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Different Levels of Repressor Activity Assign Redundant and Specific Roles to *Nkx6* Genes in Motor Neuron and Interneuron Specification

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Summary

Specification of neuronal fate in the vertebrate central nervous system depends on the profile of transcription factor expression by neural progenitor cells, but the precise roles of such factors in neurogenesis remain poorly characterized. Two closely related transcriptional repressors, Nkx6.2 and Nkx6.1, are expressed by progenitors in overlapping domains of the ventral spinal cord. We provide genetic evidence that differences in the level of repressor activity of these homeodomain proteins underlies the diversification of interneuron subtypes, and provides a fail-safe mechanism during motor neuron generation. A reduction in Nkx6 activity further permits V0 neurons to be generated from progenitors that lack homeodomain proteins normally required for their generation, providing direct evidence for a model in which progenitor homeodomain proteins direct specific cell fates by actively suppressing the expression of transcription factors that direct alternative fates.

Introduction

During the development of the vertebrate central nervous system, the assignment of regional identity to neural progenitor cells has a critical role in directing the subtype identity of post-mitotic neurons. Within the ventral half of the neural tube, the specification of progenitor cell identity is initiated by the long-range signaling activity of the secreted factor, Sonic hedgehog (Shh) (Briscoe et al., 2001; Briscoe and Ericson, 2001). Shh signaling appears to establish ventral progenitor cell identities by regulating the spatial pattern of expression of homeodomain transcription factors of the Nkx, Pax, Dbx, and Irx families (Ericson et al., 1997; Pierani et al., 1999; Briscoe et al., 2000). Members of all four gene families have been duplicated during evolution (Shoji et al., 1996; Wang et al., 2000; Hoshiyama et al., 1998; Peters et al., 2001), and the resulting homeodomain protein pairs are typically expressed in overlapping or nested domains within the neural tube (Briscoe and Ericson, 2001). Some of these homeodomain protein pairs have been proposed to have distinct, and others redundant, roles in spinal cord patterning (Mansouri and Gruss, 1998; Briscoe et al., 1999; Pierani et al., 2001), but the impact of such homeobox gene duplication on neuronal diversification has not been explored directly.

One unifying feature of this diverse array of progenitor homeodomain proteins is their subdivision into two general groups, termed class I and II proteins, on the basis of their mode of regulation by Shh signaling (Briscoe and Ericson, 2001). The class I proteins are constitutively expressed by neural progenitor cells, and their expression is repressed by Shh signaling, whereas neural expression of the class II proteins requires exposure to Shh (Ericson et al., 1997; Qiu et al., 1998; Briscoe et al., 1999, 2000; Pabst et al., 2000). Although the spatial pattern of expression of the class I proteins has revealed the existence of five ventral progenitor domains, class II proteins have been identified for only two of these domains (Briscoe et al., 2000), raising questions about the existence and identity of additional class II proteins. There is, however, emerging evidence that the combination of class I and II proteins that is expressed by neural progenitor cells directs the fate of their neuronal progeny. In support of this, misexpression of individual progenitor homeodomain proteins in the chick neural tube promotes the ectopic generation of neuronal subtypes, with a specificity predicted by the normal profile of progenitor homeodomain protein expression (Briscoe et al., 2000; Pierani et al., 2001). Conversely, the analysis of mouse mutants has provided genetic evidence that the activities of specific class I and II proteins are required to establish progenitor cell domains and to direct ventral neuronal fates (Ericson et al., 1997; Briscoe et al., 1999; Sander et al., 2000; Pierani et al., 2001).

The participation of progenitor homeodomain proteins in the conversion of graded Shh signals into all-or-none distinctions in progenitor cell identity depends on crossrepressive interactions between selected pairs of class I and II proteins (Ericson et al., 1997; Briscoe et al., 2000; Sander et al., 2000; Muhr et al., 2001). In addition, most class I and II proteins have been shown to function directly as transcriptional repressors, through the recruitment of corepressors of the Gro/TLE class (Muhr et al., 2001). These findings have suggested a derepression model of neural patterning which invokes the idea that the patterning activities of individual class I or II proteins are achieved primarily through their ability to repress expression of complementary homeodomain proteins from specific progenitor domains. A central implication of this model is that homeodomain proteins direct progenitor cells to individual neuronal fates by suppressing alternative pathways of differentiation—a view that has strong parallels with proposed mechanisms of lineage restriction during lymphoid differentia-

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tion (Nutt et al., 1999; Rolink et al., 1999; Eberhard et al., 2000).

Much of the evidence that has led to this general outline of ventral neural patterning has emerged from an analysis of members of the *Nkx* gene family. Two closely related Nkx repressor proteins, Nkx2.2 and Nkx2.9, function as class II proteins that specify the identity of V3 neurons (Ericson et al., 1997; Briscoe et al., 1999, 2000). A more distantly related class II repressor protein, Nkx6.1, is expressed throughout the ventral third of the neural tube and when ectopically expressed, can direct motor neuron and V2 neuron fates (Briscoe et al., 2000; Sander et al., 2000). These gain-of-function studies are supported by an analysis of mice lacking *Nkx6.1* function, which exhibit a virtually complete failure in V2 interneuron generation (Sander et al., 2000). *Nkx6.1* null mice also show a reduction in motor neuron generation at rostral levels of the spinal cord, but at more caudal levels, motor neurons are formed in near-normal numbers (Sander et al., 2000). This observation reveals the existence of an Nkx6.1-independent program of spinal motor neuron generation, although the molecular basis of this alternative pathway is unclear.

A close relative of *Nkx6.1*, termed *Nkx6.2* (also known as *Nkx6B* or *Gtx*), has been identified (Komuro et al., 1993; Lee et al., 2001) and is expressed by neural progenitor cells (Cai et al., 1999). In its alias of Gtx, Nkx6.2 has been suggested to regulate myelin gene expression (Komuro et al., 1993), but its possible functions in neural patterning have not been examined. The identification of an *Nkx6* gene pair prompted us to address three poorly resolved aspects of ventral neural patterning. First, do closely related pairs of repressor homeodomain proteins serve distinct or redundant roles in ventral neural patterning? Second, are class I repressor proteins always complemented by a corresponding class II repressor, and if so, is Nkx6.2 one of the missing class II proteins? Third, to what extent is the generation of spinal motor neurons dependent on the activity of Nkx6 class proteins?

To address these issues, we mapped the profile of expression of Nkx6.2 and Nkx6.1 during neural tube development, and analyzed mouse *Nkx6* mutants to determine the respective contributions of these two genes to neural patterning. We show that Nkx6.2, like Nkx6.1, functions as a class II repressor homeodomain protein. Our analysis of *Nkx6* mutants further indicates that the duplication of an ancestral *Nkx6* gene has resulted in the expression of two proteins that exert markedly different levels of repressor activity in the ventral neural tube. This differential repressor activity of these two proteins appears to provide both a fail-safe mechanism during motor neuron generation and the potential for enhanced diversification of ventral interneuron subtypes. Moreover, we find that under conditions of reduced *Nkx6* gene dosage, ventral neuronal subtypes can be generated from progenitor cells that lack the class I or class II proteins normally required for their generation. This finding supports one of the central tenets of the derepression model of ventral neural patterning—that progenitor homeodomain proteins direct particular neuronal fates by actively suppressing cells from adopting alternative fates.

Results

Distinct Patterns of Nkx6.1 and Nkx6.2 Expression in Embryonic Spinal Cord

To examine the roles of *Nkx6* class genes in ventral neuronal specification, we compared the patterns of expression of Nkx6.2 and Nkx6.1 with that of other progenitor homeodomain proteins in the spinal cord of mouse and chick embryos. In the caudal neural tube of the mouse, the expression of Nkx6.2 was first detected at ~e8.5, in a broad ventral domain that largely coincided with that of Nkx6.1 (Figure 1A). Between e8.5 and e9.5, the expression of Nkx6.2 was lost from most Nkx6.1$^+$ cells in the ventral neural tube, although expression persisted in a narrow stripe of cells just dorsal to the limit of Nkx6.1 expression (Figures 1B and 1C). At e10.0–e10.5, virtually all Nkx6.2$^+$ cells coexpressed Dbx2 (Figure 1E), and the ventral limit of expression of both Nkx6.2 and Dbx2 coincided with the dorsal limit of Nkx6.1 expression at the p1/p2 domain boundary (Figures 1D and 1E). Nkx6.2 was expressed predominantly within the p1 domain, but scattered Nkx6.2$^+$ cells were detected within the p0 domain—the domain of expression of Pax7$^-$, Dbx1$^+$ cells (Figure 1F). Within the p0 domain, however, individual Nkx6.2$^+$ cells did not coexpress Dbx1, although they did express Dbx2 (Figures 1E–1G). Thus, the scattered Nkx6.2$^+$ cells found at the dorsoventral level of the p0 domain exhibit a p1, rather than p0, progenitor cell identity. Studies in chick have similarly shown that p0 and p1 progenitors are interspersed in the most dorsal domain of the ventral neural tube (Pierani et al., 1999).

In the chick neural tube, as in the mouse, *Nkx6.1* and *Nkx6.2* are initially coexpressed in a broad ventral domain (Cai et al., 1999; data not shown). But in contrast to the mouse, *Nkx6.2* expression persists in ventral progenitor cells, with the consequence that the expression of *Nkx6.2* and *Nkx6.1* also overlaps at later developmental stages (Figures 1H and 1I). Nevertheless, expression of chick *Nkx6.2* is also detected in a thin stripe of cells dorsal to the limit of *Nkx6.1* expression, within the p1 domain (Figure 1H). Thus, in both species, p1 progenitors coexpress Nkx6.2 and Dbx2 and exclude Nkx6.1.

