The Zinc Finger Transcription Factor Sp9 Is Required for the Development of Striatopallidal Projection Neurons

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In Brief
Zhang et al. analyze Sp9 constitutive and conditional knockout mice and find that this zinc finger transcription factor is critical for the proliferation, differentiation, and survival of striatopallidal projection neurons. The development of striatonigral projection neurons is, in contrast, largely unaffected by the absence of Sp9.

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Highlights
- Sp9 promotes striatopallidal MSN progenitor division and differentiation
- Sp9 is required for the survival of striatopallidal MSNs
- Ascl1 directly regulates the Sp9 promoter in vivo
- Sp9 promotes Adora2a, P2ry1, Gpr6, and Grik3 expression in the striatum
The Zinc Finger Transcription Factor Sp9 Is Required for the Development of Striatopallidal Projection Neurons

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SUMMARY

Striatal medium-sized spiny neurons (MSNs), composed of striatonigral and striatopallidal neurons, are derived from the lateral ganglionic eminence (LGE). We find that the transcription factor Sp9 is expressed in LGE progenitors that generate nearly all striatal MSNs and that Sp9 expression is maintained in postmitotic striatopallidal MSNs. Sp9-null mice lose most striatopallidal MSNs because of decreased proliferation of striatal MSN progenitors and increased Bax-dependent apoptosis, whereas the development of striatonigral neurons is largely unaffected. ChIP qPCR provides evidence that Ascl1 directly binds the Sp9 promoter. RNA-seq and in situ hybridization reveal that Sp9 promotes expression of Adora2a, P2ry1, Gpr6, and Grik3 in the LGE and striatum. Thus, Sp9 is crucial for the generation, differentiation, and survival of striatopallidal MSNs.

INTRODUCTION

The striatum (caudate nucleus and putamen) is the largest component of the basal ganglia. The majority of striatal neurons (90%–95%) are DARPP-32-expressing (+), GABAergic, medium-sized spiny neurons (MSNs). Two major subtypes of MSNs send outputs to other components of the basal ganglia: striatonigral (direct pathway) and striatopallidal (indirect pathway). Striatonigral MSNs express the dopamine D1 receptor (Drd1) and neuropeptide Tac1 (known as substance P), whereas striatopallidal MSNs express the dopamine D2 receptor (Drd2) and neuropeptide enkephalin (Enk) (Gerfen, 1992; Gerfen et al., 1990; Gerfen and Surmeier, 2011; Kawaguchi et al., 1990). About 5%–10% of neurons in the striatum are aspiny interneurons. They are choline acetyltransferase (ChAT)+, parvalbumin (PV)+, somatostatin (SST)+ and calretinin (CR)+ (Kawaguchi et al., 1995). Interneurons in the mouse striatum are known to mainly originate from the medial ganglionic eminence (MGE) (Marin et al., 2000; Xu et al., 2006). Recent studies provide evidence that human and monkey striatal interneurons are also derived from the MGE (Wang et al., 2014). The lateral ganglionic eminence (LGE), the primordium of the striatum, contains two distinct compartments: a dorsal part, which is the source of most interneurons in the olfactory bulb (Stenman et al., 2003; Waclaw et al., 2006; Yun et al., 2001), and a ventral part, which generates striatal MSNs (Anderson et al., 1997; Deacon et al., 1994; Olsson et al., 1995, 1998; Stenman et al., 2003).

Translational profiles of striatonigral and striatopallidal MSNs have been defined using the fluorescent activated cell sorting (FACS) and translating ribosome affinity purification (TRAP) approaches. These studies identified more than 200 striatonigraly enriched genes and more than 100 striatopallidally enriched genes (Ena et al., 2013; Heiman et al., 2008; Lobo et al., 2006). Some transcriptional factors (TFs) that control striatal MSN development are beginning to be identified. Dlx1 and Dlx2 are required for generating late-born MSNs (Anderson et al., 1997), whereas Dlx6 controls striatal regional molecular properties (Wang et al., 2011). Bcl11b (also known as Ctip2) is critical for the differentiation of all MSNs (Arlotta et al., 2008), Ebf1 is essential for the differentiation of striatonigral MSNs (Lobo et al., 2006, 2008). The LIM homebox gene Isil is required for the specification of striatonigral cell identity and the correct development of the striatonigral pathway (Ehrman et al., 2013; Lu et al., 2014). Thus, we know some of the TFs involved in making the striatonical MSNs, but little is known about the molecular mechanisms controlling the development of striatopallidal MSNs.

Here we investigated the role of the Sp9 zinc finger TF in the development of MSNs. Sp9 is widely expressed in the ganglionic eminences (GEs) and in all striatopallidal MSNs. We generated a Sp9-Cre knockin mouse and found that Sp9+ progenitors give rise...
to most GABAergic neurons in the telencephalon, including cortical and olfactory bulb interneurons, striatal MSNs, and striatal interneurons. In postmitotic MSNs, Sp9 is specifically expressed in all striatopallidal neurons but not in striatonigral neurons. We also generated Sp9\textsuperscript{LacZ/LacZ mutant} mouse and observed a 97% reduction of striatopallidal MSNs but saw little change in striatonigral MSNs. Bromodeoxyuridine (BrdU) pulse-labeling and BrdU birth-dating experiments indicated that the neurogenesis ability of LGE progenitors, especially striatopallidal MSN progenitors, was compromised. Moreover, loss of Sp9 function resulted in a lack of striatopallidal neuron differentiation and Bax-dependent apoptosis in the striatum during postnatal life. Conditional inactivation of Sp9 using Drd2-Cre transgenic mice further revealed that the function of Sp9 in regulating striatopallidal MSN survival, at least in part, was cell-autonomous. Chromatin immunoprecipitation (ChiP) qPCR experiments showed that Ascl1 was upstream of Sp9 in the LGE and directly bound to its promoter. Finally, RNA sequencing (RNA-seq) and RNA in situ hybridization indicate that the prosurvival effect of Sp9 in striatopallidal MSNs may be through promoting the expression of three G protein-coupled receptors (GPCRs), including adenosine A2a receptor (Adora2a), purinergic receptor P2Y, G protein-coupled 1 (P2y1) and Gpr6, and glutamate ionotropic receptor kainate type subunit 3 (Grik3). Taken together, our studies uncover a crucial role for Sp9 in the generation, differentiation, and survival of striatopallidal MSNs.

