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The *Xenopus Irx* genes are essential for neural patterning and define the border between prethalamus and thalamus through mutual antagonism with the anterior repressors Fezf and Arx

Elisa Rodríguez-Seguel [1], Pilar Alarcón [1], José Luis Gómez-Skarmeta *

Centro Andaluz de Biología del Desarrollo (CABD), Consejo Superior de Investigaciones Científicas/Universidad Pablo de Olavide, Carretera de Utrera Km1, 41013 Sevilla, Spain

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ABSTRACT

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The *Iroquois* (*Irx*) genes encode homeoproteins conserved during evolution. Vertebrate genomes contain six *Irx* genes organized in two clusters, *IrxA* (which harbors *Irx1*, *Irx2* and *Irx4*) and *IrxB* (which harbors *Irx3*, *Irx5* and *Irx6*). To determine the precise role of these genes during development and their putative redundancies, we conducted a comparative expression analysis and a comprehensive loss-of-function study of all the early expressed *Irx* genes (*Irx1–5*) using specific morpholinos in *Xenopus*. We found that the five *Irx* genes display largely overlapping expression patterns and contribute to neural patterning. All *Irx* genes are required for proper formation of posterior forebrain, midbrain, hindbrain and, to a lesser an extent, spinal cord. Nevertheless, *Irx1* and *Irx3* seem to have a predominant role during regionalization of the neural plate. In addition, we find that the common anterior limit of *Irx* gene expression, which will correspond to the future border between the prethalamus and thalamus, is defined by mutual repression between Fezf and Irx proteins. This mutual repression is likely direct. Finally, we show that Arx, another anteriorly expressed repressor, also contribute to delineate the anterior border of *Irx* expression.

Introduction

In vertebrates, the subdivision and generation of different identities in the developing neural system depend on the combinatorial activity of a group of transcription factors, which together form the so-called *prepattern* (Gómez-Skarmeta et al., 2003). The *Iroquois* (*Irx*) genes encode homeoproteins that participate in this prepattern (Gómez-Skarmeta and Modolell, 2002). Most vertebrates contain six *Irx* genes grouped in two paralog clusters of three genes each (de la Calle-Mustienes et al., 2005; Peters et al., 2000). The *IrxA* cluster contains *Irx1*, *Irx2* and *Irx4*, while the *IrxB* cluster harbours *Irx3*, *Irx5* and *Irx6*. In all organisms analyzed, the *Irx1*/*Irx2* and *Irx3*/*Irx5* pairs have very similar expression patterns (Alarcon et al., 2008; Bellefroid et al., 1998; Bosse et al., 1997; de la Calle-Mustienes et al., 2005; Garriock et al., 2001; Gómez-Skarmeta et al., 1998; Houweling et al., 2001; Lecaudey et al., 2005). The expression of the third gene in each cluster, *Irx4* or *Irx6*, is in general more divergent. However, in some tissues all the genes of a cluster, or even of both clusters, are identically expressed (Houweling et al., 2001; Lecaudey et al., 2005). In addition, the expression of *Irx* orthologs is largely equivalent, which is consistent with cross-species conservation of regulatory elements (de la Calle-Mustienes et al., 2005).

One common expression feature of the *Irx* genes is their sharp anterior expression border at the posterior diencephalon (Bosse et al., 1997; Lecaudey et al., 2005). This border has been recently shown to be important to position the posterior limit of the zona limitants intrathalamica (ZLI) and to provide competence to the thalamic region to respond to sonic hedgehog (Braun et al., 2003; Kiecker and Lumsden, 2004; Kobayashi et al., 2002; Scholpp et al., 2007). How the *Irx* anterior border is set-up is still controversial. Mutual antagonism between *Six3* and *Irx3* has been proposed to position the ZLI (Braun et al., 2003; Kobayashi et al., 2002). However, closer examination of the expression domains of *Six3* and *Irx3* in several species has shown a gap of expression between both genes (Kiecker and Lumsden, 2005; Lecaudey et al., 2005; Puelles et al., 2004; Wilson and Houart, 2004). Recently, *Fezf1* and *Fezf2* have been shown to be also required for positioning the ZLI (Hirata et al., 2006; Jeong et al., 2007). These genes are co-expressed in the forebrain and their expression domains abut the anterior border of *Irx1* (Hirata et al., 2006). Moreover, loss or gain of *Fezf* function shifts the anterior *Irx1* and *Irx3* borders rostrally or caudally, respectively (Hirata et al., 2006; Jeong et al., 2007). The reciprocal influence of *Irx* on *Fezf* has not been reported. Thus, it is not know whether a mutual antagonism between these genes defines their precise limits of expression in the diencephalon.

Most of the information on the function of *Irx* genes derives from overexpression experiments using wild-type or dominant negative forms of Irx proteins (Bao et al., 1999; Bellefroid et al., 1998, 2000; de

* Corresponding author.
E-mail address: jlgomska@upo.es (J.L. Gómez-Skarmeta).
[1] These authors contributed equally to this work.

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la Calle-Mustienes et al., 2002; Glavic et al., 2001, 2004a,b; Gómez-Skarmeta et al., 1998; Jin et al., 2003; Kiecker and Lumsden, 2004; Kobayashi et al., 2002; Kudoh and Dawid, 2001; Matsumoto et al., 2004; Mizuguchi et al., 2001; Novitch et al., 2001). Although these experiments indicate that *Irx* genes are required during vertebrate neural development and organogenesis, this interpretation needs some caution. In zebrafish, loss of *Irx* gene function has been assayed by injection of specific morpholinos (MOs) against only three *Irx* genes, *Irx1a*, *Irx1b* and *Irx7*. These experiments indicate that these gene function in isthmic organizer development, positioning of the boundary between rhombomeres 4 and 5, neural crest formation, proneural gene activation, heart contraction and retinal development (Cheng et al., 2006; Itoh et al., 2002; Joseph, 2004; Lecaudey et al., 2004). These results support an early requirement of *Irx* genes for different developmental processes. However, the attempts to analyze loss of function of some *Irx* genes in mice (*Irx2*, *Irx4*, and *Irx5*) have only disclosed late requirements for these genes in neural patterning and organogenesis (Bruneau et al., 2001; Cheng et al., 2005; Costantini et al., 2005; Lebel et al., 2003). The lack of early patterning defects in these mutant mice may be due to redundant *Irx* functions (Lebel et al., 2003).

Here, we examine the effect of impaired function of individual or combinations of *Irx* genes in early frog development by means of MOs against the different *Irx* genes of *Xenopus*. We demonstrate that *Irx* genes have essential, but partially redundant, functions during neural patterning. We show that the sharp border between *Fezf* and *Irx* genes, which is important for positioning the ZLI, is probably formed in the early neurula by a direct mutual antagonism between the products of these genes. In addition, we find that Arx, another anterior repressor, also helps define the rostral border of *Irx* expression.

Material and methods

Plasmid constructions

Fezf constructs were made using the *Fezf2* cDNA (IMAGE7981464). The Fezf2 zinc finger domain was amplified using the primers: 5′-**ggatct**GACAGAACCGGCAAAATCC-3′ and 5′-**gagctc**ACTCTGTCCAGTCCTGGAG-3′ that included a BglII and a SacI site, respectively (bold). The PCR fragment was subcloned and sequenced. The hormone-inducible GR domain was obtained by digestion with SacI and NotI from the construct MT-Irx1-GR (Alarcon et al., 2008). The Fezf zinc fingers and the GR domain were ligated to pBS-RN3-VP16 and pBS-RN3-EnR linearized with BamHI and NotI. pBS-RN3-VP16 and pBS-RN3-EnR derived from pBS-RN3-VP16-Mix and pBS-RN3-EnR-Mix (Lemaire et al., 1998).

In situ hybridization and X-Gal

Antisense RNA probes were prepared from cDNAs using digoxigenin or fluorescein (Boehringer Mannheim) as labels. *Xenopus* specimens were prepared, hybridized and stained as described (Harland, 1991). X-Gal staining was performed accordingly to (Coffman et al., 1993).