Nkx6.2 Regulates V0 and V1 Interneuron Fates by Repression of Dbx1 Expression

The establishment and maintenance of progenitor cell domains in the ventral neural tube have been proposed to depend on mutual repressive interactions between complementary pairs of class I and II homeodomain proteins (Briscoe et al., 2000; Muhr et al., 2001). But class II proteins have been identified for only two of the five known progenitor domain boundaries (the p1/p2 and pMN/p3 boundaries) (Ericson et al., 1997; Briscoe et al., 1999, 2000; Sander et al., 2000). The mutually exclusive pattern of expression of Nkx6.2 and Dbx1 within p1 and p0 progenitors led us to consider whether Nkx6.2 might function as a class II protein that represses Dbx1 expression, and thus help to establish the identity of p1 progenitor cells and the fate of their En1$^+$ V1 neuronal progeny.

To test this idea, we analyzed the profile of expression of class I and II homeodomain proteins in *Nkx6.2* mutant

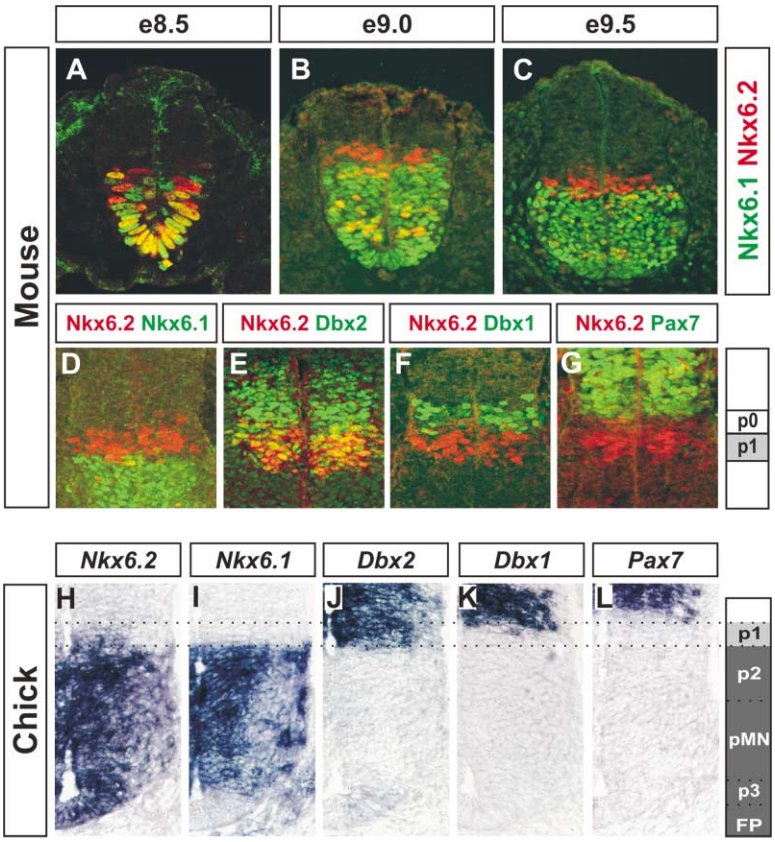

Figure 1. Expression of Nkx6.2 and Nkx6.1 in Developing Mouse and Chick Spinal Cord

(A) At e8.5, Nkx6.2 and Nkx6.1 are expressed in a broad ventral domain of the mouse neural tube. (B) At e9.0, Nkx6.2 expression is largely confined to a narrow domain immediately dorsal to the domain of Nkx6.1 expression. A few scattered cells that coexpress Nkx6.2 and Nkx6.1 are detected in more ventral positions at this stage. (C) At e9.5, Nkx6.2 is expressed in a narrow domain, dorsal to the Nkx6.1 boundary.

(D–G) Comparative patterns of expression of Nkx6.2, Nkx6.1, Dbx2, Dbx1, and Pax7 in the intermediate region of e10.5 mouse spinal cord. (E) Virtually all Nkx6.2^{+} cells coexpress Dbx2, but since the level of Dbx2 expression in individual p1 progenitors varies, some cells appear reddish rather than bright yellow.

(H–L) Expression pattern of *Nkx6.2*, *Nkx6.1*, *Dbx2*, *Dbx1*, and *Pax7* in HH stage 20 chick spinal cord. Panels on right indicate progenitor domains, defined according to Briscoe et al. (2000).

embryos. We inactivated the mouse *Nkx6.2* gene by homologous recombination in embryonic stem (ES) cells. A targeted *Nkx6.2* allele (*Nkx6.2*^{tlz}) was generated by replacing the coding sequence of *Nkx6.2* with a *tauLacZ* cassette (Figure 2A). In the spinal cord of *Nkx6.2*$^{+/tlz}$ embryos analyzed at e10.5, expression of LacZ and Nkx6.2 coincided within the p1 progenitor domain (see Figures 2E and 2F). In *Nkx6.2*$^{tlz/tlz}$ embryos, the location of LacZ^{+} cells was also similar to that in *Nkx6.2*$^{+/tlz}$ embryos (Figures 2F and 2G), but Nkx6.2 protein was not detected (Figure 2G). These data provide evidence that the *Nkx6.2*^{tlz} allele generates a null mutation, and that disruption of the *Nkx6.2* locus does not perturb the normal spatial pattern of expression of this gene.

We did observe, however, that the level of LacZ expression was markedly elevated in *Nkx6.2*$^{tlz/tlz}$, when compared with *Nkx6.2*$^{+/tlz}$, embryos (Figures 2B–2D). An elevation in level of expression of the residual 5′ *Nkx6.2* transcript was also detected in *Nkx6.2*$^{tlz/tlz}$ embryos (Figures 2H–2J). These observations provide evidence that Nkx6.2 negatively regulates its own expression level within p1 progenitor cells.

We next analyzed the pattern of expression of class I and II homeodomain proteins in the spinal cord and caudal hindbrain of *Nkx6.2*$^{tlz/tlz}$ embryos. The domains of expression of the class II proteins Nkx2.2 and Nkx6.1, and of the class I proteins Pax7, Dbx2, Irx3, and Pax6, were similar in *Nkx6.2* $^{tlz/tlz}$, *Nkx6.2*$^{+/tlz}$, and wild-type embryos (Figures 3B–3D and 3G–3I; data not shown). In addition, normal patterns of expression of Dbx2 and Nkx6.1 were detected at the p1/p2 domain boundary (data not shown), showing that establishment of the p1 progenitor domain does not require Nkx6.2 function. However, the level of Dbx2 expression in p1 domain progenitors was increased ~2-fold in Nkx6.2$^{tlz/tlz}$ mutants (Figures 2K–2M), indicating that Nkx6.2 normally limits the level of Dbx2 expression in this domain.

We also detected a marked change in the pattern of expression of the p0 progenitor cell marker Dbx1 in *Nkx6.2*$^{tlz/tlz}$ embryos. At caudal hindbrain levels, the number of ventral Dbx1^{+} progenitor cells increased 1.7- fold (Figure 3F), and the domain of Dbx1^{+} cells expanded ventrally, extending through the p1 domain to the dorsal limit of Nkx6.1 expression (Figure 3H). Moreover, in *Nkx6.2*$^{tlz/tlz}$ embryos, all of the ectopic Dbx1^{+} cells found within the p1 domain coexpressed LacZ (Figure 3J). Thus, many progenitors within the p1 domain initiate Dbx1 expression in the absence of Nkx6.2 function. Nevertheless in *Nkx6.2*$^{tlz/tlz}$ embryos, numerous LacZ^{+} progenitors still lacked Dbx1 expression (Figure 3J), implying the existence of an Nkx6.2-independent means of excluding Dbx1 expression from p1 progenitors. The ventral expansion of Dbx1 was most prominent at caudal hindbrain and cervical spinal levels of the neural tube, but a similar, albeit less marked, expansion of Dbx1 expression was detected at caudal spinal levels (data not shown; see Figure 6). Taken together, these data imply that within p1 domain progenitors, Nkx6.2 functions as a weak repressor of Dbx2 expression and a more potent repressor of Dbx1 expression.

