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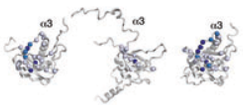

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POINT OF VIEW

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Retention and splicing complex (RES) – the importance of cooperativity

Piotr Wysoczanski[a] and Markus Zweckstetter[a,b,c]

[a]Department for NMR-based Structural Biology; Max Planck Institute for Biophysical Chemistry; Am Fassberg 11, Göttingen, Germany; [b]German Center for Neurodegenerative Diseases (DZNE); Göttingen, Germany; [c]Center for Nanoscale Microscopy and Molecular Physiology of the Brain; University Medical Center; Göttingen, Germany

ABSTRACT

One of the great challenges to structural biologists lies in explaining the complexities of the spliceosome – a ribosome-sized molecular machine that is assembled in a step-wise manner and is responsible for pre-mRNA splicing. The spliceosome is both fascinating and difficult to work with, because of its dynamic nature. At each discrete step of splicing tens of proteins come and go orchestrating the functional transition through massive structural rearrangements. The retention and splicing complex (RES) is an important splicing factor interacting with pre-mRNA at the onset of the first transesterification reaction. RES is a specific splicing factor for a number of genes and is important for controlling pre-mRNA retention in the nucleus. RES is a 71 kDa heterotrimer composed of the 3 proteins Pml1p, Bud13p and Snu17p. We solved the 3-dimensional structure of the core of the RES complex as well as the 2 dimers, Snu17p-Bud13p and Snu17p-Pml1p. Further biophysical analysis revealed an astounding cooperativity that governs the assembly of this trimeric complex as well as its interaction with pre-mRNA. The more than 100-fold cooperativity originates from the progressive rigidification of Snu17p upon coupled binding-and-folding of protein regions, which are disordered in the unbound state. Our work highlights the role of cooperativity in the spliceosome and poses new questions about the structure and assembly of the spliceosome.

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Introduction

Efforts to characterize the structure of a complete spliceosome at any stage of its catalytic cycle mostly failed so far, because of its huge size, high flexibility and time-variability, and an extremely high content of intrinsically disordered proteins (IDPs).[1,2] Only very recently the first cryo-electron microscopy structure of a full spliceosome became available.[3] Thus, divide and conquer strategies are often the method of choice for structural biologists seeking atomic resolution coupled with MS-crosslinking and single particle electron microscopy for the subsequent assembly of spliceosomal substructures.[2-7] Though X-ray crystallography was successful for a subset of those substructures, it largely failed at higher levels of organization. Over the years, NMR spectroscopy demonstrated to be a suitable alternative to some of those otherwise intractable problems with the additional advantage of access to protein dynamics with single-residue resolution,[8-12] which plays an important role in the spliceosome.[5]

Functionally, the spliceosome controls the flow and diversification of genetic information by transforming interrupted exons into mature mRNA molecules.[4] Though a subset of spliceosomal components is pre-assembled such as small-nuclear ribonucleic proteins (snRNPs), by and large the spliceosome achieves its structural and catalytic competence through a rapid cascade of multiple interactions each time it encounters a single substrate molecule.[4] The high complexity of the yeast spliceosome system, which comprises approximately 90 proteins (nearly 200 in human), necessitates cooperativity as a major player accelerating and controlling the assembly and disassembly of the spliceosome.[13,14] The major steps of the spliceosome cycle include the protein/RNA complexes denoted as E, A (pre-spliceosome), B (pre-catalytic), B^{act} (activated) and B^{*} (catalytically activated),[4] followed by C (after step 1 transesterification) and the post-spliceosomal complex.[4]

We studied the structure, dynamics and assembly of the 71 kDa trimeric complex RES.[15-17] RES interacts with the 3′ part of the intron and is close to the catalytic center at the onset of the first transesterification reaction.[16,18,19] The recently revealed structural details.[16,20,21] are consistent with earlier functional assays, which identified RES as a crucial factor for spliceosomal assembly and cell viability at elevated temperatures.[15,17,20,22] Most importantly, RES offers a glimpse into the cooperative assembly of subunits of the spliceosome.[16]

The 3D structures of RES and its dimeric intermediates

RES is composed of 3 proteins. Pml1p and Bud13p fold through their intrinsically disordered parts onto Snu17p (Fig. 1).[16,23] In our efforts to investigate each step of the assembly, we first attempted to obtain the 3D structure of the isolated Snu17p. This proved to be impossible due to its instability.[16] After establishing the minimal Bud13p and Pml1p fragments required to mimic their full-length parent proteins, we observed that addition of any of the 2 binding partners (Pml1p or Bud13p) stabilized the protein sufficiently.[16,23,24] When both proteins were present, stability was increased even further.