**RESULTS**

**Sp9 Is Widely Expressed in the LGE, MGE, and Caudal Ganglionic Eminence**

To systematically study the expression and function of the TF Sp9 in the telencephalon, we generated Sp9 polyclonal antibodies and several Sp9 mutant alleles. Based on the “knockout-first” strategy (Skarnes et al., 2011; Testa et al., 2004), we generated a Sp9 knockout-first null allele that, in turn, produced a Sp9-LacZ null allele (Sp9\textsuperscript{LacZ/LacZ}) and a Sp9 floxed allele (Sp9\textsuperscript{loxP/loxp}) following exposure to germline recombinases Zp3-Cre (de Vries et al., 2000) and ACTB-Flpe (Rodríguez et al., 2000), respectively (Figures S1A–S1D).

Sp9-LacZ expression was detected in the GEs at embryonic day (E) 10.5 (Figures 1A–1D). Immunocytochemistry of Sp9 and Sp9-LacZ (β-galactosidase [β-gal]) and in situ Sp9 RNA hybridization demonstrated that Sp9 RNA and Sp9 protein were widely expressed in the subventricular zone (SVZ) and mantle zone of the LGE, MGE, and CGE (caudal ganglionic eminence) at E13.5 (Figures 1E–1H). A subset of Sp9\textsuperscript{+} cells in the SVZ of GEs expressed the proneural protein Ascl1 and cell proliferation marker Ki67 (Figures 1I–1K). This suggests that, although Sp9 is not detectable in radial glia cells (primary stem/progenitor cells) in the ventricular zone (VZ), it is expressed in a subpopulation of dividing progenitors in the SVZ and postmitotic neurons in the mantle zone. The GEs generate neocortical interneurons, including in humans (Hansen et al., 2013; Ma et al., 2013). We observed that most migrating cortical interneurons expressed Sp9 at embryonic developmental stages (Figures 1E–1H). Sp9 protein and Sp9 RNA were not detected in Sp9\textsuperscript{LacZ/LacZ mutant} mice (data not shown).

**Sp9\textsuperscript{*} Progenitors Generate Most Neocortical and Striatal GABAergic Neurons**

To genetically identify the progeny of Sp9\textsuperscript{*} progenitors, we generated Sp9-Cre knockin mice (Figure S1). Fate mapping at postnatal day (P) 30 using Sp9-Cre; Rosa-YFP mice showed that Sp9\textsuperscript{*} progenitors generated >96% of cortical interneurons (including PV\textsuperscript{+}, SST\textsuperscript{+}, CR\textsuperscript{+}, NPY (neuropeptide Y)\textsuperscript{+}, and VIP (vasoactive intestinal polypeptide)\textsuperscript{+} subtypes; Figures S2A–S2M) and virtually all striatal interneurons (PV\textsuperscript{+}, SST\textsuperscript{+}, CR\textsuperscript{+}, and ChAT\textsuperscript{+}; Figures S2N–S2Q and S2S). Foxp1 is expressed by all postmitotic MSNs and is excluded from other striatal cell types (Arlotta et al., 2008; Tamura et al., 2004). All Foxp1\textsuperscript{+} cells were GFP\textsuperscript{*} (Figures S2R and S2S), demonstrating that Sp9-Cre is active during the development of both striatopallidal and striatonigral MSNs.

In the olfactory bulb (OB), the identities of GFP\textsuperscript{*} cells were difficult to determine because of the high density of interneurons (Figure S2A). However, from the broad expression of GFP, we speculated that Sp9\textsuperscript{*} progenitors also give rise to most (perhaps all) OB interneurons. We confirmed all of these results using Sp9-Cre; Ai14 mouse lines (data not shown).

**Sp9 Is Specifically Expressed in Drd2\textsuperscript{*} Striatopallidal MSNs**

Using Drd2-EGFP transgenic mice (Gong et al., 2003), we determined the specificity of Sp9’s striatal expression (Figure S3). Sp9 was strongly expressed in the prenatal striatum; its expression continued into adulthood, albeit at a lower level (Figures S3A–S3L). Virtually all GFP\textsuperscript{*} cells (Drd2\textsuperscript{*} striatopallidal MSNs) in the Drd2-EGFP transgenic mouse striatum expressed Sp9 at E16.5, P5, P5, P17, and P35 (Figures S3A–S3L and S3O). In contrast, only about 7% of striatonigral MSNs in Drd1-EGFP mice expressed Sp9 at P5, and Sp9 was not detectable in Drd1-GFP\textsuperscript{*} MSNs at P35 (Figure S3M). This result shows that Sp9 is expressed in both progenitors of striatopallidal and striatonigral MSNs, but, in postmitotic MSNs, Sp9 expression becomes restricted to Drd2\textsuperscript{*} striatopallidal MSNs. Next we assessed whether Sp9 regulated the development of MSNs.

