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POINT OF VIEW

When SUMO met splicing

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ABSTRACT

Spliceosomal proteins have been revealed as SUMO conjugation targets. Moreover, we have reported that many of these are in a SUMO-conjugated form when bound to a pre-mRNA substrate during a splicing reaction. We demonstrated that SUMOylation of Prp3 (PRPF3), a component of the U4/U6 di-snRNP, is required for U4/U6•U5 tri-snRNP formation and/or recruitment to active spliceosomes. Expanding upon our previous results, we have shown that the splicing factor SRSF1 stimulates SUMO conjugation to several spliceosomal proteins. Given the relevance of the splicing process, as well as the complex and dynamic nature of its governing machinery, the spliceosome, the molecular mechanisms that modulate its function represent an attractive topic of research. We posit that SUMO conjugation could represent a way of modulating spliceosome assembly and thus, splicing efficiency. How cycles of SUMOylation/de-SUMOylation of spliceosomal proteins become integrated throughout the highly choreographed spliceosomal cycle awaits further investigation.

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Introduction

The diverse organismal complexity observed among metazoans is nourished by orchestration of sophisticated molecular mechanisms operating at different tiers of gene expression, thus providing eukaryotic cells with the possibility for massive proteomic expansion from a relatively limited number of genes. This article tackles two cellular processes that are key determinants of the existing proteome diversity: splicing at the pre-mRNA level, and post-translational modifications (PTMs) at the protein level. In particular, we discuss recent findings from our laboratory regarding the modification of spliceosomal proteins by SUMO conjugation (a.k.a. SUMOylation), contextualizing these within current knowledge focused on the emerging connection between the machinery responsible for pre-mRNA processing and the SUMO conjugation pathway.

The splicing process and its complex machinery

Pre-mRNA splicing is catalyzed by the spliceosome, a highly dynamic macromolecular machine that recognizes sequence elements within pre-mRNAs, carrying out the removal of introns and the concomitant joining of exons to generate mature mRNA products.

Two types of spliceosomes have been described in metazoans, major and minor, and each one is composed of a particular set of ribonucleoprotein particles. The major spliceosome, on which we will focus here, is responsible for most splicing events. It is composed of five small nuclear (sn) ribonucleoprotein particles termed U1, U2, U5 and U4/U6 snRNPs and many associated non-snRNP splicing factors. Each snRNP consists of an snRNA (or two in the case of U4/U6), a common set of "Sm" or "LSm" proteins and a variable number of particle-specific factors [1,2].

The spliceosome assembles "on site" in a precise and stepwise manner. First, U1 is recruited to the 5' splice site (ss) and the 3′ss is recognized by non-snRNP factors known as splicing factor 1 (SF1) and U2 auxiliary factors (U2AFs). Subsequently, U2 snRNP is recruited to the branch point in the pre-mRNA, forming the A complex or pre-spliceosome. Then, complex B is generated by recruitment of the tri-snRNP U4/U6•U5. After numerous RNA and protein rearrangements, including the dissociation of the U1 and U4 snRNPs, the spliceosome adopts an activated form termed B^{act} complex, followed by the catalytically active form known as B^{*} complex, which catalyzes the first step of the splicing reaction, i.e. cleavage at the 5'ss and intron lariat formation. Further rearrangements yield the C complex, which in turn catalyzes the second step, during which the intron is excised and the flanking 5' and 3' exons are ligated. Following this two-step catalytic process, the spliceosome disassembles and its components are recycled for new rounds of splicing. Neither the snRNPs nor the different spliceosomal complexes are static structures but, instead, they are highly dynamic and are substantially remodeled during the splicing reaction [1–3].

