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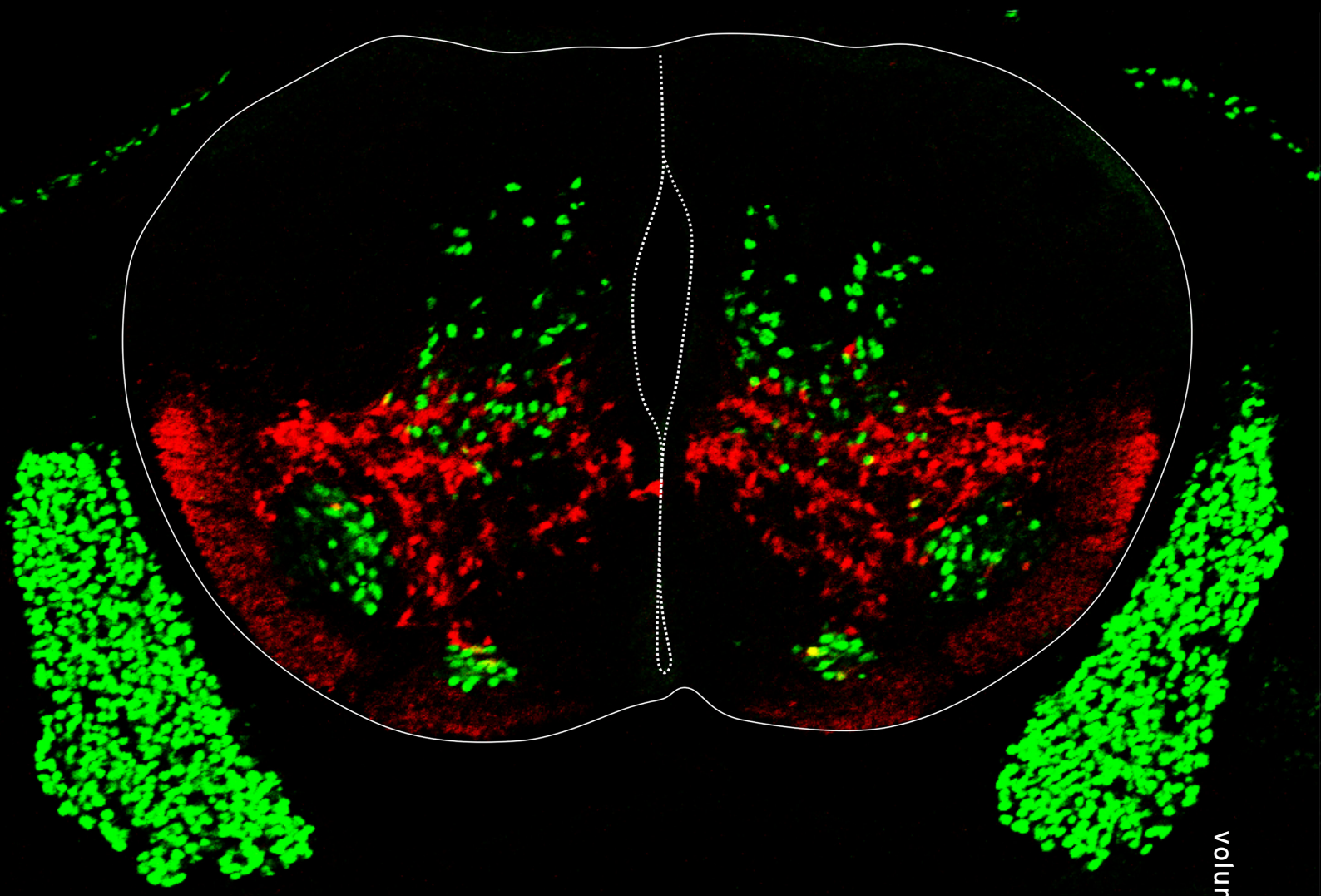

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TECHNOLOGY REPORT

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Generation and characterization of a tamoxifen-inducible *Vsx1-CreER^{T2}* line to target V2 interneurons in the mouse developing spinal cord

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Summary

In the spinal cord, ventral interneurons regulate the activity of motor neurons, thereby controlling motor activities including locomotion. Interneurons arise during embryonic development from distinct progenitor domains orderly distributed along the dorso-ventral axis of the neural tube. The p2 progenitor domain generates at least five V2 interneuron populations. However, identification and characterization of all V2 populations remain currently incomplete and the mechanisms that control their development remain only partly understood. In this study, we report the generation of a *Vsx1-CreER^{T2}* BAC transgenic mouse line that drives CreER^{T2} recombinase expression mimicking endogenous *Vsx1* expression pattern in the developing spinal cord. We showed that the *Vsx1-CreER^{T2}* transgene can mediate recombination in V2 precursors with a high efficacy and specificity. Lineage tracing demonstrated that all the V2 interneurons in the mouse developing spinal cord derive from cells expressing *Vsx1*. Finally, we confirmed that V2 precursors generate additional V2 populations that are not characterized yet. Thus, the *Vsx1-CreER^{T2}* line described here is a useful genetic tool for lineage tracing and for functional studies of the mouse spinal V2 interneurons.

KEYWORDS
embryonic spinal cord, tamoxifen-inducible lineage tracing, V2 interneurons, Vsx1

1 | INTRODUCTION

Locomotion is a stereotyped but complex behavior regulated by neural circuits located in ventral regions of the spinal cord. These circuits are made up of motor neurons (MNs), which directly innervate skeletal muscles, and of multiple populations of pre-motor interneurons (INs) whose integrated action controls the activity of the MNs (Cote, Murray, & Knikou, 2018). Several studies evidenced that different INs subpopulations have not been characterized yet (Alvarez et al., 2005; Bikoff et al., 2016; Hayashi et al., 2018; Li, Misra, & Xiang, 2010), underlining the need of lineage-tracing tools to permanently label these cells and to characterize their developmental origin, their phenotype and their activity.

