RESEARCH COMMUNICATION

RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage

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Dnmt2 proteins are the most conserved members of the DNA methyltransferase enzyme family, but their substrate specificity and biological functions have been a subject of controversy. We show here that, in addition to $tRNA^{Asp\text{-}GTC}$, $tRNA^{Val\text{-}AAC}$ and $tRNA^{Gly\text{-}GCC}$ are also methylated by Dnmt2. *Drosophila* Dnmt2 mutants showed reduced viability under stress conditions, and Dnmt2 relocalized to stress granules following heat shock. Strikingly, stress-induced cleavage of tRNAs was Dnmt2-dependent, and Dnmt2-mediated methylation protected tRNAs against ribonuclease cleavage. These results uncover a novel biological function of Dnmt2-mediated tRNA methylation, and suggest a role for Dnmt2 enzymes during the biogenesis of tRNA-derived small RNAs.

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The covalent modification of nucleic acids plays an important role in regulating the functions of DNA and RNA. DNA modifications have been analyzed in considerable detail, and the characterization of (cytosine-5) DNA methylation has been crucial for understanding the molecular basis of epigenetic gene regulation (Klose and Bird 2006). (Cytosine-5) methylation has also been documented in various RNA species, including tRNA, but the function of RNA methylation has not been firmly established yet (Motorin et al. 2010).

Dnmt2 proteins were originally assigned to the DNA methyltransferase family, because of their strong sequence conservation of catalytic DNA methyltransferase motifs (Okano et al. 1998; Yoder and Bestor 1998). A recent study has suggested that Dnmt2-mediated DNA methylation is important for transposon silencing in *Drosophila* (Phalke et al. 2009). However, only a weak and distributive DNA methylation activity has been reported in various systems (Jeltsch et al. 2006).

The ambiguities associated with the DNA methyltransferase activity of Dnmt2 have also prompted the search for alternative enzyme substrates, and resulted in the discovery of a tRNA methyltransferase activity of Dnmt2 (Goll et al. 2006). Purified recombinant human Dnmt2 methylated RNA preparations from Dnmt2 mutant mice, flies, and plants. Further experiments identified C38 in the anti-codon loop of $tRNA^{Asp}$ as the methylation target site of Dnmt2 (Goll et al. 2006). However, the functional relevance of the tRNA methyltransferase activity of Dnmt2 remains to be established. Dnmt2 mutant mice, flies, and plants were reported to be viable and fertile (Goll et al. 2006) under standard laboratory conditions. A distinct Dnmt2 mutant phenotype, caused by morpholino knockdown experiments, has so far been reported only in zebrafish, leading to lethal differentiation defects in the retina, liver, and brain (Rai et al. 2007). In addition, two studies have indicated increased stress tolerance in Dnmt2-overexpressing flies and amoebas (Lin et al. 2005; Fisher et al. 2006). However, the underlying molecular mechanisms have not been investigated yet.

Results and Discussion

The *Drosophila* Dnmt2 locus (chromosome 2, 33C4) gives rise to two transcripts: Dnmt2-RC and Dnmt2-RA. The predicted protein products Dnmt2-PC and Dnmt2-PA differ in their N termini by a peptide stretch of 21 amino acids (Supplemental Fig. S1A), which eliminates the catalytic DNA methyltransferase motif I from the putative Dnmt2-PA protein. Both transcripts were detectable during all stages of development and in adult flies (Fig. 1A), suggesting a general function throughout development.

In order to establish a mutant allele that causes the loss of both transcripts, we generated a deletion in the genomic Dnmt2 locus by mobilizing the P-element EP(2)GE15695, which is located upstream of the Dnmt2 coding sequence (Jurkowski et al. 2008). Since the P-element insertion mapped to the promoter region of both Dnmt2 and the adjacent CG6712 locus, we performed an imprecise excision screen for deletions that allowed the expression of CG6712, but deleted parts of the coding region of Dnmt2. One of the excision alleles generated ($Dnmt2^{99}$) contained a proximal deletion of the EP element and fused the remnant of the EP element to the 3′ half of the Dnmt2 coding region, thereby deleting large parts of the Dnmt2 gene, including motifs I–VII and part of motif VIII (Supplemental Fig. S1B). Quantitative PCR analysis showed that both transcripts generated from the Dnmt2 locus were undetectable in homozygous $Dnmt2^{99}$ mutants (Supplemental Fig. S1C). Dnmt2 protein could not be detected in embryonic protein extracts from homozygous $Dnmt2^{99}$ mutants, but was detectable as a 40-kDa polypeptide in wild-type embryos using Dnmt2-specific antibodies (Supplemental Fig. S1D). These results strongly suggested that $Dnmt2^{99}$ represents a null allele suitable for further analysis.

