

# Dicer Partner Proteins Tune the Length of Mature miRNAs in Flies and Mammals

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## SUMMARY

*Drosophila* Dicer-1 produces microRNAs (miRNAs) from pre-miRNA, whereas Dicer-2 generates small interfering RNAs (siRNAs) from long dsRNA. Alternative splicing of the *loquacious* (*loqs*) mRNA generates three distinct Dicer partner proteins. To understand the function of each, we constructed flies expressing Loqs-PA, Loqs-PB, or Loqs-PD. Loqs-PD promotes both endo- and exo-siRNA production by Dicer-2. Loqs-PA or Loqs-PB is required for viability, but the proteins are not fully redundant: a specific subset of miRNAs requires Loqs-PB. Surprisingly, Loqs-PB tunes where Dicer-1 cleaves pre-miR-307a, generating a longer miRNA isoform with a distinct seed sequence and target specificity. The longer form of miR-307a represses *glycerol kinase* and *taranis* mRNA expression. The mammalian Dicer-partner TRBP, a Loqs-PB homolog, similarly tunes where Dicer cleaves pre-miR-132. Thus, Dicer-binding partner proteins change the choice of cleavage site by Dicer, producing miRNAs with target specificities different from those made by Dicer alone or Dicer bound to alternative protein partners.

## INTRODUCTION

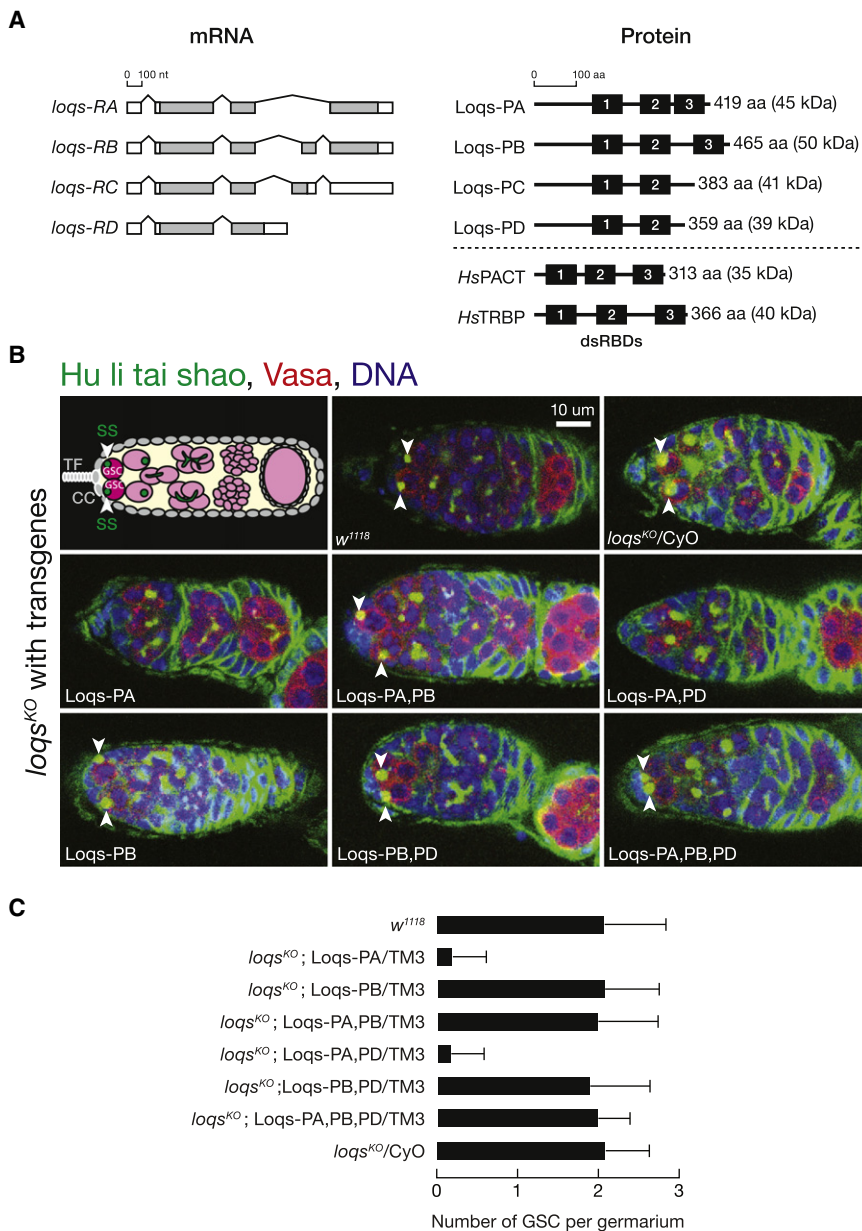
In *Drosophila melanogaster*, distinct enzymes generate 21 nt small interfering RNAs (siRNAs) and ~21–24 nt microRNAs (miRNAs). miRNA production begins with cleavage of an RNA polymerase II primary transcript by the RNase III enzyme Drosha, aided by its dsRNA-binding partner protein, Pasha. Drosha cleavage liberates a ~60–70 nt long pre-miRNA (Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004; Han et al., 2004, 2006). A second RNase III enzyme, Dicer-1 (Dcr-1), then cleaves the pre-miRNA to release a duplex comprising the mature miRNA and its miRNA\*, a partially complementary small RNA derived from the opposite arm of the pre-miRNA stem. Just as Pasha collaborates with Drosha, dsRNA-binding partner proteins assist Dicers in the production of small RNAs. In *Drosophila*, Dcr-1 partners with Loquacious-PA (Loqs-PA) or Loquacious-PB (Förstemann et al., 2005; Saito et al., 2005; Jiang et al., 2005).

*Drosophila* Dicer-2 (Dcr-2) generates siRNAs (Lee et al., 2004). Dcr-2 forms a 1:1 complex with R2D2, a paralog of Loqs (Liu et al., 2003; Tomari et al., 2004). Both R2D2 and free phosphate suppress the inherent ability of Dcr-2 to process pre-miRNAs into 21 nt duplexes, restricting it to the longer dsRNA triggers associated with RNAi (Cenik et al., 2011). The Dcr-2:R2D2 heterodimer also senses the thermodynamic asymmetry of siRNA duplexes and, together with chaperone proteins, loads them into Argonaute2 (Ago2; Liu et al., 2003, 2006; Tomari et al., 2004, 2007; Pham and Sontheimer, 2005). Dcr-2 produces siRNAs from both endogenous and exogenous double-stranded RNA (dsRNA). Exogenous siRNAs (exo-siRNAs) in flies are thought to derive from long dsRNA molecules generated during viral replication (Ding, 2010) or long dsRNA introduced experimentally (Kennerdell and Carthew, 1998, 2000; Lee and Carthew, 2003). In flies and mammals, endogenous siRNAs (endo-siRNAs) derive from self-complementary hairpin transcripts (esiRNAs), convergent mRNAs (cis-NAT endo-siRNAs), or mobile elements (Yang and Kazazian, 2006; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a, 2008b; Tam et al., 2008; Watanabe et al., 2008).

Production of esiRNAs by Dcr-2 in cultured *Drosophila* S2 cells requires the alternative partner protein, Loqs-PD, rather than R2D2 (Okamura et al., 2008b; Hartig et al., 2009; Zhou et al., 2009; Miyoshi et al., 2010; Hartig and Förstemann, 2011). However, it is not known whether the production of exo-siRNAs, cis-NAT-endo-siRNAs, and mobile element-derived endo-siRNAs also requires Loqs-PD. Moreover, an in vivo role for Loqs-PD in small RNA production has not been established.

The *loqs* gene generates four mRNA isoforms by alternative splicing: *loqs-PA*, *loqs-RB*, *loqs-RC*, and *loqs-RD*. The four mRNAs are predicted to produce four distinct proteins, Loqs-PA, Loqs-PB, Loqs-PC, and Loqs-PD (Figure 1A; Förstemann et al., 2005; Saito et al., 2005; Jiang et al., 2005; Zhou et al., 2009; Hartig et al., 2009; Miyoshi et al., 2010). The Loqs-PC protein has not yet been detected in any fly cell or tissue. In theory, an intron in the 3' UTR of *loqs-RC* may target the mRNA for nonsense-mediated decay (Moore and Proudfoot, 2009).

The largest Loqs protein isoform, Loqs-PB, comprises three double-stranded RNA-binding domains (dsRBDs); dsRBD3 is required for Loqs-PB to bind to Dcr-1 (Förstemann et al., 2005; Ye et al., 2007). Loqs-PA, which also binds to Dcr-1, lacks a part of the third dsRBD of Loqs-PB. Loqs-PD has a unique carboxy terminal sequence in place of dsRBD3 and binds to Dcr-2



**Figure 1. Loqs-PB is Necessary and Sufficient to Maintain Female Germline Stem Cells**

(A) *Drosophila* and human Dicer partners.

