Mitochondrial dysfunction and oxidative stress contribute to cognitive and motor impairment in FOXP1 syndrome

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FOXP1 syndrome caused by haploinsufficiency of the forkhead box protein P1 (FOXP1) gene is a neurodevelopmental disorder that manifests motor dysfunction, intellectual disability, autism, and language impairment. In this study, we used a Foxp1+/− mouse model to address whether cognitive and motor deficits in FOXP1 syndrome are associated with mitochondrial dysfunction and oxidative stress. Here, we show that genes with a role in mitochondrial biogenesis and dynamics (e.g., Foxo1, Pgc-1α, Tfam, Op1, and Drp1) were dysregulated in the striatum of Foxp1+/− mice at different postnatal stages. Furthermore, these animals exhibit a reduced mitochondrial membrane potential and complex I activity, as well as decreased expression of the antioxidants superoxide dismutase 2 (Sod2) and glutathione (GSH), resulting in increased oxidative stress and lipid peroxidation. These features can explain the reduced neurite branching, learning and memory, endurance, and motor coordination that we observed in these animals. Taken together, we provide strong evidence of mitochondrial dysfunction in Foxp1+/− mice, suggesting that insufficient energy supply and excessive oxidative stress underlie the cognitive and motor impairment in FOXP1 deficiency.

FOXO1 syndrome | ASD | cognitive and motor impairment | mitochondrial defect | ROS

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired social communication, restricted or stereotypical interests, and repetitive behaviors. Molecular genetic studies on ASD individuals and their families have identified several hundred ASD risk genes, including the forkhead box protein P1 (FOXO1) gene. In humans, haploinsufficiency of FOXO1 leads to FOXO1 syndrome (Online Mendelian Inheritance in Man [OMIM] 605515), a neurodevelopmental disorder characterized by delayed motor and language development, intellectual disability, autistic traits, and dysmorphic features (1, 2). Since the first report in 2010 (1), more than hundred cases with de novo mutations have been described in the literature, and FOXO1 is now among the highest ranked ASD genes according to the Simons Foundation Autism Research Initiative (SFARI) Gene database (3).

FOXO1 is a member of the Forkhead Box P (FOXO) subfamily of transcription factors, which also includes FOXP2, FOXP3, and FOXP4. Both FOXP1 and FOXP2 are highly conserved in vertebrates and form homo- and heterodimers (4, 5), and while FOXP2 mutations have been linked with a distinct speech and language disorder (6), FOXO1 mutations affect much more global neural functions. They may also form oligomers with FOXP4 (5), suggesting that these three related transcription factors regulate cognitive behavior and motor development via common pathways (7).

The complete loss of Foxp1 in mice is lethal at embryonic day 14.5 (E14.5) (8), but the phenotype of heterozygous Foxp1+/− mice resembles that of individuals with FOXP1 syndrome. Foxp1+/− animals exhibit behavioral and muscle deficits such as reduced neonatal ultrasonic vocalization and grip strength (9), that are first indications for social deficits and hypotonia in animals. Furthermore, Foxp1+/− mice display esophageal achalasia and impaired peristalsis in the colon (10), reflecting the gastrointestinal disturbances seen in many individuals with FOXP1 syndrome (2, 11).

In the brain, Foxp1 is mainly expressed in the cortex, hippocampus, and striatum (7, 12). In the cortex, Foxp1 is mainly expressed in the intratelecephalic projection neurons in layers 3 to 5, regulating neonatal vocalizations (13). In the hippocampus, Foxp1 expression is seen in the CA1 subfield and subiculum region, required for spatial learning and synaptic plasticity (14). In the striatum, Foxp1 has been shown its most abundant expression and to regulate the cellular composition and connectivity in a cell type-specific manner (15). Thus, Foxp1 has an essential role in regulating neurogenesis and synapse organization. However, the role of Foxp1 and its transcriptional regulators have not been studied on metabolic disturbances and oxidative

Significance

FOXO1 haploinsufficiency underlies cognitive and motor impairments in individuals with FOXP1 syndrome. Here, we show that mice lacking one Foxp1 copy exhibit similar behavioral deficits, which may be caused by striatal dysfunction. Indeed, Foxp1+/− striatal medium spiny neurons display reduced neurite branching, and we show altered mitochondrial biogenesis and dynamics; increased mitophagy; reduced mitochondrial membrane potential, structure, and motility; and elevated oxygen species in the striatum of these animals. As FOXO1 is highly conserved, our data strongly suggest that mitochondrial dysfunction and excessive oxidative stress contribute to the motor and cognitive impairments seen in individuals with FOXP1 syndrome. Thus, mitochondrial homeostasis is critical for normal development and can explain deficits in neurodevelopmental disorders.

