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# FMR1/FXR1 and the miRNA pathway are required for eye and neural crest development

Susanne Gessert, Verena Bugner, Aleksandra Tecza, Maximilian Pinker, Michael Kühl *

*Institute for Biochemistry and Molecular Biology, Ulm University, Albert-Einstein-Allee 11, D-89081 Ulm, Germany*

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ABSTRACT

FMR1 and FXR1 are RNA binding proteins interacting with the miRNA-induced silencing complex, RISC. Here we describe for the first time the function of these proteins during eye and neural crest (NC) development in *Xenopus laevis*. A loss of FMR1 or FXR1 results in abnormal eye development as well as defects in cranial cartilage derived from cranial NC cells. We further investigated the possible mechanism of these phenotypes by showing that a depletion of Dicer, an important enzyme for generating all mature miRNAs, in the anterior neural tissue also leads to eye and cranial cartilage defects. Furthermore, we examined the function of 12 miRNAs during anterior neural development. We show a specific requirement of six selected miRNAs during eye and cranial cartilage development. Mir-130a, -219, and -23b are involved in eye formation only whereas loss of miR-200b, miR-96 and miR-196a results in strong defects during eye as well as cranial cartilage development. Our results suggest an essential role for FMR1 and FXR1 for eye and NC development in *X. laevis* likely through an interaction with the miRNA pathway.



## Introduction

FMR1 (*f*ragile X *m*ental *r*etardation syndrome *1*) and its autosomal homolog FXR1 (*f*ragile *X* r*elated 1*) form a small gene family. In human and mouse, a third member of this family, FXR2, has been described (Penagarikano et al., 2007). It has been shown that all members are able to form homo- as well as heteromers. A loss of FMR1 protein caused by an expansion of a CGG trinucleotide sequence in the 5′UTR sequence of the FMR1 gene results in the most common form of human mental retardation, the fragile X mental retardation syndrome (Pieretti et al., 1991). All three members of this protein family contain two KH (*K* protein *h*omology) domains, an RGG box and signals for nuclear import and export. The KH domain and the RGG box can bind to G-quartet structures (Darnell et al., 2001; Schaeffer et al., 2001) and U-rich motifs (Denman, 2003) in RNA molecules. Thereby these proteins are involved in intracellular RNA transport as well as translational regulation (Zarnescu et al., 2005). Furthermore, FMR1 and FXR1 can interact with components of the miRNA pathway such as Dicer or components of RISC (mi*R*NA-*i*nduced *s*ilencing *c*omplex) (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004) linking FMR1/FXR1 to the miRNA machinery.

MicroRNAs (miRNAs) are small single-stranded RNAs with a length of 22–25 nucleotides. They represent non-protein coding RNA molecules regulating gene expression on a posttranscriptional level by binding to the 3′untranslated region (3′UTR) of target mRNAs. miRNA genes are transcribed by RNA-polymerase II generating primary transcripts called pri-miRNA. These pri-miRNAs are sequentially processed by two RNase III enzymes, Drosha and Dicer, resulting in double-stranded miRNA duplexes. These duplexes are separated into single-stranded miRNA molecules, one of which is degraded and the other one binds to RISC. This ribonucleoprotein complex binds to the 3′UTR of target RNAs which can lead to the degradation or translational regulation of the respective RNA molecule (He and Hannon, 2004). Many studies indicated that miRNA-mediated gene regulation has important roles during early embryonic development (Bushati and Cohen, 2007; Stefani and Slack, 2008). In mice, the homozygous loss of Dicer leads to lethality early during development (Bernstein et al., 2003). Dicer mutant zebrafish embryos display morphogenetic defects during gastrulation, brain formation, somitogenesis and cardiogenesis (Giraldez et al., 2005) and die after 3 weeks (Wienholds et al., 2003). Tissue-specific downregulation of Dicer resulted in diverse phenotypes. In the retina for example, a loss of Dicer causes misorganization of photoreceptor cells as well as retinal degeneration at later stages of development (Decembrini et al., 2008). Many studies revealed an involvement of miRNAs during neural development (Hornstein et al., 2005; Choi et al., 2008; Zhang et al., 2008; Papagiannakopoulos and Kosik, 2009; Peter, 2009; Qiu et al., 2009; Walker and Harland, 2009).

Here we analyzed for the first time the function of FMR1 and FXR1 during early anterior neural development in *Xenopus laevis* by use of a morpholino oligonucleotide (MO) based knock down approach. Downregulation of FMR1 as well as FXR1 led to abnormal eye and

* Corresponding author. Fax: +49 731 500 23277.
*E-mail address:* michael.kuehl@uni-ulm.de (M. Kühl).

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cranial cartilage development. Furthermore we could show a role for Dicer and some selected miRNAs in the same tissue suggesting that FMR1/FXR1 function is essential for early neural development likely by interacting with the miRNA pathway.

## Materials and methods

### *X. laevis*

*X. laevis* embryos were obtained and staged by standard protocols.

### *Morpholino oligonucleotides (MO)*

MOs were obtained by Gene Tools, LLC, OR and resuspended in DEPC-$H_2O$. For loss of FMR1 function we injected an MO with the following sequence: 5′-CTC CTC CAT GTT GGG TCC GCA CGA T-3′. To test the functionality and binding specificity of the FMR1 MO we performed a coupled transcription and translation assay (TNT-Kit) following the manufacturer's protocol (Promega). For FXR1 knock down studies, we injected an FXR1 MO as previously published (Huot et al., 2005). For knocking down Dicer function, we designed two MOs with the following sequences: Dicer MO1: 5′-GGC CTG CCA TGC TGA GGG TCTGCA A-3′; and Dicer MO2: 5′-GAG TCA TGA GCT GAA GGC CTG CCA T-3′. The sequences of the MOs targeting the formation of or the matured miRNAs are: miR-130a MO: 5′-AAT GCC CTT TTA ACA TTG CAC TGC T-3′; miR-219 MO: 5′-CAA GAA TTG CGT TTG GAC AAT CAA G-3′; miR-23b MO: 5′-GTG GTA ATC CCT GGC AAT GTG ATT T-3′; miR-200b MO: 5′-AAT CAT CAT TAC CAG GCA GTA TTA G-3′; miR-96 MO: 5′-AAG CAA AAA TGT GCT AGT GCC AAA G-3′; miR-196a MO: 5′-TCC CAA CAA CAT GAA ACT ACC TAA A-3′; miR-124: 5′-TTG GCA TTC ACC GCG TGC CTT AATT-3′; miR-98: 5′-ACA ACA ATA CAA CTT ACT ACC TCA T-3′; miR-24a: 5′-AAC TGA TAT CAG TTC AGT AGG CAC A-3′; miR-18a: 5′-ACT ATC TGC ACT AGA TGC ACC TTA G-3′; Let-7a: 5′-TCA ACT ATA CAA CCT ACT ACC TCA G-3′; and Let-7f: 5′-AAA ACT ATA CAA TCT ACT ACC TCA T-3′. The standard control MO of Gene Tools was used for control experiments. If not indicated, the injected MO concentrations are: FMR1: 30 ng, Dicer MO1 and 2: 20 ng; miR-130a MO: 40 ng; miR-219 MO: 20 ng; miR-23b MO: 30 ng; miR-200b MO: 40 ng; miR-96 MO: 30 ng; and miR-196a MO: 20 ng. For the MOs targeting miR-124, miR-98, miR-24a, miR-18a, let-7a and let-7f we used concentrations up to 42 ng. In all experiments, the MOs were injected unilaterally into one dorso-animal blastomere at 8-cell stage. GFP-mRNA was used as a tracer. Injection was controlled under a fluorescence microscope.

### *Cloning*

For all cloning steps proof reading PfuUltra II Fusion HS DNA-Polymerase (Stratagene) was used. The PCR reactions were done on cDNA obtained from *X. laevis* embryos at stage 23 (Dicer probe), stage 27 (FXR1 full length construct) and stage 31 (FMR1 full length construct). To examine the spatial expression pattern as well as rescue experiments, we cloned the full length FMR1 and the Δ5′UTR FMR1 constructs first into the pSC-B vector (Stratagene) and then into the pCS2+ or pDH105 vector using ClaI. The primer sequences based on the published *Xenopus* FMR1 sequence (Acc. No. NM_001085587) are: ClaI5FMR1_l: 5′-CCA TCG ATC GCG AAA CGG GAC CCC GTC CG-3′; ClaIFMR1_l: 5′-CCA TCG ATA TGG AGG AGC TGG CCG TGG AGG TG-3′; and ClaIFMR1_R: 5′-CCA TCG ATT TAT GGT ACG CCA TTA ACC AC-3′. The FXR1 primer sequences for the cloning of the full length and Δ5′ UTR constructs are: 5′UTR_FXR1_l: 5′-CAA TCG ATC TTT TAC CCA TCC CTT CCT TT-3′; FXR1_l: 5′-CCA TCG ATA TGG AGG ACA TGA CGG TGG AA-3′; and FXR1_r: 5′-CCA TCG ATT TAA GAC ACC CCA TTC ATT AT-3′. By cloning and sequencing the full length FMR1 construct we realized some differences in our sequence in comparison to the published *X. laevis* FMR1 sequence (Acc. No. NM_001085587). In the 5′UTR region at position 122 we observed a nucleotide exchange from G to C which can also be found in the published *X. tropicalis* sequence (Acc. No. NM_001005454). We additionally discovered three exchanges in the open reading frame resulting in amino acid exchanges: at position 312 from A to T (arginine to serine), at position 976 from A to G (asparagine to aspartate) and at position 1257 from C to G (histidine to glutamine). An amino acid alignment comparing our sequence and the published *X. tropicalis* (Acc. No. NM_001005454) and *Mus musculus* (Acc. No. NM_008031) sequences revealed that our sequence of FMR1 shows higher similarities to these orthologues than the published *X. laevis* sequence. The *Xenopus* Dicer sequence has been deposited with the Acc. No. XL414a12ex in the Gurdon Institute *X. laevis* EST database and can be found at http://www.xenbase.org/gene/showgene.do?method=displayGeneSummary&geneId=491113. To investigate the spatio-temporal expression pattern of Dicer, we cloned a Dicer fragment received by a PCR with a length of 497 bp into pSC-B vector (Stratagene). Primer sequences are: Dicer_probe_l: 5′-TTG CAG ACC CTCAGC ATG G-3′; and Dicer_probe_r: 5′-AGA TTA ATG TTT GAC AAT GAC AC-3′. To test the binding specificity of the Dicer MOs used, we cloned upon touch-down PCR the Dicer MO binding site in front to and in frame with GFP in pCS2+ by using following primers: Dicer_MO_l: 5′-GAT CCT TGC AGA CCC TCA GCA TGG CAG GCC TTC AGC TCA TGA CTC CG-3′; and Dicer_MO_r: 5′-AAT TCG GAG TCA TGA GCT GAA GGC CTG CCA TGC TGA GGG TCT GCA AG-3′. The primers contained sticky ends for BamH1 and EcoR1 and 5′ ends were phosphorylated.

