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Dicer inactivation causes heterochronic retinogenesis in *Xenopus laevis*

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ABSTRACT Maturation of miRNAs by dicer is required in vertebrates for normal neural development. Here we report that dicer inactivation in *Xenopus* affects cell cycle progression, survival and timing of the generation of retinal cells, resulting in small retinas with lamination defects. In particular, dicer inactivation delays the exit from the cell cycle and the translation of key genes of late neurogenesis, highlighting a crucial role of miRNAs in retinal development.

KEY WORDS: *Dicer, miRNA, timing, neurogenesis, retina*

Introduction

MicroRNAs play a key role in timing the development of the worm *Caenorhabditis elegans* (Ambros, 2000). In vertebrates, they are required for normal development (Bernstein *et al.,* 2003; Giraldez *et al.,* 2005; Harfe *et al.,* 2005; Wienholds *et al.,*2005), but their precise role is still not fully understood. In zebrafish, inactivation of dicer, an enzyme that is required for miRNA maturation (Bartel, 2004), causes size reduction of nervous system structures, including the neural retina (Giraldez *et al.,* 2005). However, little is known on the role of miRNAs in the control of cell cycle and cell fate determination of neural progenitor cells.

The different retinal cell types are generated from a common progenitor cell and exit from the cell cycle following an evolutionarily conserved timing schedule (ganglion cells, horizontal cells, cones, amacrine cells, rods, bipolar cells, Muller glia; Livesey *et al.,* 2001). bHLH and homeobox genes (Hatakeyama *et al.,* 2001) work together with cell cycle progression (Ohnuma *et al., 2002;* Cremisi *et al.,* 2003) to establish the different retinal cell types. Recently, we described a cell-cycle-dependent clock, which sets the time of translation of key homeobox genes supporting the generation of photoreceptors (*Xotx5b*) and bipolar cells (*Xotx2*) in *Xenopus* developing retina (Decembrini *et al.,* 2006). Since our observations indicate a key role of translational control in retinogenesis, we investigated the effects of miRNAs inactivation in *Xenopus* embryonic retina obtained by dicer knockdown.

Results

A tBlastx search identified a *Xenopus* clone (GenBank accession no. BP673528) displaying a highly significant similarity to all the described *Dicer 1* from different species. To design antisense morpholino oligonucleotides (Mos) we first defined the regions of the 5' end of *Xdicer1* transcripts showing the highest conservation among different alleles and polymorphic forms. To this aim, we amplified by RT-PCR and sequenced the 112 bp region upstream of the first ATG from different batches of embryos at various stages. This analysis identified two forms of *Xdicer1* transcripts and revealed conserved regions, which were used as target for the two Mos used in this work.

The specificity and efficiency of the two Mos, Xdcr-Mo1 and Xdcr-Mo2, was confirmed by microinjecting reporter constructs carrying a normal or mutated Mo target sequence (Fig. 1 and results not shown), and by comparing the expression of retinal miRNAs between normal and Mo-injected embryos (Supplementary Fig. 1).

We focused on the effects generated by dicer knockdown in *Xenopus* retinal development. Embryos were injected with 10 nl of 125 microM Xdcr-Mo1 or Xdcr-Mo2 in one dorsal cell at 4 cell-

Abbreviations used in this paper: BrdU, bromodeoxyuridine; GFP, green fluorescent protein; Mo, morpholino oligonucleotide; Morphant, Mo-injected embryo; St., developmental stage; Stdev, standard deviation; S.e.m., standard error of the means.

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stage. Embryos injected with the same amount of control-Mo were indistinguishable from wild type (wt) embryos (not shown). Embryos injected with Xdcr-Mos were traced and selected by GFP mRNA coinjection. Microinjection of each Xdcr-Mo at 4-cell stage produced the same effects. The eyes of Mo-injected embryos (morphants) at the stage of swimming tadpole (st. 42) were smaller compared to controls and their retinal lamination was severely affected. 61% of morphants (n= 238) showed a severe eye phenotype (Fig. 2 A, D) with extremely reduced eye size and lack of retinal lamination, 35% displayed decreased size (Fig. 2C), incomplete lamination and photoreceptors organised in rosettes-like structures (Nakagawa *et al.,* 2003; arrow in Fig. 2 C, J). The morphology of lens and pigmented epithelium (PE) was not apparently affected in morphants. However, PE thickness was significantly reduced in morphants compared to controls (58%, stdev = 12%, n= 14 retinas; Fig. 2 B-D).

