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# Onecut transcription factors act upstream of *Isl1* to regulate spinal motoneuron diversification

Agnès Roy<sup>1,\*</sup>, Cédric Francius<sup>1,\*</sup>, David L. Rousso<sup>2,†</sup>, Eve Seuntjens<sup>3,4,§</sup>, Joke Debruyne<sup>3,4</sup>, Georg Luxenhofer<sup>5</sup>, Andrea B. Huber<sup>5</sup>, Danny Huylebroeck<sup>3,4,§</sup>, Bennett G. Novitsch<sup>2</sup> and Frédéric Clotman<sup>1,¶</sup>

## SUMMARY

During development, spinal motoneurons (MNs) diversify into a variety of subtypes that are specifically dedicated to the motor control of particular sets of skeletal muscles or visceral organs. MN diversification depends on the coordinated action of several transcriptional regulators including the LIM-HD factor *Isl1*, which is crucial for MN survival and fate determination. However, how these regulators cooperate to establish each MN subtype remains poorly understood. Here, using phenotypic analyses of single or compound mutant mouse embryos combined with gain-of-function experiments in chick embryonic spinal cord, we demonstrate that the transcriptional activators of the Onecut family critically regulate MN subtype diversification during spinal cord development. We provide evidence that Onecut factors directly stimulate *Isl1* expression in specific MN subtypes and are therefore required to maintain *Isl1* production at the time of MN diversification. In the absence of Onecut factors, we observed major alterations in MN fate decision characterized by the conversion of somatic to visceral MNs at the thoracic levels of the spinal cord and of medial to lateral MNs in the motor columns that innervate the limbs. Furthermore, we identify *Sip1* (*Zeb2*) as a novel developmental regulator of visceral MN differentiation. Taken together, these data elucidate a comprehensive model wherein Onecut factors control multiple aspects of MN subtype diversification. They also shed light on the late roles of *Isl1* in MN fate decision.

**KEY WORDS:** Onecut factors, *Isl1*, *Sip1*, Neuronal diversification, Spinal cord, CNS development

## INTRODUCTION

Body movements and locomotion as well as visceral activity rely on the ability of spinal motoneurons (MNs) to activate a diversity of distinct targets. To achieve this, MNs diversify during development into distinct subtypes that are specifically dedicated to the control of particular sets of skeletal muscles or visceral organs (Jessell, 2000; Dasen and Jessell, 2009). However, certain aspects of MN diversification, including the segregation of somatic and visceral MNs at thoracic levels of the spinal cord, remain poorly understood and the contribution of certain developmental regulators to this process remains obscure.

MNs arise from a ventral progenitor domain (pMN) characterized by the expression of *Olig2*. Upon activation of several extrinsic and intrinsic signals, newly born MNs rapidly diversify into different subtypes that constitute five columnar groups, each occupying a defined position within the spinal cord and innervating a unique set of targets (Dasen and Jessell, 2009). Briefly, somatic MNs of the medial motor column (MMC)

innervate the dorsal axial musculature. At thoracic levels, somatic MNs of the hypaxial motor column (HMC) ensure innervation of the intercostal or abdominal wall muscles, whereas visceral MNs of the preganglionic column (PGC) innervate sympathetic neurons of the paravertebral ganglia. At limb levels, medial or lateral columnar subdivision of the lateral motor column (LMC) ensures innervation of the ventral (flexor muscles) or dorsal (extensor muscles) part of the limbs, respectively. MNs further diversify through the segregation of certain neurons into motor pools that innervate a specific target muscle.

*Isl1* is of paramount importance for MN development. Indeed, it is required for the survival of newly born MNs (Pfaff et al., 1996). In addition, it contributes with *Hb9* (also known as *Mnx1*) and *Isl2* to consolidate MN identity by suppressing the V2 interneuron differentiation program (Arber et al., 1999; Thaler et al., 1999; Thaler et al., 2004; Song et al., 2009). Furthermore, it also defines the identity and the axonal projections of medial LMC (LMCm) neurons (Kania et al., 2000) and may contribute to the generation of PGC neurons (Thaler et al., 2004). However, detailed analysis of the roles of *Isl1* in MN subtype specification has been partly hindered by its early requirement in MN survival and identity consolidation. In addition to *Isl1*, other transcriptional regulators have been identified to control MN subtype diversification. *Isl2* and *Foxp1* have been proposed to promote visceral MN differentiation (Thaler et al., 2004; Dasen et al., 2008; Rousso et al., 2008). Upon activation by Wnt signaling, *Lhx3* promotes MMC identity (Agalliu et al., 2009). At limb levels, *Foxp1* is required for the production of LMC neurons (Dasen et al., 2008; Rousso et al., 2008), whereas *Lhx1* defines, in opposition to *Isl1*, the identity and the axonal projections of lateral LMC (LMCl) neurons (Bonanomi and Pfaff, 2010). Thus, MN diversification depends on the coordinated action of multiple transcriptional regulators. However, how these different regulators cooperate to establish MN subtype identity remains poorly understood.

<sup>1</sup>Université catholique de Louvain, Institute of Neuroscience, Laboratory of Neural Differentiation, 1200 Brussels, Belgium. <sup>2</sup>Department of Neurobiology, Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA. <sup>3</sup>Laboratory of Molecular Biology (Celgen) of the Center for Human Genetics and of the <sup>4</sup>Department of Molecular and Developmental Genetics (VIB11), KU Leuven, 3000 Leuven, Belgium. <sup>5</sup>Institute of Developmental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Neuherberg, Germany.

\*These authors contributed equally to this work

†Present address: Harvard University, Department of Molecular and Cellular Biology, Cambridge, MA 02138, USA

§Present address: Laboratory of Molecular Biology (Celgen), Department of Development and Regeneration, University of Leuven, 3000 Leuven, Belgium

¶Author for correspondence ([frederic.clotman@uclouvain.be](mailto:frederic.clotman@uclouvain.be))

We recently discovered that the transcriptional activators of the Onecut (OC) family are also present in differentiating spinal MNs (Francius and Clotman, 2010). In mammals, the OC class of transcription factors comprises three members: hepatocyte nuclear factor 6 (Hnf6; also known as Onecut1, Oc1 or OC-1), Oc2 and Oc3 (Lemaigre et al., 1996; Vanhorenbeek et al., 2002; Jacquemin et al., 2003b). These proteins are detected during development in several endodermal derivatives including liver and pancreas, where they redundantly control different aspects of cell fate decision and morphogenetic processes (Jacquemin et al., 2000; Clotman et al., 2002; Jacquemin et al., 2003a; Pierreux et al., 2006; Vanhorenbeek et al., 2007). They are also present in the embryonic CNS (Landry et al., 1997; Jacquemin et al., 2003b; Vanhorenbeek et al., 2007), where they regulate the generation, maintenance or the projections of different encephalic structures (Hodge et al., 2007; Chakrabarty et al., 2012; Espana and Clotman, 2012a; Espana and Clotman, 2012b) and of a spinal interneuron population (Stam et al., 2012). In the ventral spinal cord, the three OC genes are differentially and dynamically expressed during the early steps of MN differentiation (Francius and Clotman, 2010), suggesting that OC factors might contribute to spinal MN development.

Here, combining loss-of-function experiments in mouse embryos and gain-of-function experiments in chick embryos, we provide evidence that direct stimulation of *Isl1* expression by the OC factors is required to maintain *Isl1* in newly born MNs and in specific MN subtypes, and that OC factors control MN subtype segregation among different columnar groups. Taken together, these observations elucidate a comprehensive model wherein OC factors cooperate with multiple regulators to control different aspects of MN subtype diversification in the developing spinal cord.

## MATERIALS AND METHODS

### Animals

Mice were raised in our animal facilities and treated according to the principles of laboratory animal care of the University Animal Welfare Committee. *Hnf6*, *Oc2*, *Foxp1* and *Brn4::Sip1* mutant mice have been described previously (Jacquemin et al., 1999; Clotman et al., 2005; Rouso et al., 2008; Seuntjens et al., 2009). To ensure accurate comparison, the developmental stage of E9.5 or E10.5 embryos was assessed by counting the number of pairs of somites.

