Corl1, a Novel Neuronal Lineage-specific Transcriptional Corepressor for the Homeodomain Transcription Factor Lbx1*

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During development, neuronal identity is determined by a combination of numerous transcription factors. However, the mechanisms of synergistic action of these factors in transcriptional regulation and subsequent cell fate specification are largely unknown. In this study, we identified a novel gene, Corl1, encoding a nuclear protein with homology to the Ski oncoprotein. Corl1 was highly selectively expressed in the central nervous system (CNS). In the embryonic CNS, Corl1 was expressed in a certain subset of postmitotic neurons generated posterior to the midbrain-hindbrain border. In the developing spinal cord, Corl1 was selectively expressed in the dorsal horn interneurons where a homeodomain transcription factor, Lbx1, is required for proper specification. Corl1 was localized in a nuclear dot-like structure and interacted with general transcriptional corepressors. In addition, Corl1 showed transcriptional repression activity in the GAL4-fusion system, indicating its involvement in the regulation of transcriptional repression. Furthermore, Corl1 interacted with Lbx1 and cooperatively repressed transcription, suggesting that it acts as a transcriptional corepressor for Lbx1 in regulating cell fate determination in the dorsal spinal cord. Corl1 corepressor activity did not depend on Gro/TLE activity, and Gro/TLE also functioned as a corepressor for Lbx1. Thus, Lbx1 can select two independent partners, Corl1 and Gro/TLE, as corepressors. Identification of a novel transcriptional corepressor with neuronal subtype-restricted expression might provide insights into the mechanisms of cell fate determination in neurons.

Neuronal patterning is regulated by extrinsic inductive signals and downstream intrinsic signals mediated by transcription factors (1–4). In early development, neural progenitor cells are specified by secreted molecules such as sonic hedgehog (Shh), bone morphogenetic proteins (BMPs),1 Wnts and fibroblast growth factors, which regulate the expression pattern of downstream transcription factors including bHLH and homeodomain factors. These signals regulate sets of transcription factors at different concentration thresholds. Thus, the neural tube is regionalized into distinct progenitor domains expressing different sets of transcription factors along the anteroposterior and dorsoventral axes depending on distance from the organizing centers expressing secreted factors. As development proceeds, different classes of postmitotic neurons emerge from the individual progenitor domains during neurogenesis.

Accumulating evidence has elucidated the mechanism of development in several central nervous system (CNS) regions. For example, in the ventral spinal cord, the gradient of Shh, which is secreted from the organizer regions, notochord and floor plate, is established in the ventral half of the neural tube during early development (2, 5). Graded Shh repressed expression of the class I homeodomain transcription factors, and instead induced the class II transcription factors (6). Cross-inhibitory interactions between complementary pairs of class I and class II factors establish a sharp boundary for each progenitor domain (6). Evidences obtained by ectopic expression and loss-of-function experiments revealed that these transcription factors specify the cell fate of neural progenitors and derived neurons (6–12). In the dorsal spinal cord, similar extrinsic and intrinsic signaling mechanisms have been revealed (1, 13). It has also been shown that BMPs- and TGFβ-related factors are secreted from the roof plate and specify dorsal neurons in a concentration-dependent manner (14–17). Distinct classes of proneural bHLH transcription factors, which can constructively induce the different types of dorsal neurons, are expressed in individual dorsal progenitor domains through cross-repressive regulation (18, 19). Thus, in both ventral and dorsal neural tubes, specification of neural progenitors by secreted signaling molecules and subsequent cross-repressive interactions between downstream transcription factors is a common mechanism for producing different classes of neurons.

Expression of most transcription factors that specify progenitor cell fate is extinguished after exit from the cell cycle (1). Instead, distinct sets of transcription factors are induced postmitotically, and these factors determine the identity of each neuron. These postmitotic factors have been shown to be able to re-specify neuronal identity by ectopic expression, but in many cases these activities are cellular context-dependent (20–22). Thus, these postmitotically expressed transcription factors might function in a combinatorial manner with other transcription factors to specify neuronal cell fate. In the case of spinal cord motor neurons, two LIM homeodomain transcription factors, Ial1 and Lhx3, have been shown to form a complex containing a common LIM domain-interacting adaptor protein, NLI, when determining cell fate (22). In addition, this complex acts cooperatively with neurogenic bHLH factors, Ngn2 or NeuroM, to synchronously regulate neurogenesis and subtype specification (23). Thus, understanding the cooperation among transcription factors is important in unmasking the mechanism of neuronal cell fate specification. However, although many factors are reportedly involved in cell fate specification,
in most cases, the cooperation among these transcription factors is not yet understood. Thus, the precise mechanism of cell fate specification by transcription factors remains mostly unknown.