In vitro RNA synthesis and microinjection of mRNA and morpholinos

All DNAs were linearized and transcribed as described by Harland and Weintraub (Harland and Weintraub, 1985) with GTP cap analog (New England Biolabs). SP6, T3 or T7 RNA polymerases were used. After DNAse treatment, RNA was extracted with phenol–chloroform, column purified and precipitated with ethanol. mRNAs for injection were resuspended in water. Synthetic mRNAs or MOs were injected into embryos at the 1- or 2-cell stage using 5–10 nl. The following morpholinos were used in this study: MO*Irx1*: 5′-CATGTCTCTCCGGCAGGGAAATCGC-3′, MO*Irx2*: 5′-AGGTAACCCTGAGGATAGGACATGG-3′, MO*Irx3*: 5′-CTGTGGGAAGGACATGGTGCAGCCG-3′, MO*Irx3.2*: 5′-AGCTGTGGGAAGGACATGGTGCAGC-3′, MO*Irx4*: 5′-GTAGCCAAACTGAGGATATGACATT-3′ and MO*Irx5*: 5′-CAAGTAGCCCTGCGGATAGGACATG-3′. MO*Irx1* and MO*Irx5* are 100% homologous to the *Irx1* and *Irx5* alleles used in this study. The second *Irx5* and *Irx1* alleles contain 1 and 2 sequence mismatches, respectively, with their corresponding MOs. The other *Irx* MOs have 100% homology with all their corresponding *Irx* alleles. The specificity of these MOs has been shown recently (Alarcon et al., 2008).

Results

Comparative analysis of Irx expression patterns in Xenopus

We first compared in *Xenopus laevis* and *X. tropicalis* the patterns of expression of the different *Irx* genes at developmental stages ranging from gastrula to late neurula. We excluded *Irx6* from this analysis because it is only expressed after the period analyzed in this work (de la Calle-Mustienes et al., 2005). Our results complement and extend those previously published (Bellefroid et al., 1998; Garriock et al., 2001; Glavic et al., 2001; Gómez-Skarmeta et al., 1998). In *X. laevis*, at early gastrula *Irx1* and *Irx2* are the only *Irx* genes expressed in the presumptive neuroectoderm (Figs. 1A1, A2; insets). In addition, *Irx1* and *Irx3* are expressed in two symmetrical bands in the dorso-lateral mesoderm (Figs. 1A1, A3; arrowheads). *Irx4* and *Irx5* are not detected at this stage (Figs. 1A4, A5). At early neurula, the five *Irx* genes show similar, but not identical, expression patterns in two patches at each side of the midline that extend from the posterior forebrain to the spinal cord with different intensities at distinct A/P levels (Figs. 1B1–B5). In addition, *Irx1* is detected in the notochord (Fig. 1B1; arrowhead), and together with *Irx2* is expressed in the placodes (red arrows in Figs. 1B1, B2). To define the anterior border of *Irx* genes at early neurula stages, we performed a double staining analysis. The expression domains of all *Irx* genes are clearly separated several cells diameters from that of *Six3* (Fig. 1C1 and not shown) and abut that of *Fezf1* and *Fezf2* (Figs. 1C2–C5 and not shown). Thus, all *Irx* genes show the same anterior border, as it has been shown for most *Irx* genes in other organisms (Bosse et al., 1997, 2000; Braun et al., 2003; Cohen et al., 2000; Kiecker and Lumsden, 2004; Kobayashi et al., 2002; Lecaudey et al., 2005). This has been further confirmed by double staining for several *Irx* genes (Figs. 1D1, D2). The facts that the rostral limit of *Irx* expression extends anteriorly to the mesencephalon marker *Pax2* (Figs. 1D3 and D4), which overlaps with the diencephalon-expressed gene *Pax6* (Fig. 1D5), and abut *Fezf1* and *Fezf2*, which are expressed in the forebrain up to the prethalamus, indicate that this limit is located within the diencephalon at the border between the prethalamus and the thalamus. At later stages, the ZLI organizer will be positioned at this border.

Another common characteristic of *Irx* genes in other vertebrates is their exclusion from the midbrain–hindbrain boundary (mhb) (Bosse et al., 2000; Cohen et al., 2000; Lecaudey et al., 2005). This is not evident in *Xenopus* at the early neurula (Figs. 1B1–B5). However, at tailbud stage, the expression of all *Irx* genes is clearly reduced at this region (Figs. 1E1–1G5, red arrowheads). In the rhomboencephalon, all *Irx* genes show similar broad expression patterns, with stronger levels in even rhombomeres, as detected by co-expression with *Krox20* (Figs. 1G1–1G5, black and blue arrowheads point at rombomeres 3 and 5, respectively). This is even more extreme for *Irx4*, which is only expressed at high levels in rhombomere 3 (Figs. 1F4, E4, G4). At tailbud stage, and outside the nervous system, *Irx* genes are detected in the otic vesicle, the branchial arches and the prospective heart region (Figs. 1E1–E5 and not shown). In addition, *Irx1*, *2* and *3* are detected in the pronephros and *Irx5* in the eye (Figs. 1E1–E3, E5).

Identical patterns were found for *Irx* genes in *X. tropicalis* (Supplementary SFig. 1 and not shown).

