The Xenopus doublesex-related gene Dmrt5 is required for olfactory placode neurogenesis

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ABSTRACT

The *Dmrt* (doublesex and mab-3 related transcription factor) genes encode a large family of evolutionarily conserved transcription factors whose function in sexual development has been well studied. In vertebrates, some *Dmrt* genes also function in nongonadal tissues. For example, *Xenopus Dmrt4* is essential for neurogenesis in the olfactory system. Here we have isolated and characterized *Xenopus Dmrt5*. *Dmrt5* is coexpressed with *Dmrt4* in the developing olfactory placodes. As *Dmrt4*, *Dmrt5* is positively regulated in the ectoderm by neural inducers and negatively by proneural factors. Both *Dmrt5* and *Dmrt4* genes are also strongly activated by the combined action of the transcription factor *Otx2*, broadly transcribed in the head ectoderm and of Notch signaling, activated in the anterior neural ridge. Knockdown of *Dmrt5* induces impaired neurogenesis in the embryonic olfactory system. *Dmrt5* deficiency also impaired Noggin-mediated activation of neuronal differentiation markers in animal caps, a phenotype that can be rescued by overexpression of *Dmrt5* or *Dmrt4*. Consistent with these findings, *Dmrt5* upregulates the expression of the proneural *Ngnr1* and *Ebf2* genes and promotes Xath5 induced neurogenesis in animal caps. Together, these data identify *Dmrt5* as a novel important upstream regulator of neurogenesis and suggest redundant roles for *Dmrt4* and *Dmrt5* during olfactory system development.
INTRODUCTION

Cranial placodes are transient thickening of the embryonic ectoderm that give rise to various sensory ganglia and contribute to the pituitary gland and paired sense organs of the head of vertebrate embryos. Despite their different developmental fates, all placodes derive from a common panplacodal primordium around the anterior neural plate, the preplacodal ectoderm. The olfactory placodes develop posterior to the adenohypophyseal placode that appears at the midline of the anterior neural ridge. The inductive events that lead to olfactory placode development remain unclear. It is believed to be a step-wise process that begins with the specification of the preplacodal ectoderm, a step that requires modulation of the BMP signaling pathway, repression of the lens ground state cell fate and induction of olfactory placode identity, processes in which Fgf5 play an important role (Schlosser, 2006; Streit, 2008; Schlosser, 2010; Park et al., 2010).

A number of transcription factors have been identified that are expressed at different times and in overlapping patterns during the course of olfactory placode specification and that are required for their development. These factors include Otx2, expressed from the end of gastrulation in all germ layers in a broad anterior domain that includes in the ectoderm the prospective adenohypophyseal, olfactory and lens placodes and the adjacent anterior neural plate (Matsuo et al., 1995; Gammill et al., 2001). Proneural genes such as Ngn1, Ebf2 and ATH5 are expressed in neurogenic placodes and absent from the regions of the prospective non neurogenic adenohypophysis and lens placodes (Wang et al., 1997; Wang et al., 2004; Nieber et al, 2009; Green and Vetter, 2011). Several Notch ligands are also expressed in the anterior neural plate border including the olfactory placodes but little is known today about their function in the developing olfactory epithelium (Chitnis et al., 1995; Peres et al., 2006; Schwarting et al., 2007).

The Dmrt (doublesex and mab-3 related transcription factor) genes encode a large family of evolutionarily conserved transcription factors sharing an unusual zinc finger DNA-binding motif known as the DM domain. Dmrt genes are well known for their function in sex determination and sexual dimorphism in invertebrates (Hong et al., 2007; Ross et al., 2005; Kimura et al., 2008). In vertebrates, most Dmrt genes are expressed in the developing gonads. Like doublesex and mab-3, vertebrate Dmrt1 has been shown to play a critical role in mammalian male sex differentiation (Raymond et al., 1998; Matson et al., 2010). However, several of them are also expressed and function in nongonadal tissues. For example, the zebrafish gene terra and its mouse homolog Dmrt2 are expressed in the presomitic mesoderm.
and newly formed somites and play an essential role in somitogenesis (Seo et al., 2005; Seo et al., 2006). *Dmrt3* in chicken and mouse embryos is expressed in the forebrain, spinal cord and nasal placode (Meng et al., 1999; Smith et al., 2002). *Dmrt4* in *Xenopus* is expressed in the developing telencephalon and olfactory epithelium and functions as an upstream regulator of proneural genes in the molecular cascade leading to neuronal differentiation in the olfactory system (Huang et al., 2005). *Dmrt5* is expressed in the developing mouse prosencephalon and in the ventral-medial mesencephalic neuroepithelium. In neuralized embryonic stem cells, Dmrt5 promotes midbrain dopaminergic identity suggesting that it plays an important role *in vivo* in midbrain ventral progenitor fate (Gennet et al., 2011). In the simple chordate *Ciona*, *Dmrt1*, the probable ortholog of vertebrate *Dmrt4* and *Dmrt5*, has been shown to be essential for the development of anterior neural plate derivatives (Tresser et al., 2010) suggesting that vertebrate *Dmrt3-5* genes may play a similar role in vertebrates.

Here we have initiated the characterization of *Dmrt5* in the frog *Xenopus laevis* to approach its function during development. We describe its expression during early embryogenesis, analyse regulatory inputs that control its expression in the ectoderm and the consequences of its knockdown and overexpression on the development of the olfactory epithelium. The data reveal that *Dmrt5* is an important upstream regulator in the cascade leading to neuronal differentiation and suggest redundant roles for *Dmrt5* and *Dmrt4* during olfactory system development.
MATERIAL AND METHODS

Isolation of Dmrt5 cDNA and plasmid constructions

The full length Dmrt5 cDNA clone was isolated by screening a Xenopus laevis tadpole head cDNA Library constructed in ZAP II (Hemmati-Brivanlou et al., 1991) using as a probe a PCR generated 580 bp fragment corresponding to the 5’ end of EST CF290698. DNA sequence alignments and phylogenetic trees were obtained using the Clustal method and the MegAlign (DNASTar, Inc) software program. Gene synten analysis was performed using Metazome (http://www.metazome.net). The ORF of the Xenopus and mouse Dmrt5 cDNA (accession number DQ329358) were subcloned by PCR into the pCS2+ Myc plasmid. The Xenopus Dmrt5ΔC mutant in which the C-terminal domain was deleted at amino acid 146 and the Xenopus Dmrt5ΔDM mutant in which the N-terminal part was deleted until amino acid 139 were generated by PCR and subcloned into the pCS2+ Myc. All constructs were sequenced and the corresponding proteins monitored by overexpression in animal caps and western blot analysis.

