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Molecular cloning and characterization of SL3: A stem cell-specific SL RNA from the planarian *Schmidtea mediterranea*

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Abstract

Spliced leader (SL) *trans*-splicing is a biological phenomenon, common among many metazoan taxa, consisting in the transfer of a short leader sequence from a small SL RNA to the 5' end of a subset of pre-mRNAs. While knowledge of the biochemical mechanisms driving this process has accumulated over the years, the functional consequences of such post-transcriptional event at the organismal level remain unclear. In addition, the fact that functional analyses have been undertaken mainly in trypanosomes and nematodes leaves a somehow fragmented picture of the possible biological significance and evolution of SL *trans*-splicing in eukaryotes. Here, we analyzed the spatial expression of SL RNAs in the planarian flatworm *Schmidtea mediterranea*, with the goal of identifying novel developmental paradigms for the study of *trans*-splicing in metazoans. Besides the previously identified SL1 and SL2, *S. mediterranea* expresses a third SL RNA described here as SL3. While, SL1 and SL2 are collectively expressed in a broad range of planarian cell types, SL3 is highly enriched in a subset of the planarian stem cells engaged in regenerative responses. Our findings provide new opportunities to study how *trans*-splicing may regulate the phenotype of a cell.

Keywords

Stem cells; SL RNA; Planarians; Regeneration; *Trans*-splicing

1. Introduction

Spliced leader (SL) *trans*-splicing is an inter-molecular splicing reaction in which the mini-exon of a small SL RNA is joined to the 5' end of pre-mRNA molecules (Lasda and Blumenthal, 2011) (Fig. 1A). This process was first discovered in the protozoan *Trypanosoma*, where the totality of transcripts shares a common 5'-terminal sequence of 35 nt (Boothroyd and Cross, 1982; Milhausen et al., 1984). Later, SL *trans*-splicing was found

in animals such as nematodes and flatworms (Krause and Hirsh, 1987; Rajkovic et al., 1990), leading to the suggestion that the process could be peculiar to lower invertebrate phyla (Davis, 1996). However, increasing sampling of genomic data from diverse species has clearly shown that SL *trans*-splicing is found in all major metazoan lineages (Pre-bilateria, Lophotrochozoa, Ecdysozoa and Deuterostomes) (Douris et al., 2010; Lasda and Blumenthal, 2011).

Despite such wide representation in the animal kingdom, SL *trans*-splicing has been studied at genetic and biochemical levels only in trypanosomes and nematodes (Blumenthal, 2005; Liang et al., 2003). In fact, a remarkable amount of research has been done in these organisms on the fundamental role of SL *trans*-splicing in resolving poly-cistronic transcripts (Evans et al., 2001), the biochemical requirements for SL *trans*-splicing (Hannon et al., 1991), the purification of factors associating with the SL ribonucleoprotein (RNP) (Denker et al., 2002), and the identification of translation factors binding the trimethylguanosine cap at the 5' of the SL (Fig. 1A) or mediating its methylation (Keiper et al., 2000; Liu et al., 2011; Takagi et al., 2007; Wallace et al., 2010). Yet, even with such systematic and rigorous molecular dissection, and with the exception of the role played by SL RNAs in operon processing, it is still unclear what the biological effect of adding an SL sequence to monocistronic transcripts in these organisms may be.

Given the current status of knowledge on this unresolved biological problem, we reasoned that the study of *trans*-splicing in a still understudied metazoan phylum may help inform our understanding of this RNA processing mechanism. Hence, we undertook a comprehensive molecular and cellular characterization of *trans*-splicing in the planarian *Schmidtea mediterranea*. These free-living flatworms are an emerging model system for stem cell biology and regeneration (Newmark and Sanchez Alvarado, 2002), and belong to the widely diverse group of rhabditophoran Platyhelminthes. Rhabditophorans display an extensive variety of body plans and physiology, and include marine worms (such as macrostomids and polyclads), freshwater planarians, as well as parasites (such as flukes and tapeworms) (Littlewood and Bray, 2001). Despite such vast diversity, *trans*-splicing has been retained in all the rhabditophoran lineages examined thus far and is most likely a plesiomorphy of the group (Cheng et al., 2006; Davis, 1997). On the contrary, other monophyletic but similarly highly diversified phyla such as Cnidaria, Arthropoda and Urochordata display a poor conservation of the spliced leader primary sequence as well as absence of *trans*-splicing altogether (Derelle et al., 2010; Douris et al., 2010; Ganot et al., 2004; Vandenberghe et al., 2001). In this respect, comparative studies of SL *trans*-splicing in Rhabditophorans could provide useful information about the biological constraints guiding the evolution of this process in metazoans. Moreover, flatworms occupy a very interesting phylogenetic position. In the classical view phylogeny, platyhelminthes were regarded as basal metazoans because of their “primitive characters” (e.g., lack of through gut, coelom, segmentation and circulatory system) (Hyman, 1951). However, based on molecular phylogeny, they were subsequently grouped in the Lophotrochozoa (Adoutte et al., 2000), and their simple traits are now regarded by the majority of scientists as secondary losses (De Robertis, 2008). Irrespective of which view may ultimately prevail, the study of *trans*-splicing in flatworms can either provide a snapshot of gene expression regulation in an ancestor-like metazoan, or

inform the evolutionary mechanisms that led to the disappearance of this process in other lophotrocozoan lineages.

