Novel small Cajal-body-specific RNAs identified in Drosophila: probing guide RNA function

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ABSTRACT
The spliceosomal small nuclear RNAs (snRNAs) are modified post-transcriptionally by introduction of pseudouridines and 2′-O-methyl modifications, which are mediated by box H/ACA and box C/D guide RNAs, respectively. Because of their concentration in the nuclear Cajal body (CB), these guide RNAs are known as small CB-specific (sca) RNAs. In the cell, scaRNAs are associated with the WD-repeat protein WDR79. We used coimmunoprecipitation with WDR79 to recover seven new scaRNAs from Drosophila cell lysates. We demonstrated concentration of these new scaRNAs in the CB by in situ hybridization, and we verified experimentally that they can modify their putative target RNAs. Surprisingly, one of the new scaRNAs targets U6 snRNA, whose modification is generally assumed to occur in the nucleolus, not in the CB. Two other scaRNAs have dual guide functions, one for an snRNA and one for 28S rRNA. Again, the modification of 28S rRNA is assumed to take place in the nucleolus. These findings suggest that canonical scaRNAs may have functions in addition to their established role in modifying U1, U2, U4, and U5 snRNAs. We discuss the likelihood that processing by scaRNAs is not limited to the CB.

Keywords: Cajal body; RNA modification; scaRNA; WDR79 protein

INTRODUCTION
Post-transcriptional modifications in spliceosomal small nuclear RNAs (snRNAs) and ribosomal RNAs (rRNAs) are essential for spliceosome assembly and splicing competency (Yu et al. 1998; Donmez et al. 2004; Zhao and Yu 2004, 2007; Yang et al. 2005; Karijolich and Yu 2010) as well as for the assembly of functional ribosomal subunits and the efficiency and fidelity of translation (Lapeyre 2005; Liang et al. 2007, 2009; Esguerra et al. 2008; Jack et al. 2011). Two common alterations in these RNAs, namely, pseudouridylation and 2′-O-methylation, are usually mediated by the so-called modification guide RNAs, the box H/ACA and box C/D RNAs, respectively. For review of the discovery and general features of guide RNAs, see Maxwell and Fournier (1995), Smith and Steitz (1997), and Yu et al. (2005). These RNAs were typically identified in isolated nucleoli or nucleolar extracts and were called small nucleolar RNAs, or snoRNAs. Because of the colocalization of snoRNAs with newly transcribed rRNAs in the nucleolus, it was assumed that the modifications themselves take place in the nucleolus. The first guide RNAs for spliceosomal snRNA modification, namely, for U6 snRNA 2′-O-methylation, were also identified in a nucleolar fraction. Furthermore, one of these guide RNAs was found to modify both U6 snRNA and 28S rRNA (Tycowski et al. 1998). Later experiments showed that U6 snRNA fragments could be modified when they were specifically targeted to the nucleolus (Ganot et al. 1999).

When guide RNAs for 2′-O-methylation and pseudouridylation of U1, U2, U4, and U5 snRNAs were discovered, they were found to concentrate not in the nucleolus but in another nuclear organelle, the Cajal body (CB). Because of their localization, these guide RNAs were referred to as small CB-specific RNAs, or scaRNAs (Darzacq et al. 2002; Kiss et al. 2002, 2004; Richard et al. 2003). Detailed analysis of scaRNA structure revealed short motifs that mediate the targeting of scaRNAs to CBs. These CB-specific localization signals are referred to as CAB boxes (Richard et al. 2003; Tycowski et al. 2009). CBs themselves were first described in neurons more than 100 years ago (Cajal 1903). In recent years, they have been found in a wide variety of tissue and cell types in all eukaryotic species that have been carefully examined. CBs are usually recognized by a high concentration of coilin, a marker protein of unknown function. Along with coilin and scaRNAs, CBs are enriched for splicing snRNAs and the core components for modification guide RNP assembly (Liu et al. 2009; Nizami et al. 2010a). The accumulation of snRNAs and their...
corresponding guide RNAs in the same nuclear organelle immediately led to the idea that U1, U2, U4, and U5 snRNAs are modified in the CB. Indeed, fragments of U2 and U5 snRNAs became modified when they were targeted to CBs, but not when targeted to nucleoli (Jády et al. 2003). In contrast, U6 snRNA, whose known guide RNAs concentrate in nucleoli (Tycowski et al. 1998; Ganot et al. 1999; Darzacq et al. 2002), could not be modified when targeted to CBs (Jády et al. 2003). The postulate that snRNA modification requires targeting to CBs became untenable when it was shown that snRNA modification still takes place in mutant cells that lack CBs (Deryusheva and Gall 2009; Nizami et al. 2010b; Deryusheva et al. 2012). Furthermore, snRNAs can be efficiently modified in vitro in cell-free systems (Jády and Kiss 2001; Ma et al. 2005; Deryusheva and Gall 2009). The question remains, however, why guide RNAs for snRNA modification often concentrate in CBs when that localization is not required for function.

The WD-repeat protein WDR79 has been identified recently in Drosophila and human cells as a protein that specifically binds to the CAB box in scaRNAs and is required for scaRNAs to concentrate in CBs (Tycowski et al. 2009; Venteicher et al. 2009). Tycowski et al. (2009) have shown that endogenous scaRNAs can be coprecipitated efficiently with WDR79 protein from both human and Drosophila cells. We decided to use this approach for identification of novel scaRNAs in Drosophila. In previous studies nine 2′-O-methylated residues and 15 pseudouridines were detected in Drosophila U1, U2, U4, and U5 snRNAs (Myśliński et al. 1984; Huang et al. 2005; Deryusheva and Gall 2009). Although most or all of these modifications are probably directed by guide RNAs, only seven scaRNAs have been described so far in Drosophila (Jády and Kiss 2001; Deryusheva and Gall 2009; Tycowski et al. 2009). Here we use coimmunoprecipitation of WDR79–RNA complexes followed by RNA extraction and cDNA analysis to identify more scaRNAs in Drosophila. We found seven new scaRNAs that interact with WDR79 protein, possess CAB-box motifs, and concentrate in CBs. Surprisingly, among the newly identified scaRNAs, we found a box H/ACA RNA that base-pairs with U6 snRNA. Moreover, two other scaRNAs from our screen form pseudouridylation pockets that can base-pair with 28S rRNA and are capable of positioning the predicted modifications. The finding of these unexpected guide RNAs poses again the question of where post-transcriptional modification of snRNAs and rRNAs occurs in the nucleus and why scaRNAs concentrate in CBs.

