



Anosmin-1 is essential for neural crest and cranial placodes formation in *Xenopus*



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ABSTRACT

During embryogenesis vertebrates develop a complex craniofacial skeleton associated with sensory organs. These structures are primarily derived from two embryonic cell populations the neural crest and cranial placodes, respectively. Neural crest cells and cranial placodes are specified through the integrated action of several families of signaling molecules, and the subsequent activation of a complex network of transcription factors. Here we describe the expression and function of Anosmin-1 (Anos1), an extracellular matrix protein, during neural crest and cranial placodes development in *Xenopus laevis*. Anos1 was identified as a target of Pax3 and Zic1, two transcription factors necessary and sufficient to generate neural crest and cranial placodes. *Anos1* is expressed in cranial neural crest progenitors at early neurula stage and in cranial placode derivatives later in development. We show that *Anos1* function is required for neural crest and sensory organs development in *Xenopus*, consistent with the defects observed in Kallmann syndrome patients carrying a mutation in *ANOS1*. These findings indicate that *anos1* has a conserved function in the development of craniofacial structures, and indicate that *anos1*-depleted *Xenopus* embryos represent a useful model to analyze the pathogenesis of Kallmann syndrome.

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1. Introduction

The vertebrate head is characterized by a complex craniofacial skeleton and paired sensory organs. These structures are derived from two embryonic cell populations, neural crest and cranial placodes, that are specified at the anterior border of the neural plate at the end of gastrulation. There is abundant literature indicating that molecules of the fibroblast growth factor (Fgf), Wnt and bone morphogenetic protein (Bmp) families must be precisely modulated to specify neural crest and cranial placodes in the embryo [1–4]. These growth factors in turn activate a unique repertoire of transcription factors that are responsible for initiating the differentiation program of each one of these cell populations [5,6]. Among these transcription factors, Pax3 and Zic1 are especially critical to promote

neural crest and cranial placode fates [7–12]. To characterize the molecular events downstream of Pax3 and Zic1, several years ago we performed a microarray screen using *Xenopus* animal cap explants expressing varying combinations of these factors [13]. Here we describe the expression and function of one of these targets Anosmin-1. Anosmin-1 is a secreted molecule of the extracellular matrix, encoded by the *anos1* gene. In human, mutations in *ANOS1* cause Kallmann syndrome, a condition characterized by craniofacial defects, anosmia, deafness and hypogonadotropic hypogonadism [14,15]. Using knockdown approaches in *Xenopus* embryos and animal cap explants we show that Anosmin-1 is required for neural crest specification and for cranial placode-derived sensory organs formation, consistent with defects observed in Kallmann syndrome patients. There is no *anos1* ortholog in mouse therefore *anos1*-depleted *Xenopus* embryos may represent a unique model to analyze the etiology and pathogenesis of Kallmann syndrome.

2. Materials and methods

2.1. Plasmid constructs and morpholino antisense oligonucleotides

The ORF of *Xenopus laevis anos1* was amplified by PCR from neurula stage embryo cDNA using the following primers, F: 5'-

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ATGTGGCTGAGGGAGCCAGGC-3' and R: 5'-TCAGTACTTCTCTGGG-GATGG-3'. *Anos1* (*anos1MO*; 5'-GAGAACCTCGCTCCCTCAGCCACAT-3'), *Pax3* (*pax3MO*; [7]), and *Zic1* (*zic1MO*; [8]) morpholino antisense oligonucleotides (MOs) were purchased from GeneTools (Philomath, OR). The *pax3GR* and *zic1GR* hormone-inducible constructs were generated as previously described [9]. Synthetic mRNAs encoding *pax3GR*, *zic1GR*, *noggin*, *wnt8a*, *anos1*, and β -galactosidase [9,16] were synthesized *in vitro* using the Message Machine kit (Ambion, Grand Island NY).

