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Xenopus Dbx2 is involved in primary neurogenesis and early neural plate patterning

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ABSTRACT

The evolutionarily conserved Dbx homeodomain-containing proteins play important roles in the development of vertebrate central nervous system. In mouse, Dbx and Nkx6 have been suggested to be cross-repressive partners involved in the patterning of ventral neural tube. Here, we have isolated *Xenopus Dbx2* and studied its developmental expression and function during neural development. Like *XDbx1*, from mid-neurula stage on, *XDbx2* is expressed in stripes between the primary motoneurons and interneurons. At the tailbud stages, it is detected in the middle region of the neural tube. XDbx2 acts as a transcriptional repressor *in vitro* and over-expression of *XDbx2* inhibits primary neurogenesis in *Xenopus* embryos. Over-expression of *XDbx* genes represses the expression of *XNkx6.2* and *vise versa*. Knockdown of either XDbx1, XDbx2 or both by specific morpholinos induces lateral expansion of *XNkx6.2* expression domains. These data reveal conserved roles for Dbx in primary neurogenesis and dorsoventral neural patterning in *Xenopus*.

1. Introduction

Vertebrate neural ectoderm is induced by the underlying dorsal mesoderm derived from the Spemann organizer during gastrulation. The neural ectoderm is then patterned along both the anterior–posterior and dorsoventral axes, which positions the subtypes of the neural progenitors [1,2]. In mouse and chick, the dorsoventral patterning of the neural tube is mediated by morphogens secreted by roof-plate and notochord. These signals set up distinct neural progenitor domains defined by specific transcription factor codes, including homeodomain proteins of the Nkx, Dbx, Pax families [3,4]. These factors are subdivided into two classes based on their different response to Shh signal: class I proteins, such as Dbx1/2, Pax6 and Irx3, are expressed at the dorsal and medial neural tube and are repressed by Shh signal; and class II proteins, including Nkx6.1/6.2 and Nkx2.2, are expressed in the ventral neural tube and are activated by Shh signal. The cross-repression between these two classes of proteins contributes to the formation of the boundaries between the adjacent neural progenitor domains [4–6].

Dbx (*developing brain homeobox*) was first isolated from a mouse 13.5 day embryonic telencephalon cDNA library in 1992 [7,8]. Two *Dbx* genes (*Dbx1* and *Dbx2*) in mammals and three in zebrafish have been reported. These *Dbx* genes show conserved expression patterns in the developing brain and spinal cord and play important roles in neural patterning and differentiation [7–11]. In mouse, *Dbx1* and *Dbx2* are expressed with different ventral limits in the medial neural tube and cross repress with *Nkx6.2* and *Nkx6.1*, respectively [12,13]. In zebrafish, however, the expression of *Dbx1a*, *Dbx1b* and *Dbx2* seems to be overlapping at the medial domain of the neural tube. In zebrafish *Dbx1a/b* morphant, the expression of *Nkx6.2* expands dorsally, suggesting a repressive role of Dbx1 on the expression of *Nkx6.2* [11].

In *Xenopus* embryos, an early phase of neurogenesis takes place at the open neural plate stage which gives rise to primary neurons in three longitudinal stripes (medial, intermediate and lateral) on either side of the midline, as marked by expression of *N-tubulin* [14,15]. XDbx1 is able to inhibit primary neurogenesis when over-expressed and has been suggested to have a role to refine the patterns of neurogenesis in the neural plate [9]. In *Xenopus* embryos, over-expression of Dbx1 or Nkx6.1/6.2 represses the expression of each other [16,17], suggesting conserved mechanisms for neural patterning. However, *Xenopus Dbx2* has not been reported and loss-of-function evidence for the roles of Dbx in *Xenopus* neurogenesis and neural patterning have been lacking.