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mitochondrial malfunction. In fact, mitochondrial function plays an important role in neurogenesis, synaptic plasticity, and neurotransmission (16–18).

Metabolic disturbances and mitochondrial malfunctions are also more common in individuals with ASD than in the general population, indicating that they play a role in the pathogenesis of ASD (19, 20). However, only certain subgroups of the ASD population are affected. To investigate whether mitochondrial impairment might underlie the cognitive and motor features of FOX1 syndrome, we analyzed mitochondrial structure and motility, and the expression of genes that regulate mitochondrial biogenesis and dynamics in the striatum of Foxp1−/− mice. We examined autophagy, production of reactive oxidative species (ROS), metabolic status, and apoptosis in these animals. In addition, we analyzed neuronal morphology, motor function, as well as emotional and spatial learning and memory.

Results

**Dysregulated Mitochondrial Biogenesis in the Foxp1−/− Striatum.**

Nervous system-specific and conventional Foxp1 knockout mice (9, 12) and MRI images of FOX1 syndrome individuals (2, 21) have revealed that the striatum is particularly affected by FOX1 deficiency. To evaluate Foxp1 expression at early postnatal development as well as adult stage in the striatum, we compared Foxp1 mRNA and protein level of Foxp1−/− to that of wild-type (WT) littermates at postnatal day (P) 1.5, P12.5, and 8 wk. The rational for the choice P1.5 is that at this time of postnatal development as well as adult stage in the striatum, we have revealed that the striatum is particularly affected (9, 12) and MRI images of FOXP1 syndrome individuals (Fig. 1 A–C) show significantly reduced protein levels in the striatum compared to WT striata (Fig. 1 G), supporting the impairment of mitochondrial biogenesis in the Foxp1−/− striatum.

**Genes Inolved in Fission and Fusion Are Dysregulated in the Foxp1−/− Striatum, Pointing to Impaired Mitochondrial Dynamics.**

A direct transcriptional target of Foxp1, Pgc-1α, showed significantly reduced protein levels in the Foxp1−/− striatum (Fig. 1 C and SI Appendix, Fig. S3). Pgc-1α regulates mitochondrial dynamics by influencing dynamin-like 120-kDa protein (Opal1), mitofusin-1 (Mfn1), and mitofusin-2 (Mfn2)—all involved in fusion—and dynamin-1-like protein (Drp1) and mitochondrial fission 1 protein (Fis1), which are involved in fission (Fig. 2 A). Expression of these genes was also altered in the Foxp1−/− striatum at one or more of the investigated time points (SI Appendix, Fig. S4).

Mfn1 protein levels, were significantly reduced at P12.5 and elevated at 8 wk in the Foxp1−/− striatum compared to WT (Fig. 2 B and SI Appendix, Fig. S5). Opal1 has two isoforms: L-Opal1 and S-Opal1. S-Opal1 protein expression was lower at all three time points. The increased L-Opal1/S-Opal1 ratio (Fig. 2 B and SI Appendix, Fig. S5) suggests disturbed Foxp1−/− mitochondrial morphology (31). Drp1 protein levels increased at P1.5 and decreased at P12.5 and 8 wk in Foxp1−/− striata (Fig. 2 C and SI Appendix, Figs. S2 C and S5C). Reversible Drp1 phosphorylation at Drp1(Ser637) blocks mitochondrial fission (32), so we measured Ser637-phosphorylated Drp1 and detected increased levels in the Foxp1−/− striatum than in the WT striatum at all examined stages (Fig. 2 C and SI Appendix, Fig. S5C). Fis1 mediates fission by recruiting Drp1 to mitochondria and also promotes mitophagy. Significantly elevated Fis1 protein levels in Foxp1−/− striata at all examined stages indicate increased mitochondrial fission (Fig. 2 C and SI Appendix, Fig. S5D). However, Drp1 must be recruited from the cytoplasm to the mitochondrial surface to mediate the mitochondrial fission (33). Higher Drp1 expression was detected in the cytosolic fraction at P12.5 and 8 wk (SI Appendix, Fig. S5E), suggesting decreased fission in the Foxp1−/− striatum.