2.2. Embryos, injections and explants culture

Xenopus laevis embryos were staged as previously described [17] and raised in 0.1X NAM (Normal Amphibian Medium; [18]). The procedures were approved by NYU Institutional Animal Care and Use Committee, under animal protocol # 150201. Embryos were injected in one blastomere at the 2-cell stage with MOs and synthetic mRNA together with 500 pg of β -galactosidase mRNA as a lineage tracer. For animal cap explant experiments, *pax3GR* (100 pg), *zic1GR* (100 pg), *noggin* (200 pg), *wnt8a* (10 pg) and *anos1* (3 ng) mRNAs and *anos1MO* (40 ng) were injected in the animal pole region at the 2-cell stage, explants were dissected at the late blastula stage and cultured for several hours in NAM 0.5X plus 10 μ M of dexamethasone (Dex; Sigma-Aldrich). The explants were subsequently analyzed by quantitative PCR (qRT-PCR). For whole embryo injections and animal cap explant assays each experiment was performed on at least three independent batches of embryos.

2.3. Lineage tracing, in situ hybridization and histology

Xenopus embryos were fixed in MEMFA and processed for Red-Gal (Research Organics, Cleveland OH) staining to visualize β -galactosidase prior to *ISH*. Whole-mount *ISH* was performed as previously described [19]. Digoxigenin (DIG)-labeled antisense RNA probes (Roche Diagnostics, Indianapolis, IN) were synthesized using template cDNA encoding *Anos1*, *Snai2* [20], *Sox10* [21], *Sox2* [22], *Six1* [23], *Foxi4.1* [24], *Dmrta1* [25], *Pax8* [26], *Emx2* [27], *Ebf2* [28] and *Foxe1* [29].

2.4. Western blot analysis

For Western blots, embryos were injected at the 2-cell stage with mRNA (500 pg) encoding a myc tagged version of *anos1* along with increasing amounts of *anos1MO* and cultured up to stage 15. Pools of 10 embryos were homogenized in lysis buffer (0.5% Triton X-100, 10 mM Tris-HCl at pH 7.5, 50 mM NaCl, 1 mM EDTA), containing Halt™ Protease Inhibitor Cocktail (ThermoFisher Scientific; Waltham, MA). Extracts were resolved on a 10% NuPAGE Bis-Tris gel and transferred onto a PVDF membrane using the iBlot system (Invitrogen, Grand Island NY). Blots were subsequently incubated overnight with anti-Myc polyclonal antibody (Abcam, Cambridge, MA; 1.5 μ g/ml dilution). The blots were then washed and incubated with donkey anti-goat IgG coupled to horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX; 1:5000 dilution). Peroxidase

activity was detected with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and imaged on a ChemiDoc MP Biorad gel documentation system (Hercules, CA).

2.5. RNA preparation and qRT-PCR

Total RNAs from embryos were extracted using the RNeasy Micro Kit (Qiagen, Valencia CA). For mRNA extraction from animal cap explants, pools of 8 explants were homogenized and mRNAs were isolated using Dynabeads® mRNA DIRECT™ Micro Kit (Invitrogen). cDNA synthesis from total RNA and mRNA were performed using Superscript VILO cDNA Synthesis Kit (Invitrogen, Grand Island, NY). qRT-PCR was performed on an Eco Real-Time PCR System (Illumina, San Diego CA) using the primers shown in Table 1, and the Power SYBR Green PCR Master Mix (Invitrogen). The reaction mixture consisted of 10 μ l of Power SYBR Green PCR Master Mix, 200 nM primers, and 2 μ l of cDNA in a total volume of 10 μ l. The PCR conditions were as follows: 95 °C for 10min; 40 cycles at 95 °C for 10sec and at 60 °C for 30sec. The $\Delta\Delta$ CT method was used to analyze the qRT-PCR results. Each reaction included a standard curve of serial dilution points (in 10-fold increments) of test cDNA. *odc1* or *ef1 α* was used for normalization.

3. Results

3.1. *Anos1* is expressed in the neural crest and cranial placodes

Ansomin-1 (*Anos1*, formerly *Kal1*; [30]), an extracellular matrix protein, was independently isolated in two microarray screens designed to identify targets of *Pax3* and *Zic1* [13,31]. Both transcription factors are necessary and sufficient to specify the neural crest and placodes [7–9]. By *ISH* *anos1* transcripts are first detected at the early neurula stage (NF stage 14) at the lateral edge of the anterior neural plate, in the prospective cranial neural crest region (Fig. 1A). As the neural plate closes, *anos1* remains spatially confined to the neural crest territory (Fig. 1B and C). As development proceed, *anos1* is expressed in the pharyngeal arches, somites, and anteriorly at the midline in the prospective pituitary gland (Fig. 1D–F). At the tailbud stage (NF stage 27) *anos1* transcripts persist in the pharyngeal arches, somites, and anterior pituitary, and appeared to accumulate in the ventral aspect of the otic vesicles (Fig. 1G and H). Using qRT-PCR, we compared the temporal expression profile of *anos1* to that of *snai2*, a well-established early neural crest gene [22]. We found that *anos1* is maternally expressed (NF stage 8), and towards the end of gastrulation (NF stage 12) the escalation of *snai2* and *anos1* expression follows a very similar pattern (Fig. 1I).

