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# Conserved and novel roles for the Gsh2 transcription factor in primary neurogenesis

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## SUMMARY

The Gsx genes encode members of the ParaHox family of homeodomain transcription factors, which are expressed in the developing central nervous system in members of all major groups of bilaterians. The Gsx genes in *Xenopus* show similar patterns of expression to their mammalian homologues during late development. However, they are also expressed from early neurula stages in an intermediate region of the open neural plate where primary interneurons form. The Gsx homologue in the protostome *Drosophila* is expressed in a corresponding intermediate region of the embryonic neuroectoderm, and is essential for the correct specification of the neuroblasts that arise from it, suggesting that Gsx genes may have played a role in intermediate neural specification in the last common bilaterian ancestor. Here, we show that manipulation of Gsx function disrupts the differentiation of primary interneurons. We demonstrate that, despite their similar expression patterns, the uni-directional system of interactions between homeodomain transcription factors from the Msx, Nkx and Gsx families in the *Drosophila* neuroectoderm is not conserved between their homologues in the *Xenopus* open neural plate. Finally, we report the identification of *Dbx1* as a direct target of Gsh2-mediated transcriptional repression, and show that a series of cross-repressive interactions, reminiscent of those that exist in the amniote neural tube, act between Gsx, Dbx and Nkx transcription factors to pattern the medial aspect of the central nervous system at open neural plate stages in *Xenopus*.

**KEY WORDS: Gsx, Gsh1, Gsh2, Interneurons, Msx, Neurogenesis, Nkx, *Xenopus***

## INTRODUCTION

The Gsx (genomic screened homeobox) genes encode a class of homeodomain transcription factors from the ParaHox family, which also includes the Pdx/Xlox and Cdx classes. ParaHox genes have been identified both in cnidarian species and in all major branches of bilaterians (Brooke et al., 1998; Finnerty and Martindale, 1999; Frobius and Seaver, 2006; Hui et al., 2009; Ryan et al., 2007), and their products play conserved roles in the regulation of tissue patterning and differentiation during development (Isaacs et al., 1998; Moreno and Morata, 1999; Offield et al., 1996; Von Ohlen et al., 2007).

We have previously cloned and characterised the expression of the Gsx genes *Gsh1* and *Gsh2* in the frog *Xenopus tropicalis* (Illes et al., 2009). Of particular interest is the observation that *Gsh2* is expressed in the open neural plate during primary neurogenesis. Primary neurogenesis is an early phase of neural development unique to non-amniotic vertebrates, which generates neurons controlling simple escape responses of the free-swimming larvae. These neurons form in three columns across the dorsoventral, or mediolateral, axis of the open neural plate, interspersed by regions of non-differentiating neuronal progenitors. Primary sensory neurons develop from the lateral column, interneurons from the intermediate column and motoneurons from the medial column. *Gsh2* was found to be expressed in the intermediate column.

The *Xenopus* open neural plate is similar, both in its architecture and in the presence of three neurogenic columns, to that of the neuroectoderm of the protostome *Drosophila*. During development of the *Drosophila* trunk neuroectoderm, early delaminating neuroblasts form within three columns on either side of the ventral midline, and the Gsx homologue *ind*, like *Gsh2*, is specifically expressed in the intermediate column. Two other homeobox genes, *msh* and *vnd*, mark the lateral and medial columns, respectively (Weiss et al., 1998). Ind is essential for the correct specification and differentiation of the neurons that arise from the intermediate column. We were interested to determine whether Gsx genes play homologous roles in the development of early intermediate neuronal populations in both vertebrates and protostomes.

A prevailing theory of metazoan evolution proposes that the last common bilaterian ancestor possessed a ventral CNS like that of protostomes, and that an inversion of the body axis occurred during the evolution of vertebrates, such that the CNS became dorsal (Arendt and Nubler-Jung, 1994; De Robertis, 2008; De Robertis and Sasai, 1996; Gerhart, 2000).

Numerous studies have provided support for this theory by demonstrating equivalent expression patterns and functions for homologous genes in the development of protostome and vertebrate nervous systems. For example, it has recently been shown that the order of expression of several homeodomain transcription factors along the mediolateral axis of the ventral CNS in the annelid *Platynereis* corresponds to that of their homologues in the vertebrate dorsal neural tube (Denes et al., 2007). Our previous findings that *Gsh2*, as well as *Msx1* and Nkx family genes, are expressed in the *Xenopus* open neural plate in similar mediolateral positions to that of the related *Drosophila* genes *ind*, *msh* and *vnd* also support this theory (Illes et al., 2009).

In the *Drosophila* trunk neuroectoderm, Ind represses expression of the lateral column gene *msh*, whereas the medial column factor Vnd represses *ind* and *msh* expression (Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). These findings led to the formulation of the 'ventral dominance' hypothesis, which proposes that the three genes *vnd*, *ind* and *msh* interact according to a system whereby the product of the more ventral (or medial) gene represses

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genes expressed more dorsally (or laterally) (Cowden and Levine, 2003). We were interested to determine whether equivalent interactions exist between the Gsx, Msx and Nkx family genes in *Xenopus*. This would provide further support for a common origin for the bilaterian CNS, and for the inversion hypothesis. To avoid confusion arising from the inversion of the *Xenopus* CNS with respect to *Drosophila*, the 'ventral dominance' hypothesis will henceforth be described as 'medial dominance'. It should be noted that *vnd*, *ind* and *msh* are also expressed in the proencephalic neuroectoderm of the *Drosophila* embryo, and that their interactions differ in this region (Urbach, 2007).

In the present study, domain-swap mutants and antisense morpholino oligos were used to manipulate the activity of the Gsx genes. These were used in combination with overexpression of the wild-type gene products to determine the role of Gsx in the development of primary interneurons. Gsx overexpression or inhibition was found to interfere with the expression of primary interneuron markers, supporting a conserved role for the Gsx family in the specification of intermediate neuronal precursors. However, we provide data to indicate that a uni-directional, medial dominance hierarchy, involving Msx, Gsx and Nkx6 family genes, does not operate in *Xenopus*. This indicates that, during vertebrate evolution, lineage-specific interactions have arisen between the components of the ancestral mediolateral neural patterning system. During the course of this study, we identified the homeobox gene *Dbx1* as a direct target of transcriptional repression by Gsh2. We show that *Dbx1*, *Gsh2* and Nkx6 family genes are components of a regulatory network that patterns the medial region of the *Xenopus* open neural plate through a series of bi-directional repressive interactions, similar to those previously documented for other homeobox genes in the neural tube of higher vertebrates (Briscoe et al., 2000; Wilson and Maden, 2005).