### Immunofluorescent labeling and in situ hybridization

Whole-mount antibody stainings were performed as described (Huetl et al., 2011) using anti-neurofilament antibody at 1:50 (DSHB #2H3). z-stack images were generated using a Zeiss LSM 510 confocal laser-scanning microscope. For immunofluorescent labeling and in situ hybridization on sections, embryos were fixed in PBS/4% paraformaldehyde (PFA) before cryosectioning (12–20  $\mu$ m). Immunofluorescent labeling was performed as described (Francius and Clotman, 2010). Antibodies were rabbit anti-caspase 3 at 1:1000 (Cell Signaling #9661S), rabbit anti-Hb9 at 1:4000 (kindly provided by T. Jessell, Columbia University, New York, NY, USA), sheep anti-Chx10 at 1:500 (Exalpha #X1190P), goat anti-Foxp1 at 1:1000 (R&D #AF4534), chick anti-GFP at 1:1000 (Aves Lab #GFP-1020), rabbit anti-Hb9 at 1:5000 (Abcam #ab26128), rabbit anti-Hnf6 at 1:50 (Santa Cruz #sc-13050), mouse anti-Isl1 at 1:1000 (DSHB #39.3F7), rabbit anti-Isl1 at 1:2000 (Abcam #ab26122-100), mouse anti-Isl1/2 at 1:6000 (DSHB #39.4D5), mouse anti-Lhx3 at 1:1000 (DSHB #67.4E12), mouse anti-Lhx1/5 at 1:1000 (DSHB #4F2), mouse anti-MNR2 (Hb9) at 1:1000 (DSHB #81.5C10), rabbit anti-nNOS at 1:4000 (ImmunoStar #24287), rabbit anti-pSmad1/5/8 at 1:2000 (kindly provided by T. Jessell), rat anti-Oc2 at 1:400 (Clotman et al., 2005), guinea-pig anti-Oc3 at 1:6000 (Pierreux et al., 2004), guinea-pig anti-Olig2 at 1:4000 (Novitsch et al., 2001), rabbit anti-Raldh2 at 1:10,000 (kindly provided by P. Mc Caffery,

Institute of Medical Science, University of Aberdeen, Aberdeen, UK) and rabbit anti-Sip1 at 1:500 (Van de Putte et al., 2003). In situ hybridizations were performed as described (Ravassard et al., 1997) using digoxigenin-labeled probes against *Ephb1* or *Epha4* (kindly provided by C. Pierreux, de Duve Institute, Université catholique de Louvain, Brussels, Belgium). Immunofluorescence and in situ hybridization images were acquired on a Zeiss Axio Observer.Z1 confocal microscope.

### Neuron number and *Isl1* level quantifications

Cell counts were performed unilaterally on three sections at three levels of brachial, thoracic or lumbar regions in embryonic ventral spinal cord (three levels along the rostrocaudal axis for embryos at E9.5), in at least three independent embryos. *Isl1* levels were determined at subsaturating antibody concentrations on three sections (at least 90 nuclei) of three independent E10.5 wild-type embryos. Mean nuclear pixel intensity was determined using ImageJ (NIH). SigmaPlot v11 software was used for statistical analyses.

### In ovo electroporation

In ovo electroporation was performed at stage HH12–14. *pCMV-Hnf6-flag* or *pCMV-Oc2-myc* (0.5  $\mu$ g/ $\mu$ l) (Pierreux et al., 2004) was co-electroporated with a *pCMV-eGFP* plasmid to visualize electroporated cells. Six hours (HH16–18), 24 hours (HH22–23) or 4 days (HH28–29) after electroporation, embryos were fixed in PBS/4%PFA for 20 minutes, 30 minutes or 1 hour, respectively. The *zCREST2-hsp70:GFP* reporter vector (3.5  $\mu$ g/ $\mu$ l) [kindly provided by H. Okamoto (Uemura et al., 2005)] was co-electroporated with the *pCAGGS-DsRed2* plasmid (gift of Y. Takahashi, Nara Institute of Science and Technology, Ikoma, Japan) to visualize electroporated cells, and embryos were collected 24 hours after electroporation and processed as above.

### Retrograde labeling of spinal MNs

Retrograde labeling of MNs was performed at E12.5 using 3000 molecular mass Rhodamine-dextran (Invitrogen #D3308). This retrograde tracer was injected into the dorsal part of the limbs or into the paravertebral ganglionic chain in 600- $\mu$ m vibratome slices. Tissues were maintained in oxygenated DMEM/F12 medium (Invitrogen #11039) for 6 hours at room temperature. The samples were then processed for immunofluorescence analysis. The number of PGC neurons labeled by Rhodamine-dextran was estimated from the ratio of the area occupied by retrograde labeling.

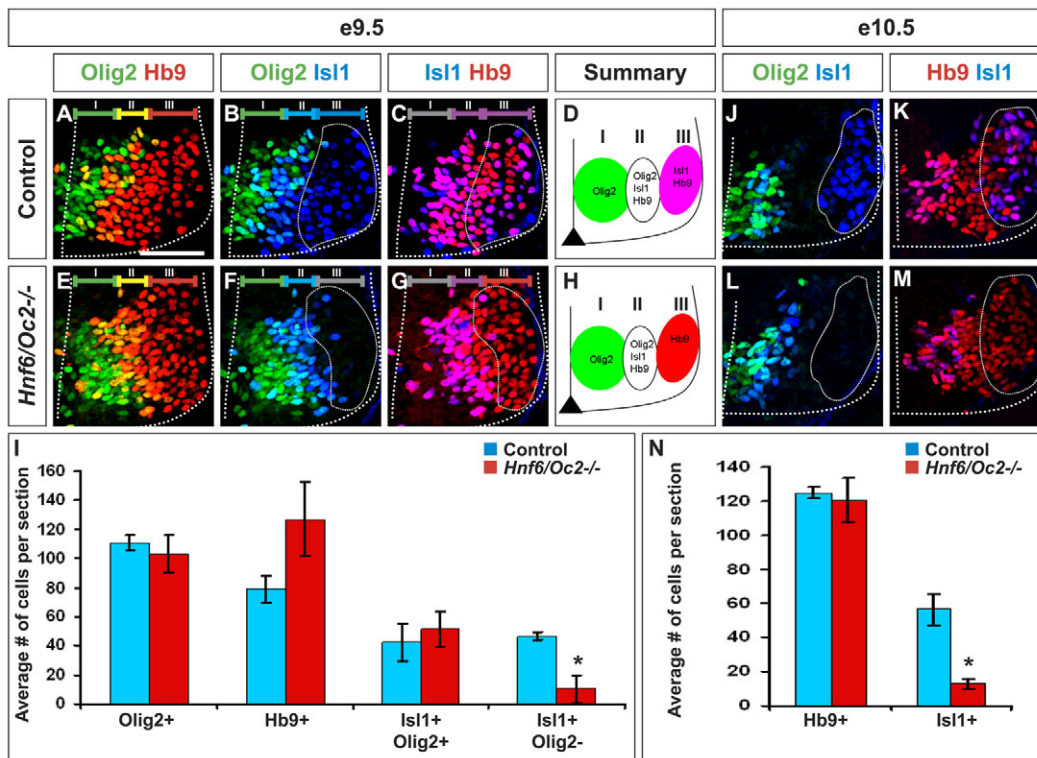
### Chromatin immunoprecipitation (ChIP) assay

Neural tubes were dissected from HH17–18 chick embryos. Cells were homogenized in DMEM/F12 using a Dounce homogenizer and treated with 1% formaldehyde at room temperature for 10 minutes. Immunoprecipitation was performed using a ChIP kit (Millipore #17-371) according to the manufacturer's instructions. Chromatin was fragmented to 200–1000 bp by sonication (high power, 30 cycles of 30 seconds with 1 minute between pulses) and incubated overnight at 4°C with antibody against Hnf6 (Santa Cruz #sc-13050) at 1:50 or species-matched IgG. Primers (5'-3') were: TTGCTGAATGCTGAAAGCAC and TTCTTTGCACAGCCAGTTTG for CREST1; AGCTAAAGC-CCACATTTTGC and CAACAGTTCCTGCATTAGCA for CREST2; TGTATGGCAGCCACAAGAGA and TCCCAAGAAGCAGGCATAAT for CREST3; CTTGCGATGCTTTTTGTGAC and CGTGGAGCAGTT-TTACAGAC for noggin. Fold enrichment was calculated over IgG using  $2^{(-\Delta\Delta CT)}$ , where  $\Delta\Delta CT = (C_{tip} - C_{input}) - (C_{tipG} - C_{input})$ .