During spinal neurogenesis, MNs and cardinal ventral INs populations arise from specific progenitor domains, pMN and p0 to p3 domains respectively, following a well-regulated differentiation process. The p2 progenitor domain, defined by the expression of *Pax6*, *Nkx6.1* and *Irx3* patterning genes, firstly gives rise to V2 INs precursors characterized by the transient expression of the CVC paired-like transcription factor *Vsx1* (Francius et al., 2016). In these cells, V2 INs identity is consolidated by a tetrameric complex composed of Lhx3 and the Nuclear Lim-Interacting protein (NLI). This V2-tetramer

Charlotte Baudouin and Barbara Pelosi contributed equally to this work.

binds a regulatory region called Tetramer Response Element (TeRE) and stimulates the expression of V2 specific genes including *Chx10*, the single paralog of *Vsx1* in the mammalian genome (Clovis et al., 2016; Lee et al., 2008; Thaler, Lee, Jurata, Gill, & Pfaff, 2002). Vsx1 and Chx10 cooperatively prevent the activation of a MN fate-promoting enhancer called Hexamer Response Element (HxRE) that stimulates the expression of MN determinants including Hb9 (Debrulle et al., 2020; Lee et al., 2008). Then, this cardinal V2 population generates at least five populations of V2 INs characterized by specific molecular identity, localization, and connectivity. V2a and V2b INs, expressing respectively *Chx10* and *Gata3*, are produced first through Notch signaling following a last mitotic division of p2 progenitors (Del Barrio et al., 2007; Kimura, Satou, & Higashijima, 2008; Li, Misra, Matise, & Xiang, 2005) and represent around 60% of the total number of V2 INs (Li et al., 2010). Functional experiments demonstrated that V2a INs regulate left–right limb alternation at high locomotion speed (Crone et al., 2008), while V2b cells contribute to the alternate contraction of reciprocal flexor or extensor muscles (Zhang et al., 2014). Other smaller populations, including V2c (expressing *Sox1*), V2d (*Shox2*), and V2-Pax6 (*Pax6*), are produced at supposedly later developmental stages (Dougherty et al., 2013; Panayi et al., 2010; Panayiotou et al., 2013). V2c INs derive from V2b (Panayi et al., 2010), V2d are related to V2a cells (Dougherty et al., 2013) and V2-Pax6 likely constitutes a late V2b subset (Panayiotou et al., 2013). V2d participate in the locomotor rhythm generation (Dougherty et al., 2013), whereas the function of the other small V2 populations remain unknown. However, these small populations only account for 10% of the total number of V2 INs (Li et al., 2010), suggesting that all the V2 IN populations are not identified and characterized yet.

Our previous studies suggested that Vsx1 is produced in all the V2 precursor cells (Francius et al., 2016). Vsx1 is a transcriptional repressor of the *paired-like:CVC (Prd-L:CVC) homeobox* gene family, with its unique paralog Chx10 (Liu et al., 1994). *Vsx1* expression is first detected at the gastrulation stage in these uncharacterized cells (Ohtoshi, Justice, & Behringer, 2001). Later, it is expressed in the hindbrain and in the spinal cord at the onset of neurogenesis (Batista, Jacobstein, & Lewis, 2008; D'Autilia et al., 2006; Francius et al., 2016; Kimura et al., 2008), while its expression in the developing retina is initiated at postnatal stages (Chow et al., 2001). In the eye, Vsx1 is detected from postnatal day 5 (P5) in the inner nuclear layer of the retina, where it controls terminal differentiation of bipolar OFF (2a and 3) or ON (7) cone INs (Chow et al., 2004; Kerschensteiner et al., 2008; Ohtoshi et al., 2004; Shi et al., 2011; Star et al., 2012). In the zebrafish, Vsx1 is produced in V2 progenitors and is maintained in differentiating V2a INs (Kimura et al., 2008). In *Xenopus* and mouse, its expression has been attributed to V2a cells (D'Autilia et al., 2006; Delile et al., 2019). However, recent data demonstrated that in the mouse spinal cord, Vsx1 is transiently detected in an intermediate V2 precursor compartment wherein progenitors stopped dividing but did not initiate neural differentiation yet, and is absent from differentiating V2 INs (Francius et al., 2016). Consistently, Vsx1 contributes to the consolidation of V2 fate by inhibiting the activation of the HxRE enhancer and of the MN differentiation program in V2 precursors, whereas its paralog takes over in differentiating V2a INs (Debrulle et al., 2020). This suggests that Vsx1 is transiently produced in all the V2 precursors and may be instrumental in lineage-tracing the V2 IN population and further characterizing its progeny.

Therefore, we generated a BAC transgenic line wherein the inducible CreER^{T2} is produced under the regulating sequences of *Vsx1*. These mice were crossed with a *Rosa26R-tdTomato* reporter line (*Vsx1|tdTomato*) to trace the lineage of *Vsx1* expressing cells. Inducible Cre was chosen to prevent any precocious activation of reporter expression in uncharacterized cells at the gastrulation stage (Ohtoshi et al., 2001). Characterization of *Vsx1|tdTomato* embryos indicated first that the *Vsx1-CreER^{T2}* transgene can mediate recombination in V2 precursors with a high efficacy and specificity. Second, lineage-tracing demonstrated that the cells expressing *Vsx1* give rise to all the V2 INs in the mouse developing spinal cord. Third, we confirmed that V2 precursors generate additional V2 populations that are not characterized yet. Hence, the *Vsx1-CreER^{T2}* mouse line is a highly efficient and specific tool for lineage tracing and might be instrumental for genetic targeting of V2 INs during spinal cord development.

2 | RESULTS

2.1 | Generation of *Vsx1-CreER^{T2}* BAC transgenic mouse line

To genetically target the embryonic V2 INs in the mouse spinal cord, we generated a *Vsx1-CreER^{T2}* allele wherein a BAC containing the *Vsx1* locus was modified by homologous recombination in *E. coli* to insert the *CreER^{T2}* coding region at the *Vsx1* translation initiation codon (Figure 1a). This transgenic construct contains 160 kb around the *Vsx1* locus, thereby increasing the probability to mimic the endogenous *Vsx1* expression pattern. Nine founder lines were obtained, and were crossed with *Rosa26R-tdTomato* mice (referred to here as *Ai14*) (Madisen et al., 2010). Pregnant *Ai14* females were injected at embryonic day (e)9.5 with tamoxifen to activate inducible CreER^{T2} activity (Figure 1b). Three founders generated embryos with higher tdTomato production in the developing spinal cord (Figure 2; Movie S1 and data not shown). Those three lines were analyzed for the presence of tdTomato in V2 INs (Figure 2 and data not shown). Two of them showed a higher recombination efficiency (mean number of tdTomato-positive V2 > 90%; Figure S1) and the line derived from founder 471 was used for further analyses.

