Msx1 and Msx2 act as essential activators of Atoh1 expression in the murine spinal cord

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ABSTRACT
Dorsal spinal neurogenesis is orchestrated by the combined action of signals secreted from the roof plate organizer and a downstream transcriptional cascade. Within this cascade, Msx1 and Msx2, two homeodomain transcription factors (TFs), are induced earlier than bHLH neuralizing TFs. Whereas bHLH TFs have been shown to specify neuronal cell fate, the function of Msx genes remains poorly defined. We describe dramatic alterations of neuronal patterning in Msx1/Msx2 double-mutant mouse embryos. The most dorsal spinal progenitor pool fails to express the bHLH neuralizing TF Atoh1, which results in a lack of Lhx2-positive and Barhl2-positive dI1 interneurons. Neurog1 and Ascl1 expression territories are dorsalized, leading to ectopic dorsal differentiation of dI2 and dI3 interneurons. In proportion, the amount of Neurog1-expressing progenitors appears unaffected, whereas the number of Ascl1-positive cells is increased. These defects occur while BMP signaling is still active in the Msx1/Msx2 mutant embryos. Cell lineage analysis and co-immunolabeling demonstrate that Atoh1-positive cells derive from progenitors expressing both Msx1 and Msx2. In vitro, Msx1 and Msx2 proteins activate Atoh1 transcription by specifically interacting with several homeodomain binding sites in the Atoh1 3' enhancer. In vivo, Msx1 and Msx2 are required for Atoh1 3' enhancer activity and ChIP experiments confirm Msx1 binding to this regulatory sequence. These data support a novel function of Msx1 and Msx2 as transcriptional activators. Our study provides new insights into the transcriptional control of spinal cord patterning by BMP signaling, with Msx1 and Msx2 acting upstream of Atoh1.

KEY WORDS: Homeodomain transcription factor, Neurogenesis, Developing spinal cord

INTRODUCTION
In vertebrates, the patterns of neuronal differentiation set during the development of the CNS form the basis of functional neural circuits in adults. For instance, the location and identity of sensory interneurons are established within the dorsal region of the developing spinal cord by the combined action of signals secreted from the roof plate (RP) organizer and a downstream gene regulatory network (Tanabe and Jessell, 1996; Liem et al., 1997). Both types of molecules orchestrate the spatiotemporal dynamics of gene expression within progenitor cells and their postmitotic derivatives (Helms and Johnson, 2003). Pax and Msx transcription factors (TFs) are among the first to appear within the dorsal proliferating neuronal progenitors (e.g. Tanabe and Jessell, 1996; Timmer et al., 2002). Later on, basic helix-loop-helix (bHLH) proneural TFs are induced within stripes organized along the dorsoventral axis of the neural tube. There, they act as specifiers by imposing a neuronal identity on the cells, assigning their future location in the spine, and controlling their connections to their target (Timmer et al., 2002; Helms and Johnson, 2003; Liu and Niswander, 2005; Chizhikov and Millen, 2004). Thus, the three dorsalmost progenitor pools, namely dp1, dp2 and dp3, with dp1 abutting the RP, are respectively specified by Atoh1 (Helms and Johnson, 1998), Neurog1 and Neurog2 (Gowan et al., 2001) and Ascl1 (Helms et al., 2005). The sharp expression profiles of these four genes emerge on the one hand in response to the morphogenetic gradients of BMP and Wnt proteins secreted from the RP (Tozer et al., 2013; Zechner et al., 2007), and, on the other hand, as the result of repressive interactions between the bHLH TFs. Atoh1 and Neurog1 cross-repress each other (Gowan et al., 2001), whereas Neurog2 restricts Ascl1 expression (Helms et al., 2005). Progenitor differentiation is achieved when a new set of homeodomain (HD) TFs, including Lhx2 and Barhl2 (Bermingham et al., 2001; Saba et al., 2005), Lhx1/5 (Gowan et al., 2001) and Isil (Helms et al., 2005), are respectively expressed by dI1, dI2 and dI3 interneurons.

Whereas the role of bHLH TFs is well documented, the function of HD TFs Msx1, Msx2 and Msx3 remains unclear. These genes are expressed as early as the neural plate stage, then during neuronal patterning and neurogenesis in the dorsal neural tube (Fig. 1A). Later on, Msx1 and Msx2 expression domains become progressively restricted to the RP, whereas Msx3 expression persists in the dorsal part of the spinal cord excluding the RP (Hill et al., 1989; Robert et al., 1989; Shimeld et al., 1996). HD sequences and Engrailed homology 1 motif (Eh1) domains in their N-terminus are highly conserved between Msx1, Msx2 and Msx3 and between species (Takahashi et al., 2008). Consistent with the presence of an Eh1 repressor domain, Msx1 and Msx2 are described as transcriptional repressors. In chick embryos, overexpression of Msx1 is sufficient to direct spinal dorsal progenitors towards an RP fate, whereas overexpression of Msx3 induces ectopic expression of Atoh1 and increases the number of dI1 cells (Liu et al., 2004).