**Most Striatopallidal MSNs Are Lost in Sp9\textsuperscript{LacZ/LacZ} Mutant Mice**

To investigate the function of Sp9 in the development of the striatum, we first analyzed Sp9\textsuperscript{LacZ/LacZ} mutant mice. Although Sp9\textsuperscript{LacZ/LacZ} mice (referred to as controls) developed normally and were fertile, Sp9\textsuperscript{LacZ/LacZ} mutant mice failed to thrive and developed general weakness from P7 onward (Figure 2A). These mice started to die at P14, and none survived beyond P22. The brain sizes and weights of the Sp9 mutants were grossly reduced compared with controls at P20 (Figures 2B and 2C).

The most prominent phenotype in the Sp9 mutant telencephalon was atrophy of the striatum; the volume of mutant striatum was reduced to 54% of controls at P9 (Figures 2D and 2E). Compared with Sp9\textsuperscript{LacZ/LacZ}; Drd2-EGFP mice (Figures 2F–2J), we observed loss 57% of Foxp1\textsuperscript{*} cells and 97% of Foxp1\textsuperscript{+/}Drd2-GFP\textsuperscript{*} cells in the striatum of Sp9\textsuperscript{LacZ/LacZ}; Drd2-EGFP mice at P9 (Figures 2K–2O, 2X, and 2Y). The remaining Foxp1\textsuperscript{+}/Drd2-GFP\textsuperscript{*} cells were not evenly distributed in the...
mutant striatum. In the dorsal medial striatum, Foxp1+/Drd2- GFP+ cells were barely detectable (Figure 2M). In contrast, most remaining Foxp1+/Drd2-GFP+ cells were located in the lateral striatum (Figure 2M), a population of MSNs that are generated at early developmental stages (Anderson et al., 1997). Drd2 in situ hybridization confirmed the loss of the cell type with this molecular signature (Figures 2P and 2T). Furthermore, the mutants showed severe loss of Enk+ cells, further evidence for a defect in striatopallidal MSNs (Figures 2R and 2V). A small population of Drd2-GFP+ cells remained in the dorsal striatum of Sp9 mutants; however, these Drd2-GFP+ cells expressed ChAT and thus were an interneuron subtype (Figures 2Q and 2U; Durieux et al., 2009).

Striatal MSNs are roughly equally divided into striatopallidal and striatonigral neurons. Although Drd1+ and Drd1-GFP+ striatonigral neurons were apparently not affected (Figures 2S and 2W), the observation of loss 57% of striatal Foxp1+ cells in Sp9LacZ/LacZ mutant striatum (Figure 2X) suggests that striatonigral MSNs are slightly affected.

The Sp9 Mutant LGE SVZ Has Reduced Proliferation
To test whether Sp9 regulates cell proliferation in the LGE, we performed 30-min BrdU pulse labeling at E13.5 and E15.5 (Figures 3A–3H). At E13.5, the number of BrdU+ cells in the Sp9lacZ/LacZ mutants was reduced in the SVZ but not the VZ (Figure 3M). At E15.5, we observed a reduction in SVZ BrdU+
Figure 2. Striatopallidal MSNs Are Lost in Postnatal Sp9LacZ/LacZ Mutants
(A) Sp9LacZ/+ and Sp9LacZ/LacZ littermates were taken at P20 to compare their sizes.
(B) P20 images of Sp9LacZ/+ and Sp9LacZ/LacZ brains.
(C) Brains were weighed at P20. The weight of Sp9LacZ/LacZ was significantly reduced compared with Sp9LacZ/+ controls (Student’s t test, ***p < 0.001, n = 6, mean ± SEM).
(D and E) Telencephalon coronal section of Sp9LacZ/+ control and Sp9LacZ/LacZ mutant counterstained with DAPI at P9. Note the reduction of striatal (Str) size in Sp9LacZ/LacZ mutants compared with Sp9LacZ/+ controls. Scale bar, 1 mm.
(F–J) Foxp1/GFP double-immunostained striatal sections from Drd2-EGFP; Sp9LacZ/+ P9 mice.
(K–O) Foxp1/GFP double-immunostained striatal sections from Drd2-EGFP; Sp9LacZ/LacZ mutant mice at P9 showed severe reduction of GFP+ striatopallidal MSNs. Note that almost all GFP+ striatopallidal MSNs were eliminated in the dorsal medial striatum.
(P and T) Severe reduction of Drd2 RNA in Sp9LacZ/LacZ mice compared with Sp9LacZ/+ mice at P9, shown by immunostaining.
(Q and U) In the dorsal striatum of Drd2-EGFP; Sp9LacZ/+ control mice (Q), less than 3% of Drd2-GFP+ cells expressed ChAT, whereas, in the dorsal striatum of Drd2-EGFP; Sp9LacZ/LacZ mutant mice (U), most of the remaining Drd2-GFP+ cells express ChAT (>67%), suggesting they are ChAT+ interneurons rather than striatopallidal MSNs.
(R and V) Severe reduction of Enk+ cells in Sp9LacZ/LacZ mice compared with Sp9LacZ/+ mice at P9, shown by immunostaining.

(legend continued on next page)
progenitor cells and in SVZ Foxp1+ postmitotic MSNs (Figures 3E–3H and 3N).

