Int. J. Dev. Biol. 55: 923-931
doi: 10.1387/ijdb.103253in

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

The RNA-binding protein Xp54nrb isolated from a Ca^{2+}-dependent screen is expressed in neural structures during *Xenopus laevis* development

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ABSTRACT In amphibian embryos, calcium (Ca^{2+}) signalling is a necessary and sufficient event to induce neural fate. Transient elevations of [Ca^{2+}]i are recorded in neural tissue precursor cells in whole embryos during gastrulation. Using a subtractive cDNA library between control ectoderm (animal caps) and ectoderm induced toward a neural fate by Ca^{2+} release, we have isolated several Ca^{2+}-induced target genes. Among the isolated genes, *Xp54nrb* encodes a protein which exhibits the RRM domains characteristic of RNA binding proteins, and is implicated in pre-mRNA splicing steps. Here we show that the *Xp54nrb* transcripts are expressed throughout early developmental stages, specifically in the neural and sensorial territories and that *Xp54nrb* could be involved in anterior neural patterning.

KEY WORDS: *Xenopus laevis, neural fate, eye development, RNA-binding protein, calcium signalling*

Introduction

The developmental process of neural induction is highly conserved among vertebrates. Early *Xenopus* embryos have been used as a model system to investigate the first steps of this important event. It has been suggested that neural induction results from the opposing action of ventralizing signals such as bone morphogenetic proteins (BMPs) from the ectoderm, which are responsible for the determination of the epidermis, and dorsalizing signals, such as noggin, chordin, follistatin, XnR3 and Cerberus, from the dorsal mesoderm. The molecular mechanisms driving neural specification depend partly on a context where BMP pathway is downregulated (review by Sasai and De Robertis 1997).

However antagonizing BMP signalling is not sufficient to explain neural induction and other signalling pathways are necessary. In particular, in amphibian embryos during gastrulation transient elevations of internal calcium concentration ([Ca^{2+}]i) are restricted to the neural tissue precursor cells. These Ca^{2+} transients require the activation of DHP-sensitive Ca^{2+} channels (Leclerc *et al.,* 1997; Leclerc *et al.,* 2000). Ectodermal explants (animal cap) isolated at blastula are multipotent cells and exhibit developmental plasticity. In the absence of an inducing signal, the ectodermal cells express markers specific to epidermis. However, in the presence of BMP antagonists, such as noggin, they express neural-specific markers. Using this assay we have shown that Ca^{2+} signaling is a necessary and sufficient event to induce neural fate (reviewed in (Moreau *et al.,* 2009). Indeed, an artificial increase in [Ca^{2+}]i obtained by an entry of Ca^{2+} through plasma membrane Ca^{2+} channels or by caffeine, known to stimulate the release of Ca^{2+} from internal stores, is sufficient to trigger the expression of proneural markers such as *Pou2* within 30 minutes (Moreau and Leclerc 2004) but also the formation of neurons and glial cells (Moreau *et al.,* 1994). In order to identify new genes early transcribed after a Ca^{2+} signal and involved in neural induction we have generated a subtractive cDNA library between untreated animal caps (i.e. fated to give epidermis) and caffeine-treated animal caps (i.e. fated to give neural tissue) (Batut *et al.,* 2003).

Among the Ca^{2+} target genes isolated from the cDNA subtractive library and involved in neural induction (Batut *et al.,* 2003; Batut *et al.,* 2005), a differentially expressed gene encoding a putative RNA-

Abbreviations used in this paper: AC, animal cap; BMP, bone morphogenetic protein; GFP, Green Fluorescent Protein; MoXp54, morpholino against Xp54nrb; nrb, nuclear RNA-binding; RBP, RNA-binding protein.

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Accepted: 3 August 2011. *Final, author-corrected PDF published online:* 10 January 2012

ISSN: Online 1696-3547, Print 0214-6282

binding protein, initially annotated as EFNA-2-prov, and renamed *Xp54nrb,* is selected for further analysis. In this study, we establish that *Xp54nrb* expression is restricted to neural tissues during *Xenopus* embryogenesis, with a strong persistence in the anterior nervous system and in the visual structures. In addition Xp54nrb knockdown by specific morpholino leads to reduced expression of proneural markers in the anterior region of the neural plate.

Results

Isolation of a new RNA binding protein from the calcium-specific subtractive library

In an attempt to identify Ca^{2+}-responding genes critical for *Xenopus* early neural development using a cDNA subtractive library between short-time Ca^{2+}-induced and non-induced ectoderms (Batut *et al.,* 2003; Batut *et al.,* 2005) together with a large-scale whole mount *in situ* hybridization, we identified a partial cDNA fragment (clone 3F4) that is exclusively present in the subtracted population issued from the neuralized ectoderms and totally absent from the subtracted non-induced population. The partial cDNA clone 3F4 is used as probe for *in silico* cDNA library screening and the corresponding cDNA is identified as a complete clone, with the initial annotation for encoding an EFNA-2 related as provisional sequence (accession number BC045128; (Klein *et al.,* 2002). Another clone sharing 98% identity in its nucleotide sequence is generated from a systematic screening library of *Xenopus* anterior structures (accession number AB238228; (Takahashi *et al.,* 2005).

As shown in Fig. 1, the resulting 464 amino acid-long polypeptide contains two highly conserved RNA Recognition Motifs (RRM1 and RRM2), a QH-rich domain and an acidic/basic region characteristic of the RNA-binding protein (RBP) family. The alignment sequence across vertebrate species reveals that our clone is homologous to the non-POU-domain containing, octamer binding protein, NonO/p54nrb in mammalians (Dong *et al.,* 1993) (i.e. 75% and 74% identity with *Homo sapiens* and *Mus musculus* respectively; Table1). The lowest similarity is with the *Drosophila melanogaster* NonA protein (Jones and Rubin 1990; Rendahl *et al.,* 1996). According to its sequence, the putative EFNA-2 is therefore renamed Xp54nrb, for *Xenopus laevis* 54 kDa nuclear RNA-binding protein.

Since *Xp54nrb* is isolated in a screen for Ca^{2+}-responding genes, we confirmed by RT-PCR that the expression of *Xp54nrb* during neuralisation of animal caps depends on Ca^{2+} signalling. Previously we have shown that the treatment of animal caps with the BMP antagonist noggin leads to a rapid and transient increase in $[Ca^{2+}]_i$ (Batut *et al.,* 2005; Leclerc *et al.,* 1997). Therefore, animal caps prepared from stage 9 embryos are incubated for 30 min with 20 μM of BAPTA-AM prior to incubation with noggin (at 2 μg/mL) and then cultured to late gastrula (stage 12). RT-PCR analyses indicate that the expression of *Xp54nrb* is strongly reduced by BAPTA and

