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The Isl1-Lhx3 Complex Promotes Motor Neuron Specification by Activating Transcriptional Pathways that Enhance its Own Expression and Formation

Transcriptional Regulation of Isl1-Lhx3 in Motor neurons

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41 **Abstract**

42 Motor neuron progenitor cells rapidly induce high expression of the transcription factors
43 Isl1, Lhx3, and the transcriptional regulator LMO4, as they differentiate. While these
44 factors are critical for motor neuron specification, the mechanisms regulating their
45 precise temporal and spatial expression patterns are not well characterized. Isl1 and
46 Lhx3 form the Isl1-Lhx3 complex, which induces the transcription of genes critical for
47 motor neuron specification and maturation. Here we report that *Isl1*, *Lhx3* and *Lmo4* are
48 direct target genes of the Isl1-Lhx3 complex. Our results show that specific genomic loci
49 associated with these genes recruit the Isl1-Lhx3 complex to activate the transcription of
50 *Isl1*, *Lhx3* and *Lmo4* in embryonic motor neurons. These findings support a model in
51 which the Isl1-Lhx3 complex amplifies its own expression through a potent
52 autoregulatory feedback loop and simultaneously enhances the transcription of *Lmo4*.
53 LMO4 blocks the formation of the V2 interneuron-specifying Lhx3 complex. In
54 developing motor neurons, this action inhibits the expression of V2 interneuron genes
55 and increases the pool of unbound Lhx3 available to incorporate into the Isl1-Lhx3
56 complex. Identifying the pathways that regulate the expression of these key factors
57 provides important insights into the genetic strategies utilized to promote motor neuron
58 differentiation and maturation.

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64 **Significance Statement:**

65 The precise temporal and spatial regulation of transcription factor expression is critical
66 for embryos to generate the appropriate number and variety of motor neurons. This
67 process dictates the formation of motor circuits, which regulate coordinated movement
68 and homeostasis. When motor neuron specification is impaired, it leads to serious
69 medical conditions such as spinal muscular atrophy. Understanding motor neuron
70 development is crucial for effectively treating pediatric motor neuron disorders and
71 neurodegenerative disorders, such as amyotrophic lateral sclerosis. Here we show that
72 three essential factors for motor neuron development, Isl1, Lhx3 and LMO4 are induced
73 directly by the Isl1-Lhx3 complex. Characterizing the pathways that direct the
74 expression of these factors provides key insights into the genetic mechanisms that
75 regulate motor neuron development.
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78 **Introduction**

79 Combinatorial expression of specific transcription factors establishes discrete progenitor
80 domains, in the embryonic spinal cord, which each generate distinct types of neurons
81 (Jessell, 2000; Lee and Pfaff, 2001). The p0-p3 domains generate ventral interneurons
82 and the pMN domain generates motor neurons (MNs) (Jessell, 2000; Lee and Pfaff,
83 2001). While the signaling cascades that establish the pMN domain are well
84 characterized, the mechanisms that promote the initiation and maintenance of
85 transcription factor expression in developing MNs remain unclear. As these factors are
86 critical for MN specification and diversification, understanding the pathways that
87 regulate their expression will provide important insights into this process.

88 Immediately prior to differentiation, pMN cells express two LIM-homeodomain
89 transcription factors, Islet-1 (Isl1) and LIM-homeobox 3 (Lhx3) (Mizuguchi et al., 2001;
90 Roy et al., 2012). Both proteins contain two LIM domains, that facilitate protein-protein
91 interactions, as well as a single homeodomain, that binds DNA (Bhati et al., 2008,
92 2012). When co-expressed, Isl1 and Lhx3 interact with each other and with Nuclear Lim
93 Interactor (NLI) to form a hexameric transcription complex, called the Isl1-Lhx3
94 complex, with a 2:2:2 stoichiometry (Thaler et al., 2002). When Lhx3 is expressed in the
95 absence of Isl1, as is the case in developing V2 interneurons, Lhx3 and NLI form the
96 tetrameric Lhx3 complex, with a 2:2 stoichiometry (Thaler et al., 2002). The Isl1-Lhx3
97 complex primarily functions through binding to the Long Hexamer Response Element
98 (HxRE-Long), and the Short Hexamer Response Element (HxRE-Short) (Lee et al.,
99 2008, 2013). The HxRE-Short is also known as the Tetramer Response Element
100 (TeRE), and it is also recognized and bound by the tetrameric Lhx3 complex (Lee et al.,

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101 2008). Binding of the Isl1-Lhx3 complex to HxRE-Long and HxRE-Short elements
102 activates the transcription of genes that are essential for MN specification such as *Hb9*,
103 and genes that are required for cholinergic neurotransmission, such as *VaCHT* (Thaler
104 et al., 2002; Lee and Pfaff, 2003; Lee et al., 2012).

105 To efficiently transition from a progenitor state to a terminally differentiated state,
106 pMN cells must rapidly upregulate and maintain the expression of Isl1 and Lhx3.
107 Deletion of Isl1 or Lhx3, or disruption of Isl1-Lhx3 complex assembly, severely impairs
108 MN specification (Pfaff et al., 1996; Sharma et al., 1998; Thaler et al., 2002; Song et al.,
109 2009; Liang et al., 2011). Following MN specification and migration, Isl1 expression is
110 maintained in many MN subtypes, but Lhx3 expression is only maintained in Medial
111 Motor Column (MMCm) neurons (Tsuchida et al., 1994; Rousso et al., 2008a). Despite
112 recent progress characterizing the spatial and temporal patterns of gene expression in
113 differentiating MNs, the genetic mechanisms that direct differentiating MNs to induce
114 high levels of Isl1 and Lhx3 transcription during MN specification, and the mechanisms
115 utilized to maintain high levels of Isl1 and Lhx3 expression in MMCm neurons remain
116 unclear.

117 Here we report that the Isl1-Lhx3 complex binds two distinct genomic regions
118 downstream of *Lhx3*, as well as a known Isl1 enhancer (Uemura et al., 2005; Kim et al.,
119 2015). Interestingly, we also found a binding site located in the second intron of *Lmo4*,
120 which encodes LIM Only Protein 4 (LMO4). LMO4 is expressed in embryonic MNs, and
121 is important for inhibiting the formation of the Lhx3 complex, indirectly increasing the
122 probability that Lhx3 will incorporate into the Isl1-Lhx3 complex (Lee et al., 2008; Song
123 et al., 2009). Using GFP-reporter studies and embryonic chick neural tube

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124 electroporation, we found that each of these Isl1-Lhx3 binding sites act as MN-specific
125 enhancers and each is activated by the Isl1-Lhx3 complex. Therefore, our results show
126 that the Isl1-Lhx3 complex activates two distinct transcription pathways in parallel to
127 enhance its own expression and formation during MN development. First, a positive
128 autoregulatory loop amplifies the expression of the complex's key components, Isl1 and
129 Lhx3. Second the Isl1-Lhx3 complex activates the expression of LMO4, which indirectly
130 promotes Lhx3 incorporation into the Isl1-Lhx3 complex.
131
132 **Materials and Methods**
133 All embryo experiments in this study were performed without determining the sex of
134 each embryo.
135
136 **Chromatin immunoprecipitation (ChIP)-qPCR assays**
137 We isolated spinal cords from E12.5 mouse (ms) embryos. Spinal cords from 5-12
138 embryos were combined for each ChIP reaction with a specific antibody. Antibodies
139 used for immunoprecipitation were rabbit (rb) anti Isl1/2 (kindly provided by Tom Jessell,
140 Columbia University) (Tsuchida et al., 1994), rb anti Lhx3 (Abcam ab14555), and non-
141 specific rabbit IgG. The tissues were dissociated completely before the ChIP process.
142 Next, cells were washed with Buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM
143 EGTA, 10 mM Hepes, pH 6.5) and Buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA,
144 10 mM Hepes, pH 6.5) sequentially. Then, cells were lysed with lysis buffer (0.5% SDS,
145 5 mM EDTA, 50 mM Tris·HCl, pH 8.0, protease inhibitor mixture) and were subjected to
146 sonication for DNA shearing. Next, cell lysates were diluted 1:10 in ChIP buffer (0.5%

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147 Triton X-100, 2 mM EDTA, 100 mM NaCl, 50 mM Tris·HCl, pH 8.0, protease inhibitor
148 mixture) and, for immunoclearing, were incubated with IgG and protein A agarose
149 beads for 1 hour at 4°C. Supernatant was collected after quick spin and incubated with
150 IgG or the afore-mentioned antibodies and protein A agarose beads overnight at 4°C.
151 After pull-down of chromatin/antibody complex with protein A agarose beads, the beads
152 were washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl,
153 pH 8.0, 150 mM NaCl), TSE II (same components as in TSE I except 500 mM NaCl)
154 and Buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM
155 Tris·HCl, pH 8.0) sequentially for 10 min at each step. Then the beads were washed
156 with TE buffer three times. Protein/chromatin complexes were eluted in elution buffer
157 (1% SDS, 1 mM EDTA, 0.1 M $NaHCO_3$, 50 mM Tris·HCl, pH 8.0) and de-crosslinked by
158 incubating at 65°C overnight. Eluate was incubated at 50°C for more than 2 hours with
159 Proteinase K. Next, DNA was purified with phenol/chloroform and DNA pellet was
160 precipitated by ethanol and resolved in water. The purified final DNA samples were
161 used for quantitative PCR reactions using the SYBR green kit (11762-500, Invitrogen)
162 and CFX Connect ™ (Bio-Rad). The total input was used for normalization. All ChIP
163 experiments were repeated independently at least three times. Data are represented as
164 the mean of duplicate or triplicate values obtained from representative experiments;
165 error bars represent standard error of the mean. Following immunoprecipitation, qPCR
166 was performed to detect peaks using the following primers: Lhx3-Peak-A Fwd:
167 GGTCTGCCTCCCGTAAAACT Rev: CACCATCAATGCTTTGTTCAG, Lhx3-Peak-B
168 Fwd: CAATGCAGGGTGACCTGG Rev: GTGGGATTGACTGGGGTC, Isl1-Peak Fwd:
169 CTGCCACTCCACTTAATAACCTAA Rev: ATGGACACACCAGCTGGATAAATC,