## MATERIALS AND METHODS

### *Xenopus* embryological methods and microinjection

Embryos were generated by in vitro fertilisation of eggs laid following injection with human chorionic gonadotrophin. *X. laevis* embryo culture was in NAM/10 (Slack and Forman, 1980) at 14-24°C. *X. tropicalis* embryo culture was in MRS/9 (Tindall et al., 2007) prior to gastrulation, and in MRS/20 thereafter, at 21.5-27°C. Jelly coats were removed with 3% L-cysteine (Sigma) in NAM or MRS/9 (pH 7.8-8.0). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Embryos were injected in NAM/3 or MRS/9 plus 3-5% Ficoll (Sigma) at the two- to four-cell stage using a Drummond microinjector. Unilateral injections were monitored using co-injection of *GFP* mRNA. Microinjected embryos were transferred to NAM/10 or MRS/20 prior to gastrulation. *X. laevis* animal cap explants were explanted at blastula stage 9. Animal caps were cultured in NAM/2 in agarose-coated wells, and fixed for in situ hybridization or snap-frozen for RT-PCR as required.

### Drug treatments

In order to activate the Gsh2-VP16-GR fusion protein, dexamethasone (DEX) was added to the culture medium at the appropriate stage to a final concentration of 10 μM. In the experiments to determine the directness of regulation by *Gsh2*, cycloheximide (CHX) was added to the culture medium at a final concentration of 10 μg/ml. Animal caps were excised from injected or control embryos and 15-20 caps per group were cultured in the presence of CHX for 30 minutes. DEX was then added to the appropriate wells and caps were cultured in CHX+DEX for 2 hours. Caps were then transferred into appropriate medium without CHX for a further 1.5 hours. At stage 10-10.5, caps were fixed for in situ hybridisation or snap-frozen for RT-PCR. This protocol has previously been shown to result in effective inhibition of protein synthesis, as measured by radio-methionine incorporation (Fisher et al., 2002).

### Photography and sectioning

Whole specimens were photographed using a SPOT 14.2 Color Mosaic camera (Diagnostic Instruments) and SPOT Advanced software, with a Leica MZ FLIII microscope. For sections, unbleached stained embryos were embedded in 4% agarose and 50 μm sections were cut in cooled PBS-A using a Leica VT1000 S vibratome and mounted in Hydromount (National Diagnostics). Sections were photographed using an 18.2 Color Mosaic camera (Diagnostic Instruments) and SPOT Advanced software with a Leica DM2500 microscope. Images were processed using Adobe Photoshop Elements 4.0.

### DNA constructs and mRNA synthesis

To generate the Gsh2-VP16-GR construct, the coding region of *Gsh2* was first subcloned into the *Xho*I/*Xba*I site of the CS2+VP16-N plasmid [described in Isaacs et al. (Isaacs et al., 1998)] to form Gsh2-VP16. The ligand-binding domain from the human glucocorticoid receptor alpha (GR) gene was then subcloned from pSP64T-MyoDGR (Kolm and Sive, 1995) into the *Xho*I site of Gsh2-VP16, between the *VP16* and *Gsh2* sequences, using the following primers: forward, 5′-GGCGCCGGACTCGAGT-CTGAAAT-3′; reverse, 5′-GCGGCGGGACTCGAGCTTTTGAT-3′.

For synthesis of functional capped mRNA, plasmids were linearised using enzymes indicated in Table 1A, and transcribed using the SP6 MegaScript transcription kit (Ambion) according to a modified protocol described by Isaacs et al. (Isaacs et al., 1998).

### Antisense morpholino oligos

Two non-overlapping translation-blocking AMOs targeted against *X. tropicalis Gsh2* were synthesized. The standard control morpholino was also used. All AMOs were designed and synthesized by GeneTools. Sequences are as follows: Gsh2-AMO (T1), 5′-CATAAAAAGACCTA-GA**CAT**TGCCGC-3′; Gsh2-AMO (T2), 5′-AGCTCAGCAGTCAGA-CAGCTCCTTC-3′; control MO, 5′-CCTCTTACCTCAGTTACAATT-TATA-3′. [Bold type in Gsh2-AMO (T1) indicates position of initiating ATG in the complementary gene sequence. Gsh2-AMO (T2) ends 5′ of the initiating ATG.]

### First-strand cDNA synthesis and RT-PCR

Embryos and animal caps for RT-PCR were snap-frozen on dry ice and total RNA extraction was carried out using Tri-Reagent (Sigma). First-strand cDNA was synthesized using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen), with OligodT primers and 2-3 μg RNA per sample. PCR reactions were conducted using 2 μl first-strand cDNA and 2× PCR Master Mix (Promega). Primers were designed to amplify a 200-500 bp mRNA fragment, when possible, from a region flanking an intron in the genomic sequence. Sequences of the primers used are given in Table S1 in the supplementary material.

### In situ hybridisation analysis

To produce the *Dbx1* in situ hybridisation probe, a 431 bp fragment of the *Dbx1* gene was amplified from *X. tropicalis* genomic DNA by PCR, using the primers given below, and cloned into the pGEM-T-Easy vector (Promega): forward, 5′-TGGGGCTGGCGGAGAGGAT-3′; reverse, 5′-GAGGGCAGGCAGGCATAACCG-3′.

Table 1B gives details of the linearisation and transcription of plasmids for generation of in situ hybridisation probes. Transcriptions were performed using 10× DIG RNA labelling mix (Roche) or 10× fluorescein labelling mix (Roche) for the *Dbx1* probe used for double in situ hybridisation.

In situ hybridisation was carried out as described previously (Harland, 1991) with the modifications given elsewhere (Pownall et al., 1996). Hybridising probes were detected using an anti-DIG AP-coupled antibody (Roche) and BM purple (Roche) as the precipitating substrate. The *Gsh2/Dbx1* double in situ hybridisation was performed as described previously (Isaacs et al., 1998), with the following adaptations. The DIG-labelled probe (*Gsh2*) was detected first, using anti-DIG antibody and BM purple, as above. The fluorescein-labelled probe (*Dbx1*) was detected using anti-fluorescein antibody (Roche) and a solution of 3.5 μl magenta phosphate (Sigma; stock 50 mg/ml in 100% DMF) + 4.5 μl Tetrazolium Red (Sigma; stock 75 mg/ml in 70% DMF) in 1.5 ml AP buffer.