Here we isolated *Xenopus Dbx2* and studied its developmental expression and functions during neural development. *XDbx2* is expressed in the middle region of the neural tube, overlapping with

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XDbx1. XDbx2 acts as a transcriptional repressor *in vitro* and overexpression of XDbx2 inhibits primary neurogenesis. Knockdown of either *XDbx1* or *XDbx2* induced the expansion of *XNkx6.2* expression domains, supporting a repressive role for Dbx on *Nkx6* expression.

2. Materials and methods

2.1. Xenopus Dbx2 cloning and phylogenetic analysis

Xenopus laevis Dbx2 was cloned by reverse transcription-polymerase chain reaction (RT-PCR) using RNAs from stage 30 embryos as template. The PCR primers were designed according to EST sequences from public databases (NCBI): forward: 5′-GAAAGCTA GGGAGCCAGACCAGAA-3′ and reverse: 5′-TAATAGCAGCCAATC-CACTCCGT-3′. The *XDbx2* sequence is submitted to GenBank under accession number JN184788. A phylogenetic tree of Dbx proteins was constructed using molecular evolutionary genetics analysis (MEGA) [18] with full-length protein sequences.

2.2. Semi-quantitative RT-PCR assays

Semi-quantitative RT-PCR was carried out as described [19] and the PCR primers and conditions were: *XDbx1*: forward: 5′-AATC-TACTGAGACCCACCCC-3’ and reverse: 5′-AGGTGAAGGGCTTTG-GATG-3′, 35 cycles; *XDbx2*: forward: 5′-GAAAGCTAGGGAGCCA GACCAGAA-3′ and reverse: 5′-TAATAGCAGCCAATCCACTCCGT-3′, 35 cycles; *H4* was used as a loading control: forward: 5′-CGGGA-TAACATTCAGGGTA-3′ and reverse: 5′-TCCATGGCGGTAACTGTC-3′, 26 cycles.

2.3. Plasmid constructs

For preparation of RNAs for microinjection, the *XDbx1, XDbx2, XNkx6.1* and *XNkx6.2* open reading frames were sub-cloned into the pCS2+ derived vector, pCS2+-c-flag, which contains a sequence encoding a flag tag at the 3′ end of the cloning site. The *XDbx2* and its deletion constructs were sub-cloned into the pBIND vector (Promega) for cell transfection and luciferase reporter assays. All of the constructs were confirmed by sequencing.

2.4. Embryos, microinjection, whole mount in situ hybridization and sections

These assays were carried out as described [20]. The mRNA encoding a nuclear-localized LacZ was co-injected to trace the injected sides. The sequences of the morpholinos (MO) used were: *XDbx1* MO: 5′-GAGCTAAGAGGCTTGGGAACATCA-3′; *XDbx2* MO: 5′-ACTTCTGATTCTGGTCTGGCTCCTA-3′. The full open reading frames of *X. laevis Dbx1* and *Dbx2* were used to prepare probes for *in situ* hybridization. The probes of *X. laevis Nkx6.1* and *Nkx6.2* were used as previously described [21]. Stained embryos were embedded in paraffin and sectioned at 20 μm. The injected areas were traced by staining of LacZ using red-gal (Research Organics).

2.5. Cell culture and luciferase reporter assay

HEK293T cell was cultured in DMEM plus 10% FBS and transfected using lipo2000 transfection reagent (Invitrogen). The pGAL4-TK-Luc (gift from Prof. Jing) and the full-length or truncated *XDbx2* constructs in the pBIND vector (Promega) which also constitutively express the Renilla luciferase were co-transfected into HEK293T cells in 96-well plates. Twenty-four hours after transfection, the cells were harvested and assayed for their luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) as described [19].