Here, by analyzing spinal cell fates within mouse embryos mutant for Msx1 and Msx2, we show that these two TFs play essential functions during dorsal spinal cord neurogenesis. They are required for Atoh1 expression and dI1 interneuron generation. In addition, in the absence of Msx1 and Msx2, Pax7, Olig3, Neurog1 and Ascl1 expression domains are mispatterned. Furthermore, by
combining several in vitro and in vivo assays, we demonstrate that Msx1 and Msx2 positively regulate the expression of Atoh1 by directly interacting with its 3' enhancer and promoting its activity. Notably, we identify homeodomain binding sites (HBSs) that mediate the interactions of Msx proteins with this enhancer and thus control its activity. These data support a model in which Msx1 and Msx2 act upstream of a bHLH neuralizing transcriptional cascade, notably by cell-autonomously regulating the induction of a dp1 specifier.

RESULTS

Msx1 and Msx2 exhibit dynamic expression patterns in the dorsal spinal cord

Msx1 and Msx2 expression profiles were studied using β-galactosidase immunostaining on spinal cord sections from Msx1nlacZ/+ or Msx2nlacZ/+ heterozygous mouse embryos (Fig. 1A). We also compared these profiles with those of Olig3, which is a dp1-dp3 progenitor marker (Müller et al., 2005), Atoh1, which is a dp1 specifier, and Pax6, which labels from dp3 to the ventral p3 domains (Ericson et al., 1997) (Fig. 1B). At E9.5 and E10.5 Msx1nlacZ displays graded expression across the dorsal third of the neural tube. At E10.5, its expression domain encompasses that of Atoh1 and its ventral boundary matches that of the Olig3 domain (Fig. 1Ba-b”). Consistently, β-galactosidase was detected in cells expressing low levels of Pax6 in the dp3 domain (Fig. 1Be-c”). From E10.5, it becomes progressively restricted to the RP (Fig. 1A). By contrast, Msx2nlacZ expression is concentrated at the RP (Fig. 1A). However, from E10.5 to E11.5, its territory also overlaps with part of the Olig3 expression domain and coincides with that of Atoh1 (Fig. 1D). Altogether, these data demonstrate that early, proliferating dorsal progenitors, including the dp3 to dp1 cells, express Msx1. As these progenitors initiate their final differentiation, Msx1 is progressively switched off (Fig. 1Bf). Msx2 is also transiently induced, but only in the Atoh1+ dp1 cells (Fig. 1Be-f). Finally, both TFs display high levels of expression in the RP.

We then performed a cell lineage analysis using embryos carrying the Msx1CreERT2 allele and the ROSA26RSTOPAlbino reporter. In these embryos, upon tamoxifen-mediated Cre activation, membrane GFP expression labels cells in which Msx1 is expressed, as well as all their progeny. Females were injected with one or several doses of tamoxifen from 8 to 9.5 days post-fertilization (Fig. 1C,D) and embryos were collected at E10.5. In agreement with Msx1 expression dynamics, the number of GFP+ cells detected within the dorsal neural tube varied according to the stage and number of injections. A single injection at E8.5 produces the lowest proportion of cells expressing GFP (15% of total cells counted). Under this condition, most of the GFP is detected in RP cells (Fig. 1Ca”) and only 5% of the GFP+ cells co-expressed Atoh1 (Fig. 1D). Strikingly, the proportion of GFP+ cells is similar in embryos that received tamoxifen twice or three times at E8.0 or E9.5 (Fig. 1Db-f, D) and in embryos that received a single dose of tamoxifen at E9.0 or E9.5 (Fig. 1Ce-f”). In these conditions, GFP is detected in the RP, in Atoh1-expressing cells as well as in the dp2 and dp3 domains (Fig. 1Df”; data not shown). Altogether, these experiments demonstrate that the GFP expression domain reaches its maximum upon single Cre activation at ~E9.0. Most importantly, they indicate...
that nearly all Atoh1+ cells detected at E10.5 are born from progenitors expressing Msx1 as early as E9.25.

**dp1 progenitors fail to be specified whereas the dp2 and dp3 domains are dorsally shifted in Msx1/Msx2 mutants**

We next assessed the functions of Msx1 and Msx2 during dorsal neurogenesis by analyzing the expression of key transcriptional specifiers in double mutants (Msx1−/−Msx2−/−), referred to as Msx1/Msx2) at E10.5 (Fig. 2). Notably, the body size of Msx1/Msx2 mutant embryos was reduced compared with controls. As a consequence, at all stages analyzed from E9.5 to E11.5, the diameter of their spinal cord was consistently reduced to 85% of that of wild-type littersmates. All the progenitor domains (from the ventralmost to the dorsalmost) appear smaller (data not shown). This suggests a role for Msx1 and Msx2 during late gastrulation in controlling embryonic growth.