### *Whole mount in-situ hybridizations (WMISH)*

Wildtype or MO injected embryos were fixed at indicated stages with MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 4% formaldehyde) over night at 4 °C. WMISH was done according to standard protocols (Hemmati-Brivanlou et al., 1990). After staining, the embryos were refixed in MEMFA and bleached in 30% $H_2O_2$ for several hours. For sections, the embryos were embedded in gelatine/BSA and cut with a thickness of 20–30 μm.

### *Alcian blue staining*

For Alcian blue staining, the cranial cartilage was dissected after removing the skin of fixed *Xenopus* embryos at stage 46. The subsequent staining was done as previously described (Gessert et al., 2007). Cartilage photos were placed in front of a homogenous background.

## Results

### *FMR 1 and FXR1 function in the developing eye and NC cells*

We first analyzed the spatial expression of FMR1 and FXR1 during *X. laevis* embryogenesis thereby completing earlier studies by others. At late embryogenesis, FMR1 is expressed in the neural tube, the eyes, the otic vesicles and the pharyngeal arches (Lim et al., 2005). We could detect FMR1 transcripts in anterior neural tissue at stage 15, and later in the eye anlage, migrating NC cells, the otic vesicle and the pronephric nephrostomes (Suppl. Fig. 1A). FXR1 protein could be mainly detected in the developing somites of tailbud stage embryos (Huot et al., 2005). In addition we found FXR1 to be also expressed in anterior neural tissue. A weak staining in the eye can also be detected at stage 28 (Suppl. Fig. 1B).

We next investigated the function of FMR1 and FXR1 performing loss of function experiments using specific *antisense* MOs. The FMR1 MO was designed to cover the AUG start codon. In an *in vitro* transcription and translation assay, we could show that the FMR1 MO used specifically blocks the translation of FMR1 RNA including the 5′ UTR (5′UTR FMR1 construct, Suppl. Fig. 2). Furthermore the FMR1 MO is unable to inhibit translation of an FMR1 construct lacking the 5′UTR and thus lacking most part of the MO binding site (Δ5′UTR FMR1

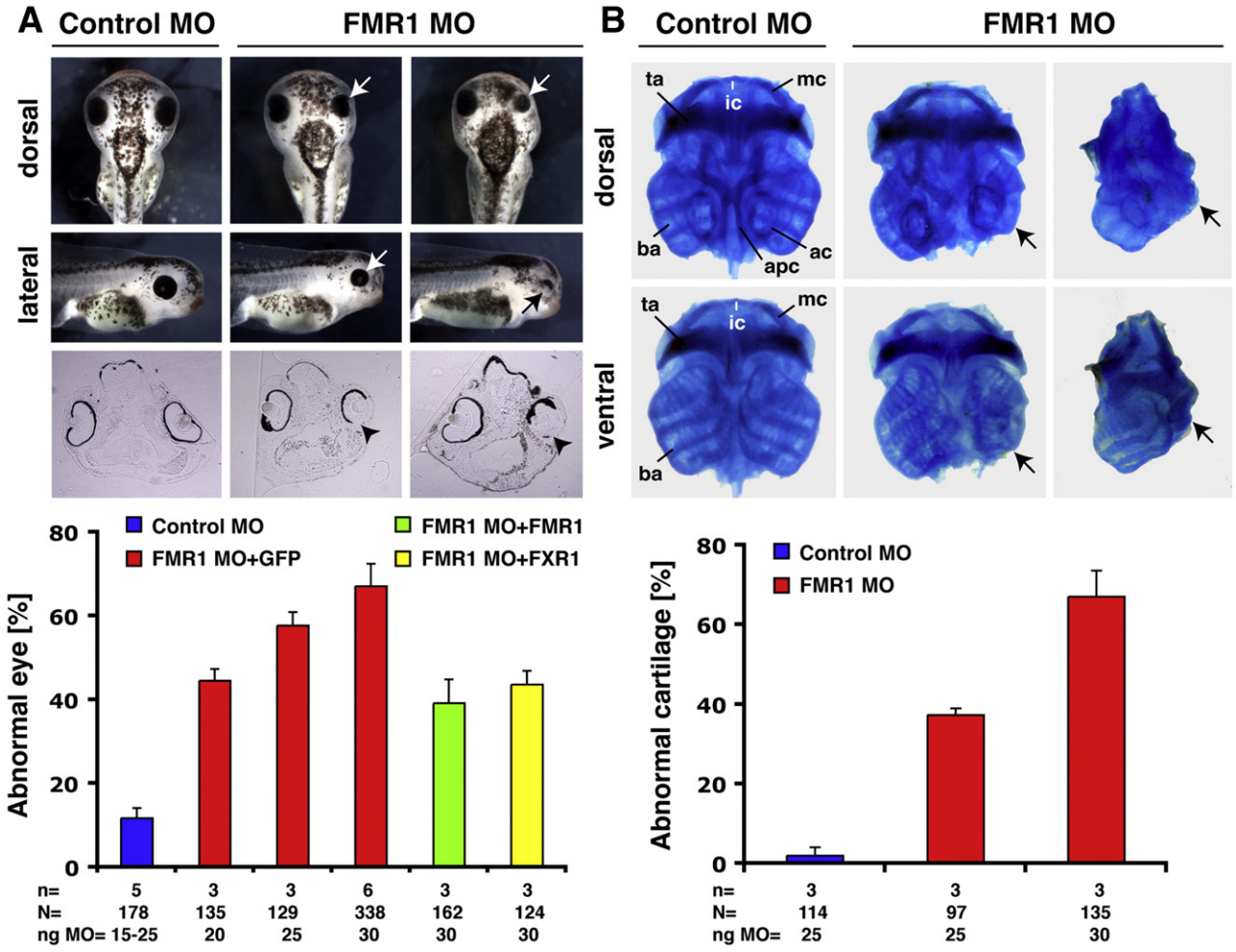



**Fig. 1.** Downregulation of FMR1 results in defects during eye as well as cranial cartilage development. A. Injection of FMR1 MO led to abnormal eye development (white and black arrows) in a dose-dependent manner as judged by the RPE. Control MO injection did not interfere with eye development. Vibratome sections showed disorganized layers of the retina as well as missing ventral RPE (black arrowheads). The coinjection of FMR1 MO with Δ5′UTR FMR1 or Δ5′UTR FXR1 RNA resulted in a rescue of the eye phenotype, see also Suppl. Fig. 4. B. Downregulation of FMR1 function resulted in reduced cranial cartilage structures on the injected side (arrows) shown by Alcian blue staining. Control MO did not interfere with cartilage development. Quantitative presentations are given. Abbreviations: ac = auditory cap; apc = anterior parachordal cartilage; ba = branchial arches; ic = infrarostral cartilage; mc = Meckels cartilage; *n* = number of independent experiments; *N* = number of analyzed embryos; ng = nanogram; ta = tectum anterius.

