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Assembly and recycling of functional splicing complexes *in vivo*.

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Content

Souhrn

Sestřih pre-mRNA je jedním z klíčových kroků genové exprese, který probíha v buněčném jádře. Je katalyzován multiproteinovým komplexem zvaným spliceosome, který je formován z pěti hlavních malých jaderných ribonukleoproteinových částic U1, U2, U4, U5 a U6 snRNP a dalších asociovaných proteinů. Rozpoznání a vystřižení intronů z pre-mRNA a následné spojení exonů za tvorby mRNA je doprovázeno mnoha konformačními změnami mezi jednotlivými částicemi spliceosomu. Kompletní sestřihová mašinerie se sice nachází v jádře, ale některé důležité kroky snRNP maturace se odehrávají v cytoplazmě. snRNP cyklují kontinuálně přes nukleoplazmu a jaderné kompartmenty, kde vykonávají své funkce. V Cajalových tělískách probíhají posttranskripční modifikace jednotlivých snRNA a některé snRNP se tady skládají do větších komplexů jako U4/U6 di-snRNP a U4/U6.U5 tri-snRNP.

Pre-mRNA sestřih probíhá za postupné asistence jednotlivých snRNP. V této práci jsem ukázala, že asociace U1 snRNP s 5´ sestřihovým místem probíhá řádově v sekundách. U2 snRNP pak rozeznává 3´sestřihové místo a takto je intron definován. Za účasti U4/U6.U5 tri-snRNP vznikne katalytické jádro spliceosomu U2.U5.U6. Ukázala jsem, že samotná sestřihová reakce až do uvolnění spliceosomu trvá přibližně 30 sekund a ve srovnání s dynamikou rozeznání intronu určuje rychlost celého procesu.

Uvolněné snRNP se recyklují, aby se mohly znovu účastnit sestřihu. Ukázali jsme, že opakované skládání U4/U6.U5 tri-snRNP komplexů probíhá převážně v Cajalových tělískách, kde U5 snRNP asociuje s U4/U6 di-snRNP komplexem. Tento proces je dynamický a vyžaduje produktivní interakci mezi specifickými proteiny U5 a U4/U6 snRNP, hPrp6 a hPrp31. Myší knockout proteinu hPrp31 je letální a RNAi znemožní zformování tri-snRNP komplexu a vede ke zvětšení Cajalových tělísek.

Některé mutace hPrp31 proteinu se vážou k onemocnění retinitis pigmentosa, které ovlivňuje specifické buňky v retině a může vést k úplné slepotě. Ukázala jsem, že mutace Ala216Pro (AD29) brání integraci tohoto proteinu do U4/U6 di-snRNP komplexu, ale zvyšuje jeho interakci s hPrp6 proteinem. Další studium AD29 interakcí s proteiny a snRNA z U4/U6.U5 tri-snRNP komplexu by mohlo prohloubit naše poznatky o molekulárním mechanismu, který spouští toto onemocnění.

Technika RB-FRET, kterou jsem zavedla, by v tomto případě byla ideálním nástrojem pro analýzu interakcí mezi RNA a proteiny v živých buňkách.

Summary

Pre-mRNA splicing is a key step in gene expression, which occurs in the cell nucleus. It is accomplished by a huge complex called the spliceosome. Within the spliceosome five major snRNPs U1, U2, U4, U5, U6 and additional splicing factors undergo dynamic interactions and rearrangements to define and excise the intronic sequences from pre-mRNA to generate translatable mRNA. The whole splicing machinery is localized to the cell nucleus and some steps in snRNPs maturation occur also in the cytoplasm. snRNPs continuosly cycle through nucleoplasm and nuclear compartments, like CBs and speckles to fullfill their functions. In CBs snRNPs obtain the necessary posttranscriptional modifications (pseudouridinylation, 2‘-O-ribose methylation) and some get pre-assembled to U4/U6 di-snRNP and U4/U6 tri-snRNP.

Pre-mRNA splicing occurs in an ordered association and dissocation of snRNPs with pre-mRNA. In the nucleoplasm the U1 and U2 snRNPs scan and recognize the intronic sequences. I showed that U1 snRNP association with 5‘ splice site is characteristic for a rapid exchange rate of 0.5 s. After the 3‘ splice site is synthetized U2 snRNP comes and this way the intron is defined. By engagement of U4/U6.U5 tri-snRNP the catalytic spliceosomal core U2.U5.U6 is generated. I showed that compared to the dynamic and fast intron recognition, the splicing reaction is the rate limiting step, which is accomplished within 30 seconds before the spliceosome disassembles.

The disassembled snRNPs are recycled and participate in the splicing process again. We showed that repeated formation of U4/U6.U5 tri-snRNP occurs mainly in Cajal bodies, where the free U5 snRNP joins the pre-assembled U4/U6 di-snRNP. This process is very dynamic and encounters productive interaction between U5 and U4/U6 snRNP specific proteins, hPrp6 and hPrp31 respectivelly. Mouse knockout of hPrp31 is lethal and RNAi leads to impaired formation of tri-snRNP and enlarged CBs.

