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Sox9 and NFIA Coordinate a Transcriptional Regulatory Cascade during the Initiation of Gliogenesis

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SUMMARY

Transcriptional cascades that operate over the course of lineage development are fundamental mechanisms that control cellular differentiation. In the developing central nervous system (CNS), these mechanisms are well characterized during neurogenesis, but remain poorly defined during neural stem cell commitment to the glial lineage. NFIA is a transcription factor that plays a crucial role in the onset of gliogenesis; we found that its induction is regulated by the transcription factor Sox9 and that this relationship mediates the initiation of gliogenesis. Subsequently, Sox9 and NFIA form a complex and coregulate a set of genes induced after glial initiation. Functional studies revealed that a subset of these genes, *Apcdd1* and *Mmd2*, perform key migratory and metabolic roles during astro-gliogenesis, respectively. In sum, these studies delineate a transcriptional regulatory cascade that operates during the initiation of gliogenesis and identifies a unique set of genes that regulate key aspects of astro-glial precursor physiology during development.

INTRODUCTION

Cell fate decisions in the developing central nervous system (CNS) are governed by transcriptional networks that control both cellular diversity and lineage progression. These networks operate in both space and time to control these distinct aspects of CNS

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SUPPLEMENTAL INFORMATION

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development. Spatial patterning of homeodomain-containing (HD) transcription factors along the dorsal ventral axis of the spinal cord is responsible for the specification of distinct subtypes of neurons in progenitor populations (Briscoe et al., 2000; Ericson et al., 1997; Pierani et al., 1999). Subsequently, these progenitor populations undergo a series of differentiative steps over time that culminates in the generation of terminally differentiated neurons (Lee and Pfaff, 2003; Novitch et al., 2001; Thaler et al., 2004). These sequential differentiative steps are governed by temporal changes in the transcription factor milieu; therefore, delineating transcriptional regulatory cascades is crucial to our understanding of the development of neural cell lineages. Although these transcriptional mechanisms have been characterized for several neuronal subtypes in the developing spinal cord, analogous relationships between transcriptional regulators of early gliogenesis remain poorly defined (Briscoe and Novitch, 2008; Lee et al., 2005; Thaler et al., 2002).

During embryonic development of the CNS, neural stem cells undergo a characteristic temporal pattern of differentiation wherein neurons are generated first followed by glial cells. This developmental transition is best characterized in the ventral region of the mouse and chick embryonic spinal cord, where neurogenesis occurs between E9.5 and E11.0 in mouse (E2–E4 in chick) and gliogenesis commences at E11.5 (E5 in chick) (Kessaris et al., 2001; Rowitch, 2004; Zhou et al., 2001). This developmental interval, herein called the “gliogenic switch,” consists of two distinct molecular processes: the cessation of neurogenesis and the initiation of gliogenesis. Importantly, while these populations undergo a change in cell fate, neurogenic capacity is maintained and gliogenic potential is acquired (Mukouyama et al., 2006; Scott et al., 2010). Previously, we used this developmental process as a model for examining the formative stages of gliogenesis and identified nuclear factor-I A (NFIA) as a crucial transcriptional determinant that regulates the initiation of gliogenesis (Deneen et al., 2006). Importantly, the de novo induction of NFIA expression in neural stem cell populations is tightly correlated with the timing of the initiation of gliogenesis at E11.5 in mouse (E5 in chick). Therefore, the identification of the transcriptional processes that control the induction of NFIA provides a starting point in defining transcriptional regulatory cascades that operate in neural stem cells during the gliogenic switch.

Another transcription factor associated with the initiation of gliogenesis is the HMG-box family member Sox9. Genetic knockout of Sox9 results in an extended period of neurogenesis, coupled with a delay in the onset of oligodendrogenesis, a phenotype consistent with a role during the gliogenic switch (Stolt et al., 2003). In addition, Sox9 has been implicated in initiating and maintaining neural stem cell populations in the embryonic and adult CNS (Cheng et al., 2009; Scott et al., 2010). Although Sox9 function has been associated with several critical aspects of CNS development, our understanding of how it contributes to the initiation of gliogenesis and coordinates these diverse functions during CNS development remain undefined. Thus, delineating these mechanisms will reveal new insight into the gliogenic switch and Sox9 function during CNS development.

To decipher the transcriptional processes that govern NFIA induction, we performed in vivo screening of NFIA enhancer elements. This screen identified an enhancer element that recapitulates NFIA induction in vivo and is directly regulated by Sox9. Subsequent studies revealed that Sox9 directly regulates NFIA, and this relationship is crucial for the initiation of gliogenesis. Next we demonstrate that Sox9 and NFIA physically associate and that this Sox9/NFIA complex directly regulates a subset of genes induced just after the initiation of gliogenesis. Functional studies revealed that two of these genes, *Apcdd1* and *Mmd2*, perform key migratory and metabolic roles during gliogenesis, respectively. In sum, these studies delineate a transcriptional regulatory cascade that operates during the initiation of

gliogenesis and identifies a unique set of genes specifically associated with astro-glial precursors that function to regulate key aspects of their physiology during development.

RESULTS

e123 Enhancer Recapitulates NFIA Induction In Vivo

NFIA is induced in the ventricular zone (VZ) of the developing spinal cord at the onset of gliogenesis in both chick (Figures 1D–1F) and mouse (Deneen et al., 2006). To elucidate the transcriptional mechanisms that govern NFIA induction, we sought to identify *cis*-acting regulatory elements by aligning 100 kb 5′ of the transcriptional start site of the chick and mouse *NFIA* gene. This analysis identified 12 regions between 100–500 bp in length that share >70% identity (Figure 1A, white lines). Next, we grouped these regions into six clusters, isolated the putative enhancers and the surrounding ~1 kb on either side from chick genomic DNA (Figure 1A, blue boxes), and cloned them upstream of a minimal promoter and a GFP reporter.

To determine whether these putative NFIA enhancer elements have activity that resembles the spatial and temporal patterns of NFIA induction, we introduced them into the embryonic chick spinal cord via electroporation and harvested during the E4–E6 NFIA induction interval (Figures 1D–1F). Each enhancer was coelectroporated with a CMV-cherry construct that served as an internal control for electorporation efficiency (Figures 1J–1L). Among six enhancer elements, e123 demonstrated activity in the VZ during the E4–E6 induction interval (Figures 1G–1I), with the remaining enhancers demonstrating activity at time points prior to NFIA induction or in motor neurons (Figure S1 available online). We chose to focus our attention on the e123 enhancer because its pattern of activity is strongly correlated with endogenous NFIA induction, where it demonstrates a sharp upregulation in VZ populations during the E4–E6 interval (Figures 1G–1I, arrows). By combining cross species genomic analysis with in vivo enhancer screening, we have identified a NFIA enhancer element that recapitulates its spatial and temporal patterns of induction.

Sox9 Directly Regulates e123 Activity

To identify transcriptional regulators of e123, we used bioinformatics to identify putative transcription factor binding sites within this region and cross-correlated this analysis with an atlas of transcription factors expressed in the VZ of the embryonic mouse spinal cord during early gliogenesis (Fu et al., 2009). This analysis identified several transcription factors, including Sox9, which contain binding sites in e123 (Figures 1C and S1). Sox9 is of particular interest because its expression is induced prior to NFIA in the embryonic spinal cord, and genetic knockout of Sox9 results in a delay in the onset of oligodendrocyte formation (Stolt et al., 2003). To determine whether Sox9 can induce e123 activity, we performed coelectroporation and assessed activation at time points prior to e123 induction (E4, see Figure 1D). As indicated in Figures 1M–1P and 1AA, ectopic expression of Sox9 is sufficient to induce precocious and ectopic activity of e123 at E4. This activation of e123 appears to be specific to Sox9, because Sox2 overexpression is not sufficient to induce e123 activity at E4 (Figure S2). Deletion mapping revealed that region 2 contains the Sox9 response site and, importantly, can recapitulate the activity of e123 (Figures 1Q–1V and S2). Together, our analysis reveals that Sox9 controls e123 activity through region 2.

To determine whether the Sox9 site within region 2 of e123 (herein called Sox9-Mu2) is responsible for the activity of e123, we deleted it in the context of the full-length e123 enhancer and assessed activation at E6 (Figure 1C, red box). Deletion of Sox9-Mu2 resulted in a loss of e123 activity at E6, indicating that this site mediates e123 activity (ΔMu2-GFP) (Figures 1X and 1BB). Further supporting the regulatory relationship between e123 and

Sox9, coelectroporation of e123 with a dominant-negative version of Sox9 (Sox9-EnR) resulted in a loss of activity at E6 (Figure S2; Scott et al., 2010). Next, we performed chromatin immunoprecipitation (ChIP) assays to determine whether Sox9 directly associates with the Mu2 site in e123 region of the endogenous NFIA promoter. To this end we electroporated HA-Sox9 into the embryonic chick spinal cord, harvested embryos at E4, and performed ChIP assays on chick spinal cord lysates. As indicated in Figure 1CC, Sox9 is able to specifically ChIP the Sox9-Mu2 site in the e123 enhancer of the NFIA promoter. Taken together, these data indicate that Sox9 is necessary and sufficient for the activity of the e123 enhancer and does so via a direct mechanism.