3. Results and discussion

3.1. Isolation and phylogenetic analysis of Xenopus Dbx2

The full length *XDbx2* cDNA was cloned by RT-PCR. The deduced XDbx2 protein contains 251 amino acids and shows 34% overall identity with XDbx1. The homeodomains of XDbx1 and XDbx2 are highly conserved but there is only very weak homology in other regions (Fig. 1A). A less conserved eh1 motif was also detected among XDbx1/2 (Fig. 1A) [22]. A phylogenetic tree was constructed for the Dbx proteins in human, rat, mouse, *X. laevis* and *Xenopus tropocalis*, zebrafish and fruit fly (Fig. 1B). The vertebrate Dbx1 and Dbx2 proteins are clearly clustered into two separate branches, suggesting that they probably originated in the common ancestor of vertebrates.

3.2. Temporal and spatial expression of XDbx2 during Xenopus early embryogenesis

Semi-quantitative RT-PCR was used to investigate the temporal expression patterns of the *Dbx* genes during *X. laevis* early development. *XDbx2* was clearly maternally expressed and its expression went up during early neurula stages and maintained stable later on (Fig. 2 A). The maternal expression of *XDbx1* was very weak if any, and its expression went up during the neurula stages and down a little bit during the following stages (Fig. 2A).

Whole mount *in situ* hybridization was carried out to determine the spatial expression pattern of *Xenopus Dbx2*. Similar to *XDbx1*, the expression of *Xenopus Dbx2* appears neural specific (Fig. 2B, F, F’). During early neurula stages, *XDbx2* was detected in bilaterally symmetric stripes at the middle of the mediolateral axis (Fig. 2B). The *XDbx2* expression domain localized between the medial and intermedial stripes of *N-tubulin* expressing cells, overlapping with that of *XDbx1* but not *XNkx6.2* (compare Fig. 2B–E). Following neural tube closure, the expression of *XDbx2* was detected in the middle region of the spinal cord along the dorsoventral axis, overlapping with that of *XDbx1*, but not in the brain region where *XDbx1* is expressed (Fig. 2F,F’,G,G’) [9]. *XDbx2* was also detected in the branchial arches at tailbud stages (Fig. 2F). In mouse and chick, *Dbx1* and *Dbx2* are expressed at the medial level of the neural tube with different ventral limits [12]. In frog, however, the two genes likely share the same expression extent at the dorsoventral axis, as in zebrafish, indicating redundant roles of Dbx1 and Dbx2 in these species [11].

3.3. Xenopus Dbx2 functions as a transcriptional repressor in vitro

The transcriptional activity of XDbx2 and a series of deletion constructs were tested in an *in vitro* transcriptional reporter assay (Fig. 3). In transiently transfected HEK293T cells, co-expression of the GAL4-Dbx2 fusion protein strongly repressed the expression of the GAL4-driving luciferase reporter. The N-terminal eh1 domains of mouse Dbx1 and Dbx2 have been suggested to mediate interactions with the Groucho-TLE (Gro/TLE) co-repressors [22]. Indeed, the Dbx2 truncates containing the eh1 domain (ΔC and ΔHDC) all showed transcriptional repression activities in reporter assays (Fig. 3C). Unexpectedly, constructs containing only the Dbx2 C-terminal domain or plus the homeodomain could also partially repress the reporter expression (Fig. 3C). The Dbx2 homeodomain alone has only very weak transcription repression activity (Fig. 3C).

