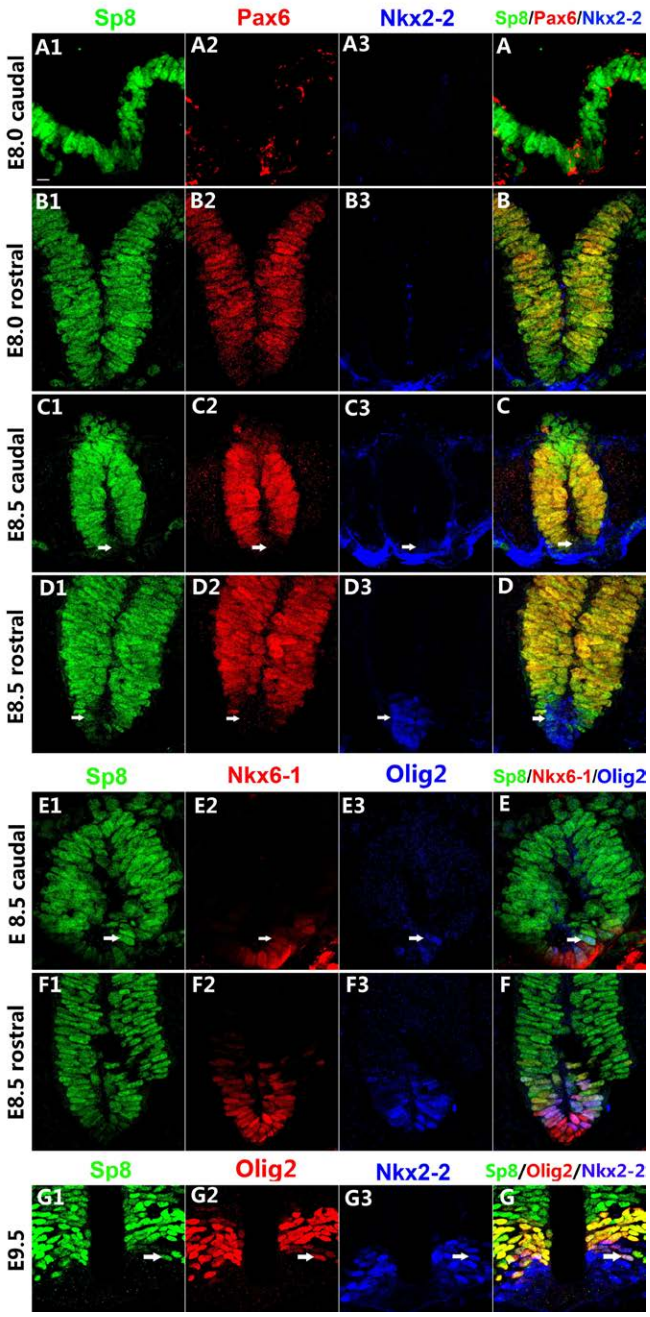

Figure S1. The temporal specification of the p3/pMN domain boundary. (A1-B) The expression patterns of Sp8, Pax6 and Nkx2-2 in the mouse caudal (A1-A) and rostral (B1-B) neural plate at E8.0. Note that only Sp8 was detected at caudal levels. (**C1-D**) At E8.5, Nkx2-2 was first detect in the ventral midline. Sp8 but not Pax6 was expressed in some Nkx2-2+ cells (arrows). **(E1-F)** The expression patterns of Sp8, Nkx6-1 and Olig2 in rostral and caudal spinal cord at E8.5. (**G1-G**) At E9.5, Sp8, Olig2 and Nkx2-2 triple-labeled cell can be still observed at the p3/pMN domain boundary (arrows). Scale bar: 10 μm (shown in **A1**, applies to **A1-G**).

