Critical Roles of the LIM Domains of Lhx3 in Recruiting Coactivators to the Motor Neuron-Specifying Isl1-Lhx3 Complex

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During spinal cord development, the LIM domains of the LIM homeodomain factor Lhx3 bind to either the LIM cofactor nuclear LIM interactor (NLI) or another LIM homeodomain factor, Isl1, assembling the tetrameric V2 interneuron-specifying Lhx3 complex (2NLI:2Lhx3) or the hexameric motor neuron-specifying Isl1-Lhx3 complex (2NLI:2Isl1:2Lhx3). However, the detailed molecular basis by which the Lhx3-LIM domains contribute to motor neuron specification still remains poorly understood. Here, we show that the Lhx3-LIM domains are essential for recruiting transcriptional coactivators to the Isl1-Lhx3 complex. Using a yeast genetic screening system, we identify Lhx3 point mutants that bind to NLI but not Isl1. Accordingly, these mutants fail to assemble the Isl1-Lhx3 complex. However, their interaction with coactivators is relatively intact, and they are fully functional in the Lhx3 complex and V2 interneuron specification. Interestingly, when these Lhx3 mutants are directly fused to Isl1, their transcriptional activity in the Isl1-Lhx3 complex is restored. We further show that this restoration reflects an unexpected role of the Lhx3-LIM domains, likely together with Isl1, to form an interaction interface for coactivators. Our results suggest that the Lhx3-LIM domains play critical roles in transactivation of the Isl1-Lhx3 complex by not only directing the assembly of the Isl1-Lhx3 complex but also recruiting coactivators to the complex.

Combinatorial transcription codes are commonly utilized in the developing central nervous system (CNS) to specify a large number of distinct cell types with only a limited number of transcription factors (1). Although this is a well-established concept, the biochemical basis for these codes has remained poorly understood, as has an understanding of the mechanisms that control the context-dependent activities of transcription factors. The specification of spinal cord motor neurons (MNs) and V2 interneurons (V2-INs), involved in the coordination of locomotor control, provides a good model system to tackle these important issues, because the combinatorial transcription code has been relatively well characterized in this context (1, 2).

Early in developing ventral spinal cord, graded signaling of Sonic hedgehog (Shh), secreted from the notochord and floor plate, establishes five progenitor domains, termed p0 to p3, and pMN domains (Fig. 1A), which express distinct sets of progenitor transcription factors (1, 2). Later in development, each progenitor domain gives rise to a group of postmitotic neurons that migrate and settle in the lateral region of the spinal cord (1, 2). V2-INs and MNs emerge from neighboring progenitor domains p2 and pMN, respectively (Fig. 1A) (1, 2). V2-INs and MNs share the expression of many transcription factors during lineage specification, although their gene expression pattern, neurotransmitter phenotypes, and axonal projections are markedly different (2–4).

LIM-homeodomain (LIM-HD) transcription factors (Fig. 1B) have a DNA-binding HD and two LIM domains for protein-protein interactions (5–9) and act as key regulators of many development pathways (6, 10–12). In developing CNS, they postmitotically specify neuronal identities (1, 13). In particular, both p2 and pMN cells upregulate LIM-HD factor Lhx3 and its redundant factor, Lhx4, during their exit from the cell cycle (14, 15). Progenitors differentiating to MNs express an additional LIM-HD factor, Isl1 (16). Consistent with these expression patterns, Lhx3/4-null mice fail to generate Chx10⁺-V2-INs and proper MNs (14), and Isl1 deletion disrupts MN development (17). Moreover, Lhx3 expres-

sion induces ectopic V2-INs, and the expression of both Lhx3 and Isl1 generates ectopic MNs (15, 18). These cell type specifications by Isl1 and Lhx3 involve a combinatorial formation of two related LIM complexes assembled by nuclear LIM interactor (NLI; also named CLIM, Ldb, and Chip) (19–22). While NLI binds the LIM domains of Lhx3 or Isl1, the Lhx3-LIM domains also bind the C-terminal region of Isl1 (Fig. 1A). These interactions, coupled with self-dimerization of NLI, result in a V2-IN-specifying Lhx3 complex (2NLI:2Lhx3) or an MN-specifying Isl1-Lhx3 complex (2NLI:2Isl1:2Lhx3) (15). Correspondingly, the DNA response elements for the tetrameric Lhx3 complex (tetramer response element [TeRE]) consist of a spacer flanked by two Lhx3-binding sites (TeRE^{1/2}s), and the DNA response elements for the hexameric Isl1-Lhx3 complex (hexamer response element, or HxRE) have two Isl1:Lhx3-binding sites (HxRE^{1/2}s) separated by a spacer (Fig. 1A).

While the biological roles for the Lhx3 complex and the Isl1-Lhx3 complex in V2-IN and MN development are relatively well defined (2–4, 15, 18), the biochemical basis underlying their assembly and function still are poorly understood. Interestingly, despite the high degree of sequence conservation through all known LIM domains (5, 7, 9, 23), only the LIM domains of Lhx3 appear to interact with both Isl1 and NLI (15). As these interac-

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FIG 1 LIM-HD complexes during embryonic spinal V2-IN and MN development. (A) Schematic representation of V2-INs and MNs derived from p2 and pMN domains, respectively, as well as the Lhx3 complex and the Isl1-Lhx3 complex, on their response elements. (B) Schematic representation of LIM-HD factors and their fusions used in the study. (C) Comparison of the LIM domains of Lhx3 and Lhx1. Two LIM domains, LIM1 and LIM2, are denoted by arrows.

tions are mutually exclusive, the Lhx3-LIM domains serve as a key assembly determinant between the Lhx3 complex and the Isl1-Lhx3 complex (3, 15).

