

miR-34 is maternally inherited in *Drosophila melanogaster* and *Danio rerio*

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ABSTRACT

MicroRNAs (miRNAs) are small, endogenous, regulatory RNA molecules that can bind to partially complementary regions on target messenger RNAs and impede their expression or translation. We rationalized that miRNAs, being localized to the cytoplasm, will be maternally inherited during fertilization and may play a role in early development. Although Dicer is known to be essential for the transition from single-celled zygote to two-cell embryo, a direct role for miRNAs has not yet been demonstrated. We identified miRNAs with targets in zygotically expressed transcripts in *Drosophila* using a combination of transcriptome analysis and miRNA target prediction. We experimentally established that *Drosophila* miRNA *dme-miR-34*, the fly homologue of the cancer-related mammalian miRNA miR-34, involved in somatic-cell reprogramming and having critical role in early neuronal differentiation, is present in *Drosophila* embryos before initiation of zygotic transcription. We also show that the *Drosophila* miR-34 is dependent on maternal Dicer-1 for its expression in oocytes. Further, we show that miR-34 is also abundant in unfertilized oocytes of zebrafish. Its temporal expression profile during early development showed abundant expression in unfertilized oocytes that gradually decreased by 5 days post-fertilization (dpf). We find that knocking down the maternal, but not the zygotic, miR-34 led to developmental defects in the neuronal system during early embryonic development in zebrafish. Here, we report for the first time, the maternal inheritance of an miRNA involved in development of the neuronal system in a vertebrate model system.

INTRODUCTION

MicroRNAs (miRNAs) are endogenously occurring small regulatory RNA molecules of ~19–22 nt in length. Cellular biosynthesis of miRNA starts with RNA polymerase-II-mediated transcription of miRNA genes to form a large capped and polyadenylated transcript called primary-miRNA (pri-miRNA) (1,2). Particular stem-loop structures in pri-miRNA serve as a substrate for Drosha-DGCR8 (microprocessor) complex, which cleaves at the base of the stem-loop to produce a smaller product called pre (precursor)-miRNA (3,4). Pre-miRNA is transported to the cytoplasm, by exportin-5 (5–7), where it undergoes a second enzymatic cleavage by Dicer to form an imperfect RNA duplex (8,9). Typically, one of the strands in the duplex is incorporated in an Argonaute-containing ribonucleoprotein complex (miRNP) (10). In several pre-miRNAs, both the arms can form functional entities (11). The composition of this complex is not fully understood, but it universally contains Dicer and Argonaute protein family members (12–16). The miRNA in the miRNP complex, by virtue of its partial complementarity, can bind to a target mRNA and provide the context for the action of the Argonaute proteins. The mRNA in the mRNA-miRNP complex may undergo degradation, especially with high affinity targets (17,18). Alternatively, translation from the mRNA is reduced, or a deadenylation complex may degrade the mRNA (19,20). The net effect of the transcript cleavage and degradation is reduction in the mRNA and protein product in the cell, whereas translational inhibition results in reduction, only of the protein (21–23).

Several miRNAs show tissue-specific expression patterns established during organogenesis (24). The tissue-specific expression pattern of the neuronal miR-124 and liver-specific miR-122 are, for instance, established in the fetal brain and liver, respectively (25). miRNAs may directly or indirectly influence the expression of hundreds of transcripts during development, thus shaping the mRNA and

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proteomic expression profile of the cell (22). Ectopic expression of miRNA can alter the transcriptional profile of the cell to recapitulate events during differentiation (26). Many miRNAs also undergo rapid and pronounced changes in expression during differentiation of specific cell types from embryonic stem cells (27). Thus, miRNA expression plays an important role during development and differentiation by directly targeting developmentally important genes or regulating growth and cell division.

The earliest gene expression events in the development of an organism are initiated even before the genome of the zygote is transcriptionally active. Maternal mRNAs deposited into the developing oocyte are translated to produce proteins that in turn regulate the expression of zygotic genes. Maternal effects, caused by cytoplasmic inheritance, were classically described in the inheritance of left-handed coiling or sinistrality in the snail *Lymnaea peregra* depending on the genotype of the mother at a single locus (28). At the molecular level, maternal effects are best understood in the establishment of the anterior-posterior axis of the *Drosophila* embryo. Cell polarity is established in the early *Drosophila* embryo through the localization of maternal mRNA transcripts deposited by nurse cells in the developing oocyte. These mRNAs are translated to produce transcription factors that dictate asymmetry along anterior-posterior axis (29,30). Concomitantly, these maternal RNAs are cleared after the mid-blastula transition when the zygote becomes transcriptionally active (31–33). Many transcripts can be expressed both maternally and from the zygotic genome; a notable example is the bicoid gene. Post-transcriptional regulation of mRNA plays an important role in the regulation of early development, as is known in *Caenorhabditis elegans* where the miRNA let-7 controls the progress of the larval stages (34). Although miRNAs are known to regulate gene expression at the post-transcriptional stage, there is currently no evidence of involvement of maternal miRNAs in the maternal zygotic transition (MZT).

Two independent facts led us to hypothesize that miRNAs may be maternally inherited. First, the mature and stable form of miRNAs, i.e. the 19–22 nt form (in association with proteins), is present in the cytoplasm of cells. Second, miRNAs in general are thought to be stable in the cell and have typically longer half-life than mRNA (35). We reasoned that like mitochondria and other cytoplasmic components, miRNA would be present in the cytoplasm in unfertilized oocytes. In support of this hypothesis, conditional knockout of Dicer in the maturing oocytes of mice, fail to survive early developmental stages, specifically the one-cell zygote to two-cell embryo transition in mouse, suggesting an important role for maternally inherited miRNAs in early development (36,37). However, Dicer can also have additional roles, over and beyond biogenesis of miRNAs, during early development. The expression profiles of miRNAs in single mouse oocytes and developing embryos were also studied, in an attempt to identify abundant maternally inherited miRNAs (36). Interestingly, miRNAs of the let-7 family, miR-30 and miR-16, were most abundantly expressed in mouse oocytes (36), but a functional role for these abundant miRNAs has remained elusive. However, miR-30 was

shown to be zygotically expressed in *Xenopus*, where it is proposed to target and degrade maternally inherited transcripts of *Xlim1/Lhx1* (38). Maternally expressed miRNA would be transferred to the zygote during fertilization and could potentially regulate the expression of mRNA transcripts during early stages of development of the zygote, especially because miRNAs have relatively slow turn-over rate (35). Alternatively, there might exist a mechanism for active clearing of miRNAs during oogenesis, to prevent maternal inheritance of such regulatory molecules, similar to the active degradation of maternal transcripts by deadenylation. To explore these possibilities, we used a bioinformatics approach to identify miRNAs with putative targets in transcripts present during early developmental stages of the zygote in *Drosophila*, and subsequently experimentally tested their presence *in vivo*.

