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Full length article

Head organizer: Cerberus and IGF cooperate in brain induction in *Xenopus* embryos

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ABSTRACT

Neural induction by cell-cell signaling was discovered a century ago by the organizer transplantations of Spemann and Mangold in amphibians. Spemann later found that early dorsal blastopore lips induced heads and late organizers trunk-tail structures. Identifying region-specific organizer signals has been a driving force in the progress of animal biology. Head induction in the absence of trunk is designated archencephalic differentiation. Two specific head inducers, Cerberus and Insulin-like growth factors (IGFs), that induce archencephalic brain but not trunk-tail structures have been described previously. However, whether these two signals interact with each other had not been studied to date and was the purpose of the present investigation. It was found that Cerberus, a multivalent growth factor antagonist that inhibits Nodal, BMP and Wnt signals, strongly cooperated with IGF2, a growth factor that provides a positive signal through tyrosine kinase IGF receptors that activate MAPK and other pathways. The ectopic archencephalic structures induced by the combination of Cerberus and IGF2 are of higher frequency and larger than either one alone. They contain brain, a cyclopic eye and multiple olfactory placodes, without trace of trunk structures such as notochord or somites. A dominant-negative secreted IGF receptor 1 blocked Cerberus activity, indicating that endogenous IGF signals are required for ectopic brain formation. In a sensitized embryonic system, in which embryos were depleted of β-catenin, IGF2 did not by itself induce neural tissue while in combination with Cerberus it greatly enhanced formation of circular brain structures expressing the anterior markers *Otx2* and *Rx2a*, but not spinal cord or notochord markers. The main conclusion of this work is that IGF provides a positive signal initially uniformly expressed throughout the embryo that potentiates the effect of an organizer-specific negative signal mediated by Cerberus. The results are discussed in the context of the history of neural induction.

1. Introduction

This year we celebrate the centennial of the most famous experiment in embryology, in which Hans Spemann and Hilde Mangold discovered that the transplantation of the dorsal blastopore lip of the amphibian gastrula to the ventral side of a host embryo resulted in induction of secondary embryonic axes (Spemann and Mangold, 1924). They found that tissue destined to become dorsal mesoderm was able to induce central nervous system (CNS), as well as dorsal mesoderm tissues such as somites, in neighboring cells of the recipient embryo. Neural induction, also called the primary induction, is an important paradigm in the study of cell-cell interactions that has attracted much attention since its discovery. In the initial publication (consisting of five lineage-marked embryos) the induced CNS lacked anterior brain tissue because relatively late blastopore lips were used as donors. Subsequently, it was discovered that early organizers, the name adopted for the dorsal blastopore lip, induced complete brains while late organizers induced trunk-tail structures (Spemann, 1931). Otto Mangold further analyzed the regional specificity of CNS induction by implanting mesoderm fragments underlying the neural plate (also called archenteron roof), into the blastocoel cavity of host gastrulae (Mangold, 1933). Anterior fragments specifically induced heads with forebrain vesicles (telencephalon and diencephalon), eyes, and noses, but lacked trunk structures. This type of induction was later designated as archencephalic in the embryological literature. Deuteroencephalic inductions, consisting of posterior head structures such as hindbrain and auditory vesicles, arise from the interaction between archencephalic and trunk-tail inducing signals (Toivonen and Saxén, 1968; Hamburger, 1988).

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While earlier researchers employed urodele (newt and salamander) embryos, modern work in neural induction utilizes *Xenopus* embryos as the preferred amphibian model system. Many genes expressed specifically in the Spemann organizer and involved in organizer activity have been isolated (Harland, 2000; De Robertis and Kuroda, 2004; De Robertis and Tejeda-Muñoz, 2022). One of them, called *cerberus*, stands out because when its mRNA is overexpressed in a ventral blastomere it induces purely archencephalic structures (Bouwmeester et al., 1996). Cerberus induces small head structures containing brain, eyes, olfactory placodes, and sometimes a second heart, that are devoid of trunk-tail structures and posterior neural tissue.

Cerberus is secreted in the leading edge of the anterior dorsal endoderm in *Xenopus*, and its identification opened the path for the discovery of the important role of the anterior visceral endoderm (AVE) in brain induction in mouse and chick (Beddington and Robertson, 1999; Stern, 2005). From a molecular mechanism standpoint, Cerberus functions in *Xenopus* as a negative signal. It is an extracellular multivalent growth factor antagonist that binds to Nodal, BMP (Bone Morphogenic Proteins) and Wnt growth factors, preventing signaling through their cognate plasma membrane receptors (Piccolo et al., 1999). The Nodal, BMP and Wnt pathways promote trunk development and must be inhibited for head development to occur.

The insulin-like growth factor (IGF) pathway is also capable of archencephalic inductions in *Xenopus*. Ventral microinjection of IGF1, 2 or 3 mRNAs, or of IGF binding protein 5 (IGFBP5), was able to induce ectopic heads (Pera et al., 2001). Like Cerberus, IGF2 ectopic heads lacked trunk structures such as somites and notochord but occurred at a lower frequency than in Cerberus overexpression. When injected into the dorsal side, IGF1 mRNA expanded brain structures and resulted in the induction of multiple eyes in the midbrain and hindbrain regions (Richard-Parpaillon et al., 2002). IGF signaling in *Xenopus* constitutes a positive signal for brain induction that involves the activation of mitogen-activated protein kinase (MAPK). However, MAPK is also activated by many other pathways, such as fibroblastic growth factors (FGFs), hepatocyte growth factor (HGF) and even cell disaggregation (Streit et al., 1995, 2000; Kuroda et al., 2005), but none of these can induce ectopic heads lacking trunks in the intact embryo. Thus, IGF signaling not only activates MAPK but likely also activates other signaling pathways that repress trunk development.

Whether the two known archencephalic inducing factors in *Xenopus* cooperate with each other has not been investigated previously. In this study, we show that Cerberus and IGF2 cooperate in embryos microinjected with mRNA. The results are discussed in the context of other signaling pathways involved in brain induction in amphibians and other vertebrates.

2. Results

2.1. IGF2 and Cerberus mRNAs microinjected into ventral blastomeres cooperate in head induction

IGF2 or *Cerberus* synthetic mRNAs microinjected into a ventral blastomere at the 4- to 8-cell stage induced protrusions with pigmented cement glands on the ventral side of embryos (Fig. 1A–C). The cement gland is an anterior differentiation marker in *Xenopus*. IGF2 head-like structures were smaller and occurred at lower frequency. When *IGF2* (2 ng) and *Cerberus* (100 pg) mRNAs were coinjected, ectopic head structures were larger and their cement glands were much expanded (Fig. 1D; see Fig. 1E–H for a panoramic view). Lower suboptimal IGF2 levels, at which ectopic head structures were lacking or induced at very low frequency, were nevertheless able to synergize with Cerberus in secondary head induction (Supplemental Fig. S1).

