

Reprogramming of microRNAs by adenosine-to-inosine editing and the selective elimination of edited microRNA precursors in mouse oocytes and preimplantation embryos

Jesús García-López, Juan de Dios Hourcade and Jesús del Mazo*

Department of Cellular and Molecular Biology, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain

Received December 13, 2012; Revised and Accepted March 15, 2013

ABSTRACT

Adenosine deaminases-acting-on-RNA (ADAR) proteins induce adenosine-to-inosine editing in double-stranded RNA molecules. This editing generates RNA diversity at the post-transcriptional level, and it has been implicated in the control of cell differentiation and development. The editing of microRNA (miRNA) precursors, along with Tudor-SN (*Snd1*) activity, could lead to the elimination of selected miRNAs and reprogram miRNA activity. Here, we report the dynamics of adenosine-to-inosine editing in miRNA precursors and their selected elimination during mouse preimplantation development. *Adar1p110* and *Snd1* were found to be strongly but differentially expressed in oocytes and zygotes with respect to later pre-implantation stages. When the biogenesis of *miR-151* was assessed, the majority of *miR-151* precursors was edited and subsequently eliminated during early development. Deep sequencing of this and other miRNAs confirmed that, in general, edited precursors were selectively eliminated at early post-zygotic stages. Moreover, in oocytes and throughout the zygote-to-blastocyst stages, Tudor-SN accumulated in newly discovered aggregates termed 'T bodies'. These results provide new insight into how editing and Tudor-SN-mediated elimination of miRNA precursors is regulated during early development.

INTRODUCTION

MicroRNAs (miRNAs) are endogenous, non-coding RNAs of ~22 nucleotides that regulate gene expression

by targeting messenger RNAs (mRNAs). By binding to complementary regions in mRNAs, miRNAs repress the translation or they induce the degradation of their target transcripts (1). The miRNAs are transcribed as longer RNAs by polymerase II, called primitive miRNAs (pri-miRNAs), with regions able to generate double-stranded structures and loops owing to their sequence complementarity. Pri-miRNAs are turned into pre-miRNAs by the DROSHA and DGCR8 protein complex, and once exported to the cytoplasm, these pre-miRNAs can be processed by the RNase DICER, which eliminates any single-stranded regions to generate a duplex miRNA molecule. The final production of active mature forms of single-stranded miRNAs involves the activity of a protein complex that includes members of the Argonaute family (2).

From *Caenorhabditis elegans* to humans, adenosine-to-inosine (A-to-I) editing represents a post-transcriptional modification of double-stranded RNA, including miRNA precursors (3–5). Adenosine (A) is converted to inosine (I) through specific deamination by the adenosine deaminases-acting-on-RNA (ADAR) family of enzymes. Inosine is recognized as guanosine (G) by the cell machinery, which affects the subsequent processing of edited molecules. Recent evidence indicates that A-to-I editing in miRNA precursors (pri- and pre-miRNAs) affects the RNA interference pathway (5–8), which may modulate specific miRNA–mRNA interactions, or lead to miRNA retargeting (9–11). When A-to-I-edited miRNA precursors are not recognized as substrates by DROSHA/DGCR8 or DICER, they may be degraded by the Tudor-SN nuclease that is encoded by the *Snd1* gene. Tudor-SN recognizes the inosine residues in these molecules, and it actively participates in the degradation of hyper-edited dsRNAs (12,13).

A-to-I editing is associated with cell differentiation and development. ADAR is most strongly expressed and active

*To whom correspondence should be addressed. Tel: +34 91 837 3112; Fax: +34 91 536 0432; Email: jdelmazo@cib.csic.es

in the brain and testis (14–16), and it is also crucial in embryonic erythropoiesis (17). In the mouse, three genes code for the different ADAR proteins: *Adar1*, *Adarb1* and *Adarb2* (also called *Adar1*, *Adar2* and *Adar3*, respectively). *Adar1* can produce two transcripts depending on the promoter used for their transcription, *Adar1p150* (also known as *Adar1L*) and *Adar1p110* (or *Adar1S*). Moreover, although both *Adar1* and *Adarb1* can be detected in many tissues, the expression of *Adarb2* has only been detected in the brain, and it is considered to be inactive (5). Significantly, *Adar1* mRNA is a target of microRNA-1 (*miR-1*) (18), which is in turn required for embryonic haematopoiesis and regulates the differentiation of skeletal myoblasts (19). However, *Adar1* regulates *miR-1* expression via the A-to-I editing of *pri-miR-1* in other cell types (11). This feedback between *miR-1* and *Adar1* may therefore be crucial in cell differentiation.

To understand the potential modulatory role of miRNA editing in fertilization and during pre-implantational development, the expression of all the ADAR genes was assessed by quantifying the accumulation of their transcripts between the oocyte and blastocyst stages of mouse development. The results obtained were compared with those for mouse brain and testis where A-to-I editing is very active. The expression of *miR-1* as a potential regulator of Adar transcripts was also examined, and the dynamics of miRNA biogenesis and elimination were studied by analysing the expression of *miR-151* (which is thought to be edited in the brain) (10).

The presence of A-to-I-edited miRNA precursor molecules, and of A-to-I-edited mature miRNAs, was recorded during the biogenesis of *mir-151* at different pre-implantation stages. The lowest levels of mature edited molecules were detected in the oocyte and zygote, coinciding with the maximal accumulation of *Adar1p110* and *Snd1* transcripts. This suggests that both active editing and the degradation of edited precursor RNA molecules occur during the perifertilization period. This was confirmed by the identification of other editable miRNAs expressed in oocytes and zygotes by deep sequencing. Immunolocalization studies showed that the distribution of Tudor-SN changed from a pattern of small, dispersed aggregates in the oocyte, to one of absolute dispersal between the zygotic and 8-cell stage, and finally to a pattern of large aggregates in the morula and blastocyst, herein referred to as ‘T bodies’.

MATERIALS AND METHODS

Animals

All procedures relating to the care and handling of C57BL6 and DBA/6J mice were carried out in the CIB-CSIC bioterium under specific pathogen-free, temperature (22 ± 1°C) and humidity-controlled (50–55%) conditions. All animals were housed on a 12h light/dark cycles with *ad libitum* access to food and water. Animal care and handling was carried out in accordance with the regulations of the Bioethics Committee of the *Consejo Superior de Investigaciones Científicas (CSIC)* that approved the study (permit #: PI071007) and adhering to the European Commission guidelines (directive 2010/63/EU).