Several mutations within hPrp31 protein were linked to cell specific disease retinitis pigmentosa, which affects rod cells in retina and ultimately leads to total blindness. I showed that Ala216Pro mutation in hPrp31 (AD29) impairs integration of this mutant form to U4/U6 di-snRNP, but streghtens its interaction with hPrp6 protein. Deeper study of AD29 interactions with U4/U6.U5 tri-snRNP proteins and snRNAs might bring more insights to the molecular mechanisms initiating this disease.

RB-FRET approach, which I introduced would be in this case an ideal tool for analysing RNA-protein interactions in living cells.

1. Introduction

The cell nucleus is the hallmark of eukaryotic cells, where genetic information - DNA is accommodated and replicated prior to cell division and where the initial steps of gene expression take place. In higher eukaryotes protein coding genes consists of coding sequences – exons and non – coding sequences – introns. Transcription of these genes results in precursors (pre-mRNAs) that must be processed before translation of the message into protein. During pre-mRNA splicing introns are excised and exons joined together to generate mature messenger RNA (mRNA). One pre-mRNA can be spliced in alternative ways and several different mRNAs and proteins can be produced from one DNA sequence. As it is currently estimated that over two-thirds of human genes produce alternatively spliced mRNAs, pre-mRNA splicing is the major source of protein diversity (Blencowe 2006).

Spliceosome - the executor of pre-mRNA splicing.

Splicing of pre-mRNA is carried out by the spliceosome in a two-step phosphodiester transesterification reaction. The spliceosome is a huge ribosome-size ribonucleoprotein complex composed of five major spliceosomal small ribonucleoprotein particles (snRNPs) U1, U2, U4, U5, and U6, plus additional splicing factors (Jurica and Moore 2003). Each snRNP consists of a unique snRNA that is associated with a specific set of proteins (Will and Luhrmann 2001).

During splicing several rearrangements must occur, which require the association, displacement and switching of snRNPs within the spliceosome (Staley and Guthrie 1998). The prevailing model of spliceosome assembly is derived from *in vitro* splicing experiments, which indicate a step-wise addition and displacement of individual snRNPs to the pre-mRNA. U1 snRNP binds to 5‘ ss of the intron followed by U2 snRNP interaction with the 3‘ss forming the A complex. U4, U5 and U6 snRNPs, pre-assembled into the U4/U6.U5 tri-snRNP, subsequently enter the A complex to form the pre-catalytic B complex. U1 and U4 snRNPs dissociate and the activated B* complex is generated. The activated spliceosome performs two transesterification reactions, the intron is excised, the exons are ligated together and mRNA is released. Finally, U2.U6.U5 snRNPs disassemble from the intron lariat to be recycled in new rounds of splicing and the intron is degraded. While the ordered assembly model of the

spliceosome is conserved from yeast to humans, based on *in vitro* experiments it has been proposed that the association of the spliceosome can occur in a pre-assembled complex also called the `holospliceosome` or the `penta-snRNP`. How the spliceosome assembles *in vivo* still remains an open question. In this thesis I analyzed *in vivo* formation of the spliceosome using fluorescent live-cell imaging techniques FRAP and FCS.

Spatial organization and compartmentalization of the splicing machinery.

The biogenesis and maturation of the U1, U2, U4, U5, and U6 snRNPs is a complex process that occurs both in the nucleus and in the cytosol. U1, U2, U4 and U5 snRNAs are synthetized by RNA polymerase II, capped at their 5' ends and transported to the cytoplasm. In the cytoplasm a ring of seven Sm proteins is assembled on snRNAs by the sequential action of pCl1 and SMN complex (Paushkin, Gubitz et al. 2002). The 5' end is hypermethylated by TGS1 methyltransferase and core snRNPs are imported back to the nucleus to participate in the splicing process. The U6 snRNA is synthetized by RNA polymerase III and its maturation occurs solely in the nucleoplasm. In the cell nucleus, prior entering splicing pathway, snRNPs first accumulate in CBs (Sleeman and Lamond 1999), where snRNAs are posttranscriptionally modified by 2′-O-ribose methylation and pseudouridylation (Jady, Darzacq et al. 2003; Kiss 2004) and likely associate with snRNP-specific proteins.

In addition, CBs serve as the sites of complex assembly steps that involve RNA-RNA annealing and the sequential addition of snRNP-specific proteins. Assembly of both 12S and mature 17S U2 snRNP, U4/U6 di-snRNP and U4/U6.U5 tri-snRNP takes place in CBs (Nesic, Tanackovic et al. 2004; Schaffert, Hossbach et al. 2004; Stanek and Neugebauer 2004). Inhibition of tri-snRNP formation by knockdown of hPrp6 (U5 snRNP) or hPrp31 (U4/U6 di-snRNP) leads to accumulation of the U4/U6 snRNPs in CBs, while levels of the U5 snRNA and U5-specific proteins remain unchanged (Schaffert, Hossbach et al. 2004). These data show that U4/U6 di-snRNP is retained in CB until the U5 snRNP joins; only when the mature tri-snRNP is formed it is released from the CB. In this thesis I addressed the role of CBs in snRNP recycling after splicing.