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                                - QH-rich region -
X.laevis        MQGNRGYRMEQQNHTPRRQQQQQQHHQQQQ--------PETTSPTNGQQATSPNEGVTID
X.tropicalis    MQGNRGYRMEQQNHAPRRQQHQQQHHHQHQQ-------AETTSPTNGQQATSPNEGVTID
M.musculus      MQSNKAFNLEKQNHTPRKHHQHHHQQHHQQQQQQQQQQPPPPIPANGQQASSQNEGLTID
H.sapiens       MQSNKTFNLEKQNHTPRKHHQHHHQQQHHQQQQQQ--PPPPPIPANGQQASSQNEGLTID
G.gallus        MQGNKGFNMEKQNHAPRKQHQHQHQQH--PPPSIPANGQQANSQKQLCTLFPSDEGLTID
D.rerio         MQGNRGPRSEQQNHGPQRQQPNP---QQEQQRKP-TGADSNGQHTDAGEQSSPNAGFTID

                                [-----------------RRM1------------------------
X.laevis        LKNFRKPSEKTFTQRSRLFVGNLPSDVTEEEMRKLFEKFGKAGEIFIHKDKGFGFIRLET
X.tropicalis    LKNFRKPGEKTYTQRSRLFVGNLPMDVTEEEMRKLFEKYGKAGEIFIHKDKGFGFIRLET
M.musculus      LKNFRKPGEKTFTQRSRLFVGNLPPDITEEEMRKLFEKYGKAGEVFIHKDKGFGFIRLET
H.sapiens       LKNFRKPGEKTFTQRSRLFVGNLPPDITEEEMRKLFEKYGKAGEVFIHKDKGFGFIRLET
G.gallus        LKNFRKPGEKTFTQRSRLFVGNLPPDITEEEMRKLFEKYGKAGEVFIHKDKGFGFIRLET
D.rerio         LQNFKKPGEKTYTQRSRLFVGNLPAGTTEEDVEKLFSKYGKASEIFINKDRGFGFIRLET

                ------------------------]         [------------------RRM2----
X.laevis        RTLAEIAKAELDNLPLRGKQLRVRFACHSAALSVKNIPQFVSNELLEEAFSMFGQVERAV
X.tropicalis    RTLAEIAKAELDNLPLRGKQLRVRFACHSASLSVKNIPQFVSNELLEEAFSIFGQVERTV
M.musculus      RTLAEIAKVELDNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAV
H.sapiens       RTLAEIAKVELDNMPLRGKQLRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAV
G.gallus        RTLAEIAKVELDNMPLRGKQLRVRFACHSASLTVRNLPQFVSNELLEEAFSVFGQVERAV
D.rerio         KTLADIAKAELDDTIFRGRQIRVRFATHGAALTVKNLPQFVSNELLEEAFSMFGPIERAI

                -------------------------------]
X.laevis        VIVDDRGRSTGKGIVEFASKPSARKALDRCKDGAYLLTAFPRPITVEPMDQLDDEEGLPD
X.tropicalis    VIVDDRGRSTGKGIVEFASKPSARKALDRCSDGAYLLTSFPRPITVEPMDQLDDEEGLPE
M.musculus      VIVDDRGRPSGKGIVEFSGKPAARKALDRCSEGSFLLTTFPRPVTVEPMDQLDDEEGLPE
H.sapiens       VIVDDRGRPSGKGIVEFSGKPAARKALDRCSEGSFLLTTFPRPVTVEPMDQLDDEEGLPE
G.gallus        VIVDDRGRSSGKGIVEFSGKPAARKALDRCSDGSFLLTTFPRPVTVEPMDQYDDEEGLPE
D.rerio         VIVDDRGRPTGKGIVEFANKPSARKALDRCGDGAFLLTAFPRPVTIEPMEQLDEEEGLPE

                                                                [-----HTH--
X.laevis        KLLVKNQMCHKEREQPPRFAQPGSFEYEYAMRWKALIDMEKQQQQQVDRNIKEAQEKMEI
X.tropicalis    KLLVKNQMCQREREQPPRFAQPGSFEYEYAMRWKALIEMEKQQQEQVDRNIKEAQEKMEI
M.musculus      KLVIKNQQFHKEREQPPRFAQPGSFEYEYAMRWKALIEMEKQQQDQVDRNIKEAREKLEM
H.sapiens       KLVIKNQQFHKEREQPPRFAQPGSFEYEYAMRWKALIEMEKQQQDQVDRNIKEAREKLEM
G.gallus        KLVIKNQQYHKEREQPPRFAQPGSFEYEYAMRWKALIEMEKQQQEQVDRNIKEAREKLEM
D.rerio         RLINKNPVYHKEREQPPRFAQPGSFEYEYAMRWKALMEMEKQQYEQVDRNIKEAKEKLET

                ----------]       [----------------------basic/acid----------
X.laevis        EMEAARHEHQVMLMRQDLMRRQEELHRMEELHNQEIQKRKQIEIRHEEERRRREEETRRQ
X.tropicalis    EMEAARHEHQVMLMRQDLMRRQEELRRMEELHSQEVQKRKQIELRHEEERRRREEETRRQ
M.musculus      EMEAARHEHQVMLMRQDLMRRQEELRRMEELHNQEVQKRKQLELRQEEERRRREEEMRRQ
H.sapiens       EMEAARHEHQVMLMRQDLMRRQEELRRMEELHNQEVQKRKQLELRQEEERRRREEEMRRQ
G.gallus        EMEAARHEHQVMLMRQDLMRRQEELRRMEELHNQEVQKRKQLEIRQEEERRRREEEMRRQ
D.rerio         ELEAARHEHQVMLMRQDLLRRQEELRRMEEAHSQEVQKRKQMELRQEEERRRREEELRAH

                ----------]
X.laevis        QEEMLRRQQEGFKNSYPDAREAD-LRMAQMAMPGAMGLNNRASLGNSNVASGAAATPGPG
X.tropicalis    QEEMLRRQQEGFKNSYPDAREAD-IRMAQMAMPGAIGMNNRPSLANSNVASGAAANAGPG
M.musculus      QEEMMRRQQEGFKGTFPDAREQE-IRMGQMAMGGAMGINNRGAMPPAPVPPGTPAPPGPA
H.sapiens       QEEMMRRQQEGFKGTFPDAREQE-IRMGQMAMGGAMGINNRGAMPPAPVPAGTPAPPGPA
G.gallus        QEEMMRRQQEGFKGNFADAREPPDMRMGQMGMGGTIGMNNRGAMGGTNVPAAAPPAAGPG
D.rerio         SEELMRRQQ-GQGGNFSEKRDPD-MRM---HMGGQGMGMNRNPMGGNASNT---------

