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ORIGINAL ARTICLE

Vsx1 and Chx10 paralogs sequentially secure V2 interneuron identity during spinal cord development

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Abstract

Paralog factors are usually described as consolidating biological systems by displaying redundant functionality in the same cells. Here, we report that paralogs can also cooperate in distinct cell populations at successive stages of differentiation. In mouse embryonic spinal cord, motor neurons and V2 interneurons differentiate from adjacent progenitor domains that share identical developmental determinants. Therefore, additional strategies secure respective cell fate. In particular, Hb9 promotes motor neuron identity while inhibiting V2 differentiation, whereas Chx10 stimulates V2a differentiation while repressing motor neuron fate. However, Chx10 is not present at the onset of V2 differentiation and in other V2 populations. In the present study, we show that Vsx1, the single paralog of Chx10, which is produced earlier than Chx10 in V2 precursors, can inhibit motor neuron differentiation and promote V2 interneuron production. However, the single absence of Vsx1 does not impact on V2 fate consolidation, suggesting that lack of Vsx1 may be compensated by other factors. Nevertheless, Vsx1 cooperates with Chx10 to prevent motor neuron differentiation in early V2 precursors although these two paralog factors are not produced in the same cells. Hence, this study uncovers an original situation, namely labor division, wherein paralog genes cooperate at successive steps of neuronal development.

Keywords Paralog genes · Vsx1 · Chx10 · V2 interneurons · Motor neurons · Spinal cord

Introduction

Robustness of a biological system is defined as the ability to maintain its functions despite perturbations. The mechanistic bases of robustness are not fully understood but seem to notably rely on the fact that several genes or groups of genes have partially overlapping functions, ensuring compensation when perturbations occur [1]. A major source of compensation is gene duplication, which results in the birth of paralog genes, and logically supposes at least partial overlapping expression of the two paralogs in the same cells. However, newly generated paralogs can evolve towards loss of function (nonfunctionalization), acquisition of novel functions (neofunctionalization) or retention of varying degrees of overlapping function (subfunctionalization) [1, 2]. Here, we uncover an original situation of labor division wherein two paralogs exert seemingly identical functions in a single cell lineage at successive steps of differentiation.

In the developing spinal cord, different neuronal populations are generated from distinct progenitor domains orderly distributed along the dorsoventral axis of the ventricular zone

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[3, 4]. Adjacent progenitor domains often share identical developmental determinants. Therefore, additional strategies have been developed to segregate and consolidate respective cell fate in neighboring populations, as observed for motor neurons (MNs) and V2 interneurons (INs) (Fig. 1a). In the adjacent progenitor domains of MNs (pMN) and of V2 INs (p2), the LIM-homeodomain transcription factor Lhx3 is upregulated shortly before the onset of neuronal differentiation. In differentiating MNs, Lhx3 associates with its LIM-homeodomain partner Isl1 and the nuclear LIM interactor (NLI, also called LDB-1 or CLIM2) to form a MN-hexameric complex. This complex binds to hexamer-response elements (HxREs), stimulates expression of a large array of MN genes including *Hb9* and promotes MN differentiation [5-11]. Additionally, it inhibits multiple IN determinants [5]. In contrast, in differentiating V2 INs, Lhx3 associates only with NLI to form a V2-tetrameric complex that binds tetramer-response elements (TeREs) [6, 11]. This complex stimulates V2 genes including *Chx10*, which promotes the differentiation of V2a INs, one of the 2 main V2 populations [6, 11, 12]. However, additional mechanisms are necessary to consolidate MN and V2 fates. In particular, MNs must be protected against aberrant activation of the V2 differentiation program by the Lhx3-NLI tetramer complex, which can also form in MNs, and by the MN hexamer, which can bind and activate the TeREs [6]. Hb9, which is specifically produced in MNs upon stimulation by the MN-hexameric complex [8, 9], silences TeREs by replacing the V2-tetrameric complex and by actively suppresses its activation, thereby preventing aberrant activation of the V2 program [6]. Consistently, the absence of Hb9 results in ectopic activation of Chx10 in early MNs and production of a hybrid lineage coexpressing MN and V2a markers [12-14]. Symmetrically, Chx10 secures cell fate in V2a INs by binding to and preventing the activation of HxREs, thereby inhibiting ectopic activation of the MN differentiation program [6] and enabling the expression of IN determinants [5]. Accordingly, absence of Chx10 in the $Chx10^{orJ/orJ}$ single mutant results in a reduction in the V2a IN population [12].

However, *Chx10* expression is activated exclusively in the V2a population after segregation of the V2a and V2b lineages [15] (Fig. 1a). This raises the question of the consolidation of V2 identity before *Chx10* activation and in the other V2 populations. Recently, we showed that *Vsx1*, the single paralog of Chx10 in the mammalian genomes, is expressed in V2 precursors before the segregation of the V2a/V2b lineages and the onset of *Chx10* expression (Fig. 1a) [16]. Vsx1 is a transcriptional repressor of the Paired-like CVC (Prd-L:CVC) homeobox gene family [17, 18]. In the mouse, it is expressed in gastrula stage embryos [18] and in several types of bipolar cone INs of the retina wherein it regulates different aspects of terminal differentiation [19-22]. In the spinal cord, it is transiently detected after cell cycle exit of p2 progenitors but before the onset of neuronal differentiation. The role of Vsx1 in the developing spinal cord remains elusive but this factor is not required for V2 IN production or for the segregation of the V2a and V2b lineages [16].

As Vsx1 is paralog to Chx10 and is expressed prior to Chx10 in the V2 lineage, we hypothesized that it may anticipate Chx10 action in V2 INs to secure V2 identity. Here, we demonstrate that Vsx1 can bind HxREs and inhibit their activation by the MN Isl1-NLI-Lhx3 hexamer. Consistently, Vsx1 is sufficient to inhibit MN differentiation and to promote V2 IN production. However, the absence of Vsx1 does not impact on V2 fate consolidation, suggesting that lack of Vsx1 may be compensated by other factors. Nevertheless, combined inactivation of *Vsx1* and *Chx10* induces MN/V2 differentiation imbalance that was not observed in single mutants, confirming that Vsx1 and Chx10 paralogs sequentially secure V2 IN identity during spinal cord development. Hence, this study uncovers an original situation, namely labor division, exerted by paralog genes at successive steps of neuronal differentiation.

Materials and methods

Ethics statement and mouse lines

All experiments were performed in accordance with the European Community Council directive of 24 November 1986 (86–609/ECC) and the decree of 20 October 1987 (87–848/EEC). Mice were treated according to the principles of laboratory animal care, and mouse housing and experiments were approved by the Animal Welfare Committee of the Université catholique de Louvain (Permit Number: 2013/UCL/MD/11 and 2017/UCL/MD/008). The day of the vaginal plug was considered to be embryonic day (e) 0.5. The embryos were collected at e10.5 and e12.5, a minimum of 3 embryos of each genotype were used in each experiment. *Olig2-Cre, Nestin-Cre*, $Vsx1^{-}$, $Chx10^{orJ}$ and $Hb9^{-}$ mutant lines have been previously described [14, 16, 23-25]. The *Rosa26-Vsx1* line was generated using a gateway- and a recombinase-mediated cassette exchange-based system targeting the G4 ROSALUC embryonic stem cells, as previously described (Fig. S1A) [26]. The *Vsx1* coding sequence, flanked by *Att* recombination sites, was amplified from embryonic cDNA at e10.5 by PCR with GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAACCATGACTGGACGGGATGGGCTTTCG and GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATGAGGCTCCCACCTGTGG primers (5′–3′). Primer sequences are available on request.

Chromatin immunoprecipitation (ChIP) and quantitative (q)-PCR assays

ChIP experiments were performed using Human Embryonic Kidney 293 (HEK293) cells. Cells were cultured in

DMEM high glucose media (Thermo Fisher Scientific #11,965,092) supplemented with fetal bovine serum 10% (Thermo Fisher Scientific #10,500,064) and Penicillin–Streptomycin (Thermo Fisher Scientific #15,070,063). For ChIP assays, cells were seeded in 6-wells plates and transfected with pEF::Vsx1-HSV (625 ng/well) or pEF::GFP plasmid (625 ng/well), and pCAGGS::DsRed2 (625 ng/well) using Lipofectamine 2000 (Thermo Fisher Scientific #11,668,027). Cells were collected 16 to 24 h after transfection and ChIP was performed using the EZ-ChIP™ kit (Millipore #17–371) according to manufacturer's instructions. Chromatin was fragmented to 200–600 bp by sonication (high power, 30 cycles of 30 s with 1 min between pulses) and incubated with anti-HSV antibody (goat; 1:500; Novus Biolabs #NB600-513) or species-matched IgG overnight at 4 °C. *HxRE* sequence enrichment was assessed by quantitative PCR assays with GCAACACTTCCAGGCTCAGCCAG and CTGTTCTTGCAGACTAGCAGG primers (5′–3′). Fold enrichment was calculated over IgG using $2^{(-\Delta\Delta CT)}$, where $\Delta\Delta CT = (Ct_{ip} - Ct_{Input}) - (Ct_{IgG} - Ct_{Input})$.

