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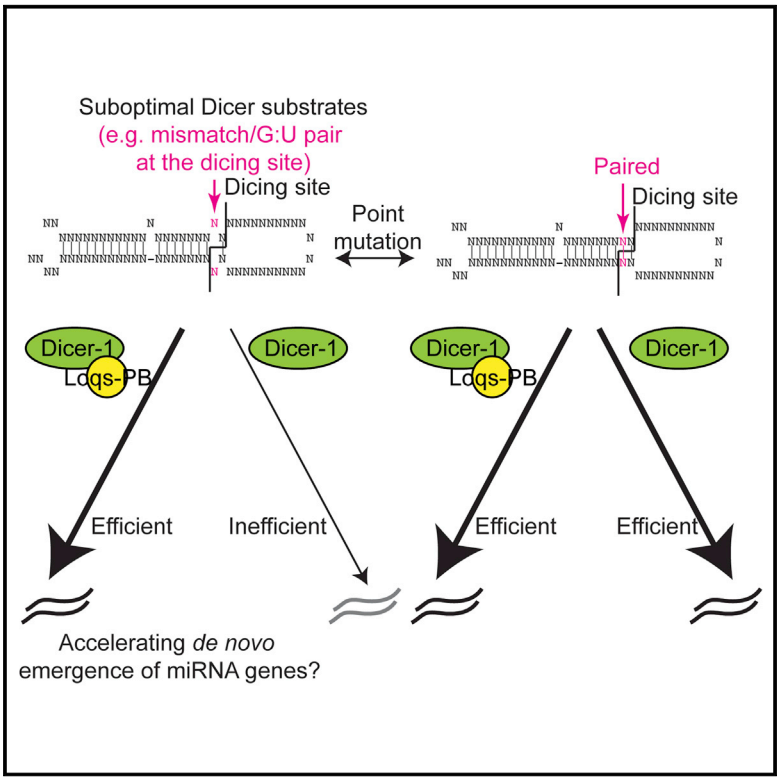
The *Drosophila* Dicer-1 Partner Loquacious Enhances miRNA Processing from Hairpins with Unstable Structures at the Dicing Site

Graphical Abstract



Highlights

- A cell line lacking Loqs was established from *loqs*-null fly embryos
- ~40% of fly miRNAs require Loqs for efficient processing
- Loqs enhances dicing of miRNA precursors with unstable structures at the dicing site
- Evolutionarily young miRNAs tend to require Loqs for efficient processing

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In Brief

By generating a *Drosophila* cell line that lacks Dicer partner Loquacious (Loqs), Lim et al. demonstrate that Loqs facilitates processing of suboptimal miRNA hairpins containing unstable structures at the dicing site. They propose a role for Loqs in accelerating de novo emergence of miRNA genes.

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The *Drosophila* Dicer-1 Partner Loquacious Enhances miRNA Processing from Hairpins with Unstable Structures at the Dicing Site

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SUMMARY

In *Drosophila*, Dicer-1 binds Loquacious-PB (Loqs-PB) as its major co-factor. Previous analyses indicated that *loqs* mutants only partially impede miRNA processing, but the activity of minor isoforms or maternally deposited Loqs was not eliminated in these studies. We addressed this by generating a cell line from *loqs*-null embryos and found that only ~40% of miRNAs showed clear Loqs dependence. Genome-wide comparison of the hairpin structure and Loqs dependence suggested that Loqs substrates are influenced by base-pairing status at the dicing site. Artificial alteration of base-pairing stability at this position in model miRNA hairpins resulted in predicted changes in Loqs dependence, providing evidence for this hypothesis. Finally, we found that evolutionarily young miRNA genes tended to be Loqs dependent. We propose that Loqs may have roles in assisting the de novo emergence of miRNA genes by facilitating dicing of suboptimal hairpin substrates.

INTRODUCTION

MicroRNAs (miRNAs) are a family of ~22-nt small regulatory RNAs that are processed from longer precursor transcripts (Ha and Kim, 2014), and dysregulation of miRNA-processing activity is often associated with human diseases (Foulkes et al., 2014; Garzon et al., 2009). In the canonical miRNA-processing pathway, miRNA hairpins residing in primary miRNA transcripts (pri-miRNAs) are cleaved by the Microprocessor complex, which contains the nuclear RNase III enzyme Drosha, to produce ~60- to 80-nt precursor- (pre-) miRNAs. Pre-miRNAs are then exported to the cytoplasm and undergo the second processing step. The cytoplasmic RNase III protein Dicer cleaves pre-miRNAs to release short RNA duplexes by removing loop regions of pre-miRNAs. Processed mature miRNAs are loaded to the effector complexes containing Argonaute proteins to execute their functions.

Eukaryotic RNase III enzymes often require partner proteins to carry out their miRNA-processing functions (Heo and Kim, 2009). Drosha and its partner protein Pasha (DGCR8 in vertebrates) form the core of the Microprocessor complex, and Pasha is essential for recognition and precise cleavage of hairpin substrates (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Herbert et al., 2016; Kwon et al., 2016; Martin et al., 2009; Nguyen et al., 2015).

Dicer also binds its partner proteins. In contrast to Pasha/DGCR8 whose activity is essential for pri-miRNA cleavage, roles of Dicer partner proteins are enigmatic. In mammals, two paralogs of Dicer partners, TRBP and PACT, have been identified (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). Both proteins were originally reported to facilitate miRNA maturation at the dicing and/or loading steps (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). However, studies showed that TRBP only has roles in modulating lengths of mature miRNA species for specific genes and is dispensable for efficient pre-miRNA processing (Fukunaga et al., 2012; Lee and Doudna, 2012). Furthermore, miRNA expression profiles of a recently established TRBP/PACT double-knockout cell line are indistinguishable from profiles of TRBP single-knockout cells, indicating that PACT does not compensate for the absence of TRBP (Kim et al., 2014). However, these results do not exclude the possibility that TRBP plays tissue-specific roles in miRNA processing or the lack of TRBP may be compensated for by alternative mechanisms including binding of ADAR1 (Adenosine deaminase acting on RNA 1) to Dicer (Ding et al., 2015; Ota et al., 2013). Besides dicing functions, TRBP plays roles in gene regulation and immune response via Dicer-independent mechanisms (Goodarzi et al., 2014; Kim et al., 2014; Nakamura et al., 2015).

In flies, the counterpart of TRBP/PACT is known as Loquacious (Loqs; also known as R3D1) (Förstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). There are three alternative splicing/polyadenylation variants expressed at detectable levels, Loqs-PA, -PB, and -PD. Loqs-PA and -PB stably bind to Dicer-1 (Dcr-1) to form the miRNA Dicing complex and have been suggested to play distinct but overlapping roles in the miRNA pathway (Förstemann et al., 2005; Fukunaga et al., 2012; Jiang et al., 2005; Saito et al., 2005). The remaining isoform Loqs-PD plays roles in the small interfering RNA (siRNA) pathway by binding to the siRNA Dicer, Dicer-2 (Hartig et al., 2009; Miyoshi et al., 2010; Zhou et al., 2009). RNAi-mediated knockdown of *loqs* often results in accumulation of pre-miRNAs, and the Dcr-1/Loqs-PB heterodimer complex shows more rapid pre-miRNA processing compared to the Dicer protein itself in in vitro assays or heterologous reconstitution assays in Dicer-knockout mammalian cells (Bogerd et al., 2014; Förstemann et al., 2005; Jakob et al., 2016; Jiang et al., 2005; Saito et al., 2005). These observations led to the notion that Loqs is generally required for efficient miRNA processing. Zygotic *loqs* mutant shows larval/pupal lethality, a phenotype that is commonly seen in mutants of core miRNA-processing enzymes (Lee et al., 2004; Martin et al., 2009; Park et al., 2007; Pressman et al., 2012; Smibert et al., 2011). In contrast, a study that tested miRNA expression levels in *loqs* mutant flies demonstrated that only a subset of miRNAs showed decreased mature miRNA levels (Liu et al., 2007; Marques et al., 2010). Due to the possible contribution of maternally deposited Loqs protein from their heterozygous mothers, it has remained unclear whether mature miRNAs present in *loqs* mutants were generated by a Loqs-independent mechanism, or a result of maternal Loqs protein and mature miRNA carryover.

