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# Sumoylation of FOXP2 regulates motor function and vocal communication through Purkinje cell development

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## Abstract

**Background—**Mutations in the gene encoding the transcription factor forkhead box P2, *FOXP2*, result in brain developmental abnormalities including reduced gray matter in both human patients and rodent models, and speech and language deficits. However, neither the region-specific function of FOXP2 in the brain, in particular the cerebellum, nor the effects of any post-translational modifications of FOXP2 in the brain and disorders have been explored.

**Methods—**We characterized sumoylation of FOXP2 biochemically, and analyzed the region-specific function and sumoylation of FOXP2 in the developing mouse cerebellum. Using *in utero* electroporation to manipulate the sumoylation-state of Foxp2 as well as Foxp2 expression levels in Purkinje cells (PCs) of the cerebellum *in vivo*, we reduced Foxp2 expression approximately 40% in the mouse cerebellum. Such a reduction approximates the haploinsufficiency observed in human patients who demonstrate speech and language impairments.

**Results—**We identified sumoylation of FOXP2 at K674 (K673 in mouse) in the cerebellum of neonates. *In vitro* co-immunoprecipitation and *in vivo* colocalization experiments suggest that PIAS3 acts as the SUMO E3 ligase for FOXP2 sumoylation. This sumoylation modifies transcriptional regulation by FOXP2. We demonstrate that Foxp2 sumoylation is required for regulation of cerebellar motor function and vocal communication, likely through dendritic outgrowth and arborization of PCs in the mouse cerebellum.

**Conclusions—**Sumoylation of Foxp2 in neonatal mouse cerebellum regulates PC development as well as motor functions and vocal communication, demonstrating evidence for sumoylation in regulating mammalian behaviors.

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**Keywords**

FOXP2; Purkinje cells; cerebellum; sumoylation; vocal communication; motor function

## Introduction

The transcription factor *FOXP2* has been implicated in human brain evolution, language, cognition, vocal-motor integration, and neural development in the central nervous system (CNS) through orchestration of transcriptional cascades that also tend to be at risk in several neurodevelopmental disorders such as autism spectrum disorder (ASD) and schizophrenia (1–3). Previous work using humanized Foxp2 mouse models has suggested that humanized Foxp2 alters cortico-striatal function (4–6), but the cerebellum appears to be a key brain region for FOXP2 function as patients with mutations in *FOXP2* demonstrate significant grey matter reduction in the cerebellum as evidenced by MRI (7), and genetic disruption of *Foxp2* in mice results in decreased cerebellar size (8–11). Recent studies have uncovered important roles for the cerebellum in higher cognitive functions such as language, cognition, emotion, and memory (12–19). In particular, function of PCs in the mouse cerebellum is critical for ASD-relevant behaviors (20). However, the cerebellar-specific function of FOXP2 has not been explored.

Sumoylation, a highly conserved post-translational modification, regulates protein function in numerous ways including subcellular localization, stability, and transcriptional activity (21, 22). In the CNS, sumoylation regulates transcription, ion channel activity, synapse formation and regulation, mRNA transport in axons, and mitochondrial function (22–24). During sumoylation, the SUMO proteins are conjugated to lysine residues of the target proteins by SUMO enzymes (E1 activating, E2 conjugating, and E3 ligase enzymes), and are subsequently removed by SUMO-specific proteases, SENPs (25). Disruption of sumoylation can affect pathology in brain disorders such as Huntington's disease (Htt), spinal and bulbar muscular atrophy (SUMO-1 positive intranuclear inclusions), spinocerebellar ataxias (Ataxin-1, 3, 7), Alzheimer's disease (APP, Tau), Parkinson's disease (α-Synuclein, Parkin, DJ-1) and ischemia (increase of SUMO-2/3, Drp1) (22, 24). A recent report has shown that FOXP2 is a substrate for sumoylation in transformed cell lines (26), however the role of sumoylation and potentially other post-translational modifications of FOXP2 in the CNS is completely unknown.

In this study, we identified sumoylation of FOXP2 in the cerebellum of neonates, a critical time for neural circuit formation and the emergence of vocal communication in mammals. Therefore, we explored the role of FOXP2 sumoylation in neuronal development and mammalian behavior related to the cerebellum. Here, we provide *in vivo* evidence demonstrating the requirement for sumoylation and cerebellar-specific expression of FOXP2 in directing complex motor behaviors and vocal communication. We found sumoylation of FOXP2 regulates dendritic outgrowth and arborization in PCs of the cerebellum, resulting in altered mammalian behavior and transcriptional regulation of FOXP2 respectively. These data demonstrate a critical role for FOXP2 in the cerebellum to regulate PC development,

motor function and vocal communication that might be relevant to neurodevelopmental disorders.

## Methods and Materials

Detailed Methods and Materials are described in the Supplemental Methods and Materials.

### Animal experiments

Wild type C57BL/6J mice were used for all *in vivo* experiments. For *in utero* electroporation (IUE), plasmid DNA (1–2 μg/μl) was microinjected into the 4th ventricles of E12.5 embryos to target PCs. The embryo was electroporated (five 50-millisecond pulses of 33 V with an interval of 950 milliseconds; CUY21SC, NEPA GENE, Ichikawa-City, Chiba, Japan) using platinum plate electrode tweezers (CUY650P5; Protech International Inc., Boerne, TX) (27–30). All procedures were approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center.

### Biochemical experiments

293T cells were transfected using FuGENE6 (#E2691, Promega, Madison, WI) and harvested 48 hrs later. 50 mM N-ethylmaleimide (NEM) (#E3876, Sigma-Aldrich, St. Louis, MO) was used as a SUMO protease inhibitor. 1 mM hydrogen peroxide ($H_2O_2$) (31) and 100 μM ginkgolic acid (#75741, Sigma-Aldrich, St. Louis, MO) (26, 32) were used as sumoylation inhibitors.

## Results

### FOXP2 is sumoylated in the neonatal cerebellum

In the course of examining Foxp2 expression in the developing cerebellum, we observed that while unmodified Foxp2 peaks in expression embryonically, a higher molecular weight band corresponding to Foxp2 peaks in expression in neonatal mouse cerebellum (Figure 1A, B). When we expressed wild type FOXP2 (FOXP2 WT) in 293T cells, we also observed a higher molecular weight band recognized by a FOXP2 antibody that is decreased by $H_2O_2$ treatment, a mechanism for reversible inhibition of SUMO conjugating enzymes (31) (Figure 1C) and ginkgolic acid (26, 32), a sumoylation-specific inhibitor (Figure 1D). Furthermore, in both 293T cells and mouse cerebellum, this high molecular weight band is recognized by either FOXP2 or SUMO-1 antibody in lysates that have undergone immunoprecipitation with an antibody recognizing SUMO-1 or FLAG (to capture FLAG-tagged FOXP2) respectively (Figure 1B–D). Based on these observations, we identified a conserved consensus sumoylation site (ψKXE) at K674 (K673 in mouse) that is outside of the annotated functional domains of FOXP2 (26) (Figure 1E, F). Upon mutation of this lysine to arginine (the non-sumoylated form of $FOXP2^{K674R}$, FOXP2 KR), the high molecular weight band was not observed (Figure 1G), however this point mutation does not affect the protein expression of FOXP2 in 293T cells, nor the amount immunoprecipitated by an antibody (Figure S1 in Supplement 1). Together, these data demonstrate that the high molecular weight modification of Foxp2 in mouse cerebellum is a sumoylated form of Foxp2.