The occurrence of SL *trans*-splicing in *S. mediterranea* was first documented in the framework of an EST sequencing project that led to the identification of two SL RNAs (SL1 and SL2) (Zayas et al., 2005a). Here, we report on the discovery of a novel planarian SL RNA that we have named SL3 and a detailed expression analysis of SL1, SL2 and SL3. Both EST sampling and deep sequencing data suggest that SL1 is the most abundant SL RNA in *S. mediterranea*. However, this RNA species cannot be distinguished from the highly similar SL2 via hybridization-based methodologies. As a result, we have studied the expression of these two SL RNAs collectively and showed their broad expression in the animal. In contrast, SL3 expression was strikingly enriched in a subset of planarian stem cells. Interestingly, SL3⁺ stem cells are recruited at wound sites during regeneration and take part in the reconstitution of the stem cell compartment after non-lethal irradiation (Wagner et al., 2011). To our knowledge, these observations represent the first detailed report of tissue-specific distribution of SL RNAs in a metazoan, and provide a novel experimental context in which to study the biological significance and evolutionary history of *trans*-splicing.

2. Material and methods

2.1. Planarians, irradiation and RNA extraction

The asexual clonal line CIW4 of *S. mediterranea* was reared and maintained as previously described (Eisenhoffer et al., 2008). Irradiation experiments were performed using a Gammacell 40 Exactor irradiator (Theratronics). Total RNA for Northern blots and SL3 cloning was extracted from whole or amputated worms using TRIzol (Invitrogen).

2.2. Northern blot analyses

20µg of total RNA was separated on a 1.8% formaldehyde agarose gel. The resolved RNA was blotted and cross-linked to a positively charged nylon membrane (Roche) and hybridized with kinase-labeled oligo probes encompassing the 42 nt-long mini-exon of SL1 or SL3. A probe specific for U6 snRNA (5' GCGTGTCATCCTTGCGCAGGGGCCATGCTAATCTTCTCTGTATCGT) was also used as a loading control. 20pmol of primer was labeled with 300 µCi of γ -ATP (6000 mCi/mmol). Hybridizations were carried out at 55 °C in 25 mL of hybridization buffer (10× Denhardt's, 6× SSC, 0.1% SDS). Excess probe was removed by three consecutive washes in 6× SSC/0.1% SDS at room temperature and one 20 min wash at 55 °C. The membrane was dried and exposed to a film (MS films, Kodak) for 2 h to 24 h at –80 °C. The blots were then stripped in 0.5% SDS at 60 °C for 1 h and hybridized with U6 probe as described above. Intensity of autoradiographic signals was quantified using ImageJ (rsbweb.nih.gov/ij/).

2.3. 5' and 3' RACE

5' and 3' RACE cloning of SL3 was carried on using a First Choice RLM-RACE Kit (Ambion) with some modifications. For 5' RACE total RNA was de-capped, linker ligated and primed with the oligo SL3RACEREVα (5' GGACCAGTCAAAAATTAGAC) for

cDNA synthesis. The reverse transcription reaction was carried on at 50 °C using Superscript III (Invitrogen). SL3RACEREVb and SL3RACEREVb (5' GATGAAAATTATCATCAAATTATTGTC) were used for amplification of the RACE product in two sequential nested PCR reactions. For 3' RACE, total RNA was polyadenylated with *Escherichia coli* Poly(A) Polymerase (New England Biolabs) and primed with a dT-anchor primer provided in the kit. SL3RACEFOR (5' GAATGGTAAGACTACGAAGAC) and anchor-specific primers were used to PCR amplify the RACE product. The obtained amplicons were cloned in a pCR4TOPO vector (Invitrogen) and sequenced. The full length SL3 sequence was deposited in GenBank with accession number JQ917423.

2.4. Bioinformatics

Approximately 86 million reads, averaging 100 nt in length, were obtained from a neoblast RNA-Seq survey (Labbe et al., 2012). Reads were searched for the last 13 nt of the known *S. mediterranea* spliced leaders. Random occurrence of the 13-mers in the RNA-Seq dataset was filtered out by removing the putative SL from the reads and adding a splicing acceptor site "AG" to their 5' ends. These sequences (AG + 25 nt downstream) were mapped to the genome assembly using BLAST (Altschul et al., 1990). Full-length alignments (starting from AG) were required to be scored as perfect matches.

The frequency of SL1, SL2 and SL3 *trans*-splicing events for each mapped acceptor site was estimated by counting the occurrence of the word "SL+ 25 nt downstream" in the RNA-Seq data. Transcriptome reads resulting from the usage of the putative *trans*-splicing acceptor sites in *cis*-splicing or retention of the outtron (an intron-like stretch upstream of the *trans*-splicing acceptor site that includes a branch site and is devoid of any 5' splice site region) (Conrad et al., 1993) were also counted. Putative *trans*-splicing events supported by less than 3 total reads at the acceptor site were discarded. This choice was based on the reads normal distribution as well as the distribution of experimentally cloned *trans*-spliced genes (NCBI library identifiers: LIBEST_027834; LIBEST_027835; LIBEST_027836; LIBEST_027837). Genes *trans*-spliced with a frequency inferior to 10% were also omitted by the annotation. To assess the statistical significance of the SL3 *trans*-splicing frequency at each acceptor site we used a binomial distribution probability that takes into account the number of total SL reads and the average probability of SL3 splicing. To identify gene annotations that correspond to each of the identified SL splice sites, MAKER predictions (Cantarel et al., 2008) located within 500 nt downstream of the splice sites were retrieved from the *S. mediterranea* genome database (Robb et al., 2008) (<http://smedgd.stowers.org>).

