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Foxo1 is a downstream effector of Isl1 in direct pathway striatal projection neuron development within the embryonic mouse telencephalon

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ABSTRACT

Recent studies have shown that the LIM-homeodomain transcription factor Isl1 is required for the survival and differentiation of direct pathway striatonigral neurons during embryonic development. The downstream effectors of Isl1 in these processes are presently unknown. We show here that Foxo1, a transcription factor that has been implicated in cell survival, is expressed in striatal projection neurons (SPNs) that derive from the *Isl1* lineage (i.e. direct pathway SPNs). Moreover, *Isl1* conditional knockouts (cKOs) show a severe loss of Foxo1 expression at E15.5 with a modest recovery by E18.5. Although Foxo1 is enriched in the direct pathway SPNs at embryonic stages, it is expressed in both direct and indirect pathway SPNs at postnatal time points as evidenced by co-localization with EGFP in both *Drd1-EGFP* and *Drd2-EGFP* BAC transgenic mice. Foxo1 was not detected in striatal interneurons as marked by the transcription factor Nkx2.1. Conditional knockout of *Foxo1* using *Dlx5/6-CIE* mice results in reduced expression of the SPN marker Darpp-32, as well as in the direct pathway SPN markers Ebf1 and *Zfp521* within the embryonic striatum at E15.5. However, this phenotype improves in the conditional mutants by E18.5. Interestingly, the Foxo family members, Foxo3 and Foxo6, remain expressed at late embryonic stages in the *Foxo1* cKOs unlike the *Isl1* cKOs where Foxo1/3/6 as well as the Foxo1/3 target *Bach2* are all reduced. Taken together, these findings suggest that Foxo-regulated pathways are downstream of Isl1 in the survival and/or differentiation of direct pathway SPNs.

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1. Introduction

The striatum (a.k.a. caudate and putamen) represents the principal component of the basal ganglia circuit and mediates its output through the striatal projection neurons (SPNs). These neurons utilize GABA as a neurotransmitter and comprise two separate pathways; the direct pathway that projects to the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr) and the indirect pathway which projects to the external segment of the globus pallidus (GPe) (Gerfen and Surmeier, 2011). In addition to the distinct axonal targets that each of these output pathways target, they can be neurochemically defined by the expression of neuropeptides as well as dopamine receptors. In this respect the indirect pathway SPNs express enkephalin (Enk) and dopamine D2 receptors, while their direct pathway counterparts express substance P (SP) and dopamine D1 receptors (Gerfen and Surmeier, 2011). Balanced output between these pathways is known to be essential for normal movements and appropriate behaviors (Albin et al., 1989; Gerfen and Surmeier, 2011).

Despite the importance of the SPN output pathways, little is known about the mechanisms that regulate their development. A number of developmental control genes have been shown to play a role in the specification and/or differentiation of SPNs including the Gsx2, Ascl1 and Dlx transcription factors (Anderson et al., 1997; Toresson and Campbell, 2001; Yun et al., 2002, 2003; Waclaw et al., 2004; Long et al., 2009; Wang et al., 2009, 2011, 2013). The zinc finger transcription factors Ikaros and Helios as well as the early B-cell factor (Ebf1) have been shown to play crucial roles in the differentiation of the late born SPNs belonging to the matrix compartment (Garel et al., 1999; Martin-Ibanez et al., 2010, 2012). Studies in recent years have begun to shed light on the molecular genetic mechanisms that control the

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development of the direct and indirect SPN pathways. In this respect, Ikaros and Sp9 are required for the normal differentiation and survival of the indirect SPNs (Martin-Ibanez et al., 2010; Zhang et al., 2016). Conversely, Ebf1 (Garel et al., 1999; Lobo et al., 2006, 2008) and the LIM homeodomain protein, Isl1 (Ehrman et al., 2013; Lu et al., 2014) have been implicated in the development of the direct pathway (i.e. striatonigral) SPNs. In particular, Isl1 is required for the survival and ultimate differentiation of a subpopulation of direct pathway SPNs, already at embryonic stages (Ehrman et al., 2013; Lu et al., 2014). It is currently unclear how Isl1 regulates SPN survival, however, it is likely that it controls a genetic pathway which results in the expression of a survival factor in embryonic direct pathway neurons.

In this respect, the Foxo transcription factors have been implicated in numerous cellular processes including metabolism, differentiation, cell death and survival (Kousteni, 2012; Puthanveetil et al., 2013). *Foxo* genes (*Foxo1*, *3* and *6*) have been shown to be expressed in either the developing or adult striatum, with *Foxo1* being highest expressed at all stages examined (Hoekman et al., 2006). While Foxo1 has been shown to mediate neuronal cell death (Yuan et al., 2008, 2009; Zhou et al., 2015), it is required for survival of certain non-neuronal cells such as cardiomyocytes (Sengupta et al., 2011; Shao et al., 2014). We have investigated here whether Foxo1 (and its family members Foxo3/6) may function downstream of Isl1 to promote the survival and/or differentiation of direct pathway SPNs.

2. Material and methods

2.1. Animals

Fate-mapping studies were carried out by mating *ROSA-*$^{CAG\text{-}tdTomato}$ (JAX stock#007914) mice with *Isl1*$^{cre/+}$ mice (Srinivas et al., 2001), generously provided by T. Jessell (Columbia University). *Isl1*$^{cre/+}$ mice were genotyped as previously described (Waclaw et al., 2010). Double transgenic embryos were collected on E15.5 and E18.5. To label the direct and indirect SPN pathways at postnatal stages, we used *Drd1-EGFP* and *Drd2-EGFP* BAC mice obtained from the Mutant Mouse Regional Resource Center (MMRRC), a NCRR-NIH funded strain repository originally donated by the NINDS funded GENSAT consortium (Gong et al., 2003), as described in Ehrman et al. (2013). These mice were genotyped using EGFP PCR primers (Pei et al., 2011).