Cell culture, transfection and immunolocalization

Cos-7 cells were grown in Dubelco’s modified Eagle’s Medium (Gibco-BRL) containing 10% fetal bovine serum (Gibco-BRL), penicillin (50 U/ml), streptomycin (50 mg/ml) and L-glutamine (2 mM) on coverslips and transfected using FUGENE-6 reagent (Roche) according to the manufacturer’s instructions. 2µg of DNA were transfected in 2 x 10^5 cells with 6µl FUGENE-6. Cells were fixed 24h or 48h after transfection in MEMFA for 15 min, washed two times in PBS and permeabilized in PBS, Triton X-100 0.5% for 10 min. min at 4°C. After two washes in PBS, cells were incubated in PBS plus 2% goat serum for 30 min. at RT. The coverslips were incubated with the primary antibody diluted in the former solution for 1h at RT (anti-Myc,1/500, Sigma) The samples were then washed three times with PBS plus 2% goat serum, and incubated for 1 h in with the secondary antibody (Alexa Fluor 488 goat anti-Mouse IgG, Invitrogen). The samples were then washed three times in PBS and incubated 2-3 min in PBS plus Hoechst 0,0005% before mounting.

EMSA
Double strand probes identical to the A, E and F probes (Yi and Zarkower, 1999) were radiolabeled with [α-32P]dATP by filling in with Klenow fragment. Myc tagged Dmrt5 cloned into the CS2+ vector was synthesized by coupled in vivo translation/transcription using Sp6 polymerase and a TNT kit (Promega). EMSA was performed with proteins incubated with 0,5 \(10^5\) cpm of probe (corresponding to about 0,1 ng DNA) in 20 µl of binding buffer as described (Pichon et al., 2004). For supershifts, 1 µl of Myc antibody (Sigma) was added to the binding buffer. Samples were loaded on 4% acrylamide gels and run in 0,5X TBE. Dried gels were exposed to X-ray films overnight at -80°C in the presence of an intensifying screen.

**Embryo culture, micro-injections and animal cap dissections**

*Xenopus* embryos were obtained from adult frogs by hormone induced egg-laying and in vitro fertilization using standard methods (Sive et al., 2000) and staged according to Nieuwkoop and Faber (1967). Synthetic mRNAs were made using Sp6 mMESSAGE mMACHINE. (Ambion). MT-xDmrt5, MT-mDmrt5, MT-Dmrt5AC and MT-Dmrt5ADM were linearized with NotI and transcribed with Sp6. Templates described previously include: Ngnr1 (Ma et al., 1996), Noggin (Smith and Harland, 1992), EBF2 and EBF3 (Green and Vetter, 2011), Ath5 (Burns and Vetter, 2002), MT-xDmrt4 (Huang et al., 2005); MT-Otx2-hGR (Gammill and Sive, 2001) and hGR-Su(H)Ank-MT (Wettstein and al., 1997). The Dmrt5 antisense morpholino (5'-ACC ATT CAG CTC CAT TGT ACA GTT G-3') and control morpholino oligonucleotides were obtained from Genetools. Dmrt4 MO was as previously described (Huang and al., 2005). For in situ analysis, embryos were injected in one cell of two-cell stage embryos, and fixed at neurula, tailbud or early tadpole stage. In all experiments, embryos were coinjected with β-gal mRNA (100 pg/blastomere) to reveal the manipulated side. For animal cap assays, synthesized mRNA was microinjected into the animal region of each blastomere of 4-cell-stage embryos. Animal caps were dissected at blastula stage (st.9) and cultured until neurula stage (st.15) for RT-qPCR. Induction of GR constructs was performed by addition of dexamethasone (Dex) around stage 12-13 (10 µM) (Sigma).

**RT-PCR and whole-mount in situ hybridization**

Total RNA (Quiagen) was extracted using the RNeasy mini kit (Quiagen) or the RNAspin Mini RNA isolation kit (GE Healthcare). All samples were tested for DNA contamination by 30 cycles of PCR amplification using histone H4 primers. cDNA was
synthesized with iScript cDNA synthesis kit (Biorad). RT-PCR was done according to Gene Amp PCR kit (Perkin Elmer). Real time RT-PCR was performed using the the Step One Plus Real Time PCR system (Applied biosystems) with Q-PCR core kits for SYBR Green I (Eurogentec). Samples were normalized with Xenopus GAPDH. The following primers were used: Dmrt4 (forward 5’-GCCCAAGATGAAACCCTAGA-3’ and reverse 5’-GCACCCCTGTCAGTGATAC-3’); Dmrt5 (forward 5’-CGGAATCACGAGGATTATG-3’ and reverse 5’-TAGGCCTTCCGAGGATCC-3’); XAG (forward 5’-CTGACTGTCCGATCAGAC-3’ and reverse 5’-GAGTTGCTCTCTCGCAT-3’); Histone H4 (5’-CGGATAACATTACAGGTATAC-3’ and reverse 5’-ATCCATGGCGTAACTGCC-3’); Ebf2 (forward 5’-TGAGGTGCAACAGAAAAACG-3’ and reverse 5’-TTTCTCTCCTACCCAGGACTG-3’); Neurogenin (forward 5’-GCCGCGTAAAGCTAACAAC-3’ and reverse 5’-GCAGGATTCTCTCTATCT-3’); Sox2 (forward 5’-TGCCTCAAACACCAGAATA-3’ and reverse 5’-TTGCTGATCTCCGAGTTTG-3’) and GAPDH (forward 5’-TAGTTGGCCAGCATGAG-3’ and reverse 5’-GCCAAAGTTGTGTTGATGA-3’).

All experiments were repeated at least two times with each assay performed in duplicate or triplicate. The values in the figures represent the mean of a representative experiment. Error bars represent s.d.

Whole-mount in situ hybridization analysis was performed as described using digoxigenin-or fluorescein labeled antisense probes (Sive et al., 2000; Bellefroid et al., 1996) generated as indicated: pCMVSport6 Dmrt5 (EST CF290698, Sall/T7), pCMVSport6 Dmrt4 (EST BQ733893, Sall, T7), pbSK(-) Sox2 (EST AF022928, EcoR1, T7). The antisense Aml riboprobe was generated from a cDNA fragment encompassing the entire ORF obtained by RT-PCR and subcloned into pZero-2 (Invitrogen) (EcoRI, T7). The following constructs were previously described: Dlx6 (Luo et al., 2001), BF-1 (Bourguignon et al., 1998), Nkx2.1 (Small et al., 2000), Slug (Mayor et al., 1995), Hes2 (Söltér et al., 2006), FoxE1 (El-Hodiri et al., 2005), FoxE3 (Kenyon et al., 1999), Delta1 (Chitnis et al., 1995), Otx2 (Pannese et al., 1995), Ebf2 and Ebf3 (Green and Vetter, 2011), Neurogenin (Ma et al., 1996), Ep.K (Jonas et al., 1985). For sections, embryos after completion of the whole-mount procedure were gelatine-embedded and vibratome-sectioned at 30 μm thickness.