**Mitophagy Is Increased in the Foxp1−/− Striatum.** Damaged or excess mitochondria are eliminated by mitophagy. The microtubule-associated protein 1A/1B-light chain 3 I (LC3I) is conjugated to phosphatidylethanolamine to form LC3II, a structural protein in autophagosome membranes. We measured both LC3I and its protein as well as Parkin protein expression in Foxp1−/− striata at one or more of the three stages investigated (SI Appendix, Fig. S1D). In particular, proliferator-activated receptor gamma coactivator 1-alpha (Pgc-1a) and mitochondrial transcription factor A (Tfam) were downregulated on protein level at all three stages in Foxp1−/− striatal tissue (Fig. 1 C and D and SI Appendix, Fig. S2 C and D), which was not the case for nuclear respiratory factor 1 (Nrf1) (SI Appendix, Fig. S2E). Moreover, mitochondrial markers Atp5a and Tom20 also showed decreased protein levels in adulthood, indicating impaired mitochondrial biogenesis (Fig. 1 E and F).

Next, we calculated mitochondrial DNA copy numbers and deletions using the mtDNA/nDNA and Ndj1/Nd4 ratio at P1.5, P12.5, and 8 wk. The mtDNA/nDNA ratio was significantly reduced and the Ndj1/Nd4 ratio was significantly increased in Foxp1−/− striata compared to WT striata (Fig. 1 G), supporting the impairment of mitochondrial biogenesis in the Foxp1−/− striatum.

**Mitochondrial Dysfunction and Excessive Oxidative Stress in the Foxp1−/− Striatum.** Proper mitochondrial dynamics, biogenesis, and mitophagy are essential for the maintenance of mitochondrial homeostasis and function. As mitochondria supply energy by generating metabolites and adenosine triphosphate (ATP) via tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS), we measured adenosine compounds (ATP, adenosine diphosphate [ADP], adenosine monophosphate [AMP], nicotinamide adenine dinucleotide [NAD], nicotinamide adenine dinucleotide-hydrogen [NADH], nicotinamide adenine dinucleotide phosphate [NADPH], S'-methylthioadenosine, adenosine, S'-adenosylmethionine) in primary striatal neurons. Foxp1−/− cells
Fig. 1. Mitochondrial biogenesis is altered in the Foxp1<sup>1-/-</sup> striatum. (A) The expression of Foxp1 isoform A and D were quantified in adult WT and Foxp1<sup>1-/-</sup> striatal tissue by Western blot. At the age of 8 wk Foxp1 A was reduced by ~57% and Foxp1 D by ~55% in Foxp1<sup>1-/-</sup> mice compared to WT animals. (B) Foxo1 protein was reduced by ~42% in adult Foxp1<sup>1-/-</sup> tissue, (C) Pgc-1α by ~24%, and (D) Tfam by ~17%. (E and F) Atp5a and Tom20 were decreased by ~33% and ~64%, respectively, compared with WT mice at adult stage. (G) Mitochondrial copy number and mtDNA deletion were evaluated by quantitative real-time PCR. The mtDNA/nDNA ratio was calculated by normalizing the expression of D-loop, 16sRNA, and Nd1 to Hk2 and B2m. mtDNA/nDNA ratios were reduced in Foxp1<sup>1-/-</sup> tissue at all three stages, indicating a decreased mitochondrial copy number. The Nd1/Nd4 ratio was elevated in the Foxp1<sup>1-/-</sup> striatum at P1.5 and P12.5, indicating increased mtDNA deletions. In each experiment, at least four animals per group were used, the exact number of animals is given in the figure. In the box-and-whisker plot, the boxes represent the first and third quartiles, the whiskers represent 95% CI, and the lines within the boxes are median values. Weak outliers are marked with a circle. Asterisks indicate a significant difference (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001); two-way ANOVA.
Fig. 2. The Foxp1<sup>+/−</sup> striatum displays altered mitochondrial dynamics and increased mitophagy. (A) Scheme illustrating alterations of the mitochondrial machinery in the Foxp1<sup>+/−</sup> striatum. Reduced Foxp1, Foxo1, Tfam, and Pgc-1α expression indicating disrupted mitochondrial biogenesis is represented by down-pointing arrows (red). Pgc-1α initiates mitochondrial dynamics by regulating genes involved in fusion (Mfn1, Mfn2, and Opa1) and fission (Drp1 and Fis1). LC3A/B and Pink-Parkin regulate mitophagy to eliminate damaged mitochondria. (B) Mfn1 expression was increased by ∼89% in Foxp1<sup>+/−</sup> striata at 8 wk compared to WT tissue. Long (L)-Opa1 expression was not altered, short (S)-Opa1 expression by ∼27% at 8 wk in the Foxp1<sup>+/−</sup> striatum. Consequently, the ratio of L-Opa1/S-Opa1 was significantly increased in the Foxp1<sup>+/−</sup> striatum than in WT tissue at adulthood by ∼20%. (C) Drp1 levels were increased by ∼51% at 8 wk in Foxp1<sup>+/−</sup> animals. pDrp1 (Ser637) showed a significantly increased expression at adulthood compared to WT mice. Fis1 displayed an increased expression by ∼109% at 8 wk in the Foxp1<sup>+/−</sup> striatum. (D) LC3A/BI and LC3A/BII isoform expression was quantified by Western blot in striatal tissue of both genotypes. LC3A/BI expression did not differ between the genotypes; however, LC3A/BII levels were significantly increased in Foxp1<sup>+/−</sup> tissue at 8 wk. (E) Mitophagy was increased by ∼95% in Foxp1<sup>+/−</sup> striatal neurons at DIV8, as shown by colocalization of Mitotracker and Lyso-tracker staining (see arrowheads) (WT = 5 mice, 7 images, 3,441 total lysosomes; Foxp1<sup>+/−</sup> = 7 mice, 8 images, 5,037 total lysosomes). In each experiment, at least five animals per group were used, the exact number of animals is given in the figure. Weak outliers are marked with a circle. Asterisks indicate significant difference (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001); n.s., nonsignificant; two-way ANOVA; DIV, days in vitro.
exhibited increased AMP and decreased NADPH levels; all other molecules tested were not altered (SI Appendix, Fig. S7).

