Identification of a quality control check-point for the assembly of mRNA-processing snRNPs

Identifizierung einer Qualitäts-Kontrollmechanismus für die Zusammenlagerung mRNA-prozessierender snRNPs

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by

Elham Paknia

from

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Submitted on:

Members of the *Promotionskomitee*:

Chairperson: …………………..………...….…..Prof. Dr. Alexander Buchberger

Primary Supervisor ....Prof. Dr. Utz Fischer

Supervisor (Second): ....Prof. Dr. Manfred Gessler

Supervisor (Third): ....Prof. Dr. Ulrich Scheer

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation **"Identifizierung einer Qualitäts-Kontrollmechanismus für die Zusammenlagerung mRNA-prozessierender snRNPs"** eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Summary

An essential step in eukaryotic gene expression is splicing, i.e. the excision of non-coding sequences from pre-mRNA and the ligation of coding-sequences. This reaction is carried out by the spliceosome, which is a macromolecular machine composed of small nuclear ribonucleoproteins (snRNPs) and a large number of proteins. Spliceosomal snRNPs are composed of one snRNA (or two in case of U4/6 snRNPs), seven common Sm proteins (SmD1, D2, D3, B, E, F, G) and several particle-specific proteins. The seven Sm proteins form a ring shaped structure on the snRNA, termed Sm core domain that forms a structural framework of all spliceosomal snRNPs. In the toroidal Sm core domain, the individual Sm proteins are arranged in the sequence SmE-SmG-SmD3-SmB- SmD1-SmD2-SmF from the first to the seventh nucleotide of the Sm site, respectively. The individual positions of Sm proteins in the Sm core domain are not interchangeable.

snRNPs are formed in vivo in a step-wise process, which starts with the export of newly transcribed snRNA to the cytoplasm. Within this compartment, Sm proteins are synthesized and subsequently transferred onto the snRNA. Upon formation of the Sm core and further modifications of snRNA, the snRNP is imported into the nucleus to join the spliceosome.

Prior to assembly into snRNPs, Sm proteins exist as specific hetero-oligomers in the cytoplasm. The association of these proteins with snRNA occurs spontaneously in vitro but requires the assistance of two major units, PRMT5- and SMN- complexes, in vivo. The early phase of assembly is critically influenced by the assembly chaperone pICln. This protein pre-organizes Sm proteins to functional building blocks and enables their recruitment onto the PRMT5 complex for methylation. Sm proteins are subsequently released from the PRMT5 complex as pICln bound entities and transferred onto the SMN-complex. The SMN complex then liberates the Sm proteins from the pICln-induced kinetic trap and allows their transfer onto the snRNA. Although the principal roles of SMN- and PRMT5 complexes in the assembly of snRNPs have been established, it is still not clear how newly translated Sm proteins are guided into the assembly line.

In this thesis, I have uncovered a new facet of pICln function in the assembly of snRNPs. I have shown that newly synthesized Sm proteins are retained at the ribosome upon termination of translation. Their release is facilitated by pICln, which interacts with the cognate Sm protein hetero-oligomers at their site of synthesis on the ribosome and recruits them into the assembly pathway. Additionally, I have been able to show that the early engagement of pICln with the Sm proteins ensures the flawless oligomerization of Sm proteins and prevents any non-chaperoned release and diffusion of Sm proteins in the cytoplasm.

In a second project, I have studied the mechanism of U7 snRNP assembly. This particle is a major component of the 3' end processing machinery of replication dependent histone mRNAs. A biochemical hallmark of U7 is its unique Sm core in which the two canonical Sm proteins D1 and D2 are replaced by so-called "like Sm proteins". The key question I addressed in my thesis was, how this "alternative" Sm core is assembled onto U7 snRNA. I have provided experimental evidence that the assembly route of U7 snRNPs and spliceosomal snRNPs are remarkably similar: The assembly of both particles depends on the same assembly factors and the mechanistic details are similar. It appears that formation of the U7- or spliceosomal- core specific 6S complex is the decisive step in assembly.

Zusammenfassung

Ein wesentlicher Schritt in der eukaryotischen Genexpression ist das Spleißen, welches nicht-kodierende Sequenzen aus prä-mRNA entfernt und kodierende Sequenzen zusammenfügt. Diese Reaktion wird durch das Spleißosom, einer makromolekularen Maschine, die aus kleinen nucleären Ribonukleoproteinpartikeln (snRNPs) und einer großen Anzahl von Proteinen zusammengesetzt ist, durchgeführt. Spleißosomale snRNPs bestehen aus einer snRNA (oder zwei im Falle von U4/6 snRNPs) und zwei Klassen von Proteinen: Die Sm Proteine SmD1, SmD2, SmD3, SmB, SmE, SmF und SmG finden sich in allen snRNPs und sind daher für allgemeine Funktionen der snRNPs verantwortlich. Dem gegenüber stehen die Partikel-spezifischen Proteinen, die für spezifische Funktionen der individuellen snRNPs verantwortlich sind. Die gemeinsamen Sm Proteine umschließen einen einzelsträngigen Bereich der snRNA (Sm-Bindungsstelle) in einer ringförmigen Struktur und bilden so die Sm-Core-Domäne aus. Diese Domäne stellt das strukturelle Grundgerüst aller spleißosomalen snRNPs dar, wobei die einzelnen Sm Proteine in der Reihenfolge SmE-SmG-SmD3-SmB-SmD1-SmD2-SmF von dem ersten zum siebenten Nukleotid der Sm-Bindungsstelle der snRNA angeordnet werden. Die einzelnen Positionen des Sm Proteine in der Sm core Domäne sind nicht austauschbar.

Die Biogenese der snRNPs erfolgt in vivo in einem mehrphasigen Prozess, der mit der Transkription der snRNA und deren Export ins Zytoplasma beginnt. In diesem Kompartiment werden Sm Proteine synthetisiert und anschließend auf die snRNA übertragen. Nach der Bildung der Sm-Core-Domäne und diversen Modifikationen der snRNA erfolgt der Kern Transport und die Integration in das Spleißosom.

Vor dem Einbau in snRNPs existieren die Sm Proteine als spezifische Hetero-Oligomere im Zytoplasma. Obwohl die Assoziation dieser Proteine mit snRNA in vitro spontan erfolgt, erfordert dieser Prozess in vivo die Unterstützung von zwei großen makromolekularen Funktionseinheiten, den PRMT5-und SMN-Komplexen. Die frühe Phase der Zusammenlagerung von snRNPs wird maßgeblich durch den PRMT5-Komplex, und hier speziell durch seine pICln-Untereinheit beeinflusst. Dieses Protein fungiert als sogenanntes Assembly-Chaperon, da es die Sm Proteine zu funktionellen

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Bausteinen zusammenfügt ohne selbst ein snRNP Baustein zu sein. Sm Proteine werden anschließend direkt von pICln als vorgefertigte Einheiten auf den SMN-Komplex übertragen. Der SMN-Komplex befreit die Sm Proteine von einer kinetischen Falle, die durch die Bindung von pICln an Sm Proteinen hervorgerufen wird und ermöglicht deren Transfer auf die snRNA. Obwohl die Wirkungsweise von SMN- und PRMT5-Komplexe bei der Zusammenlagerung von snRNPs in Grundzügen verstanden ist, bleibt es noch unklar, wie neu synthetisierte Sm Proteine Zugang zur Zusammenlagerungs-Maschinerie erhalten.

In dieser Arbeit habe ich eine neue Facette der Funktion von pICln bei der Zusammenlagerung von snRNPs aufgedeckt. Ich habe gezeigt, dass neu synthetisierte Sm Proteine nach ihrer Synthese am Ribosom gebunden bleiben. Ihre Freisetzung und Weiterverarbeitung im snRNP Biogeneseprozess wird durch pICln unterstützt. Hierbei bindet das Assembly-Chaperon kognate Sm-Hetero-Oligomere am Ribosom und überführt diese direkt in die folgende Zusammenlagerungsphase am PRMT5-Komplex. Darüber hinaus konnte ich zeigen, dass die frühe Bindung von pICln an Sm-Proteine deren spezifische Oligomerisierung sicherstellt und somit die Freisetzung ins Zytoplasma in freier, nicht Chaperon gebundener Form verhindert.

In einem zweiten Projekt, habe ich den Mechanismus der Zusammenlagerung des U7 snRNPs untersucht. Dieses Partikel ist an der Prozessierung des 3'-Endes von Replikations-abhängigen Histon mRNAs beteiligt. Eine biochemisches Merkmal des U7 snRNPs ist ihre einzigartige Sm-Core-Domäne, in der zwei kanonische Sm Proteine durch sogenannte "Sm-like" Proteine ersetzt werden. In meiner Promotion habe ich der grundsätzliche Frage adressiert wie diese "alternative" Sm-Core-Domäne des U7 snRNPs zusammengebaut wird. Ich konnte den experimentellen Nachweis erbringen, dass die Zusammenlagerung des U7 snRNPs und spleissosomalen snRNPs bemerkenswert ähnlich sind. Die Montage beider Teilchen hängt von den gleichen Faktoren ab und die mechanistischen Details sind ähnlich. Es scheint, dass die Ausbildung des 6S Komplexes, welches U7- beziehungsweise spleissosomale snRNPs spezifiziert, der massgebliche Schritt in der Bildung beider Partikel ist.

1   Introduction

1.1   Gene expression in eukaryotes

The accurate decoding of genetic information is fundamental for the impeccable function of any organism. The genetic information stored in DNA is initially converted to RNA in a process known as transcription. The product of transcription is a pool of RNAs entitled the "transcriptome". This pool not only includes protein-coding mRNAs, but also a large number of non-coding RNAs (ncRNAs) such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and small interfering RNAs (siRNAs) (Jacquier, 2009). In the transcriptome of eukaryotes, the majority of protein-coding transcripts are found as precursor mRNAs (pre-mRNAs), which undergo further modifications in the nucleus prior to their export to the cytoplasm as mature mRNAs. In the cytoplasm mRNAs are translated into proteins by translational machinery (Mendes Soares and Valcarcel, 2006).

Transcription of protein-coding genes is mediated by an RNA polymerase holo-enzyme, which uses a group of cis-regulatory elements and acts in concert with trans-acting factors. The majority of pre-mRNAs contain non-coding sequences (introns), which intervene the coding sequences (exons). A macromolecular complex, the spliceosome, removes the introns from pre-mRNA and re-ligates the exons (Montes et al., 2012). Additionally, almost all pre-mRNAs undergo cleavage and polyadenylation at their 3' end. This reaction is accomplished by yet another macromolecular complex, which requires a group of cis-regulatory elements and is assisted by trans-acting factors (Chan et al., 2011). The resulting mRNAs are then exported into the cytoplasm through the nuclear pore complex, which is mediated by factors that couple transcription to splicing, 3' end modification and export (Bjork and Wieslander, 2011; Saguez et al., 2005). In the cytoplasm, mRNA is used as a template by ribosomes to synthesize protein. Ribosomes are large complexes of RNA and proteins and are aided by trans-acting factors. Altogether, the cascade of gene expression requires approximately 400 different protein factors. These factors do not function as single

proteins but are mainly found as macromolecular machines composed of proteins or RNA and proteins (ribonucleoproteins (RNPs)).

The accurate assembly of macromolecular complexes, particularly ribonucleoproteins, is a very challenging and vital task for a living cell to ensure accurate gene expression. First, as RNAs and proteins are synthesized in two different compartments (nucleus and cytoplasm), cells have to synchronize the synthesis of both constituents. Then, the components have to be transferred to the site of assembly at the same time. Upon assembly, the complex has to be transferred to the site of function, which is usually different from the site of assembly. Using such an agenda, cells assemble different macromolecular machineries such as the spliceosome and the mRNA 3' end modifying machinery.

1.2   The spliceosome processes pre-mRNAs in the nucleus

An essential step in eukaryotic gene expression is the removal of introns (non-coding sequences) from nascent transcripts (pre-mRNAs) and the ligation of exons (coding-sequences). The spliceosome is a macromolecular complex, which accomplishes this function and is composed of small nuclear ribonucleoproteins (snRNPs) and a large group of additional proteins not tightly bound to any of the snRNPs (Hoskins and Moore, 2012; Will and Luhrmann, 2011).

The spliceosome is formed anew on introns of pre-mRNAs by the consecutive addition of snRNPs and spliceosomal proteins (Figure 1.1). Initially, the U1 snRNP binds to the 5' exon/intron boundary resulting in the formation of complex E. Then, the U2 snRNP interacts with the branch point, forming the pre-spliceosome or complex A. The subsequent association of pre-assembled U4/U6.U5 tri-snRNPs results in the formation of a pre-catalytic spliceosome (complex B). Rearrangements in RNA-RNA and RNA-protein interactions lead to the dissociation of U1- and U4 snRNPs and formation of the active spliceosome (complex B*). The catalytically active complex B* initiates the two-step splicing reaction. First, the phosphodiester bond at the 5' end of the intron is cleaved by nucleophilic attack of the 2' hydroxyl of adenosine at the branch point, which liberates the 5' exon. In the second step, the 3' hydroxyl of the released 5' exon attacks the 3' end of the intron. As a consequence, exons are ligated and the intron is

released as a lariat (figure 1.1) (Hoskins and Moore, 2012; Pandya-Jones, 2011; Will and Luhrmann, 2011).
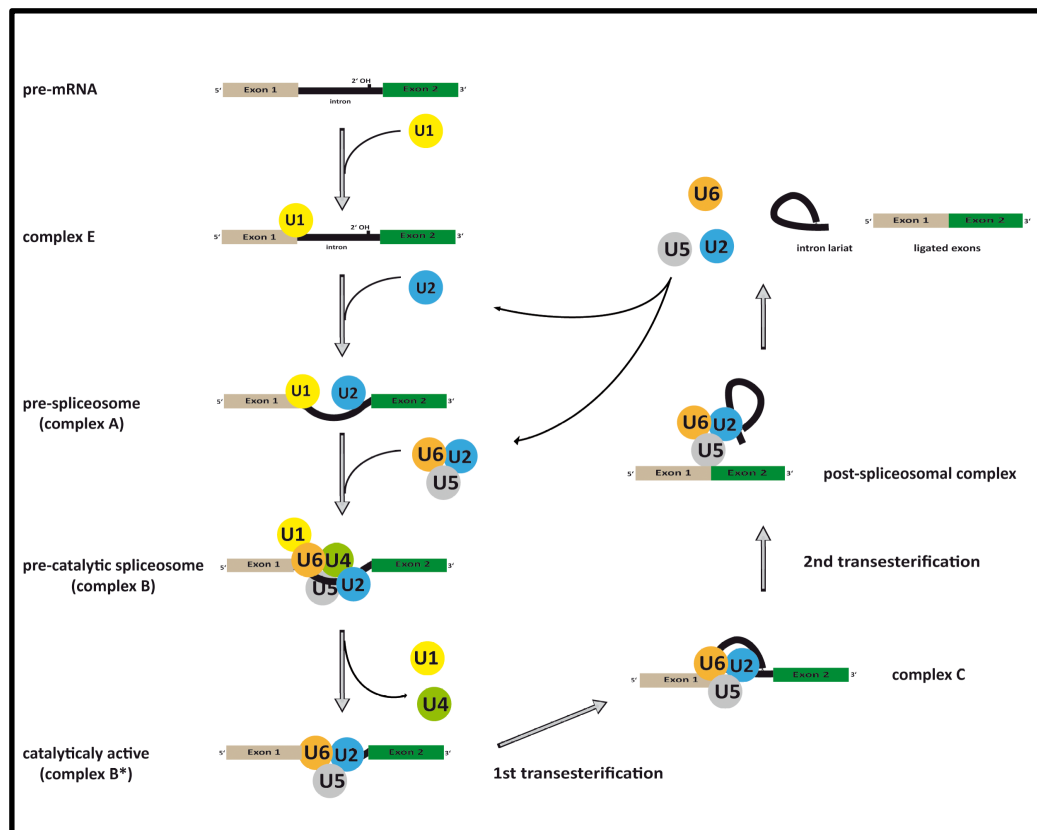
Figure 1.1. Removal of introns and re-ligation of exons occurs by spliceosome
Spliceosome is formed by sequential association of snRNPs with the pre-mRNA. Removal of the intron is accomplished in a two-step transesterification reaction catalyzed by active spliceosome (adopted from Will and Luhrmann 2011).

Thus, the most essential components of the spliceosome are snRNPs, which identify the specific cis-acting elements of the pre-mRNA and guide the remaining factors to the accurate site of cleavage. Despite their functional differences, all spliceosomal snRNPs have some remarkably similar features. Each spliceosomal snRNP is composed of one name-giving snRNA, seven common Sm proteins called SmD1, SmD2, SmD3, SmB, SmE, SmF and SmG and various numbers of proteins specific for each snRNP (figure 1.2) The only exception is the U6 snRNP, which is bound to Lsm2-8 proteins. The Lsm/Sm proteins bind to the Sm-site of the snRNA forming a toroidal structure called the "Sm core" (figure 1.2) (Fischer et al., 2011; Will and Luhrmann, 2001).
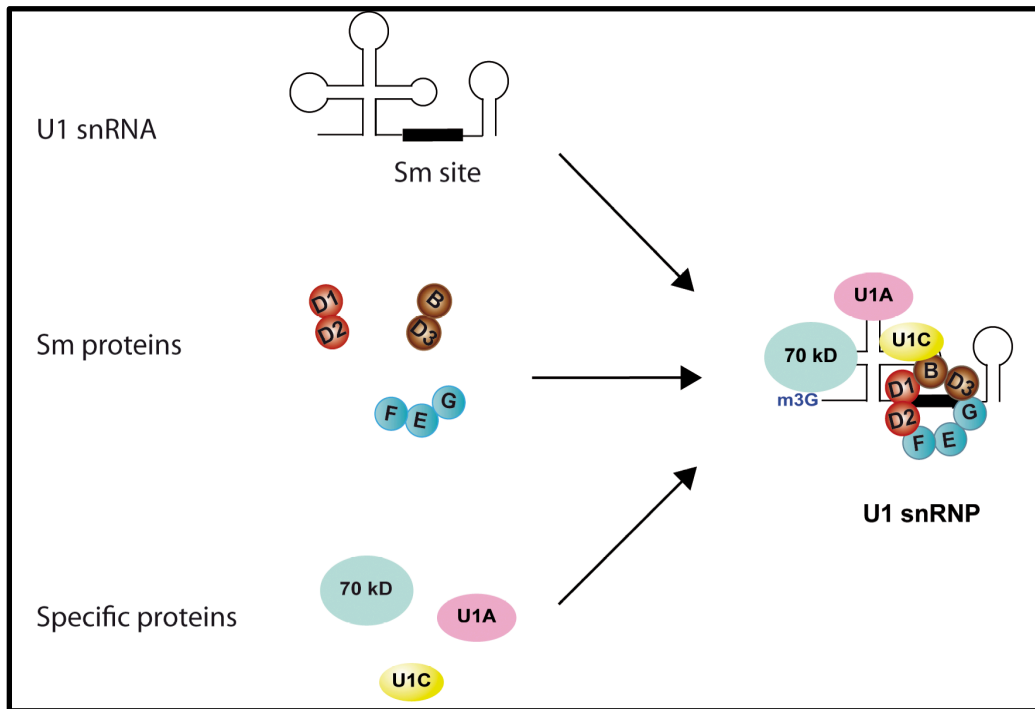
Figure 1.2. Structural features of snRNP

snRNPs are composed of one (or two in the case of U4/U6) snRNA molecule(s) and seven common "Sm proteins", which form a ring around the "Sm-site" of the snRNA. Furthermore, each snRNP comprises several specific proteins. The general architecture of an snRNP is shown for U1 snRNP.