To completely eliminate Loqs, we established a cell line that lacks the *loqs* gene locus and demonstrated that *loqs* is dispensable for processing of most miRNA genes. As previously reported, Loqs-PB altered Dicer-mediated cleavage sites in a small number of miRNA hairpins. We confirmed that a subset (~40%) of miRNAs showed reduced expression in the absence of Loqs and characterized molecular features of *loqs*-dependent miRNAs to show that Loqs-dependent miRNA hairpins often had unstable base-pairing structures around the Dcr-1 cleavage sites. Using mutated model hairpins, we further verified the importance of the base-pairing status at this position in determining the *loqs* independence. Our results provide an insight into the mechanism by which a Dicer partner protein facilitates biogenesis of a subset of miRNAs. Furthermore, we found that evolutionarily young miRNAs tended to be Loqs dependent, suggesting a role for Loqs in evolution of miRNA genes.

RESULTS

Generation of a *loqs* Mutant Cell Line

To study miRNA processing in the complete absence of Loqs, we generated a cell line from *loqs*-null mutant embryos (*loqs-KO*, a deletion allele) following a protocol for establishing *Drosophila* cell lines using expression of oncogenic Ras^{V12} (Liu et al., 2007; Simcox et al., 2008a, 2008b). Only cells mutant for *loqs* also express Ras^{V12}, which gives them a proliferation advantage (Figure 1A) (Simcox et al., 2008a). We verified the *loqs* deletion by PCR assays with primers specific for the wild-type or mutant alleles (Figure 1B). Recovering only the expected product specific to the deletion allele from the cells showed that the line is comprised of homozygous *loqs* knockout cells and any other cells, if present, are rare.

The cell line has a doubling time of about 1 day (Figure 1C) and could be passaged more than 50 times showing that it is a continuous line. The extensive cell doublings that occurred during this time in culture exclude the possibility that any residual Loqs function remains and makes the line an ideal source of *loqs* mutant cells. Moreover, the *loqs* mutant cells could also be transiently transfected with plasmids encoding other individual *loqs* isoforms allowing a molecular dissection of Loqs function (Figure 1D) (Förstemann et al., 2005; Hartig et al., 2009). Furthermore, we found that cells stably transfected with a plasmid encoding the Loqs-PB cDNA sequence under the control of a $CuSO_4$-inducible promoter (Saito et al., 2005) showed a similar growth characteristics to the mutant cells, suggesting that presence or absence of Loqs-PB function did not strongly affect cell growth (Figures 1C and 1E). In summary, we have generated a cell line with complete absence of Loqs function that is amenable to molecular analysis.

loqs Mutant Cells Show Expected Defects in Known Loqs-Dependent miRNAs

To confirm that Loqs functionalities were abolished in the *loqs* mutant cells, we first examined whether the previously reported Loqs-PB-dependent long isoform of miR-307a was produced in the *loqs* mutant cells (Fukunaga et al., 2012). We overexpressed *mir-307a* along with isoform-specific *loqs* rescue constructs (Figure 2A, upper panel). When Loqs-PA or -PD was co-expressed, we observed a mild shift of the cleavage site. A more dramatic effect was observed in cells rescued with Loqs-PB, consistent with a previous study (Fukunaga et al., 2012). In addition, the levels of two other miRNAs that are known to require Loqs-PB for efficient processing (*mir-283* and *mir-305*) were increased by expression of Loqs-PB (Fukunaga et al., 2012) (Figure 2A, middle and lower panels). Similarly to the effects on *mir-307a*, we unexpectedly observed weak but reproducible up-regulation of mature miRNA production for these genes when Loqs-PA or -PD was co-expressed. We also tested whether siRNA production was restored by Loqs-PD expression. Plasmids encoding hp-siRNA precursors were co-transfected with the rescue constructs and mature siRNA were detected by northern blotting (Figure 2B). As expected, we observed clear signals of mature siRNAs from hp-CG4068 and hp-CG18854 only in cells rescued with Loqs-PD, while the mature siRNA signals were very weak or undetectable in other lanes. These results further confirmed that all known functions of Loqs isoforms were impaired in the *loqs* mutant cell line and could be rescued by re-expression of specific Loqs isoforms.

Small RNA Library Analysis Confirms Molecular Phenotypes of *loqs* Mutant Cells

To understand global effects of Loqs-PB re-expression in *loqs* mutant cells, we sequenced small RNA libraries prepared from

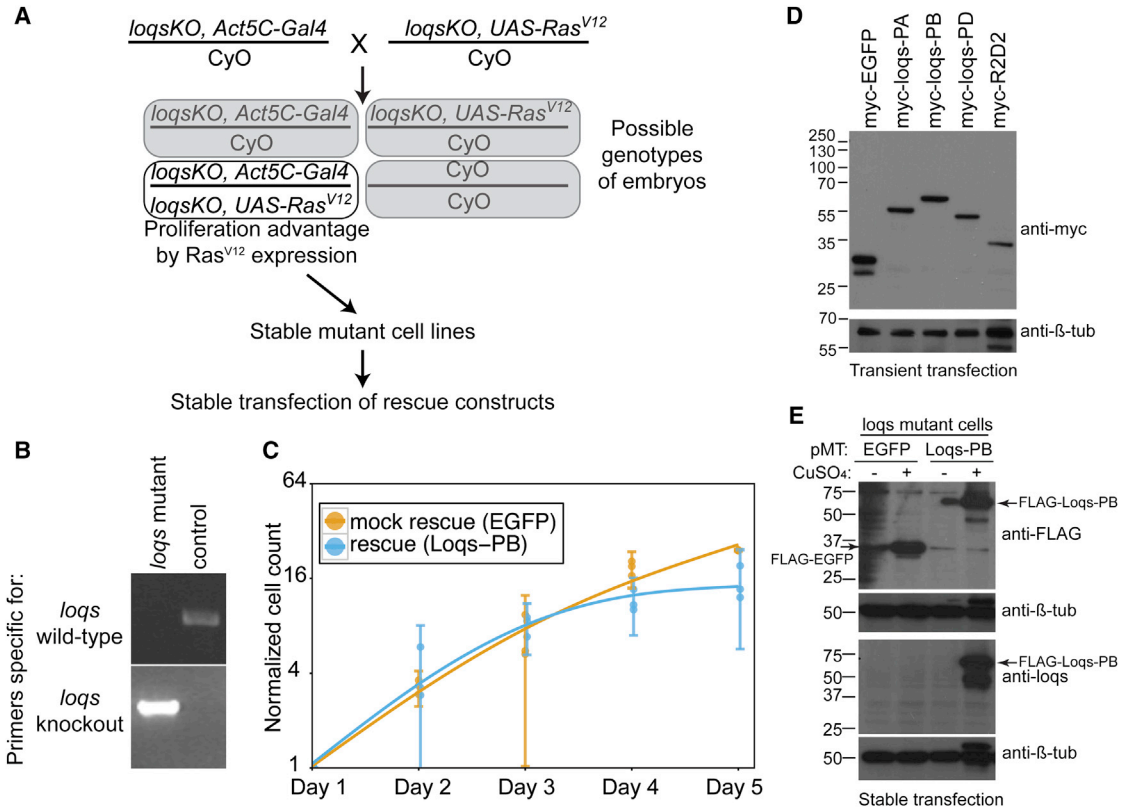


Figure 1. Generation of a *loqs* Mutant Cell Line

(A) Schematic representation of cell line establishment. Only cells mutant for *loqs* express *RAS*^{V12} and hence have a proliferation advantage.

(B) PCR genotyping. The presence of the wild-type (upper panel) or knockout (lower panel) *loqs* allele was tested by specific primers using genomic DNA templates prepared from *loqs* mutant cells or a control cell line established by the same procedure. Only the *loqs*-KO allele was detected in the *loqs* mutant cell line.

(C) Growth curves of *loqs* mutant cells rescued with EGFP control or Loqs-PB. Stably transfected *loqs* mutant cells (dark yellow, control EGFP; blue, Loqs-PB rescue) were grown under a standard condition, and cell densities were monitored for 4 days. Cell counts were normalized by the count on day 1 of each cell line and the normalized numbers (dots) were plotted on a log scale.

(D) Western blotting detection of individual myc-tagged Loqs isoforms overexpressed by transient transfection. Proteins were detected by anti-myc antibody and re-probed with anti-β-tubulin antibody for loading control.

(E) Western blotting detection of stably transfected genes. *loqs* mutant cells stably transfected with indicated plasmids were incubated with or without $CuSO_4$. Membranes were incubated with anti-FLAG (upper panel) or anti-Loqs (lower panel) and then re-probed with anti-β-tubulin antibody for loading control.

the cell lines stably transfected with the Loqs-PB rescue or EGFP control plasmid that were grown in the presence or absence of the inducer (2 mM $CuSO_4$). These yielded ~5–14 million reads mapping to the *Drosophila melanogaster* genome, ~60%–70% of which were derived from miRBase miRNA hairpins (Kozomara and Griffiths-Jones, 2014) (Table S1).