## RESULTS

### OC factors are required to maintain *Isl1* expression during MN subtype diversification

OC proteins are initially detected in MNs at ~E9.0, as soon as neural progenitors exit the cell cycle and start producing the earliest MN markers *Isl1* and *Hb9* (Francius and Clotman, 2010) (supplementary material Fig. S1A–I). They are present in an overlapping pattern in the vast majority of newly born MNs until E11.5, when their distribution becomes restricted to subsets of differentiating MNs (Francius and Clotman, 2010). Therefore, we first assessed whether



**Fig. 1. OC factors are required to maintain *Isl1* expression during MN subtype diversification.** (A-H) Triple label immunofluorescence analysis of Olig2, Hb9 and *Isl1* on a single spinal cord section of control mouse embryos or embryos double mutant for *Hnf6* and *Oc2* (*Hnf6/Oc2*<sup>-/-</sup>) at E9.5. At this developmental stage, the spinal cord is not yet divided into distinct brachial, thoracic and lumbar regions, and newly born MNs form a homogeneous column along the anteroposterior axis of the spinal cord. In the absence of OC factors, *Isl1* is present in the 'intermediate' population (II) but is almost completely absent from newly born MNs (III). I, pMN domain. (I) Quantification of Olig2<sup>+</sup> progenitors and Hb9<sup>+</sup> or *Isl1*<sup>+</sup> newly born MNs in E9.5 *Hnf6/Oc2*<sup>-/-</sup> embryos and control littermates. (J-M) In *Hnf6/Oc2*<sup>-/-</sup> embryos at E10.5, the number of thoracic Hb9<sup>+</sup> MNs is normal but *Isl1* is lost from the newly born MNs. (N) Quantification of *Isl1*<sup>+</sup> or Hb9<sup>+</sup> MNs in control and *Hnf6/Oc2*<sup>-/-</sup> spinal cord at E10.5. \*, *P* < 0.05; error bars indicate s.e.m. Scale bar: 100 μm.

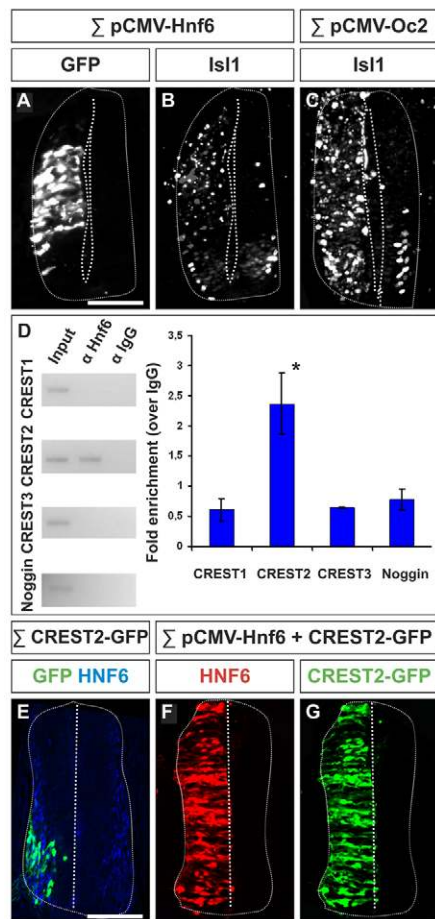
the early steps of MN differentiation were affected in embryos double mutant for *Hnf6* and *Oc2* (referred to here as *Hnf6/Oc2*<sup>-/-</sup>). The expression of *Oc3* was completely abrogated in the spinal cord of these embryos (supplementary material Fig. S1J-M; data not shown), enabling us to study the consequences of the loss of all three OC proteins on MN development.

In control embryos, Olig2 was present in MN progenitors located in the ventral portion of the spinal cord (population I in Fig. 1A,B). As these cells exited the cell cycle and emerged from the ventricular zone, they transiently contained Olig2, *Isl1* and Hb9 ('intermediate' population II in Fig. 1A,B). Then, the production of Olig2 was downregulated whereas the newly born MNs maintained Hb9 and *Isl1* (population III in Fig. 1A-D). In *Hnf6/Oc2*<sup>-/-</sup> embryos, the MN progenitor domain seemed unaffected (Fig. 1E,F,I,L). In addition, the number and distribution of newly born MNs in mutant embryos were identical to those in control embryos (Fig. 1A-M; supplementary material Fig. S1O-T, Fig. S2). Thus, OC factors are not required for the generation of MNs. However, although *Isl1* was present in the Olig2<sup>+</sup> intermediate population, it was absent from the majority of newly born MNs (Fig. 1A-N). This loss of *Isl1* was not due to MN cell death, as no increase in apoptosis was detected in the ventral spinal cord of OC mutant embryos (supplementary material Fig. S1S,T). Moreover, the number of Lhx3<sup>+</sup> V2a interneurons was also normal (supplementary material Fig. S1Q,T, Fig. S2), arguing against a

fate conversion of MNs into V2 interneurons. Taken together, these observations indicate that OC factors are required to maintain *Isl1* expression in newly born MNs and that neither MN generic differentiation nor MN identity consolidation was altered by the lack of OC proteins.

The co-detection of *Isl1* and OC factors in newly born MNs and the precocious loss of *Isl1* in OC mutant MNs suggested that OC factors might directly stimulate *Isl1* expression. Accordingly, ectopic expression of *Hnf6* or *Oc2* alone in chick embryonic spinal cord was sufficient to induce *Isl1* expression as early as 6 hours after electroporation (Fig. 2A-C). *Isl1* production in differentiating MNs is under the control of three conserved enhancers named CREST1-3 (Uemura et al., 2005). We found one potential OC binding site corresponding to the 5'-(A/T/G) (A/T) (A/G) TC(A/C)AT N (A/T/G)-3' consensus (the highly conserved core sequence is underlined) (Lannoy et al., 1998; Jacquemin et al., 2000) in CREST1 and two potential binding sites in CREST2, whereas CREST3 was devoid of such sequence (supplementary material Fig. S1U-W). ChIP assay indicated that endogenous OC proteins were bound to CREST2 but to neither CREST1 nor CREST3 (Fig. 2D). Furthermore, a reporter construct in which *GFP* expression is under the control of the CREST2 enhancer was exclusively activated in MNs expressing OC factors (Fig. 2E), and was strongly stimulated by co-electroporation with an *Hnf6*





**Fig. 2. OC factors directly stimulate the expression of *Isl1*.**

(A–C) Overexpression of *Hnf6* or *Oc2* in chick embryonic spinal cord at HH14 results in ectopic *Isl1* expression 6 hours after electroporation. (D) Binding of endogenous OC factors to potential binding sites in *Isl1* enhancers was assessed by ChIP (left, gel; right, quantitative real-time PCR) on chick embryonic spinal cord extracts with an anti-*Hnf6* antibody or species-matched IgG. The *noggin* promoter, which contains no potential OC binding site, served as negative control. OC proteins are bound to sites in the CREST2 enhancer, but not to the CREST1 or CREST3 regions. \*,  $P < 0.05$ ; error bars indicate s.e.m. (E–G) A reporter construct in which *GFP* expression is regulated by the CREST2 enhancer is exclusively activated in MNs expressing OC factors (E). This reporter construct is strongly stimulated by *Hnf6* co-expression (F,G). Scale bars: 100  $\mu$ m.

expression vector (Fig. 2F,G). Taken together, these observations are consistent with direct regulation of *Isl1* expression by OC factors in spinal MNs.

