

The doublesex-related gene, *XDmrt4*, is required for neurogenesis in the olfactory system

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The *Dmrt* genes encode a large family of transcription factors whose function in sexual development has been well studied. However, their expression pattern is not restricted to the gonad, suggesting that *Dmrt* genes might regulate other developmental processes. Here, we report the expression and functional analysis of one member of this family: *Xenopus Dmrt4* (*XDmrt4*). *XDmrt4* is initially expressed in the anterior neural ridge and then becomes progressively restricted to part of the telencephalon and the olfactory placode/epithelium. *XDmrt4* is induced at the anterior neural plate by a balance of neural inducers and caudalizing factors. Interference with *XDmrt4* function by injection of a morpholino oligonucleotide or an inhibitory mutant resulted in a similar phenotype, the specific disruption of the olfactory placode expression of *Xebf2* without affecting the expression of other placodal markers. *Xebf2* belongs to a family of helix-loop-helix transcription factors implicated in neuronal differentiation, and later in embryogenesis *XDmrt4*-deficient embryos show impaired neurogenesis in the olfactory epithelium. Consistent with this finding, *XDmrt4* is sufficient to activate neurogenin, *Xebf2*, and neural cell adhesion molecule expression in animal explants and is required for Noggin-mediated neuralization. Altogether, these results indicate that *XDmrt4* is an important regulator of neurogenesis in the olfactory system upstream of neurogenin and *Xebf2*.

placode | *Xenopus* | *Xebf2* | forebrain | neurogenin

Genes related to the *Drosophila doublesex* and *Caenorhabditis elegans mab-3* genes encode transcription factors conserved during evolution (1). They constitute the *Dmrt* (doublesex and *mab-3*-related transcription factor) gene family, a class of molecules characterized by a signature zinc finger-like DNA-binding motif known as the DM domain (2).

Dmrt genes have been shown to regulate sexual development in arthropods, nematodes, and vertebrates and, as such, represent a rare example of genes whose function in sex regulation has been highly conserved during evolution (3). *Dmrt1* was the first DM domain gene identified in vertebrates (4). In the mouse, *Dmrt1* is expressed in the genital ridge, and upon sexual differentiation its expression decreases in the ovary and is maintained in the testis (4). Mice with a targeted deletion of *Dmrt1* show normal development in XX individuals, whereas genetically male individuals have severely hypoplastic testes (5). In humans, DMRT1 maps to the short arm of chromosome 9, and hemizygous deletion of this region is associated with defective testicular development (6, 7). *Dmrt1* has also been isolated in fish (8–13), amphibians (14), reptiles (15), and marsupials (16), and *Dmrt1* expression pattern in these species is consistent with a role in testis differentiation.

There is accumulating evidence from different phyla that more than one *Dmrt* gene could be involved in sexual development. For example, *Dmrt3*, *Dmrt5*, and *Dmrt7* also exhibit sexually dimorphic expression in the early embryonic gonad (17). Conversely, several *Dmrt* family members are expressed in multiple tissues in addition to the gonads, suggesting that *Dmrt* genes are not strictly involved in sexual development. For example, the zebrafish gene *terra* and its mouse homolog *Dmrt2* both are expressed in the presomitic mesoderm and newly formed somites (18). *Dmrt3* from chick and mouse (19) has similar expression domains in the forebrain, spinal

cord, and nasal placode. However, the precise function of these genes in nongonadal tissues remains largely unknown.

Here, we analyze the expression and function of *Xenopus Dmrt4* (*XDmrt4*). *XDmrt4* is first detected in a domain within the preplacodal ectoderm and then becomes restricted to the forebrain and olfactory placode. Altered *XDmrt4* function by injection of morpholino antisense oligonucleotide or an inhibitory mutant resulted in a specific inhibition of the olfactory placode expression of *Xebf2*, leading to impaired neurogenesis in the olfactory epithelium. We propose that *XDmrt4* is a key regulator of neurogenesis in the olfactory system.

