Poly(A)-specific ribonuclease sculpts the 3' ends of microRNAs

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ABSTRACT

The 3' ends of metazoan microRNAs (miRNAs) are initially defined by the RNase III enzymes during maturation, but subsequently experience extensive modifications by several enzymatic activities. For example, terminal nucleotidyltransferases (TENTs) elongate miRNAs by adding one or a few nucleotides to their 3' ends, which occasionally leads to differential regulation of miRNA stability or function. However, the catalytic entities that shorten miRNAs and the molecular consequences of such shortening are less well understood, especially in vertebrates. Here, we report that poly(A)-specific ribonuclease (PARN) sculpts the 3' ends of miRNAs in human cells. By generating PARN knockout cells and characterizing their miRNAome, we demonstrate that PARN digests the 3' extensions of miRNAs that are derived from the genome or attached by TENTs, thereby effectively reducing the length of miRNAs. Surprisingly, PARN-mediated shortening has little impact on miRNA stability, suggesting that this process likely operates to finalize miRNA maturation, rather than to initiate miRNA decay. PARN-mediated shortening is pervasive across most miRNAs and appears to be a conserved mechanism contributing to the 3' end formation of vertebrate miRNAs. Our findings add miRNAs to the expanding list of noncoding RNAs whose 3' end formation depends on PARN.

Keywords: PARN; microRNA; isomiR; trimming; tailing

INTRODUCTION

MicroRNAs (miRNAs) are small regulatory RNAs of ~22 nucleotides (nt) that are processed from characteristic hairpin structures embedded in longer precursor transcripts (Ha and Kim 2014; Bartel 2018). In the canonical biogenesis pathway, the RNase III enzymes DROSHA and DICER sequentially cleave the hairpin to produce a miRNA duplex. An Argonaute (AGO) protein incorporates the duplex and selects one strand to form a functional miRNA-induced silencing complex (miRISC), which recognizes complementary sites within target mRNAs to direct their post-transcriptional repression. The broad influence of miRNAs on the mammalian transcriptome is manifested by the observations that each miRNA down-regulates hundreds of mRNAs (Lim et al. 2005), and more than 60% of

mRNAs have selectively maintained pairing to at least one miRNA (Friedman et al. 2009).

With the advent of high-throughput sequencing technologies, it became clear that a single miRNA can exist in multiple isoforms in cells. These so-called “isomiRs” (Morin et al. 2008) arise from diverse sources, including alternative cleavage of precursors by the RNase III enzymes, exonuclease-mediated trimming, untemplated nucleotide addition at the 3' end, and less frequently, adenosine-to-inosine RNA editing (Gebert and MacRae 2019). Because the targeting specificity of a metazoan miRNA is mainly determined by a short stretch of nucleotides located near the 5' end, called the miRNA “seed” (Bartel 2009), 5' isomiRs are expected to regulate distinct sets of mRNAs. However,

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only a few exceptional miRNA genes produce alternative 5' isoforms at levels sufficient to direct the repression, and most conserved miRNAs have very consistent 5' ends (Ruby et al. 2006, 2007; Chiang et al. 2010).

On the other hand, variations near the 3' ends of miRNAs are much more prevalent. In *Drosophila*, the 3'-to-5' exoribonuclease Nibbler trims the 3' ends of more than a quarter of miRNAs, and such 3' trimming is thought to enhance miRNA targeting, at least for miR-34 (Han et al. 2011; Liu et al. 2011). In vertebrates, miR-451 exhibits significant 3' heterogeneity, which is in part attributed to its noncanonical biogenesis: pre-miR-451 is too short to be processed by DICER and instead directly loaded into AGO2, which cleaves the 3' arm of the hairpin in the middle to generate a ~30 nt AGO2-cleaved pre-miR-451 (ac-pre-miR-451) (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang et al. 2010). Poly(A)-specific ribonuclease (PARN) finalizes the maturation of this atypical miRNA by trimming the 3' end of ac-pre-miR-451 to produce the ~23–26 nt mature miR-451 (Yoda et al. 2013). Importantly, many canonical miRNAs in vertebrates also possess heterogeneous 3' ends reminiscent of exoribonucleolytic trimming (Kozomara and Griffiths-Jones 2014; Fromm et al. 2015), such that the overall 3' heterogeneity of miRNAs is comparable in human and *Drosophila* cells (Han et al. 2011). However, it is currently unclear whether a general miRNA trimmer, similar to Nibbler in *Drosophila*, exists in vertebrates.

In addition to ribonucleases, terminal nucleotidyltransferases (TENTs) also contribute to 3' isomiR diversity by adding one or a few nucleotides, usually A or U, to the 3' end of miRNAs (Burroughs et al. 2010; Chiang et al. 2010; Wyman et al. 2011; Thornton et al. 2014). Untemplated nucleotide addition or "tailing" occasionally modulates the stability or function of miRNAs. For example, TENT2 catalyzes the monoadenylation of miR-122, which is reportedly associated with its increased stability (Katoh et al. 2009; D'Ambrogio et al. 2012). On the contrary, monoadenylation of miR-21 by TENT4B is proposed to serve as a decay signal (Boele et al. 2014). TUT4-mediated uridylation of miR-26 abrogates its regulatory activity and derepresses cytokine expression (Jones et al. 2009), while the same modification marks many miRNAs for degradation during T cell activation (Gutiérrez-Vázquez et al. 2017). The basic enzymology of untemplated nucleotide addition is established by virtue of several transcriptome-wide studies, with multiple TENTs often operating redundantly (Burroughs et al. 2010; Wyman et al. 2011; Thornton et al. 2014). On the other hand, it is less well understood whether this type of modification is generally reversible, and if so, which ribonucleases are responsible for the clearance of untemplated nucleotides. Previously, PARN has been implicated in the degradation of adenylated miR-122 and miR-21 (Boele et al. 2014; Katoh et al. 2015), but the role of PARN here is described as a "reader/effector" rather than an "eraser."

PARN is a member of the DEDD family of 3'-to-5' exonucleases with a marked nucleotide preference toward A (Zuo and Deutscher 2001). Because of its unique ability to interact with the 7-methylguanosine cap of mRNAs, PARN has long been thought to be primarily involved in mRNA metabolism (Balatsos et al. 2012; Virtanen et al. 2013). However, accumulating evidence suggests that PARN-mediated deadenylation may be limited to a small subset of mRNAs and/or may occur mostly in particular biological contexts, rather than comprising the default pathway of mRNA deadenylation and decay (Yamashita et al. 2005; Son et al. 2018; Yi et al. 2018). For example, knockdown of PARN did not evidently alter the poly(A) tail length or abundance of mRNAs in HeLa cells (Son et al. 2018; Yi et al. 2018). Instead, PARN has recently emerged as a key player in the maturation of a wide variety of noncoding RNAs, including snoRNAs and scaRNAs (Berndt et al. 2012; Son et al. 2018), telomerase RNA component (TERC) (Moon et al. 2015; Nguyen et al. 2015; Tseng et al. 2015), miR-451 (Yoda et al. 2013), 18S rRNA (Ishikawa et al. 2017; Montellese et al. 2017), and Y RNAs (Shukla and Parker 2017). The precursors of these RNAs carry small extensions at their 3' ends that are often followed by untemplated oligo(A) tails. PARN recognizes and removes the oligo(A) tails and subsequently polishes the 3' ends of these noncoding RNAs to complete their biogenesis. Because oligo(A) tails in nuclear RNAs serve as a mark for exosome-mediated destruction (Houseley et al. 2006; Vanacova and Stefl 2007), PARN-mediated deadenylation and 3' end formation play a pivotal role in maintaining the stability of many nuclear noncoding RNAs, including TERC (Berndt et al. 2012; Moon et al. 2015; Nguyen et al. 2015; Tseng et al. 2015; Son et al. 2018). On the other hand, it is not fully understood whether PARN generally targets miRNAs, a major class of cytoplasmic noncoding RNAs. Previous studies documented miRNA substrates of PARN (Yoda et al. 2013; Boele et al. 2014; Katoh et al. 2015). However, they focused on a few model miRNAs and did not provide a comprehensive view on the role of PARN in miRNA metabolism.

Here, we expand the repertoire of noncoding RNA substrates of PARN to most canonical miRNAs, the biogenesis of which depends on both DROSHA and DICER. We reaffirm the notion that the sequence of a miRNA in cells does not necessarily correspond to that defined by the RNase III enzymes, because of frequent terminal modifications. We find that some miRNAs released by DICER processing possess slightly extended 3' ends than their actual cellular populations, and that PARN is responsible for the 3' shortening of such miRNAs in cells. By ablating PARN in HeLa cells, we demonstrate that PARN-mediated miRNA shortening is widespread across miRNAs and occurs through two distinct modes, depending on the origin of the resected nucleotide. PARN functions as a "trimmer" to digest the genome-encoded 3' extensions of miRNAs, and as a

"de-tailor" to erase or reduce the size of untemplated nucleotide additions. Collectively, our findings unveil the role of PARN in sculpting the 3' ends of miRNAs, which contributes to miRNA diversity.