In contrast to the reduced eye size, the number of retinal cycling cells of morphants at three different developmental stages (st. 30, st. 33, st. 37) was dramatically higher than in control, as indicated by BrdU labelling index (LI, Fig. 2 E-G, Supplementary Fig. 2) and *XcyclinD1* expression (not shown). However, morphants displayed also an increased retinal cell death compared to controls (Fig. 2 H, Supplementary Fig. 3). In fact, cell death induced by dicer

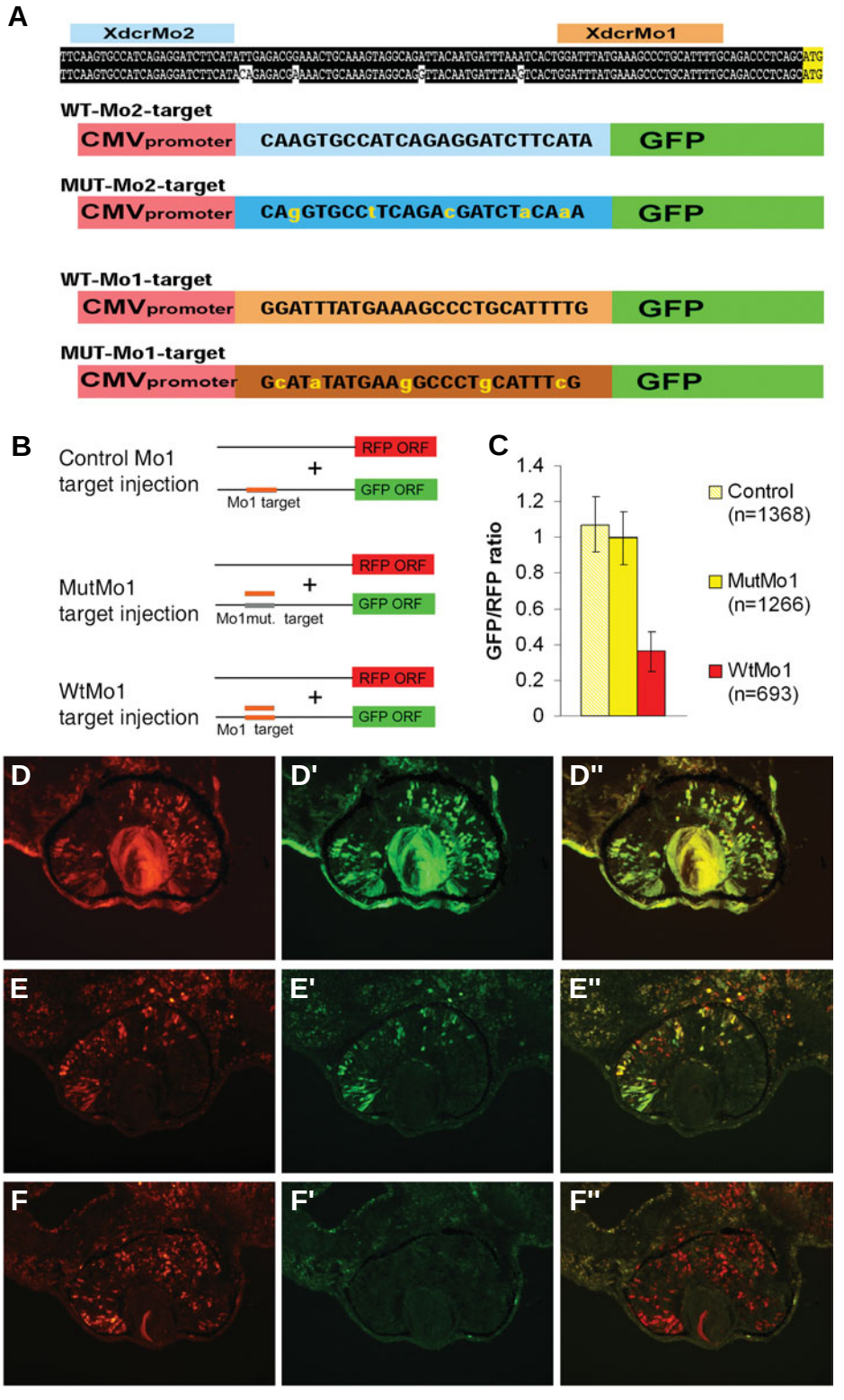

Fig. 1. *In vivo* GFP reporter activity of Xdcr-Mo-target constructs. *Sequence alignment in the top panel* **(A)** *shows the two forms of* Xdicer1 *obtained by the analysis, with mismatches indicated in white boxes. The target sequences of Xdcr-Mo1 and Xdcr-Mo2, which are the two Mos chosen for functional analysis, are shown in light blue and orange colours, respectively. Constructs carrying the GFP reporter activity under the control of normal (WT) or mutated (MUT) Xdcr-Mo target sequences were cloned in pCS2 vector under CMV transcriptional control, as shown in (A). In the MUT constructs, the small letters indicate inserted mutations.* **(B)** *Scheme of the strategy followed to assay Xdcr-Mo1 ability to specifically inhibit its target. Red Fluorescent Protein (RFP) construct was always co-injected into early embryo as an internal standard, together with WT or MUT constructs, without (Control Mo1 target injection) or with (MutMo1; Mo1 target injection) Xdcr-Mo1.* **(C)** *The ratio between GFP and RFP positive cells (n= number of cells analysed in three independent experiments) of mature embryonic retinas at st. 42. Bars indicate standard error of the mean. Notably, only WtMo1 target injection (WtMo1) significantly decreases the ratio ($p< 0.001$), indicating the specificity of Xdcr-Mo1 to inhibit its specific target. Comparable results were obtained when injecting WtMo2 or MutMo2 constructs, with or without Xdcr-Mo2 (not shown).* **(D-F'')** *Examples of retinas after Control Mo1 target injection without any Mo (D-D''), MutMo1 target injection with Xdcr-Mo1 (E-E''), or Mo1 Target injection with Xdcr-Mo1 (F-F''). (D,E,F) RFP detection; (D',E',F') GFP detection; (D'',E'',F'') merge.*

