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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 posttranslational 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 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 mice) in the cerebellum of neonates. In vitro coimmunoprecipitation and in vivo colocalization experiments suggest that PIAS3 acts as the small ubiquitin-like modifier E3 ligase for FOXP2 sumoylation. This sumoylation modifies transcriptional regulation by FOXP2. We demonstrated that FOXP2 sumoylation is required for regulation of cerebellar motor function and vocal communication, likely through dendritic outgrowth and arborization of Purkinje cells in the mouse cerebellum.

CONCLUSIONS: Sumoylation of FOXP2 in neonatal mouse cerebellum regulates Purkinje cell development and motor functions and vocal communication, demonstrating evidence for sumoylation in regulating mammalian behaviors.

Keywords: Cerebellum, FOXP2, Motor function, Purkinje cells, Sumoylation, Vocal communication

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The transcription factor forkhead box P2 (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 corticostriatal function (4–6), but the cerebellum appears to be a key brain region for FOXP2 function, as patients with mutations in *FOXP2* demonstrate significant gray matter reduction in the cerebellum as evidenced by magnetic resonance imaging (7), and genetic disruption of *Foxp2* in mice results in decreased cerebellar size (8–11). Recent studies have revealed important roles for the cerebellum in higher cognitive functions, such as language, cognition, emotion, and memory (12–19). In particular, function of Purkinje cells (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 posttranslational 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, messenger RNA transport in axons, and mitochondrial function (22–24). During sumoylation, the small ubiquitin-like modifier (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, sentrin-specific proteases (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, Ataxin-3, Ataxin-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

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and potentially other posttranslational 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. We explored the role of FOXP2 sumoylation in neuronal development and mammalian behavior related to the cerebellum. We provide in vivo evidence demonstrating the requirement for sumoylation and cerebellar-specific expression of FOXP2 to direct complex motor behaviors and vocal communication. We found that 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

Methods and materials are described in greater detail in the Supplemental Methods and Materials.

Animal Experiments

Wild-type (WT) 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 fourth ventricles of embryonic day 12.5 embryos to target PCs. Electroporation of the embryo was performed (five 50-ms pulses of 33 V with an interval of 950 ms) (CUY21 SC electroporator; Nepa Gene Co., Ltd., Ichikawa-City, Chiba, Japan) using platinum plate electrode tweezers (CUY650; Protech International, Inc., Boerne, TX) (27–30). All procedures were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center.

Biochemical Experiments

293T cells were transfected using FuGENE 6 (Product No. E2691, Promega Corporation, Madison, WI) and harvested 48 hours later. *N*-ethylmaleimide 50 mmol/L (Product No. E3876; Sigma-Aldrich, St. Louis, MO) was used as a SUMO protease inhibitor. Hydrogen peroxide 1 mmol/L (31) and ginkgolic acid 100 μmol/L (Product No. 75741; Sigma-Aldrich) (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 although 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 hydrogen peroxide treatment, a mechanism for reversible inhibition of SUMO conjugating enzymes (Figure 1C) (31), 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). On mutation of this lysine to arginine (the nonsumoylated 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 or the amount immunoprecipitated by an antibody (Supplemental Figure S1). 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 colocalization 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 and Supplemental Figure S2). 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 depends on 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 (Supplemental Figure S3). In contrast, FOXP2 KR was unable to promote the length of Tuj1-positive and MAP2-positive neurites as effectively (Supplemental Figure S3). 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