Small Cajal Body–specific RNAs of *Drosophila* Function in the Absence of Cajal Bodies

Svetlana Deryusheva and Joseph G. Gall

Department of Embryology, Carnegie Institution for Science, Baltimore, MD 21218

Submitted September 10, 2009; Revised September 29, 2009; Accepted October 9, 2009

Monitoring Editor: A. Gregory Matera

During their biogenesis small nuclear RNAs (snRNAs) undergo multiple covalent modifications that require guide RNAs to direct methylase and pseudouridylation enzymes to the appropriate nucleotides. Because of their localization in the nuclear Cajal body (CB), these guide RNAs are known as small CB-specific RNAs (scaRNAs). Using a fluorescent primer extension technique, we mapped the modified nucleotides in *Drosophila* U1, U2, U4, and U5 snRNAs. By fluorescent in situ hybridization (FISH) we showed that seven *Drosophila* scaRNAs are concentrated in easily detectable CBs. We used two assays based on *Xenopus* oocyte nuclei to demonstrate that three of these *Drosophila* scaRNAs do, in fact, function as guide RNAs. In flies null for the CB marker protein coilin, CBs are absent and there are no localized FISH signals for the scaRNAs. Nevertheless, biochemical experiments show that scaRNAs are present at normal levels and snRNAs are properly modified. Our experiments demonstrate that several scaRNAs are concentrated as expected in the CBs of wild-type *Drosophila*, but they function equally well in the nucleoplasm of mutant flies that lack CBs. We propose that the snRNA modification machinery is not limited to CBs, but is dispersed throughout the nucleoplasm of cells in general.

INTRODUCTION

The two major ribosomal RNAs (rRNAs) and the spliceosomal small nuclear RNAs (snRNAs) U1, U2, U4, U5, and U6 contain many posttranscriptionally modified nucleotides, which are crucial for RNA–RNA and RNA–protein interactions, as well as spliceosome function (Yu *et al.*, 1998; Zhao and Yu, 2004, 2007). The most abundant modifications in both rRNAs and snRNAs are 2'-O-methylation and pseudouridylation, which are directed by small box C/D and box H/ACA guide RNAs, respectively. Each guide RNA molecule is associated with a set of four core proteins: box C/D RNAs form RNP particles with fibrillarin (the methyltransferase), Nop56, Nop58, and a 15.5-kDa protein, whereas box H/ACA RNAs associate with NAP57/dyskerin (the pseudouridine synthase), GAR1, NHP2, and NOP10 proteins (reviewed in Yu *et al.*, 2004; Matera *et al.*, 2007). Most guide RNAs are concentrated in the nucleolus, where they are involved in posttranscriptional modifications of rRNA. Because of their localization, they are referred to as small nucleolar RNAs (snoRNAs). However, the guide RNAs that mediate modification of the snRNAs are preferentially found in another nuclear organelle, the Cajal body (CB). These guide RNAs are called small CB-specific RNAs (scaRNAs).

The first scaRNA to be studied in detail was U85 scaRNA (Jády and Kiss, 2001; Darzacq *et al.*, 2002). U85 is a remarkable double guide RNA that contains both box C/D and box H/ACA motifs. In a set of detailed experiments it was shown that human U85 scaRNA guides both 2'-O-methyl-

ation at C45 and pseudouridylation at U46 in U5 snRNA. Modification takes place in the nucleus after import of the U5 snRNA from the cytoplasm (Jády *et al.*, 2003). CB localization of U85 scaRNA was originally demonstrated by fluorescent in situ hybridization (FISH) in mammalian and *Drosophila* cultured cells (Darzacq *et al.*, 2002; Richard *et al.*, 2003), and subsequently in various tissues of the fly (Liu *et al.*, 2006b, 2009).

CBs are small nuclear bodies found in a wide variety of eukaryotic organisms, including mammals, amphibians, insects, and plants. They are most often recognized by immunostaining for the marker protein coilin (Andrade *et al.*, 1991; Raska *et al.*, 1991) and by their unusually high concentration of splicing snRNPs relative to the rest of the nucleus (Carmo-Fonseca *et al.*, 1991; Matera and Ward, 1993; Spector, 1993). The discovery of scaRNAs added a convenient way to identify CBs by FISH. Because *Drosophila* coilin was difficult to recognize on the basis of sequence comparison, we originally used U85 scaRNA and other markers to identify the *Drosophila* CB (Liu *et al.*, 2006b). More recently we identified *Drosophila* coilin and showed that it colocalizes with U85, snRNAs, fibrillarin, and the survival motor neuron (SMN) protein in CBs in a wide variety of larval and adult cells (Liu *et al.*, 2009). We also generated two coilin-null strains, which lack CBs detectable by probes for any of the standard CB markers. Thus coilin is essential for normal CB organization in *Drosophila*. Nevertheless, coilin-null flies are homozygous viable and fertile.

These data raise two important questions. Are the usual CB components (other than coilin) present in coilin-null flies, but are simply not concentrated in a CB? And do reactions that normally take place in the CB occur in the absence of a morphologically recognizable CB? Here we examine U85 and six other scaRNAs in both wild-type and coilin-null flies. We show that scaRNAs are present at normal levels in the mutants, although they are not concentrated in a CB. We also found methylation and pseudouridylation of U1, U2, U4, and U5 snRNAs in both wild-type and coilin-null flies.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09-09-0777>) on October 21, 2009.

Address correspondence to: Joseph G. Gall (gall@ciwemb.edu).

Abbreviations used: CB, Cajal body; GV, germinal vesicle; scaRNA, small Cajal body–specific RNA.

Thus, cytologically detectable CBs are not required for normal function of scaRNAs in *Drosophila*.

MATERIALS AND METHODS

Flies

Drosophila melanogaster strains were maintained at 21–23°C on a standard cornmeal-based medium. A *y w* stock was used as control. Two coilin null lines, *coil¹⁹⁹* and *coil²⁰³*, were generated by site-specific mutation using zinc-finger nucleases (Beumer *et al.*, 2008; Liu *et al.*, 2009). *Drosophila* S2 cells were used as an additional wild-type control.

Templates for In Vitro Transcription

Using a nucleotide database search similar to that described by Tycowski *et al.* (2009), we independently identified a guide RNA for 2'-O-methylation of *Drosophila* U4 snRNA at position A65. A standard RACE-PCR strategy was used to determine the 5' and 3' ends of the RNA.

Drosophila snRNAs and box C/D guide RNAs were amplified from genomic DNA isolated from *y w* flies using PCR with gene specific primers. The oligos we used are listed in Supplemental Table S1. Most 5' oligos contain a T3 promoter to make sense RNA, whereas 3' oligos contain a T7 promoter to make antisense RNA. PCR fragments were either cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) or pGEM-T-Easy (Promega, Madison, WI) vectors (U1, U2, U4, and U5 snRNAs; mgU2-25, mgU2-41, mgU4-65, mgU5-38, and putative mgU2-31) or were digested with EcoRI + XbaI and cloned into pUC19 (mgU2-28, mgU5-42, and U85 scaRNA). Otherwise PCR products were purified and used as templates (pug28S-3186 and pug28S-3327). To obtain mgU2-28 lacking the CB-specific localization sequence, referred to as the CAB box, oligos SD104 and SD105 were annealed and extended with Taq DNA polymerase at 55°C for 15 min. The DNA fragment was cloned into pGEM-T-Easy vector, and the plasmid was used as a template for in vitro transcription. To make a 5' truncated fragment of mgU2-28, oligo SD94 was annealed with a standard T7 oligo and used as a template for transcription.

