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Research article

### ARGININE METHYLATION ANALYSIS OF THE SPLICING-ASSOCIATED SR PROTEIN SFRS9/SRP30C

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**Abstract:** The human SFRS9/SRp30c belongs to the SR family of splicing regulators. Despite evidence that members of this protein family may be targeted by arginine methylation, this has yet to be experimentally addressed. In this study, we found that SFRS9 is a target for PRMT1-mediated arginine methylation *in vitro*, and that it is immunoprecipitated from HEK-293 lysates by antibodies that recognize both mono- and dimethylated arginines. We further observed that upon treatment with the methylation inhibitor Adox, the fluorescent EGFP-SFRS9 re-localizes to dot-like structures in the cell nucleus. In subsequent confocal analyses, we found that EGFP-SFRS9 localizes to nucleoli in Adox-treated cells. Our findings indicate the importance of arginine methylation for the subnuclear localization of SFRS9.

**Key words:** Nuclear bodies, Speckles, RGG boxes, Arginine methylation, Protein-protein interaction

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Abbreviations used: Adox – adenosine-2',3'-dialdehyde; Ki-1/57 – the 57-kDa protein antigen detected by the Ki-1 antibodies; mAb – monoclonal antibodies

## INTRODUCTION

The process of pre-mRNA splicing is an essential step in controlling the expression of most eukaryotic genes. This RNA processing activity takes place in the spliceosomes, which are large ribonucleoprotein complexes formed by the small nuclear ribonucleoprotein particles (U1, U2, U4/U6 and U5 snRNPs) and several regulatory non-snRNP splicing factors [1]. The functionally and structurally highly conserved serine- and arginine-rich proteins (SR proteins) are non-snRNP splicing factors that may affect both constitutive and alternative splicing. They may function in the early stages of spliceosome assembly, during the recruitment of U1 snRNP and U2AF to the 5' and 3' splice sites [2], and in the later steps of the splicing reaction, when they facilitate the formation of the U4/U6.U5 trimer snRNP complex. They may also be involved in the first catalytic steps of the splicing itself [3].

The SR proteins have a modular domain structure consisting of one or two RNA-binding domains (RRM) and a C-terminal region rich in arginine/serine, the RS-rich domain [1, 4]. While RRM domains determine RNA-binding specificity, the RS-rich domain mediates protein-protein interactions with other splicing factors, with the spliceosome, and also with different sequences within the pre-mRNA [4]. It has been reported that SR proteins are phosphorylated at serine residues within the RS domains, and that this modification can have a profound impact on their activity [1, 4]. Although several SR proteins have been identified as part of protein complexes related to arginine methylation activity [5], to date, no study has experimentally assessed this post-translational modification for these proteins. Arginine methylation occurs frequently in the context of RGG/RXR/RG motifs (RG-rich) and is catalyzed by a class of proteins called arginine methyl transferases (PRMTs). The methylation of these RG-rich clusters commonly occurs in RNA-binding proteins, and it has been implicated to be important for several cellular processes, such as subcellular localization, transcription, signal transduction and mRNA processing, including splicing [6]. We examined the arginine methylation of the SR protein SFRS9/SRp30c and the effects of inhibiting the methylation process on the nuclear distribution of EGFP-SFRS9 in cultured cells. Our study yielded new data concerning the relevance of the arginine methylation modification for the function of SFRS9 and other members of the SR protein family.

## MATERIALS AND METHODS

### Plasmids, recombinant proteins and *in vitro* methylation assays

The full-length SFRS9 cDNA was amplified by PCR from a fetal brain cDNA library (Clontech) and cloned into the vector pEGFP-C2 (Invitrogen) and the baculovirus transfer vector pFastBac Hta (Invitrogen). The cloning of the full-length FEZ1 cDNA into the pET 28a vector (Novagen/EMD Biosciences), and the protein expression and purification procedures were performed as previously described [28]. The recombinant PRMT1-pGEX-5X-2 has been described

elsewhere [8]. The bacteria (GST-PRMT1 and 6xhisFEZ1) and baculovirus (6xHis-SFRS9) recombinant protein production and *in vitro* methylation assays were performed as previously described [8, 9].