1.3   Formation of Histone mRNA 3' end in the nucleus

Not all pre-mRNAs go through splicing and 3' end polyadenylation prior to their export to the cytoplasm. Well-known exceptions are the replication-dependent histone mRNAs. There is a high demand for histone proteins during the S phase of the cell cycle in order to pack the DNA into chromatin. To respond to this demand, cells up-regulate the transcription of histone genes at the beginning and down-regulate them at the end of the S phase. These transcripts are called replication-dependent as they are synchronized with the cell cycle. In contrast to the majority of eukaryotic mRNAs, metazoan replication-dependent histone mRNAs are intron-less. Furthermore, they do not contain a poly (A) tail but rather have a conserved stem-loop followed by a very short tail at their 3' end (Dominski and Marzluff, 1999, 2007; Osley, 1991).

The mature 3' end of histone mRNA is formed by machinery composed of the U7 snRNP and several trans-acting factors (figure 1.3). The cleavage site at the 3' end of histone mRNA is located downstream of the translation stop codon. The two major units specifying the cleavage site are the stem-loop (SL) structure and the purine-rich histone downstream element (HDE) (Birchmeier et al., 1983; Birchmeier et al., 1982; Birchmeier et al., 1984; Mowry et al., 1989; Mowry and Steitz, 1987). The stem-loop is located about 5 nucleotides upstream of the cleavage site and is recognized by the stem-loop binding protein (SLBP). The HDE is positioned about 20 nucleotides downstream of the stem-loop and forms a base pair with the unstructured 5' end of U7 snRNA (Bond et al., 1991; Cotten et al., 1988; Schaufele et al., 1986; Spycher et al., 1994). U7 snRNA-HDE base pairing operates as a ruler and puts the cleavage site at the correct position for the endonuclease (figure 1.3) (Scharl and Steitz, 1994). Endonucleolytic cleavage occurs about five nucleotides downstream of the stem-loop, usually after an adenosine. Upon cleavage, the downstream oligonucleotide is degraded by a 5' ➔ 3' exonuclease activity and the U7 snRNP is released to function in a new round of cleavage (Walther et al., 1998).
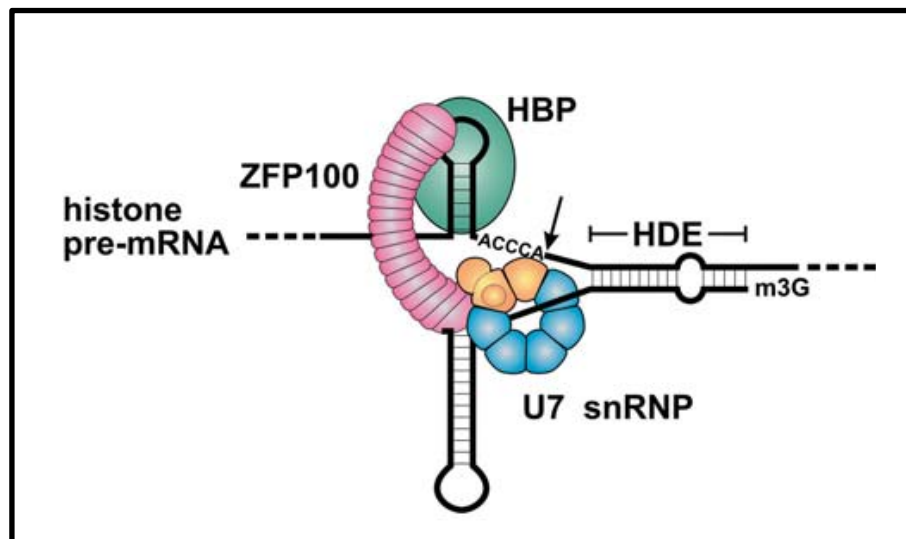
Figure 1.3. Histone mRNA 3' end processing.

Two determining sequences flanking the cleavage site are stem-loop (SL) or Hairpin (HP) and histone downstream element (HDE). Hairpin binds to Hairpin binding protein (HBP) and HDE interacts with 5' end of U7 snRNA. ZFP100 connects HBP and Lsm11 and thus stabilizes the complex. U7 snRNP functions as a ruler and locates the cleavage site at the right position for endonucleolytic attack (Schumperli and Pillai, 2004).

Consequently, though the spliceosomal snRNPs and U7 snRNP are involved in two distinctive processes, they mainly accomplish similar tasks. In both reactions, snRNPs recognize and bind specific sites on the target RNA and assure the accuracy of the catalytic reaction.

Likewise, the structures of the U7 snRNP and spliceosomal snRNPs are remarkably similar. They both contain an Sm protein-containing core formed around the Sm-site of the respective snRNA. Nevertheless, the core of the U7 snRNP differs in its protein composition from spliceosomal snRNPs. The Sm core of the U7 snRNP shares five "canonical" Sm proteins (i.e. SmD3, SmB, SmE, SmF and SmG) with spliceosomal snRNPs, but Lsm10 and Lsm11 replace the canonical SmD1 and SmD2, respectively (Gilmartin et al., 1988; Pillai et al., 2003; Pillai et al., 2001). In addition, the Sm-site in the U7 snRNP is slightly different from the canonical Sm-site in spliceosomal snRNPs (see figure 1.4) (Dominski et al., 2003; Gilmartin et al., 1988).
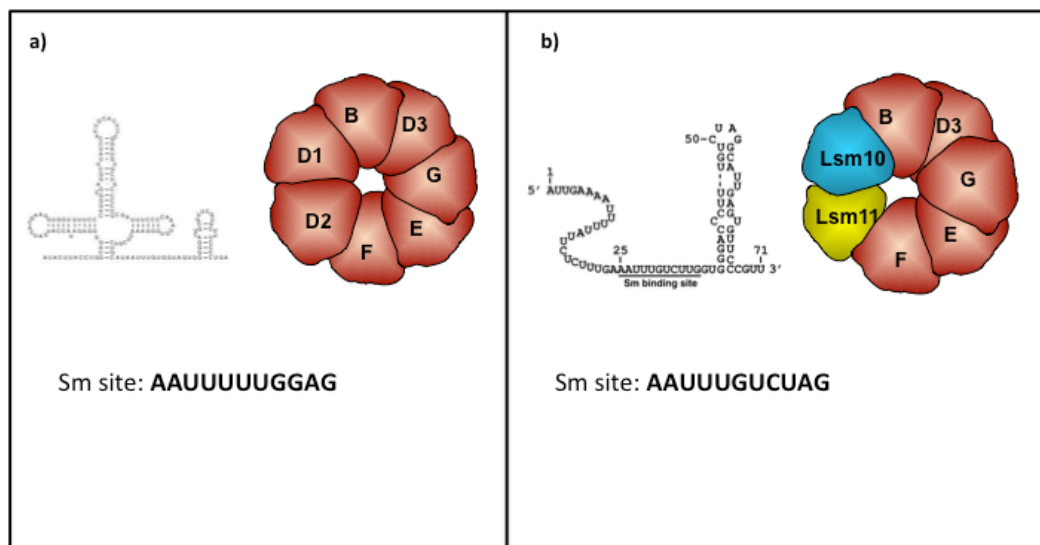
Figure 1.4. Structural characteristics of U1 and U7 snRNPs.

a) The U1 snRNP contains seven common Sm proteins, SmD1, SmD2, SmB, SmD3, SmE, SmF and SmG, which form the canonical Sm core. b) The Sm core of U7 snRNP contains five common Sm proteins but Lsm10 and Lsm11 replace SmD1 and SmD2, respectively. The Sm-site differs slightly between both snRNAs (adopted from (Dominski and Marzluff, 2007; Schumperli and Pillai, 2004)).

1.4   Biogenesis of snRNPs

1.4.1   Biogenesis of spliceosomal snRNPs

In vivo, spliceosomal snRNPs are assembled in a multi-step process that involves nucleo-cytoplasmic transports and the assistance of a number of trans-acting factors. Biogenesis of spliceosomal snRNPs initiates with the transcription of snRNA in the nucleus by RNA polymerase II (in case of U1, U2, U4 and U5 snRNAs) or RNA polymerase III (in case of U6 snRNA) (Mattaj et al., 1985; Mattaj and Zeller, 1983; Will and Luhrmann, 2001) (figure 1.5). Upon transcription of the pol II genes, the m^7G-cap structure of the snRNA is recognized and bound by the CBC (cap binding complex), which consists of CBP80 and CBP20 proteins (Izaurralde et al., 1995). CBC binds to the export adaptor protein PHAX, which links the export complex to the export receptor CRM1 (chromosome region maintenance-1) (Gorlich et al., 1996; Ohno et al., 2000). The complex is then exported into the cytoplasm in a Ran GTP(ase) dependent manner (Hamm and Mattaj, 1990). The pol III transcribed U6 follows a different route, as it acquires a γ-monomethyl phosphate cap co-transcriptionally and never leaves the nucleus (Zieve and Penman, 1976). In addition, U6 does not contain a classical Sm site but a uridine-rich sequence at the 3' end that binds to a ring formed by the Sm-homologous proteins, Lsm2-8.

In the cytoplasm, the seven common Sm proteins bind to the Sm-site of the snRNA to form the "Sm core" domain (Hamm et al., 1987; Jarmolowski and Mattaj, 1993). This common structure of snRNPs is essential for the conversion of the m^7G-cap to hypermethylated m_3G-cap and trimming of the 3' end (Mattaj, 1986; Neuman de Vegvar and Dahlberg, 1990; Plessel et al., 1994). The matured snRNP particle is eventually transported into the nucleus by virtue of a bipartite nuclear localization signal (NLS) consisting of the m_3G-cap and the Sm-core (Fischer and Luhrmann, 1990; Fischer et al., 1993; Hamm et al., 1990; Huber et al., 1998; Narayanan et al., 2004). In the nucleus, snRNPs accumulate in Cajal bodies, where they undergo further modifications of the snRNA prior to their transport to their final destinations, perichromatin fibrils and interchromatin granule clusters. Specific proteins join the

snRNP probably in the nucleus (Figure 1.5) (Carmo-Fonseca et al., 1993; Fischer et al., 2011; Sleeman and Lamond, 1999).
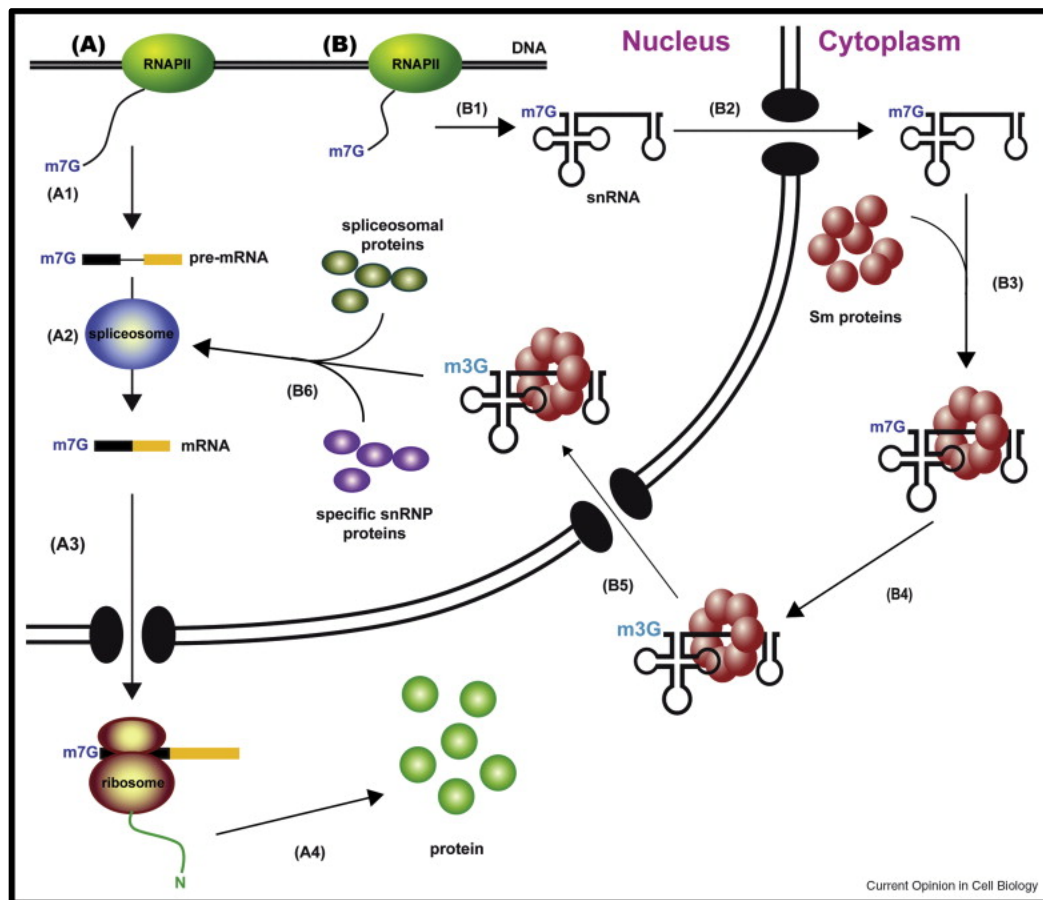
Figure 1.5. An overview of the assembly of snRNPs.

Assembly initiates by transcription of the snRNA in the nucleus, which is followed by export of the snRNA. In the cytoplasm Sm proteins are loaded onto the snRNA to form the snRNP. The cytoplasmic phase of the assembly reaction ends by the import of the snRNP into the nucleus. In the nucleus, specific proteins join the snRNP and upon further modifications of the snRNA, snRNPs are transported to their site of function in the nucleus (Chari et al., 2009).

1.4.1.1 Formation of "Sm core" domain in the cytoplasm

Initial in vitro studies with purified Sm proteins and snRNA indicated that Sm proteins form three hetero-oligomers D3/B, D1/D2, and E/F/G independent of snRNA. Association of D1/D2 and E/F/G with the snRNA leads to the formation of "Sm subcore" intermediate. Addition of D3/B dimer to the Sm subcore forms the mature

"Sm core". Therefore, in vitro, the "Sm core" domain is formed spontaneously and as a consequence of self-recognition of RNA and proteins (Chari et al., 2008; Raker et al., 1996). However, in vivo, this reaction requires a large number of trans-acting factors that are organized in two macromolecular units termed PRMT5- and SMN- complexes (figure 1.6) (Meister et al., 2001; Meister et al., 2002; Paushkin et al., 2002).

The PRMT5 complex consists of the WD repeat domain protein of 45kDa (WD45/Mep50), the chloride conductance regulatory protein (pICln) and the protein arginine methyltransferase 5 (PRMT5) (Friesen et al., 2001; Meister et al., 2001). This unit acts in the early phase of the assembly pathway and functions as an Sm protein organizer. The subunit pICln initially binds to Sm proteins, which leads to the recruitment of all Sm proteins into the PRMT5 complex. Hereafter, PRMT5 symmetrically di-methylates arginine residues in SmB, SmD3 and SmD1 (figure 1.6) (Brahms et al., 2001; Friesen and Dreyfuss, 2000). Eventually, a complex composed of pICln, SmD1, SmD2, SmE, SmF and SmG (termed 6S complex) dissociates from the PRMT5 complex. Sm proteins in the 6S complex are kinetically trapped and prevented from deleterious interactions with non-specific RNAs in the cytoplasm (figure 1.6). In the 6S complex, Sm proteins are found in the same order as in the Sm core domain of snRNPs. PICln has an intriguing function in organizing and guiding the Sm proteins through the assembly line and handing them over to SMN complex for the final step of the assembly. Therefore pICln has been recognized as an assembly chaperone for the formation of snRNPs (Chari et al., 2008).

