Intertwined pathways for Argonaute-mediated microRNA biogenesis in Drosophila

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ABSTRACT

Although Dicer is essential for general microRNA (miRNA) biogenesis, vertebrate mir-451 is Dicer independent. Instead, its short pre-miRNA hairpin is ‘sliced’ by Ago2, then 3′-resected into mature miRNAs. Here, we show that Drosophila cells and animals generate functional small RNAs from mir-451-type precursors. However, their bulk maturation arrest as Ago-cleaved pre-miRNAs, which mostly associate with the RNAi effector AGO2. Routing of pre-mir-451 hairpins to the miRNA effector AGO1 was inhibited by Dicer-1 and its partner Loqs. Loss of these miRNA factors promoted association of pre-mir-451 with AGO1, which sliced them and permitted maturation into ~23–26 nt products. The difference was due to the 3′ modification of single-stranded species in AGO2 by Hen1 methyltransferase, whose depletion permitted 3′ trimming of Ago-cleaved pre-miRNAs in AGO2. Surprisingly, Nibbler, a 3′-5′ exoribonuclease that trims ‘long’ mature miRNAs in AGO1, antagonized mir-451 processing. We used an in vitro reconstitution assay to identify a soluble, EDTA-sensitive activity that resects sliced pre-miRNAs in AGO1 complexes. Finally, we use deep sequencing to show that depletion of dicer-1 increases the diversity of small RNAs in AGO1, including some candidate mir-451-like loci. Altogether, we document unexpected aspects of miRNA biogenesis and Ago sorting, and provide insights into maturation of Argonaute-cleaved miRNA substrates.

INTRODUCTION

microRNAs (miRNAs) are an abundant and diverse class of ~22 nucleotide (nt) regulatory RNAs that collectively play essential roles during development and physiology (1,2). Generally speaking, miRNAs are precisely defined small RNAs that derive from endogenous inverted repeat transcripts (3). In animals, most miRNAs are generated via a canonical mechanism involving compartmental, sequential processing (4). Primary miRNA transcripts are first cleaved near the base of the hairpin by the nuclear Drosha RNase III enzyme to generate a ~55–70 nt pre-miRNA bearing a ~2 nt 3′ overhang. The pre-miRNA 3′ overhang, which is characteristic of RNase III cleavage, is recognized by Exportin-5, which transports it to the cytoplasm. There, the hairpin is cleaved near the terminal loop by the Dicer RNase III enzyme to yield a miRNA/miRNA* (star) duplex that is loaded into an appropriate Argonaute effector protein and matured into a single-stranded regulatory complex (5).

Since the recognition of the core canonical pathway for miRNA biogenesis, several non-canonical strategies have emerged. These include a variety of Drosha-independent mechanisms, which can exploit the splicing machinery, snoRNA pathway, RNA exosome, RNase Z or the Integrator complex to define pre-miRNA substrates (6). Conversely, a Dicer-independent mechanism for miRNA biogenesis was ascribed to the conserved vertebrate locus mir-451 (7). Cleavage of its primary transcript by Drosha generates a 42 nt pre-miRNA whose duplex is too short to serve as a Dicer substrate. Instead, direct loading of pre-mir-451 into the Slicer enzyme Ago2 results in cleavage of its 3′ arm to generate a 30 nt Ago-cleaved pre-miRNA (ac-pre-miRNA), whose 3′ terminus is resected to yield mature ~23 nt miR-451 (8–11). Since short hairpins analogous to mir-451 have not yet been identified in well-studied models such as Caenorhabditis elegans and Drosophila melanogaster, the capacity for direct Ago-mediated miRNA biogenesis in invertebrates is not known.

Pathways for small RNA biogenesis diversified in arthropods by the emergence of two Dicers; vertebrate genomes encode only one. For example in Drosophila, the 3′ terminus is resected into a mature ~23 nt miRNA bearing a ~2 nt 3′ overhang. The pre-miRNA 3′ overhang, which is characteristic of RNase III cleavage, is recognized by Exportin-5, which transports it to the cytoplasm. There, the hairpin is cleaved near the terminal loop by the Dicer RNase III enzyme to yield a miRNA/miRNA* (star) duplex that is loaded into an appropriate Argonaute effector protein and matured into a single-stranded regulatory complex (5).

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Pathways for small RNA biogenesis diversified in arthropods by the emergence of two Dicers; vertebrate genomes encode only one. For example in Drosophila,
Dcr-1 is specialized for miRNA biogenesis, whereas Dcr-2 is dedicated to siRNA biogenesis (12). This is accompanied by a division in labor for the two Drosophila Ago proteins: both have Slicer activity, but AGO1 mediates miRNA function while AGO2 mediates siRNA function (13). However, dicing and Ago loading are not directly coupled. Instead, small RNA duplexes are inspected for the relative thermodynamic energy of their termini (14–16), their 5' ends are not directly coupled. Instead, small RNA duplexes are sorted to and which strand is preferentially selected as the guide strand (17–21). In general, AGO2 prefers duplexes that are highly paired, especially at positions 9–10 of the 5' end of the strand selected as the guide, and prefers 5' C, whereas AGO1 prefers centrally unpaired duplex nucleotides, is partial to 5' U, and selects its guide strand predominantly on the thermodynamically unstable duplex end (5).

The independent assessment of duplexes for Ago sorting means that certain Dcr-1 products, including specific mature miRNAs and many miRNA* strands, are preferentially sorted to AGO2 (19–22), while some Dcr-2 products can productively load AGO1 (23,24). In particular, loss of R2D2, a double-stranded RNA binding domain (dsRBD) partner of Dcr-2 that determines siRNA strand selection, or loss of AGO2 itself, both result in substantial redirection of endo-siRNA duplexes to AGO1 (25,26). A further twist is the recent finding that invertebrate and vertebrate Ago proteins are capable of loading some endogenous single-stranded RNAs, including the terminal loops of select miRNA hairpins (27,28).

In this study, we examined the capacity of Drosophila cells and animals to mature miRNA hairpins via a Slicer-mediated mechanism. We show that mir-451-class hairpins can generate functional miRNAs with regulatory activity, but the majority of products arrest at the 30 nt ac-pre-miRNA stage. This was due to preferential loading of the well-paired pre-mir-451-class hairpins into AGO2; hairpins that loaded AGO1 were successfully resected into mature miRNAs. Surprisingly, although removal of RNAi factors results in substantial re-sorting of endo-siRNAs into AGO1 (25), this condition did not enhance accumulation of miR-451 into AGO1. Instead, the maturation of miR-451 was strongly stimulated by loss of Dicer-1, likely due to loss of the competing canonical miRNA pathway. As with mature endo-siRNAs (5,29), the 3' ends of pre-mir-451 hairpins were modified in AGO2, and depletion of Hen1 permitted resection of ac-pre-miRNAs in AGO2. Notably, the miR-451 resectase does not correspond to the 3'–5' exoribonuclease Nibbler, which was recently shown to trim the 3' ends of Drosophila miRNAs in AGO1 (30,31). In fact, loss of Nibbler actually stimulated the accumulation of mature resected miR-451. We used an in vitro reconstitution assay to identify an EDTA-sensitive activity that mediates 3' trimming of ac-pre-mir-451 molecules, which sets the stage for biochemical purification of the underlying activity. Altogether, our data demonstrate the capacity of Dicer-independent miRNA biogenesis in Drosophila and provides mechanistic insight into the sorting and maturation of this atypical class of miRNA substrate.