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170 LMO4-Peak Fwd: ATCACTCGAGGACGTGGGTCCCTTTAAGATCC Rev:
171 CTGAGTCGACGGATTCTGCCTCCTCTCCTC.
172
173 **In ovo electroporation**
174 Electroporation was performed in HH st12-14 chick embryos, by injecting DNA into the
175 embryonic neural tube. A square pulse electroporater was used to apply 5 pulses, 25V,
176 50ms with 1 second between each pulse across the neural tube. Enhancers were
177 cloned into PBS-miniCMV-eGFP or SP72-TATA-eGFP reporter plasmids. Lhx3-Peaks
178 and the LMO4-Peak were cloned from the mouse genome, and the Isl1-Peak was
179 cloned from the human genome. Embryos were injected with 2.5µg/µL of reporter
180 construct and 1.75µg/µL of LacZ or 1.75µg/µL of Isl1-Lhx3 expression construct.
181 Embryos were harvested and processed for immunolabeling 3 days post electroporation
182 (3DPE), at HH st25. Images are representative of electroporations from multiple
183 embryos.
184
185 **Cloning of Isl1-Lhx3 ChIP-Seq loci**
186 The Isl1-Lhx3 ChIP-Seq peaks were constructed using the following primer sets. We
187 have also listed the sequences for wt HxRE sequences and the sequences for mutated
188 HxREs. Mutations were introduced using PCR. *Lhx3-Peak-A (ms)* (chr2:26194774-
189 26194788): Fwd-CTAGAGGTAGCCAAGGCC Rev-TGGAGAGGGCTAGCCAC. Hx-L-
190 wt: CATTTTAACTAATGG ΔHx-L: CGCGGCCGCAGCCGG. Hx-S-wt: CTAATTAAA
191 ΔHx-S: CGGCCGCAA. *Lhx3-Peak-B* (ms) (chr2:26186472-26187246): Fwd-
192 CAATGCAGGGTGACCTGG Rev-GTGGGATTGACTGGGGTC. Hx-L-wt:

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193 ATTTGATTAATCA. ΔHx-L: AGCGGCCGCCTCA. *Isl1-Peak* (hum)(chr5:51559189-
194 51559911): Fwd-CAGATGCACCTACCTCTTAAAG. Rev-
195 GGACATATGGCTAGAGTGTGG. (1-409) Rev-CCCTACTCTGTCTGCCACTCC. Hx-
196 S1-wt: TTTTAATTAGCT ΔHx-S1: TTTCTAGAAGCT. H2-wt: ATATTAAAAT ΔH2:
197 ATCTAGAAAT. A/T motif-wt: AATTTTAGCATAT ΔA/T: ACGGTTGGCGCCT. *LMO4-*
198 *Peak (ms)*(ch3:144198960-144199257): Fwd-GACGTGGGTCCCTTTAAGATCC Rev-
199 GGATTCTGCCTCCTCTCCTC. Hx-L-wt: AATTTTGTTAATTAA ΔHx-L:
200 AACCATGGTAGGTAA.
201
202 **Immunofluorescence labeling**
203 Embryos were fixed in 4% PFA/PBS for 90 minutes, embedded in OCT and
204 cryosectioned at 12μM. Embryos were incubated in primary antibody in either 0.1% Fish
205 Gelatin or 0.3% BSA blocking buffer, overnight at 4°C. Primary antibodies used were
206 goat (gt) anti LacZ (Sigma 1:2000), rb anti LacZ (Cappel 55976 1:2000), ms anti Mnr2
207 (DSHB 5C10 1:250), and chicken (chk) anti GFP (Aves Labs 1020 1:1000). Sections
208 were imaged using a Zeiss Axio Imager.Z2 microscope.
209
210 **GFP quantification**
211 Embryos used for GFP fluorescence analysis were not immunostained for GFP. 750ms
212 exposure time was used for all images that were analyzed for GFP quantification.
213 Integrative pixel density was measured in the ventral horn of the electroporated side of
214 the spinal cord, using Image J. 4-12 embryos were analyzed for each reporter construct.
215 And for each embryo, average fluorescence intensity was calculated from analyzing 3-7

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216 sections.

217

218 **In situ hybridization**

219 Embryos were electroporated with 1.75 µg/µL pBluescript expression vectors containing

220 either mouse Isl1, rat Lhx3 or Isl1-Lhx3 fusion protein (Lee et al., 2012). Embryos were

221 harvested at 3DPE and fixed in 4% PFA/PBS for 90 minutes. They were embedded in

222 OCT and cryosectioned at 18µm. Glassware for these experiments was treated with

223 NaOH to avoid RNAase contamination. Sections were fixed in 4%

224 paraformaldehyde/PBS at room temperature for 10 minutes, then washed 2 times in

225 PBS at room temp. Sections were then digested in Proteinase K Buffer (6.25mM EDTA,

226 0.05M Tris, 1µg/mL Proteinase K) at room temperature for 5 minutes. Sections were

227 fixed again in 4% paraformaldehyde/PBS at room temperature for 5 minutes, and then

228 washed 2 times in PBS at room temperature. Next, sections were submerged in 300mL

229 acetylation buffer (1.33% triethanolamine, 0.175% HCl). 750µL of acetic acid was

230 gradually added to slides. Slides were incubated in acetic acid / acetylation buffer for 10

231 minutes at room temperature, and then washed 2 times in PBS. Slides were then

232 incubated in hybridization solution (0.75M NaCl, 75mM Sodium Citrate, 50%

233 Formamide, 5X Denhardt's solution, 1% herring sperm DNA) for 2 hours at room

234 temperature. To make in situ probes, cDNA for chick Lhx3, Isl1 and LMO4 3'

235 Untranslated Region was cloned into pBluescript vector. Digoxigenin-labeled riboprobes

236 were generated using T7 polymerase PCR. Probes were denatured in hybridization

237 solution at 80°C for 5 minutes. Slides were incubated in probe / hybridization solution at

238 68°C overnight. Slides were then washed in 5X SSC (0.75M NaCl, 75mM Sodium

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239 Citrate) at 65°C for 10 minutes. Next, slides were incubated in 0.2X SSC (30mM NaCl,
240 3mM Sodium Citrate) at 65°C for 2 hours and then washed in fresh 0.2X SSC at 65°C
241 for 5 minutes. Slides were then blocked in Buffer 1 (0.1M Tris, 0.15M NaCl) + 4%
242 Bovine Serum Albumin (BSA) at room temperature for 1 hour, and then incubated in
243 Buffer 1 + 2% BSA + 1:5000 anti-Digoxigenin antibody at 4°C overnight. Next, slides
244 were washed 3 times with Buffer 1 for 5 minutes each at room temperature, and then in
245 Buffer 2 (0.1M Tris, 0.1M NaCl, 50mM $MgCl_2$) at room temperature for 5 minutes. Slides
246 were then incubated in Buffer 3 (0.1M Tris, 0.1M NaCl, 50mM $MgCl_2$, 2.4μg/mL
247 Levamisole, 338μg/mL NBT, 175 μg/mL BCIP) at room temperature overnight, to
248 perform the colormetric reaction. To stop the reaction, slides were then washed in TE.
249 Prior to mounting, slides were dehydrated with serial ethanol washes (30%, 50%, 70%,
250 95%, and 100%) at room temperature, and incubated in xylene for 10 minutes at room
251 temp. Slides were then mounted using Permount.
252
253 **Luciferase assays**
254 Assays were performed in cultured P19 embryonic mouse carcinoma cells. Cells were
255 cultured in α-minimal essential media with 7.5% Bovine Calf Serum and 2.5% Fetal
256 Bovine Serum. For luciferase assays, cells were seeded in 48 well plates, and
257 transfected using Lipofectatmine 2000 (Invitrogen). Cells were transfected with reporter
258 constructs, transcription factor expression constructs, a CMV-beta-galactosidase
259 construct, to test transfection efficiency, and with empty plasmid to equalize the total
260 amount of DNA for each condition. Luciferase and beta-galactosidase activity was
261 measured 48 hours after transfection. Results are reported as activity fold change of

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262 each reporter construct when co-transfected with Isl1 plus Lhx3, compared to co-
263 transfection with empty plasmid. Results from each experiment were obtained from the
264 average of technical duplicates. Summarized results show the average activity fold
265 change from at least 5 independent experiments.
266

267 **Results**

268 **The Isl1-Lhx3 complex binds genomic loci associated with *Isl1*, *Lhx3* and *Lmo4***

269 As transcriptional autoregulation is a powerful mechanism utilized by a variety of
270 systems during development (Johnson et al., 1994; Belaguli et al., 1997; Smith et al.,
271 2000; Aota et al., 2003; Bai et al., 2007; Borromeo et al., 2014), we hypothesized that
272 the Isl1-Lhx3 complex may act to directly regulate its own expression. This hypothesis is
273 further supported by the observation that the Isl1-Lhx3 fusion protein induces the
274 transcription of *Isl1*, *Lhx3* and *Lmo4* in the induced MN embryonic stem cell system
275 (Lee et al., 2012). To test this hypothesis, we analyzed the previously reported data
276 from chromatin immunoprecipitation experiments, performed in conjunction with high-
277 throughput sequencing (ChIP-Seq) (Lee et al., 2013). These experiments used mouse
278 MN-inducible embryonic stem cells (iMN-ESCs), that have a doxycycline-inducible *Isl1-*
279 *Lhx3* fusion gene. Dox treatment was coupled with a MN differentiation protocol to
280 induce high levels of the Isl1-Lhx3 complex and MN differentiation (Lee et al., 2012,
281 2013).