**Cell proliferation and TUNEL assays**
Whole-mount TUNEL was performed as previously described (Hensey and Gautier, 1998; Yeo and Gautier, 2003) using to reveal the incorporated digoxygenin-dUTP an anti-digoxygenin antibody coupled to alkaline phosphatase (Roche, 1/2000).
RESULTS

Identification of Xenopus laevis Dmrt5

In a microarray screen comparing the transcriptome of wild-type and Mdm4 mutant brains, which display a high level of apoptosis and a severe deficit in neurogenesis (Martoriati et al., 2005), we identified Dmrt5 as a potential novel transcriptional factor involved in anterior neural tissue development. To approach its function during embryogenesis, we decided to identify and characterize the orthologous gene in the amphibian Xenopus laevis due to the advantages it offers for studying early vertebrate development. Through a BLAST search of the Xenopus laevis EST databases, we identified three overlapping partial cDNA clones (accession numbers BJ081978, CF290698 and BG 730357) encoding a protein with 48.2% identity over the common region to mouse Dmrt5 (data not shown). By screening with a 580 bp fragment corresponding to the 5’ end of cDNA CF290698 a tadpole head cDNA library (a gift from A. Hemmati-Brivanlou), we isolated a 2.5 kb full length cDNA clone (accession number DQ329358). The sequence obtained encodes a predicted protein of 437 amino acids that clusters into the DMRTA subfamily (Dmrt3, Dmrt4 and Dmrt5) (Suppl. Fig. S1) and is most closely related to Dmrt5 of all vertebrate species (66% identity with human, rat and mouse, 67% with medaka, 70% with zebrafish and 92% with Xenopus tropicalis) (Fig. 1A). The predicted Xenopus laevis Dmrt5 protein contains a N-terminal DM domain that is highly conserved and shares 97%-100% identity with those of the other vertebrate Dmrt5 proteins. It also contains the DMA domain (aa 250-290) that is conserved in the branch of Dmrt3, Dmrt4 and Dmrt5 and the DMB domain (aa 340-410) located near the C-terminus that appears only in the Dmrt5 branch (Guo et al., 2004). The orthologous relationship between the Xenopus tropicalis Dmrt5 gene and the other vertebrate Dmrt5 genes is also supported by synteny analyses, which show the conservation of genetic linkages between Dmrt5 and nearby Faf1 and Elav genes (Suppl. Fig.1B).

To determine the subcellular localization of Xenopus laevis Dmrt5, we constructed a plasmid encoding a 6X Myc-tagged version of the protein, transfected it in COS cells and found that the protein is exclusively detected in the nucleus (Fig. 1B). To determine whether Xenopus laevis Dmrt5 exhibits a similar DNA binding specificity to the other Dmrt proteins, we performed electromobility shift (EMSA) assays using an oligonucleotide that contains Mab3 and Dsx binding sites (Yi and Zarkower, 1999) and the Myc tagged Dmrt5 protein. Fig. 1C shows that Dmrt5 strongly shifts the Mab3/Dsx probe (Fig. 1C, lane 2), and that the
Dmrt5-specific band can be supershifted by an anti-Myc antibody (lane 3). Binding of Dmrt5 to the MAB-3 and DSX site is specific as it is competed by cold Mab3/Dbx oligonucleotides (lane 4, 5), but not by an oligonucleotide that is bound by the bHLH factor XHRT1 (Pichon et al., 2004) (lane 6, 7). Moreover, changes in the nucleotides that are absolutely required for binding severely affect the binding by Dmrt5 (lane 8, 9). These finding suggest that Xenopus laevis Dmrt5 possesses an in vitro binding specificity similar to the other Dmrts and further support its identification as a member of the Dmrt class of transcription factor.

**Temporal and spatial expression of Dmrt5 during Xenopus early embryogenesis**

Semi-quantitative RT-PCR was first used to investigate the temporal expression patterns of Dmrt5 during early Xenopus embryogenesis and to compare it to that of Dmrt4. As shown in Fig. 2A, Dmrt5 was detected maternally and zygotically while Dmrt4 was only detected zygotically. Zygotic Dmrt5 transcripts, like those of Dmrt4, increase during neurula stages and decrease slightly during the following stages.

In whole-mount in situ hybridization, Dmrt5 transcripts are first detected at early neurula stage (stage 12.5) throughout the anterior portion of the neural plate (Fig. 2B). No expression of Dmrt4 could be detected at that stage (data not shown). Double in situ hybridization with the ventral forebrain marker Nkx2.1 (Small et al., 2000) indicates that Dmrt5 expression does not overlap with Nkx2.1 (Fig. 2C). During later neurula stages, as for Dmrt4, strong Dmrt5 expression was detected in two bilateral patches of cells at the anterior neural plate border. Double in situ hybridization using Dmrt5 and Dlx6, highly expressed in the anterior neural fold in the entire preplacodal ectoderm (Luo et al., 2001; Schlosser et al., 2006) and single in situ of Dmrt4 and Dmrt5 shows that Dmrt5 is expressed in a subdomain of the preplacodal region and that its expression in the panplacodal primordium is more restricted than that of Dmrt4, which covers at early stages the adenohypophysis, olfactory and lens placodes (Schlosser et al. 2006) (Fig. 2 D-H). Single and double in situ hybridization experiments using Dmrt5 and Bf1, a dorsal telencephalic marker (Bourguignon et al., 1998) show that, in contrast to Dmrt4, Dmrt5 expression extends into the adjacent neural plate (Fig. 2 I-K). Double in situ with the neural crest Slug marker indicates that the posterior domain of Dmrt5 expression ends anterior to the rostral limit of Slug, with a gap detected between the neural crest domain of Slug and the Dmrt5 placodal expression (Fig. 2 L). After neural tube closure, Dmrt5 is strongly expressed in the olfactory placodes and in the telencephalon (Fig. 2 M-Z). Around stage 22, as revealed by single and double in situ, the expression of Dmrt5 in
the telencephalon is much stronger than that of Dmrt4 (Fig. 2 M-O). Weak Dmrt5 expression is also detected in the prospective lens ectoderm and in the mesenchyme surrounding the eyes (Fig. 2M, P and S). At stages 25-28, in contrast to Dmrt4, Dmrt5 is also highly expressed in the developing ventral mesodiencephalon (Fig. 2 S, T, U, X, Y). On sections, Dmrt5 expression in the olfactory placode and dorsal part of the telencephalon clearly segregates and expression is detected in the mesodiencephalon. In the telencephalon, Dmrt5 is restricted to the dorsal pallium compartment and is excluded from the most anterior part of it (Fig. 2R, T-V, Y). Thus, in the frog, Dmrt5 and Dmrt4 share overlapping restricted expression patterns during early embryogenesis, with strong early expression in the developing olfactory system.