Luciferase assays

Luciferase assays were performed using the Dual-luciferase Reporter Assay System (Promega #E1910) in HEK293 cells. Cells were seeded in 24-wells plates and transfected with HxRE::Luciferase (125 ng/well) [6], renilla luciferase control vector (5 ng/well) used for reporter activity normalization, Isl1-Lhx3 (30 ng/well), and pCS2::Vsx1 or pCS2::Vsx1R166W (60 ng/well) or an empty vector (60–220 ng/well). After 24 h of treatment, cells were collected and prepared according to manufacturer's instructions. Luciferase reporter activities were measured with a tube luminometer (Titertek Berthold).

In ovo electroporation

In *ovo* electroporations were performed at stage HH12 and embryos were collected 48 h after electroporation. HxRE::GFP (1 µg/µl) [6], Isl1-Lhx3 (0.25–0.5 µg/µl) [6], pCS2::Vsx1 and pCS2::Vsx1R166W (1.5 µg/µl) [27], pCAGGS::Nkx6.1 (0.25–0.5 µg/µl; kindly provided by J. Briscoe) [28], pMxig-Pax6 (0.25–0.5 µg/µl; kindly provided by M. Gotz) [29], pEF::Vsx1-HSV (0.25 µg/µl) and empty pCMV (0.25–1.5 µg/µl, kindly provided by C. Pierreux) were co-electroporated with the pCAGGS::DsRed2 plasmid (0.25 µg/µl; gift of Y. Takahashi) [30] to visualize electroporated cells. Collected embryos were fixed in ice-cold PBS/4% PFA for 25 min and processed as above. Labeled cells were counted on both sides of 5–10 sections per embryo. For *Vsx1*, *Nkx6.1* and *Pax6* overexpression experiments, the ratio between electroporated and non-electroporated sides of each embryo was calculated to normalize for developmental stage and experimental variations.

Immunofluorescence labelings

Collected embryos were fixed in ice-cold phosphate-buffered saline (PBS)/4% paraformaldehyde (PFA) for 15–25 min, according to the developmental stage. After washes in PBS, fixed embryos were incubated in PBS/30% sucrose overnight at 4 °C, embedded and frozen in PBS/7.5% gelatin/15% sucrose. Immunostainings were performed on 14 µm serial sections as previously described [16].

Primary antibodies against the following proteins were used: activated Caspase-3 (rabbit; 1:100; Cell Signaling #ASP175), Ascl1 (mouse; 1:200; BD #556,604), Chx10 (sheep; 1:500; Exalpha Biologicals #X1179P), Foxp1 (goat; 1:1000; R&D Systems #AF4534; or mouse; 1:250; Origene #UM800020), Gata3 (rat; 1:50; Absea Biotechnology #111214D02), GFP (chick; 1:1000; Aves Lab #GFP-1020), chicken Hb9 (rabbit; 1:1000; kindly provided by S. Morton), Isl1/2 (goat; 1:3000; Neuromics #GT15051; or mouse; 1:6000; DSHB #39.4D5), Lhx3 (rabbit; 1:2000; DSHB #G7.4E12), MNR2 (mouse; 1:2000; DSHB #81.5C10), Nkx2.2 (mouse; 1:1000; DSHB #74.5A5), Nkx6.1 (mouse; 1:2000; DSHB #F55A10), Olig2 (rabbit; 1:4000; Millipore #AB9610), Shox2 (mouse; 1:500; Abcam #ab55740), Sip1 (rabbit; 1:500) [31], Sox1 (goat; 1:500; Santa Cruz #sc-17318), Sox14 (guinea-pig; 1:1000) [12], Vsx1 (rabbit, 1:500; kindly provided by E. Levine) [32].

Following secondary antibodies were used: donkey anti-goat/alexaFluor 488, donkey anti-guinea-pig/AlexaFluor 594, 488 or 647, donkey anti-mouse/AlexaFluor 488, 594 or 647, goat anti-mouse, IgG1/ AlexaFluor 594, goat anti-mouse, IgG2a/AlexaFluor 488, goat anti-mouse IgG3/AlexaFluor 488, donkey anti-rabbit/AlexaFluor 488, 594 or 647, donkey anti-rat/AlexaFluor 647, donkey anti-sheep/alexaFluor 594 or 647 purchased from ThermoFisher Scientific or Jackson Laboratories and used at dilution 1:1000.

Imaging, quantitative and statistical analyses

Acquisition of immunofluorescence images was performed on Evos FL, Evos FL Auto cell imaging system or Olympus FluoView FV1000 confocal microscope. Adobe Photoshop CS3 program was used for cell quantifications and image modifications. Brightness and contrast were adjusted uniformly in all replicate panels within an experiment to match with observation. Labeled cells were counted on both sides of 3–5 sections at brachial or thoracic levels at e10.5 and at brachial, thoracic or lumbar levels at e12.5. The number of MN in each motor column was quantified according to the presence of specific marker combinations: Isl1 + Lhx3

(medial motor column, MMC) all along the spinal cord, Foxp1 + Isl1 (medial portion of the lateral motor column, LMCm) or Foxp1 alone (lateral portion of the lateral motor column, LMCl) at brachial or lumbar levels, Isl1 alone (hypaxial motor column, HMC) or Foxp1 alone (preganglionic column, PGC) at thoracic levels. Raw data were exported from Adobe Photoshop CS3 software to SigmaPlot v11.0 software and processed for statistical analyses. *T* test or Wilcoxon-Mann–Whitney test were used for statistical comparison of mouse section quantifications, luciferase assay data and qPCR data. Paired-test or Wilcoxon-signed-rank-test were used for chicken section quantifications.

Results

Vsx1 inhibits HxRE activation and stimulation of MN differentiation by the Isl1-NLI-Lhx3 complex

In early-born MNs, the Isl1-NLI-Lhx3 hexameric complex binds HxREs, stimulates Hb9 production and promotes MN differentiation. In V2a INs, Chx10 secures V2a identity by preventing activation of HxREs and the MN differentiation program [6] and by stimulating V2a gene expression (Fig. 1a) [12]. However, early V2 precursors lack the expression of *Chx10*. Recently, we showed that *Vsx1*, the