X.laevis        AMMPEAAAIGMTSPPNDRFGQAPNIEGLG--GANPPAFPRGAPGADFGPNKRRRY  464
X.tropicalis    AMMPEAAAIGMTSPPTERFGQAPNIEGLG--GANPPAFPRGTPGADFGPNKRRRY  465
M.musculus      TMMPD-GTLGLTPPTTERFGQAATMEGIGAIGGTPPAFNRPAPGAEFAPNKRRRY  475
H.sapiens       TMMPD-GTLGLTPPTTERFGQAATMEGIGAIGGTPPAFNRAAPGAEFAPNKRRRY  473
G.gallus        AMIPDGAMGMTPPPPADRFGQGSAMEGLGAMGGNPPAFNRGNPGGEFGPNKRRRY  473
D.rerio         ----------------GSTNLTSNEQGASGNPGGLPLPFPRPGPNDFGANKRRRF  441
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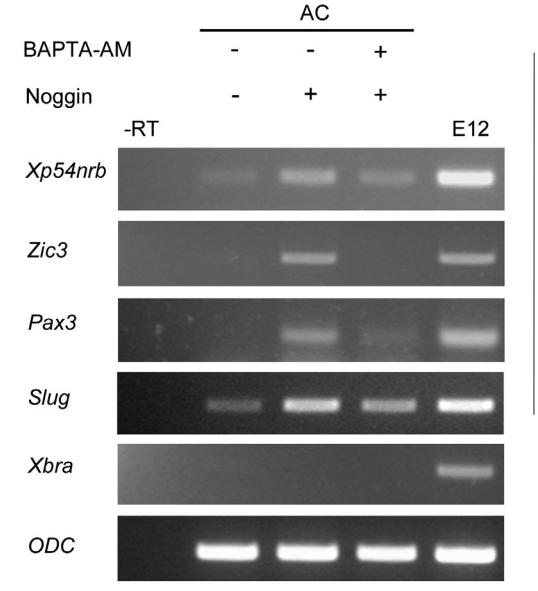
Fig. 1. 3F4 encodes an RNA binding protein. Alignment of p54nrb amino acid sequences from different species. *Sequence comparison indicates that the putative protein encoded by a full-length cDNA belongs to the RNA binding protein family, sharing the 2 conserved RRM regions (in grey) specific for this class of proteins (QH rich domain, RRM1, RRM2, acidic/basic region). They are found in several species: mouse (protein id: NP_075633), rat (protein id: NP_001012356) and human (protein id: NP_031389), and putative translated sequences from* Xenopus tropicalis *(BC066129), zebrafish* Danio reiro *(BC046880) and chicken* Gallus gallus *(AJ720639). The zebrafish sequence is more divergent in the N-terminal and C-terminal domains.*

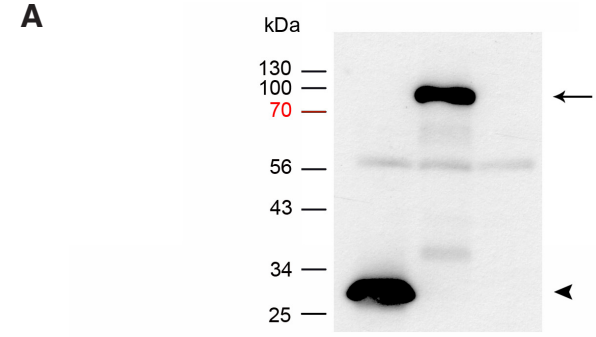
that the expression of the pan-neural marker *Zic3* is completely abolished by BAPTA (Fig. 2, n=2 independent experiments).

Recently, it has been suggested that animal caps might have pre-neural or neural border character which leads to a reconsideration of the naive properties of this model (Linker *et al.,* 2009). To verify whether animal caps possess naïve properties in our hands we assayed the expression of *Pax3,* a marker of neural border character (Wills *et al.,* 2010). As shown on Fig. 2, *Pax3* expression is undetectable in non stimulated animal caps. In addition the induction of *Pax3* expression by noggin protein is reduced when animal caps are pre-loaded with the calcium chelator BAPTA-AM. The prospective neural crest gene *Slug* (Mayor *et al.,* 1995) is weakly expressed in control explants however, it remains transcribed after Noggin treatment even in the presence of BAPTA. No expression of the mesodermal marker *Xbra* is detected in these animal caps. These data strongly suggest that the induction of *Xp54nrb* expression in animal cap cells treated with the BMP antagonist noggin requires an increase in $[Ca^{2+}]_i$.

Nuclear localisation of Xenopus *Xp54nrb protein*

Xp54nrb exhibits the RRM domains characteristic of RNA binding proteins, implicated in pre-mRNA splicing steps. This mechanism occurs within the nucleus and in mammalian cells in specialized nuclear structures (Fox *et al.,* 2002). The first indication of p54nrb/NonA nuclear localisation emanated from *Drosophila* immunohistochemistry assay (Rendahl *et al.,* 1992). A nuclear localisation sequence of p54nrb is carried by the C terminus domain, since its deletion implies the cytoplasmic retention of the truncated form (Zhang and Carmichael 2001). In order to study the subcellular localisation of Xp54nrb, we have injected a synthetic *Xp54nrb* mRNA tagged with *GFP* (see experimental procedures). We have verified by western blot experiments that this tagged protein is expressed at gastrula stage embryo (Fig.3A). As shown in Fig. 3B, the exogenous Xp54nrb-GFP tandem protein is effectively visualized within the nucleus of injected cells, whereas the GFP protein alone remains mainly cytoplasmic. To confirm this nuclear localisation, the embryos are cultivated in the presence of the DNA marker DRAQ5. Indeed, the Xp54nrb-GFP foci are ex-





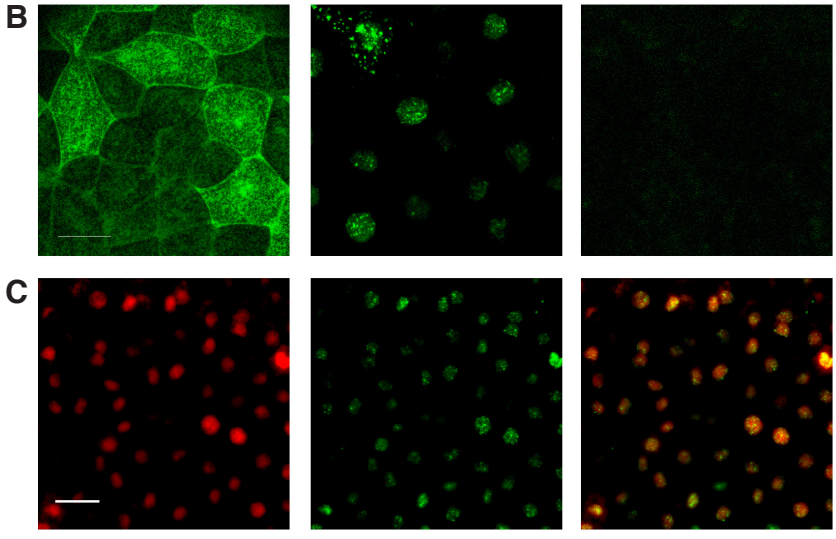


Fig. 2 (Left). *Xp54nrb* expression is induced by noggin and repressed by Ca^{2+} chelator. *The expression of* Xp54nrb, Zic3, Pax3, Slug, Xbra *and* ODC *in ten animal caps (AC) is analysed by RT-PCR. AC excised at stage 8-9 are pre-incubated (+) or not (-) for 30 minutes with BAPTA-AM (20 μM), a membrane-permeant Ca^{2+} chelator, prior to incubation with noggin protein (2 μg/mL). The RNA from one sibling embryo at stage 12 serves as positive control and PCR without reverse transcription (-RT) is performed to check the absence of contamination.* ODC *is used as a loading control.*

