Stem Cells As a Tool for Studying the Developmental Regulation of Gene Expression

Hynek Wichterle, PhD,[1] Michael Closser, BS,[1]
Esteban O. Mazzoni, PhD,[2]
Shaun Mahony, PhD,[3] Yuchun Guo, PhD,[4]
Rachel Kopunova,[1] and David K. Gifford, PhD[4]

[1]Departments of Pathology and Cell Biology,
Neurology, and Neuroscience
Center for Motor Neuron Biology and Disease,
Columbia Stem Cell Initiative
Columbia University Medical Center
New York, New York

[2]Department of Biology
New York University
New York, New York

[3]Department of Biochemistry and Molecular Biology
Center for Eukaryotic Gene Regulation
The Pennsylvania State University
University Park, Pennsylvania

[4]Computer Science and Artificial Intelligence Laboratory
Massachusetts Institute of Technology
Cambridge, Massachusetts

SfN Society for Neuroscience

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Introduction

Despite extensive functional analysis of transcription factors, the detailed mechanisms by which they regulate gene expression and specify cell identity in developing organisms remain poorly understood. Recent advances in chromatin mapping technologies have provided unprecedented insight into the organization of regulatory regions, chromatin structure, and the exact positions of transcription factor binding sites. The emerging picture of extremely plastic chromatin organization prevents simple extrapolation of a regulatory landscape from one cell lineage or even one developmental stage to another. We have developed a pluripotent stem cell–based differentiation system that facilitates systematic mapping and probing of transcriptional regulatory networks that control the specification of spinal motor neuron identity. The systematic analysis of mechanisms controlling cell type–specific regulation of gene expression is facilitated by combining inducible stem cell lines, in which gain-of-function studies can be performed, with unlimited access to relatively homogenous populations of cells differentiating along the motor neuron lineage. Identifying regulatory motifs, transcription factors, and cofactors engaged in the specification of motor neuron identity provides novel insights into ways to efficiently program and derive clinically relevant cell types.

Progress in Cell Programming

Recent progress in programming cell fate using transcription factors has given hope to those pursuing the goal of producing clinically relevant cell types for modeling disease and developing new therapeutic strategies. Muscle cells, pluripotent stem cells, pancreatic beta cells, hepatocytes, and several types of neurons have all been created by the forced expression of transcription factor combinations known as "programming modules" (Tapscott et al., 1988; Mann and Carroll, 2002; Takahashi and Yamanaka, 2006; Zhou et al., 2008; Son et al., 2011). However, the process of transcriptional programming remains largely enigmatic. Understanding the mechanism through which programming modules convert one expression profile to another would accomplish two main goals: illuminating the process of cell-fate specification during normal embryonic development, and aiding the rational design of programming modules for producing cell types that are difficult to generate using available methodologies.

Motor neurons are cholinergic cells located in the ventral and caudal CNS, whose developmental program is particularly well mapped (Jessell, 2000). Spinal somatic motor neurons innervating skeletal muscles are derived from the ventral spinal progenitor domain and are characterized by the coexpression of Isl1, Lhx3, and Hb9 (Mnx1) at the time of their birth (Jessell, 2000). The combined expression of Isl1, Lhx3, and Ngn2 transcription factors (NIL factors) is sufficient to bestow spinal motor neuron identity on dorsal spinal progenitors and on spinal progenitors derived from embryonic stem cells (ESCs) (Lee and Pfaff, 2003; Hester et al., 2011). This finding indicates that NIL factors act as a principal motor neuron identity–specifying programming module.

To study the process of motor neuron programming, we established inducible ESC lines that harbor the NIL programming module under the control of doxycycline (Dox)–regulated promoter (TetO) (Iacovino et al., 2011; Mazzoni et al., 2011). We demonstrated that NIL induction in differentiating ESCs results in rapid and highly efficient specification of spinal motor neuron identity. Taking advantage of these robust and efficient programming systems, we mapped genome-wide binding sites of programming factors in both inducible lines (Mazzoni et al., 2013). Computational analysis of occupied *cis*-regulatory elements demonstrated that Isl1 directly interacts and synergizes with Lhx3. The Isl1/Lhx3 heterodimers cooperate with additional *cis*-regulatory elements to establish active enhancers controlling the expression of motor neuron genes.