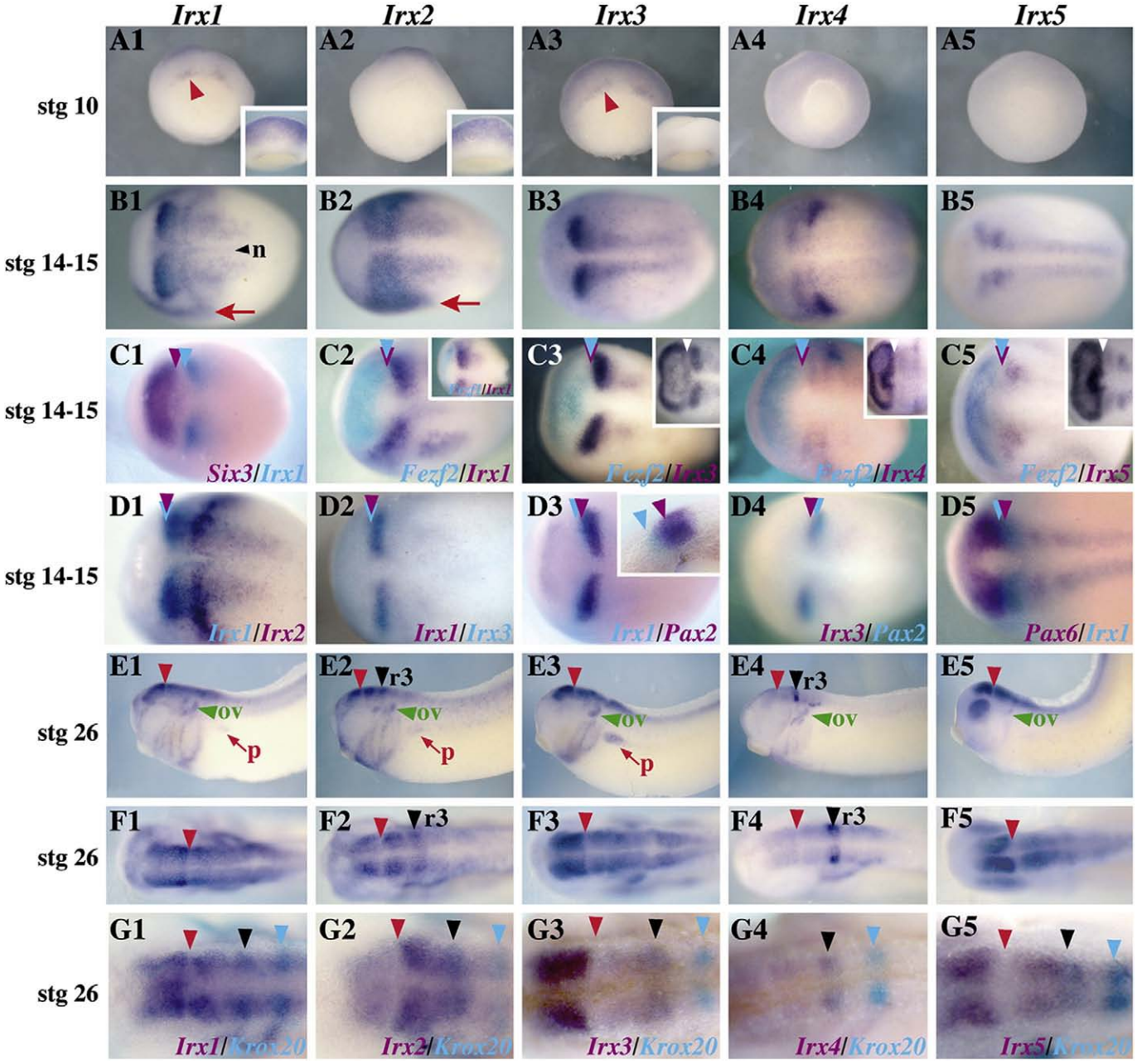

Fig. 1. Expression pattern of *Xenopus laevis Irx* genes. (A1–5) Vegetal views at early gastrula show that *Irx1* (A1) and *Irx3* (A3) are expressed in two dorso-lateral mesodermal bands (arrowhead). In addition, *Irx1* and *Irx2* are expressed in the presumptive neural ectoderm (A1 and A2, insets). (B1–5) Dorsal views of early neurula embryos illustrating the similar expression domains of all *Irx* genes (n, notochord). Arrowheads in (B1) and (B2) point at the placodal expression of *Irx1* and *Irx2*. (C1) Early neurula embryo double-stained for *Six3* (purple) and *Irx1* (cyan) genes. The expression patterns of these genes are separated a few cell diameters (arrowheads). (C2–C5) Early neurula embryo double-stained for *Irx* genes (purple) and *Fezf2* (cyan). Inset in (C2) show a similar embryo double-stained for *Irx1* (purple) and *Fezf2* (cyan). Insets in (C3–5) show double in situs developed in a single colour (purple) for *Fezf2* and *Irx3* (C3), *Irx4* (C4) and *Irx4* (C5). All *Irx* genes show similar anterior border abutting *Fezf* expression (arrowheads). (D1, D2) Double staining for *Irx1* and *Irx2* (D1) or *Irx3* (D2) also show that these genes share their anterior expression limit (arrowheads). (D3, D4). This border is rostral to *Pax2*, as shown in double-stained embryos for this gene and *Irx1* (D3) or *Irx3* (D4). Inset in (D3) show a sagittal section of a double-labelled embryo for *Pax2* (purple) and *Irx1* (cyan). Note that *Irx1* is anterior to that of *Pax2*. (D5) The *Irx* anterior limit overlaps with the posterior expression of *Pax6* in the forebrain. (E1–G5) At tailbud stage, in lateral views (E1–5) or in dorsal views (F1–5, G1–5) all *Irx* genes show similar expression patterns in the brain although with different intensities in different regions. This is more clearly seen in double-stained embryos for *Irx* genes (purple) and *Krox20* (cyan) (G1–G5). Red, black and blue arrowheads point at the M/H boundary, rhombomere 3 (r3) and rhombomere 5, respectively. Green arrowheads and red arrows point at the otic vesicle and pronephros, respectively. Stg, stage.

Irx genes are required for neural patterning

Different studies based on gain of function experiments of wild-type or dominant negative chimeras, suggest that *Irx* genes participate in the positioning of the ZLI, isthmic organizer and the rhombomere 4/5 boundary; they are also required for correct cerebellum formation and proneural gene activation (Anselme et al., 2006; de la Calle-Mustienes et al., 2002; Gómez-Skarmeta et al., 1998; Itoh et al., 2002; Kobayashi et al., 2002; Lecaudey et al., 2004; Matsumoto et al., 2004). To examine the requirement of *Irx* genes during *Xenopus* neural patterning, we used morpholinos (MOs) that specifically block the translation of each *Irx* mRNA (Alarcon et al., 2008). Each *Irx* MO was designed to block both *X. laevis* and *X. tropicalis* mRNAs and similar results were found with both organisms. Consistently with the largely overlapping expression domains of the different *Irx* genes, the effects of the different *Irx* MOs were similar although their strength varied

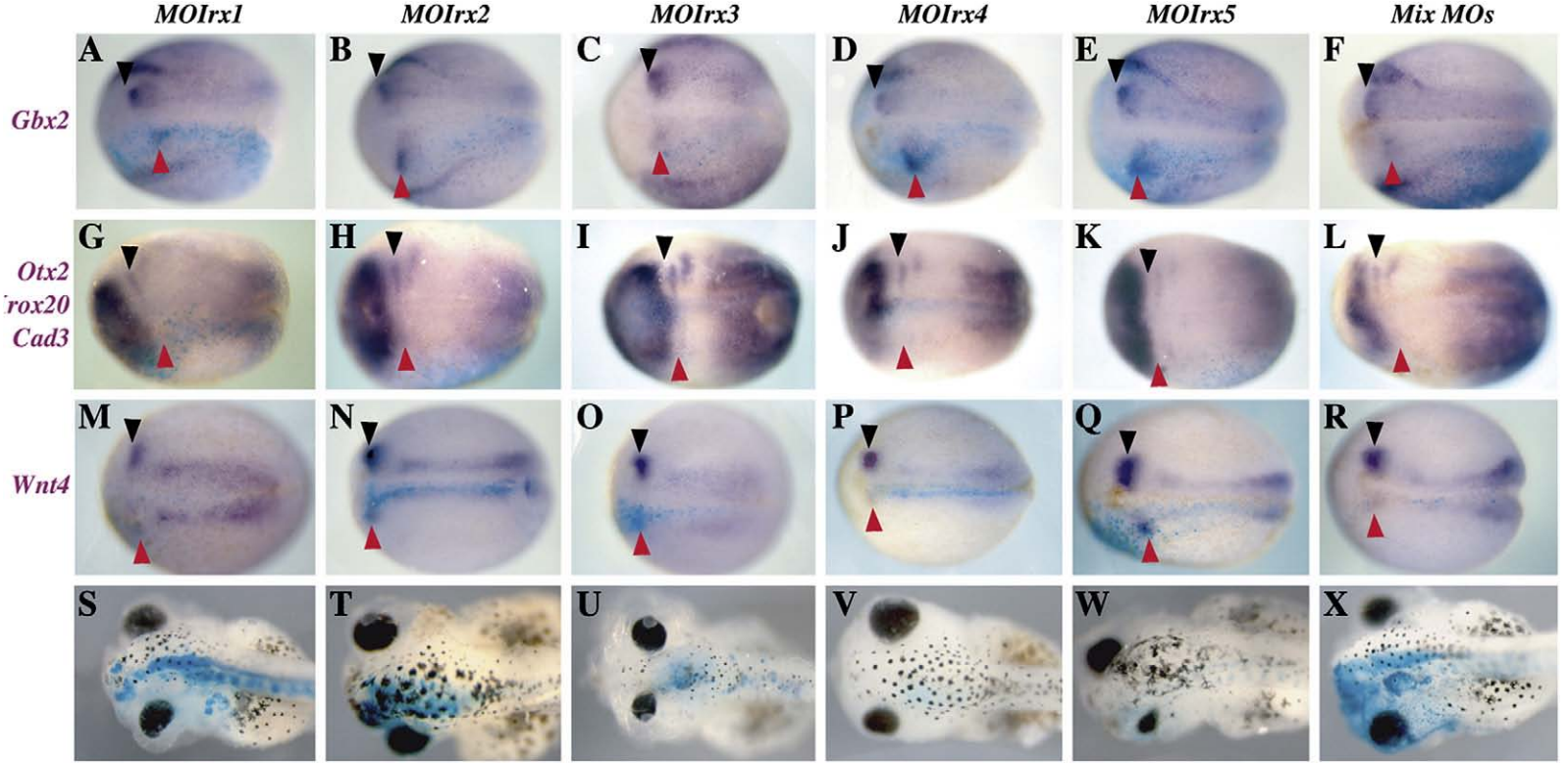

Fig. 2. *Irx* MOs causes antero-posterior neural defects. Dorsal views of *Xenopus tropicalis* embryos at early neurula (stg 14–15; A–R) or tadpoles (stg 42; S–X) injected with 10 ng of *MOIrx1* (A, G, M, S), *MOIrx2* (B, H, N, T), *MOIrx3* (C, I, O, U), *MOIrx4* (D, J, P, V), *MOIrx5* (E, K, Q, W) or a mix of 2 ng of each MO (F, L, R, X). The MOs were co-injected with *LacZ* mRNA as a tracer. In all embryos, red or black arrowheads point at the injected or control side, respectively. (A–F) In *Irx* morphant embryos, *Gbx2* is reduced and shifted caudally. (G–L). Impairment of *Irx* genes also caused *Otx2* posterior displacement and *Krox20* downregulation but does not affect *Cad3* expression. (M–R) In contrast, *Wnt4* expression in the spinal cord, as well as in the midbrain, is reduced. A caudal shift of the midbrain is also observed in some cases. (S–X) Later, all injected embryos show brain malformations and some of them eye defects.