To elucidate the molecular and biochemical basis by which the Lhx3-LIM domains function to regulate MN specification of developing spinal cord, we decided to dissect their interactions with Isl1. Because the primary sequences of LIM domains are highly conserved through evolution, identification of mutations that inactivate the specific function of a LIM domain is a challenge. However, using a powerful yeast genetic screening system (24), we identify a series of specific Lhx3 point mutants that bind to NLI but not Isl1. By further characterizing two of these mutants, we show that they separate the interaction interfaces of the Lhx3-LIM domains for Isl1 and NLI, as they mediate the assembly and transactivation of the Lhx3 complex but not those of the Isl1-Lhx3 complex. Consequently, these Lhx3 mutants support V2-IN specification but fail to specify MNs. Interestingly, these mutants restore their transcriptional activity in the Isl1-Lhx3 complex when they are directly fused to Isl1. We further show that this restoration reflects an unexpected role of the Lhx3-LIM domains, likely together with Isl1, to form an interaction interface for coactiva-

tors. Our results suggest that the Lhx3-LIM domains play dual roles in enabling transactivation by the Isl1-Lhx3 complex, as they not only mediate the assembly of the Isl1-Lhx3 complex but also direct the recruitment of coactivators to the complex.

MATERIALS AND METHODS

Plasmids. Rat Isl1, chicken NeuroM, human E47 and LacZ, and mouse Lhx3, Lhx1, and nuclear LIM interactor (NLI) in pCS2 and/or pcDNA3 (Invitrogen) containing a hemagglutinin (HA) or myc epitope tag for expression in mammalian cells and chicken embryos were as described previously (3, 15, 25–27). Isl1 and NLI were cloned to the pRS325LexA vector, and the LIM domains of Lhx3 (amino acids [aa] 33 to 153) were cloned into the pRS424UB42-GBD vector for yeast one- plus two-hybrid assay. Lhx3-F136S and Lhx3-Y116C mutant constructs were generated using two-step PCR with specific primers for mutation. MNe::LUC and MNe::GFP were described previously (26). TeRE::LUC and TeRE::GFP were created by subcloning multiple copies of the duplex oligonucleotides found from SELEX into synthetic TATA-GFP or TK-LUC vectors (3). Mouse NLI was cloned into the mammalian expression vector pEBG to generate glutathione S-transferase (GST)-NLI (27). Rat Isl1 and mouse wild-type Lhx3, Lhx3-F136S, and Lhx3-Y116C were used to generate various fusion molecules. Isl1-Lhx3, Isl1-Lhx3-F136S, and Isl1-Lhx3-Y116C

were generated by fusing full-length Isl1 and Lhx3 via the flexible linker GGSGGGSGG. L1-Lhx3 was generated by replacing the LIM domains of Lhx3 with the LIM domains of Lhx1 (aa 1 to 117), resulting in a fusion of the Lhx1 LIM domains to the Lhx3's C-terminal region (aa 152 to 402). Isl1-L1-Lhx3 was generated by fusing Isl1 to L1-Lhx3. Isl1^{HD}-Lhx3 is a fusion of the C-terminal region of Isl1 containing the HD and full-length Lhx3. Isl1^{HD}-L1-Lhx3 was generated by replacing the LIM domains of Lhx3 in Isl1^{HD}-Lhx3 with the LIM domains of Lhx1. All fusions were cloned into pCS2, pcDNA3-HA, and/or pcDNA3-Flag (27).

Yeast one- plus two-hybrid and two-hybrid assays. Yeast screening was performed as described previously (24). Random mutagenesis of the LIM domains of Lhx3 was performed using the Mn²⁺-mediated PCR mutagenesis method with 30 rounds of PCR (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s) using *Taq* polymerase and pRS424UB42-Lhx3-LIMs-GBD as templates in the presence of 0.1 mM MnCl₂. Two oligonucleotides were designed as universal primers for mutagenic PCR of the prey gene: the forward primer, 5'-CC AGC CTC TTG CTG AGT GGA GAT G-3', which corresponds to the C-terminal region of the B42 activation domain, and the reverse primer, 5'-CGG TTT TTC TTT GGA GCA C-3', which corresponds to an N-terminal portion of the GAL4 DNA-binding domain (GBD). The mutagenic PCR products obtained with these primers contained approximately 100 bp of common flanking region at each end with sequence identities to the gap plasmid prepared by EcoRI/BamHI digestions of pRS424UB42-GBD. To construct a mutant cell library of randomly mutated Lhx3-LIM domains, we used a single-step method based on the *in vivo* gap repair (28). Each of the mutagenic PCR products (1 µg) was cotransformed with the gap plasmid (4 µg) into strain YOK400 carrying the pSH18-34 reporter as well as the bait plasmid pRS325LexA-Isl1 or pRS325LexA-NLI. His⁺ transformants were obtained after a 4-day incubation at 30°C on glucose medium containing 10 mM 3-amino-1,2,4-triazole (3AT) and no histidine. More than 1,000 transformants were picked onto plate medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and no histidine, and the yeast colonies showing white or weaker blue color were isolated from the wild-type blue colonies. Approximately 122 such colonies were further processed. Prey vectors were rescued from the mutant candidates and individually transformed into the EGY48 strain expressing LexA-Isl1 or NLI (for the test of two-hybrid interaction) as well as the EGY-LG strain containing the pLGS5 plasmid (UAS_{GAL}-lacZ reporter) to check for the intactness of the GBD (one-hybrid test). Fifty prey plasmids that still conferred blue color in the one-hybrid test and white color in the Isl1 interaction test and simultaneously conferred blue color in the NLI interaction test were chosen as the final mutant candidates and subjected to DNA sequencing to identify the mutational site(s).