RNA Extraction

For Northern blot and primer extension analysis total RNA was extracted from adult females, 3rd instar larvae, pupae, and S2 cell cultures using the RiboPure kit (Ambion, Austin, TX). RNA was treated with RNase-free DNase I (Roche, Indianapolis, IN) and checked for traces of DNA by PCR. A qualitative estimate of RNA was made by RT-PCR and by electrophoresis on an agarose gel. After ethanol precipitation the RNA was dissolved in nuclease-free water, and its concentration was measured by spectrophotometry.

Northern Blot Analysis

For Northern analysis of total RNA, 20 µg samples were separated on an 8% denaturing polyacrylamide gel (8 M urea, 1× TBE buffer [89 mM Tris, 89 mM borate, and 2 mM EDTA]) and electrophoretically transferred onto a nylon membrane (GeneScreen from Perkin Elmer, Norwalk, CT, or Zeta-Probe GT from Bio-Rad, Richmond, CA). After UV cross-linking of the RNA, the membranes were stained with methylene blue to estimate the amount of transferred RNA. Hybridization was performed for 2–3 h in Rapid-Hyb Buffer (GE Healthcare, Piscataway, NJ) at 65°C for U85 RNA detection or at 50–55°C for other box C/D RNAs. Single-stranded DNAs complementary to full-length *Drosophila* scaRNAs were labeled with [α -³²P]dCTP in a reverse transcription reaction and were used as probes. Blots were washed briefly in 2× SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) and then in 2× SSC, 0.1% SDS for 20 min, followed by two times in 0.1× SSC, 0.1% SDS for 30 min at the hybridization temperature. Membranes were exposed to Kodak x-ray film (Eastman Kodak, Rochester, NY) for 1–7 d or for 2–7 h in the case of snRNA detection.

Fluorescent Primer Extension Analysis to Map 2'-O-Methylation and Pseudouridylation

For mapping 2'-O-methylation and pseudouridylation sites in *Drosophila* snRNAs we developed a nonradioactive modification of methods based on reverse transcription (Kiss and Jány, 2004). To detect 2'-O-methylation, we performed primer extension with a low concentration of deoxynucleotide triphosphate (dNTP); the dNTP concentration varied from 0.004 to 0.01 mM and was optimized for each snRNA. Control reactions were done at a high concentration of dNTPs (0.5 mM). Pseudouridines were mapped after reacting the test RNA with CMC [N-cyclohexyl-N'-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate; Sigma-Aldrich, St. Louis, MO] followed by alkali treatment. In vitro-transcribed RNAs, either untreated or treated with CMC, were used as negative controls for modifications. RNA sequencing products were used to determine the exact position of modified nucleotides. Chain termination mixes contained 270 µM dNTPs, where one dNTP was partially replaced with acyNTP (New England Biolabs, Beverly, MA) at a 1:3 (dNTP:acyNTP) ratio. Oligonucleotides terminally labeled with a 6-FAM fluorescent dye were used as primers; 3–6 pmol of 6-FAM-oligo was used per reaction (oligos are listed in Supplemental Table S1). Reaction products were purified by ethanol precipitation, lyophilized, and kept

at –20°C until use. Fragments were separated on 50-cm capillary columns in POP4 acrylamide polymer on a capillary electrophoresis instrument (ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA) using techniques and parameters suggested by the manufacturer. The Gene Scan-500 Liz Size Standard (Applied Biosystems) was included in each run to align fragments from different samples. GeneMapper version 3.7 software (Applied Biosystems) was used to screen the data, identify peaks, and precisely determine positions of modified nucleotides.

In Vitro Modification Assays

Capped *Drosophila* sense-strand RNAs were transcribed in vitro from linearized plasmids using T3 RNA polymerase according to standard procedures. After DNase I treatment and DNase I inactivation, RNAs were purified using MicroSpin G25 columns (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Xenopus oocytes were injected with 9.2 nl (85 fmol) of a guide RNA into the giant nucleus or germinal vesicle (GV), then with 18.4–23 nl (~0.5 pmol) of *Drosophila* U2 snRNA into the cytoplasm using a Nanoject microinjection apparatus (Drummond Scientific, Broomall, PA). Both RNAs were synthesized with an m⁷Gppp cap at the 5' end. As a control, U2 snRNA alone was injected into oocytes. After overnight incubation in OR2 medium (Wallace *et al.*, 1973) at room temperature, GV's were manually isolated in 5:1 KCl-NaCl isolation medium (Gall *et al.*, 1991). RNA was extracted using the RNAqueous-micro kit (Ambion) and analyzed for 2'-O-methylation mapping.

In a second type of assay we used *Xenopus* GV's isolated in mineral oil. These nuclei retain their physiological activity for hours (Lund and Paine, 1990; Paine *et al.*, 1992; Handwerger *et al.*, 2003; Deryusheva and Gall, 2004). Furthermore, U2 snRNA injected into such nuclei is properly modified (Yu *et al.*, 2001). GV's (n = 150) were collected in a minimal amount of oil in a 0.5-ml tube. Micrococcal nuclease buffer (50 mM Tris-HCl, 5 mM CaCl₂, and 0.1 mg/ml BSA, pH 7.9) was added to the GV's under oil to a final volume of 14–15 µl. The tube was then centrifuged at 20,800 × g for 2 min to break down nuclear envelopes and release the GV contents. The tube now contained three fractions: yolk on the bottom, GV extract, and oil with nuclear envelopes at the interface. While still under oil, the GV extract was treated with 1 µl micrococcal nuclease (100 gel units/µl; New England Biolabs) for 30 min at 30–35°C. Micrococcal nuclease was then inactivated by adding EGTA to a final concentration of 10 mM. RNasin, reconstitution buffer (final concentration 20 mM HEPES-NaOH, pH 8, 120 mM KCl, and 5 mM MgCl₂) and in vitro-transcribed RNAs were added to the micrococcal nuclease-treated GV extract. RNAs were either 100 ng full-length target snRNA alone or 100 ng target RNA along with a two- to threefold excess of corresponding guide RNA. After overnight incubation at room temperature, RNA was extracted and analyzed.

FISH

Various tissues from *Drosophila* third instar larvae and adult flies were examined as whole mounts. Flies were dissected in Grace's medium (Grace, 1962) and tissues were fixed for 10 min in 4% paraformaldehyde, 2.5% acetic acid in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄). Acetic acid added to the fixative improves probe penetration and increases the efficiency of hybridization. After washing in PBS, samples were equilibrated for several hours in hybridization mix (50% formamide, 5× SSC, pH 7.0, 10 mM citric acid, 50 µg/ml heparin, 500 µg/ml yeast tRNA, and 0.1% Tween 20). Fluorescent RNA probes labeled with Alexa-488-UTP, Alexa-546-UTP (Invitrogen), or Cy5-UTP (GE Healthcare) were prepared by in vitro transcription from DNA clones, PCR products, or deoxyoligonucleotides as described previously (Liu *et al.*, 2006a,b). Probes were diluted in the hybridization mix to a final concentration of 50–100 ng/ml. Tissues for in situ hybridization were incubated at 42°C overnight. DAPI (4,6-diamidino-2-phenylindole, 1 µg/ml) was added to the hybridization mix as a stain for DNA. For colocalization analysis by two-color FISH, Alexa-488 and Cy5 labels were used to obtain complete separation of the fluorescence signals. Images were taken with a 40× (NA 1.25) or a 63× (NA 1.40) planapochromatic objective on a laser-scanning confocal microscope (Leica SP2 or SP5, Deerfield, IL). Images were taken with the laser intensity and photomultiplier gain adjusted so that pixels in the region of interest were not saturated ("glow-over" display). In most cases contrast and relative intensities of the blue (DAPI), green (Alexa-488), red (Alexa-546), and far red (Cy5) images were adjusted with Photoshop (Adobe Systems, San Jose, CA).