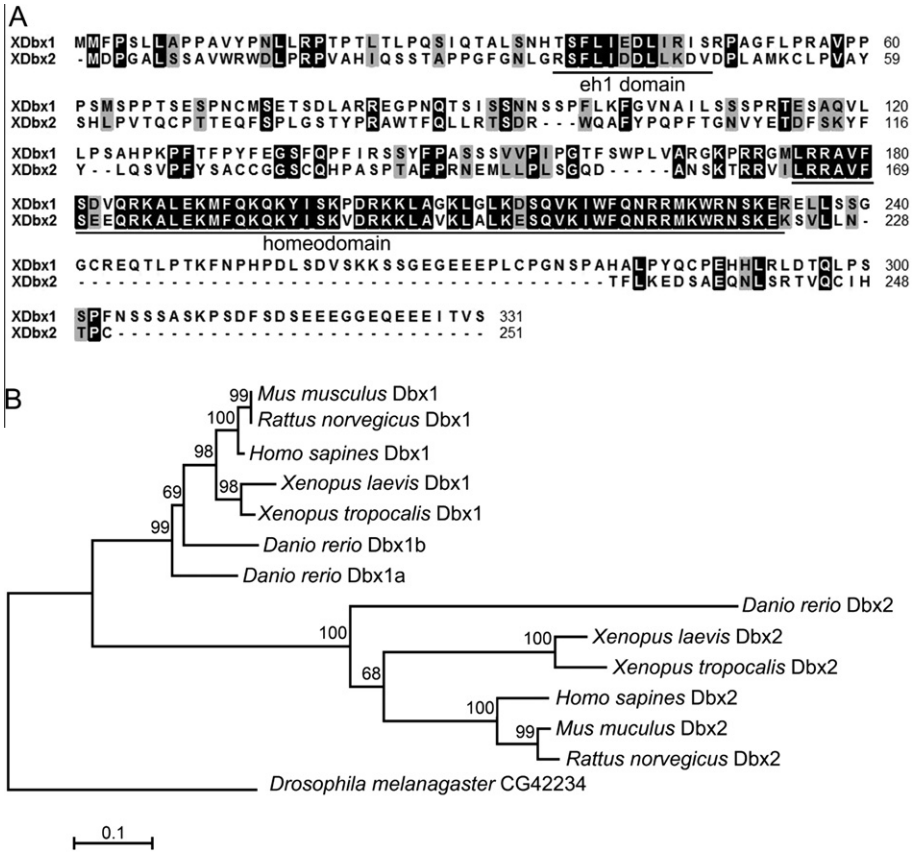

Fig. 1. Alignment and phylogenetic analysis of *X. laevis* Dbx proteins. (A) Alignment of the XDbx1 and XDbx2 protein sequences. Identical amino acids are high-lighted by black background. The conserved eh1 domain and homeodomain are underlined. (B) Phylogenetic analysis of human, rat, mouse, *X. tropicals* and *laevis*, zebrafish and *Drosophila* Dbx family proteins. ClustalW alignment, Poisson correction model, and Bootstrap test (500 replicates) were used for the neighbor-joining (NJ) tree construction. The accession number of the proteins used are *Mus musculus* Dbx1, Ensembl protein ID: ENSMUSP00000032717; *Mus musculus* Dbx2, Ensembl protein ID: ENSMUSP00000060424; *Rattus norvegicus* Dbx1, Ensembl protein ID: ENSRNOP00000019739; *Rattus norvegicus* Dbx2, Ensembl protein ID: ENSRNOP00000009143; *Homo sapines* Dbx1, Ensembl ID: ENSP00000227256; *Homo sapines* Dbx2, Ensembl protein ID: ENSP00000331470; *Danio rerio* Dbx1a, NP_571233; *Danio rerio* Dbx1b, Ensembl protein ID: ENSDARP00000013350; *Danio rerio* Dbx2, Ensembl protein ID: ENSDARP00000057729; *Xenopus tropocalis* Dbx1, XP_002940015; *Xenopus tropocalis* Dbx2, XP_002932867; *Xenopus leavis* Dbx1, NP_001079210.

3.4. Xenopus Dbx2 is involved in primary neurogenesis

In *Xenopus* embryos, over-expression of XDbx1 can inhibit primary neural differentiation by altering the neural differentiation function of Xash3 [9]. As expected, over-expression of *XDbx2* also inhibited the expression of *N-tubulin*, just like *XDbx1* (Fig. 3D–F) [9]. The homeodomain of XDbx1 has been shown to be both necessary and sufficient for the primary neurogenesis inhibition activity [9]. The Dbx2 homeodomain alone (HD), however, failed to inhibit *N-tubulin* expression. But the HDC construct, containing the HD domain plus the C-terminal domain, was active in neurogenesis inhibition (Fig. 3H,I). The Dbx2 ΔC construct also inhibited *N-tubulin* expression, consistent with its transcription repression activity (Fig. 3G).