Most importantly, Atoh1 transcripts and protein were not detected throughout the entire anteroposterior axis of the double-mutant developing spine (compare Fig. 2Aa,Ba with 2Ab,Bb and 2Ae). In addition, Atoh1 expression was reduced by more than half in developing spine (compare Fig. 2Ad,Bd) and the number of Atoh1+ cells is significantly different between either single mutant (P=7×10−4; Fig. 2Ae). Thus, Msx1 and Msx2 are both required for Atoh1 expression in the dp1 domain, but Msx1 plays a predominant role in this process.

We next analyzed the expression profiles of Neurog1 (dp2 marker), Neurog2 (dp2 and dp3 marker), Olig3 (dp1 to dp3 marker) and Ascl1 (dp3 to dp5 marker) in the absence of Msx1 and Msx2. In Msx1/Msx2 mutants, the Neurog1+ domain is included in the Olig3 territory, as in wild-type embryos (Fig. 2Aa,b,d,e,D). However, the position of the shared dorsal boundary of the Neurog1+ and Neurog2+ domains is dorsally shifted to match the dorsal limit of the Olig3+ domain (Fig. 2Cb,D; supplementary material Fig. S1A).

Similarly, the dorsal boundary of the Ascl1 expression domain almost reaches that of the Olig3+ territory (Fig. 2Ch,D). Together with the loss of Atoh1 expression, these results suggest that the dp1 (Atoh1−, Olig3+, Neurog1−, Ascl1−) cells are not specified in the absence of Msx1 and Msx2, and instead the most dorsal progenitors adopt a mixed dp2/dp3 (Neurog1+, Neurog2+, Olig3+, Ascl1+) fate. Consistent with the loss of dp1 progenitors, the Olig3+ domain in the double mutants contains fewer cells than in wild-type embryos (Fig. 2Cf). The Neurog1+ territory within the Olig3 expression domain is also smaller in the double mutants (Fig. 2Cc), although its proportion to the entire neural tube is unchanged (Fig. 2D), consistent with the overall reduction in size of the mutant embryos. Finally, the Olig3+ domain ventrally adjacent to the Neurog1+ territory expressed a similar set of markers (Neurog2, Olig3 and Ascl1) in both wild-type and Msx1/Msx2 mutant embryos (Fig. 2Cd,e,g,h,D; supplementary material Fig. S1A), indicating that dp3 cells are correctly specified in the absence of Msx1 and Msx2. However, the proportion of these cells is greater in the double mutants than in wild type (Fig. 2Ci,D).

Pax7 is one of the first TFs to be induced within the developing spine (Liem et al., 1997). In wild-type embryos, its expression encompasses the dp2 to dp6 domains, with strong expression levels in the dp6 to dp4, and lower expression in the dp3 to dp2 domains (Fig. 2Ea). In the absence of Msx1 and Msx2, at each stage analyzed (from E9.5 to E11.5), Pax7 was detected in the RP cells, as well as in dorsal ventricular precursors, including those just adjacent to the RP

![Fig. 2. Patterns of dorsal spinal specifiers in Msx1/Msx2 mutants at E10.5.](https://example.com/figure2.png)
are detected, and the absence of Barhl2 (Fig. 3Ad) transcripts persists at E14.5 (E9.5 versus E10.5), we hypothesized that it might be implicated in Atoh1 downregulation in the Msx1/Msx2 mutants. However, after mutating Pax7 (Msx1/Msx2/Pax7 triple mutants), Atoh1 was not immunodetected, indicating that ectopic Pax7 expression is not responsible for the lack of Atoh1 expression in the double-mutant embryos (Fig. 3C), which is consistent with the expansion of the dp3 progenitors upon Msx1 and Msx2 inactivation (Fig. 2C,D).

The RP and BMP signaling are functional in Msx1/Msx2 mutant embryos

Msx1 and Msx2 are highly expressed in the RP. Accordingly, gene expression within this organizer is affected in the Msx1/Msx2 mutants. For example, Msx3 (supplementary material Fig. S1B) and Pax7 (Fig. 2E) become ectopically expressed in this structure. Thus, the absence of Msx1 and Msx2 might affect the expression of RP-specific TFs or morphogens involved in patterning the dorsal spinal cord. Lmxa1a is one of these TFs (Millonig et al., 2000), the activity of which is required to exclude Atoh1 expression from the RP and prevent dI1 neuron formation at the dorsal midline (Chizhikov and Millen, 2004). Lmxa1a expression is comparable in Msx1/Msx2 mutant and wild-type embryos (Fig. 4A) and hence this gene is unlikely to act downstream of Msx proteins. The transcripts of two secreted proteins from the RP, BMP6 and Wnt1, present similar levels and distribution in Msx1/Msx2 mutants and control littersmates (Fig. 4A), suggesting that RP signaling is not perturbed by the Msx1/Msx2 mutations.