RESULTS

The cellular definition of miR-362-5p

The stem region of natural miRNA hairpins is frequently enriched for various structural imperfections, such as

wobbles, mismatches, and bulges (Fang and Bartel 2015; Liu et al. 2016). While examining the effect of such structural motifs on DICER processing, we became interested in miR-362, a mammalian miRNA whose precursor contains a relatively large bulge compared with other pre-miRNAs (Fang and Bartel 2015). Both the 5' and 3' ends of human and mouse pre-miR-362 were consistently annotated by two different miRNA databases based on small RNA sequencing (Kozomara and Griffiths-Jones 2014; Fromm et al. 2015). We also performed in vitro DROSHA

FIGURE 1. Characterization of miR-362-5p biogenesis in vitro and expression in vivo. (A) Predicted secondary structures of human and mouse pri-miR-362 (Zuker 2003). The sequences of miR-362-5p and miR-362-3p annotated in miRBase are shown in red. The purple and green arrowheads indicate the DROSHA and DICER cleavage sites determined in vitro, respectively. (B) In vitro DICER processing of pre-miR-362. Body-labeled (lanes 1–10) or 5'-labeled pre-miR-362 (lanes 11–16) was incubated with the immunopurified DICER/TRBP complex for the indicated time period. Dashed lines indicate discontinuous lanes from the same gel. (C) Characterization of DICER processing products. Cold pre-miR-362 was processed by DICER as in B for 30 min and the processing products were analyzed by small RNA northern blotting. Synthetic RNA oligonucleotides (1 fmole) corresponding to human miR-362-5p (26 nt) and miR-362-3p (22 nt) were loaded in the same gel to serve as a positive control and size reference (lanes 1–2 and 7–8). (D) miR-362-5p expression in cells. Total RNA isolated from human [BE(2)-C, MDA-MB-231, SW480] or mouse (MEF, mMSC, NIH-3T3) cell lines was subjected to northern hybridization. One femtomole of synthetic human miR-362-5p (26 nt) was loaded in the same gel to serve as a positive control and size reference (lane 1). U6 snRNA and 5S rRNA served as loading controls. (E) Primer extension analysis of miR-362-5p. A 5'-labeled DNA oligonucleotide complementary to the central region of miR-362-5p (nt 5–22) was annealed to the total RNA used in D and reverse transcribed. The capital "R" in the sequence of miR-362-5p indicates G or A in the human or mouse orthologs, respectively. Antisense DNA oligonucleotides corresponding to nt 3–22 and nt 1–22 of miR-362-5p were 5'-labeled and used as size markers. Reactions with water (No RNA) and 1 fmole of synthetic human miR-362-5p (26 nt) served as a negative and positive control, respectively (lanes 1 and 2). Ethidium bromide staining of tRNAs served as a loading control. (F) Small RNA sequencing reads mapped to the miR-362 locus. Reads from mouse tissues were obtained elsewhere (Chiang et al. 2010). For simplicity, we displayed only the reads that share the 5' end with the miRBase sequence and that do not contain untemplated nucleotides at the 3' end. The sequence of miR-362-5p defined by DICER in vitro is shown in green, with the cleavage site indicated by a green arrowhead. The sequence of miR-362-5p annotated in miRBase is shown in red. The fraction of each read was calculated and is plotted on the right side.

processing of human and mouse pri-miR-362 and sequenced the resulting pre-miR-362, which confirmed the annotations (Fig. 1A; Supplemental Fig. 1). In silico prediction of RNA secondary structure (Zuker 2003) indicated that pre-miR-362 is a 59 nt fragment containing a canonical 2 nt overhang at its 3' end and a trinucleotide bulge in the middle of the 5p strand (nt 11–13; Fig. 1A).

To characterize DICER processing of pre-miR-362 in vitro, we prepared body-labeled pre-miR-362 and incubated it with Flag-immunoprecipitate (Flag-IP) obtained from HEK293T cells overexpressing Flag-DICER1 and myc-TRBP. In vitro processing of pre-miR-362 with the immunopurified DICER complex released three major products, plausibly corresponding to the 5p strand, 3p strand, and terminal loop (Fig. 1B; lanes 1–10). When we used 5'-labeled pre-miR-362 as a substrate, only the uppermost band was produced, demonstrating that this largest product represented the 5p strand of the miR-362 duplex (Fig. 1B; lanes 11–16). Small RNA northern blot analysis of the DICER processing products corroborated this finding (Fig. 1C; left panel), and further identified the middle band as the 3p strand (Fig. 1C; right panel). Cloning and sequencing of the DICER products (~20–30 nt) allowed us to precisely map the cleavage sites on pre-miR-362, which divide the 59 nt hairpin into the 26 nt 5p strand, the 22 nt 3p strand, and the 11 nt terminal loop (Fig. 1A; Supplemental Fig. 2). Collectively, these results indicate that miR-362-5p defined by DROSHA and DICER is 26 nt in length, which is somewhat longer than the miRBase annotation of 24 nt (Fig. 1A).

Next, we investigated the expression of endogenous miR-362-5p in a panel of human and mouse cells by northern hybridization. Curiously, all cell lines tested expressed fragments that were ~2–4 nt shorter than the 26 nt miR-362-5p defined in vitro, with the heterogeneity more prominent in mouse cells (Fig. 1D). Primer extension experiments using a centrally designed primer (complementary to nt 5–22 of miR-362-5p) demonstrated that the endogenous miR-362-5p isoforms share the 5' end, suggesting the possibility of 3' end variation (Fig. 1E). To comprehensively catalog the isomiR repertoire of miR-362-5p, we analyzed our own and publically available small RNA deep sequencing data obtained from HeLa S3 cells and mouse tissues, respectively. Consistent with the results of northern blotting and primer extension assays, miR-362-5p was represented by more than two dominantly abundant isoforms of 22–26 nt that differed at their 3' ends (Fig. 1F). Taken together, these findings suggest that the “cellular definition” of a miRNA does not necessarily coincide with the definition made by the core miRNA biogenesis enzymes.

Biogenesis of miR-362-5p involves 3' exoribonucleolytic trimming

To gain insights into how the shorter isoforms of miR-362-5p are generated, we overexpressed pri-miR-362 in

HEK293T cells and analyzed the expression of miR-362-5p by northern blotting. Ectopic expression of pri-miR-362 faithfully recapitulated the production of two distinct 3' isoforms of miR-362-5p (Fig. 2A), suggesting that the observed 3' heterogeneity may be a sequence-driven feature of miR-362 biogenesis. Because pre-miR-362 was cleaved by DICER without much variation in vitro (Fig. 1A–C), we suspected that the 26 nt miR-362-5p liberated by DICER may be subsequently resected at the 3' end by unknown ribonuclease(s). To test this possibility, we transfected HeLa S3 cells with a synthetic miR-362 duplex consisting of the 26 nt 5p strand and the 22 nt 3p strand. Notably, miR-362-5p from the synthetic duplex was trimmed down to ~22–24 nt species with a pattern similar to that observed for its endogenous or ectopically expressed counterpart, implying the existence of 3' trimming activity toward this specific miRNA after DICER processing (Fig. 2B; lanes 3 and 6). Next, we incorporated an RNase-resistant 2'-O-methyl (2'-OMe) modification at the 25th or 23rd position of miR-362-5p (designated m1 or m2, respectively) and introduced a duplex bearing the mutant 5p strand into HeLa S3 cells. The m1 duplex no longer produced the shorter isoforms (Fig. 2B; lanes 4 and 7), while the m2 substitution abolished the production of miR-362-5p isoforms shorter than 24 nt (Fig. 2B; lanes 5 and 8). These results suggest that the characteristic 3' heterogeneity of miR-362-5p is conferred after DICER processing by the action of 3'-to-5' exoribonuclease(s).

To further demonstrate the existence of such 3' trimming activity in cells, we tested whether the 3' end of miR-362-5p could be resected in vitro. To this end, we incubated the miR-362 duplex containing 5'-labeled 26 nt miR-362-5p with a cytoplasmic lysate obtained from HEK293T cells overexpressing Flag-AGO2. The resulting Flag-AGO2/miR-362-5p complex was immunopurified and subjected to further incubation with HeLa whole cell lysate. Notably, WT miR-362-5p, but not the m1 mutant, was trimmed over time (Fig. 2C), with the size distribution of trimming products resembling that of miR-362-5p isoforms in cells (Figs. 1D, 2A,2B). Trimming was consistently observed when we used other human AGO proteins (AGO1, AGO3, and AGO4) expressed in HEK293T cells (Supplemental Fig. 3A,C), excluding the possibility that the identity of AGO proteins may specify the 3' trimming of miR-362-5p. Although miRNAs tend to become highly stable once loaded into AGO proteins (De et al. 2013; Park et al. 2017), because their 5' and 3' ends are anchored in MID and PAZ domains, respectively (Elkayam et al. 2012; Schirle and MacRae 2012), our data suggest that the 3' ends of certain miRNAs can be exposed to and subsequently trimmed by cellular 3'-to-5' exoribonucleases. To investigate whether the interaction between the miRNA 3' end and the AGO protein plays a role in trimming, we mutated Y311 of AGO2, which is located within the PAZ domain and makes contact with the 3' nucleotides of miRNAs (Elkayam et al.