NFIA Functions Downstream of Sox9 during the Initiation of Gliogenesis

Because Sox9 directly controls e123 enhancer activity, we reasoned that manipulation of Sox9 activity would impact expression of NFIA. To this end we introduced a dominant repressor form of Sox9, Sox9-EnR (Scott et al., 2010), into the chick spinal cord and found that it inhibited the expression of NFIA (Figure 2F). Next we introduced wild-type Sox9 or a dominant activator form of Sox9, Sox9-VP16, and found that both forms are sufficient to induce ectopic NFIA expression in regions outside the VZ (Figures 2G, 2H, and 2P, arrows). These observations indicate that Sox9 functions as a transcriptional activator to induce NFIA expression and are consistent with our findings that it regulates the activity of the e123 enhancer.

In the course of analyzing the Sox9 and the Sox9-VP16 electroporated embryos, we noticed that in regions outside the VZ demonstrating ectopic NFIA expression, there was also ectopic expression of the early astro-glial precursor marker GLAST (Figures 2L, 2M, and 2Q, arrows; Shibata et al., 1997). This observation indicates that Sox9 and Sox9-VP16 are sufficient to induce ectopic expression of glial precursor markers and is consistent with a role for Sox9 during the initiation of gliogenesis. Given that these GLAST-expressing regions contain ectopic NFIA and that NFIA is necessary for GLAST expression, we next determined whether the ability of Sox9 to induce ectopic GLAST is reliant upon its regulation of NFIA (Deneen et al., 2006). Here, we coelectroporated Sox9-VP16 along with an NFIA-shRNAi and examined the expression of GLAST and a set of other astro-glial precursor markers (Figure S3). As shown in Figures 2I, 2N, and 2Q, Sox9-VP16 is not capable of inducing ectopic GLAST in the absence of NFIA, indicating that Sox9 regulation of NFIA results in the ectopic induction of glial precursor markers. Next, we performed the converse experiment by coelectroporating Sox9-EnR and NFIA and found that NFIA can restore GLAST in the presence of Sox9-EnR (Figures 2J and 2O), further indicating that NFIA functions downstream of Sox9. Collectively, these data establish a functional hierarchy between Sox9 and NFIA during the initiation of gliogenesis, where the ability of Sox9 to promote the initiation of gliogenesis is linked to its direct induction of NFIA expression.

Sox9 Is Necessary for NFIA Induction

The foregoing data gathered in the embryonic chick spinal cord indicate that Sox9 directly regulates NFIA induction and that this relationship is crucial for the initiation of gliogenesis. We next sought to determine whether these same regulatory relationships are present in the mouse. First, we determined the temporal patterns of Sox9 and NFIA induction and found that Sox9 is induced prior to NFIA in the VZ of the embryonic spinal cord (Figures 2R–2Y). Examination of the mouse e123 enhancer revealed a Sox9 site within the conserved Sox9-Mu2 region (Figures 1B and 1C), and therefore we next determined whether Sox9 could ChIP this site in the e123 enhancer region within the endogenous mouse NFIA promoter. To this end, we performed ChIP from E12.5 mouse spinal cord and found that Sox9 is capable of interacting with the Sox9-Mu2 binding site in the e123 enhancer of the mouse NFIA

promoter (Figure 1CC). These data suggest that Sox9 and NFIA have a similar regulatory relationship in mouse and chick.

To provide genetic evidence linking Sox9 to the induction of NFIA during the initiation of gliogenesis, we intercrossed the *Sox9*$^{fl/fl}$ and *nestin-cre* mouse lines (Akiyama et al., 2002). This approach has been used previously to conditionally delete Sox9 in VZ populations of the embryonic spinal cord and revealed a delay in the generation of oligodendrocytes (Stolt et al., 2003). Given the regulatory relationship between Sox9 and NFIA, we reasoned that loss of Sox9 in this context would impact the timing and/or the expression of NFIA. To examine this possibility, we generated E11.5–E12.5 *Sox9*$^{fl/fl}$*;nestin-cre* and *Sox9*$^{fl/+}$*;nestin-cre* embryos and assessed the expression of NFIA (Figures 2Z–2GG). NFIA is normally induced in the VZ of the spinal cord at E11.5, but in the absence of Sox9, induction of NFIA was delayed by 1 day to E12.5 (Figures 2DD–2GG, arrow). Analysis at E12.5 revealed reduced levels of NFIA expression in the absence of Sox9 (Figures 2FF and 2GG, arrow). Further analysis of these embryos revealed that the induction of GLAST is also delayed from E11.5 to E12.5 and reduced in the absence of Sox9 (Figures 2HH–2KK), correlating the expression patterns of NFIA and GLAST and reinforcing the functional hierarchy established in our chick studies. These mouse studies provide genetic evidence that Sox9 is necessary for the induction and expression of NFIA during the initiation of gliogenesis in the developing spinal cord.

Identification of Common Sox9 and NFIA Target Genes

Having established that the Sox9 and NFIA regulatory axis is crucial for the initiation of gliogenesis in both the mouse and chick spinal cord, we next sought to decipher how these transcription factors operate during the next phase of gliogenesis. Given the importance of cofactors in modulating transcription factor activity during temporally distinct phases of development, coupled with the observations that Sox9 and NFIA are coexpressed in the gliogenic VZ (see Figures 2T, 2U, 2X, and 2Y), we hypothesized that Sox9 and NFIA physically interact and that this interaction regulates a repertoire of genes that define a temporally distinct phase of glial lineage development (Figure 3B). Therefore, we first examined whether there is a biochemical relationship between Sox9 and NFIA by determining whether they can physically associate. To this end, we performed immunoprecipitation (IP) experiments from E12.5 mouse spinal cord. Protein lysates from embryonic spinal cord were immunoprecipitated with antibodies to endogenous Sox9 and western blotted with antibodies to NFIA. The results of this IP-western indicate that Sox9 and NFIA physically interact in the embryonic spinal cord (Figure 3A). We confirmed this interaction by doing IP-westerns on ectopically expressed, tagged versions of NFIA and Sox9 in both p19 mouse embryonal carcinoma and HEK293 cells (Figure S4).

That Sox9 and NFIA physically associate raised the possibility that they coregulate a cohort of genes induced during the early phases of gliogenesis (Figure 3B). To identify candidate genes that are coregulated by Sox9 and NFIA, we utilized gene expression profiling data we previously generated from mouse VZ populations prospectively isolated at 24 hr intervals during the E9.5–E12.5 developmental interval (Deneen et al., 2006; Mukouyama et al., 2006). Because Sox9 and NFIA are coexpressed in the VZ from E11.5 onward, we reasoned that putative targets of the Sox9/NFIA complex are likely to be induced between E11.5 and E12.5. Analysis of our microarray data set revealed a cohort of genes specifically induced during the E11.5–E12.5 interval (Figures 3C and 3D; Table S1). Because we are seeking to identify candidate genes coregulated by the Sox9/NFIA complex, we used bioinformatics (see Experimental Procedures) to identify genes that contain Sox9 and NFIA binding sites in close proximity (i.e., ~120 bp apart) within their putative promoter region (~25 kb from the transcriptional start site). This analysis resulted in the identification of 15 candidate genes, 8 of which demonstrated specific induction in VZ populations between E11.5 and E12.5

(Figures 3E–3P and S4). The temporal patterns of induction of this cohort of genes indicate that they mark a distinct phase of gliogenesis that occurs after initiation, and, importantly, are candidate targets of the NFIA/Sox9 complex.

NFIA and Sox9 Coregulate Expression of *Apcdd1, Mmd2*, and *Zcchc24*

To determine which of the eight candidate genes are regulated by the Sox9/NFIA complex, we performed qRT-PCR on spinal cord from E12.5 *NFIA*- or *Sox9*-deficient and heterozygote control embryos, reasoning that those candidate genes demonstrating reduced levels of expression in both mutants are likely to be targets of this complex. This analysis revealed that four of the eight genes are significantly reduced in the absence of NFIA or Sox9: *Apcdd1, Mmd2, Zcchc24*, and *Hod-1* (Figure 4M and S4). Next we performed in situ hybridization on E12.5 *NFIA*- or *Sox9*-deficient and heterozygote control embryos and confirmed the reduced levels of expression of *Apcdd1, Mmd2*, and *Zcchc24* (Figures 4A–4L). These data provide genetic evidence that expression of these genes is dependent on both NFIA and Sox9.

We next sought to determine whether Sox9 and NFIA are capable of interacting with their binding sites in the promoter regions of *Apcdd1, Mmd2*, and *Zcchc24* by performing ChIP on E12.5 spinal cord. To determine whether endogenous Sox9 and NFIA interact with their binding sites, we designed primers flanking their sites and used PCR to detect ChIP of these regions (Figures 4N, 4P, and 4R, black arrows; Figure S5). These ChIP assays demonstrate that NFIA and Sox9 bind regions of the *Apcdd1, Mmd2*, and *Zcchc24* promoters that contain their consensus binding sites (Figures 4N, 4P, and 4R), indicating a direct regulatory relationship.