Next we performed a BrdU birth-dating analysis. An injection of BrdU was given at E12.5, and the number of BrdU+ and BrdU+/Foxp1+ cells were counted in the striatum of P0 in Sp9LacZ/LacZ and wild-type littermate controls (Figures 3I–3L). The mutants had reduced BrdU+ (996 ± 73 cells versus 526 ± 62.5 cells per striatal section, p = 0.0081) and BrdU+/Foxp1+ (911 ± 79.7 cells versus 465 ± 52.7 cells per striatal section, p = 0.0095; controls versus mutants) (Figures 3O and 3P). The percentage of BrdU+ and BrdU+/Foxp1+ cells was reduced in a similar ratio (47.2% versus 49%). Thus, loss of Sp9 function results in a loss of cycling progenitors (BrdU pulse label data) in the LGE SVZ, which results in loss of striatal MSNs (BrdU birth dating data).

**Sp9 Specifically Regulates the Production of Striatopallidal MSNs**

We took advantage of β-gal expression from the Sp9LacZ mutant allele to follow Sp9+ MSNs in the striatum of Sp9 mutant mice. At P0, we found that both β-gal (3638 ± 302.1 versus 1223 ± 108.6, p = 0.0016) and β-gal/Foxp1+ (2.866 ± 202.8 versus 219 ± 10.8, p = 0.0058) cells were greatly reduced in the Sp9LacZ/LacZ mutant striatum compared with Sp9LacZ+/+ control striatum (Figures S4A–S4F). Because Sp9 is expressed in all striatopallidal MSNs and only in a few striatonigral MSNs, this provides evidence that loss of Sp9 preferentially blocks production of striatopallidal MSNs.

To further assess the production of striatal MSNs at E16.5, we performed in situ RNA hybridization using ten striatal MSN markers. We used five markers for striatopallidal MSNs (Drd2, Penk, Gpr6, Adora2a, and Ptprm) and five markers for striatonigral MSNs (Drd1, Tac1, Ebf1, Potm, and Chrm4) for striatopallidal MSNs (Gerfen et al., 1990; Heiman et al., 2008; Lobo et al., 2006). All markers were detectable in Sp9LacZ+/+ controls, although Gpr6 had low expression at this time point (Figures 4A–4J). In contrast, in Sp9LacZ/LacZ mutant SVZ and striatum, striatopallidal MSN markers were either undetectable (Gpr6 and Adora2a) or greatly downregulated (Drd2, Penk, and Ptprm) (Figures 4F’–4J’). Our qRT-PCR analysis revealed similar results (Figure 4Q). On the other hand, striatonigral marker expression was modestly affected (Figure 4A’–4E’). These results further support the evidence from the BrdU birth dating and Sp9-β-gal quantification data: large population of mature striatopallidal MSNs are not produced in the absence of Sp9.

Next we compared GFP expression from the Drd2-EGFP allele in control versus Sp9 mutant E16.5 and P0 striatum (Sp9LacZ+/+ versus Sp9LacZ/LacZ). The mutant had very few Foxp1+/Drd2-GFP+ MN cells in the SVZ and striatum (Figures 4K–4P and 4R). Taken together, these results indicate that Sp9 specifically promotes the production of striatopallidal MSNs in the striatum.

**Loss of Sp9 Induces Bax-Dependent Apoptosis in the Postnatal Striatum**

We next analyzed cell death in Sp9LacZ/LacZ mutant postnatal striatum, as marked by cleaved Caspase-3 expression. The number of Caspase-3+ cells in the striatum of control mice increased from P0, reached a maximum at P3, and then declined (Figures 5A and 5C). The mutant had a robust increase in the number of Caspase-3+ cells in the striatum at all postnatal stages analyzed (P0, P3, P5, and P7) (Figures 5B and 5C). Because most mature striatopallidal MSNs were not generated (because we did not observe Caspase-3+ cells express Drd2-GFP and because we did not observe a severe reduction of striatonigral MSNs), we inferred that most of the dying cells in Sp9 mutant striatum were immature striatopallidal MSNs.

To determine whether Sp9LacZ/LacZ striatopallidal MSNs apoptosis was Bcl-2-associated X protein (Bax)-dependent, we generated Sp9LacZ/LacZ, Bax−/− double mutants. We found that striatal cell death was nearly absent in the Sp9LacZ/LacZ, Bax−/− double mutants at P3 (data not shown). We analyzed the numbers of Foxp1+ cells in the striatum of Sp9LacZ+/+, Bax−/−, Sp9LacZ/LacZ, Bax−/− and Sp9LacZ/LacZ, Bax−/− mice at P15 (Figures S5A–S5I and S5P) and found that the number of Foxp1+ cells in Sp9LacZ+/+, Bax−/− striatum was significantly larger than that in Sp9LacZ/LacZ, Bax−/− striatum (Figure S5P). Perhaps because this genetic manipulation did not rescue Sp9-mediated cell proliferation in the LGE SVZ, the number of Foxp1+ cells in Sp9LacZ+/+, Bax−/− striatum was still smaller than that in Sp9LacZ+/+, Bax−/− striatum (controls) (Figure S5P).

We noted that the “rescued” Foxp1+ cells in Sp9LacZ/LacZ, Bax−/− mutant failed to fully mature into striatopallidal MSNs as they did not express Enk, Drd2, and Adora2a at P15 (Figures S5J–S5O; data not shown), suggesting that, in addition to promoting LGE proliferation and survival, Sp9 is essential for the differentiation and maturation of striatopallidal neurons.

**Sp9 in Striatopallidal MSNs Is Required for Their Survival**

All striatopallidal MSNs in the striatum express Sp9 (Figure S3). To directly investigate the requirement of Sp9 in postmitotic striatopallidal MSNs, we utilized a conditional knockout strategy. We generated Sp9 conditional mouse mutants by breeding male Drd2-Cre; Sp9Flox/Flox mice with female Rosa-YFP Cre reporter allele. The Drd2-Cre transgene expresses Cre specifically in immature and mature striatopallidal MSNs (Gong et al., 2007). The offspring of this cross, including Drd2-Cre; Sp9Flox/Flox conditional mutants, developed normally and were fertile.