Fig. 3 (Right). Xp54nrb-GFP is localised in nucleus. (A) *Two-cell-stage embryos are injected into one blastomere with 50 pg* GFP *mRNA or 1 ng* Xp54nrb-GFP *mRNA. Lysates from stage 13 whole embryos are analysed by immunoblotting with anti-GFP antibody. Left lane: GFP protein at 27 kDa (arrowhead), middle lane: tagged protein migrating at 81 kDa (arrow), right lane: uninjected embryo.* **(B)** *Confocal observation of ectodermal cells at late gastrula stage. Left panel: 2-cell stage embryos are microinjected at the animal pole with 50 pg of* in vitro *synthetised* GFP *mRNA. The GFP protein alone is mainly localized in cytoplasm. Middle panel: the GFP-tagged Xp54nrb preferentially localises in the nucleus. Right panel: uninjected sibling embryo. Scale bar is 20 μm.* **(C)** *Nuclear localization of Xp54nrb-GFP is confirmed by nuclear staining with 10 μM DRAQ5 on a live embryo at late gastrula stage. Left panel: nuclei labeled with DRAQ5. Middle panel: GFP signal in foci. Right panel: merge, the foci are colocalised with DRAQ5 labeling. Scale bars, (B) 20 μm, (C) 50μm.*

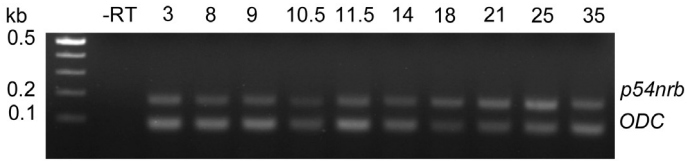


Fig. 4. Temporal distribution of *Xp54nrb* mRNA expression during *Xenopus* development. *RT-PCR analysis of RNA extracted from embryos at the indicated stages.* Xp54nrb *mRNA is present in all developmental stages tested; from stage 3 to stage 35. PCR without reverse transcription (-RT) is performed to check the absence of genomic DNA.* ODC *is used as a loading control.*

clusively restricted to the DRAQ5-labeled nuclei (Fig. 3C, merge). Taking into account that p54 proteins in *Drosophila*, mammals and diverse cell types (Amelio *et al.,* 2007; Zhang *et al.,* 2001; Rendahl *et al.,* 1992) have nuclear localisation, our data suggest that the endogenous Xp54nrb protein is likely expressed in the nucleus of *Xenopus* ectodermal cells.

Maternal and zygotic expression pattern of Xp54nrb

The spatio-temporal expression of *Xp54nrb* was also determined. *Xp54nrb* mRNA, detected by RT-PCR, is present in all the stages tested, i.e., from stage 3 through to swimming larvae (Fig. 4). The tissue-specific pattern of *Xp54nrb* transcript is revealed by whole-mount *in situ* hybridization. No staining at any stage is visible with the sense probe. Maternal expression is present at the animal pole and maintained during cleavage stages in the animal dorsal blastomeres (Fig. 5A, 5B; stage 3 and 6.5 respectively). After the midblastula transition (MBT), a faint labelling is detected in the ectodermal sheet, this labelling increases significantly during gastrulation in the dorsal ectoderm and mesoderm (Fig. 5C, stage 11). The gastrula sagittal section shown in Fig. 5D illustrates the expression of *Xp54nrb* in the ectoderm and in the involuting mesoderm. No labelling is observed in the endoderm. With subsequent development, the expression pattern of *Xp54nrb* becomes spatially restricted to the neural plate and then to the closing neural tube

TABLE 1

CONSERVATION IN P54NRB FAMILY

	identity	similarity
Xenopus tropicalis BC066129	92%	96%
Homo sapiens NP_031389	75%	86%
Mus musculus NP_075633	74%	85%
Gallus gallus AJ720639	75%	85%
Danio reiro BC046880	63%	76%
Drosophila melanogaster AAA03214	25.3 %	45%
Drosophila melanogaster AAA03214 *	38.2 %	68.5 %

*functional conserved regions. The *Xenopus laevis* Xp54nrb sequence reveals high identity and similarity with other species.