To investigate whether Dnmt2 methylates additional tRNAs, we used RNA bisulfite sequencing, a recently

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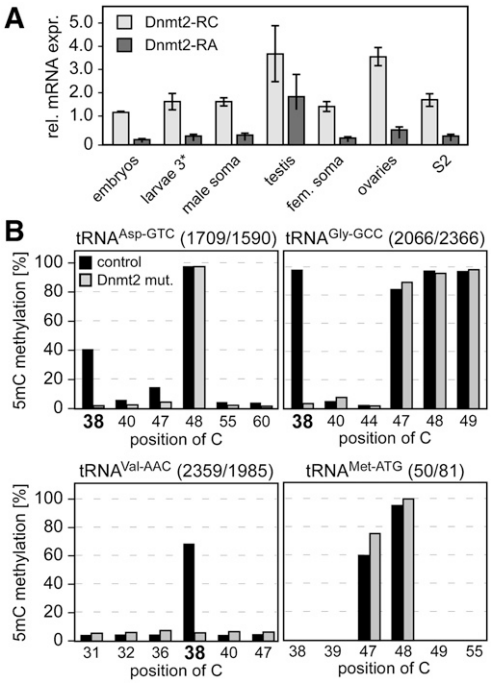

Figure 1. Dnmt2 is a multisubstrate tRNA methyltransferase. (*A*) Quantitative PCR analysis of Dnmt2 mRNA expression in *Drosophila* tissues. Expression was normalized to rp49 levels. (*B*) Identification of novel Dnmt2 substrate tRNAs. Deep bisulfite sequencing analysis of various tRNAs in wild-type (control) and Dnmt2 mutant (Dnmt2 mut.) flies. Numbers of sequence reads are indicated at the *top* of each panel. Diagrams show fractions of nondeaminated cytosines, thus revealing the pattern of m5C modification in individual tRNAs. Cytosines that were found to be methylated in a Dnmt2-dependent manner are C38 in $tRNA^{Asp\text{-}GTC}$, $tRNA^{Val\text{-}AAC}$, and $tRNA^{Gly\text{-}GCC}$ (highlighted by bold numbers).

established method that allows the direct analysis of RNA methylation patterns in their native sequence context (Schaefer et al. 2009). Obvious candidate substrates were tRNAs that also contain a cytosine residue at position 38, assuming that substrate recognition might be similar to $tRNA^{Asp}$. Among these, $tRNA^{Gly}$ and $tRNA^{Val}$ warranted particular consideration, because these RNAs have been reported to be methylated at C38 (Garel and Keith 1977; Addison et al. 1982).

Using next-generation sequencing of PCR amplicons derived from bisulfite-treated total RNA, we obtained sequence information for tRNAs from adult Dnmt2 wild-type and *Dnmt2*^{99} mutant animals. The analysis revealed robust methylation of C38 in $tRNA^{Asp\text{-}GTC}$ from control flies ($Dnmt2^{rev}$). In contrast, only background methylation levels were observed in $tRNA^{Asp\text{-}GTC}$ from *Dnmt2*^{99} mutant flies (Fig. 1B). These results confirmed previous RNA bisulfite sequencing data for $tRNA^{Asp\text{-}GTC}$ (Schaefer et al. 2009). Similarly, C38 of $tRNA^{Gly\text{-}GCC}$ and $tRNA^{Val\text{-}AAC}$ were strongly methylated in control flies, and methylation was close to background levels in *Dnmt2*^{99} mutants (Fig. 1B). Dnmt2-dependent $tRNA^{Val}$ methylation was also confirmed by site-specific, DNAzyme-mediated cleavage, and analysis by thin-layer chromatography (Hengesbach et al. 2008), as well as by a biochemical methylation assay using purified recombinant human Dnmt2 (Supplemental Fig. S2). Together, these results demonstrate that the tRNA methyltransferase activity of Dnmt2 is not limited to C38 of $tRNA^{Asp\text{-}GTC}$, and that Dnmt2 can methylate additional tRNAs both in vitro and in vivo.