(B) *Drosophila* germarium architecture. TF, terminal filament. CC, cap cells. SS, spectrosomes. Green, fusomes. Somatic cells are gray, germline are pink. Ovaries from 10-day-old females were stained with anti-Hu li tai shao (Hts) antibody (green) to detect the spectrosome and fusome, anti-Vasa antibody (red) to identify germ cells, and DAPI (blue) to highlight DNA. White arrowheads mark GSCs.

(C) The number of GSCs per germarium (mean ± SD) was measured for each genotype in 10–15 randomly selected germaria from pooled ovaries from >30 flies. Eight z axis images, spanning 20  $\mu$ m, were acquired for each germarium. See also Figure S1 and Table S1.

example, plants produce four distinct Dicer enzymes, each with its own specialized dsRBD partner (Vazquez et al., 2004; Hiraguri et al., 2005; Eamens et al., 2009).

The importance of such partnerships is well established, but the individual molecular and biological functions of dsRBD partner proteins are largely unknown. Here, we define the specific functions of the three Loqs partner proteins. Using isoform-specific rescue transgenes, we reconstituted *loqs* knockout (*loqs<sup>KO</sup>*) flies (Park et al., 2007) with one, two, or all three Loqs proteins. We find that Loqs-PA and Loqs-PB, but not Loqs-PD, can rescue the lethality of *loqs* null mutant flies. Loqs-PB is crucial for female fertility and for producing specific subsets of miRNAs. Loqs-PD enhances the production of both endo- and exo-siRNAs. In the absence of Loqs-PB, Dcr-1 produces aberrant products from pre-miR-307a, pre-miR-87, and pre-miR-316. miR-307a and miR-87

rather than Dcr-1. Thus, *loqs* encodes at least three distinct proteins that act in either the miRNA or siRNA pathways (Förstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005; Hartig et al., 2009; Zhou et al., 2009; Marques et al., 2010; Miyoshi et al., 2010; Hartig and Förstemann, 2011).

Small silencing RNA production in mammals is also catalyzed by RNase III enzymes assisted by dsRBD partner proteins. Mammalian Drosha acts with DGCR8, which contains two dsRBDs (Gregory et al., 2004). Mammalian Dicer, which produces both miRNAs and siRNAs, collaborates with TRBP and PACT, partner proteins with three dsRBDs (Figure 1A; Chendrimada et al., 2005; Gregory et al., 2004; Haase et al., 2005; Lee et al., 2006). Partnerships between dicer-like proteins and dsRBD proteins are a general theme in RNA silencing pathways. For

reside in the 3' arm of their pre-miRNA, so Dcr-1 cleavage defines their 5' end. In fly ovaries, Loqs-PB is required for Dcr-1 to generate miR-307a bearing the correct seed sequence to repress its targets *glycerol kinase* (*Gk*) and *taranis* (*tara*). Finally, in vitro dicing assays and the sequences of miRNAs from *Trbp<sup>-/-</sup>* mouse embryonic fibroblasts (MEFs) suggest that the mammalian Loqs ortholog TRBP similarly acts to ensure the correct choice of pre-miRNA cleavage sites by Dicer.

## RESULTS

### *loqs* Isoform-Specific Flies

To define the in vivo function of each *loqs* isoform, we constructed flies harboring a single-copy transgene encompassing

the cDNA of each *loqs* isoform under the control of the endogenous *loqs* promoter (Figure S1A). Double and triple transgene strains (Loqs-PA,PB; Loqs-PA,PD; Loqs-PB,PD; and Loqs-PA,PB,PD) were created by meiotic recombination (Figure S1B). The transgenic, FLAG-tagged Loqs-PA, Loqs-PB, and Loqs-PD proteins were expressed at levels equal to or greater than the level of the corresponding endogenous protein in genetically matched control ( $w^{1118}$ ) or *loqs*<sup>KO</sup>/CyO heterozygous flies (Figure S1C). *loqs*<sup>KO</sup> mutant flies are embryonic lethal (Park et al., 2007). Loqs-PA or Loqs-PB, but not Loqs-PD, restored embryonic viability, suggesting that a defect in miRNA biogenesis underlies the lethality observed in *loqs*<sup>KO</sup> embryos.

### Loqs-PB Is Necessary and Sufficient for Germline Stem Cell Maintenance

Male *loqs*<sup>KO</sup> adult flies rescued with either Loqs-PA or Loqs-PB were as fertile as control flies ( $w^{1118}$  or *loqs*<sup>KO</sup>/CyO; Table S1). In contrast, female *loqs*<sup>KO</sup> flies rescued with either Loqs-PA (4.3 eggs per female per day, none of which hatched) or Loqs-PA together with Loqs-PD (6.0 eggs per female per day, 3% hatched) laid far fewer eggs than  $w^{1118}$  or *loqs*<sup>KO</sup>/CyO (~36–45 eggs per female per day, of which 48%–54% hatched; Table S1). Few of the eggs laid by *loqs*<sup>KO</sup>;Loqs-PA/TM3 (6%) or *loqs*<sup>KO</sup>;Loqs-PA,PD/TM3 (14%) females had normal dorsal appendages; 88%–91% of the eggs laid by  $w^{1118}$  or *loqs*<sup>KO</sup>/CyO females had normal dorsal appendages. In contrast, Loqs-PB alone was sufficient to rescue the number of eggs laid per female per day (38), the rate of hatching (53%), and patterning of the dorsal appendages (91% normal; Table S1).

*loqs*<sup>100791</sup>/*loqs*<sup>KO</sup> (*loqs*<sup>100791</sup> is a hypomorphic allele) ovaries fail to maintain their germline stem cells (GSCs). A transgene expressing Loqs-PB but not a transgene expressing Loqs-PA rescues the GSC loss (Park et al., 2007). However, these experiments preceded the discovery of Loqs-PD and its role in endo-siRNA biogenesis, leaving open the possibility that Loqs-PA in combination with Loqs-PD might rescue GSC maintenance. To test this possibility, we counted the number of germline stem cells (GSCs) per germarium. Ovaries were immunostained to detect Hu Li Tai Shao (Hts), a component of the GSC-specific spectrosome (Lin et al., 1994) and Vasa, which marks germ cells (Figure 1B). GSCs were identified by their anterior-positioned, round spectrosome and by their apposition to somatic cap cells or terminal filament cells, which lack Vasa. Control ( $w^{1118}$ ) flies had  $2.1 \pm 0.8$  GSCs per germarium (mean  $\pm$  SD; Figure 1C), whereas *loqs*<sup>KO</sup>;Loqs-PA/TM3 and *loqs*<sup>KO</sup>;Loqs-PA,PD/TM3 flies had fewer ( $0.2 \pm 0.4$  GSCs per germaria for each). Ovaries from all genotypes expressing Loqs-PB had a normal complement of GSCs: *loqs*<sup>KO</sup>;Loqs-PB/TM3 ( $2.1 \pm 0.7$ ), *loqs*<sup>KO</sup>;Loqs-PA,PB/TM3 ( $2.0 \pm 0.7$ ), *loqs*<sup>KO</sup>;Loqs-PB,PD/TM3 ( $1.9 \pm 0.7$ ), *loqs*<sup>KO</sup>;Loqs-PA,PB,PD/TM3 ( $2.0 \pm 0.4$ ), and *loqs*<sup>KO</sup>/CyO ( $2.1 \pm 0.5$ ).

Our data support earlier conclusions that among the Loqs isoforms, Loqs-PB alone provides the necessary and sufficient Loqs function to maintain GSCs (Park et al., 2007). Consistent with the idea that Loqs-PA and Loqs-PB have distinct functions in the production of miRNAs, the relative abundance of their mRNAs, *loqs-RA* and *loqs-RB*, varies widely among tissues and developmental stages (Figure S1D). For example, *loqs-RA* is lower at earlier stages of development and increases with

developmental time. In adult flies, testes predominantly express *loqs-RA*, whereas ovaries express more *loqs-RB* than *loqs-RA* (Figure S1D).

### Normal Exo-siRNA Accumulation Requires Loqs-PD In Vivo

In cultured *Drosophila* S2 cells, Loqs-PD is required for efficient esiRNA production (Hartig et al., 2009; Zhou et al., 2009; Miyoshi et al., 2010). Does Loqs-PD play a role in RNAi in adult flies?