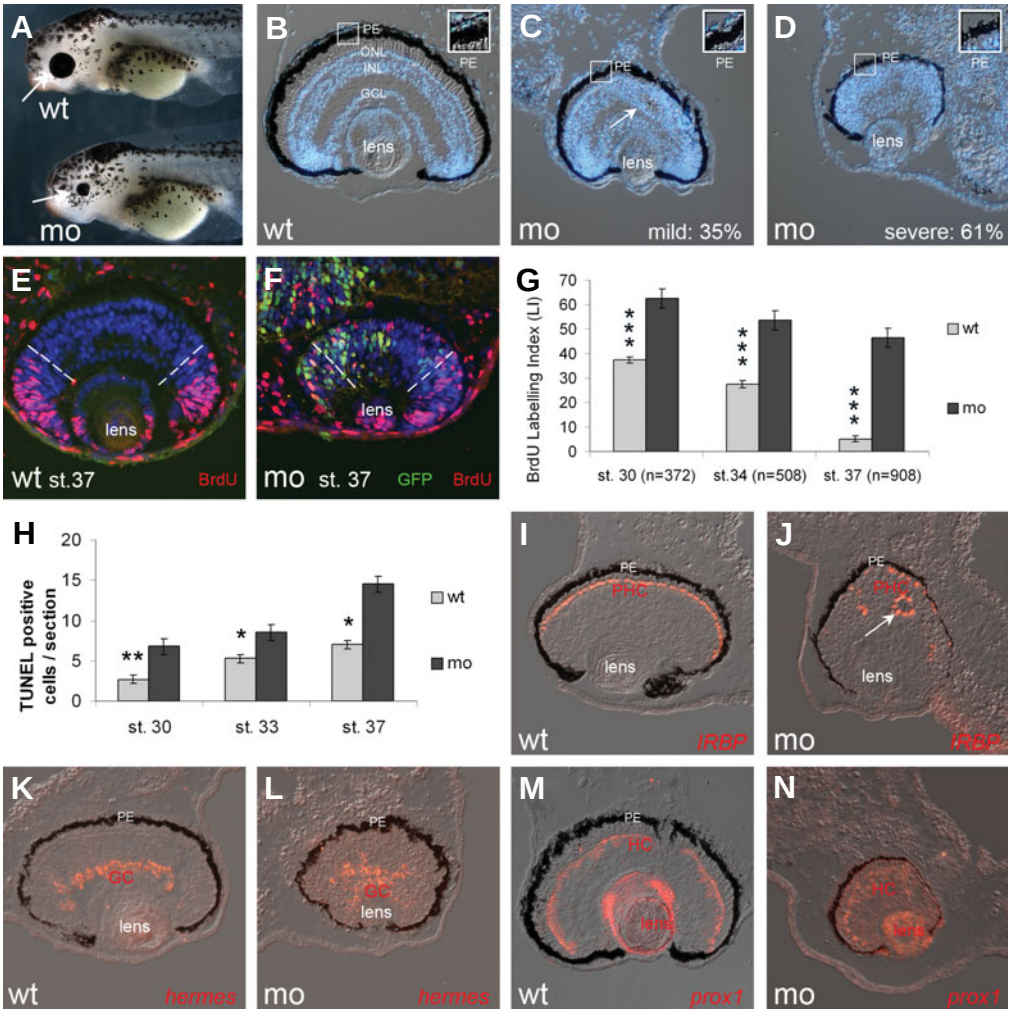

Fig. 2. Dicer down-regulation affects retinal cell lamination, delays the exit from the cell cycle and promotes cell death. (A) *Comparison between morphant (mo) and wild type (wt) eyes (arrows) at st. 42. Embryos were injected with 10 nl of 125 nM Xdcr-Mo1 in one dorsal cell at 4 cell-stage embryos. Injected embryos were traced and selected by GFP mRNA coinjection (300 ng, not shown).* **(B-D)** *Nuclear Hoechst staining of wt (B) and morphant (C,D) eye sections. PE: pigmented epithelium, ONL: outer nuclear layer, INL: inner nucler layer, GCL: ganglion cell layer. Although lens and PE morphology does not appear to be affected in morphants, the thickness of morphant PE is clearly reduced compared to control, as shown in box in (B) (control) and (C,D) (mild and severe morphant phenotypes, respectively).* **(E-G)** *BrdU labelling index (LI), obtained by the analysis of wt and mo embryos after 8 h BrdU incorporation.* **(G)** *Statistical analysis of BrdU-positive cells (red in E,F, and Supplementary Fig. 3) in the central aspect of retinal sections (delimited by dashed lines in E,F). GFP traces Xdcr-Mo1 injected cells. A significant LI increase (triple asterisk: p <0.001, student ttest) was observed in morphants compared to wt. Error bars show s.e.m., n: number of cells.* **(H)** *Statistical analysis of apoptotic TUNEL-positive cells (Supplementary Fig. 4) in mo and wt retinas (n= 69 and 482, respectively) at different stages. Mo showed a significantly higher number of apoptotic cells/section than wt (double asterisk: p < 0.01, single asterisk p < 0.05). Error bars: s.e.m.* **(I-N)** In situ *hybridisation of* Xenopus *cell-type specific markers (Decembrini* et al., *2006) on st. 42 mo and wt retinal sections:* Xirbp *(photoreceptors, PHC),* Xhermes *(ganglion cells, GC),* Xprox1 *(horizontal cells, HC). Although expressing the specific markers, retinal cells are not properly layered in mo (J,L,M) compared to wt (I,L,N).*

inactivation might account for the decreased eye size, as it occurs for limb size in mouse (Harfe *et al.,* 2005). According to previous studies (Lunardi *et al.,* 2006), control-Mo injection did not affect cell proliferation and cell death (not shown).