Results

Specification of cells expressing spinal motor markers upon inducible expression of Ngn2, Isl1, and Lhx3

To study the programming of spinal and cranial motor neuron identity, we generated two Dox-inducible ESC lines (Mazzoni et al., 2011), one of which harbors a polycistronic expression construct in which the open reading frames of spinal motor neuron determinants *Ngn2*, *Isl1*, and *Lhx3* (Lee and Pfaff, 2003; Hester et al., 2011; Lee et al., 2012) are separated by 2A peptides (the iNIL line) (Fig. 1A). NIL factors were previously shown to activate the specification of motor neuron identity in retinoic acid (RA)–treated differentiating ESCs (Hester et al., 2011; Lee et al., 2012). We established that NIL factors are sufficient to induce the expression of spinal motor neuron markers even in the absence of RA. Treating differentiating ESCs with Dox resulted

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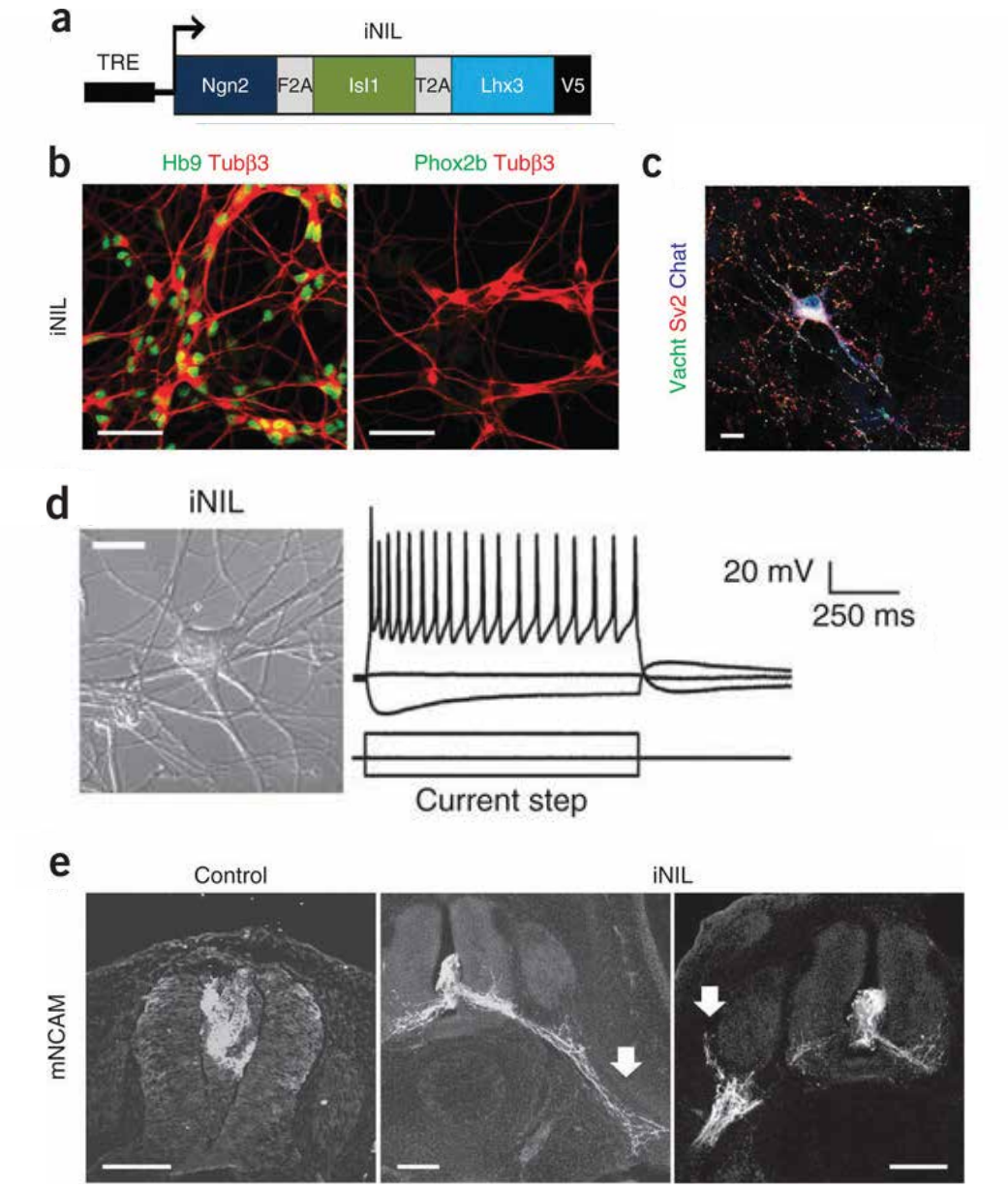