The GMR-wIR transgene produces during eye development an inverted repeat hairpin RNA corresponding to *white* exon 3. Dcr-2 processes the wIR hairpin into siRNAs (Lee and Carthew, 2003; Vagin et al., 2006), which in turn silence *white* expression, causing the eye to be white or orange instead of red. Because *white* encodes an ABC transporter required for the entry of red pigment precursor into cells, the reduction in the absorbance at 480 nm of pigment extracted from the eye provides a quantitative measure of *white* silencing. In wild-type flies (Canton S) and flies heterozygous for the RNAi pathway genes *dcr-2*, *r2d2*, or *ago2*, the wIR transgene produced white eyes ( $A_{480} = 0.02$ – $0.03$ ; Figure 2A, females; Figure S2A, males). In contrast, the eyes from wIR transgenic female flies homozygous for *dcr-2*, *r2d2*, or *ago2* mutations ( $A_{480} = 0.9$ – $1.0$ ) were similar to those of wild-type (mean  $A_{480} = 1.2$ ).

Hypomorphic *loqs*<sup>100791</sup> homozygous mutant flies show a quantitative defect in *white* silencing (Förstemann et al., 2005). *loqs*<sup>KO</sup> homozygous mutant female flies bearing the wIR and rescued with Loqs-PA or Loqs-PB or both showed similar quantitative defects in *white* silencing (Figure 2A), as evidenced by their significantly higher 480 nm absorbance (mean  $\pm$  SD ranged from  $0.11 \pm 0.00$  to  $0.16 \pm 0.01$  with p values from  $5.9 \times 10^{-11}$  to  $6.3 \times 10^{-5}$ ). The Loqs-PD transgene (*loqs*<sup>KO</sup>;Loqs-PA,PD/TM3, *loqs*<sup>KO</sup>;Loqs-PB,PD/TM3, or *loqs*<sup>KO</sup>;Loqs-PA,PB,PD/TM3) quantitatively rescued silencing ( $A_{480} = 0.01 \pm 0.01$  for all three genotypes; Figure 2A). Similar results were obtained by using male flies (Figure S2A). Thus, Loqs-PD significantly (p value =  $1.6 \times 10^{-2}$ , *loqs*<sup>KO</sup>;Loqs-PA,PB/TM3 compared to *loqs*<sup>KO</sup>;Loqs-PA,PB,PD/TM3) enhanced RNAi triggered by long dsRNA in vivo.

Next, we sequenced 18–29 nt RNA (Tables S2A and S2B) from the heads of female flies bearing the wIR transgene. The steady-state level of *white* exo-siRNAs produced from the wIR decreased in *loqs*<sup>KO</sup>;Loqs-PA/TM3, *loqs*<sup>KO</sup>;Loqs-PB/TM3, and *loqs*<sup>KO</sup>;Loqs-PA,PB/TM3 rescue flies, compared with wild-type or heterozygous controls (Figures 2B and S2C). In contrast, *loqs*<sup>KO</sup>;Loqs-PA,PD/TM3, *loqs*<sup>KO</sup>;Loqs-PB,PD/TM3, and *loqs*<sup>KO</sup>;Loqs-PA,PB,PD/TM3 flies had normal or elevated amounts of *white* siRNAs. Loqs-PD appeared to play no role in miRNA accumulation: we detected no significant change (p value  $\gg 0.05$ ; Wilcoxon test) in the global abundance of miRNAs and miRNA\* strands in the absence of Loqs-PD (*loqs*<sup>KO</sup>;Loqs-PA,PB/TM3 versus *loqs*<sup>KO</sup>/CyO). Northern hybridization of RNA from the heads of adult females expressing the wIR confirmed the sequencing results (Figure S2B). Loss of Loqs-PD decreased the abundance of *white* siRNA reads across the entire wIR hairpin sequence for both sense and antisense siRNAs, suggesting a sequence-independent role for Loqs-PD in facilitating exo-siRNA production by Dcr-2 (Figure 2C).





### Loqs-PD Decreases the $K_M$ of Dcr-2 for Pre-esiRNA

To understand how Loqs-PD enhances steady-state accumulation of endo-siRNAs in vivo, we analyzed the processing of an esiRNA precursor (pre-esi-2.1) by purified, recombinant Dcr-2 supplemented with purified, recombinant Loqs-PD (Table S4 and Figure S4A). Adding Loqs-PD decreased the  $K_M$  of Dcr-2 from  $135 \pm 32$  nM to  $31 \pm 5$  nM (p value = 0.028). In contrast, the  $k_{cat}$  of Dcr-2 alone ( $0.073 \pm 0.003$  min<sup>-1</sup>) was not significantly changed (p value > 0.05) by the addition of Loqs-PD ( $0.085 \pm 0.007$  min<sup>-1</sup>). Thus, Loqs-PD decreased the substrate concentration at which Dcr-2 was half-maximally active, but had no detectable effect on the rate of enzyme turnover. Overall, Loqs-PD increased enzyme efficiency ( $k_{cat}/K_M$ ) ~5-fold. Loqs-PD similarly enhances  $K_M$  (decreased ~14-fold) but not  $k_{cat}$  for Dcr-2 processing long dsRNA (Cenik et al., 2011). We conclude that Loqs-PD functions in both endo-siRNA and exo-siRNA production by decreasing the concentration of substrate required for Dcr-2 to efficiently produce siRNA.

### Loqs-PB Contributes Differentially to the Production of Individual miRNAs

*loqs* was discovered as a gene required for efficient miRNA production in vivo. Either Loqs-PA or Loqs-PB suffices to rescue the embryonic lethality observed in *loqs*<sup>KO</sup> homozygotes, and in ovaries some miRNAs such as *let-7*, miR-1, miR-184, miR-263a, miR-312, or miR-996, accumulated to wild-type levels when either Loqs-PA or Loqs-PB was the only Loqs protein present (Figures 2B and S3A–S3C). In contrast, the abundance of miRNAs such as miR-79, miR-283, miR-305, miR-311, and miR-318 decreased in ovaries of *loqs*<sup>KO</sup> mutant flies rescued with Loqs-PA but achieved wild-type levels when rescued with Loqs-PB. If Loqs-PB enhances the processing of these pre-miRNAs by Dcr-1 in vivo, then their pre-miRNAs might accumulate in the absence of Loqs-PB. Although pre-miRNA accumulation is diagnostic for a defect in dicing, a failure to accumulate pre-miRNA could suggest that Loqs-PB acts at a step after dicing but might simply indicate that the pre-miRNA is unstable. Neither pre-miR-283 nor pre-miR-305 accumulated in *loqs*<sup>KO</sup>;Loqs-PA/TM3 flies, relative to *loqs*<sup>KO</sup>;Loqs-PB/TM3 or in *w*<sup>1118</sup> (Figure S3A). In contrast, miR-79, miR-311, and miR-318 decreased and their corresponding pre-miRNAs increased in *loqs*<sup>KO</sup>;Loqs-PA/TM3, compared with *loqs*<sup>KO</sup>;Loqs-PB/TM3 and *w*<sup>1118</sup> (Figure S3A). For these miRNAs, our data suggest that Loqs-PB enhances the Dcr-1-catalyzed conversion of pre-miRNAs to miRNA/miRNA\* duplexes. Similarly, in heads from *loqs* mutant flies rescued with Loqs-PA rather than Loqs-PB, the abundance of miR-277 and miR-305 declined and the corresponding pre-miRNAs accumulated (Figures 2B and S2B).

### Loqs-PB Decreases the $K_M$ and Increases the $k_{cat}$ of Dcr-1 for Pre-miRNA

To understand how Loqs-PB contributes to the processing of miRNAs like miR-305, we measured the  $K_M$  and  $k_{cat}$  of purified, recombinant Dcr-1 and of Dcr-1 supplemented with purified, recombinant Loqs-PA or Loqs-PB, using pre-miR-305 and pre-*let-7* as substrates (Table S4 and Figures S4B and S4C). For pre-miR-305, Loqs-PB decreased the  $K_M$  (Dcr-1 alone,  $K_M = 2.2 \pm 0.1$  nM versus Dcr-1 + Loqs-PB,  $K_M = 1.4 \pm 0.1$  nM;

p value =  $5.5 \times 10^{-4}$ ) and increased turnover (Dcr-1 alone,  $k_{cat} = 0.11 \pm 0.00$  min<sup>-1</sup> versus Dcr-1 + Loqs-PB,  $k_{cat} = 0.15 \pm 0.00$  min<sup>-1</sup>, p value =  $2.4 \times 10^{-4}$ ), more than doubling dicing efficiency (i.e.,  $k_{cat}/K_M$ ). In contrast, Loqs-PA decreased  $K_M$  (Dcr-1 + Loqs-PA,  $K_M = 1.5 \pm 0.3$  nM, p value = 0.030), but did not alter  $k_{cat}$  (Dcr-1 + Loqs-PA,  $k_{cat} = 0.11 \pm 0.00$  min<sup>-1</sup>, p value > 0.05).