**Dmrt5 and Dmrt4 are differentially induced by BMP antagonists and both genes are downregulated by proneural genes**

Dmrt5 expression at the anterior region of the neural plate suggests that it could be regulated by neural inducers. To test this hypothesis, we injected embryos with different doses of the neural inducer Noggin. Animal cap explants were analysed at neurula stage by RT-qPCR for the expression of Dmrt5 and Dmrt4, previously shown to be induced by attenuation of Bmps (Huang et al., 2005). Noggin injected explants showed strong activation of Dmrt5. Interestingly, as expected for a neural plate marker, Dmrt5 was the strongest for the highest dose of Noggin mRNA. In contrast, Dmrt4, only expressed in the placodes at neurula stage, was induced at the highest level using the lowest dose of Noggin mRNA, consistent with the idea that placodes require a specific intermediate level of Bmp activity (Fig. 3A)(Brugmann et al., 2004). These results indicate that Dmrt5, as Dmrt4, is positively regulated by attenuation of Bmp signaling.

Several HLH transcription factors are sequentially expressed within the olfactory placode during development. In Xenopus, the first HLH gene to be expressed in the presumptive olfactory placode is Ngnr1, followed by Ebf2, Ebf3 and Ath5 factors (Burns et al., 2002). Overexpression studies in Xenopus have shown that those HLH factors can promote neurogenesis (Ma et al., 1996; Burns et al., 2002; Green and Vetter, 2011). We therefore tested whether Dmrt5 and Dmrt4 expression is regulated by those HLH proneural factors. Embryos injected with Ngnr1, Ebf2, Ebf3 or Ath5 mRNA show a reduction of Dmrt5 (96%, n = 26 for Ngnr1 ; 84%, n = 51 for Ebf2 ; 78%, n = 40 for Ebf3 and 72%, n = 18 for Ath5) and of Dmrt4 (93%, n = 15 for Ngnr1 ; 54%, n = 28 for Ebf2 ; 58%, n = 19 for Ebf3 and 74%, n = 23 for Ath5) expression on the injected side (Fig. 3B). As previously reported, the
pan-neuronal marker *N-tubulin* was in contrast upregulated in those *Ngur1, Ebf2, Ebf3* or *Ath5* injected embryos (data not shown). Thus, as *Dmrt4, Dmrt5* is induced by attenuation of BMPs and is downregulated by proneural factors.

**Otx2 and Notch activate Dmrt5 and Dmrt4 in the ectoderm**

The homeobox gene *Otx2* is a head field selector that is required in the ectoderm for anterior neural determination. It is expressed in the anterior ectoderm including the olfactory placodes and thus coexpressed with *Dmrt5* and *Dmrt4* (Fig. 4A,C). Its overexpression in embryos activates cement gland and anterior neural genes and prevents expression of posterior genes in whole embryos (Gammill and Sive, 2001). To determine whether *Otx2* plays a role in *Dmrt5* and *Dmrt4* activation at the anterior neural plate border, embryos were injected with mRNA encoding a glucocorticoid hormone inducible *Otx2* construct (hGR-*Otx2*). Dexamethasone was added at late gastrula stage (stage 12-13), and expression of *Dmrt5* and *Dmrt4*, was assayed by *in situ* hybridization at stage 25-26. In addition to *Dmrt4* and *Dmrt5*, we also examined expression of the otic placode marker *Hes2* (Sölter et al., 2006), the pituitary marker *FoxE1* (El-Hodiri et al., 2005) and the lens marker *FoxE3* (*lens1*) (Kenyon et al., 1999). We observed that *Otx2* overexpression causes at both stages moderate ectopic expression of *Dmrt5* and *Dmrt4* in the epidermis (75%, n = 57 for *Dmrt5* and 41%, n = 37 for *Dmrt4*) (Fig. 4D,G). No induction of *Hes2* (none, n = 56) and *FoxE1* (none, n = 27) was observed. As previously reported (Ogino et al., 2008), *FoxE3* was also unchanged (0%, n = 39) (Fig. 4J and data not shown). Thus, *Otx2* contributes to the activation of *Dmrt5* and *Dmrt4* in the ectoderm.

The Notch ligands *Delta1* and *Delta2* are also expressed during neurulation in the anterior part of the embryo (Fig. 4B). Both genes are detected in a ring surrounding the neural plate, with the highest expression in the region of the olfactory placodes (Chitnis et al., 1995; Bourguignon et al., 1998; Peres and Durston, 2006). To determine whether Notch plays a role in *Dmrt5* and *Dmrt4* activation, embryos were injected with mRNA encoding a glucocorticoid hormone inducible activated form of the Notch effector Su(H), hGR-Su(H)Ank (Wettstein et al., 1997). Dexamethasone was added at stage 12-13 and injected embryos were assayed by *in situ* hybridization at stage 25-26. Embryos injected with *Su(H)Ank* mRNA exhibited ectopic *Dmrt5* expression in the ectoderm (80% increased, n = 40). This upregulation of *Dmrt5* in *Su(H)Ank* mRNA injected embryos was weaker than that observed in *Otx2* overexpressing embryos, and the most robust ectopic expression was often detected in the head ectoderm. No
Su(H)Ank mRNA injected embryos with ectopic Dmrt4 could be observed (none induced, n = 57)(Fig. 4E,H). Su(H)Ank overexpression had also no significant effect on Hes2 and FoxE1 (all unaffected, n = 44 for Hes2 and 23 for FoxE1). As previously reported (Ogino et al., 2008), FoxE3 was also unchanged (all unaffected, n = 24) (Fig. 4K and data not shown). These results indicate that Notch plays a role in Dmrt5 activation in the ectoderm.

Otx2 has been shown to act in concert with Notch signaling to specify the lens-field (Ogino et al., 2008). To determine whether this combination is also involved in controlling Dmrt5 and Dmrt4 expression, embryos were coinjected with hGR-Otx2 and hGR-Su(H)Ank mRNA. Injected embryos were treated with Dex at stage 12-13 and assayed by in situ hybridization at stage 25-26. We observed in those embryos coexpressing Otx2 and Su(H)Ank extensive ectopic Dmrt5 and Dmrt4 expression in the entire ectoderm, stronger than that observed in hGR-Otx2 mRNA alone injected embryos (80%, n = 58 for Dmrt5 and 71%, n = 42 for Dmrt4) (Fig. 4F,I). Hes2 and FoxE1 were in contrast not activated by the combination of Otx2 and Notch signaling in embryos (none induced, n = 58 for Hes2 and 32 for FoxE1) (data not shown). As previously reported (Ogino et al., 2008), ectopic FoxE3 staining could also be detected in response to Otx2 and Notch signaling (28%, n = 40)(Fig. 4L). Similar results were obtained in animal cap explants cultured until stage 25 and analysed by RT-qPCR. In this assay, Dmrt4 was slightly induced by Su(H)Ank, as Dmrt5 (Fig. 4M). Upregulation of Dmrt5 and Dmrt4 expression was also detected in animal caps derived from embryos overexpressing both Otx2 and Su(H)Ank that were induced with Dex in the presence of a protein synthesis inhibitor, cycloheximide, suggesting a direct effect of hGR-Otx2, hGR-Su(H)Ank or both factors on Dmrt5 and Dmrt4 (Fig. 4N). Thus, both Otx2 and Notch play a role in Dmrt5 and Dmrt4 activation and their combined activity is sufficient to drive strong ectopic expression in the ectoderm.