RESULTS

Known and Predicted Guide RNAs for Nucleotide Modifications in *Drosophila* snRNAs

Human U85 scaRNA is a guide RNA concentrated in the CB, where it directs 2'-O-methylation of C45 and pseudouridylation of U46 in human U5 snRNA (Jány and Kiss, 2001). *Drosophila* U85 scaRNA is concentrated in the CB of the fly (Jány and Kiss, 2001; Liu *et al.*, 2006b, 2009), where it pre-

Table 1. 2'-O-methyl and pseudouridine modifications in *Drosophila* snRNAs

Internal modifications	Predicted modification guide RNA			
	Name used here	GenBank accession no.	Alternative names used	References*
U1 snRNA				
U5 Ψ				
U6 Ψ				
U22 No	pug28S-3327	AJ544026, AJ629274, AJ629275, AJ629276	Dm-586, SnoR586, Ψ28S-3327	b, c
U2 snRNA				
G25 2'-O-methyl	mgU2-25	BK006790		e
C28 2'-O-methyl	mgU2-28	AJ543999	Dm-700, SnoR700, DmmgU2-28, MeU2-C28	b, c, d, e
A31 No	mgU2-31	AJ784390	MeU2-A31	f
G34 2'-O-methyl				
U35 Ψ				
U38 Ψ				
U40 Ψ				
C41 2'-O-methyl	mgU2-41	BK006791		e
U42 Ψ				
U44 Ψ				
U45 Ψ				
U48 2'-O-methyl				
U55 Ψ				
U4 snRNA				
U36 Ψ				
U59 No	pug28S-3186	AJ809574, AJ544025	Dm-165, SnoR165, Ψ28S-3186	b, c
A65 2'-O-methyl	mgU4-65	BK006792		e, this study
U79 Ψ				
U5 snRNA				
U32 Ψ				
G38 2'-O-methyl	mgU5-38	BK006793		e
U42 2'-O-methyl	mgU5-42	AJ544002	Dm-755, SnoR755, MeU5-U42	b, c, e
U44 Ψ				
C46 2'-O-methyl	U85	AF308282	MeU5-C46	a, c
U47 Ψ				
U54 Ψ				

The first two columns list all known or predicted internal modifications in *Drosophila* snRNAs U1, U2, U4, and U5. Those in boldface are reported here for the first time. Those designated "No" were not detected in our assays. They are listed because theoretical guide RNAs for them have been described in the literature. Columns 3–6 list all predicted *Drosophila* modification guide RNAs, with the names used here, the GenBank accession number, alternative names used in other articles, and sources in which they are cited. In the notation used here "mgU2-25" stands for "methyl guide for position 25 in U2 snRNA" and pug28S-3327 stands for "pseudouridine guide for position 3327 in 28S ribosomal RNA."

* See Reference list: a. Jádý and Kiss, 2001; b. Yuan *et al.*, 2003; c. Huang *et al.*, 2005a; d. Huang *et al.*, 2005b; e. Tycowski *et al.*, 2009; f. FlyBase, 2009 (<http://flybase.bio.indiana.edu/>).

sumably directs the corresponding modifications of *Drosophila* U5 snRNA at C46 and U47. Two box H/ACA and several box C/D RNAs have been described as putative guide RNAs for modification of *Drosophila* snRNAs (Yuan *et al.*, 2003; Huang *et al.*, 2005a,b; Tycowski *et al.*, 2009; Table 1). Independently, we identified one of these *Drosophila* guide RNAs, mgU4-65. Aside from U85, however, the cytological localization of the *Drosophila* guide RNAs has not been reported. Moreover, their function as guide RNAs to direct specific snRNA modifications remains to be demonstrated.

Posttranscriptional Nucleotide Modifications in *Drosophila* snRNAs

Modifications in *Drosophila* U5 snRNA have been described in detail, but less is known about modifications in *Drosophila* U1, U2, and U4 snRNAs (Myslinski *et al.*, 1984; Reddy and Busch, 1988; Szkukalek *et al.*, 1995; Huang *et al.*, 2005b). To obtain a more complete list of modifications, we carried out primer extension reactions with fluorescently labeled oligonucleotides, followed by separation on capillary columns.

Our data confirm previously described modifications in *Drosophila* U1, U2, U4, and U5 snRNA. In addition we identified 11 new modifications in U2 and U4 (Table 1 and Figures 1–3; Supplemental Figure S1). We did not find any stage-specific modifications or differences in modification patterns between samples (Figures 1–3).

Significantly, we failed to detect modifications at certain sites for which there are putative guide RNAs (Table 1). Thus, we did not see pseudouridylation of U1 snRNA at U22 (Figure 1, C and D). The putative guide RNA for this modification, pug28S-3327, is quite abundant by Northern blot analysis (Yuan *et al.*, 2003) and concentrates in nucleoli (data not shown). We also failed to detect 2'-O-methylation at the A31 position in *Drosophila* U2 snRNA isolated from several developmental stages (Figure 2, B and D). In this case the theoretical guide RNA was not detected by RT-PCR from total RNA, again isolated from several different stages (data not shown). Finally, we did not see pseudouridylation at U59 in U4 snRNA isolated from different stages (Figure 3, C and D), even though the putative guide RNA, pug28S-3186, is abundant (Yuan *et al.*, 2003).

Figure 1. Mapping of 2'-O-methyl groups and pseudouridines in *Drosophila* U1 snRNA by primer extension. Terminations of the primer extension reaction occur one nucleotide downstream of the actual modification and appear as peaks above the baseline. The strong peak at the 5' end represents the end of the template molecule. The X-axis shows fragment length in nucleotides determined by the migration of size standards. The Y-axis shows peak intensities in arbitrary units (traces are shifted vertically to avoid overlap). Details are given in *Materials and Methods*. (A) Sequencing reaction run on in vitro-transcribed U1 snRNA allows the position of terminations to be determined. (B) There are no detectable methylation peaks in U1 snRNA derived from adult control flies (*y w*), two *coilin*-null mutants (*coil199* and *coil203*), or in vitro-transcribed U1 snRNA. (C) There are two strong pseudouridine peaks near the 5' end of U1 snRNA from adult *y w* and mutant flies (stars at $\Psi 5$ and $\Psi 6$), but not in the control in vitro-transcribed U1 snRNA (black). Multiple small peaks in all the traces represent terminations caused by uridine itself. (D) U1 snRNA derived from S2 cells, larvae, and pupae shows the same two pseudouridine peaks as U1 derived from adult flies (stars at $\Psi 5$ and $\Psi 6$). Note the absence of pseudouridylation at position U22 in all samples.

