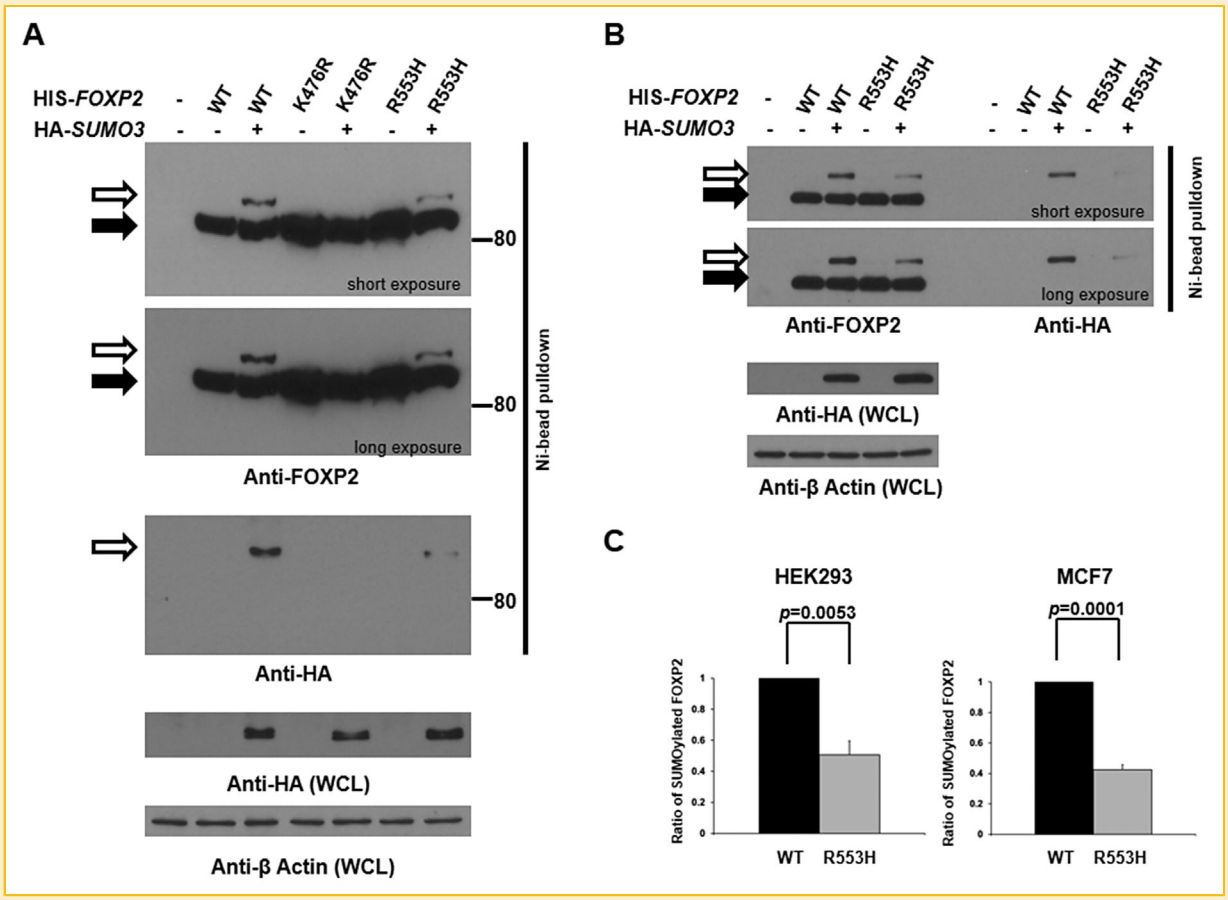

Fig. 5. SUMOylation is decreased in human etiological FOXP2 R553H point mutation. A: HEK293 cells were transiently transfected with 3 μg HIS-tagged WT *FOXP2* or K674R *FOXP2* or R553H *FOXP2* with or without 2 μg HA-tagged WT *SUMO3* expression vectors were subjected to Ni^{2+} bead pulldown, followed by anti-FOXP2 and anti-HA immunoblotting. WCL were subjected to anti-HA or anti-β-Actin immunoblotting for SUMO3 or β-Actin expression, respectively. B: MCF7 cells were transiently transfected with 3 μg HIS-tagged WT *FOXP2* or R553H *FOXP2* with or without 2 μg HA-tagged WT *SUMO3* expression vectors were subjected to Ni^{2+} bead pulldown, followed by anti-FOXP2 and anti-HA immunoblotting. WCL were subjected to anti-HA or anti-β-Actin immunoblotting for SUMO3 or β-Actin expression, respectively. C: Ratio of SUMOylated FOXP2 from HEK293 (A) and MCF7 (B) cells was determined by quantifying band intensity by ImageJ program. Experiments were performed three times with similar results.

Several studies have demonstrated that SUMOylation plays an important role in regulation of stability of its target proteins [Chen et al., 2013; Sahin et al., 2014]. To determine whether SUMOylation may influence FOXP2 stability, we compared the half-lives of WT and K674R FOXP2 by performing cycloheximide (CHX) time-course experiments on transfection into MCF7 cells. As shown in Figure 7B, the half-lives for both WT and K674R FOXP2 were similar as estimated for approximately 6 h, suggesting that SUMOylation does not affect FOXP2 stability.

Taken together, these findings indicate that SUMOylation does not affect FOXP2 cellular localization and stability.