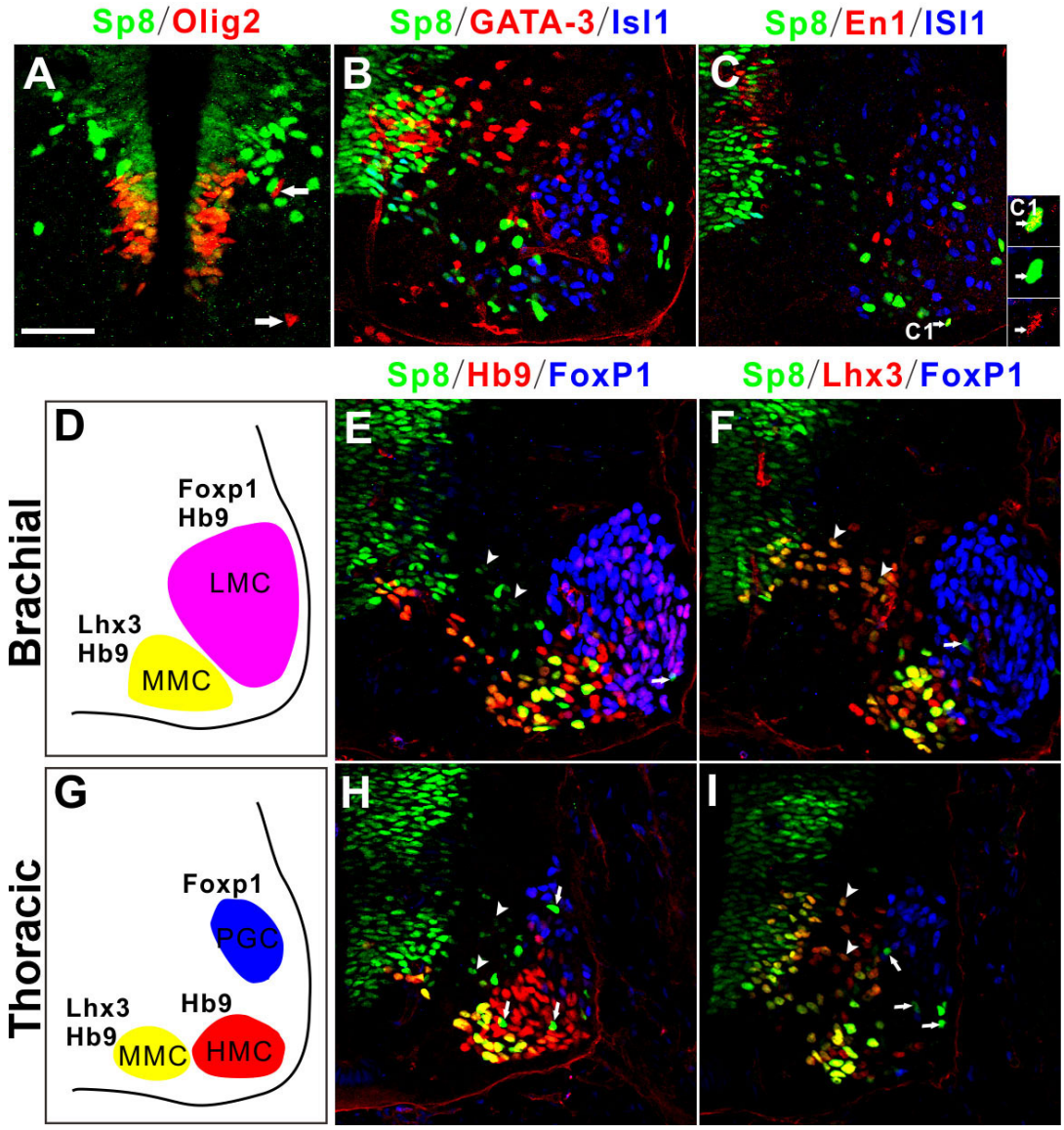

Figure S2. Sp8 is expressed by several types of ventral neurons in the mouse spinal cord at E12.0-12.5. (**A**) At E12.5, Olig2+ cells in the VZ co-expressed Sp8, but Olig2+ oligodendrocytes (arrows) that migrated outside of the VZ no longer expressed Sp8 protein. (**B**) Sp8 expression was not detected in the GATA-3+ V2 interneurons. (**C, C1**) Sp8 expression was detected in a subpopulation of En1+ V1 interneurons (arrows). (**D, G**) Schematic diagrams illustrating motor columns at the brachial levels (**D**) and thoracic levels (**G**) of the spinal cord defined by the combinatory expression of three transcription factors Lhx3, FoxP1 and Hb9. (**E, F, H, I**) At the brachial and thoracic levels, only MMC MNs expressed Sp8, but Sp8+ cells within the LMC at the brachial level and within the HMC and PGC at the thoracic level did not express any of these MN markers (arrows). Sp8 was also expressed in Lhx3+/Hb9- V2 interneurons (arrowheads). Scale bars: 50 μm (shown in **A**, applies to **A, C'**); 10 μm (shown in **B**, applies to **B, C, E, F, H, I**).

