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LMO4 functions as a co-activator of neurogenin 2 in the developing cortex

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SUMMARY

The proneural protein neurogenin 2 (NGN2) is a key transcription factor in regulating both neurogenesis and neuronal radial migration in the embryonic cerebral cortex. However, the co-factors that support the action of NGN2 in the cortex remain unclear. Here, we show that the LIM-only protein LMO4 functions as a novel co-factor of NGN2 in the developing cortex. LMO4 and its binding partner nuclear LIM interactor (NLI/LDB1/CLIM2) interact with NGN2 simultaneously, forming a multi-protein transcription complex. This complex is recruited to the E-box containing enhancers of NGN2-target genes, which regulate various aspects of cortical development, and activates NGN2-mediated transcription. Correspondingly, analysis of *Lmo4*-null embryos shows that the loss of LMO4 leads to impairments of neuronal differentiation in the cortex. In addition, expression of LMO4 facilitates NGN2-mediated radial migration of cortical neurons in the embryonic cortex. Our results indicate that LMO4 promotes the acquisition of cortical neuronal identities by forming a complex with NGN2 and subsequently activating NGN2-dependent gene expression.

KEY WORDS: LMO4, NGN2, NLI, Cortex development, Basic helix-loop-helix factor, LIM domain, Mouse

INTRODUCTION

The generation and migration of neurons during central nervous system (CNS) development is tightly regulated in time and space. Prior studies of the developing cortex have revealed numerous transcription factors that play crucial roles in neurogenesis and migration (Guillemot, 2005). However, the non-DNA binding co-factors that support the action of these transcription factors in the developing CNS remain elusive.

Proneural basic helix-loop-helix (bHLH) transcription factors are the major regulators of neurogenesis in multiple species. Neurogenin 2 (NGN2, NEUROG2), a homolog of the atonal protein in *Drosophila*, is one of the key proneural bHLH proteins responsible for initiating neuronal differentiation in vertebrates (Bertrand et al., 2002; Ross et al., 2003). This function involves direct transcriptional activation of neuronal genes such as *NeuroM* (*Neurod4* – Mouse Genome Informatics), *Delta1* (*Dll1* – Mouse Genome Informatics), *Znf238* (*Zfp238* – Mouse Genome Informatics) and *Ebf2* (Seo et al., 2005; Castro et al., 2006). In addition to its role in neurogenesis, NGN2 controls the specification of neuronal subtypes in the developing cortex, dentate gyrus, midbrain, peripheral nervous system and spinal cord (Fode et al., 1998; Zirlinger et al., 2002; Andersson et al., 2006; Kele et al., 2006; Galichet et al., 2008). Its role is particularly well established in the developing cortex, where loss of NGN2 and its redundant factor, NGN1, leads to a reduced production of cortical excitatory neurons and ectopic acquisition of a ventral telencephalic identity in presumptive cortical neurons (Fode et al., 2000; Parras et al., 2002). Notably, NGN2 also promotes the radial migration of cortical neurons. Cortical cells that lack NGN2 display retarded radial migration towards the pial surface, whereas overexpression of NGN2 in the cortex accelerates pial migration (Hand et al., 2005). The ability of NGN2 to trigger radial migration occurs at least in part through the direct transcriptional activation of the small GTP-binding protein RND2 (Heng et al., 2008).

NGN2 appears to perform its functions by dimerizing with an E-protein such as E47, binding to a consensus sequence called the E-box and activating the transcription of target genes (Ross et al., 2003). To date, only a few co-factors have been suggested to potentiate the transcriptional activity of neurogenin (NGN) family proteins. BRG1 (SMARCA4 – Mouse Genome Informatics), the catalytic subunit of the SWI/SNF chromatin remodeling complex, has been shown to mediate the neurogenic activity of NGN in *Xenopus* and mammalian P19 cells (Seo et al., 2005). The histone acetyltransferase CBP/p300 can also function as a co-activator of the NGN family in cortical cells and spinal motoneurons (Sun et al., 2001; Seo et al., 2005; Lee et al., 2009). However, the in vivo functions of the aforementioned co-factors in NGN2-dependent cortical neurogenesis and migration have yet to be addressed.

Members of the LIM domain Only (LMO) protein family are characterized by two closely spaced LIM domains for protein-protein interactions, and lack distinguishable DNA-binding or catalytic domains. LMOs interact with Nuclear LIM domain Interactor (NLI, also known as LDB, CLIM or CHIP), which is broadly expressed and involved in multiple developmental pathways, including neurogenesis (Jurata et al., 1996; Jurata and Gill, 1997; Mukhopadhyay et al., 2003; Hwang et al., 2008). The

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38 residue LIM-interaction domain (LID) near the C terminus of NLI mediates the high-affinity interaction between LMOs and NLI (Jurata and Gill, 1997). An X-ray crystal structure revealed the highly modular nature of the LMO4:NLI complex, in which the entire lengths of the LIM domains of LMO4 bind the elongated NLI-LID (Deane et al., 2003; Deane et al., 2004). Consistent with the idea that the LMO:NLI complex functions as a module, a strong association between LMO4 and NLI has been reported in many cell types (Sugihara et al., 1998; Milan and Cohen, 1999; Lee et al., 2008; Joshi et al., 2009).

LMO proteins were initially proposed as negative regulators of LIM homeodomain (LIM-HD) transcription factors because they can compete for NLI-binding in transcriptional complexes comprising NLI and LIM-HD proteins (Milan et al., 1998; Milan and Cohen, 1999; Thaler et al., 2002; Lee et al., 2008; Joshi et al., 2009; Song et al., 2009). However, mounting evidence has indicated that they are also able to activate transcription by nucleating the assembly of complexes with transcription factors such as SCL and GATA (Wadman et al., 1994; Wadman et al., 1997; Joshi et al., 2009; Song et al., 2009). Therefore, LMOs are capable of controlling transcription, both positively and negatively, depending on the cell context and their binding partners.

LMO4 has been shown to play a role in various developmental systems, including epithelial, mammary, ear and neural development (Sugihara et al., 1998; Hahm et al., 2004; Tse et al., 2004; Lee et al., 2005; Sum et al., 2005; Wang et al., 2007; Deng et al., 2010). Loss of *Lmo4* leads to embryonic lethality; a significant percentage of *Lmo4*-null mice exhibit exencephaly, while the rest show overall normal brain morphology (Hahm et al., 2004; Tse et al., 2004; Lee et al., 2005). Interestingly, LMO4 displays dynamic and complex spatiotemporal expression in the embryonic telencephalon (Hermanson et al., 1999; Bulchand et al., 2003; Lee et al., 2005; Azim et al., 2009; Huang et al., 2009), suggesting roles in multiple steps of telencephalic development. It is noteworthy that LMO4 expression is detected in areas where no LIM-HD gene expression is apparent (Bulchand et al., 2003), indicating LIM-HD-independent functions of LMO4. Indeed, LMO4 plays a key role in patterning thalamocortical connections by regulating Ca^{2+}-dependent gene transcription and in the development and function of the somatosensory cortex (Kashani et al., 2006; Huang et al., 2009). However, the contribution of LMO4 to cortical neuronal differentiation and its mode of action in this process have yet to be defined.