**MATERIALS AND METHODS**

**Plasmids**

To generate pUAST-DsRed-hsa-mir-144/451, hsa-mir-144/451 genomic fragment was PCR-amplified and cloned into the NotI/XhoI sites of UAS-DsRed plasmid. To generate MT-DsRed-hsa-mir-144/451, UAS-DsRed-hsa-mir-144/451 was cut with EcoRI/XhoI to release the DsRed-hsa-mir-144/451 fragment, which was cloned into the EcoRI/XhoI sites downstream of a copper-inducible metallothionein promoter in the pRmHa-3 plasmid.

To reprogram the mir-451 moiety of mir-144/451 constructs, we designed appropriate oligonucleotides carrying mature sequences for various miRNAs and overlapping mir-451 hairpin precursor. These primers were used in combination with either FPI or TK polyA reverse primer in standard PCR reactions using hsa-mir-144/451 constructs as template. The two overlapping PCR products were purified and mixed together with EcoRI-digested mir-144/451 plasmid, and a cold fusion reaction was carried out according to the manufacturer’s manual (System Biosciences).

For luciferase sensors, we designed oligonucleotide pairs containing two antisense miRNA sequences with 5' NotI and 3' XhoI-compatible ends. These target sequences were cloned into the NotI–XhoI sites downstream of the Renilla luciferase coding region in a modified psiCHECK2 vector (32), which carries an internal firefly luciferase gene for normalization (Promega). We used the same oligonucleotide pairs for cloning into the 3' UTR of a tubulin-GFP P-element vector (33). The sensor sequences for the different miRNAs are listed in Supplementary Table S1 under ‘miRNA sensor primers’.

**Drosophila stocks**

We generated multiple transgenic insertions of UAS-DsRed-mir-144/451, or versions in which mir-451 was reprogrammed with the mature sequence of miR-19a-3p or miR-199a-3p, using standard Δ2-3 helper plasmid injections (BestGene, Inc.). For sensor assays, we also generated tub-GFP-miR-144 and tub-GFP-miR-451 sensor transgenes, and tested these in the background of spatially localized expression of UAS-DsRed-mir-144/451 driven by ptc-Gal4, which is active at the anterior–posterior compartment boundaries of imaginal discs.

To drive ubiquitous expression of miRNA genes, balanced stocks of UAS-DsRed-mir-144/451, UAS-DsRed-mir-144/451.mir-19a and UAS-DsRed-mir-144/451.mir-199a were crossed to the ubiquitously active da-Gal4 driver. We extracted total RNA from DsRed-expressing third instar larvae of F1 progeny by TRIzol reagent and analyzed them by Northern blotting. To analyze the expression of mir-144/451 in mutant backgrounds, we introduced UAS-DsRed-mir-144/451 and da-Gal4 into the following mutant backgrounds. We used the
56 mM CuSO₄ was added to the expression in the knockdown experiments, plated for 4 days and split into another six-well plate and Drosophila cells were grown at 25°C. ago2 w; UAS-DsRed-144,451+/+; da-Gal4, ago2[454]/dicer-2 w; dcr-2[L811X] ; UAS-DsRed-144,451/da-Gal4 dicer-1 w; UAS-DsRed-144,451+/+; da-Gal4, dcr-1[Q1147X]/drosha w; drosha[21K11]/Df(2 R)exel1055 ; UAS-DsRed-S2R+/ cells were soaked with 15% tissue culture Drosophila cells were stably transformed with Flag-HA-AGO2 were previously described (21). To generate stable cells that express mir-144/451, S2R+ cells were co-transfected with MT-DsRed-mir-144/451 and pcOHygro (10:1) with Effectene reagent (QIAGEN). Four days after transfection, cells were selected with 150 μg/ml hygromycin. Cells were expanded every 3–4 days by centrifugation at 1200 rpm and resuspended with fresh medium containing 150 μg/ml hygromycin until cells started to grow. To induce expression of the miRNA construct, we added CuSO₄ into the media to a final concentration of 2 mM for 2–3 days. The expression of DsRed and mir-144/451 was examined by fluorescence microscopy and Northern blotting, respectively.

To investigate the effect of knockdowns in miRNA biogenesis, dsRNA soaking was performed in S2R+ cells. Approximately 500 bp fragments of target genes were amplified from D. melanogaster w+ genomic DNA. The PCR-amplified fragments were cloned into the XhoI/XbaI sites of pLitmus (NEB), which contains opposing T7 promoters flanking the cloning site. T7 promoter-containing templates were generated by PCR amplification of pLitmus-dsRNA constructs with Litmus primer A+B, and dsRNA were synthesized using MEGAscript T7 Kit (Ambion). The primers used to amplify these regions are listed in Supplementary Table S1, ‘dsRNA primers’. To knockdown the expression of endogenous genes, 2.5 × 10⁶ S2R+/ cells were soaked with 15 μg dsRNA in six-well plate for 4 days and split into another six-well plate and soaked with 15 μg dsRNA for another 4 days. To induce mir-144/451 expression in the knockdown experiments, 2 mM CuSO₄ was added to the MT-DsRed-mir-144/451 S2R+ stable cells on the fifth day of dsRNA soaking. For double knockdown experiments, 12 μg of each dsRNA was used; for triple knockdown experiments, 8 μg of each dsRNA was used. Knockdown efficiency of each dsRNA was analyzed by quantitative RT-PCR.

To assay the regulatory capacity of mir-451-type constructs in S2 cells, we co-transfected miRNA and sensor plasmids in 96-well plates for 72 h, and measured luciferase activities with Dual-Glo Luciferase Assay System (Promega).

Western and Northern blotting
To prepare total cell lysates, cell pellets were resuspended in 5× cell pellet volume of IP/Extract buffer [150 mM NaCl, 2 mM MgOAc₂, 20 mM Tris–HCl (pH 7.5), 5% Glycerol, 0.5% NP-40, 2 mM DTT, Complete-mini EDTA-free] and incubated on ice for 30 min. Cell lysates were cleared by centrifugation at 14 000 rpm for 10 min at 4°C. Protein samples were separated by SDS–PAGE and transferred to PVDF membrane with CAPS transfer buffer at 1.5 mA/cm² for 45 min in the cold room. Membranes were blocked with 5% non-fat milk in TBST [150 mM NaCl, 30 mM Tris–HCl (pH 7.5), 2.5% Tween-20] for 1 h at room temperature (RT), incubated with primary antibodies with gentle agitation overnight at 4°C, washed three times for 5 min each with TBST, incubated with HRP-conjugated secondary antibodies for 1 h at RT, washed three times for 5 min each with TBST. The signals were detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences).