RESULTS

WDR79 constructs

Drosophila WDR79 is a WD-repeat protein that interacts with the CAB box, a short motif found in scaRNAs (Tycowski et al. 2009). In wild-type flies, WDR79 is concentrated in CBs, where it colocalizes with scaRNAs. Despite the close association of WDR79 with scaRNAs and CBs, this association is not essential: Flies that are null for WDR79 protein lack CBs in all their cells but are otherwise apparently normal (Nizami et al. 2010b). In the following experiments, we used the interaction between WDR79 and scaRNAs in a wild-type background to verify seven previously identified scaRNAs and to discover seven new ones. Because the antibody we use for immunolocalization of WDR79 does not efficiently precipitate the protein, we made two RFP-tagged WDR79 constructs, expressed these as transgenes in flies, and used an anti-RFP antibody to immunoprecipitate WDR79 protein (Fig. 1A). In the first construct, RFP was at the carboxyl terminus of WDR79; in the second, it was at the amino terminus. In both cases, UASp was included as the most upstream element, so that WDR79 protein could be induced by GAL4 expression. Unexpectedly, the WDR79-RFP transgene expressed effectively without GAL4 induction in seven different transgenic lines. Importantly, the transgenic protein showed wild-type distribution in several different tissues, including Malpighian tubules (Fig. 1B,C), ejaculatory duct, and both germline and follicle cells in the ovary. Moreover, this transgene could rescue the formation of CBs in WDR79-null flies (Fig. 1E,F). At the same time, overexpression induced by GAL4 led to disassembly of CBs in wild-type flies (Fig. 1D). Unlike WDR79-RFP, the RFP-WDR79 construct required GAL4 induction as expected (Fig. 1H), yet without GAL4, this construct could express untagged WDR79 (Fig. 1H) and rescue the WDR79-null phenotype (Fig. 1G).

These and other data suggested that the annotation for Drosophila WDR79 was originally incomplete and that the annotated protein-coding cDNA sequence might contain a promoter. Using 5′-RACE, we detected eight transcription start sites for endogenous WDR79. These sites were different for RNA isolated from males and females, but all concentrated in a 31-nucleotide (nt) region at position 102–132 of the annotated WDR79 cDNA. The annotated mRNA is expressed as a minor fraction that appears to be testis-specific (Fig. 2).

Immunoprecipitation of previously known scaRNAs

In the earlier study of Drosophila scaRNAs (Tycowski et al. 2009), the WDR79 protein that was used to coprecipitate scaRNAs was the putative testis-specific isoform, Flag-tagged at the amino terminus. We carried out immunoprecipitation experiments with extracts from several different fly lines. First, we used flies that express WDR79-RFP from an endogenous promoter, which gives a normal level of expression from a predominantly shorter isoform. Second, we used flies that express WDR79-RFP and RFP-WDR79 from the UAS promoter driven by datGAL4. In both these cases, WDR79 expression levels are above normal and formation of CBs is disturbed (Fig. 1D).

We found that all seven of the known Drosophila scaRNAs coprecipitated equally well with WDR79-RFP and RFP-WDR79 proteins. Moreover, the interaction does not depend
FIGURE 1. Transgenic WDR79 protein behaves like endogenous WDR79 in Drosophila, and it rescues the WDR79-null phenotype. (A) Diagram of the two major WDR79 constructs used in this study. In each case, the annotated WDR79 transcript is shown in gray. Both constructs include an RFP sequence, one at the carboxyl terminus, the other at the amino terminus. Each includes an upstream UASp sequence for expression under control of GAL4. Both constructs express without GAL4 induction from an endogenous promoter within the cloned WDR79 sequence, although expression is enhanced by GAL4. (B) Endogenous WDR79 (green), detected with an antibody against the protein itself, colocalizes with coilin (red), producing a yellow Cajal body (CB) in the nucleus of a Malpighian tubule cell from a y w fly (arrow). (C) WDR79-RFP protein (green) expressed without GAL4 induction, detected with an antibody against RFP, also colocalizes with coilin (red) in the CB (arrow). (D) Ovarian follicle cells from WDR79-RFP/daGAL4 flies, stained for RFP (red) and coilin (green). Because of UAS-GAL4 variegation, two types of expression patterns are observed in the same tissue. In some cells, WDR79-RFP is overexpressed from the UAS promoter and accumulates in the cytoplasm; in most of these cells, nuclear CBs are not detectable. The remaining cells express WDR79-RFP at a lower level from the endogenous promoter. In these cells, WDR79-RFP accumulates with coilin in the CB in the nucleus. Viability of the cells seems to be unaffected by overexpression of WDR79-RFP. (E–G) Both WDR79-RFP and RFP-WDR79 transgenes can rescue the formation of CBs in WDR79-null flies. Ovarian follicle cells from WDR79-null flies (E) or from WDR79-null flies that express WDR79 from transgenes (F,G), stained for coilin (red) and Lsm11 (green). None of these flies carried a GAL4 driver, so expression is from an endogenous WDR79 promoter. The transgene in F is WDR79-RFP, whose transcript includes the RFP tag that in G is RFP-WDR79, whose transcript begins downstream from the tag. Cells from WDR79-null flies lack CBs (E). Hence, their nuclei display histone locus bodies (green Lsm11 foci), but CBs are undetectable (no red coilin foci). On the other hand, cells that express either WDR79-RFP (F) or WDR79 alone (G) may have CBs (red coilin foci) in addition to histone locus bodies (green Lsm11 foci). (H) Northern blot analysis of WDR79 mRNA expressed in y w (control) and WDR79MB10832 (mutant) flies. mRNA is expressed from the endogenous gene in y w flies and from transgenes in the mutant background. WDR79 mRNA is undetectable in RNA extracted from WDR79MB10832 mutant flies, suggesting that this is a null mutation.
on the level of WDR79 expression or even on the formation of typical CBs in the cells of the flies. The top two rows of Figure 3A show RT-PCR fragments of two known scaRNAs, U85 and mgU4-65, detected in the various immunoprecipitates. The other five known Drosophila scaRNAs were similarly detected (mgU2-25, mgU2-28, mgU2-41, mgU5-38, and mgU5-42). One more reported Drosophila scaRNA, Dm46E3 (Tortoriello et al. 2009), was undetectable in our samples.

