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Supplemental Data

The Transcription Factor NFIA Controls the Onset of Gliogenesis in the Developing Spinal Cord

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Experimental Procedures

FACS analysis and microarray experiments

To specifically isolate Olig2-expressing cells from E9.5-E12.5 embryos, an Olig2-GFP transgenic mouse (a gift from Ben Novitch) was utilized. Olig2-GFP mice were crossed with wild-type mice and embryos were isolated between E9.5-E12.5. Embryonic spinal cord was dissected out and dissociated with papain to generate a single-cell suspension, then sequentially incubated with anti-CD-15/MMA (monoclonal-IgM; Developmental Studies Hybridoma Studies-DSHB), then anti-PDGFRα (monoclonal-rat; BD Biosciences). PDGFRα-expressing cells were subtracted using a sheep-anti rat secondary antibody conjugated to magnetic beads and incubated on a magnetic column to eliminate cells reactive to PDGFRα antibody. FACS isolation was performed as described in (Mukouyama et al., 2006).

For each timepoint, cells were harvested from at least two independent litters and pooled for FACS analysis. Replicate samples of each timepoint were independently generated, and processed in parallel. For microarray analysis, RNA from approximately $1x10^4$ cells was isolated using the Stratagene Absolutely RNA-Nano Kit. Isolated RNA was subjected to two rounds of amplification using the Ambion MessageAmp kit (#1750). During the second round of amplification the cRNA was labeled using biotin-CTP and

biotin-UTP. After cRNA amplification and labeling the product was fragmented and hybridized to Affymetrix arrays, mu74vA,B,C. Analysis of the microarray data was performed by Affeymetrix suite and the Rosetta Resolver system.

Generation of RCAS/shRNAi

To identify potential cNFIA sequences that could be used as an shRNAi, we used the Promega siRNA target designer and several other applications available on the web, in order to cross compare the sequences predicted by these different algorithms. The sequences that were identified by multiple programs were tested for silencing of cNFIA. The selected sequences were integrated into oligos containing a stem-loop sequence and sites to facilitate cloning. The format of the oligo design is as follows:

Forward 5'ACC-----G(N20)------TTCAAGAGA------(20N)C-------TTTTTA 3'

Reverse 3' -----C(N20)------AAGTTCTCT-------(20N)G--------AAAAAAGCTT 5'

Oligo's were annealed, kinase treated and cloned into a Bluescript-hU6 plasmid (a gift from D. Baltimore) (Qin et al., 2003) Bbs1-HindIII. Clones were sequence verified, and the hU6-shRNAi cassette was shuttled into the Slax-12 vector (RI/Hind), then from Slax-12 shuttled into RCAS(B) (Cla1). Orientation was confirmed via sequence analysis, hU6-shRNAi was orientated with the 5' hU6 orientated towards the 5'end of the RCAS 5'LTR. RCAS-shRNAi constructs were electroporated into E2 chicks along with CMV-GFP and collected at E6. Immunostaining with the avian retroviral coat protein, AMV, was used to confirm expression of RCAS-shRNAi. Embryos were then stained with antibodies to NFIA to asses whether a given shRNAi was successful in knocking down endogenous NFIA in the chick embryo. Of the four sequences tested one gave complete

knockdown of NFIA. The sequence to cNFIA that yielded complete knockdown of endogenous protein and the mutant version are:

GCCATCGCCAACTGCATTAAA cNFIA-RNAi

GC**G**ATC**T**CCAACT**A**CATTAA**C** cNFAI-RNAi mutant

In Situ Hybridization and Immunohistochemistry

Non-radioactive in situ hybridization on frozen mouse and chicken embryos was performed as previously described (Zhou et al., 2000). Mouse and chick tissue was fixed in 4% paraformaldehyde from 1 hour to overnight, depending upon the age of the embryo. The following probes were used for in situ hybridization: cGLAST, cFGFR3, cPDGFRα, mNFIA, mNFIB, mGLAST, and cHES5.