In order to allow the assembly process to resume, the SMN-complex is required. This macromolecular unit consists of the Survival of Motor Neurons (SMN) protein mutated in the devastating neuromuscular disorder spinal muscular atrophy (SMA), seven Gemins (termed Gemins 2-8) and unrip (Otter et al., 2007). The SMN complex sequesters the pICln bound Sm proteins, releasing the assembly chaperone pICln, and transfers them onto the Sm-site of the snRNA (figure 1.6) (Chari et al., 2008; Meister and Fischer, 2002; Paushkin et al., 2002). As the SMN complex releases the Sm proteins from their kinetically trapped state and allows their transfer onto the snRNA, it acts as a catalyst in the assembly of snRNPs (Chari et al., 2008).
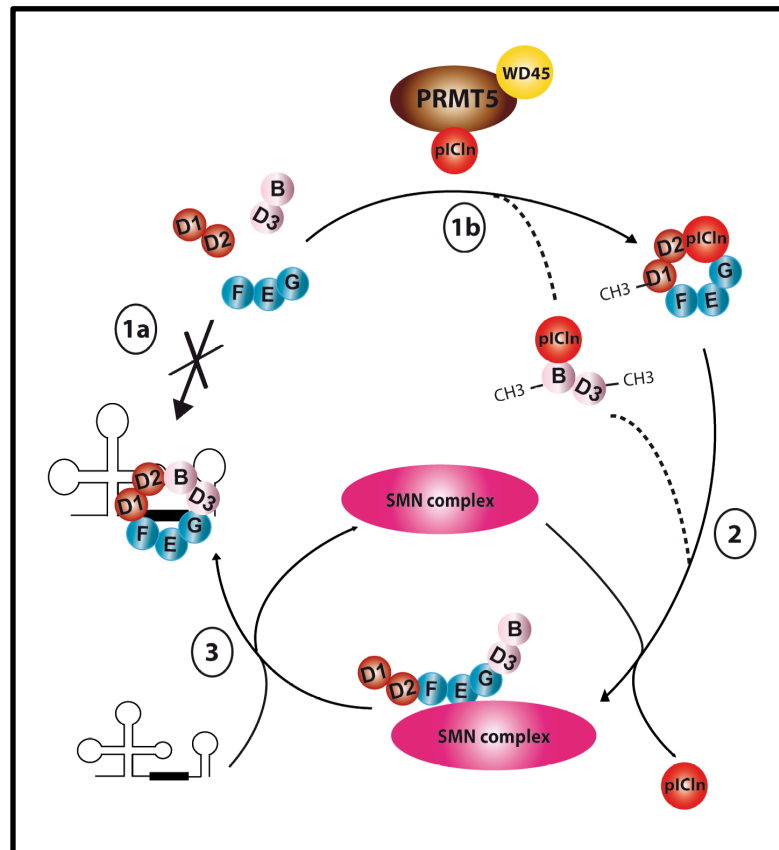
Figure 1.6. Cytoplasmic phase of snRNPs assembly, formation of the Sm core.

Upon export into the cytoplasm and prior to re-import into the nucleus, snRNA interacts with the Sm proteins. In vitro (1a), Sm proteins bind the snRNA spontaneously, but in vivo (1b), the association of Sm proteins and snRNA is a factor-mediated and highly regulated process. In the first step, the PRMT5 complex methylates the Sm proteins, SmD3, SmB and SmD1. A subset of Sm proteins, SmD1, SmD2, SmE, SmF and SmG form a complex with pICln that dissociates from the PRMT5 complex. 2) Then Sm proteins are transferred onto the SMN complex, where they adopt an open configuration. 3) The SMN complex loads the Sm proteins onto the snRNA.

1.4.2   Biogenesis of U7 snRNP

The assembly pathway of spliceosomal snRNPs has been well characterized (see above). Our knowledge regarding the biogenesis of spliceosomal snRNPs raised the question whether other snRNPs require similar assisting factors for their biogenesis. The histone mRNA 3' end-processing U7 snRNP was of particular interest as this particle contains a unique Sm core in which the Sm proteins D1 and D2 are replaced by Lsm 10 and Lsm11. Indeed, initial analyses have suggested that similar to spliceosomal

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snRNPs, the assembly of U7 snRNP is dependent on SMN complex (Azzouz et al., 2005; Pillai et al., 2003). These studies proposed the existence of two distinct populations of SMN complexes in the cell (Pillai et al., 2003). One is loaded with seven common Sm proteins and assists the formation of the spliceosomal snRNPs. The other transfers U7 snRNP-specific Sm and Lsm core proteins onto U7 snRNA and ensures the formation of this particle. However, how the different sets of Sm and Lsm proteins are loaded to their respective SMN-complexes remains unclear.

1.5　Aim of this thesis

Macromolecular complexes composed of RNA and proteins mediate almost all steps of eukaryotic gene expression. The faithful formation of these complexes is hence a vital task for all cells and often requires an elaborate system of assembly factors. However, in contrast to proteinaceous macromolecular complexes, very little is known about the mode of action of these assembly factors in the biogenesis of RNPs. In this thesis, I aimed to elucidate the mechanism of the assembly of these particles in more detail. I chose spliceosomal snRNPs and U7 snRNP as my model systems. Spliceosomal snRNPs and U7 snRNP share similar structural features and tasks, despite their involvement in two completely different pathways. Accordingly, I defined three major aims for this thesis:

1) The assembly of spliceosomal snRNPs has been studied vastly, but the mechanism of U7 snRNP formation is not clear. Consequently, in this thesis I have analyzed the assembly of U7 snRNP. Initial studies have suggested that mechanistically, U7 snRNP assembly follows a similar route as described for spliceosomal snRNPs. I initiated a series of analyses to define specific U7 snRNP assembly intermediates, which would verify this hypothesis.

2) In the past years, the trans-acting factors ensuring the precise assembly of spliceosomal snRNPs have been identified. These studies uncovered an elaborate system that organizes Sm proteins in an early phase and then mediates their targeted association with snRNA. However, a crucial aspect of the assembly process has remained entirely unanswered, namely, how newly synthesized Sm proteins are handed over from the translation machinery to the assembly factors. Elucidation of this transfer, is therefore the second aim of my thesis.

3) It has been shown that prior to their integration into snRNPs, Lsm/Sm proteins are found as hetero-oligomers in the cytoplasm (see section 1.4.1.1). Consequently, I aimed to discover the mechanism, which ensures the specificity of cognate Lsm/Sm protein hetero-oligomer formation. I will focus in this part of my thesis on the possible

role of pICln in the early recruitment of Sm proteins and cognate hetero-oligomer formation either co-translationally or upon termination of translation.

2 Materials

2.1 Devices

Device	Supplier
Äkta purifier	GE Healthcare, Giles, UK
Amersham Hyperfilm™ MP	GE Healthcare, Giles, UK
Avanti® J-20-XP Centrifuge	Beckman-Coulter, Brea, CA, USA
Avanti® J-HC Centrifuge	Beckman-Coulter, Brea, CA, USA
Biofuge pico	Heraeus Instruments, Hanau, Germany
BioPhotometer	Eppendorf, Hamburg, Germany
CEA RP NEW Medical X-ray Screen (Blue sensitive)	AGFA Healthcare, Mortsel, Belgium
Cell culture ware	BD BioSciences, Franklin Lakes, NJ USA
CERTOMAT® BS-1 Incubator shaker	Sartorius stedim biotech, Göttingen, Germany
Certomat® R shaker	Sartorius stedim biotech, Göttingen, Germany
Cryo vials	Sigma-Aldrich, St. Louis, MO, USA
Ehret Incubator KLT/S4	EHRET Labor- und Pharmatechnik, Emmendingen, Germany
Eppendorf Centrifuge 5415R	Eppendorf, Hamburg, Germany
Eppendorf Centrifuge 5424	Eppendorf, Hamburg, Germany
Eppendorf Centrifuge 5804R	Eppendorf, Hamburg, Germany
Fuchs-Rosenthal hemocytometer	Hartenstein, Würzburg, Germany
Gel Dryer Model 583	Bio-Rad, Hercules, CA, USA
GeneAmp® PCR System 9700	Applied Biosystems, Carlsbad, CA, USA
Head over tail (H.O.T.)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Heraeus TK 6060 incubator	Heraeus Instruments, Hanau,

	Germany
HT Labotron shaker	Infors AG, Bottmingen, Switzerland
Incubator BK600	Kendro Laboratory Products, Langensebold, Germany
Innova™ 4300 Incubator shaker	New Bruinswick Scientific, Edison, NJ, USA
Innova® 44 Incubator shaker	New Bruinswick Scientific, Edison, NJ, USA
LB1210B radioactive surface counter	Berthold Technologies, Bad Wildbad,
Memmert drying oven	Memmert, Schwabach, Germany
NuPAGE®Novex 4-12 % Bis-Tris Gel 1,0 mm	Invitrogen, Karlsruhe
Optima™ L-80XP Ultracentrifuge	Beckman-Coulter, Brea, CA, USA
Optima™ L-90K Ultracentrifuge	Beckman-Coulter, Brea, CA, USA
Poly-Prep columns	Bio-Rad, Hercules, CA, USA
Rotors – Centrifuges (JS 4.2, JLA 8.1000, JA 25.50)	Beckman-Coulter, Brea, CA, USA
Rotors – Ultracentrifuges (45Ti, 60Ti, 70Ti, SW41Ti, SW60Ti, SW32Ti)	Beckman-Coulter, Brea, CA, USA
Semidry blotting apparatus	Bio-Rad, Hercules, CA, USA
SilverFast32 scanner	Seiko Epson Corporation, Tokio, Japan
Slide-A-Lyzer® dialysis caps	Pierce/VWR, Radnor, PA, USA
Specord 50, UV Vis spectrophotometer	Analytik Jena, Jena, Germany
SpeedVac Concentrator	Savant/Thermo Scientific, Waltham, MA, USA
HiLoad 26/60 Superdex 200 prep grade	GE Healthcare, Giles, UK
Thermomixer 60ompact	Eppendorf, Hamburg, Germany
UVette® 220-1600 nm, disposable single sealed	Linaris, Wertheim
Vacuum pump	Greifenberger Antriebstechnik, Marktredwitz, Germany
Varioklav® Steam Sterilizer	H + P Labortechnik, Oberschleißheim
Whatman paper	VWR, Radnor, PA, USA
Zeiss Axiovert 200M Microscope	Carl Zeiss AG, Jena, Germany
Zeiss Axiovert 25 Microscope	Carl Zeiss AG, Jena, Germany

2.2 Chemicals

Chemical	Supplier
Acetic acid (99%)	VWR, Radnor, PA, USA
Acrylamide (Rotiphorese Gel A)	Carl Roth GmbH + Co. KG, Karlsruhe
Acrylamide/ bisacrylamide (37.5:1), Rotiphorese 30	Carl Roth GmbH + Co. KG, Karlsruhe
AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride)	Sigma-Aldrich, St. Louis, MO, USA
Agar	Carl Roth GmbH + Co. KG, Karlsruhe
Agarose	Carl Roth GmbH + Co. KG, Karlsruhe
Amido black	Merck, Darmstadt
Ammonium acetate	Merck, Darmstadt
Ammonium hydroxide	VWR, Radnor, PA, USA
Ammonium persulfate (APS)	Carl Roth GmbH + Co. KG, Karlsruhe
Ampicillin	Carl Roth GmbH + Co. KG, Karlsruhe
Aprotinin	Sigma-Aldrich, St. Louis, MO, USA
Bacto™ Tryptone	BD BioSciences, Franklin Lakes, NJ USA
Bacto™ Yeast Extract	BD BioSciences, Franklin Lakes, NJ USA
Beta-mercaptoethanol	Carl Roth GmbH + Co. KG, Karlsruhe
Boric acid	Carl Roth GmbH + Co. KG, Karlsruhe
Bovine Serum Albumin (BSA)	PAA Laboratories GmbH, Pasching
Bromophenol blue	Serva, Heidelberg, Germany
Calcium chloride ($CaCl_2$)	Carl Roth GmbH + Co. KG, Karlsruhe
Chloroform	Carl Roth GmbH + Co. KG, Karlsruhe
Coumaric acid	Carl Roth GmbH + Co. KG, Karlsruhe
Cycloheximide	Sigma-Aldrich, St. Louis, MO, USA
Disodium tetraborate (Borax)	Carl Roth GmbH + Co. KG, Karlsruhe
Dulbecco's Modified Eagle Medium (DMEM)	Gibco®; Invitrogen, Carlsbad, CA, USA
DMF (N,N-Dimethylformamide)	Sigma-Aldrich, St. Louis, MO, USA
DMSO (Dimethylsulfoxide)	Carl Roth GmbH + Co. KG, Karlsruhe
dNTP mix	Fermentas, St. Leon-Rot, Germany
DTT (Dithiothreitol)	Carl Roth GmbH + Co. KG, Karlsruhe

EDTA (Ethyleneaminetetraacetic acid)	Carl Roth GmbH + Co. KG, Karlsruhe
Ethanol, denatured	VWR, Radnor, PA, USA
Ethanol, p.a.	Sigma-Aldrich, St. Louis, MO, USA
Ethidium bromide	Carl Roth GmbH + Co. KG, Karlsruhe
Fetal calf serum (FCS)	Gibco®; Invitrogen, Carlsbad, CA, USA
Formaldehyde	Carl Roth GmbH + Co. KG, Karlsruhe
Galactose	Sigma-Aldrich, St. Louis, MO, USA
Gelatin	Merck, Darmstadt
GeneRuler™ 100 bp Plus DNA Ladder	Fermentas, St. Leon-Rot
Glucose	Carl Roth GmbH + Co. KG
Glutathione sepharose™ 4B	QIAGEN, Hilden
Glycerol 86% (v/v)	Carl Roth GmbH + Co. KG, Karlsruhe
Glycine	Carl Roth GmbH + Co. KG, Karlsruhe
Heparin	Sigma-Aldrich, St. Louis, MO, USA
Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Carl Roth GmbH + Co. KG, Karlsruhe
Hybond PVDF membrane	VWR, Radnor, PA, USA
Hydrogen peroxide	Merck, Darmstadt
Isopropanol	VWR, Radnor, PA, USA
Kanamycin	Carl Roth GmbH + Co. KG, Karlsruhe
Leupeptin	Sigma-Aldrich, St. Louis, MO, USA
Luminol	Carl Roth GmbH + Co. KG, Karlsruhe
Magnesium Chloride	Merck, Darmstadt
Magnesium Acetate	Merck, Darmstadt
MassRuler™ DNA Ladder Mix	Fermentas, St. Leon-Rot, Germany
Methanol	VWR, Radnor, PA, USA
NAMP100 Amplify Fluorographic Reagent	GE Healthcare, Giles
Non-Ident P40	Carl Roth GmbH + Co. KG, Karlsruhe
PageRuler™ Plus Prestained Protein Ladder	Fermentas, St. Leon-Rot
PageRuler™ Unstained Protein Ladder	Fermentas, St. Leon-Rot
Penicillin/Streptomycin	PAA, Farnborough, Hampshire
Pepstatin	Sigma-Aldrich, St. Louis, MO, USA
Phenol (Roti®-Aqua-Phenol for RNA isolation)	Carl Roth GmbH + Co. KG, Karlsruhe
PMSF (phenylmethylsulfonyl fluoride)	Sigma-Aldrich, St. Louis, MO, USA

Potassium chloride	Carl Roth GmbH + Co. KG, Karlsruhe
Potassium phosphate monobasic (KH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe
Pottasium acetate	Carl Roth GmbH + Co. KG, Karlsruhe
Puromycine	SERVA electrophoresis, Heidelberg
RNAsin	Promega, Madison, WI, USA
SDS (Sodium dodecyl sulfate)	Carl Roth GmbH + Co. KG, Karlsruhe
Serva Blue R	Serva, Heidelberg
Silver nitrate	Degussa AG, Frankfurt
Sodium acetate	Carl Roth GmbH + Co. KG, Karlsruhe
Sodium carbonate anhydrous	Fluka/Sigma-Aldrich, St. Louis, MO, USA
Sodium chloride (NaCl)	VWR, Radnor, PA, USA
Sodium hydroxide (NaOH)	Carl Roth GmbH + Co. KG, Karlsruhe
Sodium thiosulfate	Sigma-Aldrich, St. Louis, MO, USA
TEMED (N,N,N,N-Tetramethylethylenediamine)	Carl Roth GmbH + Co. KG, Karlsruhe
Trichloroacetic acid (TCA)	Carl Roth GmbH + Co. KG, Karlsruhe
TRIS (Tris (hydroxymethyl) aminomethane)	Carl Roth GmbH + Co. KG, Karlsruhe
Triton X-100	Carl Roth GmbH + Co. KG, Karlsruhe
Tween 20 (Polyoxyethylene-sorbitan monolaurate)	Carl Roth GmbH + Co. KG, Karlsruhe
Urea	Carl Roth GmbH + Co. KG, Karlsruhe
Vectashield® Mounting Medium with DAPI	VECTOR LABORATORIES, INC.

2.3 Enzymes

Enzyme	Supplier
T4 DNA ligase	Fermentas, St. Leon-Rot, Germany
Micrococcal nuclease	Fermentas, St. Leon-Rot, Germany
Restriction Enzymes	Fermentas, St. Leon-Rot, Germany
Shrimp Alkaline Phosphatase (SAP)	Fermentas, St. Leon-Rot, Germany

2.4 Antibiotics

Antibiotic	Concentration
Ampicillin	100 mg/ml
Chloramphenicol	50 mg/ml
Kanamycin	50 mg/ml
Penicillin/Streptomycin	100× stock solution (PAA)

2.5 Antibodies

Antibody	Source	Supplier
Alexa Fluor 594 anti-mouse	Goat, polyclonal	Invitrogen, Karlsruhe
α-Hemagglutinin (HA)	Mouse, monoclonal	Sigma-Aldrich
α-Flag	Mouse, monoclonal	Sigma-Aldrich
α-PRMT5	Rabbit, polyclonal	Method of the thesis
α-pICln	Rabbit, polyclonal	Method of the thesis
α- SmD1/SmD2	Rabbit, polyclonal	Method of the thesis
α- SmB/B'	Rabbit, polyclonal	Method of the thesis
α- tubulin	Mouse, monoclonal	Sigma-Aldrich
Y12	Mouse, monoclonal	Kind gift from J. Steitz
α-mouse	Goat, polyclonal	Sigma-Aldrich
α-rabbit	Goat, polyclonal	Sigma-Aldrich

2.6 Kits

Kit	Supplier
Lipofectamine™ RNAiMAX Transfection Reagent	Invitrogen, Carlsbad, CA, USA
Luciferase Assay System	PJK GmbH, Kleinblittersdorf
Passive lysis buffer, 5x	Promega, Mannheim

NucleoSpin® Plasmid QuickPure	Macherey-Nagel, Düren, Germany
NucleoSpin® Extract II	Macherey-Nagel, Düren, Germany
NucleoBond® PC100	Macherey-Nagel, Düren, Germany
NucleoBond® PC500	Macherey-Nagel, Düren, Germany
Nanofectin transfection kit	PAA Laboratories GmbH
Phusion® Master Mix with GC Buffer	FINNZYMES, NEW ENGLAND BioLabs
Phusion® Master Mix with HF Buffer	FINNZYMES, NEW ENGLAND BioLabs
PureYieldTM Plasmid Midiprep System	Promega, Mannheim

2.7 cDNAs

Full length cDNAs for human Lsm10- and Lsm11 proteins were supplied by Source BioScience (previously known as imaGenes).

cDNA	Vector	Species
Lsm10	pOTB7	Homo sapiens
Lsm11	pCR4-TOPO	Homo sapiens

2.8 siRNA

SiRNA	Supplier
SMARTpool: ON-TARGET plus CLNS1A siRNA	Thermo Scientific Dharmacon

2.9 Amplifying vectors

Initially Lsm10 and Lsm11 were cloned into pACE and pACE2 vectors.