Here, we provide a novel role of LMO4 in promoting cortical neurogenesis and neuronal migration. Furthermore, we present mechanistic and genetic evidence that the LMO4:NLI module functions as a co-activator of NGN2 in the developing cortex, uncovering a novel molecular mechanism by which NGN2 activates gene transcription in the CNS.

MATERIALS AND METHODS

Yeast-two-hybrid screen

Full-length mouse LMO4 linked with mouse NLI-LID was cloned into the pGBK-T7 vector for the bait. Yeast-two-hybrid screening was performed using the MATCHMAKER GAL4 Two-Hybrid System (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, AH109 yeast cells were transformed with LMO4-LID/pGBKT7, and the transformants were mixed for mating with Y187 yeast cells pre-transformed with the mouse E11d cDNA library in the pGADT7-Rec vector (Clontech, Mountain View, CA, USA). Diploid transformants were selected on SD/-His/-Leu/-Trp plates. Positive interactors were verified by one-on-one transformations followed by growth on SD/-Ade/-His/-Leu/-Trp plates and β-gal filter assays. The plasmids in positive clones were isolated and subjected to DNA sequencing analysis.

GST-pulldown assays

BL21 *E. coli* were transformed with pGEX/NGN2, LMO4 or NLI, induced to express the GST-fusion proteins and lyzed by sonication. The GST-fusion proteins were purified by incubating the lysates with glutathione-sepharose 4B beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The beads were washed with PBS before adding the putative interactors, which were generated and radiolabeled with [^{35}S]-methionine using the TnT T7 Quick Coupled transcription/translation system (Promega, Madison, WI, USA). Protein-protein interactions were tested by mixing the radiolabeled proteins with the GST-fusion proteins. Bound proteins were eluted by boiling and visualized through autoradiography.

For the cell-based GST-pulldowns, HEK293 cells were transfected with pCS2/3xHA-NGN2 and pEBG/LMO4 or pEBG/NLI. After 48 hours, cells were lyzed and incubated with glutathione-sepharose 4B beads. Samples were eluted by boiling and visualized by blotting with mouse anti-HA (Covance, Princeton, NJ, USA).

The sequential pulldowns were performed by first following the in vitro GST-pulldown protocol described above with bacterially produced GST-NLI and radiolabeled LMO4 and NGN2. Interactors of GST-NLI were eluted from the glutathione beads using 50 mM reduced glutathione. Eluted proteins were purified through a 3k MWCO Amicon Ultra 0.5 ml centrifugal filter (Millipore, Billerica, MA, USA) and incubated with mouse anti-Flag (Sigma-Aldrich, St Louis, MO, USA) or mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and protein A agarose beads (Invitrogen, Carlsbad, CA, USA). Interactors were eluted by boiling and visualized by autoradiography.

P19 cell culture assays

For the luciferase assays, P19 cells were seeded on a 48-well plate and transfected with the *Ebf2*:LUC reporter (100 ng/well; a gift from K. Kroll, Washington University, St Louis, MO, USA) or the *Delta1*:LUC reporter (100 ng/well; a gift from F. Guillemot, NIMR, London, UK), and combinations of pCDNA3/Flag-NGN2 (50 ng/well), pCDNA3/NLI (200 ng/well) and pEBG/LMO4 (400 ng/well). Cells were harvested 48 hours after transfection and assayed for luciferase activity. The graph shows the average fold change over the basal luciferase activity.

For the P19 cell neuronal differentiation assays, cells were seeded on gelatin-coated slides and immunostained with mouse anti-Tuj1 (Covance, Princeton, NJ, USA) 72 hours after transfection. The graph corresponds to the mean percentage of Tuj1-expressing cells based on the number of $Tuj1^+$ and GFP^+ cells counted in six fields. Neurites and neuritic branch points were also averaged from six fields.

The statistical significance for all P19 cell experiments was determined using one-way ANOVA with LSD post-hoc analysis.

For the quantitative RT-PCR (Q-RT-PCR) analyses, P19 cells were seeded on six-well plates and harvested 72 hours after transfection. RNA was extracted from the cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was generated using Superscript III (Invitrogen, Carlsbad, CA, USA). Q-RT-PCR was performed using SYBR Greener (Invitrogen, Carlsbad, CA, USA) and the following primers: *NeuroD*, forward 5′-CTTGGCCAAGAACTACATCTGG, reverse 5′-GGAG-TAGGGATGCACCGGGAA; and *CypA*, forward 5′-GTCTCCTTC-GAGCTGTTTGC, reverse 5′-GATGCCAGGACCTGTATGCT. The data represents means of duplicates of *NeuroD* (*Neurod1* – Mouse Genome Informatics) values normalized against *CypA* (*Ppia* – Mouse Genome Informatics).

Chromatin immunoprecipitation assays

Brains and spinal cords from E11.5 wild-type mouse embryos were dissociated, crosslinked with 1% formaldehyde, sonicated to fragment the chromatin and immunocleared with mouse or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and protein A agarose beads. They were then immunoprecipitated with protein A agarose beads and guinea pig anti-LMO4 (Joshi et al., 2009), rabbit anti-NLI (Thaler et al., 2002), rabbit anti-NGN2 or IgG from the same donor (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunocomplexes were eluted from the beads using 1% SDS, 0.1 M $NaHCO_3$, and reverse crosslinked at 65°C. DNA was purified by proteinase K digestion, phenol-chloroform extraction and ethanol

precipitation. This was used as a template for PCR amplification using the following primers: *Ebf2*, forward 5′-TTCGCCAATTATTAGCAAGGA, reverse 5′-CCTGCGATTGCATAACAAAA; *NeuroM*, forward 5′-CCTTGTCAGCCTTTCAGGAG, reverse 5′-AGAGTTGCTTTCAGGCCAAA; *Delta1*, forward 5′-ATGACACGCCTTTAGACG, reverse 5′-AGCTGTGGGAGTATAGAGAC; *Rnd2*, forward 5′-TGCCTCTGCTGTTGACTCCTAA, reverse 5′-CGGGTTCATCCTGACACTGA; *Zfp238*, forward 5′-GATGTGAGCTGCCTGAATTG, reverse 5′-AGAGGGACGAAGAAGGAAGC; *Sox10* intron, forward 5′-GAGAGGTGAGCGAAAAGGTG, reverse 5′-TGATCCCAACCGTCTCTAGG.

Immunofluorescence and in situ hybridization

Embryos were fixed in 4% paraformaldehyde and incubated in 30% sucrose according to embryonic stage. They were sectioned at 18 μm for in situ hybridization or 12 μm for immunofluorescence.