2.5. In situ hybridization and antibody staining

Wild type or irradiated animals were processed as previously described (Pearson et al., 2009). Riboprobes encompassing full-length SL1 and SL3 sequences and a previously described *smedwi-1* riboprobe (Eisenhoffer et al., 2008) were labeled by incorporation of digoxigenin (DIG) or dinitrophenol (DNP) rNTP (Roche). NBT and BCIP solutions (Roche) were used for chromogenic signal detection. Fluorescent detection was achieved by rhodamine and FITC tyramide amplification (Pearce). After fluorescent in situ hybridization the specimens were either incubated with DAPI (Molecular Probes) or processed for

immunostaining. The Y12 antibody (Lifespan Bioscience) was used at a 1:100 dilution. Antigen binding was detected via an Alexa 488-conjugated anti-mouse antibody (Invitrogen). For cryosectioning, worms stained via in situ hybridization were incubated in 30% sucrose PBS, embedded in OCT and sectioned at 10 μ m. Both whole-mount specimens and sections were counterstained with DAPI. Chromogenic in situ hybridizations were imaged with a Leica DM600 microscope or a Zeiss SteREO Lumar. Whole-mounted specimens processed via fluorescent in situ hybridizations were imaged with a Zeiss LSM-510-VIS, while sections were imaged with a Leica DM600. Experiments were quantified using ImageJ.

3. Results

3.1. SL3 is a novel SL RNA in *S. mediterranea*

SL *trans*-splicing (Fig. 1A) in *S. mediterranea* was first reported with the identification of two SL RNAs (SL1 and SL2) found in large cDNA collections (Zayas et al., 2005a). By further analyzing the set of planarian ESTs deposited in NCBI (Sanchez Alvarado et al., 2002; Zayas et al., 2005b), we noticed the presence of a possible third, novel spliced leader. The sequence of the parent SL RNA was inferred bioinformatically (Fig. S1), and its existence subsequently validated by 5' and 3' RACE cloning. We named this novel SL RNA SL3. While SL1 and SL2 display similar lengths and extensive nucleotide identity in both the spliced leader (95%) and the intronic portion (83%), SL3 is 19–20 nt longer and more divergent (Fig. 1B). Similar to most metazoan SL RNAs, SL3 is predicted to fold in a typical triple “stem and loop” structure (Fig. 1C). Also conserved is a single-stranded RNA stretch encompassing an Sm protein (Scofield and Lynch, 2008) binding site (Figs. 1B, C). In order to determine the abundance of SL3 relative to the other planarian SL RNAs, we performed Northern blot studies (Fig. 1D). While we could design probes specific to SL3, the sequence similarities between SL1 and SL2 precluded us from generating probes capable of discriminating between these two SL RNAs. However, based on EST sampling, SL2 was found to represent only a small fraction (~5%) of all *trans*-splicing events detected (Zayas et al., 2005a). Hence, we used SL1 for probe design, and because it will likely detect the minor SL2 fraction in the transcriptome, we refer to this probe as SL1/2. Both SL1/2 and SL3 probes hybridize to the leader portion, thereby detecting both the relative SL RNAs as well as a complex population of *trans*-spliced transcripts ranging in size from a few hundred bases to more than 3 Kb (Fig. 1D). Interestingly, the SL3 SL RNA was found to be expressed at comparatively lower levels than SL1/2 and accordingly, SL3-spliced mRNAs were also less abundant than the sum of SL1- and SL2-spliced transcripts (Fig. 1D; Fig. S2). This latter observation is in agreement with the relative paucity of SL3-spliced cDNAs deposited in NCBI (e.g., only one SL3 transcript in the nr database) as well as with the analysis of published deep sequencing data (Table S1) (Labbe et al., 2012).

Given that SL RNA genes are commonly amplified in animal genomes (Krause and Hirsh, 1987; Rajkovic et al., 1990; Yeats et al., 2010), and are often associated with 5S RNA and/or U snRNAs genes (Derelle et al., 2010; Douris et al., 2010; Ganot et al., 2004; Krause and Hirsh, 1987; Stover and Steele, 2001; Yeats et al., 2010), we explored the possibility that SL3 genes could be arranged in multi-copy arrays and in the vicinity of other non-

coding RNA genes. BLAST analysis of 1Kb reads from shotgun sequencing data of the unassembled *S. mediterranea* genome (Cantarel et al., 2008; Robb et al., 2008) detected 285 hits matching the full-length SL3 sequence with an identity of at least 95%. Given that the estimated coverage of the whole genome shotgun sequencing project was at least 10×, this result would be compatible with the presence of multiple copies of the SL3 gene. In addition, mining of the NCBI nr database revealed the presence in 140 reads of a U4 snRNA-like element in opposite orientation at a 325bp distance (Fig. S3). Future experiment involving Southern blotting, BAC library screening, PCR and chromosome FISH will help resolve the genomic organization of SL3.

3.2. SL3 expression is restricted to a mesenchymal cell population

The relatively low abundance of SL3 suggested the possibility that this SL RNA may be preferentially expressed in a small subset of cells in the animal. We tested this hypothesis by performing in situ hybridizations with riboprobes specific for SL3 and the more abundant SL1/2. Hybridization with the SL1/2 riboprobe yielded a broadly distributed punctate staining in the whole animal with a robust accumulation of signal in the brain, nerve chords, mesenchyme and a qualitatively weaker expression in the epidermis (Figs. 2A–F). In addition, tissues such as the gut, the epithelia lining the lumen, the outer side and the cavity of the pharynx were apparently devoid of signal (Figs. 2D–F). In the case of the epithelial cells lining the cavity and external wall of the pharynx, the absence of staining is consistent with the fact that their nuclei, where the bulk of the SL RNA signal accumulates (see Section 3.4), are located in the cell bodies sunken into the parenchyma (Ishii, 1964, 1966). Similarly, the gut epithelium is formed by cells with an extremely high cytoplasm/nuclear volume ratio (Forsthoefel et al., 2012) and this may therefore give the impression of lack of SL1 expression. Whether SL1 transcription and SL1 *trans*-splicing occur at some level in the gastrodermis remains to be determined using more sensitive methodologies. Strikingly, SL3 expression was detected in a discrete population of mesenchymal cells (Figs. 2G–L), confirming our initial hypothesis of a tissue-restricted compartmentalization for this SL RNA. More importantly, SL3⁺ cells were located deep in the mesenchyme (Figs. 2J–L) and were absent overall or sporadically found in the area in front of the photoreceptors (Fig. 2G) and the pharynx (Fig. 2H and Fig. 2K). Expression patterns with such topology usually identify the planarian stem cells, historically referred to as neoblasts (Eisenhoffer et al., 2008). We therefore assessed whether SL3⁺ cells are part of the stem cell compartment.

