A Novel miRNA Processing Pathway Independent of Dicer Requires Argonaute2 Catalytic Activity

Daniel Cifuentes,1 Huiying Xue,2 David W. Taylor,2 Heather Patnode,2 Yuichiro Mishima,1,2 Sihem Cheloufi,3,5 Enbo Ma,6 Shrikant Mane,7 Gregory J. Hannon,4 Nathan D. Lawson,8 Scot A. Wolfe,9,10 Antonio J. Giraldez1,10

Dicer is a central enzyme in microRNA (miRNA) processing. We identified a Dicer-independent miRNA biogenesis pathway that uses Argonaute2 (Ago2) slicer catalytic activity. In contrast to other miRNAs, miR-451 levels were refractory to dicer loss of function but were reduced in MZ ago2 (maternal-zygotic) mutants. We found that pre-miR-451 processing requires Ago2 catalytic activity in vivo. MZ ago2 mutants showed delayed erythropoiesis that could be rescued by wild-type Ago2 or miR-451-duplex but not by catalytically dead Ago2. Changing the secondary structure of Dicer-dependent miRNAs to mimic that of pre-miR-451 restored miRNA function and rescued developmental defects in MZ dicer mutants, indicating that the pre-miRNA secondary structure determines the processing pathway in vivo. We propose that Ago2-mediated cleavage of pre-miRNAs, followed by uridylation and trimming, generates functional miRNAs independently of Dicer.

MicroRNAs (miRNAs) are ~22-nucleotide (nt) small RNAs that regulate deadenylation, translation, and decay of their target mRNAs (1, 2). In animals, most miRNAs are processed from a primary transcript (termed pri-miRNA) by two ribonuclease III (RNase III) enzymes, Drosha and Dicer. Recent studies have identified several miRNA classes that bypass Drosha-mediated processing, namely miRtrons, tRNAZ, and small nucleolar RNA (snoRNA) (3–6). In contrast to Drosha, Dicer has been viewed as a central processing enzyme in the maturation of small RNAs (2). But are there functional miRNAs that bypass Dicer? To identify pathways that might process miRNAs in a Dicer-independent manner, we sequenced small RNAs (19 to 36 nt) in wild-type and maternal-zygotic dicer mutants (MZdicer) (7). We analyzed 48-hour-old embryos in two wild-type replicates and two dicer mutant alleles (8), dicerhu715 and dicerhu896 (fig. S1). Of the ~2 million reads per sample, 69 to 82% mapped to known 5′- or 3′-derived miRNAs in the wild type, whereas 4 to 9% mapped to miRNAs in the MZdicer mutants

Fig. 1. MicroRNA analysis in wild type (wt) and in MZdicer and MZago2 mutants. (A and B) Normalized reads from wild type versus MZdicer (A) or MZago2 (B) libraries for all annotated zebrafish miRNAs. Some miRNAs are shown as a reference for enhanced and reduced miRNAs (solid circles); miR-144-5′ (green) and miR-451-5′ (red) are expressed in the same pri-miRNA. (C) Scheme of miR-144/miR-451 genomic loci and predicted secondary structure of both human and zebrafish pre-miR-451 (mature miRNA in red). (D) Total number of reads that match miR-451 in wild type, MZdicer, and MZago2. Nontemplated uridines are shown in red. (E) Domain organization of Ago2. The 90-nt deletion (Δ90) results in a predicted truncated protein lacking two of three catalytic residues. Amino acid positions are based on the mammalian Ago2. (F) Northern blot of embryos to detect slicer cleavage of an injected GFP target mRNA with three complementary targets to miR-1 (3xPT-miR-1) in the presence (+) or absence (−) of miR-1 (7). Slicer activity is indicated by the higher-mobility product (fig. S1) (18).
Fig. 2. Ago2 binds and processes pre-miR-451. (A) Immunoprecipitation of FLAG-mAgo2 in wild-type and mutant embryos injected with pre-miR-451 followed by Northern blot analysis to detect bound miR-451. Input (I), supernatant (S), and immunoprecipitate (IP) are indicated. (B) In vitro cleavage assay using hAgo2 or hDicer protein and 5'-radiolabeled pre-miR-430 or pre-miR-451. (C) Ago2 processing reactions were treated with (+) or without (−) RNase I to assess protection of the processed hairpin by Ago2. (D to F) Northern blot analyses to detect mature miR-451 after injection with pre-miR-451 (+) (D) and (E) or endogenous miR-451 and miR-430 (F). Injection of wild-type mAgo2 but not a catalytically dead mAgo2D669A rescues pre-miR-451 processing in vivo (E). The processing of miR-451mm10-11 is strongly reduced. Endogenous pre-miR-451 at 48 hpf is processed in wild type and MZdicer but not in MZago2 mutants. Diagrams for predicted hairpins, cleavage intermediates, mature miR-451 (red), miR-430 (green), and miRNA* (blue) are shown. P32* indicates that injected hairpins were radiolabeled (18).