It is important to use an appropriate read normalization method when the amount of bulk miRNAs is expected to change, for example, by a mutation of a general miRNA-processing factor. To account for the change in the bulk miRNA abundance, we added synthetic RNA oligonucleotides with ten different sequences (hereafter termed spike-in oligos) to RNA samples prior to library construction and used the spike-in read counts as calibrators (Table S1). We believe that this normalization method allows for more accurate estimation of the relative bulk abundance of miRNAs than conventional normalization methods (Yi et al., 2009).

Our small RNA library analysis independently supported our northern blotting results (Figure 2C). We observed a clear shift of the major 5′ end of miR-307a-3p species, and enhanced expression of miR-305 and miR-283 mature species when cells were rescued with Loqs-PB. Unexpectedly, the effects of the Loqs-PB rescue construct were already observed in the absence of $CuSO_4$ inducer, suggesting that the low level of expression by the "leaky" metallothionein promoter in the absence of $CuSO_4$ was sufficient to support Loqs functions (Djuranovic et al., 2012). The $CuSO_4$-independent effects were confirmed by northern blotting (Figure S1).

Taken together, our northern blotting and sequencing analyses verified the expected effects of the *loqs* mutation on miRNA processing, further confirming the validity of the cell lines. Because we observed similar expression patterns in the libraries made from cells cultured with and without the $CuSO_4$ inducer (Figures 2C, S1, and S2A), we used the average miRNA

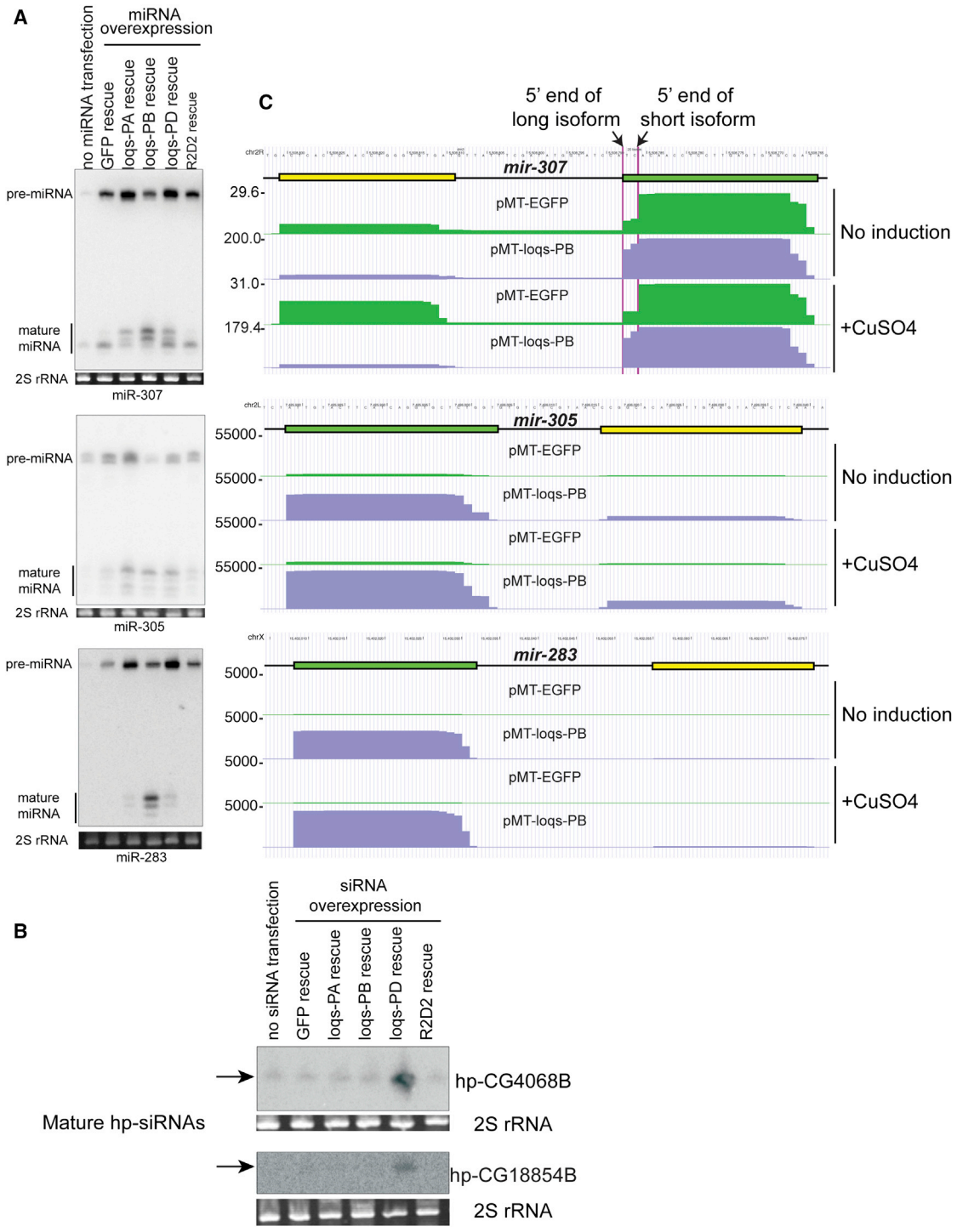


Figure 2. Known miRNA-Processing Phenotypes Can Be Rescued by Loqs-PB Expression

(A) Northern blotting verification of the Loqs-PB-dependent miRNAs. A *mir-307a*, *mir-305*, or *mir-283* overexpression plasmid was co-transfected with a control (EGFP or R2D2) or isoform-specific rescue (Loqs-PA, -PB, or -PD) plasmid in *loqs* mutant cells. Total RNA was analyzed by northern blotting. Production of the miR-307a long isoform (upper panel) and all isoforms of miR-305 and miR-283 were enhanced slightly by Loqs-PA or -PD and strongly by Loqs-PB.

(B) Production of hp-siRNAs is dependent on Loqs-PD. Plasmids encoding hp-CG4068 (upper panel) or hp-CG18854 (lower panel) were transfected in *loqs* mutant cells, and northern blotting was performed to detect siRNA species as indicated. rRNA was visualized by SYBR Green II.

(C) Small RNA read densities at the *mir-307a*, *mir-305*, and *mir-283* loci in EGFP or Loqs-PB rescue libraries. In EGFP control cells, a majority of miR-307a-3p reads have the 5′ ends shifted from the canonical cleavage site by 2 nt, in contrast to the normal 5′ position of miR-307a-3p in the Loqs-PB rescue library. Read densities of mature miR-305 and miR-283 species were higher in Loqs-PB rescue cells compared with control cells.

