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Developmentally regulated expression and expression strategies of Drosophila snoRNAs

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ABSTRACT

Small nucleolar RNAs constitute a significant portion of the eukaryotic small ncRNA transcriptome and guide site-specific methylation or pseudouridylation of target RNAs. In addition, they can play diverse regulatory roles on gene expression, acting as precursors of smaller fragments able to modulate alternative splicing or operate as microRNAs. Defining their expression strategies and the full repertory of their biological functions is a critical, but still ongoing, process in most organisms. Considering that *Drosophila melanogaster* is one of the most advantageous model organism for genetic, functional and developmental studies, we analysed the whole genomic organization of its annotated snoRNAs – whose vast majority is known to be embedded in an intronic context – and show by GO term enrichment analysis that protein-coding genes involved in cell division and cytoskeleton organization are those mostly preferred as hosts. This finding was unexpected, and delineates an unpredicted link between snoRNA host genes and cell proliferation that might be of general relevance. We also defined by quantitative RT-PCR the expression of a representative subset of annotated specimens throughout the life cycle, providing a first overview on developmental profiling of the fly snoRNA transcriptome. We found that most of the tested specimens, rather than acting as housekeeping genes with uniform expression, exhibit dynamic developmental expression patterns; moreover, intronic snoRNAs harboured by the same host gene often exhibit distinct temporal profiles, indicating that they can be expressed uncoordinatedly. In addition to provide an updated outline of the fly snoRNA transcriptome, our data highlight that expression of these versatile ncRNAs can be finely regulated.

1. Introduction

A large portion of the eukaryotic small ncRNA transcriptome is composed of small nucleolar RNAs (snoRNAs), a group of abundantly expressed RNAs of variable length (from 60 to 300 nt, on average) present from Archaeal to mammalian cells. SnoRNAs can be classified into two major classes, named box C/D and H/ACA, on the basis of common sequence motifs, structural features and sets of proteins that associate to them to form the specific small nucleolar ribonucleoprotein complexes (snoRNPs; reviewed by Henras et al. (2004), Reichow et al. (2007), Watkins and Bohnsack (2012)). A few specimens from both classes are required for pre-rRNA endonucleolytic processing, while the majority directs, by base-pairing guiding mechanism, the two most common types of nucleotide modifications present on eukaryotic RNAs, namely pseudouridylation and ribose methylation (reviewed by Henras et al. (2004), Kiss et al. (2010)). H/ACA snoRNAs direct pseudouridylation and are characterized by a hairpin-hinge-hairpin-tail secondary structure, with the H box (ANANNA) in the hinge region and the ACA motif three nucleotides from the 3′ end of the molecule. Each hairpin contains an internal pseudouridylation pocket which guides the pseudouridine synthase (dyskerin in mammals, MFL in *Drosophila*), one of the four evolutively conserved core proteins composing the H/ACA snoRNPs, in the isomerisation of specific uridines on target RNAs (reviewed by Kiss et al. (2010)). C/D snoRNAs direct ribose methylation and display a simpler structure, characterized by the presence of consensus C (5′-RUGAUGA-3′) and D (5′-CUGA-3′) motifs close to the 5′ and 3′ termini of the molecule, respectively. Additional and often