BMP signaling plays a predominant role in the establishment of differentiation patterns in the dorsal spinal cord. To monitor its state in Msx1/Msx2 mutants, phospho-Smad1/5/8 (phospho-Smad) immunostaining was performed at E9.5 (Fig. 4B) and E10.5 (Fig. 4C), i.e. before and after Atoh1 induction. Phospho-Smad-positive cells share an identical distribution in wild-type and double-mutant embryos. At both stages, Msx1/Msx2-deficient cells are positive for phospho-Smad (Fig. 4B,C, asterisks), and hence do respond to BMP. Furthermore, quantification of phospho-Smad immunostaining reveals that both the amplitude (the peak of signal at the RP ventral boundary) and the ventral extent of the phospho-Smad gradient are similar in wild-type and Msx1/Msx2 mutant embryos (Fig. 4B,C). Of note, the levels of phospho-Smad appear increased in the RP of the double mutants compared with wild-type embryos at E9.5. Altogether, these data indicate that, despite the

Loss of Msx1 and Msx2 functions alters interneuronal differentiation in the dorsal spinal cord

To assess the fate of dorsal progenitors in the absence of Msx1 and Msx2, we analyzed the expression of several TF markers of the distinct dorsal interneuron populations. Atoh1-positive progenitors give rise to dI1 interneurons, which express the HD TFs Lhx2 and Barhl2, both being downstream targets of Atoh1 (Helms and Johnson, 1998; Birmingham et al., 2001; Gowan et al., 2001; Saba et al., 2003, 2005). In E11.5 Msx1/Msx2 mutants, neither Lhx2 (Fig. 3Ab) nor Barhl2 (Fig. 3Ad) transcripts are detected, and the absence of Barhl2 transcripts persists at E13.5 (Fig. 3Af). This is in accordance with the lack of Atoh1 expression observed at E10.5. Of note, both Lhx2 and Barhl2 are still detected in the developing brain of the double mutant at each stage analyzed (data not shown). The Msx1/Msx2 mutants die soon after E14.0 (Lallemand et al., 2005), precluding the study of a possible delay in dI1 differentiation.

As expected, the dorsal shift of Neuorog1- and Ascll-positive progenitor compartments leads to a dorsal shift in the position of dI2 and dI3 interneurons (Fig. 3B). First, dI2 cells expressing Lhx1/5 were detected in an ectopic dorsal position in the double mutant compared with wild-type embryos (Fig. 3Ba,b). Furthermore, in agreement with the decrease in the number of Neurog1+ progenitors, these cells were less abundant in double mutants. Second, Islet1-positive dI3 cells were also generated in an abnormal dorsal position in the double mutants (Fig. 3Bc,d). In contrast to dI2 cells, the number of dI3 interneurons is increased in the double-mutant embryos (Fig. 3C), which is consistent with the expansion of the dp3 progenitors upon Msx1 and Msx2 inactivation (Fig. 2C,D).
ectopic expression of Pax7 and Msx3 in the RP, this signaling center remains functional in Msx1/Msx2 embryos.

Msx1 and Msx2 regulate Atoh1 3′ enhancer activity in vitro via several homeodomain binding sites

Atoh1 expression in the murine developing dorsal spinal cord is regulated by a 1500 bp sequence, termed the Atoh1 3′ enhancer, located 3 kb downstream of the Atoh1 coding sequence (Helms et al., 2000). It thus constitutes a candidate target of Msx1 and Msx2 for Atoh1 regulation. Consistently, luciferase assays in HEK293 cells indicated that firefly luciferase activity driven by the Atoh1 3′ enhancer is significantly increased upon overexpression of Msx1 or Msx2 (Fig. 5A). Activation of this enhancer by Msx1 is ∼2-fold, whereas Msx2 induces at least a 7-fold increase in its activity (Fig. 5A,Da,b).

The murine Atoh1 3′ enhancer contains 14 consensus HBSs [ATTA, the minimal consensus sequence (Catron et al., 1993)]. Interestingly, their location and the distances between them are highly conserved both in domain A (5′) and domain B (3′) among vertebrates (Fig. 5B). We next assessed, by electrophoretic migration shift assay (EMSA), whether Msx1 and Msx2 could bind these sites using nine probes encompassing all the candidate HBSs (see supplementary material Fig. S2A and Table S1). Band shifts are observed for probes 2, 3, 5, 6, 7, 8 and 9 (supplementary material Fig. S2B), but we considered as specific binding that which fulfilled the following four criteria: (1) disappearance of band shifts in competition assays (supplementary material Fig. S2C); (2) disappearance of band shifts when HBSs were mutated (Fig. 5C, right lanes); (3) presence of supershifts when Msx1HA or Msx2FLAG and their corresponding antibodies were added (left panels in Fig. 5C); (4) absence of these supershifted bands when HBSs were mutated. According to these criteria, Msx1 (Fig. 5Ca,c,e) and Msx2 (Fig. 5Cb,d,f) specifically bound to probes 2, 7 and 8. Notably, mutations of HBS3 in probe 2 (Fig. 5Ca,b, right lanes) strongly diminished, whereas mutations of HBS8 in probe 7 (Fig. 5Cc,d, right lanes) inhibited, the formation of high molecular weight complexes. Band shift signals with probe 8 disappear only when HBSs13, HBS12 and HBS11 are mutated together, although they do strongly decrease upon HBS8 mutation alone (Fig. 5Ce,f, right panels). These experiments demonstrate that Msx1 and Msx2 can physically interact with the Atoh1 3′ enhancer at three distinct sites: HBS3 in domain B, in close vicinity to the minimal enhancer sequence determined by Helms et al. (Helms et al., 2000) (Fig. 6B; supplementary material Fig. S2A); HBS8 and HBS11 in domain A.