In support of Foxp2 sumoylation in the cerebellum, a previous yeast two-hybrid screen demonstrated interaction of FOXP2 with PIAS3 (33), an E3 ligase that attaches SUMO proteins to their substrates (34). Hence, we investigated the physiological interaction of FOXP2 with SUMO-1, SUMO-2/3, or PIAS3 in 293T cells (Figure 2A–C). SUMO-2 and SUMO-3 are 97% homologous and therefore are indistinguishable (35, 36). We also examined mRNA expression of *Sumo proteins; Sumo-1*, *Sumo-2*, *Sumo-3* and *Sumo-4* (Figure 2D) and *Pias family proteins; Pias1*, *Pias2*, *Pias3* and *Pias4* (Figure 2E) and observed mostly unchanged expression throughout development of the neonatal cerebellum. We also examined expression of these sumoylation proteins in the mouse cerebellum, and observed co-localization of Foxp2, Sumo-1, Sumo-2/3 and Pias3 in PCs of mouse cerebellum at postnatal day 7 (P7) when Foxp2 is highly sumoylated (Figure 2F, Figure S2 in Supplement 1). Together, these data suggest FOXP2 is sumoylated by SUMO-1/2 and PIAS family proteins, most likely PIAS3. In particular, FOXP2 sumoylation is increased during the time points corresponding to neuronal differentiation in the cerebellum (Figure 1A, B), suggesting sumoylation of FOXP2 plays a role in neuronal development.

## Sumoylation of FOXP2 promotes neuronal differentiation through regulation of dendritic growth

To investigate whether sumoylation of FOXP2 affects its regulation of neuronal function, we assessed whether neurite outgrowth is dependent upon FOXP2 sumoylation in general in the brain using a system of mouse neural progenitors (mNPs) as previous work has demonstrated a role for FOXP2 in promoting dendrite formation (37–39). We forced expression of FOXP2 in mNPs, and found that FOXP2 WT significantly promoted the length of neurites expressing either an immature neuronal maker Tuj1 or a mature neuronal marker MAP2 (Figure S3 in Supplement 1). In contrast, FOXP2 KR was unable to promote the length of Tuj1-positive and MAP2-positive neurites as effectively (Figure S3 in Supplement 1). These data indicate that sumoylation of FOXP2 plays a role in promoting neuronal maturation potentially in any neuron expressing FOXP2.

Next, we assessed whether sumoylation of FOXP2 affects neuronal maturation *in vivo*. Extensive characterization of Foxp2 expression in the cerebellum has shown that Foxp2 expression is limited to PCs (Figures S2 and S4A in Supplement 1) (40–42). PCs send projections to the deep cerebellar nuclei and vestibular nuclei, the sole motor output of the cerebellum, indicating this neuronal pathway plays an important role in known cerebellar functions such as motor coordination and speech. The dendritic arbors of PCs are severely diminished in *Foxp2* mutant mice (8, 10), suggesting a role for Foxp2 in PC development.

To directly test the cell autonomous role of Foxp2 expression in PCs in the absence of alteration of Foxp2 in other cell types or brain regions, we, carried out directed *in utero* electroporation (IUE) experiments. We knocked down Foxp2 expression specifically in PCs of the mouse cerebellum by Foxp2 shRNA, and concurrently rescued Foxp2 expression with either a wild type FOXP2 (WT rescue) or non-sumoylated FOXP2 (KR rescue) shRNA-resistant construct (Figure 3A–D). As a control, we used a non-silencing shRNA (Control shRNA). All shRNA sequences are in the microRNA context (43). To provide IUE-specificity in targeting only PCs, we performed IUE at embryonic day 12.5 (E12.5), when

PCs arise from the ventricular zone in the mouse cerebellum (Figure 3C). The absence of Foxp2 expression in other cerebellar cell types such as the deep cerebellar nuclei at this time point (42) further supports our targeting specificity. Moreover, the uniform expression of Foxp2 in PCs throughout the entire cerebellum at this developmental time point (42) permits targeting of all cerebellar subdivisions. We confirmed IUE specificity at P7 by GFP immunostaining when FOXP2 is highly sumoylated *in vivo*, and observed GFP expression limited to only PCs and the dendrites and output fibers of the PCs, which project to the deep cerebellar nuclei and vestibular nucleus (Figure 3E, Figure S4 in Supplement 1). In line with this, approximately 30.7±2.6% (19–48%) of PCs were transfected by IUE without affecting other cerebellar cell types (Figure. 3E, Figure S4B–E in Supplement 1) (27, 44). Using this *in vivo* manipulation, we reduced Foxp2 expression approximately 40.1±2.8% *in vivo* in the mouse cerebellum at P7 (Figure 3D). Such a reduction approximates the haploinsufficiency observed in human patients who demonstrate speech and language impairments (1, 7, 45).

Using IUE to manipulate Foxp2 expression and its sumoylation, we observed a reduction in dendritic outgrowth and arborization of PCs at P7 in pups receiving Foxp2 shRNA (Figure 3F–H), consistent with published reports of *Foxp2* genetically modified mice (8). This reduction of dendritic outgrowth and arborization in PCs was restored by WT rescue but not by KR rescue (Figure 3F–H). These data indicate that sumoylation of FOXP2 plays a role in promoting neuronal differentiation through neurite/dendritic outgrowth and arborization without affecting Foxp2 expression in cortex and striatum (Figure 3F–I) and PCs viability (Figure S5 in Supplement 1).

## Sumoylation of FOXP2 regulates cerebellar motor functions

Since PC development appears to depend upon FOXP2 expression and sumoylation, we assessed whether Foxp2 sumoylation impacts cerebellar motor function. Previous studies have demonstrated a deficit in cerebellar-based motor behaviors such as righting reflex and negative geotaxis in *Foxp2* KO mice during neonatal stages (8). We found that reduction of Foxp2 specifically in the cerebellum significantly alters neonatal righting reflex and negative geotaxis at P4 and P7 (Figure 4A–C). These phenotypes were not due to a global developmental delay as has been observed in *Foxp2* KO mice (8, 10) as weights were not significantly different except for a slight decrease in weight with KR rescue at P7 that was not correlated with behavior (Figure 4D and Table S1 in Supplement 1). We also did not observe any sex differences in these behaviors (Table S2 in Supplement 1). Strikingly, both motor phenotypes were rescued by WT complementation, but not by KR complementation (Figure 4A–C). These data support a role for FOXP2 sumoylation in motor functions.