At later tadpole stages, ectopic heads induced by a combination of IGF2 and Cerberus developed a single eye structure, which in some cases was larger than the endogenous eye (Fig. 2A and B, arrow). Occasionally, second beating heart structures arose from mesoderm in the ectopic heads (Supplemental Video S1), as reported previously in the case of Cerberus (Bouwmeester et al., 1996). In no case were auditory vesicles, somites, notochord, or trunk structures observed.

When tadpoles were grown for 40 days until metamorphosis, injection of *IGF2* plus *Cerberus* mRNAs induced small ectopic heads containing a single eye and opaque internal tissue (Fig. 2C–H). Histological sections of these stage 58 tadpoles (Nieuwkoop and Faber, 1967) revealed that the ectopic heads contained brain tissue with outer gray matter (GM) and inner myelinated white matter (WM), an eye containing several layers of neurons forming neural retina (NR) surrounded by retinal pigmented epithelium (RPE), and multiple small olfactory placodes (OP) (Fig. 3). Outer GM is found in the anterior brain, while



Fig. 1. *IGF2* and *Cerberus* mRNAs cooperate in ectopic head induction in *Xenopus* embryos.
Embryos were microinjected into the ventral marginal zone of a single blastomere at the 4- to 8-cell stage. (A) Uninjected control sibling at early tailbud stage (n = 81). (B) A single ventral injection of *IGF2* mRNA caused a small ectopic head protrusion with a pigmented cement gland on the belly (n = 86, 68 % with ectopic structures). (C) *Cerberus* mRNA induced a secondary head-like structure (n = 78, 94 % with ectopic heads). (D) Co-injection of *IGF2* and *Cerberus* mRNAs induced a large ectopic head with an expanded cement gland (n = 90, 98 % with ectopic heads). (E–H) Panoramic views of control and injected embryos. Injected mRNA doses per embryo were: *Cerberus*, 100 pg; *IGF2*, 2 ng. Results from two experiments. Scale bars are 500 µm (A-D) and 2 mm (E-H).

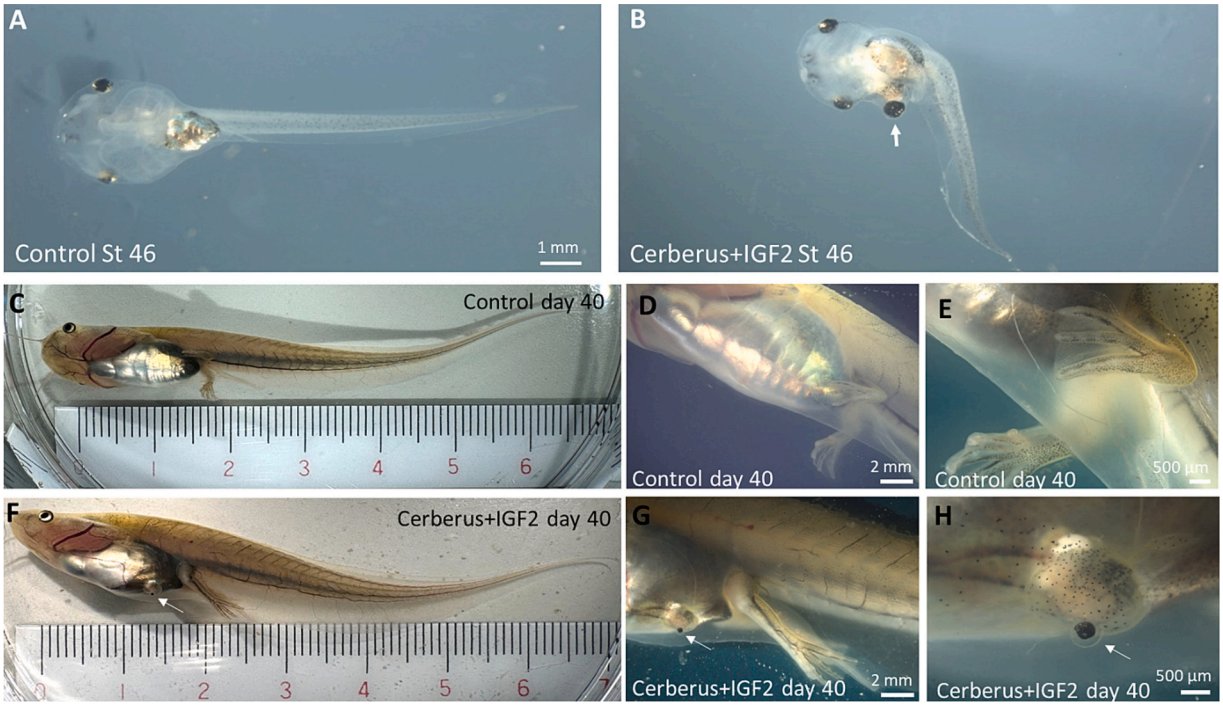


Fig. 2. Ectopic heads induced by IGF2 and Cerberus developed a cyclopic eye.
(A) Uninjected control tadpole at stage 46. (B) Ectopic head with an eye (arrow) bigger than the endogenous eyes induced by the combination of *IGF2* and *Cerberus* mRNAs, shown at stage 46. (C–E) Uninjected control tadpole at day 40. (F–H) Three magnifications of an ectopic head structure of induced by coinjection of *IGF2* and *Cerberus* in tadpole undergoing metamorphosis. Arrows indicate the ectopic eye. Injected mRNA doses per embryo were: *Cerberus*, 100 pg; *IGF2*, 2 ng. Scale bars are 1 mm (A, B), 2 mm (D, G) and 500 µm (E, H).

hindbrain and spinal cord also have segregation of nerve bodies and myelinated nerve fibers, but their organization is reversed, with WM on the outside and the GM in the inside. These results indicate that the ectopic heads induced by coinjection of IGF2 and Cerberus are typical archencephalic differentiations without traces of trunk differentiation.