**Dmrt5 is required for olfactory placode neurogenesis**

To determine whether Dmrt5 is required for olfactory placode development, we used a morpholino antisense oligonucleotide designed to interfere with Dmrt5 translation (Dmrt5-MO). The inhibitory efficiency of the Dmrt5-MO was tested in vivo by coinjection of the Dmrt5-MO with mRNA encoding a Dmrt5-eGFP fusion construct carrying the targeted sequences. Coinjection of the Dmrt5-MO inhibited the expression of the Dmrt5-reporter, but not that of a control eGFP reporter that does not contain the morpholino recognition motif (Fig. 5A). Embryos were injected unilaterally at the two to four cell-stage at the animal pole.
with 10-20 ng of Dmrt5-MO and analysed at tailbud to tadpole stages by in situ hybridization for the expression of the Ebf2 olfactory placode marker. In Dmrt5-MO injected embryos, expression of Ebf2 was reduced (70%, reduced, n = 43) as previously reported in Dmrt4 morphants (Huang et al., 2005) (Fig. 5B,C). Injection of the Dmrt5-MO also inhibited other olfactory markers such as Aml, Emx2 and Ebf3 (79% reduced, n = 29 for Aml; 64% reduced, n = 28 for Emx2; 71% reduced, n = 17 for Ebf3) (Fig. 5D-F). By contrast, expression of FoxE1 expressed in the developing pituitary was unperturbed in Dmrt5 morphants (none affected, n = 23), as was the expression of Sox2 (none affected, n = 21), and Ep Keratin (none affected, n = 13), (Fig. 5G-H and data not shown). Injection at the same dose of a control morpholino had no such effect on the expression of Ebf2, Aml and Ebf3 (12% reduced, n = 25 for Ebf2; 8% reduced, n = 12 for Aml; 8% reduced, n = 13 for Ebf3) (Fig. 5I and data not shown). As Ebf2 is expanded within the developing olfactory placode in Ath5 mRNA injected embryos (Burns and Vetter, 2002), we also asked whether Dmrt5 is required for the ability of Ath5 to expand Ebf2. Fig. 5J,K shows that while Ebf2 is expanded in Ath5 overexpressing embryos, it is downregulated in Ath5 overexpressing Dmrt5 depleted embryos. Attempts to rescue the Dmrt5 MO phenotype in embryos were unsuccessful, most probably because Dmrt5 overexpression in the conditions used (250-500 pg mRNA per blastomere) affects embryo survival. Indeed, massive dose dependent apoptosis was detected in Dmrt5 overexpressing embryos as revealed by TUNEL analysis (Suppl. Fig. S2A). Strong apoptosis was also detected in embryos overexpressing Dmrt4 (Huang et al., 2005) or mouse Dmrt5 (37% of embryos, n = 65 for Dmrt4 and 77%, n = 30 for mouse Dmrt5) (Suppl. Fig. S2B,C) and in embryos overexpressing a truncated version of Dmrt5 lacking the C-terminal region (Dmrt5ΔC) (65%, n = 43) (Suppl. Fig. S2D). In contrast, in embryos overexpressing a deletion mutant lacking the N-terminal part, including the DM DNA-binding domain (Dmrt5ΔDM), we did not detect an increase in the frequency of apoptotic cells (96% unaffected, n = 113) suggesting that it induces apoptosis through its transcriptional regulatory function (Suppl. Fig. S2E).

To further assess the role of Dmrt5 in olfactory placode development, we analysed the effects of its depletion on Noggin-mediated neuralization in animal cap explants. Animal caps were collected at stage 14 and analysed by RT-qPCR for the expression of Ngnr1, Ebf2 and Sox2. As expected, Sox2, Ngnr1 and Ebf2 were induced in Noggin injected explants. Coinjection of the Dmrt5-MO decreased the upregulation of Ngnr1 and Ebf2 while induction of Sox2 remained unchanged. To try to rescue the phenotype without inducing a high level of apoptosis, we coinjected the Dmrt5-MO with a low dose of Myc-Dmrt5 mRNA or mDmrt5.
mRNA (50 pg/blastomere). Under these conditions, coinjection of Myc-Dmrt5 mRNA or mDmrt5 was able to rescue the phenotype (Fig. 5L).

To determine whether Dmrt5 and Dmrt4 have redundant function on neurogenesis, we tested in animal caps whether Noggin mediated induction of Ngnr1 and Ebf2 that is affected by the depletion of Dmrt5 can be rescued by Dmrt4 overexpression and, conversely, whether the inhibition of these genes in the context of Dmrt4 depleted explants (Huang et al., 2005) can be rescued by Dmrt5. Fig. 5M shows that the induction of Ngnr1 and Ebf2 in the absence of Dmrt4 can be rescued by overexpression of Dmrt5 and vice versa. Altogether, these results indicate that Dmrt5 is required for Ngnr1 and Ebf2 expression and that Dmrt5 and Dmrt4 have redundant roles in olfactory placode neurogenesis. The observation that Dmrt5 overexpression induces massive apoptosis also suggests that a tight control of its expression is required during early development.

**Dmrt5 promotes neurogenesis in animal caps**

As Dmrt5 overexpression is able to rescue its depletion in neuralized caps (Fig. 5L), we further studied the role of Dmrt5 in neurogenesis by overexpressing Xenopus or mouse Dmrt5 in neuralized animal caps and analysing the caps at early neurula, tailbud, or tadpole stages (stages 14, 22 or 28) by RT-qPCR for Ngnr1, Ebf2 and Sox2 expression. Dmrt5 induces robust expression of Ngnr1 at stage 14 and to a lesser extend at stage 22. No induction was observed anymore at stage 28. Ebf2 was also activated at stage 14 and 22 but not at stage 28. In contrast, the level of Sox2 was not altered by Dmrt5 overexpression (Fig. 6A). We also tested at stage 14 in neuralized caps the consequence of the overexpression of Dmrt5ΔC and Dmrt5ΔDM on Ngnr1, Ebf2 and Sox2. No induction of Ngnr1, Ebf2 and Sox2 was observed in Dmrt5ΔC or Dmrt5ΔDM injected animal explants, indicating that both the DM DNA binding domain and C-terminal region are required for Dmrt5’s ability to induce neurogenesis.