3.2. *Anos1* is a true target of *Pax3* and *Zic1*

To confirm that *anos1* is a target of *Pax3* and *Zic1*, we performed perturbation experiments in the embryo using *pax3MO* and *zic1MO* [7–9]. *Pax3* or *Zic1* knockdown resulted in a dramatic reduction or loss of *anos1* expression in a vast majority of the embryos,

Table 1
Primers for qRT-PCR.

Gene	Forward primer	Reverse primer
<i>anos1</i>	5'- GGAGAAGGTCAGCAACAATAA -3'	5'- GGGATGGTTTGTAGTGATGAGG -3'
<i>snai2</i>	5'- CATGGGAATAAAGTGCAACCA -3'	5'- AGGCACGTGAAGGGTAGAGA -3'
<i>sox2</i>	5'-TCACCTCTTCTCCCATTCG-3'	5'-CGACATGTGCAGCTCTGCTTT-3'
<i>sox8</i>	5'- AAGGTCTCTGGTGGCTGAAA -3'	5'- CACCGCCACATTTTCAGAGTA -3'
<i>odc1</i>	5'-ACATGGCATTCTCCCTGAAG-3'	5'-TGGTCCCAAGGCTAAAGTTG-3'
<i>ef1α</i>	5'-ACCCTCTCTTGGTCGTTTT-3'	5'-TTTGGTTTTTCGCTGCTTCT-3'