The specificity of the interaction between WDR79 and the scaRNAs was confirmed by showing that several snoRNAs did not coprecipitate with WDR79. The tested snoRNAs included predicted guide RNAs for 2′-O-methylation and pseudouridylation of 28S rRNA and for 2′-O-methylation of U6 snRNA. Results for three of these snoRNAs are shown in Figure 3A (mgU6-47, pug28S-3327, and mg28S-3344). Similar negative results were obtained for two additional snoRNAs (mgU6-68 and pug28S-3186). These snoRNAs do not have CAB-box motifs and do not concentrate in CBs. They do, however, concentrate in nucleoli, as verified by FISH with antisense probes (Fig. 3C shows mgU6-47).

Spliceosomal snRNAs are substrates for scaRNA-mediated post-transcriptional modifications and are present at high concentration in CBs, along with both WDR79 and scaRNAs. Nevertheless, they are not enriched after immunoprecipitation of WDR79 (Fig. 3A, U4 snRNA). Similar results were found in the earlier study by Tykowski et al. (2009). These data suggest that WDR79 binds specifically to scaRNAs but does not interact with snRNAs.

New Drosophila scaRNAs

The seven previously known Drosophila scaRNAs direct eight of 24 known modifications in U1, U2, U4, and U5 snRNAs (U85 scaRNA directs two modifications). To find more RNAs that specifically interact with WDR79, including scaRNAs, we prepared a cDNA library from RNA that coprecipitated with WDR79-RFP protein. To avoid artifacts due to overexpression, we used a fly stock in which WDR79-RFP was expressed at a normal level from the endogenous promoter and rescued the CB phenotype of a WDR79-null mutant (Fig. 1F,H). It is worth noting that we did not obtain any clone that contained spliceosomal snRNA sequences. In addition to six of the known scaRNAs, we found seven novel small RNAs derived from introns of protein-coding genes (Table 1). We verified the existence of these small RNAs by Northern blot analysis in both wild-type and WDR79-null flies (Fig. 3B).

We also demonstrated their enrichment in WDR79 co-IP samples. Rows 3–6 in Figure 3A show RT-PCR fragments for four of these (pugU1-6, pugU2-55, mgU2-48, and mgU6-40). Three others were similarly detected (pugU2-35/45, pugU2-38/40/42, and pugU5-44). Finally, we showed by FISH that these RNAs concentrate in CBs and are thus scaRNAs by definition (Fig. 3C). We deposited the nucleotide sequences of these RNAs in GenBank; the corresponding accession numbers are listed in Table 1. These RNAs were detected previously in a genome-wide Drosophila transcriptome analysis using deep sequencing (Graveley et al. 2011), but they were not characterized as CB-specific guide RNAs.

The newly identified small RNAs could be folded into typical box H/ACA or box C/D RNA structures. Analysis of these structures allowed us to identify CAB-box motifs in all seven new scaRNAs, in good agreement with their interaction with WDR79 protein and their concentration in CBs. We analyzed potential antisense elements in these RNAs for matches with major and minor spliceosomal snRNAs, as well as for matches with rRNA. The results of this search are summarized in Table 1 and Figure 4.

Probing new scaRNAs for guide RNA activity

We have tested the seven new scaRNAs for modification activity in three ways, two involving intact Xenopus oocytes or extracts of Xenopus oocyte nuclei (Deryushova and Gall 2009) and the third making use of transformed Saccharomyces cerevisiae cells.

We first tested the seven new scaRNAs for modification activity in extracts of Xenopus oocyte nuclei (previously depleted for all RNAs). This assay involves mixing the guide RNA with its corresponding presumed substrate and then analyzing for modification of the substrate by a primer extension reaction. Only two guide RNAs were functional in this test, pugU1-6 and pugU2-38/40/42 (Fig. 5A,B; Table 1). We consider only positive results to be significant in this in vitro assay, since there are many reasons that a modification reaction involving a Drosophila scaRNA might not take place in a heterologous Xenopus nuclear extract.

The second use of Xenopus involves injection of a guide RNA along with its putative unmodified substrate into a
living oocyte, followed after a suitable time by a primer extension reaction to detect modification of the substrate. This is a limited assay because one must first deplete the oocyte of any endogenous guide activity. We have analyzed only one putative guide RNA by this method, a box H/ACA RNA that we designated \textit{Drosophila} pugU2-35/45. This is the ortholog of the well-characterized human U92 (Darzacq et al. 2002) and \textit{Xenopus laevis} pugU2-34/44 (Zhao et al. 2002). The latter can be efficiently depleted from \textit{Xenopus} oocytes by injecting an antisense oligonucleotide (Zhao et al. 2002).

We first injected unmodified \textit{Drosophila} U2 snRNA into oocytes that had been depleted for \textit{Xenopus} pugU2-34/44, an ortholog of \textit{Drosophila} pugU2-35/45. In this control experiment, we expected to see no pseudouridines at positions 35 and 45 in the injected \textit{Drosophila} U2, but normal pseudouridylation at positions modified by other endogenous \textit{Xenopus} scaRNAs (positions 38, 40, 42, and 44). Instead, we saw a normal level of modification at position 35, strongly reduced modification at position 45, and complete loss of modification at position 44 (Fig. 5C, red trace). Modification at position 35 is probably due to the activity of an endogenous pseudouridine synthase (Pus7p) that can catalyze pseudouridylation at position 35 of \textit{Drosophila} U2 snRNA (or the equivalent position 34 in vertebrates). Such an activity is known from many species including \textit{Xenopus} and \textit{Drosophila} (Ma et al. 2003; Karijolich and Yu 2010).