For pre-*let-7*, Loqs-PB decreased the  $K_M$  of Dcr-1 (Dcr-1,  $K_M = 3.0 \pm 0.2$  nM versus Dcr-1 + Loqs-PB,  $K_M = 1.8 \pm 0.2$  nM, p value =  $2.0 \times 10^{-3}$ ) without altering turnover (Dcr-1,  $k_{cat} = 0.52 \pm 0.01$  min<sup>-1</sup> versus Dcr-1 + Loqs-PB,  $k_{cat} = 0.49 \pm 0.02$  min<sup>-1</sup>, p value > 0.05). For pre-*let-7*, Loqs-PA did not detectably alter either  $K_M$  (Dcr-1 + Loqs-PA,  $K_M = 2.7 \pm 0.7$  nM, p value > 0.05) or enzyme turnover (Dcr-1 + Loqs-PA,  $k_{cat} = 0.51 \pm 0.02$  min<sup>-1</sup>, p value > 0.05). Our data suggest a model in which Loqs-PB makes a greater contribution than Loqs-PA to the dicing of “difficult” pre-miRNA substrates such as pre-miR-305, perhaps because for these pre-miRNAs, Loqs-PB enhances Dcr-1 substrate binding while favoring product release.

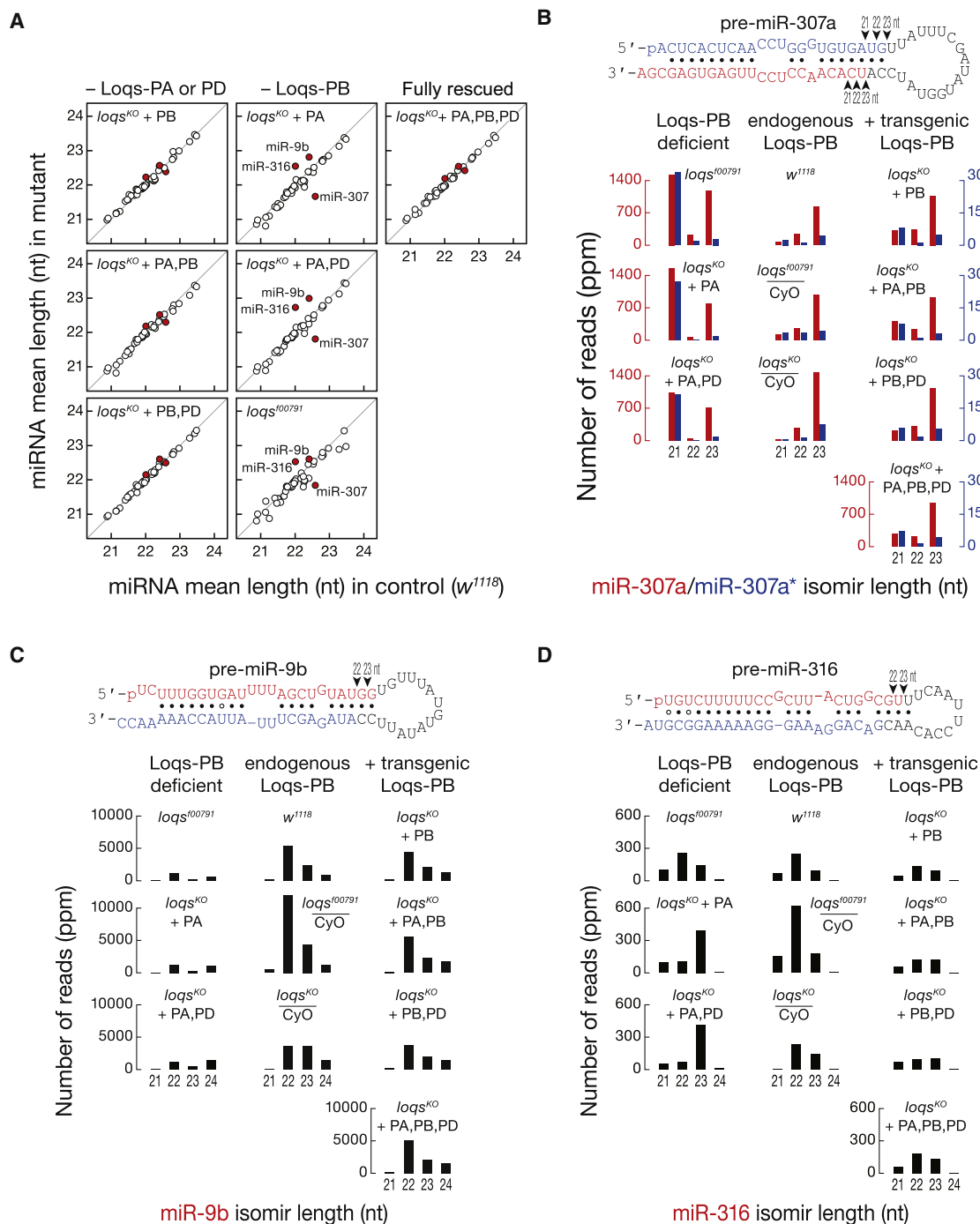
### Loqs-PB Tunes Where Dcr-1 Cleaves Some Pre-miRNAs

Dicing by Dcr-1 determines miRNA length. In addition to showing changes in miRNA abundance, sequencing of *loqs*<sup>KO</sup> mutants rescued with Loqs protein isoforms revealed changes in miRNA isoform (isomir) distributions for several miRNAs. In particular, the mean length of three miRNAs in ovaries differed between *loqs*<sup>KO</sup> mutants rescued with Loqs-PA versus Loqs-PB: in the absence of Loqs-PB, miR-307a was shorter and miR-316 and miR-9b were longer (Figure 3A–3D). miR-307a was also shorter and miR-316 was longer in *loqs*<sup>100791</sup> hypomorphic mutants.

miR-307a resides in the 3' arm of its pre-miRNA, so Dcr-1 defines its 5' end. miR-307a isomirs differ mainly at their 5' ends, a difference that alters their seed sequences, changing their repertoire of mRNA targets. In both ovaries (Figure 3B) and heads (Figure S5A), flies lacking Loqs-PB accumulate a 21 nt long miR-307a isomir (miR-307a<sup>21-mer</sup>) whose seed sequence begins at position 4 of the canonical 23 nt long miR-307a isomir (miR-307a<sup>23-mer</sup>). *loqs*<sup>KO</sup> flies rescued with Loqs-PB, *w*<sup>1118</sup> flies, and *loqs*<sup>KO</sup>/CyO heterozygotes produce mainly miR-307a<sup>23-mer</sup> (Figures 3B, S3B, and S5A). The absence of Loqs-PB also alters the 5' end of the less abundant (i.e., <100 ppm) miR-87, decreasing the canonical 24 nt miR-87<sup>24-mer</sup> and increasing the 23 nt miR-87<sup>23-mer</sup> in both heads and ovaries (Figures S5C and S5D). miR-87 resides in the 3' arm of pre-miR-87, so miR-87<sup>24-mer</sup> and miR-87<sup>23-mer</sup> have different seed sequences.

miR-316 and miR-9b reside in the 5' arm of their pre-miRNAs, so Dcr-1 cleavage defines their 3' ends. *loqs*<sup>KO</sup> mutants rescued with Loqs-PA produce miR-316 and miR-9b isomirs that differ from the canonical miRNA at their 3' termini (Figures 3C, 3D, and S5B), so their seed sequences are unchanged.

The different lengths of these miRNAs in *loqs*<sup>KO</sup>;Loqs-PA/TM3 and *loqs*<sup>KO</sup>;Loqs-PB/TM3 flies likely reflects a direct influence of Loqs-PB on the choice of cleavage site by Dcr-1. We incubated pre-miR-307a with Dcr-1 alone or Dcr-1 supplemented with Loqs-PA or Loqs-PB. (Figures 4 and S6A–S6C). Dcr-1 alone and Dcr-1 + Loqs-PA produced mostly 21 nt miR-307a; more 22 and 23 nt isomirs were made when Dcr-1 was supplemented with Loqs-PB (Figures 4 and S6A and S6B). Similarly, miR-307a\*, which derives from the 5' arm of pre-miR-307a, was longer when



**Figure 3. Loqs-PB Changes the Size Distribution of Small RNAs Cleaved from Pre-miR-307a In Vivo**

The sequence, length, and abundance of ovary small RNAs from control and mutant females bearing Loqs-expressing transgenes was measured by deep sequencing.