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degenerated internal copies of C and D elements (designated C′ and D′) are commonly present. The D/D′ upstream regions act as antisense elements able to select the residue to modify through the formation of specific duplexes with the RNA target. In the methylation process, the methyltransferase catalytic activity is furnished by fibrillarin, one of the four evolutively conserved core proteins that compose the functional C/D snoRNPs (Henras et al., 2004). The role of these abundant types of RNA modifications was initially thought to be restricted to rRNAs, then extended to snRNAs (reviewed by Karijolich and Yu (2010)), and very recently demonstrated on mRNAs and other types of ncRNAs (Schwartz et al., 2014). Intriguingly, an increasing number of "orphan" snoRNAs which lacks known target has been identified from different organisms, and it is supposed that they play regulatory roles on gene expression. For example, diverse lines of evidence hint at subtle regulatory roles on splicing. First, a subgroup of snoRNAs, termed scaRNAs, do not localize in the nucleolus but in the Cajal bodies and is typically involved in the methylation and/or pseudouridylation of the Pol II-transcribed snRNAs (Darzacq et al., 2002; Richard et al., 2003). These post-transcriptional modifications are essential for either snRNA maturation, snRNP assembly and pre-mRNA splicing (reviewed by Karijolich and Yu (2010)). Furthermore, they can also be heat-induced, as shown for U2 snRNA (Wu et al., 2011). Second, an artificial box C/D RNA targeted to a branch–point adenosine proved to be capable of impairing the splicing of the targeted pre-mRNA (Semenov et al., 2008), while orphan snoRNAs were shown to target cellular mRNAs close to alternative splice junctions (Bazeley et al., 2008). Considering that pseudouridylation has recently been shown to occur also on mRNAs, in a conserved manner from yeast to humans (Schwartz et al., 2014), this regulatory role is likely to be expanded. Third, snoRNAs can be processed in shorter RNAs, termed processed-snoRNAs (psnoRNAs) or sno-derived-RNAs (sdRNAs), which can target pre-mRNAs and affect splicing (reviewed by Khanna and Stamm (2010), Falaleeva and Stamm (2013)). While these data indicate that snoRNAs can potentially influence alternative splicing by different ways, the repertory of functional roles potentially exerted by these ncRNAs expands well beyond splicing. For example, the degree of ribosomal pseudouridylation has been found to influence the efficiency of IRES-dependent mRNA translation (Yoon et al., 2006; Rocchi et al., 2013) and the translation fidelity, by generating frameshift and influencing the recognition of *in-frame* stop codons (Jack et al., 2011; Karijolich and Yu, 2011). Moreover, it has been suggested that snoRNAs can play still uncharacterized roles in epigenetic regulatory mechanisms (reviewed by Peters and Robson (2008), Royo and Cavaillé (2008)). Consistent with this view, a number of snoRNAs has been found associated to active chromatin in either *Drosophila* and human cells, further supporting a potential role in the modulation of chromatin conformation (Schubert et al., 2012; Schubert and Längst, 2013). Intriguingly, abundance of several *Drosophila* C/D snoRNAs was found to oscillate according to circadian rhythm (Hughes et al., 2012), suggesting a possible involvement in the response to light stimulus. Finally, snoRNAs proved to act as microRNA precursors in *Drosophila* as in several other organisms (Taft et al., 2009; Politz et al., 2009; Scott et al., 2009; Brameier et al., 2011), and there is now a convincing evidence that at least a subset of them can exert a dual regulatory function. These data collectively indicate that snoRNAs are versatile molecules able to play relevant tasks in developmental processes and in cell differentiation. *Drosophila melanogaster* can provide an advantageous insect model in which exploit powerful genetic tools to dissect the variety of snoRNA functions. Focussing on the *D. melanogaster* snoRNA transcriptome might allow comprehensive functional analyses and help to expand our knowledge in this interesting field.

With this aim, we scrutinized in detail the genomic organization of *Drosophila* annotated snoRNAs., examined by GO (Gene Ontology) analysis the biological functions of their protein-coding host genes (HG) and checked the conservation of the most significant clusters in the *Drosophila* genus. Moreover, we determined the developmental expression profiling of a representative subset of the fly snoRNA transcriptome, showing that they can be dynamically regulated during the life cycle.

2. Materials and methods

2.1. Databases interrogation

Drosophila snoRNA catalogue and classification were derived from the FlyBase website (McQuilton et al., 2012). A part from a few unclassified specimens and a single snoRNA reported to contain features of both families, all specimens were included within the H/ACA or the C/D family. We noted that *snoRNA:M* (de la Pena, 2001) and *snoRNA:Me28S-G980* (Huang et a., 2005), currently annotated in FlyBase as different genes, corresponded to the same sequence, and thus counted them as one in our analysis. In the Gene Ontology analysis (Amigo 1.8; Database 2014-10-25), terms used to identify ribosome related functions were: GO:0003735 structural constituent of ribosome; GO:0003743 translation initiation factor activity; GO:0006412 translation; GO:0042254 ribosome biogenesis. In the Gene Ontology Term enrichment analysis, all *Uhgs* (*Unknown host genes*) and 5 protein-coding HGs (listed in Supplementary Table 1 A) were excluded, since they were not associated to any function or biological process, and thus were not recognized by the tool.

2.2. RNA extraction

Canton S was used as wild-type strain in all experiments. Total RNA from 0 to 24 h mixed-stage embryos, mixed population of first-second-third instar larvae, mixed-stage pupae, and adults of both sexes at 4 days after eclosion, was extracted using TRI Reagent (Sigma) following manufacturer's instruction.

2.3. RNA analysis and quantitative real-time RT-PCR

RNA was extracted using TRI Reagent (Sigma) according to manufacturer's conditions. Then 10 μg were treated with TurboDNase (Ambion) and phenol:chloroform extracted; 1 μg RNA was reverse transcribed using SuperScript III RT (Invitrogen) using manufacturer's condition and diluted 1:10. To check for gDNA elimination, 1:10 dilutions of RT plus and minus reactions were used as template for amplification of 7SL-RNA by qualitative PCR using DreamTaq (Fermentas) applying manufacturer's condition; negative amplification of RT minus reaction was used to testify the complete digestion of gDNA. Quantitative real-time RT-PCR experiments were performed in triplicate using iQ5 Multicolor Real-Time PCR Detection System (Biorad) as previously described (Tortoriello et al., 2009). All PCR reactions were carried out in a final volume of 15 μl using 1 μl of diluted cDNA, 7.5 μl of 2X SYBR-Green (Biorad) and 5 pmol of each primer. Sequences of all utilised primers were designed using Primer 3 software (Untergrasser et al., 2012) and are available under request. RpL32 was used as endogenous control for samples normalization. Quantitative PCR analysis were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Oligonucleotides utilized for HGs analysis were designed to amplify fragments common to all annotated transcripts and to span exon/exon borders in order to avoid gDNA amplification. Each qRT-PCR experiment was run in biological triplicates.