282 We found two Isl1-Lhx3 complex binding loci downstream of the *Lhx3* gene.
283 Lhx3-Peak-A is located approximately 5.1kb downstream of *Lhx3* (Fig. 1A). Lhx3-Peak-
284 B is located approximately 19.5kb downstream of *Lhx3* (Fig. 1A). We also found an

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285 Isl1-Lhx3 complex binding locus within a previously identified Isl1 enhancer (Isl1-Peak)
286 (Fig. 1A) (Uemura et al., 2005; Kim et al., 2015). The Isl1-Lhx3 complex was also found
287 to bind a locus within the first intron of *Lmo4* (LMO4-Peak) (Fig. 1A). Given the known
288 role of LMO4 in blocking the formation of the Lhx3 complex (Lee et al., 2008), this
289 finding suggests an additional regulatory pathway to indirectly facilitate the formation of
290 the Isl1-Lhx3 complex.

291 We assessed the in vivo occupancy of each peak by performing ChIP for
292 endogenous Isl1 or Lhx3, using E12.5 mouse spinal cord lysates. We precipitated with
293 either anti-Isl1, anti-Lhx3 or control IgG antibodies, followed by quantitative PCR
294 (qPCR) for Lhx3-Peak-A, Lhx3-Peak-B, the Isl1-Peak and the LMO4-Peak. Compared
295 to IgG, both anti-Isl1 and anti-Lhx3 antibodies precipitated significantly more Lhx3-
296 Peak-A, Lhx3-Peak-B, Isl1-Peak, and LMO4-Peak (Fig. 1C-G). As a negative control,
297 we also performed qPCR for the untranslated genomic locus *Untr-6* (Mali et al., 2008),
298 and saw no enrichment in the amount of *Untr-6* precipitated with anti-Isl1 or anti-Lhx3
299 antibodies, compared to IgG (Fig 1G).

300 These results show that the Isl1-Lhx3 complex specifically binds Lhx3-Peak-A,
301 Lhx3-Peak-B, the Isl1-Peak, and the LMO4-Peak during embryonic MN development in
302 vivo. This finding supports a model in which these peaks serve as enhancers for the
303 Isl1-Lhx3 complex to directly activate the transcription of *Lhx3*, *Isl1* and *Lmo4*.

304

305 **Lhx3-Peak-A is activated by the Isl1-Lhx3 complex**

306 To test whether Lhx3-Peak-A activates transcription in MNs, we performed chick neural
307 tube electroporation with a GFP-reporter construct containing two copies of Lhx3-Peak-

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308 A upstream of a minimally active TATA-box promoter and EGFP (Lhx3-Peak-A-GFP,
309 Fig. 2B). Embryos were also electroporated with a ubiquitously expressing LacZ
310 construct to mark electroporated cells. Chick embryos were electroporated at HH stage
311 14 and analyzed 3 days post electroporation (3DPE). The expression of GFP from this
312 reporter indicates the activation of Lhx3-Peak-A enhancer. We can further test the
313 importance of a specific motif within the Lhx3-Peak-A enhancer by monitoring if
314 mutation of the motif results in impaired expression of GFP.

315 Lhx3-Peak-A induced modest GFP expression, specifically in cells expressing
316 the MN-specific gene Mnr2, a homolog of Hb9 (Fig. 2C). This result is consistent with
317 the hypothesis that endogenous Isl1-Lhx3 complex activates *Lhx3* transcription in MNs
318 via Lhx3-Peak-A. To test this directly, we co-electroporated Lhx3-Peak-A-GFP with an
319 expression vector for Isl1-Lhx3 fusion protein. This construct activates ectopic
320 expression of Isl1-Lhx3 fusion protein, which complexes with endogenous NLI to form
321 the Isl1-Lhx3 complex (Lee et al., 2008; Song et al., 2009). Ectopic expression of the
322 Isl1-Lhx3 complex activated GFP expression throughout the dorsal and ventral spinal
323 cord (Fig. 2D). Further, both dorsal GFP^{+} cells and GFP^{+} cells in the ventral horn also
324 expressed the MN-specific marker Mnr2 (Fig. 2D).

325 Because forced expression of the Isl1-Lhx3 complex initiates ectopic MN cell fate
326 specification (Thaler et al., 2002; Lee et al., 2008, 2012), it was unclear if the Isl1-Lhx3
327 complex directly activates GFP expression in ectopic MNs or if the change in cell fate
328 specification indirectly activates Lhx3-Peak-A. To test if the Isl1-Lhx3 complex directly
329 activates transcription via Lhx3-Peak-A, even without initiating MN fate specification, we
330 performed luciferase reporter assays in cultured mouse embryonic carcinoma P19 cells.

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331 For these experiments, we transfected Lhx3-Peak-A-LUC reporters with expression
332 vectors for Isl1, Lhx3, Isl1+Lhx3, or with empty vector. We cultured cells for two days
333 and then performed luciferase assays to measure transcription of the luciferase
334 reporter-gene. Transfection of Isl1 plus Lhx3 significantly activated Lhx3-Peak-A-LUC
335 compared to control LUC reporter containing no enhancer (Fig 2E). Combined with the
336 ChIP-qPCR results from mouse embryonic spinal cord (Fig. 1C), these results show that
337 the Isl1-Lhx3 complex directly binds Lhx3-Peak-A to initiate the transcription of *Lhx3*.
338
339 **Lhx3-Peak-A activity is mediated by two binding sites for the Isl1-Lhx3 complex**
340 Lhx3-Peak-A contains both a putative HxRE-Long (Hx-L) motif and a putative HxRE-
341 Short (Hx-S) motif (Fig 2A). As both sequences are known binding sites of the Isl1-Lhx3
342 complex, we tested if either or both contribute to Lhx3-Peak-A enhancer activity. To do
343 this, we generated mutated versions of Lhx3-Peak-A where either the HxRE-Long, the
344 HxRE-Short, or both sites are mutated (ΔHx-L, ΔHx-S, and ΔHx-LΔHx-S, respectively)
345 (Fig. 2B). Next, we made GFP-reporter constructs with each of these mutated versions
346 of Lhx3-Peak-A and performed chick neural tube electroporation with either LacZ or
347 Isl1-Lhx3.
348 When co-electroporated with LacZ, the ΔHx-L reporter construct still activated
349 GFP expression in Mnr2-positive MNs (Fig. 2C). However, neither ΔHx-S, nor the
350 double mutant activated any detectable GFP expression in the spinal cord (Fig. 2C).
351 Co-electroporation with Isl1-Lhx3 activated both single mutant constructs throughout the
352 spinal cord, but failed to activate the ΔHx-LΔHx-S double-mutant (Fig. 2D). These
353 results show that both the HxRE-Long and the HxRE-Short in Lhx3-Peak-A contribute to

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354 its MN enhancer activity. Without the HxRE-Long, Lhx3-Peak-A is activated by
355 endogenous levels of the Isl1-Lhx3 complex. However, when the HxRE-Short is
356 ablated, the enhancer requires high levels of the Isl1-Lhx3 complex to be activated. And
357 when both sites are mutated, the Isl1-Lhx3 complex cannot activate transcription via
358 Lhx3-Peak-A.

359 We observed similar results with luciferase assays. In cells transfected with
360 luciferase reporter constructs containing wt Lhx3-Peak-A, ΔHx-L, or ΔHx-S,
361 transcription is activated by Isl1 plus Lhx3. However, when both response elements
362 were mutated, co-transfection with Isl1 plus Lhx3 failed to activate transcription (Fig.
363 2E).

364

365 **Lhx3-Peak-B is activated in embryonic MNs**

366 To test if Lhx3-Peak-B acts as a MN-specific enhancer, we electroporated Lhx3-Peak-
367 B-GFP with either LacZ or Isl1-Lhx3 in embryonic chick neural tube (Fig 3B). When co-
368 electroporated with LacZ, Lhx3-Peak-B activated GFP expression specifically and
369 robustly in Mnr2-positive MNs (Fig. 3C). Co-electroporation of Lhx3-Peak-B-GFP with
370 Isl1-Lhx3 activated GFP expression throughout the spinal cord, specifically in cells
371 expressing ectopic or endogenous Mnr2 (Fig. 3D).

372 Interestingly, when we performed luciferase assays in P19 cells, Lhx3-Peak-B
373 was not activated by co-transfection of Isl1 plus Lhx3, compared to control reporter
374 construct with no enhancer (Fig. 3E). These results indicate that Lhx3-Peak-B acts as a
375 strong MN-specific enhancer in embryonic MNs in vivo. However, the Isl1-Lhx3 complex
376 is not sufficient to activate Lhx3-Peak-B in all cellular contexts. Cultured P19 cells could

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377 lack critical co-factors that are required for the Isl1-Lhx3 complex to activate
378 transcription via Lhx3-Peak-B. However, as the Isl1-Lhx3 complex can activate other
379 MN-specific enhancers in these cells, it is more likely that P19 cells express
380 transcriptional repressors that specifically recognize Lhx3-Peak-B to block Isl1-Lhx3
381 complex binding or activity.

382 Lhx3-Peak-B contains one putative HxRE-Long motif (Fig 3A). To test if this motif
383 contributes to the enhancer activity of Lhx3-Peak-B, we generated a mutated version of
384 Lhx3-Peak-B where the HxRE-Long is mutated (ΔHx-L) (Fig. 3B). When electroporated
385 with LacZ, Lhx3-Peak-B-ΔHx-L-GFP did not activate any detectable GFP expression in
386 Mnr2^{+} MNs. Co-electroporation of Isl1-Lhx3 fusion protein also failed to activate GFP
387 expression (Fig 3D), indicating that the HxRE-Long is critical for the MN-specific
388 enhancer activity of Lhx3-Peak-B.