Most homeodomain transcription factors expressed in ventral spinal cord progenitors contain the EH1 motif, which recruits Gro/TLE family transcriptional corepressors, and act as transcriptional repressors in specifying progenitor identity (24). Thus, patterning of the ventral neural tube seems to be achieved by the derepression strategy (5, 24). In addition, some postmitotically expressed homeodomain transcription factors have a putative EH1 motif (24) and have been shown to repress the expression of transcription factors, which are normally expressed in other types of neurons, in specifying neuronal identity (25–28). Thus, the derepression mechanism might also be involved in postmitotic specification events. However, the mode of action of these transcriptional repressors and molecular mechanism of synergy among these factors remain largely unknown.

In the dorsal spinal cord, distinct interneuron populations are generated from the same progenitor domains at different developmental stages (1). During the early phase of neurogenesis (E10.5–E11.5), six classes of interneurons (d1-d6) emerge along the dorsoventral axis. At later staged (E12-E13.5), distinct populations (dILA and dILB) are generated from a common progenitor domain. A number of transcription factors that are selectively expressed in particular subsets of dorsal interneurons have been identified (1). One of these, a homeodomain transcription factor, Lbx1, is specifically expressed during early and late phases of dorsal neurogenesis, and gene-targeting experiments revealed that Lbx1 is required for proper specification of these populations (26, 28). Moreover, ectopic expression of Lbx1 repressed the expression of transcription factors such as Isl1 and LIF2A/B, which mark other classes of dorsal interneurons (28). Thus, it has been suggested that Lbx1 acts as a transcriptional repressor in specifying cell fate by blocking differentiation into other classes of neurons. However, the precise mechanism of transcriptional repression and cell fate specification by Lbx1 has not been clarified.

In this study, we identified a novel transcriptional corepressor, Corl1 (Corl: Corepressor for Lbx1). Corl1 was selectively expressed in subsets of dorsal horn interneurons in the developing spinal cord with Lbx1, which is required for proper specification. Corl1 showed a corepressor activity through binding to Lbx1, suggesting that Corl1 regulates cell fate determination in the dorsal spinal cord with Lbx1. Here, we discuss the cell fate determination strategy in the developing CNS by a combination of DNA binding factors and transcriptional corepressors with neuronal subtype-restricted expression.

**Experimental Procedures**

Isolation of Corl1—The mesencephalon region was dissected from an E12.5 mouse embryo and divided into two portions, the ventral and dorsal regions. Subtractive PCR was performed as described previously (31) using the ventral region as a tester and dorsal region as a driver. After four rounds of subtractive hybridization, the amplified cDNA fragments were cloned and sequenced. Full-length cDNAs were screened from an E12.5 mouse brain cDNA library. The nucleotide sequence of the Corl1 cDNA was deposited in the DDBJ/EMBL/GenBank™ data base under accession number AB185113.