Total RNA was extracted from cultured cells with Trizol (Life Technologies). RNA samples were separated on 20% urea polyacrylamide denaturing gels (National Diagnostics), transferred to GeneScreen Plus (Perkin Elmer), crosslinked using UV treatment and probed with γ-32P-labeled DNA or LNA oligonucleotides antisense to the small RNAs of interest at 45°C overnight. For DNA probes, the membranes were washed with 2× SSC/0.1% SDS at 45°C for four times, 15 min each time. For DNA probes, the membranes were washed with 2× SSC/0.1% SDS for three times and 0.2× SSC/0.1% SDS for two times at 45°C. The signals were exposed to Imaging Plate (Fujifilm). The sequences of the probes are listed in Supplementary Table S1 ‘Northern probes’.

To analyze Ago-associated RNA, we used an S2 cell line stably transformed with a FLAG-HA-AGO2 genomic construct (21,23). Cell lysates were cleared and then incubated with Dynabeads Protein G (Life Technologies) conjugated with AGO1 (ab5070, Abcam) or FLAG (M2, Sigma) antibodies (Supplementary Material). The Ago-associated RNA was extracted with Illustra MicroSpin G-25 Columns (GE Healthcare) or FLAG (M2, Sigma) antibodies (Supplementary Material).

mir-451 in vitro processing assays
We subjected synthetic 42-nt pre-mir-451 RNA oligo to 5′ phosphorylation using polynucleotide kinase and [γ-32P]ATP. We removed unincorporated [γ-32P]ATP with Illustra MicroSpin G-25 Columns (GE Healthcare). The radiolabeled RNA oligo was then separated on a 20% urea polyacrylamide denaturing gel, and RNA bands of the correct size were excised, crushed and extracted overnight in 400 mM NaCl at 4°C. The gel slice homogenates were collected by micropore filter (Ultrafree-MC, 0.22 μm, Millipore) and precipitated by 2.5 volumes of 100% ethanol precipitated at −20°C overnight. The RNA was analyzed as described above.
ethanol and 20 μg glycogen at −20°C for 3 h. The RNA pellets were washed with 70% ethanol and resuspended with 500 μl oligo buffer [100 mM KOAc, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. To fold the pre-mir-451 hairpin, the RNA was denatured at 95°C for 5 min followed by immediate cooling on ice for >10 min. RNA was concentrated by Vivaspin 500 MWCO 3000 (GE Healthcare Life Sciences) until the volume was <50 μl. The amount of radioactivity in the labeled samples was measured by a liquid scintillation counter.

To perform the Ago protein cleavage assay, 2000 cpm γ-32P-labeled pre-mir-451 RNA substrate was mixed with AGO1-immunoprecipitates from ~1 mg S2 cells in 25 μl reaction buffer (20 mM HEPES-KOH pH 7.3, 100 mM KOAc, 2 mM MgOAc2, 2 mM DTT, 30 U RNaseOUT). The Ago cleavage reaction was carried out at RT for ~2 h, followed by wash for two times with the reaction buffer. To test the ac-pre-mir-451 3' trimming activity in different lysates, ~100 μg total lysates from S2 cells or fly ovary tissues were incubated with the washed beads at RT for 2 h in the presence of 30 U RNaseOUT (Roche). After two times of wash with reaction buffer, RNA was extracted and analyzed as previously described.

Small RNA library cloning and analysis
dsRNA (75 μg total) knockdown experiments were carried out in 10 ml MT-DsRed-mir-144/451 S2R+ cells for 8 days, with CuSO4 added on the fifth day to induce the miRNA construct. The cells were lysed with IP/Extract buffer, and cleared by centrifugation at 14 000 rpm for 5 min. RNA was concentrated by Vivaspin 500 MWCO 3000 (GE Healthcare Life Sciences) until the volume was <10 μl. The RNA substrate was mixed with 500 μg S2 cells in 25 μl reaction buffer (20 mM HEPES-KOH pH 7.3, 100 mM KOAc, 2 mM MgOAc2, 2 mM DTT, 30 U RNaseOUT). The Ago cleavage reaction was carried out at RT for ~2 h, followed by wash for two times with the reaction buffer.

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RESULTS
mir-451-class substrates generate functional regulatory RNAs in Drosophila
Since mir-451-class substrates have not been identified outside of vertebrates, we were interested to determine if Drosophila was capable of utilizing this non-canonical type of substrate. We considered the most salient initial tests to be functional repression assays, which we performed using luciferase sensors in S2 cells and GFP sensors in transgenic animals (Figure 1A). We cloned a human mir-144/451 pri-miRNA fragment into a UAS-DsRed vector for expression in Drosophila; the mir-144 moiety serves a canonical miRNA control for these experiments. We transfected this with ub-Gal4 activator plasmid into S2 cells, and assayed for repression of a renilla luciferase 'perfect' miR-451 sensor that we had validated in human cells (8). This construct induced specific miR-451-dependent repression of its sensor (Figure 1B), suggesting that Ago-mediated miRNA biogenesis is operational in Drosophila cells. We note that this assay does not distinguish the relative contributions of the two Drosophila Argonaute proteins, both of which have slicer activity, but earlier quantitative analyses indicate that they may mostly report on the activity of AGO2 (22).

Although the only endogenous substrate of this pathway currently known is mir-451 itself, we showed that its backbone constitutes a flexible platform for the production of diverse Dicer-independent miRNAs from plasmid constructs (8,11). We therefore tested constructs in which we reprogrammed mir-451 with other mature miRNA sequences, such as miR-19a-3p and miR-19a-3p. Indeed, we successfully detected the ability of both constructs to repress cognate sensors (Figure 1C and D). Thus, the capacity for regulation by mir-451-type substrates in Drosophila is flexible to hairpin sequence.