Mitochondrial membrane potential ($\Delta \psi_m$) drives ATP generation; therefore, we compared the $\Delta \psi_m$ in WT and Foxp1$^{+/+}$/C0 striatal neurons. The $\Delta \psi_m$ was lower in Foxp1$^{+/+}$/C0 neurons than in WT neurons, as shown by decreased tetramethylrhodamine, methyl ester (TMRM; readily sequestered by active mitochondria) fluorescence, which indicates reduced OXPHOS in Foxp1$^{+/+}$/C0 striatal neurons. The $\Delta \psi_m$ was lower in Foxp1$^{+/+}$/C0 neurons than in WT neurons, as shown by decreased tetramethylrhodamine, methyl ester (TMRM; readily sequestered by active mitochondria) fluorescence, which indicates reduced OXPHOS in

Fig. 3. The Foxp1$^{+/+}$ striatum exhibits mitochondrial dysfunction and increased oxidative stress. (A) Mitochondrial membrane potential ($\Delta \psi_m$) was measured in primary striatal neurons of both genotypes by TMRM staining. The TMRM fluorescent signal was significantly lower in Foxp1$^{+/+}$ neurons compared to WT cells, indicating a lower mitochondrial membrane potential. (B) ROS abundance was quantified in living striatal neurons by MitoSOX Red, nuclei (blue) were stained with Hoechst 33324. Foxp1$^{+/+}$ cultures had ~64% more MitoSOX-red-positive neurons than WT cultures, indicating increased amounts of ROS. (C) DCF detection of striatal tissue by malondialdehyde (MDA) fluorescence. At all three stages, MDA fluorescence was significantly increased in Foxp1$^{+/+}$ tissue after 1 h and 2 h of incubation compared with WT tissue. (E) Complex I and III activity in the adult WT and Foxp1$^{+/+}$ striatum. Complex I activity was decreased by ~9% in Foxp1$^{+/+}$ tissue compared with WT, whereas complex III activity did not differ between the genotypes. (F) Measurement of reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG). Reduced GSH is decreased by ~40% and oxidized GSSG by ~27% in Foxp1$^{+/+}$ tissue, leading to an elevated GSSG/GSH ratio by ~25%. (G) mRNA expression of Gpx1, Sod2, and Prdx3 was analyzed by quantitative real-time PCR at the age of 8 wk. Foxp1$^{+/+}$ striata exhibited a reduced expression of Sod2 by ~33% compared to WT tissue. (H) Sod2 protein was quantified in striatal tissue at the age of 8 wk and in primary striatal neurons (DIV8) by Western blot. Both Foxp1$^{+/+}$ striatum and primary neurons displayed reduced Sod2 levels by ~45% and ~16%, respectively, compared to WT. In A and B, at least four animals per group were investigated and at least three replicates were performed per animal. The exact number of animals used is given in the figure. Weak outliers are marked with a circle. Asterisks indicate a significant difference (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001); n.s., nonsignificant; two-way ANOVA; DIV, days in vitro.
Thus, to directly estimate the rate of OXPHOS, we quantified oxygen consumption rate (OCR), an indicator of the mitochondrial respiration, using the Seahorse metabolic flux analyzer (SI Appendix, Fig. S8A). Baseline mitochondrial respiration (SI Appendix, Fig. S8B) as well as the rate of OXPHOS (SI Appendix, Fig. S8C) were similar between Foxp1+/− mutant and WT striatal neurons. In a pathological condition, to match the increased metabolic demands, cells tend to shift their metabolic state from OXPHOS toward aerobic glycolysis (35, 36). Hence, to estimate whether such “metabolic state” shift occurred in Foxp1+/− mutant neurons, we quantified the rate of glycolysis by estimating extracellular acidification rate (ECAR) in our Seahorse metabolic flux analysis assays (SI Appendix, Fig. S8D). We
found that Foxp1+/− and WT striatal neurons performed glycolysis at a similar level (SI Appendix, Fig. S8E).