We found miR-34 to be one of the few miRNAs that had several targets in the transcripts expressed exclusively by the zygote. Here, we report the maternal inheritance of miR-34 in *Drosophila* embryos, by demonstrating that the localization of the miRNA to the oocytes is dependent on the maternal miRNA processing machinery. We established that this miRNA is also maternally inherited in zebrafish and used microarray-based mRNA expression profiling to study the effect of the miRNA on zygotic gene expression. miRNA mimics and anti-miRNA molecules could modulate the expression of the miRNA in single-cell oocytes. We find that downregulation of maternal, but not zygotic, miR-34 at the single-cell stage in zebrafish unfertilized oocytes leads to growth defects in the brain region. This effect is specific, as genome-wide expression profiling showed that the suppression of miR-34 does not affect the extensive transcriptional changes associated with early development. We unequivocally demonstrate the maternal inheritance of miR-34 in *Drosophila* and zebrafish and its impact on Notch gene expression and neurogenesis in zebrafish.

MATERIALS AND METHODS

Drosophila strains

CantonS was used as a wild-type strain. The UAS and GAL4 line were purchased from Vienna *Drosophila* Resource Center (stock v24666) and Bloomington (stock 25378). The genotype for the stocks used are w¹¹¹⁸; P{GD11429}v24667 Dicer-1GD11429 and w [*]; P{w [+mC]=GAL4-nos.NGT}40 P{w [+mC]=lacO.256x}43 P{lacO.256x}50F P{lacO.256x}57A P{lacO.256x}60AB/CyO; P{w [+mC]=UAS-GFP.lacI}1.2/TM3,Sb [1], respectively. The GAL4 line was used to drive the expression of an RNAi against Dicer-1 under the germ line-specific nanos promoter. The transgenic flies carrying the Sb (stubble) and Cyo (curly wings) markers along with the male flies were discarded. The remaining female flies were taken for further experiments. The selected flies were fed overnight with yeast and then dissected from the abdomen to collect the unfertilized oocytes. Flies were grown on standard corn-meal-agar medium supplemented with yeast with the addition of 0.25% (w/v) sodium benzoate and 0.45% (v/v) propionic acid. The flies were grown at 25°C.

For primer extension, the embryos from CantonS flies were collected on sucrose-agar plates with yeast paste. The laid embryos were harvested at developmental time points from 0.5 to 3.5 h. The embryos were dechorionated in 50% bleach for 2–3 min followed by washing three to four times in nuclease-free water to get rid of the sticking yeast and other debris. These embryos were then transferred to Trizol for RNA isolation as per manufacturer's protocol.

Zebrafish

Danio rerio wild-type fish were maintained at the zebrafish facility at Institute of Genomics and Integrative Biology, Delhi, India under standard growth conditions. The embryos were collected and harvested at different stages of early development before and after MZT. Unfertilized embryos were collected by squeezing the abdomen of spawning females.

For creating the *her4.1* promoter expressing stable transgenic fish, the *her4.1:TetA-GBD-p2a-mCherry*ud6 plasmid was injected in wild-type fish embryos at one- to two-cell stage with Tol2 transposase mRNA to facilitate transposition and integration of the desired sequence into the fish genome (39,40). At 24 h post-fertilization (hpf), these injected embryos were scored for mCherry expression. Fish scored positive were inbred to generate stable F1 progeny. All further experiments were carried out on embryos produced by F1 progeny having stable expression of mCherry.

For locked nucleic acid (LNA) knockdown experiments to study the effect of miR-34 on neuronal development, we used a transgenic fish expressing mCherry under the regulation of *her4.1* promoter (*her4.1:TetA-GBD-p2a-mCherry*ud6). In all, 100 μ M of anti-miR-34 LNA was injected in a volume of 3 nl to knockdown miR-34 with the fish survival rate of ~50% at 24 hpf after the miRNA knockdown.

Primers used in the study

Anti-miRNAs:

anti-dme-miR-31b: CAGCTATTCCGACA; anti-dme-miR-34: CAACCAGCTAACCA; anti-dme-miR-210: AGCCGCTGTAC; anti-dme-miR-275: GCGCGCTACTTC; anti-dme-miR-278: AAACGGACGAAAG; anti-dme-miR-282: CAGACAAAGCCTAGTA; anti-dme-miR-317: CTGGATACCACCAG; anti-dre-miR-34: AC AACCAGCTAAGA; anti-dre-miR-430: CTACGCCAA CAAATA; anti-dme/dre-miR-34 LNA: ACAACCAGC TAAGA; anti-dre-pre-miR-34: CTTCGTTCTCACTC; control LNA: ACCAATCGACCAAC.