RT-PCR—RT-PCR was performed essentially as described previously (32). ExTaq polymerase (TAKARA) was used for amplification, which was carried out by denaturation at 94 °C for 30 s (2 min in the first cycle), annealing at 65 °C for 30 s and extension at 72 °C for 30 s (2 min in the last cycle). The numbers of cycles were 35 for Corl1 and 25 for G3PDH. The primer sequences were as follows: Corl1: 5’-AGT CAG AGA TCG CTA AGC TCT AC-3’/5’-AAG CGG TTG GAC TCT ACG ACC ACC TC-3’ and G3PDH: 5’-ACC ACC CCT TCA TTG ACC TCA ACT AC-3’/5’-CCA GTC GAC TCC AGC ACA TAC TCA GC-3’. Construction of Plasmids—The full-length cDNAs of Corl1, N-CoR, HIPK2, HDAC1, mSin3A, Grg1, CtBP, Lbx1, and PLZF were amplified by PCR using the following primer sets: Corl1: 5’-GAG GTG GAC ATG GCA TTG CTG TGT GGC CTG AG-3’/5’-GAG GTG GAC ATG CTA GCC CAG CAG CGG ATT GAA GC-3’; N-CoR: 5’-GAG GTG GAC ATG TCA ACT TCT CAG TAT CTT CCT ACC AA-3’/5’-GAG GGC GCT CAC GAG AAC TAC TCC ATT GCC TCA CTA TCA GAC GGT GTT C-3’; HIPK2: 5’-GCC GAA TTC ATC GGC TCA CAT GTG CAA GTT-3’/5’-GCC GGC GCT GCT ATG TAT TAA GGG TAT TGG TT-3’; HDAC1: 5’-GAG GTG GAC ATG CGG CCT AGC ACT CAG GGC ACC AAG AG-3’/5’-GAG GGC GCT CAC GAG AAC TAC TCC ATT GCC TCA CTA TCA GAC GGT GTT C-3’; HIPK2: 5’-GCC GAA TTC ATC GGC TCA CAT GTG CAA GTT-3’/5’-GCC GGC GCT GCT ATG TAT TAA GGG TAT TGG TT-3’; HDAC1: 5’-GAG GTG GAC ATG CGG CCT AGC ACT CAG GGC ACC AAG AG-3’/5’-GAG GGC GCT CAC GAG AAC TAC TCC ATT GCC TCA CTA TCA GAC GGT GTT C-3’; HIPK2: 5’-GCC GAA TTC ATC GGC TCA CAT GTG CAA GTT-3’/5’-GCC GGC GCT GCT ATG TAT TAA GGG TAT TGG TT-3’; HDAC1: 5’-GAG GTG GAC ATG CGG CCT AGC ACT CAG GGC ACC AAG AG-3’/5’-GAG GGC GCT CAC GAG AAC TAC TCC ATT GCC TCA CTA TCA GAC GGT GTT C-3’; HIPK2: 5’-GCC GAA TTC ATC GGC TCA CAT GTG CAA GTT-3’/5’-GCC GGC GCT GCT ATG TAT TAA GGG TAT TGG TT-3’; HDAC1: 5’-GAG GTG GAC ATG CGG CCT AGC ACT CAG GGC ACC AAG AG-3’/5’-GAG GGC GCT CAC GAG AAC TAC TCC ATT GCC TCA CTA TCA GAC GGT GTT C-3’; HIPK2: 5’-GCC GAA TTC ATC GGC TCA CAT GTG CAA GTT-3’/5’-GCC GGC GCT GCT ATG TAT TAA GGG TAT TGG TT-3’; HDAC1: 5’-GAG GTG GAC ATG CGG CCT AGC ACT CAG GGC ACC AAG AG-3’/5’-GAG GGC GCT CAC GAG AAC TAC TCC ATT GCC TCA CTA TCA GAC GGT GTT C-3’; HIPK2: 5’-GCC GAA TTC ATC GGC TCA CAT GTG CAA GTT-3’/5’-GCC GGC GCT GCT ATG TAT TAA GGG TAT TGG TT-3’; HDAC1: 5’-GAG GTG GAC ATG CGG CCT AGC ACT CAG GGC ACC AAG AG-3’/5’-GAG GGC GCT CAC GAG AAC TAC TCC ATT GCC TCA CTA TCA GAC GGT GTT C-3’; HIPK2: 5’-GCC GAA TTC ATC GGC TCA CAT GTG CAA GTT-3’/5’-GCC GGC GCT GCT ATG TAT TAA GGG TAT TGG TT-3’.
A schematic representation of the domain structures of Corls. The cysteine-rich, coiled-coil and Corl-homology (CH) domains are indicated. Amino acid alignment of the cysteine-rich domains of Corl and Ski/SnoN family members. Asterisks indicate conserved cysteine residues. Note that the cysteine-rich domains of Drosophila CG11093 and mouse Corls are highly conserved.
Corl1 expression was detected in the nucleus using an antibody directed against Corl1 and had a similar pattern to in situ hybridization (Fig. 2E), confirming the specificity of the signals in both in situ hybridization and immunohistochemistry, indicating that Corl1 is a nuclear protein.

**Corl1 Is Selectively Expressed in Subsets of Dorsal Interneurons in the Spinal Cord—**Next, we examined the expression profile of Corl1 during the course of neuronal differentiation. First, we compared expression of Corl1 and Pax7 as a marker for proliferating neural progenitors in the ventricular zone (VZ) of the dorsal spinal cord (28). As shown in Figs. 3A and 4A, Corl1-expressing cells were detected only outside the VZ and they did not coexpress Pax7. In addition, BrdU was not incorporated by the Corl1-positive cells (data not shown). In contrast, virtually all the Corl1-positive cells coexpressed a neuronal differentiation marker, βIII-tubulin (35) (Figs. 3B and 4B), indicating that Corl1 is specifically expressed in postmitotic neural precursor cells in the spinal cord. Similarly, in other CNS regions, such as the metencephalon and myelencephalon, Corl1 expression was observed only in the postmitotic neural precursors (data not shown). Since Corl1 was detected just outside of the Pax7-positive VZ region, Corl1 expression is induced immediately after exit from the cell cycle when cell fate is determined by postmitotic expression factors. Thus, these results suggest the possible role of Corl1 in cell fate determination.

As shown in Figs. 3A and 4A, Corl1-positive cells were mainly observed in the dorsal spinal cord. Some Corl1-positive cells were detected in the ventral region, but it is likely that they migrated from the dorsal progenitor domains, since Corl1 expression was not detected in the ventral newborn neurons located adjacent to the ventral VZ region and ventral Corl1-positive cells did not coexpress markers for ventral interneurons and motor neurons (Fig. 3D and data not shown). Therefore, to identify the neuronal identity of cells expressing Corl1, we compared the expression of Corl1 with that of markers for dorsal interneurons. In the dorsal spinal cord, two temporally distinct waves of neurogenesis generating different classes of interneurons have been reported (26, 28). In early stages (E10–11.5), six classes of dorsal interneurons are generated along the dorsoventral axis then as early neurogenesis proceeds two distinct interneurons emerge from the same progenitor domains in an intermingled manner (E12–13.5). We first examined the expression pattern of Corl1 in the dorsal spinal cord at E10.75. As shown in Fig. 3, C–E, Corl1-positive neurons were detected between regions where Isl1/2-positive dI3 and Lim1/2-positive dI6 interneurons were emerging, and neither dI3 nor dI6 neurons expressed Corl1. In contrast, the dorsal population of Corl1-positive neurons coexpressed Lim1/2, which marks dI2, dI4, and dI6 neurons, and ventral Corl1-positive populations coexpressed Brn3a, a dI1–3, and dI5 marker. Taken together, these results indicate that Corl1 is specifically expressed in dI4 and dI5 interneurons at an early phase of neurogenesis in the dorsal spinal cord (summarized in Fig. 4E).