Immunohistochemistry was performed on mouse and chicken embryonic tissue fixed as described above. The following antibodies, were used: hOlig2 (goat polyclonal), cOlig2 (rb/polyclonal-gift from T. Jessell), bovine-GFAP (rabbit-polyclonal/DAKO), m-GFAP (mouse IgG1/Chemicon), ms100β (mouse-monoclonal-IgG1/Sigma), hNFIA (rabbit polyclonal/Geneka), hNFIB (rabbit polyclonal), AMV (mouse monoclonal-IgG1, DSHB), mSox9 (mouse monoclonal-IgG1), mPax6 (mouse monoclonal-IgG1/DSHB), Pax7 (mouse polyclonal-IgG1), mNeuN (mouse-IgG1/Chemicon), GFP (chicken polyclonal/Avian Labs), HA (rat polyclonal/Roche), Lim1/2 (mouse polyclonal-IgG1/DHSB), c-Myc (rabbit polyclonal-Santa Cruz Biotechnology cat# scb-789), BrdU (rabbit polyclonal-Immunology Consultants Laboratory), and cngn2 (rabbit polyclonal).

Double In situ/Immunolabeling in Fig. 2M-R and Fig. 6L-M was performed by sequentially performing a fluorescent in situ hybridization with cGLAST or cFGFR3 followed by immuostaining with hNFIA antibody.

Electroporation of chick embryos

Expression constructs and the shRNAi cassettes were cloned into the avian retrovirus RCAS(B) vector (Morgan and Fekete, 1996). The RCAS expression constructs were injected into the embryonic chick spinal cord at stage HH13-15 (~E2) with the use of a capillary needle, micromanipulator, and dissecting microscope. Electroporation was carried out with a BTX Electro Square Porator (Momose et al., 1999). In co-electroporation experiments, empty plasmid was utilized where necessary to ensure that equal concentrations of DNA were introduced during electroporation. In the BrdU pulse-chase experiments, 250µl of a 1mg/ml BrdU-PBS solution was added directly to chick embryos at E5. Embryos were harvested 6 hours after the BrdU pulse.

Culture and transfection of cortical progenitors

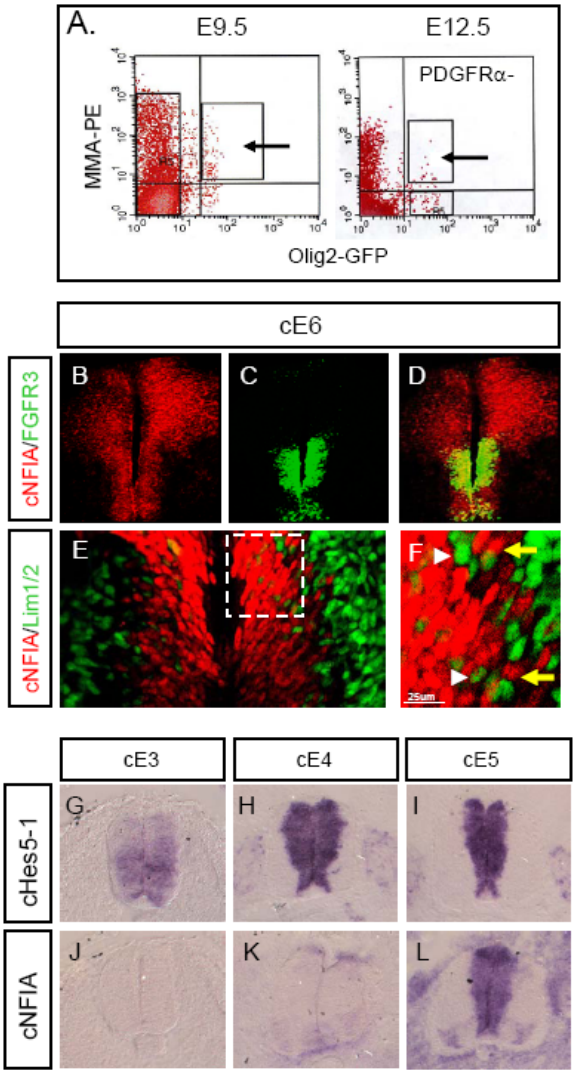
Rat cortical progenitor cells were isolated via dissection of E13.5 rat embryonic cortex and dissociation with papain. Cells were plated at density of $2x10^4$/ cm^2 on plates coated with 15µg/ml of polyornithine (Sigma) and 1µg/ml human fibronectin (Biomedical Technologies). Cells were grown in DMEM/F12 supplemented with N2 and B27 (GIBCO) and 20 ng/ml bFGF (R&D systems). Cells were expanded for 3-4 days before transfection. Cells were transfected with Lipofectamine 2000 using pCS+ or pcDNA plasmids containing GFP, HA-mNFIA, HA-mNFIB, and/or myc-mOlig2. Empty plasmid was added to bring the final total amount of DNA to 4µg where necessary. In experiments that required the removal of bFGF to promote astrocyte differentiation,

bFGF was removed 24 hours post-transfection and replaced with media described above supplemented with 2% FCS. Transfected cells were scored on images captured with a Zeiss digital camera.