SUMOylation MODULATES TRANSCRIPTIONAL ACTIVITY OF FOXP2 IN REGULATING DOWNSTREAM TARGET GENES

FOXP2 is a negative regulator of *DISC1* and *SRPX2* gene expression [Roll et al., 2010; Walker et al., 2012], which should gain insight into the role of SUMO modification of FOXP2. While *DISC1* is a leading candidate gene for bipolar disorder and schizophrenia, *SRPX2* is a gene implicated in X-linked recessive inheritance associated with developmental verbal dyspraxia (DVD) or with bilateral perisylvian polymicrogyria (BPP). Thus, we assessed the effect of this modification on FOXP2-dependent transcription using natural *DISC1* and *SRPX2* promoters. First, we tested the dose-dependent effect of FOXP2 on both promoters in two different cell lines: HEK293 and SH-SY5Y. A previous report [Walker et al., 2012] has demonstrated that both HEK293 (human embryonic kidney cells) and SH-SY5Y (neuroblastoma cells) endogenously express FOXP2. As shown in Figure S1A–C (Supplemental materials), WT FOXP2 dose-dependently represses both *DISC1* and *SRPX2* promoters in two cell lines. These results are consistent with the previous reports [Roll et al., 2010; Walker et al., 2012]. We next examined the effect of SUMOylation on FOXP2-dependent transcriptions in HEK293 and SH-SY5Y cells which both express endogenous FOXP2. As shown in Figure 8A and B (HEK293 cells) and C (SH-SY5Y cells), expression of WT FOXP2 leads to a robust reduction in the activity of *DISC1* and *SRPX2* promoter-driven luciferase reporters. Notably, expression of the SUMOylation-deficient K674R FOXP2 mutant relieved the reduction by 17–25% depending on promoters and cells. As