Splicing and diseases.

Pre-mRNA splicing is a tightly regulated process. A splicing error that adds or removes even 1nt will disrupt the open reading frame of an RNA and result in production of a non-functional or even a harmfull protein. The spliceosome must act very precisely, when recognizing the correct splice sites prior to the cut-and-paste reactions. The diseases caused by defects in pre-mRNA splicing can be caused either by *cis*-acting mutations affecting the splice sites, or by *trans*-acting mutations affecting the splicing machinery. Mutations that disrupt the assembly or function of spliceosomal snRNPs are responsible for two human diseases: retinitis pigmentosa (RP) and spinal muscular atrophy (SMA), in which two different subsets of cells are affected.

RP is a heterogeneous disease characterized by progressive retinal degeneration, night blindness, loss of peripheral vision, and ultimately total blindness. RP is caused by loss of rod photoreceptor cells and it can be inherited as autosomal dominant, autosomal recessive, or X-linked disorder. More then 30 RP-linked genes were identified, most of which have retina specific functions, like opsin. Interestingly, four genes, namely *HPRP31*, *HPRP3*, *HPRP8* and *SNRNP200*, involved in RP development encode U4/U6.U5 tri-snRNP specific proteins (McKie, McHale et al. 2001; Vithana, Abu-Safieh et al. 2001; Chakarova, Hims et al. 2002; Zhao, Bellur et al. 2009). hPrp31 and hPrp3 are U4/U6 di-snRNP associated proteins and hPrp8 and SNRNP200 are the core components of the U5 snRNP. hPrp31 promotes association between U4/U6 di-snRNP and U5 snRNP by direct interaction with hPrp6, U5-specific protein, which results in tri-snRNP formation (Schaffert, Hossbach et al. 2004). Mutations in HPRP31 gene causing RP include insertions, deletions, missence mutations, and splice site mutations. One of these mutations is AD29, a missence mutation Ala216Pro, which is not contained within the U4 or U5 snRNP interacting domains. In this thesis I examined the effect of the AD29 mutation in HPRP31 gene on snRNP metabolism and cell behaviour.

Tools for studying biological processes.

A central challenge to cell biologists is to understand the extensive networks of protein-protein, DNA-protein and RNA-protein interactions that regulate cellular processes. Recent advances in microscopy methods have made it possible to visualize the molecules using the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. In addition several *in vivo* tools, with which we can probe cellular binding interactions were introduced: fluorescence resonance energy transfer (FRET),

fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS). FRET is non-radiative transfer of energy between two fluorophores (donor and acceptor), which can occur if they are very close to each other (< 10 nm). As a protein-protein interaction usually occurs within 10nm distance, it is possible to measure FRET between two fluorescently tagged molecules, which interact with each other. For FRET to happen the fluorescence emission spectrum of the donor has to overlap with the absorption spectrum of the acceptor. In addition, the transition dipole orientations of the fluorophores must be approximately parallel for FRET to occur. The most used FRET pair is the combination of CFP and YFP (cyan and yellow fluorescent protein). In this thesis I introduced a new method for studying RNA-protein interactions in living cells based on FRET approch.

The most commonly used technique to determine the dynamic properties of proteins *in vivo* is FRAP. This approach involves photo-bleaching of a small area within the cell and monitoring the recovery of fluorescence intensity over time. Fluorescence recovery results from the movement of unbleached molecules from the surroundings into the bleached area. FRAP recovery provides not only a qualitative impression of the protein mobility, but more importantly, it contains quantitative information about the diffusion and binding characteristics of analyzed molecules (Sprague and McNally 2005). FCS provides an alternative approach to measure protein dynamics *in vivo* with microsecond time resolution (Kim, Heinze et al. 2007). In this technique, a laser beam is focused on a spot of interest in the cell and fluctuations of the fluorescence intensity are measured over time. The recorded signal reflects the movement of labelled proteins through the sample volume. The intensity record is transformed into an autocorrelation function that assesses the diffusion correlation time of the detected molecules.

Combination of all these methods provides high temporal and spatial resolution for analysing the binding properties of the proteins and biological processes of our interest in living cells.

2. Aims of the thesis

In this thesis we concentrated on assembly and recycling of functional splicing complexes in the cell nucleus. We took an advantage of using fluorescent microscopy as a powerfull tool for live cell imaging and analysis of biological events *in vivo*. First, we focused on spatial and temporal organization of snRNP dynamics within the cell nucleus during transcription and splicing. Next, we assessed the effect of a disease linked mutation in snRNP specific protein on snRNP metabolism. Finally, we introduced a method for studying RNA-protein interactions in living cells.

