Nolz1 is induced by retinoid signals and controls motoneuron subtype identity through distinct repressor activities

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The acquisition and maintenance of final neuronal identity depends in part upon the implementation of fate-specification programs in postmitotic neurons; however, the mechanisms involved remain unclear. In the developing spinal cord, retinoic acid (RA) signaling pathways specify the columnar and divisional identities of postmitotic motoneurons (MNs). Here we show that RA signals induce expression of the NET transcriptional regulator Nolz1 in differentiated chick MNs, where it regulates the progressive specification of prospective Lim3-negative motor columns. Nolz1 controls the initial formation of forelimb and thoracic Lim3-negative motor columns by downregulating Lim3 expression and maintaining the expression of key homeodomain proteins necessary for MN identity and survival. At forelimb levels, Nolz1 specifies lateral motor column (LMC) identity by inducing the expression of the postmitotic LMC determinant Hoxc6, and implements the partial specification of lateral LMC identity through Lim1 induction. The specificity of Nolz1 function depends upon distinct repressor activities that require, in part, the modulatory activity of Grg5, an atypical member of the Gro-TLE family of co-repressors. Thus, RA signals regulate diverse events in MN subtype specification by inducing the expression of a key transcriptional regulator that controls multiple developmental pathways via functionally distinct repressor complexes.

KEY WORDS: Retinoids, Nolz1, Grg5, Motoneuron, Identity, Repressor

INTRODUCTION

The establishment of a functional nervous system depends upon the coordinated generation and maintenance of diverse neuronal subtypes during embryonic and postnatal development. In progenitor cells, cross-repressive transcriptional mechanisms elicit the expression of distinct transcription factors that confer subsequent neuronal fate (Jessell, 2000; Vallstedt et al., 2001). However, neurons retain a degree of plasticity in their fates after cell cycle exit, implying that the induction or maintenance of cell fate-specification programs in postmitotic neurons is crucial for the acquisition and preservation of final neuronal identity (Gross et al., 2002; Moran-Rivard et al., 2001; Muller et al., 2002; Pearson and Doe, 2004). While the mechanisms that regulate progenitor patterning and differentiation are emerging, less is known about the molecular pathways that shape and maintain identity in postmitotic neurons.

The study of spinal motoneuron (MN) development has helped elucidate the transcriptional mechanisms that generate and consolidate postmitotic neuronal fate (Jessell, 2000). Spinal MNs derive from a discrete ventral progenitor domain termed pMN. In the chick, MNR2 is expressed in the S phase of the terminal cell cycle of pMN progenitors and triggers the expression of a cascade of factors essential for somatic MN development (Tanabe et al., 1998). All newly differentiating MNs coexpress Islet1 and Lim3, activities necessary for suppressing intrinsic interneuron specification programs in developing MNs (Pfäff et al., 1996; Arber et al., 1999; Thaler et al., 1999; Thaler et al., 2002). These ‘generic’ MNs subsequently diversify into distinct neuronal subtypes that are manifest by the organization of their cell bodies into specific motor columns, their characteristic axonal projection patterns, and their distinct LIM-HD protein expression profiles (Landmesser, 1978; Tosney et al., 1995; Tsuchida et al., 1994; Jessell, 2000). MNs form the medial division of the median motor column (MMCm) span all rostral-caudal levels and innervate axial muscles, whereas the preganglionic MNs of the column of Terni (CT) and MNs of the lateral MMC (MMCl) are found at thoracic regions (Prasad and Hollyday, 1991; Jessell, 2000). At limb levels, lateral motor column (LMC) MNs that innervate the limb form medial and lateral divisions, which innervate ventrally and dorsally derived limb muscles, respectively. MN diversification is first apparent by the downregulation of Lim3 in prospective CT, MMCl and LMC MNs (Sharma et al., 2000). Within Lim3 MNs, cross-repressive interactions between different Hox HD proteins consolidate rostral-caudal Hox protein distributions, which activate the expression of distinct LIM-HD proteins that dictate MN subtype identity and connectivity (Liu et al., 2001; Dasen et al., 2003; Sharma et al., 2000; Kania et al., 2000). For example, cross-repressive interactions between Hoxc9 and Hoxc6 define their expression domains at thoracic and forelimb levels, where they respectively regulate the formation of CT and MMCl, and of forelimb LMC columnar identities (Dasen et al., 2003).