#### **Cell culture, transfection, treatments, Western blots and immunoprecipitations**

Human HEK-293 cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Transient transfections were performed using the calcium phosphate method. The Adox treatments were performed as previously described [10]. For the Western blots, polyvinylidene difluoride (PVDF) membranes were probed with the antibodies rabbit polyclonal anti-GFP (Abcam) and monoclonal anti 5xHis (Qiagen). Horseradish peroxidase-conjugated secondary antibodies (Sigma) and the reagent luminol (Santa Cruz Biotechnology) were used to detect the primary antibodies. For the immunoprecipitations, cells were lysed in a non-denaturing NP-40 lysis buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM phenyl-methylsulfonyl fluoride; 1% NP-40) under constant homogenization. Protein-G Sepharose beads (Amersham Biosciences) and monoclonal antibodies against PRMT1 (Abcam) or against mono- or di-methylated (anti-R-methyl) arginines (Sigma) were used to immunoprecipitate the protein complexes.

#### **Microscopic analyses**

HEK-293-transfected cells growing on glass coverslips were fixed with PBS containing 2% paraformaldehyde, permeabilized with 0.3% of Triton X-100, and blocked with PBS/2% BSA. The primary antibodies (anti-SC-35 or anti-nucleophosmin/B23, both monoclonal antibodies from SIGMA) were incubated at room temperature in PBS/2% BSA followed by incubation with the Alexa594-coupled secondary antibodies (Invitrogen). The coverslips were mounted with Prolong<sup>®</sup> gold antifade medium containing DAPI (Invitrogen). The cells were examined with either a Nikon fluorescence microscope or a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems). The latter was used for the co-localization assays. For the quantitative phenotypic analysis, three independent slides were examined by fluorescence microscopy for the presence of nuclear dots upon treatment with Adox, an inhibitor of methylation. A hundred cells were counted on each microscopic slide across randomly chosen fields.

## **RESULTS**

#### **Amino acid sequence analysis of the human SR protein family reveals the presence of several RG-rich motifs**

We analyzed the amino acid sequences of various known SR protein family members looking for the presence of RG-rich clusters that might function as putative targets for arginine methylation. We found several regions containing RGG, RG or RXR motifs in all of the analyzed sequences (Fig. 1).



snRNP proteins [5]. Indeed, several spliceosomal components and splicing factors have been shown to be methylated, indicating that the splicing activity is under the control of arginine methylation [11], and supporting the hypothesis that SR proteins might also be arginine methylated.

### **Arginine methylation analysis of SFRS9**

In our earlier yeast two-hybrid studies, we found that SFRS9 is an interacting partner of the protein Ki-1/57 (unpublished observation). Although SFRS9 has not yet been directly linked to arginine methylation processes, Ki-1/57 and several other RNA-binding proteins that we found to interact with it have already been described to be methylated by the arginino methyl transferase PRMT1 [8], suggesting a common functional context shared by these proteins which may be under the control of arginine methylation. Moreover, when we approached the yeast two-hybrid system to find new protein substrates for PRMT1, we found SFRS1 [8], an SR protein highly similar to SFRS9 with regard to its amino acid sequence (SFRS1-isoform1 versus SFRS9: 72.9% sequence identity and 88.2% similarity). Interestingly, SFRS1 binds SF2p32 [12], another protein that we found to be an Ki-1/57 interacting partner in our yeast two-hybrid analysis [13]. Therefore, our protein-protein interaction data strongly suggests that SFRS9 would also be a target for arginine methylation.

The MEMO software predicted that most of the RG-clusters of the SFRS9 amino acid sequence would be methylated (Fig. 2A). To obtain information on the methylation status of SFRS9 in HEK-293 cells, we expressed it as an EGFP-fused protein and performed immunoprecipitation assays using antibodies that recognize both mono- and dimethylated arginines. We mainly detected EGFP-SFRS9 in the immunoprecipitates obtained from the lysates of the control cells, and, to a much lesser extent, in cells that had been treated with the methylation inhibitor Adox (Fig. 2B), suggesting that EGFP-SFRS9 is methylated *in vivo* or is associated with arginine-methylated complexes in HEK-293 cells.