The situation at positions 44 and 45 is more difficult to interpret. Coinjection of \textit{Drosophila} pugU2-35/45 restores the normal pattern of pseudouridines, with a very strong signal at position 45 and a somewhat weaker one at position 44 (Fig. 5C, blue trace). One interpretation is that pugU2-35/45 can modify both positions 44 and 45. Another interpretation is that a separate pseudouridine synthase exists for position 44 and that its activity is influenced by the presence or absence of pugU2-35/45. Such a synthase (Pus1p) has been identified in yeast (Massenet et al. 1999), and its orthologs are presumably responsible for the corresponding modification in higher eukaryotes (Behm-Ansmant et al. 2006).

For further analysis of \textit{Drosophila} scaRNAs in living cells, we expressed various constructs in the yeast \textit{S. cerevisiae}. One can express \textit{Drosophila} scaRNAs on a plasmid in yeast and look for modification of endogenous yeast snRNAs. Alternatively, one can coexpress the scaRNAs along with \textit{Drosophila} artificial substrates and look for modification of the substrates. Yeast offers a favorable system because of the relatively low level of endogenous snRNA modification. There are no 2′-O-methylations in any of the snRNAs, but, importantly, yeast cells have the ability to support this type of modification, since their rRNAs are methylated. There are relatively few pseudouridines (Massenet et al. 1999).
modifications have been detected in yeast U4 and U6. Only three pseudouridines are normally found in U2 at positions 35, 42, and 44 of the highly conserved branch point recognition region. Corresponding mutant strains are available for each of these positions. Two pseudouridines are known for U1 at positions 5 and 6. Finally, there is a pseudouridine at position 99 in U5, although the mechanism of its modification has not been determined (Massenet et al. 1999). Overall, when our new *Drosophila* scaRNAs were expressed in yeast cells, they were capable of modifying the predicted positions in endogenous yeast U2 or the corresponding artificial substrates coexpressed in the same cells (Figs. 6, 7; Table 1). We were not able to test our pugU1-6 and pugU5-44 because of the endogenous modifications at the corresponding positions in yeast (position 6 in U1 and position 99 in U5). We discuss three of the new *Drosophila* scaRNAs in more detail because they illustrate some unexpected features.

**pugU2-35/45**

Because the pseudouridylation pocket in the pugU2-35/45 *Drosophila* scaRNA is somewhat degenerate for modification at position 35 of U2 snRNA, we would not have been surprised to find it nonfunctional. However, expression of pugU2-35/45 in Pus7p-deficient yeast cells (*pus7Δ*) resulted in pseudouridylation of the endogenous yeast U2 (Fig. 6, top two traces). The situation at position 45 is more complicated. Because of sequence divergence between yeast and *Drosophila* U2 at position 45 (Fig. 4, second row), we expected that position to be modified inefficiently. Indeed, this was the case when pugU2-35/45 was expressed in either wild-type or *pus7Δ* yeast strains. However, when pugU2-35/45 was expressed in the *pus1Δ* strain (lacking pseudouridylation at position 44), position 45 of the endogenous yeast U2 became intensely modified (Fig. 6, third and fourth traces). These observations allowed us to exclude position 44 as a potential substrate for pugU2-35/45 modification. At the same time, they showed that an imperfect pocket for positioning pseudouridine at position 45 in yeast U2 snRNA can be fully functional under the appropriate circumstances.

**pugU2-38/40/42**

The pugU2-38/40/42 guide RNA provides another example of a fully functional but noncanonical pseudouridylation pocket. When we first assayed this guide RNA in the *Xenopus* GV extract, we saw the expected pseudouridylation at position 42 of U2 snRNA, slight modification at position 38, but no modification at the strongly predicted position 40. In fact, the same domain that forms the pseudouridylation pocket for position 40 can, when refolded, base-pair with U2 to form a pocket for position 38. However, in this configuration, not 2 but 3 nt remain unpaired in the substrate RNA (Fig. 4, third row). When yeast cells that lacked the guide RNA for pseudouridylation at position 42 (*snr81Δ*) were transformed with a plasmid expressing *Drosophila* pugU2-38/40/42, we saw rescue of pseudouridylation at position 42 as expected (Fig. 6, sixth trace). We also saw unambiguous modification at position 38. However, only a minor signal was detectable at position 40. This signal became more prominent when modification at position 42 was prevented by expressing a mutated pugU2-38/40/42Δ that lacked the guide element for that position. In addition, modification at position 40 became more prominent when pugU2-38/40/42 was...

### TABLE 1. Novel *Drosophila* scaRNAs: predicted modifications in snRNAs and rRNA and results of three modification assays

| Guide RNA      | Host gene | Accession number | Predicted modification | Guide RNA activity in modification assay |
|---------------|-----------|------------------|------------------------|------------------------------------------|
|                |           |                  | Target RNA | Position | GV extract | Xenopus oocytes | Yeast cells |
| Pseudouridylation |          |                  |            |          |            |                |            |
| pugU1-6       | dmt       | JQ963649         | U1 snRNA   | 6        | +          | n/a            | n/a         |
| pugU2-35/45   | gp210     | JQ963645         | U2 snRNA   | 35       | –          | n/a            | +           |
| pugU2-38/40/42| bur       | JN409597         | U2 snRNA   | 45       | –          | +              | +           |
|               |           |                  |            |          |            |                |             |
| pugU2-55      | prp8      | JQ963646         | U2 snRNA   | 55       | –          | n/a            | +           |
| pugU5-44      | tra2      | JQ963650         | U5 snRNA   | 44       | –          | n/a            | n/a         |
| 2’-O-Methylation |        | JQ963648         | U6 snRNA   | 40       | –          | +              | +           |

*These positions are not modified in *Drosophila* 28S rRNA (Ofengand and Bakin 1997; data not shown).

(+) Modification was detected at the position predicted by the corresponding scaRNA.

(-) Modification was not detected at the predicted position.