2.2 | The *Vsx1-CreER^{T2}* line genetically targets the cardinal V2 interneuron population in the developing spinal cord

To assess whether the selected *Vsx1-CreER^{T2}* line efficiently and specifically targets the V2 IN populations, *Vsx1|tdTomato* embryos were first collected at e10.5, a stage when tdTomato fluorescence

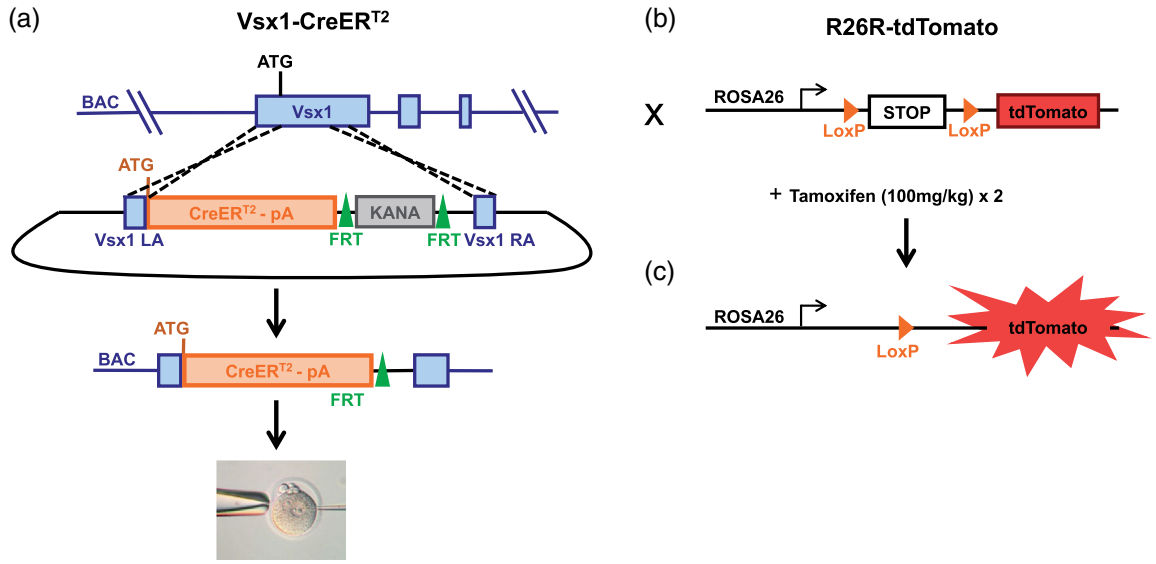

FIGURE 1 Generation of *Vsx1-CreER^{T2}* BAC transgenic mouse line. (a) A BAC containing the *Vsx1* locus was modified by homologous recombination in *E. coli* to insert the *CreER^{T2}* coding region at the Vsx1 translation initiation codon, using left-homology (LA) and right-homology (RA) arms. The kanamycin-resistance cassette (KANA) was deleted by co-transformation with a Flp-expressing vector. Circular BAC DNA was injected into C57Bl/6 F1 zygotes. (b) The *Vsx1-CreER^{T2}* line was crossed with *Rosa26R-tdTomato* mice (referred to here as *Ai14*). (c) Pregnant females were injected twice at e9.5 with 6 hr of interval with tamoxifen (100 mg/kg) to activate CreER^{T2} activity in *Vsx1*-expressing cells

was readily detected in the spinal cord (Figure 2). TdTomato was present in the ventral spinal cord in 78% of cells that contained Vsx1 (Figure 2a–d), likely corresponding to V2 IN precursors (Francius et al., 2016). We assumed that the absence of tdTomato from 22% of Vsx1-positive cells corresponded to the time interval necessary for Cre accumulation and *Rosa26R-tdTomato* locus recombination. Consistently, Cre was detected in 90% of tdTomato-positive cells but also in cells lacking fluorescence (Figure 2b,d), owing to the time necessary for excision of the STOP cassette and activation of *tdTomato* expression. In contrast, Cre was absent from V2a (not shown) and V2b INs (Figure 2b), suggesting that *Vsx1-CreER^{T2}* expression faithfully mimics that of the endogenous *Vsx1*. To confirm that tdTomato indeed labels V2 precursors, we identified the early V2a and V2b INs using their respective specific markers, Chx10 and Gata3. Virtually all the early V2a or V2b contained tdTomato (Figure 2c), suggesting that *Vsx1-CreER^{T2}* efficiently targets the V2 IN populations.

To evaluate the specificity of the model, we carried out immunolabelings for markers of other spinal neuronal population, that is, Evx1 (V0v), Foxd3 (V1), Nkx2.2 (V3), Olig2, and Isl1 (MNs) on *Vsx1|tdTomato* embryo sections at different developmental stages (e12.5 and e14.5). TdTomato was absent from Evx1^{+} V0 cells (Figure 2e) and from Nkx2.2^{+} V3 cells (Figure 2f). In contrast, it was detected in a very small minority of Foxd3^{+} V1 cells (Figure 2f; <1%), of Olig2^{+} MNs progenitors (Figure 2g; 1%) and of Isl1^{+} differentiating MNs (Figure 2h, Figure 3a; 1%), likely owing to the transient incompletely-defined boundaries between adjacent progenitor domains (Chen et al., 2011). Nevertheless, these observations indicate that the *Vsx1-CreER^{T2}* allele targets the V2 INs with a high specificity.