The specific aims were:

- to show that Cajal bodies contain also mature snRNPs
- to elucidate the role of Cajal bodies in snRNPs recycling

- to describe the snRNP diffusion properties in the cell nucleus by FCS and FRAP
- to examine the snRNP interactions with pre-mRNA and determine their dissociation rates by quantification of the FRAP data
- to elucidate how the snRNPs assemble the spliceosome and estimate the splicing kinetics in living cells

- to examine the effect of AD29 expression in human cells, where AD29 is a mutant variant of tri-snRNP specific protein hPrp31 linked to disease retinitis pigmentosa
- to analyse AD29 interactions with tri-snRNP specific proteins using immunoprecipitation and FRET

- to establish a method for detecting RNA-protein interactions *in situ* based on fluorescence resonance energy transfer (FRET)
- to test this approach and analyze binding of hnRNP H protein to its cognate RNA using acceptor photobleaching and lifetime imaging approach

3. Materials and methods

In the thesis, a broad spectrum of molecular and cell biological approaches coupled to life cell imaging and analysis was employed.

All methods are in detail commented in the following papers: Staněk et al. 2008, Huranova et al. 2009, Huranova et al. 2009, Huranova et al. 2010, J Cell Biol, *submitted*.

For all major experiments human cell lines were used. Cell maintenance required proper cell culturing and included transfections with DNA or siRNA and preparation of cell lines stably expressing proteins of interest.

The methods covered approaches enabling DNA/RNA/protein analysis, like DNA cloning, RNA isolation and chromatography, quantitative PCR, immunoprecipitation, Western blot analysis, glycerol gradient centrifugation, immunofluorescence.

The advanced microscopy methods included live cell imaging, photoactivation, FRET, FLIM-FRET, FRAP and FCS aproaches.

4. Results

4.1 Spliceosomal small nuclear ribonucleoprotein particles (snRNPs) repeatedly cycle through Cajal bodies.

To examine the role of CBs in snRNP life-cycle, we firstly demonstrated that CBs contain mainly mature snRNPs. Here we performed several experiments:

- snRNP specific proteins SmB and SmD1 were tagged with „fluorescent timer“ and both newly made and mature snRNPs were detected in CBs
- inhibition of translation resulted in inreased levels of mature snRNPs in CBs
- SmD1 was tagged with PA-GFP and its localization in CBs after photoactivation did not dramatically decrease (20minutes)
- FRET approach showed that snRNPs are not complexed with SMN in CBs, while in cytoplasm they do and that their Sm ring is correctly assembled

Furthermore, we showed that snRNPs constantly cycle between CBs. We transfected cells with SmD1-PA-GFP or SmB-PA-GFP together with CB markers SART3 or coilin to properly localize CBs. After SmD1-PA-GFP or SmB-PA-GFP photoactivation in one CB we observed rapid accumulation of fluorescence in the other CBs. These results confirmed that CB contains mature snRNPs, which constantly cycle between them.

Next we inhibited spliceosome disassembly using siRNA against hPrp22 and Ntr1 proteins, which help to release mRNA and snRNPs after the pre-mRNA is spliced. We detected increased levels of U4/U6 di-snRNP components in CBs, which resulted in CBs enlargement. U1 and U2 snRNP levels did not change and U5 snRNP levels decreased. We confirmed that after inhibition of spliceosome disassembly the enlarged CBs accomodate assembled U4/U6 di-snRNPs. Using FRET we probed the interactions between U4/U6 di-snRNP components, which did not change after siRNA treatment. In addition, inhibition of spliceosome disassembly did not result in any substantial inhibition of splicing, which suggested that the observed phenotype was due to inhibition of recycling. Analysis of spliceosomal complexes composition by glycerol gradient centrifugation revealed that inhibition of spliceosome disassembly decreased levels of mono-U5snRNP and increased its presence in post-spliceosomal complexes.

These data showed that CBs serve additionally as the U4/U6.U5 assembly sites throughout the snRNPs lifespan.

4.2 Differential interaction of snRNPs with pre-mRNA reveals splicing kinetics in living cells

To analyse the dynamic properties of individual snRNPs in living cells we established cell lines stably expressing GFP-tagged snRNP specific proteins from recombineered BACs. We showed that these GFP-tagged protein are incorporated into the particular snRNPs and so are suitable for live cell measurements.

First we employed FCS to examine the snRNPs movement at microsecond time resolution and this approach enabled us to measure diffusion properties of snRNPs mobile fraction. We found that snRNPs diffuse throught nucleoplasm with comparable diffusion times, which did not remarkably change upon inhibition of transcription with DRB indicating that this movement is not affected by interactions with pre-mRNA.

To elucidate the relatively slow interaction of snRNPs with pre-mRNA we utilized FRAP. In order to dissect diffusion from binding events in FRAP recovery curves we performed the measurements in the nucelaoplasm before and after transcription inhibition. Quantification of FRAP curves obtained from DRB treated cells revealed that the snRNPs move with comparable diffusion properties as determined by FCS. The diffusion coefficients were used as an input value in the quantification of the FRAP curves obtained from untreated cells with the diffusion-reaction model. We found that binding of individual snRNPs to pre-mRNA is described with different dissociation constants (inverse of residence times), which indicates that snRNP interact with pre-mRNA independently and supports the ordered model of spliceosome assembly. Comparison of U1 and U5 residence time revealed that while the intron recognition is accomplished within a second, the splicing reaction needs approximately ~30s to be finished. Thus, the splicing reaction is the rate limiting step of pre-mRNA splicing process and ~30s time represents the average splicing rate in living cells.