We performed more stringent regulatory tests of mir-451 in the animal. We generated UAS-DsRed-mir-144/451 transgenes, and tested their ability to repress ubiquitously expressed tub-GFP sensor transgenes linked to either miR-144 or miR-451 complementary sites. We activated the miRNAs using ptc-Gal4, which is active in a stripe of cells at the anterior–posterior compartment boundary of the wing imaginal disc. By focusing on the wing pouch region, which is relatively flat, we can obtain a clear read-out of GFP sensor expression in miRNA/DsRed-positive cells, compared to flanking DsRed-negative control cells. We first assayed canonical miR-144. Control expression of UAS-DsRed did not affect the GFP-miR-144 sensor, but similar expression of UAS-DsRed-mir-144/451 induced strong repression (Figure 1E and F). Therefore, this human miRNA construct is active in Drosophila. Similar assays of GFP-miR-451 sensor showed that it was specifically down-regulated by expression of UAS-DsRed-mir-144/451 (Figure 1G and H). Although miR-451 was not as potent as miR-144, the demonstration of regulatory activity of miR-451-type
Figure 1. *mir-451*-type substrates generate functional regulatory RNAs in Drosophila. (A) Sensor design. We assayed renilla luciferase sensors driven by the SV40 early promoter in S2 cells, and GFP sensors driven by the tubulin promoter in transgenic Drosophila. All sensors contained two sites fully complementary to the mature miRNA under assay. (B–D) Sensor assays in S2 cells that were transfected with *ub-Gal4, UAS-DsRed-mir-144/451* or variants in which the *mir-451* moiety was reprogrammed (RP) with miR-19a-3p or miR-199a-3p, and psiCHECK sensors bearing control firefly luciferase and a test renilla luciferase bearing two complementary sites for the indicated miRNA. We observed specific target repression by miR-451 (B), 451:miR-19aRP (C) and 451:miR-199aRP (D) constructs. Sensor tests using empty or non-cognate miR-451 constructs provided further evidence for the specificity of repression that we observed. (E–H) Sensor assays in third instar wing imaginal discs. Shown are the central wing pouch regions of discs that express *ptc-Gal4, UAS-DsRed-mir-144/451* or variants in which the *mir-451* moiety was reprogrammed (RP) with miR-19a-3p or miR-199a-3p, and psiCHECK sensors bearing control firefly luciferase and a test renilla luciferase bearing two complementary sites for the indicated miRNA. We observed specific target repression by miR-451 (B), 451:miR-19aRP (C) and 451:miR-199aRP (D) constructs. Sensor tests using empty or non-cognate miR-451 constructs provided further evidence for the specificity of repression that we observed. (E–H) Sensor assays in third instar wing imaginal discs. Shown are the central wing pouch regions of discs that express *ptc-Gal4, UAS-DsRed-mir-144/451* or variants in which the *mir-451* moiety was reprogrammed (RP) with miR-19a-3p or miR-199a-3p, and psiCHECK sensors bearing control firefly luciferase and a test renilla luciferase bearing two complementary sites for the indicated miRNA. We observed specific target repression by miR-451 (B), 451:miR-19aRP (C) and 451:miR-199aRP (D) constructs. Sensor tests using empty or non-cognate miR-451 constructs provided further evidence for the specificity of repression that we observed. (E–H) Sensor assays in third instar wing imaginal discs. Shown are the central wing pouch regions of discs that express *ptc-Gal4, UAS-DsRed-mir-144/451* or variants in which the *mir-451* moiety was reprogrammed (RP) with miR-19a-3p or miR-199a-3p, and psiCHECK sensors bearing control firefly luciferase and a test renilla luciferase bearing two complementary sites for the indicated miRNA. We observed specific target repression by miR-451 (B), 451:miR-19aRP (C) and 451:miR-199aRP (D) constructs. Sensor tests using empty or non-cognate miR-451 constructs provided further evidence for the specificity of repression that we observed.
substrates in Drosophila cells and animals encouraged us to examine their mechanism of biogenesis.

Biogenesis of bulk mir-451-class hairpins is arrested at the Ago cleavage stage

We collected RNA from larvae in which UAS-DsRed-mir-144/451 was activated ubiquitously using da-Gal4, and assessed the accumulation of small RNAs from this transgene using Northern blotting. Drosophila lacks orthologs of miR-144 or miR-451, allowing us to assign hybridizing bands to the introduced constructs. As shown in Figure 2, these constructs generated both ~58 nt pre-mir-144 hairpin and mature ~21–23 nt miR-144 species. We also observed accumulation of ~42 nt pre-mir-451 and ~30 nt ac-pre-mir-451, with the latter present at ~2.8-fold greater levels than the former. However, mature <30 nt miR-451 species were barely detected in total RNA samples.

We performed similar Northern blotting for RNAs generated from mir-144/451 transgenes in which miR-451 was reprogrammed with miR-19a-3p or miR-199a-3p. In these tests, we similarly observed predominant accumulation of ~30 nt ac-pre-miRNAs, with little or no fully matured species (Figure 2). We tested two independent insertions of each miR-451-class transgene, and observed similar results in all cases. Overall, these data indicate that 3′ resection of Ago-cleaved miR-451-type substrates is not as effective in Drosophila compared with vertebrates.

Biogenesis of mir-451 requires the Microprocessor complex but is stimulated by loss of the Dcr-1/Loqs complex

We analyzed the dependence of miR-451 on the canonical miRNA pathway machinery using dsRNA-mediated knockdowns in S2 cells (36). In initial tests, we found that the transfection efficiencies of cells that had been depleted of different small RNA factors was variable (data not shown). Therefore, we generated a stable S2R+ line bearing a copper-inducible mir-144/451 expression construct (MT-DsRed-mir-144/451), which enabled more uniform measurements across a panel of conditions. We soaked these cells in dsRNA for 4 days, and then replaced the media with fresh dsRNA for another 4 days to ensure strong knockdown. We induced mir-144/451 expression on the fifth day and collected RNA for analysis on the eighth day of dsRNA treatment.

As in vertebrates (8–10), the accumulation of all processed forms of miR-451 was dependent on Drosha and its dsRBD partner DGCR8/Pasha (Figure 3A). Knockdown of these factors eliminated hybridization to miR-451 probes, and also strongly depleted mature and hairpin forms of miR-144. Interestingly, not only was the biogenesis of miR-451 independent of the pre-miRNA-specific RNase III enzyme Dcr-1, depletion of this factor actually enhanced the accumulation of <30 nt miR-451 species (Figure 3A). Consistent with this, depletion of the Dcr-1 dsRBD cofactor, Loquacious (Loqs), also stimulated the accumulation of <30 nt miR-451 species (Figure 3A).

Figure 2. Bulk maturation of mir-451-type substrates in Drosophila arrests as ~30 nt species. We performed Northern blotting of third instar larvae bearing da-Gal4 and UAS-DsRed-mir-144/451, or variants in which the mir-451 moiety was reprogrammed (RP) with miR-19a-3p or miR-199a-3p. We assayed two independent transgenic insertions for each construct, and used Canton S (CS) as a control genotype. Although a modest amount of potentially trimmed <30 nt species was seen, the majority of signals for miR-451 or reprogrammed miR-451 constructs were at ~30 nt. Control blotting for mir-144 showed its expected accumulation in all transgenic animals.
miR-444, it concomitantly enhanced the maturation of miR-451 (Figure 3B).

We conclude from these experiments that full 3' resection of miR-451 is possible in Drosophila, but that this process is in competition with endogenous canonical premiRNA hairpins. Reduction of canonical hairpins generated by Dicer-1 and Loqs can thus enhance the availability of pre-miR-451 intermediates for maturation.

Maturation of mir-451 is also influenced by the RNAi machinery

We followed up this analysis by examining the requirement of RNAi factors for miR-451 biogenesis. As depletion of RNAi factors in S2 cells resulted in cell toxicity, possibly due to unleashing of persistent viruses (37), we moved directly to analyze the appropriate null mutant flies. We introduced da-Gal4 and UAS-DsRed-mir-144/451 transgenes into the homozygous viable backgrounds of der-2[88Fls] and r2d2[1], and isolated larvae for Northern blotting. As expected, these backgrounds did not affect the maturation of miR-144. However, we observed specific depletion of ~30 nt miR-451 species, whereas ~42 and ~30 nt species were relatively little affected (Figure 3B).

Taken together with the miRNA pathway manipulations, these data indicated complex genetic requirements and impact of both canonical miRNA and siRNA factors on the biogenesis of mir-451 in Drosophila. In particular, there are several sizes of miR-451 species (42, 30 and <30 nt), whose accumulations are differentially affected by miRNA and siRNA factors.