Next, we quantified ROS in living primary striatal neurons and striatal tissue. Significantly more Foxp1+/− neurons than WT neurons were stained positively with superoxide stain MitoSOX Red (Fig. 3B). More ROS were also detected in Foxp1+/− than in WT striatal tissue as shown by quantification of 2′0,7′0-dichlorofluorescein (DCF) (Fig. 3C). Moreover, significantly elevated malondialdehyde formation and higher 4-hydroxynonenal levels in the Foxp1+/− striatum points to increased lipid peroxidation (Fig. 3D and SI Appendix, Fig. S9A). A source of elevated ROS may be mitochondrial complex I and complex III, especially under pathological conditions (37, 38). The activity of complex I was significantly reduced in the Foxp1+/− striatum, while the activity of complex III was not altered in 8-wk-old animals (Fig. 3E). Excessive ROS in cells are degraded by antioxidants such as reduced glutathione (GSH). Foxp1+/− tissue exhibited decreased GSH and oxidized glutathione (GSSG) levels as well as a reduced GSH/GSSG ratio, indicating significantly increased oxidative stress (Fig. 3F).

To assess antioxidative capacity, we measured glutathione peroxidase 1 (Gpx1), superoxide dismutase 2 (Sod2), and thioredoxin-dependent peroxide reductase (Prdx3). We detected reduced Gpx1 and Prdx3 expression at P1.5 and P12.5 and reduced Sod2 levels at P12.5 and 8 wk in the Foxp1+/− striatum (Fig. 3G and SI Appendix, Fig. S9B). Elevated ROS at P12.5 and 8 wk in Foxp1+/− tissue suggests that Sod2 might play the major role in ROS accumulation. Sod2 protein analysis confirmed this, showing strongly reduced expression in striatal primary Foxp1+/− neurons at 8 days of in vitro differentiation (DIV8) and in P1.5 and 8-wk-old Foxp1+/− animals (Fig. 3H and SI Appendix, Fig. S9C).

Increased oxidative stress triggers apoptosis (39, 40). To investigate apoptosis, we measured mitochondrial cytochrome c release, which is linked to early stages of apoptosis.

Fig. 5. Foxp1+/− mice show deficits in motor performance and cognition. (A) The walking pattern of WT and Foxp1+/− mice was analyzed by the CatWalk gait analysis system. Foxp1+/− mice exhibited a reduced walking speed by ∼34% and their step number was significantly increased compared to WT mice. (B) Evaluation of endurance and muscle strength by treadmill exhaustion test. Foxp1+/− mice covered a significantly decreased distance on the treadmill (96 ± 37 m versus 245 ± 32 m), exhausted earlier (8 ± 2 min versus 16 ± 1 min), exhibited a lower maximum speed (14 ± 2 m/min versus 22 ± 1 m/min), and received significantly more electrical shocks during the same period on the treadmill than WT animals. (C) Active place avoidance test. On day 1 in the training phase (nine trials), Foxp1+/− mice displayed increased traveling and entered the shock zone more frequently than WT mice. In addition, they spent more time in there, covered a longer distance in the shock zone, and thus received more shocks than WT mice. On day 2, when memory was tested, Foxp1+/− mice entered the shock zone earlier, spent significantly more time in there, thus receiving –60% more shocks than their WT littermates. In each experiment, at least six animals per group were used, the exact number of animals is given in the figure. In C, course diagrams represent means ± SEM. Weak outliers are marked with a circle. Asterisks indicate significant difference (∗P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001); n.s., nonsignificant; three-way ANOVA. LF, left forelimb; RF, right forelimb; LH, left hindlimb; and RH, right hindlimb.
Comparable to the analysis of ROS, cytochrome c levels in the cytosolic fraction did not differ at P1.5, but were significantly elevated in Foxp1+/− tissue at P12.5 and 8 wk (SI Appendix, Fig. S10A). We further analyzed apoptosis by active (cleaved) caspase-3 immunostaining and by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay at the three developmental time points and quantified active caspase-3 in the adult striatum by Western blot. However, an increased rate of apoptosis in the Foxp1+/− striatum was not detected (SI Appendix, Figs. S10 B and C and S11). Taken together, these results suggest that Foxp1+/− neurons are able to maintain their metabolic state without switching between OXPHOS and glycolysis, but at a price of increased mitochondrial stress and ROS generation.