389 MN progenitor cells must rapidly upregulate the transcription of the Isl1-Lhx3
390 complex to promote terminal differentiation and MN cell fate specification. Immediately
391 following the onset of Isl1 and Lhx3 expression in newly specified MNs, Lhx3-Peak-A
392 and Lhx3-Peak-B likely contribute to this rapid increase in the transcription of *Lhx3.* This
393 positive feedback-loop is expected to facilitate the switch from a non-specified MN
394 progenitor cell to a fully-committed, differentiated MN.

395

396 **The Isl1-Peak is activated by the Isl1-Lhx3 complex via multiple HxRE motifs**

397 The Isl1-Peak activates transcription in newly born MNs and in mature MMCm neurons
398 in mouse, zebrafish and chick embryos (Uemura et al., 2005; Kim et al., 2015). We
399 confirmed this finding in chick embryos by electroporating an Isl1-Peak-GFP reporter

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400 construct (Fig. 4B,C). It was also reported that the Isl1-Peak is activated by ectopic
401 expression of the Isl1-Lhx3 complex (Kim et al., 2015). We tested this by electroporating
402 Isl1-Peak-GFP with Isl1-Lhx3 fusion protein. We found that, indeed ectopic expression
403 of the Isl1-Lhx3 complex expanded GFP expression to the dorsal spinal cord, and GFP
404 expression co-localized with ectopic Mnr2 expression (Fig 4D). These results are
405 consistent with our findings that both Isl1 and Lhx3, bind to the Isl1-Peak in embryonic
406 stem cells and in the mouse embryonic spinal cord (Fig. 1A, E).

407 The reported ChIP-Seq experiments (Lee et al., 2013) show that the Isl1-Peak
408 contains two distinct Isl1-Lhx3 complex binding peaks (Fig 1B), suggesting that there
409 are at least two motifs regulating Isl1-Peak enhancer activity. The Isl1-Peak is 724 base
410 pairs long. It contains motifs that are highly conserved between human and mouse,
411 including 13 TAAT sites, and two sites that closely resemble HxRE-Short motifs (Hx-S1
412 and Hx-S2) (Fig 4 A, B). TAAT sequences act as binding sites for homeodomain
413 transcription factors (H motifs) (Gehring et al., 1994) and both the HxRE-Long and the
414 HxRE-Short motifs contain TAAT sequences. Hx-S1 is located near the summit of the
415 right peak of the Isl1-Peak (Fig 1B), indicating that it may act as an Isl1-Lhx3 binding
416 site. To test if Hx-S1 contributes to the MN-specific enhancer activity of the Isl1-Peak,
417 we electroporated Isl1-Peak-GFP reporter constructs with and without a mutation of the
418 Hx-S1 motif (Fig. 4B). We found that, compared to wt-Isl1-Peak, Isl1-Peak-ΔHx-S1
419 activated significantly less GFP in embryonic MNs (Fig 4C & E). To test if Hx-S1 is
420 sufficient for the Isl1-Lhx3 complex to activate Isl1-Peak, we also made GFP reporter
421 constructs with a truncated version of the Isl1-Peak consisting of the first 409
422 nucleotides. This short version of the Isl1-Peak lacks the Hx-S2 motif [Isl1-Peak-(1-409)

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423 Fig. 4B]. In chick neural tube electroporations, Isl1-Peak-(1-409) activated GFP
424 expression specifically in Mnr2 positive embryonic MNs, but significantly less effectively
425 than full length Isl1-Peak (Fig. 4, C and E). GFP expression was further reduced when
426 Hx-S1 was mutated in Isl1-En-(1-409)-GFP reporter experiments (Fig. 4C, F). These
427 results suggest that, while Isl1-Peak-(1-409) is sufficient for the Isl1-Lhx3 complex to
428 activate the Isl1-Peak via Hx-S1, other sequences within 410-724 nucleotides of the
429 Isl1-Peak, such as the Hx-S2 motif, also contribute to its MN-specific enhancer activity.
430 Next, we co-electroporated each reporter construct with an Isl1-Lhx3 fusion
431 protein vector to activate ectopic expression of the Isl1-Lhx3 complex. Surprisingly, we
432 found that, like the wt Isl1-Peak, each mutant Peak, including Isl1-Peak-(1-409)-ΔHx-
433 S1, activated robust GFP in the dorsal spinal cord that co-localized with ectopic Mnr2
434 expression (Fig. 4D). Notably, this approach can detect even weaker enhancer activity
435 due to expression of high levels of the Isl1-Lhx3 complex. Therefore, Isl1-Peak-(1-409)
436 likely contains additional motifs that, in the absence of Hx-S1, are not active in Mnr2-
437 positive embryonic MNs but respond to high levels of the Isl1-Lhx3 complex. In support
438 of this hypothesis, many H motifs in Isl1-Peak show some homology to either HxRE-
439 Long or HxRE-Short motif. For instance, H2 shows weak homology to the HxRE-Short
440 motif. To test if H2 can independently respond to the Isl1-Lhx3 complex, we constructed
441 Isl1-Peak-GFP and Isl1-Peak-(1-409)-GFP constructs with a mutation in H2 alone or
442 combined with the ΔHx-S1 mutation. While mutation of H2 alone did not reduce GFP
443 expression (Fig. 4, C, E, F), mutating both Hx-S1 and H2 in the full-length Isl1-En
444 caused a slight reduction in GFP expression compared to mutating Hx-S1 alone (Fig 4,
445 C and E). This trend was not statistically significant, but raises the interesting possibility

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446 that the H2 motif could function as a cryptic HxRE motif, which manifests its activity only
447 in the absence of Hx-S1 motif. Hx-S1 clearly contributes substantially to the enhancer
448 activity of the Isl1-Peak, and it appears that H2 may also facilitate Isl1-Peak activation in
449 embryonic MNs. Co-electroporation of Isl1-Peak-(1-409)-ΔHx-S1ΔH2-GFP and Isl1-
450 Lhx3 activated robust GFP in the dorsal spinal cord (Fig. 4D), suggesting that additional
451 H motifs similarly function as cryptic HxRE motif(s) to activate the Isl1-Peak.
452 We also mutated the A/T-rich motif located at 470 base pairs (ΔA/T), which is
453 only present in the full length version of the Isl1-Peak (Fig. 4 A, B). Previous reports
454 have shown that this site is required for Isl1-Peak enhancer activity in endogenous MNs
455 and in ectopic MNs induced by overexpression of the Isl1-Lhx3 complex (Kim et al.,
456 2015). However, we found that the ΔA/T mutant activates robust GFP expression in
457 both endogenous and ectopic MNs (Fig 4C, D). In endogenous MNs, there was no
458 difference between ΔA/T-GFP expression and wt Isl1-Peak-GFP expression, indicating
459 that this motif does not contribute to Isl1-Peak enhancer activity (Fig 4E).
460 Overall, these findings support a model in which the Isl1-Lhx3 complex activates
461 the Isl1-Peak via HxRE-S1, likely in cooperation with additional HxRE and H motifs.
462 These results are also consistent with our findings that both Isl1 and Lhx3, bind to the
463 Isl1-Peak in embryonic stem cells and in the mouse embryonic spinal cord (Fig. 1A, E).
464

465 **The LMO4-Peak is activated by the Isl1-Lhx3 complex via a single HxRE-Long**
466 **motif**

467 During MN specification, LMO4 blocks the formation of the V2-interneuron specifying
468 Lhx3 complex, and thereby inhibits the expression of V2-specific genes in MNs (Lee et

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469 al., 2008). In addition to rapidly and robustly upregulating its own expression, we
470 hypothesized that the Isl1-Lhx3 complex also activates the transcription of LMO4 in
471 newly-differentiating embryonic MNs. To test if the LMO4-Peak (Fig. 1A) acts as an
472 enhancer in embryonic MNs, we performed chick neural tube electroporations with an
473 LMO4-Peak-GFP reporter construct (Fig. 5B). When we electroporated LMO4-Peak-
474 GFP with LacZ, we found that the LMO4-Peak activates GFP expression specifically in
475 Mnr2$^+$ MNs (Fig. 5C). Co-electroporating Isl1-Lxh3 with LMO4-Peak-GFP, expanded
476 GFP expression throughout the spinal cord, specifically in cells expressing endogenous
477 or ectopic Mnr2 (Fig. 5D).

478 Luciferase assays using LMO4-Peak-LUC with Isl1, Lhx3 or Isl1 plus Lhx3, were
479 consistent with these results (Fig. 5E). Isl1 plus Lhx3 significantly activated LMO4-Peak-
480 LUC expression, compared to control vector containing no enhancer (Fig 5E). These
481 results indicate that, in embryonic MNs, the LMO4-Peak recruits the Isl1-Lhx3 complex
482 to activate the transcription of *Lmo4*. By blocking the formation and activity of the Lhx3
483 complex, LMO4 inhibits the transcription of V2-IN specific genes in MNs and increases
484 the pool of free Lhx3 available to incorporate into the Isl1-Lhx3 complex.