2.2. *IGF2 and Cerberus cooperate in a β-catenin-depleted sensitized embryo assay*

CNS induction in *Xenopus* requires an early β-catenin signal required for subsequent Spemann organizer induction. This signal can be readily blocked by injecting radially four times antisense β-catenin morpholino (MO) at the 2- or 4- cell stage (Heasman et al., 2000). The β-catenin-depleted embryo provides a sensitized system in which, for example, depletion of BMPs results in massive brain inductions (Reversade et al., 2005). The reason for this enhanced response is the lack of expression of all Spemann organizer genes in β-catenin depleted embryos, including the anti-dorsalizing morphogenetic protein (ADMP), a BMP secreted by the organizer. ADMP has potent anti-neural effects, which are normally inhibited by co-expression of the BMP antagonist Chordin on the dorsal side (Moos Jr et al., 1995; Reversade and De Robertis, 2005).

We used the β-catenin depleted sensitized assay to further study the activities of IGF2 and Cerberus (Fig. 4). Microinjection of β-catenin MO four times radially caused ventralized embryos without any dorsal or CNS structures (Fig. 4A, B). Radial microinjection of *IGF2* mRNA + β-catenin MO did not show noticeable phenotypic effects (Fig. 4C). However, *Cerberus* mRNA + β-catenin MO resulted in embryos with radial cement glands and brains (Fig. 4D), while the combination of *Cerberus* and *IGF2* together with β-catenin MO produced even stronger phenotypes (Fig. 4E). The appearance of radial brains in Fig. 4D is reminiscent of radially dorsalized embryos resulting from treatment with Lithium chloride (LiCl) (Kao and Elison, 1988). However, there is one important difference in that the blastopore is at the center of the brain in LiCl treated embryos, while it is distal to the radial CNS in embryos injected with *Cerberus* mRNA + β-catenin MO (Fig. 4D, arrowhead); this difference will merit further study in the future.

Analysis of radially microinjected embryos by quantitative real time PCR (qRT-PCR) at late gastrula (stage 12) showed that IGF2 and Cerberus synergized in the induction of the anterior neural marker *Otx2* (Fig. 4F, compare column 4 to 5). The pan-neural marker *Sox2* was also induced but to a lower extent (Fig. 4G). *Chordin* and *ADMP*, which mark the trunk mesodermal midline and late organizer at stage 12, were not induced by IGF2, Cerberus or their combination (Fig. 4H and I).

We next examined neural induction in β-catenin-depleted embryos after a single injection of mRNA into the ventral marginal zone at the 8-cell stage. In these experiments, we included an additional dose of β-catenin MO together with the mRNAs to ensure that depletion was complete. It was found that Cerberus and IGF2 + Cerberus induced circular neural structures surrounded by cement glands (Fig. 5A–C, Supplemental Fig. S2). We also tested the two mRNAs in wild-type background and confirmed that single coinjection of β-catenin MO does not inhibit ectopic head formation (Fig. 5D).

Using qRT-PCR molecular analyses at tailbud stage 22, we found that although *IGF2* mRNA on its own did not induce the *Rx2a* eye marker or the *Otx2* forebrain and midbrain marker, in β-catenin-depleted embryos, it was able to increase brain induction by *Cerberus* mRNA in single injections (Fig. 5E and F, columns 3–5). *HoxB9/XlHbox6*, which is a spinal cord marker at this stage (Sharpe et al., 1987), was not induced by Cerberus or the combination of Cerberus and IGF2 (Fig. 5G), indicating that these secreted molecules are purely archencephalic neural inducers. At this early tailbud stage, ADMP is a posterior mesoderm marker of notochord and chordoneural hinge (the tail organizer) (Dosch and Niehrs, 2000); in keeping with the archencephalic nature of neural induction by IGF2 and Cerberus, *ADMP* was not induced by either mRNA or their combination (Fig. 5H).

We conclude that in a sensitized β-catenin depleted background IGF2 and Cerberus synergized with each other. The CNS induced by these secreted factors was exclusively of an anterior nature.