**Mitochondrial Structure, Motility, and Dendritic Arborization Are Altered in Foxp1+/− Striatal Neurons.** Mitochondrial stress and excessive ROS generation can lead to breakdown of mitochondrial network structure and influence the mitochondrial motility. To evaluate mitochondrial structure and dynamics, we stained the mitochondria revealed that Foxp1+/− mice exhibit a complete loss of Foxp1 in the nervous system (12) and to mice with a complete loss of Foxp1 in both D1- and D2-medium spiny neurons (MSNs) or D2-MSNs alone (15) revealed that reduced Foxp1 expression in Foxp1+/− mice affects gene expression differently than the complete loss of Foxp1 does. An example is Pgc-1α (28, 43), regulated by Foxo1, which is a transcriptional coactivator and master regulator of mitochondrial biogenesis. Reduced Foxo1 and Pgc-1α levels in the Foxp1+/− striatum were also detected by RNA sequencing (RNA-seq) (9). Pgc-1α coactivates nuclear respiratory factor 2 (Nrf2), and together with Nrf2 coactivates Nrf1 (44). In turn, Pgc-1α and Nrf1/2 regulate Tfam transcription in response to increased mitochondrial biogenesis (45). Most likely a reduced Pgc-1α transcriptional activation by Foxo1 in Foxp1+/− tissue is responsible for the decreased Tfam expression, a protein regulating mitochondrial genome replication and transcription (46, 47). Disrupted mitochondrial biogenesis was also shown by altered mtDNA/nDNA ratios, elevated NAD/NADH ratios, and reduced Atp5a as well as Tom20 levels in the Foxp1+/− striatum.

Pgc-1α also controls the delicate equilibrium between fusion and fission in mitochondria and stimulates Mfn1 (48), which, together with Mfn2, regulates the fusion of outer mitochondrial membranes. The adult Foxp1+/− striatum reveals a reduced Mfn1 mRNA expression, whereas Mfn1 protein levels are significantly increased. This suggests a compensatory up-regulation of Mfn1 translation in the adult Foxp1+/− striatum, which might enhance outer mitochondrial fusion. Since L- and S-Opal1 cooperate in mediating the fusion of inner mitochondrial membranes by influencing the pore opening efficiency (31, 49), we assume that reduced S-Opal1 may lead to inefficient and slow fusion of inner mitochondrial membrane as well as disrupted mitochondrial structure and energetics. In addition, Drp1 (which activates fission) was reduced, whereas phosphorylated-Ser637 Drp1 (which blocks fission) (32, 50) was increased in the Foxp1+/− striatum, pointing to reduced mitochondrial fission. Thus, we conclude that disrupted mitochondrial dynamics might impair cellular energy supply and induce cellular stress.

Mitochondrial biogenesis and dynamics as well as the removal of damaged mitochondria are essential for mitochondrial function. Interestingly, we found that Fis1 expression is increased in the Foxp1+/− striatum, and elevated Fis1 has been associated with mitochondrial fragmentation and autophagosome formation (51). LC3A/BII are microtubule-associated proteins essential for autophagosome assembly (52). Elevated LC3A/BII expression indicates increased autophagy in the Foxp1+/− striatum. However, increased autophagy was also noted, supported by increased Pink1 and Parkin, two proteins promoting mitophagy upon loss of mitochondrial membrane potential (53). Strikingly, mitochondrial membrane potential was reduced in Foxp1+/− striatal cells, which may influence ATP production. However, only AMP, a sensitive indicator of energy consumption, was altered. Elevated AMP/ATP ratios in Foxp1+/− neurons suggest a compromised energy status in the Foxp1+/− striatum (54).

The increased occurrence of ROS in the Foxp1+/− striatum is based on two different aspects: A reduced complex I activity with elevated ROS production, and a decreased degradation
due to reduced Sod2 and GSH levels. Sod2 is a direct transcriptional target of Foxo1 (55), whereas GSH production is regulated by glutathione reductase, which depends on NADPH as an indispensable cofactor (56). As NADPH is an essential electron donor, decreased NADPH levels indicate a diminished reducing force for redox balance in the Foxp1+/− striatum (57). However, the increased ROS accumulation does not result in cell death; rather in cell injury, morphological changes and cellular function deficits (58). In line with this, we observed a premature dendritic morphology of striatal neurons in Foxp1+/− animals. Altered dendritic growth and/or maintenance disrupt neural network function, ultimately leading to social and cognitive impairment. Indeed, impaired connectivity between the higher-order association areas is a major defect in ASD (59) and seems to hold true for FOX1 syndrome as well.