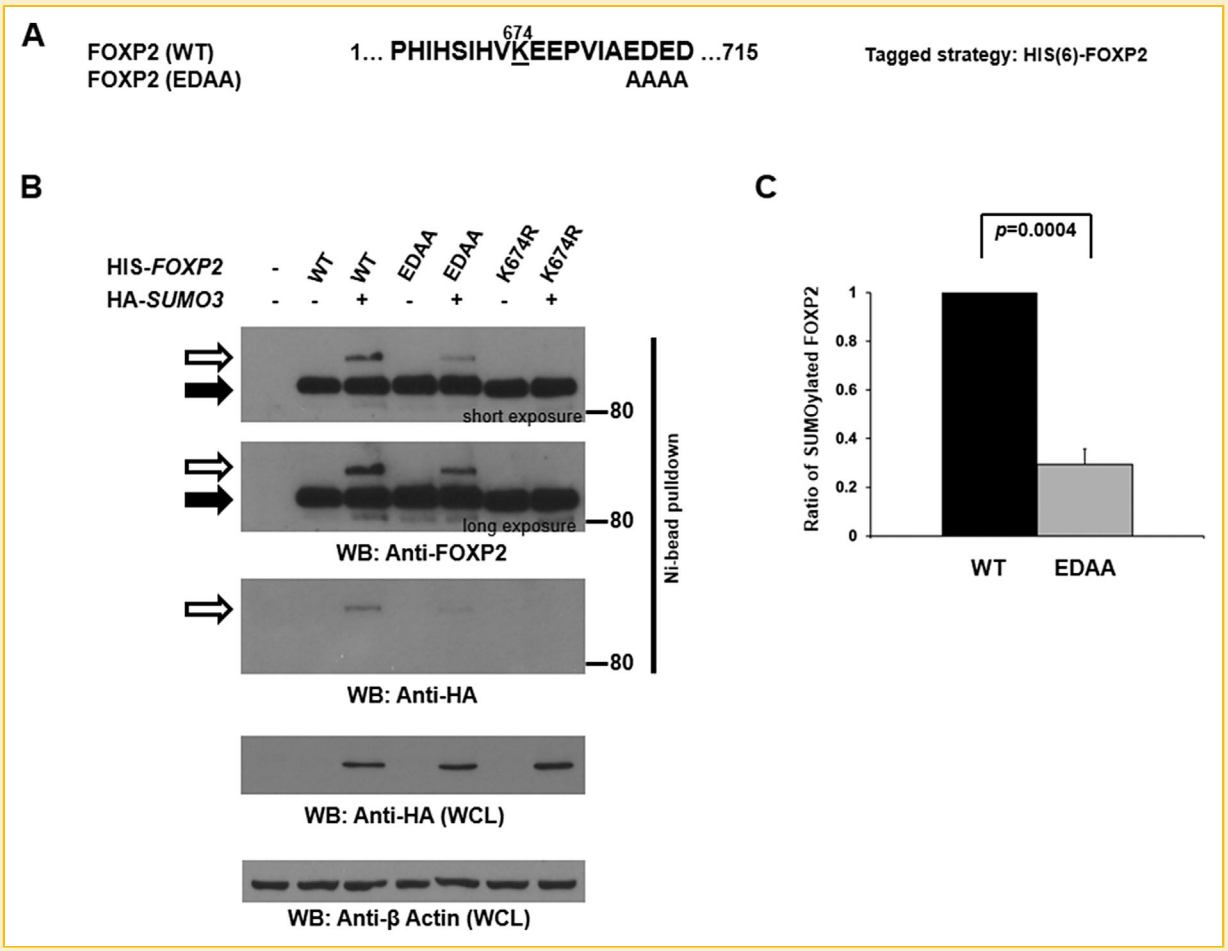

Fig. 6. NDSM motif is essential for FOXP2 SUMOylation. A: Schematic representation of the human FOXP2 protein with the EDED to AAAA (EDAA) FOXP2 mutant generated in this study to determine the effect of NDSM on FOXP2. B: MCF7 cells were transiently transfected with 3 μg HIS-tagged WT *FOXP2* or *FOXP2* in which glutamic acids/aspartic acids were mutated to alanines (EDAA) or K674R *FOXP2* with or without 2 μg HA-tagged WT *SUMO3* expression vectors were subjected to Ni^{2+} bead pulldown, followed by anti-FOXP2 and anti-HA immunoblotting. WCL were subjected to anti-HA or anti-β-Actin immunoblotting for SUMO3 or β-Actin expression, respectively. C: Ratio of SUMOylated FOXP2 from MCF7 (B) cells was determined by quantifying band intensity by ImageJ program. Experiments were performed three times with similar results.

expected and consistent with the previous reports [Roll et al., 2010; Walker et al., 2012], human etiological R553H FOXP2 mutant was not able to repress *DISC1* and *SRPX2* promoters (Fig. 8A–C).