with the gene inhibited. In all cases, the morphant embryos showed a caudally shifted forebrain, small midbrain, a reduced and caudally shifted midbrain/hindbrain boundary (MHB), alterations in the hindbrain, downregulation of some genes at the spinal cord, and eye defects (Fig. 2 and not shown). The strength of phenotypes for the different MOs (injected at 10 ng in *X. tropicalis* or at 20 ng in *X. laevis*) were Irx1 = Irx3 > Irx4 > Irx2 = Irx5 (45%, 46%, 40%, 28%, 30% of the embryos displayed patterning defects; $n = 97$, 62, 50, 51, 98, respectively). These phenotypes were not found with a control MO (not shown). Since cross-regulation could explain the similar phenotypes observed, we analyzed the expression of each *Irx* gene in morphants for all different *Irx* functions. No major effects were observed (not shown). These data confirm that all *Irx* genes participate, to some an extent, in anterior/posterior (A/P) neural patterning and brain formation.

We next compared the phenotype of the individual morphants with the effect of simultaneously blocking translation of all *Irx* genes (2 or 4 ng of each MO injected in *X. tropicalis* or *X. laevis*, respectively). The phenotypes were similar, but appeared with higher penetrance (55%; $n = 56$) (Fig. 2). This analysis was further extended by examining a broader spectrum of A/P neural markers in *X. laevis* embryos in which early *Irx* functions had been impaired (Fig. 3). In the forebrain, the morphant embryos showed a posterior expansion of the forebrain markers *Six3*, *Rx1*, *Fezf1* and *Fezf2* (Figs. 3A–C; 35–52%, $n = 29$–58; and not shown), an enlargement of the *Pax6* expression domain (Fig. 3D; 56%, $n = 27$) and a downregulation of the anterior-most expression of *Wnt1* and *Irx3* in the prospective thalamic territory (Figs. 3E, F; 48–56%, $n = 44$–56). In the midbrain, a strong down-regulation of the expression domain of *En2*, *Pax2* and *Wnt4* (Figs. 3G–J 42–50%, $n = 54$–96) and a reduction of the territory that lacks *Pax6* was observed (Fig. 3D 56%, $n = 27$). We also observed a poster-iorization of the MHB (*Otx2*, *Gbx2*, *Pax2*, *Fgf8*; Figs. 3G, J and not shown; 51–64%, $n = 48$–84) and a reduced expression of several hindbrain-expressed genes like *Wnt1*, *Gbx2*, *Krox20* and *Nhf1*β (Figs. 3E, J, K, L; 60–72%, $n = 40$–65). In the spinal cord, the embryos showed reduced *Irx3* and *Wnt4* expression (Figs. 3F, I; 55%, $n = 21$), but not a major effect on *Pax6* or *Cad3* expressions (Fig. 3D and not shown). In these injected embryos, although we did not detect a strong impairment of the pan-neural marker *Sox2* (Fig. 3M), we did observe an alteration of its expression levels in forebrain, midbrain and hindbrain that is consistent with an expanded forebrain, reduced midbrain and altered hindbrain. All these alteration can also be

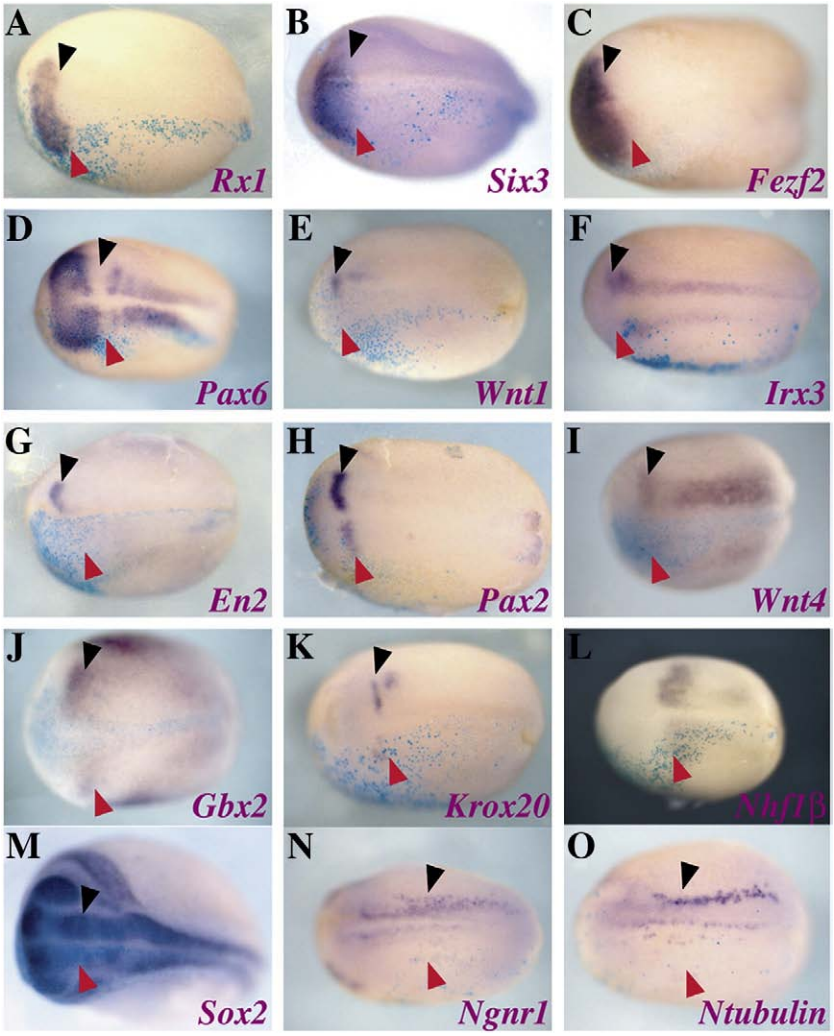

Fig. 3. *Irx* genes are required for neural patterning. All panels, except (M) that show a stage 18 embryo, show dorsal views of stage 14–16 embryos co-injected with a mix of all five *Irx* MOs and *LacZ* mRNA. Black and red arrowheads point at the control or injected side, respectively. (A–C) Forebrain markers *Rx1*, *Six2* and *Fezf2* are expanded posteriorly (compare red with black arrowheads). (D) In addition, the anterior *Pax6* domain is also shifted caudally at the expenses of the midbrain territory that lack the expression of this gene (compare red with black arrowhead). (E–F) The expression of *Wnt1* and *Irx3* in the posterior diencephalon is strongly downregulated (compare red with black arrowheads). (G–I) The midbrain is also reduced in the *Irx* morphant, as determined by the expression of *En2*, *Pax2* and *Wnt4*. In addition, *Wnt4* expression in the spinal cord in strongly impaired (I). (J–L) The rhomboencephalon markers *Gbx2*, *Krox20* and *Nhf1*β are also downregulated in the injected embryos. (M) At stage 18, *Sox2* expression shows and altered morphology of brain structures in the *Irx* impaired side. (N, O) The proneural gene *Ngnr1* (N) and the primary neurons markers *Ntubulin* (O) are also downregulated in the injected side.

observed in the morphology of stage 42 dissected brains (SFig. 2). We also examined if these patterning defects were due to alterations in the rate of cell proliferation or cell death in the neural plate of the *Irx* morphant embryos. We only detected a minor increase in cell death in the neural plate of the MO injected embryos, which cannot explain the strong patterning defects observed (SFig. 3).

We then analyzed in *Irx* morphant embryos the expression of the proneural gene *Xnrg1*, which is known to be activated by *Irx* genes (Bellefroid et al., 1998; de la Calle-Mustienes et al., 2002; Gómez-Skarmeta et al., 1998; Itoh et al., 2002). *Xnrg1* was also downregulated in *Irx* morphant embryos (Fig. 3N; 50%, $n=42$). Probably as a consequence, the differentiation of primary neurons was also impaired (Fig. 3O).