### OC factors control the ratio between somatic and visceral MNs

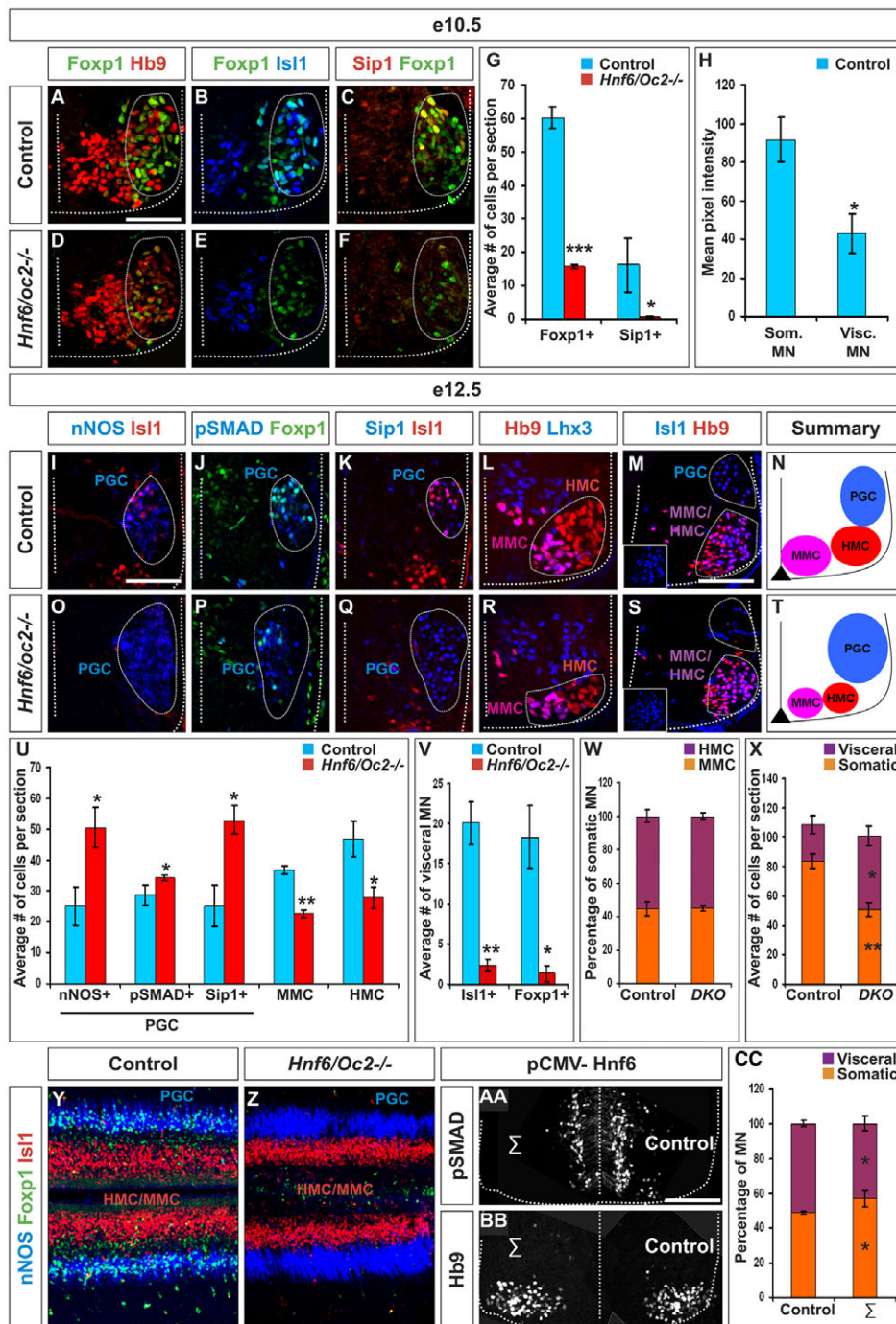
As MN survival and identity were unaffected in *Hnf6/Oc2*<sup>−/−</sup> embryos, we analyzed the consequences of the loss of OC factors and of *Isl1* on MN subtype specification in each portion of the spinal cord. At E10.5, thoracic MNs initiate the expression of columnar markers, including *Foxp1*, in the prospective visceral MNs (Dasen et al., 2008; Rouso et al., 2008). In control embryos, *Foxp1* was indeed detected in a lateral MN population containing *Isl1* and low levels of *Hb9* (Fig. 3A,B; supplementary material Fig.

S2). Furthermore, our marker analysis indicated that, in addition to its presence in spinal neural progenitors (Bassez et al., 2004; Miyoshi et al., 2006), *Sip1* (Smad-interacting protein 1; also known as *Zfhx1b* or *Zeb2*) is detected in visceral MNs. *Sip1* is a member of the *Zfhx1* family of two-handed zinc-finger/homeodomain proteins (van Grunsven et al., 2001; Bassez et al., 2004; Seuntjens et al., 2009). It was detected in prospective visceral MNs containing *Foxp1* (Fig. 3C). The absence of OC factors resulted in a strong decrease in the number of *Foxp1*<sup>+</sup> and of *Sip1*<sup>+</sup> MNs (Fig. 3D–G). These observations suggested that OC factors control visceral MN differentiation and act upstream of *Foxp1* and *Sip1* in this process. Accordingly, OC proteins are produced earlier than *Foxp1* and *Sip1* in MNs (Francius and Clotman, 2010) and were distributed normally in *Foxp1*- or *Sip1*-deficient embryos (supplementary material Fig. S3F–I, Fig. S4J–L,N). Moreover, *Hnf6* and *Oc2* induced ectopic expression of *Foxp1* and *Sip1* when overexpressed in chick embryonic spinal cord (supplementary material Fig. S3J–N, Fig. S4AA–EE).

*Isl1* and *Foxp1* have been proposed to promote visceral MN differentiation (Thaler et al., 2004; Dasen et al., 2008; Rouso et al., 2008). Therefore, we expected that their downregulation in the absence of OC factors would result in a decrease in visceral MNs. Surprisingly, an expansion of the PGC was instead observed at E12.5. Indeed, the number of nNOS<sup>+</sup> (Nos1 – Mouse Genome Informatics) and of phospho-Smad1/5/8 (pSMAD)<sup>+</sup> MNs was increased in mutant embryos (Fig. 3O,P,U,Z). Moreover, *Sip1* was detected in an increased number of visceral MNs (Fig. 3Q,U), confirming the production of supernumerary PGC neurons. By contrast, *Isl1* and *Foxp1* were absent from the vast majority of these visceral MNs (Fig. 3O–Q,V,Z). Thus, OC factors restrict the production of visceral MNs and modulate the expression of regulators of this process.

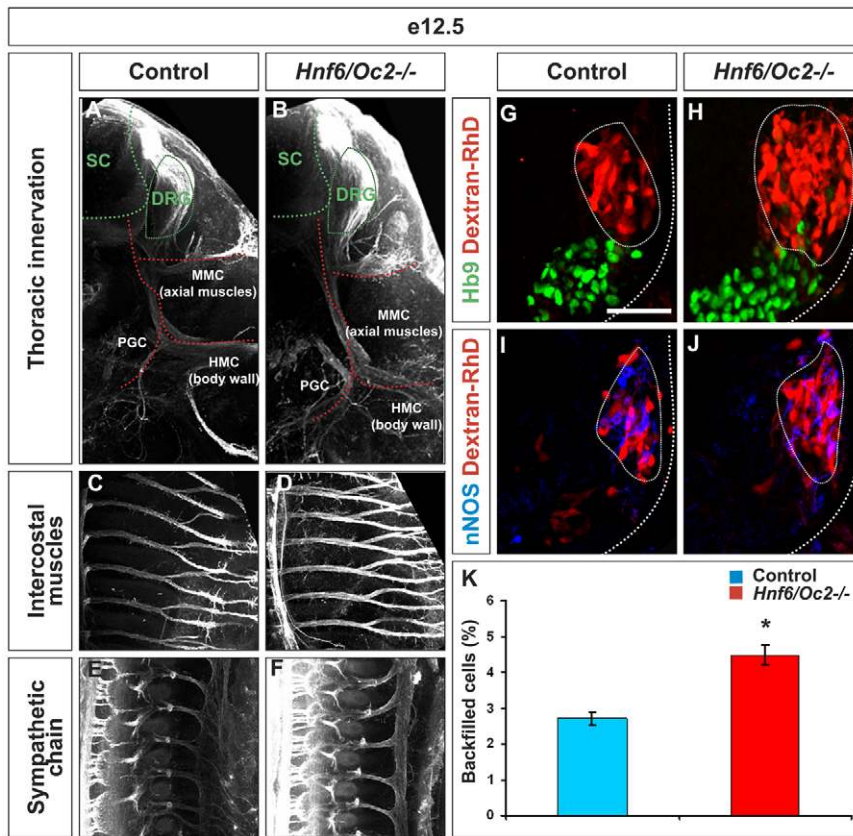
The reactivation of *Sip1* expression in visceral MNs was delayed in *Hnf6/Oc2*<sup>−/−</sup> embryos (Fig. 3F,G,Q,U). To determine the role of *Sip1* in PGC development and to assess whether delayed *Sip1* expression contributes to the visceral MN phenotype in OC mutant embryos, we studied MN development in *Brn4::Cre::Sip1lox* (*Brn4::Sip1*) embryos (Higashi et al., 2002) (*Brn4* is also known as *Pou3f4* – Mouse Genome Informatics), wherein *Sip1* was present in neural progenitors but absent from differentiating neurons (supplementary material Fig. S4A,G). However, the absence of *Sip1* reduced the number of PGC MNs (supplementary material Fig. S4), suggesting that *Sip1* exerts effects opposite to those of OC factors during visceral MN differentiation. Thus, *Sip1* is a novel marker of PGC neurons and is required to produce the proper number of visceral MNs.