We also verified if EGFP-SFRS9 would be associated with PRMT1 *in vivo* through immunoprecipitation experiments. We were able to demonstrate this association (Fig. 2C), thereby giving further support to the hypothesis of a functional association between SFRS9 and PRMT1. As immunoprecipitation assays may also involve indirect associations, we next assessed if SFRS9 could also be a direct target for arginine methylation *in vitro*. Using a catalytically active recombinant GST-PRMT1, we observed the methylation of the baculovirus-derived recombinant 6xHisSFRS9 (Fig. 2D). The methylation was highly specific, since neither an unrelated recombinant protein (FEZ1) nor any protein from a cell lysate of insect cells infected with the wild type baculo virus showed any radioactive labeling (Fig. 2D). Importantly, the PRMT1 was used as a methylating enzyme here due to the functional interconnections described above for this arginine methyl transferase and the proteins SFRS9, Ki-1/57, SFRS1 and SF2p32. This represents only a first approach, as we could not exclude the possibility that SFRS9 could be also methylated by other members of the PRMT family *in vivo*.



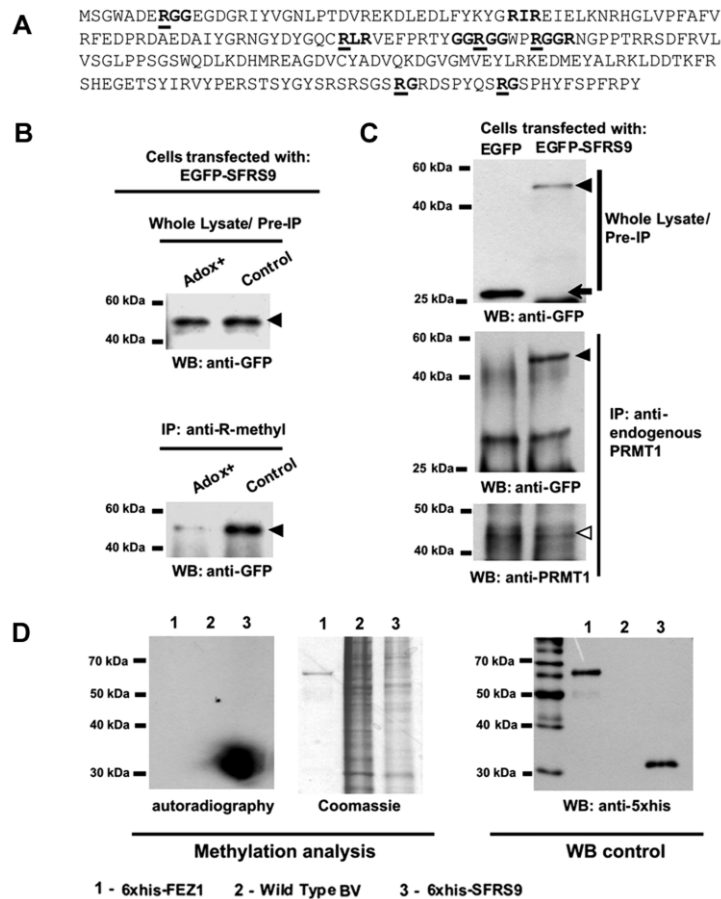


Fig. 2. A methylation analysis of SFRS9. A – The presence of RG-rich clusters in the amino acid sequence of SFRS9 (bold). The MEMO software predicted that the underlined arginines (R) would be methylated [27]. B – Immunoprecipitation with anti-R-methyl antibodies. Before the immunoprecipitation, an aliquot of each whole cell lysate was obtained to monitor the EGFP-SFRS9 expression levels in HEK-293-transfected cells (upper panel). Black arrowheads denote the detection of EGFP-SFRS9 fusion protein. C – The association of EGFP-SFRS9 with the endogenous PRMT1. Lysates of HEK-293 cells transfected with EGFP (negative control) or EGFP-SFRS9 were subjected to immunoprecipitation assays using a monoclonal antibody against the arginino-methyltransferase PRMT1 (lower panels). Endogenous PRMT1 or GFP-SFRS9 were detected by Western blot. An aliquot of each whole cell lysate was also obtained to monitor the levels of EGFP or EGFP-SFRS9 (upper panel). Black arrowhead: EGFP-SFRS9; black arrow: EGFP; white arrowhead: PRMT1. D – The SFRS9 is a substrate for PRMT1 methylation *in vitro*. The purified GST-PRMT1 was incubated in the presence of the  $^3\text{H}$ -radiolabeled methyl-donor SAM with purified 6xhis-FEZ1 (unrelated control protein [28]), or a lysate of Sf9 insect cells infected with a wild type baculovirus (BV; negative control), or a lysate of Sf9 insect cells infected with a 6xHis-SFRS9 recombinant baculovirus. The left panel shows the resulting autoradiography. A Coomassie Blue gel (middle) revealed equal loading with the lysate, and a Western blot (right) assay showed the presence of the recombinant proteins.