CONTACT Markus Zweckstetter Markus.Zweckstetter@dzne.de, mazw@nmr.mpibpc.mpg.de

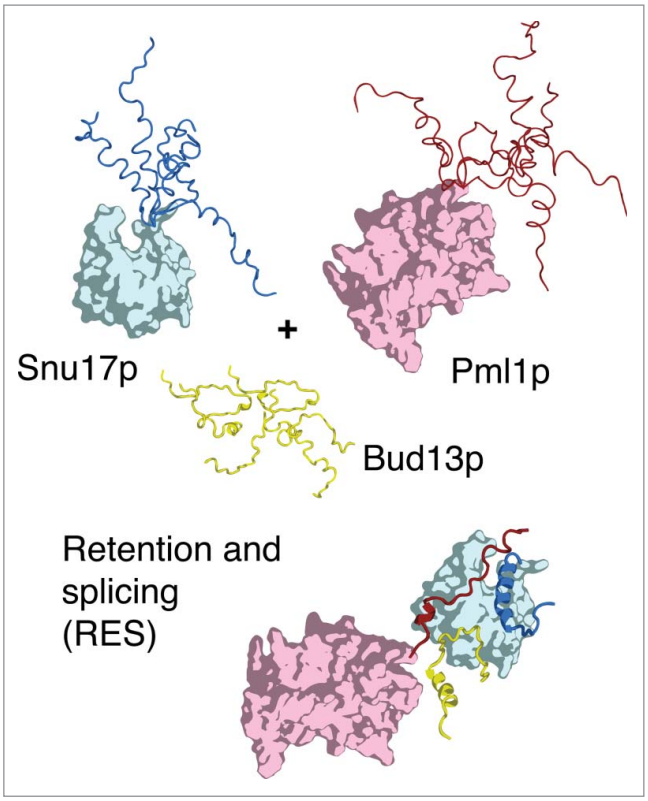

Figure 1. Cooperative RES assembly through folding and rigidification of disordered protein regions.[16,21] (Upper panel) Regions, which are disordered in free Snu17p (blue), Pml1p (pink) and Bud13p (yellow), are schematically represented as 5-membered ensembles. The RRM domain of Snu17p and the FHA domain of Pml1p are shown using a surface representation. Scales and shapes of proteins are preserved but for clarity the Bud13p representation corresponds only to the Snu17p binding part (both panels). (Lower panel) In the trimeric RES complex, the dynamic/disordered regions of Snu17p, Pml1p and Bud13p form a rigid interface.

By solving the 3D structure of the 2 dimers and the core of RES itself, we revealed a new type of interaction interface between all 3 partners (Fig. 1 and 3B).[16,21] The interface is formed by a long C-terminal extension of the RRM domain of Snu17p, which folds into an α-helix along Pml1p, as well as an extended hairpin conformation of Bud13p.[16] In particular, the Bud13p recognition mode was unusual as it did not fit the predicted so called UHM (U2AF homology motif)-ULM (UHM ligand motif) type of interaction. This finding was also independently confirmed in another structure of the Bud13p-Snu17p dimer.[19] UHM-ULM interactions are frequently observed between a subset of RRM domains (denoted UHMs) and central tryptophan containing motifs – ULMs – to which Bud13p was believed to belong.[23-25] The central tryptophan of the ULM is essential for the stability of such interactions.[11,26] Frequently ULMs shuffle between different UHMs,[27] and UHMs are capable of recognizing various ULMs.[26,28] This suggested that Bud13p might recognize a genuine UHM domain given its striking sequence similarity to other ULMs.[16]