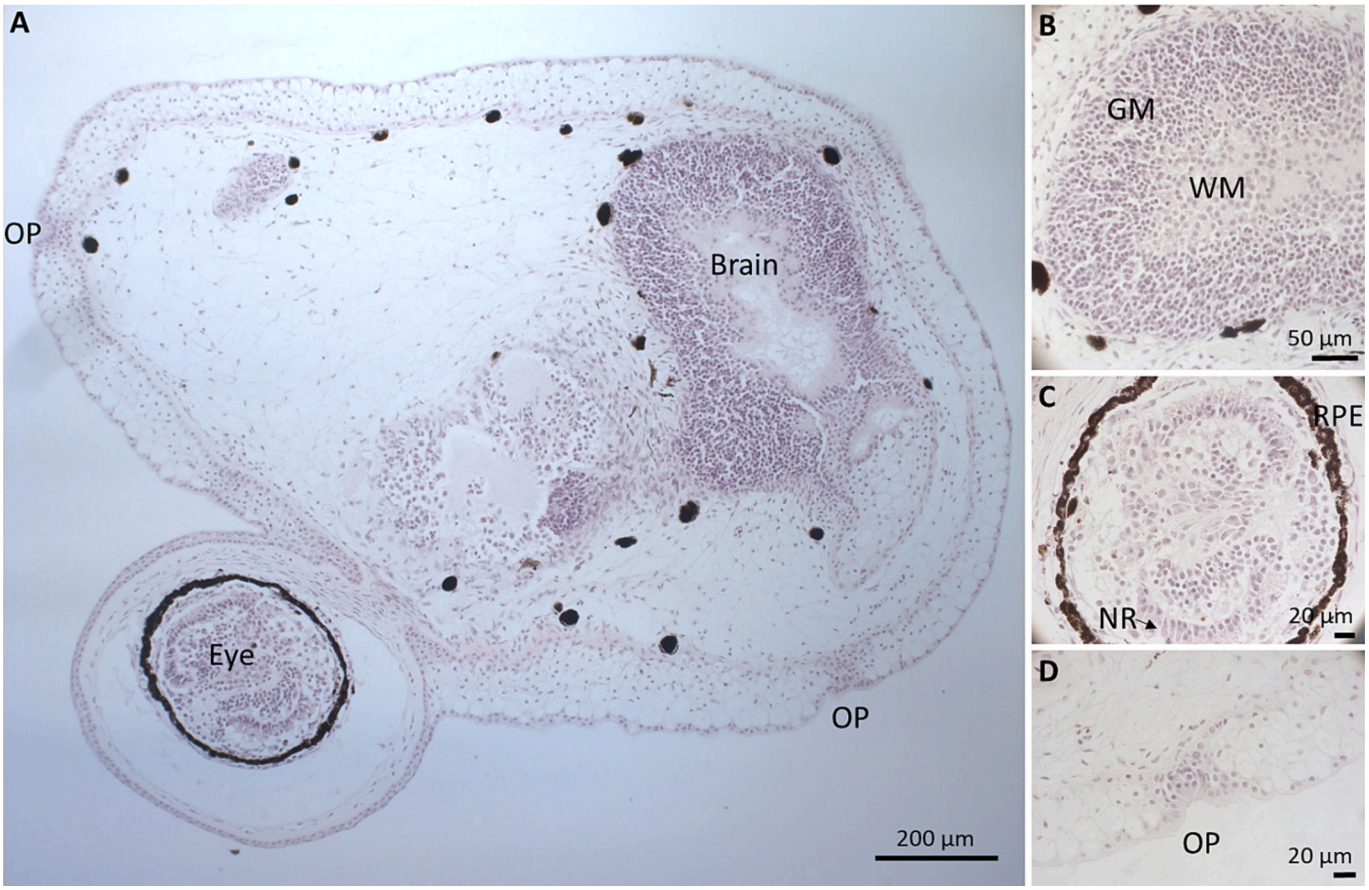


Fig. 3. Histological analysis of the ectopic head induced by IGF2 and Cerberus in 40-day old *Xenopus* tadpole embryos.
(A) Hematoxylin and eosin staining of sectioned ectopic head at low power showing brain, eye and olfactory placodes. (B) Brain at higher magnification showing gray matter (GM) consisting of neuronal bodies on the outside and white matter (WM) consisting of myelinated projections in the inside. (C) Eye including neural retina (NR) and surrounded by retinal pigmented epithelium (RPE). (D) Olfactory placode (OP) imaged at higher magnification, see scale bar. Injected mRNA doses per embryo were: *Cerberus* 100 pg, *IGF2* 2 ng. Scale bars are 200 µm (A), 50 µm (B) and 20 µm (C, D).

2.3. Cerberus ectopic heads are inhibited by dominant-negative IGF 1 receptor

We next asked whether brain induction by Cerberus, which is a negative signal for growth factors, also required a positive signal to develop ectopic heads. IGF ligands signal by binding to the transmembrane IGF 1 receptor (IGF-1R) which activates Ras-Raf-MAPK and PI3K pathways, and perhaps other downstream signaling events (Jones and Clemmons, 1995). A secreted form of the extracellular domain of IGF-1R (DN-IGFR) blocks IGF receptor signaling (D'Ambrosio et al., 1996; Pera et al., 2001). We used *DN-IGFR* in coinjections with *Cerberus* mRNA. As shown in Fig. 6, a single ventral injection of *DN-IGFR* mRNA lacked a phenotypic effect, but when injected in combination with *Cerberus* mRNA it blocked, or severely inhibited, ectopic head formation by *Cerberus* mRNA. Lineage-tracing with *LacZ* mRNA confirmed that cells injected with *Cerberus* + *DN-IGFR* did not induce ectopic brains (Fig. 6E–H). We conclude that head induction by Cerberus, a negative signal that inhibits Nodal, BMP and Wnt, also requires a positive signal downstream of IGF-1R.

3. Discussion

3.1. IGF2 cooperates with Cerberus in neural induction

In this study, we analyzed the roles of microinjected *Cerberus* and *IGF2* mRNAs in head induction in the context of the whole *Xenopus* embryo. IGF2 is uniformly expressed maternally during cleavage and in the midline at the neurula stage (Pera et al., 2001). Cerberus is expressed in the anterior endoderm at gastrula stages and in the dorsal-vegetal Nieuwkoop induction center at mid-blastula (Bouwmeester et al., 1996; Kuroda et al., 2004). When injected ventrally, Cerberus induces ectopic heads lacking trunks, forming what are called archencephalic inductions in the embryological literature. *IGF2* mRNA is a weaker inducer of ectopic heads, but greatly potentiates archencephalic head induction by Cerberus. In a loss-of-function situation, coinjection of *DN-IGFR* blocked the activity of *Cerberus* mRNA. This indicates that Cerberus, a negative signal that acts by inhibition of Nodal, BMP and Wnt growth factors, requires a positive signal *via* uniformly distributed and maternally provided IGFs. At suboptimal concentrations of *IGF2* mRNA, the synergistic cooperation with Cerberus was most evident. Using a sensitized system of β-catenin depleted *Xenopus* embryos, IGF2 alone did not induce neural markers yet strongly potentiated the induction of ectopic brain structures marked by *Otx2* and *Rx2a* while not inducing the spinal cord marker *HoxB9* or the notochord markers *ADMP* and *Chordin*.

3.2. Neural induction in animal cap explants

Explants of blastula animal cap cells normally develop into epidermis. It was discovered that the Spemann organizer secreted protein Noggin induced anterior neural tissue (Lamb et al., 1993). Overexpression of BMP4 was able to counteract neural induction in animal caps by Noggin, Chordin and Follistatin (Sasai et al., 1995). *Xenopus* animal cap cells will adopt a neural fate simply by cell dissociation for 3