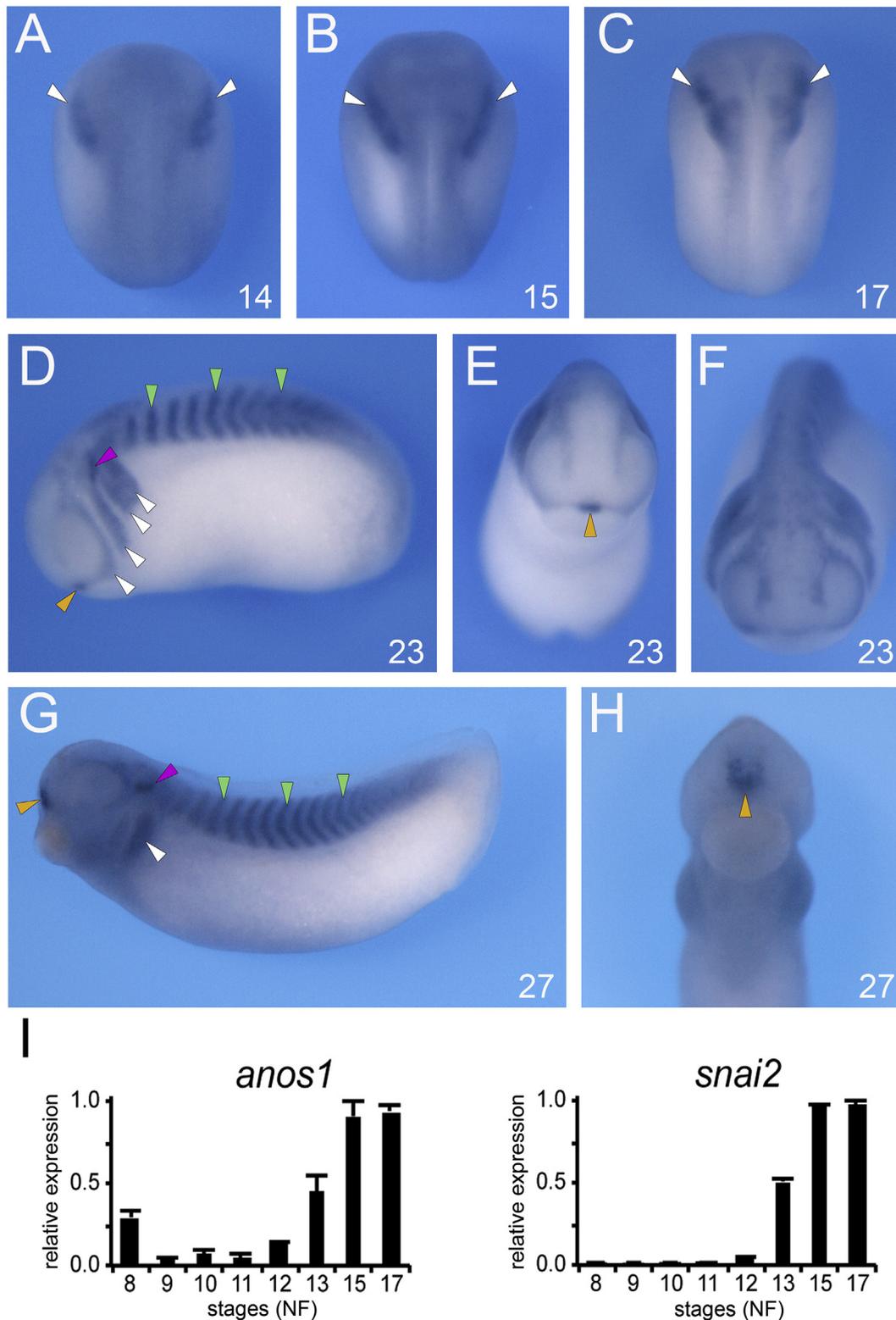


Fig. 1. Developmental expression of *anos1* by whole-mount *ISH*. (A–C) At the neurula stage (NF stage 14–17), *anos1* is detected in the prospective neural crest territory (white arrowheads). (D–F) At stage 23, *anos1* is now more broadly expressed, to include the somites (green arrowheads), otic vesicle (red arrowhead), the anterior pituitary (yellow arrowhead) in addition to the branchial arches (white arrowheads). (G–H) Later in development (NF stage 27) *anos1* persists in all these tissues. (A–C) dorsal views, anterior to top. (D, G) lateral views, dorsal to left, anterior to left. (E, F, H) frontal views, dorsal to top. The embryonic stages (NF) are indicated in the lower right corner of each panel. (I) Relative expression levels of *anos1* and *snai2* analyzed by qRT-PCR at the indicated stages. The values were normalized to *odc1*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

demonstrating that both factors are independently required for *anos1* expression (Fig. 2A). In animal cap explants (Fig. 2B), expression of *pax3GR* and/or *zic1GR* showed that Pax3 and Zic1 are sufficient to induce *anos1* expression and their activity is additive, while Pax3 and Zic1 synergistically activate *snai2* (Fig. 2C). Taken together, these results indicate that *anos1* is expressed in *Xenopus* cranial neural crest and its expression is regulated by Pax3 and Zic1.

3.3. *Anos1* is necessary but not sufficient for neural crest development

To determine whether *anos1* function is implicated in neural crest development we performed *anos1* knockdown using MOs. *anos1*MO was designed to specifically interfere with translation of *anos1* mRNA. The activity of the MO was confirmed by Western blot. In embryos injected with mRNA encoding a myc-tagged version of *Xenopus anos1*, increasing doses of *anos1*MO blocks Anos1 protein accumulation (Fig. 3A). Unilateral injection of *anos1*MO at the 2-cell stage led to a reduction of expression of several neural crest genes including *snai2* and *sox10* at stage 14

(Fig. 3B and C). The loss of neural crest genes correlated with an expansion of *sox2* expression domain in morphant embryos (Fig. 3B). We also tested the function of Anos1 in an animal cap explant assay. Activation of the Wnt/ β -catenin pathway in conjunction with attenuation of BMP signaling induces neural crest genes in animal cap explants [32]. We found that the induction of the neural crest genes, *snai2* and *sox8*, by *wnt8a* and *noggin* was significantly repressed in Anos1-depleted (MO-injected) animal cap explants (Fig. 3D). The reduction in *snai2* and *sox8* expression was associated with an increase in *sox2* expression, consistent with a loss of neural crest fate. Therefore, in the embryos and in animal cap explants Anos1 function is required to specify the neural crest.