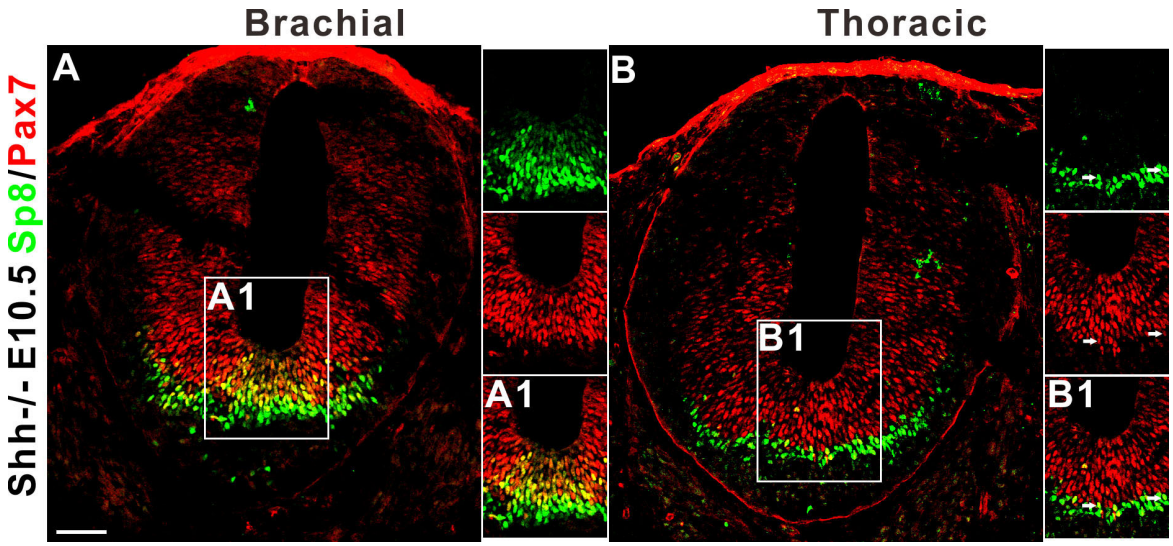

Figure S3. Sp8 expression expands into the ventral midline of the spinal cord of E10.5 Shh mutant mouse embryos. (A) At the brachial levels of the ventral midline of the spinal cord in E10.5 Shh-/- mice, Sp8 was expressed in the VZ and SVZ. In the VZ, nearly all of the Sp8+ cells expressed Pax7 and vice versa. **(B)** At the thoracic levels, Sp8 was primarily expressed in the SVZ. Sp8+ cells formed a crescent; only a small number of Sp8+ cells expressed Pax7 (arrows in B1). Scale bar: 50 μm (shown in **A**, applies to **A, A1, B, B1**).