Mitochondrial dysfunction is found more frequently in individuals with ASD than in the general population, but seems to affect only a subset of individuals with ASD (19). To define this subgroup, expression and regulation of mitochondria-related genes can be tested. Some ASD-associated risk genes (SLC25A12, MTX2, NFE, SLC25A27, SHANK3, and SOD2) have already been proposed to play a role in mitochondrial function (60–62). As Foxp1 is expressed in many tissues apart from brain, mitochondrial defects in FOX1 deficiency may also occur in other tissues. This corresponds well with the understanding that FOX1 syndrome and ASD is not only a central nervous system disorder but also causes muscular, gastrointestinal, endocrine, and immune deficiencies (19, 63). FOX1 deficiency, therefore, defines one subgroup of ASD individuals affected by mitochondrial dysfunction.

Mitochondrial dysfunction and increased oxidative stress can interplay and activate components of pathways regulating autophagy, apoptosis, and inflammation. These defects will inevitably affect all areas of cognition and motor function that rely on the basal ganglia circuitry. Moreover, mitochondrial function plays an important role in neurogenesis, synaptic plasticity, and neurotransmission (16–18). Very interesting in this context is the finding that disrupted mitochondrial activity cause GABA sequestration in mitochondria, reducing GABAergic signaling in the striatum, and resulting in social deficits (64). In this regard, we conclude that mitochondrial dysfunction does not only contribute to the pathology of FOX1 syndrome, but may also provide the impetus for the development of this disorder.

Our finding that mitochondrial function is disrupted in the Foxp1+/− striatum may also be relevant to Huntington’s disease (HD). This neurodegenerative disorder is characterized by the degeneration of GABAergic medium spiny neurons and mitochondrial dysfunction (65, 66), but the molecular mechanism by which mutant Huntington affects mitochondrial function remains elusive. Strikingly, FOX1 is down-regulated in HD individuals and mouse models of HD (67, 68). Elevating Foxp1 expression protects striatal and cortical neurons from death caused by mutant Huntington. Conversely, Foxp1 knockout promotes death in healthy neurons, indicating a neuroprotective function for Foxp1 (67). This suggests that the reduced FOX1 expression in HD contributes to mitochondrial dysfunction. Moreover, PGC-1α is also down-regulated in animal models and individuals with HD (69). In contrast, up-regulation of Pgc-1α expression prevented mitochondrial dysfunction and provided neuroprotection in the transgenic HD striatum (70), suggesting that stimulating PGC-1α function may also be beneficial in FOX1 deficiency.

The striatum plays a vital role in the coordination of motor and cognitive information from diverse brain regions into meaningful behavioral output (15). Approximately 95% of the striatal cells are Foxp1-expressing medium spiny neurons, which, at least in vitro upon current injection spike at a higher firing rate (50 to 60 Hz) than pyramidal neurons (71, 72). Under standard recording conditions in vivo, medium spiny neurons exhibit sparse firing, but up states and subthreshold depolarization may well contribute to significant calcium influx (73). Thus, it may well be that these properties make medium spiny neurons prone to a higher energy demand and neuronal vulnerability. Considering that the striatum is strongly affected by Foxp1 deficiency, mitochondrial dysfunction in the striatum presumably plays a key role in the pathogenesis of FOX1 syndrome.

Motor performance depends on proper muscle function, which is controlled by the striatum and spinal motor neurons. We have previously shown that conditional deletion of Foxp1 in the central and peripheral nervous system leads to severe striatal degeneration (12); Foxp1 also plays a crucial role in the columnar organization of spinal motor neurons (74). Furthermore, muscular malfunction leads to achalasia and altered colon motility in Foxp1+/− mice (10) and gastrointestinal problems in patients (2). Our report on reduced muscle strength as well as disrupted gait and coordination in Foxp1+/− mice, pointing to hypotonia and motor dysfunction, closely mirrors the phenotype of individuals with FOX1 deficiency (11). Impaired cognitive function is one of core features in FOX1 syndrome. It was recently shown that output of the basal ganglia via the substantia nigra pars reticulata (SNr) gates active avoidance (42), and that the striatum plays an important role in this by regulating SNr cell activity. The complete loss of Foxp1 in the striatum leads to severe morphological and behavioral disruptions (12). Apart from the striatum, cognitive function is also dependent on the proper expression of Foxp1 in the pyramidal neurons of the neocortex and the CA1/CA2 subfields of the hippocampus (13, 14). Therefore, both alterations in the basal ganglia and corticohippocampal circuitry probably contribute to the deficits in learning and memory that we report here in Foxp1+/− mice.