## Sumoylation regulates subcellular localization of Foxp2

We next explored the molecular mechanism underlying sumoylation of FOXP2 in neuronal development. As sumoylation often affects protein-protein interactions (21), we determined whether $FOXP2^{K674}$ affects interactions with known binding partners. FOXP2 homodimerizes and also heterodimerizes with other FOXP family members, FOXP1 and FOXP4 (46). We found that FOXP2 KR does not affect homo- or heterodimerization (Figure S6A–C in Supplement 1). Foxp2 has also been shown to interact with a co-repressor, c-

terminal binding protein, CTBP (46). However, this interaction was also not changed with FOXP2 KR (Figure S6D in Supplement 1).

As sumoylation can alter the subcellular localization of transcription factors (21), we next assessed the effects of sumoylation on FOXP2 localization. By inhibiting sumoylation with $H_2O_2$ treatment, we observed an increased cytoplasmic localization of FOXP2 and its co-repressor CTBP (Figure S7A in Supplement 1). Furthermore, sumoylation of CTBP has been reported to profoundly affect its subcellular localization and increase co-repressor activity (47). We therefore measured whether FOXP2 KR mutation also affects subcellular localization, and found a significant 100% increase in cytoplasmic and a significant 20% decrease in nuclear localization of FOXP2 KR compared with WT in 293T cells *in vitro* (Figure S7B–D in Supplement 1). We further investigated this shift in subcellular localization *in vivo* in PCs and cortical layer 6 neurons of mouse brain, a region where Foxp2 is also highly expressed. Consistent with our *in vitro* data, we observed increased cytoplasmic and decreased nuclear localization of FOXP2 KR *in vivo* (Figure S8 in Supplement 1). These data indicate that sumoylation modulates the subcellular localization of FOXP2 and CTBP (Figure S7 in Supplement 1), and suggest that the transcriptional function of FOXP2 is regulated in a sumoylation-dependent manner.

## Sumoylation of FOXP2 regulates vocal communication

To directly determine the potential impact of sumoylation on Foxp2 transcriptional function, we first investigated whether $FOXP2^{K674}$ is required for DNA transrepression through luciferase assays, and found that both FOXP2 WT and KR can repress a luciferase reporter equally well when presented with a canonical FOXP2 motif (AATTTG) in triplicate (48) (Figure S9 in Supplement 1). As the luciferase experiments do not utilize the endogenous chromatin state, we examined the *in vivo* regulation of a well-characterized target of FOXP2, contactin associated protein-like 2, *Cntnap2. CNTNAP2* polymorphisms are associated with specific language impairment (45, 49, 50), *CNTNAP2* promoter variants have been identified as potential ASD risk factors (50, 51). and rodent models lacking *Cntnap2* exhibit abnormal neuronal migration as well as ASD-relevant behaviors including altered ultrasonic vocalizations (USVs) (52). We observed relatively low expression of *Cntnap2* when sumoylated Foxp2 is highest in the developing cerebellum (Figures 1A, B and 5A). We therefore directly assessed the requirement of FOXP2 sumoylation on transcriptional regulation of *CNTNAP2* expression using human neural progenitors (hNPs). As previously reported (45), we confirmed repression of *CNTNAP2* by FOXP2 WT (Figure 5B). In contrast, FOXP2 KR was unable to significantly repress *CNTNAP2* (Figure 5B). These data demonstrate that transcriptional regulation of at least one ASD-associated gene, *CNTNAP2*, is dependent on sumoylation of FOXP2.

This altered regulation of *CNTNAP2* was striking as both *FOXP2* and *CNTNAP2* have been implicated in vocal motor behaviors in both human patients as well as mouse models (1, 7, 45, 49, 52). *Foxp2* KO mice have few, if any, neonatal USVs (8). It is unknown whether reduction of Foxp2 specifically in the cerebellum contributes to the diminished USVs in this mouse model; however, recent work has shown that mice containing a patient-relevant *Foxp2* mutation exhibit decreased number of USVs (10) that can be rescued with forced

cerebellar expression of FOXP2 (53). Together, these data support a role for region-specific FOXP2 function in the cerebellum related to vocal behaviors. Therefore, we examined USVs at the same developmental stages at which we examined righting reflex and negative geotaxis. A significant decrease in the number of USVs was observed in animals receiving cerebellar-directed Foxp2 shRNA at P4 and P7 (Figure 5C and Tables S1 and S2 in Supplement 1). This result suggests that normal Foxp2 expression in the cerebellum is required for neonatal USVs. We observed no difference with Foxp2 shRNA in the number of calls with frequency jumps, call duration, mean frequency or frequency range of USVs (Figure 5D–H), suggesting the calls that were present were relatively normal. As we observed with other motor behaviors, the decrease in number of USVs was rescued by the WT construct at P4 and P7, but not by the KR construct (Figure 5C). These data demonstrate sumoylation of Foxp2 in the cerebellum is also required for vocal communication in mouse.

## Discussion

In this study, we identify *in vivo* sumoylation of FOXP2 at K674 (K673 in mouse) in mouse cerebellum during neonatal stages. We demonstrate that sumoylation of FOXP2 plays a critical role in transcription regulation, neuronal development, motor functions and USVs, specifically in the cerebellum. The timing of FOXP2 sumoylation in the cerebellum occurs during a critical period in the formation of the cerebellar neuronal network when dendritic arborization, synaptogenesis, and clustering of potassium channels take place (44, 54). This network is essential for normal cerebellar functions that are at risk in neurodevelopmental disorders such as ASD (15, 16, 18, 19).