Vector	Supplier
pACE	ATG: biosynthetics GmbH, Freiburg
pACE2	ATG: biosynthetics GmbH, Freiburg

2.10 Eukaryotic expression vector

Lsm10-pACE and Lsm11-pACE2 were used for construction of the eukaryotic expression vectors.

Plasmid	Description	Supplier
pcDNA3	Modified version with an N-terminal HA-tag	Invitrogen, Carlsbad, CA, USA

2.11 Bacterial strains

Strain	Supplier
E. coli DH5α	Invitrogen, Carlsbad, CA, USA
E. coli XL1 Blue	Stratagene, La Jolla, CA, USA

2.12 Eukaryotic cell lines

Cell type	Description	Supplier
HEK 293T	Human Embryonic kidney cell line	European Collection of Cell Cultures (ECACC)
HeLa	Human cervix carcinoma cell line	European Collection of Cell Cultures (ECACC)
COS 7	Monkey Kidney Fibroblast cell line	European Collection of Cell Cultures (ECACC)

2.13 Buffers and solutions

2.13.1 Bacterial cell culture

Buffer/Solution	Composition

LB medium (Luria Broth)	1% (w/v) tryptone
	0.5% (w/v) yeast extract
	1% (w/v) NaCl
Agar plates	LB medium
	1.5% (w/v) agar
	Antibiotics
SB medium (Super Broth)	3.5% (w/v) tryptone
	2.0% (w/v) yeast extract
	0.5% (w/v) NaCl
	Adjust the pH to 7.5 using NaOH
$CaCl_2$ solution	60 mM $CaCl_2$
(preparation of chemically competent	15% (v/v) glycerol
cells)	10 mM PIPES, pH 7.0
	adjust the pH to 7.0 using NaOH
	sterile filtration (0.2 μm)

2.13.2 Eukaryotic cell culture

Medium	Description	Supplier
DMEM	Dulbecco's Modified Eagle's Medium, Culture medium for mammalian cells	PAA, Farnborough, Hampshire
FBS	Fetal bovine serum "Gold"	PAA, Farnborough, Hampshire
OPTI-MEM I	Opti-MEM® Reduced-Serum Medium	Gibco by life technologies,
DPBS	Dulbecco's phosphate buffer saline (1x) (without Ca and Mg)	PAA, Farnborough, Hampshire
HBSS	Hanks' balanced salt solution (1x) (without Ca & Mg, without Phenol Red)	PAA, Farnborough, Hampshire

2.13.2.1   Mammalian cell culture media

Buffer/Solution	Composition
HeLa (HEK293T, COS7) culture medium	Dulbecco's Modified Eagle Medium (DMEM) 10% (v/v) Fetal Bovine Serum 2 mM L-Glutamin 50 μg/ml Penicillin/Streptomycin
HEK293T culture medium (siRNA transfection)	Dulbecco's Modified Eagle Medium (DMEM) 2 mM L-Glutamin 50 μg/ml Penicillin/Streptomycin

2.13.3 Eukaryotic cell extract preparation buffers

Buffer/Solution	Composition
HEPES Lysis buffer	50 mM HEPES/KOH pH 7.5 120 mM KOAc, 5 mM Mg(AOc)$_2$, 100 μg/ml Cycloheximide, 5 mM DTT, 1/100 protease inhibitors, 10 U/ml RNasin
PBS lysis buffer	1x PBS, 5mM Mg(Cl)$_2$, 5 mM DTT, 1/100 protease inhibitors, 10 U/ml RNasin

2.13.4 Ribosomal pelleting

Buffer/Solution	Composition
Re-suspension buffer I	1x PBS
Re-suspension buffer II	50 mM HEPES/KOH pH 7.5 120 mM KOAc, 5 mM Mg(AOc)$_2$, 5 mM DTT

Re-suspension buffer III	50 mM HEPES/KOH pH 7.5
	120 mM KOAc, 5 mM Mg(AOc)$_2$,
	5 mM DTT, 10 µg/ml cycloheximide

2.13.5 Extract fractionation

Buffer/Solution	Composition
10% Galactose solution	0.55 M Galactose in 1x PBS, 5mM Mg(Cl)$_2$
	5 mM DTT
30% Galactose solution	1.6 M Galactose in 1x PBS, 5mM Mg(Cl)$_2$
	5 mM DTT
5% sucrose solution	0.15 M Sucrose in
	50 mM HEPES/KOH pH 7.5
	120 mM KOAc , 5 mM Mg(AOc)$_2$
	5 mM DTT, 10µg/ml cycloheximide
45% sucrose solution	1.3 M Sucrose in
	50 mM HEPES/KOH pH 7.5
	120 mM KOAc , 5 mM Mg(AOc)$_2$
	5 mM DTT, 10 µg/ml cycloheximide

2.13.6 Western Blotting

Buffer/Solution	Composition
10× Towbin electro transfer buffer	0.25 M Tris
	1.92 M glycine
1× transferring buffer	25 mM Tris
	192 mM glycine
	20% (v/v) methanol
Amido black staining solution	0.2% (w/v) amido black
	10% (v/v) methanol
	2% (v/v) acetic acid
De-staining solution	90% (v/v) methanol

	3% (v/v) acetic acid
10× NET	1.5 M NaCl
	0.05 M NaEDTA, pH 8.0
	0.5 M Tris, pH 7.5
	0.5 % (v/v) Triton X-100
	sterile filtered (0.45 μm)
Primary antibody solution	1× NET-gelatin solution
	primary antibody (1:5000 – 1:100)
10× PBS	1.37 M NaCl
	27 mM sodium phosphate dibasic
	(Na$_2$HPO$_4$)
	20 mM potassium phosphate
	monobasic
	(KH$_2$PO$_4$)
Western Blotting washing solution (1× PBST)	1× PBS
	0.05% (v/v) Tween 20
	0.2% (v/v) Triton X-100
Secondary antibody solution	1× NET-gelatin solution
	secondary antibody (1:5000)
Coumaric acid solution	6.8 mM coumaric acid in DMSO
Luminol solution	1.25 mM Luminol
	100 mM Tris-HCl, pH 8.5
hydrogenperoxide	30% (v/v) hydrogen peroxide

2.13.7 Immunoprecipitation (IP)

Buffer	Composition
6S-20S IP binding buffer	1x PBS, 1 mg/ml BSA
Ribosomal IP binding buffer (lysis buffer)	50 mM HEPES/KOH pH 7.5
	120 mM KOAc, 5 mM Mg(AOc)$_2$,
	100 μg/ml Cycloheximide, 5 mM DTT,
	10 U/ml RNasin, 1/100 protease inhibitors

IP wash buffer I	1X PBS buffer
	0.01% (v/v) NP40
IP wash buffer II	1x PBS buffer

2.13.8 Agarose gel electrophoresis

Buffer/Solution	Composition
5× TBE (Tris/EDTA/borate)	445 mM Tris-HCl, pH 8.3
	445 mM boric acid
	10 mM Na_2EDTA
Agarose gel	0.5% - 2.0% (w/v) agarose
	1× TBE
	5 µg/ml ethidium bromide
6× DNA sample loading buffer	10 mM Tris-HCl, pH 7.5
	60% (v/v) glycerol
	60 mM EDTA, pH 8.0
	0.03% (w/v) xylene cyanol
	0.03% (w/v) bromophenol blue
	Sterile filtered (0.2 µm)

2.13.9 SDS-PAGE

Buffer/Solution	Composition
Tris-Glycine PAGE stacking gel (5%)	5% (v/v) acrylamide/bisacrylamide (37.5:1), Rotiphorese 30
	0.125 M Tris-HCl, pH 6.8
	0.0005% (w/v) APS
	0.0025% (v/v) TEMED

Tris-Glycine PAGE separating gel (12% or 15%) (high TEMED)	12%, or 15% (v/v) Acrylamide/ bisacrylamide (37.5:1), Rotiphorese 30 0.375 M Tris-HCl, pH 8.8 0.0005% (w/v) APS 0.005% (v/v) TEMED
Bis-Tris PAGE stacking gel (5%)	5% (v/v) acrylamide/bisacrylamide (37.5:1), Rotiphorese 30 0.357 M bis-Tris pH 6.8 0.0005% (w/v) APS 0.0025% (v/v) TEMED
Bis-Tris PAGE separating gel (12%) (High TEMED)	12% (v/v) acrylamide/bisacrylamide (37.5:1), Rotiphorese 30 0.357 M bis-Tris pH 6.8 0.0005% (w/v) APS 0.0025% (v/v) TEMED
Laemmli running buffer	250 mM Tris base pH 8.3 1920 mM glycine 1% (w/v) SDS (don't adjust the pH)
MOPS SDS running buffer (20x)	1 M MOPS 1 M Tris base pH 7.7 69.3 mM SDS 20.5 mM EDTA (don't adjust the pH)

2.13.10   Protein gel staining (Coomassie staining)

Buffer/Solution	Composition
Coomassie staining solution I	50% (v/v) methanol 10% (v/v) acetic acid 0.15% (w/v) Serva blue R
Coomassie staining solution II	20% (v/v) isopropanol

	10% (v/v) acetic acid
	0.15% (w/v) Serva blue R
Coomassie de-staining solution I	30% (v/v) methanol
	10% (v/v) acetic acid
Coomassie de-staining solution II	20% (v/v) acetic acid

2.13.11 Protein gel staining (Silver staining)

Buffer/Solution	Composition
Fixation solution	50% (v/v) methanol
	12% (v/v) acetic acid
	0.185% (v/v) formaldehyde
Washing solution	50% (v/v) ethanol, degassed
Sodium thiosulfate solution (staining)	0.02 % (w/v) sodium thiosulfate
Silver solution	0.2% (w/v) silver nitrate
	0.02775% (v/v) formaldehyde
Developing solution	6% (w/v) sodium carbonate anhydrous
	0.185% (v/v) formaldehyde
Stopping solution	25% Acetic acid

3 Methods

3.1 Cloning of eukaryotic expression vectors

HA-Lsm10, HA-Lsm11, HA-Lsm11-Flag, HA-Lsm11Δβ4β5-Flag and Flag-Lsm10 were constructed according to Sambrook et al. cDNAs were inserted into pcDNA3 plasmid with an N-terminal Hemagglutinin (HA) tag or N-terminal HA-tag and C-terminal Flag-tag. Precision of the constructs was tested via sequencing analysis performed by "Eurofins Medigenomix GmbH" in case of Lsm11 constructs or "GATC Biotech" in case of other constructs.

HA-SmD1, HA-SmD2 and HA-pICln were provided by Ashwin Chari and Michael Klingenhäger. HA-tagged FireFly luciferase was a kind gift from Anja Hirmer.

3.2 Polymerase Chain Reaction (PCR)

PCR was performed with Phusion High-Fidelity PCR Master Mix with "GC buffer" in case of Lsm11 and HF buffer in case of all the other constructs. PCR reactions for Lsm11 were always performed in the presence of 3% DMSO. The reactions were carried out according to the manufacturer's recommendations.

3.3 Amplification and purification of plasmid

Plasmids were transformed into *E. coli* DH5α or XL1 blue competent cells. 50 µl- 100 µl of cells were thawed on ice and then incubated with 50ng- 100ng of DNA for 30 min on ice and then at 42°C for 75 s. Cells were transferred onto ice immediately and 1 ml LB medium was added. Cells were initially incubated on a shaker with a speed of 1000 rpm for 2h at 37°C. For large-scale purification of DNA, 500 µl of this mixture was added to 500 ml LB medium containing antibiotic and incubated at 37°C for at least 12 hours. In case of small-scale purification, 50 µl of the transformation reaction was added to 10 ml of LB medium containing antibiotic. In both cases, plasmids were purified with Macherey-Nagel kit according to the manufacturer's manual.

3.4   Eukaryotic cell culture

All eukaryotic cell lines were grown and maintained in an incubator at 37°C in the presence of 5% CO_2. Cells were plated at a density of 20% and split whenever they reached 90% density.

3.4.1   Transfection of HEK 293T cells

HEK 293T cells were seeded the day before transfection and had a density of approximately 80% at the time of transfection. Transfection was performed with Nanofectin transfection reagent (PAA laboratories) according to the manufacturer's recommendations.

3.4.2   Transfection of COS7 cells for Immunofluorescence

COS7 cells were seeded on cover slips 24 hours prior to transfection. Cells had a density of 40% at the time of transfection. Transfection was performed with Nanofectin transfection reagent according to manufacturer's manual.

3.5   Immunocytochemistry

Cells were transfected with either HA-Lsm10 or HA-Lsm11. On the day of the experiment, cells were washed with 1x PBS at room temperature and then fixed with 3.7% formaldehyde in cold PBS for 10 min at room temperature. Next, cells were washed with cold PBS for 5 min. Blocking was performed with 1% BSA and 0.2% Triton X-100 in cold PBS for 30 min. Cells were incubated with the primary antibody (anti-HA) for 1 hour at room temperature. The primary antibody was diluted 1:500 in 1x PBS containing 1% BSA. Upon completion of incubation, cells were washed twice with cold PBS for 5 min each and then incubated with the secondary antibody (anti-mouse IgG, Alexa Fluor, 1:5000 in 1x PBS containing 1% BSA). Lastly, cells were washed 2x with 1x PBS and mounted in Vectashield mounting medium and studied with Axiovert 200M Microscope.

3.6 Large-scale fractionation of the HeLa cell extract

5 g of HeLa cell pellet was used to prepare the extract. Cells were then re-suspended in 5ml of ice-cold PBS lysis buffer. The cell suspension was transferred to a pre-chilled tight dounce homogenizer. Cells were sheared by 25 strokes and the lysate was cleared by centrifugation at 13000 rpm for 30 min. Cell extract (PBS extract) was fractionated on a HiLoad 26/60 Superdex 200 gel filtration column. Prior to snap freezing the fractions in liquid nitrogen, 1 mg/ml BSA was added to each fraction. 40 µl of each fraction was checked on a 12% Tris-Glycine polyacrylamide gel.

3.7 Small-scale fractionation of the extract to resolve 6S- and 20S complexes

It had already been shown in our lab that macromolecular complexes behave differently when they are fractionated on different sugar solutions. Therefore, I prepared a HeLa cell extract. Extract was layered on top of a 10%-30% sugar (Arabinose, Galactose, Glucose, Glycerol, lactose, and Sucrose) gradient. The resolutions of peaks corresponding to 6S- and 20S complexes were studied (figure 3.1). As a result, I selected "galactose" as the best sugar to be used in fractionation of extract to resolve fractions of 6S- and 20S complexes. In this case, PBS extract from HeLa cells was layered on top of a 10%-30% galactose gradient. Centrifugation was performed at 40000 rpm at 4°C for 16 hours in SW 60Ti rotor. The gradient was harvested manually into 200 µl fractions.
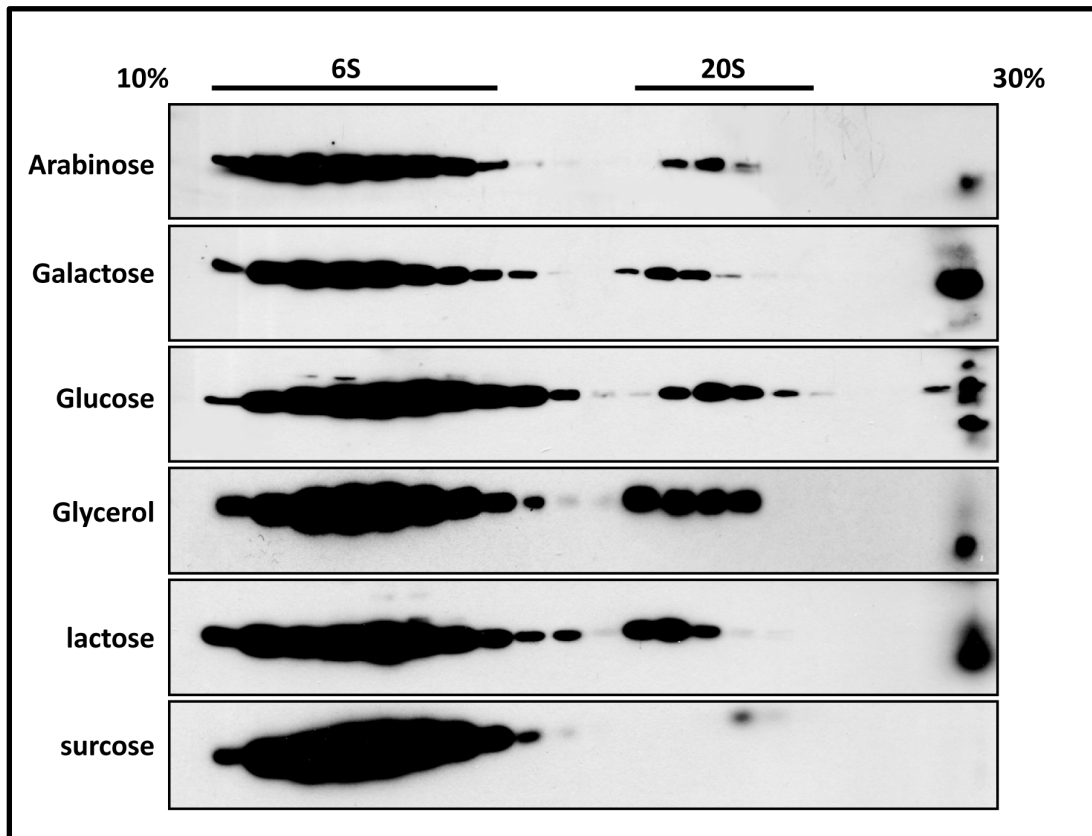
Figure 3.1. Fractionation of the extract on 10%-30% sugar gradients. PBS extract was prepared and fractionated on the gradients prepared from different sugars.

3.8 HEK293T cell extract preparation

HEK 293T cells from one to ten (depending on the experiment) 14 cm plates were used to prepare the extract. Cells were washed 1x with 1xPBS buffer at room temperature. Any additional buffer was removed from the plate. Then, 500 µl HEPES lysis buffer was added and cells were scraped and transferred to a 1.5 ml eppendorf tube. Cells were incubated for 20 min on ice in lysis buffer and inverted every 5 min. The lysate was cleared by centrifugation for 15 min at 13000 rpm. The clear lysate was transferred to a new tube for further experiments. The concentration of the RNA (OD260) in the extract was measured. The extract was prepared fresh prior to each experiment. My studies indicated that any freeze and thaw was detrimental to the extract.