Since Corl1 can be used as a marker to distinguish dI4 and dI6 neurons, which are not distinguishable by established markers (13), we analyzed the ventrally migrating populations. As shown in Fig. 3H, a subset of the ventral Corl1-positive neurons expressed Brn3a, confirming the earlier observation that some dI5 neurons migrated ventrally (28). In addition, some ventral Corl1-positive neurons coexpressed Lim1/2 (Fig. 3G), indicating that these Corl1-positive cells are a subset of dI4 neurons. These results suggest that, in addition to the dI5 and dI6 neurons, which reportedly migrate ventrally, subpopulations of dI4 neurons also migrate toward the ventral horn.

Next, we examined Corl1 expression at a later stage. As shown in Fig. 4A, Corl1 was expressed in newborn neurons lying near the VZ at E13.25, indicating that Corl1 is expressed in late type dorsal interneurons. As shown in Fig. 4B, the majority of neurons born at a late stage expressed Corl1. In addition, both Lim1/2-positive and Brn3a-positive cells coex-
pressed Corl1 (Fig. 4, C and D), indicating that Corl1 is expressed in both dILA and dILB interneurons (26, 28). Expression of Corl1 in the dorsal horn neurons persisted until E15.5 (data not shown). We therefore concluded that in the developing spinal cord Corl1 is selectively expressed in dI4 and dI5 interneurons born at an early stage, and dILA and dILB interneurons born at a later stage (summarized in Fig. 4, E). It should be noted that in all these classes of interneurons the homeodomain transcription factor Lbx1 is coexpressed with Corl1 (Fig. 3, D and F, and data not shown. see below).

Corl1 Is a Transcriptional Corepressor—To understand the function of Corl1, we first examined its subcellular localization. As mentioned above, Corl1 was localized in the nuclei in vivo (see Fig. 2E). Consistently, transfected FLAG-tagged Corl1 was localized in the nuclei of 293E cells (Fig. 5A, panel a). Furthermore, FLAG-Corl1 was concentrated in nuclear dot-like struc-
tures, where many transcriptional corepressors, including Ski, Dach1, N-CoR, and HIPK2, are localized (36–39). In addition, Corl1 was partially colocalized with N-CoR and HIPK2 in the nuclear dot-like structure. Corl1 was partially colocalized with N-CoR and HIPK2 in the nuclear dot-like structure. B, interaction of Corl1 with transcriptional corepressors. 293E cells were transiently transfected with an expression vector for each tagged protein as indicated. Cell lysates were immunoprecipitated with an anti-FLAG antibody. Immunoprecipitates and cell lysates were immunoblotted using an anti-FLAG or anti-HA antibody as indicated. C, Corl1 has a transcriptional repressor activity. Structures of the Corl1 mutants used in this study are shown. NS20Y cells were transfected with UAS-SV40-luc and the indicated expression vectors for GAL4-fusion proteins. The results show the fold repression of luciferase activity from the reporter with the GAL4 DNA binding domain alone. The mean ± S.D. of four independent experiments are shown.

**Fig. 5.** **Corl1 is a transcriptional corepressor.** A, Corl1 localized in a nuclear dot-like structure. 293E cells were transiently transfected with the expression vector for FLAG-Corl1 (panel a), GFP-Corl1 and FLAG-N-CoR (panel b), and FLAG-Corl1 and GFP-HIPK2 (panel c) and stained with anti-FLAG antibody. Corl1 was partially colocalized with N-CoR and HIPK2 in the nuclear dot-like structure. B, interaction of Corl1 with transcriptional corepressors. 293E cells were transiently transfected with an expression vector for each tagged protein as indicated. Cell lysates were immunoprecipitated with an anti-FLAG antibody. Immunoprecipitates and cell lysates were immunoblotted using an anti-FLAG or anti-HA antibody as indicated. C, Corl1 has a transcriptional repressor activity. Structures of the Corl1 mutants used in this study are shown. NS20Y cells were transfected with UAS-SV40-luc and the indicated expression vectors for GAL4-fusion proteins. The results show the fold repression of luciferase activity from the reporter with the GAL4 DNA binding domain alone. The mean ± S.D. of four independent experiments are shown.
was tethered to the promoter through the GAL4 DNA binding domain, indicating that Corl1 has an intrinsic repression domain. Together with the fact that Corl1 did not have any known DNA binding motifs, these results strongly suggest that Corl1 is a transcriptional corepressor.