Antisense in situ hybridization probes for *Lmo4* (a gift from J. Rubenstein, University of California, San Francisco, CA, USA), *Nli*, *Ngn2*, *NeuroM* (gift from Y. Nakagawa, University of Minnesota Medical School, Minneapolis, MN, USA), *Rnd2* (gift from F. Guillemot), and *Ebf2* (gift from A. Vincent, CNRS, Toulouse, France) were synthesized with digoxigenin-labeled or FITC-labeled NTPs (Roche Diagnostics GmbH, Mannheim, Germany). Fluorescence labeling and signal amplification was carried out using the TSA Plus System (Perkin Elmer, Waltham, MA, USA). For non-fluorescent labeling, tissues were incubated with anti-Digoxygenin-AP Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) and treated with a color reaction buffer.

For immunofluorescence, the tissues were incubated with the following primary antibodies: goat anti-LMO4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which we showed to be specific to LMO4 using *Lmo4*-null embryos (Lee et al., 2005), rabbit anti-Tbr2 (Chemicon, Temecula, CA, USA), mouse anti-Tuj1 (Covance, Princeton, NJ, USA), rabbit anti-Tbr1 (Chemicon, Temecula, CA, USA), rabbit anti-FoxP1 (Abcam, Cambridge, MA, USA) and chicken anti-GFP (Aves Labs, Tigard, OR, USA). Fluorophore-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Quantification of *Lmo4*-null phenotypes

The *Lmo4* knockout mice have been described previously (Lee et al., 2005). We analyzed non-exencephalic brains, using level-matched sections of at least three stage-matched embryos of each genotype, coming from at least two different litters. Quantification was normalized against the wild-type average in each litter. Significance was determined by a two-tailed one-sample *t*-test against 1.

The $Tbr1^+$ area at E12.5 was determined by outlining the immunostained region of each cortical hemisphere using AxioVision (Carl Zeiss MicroImaging GmbH, Germany). The Tbr1 and FoxP1 layer thickness at E18.5 is the average of four length measurements taken across each image using AxioVision.

For the quantification of Tbr2 and Tuj1, a custom program was written in MATLAB (The MathWorks, Natick, MA, USA) to calculate the number of pixels occupied by the markers. The boundaries of the tissue regions occupied by the cells were extracted using the graphic user interface of the program. The number of pixels occupied by the cells in the extracted regions was enumerated by counting the number of pixels with intensity values above a chosen intensity threshold. The intensity threshold was chosen based on maximum overlap between the thresholded image and the original image based on visual comparison.

In utero electroporation

Genes of interest were put under the control of the CAG promoter (Niwa et al., 1991). In utero electroporation was performed as described previously (Shimogori and Ogawa, 2008), with some modifications. Briefly, 1 μg/μl of each plasmid was injected and electroporated into the lateral ventricle of E14.5 ICR embryos using the ElectroSquarePorator ECM 830 (BTX) set at five 50 ms pulses of 30 V with 150 ms intervals. Embryos were sacrificed and processed for immunohistochemistry 72 hours after electroporation. Tissue sections were stained with DAPI to designate one bin for the VZ and SVZ, and another bin for the rest of the cortex, based on the arrangement of the nuclei. Electroporated cells marked by the expression of nGFP were counted manually to determine the percentage of electroporated cells in each bin.

The data presented shows the mean from at least three embryos per genotype based on representative experiments where LMO4 electroporation and empty vector electroporation were assessed within one litter, and NGN2 plus LMO4 was compared with NGN2 using another litter. Each set of injections was repeated in three litters. Statistical significance was determined using a one-way ANOVA, followed by LSD post-hoc analysis.

Mouse experiments

Animals were treated in compliance with the US Department of Health and Human Services and Baylor College of Medicine guidelines, in accordance to approved animal protocols.

RESULTS

LMO4 and NLI interact with NGN2

To gain insight into the functions of LMO4 in neural development, we performed a Gal4-based yeast-two-hybrid screen for LMO4 interactors in an E11.5 mouse cDNA library. We specifically targeted proteins that bind to the LMO4:NLI module (Deane et al., 2003) and prevented overrepresentation of NLI in the screen by using a construct in which LMO4 is fused to the NLI-LID (LMO4-LID) as a bait (Fig. 1A). LMO4-LID has been shown to exhibit a highly modular conformation (Deane et al., 2004). The screen identified NGN2 as an interactor of LMO4-LID, which was confirmed by subsequent in vitro GST pulldown assays (data not shown).

To test whether LMO4 and NLI can interact with NGN2 independently, we performed a series of in vitro GST pulldown experiments using bacterially expressed GST fusion proteins and in vitro translated proteins that are labeled by [^{35}S]-methionine. GST-LMO4 interacted weakly with NGN2, whereas it strongly pulled down NLI (Fig. 1B). On the other hand, GST-NLI interacted with NGN2 as well as LMO4 with high affinity (Fig. 1C). Consistently, GST-NGN2 showed a weak interaction with LMO4 and a stronger interaction with NLI (Fig. 1D). E47, a dimerization partner of NGN2, was used as a positive control (Fig. 1D). Notably, both LMO4 and NLI failed to bind to E47 (Fig. 1B,C). These results indicate that LMO4 and NLI can bind to NGN2 independently of each other.

Given that LMO4 probably forms a functional module with NLI in cells and that NGN2 was discovered as an interactor of LMO4-LID, it is plausible that NGN2, LMO4 and NLI form a multi-protein complex. To test this idea, we investigated protein-protein interactions among NGN2, LMO4 and NLI using in vitro GST-pulldown assays. The interaction between NGN2 and NLI was not significantly diminished in the presence of LMO4 (Fig. 1E,F), arguing against the possibility that LMO4 squelches NLI away from NGN2. Interestingly, the interaction between NGN2 and LMO4 was substantially strengthened by the addition of NLI (Fig. 1E,G). These results not only imply that NLI and LMO4 can simultaneously bind to NGN2 to form a complex, but further suggest that NLI stabilizes the NGN2-LMO4 interface and/or LMO4 itself. E47 did not affect the interaction of NGN2 with NLI or LMO4 (Fig. 1F,G). To directly test the formation of a multi-protein complex among NGN2, LMO4 and NLI, we performed sequential pulldown assays using GST-NLI and in vitro translated Flag-tagged LMO4 and HA-tagged NGN2 (Fig. 1H). Proteins bound by GST-NLI were eluted and subjected to a subsequent co-immunoprecipitation experiment with α-Flag antibody to pull down LMO4 and its interactors. As expected, LMO4 was