We next analyzed the generation of interneuron sub-

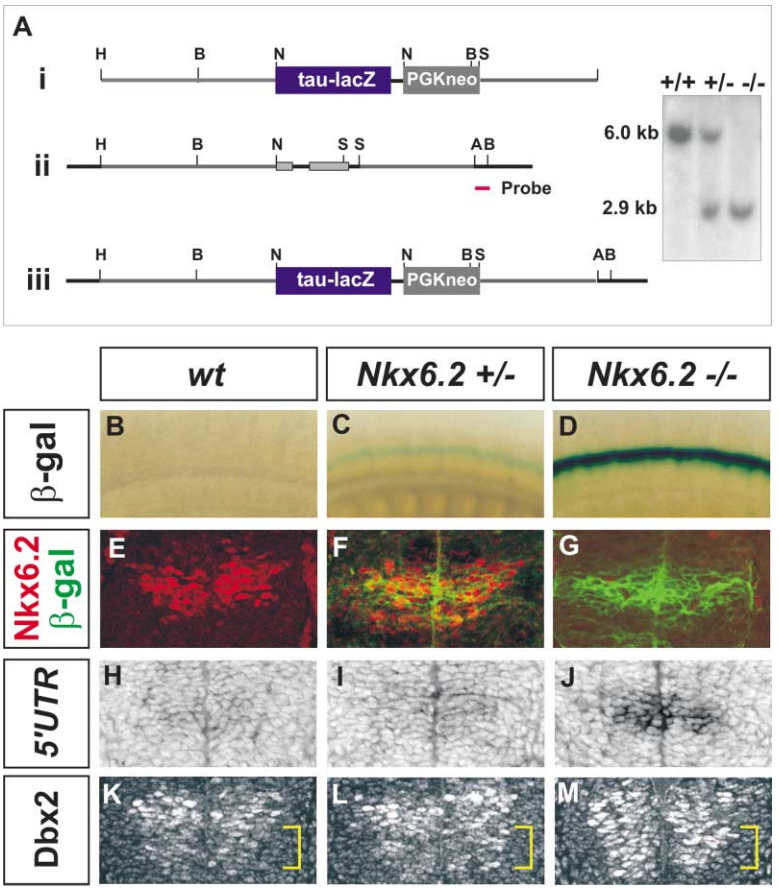

Figure 2. Elevation in *Nkx6.2* and Dbx2 Expression in p1 Domain Cells in *Nkx6.2* Mouse Mutants

(A) Diagram of the targeting construct (i) used to replace the coding sequence of *Nkx6.2* (ii) with a tau-lacZ PGK-neo cassette (iii). Red bar indicates region used as probe in genotyping.

(B–D) Sagital view of e10.5 spinal cord showing LacZ expression, detected by X-gal staining, in wild-type (*wt*), (B) *Nkx6.2*$^{+/tlz}$ (C), and *Nkx6.2*$^{tlz/tlz}$ (D) embryos.

(E–G) Nkx6.2 and LacZ expression in the p1 domain of *wt* (E), *Nkx6.2*$^{+/tlz}$ (F), and *Nkx6.2*$^{tlz/tlz}$ (G) embryos at e10.5.

(H–J) In situ hybridization with a 5′UTR probe shows that expression of *Nkx6.2* is elevated in the p1 domain of *Nkx6.2*$^{tlz/tlz}$ embryos (J), compared with *wt* (H) or *Nkx6.2*$^{+/tlz}$ (I) embryos.

(K–M) Expression of Dbx2 is upregulated ~2-fold in cells within the p1 domain (yellow bracket) in *Nkx6.2*$^{tlz/tlz}$ embryos (M), compared with *wt* (K) or *Nkx6.2*$^{+/tlz}$ (L) embryos. Abbreviations in (A): H = HindIII, B = BamHI, N = NcoI, S = SphI, A =AccI.

types in the ventral neural tube. In wild-type embryos, Dbx1^{+}, Dbx2^{+}, Nkx6.2^{-} p0 progenitors generate Evx1/2^{+} V0 neurons (Pierani et al., 1999, 2001); Nkx6.2^{+}, Dbx1^{-}, Dbx2^{+} p1 progenitors give rise to En1^{+} V1 neurons (Burrill et al., 1997; Ericson et al., 1997); and Nkx6.1^{+}, Irx3^{+}, p2 progenitors give rise to Chx10^{+} V2 neurons (Ericson et al., 1997; Briscoe et al., 2000). Dbx1 activity in p0 progenitors is required to promote V0 and suppress V1 neuronal fates (Pierani et al., 2001). The ventral expansion in Dbx1 expression in *Nkx6.2*$^{tlz/tlz}$ embryos therefore led us to examine whether the loss of Nkx6.2 function leads progenitor cells within the p1 domain to adopt a V0 rather than V1 neuronal fate.

In the caudal hindbrain of *Nkx6.2* $^{tlz/tlz}$ embryos examined at e10.5, we detected a ~2-fold increase in the number of Evx1/2^{+} V0 neurons, and the domain of V0 neuronal generation expanded ventrally to the normal position of the p1 domain (Figure 3N). Consistent with this, many Evx1/2^{+} neurons coexpressed LacZ (Figure 3P), showing directly that some V0 neurons derive from p1 progenitors in the absence of Nkx6.2 function. Conversely, the total number of En1^{+} V1 neurons generated in *Nkx6.2*$^{tlz/tlz}$ embryos was reduced by ~50% (Figure 3Q). The dorsoventral position of generation of the remaining En1^{+} V1 neurons was similar in *Nkx6.2*$^{tlz/tlz}$ embryos (Figure 3N), and these neurons expressed LacZ (Figure 3O), showing directly that Nkx6.2^{+}, Dbx2^{+} p1 progenitor cells generate V1 neurons. The total number of neurons generated from p1 domain progenitors, defined by Cyn1, TuJ1, and Lim1/2 expression, was similar in *Nkx6.2*$^{tlz/tlz}$ and *Nkx6.2*$^{+/tlz}$ embryos examined at e10.5 (data not shown). In addition, the number of TUNEL^{+} cells was similar in *Nkx6.2*$^{tlz/tlz}$ and *Nkx6.2*$^{+/tlz}$ embryos (data not shown). Chx10^{+} V2 neurons and HB9^{+}, Isl1/2^{+} motor neurons were present in normal numbers and positions in *Nkx6.2*$^{tlz/tlz}$ embryos (Figure 5; data not shown). Together, these findings show that the activity of Nkx6.2 within p1 progenitors promotes V1 neuronal generation and helps to suppress the generation of V0 neurons, a finding consistent with the proposed role of Nkx6.2 in repressing Dbx1 expression from p1 progenitors.

Repression of Nkx6.2 by Nkx6.1 Underlies *Nkx6* Gene Redundancy in Spinal Motor Neuron Generation

We next addressed the respective contributions of *Nkx6.1* and *Nkx6.2* to motor neuron and V2 neuron generation. In the ventral neural tube, p2 and pMN progenitors express Nkx6.1 and give rise to V2 neurons and motor neurons, respectively. Ectopic expression of Nkx6.1 is sufficient to induce motor neurons and V2 interneurons in dorsal regions of the neural tube, and in *Nkx6.1* mutant mice, V2 neurons are eliminated (Briscoe et al., 2000; Sander et al., 2000). Nevertheless, there is only a partial reduction in motor neuron generation in *Nkx6.1* mutants (Sander et al., 2000), revealing the existence of an *Nkx6.1*-independent pathway of motor neuron generation. Nkx6.2 does not normally contribute to motor neuron specification in the mouse since its expression is

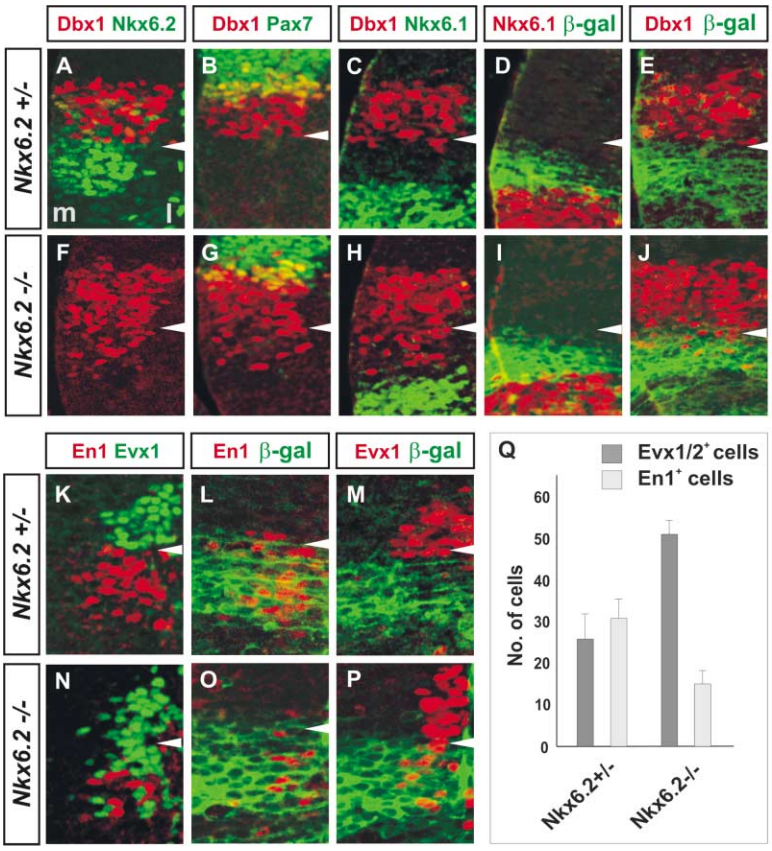

Figure 3. A Partial Switch from V1 to V0 Neuronal Fate in *Nkx6.2* Mutant Mice

(A–E) Expression of Nkx6.2 (A), Nkx6.1 (C and D), Dbx1 (B, C, and E), and Pax7 (B) appears normal at caudal hindbrain levels of e10.5 *Nkx6.2*$^{+/tlz}$ embryos. The expression of Nkx6.1 (D) and Dbx1 (E) abuts the ventral and dorsal boundaries of *LacZ* expression.

(F–J) In e10.5 *Nkx6.2*$^{tlz/tlz}$ embryos, expression of Nkx6.1 (H and I) and Pax7 (G) is unchanged, but expression of Dbx1 (F, G, and H) is expanded ventrally into the p1 domain. Many ventral ectopic Dbx1^{+} cells in *Nkx6.2*$^{tlz/tlz}$ embryos express LacZ (J).

(K–M) Evx1/2^{+} V0 neurons are generated dorsal to En1^{+} V1 neurons (K) and *LacZ*$^{+}$ cells (M) in *Nkx6.2*$^{+/tlz}$ embryos. En1^{+} neurons express LacZ in *Nkx6.2*$^{+/tlz}$ (L) and *Nkx6.2*$^{tlz/tlz}$ (O) embryos.