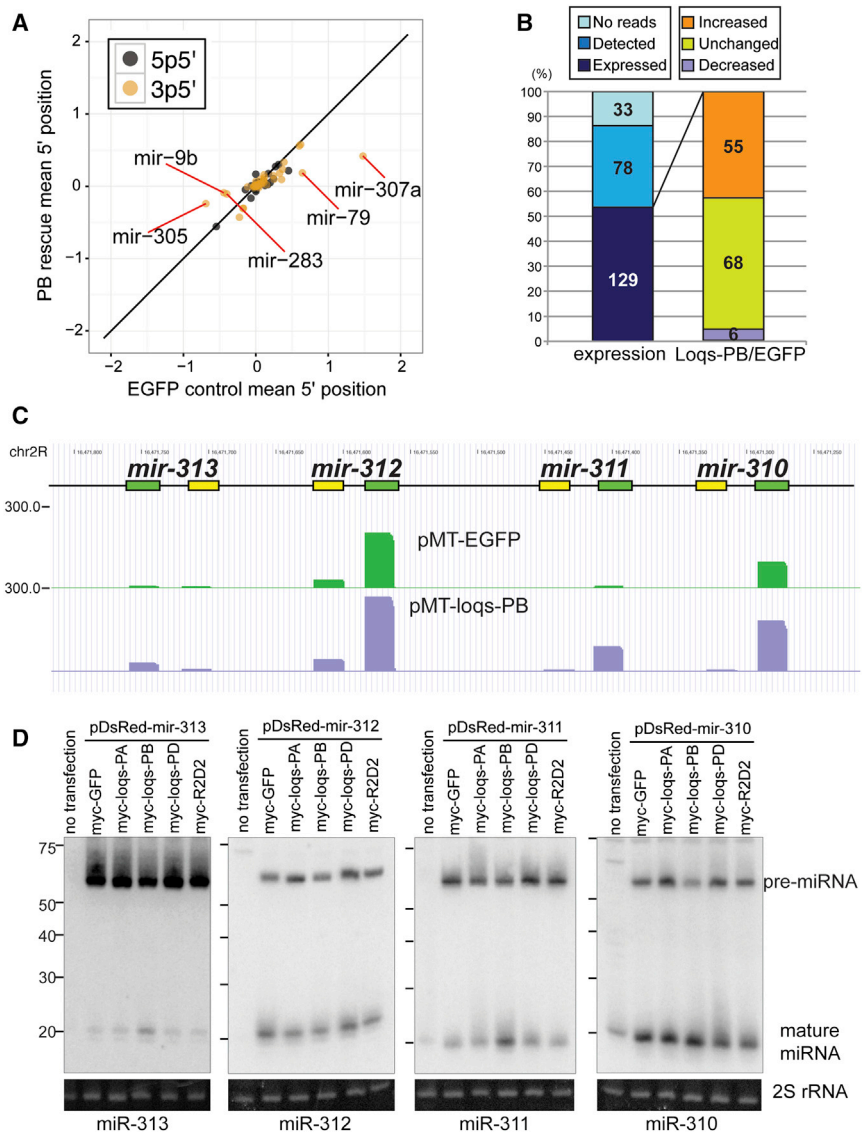


Figure 3. Subset of miRNA Genes Requires Loqs-PB

(A) 5′ position analysis. The weighted mean of the 5′ position was calculated for each miRNA arm, and the mean values from EGFP control cells and Loqs-rescue cells were plotted on the x and y axes, respectively. The perpendicular distance (D) of each plotted point to the line $y = x$ was computed. Gene names are shown for arms with D > 0.43. Note that no 5p arm satisfied our criteria.

(B) 240 miRNA genes were divided into "No reads," "Detected" (0 < average normalized reads < 2.5), and "Expressed" (>2.5 average normalized reads) based on the read count in the four libraries. The 129 expressed miRNA genes were further divided into "Increased" (>2), "Unchanged" (<2 or >0.5) and "Decreased" (<0.5) based on the ratio of read counts in Loqs rescue libraries and EGFP control libraries.

(C) UCSC Genome Browser screen shot of the *mir-310* cluster. Read counts normalized by spike-ins (reads per thousand spike-in) are shown and the maximum value is fixed at 300. Individual genes show distinct responses to Loqs-PB rescue. miR-311 is upregulated in Loqs-PB rescue cells, whereas miR-312 is not strongly changed.

(D) Northern blotting verification. Plasmids encoding individual miRNA genes from the *mir-310* cluster were co-transfected with the indicated rescue plasmids. Processed miRNAs were detected by northern blotting. The results are consistent with the library analysis results shown in (C).

See also Figure S1 and S2.

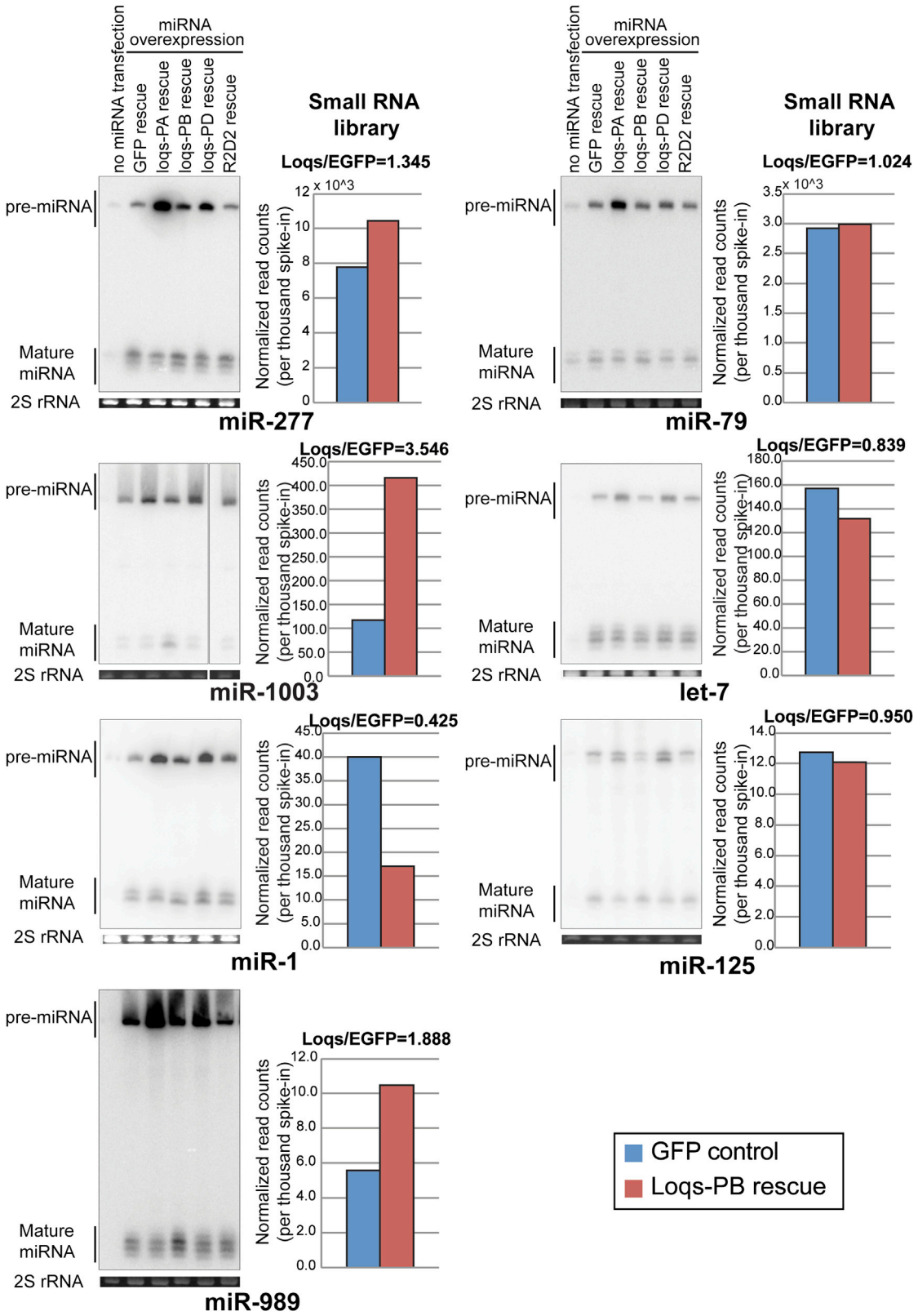
expression values of two libraries (with or without $CuSO_4$) in the following sections.

Efficient Processing of Many miRNAs in *loqs-KO* Cells

We first analyzed the mean 5′ position of each miRNA arm on a genome-wide scale (Figure 3A; Table S2). We found only five genes with shifts exceeding our threshold in cells rescued with Loqs-PB, and these genes largely overlap with those identified in the previous study using isoform-specific *loqs* mutant tissues (Fukunaga et al., 2012). As expected, no 5p species met this cutoff, consistent with the fact that the 5′ ends of 5p species are defined by Drosha-mediated cleavage (Figure 3A, black points). Therefore, these results confirmed the previous conclusion that Loqs-PB modulates dicing positions of a small number of miRNA genes.

Next, we examined expression levels of individual miRNAs. To our surprise, the bulk miRNA read abundance was only mildly (~50%) increased in cells rescued with Loqs-PB (Figure S2A). This indicated that the global miRNA-processing efficiency was not strongly enhanced by reintroduction of Loqs-PB. There were 129 miRNA genes that met our expression cutoff (>2.5 average normalized reads in the four libraries). We found that more than half of miRNA genes (68/129 miRNAs) were unchanged (0.5- to 2-fold), and about 40% (55/129 miRNAs) were upregulated at least by 2-fold in cells rescued with Loqs-PB (Figures 3B and S2B; Table S2). On the other hand, very few miRNA genes were downregulated (>2-fold; 6/129), consistent with the known roles for Loqs in enhancing Dcr-1 activity.

The behavior of clustered miRNA genes also supported the idea that differential responses of individual miRNAs to Loqs-PB expression were primarily due to differential processing efficiencies of individual miRNAs in control and rescued cells. Clustered miRNA genes are generally co-transcribed; therefore, distinct changes in the abundance of mature miRNA species derived from a cluster can be attributed to distinct processing efficiencies. As an example, individual members within the *mir-310* cluster showed distinct responses to Loqs-PB expression, with *mir-311* and *mir-312* showing the strongest and weakest enhancement of mature miRNA production, respectively (Figure 3C). Differential effects on the *mir-310* cluster genes could be verified by northern blotting analysis (Figure 3D).