Retinoic acid (RA) signaling pathways play central roles in regulating generic MN differentiation and in the specification and maintenance of forelimb LMC identity and lateral LMC divisional character (Solomin et al., 1998; Sockanathan and Jessell, 1998; Diez del Corrall et al., 2003; Novitch et al., 2003; Sockanathan et al., 2003; Vermot et al., 2005; Ji et al., 2006). RA signals directly regulate gene transcription through the activity of nuclear receptors; however, the factors that mediate RA responses to regulate postmitotic MN identity remain unknown (Maden, 2002). One candidate is Nlz2/Nolz1, a
member of the Noc, Elbow and Tlp-1 (NET) family of atypical zinc-finger-containing transcriptional regulators (Nakamura et al., 2004; Runko and Sagerstrom, 2004; Hoyle et al., 2004). Both zebrafish Nlz2 (also known as Znfs503 – ZFIN) and its mouse homolog Nolz1 (also known as Zfp503 – Mouse Genome Informatics) are expressed at known sites of RA synthesis and function in the brain (Runko and Sagerstrom, 2004; Hoyle et al., 2004; Chang et al., 2004). Nlz2 is found in the hindbrain, where it is required for the specification of rhombomeric identity, a process known to be dependent upon RA signaling, whereas Nolz1 is coincidently expressed with Raldh3 in the lateral ganglionic eminence (LGE), a region of the telencephalon that gives rise to the striatum. Nolz1 expression in cell culture is RA-inducible, a finding supported by the identification of a functional direct repeat 5 retinoic acid response element (DR5-RARE) upstream of the Nolz1 translational start site (Chang et al., 2004). Based on these collective observations, we examined if and how Nlz2/Nolz1 mediates RA-dependent events regulating postmitotic MN development.

Here, we show that Nolz1 regulates the diversification of Lim3+ motor columns from Lim3+ MNs, consolidates their formation and controls the specification and diversification of LMC MNs by regulating Hox and LIM-HD protein expression. These divergent functions of Nolz1 require distinct repressor activities that depend in part on modulatory functions of the Gro-TLE protein, Grg5 (Fisher and Caudy, 1998). This study thus reveals that RA signals regulate the progressive specification of postmitotic MN columnar and divisional identity by inducing a single pivotal molecule that executes multiple fate-specification transcriptional programs through the assembly of functionally distinct repressor complexes.

MATERIALS AND METHODS

In ovo electroproportion and luciferase transcriptional assays

All cDNAs were cloned into pCAGGS or a 3 kb Hb9 promoter-based vector (Lee et al., 2004) for in ovo electroproportion (William et al., 2003). For siRNA experiments, Nolz1 and Grg5 siRNA duplexes were electroporated as previously described (Rao et al., 2004). Target sequences are as follows: Nolz1, 5’-AAAGTGTCTGGAGTAAAA-3’ and 5’-AATTTGCTATTTGGTTATCGA-3’; Grg5, 5’-GCAGAATAACGGAGATGCA-3’ and 5’-TGGCGAAAGGGACAGAAA-3’. DsRed siRNA sequences are as published (Rao et al., 2004). Luciferase assays were carried out as described (Novitch et al., 2001; Nishihara et al., 2003).

Immunohistochemistry and in situ hybridization

Chicken embryos were prepared for immunohistochemistry and in situ hybridization as described (Sockanathan and Jessell, 1998). Primary antibodies used were as follows. Polyclonal rabbit antisera against chick Nolz1 was generated by immunizing rabbits with a GST fusion of the first 373 amino acids of Nolz1 (Covance). Polyclonal antibodies were used as follows. DAPI (Sigma)-bound beads overnight at 4°C under constant rotation. After extensive washing, the precipitated proteins were analyzed by SDS-PAGE and western blot using anti-HA antibodies (Santa Cruz).

Yeast two-hybrid screen

Yeast two-hybrid screens were carried out using the Matchmaker Two-Hybrid System (Clontech). The library was prepared from RNA generated from St 23 chick spinal cords and cotransformed into yeast strain AH109 with the GAL4 activation domain plasmid pGADT7-Rec and bait construct pGBK7-Nolz1. Only colonies growing on SD/-Ade/-His/-Leu/-Trp/X-α-Gal selective medium were picked for further analysis.

Co-immunoprecipitation

Flag or HA epitope tags were fused to the N-terminus of Nolz1 or Grg5 and cloned into pCAGGS or pCS2 vectors. Transiently transfected HEK293T cells were harvested and homogenized in lysis buffer using standard procedures. Lysates were precleared by incubation with GammaBind G Sepharose beads (GE Healthcare) followed by centrifugation, and were mixed with anti-Flag M2 (Sigma)-bound beads overnight at 4°C under constant rotation. After extensive washing, the precipitated proteins were analyzed by SDS-PAGE and western blot using anti-HA antibodies (Santa Cruz).

Reverse northern blot analysis

Two different chick Nozl EST clones (University of Delaware Chick EST Database) were electrophoresed in agarose gels and blotted according to standard procedures. Filters were probed with cDNAs derived from Hamburger Hamilton St 19 brachial chick neural explants grown for 18 hours in the absence or presence of retinol. Retinol was used to take advantage of the endogenous expression of the retinoid synthetic enzyme Raldh2, which is expressed in brachial MNs. Retinol is metabolized within the explants to substrates for Raldh2, which subsequently catalyzes the formation of endogenous retinoid metabolites (Sockanathan and Jessell, 1998). RNA from 100 explants was isolated using Trizol (Gibco BRL). cDNAs were generated and amplified using the Marathon cDNA PCR Amplification Kit (Stratagene).