485 The LMO4-Peak contains one HxRE-Long motif and one HxRE-Short motif (Fig
486 5A). To test if the HxRE-Long motif contributes to the activity of the LMO4-Peak, we
487 generated a mutated version of the LMO4-Peak where the HxRE-Long sequence is
488 ablated (ΔHx-L) (Fig. 5B). Chick neural tube electroporations with ΔHx-L-GFP did not
489 activate any detectable GFP expression in the embryonic spinal cord (Fig. 5C). Co-
490 electroporation of Isl1-Lhx3 fusion protein with the mutated reporter also failed to
491 activate GFP expression (Fig. 5D). Likewise, ΔHx-L-LUC was not activated by co-

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492 transfection with Isl1 plus Lhx3 in P19 cells (Fig. 5E). These results indicate that the
493 HxRE-Long motif is required for the Isl1-Lhx3 complex to activate transcription via the
494 LMO4-Peak.
495
496 **The Isl1-Lhx3 complex activates the transcription of endogenous Lhx3, Isl1 and**
497 **LMO4**
498 To test if the Isl1-Lhx3 complex can activate the transcription of *Isl1*, *Lhx3* and *Lmo4*, in
499 the embryonic spinal cord, we ectopically expressed mouse Isl1 and rat Lhx3, or Isl1-
500 Lhx3 fusion protein in the embryonic chick spinal cord through neural tube
501 electroporation. We harvested embryos at 3DPE and performed in situ hybridizations
502 with chicken-specific probes designed to recognize the 3' untranslated region (3'UTR) of
503 chick *Isl1*, *Lhx3* or *Lmo4*. Because the Isl1, Lhx3 and Isl1-Lhx3 expression constructs
504 lack 3'UTR sequences, these probes exclusively detect endogenous chick transcripts.
505 Embryos that were electroporated with Isl1 alone showed no change in the
506 expression of endogenous *Isl1*, *Lhx3* or *Lmo4* (Fig. 6A-C). Lhx3 electroporation slightly
507 increased the transcription of *Lmo4*, but did not affect expression of endogenous *Isl1* or
508 *Lhx3* (Fig. 6D-F). In contrast, embryos that were electroporated with Isl1-Lhx3 showed
509 robust increases in transcription of *Isl1*, *Lhx3*, and *Lmo4* throughout the dorsal spinal
510 cord (Fig. 6G-I). These results show that the Isl1-Lhx3 complex is sufficient to induce
511 the transcription of *Lhx3*, *Isl1* and *Lmo4* in the embryonic spinal cord.
512
513 **Discussion**
514 A great deal of progress has been made characterizing the activity and expression

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515 patterns of Isl1, Lhx3, and LMO4 in embryonic MNs (Thaler et al., 2002; Lee et al.,
516 2008, 2012, 2013; Rousso et al., 2008b; Song et al., 2009; Roy et al., 2012). However,
517 the mechanisms that activate the transcription of these factors in differentiating MNs,
518 and the pathways that regulate their expression in specific MN subtypes remain unclear.
519 Our results show that the Isl1-Lhx3 complex binds genomic loci associated with *Lhx3*,
520 *Isl1* and *Lmo4*, both in an inducible MN embryonic stem cell system, and in the
521 embryonic spinal cord. Each of these loci acts as a MN-specific enhancer and is
522 robustly activated by the Isl1-Lhx3 complex. Additionally, we show that overexpression
523 of the Isl1-Lhx3 complex activates the transcription of endogenous *Isl1*, *Lhx3* and *Lmo4.*

524 Together, these results show that, early in embryonic MN specification, the Isl1-
525 Lhx3 complex is recruited to loci associated with *Isl1*, *Lhx3* and *Lmo4* to directly activate
526 the transcription of each of these genes. This transcriptional activation generates a
527 positive autoregulatory feedback loop where the Isl1-Lhx3 complex activates the
528 transcription of its own components. This feedback loop contributes to the rapid
529 induction of Isl1, Lhx3 and LMO4 expression in differentiating MNs, and to the
530 maintenance of these factors in mature MMCm neurons.

531

532 **Expression of the Isl1-Lhx3 complex**

533 Onecut transcription factors, including Hnf6, activate the transcription of Isl1 in early
534 MNs and regulate the expression of Isl1 in multiple MN subtypes (Roy et al., 2012).
535 However, in the absence of Hnf6 and Onecut-2, newly generated MNs still maintain low
536 levels of Isl1 expression, and normal numbers of MNs are generated, indicating that
537 there are additional pathways contributing to the onset of Isl1 expression (Roy et al.,

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538 2012). Likewise, the activation of Lhx3 and LMO4 expression in differentiating MNs is
539 critical for MN specification, and little is known regarding the specific mechanisms that
540 regulate the expression of these two factors (Sharma et al., 1998; Lee et al., 2008).

541 Early in MN specification, Hb9 is released from transcriptional repression via
542 reduced levels of Olig2 expression (Lee et al., 2005). Reduced Olig2 expression could
543 also release Isl1 and Lhx3 from transcriptional repression, which would allow for modest
544 expression of Isl1 and Lhx3. Our results in this report suggest that the resulting low
545 levels of the Isl1-Lhx3 complex, at the onset of MN specification, activates a positive
546 transcriptional feedback loop that rapidly induces high levels of Isl1 and Lhx3
547 expression. At the same time the Isl1-Lhx3 complex concurrently activates *Lmo4*
548 transcription. LMO4 competes with Lhx3 to bind NLI, thereby blocking the formation of
549 the Lhx3 complex. This action inhibits the transcription of V2-interneuron genes in MNs
550 and increases the available pool of Lhx3, which indirectly promotes Lhx3 incorporation
551 into the Isl1-Lhx3 complex.

552 Positive and negative transcriptional feedback loops, both direct and indirect,
553 have been shown to contribute to the temporal regulation of gene expression in a
554 variety of cellular contexts (Harris and Levine, 2005; Haberland et al., 2007;
555 Svenningsen et al., 2008; DiTacchio et al., 2012; Morichika et al., 2012; Pruunsild et al.,
556 2013; Bornstein et al., 2014). In particular, transcriptional autoregulation is prominent in
557 development and cell specification (Johnson et al., 1994; Belaguli et al., 1997; Smith et
558 al., 2000; Aota et al., 2003; Bai et al., 2007; Borromeo et al., 2014). Positive
559 autoregulation of the Isl1-Lhx3 complex is an efficient mechanism to ensure the rapid
560 transition from a pluripotent, progenitor cell state to a post-mitotic, differentiated MN. It

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561 facilitates rapid induction of the Isl1-Lhx3 complex and thereby, quickly induces the
562 expression of genes essential for MN differentiation such as *Hb9* (Arber et al., 1999).
563

564 **Recruitment of the Isl1-Lhx3 complex**

565 While each peak in this study is activated by the Isl1-Lhx3 complex, the composition of
566 each peak varies. Thus, the genetic mechanisms utilized to recruit the Isl1-Lhx3
567 transcription complex also vary. Both Lhx3 peaks and the LMO4 peak contain single
568 HxRE-Long motifs, while Lhx3-Peak-A and the LMO4-Peak also contain single HxRE-
569 Short motifs. The Isl1-Peak contains two HxRE-Short motifs, but no obvious HxRE-Long
570 motifs, as well as multiple H motifs that resemble HxRE-Long and HxRE-Short motifs.
571 The enhancer activity of Lhx3-Peak-A is only completely lost when both the
572 HxRE-Long and HxRE-Short motifs are ablated. This finding shows that these two
573 motifs cooperate to recruit the Isl1-Lhx3 complex and induce transcriptional activation.
574 In contrast to this observation, the HxRE-Long motifs in Lhx3-Peak-B and the LMO4-
575 Peak are critical for the activity of these enhancers. When the HxRE-Long motifs in
576 Lhx3-Peak-B or the LMO4-Peak are ablated, neither is responsive to even high levels of
577 ectopic Isl1-Lhx3 complex expression. This result is consistent with Lhx3-Peak-B
578 containing no additional HxRE motifs. However, unlike Lhx3-Peak-A, the HxRE-Short
579 motif in the LMO4-Peak is unable to compensate for the loss of the HxRE-Long motif.
580 Further mutational analysis of the LMO4-Peak, in which the HxRE-Short is ablated, will
581 be necessary to determine whether this site can also contribute to the activity of this
582 enhancer.
583 Unlike the Lhx3 and LMO4 peaks, we found that the activity of the Isl1-Peak is

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584 mediated by cooperative action of multiple motifs. Hx-S1 contributes substantially to
585 Isl1-Peak enhancer activity, but ablating Hx-S1 is not sufficient to completely abolish its
586 enhancer activity. These findings indicate that other motifs, possibly HxRE-S2 and H
587 motifs also contribute to the activity of the Isl1-Peak. As demonstrated by H2 (Fig. 4E),
588 some motifs may function as alternative binding sites for the Isl1-Lhx3 complex only in
589 the absence of Hx-S1 or cooperate with Hx-S1 to recruit the Isl1-Lhx3 complex in
590 developing MNs. Further study of this unique enhancer could reveal interesting genetic
591 mechanisms to refine transcriptional specificity.
592 Lee et al (2008) has previously reported that HxRE-Short motifs also serve as
593 high affinity binding sites for the V2-IN-specifying Lhx3 complex. We have also shown
594 that, in embryonic MNs, Hb9 recognizes and binds HxRE-Short motifs to inhibit the
595 transcription of an Lhx3 complex target gene *Chx10* (Lee et al., 2008). Our results
596 indicate that the HxRE-Short motifs found in Lhx3-Peak-A, the Isl1-Peak and the LMO4-
597 Peak are likely not recognized by Hb9. This finding suggests that Hb9 binds only a
598 subset of high affinity HxRE-Short motifs. This is an interesting hypothesis that raises
599 questions regarding the specificity of Hb9 binding during embryonic MN development.
600 Future genome-wide analysis of Hb9 binding sites in embryonic MNs will provide critical
601 insight into this issue.
602
603 **Isl1, and Lhx3 expression in MN subtypes**
604 In addition to facilitating Isl1 and Lhx3 transcription during MN specification, the Isl1-
605 Peak and Lxh3-Peaks that we have characterized in this study likely act to maintain
606 high levels of Isl1 and Lhx3 expression in mature MMCm neurons. Following MN

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607 specification, many MN subtypes downregulate the expression of *Isl1* or *Lhx3*. LMCl
608 neurons do not express *Isl1*, and *Lhx3* expression is only maintained in MMCm neurons
609 (Tsuchida et al., 1994; Rousso et al., 2008b). To halt the expression of Isl1 or Lhx3,
610 MNs must disrupt the positive transcriptional feedback loop generated by these
611 proteins. Transcriptional repressor proteins or translational repressing pathways, such
612 as the expression of specific micro-RNAs, would be efficient mechanisms to
613 downregulate the expression of Isl1 or Lhx3. While a great deal of work has been done
614 to characterize the genetic mechanisms that activate the expression of specific
615 transcription factors and signaling molecules during MN-subtype development, the
616 pathways utilized to repress specific genes are not well understood. These repressive
617 pathways are critical for MN-subtype development, as forced expression of Lhx3 has
618 been shown to convert MNs to an MMCm fate (Sharma et al., 2000). It will therefore be
619 important to identify the mechanisms utilized to downregulate Isl1 and Lhx3 expression
620 in specific MN-subtypes and to determine if such mechanisms interrupt the positive
621 autoregulatory pathways defined in this study, to build a comprehensive model of
622 transcriptional regulation in MN development.
623