**Table 1. Plasmids used**

| Plasmid name | Enzyme for linearisation (probe) | Polymerase for antisense probe | Origin |
|---|---|---|---|
| **A Functional mRNA synthesis** | | | |
| pCS2+ nuclear GFP2 | *Not*I | | J. C. Illes (unpublished) |
| pCS2+ Xt Gsh1 (ORF) | *Not*I | | Subcloned from pCS107 Xt Gsh1 (Illes et al., 2009) |
| pCS2+ Xt Gsh2 (ORF) | *Not*I | | Subcloned from pCS107 Xt Gsh1 (Illes et al., 2009) |
| pCS2+ Xt Gsh2-myc tag | *Not*I | | Illes et al. (2009) |
| pCS107 Xt Msx1 | *Asc*I | | GenBank EST AL972643 |
| pCS2+ Gsh2-VP16-GR | *Sac*II | | This report |
| pCS2+ Dr Nkx6.1-myc tag | *Not*I | | Gift from J. S. Eisen |
| pCS2+ Xt Nkx6.2 (ORF) | *Not*I | | Subcloned from GenBank EST CT010540.2 |
| CS2+ Xt Dbx1 (ORF) | *Not*I | | Subcloned from GenBank EST CK656318 |
| **B Synthesis of in situ probes** | | | |
| pGEM-T EV Xt Dbx1 | *Spe*I | T7 | This report, genomic clone |
| pCS107 Xt Nkx6.1 | *Eco*RI + hydrolysis | T7 | GenBank EST AL894846 |
| pCS107 Xt Nkx6.2 | *Sac*I | T7 | GenBank EST CT010540.2 |
| pCS107 Xt Lbx1 | *Eco*RI + hydrolysis | T7 | GenBank EST BX727671 |
| pCS107 Xt Msx1 | *Eco*RI + hydrolysis | T7 | GenBank EST AL972643 |
| pGEM-5Zf Xt N-tubulin | *Apa*I + hydrolysis | SP6 | Gift from R. M. Harland |
| pGEM-T EV Xtgsx1fA | *Sac*II | SP6 | J. C. Illes |
| pGEM-T EV Xtgsx2fB | *Sac*II | SP6 | J. C. Illes |

## RESULTS

### Gsh2 function and interneuron development

We investigated Gsx function in primary neuron development using overexpression of wild-type Gsx proteins and a dexamethasone-inducible antimorphic Gsh2 protein (see Fig. S1 in the supplementary material). A conserved, N-terminal Eh1 Groucho interaction domain is present in *Xenopus tropicalis* Gsh1 and Gsh2, indicating that both proteins are likely to act as transcriptional repressors (Illes et al., 2009). Fusions of Gsh1 and Gsh2 to the *Drosophila* engrailed repressor domain mimic the activity of the wild-type proteins and fusions to the VP16 activation domain have antimorphic activity, supporting the notion that Gsx proteins are transcriptional repressors (data not shown).

*Lbx1* is a marker of the primary interneuron territory that, like *Gsh2*, is initially expressed in the presumptive hindbrain region of the open neural plate (Illes et al., 2009). Unilateral overexpression of Gsh2 expands the boundaries of the *Lbx1* domain (76%, *n*=33; Fig. 1A). Expression of the neuronal differentiation marker *N-tubulin* was also expanded on the injected side, and the gap of *N-tubulin* expression in the progenitor domain between the lateral and intermediate domains is frequently absent (88%, *n*=26; Fig. 1D). Similar effects result from overexpression of Gsh1 (see Fig. S2 in the supplementary material). Our data suggest that ectopic Gsx expression expands the boundaries of the primary interneuron territory.

Injection of antimorphic Gsh2-VP16-GR, with induction of activity at gastrula stage 11, caused downregulation of *Lbx1* expression on the injected side (71%, *n*=31; Fig. 1B). Furthermore, *N-tubulin* expression was downregulated in the intermediate domain (69%, *n*=16; Fig. 1E). These results are consistent with a requirement for Gsh2 in the differentiation of primary interneurons. However, it was noted that in 19% of embryos, there was also a slight downregulation and expansion of the lateral domain of *N-tubulin* expression.

We also investigated the requirement for Gsh2 activity in primary interneuron development using two translation-blocking antisense morpholino oligos, Gsh2-AMO (T1) and (T2). Both AMOs reduced translation of a myc-epitope tagged Gsh2 protein (Fig. 1I; see Fig. S3D in the supplementary material). Gsh2-AMO (T2) resulted in inhibition or loss of *Lbx1* expression on the injected side (94%, *n*=32; Fig. 1C). Gsh2-AMO (T1) also inhibited *Lbx1* expression in the open neural plate (100%, *n*=22; see Fig. S3A in the supplementary material).

Both AMOs caused slight but consistent reduction in expression of *N-tubulin* in the intermediate domain, in keeping with a role for Gsh2 in interneuron specification [Gsh2-AMO (T2): 100%, *n*=25; Fig. 1F and Gsh2-AMO (T1): 100%, *n*=18; see Fig. S3B in the supplementary material]. We note that expression in the lateral column was also downregulated in 50-56% of these embryos, and in another 11-12%, expression was reduced in all three columns. Thus, Gsh2 AMOs also appear to affect expression outside the intermediate domain, suggesting that Gsh2 may have non-cell-autonomous effects on primary neuronal differentiation.

The effects on development at later stages, resulting from manipulation of Gsh2 activity, were also investigated. Unilateral Gsh2 overexpression caused an upregulation and expansion of anterior *Lbx1* expression at early tail-bud stage 25 (94%, *n*=34; Fig. 1G and inset). Conversely, AMO-mediated Gsh2 knockdown resulted in a downregulation of anterior *Lbx1* expression [Gsh2-AMO (T2): 76%, *n*=17; Fig. 1H and Gsh2-AMO (T1): 75%, *n*=32; see Fig. S3C in the supplementary material]. Fig. S3D in the supplementary material shows that the anterior loss of *Lbx1* expression can be rescued with co-injection of Gsh2 mRNA (63%, *n*=30).

### Interactions between Gsx, Nkx and Msx factors

The data presented thus far indicate that Gsh2, and possibly Gsh1, play an essential role in the specification of interneurons, which are derived from the intermediate column of primary neurons and differentiate during the open neural plate stage in *Xenopus*.