Methylation changes in *Dnmt2*^{99} mutants were observed only at C38, and the methylation status of other cytosine residues in the tRNA fragments investigated did not become detectably altered in *Dnmt2*^{99} mutants (Fig. 1B). A certain level of enzyme specificity was also supported by our findings that other C38-containing tRNAs—including $tRNA^{Met\text{-}ATG}$, $tRNA^{Glu\text{-}CTC}$, and $tRNA^{His\text{-}GTG}$—were not detectably methylated in a Dnmt2-dependent manner (Fig. 1B; data not shown).

Dnmt2^{99} mutant flies were viable and fertile under standard laboratory conditions, as described for other mutant alleles (Goll et al. 2006; Jurkowski et al. 2008). Because a previous study suggested that Dnmt2-overexpressing flies showed increased stress tolerance (Lin et al. 2005), the viability of *Dnmt2*^{99} animals was analyzed under thermal stress conditions. When flies were raised continuously at 29°C (4°C above standard temperature), *Dnmt2*^{99} mutants showed a reduced mean lifespan (25 d) when compared with $Dnmt2^{rev}$ controls (30 d) (Fig; 2A). Importantly, transgenic flies carrying a genomic copy of the Dnmt2 locus in the *Dnmt2*^{99} background ($Dnmt2^{genTG}$) showed a partially or fully restored lifespan (Fig. 2A), indicating that this phenotype is Dnmt2-dependent.

In order to examine a potential function of Dnmt2 in oxidative stress responses, adult male flies were exposed to paraquat, and surviving flies were counted daily. The results showed that the paraquat sensitivity of *Dnmt2*^{99}

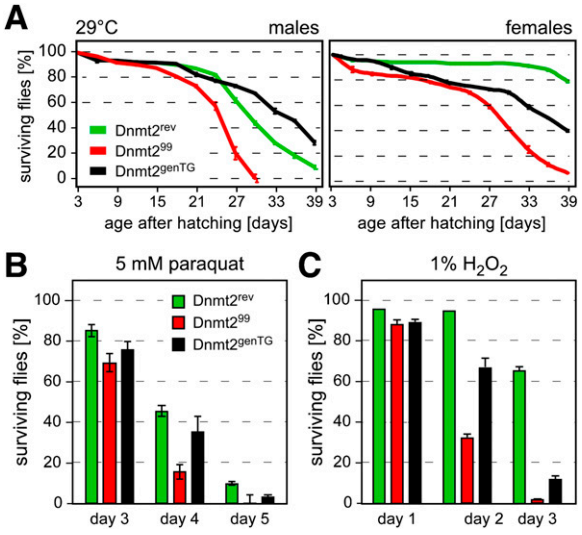

Figure 2. Loss of Dnmt2 causes increased stress sensitivity. (*A*) Viability plot of $Dnmt2^{99}$ mutant, control ($Dnmt2^{rev}$), and transgenic rescue ($Dnmt2^{genTG}$) flies reared at elevated temperature (29°C). For each genotype and sex, the survival of 20 flies (3 d old) was counted over a period of 39 d. Error bars represent standard deviations from five independent experiments. (*B*) Dnmt2 mutant flies show elevated sensitivity to paraquat. For each genotype, 20 male or female adult flies were exposed to paraquat, and live flies were counted for 5 d. Error bars represent standard deviations from five independent experiments. (*C*) Dnmt2 mutant flies show elevated sensitivity to H_2O_2. Twenty male or female adult flies were exposed to 1% H_2O_2, and live flies were counted for 3 d. Error bars represent standard deviations from five independent experiments.

mutant flies was substantially higher than that of control flies ($Dnmt2^{rev}$) after exposure to 5 mM paraquat (Fig. 2B). $Dnmt2^{genTG}$ showed a robust rescue effect (Fig. 2B), confirming that the increased sensitivity to paraquat is Dnmt2-dependent. Exposing flies to another oxidizing agent, H_2O_2, showed that *Dnmt2*^{99} mutant flies were also more sensitive to H_2O_2 than control flies ($Dnmt2^{rev}$). The increased sensitivity to H_2O_2 was partially rescued when $Dnmt2^{genTG}$ flies were analyzed (Fig. 2C), confirming that the response to H_2O_2 is mediated by Dnmt2. The appearance of Dnmt2 mutant phenotypes under stress conditions implies that Dnmt2 is associated with *Drosophila* stress response pathways.