Materials and Methods

Isolation of *XDmrt4* and Plasmid Constructions. Degenerate primers were designed within the DM domain (forward: TCSCCIMG-SMYRCCSAAITGYGC; reverse: CYMARRGCIACYTGIGC-IGCCAT) to amplify *Dmrt* genes from early neurula-stage *Xenopus* cDNA. PCR conditions were 30 s at 94°C, 40 s at 63°C, and 1 min at 72°C for 35 cycles. Several 160-bp products were isolated, cloned into pGEMTeasy (Promega), and sequenced. A second set of primers was generated (forward: CAACCATGGTGTGCGTATC-AGCC; reverse: TTTTGTCGCTGGAGGGACTGTTCA) to amplify the 3' end by RACE (Clontech). This PCR product was used to screen a stage-17 lambda ZAPII cDNA library (a gift from Michael King, Indiana University, Terre Haute) to isolate a full-length clone. The sequence of *Xenopus Dmrt4* has been deposited in the GenBank database (accession no. AY648303). The ORF of *XDmrt4* cDNA was subcloned into pCS2+ expression plasmid. A mutated version of *XDmrt4* (*muXDmrt4*) was generated by PCR. In this construct 6 bp were mutated 3' of the ATG, (TATGGATG-TAAGTCCCCCAAACATG), within the recognition motif for the morpholino oligonucleotide (see below). These mutations did not affect the amino acid composition of *XDmrt4* protein. An inhibitory mutant of *XDmrt4* (*XDmrt4ΔC*), in which the C-terminal domain was deleted at amino acid 141, was generated by PCR and cloned into pCS2+. All constructs were sequenced and the corresponding proteins were monitored by using an *in vitro* transcription/translation-coupled rabbit reticulocyte lysate system (Promega) in the presence of [³⁵S]methionine and resolved on a NuPAGE BIS-Tris gel (Invitrogen).

***Xenopus* Embryo Injections.** Embryos were staged according to Nieuwkoop and Faber (20). Xwnt-3a (0.1 ng; ref. 21), Noggin (0.2 ng; ref. 22), fibroblast growth factor 8 (Fgf8) (0.1 ng; ref. 23), Xdkk1 (0.1 ng; ref. 2), β-gal (1 ng), *XDmrt4* (1 ng), and *XDmrt4ΔC* (1 ng)

Abbreviations: *Dmrt*, doublesex and *mab-3*-related transcription factor; *XDmrt4*, *Xenopus Dmrt4*; Fgf, fibroblast growth factor; NCAM, neural cell adhesion molecule; RA, retinoic acid.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY648303).

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mRNAs were synthesized by using the Message Machine kit (Ambion, Austin, TX). For plasmid injections, a CS2+Xwnt-3a (0.1 ng) construct was used (25). *XDmrt4* (CATGTTGGGCTTTT-GCAGTCCATG), β -catenin (26), and control morpholino oligonucleotides were obtained from Gene Tools, Philomath, OR. Synthetic mRNAs, plasmid DNA, or morpholinos were injected in one animal dorsal blastomere at the eight-cell stage to target the presumptive nasal placode (27). For animal explant experiments, both blastomeres of two-cell stage embryos were injected in the animal pole, and explants were dissected at the blastula stage, cultured *in vitro*, and analyzed by RT-PCR.

Lineage Tracing and Whole-Mount *In Situ* Hybridization. In all experiments, embryos were coinjected with β -gal mRNA to identify the manipulated side. Antisense digoxigenin-labeled probes (Genius kit, Roche Diagnostics) were synthesized by using template cDNA encoding *XDmrt4*, *XBF-1* (28), *Xebf2* (29), *Xdlx5* (30), *XEmx2* (31), *Six1* (32), *Xebf3* (33), *Slug* (34), and *Pax6* (35). Embryos at the appropriate stage were successively processed for Red-Gal (Research Organics) staining and *in situ* hybridization (36).

Analysis of Gene Expression by RT-PCR. Total RNAs extracted from adult tissues or animal caps were reverse-transcribed and amplified by using primers for *XDmrt4*, *XBF1*, and elongation factor 1 α (*EF-1 α*) (see *Supporting Text*, which is published as supporting information on the PNAS web site) and the products were analyzed on agarose gel. For real-time RT-PCR, total RNAs from animal caps were extracted by using a RNeasy micro kit (Qiagen, Valencia, CA), and real-time RT-PCR (LightCycler, Roche Diagnostics) was performed by using specific primer sets to quantify *neurogenin*, *Xebf2*, neural cell adhesion molecule (*NCAM*), and *EF-1 α* transcript levels (see *Supporting Text*).