3.3. Reduced expression after irradiation suggests that SL3 is transcribed in neoblasts

Neoblasts are pluripotent stem cells required for the remarkable regenerative properties of planarians (Wagner et al., 2011). Consistently, regeneration-incompetent regions such as the area in front of the photoreceptors and the pharynx (Eisenhoffer et al., 2008) are devoid of neoblasts. In addition, neoblasts are the only dividing cell population in the asexual animal (Newmark and Sanchez Alvarado, 2000) and for this reason can be selectively and irreversibly ablated within 24 h by γ -irradiation doses of at least 6000 rads (Eisenhoffer et al., 2008) (Fig. 3). The non-dividing progeny of neoblasts displays a different radiation sensitivity profile and starts to decline only 48 h after irradiation (Eisenhoffer et al., 2008). In order to roughly estimate whether the mesenchymal SL3 expression occurs within the stem cell compartment, we first examined the in situ hybridization signal of this SL RNA 24

h after irradiation. While we could observe an obvious reduction in the number of SL3⁺ cells in irradiated animals, depletion was not complete as shown by a comparative staining with the neoblast pan-marker *smedwi-1* (Eisenhoffer et al., 2008) (Fig. 3). Similarly, Northern blots confirmed that the SL3 signal was reduced to about 50% of the RNA level detected in unirradiated animals (Fig. 3, Figs. S4A, B). Altogether, these results suggest that SL3 is expressed in the stem cell compartment, but also in differentiating or terminally differentiated cells that persist 24 h after irradiation. Consistent with the relative broad SL1/2 expression pattern, irradiation failed to produce an appreciable depletion of the signal at qualitative level (Fig. 3). Nonetheless, we noted a slight decrease (13%) of SL1/2 in the Northern blots (Fig. 3, Figs. S4A, B), which may be due to the disappearance of stem cells expressing SL1 or SL2.

3.4. SL3 expression is enriched in a subset of neoblasts

In order to conclusively demonstrate the neoblast identity of SL3⁺ cells and the possible expression of SL1/2 in the stem cell compartment, we assessed the co-localization of planarian SL RNAs with *smedwi-1* and performed double fluorescent in situ hybridization (FISH) to estimate the overlap of these markers (Fig. 4). In comparison with chromogenic in situ hybridization (Fig. 2), FISH dramatically improved the subcellular resolution of the assay allowing us to appreciate the nuclear/perinuclear distribution of both SL RNA signals (Figs. 4H–K, 4O–V and S5A). Such localization is consistent with both FISH analyses carried out in trypanosomes, and with the nucleus–cytoplasm shuttling that occurs during the biogenesis of small nuclear ribonucleoproteins (RNP), including SL RNPs (Kiss, 2004; Mandelboim et al., 2003). First, we confirmed the broad expression of SL1/2 and showed an extensive overlap with the DAPI dye used to mark the nucleus of all cells (Figs. 4A–K). Consistently, about 90% ($87.95 \pm 3.78\%$) of all *smedwi-1*⁺ cells express SL1/2 (Figs. 4H–K and Table S2) suggesting that the predominant SL RNA in planarians is expressed in both stem cells and differentiated tissues. In this sense the 13% drop in SL1/2 signal detected by Northern after irradiation may simply reflect the depletion of neoblasts from the whole complement of diverse tissues expressing SL1 and SL2. Such reduction is also consistent with the fraction of *smedwi-1*⁺ cells detected in whole organism cell macerates (Wenemoser and Reddien, 2010). As expected from the overt radiation-sensitive expression of SL3, we could detect expression of *smedwi-1* transcripts in SL3⁺ cells (Figs. 4L–R). We found that about 66% ($65.83 \pm 6.98\%$) of all SL3⁺ cells overlap with *smedwi-1* expression (Table S2). The remainder signal defines a non-neoblast component in the SL3⁺ cell population, which may explain the radiation-resistant expression detected at both cellular and RNA levels. Conversely, it is interesting to note that only a fraction of all *smedwi-1*⁺ cells ($34.02 \pm 7.06\%$) expresses detectable levels of SL3 (Table S2). The almost complete overlap of the *smedwi-1*⁺ and SL1 signals also suggests that SL1 and SL3 are simultaneously expressed in the neoblasts. Altogether, our data show a preferential expression of SL3 in neoblasts and indicate that the expression of this SL RNA can mark a still uncharacterized subset of cells in the apparently homogeneous stem cell compartment of planarians.

3.5. SL1 and SL3 do not colocalize with chromatoid bodies

One distinctive ultrastructural characteristic of neoblasts is the presence of perinuclear cytoplasmic RNP granules, referred as chromatoid bodies (CB) (Fernandez-Taboada et al.,

2010; Rouhana et al., 2012). It has been recently shown that planarian CBs are rich in proteins post-translationally modified via methylation of arginine residues to symmetrical dimethylarginine (sDMA) (Rouhana et al., 2012). sDMA is recognized by the widely used Y12 monoclonal antibody (Lerner et al., 1981), which specifically and predominantly decorates planarian CBs (Rouhana et al., 2012). Y12 is also traditionally used to pull down snRNAs, including SL RNAs (Evans and Blumenthal, 2000; Ganot et al., 2004), via binding to sDMA-modified Sm proteins (Brahms et al., 2000). Given that a planarian Sm-like protein has been shown to localize to the CBs (Fernandez-Taboada et al., 2010) and that SL RNAs are bound by this class of proteins (Fig. 1), we assessed whether SL1, SL2 and SL3 could be present in these RNP granules. Our observations (Figs. 4S–V and S5) show that the planarian SL RNAs and Y12 signals do not overlap, suggesting that CBs are not a compartment associated with the SL RNPs.