Gene-specific primers:

Dicer-1 FP: ACCAATGTACTGCGTTTGCA; Dicer-1 RP: GTTTGCTGATCACAGAACTTAACGTT;
Ago2 FP: GCACAAGTGTGCGGTCTTGATT; Ago2 RP: GTGAAGTCTTAATGCATTG;
bc12_F: GATGGCGTCCCAGGTAGATA; bc12_R: AA AACGGGTGGAACACAGAG;
e2f3_F: GGAGAGACGTCTGGATGAGC; e2f3_R: TT CGTTTCAGAGGGTGCTTT;

notch1B_F: CCTGGATGACTGTCAGACCA; notch1B_R: AAGCCATTGACTCCATCCAC;
notch1A_F: CCTGCCAGGACAGAGAGAAC; notch1A_R: TCGCAGACACACTCATAGCC;
d1D_F: TGTGCATCTGGAAGTTCTCG; d1D_R: AAC CACAAACACGTCGATCA;
d1C_F: CATCCGGTGTGTTTGAGTTG; d1C_R: GAG TCCGCGATCAGTATTTTC;
d1B_F: TGTCCTCAGGGATTCTACGG; d1B_R: GGT CCAGCCTCTTCTCACAG;
d1A_F: GAGTTTGACGACGGAGAAGC; d1A_R: CA GTTTCACCTCGTGCTTGA;
 β ActinF: GCTGTTTTCCCTCCATTGT; β ActinR: TC CCATGCCAACCATCACT.

miR-34 knockdown in zebrafish

LNA-based backbone-modified oligos against dre-miR-34 and control LNA oligonucleotide were synthesized from SBS Genetech Co. Ltd. Synthetic anti-dre-pre-miR-34 oligo with LNA backbone modification was from Exiqon. The sequences of the oligos are mentioned separately with the backbone modifications indicated in bold. This anti-miRNA is perfectly complementary to miR-34 and only partially complementary to miR-34b/c. However, as its relative targeting of the three isoforms is not established, we refer to the miRNA as dre-miR-34 and the anti-miRNA as anti-dre-miR-34. The anti-dre-miR-34 LNA and control LNA were injected into the one- to two-cell stage embryos. The embryos were collected at 1, 7 and 24 hpf post-injection. The embryos were immediately transferred to Trizol (Invitrogen) at the indicated time points, and total RNA isolation was done following the manufacturers' protocol.

For downstream experiments comparing the effect of maternal and zygotic miR-34, zebrafish embryos were injected with control LNA, anti-dre-miR-34 LNA and anti-dre-pre-miR-34 LNA at one- to two-cell stage. The embryos were monitored, collected at 0, 3, 6, 9, 12 and 24 hpf and processed for total RNA isolation as indicated earlier in the text using Trizol (Invitrogen) as per manufacturers' protocol. For each of the time point mentioned, 40 embryos were used for RNA isolation.

Microarray

Total RNA (6–8 μ g) from ~100 zebrafish embryos at the mentioned time points was processed. cRNA was prepared, and 20 μ g of cDNA was hybridized to the Zebrafish Genome array (Affymetrix). The experiment was performed as per the manufacturer's protocol at The Center for Genomic Application (TCGA), Delhi, India. The data were extracted using GCOS.

Microarray data analysis

Data were extracted from GEO for publicly available data set by Pilot *et al.* (41) (GSE3955) for characterization and analysis of *Drosophila* gene expression data. Analysis was done using mean normalization. The transcripts were classified into exclusively maternal and zygotic based on their expression levels at various time points during

early *Drosophila* development. The fold change for each transcript was calculated by dividing the expression level in the developmental stage by the average expression in all other stages. The 3'-UTRs of these transcripts were extracted from UCSC and miRNA target sites predicted using miRanda as shown in Supplementary Table S1.

For later experiments, to study the effect of downregulation of miR-34 during early time points in zebrafish development, the data generated from the zebrafish RNA after LNA treatment (as described earlier in the text) were pre-processed using Avadis. The data were log transformed and quantile normalized followed by differential expression analysis for each of the time points. Unsupervised hierarchical clustering was performed on rows for all normalized values.

Primer extension

Total RNA isolated from *Drosophila* oocytes/embryos and zebrafish embryos at desired time points was hybridized to anti-miRNA oligonucleotides for their detection (oligo sequences are indicated separately). M-MLV reverse transcriptase was added along with P³²-dCTP, and the reaction was incubated at 42°C for 30 min. The reaction mix was run on an 18% denaturing urea polyacrylamide gel electrophoresis. The blot was fixed and washed followed by an overnight exposure on the film. Radioactive bands were observed on Typhoon Scanner (Amersham).

Real-time polymerase chain reaction

Total RNA from *Drosophila* Dicer-1 RNAi fly oocytes was isolated using Trizol as per the manufacturer's protocol. In all, 1 µg of total RNA was used. Real-time polymerase chain reaction (PCR) for specific miRNAs was performed using Ago2/Loqs (internal control), for which the primers were designed as per Saito *et al.* (42) and Taqman real-time PCR probes for miR-34 supplied by Applied Biosystems, as per the manufacturer's protocol on Roche Light Cycler480. Data were extracted and analysed manually using the method described by Pfaffl (43).

Semi-quantitative reverse transcriptase-PCR

RNA from Dicer-1 RNAi *Drosophila* oocytes was analysed for expression of *Drosophila* Dicer-1. Ago2 transcript expression was measured as a positive control. PCR primer sequences and conditions were used as described previously (42). 1 µg of total RNA was reverse transcribed using random hexamers and M-MLV reverse transcriptase at 42°C for 30 min. The cDNA was subject to 35 rounds of PCR at 56°C.

In situ hybridization

LNA against dme-miR-34 was used to detect miR-34 expression in unfertilized *Drosophila* oocytes. The LNA probe was DIG-labelled at the 3'-end using the Roche 2nd Generation DIG Oligonucleotide 3'-End Labeling Kit as per the manufacturers' protocol. Hybridization was done for 48 h at 42°C, 300 rpm with gentle mixing. The embryos were then incubated with alkaline phosphatase-conjugated

anti-DIG antibody (Anti-Digoxigenin-AP Fab fragments, Roche). Detection was done using substrate solution, i.e. NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt) for 1–2 h. The oocytes were observed under the microscope at 10× objective zoom.

RESULTS

We hypothesized that the cytoplasmic inheritance of miRNAs is a theoretical possibility given their cytoplasmic localization and long half-life. Maternally inherited miRNAs can affect maternal transcripts or zygotic gene expression. We prioritized the latter case because it poses a potentially novel form of epigenetic inheritance, where in spite of the same genetic composition, two individuals may have different early gene expression patterns because of their inherited miRNA pool. To this purpose, we used a bioinformatics approach to identify miRNAs with a potential functional role in early zygotic gene expression. In accordance with the nomenclature convention for miRNAs (44), *Drosophila* and zebrafish miRNAs are prefixed with dme- and dre-, respectively.