FIGURE 2. The shorter isoforms of miR-362-5p are generated after DICER processing by 3'-to-5' exonuclease activity. (A) Recapitulation of miR-362-5p biogenesis in HEK293T cells. The cells were transfected with the indicated pri-miR-362 expression plasmid and the maturation of miR-362-5p was examined by small RNA northern blotting and primer extension assay. (B) The 3' heterogeneity of miR-362-5p arises after DICER processing. The *upper* panel shows the structure of a miR-362 duplex, with the 2'-O-methyl-modified nucleotides in the mutant 5p strands indicated by arrows. Duplexes bearing wild-type (WT) or mutant 5p strands were individually introduced into HeLa S3 cells and the processing of ectopic miR-362-5p was investigated by small RNA northern blotting and primer extension assay. (C) AGO2-loaded miR-362-5p is trimmed *in vitro*. The indicated 5'-labeled 5p strand was annealed to a cold 3p strand to produce the miR-362 duplex, which was incubated with cytoplasmic lysate from HEK293T cells overexpressing FLAG-AGO2. The resulting FLAG-AGO2/miR-362-5p ribonucleoprotein complex was immunoprecipitated and subjected to further incubation with HeLa S3 whole cell lysate for the indicated time period.

2012; Park et al. 2017). Strikingly, miR-362-5p loaded into this mutant AGO2 was trimmed more progressively and nonspecifically than when loaded into WT AGO proteins (Supplemental Fig. 3B,C), without the accumulation of the characteristic 24 nt species observed *in vivo* and *in vitro* (Figs. 1 and 2; Supplemental Fig. 3A). These results indicate that the 3' trimming of miR-362-5p takes place within the miRISC and is influenced by the miRNA 3' end-PAZ interaction. In summary, the distinguishing 3' end heterogeneity of miR-362-5p appears to result from the collaboration of multiple factors, including DICER, AGO, and an unknown "trimmer".

PARN emerges as a miR-362-5p trimmer

To identify the miR-362-5p trimmer, we individually depleted several 3'-to-5' exonucleases previously implicated in the miRNA pathway in HEK293T cells and examined the trimming of miR-362-5p. Notably, knockdown of PARN significantly decreased the fraction of the shorter miR-362-5p isoform, while knockdown of other exonucleases did not obviously change the ratio between the two isoforms (Fig. 3A; Supplemental Fig. 4A).

To rule out the possibility of off-target effects of RNAi, we rescued PARN-depleted HEK293T cells with an RNAi-

resistant PARN expression plasmid. Complementation of PARN activity restored the normal isoform ratio, suggesting that PARN is indeed responsible for the 3' trimming of miR-362-5p (Fig. 3B; lanes 3–5 and 9–11). Increasing the amount of PARN plasmid promoted the generation of even shorter isoforms of ~22 nt (Fig. 3B; lanes 6–8 and 12–14), which was evidently observed for endogenous mouse miR-362-5p (Fig. 1D). Of note, overexpression of PARN above a certain level appeared to reduce the abundance of mature miR-362-5p (Fig. 3B; compare lanes 3 and 8 or 9 and 14). However, we believe that this may not represent a true biological regulation by PARN, but may reflect experimental artifacts associated with supra-physiological expression of the enzyme, because miR-362-5p was not apparently stabilized upon PARN depletion (Fig. 3B; lanes 3–4 and 9–10; discussed further below). To corroborate these results, we overexpressed a PARN mutant, D28A, in which one of the four catalytic residues was substituted with alanine. This mutant is catalytically deficient but still retains the ability to bind substrates, and is therefore expected to act as a *trans*-dominant negative mutant (Ren et al. 2002, 2004; Lai et al. 2003). As anticipated, overexpression of D28A PARN caused a shift in the isoform ratio toward the longer one, without altering the level of miR-362-5p (Fig. 3C). These data clearly indicate that

FIGURE 3. PARN functions as a miR-362-5p trimmer. (A) Several 3'-to-5' exonucleases associated with the miRNA pathway were individually depleted in HEK293T cells and the maturation of ectopically expressed miR-362-5p was monitored. (B) Complementmentation of PARN activity restores the normal isoform ratio of miR-362-5p. HEK293T cells were first transfected with either siGFP (lanes 3 and 9) or siPARN (lanes 4–8 and 10–14). Twenty-four hours after the first transfection, a fixed amount of the pri-miR-362 expression plasmid (1 μ g per 35-mm dish) and variable amounts of the PARN expression plasmid (0.128–2 μ g) were introduced and further incubated for 48 h. (C) Overexpression of D28A PARN interferes with the trimming of miR-362-5p by endogenous PARN. (D) PARN-mediated trimming takes place after DICER processing. The indicated miR-362 duplex was introduced into HeLa S3 cells pretreated with siGFP or siPARN, and the 3' processing of miR-362-5p was analyzed. In A, C, and D, the levels of the 26 nt and $\leq$ 24 nt isoforms of mature miR-362 were individually measured by densitometry, and the fraction of the shorter isoforms ($\leq$ 24 nt) is plotted on the right side. Error bars represent standard error of the mean (SEM) from two biologically independent experiments, and asterisks indicate statistical significance of the decrease in the fraction of the shorter isoform(s) calculated by one-tailed paired t-test (*] $P < 0.05$, **] $P < 0.01$, ***] $P < 0.005$).

PARN functions as a key player in the 3' end formation of miR-362-5p.

To address whether PARN trims the 3' end of miR-362-5p after DICER processing, we reduced PARN expression in HeLa S3 cells and introduced WT or m2 miR-362 duplexes. Consistent with the results obtained in HEK293T cells, the levels of shorter miR-362-5p isoforms were markedly diminished upon PARN knockdown (Fig. 3D; Supplemental Fig. 4B). Interestingly, human and mouse miR-362-5p responded slightly differently to the reduction of PARN activity. For example, the 24 nt isoform of human miR-362-5p was barely produced in PARN-depleted cells (Fig. 3D; lanes 3–6), whereas the ~22–23 nt isoforms of mouse miR-362-5p were more prominently affected by PARN

knockdown than the 24 nt isoform (Fig. 3D; lanes 7–10). Human and mouse miR-362-5p differ by only 1 nt at the 23rd position (G and A in human and mouse, respectively; see Fig. 1A). The nucleotide identity at this position may modulate PARN-mediated trimming in cells, leading to the differential trimming patterns observed in the two orthologous miRNAs.

Finally, we tested the role of PARN as a miRNA trimmer in vitro. We incubated miR-362-5p loaded into Flag-AGO2 with lysates from HeLa S3 cells in which PARN was overexpressed or depleted (Fig. 4A). Notably, the 3' trimming of miR-362-5p was severely compromised when a lysate from PARN-depleted cells was used (Fig. 4B; lanes 7–12), suggesting that PARN is largely responsible for the trimming

FIGURE 4. PARN trims miR-362-5p in vitro. (A) Western blot analysis of lysates from HeLa S3 cells in which PARN was depleted or overexpressed. α -Tubulin served as a loading control. (B) In vitro trimming of AGO2-loaded miR-362-5p with lysates from PARN-manipulated HeLa S3 cells. (C) Coomassie brilliant blue (CBB) staining of recombinant GST-PARN produced in *E. coli*. (D) In vitro trimming of AGO2-loaded miR-362-5p with recombinant GST-PARN. In B and D, the fraction of trimmed species (~21–25 nt) was calculated by densitometry and is plotted on the right side, with error bars representing SEM from two independent experiments.

activity present in HeLa whole cell lysate. On the other hand, a lysate containing an overexpressed PARN protein greatly accelerated the trimming (Fig. 4B; lanes 13–18). In addition, recombinant PARN purified from *E. coli* to near homogeneity faithfully recapitulated the 3' trimming of miR-362-5p (Fig. 4C,D), excluding the possibility that PARN may require cofactor(s) to execute miRNA trimming. These results collectively demonstrate that PARN is a bona fide trimmer acting on the 3' end of miR-362-5p.