Having identified a role of Dnmt2 in stress responses, we analyzed the association of the protein with cellular stress compartments. To analyze the subcellular localization of Dnmt2 under stress conditions, we used flies expressing EGFP-tagged Dnmt2 from a genomic construct in the *Dnmt2*^{99} background ($Dnmt2^{genTG\text{-}EGFP}$). Activity of the tagged Dnmt2 construct was confirmed by bisulfite sequencing of tRNA from adult males, which showed that C38 methylation of $tRNA^{Asp\text{-}GTC}$ and $tRNA^{Gly\text{-}GCC}$ was restored to wild-type levels (Supplemental Fig. S3). Methylation was not rescued for $tRNA^{Val\text{-}AAC}$ (Supplemental Fig. S3), which indicates that the transgenic construct does not completely restore Dnmt2 expression or functionality in all tissues or developmental stages. These findings are consistent with the incomplete rescue of stress phenotypes observed with tagged genomic constructs (see Fig. 2). Ovaries from $Dnmt2^{genTG\text{-}EGFP}$ flies showed a homogeneous, mostly cytoplasmic distribution of Dnmt2-EGFP, which aggregated into discernable granular loci after heat shock. This increase in granular Dnmt2-EGFP signal was observed in both live and fixed ovarian tissue (Supplemental Fig. S4), thus excluding fixation artifacts. Similar results were also obtained with *Drosophila* S2 cells stably expressing Dnmt2-EGFP, and pronounced Dnmt2-EGFP-positive granules became visible after heat shock (Fig. 3A).

To confirm the association of Dnmt2 with stress-related subcellular compartments, we costained heat-shocked S2 cells for Dnmt2-EGFP and fragile X mental retardation 1 (FMR1), a ubiquitious RNA-binding protein that relocalizes to stress granules in *Drosophila* (Farny et al. 2009). The results showed that part of the Dnmt2-EGFP signal clearly colocalized with FMR1 loci (Fig. 3A). A distinct colocalization between Dnmt2-EGFP and FMR1 after heat shock was also observed in ovaries (Fig. 3B). Staining of $Dnmt2^{genTG\text{-}EGFP}$ ovaries with an antibody to ME31B, a *Drosophila* RNA processing body component in the germline (Lin et al. 2006), also showed processing body signals after heat shock with strong colocalization of Dnmt2 and ME31B (Fig. 3C). Together, these data illustrate the ability of Dnmt2 to localize to stress granules and RNA processing bodies following heat shock, and thus further illustrate the association between the subcellular localization of Dnmt2 and stress response pathways.

Stress granules and processing bodies contain high numbers of RNA molecules (Anderson and Kedersha 2009), and heat-shock stress responses have been linked to changes in RNA methylation in bacteria (Bugl et al. 2000). Interestingly, tRNA cleavage in the anticodon loop has been identified recently as a molecular mechanism associated with eukaryotic stress responses