RESULTS

Nozl1 expression is induced by RA in the developing spinal cord

We cloned the chick homolog of Nozl1 by RT-PCR using RNA prepared from dissected Hamburger Hamilton stage (St) 21-23 chick spinal cords. The predicted Nozl1 protein shares similar domains with zebrafish, mouse and human Nozl1 homologs, and is 74% and 78% identical to zebrafish and mouse Nozl1, respectively (Fig. 1A) (Nakamura et al., 2004). To test whether RA signals can regulate Nozl1 expression in the chick spinal cord, we performed reverse northerns. Densitometry analysis showed a 5-fold increase of Nozl1 expression in explants exposed to retinol (Fig. 1B). Consistent with a role for RA signals in regulating Nozl1 expression, electroporation of constructs expressing RAR-403, a potent dominant-negative retinoid receptor that disrupts RA signaling, caused a decrease in Nozl1 expression in the dorsal spinal cord (Fig. 1C,D) (Novitch et al., 2003; Sockanathan et al., 2003).

Nozl1 is expressed in subsets of developing spinal MNs

To examine the spatial and temporal distribution of Nozl1 in the chick spinal cord, we carried out a developmental time course of Nozl1 mRNA expression. Prior to St 24, MNs at all rostral-caudal levels express Nozl1; however, from St 25 onwards, Nozl1 is restricted to a subset of laterally located forelimb and lumbar MNs (Fig. 2A-H; data not shown). To determine whether Nozl1 expression correlates with the development of specific motor columns, we examined the expression of Nozl1 protein in relation to molecular markers that define the major spinal motor columns and divisions (Tsuchida et al., 1994). All newly differentiated spinal MNs coexpress Isl1 and Lim3. Lim3 expression is maintained in MMCm neurons spanning all axial levels, but it is rapidly downregulated in LMC MNs at limb levels, and in CT and MMCi neurons at thoracic regions of the spinal
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To define the requirement for Nolz1 in MN development, we ablated Nolz1 expression using short interfering RNAs (siRNAs). Oligonucleotides (21 bp) designed against the Nolz1 ORF and 3′ UTR were electroporated into chick spinal cords at St 12, prior to the onset of Nolz1 expression and MN generation (Rao et al., 2004). Plasmids expressing GFP were coelectroporated to mark the transfected side (Fig. 3A,F). Electroporated embryos showed a ~50% reduction of Nolz1 mRNA and protein when compared with the contralateral non-electroporated side, or when electroporated with unrelated siRNAs (Fig. 3A,B,F,G; data not shown; see Fig. S1 in the supplementary material). However, no changes in the number of Lim3+ MMCm MNs at either forelimb or thoracic levels of the spinal cord were observed, consistent with the lack of Nolz1 expression in MMCm MNs (Fig. 3C,E,H,J). Lim3+ MNs give rise to medial and lateral divisions of the LMC at forelimb levels, while at thoracic regions they generate MMCl and CT motor columns (Tsuchida et al., 1994; Jessell, 2000). At forelimb levels, knockdown of Nolz1 resulted in a 30% loss of LMCm neurons and a 70% loss of LMC cells, as compared with the contralateral non-electroporated side or when control unrelated siRNAs were electroporated, while at thoracic levels there was a reduction of CT and MMCl MNs by 50% and 40%, respectively (Fig. 3D,E,I,J). The percentage decrease in CT and MMCl MNs correlates well with the extent of Nolz1 knockdown; at forelimb levels, however, LMCm generation appeared less affected by the knockdown of Nolz1 than neurons of the LMC subtype. This difference might reflect the relative distribution of Nolz1 in LMC neurons, in that all LMC neurons, but only a subset of LMCm neurons, normally express Nolz1 (Fig. 2M). These results suggest that Nolz1 is specifically required for the formation of Lim3+ motor columns, consistent with its early expression in Lim3+ MN populations in the spinal cord.

Grg5 interacts with Nolz1

Nolz1 contains a single, atypical zinc finger and is thus unlikely to bind DNA directly; however, NET proteins can activate or repress transcription through the formation of multimeric complexes (Nakamura et al., 2004; Runko and Sagerstrom, 2004). We reasoned that it would be more informative to evaluate the role of Nolz1 in Lim3+ motor column development by investigating its function in the absence and presence of its associated proteins. To identify proteins that interact with Nolz1, we performed a yeast two-hybrid screen using full-length Nolz1 fused to the GAL4 DNA-binding domain as bait, together with a cDNA library generated from St 23 ventral chick spinal cords fused to the GAL4 activation domain. We identified one clone that overlapped with Nolz1 expression in chick spinal MNs (Fig. 4A). This clone corresponded to Grg5, a member of the Gro-TLE family of co-repressors that functions as a ‘derepressor’ of other Groucho (Grg) proteins owing to its inability to complex with histone deacetylases (HDACs) (Fishier and Caudy, 1998; Brantjes et al., 2001). The overlap of Grg5 and Nolz1 expression in the ventral spinal cord suggests that Grg5 is a viable candidate for mediating Nolz1 function in spinal MNs. We confirmed that Grg5 interacts with Nolz1 by co-immunoprecipitation (co-IP) assays using extracts prepared from HEK293T cells transfected with tagged versions of Nolz1 and Grg5 (Fig. 4B,C). To identify the Grg5 interaction sites on Nolz1, we generated a series of deletions within Nolz1 and examined which of these deletions lacked the ability to interact with Grg5 by co-IP (Fig. 4C). Deletion of a putative Grg consensus binding site (FKPY, Δ161-170) within Nolz1 did not abolish the interaction between Nolz1 and Grg5 (Fig. 4B,C). Instead, deletion of the C-terminal 22 amino acids significantly reduced Nolz1-Grg5 complex formation (Nolz1ΔC22) (Fig. 4B,C). Together, these results show that Grg5 can specifically interact with the C-terminus of Nolz1.

