

Isl1 Is required for multiple aspects of motor neuron development

Xingqun Liang^{a,b}, Mi-Ryoung Song^c, ZengGuang Xu^a, Guillermo M. Lanuza^d, Yali Liu^a, Tao Zhuang^a, Yihan Chen^a, Samuel L. Pfaff^e, Sylvia M. Evans^{b,c,*}, Yunfu Sun^{a,b,**}

^a Key Laboratory of Arrhythmia, Ministry of Education, East Hospital, Tongji University School of Medicine, Shanghai 200120, China

^b Department of Medicine, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA

^c Bioimaging Research Center and Cell Dynamics Research Center, School of Life Sciences Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea

^d Fundacion Instituto Leloir, Av. Patricias Argentinas 435, Buenos Aires 1405, Argentina

^e Gene Expression Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA, 92037 USA

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ABSTRACT

The LIM homeodomain transcription factor Islet1 (Isl1) is expressed in multiple organs and plays essential roles during embryogenesis. Isl1 is required for the survival and specification of spinal cord motor neurons. Due to early embryonic lethality and loss of motor neurons, the role of Isl1 in other aspects of motor neuron development remains unclear. In this study, we generated Isl1 mutant mouse lines expressing graded doses of Isl1. Our study has revealed essential roles of Isl1 in multiple aspects of motor neuron development, including motor neuron cell body localization, motor column formation and axon growth. In addition, Isl1 is required for survival of cranial ganglia neurons.

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Introduction

Functional motor circuits are dependent on generation of diverse types of neurons and establishment of precise connections of these neurons with their respective targets. Distinct subclasses of neurons in spinal cord are identified by their soma position, stereotypical axon trajectories and combinatory gene expression (Tsuchida et al., 1994; Appel et al., 1995; Tanabe and Jessell, 1996; Sharma et al., 1998). Combinatorial expression of LIM homeodomain (LIM-HD) transcription factors (LIM code) are required for specification and maintenance of distinct neuronal identities and control coordinated cell migration and axon guidance (Jessell, 2000; Shirasaki and Pfaff, 2002). In ventral spinal cord, motor neurons (MN) and V2 interneurons (IN) are derived from adjacent progenitors that share several components of their genetic programs, such as highly related LIM-HD factors Lhx3 and Lhx4. Lhx3 and Lhx4 are expressed in progenitor cells that give rise to both MNs and V2 INs and play a role in specifying the ventral MN identity (Sharma et al., 1998). Overexpression of Lhx3 alone in chick neural tube promotes the generation of V2 INs, whereas in combination with Isl1, it

promotes MN generation (Tanabe et al., 1998; Thaler et al., 2002). LIM-HD factor Isl1 and Homeodomain protein Hb9 are among the first MN genes expressed in postmitotic MNs in spinal cord. Hb9 is critical for the consolidation of MN identity by actively suppressing the V2 IN genetic program (Arber et al., 1999; Thaler et al., 1999). Mice deficient in Hb9 display aberrant expression of the V2 interneuron marker Chx10, disorganized motor columns and axon pathfinding defects (Arber et al., 1999; Thaler et al., 1999). Recent biochemical and genetic studies have provided further insights into molecular mechanisms regulating fate specification of MN and V2 IN (Thaler et al., 2002; Lee and Pfaff, 2003; Nakano et al., 2005). A MN hexamer composed of 2NLI:2Isl1:2Lhx3 binds to and directly activates the Hb9 enhancer, whereas a V2 IN tetrameric protein complex without Isl1 (2Lhx3:2NLI) drives V2 IN genesis (Thaler et al., 2002). MNs express two V2 IN repressors: LIM only protein LMO4 and Hb9. LMO4 can block V2-tetramer assembly while Hb9 binds directly V2-tetramer response elements and suppresses their activation. Similarly, in V2 INs, V2-tetramer induces Chx10, a repressor that binds MN-hexamer response elements and blocks their activation. Thus, these cross regulatory feedback loops ensure precise assignment of MN and V2 IN fates (Lee et al., 2008).

Isl1 is expressed in all postmitotic MNs and is required for various aspects of MN development (Thor et al., 1991; Ericson et al., 1992; Lundgren et al., 1995; Pfaff et al., 1996; Thor and Thomas, 1997; Segawa et al., 2001). In *Drosophila*, Islet is required for motor axon pathfinding and neurotransmitter expression (Thor and Thomas, 1997). In zebrafish, knockdown of Isl2 leads to abnormal spinal MN soma localization and defects in motor axon projection and

* Correspondence to: S.M. Evans, University of California, San Diego / Skaggs School of Pharmacy, 9500 Gilman Dr. M/C 0613C, BSB 5027, La Jolla, CA 92093, USA. Fax: +1 858 5344810.

** Correspondence to: Y. Sun, Key Laboratory of Arrhythmias, Ministry of Education, China, Shanghai East Hospital, Tongji University School of Medicine, 150 Jimo Road, Shanghai 200120, China. Fax: +86 21 56370868.

E-mail addresses: syevens@ucsd.edu (S.M. Evans), yfsunjie@gmail.com (Y. Sun).

neurotransmitter expression (Segawa et al., 2001). In mice, ablation of Isl1 results in complete elimination of spinal MNs immediately after cell cycle exit (Pfaff et al., 1996). Reduced levels of total Islet proteins lead to an increase in V2a IN generation at the expense of MN formation (Song et al., 2009). Due to early embryonic lethality and early loss of MNs in Isl1 null mice, the role of Isl1 in later MN development remains unclear.