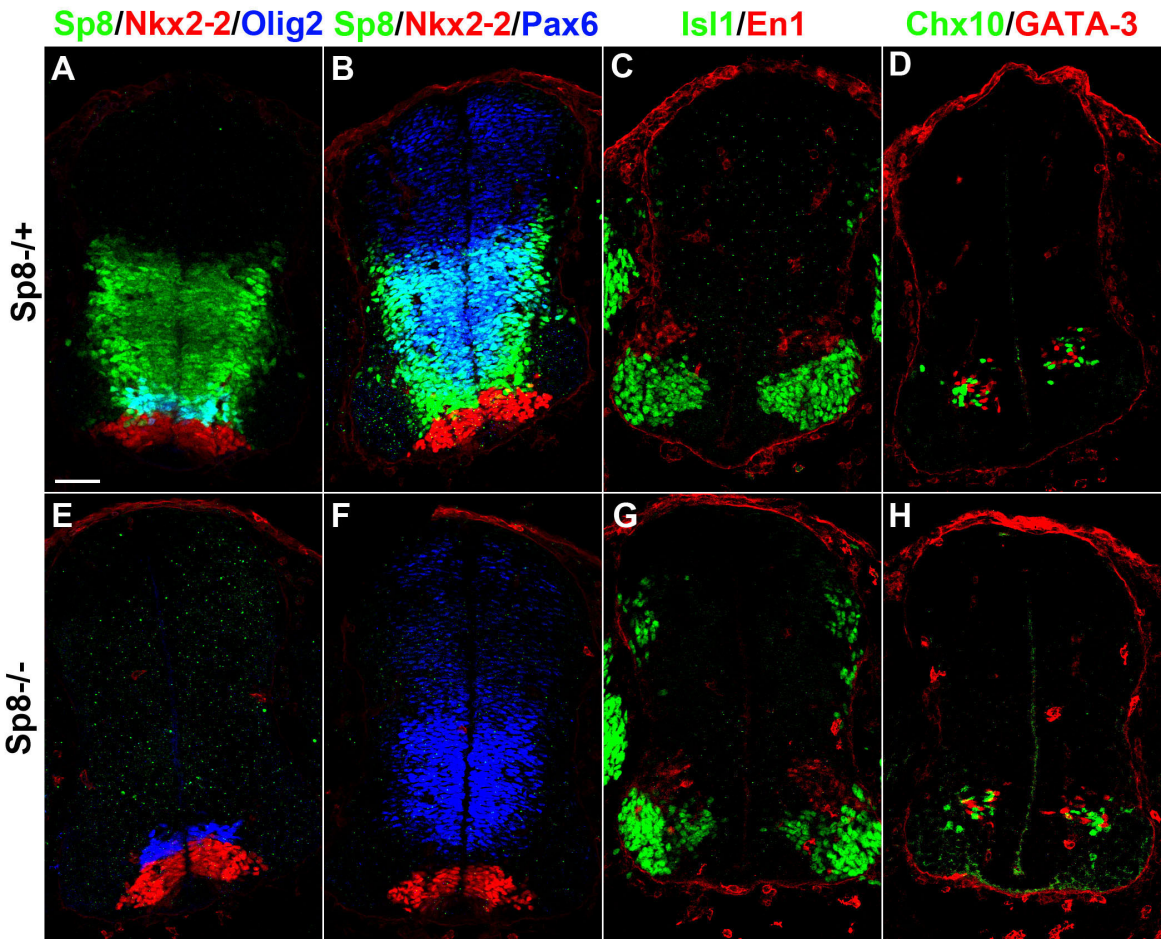

Figure S4. No pattern defects are found in Sp8 mutant mouse embryos. (A, E) While no Sp8 expression was detected in the spinal cord of Sp8-/- mouse embryos (E) compared to the Sp8+/- controls (A), the expression patterns of Olig2 and Nkx2-2 were not altered. (B, F) Pax6 expression was not altered in the Sp8-/- embryos (F) compared to the Sp8+/- control embryos (B). (C, D, G and H) The generation of Isl1+ MNs, En1+ V1 interneurons, Chx10+ and GATA-3+ V2 interneurons in the Sp8-/- embryos (G,H) was comparable to that in Sp8+/- controls (C, D). Scale bar: 50 μm (shown in **A**, applies to **A**-**H**).