Figure 1. Ngn2, Isl1, and Lhx3 (NIL) transcription factors program spinal motor neurons. ***A***, Schematic representation of Dox-inducible NIL programming modules. TRE: tetracycline response element, F2A, T2A–2A peptide sequences from foot-and-mouth disease virus. ***B***, In the absence of patterning signals, NIL-programmed spinal motor neuron exhibit neuronal morphology with multiple Tuj1 immunoreactive processes, express Hb9, but do not express the cranial marker Phox2b. Day 2 embryoid bodies treated with Dox for 48 h were dissociated, plated on laminin-coated substrate, and analyzed 24 h later. ***C***, NIL-programmed cells contain cholinergic synaptic vesicles. Dissociated iNIL cells induced with Dox were cultured on astrocyte monolayers for 7 d and stained with the synaptic marker SV2 and the cholinergic markers Vacht and Chat. ***D***, NIL-programmed neurons cultured for 7 d on astrocyte monolayers fire repetitive action potentials. Calibration: 20 mV, 250 ms. ***E***, Control and Dox-induced day 4 embryoid bodies were implanted into the stage 16 developing chick cervical spinal cord *in vivo*. Embryos were fixed 2 d later, sectioned, and stained with a mouse-specific NCAM antibody. Dense bundles of axons emanating from NIL-induced transplants were observed within the ventral root and in axial (left arrow) and limb (right arrow) nerve branches (4 of 5 successfully transplanted embryos). Scale bars: ***B***, 50 µm; ***C***, 10 µm; ***E***, 100 µm. Reprinted with permission from Mazzoni et al. (2013), their Figs. 1*a, b, f, g, h*.

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in robust induction of the tricistronic transgene 24 h later. Interestingly, despite continuing Dox treatment, Ngn2 expression was extinguished in most cells by 48 h, consistent with its transient pattern of expression in cells transitioning from progenitors to postmitotic motor neurons (Mizuguchi et al., 2001; Novitch et al., 2001).

NIL-expressing cells plated on laminin adopted typical neuronal morphology, expressed neuronal marker class III beta tubulin (Tubβ3, recognized by the Tuj1 antibody) and spinal motor neuron marker Hb9, and were negative for the cranial motor neuron marker Phox2b (Fig. 1*B*). Quantification revealed that the majority of transgenic cells (labeled by anti-V5 antibodies) expressed the postmitotic neuronal marker NeuN (99.72% ± 0.27% of $V5^+$ cells express NeuN) and the spinal motor neuron marker Hb9 (99.82% ± 0.17% express Hb9) but rarely expressed the cranial motor neuron marker Phox2b (0.24% ± 0.28% express Phox2b).

Functional characterization of induced NIL neurons

To determine whether transcriptionally programmed cells acquired key properties of mature motor neurons, we cultured induced NIL cells alone or on monolayers of primary cortical mouse astrocytes for 7–10 d. Immunostaining of NIL cells cultured on monolayers of astrocytes revealed dense arrays of synapses marked by the synaptic vesicle marker SV2 (Fig. 1C). Significantly, many of the synapses exhibited accumulation of vesicular acetylcholine transporter (Vacht, Slc18a3) and choline acetyltransferase (Chat)—markers of mature cholinergic cells (Fig. 1C). Electrophysiologically mature motor neurons fire trains of action potentials upon depolarization (Miles et al., 2004). Whole-cell patch–clamp recordings of NIL-induced cells cultured on astrocytes for 7 d demonstrated that action potentials could be evoked by 20–150 pA, 1 s current injection in all cells tested. Furthermore, nearly all patched cells (11/12 NIL cells) fired trains of action potentials, sustained for the duration of the depolarizing current step (Fig. 1*D*). Together, these observations demonstrate that inducible expression of NIL programming modules is sufficient to differentiate ESCs into electrically mature cholinergic neurons.

Motor neurons project axons outside of the CNS to innervate peripheral synaptic targets. To examine whether induced motor neurons (iMNs) acquired this defining characteristic, we implanted control, iNIL cells treated with Dox from day 2 to day 4 of differentiation into the developing cervical and brachial neural tube of developing chick embryos (Wichterle et al., 2002, 2009). Two days after implantation of iNIL neurons, we detected robust outgrowth of axons (labeled by mouse-specific neural cell adhesion molecule [NCAM] antibody) exiting spinal cord via the ventral root and extending along all major spinal motor nerves (4 out of 5 successfully transplanted embryos, Fig. 1*E*, right panels). In contrast, axons of control transplants stayed within the spinal cord and failed to project to the periphery (Fig. 1*E*, left panel). These results indicate that induced expression of the NIL module programs cell phenotypes that are by all examined criteria consistent with spinal and cranial motor neuron identities (hereafter referred to as "induced spinal motor neurons").

