The exoribonuclease Nibbler controls 3' end processing of microRNAs in *Drosophila*

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Summary

microRNAs are endogenous non-coding small RNAs with important roles in many biological pathways; their generation and activity are under precise regulation [1–3]. Emerging evidence suggests that miRNA pathways are precisely modulated with controls at the level of transcription [4–8], processing [9–11] and stability [12,13], with miRNA deregulation linked with diseases [14] and neurodegenerative disorders [15]. In the *Drosophila* miRNA biogenesis pathway, long primary miRNA transcripts undergo sequential cleavage [16–18] to release the embedded miRNAs. Mature miRNAs are then loaded into Argonaute 1 (Ago1) within the RNA-induced silencing complex (RISC) [19,20]. Intriguingly, we found that *Drosophila miR-34* displays multiple isoforms that differ at the 3'end, suggesting a novel biogenesis mechanism involving 3'end processing. To define the cellular factors responsible, we performed an RNAi screen and identified a putative 3'→5' exoribonuclease *CG9247/nibbler* essential for the generation of the smaller isoforms of *miR-34*. Nibbler (Nbr) interacts with Ago1 and processes *miR-34* within RISC. Deep sequencing analysis revealed a larger set of multi-isoform miRNAs that are controlled by *nibbler*. These findings suggest that Nbr-mediated 3' end processing represents a critical step in miRNA maturation that impacts miRNA diversity.

Results and Discussion

While miRNAs are typically annotated and observed as a single species, we found that *miR-34* showed a pattern of three major isoforms of 24, 22 and 21 nt in Northern blots from

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Supplemental Information Supplemental Information includes three figures, five tables, and Supplemental Experimental Procedures, and can be found with this article online.

adult *Drosophila* (Fig. 1A). Deep sequencing analysis [21] also showed that *miR-34* is present in multiple forms that all bear the same 5' terminus but differ at their 3' ends, presenting a nested series (Fig. 1B). To assess the relationship among these, we designed a pulse-chase experiment to follow *miR-34* biogenesis. Heat shock driven primary *miR-34* was tightly induced for 30 min, then monitored over time in adult flies. The longest isoform, isoform a (24nt), was predominant initially, while the accumulation of the shorter isoforms was delayed, but then increased over time (Fig. 1C). Moreover, as the 21 nt isoform accumulated, the 24 nt form was lost in a seemingly reciprocal manner, suggesting that the 24-mer may be converted into the 21-mer.

To define the mechanism, we treated cells with dsRNA targeting specific genes within the small RNA biogenesis pathways, and assessed the *miR-34* pattern by Northern blot. Imprecise cleavage of the precursor transcript could result in the production of the multiple forms. However, reduction of either Drosha or Dcr-1, or their binding partners Pasha and Loquacious, or Dicer-2 (Dcr-2), responsible for siRNA generation, did not alter the pattern (Fig. 2A). Therefore, we reasoned that the smaller isoforms may instead be generated by an exonuclease that sequentially processes the longest isoform into the nested series observed. To test this hypothesis, we performed an RNAi screen against the predicted 3'→5'exonucleases in *Drosophila*, including components of the RNA exosome (Table S1). This identified one gene, *CG9247* (which we named *nibbler/nbr*), with a striking effect: depletion of *nbr* led to a dramatic accumulation of the *miR-34* large isoform with a concomitant loss of the shorter isoforms (Fig. 2B; Fig. S1). In contrast, loss of *nbr* did not appear to alter the sizes or levels of miRNAs that normally show a single isoform by Northern, such as *miR-14* and *miR-277* (Fig. 2C). We also examined whether *nbr* knockdown had an effect on endogenous siRNAs, but saw no impact on *esi-2.1* (Fig. 2C). These data suggested that the novel putative exoribonuclease Nbr is required to generate the shorter isoforms of the multi-isoform miRNA *miR-34*, but is not required for general small RNA biogenesis.