We also performed gain-of-function experiments. Unilateral injection of *anos1* mRNA at the 2-cell stage did not significantly affect *snai2* and *sox10* expression levels, although their expression appeared shifted laterally (Fig. 3B and C). Moreover, in animal cap explant *anos1* expression was unable to activate *snai2* or *sox8* expression (not shown). These results support the view that *anos1* participates in neural crest formation downstream of Pax3 and Zic1, but is not sufficient to activate a neural crest development program.

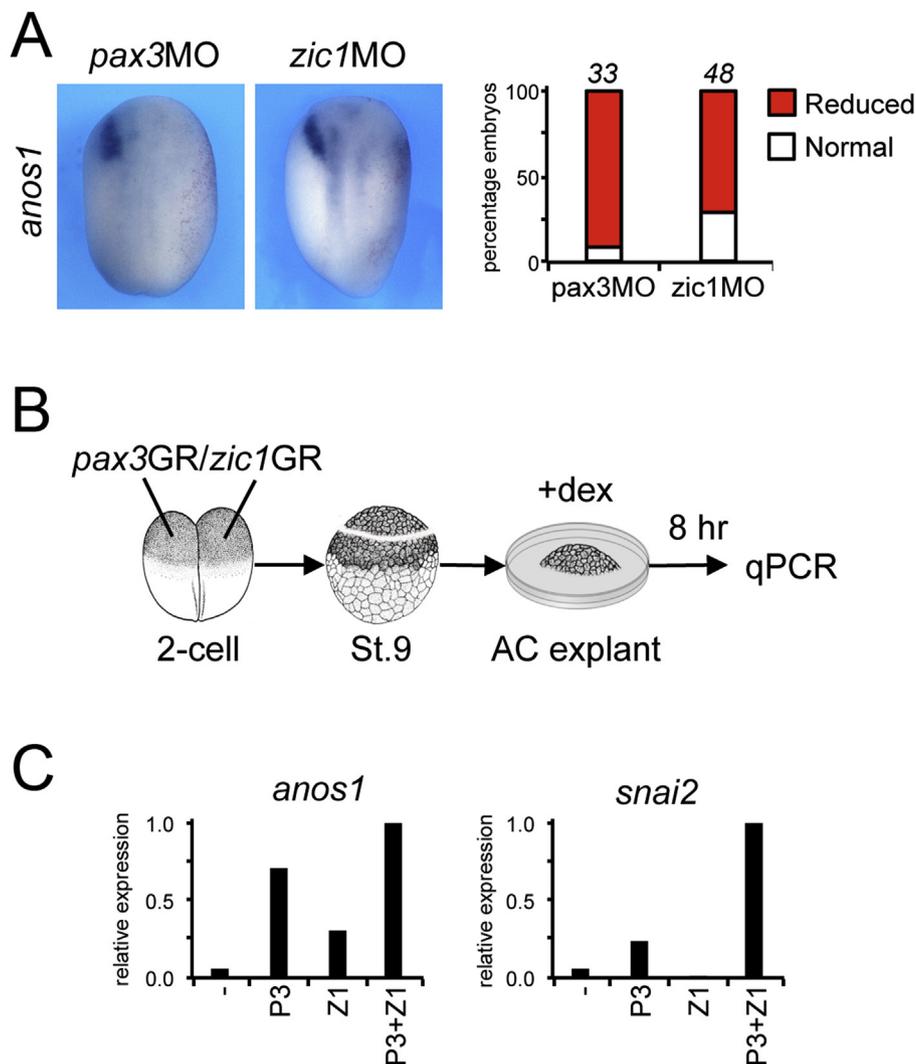


Fig. 2. *anos1* is a target of Pax3 and Zic1. (A) Injection of *pax3MO* (40 ng) or *zic1MO* (40 ng) reduces *anos1* expression at the neurula stage. Dorsal views, anterior to top. The injected side is to the right as indicated by the presence of the lineage tracer (Red-Gal). The graph indicates the percentage of embryos with normal (white) or reduced/lost (red) *anos1* expression. The number of embryos analyzed is indicated on top of each bar. (B) mRNA encoding *pax3GR* and *zic1GR* (100 pg each), alone or in combination were injected into both blastomeres in the animal pole at the 2-cell stage. At the blastula stage (stage 9), animal cap (AC) explants were dissected and cultured for 8 h in the presence of dexamethasone (+dex). (C) *anos1* and *snai2* expression in *pax3GR* and *zic1GR* injected AC explants analyzed by qRT-PCR.