3.6. SL3 trans-splicing is not restricted to a particular cohort of genes in the neoblasts

Considering the likelihood that individual neoblasts may simultaneously transcribe SL3 together with SL1 and/or SL2, we asked whether distinct subsets of mRNAs may be appended with different leaders in a mutually exclusive fashion. In order to answer this question, we took advantage of the availability of a published deep sequencing survey of the neoblast transcriptome (Labbe et al., 2012). In this work, flow cytometry was employed to produce a 70%–80% pure neoblast fraction for RNA-Seq commonly referred as X1 (Eisenhoffer et al., 2008; Hayashi et al., 2006; Higuchi et al., 2007). By analyzing the X1 transcriptome, we identified 1119 genes potentially undergoing *trans*-splicing with a frequency of at least 10% (e.g., 10 out of 100 mRNA molecules have an SL instead of a 5'UTR acquired in *cis*) (Table S1). About 73% (169/230) of the SL-cDNAs previously cloned from whole worms and different tissues (Material and methods) were represented in the survey and enriched in genes with a *trans*-splicing frequency higher than 50% (121/169), suggesting that our bioinformatics approach is quite robust. Consistent with the broad expression of SL1 in *smedwi-1*⁺ cells (Figs. 4H–K and Table S2) and the restriction of SL3 to a subset of neoblasts (Figs. 4O–R and Table S2), we found that the SL1 leader was more abundantly represented than SL3 in the analyzed dataset (18,939 and 6705 total reads, respectively; about 17 and 6 reads per gene, respectively; Table S1). On the other hand, SL2 splicing was relatively rare in the X1 transcriptome (only 366 reads detected in total in less than 15% of the genes) suggesting that this SL RNA may not be significantly transcribed in the stem cells. Finally, we analyzed the extent of SL1 and SL3 *trans*-splicing at the same acceptor site and found that in 65% of all the annotated genes both leaders were used. In these cases, SL3 accounts in average for 29% of all *trans*-splicing events at a particular site (Fig. S7). Only 11 genes displayed an SL3 representation greater than 50% with a P-value smaller than 0.01, and only 5 acceptor sites were exclusively associated to this leader (Table S1). However, RT-PCR analysis could not confirm SL3-preferential splicing in these cases (data not shown). Altogether, our data suggest that in the neoblasts most of the mRNAs competent for *trans*-splicing can be engaged by both the SL1 and SL3 RNPs. It remains to be determined whether at the single cell level specific transcripts can acquire predominantly one type of leader in a binary fashion.

3.7. SL3⁺ stem cells are deployed during regeneration

We wondered whether there could be biological attributes that could help distinguish between SL3⁺ and SL3⁻ neoblasts, and whether the transcription of this SL RNA could be dynamically regulated. As a first step to address these questions, we took advantage of the remarkable regenerative properties of planarians that allow these animals to restore any missing body part resected by amputation (Newmark and Sanchez Alvarado, 2000). Regeneration affects the proliferation, migration and differentiation of planarian stem cells (Guedelhofer and Sanchez Alvarado, 2012; Wenemoser and Reddien, 2010). After decapitation, accumulation of mainly stem cell progeny occurs at the wound site and leads to the formation of an unpigmented area, known as a regeneration blastema, where the genesis of missing organs will occur (Eisenhoffer et al., 2008; Newmark and Sanchez Alvarado, 2002). The growth of the blastema is propelled by intense proliferation in the pre-existing tissues immediately adjacent to the amputation plane, the so-called “post-blastema” region (Salo and Baguna, 1984). Similar to proliferating neoblasts, SL3⁺ cells accumulate in the post-blastema within 200–300 μ m from the amputation plane and reach a maximum density at day 2 (Wenemoser and Reddien, 2010) (Figs. 5A, C). Interestingly, Northern blot analyses also indicated increased SL3 RNA levels in six-hour to seven-day old regenerating trunks. In fact, after amputation we could detect a 1.5–2 fold up-regulation of SL3 in comparison to uncut animals (Fig. 5B, Figs. S4A, C). In contrast, and even though the evaluation of the spatial distribution of SL1/2 during regeneration was complicated by the dense expression pattern of this RNA, Northern blot analyses failed to detect any significant change in SL1/2 transcription over the time-course of regeneration (Fig. 5B; Figs. S4A, C). In order to determine whether the changes in SL3 expression reflect an augmented number of SL RNA molecules per cell or could be just explained by an increase in neoblast numbers upon proliferation, we quantified the number of *smedwi-1*⁺ and SL3⁺ cells in the post-blastema 6 h and 48 h after amputation. Consistent with the Northern blot quantification, we observed an average 1.5-fold increase of both *smedwi-1*⁺ and SL3⁺ cells across the two time points (Fig. 5C and Table S3). This figure is in agreement with previous morphological quantifications of the number of neoblasts obtained from post-blastema macerates (Salo and Baguna, 1989). At 48 h after amputation, we also observed that the percentage of total neoblasts expressing SL3 slightly increases (about 10%) (Table S3). However, we cannot resolve at this stage whether such higher percentage is the result of regeneration-induced transcription of SL3 in previously SL3⁻ cells or a higher proliferative rate in SL3⁺ neoblasts. Nonetheless, our observations suggest an involvement of SL3⁺ neoblasts in the regenerative process and indicate that the expansion of this cell subpopulation after amputation can account for the increase in the total level of SL3 RNA observed.