Although the foregoing data indicate that Sox9 and NFIA can directly regulate the expression of *Apcdd1, Mmd2*, and *Zcchc24*, they do not distinguish between individual and collaborative regulation of these genes. To determine whether Sox9 and NFIA collaborate to activate *Apcdd1, Mmd2*, and *Zcchc24* expression, we cloned their promoter regions and examined the ability of Sox9 and NFIA to activate these regulatory elements. Our reporter assays indicate that NFIA and Sox9 alone are not sufficient to activate the *Zcchc24* promoter, but combined expression resulted in a 3.5-fold induction in promoter activity (Figure 4S). Similarly, analysis of the *Apcdd1* and *Mmd2* promoters indicated that combined expression of Sox9 and NFIA resulted in a 4-fold increase in activity compared to individual expression (Figures 4O and 4Q). These data indicate that Sox9 and NFIA collaborate to drive activation of these regulatory elements. In parallel, we used two mutant versions of Sox9, one that is not capable of binding DNA (Sox9-F12L) and another that is deficient in protein dimerization (Sox9-A76E) (Mertin et al., 1999; Sock et al., 2003). We found that for all three promoters, combined induction is dependent upon both dimerization and DNA binding, as shown by the fact that synergistic activation with NFIA was significantly reduced with both Sox9 mutants (Figures 4O, 4Q, and 4S).

We next sought in vivo evidence for collaborative regulation of *Apcdd1, Mmd2*, and *Zcchc24* by Sox9 and NFIA by assessing these regulatory relationships in the chick model. Analysis of these genes in the chick spinal cord revealed similar expression dynamics and regulatory relationships with both Sox9 and NFIA, suggesting that this mechanism is conserved across species (Figures 4T–4X and S6). Our preceding data point to a collaborative model of gene activation that can be tested in the chick by examining the ability of NFIA to rescue gene expression in the presence of Sox9-EnR. Analysis of embryos coelectroporated with Sox9-EnR and NFIA revealed a similar loss of *Apcdd1, Mmd2*, and *Zcchc24* expression compared to the Sox9-EnR control (Figures 4T–4CC), indicating that NFIA is not capable of restoring gene expression in the presence of Sox9-EnR. These data suggest that fully functional Sox9 and NFIA are required for complete

expression of these genes, and in conjunction with our genetic and biochemical data support a collaborative model of gene regulation.

Apcdd1, Mmd2, and *Zcchc24* Restore Gliogenesis in the Absence of NFIA

Apcdd1 is a membrane-bound glycoprotein that can antagonize Wnt signaling, *Mmd2* (also known as PAQR 10) is a putative mitochondrial protein, and *Zcchc24* is a zinc finger-containing gene that is a putative transcription factor (Góñez et al., 2008; Shimomura et al., 2010). Because each of these genes has functions associated with cellular processes that can influence cell fate decisions, we reasoned that they participate in the early stages of gliogenesis. Therefore, to investigate the functional significance of this regulatory node within our transcriptional hierarchy, we examined whether *Apcdd1, Mmd2*, or *Zcchc24* can restore gliogenesis in the absence of NFIA. To perform these experiments, we made use of the embryonic chick spinal cord and an NFIA-shRNAi that effectively blocks the initiation of gliogenesis (Deneen et al., 2006). In these experiments, we coelectroporated the NFIA-shRNAi along with a cDNA to *Apcdd1, Mmd2*, or *Zcchc24* in the embryonic chick spinal cord and harvested embryos at early gliogenic stages (~E5.5). Our rescue experiments revealed that coelectroporation of *Apcdd1* or *Zcchc24* with the NFIA-shRNAi resulted in the restoration of GLAST and FGFR3 (Figures 5M, 5N, 5R, 5S, 5Z, and 5AA) but not Olig2 (Figures 5O, 5T, and 5BB), whereas coelectroporation of *Mmd2* resulted in rescue of GLAST, FGFR3, and Olig2 (Figures 5H–5J and 5Z–5BB). Next, we determined whether *Apcdd1, Mmd2*, or *Zcchc24* restore gliogenesis in the presence of Sox9-EnR. Here we found similar results, where *Apcdd1* and *Zcchc24* rescue GLAST and FGFR3, but not *Olig2*, whereas *Mmd2* rescues all three markers (Figure S7).

We next performed late-stage rescue to determine whether these gene can restore subsequent stages of glial lineage development in the absence of NFIA. In these studies we electroporated each gene along with the NFIA-shRNAi, harvested at E8.5, and assessed the number of migrating astrocyte and oligodendrocyte precursors outside the VZ. Consistent with our early stage rescue studies, we found that *Acpdd1* and *Zcchc24* restored migration of astrocyte precursors (ASPs) but not oligodendrocyte precursors (OLPs) (Figures 5OO–5QQ, 5TT–5VV, and 5WW–5YY), whereas *Mmd2* was able to restore migration of both ASP and OLP populations (Figures 5JJ–5LL and 5WW–5YY). Collectively, these rescue studies indicate that the Sox9/ NFIA gene regulatory network activates multiple, independent pathways that contribute to the specification and differentiation of both ASP and OLP populations.

Mmd2 Is Required for Proliferation of Glial Precursors and Energy Metabolism

The foregoing data suggest that these genes play a key role in the specification and differentiation of glial populations; therefore, we next sought to understand how they function to promote gliogenesis. To this end we performed shRNAi knockdown of *Mmd2* with an RCAS-shRNAi system. The effective knockdown of *Mmd2* at E6 was verified by in situ hybridization and resulted in decreased expression of ASP markers GLAST, FGFR3, and FABP7 and of OLP marker *Olig2* (Figures 6A–6F). Further analysis revealed a ~60% decrease in the number of Pax6-expressing progenitors, without a significant increase in cell death as measured by caspase 3 staining or concomitant increase in neurogenesis (Figure S8). To control for the specificity of the *Mmd2* knockdown phenotype, we generated a mutant version of the *Mmd2*-shRNAi containing five nucleotide substitutions, which had no effect on ASP, OLP, or Pax6 marker expression and demonstrated similarly low levels of caspase 3 staining (Figures 6H–6M and S8). Next, to confirm that these phenotypes are due to a loss of *Mmd2*, we performed a rescue experiment, where we coelectroporated a mouse cDNA to *Mmd2* with the *Mmd2* shRNAi. As indicated in Figures 6O–6T, *Mmd2* is able to

rescue the loss of ASP and OLP markers. Collectively, these data indicate that knockdown of *Mmd2* results in the loss of glial precursor populations in the embryonic spinal cord.

That knockdown of *Mmd2* resulted in a loss of precursor populations without increased cell death suggests that it influences cell proliferation. To determine whether loss of *Mmd2* effects proliferation, we electroporated the *Mmd2*-shRNAi (or mutant control) and performed BrdU labeling on chick embryos at E6. We found that knockdown of *Mmd2* results in a 70% decrease in BrdU incorporation at E6, suggesting that these phenotypes are the result of decreased proliferation (Figures 6G, 6N, and 6U).

Mmd2 (or PAQR10) contains a putative mitochondrial targeting sequence (MTS) and has previously been shown to localize to the mitochondria (Góñez et al., 2008). To confirm these findings, we overexpressed Myc-tagged *Mmd2* in U87 astrocytoma cells and found that it localizes to mitochondria (Figures 6V–6X). Because loss of *Mmd2* does not appear to influence cell death (Figure S8), we reasoned that it plays a role in energy metabolism. To determine whether *Mmd2* influences mitochondrial oxidative energy metabolism, we performed enzymologic assays of respiratory chain complexes II–IV and citrate synthase on E6 chick spinal cord electroporated with *Mmd2*-shRNAi, mutant control, and a GFP-only control. Specific knockdown of *Mmd2* resulted in a ~50% reduction in the activity of complex II and IV, compared to mutant and transfection controls, suggesting that oxidative respiration is impaired in the absence of *Mmd2* (Figure 6Y). Together, our developmental and biochemical studies in chick correlate *Mmd2* function in glial precursor proliferation to the activity of respiratory chain complexes II/IV in the mitochondria.

Apcdd1 Promotes ASP Migration

To determine the role of *Apcdd1* during gliogenesis, we generated a mutant version that has previously been shown to function as a dominant negative (*Apcdd1*-L9R) (Shimomura et al., 2010). Coexpression of *Apcdd1*-L9R (herein, L9R) with NFIA-shRNAi is not able to rescue gliogenesis (Figures 5U–5BB), suggesting that this mutant functions either as a dominant negative or null in this system. Overexpression of L9R in the chick spinal cord did not affect the generation or proliferation of ASP populations in the VZ at E5 (Figure S8), but analysis at later stages (E9.5) revealed a ~50% decrease in the number of migrating ASPs, without effecting OLP migration or cell proliferation measured by PCNA (Figures 7A–7G). Gain-of-function studies with *Apcdd1* gave complementing results, where it promoted precocious migration of ASPs, but not OLPs, and had no effect on PCNA (Figures 7G–7M). These data indicate that *Apcdd1* functions to specifically promote ASP migration during gliogenesis.

