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The RNA Methyltransferase Dnmt2 Is Required for Efficient Dicer-2-Dependent siRNA Pathway Activity in *Drosophila*

Zeljko Durdevic,[1] Mehrpouya Balaghy Mobin,[1,2] Katharina Hanna,[1] Frank Lyko,[1] and Matthias Schaefer[1,*]
[1]Division of Epigenetics, DKFZ-ZMBH Alliance, German Cancer Research Center, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany
[2]Present address: Institute of Molecular Systems Biology, ETH Zurich, HPT C53, Wolfgang-Pauli-Strasse 16, 8093 Zurich, Switzerland
*Correspondence: m.schaefer@dkfz.de
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SUMMARY

Transfer RNA (tRNA) fragmentation in response to stress conditions has been described in many organisms. tRNA fragments have been found in association with small interfering RNA (siRNA) components, but the biological role of these interactions remains unclear. We report here that the tRNA methyltransferase Dnmt2 is essential for efficient Dicer-2 (Dcr-2) function in *Drosophila*. Using small RNA (sRNA) sequencing, we confirmed that Dnmt2 limits the extent of tRNA fragmentation during the heat-shock response. tRNAs as well as tRNA fragments serve as Dcr-2 substrates, and Dcr-2 degrades tRNA-derived sequences, especially under heat-shock conditions. tRNA-derived RNAs are able to inhibit Dcr-2 activity on long double-stranded RNAs (dsRNAs). Consequently, heat-shocked *Dnmt2* mutant animals accumulate dsRNAs, produce fewer siRNAs, and show misregulation of siRNA pathway-dependent genes. These results reveal the impact of tRNA fragmentation on siRNA pathways and implicate tRNA modifications in the regulation of sRNA homeostasis during the heat-shock response.

INTRODUCTION

Transfer RNA (tRNA) fragmentation contributes to conserved stress-response mechanisms (Thompson and Parker, 2009), but the exact nature of tRNA fragment-mediated effects is poorly understood. tRNAs represent the most abundant class of small RNAs (sRNAs; 4%–10% of all RNAs). If tRNA-derived sequences relay stress responses, their fragmentation process must be tightly controlled to avoid constant stress signaling. The association of tRNA fragments with siRNA pathway components (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008b; Cole et al., 2009) indicated crosstalk between tRNA fragment production and siRNA pathway function. Dcr-2 and Ago-2 are important siRNA pathway components in flies, and their molecular function in RNA-induced silencing complexes (RISC) is well defined (Saito and Siomi, 2010). However, the impact of siRNA pathways on gene expression, especially under stress conditions and in the presence of stress-induced tRNA fragments, is less well understood.

Recent work has linked *Dnmt2* mutations to decreased tRNA stability during stress and certain developmental conditions (Schaefer et al., 2010; Tuorto et al., 2012), underscoring the importance of cytosine-5-tRNA methylation for correct RNA metabolism. Dnmt2 enzymes are highly conserved RNA cytosine-5-methyltransferases that modify the anti-codon loop of tRNAs (Goll et al., 2006; Schaefer et al., 2010), but the significance of Dnmt2-mediated tRNA methylation and stability has remained unclear.

Although an impact of tRNA fragments on protein synthesis has been established (Yamasaki et al., 2009; Ivanov et al., 2011; Sobala and Hutvagner, 2013), other tRNA-mediated functions remain to be explored. Interestingly, tRNA fragments can affect the efficiency of sRNA silencing pathways in human cells (Haussecker et al., 2010), raising the possibility that tRNA-derived RNAs play as yet unidentified roles in posttranscriptional gene silencing (Pederson, 2010; Hurto, 2011; Durdevic and Schaefer, 2013).

We report here that siRNA pathways in *Drosophila* can be transiently affected by heat-shock-induced tRNA fragmentation. Under these conditions, *Dnmt2* mutant flies showed a stronger and more prolonged inhibition of siRNA pathway activity. Increased tRNA fragmentation in *Dnmt2* mutants after heat shock and the promiscuous loading of tRNA fragments into Dcr-2 complexes blocked Dcr-2 processing of long dsRNAs, resulting in the misregulation of siRNA pathway-controlled gene expression. Our findings reveal that correct tRNA methylation contributes to the control of siRNA pathway components during the heat shock response.