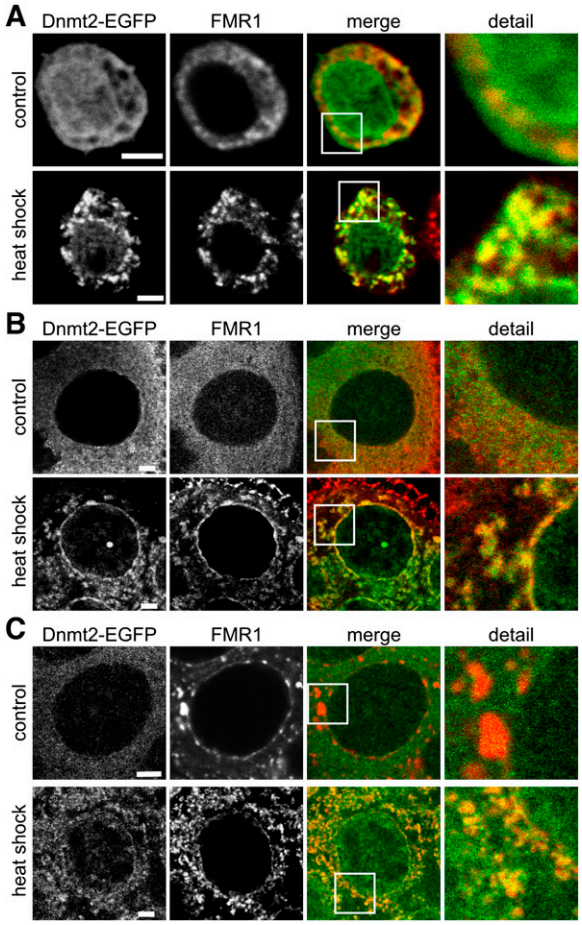

Figure 3. Dnmt2 is associated with stress compartments. (*A*) Immunofluorescence analysis of FMR1 in S2 cells expressing Dnmt2-EGFP. FMR1 localizes to the cytoplasm, whereas Dnmt2-EGFP is localized ubiquitiously to the cytoplasm and nucleus in control cells. Upon heat shock (1 h at 40°C), FMR1 concentrates in large cytoplasmic granules where the signal colocalizes with granular Dnmt2-EGFP. (*B*) Similar observations were made in ovaries after heat shock. (*C*) Immunofluorescence analysis of the P-body-associated protein ME31B on fixed Dnmt2-EGFP ovaries. After heat shock, the number of ME31B foci increased, and colocalization with Dnmt2-EGFP structures could be observed. Bars: *A*, 10 μM; *B*,*C*, 1 μM.

(Thompson et al. 2008; Yamasaki et al. 2009). We therefore established whether tRNA cleavage under thermal and oxidative stress conditions is conserved for Dnmt2 substrates and in *Drosophila*. Using *Drosophila* S2 cells, we investigated the cleavage of $tRNA^{Gly\text{-}GCC}$ as a representative Dnmt2 substrate. Northern analysis showed the rapid appearance of $tRNA^{Gly\text{-}GCC}$ fragments following heat shock (Fig. 4A), and also indicated $tRNA^{Gly\text{-}GCC}$ cleavage following oxidative stress by H_2O_2 and arsenite, respectively (Fig. 4B). A slight, heat-shock-dependent increase of $tRNA^{Gly\text{-}GCC}$ fragments was also observed by Northern analysis of RNA prepared from ovaries (Fig. 4C). Notably, this effect became much more pronounced when $tRNA^{Gly\text{-}GCC}$ cleavage was analyzed in ovaries from aged *Dnmt2*^{99} mutant females (Fig. 4C), thus suggesting a role of Dnmt2 in the regulation of tRNA cleavage. To confirm these findings, we used transgenic S2

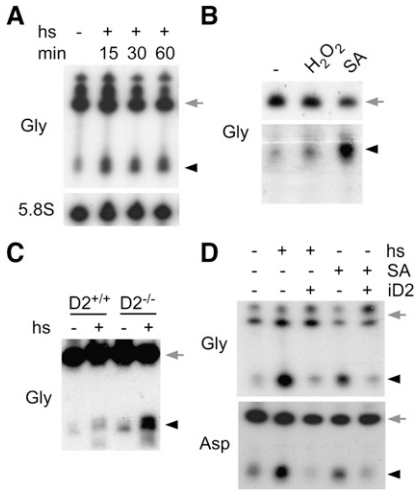

Figure 4. Stress-induced cleavage of Dnmt2 substrate tRNAs. (*A*) Northern analysis of $tRNA^{Gly\text{-}GCC}$ cleavage in S2 cells that were heat-shocked (1 h at 40°C) for the times (in minutes) indicated. 5.8S rRNA was used as a loading control. (*B*) Cleavage of $tRNA^{Gly\text{-}GCC}$ in S2 cells that were treated with H_2O_2 (1 mM H_2O_2, 60 min) or sodium arsenite (0.5 mM SA, 90 min). (*C*) Cleavage of $tRNA^{Gly\text{-}GCC}$ in wild-type ($D2^{+/+}$) or Dnmt2 mutant ($D2^{-/-}$) ovaries (resected 35 d after hatching) that were heat-shocked (+) in medium at 40°C. Controls (−) show results from parallel experiments with ovary incubation at 25°C. (*D*) Northern analysis of $tRNA^{Gly\text{-}GCC}$ and $tRNA^{Asp\text{-}GTC}$ cleavage in S2 cells that allow inducible overexpression of Dnmt2 (iD2). Full-length tRNAs are marked by gray arrows, and tRNA fragments are marked by black arrowheads.