The Nbr exoribonuclease domain shows closest sequence homology to human EXD3, falling within the *E. coli* RNAse D protein family; this includes the Werner exoribonuclease and *C. elegans* Mut-7 involved in transposon silencing (Fig. S1;[22]). Nbr, however, showed no predicted RNA binding domain, suggesting that it may function with a partner with RNA binding capacity, to bring Nbr activity to RNA substrates. To define these, we then performed a second RNAi screen genes known to bind RNA or associate with small RNA silencing pathways, including the two somatic RISC-associated Argonautes (Table S1). Strikingly, loss of Ago1 phenocopied *nbr* depletion: accumulation of the 24 nt isoform occurred, with reduction of the shorter isoforms (Fig. 3A). Controls indicated that knockdown of *Ago1* had no effect on *nbr* expression, and *nbr* knockdown had no effect on *Ago1* expression. These data suggested that Ago1 is also required for trimming, and that Nbr and Ago1 may act in a complex. Co-immunoprecipitation (IP) studies indicated that HA-tagged Nbr associates with Flag-tagged Ago1, but not with a control protein (Flag-Ran) (Fig. 3B). RNase treatment indicated the association was not RNA-dependent (Fig. S2). Proteomic studies have identified both Ago1 and Nbr as small RNA associated proteins [23], underscoring the specificity of the interaction. Since Nbr associates with Ago1, we hypothesized that *miR-34* 3'end processing may occur in the context of RISC. Indeed, IP of Ago1 revealed that all *miR-34* isoforms were bound (Fig. 3C). Furthermore, when Nbr was depleted, the longest *miR-34* isoform remained bound to Ago1 (Fig. 3C). Altogether, these data suggest that the 24 nt *miR-34* isoform is first generated by Dcr-1 then loaded into RISC. Next, Nbr, in association with Ago1, processes the long 24 nt isoform into shorter isoforms that remain loaded in RISC.

To assess the *in vivo* role of Nbr, we analyzed the expression and function of *nbr* in flies. Northerns revealed that *nbr* is expressed during development and in the adult, with peaks during the late larval/early pupal stage, and in adults (not shown). Analysis of *nbr* mRNA levels in animals with a transposon insertion in the coding region (*nbr^{f02257}*) showed that homozygous mutants (*nbr$^{-/-}$*) lacked *nbr* expression (Fig. 4A, B). *nbr$^{-/-}$* flies were semilethal, and sterile, indicating that *nbr* function is critical. Given the homology to Mut-7, we examined levels of transposons, but found no evidence linking *nbr* to transposon silencing (not shown). Assessment of *miR-34* expression in *nbr$^{-/-}$* flies phenocopied cells treated with dsRNA: the shorter isoforms were abolished, while the 24-nt form accumulated (Fig. 4C). As in cells, there was no striking effect on single-form miRNAs like *miR-277* (Fig. 4D). Furthermore, *miR-34** levels and isoform distribution appeared unaffected (Fig. S1). These data indicated that *nbr* modulates the isoform abundance of *miR-34*.

To assess the broader impact of Nbr function, we screened 65 miRNAs by Northern blot of RNA from cultured cells and flies. We identified 9 additional miRNAs with multiple isoforms: *mir-2*, miR-3, miR-12, miR-79, miR-263a/b, miR-274, miR-279, miR-281-1/2 and *miR-305*. The expression patterns of five of these were altered in *nbr$^{-/-}$* mutants, exhibiting accumulation of the longest isoforms with concomitant loss of the shorter isoforms (Fig. 4E; *miR-2* family not studied further due to cross hybridization between members). Analysis of small RNA profiling data from cells [21] confirmed that two of these (*miR-263a, miR-305*) had significant levels of multiple forms that differed at the 3'end (Table S2); *miR-3*, *miR-12*, *miR-281*, and *miR-274* were too low for analysis. Three multiple-isoform miRNAs (*miR-79*, *miR-274*, and *miR-279*) did not show an altered pattern in *nbr$^{-/-}$* flies (Fig. 4E). The deep sequencing dataset revealed that *miR-279* displays a series of isoforms that do differ at the 3'end; since *miR-279* processing is *nbr*-independent, *nbr* may be one member of a larger set of genes or mechanisms responsible for 3'end diversity. *miR-79* isoforms differed at the 5'end, suggesting that mechanisms also exist for 5' end diversity of miRNAs.