Isl1 is expressed in forebrain striatum and ablation of Isl1 in brain leads to a loss of cholinergic interneurons in the striatum and loss of cholinergic projection neurons in the nucleus basalis (Wang and Liu, 2001; Elshatory and Gan, 2008). Isl1 is expressed in sensory neurons of dorsal root ganglia and retina and is required for survival and differentiation of these cells (Elshatory et al., 2007; Pan et al., 2008; Sun et al., 2008). In addition, Isl1 is expressed in neurons of cranial ganglia and nucleus, but the role of Isl1 in these cell types has not been investigated (Thor et al., 1991; Inoue et al., 1994).

In the present study, we generated several mouse lines with graded reduction in Isl1 expression. We have shown that Isl1 is required, in a dose dependent manner, for specification and maintenance of spinal MN identity, proper cell soma settling and appropriate axonal trajectories of MNs. We have also shown that reduced Isl1 expression in Isl1 compound mutants leads to the conversion of prospective MNs to V2 INs. In Isl1 hypomorphic embryos, despite proper expression of HB9, MNs fail to form proper motor columns and motor axons which innervate axial muscles and diaphragm muscle are missing or truncated. In addition, we found that Isl1 is required for survival of cranial ganglia neurons.

Results

Generation of Isl1 hypomorphic and compound mutant Mice

Previously we generated an Isl1^{nLacZ} knock-in mouse line in which a nuclear LacZ (nLacZ) gene was introduced into the endogenous mouse Isl1 locus immediately prior to the translation initiation site (ATG) (Sun et al., 2007). Heterozygous Isl1^{nLacZ/+} are fertile and viable, thus utilized as control mice in this study. Homozygous Isl1^{nLacZ/nLacZ} mutant embryos die around E9.5 with phenotypes identical to that described previously for conventional Isl1 null mutation, and no Isl1 immunoreactivity was detected in Isl1 mutant spinal cord (not shown), thus demonstrating that the Isl1^{nLacZ} allele is a null allele. In this study, we generated a floxed Isl1 mouse line. Mice heterozygous for floxed Isl1 (Isl1^{f/+}) with or without the neo cassette, and mice homozygous for floxed Isl1 without the neo cassette (Isl1^{f/f}) are fertile and viable. However, mice homozygous for floxed Isl1 with the neo cassette (Isl1^{f;neo/f;neo}) died soon after birth with significantly reduced Isl1 expression in spinal MNs (Song et al., 2009), suggesting that the neo cassette interferes with Isl1 expression (hypomorphic allele). Although no general morphological defects were found, Isl1 hypomorphic mutant mice exhibited several motor dysfunctions, including an inability to move and breathe. Lungs of Isl1 hypomorphic mice were not inflated, or were barely filled with air (not shown).

To further compromise Isl1 expression, we crossed Isl1^{f;neo/+} mice to Isl1^{nLacZ/+} mice to generate an Isl1 compound mutant with one Isl1 null allele and one hypomorphic allele (Isl1^{nLacZ/f;Neo}). Compound mutant mice die embryonically before E12.5, due to cardiac defects (YS, SE, unpublished observation). To better visualize neuronal migration and axon projections in Isl1 hypomorphic mice, we crossed Isl1 hypomorphic mice to Hb9-GFP mice, in which GFP expression is under the control of the mouse Hb9 promoter (Wichterle et al., 2002).

Reduced Isl1 expression in Isl1 compound mutant leads to a reduction in the number of spinal motor neurons and neurons in cranial ganglia

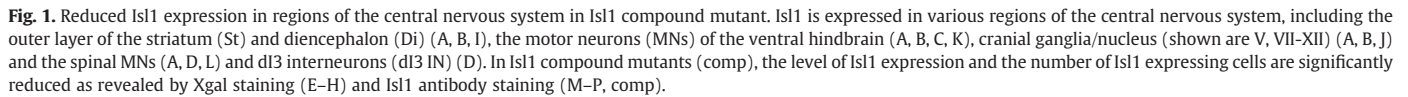
During development, Isl1 is expressed in multiple cell types and tissues and plays essential roles in these cells (Pfaff et al., 1996; Ahlgren et al., 1997; Cai et al., 2003; Laugwitz et al., 2005; Elshatory et al., 2007; Elshatory and Gan, 2008; Pan et al., 2008; Sun et al., 2008).

We first examined how Isl1 expression is affected in Isl1 compound mutant embryos by analyzing Isl1 and β -gal stainings. Wholemount β -gal staining showed that Isl1-nLacZ was expressed in a pattern similar to the Isl1 mRNA expression pattern published previously (Fig. 1A) (Pfaff et al., 1996; Cai et al., 2003). In Isl1^{nLacZ/+} control embryos at E11.5, Isl1-nLacZ was expressed in various regions of the central nervous system, including the outer layer of the forebrain striatum, diencephalon, the ventral hindbrain MNs (Figs. 1A–C), the spinal MNs and a subpopulation of dorsal spinal interneurons (dl3 INs) (Figs. 1A, D). Isl1-nLacZ was expressed in neurons of the peripheral nervous system, including neurons in the dorsal root ganglia (DRG) (Figs. 1A, D) and the sympathetic ganglia (SG) (Fig. 1D). Isl1-nLacZ was also expressed in most of the cranial ganglia with neurons derived from neural crest and/or placodes (Begbie and Graham, 2001) (Figs. 1A–C, shown are Oculomotor (III), Trigeminal (V), Facial/vestibulocochlear (VII/VIII), glossopharyngeal (IX), Vagal (X), Accessory (XI) ganglia/nerves). In compound mutant embryos, expression of β -gal and the number of β -gal expressing cells in most of these regions in central nervous system was significantly reduced (Figs. 1E–H). This result was confirmed by Isl1 antibody immunostaining (Figs. 1I–P). Compared to controls (Figs. 1I–L), there was a near absence of Isl1 immunoreactivity in forebrain striatum and hindbrain MNs (Figs. 1M, O). Isl1 immunoreactivity in the V and VII/VIII cranial ganglia and the spinal MNs was significantly reduced (Figs. 1N, P).