3.8. SL3⁺ stem cells are predominant during early phases of clonal expansion in sub-lethally irradiated animals

An interesting paradigm recently introduced to study stem cell dynamics in planarians is a colony assay that allows following the clonal expansion of single stem cells surviving a non-lethal dose of irradiation (Wagner et al., 2011; Wagner et al., 2012). Using this assay, we investigated whether there could be any particular relationship between SL3⁺ and SL3⁻ neoblasts in terms of numeric ratio or kinetic of appearance during colony growth. For

example, given that the fraction of *smewi-1*⁺/SL3⁺ cells among neoblasts is about 35% at steady state (Table S2), we would expect that in a scenario where all stem cells have an equal chance of surviving irradiation, the founder of the colony, defined as clonogenic neoblast (cNeoblast) (Wagner et al., 2011), would be SL3⁺ in the majority of the cases. To our surprise, we observed instead that in most of the colonies analyzed at early stages of expansion (1 to 258 cells, 24 colonies analyzed), cNeoblasts are predominantly SL3⁺, well above the percentage observed in wild type intact animals (Fig. 6). An inverse correlation between the colony size and the percentage of neoblasts expressing SL3 is also apparent and, remarkably, the smallest colonies counted (n=4; sizes: 1, 3, 4 and 6 cells) were entirely formed by SL3⁺ cells (Fig. 6). One possibility to explain this observation could be a higher radiation resistance of SL3⁺ over SL3[−] neoblasts. However, we found that ~90% (133/149 cell counted) of the *smewi-1*⁺ cells remaining 24 h after a 1750rads sub-lethal dose of irradiation are SL3[−] (Fig. S6). While the reason for the predominant expression of SL3 in cNeoblasts remains to be determined, these data strongly suggest that the transcription of SL3 RNA can be modulated during colony growth and may be linked to important regulatory mechanisms.

4. Discussion

Thirty years after its discovery, SL *trans*-splicing continues to be an enigma in biology (Lasda and Blumenthal, 2011). While this process is mainly used in trypanosomes to resolve polycistronic transcripts and possibly generate protein diversity via the use of alternative sites (Liang et al., 2003; Nilsson et al., 2010), a clear understanding of its significance in metazoan genomes (where monocistronic genes are predominant) is still lacking. Seminal work in *Caenorhabditis elegans* has clearly shown that SL *trans*-splicing is required for embryogenesis to proceed (Ferguson et al., 1996), and it is reasonable to assume that its crucial role in directing complex developmental processes could be generalized to other animal phyla. However, no specific cellular phenotypes have been correlated to gene expression changes deriving from *trans*-splicing events to date. Moreover in vitro and in vivo reporter assays in nematodes and schistosomes have suggested that SL and non-SL mRNAs do not likely display significant differences in translational efficiency and stability (Cheng et al., 2006, 2007; Lall et al., 2004; Wallace et al., 2010). These limitations in understanding the effect of *trans*-splicing on target mRNAs not only preclude a comprehension of the phenomenon per se, but also prevent us from gaining much needed insight about its evolutionary history (Douris et al., 2010; Nilsen, 2001). Hence, efforts to address the biological significance of SL *trans*-splicing in metazoans must extend the interrogation of this process to a greater number of phyla, and consequently, diverse biological contexts. This approach would permit the identification of developmental paradigms in which phenotypically recognizable cell types are found in binary states activating or repressing *trans*-splicing. Our characterization of this process in *S. mediterranea* opens up new avenues of investigation in this direction.

4.1. Transcription of multiple SL RNAs in planarians

The presence of multiple SL RNAs in multicellular organism is fairly common (Blumenthal, 2005; Pettitt et al., 2008; Stover and Steele, 2001; Zayas et al., 2005a). However, in most

cases specific biological functions or cell types associated with a particular SLRNA have not been uncovered. The only two exceptions are represented by SL2 and SL4 in *C. elegans*. SL2 is specialized in resolving polycistronic units (Blumenthal, 2005), while SL4 is expressed in the hypodermal cells of this nematode (Ross et al., 1995). In this paper, we have characterized the spatial expression of previously identified planarian SL RNAs (Zayas et al., 2005a) and the here identified SL3 (Fig. 1). By discovering SL3 expression in mesenchymal cells greatly enriched in neoblasts, we provide a novel and striking example of tissue-specific SL RNA transcription in metazoans (Figs. 2 and 4). Given that in all Platyhelminthes tissue homeostasis is driven by neoblast-like stem cells located in the parenchyma (Collins et al., 2013; Egger et al., 2009), it would be then interesting to analyze to what extent stem cell-specific SL RNAs are conserved among flatworms.

In contrast to the obvious compartmentalization of SL3, we found that the predominant planarian SL RNA SL1 and the highly similar SL2 are expressed in a diverse array of tissues (Figs. 2 and 4). A similar conclusion has been drawn for the probably unique SL RNA (Protasio et al., 2012) of the blood fluke *Schistosoma mansoni* (Davis et al., 1995). Our FISH experiments (Fig. 4) and analysis of the neoblast transcriptome (Table S1) indicate that in planarians, SL1 expression most likely overlaps with SL3 in the stem cells. This finding raises the important question of how specificity of *trans*-splicing is regulated. This situation has been observed also in *C. elegans* where SL1 and SL2 are co-expressed in the same cells during embryogenesis (Ferguson et al., 1996). In this paradigm, loss of SL1 can be partially compensated by the usage of SL2 in monocistronic *trans*-splicing (Ferguson et al., 1996). However, the functional specialization distinguishing SL1 and SL2 in *C. elegans* does not seem to exist in planarians. In fact, by analyzing the transcriptome of the neoblast population, we were not able to identify conspicuous sets of genes specific for either SL1 or SL3 (Table S1). It remains possible, however, that signaling events could direct specific mRNAs towards one type of splicing in individual cells. An alternative and provoking hypothesis discussed by Ferguson et al. (1996) mentioned the possibility that SL RNAs may perform functions other than *trans*-splicing to mRNAs per se. Though counterintuitive and presently unsupported by any biochemistry, this possibility could explain the apparent paradox of the transcription of two different and competing SL RNAs in the same cell.