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739 **Figure Legends:**

740 **Figure 1: The Isl1-Lhx3 complex binds genomic loci associated with *Isl1*, *Lhx3***
741 **and *Lmo4.*** (A) Isl1-Lhx3 complex binding sites, identified via ChIP-Seq, in association
742 with *Lhx3*, *Isl1* and *Lmo4*. (B) A close-up of each ChIP-Seq peak. (C-G) E12.5 mouse
743 spinal cord ChIP performed with Isl1 or Lhx3 antibodies. qPCR was performed for Lhx3-
744 Peak-A, Lhx3-Peak-B, the Isl1-Peak, the LMO4-Peak and the negative control region,
745 Untranslated Region 6 (Untr6). Experiments were performed independently 3 times.
746 Results shown are from a single representative experiment; n=3 technical replicates.
747 Results were analyzed with a One-Way ANOVA followed by Holm Multiple Comparison
748 Analysis. ** $p<0.01$, compared to non-specific IgG controls. Error bars represent the
749 standard error of the mean.
750

751 **Figure 2: Lhx3-Peak-A is activated by the Isl1-Lhx3 complex.** (A) HxRE-Long and
752 HxRE-Short sequences identified by ChIP-Seq de novo motif analysis, and the HxRE-
753 Long and HxRE-Short sequences in Lhx3-Peak-A. (B) Lhx3-Peak-A HxRE-Long and
754 HxRE-Short mutants used for GFP-reporter experiments. (C) GFP-reporter experiments
755 for Lhx3-Peak-A variants, embryos were electroporated with Lhx3-Peak-A-GFP reporter
756 constructs plus ubiquitously expressing LacZ to mark electroporated cells. Sections
757 were immunostained for GFP and Mnr2 to mark MNs. Images are representative of
758 electroporations from multiple embryos. Lhx3-Peak-A-wt: n=5, Lhx3-Peak-A-ΔHx-L:
759 n=14, Lhx3-Peak-A-ΔHx-S: n=12, Lhx3-Peak-A-ΔHx-LΔHx-S: n=15 (D) Embryos
760 electroporated with Lhx3-Peak-A-GFP reporter construct plus Isl1-Lhx3 fusion protein
761 construct. Sections were immunostained for GFP and Mnr2. Images are representative

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762 of electroporations from multiple embryos. Lhx3-Peak-A-wt: n=25, Lhx3-Peak-A-ΔHx-L:
763 n=5, Lhx3-Peak-A-ΔHx-S: n=13, Lhx3-Peak-A-ΔHx-LΔHx-S: n=15 (E) Luciferase
764 assays testing Lhx3-Peak-A wt and mutants. Lhx3-Peak variants are the same as those
765 used in GFP-reporter experiments. Luciferase assays performed in cultured P19 cells.
766 Results show the luciferase activation fold upon the addition of Isl1 plus Lhx3,
767 compared to empty vector. n = 5 independent experiments. Results were analyzed with
768 a One-Way ANOVA followed by Holm Multiple Comparison Analysis, comparing each
769 reporter construct to control reporter (no enhancer). *$p<0.05$, **$p<0.01$. Error bars
770 represent the standard error of the mean.
771
772 **Figure 3: Lhx3-Peak-B is activated by the Isl1-Lhx3 complex.** (A) HxRE-Long
773 sequence identified by ChIP-Seq de novo motif analysis, and the HxRE-Long
774 sequences in Lhx3-Peak-B. (B) Lhx3-Peak-B-wt and HxRE-Long mutant used for GFP-
775 reporter experiments. (C) GFP-reporter experiments for Lhx3-Peak-B variants. Embryos
776 were electroporated with Lhx3-Peak-B-GFP reporter constructs plus ubiquitously
777 expressing LacZ to mark electroporated cells. Sections were immunostained for GFP
778 and Mnr2 to mark MNs. Images are representative of electroporations from multiple
779 embryos. Lhx3-Peak-B-wt: n=20, Lhx3-Peak-B-ΔHx-L: n=4. (D) Embryos electroporated
780 with an Lhx3-Peak-B-GFP reporter construct plus Isl1-Lhx3 fusion protein construct.
781 Sections were immunostained for GFP and Mnr2. Images are representative of
782 electroporations from multiple embryos. Lhx3-Peak-B-wt: n=7, Lhx3-Peak-B-ΔHx-L: n=5
783 (E) Luciferase assays testing Lhx3-Peak-B wt and HxRE-Long mutant. Luciferase
784 assays performed in cultured P19 cells. Results show the luciferase activation fold upon

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785 the addition of Isl1 plus Lhx3, compared to empty vector. n = 4 independent
786 experiments. Results were analyzed with a One-Way ANOVA followed by Holm Multiple
787 Comparison Analysis, comparing each reporter construct to control reporter (no
788 enhancer). Error bars represent the standard error of the mean.
789
790 **Figure 4: The Isl1-Peak is activated by the Isl1-Lhx3 complex.** (A) Schematic
791 representation of HxRE-S1, HxRE-S2, 13 TAAT motifs and A/T-rich motif within the Isl1-
792 Peak. Yellow shading indicates the sequences included in the shortened Isl1-Peak-(1-
793 409). (B) HxRE-Short sequence identified by ChIP-Seq de novo motif analysis, and the
794 HxRE-S1 sequences in Isl1-Peak. Isl1-Peak mutants used for GFP-reporter
795 experiments. (C) GFP-reporter experiments for Isl1-Peak variants. Embryos were
796 electroporated with Isl1-Peak-GFP reporter constructs plus ubiquitously expressing
797 LacZ to mark electroporated cells. Sections were immunostained for Mnr2 to mark MNs.
798 Images are representative of electroporations from multiple embryos. Isl1-Peak-wt: n=5,
799 Isl1-Peak-ΔHx-S1: n=6, Isl1-Peak-ΔH2: n=5, Isl1-Peak-ΔA/T: n= 16, Isl1-Peak-ΔHx-
800 S1ΔH2: n= 6. Isl1-Peak-(1-409)-wt: n=17, Isl1-Peak-(1-409)-ΔHx-S1: n=10, Isl1-Peak-
801 (1-409)-ΔH2: n=9, Isl1-Peak-(1-409)-ΔHx-S1ΔH2: n= 20. (D) Embryos electroporated
802 with an Isl1-Peak-GFP reporter construct plus Isl1-Lhx3 fusion protein construct.
803 Sections immunostained for GFP and Mnr2. Images are representative of
804 electroporations from multiple embryos. Isl1-Peak-wt: n=6, Isl1-Peak-ΔHx-S1: n=2, Isl1-
805 Peak-ΔH2: n=5, Isl1-Peak-ΔA/T: n=3 , Isl1-Peak-ΔHx-S1ΔH2: n=4 . Isl1-Peak-(1-409)-
806 wt: n=5, Isl1-Peak-(1-409)-ΔHx-S1: n=4, Isl1-Peak-(1-409)-ΔH2: n=6, Isl1-Peak-(1-409)-
807 ΔHx-S1ΔH2: n= 6. (E, F) GFP fluorescence intensity for embryos electroporated with

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808 Isl1-Peak-GFP reporter constructs + LacZ. n=4-12 embryos per condition. Results were
809 analyzed with a One-Way ANOVA followed by Holm Multiple Comparison Analysis,
810 comparing each mutant reporter construct to full length wt-Isl1-Peak-GFP reporter or (E)
811 wt-(1-409)-Isl1-Peak (F), *p<0.05, **p<0.01. Error bars represent the standard error of
812 the mean.
813
814 **Figure 5: The LMO4-Peak is activated by the Isl1-Lhx3 complex.** (A) HxRE-Long
815 and HxRE-Short sequences identified by ChIP-Seq de novo motif analysis, and the
816 HxRE-Long and HxRE-Short sequences in the LMO4-Peak. (B) LMO4-Peak HxRE-
817 Long mutant used for GFP-reporter experiments. (C) GFP-reporter experiments for
818 LMO4-Peak variants. Embryos were electroporated with LMO4-Peak-GFP reporter
819 constructs plus ubiquitously expressing LacZ to mark electroporated cells. Sections
820 were immunostained for GFP and Mnr2 to mark MNs. Images are representative of
821 electroporations from multiple embryos. LMO4-Peak-wt: n=13, LMO4-Peak-ΔHx-L:
822 n=12 (D) Embryos electroporated with LMO4-Peak-GFP reporter construct plus Isl1-
823 Lhx3 fusion protein construct. Sections were immunostained for GFP and Mnr2. Images
824 are representative of electroporations from multiple embryos. LMO4-Peak-wt: n=4,
825 LMO4-Peak-ΔHx-L: n=5 (E) Luciferase assays testing LMO4-Peak-wt and mutants.
826 Luciferase assays performed in cultured P19 cells. Results show the luciferase
827 activation fold upon the addition of Isl1 plus Lhx3, compared to empty vector. n = 5
828 independent experiments. Results were analyzed with a One-Way ANOVA followed by
829 Holm Multiple Comparison Analysis, comparing each reporter construct to control
830 reporter (no enhancer). **p<0.01. Error bars represent the standard error of the mean.

831

832 **Figure 6: The Isl1-Lhx3 complex activates transcription of endogenous Isl1, Lhx3
833 and Lmo4.** (A-I) Embryos were electroporated with Isl1, Lhx3 or Isl1-Lhx3. In situ
834 hybridization shows the transcription of endogenous *Isl1*, *Lhx3* or *Lmo4*. Lightning bolts
835 indicate the electroporated side of the embryo (right side), compared to the
836 unelectroproated, control side (left side).