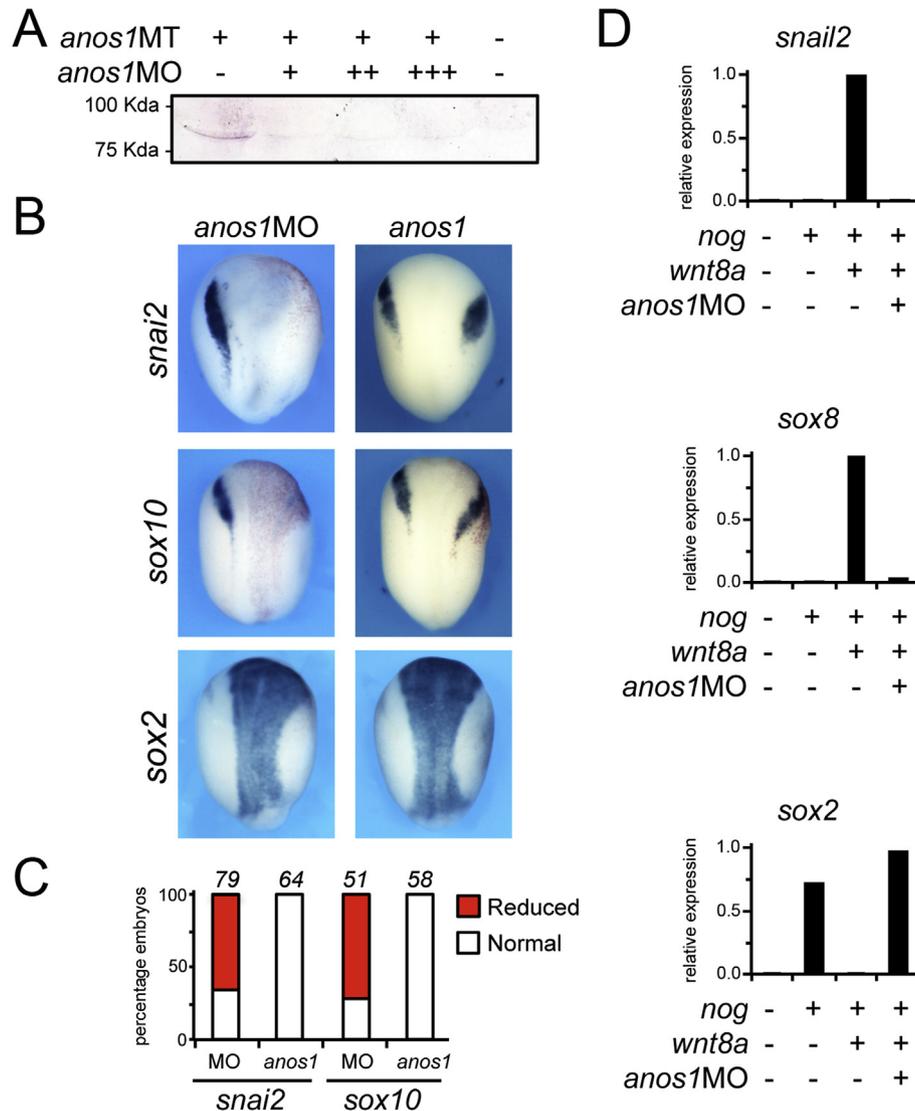


Fig. 3. Anos1 is required for neural crest formation. (A) Western blot of embryos injected with mRNA encoding a myc-tagged version of *Xenopus anos1*, alone or in combination with increasing doses of *anos1*MO, 10 ng (+), 20 ng (++), and 40 ng (+++), showed that *anos1*MO blocks Anos1 protein accumulation. (B) Unilateral injection of *anos1*MO (40 ng) at the 2-cell stage causes a reduction/loss of expression of *snai2* and *sox10*, and a lateral expansion of *sox2* expression domain. Injection of *Xenopus anos1* mRNAs (3 ng) did not significantly affect *snai2* and *sox10* expression levels, although their expression domain was shifted laterally. The expression of *sox2* was only marginally expanded in these embryos. Dorsal views, anterior to top. The injected side is to the right (Red-Gal). (C) The graphs indicate the percentage of embryos with normal (white), reduced/lost (red) expression. The number of embryos analyzed is indicated on top of each bar. (D) In explants, the induction of *snai2* and *sox8* by co-injection of noggin (200 pg) and *wnt8a* (10 pg) mRNA is dramatically reduced in the context of embryos injected with *anos1*MO (40 ng). This reduction in neural crest genes expression is associated by an increase in *sox2* expression. The values were normalized to *ef1 α* . A representative experiments is shown from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. *Anos1* is essential for sensory placodes development