Fig. 3. MZago2 mutants show reduced erythropoiesis. (A) Expression of hemoglobin (brown) visualized by the oxidation of o-dianisidine (o-das) at 48 hpf in the ducts of Cuvier. Hemoglobinized cells accumulate in wild type but are reduced in MZago2 mutants (group II (mild) and group III (severe) reduction of o-das-positive cells). (B) Percentage of embryos with hemoglobinized cells in MZago2 mutants (n = 61) compared to wild-type embryos (n = 200), showing strongly reduced (group III; light gray) and partially reduced (group II; gray) numbers of o-das (+) cells (χ² test; P < 0.001). (C) Whole-mount in situ hybridization of ago2 expression at 24 hpf. (D) May-Grünwald/Giemsa stain of erythrocytes from wild-type, MZago2 mutants, and MZago2 injected at one-cell stage with various RNAs as shown (+). Erythrocytes are representative of the mean for each group. (E) Scatterplot of the nuclear cytoplasmic ratio (N:C) for each genotype in (D) as a readout of erythrocyte maturation (17). Distributions of the N:C ratios in wild-type compared to MZago2 differed significantly (Wilcoxon rank-sum test after Bonferroni correction; P < 10⁻¹⁵). Erythrocyte maturation is rescued by miR-451-duplex (MZago2 and MZago2+miR-451, P < 10⁻¹⁵) and wild-type mAgo2 (MZago2 and MZago2+mAgo2, P < 10⁻¹⁵) but not catalytically dead mAgo2D669A (MZago2 and MZago2+mAgo2D669A, P > 0.1).
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To determine whether Ago2 participates in miRNA maturation, we generated a deletion in the Piwi domain of the ago2 gene (ago2<sup>ΔΔPiwi</sup>) with the use of zinc finger nucleases (13–15) (Fig. 1E and fig. S1). Because argonaute genes are maternally expressed (fig. S6), we generated maternal-zygotic ago2 mutants (MZago2). Indeed, slicer cleavage of an miRNA with perfectly complementary targets to miR-1 was severely reduced in MZago2 but not Zago2 relative to wild-type embryos (Fig. 1F and fig. S1).

Recent studies suggest that Ago2 binds premiRNAs and miRNA:miRNA* duplexes (19–22), where miRNA* denotes the complementary strand. Ago2 interacted with radiolabeled synthetic pre-miR-451 in vitro (fig. S7). Coexpression of Flag-mouse-Ago2 (mAgo2) with pre-miR-451 or a pre-miR-451<sup>mm10-11</sup> (with two mismatches in the predicted slicer cleavage site) followed by Ago2 immunoprecipitation showed that Ago2 bound to both mature miR-451 and pre-miR-451<sup>mm10-11</sup> (Fig. 2A). Incubation of human Ago2 (hAgo2) with pre-miR-451 but not pre-miR-430 resulted in a sharp 30-nt band corresponding with the predicted slicer

![Image](http://science.sciencemag.org/)

**Fig. 4.** A Dicer-independent miRNA. (A) Zebrafish pre-miRNAs and duplexes as indicated, pre-miR-430<sup>ago2-hairpin</sup> is a miR-430c hairpin that has been mutated and shortened to form a 42-nt hairpin mimicking pre-miR-451 (ago2-hairpin). (B) GFP-reporter mRNA (green) was co-injected at the one-cell stage with control dsRed mRNA (red). The GFP reporter contains three complementary target sites to miR-430 in its 3′-untranslated region. (C) Northern blot to detect miR-430 in wild-type embryos injected with hairpins as indicated. α-Amanitin was co-injected to inhibit transcription of endogenous pri-miR-430. (D) Northern blot to detect 5′-radiolabeled pre-miR-430<sup>ago2-hairpin</sup> after in vitro processing by recombinant hAgo2 and hDicer. (E) In vivo assay to rescue miR-430 function in MZdicer mutants. Bright-field and fluorescent images of the dorsal view of the brain after injection of TxRed dextran in the ventricles (right) in 32-hpf embryos. Brain outline (dashed line), mid-hindbrain boundary (green asterisk), and ventricles (red, white asterisk) are shown. Morphogenesis defects are rescued by injection of a Dicer-independent pre-miR-430<sup>ago2-hairpin</sup> or a miR-430-duplex but not a Dicer-dependent pre-miR-430.
cleavage product of miR-451 (Fig. 2B). Conversely, recombinant Dicer bound both pre-miRNAs (Fig. S7) but could only process pre-miR-430 (Fig. 2B). To investigate whether Ago2 processes miR-451, we injected pre-miRNAs into one-cell-stage embryos. Synthetic and endogenous pre-miR-451 hairpins were processed into ~30-nt intermediates and a ~22- to 26-nt mature miR-451 in wild-type and MZdicer mutant but not in MZago2 mutant embryos (Fig. 2, D and F). In contrast, a canonical mature miR-430 was processed in both wild-type and MZago2 mutant embryos but not in MZdicer (Fig. 2F). On the basis of the sequencing results, we hypothesized that Ago2-processed hairpin might undergo nucleolytic trimming at the 3′ end (Fig. 1D). We observed that Ago2 protected the ~30-nt slicer-cleaved intermediate from RNase I in vitro, resulting in a ~20- to 26-nt 3′-end trimmed product (Fig. 2C), similar to the mature miRNAs observed in vivo (Fig. 2, D to F). Ago2 slicer activity depends on its catalytic triad (DDH) and the pairing between the guide and the target mRNA (23–25). Expressing wild-type but not catalytically dead (D669A) m Ago2 in MZago2 mutants rescued pre-miR-451 processing in vivo (Fig. 2E). Furthermore, a hairpin with mismatches that disrupt pairing in the predicted slicer cleavage would be bound by Ago2 (Fig. S7) but was inefficiently processed into mature miR-451 (Fig. 2E). These results indicate that Ago2 binds and cleaves pre-miR-451 in a process that requires the slicer catalytic activity and is independent of Dicer.