The Dynamic Nature and Cooperativity in RES

The observation that the stability of Snu17p increases dramatically with the addition of its binding partners has its origin in the cooperativity and rigidification of this system.[16,21] All 3 structures - 2 dimers with either Pml1p or Bud13p as well as the RES trimer - show subtle adjustments such as the position of Bud13p and Pml1p. In addition, pronounced differences in the mobility of the C-terminus of Pml1p and the L63–F73 loop of Snu17p were observed. Undoubtedly, the largest scale rearrangement is the folding of the C-terminal extension of Snu17p into a rigid α-helix.[16,21] A binding-induced rigidification of Snu17p was further supported by hydrogen/deuterium exchange studies, which revealed differences in hydrogen/deuterium exchange rates between the complexes by 6 orders of magnitude.[16] The rigidification of Snu17p from the monomer through the dimeric state to the trimer follows the strong cooperativity in this system where secondary to tertiary interactions show at least 100-fold differences in affinity.[16] For example, the binding of Pml1p to free Snu17p has a K_d of 1328 nM, which decreases to 7.8 nM when Bud13p is present.[16] Notably, no direct interaction between the Pml1p and Bud13p fragments was detected, suggesting that the cooperative effect is mediated by the central binding platform – Snu17p. In addition, Snu17p is most capable to bind to mRNA, when the protein is in complex with Pml1p and Bud13p.[16] Fig. 2 illustrates the pre-mRNA induced chemical shift perturbation, which was observed in Snu17p either in the free state or bound to Pml1p and/or Bud13p.[16] Clearly, when the C-terminal α-helix of Snu17p is unfolded (in either the free state or bound to Bud13p) a smaller number of amide resonances was affected.[16]

The three 3D structures in combination with the hydrogen/deuterium exchange data of Snu17p allowed us to formulate a model in which the binding-mediated rigidification of the Snu17p structure is responsible for the progressive increase in affinity,[16] consistent with the hypothesis that most dynamic proteins exhibit cooperative behavior.[29] Notably, entropic contributions to cooperativity were unfavorable and it was primarily enthalpy, which contributed to the increase in binding affinity.[16] Cooperative behavior in pre-mRNA binding was also shown to be important in other snRNP complexes,[30,31] as well as in transcription factors and operon binding. In addition, cooperativity was shown to be important for the function of IDPs.[32] The ability of many IDPs to form promiscuous interaction hubs controlled by cooperativity is in line with the enrichment of the spliceosomal proteome by IDPs – 47% of all yeast spliceosomal proteins are predicted to be disordered whereas the average for the entire yeast proteome is only 13%.[1,2] The advantage of IDPs in terms of reach and promiscuity can be complemented by different types of cooperative behavior. For instance, cooperativity induced by the presence of multiple identical or similar motifs (a form of pseudo-homotropic allostery), similar to the one described in,[27] could be a general way by which rapid recruitment of proteins is achieved. In addition, heterotropic cooperativity, which involves different ligands and binding sites, has been reported.[16,21]

Remaining questions

Pml1p and the phosphorylated motifs

Approximately 25% of all serine/threonine/tyrosine residues in the spliceosome, most of which are located in disordered parts, are believed to be phosphorylated.[5] For example, phosphorylation of arginine/serine-rich (RS) proteins influences their

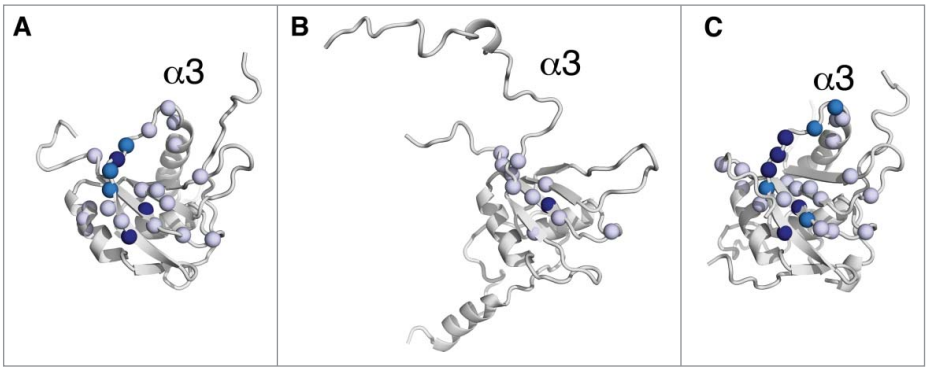

Figure 2. Interaction of Snu17p with pre-mRNA.[16,21] Mapping of the chemical shift perturbation (CSP) experienced by Snu17p upon addition of CUUCAUUCUUUUUG RNA to the Pml1p–Snu17p dimer (A), the Bud13p–Snu17p dimer (B) and the RES trimer (C). Gray, CSP $< 1\sigma$; light-blue, $1\sigma <$ CSP $< 2\sigma$; blue, $2\sigma <$ CSP $< 3\sigma$; dark blue, $3\sigma <$ CSP.