To determine the domains involved in transcriptional repression, we examined the activity of deletion mutants. As shown in Fig. 5C, the N-terminal cysteine-rich domain was not required for transcriptional repression. Consistently, a mutant containing only a cysteine-rich domain showed only weak repressor activity. These results indicate clear difference between Corl1 and members of the Ski/SnoN and Dach subfamilies, since Ski and Dach1 repress transcription through binding to N-CoR via the N-terminal cysteine-rich domain. In contrast, deletion of the CH1 region significantly reduced repressor activity (N1 and N2, C1 and C2, WT, and ΔCH1 were compared), indicating its involvement in transcriptional repression and suggesting that Corl1 represses transcription by a Corl subfamily-specific mechanism. However, the mutant containing only a CH1 domain could not affect transcriptional activity, indicating that the CH1 region alone cannot recruit the corepressor complex. In addition, a mutant without the cysteine-rich and CH1 regions (C2 mutant) showed repressor activity, suggesting that a middle region is also involved in the regulation of transcriptional repression. Taken together, these results demonstrate that at least two regions, CH1 and middle regions, are involved in transcriptional repression and that Corl1 represses transcription via distinct mechanisms of Ski/SnoN and Dach subfamilies.

Corl1 Can Act as a Corepressor for Lbx1—Given the fact that Corl1 is a transcriptional corepressor selectively expressed in subsets of the spinal interneurons, we next elucidated the DNA-binding transcription factor for which Corl1 acts as a corepressor. Expression of Corl1 in subsets of the dorsal horn interneurons, where Lbx1 is selectively expressed and its activity is required for specification (26, 28), raised the possibility that Corl1 cooperates with Lbx1. To test this possibility, we first examined whether Corl1 can interact with Lbx1. 293E cells transiently expressing Corl1 and Lbx1 were crosslinked with DSP reagent, lysed with SDS-containing buffer, and subjected to immunoprecipitation assay. As shown in Fig. 6A, Corl1 and Lbx1 were reciprocally coimmunoprecipitated, clearly demonstrating that Corl1 can associate with Lbx1 in intact cells.

Given the fact that Corl1 has transcriptional repressor activity and that it interacts with Lbx1, we next examined whether Corl1 cooperatively regulates transcriptional repression with Lbx1. When the GAL4-Lbx1 fusion protein was expressed, transcriptional activity of the reporter containing GAL4-binding sites was slightly reduced (Fig. 6C). In contrast, coexpression of Corl1 with GAL4-Lbx1 strongly reduced the transcriptional activity of the reporter, demonstrating that Corl1 can cooperatively repress transcription with Lbx1. Together with the observations that Corl1 is selectively coexpressed with Lbx1 in subsets of the dorsal spinal interneurons, these results suggest that Corl1 regulates transcriptional repression and subsequent cell fate determination in dorsal spinal cord interneuron by acting as a corepressor for Lbx1.

Lbx1 is selectively expressed in and required for proper differentiation of limb muscle precursors as well as dorsal spinal interneurons (26, 28, 40). Therefore, we next examined whether Corl1 is coexpressed with Lbx1 in muscle precursors. Lbx1 expression was detected in an E10.5 forelimb as previously reported (40). However, in the same region, Corl1 expression was not observed (data not shown), indicating that Corl1 is not required for Lbx1 function in the muscle precursors. Thus, Corl1 is not required for all Lbx1 functions; rather Corl1 might be involved in the modulation of Lbx1 activity depending on cellular context.

To determine the domain required for interaction between Corl1 and Lbx1, we performed immunoprecipitation experi-
ment using deletion mutants of Corl1 (Fig. 6B). Mutants containing only the N- or C-terminal regions of Corl1 (N1 and C2 mutants) could still interact with Lbx1 like the wild-type Corl1, suggesting that at least two regions are involved in the interaction. In addition, a mutant lacking the CH1 region could still efficiently interact with Lbx1, indicating that the CH1 region is not required for association with Lbx1.

We next examined the corepressor activity of the Corl1 mutants. As shown in Fig. 6C, the ΔCH1 mutant, which can interact with Lbx1 with high affinity but did not show efficient transcriptional repressor activity by itself (see Fig. 5C), could not repress transcription through GAL4-Lbx1, further supporting the idea that Corl1 acts as a corepressor for Lbx1. In addition, the N2 and C1 mutants, which showed transcriptional repressor activities by themselves (see Fig. 5C) and abilities to interact with Lbx1, showed only weak corepressor activities with GAL4-Lbx1. These results indicate that recruitment of the transcriptional repressor domain of Corl1 onto Lbx1 is not sufficient for efficient cooperative transcriptional regulation by these factors.