As the total number of MNs initially produced in OC mutant embryos was normal, we wondered whether the production of supernumerary visceral MNs observed at E12.5 correlated with a decrease in somatic MNs. Somatic MNs and their columnar subdivision were detected in the thoracic spinal cord of *Hnf6/Oc2*<sup>−/−</sup> embryos, as evidenced by the normal distribution of *Hb9* and *Lhx3* (Fig. 3R; supplementary material Fig. S2). Surprisingly, *Isl1* was also detected in all the somatic MNs of the MMC and HMC (Fig. 3S,Z; supplementary material Fig. S2), suggesting that the requirement for OC factors in *Isl1* expression is MN subtype specific. However, the number of somatic MNs was reduced in the absence of OC factors (Fig. 3R,U,Z; supplementary material Fig. S2). This reduction was similar for MMC and for HMC neurons, preserving a normal MMC/HMC ratio (Fig. 3W). Loss of somatic MNs was not due to cell death, as apoptosis was not increased at E10.5 or E12.5 (supplementary material Fig.



**Fig. 3. OC factors control the ratio between somatic and visceral MNs.** (A-F) Distribution of visceral MN markers at thoracic levels in control and *Hnf6/Oc2<sup>-/-</sup>* mouse embryos at E10.5 unveils a loss of Foxp1 and Sip1 in the absence of OC factors. (G) Quantitative analysis of prospective visceral MN markers at E10.5 on thoracic spinal cord sections of control and *Hnf6/Oc2<sup>-/-</sup>* embryos. (H) Comparison of Isl1 protein levels in prospective somatic or visceral MNs in wild-type embryos at E10.5 demonstrates that Isl1 content in prospective visceral MNs is half that in prospective somatic MNs. (I-T) Distribution of MN columnar subtype markers at thoracic levels in control and *Hnf6/Oc2<sup>-/-</sup>* spinal cord at E12.5 shows an expansion of visceral MNs at the expense of somatic MNs. (U) Quantitative analysis of MN columnar subtype markers on thoracic spinal cord sections of control and *Hnf6/Oc2<sup>-/-</sup>* embryos at E12.5. (V) Quantitative analysis of Isl1 and Foxp1 in visceral MNs on thoracic sections of control and *Hnf6/Oc2<sup>-/-</sup>* spinal cord at E12.5. (W) Although the somatic MN population is reduced in *Hnf6/Oc2<sup>-/-</sup>* (DKO) spinal cord at E12.5, the ratio between MMC and HMC neurons is unchanged. (X) In *Hnf6/Oc2<sup>-/-</sup>* embryos, the total number of MNs at E12.5 is normal but the ratio between somatic and visceral MNs is modified. (Y,Z) Open-book view of control and *Hnf6/Oc2<sup>-/-</sup>* spinal cord summarizes the observations obtained at thoracic levels of the spinal cord, i.e. the modification of the ratio between somatic and visceral MNs, the absence of Isl1 and Foxp1 in PGC neurons and the context-dependent regulation of Isl1 by OC factors. (AA-CC) Overexpression of *Hnf6* in chick embryonic spinal cord at HH14 results, 96 hours after electroporation ( $\Sigma$ ), in a reduction in visceral MNs of the column of Terri and in an expansion of somatic MNs. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; error bars indicate s.e.m. PGC, preganglionic column; MMC, median motor column; HMC, hypaxial motor column. Scale bars: 100  $\mu$ m in A-F; 125  $\mu$ m in I-L,O-R; 170  $\mu$ m in M,S; 200  $\mu$ m in AA, BB.





**Fig. 4. OC factors are dispensable for axonal projections of thoracic MNs.** (A-F) Whole-mount neurofilament labeling indicates that axonal projections at thoracic levels of the mouse spinal cord are preserved in *Hnf6/Oc2<sup>-/-</sup>* embryos. (G-K) MN projections to the paravertebral ganglionic chain were studied by retrograde transport of Rhodamine-dextran in E12.5 control and *Hnf6/Oc2<sup>-/-</sup>* embryos. Accuracy of the injection was confirmed by the presence of Rhodamine-dextran in nNOS<sup>+</sup> PGC neurons but not in Hb9<sup>+</sup> somatic MNs. Supernumerary visceral MNs in *Hnf6/Oc2<sup>-/-</sup>* embryos innervate paravertebral ganglia. \*,  $P < 0.05$ ; error bars indicate s.e.m. SC, spinal cord; DRG, dorsal root ganglion; PGC, preganglionic column; MMC, median motor column; HMC, hypaxial motor column. Scale bar: 85  $\mu$ m.

S1S,T, Fig. S5C-E), nor to a fate conversion of MNs into V2a interneurons, as the number of Chx10<sup>+</sup> (Vsx2 – Mouse Genome Informatics) V2a cells was normal and no Hb9<sup>+</sup> Chx10<sup>+</sup> hybrid cells were detected (supplementary material Fig. S5A,B,E). Thus, the absence of OC factors most likely modifies the differentiation balance between somatic and visceral MNs, leading to the production of supernumerary visceral MNs at the expense of somatic MNs (Fig. 3X,Z). To assess the respective contribution of *Hnf6* and *Oc2* to this process, similar analyzes were performed in *Hnf6* or *Oc2* single-mutant embryos (in which *Oc3* expression was preserved; supplementary material Fig. S1J-M; data not shown). Imbalance in somatic versus visceral MNs was observed in *Hnf6* but not in *Oc2* mutant embryos, suggesting that, among the OC factors, *Hnf6* is the major contributor to this process (supplementary material Fig. S5F-N).

To determine whether increased OC expression would be sufficient to shift the somatic versus visceral MN ratio in the opposite direction, *Hnf6* was overexpressed in chick embryonic spinal cord at the time of MN specification (HH14) and embryos were analyzed 96 hours later (HH29-30). Despite a partial loss of MNs due to the overexpression of *Hnf6* in newly born MNs (data not shown), analysis of MN columnar markers indicated that the proportion of somatic MNs was increased at the expense of visceral MNs (Fig. 3AA-CC). These observations confirm that OC factors control the differentiation balance between somatic and visceral MNs.