In vitro and in vivo CoIP assays and GST pulldown assays. For *in vitro* coimmunoprecipitation (CoIP), Flag-tagged wild-type Lhx3, Lhx3-F136S, and Lhx3-Y116C, as well as HA-NLI and HA-Isl1, were *in vitro* transcribed and translated using a TnT quick coupled transcription/translation system (Promega). Mouse anti-Lhx3 (14) antibody was used for immunoprecipitation, followed by Western blotting with mouse anti-HA (Babco) antibody. For *in vivo* CoIP and GST pulldown assays, HEK293T cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. Cells were seeded and 24 h later, HA-tagged wild-type Lhx3, Lhx3-F136S, Lhx3-Y116C, Lhx1, L1-Lhx3, Isl1-Lhx3, Isl1-Lhx3-F136S, Isl1-Lhx3-Y116C, Isl1-L1-Lhx3, GST-NLI, HA-Isl1^{HD}-Lhx3, HA-Isl1^{HD}-Lhx3-F136S, HA-Isl1^{HD}-Lhx3-Y116C, and HA-Isl1^{HD}-L1-Lhx3 were transiently transfected using Superfect (Qiagen). Mouse anti-HA (Babco) antibody was used for immunoprecipitation, and rabbit anti-RbBP5 (Bethyl) and rabbit anti-p300 (Santa Cruz) antibodies were used for Western blotting assays. For GST pulldown, glutathione-Sepharose was added, and pull downed proteins were detected by Western blotting assays with mouse anti-GST (Santa Cruz) and mouse anti-HA (Babco) antibodies.

In ovo electroporation and immunohistochemistry. *In ovo* electroporation and immunohistochemistry were performed as described previ-

ously (14). In chick electroporation assays, DNAs were injected into a Hamburger-Hamilton (HH) stage 13 to 14 chick neural tube. The embryos were harvested 3 or 4 days postelectroporation and fixed in 4% paraformaldehyde, embedded in OCT, cryosectioned at 12-µm thickness, and analyzed using immunohistochemistry assays. The following antibodies were used for immunohistochemistry: mouse anti-Mnr2/Hb9 (5C10; DSHB), guinea pig anti-Chx10 (29), rabbit anti-Lhx3 (14), and rabbit anti-green fluorescent protein (anti-GFP) (Molecular Probes).

Luciferase assays. Luciferase assays were performed as previously described (25). P19 mouse embryonic carcinoma cells were cultured in α-minimal essential media supplemented with 2.5% fetal bovine serum (FBS) and 7.5% bovine calf serum. For luciferase assays, cells were seeded and incubated for 24 h, and transient transfections were performed using Lipofectamine 2000 (Invitrogen). An actin promoter β-galactosidase plasmid was cotransfected for normalization of transfection efficiency, and empty vectors were used to equalize the total amount of DNA. Cells were harvested 36 to 40 h after transfection. Cell extracts were assayed for luciferase activity, and the values were normalized with β-galactosidase activity. Data are presented as means of triplicate values obtained from representative experiments. Luciferase reporter data are shown as relative fold activation (means ± standard deviations [SD]). All transfections were repeated independently at least three times.

EMSA and ChIP. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) were performed as described previously (25). The sequences of the oligonucleotides used in EMSA were previously described (3). ChIP-PCR was carried out using primers specific to the *Chx10*-TeRE region upstream of the mouse *Chx10* gene: forward, 5'-CTT TGG GGT TGG AAG AGC TGG-3'; reverse, 5'-CAC AAT GGT CTG CTT TGT GCC-3'. ChIP-PCR primers for *Hb9*-MNe in the *Hb9* gene were the following: forward, 5'-TAC TCT CCC TAC AGT CTC TGG GGT-3'; reverse, 5'-TGT CCA GAA ATC CAC AGG CCT GCG-3'. Primers for the HxRE negative region of the *Hb9* gene were the following: forward, 5'-CCA ACC AGG TGA GAG GTT AAT AAA-3'; reverse, 5'-CCT ACT CAC TCA TCC CAA CAG AAT-3' (3, 30).

RESULTS

Identification of Lhx3 mutants that interact with NLI but not with Isl1 using the yeast one- plus two-hybrid system. During spinal cord development, the Lhx3-LIM domains interact with either NLI or Isl1. Because these two interactions are mutually exclusive, the Lhx3-LIM domains function as a primary assembly determinant between the Lhx3 complex and the Isl1-Lhx3 complex (15). Thus, dissection of the Lhx3-LIM domains should provide important insights into V2-IN and MN specification. However, residues responsible for this unique dual interaction property of the Lhx3-LIM domains are not predictable from sequence comparison to other LIM domains, because all known LIM domains are highly conserved in primary amino acid sequences (e.g., approximately 78% homology between Lhx3 and Lhx1) (Fig. 1C) (7, 31). Thus, we decided to dissect these interactions using a yeast-based nonbiased screening method, named the yeast one- plus two-hybrid system (24). In brief, we expressed Isl1 as a fusion to the LexA DNA-binding domain, along with the Lhx3-LIM domains, randomly mutated using Mn²⁺ PCR, as a fusion between the transactivation domain B42 and the Gal4 DNA-binding domain (B42-Lhx3/LIMs-GBD) in yeast (Fig. 2A). The following two reporters allowed rapid selection of specific missense mutations in the Lhx3-LIM domains, which disrupt their interactions with Isl1, as white/viable yeast colonies on X-Gal-His⁻ plates. First, loss of interactions between Isl1 and the mutated Lhx3-LIM domains blocked recruitment of B42-Lhx3/LIMs-GBD to the episomal reporter LexAop:LacZ, leading to white colonies on X-Gal amid blue colonies with wild-type and