3.9 Immunoprecipitation assay (IP)

3.9.1 Immunoprecipitation with anti-HA antibody

Extract prepared in section 3.8 or ribosomal/PEG pellets were used for immunoprecipitation assay. 0.5ml-1ml of the extract was used in each experiment. 50 µl of protein G-sepharose was added to the extract and incubated for 30 min on a rotator at 4°C. Extract was cleared by centrifugation at 5000 rpm for 5 min and transferred to a new tube. 20 µl-50 µl anti-HA beads were added and incubated for 2 hours at 4°C on a rotator. Afterwards, the extract was spun down for 5 min at 5000 rpm. The extract was transferred to a new tube and stored at 4°C. The beads were washed 5x with 1x PBS containing 0.01% NP40. At last, beads were transferred to a new tube and washed 1x with 1x PBS. I checked the IP by boiling the beads at 95°C for 5 min after addition of 1x SDS loading buffer.

3.9.2 Small-scale immunoprecipitation with anti-pICln from extract

Initially anti-pICln antibody was added to the protein G-sepharose beads at a final concentration of 1 mg/ml. Antibody and beads were incubated for 30 min at 4°C on a rotator. The mixture of beads and antibody was then added to 1 ml of the extract and incubated for at least 2 hours at 4°C on a rotator. Tube was centrifuged for 5 min at 5000 rpm at 4°C. Extract was transferred to a new tube and stored at 4°C (flow through). The beads were briefly washed 2x with IP wash buffer I and then transferred to a new tube. Beads were then washed with IP wash buffer I for 1h at 4°C on a rotator. Then, beads were washed two more times with the buffer containing NP40 and once with the IP wash buffer II. Beads were boiled at 95°C in the presence of 1x SDS loading buffer and checked on a 15% Tris-Glycine polyacrylamide gel.

3.9.3 Large-scale immunoprecipitation with anti-pICln from 6S and 20S fractions

Immunoprecipitation assay for purification of 6S and 20S complexes was performed as follows. Upon fractionation of the extract on the gel filtration column or galactose gradient, fractions of 6S and 20S complexes were pooled separately in two 15 ml or 50 ml falcon tubes. 75 µl of anti-pICln beads were added to the extract and incubated for 4 hours on a rotator at 4°C. Then the reaction was centrifuged for 5 min at 5000 rpm at 4°C. Extract was transferred to a new tube and stored at 4°C. The beads were washed 2x with IP wash buffer I and then transferred to a new tube. I washed the beads with IP wash buffer I overnight at 4°C on a rotator. The next morning beads were washed twice with the IP wash buffer I and once with IP wash buffer II. Beads were boiled at 95°C in the presence of 1x SDS loading buffer and checked on a 15% Tris-Glycine polyacrylamide gel.

3.10 Eukaryotic ribosomal profiles

For ribosome profiles, 293T extract was layered on top of 5%-45% sucrose gradient. Gradients were subjected to centrifugation at 34500 rpm for 2 hours at 4°C in SW 41Ti rotor. Gradients were harvested on Piston Gradient Fractionator from biocomp. 40 µl of each fraction was checked on a 12% Bis-Tris polyacrylamide gel.

3.11 Purification of the ribosomes

Upon fractionation of the extract on a 5%-45% gradient, polysomal fractions were pooled and ribosomes were purified as follow. Ribosomes were pelleted from the polysomal fractions with addition of 10% polyethylenglycol 6000. The PEG pellet was carefully washed and re-suspended. Purified ribosomes were analyzed by gel electrophoresis and western blotting.

3.12 Ribosomal pelleting

Prior to pelleting the ribosomes, 293T extract was cleared through a 0.45 µm filter and then layered on a 45% sucrose cushion. The cushion was subjected to centrifugation at 70000 rpm at 4°C for 2 hours in a TLA 100.3 rotor. Afterwards the supernatant was carefully removed and transferred to a new tube (Usually I removed 4x the volume of the input). The pellet was cautiously washed with re-suspension buffer I or re-suspension buffer II and then gently re-suspended in re-suspension buffer I (in case where the pellet was checked on the gel) or re-suspension buffer II (in case where re-suspended pellet was used for further experiments). For re-suspension, 100 µl-500 µl (depending on the size of the pellet) of re-suspension buffer was added to the pellet and tube was fixed on a shaker for 2 hours at 4°C. The re-suspended pellet was checked on a 12% Bis-tris gel or used for further experiments. Proteins in the supernatant of the cushion were precipitated by addition of 10% TCA.

3.13 Treatment of the extract with Micrococcal nuclease

293T extract prepared from cells transfected with HA-Lsm10 and HA-Lsm11-Flag was used. Extract was divided into three equal parts of 150 µl. $CaCl_2$ was added to all three parts to a final concentration of 2 mM. One part was used as control and to the other two parts, 150 units of micrococcal nuclease were added. All three parts were incubated at 20° C for 30 min. EDTA was added to the first and second parts to a final concentration of 5 mM and to the third part to a final concentration of 50 mM. All three parts were incubated at room temperature for 10 min and then layered on 5%-45% sucrose gradient. Gradients were run at 17000 rpm for 16 h in a SW 60Ti rotor. Fractions were harvested manually and 40 µl of each fraction was loaded on 12% Bis-Tris polyacrylamide gel. Proteins were then transferred to PVDF membrane and western blot analysis was performed.

3.14 Treatment of the extract with EDTA and puromycin

293T cells co-transfected with HA-Lsm10 and HA-Lsm11-Flag were used. Extract was prepared with the standard HEPES lysis buffer containing 50 mM EDTA or 100 µg/ml puromycin. Extracts were incubated at 25° C for 15 min and spun down briefly at 13000 rpm. Next, extracts were fractionated on 5%-45% sucrose gradient at 34500 rpm at 4° C in SW 41 Ti rotor. Fractions were harvested on gradient harvester and 40 µl of each fraction was loaded on 12% Bis-Tris polyacrylamide gel.

3.15 In vitro reconstitution of Lsm10/Lsm11 release with pICln

HEK 293T cells were transfected with HA-Lsm10 and HA-Lsm11-Flag constructs and extract was prepared as mentioned in section 3.8. Extract was layered on top of a 45% sucrose cushion and ribosomes were pelleted at 70000 rpm for 2h in a TLA 100.3 rotor. Pellets were washed and re-suspended with re-suspension buffer II. The concentration of the ribosomes was calculated by measuring the optical density at 260 nm. The final concentration of the ribosome in the re-suspension was measured as follow. 1 unit OD260 is equal to 20 pmol of ribosomes. Upon calculating the concentration of the ribosomes, increasing quantity of recombinant pICln was added to the re-suspended ribosomes. The reaction was incubated for 30 min at 37^oC. Immediately after completion of the incubation time, reaction tubes were transferred onto ice and equal volume of re-suspension buffer III was added. The whole reaction was then loaded on a 45% sucrose cushion and ribosomes were pelleted. The first and second supernatants and pellets were checked on a 12% Bis-Tris polyacrylamide gels.

3.16 Treatment of the extract with proteasome inhibitors

293T cells were transfected with HA-Lsm11-Flag. 48h upon transfection, medium was cautiously removed and fresh medium was added to the cells. Proteasome inhibitors, MG-132 and Bortezomib were added to the medium to a final

concentration of 50 µM and 10 µM respectively. Cells were incubated with the inhibitors for 2 hours at 37° C.

After 2 h of incubation, medium was removed, cells were washed and extract was prepared as already mentioned in section 3.8. Next, extract was fractionated on a 5%-45% sucrose gradient as been discussed before.

3.17 Luciferase assay

HEK 293T cells expressing firefly luciferase were transfected with HA-Lsm11, HA-Lsm10/HA-Lsm11-Flag or HA-Lsm10/HA-Lsm11-Flag/HA-pICln. Cells were harvested after 20h, 40h or 70h as follow. They were washed once with 1x DPBS. Cells were treated with trypsin for 30 s and then transferred to a tube with 1x DPBS. Cells were washed twice with DPBS and then snap frozen in liquid nitrogen.

At the day of the experiment, 500 µl of 1x passive lysis buffer was added to the pellet of the cells. Cells were re-suspended in the buffer by pipetting up and down and incubated on ice for 20 min. At the end, lysates were cleared by centrifugation at 13000 rpm for 15 min at 4° C. Concentration of the protein was measured in each sample. Accordingly, all samples were diluted to an equal final concentration by addition of 1x passive lysis buffer. Luciferase activity was measured after addition of Beatle-juice as substrate.

3.18 Metabolic labeling

293T cells were seeded into three 12-well plates. One plate was used as control (control). In the second plate, cells were transfected only with Lipofectamine RNAiMAX (lipofectamine) and in the third plate with siRNA against pICln (+siRNA). After 24h, all the cells were transferred into a 6-well plate. After another 24h, cells were transfected as follow. In each plate, two wells were used as control. Two wells were transfected with Lsm10/Lsm11 and two wells with Lsm10/Lsm11/pICln.

72h after initial transfection, medium was cautiously and completely removed. 4 ml of medium without methionine was added to each well and incubated for 1h in the incubator at 37° C (starving stage). The medium was removed carefully and 5 ml of

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medium without methionine, which contains ^{35}S-methionine at a concentration of 5 μCi/ml was added to each well (labeling stage). Cells were incubated for 2h at 37°C. At the end, medium was carefully removed and cells were once washed with 1x HBSS. The extracts were prepared as before.

3.19 General methods

3.19.1 Electrophoretic separation of proteins

Proteins were separated on a one-dimensional poly-acrylamide gel electrophoresis (PAGE). Sm proteins were resolved on a 15% Tris-Glycin system introduced by Laemmli (Laemmli, 1970). Lsm proteins were separated on lab-made 12% (or 13%) Bis-Tris gel system (introduced by Invitrogen).

3.19.2 Western blot analysis

Generally 15-100 μg of total protein was loaded on the gel. Then proteins were transferred from the gel to PVDF membrane with 0.45 μm pore size. Transfer was performed in a semi-dry western blot chamber for 2 hours at 0.8 mA/cm^2. Afterwards, the membrane was blocked in the blocking solution for 1 hour at room temperature. Incubation with the first antibody was done overnight at 4°C. The membrane was washed after incubation with the first antibody with three washes of PBS-Tween buffer for 30 min. Incubation with the secondary antibody was performed for 2 hours at room temperature. Prior to development, the membrane was washed with three washes of PBS-Tween for 30 min. To develop the membrane 10 ml of luminol solution was mixed with 100 μl of Coumaric acid and 10 μl of hydrogen peroxide. The developing mixture was added to the membrane and incubated for 10 seconds. Membrane was transferred to a transparent plastic wrap and excess of the reagent was removed. Film was exposed between 5 second to 5 min according to the strength of the signal.

3.19.3 Trichloroacetic acid (TCA) precipitation of proteins

1 volume of 100% TCA was added to 9 volumes of protein sample. Reactions were incubated on ice at 4^oC overnight. The next morning, tubes were centrifuged at 13000 rpm for 30 min at 4^oC. Protein pellets were washed extensively with the TCA wash solution. Prior to addition of Urea and 1x SDS loading buffer, pellets were dried at 37^oC. Protein pellets were re-suspended in urea and then checked on the gel.

3.19.4 Phenol extraction of protein, DNA or RNA

Equal volume of Phenol was added to the sample and was shaken for 5 min at high speed. Then, it was centrifuged for 10 min at 13000 rpm at room temperature. Centrifugation led to separation of two phases. The upper phase or aqueous phase included the nucleic acids and lower phase or organic phase contained the proteins. The aqueous phase was removed carefully and transferred to a new tube. 2.5 volumes absolute ethanol and 1/10 volume ammonium acetate was added to the aqueous phase. To the protein phase, 2.5 volumes acetone was added. Both were incubated at -20^oC overnight. The next morning, nucleic acids and proteins were precipitated by centrifugation at 4^oC for 10 min at 13000 rpm. Protein- and nucleic acid pellets were once washed with 70% absolute ethanol and dried at room temperature. RNA and DNA were re-suspended in 10 mM Tris/HCl pH 7.4 or ddH$_2$O depending on the further analysis. Proteins were re-suspended in 8.75 mM Urea.

3.19.5 Purification of antibodies

Anti-pICln and Anti-PRMT5 antibodies were purified according to Chari et al 2008, supplemental data. Anti SmD1/SmD2 and anti-SmB/SmB' were provided by Ashwin Chari.

3.19.6 Purification of recombinant pICln

pICln was purified according to Chari et al. 2008, supplemental data.

4   Results

4.1   Assembly of the U7 snRNP

PRMT5- and SMN complexes act sequentially in the assembly pathway of spliceosomal snRNPs. A hallmark of the early assembly phase is the formation of two intermediates with sedimentation coefficients of 6S- and 20S, respectively. Whereas, the 20S complex consists of the PRMT5 complex (PRMT5, WD45 and pICln) and all seven Sm proteins, the 6S complex is a stable dissociation product of the 20S complex and consists of the assembly chaperone pICln bound in a toroidal structure to Sm proteins D1, D2, E, F and G. The pre-assembled Sm proteins are then transferred en bloc onto the SMN complex and eventually loaded onto snRNA. Although the general architecture of the U7 snRNP is very similar to spliceosomal snRNPs, the Sm core of U7 snRNP is unique in its composition. The canonical core of splicing snRNPs consists entirely of Sm proteins (i.e. Sm proteins D1, D2, D3, B, E, F and G). However, in the Sm core of U7 snRNP, the Sm proteins D1 and D2 are replaced by the like-Sm (Lsm) proteins 10 and 11. Initial studies on U7 snRNP biogenesis have revealed similarities to the assembly pathway of canonical snRNPs. Specifically, a distinct SMN complex containing the set of Lsm and Sm proteins found in the U7 snRNP core has been described (Azzouz et al., 2005; Pillai et al., 2003). In addition, an interaction of pICln with Lsm10/Lsm11 has been documented (Azzouz et al., 2005). While these data point to an involvement of spliceosomal snRNP assembly factors in the formation of the U7 snRNP, their mode of action has remained unclear. To achieve a detailed characterization of the assembly route of U7 snRNP, in the first part of my thesis, I conducted studies aimed to identify the assembly intermediates formed in the U7 snRNP assembly pathway.

4.1.1 Identification of 6S- and 20S assembly intermediates containing Lsm10 and Lsm11

To search for U7 snRNP specific assembly intermediates (i.e. U7 snRNP assembly intermediates that are equivalent to spliceosomal 6S- and 20S complexes and contain Lsm10 and Lsm11 instead of SmD1 and SmD2), assembly active extract prepared from HeLa cells was fractionated by gel filtration using a column that separates complexes in this size range (Figure 4.1a).
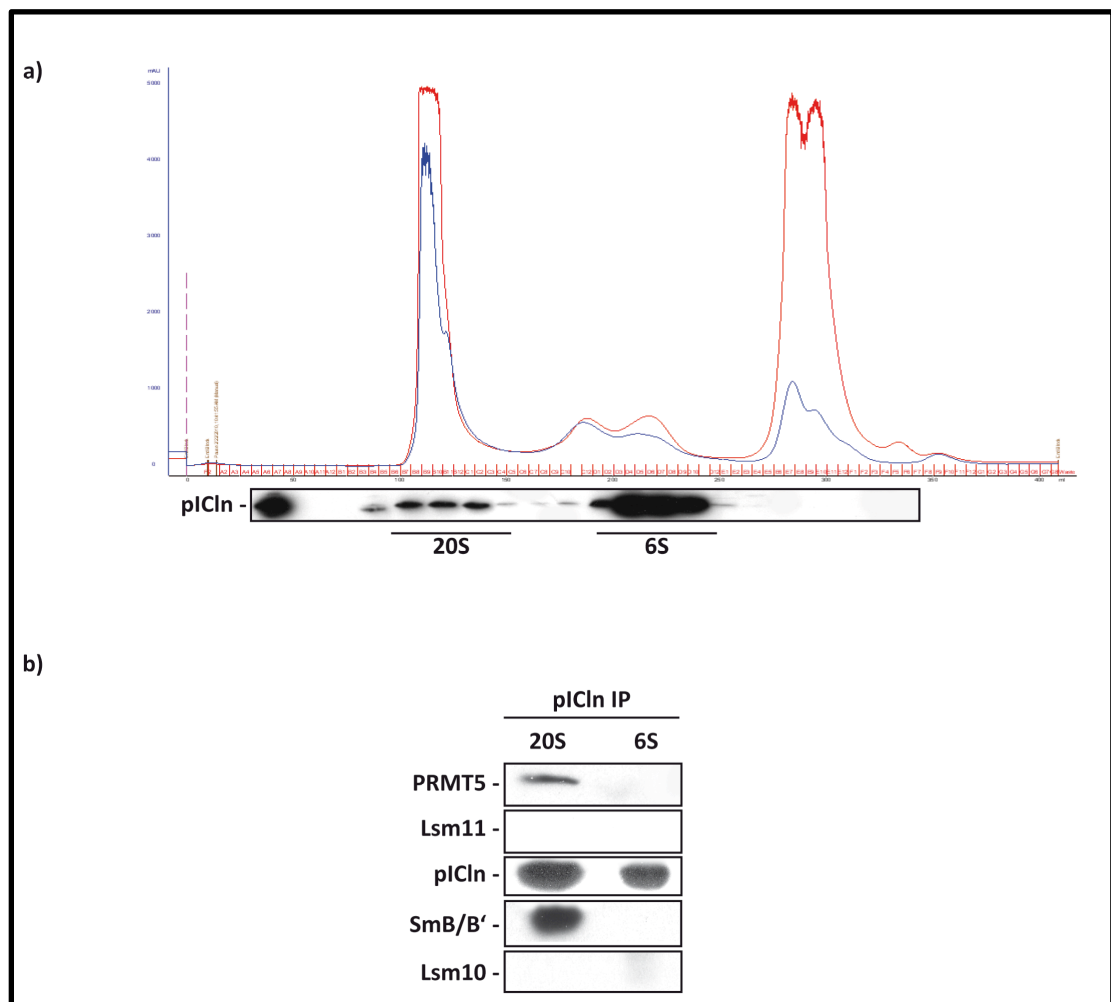
Figure 4.1. Large-scale fractionation of the extract using a HiLaod 26/60 Superdex 200 column.

a) pICln is found in fractions of 20S- and 6S peaks. b) Immunoprecipitation with anti-pICln antibody from 20S- and 6S fractions followed by Western blot analysis with α-PRMT5, α-

Lsm11, α-pICln, α-SmB/B' and α-Lsm10. PICln was detected in the 6S and 20S lanes. PRMT5 and SmB/B' were found in the purified 20S complex. I was not able to detect Lsm10 and Lsm11 in either 6S- or 20S complexes.