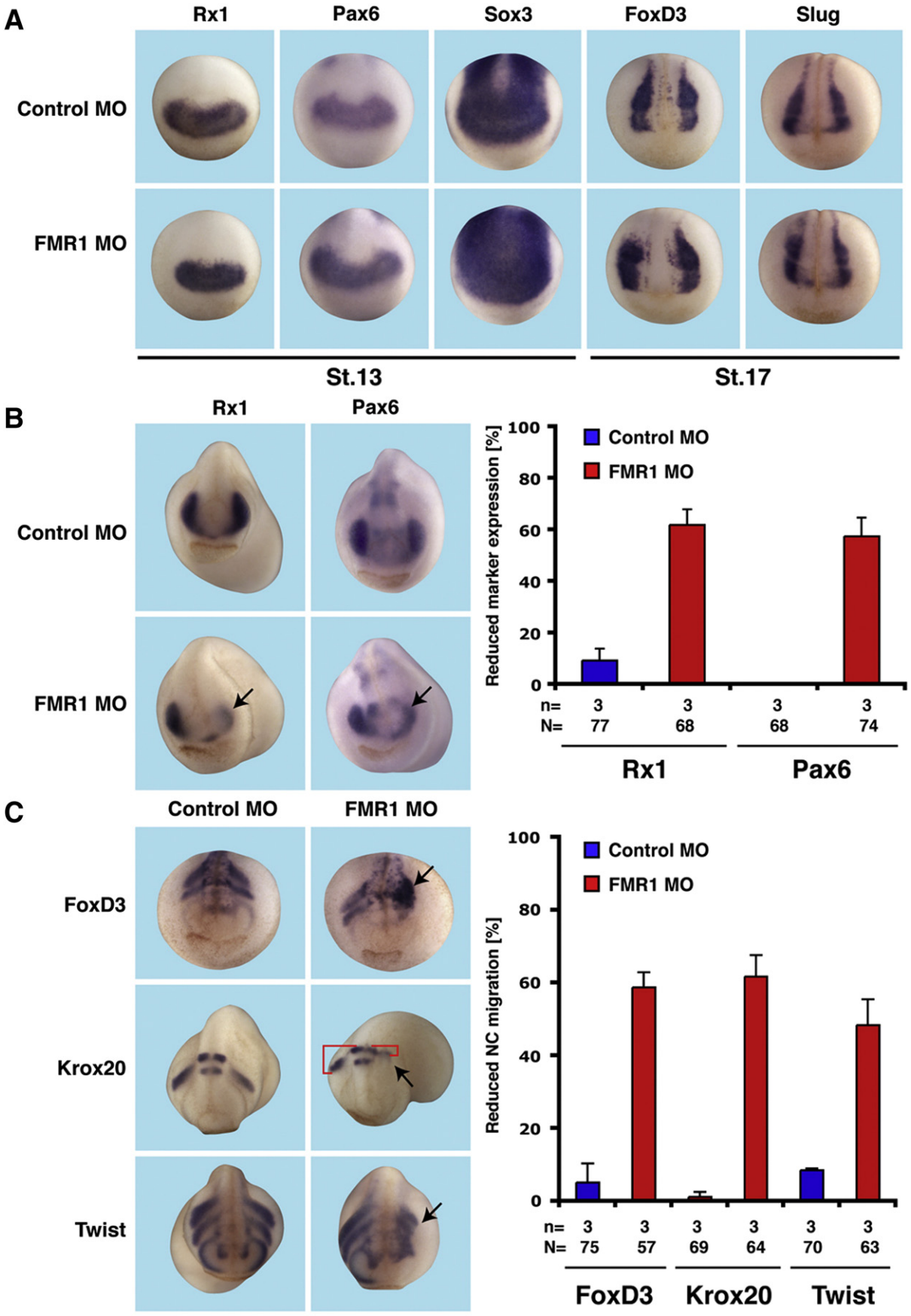
construct, Suppl. Fig. 2). This construct therefore can be used for subsequent rescue approaches.

In all experiments we injected the FMR1 MO unilaterally into one animal-dorsal blastomere of 8-cell stage embryos. Fate mapping studies had shown that this blastomere is contributing to anterior neural tissue, including the eye, brain and NC cells (Moody, 1987). In all experiments coinjection of GFP RNA served as an injection control. GFP expression was monitored at stage 13 or 23. Embryos negative for GFP in anterior neural tissue were not further considered.

In a first set of experiments we injected 20, 25 and 30 ng of the FMR1 MO. Loss of FMR1 function resulted in an eye phenotype including smaller, abnormal and absent eyes as judged by the size of the retinal pigment epithelium (RPE) (Fig. 1A). The individual layers of the retina were not distinguishable and the ventral RPE was missing giving rise to a coloboma. The same phenotype was observed after injecting a previously characterized FXR1 MO (Suppl. Fig. 3). Control MO injection had no influence on eye development. This eye phenotype could be partially reverted by coinjection of Δ5′UTR FMR1 and Δ5′UTR FXR1 RNA which are both not targeted by the FMR1 MO (Fig. 1A and Suppl. Fig. 4). This indicates that the observed eye phenotype is specific and that FMR1 and FXR1 are at least partially functionally redundant. Since FMR1 is expressed in migrating NC cells we also investigated the development of cranial cartilage as one derivative of the NC cells and analyzed this structure by Alcian blue staining. Downregulation of FMR1 resulted in reduced and deformed cranial cartilage structures on the injected side in a dose-dependent manner (Fig. 1B). Similar, the downregulation of FXR1 resulted in a cranial cartilage phenotype (Suppl. Fig. 3).

Next, we investigated the molecular basis of the defects in eye and cranial cartilage structures upon FMR1 inhibition. For all following experiments we injected 30 ng of the FMR1 MO. The injection of FMR1 MO did not influence the induction of neural tissue shown by the expression of the pan-neural marker gene Sox3 and the early eye marker genes Rx1 and Pax6 (Fig. 2A). Also the induction of NC cells was unaffected as indicated by the expression of FoxD3 and Slug at stage 17 (Fig. 2A). Later, however, loss of FMR1 function resulted in a

**Fig. 2.** FMR1 interferes with late eye and NC cell development. A. Injection of FMR1 and Control MO had no effect on the expression of neural marker genes at stages 13 and 17. B. FMR1 deficient embryos showed a reduced expression of eye marker genes Rx1 and Pax6 on the injected side (arrows) at stage 23. A quantitative representation is given. C. Inhibition of FMR1 resulted in defects in NC cell migration at stage 20 (FoxD3 expression, arrow) and stage 23 (expression of Krox20 and Twist, arrows). The red brackets highlight the difference of migrating Krox20-positive NC cells between uninjected and FMR1 MO injected sides. A quantitative presentation is shown. *n* = number of independent experiments; *N* = number of analyzed embryos.


A
Rx1
Pax6
Sox3
FoxD3
Slug
Control MO
FMR1 MO
St.13
St.17
B
Rx1
Pax6
Control MO
FMR1 MO
Reduced marker expression [%]
100
80
60
40
20
0
Control MO
FMR1 MO
n= 3 3 3 3
N= 77 68 68 74
Rx1
Pax6
C
Control MO
FMR1 MO
FoxD3
Krox20
Twist
Reduced NC migration [%]
100
80
60
40
20
0
Control MO
FMR1 MO
n= 3 3 3 3 3 3
N= 75 57 69 64 70 63
FoxD3
Krox20
Twist

smaller expression domain of Rx1 and Pax6 (Fig. 2B). At stage 20 cranial NC cells start to migrate from the dorsal side of the neural tube contributing to structures such as cranial nerves and cartilage. Therefore we analyzed whether FMR1 depletion leads to defects in NC cell migration by investigating the expression of FoxD3 at stage 20 and Krox20 as well as Twist at stage 23. The injection of FMR1 MO resulted in a severe migration defect as indicated by all three marker genes analyzed (Fig. 2C). In addition we investigated the expression of brain marker genes Emx1 (forebrain), En2 (isthmus), and Krox20 (rhombomeres 3 and 5 of the hindbrain). Loss of FMR1 function did not interfere with the expression of these genes in the brain (Suppl. Fig. 5) indicating the specificity of the observed effects.

In summary, these data suggest that FMR1 is neither required for neural induction in general nor for induction of the eye anlage or NC in particular. In contrast, FMR1 is essential for further development of the eye and for migration of NC cells.

*Loss of Dicer phenocopies the effect of FMR1 downregulation in the eye and NC cells*

FMR1 and FXR1 have been shown to interact with RISC and RNA molecules and to regulate RNA stability, transport and localization in neurons (Jin et al., 2004; Zarnescu et al., 2005). As the observed phenotype was accompanied by changes in gene expression on the RNA level, we hypothesized that FMR1 and FXR1 function through miRNAs in this context. If a loss of FMR1/FXR1 results in deficits of miRNA function, then a general loss of miRNA production should result in the same or a similar phenotype. We therefore focused our further attempts on the functional characterization of Dicer in anterior neural tissue.

We first analyzed the spatial expression pattern of Dicer. Dicer is ubiquitously expressed with an accumulation of Dicer transcripts in the anterior neural tissue during early *X. laevis* development (Suppl. Fig. 6). For the functional analyses of Dicer we designed two *antisense* MOs (Dicer MO1 and MO2) for knock down experiments. First we tested the functionality of the designed MOs by cloning the MO binding sites in front of and in frame with GFP. RNA of these constructs was injected together with the respective MO into early *Xenopus* embryos. Both Dicer MOs but not a Control MO blocked the translation of the GFP reporter (Suppl. Fig. 7).