4.2. SL3 expression defines a novel subpopulation of stem cells in planarians

Neoblasts have been historically considered a morphologically homogeneous and collectively totipotent stem cell population. However, the continuous identification of novel molecular markers and a better resolution in the study of organ development in planarians are uncovering a previously unsuspected heterogeneity in the stem cell compartment (Reddien, 2013). In particular, while the existence of pluripotent or “clonogenic” neoblasts has been clearly demonstrated (Wagner et al., 2011), it is also true that not all planarian stem cells are endowed with an unlimited potential but can instead display lineage-restricted characteristics (Reddien, 2013). Here, we have shown the expression of the newly characterized SL3 in a fraction of *smedwi-1*⁺ cells, identifying a novel element of heterogeneity in the planarian stem cell population (Fig. 4). What attributes could then distinguish SL3⁺ and SL3[−] neoblasts at steady state, upon wounding or during the repopulation of the stem cell compartment? First, SL3 transcription may be cell-cycle-

dependent and occur only at particular phases of the cycle. In this regard, the differential radiation sensitivity associated with different phases of the cycle (Sinclair and Morton, 1966) may explain the drop in colocalization of SL3 with *smewi-1* observed 24 h after irradiation (Fig. S6). On the other hand, if the apparent existence of SL3⁺ and SL3⁻ neoblasts results purely from cell cycle-dependent transcription, a more stochastic SL3 expression during the colony assay should have been expected (Fig. 6). This issue could be explored by studying SL3 transcription upon perturbation of the cell-cycle and in conjunction with appropriate markers.

Second, SL3 expression may correlate with the potency of neoblasts. In this scenario SL3 would identify cNeoblasts and *smewi-1*⁺/SL3⁻ cells would correspond to specialized progenitors (Reddien, 2013). This hypothesis would be in agreement with the predominance of SL3⁺ cells during the early phases of colony expansion (Fig. 6) and could be tested by comparing the rescuing efficiency of SL3⁺ and SL3⁻ neoblasts via single cell injections (Wagner et al., 2011). However, it is not yet possible to specifically purify these two cell sub-types.

Finally, an indispensable but rarely discussed property of neoblasts in particular, and stem cells in general, is their ability of switching from asymmetric to symmetric cell division in contexts requiring an increase in cell numbers (Morrison and Kimble, 2006). Such events must occur during both planarian regeneration and the reconstitution of the stem cell compartment and may correlate with the increased prevalence of *smewi-1*⁺/SL3⁺ cells observed after amputation (Fig. 5 and Table S3) and during colony expansion (Fig. 6). In this scenario SL3⁺ neoblasts would be capable of dividing symmetrically to amplify the pool of stem cells while SL3⁻ neoblasts would produce post-mitotic progeny via asymmetric cell division. While more work and tools are clearly needed to start examining these possibilities, the heterogeneity defined by SL3 in the planarian stem cell compartment provides a novel opportunity to study the biological effects of *trans*-splicing on cell fate determination and stem cell behavior.

4.3. Conclusion

Our study provides a detailed and unique analysis of SL *trans*-splicing in *S. mediterranea* at cellular level. Given that the only attempts to visualize the spatial distribution of SL RNAs in metazoan were carried out nearly 20 years ago with suboptimal *in situ* hybridization methodologies (Davis et al., 1995; Ross et al., 1995), we hope that our work will stimulate similar analyses in other organisms in which *trans*-splicing is known to occur. This kind of studies will be extremely beneficial in reconstructing the evolutionary history of this process. Additionally, we reported that the expression of a newly discovered SL RNA (SL3) is enriched in a subpopulation of neoblasts actively deployed during regeneration and clonal expansion. The identification of SL3⁺ and SL3⁻ neoblasts provides a unique opportunity to analyze the correlation between the biological attributes of these cell subpopulations and the proteomic output of the transcripts subject to SL3 *trans*-splicing. The discoveries reported here, combined with recent advances in RNA detection in live cells (Prigodich et al., 2012), planarian stem cell transplantation (Wagner et al., 2011) and proteomics (Onal et al., 2012),

pave the way for a mechanistic interrogation of how SL *trans*-splicing may regulate both gene expression, and ultimately the phenotype of cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A	adenosine
ATP	adenosine triphosphate
b	brain
BAC	bacterial artificial chromosome
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair(s)
C	cytidine
CB	chromatoid bodies

cDNA	DNA complementary to RNA
cNeoblast	clonogenic neoblast
d	deoxyribo
DAPI	4',6-diamidino-2-phenylindole
DIG	digoxigenin
DNP	dinitrophenol
EST	expressed sequence tag
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
g	gut
G	guanosine
IRR	irradiation
Kb	kilobase(s) or 1000 bp
MMG	7-methylguanosine
mRNA	messenger RNA
NBT	Nitro Blue tetrazolium
nc	nerve chords
nt	nucleotide(s)
OCT	Optimal Cutting Temperature compound
p	pharynx
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pr	photoreceptors
R	purine
RACE	rapid amplification of cDNA ends
RNP	ribonucleoprotein
rNTP	ribonucleotide
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-PCR
S	in vitro transcribed standard
sDMA	symmetrical dimethylarginine
SDS	sodium dodecyl sulfate

SL	spliced leader
SL RNA	spliced leader RNA
SL RNP	spliced leader RNA ribonucleoprotein
Sm	Smith's antigen
<i>smedwi-1</i>	<i>Schmidtea mediterranea</i> piwi-like protein
snRNA	small nuclear RNA
SSC	0.15 M NaCl/0.015 M Na ₃ -citrate pH 7.6
T	thymidine
TMG	2,2,7-trimethylguanosine
U	uridine
UTR	untranslated region(s)
WT	wild type

Fig. 1.