RESULTS

tRNA Fragmentation Is Specifically Affected in *Dnmt2* Mutants

To globally quantify the extent of Dnmt2-dependent RNA fragmentation, we heat shocked adult male flies, extracted sRNAs

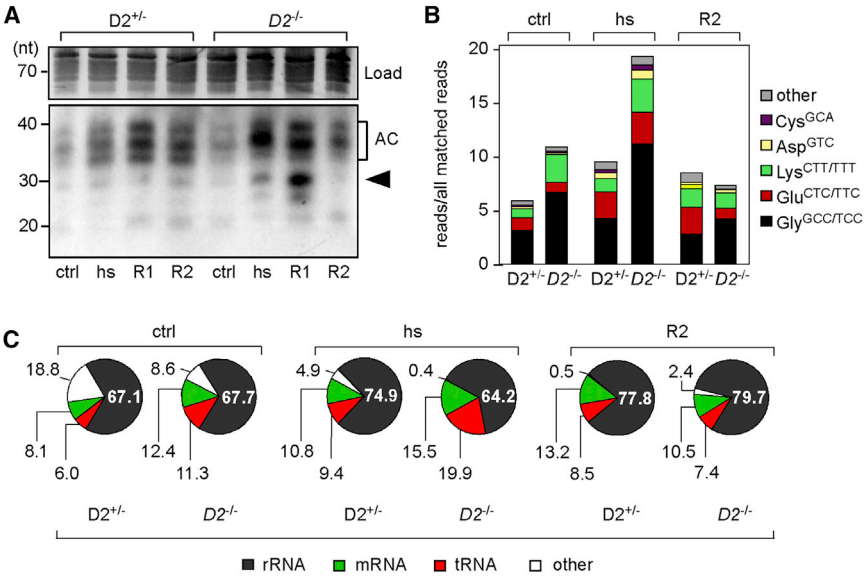

Figure 1. RNA Fragmentation Patterns in *Dnmt2* Mutant Flies Indicate Specificity for tRNA

(A) Northern blot (15 μg RNA) for 5′-fragments (<70 nt) of $tRNA_{Asp}^{GTC}$ before and after heat shock (37°C, 1 hr) of control ($D2^{+/-}$) and *Dnmt2* mutant ($D2^{-/-}$) male flies, followed by 2 days of recovery (R). AC, cleavage in the anti-codon; smaller tRNA-derived fragments are marked by black arrowhead.

(B) Presentation of tRNA-derived reads (normalized to the total read number of individual experiments; see Tables S1, S2, S3, and S4) after sRNA (25–70 nt) sequencing of a heat-shock experiment as in (A).

(C) Overview of sRNA sequencing data from a heat-shock experiment as in (A). Percentages of mapped reads from individual experiments are displayed as pie charts and color-coded for rRNA, mRNA, tRNA, and RNAs from other sources (i.e., microbes).

See also Figure S1 and Tables S1, S2, S3, and S4.

from somatic tissues (Figure 1A), and sequenced complementary DNA (cDNA) libraries on an Illumina platform. Mapping of tRNA-derived reads revealed significant Dnmt2-dependent effects on the fragmentation of various tRNAs (Figures 1B, S1A, and S1B; Tables S1, S2, and S3), including known Dnmt2 substrates ($tRNA_{Asp}^{GTC}$ and $tRNA_{Gly}^{GCC}$). Analysis of individual $tRNA_{Asp}^{GTC}$ halves showed that *Dnmt2* mutant tissues accumulated shorter 5′ fragments than controls after heat shock (Figure S1C; Table S4), indicating that tRNA cleavage-site accessibility or further processing of tRNA fragments was Dnmt2 dependent.

Ribosomal RNA fragmentation was not significantly changed in *Dnmt2* mutants after heat shock (Figures 1C, S1D, and S1E). Also, most messenger RNA (mRNA) fragments were derived from exons of large genes (Figures S1F and S1G) and no significant differences were detectable between genotypes (Figure S1H). These results indicate that Dnmt2 function specifically affected tRNA stability during the heat-shock response.

Dicer-2 Cleaves tRNA Sequences into sRNAs

Angiogenin-dependent tRNA cleavage has been shown in mammalian cells (Yamasaki et al., 2009), but the responsible enzymes in *Drosophila* remain to be identified. Importantly, DICER-dependent tRNA cleavage has also been observed in mammals (Babiarz et al., 2008; Cole et al., 2009), suggesting that Dicer proteins accept tRNAs as substrates. RNA immunoprecipitations confirmed the association of Dcr-2 with tRNA fragments in *Drosophila* S2 cells (Figure 2A). In vitro cleavage assays using purified tRNAs and ovary protein extracts showed that Dcr-2 activity contributed to tRNA fragmentation (Figure 2B). Fragmentation was enhanced in heat-shocked extracts, indicating stress-induced changes in Dcr-2 activity. tRNA cleavage could be partially blocked by RNase inhibitors (Figure 2B), suggesting also the presence of stress-induced but Dcr-2-independent nuclease activities. Of note, overexpression of Dcr-2 in S2 cells also caused tRNA fragmentation (Figure 2C), indicating that changes in Dcr-2 protein concentration supported Dcr-2-mediated tRNA cleavage.