**The inhibition of methylation by Adox causes the re-localization of EGFP-SFRS9**

In previous fluorescence microscopy studies, we found that the nuclear and cytoplasmic protein Ki-1/57 predominantly re-localizes to the nucleus, where it accumulates as discrete, dot-like substructures upon the inhibition of methylation [8]. We therefore reasoned that a similar re-distribution may also be found for SFRS9. Indeed, we observed that when methylation is inhibited by Adox in human HEK-293 cells, a great proportion of EGFP-SFRS9 localizes to several nuclear dots (Fig. 3A). We found that more than 90% of the analyzed cells presented such substructures (Fig. 3D). Moreover, we found such dot-like structures even in control cells, but in this case, the observed bodies constituted fainter structures, being found in only around 29% of the counted cells (subpopulation 1) (Fig. 3B and D). The remaining transfected cells (around 71%; subpopulation 2) present a predominantly diffuse EGFP-SFRS9 nuclear distribution, without any observable granules (Fig. 3C and D). These findings suggest that SFRS9 is not only a target for arginine methylation, but that this activity is also important for its localization and trafficking in mammalian cell nuclei. It has been shown that arginine methylation can influence the subcellular localization of a number of RNA-binding proteins [9, 14], which is in close agreement with our observations for this member of the SR protein family.

**The EGFP-SFRS9 can co-localize to the nucleoli in HEK-293 cells treated with Adox**

It is already known that SFRS9 and many other SR-proteins localize to splicing speckles [15]. These nuclear dot-like substructures are functionally associated with pre-mRNA splicing, and their composition and localization in the cell nucleus is highly dynamic, being influenced by several conditions such as heat shock treatment and drug-mediated inhibition of protein dephosphorylation or transcription [16, 17]. In order to verify if the inhibition of methylation can also affect the structure of speckles or the localization of EGFP-SFRS9 into this organelle, we performed co-localization experiments of EGFP-SFRS9 with the speckle marker protein SC-35, another member of the SR-protein family.

Through confocal microscopy analyses, we observed that in untreated control cells, the EGFP-SFRS9 was principally diffuse in the nucleoplasm, although there was some weak and partial co-localization with SC-35 splicing speckles (Fig. 4A, 1-3). We did not find any apparent changes in the distribution and overall structure of the speckles in cells submitted to Adox treatment, suggesting that this compartment may not suffer structural modifications under such conditions (Fig. 4A, compare panels 2 and 5). Also, this treatment did not seem to cause any detectable modifications in the partial association between EGFP-SFRS9 and this nuclear substructure (Fig. 4A, 1-3 and 4-6). On the other hand, upon confocal analysis, the previously observed EGFP-SFRS9-associated bodies appeared as structures that resemble different juxtaposed rings in regions possibly related to the nucleoli (Fig. 4A, white arrows in the insets of the panels 4 and 6).