As Ath5 regulates neurogenesis in the olfactory placodes (Burns and Vetter, 2002), we tested the ability of Xenopus Dmrt5 to influence neuronal differentiation induced by Xenopus Ath5 in neurula stage naïve animal caps. Injection of Dmrt5 mRNA alone slightly induced Ngnr1 and Ebf2 and had no effect on Sox2. As reported previously, Ath5 overexpression induces strong Ebf2 expression (Logan et al., 2005). Sox2 was also strongly induced by Ath5 and Ngnr1 was only weakly activated. Interestingly, coexpression of Dmrt5 together with Ath5 increases the level of induction of Ngnr1 and Ebf2 while the level of Sox2 induction
remains unchanged suggesting that Dmrt5 cooperates with Ath5 to induce olfactory neurogenesis.
DISCUSSION

The results presented here strongly suggest that Dmrt5 plays an important role in neurogenesis during olfactory placode development. We showed that Dmrt5 is initially coexpressed with Dmrt4 in the anterior neural ridge in regions that segregate into olfactory placodes and dorsal telencephalon and that, in contrast to Dmrt4, it is also later detected in the ventral mesodiencephalon. Homologs of Dmrt5 have been identified in Platifish, mouse, and zebrafish (Veith et al., 2006; Yoshizawa et al., 2011; Gennet et al., 2011). In all three species, Dmrt5/Dmrt2 is detected, like in the frog, in the developing olfactory system, forebrain and ventral mesodiencephalon. Homologs of Dmrt4 have been identified in medaka and mouse. In medaka, as in Xenopus, Dmrt4 is expressed in the olfactory placodes and the dorsal telencephalon (Winkler et al., 2004). In the mouse Dmrt1/Dmrt4 is expressed in the developing brain (Kim et al., 2003) but its spatial expression has not been investigated. The closely related Dmrt3 gene is also detected in the olfactory placodes and dorsal forebrain in zebrafish, mouse and chick. It is also transcribed in spinal cord dorsal interneurons in those species and in the presomitic mesoderm in chicken (Smith et al., 2002; Winkler et al., 2004; Li et al., 2008). Xenopus laevis Dmrt3 has not been identified. We found Dmrt3 however in Xenopus tropicalis but could not detect its expression by in situ hybridization during early embryogenesis (data not shown). Thus, Dmrt3-5 genes have highly overlapping expression patterns and are coexpressed in the olfactory placodes. Among them, Dmrt5 expression pattern appears the most conserved in vertebrates.

Little is known about the inductive events that initiate olfactory development. In addition to the attenuation of Bmps known to be required for preplacodal ectoderm formation (Brugmann et al., 2004; Glavic et al., 2004), we found that Otx2 and Notch signaling positively regulate the expression of Dmrt5 in the ectoderm. Consistent with this idea, Otx2 is expressed in the mouse as in Xenopus in a broad anterior domain of the embryo that includes in the ectoderm the prospective olfactory placodes (Simeone et al., 1993; Pannese et al., 1995) and blocking Otx2 function in early embryogenesis results in lack of many head structures including the olfactory placodes (Acaimora et al., 1995; Matsuo et al., 1995; Gammil and Sive, 2001). Consistent with our data, Dmrt5 (identified as EST-34) has been also found induced in an oligonucleotide-based microarray screen performed in animal caps as a target of the endodermal Sox17β transcription factor, a known inducer of Otx2 (Dickinson et al., 2006). Several members of the Notch pathway are expressed in the mouse olfactory sensory epithelium (Schwarting et al., 2007) and a recent study has shown that Notch activity is
required for the maintenance of the proliferative pools of progenitor cells within the olfactory epithelium (Maier et al., 2010). However, the role of Notch signaling in mouse olfactory placode induction remains unknown.

Otx2-Notch interactions have been shown previously to be important in lens determination program (Ogino et al., 2008). Interestingly, we found that the combined overexpression of Otx2 and activation of Notch induces widespread Dmrt5 and Dmrt4 expression in the ectoderm, stronger than that observed in embryos overexpressing Otx2 or Su(H)Ank alone, suggesting that this combination may be also used in olfactory placode induction. Further analysis will be needed to determine whether these factors are required in the frog for Dmrt4 and Dmrt5 activation and whether they input directly on Dmrt5 cis-regulatory elements. Whether this combination is sufficient to activate later olfactory placode markers remains also to be determined.

Early cell death occurs after neural induction within the neurectoderm and has been shown to be dependent on neurogenesis (Yeo et al., 2005). We found that overexpression of Dmrt5 and Dmrt4 induces rapid apoptosis in the embryo. In zebrafish, Dmrt2/terra overexpression also induces apoptosis. Whether apoptosis induced by high level of Dmrt5 expression has no relevance in normal physiological conditions or whether it reflects a role in neurogenesis in the control of cell cycle or proliferation of neural precursors requires further investigation.

The olfactory neuronal transcription factor Olf2/Ebf2 plays an important role in neurogenesis during CNS and olfactory placode development downstream of Ngnr1 (Dubois et al., 1998; Wang et al., 2003). Dmrt4 is thought to function upstream of these factors in the molecular cascade leading to neuronal differentiation (Huang et al., 2005). In accordance, we found that Dmrt4 is not activated by Ngn1, Ebf2 and Ebf3. The observations that i) Ngn1, Ebf2 and Ebf3 do not induce Dmrt5 ii) Ngnr1, Ebf2 and other olfactory markers are blocked in the absence of Dmrt5 iii) Dmrt5 depletion can be rescued by Dmrt4 and vice versa and iv) Dmrt5 activates Ngnr1 and Ebf2 in animal caps and promotes their induction by Ath5 support the hypothesis that Dmrt5 and Dmrt4 may function redundantly upstream of Ngnr1 and Ebf2 in olfactory placode neurogenesis. In the mouse, Dmrt4 deficient mice have a histologically normal olfactory epithelium and general olfaction (Balciunienne et al., 2006). Dmrt4 expression however differs from that in the frog and the *Xenopus* Dmrt4 expression pattern more closely resembles that of murine Dmrt3, whose loss of function has not yet been reported. Interestingly, we found that the olfactory epithelium is reduced in Dmrt5 mutants and is almost completely absent in Dmrt3:Dmrt5 mutants (Parlier et al., unpublished data),
which further demonstrates the importance of *Dmrt* genes and suggests redundant functions in vertebrate olfactory placode development. To better understand the function of *Dmrt* genes in olfactory system development, it will be important to analyse the phenotype of the *Dmrt5* mutants as well as, due to possible genetic redundancy, that of Dmrt3; Dmrt5 double mutants. Further studies are also required to understand the nature of the regulation of *Ngnr1* and *Ebf2* by Dmrt4-5. Our observation that Dmrt5 induction of *Ebf2* is efficiently blocked by MO depletion of Ngnr1 (data not shown) suggest that Dmrt5 may indirectly control *Ebf2*, via the activation of Ngnr1.