Previous reports including ours [Bao et al., 2011; Wang et al., 2014a] have demonstrated that FOXM1 is a negative regulator of *MiR200b/c* gene expression. Since most FOX proteins bind to conserved response element (TAAACA), we therefore tested whether FOXP2 regulates MiR-200b/c gene expression using natural *MiR-200b* and *MiR-200c* promoters. As shown in Figure S1D (Supplemental materials), WT FOXP2 dose-dependently activates *MiR-200c* but not *MiR-200b* (data not shown). We next examined the effect of SUMOylation on FOXP2-dependent transcriptions in HEK293 cells. As shown in Figure 8D, expression of WT FOXP2 leads to an increase (threefolds) in the activity of *MiR-200c* promoter-driven luciferase reporters. Notably, expression of the SUMOylation-deficient K674R FOXP2 mutant reduced the activation by 25%. As expected, human etiological R553H FOXP2 mutant was not able to activate *MiR-200c* promoter.

These findings indicate that recruitment of SUMO to FOXP2 plays a functional role, at least in part, for its full inhibitory effect on natural *DISC1* and *SRPX2* transcription and full activation effect on natural *MiR200c* transcription.

DISCUSSION

Here, we have demonstrated for the first time that FOXP2, a critical protein for speech and language development, can be SUMOylated and lysine 674 is the major SUMO acceptor site. Interestingly, we showed that FOXP2 R553H mutation, found in all affected members of the KE family with speech-language disorder [Lai et al., 2001], significantly lost SUMOylation capacity. We further demonstrated that SUMOylation may modulate transcriptional activity of FOXP2 in regulating downstream target genes (*DISC1*, *SRPX2*, and *MiR200c*), providing the underlying molecular mechanism, at least in part, how FOXP2 plays a functional role in regulating target gene activities.