Interplay of miRNA and siRNA sorting pathways for mir-451 biogenesis

We hypothesized that the complex genetic requirements for miR-451 maturation might reflect the possibility that short hairpins assort into both miRNA and siRNA pathways, and thus access both Argonaute effectors. In mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNAs for miR-451 maturation might reflect the possibility that miRNA effector AGO1 also exhibits detectable cleavage of mir-451 further. Although the siRNA effector AGO2 is selective reduced the 30 nt miR-451 species, whereas mutualization of AGO1 may permit the re-sorting of RNAi factors for miR-451 biogenesis. As depletion of canonical hairpins in mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNAs for miR-451 maturation might reflect the possibility that miRNA effector AGO1 also exhibits detectable cleavage of mir-451 further. Although the siRNA effector AGO2 is selective reduced the 30 nt miR-451 species, whereas mutualization of AGO1 may permit the re-sorting of RNAi factors for miR-451 biogenesis. As depletion of canonical hairpins in mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNAs for miR-451 maturation might reflect the possibility that miRNA effector AGO1 also exhibits detectable cleavage of mir-451 further. Although the siRNA effector AGO2 is selective reduced the 30 nt miR-451 species, whereas mutualization of AGO1 may permit the re-sorting of RNAi factors for miR-451 biogenesis. As depletion of canonical hairpins in mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNAs for miR-451 maturation might reflect the possibility that miRNA effector AGO1 also exhibits detectable cleavage of mir-451 further. Although the siRNA effector AGO2 is selective reduced the 30 nt miR-451 species, whereas mutualization of AGO1 may permit the re-sorting of RNAi factors for miR-451 biogenesis. As depletion of canonical hairpins in mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNAs for miR-451 maturation might reflect the possibility that miRNA effector AGO1 also exhibits detectable cleavage of mir-451 further. Although the siRNA effector AGO2 is selective reduced the 30 nt miR-451 species, whereas mutualization of AGO1 may permit the re-sorting of RNAi factors for miR-451 biogenesis. As depletion of canonical hairpins in mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNAs for miR-451 maturation might reflect the possibility that miRNA effector AGO1 also exhibits detectable cleavage of mir-451 further. Although the siRNA effector AGO2 is selective reduced the 30 nt miR-451 species, whereas mutualization of AGO1 may permit the re-sorting of RNAi factors for miR-451 biogenesis. As depletion of canonical hairpins in mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNAs for miR-451 maturation might reflect the possibility that miRNA effector AGO1 also exhibits detectable cleavage of mir-451 further. Although the siRNA effector AGO2 is selective reduced the 30 nt miR-451 species, whereas mutualization of AGO1 may permit the re-sorting of RNAi factors for miR-451 biogenesis. As depletion of canonical hairpins in mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNAs for miR-451 maturation might reflect the possibility that miRNA effector AGO1 also exhibits detectable cleavage of mir-451 further. Although the siRNA effector AGO2 is selective reduced the 30 nt miR-451 species, whereas mutualization of AGO1 may permit the re-sorting of RNAi factors for miR-451 biogenesis. As depletion of canonical hairpins in mammalian cells, multiple Ago proteins can load pre-miRNAs and siRNAs (13, 25). Activity that can be programmed by endogenous miRNA
but the reciprocal loss of AGO2 does not effectively permit re-sorting to AGO1, possibly because they are out-competed by miRNA hairpins.

To examine this more directly, we sequentially purified AGO1 and Flag-AGO2 complexes from S2R+ cells stably expressing FLAG-HA-AGO2, and examined their immunoprecipitates for different small RNAs. Mature miR-144 was preferentially associated with AGO1 relative to AGO2, as expected for a canonical miRNA (Figure 4). We note that the dominant miR-144 species in AGO2 was 1 nt longer than in AGO1, in line with our finding that depletion of ago1 causes a slightly larger mature miR-144 species to accumulate (Figure 3A). By comparison, the endo-siRNA product hp-CG4068B preferentially associated with AGO2, validating the purity of our effector complexes (Figure 4).

Consistent with earlier results, total RNA from cells treated with gfp dsRNA predominantly showed the ~42 and ~30 nt bands (Figure 2). Interestingly, AGO1-IP complexes from these cells contained the pre-mir-451 and a species that appeared to be slightly shorter than the 30 nt species seen in total RNA; moreover they also contained shorter miR-451 species corresponding to 3\' resected mature miR-451 (Figure 4). Since S2 total RNA samples only modestly accumulated <30 nt miR-451 species, we infer that these are rarer products that were enriched by purification of AGO1 complexes. Likewise, the mild excess of <30 nt species over ~42 nt species in AGO1 complexes suggested that the majority of 42 nt species observed in total RNA are not associated with AGO1. In contrast, AGO2 complexes from these cells contained exclusively the ~30 nt species, which migrated slightly slower than the largest <30 nt species in AGO1 complex.

These data allow us to infer that the various mir-451-hybridized products actually correspond to a mixture of distinct populations: (i) a ~42 nt Drosha/Pasha-cleaved hairpin that is in transit for further maturation, (ii) a minor population of ~42 nt species that associates with AGO1 and can be cleaved into ~30 nt hairpins and 3\' resected into <30 nt species and (iii) a major population of 30 nt species that are associated with AGO2. Moreover, these data indicate that full maturation of miR-451 is possible provided that its pre-miRNA is directed into AGO1; hairpin loading to AGO2 results in efficient cleavage, but this product is apparently blocked for further maturation.

**Influence of the two *Drosophila* Dicers on mir-451 sorting and maturation**

We next tested the impact of depleting Dicer-class enzymes on these sorting patterns. Knockdown of dcr-1 strongly enhanced the steady-state association of all three forms of mir-451 species with AGO1 (Figure 4). Since even the 42 nt species increased in AGO1 complexes, we infer that pre-mir-451 may load AGO1 more efficiently when it is no longer competing with loading of endogenous miRNA duplexes. We observed a minor decrease in all mir-451 species associated to AGO1 upon depletion of dcr-2, perhaps suggesting that it plays a role in trafficking of pre-mir-451 hairpins. Reciprocally, the pure 30 nt miR-451 products in AGO2 complexes were unaffected by depletion of dcr-1, while single dcr-2-KD or double dcr-1/2-KD nearly abolished its accumulation in AGO2 (Figure 4). Since 42 nt pre-mir-451 still associates with AGO1 in dcr-2-KD conditions, we infer that miR-451 processing is independent of Dcr-2, but that it requires Dcr-2 in its capacity as an AGO2 loading factor (18).