We quantified the number of Foxp1+ and Foxp1+/GFP+ cells in the striatum of Drd2-Cre; Sp9Flox/Flox, Rosa-YFP mice and Drd2-Cre; Sp9Flox/Flox, Rosa-YFP triple-transgenic mice at P30.
The mutants had decreased numbers of Foxp1+ and Foxp1+/GFP+ cells (Figure 5F). Next we analyzed cell death in the striatum of these mice. Similar to Sp9 constitutive mutants, the striatum of Sp9 conditional mutants had increased numbers of Caspase-3+ cells at P0, P3, and P5 (Figures 5G–5I). Thus, the loss of Drd2+ striatopallidal MSNs in Sp9 conditional mutants was due to cell programmed death, demonstrating that Sp9 expression in postmitotic striatopallidal MSNs is necessary for their survival.

Loss of Striatopallidal MSNs Results in Hyperlocomotion

Activation of the striatonigral (direct) pathway promotes locomotion, whereas activation of the striatopallidal (indirect) pathway inhibits locomotion (Durieux et al., 2009, 2012; Kravitz et al., 2010). Therefore, the loss of striatopallidal MSNs observed in the Drd2-Cre; Sp9LacZ/LacZ conditional mutant mice could influence locomotive behaviors. To test this, we examined the locomotive activity of 2-month-old Drd2-Cre; Sp9LacZ/LacZ, Drd2-Cre; Sp9Flox/Flox (control) and Sp9Flox/+ (control) mice in an open field.
Figure 4. Most Mature Striatopallidal MSNs Are not Generated in Sp9 Mutants

(A–E) In situ RNA hybridization of striatonigral MSN markers on E16.5 brain sections showing that the development of striatonigral MSN was less affected.

(F–J) RNA expression of striatopallidal markers was significantly reduced. Note the complete loss of Adora2a RNA in the Sp9 mutant LGE SVZ and mantle zone (striatum) except for a few cells in the lateral striatum (H').

(K–P) Most Drd2-EGFP+ cells were lost in the SVZ and striatum at E16.5 and P0.

(Q and R) Validation of striatopallidal MSN loss in the E16.5 LGE by qPCR (Q) and quantification of Foxp1+, Foxp1+/Drd2-GFP+, and Foxp1+ but Drd2-GFP− (negative) cells in the LGE SVZ at E16.5 (R) (Student’s t test, *p < 0.05, **p < 0.01, ***p < 0.001, n = 3, mean ± SEM.

Scale bars, 100 μm in (P) for (A)–(K), (M), (N), and (P) and 50 μm in (O) for (L) and (O).
Figure 5. Programmed Cell Death Eliminates Striatopallidal MSNs in Postnatal Sp9 Constitutive and Conditional Mutants

(A and B) Significant increase in expression of cleaved Caspase-3 in Sp9 mutant striatum compared with controls at P3. (C) Temporal profile of numbers of Caspase-3+ cells in postnatal striatum in Sp9 mutant and control mice (one-way ANOVA followed by Tukey-Kramer post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, n = 3, mean ± SEM).

(D–F) Foxp1/GFP double-immunostaining revealed a significant decrease in numbers of Foxp1+ and Foxp1+/GFP+ cells in the striatum of Drd2-Cre; Sp9$^{flox}$; Rosa-YFP conditional mutants compared with Drd2-Cre; Sp9$^{flox/+}$; Rosa-YFP (controls) at P30 (Student’s t test, **p < 0.01, ***p < 0.001, n = 3, mean ± SEM).

Scale bar, 50 μm.

(G–I) Caspase-3+ cells in controls and Sp9 conditional mutants at P3 showing a significant increase in the numbers of Caspase-3+ cells in the striatum of Sp9 conditional mutants compared with controls at P0, P3, and P5 (one-way ANOVA followed Tukey-Kramer post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, n = 3, mean ± SEM).

(J and K) In the open field test, adult Drd2-Cre; Sp9$^{flox}$ mice exhibited increased locomotor activity compared with Sp9$^{flox/+}$ or Drd2-Cre; Sp9$^{flox/+}$ littermate controls (J, measured by the total distance), but they exhibited similar anxiety related behavior (K, measured by the center distance/total distance ratio) (one-way ANOVA followed Tukey-Kramer post hoc test, *p < 0.05, mean ± SEM).

(L) In the rotarod test, adult Drd2-Cre; Sp9$^{flox}$ mice and littermate control mice displayed similar motor coordination and learning ability in the latency to fall during 3 training days (days 1, 2, 3) and one trial day (day 4).

Scale bar, 100 μm in (H) for (A), (B), (G), and (H).
Compared with controls, Drd2-Cre; Sp9\textsuperscript{Flox/Flox} mice exhibited a significant increase in locomotion based on the total distance traveled (Figure 5J). However, on the basis of the ratio of the center to the total distance in the open field test, we did not observe any significant difference in anxiety-related behaviors (Figure 5K). In addition, Drd2-Cre; Sp9\textsuperscript{Flox/Flox} mice showed normal performance in a rotarod test (Figure 5L), indicating normal motor coordination and motor learning. Overall, these experiments indicate that loss of striatopallidal MSNs in Drd2-Cre; Sp9\textsuperscript{Flox/Flox} mice resulted in hyperlocomotion but no measurable change in anxiety or motor coordination/learning.