To elucidate molecular mechanisms of FOXP2 sumoylation, we demonstrate FOXP2 physically interacts with SUMO-1, SUMO-2 and PIAS3 in 293T cells. To demonstrate *in vivo* evidence that Pias3 sumoylates Foxp2, we performed endogenous co-immunoprecipitation using mouse cerebellum at P10 but we were unable to detect such an interaction (data not shown). In order to investigate the possibility that Foxp2 is sumoylated by other Pias family proteins, we also examined Pias1, Pias2, and Pias4, but again could not detect an interaction (data not shown). Therefore, we hypothesize that the interaction of Foxp2 and the Pias family proteins is a dynamic interaction during the catalytic reaction of an enzyme and a substrate, and therefore it would be challenging to detect endogenous interaction of Foxp2 and Pias family proteins by co-immunoprecipitation. By immunostaining, we observed *in vivo* co-localization of Foxp2 and Pias3 in PCs of mouse cerebellum at P7 (Figure 2F and Figure S2 in Supplement 1), suggesting an opportunity for Foxp2 to be sumoylated by Pias3. However, we cannot rule out possible sumoylation of Foxp2 by other factors *in vivo*. Future studies using knockdown or knockout of Pias3 *in vivo* should determine the requirement of Pias3 in Foxp2 sumoylation. Moreover, loss of the lysine residue of FOXP2 at K647 could have additional detrimental effects in addition to loss of sumoylation, such as conformational changes of FOXP2 leading to altered protein-protein interaction or DNA binding, alterations of other post-transcriptional modifications like ubiquitination, or protein stability. We investigated a number of these possibilities, but there were no differences between FOXP2 WT and KR in our experiments (Figures S6 and S9 in Supplement 1, data not shown), consistent with a recent study showing that FOXP2 sumoylation does not affect protein stability (26).

This is the first examination of the role of FOXP2 post-translational modification in brain development and function. We observe Foxp2 sumoylation occurs specifically during neonatal stages of mouse cerebellar development. We demonstrate FOXP2 sumoylation plays a role in promoting neuronal development through neurite/dendritic outgrowth in both mNPs *in vitro* and PCs *in vivo*. In support of our results, previous studies have shown that FOXP2 transcriptional targets are enriched for genes involved in neuronal differentiation and axon guidance (37, 39, 48). Our data suggest that sumoylation of FOXP2 is spatiotemporally regulated for promoting maturation of neural networks. We further investigated the molecular mechanisms whereby FOXP2 sumoylation may affect transcriptional regulation. We found that sumoylation of FOXP2 does not affect DNA transrepression of a canonical FOXP2 motif. However, we did find that a FOXP2 target gene, *CNTNAP2,* was derepressed by FOXP2 KR. In addition, a recent study has shown that FOXP2 sumoylation modulates transcriptional activity of FOXP2 in regulating target genes such as *DISC1*, *SRPX2* and *MIR200C* (26). In support of these results, sumoylation of L3MBTL2, a protein implicated in transcriptional repression and chromatin compaction, affects only a subset of its target genes, due to derepression by a non-sumoylated form of L3MBTL2, but does not affect chromatin binding as evidenced by ChIP-seq (55). Finally, we found sumoylation of FOXP2 altered the subcellular localization of FOXP2, suggesting that non-nuclear FOXP2 is unable to act as a transcriptional repressor. In contrast, another FOXP2 sumoylation study has reported non-quantitative evidence that sumoylation does not affect subcellular localization of FOXP2 *in vitro* in MCF7 cells (a breast cancer cell line) (26). It is possible that different phenotypes will be observed depending on cell type. In addition, we confirmed that sumoylation modulates the subcellular localization of FOXP2 using quantification of high-resolution confocal imaging both *in vitro* and *in vivo*. Together, these findings suggest sumoylation of FOXP2 provides specificity and selectivity in transcriptional regulation. However several unanswered questions remain: 1) is sumoylation of other transcription factors important for PC function? And 2) is sumoylation of Foxp2 important outside of the cerebellum? We believe this study is just the first of many future studies delineating these distinctions

The cerebellum has been typically thought to be involved in motor functions in the CNS, however recent studies have uncovered important roles for the cerebellum in higher cognitive functions such as language, cognition, emotion, and memory (12–19). We demonstrate that Foxp2 expression is required for normal cerebellar development through dendritic outgrowth and arborization of PCs. Consequently, abnormal PC development in the mouse cerebellum leads to motor and vocal impairments, consistent with previous whole body *Foxp2* KO and mutant mice studies (8, 10). *Foxp2* KO mice also reportedly exhibit a reduction in cerebellar size (8–11). This is consistent with a reduction in both cerebellar size and PCs in patients with ataxia (14, 16–19, 56), language disorders (7, 16–19), ASD (14–16, 18, 19, 57, 58) and schizophrenia (16, 59–61). In this study, we could not ascertain a significant reduction in cerebellar size in our mouse models due to the transfection efficiency of IUE. However, given this inherent variability of IUE it is even more remarkable that we demonstrate consistent and robust developmental disruption to PCs, and impairments of motor functions and USVs with FOXP2 knockdown. PCs play a critical role in modulating and integrating all cerebellar inputs into a unified output: a single PC receives

synaptic inputs from up to 200,000 parallel fibers (62). Therefore, developmental disruptions of even a limited number of PCs can significantly alter cerebellar function in this study. Future studies should investigate the physiological consequences of altered PC development with loss of Foxp2 function. In addition, the other region-specific roles of FOXP2 in brain areas such as cortex and striatum, where FOXP2 is also highly expressed, are still unknown and need to be investigated. However, our *in vitro* data using mNPs and previous *in vitro* studies (38, 39, 45) suggest that FOXP2 and its modification may play a role in neuronal differentiation, which could universally affect neural circuit formation in regions with FOXP2 expression.

Our data using mouse models show that sumoylation can regulate motor behaviors such as USVs. Measuring USVs is widely carried out in genetic ASD model mice (63), with phenotypes observed in models of *Foxp1* (64), *Cntnap2* (52), *Tsc1* (20), *Tsc2* (65), *Nlgn3* (66), *Nlgn4* (67), *Shank1* (68), *Shank2* (69), *Shank3* (70) and *Mecp2* (71). In support of our behavioral results, two recent studies have demonstrated that sumoylation in hippocampus can modify mammalian cognitive behaviors by altering hippocampal-dependent learning and memory by Ubc9, a E2 conjugating enzyme (72), and spatial memory by sumoylation of CREB (73). In addition, neuron-specific knockdown of all *SUMO-1/2/3* in RNAi transgenic mice leads to anxiety-like behavior, and impairs episodic and fear memories (74). Many genes implicated in ASD encode synaptic proteins important for regulating synaptic homeostasis involved in cognition (3, 75, 76). Sumoylation of ASD genes (e.g. MEF2A (77), CASK (78), MeCP2 (79)) has been reported to play roles in regulating synaptic development and function (22, 24, 80), but direct evidence for the requirement for sumoylation in regulating ASD-relevant behaviors has not been shown. In contrast, hyper-sumoylation has been shown to cause functional abnormalities in the brain including seizures by affecting potassium channel function (81). As there is high co-morbidity of seizure disorders and ASD, these results further support a potential role for sumoylation in ASD pathophysiology.