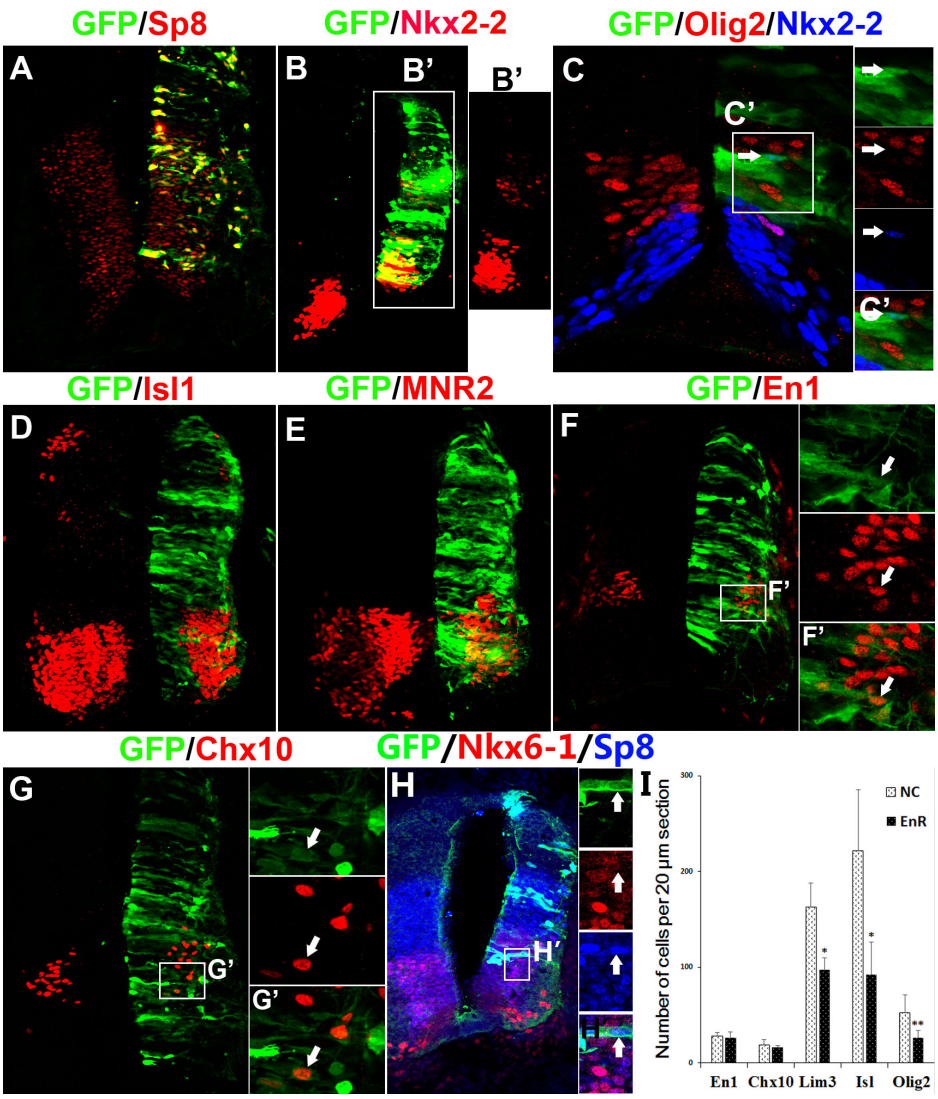

Figure S5. A repressor form of Sp8 (Sp8ZnF::EnR) inhibits MN generation. (A) Nearly all GFP-labeled cells expressed the fusion protein Sp8ZnF::EnR following electroporation. **(B, B')** The expression of Sp8ZnF::EnR resulted in ectopic Nkx2-2 expression dorsal to the p3 domain in 3/13 embryos (B'). (**C, C'**) Olig2 expression was greatly impaired by Sp8ZnF::EnR expression, but only a subset of Olig2-expressing pMN domain cells exhibited ectopic Nkx2-2 expression (**C'**). (**D, E**) The expression of the MN lineage genes Isl1 and MNR2 was reduced following Sp8ZnF::EnR overexpression. (**F, G**) The overexpression of Sp8ZnF::EnR did not affect the generation of En1+ V1 (arrows in F) or Chx10+ V2 (arrows in G) interneurons. (**H**) The expression of Sp8ZnF::EnR did not result in ectopic Nkx6-1 expression. Arrows indicate one cell that expressed GFP and Sp8 but not Nkx6-1. (**I**) Quantification of the above experiments (**C-G**). Error bars = SEM from 3 chickens per group; * $p<0.05$; ** $p<0.01$; Student's *t* test. Scale bars: 50 µm (shown in **A**, applies to **A, B, B', D-G**); 10 µm (shown in **C**, applies to **C, C', F', G'**).