Results

Cloning of *Xenopus Dmrt4*. We used degenerate PCR to isolate *Dmrt* family members from neurula-stage *Xenopus* embryos. Among the several PCR products obtained, one was identified as *Dmrt4*, based on its homology to the human (37) and murine (17, 38) *Dmrt4* DM domains. A 660-bp product was subsequently isolated by RACE and used to screen a stage-17 *Xenopus* cDNA library. All positive clones isolated contained a 1,344-bp ORF encoding a predicted 448-aa protein (see Fig. 7A, which is published as supporting information on the PNAS web site). The longest clone also contains 209 bp of 5' UTR and 230 bp of 3' UTR. At the amino acid level this clone shares highest identity with human *Dmrt4* (46%; ref. 37), mouse *Dmrt4* (43%; refs. 17 and 38), platyfish *Dmrt4* (43%; ref. 39), and Medaka *Dmrt4* (40%; ref. 39). Based on these observations we named this gene *XDmrt4* (see Fig. 7B). RT-PCR analysis of adult organs (see Fig. 7C) reveals abundant *XDmrt4* transcripts in the brain and the testis, whereas *XDmrt4* is undetectable in the lung, liver, skeletal muscle, or heart. This finding is consistent with the adult tissue distribution reported for other *Dmrt* family members across species (17).

***XDmrt4* Is Expressed in the Developing Olfactory System.** In whole-mount *in situ* hybridization *XDmrt4* is first detected at the early neurula stage (stage 14) in the anterior-most region of the neural plate (Fig. 1A) in a subdomain of the preplacodal ectoderm known as the anterior neural ridge (40). *Six1*, a gene homologous to *Drosophila sine oculis*, is expressed in the entire preplacodal ectoderm (Fig. 1B and ref. 32). Double *in situ* hybridization using *XDmrt4* and *Six1* probes illustrates that *XDmrt4* is expressed in a subdomain of the preplacodal ectoderm (Fig. 1E). Around stage 17, expression begins to fade at the midline but persists bilaterally in the prospective region of the olfactory placodes (Fig. 1F). The paired box gene *Pax6* is expressed in the developing eye field and the prospective olfactory placode (35) and overlaps with *XDmrt4* in the latter region (Fig. 1F and G). Double *in situ* hybridization using