*Ind* is a Gsx family member that is required for the development of the intermediate column of neuroblasts in *Drosophila* and is a component of a regulatory network, involving the Msx family gene, *msh* and the Nkx family gene, *vnd* (Cowden and Levine, 2003; McDonald et al., 1998; Weiss et al., 1998). In *Drosophila*, these homeobox genes operate in a hierarchy by which the product of the more medial gene represses the expression of the more

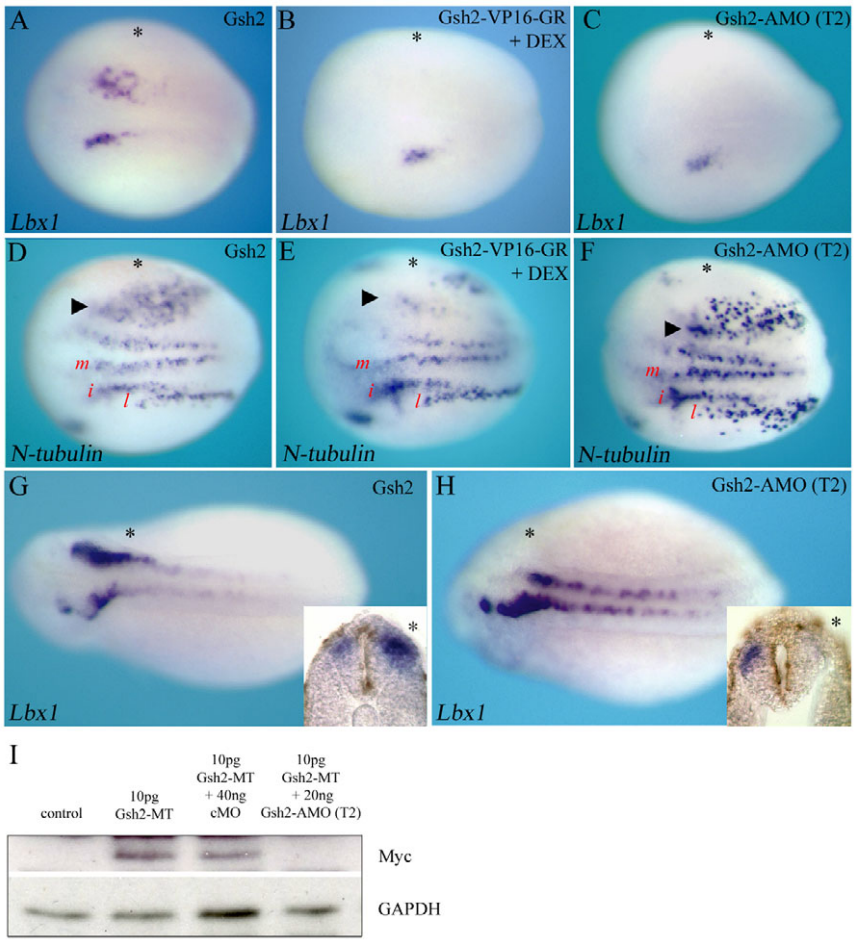


**Fig. 1. Effect of Gsx on primary interneuron development.** (**A-F**) Whole-mount in situ hybridisation to show expression of (A-C) *Lbx1* or (D-F) *N-tubulin* in stage 14 *X. tropicalis* embryos unilaterally injected with (A,D) 5 pg Gsh2; (B,E) 15 pg Gsh2-VP16-GR with addition of dexamethasone at stage 11, or (C,F) 20 ng Gsh2-AMO (T2). Dorsal views, anterior towards the left. Asterisks indicate injected side. Arrowheads indicate interneuron column on injected side. *i*, intermediate column (primary interneurons); *l*, lateral column (primary sensory neurons); *m*, medial column (primary motoneurons). (**G**,**H**) Expression of *Lbx1* in stage 25 *X. tropicalis* embryos unilaterally injected with (G) 5 pg Gsh2 or (H) 10 ng Gsh2-AMO (T2). Main pictures are dorsal views with anterior towards the left; insets show cross-sections at the hindbrain level. Asterisks indicate injected side. (**I**) Western blot to show ability of Gsh2-AMO (T2) to inhibit translation of a myc-tagged Gsh2 mRNA in stage 10.5 *X. tropicalis* embryos. Embryos were injected with Gsh2 mRNA and Gsh2-AMO (T2) or a control AMO as indicated, and blotted with antibodies against Myc or GAPDH as a loading control. cMO, control morpholino.

lateral genes; a property that has been termed ventral or medial dominance. There has been considerable speculation that such a uni-directional interaction, involving homologues of *vnd*, *ind* and *msh* might represent a conserved regulatory pathway that was required for dorsoventral patterning of the nervous system in the common ancestor of both protostome and deuterostome lineages (Cornell and Ohlen, 2000; Mizutani et al., 2006). With this in mind, we investigated the interactions operating between the Gsx, Msx and Nkx families of transcription factors in the *Xenopus* open neural plate.

A prediction of the medial dominance hypothesis is that the product of the intermediate gene *Gsh2* should repress the expression of the lateral gene *Msx1* (Fig. 2A). When Gsh2 is overexpressed, the effect on *Msx1* expression is somewhat complex, resulting in a slight downregulation of *Msx1* expression in the anterior, as predicted; however, this is accompanied by a general expansion in more posterior regions (100%, *n*=24; Fig. 2B). Furthermore, in the presence of antimorphic Gsh2, *Msx1* expression is downregulated (80%, *n*=15; Fig. 2C). We also observed that Msx1 overexpression resulted in mild expansion in *Gsh2* expression (45%, *n*=20; Fig. 2D). These observations argue against the conservation of a simple, unidirectional medial dominance-based regulatory pathway operating in the amphibian open neural plate.

We also investigated interactions between Gsh2 and the Nkx6 subfamily of genes. The medial dominance hypothesis predicts that the medial Nkx6 genes should repress *Gsh2* expression. It has previously been reported that *Xenopus tropicalis Nkx6.1* is expressed in a medial domain at open neural plate stages (Illes et al., 2009). In order to address the potential repression of *Gsh2* by Nkx6 family proteins, we have overexpressed Nkx6.1. Zebrafish Nkx6.1 was used because we have been unable to obtain a *Xenopus tropicalis* cDNA encoding the full-length Nkx6.1 protein. We find that injection of *Nkx6.1* strongly downregulates the expression of *Gsh2* (95%, *n*=22; Fig. 2E). *Xenopus tropicalis Nkx6.2* is expressed in a similar midline domain (see Fig. S4A,B in the supplementary material) to that reported for *Xenopus tropicalis Nkx6.1* (Illes et al., 2009). Overexpression of *Xenopus tropicalis Nkx6.2* also strongly downregulated the expression of *Gsh2* (100%, *n*=32; see Fig. S4C in the supplementary material). However, in conflict with the unidirectional interactions predicted by the medial dominance hypothesis, we find that Gsh2 overexpression results in expansion of the *Nkx6.1* expression domain (88%, *n*=26; Fig. 2F) and antimorphic Gsh2 downregulates *Nkx6.1* (84%, *n*=31; Fig. 2G).