To further substantiate these finding, we examined the effect of *Apcdd1* overexpression on a cellular level by assessing F-actin dynamics in vitro. Here we found that overexpression of *Apcdd1* resulted in an increase in F-actin polymerization, a phenotype associated with cell migration and motility, whereas L9R did not (Figures 7M–7R; Le Clainche and Carlier, 2008). Because assembly of F-actin fibers is associated with Rho-GTPases (Machesky and Hall, 1997; Nobes and Hall, 1995), we next investigated whether *Apcdd1* can associate with Rho-GTPases by performing co-IP studies on ectopically expressed, tagged versions of *Apcdd1* and GFP-fusion versions of *Rho, Rac1*, or *cdc42* in HEK293 cells. We found that both *Apcdd1* and L9R co-IP with *Rac* and *cdc42*, suggesting this association mediates their respective phenotypes (Figure 7S). The *Apcdd1*-L9R mutation disrupts normal membrane localization and so we next examined intracellular localization of *Apcdd1* or L9R with *Rac1* and *cdc42* (Shimomura et al., 2010). Coexpression of *Apcdd1* with *Rac1* or *cdc42* and subsequent immunolocalization revealed that *Apcdd1* colocalizes with each of these Rho-GTPases at the cell membrane, whereas L9R colocalization appears to be mostly cytosolic (Figures 7T–7Y and S8). These data implicate *Apcdd1* function in ASP migration and correlate this in vivo activity with actin polymerization and association with Rho-GTPases.

DISCUSSION

Here we delineated a transcriptional regulatory cascade that operates during the initiation of gliogenesis in the developing spinal cord and identified a unique set of genes that regulate key aspects of astro-glial precursor physiology. Although analogous transcriptional cascades have been elucidated during neurogenesis, our studies demonstrate a transcriptional hierarchy that functions during neural stem cell commitment to the glial lineage in vivo. In the course of these studies, we found that key members of this hierarchy, Sox9 and NFIA, physically associate and collaborate to control induction of glial-specific genes. Functional studies revealed that a subset of these genes, *Apcdd1* and *Mmd2*, perform key migratory and metabolic roles during gliogenesis, respectively. Together, these studies link the Sox9/NFIA regulatory complex to multiple genetic programs that regulate the physiology of astro-glial precursors, suggesting that they have unique metabolic and migratory properties that distinguish them from their neuronal counterparts.

Regulation of NFIA Induction during the Gliogenic Switch

Our enhancer screen identified e123 as a regulatory element whose activity recapitulates the spatial and temporal patterns of NFIA induction. Analysis of this enhancer revealed that Sox9 is responsible for its activity and controls the induction of NFIA expression in both mouse and chick spinal cord. Recently, Notch signaling has been implicated in the upregulation of NFIA during astrocyte differentiation in cortical cultures (Namihira et al., 2009). However, studies on Notch signaling during the gliogenic switch in the embryonic spinal cord indicate that it does not result in the induction of NFIA or gliogenesis in vivo (Deneen et al., 2006; Park and Appel, 2003; Zhou et al., 2001). Thus, regulation of NFIA by Notch may reflect a stage-specific phenomenon in differentiated astrocytes or a region-specific mechanism of regulation (i.e., cortex versus spinal cord). Indeed, regulation of the proneuronal gene neurogenin 2 (*ngn2*) is both domain and region specific (Henke et al., 2009; Novitch et al., 2001; Stoykova et al., 2000; Yun et al., 2001). Alternatively, given its well-established role in maintaining the progenitor pool, Notch may function as a permissive factor rather than an instructive cue for NFIA induction in vivo (Androutsellis-Theotokis et al., 2006; Imayoshi et al., 2010; Shimojo et al., 2008).

Although Sox9 directly controls NFIA induction, it is eventually expressed, albeit in a delayed and reduced manner, in the absence of Sox9. This raises the question of what other factors contribute to the regulation of NFIA induction or expression during gliogenesis. One possibility is partial compensation by other Sox proteins. Several Sox proteins are expressed in spinal cord VZ populations during gliogenesis and play active roles in glial differentiation (Bylund et al., 2003; Graham et al., 2003; Stolt et al., 2002, 2005; Stolt and Wegner, 2010). Another possibility is that Sox9 controls the timing of NFIA induction but other factors are responsible for maintaining its expression during later stages of gliogenesis, and in the absence of Sox9, these factors are able to partially compensate for its absence. Sox proteins have been implicated in stage-specific gene regulation during lens development, where Sox2 controls expression of N-cadherin in preplacode ectoderm, and later in development, regulation of N-cadherin becomes dependent upon Pax6 (Smith et al., 2009). An analogous mechanism may be controlling NFIA expression during astro-glial development.

Another key consideration in our understanding of the transcriptional mechanisms controlling the induction of NFIA is the role of epigenetics. Chromatin-modifying factors, PcG genes *Ring1b* and *Ezh2*, have been implicated in the repression of neurogenesis, a key process in the gliogenic switch, in the embryonic cortex, and DNA methylation has been implicated in regulating the expression of GFAP during astrocyte differentiation (Fan et al., 2005; Hirabayashi et al., 2009; Takizawa et al., 2001). Future studies will be aimed at examining the link between epigenetic modifiers and NFIA induction.

Sox9 and NFIA Complex Formation—Managing Sox9's Diverse Roles

Biochemical studies demonstrate that NFIA and Sox9 physically associate and collaborate to induce the expression of a subset of genes just after the initiation of gliogenesis. Given that Sox9 function is associated with neural stem cell maintenance, initiation of gliogenesis, and various aspects of glial differentiation during CNS development, its interaction with NFIA may mediate a subset of these diverse roles. Although Sox9 induction of NFIA may trigger the generation of glial fates, it does not result in a loss of neurogenic potential from these populations, as Sox9 expression is required at these stages for neurosphere formation in vitro, and NFIA is not sufficient to suppress neurogenesis (Deneen et al., 2006; Scott et al., 2010). Therefore, we propose a model whereby Sox9 function during the gliogenic switch evolves from maintaining neural stem cells and initiating gliogenesis (E10.5–E11.5) to promoting glial lineage progression (E11.5–E12.5) by controlling a set of genes that contribute to early gliogenesis (Figure 8). This shift in Sox9 function during glial lineage progression is facilitated by a feedforward mechanism, where Sox9 induces NFIA expression during glial initiation and subsequently associates with NFIA to drive lineage progression. Hence, Sox9 coordinates glial initiation and glial lineage progression via regulation and association with NFIA, respectively.

Our rescue analysis of targets of the Sox9/NFIA complex found that these genes restore panglial or ASP-specific identity during gliogenesis. The role of this complex in ASP formation is supported by specific defects at later developmental stages in astrocyte differentiation in both Sox9 and NFIA knockout mice (Deneen et al., 2006; Stolt et al., 2003). That this complex appears to influence ASP development raises the question of whether it also has a specific role in oligodendrocyte precursor (OLP) development. Given that both NFIA and Sox9, and the targets we identified, are also expressed in OLPs, it is possible that a subset of their targets specifically contribute to OLP development. Alternatively, if the Sox9/NFIA complex plays an ASP-specific role, it is likely that Olig2 interferes with the ability of this complex (or its targets) to activate ASP-specific genetic programs in OLPs. Indeed, Olig2 is a known antagonist of astrocyte development and has been shown to physically interact with NFIA and inhibit its ability to promote astrocyte differentiation (Deneen et al., 2006; Hochstim et al., 2008).

Markers of Early Gliogenesis

In the course of these studies, we utilized temporal profiling of neural stem cell populations and identified a subset of genes that are specifically induced between E11.5 and E12.5, just after the initiation of gliogenesis. Given that the paucity of reliable markers of early gliogenesis has hindered the study of these formative stages of gliogenesis and the intermediate stages of astro-glial development in vivo, this group of genes represents a unique set of markers that designates such stages of the glial lineage and may facilitate these studies. Indeed, there has been considerable effort to identify new markers of glial lineages, especially those that specifically mark astrocytes and subpopulations of astrocytes (Cahoy et al., 2008; Garcia et al., 2010; Hochstim et al., 2008; Yang et al., 2011).

Comparison of the genes we found to be induced after the initiation of gliogenesis with a transcriptome database of astrocyte and oligodendrocyte populations from the brain found that *Hod-1* and *Fgfbp3* are specifically expressed in astrocytes (Cahoy et al., 2008). Recent studies found that *Ndrg2* is expressed in astrocyte populations in the adult mouse brain (Shen et al., 2008). These observations suggest that these genes are expressed in multiple regions of the CNS (i.e., brain and spinal cord) and throughout astrocyte lineage development and, consequently, may be general markers of astrocytes. Functionally, both *Hod-1* and *Ndrg2* are incapable of restoring ASPs or OLPs in the absence of NFIA, suggesting that they may contribute to later stages of ASP development (data not shown).

Consistent with this, *Ndrg2* expression has been linked to proliferating astrocytes in vitro (Shen et al., 2008).