(N–P) Evx1/2^{+} V0 neurons are generated in increased numbers and at ectopic ventral positions in the caudal hindbrain of *Nkx6.2*$^{tlz/tlz}$ embryos. (N) The number of En1^{+} V1 neurons is reduced and the remaining En1^{+} neurons are intermingled with ectopic Evx1/2^{+} cells. (P) Many Evx1/2^{+} neurons in *Nkx6.2*$^{tlz/tlz}$ embryos coexpress LacZ.

(Q) Quantitation of Evx1/2^{+} V0, and En1^{+} V1 neurons at the caudal hindbrain of *Nkx6.2*$^{+/tlz}$ and *Nkx6.2*$^{tlz/tlz}$ embryos at e10.5. Counts from 12 sections, mean ± SD. In panels (A)–(P), the white arrowhead indicates the p0/p1 boundary. The mouse *Nkx6.2* gene has also been inactivated by Cai et al. (2001), but a similar V1 to V0 interneuron fate change was not noted.

extinguished from ventral progenitors well before the appearance of post-mitotic motor neurons (Figures 1A–1C), and there is no change in the number of motor neurons generated in *Nkx6.2*$^{tlz/tlz}$ embryos (see Figure 5G).

Three lines of evidence, however, led us to consider a cryptic role for Nkx6.2 in motor neuron generation. First, Nkx6.2 and Dbx2 share the same ventral limit of expression at the p1/p2 domain boundary, and the expression of Dbx2 is repressed by Nkx6.1 (Briscoe et al., 2000; Sander et al., 2000). Second, Nkx6.2 negatively regulates its own expression level within p1 domain progenitors (Figures 2D, 2G, and 2J). Third, Nkx6.1 and Nkx6.2 possess similar Gro/TLE recruitment activities and DNA target site binding specificities (Muhr et al., 2001). We reasoned therefore that under conditions in which Nkx6.1 activity is reduced or eliminated, Nkx6.2 expression might be derepressed in p2 and pMN progenitors.

In support of this idea, in *Nkx6.1*$^{+/-}$ embryos examined at e10.5, we detected a marked increase in the number of Nkx6.2^{+} cells within the p2 and pMN domains (Figure 4B). And in *Nkx6.1*$^{-/-}$ embryos, expression of Nkx6.2 was detected in virtually all progenitor cells within the p2 and pMN domains (Figure 4C). Indeed, in *Nkx6.1*$^{-/-}$ embryos, the level of Nkx6.2 expression in the nuclei of progenitor cells within the p2 and pMN domains was 1.9-fold greater than that in progenitor cells located within the p1 domain (Figure 4C; data not shown). Together, these data show that Nkx6.1 activity normally represses Nkx6.2 expression from p2 and pMN progenitors in the mouse embryo.

In turn, these findings raised the possibility that in *Nkx6.1*$^{-/-}$ embryos, the derepression of Nkx6.2 expression substitutes for the loss of Nkx6.1 during motor neuron generation. If this is the case, Nkx6.2 would be predicted to mimic the ability of Nkx6.1 to induce motor neurons in vivo. Expression of chick or mouse *Nkx6.2* in the neural tube of HH stage 10–12 chick embryos repressed Dbx2 and Dbx1 expression (Figures 4D–4F), and induced ectopic motor neuron differentiation (Figures 4G–4I and 4L–4N) with an efficacy similar to that of *Nkx6.1* (Briscoe et al., 2000). These data show that Nkx6.2 can induce ectopic motor neurons when expressed at high levels in the dorsal neural tube, supporting the idea that both Nkx6 proteins can exert similar patterning activities in vivo (Figures 4D–4O; Briscoe et al., 2000). In addition, misexpression of Nkx6.2 in the p0 and p1 progenitor domains suppressed the generation of Evx1/2^{+} V0 and En1^{+} V1 neurons and promoted the generation of Chx10^{+} V2 neurons (Figures 4J, 4K, 4O, and 4P). Thus, a high level of expression of Nkx6.2 is not compatible with the generation of either V0 or V1 neurons (Figures 4O and 4P).

Based on these findings, we examined whether Nkx6.2 has a role in motor neuron generation in *Nkx6.1* mutant mice by testing the impact of removing Nkx6.2 as well as Nkx6.1 on the generation of spinal motor neurons. In *Nkx6.2*$^{tlz/tlz}$ embryos, there was no change in the number of motor neurons generated at any level of the spinal cord or hindbrain (Figures 5G, 5N, and 5O; data not shown). In *Nkx6.1*$^{-/-}$ mutants, the number of spinal motor neurons was reduced by ~60% at cervical

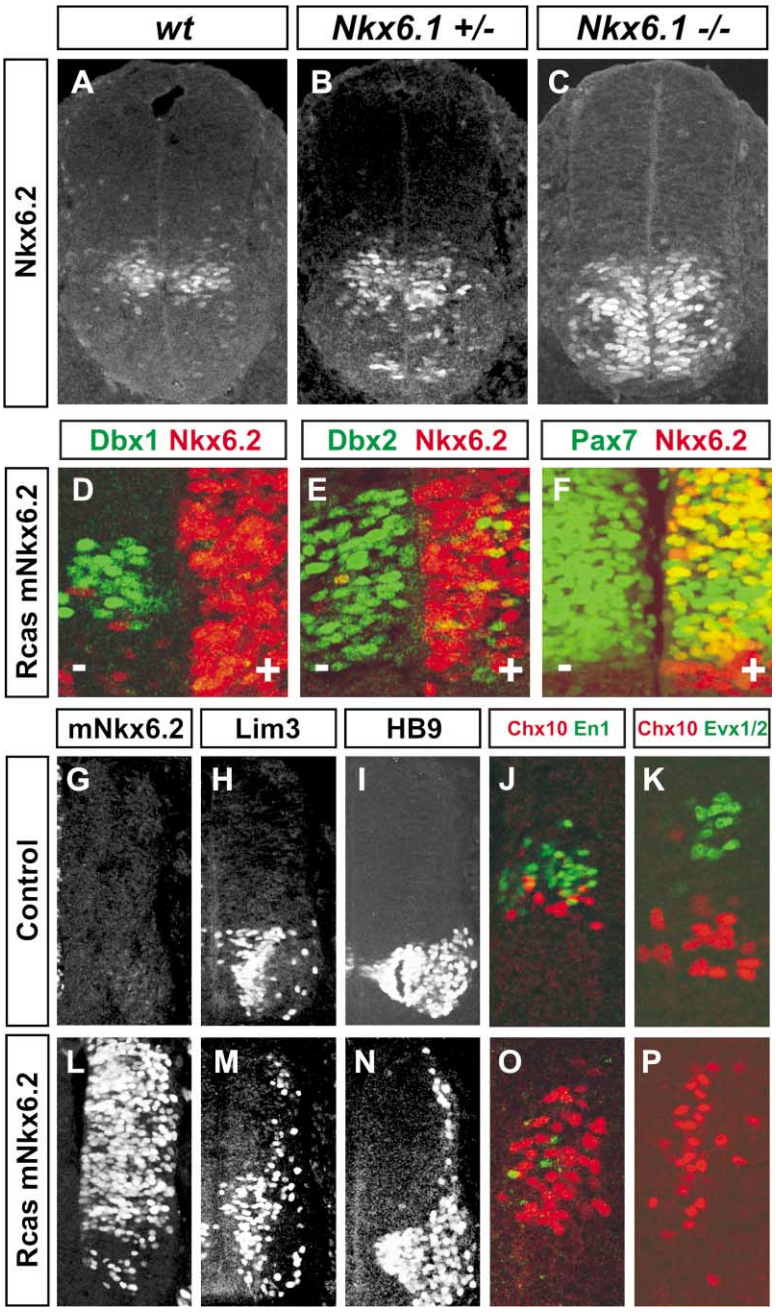

Figure 4. Deregulated Expression of Nkx6.2 in *Nkx6.1* Mutant Mice, and Similar Patterning Activities of Nkx6 Proteins in Chick Neural Tube

(A) In e10.5 *wt* embryos, Nkx6.2 expression is confined to the p1 progenitor domain. (B) In *Nkx6.1*$^{+/-}$ embryos, scattered Nkx6.2^{+} cells are detected in the p2, pMN, and p3 domains. (C) In Nkx6.1$^{-/-}$ embryos, Nkx6.2 is expressed in most progenitors in the p2, pMN, and p3 domains.

(D–F) Misexpression of Nkx6.2 at high levels represses the expression of Dbx1 (D) and Dbx2 (E), but not Pax7 (F).

(G–P) Expression of Nkx6.2 in dorsal positions of the chick neural tube result in ectopic dorsal generation of motor neurons, as indicated by ectopic induction of Lim3 and HB9 expression (G–I and L–N). Forced expression of Nkx6.2 at high levels in the p0 and p1 progenitor domains promotes the ectopic generation of Chx10^{+} V2 neurons (J, K, O, and P) and suppresses Evx1/2^{+} V0 (K and P) and En1^{+} V1 (J and O) neurons.

levels, but by only 25% at lumbar levels (Figures 5H, 5N, and 5O; Sander et al., 2000). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ embryos, motor neuron generation was reduced to ~25% of controls at both cervical and lumbar levels (Figures 5I, 5N, and 5O; data not shown). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{tlz/tlz}$ embryos, the generation of motor neurons was reduced to <10% of wild-type numbers, at all levels of the spinal cord (Figure 5J). In these *Nkx6* double mutant embryos, residual motor neurons were detected at e10.0, and no further increase in motor neuron number was evident at e12 (Figures 5M and 5P; data not shown). Since there was no increase in apoptotic cell death in the ventral neural tube over this period (data not shown), we infer that the few spinal motor neurons present in *Nkx6* double mutants are generated prior to e10. Together, these findings demonstrate that Nkx6.2 substitutes for the loss of Nkx6.1 in spinal motor neuron generation, and reveal a link between *Nkx6* gene dosage and the incidence of motor neuron generation.