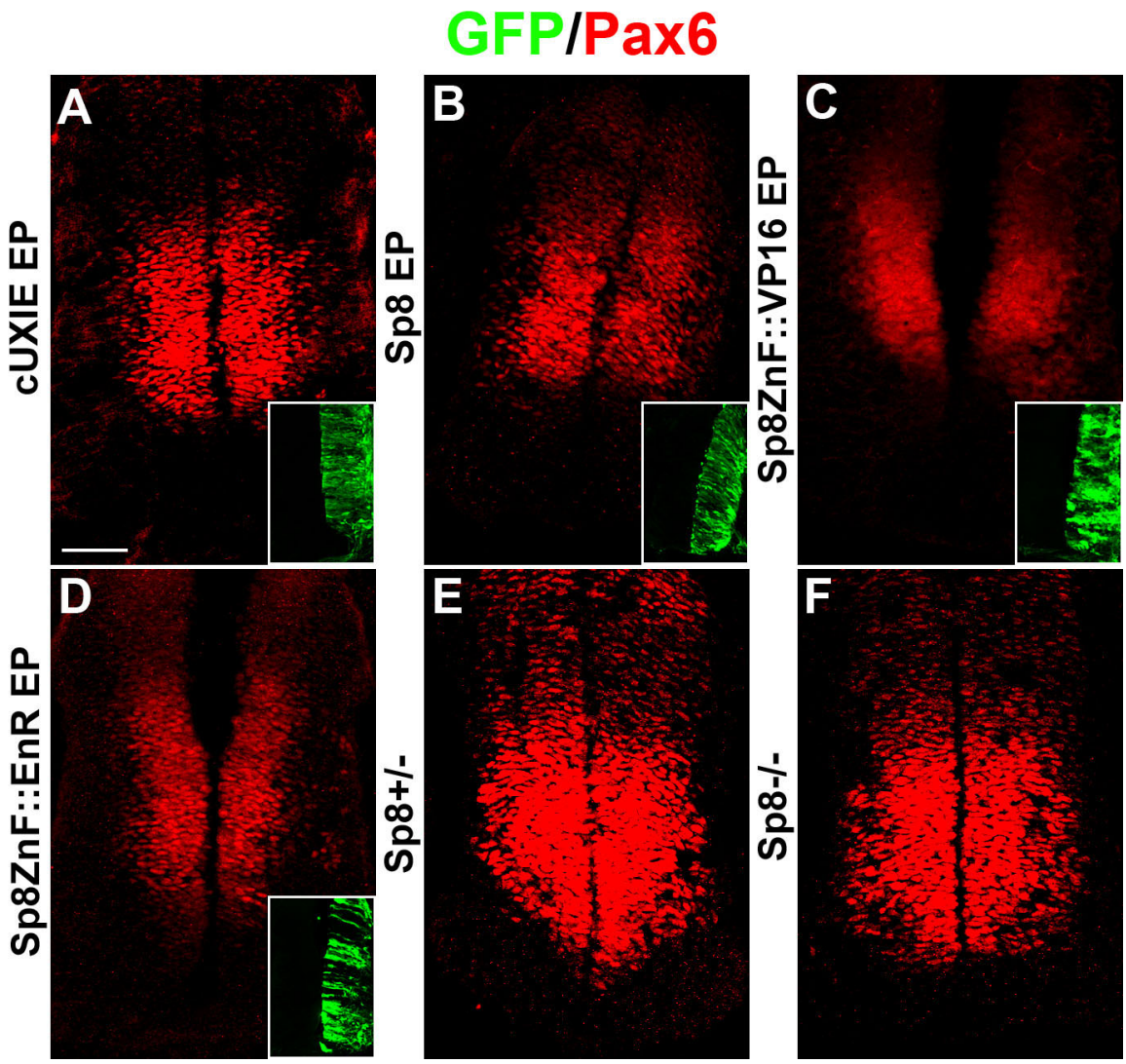

Figure S6. Sp8 does not regulate the expression of Pax6. (A-D) Pax6 expression in the electroporated side was unaffected compared to the unelectroporated control side following electroporation with cUXIE (A), Sp8 (B), Sp8ZnF::VP16 (C) and Sp8ZnF::EnR (D). GFP expression is shown in the insets in (A-D), indicating the expression levels of the exogenous gene. **(E, F)** The expression pattern of Pax6 in Sp8-/- embryos was very similar to the pattern in the Sp8+/- embryos. Scale bar: 50 μm (shown in **A**, applies to **A-F**).