Purified Dcr-2 has been shown to cleave noncanonical substrates such as pre-microRNAs (pre-miRNAs) (Cenik et al., 2011). To analyze whether Dcr-2 was also able to cleave tRNA fragments, S2 cell protein extracts were incubated with $tRNA_{Asp}^{GTC}$ fragments (nt 1–38). This revealed the production of distinct sRNAs (Figure S2A), specifically in heat-shocked protein extracts, which was dependent on Dcr-2 but not on Dcr-1 (Figures S2B–S2D). RNAi-mediated depletion of annotated *Drosophila* nucleases confirmed the specificity for Dcr-2 (Figure S2E). Furthermore, extracts from wild-type ovaries, but not catalytically inactive *Dcr-2* mutant ovaries, degraded tRNA halves (Figures 2D and S2F). This confirmed that Dcr-2 accepted tRNA-derived sequences, especially if provided in excess, which indicates that Dcr-2, tRNAs and their fragments interact under specific conditions.

Dcr-2 Activity on Long dsRNA Is Inhibited by tRNA Fragments

Dcr-2 processes long dsRNA originating from self-complementary transcripts, convergent mRNAs, and mobile elements (Carthew and Sontheimer, 2009). To analyze Dcr-2 activity, defined long dsRNAs were incubated with ovary protein extracts and the production of small RNAs (sRNAs) was monitored in vitro. sRNAs (21 nt) were produced in a time- and Dcr-2 activity-dependent manner (Figure 3A). Addition of $tRNA_{Asp}^{GTC}$ fragments (nt 1–38) resulted in a concentration-dependent loss of sRNAs (Figure 3B), indicating that tRNA fragments interfered with Dcr-2 activity. Heat shock of S2 cells reduced Dcr-2 protein levels transiently and Dcr-2 activity on dsRNA was diminished (Figures S3A and S3B). Fragmentation of $tRNA_{Asp}^{GTC}$ into halves was highest when Dcr-2 activity levels were low (Figure S3C), indicating that Dcr-2 was not primarily responsible for tRNA fragmentation. Instead, these results suggest competition between dsRNA precursors and tRNA fragments for reduced Dcr-2 protein levels during the heat-shock response.

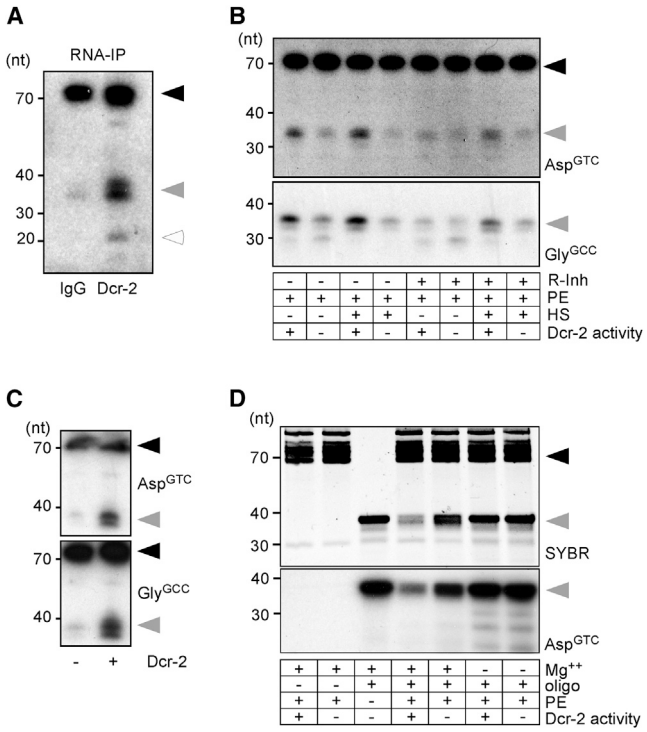

Figure 2. Dcr-2 Processes tRNAs into Halves and siRNA-Sized Fragments

(A) Northern blot (using 5′ probes) for $tRNA_{Asp}^{GTC}$ and $tRNA_{Gly}^{GCC}$ in Dcr-2-FLAG complexes after RNA immunoprecipitation. Arrowheads indicate unspecific tRNA binding (black), tRNA halves (gray), smaller tRNA-derived RNAs (white). IgG, immunoglobulin G control.