We further investigated the extent to which trimming is involved in miRNA processing by deep sequencing the small RNAs from flies, comparing *nbr* mutants to controls. There was no major impact on the size distribution of small RNAs as a whole or miRNAs in particular (Fig. S3A–B). To more carefully assess isoforms, reads were mapped to the miRNA stem-loop sequences and analyzed for length. For each miRNA, we calculated a ratio of the most frequent length in wild type to the sum of all other lengths, and compared this ratio between *nbr* and control. The distribution of the length ratios highlighted a cohort of miRNAs with extreme differences between *nbr$^{-/-}$* and control. At the two ends of the plot were miRNAs where the most common length isoform of the miRNA was present at a much higher or much lower level in *nbr$^{-/-}$* than in wild type, reflecting an altered pattern of isoform distribution or relative abundance for these miRNAs in the absence of *nbr*. These included miRNAs we had defined as trimmed and modulated by *nbr* (*miR-34, miR-263a, miR-263b*), with additional candidates (Fig. 4F, red boxes). Northern blots were performed on the top and bottom 8 miRNAs that we had not tested; we confirmed 7 new *nbr*-dependent miRNAs (*miR-7, miR-10, miR-11, miR-31b, miR-100, miR-190, miR-317*; Fig. S3, Table S3, S4). Northern revealed some miRNAs that were trimmed that were not detected as so by deep sequencing and the reverse: for any given miRNA, the extent of trimming had to be greater than ~10% in isoform level to detect a consistent change by Northern blot, while deep sequence analysis suggested that not all isoforms were cloned with equal efficiency.

Trimming exerts a profound and diverse impact on miRNA sequence profiles: *nbr* promotes the diversity of some miRNAs (*miR-34, miR-7, miR-317*), and alters the relative abundance of the most prominent isoform of others (*miR-190* and *miR-10*; Fig. S3C-F, Table S3). To identify potential Nbr-dependent miRNA targets, we performed transcriptional profiling of cells. This would allow identification of mRNA targets whose stability was altered by

miRNA trimming, but not targets primarily controlled by translational repression [24]. This identified 12 genes whose levels were affected by *nbr* depletion by >1.5-fold (Fig. 4G, Table S5); of these, one was reduced (*nbr*), the others were upregulated. Assessing the levels of 8 of these by realtime PCR confirmed increased expression of 6/8 mRNAs (75%) in *nbr*-depleted cells (Table S5). Next, we assessed expression of 9 of these genes in $nbr^{-/-}$ flies, compared to wild type and *loquacious* mutant flies. $loqs^{f00791}$ mutants are viable and show deficiency in miRNA maturation and function, thus allow assessment of miRNA function in adults [25]. We reasoned that genes regulated by miRNAs that are impacted by *nbr*-processing would also show dependence on *loqs*. We validated 5/9 genes (55%) as upregulated in both $nbr^{-/-}$ and $loqs^{f00791}$ (two additional genes were upregulated, although did not reach statistical significance in $nbr^{/-}$) (Fig. 4H, Table S5). Sequence analysis of these mRNA targets revealed that 4/7 genes (57%) have potential sites for the miRNAs that showed *nbr*-dependent processing (Table S5). It is unclear, however, if existing algorithms for miRNA targeting efficiently predict binding sites for miRNAs with 3'end diversity; targets for trimmed miRNAs may use non-canonical recognition motifs that are more dependent on 3'end pairing than seed complementarity.

These data provide evidence for a novel step in miRNA biogenesis: miRNA 3' end terminal trimming mediated by the 3'→5' exoribonuclease Nbr. Notably, small RNA deep sequencing has unveiled a rich pattern of miRNA sequence isoforms, although miRNAs have routinely been annotated as a single mature form. Our findings suggest that miRNA processing by Nbr alters the repertoire of at least a subset of miRNAs in cells and whole animals, contributing to the diversity of the small RNA profile and potentially impacting post-transcriptional gene regulation in *Drosophila*. Mechanistically, our data indicate that, upon *nbr* knockdown, *miR-34* is still associated with RISC, thus trimming is not a pre-requisite to *miR-34* loading, and likely occurs after loading.