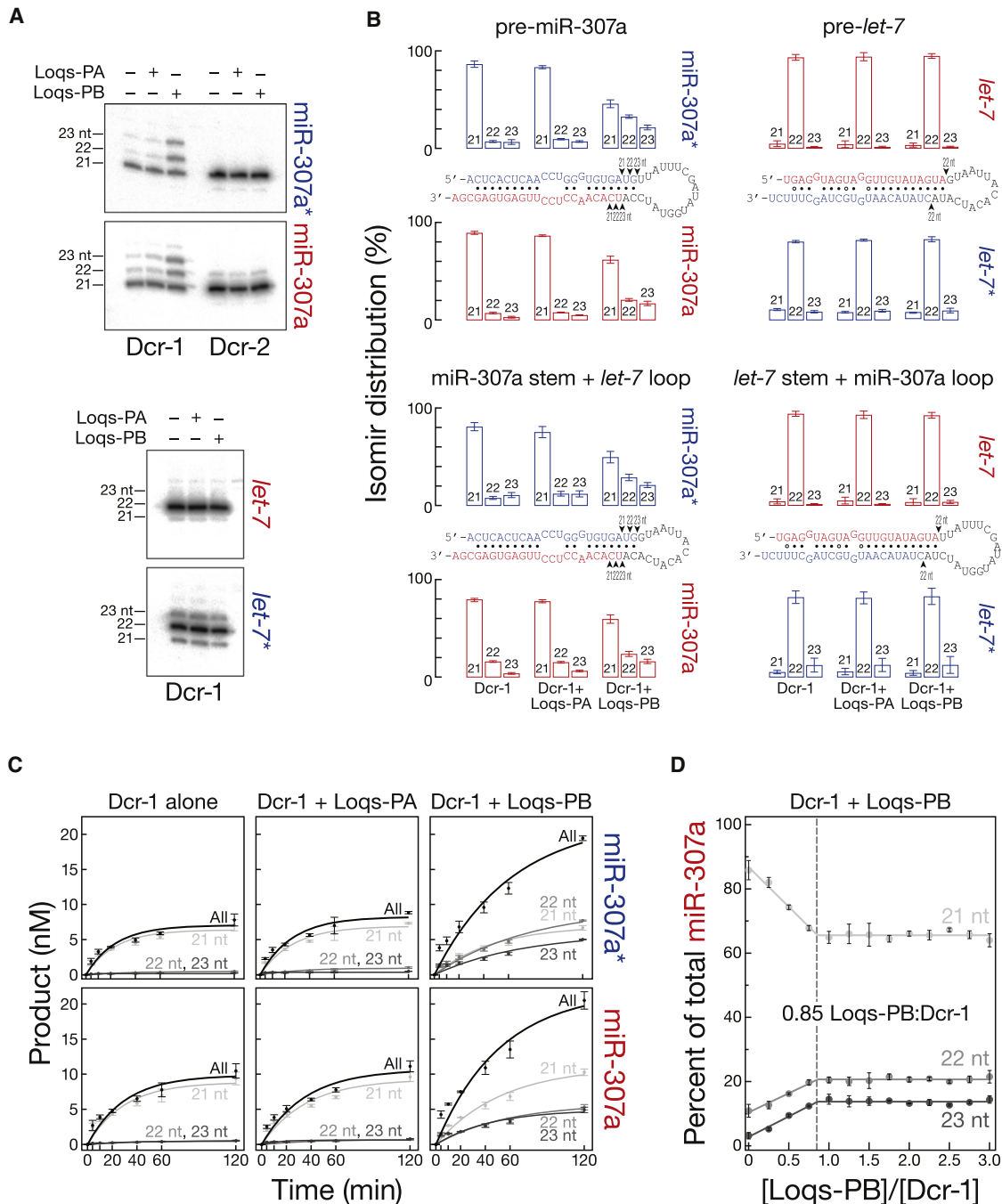
(A) Mean length of all miRNA isomirs in the transgenic, mutant flies compared to control ( $w^{1118}$ ). Only miRNAs whose total isomir abundance was more than 100 ppm in  $w^{1118}$  were analyzed.

(B) Normalized abundance of each isomir of miR-307a\* (blue) and miR-307a (red) for each genotype. Here and below, we considered only isomirs whose sequence variation reflected a change solely in the site Dicer cleaved.

(C) Abundance of miR-9b isomirs.

(D) Abundance of miR-316 isomirs.

See also Figure S5.



**Figure 4. Purified Loqs-PB, but Not Loqs-PA, Tunes the Length of Small RNAs from Pre-miR-307a**

(A) Pre-miR-307a and pre-let-7 (100 nM) were incubated with recombinant Dcr-1 or Dcr-2 (10 nM) supplemented with Loqs-PA or Loqs-PB (10 nM) for 2 hr. The products were resolved by denaturing polyacrylamide electrophoresis and detected by northern hybridization.

(B) Quantification of three independent replicates of the experiment in (A) and Figures S6H and S6I. Chimeric pre-miRNA comprised the pre-miR-307a stem and the pre-let-7 loop or the pre-let-7 stem and the pre-miR-307a loop.

(C) Pre-miR-307 (30 nM) was incubated with purified, recombinant Dcr-1 alone (8 nM) or supplemented with Loqs-PA or Loqs-PB (8 nM). For sites of the  $^{32}$ P-radiolabel see Figure S6A.

(D) The stoichiometry of Loqs-PB in complex with Dcr-1 was determined by titration. Products were detected by quantitative northern hybridization. The inflection point in the curves, 0.85 molecules of Loqs-PB per molecule of Dcr-1, suggests the two proteins form a 1:1 complex.

Data are reported as mean  $\pm$  SD for three independent trials. See also Figures S6 and S7.

Loqs-PB, but not Loqs-PA, was included (Figures 4A–4C and S6A–S6C). The addition of Loqs-PA or Loqs-PB had no effect on the length of *let-7* or *let-7\** produced by Dcr-1 from pre-*let-7* (Figures 4A and 4B). Our data suggest that Loqs-PB repositions pre-miR-307a on Dcr-1 so as to favor production of miR-307a<sup>23-mer</sup> over miR-307a<sup>21-mer</sup>.

Inclusion of Loqs-PB similarly favored production of miR-87<sup>24-mer</sup> over miR-87<sup>23-mer</sup> from pre-miR-87 (Figures S6D and S6E). In contrast, Loqs-PB favored production from pre-miR-316 of a shorter, 22 nt isomir of miR-316 (Figure S7); miR-87<sup>24-mer</sup> and the shorter isomir of miR-316 predominate in vivo in wild-type flies (Figures 3D, S5B, S5C, and S5D).

The effect of Loqs-PB on cleavage site choice within the pre-miRNA stem was specific for Dcr-1. Neither Loqs-PA nor Loqs-PB affected the cleavage site chosen by Dcr-2 (Figure 4A).

### Loqs-PB Forms a 1:1 Complex with Dcr-1

The precise stoichiometry between Dicer partners and Dicer has been established only for the 1:1 R2D2:Dcr-2 heterodimer (Tomari et al., 2004, Supporting Online Material, page 14). We used titration to estimate the stoichiometry of Loqs-PB bound to Dcr-1 during pre-miRNA processing. We incubated 5 nM Dcr-1 with increasing concentrations of Loqs-PB (0–15 nM) and 100 nM pre-miR-307a and measured the isomirs produced (Figures 4D, S6B, and S6C). After 2 hr, 86% ± 3% of the miR-307a made by Dcr-1 alone was the 21 nt isoform (Figure 4D). The percentage of 22 and 23 nt miR-307a isomirs increased with increasing Loqs-PB, then plateaued once Dcr-1 and Loqs-PB were essentially equimolar. The results were similar when we monitored the production of miR-307a\* isomirs (Figures S6B and S6C). We conclude that Dcr-1 and Loqs-PB form a 1:1 complex.

### Tuning of Dcr-1 Cleavage by Loqs-PB Requires the Pre-miR-307a Stem but Not the Loop

To begin to determine the features required for Loqs-PB to alter where Dcr-1 cleaves pre-miR-307a, we created two chimeric pre-miRNAs: one combined the pre-miR-307a stem and the pre-*let-7* loop, the other combined the pre-*let-7* stem and the pre-miR-307a loop (Figure 4B, S6H, and S6I). Loqs-PB changed the site at which Dcr-1 cleaved the chimera containing the pre-miR-307a stem but not the chimera with the pre-*let-7* stem. Thus, the pre-miR-307a stem, not the loop, is necessary and sufficient for Loqs-PB to redirect where Dcr-1 cleaves.

### Loqs-PB Does Not Require a 5' Terminal Phosphate to Influence Dcr-1 Cleavage Site Choice

Both human Dicer and *Drosophila* Dcr-1 use the 5' terminal phosphate to align a pre-miRNA in their active sites (Park et al., 2011); without a 5' phosphate, cleavage site choice is less precise. To ask whether Loqs-PB requires a 5' terminal phosphate to influence the choice of cleavage site by Dcr-1, we compared the products from 5' phosphorylated (Figures 4A, 4B, S6A, and S6B) and hydroxy pre-miR-307a (Figures S6J and S6K). Loqs-PB did not require a 5' terminal phosphate to influence where Dcr-1 cuts pre-miR-307a: for Dcr-1 alone, 66% ± 1% of 5' hydroxy miR-307a\* was 21 nt and 25% ± 1% was 23 nt, but when Dcr-1 was supplemented with Loqs-PB,

24% ± 2% of 5' hydroxy miR-307a\* was 21 nt and 65% ± 4% was 23 nt (Figures S6J and S6K).

