ADAR mediates differential expression of polycistronic microRNAs

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ABSTRACT

Adenosine deaminases acting on RNAs (ADARs) convert adenosine residues to inosines in primary microRNA (pri-miRNA) transcripts to alter the structural conformation of these precursors and the subsequent functions of the encoded microRNAs (miRNAs). Here we show that RNA editing by *Drosophila* ADAR modulates the expression of three co-transcribed miRNAs encoded by the evolutionarily conserved *let-7-Complex* (*let-7-C*) locus. For example, a single A-to-I change at the −6 residue of pri-miR-100, the first miRNA in this *let-7-C* polycistronic transcript, leads to enhanced miRNA processing by Drosha and consequently enhanced functional miR-100 both *in vitro* as well as *in vivo*. In contrast, other editing events, including one at the +43 residue of the pri-miR-125, destabilize the primary transcript and reduce the levels of all three encoded miRNAs. Consequently, loss of *adar in vivo* leads to reduced miR-100 but increased miR-125. In wild-type animals, the destabilizing editing events in *pri-let-7-C* increase during the larval-to-adult transition and are critical for the normal downregulation of all three miRNAs seen late in metamorphosis. These findings unravel a new regulatory role for ADAR and raise the possibility that ADAR mediates the differential expression characteristic of many polycistronic miRNA clusters.

INTRODUCTION

MicroRNAs (miRNAs) are a class of noncoding RNAs that silence target mRNAs by preventing their translation or by causing their decay (1–6). Biogenesis of miRNAs is initiated by transcription of either monocistronic or polycistronic loci. Resulting primary microRNA (pri-miRNA) transcripts undergo processing via two steps to generate functional 'mature' miRNAs that guide the RNA-induced silencing complex to specific target mRNAs. In the first step, pri-miRNAs are cleaved by a nuclear RNAse III enzyme, Drosha, to generate stem loop precursor miRNAs (pre-miRNAs). Pre-miRNAs undergo a second cleavage by the cytoplasmic RNAse III enzyme Dicer to generate functional miRNAs. A number of RNA binding proteins associate with pri- and/or pre-miRNA intermediates to modulate this processing of miRNAs in a context-dependent manner (7–14).

RNA editing by adenosine deaminases acting on RNA (ADAR) is a widespread post-transcriptional mechanism that regulates the processing and functioning of miRNAs (15,16). Adenosine-to-inosine (A-I) editing of pri-miRNA transcripts usually inhibits miRNA processing, redirects edited miRNAs to new mRNA targets and suppresses RNA-induced silencing complex loading (17–21). Recently, mouse ADAR1 has been also shown to globally promote miRNA biogenesis, but this is via its RNA-binding independent interaction with Dicer rather than its editing function (22). Differential expression is a hallmark of clustered miRNAs and although RNA binding proteins have been shown to bind and facilitate processing of specific miRNAs, the effect of ADAR-mediated editing on the processing of adjacent miRNAs in polycistronic miRNA transcripts has not been explored (7).

We investigated whether RNA editing mediates the differential expression of miR-100, let-7 and miR-125 during *Drosophila* development. Because these miRNAs are co-transcribed from a single locus termed the *let-7-Complex* (*let-7-C*) (Figure 1A) (23), differential processing and/or turnover likely explain their sharply different expression levels *in vivo* (24,25). Previous deep sequencing of small RNAs derived from *Drosophila* tissues and cell lines identified a limited set of edited processed miRNAs that included *let-7-C* miRNAs (24), but key editing events in primary transcripts that occur outside of the mature miRNA sequence were likely missed. Here, we have identified additional editing events in the *pri-let-7-C* transcript, and found that one of these can affect the efficiency of Drosha cleavage and thereby enhance miR-100 processing. In addition, other editing events in the miR-125 precursor reduce the stability of the pri-miRNA transcript,

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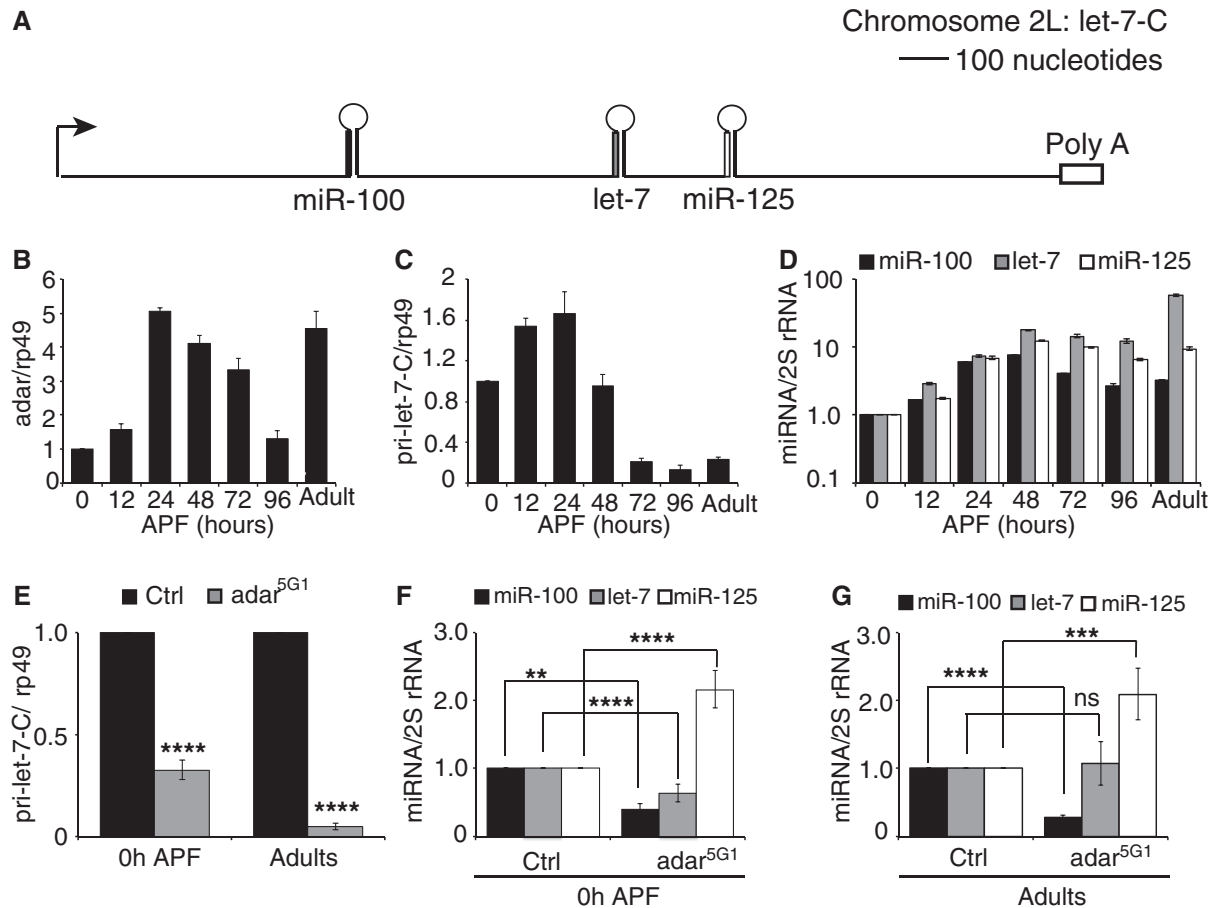
Figure 1. ADAR regulates expression of *let-7-Complex* miRNAs. (**A**) Schematic representation of *let-7-C* cDNA that encodes three evolutionary conserved miRNAs, miR-100, let-7 and miR-125 (black, dark gray and white lines, respectively). (**B–D**) Developmental profile of *adar* (B), *pri-let-7-C* (C) and processed *pri-let-7-C* miRNAs (D) as determined by qRT-PCR of total RNA extracted from pupae staged relative to puparium formation (APF) and adults. (**E**) Expression of *pri-let-7-C* in *wild type* (Ctrl) and *adar^5G1* null mutant prepupae (0 h APF) and adults, as determined by qRT-PCR. (**F, G**) Expression levels of processed miR-100 (black column), let-7 (gray column) and miR-125 (white column) in *wild type* (Ctrl) and *adar^5G1* null mutant prepupae (F) and adults (G), as determined by qRT-PCR. Assays were performed in triplicate for each experiment. Control *rp49* transcript (B, C, E) or *2S* rRNA (D, F, G) was used for normalization.