In conclusion, our data demonstrate three novel findings: 1) sumoylation of FOXP2 regulates PC development, 2) sumoylation can direct cerebellar-specific motor behaviors, in particular vocal communication, and 3) cerebellar-specific expression of FOXP2 is required for rodent vocalizations. These findings support a critical role for FOXP2 in the cerebellum. This is compelling given the mounting evidence for cerebellar dysfunction in ASD (15, 16, 18–20, 57, 58) and the identification of numerous ASD-relevant genes regulated by FOXP2 (2). Our data suggest that FOXP2 sumoylation at a single amino acid orchestrates a switch in FOXP2 function in the brain. These findings provide insight into understanding the mechanisms underlying functional diversification of FOXP2 across circuits mediating distinct behaviors in the brain. Further understanding of sumoylation in the CNS should give rise to novel insights and targets for understanding the molecular mechanisms underlying neurodevelopmental disorders.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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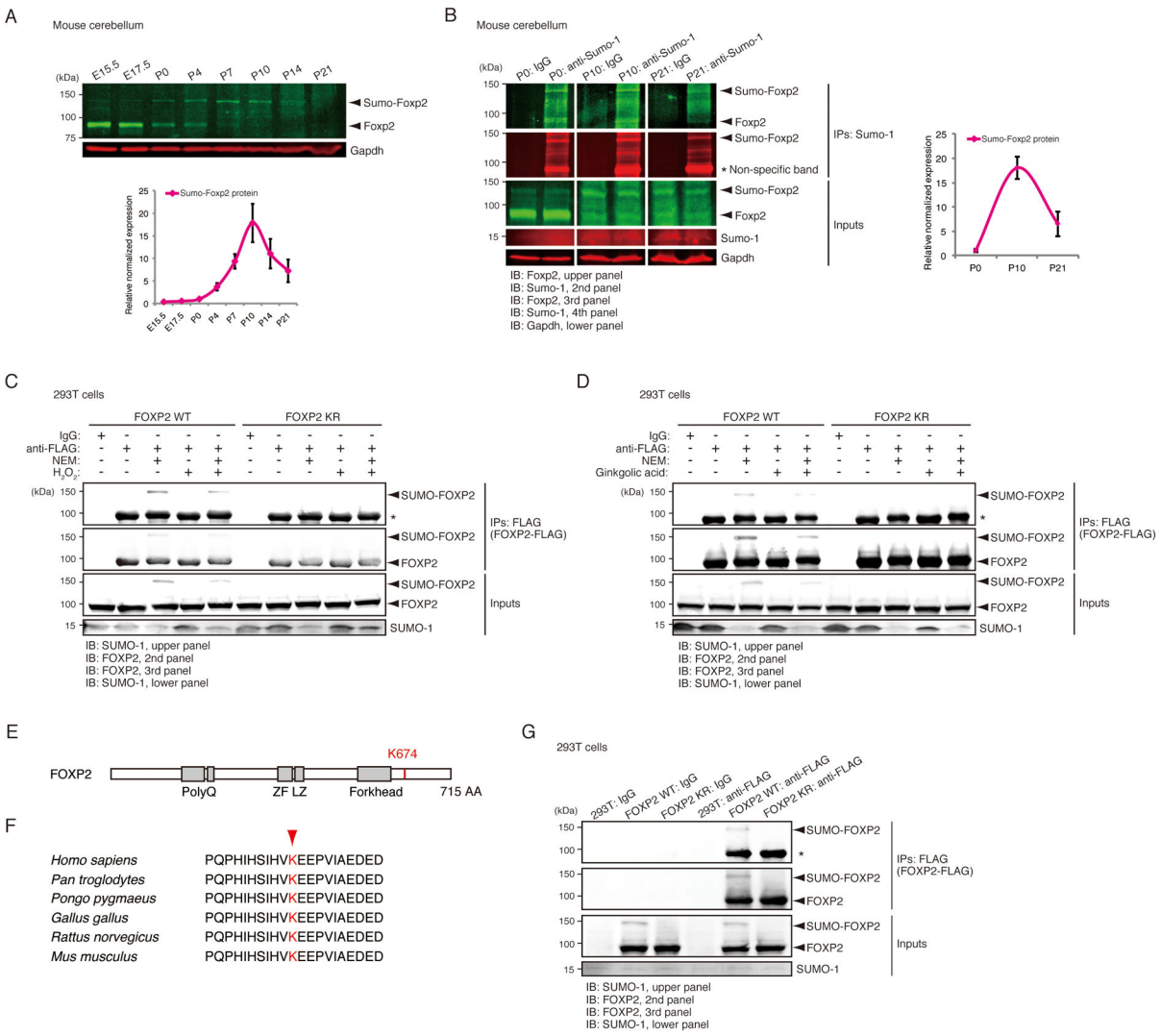


**Figure 1. Sumoylation of FOXP2 in the cerebellum**

**(A)** Endogenous Foxp2 immunoblot of mouse cerebellum using an anti-Foxp2 antibody over time demonstrates an increase in sumoylated Foxp2 protein in early postnatal life. Right panel is quantification of left panel (Sumo-Foxp2 protein). Immunoblot results were normalized to Gapdh and Foxp2 at each time point and then subsequently normalized to non-sumoylated Foxp2 levels at P0. Data are represented as means (±sem), n=4/condition. **(B)** Endogenous co-immunoprecipitation of Foxp2 and Sumo-1 in the mouse cerebellum at P0, P10 and P21 using an anti-Sumo-1 antibody. Right panel is quantification of left panels (Sumo-Foxp2 protein). Immunoblot results were normalized to Foxp2 at each time point and then subsequently normalized to non-sumoylated Foxp2 levels at P0. Data are represented as means (±sem), n=3/condition. **(C, D)** A high molecular weight band is observed blotting for SUMO-1 and FOXP2 in 293T cells. 293T cells were transfected with FLAG-tagged FOXP2 WT or KR construct, and then immunoprecipitation was performed with an anti-FLAG antibody. The intensity of this band is reduced by 1 mM hydrogen peroxide ($H_2O_2$) for 1 hour (**C**) or 100 μM ginkgolic acid for 6 hours (**D**), and increased by N-ethylmaleimide

(NEM). **(E)** Schematic of FOXP2 protein showing K674 location. *PolyQ: polyglutamine motif, ZF: zinc finger, LZ: leucine zipper*. **(F)** K674 is conserved across species. **(G)** Immunoblot of immunoprecipitated FOXP2 showing that mutation of K674 to an arginine results in disappearance of a sumoylated high molecular weight band recognized by an anti-SUMO-1 antibody. 293T cells were transfected with FLAG-tagged FOXP2 WT or KR construct except the 293T control, and then immunoprecipitation was performed with an anti-FLAG antibody. FOXP2 WT: wild type FOXP2, FOXP2 KR: non-sumoylated form of $FOXP2^{K674}$. Asterisk indicates non-specific band of SUMO-1 antibody.