### miR-307a<sup>21-mer</sup> and miR-307a<sup>23-mer</sup> Have Distinct Target Specificities

miR-307a<sup>21-mer</sup> and miR-307a<sup>23-mer</sup> have overlapping but distinct seed sequences. To test whether both miR-307a isoforms are functional and whether they have different target specificities, we measured their ability to silence a *Renilla reniformis* (*Rr*) luciferase reporter in cultured *Drosophila* S2 cells.

Endogenous miR-307a in S2 cells is below the limit of detection of northern hybridization (data not shown), and only 28 pre-miR-307a-mapping sequence reads per million (ppm) were detected in S2 cells (Han et al., 2011). For comparison, the tenth most abundant miRNA in S2 cells, miR-305, was present at 11,523 ppm in the same library, and 46 miRNAs were more abundant than miR-307a. The low level of miR-307a allowed us to introduce the 21-mer and 23-mer isoforms as miR-307a/miR-307a\* duplexes in S2 cells by transfection.

We constructed *Rr* luciferase reporters containing in their 3' UTRs six tandem, bulged sites (i.e., fully complementary sites containing mismatches at positions 9–11; Figure 5A). Each reporter plasmid was cotransfected with an increasing amount of 21-mer or 23-mer miR-307a/miR-307a\* duplex. miR-307a<sup>21-mer</sup> and miR-307a<sup>23-mer</sup> each suppressed the miR-307a<sup>21-mer</sup> and the miR-307a<sup>23-mer</sup> complementary reporters (Figure 5B). This was expected, because the two isomirs are both complementary to the two reporters.

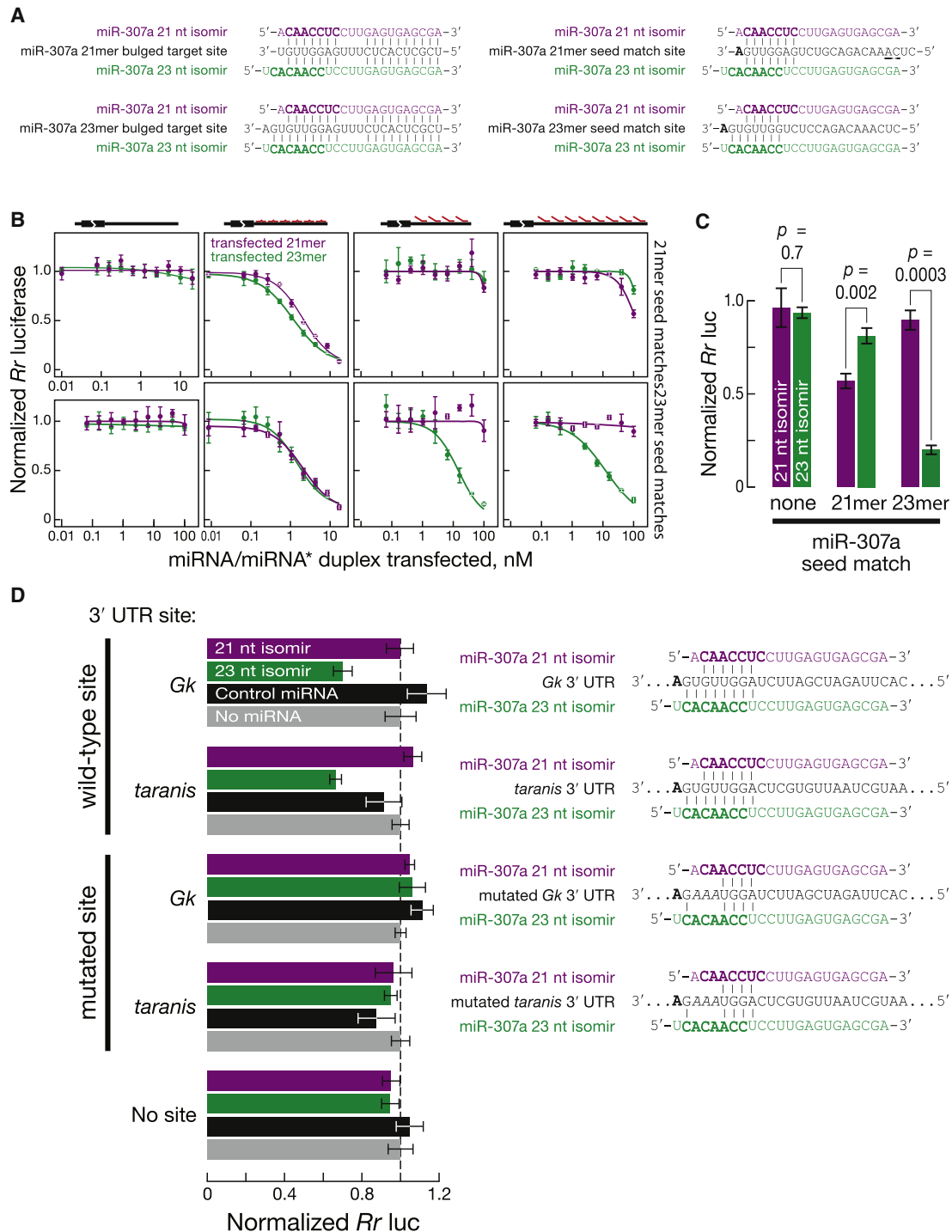
To test whether miR-307a<sup>21-mer</sup> and miR-307a<sup>23-mer</sup> have distinct targeting specificities, we constructed reporter constructs with four or eight t1A, 7 nt seed-matching sites. Each seed match was complementary to the seed sequence of one of the two miR-307 isoforms (Figure 5A). The miR-307a<sup>21-mer</sup> seed-match reporter was more efficiently repressed by the 21-mer than by the 23-mer (Figures 5B and 5C). Conversely, the miR-307a<sup>23-mer</sup> seed-match reporter was more efficiently repressed by the 23-mer than by the 21-mer. Control reporters were not repressed by either isoform. Thus, miR-307a<sup>21-mer</sup> and miR-307a<sup>23-mer</sup> have distinct target specificities.

### miR-307a<sup>23-mer</sup>, but Not miR-307a<sup>21-mer</sup>, Represses *Gk* and *tara*

The mRNAs for *glycerol kinase* (*Gk*) and *taranis* (*tara*), a trithorax group protein, are the only 8-mer (t1A plus 7 seed-matching nucleotides) targets predicted by TargetScan (Lewis et al., 2005) for miR-307a<sup>23-mer</sup>. To test whether *Gk* and *tara* mRNAs are direct targets of miR-307a<sup>23-mer</sup>, we constructed luciferase reporters with the 3' UTR of *Gk* or *tara*. Both reporter constructs were silenced by transfection of miR-307a<sup>23-mer</sup> but not by miR-307a<sup>21-mer</sup> or a control miRNA (Figure 5D; for miR-307a<sup>23-mer</sup>, *p* value = 0.004 for *Gk* and  $2 \times 10^{-4}$  for *tara*; for miR-307a<sup>21-mer</sup>, *p* value > 0.05 for both). Mutating the seed-matching sites in the *Gk* or *tara* 3' UTR eliminated silencing by miR-307a<sup>23-mer</sup> (all *p* values > 0.05).

To further test the idea that miR-307a<sup>23-mer</sup> but not miR-307a<sup>21-mer</sup> represses *Gk* and *tara*, we used RNA sequencing to measure the consequences of changing the miR-307a isoform distribution in vivo. We sequenced poly(A)<sup>+</sup> RNA from ovaries