Inhibition of splicing with isoginkgetin compound increased levels of pre-spliceosomal complex A (containing U1 and U2 snRNPs) and revealed their higher residence time on pre-mRNA, which clarified the proper use of k_{off} value for description of snRNPs interaction with pre-mRNA. We employed immunoprecipitation to analyse the snRNP composition after treatment with DRB and isogingetin. We found that di-

snRNP and tri-snRNP formation was impaired in both cases indicating that ongoing transcription and splicing is necessary for their integrity.

In order to analyze snRNP interactions in a specific gene loci we established the inducible E3 U2-OS Tet-On cell line. The model gene β-globin is expressed from an inducible promoter and its transcripts are vizualized *in vivo* via interaction of their 3`UTR localized MS2 loops with the MS2-mRED protein. Upon induction of the β-globin gene expression with doxycycline snRNPs accumulate in its transcription site reflecting the demand for splicing machinery. We performed FRAP in this loci and found that snRNPs interact independently also with transcripts in a specific gene loci further supporting the step-wise assembly model of the spliceosome.

4.3 A mutation linked to retinitis pigmentosa in HPRP31 causes protein instability and impairs its interactions with spliceosomal snRNPs.

To examine the effect of expression of an RP linked mutant variant of hPrp31 protein in human cells we prepared Hela cell lines stably expressing the hPrp31 wild-type (WT31) and Ala216Pro mutant (AD29) proteins tagged with YFP. We employed life cell imaging approach and comparisson of WT31 and AD29 cell lines growth rate revealed that AD29 cell line divided 10% slower compared to WT31.

As hPrp31 is necessary for tri-snRNP formation we decided to analyse snRNP metabolism and especially the CBs as the major sites of tri-snRNP assembly in the cell nucleus. We stained cells with coilin, the CB marker and found that CBs are smaller or even disappeared in AD29 cell line. In cells, having at least the residual CBs we examined localization of snRNP specific proteins. We detected reduced localization of U4/U6 di-snRNP specific proteins in AD29 cell line, whereas localization of U5 and U2 snRNP specific proteins remained unchanged.

To examine the AD29 association with tri-snRNP proteins we employed FRET and immunoprecipitation assay. With FRET we probed AD29 interactions with SART3 (U4/U6), hPrp4 (U4/U6, U4/U6.U5) and hPrp6 (U4/U6.U5) proteins, each marking different stage of tri-snRNP formation (in parenthesis). We found that AD29 interaction with U4/U6 di-snRNP is compromised, which was confirmed by immunoprecipitation as we did not pull down with AD29 any U4/U6 specific protein. Interaction between AD29 and U5 specific proteins Snu114 or hPrp6 remained preserved. We analysed the AD29 association with snRNP complexes via glycerol gradient centrifugation and found

that AD29 exists predominantly in free form, little associates with U4/U6 and U5 snRNPs, but does not associate with tri-snRNP. These data suggested that AD29 exerts its negative function by retaining the functional U5 snRNP. We overexpressed the hPrp6 in AD29 cell line and consequenlty detected more CBs and increased proliferation rate.

During live cell observations the AD29 YFP levels differed, but were not connected to any particular cell cycle phase implying for a dynamics in AD29 expression and degradation. We inhibited translation using cycloheximide and monitored AD29 fluorescence over time and compared it to WT31. We found that the AD29 mutation likely destabilizes the AD29 protein and confirmed the rapid degradation of AD29 protein also with Western blot analysis.

4.4 In vivo detection of RNA-binding protein interactions with cognate RNA sequences by fluorescence resonance energy transfer.

To analyze interaction of hnRNP H protein with its cognate RNA using FRET acceptor photobleaching and lifetime imaging approach we prepared the following constructs:

- RNA binding protein hnRNP H was tagged at its N terminus with ECFP or Cerulean generating the constructs pECFP-hnH and Cerulean-hnH, used as the FRET donor
- bacteriophage MS2 coat protein was tagged at its C terminus with EYFP generating the construct pMS2-EYFP, used as the FRET acceptor
- pRed vector series contained 4 or 6 binding sites of MS2 coat protein and 1 or 2 binding sites for hnRNP H protein inserted to 3`UTR of the HcRed1 gene generating constructs pRed-M4x-H3`, pRed-M4x-H5`, pRed-M6x-H5` and pRed-M1x-H1x, negative control pRed-M4x represented construct without hnRNP H binding sites

To confirm direct recruitment of ECFP-hnH and MS2-EYFP onto the target RNA *in vitro* we performed RNA affinity chromatography. RNA substrates contained within the 3`UTR of the HcRed1 gene were amplified in vitro and incubated with cell extract from Hela cells transfected either with ECFP-hnH or MS2-EYFP or with both. Proteins bound to the bait RNAs were specifically eluted and analyzed by Western blot.