**Fig. 7.** Gro/TLE-independent transcriptional repression by Corl1. A, schematic representation of the domain structures of Lbx1 and an Lbx1ΔN mutant. The EH1 motif and homeodomain are indicated. B–D, binding of Grg1 to Lbx1 is not required for the corepressor activity of Corl1. E–G, Gro/TLE factors are not involved in Corl1 corepressor activity. B, C, and E, immunoprecipitation experiments were carried out as described in the legend to Fig. 5. D, F, and G, reporter assay was carried out as described in the legend to Fig. 6. The results show the fold repression of luciferase activity from the reporter with the GAL4-fusion protein alone (D and F) or GAL4-Lbx1 + FLAG-GrG1ΔC (G). The mean ± S.D. of four independent experiments are shown.

**Corl1 Represses Transcription through Lbx1 by a Gro/TLE-independent Mechanism—**Many homeodomain transcription factors that regulate neuronal differentiation have been shown
to have an EH1 motif, which recruits Gro/TLE corepressors, and to act as transcriptional repressors (24). As previously suggested, Lbx1 has an EH1 motif at its N-terminal region. We first examined whether the EH1 motif of Lbx1 functions as a repression domain via recruitment of Gro/TLE family corepressors. As shown in Fig. 7B, one of the Gro/TLE family members, Grg1, was efficiently coimmunoprecipitated with Lbx1. In contrast, an ΔN mutant lacking the EH1 motif (Fig. 7A) could not interact with Grg1, indicating that Lbx1 interacts with Grg1 through the EH1 motif. In addition, Grg1 cooperatively repressed transcription through GAL4-Lbx1, but not through GAL4-Lbx1ΔN (Fig. 7D), strongly suggesting that Grg1 acts as a functional corepressor for Lbx1.

We next examined whether Grg1 is involved in the corepressor function of Corl1 on Lbx1. To test whether Grg1 is required for the interaction between Corl1 and Lbx1, we examined the ability of an Lbx1ΔN mutant to associate with Corl1. As shown in Fig. 7C, the EH1 motif of Lbx1 was dispensable for the interaction with Corl1, indicating that association of Grg1 with Lbx1 is not necessary for Corl1-Lbx1 complex formation. Furthermore, Corl1 cooperated with GAL4-Lbx1ΔN during transcriptional repression (Fig. 7D). These results suggest that Corl1 can repress transcription through Lbx1 in a Corl1-independent fashion. However, the observation that Corl1 could interact with Grg1 (see Fig. 5B) raises an alternative possibility that Grg1 is involved in the repressor activity of the Lbx1-Corl1 complex by acting as a corepressor without directly binding to Lbx1. To test this possibility, we used a Grg1 mutant lacking the C-terminal region, which has been shown to act as a dominant negative mutant (41, 42). We confirmed that the Grg1ΔC mutant could interact with Lbx1 and inhibit transcriptional repression through GAL4-Lbx1 (Fig. 7, E and F). As shown in Fig. 7G, even in the presence of the Grg1ΔC mutant, Corl1 could repress transcription through GAL4-Lbx1 at a similar efficiency as in the absence of Grg1ΔC. Taken together, we concluded that Grg1 activity is not required for transcriptional repression by cooperation between Corl1 and Lbx1. Thus, Lbx1 can select two distinct and independent corepressors, Corl1 and Gro/TLE, to repress transcription in a cellular context-dependent manner.

Corl1 Can Function as an Adapter Molecule Linking Two DNA-binding Transcription Factors—The N-terminal cysteine-rich domain of Corl1 is highly conserved among Corl family members, suggesting the important role of this domain. However, it was not necessary for transcriptional repressor activity or interaction with Lbx1. Therefore, we searched for molecules interacting with the N-terminal cysteine-rich domain of Corl1 by yeast two-hybrid screening to understand the possible role of this domain. A Corl1 N2 construct was used as a bait to screen a mouse E12.5 brain cDNA library. One of the clones obtained encoded a transcriptional repressor protein, PLZF (43). First, we confirmed the interaction between Corl1 and PLZF by immunoprecipitation assay. As shown in Fig. 8A, full-length PLZF was efficiently coimmunoprecipitated with Corl1, indicating that Corl1 can bind to PLZF in intact cells. These results prompted us to examine whether Corl1 and PLZF are coexpressed in dorsal spinal cord neurons. We analyzed the expression pattern of PLZF in the developing spinal cord by in situ hybridization. At E12.5, PLZF expression was detected in newborn neurons lying near the VZ in the dorsal spinal cord (Fig. 8B). This pattern apparently overlapped with that of Corl1 expression. Since Corl1 is expressed in all populations of late-type dorsal interneurons (see Fig. 4), PLZF appears to be coexpressed with Corl1 in differentiating dorsal spinal neurons. Furthermore, PLZF was expressed in early-type interneurons including d14 and d15 neurons (data not shown). Taken together, these results suggest the possible cooperative function of Corl1 and PLZF in specification of the dorsal neurons.

Next, we analyzed the domain of Corl1 responsible for the association with PLZF. As expected from the yeast two-hybrid screening results, a Corl1 mutant containing only the cysteine-rich domain (N1) could bind to PLZF (Fig. 8A), indicating that Corl1 interacts with PLZF through the N-terminal cysteine-rich domain. Thus, PLZF and Lbx1 can associate with distinct domains of Corl1. We could not detect the formation of a ternary complex containing Corl1, Lbx1, and PLZF in our experimental immunoprecipitation assay conditions using transfected 293E cells. However, these results raise the possibility that Corl1 might function as an adapter molecule linking two DNA-binding transcription factors, Lbx1 and PLZF, for regulation of transcription and neuronal cell fate determination.