Oocyte/embryo collection

Oocytes, zygotes and developing pre-implantation embryos were collected and processed as described previously (20). Groups of 10 cells or embryos were used for each reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Oligonucleotides

The oligonucleotides used for PCR were designed using ProbeFinder v.2.20 software (Roche), and they were as follows: *GGGTCTTGATCGGGGAGA* (*Adar1p110-F*); *GCTGCCAGAGAGAGGAAGTG* (*Adar1p110-R*); *GCCGGCACTATGTCTCAAG* (*Adar1p150-F*); *GCTGGAACTCCTAGGGTAAGAC* (*Adar1p150-R*); *ATCCCGCCTGTGTAAGCA* (*Adar2-F*); *GACTCGTGGTATGTGTAGGC* (*Adar2-R*); *CCACAGGATGGAGGGAATC* (*Adar3-F*); *GCACTGAAGTGGGGAGACTT* (*Adar3-R*); *TCTACATCGACTACGGCAACA* (*Snd1-F*); *GTGCTGAAGGCAGGTGTA* (*Snd1-R*), *ACAGCGAGCCATCCTGGCGTA* (*H2afz-F*); *TTCCCGATCAGCAGATTTGTGGA* (*H2afz-R*); *CGCGTCTCCTTCGAGCTGTTT* (*Ppia-F*); *TGTA AAGTCAACCACCCTGGCACAT* (*Ppia-R*); *GCATGACGTCTGCTTTGGA* (*U6-F*); *CCACAATCATTCTGCCATCA* (*U6-R*); *TGATGGAGCGCTTTCCTG* (*pri-miRNA-151-F*); *CTCGAGGAGCTCACAGTCTAGTA* (*pre-miRNA-151-F*); *GTCCTCAAGGAGCCTCAGTC* (*pri- and pre-miR-151-R*). The primers and specific Taq-Man® probes for miRNA analysis were acquired from Applied Biosystems, and the IDs for each primer are as follows: *miR-151-3p* (ID#001190); *miR-151-3p* (edited at the +3 position: SO#65632707); *miR-151-3p* (edited at the –1 and +3 positions: SO#186307388); *miR-1* (ID#002222).

Expression of ADAR and *Snd1* genes by RT-qPCR

RT reactions, followed by qPCR, were performed on whole oocyte or pre-implantation embryo lysates in the presence of NP-40 (21). After denaturation at 95°C for 5 min, RNA was retrotranscribed by adding the lysate to 10 µl of a mixture containing the following: 2.5 µM Oligo dT17, 1X First-Strand Buffer (Invitrogen), 0.01 M dithiothreitol, 2U of RNasin (RNase inhibitor: Promega), 0.5 mM of each dNTP and 200 U of superscript II (Invitrogen). The reaction was made up to a final volume of 20 µl with RNase-free water.

RNA was also isolated from testis and brain using the TRIzol® Reagent (Invitrogen), according to the manufacturer’s instructions. The RNA was resuspended in RNase-free water, the concentration was verified by absorbance (A260/280 ratio) on a NanoDrop ND-1000 spectrophotometer (NanoDrop), and the samples were stored at –80°C. Subsequently, complementary DNA (cDNA) was synthesized from 0.5 µg of total RNA as described earlier in the text, and the resulting cDNAs were amplified by real-time qPCR.

PCR was performed by adding 10 µl of 2X SYBR Green PCR supermix (Bio-Rad) to each well, along with 4 µl of the template and 0.0625 µM of each specific primer in a total reaction volume of 20 µl. PCR profiles were obtained using the iQ5 Detection System (Bio-Rad) as

follows: denaturation at 95°C for 10 min; 50 amplification cycles of 15 s at 95°C, 30 s at 61.4°C and 1 min at 72°C. At the end of each reaction, the cycle threshold (Ct) was manually set to the level that reflected the best PCR kinetics. Melting curves were then acquired and analysed.

Primer design to quantify the miRNA precursor and mature molecules

The primers used to analyse the miRNA precursor molecules were designed as described previously (22). The same reverse primer, but different forward primers, was used to amplify each pri- and pre-miRNA. To quantify the relative expression of the pri-miRNA molecules, the forward primer was designed 5' to the pre-miRNA hairpin region. However, as the pre-miRNA molecules are the result of pri-miRNA cleavage, primers designed to the stem portion simultaneously amplify both precursor molecules (pri- and pre-miRNAs). Therefore, to determine the amount of pre-miRNA, we used the equation reported previously (22): $\text{pre-miRNA} = 2^{-CT(\text{pri-miRNA} + \text{pre-miRNA})} - 2^{-CT_{\text{pri-miRNA}}}$.

Commercial stem-loop RT-primers and TaqMan® (Applied Biosystems) qPCR primers were used to amplify the mature miRNAs, *miR-151-3p* and *miR-1*. Specific RT primers for the A-to-I-edited *miR-151-3p* forms were designed in collaboration with Applied Biosystems.

RT-qPCR amplification of miRNA precursor and mature molecules

RNA from 10 oocytes or embryos was used to generate cDNAs by reverse transcription. All reactions were performed at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and they were then held at 4°C. The reactions were performed in a total volume of 15 µl, containing: NP-40 0.1%, 1X RT buffer, 0.25 mM of each dNTP, 50 U of MultiScribe™ Reverse Transcriptase (Applied Biosystems), 38 U of RNase inhibitor and the specific primer. For precursor molecules, the same primers were used to retrotranscribe the RNA isolated and to amplify the cDNAs in qPCR reactions, and they were added at a final concentration of 0.25 µM. To reverse transcribe the mature miRNA molecules, commercial stem-loop RT primers were used following the manufacturer's recommendations (Applied Biosystems).

The qPCR reactions were performed using specific TaqMan® real-time PCR primers and precursor primers in an iQ5 thermocycler Detection System (Bio-Rad). The 20 µl PCR reaction volume contained 4.5 µl RT product, 1X TaqMan® Universal PCR master mix, 1 µl of primers and the probe mix of the TaqMan® MicroRNA Assay kit. Reactions were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 10 min. The expression of mature miRNAs was determined using the $2^{-\Delta\Delta Ct}$ method, using *U6* as a reference gene.

Cloning and sequencing of the *miR-151-3p* precursor molecules

To identify A-to-I changes in the precursor sequences, PCR products from the analysis of *pri-* and

pre-miRNA-151 were cloned into pCR®2.1-TOPO vectors (Invitrogen). Random colonies were selected from each embryonic stage, and the cloned inserts were sequenced on a GS-FLX sequencer (Roche) to quantify the A to G (I) changes. We detected three types of precursor molecules: unedited, edited at one specific position (at the +3 position) and with two edited nucleotides (at the -1 and +3 positions). The editing rate was estimated after sequencing, as the proportion of the cDNA clones containing the A-G change out of the total number of clones sequenced. From the sequencing data, primers were designed in collaboration with Applied Biosystems to amplify the different unedited and edited miRNA forms.

Specificity of the probes and primers used to differentiate unedited versus edited *miR-151-3p* molecules

The RT-qPCR products were sequenced to evaluate the specificity of the primers and TaqMan® probes used to distinguish between unedited and edited *miR-151-3p* molecules, which confirmed the specificity of amplification (Supplementary Figure S2). In addition, the PCR products identified were cloned into TOPO vectors, and DNA from individual clones was extracted using High Pure Plasmid Isolation Kit (Roche). The DNA extracted was used as a template to check the primer/probe specificity, performing PCR assays in which the different DNA templates were cross-reacted with the different primers/probes to discriminate between the unedited and edited *miR-151-3p* isoforms. PCR amplification only occurred when the primers and probes corresponded to their respective template.