The impact of PARN on the miRNAome

PARN is involved in the biogenesis and 3' end formation of miR-451, an atypical miRNA whose precursor bypasses DICER processing (Yoda et al. 2013). Notably, our findings suggest that PARN may also polish the 3' ends of canonical miRNAs supposedly longer than the average (Figs. 1–4). To examine the impact of PARN on the mammalian miRNAome, we generated PARN knockout (KO) HeLa S3 cells using CRISPR/Cas9 technology. A guide RNA was designed to target the second coding exon of the PARN gene (Fig. 5A). Following clonal selection, we obtained two independent clones with a complete absence of the PARN protein (Fig. 5B). Sequencing-based genotyping confirmed the presence of frame-shifting indels on the targeted locus (Fig. 5C). PARN KO cells exhibited slightly retarded proliferation but were still viable throughout multiple passages (data not shown), suggesting that the activity of PARN is not essential, at least in cultured cells.

We performed small RNA deep sequencing on ~18–30 nt RNA from parental cells and individual PARN KO clones.

With the idea that PARN-mediated trimming of 3' ends would reduce the size of miRNAs, we first analyzed the length distribution of miRNAs in each sample. Notably, the distribution was shifted by ~1 nt toward longer species upon ablation of PARN, such that the length of miRNAs from PARN KO cells peaked at 23 nt rather than 22 nt (Fig. 5D). On the other hand, the steady-state levels of miRNAs were not altered apparently in PARN KO cells (Fig. 5E), suggesting that PARN regulates the length, but not the abundance, of mature miRNAs.

Mechanisms of PARN-mediated miRNA shortening

Our data from biochemical and cell-based assays suggest a model for the biogenesis of miR-362-5p: DICER cleaves pre-miR-362 asymmetrically, perhaps because of the internal bulge, such that the resulting miRNA duplex contains the 26 nt miR-362-5p. Once selected by AGO proteins, this strand is subsequently trimmed at the 3' end by PARN to generate the ~22–24 nt isoforms (Figs. 1–4). Thus, although their upstream effectors are quite different, the genome-matching (GM) portions of both miR-451 and miR-362-5p are actively digested by PARN (Yoda et al. 2013). On the other hand, PARN is known to remove post-transcriptionally added mono or oligo(A) tails from miR-21 and miR-122 to promote their destabilization (Boele et al. 2014; Katoh et al. 2015).

Based on these observations, we hypothesized that PARN may shorten the length of miRNAs by two distinct modes, depending on the origin of the nucleotide being resected. PARN may function as a “trimmer” to nibble

FIGURE 5. The impact of PARN on the miRNAome. (A) Domain architecture of human PARN. The four catalytic residues are indicated. The red scissors mark the position of the site targeted by CRISPR/Cas9 nuclease. (B) Western blot analysis of lysates from parental HeLa S3 cells and two independent *PARN* KO clones. α -Tubulin and β -actin served as loading controls. (C) PCR-based genotyping of the targeted locus in *PARN* KO cells. Exonic and intronic sequences are indicated by black uppercase and gray lowercase letters, respectively. The nucleotide sequence complementary to the guide RNA and the protospacer adjacent motif (PAM) are shown in red and blue, respectively. The type and frequency of indels identified by Sanger sequencing is indicated on the right. (D) PARN reduces the length of mature miRNAs. The length distribution of total small RNA sequencing reads mapped to the miRNA loci is presented. (E) PARN does not affect the abundance of mature miRNAs. Normalized read counts for individual miRNAs in each cell line, presented as reads per 30 million (RP30M), were compared. The boundaries for twofold expression changes are indicated by red dashed lines.

FIGURE 6. Two distinct modes of PARN-mediated miRNA shortening. (A) Illustration of the roles of PARN as a "trimmer" and a "de-tailor" in miRNA metabolism. The dominantly abundant miRNA isoform in cells, which would be the probable sequence annotated by sequencing-based databases, is shown in red. As a trimmer, PARN digests the genome-encoded 3' extensions of miRNAs such that the shorter species occasionally becomes most abundant. As a de-tailor, PARN removes untemplated nucleotides from the 3' end of miRNAs. (B) Separation of small RNA sequencing reads into two categories. GM reads refer to those that are perfectly and contiguously mapped to the genome. PM reads indicate those containing one or more non-GM nucleotides at the 3' end. The terms ("genome-matching" and "prefix-matching") and the illustration for each category were adopted or modified from a previous report (Reimão-Pinto et al. 2015). (C) Fraction of genome-matching (GM) and prefix-matching (PM) reads in the small RNA libraries of parental and *PARN* KO cells. (D) PARN functions as both a trimmer and a de-tailor. The length distributions of GM and PM reads are presented.

the portion of miRNA sequence encoded by the genome, and/or as a “de-tailor” to erase untemplated nucleotide addition (Fig. 6A). To investigate whether both modes do indeed operate, we divided small RNA sequencing reads into two categories: GM reads and prefix-matching (PM) reads (Fig. 6B). PM reads refer to those containing one or more non-GM nucleotides at the 3' end, and are therefore expected to represent unambiguous 3' nt addition events (Reimão-Pinto et al. 2015). Approximately 14% of total reads from parental HeLa S3 cells fell into this category, reaffirming widespread 3' modification of miRNAs by untemplated nucleotide incorporation (Fig. 6C). We individually examined the length distribution of reads from

each category, with the idea that the lengthening of miRNAs upon *PARN* deletion should be observed only in one category if one of the two modes dominates. This was not the case, however, because the lengths of both the GM reads and the PM reads were elongated in *PARN* KO cells to a similar extent (Fig. 6D). These data indicate that *PARN* functions as both a trimmer and a de-tailor in miRNA metabolism.

PARN as a general miRNA trimmer

To investigate the role of *PARN* as a miRNA trimmer in more detail, we calculated for each miRNA the fraction of

FIGURE 7. PARN as a general miRNA trimmer. (A) For each miRNA, “Fraction +1” was defined by the fraction of reads that contain one or more additional nucleotides at the 3' end, compared with the reference sequence annotated in miRBase. “Fraction +2” and “Fraction +3” were defined similarly, by the fraction of reads that contain two or more and three or more nucleotides at the 3' end, respectively. For simplicity and reliability, we considered GM reads only in these analyses and selected 247 miRNAs whose GM read counts exceed 1000 in parental HeLa S3 cells. See also Supplemental Table 2. (B) PARN-mediated miRNA trimming is prevalent. Changes in “Fraction +1”, “Fraction +2”, and “Fraction +3” upon *PARN* deletion were calculated for each miRNA by subtracting the fraction in parental cells from the fraction in each *PARN* KO clone. The 5p and 3p miRNAs are colored blue and turquoise, respectively. The miRNAs with more than 25% point changes in the 3'-extended fraction are shown in red. (C) Validation of PARN-mediated miRNA trimming. The expression and length distribution was examined by small RNA northern blotting for six miRNAs that exhibited significant changes in the 3'-extended fraction (miR-224-5p, miR-182-5p, miR-425-5p, miR-361-3p, miR-301a-3p, and miR-454-3p; see Fig. 7B) and two miRNAs with negligible changes (miR-16-5p and miR-27a-3p). For miRNAs trimmed by PARN, the length distribution of sequencing reads for each miRNA in parental and *PARN* KO#2 cells is shown on the right side of the blot image. U1 snRNA, U6 snRNA, lysyl tRNA ($tRNA^{Lys}$), and 5S rRNA served as loading controls. (D) PARN-mediated 3' trimming of miRNAs does not affect their association with AGO proteins. Endogenous miRISC was purified from lysates of parental or *PARN* KO#2 cells by AGO-APP, and the enrichment and length distribution of copurifying miRNAs was examined by northern blotting. $tRNA^{Lys}$ and 5S rRNA served as loading controls for input lysates and negative controls for AGO-APP.

GM reads that have extensions beyond the 3' end of the reference sequence annotated in miRBase (designated "Fraction +1") (Fig. 7A). Because miRBase entries are deposited mainly on the basis of high-throughput sequencing, we reasoned that the miRBase sequence would represent miRNA species that had undergone post-processing modifications such as 3' trimming, as illustrated by the case of miR-362-5p (Fig. 1). Keeping this in mind, we examined how the fraction of 3'-extended reads changed in *PARN* KO cells, with the expectation that the difference would reflect the extent to which a given miRNA is subjected to *PARN*-mediated trimming. Among the 247 miRNAs examined, 179 (~72%) were more frequently 3'-extended in both *PARN* KO clones than in parental cells, and 77 (~31%) exhibited average changes in the 3'-extended fraction of more than 5 percent points, demonstrating widespread *PARN*-mediated trimming of miRNAs (Fig. 7B; [Supplemental Fig. 5A](#)). Both 5p and 3p miRNAs were lengthened at their 3' ends in *PARN* KO cells (Fig. 7B; [Supplemental Fig. 5A](#)), supporting the notion that *PARN* trims mature miRNAs liberated by DICER processing (Figs. 1–4). Some miRNAs appeared to be efficiently trimmed by *PARN*, with the changes in the 3'-extended fraction even exceeding 25 percent points, as exemplified by miR-425-5p, miR-361-3p, miR-182-5p, miR-301a-3p, and our experimental model miRNA, miR-362-5p (Fig. 7B). We extended our analysis to the GM reads containing two or more nucleotides at the 3' end ("Fraction +2"), because some miRNAs could potentially be missing from the current analysis if their *PARN*-mediated trimming mostly occurs outside of the reference sequence. As one such miRNA, miR-224-5p ranked 92nd in the first analysis with an average change of ~2.8 percent points, but ranked 1st in the second analysis with an average change of ~40 percent points (Fig. 7B; [Supplemental Fig. 5A,B](#)). The reference sequence of miR-224-5p is 21 nt in length, but this miRNA existed in two dominantly abundant isoforms of 22 and 24 nt in parental cells, the latter of which was likely trimmed by *PARN* to generate the former (Fig. 7C; [Supplemental Fig. 5B](#)). The fraction of reads with three or more GM nucleotides ("Fraction +3") was generally low across most miRNAs and barely changed upon *PARN* deletion, except for miR-224-5p (Fig. 7B; [Supplemental Fig. 5](#)), suggesting that *PARN* tailors miRNAs to the reference sequence by trimming their 3' ends by ~1–2 nt.