(? Assay was inconclusive for the predicted modification.

(n/a) Not analyzed.
expressed in the pu7Δ yeast strain, which cannot modify position 35 (Fig. 6, fifth trace).

**pugU1-6**

The most striking example of guide-substrate plasticity was observed when we tested the ability of pugU1-6 scaRNA to modify the fragment of 28S rRNA inserted in huU87 RNA. In this case, two pseudouridines were clearly detectable in the artificial substrate, as if an additional target for the guide RNA had been accidentally created (Fig. 7, bottom two traces). Formation of the pocket for this second pseudouridylation produces a quite unstable stem–loop structure (Fig. 4, first row), which would never be predicted by standard free-energy-based RNA folding software.

In summary, our analysis of *Drosophila* scaRNAs in yeast and *Xenopus* verified nine of 10 predicted “conventional” modifications of snRNAs and two unexpected modifications of 28S rRNA. In addition, we experimentally demonstrated modifications by a guide RNA with imperfect antisense elements (pugU2-35/45), by a guide RNA with a dual pseudouridylation pocket preferentially functional in a noncanonical configuration (pugU2-38/40/42), and by a guide RNA that functioned with an unusual folding pattern (pugU1-6). Finally, we demonstrated that a genuine scaRNA (pugU6-40) can modify its putative target in U6 snRNA.

**DISCUSSION**

**New scaRNAs**

The major finding of our study is the identification of seven new scaRNAs in *Drosophila* in addition to the seven previously identified.
known. The approach we used was to immunoprecipitate the
WDR79 protein from lysates of fly tissue and to look for
scaRNAs in the precipitate. We identified all seven of the pre-
viously known scaRNAs by RT-PCR. Importantly, we showed
that the immunoprecipitate did not contain snRNAs, which
are both abundant in the nucleus and concentrated in the
CB, the same nuclear organelle where the scaRNAs are con-
centrated. New scaRNAs were identified in a cDNA library
prepared from the immunoprecipitate and were verified by
Northern blot analysis of Drosophila RNA. To make sure
that all the new RNAs met the definition of an scaRNA, we
demonstrated their presence in the CB by in situ hybridization
on Drosophila tissues. Thus, they are scaRNAs by virtue of
their nuclear localization as well as their association with
WDR79.

We used three systems to verify the function of the newly
identified scaRNAs: an extract of Xenopus GVs, injection into
Xenopus oocytes, and expression from exogenous plasmids
in yeast. We verified that six of the seven newly identified
scaRNAs can function as guide RNAs for modification of
other RNAs at the predicted positions (Table 1). The excep-
tion is pugU5-44, which did not modify U5 snRNA in an ex-
tact of Xenopus GVs and could not be tested in yeast.

Unusual guide functions

A surprise was the demonstration of pugU6-40 among the
RNAs associated with WDR79. As with all the new RNAs,
we verified that it is concentrated in the CB by in situ hybrid-
ization and meets the cytological definition of an scaRNA (Fig.
3C). Furthermore, it mediates the pseudouridylation of posi-
tion 40 in U6 snRNA in a yeast assay. Guide RNAs for this
modification have also been identified in human (ACA12
and HBI-100), mouse (MBI-100 and MBI-114), and a wide
variety of other vertebrate species (Huttenhofer et al. 2001;
Kiss et al. 2004; Lestrade and Weber 2006). Although there are
no FISH data for these vertebrate guide RNAs, they have
been annotated as scaRNAs based on the possession of
CAB-box motifs (Lestrade and Weber 2006) (http://www-
snorna.biotoul.fr/). In addition, human ACA12, HBI-100,
and ACA65 (which presumably directs U6 pseudouridylation
data at positions 31 and 86) coprecipitate with WDR79 protein
(Tycowski et al. 2009). Thus, there is good reason to suppose
that the situation in Drosophila is not unique and that some
positions in mammalian U6 snRNA are modified by canonical
scaRNAs. How is this observation to be reconciled with the
general consensus that modification of U6 snRNA takes
place in the nucleolus? One possibility is that modification
of U6 snRNA requires shuttling between the CB and the nu-
cleolus. Alternatively, the fact that a guide RNA is concentrat-
ed in either the nucleolus or the CB may be misleading.
High concentration makes the organelle prominent after FISH, but
need not correlate with total amount in the nucleus. It is
known, for instance, that both coilin-null and WDR79-null
flies lack cytologically detectable CBs, yet they have normal
levels of scaRNAs and their snRNAs are normally modified
(Deryusheva and Gall 2009; Nizami et al. 2010b). The clear
implication is that the majority of modification either occurs
normally in the nucleoplasm or at least can occur there when
the CBs missing. For reasons specific to the nucleolus—it is a
gene locus and not a “free-floating” organelle—the compara-
table ablation experiment cannot be carried out. Nevertheless, it
remains possible that snorRNAs are concentrated in the nucle-
olus and function there primarily because that is the only place
where their RNA substrate resides. The site of U6 snRNA
modification may be more flexible because the substrate is
free to move about in the nucleus, being found in the nucleo-
lus, the CB, or the nucleoplasm.

FIGURE 6. (Legend on next page)
We found two unusual scaRNAs with dual modification functions, pugU1-6 and pugU2-55. pugU1-6 modifies position 6 in Drosophila U1 snRNA in GV extract, but it also can modify position 2838 in Drosophila 28S rRNA in a yeast assay. Similarly, pugU2-55 modifies position 55 in Drosophila U2 snRNA as well as position 1960 in Drosophila 28S rRNA, both in yeast assays. Although the modifications in U1 and U2 snRNA occur normally in Drosophila and other organisms, modifications at positions 2838 and 1960 of Drosophila rRNA have not been detected (Ofengand and Bakin 1997). It is possible that the modifications in rRNA somehow result from use of an exogenous assay system. It is known, for instance, that guide RNA snR81 of yeast concentrates in the nucleolus and directs modifications in yeast U2 snRNA and 25S rRNA (Ma et al. 2005). Alternatively, scaRNAs may recognize noncanonical substrates only under certain conditions, such as in certain tissues or under special circumstances. Such an example is known from yeast, where two pseudouridines in U2 snRNA appear only under stress conditions (Wu et al. 2011).