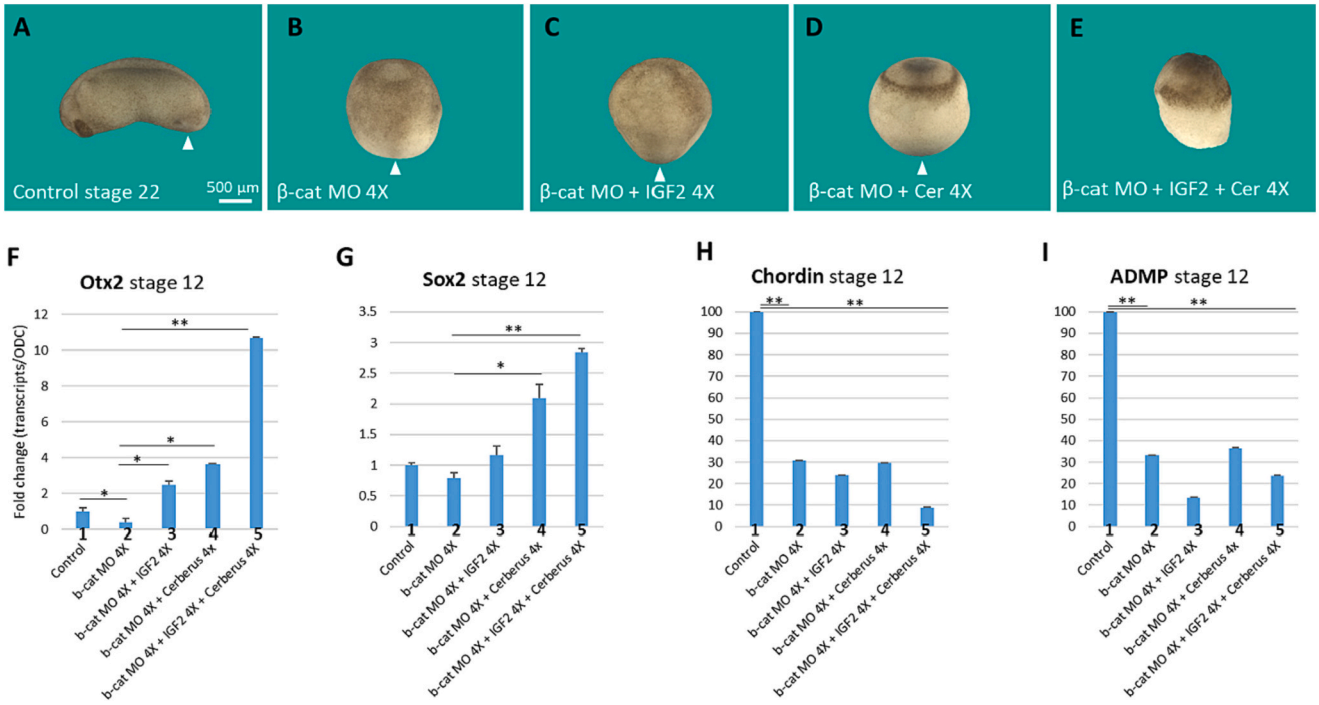


Fig. 4. In β-catenin depleted embryos, 4× coinjection of *Cerberus* mRNA or in combination with *IGF2* mRNA induced circular radial brain structures; IGF2 and Cerberus cooperated in brain marker induction but did not induce notochord markers at stage 12 late gastrula.
(A) Control embryo at stage 22 (n = 55). (B) Radial 4× β-catenin MO injection resulted in complete ventralization (n = 15). (C) Embryo with 4× microinjection of β-catenin MO together with *IGF2* mRNA (n = 35, 83 % completely ventralized, 17 % with very weak axis). (D) β-catenin MO and *Cerberus* radial coinjection induced radial cement gland and brain (n = 17, all radially dorsalized). (E) Embryos injected 4× with a combination of β-catenin MO, *IGF2* mRNA and *Cerberus* mRNA with enlarged radial cement gland and brain (n = 30, all with radial phenotype). Arrowheads indicate the position of blastopore. Scale bar, 500 µm. qRT-PCR results at late gastrula of siblings (three embryos per sample in triplicate). (F) Anterior neural marker *Otx2*. (G) pan-neural marker *Sox2*, (H–I) The trunk mesodermal midline and late organizer markers *Chordin* and *ADMP* at stage 12. Error bars denote SD (n = 3) (*P < 0.05 and **P < 0.01). Coinjections were performed four times radially at the 2- to 4-cell stage with the following doses per embryo: β-catenin MO, 34 ng total; *IGF2* mRNA, 8 ng total; *Cerberus* mRNA, 400 pg total.

h followed by reaggregation (Grunz and Tacke, 1989; Kuroda et al., 2005), and this process can be reversed by the addition of BMP4 protein (Wilson and Hemmati-Brivanlou, 1995). This led to the idea that during cell dissociation endogenous BMP4 signals are diluted into the culture medium and to the default model of neural induction (Muñoz-Sanjuán and Brivanlou, 2002).

It was later found that the BMP downstream transcription factor Smad1 was still phosphorylated by BMP receptor (BMPR) in dissociated animal cap cells (Kuroda et al., 2005). Dissociation causes a sustained activation of the Ras/MAPK pathway, and the neural induction in *Xenopus* animal caps could be blocked by inhibiting this pathway (Kuroda et al., 2005). In salamander (*Ambystoma maculatum*), anterior CNS structures including eyes could be induced in animal cap explants simply by culturing them in saline solution attached to a glass surface (Barth, 1941; Holtfreter, 1944). We reproduced this experiment and found that this neural induction required MAPK activity (Hurtado and De Robertis, 2007). Thus, a positive signal mediated by MAPK is required for anterior neural induction in animal caps of *Xenopus* and *Ambystoma*. Anterior neural induction in animal cap cells can be induced by FGF8 signaling (Hongo et al., 1999; Hardcastle et al., 2000), by IGF1 (Richard-Parpaillon et al., 2002), or by IGF2 (Pera et al., 2001). All of these signals activate MAPK and are likely to provide the positive signal required in addition to BMP signaling antagonism in animal caps.

3.3. Neural induction in intact Xenopus embryos

BMP antagonism is important in the context of the whole embryo, for the triple depletion of Noggin, Chordin and Follistatin results in ventralized embryos lacking all neural structures (Khokha et al., 2005). Conversely, simultaneously depletion of BMP2/4/7 in β-catenin-depleted embryos results in massive brain inductions. When ADMP, which is expressed in the Spemann organizer, is depleted together with BMP2/4/7, the entire ectoderm becomes neural tissue (Reversade and De Robertis, 2005). However, when only a small patch of ventral ectodermal cells is targeted, neural differentiation requires the inhibition of both the BMP Smad1/5/8 and Nodal Smad2/3 pathways in the intact embryo (Chang and Harland, 2007). This is because explanted animal caps have very low endogenous levels of Nodal/TGF-β signaling (transduced by Smad2/3), and the Smad1/5/8 pathway predominates in these explants (Linker and Stern, 2004; Delaune et al., 2005; Chang and Harland, 2007).

3.4. Integration of signaling pathways at the level of Smad linker region phosphorylations

As indicated in the model in Fig. 7, Smad1/5/8 are phosphorylated by BMPR and Smad2/3 by the Nodal/TGF-β receptor complex at the carboxy terminus. The carboxy-terminal phosphorylations activate these transcription factors. In addition, Smads are phosphorylated in their linker (middle) region by MAPK phosphorylations that prime additional phosphorylations by GSK3 (Glycogen Synthase Kinase 3) (Kretzschmar et al., 1999; Fuentealba et al., 2007). The net effect of the linker phosphorylations is to inhibit Smad transcriptional activity by preventing nuclear localization and promoting proteasomal degradation.