Role of *Apcdd1* and *Mmd2* in Glial Development

Functional studies in the embryonic chick spinal cord demonstrate that *Apcdd1* specifically rescues ASP populations, whereas *Mmd2* rescues both ASP and OLP populations in the absence of NFIA. These data, coupled with our observations that Sox9 and NFIA coregulate their expression, indicate that *Apcdd1* and *Mmd2* are functionally downstream of Sox9 and NFIA in the gliogenic cascade. Functional analysis of both genes revealed that they contribute to key physiological processes germane to glial precursor maintenance and differentiation.

Mmd2 (or PAQR10) contains a putative MTS and localizes to the mitochondria, though its precise function there has remained undefined (Góñez et al., 2008). We found that knockdown of *Mmd2* in the chick spinal cord resulted in reduced numbers of glial progenitor populations, because of a decrease in their proliferative capacity. Alternatively, it is also possible that these changes in proliferation are a consequence of direct changes in cell fate; future studies will be aimed at delineating these models of *Mmd2* function. Biochemical analysis revealed that loss of *Mmd2* in the chick spinal cord results in decreased activity of respiratory chain complexes II and IV, thus correlating the proliferation of glial progenitors with energy metabolism. Indeed, electron transport chain function has previously been linked to cell cycle regulators and proliferation; therefore, it will be important to decipher the relationship between complex II/IV, cell proliferative mechanisms, and glial precursor biology (Mandal et al., 2010; Schauen et al., 2006). Moreover, that *Mmd2* appears to regulate energy metabolism via complex II/IV and is induced just after glial specification suggests that glial precursors have unique energy and/or metabolic requirements that are distinct from neural stem cells and committed neuronal progenitors. It is likely that each of these cell populations have unique metabolic profiles that reflect their biology and/or impending lineage commitments; indeed, neurons, astrocytes, and oligodendrocytes each have distinct metabolic requirements. Interestingly, the timing of cardiac myocyte differentiation has been linked to mitochondria maturation and function, indicating that metabolic function participates in lineage development (Hom et al., 2011). Therefore, in the future it will be important to identify distinct metabolic features of these precursor populations and to further delineate how these processes are coordinated with transcriptional cascades that specify their identity.

Apcdd1 is a membrane-bound glycoprotein that can inhibit canonical Wnt signaling through association with Wnt receptor complexes, though its exact role during spinal cord development remains undefined (Shimomura et al., 2010). These previous studies revealed a mild effect of *Apcdd1*-L9R on proliferation and specification during neurogenesis, phenotypes that we did not observe during gliogenesis (Figures 7 and S8), probably reflecting stage-specific effects of *Apcdd1*-L9R. Our studies indicate that *Apcdd1* plays a key role in the migration of ASP populations, probably through an association with Rho-GTPases. The observation that *Apcdd1* can influence Wnt receptor complexes, coupled with the role of noncanonical Wnt signaling in cell migration and regulation of Rho-GTPases, suggest a model whereby *Apcdd1* could function to promote ASP migration via noncanonical Wnt signaling (Schlessinger et al., 2009). That L9R overexpression does not effect the generation of ASP populations in the VZ suggests that *Apcdd1* is either not necessary for the generation of these populations or functions through other mechanisms. Alternatively, the epithelial to mesenchymal transition (EMT) has been shown to promote migration and the acquisition of progenitor-like states (Mani et al., 2008; Acloque et al., 2009). Therefore, one possibility is that *Apcdd1* activates an EMT program that is sufficient to restore ASP populations in the absence of NFIA. Nevertheless, a more comprehensive

understanding of signaling pathways associated with *Apcdd1* function will provide further insight into its role during astro-glial development.

EXPERIMENTAL PROCEDURES

Chick and Mouse Experiments

Expression constructs were cloned into the RCAS(B) (Morgan and Fekete, 1996) or pCIG vector (Megason and McMahon, 2002). Constructs were injected into the chick spinal cord at stage HH13–HH15 (~E2). See Supplemental Information for construct information. Electroporation was carried out with a BTX Electro Square Porator (Momose et al., 1999). *NFIA*$^{+/-}$ (das Neves et al., 1999), *Sox9*$^{fl/fl}$ (Akiyama et al., 2002), and *nestin-cre* (Betz et al., 1996) were used. The *Sox9*$^{fl/fl}$ mice were intercrossed with the *nestin-cre* mice to generate *Sox9*$^{fl/fl}$*;nestin-cre* and *Sox9*$^{fl/+}$*;nestin-cre* mice. Care of all animals and procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

ChIP and Immunoprecipitation

Mouse E12.5 spinal cord was dissected, dissociated, and processed for ChIP assays. Similarly, the electroporated chick spinal cords was dissected and used in ChIP assays. See Supplemental Information for details and ChIP primer sequences. Co-IP was performed by combining five E12.5 mouse spinal cords per experiment. Spinal cords were homogenized and the cell lysates were subject to immunoprecipitation with a specific antibody or IgG control and protein G agarose beads. See Supplemental Information for additional information.

In Situ Hybridization and Immunostaining

In situ hybridization on frozen mouse and chicken embryos was performed as previously described (Deneen et al., 2006). Mouse and chick tissue was fixed in 4% paraformaldehyde. The following probes were used for in situ hybridization: cGLAST, cFGFR3, cFABP7, cPDGFRα, mGLAST, cApcdd1, cMmd2, and cZcchc24. DNA to generate probes for the candidate gene in situs in Figures 3 and S4 was purchased from Open Biosystems. See Supplemental Information for probe and antibody information.

Reporter Assays and qPCR

Mouse *Apcdd1, Mmd2*, and *Zcchc24* promoter fragments were generated from mouse genomic DNA. Each promoter was cloned into a pGL3-basic vector. HEK293 cell line was transfected with reporter constructs and CMV-β-galactosidase vector and harvested, and cell lysate was mixed with luciferin to measure luciferase activity. For normalization of transfection efficiency, β-galactosidase was measured by the absorbance at 430 nm. Total RNA was isolated from E12.5 mouse spinal cord with a RNeasy mini isolation kit (QIAGEN). Quantitative RT-PCR was performed with PerfeCta SYBR Green Fast Mix (Quanta Biosciences) and a LightCycler 480 (Roche). See Supplemental Information for primer sequences used in these studies.

Analysis of Respiratory Chain Complexes

For enzymologic assays of respiratory chain complexes I–IV and citrate synthase, individual dissected chick embryonic spinal cords were lysed by sonication and spectrophotometric kinetic assays were performed with a monochromator microplate reader (Tecan M200). Complex II (succinate dehydrogenase), Complex III (ubiquinol: cytochrome c oxidoreductase), Complex IV (cytochrome *c* oxidase), and Citrate synthase activities were measured as described (Graham et al., 2010). Activities were calculated as nmol/min/mg

protein, normalized to citrate synthase activity, and expressed as a percentage of wild-type activity. For each group, spinal cords from six embryos were tested.

In Vitro Immunolocalization

For mitochondria localization, human U87 cells were transfected with myc-tagged *Mmd2* plasmid with lipofectamine. After 2 days transfection, cells were stained with 100 nM MitoTracker (Molecular Probes) and then fixed for immunostaining to detect Myc expression. For actin studies, HeLa cells were transfected with Flag-tagged *Apcdd1* wild-type or mutant (L9R). One day after trasfection, cells were moved to serum-free medium for 18 hr, fixed, and immunostained with Flag- and Alexa 488-conjugated phalloidin antibodies (Molecular Probes).

Bioinformatics

NIH Vista and ECR browser genomic alignment programs were used to compare 100 kb upstream of the mouse and chick NFIA gene. Upon isolation from chick genomic DNA, enhancer fragments were cloned into Topo2.1 vector, followed by subcloning of GFP with a minimum TATA box. Chick e123 genomic location: chromosome8: 27803349-27804949; mouse e123 genomic location: chromosome4: 97385017-97386617.