Quantification of the absolute number of molecules

Once the specificity of the *miR-151-3p* probes had been tested, we quantified the absolute number of molecules by qPCR (23–25). The DNA standards necessary to elaborate the calibration curves for the absolute quantification were obtained from PCR products cloned in TOPO vectors as described earlier in the text. Based on the known DNA concentrations, a standard curve with different dilutions was generated to interpolate the Ct values for qPCR amplification at each biological stage, and consequently, to derive the copy number of each mature miRNA. The initial number of molecules for standard DNAs was calculated by Avogadro's number.

Immunocytochemistry

Oocytes and pre-implantation embryos were fixed with 4% paraformaldehyde for 20 min at room temperature (r.t.). All samples were then treated with 0.2% Triton X-100 for 10 min, washed for 45 min in PBS + 5% BSA, and incubated for 1 h at r.t. with a goat polyclonal anti-Tudor-SN antiserum diluted 1:100 (sc-34753 Santa Cruz Biotechnology INC). After washing in PBS 1X, the samples were incubated for 45 min at r.t. with a donkey anti-goat immunoglobulin G-TR secondary antibody diluted 1:100 (sc-2783 Santa Cruz Biotechnology INC). After mounting with UltraCruz™ Mounting Medium (sc-24941 Santa Cruz Biotechnology INC) containing

DAPI, the samples were visualized on a TCS SP2 Confocal Laser Scanning Microscope (LEICA), and the images were recorded.

Data analysis

All expression studies involved four biological replicates, each of which provided sufficient material for four technical replicates. All the data were normalized using the delta-Ct method (26), and *H2afz* and *Ppia* were used as the reference genes to normalize the expression of the ADAR genes and *Snd1*. *U6* was used to normalize the expression of precursors and mature miRNAs.

Statistical analyses were performed using the PASW statistics 18 software (SPSS Inc., Chicago, IL). Parametric statistical analysis [(mean and standard deviation (SD))] were performed on a total of 16 measurements (four biological replicates each producing four technical replicates) for each gene studied, at each stage analysed. One-way ANOVA was performed for multiple comparisons. The results of descriptive statistics and *post hoc* Tukey's test with a 95 and 99% mean confidence interval are shown in the Supplementary Tables S3–S7.

Small RNA isolation, library construction and deep sequencing analysis

The TRIzol reagent (Invitrogen) was used to isolate total RNA from each sample, permitting the recovery of small RNAs (<200 nt). This total RNA was quantified on a Nanodrop ND-1000 apparatus (Thermo Scientific), and the total RNA used was 0.96 µg for oocytes (from 15 210 cells) or 1.6 µg for zygotes (from 15 416 cells). The cDNA libraries were prepared using the Illumina Small RNA kit (Illumina), according to the manufacturer's instructions. Briefly, small RNAs in the 18–30 nt range were purified from each library and recovered on a denaturing polyacrylamide gel electrophoresis gel. Adaptors for reverse transcription and amplification were then ligated to the 5' and 3' ends, and the modified RNAs were reverse transcribed to cDNA and PCR-amplified. After the cDNAs were purified and recovered on a denaturing polyacrylamide gel electrophoresis gel, the purity and concentration of the samples were determined using a Nanodrop ND-1000 apparatus. Sequencing was performed, and libraries were constructed using an Illumina HiSeq2000 Genome Analyzer (DNAScience).

Sequential alignments of the sequenced reads against a number of miRBase database v.16 entries were performed using CLC Genomics Workbench v.4.06 software (CLCbio). Target prediction was analysed using Target Scan algorithms (http://www.targetscan.org/mmu_50/).

RESULTS

Regulated expression of the ADAR gene family

To assess the regulation and involvement of ADAR proteins in the editing of miRNAs in oocytes, and during the different stages of pre-implantation development (from the zygote to blastocyst), the expression of the ADAR genes was quantified by RT-qPCR. As the strongest

expression of ADAR genes was detected in the brain and testis, and as most targets of A-to-I editing are found in the nervous system (27), the expression of the ADAR genes in oocytes and embryos was compared with that in these tissues. These analyses showed that unlike the brain, where all the genes of this family were expressed abundantly, *Adar1p110* was the only ADAR gene expressed at the developmental stages examined, mainly in oocytes and zygotes (Figure 1). However, the accumulation of *Adar1p110* transcripts in oocytes and zygotes was around twice that detected in the brain, and >10 times that detected in testis. To our knowledge, these are the highest levels of *Adar1p110* detected in any tissue or cell type, indicative that there is very active RNA editing by ADAR1p110 during the periferalization period.

Through translational repression followed by mRNA decay, miRNAs can regulate the accumulation of their target transcripts (28–30). Interestingly, *miR-1* is a negative regulator of both *Adar1* mRNAs (18). Hence, *Adar1* gene regulation was studied in oocytes and early embryos, and the participation of *miR-1* in the rapid decline of *Adar1p110* transcripts after fertilization was analysed. An inverse correlation was detected between *Adar1p110* and *miR-1* expression, except in the morula where both *Adar1p110* transcripts and *miR-1* were present at very low levels for reasons that are not yet clear. By contrast, the highest levels of *miR-1* were detected in 4-cell embryos, in which very little *Adar1p110* mRNA accumulated (Supplementary Figure S1). This suggests an active regulatory role for *miR-1* in the editing of miRNAs in the early embryo, mediated by the negative regulation of *Adar1* transcripts.

The biogenesis and editing dynamics of *miR-151-3p*

To determine whether the dynamics of *Adar1p110* regulation had any impact on the editing of specific miRNAs during pre-implantation development, the biogenesis and editing of *miR-151-3p*, a representative miRNA in which

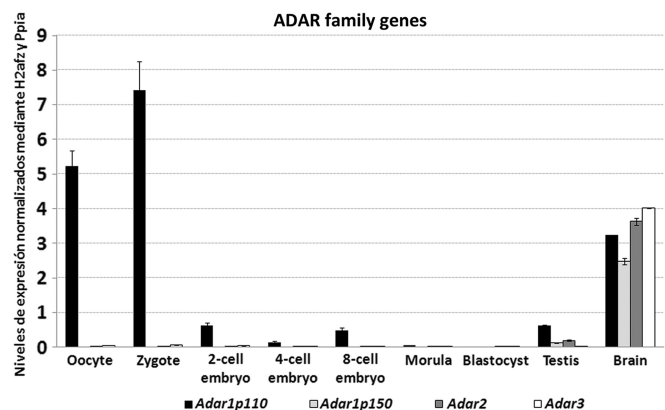


Figure 1. Relative expression of genes in the Adar family. Expression of the *Adar1p110* (*Adar1s*), *Adar1p150* (*Adar1l*), *Adar2* (*Adarb1*) and *Adar3* (*Adarb2*) genes in oocytes and during pre-implantation development. RT-qPCR data were obtained from oocytes, and all pre-implantation development stages up to the blastocyst, as well as from testis and brain. *Ppia* and *H2afz* were used as reference genes, and in all the samples, the data represent the mean of 16 replicates \pm SD.

editing has been described in nerve cells (10,31,32) and which we confirmed to be present in early embryos, was studied from the oocyte to blastocyst stages. The accumulation of the successive forms of this miRNA (i.e. pri-miRNA, pre-miRNA and the canonical non-edited mature form) was analysed by RT-qPCR, with the highest levels of *pri-miRNA-151* detected in oocytes and zygotes. However, the relatively low levels of pre- and mature forms detected at these stages were not consistent with these high levels of the pri-miRNA form (Figure 2A). An analysis of the accumulation of the precursor and mature forms showed an increase in pre-miRNA in 2-cell stage embryos, and an increase in the mature form

at the 4-cell stage (Figure 2A). These results might reflect the processing of pri-miRNA accumulated in oocytes and zygotes over successive developmental stages.