To validate our analysis, we performed small RNA northern blotting with total RNA prepared from parental and *PARN* KO cells. Trimming was clearly manifested by the shift of major miRNA isoforms toward ~1–2 nt longer species in *PARN* KO cells, which was in good agreement with the observed changes in the length distribution of sequencing reads for individual miRNAs (Fig. 7C). One prominent example was miR-182-5p, the dominantly abundant isoform of which was 24 nt in parental cells, but became 26 nt in both *PARN* KO clones. To address whether

PARN-mediated trimming of miRNAs operates in other cell types, we depleted *PARN* in four different cell lines and monitored changes in the isoform distribution of miRNAs by northern blotting. Notably, the miRNAs that were trimmed in HeLa S3 cells were also consistently trimmed in all cell lines tested ([Supplemental Fig. 6A](#)), suggesting that the trimming of a specific miRNA is an inherent feature of its maturation rather than a cell-type-specific event. It is noteworthy that the composition of GM 3' nucleotides resected by *PARN* is surprisingly diverse and is not particularly enriched for A ([Supplemental Fig. 6B](#)), the nucleotide most preferred by *PARN* in vitro (Balatsos et al. 2012; Virtanen et al. 2013). The steady-state levels of all miRNAs tested were comparable in control and *PARN*-manipulated cells, reaffirming that *PARN*-mediated trimming does not generally lead to changes in miRNA stability (Fig. 5E).

To examine whether untrimmed miRNAs in *PARN* KO cells associate with AGO proteins as efficiently as trimmed miRNAs in parental cells, we utilized peptide-based affinity purification of AGO proteins (termed "AGO-APP") to isolate endogenous miRISC from HeLa whole cell lysate (Hauptmann et al. 2015). A small peptide derived from the AGO-binding domain of human TNRC6B (T6B) was fused to GST and used as a bait for AGO affinity purification ([Supplemental Fig. 7A](#)). As reported previously (Hauptmann et al. 2015), GST-T6B was capable of precipitating all human AGO proteins (AGO1–4) exogenously expressed in HEK293T cells ([Supplemental Fig. 7B](#)), and isolating the endogenous pool of AGO proteins equipped with miRNAs from HeLa S3 lysate ([Supplemental Fig. 7C](#)). Notably, AGO-APP from parental or *PARN* KO cells enriched the miRNAs to a similar extent, without altering the overall length distribution of the miRNA isoforms (Fig. 7D), suggesting that *PARN*-mediated trimming does not affect the interaction of miRNAs with AGO proteins.

To address whether 3' trimming affects miRNA-mediated gene silencing, we focused on miR-182-5p, one of the miRNAs most efficiently trimmed by *PARN* in HeLa S3 cells (Fig. 7B,C). We interfered with the function of miR-182-5p in mock- or *PARN*-depleted HeLa S3 cells by introducing 2'-OMe-modified anti-miR-182-5p, and measured the level of one of its target mRNAs, *FRS2*, which contains four canonical target sites for miR-182-5p in its 3'-UTR (Agarwal et al. 2015). The *FRS2* mRNA level was significantly increased upon transfection of anti-miR-182-5p, but the extent of its derepression was comparable in mock- and *PARN*-depleted cells ([Supplemental Fig. 8A,B](#)). A similar trend was observed for the expression of a luminescence-based reporter bearing one of the miR-182-5p target sites ([Supplemental Fig. 8C,D](#)), suggesting that *PARN*-mediated trimming is dispensable for miRNA-mediated regulation. Given that miRNA-mediated repression of mRNAs involves deadenylation followed by destabilization

FIGURE 8. PARN as a miRNA de-tailor. (A) Elevated miRNA tailing frequency in *PARN* KO cells. We restricted our analysis to the 267 miRNAs whose total read counts exceeded 1000 in parental HeLa S3 cells, which are listed in [Supplemental Table 3](#). The tailing frequency was calculated as the fraction of PM reads for each miRNA. Changes in the tailing frequency in *PARN* KO cells are shown. (B) Nucleotide composition of mono-nucleotide and dinucleotide miRNA tails in parental and *PARN* KO cells. (C) Changes in the frequencies of monoadenylation and monouridylation of individual miRNAs in *PARN* KO cells. Modification frequency was calculated for the 267 miRNAs used in A, as the fraction of reads containing the indicated untemplated nucleotide addition. (D) Changes in the frequencies of six types of modification (A, AA, AAA, U, UU, UUU) in *PARN* KO cells. The fold increases in the modification frequency are shown on the right. (E) Changes in the mean length of miRNA adenosine tails. We calculated the mean length of adenosine tails based on the frequencies of mono-, di-, and triadenylation, because the fraction of reads containing four or more untemplated nucleotides was very low across all small RNA libraries ([Supplemental Fig. 9A](#)). The miRNAs with monoadenylated read counts exceeding 100 in parental cells were used for the analysis ($n = 198$). See also [Supplemental Table 4](#). (F) Some miRNAs are highly deadenylated by PARN. The expression, length distribution, and adenylation status of miR-186-5p in parental and *PARN* KO cells was examined by small RNA northern blotting and sequencing reads. SNORA63, a known substrate of PARN (Berndt et al. 2012; Son et al. 2018), served as a positive control for *PARN* deletion.

(Jonas and Izaurralde 2015), these results are in part consistent with the recent findings that down-regulation of PARN causes no significant change in the steady-state abundance or poly(A) tail length of mRNAs (Moon et al. 2015; Son et al. 2018; Yi et al. 2018).

PARN as a miRNA de-tailor

The 3' ends of miRNAs are extensively modified by the action of TENTs (Burroughs et al. 2010; Chiang et al. 2010; Wyman et al. 2011; Thornton et al. 2014). However, it is unclear whether such "tailing" is a biologically reversible process. Notably, we observed that ablation of *PARN* markedly increased the fraction of PM reads in small RNA sequencing libraries (Fig. 6C), which represent miRNA species that are unambiguously modified at their 3' ends by one or more non-GM nucleotides (Fig. 6B). Concurrent with the elevated tailing frequency, the overall length of miRNA tails also increased in the absence of PARN ([Supplemental Fig. 9A](#)). The mRNA levels of TENTs were comparable in parental

and *PARN* KO cells (data not shown), excluding the possibility of their potential up-regulation by *PARN* deletion. These findings suggest that PARN may be responsible for the reversal of untemplated nucleotide addition to miRNAs.

The frequency of untemplated nucleotide addition varies among miRNAs, with some miRNAs being much more frequently modified than others (Burroughs et al. 2010; Chiang et al. 2010; Wyman et al. 2011; Thornton et al. 2014). Furthermore, two miRNAs previously reported to be de-tailed by PARN, miR-21 and miR-122 (Boele et al. 2014; Katoh et al. 2015), tend to be expressed at high levels in their respective niches (Chang et al. 2004; McCall et al. 2017). To investigate whether the observed increase in the fraction of PM reads in *PARN* KO cells is mostly accounted for by de-tailing of such few miRNAs that are abundant and/or highly modified, we calculated the fraction of tailed species for each miRNA in parental and *PARN* KO cells. Notably, 229 of 267 miRNAs (~86%) were more frequently tailed in both *PARN* KO clones

than in parental cells (Fig. 8A; Supplemental Fig. 9B,C), demonstrating that PARN-mediated de-tailing is not confined to the few, but is pervasive across most miRNAs. Of note, we noticed that the *PARN* KO#2 clone exhibited stronger molecular phenotypes than the KO#1 clone in small RNA sequencing analyses (Fig. 8A; Supplemental Fig. 9B).