Changes in gene expression profiles accompanying motor neuron programming

Effective programming of ESCs into motor neurons should be accompanied by a repression of the stem cell expression program and induction of the spinal or cranial motor neuron–specific transcriptome. Global expression profiling using GeneChIP ST arrays (Affymetrix, Santa Clara, CA) revealed that 48 h Dox treatment of iNIL cells resulted in a dramatic change in gene expression profile (3185 genes > twofold differentially expressed following NIL induction; $p < 0.001$) (Figs. 2*A*, *B*). Induction of the NIL programming module extinguished the expression of pluripotency genes (*Oct4*, *Nanog*) and upregulated generic motor neuron genes (endogenous *Isl1*, *Ebf1/3*, *Onecut1/2*), cholinergic genes (*VAChT, Chrnb4*), and genes encoding axon guidance molecules (*Nrp1*, *Robo1/2*, *Dcc*) (Fig. 2*A*).

We set out to examine how closely programmed neurons correspond to motor neurons differentiated from ESCs using the normal patterning signals RA and sonic hedgehog (Hh). To do so, we compared the expression profiles of fluorescence-activated cell sorting (FACS)–purified Hb9-GFP^+ RA/Hh motor neurons on day 5 of differentiation with Hb9-GFP+ cells purified from iNIL cultures treated with Dox for 48 h. We found that the iMNs were remarkably similar to RA/Hh–generated motor neurons (Fig. 2B). Most genes (97.4%) were expressed at levels that were not significantly different between the two samples ($p < 0.001$), and only 1.6% of all

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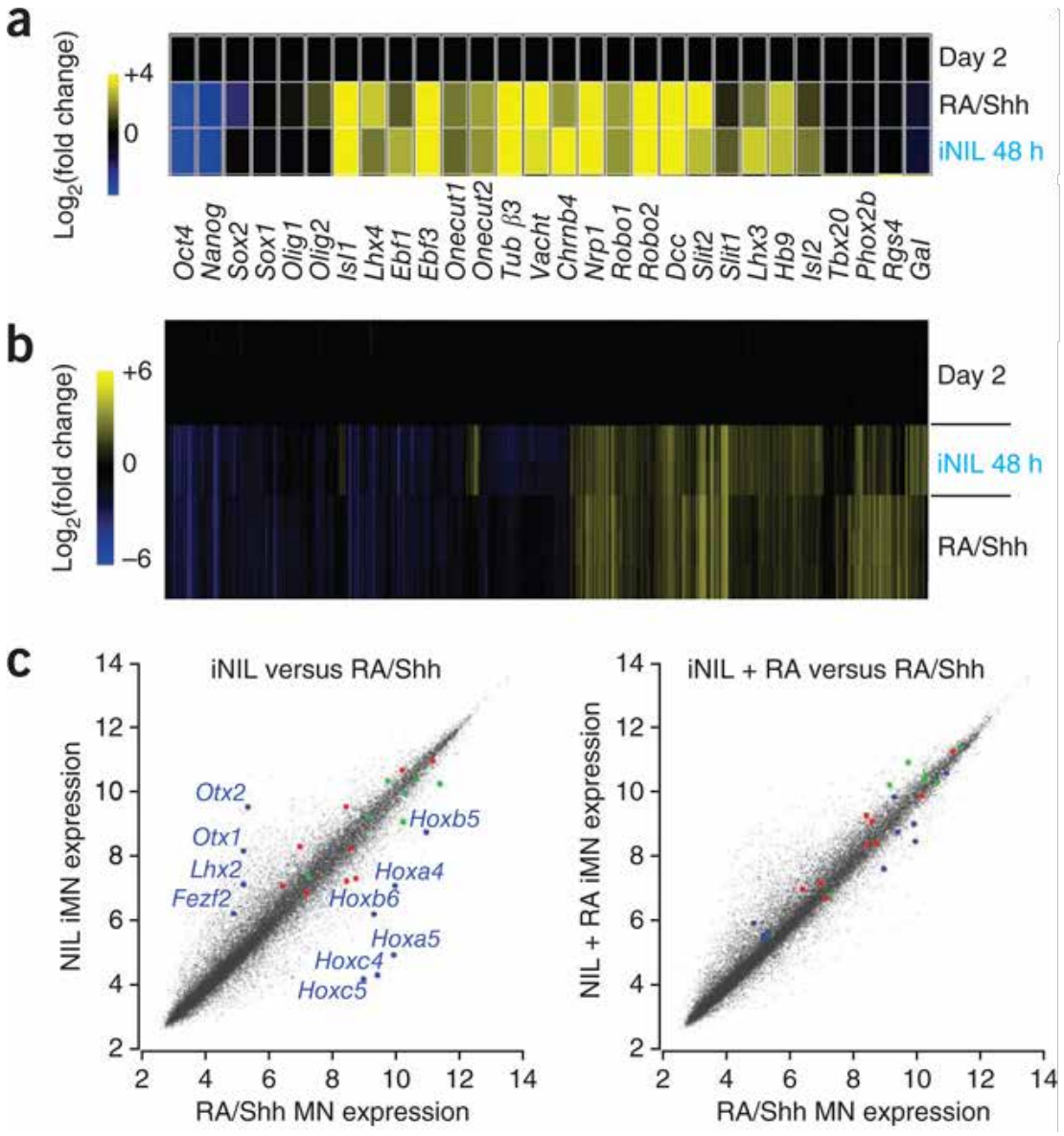