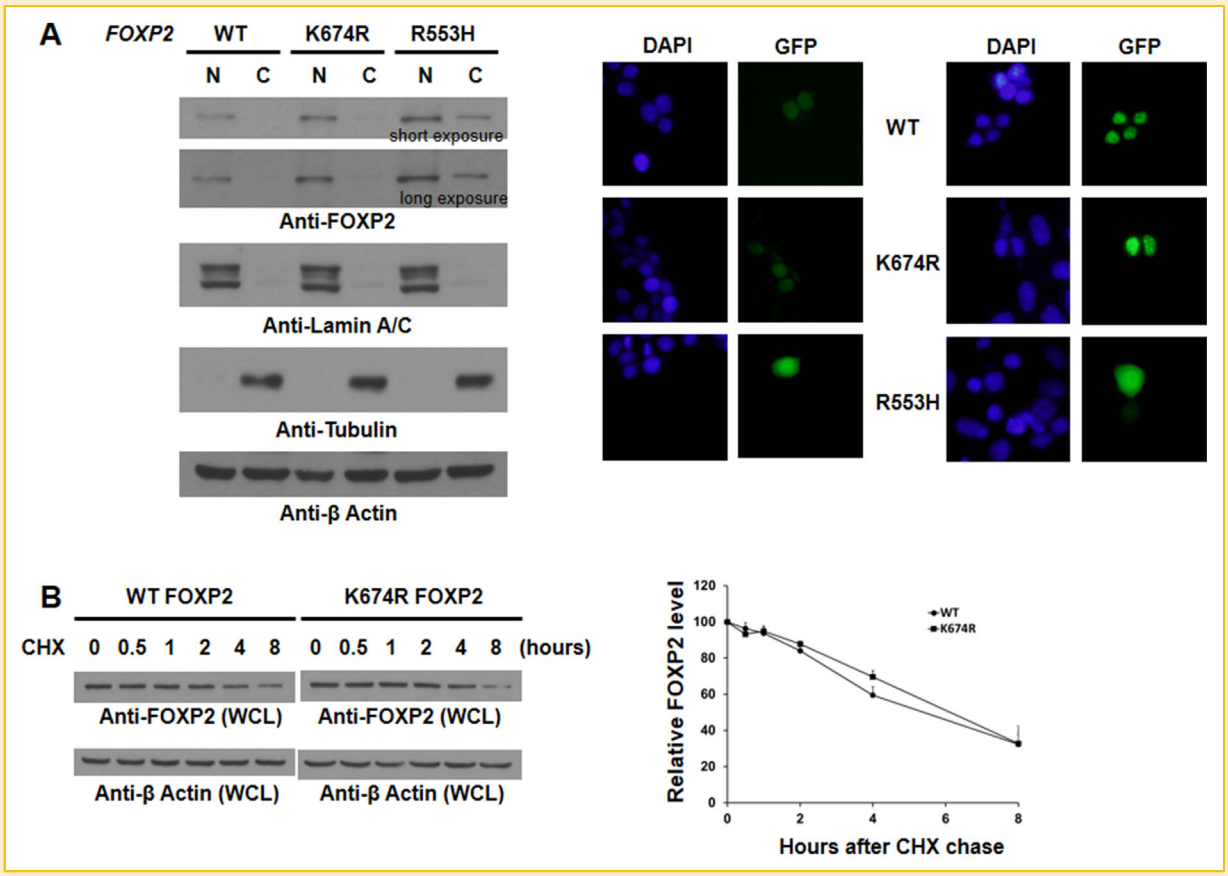

Fig. 7. The K674R mutation does not alter FOXP2 subcellular localization and stability. A: Nuclear (N) and cytoplasmic (C) fractions of MCF7 cells expressing HIS-tagged WT FOXP2, K674R FOXP2, or R553H FOXP2 were subjected to anti-FOXP2, anti-β-Actin, anti-Lamin, and anti-Tubulin immunoblotting (left). MCF7 cells were transfected with WT *FOXP2-GFP*, or K674R *FOXP2-GFP*, or R553H *FOXP2-GFP* expressing plasmids for 2 days. Cells were then counterstained with DAPI and observed under a fluorescence microscope (right). B: MCF7 cells were transfected with expression plasmid bearing WT or K674R *FOXP2*, where indicated, and then treated with cycloheximide. Cells were harvested at the indicated time points, and cell lysates were subjected to anti-FOXP2 and anti-β-Actin immunoblotting (left). Relative FOXP2 levels were determined by quantifying band intensity by ImageJ program (right). Experiments were performed three times with similar results.

In the present study, we demonstrated that both SUMO1 and SUMO3 are capable of conjugating FOXP2 in a mammalian cell system with similar efficiency. Furthermore, while SUMO1/3 dramatically enhances FOXP2 SUMOylation, SENP2 (one of the SUMO proteases) significantly reduces FOXP2 SUMO modification. These findings suggest that the typical SUMO machinery anticipates on the post-translational modification of FOXP2. Although UBE2I, the only E2 enzyme for SUMO conjugation cycle, has been shown to enhance SUMOylation on certain target proteins [Wang et al., 2013], we found that UBE2I alone does not increase FOXP2 SUMOylation (data not shown). The potential SUMOylation site in FOXP2 is located at lysine 674 based on the core SUMOylation motif (PsiKXE). Our data showed that lysine 674 was efficiently conjugated by both SUMO1 and SUMO3 in mammalian cell system and FOXP2 SUMOylation was totally lost when mutation of lysine 674 to arginine (Fig. 4). Unlike R553 is located within the fork-head DNA binding domain of FOXP2 and severely damages FOXP2 activity, lysine 674 is located further downstream of the fork-head DNA binding domain and SUMO conjugation at lysine 674 of FOXP2 would unlikely disrupt its binding ability and affinity to DNA. However, further studies are indeed needed to dissect whether SUMO conjugation at lysine 674 of FOXP2 could play an important role in regulating the interactions between FOXP2 and other transcriptional regulators and cofactors, such as FOXP1 and FOXP4.

DNA recognition by FOXP2 is mediated predominately by helix H3, where R553 and H554 form strong hydrogen bonding and hydrophobic interactions with the DNA. R553 is specially conserved among all forkhead proteins, including FOXP1-4, FOXC1-2, and FOXA3 [Stroud et al., 2006]. In fact, FOXP2 R553H is an etiological point mutation found in all 15 affected members of the KE family with speech-language disorder [Lai et al., 2001]. Because R553 is located within the highly conserved DNA-binding domain, FOXP2 R553H mutation lost its transcriptional repressor activity ([Walker et al., 2012], and Figure 8 of the current study). Interestingly, we also observed that FOXP2 R553H mutation reduces its SUMOylation capacity by approximately 50%, suggesting that R553H mutation not only interferes FOXP2's DNA binding but also affects SUMO conjugation possibly due to a 3D conformational change. Further