In Vitro Assays to Test the Predicted Role of Novel Box C/D RNAs in snRNA Modification

We used *Xenopus* oocytes and their GVs for two assays of snRNA modification. In the first assay we injected in vitro-transcribed *Drosophila* snRNAs into the cytoplasm of *Xeno-*

Figure 2. Mapping of 2'-O-methyl groups and pseudouridines in *Drosophila* U2 snRNA. Conditions as in Figure 1. (A) Sequencing reaction on in vitro-transcribed U2 snRNA. (B) Six 2'-O-methyl groups are detectable in U2 snRNA derived from *y w* and *coilin*-null mutant flies (stars at Am1, Gm25, Cm28, Gm34, Cm41, and Um48). (C) Seven pseudouridine modifications are detectable in U2 snRNA derived from *y w* and *coilin*-null flies (stars at $\Psi 35$, $\Psi 38$, $\Psi 40$, $\Psi 42$, $\Psi 44$, $\Psi 45$, and $\Psi 55$). (D) U2 snRNA derived from S2 cells, larvae, and pupae shows the same six 2'-O-methyl peaks as U2 derived from adult flies (stars at Am1, Gm25, Cm28, Gm34, Cm41, and Um48). (E) U2 snRNA from *y w* flies shows no peaks when treated with high concentration of dNTP during the extension reaction. Note the absence of methylation at A31 in all samples.

pus oocytes. We isolated GVs from the injected oocytes after overnight incubation and tested for 2'-O-methylation of the injected RNAs by primer extension. By choosing suitable

Figure 3. Mapping of 2'-O-methyl groups and pseudouridines in *Drosophila* U4 snRNA. Conditions as in Figure 1. (A) Sequencing reaction on in vitro-transcribed U4 snRNA. (B) Two 2'-O-methyl groups are detectable in U4 snRNA derived from *yw* and coilin-null mutant flies (stars at Am1 and Am65). (C) Two pseudouridine modifications are detectable in U4 snRNA derived from *yw* and coilin-null flies (stars at Ψ36 and Ψ79). The prominent peak at A65 (no star) is due to methylation at this site, which can cause termination of the extension reaction in CMC-treated RNA. (D) U4 snRNA derived from S2 cells, larvae, and pupae shows the same two pseudouridine peaks as U4 derived from adult flies (stars at Ψ36 and Ψ79). (E) U4 snRNA from *yw* flies shows one tiny peak (Am65) when treated with high concentration of dNTP during the extension reaction. Note the absence of pseudouridine at U59 in all samples.

oligonucleotides as primers, we could selectively distinguish the injected *Drosophila* snRNA from endogenous *Xenopus* snRNAs. When in vitro-transcribed *Drosophila* U2, U4, or U5 snRNAs were injected into oocytes, they became methylated, showing that endogenous *Xenopus* guide RNPs

Figure 4. Modification of *Drosophila* U2 snRNA injected into *Xenopus* oocytes. When U2 was injected alone, it became methylated in the pattern expected for *Xenopus* U2 (black trace); that is, at sites common to the two species (stars at Cm25, Gm34, Cm41, and Um48), at *Xenopus*-specific sites (stars at Gm11 and Am30), but not at a *Drosophila*-specific site (star at Cm28). When U2 was coinjected with the *Drosophila* guide for C28 (mgU2-28), an extra peak corresponding to methylation at C28 was observed (blue trace). Methylation at C28 was also observed when the CAB box of its guide RNA was mutated (green trace; mgU2-28ΔCAB). Coinjection of U2 and the putative guide "mgU2-31" did not change the modification pattern (purple trace). If this guide RNA were functional, an additional peak at A31 would be present.

can recognize and direct the modification of in vitro-transcribed *Drosophila* snRNAs. U4 and U5 were modified in the pattern shared by *Xenopus* and *Drosophila* (data not shown). However, when *Drosophila* U2 snRNA was injected into oocytes, it was methylated in the pattern expected for *Xenopus* U2 rather than that of *Drosophila* U2. Thus, 2'-O-methylation occurred at two *Xenopus*-specific sites (A30 and G11) and at all sites shared between *Xenopus* and *Drosophila*. Significantly, the *Drosophila*-specific methylation at C28 was not detected (Figure 4). The predicted guide RNA for methylation of C28 in *Drosophila* is mgU2-28. Thus, we could test the ability of *Drosophila* mgU2-28 to function as a guide RNA by injecting it into the *Xenopus* oocyte along with unmodified *Drosophila* U2 snRNA. In this case we injected U2 into the cytoplasm and mgU2-28 directly into the GV. After overnight incubation the injected U2 was tested for modification by primer extension. We detected an extra peak corresponding to 2'-O-methylation of C28 (Figure 4). This experiment demonstrates that in vitro-transcribed *Drosophila* mgU2-28 can be assembled into an efficient modification RNP in the *Xenopus* GV and act as the guide for its predicted site in *Drosophila* U2 snRNA. As a negative control we injected a 30-nt fragment of mgU2-28 that contains only the region complementary to the U2 target (underlined) and adjacent sequences (GgUUACCUUGCACUUUGAUCGCGU-GAGGCAG). This truncated RNA did not support methylation at C28 (data not shown).

Our second assay for snRNA modifications involves a *Xenopus* GV extract to which we add unmodified *Drosophila* snRNAs and putative guide RNAs. It has been known for a long time that intact GVs isolated in mineral oil (as opposed to a saline solution) retain physiological activity for at least 16 h, including RNA transcription (Lund and Paine, 1990; Paine *et al.*, 1992) and modification of injected U2 snRNA (Yu *et al.*, 2001). When we work with a GV extract, as opposed to intact GVs, we can remove endogenous *Xenopus* RNAs with micrococcal nuclease. Thus we can use a depleted *Xenopus* GV extract to examine the modification of in vitro-transcribed *Drosophila* snRNAs by in vitro-transcribed guide RNAs. In this way we can directly test whether a

Figure 5. Modification of *Drosophila* U2 snRNA in RNA-depleted *Xenopus* GV extract. In each case in vitro-transcribed U2 snRNA was added to the extract along with a putative in vitro-transcribed guide RNA. (A) Addition of guide RNA mgU2-41 resulted in a peak of methylation at C41 (yellow trace, star) that was higher than in the control without the guide (black trace). (B) Guide RNA mgU2-28 produced a strong methylation peak at C28 (blue trace, star). (C) Methylation at C28 was also observed when the CAB box of guide RNA mgU2-28 was mutated (mgU2-28ΔCAB, green trace, star). (D) The putative guide RNA mgU2-25 had no effect on methylation (red trace). (E) The putative guide RNA “mgU2-31” had no effect on methylation (purple trace). (F) Modification of *Drosophila* U4 snRNA in RNA-depleted *Xenopus* GV extract. Each trace represents a primer extension reaction in which in vitro-transcribed U4 snRNA was added to the extract. Addition of guide RNA mgU4-65 resulted in a peak of methylation at A65 (green trace, star). No peak was seen in the control without the guide (red trace) or in a reaction with the guide but carried out at high concentration of dNTPs (black trace).

putative guide RNA mediates a specific modification. A modification reaction was scored as positive if the peak corresponding to the predicted modification was higher than the control (no guide RNA) when the primer extension reaction was performed at low dNTP concentration, but was similar to the control at high dNTP concentration. The in vitro method was successful with U2 and U4 snRNAs, but for some reason in vitro-transcribed U5 snRNA was unstable when incubated in *Xenopus* GV extracts. We examined five putative guide RNAs using the in vitro method, three of which gave positive results.

mgU2-28. Methylation at C28 is *Drosophila* specific, not being found in vertebrate U2. This modification was carried out by *Drosophila* mgU2-28 in the in vitro assay (Figure 5B), as it was in the injection experiment described earlier (Figure 4).

mgU2-41. Methylation at C41 occurs normally in *Drosophila* U2 (Figure 2, B and D). The modification was carried out by *Drosophila* mgU2-41 in the in vitro assay (Figure 5A).