Linker phosphorylations provide a very attractive mechanism for integrating signaling pathways because it is hard-wired at the protein level. Linker phosphorylations can potentially integrate the BMP, Nodal, Wnt (through GSK3) and the IGF and FGF pathways (through MAPK activation). Using Smad1 mutants resistant to MAPK phosphorylation it

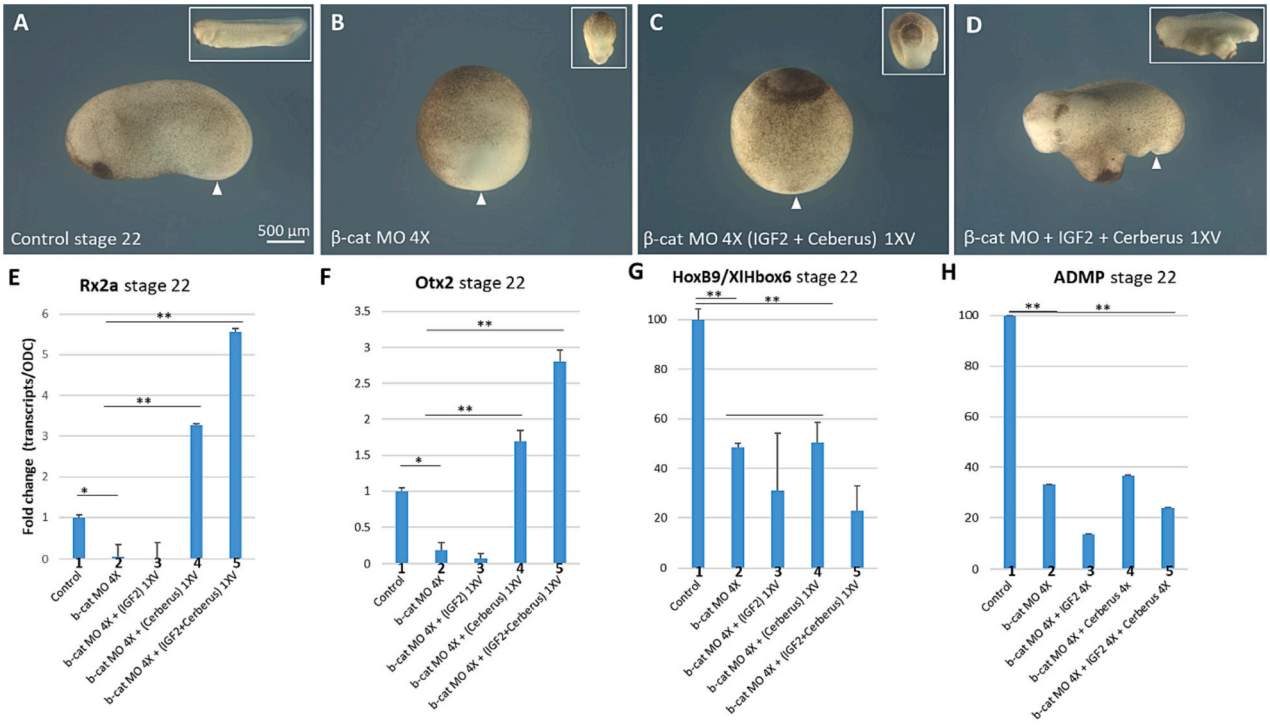


Fig. 5. In β-catenin MO ventralized embryos a single injection of *IGF2* and *Cerberus* induced circular brains; IGF2 alone did not induce neural markers at early tailbud but cooperated with Cerberus inducing anterior markers while spinal cord or posterior trunk mesoderm markers were not induced.
(A) Uninjected control at tailbud stage 22 (n = 100). (B) 4× β-catenin MO injected embryos (n = 40, all completely ventralized). (C) Radial β-catenin MO followed by 1-time ventral injection of *IGF2* and *Cerberus* plus β-catenin MO resulted in ectopic radial cement gland and brain structures (n = 46, all radial brain). (D) β-catenin MO did not have any discernable effect on wild type embryos injected 1 time ventrally with *IGF2* mRNA and *Cerberus* mRNA (n = 91, all with ectopic head). Arrowheads indicate the position of blastopore. Insets show embryos from the same experiment photographed one day later at stage 34. Scale bar, 500 µm. qRT-PCR results for (F) eye marker *Rx2a*, (G) anterior neural marker *Otx2*, (H) spinal cord marker *HoxB9/XlHbox6*, and (I) trunk mesodermal midline and late organizer marker *ADMP* at stage 22. The qRT-PCR experiments represent biological triplicates. Error bars denote SD (n = 3) (**P < 0.01 and P < 0.1). Doses per injection were: β-catenin MO, 8.5 ng; *IGF2* mRNA, 2 ng; *Cerberus* mRNA, 100 pg.

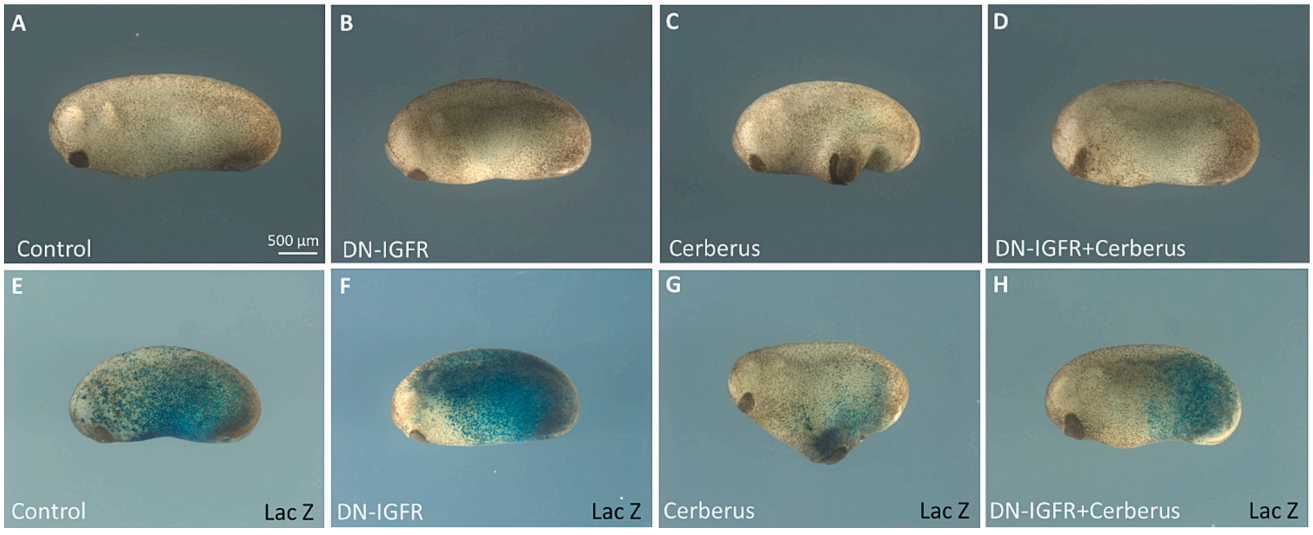


Fig. 6. Dominant-negative IGF receptor 1 blocked ectopic head formation by *Cerberus* mRNA in single ventral injections.
(A) Control embryo at stage 24 injected with *LacZ* (100 pg) mRNA but not stained for β-galactosidase (n = 50). (B) *DN-IGFR* (600 pg) and *LacZ* injected embryos (n = 20, all normal). (C) *Cerberus* (100 pg) injected embryos with ectopic heads (n = 56, 96 % ectopic heads). (D) *DN-IGFR* blocked Cerberus ectopic heads (n = 33, 88 % with no ectopic structures, 12 % with small cement glands). (E–F) LacZ staining for (A–D). Scale bar, 500 µm.