Reduced Isl1 expression leads to malformation of cranial ganglia with increased cell death

To assess the dose dependent effects of Isl1 on cranial ganglia development, we performed wholemount neurofilament antibody staining on E11.5 control and Isl1 mutant embryos with graded reductions in Isl1 expression (Figs. 2A–D). In Isl1 hypomorphic mutant embryos, the size and morphology of cranial ganglia/nerves III, V, VII/VIII, IX, X and XI appeared relatively normal (Fig. 2B). However, axons of cranial nerve XII were significantly thinner and blunted (Fig. 2B, bracket and arrowhead). An ectopic axonal projection was observed that extended from glossopharyngeal ganglion (IX) and connected to vagal (X) and accessory ganglia (XI) (Fig. 2B, white arrow). Further reduction in Isl1 expression in Isl1 compound mutants led to a significant reduction in the size of cranial ganglia V, VII/VIII, IX and X, and axonal projections of these ganglia were significantly thinner (VII, IX, X and IX), blunted (V, VII) or nearly absent (V, XII) (Fig. 2C). Spinal motor axons in Isl1 compound mutant were also significantly thinner or blunted (Fig. 2C). In addition, conditional knockout of Isl1 using Nestin-Cre, which is expressed in neural progenitors (Lendahl et al., 1990; Song et al., 2009), led to a severe loss of cranial ganglia. Cranial nerves and spinal motor axons were blunted or lost (Fig. 2D).

Reduced size of cranial ganglia might be attributed to decreased neurogenesis or increased neuronal apoptosis. To assess apoptosis in cranial ganglia of Isl1 compound mutant embryos, we performed TUNEL staining and Sox10 immunostaining. Sox10 is an HMG-box transcription factor expressed in neural crest and placodal progenitors that give rise to neurons and glial cells in cranial ganglia. In Isl1 compound mutant embryos at E11.5, Sox10 expression, which outlines cranial ganglia, was similar to that of control embryos, suggesting that migration and proliferation of the neural progenitors are not affected in Isl1 compound mutant embryos (Figs. 3E–H). However, significantly increased apoptosis in cranial ganglia V, VII, IX and XI was observed in Isl1 compound mutant embryos (Figs. 3E–H and E'–H'), indicating that Isl1 is required for survival of cranial ganglia neurons. We did not observe significantly increased apoptosis in MNs of the hindbrain and spinal cord of Isl1 compound mutant embryos, despite significantly reduced Isl1 expression in these regions (data not shown).



number of Lhx3 expressing cells were observed ectopically in more ventrolateral region, a few of which coexpressed Hb9 (Figs. 3J, L).

Reduced *Isl1* expression leads to conversion of MNs to V2 INs

Isl1 plays an essential role in dictating the fate choice between MNs and V2 INs. We examined coexpression of MN markers Isl1 and Hb9 with the V2a IN marker Chx10 in Isl1 compound mutant embryos. At E11.5, the number of Isl1 and Hb9 expressing cells was significantly reduced with a concomitant increase in the number of Chx10+ V2a INs in Isl1 compound mutant embryos (Figs. 4A–D and M). A large number of Chx10 expressing cells in Isl1 compound mutant embryos were found ectopically in more ventral positions, mixed with remaining MNs (Figs. 4A–D, below the bar), suggesting a conversion of MNs to INs. However, we did not detect any cells that coexpressed MN markers and Chx10. We examined co-expression of Chx10 and Hb9 at E10, the earliest time of V2 IN genesis, to examine whether prospective MNs altered their identity immediately after birth. In control embryos, a few Chx10+ V2a INs have emerged in the spinal cord, dorsal to Hb9+ MNs (Fig. 4E). In Isl1 compound mutant embryos, there was a marked increase in the number of Chx10+ INs at this stage, which assumed a more ventral position relative to controls, and were mixed with Hb9 MNs (Fig. 4F, below the bar, and L). Some cells started to express Chx10 immediately after they leave