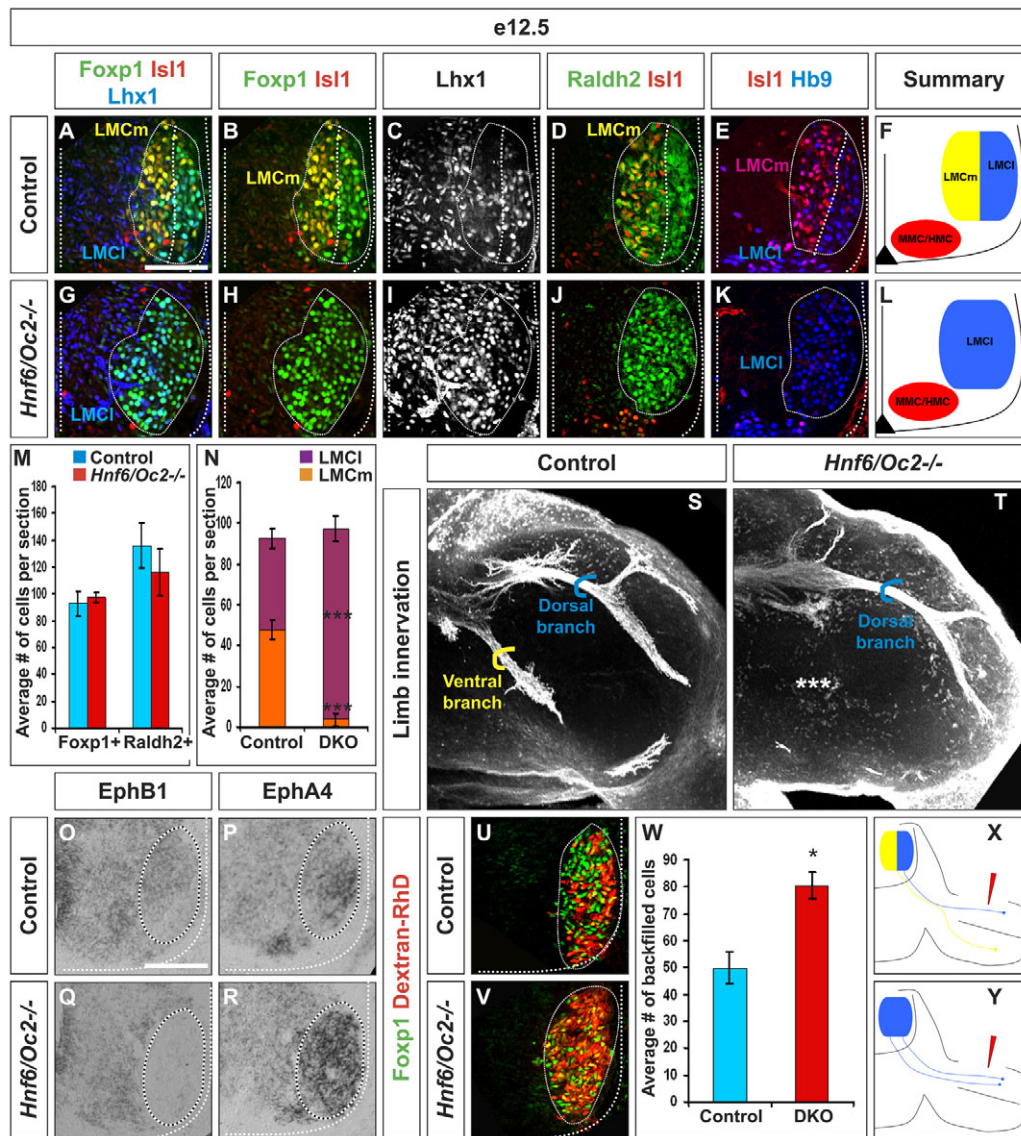
The absence of OC factors resulted in a loss of *Isl1* in newly born MNs and in an increase in PGC neurons. Conversely, OC overexpression stimulated *Isl1* expression and increased the somatic MN population. This suggested that low levels of *Isl1* in newly born MNs promote visceral MN differentiation, whereas high *Isl1* levels favor somatic MN differentiation. Therefore, we

compared *Isl1* levels in prospective PGC neurons characterized by the presence of *Foxp1* and in prospective MMC/HMC neurons devoid of *Foxp1* in wild-type embryos at E10.5. *Isl1* levels in prospective visceral MNs were half those in prospective somatic MNs (Fig. 3B,H), consistent with the hypothesis that differential amounts of *Isl1* contribute to MN subtype specification at thoracic levels of the spinal cord. However, overexpression of *Isl1* alone was not sufficient to modify the somatic-to-visceral MN ratio in chick embryonic spinal cord (data not shown), suggesting that additional OC targets cooperate with *Isl1* in this process.

#### OC factors are dispensable for axonal projections of thoracic MNs

As OC factors control the MN cell fate decision at thoracic levels of the spinal cord, we examined whether the absence of OC proteins influences thoracic MN axonal projections. Axon trajectories were analyzed by whole-mount immunofluorescence labeling for neurofilament at E12.5. The major peripheral thoracic nerve pathways were preserved in *Hnf6/Oc2<sup>-/-</sup>* embryos. Indeed, MMC axons grew dorsally towards the axial muscles of the back and HMC axons progressed ventrally towards the body wall musculature (Fig. 4B). Furthermore, the thoracic axons from the HMC population respected the strict segmental boundaries without forming any axonal bridges between segmentally arrayed motor nerves (Fig. 4D). Finally, despite the loss of *Isl1* and *Foxp1*, the PGC neurons were able to project their axons towards the sympathetic chain (Fig. 4F). These observations indicated that the OC factors are dispensable for MN axonal projections at thoracic levels of the spinal cord.

As shown above, supernumerary visceral MNs were produced in *Hnf6/Oc2<sup>-/-</sup>* embryos. We assessed whether all of these MNs acquired the target specificity of PGC cells, namely innervation of



**Fig. 5. OC factors are required for LMC subdivisions.** (A-L) Colabeling analyses of LMC markers unveil production of supernumerary LMCI neurons at the expense of the LMCm population at E12.5 in the brachial spinal cord of *Hnf6/Oc2<sup>-/-</sup>* mouse embryos. (M) Quantification of LMC neurons at E12.5 demonstrates that total MN numbers at brachial levels are not significantly changed in *Hnf6/Oc2<sup>-/-</sup>* embryos. (N) Quantitative analysis of LMC subtype markers indicates a conversion of LMCm to LMCI identity in E12.5 *Hnf6/Oc2<sup>-/-</sup>* embryos. (O-R) In control embryos at E12.5, *Ephb1* mRNA is enriched in the LMCm, whereas high *Epha4* mRNA levels are detected in the LMCI. In *Hnf6/Oc2<sup>-/-</sup>* embryos, *Ephb1* mRNA in the LMC is lost, whereas *Epha4* expression is high throughout the LMC. (S,T) Axonal projections of LMC neurons in the limbs of control and *Hnf6/Oc2<sup>-/-</sup>* embryos at E12.5. In *Hnf6/Oc2<sup>-/-</sup>* embryos, the motor nerve branch that innervates the ventral mesenchyme of the limb is absent (marked by three asterisks). (U,V) LMC neuron projections were studied by retrograde transport of Rhodamine-dextran injected into the dorsal part of the limbs of control and *Hnf6/Oc2<sup>-/-</sup>* embryos at E12.5. Foxp1 was used to identify LMC neurons. In the absence of OC factors, most of the LMC neurons innervate dorsal limb mesenchyme. (W-Y) Quantification of LMC neurons labeled after retrograde Rhodamine-dextran transport in control and *Hnf6/Oc2<sup>-/-</sup>* embryos demonstrates that the vast majority of LMC axons are redirected towards the dorsal part of the limbs in the absence of OC factors (Y). \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ; error bars indicate s.e.m. LMCm, medial portion of the lateral motor column; LMCI, lateral portion of the lateral motor column. Scale bars: 150  $\mu\text{m}$  in A-E,G-K; 170  $\mu\text{m}$  in O-R,U-V.

the paravertebral ganglia. After injection of the retrograde tracer Rhodamine-dextran into the sympathetic chain, the backfilled PGC population was indeed expanded in *Hnf6/Oc2<sup>-/-</sup>* embryos and corresponded to visceral MNs as evidenced by the presence of nNOS (Fig. 4G-K). This suggested that, in the absence of OC factors, supernumerary visceral MNs consistently innervate the paravertebral ganglia.

