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: TCSCCIMGSMYRCCSAAITGYGC; reverse: CYMARRGCIACYTGIGCIGCCAT) 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: CAACCATGGTGTCGTATCAGCC; 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, (T**ATG**GATTGTAAGTCCCCCAAACATG), 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* (*XDmrt4*Δ*C*), 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 [^{35}S]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 (CATGTTTGGGCTTTTGCAGTCCATG), 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-1α*) (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-1α* 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. 7*A*, 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. 7*B*). RT-PCR analysis of adult organs (see Fig. 7*C*) 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. 1*A*) 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. 1*B* 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. 1*E*). Around stage 17, expression begins to fade at the midline but persists bilaterally in the prospective region of the olfactory placodes (Fig. 1*F*). 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. 1 *F* and *G*). Double *in situ* hybridization using

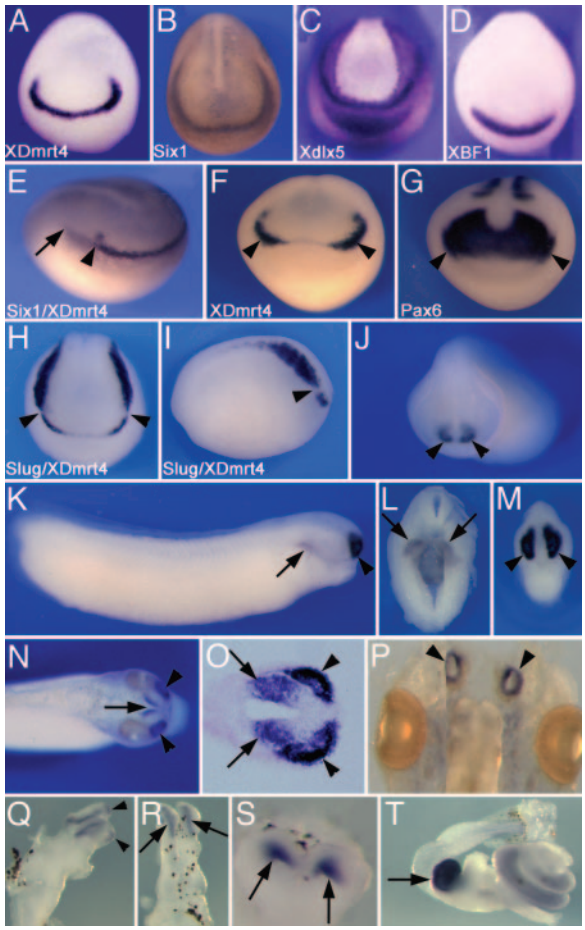

Fig. 1. Developmental expression of *XDmrt4* by whole-mount *in situ* hybridization. (*A*) *XDmrt4* expression at the early neurula stage is detected at the anterior edge of the neural plate. (*B–D*) Six1 (*B*), Xdlx5 (*C*), and XBF1 (*D*) expression at a similar stage are shown for comparison. (*E*) 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: ×10 in *A–D*, *H*, and *I*; ×12 in *E*, *F*, and *J–N*; ×20 in *O* and *S*; and ×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. 1 *H* and *I*). After neural tube closure *XDmrt4* is strongly expressed in the olfactory placode

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

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. 1 *C* and *D*). However, *XBF1* expression appears broader than that of *XDmrt4* at the anterior neural plate (Fig. 1 *A* 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. 1*C* 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. 2*A*). Noggin-injected explants showed strong activation of *XDmrt4* and *XBF1* (Fig. 2*B*) 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 coninjection of Wnt-3a or Fgf8 mRNAs blocked Noggin-mediated induction of *XDmrt4* and *XBF1* in these explants (Fig. 2*B*). 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