mgU4-65. Methylation at A65 occurs normally in *Drosophila* U4 (Figure 3B). The modification was carried out by mgU4-65 in the in vitro assay (Figure 5F).

mgU2-25. Methylation of *Drosophila* U2 at G25 was detectable in all RNA samples that we analyzed from several different stages and strains of flies (Figures 2, B and D). Furthermore, the putative guide RNA, mgU2-25 is easily demonstrable by Northern blotting (Tycowski *et al.*, 2009; Figure 6). Nevertheless, we failed to detect this modification when in vitro-transcribed U2 was incubated with in vitro-transcribed mgU2-25 (Figure 5D). It is possible that some posttranscriptional modifications must occur in a defined sequence and that a partially modified substrate might be required for mgU2-25 to function. Alternatively, methylated G25 may fail to arrest reverse transcriptase in the primer extension reaction. We noted that the G25 modification was difficult to detect in *Drosophila* U2 that had been injected into *Xenopus* oocytes (Figure 4). Similarly, methylation of G25 (and G19) was not detected in an earlier study in which

transiently expressed human U2 was analyzed in simian COS-7 cells (Jády *et al.*, 2003).

mgU2-31 (MeU2-A31). FlyBase (<http://flybase.org>) lists snoRNA:MeU2-A31 as a putative guide RNA for methylation of A31 in U2 snRNA. However, we were not able to detect an endogenous RNA corresponding to this sequence by RT-PCR, nor did we see the A31 modification itself in U2 RNA isolated from flies at different stages (Figure 2, B and D). Thus we were not surprised to find that in vitro-transcribed MeU2-A31 failed to support modification of U2 in our in vitro assays, both by injection and in GV extract (Figures 4 and 5E). Furthermore, the predicted sequence of this box C/D RNA does not have canonical C- and D-box motifs or typical secondary structure. For these reasons we think it is unlikely that the sequence identified as MeU2-A31 is a genuine guide RNA in *Drosophila*.

Figure 6. Northern blots of *Drosophila* scaRNAs. For each scaRNA there are three lanes that contain total RNA from adult *y w*, *coil*¹⁹⁹, and *coil*²⁰³ flies, respectively. Both *coil*¹⁹⁹ and *coil*²⁰³ are coilin-null mutants that have no detectable CBs in their cells; *y w* flies serve as controls. In each case the abundance of the scaRNA is approximately the same for mutant and control flies. Seven Northern blots were run separately with their own size markers. Images of the autoradiographs were grouped into one figure with a common size scale in nucleotides.

Figure 7. Confocal images of Malpighian tubule nuclei after two-color FISH for scaRNAs and their cognate snRNAs. The three images in each column show nuclei hybridized with a probe for the scaRNA listed at the top of the column (red probe). The six nuclei in each row were hybridized with U85 scaRNA (A) or the cognate snRNA for the guide RNA (B and C). For instance, the first nucleus in row A was hybridized with probes for mgU2-25 scaRNA (red) and U85 scaRNA (green), whereas the last nucleus in row B was hybridized with probes for mgU5-42 (red) and U5 snRNA (green). Nuclei in rows A and B are from control *y w* flies and show an easily detectable CB that is labeled with both probes. Nuclei in row C are from *coilin*-null flies in which a CB is not detectable. All nuclei are stained with the DNA-specific dye DAPI (blue).

Box C/D RNAs That Guide Modifications in snRNAs U2, U4, and U5 Are Concentrated in CBs

Drosophila box C/D guide RNAs possess a conserved motif called the CAB box, which is assumed to be a CB-specific localization signal (Tycowski *et al.*, 2009). To determine whether these RNAs are all found in the CB of *Drosophila*, we carried out two-color FISH experiments, using *Drosophila* U85 scaRNA as a CB marker. In earlier studies we showed that U85 is specifically localized in the CB of various somatic and germline cells (Liu *et al.*, 2006b, 2009). In every case the guide RNAs were colocalized with U85 in the CB. Representative images of Malpighian tubule nuclei are shown in Figure 7A. In addition we carried out two-color FISH experiments using the guide RNAs and their corresponding target snRNAs. Again, we saw colocalization in the CB of Malpighian tubule cells (Figure 7B). Unlike scaRNAs, which can be detected only in the CB, the snRNAs are concentrated in the CB but also occur diffusely throughout the nucleoplasm (Figure S2). We also carried out two-color FISH experiments with similar results on a variety of other tissues, including adult ovary and testis, as well as larval salivary glands, Malpighian tubules, fat bodies, and brains (data not shown).

CB Localization Is Not Required for Modification of snRNAs

The high concentration of guide RNAs and their corresponding snRNA substrates in the CB suggests that modification itself normally occurs in the CB. If this is the case, the question arises whether CB localization is required for snRNA modification. To address this issue, we examined snRNA modification in flies mutant for the gene that codes for coilin protein. Coilin is widely used as a marker for the CB and occurs at high concentration in *Drosophila* CBs. We recently described two coilin mutants (*coil*¹⁹⁹ and *coil*²⁰³) that are homozygous viable and fertile despite being completely null for coilin. These flies not only lack coilin, but they fail to form distinct foci of other CB markers, including fibrillarin, the survival motor neuron (SMN) protein, U2, U4, and U5 snRNA, and U85 scaRNA (Liu *et al.*, 2009; Figure 7C). Distinct foci are also lacking for the Box C/D scaRNAs described here: mgU2-25, mgU2-28, mgU2-41, mgU4-65, mgU5-38, and mgU5-42 (Figure 7C). Despite their failure to concentrate in cytologically detectable CBs, the guide RNAs occur at normal levels in tissues of the coilin null mutants, as shown by Northern blots (Figure 6). Furthermore, snRNAs from the mutants are modified at all of the usual sites (Figures 1, B and C, 2, B and C, and 3, B and C; Supplemental Figure S1). These data show that efficient posttranscriptional

modification of snRNAs does not depend on concentration of the snRNAs or their modification guide RNAs in cytologically detectable CBs. Furthermore, snRNA modification in coilin-null flies shows that coilin itself is not involved in the modification process, as previously demonstrated in cells from coilin knockout mice (Jády *et al.*, 2003).

The CAB Box Is Not Required for Modification of an snRNA

Earlier experiments showed that the CAB box of U85 scaRNA is required for appropriate localization to the CB (Richard *et al.*, 2003). Furthermore, localization of human scaRNA ACA57 to the CB was recently shown to require binding of a conserved WD40 protein to the CAB box (Tycowski *et al.*, 2009). These experiments raise the question whether the CAB box and the WD40 protein might also be required for the modification reactions themselves. Our in vitro modification assays allow us to test this question directly. We synthesized a mgU2-28 molecule in which the *Drosophila* CAB box (CGC-CUUAAG) was replaced with the sequence CGCG. When injected into *Xenopus* oocytes along with unmodified U2 snRNA, the mutated form of mgU2-28 was fully functional as a guide RNA for methylation of C28 (Figure 4). Similarly, the mutated mgU2-28 was functional in our second assay system, which uses a cell-free extract made from oil-isolated GVs (Figure 5C). Thus, the CAB box is not required for mgU2-28 to function as a guide RNA. It is probable, therefore, that the WD40 protein does not play a direct role in the modification reaction.