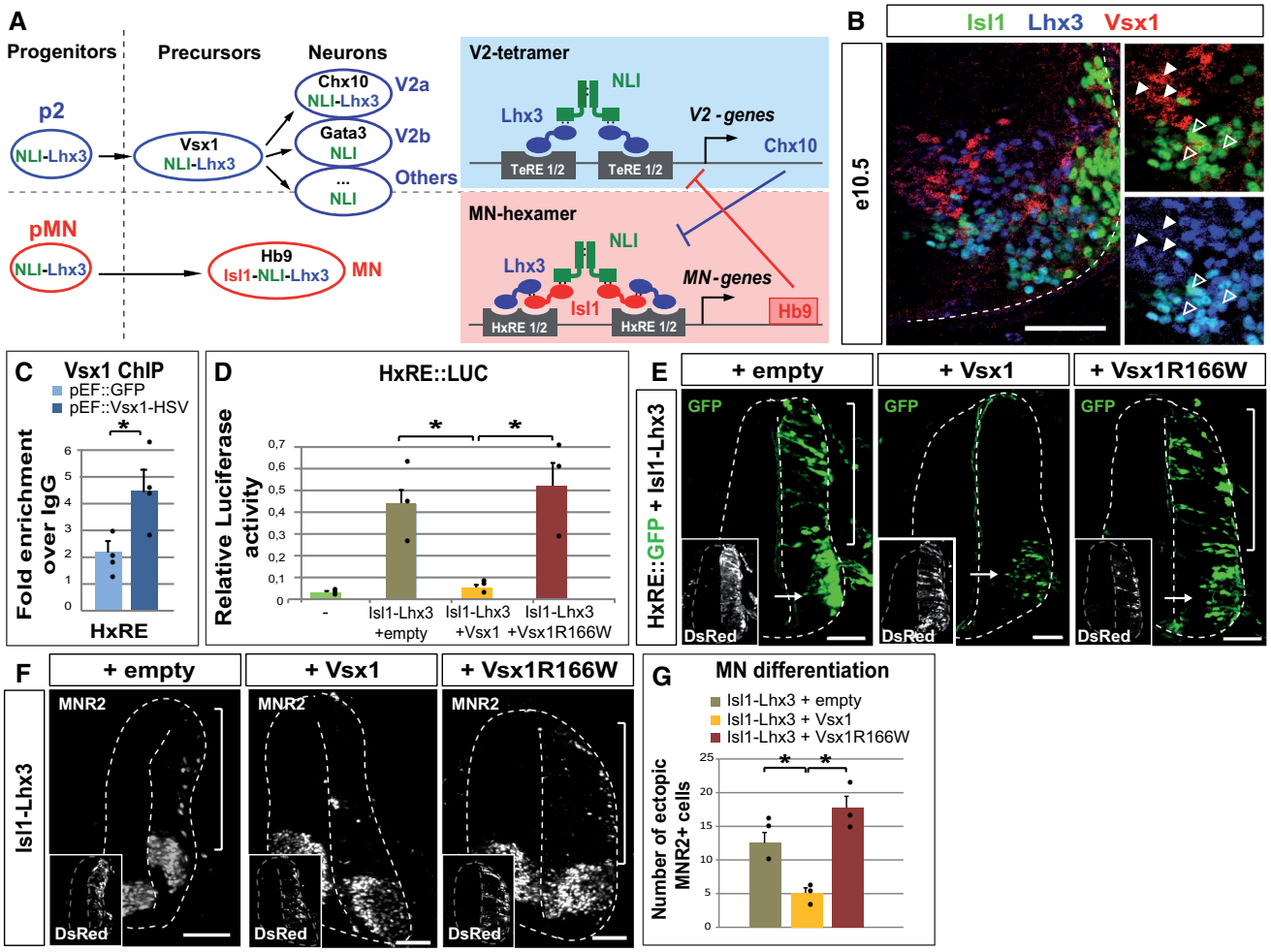

Fig. 1 Vsx1 binds the *Hb9* HxRE and prevents its activation and stimulation of MN differentiation by Isl1-Lhx3 containing complexes. **a** Schematic representation of V2 IN and MN specification during spinal cord development. **b** By immunofluorescence on transverse sections of wild type spinal cord, Vsx1 is co-detected with Lhx3 in V2 precursors (plain arrowheads) but is not present in Isl1 + MNs (open arrowheads). **c** Chromatin immunoprecipitation (ChIP) experiment demonstrates that Vsx1 can bind the *Hb9* HxRE ($n = 3$). **d** In HEK293 cells, activation of the *Hb9* HxRE by the Isl1-Lhx3 fusion protein is suppressed by wildtype Vsx1 but not by the DNA binding-deficient Vsx1R166W mutant ($n = 3$). **e** Following chicken embryonic spinal cord electroporation, activation of the HxRE by the Isl1-Lhx3 fusion protein (brackets) as well as endogenous activation in MNs (arrow) is inhibited by wildtype Vsx1 but not by the Vsx1R166W mutant (co-electroporated DsRed is shown as an electroporation control). **f–g** In electroporated chicken embryonic spinal cord, ectopic motor neuron differentiation induced by the Isl1-Lhx3 fusion protein (brackets) is reduced by Vsx1 but not by its Vsx1R166W variant ($n = 3$). Mean values ± SEM; $^*p < 0.05$. Scale bars = 50 µm

single paralog of *Chx10* in the mammalian genomes, is transiently expressed in V2 precursors during spinal cord development (Fig. 1b) [16]. Therefore, we reasoned that Vsx1 may anticipate V2a-restricted Chx10 action and consolidate V2 fate in V2 precursors. To test this hypothesis, we first investigated whether Vsx1 can bind the *Hb9* HxRE and prevent its activation. Chromatin immunoprecipitation assays in HEK293 cells transfected with a pEF::Vsx1-HSV expression vector showed that Vsx1 is able to bind the HxRE (Fig. 1c). In MNs, the *Hb9* HxRE is activated by the MN-specific hexamer complex Isl1-NLI-Lhx3 (Fig. 1a), which promotes *Hb9* expression and MN differentiation [6]. Using a HxRE::LUC reporter in HEK293 cells, we showed that Vsx1 is able to inhibit the activation of the HxRE by the Isl1-NLI-Lhx3 complex (Fig. 1d). This inhibition required binding of Vsx1 to DNA as a binding-defective version of this protein, Vsx1R166 [27], failed to downregulate HxRE activity (Fig. 1d). Thus, Vsx1 can bind the *Hb9* HxRE and prevent its activation by the MN Isl1-NLI-Lhx3 complex.

To assess whether a similar regulation can take place in spinal neuronal populations, we studied the activation of an HxRE::GFP reporter construct after chicken embryonic spinal cord electroporation. Consistently, Vsx1 was able to inhibit ectopic HxRE activation by the Isl1-NLI-Lhx3 complex and endogenous HxRE activity in MNs (Fig. 1e). In contrast, the presence of the mutated Vsx1R166W did not affect HxRE activity (brackets and arrows in Fig. 1e). To evaluate the impact of HxRE regulation on MN production, we studied the distribution of MNR2, an early marker of chicken MNs, in similar experimental conditions. Wild type Vsx1, but not the Vsx1R166W mutant, inhibited the ectopic production of MNR2-positive cells induced by the Isl1-Lhx3 fusion protein (brackets in Fig. 1f–G). Taken together, these results demonstrate that Vsx1 is able as its paralog Chx10 to bind the *Hb9* HxRE, to inhibit its activation and to prevent the stimulation of MN differentiation by Isl1-Lhx3-containing complexes.

Vsx1 inhibits MN differentiation and promotes V2 IN identity

To assess the impact of Vsx1 on MN and V2 IN differentiation in vivo, we first crossed a conditional *Rosa26R::Vsx1-IRES-EGFP* line (Fig. S1A) [26] with *Olig2::Cre* mice [23] to ectopically express *Vsx1* in the spinal MNs (Fig. S1). Immunofluorescence for Vsx1 and for EGFP evidenced ectopic expression in MNs but also in more ventral populations of the spinal cord including V3 INs (Fig. S1B-C"), likely owing to the transient expression of *Olig2* in the p3 progenitor domain [33]. Combined immunofluorescence analyses at e12.5 for the MN markers Isl1, Lhx3, Foxp1 and Sip1 demonstrated that ectopic Vsx1 significantly inhibits the differentiation of spinal MNs (Figs. 2a–c; S1). To confirm that this effect was specific to MNs and did not result from altered neurogenesis or general impairment of neuronal differentiation, we quantified the number of V3 INs also subjected to ectopic Vsx1 production (Fig. S1 B-C"). Nkx2.2 immunofluorescence labeling unveiled no change in V3 numbers between control and mutant embryos (Fig. S1Q-S; $n=3$, $p=0.26$), supporting the interpretation that the reduction in MNs upon ectopic Vsx1 production resulted from specific alteration of MN differentiation. Altered MN differentiation in the presence of Vsx1 may be counterbalanced by increased differentiation of V2 INs or production of MN/V2 hybrid cells [6, 12-14]. To assess this possibility and to evaluate whether Vsx1 is sufficient to stimulate V2 differentiation in MN precursors, we analyzed the distribution of Chx10 and Gata3, specific markers of V2a and V2b INs, respectively, in *Olig2::Cre* x *Rosa26R::Vsx1-IRES-EGFP* mutant embryos. However, no change was observed in the number of V2a or V2b INs (Fig. 2d–f; $n=3$, $p=0.65$ or 0.69 for V2a or V2b INs, respectively). Moreover, hybrid cells containing MN and V2 IN markers were not detected (Fig. S1H-I). These observations suggest that Vsx1 is not sufficient to stimulate V2 differentiation or the expression of V2 markers in a MN context.