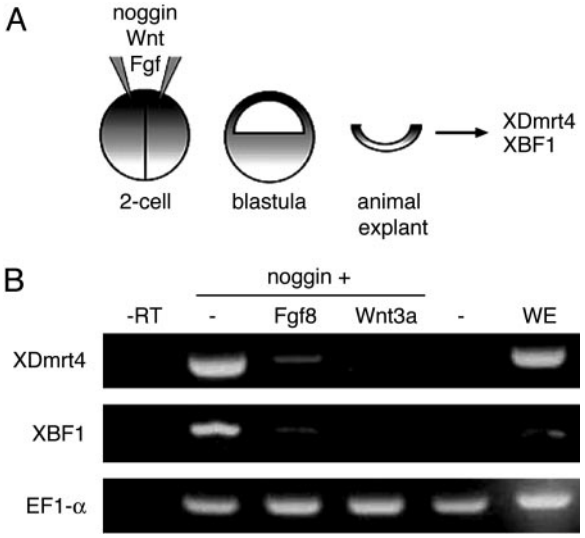

Fig. 2. Signaling pathways involved in inducing *XDmrt4*. (*A*) Two-cell stage embryos are injected in both blastomeres with a combination of Noggin, Wnt3a, and Fgf8 mRNAs. Animal explants are dissected at the blastula stage, cultured until neurula stage, and analyzed by RT-PCR for expression of *XDmrt4* 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 1α 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. 4*A*).

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

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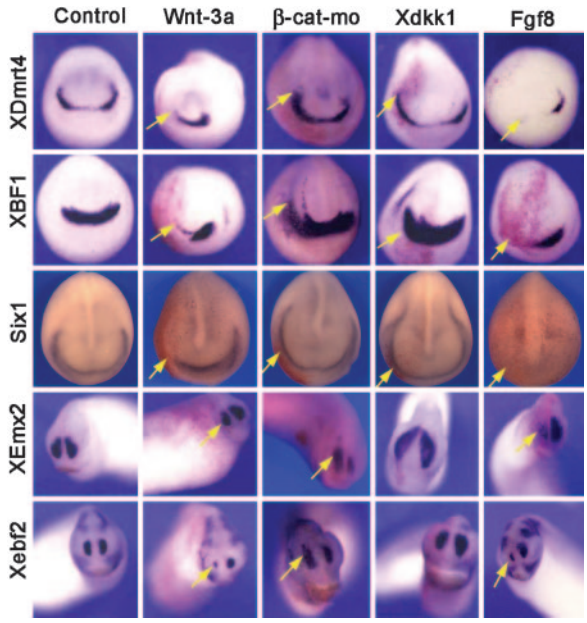

Fig. 3. Caudalizing factors restrict *XDmrt4* expression domain. Activation of Wnt signaling pathway by injection of Wnt-3a blocks *XDmrt4*, *XBF1*, and *Six1* expression at the neurula stage. Conversely, inhibition of Wnt signaling pathway by injection of β-catenin morpholino (β-cat-mo) or Xdkk1 mRNA 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: ×8.)

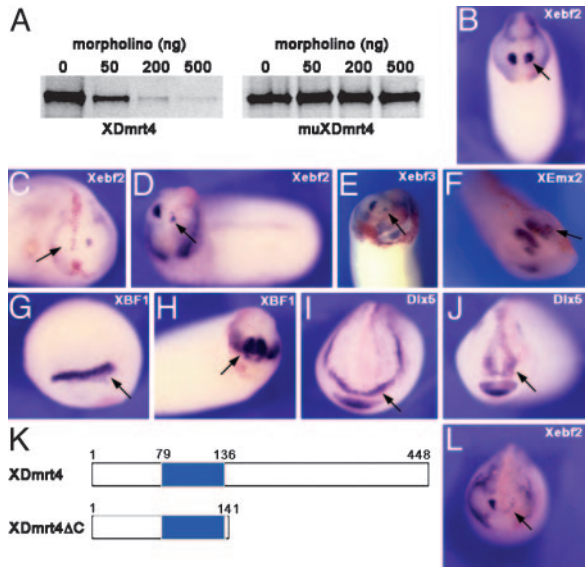

Fig. 4. *XDmrt4-mo* and *XDmrt4*Δ*C* specifically blocks Xebf2 expression. (*A*) *In vitro*-coupled transcription/translation reactions with plasmid encoding WT *XDmrt4* and a mutated version of *XDmrt4* (*muXDmrt4*) in the presence of increasing amounts of *XDmrt4-mo*. (*B*) Embryo injected in with 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: ×10.)