Bioinformatics prediction of miRNAs that target zygotic transcripts

Pilot *et al.* (41) have previously reported mRNA expression profiles for *Drosophila* genes through four developmental stages of which the earliest time point, T0, is well before the initiation of zygotic transcription, whereas T1 and T2 represent stages after zygotic transcription has been initiated. At T0, the mRNAs present are expected to be maternally inherited. We re-analysed this microarray data to calculate fold enrichment of each transcript at a particular stage by comparing it with the expression of the transcript in the other three stages. We identified 27 transcripts that showed exclusively zygotic expression. Further, using the target prediction algorithm, miRanda (45), we identified miRNAs whose targets occur at high frequency in the exclusively zygotic transcripts (Supplementary Table S1). Seven *Drosophila* miRNAs, namely, dme-miR-31 b, dme-miR-34, dme-miR-210, dme-miR-275, dme-miR-278, dme-miR-282 and dme-miR-317, had multiple targets in these zygotic mRNA transcripts.

miRNAs are maternally deposited in unfertilized *Drosophila* oocytes

We performed primer extension-based assays for the presence of these miRNA in the RNA samples prepared from embryos at early developmental stages in *Drosophila*. Timed collection of embryos was carried out, and RNA was isolated from pools of embryos at the same stage. Using LNA-modified oligonucleotide probes, primer extensions were performed to label the product by incorporation of radioactively labelled nucleotides. Finally, the products were visualized using electrophoresis. We have previously used this primer extension method for detection of miRNAs in mammalian cells (46). Among the seven miRNAs tested, only miR-34 and miR-31 showed detectable expression (Figure 1A). Further experiments

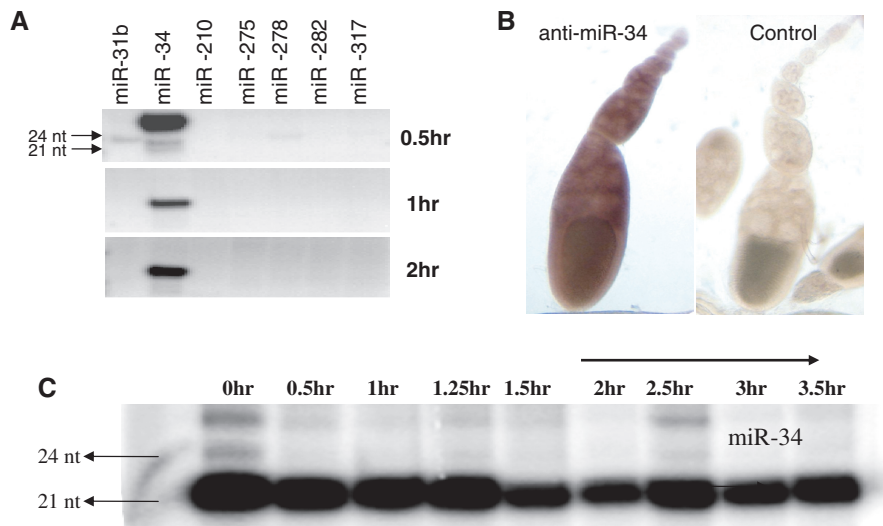


Figure 1. Spatio-temporal expression of dme-miR-34 in early developmental stages. (A) Detection of putative maternally inherited miRNAs. RNA prepared from embryos collected at indicated time points was used for primer extension as described in 'Materials and Methods' section. (B) *In situ* hybridization of dme-miR-34 in unfertilized *Drosophila* oocytes to show the localization of maternal miR-34. (C) Time-dependent expression of dme-miR-34 detected using primer extension. Arrow represents the time points after MZT.

were done using miR-34-5p (sequence derived from the 5' arm of the pre-miR-34 precursor), as its expression was relatively high, and it is already known to have an important role in cell division and differentiation. The targets predicted for miR-34 are described in Supplementary Table S1. The binding patterns of the miRNA:mRNA pairs are provided in Supplementary Table S2. To spatially detect miR-34, we carried out *in situ* hybridization in *Drosophila* oocytes within late stage egg chambers. We found clear expression of miR-34, detectable using LNA probes, whereas a control probe with unrelated sequence showed no such pattern (Figure 1B). The nurse cells known to transfer maternally inherited RNAs to the developing oocytes as well as follicle cells showed expression of the miRNA. We further monitored the temporal expression pattern of miR-34 (Figure 1C). The miRNA is detected highly at 2 and 3.5 h, both time points beyond MZT, suggesting that either maternal miR-34 persists at these time points or it is zygotically expressed.

Maternal Dicer-1 is required for expression of miR-34

The presence of miR-34 in *Drosophila* oocytes suggests that the maternal miRNA processing machinery is required for its expression. To investigate whether miR-34 expression in the oocytes is indeed dependent on the maternal processing machinery, we used tissue-specific knockdown of Dicer-1 using RNAi. Dicer-1 in *Drosophila* is known to be essential for processing of miRNAs. We created Dicer-1 knockdown in oocytes of flies by driving the expression of a shRNA against Dicer-1 from a nanos promoter using the UAS/GAL4 system to ensure the germ line-specific knockdown of Dicer-1. The Dicer-1 knockdown was confirmed by semi-quantitative reverse transcriptase (qRT)-PCR (Figure 2A). *Drosophila in situ* hybridization from the Dicer-1 knockdown embryo clearly demonstrated the absence of miR-34, whereas the

wild-type showed a clear expression of the miRNA in the oocyte (Figure 2B). We further confirmed the results using qRT-PCR for miR-34 (Figure 2C). Thus, we confirmed that the maternal Dicer gene product is required for the expression of the miRNA in the oocytes.

Temporal expression of miR-34 in zebrafish embryos during early development

Zebrafish as a model system is used extensively in developmental biology because of its optically clear embryos, high fecundity, rapid development and the recent availability of genomic tools. In the study of maternally inherited miRNAs, it provides an additional advantage that fertilization is external; therefore, it is easy to collect unfertilized oocytes from the female, thus ruling out any possibility of zygotic transcription. Sequence comparison of miR-34 family members from zebrafish, mouse and human homologues reveals that this miRNA is highly conserved in evolution. As shown in Supplementary Figure S1, *Drosophila* has a single paralogue of miR-34, whereas in higher organisms, including fish and mammals, the miRNA family has diversified to include miR-34a, b and c. dme-miR-34 and dre-miR-34 are orthologues of the miR-34a isoform of mouse and human (47).