Figure 2. NIL factors induce spinal motor neuron transcriptome but fail to specify caudal identity. ***A***, The expression of a relevant subset of genes reveals the identity of NIL-programmed cells. Heat map of average expression of genes associated with motor neuron identity in day 2 embryoid bodies, RA/Hh–derived spinal motor neurons (day 6 FACS-purified spinal motor neurons following 4 d of differentiation by RA/Hh treatment), and NIL-programmed neurons induced for 48 h with Dox. ***B***, NIL expression induces a spinal motor neuron–specific transcriptome. Clustergram of all differentially expressed genes in day 2 embryoid bodies, Dox-treated iNIL cells, and RA/Hh differentiated motor neurons. ***C***, RA imposes cervical identity onto NIL-programmed spinal motor neurons. Left: scatter plot of mRNA expression intensities in Dox-induced iNIL cells versus RA/Hh–differentiated spinal motor neurons. Right: scatter plot of mRNA expression intensities in Dox-induced iNIL cells treated with 1 μM RA for 48 h versus RA/Hh–differentiated spinal motor neurons. Color code of highlighted genes: Rostrocaudal patterning genes (blue); spinal motor neuron–associated transcription factors (red); spinal motor neuron–associated receptors and enzymes (green). Reprinted with permission from Mazzoni et al. (2013), their Figs. 2*b, d, e*.

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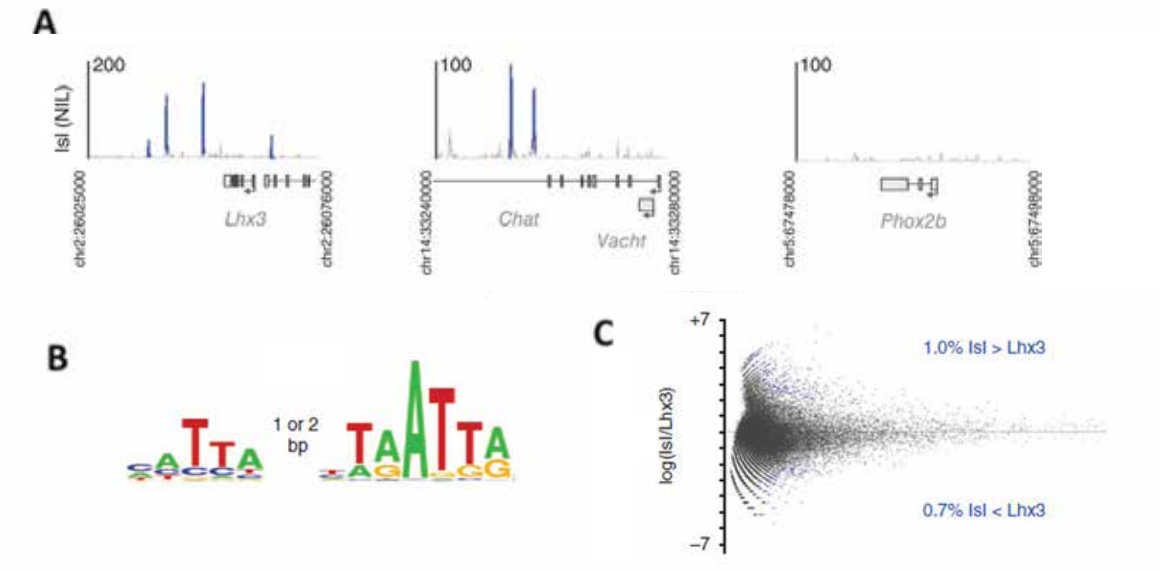