### Ascl1 Directly Binds Sp9’s Promoter and Enhances Its Expression In Vivo

Previous studies have shown that the proneural factor Ascl1 is required for the production of striatal Drd2\textsuperscript{+} MSNs at E18.5 (Casarosa et al., 1999). We confirmed this result using in situ RNA hybridization. Compared with Ascl1\textsuperscript{GFP/+} control mice, the expression level of Drd2 in P0 Ascl1\textsuperscript{GFP/GFP} mutant mice was severely reduced (Figures S6E and S6F), whereas the expression of Drd1 was less affected (Figures S6G and S6H). We also observed that the expression of Sp9 (both protein and RNA) was reduced in the ventral LGE SVZ of Ascl1\textsuperscript{GFP/GFP} mutants at E14.5 and E16.5 (Figures 6A–6D; Figures S6A–S6D), suggesting that Ascl1 positively regulates Sp9 expression. This is consistent with previous reports (Long et al., 2009a, 2009b).

Ascl1 promotes cell differentiation and proliferation in GEs (Castro et al., 2011). Thus, it is possible that Ascl1 controls striatopallidal MSN progenitor proliferation in the LGE SVZ through Sp9. To test this hypothesis, we informatically studied the putative promoter and enhancers near the Sp9 locus. We found the consensus Ascl1 binding motif (E-box sites, CAGCTG or CACCTG) in the putative promoter and in two enhancers (E953 and E245) (Castro et al., 2011; Pacary et al., 2011; Figure 6E). To determine whether Ascl1 physically associates with these regulation regions, we carried out ChIP-qPCR using an Ascl1 rabbit polyclonal antibody and chromatin prepared from E13.5 LGE of wild-type mice. The Rnd3 E1 enhancer (Rnd3 E1) was used as a positive control because previous studies demonstrated that Ascl1 promotes Rnd3 expression in the embryonic brain by a direct regulation of Rnd3 E1 (Pacary et al., 2011). We found that the Rnd3 E1, Sp9 putative promoter, E953, and E245 were all enriched in the immunoprecipitated genomic DNA over input (Figure 6F).

We performed a dual-luciferase transcription activation assay using the P19 embryonal carcinoma cell line. Ascl1 activated transcription from Sp9’s putative promoter and, to a lesser extent, from Sp9 E245 (Figure 6G), whereas it did not activate Sp9 E953 (Figure 6G). Finally, we found that the Ascl1 binding motif (CAGCTG) contributed to the regulation of the Sp9 promoter because deletion CAGCTG attenuated Ascl1-mediated activation (Figure 6H). Together, these results provide evidence that Ascl1 promotes Sp9 expression in the LGE SVZ, at least in part, through its direct binding to the promoter and, possibly, other regulatory elements within the Sp9 locus (e.g., E245).

### Sp9 Promotes Adora2a Expression in the Striatum

To investigate the cause of striatopallidal MSN apoptosis in Drd2-Cre; Sp9\textsuperscript{Flox/Flox} conditional knockout mice, we performed RNA-seq analysis. We compared gene expression profiles from the P0 littermate Sp9\textsuperscript{Flox/Flox} control striatum (including SVZ) and conditional mutant striatum of Drd2-Cre; Sp9\textsuperscript{Flox/Flox} mice and identified about 90 genes that were either significantly upregulated or downregulated in Drd2-Cre; Sp9\textsuperscript{Flox/Flox} mice (Table S1) (GEO: GSE83373). Of these, we found that the expression of three GPCRs (Adora2a, Gpr6, and P2ry1) was significantly decreased in Sp9 conditional mutant striatum, especially Adora2a (p = 0.0000000134, Q value = 0.00000115) (Table S2). Two of them (Adora2a and Gpr6) are markers for striatopallidal MSNs (Heiman et al., 2008; Lobo et al., 2006); the third one (P2ry1) is predominately expressed in striatopallidal MSNs during development. In addition, Grik3 expression was also greatly reduced in Sp9 conditional mutant striatum. Because most striatopallidal MSNs have not yet died at P0, the majority of striatopallidal MSN-specific genes were not significantly downregulated (Table S2), and none of the striatonigral MSN-specific genes were significantly downregulated (Table S2). We postulate that genes with an expression difference prior to cell death may lead to apoptosis.

To validate our RNA-seq results, we performed RNA in situ hybridization on E16, E18, and P4 sections with a number of these genes (Figure 7; Figure S7). First, we confirmed the absence of Sp9 in the mutant LGE mantle zone (striatum) at E16 (normal expression in the SVZ is present because the Cre eliminates Sp9 in the mantle zone). GFP\textsuperscript{+} and Cre\textsuperscript{+} cells were present in the striatum as apoptosis had not begun (Figures S7A–S7C). The development of striatonigral MSNs appeared normal based on expression of their markers Drd1 and Ebf1 (Figure S7D–S7E). Expression of the striatopallidal MSN markers Drd2, Penk, and Ptprm was slightly reduced (Figures S7F, 7F', and 7I–7J'). Strikingly, Adora2a RNA striatal expression was almost undetectable (Figures S7G and S7G'), and Gpr6 expression was severely reduced (Figures 7H and S7H'; Table S2). Similar results were also observed in E18 striatum (Figures 7A–7H'). In addition, we observed that the expression of P2ry1 and Grik3 was greatly reduced (Figures 7I–7J'), consistent with our RNA-seq data. At P4, we began to observe the loss of Drd2\textsuperscript{+} striatopallidal MSNs in Sp9 conditional mutant striatum (Figures S7K and S7K'). However, based on our in situ RNA hybridization, Adora2a RNA expression remained almost undetectable (Figures S7L and S7L'), and Gpr6, P2ry1, and Grik3 expression was still severely reduced in Sp9 conditional striatum (Figures S7M–S7O'). These data indicate that Sp9 is required to drive Adora2a expression in striatopallidal MSNs. Furthermore, the Sp9 conditional mutants had reduced expression of two other GPCRs, Gpr6 and P2ry1, and the glutamate ionotropic receptor Grik3 in striatopallidal MSNs.