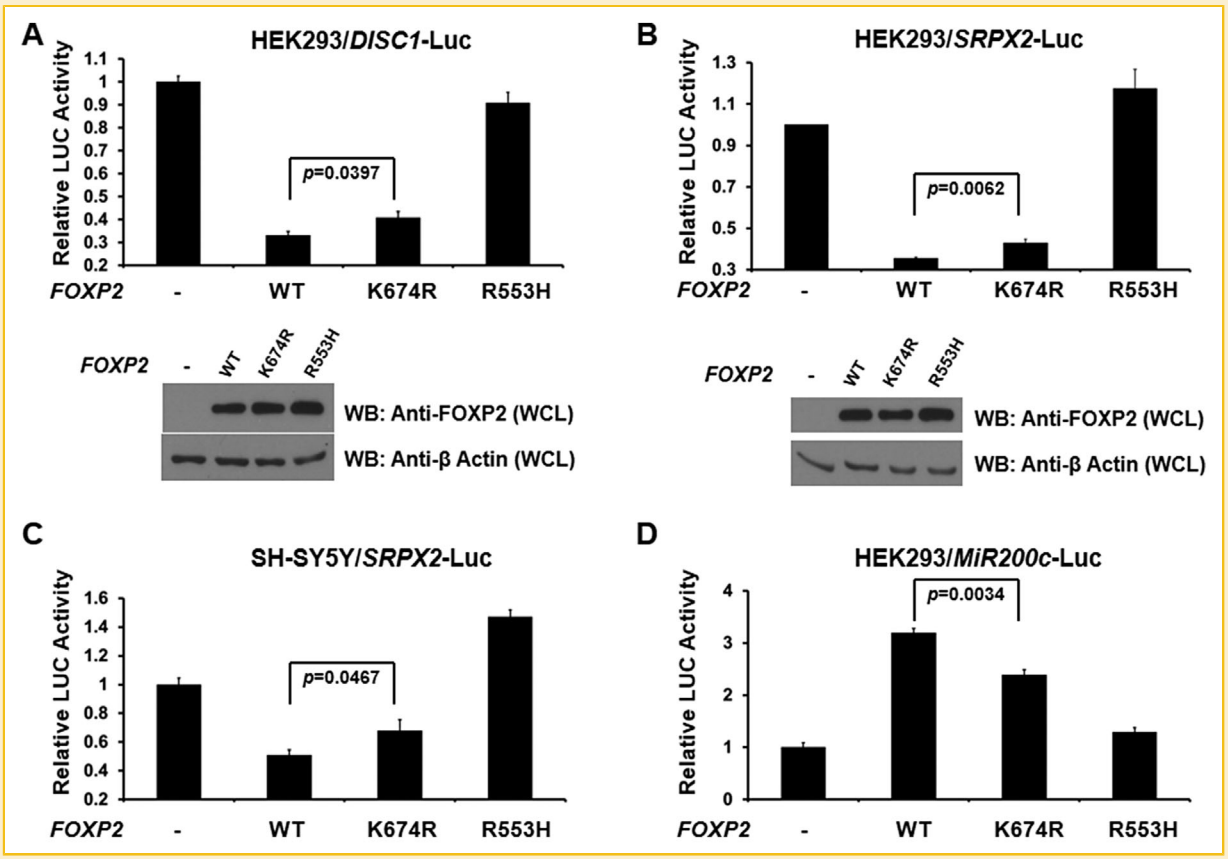

Fig. 8. Loss of SUMOylation on FOXP2 alters its transcriptional activity. A: HEK293 cells were transfected with WT or K674R or R553H *FOXP2* expression plasmid (0.2 μg) and a reporter plasmid with *DISC1* natural promoter (0.1 μg). B: HEK293 cells were transfected with WT or K674R or R553H *FOXP2* expression plasmid (0.2 μg) and a reporter plasmid with *SRPX2* natural promoter (0.1 μg). C: SH-SY5Y cells were transfected with WT or K674R or R553H *FOXP2* expression plasmid (0.2 μg) and a reporter plasmid with *SRPX2* natural promoter (0.1 μg). D: HEK293 cells were transfected with WT or K674R or R553H *FOXP2* expression plasmid (0.2 μg) and a reporter plasmid with *MiR-200c* natural promoter (0.1 μg). Luciferase activities were measured 48 h after transfection. Luciferase activity was measured and normalized with Renilla activity. Relative LUC activity (fold activation) was calculated and plotted. Experiments were performed three times in triplicate. Error bars indicate standard errors.