Figure 3. Isl1 and Lhx3 bind to many common sites harboring a complex homeodomain motif. ***A***, Isl ChIP-seq signals over Lhx3, Chat, and Phox2b. Blue peaks represent significant ($p < 0.01$) read enrichment over control. Genomic loci coordinates are shown next to the *x*-axis. ***B***, Primary DNA motifs overrepresented under enriched peaks obtained from Isl ChIP-seq experiments in iNIL cells treated for 48 h with Dox. ***C***, Lhx3 colocalizes with Isl genomic binding sites in iNIL cells. Comparison of read enrichment from Isl with Lhx3 at all detected peaks. Blue represents peaks significantly differentially enriched for Isl or Lhx3 binding. Adapted with permission from Mazzoni et al. (2013), their Figs. 4*a, 5b, c*.

genes exhibited divergent expression (i.e., they were induced in one cell type but repressed in the other). While key motor neuron–specific genes were correctly regulated, a set of genes controlling rostrocaudal neural identity and motor neuron subtype identity was differentially expressed in RA/Hh and induced iNIL cells (Fig. 2C). Induced iNIL motor neurons expressed low levels of *Hox* transcription factors and high levels of rostral neural markers (*Otx1*, *Otx2*). To rectify this difference, we asked whether programmed iNIL motor neurons would be responsive to the caudalizing RA signal (Wichterle et al., 2002; Mahony et al., 2011). Treatment of iNIL cells with RA during Dox treatment resulted in correct specification of cervical spinal identity, marked by the expression of *Hox* genes from paralogous groups 4 and 5 and suppression of rostral markers *Otx1/2* (Fig. 2C). Thus, although programmed cells acquire generic motor neuron identity following induction of NIL factors, the specification of rostrocaudal subtype identity depends on the treatment of the cells with caudalizing patterning signals.

Isl binds to a large number of genomic regions

Efficient and rapid transcriptional programming of ESCs into cells exhibiting fundamental motor neuron properties provides an ideal system in which to study whether individual transcription factors act independently or engage in synergistic interactions. We performed chromatin immunoprecipitation–sequencing (ChIP-seq) analyses of Isl1 in iNIL cells 48 h after Dox induction. Inducible Isl1 factor was not epitope-tagged, and therefore, we optimized ChIP using a pool of monoclonal antibodies raised against Isl1. Because these antibodies cross-react with both Isl1 and the closely related Isl2 transcription factor, we refer to the data as Isl ChIP-seq. We observed extensive Isl recruitment to genomic loci in the iNIL-induced cells (Fig. 3A). We identified approximately 22,000 significant Isl binding events characterized by the presence of a canonical homeodomain binding motif (Fig. 3B) at the majority of binding sites.

Next, we examined whether identified Isl binding sites are distributed randomly across the genome or whether their position correlates with tissue-specific *cis*-regulatory elements. We took advantage of project data from ENCODE (Encyclopedia of DNA Elements) that identified putative regulatory regions in mouse ESCs, whole brain, heart, kidney, liver, and spleen, defined using combinations of DNaseI hypersensitivity and enrichment in H3K4me1 and H3K27ac histone modifications. Of all tissues examined, Isl binding sites correlated best with

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whole-brain putative regulatory regions. Interestingly, the overlap with regulatory regions in ESCs was as low as in unrelated tissues. These findings indicate that expressed NIL factors are not passively recruited to existing stem cell regulatory regions, but rather, actively engage neuronal regulatory regions.