Even though the catalytic core of the spliceosome is RNA-based, determined basically by the structure and annealing of its snRNAs, the importance of its protein components should not be underestimated. For instance, the proteins Prp8 (PRPF8) and Brr2 (SNRNP200) are essential for the formation

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of the spliceosome active site. Prp8 is the most evolutionarily conserved protein within the spliceosome and rearrangements of its domains support catalysis activation. The Brr2 helicase, in complex with the Jab1/MPN domain of Prp8, is responsible for unwinding the U4/U6 duplex; while its re-association after splicing requires Prp24, an assembly chaperone in yeast, or its ortholog SART3 in humans. Prp3 (PRPF3) is also required for U4/U6 di-snRNP and U4/U6●U5 tri-snRNP formation, by interacting with Prp4 (PRPF4) and U5-specific proteins, and directly with the U4/U6 snRNAs. As mentioned previously, the transition between the subsequent spliceosomal complexes requires compositional and conformational remodeling, which relays on RNA-RNA, RNA-protein and protein-protein interactions both intra- and inter-snRNPs. This is not only crucial for spliceosome assembly, but also for its recycling after each splicing event in order to engage in further rounds of splicing [4].

Efficient pre-mRNA splicing not only requires snRNPs; numerous auxiliary factors also play essential roles during the reaction. Two protein families stand out for their involvement in both constitutive and alternative splicing: serine-arginine rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNP). These RNA-binding proteins (RBPs) interact with specific RNA sequence motifs located either within exons or introns, and termed splicing enhancers or silencers, according to whether they recruit factors that favor or inhibit the splicing event. It is worth mentioning that the mere presence of such a motif within a transcript does not guarantee its occupancy by the cognate RBP, as several contextual features influence RBP binding both *in vitro* and *in vivo* [5].

A modular structure and at least one RNA recognition motif (RRM) are common characteristics of all members of these protein families and are crucial for supporting their function. Given the commonalities among these factors, it is therefore possible that hnRNP and SR proteins may share a common ancestor [6].

SR and hnRNP proteins not only participate in splicing regulation but also play important roles in nuclear and cytoplasmic steps of mRNA metabolism, from synthesis to degradation, and have been associated to different cellular processes both in health and disease [7–10]. Focusing on SR proteins, they have been linked to the regulation of genome stability [11], transcriptional elongation [12], microRNA processing [13], mRNA export [14], mRNA stability [15] and mRNA translation [16]. Accordingly, these factors are currently considered as multifaceted, master regulators of gene expression instead of mere splicing factors [7,8]. Moreover, work from our laboratory has identified the SR protein SRSF1 (previously known as SF2/ASF) as a regulator of the SUMO conjugation pathway [17]. Further characterization of this novel role of SRSF1 is currently ongoing.

Many of the described activities are shared by different members of the family: both SRSF1 and SRSF2 were reported to stimulate transcriptional elongation [18]; SRSF1, SRSF2, SRSF3, SRSF7, and SRSF10 share the capacity to migrate into cytoplasmic stress granules [8,19] while SRSF1, SRSF7, and SRSF9 are recruited to nuclear stress bodies upon heat shock [20]; viruses exploit SRSF3, SRSF5, and SRSF6 to promote the translation of their mRNAs by different mechanisms [21,22]; a subset of SR proteins such as SRSF1 and SRSF2 strongly enhances NMD [15]; a more recent study points to SRSF1, SRSF3, and SRSF7 as responsible for the recognition of specific motifs identifying pri-miRNA hairpins from other hairpins present within RNA transcripts [23].

Structural similarities among the members of the SR family may be the cause for these overlapping activities. On the other hand, differential recognition of RNA targets, the ability to establish particular interaction networks and engage in different multimeric complexes, as well as their sub-cellular and sub-nuclear localization may provide specificity of action to each of these proteins.

How the variety of functions described for SR proteins is controlled and coordinated within cells and tissues is still scarcely understood. These activities seem to depend on different, but interconnected features of these proteins such as their RRMs (RRM1 and RRM2), the phosphorylation status of their arginine/serine-rich C-terminal RS domain [24], and their shuttling capacity, among others. Recent work has demonstrated that the nucleocytoplasmic shuttling ability of SR proteins needs to be revisited within the context of cell type and cellular differentiation state, and that this capacity is modulated by varying levels of serine phosphorylation and arginine methylation. Consequently, nuclear and cytoplasmic availability, and thus nuclear and cytoplasmic activities of individual SR proteins, can be fine-tuned in response to changing cellular conditions [25].