To examine the function of Dicer during anterior neural development, we injected both Dicer MOs independently into one animal-dorsal blastomere of 8-cell stage embryos. Upon Dicer downregulation, we observed strong defects in eye as well as cranial cartilage development at stages 42 and 46 in a concentration dependent manner (Fig. 3). In case of the eye, the injection of Dicer MO1 as well as MO2 led to smaller, abnormal and absent eyes as determined by morphology of the embryos (Fig. 3A) as well as transversal sections (data not shown). The injection of a Control MO had no effect on eye development. Due to the large size of the Dicer transcript (5.7 kb) rescue experiments could not be performed. We considered this phenotype nevertheless to be specific as two independent MOs resulted in the same outcome. All further experiments were performed with 20 ng of MO. To analyze cartilage structures after Dicer MO injections, we isolated the cranial cartilage at stage 46 and performed an Alcian blue staining. After Control MO injection all

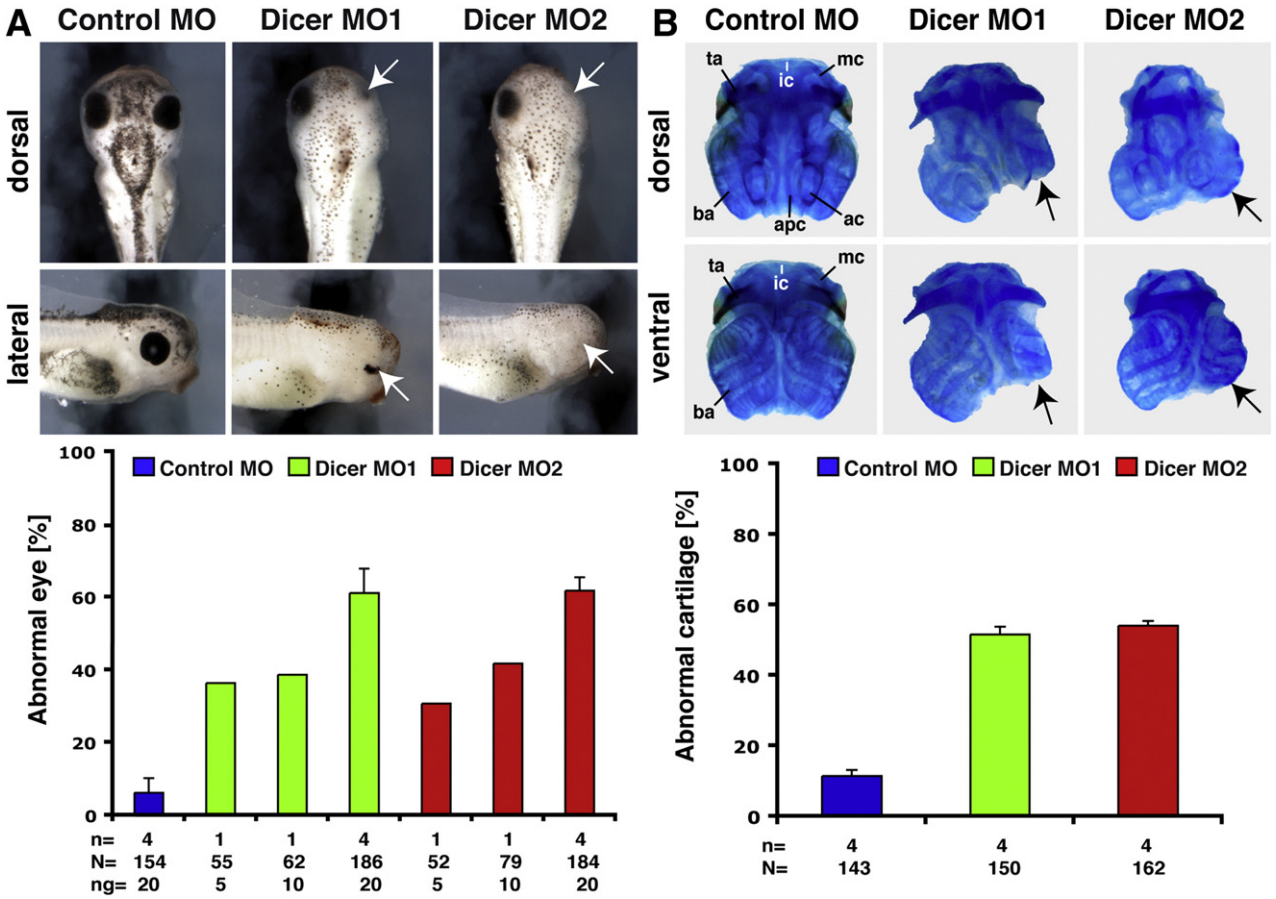



**Fig. 3.** Loss of Dicer function leads to eye and cartilage malformations. A. Dicer deficient embryos showed defects in eye development (white arrows) in a dose-dependent manner. Injection of 20 ng Control MO had no effect on eye development. A quantitative presentation is given. B. Injection of 20 ng Dicer MO1 or MO2 resulted in abnormal cranial cartilage structures on the injected side (black arrows). Control MO injection did not influence cartilage development. A quantitative evaluation is given. Abbreviations: ac = auditory cap; apc = anterior parachordal cartilage; ba = branchial arches; ic = infrarostral cartilage; mc = Meckels cartilage; *n* = number of independent experiments; *N* = number of analyzed embryos; ng = nanogram; ta = tectum anterius.

cartilage structures developed normal. In contrast, the depletion of Dicer resulted in a strong deformation of cranial cartilage on the injected side (Fig. 3B).

We next aimed to analyze these two phenotypes in more detail. A similar eye phenotype after Dicer depletion was also reported earlier by others (Decembrini et al., 2008), however, a molecular analysis at different stages of development, in particular earlier stages, was not performed. In a first step we therefore investigated the effect of Dicer knock down on the expression of early eye marker genes such as Rx1 and Pax6 and the pan-neural marker gene Sox3 at stage 13 (Fig. 4A). Neither the expression of eye marker genes nor the expression of Sox3 was affected after loss of Dicer function. NC induction was also not affected as indicated by the expression of FoxD3 and Slug (Fig. 4A).

We next analyzed the expression of eye marker genes at stage 23 (Fig. 4B) and observed a reduction of Pax6 and Rx1 expression upon Dicer depletion. We also analyzed the expression of brain-specific marker genes such as Emx1, En2 and Krox20. In case of Emx1 and En2, we observed a slight decrease in only some embryos whereas Krox20 expression in the brain was not affected (Suppl. Fig. 8). The injection of a Control MO had no effect. A stage 20, inhibition of Dicer resulted in defects in the migration of NC cells as indicated by FoxD3 expression. This was further confirmed by an altered expression of Krox20 and Twist at stage 23 (Fig. 4C and Suppl. Fig. 9).

Taken together these data indicate that Dicer is required for the maintenance of eye marker genes, for the migration of NC cells and for the development of eye and crainal cartilage structures in *X. laevis*. Furthermore, the phenotypes observed after knocking down FMR1/FXR1 and Dicer are remarkably similar.

### *miRNAs in Xenopus anterior neural development*

We next analyzed the function of particular miRNAs during anterior neural development. Watanabe et al. (2005) investigated the temporal expression of some miRNAs during *X. laevis* development and in a recent report, the spatial expression pattern of some miRNAs during *X. tropicalis* development was described (Walker and Harland, 2008). Out of this set of miRNAs we chose miRNAs which show a specific expression pattern in anterior neural tissue: miR-219, -130a, -124, -98, -96, -24a, -23b, -18a and Let7a. Additionally, we selected miR-200b (Burk et al., 2008), miR-196a (Hornstein et al., 2005; Qiu et al., 2009) and Let-7f (Wulczyn et al., 2007) as these miRNAs were linked to epithelial–mesenchymal transition (EMT), sonic hedgehog (shh) signaling or neural development in general. The function of miRNAs can be blocked by generating *antisense* morpholino oligonucleotides covering the mature miRNAs as well as the Dicer cleavage site (Martello et al., 2007; Yin et al., 2008; Rosa et al., 2009; Walker and Harland, 2009). Accordingly, we designed MOs against all selected miRNAs.

In total, we tested 12 miRNA MOs in different concentrations. For a first screening, we analyzed the eye and cartilage development as well as the expression of Rx1, Pax6 and Twist at stage 23. In case of six MOs, we did not observe any phenotype in any of the investigated contexts (miR-124, -24a, -98, -18a, Let7a and Let7f). This could be due to several reasons. First, it might well be that the analyzed miRNAs are not required for the analyzed morphological or molecular features. A more detailed analysis on a cellular level or using other marker genes might solve this issue. Second, it might be that the morpholinos are not functional due to structural problems. For the 6 other microRNAs, defects in eye and/or NC development were observed and we completed the marker gene studies similar to those for the FMR1 and Dicer phenotypes, analyzing Rx1, Pax6 and Sox3 at stage 13, Slug at stage 17, FoxD3 at stage 17 and 20 as well as Emx1, En2 and Krox20 at stage 23. Based on the phenotypes, we classified the different miRNAs examined in two functional groups. Three of these miRNAs (miR-130a, -219 and -23b) showed a phenotype only during eye development (group one) whereas three other miRNAs (miR-200b, -96, and -196a) revealed an eye as well as cranial cartilage phenotype (group two).

### *Phenotypes upon knocking down miR-130a, miR-219 and miR-23b*

MiR-130a is expressed in anterior neural tissues like the brain, the eyes and the branchial arches (Walker and Harland, 2008). Down-regulation of miR-130a led to smaller eyes (Fig. 5A). On a molecular level, the injection of miR-130a MO resulted in a reduced expression of the eye specific marker genes Rx1 and Pax6 already at stage 13 (Fig. 5B). The pan-neural marker Sox-3, however, was not affected at this stage. At stage 23 miR-130a depleted embryos showed a decrease in the expression of Rx1 and Pax6, whereas Emx1, En2 and Krox20 were not affected (Fig. 5C).