FIG 2 Isolation of Lhx3 mutants that interact with NLI but not with Isl1. (A) Schematic illustration for the yeast one- plus two-hybrid system to identify Lhx3 mutants that lose interactions with Isl1. (B) Sequences of mutants that bind NLI but not Isl1. The listed mutants, m1 to m12, lacked interactions with Isl1 but retained interactions with NLI. For wild-type sequences, two LIM domains are denoted as arrows, and the residues conforming to the consensus zinc finger motifs [CX₂CX₁₆₋₂₃HX₂CX₂CX₁₆₋₂₁CX₂(CHD)] are highlighted in green. (C) Isl1 interaction-null mutants isolated from the yeast one- plus two-hybrid screening were tested for their interactions with NLI. Yeast two-hybrid assays on LexA fused to full-length Isl1 or NLI and B42 fused to full-length wild-type Lhx3 or the Lhx3-Y116C or Lhx3-F136S mutant. +++ and – represent strong (bright blue) and no (white to very weak blue) interactions, as visualized in the X-Gal plate. (D) CoIP experiments with *in vitro*-translated Isl1, NLI, wild-type Lhx3, and the Lhx3-Y116C and Lhx3-F136S mutants. Immunoprecipitation was carried out by IgG or anti-Lhx3 antibody, followed by immunoblotting by anti-HA antibody for Isl1 and NLI as well as Lhx3 antibody.

interaction-competent B42-Lhx3/LIMs-GBD constructs. A second chromosomal reporter has upstream Gal4 sites, which drive HIS3 expression. Thus, cells with nonsense and frameshift mutations in the Lhx3-LIM domains, which lead to the lack of functional GBD, resulted in cell death on His[–] plates. This initial screening resulted in numerous white/viable yeast colonies on X-Gal-His[–] plates. Mutated B42-Lhx3/LIMs-GBDs isolated from these cells subsequently were introduced into yeast cells expressing LexA-NLI, along with the episomal reporter LexAop:LacZ. DNA sequencing analysis of blue colonies from this second screening resulted in 12 specific missense mutations in the Lhx3-LIM domains, which disrupt the interactions with Isl1 but still maintain intact interactions with NLI (Fig. 2B). Among these missense mutations, we selected Y116C and F136S (m6 and m12 and arrows in Fig. 2B) for further characterization based on the following two considerations. First, we wanted to use mutants with only a single amino acid change, as most mutants have changes in multiple amino acids (Fig. 2B). Second, based on the findings that the Lhx3 Y116C mutation was identified in human combined pituitary hormone deficiency patients and shown to display a rigid cervical spine leading to limited head rotation, we suspected that this particular Lhx3 residue (Y116) is important for the proper motor circuit formation in muscle contraction (32–34). As shown in Fig. 2C, these two mutations in full-length Lhx3 were confirmed to interact with NLI but not with Isl1 in the yeast two-hybrid tests. We also expressed these mutants and wild-type Lhx3, along with Isl1 and NLI, using *in vitro* translation, incubated them together, and immunopurified Lhx3s and their associated proteins using anti-Lhx3 antibody, and we immunoblotted (IB) them with anti-HA antibody for Isl1 and NLI and anti-Lhx3 antibody for inputs (Fig. 2D). These experiments validated that both Y116C

and F136S mutations in Lhx3 selectively disrupt the interactions with Isl1 while leaving the interactions with NLI relatively intact (Fig. 2D). Of note, in contrast to the yeast results (Fig. 2C), Lhx3-Y116C and Lhx3-F136S mutants copurified slightly lower levels of NLI in the *in vitro* CoIP experiments (Fig. 2D). It is possible that the *in vitro* CoIP experiments are less sensitive than the yeast two-hybrid system and were able to uncover weaker interactions of these mutants with NLI. Alternatively, although both mutants have no deficits in NLI interactions, NLI in the hexameric Isl1-Lhx3 complex could be more stable than NLI in the tetrameric Lhx3 complex, leading to a smaller amount of NLI being detected with these mutants, which can only form the tetramer. In either case, our subsequent experiments show that we identified specific mutations in the Lhx3-LIM domains, which at least functionally (if not physically completely) separate the interaction interface for Isl1 from that for NLI. Our results also demonstrate the utility of the yeast one- plus two-hybrid system in dissecting a highly conserved domain containing multiple protein-protein interaction interfaces.