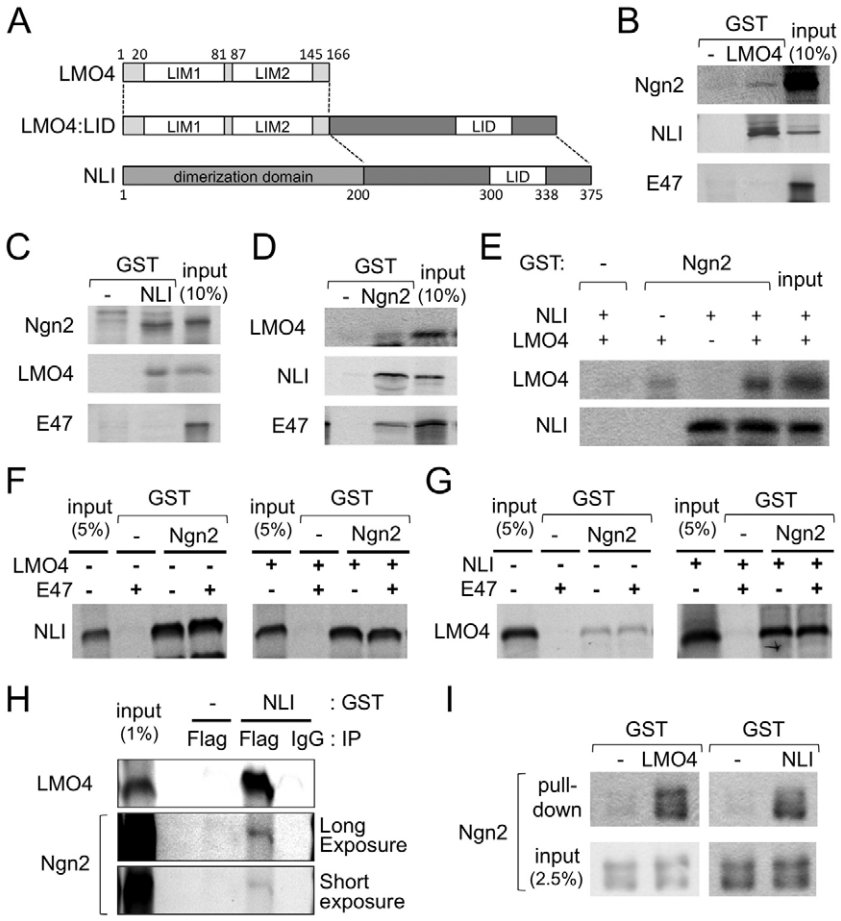

Fig. 1. LMO4 and NLI interact with NGN2. (**A**) The schematic diagram shows the LMO4-NLI module used as bait for the yeast two-hybrid screen. LIM, LIM domain; LID, LIM interaction domain of NLI. (**B-G**) GST-pulldown assays using GST proteins and radiolabeled, in vitro translated putative interactors. (B-D) NGN2 interacts with NLI and LMO4 in vitro. (E) GST-NGN2 forms a complex with LMO4 and NLI. (F) The interaction between GST-NGN2 and NLI is not significantly affected by LMO4 or E47. (G) The interaction between GST-NGN2 and LMO4 is strengthened by NLI, but not by E47. (**H**) Co-immunoprecipitation with α-Flag antibody performed after a GST-pulldown using GST-NLI and radiolabeled NGN2 and Flag-LMO4. NGN2 binds simultaneously to NLI and LMO4. (**I**) GST-pulldown assays using HEK293 cells transfected with NGN2 and GST alone, GST-LMO4 or GST-NLI. Both LMO4 and NLI interact with NGN2 in cells.

precipitated by the sequential pulldown with GST-NLI, but not with GST alone. Intriguingly, NGN2 was also immunopurified along with LMO4. These results demonstrate that NGN2 assembles a multi-protein complex with LMO4 and NLI.

To test whether NGN2 interacts with LMO4 and NLI in cells, we performed GST-pulldown assays in HEK293 cells transfected with HA-tagged NGN2 and GST-LMO4 or GST-NLI (Fig. 1I). Immunoblotting against HA revealed that NGN2 associates with both LMO4 and NLI in cells.

Together, these data indicate that NGN2, LMO4 and NLI form a multi-protein complex in cells.

LMO4 enhances neuronal differentiation triggered by NGN2

The association between the LMO4:NLI module and NGN2 led us to ask whether LMO4 and NLI regulate the activity of NGN2 in cells. To address this issue, we used P19 mouse embryonic carcinoma cells, which undergo neuronal differentiation upon NGN2 expression (Farah et al., 2000; Lee and Pfaff, 2003). We transfected the cells with NGN2, LMO4 or a combination of the two, along with GFP as an indicator of transfection, and assayed neuronal induction 3 days after transfection. NLI was not supplied exogenously, because NLI is expressed in P19 cells (Lee and Pfaff, 2003). To analyze the extent of neuronal differentiation, we monitored the expression of the post-mitotic neuronal marker, β-III tubulin, using a Tuj1 antibody. In addition, to score the maturation of the neurons, we quantified neuritogenesis and neurite branching in Tuj1$^+$ neurons. The expression of LMO4 alone did not trigger significant neuronal differentiation, whereas NGN2 alone showed neurogenic activity as expected (Fig. 2A,B). Interestingly, co-expression of LMO4 and NGN2 strongly induced neuronal differentiation in P19 cells (Fig. 2A,B). Moreover, neurite formation and neurite branching became much more prominent in cells co-expressing LMO4 and NGN2 than in cells transfected with only NGN2 (Fig. 2A,C,D). NLI expression further facilitated neuronal differentiation driven by co-expression of LMO4 and NGN2 (see Fig. S1 in the supplementary material). These results establish that LMO4 and NLI potentiate the neurogenic properties of NGN2.

Next, to test the impact of LMO4 on NGN2-dependent transcriptional activation, we also monitored the expression of *NeuroD*, a target gene of neurogenins (Seo et al., 2007). Similar to neuronal differentiation, LMO4 enhanced the induction of *NeuroD* by NGN2, whereas LMO4 alone did not activate *NeuroD* expression (Fig. 2E). These results suggest that LMO4 stimulates NGN2-dependent transcription.

LMO4 and NLI are co-expressed with NGN2 in the developing cortex

NGN2 plays a crucial role in neuronal differentiation and migration in the developing cortex. Given the association of NGN2 with LMO4 and NLI, and the augmentation of NGN2-dependent neurogenesis by LMO4 and NLI, we considered the possibility that LMO4 and NLI form a complex with NGN2 in the embryonic cortex. To test this possibility, we analyzed the expression patterns of *Lmo4*, *Nli* and *Ngn2* in the telencephalon using double fluorescence in situ hybridization with tyramide signal amplification. At E11.5 when NGN2 is triggering the rapid