Nolz1 repressor function is modulated by Grg5 activity

To investigate the transcriptional properties of Nolz1, we assayed its function using an in vitro reporter-based assay. Full-length Nolz1 was fused to the GAL4 DNA-binding domain (GalNolz1) and cotransfected into COS-7 cells with a reporter plasmid containing
GAL4 DNA-binding sequences cloned upstream of a basal E1B promoter and the luciferase reporter gene (GAL4x5-E1b-luciferase) (Novitch et al., 2001). Transfection of GAL4x5-E1b-luciferase into COS-7 cells generated basal transcriptional activity that was repressed ~11-fold by GalNolz1 (Fig. 4D). Cotransfection of Grg5 expression plasmids caused a reproducible decrease in Nolz1-dependent repressor activity (Fig. 4D). Consistent with this function, transfection of Nolz1ΔC22 fused to the GAL4 DNA-binding domain resulted in a marked increase of Nolz1 repressor activity (GalNolz1ΔC22; Fig. 4D). To determine the transcriptional properties of Nolz1 and Grg5 in vivo, we repeated these experiments using extracts prepared from dissected chick spinal cords electroporated with the same series of expression plasmids (Nishihara et al., 2003). However, in this case we utilized MH100, which contains GAL4 DNA-binding sequences upstream of a minimal thymidine kinase promoter and the luciferase reporter gene (Muhr et al., 2001). Similar to the assays carried out in COS-7 cells, GalNolz1 repressed basal MH100 expression (Fig. 4E). But, coelectroporation of Grg5 caused a marked ‘derepression’ of GalNolz1-dependent transcriptional activity (Fig. 4E). GalNolz1ΔC22 showed increased repressor activity compared with GalNolz1, consistent with the ability of Grg5 to modulate Nolz1 repressor function.

Taken together, these data suggest that Nolz1 functions as a repressor molecule, and that Grg5 interactions with Nolz1 serve to modulate Nolz1 repressor function. Mechanistically, Grg5 is known to modulate Grg co-repressor activity, raising the possibility that classical Grg proteins mediate Nolz1-dependent transcriptional repression (Fisher and Caudy, 1998; Brantjes et al., 2001; Muhr et al., 2001).

**Nolz1 overexpression leads to the downregulation of Lim3 and to MN loss**

To investigate how Nolz1 regulates the formation of Lim3 MNs, we overexpressed Nolz1 under the control of a 3 kb promoter derived from the mouse Hb9 (Mnx1 – Mouse Genome Informatics) gene, which initiates expression in newly differentiating MNs (HB9-Nolz1) (Lee et al., 2004). Embryos electroporated with plasmids expressing GFP under the Hb9 promoter (HB9-GFP) expressed Lim3 in ~80% of electroporated MNs (Fig. 5D,G). By contrast, less than 5% of electroporated MNs expressing Nolz1 were Lim3+, suggesting that Nolz1 is sufficient to cause the downregulation of Lim3 expression (Fig. 5A-C,G). Although the Nolz1-expressing cells had initiated MN differentiation, which was evident by the induction of Nolz1 from the Hb9 promoter, they lacked...
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Islet1/2 and HB9 expression (Fig. 5B,C; data not shown). Furthermore, a number of the cells were labeled by TUNEL, indicating that they had initiated apoptosis (data not shown).

To examine whether the ability of Nolz1 to downregulate Lim3 expression utilizes endogenous Grg5 activity, we electroporated HB9 promoter constructs driving Nolz1ΔC22 expression into chick spinal cords. Quantification of the percentage of Lim3+ cells expressing Nolz1 and Nolz1ΔC22 showed that removal of the Grg5-interaction domain resulted in a minor increase in the number of Nolz1 and Lim3 coexpressing cells, but did not restore Lim3 coexpression to control levels (Fig. 5E,G). This observation suggests that the Nolz1-dependent repression of Lim3 expression does not require its interaction with Grg5. In vivo transcriptional assays indicated that Nolz1 functions as a transcriptional repressor (Fig. 5E). To confirm that Nolz1 downregulates Lim3 through repressor activity and not through activator functions derived from interactions with unidentified binding partners in vivo, we fused the VP16 activator domain to the C-terminus of full-length Nolz1 (Nolz1-VP16). We verified that Nolz1-VP16 functions as an activator using in vitro and in vivo transcriptional reporter assays (Fig. 4D,E). HB9-Nolz1-VP16 constructs failed to downregulate Lim3 expression in electroporated chick spinal cords (Fig. 5F,G). These collective observations indicate that Nolz1 repressor activity downregulates Lim3 expression in spinal MNs and that this repressor function is not dependent on its association with Grg5. These findings suggest that Nolz1 repressor function plays crucial roles in the early segregation of Lim3+ and Lim3− MN populations by its ability to suppress Lim3 expression in postmitotic MNs.