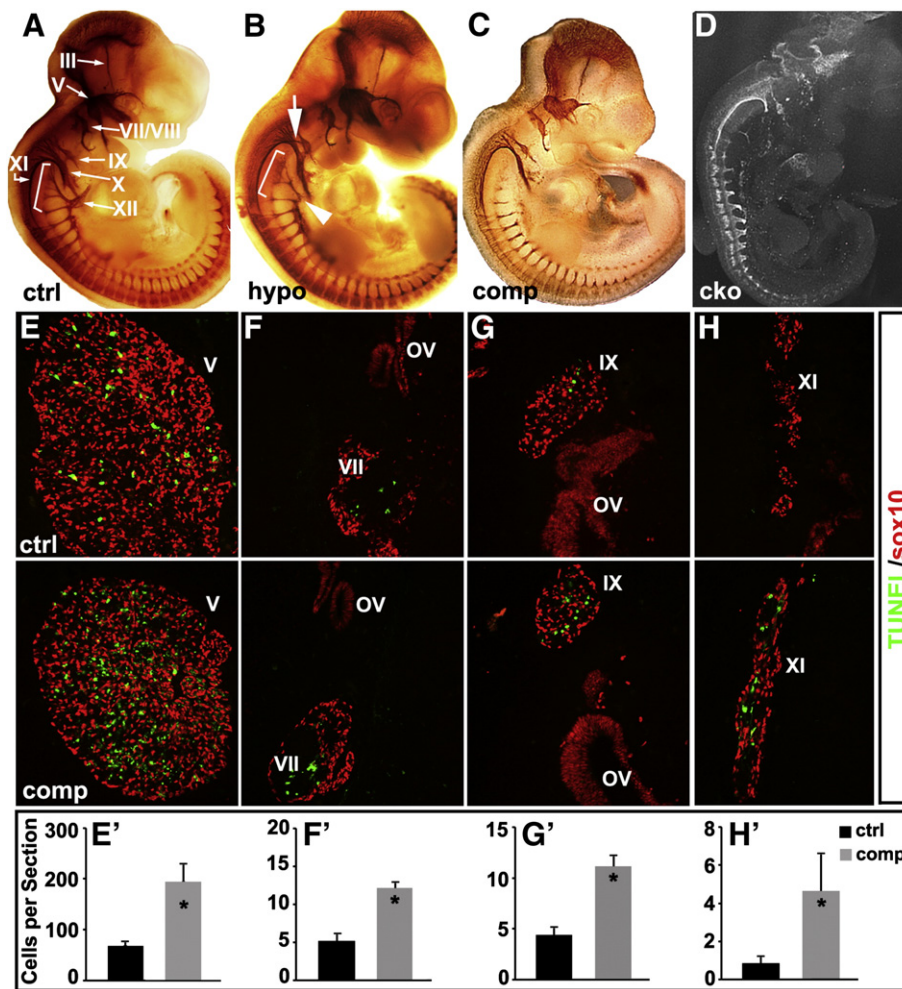


Fig. 2. Reduced *Isl1* expression in *Isl1* compound mutant leads to increased apoptosis and abnormal development of cranial ganglia. Wholemount neurofilament staining of control (ctrl) (A), *Isl1* hypomorphic (hypo) (B), *Isl1* compound mutant (comp) (C) and Nestin-Cre; *Isl1* conditional knockout (cko) (D) embryos at E11.5. Compared to control embryos, *Isl1* hypomorphic mutant embryos displayed a relative normal size of cranial ganglia III, V, VII/VIII, X and XI (B). However, ectopic axonal projections from the cranial ganglion IX were observed (B, white arrow). Axonal projections of XI and XII ganglia were significantly reduced and blunted (B, bracket and arrowhead). In *Isl1* compound mutant (C), further reduction in *Isl1* expression led to reductions in the size of the cranial ganglia (V, VII/VIII, IX and X). Reductions in axon projections were observed in ganglia V (ophthalmic, maxillary and mandibular branches), VII, IX, X, XI and XII (C). Nestin-Cre; *Isl1* conditional knockout embryos displayed an overall developmental defect with severe reduction in the size of cranial ganglia and their axon projections (D). TUNEL staining at E11.5 revealed significantly increased apoptosis in the cranial ganglia V, VII, IX and XI of *Isl1* compound mutant compared to control embryo (E–H and E'–H').

the ventricular zone and start to differentiate (Fig. 4F, arrowhead). However, no coexpression of *Chx10* and *Hb9* was observed in either *Isl1* compound mutants or control embryos at this early stage. We performed *Isl1* lineage analysis with *Isl1*-Cre; Rosa-YFP and confirmed that *Chx10* was never expressed in *Isl1* lineages (YFP) in normal spinal cord (Fig. 4G), suggesting a complete segregation of these two cell types presumably at the progenitor level. These results suggested that the increase in V2a INs is due either to the conversion of MNs to INs immediately after their generation, or to increased genesis of V2a IN at the expense of MN genesis. Taking advantage of the fact that β -galactosidase is more stable and persists longer in cells (Thaler et al., 2004), we examined whether *Isl1*- β -gal was ever expressed in ectopic V2a INs. In control embryos, *Isl1*- β -gal was never coexpressed with *Chx10* (Fig. 4H). However, in *Isl1* compound mutant embryos, a substantial proportion of *Chx10*+ V2a INs coexpressed *Isl1*- β -gal (Fig. 4I), suggesting a conversion of prospective MNs to V2a INs in *Isl1* mutants. To examine whether these aberrant *Chx10*+ V2a INs follow characteristic axonal trajectories of normal V2 INs, we performed intraspinal cord Rhodamine-dextran labeling. *Chx10* V2a INs were similarly labeled by Rhodamine-dextran (Rhd) in both control ($16.6 \pm 0.5\%$ of total *Chx10*+ cells) and *Isl1* compound mutant spinal cords

($17.2 \pm 3.44\%$) (Fig. 4J, K—arrowhead and N-a). A significant number of ectopic V2a INs in *Isl1* compound mutant spinal cords, marked by the coexpression of *Chx10* and *Isl1*- β -gal, were also labeled by Rhodamine-dextran (Fig. 4K—arrow and N-b), suggesting these ectopic INs extended axons intraspinally similarly to normal V2 INs. However, aberrant *Chx10* V2 INs populated normal sites of MNs and intermingled with MNs, suggesting that these aberrant *Chx10* V2 INs maintained a MN soma settling pattern.

Isl1 is required for MN migration, motor column formation and axon growth

In *Drosophila* and zebrafish, *Isl* is required for correct soma settling and axon trajectory of the MNs (Thor et al., 1999; Segawa et al., 2001; Tanaka et al., 2011). We analyzed *Isl1* hypomorphic mice, which survive perinatally. Despite a significant reduction in *Isl1* expression in spinal MNs of *Isl1* hypomorphic embryos, the level of *Hb9* expression and the number of *Hb9* expressing MNs did not significantly change during development (Figs. 5A, B and C). At E16.5, *Hb9*+ MNs in the spinal cord of control embryos were segregated into medial (MMC) and lateral (LMC) motor columns at