DISCUSSION

It has been known for a long time that rRNA and splicing snRNAs undergo 2'-O-methylation and pseudouridylation at specific nucleotides. These modifications are controlled by guide RNAs acting in concert with a corresponding methyltransferase (fibrillarin) and pseudouridylylase (NAP57/dyskerin; reviewed in Reddy and Busch, 1988; Matera *et al.*, 2007). It is generally believed that modification of rRNA takes place in the nucleolus. This view is consistent with the fact that rRNA is transcribed in the nucleolus, where it is rapidly assembled into ribosomal subunits and permanently exported to the cytoplasm. On the other hand, four of the snRNAs, U1, U2, U4, and U5, are transcribed in the nucleus and exported to the cytoplasm for assembly into snRNP particles, and then they return to the nucleus. Thus the cytological site of their modification is not obvious from the maturation pathway.

That modification of U2 snRNA occurs in the nucleus was shown by injection experiments using *Xenopus* oocytes (Yu *et al.*, 2001) and confirmed by transient expression of U2 constructs in COS-7 cells (Jády *et al.*, 2003). Specific evidence for involvement of the CB in snRNA modification came from the discovery of U85 scaRNA (Jády and Kiss, 2001) and the demonstration that it and other scaRNAs are localized in the CB of HeLa cells (Darzacq *et al.*, 2002; Jády *et al.*, 2003; Richard *et al.*, 2003). Localization in the CB is mediated by an evolutionarily conserved sequence (UGAG), referred to as the CB-specific localization sequence or CAB box. This sequence was identified in U85 and several other scaRNAs from human, *Drosophila*, and *Arabidopsis* (Richard *et al.*, 2003). Recently, several new box C/D scaRNAs have been described from *Drosophila*. These contain a related but somewhat different CAB box, which was shown to bind a conserved WD40 protein. Furthermore the CB localization of human ACA57 scaRNA was found to depend on its association with the WD40 protein (human WDR79; Tycowski *et al.*, 2009).

The conventional view, therefore, is the following. U1, U2, U4, and U5 are transcribed in the nucleus and quickly exported to the cytoplasm, where they assemble into snRNP particles. They then return to the nucleus, specifically to the CB (Sleeman and Lamond, 1999). In the CB they undergo 2'-O-methylation and pseudouridylation, guided by scaRNAs. The scaRNAs themselves are targeted to the CBs by their CAB boxes in association with the WD40 protein.

Elegant experiments from the Kiss laboratory provided strong evidence that snRNA modification takes place in the CB itself. Their key experiments involved targeting short fragments of snRNAs to the CB and to the nucleolus. Fragments of U2 and U5 snRNA were targeted to the CB by inserting them into the coding region of U87 scaRNA and transfecting into mouse cells. Localization of the construct in the CB was demonstrated by FISH, and modification at the expected positions in the fragments was revealed by modification mapping (Jády *et al.*, 2003). On the other hand, when the U2 and U5 fragments were targeted to the nucleolus by insertion into the mouse pW ribosomal minigene, they did not become modified (Ganot *et al.*, 1999; Jády *et al.*, 2003). These experiments demonstrate that the activity responsible for modification of U2 and U5 snRNAs is absent from the nucleolus. They also show clearly that modification occurs when the U2 or U5 fragment is attached to a scaRNA (U87), some of which is detectable in the CB. We would argue that the experiment does not formally rule out the possibility that some of the modification machinery exists at lower concentration in the nucleoplasm, where it could be functional.

We will discuss evidence from three sources that modification of snRNAs can occur in the nucleoplasm: coilin mutants, *Xenopus* GVs, and scaRNAs in the nucleoplasm.

Coilin Mutants

Our earlier studies showed that almost all cells in wild-type *Drosophila* have prominent CBs detectable with probes for a variety of standard CB components, including coilin, fibrillarin, SMN, U2 and U5 snRNAs, and U85 scaRNA (Liu *et al.*, 2006b, 2009). In the present study we show concentration of six additional scaRNAs in *Drosophila* CBs: mgU2-25, mgU2-28, mgU2-41, mgU4-65, mgU5-38, and mgU5-42. By showing colocalization of the snRNAs with their guide RNAs, these observations on wild-type flies fit the hypothesis that snRNA modification itself occurs in the CB.

Questions began to arise when we first obtained two coilin-null mutants, *coil*¹⁹⁹ and *coil*²⁰³. CBs were not detectable in cells of the mutants using antibody or FISH probes

for any of the usual CB components (Liu *et al.*, 2009 and Figure 7C). Because the mutant flies were fully viable and fertile, it seemed unlikely that they were deficient in important aspects of U snRNP biogenesis. The results presented here show that they are not: scaRNAs are present at the same level as in wild-type flies (Figure 6), and more importantly, U1, U2, U4, and U5 snRNAs are properly modified in the mutants (Figures 1–3; Supplemental Figure S1). Clearly, therefore, the modification machinery exists and is functional in *Drosophila* cells that lack CBs by standard morphological and molecular criteria.

Coilin-null mutants are also known for two other organisms, *Arabidopsis* and the mouse. As in *Drosophila*, the *Arabidopsis* coilin mutant (*no cajal body-1* or *ncb-1*) lacks morphologically detectable CBs, yet the plant is perfectly viable (Collier *et al.*, 2006). Biochemical data are not available, but the absence of a gross organismal phenotype suggests that splicing is not seriously affected, as it would be if snRNA modification were impaired (Yu *et al.*, 1998; Zhao and Yu, 2004, 2007). The evidence from the coilin knockout mouse is less clear. These mice exhibit a semilethal phenotype in which some individuals die as embryos, and the surviving adults have significant fertility and fecundity defects (Walker *et al.*, 2009). Cultured cells derived from the knockouts exhibit three types of “residual” bodies, each of which contains a subset of CB components. In principle, therefore, snRNA modification could take place in the residual bodies that contain snRNAs and their guide RNAs (Tucker *et al.*, 2001; Jády *et al.*, 2003).

Xenopus GV

The *Xenopus* GV provides additional evidence that scaRNAs can function in the nucleoplasm. Shortly after the first description of scaRNAs, a *Xenopus* H/ACA guide RNA was found that directs pseudouridylation of U2 snRNA at two different sites. This guide RNA, designated pugU2-34/44, was localized in the nucleoplasm of the GV, not in the nucleoli or CBs (Zhao *et al.*, 2002). The corresponding human guide RNA is scaRNA U92 (Darzacq *et al.*, 2002). Our experiments with *Xenopus* GV extract demonstrate clearly that snRNA modification can take place in the nucleoplasm in the absence of CBs. In this case, GVs are collected from living oocytes under oil and centrifuged to remove the lampbrush chromosomes, nucleoli, CBs, and other nuclear organelles. The resulting extract can be depleted of endogenous snRNAs and scaRNAs by treatment with micrococcal nuclease. The extract supports modification of in vitro-transcribed *Drosophila* U2 and U4 snRNAs by *Drosophila*-specific guide RNAs. Modification even takes place when the guide RNA mgU2-28 lacks the canonical CB localization sequence (CAB box). These data show conclusively that modification can take place in the nucleoplasm of *Xenopus* GVs and that the CB localization signal itself is not required.

scaRNAs in the Nucleoplasm?

How can the data from coilin mutants and the *Xenopus* GV be reconciled with the strong evidence that CBs are involved in snRNA modification? We believe that the answer is reasonably straightforward. We suggest that under normal physiological conditions scaRNAs occur both in CBs and in the nucleoplasm. Because their concentration is high in CBs, they are detectable there by in situ hybridization, whereas their low concentration in the nucleoplasm puts them below the usual level of detection. This hypothesis simply extends to scaRNAs what is well known for other CB components, particularly from FRAP (fluorescence recovery after photobleaching) experiments (Handwerker *et al.*, 2003; Deryush-

eva and Gall, 2004; Dundr *et al.*, 2004). Even coilin, the canonical CB marker, is widely distributed in the nucleoplasm, although it is concentrated in CBs (Bellini and Gall, 1998; Matera, 1998). We suggest that technical issues lead to the mistaken impression that scaRNAs are limited to CBs and by extension must function there.