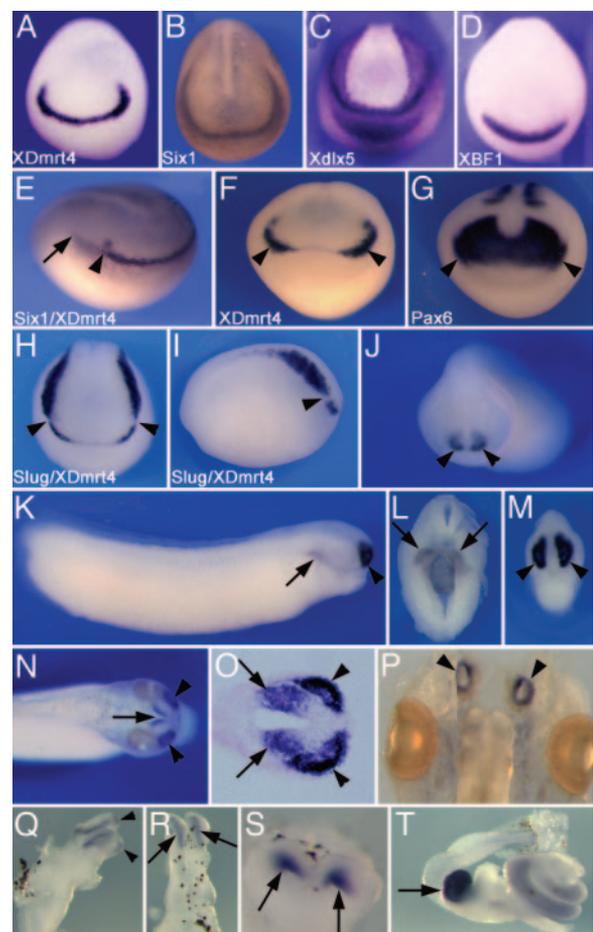


Fig. 1. Developmental expression of *XDmrt4* by whole-mount *in situ* hybridization. (A) *XDmrt4* expression at the early neurula stage is detected at the anterior edge of the neural plate. (B–D) *Six1* (B), *Xdlx5* (C), and *XBF1* (D) expression at a similar stage are shown for comparison. (E) Early neurula stage embryo stained with both *XDmrt4* and *Six1* probes show that *XDmrt4* is expressed in a subdomain of the preplacodal ectoderm. The arrowhead indicates the posterior boundary of *XDmrt4*, and the arrow marks the posterior domain of *Six1*. (F and G) Comparison of *XDmrt4* (F) and *Pax6* (G) expression in sibling embryos illustrates that both genes are expressed in the presumptive olfactory placode (arrowheads). (H and I) Double *in situ* hybridization showing the relationship of the expression domain of the neural crest-specific gene *Slug* and *XDmrt4* at stage 17. The arrowheads indicate the posterior and anterior boundary of *XDmrt4* and *Slug*, respectively. Views are dorsal-anterior (A–D and F–H) and lateral (E and I, anterior to right). (J) At stage 23, *XDmrt4* is detected bilaterally in the prospective nasal placode (arrowheads), anterior view. (K–M) By stages 29/30 *XDmrt4* is strongly detected in the developing olfactory organ and the telencephalon (arrowheads). At this stage *XDmrt4* is also detected in the foregut (arrows, K and L). Lateral (K) and anterior (M) views, dorsal to top. (L) Transverse section. (N and O) In stage-35 embryos *XDmrt4* expression domains in the telencephalon (arrows) and the olfactory organ (arrowheads) are segregated. (N) Dorsal view, anterior to right. (O) Longitudinal section through the olfactory epithelium and the telencephalon. (P–S) At stage 45, *XDmrt4* expression persists in the olfactory epithelium (arrowheads, P), the olfactory bulb (arrowheads, Q), and forebrain (arrows, R and S). Whole-brain lateral (Q) and dorsal (R) views. (S) Transverse section through the forebrain. (T) *XDmrt4* is also strongly expressed in the gall bladder (arrow) and more diffusely throughout the intestine. (Magnifications: $\times 10$ in A–D, H, and I; $\times 12$ in E, F, and J–N; $\times 20$ in O and S; and $\times 15$ in P, Q, and T.)

the neural crest marker *Slug* (34) indicates that the posterior domain of expression of *XDmrt4* abuts *Slug* anterior expression domain without any apparent overlap (Fig. 1H and I). After neural tube closure *XDmrt4* is strongly expressed in the olfactory placode

and the forebrain (Fig. 1J). Around stage 29/30 *XDmrt4* expression persists in the developing olfactory organ and forebrain and is also detected in the wall of the foregut (Fig. 1K–M). At stage 35 the *XDmrt4* expression domains in the olfactory organ and the telencephalon can be clearly identified (Fig. 1N and O). At the swimming tadpole stage (stage 45), *XDmrt4* is still detected in the olfactory epithelium (Fig. 1P), the olfactory bulb, and part of the telencephalon (Fig. 1Q–S). *XDmrt4* is also strongly expressed in the gall bladder and more diffusely throughout the intestine (Fig. 1T).

Several genes are expressed in the developing olfactory placodes (41). Among these are the winged helix transcription factor *XBF1* (28), the distal-less related homeobox gene *XDlx5* (30), a gene related to *Drosophila* empty spiracles, *XEmx2* (31), and the helix-loop-helix factor *Xebf2* (29). At the neurula stage *XDlx5* and *XBF1* are also detected in the anterior neural ridge (Fig. 1C and D). However, *XBF1* expression appears broader than that of *XDmrt4* at the anterior neural plate (Fig. 1A and D), probably reflecting its expression in most of the prospective dorsal telencephalon (28). *XDlx5* expression extends more posteriorly around the entire neural plate and shows expression in the prospective cement gland (Fig. 1C and ref. 30). The expression of *Xebf2* and *XEmx2* in the presumptive olfactory placode is weak at the neurula stage; however, expression becomes more prominent around stage 20 (data not shown and refs. 29 and 33).

We examined sections of stage-35 embryos stained by whole-mount *in situ* hybridization to further analyze the forebrain and placodal expression domains of these marker genes. *XDmrt4*, *XBF1*, and *XEmx2* are expressed in the olfactory placode and telencephalon. Whereas *XBF1* expression in the forebrain includes the entire dorsal telencephalon, *XEmx2* is excluded from the most anterior tip of the dorsal telencephalon and *XDmrt4* localizes to the posterior half of the dorsal telencephalon (see Fig. 8, which is published as supporting information on the PNAS web site). *Xebf2* is expressed in the olfactory placode and appears to be excluded from the telencephalon (see Fig. 8); however, it is also detected in the mesencephalon and the rhombencephalon (data not shown and ref. 29).

***XDmrt4* Is Induced by Noggin and Repressed by Wnt and Fgf.** *XDmrt4* is expressed in the anterior region of the neural plate, suggesting that *XDmrt4* is induced in response to neural-inducing signals. To test this possibility, blastula-stage animal explants were injected with mRNA encoding the neural inducer Noggin (22) and analyzed by RT-PCR at equivalent stage 17 (Fig. 2A). Noggin-injected explants showed strong activation of *XDmrt4* and *XBF1* (Fig. 2B) consistent with the view that induction of anterior fates requires inhibition of Bmp signaling in the ectoderm. Wnt and Fgf signaling have been shown to suppress anterior neural fate to generate posterior neural structures (42), and coinjection of Wnt-3a or Fgf8 mRNAs blocked Noggin-mediated induction of *XDmrt4* and *XBF1* in these explants (Fig. 2B). These results suggest that a balance of both neuralizing and caudalizing signals is likely to be involved in defining the *XDmrt4* expression domain *in vivo*.