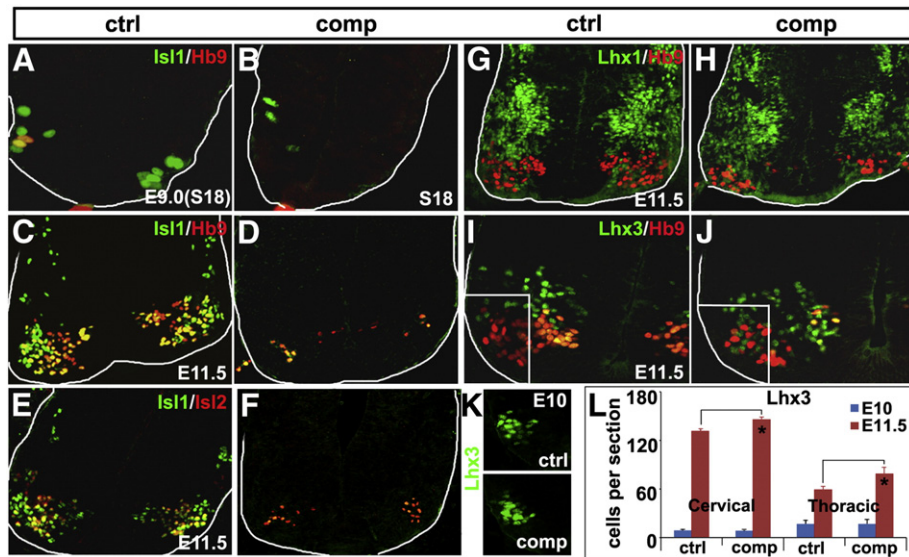


Fig. 3. Decreased MN genesis in *Isl1* compound mutant embryos. At E9 (18 Somites), a few *Isl1* expressing MNs are generated in the anterior segments of the control spinal cord, a subpopulation of which coexpress *Hb9* (A). However, in *Isl1* compound mutant spinal cord, significantly fewer *Isl1* expressing MNs are generated and none of which expresses *Hb9* (B). At E11.5, compared to that of control mice (C, E), there is a dramatic reduction in the number of MNs marked by the expression of *Isl1*, *Isl2* and *Hb9* in *Isl1* compound mutant (D, F). At E11.5, *Lhx1* expression in the spinal cord of *Isl1* compound mutant is comparable to that of control embryo (G, H). *Lhx3* is expressed in V2 INs, and the MNs of the medial motor column where it is coexpressed with *Hb9* (I). However, the number of *Lhx3* expressing cells in the spinal cord of *Isl1* compound mutant embryos is significantly increased at E11.5 (J, L—red). Ectopic *Lhx3* expressing cells were observed to invade into more ventrolateral region, a few of which coexpress *Hb9* (J). However, at E10.5 the number of *Lhx3* expressing cells is comparable in mutant and control samples (K, L—blue).

the cervical level or MMC at the thoracic level (Figs. 5D, F). However, in *Isl1* hypomorphic mice, *Hb9*+ MNs appeared to be scattered and ectopically positioned or abnormally clustered and failed to form distinct motor columns (Figs. 5E, G). In *Isl1* compound mutant embryos, MNs were frequently observed to migrate along ventral roots from the neural tube (Fig. 5I arrow). These results demonstrated

that *Isl1* is required for MN soma settling or migration, and the formation of the motor column.

To examine MN axonal projections, we utilized *Isl1* hypomorphic mice crossed into an *Hb9*-GFP transgenic mouse background that allowed us to visualize motor axon trajectories. In control embryos, spinal MNs extended motor axons that, after exiting from the