A Dissociation in Neuronal Fate and Progenitor Cell Identity in *Nkx6* Mutant Mice

We next examined whether a reduction in *Nkx6* gene dosage results in ectopic Dbx protein expression and V1 and V0 neuron generation in the p2 and pMN domains of the ventral spinal cord.

En1^{+} V1 neurons are normally generated from Dbx2^{+}, Dbx1^{-} p1 progenitor cells, and we therefore analyzed the relationship between Dbx2 expression and En1^{+} V1 neuronal generation in *Nkx6.1* and *Nkx6.2* compound

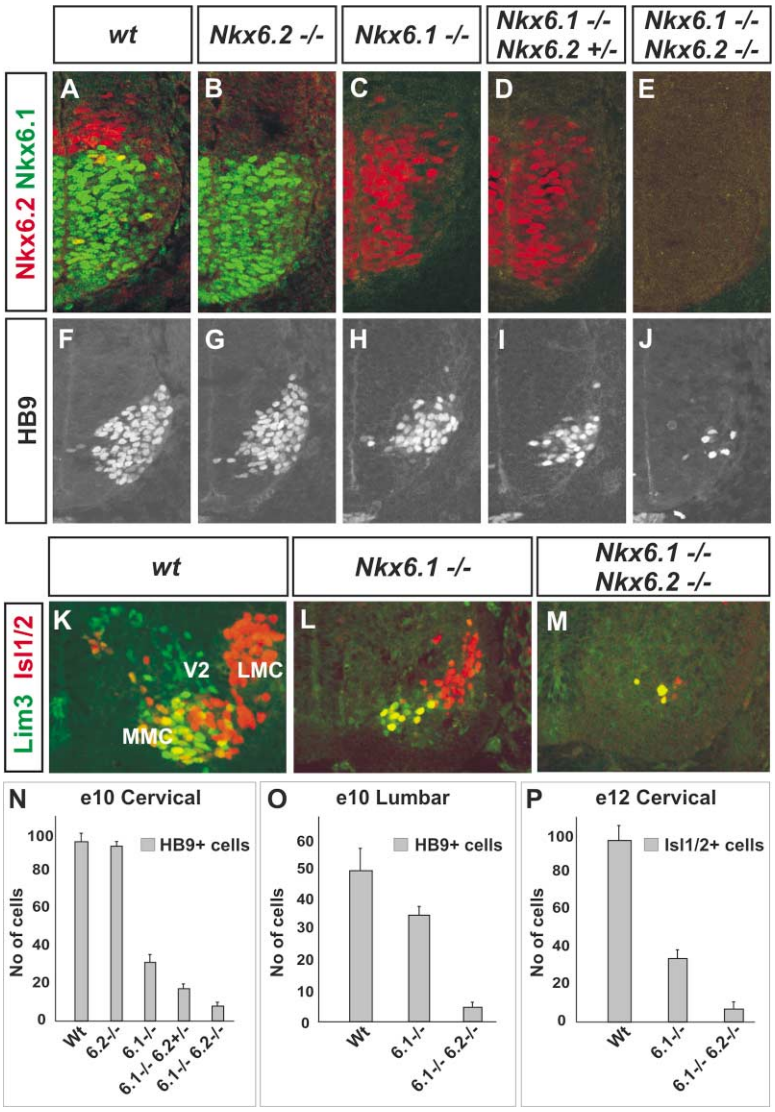

Figure 5. The Deregulated Expression of Nkx6.2 Underlies Motor Neuron Generation in *Nkx6.1* Mutants

(A) In e10.5 *wt* embryos, Nkx6.2 expression is confined to the p1 domain and Nkx6.1 is expressed in the p2, pMN, and p3 domains.
(B) No change in the expression of Nkx6.1 is detected in *Nkx6.2* $^{tlz/tlz}$ embryos.
(C and D) In *Nkx6.1* $^{-/-}$ and *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{+/tlz}$ embryos, Nkx6.2 expression is derepressed in the p2, pMN, and p3 domains.
(E) No expression of Nkx6.2 or Nkx6.1 protein is detected in *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ embryos.
(F and G) HB9^{+}, Isl1/2^{+} motor neurons are generated in normal numbers in *Nkx6.2* $^{tlz/tlz}$ embryos. The number of motor neurons is reduced by ~60% in *Nkx6.1* $^{-/-}$ embryos (H), by ~80% in *Nkx6.1* $^{-/-}$*;Nkx6.2* $^{+/tlz}$ embryos (I), and by >90% in *Nkx6.1* $^{-/-}$*;Nkx6.2* $^{tlz/tlz}$ at cervical levels of e10.5 spinal cord (J).
(K–M) At e12, the number of motor neurons of medial (MMC) (Isl1^{+}, Lim3^{+}) and lateral (LMC) (Isl1^{+}) subtype identity is reduced in similar proportions in *Nkx6.1* $^{-/-}$ and *Nkx6.1* $^{-/-}$*; Nkx6.2* $^{tlz/tlz}$ embryos. Lim3^{+} V2 neurons are missing in *Nkx6.1* $^{-/-}$ embryos and *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ embryos at this stage.
(N–P) Quantitation of HB9^{+} and Isl1/2^{+} motor neurons at cervical and lumbar levels in *wt*, *Nkx6.2*, and *Nkx6.1* single mutants and in *Nkx6.2; Nkx6.1* compound mutants at e10 and e12. Counts from 12 sections, mean ± SD.

mutants. As reported previously (Sander et al., 2000), in *Nkx6.1* $^{-/-}$ embryos examined at e10.5, ectopic ventral expression of Dbx2 was detected at high levels in the p2 and p3 domains, although cells in the pMN expressed only very low levels of Dbx2 (Figure 6H; see Sander et al., 2000). Moreover, in *Nkx6.1* $^{-/-}$ embryos, ectopic En1^{+} neurons were generated in the p2 and pMN domains of the ventral neural tube (Figure 6R). In *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{+/tlz}$ embryos, Dbx2 expression was detected at intermediate levels in the pMN domain (Figure 6I), and in *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ double mutant embryos, Dbx2 was detected at uniformly high levels in the p2 and pMN domains (Figure 6J). Strikingly, in these *Nkx6.1* and *Nkx6.2* compound mutant backgrounds, and despite the enhanced ectopic expression of Dbx2, the number of ectopic ventral En1^{+} V1 neurons was reduced rather than increased, when compared with the number generated in *Nkx6.1* single mutants (Figures 6R and 6T).

Since Evx1^{+} V0 neurons are normally generated from Dbx1^{+}, Dbx2^{+} p0 progenitors, we examined whether the reduction in ectopic ventral En1^{+} V1 neuron generation at low *Nkx6* gene dosage might reflect a change in the pattern of expression of Dbx1, and the ectopic generation of V0 neurons. Consistent with this idea, in *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ mutants, scattered Dbx1^{+} cells were detected in the p2, pMN, and p3 domains (Figure 6O), and ectopic ventral Evx1/2^{+} V0 neurons were detected throughout the ventral neural tube (Figures 6T and 6Z). Thus, in *Nkx6* double mutants, the loss of V1 neurons is associated with the ectopic ventral expression of Dbx1 and the generation of ectopic V0 neurons.

But in *Nkx6.1* single and *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{+/tlz}$ compound mutant backgrounds, the normal link between expression of Dbx1 in progenitor cells and the generation of Evx1/2^{+} V0 neurons was severed. In both these *Nkx6* compound mutants backgrounds, the domain of expression of Dbx1 was unchanged (Figures 6M and 6N): a result that can be accounted for by the maintained expression of Nkx6.2 within the p1 domain, and the deregulated expression of Nkx6.2 within the p2 and pMN domains. Nevertheless, Evx1/2^{+} V0 neurons were generated from progenitor cells in the position of p2 and pMN domains (Figures 6R, 6S, 6X, and 6Y).