was found that this protein was a much stronger inhibitor of FGF8 or IGF2 neural induction than wild-type Smad1 (Pera et al., 2003; Sater et al., 2003). However, enthusiasm for this crosstalk was diminished by the finding that mice with Smad1 MAPK phosphorylation-resistant mutations were born and had only late defects (Aubin et al., 2004). We note that Smad5 and Smad8 were still present in these mice, and that Smad2 also has inhibitory linker phosphorylations (Grimm and Gurdon, 2002).

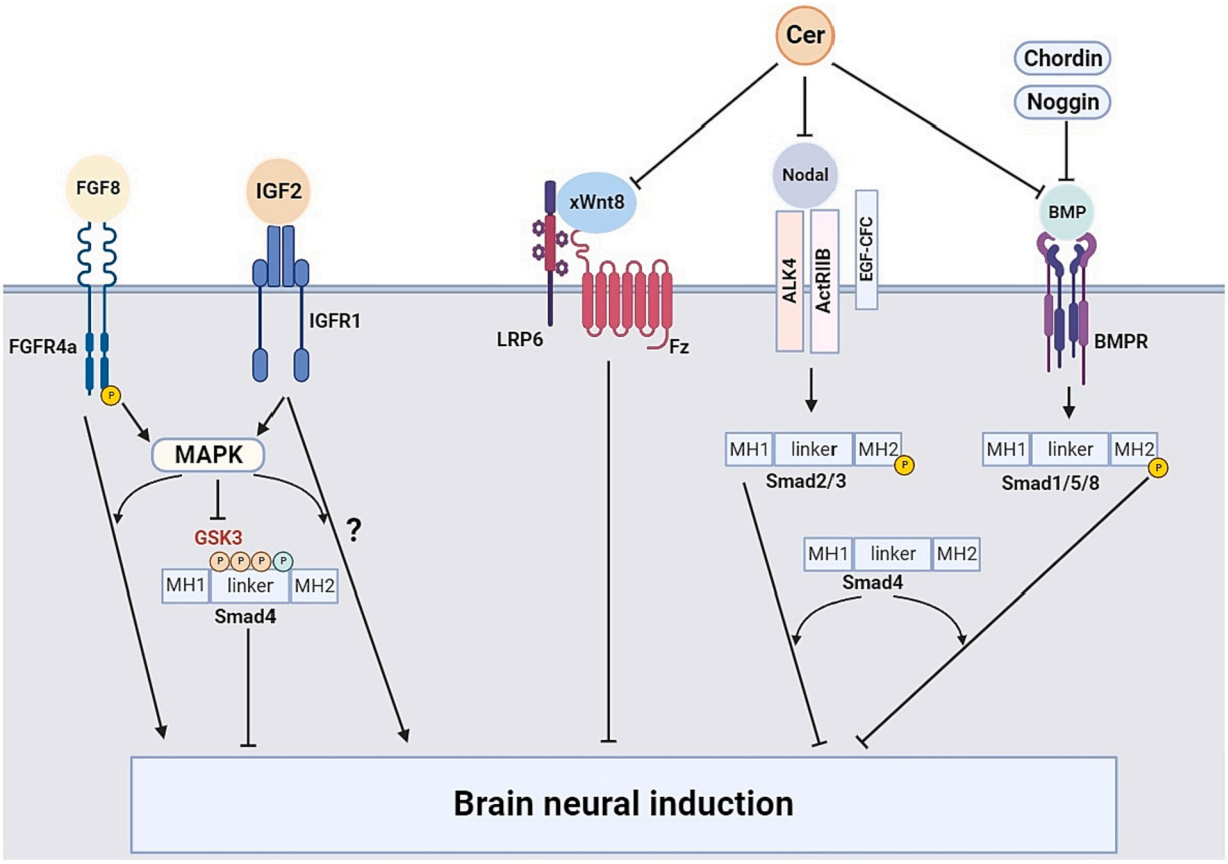


Fig. 7. Diagram of signaling pathways involved in brain induction in *Xenopus* embryos.
Cerberus is a multivalent inhibitor of BMP, Nodal and xWnt8 growth factors, inhibiting their binding to plasma membrane receptors. FGF8 and IGF2 are important positive signals that activate MAPK activity. Smads are transcription factors activated by BMP or Nodal/TGF-β carboxy terminal phosphorylation. They are also phosphorylated by MAPK, which primes phosphorylation by GSK3 resulting in the net inhibition of Smad4. The receptor Smads 1/5/8 and 2/3 also have linker inhibitory MAPK/GSK3 sites. Linker phosphorylations provide a hard-wired mechanism for the integration of signaling pathways at the protein level. Note that FGF8 and IGF2 also have Smad-independent positive mechanisms for neural induction and trunk inhibition. Figure drawn using BioRender.

An important additional integration node was provided by the discovery that Smad4, a co-Smad required for both the Nodal/TGF-β and the BMP pathways, is phosphorylated by MAPK and GSK3 in the linker region (Demagny et al., 2014). Previously, Smad4, which is a very potent tumor suppressor, was thought to not be regulated by phosphorylation.

It seems to us that the integration of MAPK and Smads signaling through linker phosphorylations remains an important node for regulation of neural induction. As indicated in Fig. 7, FGFs and IGFs likely also have other positive mechanisms in the induction of brain tissue in *Xenopus*.