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. 4*E*). By contrast, *XEmx2* expression was unperturbed in these embryos (Fig. 4*F*), 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. 4*B* 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. 4*K*, XDmrt4ΔC). 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. 6*A*). Embryos injected with *XDmrt4*Δ*C* mRNA displayed a strong reduction of *Xebf2* expression at stages 25–28 (Fig. 4*L* 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 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

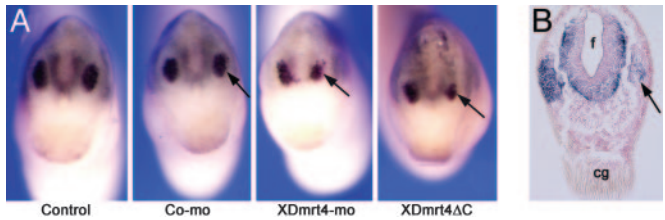

Fig. 5. Impaired neurogenesis in the olfactory epithelium of embryos lacking *XDmrt4* function. (*A*) *XDmrt4-mo* or *XDmrt4*Δ*C* mRNA-injected embryos show a marked reduction in NCAM expression in the olfactory epithelium (arrows). Injection of a control morpholino (Co-mo) has no effect on NCAM expression 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: ×15.)

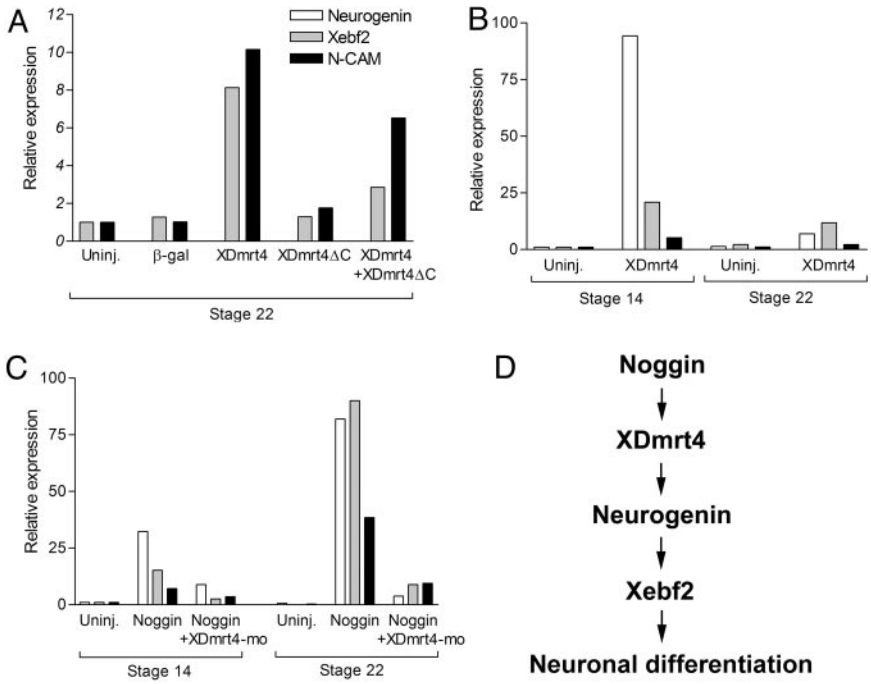

Fig. 6. Regulation of neurogenin, Xebf2, and NCAM expression by *XDmrt4*. (*A–C*) Real-time RT-PCR of animal explants isolated from embryos injected with various combinations of mRNA and morpholinos as indicated and collected when sibling embryos reached stages 14 or 22. (*A*) Injection of *XDmrt4* induces Xeb2 and NCAM expression. This activity is inhibited by coinjection of *XDmrt4*Δ*C*. (*B*) *XDmrt4* induces robust expression of *neurogenin* at stage 14 and to a lesser extend at stage 22. (*C*) Noggin-mediated activation of *neurogenin*, *Xebf2*, and *NCAM* expression is blocked in the context of *XDmrt4*-depeleted embryos (+XDmrt4-mo). (*D*) Based on these findings and other studies that have 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 in the olfactory system.

within the olfactory epithelium (Fig. 5), implying that the differentiation of olfactory neurons was impaired in these embryos. Injections of a control morpholino or a control mRNA (β-gal) had no effect on the level of NCAM (Fig. 5*A* and Table 1). Importantly, we found a strong correlation between the incidence of this phenotype at stage 35 (reduced NCAM expression) and the number of embryos that showed reduced *Xebf2* expression at stages 25–28 (see Table 1), suggesting a link between *XDmrt4*, *Xebf2*, and active neurogenesis in the olfactory epithelium.