In contrast, Vsx1 may promote V2 differentiation in a more endogenous context. To address this hypothesis, we increased Vsx1 production in all the spinal neurons by crossing the *Rosa26R::Vsx1-IRES-EGFP* line with a *Nestin::Cre* line (Fig. S2) [24]. Consistent with our previous observations, we detected a decrease in the number of MNs at e12.5, although to a lesser extent than with the *Olig2-Cre* driver (Fig. 2g–i). In contrast, quantifications of Chx10 + and Gata3 + cells unveiled a significant increase in V2a and V2b INs (Fig. 2j–l), supporting the hypothesis that upregulated *Vsx1* expression in IN populations stimulates V2 differentiation. Lack of change in progenitor or other IN populations and in cell death (Fig. S2) suggested that additional V2 cells are generated from the p2 domain. Taken together, these data demonstrate that Vsx1 is able to prevent MN differentiation and to promote V2 IN fate during spinal cord development.

Vsx1 is not necessary for proper V2 IN differentiation

To assess whether Vsx1 is necessary for V2 fate consolidation, we studied in detail V2 production in the absence of Vsx1 (Figs. 3a, b; S3) at e10.5 and e12.5. In control embryos, Vsx1 is present in an intermediate V2 precursor compartment [16] wherein it partly overlaps with Sox14, which also labels V2a INs (Fig. 3a) [12]. Therefore, we first studied Sox14 distribution in the absence of Vsx1. However, the number of Sox14 + cells was not significantly altered in mutant embryos (Fig. 3a, b, e, k–l, o). Second, we evaluated whether the lack of Vsx1 impacts on the dual production of V2a and V2b INs from common

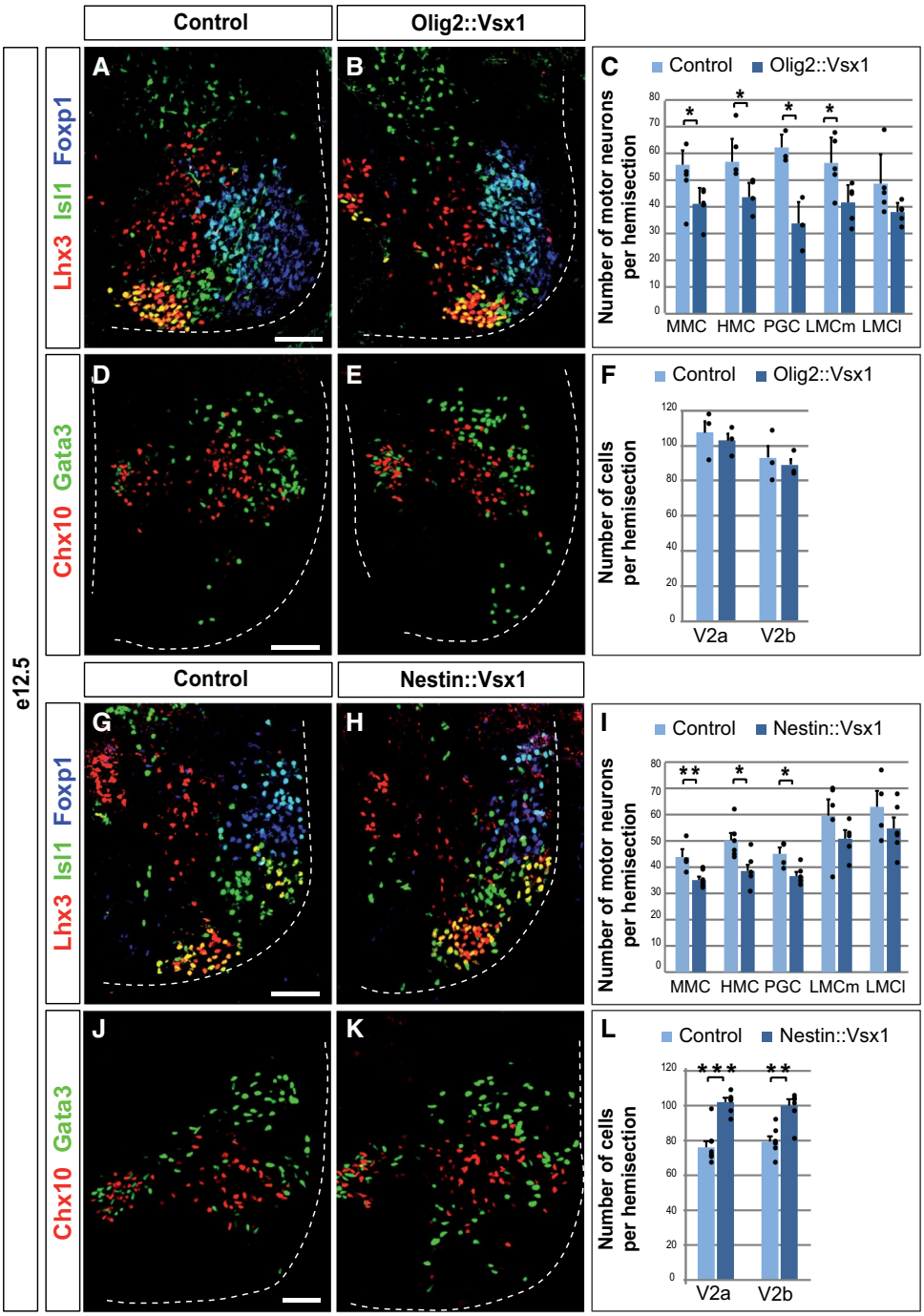

Fig. 2 Vsx1 can inhibit MN differentiation and stimulate V2 IN production. Immunofluorescence for MN (Isl1, Lhx3, Foxp1) or V2 IN (Chx10 for V2a and Gata3 for V2b) markers on transverse spinal cord sections of Vsx1 gain-of-function embryos. **a–c** In *Olig2::Cre* x *Rosa26R::Vsx1* embryos at e12.5, ectopic production of Vsx1 in MNs inhibits MN generation in each motor column ($n=3$). **d–f** Inhibition of MN production is not compensated by increased V2 IN generation ($n=3$). (**g–i**) In *Nestin::Cre* x *Rosa26R::Vsx1* embryos at e12.5, ectopic production of Vsx1 in MN also inhibits MN generation ($n=4$). **j–l** In the same embryos, increased expression of Vsx1 in V2 precursors promotes V2 IN generation ($n=4$). Mean values ± SEM; ***$p<0.001$, **$p<0.01$ and *$p<0.05$. Scale bars = 50 µm. *MMC* medial motor column; *HMC* hypaxial motor column; *PGC* pre-ganglionic motor column; *LMCm* medial portion of the lateral motor column, *LMCl* lateral portion of the lateral motor column

precursors [34, 35]. However, the number of V2a and V2b cells as well as the ratio between these two V2 subtypes were preserved (Fig. 3c–e, i–j, m–o). Consistently, production of other V2 subsets including V2c and V2d was unaffected (Fig. 3i–j, m–o). Third, to exclude any impact of Vsx1 on the p2 progenitor domain that could mask an influence on V2 differentiation, we labeled the p2 domain at e10.5 using a triple immunolabeling of Nkx6.1, Sox1 and Olig2. However, the number of p2 progenitors (Sox1 + Nkx6.1 + Olig2- cells) was similar between control and mutant embryos (Fig. S3; $n=3$, $p=0.46$). Moreover, no change was observed in the distribution of Ascl1, a key determinant of V2 differentiation [35, 36] (Fig. S3; $n=4$, $p=0.55$). Thus, Vsx1 is not required for p2 domain integrity or for proper production of the multiple V2 IN subsets. Consistently, the absence of Vsx1 had no effect on the production of MNs (Fig. 3f–h, p–r; $n=3$, $p=0.93$ or 0.77 for Isl1 + or Hb9 + cells, respectively). Taken together, these observations suggest that Vsx1 is not necessary for early differentiation of V2 INs or to prevent