We next considered whether these ectopic V0 neurons were generated from the position of the p2 and pMN domains, or whether they simply migrated ventrally

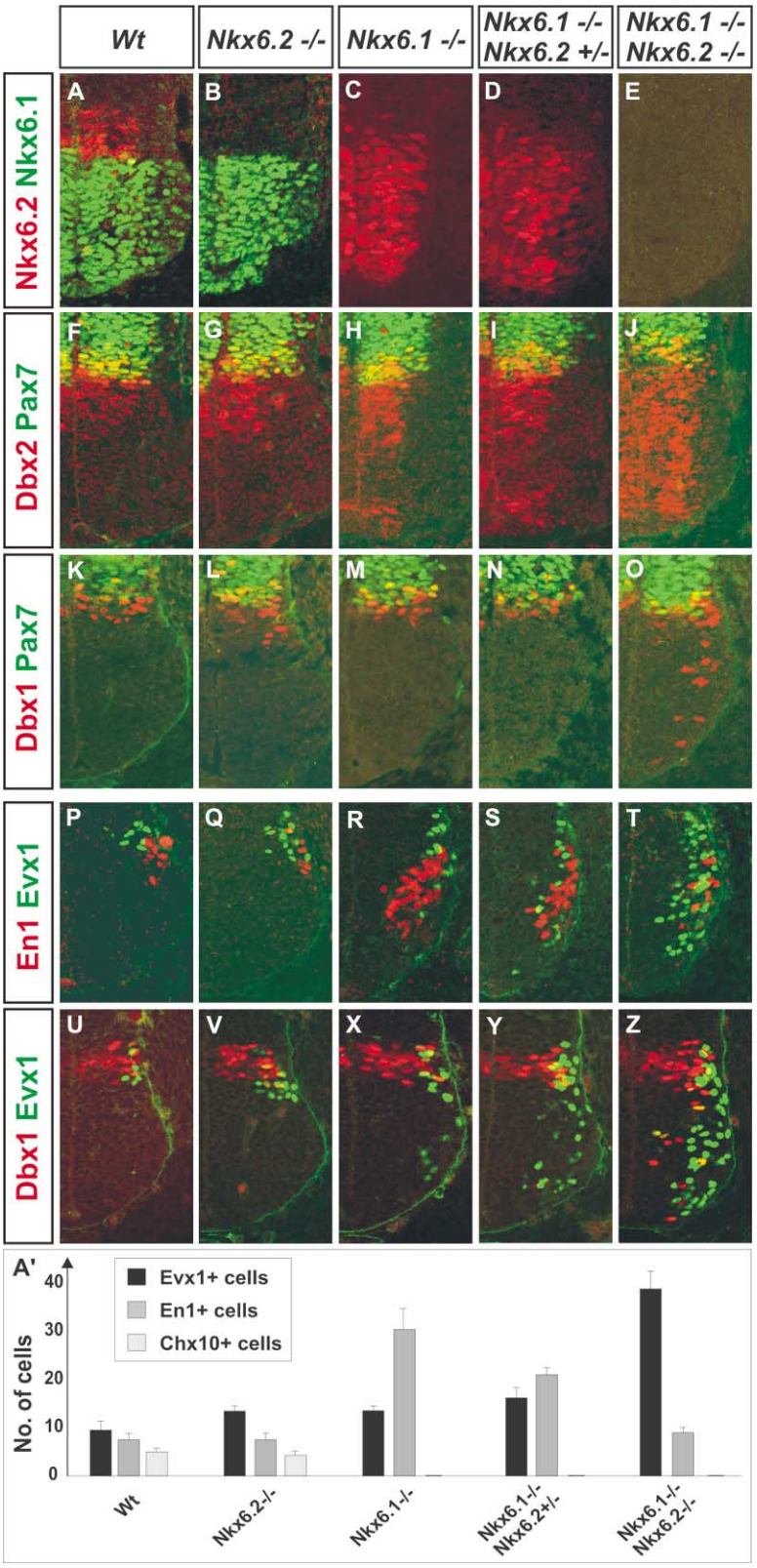

Figure 6. Changes in Class I Protein Expression and Ventral Interneuron Generation in *Nkx6* Mutants

(A–E) Expression of Nkx6.1 and Nkx6.2 in the spinal cord in different *Nkx6* mutant backgrounds at e10.5.

(F–J) Spatial patterns of Pax7 and Dbx2 expression in different *Nkx6* mutant backgrounds. Note that the level of Dbx2 expression in the pMN domain of Nkx6.1$^{-/-}$; Nkx6.2$^{+/tlz}$ is very low, implying the existence of a pMN domain-restricted gene that has the capacity to repress Dbx2 expression. Recent studies have provided evidence that the bHLH protein Olig2 possesses these properties (Novitch et al., 2001).

(K–O) Spatial patterns of expression of Pax7 and Dbx1 in different *Nkx6* mutant backgrounds.

(P–T) Spatial patterns of generation of Evx1/2^{+} V0 neurons and En1^{+} V1 neurons in different *Nkx6* mutant backgrounds. (Q) The generation of V0 neurons expands ventrally into the p1 domain in *Nkx6.2*$^{tlz/tlz}$ mutants at caudal spinal levels. (R and A′) The number of En1^{+} V1 neurons increases ~3-fold in the ventral spinal cord of *Nkx6.1*$^{-/-}$ mutants, and ectopic Evx1/2^{+} cells are detected in position of the pMN domain in these mice (see also Sander et al., 2000). (S, T, and A′) There is a progressive increase in Evx1/2^{+} V0 neurons and a loss of En1^{+} V1 neurons in the ventral spinal cord of *Nkx6.1*$^{-/-}$*;Nkx6.2*$^{+/tlz}$ and *Nkx6.1*$^{-/-}$*;Nkx6.2*$^{tlz/tlz}$ embryos. (U,V, and Z) The generation of Evx1/2^{+} V0 neurons correlates with the pattern of expression of Dbx1 in progenitors in *wt*, *Nkx6.2*$^{tlz/tlz}$, and *Nkx6.1*$^{-/-}$*;Nkx6.2*$^{tlz/tlz}$ mutant backgrounds. Note that only the most lateral progenitor cells express Dbx1 in Nkx6.1$^{-/-}$; Nkx6.2$^{tlz/tlz}$ embryos, suggesting that expression of Dbx1 in more medially positioned progenitors is repressed by an as yet undefined gene. (X and Y) Ectopic ventral Evx1^{+} V0 neurons derive from Dbx1^{-} progenitors in *Nkx6.1*$^{-/-}$ and *Nkx6.1*$^{-/-}$*;Nkx6.2*$^{+/tlz}$ mutant embryos. Chx10^{+} V2 neurons are generated at normal numbers in *Nkx6.2*$^{tlz/tlz}$ mutants, but are missing at spinal cord levels in *Nkx6.1*$^{-/-}$, *Nkx6.1*$^{-/-}$*;Nkx6.2*$^{+/tlz}$, and *Nkx6.1*$^{-/-}$*;Nkx6.2*$^{tlz/tlz}$ mutants (A′; Figure 5, see Sander et al., 2000).

from a more dorsal position of origin. Ectopic ventral Evx1/2^{+} V0 neurons were detected as early as e10.0 (Figure 7B), and many of them coexpressed LacZ (Figures 7C and 7D), providing evidence that many of these neurons derive from progenitor cells within the position of the p2 and pMN domains. The finding that Evx1/

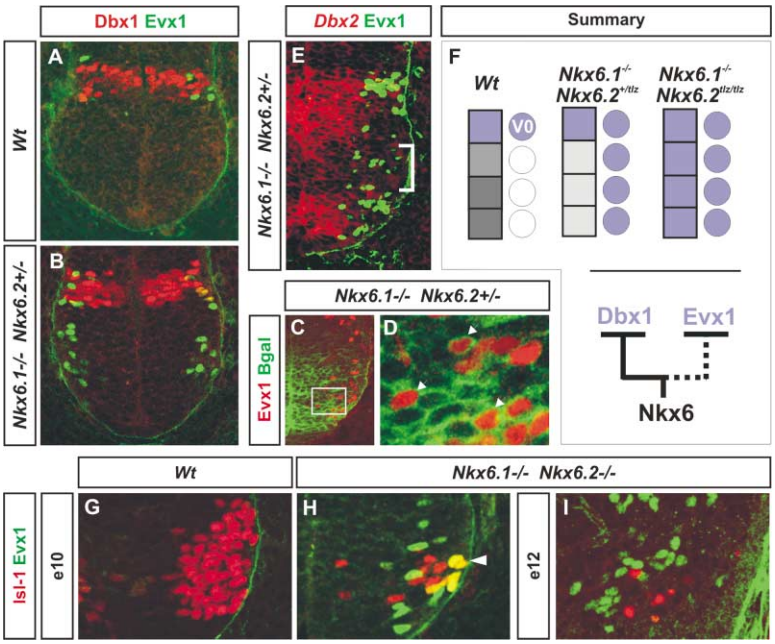

Figure 7. Dissociation of Dbx Expression and V0 Neuronal Fate in Mice with Reduced Nkx6 Protein Activity

(A) In e10.0 *wt* embryos, p0 progenitor cells express Dbx1 and generate Evx1/2^{+} V0 neurons. (B) In e10.0 *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ embryos, there is no change in the domain of expression of Dbx1, but Evx1/2^{+} V0 neurons are generated in lateral positions, along much of the ventral neural tube.

(C and D) In Nkx6.1$^{-/-}$; Nkx6.2$^{+/tlz}$ embryos examined at e10.0, many ectopic ventral Evx1/2^{+} neurons express LacZ. Framed area in (C) is shown at high magnification in (D) and indicates Evx1/2^{+} neurons that coexpress LacZ.

(E) Evx1/2^{+} neurons located at the level of the pMN domain (bracket) derive from progenitors that express low or negligible levels of *Dbx2* mRNA.

(F) Summary of Dbx1 expression and V0 neuron generation in *wt*, *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$, and *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{tlz/tlz}$ embryos. The dissociation of Dbx1 and Evx1/2 expression in *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ embryo suggests that reduced Nkx6 repressor activity is sufficient to repress Dbx1, but insufficient to repress Evx1 expression.

2^{+} V0 neurons are generated from the pMN domain in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ embryos is especially significant since these progenitors express negligible levels of Dbx2 (Figures 7E and 8), arguing against the possibility that Dbx2 expression compensates for the absence of Dbx1 during ectopic V0 neuronal generation. These results therefore provide evidence that even though Dbx1 activity is normally required for the generation of V0 neurons (Pierani et al., 2001), under conditions in which *Nkx6* gene dosage is markedly reduced, V0 neurons can be generated from progenitor cells that lack Dbx1 expression.