This impression is further fostered by the common assertion that CBs are “universal nuclear organelles,” of which we are as guilty as any (Gall, 2003). CBs have been identified in many eukaryotic organisms including mammals, amphibians, insects, angiosperm plants, and probably budding yeast. In the sense of *phylogenetic* distribution, it is probably safe to speak of CBs as universal nuclear organelles. On the other hand, it is easy to find individual cells or cell types that lack morphologically detectable CBs, and in this important sense CBs are not universal organelles. In the first articles to describe coilin, the authors examined five human and one marsupial cell lines, and five tissue types from the mouse. The number of CBs per nucleus varied from 0 to 6, with up to 20% of some cell types having no detectable body (Andrade *et al.*, 1991; Raska *et al.*, 1991). In a recent study only 15% of human WI-38 cells showed focal staining for coilin (Ghule *et al.*, 2008). CBs are absent from spermatocytes and from germline stem cells in the female germarium of *Drosophila* (Liu *et al.*, 2009). CBs are absent or are difficult to demonstrate in some adult vertebrate tissues (Young *et al.*, 2000), whereas they are prominent in others, such as nerve cells (Pena *et al.*, 2001). It is reasonable to suppose that snRNA modification and splicing occur in all of these normal cells that lack CBs. Thus, general considerations have never favored the view that specific nuclear functions must invariably take place in cytologically defined CBs.

Concluding Remarks

We suggest that scaRNAs and other CB components normally exist in the nucleoplasm as macromolecular complexes, which are too small to be resolved individually by conventional light microscopy, but appear as background fluorescence after immunostaining or FISH. These complexes can assemble into larger aggregates, which are then resolved as CBs of various sizes (Kaiser *et al.*, 2008). Coilin is essential for the assembly process, as shown by the absence of CBs in coilin-null *Drosophila*, *Arabidopsis*, and mouse. However, assembly into cytologically detectable CBs is not required for function, at least not for the scaRNA-dependent modification of splicing snRNAs.

Given the fact that cells can manage without CBs, the question remains: what role do CBs play when they are present? A common suggestion is that CB formation leads to an increase in effective concentration of macromolecules and hence increased activity based on mass action. Another possibility is that CBs might sequester specific components and thereby regulate the rate of reactions in the nucleoplasm. Future studies will need to address the extent to which CBs act as positive or negative regulators of specific biochemical reactions.

ACKNOWLEDGMENTS

We thank Allison Pinder and Alison Singer for technical help. This work was supported by National Institutes of Health Grant GM-33397 to J.G.G. J.G.G. is American Cancer Society Professor of Developmental Genetics.

REFERENCES

- Andrade, L.E.C., Chan, E.K.L., Raska, I., Peebles, C. L., Roos, G., and Tan, E. M. (1991). Human autoantibody to a novel protein of the nuclear coiled body: immunological characterization and cDNA cloning of p80-coilin. *J. Exp. Med.* 173, 1407–1419.
- Bellini, M., and Gall, J. G. (1998). Coilin can form a complex with the U7 small nuclear ribonucleoprotein. *Mol. Biol. Cell* 9, 2987–3001.
- Beumer, K. J., Trautman, J. K., Bozas, A., Liu, J.-L., Rutter, J., Gall, J. G., and Carroll, D. (2008). Efficient gene targeting in *Drosophila* by direct embryo injection with zinc-finger nucleases. *Proc. Natl. Acad. Sci. USA* 105, 19821–19826.
- Carmo-Fonseca, M., Tollervey, D., Barabino, S.M.L., Merdes, A., Brunner, C., Zamore, P. D., Green, M. R., Hurt, E., and Lamond, A. I. (1991). Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. *EMBO J.* 10, 195–206.
- Collier, S., Pendle, A., Boudonck, K., van Rij, T., Dolan, L., and Shaw, P. (2006). A distant coilin homologue is required for the formation of Cajal bodies in *Arabidopsis*. *Mol. Biol. Cell* 17, 2942–2951.
- Darzacq, X., Jády, B. E., Verheggen, C., Kiss, A. M., Bertrand, E., and Kiss, T. (2002). Cajal body-specific small nuclear RNAs: a novel class of 2'-O-methylation and pseudouridylation guide RNAs. *EMBO J.* 21, 2746–2756.
- Deryusheva, S., and Gall, J. G. (2004). Dynamics of coilin in Cajal bodies of the *Xenopus* germinal vesicle. *Proc. Natl. Acad. Sci. USA* 101, 4810–4814.
- Dundr, M., Hebert, M. D., Karpova, T. S., Stanek, D., Xu, H., Shpargel, K. B., Meier, U. T., Neugebauer, K. M., Matera, A. G., and Misteli, T. (2004). In vivo kinetics of Cajal body components. *J. Cell Biol.* 164, 831–842.
- FlyBase. (2009). A Database of *Drosophila* Genes & Genomes. MeU2–A31. FlyBase Annotation ID: CR34705. <http://flybase.org> (accessed 29 October 2009).
- Gall, J. G. (2003). The centennial of the Cajal body. *Nat. Rev. Mol. Cell Biol.* 4, 975–980.
- Gall, J. G., Callan, H. G., Wu, Z., and Murphy, C. (1991). Lampbrush chromosomes. In: *Xenopus laevis: Practical Uses in Cellular and Molecular Biology*, vol. 36, ed. B. K. Kay and H. B. Peng, San Diego, CA: Academic Press, 149–166.
- Ganot, P., Jády, B. E., Bortolin, M. L., Darzacq, X., and Kiss, T. (1999). Nucleolar factors direct the 2'-O-ribose methylation and pseudouridylation of U6 spliceosomal RNA. *Mol. Cell. Biol.* 19, 6906–6917.
- Ghule, P. N., Dominski, Z., Yang, X. C., Marzluff, W. F., Becker, K. A., Harper, J. W., Lian, J. B., Stein, J. L., van Wijnen, A. J., and Stein, G. S. (2008). Staged assembly of histone gene expression machinery at subnuclear foci in the abbreviated cell cycle of human embryonic stem cells. *Proc. Natl. Acad. Sci. USA* 105, 16964–16969.
- Grace, T. D. (1962). Establishment of four strains of cells from insect tissues grown in vitro. *Nature* 195, 788–789.
- Handwerger, K. E., Murphy, C., and Gall, J. G. (2003). Steady-state dynamics of Cajal body components in the *Xenopus* germinal vesicle. *J. Cell Biol.* 160, 495–504.
- Huang, Z. P., Zhou, H., He, H. L., Chen, C. L., Liang, D., and Qu, L. H. (2005a). Genome-wide analyses of two families of snoRNA genes from *Drosophila melanogaster*, demonstrating the extensive utilization of introns for coding of snoRNAs. *RNA* 11, 1303–1316.
- Huang, Z. P., Zhou, H., and Qu, L. H. (2005b). Maintaining a conserved methylation in plant and insect U2 snRNA through compensatory mutation by nucleotide insertion. *IUBMB Life* 57, 693–699.
- Jády, B. E., Darzacq, X., Tucker, K. E., Matera, A. G., Bertrand, E., and Kiss, T. (2003). Modification of Sm small nuclear RNAs occurs in the nucleoplasmic Cajal body following import from the cytoplasm. *EMBO J.* 22, 1878–1888.
- Jády, B. E., and Kiss, T. (2001). A small nucleolar guide RNA functions both in 2'-O-ribose methylation and pseudouridylation of the U5 spliceosomal RNA. *EMBO J.* 20, 541–551.
- Kaiser, T. E., Intine, R. V., and Dundr, M. (2008). De novo formation of a subnuclear body. *Science* 322, 1713–1717.
- Kiss, T., and Jády, B. E. (2004). Functional characterization of 2'-O-methylation and pseudouridylation guide RNAs. *Methods Mol. Biol.* 265, 393–408.
- Liu, J.-L., Wu, Z., Nizami, Z., Deryusheva, S., Rajendra, T. K., Gao, H., Beumer, K. J., Carroll, D., Matera, A. G., and Gall, J. G. (2009). Coilin is essential for Cajal body organization in *Drosophila melanogaster*. *Mol. Biol. Cell* 20, 1661–1670.
- Liu, J. L., Buszczak, M., and Gall, J. G. (2006a). Nuclear bodies in the *Drosophila* germinal vesicle. *Chromosome Res.* 14, 465–475.
- Liu, J. L., Murphy, C., Buszczak, M., Clatterbuck, S., Goodman, R., and Gall, J. G. (2006b). The *Drosophila melanogaster* Cajal body. *J. Cell Biol.* 172, 875–884.