In a very recent report, Yoshizawa et al. describes the identification and analysis of a zebrafish mutant with a mutation that alters one cysteine residue in the zinc finger domain of Dmrtalpha2/dmrt5. This analysis revealed that Dmrtalpha2/Dmrt5 regulates neurogenin, possibly via the repression of her6 in the telencephalon (Yoshizawa et al., 2011). Whether Dmrt3-5 play crucial roles in telencephalon development in the frog and in mammals remains to be investigated.
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REFERENCES


Figure 1. Sequence, subcellular localization and DNA binding specificity of *Xenopus* Dmrt5. (A) Amino acid sequence alignment of predicted *Xenopus laevis* (DQ329358), *Xenopus tropicalis* (NP 001096543), medaka (BAD00703), zebrafish (NP 001007065), mouse (NP 758500), rat (NP 001101421) and human (NP 115486) Dmrt5 proteins. Amino acids conserved in all species are highlighted in black. Dots represent gaps introduced into the sequence in order to obtain optimal sequence homology. The conserved DM, DMA and DMB domains are indicated. (B) pCS2 MT-Dmrt5 transfected COS cells show immunoreactivity restricted to the nucleus (left panel). Nuclei counterstained with DAPI and merge images of the Myc immunostaining and DAPI staining are shown (right panels). (C) EMSA of tagged *Xenopus* Dmrt5 on a labeled Mab3 and Dsx binding site (Yi and Zarkower, 1999). Consensus DNA binding site of Mab3 and Dsx is indicated in bold. The position of free probe and shifted complexes containing Dmrt5 are indicated. $^{32}$P labeled probe A containing MAB-3 and DSX binding sites was used in lane 1 to 7. Lane 1: reticulocyte lysate only; 2, Myc-tagged Dmrt5; 3 Myc-tagged Dmrt5 plus anti-Myc 9A10 antibody; 4, Myc-tagged Dmrt5 plus 10x probe A competitor; 5, Myc-tagged Dmrt5 plus 100x probe A competitor; 6, Myc-tagged xDmrt5 plus 10x probe HRT1 competitor; 7, Myc-tagged xDmrt5 plus 100x probe HRT1 competitor. $^{32}$P mutated probes E and F were used in lanes 8 and 9, respectively, with Myc-tagged Dmrt5.

Figure 2. *Dmrt5* temporal and spatial expression pattern. (A) Temporal expression of *Dmrt5* by RT-PCR. RNA was extracted from embryos at the indicated stages. Histone H4 was used as a loading control. (B-Z) Spatial expression pattern of *Dmrt5* compared to that of *Dmrt4* analysed by whole-mount in situ hybridization. Unless indicated, all embryos are shown in anterior views. Nieuwkoop-Faber stages are indicated. (B) Initial expression of *Dmrt5* is observed at stage 12.5 in a horse-shoe shaped area in the anterior region of the neurectoderm. (C) Embryo at the stage as in (A) double stained with NBT-BCIP for *Dmrt5* and *Nkx2.1* showing that *Dmrt5* and *Nkx2.1* do not overlap. (D-G) At stage 13-15, both *Dmrt5* and *Dmrt4* genes are detected bilaterally at the anterior neural plate border, the *Dmrt5* staining region being broader and extending less medially than the *Dmrt4* stained area. Weak *Dmrt5* expression is also detected at stage 13 in a stripe bordering the posterior side of the initial horse-shoe shaped stained zone (arrowhead). (H) Stage 15 embryo double stained with *Dlx6* (dark blue) and *Dmrt5* (light blue) showing that *Dmrt5* is expressed in a subdomain of the
preplacodal ectoderm and that its expression extends to the adjacent neural plate (arrowhead). (I-L) Single (I,J) and double (K,L) in situ hybridization showing that at stage 17, Dmrt5, but not Dmrt4, is coexpressed with BF-1 in the developing dorsal telencephalon. Note also that Dmrt5 staining is detected anterior to the domain of expression of the neural crest Slug marker. Arrowheads indicate posterior and anterior boundaries of Dmrt5 and Slug, respectively. (M-Z) Relationship between the Dmrt5 and Dmrt4 genes at stages 22-28, as revealed by single (M, N, P, Q, W, X, Z) and double in situ hybridization (O). (P, Q, X and Z) lateral and (W) dorsal views, anterior to the right. A high magnification view of the anterior head fold of a Dmrt5 and Dmrt4 double stained embryo is shown in panel O, with Dmrt5 in light blue and Dmrt4 in dark blue. (R-T) Transverse sections through the olfactory epithelium and dorsal telencephalon (R), the anterior (S) or posterior (T) mesodiencephalon. (U,V) Horizontal sections though the dorsal telencephalon and mesodiencephalon (U) or through the olfactory placodes and dorsal telencephalon (V). (Y) Sagittal section. Note Dmrt5 strong expression in the dorsal telencephalon, olfactory placodes and mesodiencephalon and weak expression in the prospective lens ectoderm and in the mesenchyme surrounding the eyes. Abbreviations: dt, dorsal telencephalon; hm, head mesenchyme; le, lens ectoderm; m, mesodiencephalon; op, olfactory placodes; ppe, preplacodal ectoderm.

Figure 3. Dmrt5 is upregulated by attenuation of Bmps and downregulated by overexpression of the proneural factors Ngnr1, Ebf2 and Ebf3. (A) Real time RT-PCR analysis of animal cap explants isolated from embryos injected with different doses of Noggin mRNA and collected at stage 16. Note that Dmrt5 expression is activated by higher doses of Noggin mRNA as compared to Dmrt4. Each value has been normalized to the level of GAPDH expression. (B) Overexpression of Ngnr1, Ebf2 and Ebf3 blocks Dmrt5 and Dmrt4 in the embryo. Anterior views of whole-mount in situ stained embryos are shown, with dorsal to the top. In all case, LacZ mRNA was used as a lineage tracer to identify the injected side.

Figure 4. Combined overexpression of Otx2 and Su(H)Ank efficiently induces ectopic Dmrt5 and Dmrt4 expression in the ectoderm. (A-C) Anterior views of neurula stage embryos hybridized with Otx2, Delta1 or Dmrt5. Arrows indicate the region of the olfactory placodes. (D-L) Otx2-Notch combined activation in the ectoderm efficiently induces Dmrt5 and Dmrt4 expression in the ectoderm. Embryos injected with the indicated mRNA (500 pg) were induced with Dex at stage 12-13, and then subjected to LacZ staining and in situ hybridization with the indicated probes. (D-I) Dorsal views with anterior to the right. (J-L) Lateral views of
the injected side. Moderate ectopic Dmrt4 and Dmrt5 expression was detected in embryos injected with Otx2 mRNA alone throughout the ectoderm. While moderate ectopic Dmrt5 ectopic expression mainly restricted to the head ectoderm was also detected in Su(H)Ank mRNA injected embryos, no ectopic Dmrt4 was observed in Su(H)Ank mRNA injected embryos. Embryos coexpressing Otx2 and Su(H)Ank show robust ectopic Dmrt4 and Dmrt5 staining throughout the ectoderm. FoxE3 used as control was as previously reported not induced in embryos injected with Otx2 alone or Su(H)Ank alone injected embryos but was ectopically activated in embryos injected with both constructs. (M) Real time RT-PCR analysis of Dmrt5 and Dmrt4 expression in animal caps derived from four-cell stage embryos injected with Otx2 or Su(H)Ank mRNA, alone or in combination (500 pg per blastomere). Note that their combination induce stronger expression of Dmrt5 and Dmrt4 than that induced by Otx2 or Su(H)Ank alone. Each value has been normalized to the level of GAPDH expression. (N) Real time RT-PCR analysis of the expression of Dmrt5, Dmrt4 and XAG in animal caps derived from embryos injected with hGR-Otx2 and hGR-Su(H)Ank mRNA, treated or not with dexamethasone (Dex) and cycloheximide (CHX). Note that both Dmrt5 and Dmrt4, but not XAG, are activated by hGR-Otx2 and hGR-Su(H)Ank in the presence of CHX.