Nevertheless, the pattern of ventral neurogenesis observed in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ mutants indicated that residual Isl1/2^{+}, HB9^{+} neurons and ectopic Evx1^{+} neurons were each generated from progenitors located in the position of the pMN domain. This observation raised the question of whether these two neuronal populations are, in fact, distinct. Strikingly, we found that in this compound *Nkx6* mutant background, many of the resid-

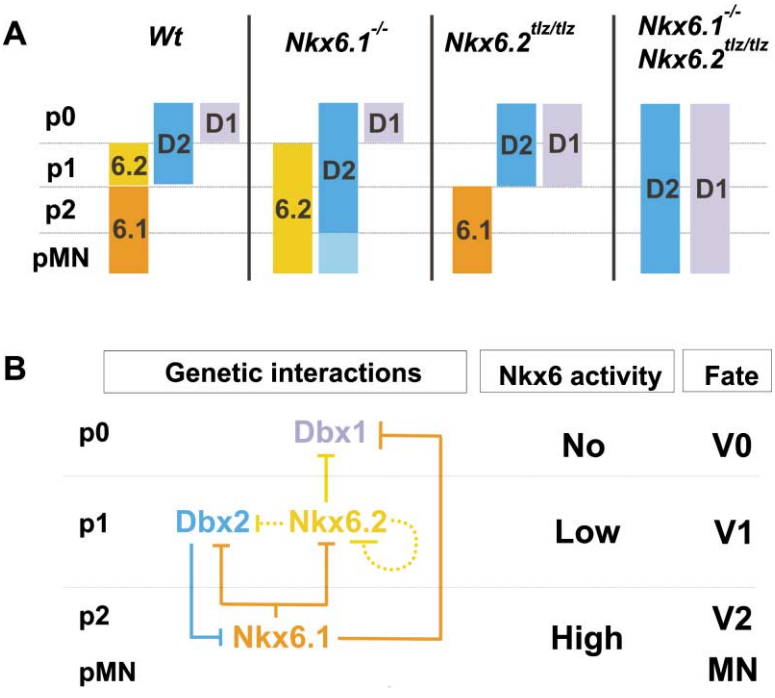

Figure 8. Genetic Interactions between Nkx6 and Dbx Proteins during the Assignment of Motor Neuron and Interneuron Fate in the Mouse Neural Tube

(A) Summary of domains of expression of Nkx6.1 (6.1), Nkx6.2 (6.2), Dbx1 (D1), and Dbx2 (D2) in the ventral neural tube of wild-type (wt) and different *Nkx6* mutant embryos.

(B) Regulatory interactions between Nkx and Dbx proteins in the ventral neural tube. These interactions result in different levels of Nkx6 protein activity in distinct ventral progenitor domains, and thus promote the generation of distinct neuronal subtypes.

For details see text.

ual Isl1/2$^+$, HB9$^+$ neurons transiently expressed Evx1 (Figures 7H and 7I). Thus, under conditions of reduced *Nkx6* gene dosage, progenitor cells at the position of the pMN domain initially generate neurons with a hybrid motor neuron/V0 neuron identity.

Discussion

The patterning of cell types in the ventral neural tube depends on the actions of a set of homeodomain proteins expressed by neural progenitor cells. Duplication of many of these genes has resulted in the overlapping neural expression of pairs of closely related homeodomain proteins, and raises the question of whether these proteins have distinct or redundant roles during ventral neurogenesis. We have used genetic approaches in mouse to examine the respective contributions of one such homeodomain protein pair, Nkx6.1 and Nkx6.2, in ventral neural patterning. Our results imply that the duplication of an ancestral *Nkx6* gene confers both redundant and distinct roles for Nkx6.1 and Nkx6.2 in ventral neuronal patterning. We discuss below how the specificity and efficacy of Nkx6-mediated transcriptional repression underlies the overlapping divergent patterning activities of the two proteins.

Redundant Activities of Nkx6 Proteins in Motor Neuron and V0 Neuron Generation

Our genetic studies in mice indicate that Nkx6.1 and Nkx6.2 have qualitatively similar activities in promoting the generation of motor neurons and in suppressing the generation of V0 neurons. How are these overlapping patterning activities achieved, given the distinct profiles of expression of these two genes?

Nkx6.1 has been shown to have a role in motor neuron generation (Sander et al., 2000), but the finding that large numbers of motor neurons are generated at caudal levels of the spinal cord in *Nkx6.1* mutant mice points to the existence of an Nkx6.1-independent pathway of motor neuron generation. At face value, Nkx6.2 would appear a poor candidate as a mediator of the Nkx6.1-independent pathway of motor neuron specification since it is not expressed by motor neuron progenitors, nor is motor neuron generation impaired in *Nkx6.2* mutant mice. Nevertheless, the activity of Nkx6.2 is responsible for the efficient generation of spinal motor neurons in *Nkx6.1* mutants. The basis of this redundant function resides in the derepression of Nkx6.2 expression in motor neuron progenitors in *Nkx6.1* mutant mice. Strikingly, Nkx6.2 is even derepressed in *Nkx6.1*$^{+/-}$ embryos, whereas there is no change in the patterns of expression of Dbx2 and other homeodomain proteins implicated in the repression of motor neuron generation. The propensity for Nkx6.2 derepression thus appears to establish a "fail-safe" mechanism that ensures that the net level of Nkx6 protein activity is maintained in motor neuron progenitors under conditions in which Nkx6.1 levels decrease. A similar "fail-safe" regulatory mechanism may operate with other Nkx protein pairs. During pharyngeal pouch development, for example, the loss of Nkx2.6 expression appears to be compensated for by the upregulation of Nkx2.5 (Tanaka et al., 2000).

The finding that Nkx6.2 is derepressed in the absence of Nkx6.1 function also offers a potential explanation for the divergent patterns of expression of Nkx6.2 in the ventral neural tube of mouse and chick embryos. We infer that the chick *Nkx6.2* gene is not subject to repression by Nkx6.1, permitting its persistent expression in p3, pMN, and p2 domain progenitor cells. Thus, in chick, the overlapping functions of Nkx6.1 and Nkx6.2 in motor neuron generation are associated with the coexpression of both genes by motor neuron progenitors, whereas in the mouse, Nkx6.2 activity is held in reserve, through its repression by Nkx6.1.

Nkx6.1 and Nkx6.2 also have an equivalent inhibitory influence on the generation of V0 neurons, albeit through activities exerted in different progenitor domains. In p1 progenitors, the repression of p0 identity and V0 neuron fate is accomplished by Nkx6.2. But ventral to the p1/p2 domain boundary, it is Nkx6.1 that prevents Dbx1 expression and V0 neuronal generation. Thus, Nkx6.1 is a potent repressor of Dbx1 expression, despite the fact that these two proteins lack a common progenitor domain boundary. The repression of genes that are normally positioned in spatially distinct domains has been observed with other class I and II proteins (Sander et al., 2000). This feature of neural patterning also parallels the activities of gap proteins in anteroposterior patterning of the *Drosophila* embryo, where the repressive activities of individual gap proteins are frequently exerted on target genes with which they lack a common boundary (Kraut and Levine, 1991; Stanojevic et al., 1991).

Distinct Functions of Nkx6.1 and Nkx6.2 in Ventral Interneuron Generation

We now turn to the question of how Nkx6.1 and Nkx6.2 can exert distinct roles in interneuron generation, given the similarities of the two proteins in DNA target site specificity (Jörgensen et al., 1999; Muhr et al., 2001), and their overlapping functions in the patterning of motor neurons and V0 neurons.

One factor that contributes to the opponent influence of Nkx6.1 and Nkx6.2 on the specification of V1 interneuron fate is a distinction in the dorsal limit of expression of the two proteins in the neural tube, presumably a reflection of differences in the regulation of expression of the two proteins by graded Shh signaling. Nkx6.1 expression stops at the p1/p2 domain boundary. And within the p2 domain, Nkx6.1 suppresses p1 progenitor identity through repression of Dbx2 and Nkx6.2 expression, in this way ensuring the generation of Chx10$^+$ V2 neurons. Nkx6.2, in contrast, occupies the p1 domain, where it is coexpressed with Dbx2. In p1 domain cells, Nkx6.2 promotes the generation of En1$^+$ V1 neurons by repressing the expression of Dbx1 and Evx1, determinants of V0 neuronal fate (Pierani et al., 2001; Moran-Rivard et al., 2001). Nevertheless, only a fraction of p1 progenitors initiate Dbx1 expression and acquire V0 neuron fate in the absence of Nkx6.2 function, raising the possibility that Dbx2 may also have a role in repressing Dbx1 expression within p1 progenitors (see Pierani et al., 1999).

The second major factor that underlies the opponent activities of Nkx6.1 and Nkx6.2 in V1 interneuron specification appears to be a difference in the potency with

which the two Nkx6 proteins repress a common set of target genes. This view is supported by several observations. Nkx6.1 completely represses Nkx6.2, whereas Nkx6.2 exerts an incomplete negative regulation of its own expression in p1 domain progenitors. Thus, Nkx6.1 is evidently a better repressor of Nkx6.2 than is Nkx6.2 itself. Similarly, Nkx6.2 is coexpressed with Dbx2 in p1 domain progenitors, whereas Nkx6.1 excludes Dbx2 from p2 domain progenitors, indicating that Nkx6.1 also is a more effective repressor of Dbx2 expression than is Nkx6.2. Consistent with this view, Nkx6.2 fails to repress Dbx2 expression completely from ventral progenitors in *Nkx6.1* mutants. The fact that Nkx6.2 is only a weak repressor of Dbx2 is critical for the formation of the p1 domain since the maintained expression of Dbx2 in these cells ensures the exclusion of Nkx6.1 expression (Briscoe et al., 2000).