(B) RNA in vitro cleavage assay using wild-type (+) and *Dcr-2* catalytic mutant (−) ovary protein extracts (15 μg) on purified tRNA preparations followed by northern blotting for tRNAs using 5′ probes against $tRNA_{Asp}^{GTC}$ (upper) and $tRNA_{Gly}^{GCC}$ (lower). Arrowheads indicate tRNA (black) and tRNA fragmentation products (gray). HS, heat shock; PE, protein extract; R-Inh, RNase inhibitor.

(C) Northern blot on RNA (15 μg) from S2 cells that express endogenous (−) or ectopic Dcr-2-FLAG protein (+) using probes against $tRNA_{Asp}^{GTC}$ and $tRNA_{Gly}^{GCC}$.

(D) RNA in vitro cleavage assay using protein extracts as in (B) on $tRNA_{Asp}^{GTC}$ oligonucleotides (nt 1–38; 1 μM). Upper part shows SYBR-stained Urea-PAGE-gel. Lower part shows northern blot using 5′ probes against $tRNA_{Asp}^{GTC}$. Arrowheads indicate tRNA (black) and tRNA oligonucleotide (gray). Mg++, Mg ions.

See also Figure S2.

Dcr-2 Activity Is Impaired in *Dnmt2* Mutant Protein Extracts

Next, adult flies were heat shocked to analyze Dcr-2 activity in vivo. Expression analysis revealed transient upregulation of *Dcr-2* and *Dcr-1* in controls (Figure S3D), suggesting positive feedback on Dicer transcription in response to heat shock. In contrast, *Dnmt2* mutants displayed constantly increasing *Dcr-2* and *Dcr-1* transcript levels, which indicated prolonged transcriptional feedback. Western blotting showed increased Dcr-2 protein levels in both genotypes after heat shock of adult flies, whereas Dcr-1 or Ago-2 expression was not affected (Figure S3E and S3F). Wild-type ovary extracts showed reduced Dcr-2 activity after heat shock, which could be restored to baseline levels by adding three times more Dcr-2 protein to the reaction (Figure 3C). Importantly, extracts from *Dnmt2* mutant ovaries were unable to restore Dcr-2 activity (Figure 3C), indicating impaired Dcr-2 activity in *Dnmt2* mutants. Dcr-2 activity in heat-shocked wild-type ovaries could also be restored by increased cleavage reaction times (Figure 3D), indicating Dcr-2 processivity on dsRNA substrates, which was not the case in *Dnmt2* mutant extracts (Figure 3D). Lower Dcr-2 activity in *Dnmt2* mutant ovaries did not correlate with lower Dcr-2 protein levels (Figure 3E), suggesting that Dcr-2 activity was present but limited in *Dnmt2* mutants. Northern blotting confirmed increased levels of tRNA fragments in *Dnmt2* mutant ovaries after heat shock (Figure 3F), supporting the notion that increased tRNA fragmentation in *Dnmt2* mutants interfered with Dcr-2 activity, especially after heat shock.

dsRNAs Accumulate in *Dnmt2* Mutants After Heat Shock

To test whether *Dnmt2* mutant conditions affected Dcr-2 substrate levels globally, antibodies against dsRNAs (Schönborn et al., 1991) and RNA dot blotting were used. Antibody specificity was confirmed using *Dcr-2* mutant RNA, which revealed high levels of dsRNA (Figure 4A). RNA from heat-shocked *Dnmt2* mutants contained increasing dsRNA concentrations (Figure 4B), indicating that dsRNAs, which were efficiently processed by Dcr-2 in wild-type tissues, accumulated in *Dnmt2* mutants. These results confirm that Dcr-2 activity was globally impaired in *Dnmt2* mutant tissues, especially after heat shock.

sRNA Production Is Affected in *Dnmt2* Mutants

Dcr-2 processes long dsRNAs into sRNAs. To analyze the production of sRNAs, northern blotting for a highly expressed endo-siRNA (esi-2.1) and a miRNA (miR-bantam) was performed. As previously shown, *Dcr-2* or *Ago-2* mutants did not produce mature esi-2.1, and only *Ago-2* mutants affected miR-bantam production (Figure 4C). Of note, *Dnmt2* mutant flies showed reduced esi-2.1 production and also miR-bantam levels were lower than in controls (Figure 4C), which indicated a role for Dnmt2 in endo-siRNA and miRNA maturation. Heat shock caused a decrease of esi-2.1 in controls, whereas *Dnmt2* mutant flies showed lower esi-2.1 levels during all time points of the experiment (Figure 4D). These results are consistent with the notion that Dcr-2 activity in *Dnmt2* mutant flies was impaired, causing inefficient processing of long dsRNAs and consequentially reduced production of Dcr-2-dependent siRNAs. In addition, changes in miR-bantam levels in *Dnmt2* mutants after heat shock (Figure 4D) also suggest Dnmt2-mediated effects on Dcr-1-dependent miRNA production.