We, therefore, tried to establish the maternal inheritance of miR-34 in zebrafish by checking its expression in unfertilized zebrafish oocytes and during early developmental stages. We used dre-miR-430 as a marker of zygotic transcription, as it is known to be expressed zygotically to degrade maternal mRNAs in the zebrafish embryo. As expected, miR-430 was not seen in the unfertilized oocytes but was expressed in later time points spanning 12 hpf to 3 days. On the other hand, miR-34 was expressed highly in unfertilized embryos, and its expression steadily decreased up to 3 days (Figure 3A and B).

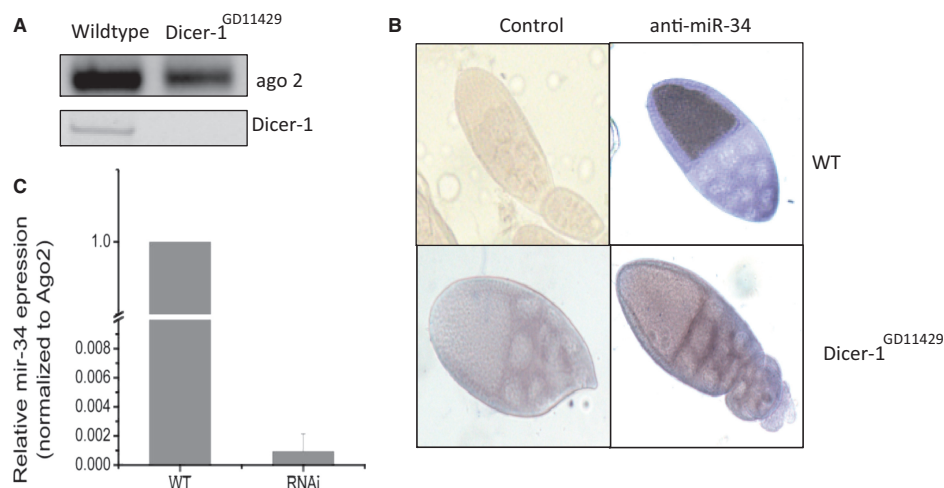


Figure 2. Maternal Dicer is essential for early dme-miR-34 expression. (A) RT-PCR to confirm Dicer-1 knockdown after the induction of RNAi driven against Dicer-1 using the UAS/GAL4 inducible RNAi system as described in ‘Materials and Methods’ section. (B) *In situ* hybridization to detect the presence of dme-miR-34 after Dicer-1 knockdown in the late stage egg chambers. Dicer-1^{GD11429} indicates the Dicer RNAi allele. (C) Relative quantification using qRT-PCR was performed for determining the levels of dme-miR-34 after knockdown. The experiment was performed from 20 late stage egg chambers from wild-type and RNAi transgenic flies each.

Knockdown of miR-34 at early developmental stages in zebrafish

Anti-sense oligonucleotides stabilized by LNA modifications have been used extensively by our and other groups to knockdown miRNAs (46,48,49). We first designed LNA-modified anti-miR-34 oligonucleotides that can differentiate between the maternal and zygotic forms of miR-34.

The primer extension assays (Figure 3A) show that the maternally inherited miR-34 is in the mature form, as it shows a size of ~24 nt. We reasoned that an anti-miR-34 designed to target the pre-miR-34 loop region will prevent processing of the zygotic miRNA, but leave the maternal miR-34 unaffected. We designed this anti-pre-miR-34 oligonucleotide to correspond to 47–61 nt of the pre-miRNA spanning the stem and loop region. We also used an LNA-modified anti-miR-34 oligonucleotide, complementary to the mature form that we have earlier used to knockdown miR-34a in mouse cell lines (48), expecting it to target only the maternal miR-34. We injected the anti-pre-miR-34 or the anti-miR-34 (against the mature form) into zebrafish embryos at single-cell stage and monitored the kinetics of miR-34 knockdown. As shown in Figure 4, the anti-miR against the mature form resulted in reduced miR-34 levels up to 6 hpf, after which expression was recovered by 12 hpf. However, the anti-miR against the pre-miR-34 did not result in loss of miR-34 expression until 3 hpf, confirming our expectation that it would target only the zygotic form. Targeting of the pre-miRNA resulted in maximum downregulation at 12 hpf (Figure 4). Interestingly, the anti-pre-miR-34 was highly toxic to the embryos, resulting in ~85% death by 24 hpf (data not shown). We used the anti-miRNA against the mature form in all further experiments to selectively target the maternally inherited form.

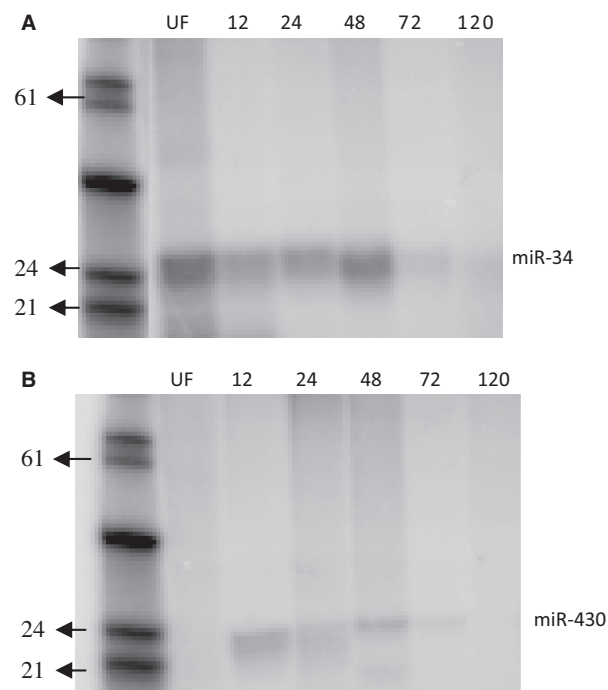


Figure 3. Expression of miR-34 through early stages of zebrafish embryonic development. (A) Detection of dre-miR-34 from RNA isolated from zebrafish embryos at the indicated time. (B) dre-miR-430 expression as an indicator of zygotic transcription. The expression was detected using primer extension-based assay as described in ‘Materials and Methods’ section. The marker sizes (in no of nucleotides) are shown by arrows.