Isl1$^{fl/+}$ mice (Mu et al., 2008) were used to generate the *Isl1* conditional knockouts (cKOs) together with *Dlx5/6-CIE* mice (Stenman et al., 2003). These mice were genotyped as previously described (Ehrman et al., 2013). To generate *Foxo1* cKOs, we crossed *Foxo1*$^{fl/fl}$ mice that were generously provided by R.A. DePinho (MD Anderson Cancer Center) with *Dlx5/6-CIE* mice. The *Foxo1* mice were genotyped as described in Paik et al. (2007).

For the staging of embryos, the morning of the vaginal plug was considered E0.5. Embryos were fixed in 4% paraformaldehyde overnight, washed in PBS, cryoprotected in 30% sucrose in PBS, and sectioned at 12 μm on a cryostat. Postnatal day 21 brains were fixed overnight in 4% paraformaldehyde, washed in PBS, cryoprotected in 20% sucrose in PBS, and sectioned at 35 μm on a freezing sliding microtome. All of the mouse studies were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Research Foundation and were conducted in accordance with US National Institutes of Health guidelines.

2.2. Immunohistochemistry (IHC)

IHC (including tyramide amplification) was carried out as previously described (Waclaw et al., 2010). Primary antibodies were used at the following concentrations: rabbit anti-cleaved caspase 3 (1:250: Cell Signaling), goat anti-Darpp-32 (1:200; Santa Cruz), rabbit anti-dsRed (1:500: Clontech), rabbit anti-Ebf1 (1:500: Millipore), rabbit anti-EGFP (1:500; Thermo Fisher Scientific), goat anti-EGFP (1:5000, Abcam), rabbit anti-Foxo1 (1:500; Cell Signaling), rabbit anti-Foxo3 (1:333; Cell Signaling), rabbit anti-Foxo6 (1:1000; gift from A. Brunet, Stanford University) (Salih et al., 2012), rabbit anti-Foxp1 (1:4000; gift from E. Morrisey, University of Pennsylvania, Philadelphia, PA), goat anti-Isl1 (1:1000, R&D Systems), rabbit anti-Nkx2.1 (1:1000; Seven Hills Bioreagents). Di-amino-benzidine (DAB) colorimetric reaction for bright-field immunostaining was developed as previously described (Waclaw et al., 2006). The following secondary antibodies were used for immunofluorescence: donkey anti-rabbit antibodies conjugated to Cy2 or Cy3 (Jackson ImmunoResearch); donkey anti-goat antibodies conjugated to Cy2 (Jackson ImmunoResearch); donkey anti-guinea pig antibodies conjugated to Cy3 (Jackson ImmunoResearch); donkey anti-mouse antibodies conjugated to Cy2 (Jackson ImmunoResearch). Tyramide Signal Amplification kits to Alexa Fluor 488 or Alexa Fluor 568 were purchased from ThermoFisher Scientific.

2.3. Quantification

Foxo1/tdTomato and Foxo1/Ebf1 double stains were quantified on confocal images taken at 400× power from E18.5 dorsolateral striatum on a Nikon C2 Confocal microscope. Images were taken from 4 serial sections of dorsolateral striatum in 3 *Isl1* fate map embryos for Foxo1/tdTomato double stains or in 3 control and 3 *Isl1* cKO embryos for the Foxo1/Ebf1 double stains.

In single IHC stained sections of Ebf1 (400× power), Foxo3 (400× power) and cleaved-caspase 3 (200× power) positive cells were counted individually in 3–4 striatal sections per embryo (at least 3 control, *Isl1* cKO or *Foxo1* cKO embryos analyzed) and are represented as cells/mm^2.

Foxo1, Foxo6, and Darpp-32 single IHC stains were quantified by the area of striatal expression using ImageJ, as previously described (Waclaw et al., 2009; Ehrman et al., 2013). Striatal area was defined and calculated in ImageJ. Signal Intensity was measured after thresholding in at least 3 to 4 serial sections at mid-striatal levels per control/mutant group (at least 3 embryos for each genotype and embryonic stage). Average intensity per unit area was converted to % difference between mutant and control. The average intensity per unit area of control samples was set to 100%. Statistics were performed between control and *Isl1* cKO or control and *Foxo1* cKO using a Student's unpaired *t*-test.

2.4. In situ hybridization

The in situ hybridization procedure was performed as previously described (Toresson et al., 1999). Digoxigenin-labeled antisense probes against *Bach2* (Clone ID: 4218490: Dharmacon) and *Zfp521* (Clone ID: 5038671: Dharmacon) were used on 12 μm sections of E15.5 and E18.5 embryos.

3. Results

3.1. Foxo1 marks direct pathway SPNs at embryonic stages

Foxo1 is known to be expressed in the developing and mature striatum (Hoekman et al., 2006), however, it is unclear whether it is restricted to specific subtypes of striatal neurons such as direct versus indirect SPNs or interneurons. To address this, we made use of an *Isl1 cre/loxP* fate map strategy, which labels neuronal cell bodies and axons of the direct pathway as well as the cholinergic interneurons (Ehrman et al., 2013; Lu et al., 2014) (Fig. 1A and D). Immunostaining for Foxo1 in the fate mapped embryonic striatum, shows that a large portion of the Foxo1-positive neurons co-express tdTomato, indicating that they derive from the *Isl1* lineage and at least, in part, represent direct pathway SPNs (Fig. 1B and E). In fact, quantification of the dorsolateral region of the striatum (the highest striatal Foxo1 expression area) reveals that 89% of the tdTomato$^+$ neurons (i.e. *Isl1* lineage) were Foxo1$^+$ (753

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