Immunoprecipitation and Immunobloting

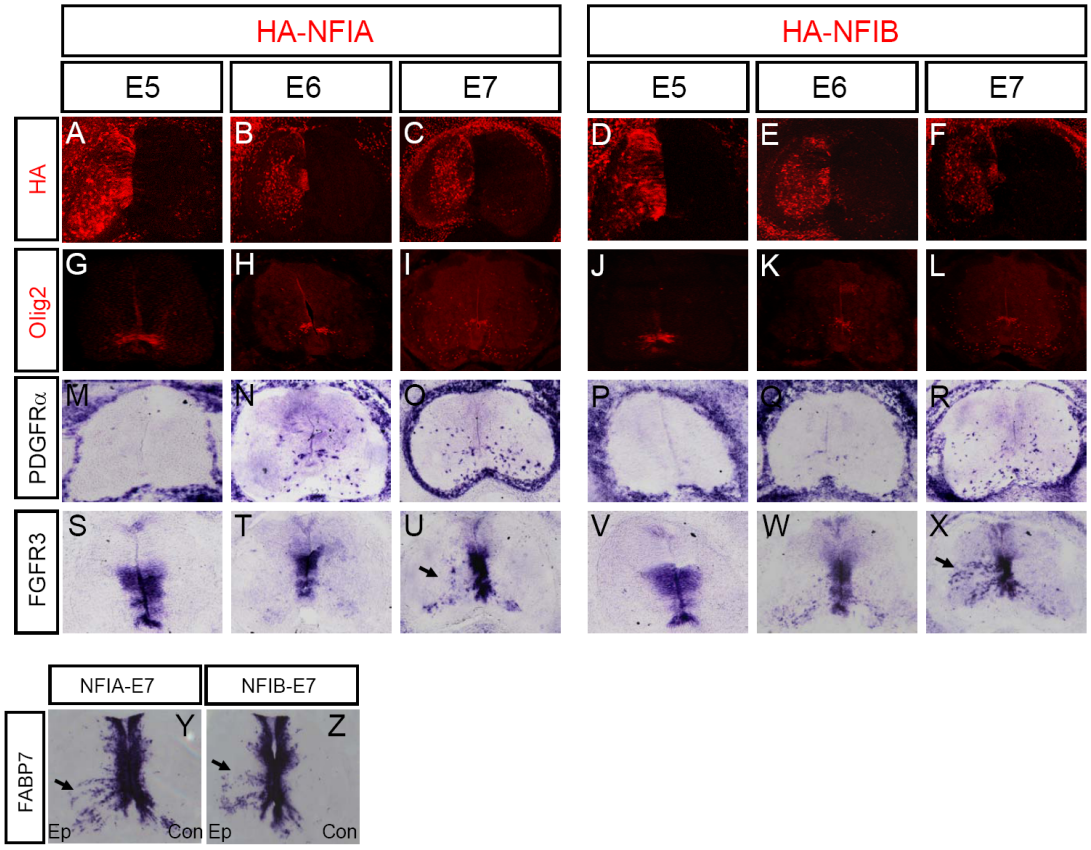
Human embryonic kidney cells (HEK293) cells were transfected using calcium phosphate precipitation, with CMV-based expression plasmids containing myc-mOlig2 and/or HA-mNFIA. Cells were harvested three days post transfection in a lysis buffer containing 100mM NaCl, 25mM Hepes pH 7.5, 5mM TritonX-100, 2mM KCl, and 0.5mM PMSF. Cells were briefly sonicated and lysates were incubated overnight with agarose bead-conjugated antibodies to myc and HA; Profound^{TM} HA and c-Myc Tag IP/Co-IP Kit (Pierce cat# 23610, 23620). Following incubation, beads were washed and incubated at 100^{o}C to dissociate the beads from the IP-complexes. IP samples were run on an 8% polyacrylamide gel, transferred to nitrocellulose membranes, and immunoblotted with either c-myc or NFIA antibodies. Immunoblots were developed using ECL development reagent (Pierce cat# 32106).