To characterize the role of PARN as a miRNA de-tailor in more detail, we examined how the composition of miRNA tails changed in *PARN* KO cells. In agreement with previous reports (Burroughs et al. 2010; Chiang et al. 2010; Wyman et al. 2011), monoadenylation and monouridylation were the two most frequent types of modification, the combined reads of which comprised more than 70% of PM reads in parental HeLa S3 cells (~6.7% and ~2.9% of total reads, respectively) (Fig. 8B). Remarkably, the frequency of monoadenylation was substantially elevated in *PARN* KO cells (~6.7% in parental cells to ~9.8% in KO#1 cells and ~12% in KO#2 cells), while those of monouridylation and monocytidylation remained largely unchanged (Fig. 8B). Interestingly, the frequency of monoguanylation was also elevated by *PARN* ablation, with a similar fold increase as that of monoadenylation, but still remained low compared with that of other types of modification (~0.22% in parental cells to ~0.32% in KO#1 cells and ~0.40% in KO#2 cells) (Fig. 8B). Next, we extended our analysis to the composition of dinucleotide miRNA tails. Although dinucleotide addition appeared a fairly rare event (~2.6% of total reads in parental cells), a preference of PARN for A was clearly observed, such that all seven types of dinucleotide tails containing at least one A were more frequently added to miRNAs in both *PARN* KO clones than in parental cells (Fig. 8B). Examination of the modification status of individual miRNAs corroborated these results, with the frequency of monoadenylation, but not that of monouridylation, being prominently increased in the absence of PARN (Fig. 8C; Supplemental Fig. 9D). To validate our analysis, we investigated the isoform distribution of members of the miR-17~92 cluster, which were previously reported to undergo TENT2-catalyzed adenylation (Burroughs et al. 2010) and appeared extensively deadenylated by PARN in our analysis. Northern hybridization indeed demonstrated the appearance of high-molecular-weight species of these miRNAs in *PARN* KO cells, which were barely or not visible in parental cells (Supplemental Fig. 9E). Collectively, these findings suggest that PARN exhibits a degree of nucleotide preference in the de-tailing of miRNAs.

PARN catalyzes the shortening of untemplated oligo(A) tails in diverse types of noncoding RNA transcripts (Berndt et al. 2012; Moon et al. 2015; Nguyen et al. 2015; Tseng et al. 2015; Son et al. 2018). To address whether PARN similarly modulates the length of adenosine tails in miRNAs, we examined the frequencies of mono-, di-, and triadenylation in parental and *PARN* KO cells. Notably,

the frequencies of di- and triadenylation were more prominently affected than that of monoadenylation, such that miRNAs acquired longer adenosine tails in *PARN* KO cells (Fig. 8D). On the other hand, the uridylation frequencies remained stable or slightly decreased upon *PARN* deletion (Fig. 8D). To be certain that PARN globally shortens the adenosine tails of miRNAs, we calculated the mean tail length of individual miRNAs. The adenosine tails, but not the uridine tails, were significantly lengthened in *PARN* KO cells (Fig. 8E; Supplemental Fig. 9F,G), suggesting that PARN plays a pivotal role in shaping the length distribution of miRNA adenosine tails. To illustrate our findings, we focused on miR-186-5p, the adenosine tail of which was extensively deadenylated by PARN (mean adenosine tail length of ~1.31 nt in parental cells to ~1.59 nt in KO#1 cells and ~1.75 nt in KO#2 cells) (Fig. 8E). Northern blotting and examination of sequencing reads clearly demonstrated the accumulation of miRNA species harboring longer adenosine tails in *PARN* KO cells (Fig. 8F). Taken together, these results add miRNAs to the rapidly expanding list of noncoding RNAs whose 3' adenosine tails are targeted and regulated by PARN activity.

DISCUSSION

In this study, we report that the 3'-to-5' exoribonuclease PARN sculpts the 3' ends of miRNAs in human cells. We find that some canonical miRNAs are represented in cells with the sequences shorter at the 3' ends than those defined by the RNase III enzymes, and link this discrepancy to the 3' trimming of such miRNAs by PARN (Figs. 1–4). By generating *PARN* KO cells and characterizing their miRNAs, we demonstrate that PARN-mediated shortening is pervasive across miRNAs and involves two distinct modes, “trimming” and “de-tailing”, depending on whether the nucleotide being resected by PARN is encoded by the genome or post-transcriptionally acquired (Figs. 5–8).

Although the biochemical properties of PARN were extensively studied over the three decades (Balatsos et al. 2012; Virtanen et al. 2013), comprehensive characterization of its *in vivo* targets began to emerge only recently (Berndt et al. 2012; Moon et al. 2015; Nguyen et al. 2015; Tseng et al. 2015; Shukla and Parker 2017; Son et al. 2018). These studies have primarily associated PARN with the biogenesis and 3' end formation of a wide variety of noncoding RNAs, which is unexpected given its preference for a long stretch of adenosines and the 7-methylguanosine cap, the two hallmarks of a eukaryotic mRNA (Balatsos et al. 2012; Virtanen et al. 2013). Our current study further expands the repertoire of noncoding RNA substrates for PARN to many canonical miRNAs. Despite a certain degree of nucleotide preference (Fig. 8B–F), PARN is versatile enough to digest the 3' extensions of miRNAs derived from the genome (Fig. 7; Supplemental Fig. 6B), which is consistent with its ability to process the

highly GC-rich segment of 18S pre-rRNA (Ishikawa et al. 2017; Montellese et al. 2017).

Previously, three miRNAs have been reported as PARN substrates, including ac-pre-miR-451 (Yoda et al. 2013), miR-21 (Boele et al. 2014), and miR-122 (Katoh et al. 2015). To reconcile these studies with our findings, we focused on miR-21, because neither miR-451 nor miR-122 are expressed in HeLa S3 cells. Boele et al. (2014) claimed that DICER processing of pre-miR-21 releases miR-21+C, a 23 nt species, which is adenylated by TENT4B and subsequently trimmed by PARN into the 22 nt reference sequence of miR-21. Indeed, we found that both “Fraction +1” and the adenylation frequency of miR-21 increased in both PARN KO clones (~78% in parental cells to ~85% in KO#1 cells and ~87% in KO#2 cells for “Fraction +1”; ~5.4% in parental cells to ~13% in KO#1 cells and ~15% in KO#2 cells for the adenylation frequency). Of note, the authors defined the ratio of the miR-21 count to the miR-21+C count as the “degradation ratio” to conclude that PARN promotes decay of this specific miRNA. However, we observed no significant changes in the steady-state levels of the vast majority of miRNAs upon PARN ablation, including those more efficiently trimmed by PARN than miR-21 (Figs. 5 and 7). Our data suggest that PARN-mediated 3′ trimming likely operates in the maturation pathway of miRNAs rather than in the decay pathway, at least in our model cell lines.

PARN is conserved among most eukaryotes, with the notable exception of the two model organisms, *Drosophila melanogaster* and *Saccharomyces cerevisiae* (Balatsos et al. 2012; Virtanen et al. 2013). In *Drosophila*, Nibbler, a 3′-to-5′ exoribonuclease distinct from PARN, appears to have largely substituted for the role of PARN in miRNA metabolism (Han et al. 2011; Liu et al. 2011). To gain insights into the evolutionary conservation of PARN-mediated miRNA trimming, we focused on miR-182-5p, a miRNA that is efficiently trimmed by PARN (Fig. 7B,C) and is broadly found in the genomes of bilaterian organisms (Dambal et al. 2015). The primary sequence and secondary structure of pre-miR-182 was strikingly similar among the vertebrate orthologues (Supplemental Fig. 6C; Kozomara and Griffiths-Jones 2014; Fromm et al. 2015), plausibly suggesting that they may undergo conserved DICER processing. We examined the length distribution of miR-182-5p sequencing reads obtained from a panel of vertebrate species (Fromm et al. 2015), and compared them with those obtained from our small RNA libraries. PARN ablation in HeLa S3 cells caused a shift in the length of the most abundant miR-182-5p isoform from 24 to 26 nt, suggesting the last two nucleotides of the 26 nt isoform are subjected to PARN-mediated trimming (Fig. 7C; Supplemental Fig. 6B). Notably, in all species examined, the dominantly abundant isoform of miR-182-5p was shorter than 26 nt in length (25 nt in mouse, 24 nt in chicken and frog, and 22 nt in zebrafish) (Supplemental Fig. 6D). Given that the miR-

182-5p orthologues in these species possess nearly identical sequences to human miR-182-5p (Supplemental Fig. 6C), it is tempting to speculate that PARN may contribute to the 3′ end formation of this specific miRNA, and possibly other miRNAs, in vertebrates.