Western blot analysis of the individual fractions showed that pICln is found mainly in two major peaks corresponding to the previously described 6S- and 20S complexes (Chari et al., 2008). To identify proteins that are bound to pICln, these fractions were pooled and an immunoprecipitation (IP) was performed with a mono-specific anti-pICln antiserum (figure 4.1b). The immunoprecipitates were then analyzed by Western blotting with antibodies directed against PRMT5, pICln, SmB/B', Lsm10 and Lsm11, respectively. As shown in Figure 4.1b, PRMT5, pICln and SmB/B' were readily detectable in the immunoprecipitate, in contrast neither Lsm10 nor Lsm11 could be detected. This observation could be explained by either the low concentration of Lsm10 and Lsm11 proteins and/or their absence in these fractions.

To test either possibility, I alternatively fractionated the extract by size on a galactose gradient and proteins in each fraction were concentrated by TCA precipitation (Figure 4.2a). Similar to the results obtained by gel filtration, pICln was found mainly in two peaks with sedimentation coefficients of 6S (fractions 4-8) and 20S (fractions 13-16). PRMT5 (i.e. the catalytic unit of the PRMT5 complex), in contrast, was detected only in the 20S fractions. Of note, after concentration of the fractions, now also Lsm10 and Lsm11 were detectable in the 6S peak but were absent from the 20S peak (Figure 4.2a).

Fractions containing either the 6S complex (fractions 4-8) or the 20S complex (fractions 13-16) were then separately pooled and an IP was performed using an anti-pICln antiserum. A Western blot analysis of both immunoprecipitates indicated that pICln was successfully purified from both fractions (Figure 4.2b). In agreement with earlier studies, PRMT5 could be found only in the immunoprecipitation of the 20S complex, but was absent from the 6S complex. In contrast, both Lsm10 and Lsm11 could be detected only in the 6S immunoprecipitate, albeit at low levels. In the purified 20S complex, Lsm10 could be detected, whereas Lsm11 was absent. Thus, these results indicated that Lsm10 and Lsm11 are found in a complex with pICln, which sediments at 6S. However, these initial results left open the question whether the

Lsm10/11 containing 6S complex is a variant of the canonical 6S complex and hence is lacking SmD1 and SmD2. In addition, the results obtained for the integration of Lsm10 and Lsm11 into a 20S complex was not conclusive.

However, further characterization of the U7 snRNP assembly intermediates failed mainly due to the low abundance of these proteins in the cell and/or low sensitivity of the available antibodies (data not shown). To overcome this problem, I decided to analyze U7 snRNP assembly in cells over-expressing tagged Lsm proteins.
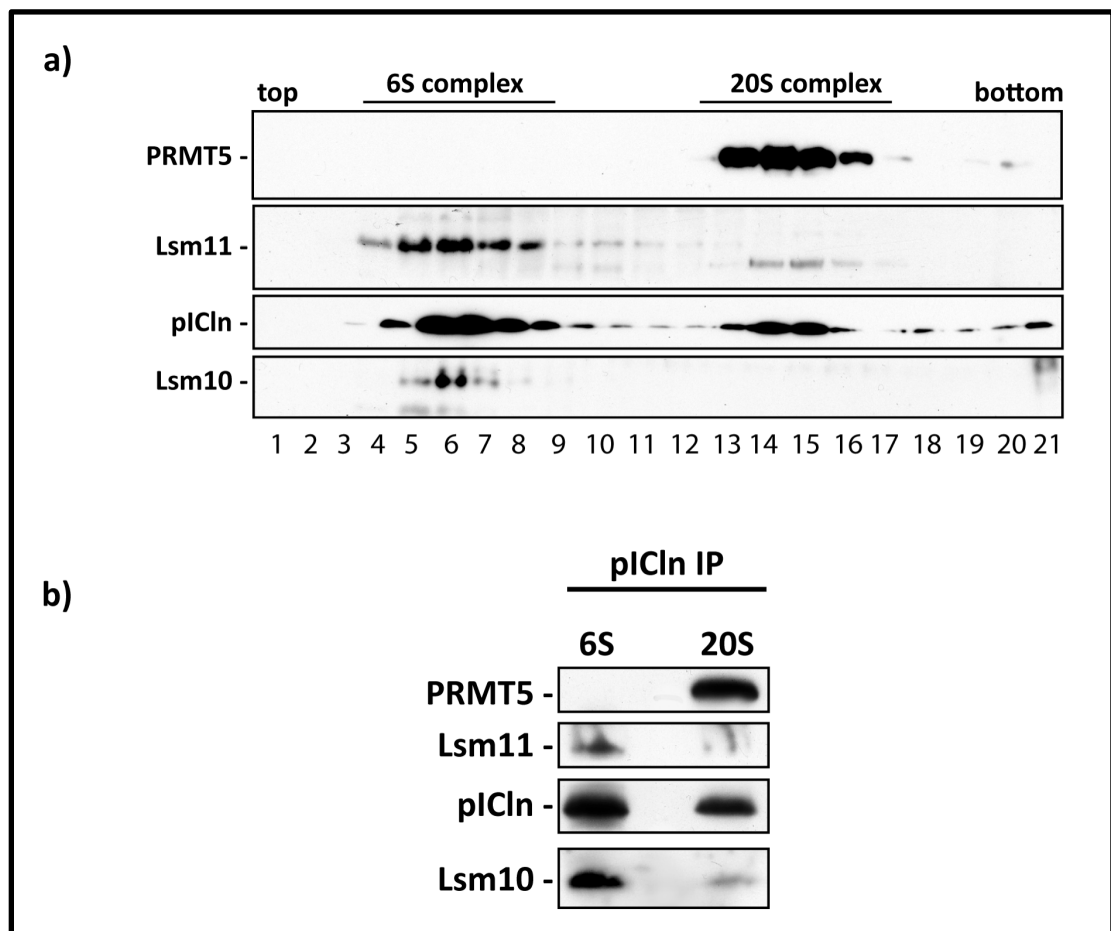
Figure 4.2. Small-scale fractionation of the HEK 293T extract.

a) Fractionation of the extract on a 10%-30% galactose gradient followed by Western blot analysis with α-PRMT5, α-pICln, α-Lsm10 and α-Lsm11 antibodies. b) Immunoprecipitation with an anti-pICln antibody from pooled fractions of 6S- and 20S complexes. In the 6S lane, I observed pICln and both Lsm10 and Lsm11. In the 20S lane, pICln and PRMT5 were found but I was hardly able to detect Lsm10 and Lsm11.

In the first set of experiments, cells were transfected with plasmids encoding either N-terminally HA-tagged Lsm10 alone, HA-tagged Lsm11 alone or both. Western blotting and immunofluorescence using an anti-HA antibody verified the expression of proteins. As shown in Figure 4.3a and b, both proteins were expressed at a similar level, and showed a predominant nuclear localization as has been reported previously for the endogenous proteins. I then addressed the question whether the over-expressed Lsm proteins integrated into cellular 6S- and 20S complexes. For this purpose, extracts from cells expressing either HA-Lsm10 alone, HA-Lsm11 alone or both proteins simultaneously were precipitated with a polyclonal anti-pICln antibody (Figure 4.3). Surprisingly, Lsm11 was found in association with pICln, whereas Lsm10 was not. However, when Lsm10 was co-expressed with Lsm11, both proteins integrated into a complex containing pICln (Figure 4.3c, left). Analysis of the unbound fraction of the purification (flow through, Figure 4.3c, right) revealed that in the absence of co-transfected exogenous Lsm11, exogenous Lsm10 did not form a complex with pICln and hence existed most likely as a free protein.
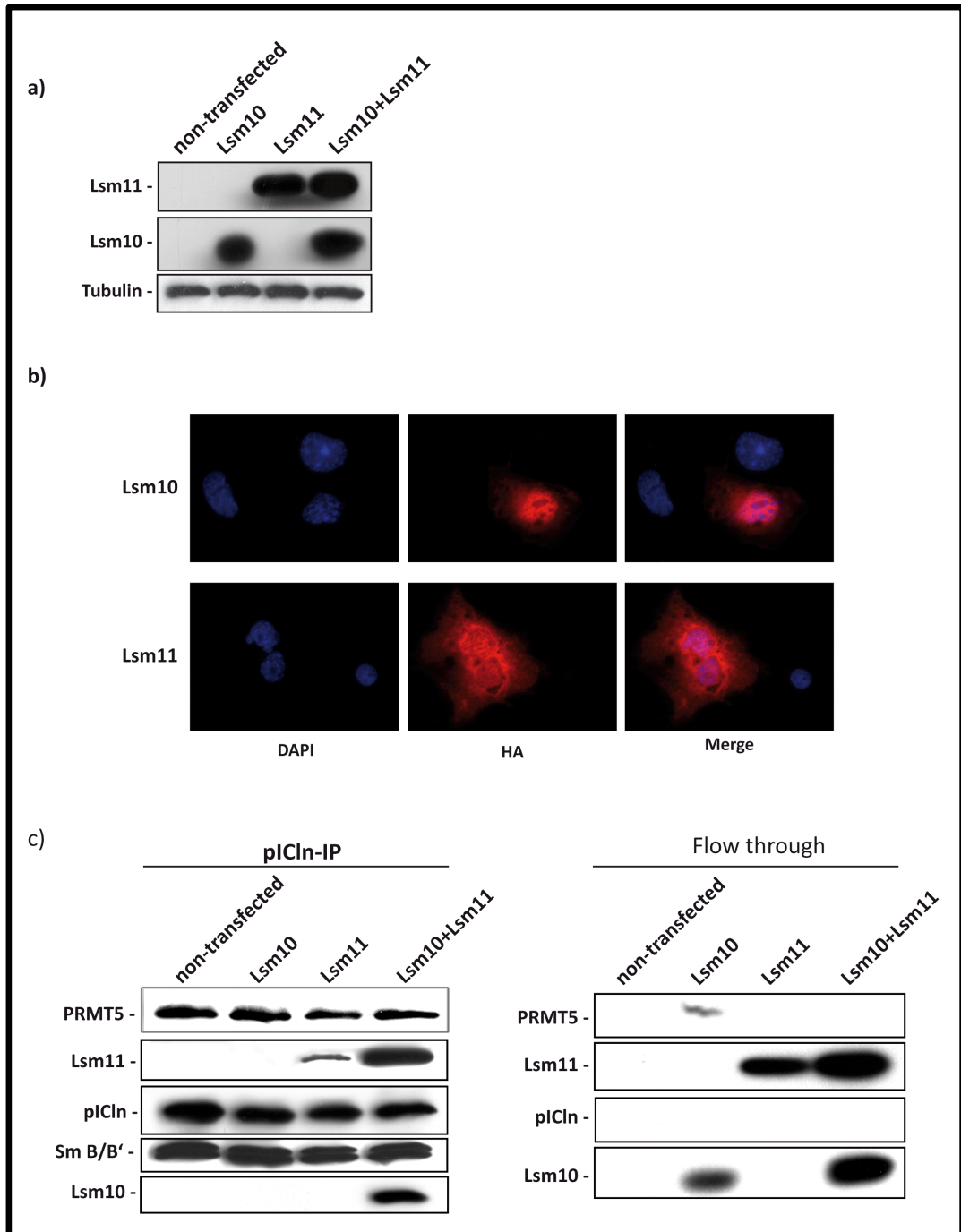
Figure 4.3. Over-expression of Lsm10, Lsm11 and Lsm10/Lsm11 in 293T cells.

a) Expression of the proteins was checked by Western blot analysis with α-HA antibody. b) Immunocytochemistry with anti-HA antibody in the transfected cells. Both Lsm10 and Lsm11 were located mainly in the nucleus. c) Immuno-blot analysis of the pICln immunoprecipitate (left) and the corresponding flow through fractions (right). Lsm11, when expressed alone, was found in a complex with pICln. Lsm10 co-purified with pICln only in the presence of Lsm11.

4.1.2 Unexpected sedimentation pattern of transfected Lsm10 and Lsm11

Having shown that co-expressed Lsm10 and Lsm11 proteins are found in a complex with pICln, I next wished to study how they are further handled by the cellular assembly machinery. For this purpose, cells were transfected either with HA-Lsm10, HA-Lsm11, or both plasmids simultaneously. Extracts from the transfected cells were subsequently fractionated on a 10%-30% galactose gradient and individual fractions were analyzed by Western blotting with an anti-HA antibody. As shown in Figure 4.4a, Lsm10 sedimented either at the top of the gradient or in the 6S peak (fractions 2-7). In contrast, Lsm11 was found in the peak of the 20S complex (fractions 12-18) and fractions that correspond to even larger S-values (fractions 20-22) (Figure 4.4b). Interestingly, co-transfection of Lsm11 and Lsm10 led to the shift of a fraction of Lsm10 into this high molecular weight region of the gradient (fractions 19-22) and also a shift of Lsm11 toward the 6S peak (Figure 4.4c). This observation indicated that co-expression of Lsm11 with Lsm10 resulted in the recruitment of Lsm10 into a heavy complex, which based on its S-value was neither the 6S- nor the 20S complexes.
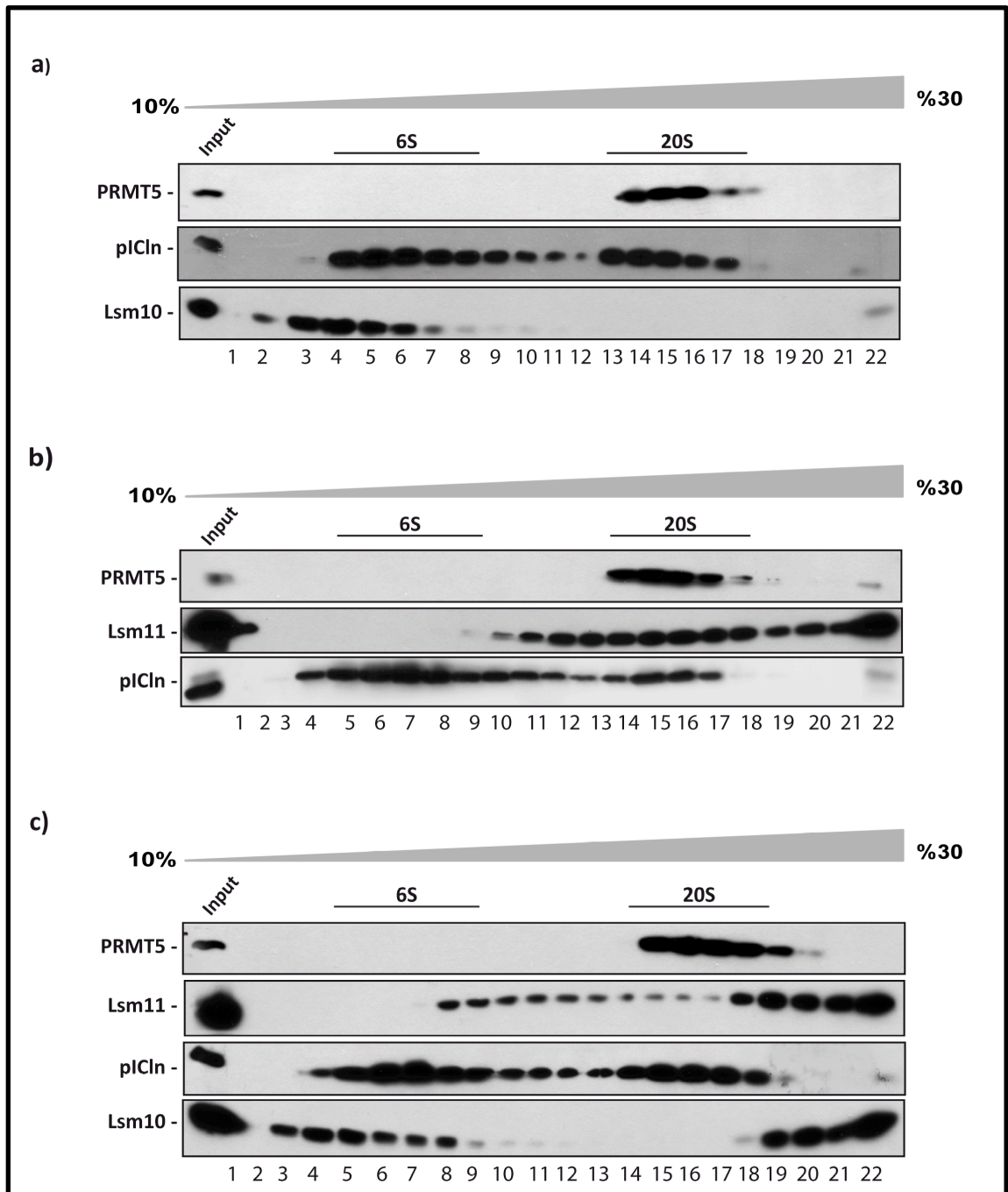
Figure 4.4. Sedimentation pattern of transfected Lsm10 and Lsm11 on 10%-30% galactose gradient.

a) Cells transfected with HA-Lsm10. The protein was found mainly as a free protein or in the peak of 6S complex (fractions 2-7). b) Cells transfected with HA-Lsm11. Some of the protein sedimented in the heavy range of the gradient (fractions 20-22). The rest of the protein was found in the peak of 20S complex (fractions 12-18). c) Cells co-transfected with HA-Lsm10 and HA-Lsm11. Upon co-transfection of Lsm11, a population of Lsm10 shifted into the heavy complex along with Lsm11 (fractions 19-22).