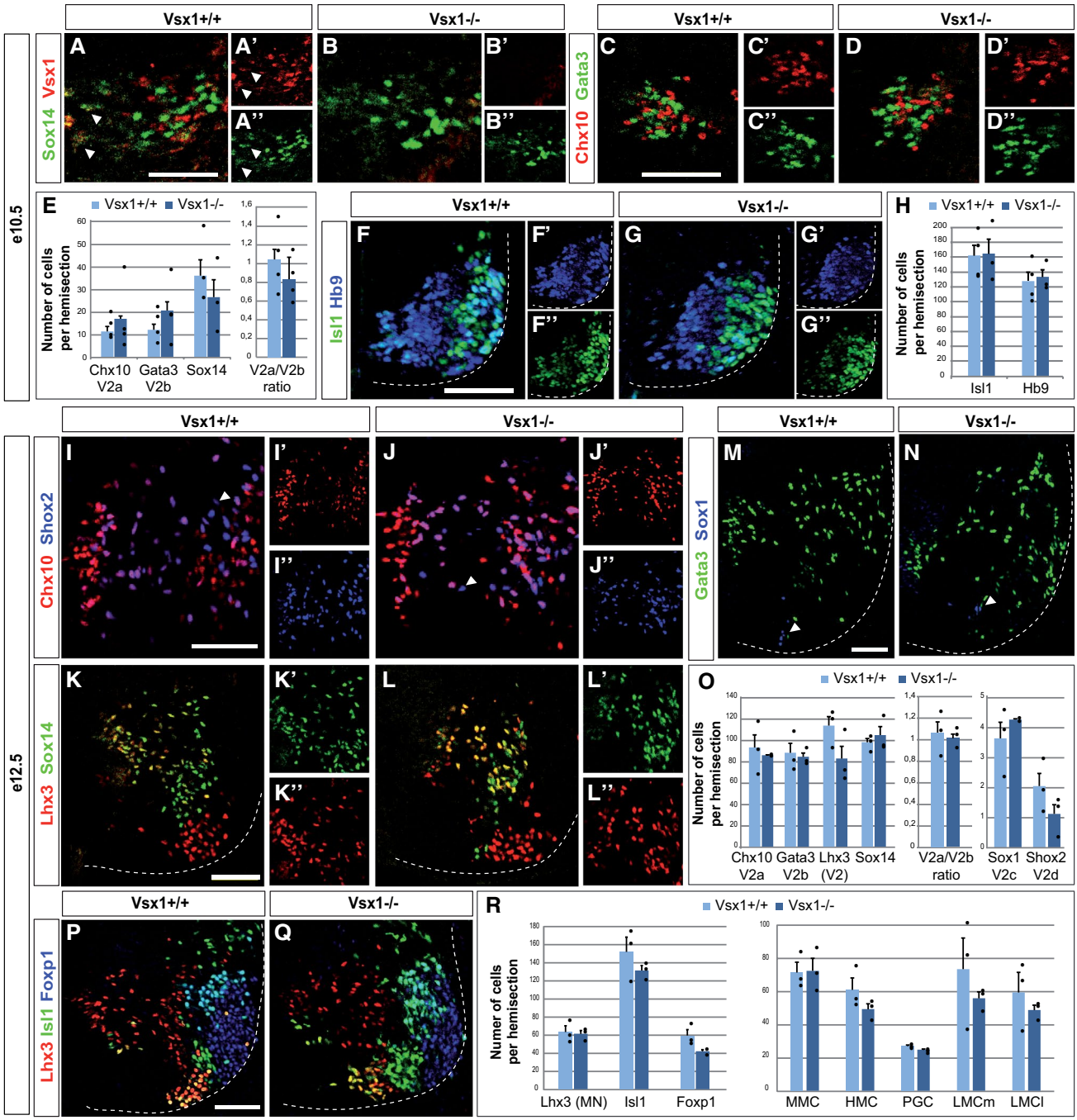

Fig. 3 Vsx1 is not necessary for V2 IN production and diversification. Immunofluorescence for MN (Hb9, Isl1, Lhx3, Foxp1) or V2 IN (Chx10, Sox14, Lhx3, Gata3, Sox1, Shox2) markers on transverse spinal cord sections of Vsx1 loss-of-function embryos. **a** At e10.5 in control embryos, Sox14 in detected in part of the V2 precursors (arrowheads), which contain Vsx1. **b**, **e** In *Vsx1-/-* mutant embryos, Vsx1 is lost whereas Sox14 distribution is not altered ($n=3$). **c**–**e** Similarly, the development of V2a and V2b INs is unaffected ($n=4$). **f**–**h** Absence of Vsx1 does not impact on MN production ($n=4$). **l**–**o** At e12.5, the lack of Vsx1 does not alter the production of V2 INs nor the diversification of V2 INs into V2a, V2b, V2c (arrowheads in **m**, **n**) and V2d (arrowheads in **i**–**j**") subsets ($n=3$). **p**–**r** Consistently, absence of Vsx1 does not impact on MN differentiation ($n=3$). Mean values ± SEM; Scale bars = 50 µm. MMC = medial motor column; HMC = hypaxial motor column; PGC = pre-ganglionic motor column; LMCm = medial portion of the lateral motor column; LMCl = lateral portion of the lateral motor column

activation of the MN differentiation program in the V2 populations.

However, the impact of Vsx1 on V2/MN production might appear in a context wherein MN differentiation is eroded. As an example, the *Vsx1* paralog *Chx10* is ectopically expressed in MNs upon inactivation of *Hb9* [12-14]. Interestingly, the number of Vsx1 + cells was also increased in the absence of Hb9 (Fig. 4a–c). However, no Vsx1/Isl1 hybrid cells were observed in *Hb9* mutants (Fig. S4A-B") and Vsx1 was not co-detected with Chx10 in MN/V2 hybrid cells (Fig. S4C-D"), possibly owing to the repressive activity of Chx10 on *Vsx1* expression [12, 32, 37] (and see below). Nevertheless, this suggested that the absence of Hb9 or impairment of the MN differentiation program releases *Vsx1* repression in prospective MNs. Therefore, we assessed whether the absence of Vsx1 may rescue the MN differentiation defects observed in *Hb9* mutant embryos. However, reduction in the number of MNs, expansion of V2 populations and aberrant production of Isl1/Chx10 hybrid cells were similar in $Hb9^{-/-}Vsx1^{-/-}$ double mutant embryos and in $Hb9^{-/-}$ littermates (Fig. 4d–s), indicating that Vsx1 does not contribute to decrease MN production or to stimulate V2 differentiation in the absence of Hb9. Surprisingly, V2b INs were not impacted by the absence of Hb9 (Fig. 4l–o), suggesting that the ectopic activation of Vsx1 and Chx10 biases excessive V2 differentiation towards the V2a lineage. Taken together, these data indicate that Vsx1 is not required for early consolidation of V2 identity, suggesting that the lack of Vsx1 may be compensated by other factors. Interestingly, Nkx6.1 and Pax6 are maintained in the V2 precursors containing Vsx1 [16]. Nkx6.1 and Pax6 are able to inhibit *Hb9* expression in transient transfection experiments [8] and Nkx6.1 can inhibit MN differentiation in the developing spinal cord [38], opening the hypothesis that Pax6 and/or Nkx6.1 could compensate for the absence of Vsx1. Using chicken embryonic spinal cord electroporation, we confirmed that Nkx6.1 and Pax6 alone can inhibit MN differentiation and we observed that combination of Vsx1 and Nkx6.1, but not Pax6, did reduce MN production (Fig. S5), suggesting that Nkx6.1 may cooperate with Vsx1 to consolidate V2 identity. However, this hypothesis could not be tested further due to the critical role of Pax6 and Nkx6.1 in ventral spinal cord patterning [28, 39-41].

Vsx1 and Chx10 act successively to secure V2 IN identity

Our data demonstrated that Vsx1 and Chx10 can use the same mechanism to prevent activation of the HxRE and of the MN differentiation program [6]. In normal conditions, Vsx1 and Chx10 are not detected in the same cells [16]. Therefore, they would exert this activity in distinct although lineage-related cell types, namely V2 precursors and V2a differentiating INs, respectively. However, Vsx1 represses *Chx10* expression in type-7 ON bipolar cells [37] and Chx10 represses *Vsx1* in retinal progenitor cells [32] and in ES cell-derived neuronal populations [12]. Therefore, we reasoned that each paralog may partly compensate for V2 differentiation defects caused by the lack of the other. The number of Chx10 + cells was not increased in the absence of Vsx1 (Fig. 3), suggesting that Chx10 does not compensate for the absence of Vsx1. In contrast, the number of Vsx1 + cells was significantly increased in $Chx10^{orJ/orJ}$ single mutant spinal cord (Fig. 5a–c). This indicates that, as previously reported in other cell types [12, 32], Chx10 prevents Vsx1 production in V2a IN, and explains the mutually exclusive expression of the 2 paralogs in wildtype [16] and in *Hb9* mutant spinal cord (Fig. S4). This additionally suggests that prolonged *Vsx1* expression may prevent MN vs V2 differentiation defects in the absence of Chx10.