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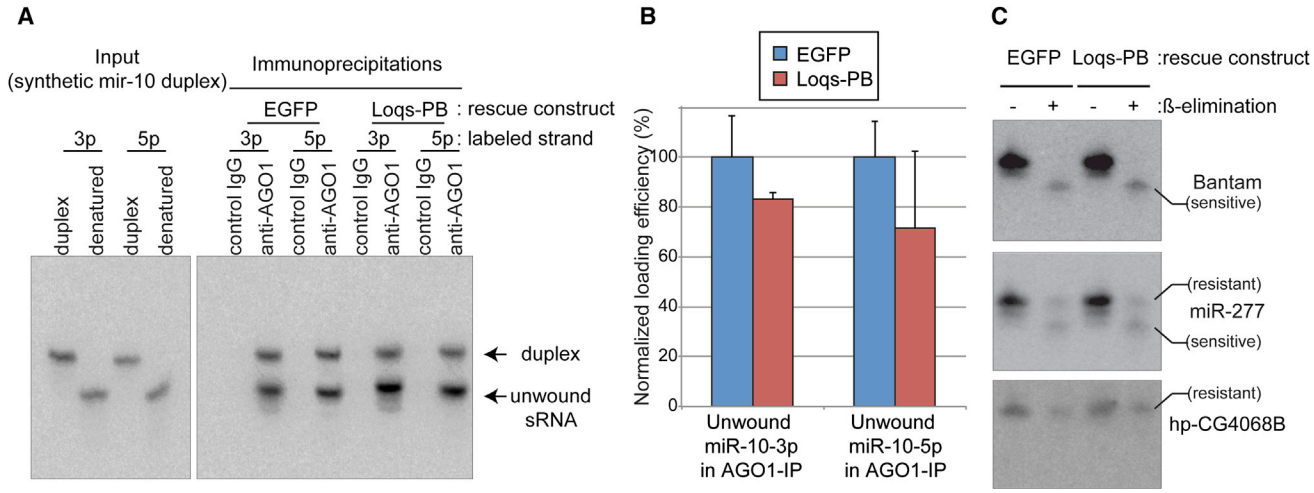

Figure 5. Loqs Is Dispensable for miRNA Duplex Loading and Strand Selection

(A) *mir-10* duplex loading in vitro. Synthetic RNA oligonucleotides corresponding to the *mir-10* duplex sequence were incubated in cell extracts prepared from *loqs* mutant cells expressing EGFP or Loqs-PB. Loaded RNA was precipitated by anti-AGO1 antibody and analyzed by native PAGE.

(B) Quantification of loaded RNA. In vitro loading was performed in triplicates and bands were quantified. Columns and error bars show averages and SDs of the loading efficiency normalized by the value in the EGFP control sample. No significant difference was observed between loading efficiencies in EGFP and Loqs-PB lysates ($p > 0.15$). See also Figure S3 for single-stranded RNA loading results.

(C) Small RNA sorting is not affected in *loqs* mutant cells. RNA samples from *loqs* mutant cells rescued with EGFP or Loqs-PB cultured in the presence of $CuSO_4$ were subjected to oxidation and β-elimination. RNA was separated by denaturing PAGE and probed with the indicated probes. The β-elimination sensitivities of strongly AGO1-sorted (Bantam), intermediate (miR-277), and strongly AGO2-sorted (hp-CG4068) species were similar in EGFP control and Loqs-PB rescue cells.

The northern blotting results for additional seven randomly chosen miRNA genes of the overexpressed miRNAs were consistent with the results of small RNA library analysis (Figure 4). These results suggested that the changes in mature miRNA levels in small RNA libraries primarily reflected enhanced processing efficiencies in rescued cells, not the secondary effects caused by Loqs-PB expression.

In summary, the overall miRNA abundance was only marginally (~50%) increased by reintroduction of Loqs-PB in *loqs* mutant cells. It is possible that the loss of Loqs activity was somehow compensated for by other mechanisms, such as increased levels of miRNA-processing factors. However, this possibility is unlikely because our qPCR analysis did not detect consistent upregulation of miRNA-processing factors in EGFP control cells (Figure S2C). The genome-wide data indicated that individual miRNAs exhibit variable responses to *loqs* deficiency, with ~40% of miRNA genes showing an increase after Loqs-PB re-expression.

Loqs Is Dispensable for miRNA Duplex Loading

In the *Drosophila* siRNA pathway, loading of siRNAs to the AGO2 complex and selection of loading siRNA strand require Dicer-2 and its partner dsRBD protein, R2D2 (Liu et al., 2003, 2006; Okamura et al., 2011; Tomari et al., 2004). The mechanism of miRNA duplex loading is less well understood. Although a previous study showed that Loqs was dispensable for miRNA duplex loading in flies, it has remained controversial whether Dicer partner proteins are involved in miRNA duplex loading in general (Betancur and Tomari, 2012; Liu et al., 2007; Noland et al., 2011).

We attempted to directly test whether miRNA duplex loading and strand selection were affected in *loqs* mutant cells using extracts prepared from *loqs* mutant cells stably transfected with the EGFP or Loqs-PB plasmid. We chose the *mir-10* duplex for this test, because both of these strands accumulate at similar levels in flies while the relative accumulation levels of the 5p and 3p species vary in other organisms (Griffiths-Jones et al., 2011; Okamura et al., 2008b). It is also conceivable that weakly asymmetric duplexes would be more sensitive to any slight changes in the strand selection mechanisms than highly asymmetric duplexes. Therefore, we expected that the *mir-10* duplex would provide a sensitive means to detect changes in strand selection. We labeled one of the duplex strands with ^{32}P at the 5′ end and incubated the labeled duplex in the cell lysate. The major miRNA effector complex was precipitated using an anti-AGO1 antibody and co-precipitated RNA was analyzed on a native gel that could separate double-stranded and unwound small RNA species (Figure 5A). To exclude the possibility of peripheral binding of small RNAs to AGO1, we focused our attention on the unwound

Figure 4. Northern Blotting Validation of Library Data

Northern blotting was performed with *loqs* mutant cells transfected with the indicated miRNA-overexpression plasmid along with the control or isoform-specific *loqs* rescue plasmid. The bar charts next to the northern blotting panels show normalized read counts in EGFP control (blue) or Loqs-PB rescued (red) *loqs* mutant cells. The relative levels of overexpressed mature miRNAs (Loqs-PB and EGFP) in northern blotting analysis and endogenous mature miRNA expression levels revealed by the small RNA library analysis are highly consistent, suggesting that increased mature miRNA levels in our small RNA libraries primarily reflect the miRNA-processing efficiency in the rescued cell line.

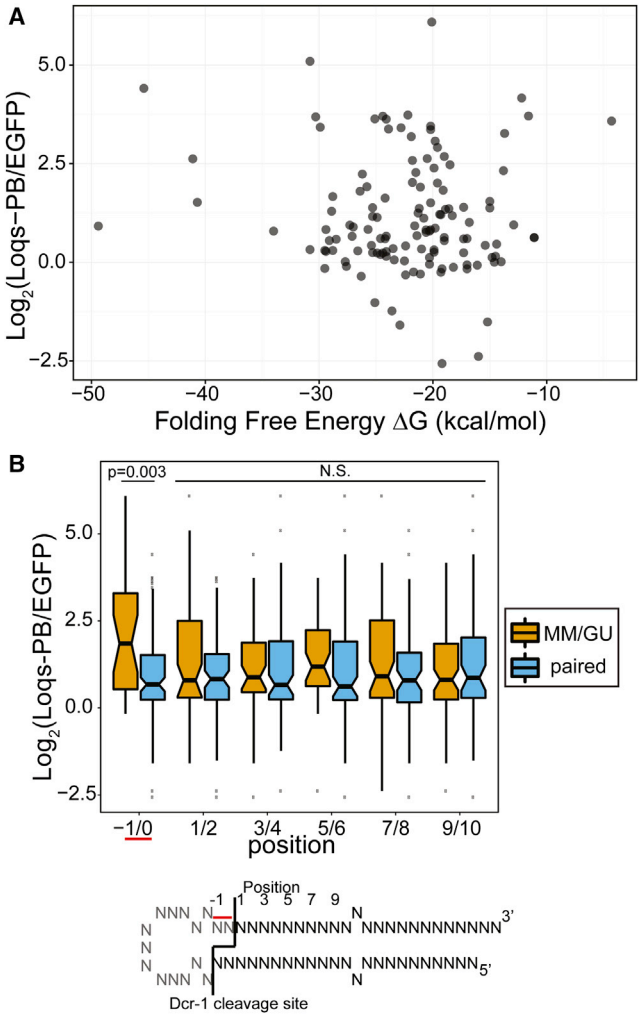

Figure 6. Features of Loqs-Sensitive miRNAs

(A) Overall folding free energy and Loqs dependence. Pre-miRNA ends were defined by 5′ ends of 5p and 3′ ends of 3p, and folding free energies (ΔG) were predicted by mfold. ΔG values were plotted against Log2 ratios of read counts in Loqs-PB rescue libraries and those in EGFP control libraries. The p value associated with the correlation is 0.408 and Bootstrap 95% confidence interval span the value zero, suggesting that there was no clear trend.