To investigate the role of endogenous XDbx during *Xenopus* primary neurogenesis, knockdown experiments were carried out using specific morpholinos (MO) against *XDbx1* and *XDbx2*. The inhibitory efficiency of the MOs was checked by co-injection of the MOs with EGFP mRNAs carrying the targeted sequences. Both MOs inhibited the expression of the reporter EGFP effectively (data not shown). In *XDbx1* MO injected embryos, the medial and intermediate stripes of *N-tubulin* were reduced. Interestingly, the lateral stripe of *N-tubulin* was expanded compared with the un-injected sides (93%, $n = 28$; Fig. 4J), partially supporting an inhibitory role of XDbx1 in neurogenesis *in vivo*. The expression of *N-tubulin* was down-regulated in the injected areas in *XDbx2* morphants (75%, $n = 28$; Fig. 4K). When both *XDbx1* and *XDbx2* were knocked-down, the primary neurogenesis was inhibited more severely compared to single gene knock-down (100%, $n = 34$; Fig. 4L). *Islet1* is another marker for primary neurogenesis which marks the medial motor neurons and the dorsal interneurons [20]. In *XDbx1*, *XDbx2* and *XDbx1/2* morphants, the expression of *Islet1* in the medial motor neurons was also inhibited (Fig. 4M,N,O). These results indicate that the Dbx genes are likely involved in a finely tuned network regulating primary neurogenesis in *Xenopus,* either over-expression or knockdown of Dbx will interfere with neurogenesis.

3.5. Conserved function of XDbx in the dorsoventral patterning of neural tube

In mouse, Dbx1–Nkx6.2 and Dbx2–Nkx6.1 are suggested to be cross-repressing partners, the cross-repression of which limit their expression boundaries at different level of the neural tube and define the neural progenitor domains [12,13]. Over-expression of *XDbx1* and *XNkx6* repress the expression of each other (Fig. 4A,C,D) [16,17]. Like *XDbx1*, over-expression of *XDbx2* also inhibited the expression of *Nkx6.2* (Fig. 4B). In knockdown experiments, the expression domains of *XNkx6.2* expanded laterally in *XDbx1* or *XDbx2* morphants (60%, $n = 35$ and 61%, $n = 33$; Fig. 4E,F,H), suggesting a requirement of XDbx1 and XDbx2 in the limitation of dorsal boundary of *XNkx6.2*. When *XDbx1* MO and *XDbx2* MO were injected