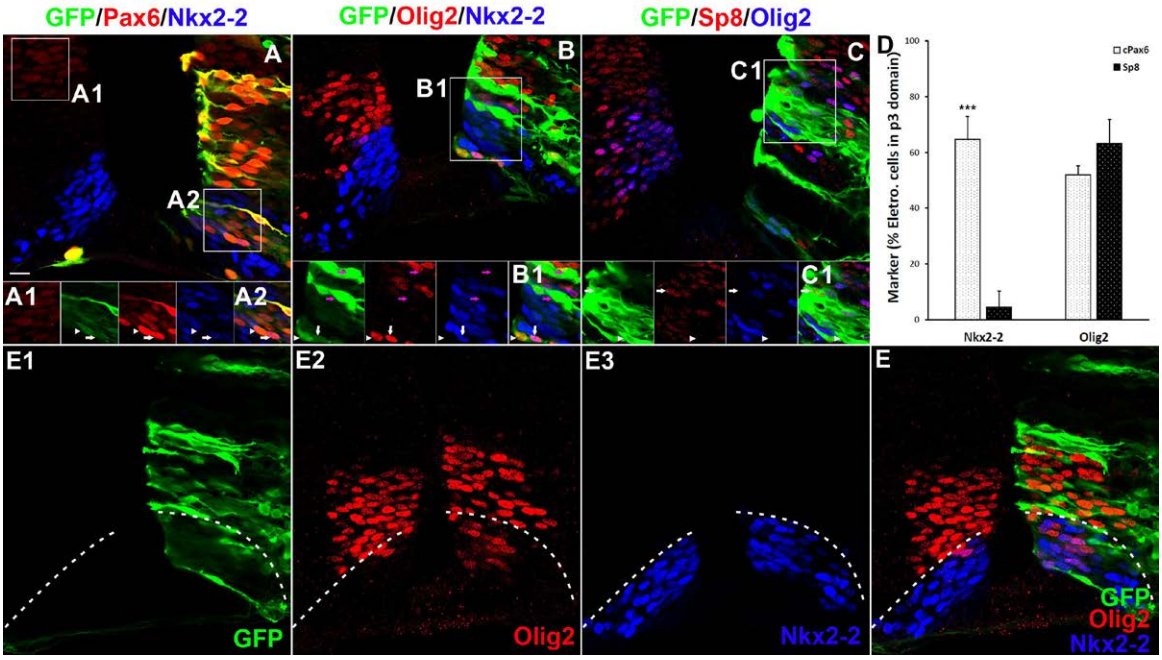

Figure S7. Pax6 misexpression represses Nkx2-2 and induces ectopic Olig2 and Sp8 expression in the p3 domain in a dose-dependent manner. (A-A2) Physiological Pax6 protein levels were defined by the highest Pax6 levels in the unelectroporated side (A1). Pax6 electroporation resulted in the expression of different levels of Pax6 in transfected cells (A2). The expression of Pax6 at much higher than physiological levels extinguished Nkx2-2 expression from p3 progenitors (arrowheads in A2). The cells that expressed physiological levels of Pax6 continued to express lower levels of Nkx2-2 (arrows in A2). **(B, B1)** One strongly GFP+ cell that expressed Olig2 but not Nkx2-2 (white arrowheads in B1) and one weakly GFP+ cell that expressed both Olig2 and Nkx2-2 (white arrows in B1). The expression of extremely high levels of Pax6 repressed both Olig2 and Nkx2-2 expression in the p3 and pMN domains (magenta arrows in B1). **(C, C1)** Higher levels of Pax6 repressed both Sp8 and Olig2 expression in the pMN domain (arrowheads in C1). However, Sp8 expression was more commonly observed than Olig2 expression in cells expressing higher levels of Pax6 in the VZ of the pMN domain (arrows in C1). **(D)** The proportion of the transgenic cells within the p3 domain that expressed Nkx2-2 and Olig2 following cPax6 and Sp8 electroporation. Error bars = SEM from 3 chickens per group; *** $p<0.001$; Student's *t* test. **(E)** The expression of the physiological levels of Pax6 induced Olig2 expression in the p3 domain without abolishing Nkx2-2 expression, resulting in the co-expression of Olig2 and Nkx2-2 in the same cells. Scale bar: 10 μm (shown in **A**, applies to all images).