***XDmrt4* Is Sufficient to Promote Neurogenesis in Animal Explants.** To determine whether *XDmrt4* is sufficient to activate Xebf2 expression, embryos at the two-cell stage were injected in the animal pole region with *XDmrt4*, *XDmrt4*Δ*C*, or a combination of both *XDmrt4*+*XDmrt4*Δ*C* mRNAs. Animal explants were dissected at the blastula stage, cultured until stage 22, and analyzed by real-time RT-PCR for *Xebf2* and *NCAM* expression. Strong induction of *Xebf2* and *NCAM* was observed in *XDmrt4*-injected animal explants as compared with explants injected with *XDmrt4*Δ*C* or a control mRNA encoding β-gal (Fig. 6*A*). These results provide further evidence that *Xebf2* and *XDmrt4* are acting in the same pathway to promote neurogenesis. *XDmrt4*-mediated induction of *Xebf2* and *NCAM* was inhibited by coinjection of *XDmrt4*Δ*C*, indicating that *XDmrt4*Δ*C* acts as a dominant interfering mutant for *XDmrt4* function (Fig. 6*A*).

The basic helix–loop–helix factor *neurogenin* is an early neuronal determination gene in vertebrate (44). To further evaluate *XDmrt4* neurogenic activity in animal explants we analyzed neurogenin expression in explants injected with *XDmrt4* mRNA and collected at the early neurula stage (stage 14) or tailbud stage (stage 22). *XDmrt4* induces strong expression of *neurogenin* at stage 14 and to a lesser extend at stage 22, thereby positioning *XDmrt4* as a key regulator of neurogenesis in *Xenopus* (Fig. 6*B*). As Noggin is a strong inducer of *XDmrt4* in animal explants (Fig. 2*B*), we tested whether Noggin-mediated neuralization depended on *XDmrt4* function. Injection of Noggin mRNA induces strong expression of *neurogenin*, *Xebf2*, and *NCAM* in explants collected at stages 14 or 22; however, in the context of *XDmrt4*-depleted animal explants the induction of these genes by Noggin was strongly inhibited (Fig. 6*C*), suggesting that *XDmrt4* function is required upstream of *neurogenin* and *Xebf2* for Noggin-mediated neurogenesis.

Discussion

The results presented here strongly argue for a critical role of *XDmrt4* in the development of the olfactory system in *Xenopus*. *XDmrt4* is initially expressed in the anterior neural ridge and later becomes restricted to part of the developing telencephalon and the olfactory placode/epithelium where it promotes neurogenesis by regulating neurogenin and Xebf2 expression. Although *Dmrt* genes are well established regulators of sexual development, we demonstrate here a function for a Dmrt family member in nongonadal tissue.

The anterior neural ridge defines a subdomain of the preplacodal ectoderm at the rostral boundary of the neural plate, and the prospective olfactory placode and some forebrain tissues will develop from this region (40). At the early neurula stage *XDmrt4* expression domain is restricted to the anterior neural ridge and abuts but does not overlap with the anterior domain of the cranial neural crest. As the neural tube closes, *XDmrt4* expression is down-regulated at the midline and persists bilaterally in a region that eventually will segregate into olfactory placodes and part of the dorsal telencephalon. In the fish Medaka, *Dmrt4* is also detected in the olfactory placode and the dorsal telencephalon (45). The expression pattern of *Dmrt4* has not yet been described in other organisms to our knowledge. However, in both mouse and chick, *Dmrt3* appears to be expressed in the telencephalon and the nasal pits (19). Interestingly, whereas *Dmrt3* genes have additional expression domains in the neural tube of mouse, chick, and Medaka, the chicken *Dmrt3* gene is also expressed in the presomitic mesoderm (19). This is not the case in Medaka in which the only *Dmrt* gene expressed in the presomitic mesoderm is *Dmrt2*, similarly to its zebrafish (18) and human (46) orthologs. These divergences in the expression of *Dmrt*s across species illustrate a remarkable shift in expression pattern of this gene family during evolution.

Little is known about the signaling factors involved in olfactory placodes induction. However, given that these placodes derive from the anterior neural ridge, signals that induce forebrain are likely to be important for olfactory placodes induction. Conversely, factors repressing forebrain fate are expected to block olfactory placodes

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 XDIx5 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. 6*D*). 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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