Our analysis demonstrates an unexpected degree of plasticity between guide RNAs and their substrates. The canonical pseudouridylation pocket involves a region of perfect base-pairing between the guide RNA and its substrate, surrounding two unpaired nucleotides, one of which is the target uridine. We showed that in living cells box H/ACA RNAs (pugU2-35/45, pugU2-38/40/42, and pugU1-6) can support pseudouridylation at position 45 (star). The pseudouridylation at position 45 is much more prominent when pugU2-35/45 is expressed in the pus1Δ strain (pus1Δ + pugU2-35/45). Similarly, expression of Drosophila pugU2-38/40/42 in the snr81Δ strain (snr81Δ + pugU2-38/40/42) can rescue pseudouridylation at position 42 (star), and additionally positions 38 and 40 (stars) become modified. Pseudouridylation at position 40 is weak in wild type, pus1Δ (data not shown) and snr81Δ strains but appears more prominent when pugU2-38/40/42 is expressed in the pus1Δ strain (pus1Δ + pugU2-38/40/42). Control primer extension reactions without CMC treatment were run on all RNA samples. They showed no stop signals, as observed for RNA from the BY4741 strain. (B) Only three pseudouridines are detectable in U2 snRNA from BY4741 yeast cells that express either Drosophila pugU2-55 guide RNA (BY4741 + pugU2-55, red trace) or its substrate chimeric yeast–Drosophila U2 (yΔU2) snRNA alone (BY4741 + yΔU2, black trace). However, an extra peak (star) corresponding to pseudouridine at position 55 in Drosophila U2 snRNA appears when both pugU2-55 guide RNA and its substrate are coexpressed in the same yeast strain (BY4741 + yU2 + pugU2-55, blue trace). (C) Expression of Drosophila mgU2-48 guide RNA in yeast cells induces 2′-O-methylation at position 48 in coexpressed chimeric yeast–Drosophila U2 snRNA (star, top green trace). Extra stop signals of unknown origin (question mark) were observed in this trace, where the primer extension reaction was performed at a low concentration (LC) of dNTP. No stop signals were produced in control reactions run at high concentration (HC) of dNTPs with the same RNA sample (black trace) or at low concentration with RNA extracted from yeast cells that express only one of the two exogenous RNAs: either mgU2-48 (blue trace) or chimeric yeast–Drosophila U2 snRNA (red trace). Alignment with U2 snRNA sequences is shown with colored dots as in Figure 5 (yeast U2 snRNA at the top of the figure and chimeric yeast–Drosophila U2 snRNA at the bottom).

Our analysis demonstrates an unexpected degree of plasticity between guide RNAs and their substrates.
been described. Our experiments do not, however, address several important questions about the interaction of these molecules in the cell. The notion that WDR79 binds to scaRNAs by means of the CAB box and targets them to their snRNA substrates in the CB, where the modification reactions occur, is too simplistic. In fact, neither WDR79 protein nor CBs themselves are required for post-transcriptional modification of Drosophila snRNAs. We showed earlier that Drosophila scaRNAs can function without the CAB box, suggesting that WDR79 does not necessarily play a direct role in the modification reaction (Deryusheva and Gall 2009). Later we confirmed this conclusion by demonstrating normal snRNA modification (Nizami et al. 2010b) and normal levels of scaRNAs in WDR79-null flies (Fig. 3B). These flies completely lack cytologically detectable CBs. Furthermore, there is evidence that the association of WDR79 with the CAB box is not limited to CBs. The recently identified human Alu-repeat box H/ACA-like RNAs contain CAB-box motifs and interact with WDR79 protein, but they do not accumulate in CBs (Jády et al. 2012). In our experiments, overexpression of WDR79 protein in transgenic flies causes disassembly of CBs (Fig. 1D), yet does not affect WDR79 binding to scaRNAs (Fig. 3A). In summary, the scaRNAs may need to associate with WDR79 for targeting to CBs, but that association is not sufficient for CB localization, nor is it required for snRNA modification.

Concluding remarks

The analysis of new scaRNAs in Drosophila underscores the necessity to verify the putative guide function(s) of newly isolated or predicted guide RNAs. Bioinformatics predictions, especially base-pairing, provide strong circumstantial evidence of scaRNA function. Nevertheless, our analysis demonstrates that not all predicted modifications occur with high efficiency and that some scaRNAs can carry out unexpected reactions. It is also clear that the relationship between WDR79, scaRNAs, and CBs is not simple, since modification reactions occur normally in flies that lack both WDR79 and CBs. When present in the cell, WDR79 interacts preferentially with scaRNAs and mediates targeting to the CB, but neither the interaction nor the targeting is necessary for scaRNA function or for viability of the organism. Finally, the ability of scaRNAs to modify U6 snRNA suggests that the traditional sharp distinction between snoRNAs and scaRNAs cannot be maintained in its simplest form.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster stocks were maintained at room temperature (21°C–23°C) on a standard cornmeal-based medium.

Transgenic flies

The annotated full-length protein coding sequence for Drosophila WDR79 was amplified from the cDNA clone AT03686. This was first cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen). It was then swapped into the pPRW or pPWR vector to make P-element constructs in which protein expression is under control of the yeast upstream activating sequence (UASp) and the expressing protein has an RFP-tag at either the amino or carboxyl terminus, respectively. Injections into Drosophila embryos were done by Genetic Services. Seventeen transgenic lines with random insertions of WDR79 cDNA were obtained: 10 lines with RFP at the amino terminus and seven lines with RFP at the carboxyl terminus.

Other fly stocks

y w served as a control strain. For a WDR79-null background, we used [w^118; Mi[ET1]WDR79^{H10832}] (Bloomington #29159). As a negative control for anti-RFP immunoprecipitation assays, we used a line that expresses mRFP with a nuclear localization signal under the ubiquitin promoter [w^118; P[Ub-mRFP,nls]3L P[neoFRT]80B] (Bloomington #30852). A stock that expresses GAL4 from the daughterless promoter (daGAL4) was used to drive UASp-WDR79 transgenes in all tissues.