**Figure 5. miR-307a<sup>21-mer</sup> and miR-307a<sup>23-mer</sup> Have Distinct Targets**

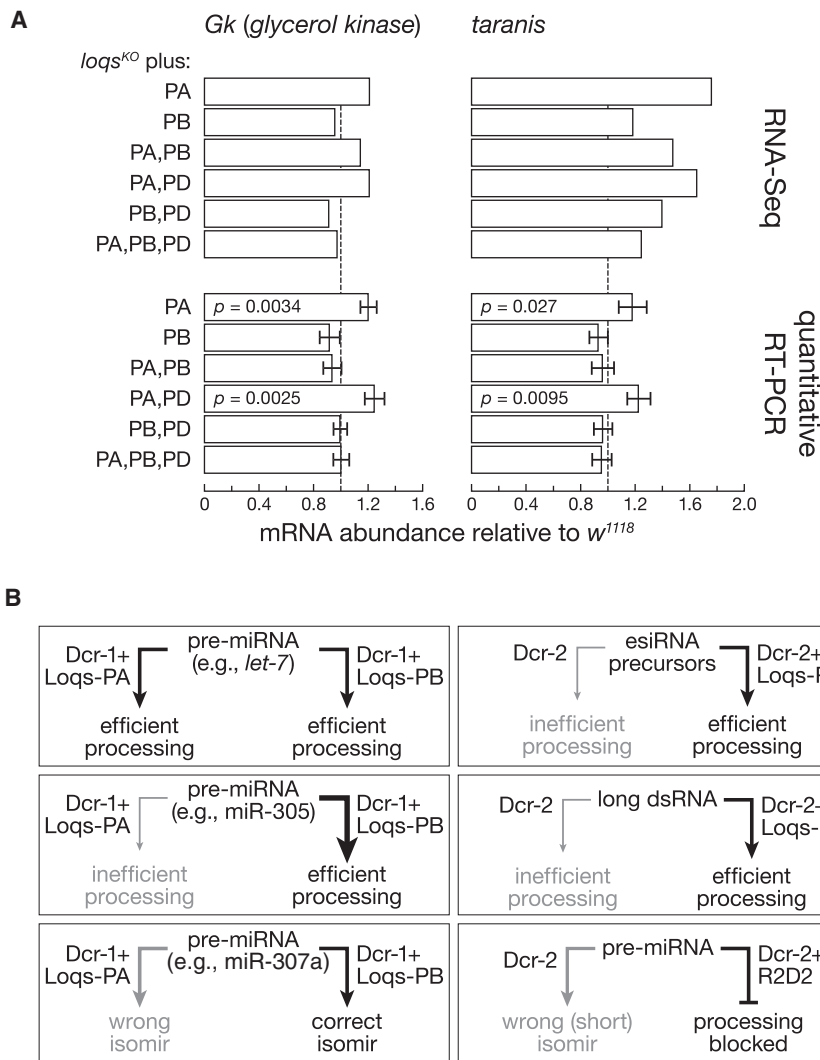
(A) Pairing of miR-307a<sup>21-mer</sup> (purple) and miR-307a<sup>23-mer</sup> (green) to luciferase reporter target sites (black).

(B) Silencing of *Renilla* luciferase reporters bearing 3' UTR target sites for miR-307a<sup>21-mer</sup> or miR-307a<sup>23-mer</sup>. Reporters had six sites with mismatches at positions 9–11, or four or eight sites matching the seed of miR-307a<sup>21-mer</sup> or miR-307a<sup>23-mer</sup>. *Renilla* luciferase expression relative to a firefly luciferase internal control was determined at different concentrations of transfected miRNA/miRNA\*. Data were normalized to a transfection control without miR-307a/miR-307\*.

(C) The reporter data for *Renilla* mRNA bearing eight or no target sites matching the seeds of miR-307a<sup>21-mer</sup> or miR-307a<sup>23-mer</sup> was analyzed to determine the extent of repression at the highest concentration of transfected miR-307/miR-307\* duplex.

(D) Data for *Renilla* mRNA bearing the 3' UTR of *Gk* or *tara* mRNA. miRNAs were transfected at 20 nM.

Data are mean ± SD for three independent trials.



of control and *loqs<sup>KO</sup>* mutant flies rescued with Loqs isoform-specific transgenes (Figure 6A). Both *Gk* and *tara* mRNAs were depressed in the mutant flies lacking Loqs-PB, compared with control and rescue flies expressing Loqs-PB; quantitative RT-PCR confirmed these results (Figure 6A). We conclude that Loqs-PB is required to produce the correct miR-307a isomir to regulate specific mRNAs in vivo (Figure 6B).

#### TRBP Changes Where Dicer Cleaves Pre-miR-132

Mammals produce two partner proteins, PACT and TRBP (Chen-drimada et al., 2005; Haase et al., 2005; Lee et al., 2006) for their single Dicer, whose main role in somatic cells is the production of miRNAs (Hutvagner et al., 2001). To assess the role of PACT and TRBP in establishing where mammalian Dicer cleaves pre-miRNAs, we sequenced small RNAs from wild-type, *Pact<sup>-/-</sup>*, *Trbp<sup>-/-</sup>*, and *Trbp<sup>-/-</sup>* MEFs (Tables S5A and S5B). Of the 809 miRNAs detected in wild-type MEFs, the 21 nt isomirs of miR-132 and miR-132\* were more abundant and the 22 nt isomirs were less abundant in *Trbp<sup>-/-</sup>* MEFs compared to *Trbp<sup>-/-</sup>*, *Pact<sup>-/-</sup>*, or wild-type MEFs. The 22 nt miR-132 isoform, which is the anno-

#### Figure 6. miR-307a<sup>23-mer</sup>, but Not miR-307a<sup>21-mer</sup>, Represses *Gk* and *tara*

(A) Relative abundance of *Gk* and *tara* mRNA in ovaries of control and mutant flies with Loqs-expressing transgenes, determined by quantitative RT-PCR (mean  $\pm$  SD for three biological replicates) and mRNA-Seq. (B) Proposed functions of Dicer partner proteins in flies.

tated form in miRBase, predominated in all MEF genotypes, but was lowest in the cells lacking TRBP (Figure 7A). miR-132 derives from the 3' arm of its pre-miRNA. Thus, changing where Dicer cleaves changes its seed sequence and alters the repertoire of targets it regulates.

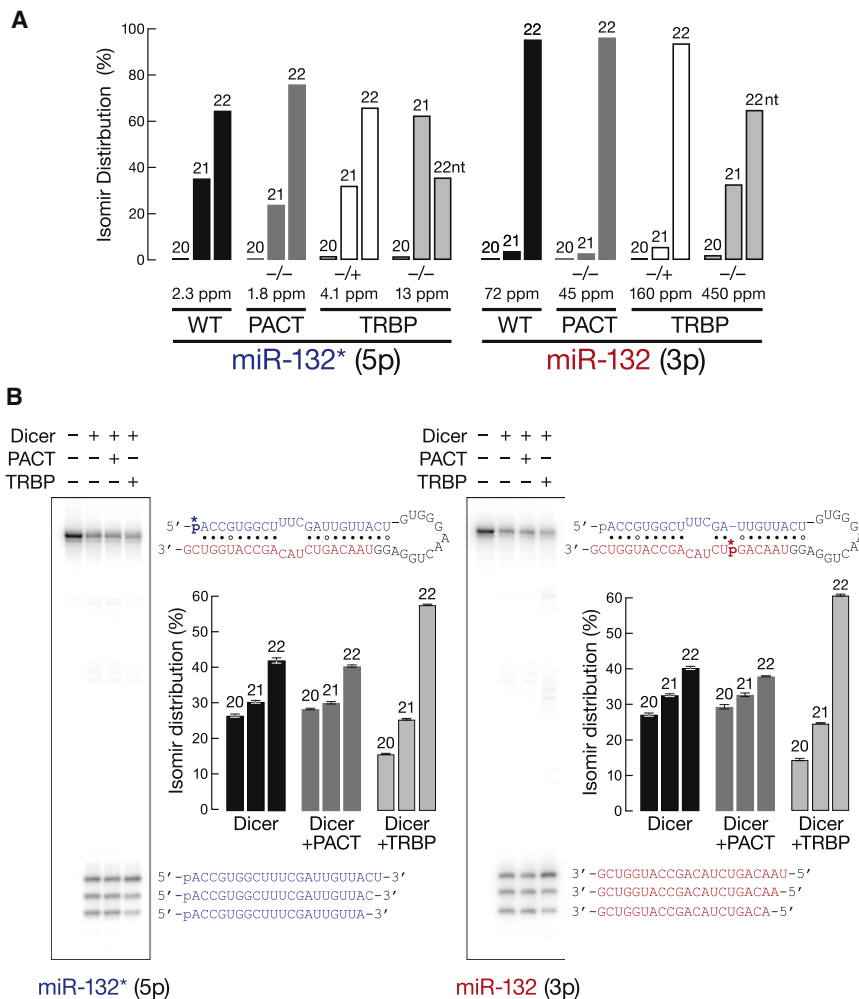
To test whether partner proteins directly influence where mammalian Dicer cleaves, we monitored the production of small RNAs from human pre-miR-132. (Human pre-miR-132 differs from mouse pre-miR-132 at only a single loop nucleotide.) In the presence of TRBP, purified, recombinant human Dicer produced more 22 nt and less 20 nt miRNA and miRNA\* products from pre-miR-132, compared with Dicer alone or Dicer plus PACT (Figure 7B). We conclude that, like Loqs-PB in flies, TRBP in mammals can directly influence where Dicer cleaves a pre-miRNA.