During *X. tropicalis* development, miR-219 is expressed in anterior neural tissue at stage 19. At stage 30, miR-219 could be visualized in the brain and the eye (Walker and Harland, 2008). For miRNA-219 we observed an eye phenotype upon MO injection in a dose-dependent manner (Fig. 5D). Loss of miR-219 led to smaller eyes without any apparent changes in the different layers of the retina. At stage 13, miR-219 knock down resulted in a slight decrease of Rx1 and Pax6 (Fig. 5E). Sox3 was not affected at this stage. The effect on Rx1 and Pax6 expression was stronger at stage 23 (Fig. 5F). The expression of brain marker genes Emx1, En2, and Krox20, however, was unchanged after miR-219 MO injection.

MiR-23b is specifically expressed in the hindbrain and the eye of *X. tropicalis* (Walker and Harland, 2008). Injecting miR-23b MO resulted in abnormalities during eye development (Fig. 5G). Histological sections reveal a misorganization of the RPE as well as the layers of the retina. Fig. 5H shows that the injection of 30 ng miR-23b MO has only a slight effect on early eye marker gene expression. At stage 23, Rx1 and Pax6 were strongly reduced in miR-23b deficient embryos (Fig. 5I). The analyzed brain markers were only slightly affected after miR-23b depletion.

### *Phenotypes upon knocking down miR-200b, miR-96 and miR-196a*

Recently published reports allocated different members of the miR-200 family as important regulators in EMT (Peter, 2009) which plays also an important role during NC cell development. In a first set of miR-200b knock down experiments, we observed abnormal or absent eyes (Fig. 6A). The induction of the eye field, however, was unaffected (Fig. 6B). At later stages, loss of miR-200b led to a reduction in Rx1 and Pax6 (Fig. 6C). Emx1 and En2 were only faintly affected. Additionally, miR-200b depletion resulted in abnormal cranial cartilage structures (Fig. 6D). Surprisingly, the induction as well as the migration of NC cells was not disturbed as indicated by the expression of specific NC marker genes (Figs. 6E and F).

Walker and Harland (2008) indicated that the expression of miR-96 is correlated with anterior neural tissue in *X. tropicalis* and that this miRNA is specifically expressed in the brain, the eyes and the branchial arches at stage 30. In our functional approaches we observed defects in eye as well as cranial cartilage development after inhibition of miR-96 (Figs. 7A and D). At stage 13, the pan-neural marker gene Sox3 was unaffected, but the induction of the eye field was impaired in some of the embryos (Fig. 7B). The reduction of Rx1 and Pax6 was more prominent during later development (Fig. 7C). At stage 23, the expression domain of En2 was slightly expanded in around 27% of the embryos. In addition, the induction of NC cells indicated by the expression of Slug and FoxD3 at stage 17 was affected by the depletion of miR-96 (Fig. 7E). At later stages we could also observe a downregulation of FoxD3 (stage 20) as well as Krox20 and Twist (stage 23) (Fig. 7F).

Qiu et al. (2009) recently demonstrated that the overexpression of miR-196a induces eye defects in *X. laevis*. MiR-196a was also implicated in shh signaling (Hornstein et al., 2005) which is also

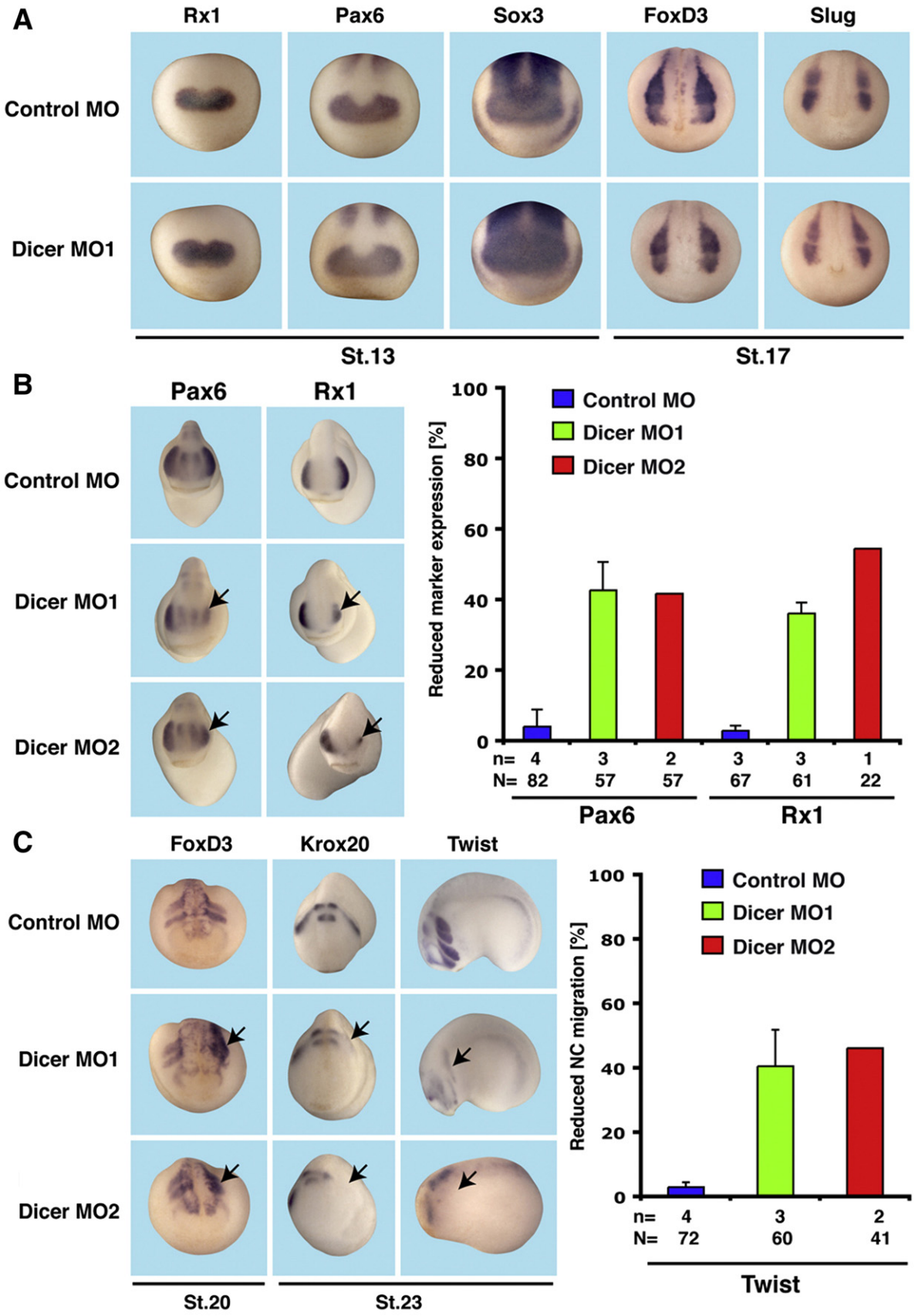



**Fig. 4.** Dicer function on a molecular level. A. The injection of 20 ng Dicer MO1 had no effect on Rx1, Pax6 and Sox3 at stage 13 as well as FoxD3 and Slug at stage 17. B. At stage 23, loss of Dicer function by injecting Dicer MO1 and MO2 led to the reduction of eye marker gene expression (black arrows). Control MO injected embryos showed a normal expression pattern. A quantitative presentation is given. C. The migration of NC cells was disturbed upon Dicer MO1 and MO2 injection (black arrows). FoxD3 at stage 20 and Krox20 and Twist at stage 23 were reduced upon loss of Dicer function (black arrows). Control MO injected embryos served as control. A quantitative presentation is given. $n$ = number of independent experiments; $N$ = number of analyzed embryos.