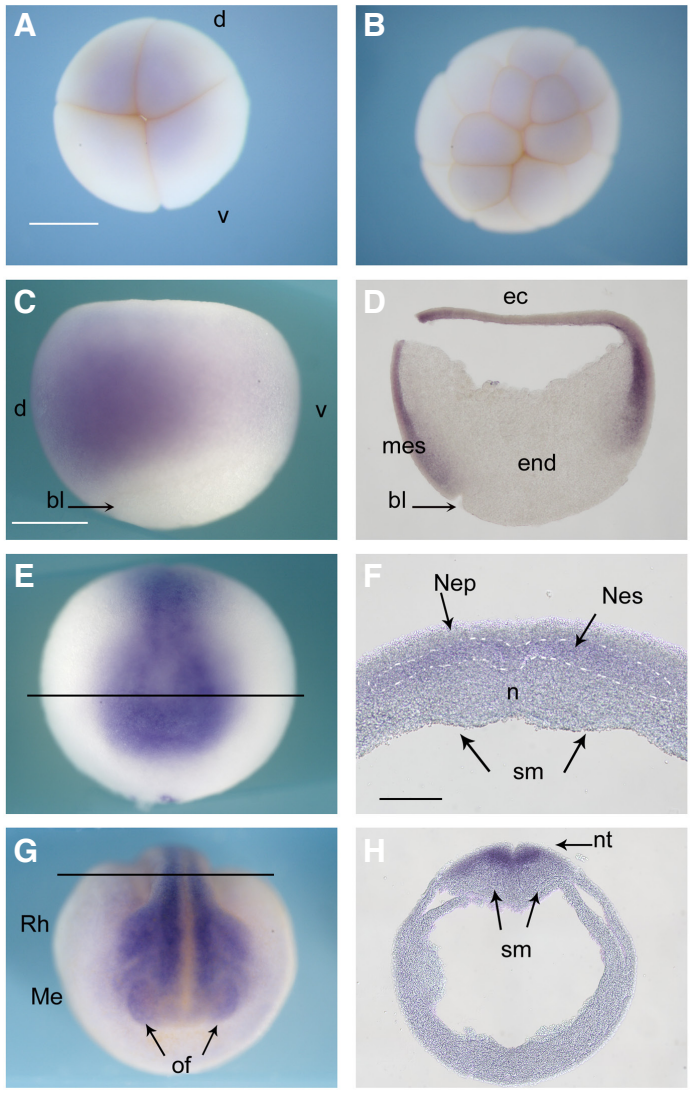


Fig. 5. Spatial distribution of *Xp54nrb* mRNA during *Xenopus* early development. *Whole mount* in situ *hybridization is performed on embryos from stage 3 to stage 21. Photomicrographs of whole-mounts and sections through the corresponding whole mounts for stage 3* **(A)**, *stage 6.5* **(B)**, *stage 11 (C,D), stage 14 (E,F) and stage 21 (G,H) are shown.* **(A,B)** *Xp54nrb transcript is detected at the animal pole of cleaving embryos (animal views).* **(C)** Xp54nrb *expression in mid-gastrula is found in the ectoderm and mesoderm with a strong expression in dorsal side (lateral view, dorsal on the left). This pattern is confirmed by section analysis* **(D)** *which showed labelled of the ectoderm (ec) and in the involuting mesoderm (mes).* **(E)** *At early neural stage, the expression is restricted to the developing nervous system (anterior view, dorsal side is up) with a strong expression underlying the neural plate.* **(F)** *Transverse section analysis of the stage 14 shows that* Xp54nrb *is expressed in the sensorial layer of the neuroectoderm (Nes) and absent in the epithelial layer of the neuroectoderm (Nep), in the notochord (n) and in the somitic mesoderm (ms).* **(G)** *At neurula (stage 21, anterior view, dorsal side is up)* Xp54nrb *expression is high in the anterior neural territories; optic field (of), mesencephalon (Me) and rhombencephalon (Rh) are labelled. Transverse section analysis* **(H)** *in a posterior position shows that only the neural tube (nt) is stained at this level.* Abbreviations: *bl; blastoporal lip, d; dorsal, Ec; ectoderm, end; endoderm, mes; mesoderm, Me; mesencephalon n; notochord, Nes; sensorial layer of the neuroectoderm, Nep; epithelial layer of the neuroectoderm, of; optic field, nt; neural tube, Rh; rhombencephalon, sm; somitic mesoderm, v; ventral. Scale bars 0.5 mm in (A,B), 0.3 mm in (C,D,E,G,H) and 0.1 mm in (F). Black bars in (E,G) represent the position of the corresponding sections in (F,H) respectively.*

(Fig. 5E, stage 14; and Fig. 5F, transverse section). The *Xp54nrb* transcript is highly expressed in the anterior neural territories, involved in the regionalisation of the future brain structures and the eye anlagen (Fig. 5G, stage 21, anterior view). The transcript is also located more caudally in the spinal cord as illustrated in the transverse section, whereas it is excluded from the notochord and the somitogenic mesoderm (Fig. 5H). At stage 23, the staining is observed in the mesencephalon and in the eye vesicles, which invaginate from the diencephalic region. The prospective retinal layer and prospective pigment layer are both stained (Fig. 6A and B). Later, during organogenesis at tailbud stages, robust expression is maintained in the anterior central nervous system and in the developing neuroretina (Fig. 6C, stage 35). Other sensorial structures, such as the olfactory placodes, the otic vesicle, and the branchial arch derivatives are also labelled. Labelling is absent from the cement gland (Fig. 6C). The associated anterior transverse section (Fig. 6E) shows the localised expression of *Xp54nrb* in the dorsal part of the encephalic epithelium, with a gradient of expression along the dorso-ventral axis. The entire overlying epidermis is free of staining. Section through the eye shows that *Xp54nrb* mRNA is specifically transcribed in the inner nuclear cell layer and ganglion cell layer (Fig. 6F). *Xp54nrb* is not expressed in the ciliary marginal zone, or in the lens. In a more posterior position, *Xp54nrb* expression is only detected in the spinal cord, and the staining is clearly restricted to the ventricular zone (Fig. 6D), where the undifferentiated neural progenitors proliferate.

Thus, during *Xenopus* development, the expression pattern of the new RBP mRNA *Xp54nrb* is associated with the neural and sensorial territories, with an important expression in the retinal structures.

Xp54nrb *downregulation affects neural gene expression*

While *Xp54nrb* is expressed all along *Xenopus* embryogenesis, especially in the neuroectodermal tissues during the early steps of development, we wondered to determine if this RNA binding protein was implicated in the early neurogenesis. To answer this question we have analysed the effects of its loss of function by morpholino injection. We firstly verified by western blot, the specificity of the morpholino directed against *Xp54nrb* (MoXp54) (Fig. 7A) and on developing embryo (Fig. 7B). Co-injections of MoXp54 with the *Xp54nrb-GFP* mRNA previously used (Fig. 3) inhibit the fusion protein expression in living embryos (Fig. 7B, left lower panel), whereas control morpholino (MoC) or co-injection of MoXp54 with *GFP* mRNA have no effect on protein translation (Fig. 7B, right and left panels respectively). Thereby, MoXp54 is able to recognize the 5' sequence of the *Xp54nrb* messenger and therefore to efficiently block the translation of the *Xp54nrb-GFP* fusion transcript. We then targeted morpholino microinjections directed against *Xp54nrb*, in one dorsal blastomere at 4-cell stage, allowed the embryos to develop until stage 14 (neural plate) or stage 17 (neural fold stage) and analyzed the expression pattern of *Zic3* (Nakata *et al.,* 1997), *POU2* (Witta *et al.,* 1995) which are early proneural markers shown to be sensitive to Ca^{2+}-induced neuralisation (Leclerc *et al.,* 2003; Leclerc *et al.,* 2000) and of *Sox2,* a pre-neural marker gene expressed at the onset of neural induction downstream to the BMP4 antagonist Chordin (Mizuseki *et al.,* 1998) and which is maintained in neural progenitors.

At stage 14, the expression of *POU2* on the injected side is diminished at the anterior part of the neural plate (n = 8; 5 embryos with a decreased staining; Fig. 8B), compared to the uninjected side or to control morpholino injected embryo (n = 8; 100% normal; Fig. 8A). This impairment remains clearly visible at stage 17 as neural tube closure progresses (n = 30/39 embryos; Fig. 8D), when *POU2* mRNA strongly stains the mesencephalon-rhombencephalon boundary. The proneural gene *Zic*3 also shows a reduced expression level in the Xp54nrb knockdown side of stage 14 embryo (n = 16/22 embryos; Fig. 8F). *Zic3* expression pattern is also affected at stage 17 (n = 16/24 embryos; Fig. 8H). Similarly, *Sox2* staining is affected in the anterior domain of the MoXp54 injected side (n = 7/12 embryos with faded staining; Fig. 8J). The effect at the anterior part of the brain anlagen is more clearly visible later at stage 17 (n = 19/23 embryos; Fig. 8L). The reduced expressions also observed at stage 17 for *Pou2, Zic3* and *Sox2* indicate that