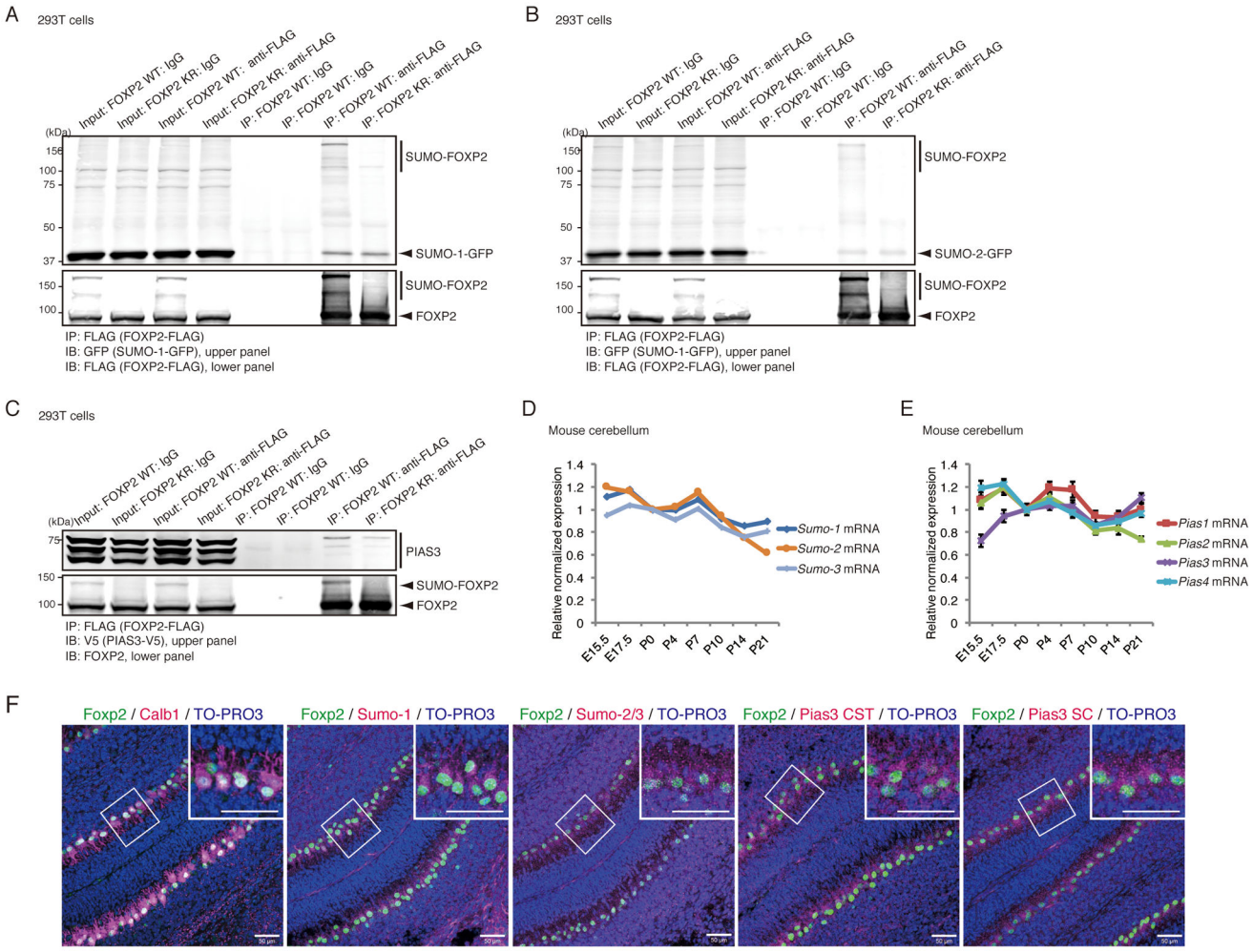


**Figure 2. Sumoylation of FOXP2 by SUMO-1/2 and PIAS3**

**(A, B)** Co-immunoprecipitations of FOXP2 WT, KR and SUMO-1, SUMO-2 proteins in 293T cells. 293T cells were transfected with FLAG-tagged FOXP2 WT or KR construct together with GFP-tagged SUMO-1 (**A**) or SUMO-2 (**B**) construct, and then immunoprecipitation was performed by an anti-FLAG antibody. FOXP2 is sumoylated by both SUMO-1 (**A**) and SUMO-2 (**B**), however these interactions are not observed with FOXP2 KR. **(C)** Co-immunoprecipitations of FLAG-tagged FOXP2 and V5-tagged PIAS3 in 293T cells. 293T cells were transfected with FLAG-tagged FOXP2 WT or KR construct together with V5-tagged PIAS3, and then immunoprecipitation was performed by an anti-FLAG antibody. FOXP2 physically interacts with SUMO E3 ligase PIAS3. **(D, E)** qRT-PCR of key sumoylation genes during cerebellar development. Expression of *Sumo-1*, *Sumo-2* and *Sumo-3* mRNAs (**D**), *Pias1*, *Pias2*, *Pias3* and *Pias4* mRNAs (**E**) remain relatively stable across postnatal mouse cerebellar development. qRT-PCR results were normalized to expression of each gene at P0. Data are represented as means (±sem), n=3/condition. FOXP2 WT: wild type FOXP2, FOXP2 KR: non-sumoylated form of FOXP2$^{K674}$. **(F)** Fluorescent images of in sagittal sections of mouse cerebellum at P7. Co-expression of Foxp2, Sumo-1, Sumo-2/3 and Pias3 are observed in Purkinje cells (PCs) of mouse cerebellum (see also

Figure S2 in Supplement 1). Insets show a higher magnification of the boxed area depicted in each fluorescent image. CST: Pias3 antibody from Cell Signaling Technology, Pias3 SC: Pias3 antibody from Santa Cruz Biotechnology. Scale bars: 50 μm.