Analysis of microarray data was performed with Rosetta Resovler software as previously described (Hochstim et al., 2008). Please also see Table S1. We used the MAPPER search engine and database to identify putative Sox9 and NFIA binding sites (Marinescu et al., 2005). For details of screening procedure, please see Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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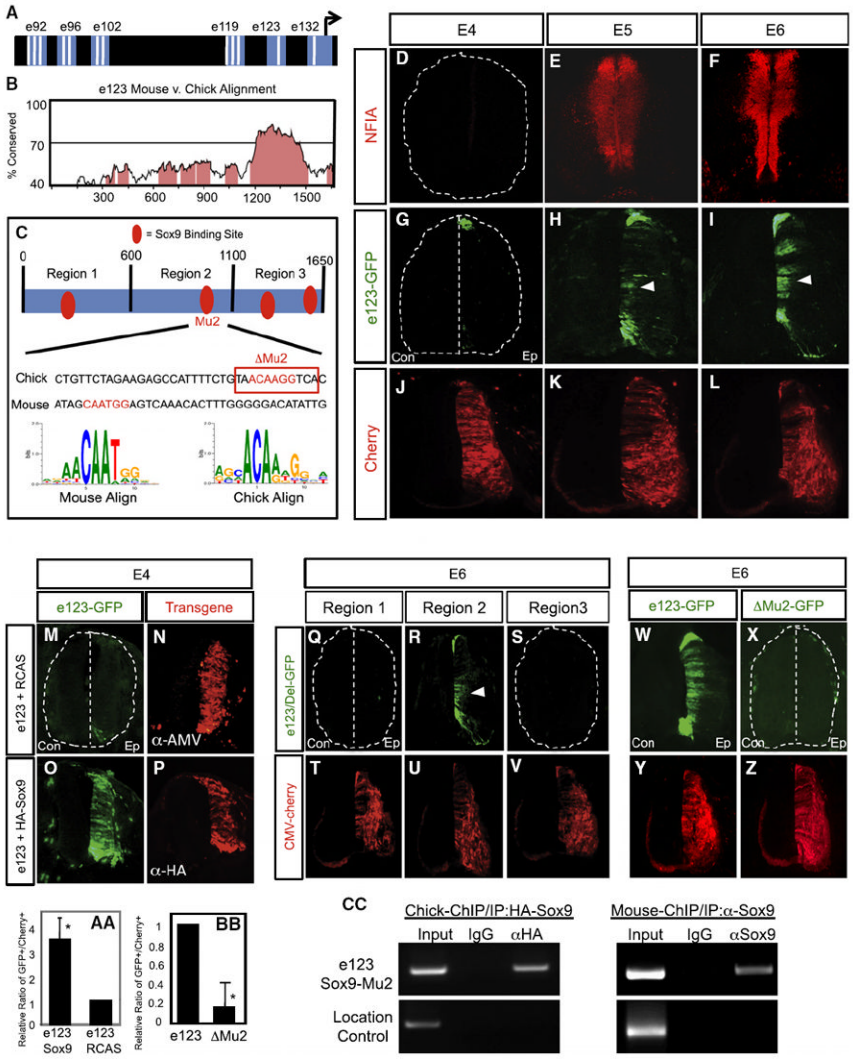

Figure 1. Sox9 Directly Regulates the Activity of the NFIA-e123 Enhancer

(A) Schematic illustrating NFIA enhancer elements. White lines are conserved regions; blue boxes the enhancer.

(B) Conservation of mouse and chick e123 sequence.

(C) Map of chick e123 enhancer. Alignment of chick and mouse Sox9-binding sites. Red box denotes region deleted in chick e123-ΔMu2.

(D–F) Expression of NFIA detected by immunostaining.

(G–L) Activity of e123; arrow indicates activity in the VZ and cherry is electroporation control.

(M–P) Ectopic Sox9 induces e123 activity at E4. Immunostaining for AMV (N) and HA-Sox9 (P).

(Q–V) Region 2 recapitulates activity of e123 at E6.

(W–Z) Loss of activity in ΔMu2-GFP at E6.

(AA and BB) FACS quantification of experiment in (M)–(P) (AA) and (W)–(Z) (BB) from four independent experiments. In (AA), *p = 0.012; in (BB), *p = 0.0002. Error bars represent SD.

(CC) PCR detection of Sox9-Mu2 site in Sox9 ChIP experiment in chick and E12.5 mouse spinal cord.

Please also see Figures S1 and S2.

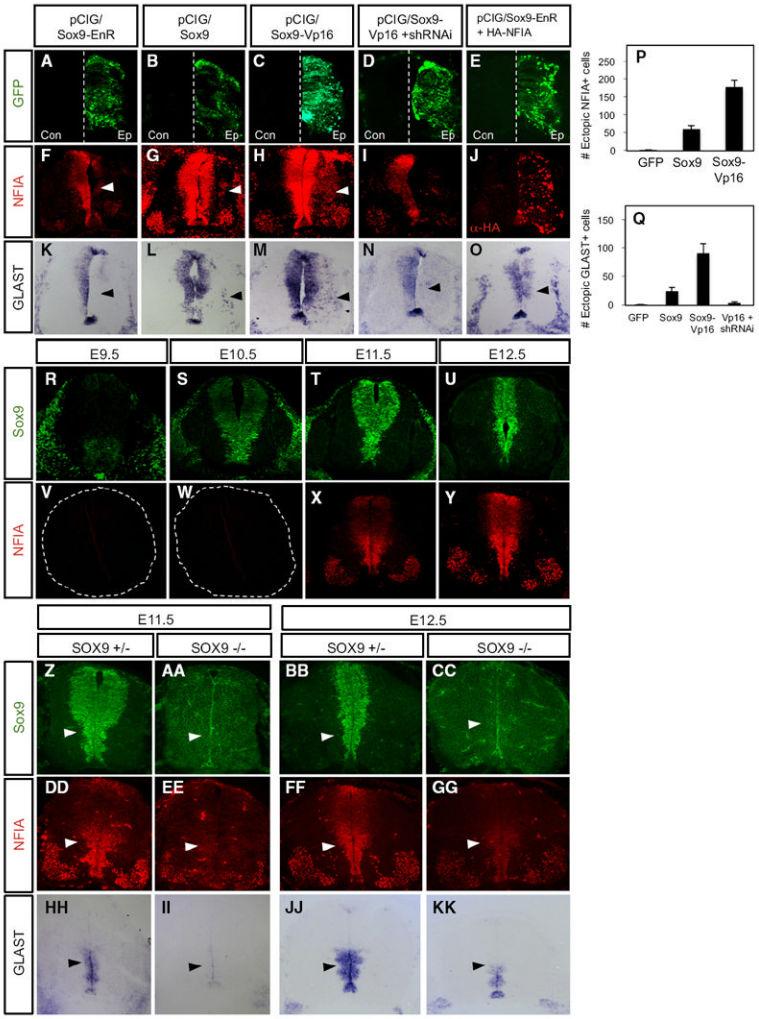

Figure 2. NFIA Expression Is Regulated by Sox9 in Embryonic Spinal Cord

(A–O) Sox9-EnR blocks NFIA and GLAST expression (A, F, K); Sox9 and Sox9-VP16 promote ectopic NFIA and GLAST (Sox9, B, G, L; Sox9-VP16, C, H, M); NFIA-shRNAi blocks Sox9-VP16 induction of ectopic GLAST (N); and NFIA rescues GLAST in the presence of Sox9-EnR (J, O).

(P and Q) Quantification of the number of ectopic NFIA- and GLAST-expressing cells, from three experiments. Error bars represent SD.

(R–Y) Sox9 is induced in the VZ prior to NFIA. Immunostaining for Sox9 (R–U) and NFIA (V–Y) in the embryonic mouse spinal cord from E9.5 to E12.5.

(Z–KK) *Sox9*$^{-/-}$ embryos demonstrate delayed and reduced NFIA and GLAST expression at E11.5 (EE, II) and E12.5 (GG, KK). Images presented are adjacent sections from a single embryo; heterozygote and homozygote images are from littermates and are representative of five embryos for each condition.

Please also see Figure S3.

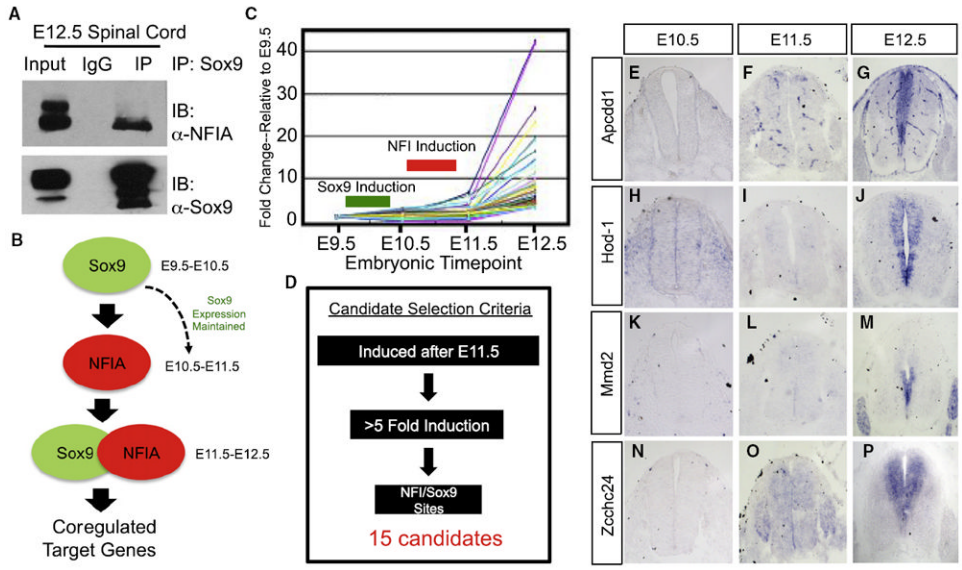

Figure 3. Identification of Candidate Targets of the Sox9/NFIA Complex

(A) Sox9 and NFIA co-IP from E12.5 spinal cord extracts.

(B) Schematic illustrating the possible mode of Sox9/NFIA function during early gliogenesis.