**OC factors are required for LMC subdivisions**

Foxp1 is required for the differentiation of LMC neurons (Dasen et al., 2008; Rousso et al., 2008). As the production of Foxp1 was strongly downregulated at thoracic levels of *Hnf6/Oc2<sup>-/-</sup>* embryos, we first examined whether LMC neurons were produced normally in the absence of OC factors. Surprisingly, Foxp1 was present and distributed normally in the brachial spinal cord of *Hnf6/Oc2<sup>-/-</sup>*



embryos (Fig. 5G,H,M; supplementary material Fig. S6A-I). Similarly, the distribution of retinaldehyde dehydrogenase 2 (Raldh2; Aldh1a2 – Mouse Genome Informatics), which is restricted to LMC neurons, was normal (Fig. 5J,M; supplementary material Fig. S6D-H). Therefore, the generation of LMC neurons was unaffected by the absence of OC factors. By contrast, Isl1 was severely depleted throughout LMC formation (Fig. 5G,H,J,K; supplementary material Fig. S6A-M). Concomitantly, the distribution of Lhx1 and Isl2 and the expression of high levels of Hb9 expanded throughout the LMC (Fig. 5G,I,K; supplementary material Fig. S6E,G; data not shown). This suggested that, in the absence of OC factors, the molecular identity of LMCm neurons was converted into that of LMCl neurons (Fig. 5N; supplementary material Fig. S6J). Similar investigations in single *Hnf6*<sup>-/-</sup> or *Oc2*<sup>-/-</sup> embryos indicated that *Hnf6* also plays a prominent role in this process and that the OC factors act redundantly to control the LMCm/LMCl ratio (supplementary material Fig. S6N-V).

As Lhx1 and Isl1 coordinate LMC neuron identity with the dorsoventral selection of axon pathways, we assessed whether this change of identity observed in OC mutants resulted in a modification of limb innervation. In wild-type embryos, Lhx1 directs the dorsal projection of LMCl axons in the developing limb through its ability to regulate *Epha4* expression, a guidance receptor required for axons to avoid the ventral limb mesenchyme. By contrast, Isl1 promotes *Ephb1* expression, which ensures ventral projection of LMCm axons (Bonanomi and Pfaff, 2010). In *Hnf6/Oc2*<sup>-/-</sup> embryos, severe downregulation of Isl1 in the LMCm correlated with a loss of *Ephb1* expression, whereas the increase in the number of Lhx1<sup>+</sup> cells was accompanied by increased *Epha4* expression levels (Fig. 5O-R). Consistently, the segregation of LMC axons at the base of the limb was altered, and the ventral branch was completely absent (Fig. 5T). This suggested that the vast majority of LMC neurons projected dorsally in the absence of OC factors. To confirm this, Rhodamine-dextran was injected into the dorsal part of the limbs and retrogradely labeled LMC neurons were quantified (Fig. 5V,W). In control embryos, neurons that innervated the dorsal portion of the limbs accounted for 50±6% of total LMC neurons. By contrast, in *Hnf6/Oc2*<sup>-/-</sup> littermates 81±5% of LMC neurons innervated the dorsal part of the limb ( $n=3$ ,  $P<0.05$ ).

Together, these results indicate that the absence of OC factors results in a switch from LMCm to LMCl molecular identity with subsequent redirection of the corresponding axons towards the dorsal mesenchyme of the limbs (Fig. 5Y).

## DISCUSSION

Despite the tremendous progress made during the last 20 years in understanding the molecular mechanisms that control MN diversification, several questions remain concerning the fine-tuned control of the expression of key developmental regulators, their exact contribution to some aspects of MN diversification and how they cooperate to exert their role. Here, we show that members of the OC transcription factor family regulate the expression of *Isl1* and control different aspects of MN subtype diversification.

### Context-dependent transcriptional regulation controls MN differentiation

Isl1 plays prominent roles during spinal MN development (Pfaff et al., 1996; Thaler et al., 2004; Luria et al., 2008; Song et al., 2009). During early phases of MN differentiation, Hb9 stimulates, probably indirectly, *Isl1* expression (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999; William et al., 2003). Conversely, Isl1

stimulates *Hb9* transcription through its participation in the formation of Isl1-Lhx3-NLI (nuclear LIM interactor; also known as Ldb1) complexes that consolidate MN identity (Lee et al., 2008). This establishes a regulatory loop in which there is mutual support of Isl1 and Hb9 expression. Factors that directly activate *Isl1* transcription in MNs remain unknown. Here, we show that OC factors stimulate, probably directly, the expression of *Isl1* in spinal MNs. However, this regulation is strongly context dependent. Indeed, in OC mutant embryos, Isl1 was almost completely lost in newly born MNs and in PGC and LMC neurons but was preserved in MMC and HMC cells, whereas OC factors are present in all MN subtypes in control embryos (Francius and Clotman, 2010). Similarly, the absence of OC factors resulted in the loss of *Foxp1* in PGC neurons but not in LMC cells. This suggests that subtype-specific co-factors that modulate either OC binding to their target sequences or OC transcriptional activity might contribute to the regulation of *Isl1* or *Foxp1* expression in specific MN subtypes. The binding of OC factors to the *Isl1* CREST2 enhancer but not to CREST1, which also contains a potential OC binding site, supports the first hypothesis.

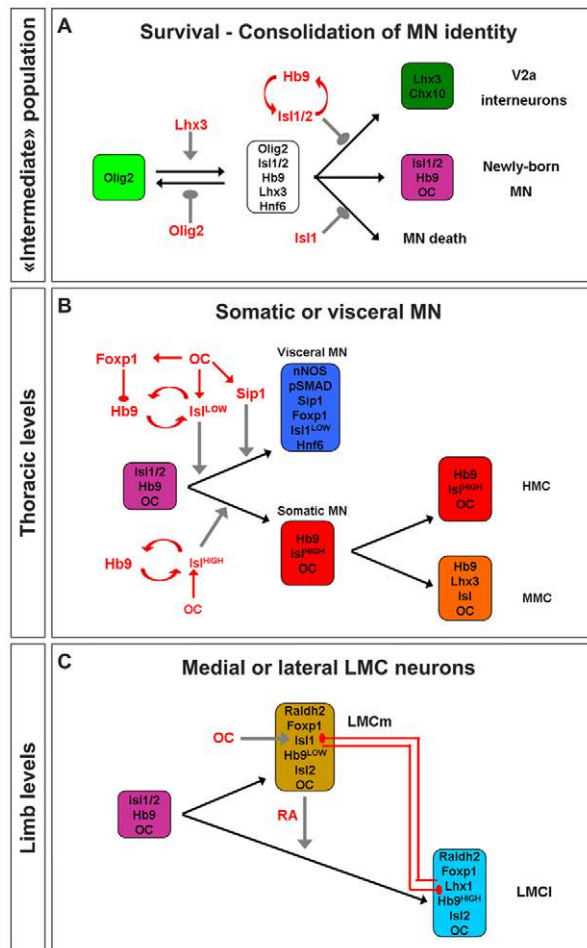
CREB binding protein (CBP, or Crebbp), p300 (Kat2b) and PGC1 (Ppargc1) behave as co-factors for OC proteins in hepatic cells (Lannoy et al., 1998; Beaudry et al., 2006). CBP and p300 are widely produced in the developing spinal cord, where they coordinately cooperate with retinoic acid signaling and neurogenin 2 to promote MN differentiation (Lee et al., 2009). Whether they also contribute to later steps of MN development and interact with OC factors remains to be investigated. PGC1 is also present in the spinal cord (Diez-Roux et al., 2011) but its role during MN development remains to be investigated. Similarly, the Hb9-Isl1 regulatory loop is strongly context dependent, as it seems to operate in newly born and in MMC/HMC MNs but not in LMC neurons (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999; William et al., 2003). Furthermore, *Foxp1* controls MN subtype specification through interactions with different Hox proteins at different levels of the spinal cord (Dasen and Jessell, 2009). Thus, context-dependent transcriptional regulation is likely to contribute to multiple aspects of spinal MN diversification.