leading to a decrease in expression of all three encoded miRNAs. Furthermore, we provide evidence that these editing events that control *pri-let-7-C* cleavage and stability are critical for the dynamic expression of this conserved miRNA cluster during normal development.

MATERIALS AND METHODS

Fly strains and husbandry, clone generation and immunostaining

Strains used include *adar^5G1* (gift from T. Jongens), *let-7-C^GKI* (23), *P(GawB)elav[C155]* [Bloomington *Drosophila* stock center (BDSC) stock 5147], *UAS-adar-RNAi*, *UAS-let-7-C^wt*, *UAS-let-7-C pri-miR-100^ed-6*, *UAS-let-7-C pri-miR-100no edit* and *UAS-let-7-C pri-miR-125^ed+43*. *Canton S* was used as control strains, and *w^1118* was used as the *wild type* strain for experiments in Figure 5. The *UAS-let-7-C^wt*, *UAS-let-7-C pri-miR-100^ed-6*, *UAS-let-7-C pri-miR-100no edit*, *UAS-let-7-C pri-miR-125^ed+43* transgenes were inserted into the *PBac {y [+]-attP-3B}VK00033*

(BDSC 9750) landing site. The miR-100 sensor transgene was inserted into the *P{CaryIP}su(Hw)attP8* (BDSC 32233) landing site. Transgenesis was performed by Rainbow Transgenic Services (CA, USA) and BestGene INC (CA, USA). Standard genetic crosses were used to obtain flies of indicated genotypes. All flies were cultured on standard cornmeal medium at 25°C under 12 h light, 12 h dark cycles. Staging of pupae was performed as previously described (25).

Clones were generated by crossing males harboring *hs-Flp^122* and one of the *UAS* transgenes to virgin females bearing *Act5c>CD2>Gal4, UAS-RFP* (BDSC 30558) on the third chromosome and the miR-100 sensor on the X chromosome. After one day, adults were removed and the progeny were aged for another day, heat shocked for 5 min at 37°C and further maintained at 25°C for 3–5 days before dissection. Immunostaining was performed as described previously (25). Antibodies used for staining included chicken anti-GFP (Rockland Immunochemicals, 1: 4000) and rabbit anti-dsRed (Clontech, 1:30 000). Images were collected on a Leica

SP5 confocal microscope (Light Microscopy Imaging Center, Indiana University).

Plasmids and transgenes

Tagged protein plasmids. Drosophila melanogaster ADAR cDNA was polymerase chain reaction (PCR) amplified from the SD06892 plasmid obtained from *Drosophila* Genomic Resource center (DGRC, Bloomington, IN, USA). The complete cDNA was generated by SOE-PCR (26). The first PCR product was amplified with oligonucleotides (oligos) 2208 and 2209 (Supplementary Table S1 for all oligo sequences). The second PCR product was amplified with oligos 2210 and 2211. The two PCR products were spliced together using the 2208 and 2211 oligos. *Drosophila melanogaster* Drosha and Pasha cDNAs were amplified from LD20030-GOLD and LD23072-GOLD (DGRC) by PCR using oligo pairs 2113, 2114 and 2115, 2116, respectively. The PCR products were cloned into the pENTR/D-TOPO plasmid (Life Technologies). Plasmids encoding N-terminal Flag tagged version of ADAR and Pasha and the C-terminal tagged version of Drosha were generated by recombining pENTR-ADAR and pENTR-Pasha with pAFW gateway plasmid (T. Murphy; obtained from DGRC) and the pENTR-Drosha with pAWF gateway plasmid (T. Murphy; obtained from DGRC) using the LR Clonase enzyme (Life Technologies), respectively.

UAS transgenes. Pri-miR-100, pri-let-7 and pri-miR-125 hairpin constructs were generated by designing forward and reverse oligos encoding the precursor miRNA sequences as well as ∼50 nucleotides of conserved flanking sequences (Supplementary Table S1 for oligo sequences). Oligo pairs 970 and 971, 948 and 949, 968 and 969 and 1055 and 1056 were annealed and cloned into the *XbaI* site of pUASTattB (a gift from Konrad Basler). The edited versions of miR-100, let-7 and miR-125 primary transcripts were generated using the QuikChange Lightning Multi mutagenesis kit (Agilent Technologies). The pri-let-7-C cDNA clone was generated by reverse transcription with total RNA extracted from BG3c2 cells mixed with RNA from 24 h 20E treated Kc-167 cells. The reverse transcription was done with random hexamers and SOE-PCR with two sets of oligos. The first PCR product was amplified with oligos 144 and 317. The second PCR product was amplified with oligos 316 and 145. The two PCR products were spliced together using oligos 316 and 317. The PCR product was cloned into TOPO vector and then the *BamHI-XbaI* fragment was cloned into *BglII-XbaI* sites of pUASTattB. All PCRs were done with Pfu polymerase.

miR-100 Sensor Transgene. The miRNA sensor transgene is a pattB-based plasmid that contains the codon optimized GFP from pJFRC7 (a gift from B. Pfeiffer) under the control of the *Drosophila ubiquitin-63e* promoter and the SV40 3′ untranslated region (3′UTR). A 2-kb fragment directly upstream of the *ubiquitin-63e* transcription start site was PCR amplified with oligos 862 and 863 from genomic DNA, subcloned and verified by sequencing (Supplementary Table S1 for oligo sequences). Oligo pairs 2053, 2054 containing six tandem repeats of miR-100 antisense sequence were subsequently inserted into *NotI-XbaI* sites located between the GFP open reading frame and the SV40 3′UTR. The GFP-SV40 junction was sequenced before transgenesis.

Cloning of edited *pri-let-7-C* transcripts

First-strand cDNA was synthesized using 1–4 μg of total RNA and oligo 2213 (Supplementary Table S1 for oligo sequences). The resultant cDNA was then amplified by PCR using PCR oligos 2214 and 2215. Reverse transcription-PCR (RT-PCR) products were subcloned using the TOPO XL cloning kit (Invitrogen, Carlsbad, CA, USA), and ≥35 cDNA isolates were sequenced (Indiana Molecular Biology Institute).