We next introduced mutations within these candidate HBSs and tested their effects on Msx1- or Msx2-mediated induction of Atoh1

Fig. 4. Unaffected roof plate signaling in Msx1/Msx2 mutants. (A) ISH at E10.5 for Bmp6, Wnt1 and Lmx1a on wild type and Msx1/Msx2 mutants. (B,C) Immunostaining for phospho-Smad1/5/8 and β-galactosidase on E9.5 and E10.5 wild type and Msx1/Msx2 mutant. Asterisks highlight selected cells doubly positive for phospho-Smad1/5/8 and β-galactosidase. (B′: b′′′) Higher magnifications of b,b′. (Bc,Cc) Phospho-Smad1/5/8 signal distribution along the dorsoventral axis of the neural tube. Bc: WT, n=2; dKO, n=3. Cc: WT, n=2; dKO, n=5; at least two sections were analyzed per embryo; error bars indicate s.e.m. Gray shading mark the RP. Scale bars: 20 μm in A,B,a,b,Ca,b; 5 μm in Bb′; 10 μm in Cb′.
Msx1 fails to activate Atoh1 3′ enhancer activity when HBS2 and HBS3 are mutated, and when all candidate HBSs are mutated in combination (experimental condition T; Fig. 5Da). Mutations in HBS8, HBS11 and HBS12 do not modify Msx1 activation properties. Second, Msx2-mediated Atoh1 3′ enhancer activity is significantly decreased by mutations in HBS2 and HBS3, or HBS11 and HBS12, or when all sites are mutated together (Fig. 5Db). This indicates that these HBSs, notably HBS2 and HBS3, mediate the ability of Msx1 and Msx2 to promote Atoh1 3′ enhancer activity.

Msx1 and Msx2 might act as activators of the Atoh1 3′ enhancer or as repressors of a repressor. Since amino acids F and S of the Msx Eh1 motif are required for its interaction with Groucho/TLE family members (Takahashi et al., 2008; Rave-Harel et al., 2005; Goldstein et al., 2005), they were replaced by G and A in Msx1 (Msx1*) and Msx2 (Msx2*) to abolish their repressive activity (Fig. 5Dc).

Fig. 5. Msx1 and Msx2 interact with the Atoh1 3′ enhancer and potentiate its activity. (A) Relative activity (RLU/μg) of firefly luciferase driven by a minimal promoter (−enh) or associated with the Atoh1 3′ enhancer (+enh) in response to Msx1 or Msx2 (n=8). (B) Scheme showing the position of conserved homeodomain binding sites (HBSs) along the Atoh1 3′ enhancer (x-axis: 5′ to 3′, bp) among 17 vertebrates: H. sap, human; P. tro, chimpanzee; M. lem, mouse lemur; M. mus, mouse; R. nor, rat; P. cap, hyrax; E. tel, lesser hedgehog tenrec; C. por, cobaye; E. cab, horse; V. pac, alpaca; L. afr, elephant; B. tau, cow; C. fam, dog; M. luc, microbat; T. tru, dolphin; G. gal, chicken; and O. lat, medaka. Each symbol corresponds to a specific HBS. (C) EMSAs with probes 2, 7 and 8 containing wild-type or mutated HBSs (top panels) and Msx1, Msx1-HA (a,c,e), Msx2, Msx2FLAG (b,d,f). Arrows indicate specific complexes. Asterisks indicate supershifts in the presence of anti-Tag antibodies (+Ab). F.P., free probe; NT, not transfected. (D) Luciferase assays expressed as relative fold activation. The Atoh1 3′ enhancer, intact or mutated for HBSs, was co-transfected with Msx1 (a) or Msx2 (b) (n=7). (c) The effect of Eh1 mutations in Msx1 and Msx2 (Msx1* and Msx2*) on Atoh1 3′ enhancer activity (n=3). Error bars indicate s.e.m. 0.01<**P<0.05; 0.001<***P<0.01; ***P<0.0001; ns, not significant.
Msx1* increases the activity of the Atoh1 3' enhancer, similar to Msx1. Msx2* is also able to increase Atoh1 3' enhancer activity, but to a lower level than Msx2. Thus, the Eh1 motif in Msx1 and Msx2 plays a limited role in their ability to activate the Atoh1 3' enhancer.

Taken together with results above, this strongly suggests that Msx1 and Msx2 directly activate Atoh1 expression via its 3' enhancer.