(C) Microarray analysis reveals a set of genes induced between E11.5 and E12.5; the green bar represents the induction of Sox9 (Figures 2R-2U) and red bar the induction of NFIA (Figures 2V-2Y).

(D) The selection criteria that resulted in the 15 candidate genes (see Experimental Procedures and Supplemental Experimental Procedures).

(E–P) In situ hybridization on E10.5–E12.5 embryonic spinal cord sections for four of the candidate genes identified in the screen. *Apcdd1* is also expressed in endothelial cells outside the VZ.

Please also see Figure S4 and Table S1.

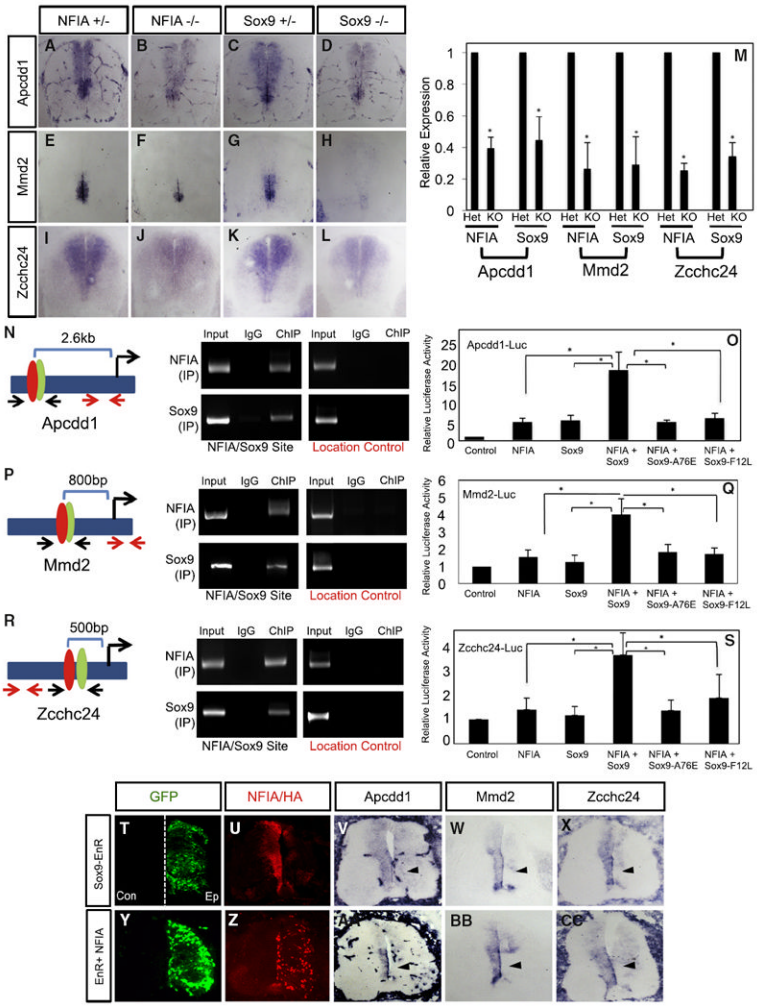

Figure 4. Sox9 and NFIA Coregulate *Apcdd1, Mmd2*, and *Zcchc24*

(A–L) *Apcdd1, Mmd2*, and *Zcchc24* expression is reduced in E12.5 spinal cord from $Sox9^{-/-}$ or $NFIA^{-/-}$ embryos.

(M) qPCR analysis from mRNA extracted from E12.5 $Sox9^{+/-}$ or $Sox9^{-/-}$ and E12.5 $NFIA^{+/-}$ or $NFIA^{-/-}$ embryos. Analysis for both experiments is representative of three embryos for each genotype (*p < .004).

(N, P, R) E12.5 mouse spinal cord ChIP demonstrating that Sox9 and NFIA associate with the *Apcdd1, Mmd2*, and *Zcchc24* promoters. Green ovals are Sox9 binding sites and red ovals are NFIA binding sites.

(O, Q, S) Sox9 and NFIA collaborate to activate the *Apcdd1, Mmd2*, and *Zcchc24* promoters. Values presented are the average of a least five independent experiments performed in triplicate. Error bars represent SD; *p < .007.

(T–CC) NFIA does not rescue loss of *Apcdd1* (V, AA), *Mmd2* (W, BB), or *Zcchc24* (X, CC) in the presence of Sox9-EnR. Arrows denote loss of gene expression.

Please also see Figures S5 and S6.

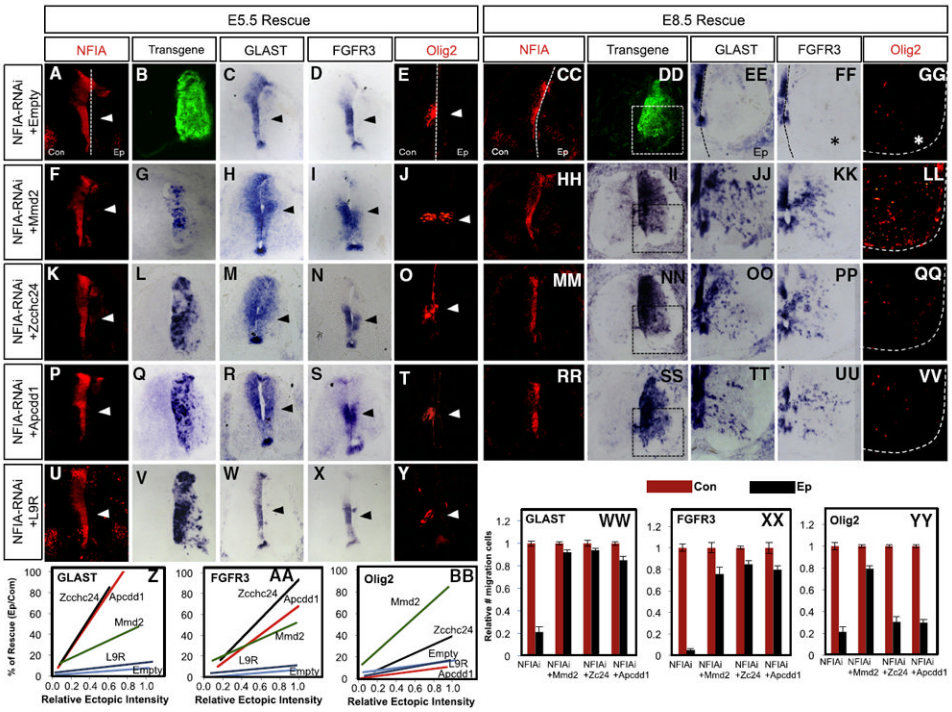

Figure 5. *Apcdd1, Mmd2*, and *Zcchc24* Rescue NFIA Knockdown in Chick

(A–E) NFIA-shRNAi + RCAS control results in loss of glial markers in the chick at E5.5.

(F–J) Ectopic expression of RCAS-*Mmd2* restores expression of glial markers.

(K–O) Ectopic expression of RCAS-*Zcchc24* restores ASP (M, N) but not OLP markers (O).

(P–Y) Ectopic expression of RCAS-*Apcdd1* rescues ASP (R, S) but not OLP markers (T).

(Z–BB) Ectopic expression of RCAS-*Apcdd1*-L9R does not rescue NFIA-shRNAi.

(CC–YY) Late-stage rescue reveals similar results; *Mmd2* rescues migration of all glial precursor populations (JJ–LL); *Zcchc24* and *Apcdd1* rescue migration of ASP (OO, PP, TT, UU) but not OLP (QQ, VV) populations. Boxed areas in (DD), (II), (NN), (SS) represent regions in adjacent sections shown at higher power in (EE)–(VV).

Quantification methods in (Z)–(BB) and (WW)–(YY) are described in Supplemental Experimental Procedures. The values in (Z)–(BB) are derived from eight embryos and ten adjacent sections series' per embryo. For (WW)–(YY), each condition the values represent eight sections from at least five embryos. Error bars represent SD. Please also see Figure S7.