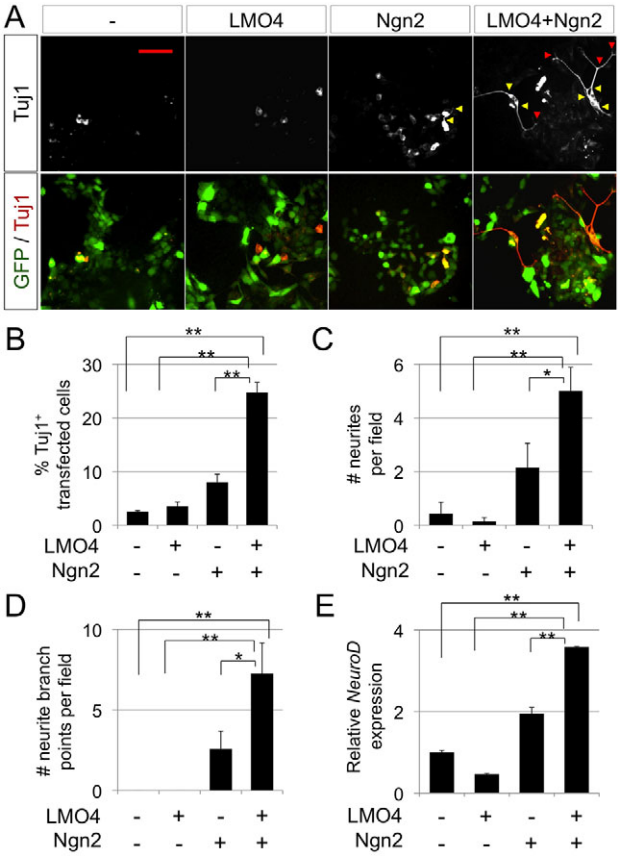

Fig. 2. LMO4 enhances NGN2-mediated neuronal induction in P19 cells. (**A**) Immunohistochemical analyses using the neuronal marker Tuj1 in P19 cells transfected with a GFP expression vector and the constructs indicated above the images. (**B-D**) Quantification of the various aspects of neuronal differentiation in P19 cells transfected with constructs indicated below the graphs. (**E**) Quantitative RT-PCR results to monitor the expression levels of *NeuroD* in P19 cells transfected with constructs indicated below the graph. LMO4 facilitated induction of *NeuroD* by NGN2. (B-E) Error bars show s.e.m. Tuj1 expression, P=7.7E^{-11}; neurite formation, P=9.6E^{-5}; neurite branching, P=4.1E^{-4}; *NeuroD* expression, P=1.03E^{-5}. *P<0.05 and **P<0.0005 in post-hoc analysis.

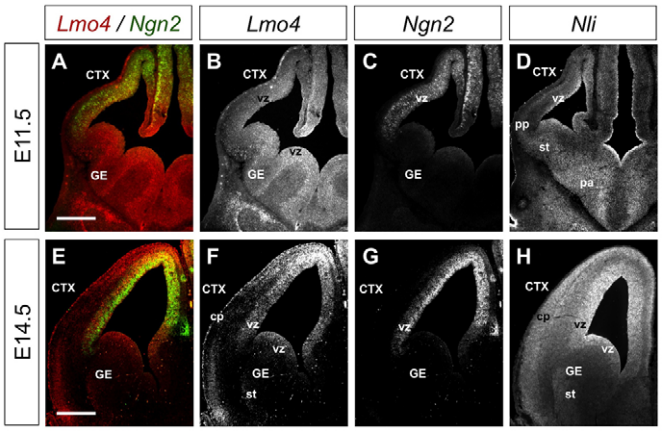

Fig. 3. Expression analyses of *Lmo4*, *Ngn2* and *Nli* in the developing cortex. (**A-C**,**E-G**) Double fluorescence in situ hybridization shows that the transcripts of *Lmo4* and *Ngn2* are co-expressed in the cortex (CTX) at E11.5 and E14.5. (A,B) *Lmo4* is expressed in the VZ and preplate at E11.5. (E,F) At E14.5, *Lmo4* is expressed in the VZ and SVZ of the cortex, along with specific post-mitotic neuronal populations. (C,G) *Ngn2* is expressed in the VZ of the cortex at E11.5 and E14.5. (**D**,**H**) *Nli* is ubiquitously expressed in the forebrain at both stages, encompassing the *Lmo4* and *Ngn2* expression domains. Images show coronal sections of the left hemisphere of the telencephalon, dorsal side upwards and lateral side towards the left. Scale bars: 400 μm. GE, ganglionic eminences; cp, cortical plate; st, striatum; pa, pallium; pp, preplate; vz, ventricular zone.

differentiation and specification of cortical neurons, *Lmo4* is co-expressed with *Ngn2* in the ventricular zone (VZ) of the cortex, and is strongly expressed in the preplate (Fig. 3A-C). Consistently, LMO4 expression in the VZ was also detected by immunostaining (see Fig. S2 in the supplementary material). *Nli* is also expressed in the *Ngn2*$^{+}$ cortical VZ (Fig. 3D). At E14.5 when most neurons of the cortex are being born, *Lmo4* is expressed in the *Ngn2*$^{+}$ VZ, as well as in the subventricular zone (SVZ) (Fig. 3E-G). *Nli* is likewise expressed throughout the cortex with a high level of expression in the cortical plate and the VZ (Fig. 3D,H). Thus, *Lmo4*, *Nli* and *Ngn2* are co-expressed in the neurogenic regions of the developing cortex, supporting the action of LMO4 and NLI as co-factors of NGN2.

LMO4 and NLI function as co-activators of NGN2

To demonstrate the role of LMO4 and NLI as transcriptional co-activators of NGN2, it is crucial to test whether LMO4 and NLI are recruited to the target enhancers of NGN2 in the embryonic CNS. Previous studies have identified NGN2-bound E-boxes in the enhancers of neuronal genes such as *Ebf2*, *NeuroM*, *Delta1*, *Rnd2* and *Znf238* genes (Castro et al., 2006; Seo et al., 2007; Heng et al., 2008). Ebf2, NeuroM and Znf238 are involved in neuronal differentiation or survival, whereas Ebf2, Rnd2 and Znf238 control neuronal migration (Roztocil et al., 1997; Tomita et al., 2000; Garcia-Dominguez et al., 2003; Nakamura et al., 2006; Okado et al., 2009). Delta1 allows the activation of the Notch pathway to maintain a population of neural progenitors (Louvi and Artavanis-Tsakonas, 2006). To test the in vivo recruitment of LMO4 and NLI to NGN2-target enhancers, we dissected the brains and spinal cords from E11.5 mouse embryos and immunopurified LMO4-, NLI- and NGN2-bound chromatin fragments using α-LMO4, α-NLI and α-NGN2 antibodies. Immunoprecipitation with IgG was carried out in parallel as a negative control. Then we tested the presence of NGN2-target enhancer regions through subsequent PCR amplification. Intriguingly, the chromatin immunoprecipitation (ChIP) assays revealed that LMO4 and NLI are recruited to the NGN2-target enhancers of *Ebf2*, *NeuroM*, *Delta1*, *Rnd2* and *Znf238* genes, like NGN2 (Fig. 4A). By contrast, NGN2, LMO4 and NLI did not interact with an intronic region of *Sox10* that lacks NGN2-binding E-boxes. These data demonstrate that LMO4 and NLI bind to NGN2 targets in the developing CNS.