Next we tested whether we are able to detect this interaction directly in cells using FRET acceptor photobleaching approach *in situ*. As a positive control was used

sample expressing CFP and YFP tagged protein coilin, which is known to self-interact. The cells were transfected with aforementioned constructs and their expression was monitored by vizualizing the ECFP, EYFP tags and HcRed1 protein. FRET efficiency between MS2-YFP and ECFP-hnH correlated with expression of the RNA substrate containing binding sites for both proteins and was comparable to the coilin positive control. We next confirmed the FRET measurements utilizing the fluorescence lifetime imaging approach. We expressed either coilin-ECFP or hnRNP H-ECFP/Cerulean alone or co-expressed with the acceptor molecules. In case of our positive control we detected strong decrease in coilin-ECFP lifetime in presence of the acceptor molecule coilin-EYFP, which indicated efficient FRET. We detected also decrease in ECFP-hnH or Cerulean-hnH lifetime in presence of MS2-YFP, likely bound to the RNA substrate. These positive FRET measurements indicated that the stable interaction occuring between hnRNP H and its binding sequence in the pRed constructs could be vizualized *in situ*.

5. Discussion

In this thesis I presented results that contribute to understanding the dynamic behaviour and assembly of the spliceosomal complexes in the context of the cell nucleus. I showed how the disease linked mutation in a particular splicing factor affects the dynamic interactions between the spliceosomal subcomplexes. In addition, I introduced a novel method for studying RNA-protein interactions in living cells. The results were separately described and discussed in chapters 2, 3, 4, and 5 of the thesis.

Mature splicing machinery is exclusively restricted to the cell nucleus, where the pre-mRNA processing takes place. The pre-mRNA splicing is accomplished by the dynamic network of snRNPs within spliceosome. Once the intron is defined by interactions of U1 and U2 snRNPs, the pre-assembled U4/U6.U5 tri-snRNP joins this complex and after extensive rearrangements the active spliceosome core is generated (Wahl, Will et al. 2009). Recently two models of spliceosome assembly were proposed based mostly on *in vitro* approches (reviewed in (Rino and Carmo-Fonseca 2009)). One model proposes a step-wise assembly of the spliceosome exerted by sequential association and dissociation of snRNPs with pre-mRNA, while the second one proposes that spliceosome is already a pre-assembled complex and in the form of `penta-snRNP` associates with pre-mRNA to promote splicing.

In this thesis I addressed the question how the spliceosome assembles *in vivo* by analysing and quantifying snRNP interactions with pre-mRNA. The dynamic behaviour of snRNPs in the nucleoplasm reflects their splicing activity and so is determined by their diffusion and interactions with pre-mRNA. Inhibition of transcription leads to relocalization of snRNPs to speckles implying that inactive snRNPs are stored to these structures. The fraction of snRNPs that remained in the nucleoplasm showed faster movement which indicated a lack of a substrate to bind. Similarly faster movement of several splicing factors due to transcription inhibition was already reported (Rino, Carvalho et al. 2007). This movement is characterized as an effective diffusion, because even in the absence of pre-mRNA the snRNPs scan and transiently interact with the nuclear environment (Phair and Misteli 2001). I quantified diffusion rates describing snRNPs effective diffusion ranging between 0.2 - 0.8 $\mu m^2 s^{-1}$.

While measurements performed after transcription inhibition provide an information about snRNPs movement, it does not answer the question how the spliceosome assembles on the pre-mRNA. Here, I quantified the interactions of individual snRNPs with pre-mRNA in cells with ongoing transcription. The strenght of a particular interaction is characterized by the dissociation constant, which specifies the time the snRNP is bound to the pre-mRNA and released afterwards – the residence time. I found that U1 and U4/U6 snRNPs stay bound to the pre-mRNA around 1s, but U2 and U5 snRNPs associated with pre-mRNA at least 10 times longer, approximately tens of seconds. Thus, these differantial interactions between snRNPs and pre-mRNA let me assume that snRNPs interact with pre-mRNA independently, which supports the step-wise model of spliceosome assembly. Importantly, the residence time of the core spliceosomal components U2 and U5 snRNPs provided me also the estimate of an average splicing rate, as the aforementioned 12-30s reflect the time the U2 and U5 snRNPs stay associated with pre-mRNA till it is spliced. This data are consistent with previous studies from Miller spreads from Drosophila embryos and ChIP data from yeast, where both show that splicing is accomplished within a minute after spliceosome formation (Beyer and Osheim 1988; Wetterberg, Zhao et al. 2001; Gornemann, Kotovic et al. 2005). Conclusivelly, I showed that individual snRNPs and pre-assembled snRNPs move throughout the nucleoplasm and in an ordered pathway assemble the spliceosome, which likely allows to accomodate many regulatory events to control the process of pre-mRNA splicing.