In vitro editing, *in vitro* processing assays and northern blot analysis

In vitro editing assays were performed as described in (19) with the exception that purified Flag-tagged *Drosophila* ADAR was used in the assays. *In vitro* processing assays was performed as described in (27) with the exception that the reaction contained 15 μl of Flag-Drosha-Pasha beads immunoprecipitate, 6.4 mM $MgCl_2$, 1U/μl of RNAse Inhibitor(Invitrogen) and the refolded labeled transcripts(0.5×10^5 cpm). Northern blot analysis was performed as described in (25).

Quantitative real time PCR

Total RNA was extracted with Trizol and treated with DNAse I. The purified RNA was used in reverse transcription using Superscript III (Life Technologies). The first-strand cDNA was used as a template for quantitative real time PCR (qRT-PCR) in a volume of 10–15 μl containing oligos and SYBR green master mix or Taqman Universal PCR master mix. For mature miRNAs, expression levels were measured by qRT-PCR analysis with TaqMan miRNA assays containing specific oligos for mature miR-100, let-7 and miR-125 (Life technologies) using a StepOnePlus Real time PCR machine (Life Technologies). A standard curve was run in each PCR. Individual values were normalized to 2S rRNA or Snu442 levels for Taqman miRNA assays and Rp49 levels for Sybr green assays. For qRT-PCR analysis, oligos 2515, 2215, 2599, 2530, 2531, 2500 and 2501 listed in Supplementary Table S1 were used. All reactions were done three times in triplicate, and relative expression of RNAs was calculated using the Pfaffl method (28).

Luciferase sensor assays

For luciferase assays, psiCHECK plasmids (50 ng/well) bearing six perfect sites for either miR-100, let-7 or miR-125 downstream of a *Renilla* luciferase gene (29) were cotransfected with a Tubulin-GAL4 plasmid (50 ng/well) as well as plasmids encoding either unmodified or edited versions of pri-let-7-C (50 ng/well) in 48-well plates. After 68–72 h, luciferase activity was measured with the Dual-Glo luciferase Assay system (Promega). Fold repression was calculated by dividing the ratio of *Renilla* luciferase and firefly luciferase in cells transfected with an empty

pUAST attB plasmid with the ratio of *Renilla* luciferase and firefly luciferase in cells transfected with pUAST attB plasmid containing *let-7-C* cDNAs.

Statistical analyses

Quantified data are expressed as the mean ± S.E. values. Two-tailed t tests were used for significance testing in Figures 1, 3 and 4, and an unpaired t test with Welch's correction was used to calculate significance of the frequency of editing events in adults versus prepupae in Figure 5E. For all figures, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$.

RESULTS

ADAR regulates processing of *pri-let-7-C*

A-to-I editing in *Drosophila* is catalyzed by a single neurally enriched enzyme, ADAR, which plays an important role in the maintenance of neuronal homeostasis (30–32). Consistent with this physiological role, *adar* mutant adults display numerous behavioral defects as well as age-dependent neurodegeneration (33,34). The expression levels of the *adar* transcript increased during metamorphosis (Figure 1B), suggesting a developmental basis for these defects, and coincided with the onset of the *let-7-C* transcript (Figure 1C), a locus required for the formation of the adult nervous system (23,25,29). The three miRNAs encoded by *pri-let-7-C* displayed a dynamic expression pattern during metamorphosis, with a peak at 48 h after puparium formation (APF) followed by a decline at 72 h that persisted until adulthood (Figure 1D). Deep sequencing analysis of small RNAs from tissues and cell lines (24) as well as northern blot and Taqman qRT-PCR assays of staged animals (25) (Figure 1D, log scale graph) indicated that the relative levels of the three miRNAs encoded by this locus vary drastically; let-7 was most highly expressed, followed by miR-125 and then miR-100. The overlapping expression of *adar* and *pri-let-7-C* suggested that editing may contribute to this differential expression of *let-7-C* miRNAs.

To investigate this possibility, we compared the levels of processed miR-100, let-7 and miR-125 in total RNA isolated from control and genetic null *adar^{5G1}*. The elimination of *adar* transcript had opposing effects on miR-100 and miR-125, since miR-100 levels were reduced 2.5-fold, while miR-125 levels were increased 2-fold in *adar* mutant samples compared with age-matched controls (Figure 1F and G). These data were recapitulated in central nervous tissue derived from *adar* mutants as well as *adar* knockdown lines (Supplementary Figure S1). The increased miR-125 levels were particularly striking, as *pri-let-7-C* levels were globally reduced in *adar* mutant samples (Figure 1E). These data indicated that ADAR regulated *let-7-C* miRNAs, and also suggested that ADAR functioned simultaneously to enhance miR-100 expression and repress miR-125 expression.

All three *let-7-C* miRNAs are edited by ADAR

To identify edited adenosine residues in the *pri-let-7-C* transcript that were responsible for these effects, we searched for nucleotide changes in *pri-let-7-C* that occurred under three conditions where ADAR activity was likely enhanced. These conditions included (i) incubation of purified flag-tagged *Drosophila* ADAR (Supplementary Figure S2A and B) with *in vitro* transcribed *pri-let-7-C* transcript, (ii) overexpression of *Drosophila* ADAR in cultured neuronal BG3c2 cells that ordinarily transcribe *pri-let-7-C* and (iii) co-expression of *Drosophila* ADAR and *pri-let-7-C* in cultured Kc-167 cells that do not ordinarily transcribe *pri-let-7-C*. RT-PCR was performed on RNA extracted from these *in vitro* reactions and transfected cells to amplify cDNAs that spanned the miR-100, let-7 and miR-125 precursors. These RT-PCR products were subsequently cloned and sequenced and editing events were detected as A-to-G changes in the cDNA sequence.

This analysis revealed only a handful of editing events in the three pri-miRNAs that occurred in >30% of cDNAs recovered from the *in vitro* reactions and in >10% of cDNAs recovered from at least one cell line (Figures 2A–F). Pri-miR-100 was most frequently edited at the −6 and +42 positions: 48 and 45% of clones from Kc-167 cells and *in vitro* editing reactions contained −6 edits, respectively, while 55% of clones from *in vitro* editing reactions contained the +42 edit (Figure 2A and B). Two let-7 residues, +19 and +53, were also frequently edited *in vitro* by recombinant ADAR, and to a lesser degree in transfected cells (Figure 2C and D). Finally, a single editing event at the +43 position in the miR-125 stem loop was identified that occurred at a substantial frequency of 28% in both BG3c2 and Kc-167 cells and at an even higher frequency of 61% in *in vitro* editing reactions (Figure 2E and F). These results were supported by the previous recovery of let-7 miRNAs that contained A-to-G changes at the +19 position in a large-scale sequencing study of processed small RNAs (24). However, we noted that most of the editing events we identified occurred outside of the mature 5p miRNA sequences (Figure 2B, D and F), which are the dominant products of all three of these hairpins. Thus, such deep sequencing analysis of processed small RNAs could not detect editing events that are likely physiologically relevant. These data suggested that the absence of editing of pri-miR-100 at residues −6 and +42 and of pri-miR-125 at residues +43 might be responsible for the altered levels of miR-100 and miR-125 in *adar* mutant prepupae and adults.