The esi-2.1 Target *mus308* Is Upregulated in *Dcr-2* and *Dnmt2* Mutants

esi-2.1 matches the coding sequence of the DNA repair enzyme *mus308* (Czech et al., 2008; Okamura et al., 2008b), suggesting endo-siRNA-mediated repression of Mus308-mediated DNA repair. *Mus308* RNA levels were slightly elevated in adult *Dcr-2* and *Dnmt2* mutant flies during a heat-shock experiment (Figure 4E), indicating that Dnmt2 function affects *mus308* expression. Separation of male somatic and germline tissues followed

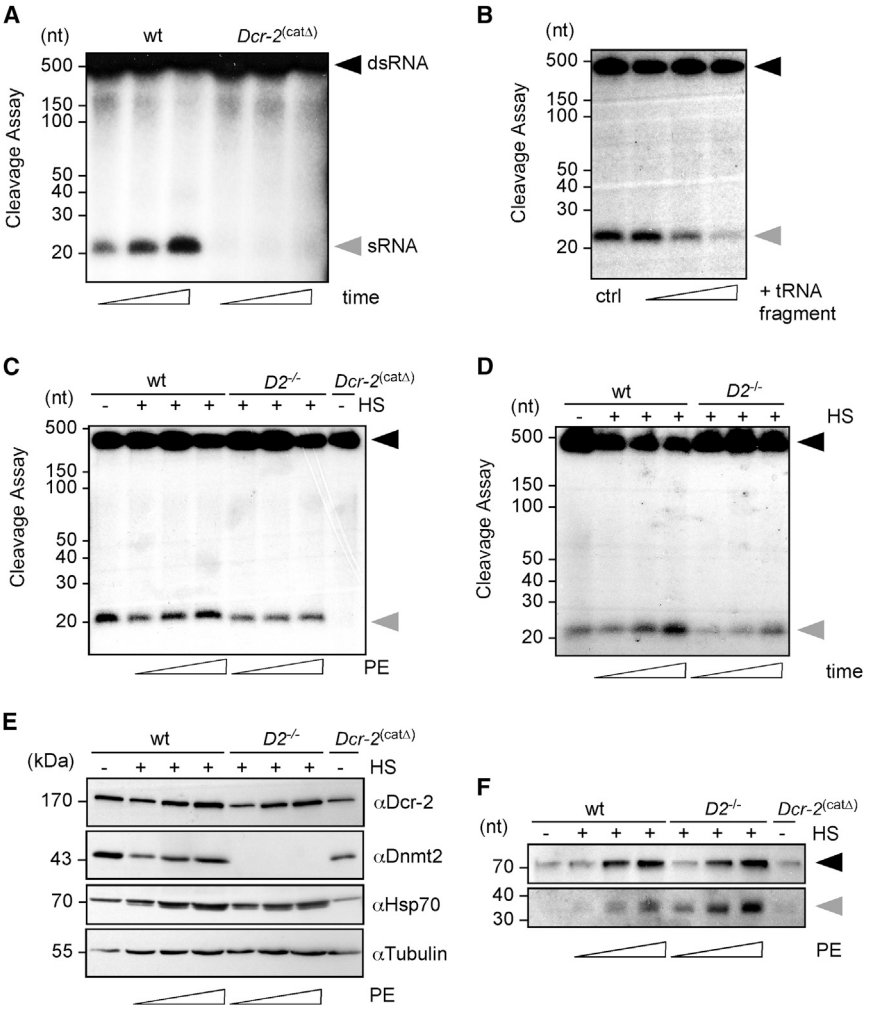

Figure 3. *Dnmt2* Mutants Show Reduced Dcr-2 Activity on Long dsRNA

(A) Dcr-2 cleavage assay on ^{32}P-labeled *egfp*-derived dsRNA (5 ng) using protein extracts (15 μg) from wild-type (WT) and *Dcr-2* catalytic mutant (*Dcr-2*^{catΔ}) ovaries. Arrowheads indicate long dsRNAs (black) and sRNA duplexes (sRNA, gray).

(B) Dcr-2 cleavage assay on *egfp*-derived dsRNAs using protein extracts (15 μg) from WT ovaries and increasing amounts of tRNA$_{Asp}^{GTC}$ oligonucleotides (3, 6, 10 μM). Arrowheads indicate RNAs as in (A).