Anterior Expression of *XDmrt4* Requires Inhibition of Caudalizing Factors. The exclusion of Wnt signaling from the anterior neural plate is required to establish anterior neural character (42). To investigate whether Wnt signaling functions in restricting *XDmrt4* to the anterior neural ridge, we analyzed *XDmrt4* expression in embryos in which Wnt signaling was experimentally manipulated. Embryos injected with Wnt-3a expression plasmid (used in lieu of RNA to avoid axis duplication) show a dramatic reduction of *XDmrt4* (70% of the embryos; $n = 49$) on the injected side (Fig. 3). A reduction in the expression of *XBF1* (71%, $n = 63$), *Six1* (100%, $n = 26$), *Xebf2* (58%, $n = 36$), and *XEmx2* (85%, $n = 39$) was also observed in these embryos (Fig. 3). β -Catenin is a downstream component of the canonical Wnt pathway. Blockage of Wnt signaling by injection of a β -catenin morpholino in one animal

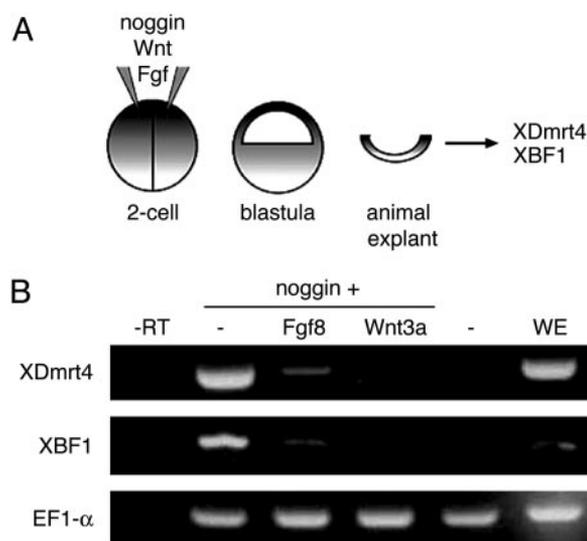


Fig. 2. Signaling pathways involved in inducing *XDmrt4*. (A) Two-cell stage embryos are injected in both blastomeres with a combination of Noggin, Wnt3a, and Fgf8 mRNAs. Animal explants are dissected at the blastula stage, cultured until neurula stage, and analyzed by RT-PCR for expression of *XDmrt4* and *XBF1*. (B) Noggin induces strong expression of *XDmrt4* and *XBF1*. Coinjection of Fgf8 or Wnt3a severely reduces Noggin-mediated *XDmrt4* and *XBF1* induction. Controls include uninjected animal explant (–), whole embryo (WE) at stage 17, and reaction minus reverse transcriptase (–RT). Elongation factor 1 α is used as a loading control.

dorsal blastomere at the eight-cell stage resulted in an enlargement and a posterior expansion of the *XDmrt4* expression domain (Fig. 3) in 69% of the embryos ($n = 77$). A posterior expansion of *Six1* (80%, $n = 51$) and *XBF1* (92%, $n = 12$) was also observed in these embryos, which correlates with an enlargement of the olfactory placode expression domain of *Xebf2* (47%, $n = 114$) and *XEmx2* (68%, $n = 41$) at the tail bud stage (Fig. 3). Similarly, overexpression of Dickkopf1 (*Xdkk1*), a secreted Wnt inhibitor expressed in the head region (24), resulted in embryos with a dramatic expansion of all five marker genes, usually on both sides of the injected embryos (Fig. 3), presumably because of *Xdkk1* protein diffusion.

Other factors involved in establishing anterior-posterior patterning in the neuroectoderm include Fgf and retinoic acid (RA) (42). Injection of Fgf8 mRNA resulted in a reduction of *XDmrt4* (83%, $n = 123$), *XBF1* (90%, $n = 102$), and *Six1* (100%, $n = 32$) expression at stage 15 (Fig. 3), as well as a reduction of *Xebf2* (82%, $n = 28$) and *XEmx2* (98%, $n = 41$) at stage 25. Similarly, exposure to RA (see *Supporting Text*) led to a complete loss of *XDmrt4* (see Fig. 9, which is published as supporting information on the PNAS web site). Altogether, these results indicate that *XDmrt4* is induced in the anterior neural ridge by neural inducers (Bmp antagonists) and that a proper balance of caudalizing factors (Wnt, Fgf, and RA) is required for restricting the *XDmrt4* expression domain anteriorly.