Lhx3 co-occupies binding sites with Isl to specify motor neuron cell fate

Previous analysis of the spinal motor neuron–specific *Hb9* enhancer revealed that Isl1 forms a multimeric complex with Lhx3, Ldb1, and Ngn2 or Neurod4 (Lee and Pfaff, 2003). We therefore asked whether Lhx3 co-occupies other sites selectively bound by Isl in the iNIL cell line. Taking advantage of the V5 epitope tag on the *Lhx3* transgene (Mazzoni et al., 2011), we performed ChIP-seq analysis of Lhx3 binding in the iNIL cells 48 h after Dox induction. We identified 47,908 Lhx3 binding sites in the genome and found that these sites are highly coincidental with the sites occupied by Isl in the iNIL cell line. We observed that only 1.7% of all Isl sites were significantly differentially enriched ($p < 0.001$) (Fig. 3C). These findings suggest that Isl1 and Lhx3 bind to DNA as a heterodimer during spinal motor neuron differentiation. Previously, it had been shown that purified Isl1 and Lhx3 transcription factors interact in solution (Lee and Pfaff, 2003). Coimmunoprecipitation experiments confirmed this Isl1/Lhx3 interaction in induced iNIL cells, indicating that motor neuron identity is encoded by cooperative recruitment of Isl/Lhx3 transcriptional complexes to cell type–specific enhancers.

A subset of Isl/Lhx3–cobound sites is characterized by a gain of H3K27ac modification

Programming factors bind throughout the genome in iMNs, suggesting regulatory potential, but how Isl/Lhx3 binding correlates with function has yet to be examined. To investigate this correlation, we performed ChIP-seq for enhancer-associated histone modification H3K4me1 and a modification associated with activated enhancers H3K27ac (Fig. 4). A comparison of these modifications' locations in

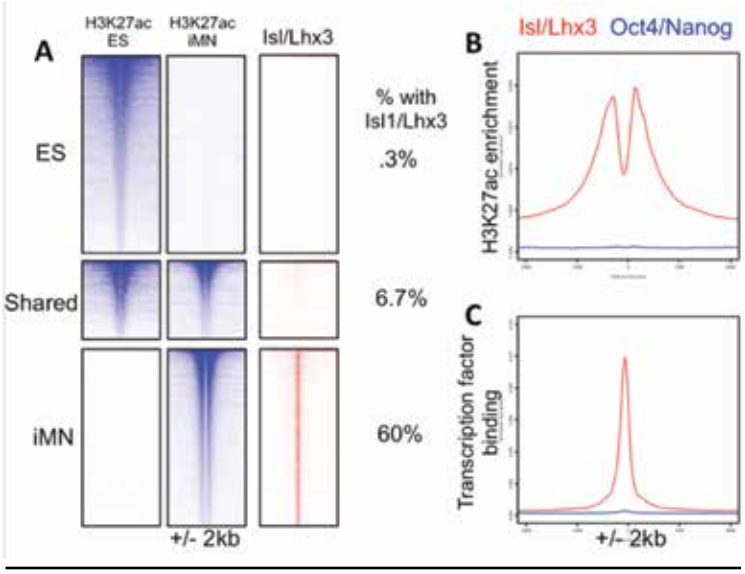

Figure 4. Isl1 and Lhx3 binding is accompanied by acetylation of H3K27 histone. ***A***, Line plots of ChIP-seq profiling of H3K27ac modification revealed a dramatic shift in these activated regulatory regions between ESCs and iMNs. The majority of Isl/Lhx3 binding sites coincides with the newly gained H3K27ac mark. ***B***, Cumulative quantitative analysis of H3K27ac level at Isl/Lhx3 binding sites in iMNs (red line) and in ESCs (blue line). ***C***, Genomic sites bound by Isl and Lhx3 (red line) in iMNs are not occupied by Oct4, Nanog, or Sox2 transcription factors in ESCs (blue line).

ESCs and iMNs revealed dramatic remodeling of the chromatin regulatory landscape. Remarkably, a large fraction of newly gained active enhancers (H3K27ac) coincided with Isl/Lhx3 binding (Figs. 4*A*, *B*), yet a significant fraction of Isl/Lhx3 binding sites lacked the H3K4me1 or H3K27ac modifications associated with functional regulatory regions. We identified 14,000 or 63% of Isl/Lhx3 binding events to coincide with putative regulatory elements marked by enhancer-associated histone modifications. Interestingly, approximately 6000 (40%) distal regulatory elements contained H3K27ac, the mark of active enhancers. Together, these data suggest that Isl/Lhx3 binding has a global regulatory function in enhancer recruitment and activation during iMN identity programming. We were able to annotate transcription factor binding into three categories, based on chromatin modifications around transcription factor binding sites. Active regions were marked by H3K4me1 and H3K27ac, primed regions displayed H3K4me1 but no H3K27ac, and inactive regions were identifiable by transcription factor binding in the absence of chromatin modifications. Significantly, transcription factor binding alone was not sufficient to bring about enhancer-associated chromatin modifications, and therefore, it was not sufficient to identify active enhancers in a given cell type.