In contrast to miR-451, mature mir-144 species in AGO2 complexes were strongly depleted in all three conditions (dcr-1-KD, dcr-2-KD and dcr-1/2-KD), since it should require Dcr-1 for pre-miRNA cleavage and Dcr-2 for loading to AGO2 (Figure 4). The endo-siRNA hpCG4068B associated with AGO2 to similar levels in

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**Figure 4.** Distinct requirements of two Dicer proteins for miR-451 sorting to the two Ago proteins. We depleted stable Flag-AGO2 S2R+ cells of the indicated factors using dsRNA, and then transfected them with ub-Gal4 and UAS-DsRed-mir-144/451. We then immunoprecipitated AGO1 and Flag-AGO2 complexes and examined their associated small RNAs using Northern blotting. Consistent with previous results, the input lanes show predominant accumulation of ~42 nt (pre-mir-451) and ~30 nt (ac-pre-mir-451) species in control knockdown cells, while the accumulation of <30 nt trimmed miR-451 species was enhanced by depletion of Dcr-1 and the accumulation of ac-pre-mir-451 species was eliminated by depletion of Dcr-2. Analysis of Ago complexes revealed that AGO1 associates with ~42, ~30 and <30 nt miR-451 species, whereas AGO2 selectively accumulates ~30 nt miR-451 species. Note that the ac-pre-mir-451 species in AGO2 are slightly larger than the comparable species in AGO1; we later show that ac-pre-mir-451 species in AGO2 are 3\' modified by Hen1 methyltransferase (Figure 5). The Dcr-1-dependent canonical miRNA miR-144 preferentially accumulates in AGO1 complex while the Dcr-2-dependent endo-siRNA hp-CG4068B preferentially accumulates in AGO2 complex, validating the knockdown and AGO-IP procedures. Note though that the largest miR-144 isoform preferentially associates with AGO2, and its accumulation therein is dependent on Dcr-2.
cells treated with gfp and der-1 dsRNAs, as expected for an endo-siRNA. Altogether, these studies demonstrate distinct requirements for Dicer enzymes for accumulation of small RNAs from an endo-siRNA hairpin, a canonical miRNA hairpin and a Dicer-independent hairpin in the RNAi effector AGO2.

**Depletion of Hen1 methyltransferase permits 3’ resection in AGO2**

Single-stranded RNAs in AGO2, but not in AGO1, are modified at their 3’ ends by the Hen1 methyltransferase (39). This modification renders matured RNAs in AGO2 resistant to β-elimination, while mature RNAs in AGO1 are sensitive resulting in their increased mobility. For example, the bulk population of the canonical miRNA bantam is sensitive to β-elimination, and this is due to its dominant sorting to AGO1. On the other hand, miR-277 is an atypical miRNA that is sorted into both AGO1 and AGO2 effectors (22), for which the AGO1-loaded population is sensitive to β-elimination while the AGO2-loaded population is resistant (Figure 5A).

Consistent with our observations of preferential loading to AGO2, bulk ~30 nt ac-pre-mir-451 species in total RNA were resistant to β-elimination (Supplementary Figure S1). In addition, analysis of mir-451 species in AGO2-IP and AGO1-IP complexes showed that the former were sensitive while the latter were resistant (Supplementary Figure S1). We performed similar analysis using mir-451 constructs reprogrammed with mature miR-199a or miR-19a, and found that bulk ~30 nt small RNAs from both constructs were resistant to β-elimination, and that this was due to their dominant sorting to AGO2 (Figure 5A). Western analysis confirmed the selectivity of the AGO1 and Flag-AGO2 immunoprecipitations (Figure 5B).

We inferred that the free hydroxyl groups on the 3’ end of ac-pre-mir-451 in AGO1 complexes permitted its resection. This was indeed the case: cells treated with control gfp dsRNA maintained a single 30 nt ac-pre-mir-451 in AGO2-IP, but treatment with henl dsRNA permitted a ladder of shortened miR-451 species to accumulate in AGO2 complexes (Figure 5C).

These data indicate that 3’ resection obligately occurs within a mature Ago complex, and that miR-451 intermediates and products are not exchanged between Ago complexes. This notion is supported by the observation of distinctly-sized miR-451 species in AGO1 and AGO2 (Figure 4). Moreover, they indicate that the modest processing of mir-451 observed in wildtype cells is a consequence of the separation of siRNA and miRNA pathways in Drosophila, such that default sorting of pre-mir-451 into AGO2 leads to its 3’ modification and blockage from further resection. Enhancement of pre-mir-451 loading into AGO1 by genetic means (e.g. by loss of Dcr-1 or Loqs), or prevention of 3’ modification of ac-pre-mir-451 in AGO2 (e.g. by loss of Hen1), are both compatible with full maturation of miR-451.

![Figure 5](https://academic.oup.com/nar/article-abstract/42/3/1987/1054366)

**Figure 5.** Hen1-dependent modification of ac-pre-mir-451 species in AGO2 complexes. (A) We analyzed total RNAs before and after β-elimination. Small RNAs that have reacted exhibit increased mobility and/or decreased apparent level. Most of the pool of the canonical miRNA bantam, which is preferentially loaded in AGO1, is amenable to β-elimination. A substantial portion of miR-277, which is sorted into both AGO1 and AGO2, was not β-eliminated. The bulk of small RNAs from mir-144/451 constructs reprogrammed with miR-199a-3p or miR-19a-3p, which accumulate as ac-pre-miRNAs, were resistant to β-elimination. (B) Western blotting confirms the selective isolation of AGO1 and AGO2 complexes. (C) We soaked S2R+ cells stably expressing Flag-HA-AGO2 with gfp or henl dsRNAs, then transfected them with ub-Gal4 and UAS-DsRed-mir-144/451. As before, AGO1 complexes normally contain ~30 nt miR-451 species while AGO2 complexes strictly contain 30 nt ac-pre-mir-451. However, depletion of henl permits the accumulation of 3’ trimmed miR-451 species in AGO2.
Depletion of Nibbler 3′–5′ exoribonuclease enhances 3′ resection of miR-451

The inhibition of ac-pre-mir-451 maturation by Hen1-dependent 3′ modification suggested that its trimming is performed by a 3′–5′ exoribonuclease, as opposed to an endoribonuclease. We attempted to identify a cognate factor by depleting candidate 3′–5′ exoribonucleases in S2 cells, and assaying for effects on miR-451 maturation. We soaked MT-DsRed-mir-144/451 cells with dsRNAs against various RNases, then induced miRNA expression and analyzed the resultant RNAs by Northern blotting.

These tests did not identify any individual ribonuclease whose depletion caused a substantial effect on miR-451 3′ trimming (data not shown). To sensitize these tests, we then performed double knockdowns of exoribonucleases with dcr-1, whose depletion enhances the signals for mature miR-451 in total RNA (Figures 3 and 4). However, these tests did not clearly yield a condition under which ac-pre-mir-451 preferentially accumulated (Supplementary Figure S2), as might be expected upon loss of the 3′ resectase. For example, although the RNA exosome removes the unstructured ends of a subclass of splicing-derived hairpins that require 3′ trimming to generate the pre-miRNA substrate, since precursor 3′ tailed mirtrons accumulate following loss of the exosome (40). However, the exosome RNases Rrp6 and Dis3 did not appear to be required for miR-451 maturation, and neither was the Dis3-related protein CG16940 (Figure 6A). It is unclear whether the nucleases tested were insufficiently depleted, whether the relevant nuclease was not tested, or whether there might be redundant activities. However, these tests did yield an unexpected finding, namely that depletion of Nibbler (30,31) substantially enhanced the maturation of miR-451. Whereas little <30 nt miR-451 species was in total RNA from control gfp/dcr-1 dsRNA-treated S2 cells, these were more abundant in cells codepleted of dcr-1 and nhibbler (Figure 6A).

The Nibbler 3′–5′ exoribonuclease mediates broad 3′ trimming of mature miRNAs in AGO1 complexes (30,31), especially in cases where the species produced by Dicer-1 cleavage are relatively long (41). For example, miR-34 is initially produced as a 24 nt product, but Nibbler resects 3–4 nt from its 3′ end. We verified that in our nhibbler knockdowns, miR-34 was stabilized at the length generated by Dicer-1 cleavage (Figure 6A, miR-34 blot, arrowhead). We performed further single and double knockdowns of gfp, dcr-1 and nhibbler dsRNAs to demonstrate that dcr-1/nhibbler double depletion synergize in promoting miR-451 maturation (Supplementary Figure S3). Moreover, knockdown of nhibbler alone could enhance the accumulation of 3′-resected miR-451 (Supplementary Figure S3).