Even if dicer knockdown dramatically affects retinal development and differentiation, it does not prevent the expression of a number of retinal cell markers such as *Xirbp*, *Xhermes, Xprox1* (Fig. 2 I-N), *Xotx5b* (Fig. 3 C,D) and *Xotx2* (Fig. 3 G,H). This observation suggests that dicer downregulation is somehow compatible with the capability of retinal progenitor cells to progress toward neural differentiation. Thus, an interesting question is whether in vertebrates, as in *C. elegans* (Moss *et al.,*1997; Slack *et al.,* 2000), miRNAs may affect the developmental timing. We compared the time of translation of *Xotx5b* and *Xotx2,* two key factors controlling the timing of retinal histogenesis, between morphants and control embryos. Whereas *Xotx5b* and *Xotx2* mRNAs were present from early developmental stages both in morphants and in controls (st. 33, Supplementary Fig. 4;Viczian *et al.,* 2003), the onset of both Xotx5b and Xotx2 proteins detection was delayed in morphants compared to controls. In partly injected embryos, we compared non-injected (GFP-negative) aspects of retinas to Mo-injected (GFP-positive) regions within the same eye. Non-injected (GFP-negative) aspects of retinas showed normal layering (Fig. 3 A-F). In these parts of the retinas, Xotx5b and Xotx2 expression is comparable to that of wt retinas (Decembrini *et al.,* 2006). Conversely, neither Xotx5b nor Xotx2 are detectable in GFP-positive cells at st. 37 (Fig. 3 A,B) and 42 (Fig. 3 E,F), respectively. Protein detection is delayed in Mo-injected cells to later stages, namely st. 42 (Xotx5b, Fig. 3 C,D) and st. 45 (Xotx2, Fig. 3 G,H). Embryos injected with control morpholino showed no such delayed expression of the two proteins (Supplementary Fig. 5).

Discussion

By dicer inactivation in *Xenopus* retinal progenitor cells, we found that miRNAs play a crucial role in cell cycle control and survival and are necessary to set the correct timing of translation of key genes of retinal histogenesis. Although miRNAs are expected to directly inhibit protein translation, our results indicate a