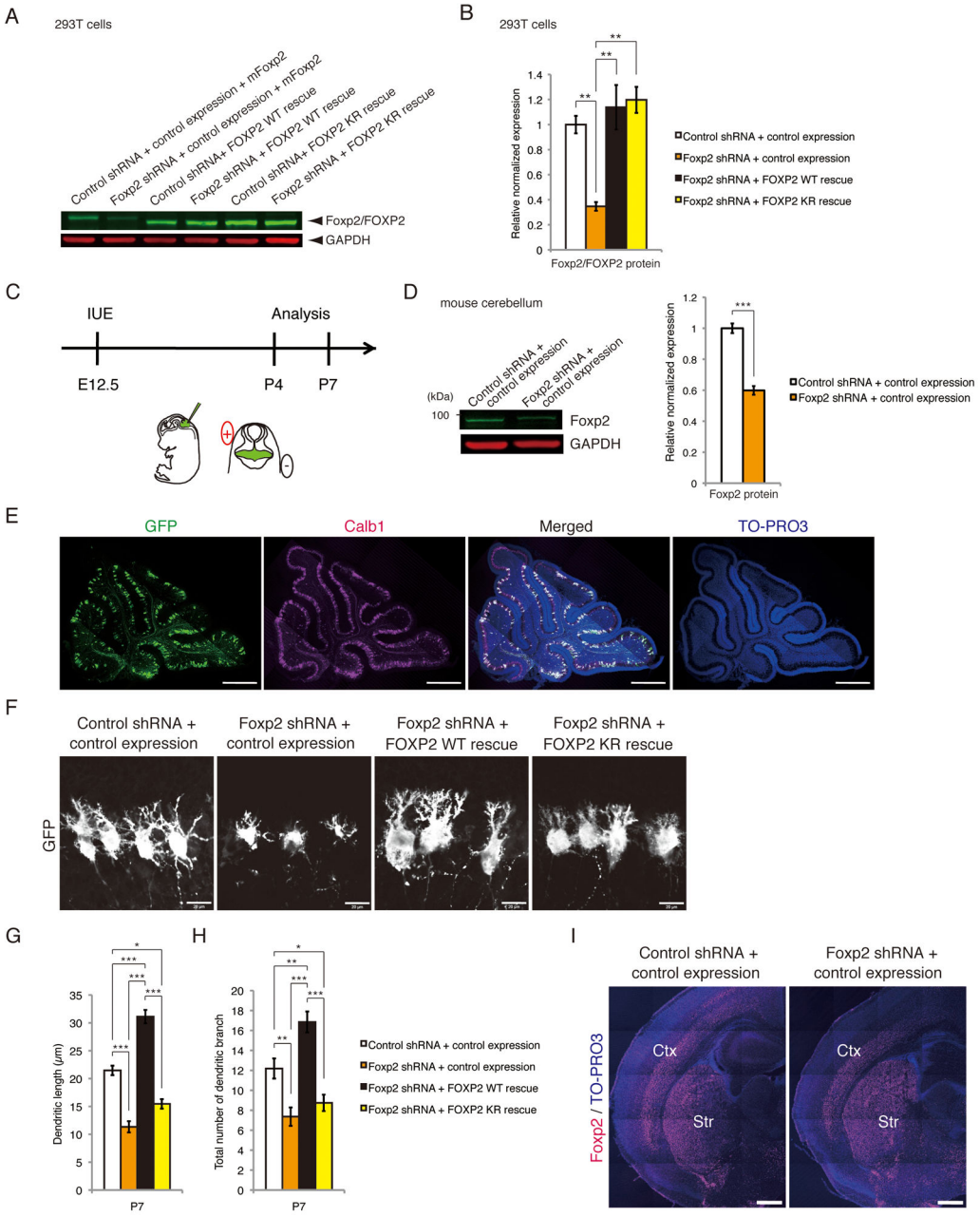


**Figure 3. Foxp2 sumoylation regulates Purkinje cell development *in vivo***

**(A)** Representative immunoblot for Foxp2 demonstrating knockdown of mouse (m) Foxp2 protein with a specific shRNA. Co-expression with either FOXP2 WT or KR rescue constructs that are modified to resist the hairpin rescues the knockdown using 293T cells. 293T cells were transfected with control shRNA or Foxp2 shRNA construct together with empty vector for control with mFoxp2 construct, FOXP2 WT or KR rescue construct. **(B)** Quantification of knockdown and rescue of Foxp2/FOXP2 protein in 293T cells. Data are represented as means (±sem). Asterisks indicate P<0.01, one-way ANOVA with a Tukey's multiple comparison test (P<0.0018), n=3/condition. **(C)** Schematic and time line of *in utero* electroporation (IUE) of Foxp2 manipulation into the mouse cerebellum followed by

neonatal morphological assessment of PCs. **(D)** Immunoblot and quantification of Foxp2 protein knockdown in electroporated mouse cerebellum at P7. Approximately 40.1±2.8% knockdown in cerebellum by IUE is observed. Data are represented as means (±sem), Asterisks indicate P=0.0007, *t*-test, n=3/condition. **(E)** An example of IUE specificity is confirmed by GFP expression limited to only PCs in sagittal section of electroporated mouse cerebellum at P7. Calb1 is a marker for PCs. **(F)** Fluorescent images of PCs in sagittal sections of mouse cerebellum at P7 for each condition. **(G, H)** Quantification of dendritic length (**G**) and branching (**H**) in PCs for each condition. Decreased dendritic outgrowth and arborization of PCs are observed after Foxp2 knockdown, and FOXP2 WT and KR forms rescue dendritic outgrowth, but KR is unable to fully rescue the arborization. Data are represented as means (±sem). Asterisks indicate ***P<0.001, **P<0.01, *P<0.05, one-way ANOVA with a Tukey's multiple comparison test (P<0.0001 for dendritic length, P<0.0001 for dendritic branching), n=70–155 cells for dendritic length, 50–77 cells for branching/condition from 3–4 animals. **(I)** Fluorescent images of mouse cortex at P7, which are electroporated into cerebellum. No differences in Foxp2 expression by IUE of Foxp2 knockdown are observed in both cortex and striatum. Ctx: cortex, Str: striatum. Scale bars: 500 μm in **E**, 20 μm in **F**, 500 μm in **I**.