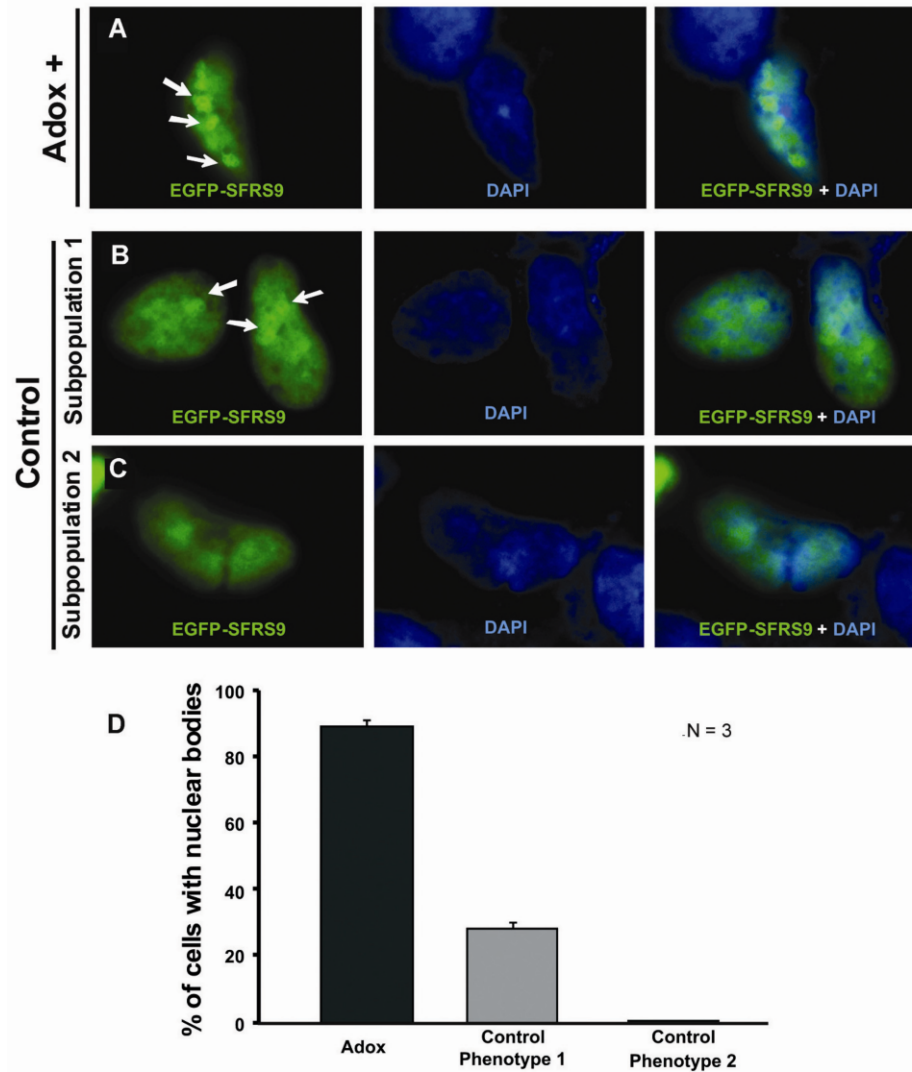


Fig. 3. The localization of EGFP-SFRS9 in cells with inhibited methylation. A-C – Fluorescence microscopy analyses of human HEK-293 cells expressing the construct EGFP-SFRS9, and either treated with Adox or untreated. Nuclear DNA labeled with DAPI is shown in blue. D – The proportion of HEK-293 cells transfected with EGFP-SFRS9 displaying nuclear bodies. A hundred cells were counted across randomly chosen fields in each of three independent slides analyzed for each condition ( $n = 3$ ): Adox-treated cells or untreated control cells (a total of 600 events/transfected cells counted). Two different subpopulations of cells were found in the control slides. They are marked as phenotype 1 (with the presence of granules, normally discrete, shown as subpopulation 1 in B) or phenotype 2 (without granules, shown as subpopulation 2 in C).