In human, mutations in *ANOS1* have been associated with sensory deficits, including anosmia and hearing loss, and hypogonadotropic hypogonadism due to malfunction of the gonadotropin releasing hormone (GnRH) system [15]. To determine whether Anosmin-1 is also critical for sensory organs development in *Xenopus* we performed unilateral injection of *anos1*MO at the 2-cell stage. These injections resulted in the reduction or loss of expression of two placodal genes, *six1* and *foxi4.1*, as well as genes restricted to individual placodal domains such as *dmrta1* for the adenohypophyseal and olfactory placodes, and *pax8* for the otic and lateral line placodes (Fig. 4A–D). Later in development, these embryos exhibited reduced *dmrta1*, *emx2* and *ebf2* expression in the presumptive olfactory epithelium (Fig. 4E–G), and a marked decrease of *foxe1* expression in

the prospective pituitary (Fig. 4I). These observations are consistent with a broad function of *anos1* in the regulation of cranial placode-derived sensory organs development.

4. Discussion

Here we describe the expression and function of Anosmin-1 (*Anos1*) during early *Xenopus laevis* development. *Anos1* was identified as a target of Pax3 and Zic1 [13,31], two important regulators of neural crest and cranial placode fates in the embryo [7–12]. *anos1* is first detected at the early neurula stage at the lateral edges of the anterior neural plate, in the cranial neural crest forming region. Later in development, *anos1* expression persists in neural crest cells as they populate the branchial arches, and is activated in the prospective nasal and otic placodes, anterior