To test whether LMO4 and NLI can influence NGN2-mediated transcription in NGN2-target enhancers, we monitored the transcriptional activity of NGN2 in P19 cells, using luciferase reporter assays with an *Ebf2*:LUC reporter, in which the *luciferase* gene is driven by the *Ebf2* gene enhancer containing NGN2-binding E-box sites (Seo et al., 2007). NGN2 caused a 30-fold activation the *Ebf2*:LUC reporter (Fig. 4B). By contrast, the expression of LMO4 and NLI without NGN2 stimulated the reporter only modestly (Fig. 4B). Interestingly, expression of

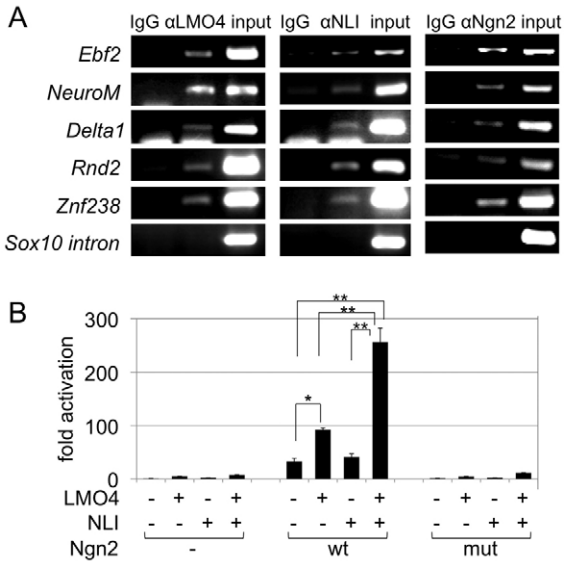

Fig. 4. LMO4 and NLI are co-activators for NGN2 targets in the cortex. (**A**) ChIP assays with anti-LMO4, anti-NLI and anti-NGN2 antibodies using mouse E11.5 brains and spinal cords. NGN2, LMO4 and NLI are recruited to the NGN2-target enhancers in the *Ebf2*, *NeuroM*, *Delta1*, *Rnd2* and *Znf238* genes, whereas they did not bind to the *Sox10* intron that lacks NGN2-binding E-boxes. (**B**) Luciferase assays using an *Ebf2* enhancer-driven luciferase reporter in P19 cells transfected with the cDNAs specified below each graph. Co-expression of LMO4 and NLI strongly enhances the transcriptional activation by wild-type (wt) NGN2, but not by the DNA-binding defective form of NGN2 (mut). Error bars indicate s.e.m. ANOVA: P=1.1E^{-9}, *P<0.005 and **P<0.0005 in the post-hoc analysis.

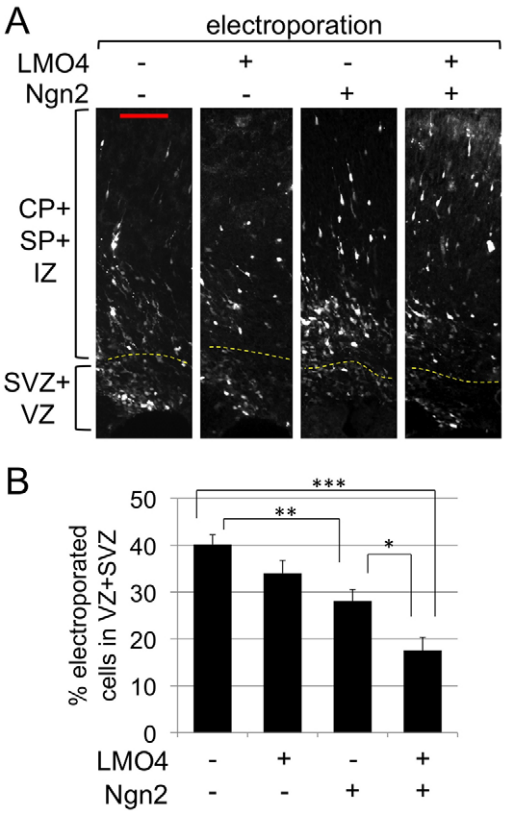

Fig. 5. LMO4 enhances NGN2-mediated neuronal differentiation in utero. (**A**) In utero electroporation of LMO4, NGN2 and a combination of LMO4 and NGN2, along with the marker nGFP, in E14.5 mouse embryonic cortex. The behavior of GFP$^+$ electroporated cells was analyzed 3 days after electroporation. Expression of NGN2 promotes the migration of electroporated cells away from the VZ and SVZ (shown below the yellow dotted line). The combination of LMO4 and NGN2 results in more efficient migration of cells compared with NGN2 alone. (**B**) Quantification of these results. Error bars indicate s.e.m. ANOVA: P=0.00034, *P<0.05, **P<0.005 and ***P<0.0005 in the post-hoc analysis. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate. Scale bar: 100 μm.

LMO4 facilitated the transcriptional activity of NGN2, resulting in a 90-fold activation of *Ebf2*:LUC. Moreover, co-expression of LMO4 and NLI with NGN2 led to a synergistic 250-fold activation of the *Ebf2* enhancer (Fig. 4B), consistent with the formation of a complex among LMO4, NLI and NGN2. It is noteworthy that LMO4 and NLI failed to synergize with the NGN2-AQ mutant that lacks DNA-binding activity (Fig. 4B) (Sun et al., 2001; Lee and Pfaff, 2003). Thus, E-box recognition by NGN2 is required for LMO4 and NLI to robustly activate NGN2-target enhancers. We also found that LMO4 and NLI similarly cooperate with NGN2 to activate a *Delta1* enhancer:LUC reporter (see Fig. S3 in the supplementary material).

Taken together, these data suggest that LMO4 and NLI are recruited to the NGN2-target enhancers in vivo and stimulate NGN2-mediated activation of neuronal genes in the developing cortex.

LMO4 enhances NGN2-mediated neuronal differentiation in the embryonic cortex

Migration of cells towards the pial surface is one of the hallmarks of neuronal differentiation in the developing cortex. NGN2 promotes both neurogenesis and the radial migratory properties of cortical neurons. The in vivo recruitment of LMO4 to NGN2-target enhancers and the potentiation of the transcriptional activity of NGN2 by LMO4 prompted us to ask whether LMO4 can facilitate NGN2-mediated differentiation and migration of cortical neurons within embryos. To address this issue directly, we implemented in utero electroporation techniques, which allow us to investigate the behavior of transfected cortical cells during embryonic development. We electroporated NGN2, LMO4 or a combination of the two, along with nuclear GFP (nGFP) as the electroporation marker, into the cerebral cortex of E14.5 embryos in utero. The embryos were collected 3 days after electroporation, and the migratory behavior of transfected cortical cells was assayed by counting the proportion of GFP$^+$ cells within the VZ and SVZ, and in the rest of the cortex (Fig. 5A,B). The electroporation of nGFP alone resulted in 40% of the electroporated cells remaining in the VZ and SVZ. As expected, the expression of NGN2 promoted differentiation and pial migration of cortical neurons, leading to a substantial reduction of nGFP$^+$ cells in the VZ and SVZ to 28%. Electroporation of LMO4 alone caused a slight and insignificant increase in migration. However, intriguingly, co-expression of LMO4 and NGN2 led to a striking increase in the radial migration of cortical neurons, as reflected in the observation that only 17% of nGFP$^+$ cells remained in the VZ and SVZ. These results indicate that LMO4 enhances NGN2-mediated neuronal differentiation within the developing cortex.