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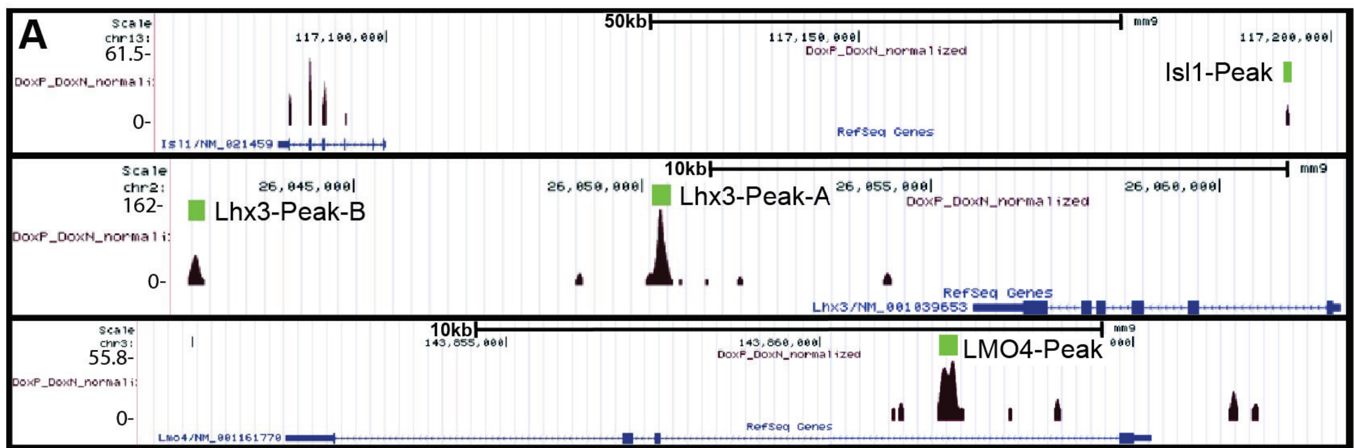

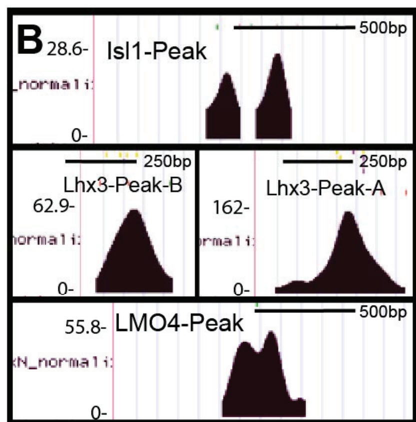

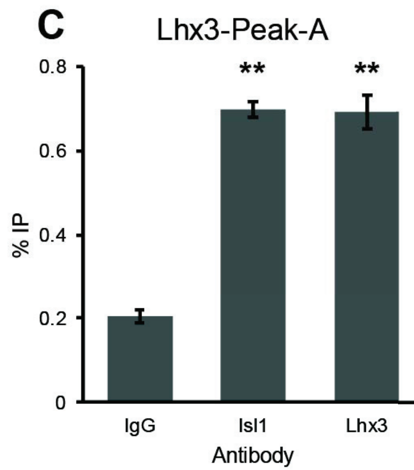

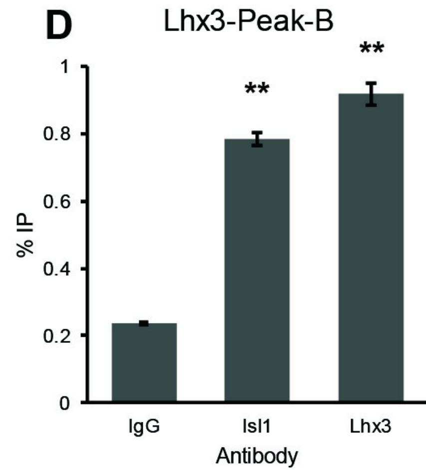

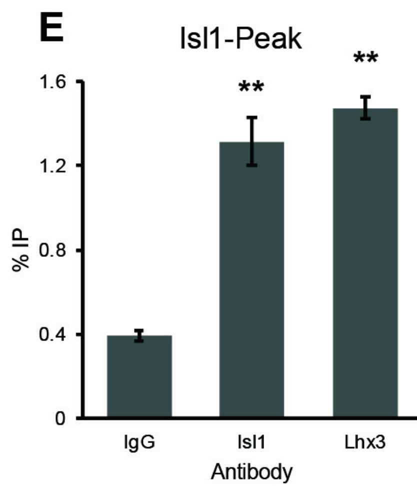

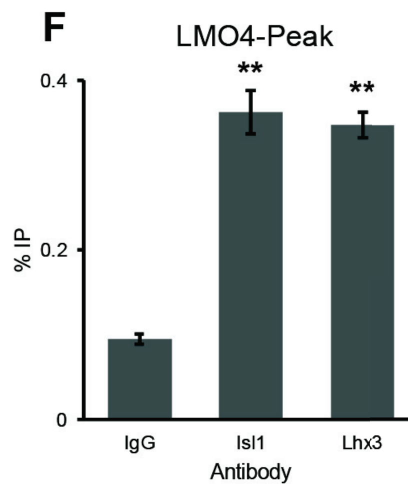

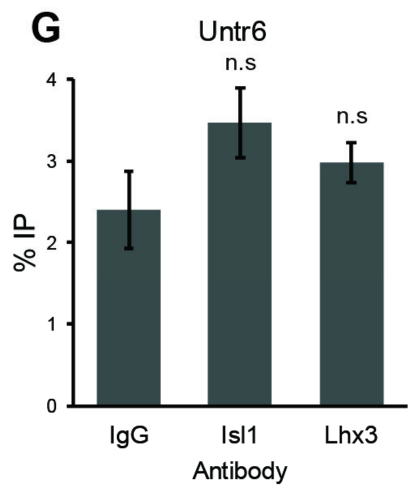

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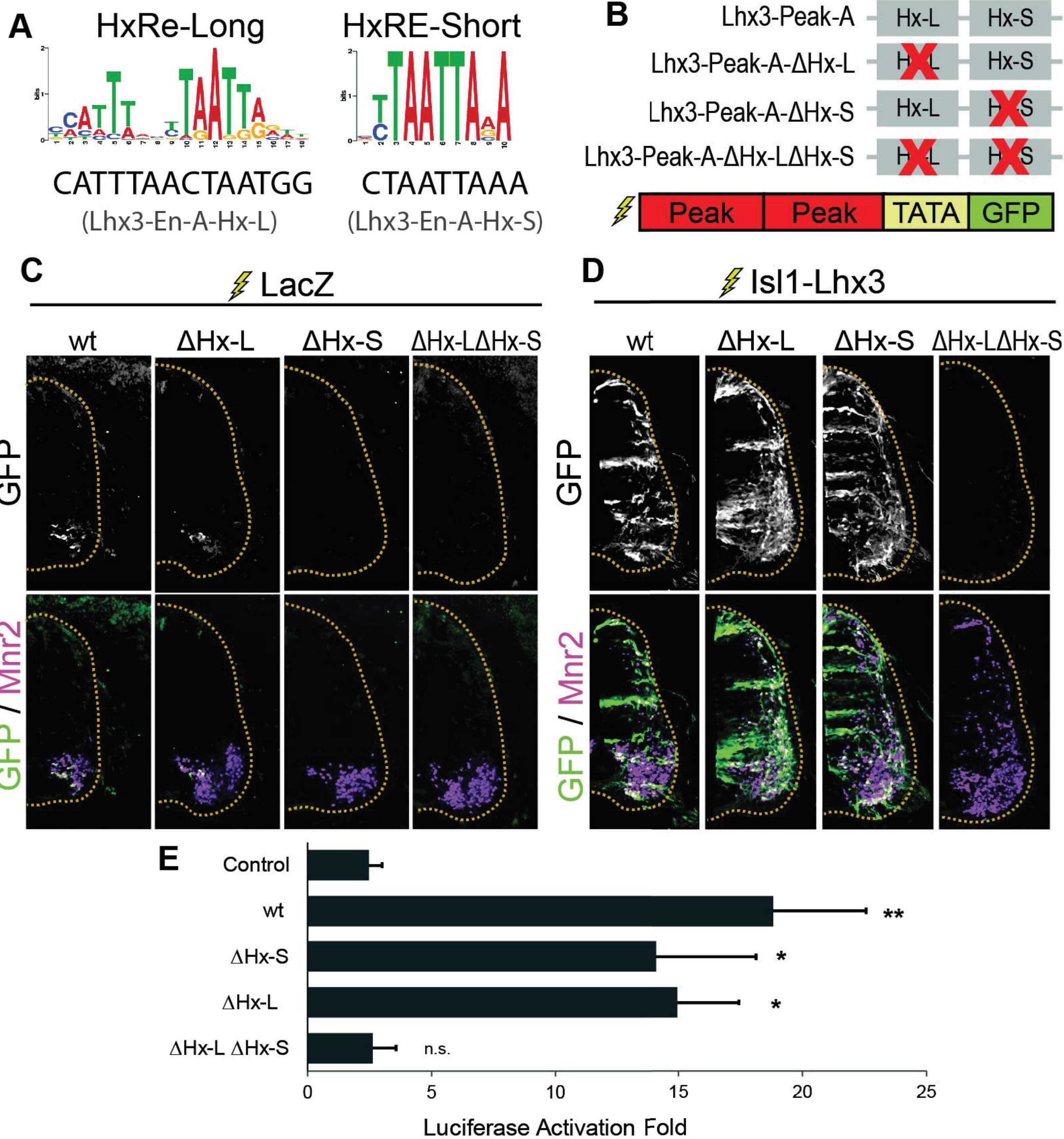