Supplemental Figure S1



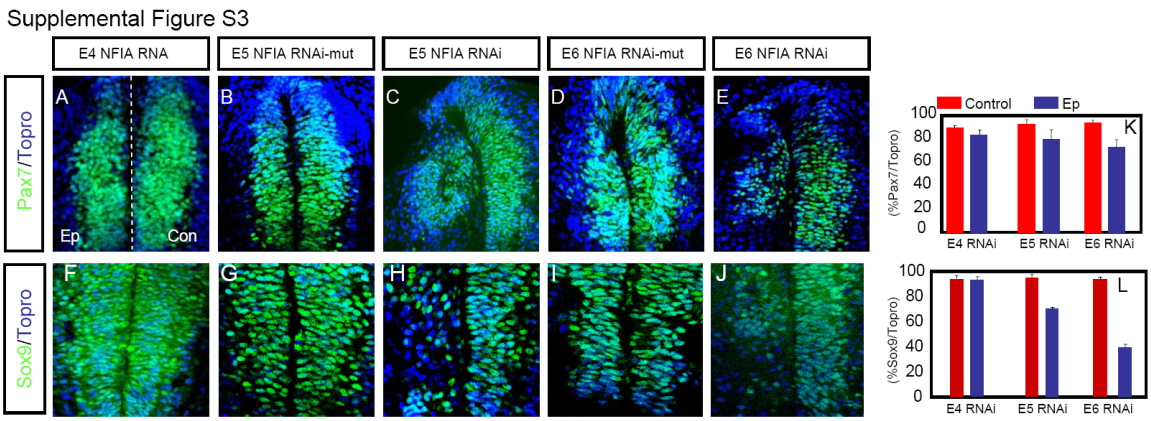
Supplemental Figure S1. <u>FACS plots and expression characteristics of various developmental markers with respect to NFIA.</u> (S1A) FACS plots illustrating isolation of Olig2-GFP^{+}/CD15-MMA^{+}/PDGFRα^{-} progenitors from E9.5 and E12.5 murine spinal cord. (S1B-D) Double Fluorescent in situ for NFIA and FGFR3. (S1E-F) Double immunolabeling for NFIA and Lim1/2. (S1F) Higher magnification image of the boxed region in (S1D). Lim1/2^{+} (white arrowheads) and NFIA^{+} (yellow arrows) -expressing cells are distinct. (S1G-I) cHes5 in situ hybridization and (S1J-L) cNFIA in situ hybridization on chick embryonic spinal cord. Sections in (S1G-I) and (S1J-L) were processed in parallel and developed for the same length of time.

Supplemental Figure S2

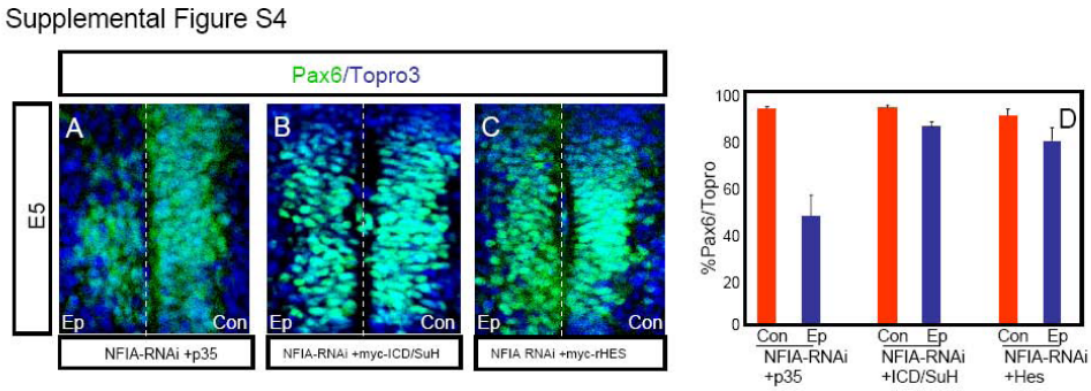


Supplemental Figure S2. <u>Ectopic overexpression of NFIA or NFIB in the embryonic chick spinal cord does not effect the generation of oligodendrocyte precursors.</u>

Overexpression of NFIA (S2A-C) or NFIB (S2D-F) from embryos collected at E5-E7 did not influence the temporal or spatial expression patterns of oligodendrocyte precursor markers Olig2 (S2G-L) or PDGFRα (S2M-R). Overexpression of NFIA or NFIB promotes the precocious migration of $FGFR3^{+}$ APs (S2S-X) and $FABP7^{+}$ (S2Y-Z) APs at E7. The analysis presented is representative of at least four embryos from three independent electroporation experiments.