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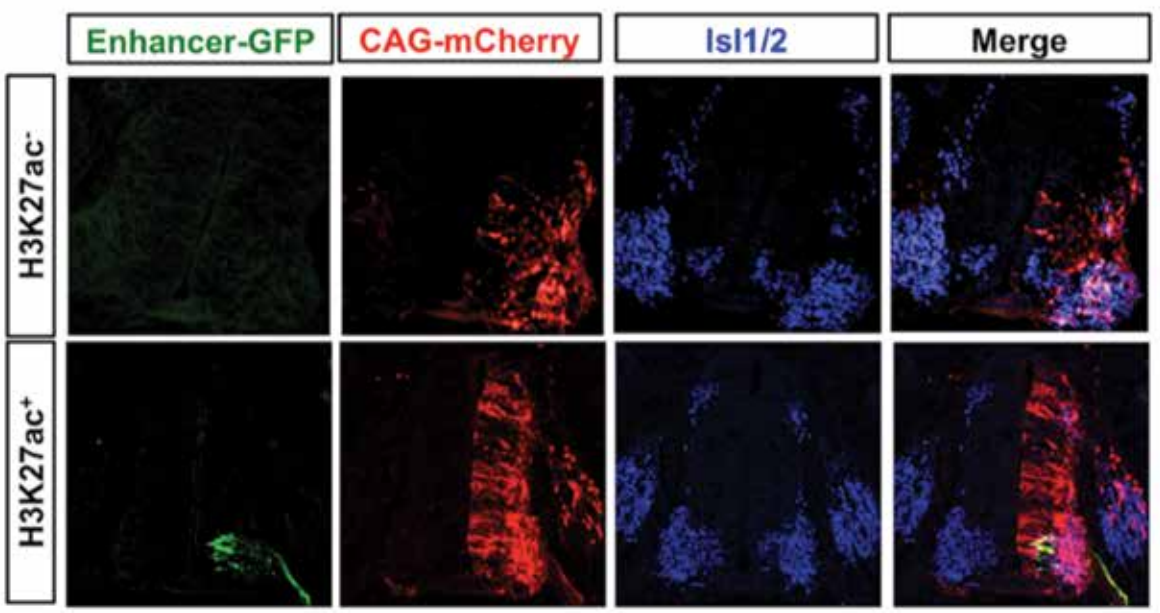

Figure 5. *Cis*-regulatory motifs contribute to the activity of Isl/Lhx3 binding sites. Expression of GFP reporter plasmids carrying distal Isl/Lhx3–bound enhancer lacking H3K27ac modifications (top series) or containing a high level of H3K27ac modifications (bottom series). Retention of enhancer activity in a novel genomic context (proximal enhancer) indicates that local *cis*-regulatory elements control the activity of individual Isl/Lhx3 binding sites.

Cis-regulatory elements distinguish between active and inactive Lhx3/Isl–bound enhancers

The identification of Lhx3/Isl binding sites with distinct chromatin signatures raised the possibility that either a global chromatin architecture or the presence of local *cis*-regulatory elements might modify the active/inactive status of individual binding sites. To test this hypothesis, we performed reporter assays *in vivo* using electroporation of cloned enhancer constructs driving a green fluorescent protein (GFP) reporter (Fig. 5). Upon electroporation into the developing chick spinal cord, we observed robust expression of active enhancers with little to no induction of inactive enhancers. These results suggest that even when Isl/Lhx3 enhancers are taken out of their genomic context, they maintain their levels of activity, suggesting a role for *cis*-regulatory sequences in Isl/Lhx3–mediated enhancer activation. These data also indicate a potential role for additional *cis*-regulatory factors in activating motor neuron enhancers that are bound by Isl/Lhx3.

Conclusions

Pluripotent stem cells have been used during the past three decades as a convenient tool to model and study aspects of normal embryonic development. The recent development of powerful sequencing-based approaches for studying transcription factor function has opened the door to systematically analyzing the mechanisms that underlie the developmental programming of gene expression and the specification of cell identity. However, the effective deployment of these biochemical approaches will critically depend on access to a significant quantity of homogenous cell populations. Here we demonstrated how combining an inducible stem cell differentiation system with transcription factor binding studies, chromatin analysis, and gene expression profiling can reveal the fundamental molecular mechanisms underlying the specification of spinal motor neuron identity during embryonic development.

Acknowledgments

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