The spinal cord is subdivided by a combination of transcription factors activated in different domains of the dorsal/ventral (D/V) axis. Gain of function experiments have shown that *Irx* genes participate in this process (Briscoe and Ericson, 2001). Thus, mutual antagonism between dorsally expressed *Irx3* and ventrally expressed *Olig2* set the border between the domains where dorsal V2 and ventral motor neurons arise (Briscoe et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001; Zhou and Anderson, 2002). Since in the developing spinal cord of early neurula all *Irx* genes display similar expression patterns (Figs. 1B1–B5), we determined how D/V patterning was affected in *Irx* morphant embryos. The expression of five D/V spinal cord genes (*Pax3*, *Pax6*, *Olig2*, *Nkx6* and *Nkx2*) was examined in stage 25 *X. tropicalis* morphants for individual *Irx* genes or for a combination of all *Irx* MOs (SFig. 4). Similar patterning defects were observed with the individual *Irx* MOs, although the phenotypes were strongest with *Irx1*, *Irx2* and *Irx3* MOs, and for the mix of all MOs. Interference with any of the *Irx* genes had no effect either in the dorsal-most (*Pax3*), intermediate (*Pax6*) or ventral-most expressed gene (*Nkx2*) (SFig. 4 A, D, G, J, M, P and not shown). However, the expression of *Olig2* was shifted dorsally, a result expected if Irx proteins repress this gene, (SFig. 4 B, E, H, K, N, Q). Unexpectedly, in some *Irx* morphants, the *Nkx6* domain of the injected side was ventrally shifted (SFig. 4 C, F, I, L, O, R). These results suggest that Irx proteins are not only required to repress *Olig2*, but they may also be necessary to set the dorsal limit of the *Nkx6* domain. Thus, the *Irx* genes are necessary for patterning the neuroectoderm in the A/P and D/V axes.

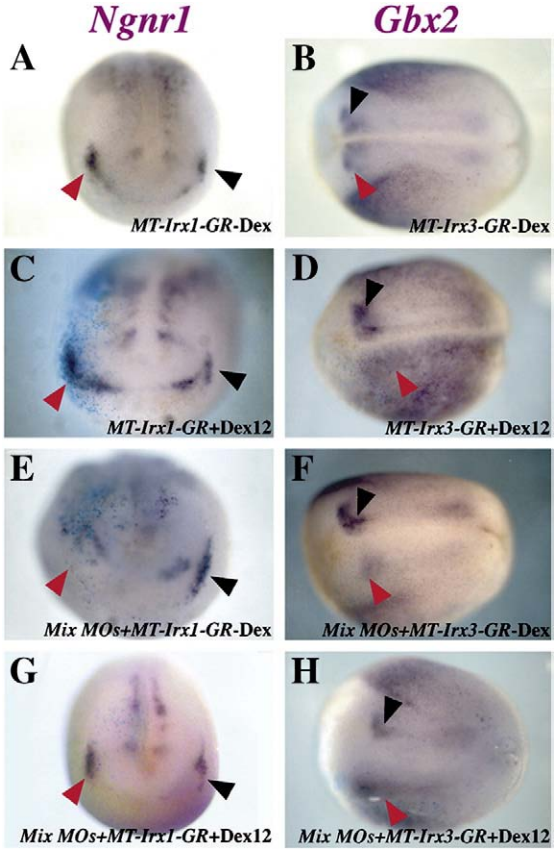

Fig. 4. Rescue of *Irx* MOs defects. All embryos are at stage 14–16. (A, C, E and G) are anterior and (B, D, F, H) are dorsal views, respectively. Black and red arrowheads point at control and injected sides, respectively. Embryos injected with *MT-Irx1-GR* (A, C, E, G) or *MT-Irx3-GR* (B, D, F, H) mRNAs alone (A–D) or with a mix of *Irx* MOs (E–H). (A–D) In the presence of Dexamethasone, the injected mRNAs caused expansion of *Ngnr1* (C) and *Gbx2* (D). This is not observed in the absence of the hormone (A, B). (E–H) In embryos co-injected with *MT-Irx-GR* mRNA and *Irx* MOs, in the absence of Dex, *Ngnr1* (E) and *Gbx2* (F) are downregulated. (G, H) This phenotype is rescued in the presence of Dex.

The MOs defects are rescued by overexpression of any Irx gene

Since the loss of function of each *Irx* gene produces similar neural defects, although of different intensities, it is likely that the functions of these genes are partially interchangeable. This is supported by the fact that misexpression of different *Irx* genes cause similar phenotypes (de la Calle-Mustienes et al., 2002). To test this hypothesis, we examined the capacity of individual *Irx* genes to rescue the defects induced by simultaneous impairing of all five genes. Accordingly, we generated hormone-inducible forms of the different *Irx* proteins (MT-Irx-GR) that were insensitive to the MOs (Alarcon et al., 2008). These constructs allowed activation of the Irx proteins after gastrulation, thus eliminating possible earlier effects of *Irx* genes on mesoderm formation (Glavic et al., 2001). The different MT-Irx-GR proteins behave similarly in overexpression studies (see below and not shown). We next injected *MT-Irx1-GR* or *MT-Irx3-GR* mRNAs with or without a mix of all *Irx* MOs. Overexpression of these mRNAs caused upregulation of *Xngnr1* and *Gbx2* when Dexamethasone (Dex) was added at stage 12 (Fig. 4C, D; 40–52%, $n=48$–60), but not in the absence of the hormone (Figs. 4A, B). In contrast, embryos co-injected with the mix of *Irx* MOs and any of the MT-Irx-GR mRNAs showed, in the absence of Dex, the expected downregulation of these genes (Figs. 4E, F; 50–52%, $n=30$–44). This effect was rescued upon the addition of the hormone to the co-injected embryos at stage 12 (Fig. 4G,H: 15–17% downregulation and 38–60% upregulation, $n=26$–28). These data indicate that overexpression of any *Irx* gene compensates to a large an extent the impairment of all *Irx* genes, pointing out again to a partially redundant function of these genes.

Mutual repression between Irx and Fez defines the border between the prethalamus and the thalamus at early neurula

The anterior limit of expression of *Irx* genes defines the border between the prethalamus and thalamus and the posterior limit of the zona limitants intrathalamica (ZLI) (Braun et al., 2003; Kiecker and Lumsden, 2004; Kobayashi et al., 2002; Scholpp et al., 2007; Staudt and Houart, 2007; and this work). Our work also shows that the reduction of *Irx* function impairs the formation of all neural territories located posteriorly from the prospective thalamus and expands more anterior territories. Thus, the definition of a sharp anterior *Irx* expression border is essential for brain regionalization. *Fezf* genes, which encode zinc finger transcription factors, have been implicated in defining this border in mice (Hirata et al., 2006). In *Xenopus*, the expression of *Fezf* genes abuts that of *Irx* genes (Figs. 1C2–C5), which is also compatible with this function. Accordingly, overexpression of 250 pg of *Fezf1* mRNA (Matsuo-Takasaki et al., 2000) downregulated *Irx1* and *Irx3* (Figs. 5A, B; 55% $n=57$). Fezf proteins contain an Engrailed-like repressor domain at their amino-termini and are thus believed to act as transcriptional repressors (Hashimoto et al., 2000). We have examined this possibility by injecting hormone-inducible constructs containing either the Engrailed repressor or the VP16 activator domains fused to Fezf1 zinc fingers (*EnR-Znf-GR* and *VP16-Znf-GR*, see material and methods). Embryos injected with *EnR-Znf-GR*