Since Nibbler does not correspond to the miR-451 ‘Resector’, these findings imply the existence of multiple, potentially competing, ribonucleases that can shape the 3′ ends of different miRNA substrates in Drosophila.

In vitro recapitulation of the 3′ resection of AGO1-cleaved pre-mir-451

Since we were not able to identify the miR-451 3′ trimming enzyme genetically, we attempted to gain insight into its properties using a biochemical approach. Inspired by the

Figure 6. 3′ resection of miR-451 is antagonized by Nibbler and promoted by a soluble EDTA-sensitive ribonuclease. (A) We treated S2R+ cells stably transformed with MT-DsRed-mir-144/451 with dsRNAs against the indicated RNases, and processing of miR-451 was enhanced by co-depletion of dicer-1. Following treatment with copper to induce miRNA expression, we analyzed total RNAs using Northern blotting. Interestingly, depletion of the 3′–5′ exoribonuclease Nibbler, which shortens the 3′ tails of ‘long’ miRNAs in AGO1 complexes, actually enhanced the 3′ resection of miR-451. Effective depletion of Dicer-1 was evidenced by the loss of mature mir-144, accumulation of pre-mir-34, and shift in the size distribution of the remaining mature miR-34 to its longest isoform. (B) Scheme for in vitro processing assay for the miR-451 3′ resectase. (C) Incubation of 5′ radiolabeled pre-mir-34 with AGO1 complexes immunopurified from dsDcr-1 cells generates a detectable amount of stable 30 nt ac-pre-mir-451 species. Following treatment with S2 cell lysate, we observed a modest amount of 3′ trimming (~1 nt); this was blocked by pretreatment of lysate with EDTA. Treatment with ovary lysate yielded a greater amount of trimmed products, and this activity was again EDTA sensitive.
Recent in vitro recapitulation of piRNA 3’ end formation (42), we set up an analogous assay for miR-451 processing (Figure 6B). We first immunoprecipitated AGO1 from S2 cells, and loaded it with 5’ radiolabeled synthetic pre-mir-451. However, we were not able to observe in vitro cleavage to generate ac-pre-mir-451 (Supplementary Figure S4A). Subsequent reactions showed that some pre-mir-451 associated with control Myc-Ab beads despite extensive washing (Supplementary Figure S4B). Therefore, a fraction of visualized substrates were not genuinely loaded in AGO1, which might obscure our ability to detect mir-451 processing.

Based on our observation that loss of canonical miRNA biogenesis enhances mir-451 maturation, we then isolated AGO1 complexes from S2 cells depleted of Dcr-1. Following loading of pre-mir-451, we were now able to observe the generation of 30 nt ac-pre-mir-451 (Figure 6C, ‘no lysate’ lane). Even though the loss of Dcr-1 substantially decreases the steady-state level of AGO1 (43), these data support the interpretation that absence of canonical miRNA biogenesis enhances the assembly of AGO1:pre-mir-451 complexes.

We then treated this with lysates of S2 cells or Drosophila ovary. We observed only mild 3’ trimming in the S2 lysate, perhaps of a single nucleotide (Figure 6C). However, we obtained reasonably robust trimming using soluble ovary lysate, such that most of the 30 nt species was reduced to mature miRNA-sized species (Figure 6C). This activity was notable given that the piRNA ‘Trimmer’, which also exists in ovarian (silkworm) cells, is completely insoluble (42); thus, our assay may potentially detect the activity of a distinct resenase. We tested the dependence of this activity on divergent cations by supplementing the lysates with EDTA. Even though S2 lysate was only capable of removing 1 nt, this was blocked by EDTA. The more substantial 3’ trimming obtained with ovary lysate was also fully blocked by EDTA. Therefore, a soluble miR-451 3’ trimming activity can be detected in vitro, and it appears to act by a metal ion-dependent mechanism.

Deep sequencing of AGO1 complexes from cells depleted of dcr-1

No endogenous mir-451-type substrates are yet known in Drosophila. We attempted to identify such loci using extant AGO1/2-IP small RNA data from S2 cells (27), ovaries (23,25) and heads (20), but did not find convincing evidence for mir-451-type loci (data not shown). Still, in light of the fact that conserved vertebrate miR-451 exhibits narrow tissue specificity, being largely restricted to red blood cells (44,45), it is conceivable that the few extant sources of data from Drosophila Argonaute complexes do not include the expression domain of a putative member of this class. Our studies provided a potentially useful lead, in that loss of Dicer-1 enhances the maturation of miR-451 in AGO1 complexes (Figure 4).

We tested this notion by soaking MT-DsRed-mir-144/451 S2R+ cells for two rounds of 4 days each with dsRNAs, and induced the miRNA construct on the fifth day of the regimen. Under these conditions, we confirmed substantial depletion of mature bantam and strong accumulation of pre-bantam (Figure 7A). We proceeded to generate AGO1-IP libraries from the gfp and dcr-1 samples. Given that miRNAs were so strongly depleted, it was perhaps surprising that both libraries were mostly composed of mature canonical miRNAs (Figure 7B, left graphs). However, we can rationalize this result in light of our recent appreciation that loss of canonical miRNA biogenesis factors concomitantly destabilizes AGO1 protein (43). Thus, the process of purifying AGO1 complexes for library construction obscures the fact that the absolute amount of miRNAs (and AGO1) is reduced by dcr-1 knockdown. These data illustrate the challenge in fully depleting the capacity for miRNA biogenesis using RNAi strategies, which in any case has associated consequences for cell survival.

Nevertheless, by focusing in on the non-miRNA reads, we do observe that the contribution of these species was about twice as large in the dcr-1 library relative to the gfp library (Figure 7B, right graphs). For example, hpRNA-derived siRNAs, which are generated by Dcr-2, increased by 2.75-fold in the dcr-1 library (Supplementary Table S2). Therefore, there is increased promiscuity in substrate acceptance by AGO1 upon depletion of Dcr-1. This interpretation was bolstered by quantification of mature miR-451 reads, which increased 8.34-fold from the gfp dsRNA library to the dcr-1 dsRNA library (from 1772 reads/56.1 RPM to 17 829 reads/468 RPM).

With this in mind, we rescanned the genome with these AGO1-IP data in search of reads that mapped to short hairpins that furthermore (i) yielded dominant 5’ species exhibiting 3’ heterogeneity, (ii) generated longer reads that extend across the terminal loop and (iii) exhibited enhanced accumulation in dcr-1 knockdown relative to gfp knockdown conditions. This analysis revealed some potential candidates. The locus depicted in Figure 7C was enriched 9.13-fold upon dcr-1 knockdown and generated typical 21–22 nt miRNA-sized reads that crossed the terminal loop. As well, we observed 28 nt reads that shared the 5’ end of the shorter dominant reads, and these extended down the 3’ arm to a position that was reminiscent of a potential hairpin cleavage product. Although the hairpin pairing is not perfect, the unpaired nucleotides are in positions analogous to ones that are compatible with miR-451 biogenesis (11). Another locus with similar structural properties and dcr-1 enrichment is shown in Supplementary Figure S5. We recognize that these loci are not very abundantly expressed, with the former locus reaching 1.37 RPM and the latter reaching 1.44 RPM in dcr-1 AGO1-IP libraries. On the other hand, these experiments provide plausibility for the scenario that the miR-451 biogenesis tests we conducted may reflect the potential existence of endogenous Dicer-independent substrates in Drosophila.