cells that allow inducible overexpression of Dnmt2. When these cells were subjected to heat shock or oxidative stress, Northern blot analysis showed the stress-dependent appearance of $tRNA^{Gly\text{-}GCC}$ fragments (Fig. 4D). Ectopic expression of Dnmt2 substantially decreased the amount of detectable cleavage products (Fig. 4D). Essentially similar results were also obtained when analyzing $tRNA^{Asp\text{-}GTC}$ (Fig. 4D). Together, these findings indicate that either Dnmt2 binding to tRNAs or ectopic Dnmt2-mediated tRNA methylation protected $tRNA^{Gly\text{-}GCC}$ and $tRNA^{Asp\text{-}GTC}$ molecules from cleavage.

The RNase A-like ribonuclease angiogenin has been shown recently to mediate stress-induced tRNA cleavage in human cells (Yamasaki et al. 2009). Since the endonuclease targeting the anti-codon loop of *Drosophila* tRNAs has not been identified yet, we treated *Drosophila* S2 cells with recombinant human angiogenin and analyzed cleavage of $tRNA^{Asp\text{-}GTC}$ and $tRNA^{Gly\text{-}GCC}$ by Northern blot analysis. The results clearly showed that exogenous angiogenin induces the generation of small tRNA fragments (Fig. 5A). Importantly, Dnmt2 overexpression protected Dnmt2 substrate tRNAs from angiogenin cleavage, while $tRNA^{Met\text{-}ATG}$, which is not a substrate of Dnmt2, remained unaffected by Dnmt2 overexpression (Fig. 5A). These experiments indicated that Dnmt2 functions to protect against angiogenin-mediated tRNA cleavage.

To test whether Dnmt2-mediated tRNA methylation at C38 inhibits angiogenin-catalyzed tRNA cleavage, we purified tRNAs from wild-type and Dnmt2 mutant embryos, and incubated these RNAs with recombinant angiogenin in a biochemical assay. Northern blot analysis showed very low levels of angiogenin cleavage of $tRNA^{Asp\text{-}GTC}$, $tRNA^{Gly\text{-}GCC}$, and $tRNA^{Val\text{-}AAC}$ when the tRNAs were isolated from wild-type embryos (Fig. 5B). In contrast, all three Dnmt2 substrates were cleaved efficiently when the tRNAs were purified from Dnmt2 mutant embryos (Fig. 5B). The cleavage kinetics on $tRNA^{Met\text{-}ATG}$ was not affected by the genotype of the RNA preparation (Fig. 5B), which confirmed that C38 methylation functions to protect Dnmt2 substrate tRNAs from endonucleolytic cleavage. While small RNA sequencing studies have indicated cleavage sites in the vicinity of the Dnmt2 target nucleotide C38 (Kawaji et al. 2008; Fu et al. 2009), angiogenin is also known to be a ribonuclease with comparably low specificity (Rybak and Vallee 1988). We therefore determined the cleavage sites by sequencing of 5′-tRNA fragments from $tRNA^{Asp\text{-}GTC}$, $tRNA^{Gly\text{-}GCC}$, and $tRNA^{Val\text{-}AAC}$. The results showed cleavage at various positions in the anticodon stem–loop (Supplemental Fig. S5), suggesting an effect of C38 methylation on the structure and endonuclease accessibility of this tRNA region.

Because of the remarkable evolutionary conservation of Dnmt2, we sought to confirm the function of Dnmt2 in endonuclease cleavage protection in a different model