Edited residues of *pri-let-7-C* alter miR-100 and miR-125 levels

To test whether these A-to-I editing events affected the expression levels of miR-100, let-7 and miR-125, northern blot analysis was performed on Kc-167 cells that were transfected with expression plasmids encoding wild type or modified versions that contained A-to-G changes at appropriate residues. Such changes mimicked the consequences of ADAR editing and were included at
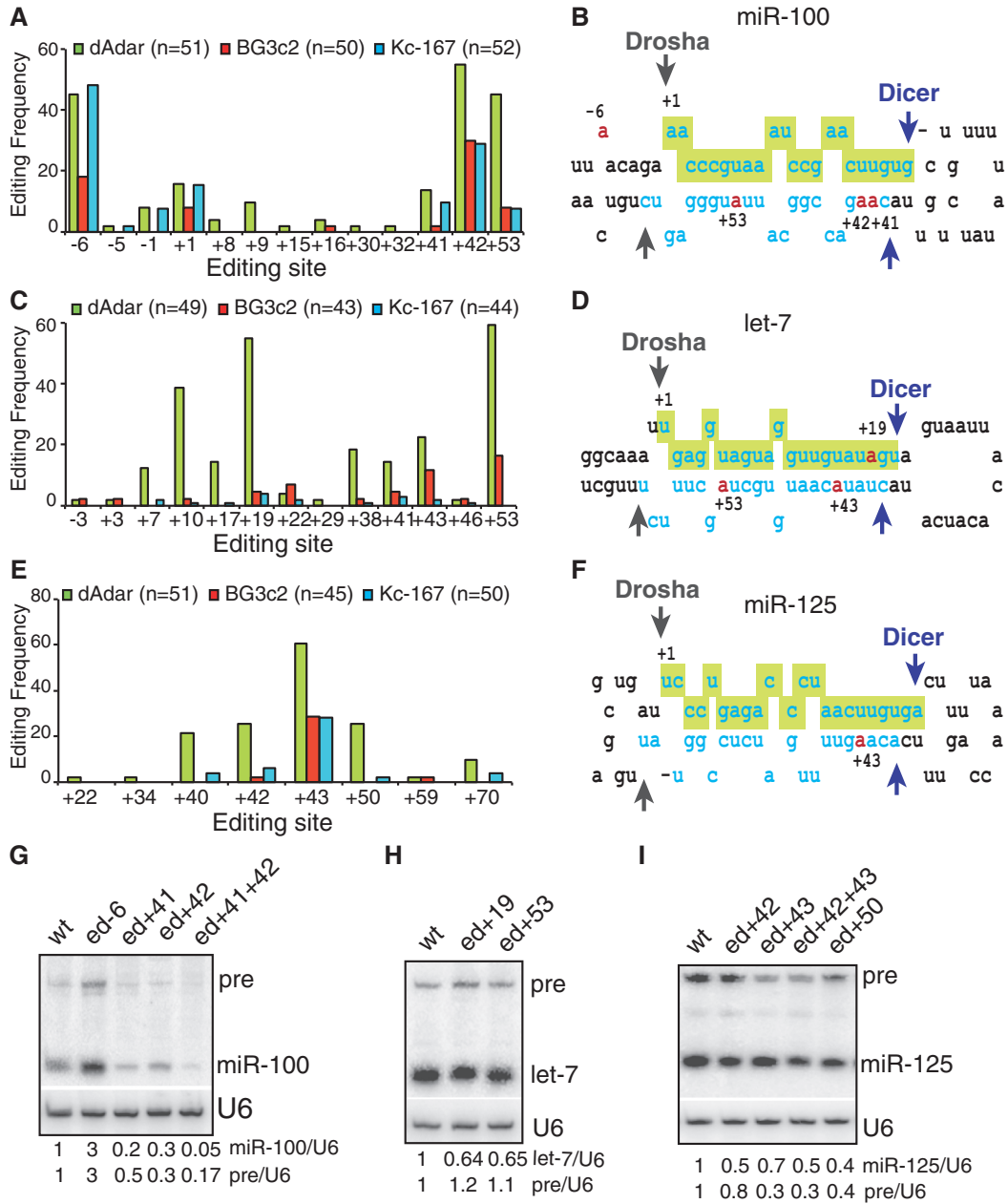
Figure 2. A-to-I editing of *let-7-Complex* pri-miRNAs by ADAR. (**A**, **C**, **E**) Bar graphs displaying editing frequency of pri-miR-100 (A), pri-let-7 (C) and pri-miR-125 (E) as determined by sequencing of cDNA clones from *in vitro* ADAR editing reactions (green bar), BG3c2 cell lines (red bar) and Kc-167 cell lines (blue bar). Editing frequency is represented as a percentage of independent clones representing the edited pri-miRNA sequence at a particular position relative to the total number of clones analyzed. (**B**, **D**, **F**) Schematics of pri-miR-100 (B), pri-let-7 (D) and pri-miR-125 (F) showing editing sites. Mature miRNAs are highlighted in green and edited adenosine residues are indicated in red. The 5′ end of mature 5p miRNA is numbered as +1; blue font indicates 5p and 3p mature miRNAs. Gray and blue arrows indicate Drosha and Dicer cleavage sites, respectively. (**G–I**) Northern blot analysis of transfected Kc-167 cells probed for miR-100 (G), let-7 (H) and miR-125 (I). Modified expression plasmids contained A-to-G changes at indicated residues. *U6* was used for normalization and normalized levels of precursor and processed miRNAs are indicated below each gel.

the −6, +41, +42 or at both +41 and +42 residues of pri-miR-100, at the +19 and +53 residues of pri-let-7 and at the +42, +43, +50 or at both +42 and +43 residues of pri-miR-125. Residues +41 of pri-miR-100 and +42 of pri-miR-125 were included in this analysis, as they neighbored residues that were frequently edited. RNA was extracted from transfected Kc-167 cells, and expression levels of pre-miRNA as well as processed miRNA were

quantitatively monitored for miR-100, let-7 and miR-125 (Figure 2G–I).

Most changes resulted in reduced miRNA levels, but the A-to-G change at the −6 position of pri-miR-100 resulted in a striking 3-fold increase of both pre-miR-100 and miR-100 levels compared with the control, suggesting increased cleavage of pri-miR-100 by Drosha (Figure 2G). Meanwhile, changes at the +42 or +41

positions of pri/pre-miR-100 resulted in a ≥3-fold decrease of processed miR-100, and combined changes at both +41 and +42 positions further reduced miR-100 levels dramatically (Figure 2G). Similarly, the change at the +43 residue of pri-miR-125 reduced levels of pre-miR-125 by 70% and processed miR-125 by 30% (Figure 2I). Editing at +42 and +50 positions of pri-miR-125 also resulted in reduced pre-miR-125 and miR-125 levels. Changes in pri-let-7 had the most modest effects: A-to-G changes at position +19 and +53 resulted in a slight reduction in processed let-7 levels and a slight increase in pre-let-7 levels (Figure 2H). This analysis indicated that editing events in pri-miR-100 and pri-miR-125 could repress miRNA production, but also suggested that miR-100 was a rare miRNA whose Drosha processing could also be enhanced by ADAR editing.