Given the predominant expression of *Adar1p110* in oocytes and zygotes, the maternally inherited pri-miRNAs might be subject to a high rate of A-to-I editing and removal such that they are consequently not processed to pre-miRNAs. By contrast, the relative increase in the rate of unedited pri-miRNA accumulation in successive developmental stages might allow processing to pre- and mature miRNAs, as detected in the 2- and 4-cell embryos. To test this hypothesis, we first cloned and sequenced 85 cDNAs from the *miR-151* precursor

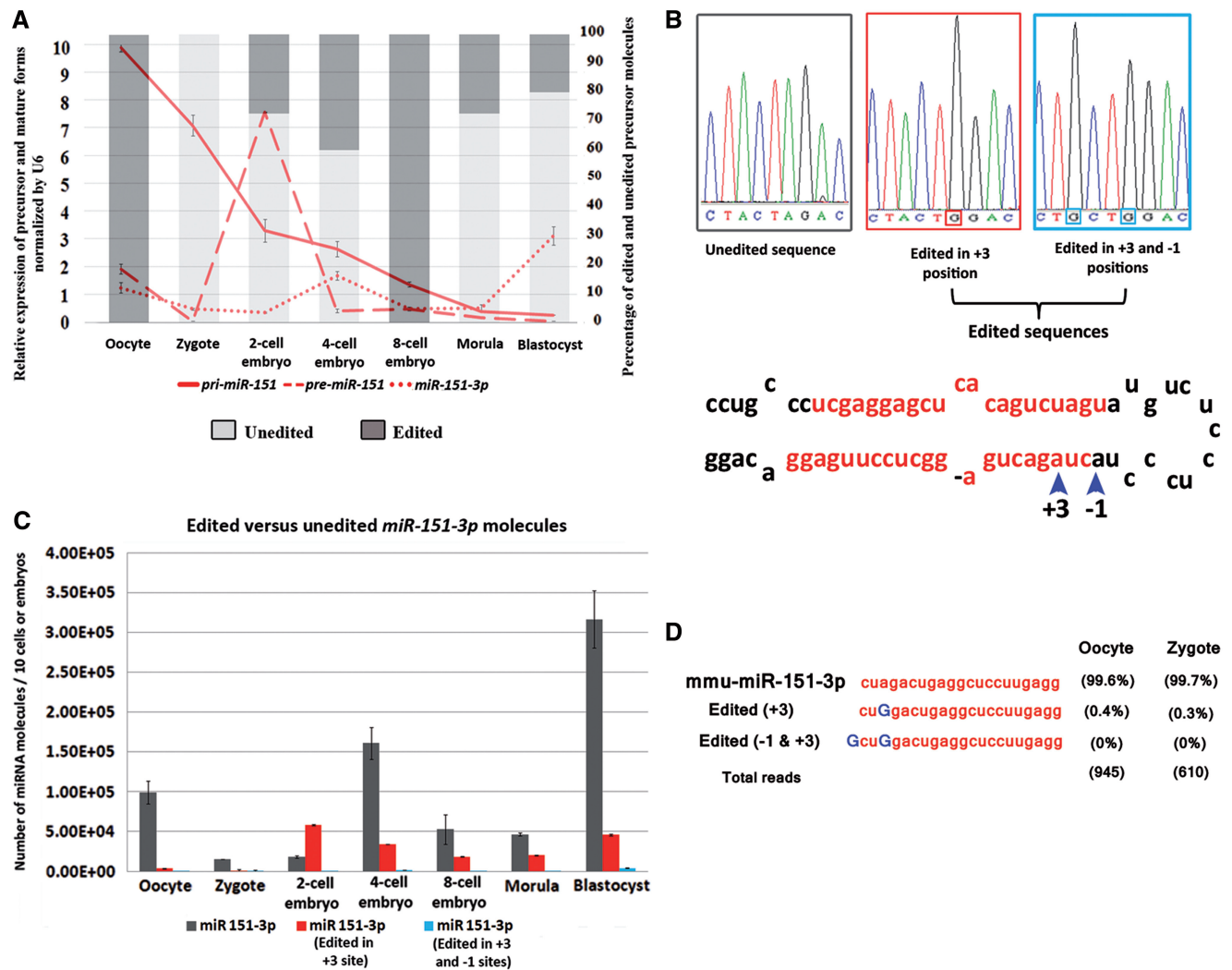


Figure 2. Analysis of *miR-151* biogenesis. (A) The expression of precursor (pri- and pre-) and mature forms of *miR-151-3p* was assessed by RT-qPCR to evaluate the biogenesis of *miR-151-3p*. The variation in the expression of the precursor forms did not correspond to that of the mature forms, suggesting the elimination of potentially edited precursor molecules at early embryonic stages. The right-hand scale represents the proportion of edited precursor molecules found by the cloning and sequencing of 85 cDNAs from *miR-151* precursors. (B) The dynamics of A-to-I editing in the precursor molecules of *miR-151* was analysed from the oocyte to blastocyst stage of development. The edited sites found in *miR-151* precursor molecules, which were detected as A to G changes in the DNA sequencing chromatogram. The edited sites are located at position +3 within the sequence of mature *miR-151-3p* and at position -1 outside the mature miRNA sequence. (C) Comparative analysis of *miR-151-3p* expression and the absolute quantification of mature unedited and edited *miR-151-3p* molecules. Grey bars represent the unedited mature *miR-151-3p* expression, whereas the red and cyan bars represent the 'one edition' site (position +3) and the 'two edition' sites (positions +3 and -1) of *miR-151-3p* molecules, respectively. (D) Editing rates detected in mature *miR-151-3p* molecules from oocytes and zygotes using deep sequencing approaches.

corroborated those of the relative distribution during development for the unedited form of *miR-151-3p* (Figure 2C), providing an estimation of the molecular dynamics of *miR-151-3p* biogenesis and editing. The detailed analysis of the three forms at each pre-implantation stage indicated that only a small proportion of *miR-151-3p* molecules were edited at the +3 position, and very few molecules were edited at the two positions, except at the 2-cell stage. In these embryos, there was a higher proportion of *miR-151-3p* with one edition at the +3 position than of the unedited miRNA form (Figure 2C). This increase in the edited form was coincident with the decline seen in pri-miRNA and the increase seen in pre-miRNA, suggesting that the abundance of pre-miRNA forms circumvented the potential elimination of the edited precursors, which become edited mature miRNAs.