structure and interaction with other spliceosomal proteins and RNA.[33,34] In contrast, little is known about the importance of phosphorylation for the structure and function of the RES complex. The most obvious phosphorylation-specific site of regulation in RES might be the forkhead-associated domain (FHA domain) of Pml1p, because FHA domains are phosphopeptide recognition domains found in many regulatory proteins.[25,35] Unfortunately, our efforts to identify phosphopeptides, which bind to the FHA domain of Pml1p, have failed so far. In a high throughput assay using a large selection of spliceosomal peptides, we identified a possible peptide sequence and could see specific sequence enrichment, consistent with a distinct recognition motif (Fig. 3A). However, binding of the same peptide sequences to the FHA domain of Pml1p in solution could not be detected by NMR spectroscopy (data not shown). It therefore remains an open question which phosphorylated protein is responsible for the interaction with the FHA domain of Pml1p and why it has not yet been picked up in pull-down assays. The latter suggests a transient interaction rather than a stable complex. Also, given the short length of the linker between the

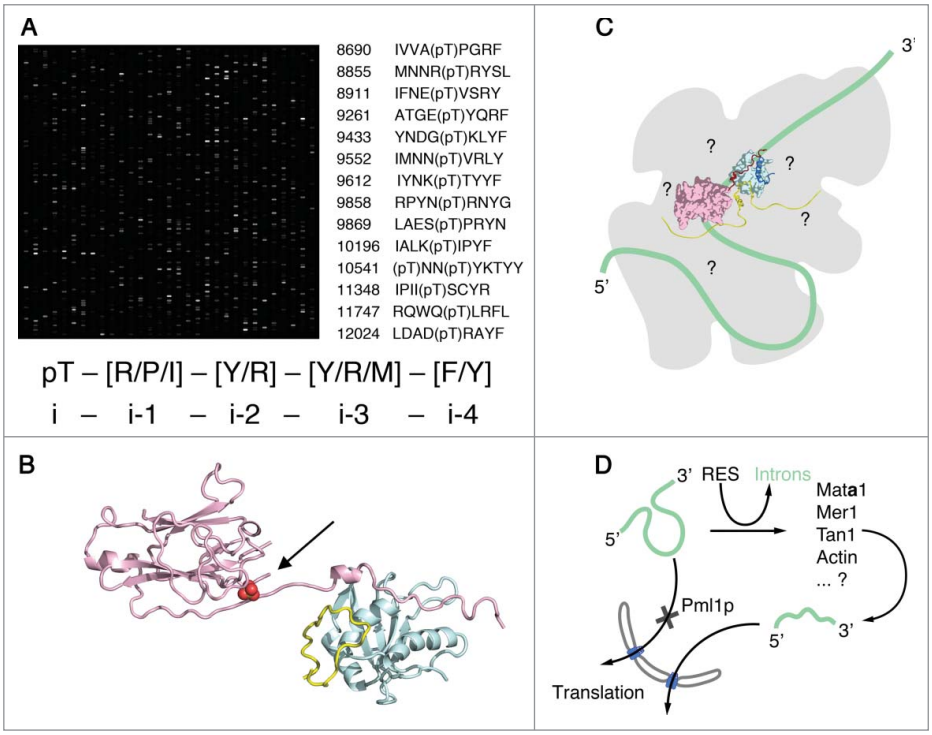

Figure 3. Toward the function of RES. (A) High-throughput phosphopeptide assay aiming at identification of potential binding partners of the FHA domain of Pml1p (left).[25,35] 14 top scoring peptides identified by the assay (right). Below a frequency-based consensus motif is shown listing the 4 residues downstream of the phosphorylated threonine. Residues were selected based on the 40 top scoring sequences with at least 2-fold higher frequency as compared to all (2553) sequences in the assay. (B) Model of the complex of full-length Pml1p (pink) with Snu17p (cyan) and Bud13p (yellow). The orientation of the structures is based on the 2MKC and 3ELV structures as deposited in the PDB. Pml1p residues in between the FHA domain of Pml1p and the N-terminal end of the Pm1p peptide in complex with Snu17p were added using Pymol.[44] (the "reverse" orientation was selected, because it results in the largest distance between the FHA domain of Pml1p and the RRM domain of Snu17p). The likely position of a phosphorylated threonine in a yet to be determined phosphopeptide is indicated with an arrow. (C) Schematic representation of full-length RES in the context of the B^{act} spliceosome (gray). The bound pre-mRNA is represented in green. The exact position of potential RES binding partners such as Spp2, Hsh155 or Prp2 is currently not known (indicated with question marks). (D) Diagram illustrating the current knowledge about the function of RES. Proteins, for which pre-mRNA splicing is controlled by RES, are indicated. The function of Pml1p as an inhibitor of pre-mRNA export is also represented.

FHA domain of Pml1p and its Snu17p-interaction site (Fig. 3B), binding of a phosphopeptide at the predicted binding site in the FHA domain of Pml1p might modulate the interaction of Pml1p with Snu17p.

The structure of full-length RES in solution and bound to the spliceosome