Validation of Lhx3-F136S and Lhx3-Y116C for their inability to form a functional Isl1-Lhx3 complex. We have deduced the consensus response elements for the Lhx3 complex, the Isl1-Lhx3 complex, and Isl1 (3). We further showed that in EMSA, while the Isl1-Lhx3 complex binds both HxRE and TeRE, the Lhx3 complex and Isl1 bind only TeRE and the Isl1 response element (Isl1-RE), respectively (3), indicating that the Isl1-Lhx3 interaction, which likely is critical for the Isl1-Lhx3 recognition of HxRE, excludes Isl1 from binding Isl1-RE but allows Lhx3 to bind TeRE (3). After examining these *in vitro* DNA-binding properties of wild-type Lhx3 and Isl1, we assessed the DNA-binding capacity of our mutant Lhx3s using EMSA (Fig. 3A). As we previously reported (3),

FIG 3 Lhx3-Y116C and Lhx3-F136S mutants are specifically impaired for Isl1-Lhx3 complex assembly. (A) EMSA with MNe, TeRE^{1/2}, and Isl1-RE as probes. Arrows in MNe and TeRE^{1/2} panels indicate binding by a complex of Isl1 plus Lhx3. The arrow in the Isl1-RE panel indicates binding by Isl1. The arrowhead indicates binding by Lhx3 alone. (B) ChIP assays for Hb9 MNe and Chx10 TRE using P19 cells transfected with Isl1 and wild-type or mutant Lhx3. (C and D) Luciferase reporter assays with TeRE::LUC (C) and MNe::LUC (D) in P19 cells, shown as relative fold activation.

wild-type Lhx3 bound TeRE (arrowhead in Fig. 3A), but neither Isl1-RE nor the MN-specific enhancer (MNe) of the Isl1-Lhx3 complex targets gene *Hb9*, which contains HxRE; wild-type Isl1 bound only Isl1-RE (Fig. 3A, arrow), and a mixture of Isl1 and Lhx3 bound MNe (Fig. 3A, arrow) and TeRE^{1/2} (Fig. 3A, arrow) but not Isl1-RE. Interestingly, Lhx3-F136S or Lhx3-Y116C alone behaved like wild-type Lhx3, as it bound TeRE but neither MNe nor Isl1-RE (Fig. 3A). However, binding of MNe by a mixture of Isl1 and Lhx3-F136S or Lhx3-Y116C was significantly weaker than that by a mixture of Isl1 and Lhx3 (Fig. 3A). We also observed a much weaker band intensity for a complex of TeRE, Isl1, and either Lhx3-F136S or Lhx3-Y116C than that for a complex of TeRE, Isl1, and Lhx3 (Fig. 3A, arrow). Lhx3-F136S and Lhx3-Y116C also were not as efficient as wild-type Lhx3 in blocking Isl1 from binding Isl1-RE (Fig. 3A).

To monitor the ability of Lhx3-Y116C and Lhx3-F136S to bind HxRE *in vivo*, we carried out ChIP using P19 mouse embryonic carcinoma cells expressing Isl1 and wild-type or mutant Lhx3. Wild-type Lhx3 bound MNe (but not the negative-control region) only when Isl1 is coexpressed (Fig. 3B). In contrast, even in the presence of Isl1, Lhx3-F136S and Lhx3-Y116C showed no binding to MNe (Fig. 3B). Both mutants showed strong recruitment to the TeREs of the Lhx3 complex target gene *Chx10*, confirming their ability to form the Lhx3 complex (Fig. 3B).

To monitor the functional consequences of Y116C and F136S mutations in Lhx3, we carried out luciferase assays using the Lhx3 complex-responsive TeRE::LUC and the Isl1-Lhx3 complex-responsive MNe::LUC reporters (3, 25). Both Lhx3-

F136S and Lhx3-Y116C mutants were even more efficient than wild-type Lhx3 in stimulating TeRE::LUC transactivation (Fig. 3C), consistent with their ability to form the Lhx3 complex (Fig. 3A). However, both mutants were significantly impaired in the ability to stimulate HxRE::LUC transactivation when coexpressed with Isl1 (data not shown). Moreover, while the Isl1-Lhx3 complex (i.e., Isl1 plus Lhx3) displayed the reported synergy with Ngn2 in stimulating transactivation of a reporter directed by MNe (MNe::LUC), which consists of HxRE and Ngn2-binding E-boxes (25), Lhx3-F136S and Lhx3-Y116C mutants lacked this synergy (Fig. 3D).

Collectively, these results demonstrate that Lhx3-Y116C and Lhx3-F136S indeed are significantly impaired in their ability to form the Isl1-Lhx3 complex with Isl1 and NLI, while they are competent to form the Lhx3 complex with NLI.

Lhx3-F136S and Lhx3-Y116C do not support MN specification in developing spinal cord. Using chick electroporation, we further validated the ability of Lhx3-F136S and Lhx3-Y116C to assemble the Lhx3 complex but not the Isl1-Lhx3 complex *in vivo*. Both mutants were as efficient as wild-type Lhx3 in inducing the expression of GFP from the TeRE::GFP reporter in developing chick neural tubes (Fig. 4A). In contrast, both mutants were unable to induce GFP expression from MNe::GFP when they were coexpressed with Isl1 (Fig. 4B). Moreover, while Lhx3-F136S and Lhx3-Y116C were as efficient as wild-type Lhx3 in ectopically inducing the expression of the V2-IN marker *Chx10* in developing chick spinal cord (Fig. 4C), both mutants failed to trigger ectopic MN specification (marked by

FIG 4 Failure to specify MNs by Lhx3-Y116C and Lhx3-F136S. (A and B) Ectopic GFP expression from TeRE::GFP (A) and MNe::GFP (B) in the dorsal spinal cord. (C and D) Electroporation to assess ectopic Chx10⁺ V2 differentiation (C) and Hb9⁺ MN differentiation (D) in the dorsal spinal cord. The efficiency of V2 and MN induction was determined by the number of ectopic Chx10⁺ V2-INs (red) and Hb9⁺ MNs (red) among all electroporated cells expressing Lhx3 (green). Quantification results are shown. *, *P* values less than 0.001.

the MN marker Hb9) in chick neural tubes when they were coexpressed with Isl1 (Fig. 4D). These results demonstrate that our Lhx3 mutants are selectively impaired in interactions with Isl1; thus, they are inefficient in forming the Isl1-Lhx3 complex, while they still retain the ability to interact with NLI and assemble the Lhx3 complex.