To confirm that A-to-G changes at residues −6 and +42 in pri-miR-100 had opposing effects on miR-100 levels, even within the context of its host polycistronic transcript, we prepared a second set of expression plasmids containing the full-length ∼2.4 kb *pri-let-7-C* sequence with A-to-G changes at residues −6, +42 or at both +41 and +42 of pri-miR-100. We also prepared two additional plasmids to further characterize these residues: one that contained A-to-G changes at both −6 and +42 residues and one that contained an A-to-C change at −6, which should mimic the absence of ADAR activity because it cannot be edited. We referred to such A-to-C changes as 'no edit'. As before, the effects of these base pair changes on mature miR-100 levels were evaluated by northern blot analysis of transfected Kc-167 analysis. In addition, we evaluated the effectiveness of a subset of these plasmids to repress the expression of a luciferase reporter that harbored six miR-100 binding sites in its 3′UTR to confirm that any changes in processed miR-100 levels also had functional consequences. As expected, changes at the +41 and/or +42 residues resulted in decreased miR-100 levels, as well as a significant decrease ($P = 0.0008$) in the fold repression of the luciferase reporter (Figure 3A and B). Likewise, the A-to-G changes at residue −6 resulted in a 1.6-fold increase in miR-100 levels, as well as significantly stronger repression ($P = 0.028$) of the luciferase reporter. Combined changes at both the −6 and +42 residues resulted in increased levels of pre miR-100 and miR-100, indicating that the editing event at −6 position was dominant over the editing event at +42 position. Finally, the A-to-C change at −6 resulted in a 2.5-fold decrease in processed miR-100 as well as significantly less repression ($P = 0.032$) of the luciferase reporter, consistent with the decrease in miR-100 levels detected in the *adar* mutant (Figures 1F and G). These results confirmed that editing of *pri-let-7-C* affected miR-100 levels, and suggested that miR-100 targets were misregulated in *adar* mutant tissue.

To test whether editing of pri-miR-100 affected the processing of its co-transcribed neighbors, let-7 and miR-125, we repeated these analyses with let-7 and miR-125 probes and luciferase reporters. Northern blot analysis revealed that altered miR-100 production had no obvious effects on processed let-7 or miR-125 levels (Figure 3A). Repression of let-7 and miR-125 luciferase reporters was also largely unaffected, although a slight but significantly increased repression of the let-7 reporter was detected with plasmids containing an A-to-G at the −6 residue of pri-miR-100 (Figure 3B). Nonetheless, these data indicated that editing-mediated changes in pri-miR-100 levels did not substantially affect the production of miR-125 or let-7.

Edited −6 residue of pri-miR-100 enhances miR-100 levels *in vivo*

Although these experiments indicated that editing of *pri-let-7-C* affected *let-7-C* miRNA production, they were performed in cells that did not ordinarily transcribe *let-7-C* and perhaps did not reflect endogenous regulation of the *let-7-C* locus. We therefore analyzed *let-7-C* miRNA expression in transgenic flies containing the full-length *pri-let-7-C* expression constructs under the control of the endogenous *let-7-C* regulatory region and in a *let-7-C* mutant background, so that endogenous *let-7-C* miRNAs would not interfere with our analysis. Total RNA was extracted from adult males harboring one copy of each transgene, and the levels of the three *let-7-C* miRNAs were analyzed by Taqman qRT-PCR. Consistent with our *in vitro* results, the A-to-G change in residue −6 of pri-miR-100 resulted in a 10-fold increase in miR-100 levels compared with the control (Figure 3C), while the A-to-C change displayed the opposite trend. Although miR-125 levels were unaffected by these changes in *pri-let-7-C*, an increase in let-7 levels was observed in both cases. These data indicated that editing of pri-miR-100 enhanced miR-100 expression in adult males, and likely in their nervous systems where *let-7-C* is predominantly expressed.

To determine whether this altered expression had functional consequences, we compared the effects of changing residue −6 of pri-miR-100 from A to either G or C on the expression of a miR-100 sensor transgene. For this analysis, the *pri-let-7-C* constructs were ectopically expressed in larval imaginal disc cells, where *let-7-C* is not normally expressed, and the expression levels of a GFP transgene that contained six miR-100 binding sites were examined. Expression of the A-to-G version of pri-miR-100 resulted in a far greater repression of the GFP sensor than the A-to-C version of pri-miR-100 (Figure 3D). These data indicated that ADAR editing of the −6 residue of pri-miR-100 resulted in greater miR-100 production and consequently less expression of miR-100 target genes *in vivo*.