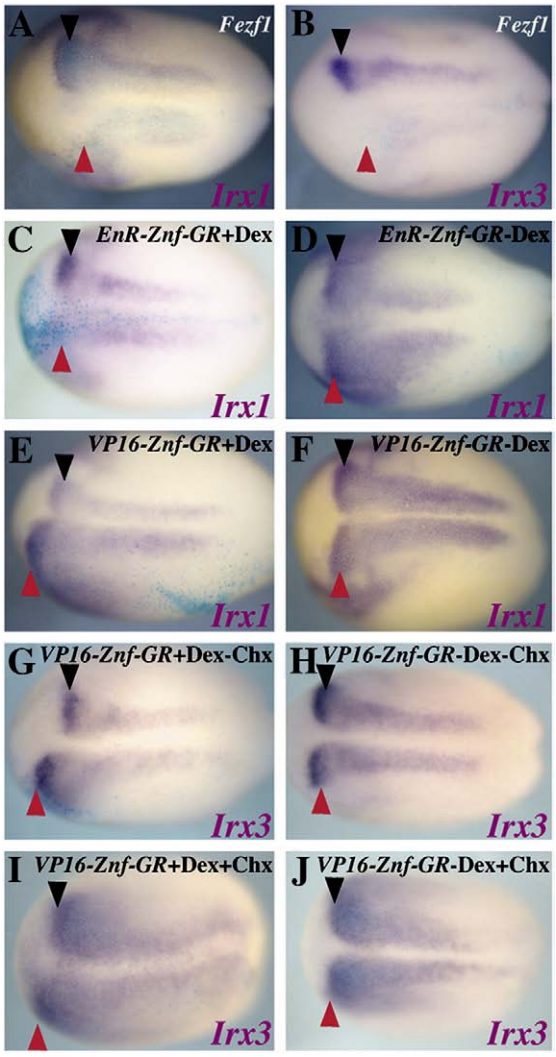

Fig. 5. Fezf represses *Irx* genes. All embryos are dorsal views at stage 14–15. Black and red arrowheads point at the control or injected side, respectively. (A, B) In embryos injected with *Fezf1* mRNA *Irx1* (A) and *Irx3* (B) are downregulated. (C, D) Injection of *EnR-Znf-GR* mRNA caused similar *Irx1* downregulation in the presence of Dex (C) but not in its absence (D). (E, F) Injection of *VP16-Znf-GR* mRNA caused anterior *Irx1* expansion in the presence of Dex (E) but not in the absence of the hormone (F). (G–J) *Irx3* is also activated by *VP16-Znf-GR* mRNA even in the presence of cycloheximide.

mRNA (0.25 ng) showed a downregulation of *Irx1* and *Irx3* similar to that observed in *Fezf*-injected embryos, but only upon hormone addition at stage 12 (Figs. 5C, D and not shown). In contrast, injection of *VP16-Znf-GR* mRNA expanded anteriorly the expression of *Irx1* and *Irx3* (Figs. 5E–H; 52% $n=62$), an effect similar to that observed in mice deficient for both *Fezf* genes (Hirata et al., 2006). These data indicate that Fezf proteins act as repressors during early neural patterning by downregulating *Irx* expression. This could be a direct effect. If so, VP16-Znf-GR should directly activate *Irx* genes in the absence of protein synthesis. This was the case since expression of *Irx1* and *Irx3* was expanded anteriorly in *VP16-Znf-GR*-injected embryos treated with cycloheximide (CHX) for 30 min, and then for 3 h with CHX and Dex (Figs. 5I–J and not shown; 56%, $n=46$). Thus, Fezf proteins are likely direct repressors of *Irx* genes and delimit their anterior border of expression.

We have shown that interference with *Irx* function caudally extends *Fezf* expression (Fig. 3). This suggests that Irx proteins probably define, by repression, the posterior border of the *Fezf* domain. Accordingly, overexpression of inducible Irx proteins (Alarcon et al., 2008) caused hormone-dependent downregulation of *Fezf* genes (Figs. 6A, B and not shown; 62% $n=32$). This effect was mimicked by overexpressing an mRNA encoding an inducible chimera (300 pg of *HD-GR-EnR*) that contained the Irx1 homeodomain fused to the EnR domain (Gómez-Skarmeta et al., 2001) (Figs. 6C, D; 67% $n=27$). In contrast, a similar chimeric protein fused to the E1A activator domain (300 pg of *HD-GR-E1A* mRNA; Gómez-Skarmeta et al., 2001) caused a clear posterior expansion of *Fezf* expression (Figs. 6E–H; 53% $n=34$). This indicates that Irx proteins repress *Fezf* expression. To test whether this effect is direct, we analyzed if HD-GR-E1A-mediated *Fezf* expansion depends on protein synthesis. In the presence of the protein synthesis inhibitor CHX, embryos injected with *HD-GR-E1A* mRNA still showed *Fezf* caudalization (Figs. 6I, J; 57%, $n=44$).

Our results therefore indicate that *Fezf* and *Irx* genes are directly repressing each other.

The repressor Arx participates in anteriorly delimiting Irx expression

In zebrafish, *fezf2*, but not *fezf1*, is expressed in the prethalamus, abutting the domain of *Irx* expression (Jeong et al., 2007; Scholpp et al., 2007; Staudt and Houart, 2007). Impairment of zebrafish *fezf2* causes only moderate *irx3a* anterior expansion (Jeong et al., 2007). Thus, it is possible that other anteriorly expressed genes help define,

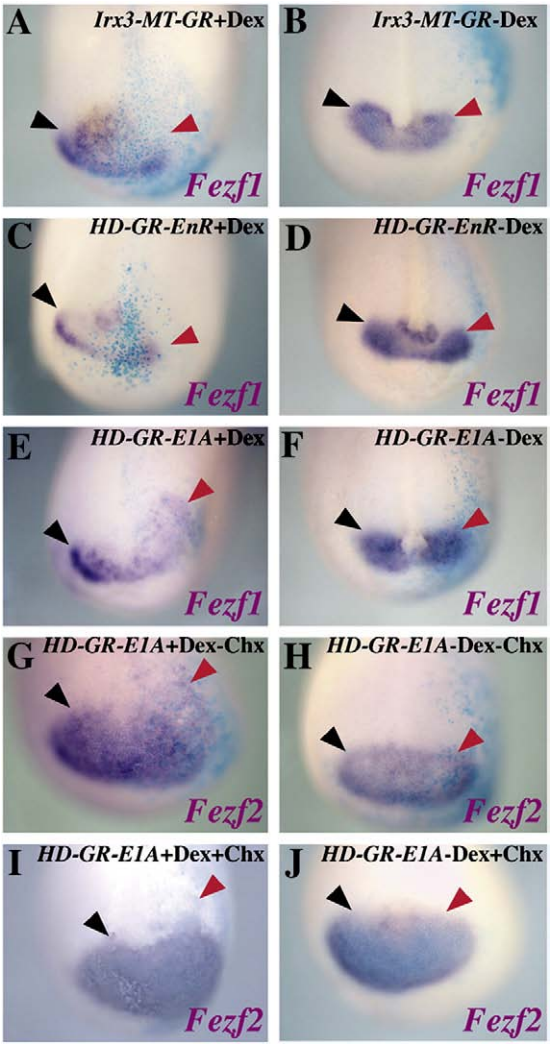

Fig. 6. Irx repress *Fezf* genes. All embryos are dorsal views at stage 14–15. Black and red arrowheads point at the control or injected side, respectively. (A, B) In embryos injected with *Irx3-MT-GR* mRNA *Fezf1* is downregulated in the presence (A) but not in the absence (B) of Dex. (C, D) Injection of *HD-GR-EnR* mRNA caused similar *Fezf1* downregulation. (E, F) In contrast, injection of *HG-GR-E1A* mRNA caused posterior *Fezf1* expansion in the presence (E) but not in the absence of the hormone (F). (G–J) *Fzf2* is also activated by *HG-GR-E1A* mRNA even in the presence of cycloheximide.

in addition to *Fezf*, the anterior limit of the *Irx* domain and therefore the prethalamus–thalamus border. One candidate is the forebrain gene *arx*, whose posterior expression border abuts that or *irx7* in zebrafish (Staudt and Houart, 2007). We have determined the spatial relationship between *Fezf*, *Arx* and *Irx* genes in *Xenopus* (Figs. 7A,B). *Arx* is expressed within the *Fezf* territory and both genes share their posterior borders (Fig. 7A). This border abuts the anterior limit or *Irx* territory (Fig. 7B). Since the expression domain of *Fezf* genes is broader and initiates before that of *Arx* (El-Hodiri et al., 2003; Matsuo-Takasaki et al., 2000), it is possible that *Arx* lies downstream of *Fezf*. We therefore analyzed whether manipulation of *Fezf* activity altered *Arx* expression. Overexpression of *Fezf1* expanded *Arx* expression posteriorly (Fig. 7C; 50% $n=39$). A similar effect was found upon overexpressing *EnR-Znf-GR* mRNA (not shown). Conversely, embryos injected with the dominant negative *Fezf* construct (*VP16-Znf-GR*) shifted anteriorly the expression of *Arx* (Fig. 7D; 65% $n=46$). No effect on *Fezf* genes could be detected by modulating *Arx* activity (not shown). This suggests that *Arx* lies downstream of *Fezf*.