## Gsh2 regulation of *Dbx1*

Our data show that overexpression of Gsh2 resulted in upregulation of *Nkx6.1* expression, whereas over-expression of antimorphic Gsh2 (a Gsh2-activator fusion) leads to downregulation. As this cannot be the result of direct transcriptional regulation, we hypothesise that the basis of this observation is that a target of Gsh2 repression is itself a repressor of *Nkx6.1* expression.

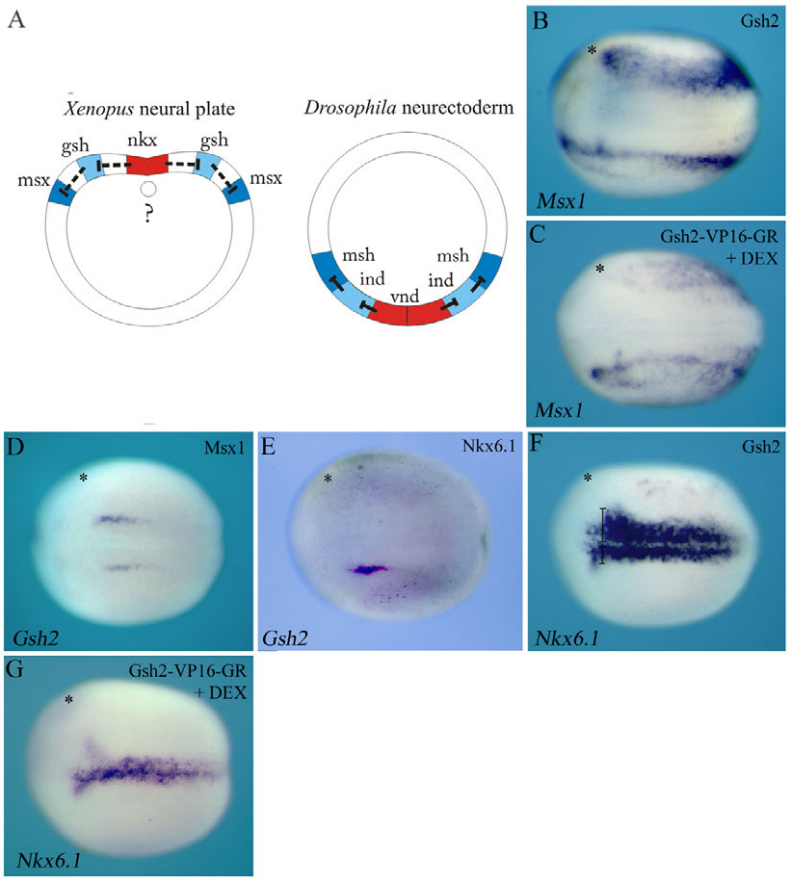


**Fig. 2. Interactions between Gsx, Msx and Nkx factors.** (**A**) Schematic cross-section diagrams to show expression of homeobox genes *Msx1*, *Gsh2* and *Nkx6.1* in *Xenopus* open neural plate, and *msh*, *ind* and *vnd* in the *Drosophila* neuroectoderm. Uni-directional repressive interactions in *Drosophila* and putative equivalent interactions in *Xenopus* are indicated by solid and broken lines, respectively. (**B-D**,**F**,**G**) Whole-mount in situ hybridisation of stage 14 and (**E**) stage 13 *X. tropicalis* embryos to show expression of (B) *Msx1* in 5 pg Gsh2-injected embryos; (C) *Msx1* in 15 pg Gsh2-VP16-GR-injected embryos, plus dexamethasone at stage 11; (D) *Gsh2* in 10 pg Msx1-injected embryos; (E) *Gsh2* in 20 pg Nkx6.1-injected embryos; (F) *Nkx6.1* in 5 pg Gsh2-injected embryos; and (G) *Nkx6.1* in 15 pg Gsh2-VP16-GR-injected embryos, plus dexamethasone at stage 11. All embryos are unilaterally injected; asterisks indicate injected side. Dorsal views, anterior towards the left.

A good candidate for this target is the homeobox gene *Dbx1*, which is expressed in the progenitor domain between the medial and intermediate columns of primary neurons at the open neural plate stage. In keeping with *Dbx1* being a Gsh2 target, overexpression of wild-type Gsh2 inhibits *Dbx1* expression (97%, *n*=27; Fig. 3A), whereas injection of Gsh2-VP16-GR causes massive upregulation and expansion of the *Dbx1* expression domains (100%, *n*=28; Fig. 3B). AMO-mediated knockdown of Gsh2 caused a slight expansion of the *Dbx1* expression domain (43%, *n*=28; Fig. 3C,D).

These data indicate that *Dbx1* is a good candidate to be a target of Gsh2-mediated transcriptional repression. To determine whether *Dbx1* is directly regulated by Gsh2, *Dbx1* expression was analyzed in animal cap explants in the presence of the protein synthesis inhibitor cycloheximide, following activation of inducible antimorphic Gsh2. *Dbx1* was strongly induced above control levels by Gsh2-VP16-GR in the presence of dexamethasone, and this induction was still seen in the presence of cycloheximide (Fig. 3E). Therefore, regulation of *Dbx1* by Gsh2 requires no intermediate proteins to be synthesized, and is likely to be direct.

Previous studies have indicated that *Dbx1* is expressed in the open neural plate in the zone of non-differentiating cells between the intermediate and medial columns of primary neurons (Gershon et al., 2000). In order to precisely determine the relationship between *Gsh2* and *Dbx1* expression, double in situ hybridisation was carried out. Our data show that the neural plate domains of *Gsh2* and *Dbx1* expression at stage 14 are directly juxtaposed (Fig. 3F and inset), indicating that *Dbx1* is expressed between the domains of *Gsh2* and *Nkx6.1/6.2* expression at open neural plate stages (Fig. 3G).

### Cross-repressive interactions between Gsx, Dbx and Nkx transcription factors in the open neural plate

Based upon our observations, we predict that Dbx1 represses *Nkx6.1*. In support of this hypothesis, we observed that *Nkx6.1* expression was strongly repressed by Dbx1 overexpression (61%, *n*=23; Fig. 4A). Furthermore, we showed that Dbx1 overexpression repressed *Gsh2* (100%, *n*=19; Fig. 4B).