Figure 5. Knockdown of Dmrt5 reduces Ngnr1 and Ebf2 expression. (A) In vivo translation of a Dmrt5-eGFP reporter construct is specifically inhibited by the Dmrt5 MO. Embryos were injected with 500 pg of Dmrt5-eGFP mRNA or, as a control, 500 pg of eGFP mRNA, alone or in combination with 20 ng of the Dmrt5-MO, as indicated. (B-I) Whole-mount in situ hybridization of early tadpole embryos injected with 20 ng of Dmrt5-MO or a control standard MO and analysed with the indicated probes. (B, D-I) Anterior views with dorsal to the top. (C) Horizontal section through the head, anterior to the top. The injected side marked by Xgal staining in light blue is on the right. Note that Dmrt5 morphants show reduced Ngnr1, Ebf2, Aml, and Ebf3 expression in the olfactory epithelium while Ep.Keratin and Sox2 expression is unaffected. Injection of a control MO has no effect on Ebf2. (K-L) Anterior views of early tadpole stage embryos injected with Ath5 mRNA, with or without 20 ng of DMRT5-MO and hybrided with Ebf2. Note that Ebf2 is expanded in Ath5 injected embryos and reduced in Ath5 overexpressing Dmrt5 depleted embryos. (L, M) RT-qPCR analysis of Ngnr1, Ebf2 and Sox2 expression in animal caps derived from embryos injected with Noggin (100 pg), Xenopus MT-Dmrt5 (50 pg), mouse MT-Dmrt5 (mMT-Dmrt5, 50 pg), Xenopus MT-Dmrt4 (xDmrt4, 50 pg), Dmrt4-MO (20 ng) and Dmrt5-MO (20 ng), as indicated. Expression
levels were normalized to GAPH and compared to the level observed in Noggin injected caps, which was set to 1. Note that Noggin-mediated activation of *Ngnr1* and *Ebf2*, but not that of *Sox2*, is blocked in caps derived from Dmrt5 or Dmrt4 depleted embryos and that their absence can be indifferently rescued by *Dmrt5* or *Dmrt4* overexpression.

Figure 6. Regulation of *Ngnr1*, *Ebf2* and *Sox2* by *Dmrt5*. (A) Animal caps were isolated from embryos injected with *Noggin* (100 pg), *Xenopus MT-Dmrt5* (Dmrt5, 50 pg) as indicated, cultured until sibling embryos reached stages 14, 22 or 28 and subjected to RT-qPCR analysis. Note that injection of *Xenopus* or mouse *Dmrt5* upregulates *Ngnr1* and *Ebf2* in neuralized caps at stages 14 and 22 while it has no effect on *Sox2*. (B) Animal caps from embryos injected with *Noggin* (100 pg), *MT-Dmrt5* (50 pg), *MT-Dmrt5ΔC* (50 pg) and *MT-Dmrt5ΔDM* (50 pg) as indicated harvested at stage 14 and analysed by RT-qPCR. Note that neither *Dmrt5ΔC* nor *Dmrt5ΔDM* are able to induce *Ngnr1* and *Ebf2*. (C) Animal caps from embryos injected with *MT-Dmrt5* (50 pg) and *Ath5* (250 pg) as indicated and analysed at stage 14 by RT-qPCR. Note that Dmrt5 slightly induces *Ngnr1* and *Ebf2* in naïve caps and that it promotes their expression in *MT-Ath5* overexpressing caps. In all cases, expression levels were normalized to *GAPDH*. In A and B, expression levels were compared to the level observed in *Noggin* injected caps, which was set to 1. In C, expression levels were compared to the level observed in non injected caps, which was set to 1.
Suppl. Fig. S1. Identification of *Xenopus Dmrt5* by phylogeny and synten analysis. (A) Phylogenetic tree of the Dmrt proteins showing that *Xenopus* Dmrt5 clusters into the clade of *Dmrt5* from all species. The gene names, organism names, and the accession numbers of the analysed sequences are shown. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events per 100 amino acid residues. (B) Conserved syntenic regions between human (Hs), mouse (Mm), rat (Rno), Monodelphis domestica (Mdo) and *Xenopus tropicalis* (Xtr) chromosome regions containing the *Dmrt5* locus. Genes are represented as colored boxes with the arrow indicating the orientation of the transcription unit. Boxes with the same color correspond to ortholog genes. The drawing is not on scale to avoid complexity.

Suppl. Fig. S2. Increased apoptosis in *Dmrt5* overexpressing embryos. (A-F) Embryos (stage 15) injected with the indicated Myc-tagged constructs analysed by TUNEL staining. Dorsal views are shown with anterior to the bottom. An increase of staining was detected on the injected side in 93% of embryos, n = 45 for MT-Dmrt5; 51%, n = 74 for MT-Dmrt4; 85%, n = 39 for MT-mDmrt5; 56%, n = 43 for MT-Dmrt5ΔC and 12%, n = 113 for MT-Dmrt5ΔDM.
### Fig. 1

#### A

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#### B

![Image of Dmrt5, DAPI, and MERGE](image)

#### C

Probes: A, ACTACAATATT010AATCA

Probe: E, CACCGAAATATT010AATTT

Probe: F, CACCGAAATATT010AATTT

Delmt5 + F1E1

[Table and diagram contents]

#### Diagram

- **A**: Table format
- **B**: Image of cellular localization
- **C**: Gel electrophoresis with bands labeled A, E, and F

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**Note:** The table and diagram represent genetic and molecular data, with specific sequences and probes for analysis.
Fig. 3

A

Relative expression

Dmrt4 Dmrt5

B

Ngnr1

Dmrt4 Dmrt5

Bbf2

inj. inj.

Bbf3

inj. inj.

ATH5

inj. inj.
Fig. 5

A

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B

Dmrt5-MO

C

Dmrt5-MO

D

Dmrt5-MO

E

Dmrt5-MO

F

Dmrt5-MO

G

Dmrt5-MO

H

Dmrt5-MO

I

Control-MO

J

ATH5

K

ATH5 + Dmrt5-MO

L

M

Relative expression

- Ebf2
- Sox2
- Ngr1
Fig. 6

A

B

C

Relative expression

Relative expression

Relative expression

Ebf2

Sox2

Ngmi1

Ebf2

Sox2

Ngmi1

Relative expression

Relative expression

Relative expression

Control 1

MT-Dmm5

AT16

Dmm5-AT16

Control 1

MT-Dmm5

AT16

Dmm5-AT16

Control 1

MT-Dmm5

AT16

Dmm5-AT16