Effect of miR-34 knockdown on target genes

dre-miR-430 has been shown to localize the deadenylation machinery to the mRNAs bearing target sites and mediate large scale and rapid clearance of maternal transcripts (20). As gene expression changes during early

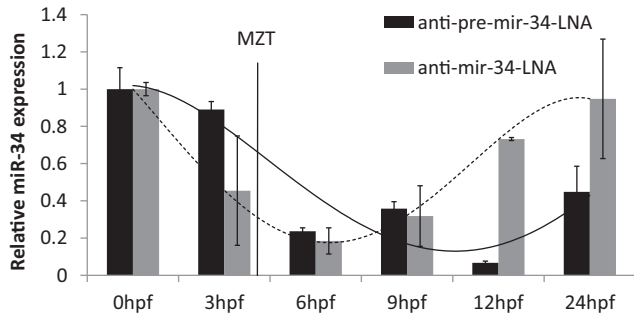


Figure 4. Kinetics of maternal and zygotic miR-34 knockdown: anti-miRNAs specific to the zygotic or maternal form were injected into zebrafish embryos, and embryos were monitored for miR-34 expression level using Taqman-based real-time PCR for miR-34a and mir-92 (normalization control). MZT at 4 hpf is marked by a vertical line. Error bars indicate standard deviation of three biological replicates. Fitted curves indicate the maximum knockdown for anti-miR-34 LNA (dashed line) and anti-pre-miR-34 LNA (solid line).

developmental stages are marked by the concomitant induction and repression of large groups of genes, we tested the effect of knocking down the miRNA on the global mRNA expression profile of developing zebrafish embryos. We did not see any gross morphological defects or developmental arrest after knockdown of miR-34 (Figure 5A). RNA was collected at three time points after injection of the anti-miR-34 LNA or a control LNA. The knockdown of the miRNA was confirmed using qRT-PCR using Taqman assay for miR-34. The knockdown effect was most prominent at 7 hpf with a reduction of 90%, whereas 50 and 70% reduction was seen at 1 and 24 hpf (Figure 5B). The first time point sampled, 1 hpf, is well before MZT, thus accounting for maternal transcripts. As MZT takes place ~3–4 hpf, we used samples collected at 7 hpf to study early zygotic expression. Finally, at 24 hpf, most of the organ systems are developed, and transcription is completely zygotic. Clustered expression profiles clearly showed expected changes during developmental transitions like the