3.5. Neural induction in other organisms

Neural induction has been extensively studied in many organisms, particularly in the chick (Stern, 2005), in which multiple FGFs and HGF play fundamental roles in neural plate induction (Streit et al., 1995, 2000). There are important developmental differences between species, for example, the initial dorsal signal in *Xenopus* occurs at the 32-cell stage (Tejeda-Muñoz and De Robertis, 2022), while the unincubated chick egg already has several thousand cells. The brain is the most complex organ in the vertebrates and the development of neural tissue can also be expected to be very complex. In the chick, an extensive gene regulatory network of over 100 transcriptional regulators induced by organizer tissue has been recently described (Trevers et al., 2023). In the mouse, in which development is much slower than in *Xenopus*, it is necessary to knock out both Chordin and Noggin to prevent forebrain development (Bachiller et al., 2000). Knockout mice for IGF1, IGF2 or IGF-1R have growth retardation but develop a CNS (Baker et al., 1993). However, in *Drosophila* embryos loss-of-function mutations in the IGF/Insulin receptor homolog *inr* strongly impair CNS development (Fernandez et al., 1995). Cerberus mouse knockouts are perfectly viable (Belo et al., 2000), while in *Xenopus* Cerberus MO in combination with Chordin MO essentially eliminates CNS formation (Kuroda et al., 2004). In brain organoids induced from stem cells, addition of FGF plays an essential role (Watanabe et al., 2005; Lancaster et al., 2013). In addition, in adult mouse brains IGF2 plays an essential role in maintaining neural stem cells (Ziegler et al., 2019; Lozano-Ureña et al., 2023).

3.6. Conclusions

In intact embryos, neural induction is more difficult to obtain than in isolated ectodermal animal cap explants. Cerberus will readily induce ectopic heads when introduced into the ventral side of the embryo through a negative molecular mechanism that involves triple inhibition of Nodal, BMP and Wnt signals required for trunk formation. The investigations presented here show that a positive IGF signal strongly cooperates with the negative Cerberus signal. There is also evidence that FGFs are important as positive signals that activate MAPK. While differences exist among the various species used to study CNS formation, the interest of the investigations presented in this paper is enhanced by the fact that amphibians were historically where the paradigm of head and trunk-tail organizers was initially described by Spemann in 1931. Developmental biology has a rich historical heritage, and old questions

about the nature of embryonic induction posed a century ago are still the subject of continuing inquiry.

4. Materials and methods

4.1. mRNA synthesis and β-catenin depletion

pCS2-*nLacZ* (nuclear LacZ), pCS2-*Cerberus*, pCS2-*IGF2* (wild type *Xenopus* IGF2; open reading frame with shortened, 24 amino acid long signal peptide), and pCS2-*DN-xIGFR* (dominant negative *Xenopus* IGF type I receptor consisting of only the extracellular domain) (Piccolo et al., 1999; Pera et al., 2001) were linearized by digestion with *Not* I. mRNAs were synthesized with SP6 RNA polymerase, using the mMessage mMachine SP6 kit (Invitrogen, Massachusetts, USA), and purified with the MegaClear kit (Invitrogen, Massachusetts, USA) as described (Tejeda-Muñoz and De Robertis, 2022). The β-catenin MO (5′ TTTCAACCGTTTCCAAAGAACCAGG 3′) was purchased from Gene Tools, LLC (Philomath, OR, USA).

4.2. Xenopus embryo manipulations

Animal experiments were done according to the recommendations in the Guide for the "Care and Use of Laboratory Animals of the National Institutes of Health". All experimental procedures followed the institutional animal care and use committee (IACUC) protocols (#D16-00124) and were approved by the University of California Animal Research Committee Los Angeles, Medical School (Permit Number: ARC-1995-129). Mature pigmented *Xenopus laevis* frogs were purchased from Xenopus-1 Inc. (Dexter, MI, USA). Eggs were fertilized *in vitro* using dissected testis. At the 2- or 4-cell stage, embryos in 1× MMR (Marc's modified Ringers) solution were injected four times radially with 8.5 ng of β-catenin MO (34 ng total). At the 4- or 8-cell stage, synthetic mRNA was injected into the margin of a single ventral blastomere or four times radially. Following culture in 0.1× MMR, the embryos were fixed with 4 % PFA (paraformaldehyde, Sigma, Missouri, US) diluted in PBS (phosphate buffer saline, Fisher BP339).

4.3. LacZ staining

Whole embryos were fixed with 0.5 × MEMFA (0.05 M MOPS pH 7.4, 1 mM EGTA, 0.5 mM $MgSO_4$ and 3.7 % formaldehyde) for 1 h and washed with PBS 3 times for 10 min at room temperature. Embryos were then stained in X-Gal solution (2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM X-Gal in PBS) overnight at 4 °C. Stained embryos were washed with PBS for 5 min, re-fixed in 0.5 × MEMFA at room temperature for 2 h and washed with PBS three times for 5 min.

4.4. qRT-PCR

The qRT-PCR analyses of *Xenopus* embryos at stages 12 or 22 were performed as previously described (Colozza and De Robertis, 2014). Primer sequences are listed in Table 1.

4.5. Imaging and histology

Embryos were imaged and filmed, using ZEISS Axio Zoom.V16. The 40-day old tadpoles (stage 58) were documented using a mobile phone camera. Ectopic heads were dissected, embedded in paraffin, sectioned, and stained in hematoxylin & eosin as described (Piccolo et al., 1999; https://hhmi.ucla.edu/derobertis/wp-content/uploads/2023/02/Hematoxylin.pdf). Fig. 7 and graphical abstract were drawn using the BioRender program.

4.6. Statistical analysis

Data were expressed as standard deviation of the mean. Statistical analysis was done using the student *t*-test of Excel. P values of <0.05* and <0.01** were considered statistically significant between means of samples.

Table 1
Primer sequences for qRT-PCR.

Gene	Forward primer sequence	Reverse primer sequence
Otx2	5′-GGA TGG ATT TGT TAC ATC CGT C-3′	5′-CAC TCT CCG AGC TCA CTT CCC-3′
Sox2	5′-GAG GAT GGA CAC TTA TGC CCA C -3′	5′-GGA CAT GCT GTA GGT AGG CGA-3′
Rx2a	5′-AGA CTG GTG GCT ATG GAG-3′	5′-ATA CCT GCA CCC TGA CTT-3
ADMP	5′ GAT GAT GGA AGG AGA GGA 3′	5′ TCA TGT TCT GAC CCA AAG 3′
Chordin	5′-CCT CCA ATC CAA GAC TCC AGC AG-3′	5′-GGA GGA GGA GGA GCT TTG GGA CAA G-3′
HoxB9/ XlHbox6	5′-TAC TTA CGG GCT TGG CTG GA-3′	5′-AGC GTG TAA CCA GTT GGC TG-3′

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cdev.2023.203897.

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CRediT authorship contribution statement

All authors performed experiments and wrote the manuscript.

Data availability

No data was used for the research described in the article.

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