Finally, we investigated the effects resulting from overexpression of Nkx6 family proteins on *Dbx1* expression. We observed that Nkx6.1 strongly repressed *Dbx1* expression (100%, *n*=12; Fig. 4C). Nkx6.2 also repressed *Dbx1* (100%, *n*=37; see Fig. S4D in the supplementary material). Thus, a complex series of cross-repressive interactions exists between these three classes of homeodomain transcription factors in the open neural plate (Fig. 4D). Our observations contradict the simple predictions of the uni-directional, repressive interactions of the medial dominance model, and support a model in which bi-directional repressive interactions, similar to those occurring in the amniote neural tube, operate to establish the boundaries of expression between the conserved homeodomain transcription factors in the *Xenopus* open neural plate.

## DISCUSSION

### Evolutionarily conserved roles for Gsh2 in the development of intermediate column neurons

The *Drosophila* Gsx homologue Ind plays a crucial role in the specification and differentiation of early neuroblasts in the intermediate column of the neuroectoderm (Weiss et al., 1998). Our

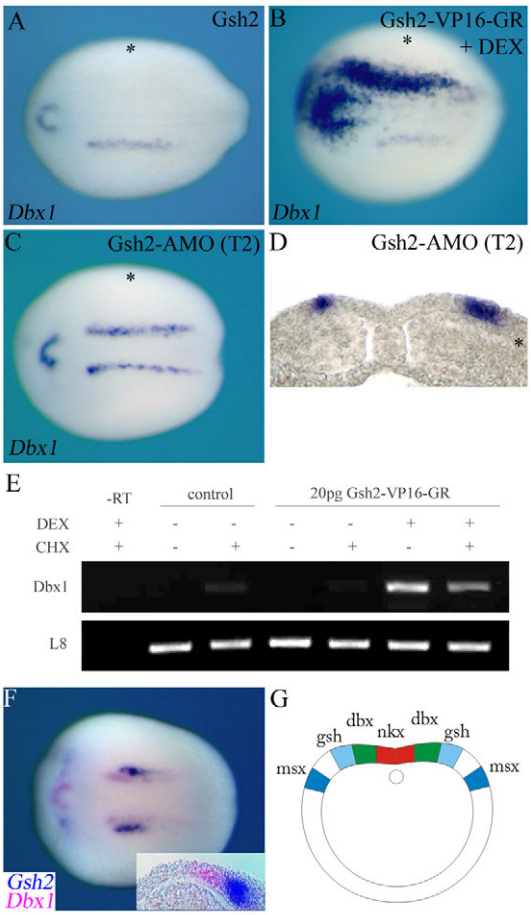


**Fig. 3. Gsh2 and Dbx1 in the open neural plate.** (**A-C**) Whole-mount in situ hybridisation to show expression of *Dbx1* in stage 14 *X. tropicalis* embryos unilaterally injected with (A) 5 pg Gsh2; (B) 15 pg Gsh2-VP16-GR, plus dexamethasone at stage 11; or (C) 10 ng Gsh2-AMO (T2). Dorsal views, anterior towards the left, asterisks indicate injected side. (**D**) *Dbx1* expression in a cross-section at the hindbrain level of an embryo unilaterally injected with 10 ng Gsh2-AMO (T2). (**E**) RT-PCR analysis of *Dbx1* expression in animal cap explants from *X. laevis* control embryos or embryos injected with 20 pg Gsh2-VP16-GR, and treated with cycloheximide (CHX) and/or dexamethasone (DEX) as indicated. L8 is a loading control. (**F**) Double whole-mount in situ hybridisation to show adjacent expression of *Gsh2* and *Dbx1* in the open neural plate. Inset shows a half cross-section at hindbrain level. (**G**) Schematic cross-section diagram to show relative positions of homeobox gene expression domains in the *Xenopus* open neural plate.

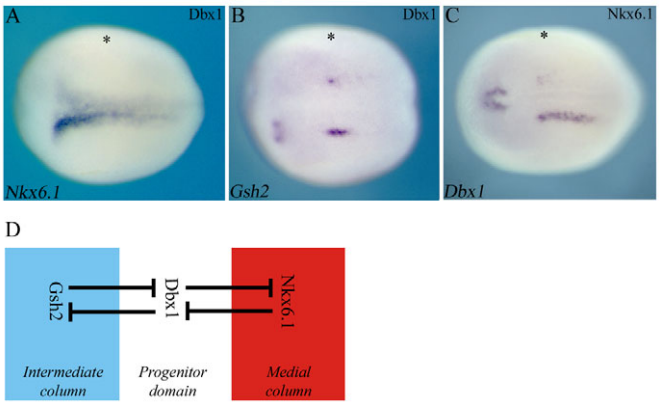


**Fig. 4. Interactions between Gsx, Nkx and Dbx factors.** (**A-C**) Whole-mount in situ hybridisation of stage 14 *X. tropicalis* embryos to show expression of (A) *Nkx6.1* and (B) *Gsh2* in 5 pg Dbx1-injected embryos, or (C) *Dbx1* in 20 pg Nkx6.1-injected embryos. Asterisks indicate injected side. (**D**) Proposed model of bidirectional repressive interactions between Gsh2, Dbx1 and Nkx6.1 transcription factors in the *Xenopus* open neural plate.

results demonstrate that Gsh2 is required for the development of *Lbx1*-positive interneurons derived from the intermediate column of primary neurons during open neural plate stages in *Xenopus*. However, it was noted that effects of Gsh2 manipulation on the expression of the neuronal differentiation marker *N-tubulin* in the interneuron domain were less dramatic, suggesting that some primary interneurons may develop independently of Gsh2. This suggests that the role of Gsx factors in the *Xenopus* open neural plate may more closely resemble that of its homologues in the amniote neural tube, which specify only some subsets of interneurons, rather than that of Ind in *Drosophila*. In this regard, it is of note that although the Gsx homologue in the annelid *Platynereis* is expressed in a corresponding intermediate domain of the CNS, this expression is seen only in differentiating neurons, and not during their initial specification (Denes et al., 2007). Thus, although these cumulative data strongly support an ancestral role for Gsx genes in intermediate neural development, there is considerable variation in exactly how they have been subsequently deployed during animal evolution.