We observed that the degree of PARN-mediated trimming varies among miRNAs (Fig. 7). Then, what specifies the trimming of a specific miRNA by PARN? We failed to find common primary-sequence motifs or compositional biases among the miRNAs experiencing efficient trimming in HeLa S3 cells (Supplemental Fig. 6B). However, we noticed that they were slightly longer than the typical size of miRNAs of ~22 nt, ranging from 24 to 26 nt in length (Figs. 1–4 and 7). Furthermore, PARN failed to trim the miRNAs to less than a certain size in vitro and in cells, which was ~22 nt in the case of miR-362-5p (Figs. 3 and 4). Perhaps the longer miRNAs readily dissociate their 3′ ends from the PAZ domain and PARN trims them to the sizes that perfectly fit in and are fully protected by AGO proteins. Notably, similar observations were made in previous studies of Nibbler-mediated miRNA trimming in *Drosophila* (Han et al. 2011; Liu et al. 2011), suggesting that the length of miRNAs may play a role in specifying exoribonuclease-directed 3′ trimming in both human and *Drosophila*. Nevertheless, miRNA length is apparently not the sole determinant of trimming: For example, miR-182-5p is trimmed by PARN from 26 to 24 nt, which is the size of untrimmed miR-224-5p (Fig. 7C). miR-186-5p, one of the miRNAs highly deadenylated by PARN, also undergoes a degree of trimming, from 22 to 21 nt (Fig. 8F). These observations suggest the existence of more complicated determinant(s) beyond the length of miRNAs for PARN-mediated trimming.

A previous study emphasized the role of the RNA-binding protein, CUGBP1, in guiding PARN to its substrate miRNAs for destruction (Katoh et al. 2015). However, overexpression or knockdown of CUGBP1 did not obviously affect the 3′ trimming of miR-362-5p in HEK293T cells (Supplemental Fig. 10). Furthermore, we found that recombinant PARN produced in *E. coli* effectively trimmed the 3′ end of AGO-loaded miR-362-5p in vitro (Fig. 4C,D), which, together with an earlier report (Yoda et al. 2013), suggests that PARN does not require essential cofactor(s) to execute miRNA trimming. Nevertheless, we do not exclude the possibility that some *trans*-acting factor(s) may modulate the activity of PARN in miRNA metabolism, for example, in a cell-type-specific manner, because the degree of trimming of a specific miRNA varied across multiple cell types (Supplemental Fig. 6A). Another appealing possibility for the specificity of PARN-mediated miRNA trimming in vivo is “tailing.” Some noncoding RNA substrates of PARN, including snoRNAs and TERC, are primed with oligo(A) tails by the action of TENTs prior to being trimmed (Berndt et al. 2012; Moon et al. 2015; Nguyen et al. 2015; Tseng et al. 2015; Son et al. 2018), and similar mechanisms were

suggested to operate in the cases of miR-21 and miR-122 (Boele et al. 2014; Katoh et al. 2015). Indeed, we found that many miRNAs subjected to a high degree of trimming also acquired longer tails upon PARN deletion (Fig. 7C; Supplemental Fig. 6A). However, the adenylation frequency of these miRNAs in PARN KO cells was not particularly prominent compared with that of other miRNAs (Fig. 8 and data not shown), making it difficult to generalize the association of this type of modification with PARN-mediated trimming. Considering all these observations, we speculate that whether a given miRNA is trimmed by PARN, and if so, to what extent, may be determined by a combination of multiple factors, including the length, nucleotide composition, and/or modification status of the miRNA and *trans*-acting factor(s), if any, modulating PARN activity.

PARN-mediated shortening of miRNA 3' ends changes neither the seed nucleotides nor the stability of miRNAs, and is therefore not expected to substantially influence the vast majority of canonical targeting events relying on the miRNA seed (Supplemental Fig. 8). This, together with the notable conservation of a similar process in *Drosophila* (Han et al. 2011; Liu et al. 2011), raises the question as to why some miRNAs have evolved to be trimmed by these 3'-to-5' exoribonucleases. We speculate that PARN-mediated 3' trimming may prove its relevance at a minority of target sites, which extend pairing to the 3' region of the miRNA to enhance the specificity of miRNA-target interaction (3'-supplementary sites) or to compensate for imperfect pairing to the seed (3'-compensatory sites) (Bartel 2009). Alternatively, but not mutually exclusively, trimming may differentially contribute to the fate of miRNAs in a spatiotemporal manner or in response to external cues. For example, we recently reported that seedless, noncanonical target RNAs promote degradation of the cognate miRNA through releasing it from and/or destabilizing its 3' end within AGO proteins (Park et al. 2017). Given its ability to trim most miRNAs (Fig. 7B), PARN may be the culprit enzyme that destructs free miRNAs released from AGO proteins or destabilizes AGO-bound miRNAs in this context. It will be of interest to investigate the role of PARN in a larger paradigm of target RNA-directed miRNA degradation (TDMD), in which the 3' region of miRNAs plays a key role in specifying and eliciting regulatory effects (Fuchs Wightman et al. 2018). Finally, we point out that PARN-mediated 3' trimming may influence the recently discovered length-related functions of miRNAs (Yamane et al. 2017; Yu et al. 2017). For example, longer 3' isoforms of miR-122, resulting from either untemplated nucleotide addition or lack of 3' trimming, interact with the genomic RNA of hepatitis C virus more efficiently than the reference species (Yamane et al. 2017). Similarly, 3' heterogeneity of miR-222 appears to have functional consequences, with longer 3' isoforms exhibiting increased apoptotic activity and nuclear enrichment in a length-dependent manner (Yu et al. 2017). It will be interesting to investigate whether such

length-related effects are regulated by PARN-mediated trimming. In conclusion, further studies are required to uncover the full extent and the biological relevance of PARN-mediated miRNA shortening, and its possible contribution to human pathologies related to PARN mutations (Stuart et al. 2015; Tummala et al. 2015).

MATERIALS AND METHODS

Plasmids

To generate pri-miRNA expression plasmids, 300 bp fragments containing the pre-miRNA and genomic sequences on each side of the hairpin (~110–120 bp) were amplified from HeLa S3 or MEF genomic DNA and subcloned into the NheI/XbaI sites of pcDNA3.1(+) (Thermo Fisher Scientific). For high-level protein expression, the coding sequences of candidate genes were subcloned into a modified pCK vector using the Overlap Cloner DNA Cloning Kit (Elpis Biotech). Site-directed mutagenesis was carried out by standard inverse PCR with Phusion High-Fidelity DNA Polymerase (New England Biolabs). Primer sequences are listed in Supplemental Table 1.

Cell culture and transfection

HeLa S3 (and its PARN KO derivatives), HEK293T, A549, Huh7, and MCF7 cells were cultured in DMEM (Welgene) supplemented with 9% FBS (Welgene). For RNAi-mediated knockdown, cells were transfected with ~18 nM siRNA (Bioneer) twice over the course of 72-h incubation. For transfection of miRNA duplexes, synthetic 5p and 3p RNA strands (Bioneer) were mixed at an equimolar ratio and annealed immediately before transfection by boiling the mixture at 90°C for 3 min then slowly cooling it down to 25°C at a rate of –1°C per 4 min. The resulting duplexes were introduced at ~0.1 nM and harvested 48 h after the transfection. For plasmid transfection, cells were cotransfected with 1 µg of pri-miRNA expression plasmid and the indicated amount of effector plasmid per 35-mm dish and incubated for 48 h. All transfection procedures were performed with Lipofectamine 2000 (Thermo Fisher Scientific). The sequences of oligonucleotides used for transfection are listed in Supplemental Table 1.

CRISPR/Cas9-mediated gene knockout

To generate KO cells, pSpCas9(BB)-2A-Puro (Addgene; ID 48139) was equipped with a single guide RNA targeting the second exon of the human PARN gene and used for KO procedures in HeLa S3 cells, essentially as described elsewhere (Ran et al. 2013). Single-cell colonies were screened for the absence of the PARN protein by western blotting and candidate clones were genotyped to confirm the frame-shifting mutations.

In vitro DROSHA or DICER processing

In vitro DROSHA processing of pri-miR-362 was performed as described elsewhere (Lee et al. 2017), except that Flag-immunopurified Microprocessor was used instead of Microprocessor lysate. Pre-miRNA substrates for in vitro DICER processing were generated as described in the Supplemental Material. To immunopurify the DICER complex, we transiently cotransfected HEK293T cells

grown on a 100-mm dish with 6 μ g of pCK-Flag-DICER1 and 3 μ g of pCK-myc-TRBP and incubated for 2 d. Approximately one quarter of the cells from a single 100-mm dish were used for immunoprecipitation. The cells were lysed in 300 μ L of ice-cold Hypotonic Lysis Buffer [10 mM Tris-HCl at pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 0.1%(v/v) NP-40, and 1 $\times$ cOmplete Protease Inhibitor Cocktail (Roche Life Science)] on ice for 10 min and spun at 15,000 rpm at 4°C for 10 min. The resulting supernatant was incubated with 5 μ L of anti-Flag M2 Affinity Gel (Sigma) at 4°C for 2 h with constant rotation. The beads were washed with 1 mL of Buffer T2000 [20 mM Tris-HCl at pH 7.4, 2 M KCl, 0.2 mM EDTA, and 10%(v/v) glycerol] twice, then with 1 mL of Buffer T100 [20 mM Tris-HCl at pH 7.4, 100 mM KCl, 0.2 mM EDTA, and 10%(v/v) glycerol] four times. After washing, the volume of immunoprecipitate was adjusted to 100 μ L with Buffer T100. In vitro DICER processing was performed in 10- μ L reactions containing $\sim$ 10 nM pre-miRNA substrate, 50%(v/v) Flag-IP, and 2 mM Mg(OAc)₂ with incubation at 37°C for the indicated time period. The reactions were quenched by treating with Proteinase K (Roche Life Science) at 60°C for 20 min and then purified by phenol extraction and ethanol precipitation. The reaction products were resolved on a 15% urea-polyacrylamide gel and analyzed by direct phosphorimaging with the Typhoon FLA 7000 laser scanner (GE Healthcare) or small RNA northern blotting.