Edited −6 residue results in enhanced Drosha processing of pri-miRNA

To determine the mechanistic basis for the enhanced production of miR-100 from edited versions of *pri-miR-100*, we performed *in vitro* Drosha processing assays on transcripts containing either an A or a G at the −6 residue of the pri-miR-100 stem-loop base. Because of its inefficient processing *in vitro*, we could not perform this experiment on pri-miR-100. We therefore prepared chimeric constructs that encoded the let-7 hairpin flanked by the pri-miR-100 stem-loop base containing either an A or a G at the −6 residue (Supplementary Figure S3A). We first
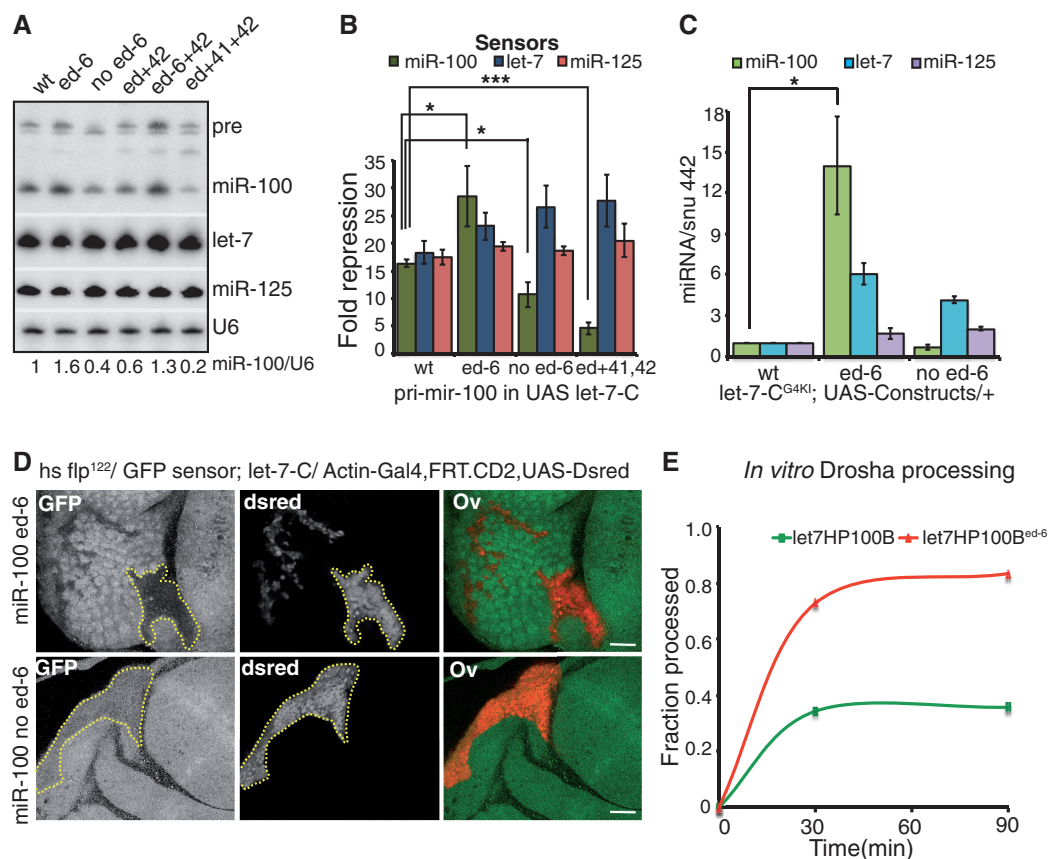
Figure 3. Processing of miR-100 is enhanced by ADAR editing. (**A**) Northern blot analysis of Kc-167 cells transfected with expression plasmids and probed for miR-100, let-7 and miR-125. Pri-let-7-C expression plasmids contain A-to-G (ed) or A-to-C (no ed) changes at indicated residues. U6 was used as a control for normalization. (**B**) Fold repression of miR-100 (green bar), let-7 (blue bar) and miR-125 (pink bar) luciferase sensors in Kc-167 cells transfected with the *pri-let-7-C* constructs. Assays were performed in triplicates. (**C**) Expression of miR-100 (green bar), let-7 (blue bar) and mir-125 (purple bar) in transgenic lines expressing *UAS-let-7-C* cDNAs under the control of the *let-7-C* Gal4 knock-in driver (*G4KI*) as determined by Taqman qRT-PCR. Expression plasmids contain A-to-G (ed) or A-to-C (no ed) changes at indicated residues. Total RNA was extracted from adult flies of the following genotype: *let-7-C^{G4KI}/let-7-C^{G4KI}*; *UAS-transgene/+*. *let-7-C^{G4KI}* is a null *let-7-C* allele (23). *Snu 442* was used as a normalization control. (**D**) Third instar larval imaginal discs containing dsRed-labeled clones (red in overlay) expressing *UAS-let-7-C* cDNA transgenes with A-to-G (ed, upper row) or A-to-C (no ed, lower row) changes at −6 position pri-miR-100 as well as a miR-100 GFP sensor (green in overlay). Clones (outlined in yellow) have lower GFP levels in upper row compared with lower row. (**E**) Line graph depicting kinetics of processing of chimeric miR-100/let-7 pri-miRNAs containing an A (green line, let-7HP100B) or a G (red line, let-7HP100B^{ed-6}) at the −6 residue. Pri-let-7HmiR-100B and pri-let-7HmiR-100B^{ed-6} were subjected to Drosha cleavage and the fraction of processed pre-miRNA was calculated as the ratio of pre/pre+pri.

confirmed that let-7 expression was enhanced in Kc-167 cells transfected with the chimeric construct that contained a G at the −6 position (Supplementary Figure S3B). Then, the rate of processing of these two chimeric transcripts was examined by incubating them with Drosha-Pasha complex immunoprecipitated from BG3c2 cells (Figure 3E and Supplementary Figure S3C). Roughly 73% of the transcripts containing a G at the −6 position were efficiently processed within 30 min (Figure 3E). In contrast, only 34% of the transcripts containing an A at −6 were efficiently processed within 30 min, and processing of these transcripts did not increase with time. Together, these data confirmed that ADAR editing of the pri-miR-100 base enhanced its processing by Drosha.

Edited +43 residue of pri-miR-125 destabilizes the primary *let-7-C* transcript

Because our analysis had also identified the +43 position of pri-miR-125 as another frequently edited residue in the

pri-let-7-C transcript (Figure 2E, F and I), we also characterized this editing event in context of the full-length *pri-let-7-C* transcript. As before, we analyzed the expression of *let-7*-C miRNAs in Kc-167 cells transfected with *let-7*-C expression plasmids containing A-to-G changes at positions +43, +42 and +50 of pri-miR-125 by qRT-PCR. As suggested by our previous analysis, these nucleotide changes resulted in reduced levels of miR-125 (Figure 4A). However, unlike the A-to-G changes in pri-miR-100, these changes affected the expression of all three let-7-C miRNAs, suggesting that they caused instability of the pri-let-7-C transcript. Indeed, pri-let-7-C levels were reduced in RNA from transfected Kc-167 cells with all three modified expression constructs (Figure 4B). Consistent with a reduction in mature miRNA levels, luciferase reporters for miR-100 and miR-125 miRNAs were less repressed in cells transfected with a plasmid containing an A-to-G change at +43 of pri-miR-125 (Figure 4C). Expression of the let-7 sensor was
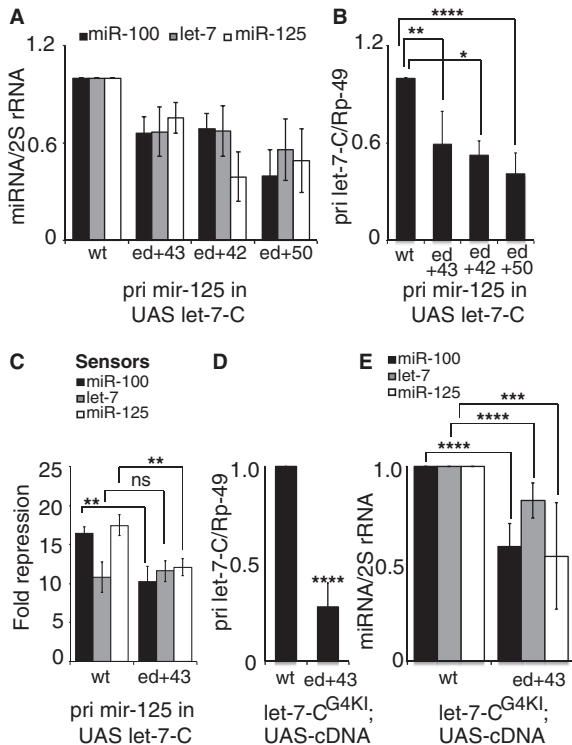
Figure 4. ADAR editing of miR-125 precursor destabilizes *pri-let-7-C* transcript. (**A**) Taqman qRT-PCR of miR-100 (black bar), let-7 (gray bar) and miR-125 (white bar) in RNA from Kc-167 cells transfected with either unmodified *let-7-C* cDNA constructs or ones that contained A-to-G changes at indicated residues. Reduction of miR-100, let-7 and miR-125 levels in cells transfected with each modified versus unmodified construct are statistically significant ($P < 0.05$). (**B**) qRT-PCR of *pri-let-7-C* in Kc-167 cells transfected with either unedited or pre-edited UAS-let-7-C cDNA constructs. *Rp49* was used as control for normalization. (**C**) Fold repression of miR-100, let-7 and miR-125 luciferase sensors in Kc-167 cells transfected with *pri-let-7-C* constructs. Assays were performed in triplicates. (**D** and **E**) qRT-PCR analysis of *pri-let-7-C* (D) and processed *let-7-C* miRNAs (E) in transgenic lines expressing *UAS-let-7-C* cDNAs under the control of the *let-7-C* Gal4 knock-in driver (*G4KI*). Expression plasmids contain an A-to-G change at indicated residues. Total RNA was extracted from pharate adults of the following genotype: *let-7-C^{G4KI}; UAS-transgene. Rp49* (D) and *Snu 442* (E) were used as a normalization control.

probably not affected owing to the abundance of let-7 in these cells: a modest reduction in let-7 levels was not sufficient to affect luciferase levels. Nevertheless, these data indicated that editing at +43 residue of pri-miR-125 destabilized the entire *pri-let-7-C* transcript in the Kc-167 cell line.