Our data showed that Gsh2 knockdown also influenced *N-tubulin* expression in the medial and lateral domains of the neural plate. Perhaps the most likely interpretation of this observation is that Gsh2 regulates the expression of downstream diffusible signalling molecules involved in regulating the pattern of neuronal differentiation. Another possibility is that Gsx factors function, in part, by direct trafficking of the proteins from expressing to adjacent, non-expressing cells. Such intercellular movement has been observed for other homeodomain-containing transcription factors, including Hox and Pax family members (Le Roux et al., 1993; Lesaffre et al., 2007; Prochiantz, 1999).

## *Dbx1*: a novel Gsh2 target

In this report, the *Dbx1* gene was shown to be a direct target of Gsh2-mediated transcriptional repression. The Dbx (developing brain homeobox) genes encode homeodomain transcription factors from the Hlx (H2.0-like) class. Mammals have two Dbx genes, *Dbx1* and *Dbx2*, whereas three Dbx genes, *Dbx1a*, *Dbx1b* and *Dbx2*, have been described in zebrafish. Thus far, only one Dbx gene, *Dbx1*, also known as *Dbx-A*, has been cloned and characterised in *Xenopus*, although the *X. tropicalis* genome reveals a probable *Dbx2* homologue (encoding a protein with a homeodomain 90% identical to that of mouse *Dbx2*).

In the mouse embryo, *Dbx1* and *Dbx2* are first expressed after neural tube closure in discrete regions of the central nervous system, including four central interneuron progenitor domains, vI1 to dI5, in the hindbrain and spinal cord (Lu et al., 1992; Pierani et al., 2001; Shoji et al., 1996). The Dbx genes are involved in dorsoventral patterning of the neural tube by cross-repressive interactions with other homeodomain transcription factors, including members of the Nkx6 family (Gribble et al., 2007; Vallstedt et al., 2001). Direct genetic interactions between Gsx and

Dbx family transcription factors have not previously been reported, although, in the mouse telencephalon, Gsh2 is thought to act in the lateral ganglionic eminence to restrict *Dbx1* expression to the adjacent ventral pallium (Yun et al., 2001). In the mouse neural tube, inhibition of Gsh2 (or of both Gsh1 and Gsh2) has no effect on the expression of *Dbx2*, but possible effects on *Dbx1* have not been investigated (Kriks et al., 2005).

Although the later expression patterns of zebrafish *Dbx1a/1b* and *Xenopus Dbx1* are very similar to those in mammals, expression is also seen before neural tube closure, in an anterior domain and in two parallel columns within the open neural plate (Fjose et al., 1994; Gershon et al., 2000). In *Xenopus*, these columns have been shown to fall between the medial and intermediate columns of primary interneuron progenitors. Overexpression of *Dbx1* leads to downregulation of the early proneural gene *Ngnr1* and the differentiation marker *N-tubulin* (Gershon et al., 2000). Thus, *Dbx1* has been proposed to act during primary neurogenesis in *Xenopus* to maintain proliferating cell populations and limit the extent of neuronal differentiation. Our findings point to a model where Gsh2 acts in the intermediate domain to directly repress *Dbx1* expression, thus permitting the differentiation of primary interneurons. This interaction has not been previously reported in any organism.

A homologue of the Dbx genes, *H2.0*, does exist in *Drosophila*, but is not expressed in longitudinal columns, and therefore is unlikely to play a role in mediolateral patterning (Kriks et al., 2005). However, in the annelid *Platynereis*, a *Dbx* homologue is expressed in a population of differentiating neurons in an equivalent region to that of its homologues in the vertebrate neural tube and *Xenopus* neural plate. This suggests that Dbx genes may also be components of an ancestral network of genes involved in nervous system development, although, as for Gsx genes, their precise function appears to vary between species (Denes et al., 2007).

## Medial dominance and the *Xenopus* open neural plate

In *Drosophila* the *msh*, *ind* and *vnd* genes, which are members of the Msx, Gsx and Nkx families respectively, play a crucial role in regulating the tripartite dorsoventral pattern of the neuroectoderm (Cowden and Levine, 2003). We have previously shown that Msx, Gsx and Nkx family genes are expressed in equivalent regions of the *Xenopus* neural plate during primary neurogenesis (Illes et al., 2009). These observations raise questions about whether the tripartite dorsoventral organization of the early neuroectoderm, and the gene hierarchy regulating neuronal specification in the neuroectoderm of protostome and deuterostome lineages, was also present in the common bilaterian ancestor.

During development of the *Drosophila* trunk neuroectoderm, products of the more medial genes repress the expression of those expressed more laterally, whereas, at least initially, the lateral proteins do not repress the more medial genes (McDonald et al., 1998; Von Ohlen and Moses, 2009; Weiss et al., 1998). This mode of interaction has been termed ‘ventral’ or, as we suggest, ‘medial’ dominance (Cowden and Levine, 2003). One aim of the present study was to determine whether these genes and their products interact in accordance with the medial dominance hypothesis in *Xenopus*.

We find that overexpression of medially expressed Nkx6 genes repress the interneuron expression of a Gsx family gene (*Gsh2*). It could be argued that these observations constitute evidence for a conserved regulatory interaction between Nkx and Gsx genes. However, it would perhaps be misleading to describe these interactions as truly ‘conserved’. It is important to note that *Drosophila vnd* is more closely related to vertebrate Nkx2 genes than to the Nkx6 group, and, unlike Nkx6 genes, Nkx2 genes are not expressed in the *Xenopus* open neural plate (Hollemann and Pieler, 2000; Saha et al., 1993; Small et al., 2000; Zhao et al., 2007). Despite this, a role for Nkx6 genes is indicated in setting up the mediolateral pattern of motoneurons and interneurons. In zebrafish Nkx6.1 promotes motoneuron development but inhibits interneuron development, a function that it shares with *Drosophila* Nkx6 (Cheesman et al., 2004).

It is interesting to note that, whereas at initial stages of *Drosophila* development the expression domain of *vnd* extends to the midline, it is rapidly excluded from the most medial region. Similarly, the expression of *Xenopus* Nkx6.1 and Nkx6.2 is somewhat lower in the most medial cells, which are fated to form the floor plate of the neural tube. In *Drosophila*, medial exclusion of *vnd* expression is mediated by the indirect action of the Sim (single-minded) transcription factor (Estes et al., 2001; Kim et al., 2005). Although a *sim* homologue is expressed during early *Xenopus* development, expression is broad throughout the CNS, suggesting that this regulatory interaction is not conserved (Coumailleau et al., 2000).