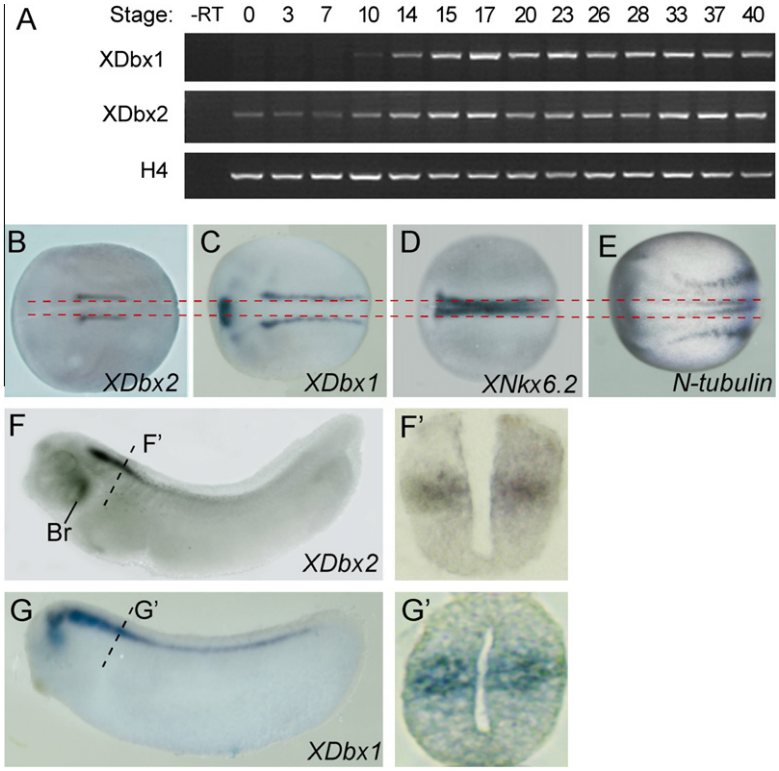

Fig. 2. The temporal and spatial expression patterns of *XDbx*. (A) RT-PCR analysis of the developmental expression of *XDbx1* and *XDbx2*. (B–G) The expression patterns of *XDbx1* and *XDbx2* revealed by *in situ* hybridization. (B–E) Stage 15, dorsal view, anterior to the left. The red broken lines indicate the expression boundary of *XDbx2*. (F–G) Stage 32, lateral view, anterior to the left. The black broken lines indicate the position of corresponding sections showed in F' and G'. Br, branchial arches.