To confirm this same effect *in vivo*, *pri-let-7-C* and processed *let-7-C* miRNA levels were analyzed in transgenic lines expressing either an unmodified *wild type* version of *pri-let-7-C* or one that contained an A-to-G change at +43 of pri-miR-125 (Figure 4D and E). As with our previous analysis, total RNA was isolated from pharate adults containing the expression constructs under the control of the endogenous *let-7-C* promoter and in a *let-7-C* mutant background. The A-to-G change resulted in a 70% reduction in pri-let-7-C levels (Figure 4D). This reduction in *pri-let-7-C* levels was reflected by a decrease in miR-100, let-7 and miR-125 levels (Figure 4E). These findings suggested

that RNA editing had indirect effects within polycistronic miRNA transcripts, and that a single editing event could modulate the expression of adjacent miRNAs in the same primary transcript.

RNA editing regulates expression of *let-7-C* miRNAs during metamorphosis

To assess whether ADAR editing shaped the distinctive temporal expression pattern of *let-7-C* miRNAs in developing animals, we analyzed the impact of editing at either the −6 residue of pri-miR-100 or the +43 residue of pri-miR-125 on the developmental profile of *let-7-C*. For this analysis, we characterized the previously described strains expressing either an unmodified version of the *let-7-C* cDNA, or one that contained an A-to-G change at +43 of pri-miR-125 or A-to-G or A-to-C changes at residue −6 of pri-miRNA-100. Total RNA was extracted from prepupae and staged pupae (6, 12, 24, 48 and 72 h APF) harboring two copies of each transgene, and levels of *pri-let-7-C* and processed let-7-C miRNAs were analyzed by qRT-PCR (Figure 5A–D, log scale graphs). This analysis revealed that the A-to-G change at +43 of pri-miR-125 drastically dampened the profile not only of miR-125 but also miR-100 and let-7 as well as *pri-let-7-C*, consistent with our previous findings that this change destabilized the *pri-let-7-C* transcript (Figure 5A–D, purple columns). The A-to-G or A-to-C changes at residue −6 of pri-miRNA-100 also had the expected effects on miR-100 levels: the A-to-G change resulted in significantly elevated miR-100 levels at all time points, while the A-to-C change resulted in decreased miR-100 levels in the 24, 48 and 72 h time points as well (Figure 5B, blue and green columns). These effects were largely specific to miR-100, as let-7 and miR-125 levels were normal at most time points (Figure 5C and D, blue and green columns). We also noted a decrease in *pri-let-7-C* levels caused by the A-to-G change, likely a consequence of the enhanced processing of the primary transcript. These data highlighted the role of RNA editing in the temporal control of miR-100, and suggested that the precise levels of each miRNA is likely to be governed by the combined effects of all the editing events that occur at a particular stage during development.

To obtain direct evidence that ADAR edited endogenous *pri-let-7-C* transcripts, we sequenced *let-7-C* cDNA clones amplified from staged *wild type* animals. We recovered clones containing edited residues at +41 and +42 of pri-miR-100, +22 of pri-let-7 and +43 of pri-miR-125 (Figure 5E). The frequency of editing of *pri-let-7-C* increased during metamorphosis, as no edited cDNA clones were observed in prepupae or 24 h to 48 h pupae. We also noted the absence of clones containing an edited −6 residue of pri-miR-100, likely because *pri-let-7-C* transcripts with this change are rapidly processed and therefore difficult to recover. However, our expression analysis of miR-100 levels in *adar* mutants is consistent with such an event in both prepupae and adults (Figure 1F and G).
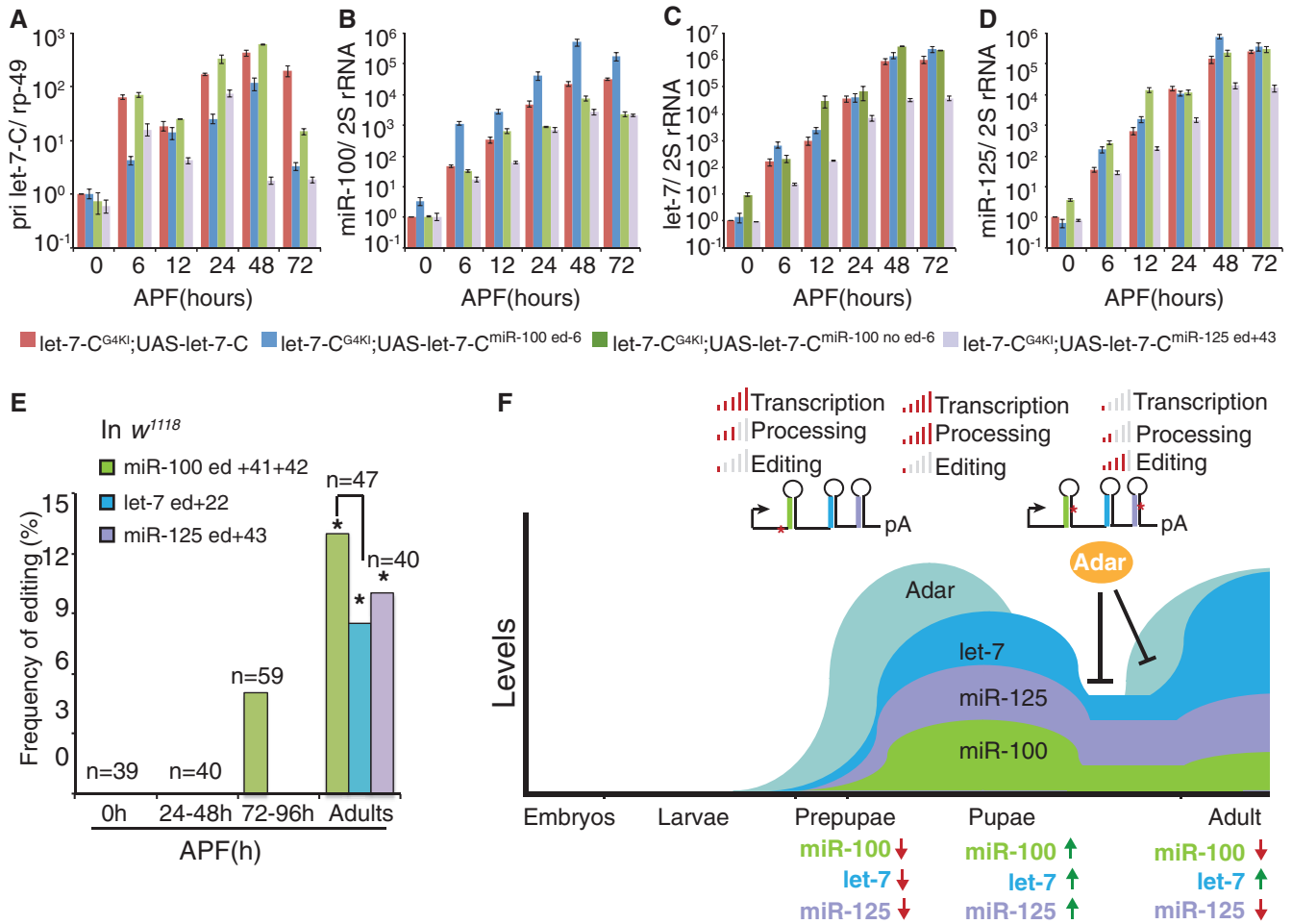
Figure 5. ADAR editing shapes developmental profiles of *let-7-C* miRNAs. (**A–D**) Developmental profile of *pri-let-7-C* (A), miR-100 (B), let-7(C) and miR-125 (D) as determined by qRT-PCR of total RNA extracted animals collected 0, 6, 12, 24, 48 and 72 h APF. The genotypes of the strains used are indicated below the graphs (D). *Rp49* was used as a normalization control for *pri-let-7-C* and *2S* rRNA was used as normalization control for miR-100, let-7 and miR-125. The bar graphs are represented on a logarithmic scale and error bars represent s.d. (**E**) Editing frequency detected in *pri-let-7-C* cDNA clones from wild type (*w^{1118}*) pupae and adult. Pupae were collected at 0, 24–48, 72 and 96 h APF. Significance of frequency of editing events detected in adult samples relative to prepupal samples is indicated. (**F**) Schematic model for post-transcriptional regulation of *let-7-C* miRNAs by ADAR. The *let-7-C* miRNAs expression is triggered by ecdysone peaks that occur in mid-late third larval instar (25), and levels of the three miRNAs encoded by this locus peak at 48 h APF. No editing of the *let-7-C* primary transcript is detected until 48 h APF, suggesting that both transcription and efficient processing contribute to the build up of miR-100, let-7 and miR-125. The subsequent increase in the frequency of ADAR editing of *pri-let-7-C* in older pupae and adults contributes to the rapid decline in miR-100 and miR-125.