**Nolz1 and Grg5 coexpression induces ectopic MNs**

The loss of Lim3− motor columns in Nolz1 knockout embryos is unlikely to result from Lim3 derepression, as forced expression of Lim3 in MNs is not detrimental to MN survival (Sharma et al., 2000; William et al., 2003). These observations suggest additional roles for Nolz1 in regulating Lim3− motor column development that are distinct from its function in downregulating Lim3 expression. MN generation and maintenance are orchestrated by key HD transcription factors that include MNR2, Lim3, Islet1 and HB9 (Pfaff et al., 1996; Tanabe et al., 1998; Sharma et al., 1998; Arber et al., 1999; Thaler et al., 1999; Thaler et al., 2002; William et al., 2003; Thaler et al., 2002). We reasoned that Nolz1 might function to maintain the expression of these key transcription factors in prospective Lim3− MNs. If so, then Nolz1 should be capable of regulating their transcription, and thus might induce their expression in gain-of-function assays. We tested this possibility by expressing Nolz1 in the dorsal spinal cord by in ovo electroporation of Nolz1 expression constructs driven by the chick β-actin promoter (pCAGGS-Nolz1).

Electroporation of Nolz1 alone did not lead to the ectopic expression of MN factors dorsally (see Fig. S2 in the supplementary material). By contrast, coexpression of Nolz1 and Grg5 induced the cell-autonomous expression of Lim3, Islet1 and HB9 (Fig. 6A-D). A small number of cells expressed Islet2 days after electroporation, reflecting the temporal profile of Islet2 expression in mature MNs (data not shown) (Tsuda et al., 1994). Nolz1 and Grg5 coexpression failed to induce MNR2, consistent with the restriction of endogenous Nolz1 and Grg5 expression to postmitotic MNs (Fig. 4A, Fig. 6C). Consistent with previous reports, Grg5 overexpression alone did not lead to ectopic MNR2, HB9, Islet1, Islet2 or Lim3 expression (see Fig. S2 in the supplementary material) (Muhr et al., 2001). Coelectroporation of Nolz1ΔC22 and Grg5 did not elicit the ectopic expression of MN HD proteins, suggesting that Nolz1-Grg5 complex formation is necessary for these events (Fig. 6E,F; data not shown). Furthermore, Nolz1-VP16 expression did not induce Lim3, HB9, Islet1 or Islet2, consistent with the inability of Nolz1-Grg5 complexes to implement MN determinant expression through repressor activities (Fig. 6G,H).

HB9 expression is directly induced by Islet1-Lim3 multimers, and HB9 can upregulate the expression of Islet1 and Lim3 (Arber et al., 1999; Thaler et al., 1999; Thaler et al., 2002; William et al., 2003). To examine the hierarchy of MN determinant expression induced by Nolz1-Grg5, we compared the expression profiles of these markers relative to each other. Nolz1 and Grg5 coexpression induced many HB9+ cells in the absence of Lim3 or Islet1, suggesting that Nolz1-Grg5 provides an alternative pathway to upregulate HB9 expression that does not require Islet1 and Lim3 (Fig. 6B-D). Furthermore, 35% of ectopic Islet1+ cells and 62% of ectopic Lim3+ cells lacked detectable HB9 expression (Fig. 6B,D; n=126 Islet1+ cells, n=42...
Lim3+ cells analyzed). Taken together, these results suggest that Nolz1-Grg5 complexes can independently upregulate the expression of HB9, Islet1 and Lim3.

Dorsal cells that express the MN HD transcription factors when Nolz1 and Grg5 are coexpressed consistently occupied lateral positions, suggesting that the induction of these HD proteins occurred in the context of normal neurogenesis (Fig. 6A-D). Ectopic cells expressed choline acetyltransferase (ChAT), the enzyme responsible for acetylcholine synthesis, and the MN surface marker SC1, consistent with their adopting MN fates (Fig. 6I,J) (Tanabe et al., 1998). In support of their MN identity, none of the ectopic Islet1+ cells expressed the V0 or V1 interneuron markers Evx1/2 or En1 (Fig. 6L,M) (Pierani et al., 1999). However, 1-2 ectopic cells expressed the V2 marker Chx10, probably owing to the absence of HB9, which is required to repress V2-specific fates in MNs (Fig. 6K) (Arber et al., 1999; Thaler et al., 1999). To determine whether Nolz1-Grg5-induced MNs adopt a Lim3– columnar identity, we examined electroporated embryos 48 hours after coelectroporation of Nolz1 and Grg5. We found that 98% of ectopic Islet1+ MNs lacked Lim3 expression (n=120 Islet1+ cells analyzed).