Our results do not resolve why Nkx6.2 is a weaker repressor than Nkx6.1 in vivo. Differences in the primary structure of Nkx6.2 and Nkx6.1 (Cai et al., 1999; Muhr et al., 2001) could result in an intrinsically lower repressor activity of Nkx6.2, when compared with that of Nkx6.1. But our findings are also consistent with the possibility that the two Nkx6 proteins have inherently similar repressor activities, and that the Nkx6.2 protein is merely expressed at a lower level. Indeed within p1 progenitors, the level of Nkx6.2 expression is clearly subject to tight regulation, with significant consequences for neuronal specification. The selective expression of Nkx6.2 in p1 progenitors, coupled with its weak negative autoregulatory activity, ensures a level of Nkx6 activity that is low enough to permit Dbx2 expression but is still sufficient to repress Dbx1 expression, thus promoting the generation of V1 neurons.

Our findings therefore reveal that a gradient of extracellular Shh signaling is translated intracellularly into stepwise differences in the level of Nkx6 activity along the ventral-to-dorsal axis of the neural tube. Moreover, the different Nkx6 protein activity levels within ventral progenitor cells are a critical determinant of ventral neuronal fate. Cells that express low or negligible levels of Nkx6 activity (p0 progenitors) are directed to a V0 neuronal fate, cells that express an intermediate Nkx6 activity level (p1 progenitors) are directed to a V1 fate, and cells that express a high Nkx6 activity level (pMN and p2 progenitors) are directed to a motor neuron or V2 fate (Figure 8).

Nkx6 Repressor Function and Neuronal Patterning by Derepression

The finding that many progenitor homeodomain proteins exert mutual-cross repressive interactions has led to a model of spinal neuronal patterning based on transcriptional derepression (Muhr et al., 2001). Similar crossrepressive interactions may establish regional progenitor domains in more rostral regions of the developing CNS (Toresson et al., 2000; Yun et al., 2001). A premise of this model is that transcriptional repression is exerted at two sequential steps in neurogenesis. One repressive step operates at the level of the progenitor homeodomain proteins themselves, but a second repressive step is exerted on neuronal subtype determinant factors that have a downstream role in directing neuronal subtype fates (Briscoe et al., 2000; Muhr et al., 2001).

Our analysis of *Nkx6* compound mutant mice provides direct support for this two-step repression model, and in addition indicates that progenitor homeodomain proteins and neuronal subtype determinants differ in their sensitivity to repression by the same class II protein. Normally, the functions of *Dbx1* and *Evx1* are required sequentially during the generation of V0 neurons (Pierani et al., 2001; Moran-Rivard et al., 2001). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ mutants, however, the generation of Evx1/2^{+} V0 neurons occurs in the absence of expression of Dbx1 by neural progenitor cells. Dbx1 expression is therefore dispensable for V0 neuron generation under conditions of reduced *Nkx6* gene dosage. From these results, we infer that the net level of Nkx6 protein activity in ventral progenitor cells is still above threshold for repression of Dbx1 expression, but is below the level required for repression of Evx1 expression. These data therefore support the idea that Nkx6 proteins normally inhibit V0 neuronal fate by repressing the class I progenitor homeodomain protein Dbx1, and independently by repressing expression of the V0 neuronal subtype determinant Evx1.

A differential sensitivity of progenitor homeodomain proteins and neural subtype determinants to repression appears therefore to underlie the dissociation of progenitor cell identity and neuronal fate observed in *Nkx6* mutants. Such two-tiered repression is, in principle, necessary to specify neuronal fate through transcriptional derepression. In the case of Nkx6.1, for example, repression of Dbx1 and Dbx2 (and possible other unidentified repressors) should be sufficient to derepress motor neuron subtype determinants such as MNR2 and Lim3 in pMN progenitors. But, unless Nkx6.1 also represses the expression of V0 determinants, Evx1 expression would also be initiated in differentiating motor neurons, resulting in a hybrid neuronal phenotype. Indeed, under conditions in which *Nkx6* gene dosage is reduced or eliminated, some of the neurons generated from the position of the pMN domain do transiently express a hybrid motor neuron/V0 neuron phenotype.

The derepression model also invokes the idea that a major role of Nkx6 class proteins is to exclude the expression of Dbx2 and other proteins that inhibit motor neuron generation. This view offers a potential explanation of why a few residual motor neurons are generated in *Nkx6* double mutants. We find that in the absence of *Nkx6* gene function, residual motor neurons are generated only at early developmental stages, suggesting that progenitor cells within the position of the pMN domain have committed to a motor neuron fate prior to the onset of the deregulated ventral expression of Dbx2 and other motor neuron repressors. We note that a third *Nkx6*-like gene exists in the mouse, but this gene is not expressed in the spinal cord of wild-type or *Nkx6* mutant embryos (E. Anderson and J.E., unpublished data), and thus its activity appears not to account for the residual motor neurons generated in *Nkx6* double mutants. Importantly, the detection of residual motor neurons in *Nkx6* double mutants also provides evidence that Nkx6 proteins do not have essential functions as transcriptional activators during motor neuron specification, further supporting their critical role as repressors.

Finally, the present studies and earlier work on neurogenesis in the ventral spinal cord (Ericson et al., 1996;

Thaler et al., 1999; Arber et al., 1999; Sander et al., 2000) have provided evidence that newly generated neurons can sometimes express mixed molecular identities. These observations raise the possibility that repressive interactions that select or consolidate individual neuronal identities are not restricted to progenitor cells. Consistent with this view, Evx1 is required to establish V0 and repress V1 neuronal identity through an action in post-mitotic neurons (Moran-Rivard et al., 2001), although it remains unclear whether Evx1 itself functions in this context as an activator or repressor. Similarly, the homeodomain protein HB9 has been implicated in the consolidation of motor neuron identity, through repression of V2 neuronal subtype genes (Arber et al., 1999; Thaler et al., 1999). HB9 possesses an eh-1 Gro/TLE recruitment domain (Muhr et al., 2001), suggesting that HB9 controls the identity of post-mitotic motor neurons through a direct action as a transcriptional repressor. The consolidation of neuronal subtype identity in the spinal cord may therefore depend on transcriptional repressive interactions within both progenitor cells and post-mitotic neurons.

Experimental Procedures

Generation of *Nkx6.2* Mutant Mice

Mouse *Nkx6.2* genomic clones were isolated from a 129/Ola mouse genomic library. A targeting construct was constructed by inserting a tau-lacZ/pGKneo cassette into a 5 kb 5′ HindIII-NcoI fragment and a 2.7 kb 3′ SphI-AccI fragment. The linearized targeting construct was electroporated into E14.1 (129/Ola) ES cells. Cells were selected with G418 and screened by Southern blot analysis using a 200 bp 3′ AccI fragment, which detected a 6 kb wild-type band and a 2.9 kb mutant band. Recombinant clones were injected into C57BL/6J blastocysts to generate two chimeric founders, both of which transmitted the mutant allele. Mice homozygous for the mutant alleles were born at Mendelian frequency and survived through adulthood. All experiments involved mice maintained on a C57BL/6 background. The generation and genotyping of *Nkx6.1* mutant mice have been described previously (Sander et al., 2000). Compound *Nkx6* mutant mice were obtained by crossing *Nkx6.2*$^{+/ltz}$; *Nkx6.1*$^{+/-}$ double heterozygous mice. Genotyping was performed using Southern blot analysis.

Chick In Ovo Electroporation

Mouse *Nkx6.2* was isolated by PCR (Komuro et al., 1993) and chick *Nkx6.2* from a chick spinal cord library (Basler et al., 1993) using mouse *Nkx6.1* and *Nkx6.2* as probes. cDNAs encoding full-length mouse and chick *Nkx6.2* were inserted into a RCASBP(B) retroviral vector and electroporated into the neural tube of stage HH (Hamburger and Hamilton, 1953) 10–12 chick embryos (Briscoe et al., 2000). After 24–48 hr, embryos were fixed and processed for immunohistochemistry.

Immunohistochemistry and In Situ Hybridization Histochemistry

Immunohistochemical localization of proteins was performed as described (Yamada et al., 1993; Briscoe et al., 2000). Guinea-pig antisera were generated against an 11 amino acid N-terminal sequence of mouse Nkx6.2. Other antibodies used were rabbit anti-Lim3 (Ericson et al., 1997), mAb Hb9 (Tanabe et al., 1998), rabbit anti-Isl1/2 (Tsuchida et al., 1994), rabbit anti-Chx10 (Ericson et al., 1997), rabbit anti-En1 (Davis et al., 1991), mAb anti-Evx1/2, rabbit anti-Dbx1, rabbit anti-Dbx2 (Pierani et al., 1999), rabbit anti-Nkx6.1 (Jörgensen et al., 1999), mAb anti-Pax7 (Ericson et al., 1996), rabbit anti-βgal (Cappel), and goat anti-βgal (Biogeneseis). Images were collected on a Zeiss LSM510 confocal microscope. In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993), using chick probes for *Dbx1*, *Dbx2* (Pierani et al., 1999), *Nkx6.1* (Briscoe et al., 2000), and *Nkx6.2*. A mouse probe for the 5′UTR of *Nkx6.2* comprised 346 bp upstream of the start ATG site. Whole-mount X-gal staining was performed as described (Mombaerts et al., 1996).

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