To address this hypothesis, we studied V2 IN and MN production in $Vsx1/Chx10^{orJ}$ double mutant embryos at e12.5. Using Sox14 as marker to label V2 precursors and V2a INs (Fig. 3) [12], we observed that combined absence of both Prd-L:CVC factors resulted in a reduction in the number of Sox14 + cells (Fig. 5d–f, p) that was not observed in single mutants (Fig. 3; Fig. 5e, p). Consistently, the number of cells containing Lhx3 but not Hb9 or Isl1, corresponding to V2a, was similarly smaller (Fig. 5j–p). Furthermore, cells containing Shox2, which consists of a majority of V2a and in the V2d INs [42], were also significantly reduced (Fig. 5d–f, p). This demonstrates that Vsx1 and Chx10 act successively to promote V2a IN differentiation. Surprisingly, although they derive from Vsx1-containing cells and although their number was increased upon *Vsx1* overexpression in V2 INs (Fig. 2j–l), the number of V2b cells was not affected by the combined absence of Vsx1 and Chx10. Similarly, V2c INs, which derive from V2b [43], were not impacted by the lack of Prd-L:CVC factors (Fig. 5g–i, P). These observations confirm a bias in Vsx1 activity towards V2a fate (Fig. 4d–g; l–o), as observed for Chx10 [12]. To assess whether this decrease in V2a INs was compensated by increased MN production, MN were quantified in $Vsx1/Chx10^{orJ}$ double mutant embryos. A significant increase in the number of MNs (Fig. 5j–o, q) was observed, that is neither detected in the absence of Chx10 alone (Fig. 5k, n, q) [12] nor in the absence of Vsx1 (Fig. 3). Taken together, these observations indicate that Vsx1 and Chx10 cooperate during spinal cord development to prevent MN differentiation and to activate the V2a differentiation program in the V2 lineage.

Discussion

Paralog genes are usually reported to evolve towards non-functionalization, neofunctionalization or subfunctionalization [1, 2]. Here, we showed that two paralogs of the

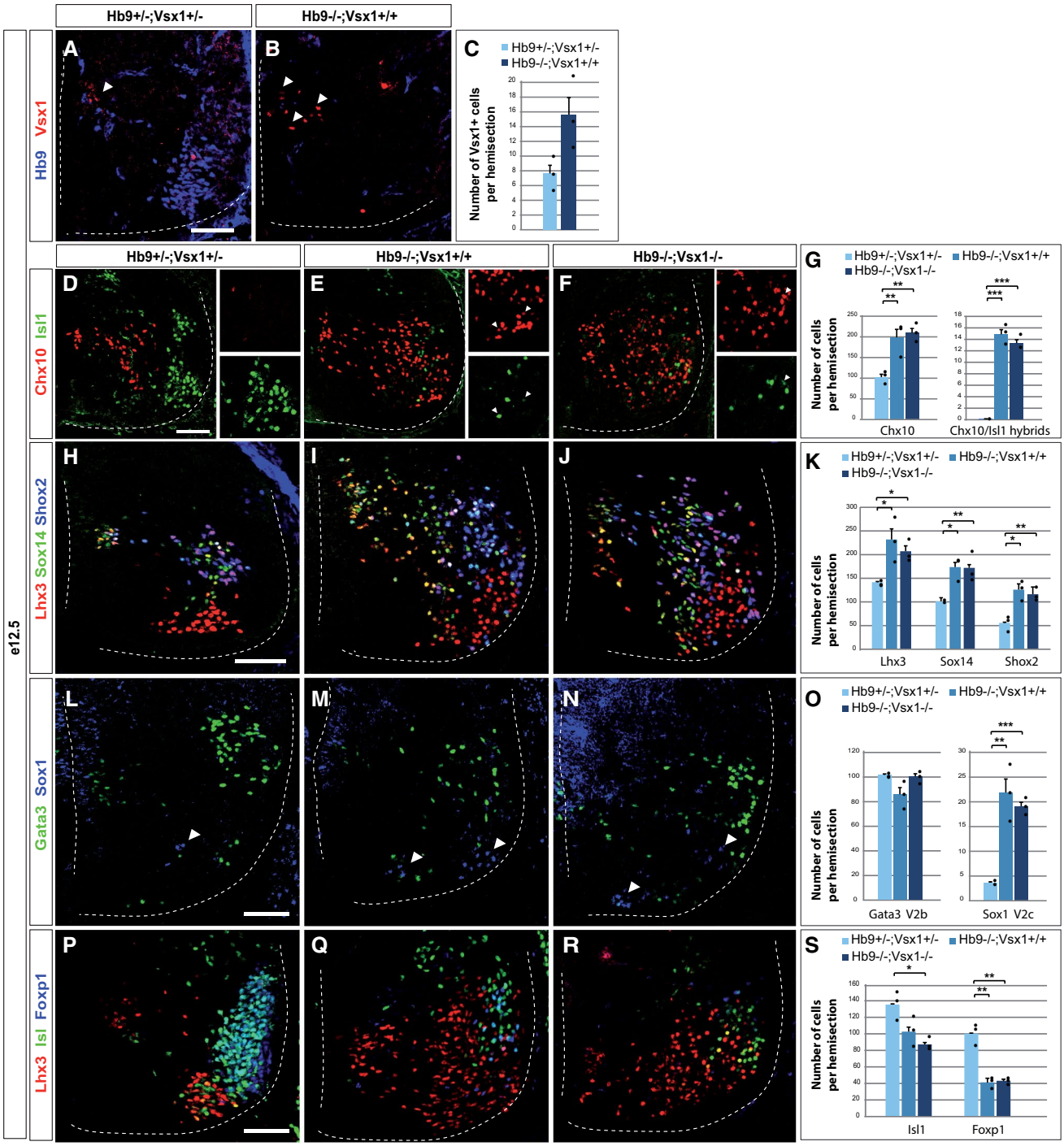

Fig. 4 Vsx1 does not contribute to decrease MN production or to stimulate V2 differentiation in the absence of Hb9. Immunofluorescence for MN (Hb9, Isl1, Lhx3, Foxp1) or V2 IN (Chx10, Sox14, Lhx3, Gata3, Sox1, Shox2) markers on transverse spinal cord sections of *Hb9* single mutant or *Hb9/Vsx1* double mutant embryos. (**a**–**c**) In *Hb9-/-* single mutants at e12.5, the number of Vsx1 + cells is increased (arrowheads), indicating that Hb9 prevents Vsx1 production in MNs ($n = 3$). **d**–**e**, **g** Absence of Hb9 results in increased production of cells containing Chx10 and in the aberrant generation of hybrid MN/V2 cells (arrowheads) containing the MN marker Isl1 and the V2a marker Chx10 ($n = 3$). **f**–**g** Chx10 expansion and hybrid cell production (arrowheads) are similar in double *Hb9/Vsx1* mutant embryos and in single *Hb9* mutants ($n = 3$). **h**–**k** Accordingly, other markers of V2a INs are similarly expanded in *Hb9* single mutant and in *Hb9/Vsx1* double mutant embryos ($n = 3$). **l**–**o** Surprisingly, the V2b marker Gata3 is not upregulated in the absence of Hb9 or in the combined absence of Hb9 and Vsx1 ($n = 3$) whereas the V2c marker Sox1 (arrowheads) is similarly expanded in both mutants ($n = 3$). **p**–**s** Consistently, the number of MNs is similarly decreased both in single and in double mutants ($n = 3$; Isl1 + cells: $p = 0.08$ for single mutants). Mean values ± SEM; $***p < 0.001$, $**p < 0.01$ and $*p < 0.05$. Scale bars = 50 µm