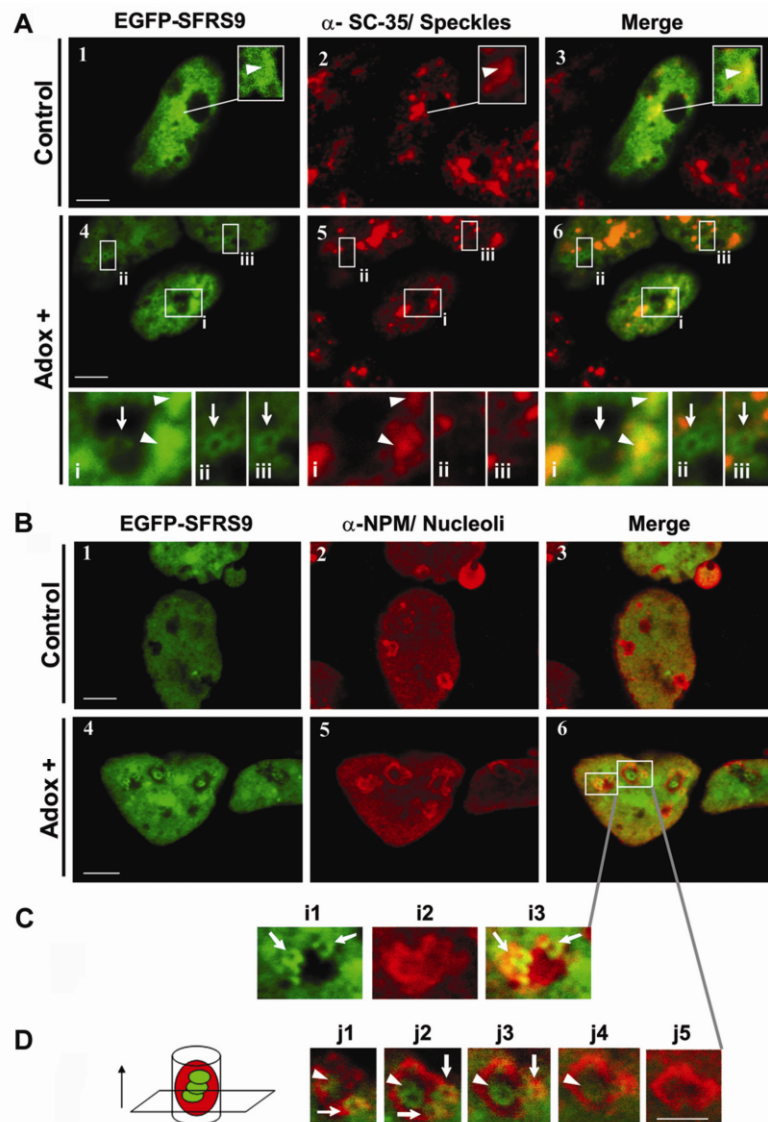


Fig. 4. Confocal analysis of EGFP-SFRS9 in the splicing speckles and nucleoli. A – Co-localization of EGFP-SFRS9 to the splicing speckles (SC-35). Selected regions in panels 4-6 are shown as separated figure insets (i, ii, and iii). White arrowheads show regions of partial co-localization between EGFP-SFRS9 and the speckles, while white arrows indicate the ring-like structures formed upon Adox treatment. B – Co-localization of EGFP-SFRS9 to the nucleoli (Nucleophosmin, NPM). C – A selected region of image 6 is shown in detail as separated insets. The arrows show EGFP-SFRS9 localization (green) in the outer subregions of the nucleolar space delimited by nucleophosmin (red). D – Confocal laser microscopic sections taken 0.3  $\mu$ m apart reveal that EGFP-SFRS9 can be also found (green) in the inner subregion of nucleoli (arrowheads). Scale bar, 3  $\mu$ m. Arrows show EGFP-SFRS9 substructures in the more external nucleolar subregion.