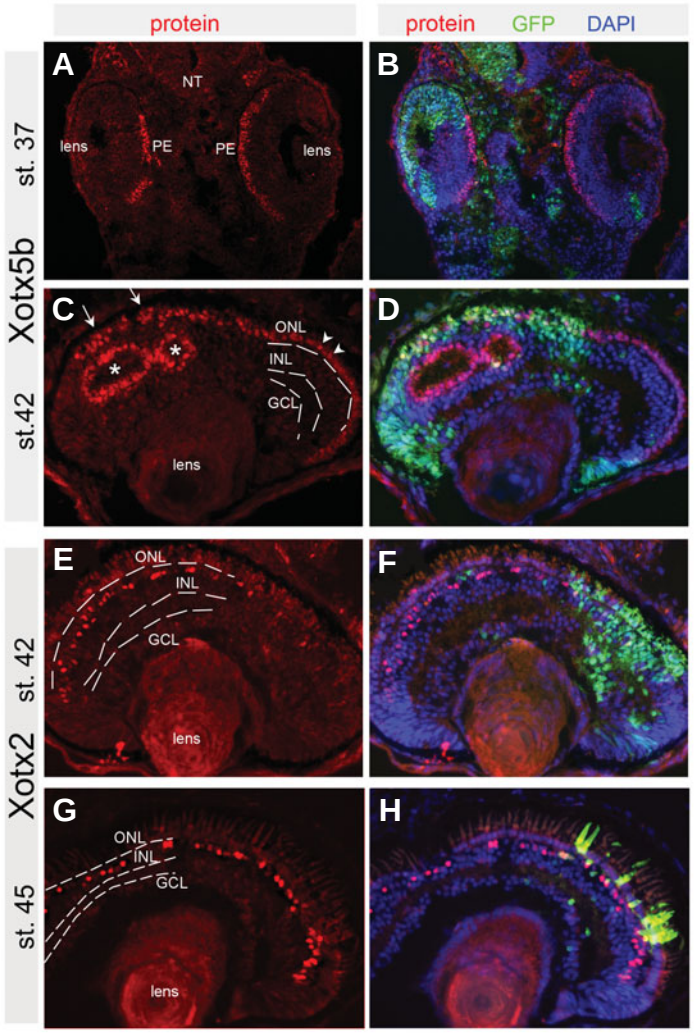

Fig. 3. Dicer down-regulation delays the translation of Xotx5b and Xotx2. (A-H) *Typical immunodetections (red staining) of Xotx5b* **(A-D)** *and Xotx2* **(E-H)** *on retinal sections of morphants at different developmental stages, obtained in three different experiments (n > 15 embryos in each experiment). GFP (green) traces injected cells in (B,D,F,H); DAPI (blue) stains nuclei in (B,D,F,H). NT: neural tube; PE pigmented epithelium; ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. The non-injected (GFP-negative) aspects of mo retinas showed normal layering, which is highlighted by dashed lines. In these parts of the retinas, Xotx5b and Xotx2 expression is comparable to that of wt retinas (Decembrini* et al., *2006). Conversely, Xotx5b and Xotx2 are not detectable in GFP-positive cells at st. 37 (A,B) and 42 (E,F), respectively. Protein detection is delayed in Mo-injected cells to later stages, namely st. 42 (Xotx5b, C,D) and st. 45 (Xotx2, G,H). Asterisk in* **(C)** *shows rosette-like structures. In* **(H)**, *the Mo-injected aspect of the retina, which is GFP-labelled, shows Xotx2-positive nuclei (labelled in red). In* **(C)**, *arrowheads indicate a typical monolayer of Otx5b-positive, normal photoreceptor nuclei, whereas arrows point at multilayered GFP/Xotx5b double-positive nuclei.*

facilitating rather than an inhibitory role of miRNAs on the translation of the key cell fate factors Xotx5b and Xotx2. This is reminiscent of the mode of action of the let-7 (Slack *et al., 2000*) and lin-4 (Moss *et al.,* 1997) miRNAs, which generate heterochronic phenotypes in *C. elegans* mutants. Indeed, these two miRNAs indirectly facilitate the translation of developmental genes, by repressing the translation of inhibitory RNA binding proteins. We speculate that such kind of miRNAs could exist also in vertebrates and that in *Xenopus* they could indirectly support the translation of Xotx5b and Xotx2, and therefore the generation of photoreceptors and bipolar cells. Indeed, in a preliminary study, we for instance observed that the vertebrate counterpart of lin-4 (miR-125b) is expressed in the embryonic retina and that its inactivation by antisense oligonucleotide lipofection dramatically reduces the proportion of bipolar neurons in lipofected retinas. However, the overexpression of this miRNA alone is not sufficient to rescue the effects of dicer knockdown (unpublished), suggesting that a complex miRNA regulatory network may in fact exist for retinogenesis.

A crucial link between cell cycle and cell fate has been shown in *Xenopus* retinal progenitor cells (Moore *et al.,* 2002; Ohnuma *et al.,* 2002; Casarosa *et al.,* 2003; Cremisi *et al.,* 2003). In fact, there is a close relation between the time a retinal progenitor cell exits from the cell cycle (cell birth date) and its differentiation fate. As a consequence, anticipating or delaying the cell birth date of retinal progenitors affects the type of neurons they generate. Cdk2/cyclinA2 overexpression delays retinal cell birth date and supports the expression of Xotx2 protein, thus causing an increase of the proportion of bipolar neurons (Casarosa *et al.,* 2003; Decembrini *et al.,* 2006). Similarly to cdk2/cyclinA2 overexpression, dicer morpholino injection also delays the average retinal cell birth date (Fig. 2G, Supplementary Fig. 2). However, dicer downregulation does not support Xotx2 expression, rather it delays it, as it occurs for Xotx5b (see Fig. 3).