#### DISCUSSION

The simplest interpretation of our data that Loqs-PA and Loqs-PB both

decrease the  $K_M$  of Dcr-1 for pre-miRNA substrates is that Loqs partner proteins increase the affinity of Dcr-1 for some pre-miRNA substrates. For other pre-miRNAs, Loqs-PB can also increase Dcr-1 enzyme turnover. Figure 6B summarizes the functions of Dicer partner proteins in small RNA biogenesis in flies: preventing Dcr-2 from processing pre-miRNAs (R2D2), promoting the processing of "foreign" dsRNA and of esiRNAs and other endo-siRNA precursors (Loqs-PD), enhancing the efficiency of pre-miRNA processing by Dcr-1 (Loqs-PA and PB), and tuning cleavage site choice by Dcr-1 for a small number of pre-miRNAs (Loqs-PB).

The last two functions are surprising because the number of pre-miRNAs that require Loqs-PB and not Loqs-PA for the efficient production of their miRNAs is small and even fewer require Loqs-PB to produce the correct isomir. Nonetheless, our results suggest that the loss of GSCs in flies lacking Loqs-PB reflects the reduced abundance of specific miRNAs such as miR-79, miR-283, miR-305, miR-311, and miR-318 or perhaps the production of the wrong isomirs for miR-307a, miR-87, or miR-316. Among these, miR-318 is the most



**Figure 7. Mammalian TRBP Tunes Where Dicer Cleaves Pre-miR-132**

(A) Length distribution of miR-132 and miR-132\* isomirs in MEFs measured by sequencing.

(B) Pre-miR-132 (100 nM), bearing a single  $^{32}\text{P}$ -radiolabel (asterisk), was incubated for 2 hr with purified, recombinant human Dicer (10 nM) or Dicer supplemented with PACT or TRBP (10 nM). Data are mean  $\pm$  SD for four independent trials.

See also Table S5.

how Loqs-PB and TRBP change where Dcr-1 and Dicer cleave is that the mismatches and internal loop causes the stems of pre-miR-307a, pre-miR-87, and mammalian pre-miR-132 to be longer than the corresponding A-form helix; binding of Loqs-PB or TRBP might then “shrink” the stems. In contrast, Loqs-PB binding may extend of the stem of pre-miR-316. Because Loqs-PB does not change where Dcr-2 cleaves pre-miR-307a, Loqs-PB likely acts only when bound to Dcr-1.

Our data suggest that the effect of Loqs-PB is biologically relevant. The long miR-307a isomir predominates in wild-type flies, and miR-307a<sup>23-mer</sup> but not miR-307a<sup>21-mer</sup> can repress the *Gk* and *tara* mRNAs in vivo. Why has evolution failed to select for easier-to-dice variants of pre-miR-307a and pre-miR-87 in flies and pre-miR-132 in mice, so that a specific partner is no longer

abundant miRNA in ovaries (140,000 ppm in *w<sup>1118</sup>* ovaries but only 8 ppm in heads). Future experiments using Loqs-independent versions of pre-miR-318 and other miRNAs should help test this idea.

The amino acid sequence of Loqs-PB suggests it is slightly more related to PACT (28% identity; 34% similarity) than to TRBP (24% identity; 33% similarity). Nonetheless, our data suggest that the functional homolog of Loqs-PB in mammals is TRBP. Like Loqs-PA and Loqs-PB, TRBP may act generally to enhance the binding of Dicer to pre-miRNA. Such a role for TRBP is consistent with earlier observations that the two cleavage reactions catalyzed by mammalian Dicer are less well coordinated in the absence of TRBP (Koscianska et al., 2011). But like Loqs-PB, TRBP also helps Dicer produce specific isoforms of a one or more miRNAs. This novel function of Dicer partner proteins may be widely conserved, enabling plants and animals to effectively and accurately dice difficult but important pre-miRNA substrates. Perhaps the Drosha-binding partner Pasha in flies or DGCR8 in mammals similarly tunes pri-miRNA cleavage site choice by Drosha.

The stem, but not the loop, of pre-miR-307a enables Loqs-PB to influence where Dcr-1 cleaves. One possible explanation for

needed to ensure production of the “right” isomir? The persistence of the miR-307a<sup>21-mer</sup> in wild-type flies, for example, suggests that most, if not all, abundant isomirs have distinct biological functions and that the optimal function of miRNAs like miR-307a, miR-87, and miR-132 requires a defined ratio of isomirs.

Although we failed to detect altered regulation of predicted miR-307a<sup>21-mer</sup> targets in ovaries from flies lacking Loqs-PB, miR-307a<sup>21-mer</sup> may have functions in other tissues or times in development. In flies, mice, and humans, the relative abundance of miRNA isomirs generated by different Dicer cleavage sites, including 5' isomirs with distinct seed sequences, varies among tissues and developmental stages (Azuma-Mukai et al., 2008; Fernandez-Valverde et al., 2010; Lee et al., 2010). Perhaps the abundance of Loqs-PB versus Loqs-PA or TRBP versus PACT is regulated across development and differentiation to ensure the correct relative abundance of isomirs from various pre-miRNAs, much as the ratios of alternatively spliced mRNAs are regulated in different tissues and cell types. Our *loqs<sup>KO</sup>* flies rescued with transgenes producing individual Loqs isoforms should facilitate the testing of this idea in vivo.

## EXPERIMENTAL PROCEDURES

### Transgenic Flies

P{Loqs-PA}, P{Loqs-PB}, and P{Loqs-PD} rescuing transgenes containing the *loqs* promoter were generated by appending the ~2.5 kbp upstream and ~0.8 kbp downstream genomic sequences to the Loqs-PA, Loqs-PB, or Loqs-PD cDNA in pattB (Bischof et al., 2007). A 3x FLAG tag was added to the amino-terminus of each protein. Transgenes were integrated into a single site in the genome (Bischof et al., 2007), and the mini-*white* gene (*w<sup>mc</sup>*) removed by using Cre-Lox. Double and triple rescue flies were generated by meiotic recombination.

### Immunohistochemistry and Microscopy

Egg chamber fixation and whole-mount antibody labeling were performed as described (Theurkauf, 1994; Li et al., 2009). Samples were analyzed with a Leica TCS-SP inverted, laser-scanning microscope. Images were acquired in 2.89  $\mu$ m increments along the z axis in order to determine the number of GSC within the germarium. All images were acquired at the same settings and are presented in the figure at the same scale.

### Recombinant Protein Expression and Purification

His<sub>6</sub>-Dicer-1 containing the HRV3C protease recognition site after the amino-terminal His<sub>6</sub>-tag was expressed in Sf9 insect cells by using the Bac-to-Bac system (Invitrogen). cDNA encoding Loqs-PA, Loqs-PB, PACT, or TRBP was inserted between the Sall and NotI restriction sites of a modified pCold I vector (Takara, Otsu, Shiga, Japan) containing the HRV3C protease recognition site. Amino-terminally His<sub>6</sub>-tagged Loqs-PA, Loqs-PB, PACT, and TRBP proteins were expressed in *Escherichia coli* BL21 codon Plus RIL (Agilent Technologies). Proteins were purified by using Ni-Sepharose (GE Healthcare, Pittsburgh, PA, USA), followed by HRV3C protease cleavage to remove the His<sub>6</sub>-tag, and then further purified by chromatography by using HiTrap SP and HiTrap Heparin (GE Healthcare). Recombinant protein concentrations were determined by quantitative amino acid analysis of protein isolated from an SDS-polyacrylamide gel (Keck Biotechnology Resource Laboratory, New Haven, CT, USA).

### Dicing

Dicing reactions were performed as described (Cenik et al., 2011) at 25°C for fly and 37°C for human Dicer. Dcr-1, Dcr-2, or human Dicer was used with or without an equimolar amount of purified recombinant partner protein.

To determine the inflection point *n* in the stoichiometric titration (Figures 4D and S6C), the data were fit to:

$$f(r) = m \times \frac{r + n - \sqrt{(r - n)^2}}{2n} + b.$$

When  $r \geq n$ ,  $f(r) = m + b$ ; when  $r < n$ ,  $f(r) = m \cdot r/n + b$ ; where  $b = y$  intercept and  $m + b = y_{\text{plateau}}$ , the  $y$  intercept of the plateau.

### Sequencing

miRNA libraries were as described (Li et al., 2009; Ghildiyal et al., 2010; Ameres et al., 2010; Han et al., 2011; Zhang et al., 2011). Strand-specific mRNA libraries were prepared (Wang et al., 2011) by using adapters and primers in Table S6. Data are available from SRA (accession number SRP014526).

## ACCESSION NUMBERS

The GEO accession number for the small RNA libraries reported in this paper is GSE37443, and the SRA accession number for the RNA-Seq libraries reported in this paper is SRP014526.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2012.09.027>.

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#### Note Added in Proof

Lee and Doudna recently reported that human TRBP alters where purified Dicer cleaves pre-miRNAs, producing isomirs 1 nt longer than those made by Dicer alone: Lee, H.Y. and Doudna, J.A. (2012). TRBP alters human precursor microRNA processing in vitro. *RNA*. Published online September 24, 2012, <http://dx.doi.org/10.1261/rna.035501.112>.