2.3 | Lineage-tracing of the V2 interneuron populations

To confirm the efficacy of the *Vsx1-CreER^{T2}* line and to assess whether all the V2 INs derive from *Vsx1*-expressing precursors, the presence of tdTomato was quantified at later developmental stages in V2a and in V2b cells, as well as in later V2c and V2d populations. At e12.5 (Figure 2i–l) and e14.5 (Figure 2m–o), tdTomato was present in the vast majority of these four V2 IN populations. At e14.5, it was detected in >95% of V2a, V2b, and V2c cells (Figure 2o), but only in 85% of V2d INs (Figure 2o). This is possibly due to later generation of the V2d cells (Dougherty et al., 2013) at a stage when *CreER^{T2}* expression or CreER^{T2} activity was already reduced. These data suggest that the *Vsx1-CreER^{T2}* allele efficiently targets all the V2 IN precursors and that all the V2 INs derive from Vsx1-positive precursors (Francius et al., 2016). Specificity of *Vsx1-CreER^{T2}* expression for the V2 lineage was confirmed by very limited (1%) activation outside of the V2 populations (Figure 2m and data not shown). Lack of tdTomato fluorescence in control embryos (tdTomato only, *Vsx1|tdTomato* without tamoxifen administration and *Vsx1|tdTomato* with tamoxifen administration at e13.5, that is, after the *Vsx1* expression period) demonstrated absence of leaky *Vsx1-CreER^{T2}* expression or uncontrolled CreER^{T2} activation (Figure 2n and data not shown). Taken together, these results demonstrate that the *Vsx1-CreER^{T2}* allele very efficiently targets V2 INs and enables labeling, tracing and recombination of a vast majority of cells in each V2 population.

In addition to cell bodies, the *Vsx1|tdTomato* line also enables fluorescent labeling of axonal projections. At e14.5, tdTomato-positive axonal projections or terminals were observed at high density in ventrolateral regions of the white matter (Figure 3a,b arrowheads;

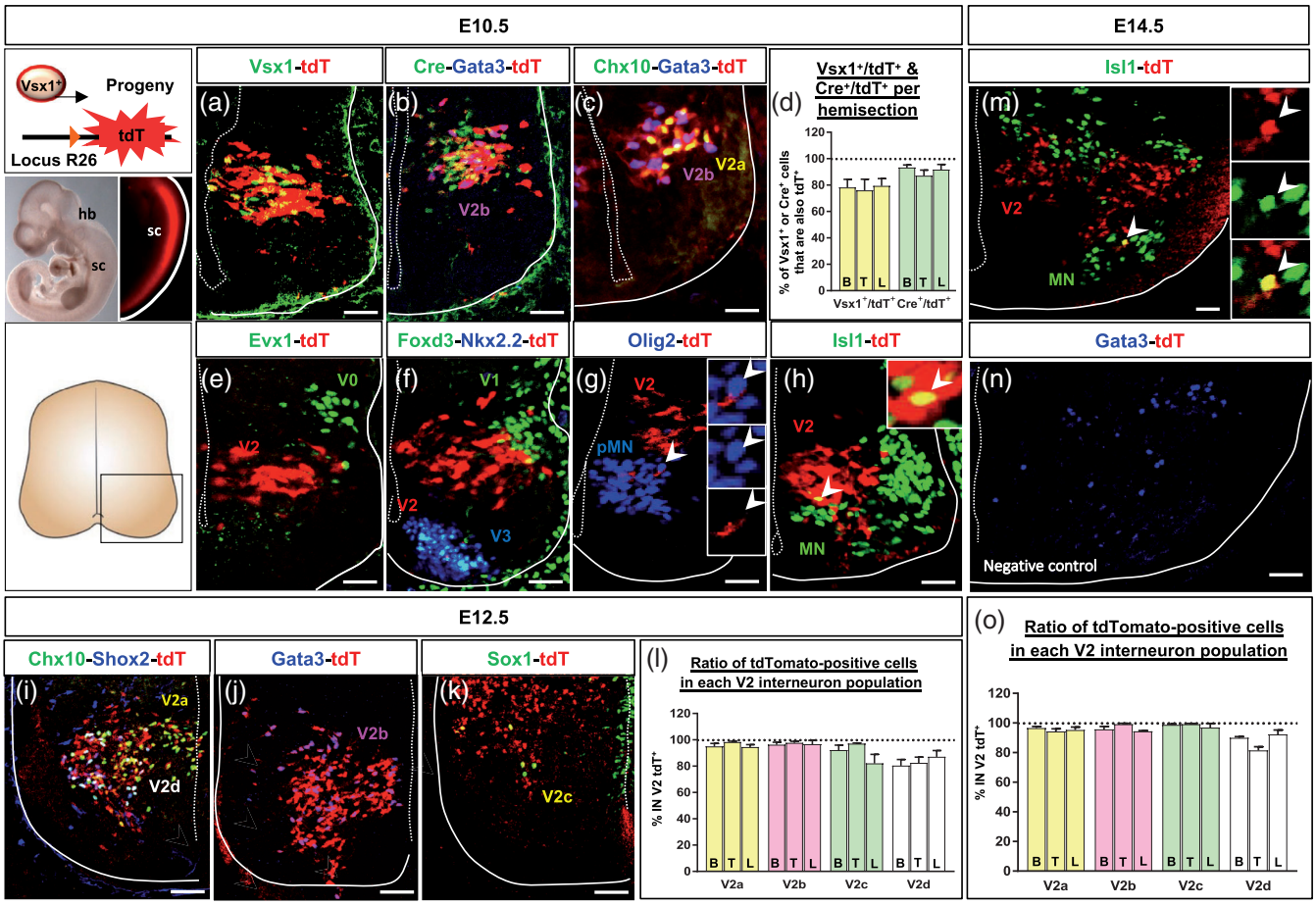

FIGURE 2 The *Vsx1-CreER*^{T2} line genetically targets the cardinal V2 interneuron population in the developing spinal cord. *Vsx1|tdTomato* embryonic spinal cord brachial sections were labeled by immunofluorescence using the indicated antibodies at e10.5 (a–h), e12.5 (i–l) or e14.5 (m–o). tdTomato colocalized with Vsx1 (yellow in a), Cre (yellow in b), Chx10 in V2a interneurons (yellow in c, i), Gata3 in V2b interneurons (magenta in b and c, j), Sox1 in V2c interneurons (yellow in k) and Shox2 in V2d interneurons (white in i). (d) Quantification of Vsx1- or Cre-positive cells containing tdTomato at each level of the spinal cord (brachial, thoracic, lumbar) (mean values ± SEM, $n = 3$). TdTomato was not detected with Evx1 in V0 neurons (e) or with Nkx2.2 in V3 neurons (f), but localized in few cells containing Foxd3 (V1 neurons, f), Olig2 (motor neuron progenitors, g) or Isl1 (differentiating motor neurons, h and m). (l, o) Quantification of V2a, V2b, V2c, and V2d interneurons containing tdTomato at e12.5 (l) or e14.5 (o) (mean values ± SEM, $n = 5$). (n) As a control, tdTomato was undetectable in e14.5 *Vsx1|tdTomato* embryos after tamoxifen injection at e13.5. Scale bars = 50 μm. B = brachial; T = thoracic; L = lumbar