Msx1 and Msx2 proteins regulate Atoh1 3' enhancer activity in vivo

We next assessed direct interactions of the Msx proteins with the Atoh1 3' enhancer in vivo. We designed an Msx1 allele containing HA and His tags upstream of its 3'UTR (supplementary material Fig. S3). Immunoprecipitations using an anti-HA antibody were performed on chromatin isolated from spinal cord-enriched dissected tissues of E10.5 Msx1Tag/Tag and wild-type embryos (supplementary material Fig. S3D), followed by Q-PCR to amplify three parts of the Atoh1 3' enhancer (enh A, enhB1 and enhB2; Fig. 6B). Significant enrichment of these sequences was observed in Msx1Tag/Tag embryo samples compared with wild type (Fig. 6A).

Conversely, the Atoh1 transcription initiation region, which is devoid of any potential HBSs, was not enriched (Atoi, Fig. 6A). Furthermore, known Msx1 targets in the promoters of connexin 43 (Cx43; also known as Gja1) (Boogerd et al., 2008) and Stra8.2 (Le Bouffant et al., 2011) were not amplified (Fig. 6A), consistent with the absence of heart primordium or developing gonads from the samples analyzed. These experiments indicate that Msx1 directly binds the Atoh1 3' enhancer in vivo.

Finally, regulation of Atoh1 3' enhancer activity by Msx1 and Msx2 was addressed in vivo. For this, we bred transgenic mice in which mCherry expression is under the control of the Atoh1 3' enhancer [Tg(Math1/mCherry) strain (Lepelletier et al., 2013)] with Msx1nlacZ/+ Msx2GFP/+ animals. At E10.5, in wild-type embryos the mCherry fluorescence recapitulated endogenous Atoh1 expression in the dorsal spinal cord (Fig. 6Ca,a'). Some activity was observed in limb buds, where Atoh1 is not expressed (Lumpkin et al., 2003). In Msx2 mutant embryos, the fluorescence pattern was similar to that...
of wild-type embryos (Fig. 6Cc,c’). By contrast, mCherry fluorescence was abolished in the dorsal spinal cord of Msx1 mutants, although it remained detectable in the limb buds and rombomeres (Fig. 6Cb,b’). In Msx1/Msx2 mutants, mCherry signal was absent from the dorsal neural tube except for residual activity in the dorsal part of the first two rombomeres (Fig. 6Cd,d’). This further confirms that Msx1 and Msx2 control Atoh1 expression by promoting the activity of its 3’ enhancer.

**DISCUSSION**

**Msx1 and Msx2 are required for Atoh1 expression, dl1 specification and restriction of Pax7 expression**

The series of *in vivo* and *in vitro* experiments described in this study thoroughly demonstrates that Atoh1 is a direct target of both Msx1 and Msx2. Msx3 is also expressed in the dorsal part of the murine embryonic spinal cord (Wang et al., 1996; Shimeld et al., 1996). However, although Msx3 expression persists in Msx1/Msx2 mutants, it is not able by its own to maintain Atoh1 expression. The Msx3 gene is absent from many mammalian genomes, except for mouse, rat, opossum (Finnerty et al., 2009) and a number of unguulates (our unpublished observations); further supporting the idea that Msx3 is dispensable for Atoh1 expression in mammals. Importantly, the loss of activity of Atoh1 in Msx1/Msx2 mutants is highly likely to account for the lack of dl1 neurons in these embryos. Atoh1 has indeed been shown to directly induce Barhl2 expression (Bermingham et al., 2001; Saba et al., 2005), which, in turn, activates Lhx2 expression (Kawauchi et al., 2010). This transcriptional cascade that specifies dl1 interneurons fails to be activated in the absence of Msx proteins.

Furthermore, other neuronal patterning alterations displayed by Msx1/Msx2 mutants are identical to those found in Atoh1 mutants, such as the dorsalization of the Neurog1 and Ascl1 expression domains and of the dl2 and dl3 populations (Bermingham et al., 2001; Gowen et al., 2001; Miesegaes et al., 2009). Consistent with a dorsalization of the territories of several key spinal specifiers upon Msx1 and Msx2 gene inactivation, Pax7 is found in the dorsalmost domain of the spinal cord including the RP. As Atoh1 expression is not rescued in Msx1/Msx2/Pax7 mutant embryos, and as the RP signals operate normally in Msx1/Msx2 mutants, this ectopic expression of Pax7 is not the cause for Atoh1 silencing, either directly or indirectly. Dorsal ectopic expression of Pax7 has been described in Pax3 mutant neural tube (Borycki et al., 1999) and in the Mxs1”−/−” mutant (Bach et al., 2003). As the Pax3 expression pattern is unaltered in Msx1/Msx2 mutants (not shown), ectopic expression of Pax7 is most likely a direct consequence of Msx1 and Msx2 inactivation. Our results indicate that Atoh1 and Pax7 expression is controlled by Msx1 and Msx2 in the dorsal spinal cord. Whether this control is exerted by direct binding to regulatory sequences of the murine Pax7 gene remains to be elucidated.