The Lhx3-LIM domains play critical roles in recruiting co-activators to the Isl1-Lhx3 complex. To independently test whether Lhx3-Y116C and Lhx3-F136S are specifically impaired for the interactions with Isl1, we adopted a strategy to fuse these mutants directly to Isl1 (Fig. 1B), which should bypass the requirement for the Lhx3-LIM domains to interact with Isl1 during Isl1-Lhx3 complex assembly. We have previously shown that wild-type Lhx3 fused to full-length Isl1 (Isl1-Lhx3) forms a structural and fully functional homologue of the Isl1-Lhx3 complex with endogenous NLI (27). Indeed, expression of Isl1-Lhx3 in chick embryos resulted in ectopic generation of MNs, as shown by immunostaining with anti-Hb9 antibody (Fig. 5A). Remarkably, while both Lhx3 mutants failed to specify ectopic MNs in chick neural tubes when they were coexpressed with Isl1 (Fig. 4D), these MN defects were completely rescued by fusing them to Isl1, as Isl1-Lhx3-F136S and Isl1-Lhx3-Y116C fusions generated ectopic MNs as efficiently as Isl1-Lhx3 fusion (Fig. 5A). In support of these results, Isl1-Lhx3-Y116C and Isl1-Lhx3-F136S fusions synergized with NeuroM in stimulating transactivation of a reporter

directed by MNe (MNe::LUC) as efficiently as Isl1-Lhx3 fusion (Fig. 5B).

We have previously reported that the Lhx3-LIM domains are required to direct transactivation by the Isl1-Lhx3 complex, and this requirement extends to the Isl1-Lhx3 fusion protein, in which the protein-protein interactions between Isl1 and the Lhx3-LIM domains are no longer needed. We hypothesized that this reflects a novel role of the Lhx3-LIM domains in recruiting coactivators to the Isl1-Lhx3 complex. To test this hypothesis, we first examined the identity of specific coactivators that may interact with the Isl1-Lhx3 complex. We found that ectopically expressed Lhx3 in HEK293T cells fails to interact with the endogenous histone acetyltransferase p300 (35–37) (data not shown) but is strongly associated with the endogenous RbBP5 (Fig. 6A). RbBP5 is a common subunit of a family of six multiprotein coactivator complexes, and each complex contains one of the histone H3 lysine-4 methyltransferases Set1 α , Set1 β , MLL1, MLL2/WBP7, MLL3, and MLL4/KMT2D (38, 39). Interestingly, Lhx1 and Isl1 showed very weak interactions with RbBP5 (Fig. 6A). Consistent with these results, both Lhx1 and Isl1 showed almost no transcriptional activity in luciferase reporter assays (Fig. 6B and C). Surprisingly, Lhx3-Y116C and Lhx3-F136S still showed significant interactions with RbBP5, although these interactions were weaker than those of wild-type Lhx3 (Fig. 6D). These results explain why these mutants acted as functionally intact Lhx3 complexes (Fig. 3 and 4). In

FIG 5 Direct fusion of Lhx3-Y116C and Lhx3-F136S to Isl1 enables them to specify MNs. (A) Electroporation to assess formation of ectopic Hb9⁺ MN differentiation in the dorsal spinal cord by Isl1-Lhx3, Isl1-Lhx3-F136S, and Isl1-Lhx3-Y116C fusions. (B) Luciferase reporter assays with MNe::LUC in P19 cells. In both types of assays, it is evident that Lhx3-Y116C and Lhx3-F136S directly fused to Isl1 act as a fully functional Isl1-Lhx3 complex. RLU, relative luciferase units.

contrast, L1-Lhx3, in which the Lhx3-LIM domains were replaced by the Lhx1-LIM domains (Fig. 1B), completely lost the interactions with RbBP5 (Fig. 6D). These results strongly suggest that Lhx3 recruits coactivators through its LIM domains and that this coactivator binding function in the Lhx3-LIM domains is not conserved in the LIM domains of Isl1 and Lhx1.

We next tested the interactions of Isl1-Lhx3 fusions with coactivators. Interestingly, while Isl1-L1-Lhx3 failed to interact with RbBP5, Isl1-Lhx3-F136S and Isl1-Lhx3-Y116C fusions interacted with RbBP5 as effectively as Isl1-Lhx3 (Fig. 7A). Moreover, Isl1-Lhx3-F136S and Isl1-Lhx3-Y116C fusions also interacted with p300 as effectively as Isl1-Lhx3 (Fig. 7A). To test whether RbBP5 and p300 are recruited to the MNe of the *Hb9* gene, we performed ChIP experiments in P19 cells transfected with Isl1-Lhx3 fusions. Both coactivators indeed were recruited to the MNe region in the presence of Isl1-Lhx3, Isl1-Lhx3-F136S, or Isl1-Lhx3-Y116C but not in the presence of Isl1-L1-Lhx3 (Fig. 7B), supporting the notion that the Lhx3-LIM domains contribute to the interaction interface for coactivators of the Isl1-Lhx3 complex. We also performed the ChIP experiments using embryonic day 12.5 (E12.5) mouse embryonic spinal cord extracts and discovered that both coactivators are recruited to the MNe region of the *Hb9* gene but not the negative-control region (Fig. 7C).