(B) Analysis of mismatch positions. The 5′ nucleotide of 3p species was set as position 1. Each nucleotide on the 3p arm was numbered according to the nucleotide distance from position 1 (see hairpin structure). miRNA hairpins were grouped according to base-pairing status in each 2-nt window, and the distribution of read count ratios in Loqs-PB/EGFP libraries for each group was plotted. Windows from −1/0 to 9/10 are shown in this figure. Only position "−1/0" (red underline) showed a significant difference ($p = 0.003$), and other positions showed no statistical significance (N.S.: $p > 0.05$; *k*-sample Anderson-Darling test) on mismatched/G:U group versus paired group for each position. See also Figure S4 for other windows.

species because duplex unwinding occurs only after proper loading (Kawamata and Tomari, 2010). To account for the amount of available empty AGO1 proteins in the lysate, we performed a control experiment using a single-stranded RNA (ssRNA) oligonucleotide corresponding to the *mir-34* loop sequence (Figure S3). Because ssRNA loading to Argonaute proteins generally occurs in passive mechanisms without additional loading factors (Chak and Okamura, 2014; Okamura et al., 2013; Rivas et al., 2005), we assumed that the amount of ssRNA loading would reflect the amount of AGO1 available for loading. After this normalization, the loading efficiencies of miR-10-5p and miR-10-3p showed no significant difference (Figure 5B). These results supported the previous conclusion that miRNA duplex loading occurs independently of Loqs in flies (Liu et al., 2007), whereas its mammalian homolog TRBP is known to sense duplex thermodynamic stability (Noland et al., 2011).

In *Drosophila*, small RNAs are sorted to AGO1 and AGO2 according to their duplex structures, and these two small RNA loading pathways compete with each other (Förstemann et al., 2007; Nishida et al., 2013; Okamura et al., 2011; Tomari et al., 2007). Although our in vitro experiments did not detect defects in miRNA loading to AGO1, we were interested to test whether small RNA sorting to the two Argonautes was affected in the *loqs* mutant cells. We took advantage of the difference in the chemical structure of the 3′ nucleotide between AGO1- and AGO2-loaded species. Small RNAs in the AGO1 and AGO2 complexes have 2′-OH and 2′-O-methyl groups at their 3′ ends, respectively (Han et al., 2011; Horwich et al., 2007). $NaIO_4$ selectively oxidizes vicinal 2′- and 3′-OH groups in RNA molecules; therefore, AGO1-loaded species but not AGO2-loaded small RNAs are sensitive to oxidation by $NaIO_4$. β-elimination after the oxidation reaction removes the oxidized 3′ nucleotide, resulting in higher mobility of AGO1-loaded small RNAs on denaturing gels (Han et al., 2011; Horwich et al., 2007). We carried out the oxidation and β-elimination experiment using RNA from *loqs* mutant cells stably transfected with the EGFP- or Loqs-PB-expression plasmid and probed the membrane for highly AGO1-enriched (Bantam), intermediate (miR-277), or highly AGO2-enriched (hp-CG4068B) small RNAs (Figure 5C). We observed no difference in the ratios of these small RNA populations in the two Argonaute complexes, supporting the notion that Loqs-PB is dispensable for proper small RNA sorting.

Structural Features of Loqs-Dependent miRNAs

Our genome-wide analyses revealed that a subset of miRNA genes require Loqs for their normal expression (Figures 2 and 3). We sought to determine common features of Loqs-dependent miRNA hairpins. We first considered overall stability of their hairpin structures by calculating folding free energy using mfold (Zuker, 2003). The predicted folding free energy (ΔG) was plotted against the ratio of read abundances in Loqs-PB rescue and control EGFP mock rescue cells (Loqs-PB/EGFP) (Figure 6A). However, no significant correlation between the overall structural stability and the Loqs dependence was observed, suggesting that overall hairpin stability is not a major determinant of Loqs dependence.

We decided to examine the hairpin structures more closely and asked whether the base-pairing status at any specific position showed a correlation with Loqs dependence. For this analysis, the 5′ nucleotide position of the most abundant 3p was defined as the standard position (position +1), and the nucleotides on the 3p arm were numbered from −1 to 20 (Figure 6B, hairpin structure). The base-pairing status at each position was analyzed based on the structures predicted by mfold

(Table S3). miRNA genes were then grouped based on the base-pairing status within 2-nt windows across the entire stem region. If the miRNA hairpin had at least one mismatch or a G:U wobble pair in the 2-nt window, the hairpin was considered to have "unpaired" nucleotides in the window. If both nucleotides in the window were paired, the gene was considered as a "paired" gene for the window. The distributions of Loqs-PB/EGFP read count ratios were plotted for all these groups (Figures 6B and S4A). Almost all windows showed no significant differences between the paired and unpaired groups, with the striking exception of window −1/0. The unpaired group at window −1/0 showed significantly higher Loqs-PB/EGFP read ratios than those of the paired group ($p = 0.003$, Anderson-Darling test). This position coincides with the site of Dicer-mediated cleavage, suggesting the hypothesis that Loqs-PB may be required for processing of miRNA hairpins with unstable base-pairing structures at the dicing sites.

We further analyzed base-pairing status at a single-nucleotide resolution for window −1/0 and found that genes with a mismatch or G:U wobble pair at position −1 and genes with those at position 0 showed similar distributions (Figure S4B). Having two mismatches/wobble pairs did not further shift the distribution (Figure S4B). These results suggested that one mismatch/G:U pair at either nucleotide in window −1/0 was sufficient to alter the behavior. In summary, our secondary structure analysis identified the base-pairing status at the dicing site as a potentially important feature that distinguishes Loqs-dependent and independent hairpins.

Base-Pairing Status at the Dicing Site Affects the Loqs Dependence

Our genome-wide analysis raised the possibility that the base-pairing status at the dicing site may have active roles in determining the Loqs dependence. To test this possibility, we mutated the *mir-283* hairpin that exhibited the strictest Loqs dependence (Figure 2A) (Fukunaga et al., 2012) and contained a G:U wobble pair at position 0 (Figure 7A).

We introduced a single-nucleotide mutation to *mir-283* to change the U at position 0 to a C, converting the Dicer cleavage site to fully canonical base pairing (Figure 7A). This mutant *mir-283* construct (U-to-C mut) was transfected along with the rescue constructs in *loqs* mutant cells. In contrast to the very weak mature miRNA signal produced from wild-type *mir-283* in EGFP control cells, the mature miR-283 product could be easily detected when expressed from the U-to-C mutant, reaching ~10% of the amount that could be processed in cells rescued with Loqs-PB (Figures 7B and 7C, U-to-C mutant, EGFP rescue).

To ensure that the weaker Loqs dependence was caused by the change in the structure, not in the sequence of the *mir-283* hairpin, we made an additional mutant that reverts the G:C pair in the U-to-C mutant to an A:C mismatch (U-to-C, G-to-A mutant; Figures S5A, S5B, and S5D). As expected, no clear production of mature miR-283 from this double mutant was observed in the absence of Loqs-PB. Furthermore, we confirmed that the position of the G:U pair is important, because a mutant changing the G:U pair at position 2 to a canonical pair did not enhance miRNA processing in the absence of Loqs (mutant A; Figures S5A, S5C, and S5D). To generalize our finding, we used another Loqs-dependent miRNA *mir-311*, whose dicing site contains an asymmetric bulge (Figure S6A). Restoring the double-stranded structure in this region weakened its Loqs dependence, again confirming our conclusion (Figures S6B and S6C).

Because the Loqs dependence could be alleviated by point mutations stabilizing base-pairing structures of dicing sites, we sought to test whether the opposite is also true. We chose *mir-277* based on its paired dicing site and very weak Loqs dependence (Figure 7D; Tables S2 and S3). Consistent with our library data, production of mature miR-277 was not strongly enhanced by co-expression of Loqs-PB (Figures 7E and 7F). We mutated the *mir-277* hairpin by changing the G:C pair at position 0 to introduce a G:U pair (Figure 7D). Supporting our hypothesis, the mutant hairpin showed higher sensitivity to the *loqs* mutation (Figures 7E and 7F).