In vitro trimming assay

The strand of interest was 5'-radiolabeled and annealed to the cold opposite strand to form a miRNA duplex. Approximately one-eighth of the HEK293T cells transiently overexpressing Flag-AGO from a 100-mm dish were lysed in 25 μ L of ice-cold Hypotonic Lysis Buffer. Small RNA loading was performed in 50- μ L reactions containing $\sim$ 20 nM miRNA duplex, 50%(v/v) AGO cytoplasmic lysate ($\sim$ 18 μ g/ μ L), 50 mM KCl, 1 mM ATP, 25 mM creatine phosphate, and 2 mM Mg(OAc)₂ with incubation at 25°C for 1 h. The reactions were then incubated with 5 μ L of anti-Flag M2 Affinity Gel at 4°C for 2 h with constant rotation. The beads were washed with 1 mL of Buffer T2000 twice, then with 1 mL of Buffer T100 four times. After washing, the volume of immunoprecipitate was adjusted to 50 μ L with Buffer T100. To prepare HeLa S3 whole cell lysate, cells grown on a single 100-mm dish were dispersed in 250 μ L of ice-cold Hypotonic Lysis Buffer, sonicated on ice, and cleared by centrifugation. In vitro trimming was carried out in 10- μ L reactions containing 50%(v/v) Flag-IP, 40%(v/v) HeLa S3 whole cell lysate ($\sim$ 18 μ g/ μ L), and 2 mM Mg(OAc)₂ at 37°C for the indicated time period. For Figure 4D, recombinant GST-PARN was used at a concentration of $\sim$ 700 nM instead of whole cell lysate. The reactions were purified, separated on a 15% urea-polyacrylamide gel, and analyzed by phosphorimaging.

Western blot analysis

Cells were dispersed in ice-cold Hypotonic Lysis Buffer, lysed by sonication, and spun at 15,000 rpm at 4°C for 10 min to pellet insoluble debris. Thirty to fifty micrograms of the cleared lysate was separated on a 10% SDS-polyacrylamide gel and electrotransferred to an Immobilon-P PVDF membrane (EMD Millipore). The primary antibodies used in this study include rabbit anti-PARN (Abcam, ab27778), mouse anti-myc (9E10; Santa Cruz Biotechnology, sc-40), rabbit anti-Flag (Sigma, F7425), rabbit

anti- α -tubulin (Abcam, ab52866), and mouse anti- β -actin (Abcam, ab8224).

Expression and purification of recombinant proteins

We replaced the coding sequence of MBP present in pMAL-c2x (New England Biolabs) by that of hexahistidine and GST to generate pHis-GST. For bacterial expression of recombinant proteins, the coding sequence of full-length human PARN (WT or D28A) or the human TNRC6B (T6B) peptide (599–683) was subcloned into pHis-GST. The resulting plasmid was introduced into Rosetta2(DE3) *E. coli* cells (EMD Millipore), which were grown at 37°C to an OD₆₀₀ of $\sim$ 0.7 and treated with 0.5 mM IPTG at 16°C overnight to induce protein expression. His-GST (from empty pHis-GST) and His-GST-T6B were purified with HisTrap HP columns (GE Healthcare) and dialyzed against 1 $\times$ PBS/20% glycerol supplemented with 1 mM DTT. His-GST-PARN was batch-purified using GST-Bind Agarose Resin (Elpis Biotech) and dialyzed against Storage Buffer II [20 mM Tris-HCl at pH 7.4, 100 mM KCl, 0.2 mM EDTA, 20%(v/v) glycerol, and 1 mM DTT]. The detailed methods for protein purification are described in the Supplemental Material.

Small RNA northern blot analysis and primer extension assay

Total RNA was prepared using Tri Reagent (Thermo Fisher Scientific). For small RNA northern blotting, 5–50 μ g of total RNA was resolved on a 12.5% urea-polyacrylamide gel, electrotransferred to a Hybond N+ membrane (GE Healthcare), and fixed by UV crosslinking (0.12 J). DNA oligonucleotides complementary to the miRNA sequences were 5'-radiolabeled using T4 Polynucleotide Kinase (Takara) and used as probes for hybridization. To prepare high-specific-activity probes to detect miRNAs expressed at low levels, we radiolabeled both the 5' and 3' ends of the probe. For this purpose, a DNA oligonucleotide was designed such that a small extension (5'-ACCCGAGG-3') was added to the 3' end of the standard probe sequence, 5'-radiolabeled, and desalted or gel-purified to remove unincorporated [γ -³²P]ATP. The resulting 5'-labeled probe was annealed to a universal template oligonucleotide (HS_universal) and incubated with [α -³²P]dATP and Klenow Fragment, exo- (Elpis Biotech) at 25°C for 1 h. The sequences of probes are listed in Supplemental Table 1. For primer extension of miR-362-5p, 1 pmole of the 5'-radiolabeled DNA oligonucleotide complementary to nt 5–22 of miR-362-5p was mixed with 5–10 μ g of total RNA, denatured at 65°C for 5 min, and annealed at 50°C for 20 min. Reverse transcription was carried out with PrimeScript Reverse Transcriptase (Takara) at 42°C for 30 min. The reactions were quenched at 70°C for 15 min, purified by ethanol precipitation, and resolved on a 15% urea-polyacrylamide gel.

Small RNA sequencing and analysis

The TruSeq Small RNA Library Preparation Kit (Illumina) was used to prepare small RNA sequencing libraries, with minor modifications. Total RNA isolated with Tri Reagent was further enriched for small RNA using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific). Five micrograms of small RNA was fractionated on a 15% urea-polyacrylamide gel, and $\sim$ 18–30 nt RNA was gel-

purified and ligated to the 3' adaptor with T4 RNA Ligase 2, truncated (New England Biolabs). The ligation reaction was separated on a 15% urea-polyacrylamide gel; ~35–52 nt RNA was gel-purified and ligated to the 5' adaptor using T4 RNA Ligase (Ambion). The ligated RNA was reverse transcribed with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), amplified with Phusion High-Fidelity DNA Polymerase, and sequenced on the HiSeq 2500 platform (Illumina). For sequencing data analysis, adaptor sequences were trimmed using the cutadapt tool (Martin 2011) and reads shorter than 18 nt were discarded. After merging technical replicates, tRNA and rRNA were filtered out through mapping onto the human tRNA and rRNA sequences with BWA (version 0.6.2) (Li and Durbin 2009). These preprocessed reads were locally aligned to human miRBase 21 using BOWTIE2 (version 2.2.6) with the following parameters: -D 50 -R 5 -N 0 -L 10 -i C,1,0 --score-min=C,32,0 (Langmead and Salzberg 2012). The reads that include soft-clip (S) at the 3' end in the CIGAR string were defined as PM miRNA reads, whereas those consisting of only M in the CIGAR were considered as GM miRNA reads. To explore the role of PARN as a miRNA de-tailor, the PM nucleotide sequences were extracted using regular expression based on the parsed CIGAR strings. Finally, read coverages were calculated for 5p and 3p using the mirUtils package (version 1.0.0-r27). The small RNA sequencing data generated in this study were submitted to the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE121466.

AGO protein affinity purification by peptides (AGO-APP)

AGO-APP was performed as described previously (Hauptmann et al. 2015), with minor modifications. Briefly, ~1 mg of His-GST or His-GST-T6B was incubated with ~300 μ L of GST-Bind Agarose Resin, which was prewashed with 1 $\times$ PBS, at 4°C for at least 1 h with constant rotation. The beads were then washed with Buffer APP [50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1%(v/v) NP-40, 1 mM DTT, and 10%(v/v) glycerol] to remove unbound proteins. HeLa S3 whole cell lysate was prepared as described above, with the exception that Buffer APP was used instead of Hypotonic Lysis Buffer. It is noted that salt concentrations as low as that in the Hypotonic Lysis Buffer significantly impair the efficiency of AGO-APP, probably due to the hydrophobic nature of AGO-TNRC6 interactions. Approximately 8–10 mg of HeLa S3 whole cell lysate was incubated with the washed beads in a total volume of 1.5 mL at 4°C for 4 h with constant rotation. The beads were washed with 1 mL of Buffer APP six times and treated with Tri Reagent to isolate copurifying RNAs.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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