Movie S1). We took advantage of this observation to confirm that, as previously suggested, V2 IN projections are ipsilateral (Al-Mosawie, Wilson, & Brownstone, 2007; Dougherty et al., 2013; Lundfald et al., 2007). Indeed, tdTomato-positive projections were completely absent from the ventral commissure (Figure 3a′,b′ arrow; Movie S1), suggesting that all the V2 INs projections including those of the uncharacterized V2c and V2-Pax6 cells remain ipsilateral in the developing spinal cord.

2.4 | Vsx1^{+} cells give rise to yet-uncharacterized V2 interneuron subsets during spinal cord neurogenesis

Previous publication suggested that all the V2 IN subsets are not characterized yet (Li et al., 2010). To confirm that additional V2 populations further exist, e12.5 or e14.5 spinal cord sections of *Vsx1|tdTomato* embryos were labeled with a cocktail of antibodies against markers of the known V2 populations, that is, Chx10, Gata3, Sox1, and Shox2 proteins, and each cell population was quantified (Figure 4a–d). At e12.5, ~10% of the tdTomato^{+} V2 INs were not labeled for known V2 markers (Figure 4a,b), corresponding to single or multiple additional V2 population(s) generated at earlier developmental stages. At e14.5, ~35% of the tdTomato^{+} cells were not labeled for another V2 marker (Figure 4c, d). These cells were mainly located ventrally at e12.5 (Figure 4a) but were more numerous dorsally at e14.5 (Figure 4c). They likely contain V2a INs that downregulated the expression of *Chx10* (Hayashi et al., 2018). However, at this stage, 50% of V2a cells retain *Chx10* expression at brachial level of the spinal cord, and 90% at lumbar level (Hayashi et al., 2018). Therefore, these tdTomato-only cells are too numerous to only consist in Chx10-negative V2a INs. Thus, V2 IN subsets that are different from the known V2 populations and that remain to be characterized exist, at least

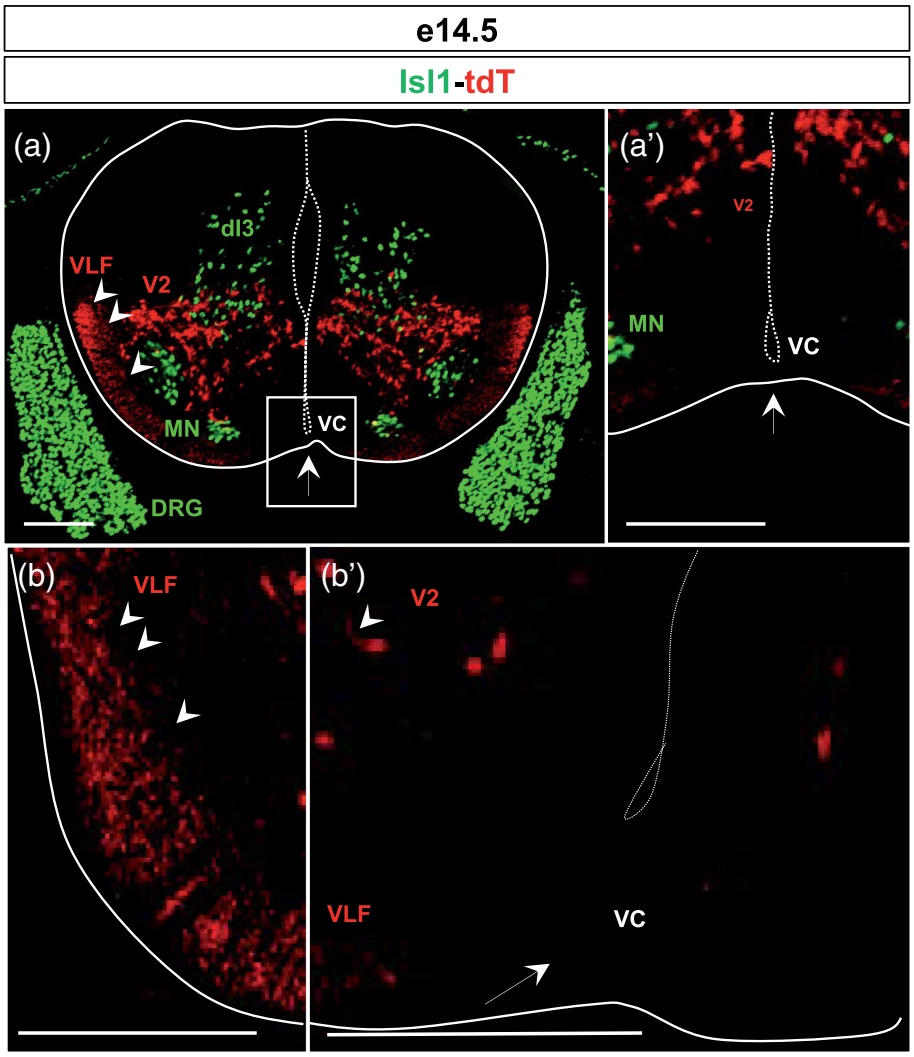

FIGURE 3 All the spinal V2 interneurons project their axon ipsilaterally. *Vsx1|tdTomato* embryonic spinal cord brachial sections at e14.5 were stained by immunofluorescence for Isl1 and imaged using regular (a, a′) or confocal (b, b′) microscopy. (a) tdTomato was detected in V2 interneurons but only in very few ventral Isl1$^+$ cells (motor neurons, MN, yellow), and not in dorsal (dI3 interneurons) or sensory (in the dorsal root ganglia, DRG) Isl1$^+$ neurons. (a, b) Furthermore, tdTomato localized in axons or axon terminals in the ventral gray matter (arrowhead in b′) and in ventrolateral regions of the white matter (arrowheads in b), but was completely absent from the ventral commissure (VC, arrow in a′ and b′), indicating that all the V2 interneurons project their axon ipsilaterally. Scale bars = 50 μm