Taken together, our small RNA sequencing and structure-function analyses demonstrated active roles of structural stability at the dicing site in determining the Loqs dependence of pre-miRNA processing.

Distinct Responses of Young and Old miRNAs to *loqs* Mutation

Evolutionarily "young" and "old" miRNA genes often exhibit distinct properties. For example, for young miRNAs, expression levels are generally lower and the impact of their ectopic expression on gene regulatory networks tends to be smaller (Bejarano et al., 2012; Berezikov et al., 2011; Mohammed et al., 2014; Ruby et al., 2007). These features of young miRNAs may reflect evolutionarily transitional states where miRNA loci are in the process of acquiring hairpin features that promote efficient processing and gaining effective target sites in the transcriptome (Bartel and Chen, 2004; Berezikov, 2011).

We asked whether "young" and "old" miRNA genes generally had distinct sensitivities to the *loqs* mutation. miRNA genes were grouped according to their evolutionary ages as defined in a previous study (Mohammed et al., 2013), and we plotted the distributions of Loqs dependence values of the two groups (Figure 7G; Table S2, sheet 2). The "young" miRNA gene group tended to show higher sensitivity to the *loqs* mutation ($p = 0.003$; Anderson-Darling test).

Our biochemical and genomics analyses indicated that Loqs-PB enhances dicing of suboptimal hairpins including ones with mismatches at the dicing site (Figure 7H). The enrichment of Loqs-sensitive miRNAs in young miRNA genes may reflect a role for Loqs in the emergence of new miRNA genes by facilitating dicing of substrates that have not acquired hairpin features essential for efficient miRNA processing.

DISCUSSION

Utility of the *loqs* Mutant Cell Line

In *Drosophila*, Loqs protein is maternally deposited and may mask the role of the gene in miRNA processing when studied in zygotic mutants (Fukunaga et al., 2012; Liu et al., 2007). The recovery of complete loss-of-function animals derived from germline clones is compounded by a stem cell loss phenotype (Liu et al., 2007; Park et al., 2007). To circumvent these

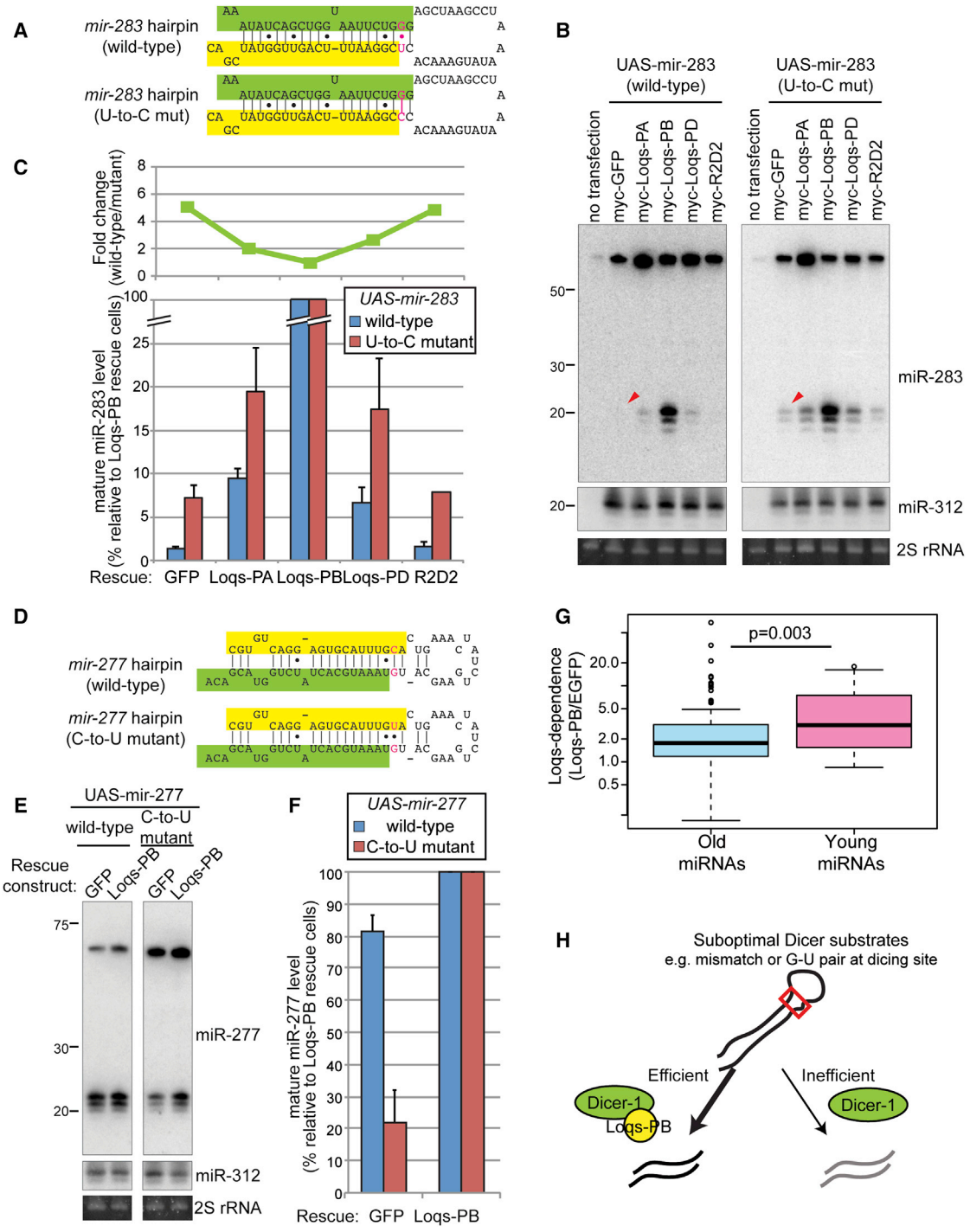

Figure 7. Roles for Dicing Site Structures and Significance of Loqs in miRNA Gene Evolution

(A–F) Base-pairing status at position 0 of *mir-283* (A–C) or *mir-277* (D–F) hairpin affects Loqs dependence. See also Figure S6.

(A) Structures of wild-type (upper) or "U-to-C" mutant (lower) *mir-283* hairpin. The G:U pair at position 0 (magenta) is changed to a G:C pair in the "U-to-C" mutant *mir-283* hairpin.

(B) Mature miRNA production from wild-type and U-to-C mutant *mir-283* hairpin with or without individual Loqs isoforms. The Loqs-independent *mir-312* was co-transfected as an internal control (Figure 3D). *UAS-mir-283* wild-type or U-to-C mutant was transfected along with the indicated rescue plasmid in *loqs* mutant cells. miR-283 and miR-312 products were detected by northern blotting. A representative result of three independent trials is shown. A clear signal from mature miR-283 is seen even in EGFP control cells when transfected with the mutant *mir-283* plasmid (red arrowheads).

(legend continued on next page)

problems, we established a cell line that is null for the *loqs* gene (Figure 1). The *loqs* mutant cell line has been continually passaged (>50 passages) demonstrating that cells are viable in the complete absence of Loqs. By analyzing the *loqs* mutant cells, we found that most miRNA genes do not require *loqs* for efficient processing in the cell line (Figure 3). Analysis of these cells, which are devoid of Loqs function, allows a more rigorous test of gene function than RNAi experiments or the analysis of zygotic mutant animals that may have residual functions. The cells also provided a convenient platform for structure-function analysis by simple transfection experiments (Figures 7, S5, and S6).

Given that loss-of-function mutant flies have been established for all of the core miRNA/siRNA-processing factors, generation of mutant cell lines from these mutants, as we have done for *loqs*, should be possible and will allow comprehensive analysis of molecular phenotypes that result from complete depletion of gene products in these pathways.

Gene-Specific Regulation of miRNAs via Control of General miRNA-Processing Factors

Using bioinformatics analysis, we were able to identify a feature of Loqs-dependent miRNA hairpins that could be experimentally validated (Figures 6 and 7). Together with a recent study identifying features of miRNA hairpins that are sensitive to Drosha levels (Sperber et al., 2014), our study encourages similar approaches in different contexts, such as disease conditions with altered general miRNA-processing activity.