Post-translational modifications: SUMO conjugation

PTMs are covalent but mainly reversible alterations to already synthesized proteins that allow extremely fast responses to both internal and external cues. Protein structure, subcellular localization, engagement in multimeric complexes, protein turnover, among other aspects that are key determinants of protein function, are deeply shaped by the combinatorial attachment of small chemical functional groups (i.e.: phosphate, acetyl, methyl), lipids and sugar moieties, as well as by the conjugation of peptides from the Ubiquitin (Ub) family to specific target residues within each particular protein. Among these modifying peptides or Ubls (Ubiquitin-like proteins), SUMO (small-ubiquitin related modifier) is one of the best-characterized family members [26,27]. SUMO is synthesized as an immature precursor that is subject to proteolytic cleavage by specific SUMO proteases. After this maturation step, an enzymatic cascade involving an E1 activating enzyme (SAE1/SAE2), an E2 conjugating enzyme (Ubc9) and, in most cases, an E3 ligase (several have been described so far) allows the conjugation of SUMO to a lysine residue in the target protein. SUMO can be attached as a single molecule to one or several conjugation sites within a given protein, or to an already conjugated SUMO peptide leading to the formation of SUMO chains. This varies among different target proteins and depends on the chain-formation ability of the SUMO isoforms. To date, evidence supports the existence of five distinct SUMO isoforms. The mature forms of SUMO2 and SUMO3 share 97% amino acid sequence identity and both share only 50% identity with SUMO1 which, unlike the previous two, cannot form chains. SUMO5 has been recently described. While this isoform shares

a high degree of identity with SUMO1, they differ in several aspects. First of all, SUMO5 is conserved in primates but does not seem to be expressed in mice. Second, its gene is only transcriptionally active in particular tissues, with high expression in testis and blood cells [28]. Regarding SUMO4, it is not yet clear whether it is even processed, although a mutation that leads to an aminoacidic substitution (M55V) has been associated to type 1 diabetes [29]. Reversibility and regulation is sustained by SUMO proteases, which not only carry out the SUMO maturation step but also de-conjugate SUMO from the substrate and/or de-polymerize SUMO chains. In most cases, SUMOylation regulates intra and/or intermolecular interactions of the SUMO-modified protein, either disrupting interactions or generating new interaction surfaces that can favor the non-covalent recruitment of specific protein partners. This recruitment is often mediated by SUMO-interacting motifs (SIMs) present within the partners. Additionally, this PTM is known to regulate nucleic acid-binding activity of target protein [30,31].

Post-translational modifications affecting the splicing machinery

Different PTMs have been shown to promote critical structural rearrangements in splicing-associated ribonucleoprotein particles [3]. The essential role of phosphorylation/de-phosphorylation of SR proteins has been extensively reported as a modulator of protein-protein and RNA-protein interactions [32–37]. On the other hand, de-phosphorylation of many spliceosomal proteins is required for the catalytic steps of the splicing reaction, being PP1 and PP2A phosphatases important players [38]. It has been proposed that phosphorylation of many tri-snRNP proteins contributes to its stable integration during complex B formation. Particularly, hPrp28 (DDX23) is phosphorylated by the SR protein kinase SRPK2 and, in the absence of this modification, this step is blocked [39]. Since this kinase is absent from *S. cerevisiae*, is it likely that in higher eukaryotes, numerous phosphorylation events, as well as other PTMs, contribute to spliceosome assembly, suggesting a greater flexibility for interactions and an increased susceptibility for fine-tuning.

Beyond phosphorylation, other PTMs have been linked to splicing. Proteomic analysis revealed that many spliceosomal proteins are acetylated [40], and acetylation inhibitors block *in vitro* spliceosome assembly at different steps [41], suggesting a role for acetylation during the splicing process. In fact, and based on the co-transcriptional nature of the splicing process, it has been demonstrated that histone acetyl-transferase Gcn5 is required for U2 snRNP association with the branch point and impairment of histone acetylation, by mutation of either Gcn5 catalytic site or target lysine residues within histone H3, is lethal when combined with deletion of U2 snRNP components, such as Lea1 or Msl1 [42]. These studies suggest that rearrangements occurring during co-transcriptional spliceosome assembly are linked to dynamic changes in histone acetylation state.