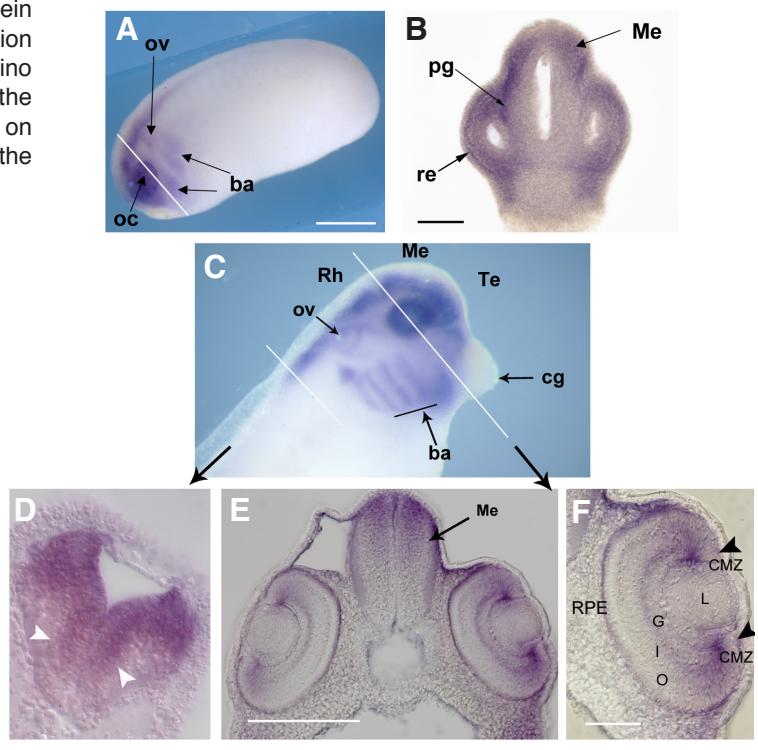


Fig. 6. Spatial distribution *of Xp54nrb* mRNA during *Xenopus* early organogenesis. *Photomicrographs of whole-mounts and sections through the corresponding whole mounts for stage 23 (A, B) and stage 35 (C-F) embryos are shown.* **(A)** *Whole mount and section analysis* **(B)** *at stage 23 show* Xp54nrb *expressed in optic cup (oc), otic vesicle (ov), branchial arches (ba) and mesencephalon (Me) (whole mount, lateral view, anterior is left).* **(C)** *Anterior view at stage 35 shows similar labelling patterns to the stage 23 embryo with neuroretina, encephalon and spinal chord labelled.* **(D)** *In the transverse section at the trunk level, only the ventricular zone of the spinal cord is stained (white arrowheads).* **(E)** *Section at the head level through the eye* **(F)***, enlargement of E) the dorsal part of the spinal chord, and the inner layer of the neuroretina are labelled. No staining is detected in the lens or in the ciliary marginal zone (CMZ, black arrowhead in F). Abbreviations: ba; branchial arches, cg; cement gland, CMZ, ciliary marginal zone, G, ganglion cell layer, I, inner nuclear cell layer, L, lens, Me; mesencephalon, O, outer nuclear cell layer, ov; otic vesicle, oc; optic cup, Rh; rhombencephalon, RPE retinal pigmented epithelium, Te; telencephalon, Scale bars 0.5 mm (in A-D), and 0.1 mm (in E, F).*

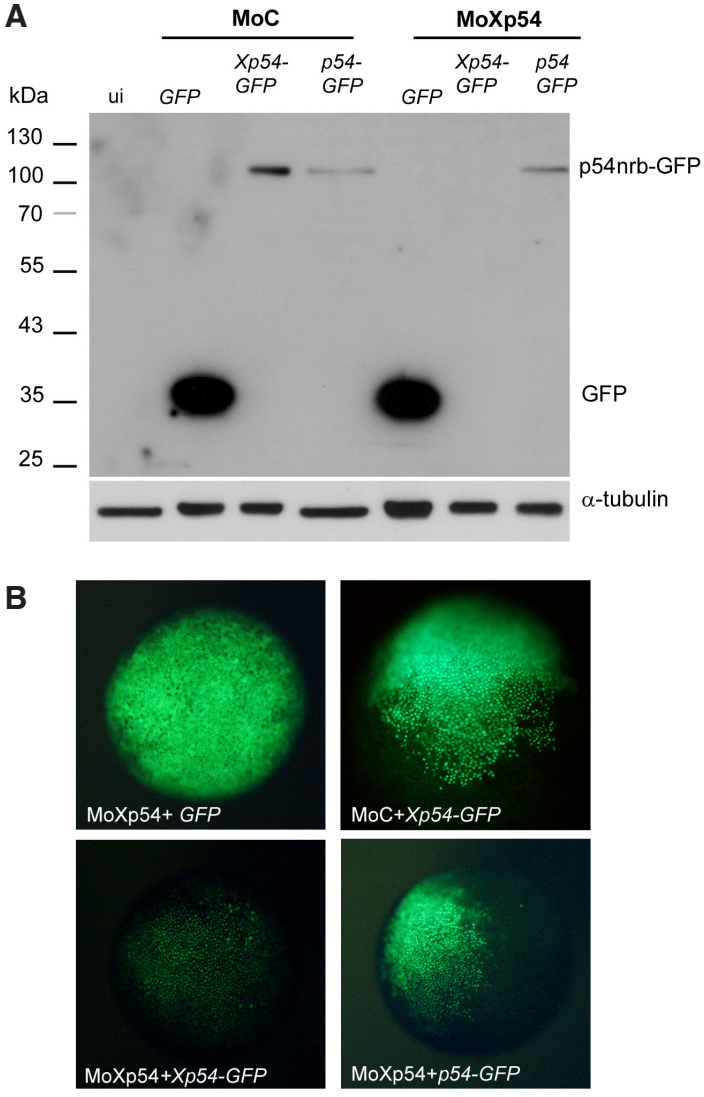


Fig. 7. Morpholino MoXp54 blocks Xp54nrb-GFP translation, but not human p54nrb-GFP translation. (A) *Western blot. Two-cell-stage embryos are injected into the animal pole of both blastomeres. Lysates from stage 13 whole embryos are analysed by immunoblotting with anti-GFP antibody. Anti* α*-tubulin antibody attests equivalent loading.* ui*; non injected embryo extract (Lane1). Embryos were injected with 4 ng of control morpholino (MoC, lanes 2, 3, and 4) or 4 ng of morpholino against Xp54nrb (MoXp54, lanes 5, 6, and 7) and with 50 pg* GFP *mRNA (lanes 2, 5), 2ng of* Xp54nrb-GFP *mRNA (lanes 3, 6), or 2 ng* hp54nrb-GFP *mRNA (lanes 4, 7) respectively. GFP protein is expressed in embryos injected with Moc or with MoXp54. The Xp54nrb-GFP fusion protein is translated in embryos injected with MoC (lane 3) but not translated in embryos injected with MoXp54 (lane 6). While the morpholino against Xp54nrb inhibits Xp54nrb-GFP expression (lane 6), the human tagged p54nrb-GFP is still synthesised (lane 7).* **(B)** *Micrographs of stage 13 embryos showing GFP fluorescence. Left top panel: embryo injected with 4 ng MoXp54 and 50 pg* GFP *mRNA, Left lower panel: embryo injected with 4 ng MoXp54 and 1 ng* Xp54nrb-GFP *mRNA (*Xp54-GFP*), Right top panel: embryo injected with 4 ng MoC and 1 ng* Xp54nrb-GFP *mRNA, Right lower panel, embryo injected with 4 ng MoXp54 and 1 ng human* p54nrb-GFP *mRNA (*p54-GFP*). Xp54nrb morpholino (MoXp54) allows human p54nrb-GFP expression but blocks Xp54nrb-GFP signals.*

the loss of function does not induce a delay of neural development in the morphants, but a real impairment of neurogenesis.