at lumbar levels of the spinal cord. In the developing nervous system, Sox1 is detected in progenitor cells of the ventricular zone and in ventral V2 INs corresponding to V2c (Panayi et al., 2010). Surprisingly, at e14.5, Sox1 was additionally detected in td-Tomato$^+$ V2 INs located dorsally (arrowheads in Figure 4e,f), unlikely to correspond to the ventral V2c population (arrows in Figure 4e,f). These cells were more dorsal than a majority of V2 INs, and expanded from medial to lateral positions as observed for V2a and V2b populations (Figure 4e,f). They amounted to ~10% of td-Tomato$^+$ cells. Taken together, these observations confirm that V2 precursors generate multiple INs subsets that have not been characterized yet, one of which expresses *Sox1* at e14.5. These dorsal Sox1$^+$ V2 cells were called V2e INs (Figure 4). The *Vsx1|tdTomato* transgenic line described in the present study will contribute to further study these uncharacterized V2 populations.

3 | DISCUSSION

In this study, we described the generation of *Vsx1-CreER^{T2}* BAC transgenic mice that express tamoxifen-activated *Cre* recombinase in all V2 precursors during spinal cord development. These mice constitute a unique tool to induce efficient CreER^{T2}-dependent recombination restricted to the V2 INs following a dual IP injection of tamoxifen at e9.5. By crossing *Vsx1-CreER^{T2}* BAC transgenic males with females of the Ai14 conditional reporter line, and activating Cre with tamoxifen, we were able to track the fate of Vsx1$^+$ precursors-derived cells at different embryonic stages.

Given its unique and restricted expression domain in the murine spinal V2 precursors (Francius et al., 2016), we chose the *Vsx1* locus to drive *Cre* expression. Inducible Cre activation with tamoxifen in a well-defined time window was necessary because *Vsx1* expression was detected during gastrulation (Ohtoshi et al., 2001). Initial characterization of this transgenic line demonstrated Cre-mediated recombination at the *Rosa26* locus in approximately 50% of V2 INs 24 hr after a single injection of tamoxifen at e9.5 (data not shown). As Cre-dependent recombination is a stochastic event (Nagy, 2000), we used dual injection of tamoxifen (two injections at e9.5 with a 6 hr interval) to induce highly efficient recombination in more than 90% of the V2 cells. Previous studies from our laboratory showed that transient *Vsx1* expression lasts from e9.5 to e12.5 and is absent from fetal or adult spinal cord (Francius