DISCUSSION

We have presented a detailed molecular analysis of ADAR editing events that occur in the *pri-let-7-C* transcript. Loss of *adar* causes diverging effects on the expression levels of miRNAs encoded by this transcript: processed miR-100 is drastically reduced, while processed miR-125 is increased in both prepupal and adult stage *adar^{5G1}*. These data were corroborated by the identification of ADAR editing sites that altered the processing of *let-7-C* miRNAs. Edited residues were conserved across all *Drosophilid* genomes, suggesting that ADAR editing is conserved mechanism that regulates *pri-let-7-C* processing.

Based on our findings, we propose a model for the post-transcriptional regulation of *let-7-C* miRNAs by ADAR (Figure 5F). *Pri-let-7-C* is transcriptionally induced by

pulses of the steroid hormone ecdysone in mid-late third larval instar (25). During the larval-to-adult transition, rising levels of ADAR and *pri-let-7-C* lead to an increase in the frequency of ADAR editing of *pri-let-7-C* in older pupae and young adults. Editing of pri-miR-100 at positions +41 and +42 and pri-miR-125 at position +43 lead to a sharp decrease in miR-100 and a moderate decrease in miR-125 and let-7. The frequency of editing further increases in aging adults and this is likely to result in additional differential processing of *let-7-C* miRNAs at this stage. We propose that combinatorial effects of these editing events together with a change in their frequency during development results in differential levels of the three miRNAs encoded by the *let-7-C* locus (Figure 5F).

Pri-miR-100 provides a vivid example of these combinatorial effects. ADAR editing at the −6 position of pri-miR-100 enhances miR-100 production, while editing at

+42 and/or +41 reduces it. We obtained direct evidence of the +41/+42 editing event of pri-miR-100 (Figure 5E) and indirect evidence of the −6 editing event, as *adar* mutants express significantly reduced amounts of miR-100 (Figure 1F and G). Combining both of these editing events leads to an increase in miR-100, indicating that the effects of editing at the −6 position supersede those at the +41/+42 positions. Editing-mediated enhancement of processing may be particularly important for miR-100, as it is the most inefficiently processed and weakly expressed member of the three *let-7-C* miRNAs: we found, for example, that processed miR-100 was ∼100-fold less abundant than let-7 in adults (Figure 1D), consistent with previous miRNA expression analyses (24,25). Thus, ADAR-mediated editing is probably critical in boosting miR-100 levels in response to critical developmental or environmental cues. MiR-100 is also considered the most ancient animal miRNA (35), suggesting that processing enhancement is an ancestral feature that most miRNAs have lost.

This work presents strong evidence that ADAR editing enhances the production of a mature miRNA *in vivo*, the demonstration of a novel function of ADAR that has only been previously hinted at by *in vitro* processing assays of mouse and human *pri-miR-203* (18). Although ADAR-mediated editing of pri-miR-203 resulted in a 2-fold increase in its processing by Drosha *in vitro*, neither edited nor unedited miR-203 were detected in tissues where edited pri-miR-203 was identified (18). In contrast, in addition to finding elevated miR-100 levels in *adar* mutants, we not only show that editing at the −6 position enhances Drosha processing but also, importantly, that edited pri-miR-100 yields higher miR-100 both *in vitro* (Figure 2G, 3A and B) and *in vivo* (Figure 3C and D). ADAR likely enhances the processing of additional miRNAs, but identifying these miRNAs is a challenge. For miR-100, the relevant residue is in the pri-miRNA stem at the −6 position, a portion of the miRNA transcriptome that is difficult to recover and comprehensively query for editing events, particularly because editing events would enhance miRNA processing and make these RNA fragments even more transient. Nevertheless, the mechanism reported here, that editing of one pri-miRNA can enhance or repress its processing, probably affects other polycistronic miRNAs as well. The mechanisms underlying the 10-fold increase of muscle-specific miR-1 relative to its co-transcribed neighbor miR-133 in heart tissue (36) as well as the differential expression of the oncogenic miR-17-92 cluster (37,38) are currently unknown. ADAR editing of these polycistronic miRNAs may be relevant to their differential expression as well.

This analysis also identified an A-to-I modification in the pre-miR-125 sequence at position +43, +42 and +50 that affects the stability of the entire *pri-let-7-C* transcript. This editing consequently leads to decreased expression of all three encoded miRNAs, perhaps by Tudor-SN–mediated degradation known to target other edited miRNAs (19). Although ADAR editing events in clusters of miRNAs have been reported previously (21,39), these analyses have focused on editing of isolated pri-miRNAs and not on their indirect effects on the entire polycistronic transcript. This mechanism could be important for rapidly clearing a set of miRNAs from a system.

The frequency of editing events varies throughout development (18,40). Our analysis of ADAR editing of *pri-let-7-C* reveals an increase in specific editing events that reduce processing of mir-100 and miR-125 during late metamorphosis and into adulthood. Combinatorial effects of these different editing events are likely to result in the precise spatiotemporal expression of clustered miRNAs during normal development. This analysis therefore reveals a previously uncharacterized ADAR-mediated mechanism to fine-tune expression of polycistronic miRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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