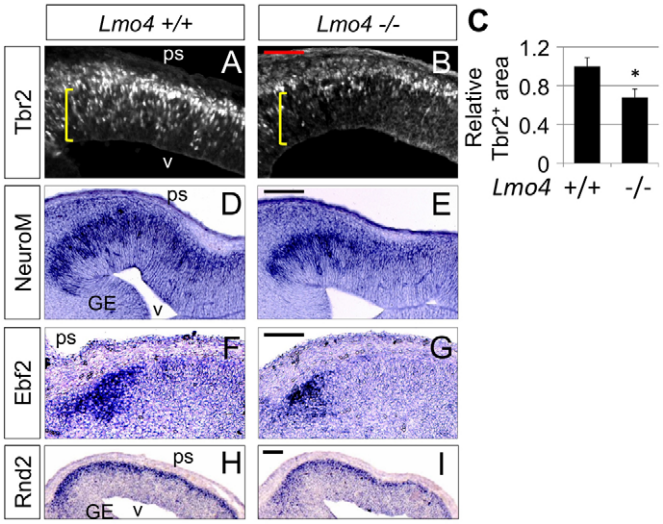

Fig. 6. Expression of NGN2 targets are reduced in the *Lmo4*-null cortex. (A-I) The marker analyses of E12.5 cortices in *Lmo4*-null and wild-type embryos using immunohistochemistry (A,B) or in situ hybridization (D-I). The images show the cortex from left hemisphere, with the dorsal side towards the right, the pial surface (ps) towards the top and the ventricle (v) towards the bottom. GE, ganglionic eminences. (**A**,**B**) The number of Tbr2$^+$ cells is drastically reduced in the *Lmo4* knockout cortex and the Tbr2$^+$ cells in the VZ (brackets) are mostly missing. (**C**) In addition, immunofluorescence of Tbr2 is generally weaker in the *Lmo4*-null cortex. The error bars represent s.e.m. **P*<0.05 in a two-tailed one-sample *t*-test. (**D**,**E**,**H**,**I**) The *NeuroM* and *Rnd2* expression domains are narrower and are more difficult to detect in more dorsal regions of the cortex in the *Lmo4*-null mice. (**F**,**G**) Strong Ebf2 expression at the base of the preplate is diminished in the *Lmo4*-null mice. Scale bars: 100 μm.

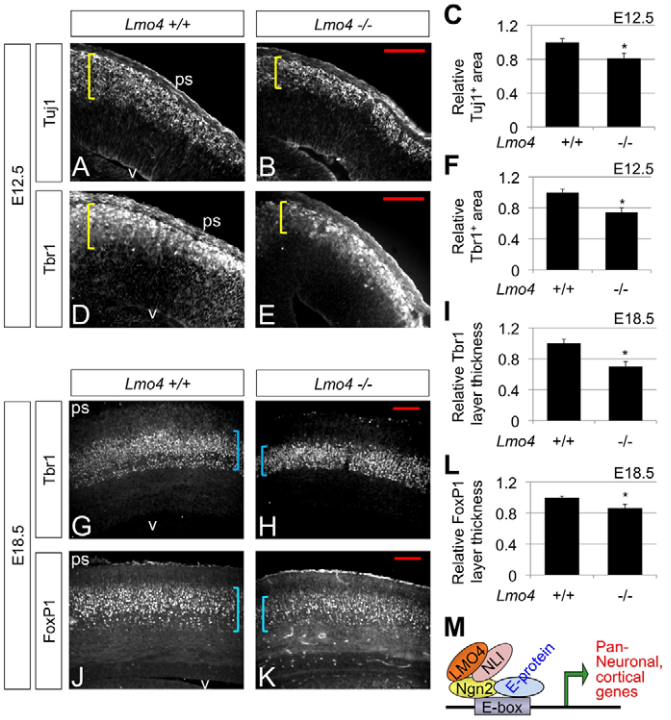

Fig. 7. Cortical neurogenesis is impaired in the *Lmo4*-null cortices. For all images, the pial surface (ps) is positioned at the top, the ventricle (v) is found on the bottom. (**A-F**) Immunohistochemical analyses of E12.5 cortices in *Lmo4*-null and wild-type embryos. The images show the cortex from the left hemisphere, with the dorsal side towards the right and the ganglionic eminences (GE) towards the left. The neuronal zone marked by Tuj1 and Tbr1 (brackets) is reduced in the *Lmo4*-null cortex. (**G-L**) Immunohistochemical analyses of E18.5 cortices. The Tbr1$^+$ and FoxP1 neuronal layers (brackets) are narrower in the *Lmo4* knockouts. (C,F,I,L) Error bars represent s.e.m. **P*<0.05 in a two-tailed one-sample *t*-test. Scale bars: 100 μm. (**M**) Model for transcriptional regulation by NGN2, LMO4 and NLI. LMO4 and NLI bind to NGN2 as a module to promote the activation of neuronal genes.

Neurogenesis is impaired in the *Lmo4*-deficient cortex

The cooperative effect of LMO4 on NGN2-mediated gene expression and neuronal differentiation raises the possibility that the loss of LMO4 results in neurogenesis defects in the dorsal telencephalon where NGN2 plays key roles for neurogenesis and specification of excitatory projection neurons (Fode et al., 2000; Parras et al., 2002). To address this possibility, we first monitored the expression of Tbr2 and *NeuroM*, which are targets of NGN2 and markers of neuronal differentiation (Seo et al., 2007; Mattar et al., 2008; Ochiai et al., 2009). We focused our analysis on non-exencephalic *Lmo4*-null cortices because exencephaly alters brain anatomy. At E12.5, Tbr2 was decreased in the SVZ, and more strikingly in the VZ of the *Lmo4*-null cortex (Fig. 6A,B). Quantification shows the Tbr2$^+$ cells were reduced by ~30% (Fig. 6C). Similarly, the expression level of *NeuroM* was reduced substantially in the *Lmo4* knockout cortex (Fig. 6D,E). We then analyzed expression of *Rnd2* and *Ebf2*, NGN2 targets that are expressed in the post-mitotic neurons of the preplate (Garel et al., 1997; Seo et al., 2007; Heng et al., 2008). The strong *Ebf2* expression at the base of the cortex was significantly reduced in the *Lmo4*-null cortex (Fig. 6F,G). *Rnd2* was expressed in a thinner domain in the *Lmo4* knockout cortex, which was most apparent in the medial region of the cortex (Fig. 6H,I). These results suggest that NGN2-target gene expression is compromised in the *Lmo4*-deficient cortex.

The Tuj1$^+$ neuronal domain was reduced by ~20% in *Lmo4*-null mice (Fig. 7A-C), underlining an impairment of neurogenesis in the *Lmo4* knockout cortex. We also analyzed the specification of cortical projection neurons, which requires NGN2 function, using a cortical neuronal specific marker Tbr1. At E12.5, the Tbr1 expression domain encompassing the neurons of the subplate is decreased by ~25% in the *Lmo4*-null embryo (Fig. 7D-F). By E18.5, the cortex is characterized by several molecularly and functionally distinct neuronal layers, and Tbr1 marks the subplate and layer VI of the cortical plate (Hevner et al., 2001). At this stage, we found that the Tbr1 expression domain remains significantly thinner in the *Lmo4* null compared with the wild type (Fig. 7G-I), indicating that the decrease in early-born cortical neurons at E12.5 is maintained until later stages of development in the *Lmo4*-deficient mice. Notably, the FoxP1$^+$ domain, which marks cortical layers III to V (Ferland et al., 2003), also became ~15 % narrower in the *Lmo4*-null cortex compared with the wild-type cortex (Fig. 7J-L), suggesting that neuronal reduction in the *Lmo4*-null cortex is not limited to layer VI or the subplate, but encompasses early-born and later-born neurons.