We have previously reported that the Lhx3-LIM domains may still interact with Isl1 within the Isl1-Lhx3 fusion based on our GST pulldown results showing that the Lhx3-LIM domains in a fusion protein of Isl1^{HD} and Lhx3 (Fig. 1B) failed to recognize NLI

(27). Consistent with the fact that the LIM domains of all LIM-HD factors interact with NLI, our GST pulldown assay revealed that wild-type Lhx3, Lhx3-F136S, Lhx3-Y116C, and L1-Lhx3 interacted with NLI (Fig. 8A). Interestingly, while Isl1^{HD} fused to L1-Lhx3 interacted strongly with NLI, Isl1^{HD} fused to wild-type Lhx3, Lhx3-F136S, or Lhx3-Y116C did not (Fig. 8B). Overall, these results suggest that both mutants are capable of forming the putative interaction interface for coactivators when they are directly fused to Isl1, and formation of this interface blocks the Lhx3-LIM domains from interacting with NLI.

DISCUSSION

The LIM domain, a tandem zinc finger structure (Fig. 2B) that functions as a modular protein-binding interface, is present in many proteins that play diverse cellular roles as regulators of gene expression, cytoarchitecture, cell adhesion, cell motility, and signal transduction (7, 9, 23). However, the evolutionarily highly conserved nature of LIM domains poses a challenge to understanding the specific function of a particular LIM domain over others at the molecular level. In this report, we utilized a powerful yeast genetic method, named the yeast one- plus two-hybrid system (24). This system rapidly identifies specific missense mutations for residues essential for protein-protein interactions by instantly eliminating nonsense and frameshift mutations, which constitute the bulk of unwanted mutations in any random mutagenesis method. As demonstrated by our success in isolating specific mutations that disrupt only the Isl1 interactions, this yeast

FIG 6 Lhx3-Y116C and Lhx3-F136S interact with coactivators. (A) CoIP assays between coactivator RbBP5 with Lhx1, Isl1, and Lhx3 in HEK293T cells. (B and C) Luciferase reporter assays with TeRE::LUC (B) and HxRE::LUC (C) in P19 cells expressing Isl1, Lhx1, and Lhx3, alone or in combination as indicated. (D) CoIP assays between RbBP5 and L1-Lhx3, Lhx3, Lhx3-F136S, and Lhx3-Y116C in HEK293T cells.

genetic system is suited for a difficult task of analyzing a protein-protein interaction interface embedded within highly conserved protein domains. The power of this system is evident from the fact that Y116C, F136S, and other mutations in the Lhx3-LIM domains that we have identified (Fig. 2B) involve highly conserved residues throughout diverse LIM domains, which would not be

obvious from simple comparative sequence analyses. Interestingly, our effort to identify specific mutations in the Lhx3-LIM domains that destroy NLI interactions without affecting Isl1 interactions failed, as all of the mutations identified in the yeast one- plus two-hybrid screening for LexA-NLI also impaired interactions with LexA-Isl1 (data not shown). These results

FIG 7 Recruitment of coactivators to motor neuron enhancers via Isl1-Lhx3 complex. (A) CoIP assays between coactivators RbBP5 and p300 with Isl1-Lhx3, Isl1-Lhx3-F136S, Isl1-Lhx3-Y116C, and Isl1-L1-Lhx3 in HEK293T cells. (B) ChIP assays with anti-p300 and anti-RbBP5 antibodies to show the recruitment of coactivators to the motor neuron enhancers in P19 cells transfected with the indicated Isl1-Lhx3 fusion constructs. (C) ChIP assays with anti-p300 and anti-RbBP5 antibodies to show the recruitment of coactivators to the motor neuron enhancers in developing E12.5 mouse spinal motor neurons.

FIG 8 Lhx3-Y116C and Lhx3-F136S do not interact with NLI within the Isl1-Lhx3 complex. (A) GST-pulldown assays to examine the interaction between NLI and Lhx3, Lhx3-F136S, Lhx3-Y116C, or L1-Lhx3 in HEK293T cells. (B) GST pulldown assays to examine the interaction between NLI and Isl1^{HD} fused to Lhx3, Lhx3-F136S, Lhx3-Y116C, or L1-Lhx3 in HEK293T cells.

suggest that disruption of NLI interactions while preserving the Isl1 interactions is not possible, coinciding with the finding that the LIM interaction domain of NLI binds to the N-terminal LIM domain of LMO2 and LMO4 in an unusually extended conformation (40). In fact, this rather loose specificity was suggested as a mechanism by which NLI can bind a variety of diverse LIM domains (31, 40). Nonetheless, our results show that the Isl1 interaction interfaces in the Lhx3 LIM domains can be at least functionally (if not physically completely) separated from the NLI interaction interfaces through specific mutations. Because both Lhx3-Y116C and Lhx3-F136S form functional Lhx3 complex but not Isl1-Lhx3 complex, our mutational strategy resulted in V2-specific Lhx3 mutants.