(C) Dcr-2 cleavage assay on *egfp*-derived dsRNA using protein extracts from WT, *Dnmt2* mutant (*D2*$^{-/-}$), and *Dcr-2* catalytic mutant (*Dcr-2*^{catΔ}) ovaries before and after a heat shock (37°C, 1 hr). Increasing amounts of protein extract (7.5, 15, and 30 μg) from WT and *Dnmt2* mutants were added to the reaction to recover the heat-shock-induced decrease of Dcr-2 activity on dsRNA (see Figure S3B). Arrowheads indicate RNAs as in (A). HS, heat shock; PE, protein extract.

(D) Dcr-2 cleavage assay on *egfp*-derived dsRNA using protein extracts (15 μg) from WT and *Dnmt2* mutant (*D2*$^{-/-}$) ovaries after a heat shock (37°C, 1 hr) and increasing incubation times (1, 2, and 3 hr). Arrowheads indicate RNAs as in (A).

(E) Western blot of protein extracts (25, 50, 80 μg), which were used in Dcr-2 cleavage assays as in (C) and probed for Dcr-2, Dnmt2, Hsp70, and Tubulin levels.

(F) Northern blot of RNA (5, 10, and 15 μg) extracted from protein extracts used in Dcr-2 cleavage assays (C) with 5′ probes against tRNA$_{Asp}^{GTC}$. Arrowheads indicate full-length tRNA (black) and tRNA halves (gray).

See also Figure S3.

by expression analysis showed transiently increased *mus308* expression in controls after heat shock, supporting the notion that temporary inhibition of Dcr-2 activity caused reduced esi-2.1 production and concomitantly the derepression of *mus308* transcription. In contrast, constantly elevated *mus308* levels were observed in *Dnmt2* mutants (Figure 4F). These results are consistent with the notion that molecular inhibition of Dcr-2 activities in *Dnmt2* mutant tissues resulted in inefficient endo-siRNA production, leading to long-term derepression of *mus308*.

Dnmt2-Dependent RNA Fragments Affect siRNA Pathway-Regulated Gene Expression

Dcr-2 and *Ago-2* mutant animals are viable and fertile (Lee et al., 2004; Okamura et al., 2004), suggesting a predominant function for siRNA pathways under nonstandard conditions. Previous expression analyses showed that Dcr-2- and Ago-2-regulated genes include various stress-induced factors and DNA repair enzymes (Lim et al., 2013; Rehwinkel et al., 2006). Gene-expression analysis of a representative number of these genes using quantitative PCR (qPCR) confirmed that Dcr-2 or Ago-2 depletion from S2 cells did not significantly affect transcript levels (Figures S4A and S4B). In contrast, heat shock caused gene-expression changes in Dcr-2- and Ago-2-depleted cells during stress recovery, supporting the notion that loss-of-function phenotypes caused by Dcr-2 and Ago-2 depletion become detectable under stress conditions (Figure S4B). To test whether heat-shock-induced sRNAs (including tRNA fragments) caused these gene-expression changes, we extracted sRNAs (10–60 nt) from heat-shocked adult flies (Figures S4C) and incubated them with S2 cells. The results showed the induction of Dcr-2- and Ago-2-regulated genes by sRNAs that were derived from heat-shocked *Dnmt2* mutants but not from control flies (Figures S4D). Finally, incubation of S2 cells with specific tRNA$_{Asp}^{GTC}$ fragments also induced the expression of Dcr-2- and Ago-2-regulated genes (Figure S4E), indicating that ectopic tRNA fragments were able to mimic heat-shock-induced effects on siRNA pathway components.

DISCUSSION

siRNA pathways function to silence mobile elements and regulate mRNA expression (Carthew and Sontheimer, 2009).