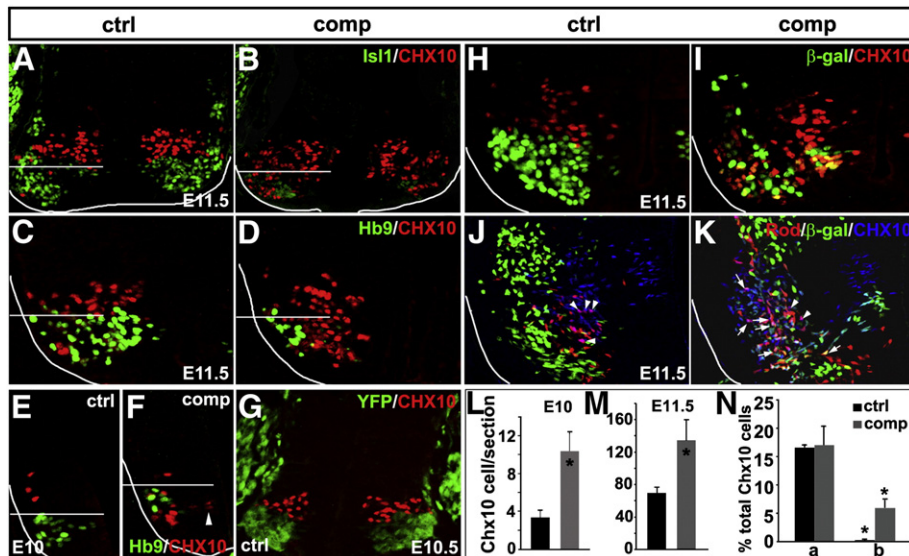


Fig. 4. Reduced *Isl1* expression leads to conversion of prospective MNs to V2a INs. A significant increase in the number of *Chx10* expressing V2a interneurons (INs) was observed in the spinal cord of *Isl1* compound mutant embryos at E11.5 (A–D, M). These *Chx10* cells found ectopically in more ventrolateral position, mixed with remaining MNs (B, D, below the bar). At E10, a few *Chx10*+ V2a INs start to emerge in the domain dorsal to that of *Hb9*+ MNs (E). A significant increase in the number of *Chx10*+ INs was readily detectable at this stage, which assumed a more ventral position and was mixed with *Hb9* MNs (F, L). Some mutant cells started to express *Chx10* immediately after they left the ventricular zone and started to differentiate (F, arrowhead). No coexpression of V2a IN marker *Chx10* with MN marker *Isl1* (A, B) and *Hb9* (C–F) were observed in both *Isl1* compound mutant and control embryos. *Chx10* is never expressed in *Isl1* lineage (YFP) in the normal spinal cord (G). *Isl1*- β gal is not coexpressed with *Chx10* in control embryos (H). However, a significant number of ventral ectopic *Chx10* V2a INs in *Isl1* compound mutant embryos coexpress *Isl1*- β gal (45.4 \pm 23.4 per section) (I). Intraspinal cord labeling revealed that *Chx10* V2a INs were similarly labeled by Rhodamine-dextran (Rhd) in both control (16.6 \pm 0.5% of total *Chx10*+ cells) and *Isl1* compound mutant (17.2 \pm 3.44%) (J, K, arrowhead and N-a). A significant number of ectopic V2a INs in *Isl1* compound mutant, marked by the coexpression of *Chx10* and *Isl1*- β gal, were also labeled by Rhodamine-dextran (K-arrow and N-b). a: Rhd+/+ *Chx10*+; b: Rhd+/+ *Chx10*+/ β gal+.

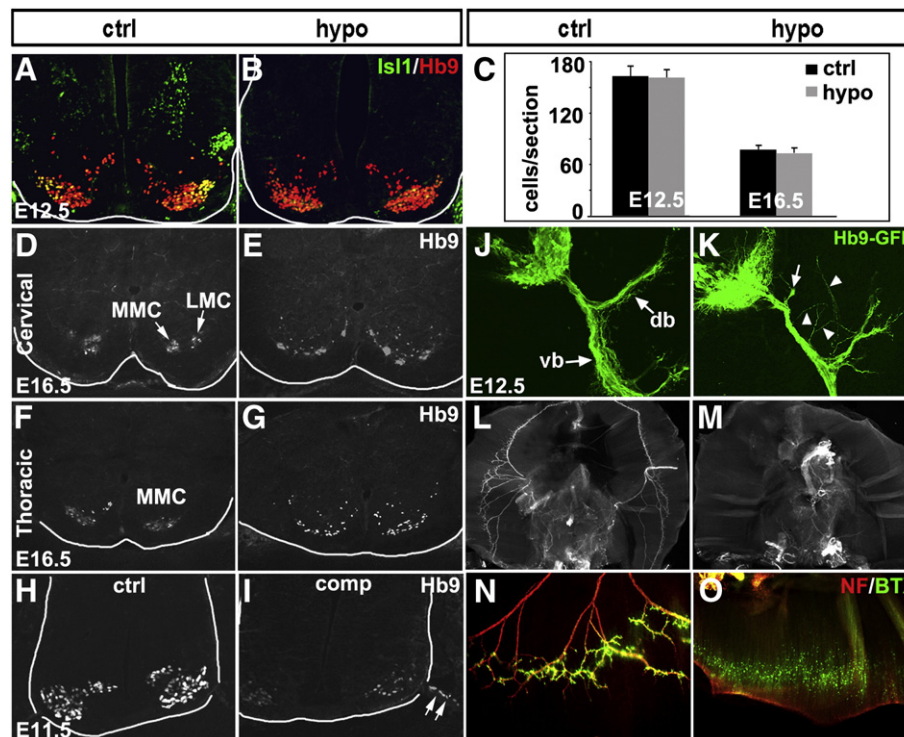


Fig. 5. Defects in MN migration, motor column formation and axon growth in *Isl1* hypomorphic embryos. Despite significant reduction in *Isl1* expression in spinal MNs of *Isl1* hypomorphic mutant, the level of Hb9 expression and the number of Hb9 expressing MNs during development are not changed (A, B and C). At E16.5, Hb9+ MNs are segregated into medial (MMC) and lateral (LMC) motor columns at the cervical spinal cord, or MMC at the thoracic spinal cord of control mice (D, F). However, in *Isl1* hypomorphic embryos, Hb9+ MNs are scattered and ectopically positioned or abnormally clustered and fail to form distinct motor columns (E, G). In *Isl1* compound mutant spinal cord, MNs were frequently observed to migrate along ventral roots out of neural tube (H, I arrow). In Hb9-GFP transgenic mice, GFP expression marks the axons of the spinal MNs, which after exit from vertebral column, are divided into dorsal branch (db) and ventral branch (vb) (J). In *Isl1* hypomorphic mice that have been crossed onto Hb9-GFP transgenic background, the dorsal branch of the spinal motor axons appeared to be blunt (K, arrow) and frequent ectopic motor projections were observed (K, arrowhead). Wholemount neurofilament immunostaining and Alexa 488- α -bungarotoxin (BTX) staining revealed motor innervation of diaphragm by phrenic nerve and the formation of neuromuscular junctions on the diaphragm of control mice (L, N). However, diaphragm of *Isl1* hypomorphic mutant mice were deinnervated and no functional neuromuscular junctions were detected (M, O).

vertebral column, divided into the dorsal branch (db) and ventral branch (vb) (Fig. 5J). In *Isl1* hypomorphic mutants, dorsal branches of motor axons were lost or blunted (Fig. 5K, arrow) and frequent ectopic motor projections were observed (Fig. 5K, arrowhead).

Isl1 hypomorphic mice died soon after birth, presumably due to defects in respiratory motor activity. Therefore we examined phrenic nerve innervation of diaphragm muscle. Wholemount neurofilament immunostaining and rhodamine- α -bungarotoxin (BTX) staining revealed phrenic motor innervation and the formation of neuromuscular junctions in the diaphragm of control mice (Figs. 5L, N). However, the diaphragm of *Isl1* hypomorphic mutant mice was deinnervated and no functional neuromuscular junctions were observed (Figs. 5M, O). Together, these results suggested that *Isl1* is required for correct axon pathfinding and growth. It is likely that *Isl1* hypomorphic mice died of respiratory defects and a lack of diaphragm innervation.