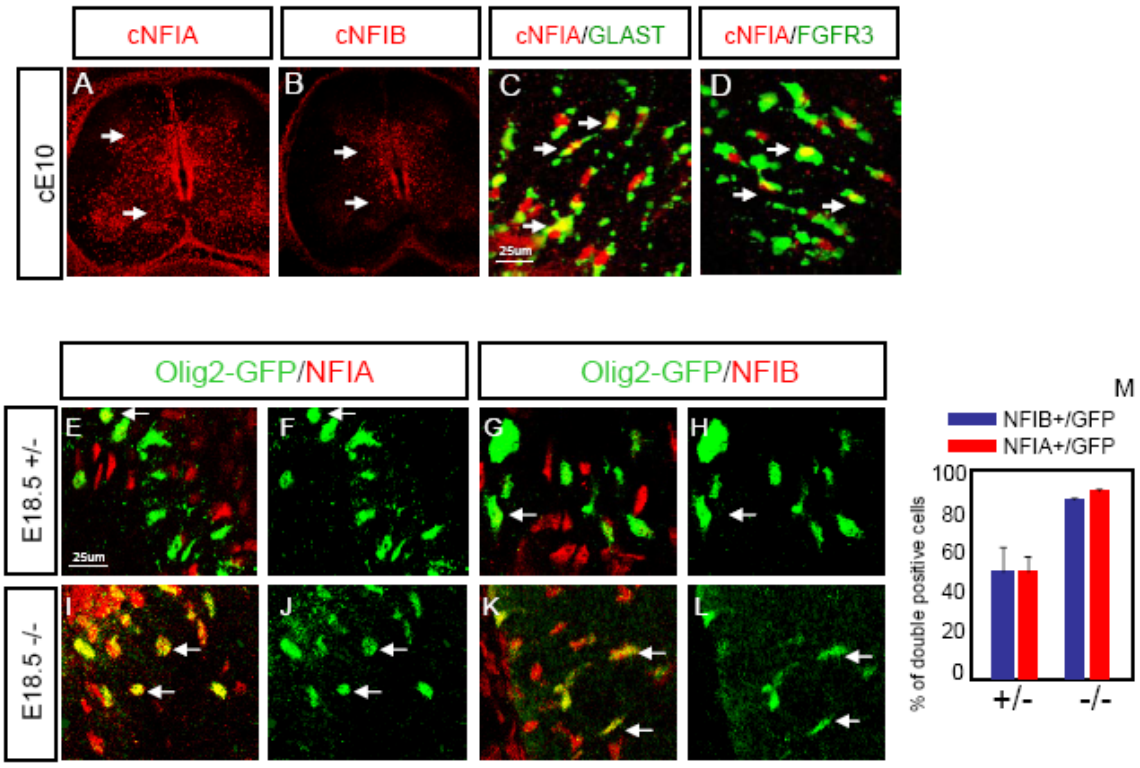
Supplemental Figure S3. **<u>RNAi knockdown of cNFIA leads to a reduction in the expression of VZ progenitor markers at E5 and E6.</u>** Imunostaining with Pax7 counterstained with nuclear marker Topro-3 is indicated in S3A-E, K. Pax7 marker expression is unaffected at E4, E5, and in the presence of the cNFIA-shRNAi-mutant (S3A-D, K), however by E6 there is a significant decrease in the number of Pax7-expressing cells, (S3E, K) in the presence of the cNFIA-shRNAi. (S3F-J, L) Immunostaining with Sox9 counterstained with Topro3 in regions dorsal to the pMN domain.



Supplemental Figure S4. **<u>Co-electroporation of Hes or Notch^{ICD}/SuH with NFIA-RNAi rescues loss of Pax6 expression.</u>** (A-C) Immunostaining with Pax6 counterstained with Topro3 on NFIA-RNAi+p35 (A), NFIA-RNAi+Notch^{ICD}/SuH (B) or

NFIA-RNAi+Hes (C) -electroporated embryos. (D) Quantitative analysis presented is representative of at least five sections x three electroporated embryos for each condition.

Supplemental Figure S5



Supplemental Figure S5. **<u>NFI genes are derepressed in the absence of Olig2.</u>** (A-D) Analysis in chick at cE10. (A, B) Immunostaining for NFIA (A) or NFIB (B). Arrows indicate presumptive migrating cells. (C, D) Double-label in situ reveals co-expression of cNFIA with GLAST (C) and FGFR3 (D). (E-M) Double-immunolabeling for NFIA and Olig2-GFP, in *Olig1/2* heterozygous (E-H), or homozygous (I-L), embryos. Note increased proportion of double-positive cells in homozygotes (M). Data are derived from four sections per embryo x 3 embryos of each genotype, from three different litters.