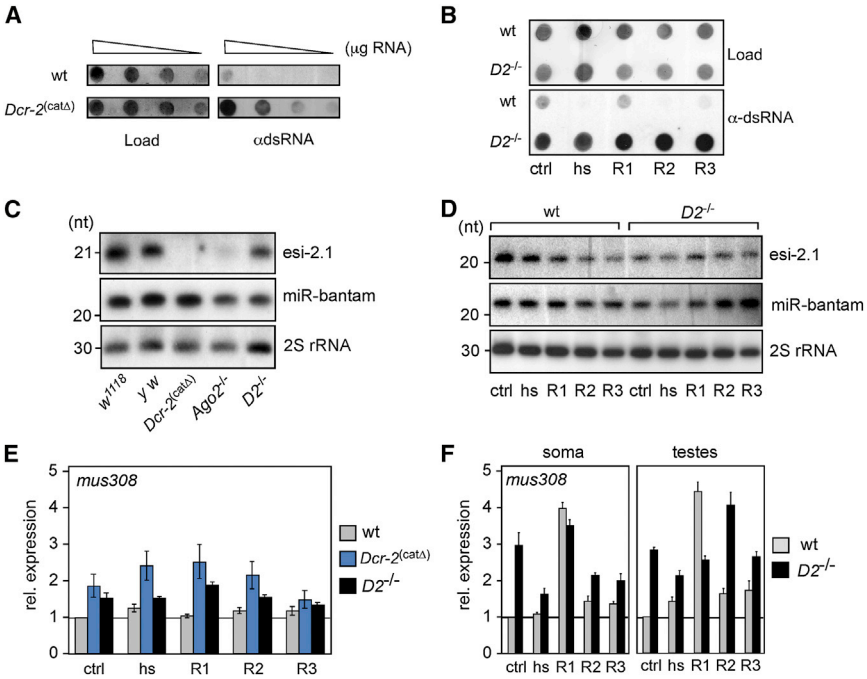

Figure 4. *Dnmt2* Mutants Accumulate DsRNAs and Show Reduced sRNA Production

(A) Dot-blot analysis of decreasing amounts of total RNA (1 μg to 125 ng) from WT and *Dcr-2* catalytic mutant (*Dcr-2*^{catΔ}) flies. Blots were stained with methylene blue for loading control, followed by probing with antibodies against dsRNA.

(B) Dot-blot analysis of total RNA (2 μg) from a heat-shock experiment with WT and *Dnmt2* mutant (*D2*$^{-/-}$) flies. Blots were stained with methylene blue to control loading, followed by probing with antibodies against dsRNA.

(C) Northern blot of RNA (15 μg) from adult WT (*w*^{1118}, *yw*), *Dcr-2*, *Ago-2*, and *Dnmt2* mutant flies using probes for esi-2.1 RNA, miR-bantam, and 2S rRNA.

(D) Northern blot of RNA (15 μg) from WT and *Dnmt2* mutant (D2$^{-/-}$) flies during a heat-shock experiment probed for esi-2.1, miR-bantam and 2S rRNA.

(E) qPCR analysis for *mus308* mRNA in adult WT, *Dcr-2* (*Dcr-2*^{catΔ}) and *Dnmt2* mutant (D2$^{-/-}$) flies during a heat-shock experiment (hs = 37°C, R = recovery in days). Three independent experiments were performed for quantification (mean ± SD).

(F) qPCR analysis for *mus308* mRNA in WT and *Dnmt2* mutant (*D2*$^{-/-}$) male soma (left) and germline tissue (right) during a heat-shock experiment as in (E). Three independent experiments were performed for quantification (mean ± SD).

See also Figure S4.

Predicted mRNA targets encode proteins with roles in cell-cycle control, DNA repair, and stress response (Czech et al., 2008; Okamura et al., 2008a), and profiling of both RNA and protein expression indicated that Ago-2 and Dcr-2 function impacts stress and metabolic processes (Rehwinkel et al., 2006; Lim et al., 2011, 2013). These observations suggest that siRNA pathways downregulate stress-related gene products under non-stress conditions. However, how such siRNA-mediated mRNA suppression is relieved during stress conditions to accommodate efficient stress responses has remained unclear.

Our results define a mechanism for the regulation siRNA pathways during the heat-shock response. Our findings show that heat shock causes a transient reduction in Dcr-2 protein levels that leads to reduced Dcr-2 activities on long dsRNA substrates, resulting in lower levels of mature sRNAs and the derepression of siRNA pathway-controlled genes. Concomitantly with the reduction of Dcr-2 activity, we observe an increase in tRNA fragmentation, which is known to contribute to stress-response mechanisms (Thompson and Parker, 2009). Dcr-2 binding to tRNA-derived sequences and Dcr-2-dependent processing of tRNA fragments into sRNAs confirms that the competition of small dsRNAs with long dsRNA substrates contributes to the transient reduction of siRNA pathway function during stress conditions. In contrast to the stress recovery in wild-type flies, *Dnmt2* mutant flies show signs of constant or prolonged siRNA pathway inhibition, and thereby resemble *Dcr-2* or *Ago-2* mutant animals, especially after heat shock. Because *Dnmt2* mutants produce more tRNA fragments during the heat-shock response, these findings strongly indicate a connection between Dnmt2-mediated tRNA stability and correct siRNA pathway function.

tRNA fragments have also been found in Ago-2 complexes under normal conditions (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008b; Cole et al., 2009). Specific tRNA fragments affected siRNA pathways in human cells (Haussecker et al., 2010), implicating tRNA fragments in the fine-tuning of sRNA-mediated gene regulation. Whether tRNA fragments could also function as bona fide miRNAs or siRNAs has been debated (Pederson, 2010), and in vivo tRNA fragment targets remain to be defined. However, matching of various tRNA fragments to human endogenous virus sequences (Li et al., 2012) suggests a putative biological role for constitutive tRNA fragmentation to target siRNA pathway activities after leaky virus expression.