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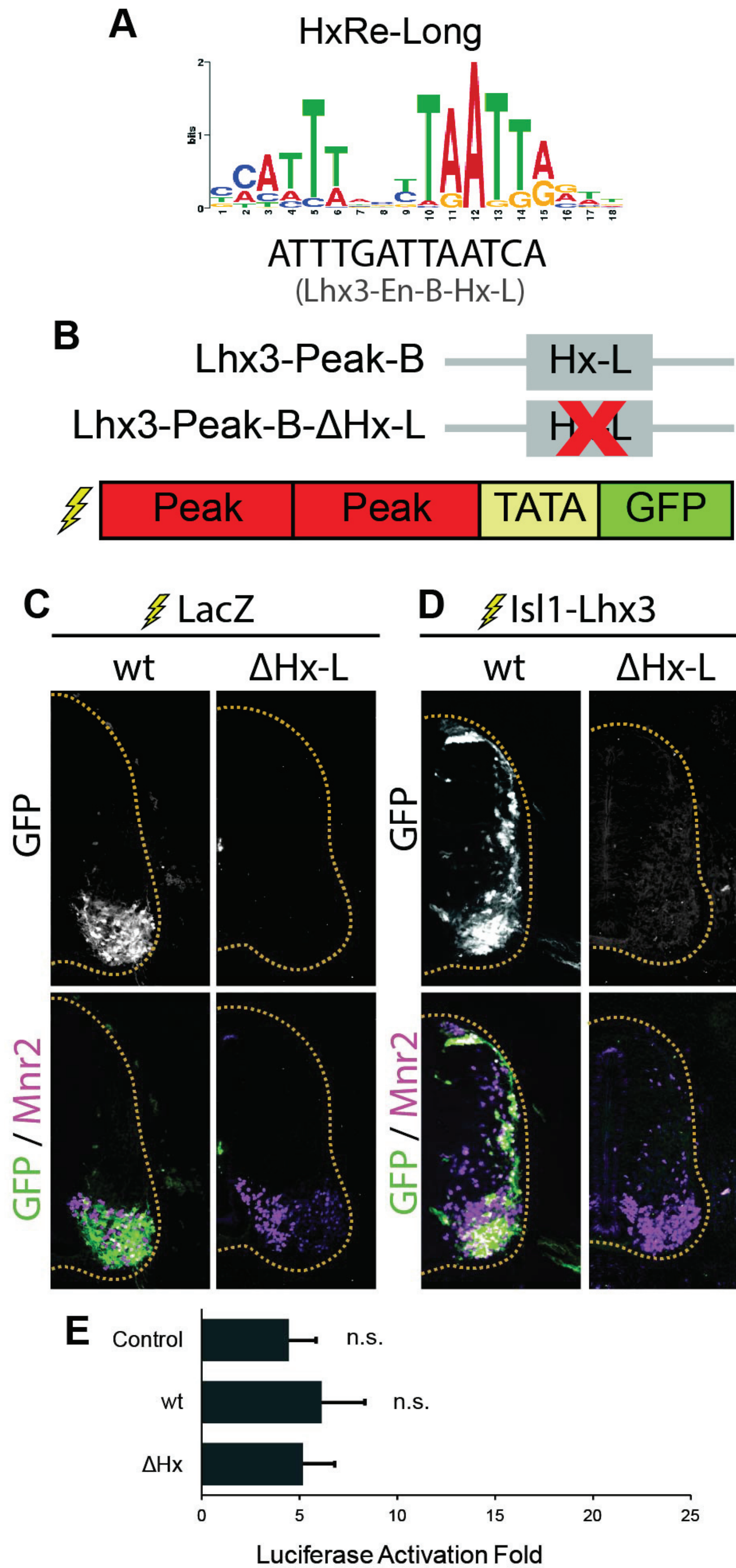

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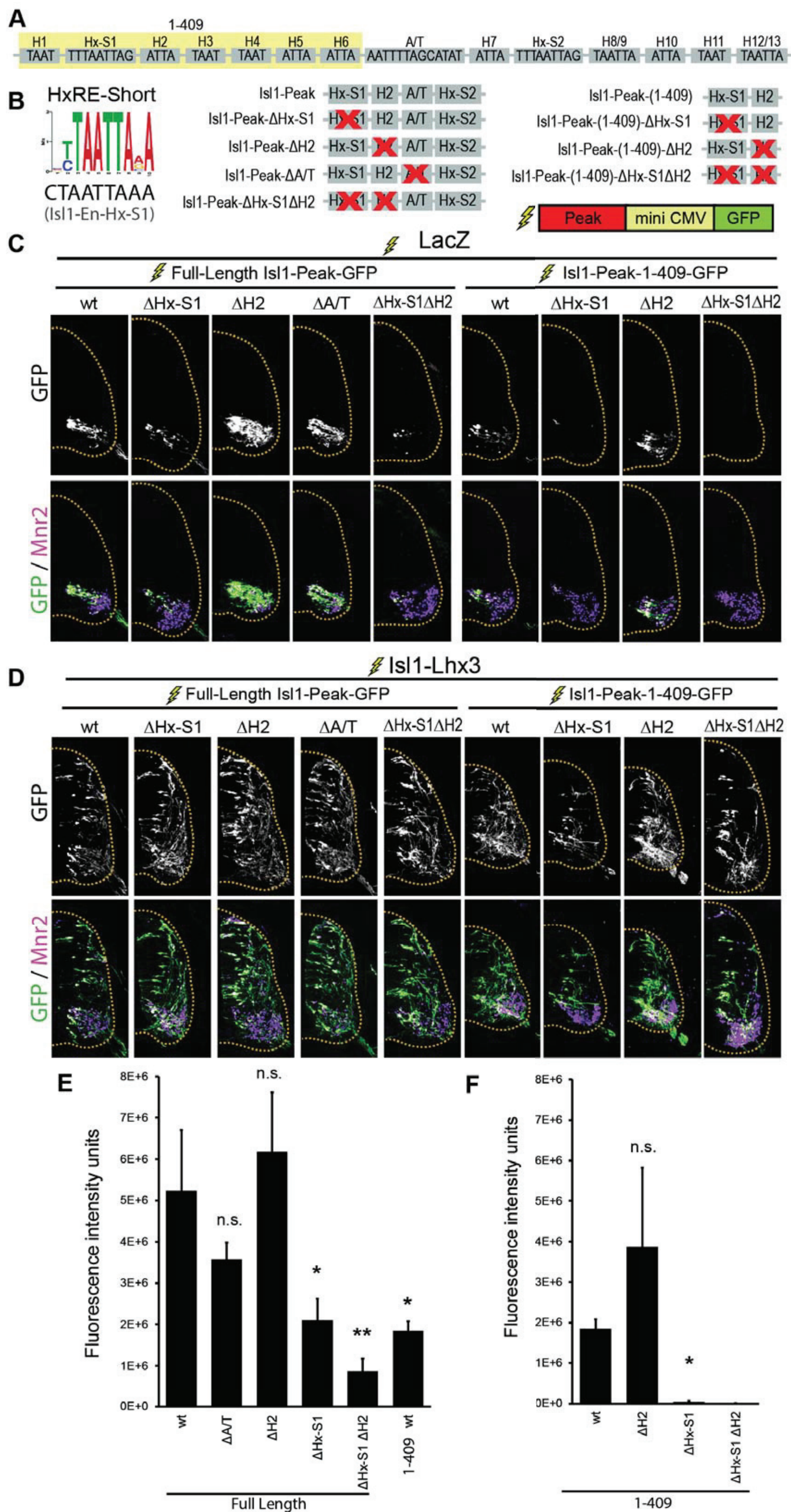

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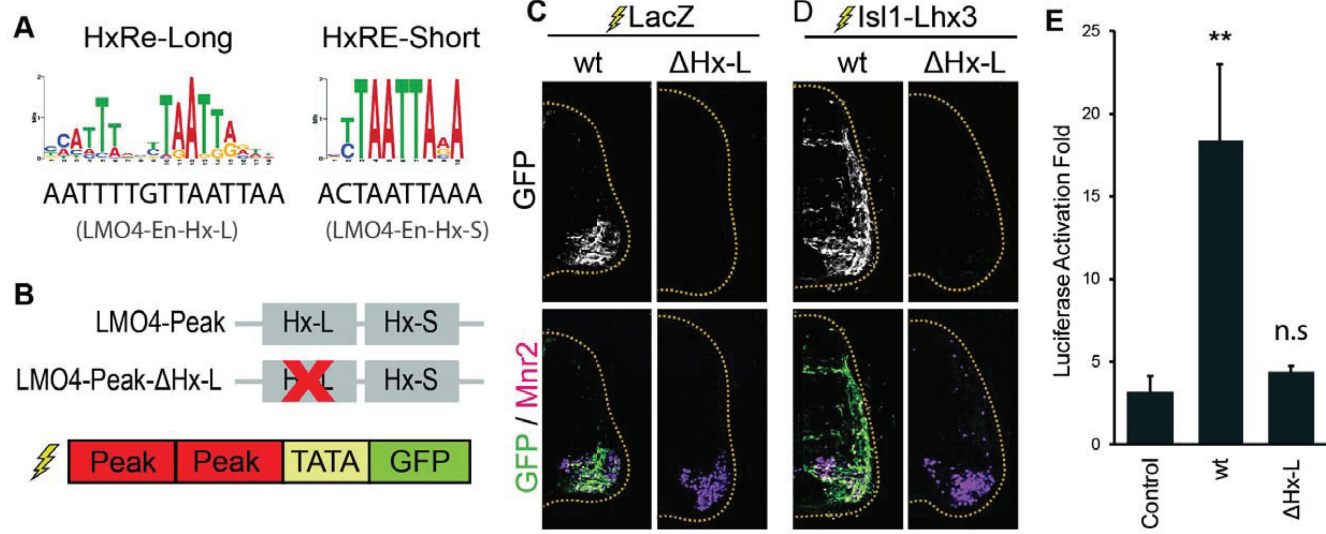

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