MZago2 mutant embryos displayed normal morphology during gastrulation, brain development, and heart development (Fig. S8). Ago2 is maternally expressed, and later in development it acquires tissue-specific expression in the brain and intermediate cell mass (ICM) (Fig. 3C and Fig. S6). The ICM corresponds to the hematopoietic precursors and responds to the hematopoietic precursors and ICM (Fig. 3C and fig. S6). The ICM corresponds to the hematopoietic precursors and is independent of Dicer. MiR-430 mutant embryos displayed normal morphogenesis during gastrulation, brain development, and heart development (Fig. S8). Ago2 is maternally expressed, and later in development it acquires tissue-specific expression in the brain and intermediate cell mass (ICM) (Fig. 3C and Fig. S6). The ICM corresponds to the hematopoietic precursors and responds to the hematopoietic precursors and ICM (Fig. 3C and fig. S6). The ICM corresponds to the hematopoietic precursors and is independent of Dicer.

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Control of Membrane Protein Topology by a Single C-Terminal Residue

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The mechanism by which multispanning helix-bundle membrane proteins are inserted into their target membrane remains unclear. In both prokaryotic and eukaryotic cells, membrane proteins are inserted cotranslationally into the lipid bilayer. Positively charged residues flanking the transmembrane helices are important topological determinants, but it is not known whether they act strictly locally, affecting only the nearest transmembrane helices, or can act globally, affecting the topology of the entire protein. Here we found that the topology of an Escherichia coli inner membrane protein with four or five transmembrane helices could be controlled by a single positively charged residue placed in different locations throughout the protein, including the very C terminus. This observation points to an unanticipated plasticity in membrane protein insertion mechanisms.

Integral α-helical membrane proteins carry out a wide range of central biological functions. They have two conspicuous structural features: hydrophobic transmembrane α helices and a strong bias in the distribution of positively charged arginine (Arg) and lysine (Lys) residues between cytoplasmic and extracytoplasmic loops, with up to three times the frequency of Arg and Lys found in the cytoplasmic loops (1). Positively charged residues exert local control over the orientation of transmembrane helices in their immediate neighborhood (2, 3), but whether they can also affect the global topology of a protein is unknown. Multispanning membrane proteins insert into their target membrane cotranslationally; therefore, positively charged residues in a more C-terminal region of the protein might be expected not to be able to influence the orientation of distant N-terminal transmembrane helices. How-

Fig. 1. (A) The dual-topology protein EmrE and the EmrE(Nin) and EmrE(Nout) constructs (22). Positively charged Arg and Lys residues are shown as black circles and the functionally important Glu14 residue (29) is shown as a white circle. (B) Growth of serial log_{10} dilutions of cells expressing the indicated constructs on a pH 7 ampicillin plate supplemented with 45 μg EtBr per ml. The normalized growth of a particular construct is calculated as the ratio of the area under its growth-dilution curve relative to that obtained for wild-type EmrE, after subtraction of the area for the empty vector control. (C) Normalized growth values for EmrE constructs discussed in the text. Error bars indicate ±1 SEM.
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No Dicer for Me

MicroRNAs (miRNAs) are small noncoding RNAs found in most eukaryotes. Most are processed from primary transcripts in the nucleus by the microprocessor enzyme complex, which includes the nuclease Drosha, with a small number being generated by the messenger RNA splicing machinery. All pre-miRNAs are then exported into the cytoplasm where they are cleaved further by a second nuclease, Dicer, into the mature, functional miRNA. Cifuentes et al. (p. 1694, published online 6 May), now show that in a Dicer mutant fish at least one miRNA, miR-451, is still formed from pre-miR-451. The processing of pre-miR-451 requires the slicing activity of another protein in the miRNA pathway, Argonaute2. The unusual secondary structure of the pre-miR-451 determines its noncanonical processing pathway, which suggests that other miRNAs might also be processed in this way.