DISCUSSION

We identified a novel transcriptional corepressor, Corl1, and revealed several lines of evidence demonstrating that Corl1 can
Corl functions in a similar pathway, possibly with Lbx1 and the other subfamilies, suggests that mammalian and the cysteine-rich domain is highly conserved among mouse Corls and in the same pathway (34, 44, 45, 46). The fact that the N-terminal interacts with Six through Eya, and the in which each subfamily acts. For instance, vertebrate Dach from nematodes to mammals (34), as are the signaling pathways for Dach, c-Myb for Ski, Lbx1 for Corl1) (this study and Refs. 48–51). Similarly, the nematode Ski orthologue DAF-5 is reportedly in- volved in the TGFβ pathway as vertebrate Ski and SnoN function

Corl1 as a Protein with Homology to Ski/SnoN and Dach Families—The overall domain structures and amino acid se- quences of the N-terminal cysteine-rich domain were shown to be highly conserved among Corl, Ski/SnoN, and Dach subfamily proteins. In addition, similar subcellular localization, po- tency to interact with general corepressors and transcriptional repression activity are also conserved among these factors (this study and Refs. 38 and 39). Thus, members of these three subfamilies appear to be commonly involved in regulating transcriptional repression. Conservation of the N-terminal cysteine-rich domain among the Ski/Dach/Corl family and obser- vation that Ski and Dach1 recruited HDAC1 through N-CoR via the cysteine-rich domains (38, 39) suggest the possible role of this domain as a transcriptional repressor. However, Corl1 did not efficiently interact with N-CoR and the cysteine-rich domain of Corl1 did not show repressor activity, indicating that this domain does not generally function as a repressor domain. Rather, it might act as a protein-protein interaction domain, which selects partners specific for each subfamily. In addition to the cysteine-rich domain, other regions are also involved in the protein-protein interaction (38, 39, 44–47). Each subfamily in- teracts with different partner proteins (for example, Eya family for Dach, c-Myb for Ski, Lbx1 for Corl1) (this study and Refs. 48 and 49), thus the physiological functions of these proteins seem to be different. Highly selective expression of Corl1 in subsets of neurons, compared with relatively broad expression of the other families, suggests a specific function of Corl1 in regulating neuronal identity specification and/or differentiation.

Ski/Dach/Corl family proteins are evolutionally conserved from nematodes to mammals (34), as are the signaling pathways in which each subfamily acts. For instance, vertebrate Dach interacts with Six through Eya, and the Drosophila orthologues of these factors, Dac, So and Eya, form a complex that regulates muscle development and eye formation, respectively (48, 50). Similarly, the nematode Ski orthologue DAF-5 is reportedly in- volved in the TGFβ pathway as vertebrate Ski and SnoN function in the same pathway (34, 44, 45, 46). The fact that the N-terminal cysteine-rich domain is highly conserved among mouse Corls and the Drosophila orthologue CG11093, compared with among the other subfamilies, suggests that mammalian and Drosophila Corls function in a similar pathway, possibly with Lbx1 and the orthologue Lady bird, respectively.

It has been reported that the Ski/SnoN and Dach family proteins interact with Smad proteins and inhibit TGFβ and BMP signaling (44–47). In the dorsal spinal cord, TGFβ-related signals derived from the roof plate are required for speci- fication of the d11, d12 and d13 interneurons (15). In contrast, the d14 and d15 neurons can be generated without roof plate signals (28), suggesting that blocking these signals distin- guishes the differentiation program of these classes of inter- neurons. Therefore, it is possible that Corl1, which is selec- tively expressed in d14 and d15 neurons, regulates cell fate specification by inhibiting roof plate-derived signals. In agree- ment with this hypothesis, a weak interaction between Corl1 and Smad3 was observed by immunoprecipitation assay using transfected cells (data not shown). However, TGFβ- and Smad3-induced transcriptional activations were not inhibited by Corl1 in the condition where Ski completely inhibited these activations, although we could not exclude the possibility that Corl1 regulates BMP-mediated signaling (data not shown).

Since neuronal identity in the dorsal spinal cord is thought to be determined at the proliferating progenitor state through the Msx class of homeodomain transcription factors and the bHLH transcription factors acting downstream of roof plate signals (18, 51, 52), and since Corl1 is specifically expressed in post- mitotic neurons, it is unlikely that Corl1 is involved in inhibi- tion of the roof plate signals in specifying d14 and d15 cell fates.