Thus, Nolz1/Grg5 repressor activity can initiate MN differentiation programs downstream of MNR2, resulting in MNs with a Lim3– expression profile. The transient expression of Lim3 mediated by Nolz1 and Grg5 coexpression reflects the profile of Nolz1 expression in developing spinal MNs, where Nolz1 is coexpressed with Lim3 in a lateral band of newly generated MNs, prior to its rapid segregation with Lim3– spinal MNs (Fig. 2S).

Grg5 is required for Lim3– motor column development

Our gain-of-function studies suggest that Grg5 plays central roles in regulating the development and formation of Lim3– motor columns. To examine whether Grg5 is necessary for Lim3– motor column development, we electroporated 21 bp siRNAs designed against the...
Grg5 ORF into chick embryonic spinal cords at St 14, and analyzed electroporated embryos at St 23/24 (Rao et al., 2004). Grg5 mRNA levels were efficiently decreased in siRNA-treated embryos; however, owing to the lack of available antibodies against chick Grg5, we were unable to assess the efficiency of reducing Grg5 protein (Fig. 7A,B). Nevertheless, we evaluated embryos electroporated with Grg5 siRNAs for the formation of Lim3+ MMCm MNs and Lim3– LMCl and LMCm MNs at forelimb levels of the spinal cord. No changes in the number of Lim3+ MMCm MNs were detected in embryos that showed efficient knockdown of Grg5 mRNA. By contrast, we consistently detected a 30% reduction in the number of Lim3– LMCl and LMCm MNs, as compared with the non-electroporated contralateral side, or when control siRNAs were electroporated (Fig. 7C-E). These results indicate that Grg5 is required for the formation of Lim3– MNs, and further supports our model that Nolz1-Grg5 complexes regulate the generation of Lim3– motor columns in the spinal cord.

**Nolz1 interacts with Grg5 to induce Hoxc6 expression in the spinal cord**

The retention of Nolz1 expression in LMC neurons suggests that Nolz1 might be required for the development of LMC motor columns. Since RA signaling pathways are necessary for specifying forelimb LMC identity, we examined whether Nolz1 mediates the transcriptional programs required for triggering forelimb LMC specification (Sockanathan et al., 2003). We first examined the onset of Nolz1 expression in prospective forelimb LMC neurons in relation to that of Hoxc6, a key postmitotic determinant of forelimb LMC identity (Dasen et al., 2003). At St 17, we detected many Nolz1+ MNs at forelimb levels, but very few, weakly expressing Hoxc6+ cells (Fig. 8A,B). By St 19, many Nolz1+ MNs were distributed along the mediolateral axis, a lateral subset of which coexpressed Hoxc6 (Fig. 8C). These data indicate that Nolz1 expression initiates prior to the induction of Hoxc6 in prospective forelimb LMC neurons.

To test whether Nolz1 or Grg5 is capable of inducing Hoxc6 expression, we electroporated pCAGGS-Nolz1 into chick spinal cords and analyzed dorsal thoracic spinal cords at St 24, when Hoxc6 is normally absent. Expression of Nolz1 or Grg5 alone failed to elicit Hoxc6 expression (see Fig. S2 in the supplementary material). By contrast, coelectroporation of Nolz1 and Grg5 induced many dorsal Hoxc6+ cells, and upregulated Hoxc6 expression in a small number of thoracic MNs (Fig. 8D,E,H,I). Notably, dorsal Hoxc6 expression rarely coincided with cells coexpressing GrG5 and Nolz1, implying that Nolz1/Grg5-dependent induction of Hoxc6 occurs non-cell-autonomously. Grg5 and Nolz1AC22 coexpression did not induce Hoxc6, suggesting that Nolz1 and Grg5 interact to upregulate Hoxc6 expression (Fig. 8F; see Fig. S2 in the supplementary material). Electroporation of Nolz1-VP16 failed to elicit Hoxc6 expression (Fig. 8G). Taken together, these findings suggest that Nolz1-Grg5 complexes induce Hoxc6 expression.
through transcriptional repressor functions, and provide a mechanism that links Hoxc6-dependent specification of forelimb LMC identity with RA signaling pathways.