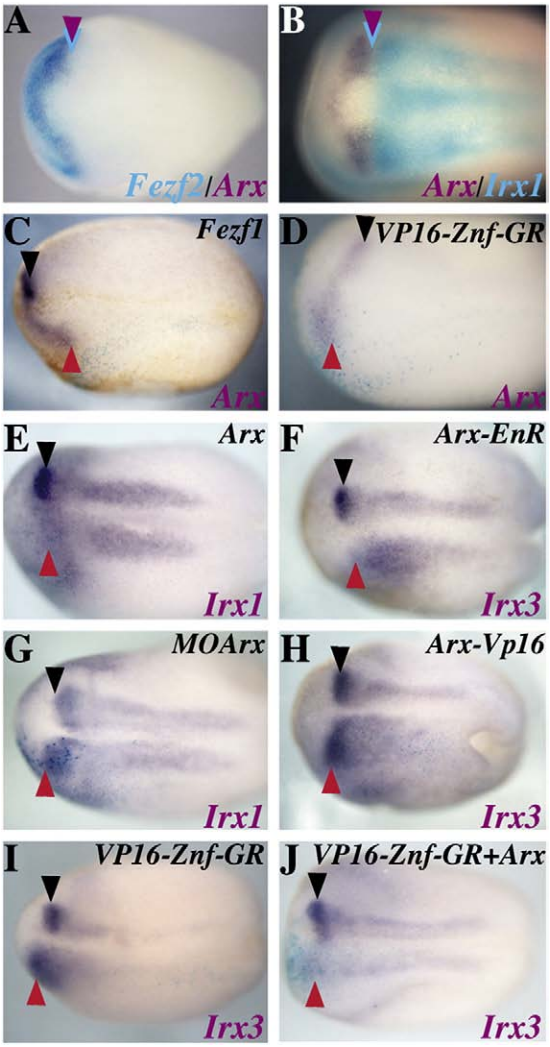

Fig. 7. Arx represses *Irx* genes. All embryos are dorsal views at stage 14–15. Black and red arrowheads point at the control or injected side, respectively. (A) Double staining of *Fezf2* (cyan) and *Arx* (purple). Both genes share their posterior limits (arrowheads). (B) Double staining of *Irx1* (cyan) and *Arx* (purple) showing the complementary expression domains of these genes. (C) Injection of *Fezf1* mRNA expanded *Arx* posteriorly. (D) In embryos injected with *VP16-Znf-GR* mRNA *Arx* expression is shifted anteriorly. (E–F) Overexpression of *Arx* (E) or *Arx-EnR* (F) mRNAs downregulated *Irx1* (E) and *Irx3* (F). (G) Impairment of *Arx* function with a specific MO expands *Irx1* anteriorly. (H) Injection of *Arx-VP16* mRNA caused a similar rostral expansion of *Irx3*. (I) Injection of *VP16-Znf-GR* mRNA expands anteriorly *Irx3* expression. (J) This effect is reverted by overexpressing *Arx*.

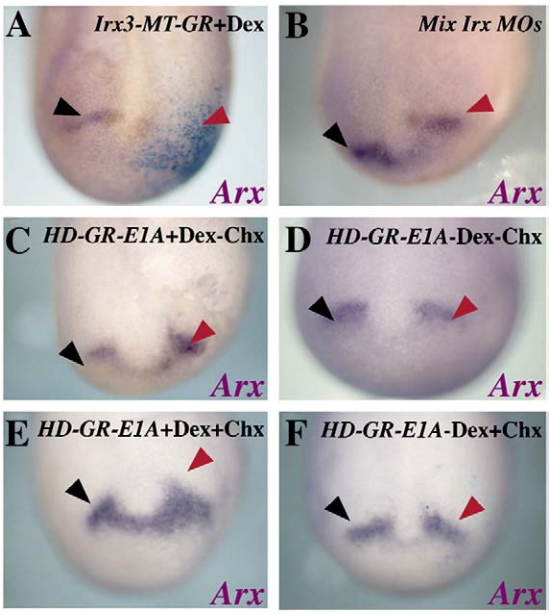

Fig. 8. Irx repress *Arx*. All embryos are dorsal views at stage 14–15. Black and red arrowheads point at the control or injected side, respectively. (A) In embryos injected with *Irx3-MT-GR* mRNA *Arx* is downregulated in the presence of Dex. This effect was not observed in the absence of the hormone (not shown). (B) Impairment of *Irx* activity caused caudal expansion of *Arx*. (C, D) Injection of *HG-GR-E1A* mRNA caused posterior *Arx* expansion in the presence (C) but not in the absence of the hormone (D). (E–F) This expansion also occurred in the presence of cycloheximide.

We next assayed whether *Arx* could repress *Irx* expression. Injection of *Arx* mRNA (500 pg) strongly downregulated the anterior-most expression domain of *Irx1* and *Irx3* (Fig. 7E and not shown; 80% $n=52$). Arx can act as an activator or a repressor during forebrain development (Seufert et al., 2005). Embryos injected with 250 pg of an mRNA encoding a chimeric Arx construct fused to the EnR repressor domain (Seufert et al., 2005), similarly downregulated *Irx1* and *Irx3* (Fig. 7F and not shown; 75% $n=39$). In contrast, embryos injected with 15 ng of a morpholino against *Arx* or 250 pg of a mRNA encoding a Arx-VP16 chimeric protein (Seufert et al., 2005), expression of *Irx* genes was similarly expanded and slightly shifted rostrally (Figs. 7G, H and not shown; 60% $n=58$). These results indicate that Arx is a repressor of *Irx* expression. To analyze whether *Arx* could repress *Irx* when *Fezf* function is impaired, we co-injected *Arx* mRNA and the dominant negative *Fezf* encoding mRNA (*VP16-Znf-GR*). These co-injected embryos showed a clear *Irx* downregulation (Figs. 7I, J and not shown; 45% $n=35$). These results indicate that Arx acts as a repressor independent of Fez function and that it contributes to limit the anterior expression of *Irx* genes. The lack of an *Arx* inducible construct prevented us to determine whether this repression occurs in the absence of protein synthesis.

We also determined whether *Irx* genes could repress *Arx*. Indeed, overexpression of *Irx* or *HD-GR-EnR* mRNAs downregulated *Arx* or caudally displaced its expression (Fig. 8A and not shown). In contrast, impairment of *Irx* activity with a mix of *Irx* MOs or by overexpressing *HD-GR-E1A* mRNA caused a posterior shift of *Arx* expression (Figs. 8B, C). The ability of *HD-GR-E1A* mRNA to promote a caudal displacement was also observed in the absence of protein synthesis (Figs. 8C–F). These results suggest that Irx proteins likely bind directly to *Arx* regulatory elements to repress this gene.

Discussion

We found that *Xenopus Irx* genes display expression patterns largely similar to those of their orthologous genes in other species. This is probably due to evolutionary conserved regulatory regions (de

la Calle-Mustienes et al., 2005). In each organism, these patterns partially overlap suggesting at least some degree of redundant functions.