Adora2a, among its many functions, participates in protecting cells from neuronal death after brain insults (Chen et al., 2007; Rivera-Oliver and Diaz-Rios, 2014). We propose that the apoptotic cell death of striatopallidal MSNs in the Sp9 conditional mutant is likely in part a consequence of the loss of Adora2a expression along with attenuated expression of Gpr6, P2ry1, and Grik3.
Here we find that Sp9 has several critical roles in striatal MSN development. Sp9+ LGE progenitors generate most MSNs, and Sp9 expression is maintained in postmitotic striatopallidal MSNs but not striatonigral MSNs. In the absence of Sp9, striatal development was disrupted for three main reasons: LGE SVZ cell proliferation was reduced, striatopallidal MSN differentiation and maturation were arrested, and Bax-dependent apoptosis of these neurons occurred. The latter two processes largely did not affect striatonigral neurons. We suggest that Ascl1 drives Sp9 expression in the SVZ through binding and activation of the Sp9 promoter. Finally, striatopallidal MSN survival is mediated in part through Sp9 driving the expression of several GPCRs, especially Adora2a.

**Sp9 Specifically Promotes Striatopallidal MSN Progenitor Cell Division in the LGE SVZ**

Sp9 expression is detected in the SVZ but not the VZ (primary neural stem/progenitors) of the GEs. Sp9 is co-expressed with the progenitor cell markers Ascl1 and Ki67 particularly at the VZ-SVZ boundary (also known as SVZ1) (Petryniak et al., 2007; Figures 1I–1K). The reduced numbers of S-phase cells labeled by BrdU pulse at E13.5 and E15.5 in the constitutive null mutant
mice indicate that Sp9 promotes proliferation of the progenitors. This reduced proliferation leads to reduced LGE neurogenesis, as revealed by our BrdU birth-dating analysis, based on a significant reduction of BrdU+ and BrdU+/Foxp1+ cells in the Sp9 mutant striatum at P0 (Figures 3I–3L, 3O, and 3P). Thus, decreased progenitor proliferation contributes to reduced numbers of MSNs in the mutant mice. Although Sp9+ progenitors in the LGE SVZ give rise to all striatal MSNs, Sp9 expression is restricted to striatopallidal MSNs in the striatum because only a few of striatonigral MSNs express Sp9. Using the Sp9-LacZ mutant allele, we found that there was a significant reduction of β-gal+ cells in P0 Sp9 mutant striatum compared with Sp9Flox/Flox controls (Figure S4). This strongly suggests that the function of Sp9 in the LGE SVZ is mainly promoting striatopallidal MSN progenitor cell proliferation.

**Regulation of Sp9 Expression in the LGE by Ascl1**

Ascl1 is a basic helix-loop-helix proneural TF that is expressed in the VZ and SVZ of the GEs. Ascl1 expression has been shown to promote both cell proliferation (Castro et al., 2011) and cell-cycle exit (Castro et al., 2006; Farah et al., 2000; Nakada et al., 2004). In this study, we showed that Ascl1 is co-expressed with Sp9 in the SVZ of the LGE (Figures 1I and 1J). In Ascl1 mutant mice, the expression of Sp9 is reduced (Figures S6A–S6D; Figures 6A–6D). These data indicate that Sp9 is genetically downstream of Ascl1. We also found that loss of striatopallidal MSNs was more severe than loss of striatonigral MSNs in Ascl1 mutant striatum (Figures S6E–S6H). Thus, Ascl1 mutants phenocopy Sp9 mutants; both have reduced LGE cell proliferation and Drd2 striatal expression (Casarosa et al., 1999; Castro et al., 2011). Ascl1 ChIP-chip
Sp9 Controls the Survival of Striatopallidal MSNs Likely via Positively Regulating GPCR Expression

Sp9 expression and function starts from LGE progenitors and then becomes restricted to differentiating striatopallidal MSNs. Consistent with this expression pattern, reduction of cycling progenitors was observed in the Sp9 constitutive mutant LGE SVZ, and a reduction of markers of striatopallidal MSNs was observed in the LGE mantle zone (striatum) of Sp9 constitutive and conditional mutants. The Sp9 constitutive mutant may result in the premature cell-cycle exit of striatopallidal MSN. These neurons have a differentiation defect (fail to express markers of striatopallidal MSNs) and undergo Bax-dependent apoptosis largely in the early postnatal period. Indeed, we never observed striatal Caspase-3+ cells that expressed Drd2-GFP. Conditional inactivation of Sp9 in striatopallidal MSNs using Drd2-Cre transgenic mice largely phenocopied the striatal defects of Sp9 constitutive mutants, showing that elimination of Sp9 beginning at E13.5 in striatal postmitotic neurons leads to the failure of striatopallidal MSN differentiation followed by their apoptotic elimination.