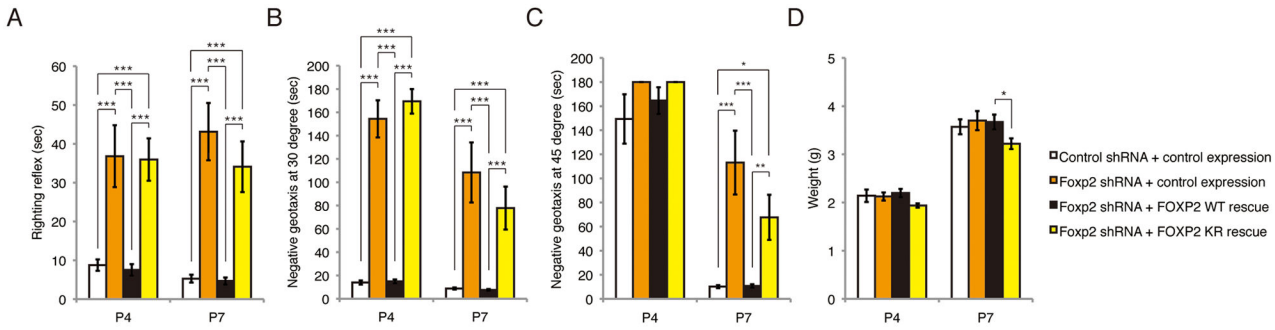


**Figure 4. Foxp2 sumoylation regulates motor functions**

**(A–C)** Knockdown of endogenous Foxp2 results in deficiencies of righting reflex (**A**) and negative geotaxis at 30 (**B**) and 45 degrees (**C**) at P4 and P7. These Foxp2 knockdown phenotypes can be rescued with FOXP2 WT, but not KR. The 45 degree angle of our system is difficult for P4 pups, but not for P7 pups. Asterisks indicate ***P<0.001, **P<0.01, *P<0.05, two-way ANOVA with a Tukey's multiple comparison test (righting reflex: interaction, P=0.69; age, P=0.89, genotype, P<0.0001; negative geotaxis at 30 degrees: interaction, P=0.0011; age, P<0.0001, genotype, P<0.0001; negative geotaxis at 45 degrees: interaction, P=0.0089; age, P<0.0001, genotype, P<0.0001; n=9–15/condition). **(D)** Mouse weight was measured at P4 and P7 after behavioral testing. There is no statistical difference among conditions at both stages. Data are represented as means (±sem). Asterisk indicates P<0.05 two-way ANOVA with a Tukey's multiple comparison test (interaction, P=0.71; age, P<0.0001, genotype, P=0.023), n=9–15/condition.

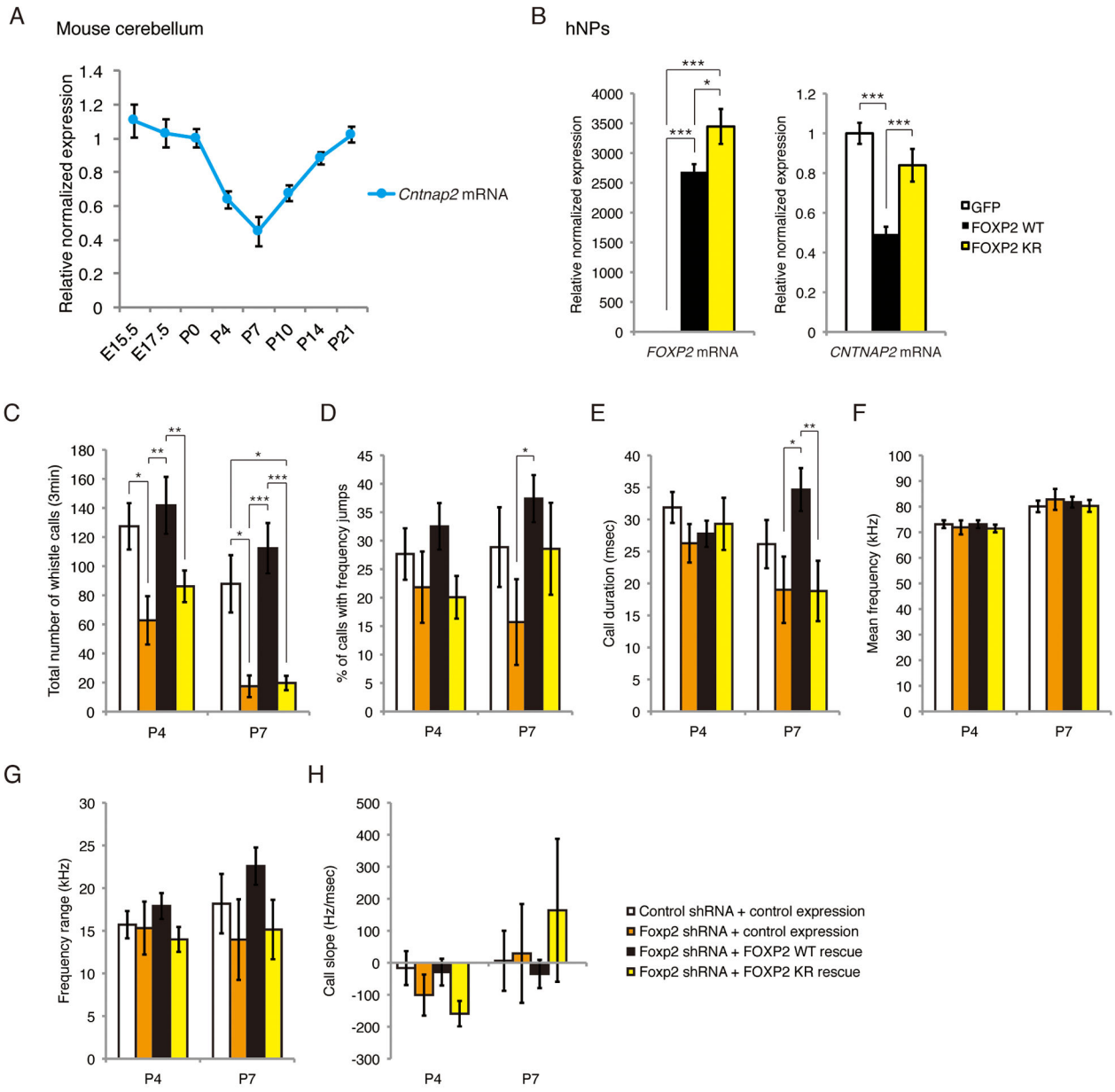


**Figure 5. Foxp2 sumoylation regulates vocal communication**

(A) qRT-PCR of *Cntnap2* during mouse cerebellar development. *Cntnap2* mRNA expression is decreased at the time point when sumoylation of Foxp2 is strongly observed in developing mouse cerebellum at P7. qRT-PCR results were normalized to *Cntnap2* expression at P0 (Figure 1A). Data are represented as means (±sem), n=3/condition. (B) Quantification of qRT-PCR in human neural progenitors (hNPs) expressing either FOXP2 WT or FOXP2 KR. FOXP2 WT but not KR can repress expression of *CNTNAP2*. Data are represented as means (±sem). Asterisks indicate ***P<0.001, *P<0.05, one-way ANOVA with a Tukey's multiple comparison test (P<0.0001 for *FOXP2*, P<0.0001 for *CNTNAP2*), n=12/condition. (C–H) USVs were analyzed in detail. Foxp2 knockdown results in a decrease in USVs at P4 and P7. This USV deficiency can be rescued with FOXP2 WT construct, but not KR construct.

(C), Total number of whistle calls (USVs: interaction, P=0.70; age, P=0.0001, genotype, P<0.0001); (D), percentage of calls with frequency jumps (interaction, P=0.63; age, P=0.61, genotype, P=0.048); (E), call duration (interaction, P=0.11; age, P=0.12, genotype, P=0.08); (F), mean frequency (interaction, P=0.86; age, P<0.0001, genotype, P=0.89); (G), frequency range (interaction, P=0.74; age, P=0.37, genotype, P=0.14); and (H), mean call slope (interaction, P=0.44; age, P=0.14, genotype, P=0.98). Data are represented as means (±sem). Asterisks indicate ***P<0.001, **P<0.01, *P<0.05, two-way ANOVA with a Tukey's multiple comparison test, n=9–15/condition.