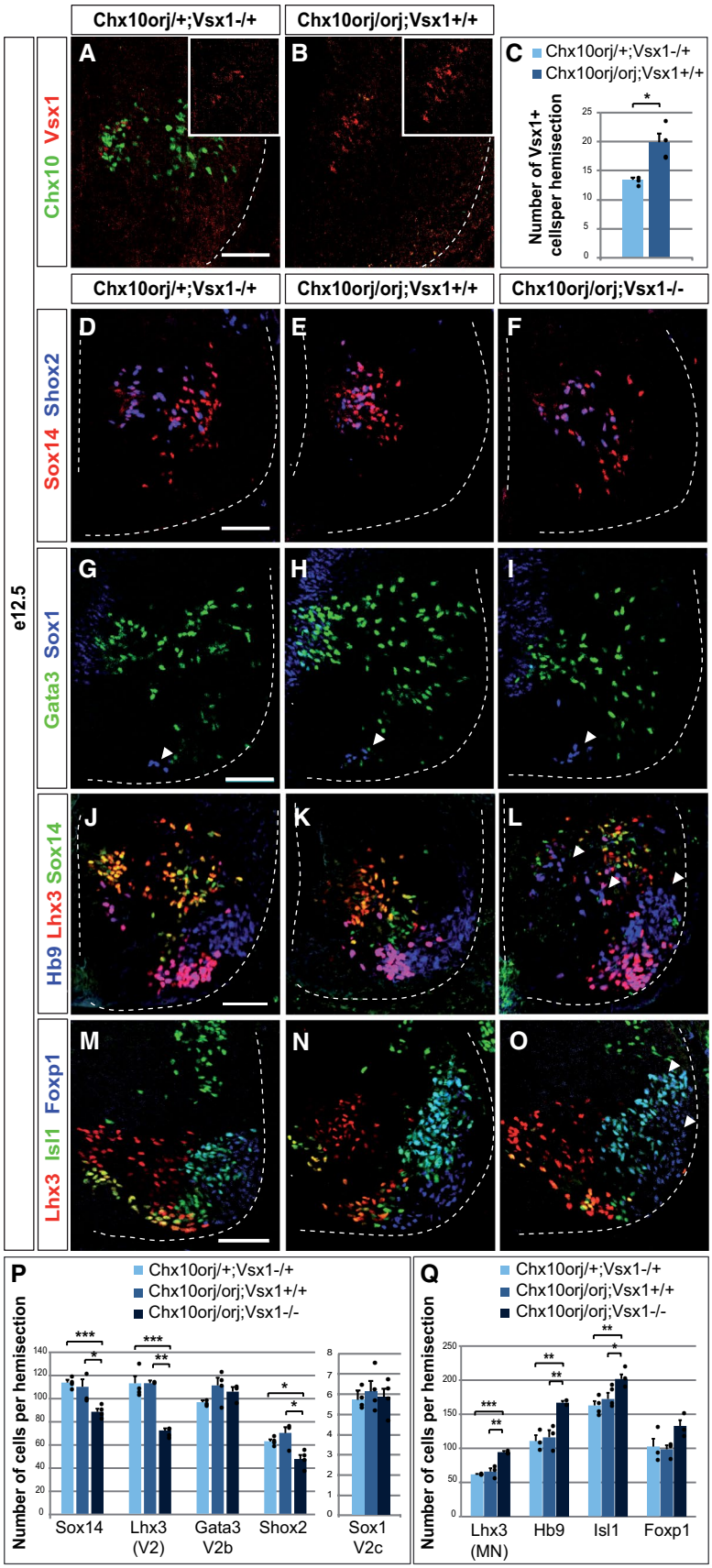

Fig. 5 Vsx1 and Chx10 cooperate to prevent MN differentiation and to activate the V2a differentiation program in the V2 lineage. Immunofluorescence for MN (Hb9, Isl1, Lhx3, Foxp1) or V2 IN (Chx10, Sox14, Lhx3, Gata3, Sox1, Shox2) markers on transverse spinal cord sections of *Chx10*^{orJ} single mutant or *Chx10*^{orJ}/*Vsx1* double mutant embryos. **a–c** In *Chx10*$^{orJ/orJ}$ single mutants at e12.5, the number of Vsx1 + cells is increased, indicating that Chx10 represses Vsx1 production in V2a INs ($n = 3$). **d–f**, **p** The number of V2a INs labeled by Lhx3 (Lhx3 + Isl1- cells), Sox14 or Shox2 is not changed in the *Chx10*$^{orJ/orJ}$ single mutant but is significantly reduced in the combined absence of Vsx1 and Chx10 ($n = 3$). **g–i**, **p** In contrast, the generation of V2b and of V2c (arrowheads) INs is unaffected ($n = 3$). **j–o**, **q** Consistently, the number of MNs is not modified in the *Chx10*$^{orJ/orJ}$ single mutant but is significantly increased (arrowheads) in the combined absence of Vsx1 and Chx10 ($n = 3$). Mean values ± SEM; ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$. Scale bars = 50 µm

Prd-L:CVC homeobox gene family use identical mechanisms to prevent activation of the MN differentiation program and secure V2 identity at successive stages of V2 IN differentiation (Fig. 6). Hence, we uncover an original situation of labor division wherein two paralogs exert seemingly identical functions in a single cell lineage at successive steps of development.

Prd-L:CVC paralogs sequentially secure V2 interneuron identity

We demonstrated that Vsx1 and Chx10 are jointly required to prevent ectopic activation of the MN differentiation program in V2 cells and to secure V2 identity. Indeed, combined absence of these 2 Prd-L:CVC factors resulted in an increase in MN production that is not observed in corresponding single mutants. This suggests that, although the hexameric Isl1-NLI-Lhx3 complex cannot form in V2 interneurons since Isl1 is not produced in these cells, Vsx1 and Chx10 are necessary to prevent activation of HxREs and of the motor neuron differentiation program in the V2 lineage. Several observations support this hypothesis. First, Vsx1 and Chx10 are able to bind the *Hb9* HxRE and to prevent its activation by the MN-hexameric complex. Second, both factors suppress MN production induced by the hexameric complex. Third, ectopic production of Vsx1 in MN in 2 independent transgenic mouse lines results in a reduction in MN differentiation. Taken together, these observations suggest that Vsx1 and Chx10 actively suppress HxRE activation and MN differentiation in V2 INs. Since HxRE activation results in inhibition of the expression of multiple INs determinants [5], joined repression of HxREs is also likely required to enable IN differentiation. However, Chx10 is present in the V2a INs but is not produced in earlier V2 precursors cells (Fig. 6) [16]. Therefore, we hypothesized that Vsx1 could anticipate V2a-restricted Chx10 action and secure V2 fate in V2 precursors. In support of this possibility, increased *Vsx1* expression in spinal INs resulted in increased production of V2 cells. However, loss-of-function experiments demonstrated that Vsx1 alone is not required for proper V2 IN generation. In contrast, combined inactivation of the 2 Prd-L:CVC genes *Vsx1* and *Chx10* resulted in V2 and in MN defects that were not observed in single mutants of the same mouse line, although the lack of Chx10 induced a mild reduction in V2 cells in a different genetic background [12]. This apparent cooperativity between these 2 factors is surprising since Vsx1 and Chx10 are not present in the same cell compartment. However, Chx10 represses *Vsx1* expression in differentiating V2a INs and loss of Chx10 results in prolonged production of Vsx1. Given their similar activity, the persistence of Vsx1 in V2a likely compensate for the loss of Chx10 [12]. In contrast, *Chx10* expression is not anticipated in embryos lacking Vsx1 and can therefore not substitute for its absent paralog in V2 precursors. However, the loss of Vsx1 could be compensated by the homeodomain-containing transcription factors Nkx6.1 and Pax6 [8, 38]. Hence, alterations of MN and of V2 IN development in *Vsx1/orJ* double mutants support the hypothesis that Vsx1 and Chx10 cooperatively

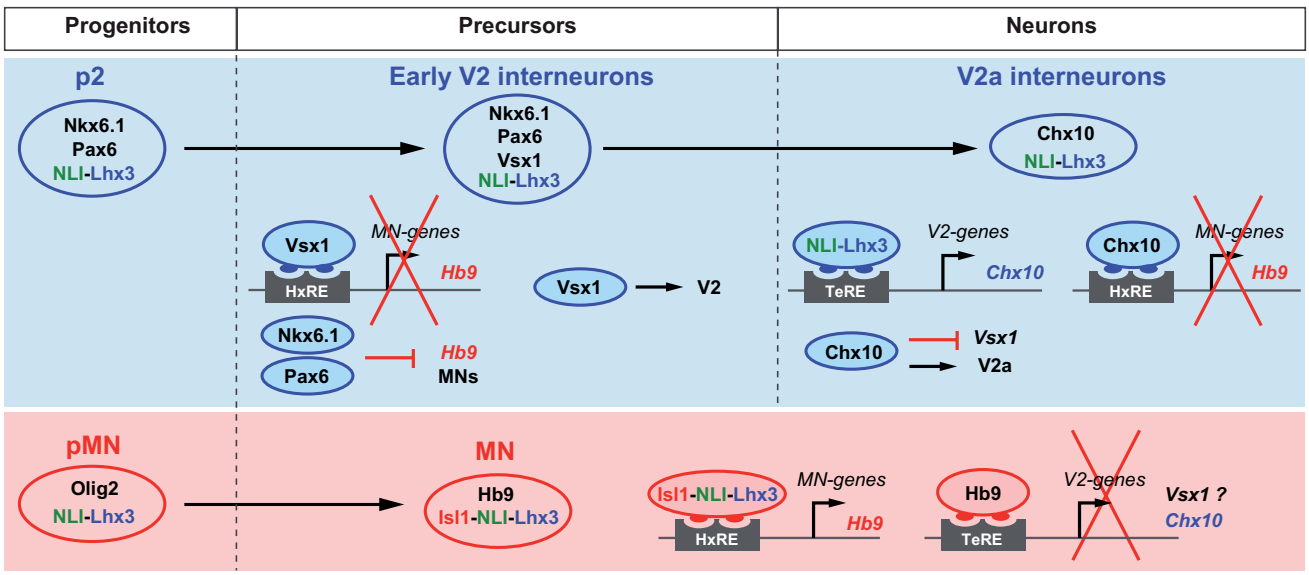

Fig. 6 Labor division between Vsx1 and Chx10 at successive stages of V2 IN differentiation. Schematic representation of MN and V2 IN identity specification and consolidation. In V2 precursors (early V2 INs), Vsx1 binds HxREs and inhibits their activation and *Hb9* and MN gene expression, thereby securing V2 identity. Nkx6.1, and possibly Pax6, may cooperate with Vsx1 in this process. In addition, Vsx1 can stimulate V2 differentiation

contribute to inhibit MN differentiation and to promote V2 fate at successive stages of differentiation in the developing V2 INs (Fig. 6).