RNA extraction and PCR-based analysis

RNA was extracted from whole flies, isolated testes, or male carcasses from which the testes had been removed manually. Either the RiboPure kit or the RNeAqueous Micro kit (Ambion) was used depending on the sample size. RNA samples were treated with RNase-free DNase I (Ambion) when used for RT-PCR. RT-PCR was performed using the One-Step RT-PCR Kit (QIAGEN). All amplified fragments were sequenced to verify the specificity of the RT-PCR.

To detect the 5’ end of Drosophila WDR79 mRNA, we used the FirstChoice RLM-RACE Kit (Ambion). We followed the recommended protocol, including tobacco acid pyrophosphatase (TAP)–minus reactions as negative controls. The 3’-gene-specific primers annealed either to exon 2 (to detect major mRNA variants) or to a region upstream of major transcription start sites (to detect testis-specific mRNA). PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced.

Northern blot analysis

RNA samples were separated either on formaldehyde agarose gels (for mRNAs) or on 8% polyacrylamide–8 M urea gels (for small RNAs). The RNA was transferred onto a nylon membrane (Zeta Probe GT, Bio-Rad) and probed with single-stranded antisense DNA labeled with digoxigenin (DIG)–dUTP. DIG was detected using an anti-DIG antibody conjugated with alkaline phosphatase and the chemiluminescent substrate CDPh-Star (Roche) according to the manufacturer’s protocols.

Western blot analysis and immunofluorescent staining

Western blot analysis of Drosophila protein extracts was performed according to standard procedures. Whole-mount immunofluorescent staining of various Drosophila tissues was as previously
described (Liu et al. 2009). In both cases, the primary antibodies were the same: an affinity-purified rabbit polyclonal antibody generated against an 18-amino-acid peptide corresponding to residues 136–153 of the predicted Drosophila WDR79 protein, guinea pig antibodies against Drosophila coillin (Liu et al. 2009), rabbit anti-RFP either unconjugated or HRP-conjugated (MBL Medical and Biological Laboratories Co., Ltd.), or mouse anti-a-tubulin (clone DM1A, Sigma-Aldrich). Secondary antibodies for Western blots were conjugated with HRP, whereas those for tissue immunostaining were conjugated with Alexa 488, Alexa 594, or Alexa 633 fluorescent dyes. Antibodies were used at a dilution of 1:30,000 in 5% nonfat dry milk for Western blots and 1:1000 in 10% horse serum for immunofluorescent staining.

**Fluorescent in situ hybridization (FISH)**

Fluorescent RNA probes were labeled with Alexa 488-UTP or Cy5-CTP in in vitro transcription reactions using DNA clones or PCR products as templates. These dyes were chosen to obtain complete separation of fluorescent signals when two-color FISH was used for colocalization analysis. The procedure for whole-mount FISH on Drosophila tissues was described previously (Deryusheva and Gall 2009). Images were captured using a laser-scanning confocal microscope (Leica SP2 or SP5) with a 63× (NA 1.40) planapochromatic objective. Images were taken with the laser intensity and photomultiplier gain adjusted to avoid saturated pixels in the region of interest (“glow-over” display). In some cases, the relative intensities and colors of the channels were adjusted with Photoshop (Adobe).

**Immunoprecipitation**

For each coimmunoprecipitation (co-IP) reaction, 50–60 pairs of Drosophila ovaries were manually dissected in ice-cold Grace’s medium. Cells were lysed in 500 μL of protein extraction buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.1% NP-40) supplemented with Protease and Phosphatase Inhibitor Cocktail (Roche) and RNasin (Promega). Lysates were precleared using protein G agarose beads (Roche). Then 3 μL of anti-RFP antibody was added, and the lysates were incubated for 1 h at 4°C. To capture the immunocomplexes, protein G agarose beads were added, and the incubation was continued for another 1 h (beads were pre-blocked with bovine serum albumin and calf intestinal alkaline phosphatase–treated yeast tRNA). After incubation, the beads were washed five times with 1 mL of the protein extraction buffer. To determine the efficiency of the WDR79 IP, immunocomplexes were dissociated from the beads by boiling in sample buffer and then subjected to Western blot analysis. RNA was extracted from the beads with the RiboPure kit (Ambion).

RNA samples from co-IP reactions were treated with RNase-free DNase I and used for RT-PCR. Oligos specific for Drosophila scaRNAs and snRNAs were as previously described (Deryusheva and Gall 2009). To make a cDNA library of RNAs to which WDR79 protein binds, RNA released from the beads was decapped and polyadenylated, and 5′ and 3′ adapters were added (FirstChoice RLM-RACE Kit). cDNA fragments were amplified using 5′- and 3′-RACE primers from the same kit. The resulting PCR products were cloned into the pGEM-T Easy vector, and 96 randomly picked colonies were sequenced. To verify the 5′ and 3′ ends of newly identified small RNAs, a standard RACE procedure was performed on total RNA isolated from whole flies of the y w strain.

**RNA sequence analysis**

An initial prediction of scaRNA folding was carried out using the Mfold web server (http://mfold.rna.albany.edu/). Secondary structures predicted by the software were then analyzed and adjusted manually. Putative antisense elements were screened for matches with all regions of Drosophila spliceosomal snRNAs and rRNAs.

**In vitro modification assays**

Full-length coding sequences of Drosophila scaRNAs were amplified from genomic DNA and ligated into the pGEM-T Easy vector. A T3 promoter was included in the forward primer upstream of the first coding nucleotide; a BamHI site was included in the reverse primers immediately following the last coding nucleotide. Plasmids were linearized with BamHI, and sense-strand RNAs were transcribed using T3 RNA polymerase. The same strategy was used to clone fragments of Drosophila 28S rRNA; specific oligos were designed to amplify regions 1904–2062 and 2747–3055 in 28S rRNA. In vitro–transcribed snRNAs were made from previously described constructs of Drosophila U1, U2, U4, and U5 snRNAs (Deryusheva and Gall 2009). The coding sequence of Drosophila U6 snRNA was also cloned.