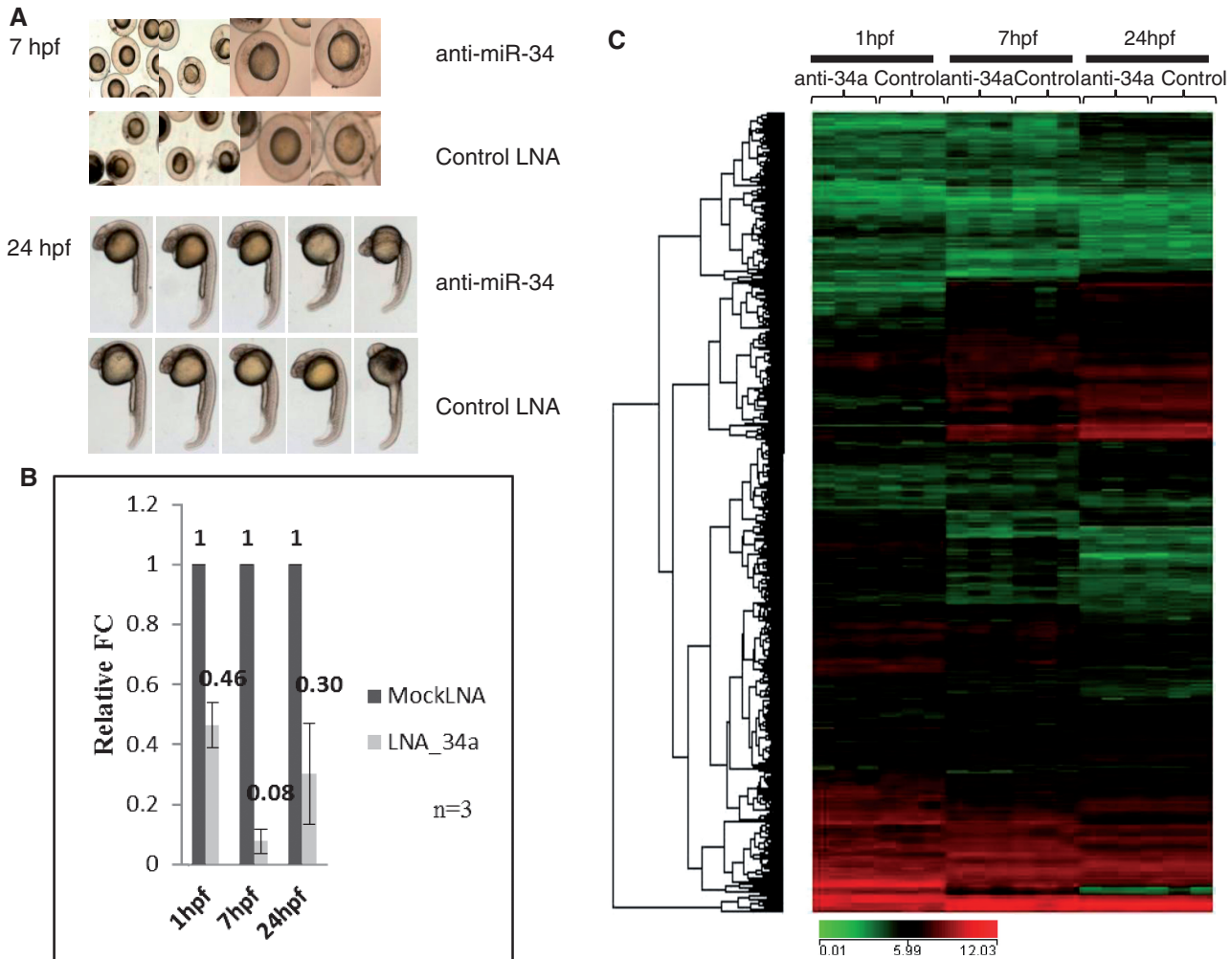


Figure 5. Knockdown of maternal dre-miR-34 followed by expression profiling to show the role of maternal miR-34 in early zebrafish development. (A) Microinjections done at single-cell stage in zebrafish embryos at 1, 7 and 24 hpf. Shown are the representative images at 7 and 24 hpf. (B) qRT-PCR to detect the changes in levels of miR-34 after injection of embryos with anti-miR-34 LNA relative to control LNA at the indicated time points. Standard deviation was calculated on average of three biological replicates. (C) Hierarchical clustering performed on rows for gene expression data for the three time points. Scale bar for expression levels is shown. Data analysis was performed as described in ‘Materials and Methods’ section.

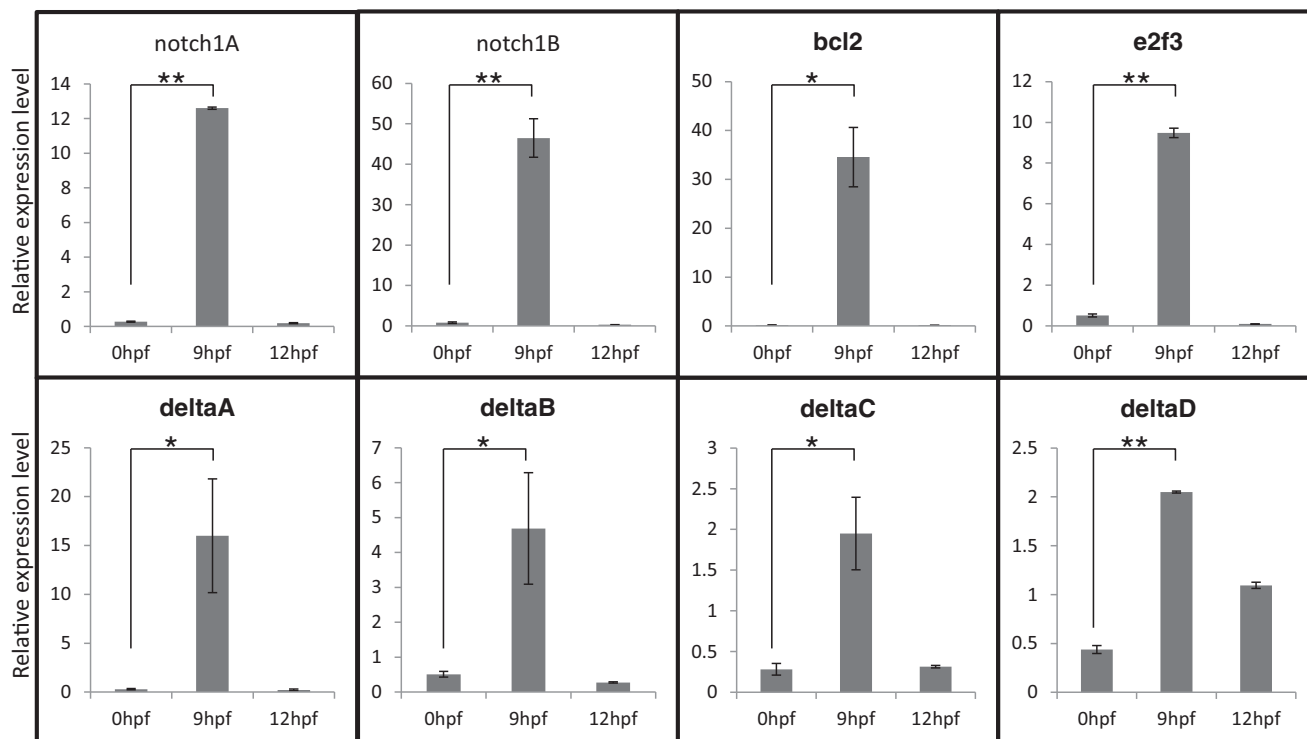


Figure 6. Differential expression of known mammalian targets of miR-34 in zebrafish: qRT-PCR analysis was performed to detect differentially expressed known mammalian miR-34 targets in zebrafish after knockdown of dre-miR-34 at the indicated time points. The relative fold changes indicated are normalized to β -actin as the internal reference control. The values are represented as relative fold changes (* $P < 0.05$, ** $P < 0.001$, Student's t -test).

clearance of maternal transcripts at 7 hpf, induction of zygotic genes, especially the ribosomal genes at 7 hpf. However, there were no gross changes in gene expression profiles after the knockdown of the miR-34 (Figure 5C). Earlier reports have shown Notch and its ligand Delta to be regulated by miR-34 in human cell lines (50,51). We, therefore, monitored the expression of the Notch-Delta homologues in zebrafish. Using qRT-PCR (Figure 6), we found these targets upregulated after miRNA knockdown. They were also upregulated in the microarray experiment (data not shown). Thus, we confirmed that knockdown of maternal miR-34 results in specific dysregulation of target genes without any generalized effect on the transcriptional program of development.

***In vivo* model to validate role of zebrafish miR-34 in early neuronal development**

Zebrafish miR-34 is known to be expressed in the developing brain (52). Notch-delta pathway is also involved in early neuronal differentiation (53). To elucidate the involvement of miR-34 in neuronal development, we used a transgenic zebrafish harbouring mCherry expressed by her4.1 promoter. This transgenic line expresses mCherry protein throughout the nervous system (54). We knocked down miR-34 in these transgenic zebrafish and found a reduction in the her4.1 promoter-driven mCherry expression in the zebrafish neuronal system at 24 hpf, establishing the involvement of miR-34 in neurogenesis (Figure 7A). Bright field images of these zebrafish

also show an enlargement of the hindbrain (Figure 7B). The neuronal growth abnormalities were observed in ~35% of the surviving injected embryos with a survival rate of 60–80% post-injection. These observations suggest an important role for miR-34 in establishment of early lineage of neuronal cells and may provide clues to understand neuronal defects in an organism.