4.2   Lsm10 and Lsm11 proteins associate with ribosomes

The high molecular weight complex found in cells transfected with Lsm11 or co-transfected with Lsm10/Lsm11 described in the previous chapter might correspond to either the SMN-complex, which sediments at 30S, or another yet uncharacterized assembly intermediate. In the next set of experiments, I therefore aimed to characterize this large complex. As a first approach, I fractionated the corresponding extracts on 5%-45% sucrose gradients that enabled me to resolve the observed high molecular weight species. In agreement with previous results shown in figure 4.4a, Lsm10 was found merely at the top of the gradient when transfected alone. These fractions (fractions 1-2) contained either the free protein, 6S- and/or 20S complexes (Figure 4.5a). Surprisingly, the over-expressed Lsm11 protein sedimented well into the gradient including polysomal fractions (fractions 1-22) (Figure 4.5b) and its co-transfection with Lsm10 led to the co-sedimentation of both proteins with polysomes (mainly fractions 11-22) (Figure 4.5c). Altogether, these results showed that transfected Lsm11 or co-transfected Lsm10/Lsm11 but not Lsm10 alone co-sedimented with polysomes in sucrose gradients.
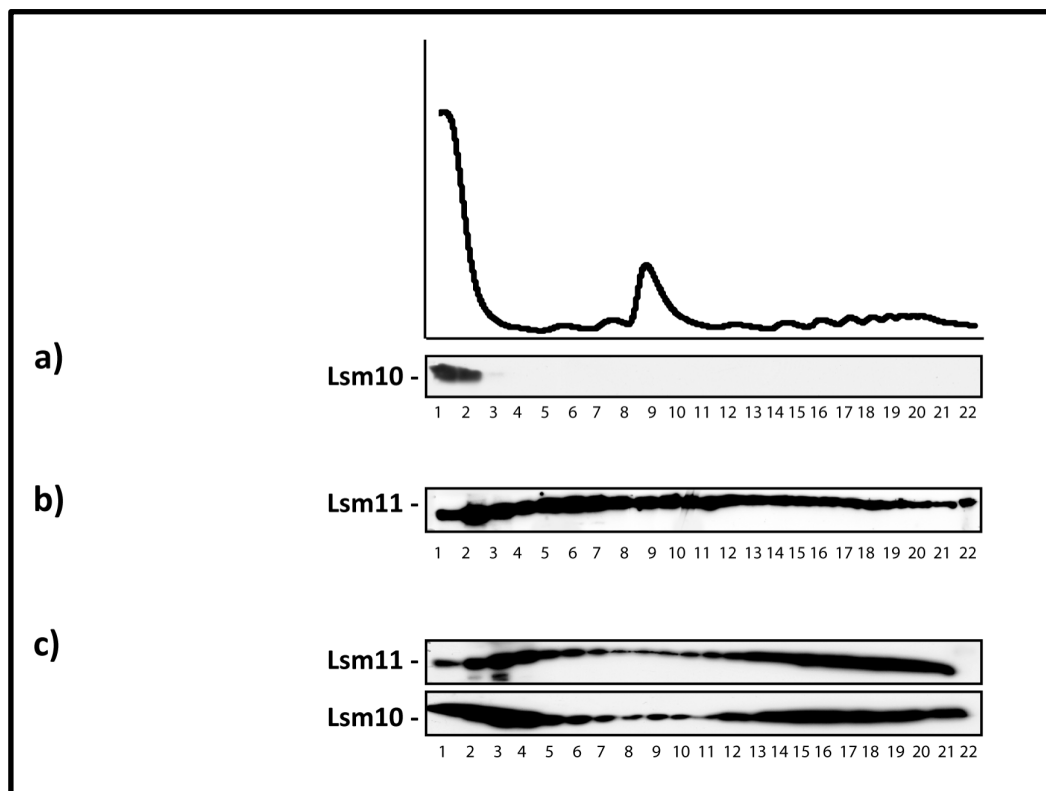
Figure 4.5. Sedimentation of transfected HA-Lsm10 and HA-Lsm11 on a 5%-45% sucrose gradient.

a) Cells transfected with HA-Lsm10. The over-expressed protein was found in the top fractions of the gradient where the free protein, 6S- and/or 20S complexes sediment (fractions 1-2). b) Cells transfected with HA-Lsm11. The protein sedimented throughout the gradient into polysomal fractions (fractions 1-22). c) Cells co-transfected with HA-Lsm10 and HA-Lsm11. Co-transfection of Lsm11 led to co-sedimentation of Lsm10 with polysomes (fractions 11-22).

4.2.1 Direct interaction of Lsm10 and Lsm11 proteins with the ribosomes

In the next step, a series of experiments were performed to test whether Lsm10 and Lsm11 physically associate with ribosomes as the sedimentation experiments presented in the preceding section had suggested.

In the first approach, I purified the ribosomes from the polysomal fractions of the gradient (see figure 4.5c, fractions 11-21). The purification was performed under low salt conditions to allow the co-purification of ribosome-associated factors. The obtained fraction was then separated by SDS-PAGE and analyzed by Western blotting with antibodies directed against ribosomal protein L7 (rpL7), Lsm10, Lsm11 and pICln. As shown in figure 4.6a, Lsm10 and Lsm11 co-purified with ribosomes as revealed by Western blotting. This biochemical experiment provided a first indication that both proteins associate with the ribosome under low salt conditions.

To further support the finding described above, I next studied the effect of EDTA on the sedimentation pattern of transfected Lsm10 and Lsm11 proteins. EDTA causes the dissociation of ribosomal subunits and the disruption of polysomes. As shown in Figure 4.6b, EDTA treatment of the extract altered the sedimentation of both Lsm proteins considerably. Under these conditions, Lsm10 and Lsm11 proteins were mainly found to co-sediment with the large ribosomal subunit (60S) and remaining monosomes (80S ribosomes).
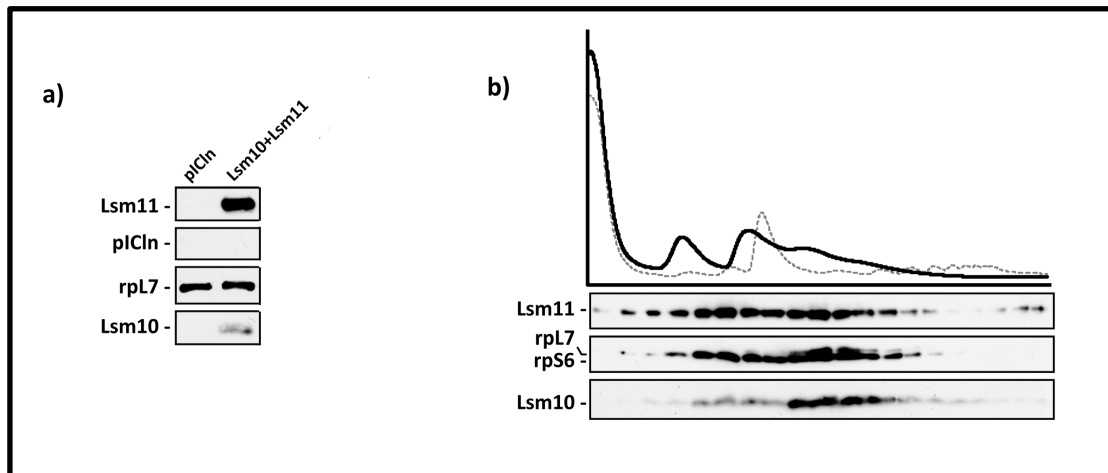
Figure 4.6. Purification of ribosomes from transfected cells and EDTA treatment of the extract

a) Ribosomes were purified under low salt condition from cells transfected with pICln or co-transfected with Lsm10/Lsm11. Purified ribosomes were analyzed by Western blotting. Cells transfected with pICln were used as a control. Lsm10 and Lsm11 but not pICln were co-purified with the ribosomes. b) Association of Lsm10 and Lsm11 with the polysomes was sensitive to EDTA treatment of the extract. Gray line indicates the ribosome profile of the non-treated and black line of EDTA-treated cells.

Finally, I analyzed the effect of limited micrococcal nuclease treatment on the sedimentation pattern of Lsm10 and Lsm11. Under these conditions, the nuclease digests the mRNA selectively, whereas ribosomal RNA is unaffected. As a result, polysomes are converted into monosomes (80S ribosomes). Upon treatment of these monosomes with EDTA, 40S and 60S ribosomal subunits are obtained. For this specific experiment, the extract prepared from cells transfected with both Lsm10 and Lsm11 was divided into three equal parts. The first part was used as control, while the second and third parts were treated with micrococcal nuclease. The third part was additionally treated with EDTA post nuclease treatment. The differentially treated extracts were then analyzed by gradient centrifugation, which resolved 40S, 60S and 80S ribosomal species (figure 4.7). In the control gradient, Lsm11 was found in both the 80S and polysomal fractions of the gradient (figure 4.7a). Upon treatment of the extract with micrococcal nuclease, the majority of Lsm11 and Lsm10 shifted to the 80S peak (figure 4.7b). Further treatment of the extract with EDTA, which dissociated the ribosomal

subunits, led to the accumulation of both Lsm proteins mainly in the 60S peak and to a lesser extent in the 40S peak (figure 4.7c).

In sum, all three approaches described in this section strongly indicated that Lsm10 and Lsm11 associate with ribosomes. Furthermore, the results from the EDTA and micrococcal nuclease treatment implied that both Lsm proteins associate with the large subunit of the ribosome.
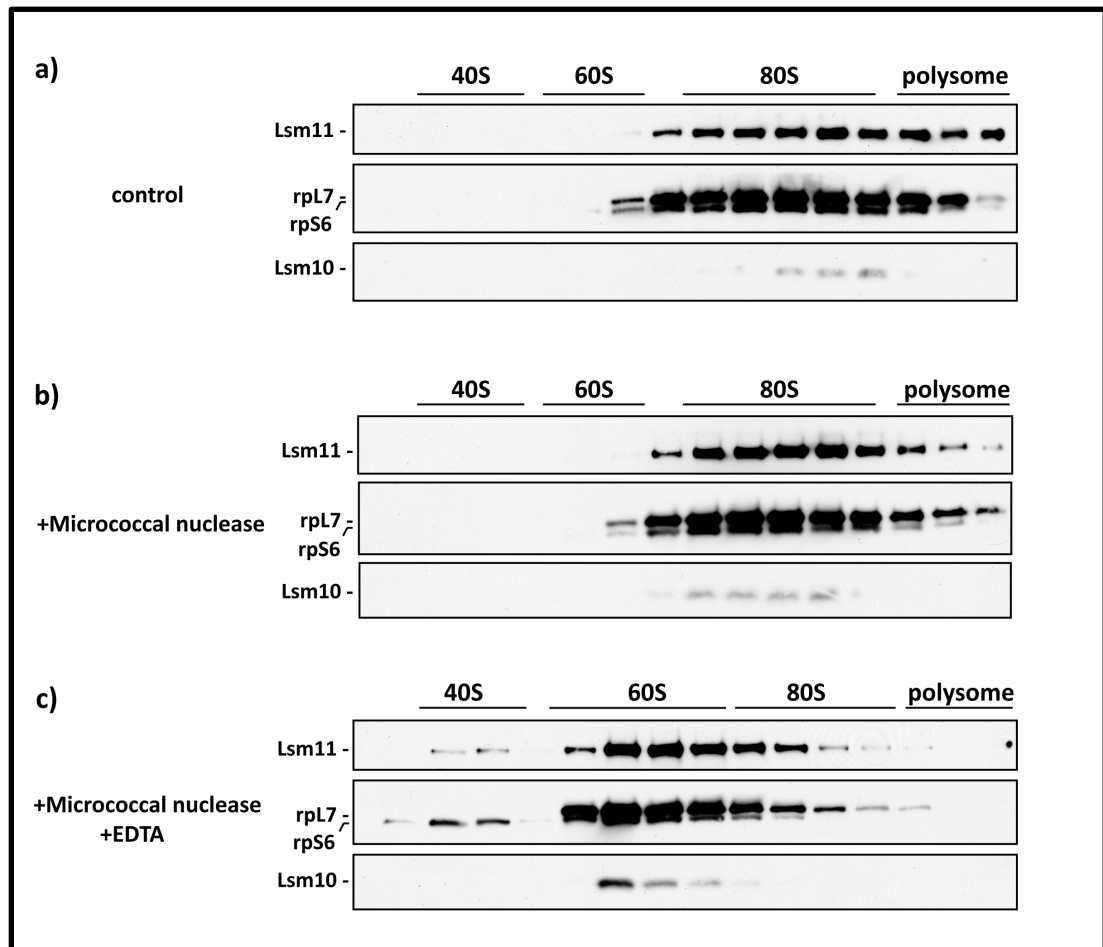
Figure 4.7. Micrococcal nuclease treatment of the extract prepared from cells co-transfected with Lsm10 and Lsm11.

Extracts prepared from cells co-transfected with Lsm10 and Lsm11 were treated with micrococcal nuclease (b and c) and additionally further treated with EDTA (c).

a) In the control extract, Lsm10 and Lsm11 were associated with the monosomes (80S) as well as polysomes. b) Upon treatment with micrococcal nuclease, I observed an increase in the monosomal (80S) association and decrease in the polysomal association of Lsm11. c) After treatment with EDTA, both proteins were predominantly found in the 60S peak.

4.3 Mechanism of Lsm protein association with the ribosomes

The experiments I have described thus far provided strong evidence that Lsm proteins associate with ribosomes. In the next set of experiments, I aimed to investigate the mechanistic nature of this association. Hence, I initially tested the effect of puromycin on the sedimentation pattern of the transfected Lsm proteins. Puromycin is an inhibitor of translation, which causes the premature release of nascent chains from ribosomes and the dissociation of polysomes. Interestingly, treatment of the extract with puromycin did not affect the ribosomal association of the Lsm proteins (i.e. Lsm proteins were not released from polysomes after puromycin treatment) (figure 4.8). This observation was surprising, as the insensitivity of polysomal association of a given protein to the presence of puromycin is an indication of ribosomal stalling on mRNA.
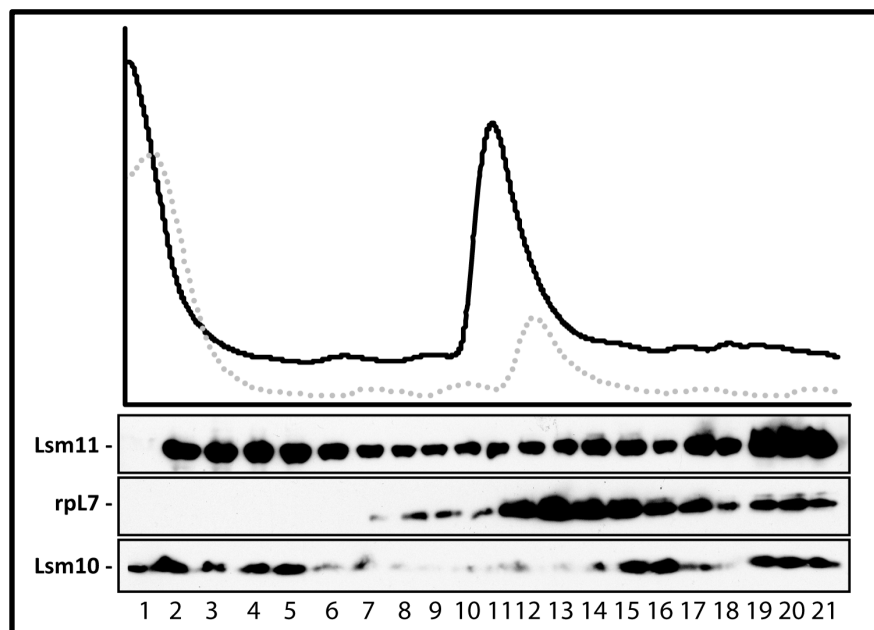
Figure 4.8. Effect of puromycin on the co-sedimentation of Lsm10 and Lsm11 with polysomes.

Extract from cells co-transfected with Lsm10 and Lsm11 was treated with puromycin prior to fractionation on the sucrose gradient. Puromycin had hardly any effect on the sedimentation pattern of the Lsm proteins. The gray line indicates the ribosome profile of the control extract and the black line indicates the ribosome profile of the puromycin treated extract.

Several scenarios appeared reasonable to explain the nature of the association of Lsm11 and Lsm10 with the ribosomes and hence were investigated experimentally.

1) *Translational stalling as a cause for ribosome association of Lsm proteins*: Because co-sedimentation of Lsm proteins with polysomes was shown to be insensitive to puromycin (see figure 4.8), ribosomes might stall on the Lsm11 message. To investigate this hypothesis, I appended a C-terminal Flag-tag to the protein. Synthesis of the tag could only occur if translation continued downstream of the Lsm11 open reading frame of the message. Extract from Lsm11 transfected cells were fractionated on a 5%-45% sucrose gradient. I observed that the Flag-tagged protein co-sedimented with ribosomes in a similar manner as the HA-tagged protein (see Figure 4.9a and compare it with Figure 4.5b). This indicated that ribosomes are not stalled on the Lsm11 mRNA but that the Lsm11 protein is retained on ribosomes upon termination of translation.

2) *Electrostatic or hydrophobic association of Lsm proteins with the ribosomes*: It is well known that Lsm11 is a basic protein (pI=10.9). It is therefore conceivable that Lsm11 remains associated with the ribosome after completion of translation as a result of electrostatic interactions. In this case, one would predict the dissociation of Lsm protein from the ribosome at elevated salt concentrations. To test this scenario, I transfected cells with plasmids encoding for either Lsm11 or both Lsm10 and Lsm11. Ribosomes were afterward pelleted from the extract through a sucrose cushion. The ribosomal pellets were then re-suspended in increasing concentrations of KOAc and pelleted again on a sucrose cushion. As shown in figure 4.9b, the Lsm proteins failed to dissociate from ribosomes even in the presence of 1 M salt. This indicated that ribosomal association of Lsm proteins is not primarily based on an electrostatic interaction of the proteins with the ribosome.

If on the other hand Lsm11 associate with the ribosome through hydrophobic interactions, I should have been able to dissociate Lsm11 from ribosomes, as the extract was prepared in the presence of 1% detergent. This amount of detergent would be able to disrupt hydrophobic protein-protein or RNA-protein interactions as well as solubilize membranes to liposomes.

3) *The release of Lsm proteins has a strong energy requirement*: It was possible that the release of Lsm11 from ribosomes was inefficient due to a strong energy requirement, supplied by either GTP or ATP. To test for this scenario, I co-transfected cells with plasmids encoding Lsm10 and Lsm11. After overexpression of both proteins, extracts were prepared and ribosomes pelleted on a sucrose cushion. The ribosomal pellets were then re-suspended and incubated with either ATP or GTP prior to the second pelleting. As shown in figure 4.9c, neither GTP nor ATP dissociated the proteins. It is therefore unlikely that Lsm11 binding to ribosomes is regulated in an ATP and/or GTP dependent manner.