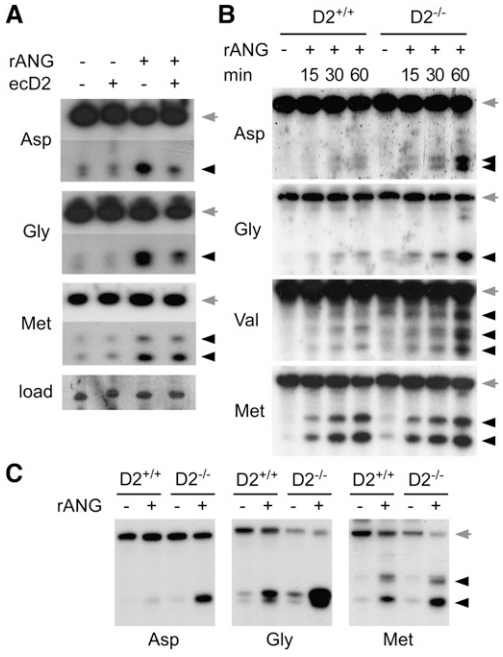

Figure 5. Dnmt2-mediated tRNA methylation inhibits endonucleolytic cleavage by angiogenin. (*A*) Dnmt2 overexpression (ecD2) inhibits angiogenin-induced tRNA cleavage. tRNA cleavage was induced in S2 cells by the addition of recombinant angiogenin (rANG) to the medium (1 μg/mL), and was analyzed by Northern blotting. Full-length tRNAs (gray arrows) and cleavage products (black arrowheads) are shown from the same original blot. (*B*) Dnmt2 substrate tRNAs isolated from wild-type embryos ($D2^{+/+}$) are protected against angiogenin cleavage in vitro. No protection effect was observed with Dnmt2 substrate tRNAs isolated from Dnmt2 mutant embryos ($D2^{-/-}$), or with $tRNA^{Met\text{-}ATG}$, which is not a Dnmt2 substrate. (*C*) Dnmt2-mediated protection against angiogenin cleavage is conserved in mice. tRNA was purified from wild-type ($D2^{+/+}$) or Dnmt2 mutant ($D2^{-/-}$) MEFs, incubated with recombinant angiogenin (1 μM, 60 min), and analyzed by Northern blotting. Full-length tRNAs are marked by gray arrows, and tRNA fragments are marked by black arrowheads.

system. To this end, we derived embryonic fibroblasts from wild-type and Dnmt2 homozygous mutant mice (Goll et al. 2006). tRNAs were isolated from both cell lines, and Dnmt2-dependent $tRNA^{Asp\text{-}GTC}$ methylation at C38 was confirmed by RNA bisulfite sequencing (Supplemental Fig. S6). Purified tRNAs from wild-type and Dnmt2 mutant mouse embryonic fibroblasts (MEFs) were incubated with recombinant angiogenin, and tRNA cleavage of $tRNA^{Asp\text{-}GTC}$, $tRNA^{Gly\text{-}GCC}$, and $tRNA^{Met\text{-}ATG}$ was analyzed by Northern blot. The results showed a clear protection against angiogenin cleavage for the Dnmt2 substrates $tRNA^{Asp\text{-}GTC}$ and $tRNA^{Gly\text{-}GCC}$ if derived from Dnmt2 mutant MEFs, but not for $tRNA^{Met\text{-}ATG}$ (Fig. 5C). These data provide important confirmation for a role of Dnmt2-mediated tRNA methylation in protection from endonucleolytic cleavage.

The lack of phenotypic and molecular data has been a major obstacle in defining the biological function of Dnmt2. The distributive Dnmt2-mediated DNA methylation that was reported in several studies (Jeltsch et al. 2006) has been difficult to interpret as biologically relevant. Similarly, the methylation of a single cytosine residue in a single tRNA made it difficult to envisage a fundamental biological role for Dnmt2 enzymes. However, the initial assumption that Dnmt2 activity is restricted to $tRNA^{Asp}$ was based largely on a low-resolution analysis of cellular RNAs (Goll et al. 2006). The recent development of RNA bisulfite sequencing (Schaefer et al. 2009), and the use of methods for the purification and site-specific methylation analysis of defined tRNAs (Hengesbach et al. 2008) allowed us to identify additional tRNAs that were methylated by Dnmt2 in *Drosophila*, thus providing evidence for multisubstrate methyltransferase activity of Dnmt2. Multisubstrate specificity has been described for other RNA modification enzymes, including pseudouridine synthase 1 (Behm-Ansmant et al. 2006), and, significantly, TRM4, the only other characterized cytosine-5 tRNA methyltransferase (Motorin and Grosjean 1999). Considering that TRM4 methylates minisubstrates as small as 40 nucleotides, it remains a possibility that other unidentified substrate RNAs (e.g., small regulatory RNAs) might be methylated by RNA methyltransferases such as Dnmt2. Furthermore, since we were unable to reproduce Dnmt2-dependent DNA methylation of Invader4 transposons in *Drosophila*, it remains possible that the reported role of Dnmt2 in transposon silencing is mediated by the RNA methyltransferase activity of the enzyme (M Schaefer and F Lyko, in prep.).