In conclusion, mitochondrial dysfunction and oxidative stress in the striatum may explain the behavioral and motor deficits seen in mice and individuals with FOX1 deficiency and provide an insight into the pathomechanisms underlying FOX1 syndrome. Further studies, using cell type- and region-specific deletion or replenishment of Foxp1 are needed to determine whether defects in FOX1 syndrome are restricted to the striatum or whether mitochondrial dysfunctions in other tissues also contribute to its pathology. Current medical interventions for FOX1 syndrome and autism are not aimed at improving mitochondrial function. Strategies targeting dysregulated proteins involved in mitochondrial function, electron transport chain, or antioxidants may therefore offer new therapy options.

Methods and Materials

Animals. Foxp1+/− mice (8) were backcrossed with C57BL/6J mice for at least 12 generations to obtain congenic animals. Mice were kept in a specific pathogen-free biomedical animal facility under a 12-h light-dark cycle and given ad libitum access to water and food. All procedures were conducted in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and were in accordance with the German Animal Protection Law (TierSchG). The day of birth was considered as postnatal day (P) 0. Animal studies were approved by the Governmental Council Karlsruhe, Germany (license Nos.: 35.9185.81-G-10016 and 35.9185.81-G-10216).

Behavioral Analysis. CatWalk gait analysis, treadmill exhaustion test, and active place avoidance were performed as described in SI Appendix, Methods and Materials. Analysis of mRNA and protein expression levels as well as the calculation of mitochondrial DNA copy number and DNA deletion are described in SI Appendix, Methods and Materials. Primer sequences are listed in SI Appendix, Table S2, primary and secondary antibodies in SI Appendix, Table S3.
Detection of Mitochondrial Membrane Potential. Mitochondrial membrane potential of primary neurons was measured by fluorescent staining of tetramethylrhodamine methyl ester (Image-IT TMRM reagent, Thermo Fisher Scientific) according to the manufacturer’s instructions.

Measuring ROS in Striatal Tissue. Striatal ROS was marked by 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes H2DCFDA) until pH equals 4 to 6. The deproteinized and neutralized supernatant was required for spatial learning and synaptic plasticity. J. Neurosci. 37, 10917–10931 (2017).

Analysis of GSH and GSSG. GSH/GSSG ratio was calculated as manufacturer instructions. The tissue was homogenized in PBS-containing butylated hydroxytoluene (BHT) to prevent sample oxidation during homogenization.

Supernatant was collected and performed on a TCA/NaHCO3 deproteinization protocol (75) and assessed by DSI-11 Series Spectrophotometer/Fluorometer (DeNovix Inc.).

Detection of Lipid Peroxidation Products. Lipid peroxidation products were analyzed in striatal tissue using the Bioxytech LPO-586 kit (OxisResearch) according to the manufacturer’s instructions. The tissue was homogenized in PBS-containing butylated hydroxytoluene (BHT) to prevent sample oxidation during homogenization.

Analysis of Mitochondrial Dynamics, Mitophagy, and Morphology. Mitophagy was measured by the ratio of Mitotracker/Lysotracker colocalization events to the total number of lysosomes. To analyze mitochondrial dynamics, kymographs were generated from the time series images using the Icy Kymograph Tracker tool (version 2.1.2.0) (76, 77). To analyze mitochondrial structure, single frame images were segmented with Ilastik (78) and the analysis was performed using the Region Properties MATLAB function (Mathworks, 2020a). Mitochondrial circularity was measured by ImageJ Analyze Particles tool.

Isolation of mitochondria, primary striatal neuron culture, Sholl analysis, immunoprecipitation (Co-IP), immunofluorescence, cellular adenosine compound, complex I and III activities, TUNEL assay, measurement of cytochrome c release, determination of the OCR and ECAR were performed using standard protocols (SI Appendix, Methods and Materials).

Statistics. Mice were randomly assigned to tests and investigators were blinded to genotypes. IBM SPSS Statistics 25 and Microsoft Office Excel software were used to analyze primary data. Outliers were determined via IBM SPSS Statistics 25 and strong outliers (≥3 SDs above mean) were excluded from further analysis. All data were checked for normal distribution via the Kolmogorov-Smirnov test. Two-tailed t test, ANOVA was performed with the litter as a second factor. In the analysis of mitochandrial dynamics, Mann-Whitney test was applied. P values of ≤0.05 were considered significant.

Data Availability. All study data are included in the article and/or supporting information.

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