**Msx genes as neuronal specifiers**

As shown here, Msx1 and Msx2 are required for Atoh1 expression and thus control dl1 interneuron specification in the developing spinal cord. Msx genes are known to be involved in neuronal specification (reviewed by Ramos and Robert, 2005). Loss of function of msh (the Drosophila Msh gene ortholog, also known as *Drop*) leads to differentiation defects of dorsal neuroblast progeny (Ishii et al., 1997). *Msh* function has been shown to be required for the proper differentiation of neuronal cells in *Cupiennus salei* spider embryos (Döffinger and Stollewerk, 2010). Dorsal ectopic overexpression of murine Msx1 and Msx3 in chick embryo spinal cord leads, respectively, to cell death and dl1 misspecification into RP cells and to increased Atoh1 expression and dl1 differentiation (Liu et al., 2004). This demonstrates that Atoh1 expression and dl1 specification depend on the proper expression levels of each Msx gene. Spatiotemporal regulation of Gnrh1 (Gnrh1) transcription by Msx1 has been demonstrated in the murine developing CNS (Givens et al., 2005), although in this case Msx1 repressor activity has been proposed to control neuronal differentiation (Rave-Harel et al., 2005). Our data, showing that Msx1 and Msx2, expressed by undifferentiated neuronal progenitors, are required for dl1 specification, are consistent with a conserved function of these genes in neuronal specification in many animal phyla.

**Regulation of Atoh1 transcription**

Atoh1 controls its own expression (Helms et al., 2000), but Zic1 is able to block this activity by directly binding the Atoh1 3’ enhancer close to its autoregulatory site (Ebert et al., 2003). The Zic1 expression pattern is not altered in Mxs1/Mxs2 mutants (not shown), precluding an interplay between Msx and Zic1 in Atoh1 regulation. Similarly, expression of *Lmx1a*, which is responsible for exclusion of Atoh1 expression from the RP (Chizhikov and Millen, 2004), is unchanged in these mutants. BMP signaling from the RP is required for the strict spatiotemporal control of neuronal patterning in the dorsal developing spinal cord (as summarized in Fig. 6D). This signaling pathway is thought to be responsible for the initiation of Atoh1 expression in the developing spinal cord. Recently, it has been shown that a dynamic gradient of BMP activity confers progressively more dorsal neural identities in the chick embryonic spinal cord and that both high levels and prolonged duration of BMP activity are required for Atoh1 induction (Tozer et al., 2013). Consistently, Atoh1 expression is abolished and differentiation of dl1 does not occur in Gdf7 null embryos (Lee et al., 1998) as well as when RP is ablated (Lee et al., 2000). Conditional double knockout of type 1 BMP receptor genes (*Bmpr1a* and *Bmpr1b*) in the neural tube precludes Atoh1 expression and dl1 differentiation, while dl2 are reduced in number and dl3 and dl4 are dorsally shifted (Wine-Lee et al., 2004). Interestingly, Msx1 and Msx2 expression is also abolished or strongly reduced under these experimental conditions, indicating that BMP signaling acts upstream of the Msx genes. As demonstrated by phospho-Smad immunostaining before and when Atoh1 expression is induced (at E9.5 and E10.5), absence of Msx1 and Msx2 expression does not notably perturb BMP signaling. Hence, Msx1 and Msx2 are proposed to relay BMP signaling from the RP and are responsible for the transcriptional activation of Atoh1 in neuronal progenitors (as illustrated in Fig. 6E).

**Msx1 and Msx2 as Atoh1 transcriptional activators**

Our data demonstrate for the first time that the two HD TFs Msx1 and Msx2 activate transcription of Atoh1 via direct binding to its 3’ enhancer. Msx1 and Msx2 bind *in vitro* to HBS3, HBS8 and HBS12. Interestingly, HBS3 is located 84 bp upstream of the minimal enhancer described by Helms et al. (Helms et al., 2000). Atoh1 3’ enhancer B sequence conservation, including the E-box that binds Atoh1 itself (Helms et al., 2000) and a nearby Zic1 binding site (Ebert et al., 2003), suggests that Atoh1 regulation is under selective pressure. HBS3 is conserved in chicken and mammals, whereas HBS12 and HBS8 are present in fish (supplementary material Fig. S2D).