Corl1 Functions in the Specification of Neuronal Identity—

During development, specification of cell fate is achieved by the derepression strategy in many tissues including the CNS (24, 53). In the developing CNS, regional identity of neural progen- itor cells is defined by sets of transcription factors, whose expression is regulated by a cross-repressive mechanism (3, 5, 18, 24). In addition to progenitor fate, postmitotic cell fate is determined by the derepression mechanism at least in some neuronal subtypes (25, 26, 28–30, 53).

During early stage neurogenesis, six classes of interneurons emerge from the VZ of the dorsal spinal cord. These neuronal classes can be subdivided into two major classes, class A, a dorsally generating class, which requires TGFβ signals derived from the roof plate for their specification, and class B, a ven- trally generating class, which is not dependent on either roof or floor plate signals and is marked by the expression of Lbx1 (26, 28, 54). Loss- and gain-of-function studies have revealed that Lbx1 specifies class B neurons by blocking the acquisition of class A cell fate (26, 28, 54). Thus, the identity of dorsal interneurons seems to be regulated by the derepression strategy through Lbx1. Consistent with this, we showed that Lbx1 has a transcriptional repressor activity. In addition, Lbx1 has a conserved EH1 motif, which recruits Gro/TLE co repressors, as do many homeodomain transcription factors regulating neuronal cell fate.

Gro/TLE factors are required for specification of most ventral spinal cord neurons through homeodomain transcription factors (24). It has been reported that Gro/TLEs are expressed in the ventral spinal cord at high levels, but at relatively lower levels in the dorsal region (24). However, ectopic expression of Nkx2.2, whose EH1 motif is required for transcriptional repression, can induce ectopic V3 neuron generation in the dorsal spinal cord (24). The homeodomain of Nkx2.2 fused to the engrailed repression domain showed similar activity, suggesting that Gro/TLE factors, rather than the specific corepressor for Nkx2.2, is in- volved in this Nkx2.2 activity. Thus, Gro/TLE factors appear to be active in the developing dorsal spinal cord, supporting the idea that Gro/TLEs are involved in the Lbx1 function.

Broad expression and the requirement of Gro/TLE factors for many transcription factors suggest that Gro/TLE factors might play a permissive rather than a regulatory role in specification.
of spinal cord neurons. However, the question remains as to whether Corl1 plays a similar role as Gro/TLE factors in transcriptional repression and cell fate determination through Lbx1. Our observations show that Corl1 interacts with HDAC1 and acts as a corepressor for Lbx1 independent of Gro/TLE activity. Some distinct classes of transcriptional corepressor complexes containing different sets of components have been reported (55, 56). Thus, one possibility is that Corl1 acts as a corepressor for Lbx1, like Gro/TLEs, but that Corl1- and Gro/TLE-mediated transcriptional repression through Lbx1 are regulated by different mechanisms. In this regard, Corl1- and Gro/TLE-containing corepressor complexes might synergistically act to repress transcription on Lbx1. However, in our experimental conditions, a synergistic effect between Gro/TLE and Corl1 was not observed (data not shown). Since direct downstream target genes for Lbx1 have yet to be identified, we used the GALA-Lbx1 system to examine the corepressor activity of Corl1 and Gro/TLE. On native target promoters, in contrast to artificial reporters, many transcription factors might be involved and synergistically act together. In these conditions, Corl1 and Gro/TLE factors might play distinct or synergistic roles in transcriptional repression. Further studies are needed to define the mechanism of action of these corepressors for Lbx1 function.

An alternative possibility of Corl1 function is modulation of Lbx1 activity. It has been reported that ectopic expression of Lbx1 in the dorsal spinal cord, where Lbx1 is not expressed, repressed expression of class A neuron markers and induced class B neuron genes (26, 28). Thus, Corl1 activity might not be required for the initial specification between class A and class B interneurons by Lbx1. In addition, Corl1 was not coexpressed with Lbx1 in developing limb buds, where Lbx1 function is required for proper specification of muscle precursors (40). These observations indicate that Corl1 activity is not required for all Lbx1 functions. General corepressors, such as Gro/TLE, are broadly expressed, whereas Corl1 is selectively expressed in some lineages, further supporting the idea that Corl1 does not simply act as a transcriptional corepressor recruiting general repressor complexes containing HDAC activity. Lbx1 might regulate distinct sets of downstream target genes in different lineages, such as neurons and muscle precursors. Our observation that two DNA binding factors, Lbx1 and PLZF, could interact with distinct regions of Corl1, suggests that Corl1 might determine the target genes for Lbx1 by selecting a partner, which can synergistically act with Lbx1. A similar role of Dach family proteins in synergistic action between Pax and Six classes of transcription factors in Drosophila eye formation and vertebrate muscle development has also been proposed (48, 50).

In any case, our observations suggest a possible strategy of neuronal subtype specification that uses not only lineage-specific DNA binding factors but also transcriptional cofactors with lineage-selective functions. More detailed analyses including loss-of-function experiments will be needed to understand the precise function of Corl1 in cell fate specification and/or differentiation of dorsal spinal cord neurons.

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Corl1, a Novel Neuronal Lineage-specific Transcriptional Corepressor for the Homeodomain Transcription Factor Lbx1
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