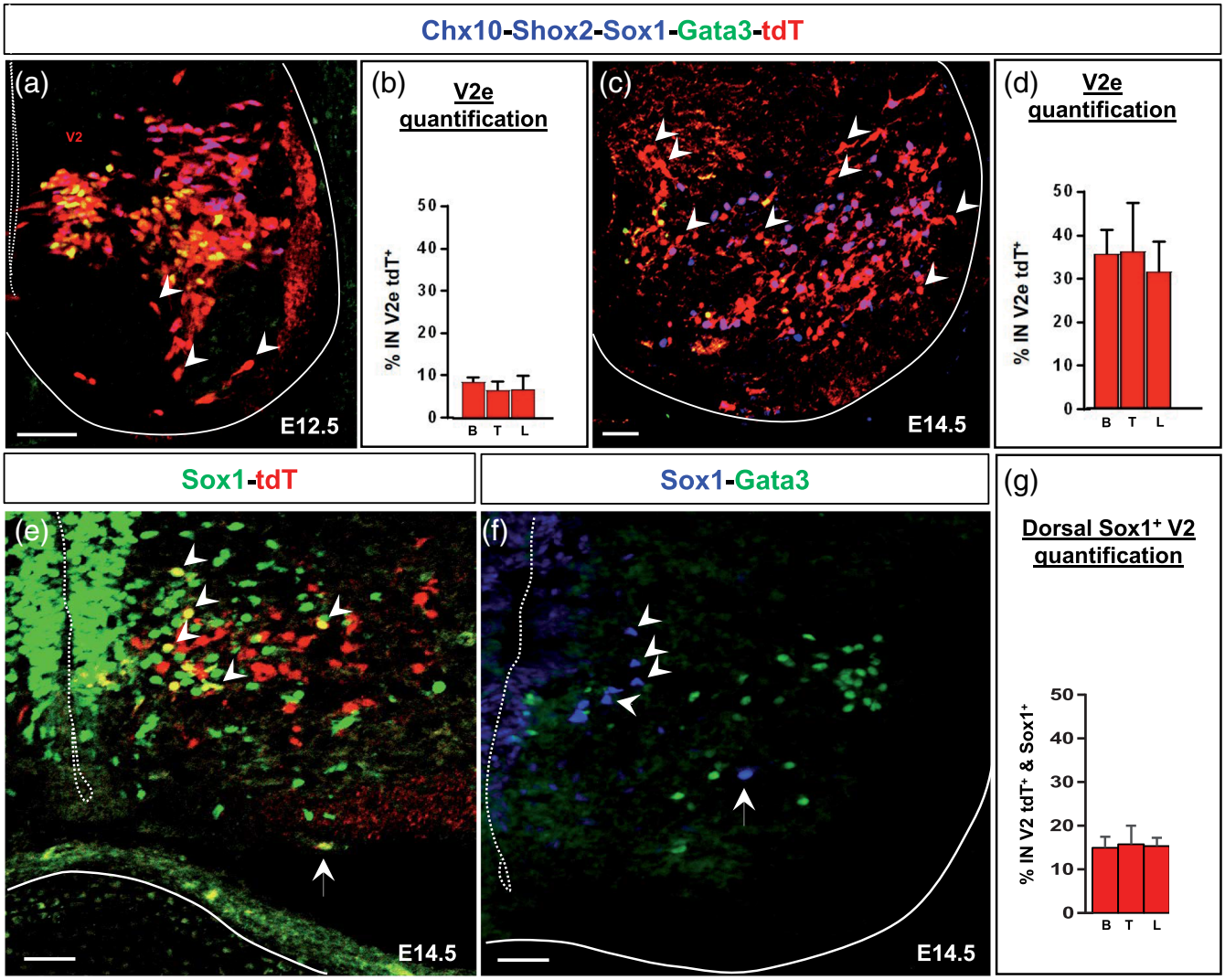

FIGURE 4 Vsx1^{+} cells give rise to uncharacterized subsets of V2 interneurons. *Vsx1|tdTomato* embryonic spinal cord brachial sections were stained by immunofluorescence using the indicated antibodies. At e12.5 (a, b) and e14.5 (c, d), tdTomato was detected in cells (red only, referred as V2e interneurons) that do not belong to known V2 interneuron populations (yellow and magenta). (b, d) Quantification of V2e interneurons at each level of the spinal cord (brachial, thoracic, lumbar) (mean values ± SEM, $n = 3$). (*e*–g) A dorsal subset of V2 interneurons, distinct from ventral V2c cells (arrows), contains Sox1 (yellow in e and blue in f) at e14.5. (g) Quantification of dorsal Sox1^{+} V2 interneurons at each level of the spinal cord (brachial, thoracic, lumbar) (mean values ± SEM, $n = 3$). Scale bars = 50 μm. B = brachial; T = thoracic; L = lumbar

et al., 2016; data not shown). Characterization of the *Vsx1-CreER*^{T2} transgenic line indicated that Cre-dependent recombination reflects the expected pattern of *Vsx1* expression over the course of neurodevelopment. Cre production was initiated at e9.5, as observed for Vsx1 (Francius et al., 2016), and td-Tomato activation followed rapidly, although with a short delay. A vast majority of V2 INs in each population was labeled by td-Tomato, demonstrating the efficacy of the model. Furthermore, Cre activity was highly specific and only labeled few cells other than V2 INs. Thus, this tool will enable to characterize all the V2 INs subsets in combination with global or single-cell RNA sequencing techniques, and to assess their phenotype, their activity and their contribution to the spinal motor circuits.

In addition, this line enabled to confirm previous hypotheses. Vsx1 was proposed to be transiently produced in the precursors that give rise to all the V2 populations (Francius et al., 2016). The observations presented here demonstrate that *Vsx1* is expressed before V2 IN diversification and that the intermediate compartment containing Vsx1^{+} cells gives rise to all the V2 subsets. Furthermore, they confirm that all the V2 INs project ipsilaterally in the spinal cord, suggesting that V2c INs contribute to regulate the activity of MNs located in their direct vicinity (Panayi et al., 2010). Importantly, they also verified the hypothesis that some V2 IN populations remain to be characterized (Li et al., 2010). We showed that at least 10% of td-Tomato^{+} cells could not be attributed to any known V2 population. Part of these is dorsal Sox1^{+} V2 cells that we called V2e INs, which are therefore distinct from the Sox1^{+} ventral V2c population. However, further characterization of these cells will necessitate identification of specific markers to distinguish them from V2c INs.

In conclusion, *the Vsx1-CreER^{T2}* line described here is a useful genetic tool for lineage tracing and for functional studies of the mouse spinal V2 INs. To our best knowledge, it is the only transgenic line that currently enables to target all the spinal V2 interneurons.

4 | MATERIALS AND METHODS

4.1 | Animals

All experiments were strictly performed in accordance with the European Community Council directive of November 24, 1986 (86-609/ECC) and the decree of October 20, 1987 (87-848/EEC). Mice were raised in our animal facilities and were treated according to the principles of laboratory animal care, and experiments and mouse housing were approved by the Animal Welfare Committee of Université catholique de Louvain (Permit Number: 2017/UCL/MD/008). *Vsx1-CreER*^{T2} mouse line was generated using a BAC recombination strategy, as previously described (Pelosi, Migliarini, Pacini, Pratelli, & Pasqualetti, 2014). Briefly, a *Vsx1*-containing BAC (Clone-ID: RP23-341J5) was obtained from the Children's Hospital Oakland Research Institute (CHORI). Two adapters located in exon 1 containing the translation start site (*Vsx1LA* and *Vsx1RA*; Figure 1a) were used for homologous recombination, to favor faithful regulation of *CreER*^{T2} expression by the endogenous *Vsx1* regulating sequences. The *Vsx1LA* sequence (420 bp) was amplified by overlapping PCR with the *Vsx1* sequence directly upstream of the ATG amplified using 5′-ATTATGTCGACATCTTACAGGTTAGTGGGGAGTG-3′ and 5′-GTGGTACGGTCAGTAAATTGGACATGCTTCTCAGTCCTCTAGGCTAG-3′ combined to the CreER^{T2} sequence amplified using 5′-CTAGCCTAGAGGACTGAGAAGCATGTCCAATTTACTGACCGTACAC-3′ and 5′-TAGAGTCGACCAGACATGAT-3′. The *Vsx1RA* sequence (430 bp) was amplified using 5′-ATTATCTCGAGGAAGCTTTGCCATCAATGACCTG-3′ and 5′ ATATACTCGAGAGCGAGCAGGTAACCCAGGTG-3′. The kanamycin-resistance cassette flanked by FRT sites was deleted by co-transformation with a Flp-expressing vector. Circular BAC DNA was injected into C57Bl/6 F1 zygotes at the Transgenesis Facility of the Université catholique de Louvain, and 9 *Vsx1-CreER*^{T2} founder lines were obtained. *Vsx1-CreER*^{T2} males were crossed with females of the *Ai14 Rosa26R-tdTomato* reporter line ((Madisen et al., 2010); a kind gift of M. Goulding). Pregnant females were injected intraperitoneally twice (6 hr interval) at e9.5 with 100 mg/kg of body weight tamoxifen (Sigma T5648) previously dissolved by sonication in corn oil. Founders and subsequent offspring were genotyped using the following primer set: *Cre*: 5′-CTGAGAGATGGTCACAAGATCC-3′ and 5′-AGGCATGGAGCATCTGTACAGC-3′; *Ai14*: *wild-type locus* 5′-AAGGGAGCTGCAGTGGAGTA-3′ and 5′-CCGAAAATCTGTGGGAAGTC-3′ or modified *Vsx1-CreER*^{T2} *locus* 5′-GGCATTAAA GCAGCGTATCC-3′ and 5′-CTGTTCCTGTACGGCATGG-3′. Useful lines were selected based on td-Tomato fluorescence and immunofluorescence for V2 IN markers (see Section 2). Pregnant females were sacrificed at the expected developmental stage, considering the day of the vaginal plug as the embryonic day (e) 0.5. A minimum of three embryos of the same genotype was analyzed for each experimental condition. The embryos were collected at embryonic day (e)10.5, e12.5, e14.5.