Discussion

Due to early lethality and loss of MNs in *Isl1* conventional knockout mice, the role of *Isl1* in MN development remains unclear. In this study, we have generated two *Isl1* mutant mouse lines with graded reductions in *Isl1* expression. Analysis of these mice has revealed essential roles of *Isl1* in MN soma settling and axon projection, and provided further evidence that reduced *Isl1* expression leads to conversion of prospective MNs to V2a INs. In addition, our study has demonstrated a requirement for *Isl1* in survival of the cranial ganglia neurons.

Isl1 plays essential roles in MN survival and MN cell fate choice. It has been shown that reduced level of total *Islet* proteins leads to

reduced number of MNs and conversion of MNs to V2a INs (Song et al., 2009). Consistent with these studies, we found that reduced *Isl1* expression in *Isl1* compound mutant lead to a reduction in the number of MNs with a concomitant increase in the number of V2 INs. Loss of Hb9 expression and thus MNs occur at the very beginning of MN genesis (E9.0). However, at this stage no premature generation of Chx10 cells was detected. We observed a significantly increased number of Chx10 cells in *Isl1* compound mutant spinal cord at E10 (30–somite), a time when V2 IN genesis is just beginning. A few mutant cells started to express Chx10 immediately after their emergence from the ventricular zone of the spinal cord. These results suggest an early conversion of MNs to V2 INs, or an increase in V2a IN genesis at the expense of MN genesis. We did not observe coexpression of MN marker Hb9 or *Isl1* with V2 IN marker Chx10 in either control or *Isl1* compound mutant embryos. Lineage analysis with *Isl1*-Cre; Rosa-YFP confirmed that Chx10 was never expressed in *Isl1* lineages in normal spinal cord. However, by using an *Isl1*-LacZ mouse line, in which *Isl1* nuclear LacZ expression recapitulates endogenous *Isl1* expression, but nuclear LacZ persists longer than *Isl1* protein in cells, we found that a substantial proportion of *Isl1* mutant cells coexpressed *Isl1*-LacZ and Chx10, thus providing direct evidence that with reduced *Isl1* expression, prospective MNs are converted to V2 INs, which are capable of projecting axon intraspinaly.

The phenotype seen in the *Isl1* mutant embryos is similar but distinct to that of Hb9 mutant mice. Hb9 is not required for initial MN development. Deletion of Hb9 leads to a gradual conversion of MNs to V2 INs over a period between E10.5 and E13.5, and this conversion is less complete, thus resulting in hybrid cells with mixed identities (Arber et al., 1999; Thaler et al., 1999). In contrast, the conversion of MNs to V2 INs in *Isl1* compound mutant embryos occurs earlier,

suggesting that *Isl1* and *Hb9* may not function in the same pathway, and other targets may exist downstream of *Isl1* in specifying MN fate. Interestingly, in *Drosophila*, *dHb9* and *Isl1* do not regulate each other, but rather, function in parallel to specify MN fates (Broihier and Skeath, 2002). However, overexpression of *Hb9*, but not *Isl1*, is sufficient to induce MN differentiation, suggesting that *Isl1* must function together with other factors. In this context, delayed down-regulation of *Isl1* in *Hb9* mutants, but early loss of *Hb9* expression in *Isl1* compound mutants may contribute to phenotypic differences in these two mutant mice. In the future, it would be interesting to see to what extent *Hb9* overexpression could rescue the MN phenotype caused by loss of *Isl1*.

Lhx3 is expressed transiently in all MNs, but downregulated when MNs migrate laterally. Overexpression of *Lhx3* is sufficient to induce ectopic V2 IN differentiation (Tanabe et al., 1998; Thaler et al., 2002). We found that, in contrast to *Hb9* mutants, initial expression of *Lhx3* in *Isl1* compound mutants was comparable to that of controls, but increased at E11.5, mainly due to conversion of MNs to V2a INs. Interestingly, a previous study has shown that deletion of *Isl1* leads to ectopic *Lhx3* expression in DRG sensory neurons, suggesting a role of *Isl1* in repressing *Lhx3* expression (Sun et al., 2008). Thus, increased *Lhx3* expression in certain cellular contexts with reduced *Isl1* and *Hb9* promotes conversion of MNs to V2a INs.

Subclasses of spinal MNs are identified by their distinct soma position and stereotypical axonal trajectories. Studies in *Drosophila* and zebrafish have demonstrated that *Isl1* is required for MN migration and axon pathfinding (Thor and Thomas, 1997; Segawa et al., 2001; Tanaka et al., 2011). However, due to the early loss of MNs in *Isl1* null mice, the role of *Isl1* in these later developmental events remains unclear. In this study, we have generated *Isl1* hypomorphic mice, which survive until birth, allowing us to address these questions. During early development, the number and distribution of *Hb9*⁺ MNs in *Isl1* hypomorphic mice was comparable to that of control mice. At later stages, control MNs are further sorted and consolidated into distinct motor columns. Although similar numbers of *Hb9* MNs were observed in *Isl1* hypomorphic mutant and control mice at this stage, *Isl1* mutant MNs failed to form distinct motor columns and remained dispersed or formed small clusters. Some mutant MNs migrated ectopically and extraspinally, similar to behaviors observed in *Hb9* and *Isl2* null mice (Arber et al., 1999; Thaler et al., 2004). Our results demonstrated that *Isl1* is required for MN soma settling. In addition, *Isl1* hypomorphic mutant MNs projected axons along ventral pathway to peripheral targets. However, dorsal branches of *Isl1* mutant motor axons and phrenic nerve were lost. So far the underlying mechanisms for this remain unclear. Although initially *Isl1* is expressed in all spinal MNs, its expression level varies among motor columns (Tsuchida et al., 1994). Thus the dose-dependent requirement for *Isl1* in each of the motor columns may vary significantly, but higher levels of *Isl1* expression may be required for MNs of MMC to elaborate axons properly. Phrenic MNs that innervate diaphragm express *Isl1*, but not *Isl2* (Ericson et al., 1997). Thus development of this population of MNs may also be critically dependent on high levels of *Isl1* expression.