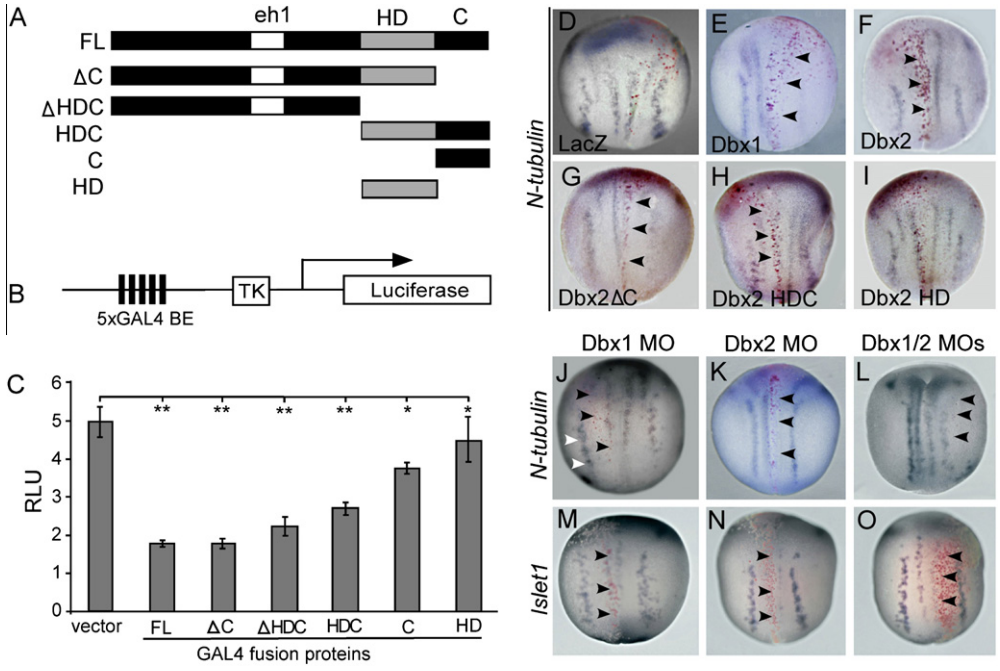

Fig. 3. XDbx2 acts as a transcriptional repressor *in vitro* and is involved in *Xenopus* primary neurogenesis. (A) The schematic structures of XDbx2 and its truncated constructs. (B) Schematic diagram of the pGAL4-TK-Luc reporter construct. GAL4 BE, GAL4 binding elements. (C) Luciferase reporter assay showing the transcriptional repressor activities of XDbx2 and its truncates. HEK293T cells were transiently transfected with 100 ng pGAL4-TK-Luc; 10 ng pRL-TK and 90 ng indicated plasmids per well of 96-well plate. RLU, relative light units. $^{*}p < 0.05$; $^{**}p < 0.001$. (D–I) *In situ* hybridization with *N-tubulin* in embryos injected on one side with control *LacZ* mRNA and the indicated XDbx constructs. The arrowheads indicate the reduction of *N-tubulin* expression in the injected areas. (J–L) The expression of *N-tubulin* in embryos injected on one side with *XDbx1* MO (J), *XDbx2* MO (K) and *XDbx1/2* MOs (L). The black arrowheads indicate the reduction of *N-tubulin* expression while the white arrowheads in (J) indicate the expansion of the lateral *N-tubulin* expression domain. (M–O) The expression of *XIslet1* in the medial stripes was reduced on the sides injected with *XDbx1* MO (M), *XDbx2* MO (N) or *XDbx1/2* MOs (O). *LacZ* mRNA was co-injected for tracing the injected sides which were stained in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

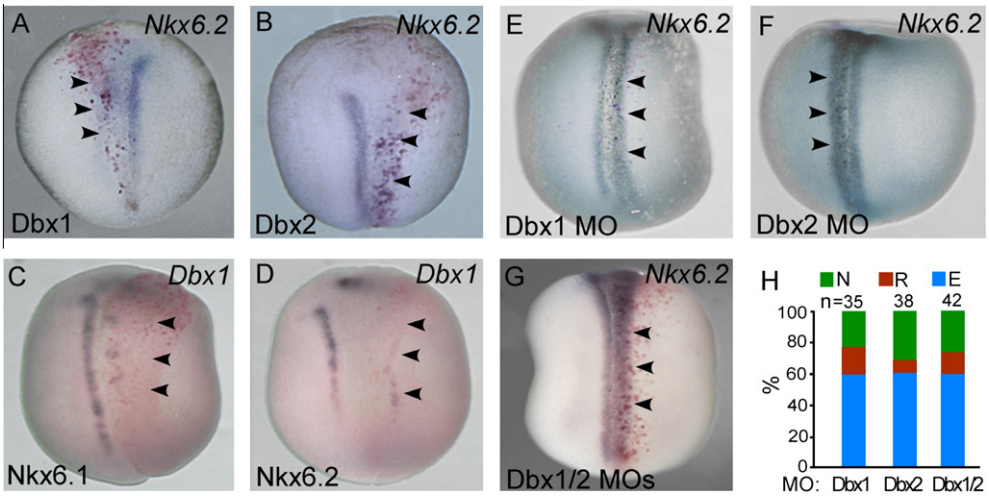

Fig. 4. Cross-repression between *XDbx* and *XNkx6* during neural patterning in *Xenopus* embryos. (A, B) Exogenous XDbx1 or XDbx2 repressed the expression of *XNkx6.2*. (C, D) *XDbx1* was down regulated in *XNkx6.1* or *XNkx6.2* mRNA injected area. (E, F) The expression domains of *XNkx6.2* expanded on the sides injected with *XDbx1* MO (E), *XDbx2* MO (F) or *XDbx1/2* MOs (G). (H) Statistics of the embryos shown in E–G. The percentage of embryos with normal (N), reduced (R) and expanded (E) *Nkx6.2* expression are presented by green, red and blue boxes, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

together, similar expansion of *XNkx6.2* expression was observed with similar percentage (60%, $n = 42$ and Fig. 4G,H). These data support a repressive role for Dbx on the expression of *Nkx6* during the patterning of the ventral neural tube in *Xenopus*. Unexpectedly, reduction of *XNkx6.2* expression was also observed in some Dbx-MO injected embryos at a low percentage (5–17%) (Fig. 4H).

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