Identification of the novel SL RNA SL3 in *S. mediterranea*. (A) Schematic representation of the SL *trans*-splicing reaction. The SL RNA is a small RNA containing a splicing donor site (GU) downstream of the spliced leader (in red). The donor site is engaged in a splicing reaction with the acceptor sites (AG) of target pre-mRNAs. In most of the metazoan examined to date the SL RNA is capped with a 2,2,7-trimethylguanosine (TMG) group. Consequently, mRNAs appended to the SL are also TMG-capped (Lasda and Blumenthal, 2011). Conversely, non *trans*-spliced transcripts possess a mono-methylated cap (MMG). In planarians, mRNAs competent for *trans*-splicing can also acquire a 5' UTR in *cis* via splicing to an upstream exon or retention of an outtron. (B) ClustalW alignment of the three known planarian SL RNAs. (C) MFOLD prediction of SL3 secondary structure. (D) Northern blot analysis of SL RNAs and *trans*-spliced mRNAs. The SL RNAs are indicated by the black arrow, while the rest of the signal corresponds to *trans*-spliced transcripts. (B–C) The spliced leader–intron boundary is indicated by the red arrowhead and the Sm protein binding site (consensus RAU₄₋₆GR) (Scofield and Lynch, 2008) is underlined in red.

Fig. 2.

SL1 and SL3 are tissue-specifically expressed in *S. mediterranea*. Whole mount in situ hybridization of wild type worms hybridized with SL1/2 (A–C) and SL3 (G–I) riboprobes. In situ processed worms were also sectioned (D–F, J–L). Different areas of the same specimen and representative transverse sections across the same region are shown as indicated: A, D, G, and J cephalic region. B, E, H, and K trunk region. C, F, I, and L caudal region. Indicated in red characters are: brain (b); nerve chords (nc); pharynx (p); gut (g); photoreceptors (pr). Scale bars, 50 μm.

Fig. 3.

SL3 expression is affected by irradiation. (A) Wild type worms (WT) were subjected to 10Krad of γ -irradiation (IRR) and processed for in situ hybridization and Northern blot 24 h later. Irradiation completely depletes neoblasts under these conditions (*smedwi-1*, IRR). RNA levels for *SL1/2* and *SL3* were quantified by Northern blot (bottom of the situ hybridization panels).

Scale bars, 100 μ m.

Fig. 4.

FISH analysis confirms the overall broad expression of SL1 and the transcription of SL1 and SL3 in the neoblasts. Wild type specimens were processed for double FISH with either SL1/*smedwi-1* (A–K) or SL3/*smedwi-1* (L–M, O–R) riboprobe combinations. In N and S–V panels SL3 FISH was followed by immunostaining with the Y12 antibody. At the end of the staining procedures nuclei were counterstained with DAPI (D, H, O, S). (A–C) Low magnification images of a specimen hybridized with SL1 (red) and *smedwi-1* (green) riboprobes (C, merge). (D–G) Higher magnification images of SL1 expression in the brain lobes (b) and in the mesenchyme between the brain (indicated by *). The SL1 riboprobe (red) decorates the nuclei (blue) of most cells including the neoblasts (green) (G, merge). (H–K) Most of the neoblasts (green) in the mesenchyme express SL1 (red) (K, merge). (L–M) A specimen double labeled with SL3 (red) and *smedwi-1*⁺ (green) imaged at low magnification. (N) The Y12 antibody (green) preferentially reacts with the chromatoid bodies (CBs) in the neoblasts, yielding a pattern similar to *smedwi-1* in situ hybridizations. Shown is a worm stained after SL3 FISH (signal not shown at this magnification). (O–R) SL3 (red) is expressed in a subset of *smedwi-1*⁺ cells (green) (R, merge). Indicated in O are the nuclei of *smedwi-1*[−]/SL3⁺ (1), *smedwi-1*⁺/SL3⁺ (2) and *smedwi-1*⁺/SL3[−] (3) cells. (S–V) SL3 RNA (red) does not colocalize with CBs (green) (V, merge). Images were taken with 5× (A–C and L–N), 20× (D–K and O–R) or 40× (S–V) objectives. Scale bars: A–C and L–N, 165μm; D–G, 25μm; H–K and O–V, 10μm.

Fig. 5.

Regeneration induces accumulation of $SL3^{+}$ cells at wound sites and an increase in whole animal $SL3$ RNA levels. (A) Wild type worms were amputated in pre- and post-pharyngeal regions. Resulting trunks were allowed to regenerate and processed for in situ hybridization at the indicated time points. Regenerating photoreceptors at day 7 are circled. Scale bars, 50 μm . (B) Northern blot analysis of RNA obtained from uncut worms and regenerating trunks. Fold changes relative to uncut animals and normalized to the U6 loading control are indicated for $SL1/2$ and $SL3$ blots. (C) Example of coronal sections used to quantify the number of $smedwi-1^{+}$ (green) and $SL3^{+}$ (red) in the post blastema (Table S3). Nuclei (blue) are stained with DAPI and brain lobes (b) are indicated. Scale bars, 25 μm .

Fig. 6.

Expression of SL3 during colony expansion. Shown are representative colonies of increasing size and the percentage of *smedwi-1*⁺ cells (green) expressing SL3 (red). The size of 24 colonies from worms fixed 5–12 days after non-lethal irradiation and their content in SL3⁺ neoblasts were plotted. The percentage of *smedwi-1*⁺ cells expressing SL3 in wild type animals is indicated by the dotted line.