From the deep sequencing of small RNAs in oocytes and zygotes (>50 million reads for each cell type: manuscript in preparation), we extracted data for *miR-151-3p* and other editable miRNAs. The results confirmed the low levels of A-to-I edited *miR-151-3p* compared with the unedited molecules (Figure 2D), corroborating the RT-qPCR analysis presented here. Additionally, the editing rate of other miRNAs previously shown to be edited in the brain was examined in oocytes and zygotes where *Adar1p110* is expressed most strongly (Figure 1). From 33 mouse homologues of human miRNAs (32), another 7 miRNAs were expressed in oocytes or zygotes and were examined (>100 reads): *miR-376a*, *let7-g*, *miR-27a*, *miR-411*, *miR-379*, *miR-376b-3p* and *miR-376b-5p* (Figure 3). Among all these miRNAs, few or no mature edited forms were expressed in oocytes and zygotes as opposed to the unedited molecules (Figure 3). Globally, these results strongly suggest a reprogramming of A-to-I edition and the degradation of these miRNA precursors after fertilization.

The finding of edited nucleotides inside and outside the seed regions suggests that edition may cause the retargeting of some miRNAs (31,34). To test this hypothesis, we examined the retargeting that might occur after *miR-411* and *miR-376b-3p* editing in oocytes and zygotes, where these miRNAs showing the highest rate of editing in the seed region (Figure 3D and F). The use of TargetScan prediction software identified 48 genes as putative targets for canonical *miR-411* and 112 genes for *miR-376b-3p*. By contrast, we found 32 genes that could be the targets of edited *miR-411* and 212 genes targeted by the edited *miR-376b-3p* (Supplementary Tables S1 and S2). Significantly, there were no common target genes for unedited and edited *miR-411*, and just three of genes were identified in both the unedited and edited *miR-376b-3p* target lists. This indicates that editing mechanisms may also generate alternative seed regions for some miRNAs, and that these mechanisms would influence the gene regulation mediated by miRNAs, increasing the number of targets potentially subjected to such regulation.

Tudor-SN and the degradation of miRNA precursors

As editing by ADAR could reflect the rate of degradation of edited double-stranded RNAs, the expression of *Snd1*,

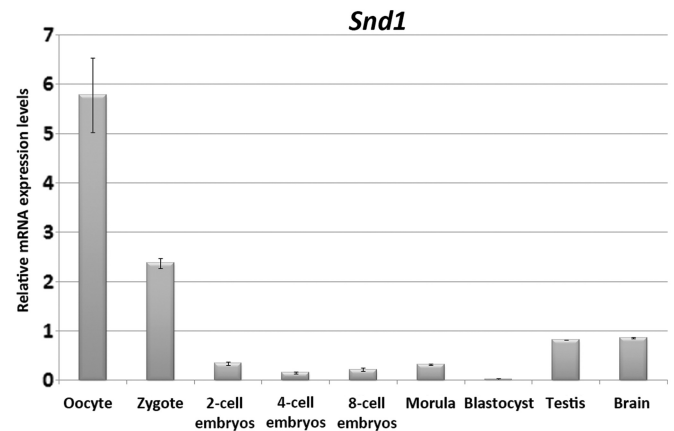


Figure 4. *Snd1* expression levels. Relative expression of the gene encoding the Tudor-SN protein obtained by RT-qPCR analysis using *Ppia* and *H2afz* as reference genes to normalize the data. The results are the mean of 16 replicates \pm SD.

the gene encoding the protein Tudor-SN, was assessed. Through the recognition of inosine residues, Tudor-SN is involved in the degradation of hyperedited RNAs, including miRNAs, (12). The *Snd1* gene was most strongly expressed in oocytes and zygotes, ~6- and 2-fold the expression detected in brain or testis (Figure 4). These results were consistent with the co-regulation by hyperediting and the degradation of tagged molecules as a post-transcriptional mechanism to modulate miRNA precursors, mainly in the oocyte-to-zygote transition.

The cellular distribution of Tudor-SN was examined by immunofluorescence in oocytes and embryos up to the blastocyst stage. Tudor-SN immunostaining showed a speckled pattern in the nucleus and cytoplasm of oocytes, zygotes and embryos up to the 8-cell stage, as well as accumulating in small granules in the cytoplasm of oocytes and the early zygote. In the morula and blastocyst stages, large aggregates of Tudor-SN were observed (Figure 5), which we describe here for the first time, and we refer to as ‘T-bodies’ (from Tudor-SN-bodies). The functional role of these changes in the distribution and conformation of T-bodies is unknown, and nor is it clear whether they might appear during the differentiation or development of other cell types. However, the role of Tudor-SN suggests that T-bodies could be foci for the accumulation and degradation of hyperedited molecules.

DISCUSSION

The role of ADAR proteins in the editing of mRNAs has been mainly studied in the nervous system (35–38). More recently, ADAR-mediated modulation of A-to-I editing in miRNAs generated new perspectives on gene regulation during development and differentiation (5,39–42), even during the embryonic period (34). However, to our knowledge, this is the first time such modulation has been analysed in fertilization and pre-implantation development.