***XDmrt4* Is Required for *Xebf2* Expression in the Olfactory Placode.** To determine whether *XDmrt4* is required for olfactory placode development, we used a morpholino antisense oligonucleotide (*XDmrt4*-mo) designed to interfere with *XDmrt4* translation. The specificity of *XDmrt4*-mo was tested in an *in vitro* transcription/translation reaction directed by WT *XDmrt4* and *muXDmrt4* (a construct that has 6-bp mutations within the morpholino recognition motif). Increasing amounts of morpholino can completely block translation of *XDmrt4* mRNA in this assay, but failed to interfere with translation of *muXDmrt4* mRNA (Fig. 4A).

Upon injection of 20–40 ng of *XDmrt4*-mo in one animal dorsal blastomere at the eight-cell stage, 40% of the embryos ($n = 163$) showed a dramatic reduction or a complete loss of *Xebf2* expression

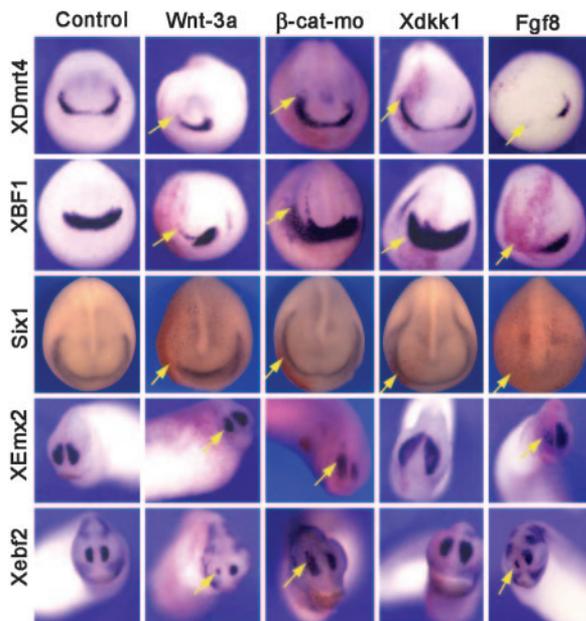


Fig. 3. Caudalizing factors restrict *XDmrt4* expression domain. Activation of Wnt signaling pathway by injection of Wnt-3a blocks *XDmrt4*, *XBF1*, and *Six1* expression at the neurula stage. Conversely, inhibition of Wnt signaling pathway by injection of β -catenin morpholino (β -cat-mo) or *Xdkk1* mRNA expands posteriorly *XDmrt4*, *XBF1*, and *Six1* placodal expression domains. At the tail bud stage, the same manipulations of the Wnt signaling pathway resulted in a reduction or an expansion of *Xebf2* and *XEmx2* expression in the nasal placode. In most cases, *Xdkk1* injection leads to an expansion of these markers on both sides of the injected embryos presumably because of *Xdkk1* protein diffusion. Overexpression of *Fgf8* completely blocks *XDmrt4*, *XBF1*, and *Six1* placodal expression at the neurula stage, as well as the nasal placode expression of *Xebf2* and *XEmx2* at the tail bud stage. Anterior views are shown, dorsal to the top. RNA encoding the lineage tracer β -gal was coinjected in all cases to identify the injected side (red staining, left, arrows). (Magnifications: $\times 8$.)

at stages 25–28 (Fig. 4 C and D and see Table 1, which is published as supporting information on the PNAS web site). *Xebf3*, another member of the Olf-1/Ebf family of helix–loop–helix transcription factors (33), was also reduced in 48% ($n = 40$) of *XDmrt4*-mo-injected embryos (Fig. 4E). By contrast, *XEmx2* expression was unperturbed in these embryos (Fig. 4F), as was the early (stage 14/17) and late (stage 25) expression of *XBF1* and *Dlx5* (Fig. 4 G–J). Similarly, *Six1* expression was unaffected in these embryos (data not shown; 98%, $n = 83$). Importantly, injection of a control morpholino at the same concentration had no effect on the expression of *Xebf2* (Fig. 4B and Table 1).