- Lund, E., and Paine, P. L. (1990). Nonaqueous isolation of transcriptionally active nuclei from *Xenopus* oocytes. *Methods Enzymol.* **181**, 36–43.
- Matera, A. G. (1998). Of coiled bodies, gems, and salmon. *J. Cell Biochem.* **70**, 181–192.
- Matera, A. G., Terns, R. M., and Terns, M. P. (2007). Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat. Rev. Mol. Cell Biol.* **8**, 209–220.
- Matera, A. G., and Ward, D. C. (1993). Nucleoplasmic organization of small nuclear ribonucleoproteins in cultured human cells. *J. Cell Biol.* **121**, 715–727.
- Myslinski, E., Branlant, C., Wieben, E. D., and Pederson, T. (1984). The small nuclear RNAs of *Drosophila*. *J. Mol. Biol.* **180**, 927–945.
- Paine, P. L., Johnson, M. E., Lau, Y. T., Tluczek, L. J., and Miller, D. S. (1992). The oocyte nucleus isolated in oil retains in vivo structure and functions. *Biotechniques* **13**, 238–246.
- Pena, E., Berciano, M. T., Fernandez, R., Ojeda, J. L., and Lafarga, M. (2001). Neuronal body size correlates with the number of nucleoli and Cajal bodies, and with the organization of the splicing machinery in rat trigeminal ganglion neurons. *J. Comp. Neurol.* **430**, 250–263.
- Raska, I., Andrade, L.E.C., Ochs, R. L., Chan, E.K.L., Chang, C.-M., Roos, G., and Tan, E. M. (1991). Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. *Exp. Cell Res.* **195**, 27–37.
- Reddy, R., and Busch, H. (1988). Small nuclear RNAs: RNA sequences, structure, and modification. In: *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*, ed. M. L. Birnstiel, Berlin: Springer, 1–37.
- Richard, P., Darzacq, X., Bertrand, E., Jady, B. E., Verheggen, C., and Kiss, T. (2003). A common sequence motif determines the Cajal body-specific localisation of box H/ACA scaRNAs. *EMBO J.* **22**, 4283–4293.
- Sleeman, J. E., and Lamond, A. I. (1999). Newly assembled snRNPs associate with coiled bodies before speckles, suggesting a nuclear snRNP maturation pathway. *Curr. Biol.* **9**, 1065–1074.
- Spector, D. L. (1993). Nuclear organization of pre-mRNA processing. *Curr. Opin. Cell Biol.* **5**, 442–447.
- Szkukalek, A., Myslinski, E., Mougin, A., Luhrmann, R., and Branlant, C. (1995). Phylogenetic conservation of modified nucleotides in the terminal loop 1 of the spliceosomal U5 snRNA. *Biochimie* **77**, 16–21.
- Tucker, K. E., Berciano, M. T., Jacobs, E. Y., LePage, D. F., Shpargel, K. B., Rossire, J. J., Chan, E.K.L., Lafarga, M., Conlon, R. A., and Matera, A. G. (2001). Residual Cajal bodies in coilin knockout mice fail to recruit Sm snRNPs and SMN, the spinal muscular atrophy gene product. *J. Cell Biol.* **154**, 293–307.
- Tycowski, K. T., Shu, M. D., Kukoyi, A., and Steitz, J. A. (2009). A conserved WD40 protein binds the Cajal body localization signal of scaRNP particles. *Mol. Cell* **34**, 47–57.
- Walker, M. P., Tian, L., and Matera, A. G. (2009). Reduced viability, fertility and fecundity in mice lacking the Cajal body marker protein, coilin. *PLoS One* **4**, e6171.
- Wallace, R. A., Jared, D. W., Dumont, J. N., and Segal, M. W. (1973). Protein incorporation by isolated amphibian oocytes: III. Optimum incubation conditions. *J. Exp. Zool.* **184**, 321–333.
- Young, P. J., Le, T. T., Man, N. T., Burghes, A.H.M., and Morris, G. E. (2000). The relationship between SMN, the spinal muscular atrophy protein, and nuclear coiled bodies in differentiated tissues and cultured cells. *Exp. Cell Res.* **256**, 365–374.
- Yu, Y.-T., Terns, R. M., and Terns, M. P. (2004). Mechanisms and functions of RNA-guided RNA modification. In: *Topics in Current Genetics: Fine-Tuning of RNA Functions by Modification and Editing*, vol. 12, ed. H. Grosjean, Berlin: Springer, 223–262.
- Yu, Y. T., Shu, M. D., Narayanan, A., Terns, R. M., Terns, M. P., and Steitz, J. A. (2001). Internal modification of U2 small nuclear (sn)RNA occurs in nucleoli of *Xenopus* oocytes. *J. Cell Biol.* **152**, 1279–1288.
- Yu, Y. T., Shu, M. D., and Steitz, J. A. (1998). Modifications of U2 snRNA are required for snRNP assembly and pre-mRNA splicing. *EMBO J.* **17**, 5783–5795.
- Yuan, G., Klambt, C., Bachellerie, J. P., Brosius, J., and Huttenhofer, A. (2003). RNomics in *Drosophila melanogaster*: identification of 66 candidates for novel non-messenger RNAs. *Nucleic Acids Res.* **31**, 2495–2507.
- Zhao, X., Li, Z. H., Terns, R. M., Terns, M. P., and Yu, Y. T. (2002). An H/ACA guide RNA directs U2 pseudouridylation at two different sites in the branch-point recognition region in *Xenopus* oocytes. *RNA* **8**, 1515–1525.
- Zhao, X., and Yu, Y. T. (2004). Pseudouridines in and near the branch site recognition region of U2 snRNA are required for snRNP biogenesis and pre-mRNA splicing in *Xenopus* oocytes. *RNA* **10**, 681–690.
- Zhao, X., and Yu, Y. T. (2007). Incorporation of 5-fluorouracil into U2 snRNA blocks pseudouridylation and pre-mRNA splicing in vivo. *Nucleic Acids Res.* **35**, 550–558.