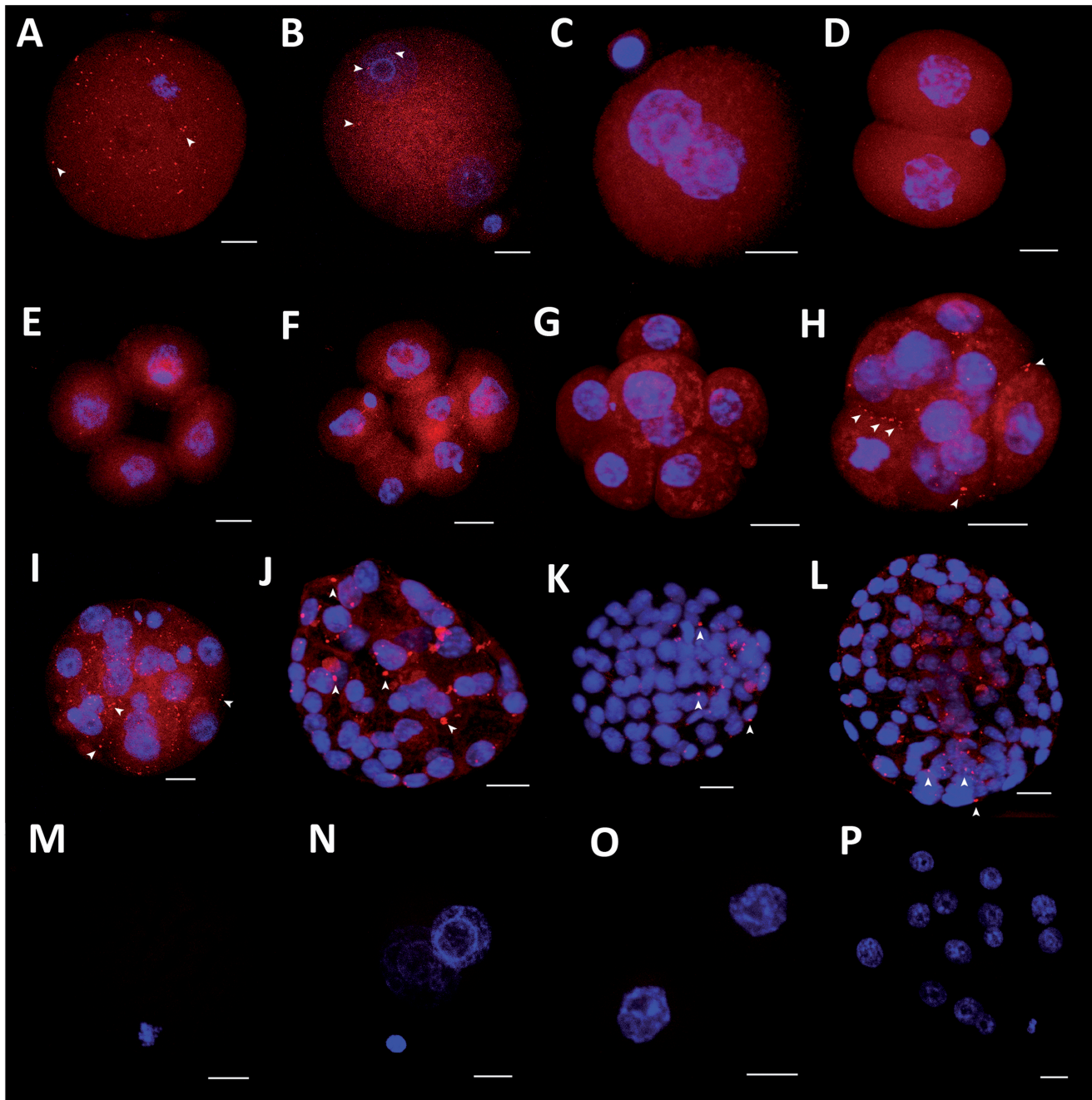


Figure 5. Immunolocalization of Tudor-SN (red) during pre-implantation development. The white arrowheads indicate the presence of 'T-bodies'. All nuclei (blue) were counterstained by DAPI (4,6-diamidino-2-phenylindole). Pre-implantation stages analysed: (A) Oocyte; (B) early zygote; (C) syngamy zygote; (D) 2-cell embryo; (E) 4-cell embryo; (F-G) 6 to 9-cell embryo; (H-I) early and late morula; (J-L) blastocyst; (M-P) negative control. Scale bars represent 20 μ m.

Adar1p110 and *Snd1* are strongly expressed by oocytes and zygotes. It was previously suggested that miRNA activity is largely suppressed in the early stages of pre-implantation development (43). Therefore, it is not strange that mechanisms such as A-to-I editing followed by the elimination of hyperedited miRNA precursors should be upregulated during the periferilization period.

ADAR1 has been demonstrated to be essential during embryogenesis (44). Although *Adar1p150* is induced by interferon, and this protein is present in both the

nucleus and cytoplasm, *Adar1p110* is constitutively expressed, and this protein is found in the nucleus (45–47). We have detected miRNA editing in conjunction with strong *Adar1p110* expression in oocytes and zygotes but not of that of other ADAR genes, suggesting that this ADAR isoform seems to be sufficient for the editing that occurs in the pre-implantation stages examined.

The biogenesis and function of miRNAs can be altered by A-to-I editing. However, *Adar* transcripts are themselves the targets of several miRNAs, one of

which is *miR-1*. The expression of *Adar1* is downregulated by this miRNA (18), which might possibly play a key role in the development of the embryonic heart (19). In the present work, increased *miR-1* levels were observed in embryos after the 2-cell stage, coinciding with a fall in *Adar* transcripts. These results corroborate the active regulation of ADAR by miR-1, even when only a single isoform of ADAR is expressed, as observed during pre-implantation development.

It has been reported that disrupting both *Adar1* isoforms causes embryonic lethality in mice at about 11.5 days post coitum (44,48). Thus, although the data suggest a minor role for ADAR proteins in pre-implantation development, they appear to fulfill an essential role during the development of the haematopoietic system (44,48). Here, RT-qPCR studies showed *Adar1p110* to be strongly expressed in oocytes and zygotes, diminishing at successive pre-implantation stages. This suggests that precursor editing may preferentially occur in the oocyte and zygote. ADAR1^{-/-} mice could circumvent the absence in ADAR during the very early stages of pre-implantation development owing to the maternal provision of *Adar1p110* in oocytes from the ADAR1^{+/-} heterozygotic dams used to generate the null mutant offspring (44).

Although ADAR proteins mark the miRNA precursor molecules with inosine, the degradation of hyperedited precursors, as for other double-stranded RNAs, is carried out by Tudor-SN (encoded by *Snd1*) (12). Like *Adar1p110*, the highest level of *Snd1* expression was detected in the oocyte and zygote, although immunocytochemical analysis revealed Tudor-SN protein to be present at all pre-implantation stages. The high maternal and zygotic expression, coupled to the stability of Tudor-SN, might explain the distinct distribution of the mRNA transcripts and protein. Other proteins behave similarly during pre-implantation development. For example, *Nalp5* transcripts accumulate strongly in oocytes; yet, they diminish over successive stages (49), whereas the corresponding protein is detected at all stages. Nevertheless, the presence of discrete granules of Tudor-SN in oocytes and zygotes, and later in morulae and blastocysts, might reflect different activities of the protein.

If these T-bodies represent the cellular foci where edited precursor miRNA molecules are eliminated, there may be two important moments during mouse pre-implantation development when edited RNAs are erased, the first during the transition from the oocyte to zygote, and the second in the morula and blastocyst stages. It has been postulated that Tudor-SN may degrade RNA in association with other components of the cell machinery, the products being grouped in discrete foci known as P-bodies (50). However, Tudor-SN has not been identified in P-bodies (51), and we were unable to find Tudor-SN in such structures [identified with an antibody against DCP1a: (52,53)] during pre-implantation development or in cultured cells (data not shown). Consequently, the dynamic configuration and cellular localization of Tudor-SN appears to be distinct to that of other RNA degradation foci, displaying an independent configuration as 'T-bodies' in pre-implantation embryos.

The activity of ADAR1 in meiotic progression after fertilization might also be mediated by NF90/NF45 (encoded by the *Ilf3* and *Ilf2* genes, respectively). NF90 and NF45 contain a double-stranded RNA-binding motif, as do DROSHA and DGCR8 (54–56), key elements for pri-miRNA processing. NF90/NF45 are negative regulators of the first step in the transition from pri-miRNAs to pre-miRNAs (57). Consequently, the accumulation of pri-miRNAs might establish a feedback loop and facilitate the activity of ADAR. However, ADAR also regulates nuclear factors of the NF90 protein family, and ADAR1 interacts with NF45/NF90 leading to the upregulation of NF90 target genes (58). *DNA-Pk* (encoding DNA-dependent protein kinase) is in turn regulated by NF90/NF45 (59,60). This kinase is involved in double-strand break repair, which represents a key check point in meiotic progression. We previously reported that *NF90* (*Ilf3*) and *NF45* (*Ilf2*) are expressed strongly in mouse meiotic cells, and that the proteins they encode are localized in the nucleus of both spermatocytes and oocytes. Interestingly, NF90 and NF45 only associate with the chromatin involved in chromosome synapses, and consequently double-strand breaks, but not in the XY body of spermatocytes (61). Thus, although the role of these proteins in the editing and processing of miRNAs during oogenesis and early embryo development appears to be of great interest, extensive work will be required to fully decipher how these events are controlled.