We propose that the precise spatiotemporal control of Atoh1 expression occurs upon Msx1 and Msx2 binding to HBS2/3, 8 and 11/12 in order to initiate Atoh1 transcription. *In vitro*, Msx1 activation potency might seem weak compared with Msx2, considering the effect of their respective mutations in vivo. We do not exclude the possibility that other partners potentiating Msx1 activation may contribute to Atoh1 transcription initiation in vivo. However, even
when weakly temporally initiated by Msx1 and Msx2, Atoh1 would by itself sustain its own expression by binding its enhancer E-box. This implies that Msx1 and Msx2 should be expressed in neuronal progenitors before (E9.5) and during (E10.5) activation of Atoh1 expression. β-galactosidase expression patterns show that they are indeed expressed in dorsal progenitors at E9.5 and E10.5 and cell lineage analyses indicate that Atoh1-positive cells derive from progenitors expressing Msx1 as early as E9.25, a stage compatible with Atoh1 initiation of transcription at -E10.0. Msx1 and Msx2 act as transcriptional repressors in various contexts, either by directly interfering with the transcriptional machinery (Shetty et al., 1999; Hovde et al., 2001) or via a repressor complex containing Groucho factors (Takahashi et al., 2008; Rave-Harel et al., 2005). Activation of transcription by Msx1 and Msx2 is much less documented: Msx1 and Msx2 proteins have been shown to be active to activate Hspa1b promoter by interacting with heat shock factors Hsf1 and Hsf2 in a DNA binding-independent manner (Zhuang et al., 2009). Furthermore, direct activation of Stra8 transcription by Msx1 and Msx2 has been described during meiosis in the female germ line (Le Bouffant et al., 2011). Our data do not rule out the possible repression of an Atoh1 repressor by Msx1 and Msx2 (Neurog1, for instance). However, we demonstrated a direct interaction with the Atoh1 3′ enhancer. Furthermore, Atoh1 3′ enhancer activity is slightly affected by mutations in the E1 Groucho/TLE binding site, which would not be expected if the function of Msx1 and Msx2 were to repress a repressor. Therefore, a simpler mechanism consisting of the initiation of Atoh1 transcription by Msx1 and Msx2 is proposed.

### MATERIALS AND METHODS

#### Mice and tamoxifen treatment

Experiments were performed in accordance with European Community guidelines (2010/63/UE) and with French national regulations for the care and use of laboratory animals. The following mouse lines were used: Msx1CreERT2 (Houzelestein et al., 1997), Msx2Pax7lacZ (Lallemand et al., 2005), Msx2GFP (Bensoussan et al., 2008), Pax3Pax7lacZ (Mansouri et al., 1996), Msx1CreERT2 (Lallemand et al., 2013), ROSAmTDLacZ (Muzumdar et al., 2007) and Tg(Math1/mCherry) (Le Roux et al., 2009). The Msx1 allele with a transgenic version of Msx1 as described in supplementary material Fig. S3 and Table S2. Tamoxifen was administrated at 4 mg per embryonic injection. The tamoxifen treatment were performed using Lipofectamine 2000 (Invitrogen), nuclear extracts were prepared (Papin et al., 2003) from HEK293T cells transfected with empty or Msx2-containing pCMVTagTNT plasmid. Tagged protein expression was checked by western blot. 5 μg of nuclear protein extract was used per EMSA assay (Bajard et al., 2006). Sequences of radiolabeled oligonucleotides are listed in supplementary material Table S1. For supershifts, 2 μl of anti-HA (clone 3F10, Roche) or anti-FLAG M2 (Sigma) were added.

#### Luciferase assays

HEK293T cells were transfected using Lipofectamine 2000 with 700 ng Mx expression vectors, 700 ng carrier DNA (when needed), 20 ng Renilla luciferase expression vector and 200 ng plasmid containing the firefly luciferase driven by the Atoh1 3′ enhancer (Helms et al., 2000). Luciferase activity assays were performed 48 h post-transfection. As expression of Renilla luciferase activity was repressed by Msx1 and Msx2, the firefly luciferase activity was expressed as relative light units (RLU) per μg protein in cell extracts. Fold induction corresponds to the ratio of firefly luciferase activities in the presence and absence of Msx proteins.

#### Chromatin immunoprecipitation followed by quantitative real-time PCR

Tissues from E10.5 mouse embryos (see supplementary material Fig. S3D) were collected in cold PBS and crosslinked in 1% formaldehyde for 5 min at room temperature. Chromatin was prepared as described (Daubas and Buckingham, 2013). The chromatin immunoprecipitation (ChIP) protocol was derived from Navarro et al. (Navarro et al., 2010). Rabbit polyclonal anti-HA (ChIP grade; Abcam, ab 71113) was used. A StepOnePlus PCR machine (Applied Biosystems) and the FastStart Universal SYBR Green Master (Roche) were used for quantitative real-time PCR (Q-PCR) using 1.0 and 0.1 μl of immunoprecipitated and input DNA, respectively. Primer sequences are listed in supplementary material Table S3. PCR quantifications were calculated by the ΔΔCt method (Livak and Schmittgen, 2001) and results are expressed as percentage of input.

### Statistical analysis

Experimental groups were analyzed using one-way ANOVA. Multiple comparisons were performed using Student’s t-test (for cell quantifications) and Newman-Keuls (for luciferase assays) post-hoc tests. One-tailed paired t-test was used for ChIP-Q-PCR analysis.

### Competing interests

The authors declare no competing financial interests

### Author contributions

N.D. and B.R. designed the experiments. N.D., P.D., C.B.deC., C.StC. and V.R. performed the experiments. M.L. conceived the cell lineage experiments. N.D., V.R. and J.-Y.T. analyzed the data. N.D., V.R. and B.R. wrote the paper.

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