Xp54nrb morpholino effects are partially rescued by the human p54nrb

Taking account that the 5' sequence of human p54nrb presents 9 mismatches compared to the 25 nucleotides targeted by the morpholino chosen against the *Xenopus* sequence, we used the human p54nrb for rescue experiments. We firstly verified that the human *p54nrb* is effectively resistant to Xp54nrb morpholino. Indeed, as shown by western blot (Fig. 7A) as well as by fluorescence detection (Fig. 7B), the morpholino directed against *Xp54nrb* does not inhibit translation of the *in vitro* synthesised mRNA for human *p54nrb* tagged with *GFP*, whereas the morpholino efficiently blocks Xp54nrb-GFP expression.

We therefore co-injected Xp54nrb morpholino with mRNA encoding human *p54nrb* that does not contain the morpholino recognition site and analysed by *in situ* hybridization the resulting expression pattern on neural markers at stage 17 (Fig. 8). The human p54nrb is able to restore *Sox2* and *Zic3* expression (Fig. 8O; n=6/11 embryos and Fig. 8N, n=7/12 embryos, respectively) previously affected in morphants. Compared to *Pou2* pattern obtained with morpholino treatment, co-injection with human *p54nrb* allows *Pou2* re-expression even with a weak posteriorization shift (Fig. 8M; n=5/8 injected embryos).

These data suggest that *Xp54nrb* is required for neural development, since loss of function by morpholino leads to a reduction of early proneural gene expressions which is restricted to the anterior part of the future central nervous system in *Xenopus* embryo.

Discussion

In this work, we show, for the first time, the detailed spatial and temporal expression pattern of *Xp54nrb* during embryonic development in *Xenopus laevis*. One important point is that *Xp54nrb* is a Ca^{2+} target gene isolated from the cDNA subtractive library and involved in neural induction. This further confirms the essential role played by Ca^{2+} during early neurogenesis (Batut *et al.,* 2005). The expression of *Xp54nrb* is highly specific, namely early restricted to neural tissue and maintained in the neural progenitors in the ventricular zone. Thus, *Xp54nrb* is likely associated with neural progeny in undifferentiated state.

Xp54nrb encodes *Xenopus* homologue *of* p54nrb/NonO protein, which has multifunctional roles in the nucleus, such as DNA binding protein (Yang *et al.,* 1993), interaction with the RNA polymerase II (Emili *et al.,* 2002), association with another RRM-containing protein PSF the polypyrimidine tract-binding protein-associated splicing factor (Shav-Tal and Zipori 2002), modulation of the androgen receptor activity in cell culture (Dong *et al.,* 2007). It has also been shown that p54nrb acts as transcriptional repressor to regulate Connexin-43 expression when it interacts with the progesterone receptor during the labour (Dong *et al.,* 2009), or with the Malignant Inhibitor Activity (MIA) in melanoma progression (Schiffner *et al.,* 2011). Interestingly, (Amelio *et al.,* 2007) showed that p54nrb is implicated in transcription regulation of *c-fos* mediated by CREB pathway. In addition p54nrb can bind to RNA and function in RNA processing activities in pre-mRNA splicing (Dong *et al.,* 1993; Kameoka *et al.,* 2004), in polyadenylation steps (Liang and Lutz

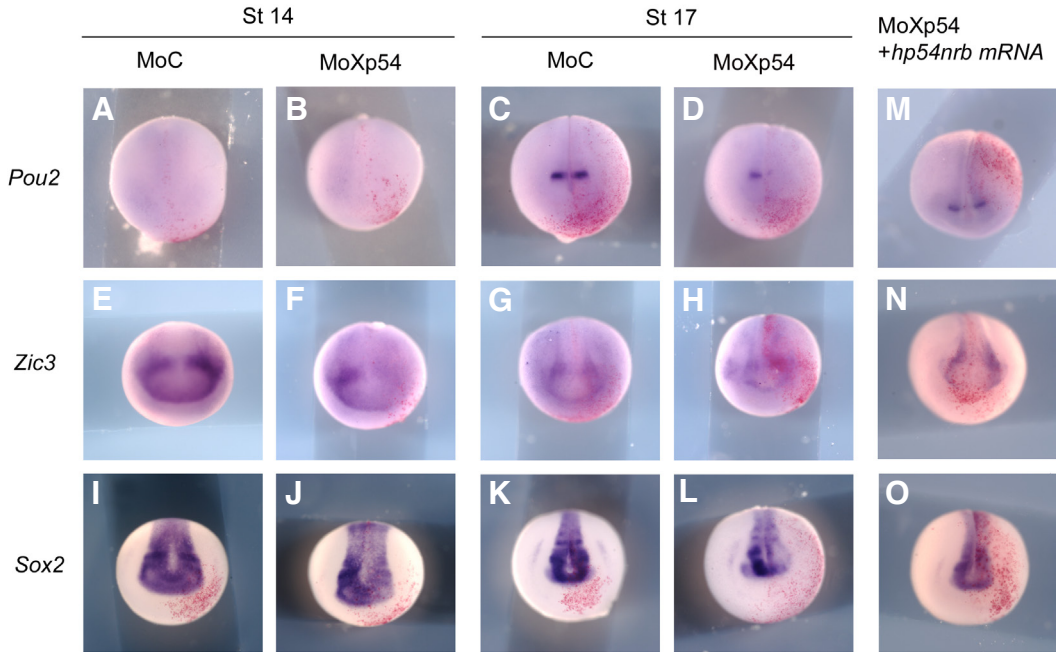


Fig. 8. Morpholino MoXp54 impairs expression pattern of proneural genes. *Embryos are injected at the two-cell stage into one blastomere with either 4 ng of a control morpholino (MoC), 4 ng of Xp54nrb morpholino (MoXp54) or co-injected with 4 ng of MoXp54 and 2 ng of human* p54nrb *mRNA. Embryos are raised to stage 14 or to stage 17, fixed and analysed by whole mount* in situ *hybridization for the expression of* POU2 **(A-D,M)**, Zic3 **(E-H,N)** *and* Sox2 **(I-L,O)**. *The side of injection is visualized by the* β*-galactosidase enzymatic reaction with Red-Gal substrate. Injected side is to the right. The embryos are shown in anterior views, dorsal is up. The patterns observed are representative of the embryos analysed.*

2006), and in transcription termination (Kaneko *et al.,* 2007). To date, the major *in vivo* implication of p54nrb in mammals is demonstrated in chondrogenesis where it controls the splicing of the *Col2a1* gene in association with the transcription factor Sox9 (Hata *et al.,* 2008) and in cartilage regeneration (Schmid *et al.,* 2010).

An important point of this work is the specific expression of *Xp54nrb* mRNA in the inner nuclear cell layer and ganglion cell layer while *Xp54nrb* does not seem to be expressed in the ciliary marginal zone, or in the lens. Accordingly to *Drosophila* and mouse patterning, *Xp54nrb* is notably expressed in eye structures. Loss of function of Xp54nrb affects the expression pattern of pro-neural genes, particularly at the anterior part of the developing central nervous system, from where emerges the neuroretina.