Together, the data presented here indicate that ADAR and Tudor-SN participate in the A-to-I editing and elimination of miRNA precursors, respectively, which occurs in the progression from the oocyte to zygote, before the activation of the zygotic genome that occurs in 2 cell stage mouse embryos. The data reported here suggest that there is significant erasure of edited miRNA precursors post-fertilization such that most edited precursors do not generate mature molecules. However, some edited miRNAs, such as *miR-411*, *miR-376b-3p* (34,62,63), may acquire new targets and perhaps act in new biological pathways. Thus, the expression of *Adar1* and *Snd1* during oogenesis could be decisive, both in the formation of the oocyte and in the initial stages of differentiation after syngamy.

SUPPLEMENTARY DATA

Supplementary Data available at NAR Online: Supplementary Tables 1–7 and Supplementary Figures 1 and 2.

ACKNOWLEDGEMENTS

The authors thank M. Quesada and M. Moreno for technical assistance, and J.B. Schwartzman and M. Sefton for revision of the article. Specific RT primers for the A-I-edited mature miRNAs were a gift from Applied Biosystems.

FUNDING

European Chemical Industry Council Long-range Research Initiative (CEFIC-LRi); MEDDTL, France

[11-MRES-PNRPE-9-CVS-072]; CSIC, Spain [PIE 201020E016]. Funding for open access charge: MEDDTL, France [11-MRES-PNRPE-9-CVS-072]; CSIC, Spain [PIE 201020E016].

Conflict of interest statement. None declared.

REFERENCES

1. Chekulaeva, M. and Filipowicz, W. (2009) Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr. Opin. Cell Biol.*, **21**, 452–460.
2. Perron, M.P. and Provost, P. (2009) Protein components of the microRNA pathway and human diseases. *Methods Mol. Biol.*, **487**, 369–385.
3. Bass, B.L. (2002) RNA editing by adenosine deaminases that act on RNA. *Annu. Rev. Biochem.*, **71**, 817–846.
4. Keegan, L.P., Leroy, A., Sproul, D. and O'Connell, M.A. (2004) Adenosine deaminases acting on RNA (ADARs): RNA-editing enzymes. *Genome Biol.*, **5**, 209.
5. Nishikura, K. (2010) Functions and regulation of RNA editing by ADAR deaminases. *Annu. Rev. Biochem.*, **79**, 321–349.
6. Das, A.K. and Carmichael, G.G. (2007) ADAR editing wobbles the microRNA world. *ACS Chem. Biol.*, **2**, 217–220.
7. Faller, M. and Guo, F. (2008) MicroRNA biogenesis: there's more than one way to skin a cat. *Biochim. Biophys. Acta*, **1779**, 663–667.
8. Nishikura, K. (2006) Editor meets silencer: crosstalk between RNA editing and RNA interference. *Nat. Rev. Mol. Cell Biol.*, **7**, 919–931.
9. Gott, J.M. (2003) Expanding genome capacity via RNA editing. *C. R. Biol.*, **326**, 901–908.
10. Kawahara, Y., Zinshteyn, B., Chendrimada, T.P., Shiekhattar, R. and Nishikura, K. (2007) RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. *EMBO Rep.*, **8**, 763–769.
11. Yang, W., Chendrimada, T.P., Wang, Q., Higuchi, M., Seeburg, P.H., Shiekhattar, R. and Nishikura, K. (2006) Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat. Struct. Mol. Biol.*, **13**, 13–21.
12. Scadden, A.D. (2005) The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. *Nat. Struct. Mol. Biol.*, **12**, 489–496.
13. Scadden, A.D. and O'Connell, M.A. (2005) Cleavage of dsRNAs hyper-edited by ADARs occurs at preferred editing sites. *Nucleic Acids Res.*, **33**, 5954–5964.
14. Hogg, M., Paro, S., Keegan, L.P. and O'Connell, M.A. (2011) RNA editing by mammalian ADARs. *Adv. Genet.*, **73**, 87–120.
15. Wahlstedt, H., Daniel, C., Enstero, M. and Ohman, M. (2009) Large-scale mRNA sequencing determines global regulation of RNA editing during brain development. *Genome Res.*, **19**, 978–986.
16. Alon, S., Mor, E., Vigneault, F., Church, G.M., Locatelli, F., Galeano, F., Gallo, A., Shomron, N. and Eisenberg, E. (2012) Systematic identification of edited microRNAs in the human brain. *Genome Res.*, **22**, 1533–1540.
17. Wang, Q., Khillan, J., Gadue, P. and Nishikura, K. (2000) Requirement of the RNA editing deaminase ADAR1 gene for embryonic erythropoiesis. *Science*, **290**, 1765–1768.
18. Lim, L.P., Lau, N.C., Garrett-Engle, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S. and Johnson, J.M. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, **433**, 769–773.
19. Srivastava, D. (2006) Making or breaking the heart: from lineage determination to morphogenesis. *Cell*, **126**, 1037–1048.
20. Garcia-Lopez, J. and del Mazo, J. (2012) Expression dynamics of microRNA biogenesis during preimplantation mouse development. *Biochim. Biophys. Acta*, **1819**, 847–854.
21. Bonilla, E. and del Mazo, J. (2003) Deregulation of gene expression in fetal oocytes exposed to doxorubicin. *Biochem. Pharmacol.*, **65**, 1701–1707.
22. Schmittgen, T.D., Jiang, J., Liu, Q. and Yang, L. (2004) A high-throughput method to monitor the expression of microRNA precursors. *Nucleic Acids Res.*, **32**, e43.
23. Bustin, S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.*, **25**, 169–193.
24. Rutledge, R.G. and Cote, C. (2003) Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Res.*, **31**, e93.
25. O'Callaghan, N., Dhillon, V., Thomas, P. and Fenech, M. (2008) A quantitative real-time PCR method for absolute telomere length. *Biotechniques*, **44**, 807–809.
26. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402–408.
27. Tan, B.Z., Huang, H., Lam, R. and Soong, T.W. (2009) Dynamic regulation of RNA editing of ion channels and receptors in the mammalian nervous system. *Mol. Brain*, **2**, 13.
28. Bethune, J., Artus-Revel, C.G. and Filipowicz, W. (2012) Kinetic analysis reveals successive steps leading to miRNA-mediated silencing in mammalian cells. *EMBO Rep.*, **13**, 716–723.
29. Bazzini, A.A., Lee, M.T. and Giraldez, A.J. (2012) Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. *Science*, **336**, 233–237.
30. Djuranovic, S., Nahvi, A. and Green, R. (2012) miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. *Science*, **336**, 237–240.
31. Kawahara, Y., Zinshteyn, B., Sethupathy, P., Iizasa, H., Hatzigeorgiou, A.G. and Nishikura, K. (2007) Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. *Science*, **315**, 1137–1140.
32. Kawahara, Y., Megraw, M., Kreider, E., Iizasa, H., Valente, L., Hatzigeorgiou, A.G. and Nishikura, K. (2008) Frequency and fate of microRNA editing in human brain. *Nucleic Acids Res.*, **36**, 5270–5280.
33. Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R. et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.*, **33**, e179.
34. Vesely, C., Tauber, S., Sedlazeck, F.J., von Haeseler, A. and Jantsch, M.F. (2012) Adenosine deaminases that act on RNA induce reproducible changes in abundance and sequence of embryonic miRNAs. *Genome Res.*, **22**, 1468–1476.
35. Brusa, R., Zimmermann, F., Koh, D.S., Feldmeyer, D., Gass, P., Seeburg, P.H. and Sprengel, R. (1995) Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. *Science*, **270**, 1677–1680.
36. Burns, C.M., Chu, H., Rueter, S.M., Hutchinson, L.K., Canton, H., Sanders-Bush, E. and Emeson, R.B. (1997) Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature*, **387**, 303–308.
37. Hoopengardner, B., Bhalla, T., Staber, C. and Reenan, R. (2003) Nervous system targets of RNA editing identified by comparative genomics. *Science*, **301**, 832–836.
38. Ohlson, J., Pedersen, J.S., Haussler, D. and Ohman, M. (2007) Editing modifies the GABA(A) receptor subunit alpha3. *RNA*, **13**, 698–703.
39. Wu, D., Lamm, A.T. and Fire, A.Z. (2011) Competition between ADAR and RNAi pathways for an extensive class of RNA targets. *Nat. Struct. Mol. Biol.*, **18**, 1094–1101.
40. Wulff, B.E., Sakurai, M. and Nishikura, K. (2011) Elucidating the inosinome: global approaches to adenosine-to-inosine RNA editing. *Nat. Rev. Genet.*, **12**, 81–85.
41. Zinshteyn, B. and Nishikura, K. (2009) Adenosine-to-inosine RNA editing. *Wiley Interdiscip. Rev. Syst. Biol. Med.*, **1**, 202–209.
42. Galeano, F., Tomaselli, S., Locatelli, F. and Gallo, A. (2012) A-to-I RNA editing: the “ADAR” side of human cancer. *Semin. Cell Dev. Biol.*, **23**, 244–250.
43. Ma, J., Flemer, M., Stein, P., Berninger, P., Malik, R., Zavolan, M., Svoboda, P. and Schultz, R.M. (2010) MicroRNA activity is suppressed in mouse oocytes. *Curr. Biol.*, **20**, 265–270.
44. Wang, Q., Miyakoda, M., Yang, W., Khillan, J., Stachura, D.L., Weiss, M.J. and Nishikura, K. (2004) Stress-induced apoptosis