An in vitro modification assay based on a Xenopus oocyte nuclear extract was previously described (Deryusheva and Gall 2009). Briefly, 150 nuclei (germinal vesicles or GV’s) were manually isolated and collected in mineral oil. The nuclei were centrifuged to disrupt the nuclear envelope and sediment nuclear organelles, and the resulting extract was treated with micrococcal nuclease (New England Biolabs) to destroy endogenous RNA. The nuclease was then inactivated by adding EGTA. Finally, an in vitro–transcribed snRNA or rRNA was added to the nuclear extract along with its putative guide RNA. RNA was extracted after 6–12 h of incubation, and the substrate RNA was tested for modification(s) introduced by the guide RNA.

In a second assay based on Xenopus oocytes, an in vitro–transcribed guide RNA (Drosophila pugU2-35/45) was injected into the GV and an in vitro–transcribed snRNA (Drosophila U2) into the cytoplasm (Deryusheva and Gall 2009). First, however, it was necessary to destroy the endogenous guide RNA pugU2-34/44. To do this, 230 ng of a DNA oligonucleotide, aT2 (GTTATCTGTCGT CATCAA), was injected into the cytoplasm and the oocyte was incubated overnight (Zhao et al. 2002). Then Drosophila U2 snRNA and its corresponding guide RNA, pugU2-35/45, were injected, and the oocytes were incubated a further 6–7 h. Finally, nuclei were manually isolated and the Drosophila U2 snRNA was tested for modification.

**Expression of Drosophila guide RNAs and their substrates in yeast**

pugU2-35/45 and pugU2-38/40/42

The pugU2-35/45 and pugU2-38/40/42 guide RNAs reside in the introns of Drosophila gp210 and bur genes, respectively. Fragments that contained the guide RNAs and the flanking exons were
amplified from genomic DNA of y w flies. Xmal and XhoI restriction sites were added to the fragments, which were then cloned into the yeast p425Gal1 vector.

**mgU2-48, pugU2-55, and pugU1-6**

Constructs were made in which *Drosophila* mgU2-48 replaced a box C/D snoRNA (snR18) in the yeast *EFB1* gene, and *Drosophila* pugU2-55 or pugU1-6 replaced a box *HACA* snoRNA (snR191) in the yeast *NOG2* gene. In each case, overlap extension PCR was used to carry out the replacement. Fragments of the yeast genes were amplified from genomic DNA of the BY4741 yeast strain; Xmal and XhoI restriction sites were included in the primers. The *Drosophila* scaRNAs were amplified from genomic DNA of y w flies. The chimeric fragments resulting from the overlap extension PCR were digested with Xmal and XhoI and cloned into the yeast p425Gal1 vector.

**pugU6-40, pugU2-35/45, pugU2-38/40/42, and pugU2-38/40/42A**

The coding sequences of the pugU6-40, pugU2-35/45, pugU2-38/40/42, and pugU2-38/40/42Δ *Drosophila* guide RNAs were cloned into the YEplac181 vector, which contains a *GPD* promoter, an RNT1 cleavage site, and an snR13 terminator (Huang et al. 2011) (a gift from Yi-Tao Yu, University of Rochester Medical Center). pugU2-38/40/42Δ is a variant guide RNA with a mutated pseudouridylation pocket for position 42.

**Modified yeast U2 snRNA**

Sequences from *Drosophila* U2 snRNA were introduced into the yeast U2 snRNA gene as follows. A plasmid containing the yeast U2 snRNA gene with a unique EcoRI site at the 5′ end was cut with EcoRI and EcoNI and religated with a fragment of the *Drosophila* U2 gene (nucleotides 9–70 with EcoRI and EcoNI sites immediately flanking the coding sequence). The yeast U2 plasmid pRS316[URA3 CEN SNR20] was a gift from Yi-Tao Yu.

**Substrates for Drosophila pugU6-40, pugU2-55, and pugU1-6**

A fragment of *Drosophila* U6 snoRNA (nucleotides 33–46) and two fragments of *Drosophila* 28S rRNA (nucleotides 1952–1965 and 2831–2844) were inserted into the human U87 scaRNA by replacing nucleotides 210–217. A somewhat similar strategy was used by Jady et al. (2003). In addition, a lower-complexity substrate was made by deleting the *HACA* domain (nucleotides 27–183) of human U87 scaRNA. These six chimeric sequences were then substituted for snR18 in the *EFB1* gene and cloned into the p426Gal1 vector.

The various constructs described above were introduced into yeast *S. cerevisiae* by the standard lithium acetate method. Wild type (BY4741) and three mutant strains (*pup1Δ, pup7Δ, and snr81Δ*) were used. Expression of *Drosophila* scaRNAs under the *GAL1* promoter was obtained by growing cells for 24 h in appropriate selective media with galactose as a source of sugar. Expression and processing of the exogenous RNAs were verified by Northern blot analysis.

**Primer extension-based modification analysis**

2′-O-Methylation and pseudouridylation were analyzed by fluorescent primer extension assays as described (Deryusheva and Gall 2009). Single-stranded DNA fragments were separated using a capillary electrophoresis instrument (ABI Prism 3100 Genetic Analyzer). The Gene Scan-500 Liz Size Standard (Applied Biosystems) was included in each sample.

Oligonucleotides for *Drosophila* U1, U2, U4, and U5 snRNA modification mapping were previously described (Deryusheva and Gall 2009); in vitro-transcribed U6 snRNA was analyzed using an oligonucleotide: ATACGACTCACTATAGGATCCAAAA. To analyze yeast U2 snRNA, an oligonucleotide complementary to nucleotides 99–125 was used: GGGTGCCAAAAATGTGTATTGTAACA. *Drosophila* 28S rRNA was probed for modification using two oligonucleotides: [2009–2033] CCGAGGAAAAATATCGACACAA CA and [2986–3022] TTATACCAATTTTCAATCAAAAATACA TAAAATGCA. Artificial substrates inserted into the huU87 sequence were analyzed using an oligonucleotide complementary to the 3′ end of huU87: AGGTCTCAGATGAAAAACTTGAGATCA.

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