Attempts to rescue the *Xebf2* phenotype by injection of an *XDmrt4* expression plasmid or mRNA with a mutated morpholino recognition motif (*muXDmrt4*) were unsuccessful, presumably because sufficient levels of *XDmrt4* proteins could not be obtained under these conditions without compromising the embryos survival. However, to further validate the phenotype of *XDmrt4*-depleted embryos we generated a truncated version of *XDmrt4*, lacking the C-terminal region (Fig. 4K, *XDmrt4* Δ C). This construct, which retains the DNA binding (DM) domain, is predicted to block *XDmrt4* function, and possibly other *Dmrt* proteins, by acting as a dominant negative (see Fig. 6A). Embryos injected with *XDmrt4* Δ C mRNA displayed a strong reduction of *Xebf2* expression at stages 25–28 (Fig. 4L and Table 1) without affecting the expression of other early and late placodal markers (data not shown), thereby mimicking the phenotype of morpholino-treated embryos. Because *XDmrt4* morpholino and the *XDmrt4* Δ C inhibitory mutant both specifically interfere with *Xebf2* expression, a well

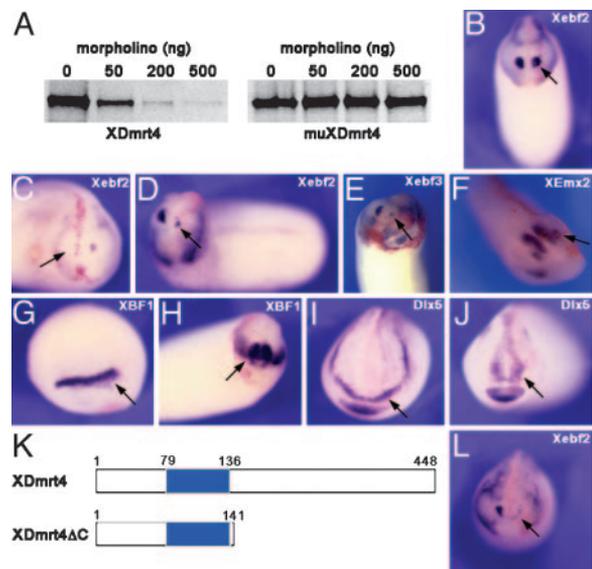


Fig. 4. *XDmrt4*-mo and *XDmrt4* Δ C specifically blocks *Xebf2* expression. (A) *In vitro*-coupled transcription/translation reactions with plasmid encoding WT *XDmrt4* and a mutated version of *XDmrt4* (*muXDmrt4*) in the presence of increasing amounts of *XDmrt4*-mo. (B) Embryo injected with a control morpholino shows normal expression of *Xebf2* on the injected side. (C and D) Injection of 20 ng of *XDmrt4*-mo in one blastomere at the eight-cell stage results in a reduction or loss of *Xebf2* on the injected side. (E) Another Olf/Ebf family member, *Xebf3*, is also reduced upon *XDmrt4*-mo injection. (F) However, the olfactory placode marker, *XEmx2*, remains unaffected under these conditions. (G–J) *XBF1* (G and H) and *Dlx5* (I and J) expression at stage 17 (G and I) or stage 25 (H and J) are unperturbed in *XDmrt4*-mo-injected embryos. (K) Schematic representation of WT and *XDmrt4* deletion construct (*XDmrt4* Δ C). The blue box indicates the position of the DM domain. (L) Injection of 1 ng of *XDmrt4* Δ C mRNA results in a loss of the *Xebf2* expression domain. (B–J and L) RNA encoding the lineage tracer β -gal was coinjected in all cases to identify the injected side (red staining, arrows). Anterior views are shown, dorsal to the top. (Magnifications: $\times 10$.)

established regulator of neurogenesis (43), we predict that *XDmrt4* is likely to function in the differentiation of the placode into an olfactory epithelium.

***XDmrt4* Is Required for Neurogenesis in the Olfactory Epithelium.** To further evaluate the consequences of compromised *XDmrt4* function on neurogenesis in the olfactory system, embryos injected with either *XDmrt4*-mo or *XDmrt4* Δ C mRNA were analyzed for expression of the neuronal marker NCAM by immunocytochemistry at stage 35 (see *Supporting Text*). Both experimental manipulations of *XDmrt4* resulted in embryos with reduced NCAM expression