The impact and biological consequences of trimming may be complex. Nbr may impact strand selection within RISC because strand selection is influenced by the extent of 3' overhang and degree of pairing for any miRNA-miRNA* duplex [26,27]. Nbr may impact miRNA stability, as previous studies have demonstrated that tailing and trimming of mature *Drosophila* miRNAs influences their turnover [28]. Trimming may also impact mRNA silencing by favoring alterative miRNA sites within mRNA targets. Although canonical miRNA-target specificity is thought to be driven largely by complementarity within the seed, non-canonical interactions can depend more heavily on 3' compensatory sites [29,30]. Therefore, differences in the length of the 3' end of miRNAs may influence both target selection, as well as silencing efficiency of targets that require extensive 3'end pairing. Future analysis of trimmed miRNAs and their range of targets will reveal rules governing miRNA-mRNA pairing specificity that may be impacted by 3'end heterogeneity. Given that some mammalian miRNAs also display multiple isoforms [31,32], miRNA 3'end processing may be conserved. Our studies focused on the role of *nbr* in miRNA pathway function; whether *nbr* plays a role in additional small RNA pathways remains an open question, although we did not observe effects on transposons suggesting it does not globally impact esiRNA or piRNA pathways. The modification of mature miRNAs and their precursors is an emerging facet of miRNA-mediated gene regulation [33]. Nbr may represent a central player in a larger spectrum of factors that shape miRNA repertoire and function, through the generation of multi-isoform miRNAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. A new fly gene, *nibbler/CG9247 (nbr)*, is identified in *miR-34* 3'end processing.

2. Nbr forms a protein complex with Ago1 and both are required for trimming.

3. Nbr-mediated 3'end processing modulates a large number of miRNAs.

4. Nbr modulation of the miRNA profile impacts gene expression.
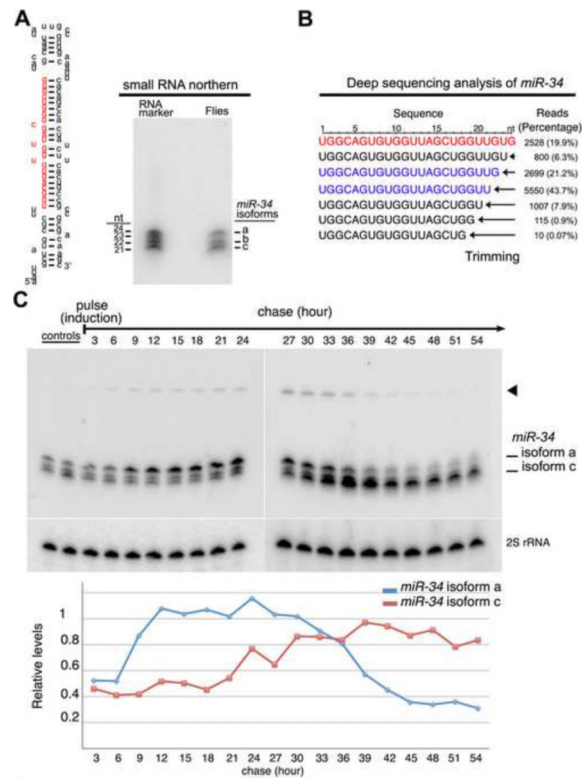
Figure 1. Fly *miR-34* Shows Multiple Isoforms Whose Generation Appears Dependent on 3' End Trimming

(A) *miR-34* has multiple forms in adult flies. Left, *miR-34* precursor, mature 24-nt sequence in red. Right, Northern for *miR-34*. Isoforms of 24, 22 and 21nt, labeled a, b, c, respectively.

(B) *miR-34* isoforms from a deep sequencing fly S2 cell dataset [21]. In red, 24nt isoform a. In blue, isoforms b and c. These are 99.1% of the *miR-34* reads.