DISCUSSION

Shpiz and Kalmykova (55) have previously shown that piRNAs are inherited through the female germ line. These RNA, found in association with PIWI proteins, are often derived from repeats, expressed abundantly in germ line cells and seem to play a role in regulating transposon activity, thus imparting stability to the genome (56). Besides the maternal inheritance of piRNAs, Tang *et al.* (36) have earlier attempted to explore maternal inheritance of mouse miRNAs by studying Dicer knockout mice. Dicer knockout in developing oocytes results in infertility. Because of the diverse roles played by Dicer, it is difficult to ascribe a direct role for miRNAs in this experiment. For instance, Murchison *et al.* (37) have shown that tissue-specific knockout of Dicer in oocytes allows their normal growth but does not allow completion of meiosis because of defects in spindle organization. Although Tang *et al.* (36) identified several miRNAs that are present at high levels in single mouse oocytes, miR-34 was not reported in this list. We checked the expression level of miR-34 in the data generated during these

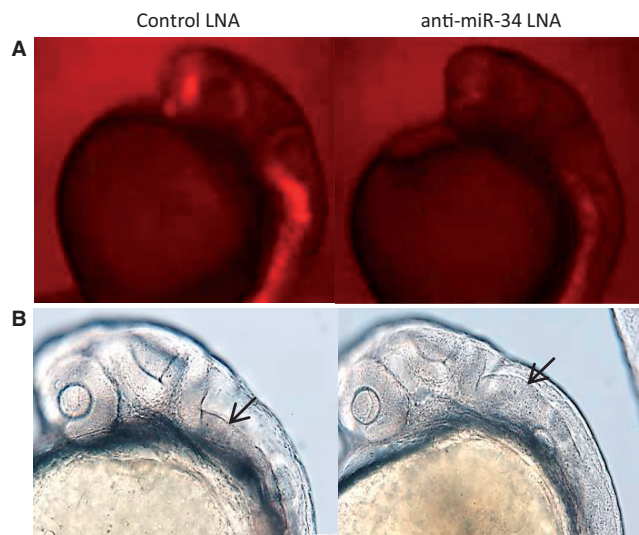


Figure 7. Knockdown of maternal miR-34 results in defects in brain development. Anti-miR-34 LNA was injected into *her4.1*-driven mCherry expressing transgenic zebrafish at one- to two-cell stage. The fish were monitored for defects in neuronal development. (A) A visible reduction in mCherry levels in the brain and throughout the neuronal system was observed. (B) Bright field image of anti-miR-34 LNA-injected fish as compared with a control LNA-injected zebrafish with defects in the development of hind brain. The swelling in the hindbrain is marked by an arrow.

experiments and found that mouse miR-34 was detected in two of the three oocytes used for single-cell miRNA profiling. However, compared with the abundant miRNAs like *let-7* and miR-30, the expression of miR-34 was lesser by couple of orders of magnitude. mRNA expression profiling has been carried out earlier in developing zebrafish embryos using microarrays and RNA-seq to study changes in transcripts during early development, especially MZT (57). However, barring the isolated case of *dre-miR-430*, there is currently no data on the expression profile of miRNAs or their role in unfertilized oocytes in zebrafish.

Knockdown of mature *dre-miR-34* resulted in swelling of the hindbrain in only ~30% of the embryos. Further, miRNA knockdown did not result in non-specific effects on transcriptional changes during development or the kinetics of maternal mRNA clearance. The kinetics of the miR-34 knockdown shows a maximum of 80% reduction, implying that inefficient knockdown was not the reason for the limited penetrance. Therefore, we believe that the low penetrance of the phenotype is likely to be because of the effect of redundant pathways that can buffer the effect of individual miRNAs. Moreover, miRNAs have been previously implicated in incomplete penetrance and variable expressivity (58,59).

miR-34 has been shown to have a critical role in the early determination of *Xenopus* body axis polarity by targeting the β -catenin UTR (60). It has also been shown that miR-34 has an important function in the reprogramming of somatic cells to form iPSCs (induced pluripotent stem cells) in a regulated manner through regulation of pluripotency genes like *Nanog*, *Sox2* and *N-myc*. The expression of p53-dependent miR-34 along

with other genes in the pathway restrains the reprogramming of somatic cells to iPSCs (61). Other important functions of miR-34 include the regulation of epithelial to mesenchymal transition in cancers and mouse neuronal stem cell differentiation (62–65).

Mammalian miR-34a is known to inhibit cell proliferation, enhance apoptosis and induce cell differentiation (66). Loss of expression of miR-34 is known to occur in a variety of cancers, including medulloblastoma, hepatocellular carcinoma colon cancer and gastric cancer. In mouse, miR-34a is implicated in neural stem cell differentiation, as its ectopic expression promotes the formation of post-mitotic neurons (63). In zebrafish, the spatial expression pattern of miR-34 shows localization to the hindbrain, whereas in the developing and adult rat brain, it shows high expression in the cerebellum (67,68). Thus, we speculate that maternally inherited miR-34 could influence the translational regulation of genes during neurogenesis in the embryo.

In summary, miR-34, a developmentally important miRNA, is inherited maternally in zebrafish and *Drosophila*. The miR-34 expression in oocytes is dependent on the maternal miRNA processing machinery. The mammalian homologue of *dme-miR-34* has targets in the cell cycle, and its aberrant expression is associated with several cancers. In the light of the results reported here, it would be interesting to explore the possibility of maternal inheritance of miR-34 in mammals, in future. Further, the role of maternally inherited miR-34 in neurogenesis is also likely to provide insights into the biological relevance of the maternal inheritance of miR-34.

ETHICAL CLEARANCE

The zebrafish work described in this study was conducted in accordance with the guidelines set by the CSIR Institute of Genomics and Integrative Biology Animal Ethical Committee Board (Proposal No. 45a).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figure 1.

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