Thus, the effects of dicer knockdown can not be simply explained as a consequence of delayed cell birth date.

Although delayed, the translation of Xotx5b and Xotx2 in morphants is not repressed and, at a qualitative analysis, the proportion of cells expressing the two proteins eventually appears to be comparable in morphants and controls. This favours the hypothesis that both photoreceptors and bipolar cells may form properly in morphants, even if later compared to controls. Normal photoreceptors and bipolar cells segregate in two distinct cell layers in a timely ordered process and in coordination with the other retinal cell types. Thus, the heterochronic generation of these two cell types is compatible with a general disorganisation of layers, including the formation of rosette-like structures.

In conclusion, dicer inactivation generates a retinal heterochronic phenotype, namely delay of both retinal cell birth-date and translation of key genes required for the generation of late retinal cell types. This supports the idea that specific miRNAs are part of a cell clock machinery timing retinal neurogenesis.

Materials and Methods

Design and use of morpholino oligonucleotides

RT-PCR of *Xenopus laevis* dicer was carried out using forward 5'atgcatttcaagtgccatca and reverse 5'aaaagggacccattggagag primers. Mos, including a standard control morpholino oligonucleotide (control morpholino), were designed and supplied by Gene-Tools. A master mix of 30 pg/nl each of pCs2-RFP (a kind gift of Dr. Marina Mione) and either Wt-Mo-target or Mut-Mo-target GFP reporter constructs was previously prepared. Either Xdcr-Mo1, Xdcr-Mo2 or control Mo was added at the final concentration of 125 microM, when indicated, and 10 nl of the mix were injected at 4-cell stage into one dorsal blastomere. The analysis of RFP and GFP positive cells was carried out at st. 42 on 12 micron cryosections, under

normal epifluorescence.

Microarray screening

miRNAs expressed in the developing *Xenopus* retina were selected by probing an Exiqon miRCURY™ LNA Array version 7.1 with 2 micrograms of total RNA extracted from a pool of st.33 dissected retinas (n=40), following the manufacturer method. The capture probes showing highest signals were chosen for further validation by ISH.

Morphological analysis, immunodetection and ISH

PE analysis was performed on microphotographs of either control or dcr-Mo injected embryos (including both mild and severe phenotype), using the ImageJ software. The thickness of three different pictures of each retina, for a total number of 14 retinas, was measured by the measure tool and the average PE thickness of morphants was calculated as percentage of the control average thickness.

The whole mount protocol of ISH of miRNAs (Wienholds *et al.,* 2005; Kloosterman *et al.,* 2006) is a slight modification of the common ISH protocol. The significant difference is the probe nature and the temperature of hybridisation and of the washes, which is usually set 20-22°C lower than the Tm of the LNA modified oligonucleotide used as probe. We used 15-22 nt long, Dig-pre-labelled LNA™ probes (Exiqon), complementary to the mature sequence of miRNAs. Detection was carried out at RT by BMP purple staining for 24-36 hrs. Negative control probes did not significantly stain (not shown). List of probes used:

mir-298: 5' GGAAGAACAGCCCTCCTCTGCC
mir-375: 5' TCACGCGAGCCGAACGAACAAA
mir-452: 5' GTCTCAGTTTCCTCTGCAAACA
mir-381: 5' ACAGAGAGCTTGCCCTTGTATA
mir-125a: 5' CACAGGTTAAAGGGTCTCAGGGA
mir-347: 5' TGGGCGACCCAGAGGGACA.

mRNA ISH and immunodetection were performed as previously described (Casarosa *et al.,* 2003; Decembrini *et al.,* 2006).

BrdU labelling index

Embryos were co-injected at 4-cell stage in a dorsal blastomere with Xdcr-Mo1 and GFP as tracer. Embryos were then injected with BrdU (100 nl of 15 mg/ml BrdU solution) at the stages indicated, fixed after 8 hrs and eventually processed for further analysis as described (Decembrini *et al.,* 2006).

TUNEL assay

TUNEL assay was performed by an ApopTag Peroxidase Kit (Serologicals) on 12 micron cryostat sections, following the manufacturer's instruction.

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