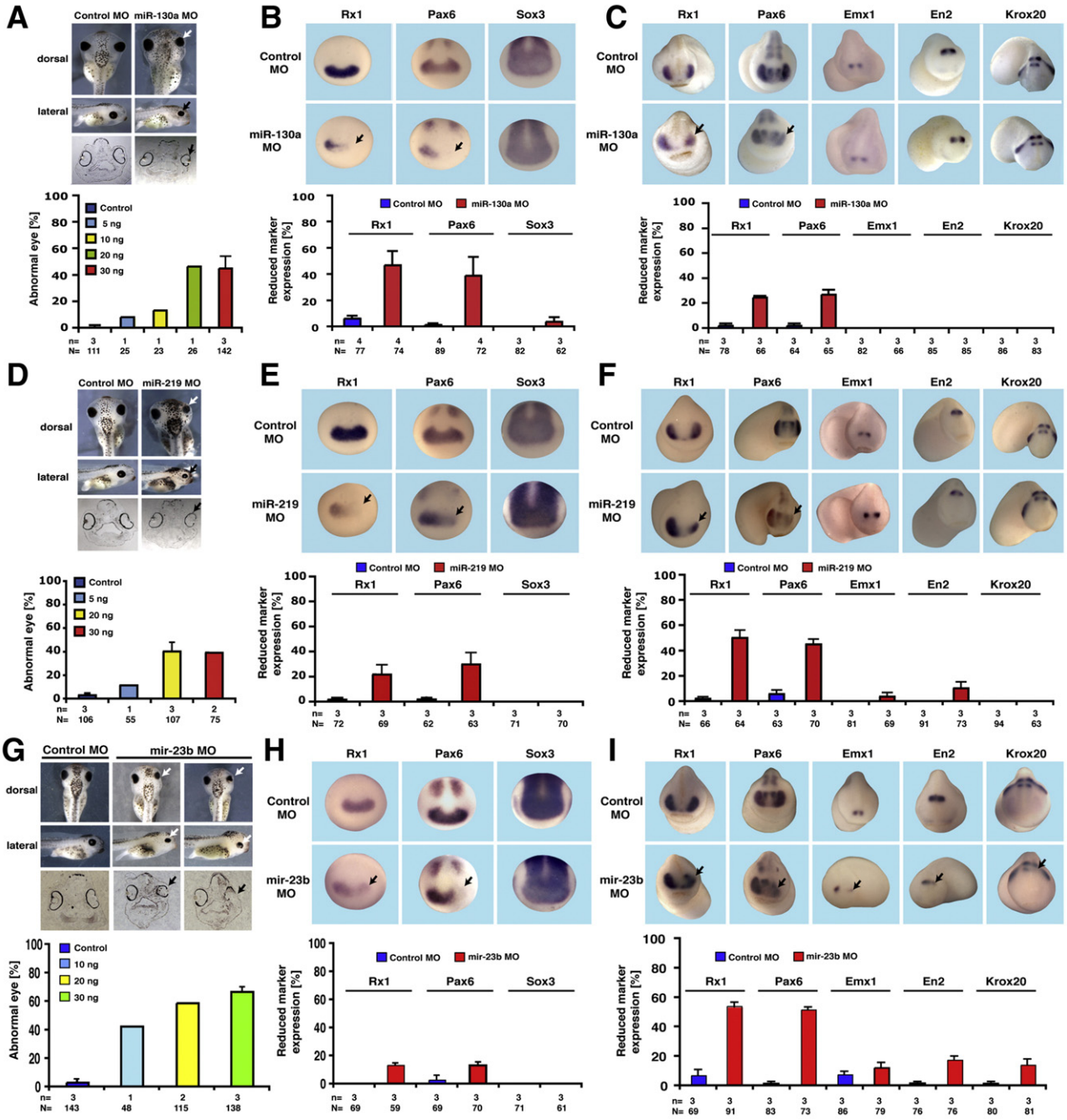



**Fig. 5.** Phenotype of miR-130a, miR-219 and miR-23b MO injections. A. Injection of miR-130a MO resulted in smaller eyes on the injected side (white and black arrows) in a dose-dependent manner. Histological sections showed that the different layers of the retina were not disturbed (black arrow). Control MO injection did not affect eye development. B. Loss of miR-130a function did not alter the expression of the pan-neural marker gene Sox3 at stage 13, but the expression of Rx1 and Pax6 (back arrows). C. MiR-130a MO injected embryos revealed a downregulation of Rx1 and Pax6 at stage 23 (black arrows), but no chance in the expression of brain marker genes. D. MiR-219 deficient embryos showed smaller eyes (white and black arrows) in contrast to the control embryos (Control MO). E. The injection of 20 ng miR-219 MO led to a reduced expression of Rx1 and Pax6 (black arrows), but not of Sox3 at stage 13. F. The expression of Rx1 and Pax6 (black arrows) at stage 23 was downregulated upon loss of miR-219, whereas Emx1, En2 and Krox20 were not affected. G. The injection of miR-23b MO resulted in the development of abnormal eyes which are completely deformed (white arrows). In many embryos, the RPE was not closed at the ventral side of the embryo (right panel). The quantitative representation shows that this effect was dose-dependent. H. Expression of Rx1, Pax6 and Sox3 at stage 13. Loss of miR-23b led to a slight reduction in Rx1 and Pax6 expression. Sox3 was not affected. I. MiR-23b deficient embryos showed a strong downregulation of Rx1 and Pax6 and a slight reduction of Emx1, En2 and Krox20 on the injected side at stage 23 (black arrows). For all experiments a quantitative representation is shown. $n$ = number of independent experiments; $N$ = number of analyzed embryos.

required for splitting the early eye field into two lateral domains. We here addressed the question whether the downregulation of this miRNA has an effect on anterior neural development. The depletion of miR-196a led to a strong eye phenotype (Fig. 8A). Histological sections of the embryos clearly present that the different eye structures including the different layers of the retina and the lens are completely disorganized. By investigating the role for miR-196a on a molecular level, we realized that neural induction was normal as indicated by the expression of Sox3 at stage 13 (Fig. 8B). At this stage of development, Rx1 and Pax6 were only slightly downregulated. Fig. 8C reveals that the knock down of miR-196a results in a diffused and expanded expression pattern of all examined neural marker genes Rx1, Pax6, Emx1, En2 and Krox20 in contrast to the expression pattern in Control MO injected embryos. Furthermore, miR-196a MO

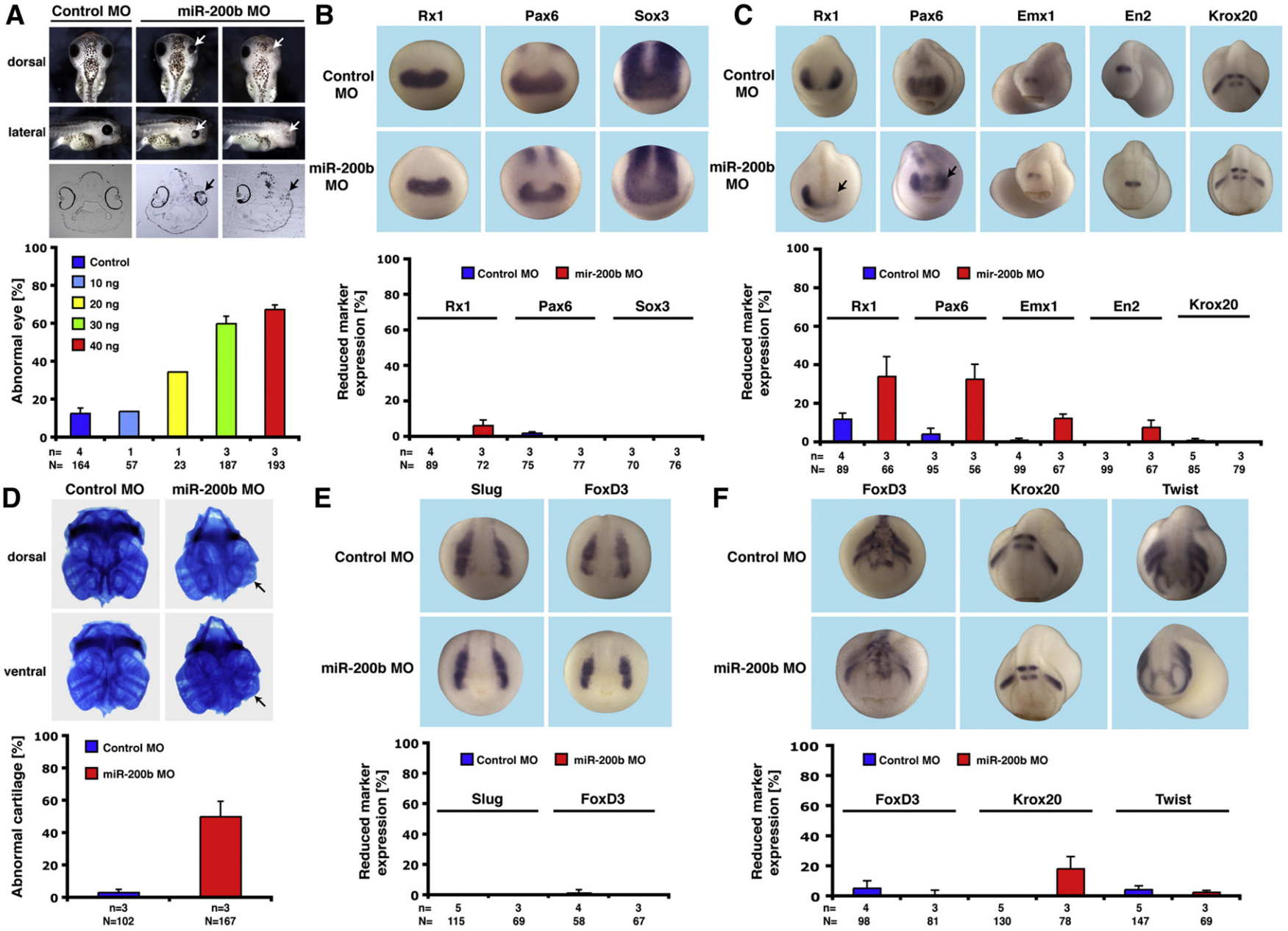



**Fig. 6.** miR-200b MO phenotype. A. The injection of miR-200b MO led to abnormal eye structures or even absent eyes on the injected side (white arrows). Histological sections confirmed this observation. A quantitative representation depicts the dose-dependency of this MO effect. B. The expression of Rx1, Pax6 and Sox3 was not affected upon injection of 40 ng miR-200b MO. C. At stage 23, Rx1 and Pax6 expression are downregulated after loss of miR-200b (black arrows). Other examined brain markers were not affected. D. Alcian blue staining at stage 46. MiR-200b deficient embryos showed smaller cranial cartilage structures on the injected side (black arrows). A quantitative representation is given. E. Slug and FoxD3 expression at stage 17 was not affected upon miR-200b depletion. F. The injection of 40 ng miR-200b MO had no effect on the expression of FoxD3 at stage 20 and Krox20 and Twist at stage 23. A quantitative representation is shown. $n$ = number of independent experiments; $N$ = number of analyzed embryos.