Irx genes play essential functions during neural development

Our loss-of-function study demonstrates that *Irx* activity is required for proper neural patterning in both the anterior–posterior and the dorsal–ventral axes. Consistent with their largely overlapping expression domains, all *Irx* genes seem to collaborate for the proper specification of different neural territories. Thus, interference with any *Irx* gene caused similar, albeit quantitatively different, defects: posterior shift of the forebrain, reduction of midbrain and hindbrain structures, downregulation of some genes in the spinal cord, dorsal shift of the ventral neural tube domain expressing *Olig2* and eye defects. All *Irx* genes are largely co-expressed in these tissues, excepting in the eye, where only *Irx5* is detected. Interestingly, all *Irx* genes are co-expressed with *Wnt4* in the mesencephalon, and all of them are required for its expression. Given that *Wnt4* is necessary for eye development (Maurus et al., 2005), it is likely that the eye defects associated with *Irx* morphants are indirect. We have not detected major differences in the rate of cell proliferation or cell death in the *Irx* morphant embryos. Thus, the major defects are due to patterning alterations. The fact that *Fezf*, *Arx*, *Rx* and *Six3* are expanded posteriorly, and that the anterior expression of *Irx3* and *Wnt1* are strongly reduced, indicates that in *Irx* deficient embryos there is a caudal enlargement of forebrain structures anterior to the thalamus, at least in part, at the expense of this territory. This enlargement also affects the mesencephalon, since *En2*, *Wnt4* and *Pax2* are reduced, while *Pax6* is caudally extended. The mesencephalon is not only reduced, but it is also displaced posteriorly, shifting the midbrain–hindbrain boundary (MHB) posteriorly and reducing the cerebellum territory. In the dorsal–ventral axis of the neural tube, there are also patterning defects in the intermediate region. Thus, a dorsal shift of the territory expressing *Olig2*, in which motorneurons develop, occurs at the expense of the V2 domain, which expresses *Irx* genes. In contrast, the dorsal and ventral-most territories are not affected in *Irx* morphants.

The phenotypes observed are largely similar to those obtained in mice homologous for the *Ft* deletion, which eliminates the *IrxB* complex and three adjacent genes (Anselme et al., 2006; Gotz et al., 2005). Therefore, many phenotypic defects associated with this mutation are most likely due to the simultaneous removal of the *IrxB* genes. Our data also confirms previous loss-of-function *Irx* studies in zebrafish (*Irx1a*, *Irx1b* and *Irx7*), and those based on the overexpression of wild-type and dominant negative *Irx* molecules in different organisms (Briscoe et al., 2000; Cheng et al., 2006; de la Calle-Mustienes et al., 2002; Gómez-Skarmeta et al., 1998; Itoh et al., 2002; Joseph, 2004; Kiecker and Lumsden, 2004; Kobayashi et al., 2002; Lecaudey et al., 2004; Matsumoto et al., 2004). These studies, together with our loss-of-function analysis in *Xenopus*, indicate that the *Irx* genes have important functions in neuroectoderm regionalization.

While interference with any *Irx* gene affects patterning to a different an extent, the depletion of *Irx1* and *Irx3* induced the strongest defects. These genes appear to have the most relevant functions during neural patterning. This could explain why the available *Irx* knock-out mice (for *Irx2*, *Irx4* and *Irx5*) display only minor neural defects (Bruneau et al., 2001; Cheng et al., 2005; Lebel et al., 2003). If this were the case, we expect that mice lacking *Irx1* or *Irx3* would have more severe phenotypes. Another explanation for the minor defects of the available *Irx* mouse mutants is a compensatory upregulation of other *Irx* genes when *Irx2* or *Irx4* are eliminated (Bruneau et al., 2001; Lebel et al., 2003). This compensatory effect is not observed in *Xenopus*. The compensatory upregulation observed in mice mutant for *Irx* genes may reflect the presence of new cross-regulatory modules not found in *Xenopus* or fish. These modules may lie within the highly conserved non-coding regions common to human and mouse genomes but absent in *Xenopus* (de la Calle-Mustienes et al., 2005).

In many processes, due to their largely overlapping expression patterns, different *Irx* genes have equivalent functions. In that sense, they behave as redundant genes. However, since it seems that for many processes a high level of *Irx* activity is required, interference with any *Irx* genes would decrease *Irx* activity below the required level, causing similar defects. This is most evident in *Xenopus* or zebrafish, organisms that do not seem to have the compensatory cross-upregulation mechanisms that operate in mice and make *Irx* genes much more redundant between them. Despite these compensatory mechanisms, the full set of *Irx* genes is conserved in evolution, pointing out to essential specific functions for individual *Irx* genes. The available data for the three *Irx* mouse mutant indicate that this is the case for *Irx4* and *Irx5*, which are necessary for the correct contractile functions of the developing heart. Moreover, although *Irx2* deficient mice appear normal, abnormalities may be beyond the detection procedures. We conclude that *Irx* genes have many essential functions during different stages of development.

The prethalamus–thalamus border (PTB) is defined at early neurula stages by cross-repression of Fezf/Arx and Irx genes

The zona limitants intrathalamica (ZLI) is an organizing center, localized at the PTB, that plays essential function in forebrain patterning (Hashimoto-Torii et al., 2003; Scholpp et al., 2007; Scholpp et al., 2006; Vieira and Martinez, 2006). Several reports have shown that the expression of *Irx* genes in the thalamus is required to define this border and to set the posterior limit of the ZLI (Braun et al., 2003; Kiecker and Lumsden, 2004; Kobayashi et al., 2002; Scholpp et al., 2007). However, there is still some controversy about the gene/genes required at the prethalamus side to define this border. Although mutual antagonisms between *Six3* and *Irx3* genes have been proposed to position this border and the ZLI in chick (Braun et al., 2003; Kobayashi et al., 2002), recent re-examination of the expression domains of these genes in several species indicated a gap of expression between both genes (Kiecker and Lumsden, 2005; Lecaudey et al., 2005; Puelles et al., 2004; Wilson and Houart, 2004). In contrast, studies in mice and zebrafish have shown that *Fezf1* and *Fezf2* genes abut *Irx* genes (Hirata et al., 2006; Scholpp et al., 2007), are necessary for prethalamus formation, prevent anterior *Irx* expansion and are required for positioning the ZLI (Hirata et al., 2006; Jeong et al., 2007). We show that in *Xenopus*, all *Irx* genes share the same anterior border at the PTB and are required for proper development of the thalamic territory as well as all other posterior located brain structures. We also show that both *Fezf* genes are expressed in an anterior domain abutting *Irx* genes. Our overexpression and loss-of-function analyses demonstrate that a mutual repression between Fezf and Irx proteins takes place during early neurula stages to define what will become the future PTB. Our results are consistent with a recent report that, by means of transplantation assays, proposed that the prethalamus is established at late zebrafish gastrula (Staudt and Houart, 2007). Moreover, our studies suggest that the mutual antagonism between Fezf and Irx can occur in the absence of protein synthesis and therefore is likely to be direct.

We have identified another anteriorly expressed repressor, Arx, that helps delimit the anterior expression of *Irx* genes and therefore is likely important to set the PTB. Our experiments indicate that *Arx* lies downstream of *Fezf* genes in the genetic cascade required for anterior forebrain formation. Thus, gain or loss of *Fezf* activity expands or impairs *Arx* expression, respectively. In contrast, manipulation of *Arx* function does not affect the expression of *Fezf* genes. Fezf proteins are required as repressors to expand *Arx* expression. Thus, the effect of *Fezf* on *Arx* should be an indirect consequence of the enlargement of forebrain structures in *Fezf*-overexpressing embryos. Remarkably,

under conditions in which *Fezf* function is impaired, Arx can still downregulate *Irx* genes. Thus Arx repress *Irx* genes through a mechanism independent on Fezf proteins.

Many parallels can be established between the PTB and the midbrain–hindbrain boundary (MHB). Both are sources of signaling molecules that pattern adjacent tissues, have lineage restriction properties, are positioned by mutual direct antagonism between repressors (Otx2 vs Gbx2 in MHB; Fezf1, Fezf2 and Arx vs Irx1–5 in PTB) and factors at each side of the border confer specific competences to these territories to respond to the signaling molecules emanating from the border (Kiecker and Lumsden, 2005). Interestingly, *Irx* genes also participate in positioning the MHB (Glavic et al., 2002) and provide competence to the anterior hindbrain to form cerebellum (Matsumoto et al., 2004). An addition, a parallel can be established between subdivision of the brain and the spinal cord. Thus, combinations of cross-repression between pairs of transcription factors subdivide the neural tube in different dorsal–ventral territories (reviewed in Dessaud et al., 2008). *Irx* genes also participate in this process (Briscoe et al., 2000; Lu et al., 2002; Mizuguchi et al., 2001; Novitch et al., 2001). Thus a common mechanism to subdivide the developing neural ectoderm is the cross-repressive interactions between different set of transcription factors. Our comprehensive *Irx* loss-of-function study clearly demonstrates the *Irx* genes participate in many of these neural regionalization events.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.02.028.

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