DISCUSSION

Ago-mediated miRNA biogenesis in invertebrate species

Bulk miRNAs in plants and animals are produced by canonical pathways, consisting of a series of seemingly
essential biogenesis steps (3). However, such rules have proven made to be broken, and the recognition of diverse non-canonical strategies for miRNA biogenesis indicates a great deal of flexibility in the possibilities for the processing and metabolism of short RNA molecules (6). Notably, it has sometimes proven possible to import a completely foreign small RNA system into a naïve host and have it be functional. For example, the human RNAi machinery is functional when introduced into Saccharomyces cerevisiae (46), and the bacterial CRISPR-Cas9 system has now been exploited in diverse eukaryotic systems (47). In this study, we imported the vertebrate Dicer-independent, Ago-dependent mir-451 system into Drosophila cells and animals, and found it to be indeed functional. The maturation of mir-451-class substrates in vertebrate Ago2 and fly AGO1 appears similar. However, the segregation of the Drosophila miRNA and siRNA pathways introduces some unexpected twists, since mir-451-type hairpins are preferentially routed to fly AGO2 (Figure 8). In this effector, the biogenesis of ac-pre-mir-451 is arrested, due to methylation of its 3' end by Hen1.

The mir-451 system is not the only Dicer-independent pathway known. Several species of budding yeast, such as Saccharomyces castellii and Candida albicans, utilize non-canonical ‘Dicer’ proteins to generate small RNAs that load Ago (48). These species use RNase III proteins that are not evolutionarily related to canonical Dicer proteins, and they utilize a distinct mechanism to measure cleavage sites for small RNA duplexes (49). In addition, Schizosaccharomyces pombe is capable of loading Dicer-independent ‘primal RNAs’ directly into Ago1 (50). These yeast pathways differ from mir-451-type substrates in that they do not generate specific miRNAs, but instead sample short RNAs from across transposons, or from the transcriptome in general. However, Neurospora crassa can utilize ribonucleases other than Dicer, such as the mitochondrial RNase MRPL3, to generate specific small RNAs that load into its Argonaute proteins (51). In addition, Neurospora Argonaute QDE-2 serves essential
non-catalytic functions for the biogenesis of certain noncanonical miRNAs (52). Most recently, we used a ‘mix-and-match’ approach to combine Drosha-independent viral miRNA biogenesis strategies with Dicer-independent mir-451 biogenesis, yielding multiple types of RNase III-independent miRNA substrates in mammalian cells (53). Studies such as these suggest that there may yet be additional endogenous Dicer-independent miRNA pathways still to be recognized.

Another theme that grows from the mir-451 system is the appreciation that Ago proteins are not only receptacles of processed short RNAs, but can play important roles in...
their biogenesis. For example, it was recently found that the Slicer activity of C. elegans ALG1/2 is required, in some still undefined role, to promote miRNA biogenesis (54). Altogether, such findings may be potentially interpreted to reflect a precedent ‘Ago-first’ small RNA world that may have existed before the molecular coupling of RNase III enzymes with Ago proteins that is currently deeply embedded in most species with the capacity for RNAi. Our initial sequencing analyses of der-1 knockdown in S2 cells indicate that this condition permits non-canonical substrates to accumulate in AGO1 complexes (Figure 7). Therefore, it should be interesting in the future to sequence AGO1-IP contents from der-1 depletions of other types of cultured cells, or perhaps by examining der-1 mutant tissues. Such profilings might reveal endogenous Ago-dependent substrates that are not predominantly expressed in S2 cells.

Unexpected interplay of 3’–5’ exoribonucleases for maturation of ac-pre-miRNAs

Although the identity of the 3’ resesetase that matures miR-451 is presently elusive, our studies provide useful information regarding this process. First, our finding that the maturation of ac-pre-miR-451 in AGO2 is blocked in a Hn1-dependent manner, provides strong evidence that 3’ reseration is performed by a 3’–5’ exoribonuclease. Moreover, these data indicate that fully matured miR-451 species do not exchange between Drosophila AGO1 and AGO2 complexes, similar to our previous findings that mature miR-451 species do not exchange between mammalian Ago2 and non-slicing Argonautes such as Ago1 and Ago3 (11).

Second, we used knockdown assays to rule out that the Nibbler 3’–5’ exoribonuclease, which trims mature single-stranded miRNAs in AGO1 (30,31), is required for maturation of miR-451. It is currently difficult to imagine how Nibbler distinguishes these classes of ‘long’ RNAs associated with AGO1, and indicates mechanistic sophistication by this enzyme that remains to be understood. Beyond this, we unexpectedly observed that depletion of Nibbler actually increased the 3’ reseration of miR-451. Taken at face value, these data suggest a competition for substrate access between Nibbler and the ‘Resector’ enzyme for miR-451-type molecules, and conversely, the existence of preferred substrates of these exoribonucleases (Figure 8). Since nibbler knockdown also seemed to increase the total amount of mir-451-associated species in AGO1 (Figure 6), it also seems that Nibbler may also regulate the flux of loading or perhaps turnover of AGO1 complexes. Either outcome would represent a novel function for Nibbler, which has thus far been implicated only in the trimming of species in mature AGO1 complexes (30,31). These scenarios deserve further study.

Third, we used an in vitro assay to show that miR-451 resesetase activity resides in a soluble fraction and is likely Mg sensitive. Curiously, we were barely able to detect ‘Resector’ activity from S2 cell lysates, but it was more robust in ovary lysates. This is notable, since a conceptually similar activity in silkworm ovarian cells trims the 3’ ends of piRNAs in Piwi proteins (‘Trimmer’), but is apparently completely insoluble (42). To our knowledge, Trimmer activity has not been investigated in Drosophila, but these observations suggest that ovarian cells may harbor distinct 3’–5’ exoribonucleases, with specificity for immature RNAs loaded into Piwi proteins and immature RNAs loaded into Slicer Argonaute proteins. Such a conjecture awaits knowledge of the cognate molecular identities of these enzymes. Along with our previous finding that the RNA exosome trims the 3’ ends of a subclass of splicing-derived hairpins that require trimming to generate the pre-miRNA substrate (40), and the recent report that the Perlman syndrome 3’–5’ exoribonuclease Dis3L2 specifically degrades uridyalted pre-let-7 hairpins (55), there is clearly a diversity of exoribonucleases that shape the 3’ ends of small RNAs relevant to Argonaute pathways (56). It will be productive in the future to understand the full range of RNases that regulate small RNA sequence, abundance, and function, as well as to understand the nature of their potentially competitive or collaborative activities.

ACCESSION NUMBERS

The sRNA sequencing data were deposited at the NCBI Gene Expression Omnibus under accession GSE51585.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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