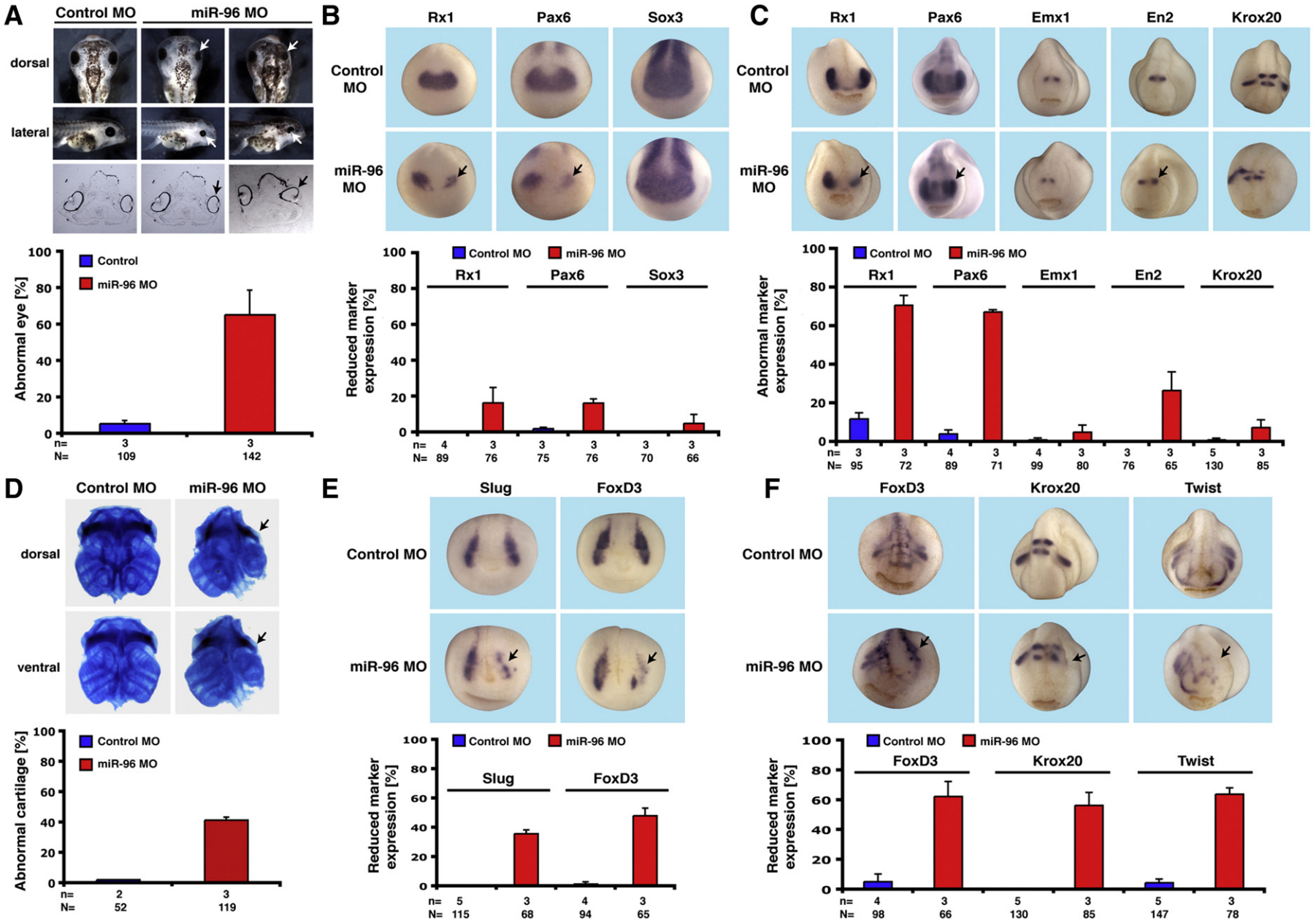



**Fig. 7.** miR-96 MO phenotype. A. Loss of miR-96 function was accompanied by eye malformations (white arrows). B. Injection of 20 ng miR-96 MO resulted in a slight downregulation of Rx1 and Pax6 on the injected side at stage 13 (black arrows). C. At stage 23, depletion of miR-96 led to a strong downregulation of Rx1 and Pax6 on the injected side (black arrows). Emx1 and Krox20 were not affected, whereas the expression domain of En2 was broadened in around 27% of the analyzed embryos (black arrow). D. The downregulation of miR-96 was followed by a reduction of the cranial cartilage on the MO-injected side of the embryo (black arrows). The cartilage preparation of the control MO injected embryo is identical to Fig. 6D. E. At stage 17, the expression of Slug and FoxD3 was disturbed after loss of miR-96 function (black arrows). E. At stage 20, FoxD3 expression was downregulated on the injected side. At stage 23, Krox20 and Twist were reduced after the injection of 20 ng miR-96 MO (black arrows). For all shown experiments, quantitative representations are given. $n$ = number of independent experiments; $N$ = number of analyzed embryos.

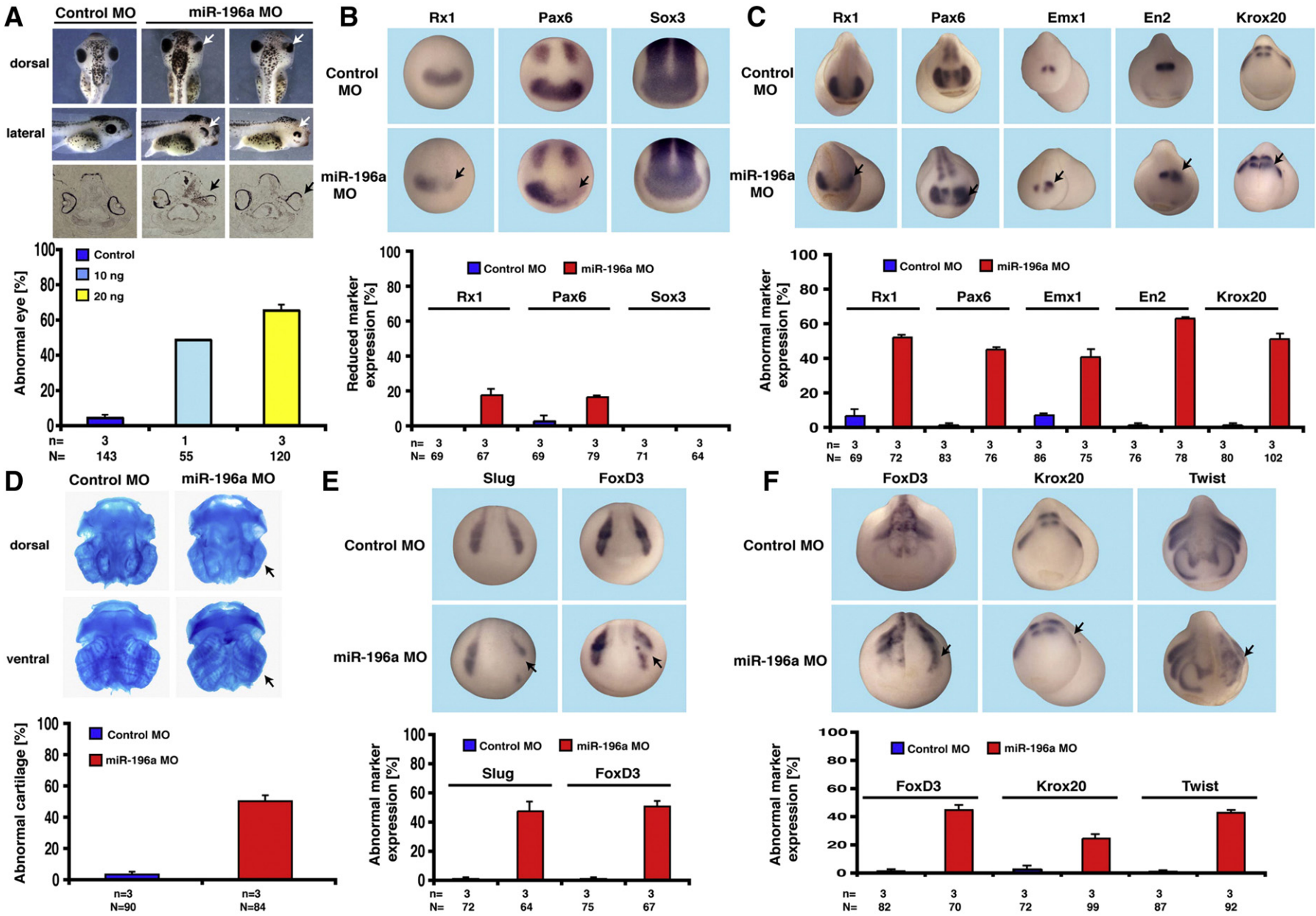



**Fig. 8.** miR-196a MO phenotype. A. The injection of miR-196a MO led to a strong eye phenotype on the injected side in a dose-dependent manner (black and white arrows). B. The early eye marker genes Rx1 and Pax6 were slightly affected upon loss of miR-196a function. C. The injection of 20 ng miR-196a MO resulted in a diffused and broadened expression pattern of Rx1, Pax6, Emx1, En2 and Krox20 at stage 23. D. Loss of miR-196a led to reduced cranial cartilage at stage 46 (black arrows). E. At stage 17, the expression of Slug and FoxD3 was strongly affected upon miR-196a depletion. E. FoxD3 at stage 20 and Krox20 and Twist at stage 23 were downregulated after injection of 20 ng miR-196a MO. For all shown experiments, quantitative representations are given. $n$ = number of independent experiments; $N$ = number of analyzed embryos.

injection led to smaller cartilage on the injected side (Fig. 8D). By looking on molecular marker genes important for NC cell development, we observed an inhibition of Slug (stage 17), FoxD3 (stages 17 and 20), Krox20 and Twist (stage 23) (Figs. 8E and F).