In mouse, *p54nrb/NonO* itself is regulated by splicing, as it exhibits two transcripts in brain and a third form which is specific of the adult retina (Yang *et al.,* 1993). In *Drosophila melanogaster,* two isoforms of *NONA/BJ6* are found (Jones and Rubin, 1990). This gene is expressed in the ocular structures of the fly and several mutants are described in which the vision is affected (Rendahl *et al.,* 1996). The Sfrs1 alternative splicing factor, for example, is necessary for murine retina development and retinal neuron survival (Kanadia *et al.,* 2008). The involvement of several RNA-binding proteins (RBPs) in *Xenopus* eye development has been also demonstrated for retinal cell fate decision (Boy *et al.,* 2004). Furthermore, the expression patterns of five neural RNA binding proteins belonging to different RBP families, showed distinct spatial-temporal distributions in the multilayered retina in *Xenopus laevis* (Amato *et al.,* 2005). The authors suggested that these posttranscriptional regulators may play important roles in the multiple steps occurring for cell-type specification and/or differentiation during retinogenesis.

In this study, we show that *Xp54nrb* transcription exhibits a typical neural specificity of expression throughout *Xenopus* early development, and its presence is required for the correct patterning of the anterior neural structures. Furthermore *Xp54nrb* is a calcium target gene. Whether its regulation by Ca^{2+} is direct or indirect remains a mechanism to be investigated.

Materials and Methods

Animals and explanted animal caps

Xenopus laevis eggs are collected, fertilized, and embryos are cultured by standard procedures (Batut *et al.,* 2005). Embryos are staged according to (Nieuwkoop and Faber 1967). Animal caps are dissected in 0.5 x NAM from stage 8-9 embryos, preincubated for 30 minutes with the calcium chelator BAPTA-AM (20 μM) prior to incubation with noggin protein (2 μg/mL) and cultured until sibling embryo reached to stage 12.

Xp54nrb cloning

A subtractive library (PCR-Select cDNA Subtraction kit, Clontech) was constructed between untreated animal caps and animal caps neuralized by caffeine-triggered Ca^{2+} release (Batut *et al.,* 2003; Batut *et al.,* 2005). A 820 bp-long fragment (3F4) was isolated and used to screen in silico libraries. Two complete cDNA exhibited perfect homology in their 3'UTR part with the 3F4 sequence, one isolated from stage 31-32 cDNA library (Klein *et al.,* 2002) corresponds to the accession number BC045128, and the second issued from a systematic screen of anterior genes library (Takahashi *et al.,* 2005) is referred under the accession number AB238228. The full length cDNA BC045128 was purchased from RZPD Consortium, Germany (http://image.llnl.gov).

Morpholino

Morpholino oligonucleotide (GeneTools, Corvalis, USA) was designed to block translation of *X. laevis* p54nrb. The sequence enclosed the *AUG* start codon (in bold): GTACCCTCTGTTTCCCTGCATGTTT. The standard control Morpholino (MoC) was provided by the manufactor. Typically 4 ng of Morpholinos per embryo are injected in the presence of in vitro-synthesized nuclear β-galactosidase mRNA (50 pg) as a tracer, revealed at the desired stages of development with the Red-Gal substrate (6-chloro-3-indoyl-β-D-galactoside, Research Organics).

Plasmid constructs, in vitro transcription for microinjections, GFP detection

For *in vivo* expression, pCS2-Xp54nrb-GFP was constructed by PCR-

amplified ORF of *Xp54nrb* without stop codon introduced in frame into pCS2-GFP plasmid. The human *p54nrb* ORF was amplified by PCR from cDNA pool of HeLa cells (a gift from Dr P. Belenguer) and introduced in the pCS2 vector, with or without *GFP*, in place of *Xp54nrb*. These construts were linearised at the NotI site, and the capped synthetic mRNAs were generated by using SP6 mMessage mMachine kit (Ambion).

Embryos were pressure-injected in one blastomere at the 2-cell to 4-cell stages with *GFP*, or *Xp54nrb-GFP* and *p54nrb-GFP* mRNAs, at 50 pg or 1 ng respectively, and allowed to develop at 22°C. *In vivo* expression of GFP was imaged on stage 13 embryo using confocal facilities (Leica, TCS SP5, TRI platform, Toulouse). The nuclei were stained by pre-incubating the embryos 1 hour with 10 μM DRAQ5 (BioStatus Ldt, UK).

For western blot analysis, control and microinjected embryos were lysed at stage 13 (lysis buffer: 20 mM Tris pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 1 mM DTT, 0.5% NP40) and 10K supernatants equivalent to 1 embryo, were applied on SDS-PAGE. The GFP and GFP-tagged proteins were revealed by immunoblotting with rabbit anti-GFP polyclonal antibody (1/3000, TP401, Torrey Pines, Biolabs), Goat peroxidase-conjugated anti-rabbit antibody (1/5000, Promega) was detected with the enhanced chemiluminescent kit (ECL, Amersham).

RT-PCR and in situ hybridization

Extraction of total RNA, Reverse-Transcription and PCR assays with primer sets for *ODC*, *Xbra* and *Zic3* were performed as described (Batut *et al.,* 2003). *Pax3* primer set is according to *Xenopus* Molecular Marker Resources, *Slug* primers to (Aybar *et al.,* 2003). We designed the following primers for X*p54nrb*: Forward (at 1511 bp) 5'-AGGTCAGTCTCTAGTG-CAGATGG-3' and Reverse (at 1671 bp) 5'-AACGGACAGTATACTAC-GACTGG-3'. RT-PCR analyses with these primers were performed for 32 cycles (thermocycler Flexigene, Techne). The absence of genomic contamination was systematically checked with *ODC* amplification of the RNA samples without reverse transcriptase. Similar results were obtained from three independent experiments in each assay.

Whole-mount *in situ* hybridization (ISH) was carried out according to (Harland 1991). Antisense RNA digoxigenin-labeled probes were synthesized by using cDNA templates encoding *POU2* (Witta *et al.,* 1995), *Sox2* (Mizuseki *et al.,* 1998) and *Zic3* (Nakata *et al.,* 1997). For *Xp54nrb* ISH, we used the complete cDNA as a template. For *Xp54nrb* antisense, the vector was linearised at the KpnI site and transcribed by using the T7 promoter. The sense probe was obtained by SP6 transcription after NotI linearization. For histology, stained embryos were embedded in 3% agarose and sectioned on a vibratome at 70 μm.

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique (CNRS). We thank Dr Julie Batut for her collaboration in the subtractive library construction, the imaging plateform (TRI, Toulouse, http://tri.ups-tlse.fr), Anne Bibonne for her technical assistance, Boris Demain for his help, Dr Sarah Webb for correcting the English and pertinent suggestions for the manuscript. N.D. was supported by a post-doctoral grant from FYSSEN Foundation and P.S. by ERASMUS grant.

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