Our studies have demonstrated that *Isl1* is required for MN soma settling and motor column formation and axonal growth, however, underlying mechanisms remain to be defined. Motor axon growth is regulated by a series of long-range and cell-contact chemoattractive or repulsive signals that involve several families of molecules and their respective receptors, including netrins/Unc, semaphorins/NRPs and several growth factor signaling pathways (Dickson, 2002; Klein, 2004). Mice deficient in each of these genes present defects in motor axon trajectories and/or motor neuron soma settling, which partially resemble defects observed here in *Isl1* hypomorphic mice (Kawasaki et al., 1999; Chen et al., 2000; Giger et al., 2000; Haase et al., 2002; Takashima et al., 2002; Burgess et al., 2006). It has been shown that MMC axons express FGFR1 and are chemoattracted by FGFs

expressed in dermomyotome (Shirasaki et al., 2006). In addition, differential expression of cell adhesion molecules such as cadherins and NCAM in subclasses of spinal motor neurons are involved in motor pool formation and axon guidance (Price et al., 2002). Future study is needed to examine whether alternations in expression of these genes are responsible for the phenotypes observed in *Isl1* hypomorphic embryos.

Our study revealed a role of *Isl1* in the survival and axon projection of cranial ganglia neurons, similar to that found in sensory neurons (Elshatory et al., 2007; Pan et al., 2008; Sun et al., 2008). We found that reduced *Isl1* expression in *Isl1* hypomorphic embryos resulted in misprojection of glossopharyngeal nerve (XI) and significant loss of hypoglossal nerve (XII). Further reduction or deletion of *Isl1* lead to markedly increased cell death in neurons of multiple cranial ganglia and loss of axonal projections. However, loss of axons in these instances is probably secondary to the loss of neurons.

Experimental methods

Transgenic mice

Isl1^{nLacZ} knock-in mouse line was generated by insertion of nuclear LacZ (nLacZ) gene into mouse *Isl1* gene locus just before the *Isl1* translation initiation site (ATG) (Sun et al., 2007). Floxed *Isl1* mouse line was generated as described (Sun et al., 2008). Briefly, exon 4 of *Isl1* gene was floxed by the insertion of a loxP site on one end and a loxP site and frt-flanked neomycin cassette (neo) on the other end that serves as an ES cell selection marker. By backcrossing mice heterozygous for floxed *Isl1* with neo gene (*Isl1*^{f;Neo/+}) to get mice homozygous for floxed *Isl1* with neo gene (*Isl1*^{f;neo/f;neo}) (*Isl1* hypomorphic mice). *Isl1* compound mutant mice (*Isl1*^{nLacZ/f;Neo}) were generated by crossing *Isl1*^{f;Neo/+} mice with *Isl1*^{nLacZ/+} mice. To better visualize the neuronal migration and axon projections in *Isl1* hypomorphic mice, we crossed *Isl1* hypomorphic mice with *Hb9*-GFP mice (Lee et al., 2004).

X-gal staining and histological analysis

Embryos were harvested at the desired time points during gestation and fixed for 1–2 h in 4% PFA. Embryos were stained several hours to overnight at 37 °C in X-gal substrate solution consisting of 5 mM K₄Fe (CN)₆, 5 mM K₃Fe (CN)₆, 2 mM MgCl₂, 0.01% NP-40, 0.1% deoxycholate, and 0.1% X-gal in PBS. Wholemount samples were fixed with 4% paraformaldehyde, paraffin-embedded and sectioned, and counter stained with hematoxylin and eosin (H&E).

Immunostaining, TUNEL staining and retrograde labeling

Embryos were fixed for 1–3 h in 4% PFA, embedded in OCT, and sectioned at 10 μm. Immunofluorescence was performed with the following primary antibodies: mouse monoclonal anti-Islet1/2 (DSHB), rabbit anti-Islet1 (abcam), guinea pig anti-*Isl2*, guinea pig anti-*Hb9*, guinea pig anti-*Sox10*, mouse anti-*Lhx1* and mouse anti-*Lhx3*, rabbit anti-*Chx10*, rabbit anti-caspase-3 (Cell Signal Tech. #9661), mouse anti-neurofilament (DSHB) and rabbit anti-β-gal (Cappel, #55976). Sections were washed then incubated with the appropriate secondary antibodies fluorescently labeled with Alexa 488 or 594 (Invitrogen), and mounted in Vectashield DAPI medium (Vector Laboratories). TUNEL staining was performed as recommended (Roche). For the wholemount neurofilament immunostaining, E11.5 embryos were harvested and processed for immunostaining using rabbit anti-neurofilament 150 (DSHB and Chemicon) antibody. For the phrenic nerve labeling, the diaphragm muscles were fixed and processed for immunostaining using rabbit anti-neurofilament 150 antibody and Alexa 488-conjugated Bungarotoxin (Invitrogen) (Thaler et al., 1999).

Intraspinal labeling of the spinal interneurons was performed with rhodamine-dextran (3000 MW, Molecular Probes). Embryos were cultured in oxygenated Ringer's buffer overnight at room temperature and then fixed for immunocytochemistry.

Statistical analysis

Data are presented as mean \pm SEM and student *t*-test was used for 2-group comparisons. Differences were considered statistically significant at a value of $P < 0.05$.

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