(C) Northern blot analysis of *miR-34* isoform accumulation *in vivo*. Transient induction of *pri-miR-34* by *hs-GAL4* in adult flies leads to initial accumulation of isoform a, which is lost over time while the shorter isoforms accumulate. Arrowhead, *pre-miR-34*.

(D) Quantification of *miR-34* isoforms from pulse-chase in C. Values normalized to 2S rRNA.
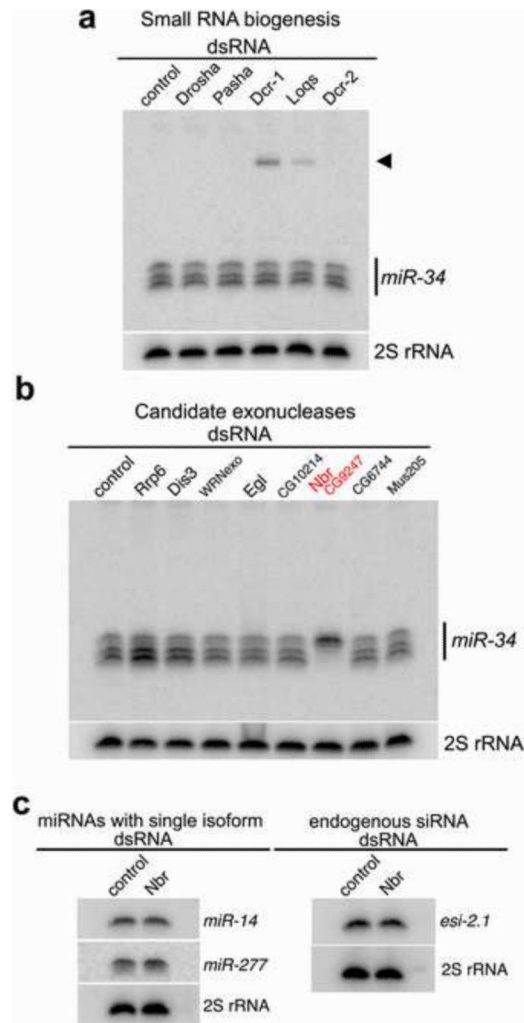
Figure 2. *nbr* is Required to Generate the Isoforms of *miR-34*
(A) Depletion of known factors in the small RNA biogenesis pathways has no effect on *miR-34*.
(B) Depletion of candidate exoribonucleases shows that loss of CG9247/Nbr (red) leads to an accumulation of the 24nt isoform, with dramatic reduction of the shorter isoforms.
(C) Cells depleted of Nbr are not altered in single isoform miRNAs or endogenous siRNA *esi-2.1*.
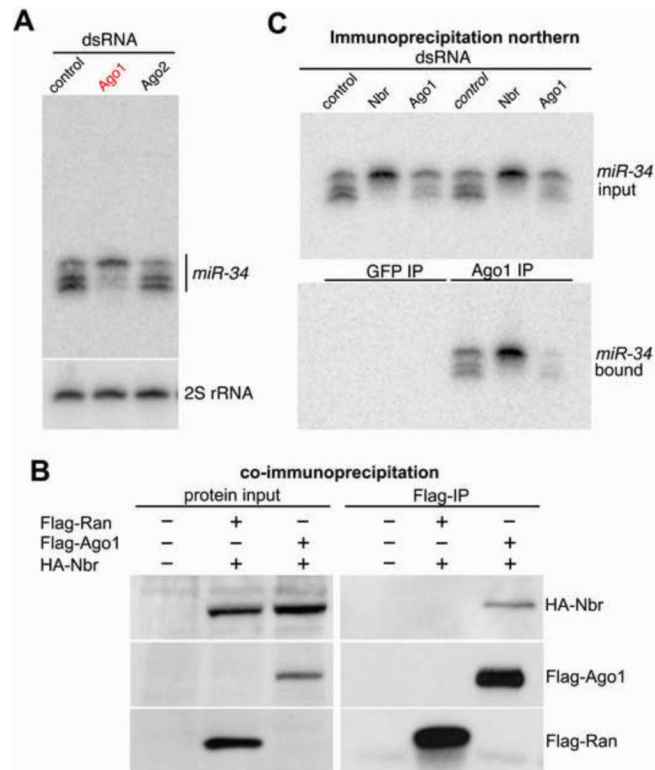
Figure 3. Nbr Interacts with Ago1-RISC

(A) Small RNA Northern blot analysis of *mir-34* isoforms. Depletion of Ago1 phenocopies Nbr knockdown.