Regarding arginine methylation, it has been proposed to be required for maturation of snRNPs. Symmetric dimethylation of the four Sm/LSm proteins (SmD1, SmD3, SmB/B', and LSm4) achieved by the methylase PRMT5 (protein arginine methyltransferase 5) appears to facilitate their recognition by the survival motor neuron (SMN) protein during the assembly of snRNP core particles. As for PRMT4/CARM1, it methylates three spliceosomal components, SmB, U1-C, and SAP49 [43,44]. In addition, proteomic studies evidenced that U1-70K (SNRNP70) also contains dimethylated arginines, but the responsible PRMT remains unknown [45]. Furthermore, methylation of both hnRNP and SR proteins has been reported to play a role in their localization within the cell [46–48]. Finally, taking into account the co-transcriptionality of the splicing process, the elongation factor CA150 is methylated by both PRMT4/CARM1 and PRMT5 and it is thought that its methylation promotes exon skipping. Last, but not least, histone modification is another potential area in which arginine methylation could influence RNA processing events by modulating transcription and mRNPs recruitment [49].

Also in the context of co-transcriptional splicing, recent experimental evidence obtained in yeast has associated ubiquitination of RNA polymerase II (Pol II) to slowed elongation and transcriptional pausing linked to pre-mRNA splicing [50]. This work suggests that the presence of ubiquitin at K1246 residue within the catalytic domain of Pol II interferes with the interaction between the enzyme active site and the DNA template, transiently pausing the polymerase to promote efficient splicing. On the contrary, Pol II de-ubiquitination triggered by Bre5-Ubp3 ubiquitin protease complex allows elongation to resume. The identity of the E3 ligase/s responsible for regulating Pol II ubiquitination remains an intriguing question.

Moreover, a role for ubiquitin in the dynamics of spliceosome assembly has also been postulated [51]. Specifically, it has been demonstrated that this PTM is required to maintain tri-snRNP levels, apparently inhibiting premature un-winding of U4/U6 [52]. These studies also revealed that Prp8 is ubiquitinated in the context of the tri-snRNP. Given the well-documented role of Prp8 in regulating helicase Brr2 activity, it has been proposed that ubiquitination/de-ubiquitination of Prp8 is probably involved in U4/U6 un-winding during spliceosome catalytic activation [51]. Moreover, it has been proposed that Prp19 complex, which has been shown to function not only as a splicing factor but also as an E3 ubiquitin ligase, promotes Prp3 ubiquitination increasing its affinity for Prp8 and stabilizing tri-snRNP formation [53], being later de-ubiquitinated for recycling by Usp4/Sart3 dimer. This weakens its interaction with Prp8, promoting U4 snRNP dissociation from the spliceosome. In this context, reversible Prp3 ubiquitination would modulate interactions between different snRNP complexes during the spliceosome catalytic cycle.

The proteomic-mediated identification of RNA binding proteins as the predominant group among SUMO conjugation targets clearly points to a role for SUMO at distinct steps of mRNA metabolism [54,55] where multi-subunit protein and RNA-protein complexes are assembled in a precise and stepwise manner allowing mRNA synthesis and processing. This proposed regulatory function of SUMO during mRNA metabolism is further supported by the *in silico* identification of SIMs in numerous RNA processing factors. Interestingly, SUMO conjugation has been found to regulate pre-mRNA 3'end processing, RNA editing, and mRNA packaging into messenger ribonucleoparticles (mRNPs) [56–58]. However, little is known about the involvement of SUMO in spliceosome biogenesis and splicing regulation. It is conceivable that the well-documented impact of SUMO conjugation on protein-protein protein-nucleic acid interactions also affects the assembly and activity of one of the