Expression of miRNA processing factors is tightly regulated, often involving auto-regulatory loops to keep miRNA-processing activity in appropriate ranges (Han et al., 2009; Kadener et al., 2009; Ristori et al., 2015; Zisoulis et al., 2012). Failure to precisely control levels of miRNA-processing activity can be a cause of diseases (Herbert et al., 2013; Stark et al., 2008; Torrezan et al., 2014; Walz et al., 2015). Although the complete removal of core miRNA-processing factors would result in universal depletion of mature miRNAs, mild modulation of miRNA-processing activity may cause misregulation of a subset of miRNA genes (Ding et al., 2015; Paroo et al., 2009; Stark et al., 2008). Interestingly, in some cases, aberrant miRNA-processing activity only affects expression of miRNAs having related biological functions, such as cell proliferation (Paroo et al., 2009). Therefore, an attractive possibility is that miRNA structures may have evolved in such a way that miRNA expression profiles can be changed by altering the level of general miRNA-processing activity. It will be interesting to further study structural features of the miRNA hairpins that determine the sensitivity to the level of general miRNA-processing activity.

Biological Roles for Loqs Isoforms in Fly Development

Due to the similarity of *loqs* and *dcr-1* mutant phenotypes, *loqs* was believed to play essential roles in miRNA processing (Jin and Xie, 2007; Park et al., 2007). Furthermore, the lethal phase of *loqs-KO* mutant (late pupae) is only slightly later than those in other core miRNA-processing factor mutants such as *drosha* and *pasha* (late third-instar larvae/pupae) or *dcr-1* mutant (larvae/pupae) (Lee et al., 2004; Martin et al., 2009; Park et al., 2007; Pressman et al., 2012; Smibert et al., 2011). Therefore, it was assumed that the lethality of *loqs-KO* mutant was caused primarily by miRNA dysfunction.

However, according to our results, expression of only a small number of miRNAs was strongly enhanced by Loqs-PB expression in the *loqs* mutant cells (27 out of 129 expressed miRNA genes at the cutoff of 5-fold enhancement by Loqs-PB expression). Although our cell-based analysis does not exclude possible tissue-specific Loqs functions as seen in mammalian TRBP mutant (Ding et al., 2015), it will be interesting to test whether these highly Loqs-dependent miRNAs identified in this study can explain the described organismal phenotypes in *loqs* mutant animals. It is also interesting to note that Loqs isoforms have distinct functions; only Loqs-PA and Loqs-PB, but not Loqs-PD, could rescue the lethality of *loqs* mutant (Fukunaga et al., 2012). On the other hand, our northern blotting experiments demonstrated that only Loqs-PB could strongly enhance processing of Loqs-dependent miRNAs, whereas the effect of Loqs-PA expression on miRNA processing was very weak and often not greater than that of Loqs-PD expression (Figures 2, 3, and 4). The fact that the lethality could be partially rescued by Loqs-PA suggests that the reduction of miRNA production in *loqs* mutant flies may not be the major cause of lethality and Loqs may play additional roles besides miRNA biogenesis. In fact, recent studies have uncovered miRNA-independent functions of TRBP through direct interaction with protein kinase R or mRNAs (Goodarzi et al., 2014; Kim et al., 2014; Nakamura et al., 2015). To investigate this idea, it will be important to understand the molecular mechanisms underlying the organismal phenotypes of *loqs* mutant flies.

Roles for Loqs-PB in Evolution of miRNA Genes

We experimentally demonstrated that Loqs facilitates processing of suboptimal substrates, such as hairpins containing mismatches at the dicing site (Figures 6 and S6). Moreover, small RNA expression profiling indicated that evolutionarily young miRNAs tended to be more strongly dependent on Loqs-PB than old miRNAs (Figure 7G). These results collectively suggested that Loqs-PB broadens the range of structures that can be processed by Dcr-1, and this activity of Loqs-PB may

(C) Quantification of (B). Signals of mature miR-283 products were quantified and normalized by the miR-312 signals. Values were further normalized by setting the signal in Loqs-PB rescue cells as 100%. Columns and error bars indicate averages and SDs of three replicates (except for U-to-C mutant with R2D2 control; n = 2).

(D) Structures of wild-type (upper) or "C-to-U" mutant (lower) *mir-277* hairpin. The G:C pair at position 0 was changed to a G:U pair in the "C-to-U" mutant (magenta).

(E and F) Mature miRNA production from wild-type and C-to-U mutant *mir-277* hairpin with or without Loqs-PB. A representative northern blotting result (E) and quantified triplicate results (F). The figure format is same as (B) and (C).

(G) Distributions of Loqs dependence values for evolutionarily "old" (Pan-Drosophilid) and "young" (Sophophoran or melanogaster-subgroup specific) genes were plotted (p values: Anderson-Darling test).

(H) Working hypothesis. Loqs-PB helps Dcr-1 process a subset of miRNA hairpins, particularly those with unstable structures at the dicing site.

promote the emergence of novel miRNA genes. Dicer binds a variety of cellular RNA molecules other than pre-miRNAs, but a small fraction of them appear to be efficiently cleaved (Rybak-Wolf et al., 2014). Modulating cleavage specificity of Dicer may have a considerable impact on the repertoire of small RNAs produced from suboptimal substrates. Such structured RNA molecules are a plausible source of novel miRNA genes when they emerge de novo, and the range of RNA structures that can be cleaved by Dicer would be an important determinant of the birth rate of miRNA genes (Wen et al., 2015).

Newly emerged miRNAs would more frequently have deleterious effects than beneficial effects on fitness (Bartel and Chen, 2004; Chen and Rajewsky, 2007). Indeed, organisms are equipped with mechanisms suppressing excessive emergence of active miRNA genes produced via the mirtron pathway (Bortolamiol-Becet et al., 2015; Reimão-Pinto et al., 2015). On the other hand, the miRNA pathway has to be flexible enough to support the emergence of beneficial genes as well. There must be a balance between negative and positive regulators determining the birth rate of miRNA genes. We propose that Loqs-PB is part of this regulatory system keeping the frequency of miRNA gene emergence within an optimal range.

EXPERIMENTAL PROCEDURES

Generation of *loqs* Mutant Cells

The *loqs* mutant cell line was established from *loqs* mutant embryos using expression of Ras^{V12} as described previously by crossing heterozygous flies carrying the *loqsKO, Act5C-Gal4, P[attP.w$^+$.attP]* or *loqsKO, UAS*-Ras^{V12}, *P[attP.w$^+$.attP]* chromosomes (Park et al., 2007; Simcox et al., 2008a). The detailed procedure can be found in the Supplemental Information.

Molecular Biology

pDsRed-miRNA hairpin constructs were described previously (Bejarano et al., 2012). Plasmids encoding mutant hairpins were generated by site-directed mutagenesis. Transfection was performed using the protocol for S2 cells with minor modifications (Okamura et al., 2007). Total RNA was extracted by Trizol and analyzed by northern blotting as described previously (Okamura et al., 2007). For northern blotting analysis of mutated miRNAs, a Loqs-independent miRNA miR-312 was co-transfected and used as an internal control to account for variations in transfection efficiency and gel loading. β-elimination and qRT-PCR protocols were described previously (Okamura et al., 2007, 2008a), and in vitro loading assays were performed as previously described (Okamura et al., 2013). Oligos used in this study are shown in Table S4. Detailed information of all experiments can be found in the Supplemental Information.

Library Construction and Bioinformatics Analyses

Small RNA libraries were constructed using a modified version of a previously published method (Brennecke et al., 2007). Bioinformatics analysis was performed as previously described (Chak et al., 2015). Briefly, adaptors were removed using cutadapt (Martin, 2009), mapped to the dm3 *Drosophila* genome and the spike-in sequences using bowtie -q -S -v 0 -a-best -M 1 (Langmead et al., 2009). miRNA sequences were defined based on mirbase20 (Kozomara and Griffiths-Jones, 2014). The spike-in read count was used for normalization. Sequences with the most abundant reads mapped to each arm in the Loqs-PB rescue library (+$CuSO_4$) were used as the standard 5p and 3p sequences. Reads having 5′ ends falling in the ±4-nt range were counted. Structural analysis was performed as described in a previous study with some modifications (Okamura et al., 2009; Zuker, 2003). The "old" and "young" miRNA groups were defined previously (Mohammed et al., 2013). Detailed bioinformatics procedure can be found in the Supplemental Information.

ACCESSION NUMBERS

The small RNA library sequencing data generated in this study are available at NCBI SRA: SRP066647.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.059.

AUTHOR CONTRIBUTIONS

M.Y.T.L., A.W.T.N., A.S., G.T.-K., and K.O. designed the study. Y.C., A.S., and K.O. established the cell line. M.Y.T.L. performed all other experiments. A.W.T.N. and T.P.L. performed bioinformatics analysis. All authors contributed to the writing and editing of the manuscript.

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