Our 3D structure of the core of the RES complex and a previously determined structure of the isolated FHA domain of Pml1p.[25,35] puts tight constraints on the structure of the full-length RES complex. This allows preparation of a model of the 3D structure of full-length RES (Fig. 3B). At the same time, the model presented in Fig. 3B only represents a crude approximation and an experimentally derived structure of full-length RES is needed. Even more interesting would be 3D structures of RES in complex with possible partners such as mRNA, Hsh155 and Spp2,[16] with the final goal of an atomic resolution structural description of the B^{act} complex.(Fig. 3C)

Further cooperativity

Equally important and related to the above static picture is to expand the quantitative RES interaction studies with a focus on cooperative contributions (Fig. 3C). Given the ever-increasing recognition of the role of cooperativity, which is often linked to coupled folding and binding, in the transcription factors–DNA interactome,[32,36,37] similar investigation into spliceosomal interaction networks is surprisingly scarce. An interesting starting point could be a study of the stoichiometry of the interaction of Bud13p with the Pml1p-Snu17p dimer. Tripsianes *et al.* observed a 1:2 stoichiometry with a weaker second binding side being evident.[20] We could not observe a similar behavior but our constructs were sufficiently different.[16] Nonetheless, those results suggest that RES assembly might be even more complex opening a possibility for another molecule of Bud13p influencing the cooperative nature of the interaction.[20]

Considering the moderate binding affinity of RES to RNA.[16] and given the still poorly characterized interaction with additional RES binding partners such as Spp2 and Hsh155, it is conceivable that the interaction with mRNA within the spliceosome is orders of magnitude stronger (Fig. 3C).

The function of RES

So far 4 pre-mRNAs, which correspond to the proteins actin, Tan1, Mer1 and Mata1, were demonstrated to exhibit RES-controlled splicing (Fig. 3D).[22,38-40] Although it was suggested that a weak 5' splice site might be a defining feature, the recognition motif of introns for RES is still unknown.[15,22,38,41] Additional complication may arise from the fact that RES – beyond its specific activity – also seems to be a general (unspecific) splicing factor. To which degree those 2 roles overlap remains to be established.

The exact role of Pml1p is even less clear. Though its involvement in mRNA retention is established,[15] how and with which additional factors is currently unknown (Fig. 3D). Both Tripsianes *et al.* and we noticed the structural similarity between the complexes of REF2/ICP27 and Bud13p/Snu17p, as well as ORF57/REF2 and Bud13p/Snu17p.[16,20] Considering that all of these proteins are involved in the same mRNA export pathway, a possible cross-talk with REF2 (Yra1 for *Saccharomyces Cerevisiae*) might occur.[16,20,42,43] How and at which stage RES or constituents of RES influence mRNA, would require further attention.[19]

Conclusion

Understanding the catalytic cycle of the spliceosome is one of the biggest challenges of structural biology in the 21st century. What appears to be the major hurdle for X-ray techniques – dynamics, flexibility and abundance of IDPs – is in fact what makes the properties of the spliceosome even more fascinating and highlights the functional importance of cooperativity. Our structural and dynamic studies of the RES complex, which is an essential part of this puzzle, is a step toward further understanding the function of the spliceosome at one of its crucial transition points.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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