Taken together, these data highlight that LMO4 plays an important role in cortical neurogenesis.

DISCUSSION

The timely generation of numerous types of neurons at precise locations is determined by complex genetic networks that involve many transcription factors, often functioning in combination with each other. Families of bHLH and LIM-HD transcription factors play crucial roles in the regulatory networks for neurogenesis and neuronal subtype specification in telencephalic development. However, the molecular events that are controlled by LMO genes in this context are poorly understood.

The lack of DNA-binding activity of LMO proteins and the strong interactions between LMOs and NLI led to a prediction that LMOs influence transcription negatively by inhibiting the formation of complexes consisting of LIM-HD factors and NLI. This prediction is supported by studies in *Drosophila* and in the vertebrate spinal cord (Milan et al., 1998; Milan and Cohen, 1999; Thaler et al., 2002; Lee et al., 2008; Joshi et al., 2009; Song et al., 2009). Interestingly, several studies revealed that LMOs can also act as a linker to recruit NLI and other transcription factors in a multi-protein complex, which binds to target genes and stimulates the transcription of target genes (Wadman et al., 1994; Wadman et al., 1997; Joshi et al., 2009).

In this study, we set out to find proteins that associate with the LMO4:NLI module and discovered NGN2 as a novel interactor. Based on our results, we propose that LMO4 and NLI bind to each other while simultaneously associating with NGN2, thereby forming a transcriptional complex (Fig. 7M). We also show that LMO4 and NLI promote NGN2-mediated transactivation of pan-neuronal and cortex-specific genes by binding to NGN2-target enhancers in the developing cortex. Consistently, we found that the loss of LMO4 results in neurogenesis defects in the cortex, whereas expression of LMO4 facilitates NGN2-mediated differentiation of cortical neurons. Our studies provide a seldom-studied in vivo link between bHLH factors and their co-factors, and suggest an additional layer of regulation in bHLH factor-mediated gene transcription in CNS development.

LMO4 function in the cortex

In the *Lmo4*-null cortex, the expression of multiple NGN2-target genes, β-III tubulin, FoxP1 and Tbr1 is substantially reduced, suggesting that LMO4 plays an important role in cortical neuronal differentiation at least partly by supporting NGN2-mediated gene transcription. However, it is noteworthy that LMO4 expression in the cortical plate varies from the anterior to the posterior end at P0, leaving a prominent gap in expression in the medial cortex (Sun et al., 2005). This expression pattern appears to be important for the proper division of cortical areas as loss of LMO4 causes the expression of cortical regionalization markers to shift along the anteroposterior axis and alters the shape of the somatosensory cortex (Huang et al., 2009). Given that Tbr1 is also involved in cortical regionalization (Rubenstein and Rakic, 1999), our observation of the thinner $Tbr1^+$ layer in the E18.5 *Lmo4*-null cortex may be linked to a defect in cortical regionalization as well as an impairment of neuronal differentiation. Combined with the previous report (Huang et al., 2009), our findings raise interesting questions about whether the neurogenic activity of LMO4 varies along the anteroposterior axis and whether it has a bearing on its patterning function. Further studies will be needed to resolve these issues.

LMO4 and NLI may function as common co-factors of proneural bHLH factors

Members of the proneural bHLH family of transcription factors share similar structures and functions. During development, a number of them are expressed in different domains that encompass the nervous system, triggering neurogenesis on a broad scale. This is best illustrated by NGN2 and mammalian achaete-scute homolog (MASH1, ASCL1), which is also responsible for initiating neurogenesis. NGN2 and MASH1 often share pan-neuronal targets such as *Delta1*, and the complementary expression of these two genes allows expression of *Delta1* throughout the SVZ (Castro et al., 2006). Through the course of this study, we found that LMO4 and NLI interact with MASH1 and potentiate MASH1-mediated transcription (data not shown). Thus, it will be interesting to test whether the LMO4:NLI module plays a role in MASH1-dependent neuronal differentiation, functioning as a key co-factor module for neurogenesis.

Interestingly, NLI has been reported to interact with a few bHLH proteins such as NGN2, NeuroM and SCL during the specification of motoneurons and V2 interneurons in the spinal cord (Lee and Pfaff, 2003; Ma et al., 2008; Joshi et al., 2009). Moreover, CHIP and dLMO (BX – FlyBase), the *Drosophila* homologues of NLI and LMO4, respectively, bridge the components of a transcriptional complex containing the proneural bHLH protein achaete-scute, during thorax compartmentalization and thoracic sensory bristle development (Ramain et al., 2000; Asmar et al., 2008; Zenvirt et al., 2008). Thus, it is possible that the LMO4:NLI module functions as a co-factor of multiple proneural bHLH factors, allowing LMO4 to affect neurogenesis broadly. For example, considering the co-expression of NGN2, LMO4 and NLI in differentiating motoneurons (Lee and Pfaff, 2003; Lee et al., 2005; Lee et al., 2008; Ma et al., 2008), the NGN2:LMO4:NLI complex may play a role in this context.

Our data support a model in which a weak interaction between LMO4 and NGN2 is stabilized by NLI, leading to the formation of a multi-protein complex. In the future, it will also be interesting to test whether the complex becomes further stabilized when bound to E-box elements.

LMO proteins and neural development

LMO family members bear significant similarity to one another within the LIM domains that constitute the bulk of the proteins (Kenny et al., 1998), pointing to the possibility that they have similar functions. Supporting this possibility, we discovered that LMO1 interacts with NGN2 and activates the *Delta1* enhancer in a NGN2-dependent manner, similar to LMO4 (data not shown). Previous studies have also shown that LMO1, LMO3 and LMO4 interact with another neural bHLH factor, HEN1, and that *Xenopus* LMO3 cooperates with HEN1 during the activation of neuronal gene expression (Bao et al., 2000; Manetopoulos et al., 2003). Thus, it is possible that multiple LMO-proneural bHLH combinations form during CNS development and that these combinations contribute to the regulation of neuronal differentiation and subtype specification in specific areas of CNS. In addition, each LMO may have its own unique function in addition to their shared activity in neurogenesis, similar to the relationship between NGN2 and MASH1 in the cortex. Of note, LMO1, LMO2 and LMO4 play different roles in regulating glycoprotein hormone α-subunit gene in the pituitary glands (Susa et al., 2010). The specific and shared functions of LMO proteins need to be explored further using genetic and molecular tools, which would provide a better understanding of the transcriptional control of neuronal differentiation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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