studies are indeed needed to fully dissect the mechanism(s) how SUMOylation capacity influences the function of R553H mutant and vice versa.

Several lines of evidence suggest that amino acids downstream or upstream from the SUMOylation consensus tetrapeptide also facilitate substrate SUMO conjugation. For example, negatively charged amino acid-dependent SUMOylation (NDSM) and phosphorylation-dependent SUMOylation motif (PDSM) contain additional negatively charged and phosphorylated amino acids sequences downstream to the consensus motif, respectively [Hietakangas et al., 2006; Yang et al., 2006; Wang et al., 2013a]. In NDSM's case, the negatively charged amino acids downstream from the core consensus motif are believed to significantly increase UBE2I interaction (by binding to the basic patch on UBE2I) and subsequently enhance SUMOylation process. For FOXP2, we found four negatively charged amino acids (EDED) downstream to the main SUMO acceptor site (K674). In the present study, we demonstrated that EDED residues downstream from the core K674 SUMO site are essential for FOXP2 SUMOylation because mutation of EDED to AAAA dramatically reduces FOXP2's SUMOylation, suggesting EDED's role in strongly enhancing UBE2I interaction and subsequently facilitating FOXP2 SUMO conjugation.

It has been suggested that SUMO modification could alter stability and subcellular localization of proteins. For instance, SUMOylation of human argonaute 2 regulates its stability [Sahin et al., 2014], and SUMOylation of CDK6 stabilizes the protein and drives the cell cycle for the cancer development and progression [Bellail et al., 2014]. SUMO modification of numerous nuclear proteins keep them away from the DNA and thus taking them out of *trans*-repression or *trans*-activation action [Zhong et al., 2000; Nagai et al., 2011]. Furthermore, the status of SUMOylation can affect the trafficking of the target proteins and influence the transport of proteins between the cytoplasm and the nucleus [Du et al., 2008; Truong et al., 2012]. In the current study, SUMO modification does not alter subcellular localization and stability of FOXP2, suggesting the transcriptional effects of FOXP2 SUMOylation are not likely to be due to alteration in subcellular localization and stability, and argue in favor of an intra-nuclear action for this modification.

The cellular SUMO modification system affects the transcriptional function of wide-range proteins including nuclear transcription

factors, co-factors, and cell cycle regulators. SUMO modification of transcription factors generally play a functional role in regulating the transcriptional repression, such as SUMOylation of NR5A1, FOXM1, and FOXA1 [Wang et al., 2013; Sutinen et al., 2014; Wang et al., 2014a], and transcriptional activation, such as SUMOylation of p53 and PAX-6 [Rodriguez et al., 1999; Yan et al., 2010]. In current study, we observed that loss of SUMOylation on FOXP2 reduces its full repression on *DISC1* and *SRPX2* genes and full activation on *MiR-200c* genes, suggesting that SUMOylation of FOXP2 is essential, at least in part, in regulating gene activities. Therefore, loss of SUMOylation on FOXP2 may interfere FOXP2′s ability to recruit co-factors and other proteins in transcriptional activity. Future studies should assess whether SUMOylation of FOXP2 alters the recruitment of co-factors for FOXP2′s transcriptional repression and activation, and if so, what molecular mechanisms contribute to transcriptional repression and activation.

In conclusion, FOXP2 is post-translationally modified by specific enzymes mediating the SUMO cycle of FOXP2 and SUMOylation of FOXP2 influences *DISC1*, *SRPX2*, and *MiR200c* gene activities. Our study adds a new layer to the previous understanding of how FOXP2 functions to regulate neural coordination, synapse formation, brain maturation, language development, as well as cancer progression and metastasis.

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