largest ribonucleoprotein machine, the spliceosome. In this respect, a putative connection between SUMO and splicing was suggested more than a decade ago by the proteomic-based identification of the SUMO E3 ligase PIAS1 co-purifying with the spliceosome [59]. Furthermore, it has been found that nuclear bodies such as speckles and Cajal bodies that are enriched in splicing factors, also contain SUMO pathway components [60–62]. In addition, our laboratory has shown that the splicing factor SRSF1, which displays various functions along mRNA metabolism, regulates SUMO conjugation in mammalian cells by acting in a SUMO E3 ligase-like manner and also regulating PIAS1 E3 ligase activity. This suggests that SRSF1 could function as a co-regulator of the SUMO pathway to modulate the specificity and efficiency of SUMOylation of splicing-related proteins [17,63,64]. Supporting these results, SRSF1 has been described as a cofactor of PIAS1, with both proteins being necessary and mutually dependent for the SUMOylation of DNA topoisomerase I (Top1). This particular modification of Top1 appears to be important for efficient recruitment of RNA processing factors to actively transcribed DNA regions, which in turn contributes to suppressing genome instability [65].

Spliceosomal proteins as SUMO conjugation substrates

Different laboratories, including our own, have reported a long list of spliceosomal proteins as SUMOylation targets (Fig. 1 and Supplementary Table) [54,63,66]. In particular, recent work from our laboratory has shown that many of them are present in a SUMO-conjugated form within spliceosomal complexes formed during an *in vitro* splicing reaction. Taking advantage of this *in vitro* splicing reconstituted system, we were able not only to identify splicing-related SUMO substrates, but also to observe that the level of SUMO conjugation within the pre-mRNA-bound protein fraction increases during the splicing reaction. This