4.2 | Immunohistochemistry

Mouse embryos were dissected and fixed in PBS/4% PFA at 4°C for 15–30 min depending on the developmental stage. Fixed mouse embryos were washed in PBS before incubation in PBS/30% sucrose overnight at 4°C. They were embedded in PBS/7.5% gelatin/15% sucrose and frozen at −55°C. Embryos were cut at 14 μm in a CryoStar NX50 cryostat. Cryosections were saturated with PBS/0.1% Triton/10% horse serum for 30 min and incubated with the primary antibodies diluted in the same solution at 4°C overnight. For Vsx1 labeling, cryosections were permeabilized with PBS/1% Triton for 30 min at room temperature and saturated for 30 min with PBS/0.1% Triton/1% horse serum. Anti-Vsx1 antibody diluted in the same solution was incubated for 2 hr at room temperature. After three washes in PBS/0.1% Triton, the secondary antibodies, diluted in PBS/0.1% Triton/10% horse serum, were added for 30 min at room temperature. Slides were washed three times in PBS/0.1% Triton before a final wash in PBS/DAPI and mounted with Fluorescent mounting medium (DAKO).

The following primary antibodies and dilution were used: sheep anti-Chx10 at 1:500 (Exalpha Biologicals #X1179P), mouse anti-Cre (Abcam ab24607) at 1:500, mouse anti-Evx1 (DSHB 99.1-3A2) at 1:500, guinea pig anti-Foxd3 at 1:5000 (Muller et al., 2005) kindly given by T. Müller, rat anti-Gata3 (Absea #111214D02) at 1:20 (Panayi et al., 2010), goat anti-Isl1 at 1:1000 (Neuromics, GT15051), Olig2 (rabbit; 1:4000; Millipore #AB9610), mouse anti-Shox2 at 1:500 (Abcam #ab55740), goat anti-Sox1 at 1:500 (R&D #AF3369), rabbit anti-Vsx1 at 1:500 ([Clark et al., 2008]; kindly provided by E. Levine). Secondary antibodies were donkey anti-sheep/AlexaFluor 488, 594 or 647, donkey anti-mouse/AlexaFluor 488 or 647, donkey anti-guinea-pig/AlexaFluor 594, donkey anti-rat/AlexaFluor 647 and donkey anti-rabbit/AlexaFluor 594 purchased from Jackson Laboratories or Thermo Fisher used at 1:1000 or 1:2000, respectively. All secondary antibodies gave signals in the spinal cord only in the presence of corresponding primary antibodies.

Whole mount immunostainings were performed as previously described (Belle et al., 2017). All embryos were dehydrated in methanol, treated overnight at 4°C with a 30% hydrogen peroxide solution in 100% methanol, and rehydrated in methanol. Next, embryos were blocked for 24 h in PBS containing 0.2% gelatin (VWR2460.233), and 0.5% Triton X-100 (Sigma-Aldrich) (PBSGT) under agitation at room temperature. They were then transferred in PBSGT solution containing 0.1% saponin (Sigma-Aldrich S-7900; 10 mg/ml) and incubated at 37°C with rotation at 70 rpm, for 5 days. Samples were finally washed over 1 day at room temperature with agitation with multiple PBSGT baths. Samples were then cleared using the protocol detailed below.

4.3 | Tissue clearing

All tissue was cleared using adapted iDISCO clearing protocol as previously described (Renier et al., 2014). Briefly, all incubation steps were performed in dark conditions at RT in a fume hood, on a tube rotator (SB3, Stuart) at 14 rpm, using a 5 ml amber TPP Eppendorf tube (Eppendorf, VWR # 75874-634). Samples were first dehydrated in ascending concentrations (50, 80, 100, and 100%) of tetrahydrofuran (THF; anhydrous, containing 250 ppm butylated hydroxytoluene inhibitor, Sigma-Aldrich #186562) diluted in H_2O. The initial 50% THF bath was done overnight while the 80 and 100% THF incubations were left for 45 min for e12.5 embryos, respectively. Samples next underwent a delipidation step of either 15 min in dichloromethane (Sigma-Aldrich #270997) followed by a 30 min clearing step in dibenzyl ether (Sigma-Aldrich #108014). The next day, samples were stored in individual light protected glass vials (Rotilabo, Roth #XC40.1 and #XC44.1) at RT. In these conditions, samples could be stored and imaged for up to 9 months without any significant fluorescence loss.

4.4 | Imaging and quantitative analyses

Immunofluorescence images of cryosections were acquired on an EVOS FL Auto Imaging System (ThermoFisher Scientific) or on a Confocal laser Scanning biological microscope FV1000 Fluoview with the FV10-ASW 01.02 software (Olympus). The images were treated with Adobe Photoshop CS6 software to match brightness and contrast with the observations. For quantifications, cells were counted on five sections of spinal cord for each level (brachial, thoracic and lumbar) in at least three independent embryos ($n \geq 3$) using the count analysis tool of Adobe Photoshop CS6 software.

3D imaging (Supporting Information) was performed with an ultramicroscope I (LaVision BioTec) using ImspectorPro software (LaVision BioTec). The lightsheet was generated by a laser (wavelength 640 nm, Coherent OBIS 640-100LX laser, LaVision BioTec). A binocular stereomicroscope (MXV10, Olympus) with a 2X objective (MVPLAPO, Olympus) was used at different magnifications (0.8x, 1x, 1.25x, 1.6x, 2x, 2.5x, 3.2x, 4x, 5x, and 6.3x). Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec) filled with DBE and illuminated from the side by the laser light. Images were acquired with a PCO Edge SCMOS CCD camera (LaVision BioTec). The stepsize between each image was fixed to 3 μm. Image processing was performed as previously described (Belle et al., 2017) using Imaris x64 software version 9.2.1, Bitplane.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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