Distribution and function of Prd-L:CVC paralogs during V2 differentiation

In the zebrafish embryonic spinal cord, Vsx1 is initially detected in V2 precursors before the V2a/V2b segregation and is retained in V2a but not in V2b interneurons [34, 44, 45]. In contrast, in the mouse, Vsx1 is exclusively detected in V2 precursors but not in V2a or V2b cells [16], suggesting that the functions of the two paralogs have progressively been segregated during evolution. This raises the question of how mutually exclusive production of Vsx1 and Chx10 during murine V2 differentiation is ensured. Intriguingly, in the retina, Prd-L:CVC factors show an opposite sequence of expression, as Chx10 is present in progenitors before the onset of Vsx1 production, which is restricted to differentiating cone bipolar cells [17]. Inverse relationship between *Chx10* and *Vsx1* expression levels suggested that mutual repression could contribute to segregate production of the Prd-L:CVC paralogs. Accordingly, Vsx1 represses *Chx10* expression in type 7 ON bipolar cells [37] whereas Chx10 represses *Vsx1* in retinal progenitor cells [32]. Furthermore, *Vsx1* is also downregulated by Chx10 in ES cell-derived neuronal populations [12]. Taken together, these observations suggest that, in the developing spinal cord, Chx10 may restrict Vsx1 production to V2 precursors, i.e. before the segregation of V2a and V2b subsets. This is in agreement with our observation that the number of Vsx1-containing cells was increased in *orJ* homozygous embryos. It also explains the absence of Vsx1/Isl1 hybrid cells in *Hb9* mutant embryos, as ectopic production of Chx10 in prospective MNs likely prevents *Vsx1* expression in these cells. This could account for the preferential production of supernumerary V2a interneurons instead of a combination of V2a and V2b cells in the absence of Hb9, as could be expected if Vsx1 would have been present in these cells. Hence, we propose a scenario wherein two paralog genes that were initially expressed in the same cells, i.e. V2 precursors and V2a differentiating interneurons as observed in zebrafish, have been progressively segregated into successive but distinct populations of the same lineage to exert seemingly identical function at different stages of development (Fig. 6). Consistent with this model, phylogenetic analyses have suggested that Chx10 and Vsx1 did evolve rapidly [17]. How Vsx1 expression is inhibited in the other V2 subsets, particularly in V2b interneurons, remains to be investigated.

Evolution of paralog genes is supposed to result in non-functionalization, neofunctionalization or subfunctionalization [1, 2]. Despite their rapid evolution [17], multiple pieces of evidence suggest that Vsx1 and Chx10 retained broadly overlapping functions in the mammalian spinal cord. Both factors can bind HxREs, prevent their activation and inhibit MN differentiation ([6, 11, 12] and this study). Furthermore, they are both able to promote V2 production ([12] and this study). Surprisingly, the absence of Vsx1 did not impact the number of V2b INs in any of the mouse lines we studied. Two hypotheses can support these observations. Firstly, Vsx1 may retain the capacity of Chx10 to specifically stimulate V2a IN fate [12]. However, increased Vsx1 production in spinal INs stimulated both V2a and V2b production, consistent with the idea that Vsx1 consolidate V2 identity before V2 diversification. Secondly, Chx10 may contribute to inhibit V2b differentiation, as observed after overexpression in the chicken embryonic spinal cord (data not shown). This could account for the lack of V2b perturbations in the single *Hb9* or compound *Vsx1/Hb9* mutants, wherein Chx10 is ectopically produced, and in the *Vsx1/orJ* compound mutant wherein the absence of Chx10 may release the inhibition on V2b IN production. V2c INs, which derive from early V2b cells [43], seem to escape this Chx10-dependant inhibition, as the number of V2c was strongly increased in *Hb9* and in *Vsx1/Hb9* mutant embryos, suggesting that Chx10 specifically control the differentiation of mature V2b INs. Careful investigations of the respective roles of Vsx1 and Chx10 in V2 precursors and regarding V2b differentiation will be required to address these hypotheses.

Hb9 represses Prd-L:CVC gene expression to secure HxRE activation and MN differentiation

Hb9 is critical for proper differentiation of the spinal MN. Accordingly, recent studies indicate that the MN hexameric complex initially binds a specific set of enhancers, including regulatory *Hb9* sequences, at early stages of MN development [46, 47], then is recruited by Ebf and Onecut transcription factors to another set of targets that promote terminal MN differentiation [47]. Hb9, which is an early target of the MN-hexameric complex [46], secures MN differentiation using two complementary strategies. Firstly, it prevents activation of the TeRE by the Lhx3-NLI tetrameric complex, which can also form in MN, and by the MN Isl1-NLI-Lhx3 hexameric complex, which is also able to bind and activate TeREs [6]. It is assisted in this function by STAT factors, which enhance the transcriptional activity of the MN–hexamer in an upstream signal-dependent manner [7], and by LMO4, which blocks V2-tetramer assembly [6, 48]. Secondly, doing so, it also prevents possible inhibition of the HxRE by the Prd-L:CVC paralogs. Indeed, the absence of Hb9 results in ectopic activation of Vsx1 (this study) and of Chx10 [12-14], indicating that Hb9 inhibits *Vsx1* and *Chx10* expression in early MNs. Repression of *Chx10* is direct, as Hb9 binds to the *Chx10*-TeRE and prevents its activation by the MN hexameric complex [6]. Whether a similar direct

mechanism accounts for *Vsx1* repression in MNs remains to be investigated. Hence, Hb9 secures MN fate by preventing Vsx1 and Chx10 activation (Fig. 6).

Labor division between Prd-L:CVC paralog genes

Within multigenic families, pairs of paralogs including Emx, Otx, Dlx or Dvl family members have been repeatedly shown to act redundantly in the regulation of CNS development. These pairs of genes are usually expressed in partly overlapping expression patterns, and often display redundant functionality in cells or tissues wherein they are coexpressed and divergent functionality in specific expression areas [49-53]. Here, we provide evidence that the murine Prd-L:CVC paralogs Vsx1 and Chx10 retained redundant functionality although their expression has been segregated at distinct stages of V2 interneuron differentiation. Active paralogous compensation by transcriptional reprogramming is at least one of the strategies used by the Prd-L:CVC factors to secure V2 identity. Direct or indirect repression of *Vsx1* expression by Chx10 enables compensation for a loss of Chx10 in V2a interneurons, reminiscent of similar compensation previously shown for *PDC1* and *5* or *NHP6A* and *B* in yeast or *RPL22* and *RPL22l1* in mouse [1]. However, our data also suggest that the Prd-L:CVC paralogs additionally acquired divergent functionalities, as Vsx1 can stimulate V2b differentiation whereas Chx10 rather represses it. Although showing conserved primary sequences, functional promiscuity of these factors may rely on versatile conformational flexibility [54] enabling variable interactions with cofactors or the transcriptional machinery. Thorough examination of the respective Vsx1 and Chx10 target genes will be necessary to address this question. Hence, our observations emphasize the critical importance of paralog redundancy for the robustness of biological systems. They are also consistent with surveys demonstrating in *S. cervisiae* or in *C. elegans* that redundancy is often an evolutionary stable state [55] and showing in mouse or human that paralog genes are less likely to harbor mutations associated to lethality or diseases, respectively [56, 57].

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