When the spliceosome carries out splicing, it is disassembled by assistance of hPrp22 and the hPrp43/Ntr1/Ntr2 complex (Company, Arenas et al. 1991; Arenas and Abelson 1997; Tsai, Tseng et al. 2007). It has not been shown so far, what happens with the disassembled U2, U5 and U6 snRNPs. In addition, snRNPs dissociate from the RNA substrate independently (discussed above) and three of them U4, U5 and U6 must be re-assembled again into the tri-snRNP to participate in the splicing process, which implies for possible recycling events in the snRNP lifecycle. In this thesis I presented a study showing an additional „recycling“ role for CBs in the snRNP metabolism pathway. As mentioned at the beginning, CBs are the sites of snRNPs biogenesis and maturation. There are two to four CBs per one cell and it was reported that the size and number of CBs depend on the cell metabolic activity and cell cycle (Andrade, Tan et al. 1993; Boudonck, Dolan et al. 1998). It was shown that snRNPs do not accumulate in CBs, when transcription or splicing is inhibited (Carmo-Fonseca, Pepperkok et al. 1992;

Kaida, Motoyoshi et al. 2007), which was consistent with our data showing that CBs accomodate also mature snRNPs and that these snRNPs rapidly exchange between them. This raised a question, why the recruitment of snRNPs to CBs depends on ongoing transcription. Probably, the cycling of snRNPs through CBs could reflect snRNPs reassembly after pre-mRNA splicing. I inhibited spliceosome disassembly by depleting cells of hPrp22 or Ntr1, which resulted in accumulation of U4/U6 di-snRNP and decreased levels of U5 snRNP in CBs implying for impaired tri-snRNP formation, fenotype recently demonstrated by depleting either of hPrp31 and hPrp6 proteins (Schaffert, Hossbach et al. 2004). The impaired formation of tri-snRNP likely reflected the lack of U5 snRNP, which might be entrapped in the stalled post-spliceosomal complexes (Turner, Norman et al. 2004). The fact that di-snRNP is still formed in CBs is the result of high levels of U6 snRNPs present in the cell and fast dissociation of U4 snRNP from pre-mRNA during splicing (mentioned above). Thus, the nuclear CBs serve as the major sites of repeated di-snRNP and tri-snRNP reassembly throughout the snRNPs life-span.

Surprisingly, the basal tri-snRNP metabolism seems to have an important role in development of such a specific disease like retinitis pigmentosa (RP), which affects only rod cells in eye retina. Although it is a heterogenous disease and many mutations are located in retina metabolism linked genes, mutations in tri-snRNP specific proteins were discovered to initiate this disease, too. In this thesis, I present results showing how the AD29 (A216P) mutant variant of hPrp31 protein affects tri-snRNP metabolism and possibly causes the RP. We expressed AD29 in cells and found that the cell proliferation was reduced. Beside its nuclear localization, this protein was also present in cytoplasm, which was similar to A194E mutant form of the hPrp31 protein (Deery, Vithana et al. 2002). Unlike the wildtype U4/U6 snRNP specific proteins, AD29 was only little accumulated in CBs suggesting its inability to be incorporated into U4/U6 di-snRNP based on the results discussed above. We confirmed that interactions between AD29 and U4/U6 snRNP specific proteins were reduced. Interestingly, interactions with U5 specific proteins Snu114 and hPrp6 were stronger and this stronger interaction of AD29 and hPrp6 was previously shown also *in vitro* (Wilkie, Vaclavik et al. 2008). AD29 likely inhibits tri-snRNP assembly by retaining U5 snRNP and preventing its association with U4/U6 di-snRNP. Overexpression of hPrp6 in AD29 cells compensated the AD29 induced phenotype and resulted in rescue of CBs morphology and increased cell proliferation rate.

Future experiments should involve detailed analysis of AD29 interaction with U4/U6 and U5 snRNPs. Particularly the AD29-hPrp6 and AD29-SART3 interactions need to be resolved. It is not clear, whether AD29 and hPrp6 form AD29-U5 snRNP complex, albeit U5 snRNP specific protein hSnu114 was also detected in the AD29 precipitate. Additionally, AD29 was shown to interact with U4/U6 di-snRNP *in situ*, however, we did not pull-down with AD29 neither hPrp3 nor hPrp4 (U4/U6 di-snRNP specific proteins). Thus, it is of importance to examine also the interactions between AD29 and U5, U4 and U6 snRNAs. As the fast degradation of AD29 could make the complexes sensitive for any *in vitro* analysis, we need an aproach, which would enable us to vizualize RNA-protein interactions *in vivo*.