(B) Ago1 and Nbr interact by co-IP. Cells were untreated or transfected with HA-Nbr and Flag-Ago1 or Flag-Ran (control). Following IP, interacting proteins were probed by immunoblot. Input, 10% of Flag-IP.

(C) All *miR-34* isoforms co-IP with Ago1. Cells were treated with dsRNA to control (LacZ), Nbr or Ago1, and IPs were performed anti-GFP (control) or Ago1 antibodies. Input and IP-ed RNA were analyzed by Northern blotting for *miR-34*.
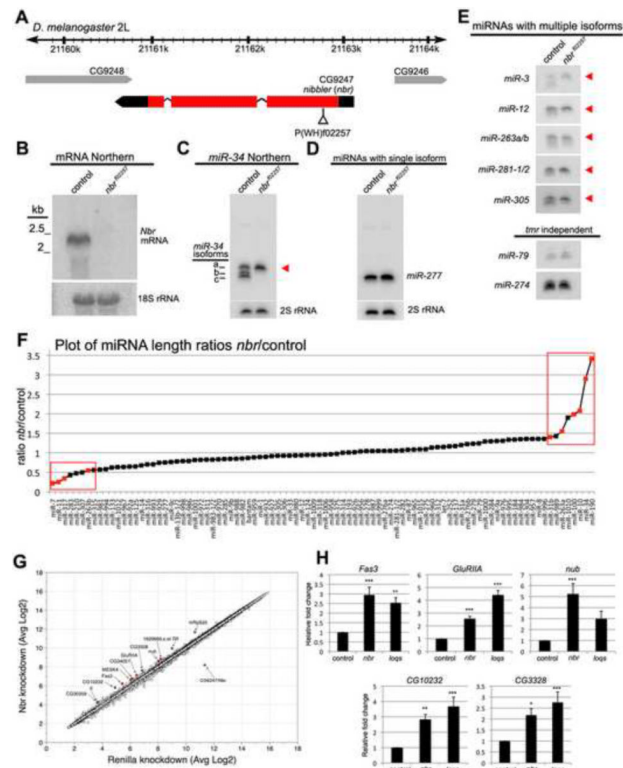
Figure 4. *nbr* is Required *in vivo* to Process Select miRNAs and Silence Target mRNAs
(A) Genomic map of the *nbr* locus. Coding region in red, with transposon insertion highlighted.
(B) Northern blot for *nbr*. The *nbr^f02257^* mutant shows complete mRNA loss.
(C) Shorter isoforms of *miR-34* are abolished in the *nbr* mutant. Arrow, isoform a.
(D) Northern blot of single-isoform *miR-277*, which is not altered in *nbr^−/−^*.
(E) Comparison of multiple-isoform miRNAs from control and *nbr^f02257^* flies. Some miRNAs require *nbr* (red arrowheads), while others are *nbr*-independent.
(F) The ratio of the most frequent form of the miRNA in wild type, compared to the sum of all other forms, was generated for *nbr* and control. The ratios were compared (*nbr* ratio/control ratio), and plotted. The ratio was excessively large or low when isoform biogenesis is defective. Red boxes, miRNAs with extreme ratios that were further analyzed. Red symbols are confirmed Nbr-targets (Fig. S4).
(G) Scatterplot of microarray data from cells treated with dsRNA against Nbr or Renilla control. Highlighted, all of the genes >1.5 fold changed in either direction.
(H) Realtime PCR for mRNAs from *nbr* and *loqs* mutant flies. (4–6 experiments; *$p<0.05$, **$p<0.01$, ***$p<0.001$).