Previous studies have shown that Ntrk2 (known as TrkB), a receptor for brain-derived neurotrophic factor (BDNF), is required for the development of striatum and survival of striatopallidal MSNs (Bayduyk et al., 2011; Li et al., 2012). However, we did not find altered RNA expression of Ntrk2 in the embryonic striatum of Sp9 mutants (Figures S7C and S7D). This suggests that programmed cell death occurring in the Sp9 mutant striatum is not due to the lack of Ntrk2. By contrast, we observed that the expression of GPCRs in striatopallidal MSNs was downregulated in the absence of Sp9 at E16.5, E18, and P4 stages. These GPCRs are Adora2a, Gpr6, and P2ry1. Adora2a and Gpr6 are only expressed in striatopallidal MSNs; P2ry1 is mainly expressed in striatopallidal MSNs during development. We also noted that the expression of Adora2a remained barely detectable in Sp9 constitutive and conditional mutants from E16 to P4. This demonstrated that the expression of Adora2a in striatopallidal MSNs was dependent on Sp9.

Adora2a mediates neuroprotection against brain injuries in various animal models of neurological disorders. Adora2a can either promote or protect from cell death depending on the nature of brain injuries (Chen et al., 2007; Rivera- Oliver and Díaz-Ríos, 2014). We show that Sp9 is required for Adora2a expression in differentiating and mature striatopallidal MSNs. We propose that the nearly complete loss of Adora2a, combined with partial loss of Gpr6, P2ry1, and Grik3 in striatopallidal MSNs during development, contributed to the striatal apoptotic cell death in Sp9 conditional mutants.

Loss of Striatopallidal MSNs and Human Disease

Aberrant basal ganglion circuitry leads to locomotor dysfunction in humans. Many diseases, such as Huntington’s disease (HD) and attention deficit/hyperactivity disorder (ADHD), are associated with abnormal striatal MSNs (Russell, 2007; Vonsattel et al., 1985). A dramatic decrease in the expression of Adora2a in the striatum is evident at a very early stage of HD (Glass et al., 2000). The preferential loss of striatopallidal MSNs in the early and middle phases of HD contributes to choreiform movements (Mitchell et al., 1999; Reiner et al., 1988). Because Drd2-Cre; Sp9<sup>Flox/Flox</sup> mutants have hyperlocomotion, this further supports the idea that striatopallidal MSNs promote movement inhibition (Kravitz et al., 2010). Thus, Drd2-Cre; Sp9<sup>Flox/Flox</sup> mutants may serve as a model to study basal ganglion disorders caused by abnormal development and survival of striatopallidal MSNs.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed in accordance with institutional guidelines. We generated Sp9<sup>−/−</sup> mice, Sp9<sup>Flox/+</sup> mice and Sp9-Cre knockin mice in this study. See the Supplemental Experimental Procedures for detailed methods. Other mouse strains used in this study were Drd2<sup>EGFP</sup>, Drd2<sup>Cre</sup>, and Drd1<sup>EGFP</sup> (Song et al., 2003, 2007) (from Mutant Mouse Research Centers [MMRC]), Ascl1<sup>Flox/+</sup> knockin knockout (Kim et al., 2007; Leung et al., 2007); and Bar<sup>+</sup> (Kudson et al., 1993). We used two Cre-reporter mouse lines in this study: Rosa-YFP (Srinivas et al., 2001) and Ai14 (Madisen et al., 2010). All lines in this study were maintained in a mixed genetic background of C57BL/6J, 129S6, and CD1.

RNA-Seq

The striatum (including the SVZ) from P0 Sp9<sup>Flox/Flox</sup> littermate controls and Drd2-Cre; Sp9<sup>Flox/Flox</sup> conditional mutants were dissected (n = 3 in each group). Total RNA was purified with a mini RNA isolation kit (ZymoGenetics). RNA-seq was performed as recommended by the manufacturer (Illumina). Levels of gene expression were reported in fragments per kilobase of exon per million fragments mapped (FPKM) (Trapnell et al., 2012). A gene was considered to be expressed when it had FPKM > 1. For a gene to be called as differentially expressed, it required p < 0.05. Data from this experiment were deposited in GEO: GSE83373.

ChIP and qPCR

ChIP was performed on E13.5 LGE using Ascl1 rabbit polyclonal antibody (Cosmo Bio, SK-T01-003). Co-precipitated DNAs were purified with phenol-chloroform and detected by qRT-PCR (Vogt et al., 2014). ChIP and qPCR methods. Other mouse strains used in this study were

Luciferase Assays

The DNA fragments of the E953 and E245 enhancers of the Sp9 gene were created by PCR and subsequently cloned into the pGL4.23 firefly luciferase vector (Promega) upstream (U) or downstream (D) of the Luc2 gene (e.g., pGL4.23-E953U or pGL4.23-E953D). The putative Sp9 promoter was amplified by PCR and cloned into the pGL4.10 promoterless firefly luciferase vector (Promega). Cells from the mouse embryonal carcinoma cell line P19 were grown in medium MEM (Gibco, 12571-063) supplemented with 10% fetal bovine serum (FBS) (Gibco, 10099-141). For the luciferase assay, P19 cell transfections were performed in triplicate in 24-well plates by using Fugene HD transfection reagent according to the manufacturer’s protocol (Promega, E2311). Luciferase sparks were quantified by a microplate luminometer (Turner BioSystems, Modulus microplate reader).
The accession number for the RNA-seq data reported in this paper is GEO: GSE83373.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.090.

AUTHOR CONTRIBUTIONS

Q.Z. and Y.Z. performed all experiments and analyses except RNA in situ hybridization, which was performed by C.W. L.A. helped to perform and analyze the ChIP-qPCR experiments. Z.X., Q.L., J.L., Z.Z., and Y.Y. helped to conduct experiments and analyze the data. M.H., Y.M., B.C., Z.Q.X., and J.L.R. helped to guide the project and analyzed the results. Z.Y. designed the experiments and analyzed the results. J.L.R. and Z.Y. wrote the manuscript.

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