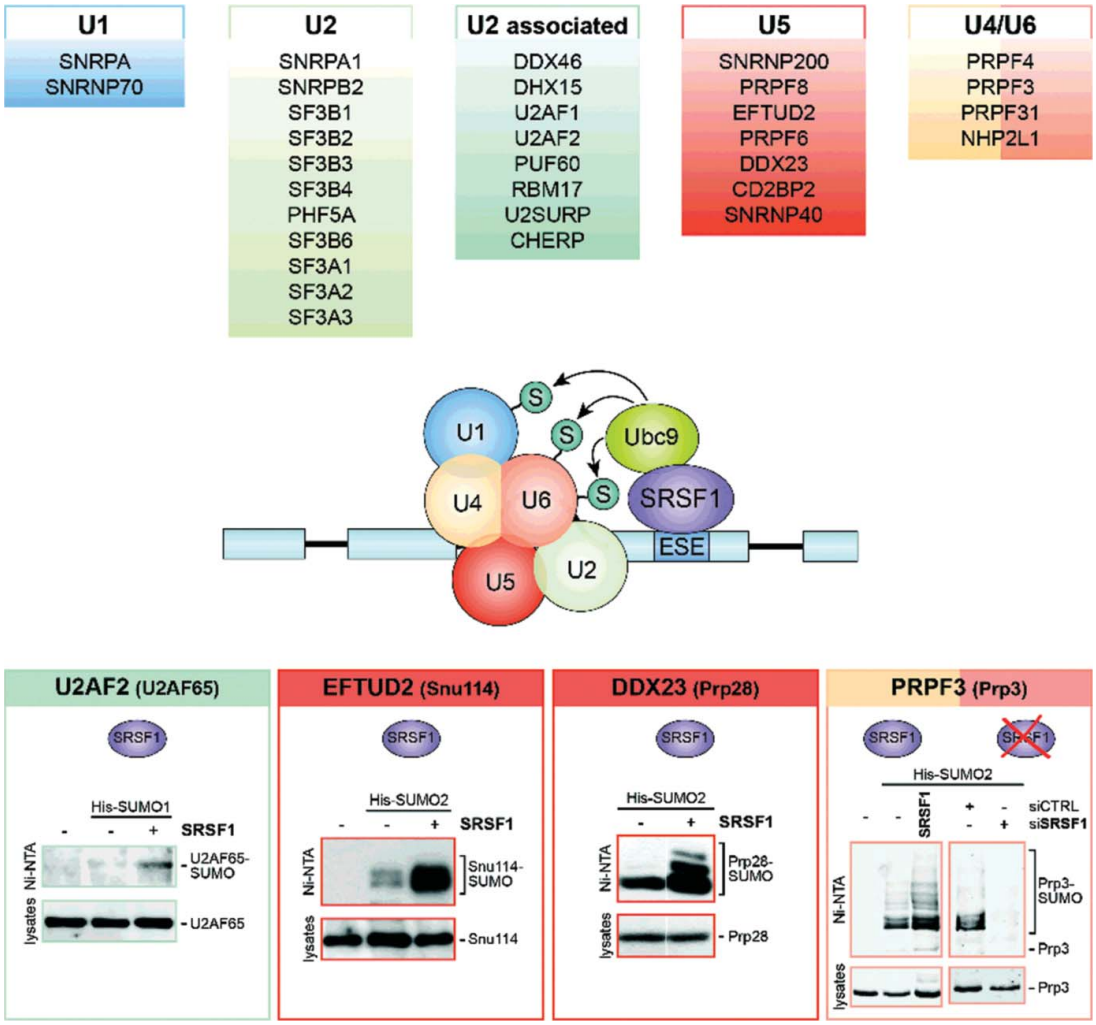

Figure 1. SRSF1 regulates SUMO conjugation to different spliceosomal proteins. Top panels indicate the snRNP protein components that have been so far described as SUMO conjugation targets by proteomic studies [54, 63, 66]. Middle scheme represents a proposed model of action of SRSF1 in which this RBP, by binding to a specific exonic splicing enhancer (ESE) assists with spliceosome recruitment and, at the same time, by its ability to interact with SUMO pathway components can regulate the SUMOylation of spliceosomal protein components. For the sake of simplicity, the cartoon shows only one spliceosomal complex. The precise step along the spliceosome assembly cycle at which each of the listed proteins is conjugated to SUMO, it is recruited to the spliceosome in its SUMO-conjugated form or interacts with SRSF1 that enhances its SUMOylation, remains unknown. Bottom panels correspond to western blot analysis of SUMO conjugation to particular spliceosomal proteins (U2AF2 – U2-associated protein-; Snu114 and Prp28 –U5 components; and Prp3 –U4/U6 component) upon SRSF1 overexpression or siRNA-mediated depletion in human cultured cells. The effect of SRSF1 depletion is only shown for Prp3 SUMOylation. HEK 293T cells were transfected with the siRNAs and/or DNA expression vectors as indicated at the top of each panel. After 48 h, cells were lysed and cell lysates were subjected to Nickel affinity chromatography (Ni-NTA). Aliquots of the cell lysates and eluates (Ni-NTA) were analyzed by western blot with antibodies against over-expressed HA-U2AF65; T7-Prp28; T7-Prp3 or endogenous Snu114. *[Reprinted in part from Pozzi B., et al. SUMO conjugation to spliceosomal proteins is required for efficient pre-mRNA splicing. Nucleic Acids Research, Oxford University Press. 2017 Jun 20;45(11):6729-6745].*

increase could be due to enhanced SUMOylation of pre-mRNA associated proteins and/or the recruitment of additional SUMO-conjugated proteins to spliceosomal complexes during the integration of the different snRNPs. Furthermore, we have reported that *in vitro* splicing efficiency can be affected by altering SUMO conjugation levels of nuclear extracts [63]. Taking into account that we have also demonstrated the SUMO conjugating activity of nuclear extracts, these results indicate that spliceosomal components and/or associated proteins may be actively modified by SUMO during the splicing process.

Recent work from the Lamond laboratory has identified a plant-derived flavonoid compound that not only inhibits splicing *in vitro* and modulates alternative splicing in cultured cells, but also increases SUMO conjugation levels. This latter effect seems to be exerted by inhibiting the SUMO protease SENP1. Proteomic analysis allowed the identification of several spliceosome components, in particular those belonging to the U2 snRNP, whose SUMOylation increases upon treatment of cultured cells with this compound [66]. These results clearly support the notion that SUMO conjugation/de-conjugation cycles are involved in spliceosome assembly and catalytic activity and may also affect alternative splicing regulation.