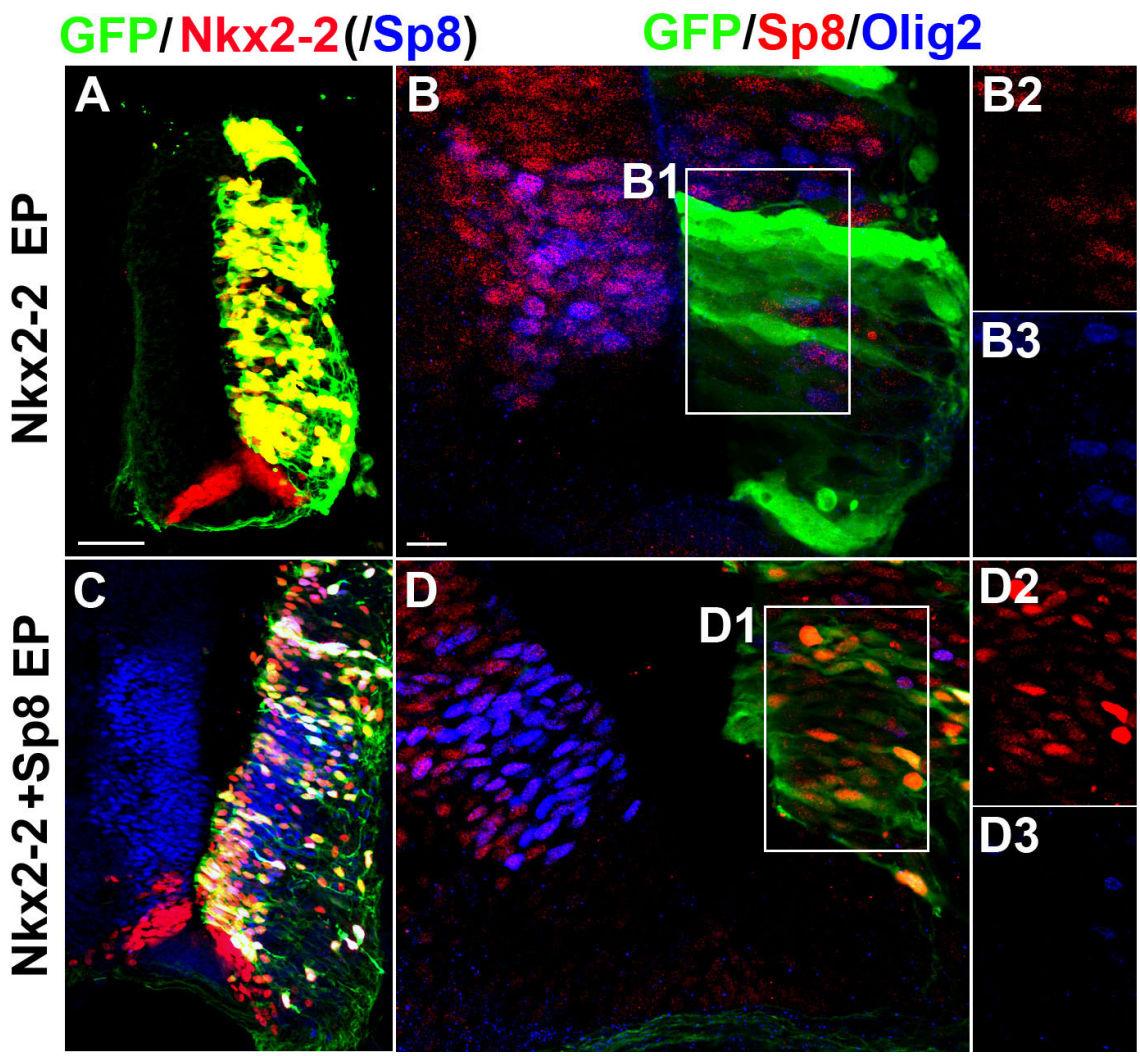

Figure S8. The clearance of Nkx2-2 expression from pMN progenitors is critical for the specification of MN fate. (A) Following Nkx2-2 electroporation, nearly all GFP+ cells expressed Knx2-2. **(B-B3)** Nkx2-2 misexpression within the pMN domain inhibited the expression of Olig2. **(C)** Co-electroporation of Sp8 and Nkx2-2 resulted in 90% GFP-labeled cells co-expressing both Sp8 and Nkx2-2. (**D-D3**) Sp8 re-expression in pMN progenitors that ectopically expressed Nkx2-2 did not rescue the expression of Olig2. Scale bars: 50 µm (shown in **A**, applies to **A, C**); 10 µm (shown in **B**, applies to **B-B3, D-D3**).

Table S1: List of primary antibodies

Antigen	Host	Dilution	Source	Catalogue #
Pax7	mouse	1/20	DSHB, The University of Iowa (Iowa, USA)	PAX7
MNR2/Hb9	mouse	1/40	DSHB, The University of Iowa (Iowa, USA)	81.5C10
Lhx3/Lim3	mouse	1/30	DSHB, The University of Iowa (Iowa, USA)	67.4E12
En1	mouse	1/20	DSHB, The University of Iowa (Iowa, USA)	4G11
Nkx2-2	mouse	1/40	DSHB, The University of Iowa (Iowa, USA)	74.5A5
Pax6	mouse	1/20	DSHB, The University of Iowa (Iowa, USA)	PAX6
Shh	mouse	1/20	DSHB, The University of Iowa (Iowa, USA)	5E1
FoxA2/Hnf3b	mouse	1/20	DSHB, The University of Iowa (Iowa, USA)	4C7
Islet 1	mouse	1/50	DSHB, The University of Iowa (Iowa, USA)	39.4D5
Chx10	sheep	1/500	Exalpha Biologicals, Inc.（Shirley，MA）	X1179P
Islet 1	rabbit	1/1000	Abcam Inc. (Cambridge, USA)	ab20670
Ki67	rabbit	1/400	Vector Laboratories. (Burlingame, USA)	VP-K451
Mash-1	rabbit	1/1000	Cosmo Bio Co.,Ltd. (Tokyo, Japan)	SK-T01-003
NeuN	mouse	1/400	Chemicon, Millipore. (Temecula, CA)	MAB377
TUJ 1	mouse	1/100	Covance (Princeton, New Jersey)	MMS-435P
Mash-1	mouse	1/100	BD Bioscience (California, USA)	556604
Olig2	rabbit	1/1000	Chemicon, Millipore. (Temecula, CA)	AB9610
Pax6	goat	1/500	Santa Cruz Biotechnology (California, USA)	sc-7750
Pax6	rabbit	1/1000	MBL International Corporation (Woburn, MA)	PD022
GFP	rabbit	1/1000	Aves Labs (Oregon, USA)	GFP-1020
Sp8	goat	1/500	Santa Cruz Biotechnology (California, USA)	sc-104661
GATA-3	mouse	1/200	Santa Cruz Biotechnology. (California, USA)	sc-269
Foxp1	rabbit	1/1000	Abcam Inc. (Cambridge, USA)	ab16645
Nkx6-1	Mouse	1/50	DSHB, The University of Iowa (Iowa, USA)	F55A10