It has been established recently that ribonucleases play an important role in the stress-induced cleavage of tRNAs (Yamasaki et al. 2009), a mechanism that promotes the assembly of stress granules (Emara et al. 2010). Our results suggest that endonucleolytic cleavage of Dnmt2 substrate tRNAs can be inhibited by the methylation mark at C38. While it is known that the mcm^5s^2 modification of U34 can promote the cleavage of certain yeast tRNAs by the *Kluveromyces lactics* γ-toxin endonuclease (Lu et al. 2008), our findings represent the first example for the inhibition of ribonucleases by cytosine-5 RNA methylation.

Defined tRNA fragments have been identified through small RNA cloning approaches (Kawaji et al. 2008; Cole et al. 2009; Haussecker et al. 2010), suggesting a physiological role for tRNA fragments. Interestingly, tRNA fragments can be processed to smaller RNAs by the Dicer RNase, indicating a potential interaction of tRNA-derived sequences and canonical small RNA processing pathways (Cole et al. 2009). Indeed, a recent study provided evidence that tRNA-derived small RNAs act to down-regulate target mRNAs, and affect different RNA silencing pathways by associating with effector core components (Haussecker et al. 2010). This suggests that altered tRNA cleavage (e.g., under stress conditions) could have phenotypic consequences.

Materials and methods

tRNA bisulfite sequencing

Total RNA isolation and bisulfite conversions were carried out as described before (Schaefer et al. 2009). Primer sequences are indicated in Supplemental Table S1. Amplicons were analyzed on a GS FLX 454 sequencer (Roche). Bioinformatic analysis was performed using a mapping script for individual bar-coded reads developed in Biopython.

Phenotype analysis

For survival assays, 20 flies were maintained on standard medium at 25°C or 29°C, 60% humidity, under a 12-h light–dark cycle, and were transferred to new medium every 3 d. Mortality was scored at transfer times. Twenty flies were tested for each genotype in five biological replicates. Paraquat (methyl viologen) and H_2O_2 toxicity assays were performed on 2-d-old animals placed in vials (20 per vial) with food media made of 1.3% agar containing 1% sucrose and 5 mM paraquat (Sigma) or 1% H_2O_2, respectively. Surviving animals were scored daily. Twenty flies were tested for each genotype in five biological replicates.

tRNA cleavage assays

S2 cells containing pRmHA3-Dnmt2-Flag constructs were induced with 0.7 mM $CuSO_4$, chased with medium without copper for 5 h, and treated with 1 μg/mL angiogenin (R&D Systems) for 1 h at 25°C. RNA was extracted and separated on 15% urea-PAGE, transferred to Nytran SuperCharge membranes (Schleicher and Schuell Bioscience), and hybridized overnight at 47°C with ^{32}P-end-labeled oligonucleotides (detecting both 5′ and 3′ ends of tRNAs) in hybridization solution (5× SSC, 20 mM Na_2HPO_4 at pH 7.4, 7% SDS, 1× Denhardt's). After washing with 3× SSC/5% SDS (15 min at 47°C) and with 1× SSC/1% SDS (15 min at room temperature), membranes were exposed to film at −80°C. For in vitro cleavage assays, 1.3 μg of urea-PAGE-purified tRNA was heated in water for 5 min at 80°C, followed by addition of cleavage buffer (30 mM Hepes at pH 6.8, 30 mM NaCl, 10 mM $MgCl_2$). tRNA was allowed to renature for 15 min to room temperature. BSA (to 0.001%) and angiogenin (to 1 μM) were added, and the reaction was incubated for the indicated times at 37°C, followed by separation on 15% urea-PAGE, blotting, and hybridization as described above.

MEFs

MEFs were derived from day 13.5 isogenic $Dnmt2^{-/-}$ (Goll et al. 2006) and wild-type embryos. Single embryos were genotyped, dispersed, and trypsinized in a 10-cm-diameter dish. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and a commercial cocktail of antibiotics (Invitrogen), and were passaged serially according to the 3T3 protocol (Todaro and Green 1963) until spontaneus immortalization.

See the Supplemental Material for additional details.

Acknowledgments

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