In summary, half of the analyzed miRNA MOs resulted in a phenotype in anterior neural tissue affecting brain, eye and/or cartilage development. In contrast to the observed FMR1/FXR1 and Dicer knock down phenotypes, the downregulation of some miRNAs additionally led to earlier defects already during eye field and NC induction. Similar to the results of FMR1/FXR1 and Dicer MO injections, the loss of some miRNA functions resulted in eye and cranial cartilage abnormalities. These data are in line with the idea that the FMR1/FXR1 loss of function phenotype might be due to defects in the miRNA pathway.

## Discussion

### *FMR1 and FXR1 function in eye and cranial cartilage development*

Here we show for the first time a requirement for FMR1/FXR1 during eye and cranial cartilage development in *X. laevis* as determined by knock down experiments. Analyses on a molecular level indicated that FMR1 is not required for neural induction in general including establishment of the early eye field and formation of NC cells. Later during development, however, changes in gene expression as well as in eye morphogenesis and NC cell migration could be observed. It should be noted that our data do not exclude an earlier function of FMR1/FXR1 as maternal protein stores of these proteins are not affected by MO based knock down approaches. How do these phenotypes in *Xenopus* relate to those observed in mouse models?

FXR1 knock out mice have been generated (Mientjes et al., 2004) but die early during embryogenesis. The molecular basis of this lethality has not been completely investigated. Loss of function analyses in *X. laevis*, however, already indicated deficits in somitogenesis, which is in agreement with the major expression domain of FXR1. Microarray gene expression analyses have been performed in *Xenopus* and implied numerous neural genes to be affected in FXR1 knock down embryos including signaling molecules and transcription factors required for early embryonic and neural development (Huot et al., 2005).

An FMR1 knock out mouse has also been generated without any gross morphological defects. This difference in phenotypes (*Xenopus* versus mouse) might be explained by functional redundancies between different members of the FMR1 gene family. Whereas in mammals three members of the family were described, only FMR1 and FXR1 are present in *Xenopus* (Blonden et al., 2005). Of note, FMR1 KO stem cells display defects in neural differentiation (Castren et al., 2005). Also in zebrafish, morpholino mediated knock down of FMR1 resulted in craniofacial cartilage defects (Tucker et al., 2006), although this has recently been challenged by the generation of FMR1 mutant fish (den Broeder et al., 2009). In *Drosophila*, only one member of the family, dFMR, exists. dFMR has been shown to be involved in proper eye development (Wan et al., 2000). Furthermore, as in case of FXR1, RNA binding partners for FMR1 have been identified and thus might reflect transcripts that are potentially regulated by FMR1 (Sung et al., 2000; Darnell et al., 2001; Denman, 2003) including genes involved in regulating cellular differentiation or cell migration.

### *Molecular basis of the FMR1/FXR1 phenotype*

Previous publications linked FMR1/FXR1 to the miRNA machinery. We therefore analyzed the potential involvement of miRNAs in the observed FMR1/FXR1 phenotype by investigating the function of Dicer, which is required for the generation of all mature miRNAs. We here demonstrated for the first time that a neural-specific loss of Dicer function leads to eye as well as cranial cartilage defects during *Xenopus* development by the injection of two independent MOs. In mice and zebrafish, Dicer knock out embryos die very early in development (Bernstein et al., 2003; Wienholds et al., 2003) showing the importance of this gene and miRNAs for early steps of vertebrate development. Most likely, this early requirement for Dicer is obscured in *Xenopus* due to the maternal stores of Dicer protein (Fruscoloni et al., 2003). Two recently published studies in mouse and *Xenopus* also showed defects during late eye development when the function of Dicer was depleted specifically in retina cells (Damiani et al., 2008; Decembrini et al., 2008). Since nothing is known about the molecular basis of the eye phenotype, we analyzed the induction of the neural plate and the eye field at stage 13 and did not observe any defects. Later in *Xenopus* development, however, eye marker genes are reduced in Dicer deficient embryos. During zebrafish development, Dicer possesses a function in the morphogenesis of the brain (Giraldez et al., 2005) without major defects in gene expression. This is in agreement with our observation of unaltered brain-specific marker gene expression in early *Xenopus* development.

In addition to the eye phenotype we observed a cranial cartilage phenotype upon Dicer downregulation. Dicer has been shown to be essential for skeletal development in mice due to a decreased proliferation rate and abnormal differentiation of chondrocytes (Kobayashi et al., 2008). The development of the cranial cartilage in *Xenopus* requires the migration of NC cells from the dorsal neural tube. We here show that NC cells were correctly induced but migration was impaired upon Dicer knock down pointing towards a function of Dicer in regulating cellular behavior.

When comparing the phenotypes of FMR1/FXR1 and Dicer it becomes evident that they are very similar: no deficits in early neural induction and specification steps. Later, however, both knock downs are characterized by defects in eye morphology including altered gene expression and an altered migration behavior of NC cells. Our findings are therefore in good agreement with previous findings indicating FMR1/FXR1 to be involved in regulating mRNA stability and mRNA translation through interaction with RISC and the miRNA pathway.

### *Downregulation of miRNAs during Xenopus development*

It can be assumed that the observed phenotypes upon FMR1/FXR1 depletion are due to the function of some specific miRNA molecules. We therefore interfered with the function of 12 selected miRNAs by use of MOs as previously done for other miRNAs (Martello et al., 2007; Yin et al., 2008; Rosa et al., 2009; Walker and Harland, 2009). The injection of six miRNA MOs resulted in a phenotype in eye or cranial cartilage development. Three of the miRNAs only showed abnormal eyes (miR-130a, -219 and -23b) whereas the other three are involved in eye as well as cranial cartilage development (miR-200b, -96 and -196a).

Some of these miRNAs deserve a more detailed discussion. Depletion of miR-200b results in strong eye defects such as misorganized or missing eyes and reduced cartilage structures. Recently, several studies suggested a role for members of the miR-200 family in EMT (Burk et al., 2008; Korpal and Kang, 2008; Korpal et al., 2008; Paterson et al., 2008). We could not observe, however, any defects in NC migration suggesting no disturbance in EMT by loss of miR-200b. The molecular effect of miR-200b during development of NC thus remains unclear. Earlier studies suggested a function of miR-96 in eye development (Loscher et al., 2007; Xu et al., 2007; Loscher et al., 2008; Lewis et al., 2009; Mencia et al., 2009; Soukup, 2009). By *in vitro* assays and target prediction, Xu et al. (2007) showed that the transcription factor Mitf (*mi*crophthalmia-associated *t*ranscription *f*actor) is a direct target of miR-96. A loss of MITF affects development of the eye and NC-derived melanocytes (Nakayama et al., 1998; Kumasaka et al., 2005). The misregulation of Mitf through miR-96 might thus well contribute to the phenotype observed here. In mouse, zebrafish and *Xenopus*, miR-196a is expressed in the posterior

trunk during later development (Mansfield et al., 2004; Wienholds et al., 2005; Qiu et al., 2009). The expression of miR-196a in early development is not described yet. In our experiments, the depletion of miR-196a resulted in malformed eyes and reduced cartilage. Moreover, marker genes for eye, brain as well as NC cells are affected. In chicken, miR-196a is regulating Hox genes as well as shh during limp development (Hornstein et al., 2005). Shh is also involved in eye, cranial cartilage and neural tube development (Chiang et al., 1996; Zhang and Yang, 2001). Thus, the observed phenotype could be due to a misregulation of shh signaling.

In all cases analyzed, loss of miRNA function could well contribute to the observed FMR1/FXR1 phenotype. It should be mentioned that the FMR1/FXR1 or Dicer phenotype might be more complex than here described and that other miRNA will surely contribute to this more complex phenotype. Moreover, it should be noted that a more causal link cannot be established by experimental means. If FMR1/FXR1 interact with RISC, a loss of FMR1/FXR1 cannot simply be rescued by providing an excess of the miRNA of interest.

In our analysis we recognized some differences between the FMR1/FXR1 and Dicer knock down phenotypes on the one side and the miRNA knock down phenotypes on the other side. For FMR1/FXR1 and Dicer downregulation we only observed a late phenotype starting around stage 20. In contrast the depletion of some miRNAs also interfered with the induction of early eye (stage 13) and NC marker genes (stage 17). We think that Dicer is also essential for early development including neural induction in *Xenopus* like in other organisms (Bernstein et al., 2003; Wienholds et al., 2003) but because of huge maternal storage of Dicer protein, we do not interfere with Dicer function early in development. Later, however, the Dicer MO mediated loss of Dicer protein resulted in abnormal eye and cartilage development. The maternal storage of Dicer proteins allows furthermore the generation of mature miRNAs during early embryogenesis. By the injection of miRNA MOs we interfere with their regulation of target mRNAs. Thereby the function of miRNAs is also depleted in early development leading to an early effect upon downregulation of some miRNAs.

Taken together, our here described data argue that FMR1/FXR1 are important for early eye and NC development likely due to a function in RISC and the miRNA machinery. The described miRNA phenotypes provide a good starting point towards a more detailed analysis of the observed phenotypes.

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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.2010.02.031.

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