**Grg5 and Nolz1 cooperate to induce Lim1 expression in ectopic MNs**

At later stages of development, Nolz1 is localized to LMCi neurons, suggesting potential roles for Nolz1 in regulating their development (Fig. 2L,M). RA signaling pathways induce LMCi identity in particular by upregulating Lim1, which directs the dorsal trajectories of LMCi axons in the limb (Sockanathan and Jessell, 1998; Kania et al., 2000; Vermot et al., 2005; Ji et al., 2006). Nolz1 and Grg5 coexpression induces Lim3– MNs in the dorsal spinal cord (Fig. 6). To examine whether a subset of these neurons acquires lateral LMC fates, we examined embryos 2 days after coelectroporation with Nolz1 and Grg5. LMCi neurons are distinguished by their coexpression of Islet2 and Lim1, and by their downregulation of Islet1 (Tsuchida et al., 1994). Sixty-three percent of ectopic Islet1/2+ MNs coexpressed Lim1 when embryos were coelectroporated with Nolz1 and Grg5. LMCi neurons are distinguished by their coexpression of Islet2 and Lim1, and by their downregulation of Islet1 (Tsuchida et al., 1994). Sixty-three percent of ectopic Islet1/2+ MNs coexpressed Lim1 when embryos were coelectroporated with Nolz1 and Grg5 (Fig. 8J,K; n = 374 Islet1/2+ cells). However, staining with an Islet2-specific antibody showed that the majority of these cells did not express Islet2, demonstrating that they had induced Lim1 expression but failed to downregulate Islet1 (Fig. 8K,L). Ectopic Islet1/2+ cells did not express interneuron markers such as Evx1/2, En1 and Chx10, but were ChAT+ and SC1+ (Fig. 6I-M). These observations suggest that Nolz1 and Grg5 coexpression results in Islet1+ Islet2– MNs that had initiated a partial LMCi specification program. Hoxc6 triggers a temporal sequence of LMC formation, whereby Raldh2-derived RA induces LMCi subtype identity (Sockanathan and Jessell, 1998; Dasen et al., 2003; Vermot et al., 2005). Nolz1 and Grg5 coexpression does not induce dorsal Raldh2 expression, suggesting that Lim1 induction in ectopic MNs arises independently of Raldh2 function (data not shown). Moreover, many dorsal Lim1+ MNs did not coexpress Hox6 and overexpression of Hoxc6 in the dorsal spinal cord did not induce Islet1/2+ Lim1+ MNs (Fig. 8M; see Fig. S3 in the supplementary material). Taken together, these results argue that Nolz1 can induce MNs that are capable of initiating partial programs of LMCi specification downstream of Hoxc6 and Raldh2 activity.
DISCUSSION
Here, we show that RA signals induce Nolz1 expression in the chick ventral spinal cord, and that Nolz1 mediates novel and known RA-dependent events that specify columnar and divisional identities in postmitotic MNs (Fig. 8N). Our results suggest a model in which paraxial mesoderm-derived RA signals induce Nolz1 in postmitotic MNs, where it regulates the diversification and maintenance of Lim3+ MNs, prior to triggering forelimb LMC specification through Hoxc6 induction. At limb levels, RA synthesized in paraxial mesoderm and in MNs maintains Nolz1 expression, which acts to upregulate Lim1 expression in late-born prospective LMC1 neurons. We discuss below how Nolz1 regulates the specification, function and survival of postmitotic Lim3+ MNs in the context of known transcriptional hierarchies that orchestrate these events.

Nolz1 regulates the segmentation and development of Lim3+ MNs
We show here that Nolz1 is sufficient to downregulate Lim3 expression in postmitotic MNs by Grg5-independent repressor functions. Lim3 downregulation is an essential prerequisite for the acquisition of LMC, CT and MMCm columnar fates, as sustained Lim3 expression suppresses CT and LMC motor column formation, and causes MNs to adopt cell body settling positions, gene expression profiles and axonal projections characteristic of MMCm MNs (Sharma et al., 2000; William et al., 2003). Previous studies have shown that HB9 also functions to suppress Lim3 expression (Arber et al., 1999; Thaler et al., 1999). We find that Nolz1 can upregulate HB9 expression through a separate repressor activity involving association with Grg5. Thus, our studies support a model in which Nolz1 implements at least two pathways that converge to control the timely repression of Lim3 expression in prospective LMC, CT and MMCm MNs. These findings invoke crucial roles for Nolz1 in regulating the early segregation of Lim3+ motor columns from Lim3+ MMCm MNs.

Ablating Nolz1 causes the loss of Lim3+ MNs, suggesting that Nolz1 has additional functions in the development and survival of CT, MMC1 and LMC motor columns besides downregulating Lim3 expression. We find that Nolz1-Grg5 repressor complexes can induce terminally differentiated MNs with a Lim3+ molecular identity, through upregulating a series of key HD transcription factors. Since endogenous Nolz1 expression initiates in postmitotic MNs and not in progenitor cells, this finding is consistent with the model that Nolz1-Grg5 complexes implement HD-dependent transcriptional programs that are necessary for consolidating and/or maintaining the properties and survival of postmitotic Lim3+ MNs. Accordingly, Nolz1-Grg5 complexes are not sufficient to activate MNR2 expression, consistent with their role in MN consolidation or maintenance rather than induction (Tanabe et al., 1998). The involvement of Nolz1-Grg5 repressor complexes in this regard parallels the requirement for Nkx2-5 expression in prospective LMC1 differentiation programs during MN generation (Muhr et al., 2001). These observations suggest that continuous derepression strategies are required in progenitors and postmitotic MNs to generate and actively preserve the properties and survival of postmitotic MNs. The function of Nolz1-Grg5 complexes might differ between limb-level and thoracic motor columns. The prolonged expression of Nolz1 at limb levels suggests that Nolz1-Grg5 complexes play more sustained roles in the specification and maintenance of LMC neurons as compared with CT and MMC1 neurons, in which Nolz1 is downregulated after St 24. Indeed, prolonged retinoid receptor activation is detrimental to thoracic MN survival, suggesting that retinoid-dependent pathways operate transiently during thoracic motor column development (Sockanathan et al., 2003).