In this thesis I present a study employing the FRET based method (RB FRET) for studying RNA-protein interactions *in situ*. I tested RB-FRET approach on the previously shown interaction of hnRNP H protein with its cognate RNA sequence and confirmed that *in vitro* interaction happens also *in situ* (Schaub, Lopez et al. 2007). I tagged hnRNP H protein with CFP and the RNA sequence with YFP using the MS2 system, which is an elegant tool for RNA labeling (Querido and Chartrand 2008). This approach allows to examine interactions over time and in response to cellular signals. The previously shown trimolecular fluorescence complementation assay (TriFC) also allows for RNA-protein interactions *in vivo* (Hu, Chinenov et al. 2002), however lacks the temporal resolution because of the permanent cross-link among the tagged molecules and the time needed for the maturation of the fluorescent protein. Another recently presented work studying RNA-protein interactions employed the FRET-FLIM approach and introduced RNA labeling with SytoxOrange (Lorenz 2009). Since SytoxOrange labels all RNA within the cell, using this methods we cannot determine the direct target RNA sequence of the studied protein. As the RNA-protein interactions rule almost all cellular processes, like transcription, splicing, translation, RNA editing, iRNA, etc., my technique allows for labeling of a particular RNA sequence and a particular protein of interest, and detect the RNA-protein interaction *in vivo* with high temporal and spatial resolution.

6. Conclusions

- In contrast to previous studies that viewed CBs as sites of newly synthetized snRNP accumulation we showed that CBs consist mainly of mature snRNPs.

- We showed that snRNPs constantly cycle between CBs. We photoactivated SmD1-PA-GFP or SmB-PA-GFP in one CB and observed rapid accumulation of fluorescence in the other CBs. Co-transfection with CB markers SART3 or coilin was performed to properly localize CBs.

- We demonstrated that CBs are the sites of snRNP recycling and repeated tri-snRNP formation. Inhibition of spliceosome disassembly resulted in accumulation of U4/U6 di-snRNP components in CBs reflecting the lack of U5 snRNP stalled in post-spliceosomal complexes.

- We demonstrated that continuous snRNPs cycling is important for maintaining the CB structure. Inhibition of spliceosome disassembly resulted in CBs enlargement due to U4/U6 di-snRNP accumulation.

- GFP-tagged snRNP specific proteins expressed from recombineered BACs are properly incorporated into snRNPs.

- FCS measurements revealed diffusion properties of snRNPs mobile fraction. We found that snRNPs diffuse throught nucleoplasm with comparable diffusion times and that this movement is not affected by interactions with pre-mRNA.

- Quantification of FRAP curves obtained from untreated cells revealed differential interaction of snRNPs with pre-mRNA and supported ordered model of spliceosome assembly.

- Based on residence times of individual snRNPs on pre-mRNA we estimated average splicing rate in living cells (approximately 30s) and found that not the intron recognition, but the splicing reaction is the rate limiting step of pre-mRNA splicing process.

- Inhibition of splicing with isoginkgetin compound increased levels of pre-spliceosomal complex A (containing U1 and U2 snRNPs) and revealed their higher residence time on pre-mRNA, which clarified the proper use of k_{off} value for description of snRNPs interaction with pre-mRNA.

- Employing snRNA co-immunoprecipitation we found that spliceosome as well as di-snRNP and tri-snRNP integrity depends on ongoing transcription and splicing.

- Employing FRAP we found that snRNPs interact independently also with transcripts in a specific gene loci further supporting the step-wise assembly model of the spliceosome.

- We compared the growth rate of the WT31 and AD29 cell lines and found that AD29 has a dominant negative effect on cell proliferation. AD29 cell line divided 10% slower compared to WT31.

- We analysed CBs integrity and found that CBs are smaller or even disappear in AD29 cell line. We showed reduced localization of U4/U6 di-snRNP in AD29 cell line, whereas localization of U5 and U2 snRNPs remained unchanged.

- We employed FRET and immunoprecipitation and analysed AD29 association with tri-snRNP proteins. We found that AD29 interaction with U4/U6 proteins decreased, while it increased in the case of U5 specific proteins Snu114 and hPrp6.

- Analysis of the AD29 association with snRNP complexes using gradient centrifugation revealed that AD29 exists predominantly in free form, little associates with U4/U6 and U5 snRNPs, but does not associate with tri-snRNP.

- Overexpression of hPrp6 in AD29 cell line compensated the AD29 induced phenotype. We detected more CBs and proliferation rate was similar to WT31 cells.

- Inhibition of translation enabled us to vizualize and examine the stability of AD29 protein. We found that AD29 protein is rapidly degraded compared to hPrp31 protein.

- We employed RNA affinity chromatography and confirmed that ECFP-hnH and MS2-EYFP are efficiently recruited onto the target RNAs *in vitro.*

- We used FRET acceptor photobleaching approach and FLIM approach to test the RNA-protein interaction *in situ.* In both cases we used control sample expressing CFP and YFP tagged protein coilin, which is known to self-interact.

- FRET efficiency between MS2-YFP/ECFP-hnH correlated with expression of substrate containing binding sites for both proteins and was comparable to the coilin positive control.

- FLIM approach validated the results obtained by acceptor photobleaching approach. We detected decrease in ECFP-hnH or Cerulean-hnH lifetime in presence of MS2-YFP indicating the stable interaction occuring between hnRNP H and its binding sequence in the pRed constructs.

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