Thereafter, aiming to test if such EGFP-SFRS9-associated substructures localize to the nucleolar region upon Adox treatment, we analyzed the co-localization of EGFP-SFRS9 with the marker protein nucleophosmin/B23. Overall, we observed that EGFP-SFRS9 in the control cells localized to the whole nucleoplasm with the exception of the nucleoli, from which it is excluded in most of the analyzed cases (Fig. 4B, 1-3). However, upon the inhibition of methylation, we observed that EGFP-SFRS9-associated bodies localized inside of the region delimited by nucleophosmin (Fig. 4B, 4-6) at different nucleolar subregions: more externally and, curiously, also more internally (selected regions in Fig. 4B, 6 and insets in Fig. 4C, i1-i3). The nucleolus is known to be constituted by distinct internal subregions which are involved in different stages of ribosome biogenesis or other more speculative functions [18]. To gain further insight into the localization of EGFP-SFRS9 in the inner nucleolar region, we obtained images at different planes of the cell (Fig. 4B). We verified that EGFP-SFRS9 seems to have a disposition in the shape of a tube inside the nucleolus (Fig. 4D, j1-j5). Moreover, depending on the image planes analyzed, the localization of the EGFP-SFRS9 substructure could also be observed in the outer nucleolar region, coexisting with the central structure in the same analyzed nucleolus (Fig. 4D, j1, j2). Together, these observations suggest that SFRS9 might be involved in different steps of the maturation of ribosomal components, or, alternatively, in the post-transcriptional modification of snRNAs.

## DISCUSSION

The nucleoli are primarily involved in ribosome biogenesis in eukaryotic cells. However, several lines of evidence point to a more complex and multifunctional role for this nuclear organelle [18]. As with the maturation of rRNA, later steps in snRNA maturation require several snoRNA-guided modifications as 2'-O-ribose methylation and pseudouridination [18, 19]. It has been reported that before reaching the nuclear speckles, snRNAs pass through the nucleoli and undergo such modifications [20, 21]. SFRS9 may be part of these maturation complexes, and its release from the nucleoli to the nucleoplasm may be under the control of arginine methylation. Independently of the involvement of SFRS9 in ribosomal biogenesis or snRNA maturation, our observations raise new questions on the function of this SR protein. Several proteome studies have identified more than 700 human proteins that co-purify with isolated nucleoli. The functional classification of these proteins surprisingly revealed that only 30% of them are related to the production of ribosome subunits. The other fraction reveals the additional processes that may occur within these structures. It includes factors involved in splicing/pre-mRNA processing, cell-cycle control, and DNA replication and repair [18]. These observations indicate that the localization of a splicing factor like SFRS9 inside the nucleolar region may not be an artifact resulting from over-expression experiments, but may indeed have functional meaning. Besides the nucleolar localization of snRNPs upon okadaic acid treatment [16], the link between splicing activity and the nucleolar compartment

was reinforced by several other reports that show the transient accumulation of splicing factors in these nuclear compartments [16, 22, 23]. Moreover, several splicing proteins have been identified in such proteomic studies, including SFRS9 and other SR proteins [24], in agreement with our observations here. Many studies have revealed that various additional components within the nucleolus are not constitutive but are rather dynamic components of the nucleolus, in such a way that it may have not been possible to detect them using the mentioned nucleolar proteome analyses [25]. The observed accumulation of SFRS9 within the nucleoli in Adox-treated cells may be a consequence of a misbalance in the entry and exit rates in cells under equilibrium conditions. The inhibition of methylation may for instance disfavor the nucleolar exit of EGFP-SFRS9, which would depend on its arginine methylation. This may be reinforced by the observation that around 29% of untreated control cells also presented such nucleolar EGFP-SFRS9-associated structures (Fig. 3B and D), reflecting a normal equilibrium state between the entrance and exit from the nucleoli. In cells subjected to the inhibition of protein dephosphorylation by okadaic acid, a similar pattern of localization in the intra-nucleolar compartment has been observed for the p80 protein coilin, and for splicing-associated snRNPs [16]. Intriguingly, in this case, the same was not observed for the SR protein SC-35 in these treated cells [16]. That in our experiments with EGFP-SFRS9 in Adox-treated cells, we could not observe SC-35 in the nucleolus suggests not only the selectivity of Adox treatment, but also that these two SR proteins are involved in slightly different functional mechanisms which may be physically separated by nuclear sub-compartmentalization.

In conclusion, we described here for the first time the arginine methylation of a SR-protein family member, SFRS9, and demonstrated how this post-translational modification may affect its subnuclear localization. Future experiments will address the functional significance of the association of SFRS9 to the nucleolar compartment, and the details of this important post-translational modification for it and possibly other SR proteins.

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