Another prediction that leads from the medial dominance hypothesis is that Gsh2 will repress *Msx1*. Again, there seems to be no simple conservation of this regulatory interaction. We find that Gsh2 overexpression does indeed downregulate the anterior of the *Msx1* domain. However, this is accompanied by a general upregulation in more posterior regions. Furthermore, our observation that antimorphic Gsh2 downregulates, rather than upregulates, *Msx1* expression argues against this interaction being direct.

These results indicate that there are significant differences in the regulatory interactions operating between the Msx, Gsx and Nkx gene families during neural development in *Drosophila* and *Xenopus*. One possible interpretation of this result is that the similarities in the order of expression of these related genes are the result of convergent evolution. However, members of these families have been identified in equivalent regions of the central nervous systems of diverse bilaterians, including vertebrates, arthropods and annelids. The presence of a pathway involving these genes and regulating mediolateral patterning in the central nervous system of the common bilaterian ancestor is a more parsimonious scenario.

In considering our data, it should be noted that the *vnd*, *ind* and *msx* genes are also expressed in the *Drosophila* procephalic neuroectoderm. In this region, Vnd does not repress the expression of *ind*, but is in fact necessary for its activation (Urbach et al., 2006). Furthermore, in the trunk, although Ind does not initially repress *vnd*, it is required to maintain the lateral boundary of the *vnd* expression domain at later stages. Thus, interactions between these transcription factors can vary even within a single organism between different body regions and over the course of development. This may be relevant to the interpretation of our results, especially in view of the differing effects of Gsh2 manipulation on *Msx1* at different levels along the anteroposterior axis.

## Cross-repressive interactions between homeodomain transcription factors pattern the medial neural plate

In contrast to the uni-directional interactions predicted by the medial dominance hypothesis, we find that the medial gene *Nkx6.1* was upregulated by overexpression of the more lateral gene *Gsh2*,

and downregulated by its inhibition. Further experiments indicated that this interaction is likely to result from direct transcriptional regulation of *Dbx1* by Gsh2.

Our experiments provide additional evidence that bi-directional interactions operate in the open neural plate. For example, we observed that overexpression of Dbx1 repressed the expression of both *Gsh1* and *Nkx6.1*. Thus, Dbx1 negatively regulates the expression of both Nkx6 and Gsx genes at its medial and lateral boundaries of expression, respectively. These results are in keeping with a model whereby the extents of the medial and intermediate columns of the open neural plate, and the intervening region of non-differentiating cells is patterned by a series of reciprocal interactions between members of the Gsx, Dbx and Nkx families.

Of course, it is important to bear in mind that the directness of these interactions, with the exception of Gsh2 regulation of *Dbx1*, has not been established. However, interactions between genes from these families have been seen in other vertebrates. For example, in the mouse neural tube, Nkx6.2 represses *Dbx1* expression and Nkx6.1 represses the expression of *Gsh1*, *Gsh2* and *Dbx2* (Sander et al., 2000; Vallstedt et al., 2001). Furthermore, in the zebrafish neural tube, the two Dbx1 homologues act redundantly to repress the expression of *Nkx6.2* (Gribble et al., 2007).

These reciprocal repressive interactions operating between homeobox genes during primary neurogenesis in the amphibian neural plate are similar to those that have been shown to be important for patterning of the neural tube in amniotic vertebrates (Briscoe et al., 2000; Wilson and Maden, 2005). However, this is the first report of such interactions occurring at the open neural plate stage in a non-amniote. These results suggest that cross-repressive interactions are an integral component of mediolateral neural patterning by homeodomain transcription factors in all phases of vertebrate neurogenesis.

## Gsx in central nervous system patterning and evolution

Our data suggest that the regulatory interactions between the Msx, Gsx and Nkx gene families in *Drosophila* are not conserved in the *Xenopus* open neural plate. Despite the similarities in expression of the highly conserved homeobox genes there are important differences. In *Drosophila* the neurogenic columns are directly abutting, whereas in *Xenopus* each column is eventually separated by a region of non-differentiating neural progenitors. This does not rule out direct cross-regulatory interactions operating between Msx, Gsx and Nkx genes, particularly during early open neural plate stages when expression patterns are very dynamic. However, direct repressive interactions between the Msx, Gsx and Nkx genes cannot be primarily responsible for maintaining their expression boundaries once the intervening progenitor domains have been established.

It seems likely that Msx, Gsx and Nkx homologues were expressed in three columns of the ancestral CNS and, based upon our data, we cannot exclude the possibility that the regulatory interactions observed in *Drosophila* were also present in the common bilaterian ancestor. However, during the evolution of vertebrates, the appearance of proliferative regions between the Msx, Gsx and Nkx domains may have necessitated the evolution of additional bi-directional interactions necessary for the establishment of the medial and lateral boundaries of the progenitor domains. One factor that excludes expression of the conserved, columnar homeobox genes from the proliferative region between the motoneurons and interneurons is the Gsh2 target gene *Dbx1*. A Dbx homologue is expressed in an intermediate population of differentiating neurons in the annelid, *Platynereis*, suggesting that an ancestral Dbx gene may have been appropriated to perform an earlier role in the vertebrate lineage (Denes et al., 2007). Our observations of indirect regulatory interactions between Msx1 and Gsh2 suggest that similar mechanisms, involving an unknown Gsh2 target gene, also define the boundaries of the progenitor domain between the interneurons and sensory neurons.

It should be noted that the central nervous system of Platynereis has also been shown to include domains of *Pax3/7*, *Pax6*, *Nkx2* and *sim* expression, in addition to *Msx*, *Nkx6* and *Gsx*, in equivalent domains to their homologues in the vertebrate neural tube, although their individual roles and potential crossregulatory interactions in the annelid have not been investigated (Briscoe et al., 2000; Denes et al., 2007; Wilson and Maden, 2005). Thus, it is arguable that the regulatory system for mediolateral neural patterning in the bilaterian ancestor was in fact much more complex than previously thought, and that the tripartite system seen in *Drosophila* is a secondary simplification of the ancestral system, perhaps as a result of its rapid developmental program. Our data suggest that the expression patterns and interactions of homeodomain transcription factors in the open neural plate resemble those in the neural tube.

Our data provide further evidence to suggest that not only was the nervous system of the last common ancestor centralised, but that a large number of homeodomain transcription factors, including a Gsx homologue, were already involved in regulating its mediolateral pattern. However, the variation in precise timing of expression and roles of these conserved families of homeodomain transcription factors in CNS development in different lineages makes establishment of their roles in the common ancestor a complex task.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at
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