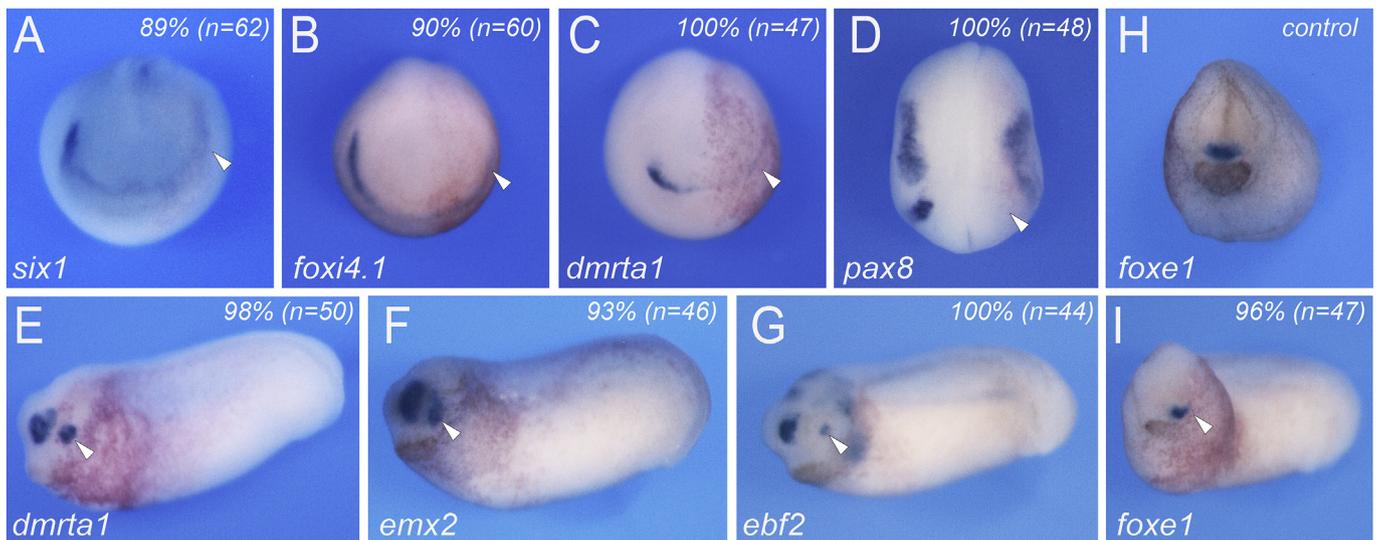


Fig. 4. *Anos1* is essential for the formation of cranial placodes and their derivatives. (A–D) Unilateral injection of *anos1*MO (40 ng) at the 2-cell stage causes a reduction/loss of expression of two pan-placodal genes, *six1* (A) and *foxi4.1* (B), as well as genes restricted to individual placodal domains such as *dmrta1* (C) and *pax8* (D). (E–G) Later in development, the injected embryos exhibited reduce *dmrta1* (E), *emx2* (F) and *ebf2* (G) expression in the olfactory epithelium. (H–I) The expression of *foxe1* in the developing pituitary was also reduced (I) as compared to sibling control (H). (A–I) Anterior views, dorsal to top. The injected side (arrowheads) is to the right. The percentage of affected embryos is indicated in the upper right corner of each panel.

pituitary, and in the somites. MO-mediated knockdown of Anosmin-1 resulted in a reduction of neural crest and cranial placode genes expression suggesting that Anosmin-1 is required for the specification of both cell populations.

ANOS1 encodes a 100-kDa glycoprotein of the extracellular matrix with great affinity for cell membrane heparin sulfate proteoglycans. It is highly conserved across species, with *anos1* orthologs present in *Drosophila*, *C. elegans*, zebrafish, and most mammals. Interestingly, *anos1* has not been identified in rat and mouse [30]. *Anos1* is composed of several functional domains including a cysteine rich region, a whey acidic protein-like domain (typically present in serine protease inhibitors), four consecutive Fibronectin-like type III domains (implicated in cell-cell adhesion) and an histidine-rich C-terminal region [30,33]. *Anos1* has been implicated in cell-cell adhesion, cell migration and differentiation of multiple cell types in the nervous system. *Anos1* regulates migration of immortalized GnRH producing neurons as well as neuronal and oligodendrocyte precursors. It has also a role in axon guidance, neurite outgrowth and the genesis of axon collaterals from neurons in the olfactory system [34–36].

Molecules of the Bmp, Fgf and Wnt families have been implicated in neural crest and cranial placode induction [1–4], however little is known how the activity of these growth factors is controlled in the extracellular space. Functional studies *in vivo* and *in vitro* have shown that *Anos1* physically interacts with fibroblast growth factor receptor 1 (FgfR1) and its ligand Fgf2 to activate signaling [37–40]. More recently, the role of *Anos1* was evaluated during cranial neural crest formation in chicken embryos. This study showed that *Anos1* upregulates Fgf8 and Bmp5 gene expression, and binds directly to Fgf8, Bmp5 and Wnt3a to modulate their activity locally and promote cranial neural crest formation [41]. While *anos1* is also expressed in the anterior neural fold region in chicken embryos, it is unclear whether its activity is also involved in the regulation of cranial placode development. Our observations indicate that in addition to the cranial neural crest, *Anos1* plays a critical role in cranial placodes formation, as *Anos1* knockdown affects the development of adenohiphyseal, olfactory and otic placode derivatives. While we did not detect *anos1* transcripts in the placodal region at the early neurula stage, *Anos1* knockdown

resulted in the reduction of two pan-placodal genes, *six1* and *foxi4.1*. Since *Anos1* is a secreted protein it is likely that knocking down its function in the cranial neural crest will also affect adjacent cell populations, including the formation of cranial placode progenitors, which depends on similar signaling events for their specification.

ANOS1 is the gene responsible for X-linked Kallmann's syndrome [42,43]. This condition is the result of defects in the development of GnRH and olfactory neurons, both cell types originating from the nasal placode. As a consequence these patients suffer from hypogonadotropic hypogonadism and anosmia. Associated defects include renal agenesis, cleft lip with or without cleft palate, hearing loss and abnormal tooth development. The genetics and cellular pathogenesis of Kallmann syndrome are still incompletely understood [15,44], therefore it is essential to develop suitable experimental model systems to gain insights into the molecular basis underlying this condition. We propose that *anos1*-depleted *Xenopus* embryos may represent an excellent model to investigate the pathogenesis of Kallmann syndrome and the mechanisms of action of *Anos1*.

Conflicts of interest

The authors declare no conflict of interest.

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