Nolz1 regulates forelimb LMC specification
Disrupting RA signals in prospective forelimb MNs causes putative LMC MNs to adopt thoracic identities, suggesting that RA signals have dual roles in specifying LMC fates and suppressing thoracic-specific columnar differentiation programs (Sockanathan et al., 2003). We show that Nolz1-Grg5 repressors induce Hoxc6, which can drive LMC-specific programs of differentiation and suppress thoracic columnar identity in limb-level MNs (Liu et al., 2001; Dasen et al., 2003). These findings provide a mechanism that links RA signaling with the specification of forelimb LMC identity through the Nolz1-dependent induction of Hoxc6 expression. How do Nolz1-Grg5 repressor complexes upregulate Hoxc6? Hoxc9 can repress Hoxc6 expression cell-autonomously in spinal MNs (Dasen et al., 2003). However, our results argue against the possibility that Nolz1 derepresses Hoxc6 expression through inhibition of Hoxc9, as Nolz1 functions non-cell-autonomously to upregulate Hoxc6. Instead, Nolz1 might induce Hoxc6 through the derepression of additional signaling pathways that remain to be identified.

A second question is how Nolz1-Grg5 complexes might regulate the restricted expression of Hoxc6 at forelimb levels, when Nolz1 and Grg5 are expressed at all axial levels at the time of Hoxc6 induction. One option is that the intermediary components that relay Nolz1-Grg5 effects are restricted primarily to spinal MNs at forelimb levels. In support of this idea, very few thoracic MNs induce Hoxc6 when Nolz1 is overexpressed, as compared with those in the dorsal spinal cord. Alternatively, the restricted expression of Hoxc6 by Nolz1-Grg5 activity might depend on the function of other Hox proteins. Hoxc9 is induced by FGFs and is expressed in thoracic progenitors and spinal MNs (Dasen et al., 2003). Thus, early Hoxc9 expression in progenitors and MNs could override the later inductive capabilities of Nolz1-Grg5 complexes, thereby repressing Hoxc6 at thoracic levels. This Hox-dependent mechanism could potentially operate at lumbar regions via Hox10 proteins, thus confining the ability of Nolz1-Grg5 complexes to induce Hoxc6 expression to forelimb levels of the spinal cord (Dasen et al., 2003; Wu et al., 2008).

Nolz1 induces Lim1 expression in prospective lateral LMC neurons
Our expression analyses and functional experiments collectively support a role for Nolz1 in lateral LMC neuronal development. Ectopic MNs induced by Nolz1-Grg5 complexes coexpress Lim1, a LIM-HD protein that partly defines LMC1 molecular identity and directs their dorsal axonal trajectory (Tsukida et al., 1994; Kania et al., 2000). However, the Lim1+ MNs induced by Nolz1-Grg5 complexes coexpress Islet1, which is normally downregulated in LMC1 neurons (Tsukida et al., 1994). These observations indicate that Nolz1-Grg5 complexes implement a subset of LMC1 differentiation pathways, specifically those regulating LMC1 axonal projections. They also imply that Lim1 induction can be uncoupled from the downregulation of Islet1, suggesting that independent pathways function to execute these previously linked events. We show that the Lim1+ MNs induced by Nolz1-Grg5 complexes are generated independently of prior Raldh2 and Hoxc6 function. These results support a mechanism whereby Nolz1-Grg5 repressors function downstream of forelimb LMC specification and Raldh2 activity, to induce Lim1 expression in prospective LMC1 MNs. Nolz1 is expressed in LMC1 neurons until at least St 29, suggesting that Nolz1-Grg5 complexes might function to maintain Lim1 expression in LMC1 cells.
In conclusion, our study suggests that RA signals specify multiple events governing MN subtype identity through the induction of Nolz1. Nolz1 exhibits strong transcriptional repressor activity, and this repressor function is modulated by interactions with Grg5. Importantly, Nolz1 repressor complexes are functionally distinct, suggesting that their individual components differ. Notably, our observation that Grg5 modulates Nolz1 repressor activity invokes the possibility that graded Nolz1 repressor activities are a crucial component in implementing selective transcriptional pathways that direct different aspects of MN development. The induction of a single key factor with functional flexibility, such as Nolz1, circumvents the need to generate large numbers of proteins that individually execute separate developmental pathways, and provides a useful and effective strategy to regulate diverse events in neuronal development.

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Supplementary material
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References