### OC-independent MN generation and consolidation of MN identity

Isl1 is necessary for spinal MN survival (Pfaff et al., 1996). In addition, it plays a prominent role in the consolidation of MN identity (Thaler et al., 2004; Song et al., 2009). Isl2 seems to exert functions similar to that of Isl1 in the latter process (Segawa et al., 2001; Thaler et al., 2004; Hutchinson and Eisen, 2006). Therefore, it has been proposed that the global level of Isl proteins, rather than a specific function attributed to each Isl factor, is crucial for the consolidation of MN identity as well as for other Isl-dependent processes in MN development (Thaler et al., 2004). In the absence of OC factors, although *Isl1* expression in newly born MNs was severely downregulated, MNs were generated in normal numbers and MN identity was maintained. This indicates that OC factors are not required for MN generation nor for MN identity consolidation. It also suggests that the requirement for Isl1 in these two processes is restricted to very early steps of MN development, probably at the stage when *Olig2* and Isl1 are jointly detected in the ‘intermediate’ population (Fig. 6A), as previously proposed (Song et al., 2009).

Hb9 also participates in the consolidation of MN identity by repressing V2a determinants including *Chx10* (Arber et al., 1999; Thaler et al., 1999; Song et al., 2009). In mouse mutants for *Hb9*





**Fig. 6. Re-evaluated model for the control of MN differentiation.**

(A) *Isl1* ensures the survival of newly born MNs, whereas *Hb9* and *Isl* cooperate to consolidate MN identity and simultaneously repress the V2a differentiation program. (B) At thoracic levels of the spinal cord, newly born MNs segregate into visceral or somatic MN populations. The distinction between these two classes is likely to depend on the level of *Isl* proteins. *Sip1* also promotes visceral MN differentiation and is regulated by the OC factors. (C) At brachial or lumbar levels of the spinal cord, retinoic acid (RA) signals provided by early-born LMCm neurons direct the acquisition of LMCi divisional identity. The crossrepressive interaction between *Isl1* and *Lhx1* reinforces the segregation between LMCm and LMCi. Because they control *Isl1* expression in LMCm cells, the OC factors are required for LMC columnar subdivision and LMCm identity. See text for further details.

or *Isl2*, increased expression of *Lhx3* and *Chx10* led to fate conversion of newly born MNs into V2a interneurons (Arber et al., 1999; Thaler et al., 1999; Thaler et al., 2004). As *Lhx3* inhibits expression of the visceral MN determinant *Foxp1* (Dasen et al., 2008; Rousso et al., 2008) and visceral MN differentiation (William et al., 2003; Thaler et al., 2004), *Lhx3* expansion is likely to underlie the loss of PGC neurons observed in these mutant animals, as previously proposed (Rousso et al., 2008).

### Versatile genetic networks control MN subtype diversification in the thoracic spinal cord

Although newly born MNs were properly generated in the absence of OC factors, their further diversification was altered. At thoracic levels, an excessive number of newly born MNs did differentiate

into PGC cells at the expense of MMC/HMC neurons. This suggests that OC factors participate in the differentiation balance between somatic and visceral MNs. Surprisingly, the expression of regulators previously proposed to promote PGC neuron differentiation, including *Isl1* and *Foxp1* (Thaler et al., 2004; Dasen et al., 2008; Rousso et al., 2008), was severely downregulated. This led us to a re-evaluated model for thoracic MN diversification (Fig. 6B).

As the absence of OC factors did not alter MN consolidation, expression of the V2a interneuron determinants *Lhx3* and *Chx10* did not expand and visceral MN differentiation could occur free of expanded *Lhx3* constraints (William et al., 2003; Thaler et al., 2004). *Isl1* expression was lost from newly born MNs in *Hnf6/Oc2*<sup>-/-</sup> embryos. Subsequently, fewer somatic MNs were produced at thoracic levels of the spinal cord. This suggests that high levels of *Isl* proteins are required in newly born MNs to promote their differentiation into somatic MNs. By contrast, supernumerary PGC neurons were produced, suggesting that low levels of *Isl* proteins are permissive for the generation of visceral MNs (Fig. 6B). Consistently, *Hb9* overexpression stimulates both *Isl1* expression and somatic MN differentiation, whereas it inhibits visceral MN production (Tanabe et al., 1998; William et al., 2003). Furthermore, the levels of *Isl1* protein in prospective somatic MNs were double those in their visceral counterparts. Thus, we propose that differential levels of *Isl* proteins contribute to somatic versus visceral MN subtype specification of newly born MNs in the thoracic spinal cord.

Lower *Isl1* content could result from the presence of *Foxp1* in the prospective visceral MNs. Indeed, *Foxp1* inhibits *Hb9* expression (Dasen et al., 2008; Rousso et al., 2008), which could weaken the mutual stimulatory loop between *Hb9* and *Isl1* and reduce *Isl* levels in prospective PGC neurons. Accordingly, the expression of *Hb9* and *Isl1* is progressively downregulated during visceral MN differentiation and this is required for visceral MN production (William et al., 2003). Thus, in newly born MNs, OC factors participate in genetic networks that, probably depending on the involvement of *Foxp1*, promote either somatic or visceral MN differentiation (Fig. 6B).

The stimulation of *Sip1* expression by OC factors might provide an additional feedback mechanism to adjust visceral MN production. However, as *Isl1* overexpression did not mimic the increase in somatic MNs at the expense of visceral MNs observed after *Hnf6* overexpression in chick embryonic spinal cord, OC factors are likely to regulate the expression of other targets involved in MN diversification. Although we cannot formally exclude the possibility that non-cell-autonomous mechanisms contribute to the MN subtype defects observed in *Hnf6/Oc2*<sup>-/-</sup> embryos, the proposed model is consistent with our data and with published findings (Arber et al., 1999; Thaler et al., 1999; William et al., 2003; Thaler et al., 2004; Dasen et al., 2008; Rousso et al., 2008; Song et al., 2009). Of note, this model challenges the view that MMC neurons are generated before HMC and PGC cells (Dasen et al., 2008; Rousso et al., 2008), and instead favors the hypothesis that these three cell populations are simultaneously produced (Arber et al., 1999; William et al., 2003; Thaler et al., 2004).

### OC factors control the identity of LMCm neurons

Absence of OC factors does not alter the generation of MNs dedicated to limb innervation, as evidenced by the proper number and distribution of *Raldh2*<sup>+</sup> and *Foxp1*<sup>+</sup> MNs at brachial or lumbar levels of the spinal cord. By contrast, it results in an expansion of

the LMCI at the expense of the LMCm, as shown by the conversion of molecular identity (Tsuchida et al., 1994), by the switch in the expression of guidance molecules and by the consequent redirection of the axonal projections towards the dorsal portion of the limbs (Kania et al., 2000; Kania and Jessell, 2003; Luria et al., 2008). Progenitors destined to form the LMCm exit the cell cycle before those that contribute to the LMCI. All these MNs initially express *Isl1* (Sockanathan and Jessell, 1998). However, *Raldh2* expression in early-born LMCm neurons generates a retinoic acid signal that imposes a lateral phenotype to later-born neurons through initiation of *Lhx1* and repression of *Isl1* expression (Sockanathan and Jessell, 1998).

The distinct identities of these LMC divisions are further reinforced by crossrepressive interactions between these two transcription factors (Kania et al., 2000). In the absence of OC factors, *Isl1* expression in newly born LMC neurons was substantially altered. However, our observations indicate that OC factors and *Isl1* are dispensable for *Raldh2* expression and for normal production of LMCI neurons. By contrast, the absence of *Isl1* released the repression on *Lhx1* expression, which resulted in a complete switch from LMCm to LMCI identity (Fig. 6C). The crossrepressive interaction between *Lhx1* and *Isl1* also determines the respective position of LMCm or LMCI cell bodies within the LMC by gating reelin signaling (Palmesino et al., 2010). However, despite the absence of *Isl1* and the expansion of *Lhx1* in OC mutant embryos, the LMC cells were positioned normally and ectopic LMCI neurons did not tend to occupy a lateral position. This suggests that OC factors might cooperate with reelin signaling to control LMC cell body position.

## Conclusions

We have shown that OC factors control different aspects of the MN fate decision during spinal cord development. The OC proteins act in this process in a redundant manner, with *Hnf6* making the major contribution. Part of this activity is likely to rely on the direct stimulation of *Isl1* expression by the OC factors, although we provide evidence that other, as yet unidentified, OC targets are likely to contribute to define MN subtype identity. The identification of additional OC target genes and co-factors would enable further dissection of the genetic networks that regulate spinal MN diversification.

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## Competing interests statement

The authors declare no competing financial interests.

## Supplementary material

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