Taken together, our findings suggest that tRNA fragmentation is not only required to inhibit protein synthesis but also contributes to safeguard the transient downregulation of siRNA pathways during the heat-shock response. tRNA-based interference with siRNA pathway activities might rapidly block the inhibitory effects of sRNAs on various stress-relevant mRNAs, which would facilitate the restarting of important cellular processes during the stress recovery (Durdevic and Schaefer, 2013). We conclude that an important biological role for Dnmt2 enzymes is the suppression of aberrant tRNA fragmentation to ensure the correct regulation of sRNA pathways.

EXPERIMENTAL PROCEDURES

RNA Extraction, Reverse Transcription, and qPCR

Total RNA was extracted from S2 cells, whole flies, or germline tissues (ovaries) using Trizol (Invitrogen). For first-strand cDNA synthesis, RNA was either reverse transcribed using the QuantiTect Reverse Transcription Kit

(QIAGEN) or treated with TurboDNase (Ambion) before reverse transcription using SuperScript III (Invitrogen). qPCR analyses were performed on a LightCycler 480 Real Time PCR System (Roche) using the ABsolute qPCR SYBR Green Mix (Thermo Scientific).

Northern Blotting

RNA was extracted using Trizol (Invitrogen) and separated on denaturing urea-PAGE, transferred to nylon membranes (Roche), and hybridized overnight at 30–40°C with ^{32}P-end-labeled oligonucleotides in hybridization solution (5× saline sodium citrate [SSC], 20 mM Na_2HPO_4 pH 7.4, 1% SDS, 1× Denhardt's reagent). After washing at 40°C with 1× SSC, 0.1% SDS (3× 15 min), the membranes were exposed to film at −80°C.

sRNA Sequencing

RNA extraction, library preparation, and deep sequencing are described in detail in the Extended Experimental Procedures.

tRNA and tRNA Fragment Cleavage Assay

We heated 2 μl of a 5′-end phosphorylated RNA oligonucleotide (20 μM; Sigma; see Extended Experimental Procedures) or 300 ng of gel-purified tRNA from *Dnmt2* mutant flies in water (total volume of 10 μl) to 75°C for 2 min. An equal volume of 2× reaction buffer S2 (200 mM potassium acetate [KOAc]; 10 mM dithiothreitol [DTT]; 1 mM ATP, 0.1 U/μl RNase inhibitor; Promega) was added for cleavage reactions in S2 cell extracts; an equal volume of 2 × reaction buffer (200 mM KOAc; 30 mM HEPES-KOH, pH 7.4; 6 mM $Mg(OAc)_2$; 10 mM DTT; 0.5% glycerol, 60 μg/ml creatine kinase, 2 mM creatine phosphate, 2 mM ATP, 0.1 U/μl RNase inhibitor; Promega) was added for cleavage reactions in ovary extracts, followed by incubation for 10 min at 37°C. Reactions were assembled by adding hypotonic protein extracts (15 μg total protein) in the respective reaction buffer (final reaction volume 30–50 μl). Reactions were incubated for 1 hr at 25°C. RNA was recovered using phenol/chloroform extraction and analyzed by denaturing urea-PAGE (15%) and northern blotting.

dsRNA Cleavage Assay

For cleavage reactions, 5–10 ng of a ^{32}P-end-labeled *egfp*-derived dsRNA was incubated in 1× reaction buffer (100 mM KOAc; 15 mM HEPES-KOH, pH 7.4; 3 mM $Mg(OAc)_2$; 5 mM DTT; 0.25% glycerol, 30 μg/ml creatine phosphate kinase, 1 mM creatine phosphate, 1 mM ATP, 0.1 U/μl RNase inhibitor; Promega), and 7.5–30 μg ovary protein extract at 29°C for the indicated times. Reactions were stopped by adding 1 vol of 2× RNA loading dye (Thermo Scientific). Samples were heated to 80°C and subjected to denaturing urea-PAGE (15%). Gels were blotted onto nylon membranes (Roche) and membranes were exposed to film at −80°C.

ACCESSION NUMBERS

The sRNA sequencing data have been deposited in the GEO database under the accession number GSE35981.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.07.046.

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