Our previous results suggested yet another novel function for the Lhx3-LIM domains in MN specification, in addition to their roles in recruiting Isl1 to the Isl1-Lhx3 complex (27). In this report, we identified recruitment of coactivators to the Isl1-Lhx3 complex as the previously unappreciated second function of the Lhx3-LIM domains in MN specification. Although we have previously reported that CBP is recruited to the MNe region of the *Hb9* gene via the retinoic acid receptor and Ngn2 (37), both of which also play important roles in motor neuron specification, specific coactivators of the Isl1-Lhx3 complex have not been characterized. Our results identify the histone acetyltransferase p300 and one or more of the family of six mammalian histone H3-lysine 4-methyltransferase complexes, which commonly contain RbBP5, as coactivators of the MN-specifying Isl1-Lhx3 complex (Fig. 7). These two types of histone-modifying coactivators are expected to cooperatively lead to an open chromatin structure, leading to an enhancement of the transcription of the target genes of the Isl1-Lhx3 complex, such as *Hb9*. Previously we reported that the absence of CBP and p300 from the developing mouse spinal cord results in reduced histone acetylation and H3 lysine 4-trimethylation in *Hb9* enhancers, suggesting that these coactivators are essential for the Isl1-Lhx3 complex to function as a transcriptional

activator of motor neuron target genes (37). In this report, we also identified the RbBP5-containing complex(es) as a novel coactivator of the Lhx3 complex (Fig. 6).

Our finding that Isl1 fused to our Lhx3 mutants binds to co-activators as efficiently as Isl1 fused to wild-type Lhx3 (Fig. 7A) strongly suggests that both F136S and Y116C mutations in the Lhx3-LIM domains do not affect the putative interaction interface for coactivators within the Isl1-Lhx3 complex. Moreover, our results strongly suggest that Isl1 also participates in the interface for coactivators within the Isl1-Lhx3 complex. First, while Lhx3-F136S and Lhx3-Y116C alone showed still significant but relatively weaker interactions with RbBP5 than wild-type Lhx3 (Fig. 6D), these interactions were further enhanced when our mutants were directly fused to Isl1 (Fig. 7A). Second, while we failed to observe interactions of p300 with Lhx3 alone (data not shown), the fusion of Lhx3 to Isl1 (i.e., Isl1-Lhx3) as well as Isl1-Lhx3-F136S and Isl1-Lhx3-Y116C fusions strongly interacted with p300 (Fig. 7A). Of note, the unique coactivator-binding function of the Lhx3-LIM domains cannot be replaced by the highly related LIM domains of Lhx1 (Fig. 7A). In addition, when fused to the HD of Isl1, while the Lhx1-LIM domains interacted with NLI, the Lhx3-LIM domains (both wild type and mutants) did not (Fig. 8B). These results suggest that within the Isl1-Lhx3 complex the Lhx3-LIM domains are not accessible to the LIM interaction domain of NLI for binding. We have previously suggested that Isl1 still interacts with the Lhx3-LIM domains within the Isl1-Lhx3 fusion based on the inability of the Lhx3-LIM domains to interact with NLI. However, given our results that the LIM domains of our two Lhx3 mutants, which are unable to interact with Isl1, still fail to interact with NLI within the Isl1-Lhx3 fusion, we propose that the LIM domains of Lhx3 are simply aligned with reference to the C-terminal region of Isl1 in a way that blocks their access to NLI, and it is likely that this alignment results from (or is forced or induced by) sequential recognition of the putative interaction interfaces (in coactivators) for the Lhx3-LIM domains and Isl1. Because Lhx1 shows only very weak

binding to coactivators (Fig. 6A), it is an interesting possibility that Lhx1 is not able to induce this alignment and the LIM domains of Lhx1 still interacts with NLI within the Isl1-L1-Lhx3 fusion. We have previously shown that the Lhx3-LIM domains are essential for the DNA-binding activity of the Isl1-Lhx3 complex, likely by helping align the DNA-binding interaction domains of Isl1 and Lhx3 (27). Therefore, both DNA bindings by Isl1 and Lhx3 as well as coactivator bindings by Isl1 and Lhx3 are likely to help induce the proper alignment of Isl1 and Lhx3 within the Isl1-Lhx3 complex. Future studies should be directed at uncovering the exact molecular basis underlying the structural uniqueness of the Lhx3-LIM domains relative to those of other LIM domains in MN specification through more mutational studies as well as X-ray absorption and nuclear magnetic resonance spectroscopic studies.

Finally, the Y116C mutation in Lhx3 also was identified in patients displaying a syndrome called combined pituitary hormone deficiency (32). These patients were characterized by the loss of all but one (adrenocorticotropin) of the five hormones produced in the anterior pituitary, leading to severe growth retardation (32). Consistent with these results, subsequent studies demonstrated that this mutant is impaired in the ability to support the activation of pituitary target genes of Lhx3 (33, 34). Our results presented in this study suggest that patients with this mutation would have motor defects derived from selective impairment of MN development (while V2 development is intact). In support of this prediction, these patients were found to have a rigid cervical spine, leading to limited head rotation (32). More severe motor defects may have been prevented in these patients by Lhx4, a functional homologue of Lhx3, which is also expressed in developing spinal MNs (1, 2). A more interesting hypothesis is that inactivation of the Isl1-Lhx3 complex (while having the Isl1-Lhx4 complex intact) selectively impacts MNs controlling head rotation. Construction of knock-in mouse models for our Lhx3 mutants will allow us to test this idea.

In summary, our analyses of the Lhx3 LIM domains revealed that these domains play at least dual roles in the Isl1-Lhx3 complex transactivation and MN specification as well as postmitotic MN columnar diversification. These involve the previously described function for the Lhx3-LIM domains to recruit Isl1 to the Isl1-Lhx3 complex (27) as well as our newly found roles for these domains to recruit coactivators to the complex.

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