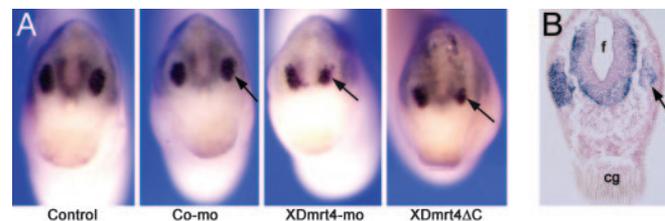


Fig. 5. Impaired neurogenesis in the olfactory epithelium of embryos lacking *XDmrt4* function. (A) *XDmrt4*-mo or *XDmrt4* Δ C mRNA-injected embryos show a marked reduction in NCAM expression in the olfactory epithelium (arrows). Injection of a control morpholino (Co-mo) has no effect on NCAM expression levels. Anterior views are shown, dorsal to the top. (B) Transverse section through the head of an *XDmrt4*-mo-injected embryo stained for NCAM shows a reduced number of neurons in the olfactory epithelium on the injected side (arrow). cg, cement gland; f, forebrain. (Magnifications: $\times 15$.)

formation (47). Our results indicate that attenuation of Bmp signaling in the ectoderm and anterior activity of Wnt, Fgf, and RA antagonists (42) is likely to be involved in inducing and restricting *XDmrt4* expression to the anterior neural ridge. The neural crest is found lateral to the neural plate but excluded from its most anterior region. Interestingly, the set of signals involved in restricting *XDmrt4* anteriorly has also been implicated in the induction of the neural crest at the lateral neural plate border (48). By looking at the expression of the preplacodal gene *Six1* and the neural crest marker FoxD3, a recent study indicates that these signaling pathways have opposite effects on placodal tissue and neural crest formation. Whereas Wnt and Fgf promote neural crest over placodal fate, inhibition of these signaling pathways favors placodal at the expense of neural crest fate (49). Therefore, the relative levels of these factors may influence cells at the neural plate border to become placode or neural crest cells.

Interference with *XDmrt4* function by injection of a morpholino oligonucleotide or an inhibitory mutant resulted in a similar phenotype, the specific reduction/loss of the olfactory expression of *Xebf2*, suggesting a direct relationship between *XDmrt4* and *Xebf2* expression in the olfactory epithelium. Although *Six1*, *XBF1*, *XDlx5*, and *XDmrt4* are coexpressed in the anterior neural ridge, loss of *XDmrt4* did not affect the early or late expression of these genes, suggesting that *XDmrt4* may act downstream of *Six1*, *XBF1*, and *XDlx5* or that these factors are functioning in different pathways during olfactory placode development.

Although *XDmrt4* is detected in the presumptive olfactory placode it appears to be dispensable for olfactory placode induction but required for the later development of the olfactory system. Compensatory activity of other *Dmrt* family members could account for this lack of an early phenotype in *XDmrt4*-deficient animals. Alternatively, this late phenotype may reflect the true function of this class of molecules. In that respect, there is an interesting parallel between our results and *Dmrt1* function in the gonad. In mammals, *Dmrt1* is expressed in the indifferent gonad; however, early events in testis differentiation occur normally in the knockout male mice (which

have defects only in postnatal testis differentiation), indicating that *Dmrt1* may not play a major early role in testis development in mammals (5).

Xebf2 belongs to the Olf/Ebf family of helix-loop-helix transcription factors (43). Both gain-of-function (29, 33) and loss-of-function (50–53) obtained in various model organisms have implicated *Olf/Ebf* genes in key stages of neural development (43). The loss of *Xebf2* expression in *Dmrt4*-deficient embryos correlates with reduced NCAM expression in the olfactory epithelium, suggesting that *Xebf2* controls neurogenesis in the olfactory system downstream of *XDmrt4*. Further analysis will be needed to determine whether these factors regulate the production, differentiation, and/or survival of olfactory neurons and whether other cell types within the olfactory epithelium (basal cells or support cells) are also affected.

The observation that *XDmrt4* is sufficient to activate *neurogenin*, *Xebf2*, and *NCAM* expression in animal explants further supports the view that these factors are acting in the same pathway to promote neurogenesis in the olfactory epithelium. Moreover, Noggin-mediated induction of *neurogenin*, *Xebf2*, and *NCAM* is blocked in the absence of *XDmrt4* function, further demonstrating that *XDmrt4* is a key regulator of neurogenesis in the vertebrate embryo. Based on these findings and other studies that positioned *Xebf2* downstream of neurogenin (29), we propose that *XDmrt4* is an upstream regulator of neurogenin and *Xebf2* in the molecular cascade leading to neuronal differentiation (Fig. 6D). Future studies should be directed at understanding the nature of the regulation of neurogenin and *Xebf2* by *XDmrt4* and defining the mechanism by which these factors control olfactory neuron development.

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