- associated with null mutation of ADAR1 RNA editing deaminase gene. *J. Biol. Chem.*, **279**, 4952–4961.
45. Patterson, J.B. and Samuel, C.E. (1995) Expression and regulation by interferon of a double-stranded-RNA-specific adenosine deaminase from human cells: evidence for two forms of the deaminase. *Mol. Cell Biol.*, **15**, 5376–5388.
 46. Patterson, J.B., Thomis, D.C., Hans, S.L. and Samuel, C.E. (1995) Mechanism of interferon action: double-stranded RNA-specific adenosine deaminase from human cells is inducible by alpha and gamma interferons. *Virology*, **210**, 508–511.
 47. George, C.X., Gan, Z., Liu, Y. and Samuel, C.E. (2011) Adenosine deaminases acting on RNA, RNA editing, and interferon action. *J. Interferon Cytokine Res.*, **31**, 99–117.
 48. Hartner, J.C., Schmittwolf, C., Kispert, A., Muller, A.M., Higuchi, M. and Seeburg, P.H. (2004) Liver disintegration in the mouse embryo caused by deficiency in the RNA-editing enzyme ADAR1. *J. Biol. Chem.*, **279**, 4894–4902.
 49. Penner, S., Perreau, C., Uzbekova, S., Thelie, A., Delaleu, B., Mermillod, P. and Dalbès-Tran, R. (2006) MATER protein expression and intracellular localization throughout folliculogenesis and preimplantation embryo development in the bovine. *BMC Dev. Biol.*, **6**, 26.
 50. Liu, J., Valencia-Sanchez, M.A., Hannon, G.J. and Parker, R. (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat. Cell Biol.*, **7**, 719–723.
 51. Weissbach, R. and Scadden, A.D. (2012) Tudor-SN and ADAR1 are components of cytoplasmic stress granules. *RNA*, **18**, 462–471.
 52. Ingelfinger, D., Arndt-Jovin, D.J., Luhrmann, R. and Achsel, T. (2002) The human LSM1-7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrn1 in distinct cytoplasmic foci. *RNA*, **8**, 1489–1501.
 53. Sheth, U. and Parker, R. (2003) Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science*, **300**, 805–808.
 54. Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. and Shiekhattar, R. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature*, **432**, 235–240.
 55. Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H. and Kim, V.N. (2004) The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev.*, **18**, 3016–3027.
 56. Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F. and Hannon, G.J. (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature*, **432**, 231–235.
 57. Sakamoto, S., Aoki, K., Higuchi, T., Todaka, H., Morisawa, K., Tamaki, N., Hatano, E., Fukushima, A., Taniguchi, T. and Agata, Y. (2009) The NF90-NF45 complex functions as a negative regulator in the microRNA processing pathway. *Mol. Cell Biol.*, **29**, 3754–3769.
 58. Nie, Y., Ding, L., Kao, P.N., Braun, R. and Yang, J.H. (2005) ADAR1 interacts with NF90 through double-stranded RNA and regulates NF90-mediated gene expression independently of RNA editing. *Mol. Cell Biol.*, **25**, 6956–6963.
 59. Parker, L.M., Fierro-Monti, I. and Mathews, M.B. (2001) Nuclear factor 90 is a substrate and regulator of the eukaryotic initiation factor 2 kinase double-stranded RNA-activated protein kinase. *J. Biol. Chem.*, **276**, 32522–32530.
 60. Shamanna, R.A., Hoque, M., Lewis-Antes, A., Azzam, E.I., Lagunoff, D., Pe'ery, T. and Mathews, M.B. (2011) The NF90/NF45 complex participates in DNA break repair via nonhomologous end joining. *Mol. Cell Biol.*, **31**, 4832–4843.
 61. Lopez-Fernandez, L.A., Parraga, M. and del Mazo, J. (2002) Ilf2 is regulated during meiosis and associated to transcriptionally active chromatin. *Mech. Dev.*, **111**, 153–157.
 62. Borchert, G.M., Gilmore, B.L., Spengler, R.M., Xing, Y., Lanier, W., Bhattacharya, D. and Davidson, B.L. (2009) Adenosine deamination in human transcripts generates novel microRNA binding sites. *Hum. Mol. Genet.*, **18**, 4801–4807.
 63. Blow, M.J., Grocock, R.J., van Dongen, S., Enright, A.J., Dicks, E., Futreal, P.A., Wooster, R. and Stratton, M.R. (2006) RNA editing of human microRNAs. *Genome Biol.*, **7**, R27.