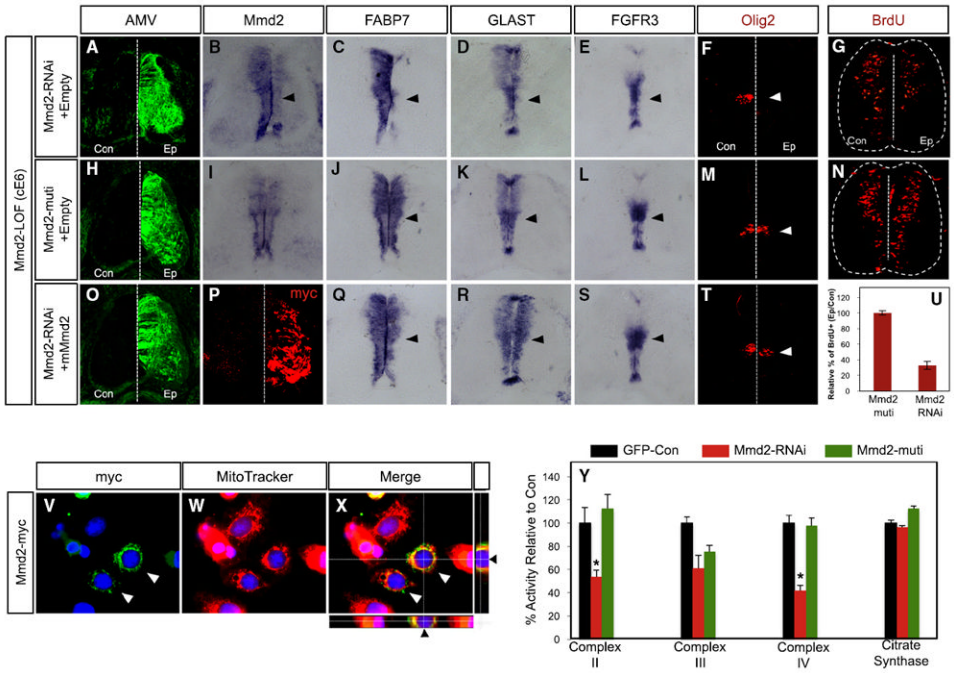

Figure 6. *Mmd2* Knockdown Results in Decreased Proliferation of Glial Precursors and Respiratory Chain Activity

(A–F) shRNAi knockdown of *Mmd2* (A, B), results in decreased expression of all glial markers.

(H–M) Mutant *Mmd2*-shRNAi does not effect expression of these markers.

(O–T) Ectopic expression of *Mmd2* with *Mmd2*-shRNAi rescues loss of glial markers.

(G, N, U) Reduced BrdU incorporation in *Mmd2*-shRNAi knockdown (G, U) compared to mutant shRNAi control (N, U).

(V–X) Expression of myc-tagged *Mmd2* in U87 cells colocalizes with mitochondrial marker, MitoTracker.

(Y) Knockdown of *Mmd2* in chick spinal cord results in decreased activity of respiratory chain complexes II and IV.

Error bars represent SEM. Please also see Figure S8.

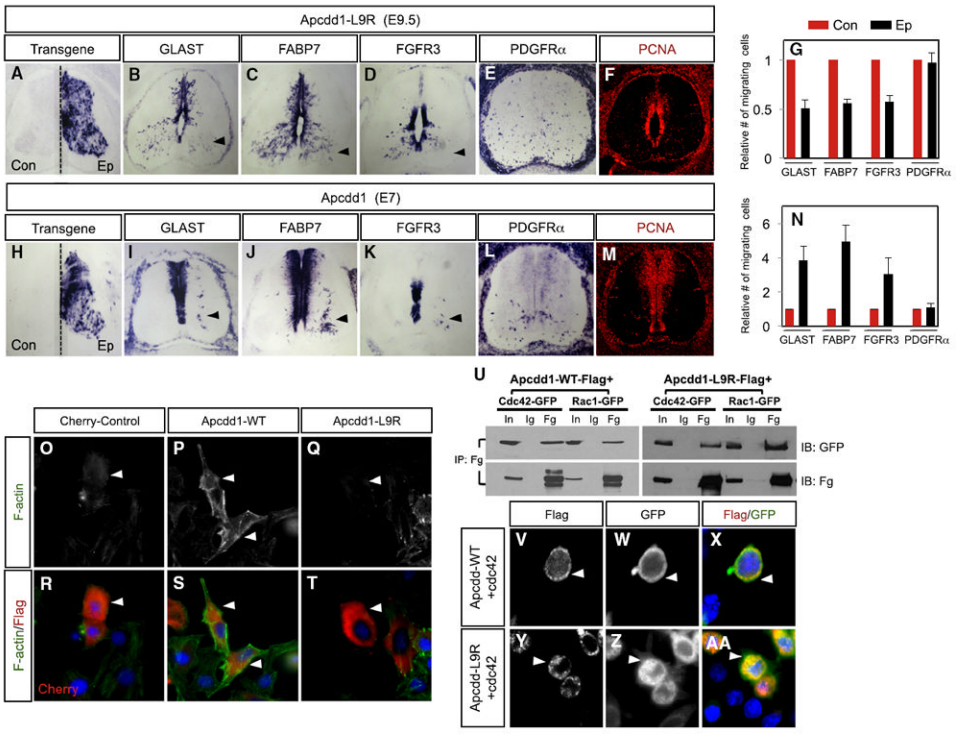

Figure 7. *Apcdd1* Promotes Astrocyte Precursor Migration

(A–F) Overexpression of *Apcdd1*-L9R inhibits migration of ASP populations at E9.5, without effecting the migration OLP (E) populations or expression of PCNA (F).

(H–M) Overexpression of *Apcdd1* promotes precocious migration of ASP populations at E7, without effecting OLP populations or expression of PCNA (L, M).

(O–T) Expression of *Apcdd1* in HeLa cells promotes actin polymerization (P, arrow), while *Apcdd1*-L9R does not (Q, arrow).

(U) *Apcdd1* and *Apcdd1*-L9R co-IP with Rac1 and *cdc42* in HEK293 cells transfected with a flag-tagged version of *Apcdd1* and GFP-fusion versions of Rac1 and *cdc42*.

(V–AA) *cdc42* colocalizes with *Apcdd1* at membrane (V–X, arrow), while *Apcdd1*-L9R colocalization is increased in the cytosol (Y–AA, arrow).

Quantification methods in (G) and (N) are described in Supplemental Experimental Procedures. For each condition, the values represent eight sections from at least eight embryos. Error bars represent SD. Please also see Figure S8.

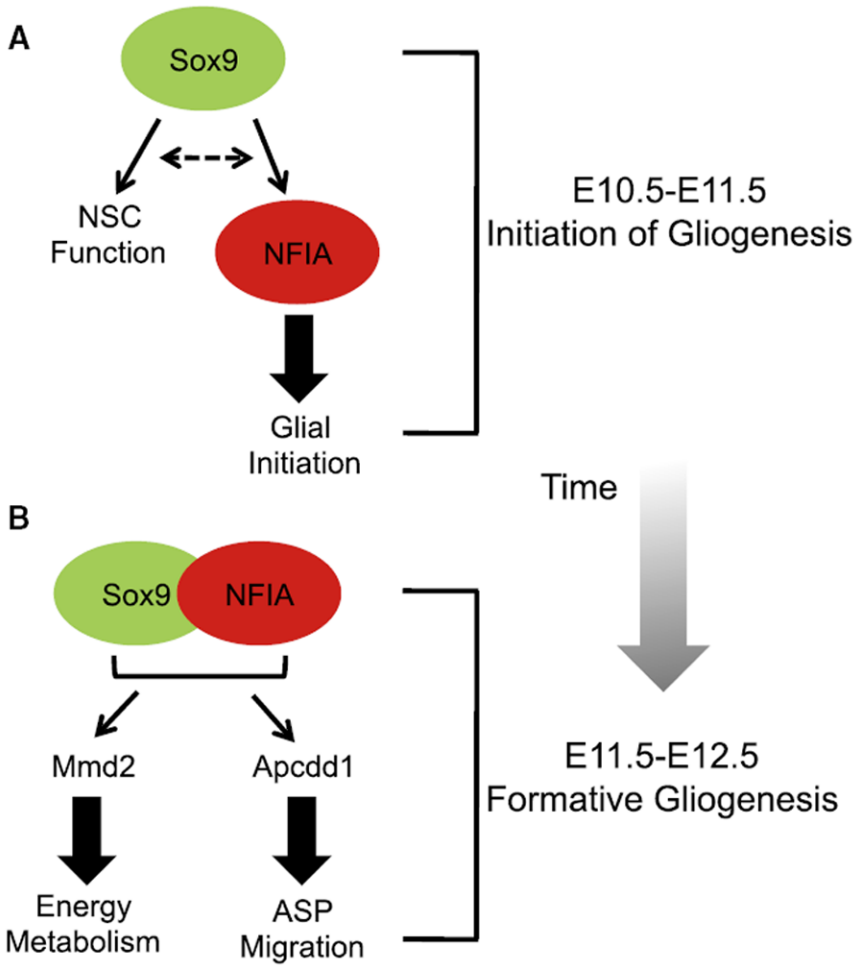

Figure 8. Sox9 and NFIA Comprise a Regulatory Hierarchy that Operates during Multiple Phases of Early Gliogenesis

(A) Sox9 induction of NFIA regulates the initiation of gliogenesis. Based on the timing of the induction patterns of Sox9 and NFIA, this event occurs between E10.5 and E11.5 and represents the initiative steps of neural stem cell commitment to the glial lineage. It remains to be determined whether Sox9 initiation of gliogenesis can be dissociated from its role in NSC maintenance, represented by the double-headed arrow.

(B) The Sox9/NFIA complex controls the induction of *Apcdd1* and *Mmd2*, which function to promote ASP migration and energy metabolism, respectively. Based on the timing of *Apcdd1* and *Mmd2* induction (and other candidates described in Figure 3) and their coregulation by Sox9 and NFIA, these events occur between E11.5 and E12.5 and represent a distinct step in early gliogenesis, which we termed formative gliogenesis.