As mentioned above, several years ago we reported that the SR protein SRSF1 functions as a regulator of protein modification by SUMO conjugation, both *in vitro* and in living cells [17]. In agreement with those previous findings, we have recently shown that SRSF1 overexpression and/or siRNA-mediated depletion affects SUMO conjugation to different spliceosomal proteins [63], as illustrated in Fig. 1 for U2AF65 (U2AF2), Snu114 (EFTUD2), Prp28 (DDX23) and Prp3 (PRPF3).

Despite proteomics-based studies that uncover an ever-growing list of SUMO substrates, the enzymes and the cellular conditions that regulate their SUMOylation are often poorly characterized. In our work, we have revealed the splicing factor SRSF1 as a regulator of SUMO conjugation to spliceosomal proteins. Further work will be required in order to understand whether this role of SRSF1 is part of its mechanism of action as a splicing regulator or beyond this.

SUMOylation at the tri-snRNP

As described in our recent publication [63], we mapped *bona fide* SUMO target sites within the spliceosomal protein Prp3 and we generated a SUMOylation-deficient mutant by site directed mutagenesis. We further showed that the interaction of this SUMOylation-deficient mutant with U4/U6 snRNA and protein components is not significantly affected. However, this mutant displayed diminished interaction with U2 and U5 snRNP components. These results suggest that whereas Prp3 SUMOylation is dispensable for the proper assembly of U4/U6 di-snRNP, it may be required for tri-snRNP formation, and/or for the proper recruitment of U4/U6 di-snRNP as part of the tri-snRNP to active spliceosomes. The Prp3 SUMOylation-deficient mutant also showed a diminished recruitment to active spliceosomes, as evidenced by a reduced association to chromatin. Consistently, splicing efficiency analyzed for several endogenous transcripts in cultured cells was severely compromised when Prp3 depletion was rescued by the Prp3 SUMOylation-deficient mutant, rather than by the wild type protein.

Concluding remarks and future perspectives

As aforementioned, Prp3 is ubiquitinated and this modification seems to increase its affinity for the U5 snRNP component Ppr8, stabilizing U5 recruitment in tri-snRNP formation [53]. As might be the case with SUMOylation, it is likely that lack of ubiquitination reduces tri-snRNP levels, consequently inhibiting the formation of pre-catalytic spliceosomes. Even though ubiquitination sites have not been identified, our results suggest that they do not overlap with SUMOylation sites [63]. However, a possible cross-talk between both modifications cannot be ruled out and warrants further investigation.

As ubiquitination, SUMOylation is an attractive mechanism to be proposed as a regulator of spliceosome structural rearrangements. Considering this modification usually affects interactions, it is possible that its conjugation/de-conjugation to splicing factors is involved in spliceosome structural and compositional changes observed in almost all of it stages [67]. Besides, spliceosome recycling after each round of splicing would require reversibility of this kind of modifications, which in the case of SUMOylation can be achieved by SENPs.

From the perspective of the "group SUMOylation" concept, which refers to the requirement of the simultaneous modification of multiple targets involved in the same process [68], it is tempting to suggest that altering the SUMOylation of various spliceosomal components, in addition to the one studied in our recent work, could have even more dramatic consequences for the splicing process.

Altogether, these data led us to postulate that SUMO conjugation may have an impact on spliceosome assembly/disassembly cycle through regulating the biogenesis of its components, as well as their interactions, consequently affecting spliceosome catalytic activity and thus pre-mRNA splicing. To further examine this hypothesis, it could be interesting to identify specific pre-mRNA bound proteins that are subjected to SUMO conjugation/de-conjugation cycles along the different steps of the splicing reaction as well as the possible involvement of SR proteins, such as SRSF1, as regulators of SUMO conjugation along these cycles.

Untangling the splicing process as well as the mechanism of action of its regulators is not only important for understanding how gene expression is orchestrated in multicellular organisms, but it is also of biomedical interest, as aberrant pre-mRNA splicing and also mutations affecting the splicing machinery have been linked to a variety of human disease, from retinal and developmental disorders to different forms of cancer [69–72].

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed

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