4) *Factor-mediated association of Lsm proteins with the ribosomes*: I assumed that there exists a factor, which mediates the interaction between Lsm11 and ribosomes. Elucidation of this factor and its role required further investigation, which will be discussed in the chapter 6 (outlook).

Taken together, these experiments suggested that association of Lsm11 with the ribosomes is not the result of ribosome stalling on Lsm11 mRNA or any defect in the energy-dependent release. Likewise, a mere electrostatic or hydrophobic interaction of Lsm11 with the ribosomes could be ruled out. Thus, it appeared that the ribosome association of Lsm11 is an intermediate in the biogenesis pathway of U7 snRNP.
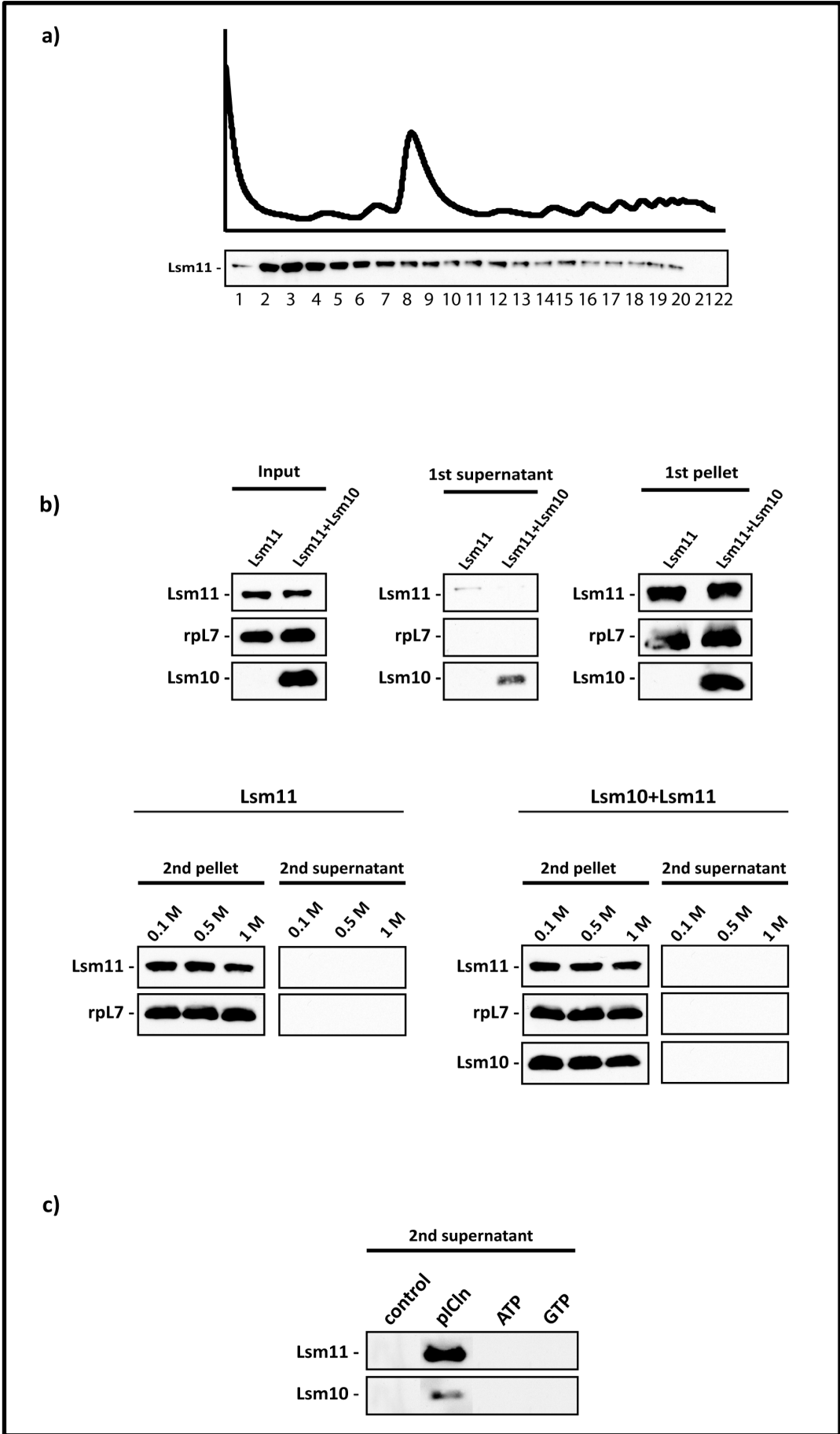
Figure 4.9. The nature of Lsm10/Lsm11 association with the ribosomes.

a) 293T cells were transfected with C-terminally Flag-tagged Lsm11. Western blotting with anti-Flag antibody confirmed that full-length Lsm11 associated with the ribosomes. b) 293T cells were transfected with Lsm11 or Lsm10/Lsm11. Ribosomes were pelleted on a sucrose cushion. Western blot analysis for the supernatants and pellets from the first and second pelleting are shown. Association of the proteins with the ribosomes was not sensitive to increasing concentration of salt. c) Ribosomal pellet from Lsm10/Lsm11 transfected cells were incubated with pICln, ATP or GTP. Western blot analysis of the supernatant of the second pelleting is shown. In contrast to pICln, incubation of ribosomal pellet with either ATP or GTP did not release the Lsm11 and Lsm10.

4.4 Release of Lsm11 from the ribosomes

The experiments described in the previous sections indicated that Lsm10 and Lsm11 physically associate with ribosomes upon completion of translation. Based on these findings, further studies on the mechanism of the release of these proteins from the ribosomes were conducted. Two major questions were addressed experimentally: 1) How do Lsm proteins dissociate from the ribosomes and 2) what is the functional importance of this mechanism?

4.4.1 The assembly chaperone pICln functions as a release factor

pICln is the earliest known assembly factor to associate with canonical Sm proteins. pICln pre-assembles Sm proteins into specific intermediates and prevents them from non-specific interactions in the cytoplasm. Therefore, it appeared plausible that it also functions in the release of the Lsm10 and Lsm11 from ribosomes. To address this hypothesis, pICln was co-transfected with Lsm11 alone or both Lsm10 and Lsm11. The extracts from these cells were size-fractionated on a sucrose gradient. As shown in figure 4.10a, pICln did not release co-transfected Lsm11 from ribosomes. Interestingly however, co-transfection of pICln with Lsm10 and Lsm11 led to the release of both proteins from the ribosomes (figure 4.10b).
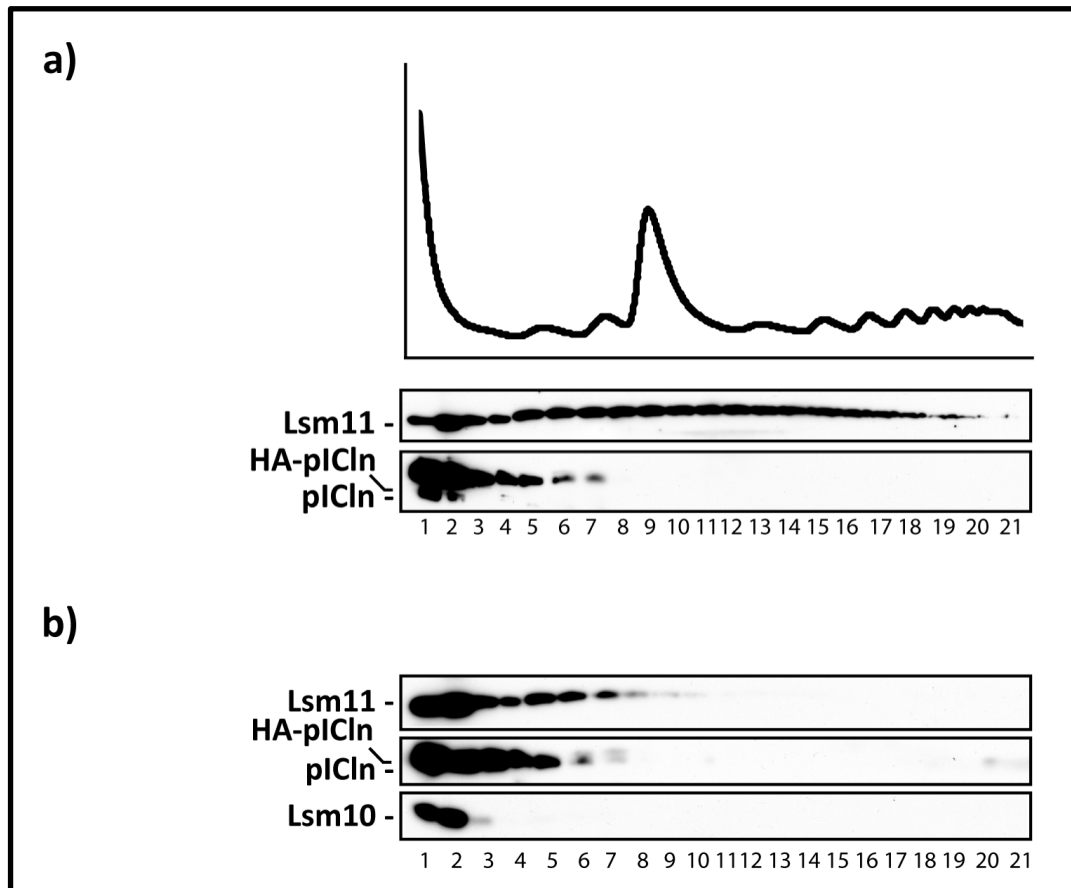
Figure 4.10. pICln functions as a release factor for Lsm10 and Lsm11.

Extracts from the transfected cells were fractionated on a 5%-45% sucrose gradient followed by Western blot analysis with anti-HA antibody. a) Cell transfected with Lsm11 and pICln. Co-transfected pICln did not release Lsm11. b) Co-transfection of pICln with Lsm10 and Lsm11 led to the release of both Lsm proteins from the ribosomes.

What might be the basis of this selectivity in the release of the co-transfected Lsm proteins? A possible explanation for the selective capability of pICln to dissociate the Lsm10 and Lsm11 from the ribosome might be obtained by early studies conducted by the Lührmann lab (Raker et al., 1996). By analyzing the properties of Sm proteins dissociated from mature snRNPs, Sm proteins were documented to exist as pre-formed hetero-oligomers consisting of SmD1/D2, SmD3/B and SmE/F/G. The Lsm10/11 dimer is known to replace the SmD1/D2 dimer in the canonical Sm core, to generate the U7-specific core. In the canonical 6S complex, pICln has been shown to directly interact only with SmD1 and SmG both by biochemical experiments and a recent crystal structure (Grimm et al., 2013). In contrast, no direct interaction of pICln with

SmD2 is detectable. Assuming that a similar architecture exists for the U7-specific 6S complex, one may predict that pICln can interact with Lsm10, which by sequence homology is presumed to be the paralog of SmD1 in the U7 snRNP. As is the case with SmD2, Lsm11, which by sequence homology is presumed to be the paralog of SmD2, would not be capable of direct interaction with pICln. This in turn would imply that, the presence of Lsm10, which links pICln to Lsm11, is essential for the release of Lsm11. Therefore, this result indicated that the function of pICln in the release of heterodimer is dependent on interactions, which are essential at a later stage of assembly (i.e. formation of the 6S complex and Sm core). Moreover, it appears the order of Lsm proteins, which is present in the assembled U7 snRNP is already established upon release from their site of synthesis, the ribosome.

The findings above indicated that co-transfection of pICln led to the release of the Lsm10/Lsm11 heterodimer from the ribosome. However, as the co-transfection experiment was conducted in vivo, the question arose whether pICln alone was necessary and sufficient to perform this reaction. To address this issue, I established an in vitro system that allowed me to recapitulate the release of Lsm proteins from the ribosomes. For this purpose, ribosomes from Lsm10/Lsm11 co-transfected cells were pelleted on a sucrose cushion. Ribosomes were then re-suspended and incubated with pICln prior to a second round of pelleting on the sucrose cushion. I was able to show that increasing concentrations of pICln led to the gradual release of Lsm10/Lsm11 heterodimer from the ribosomes (figure 4.11a). To test the specificity of pICln function in the reconstituted release reaction described above, I examined the ability of several other proteins and/or substances in the release of the heterodimer. BSA, Heparin, GroEL and spermidine were tested and none of these proteins was able to release the heterodimer from the ribosomes (Figure 4.11b). In addition, I purified the ribosomes from cells transfected with Lsm10/Lsm11 or Lsm10/Lsm11/pICln under low salt conditions. Purified ribosomes were then analyzed by Western blotting. As expected, co-transfected pICln was able to release the heterodimer from the ribosomes (figure 4.11c).

Altogether, the in vivo and in vitro results described above uncovered a thus far unknown function of pICln in the specific release of the Lsm10/Lsm11 heterodimer from the ribosomes.
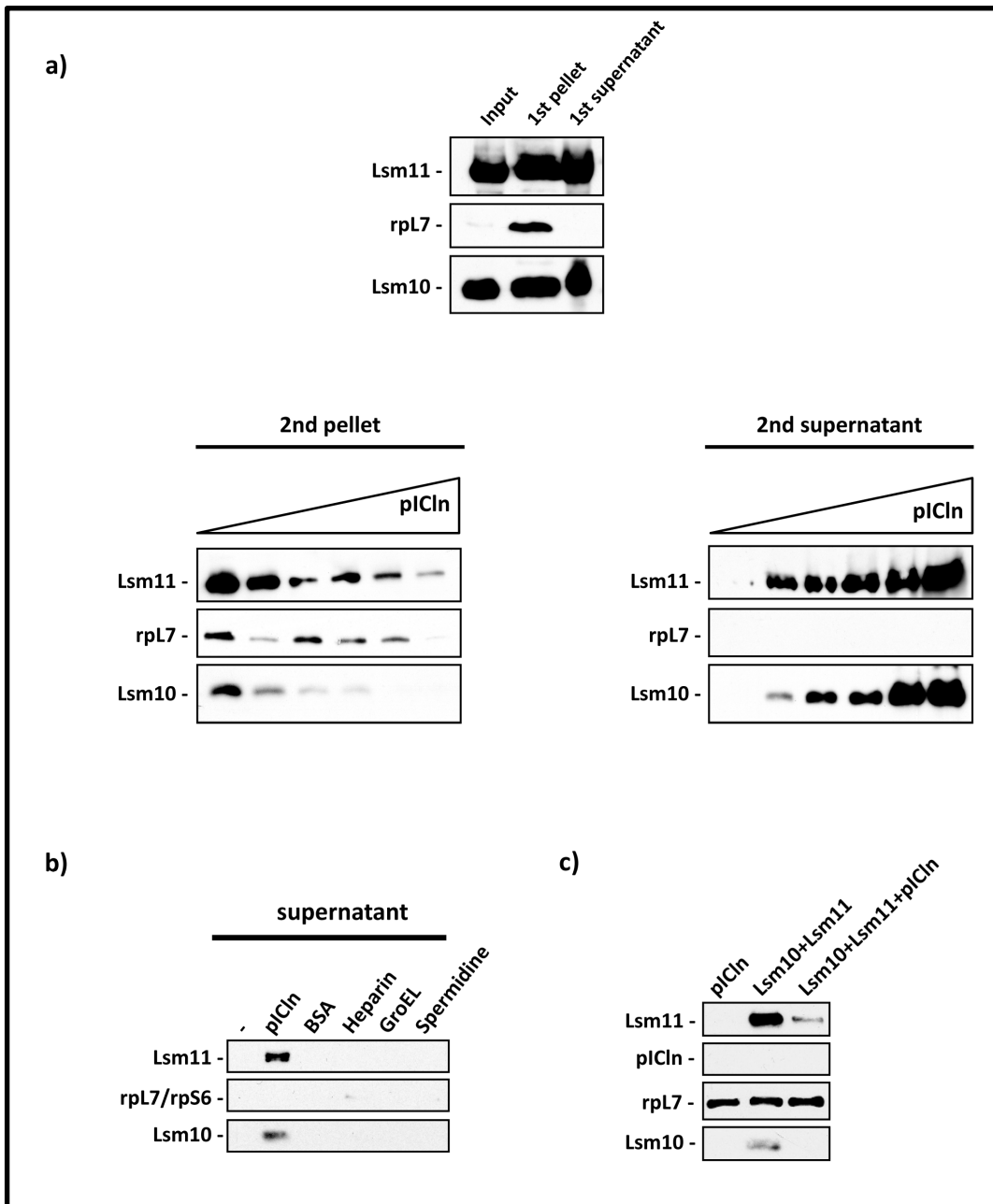
Figure 4.11. Re-constitution of the release of Lsm10/Lsm11 heterodimer in vitro.

a) Ribosomes were pelleted from Lsm10/Lsm11 transfected cells. Ribosomal pellet was re-suspended and increasing amount of pICln was added to the ribosomes. Re-suspended ribosomes were incubated with pICln and pelleted on a sucrose cushion for the 2nd time. Lsm10 and Lsm11 sedimentation was tracked by Western blot analysis with anti-HA antibody. b) In a similar experiment, role of several other proteins/substances in the release of Lsm10 and Lsm11 were studied. Western blot analysis of the second supernatant is shown. Only pICln was able to release the Lsm10/Lsm11 proteins. c) Ribosomes purified from cells transfected with pICln, Lsm10/Lsm11 or Lsm10/Lsm11/pICln under low salt condition. Co-transfection of pICln led to the release of Lsm10/Lsm11 heterodimer from the ribosomes in vivo.

4.4.2 Formation of the cognate heterodimer is prerequisite for the release

So far I was able to show that Lsm10 and Lsm11 are retained at the translational machinery after their synthesis. Furthermore, the assembly chaperone pICln was shown to be both necessary and sufficient for their release. In the next step, I aimed to understand the prerequisites for the retention and release of the proteins. Initially, I investigated whether the formation of the cognate Lsm heterodimer was important for their release from the ribosome.

Based on the similarity of SmD1 and SmD2 to Lsm10 and Lsm11, respectively ((Pillai et al., 2003; Pillai et al., 2001) and see above), it is assumed that Lsm10 and Lsm11 interact with each other in a similar manner as SmD1 and SmD2. The crystal structures of isolated SmD1/D2 and SmD3/B have revealed that the Sm-Sm interaction interface is characterized by a high proportion of hydrophobic residues (Kambach et al., 1999). Moreover, in the SmD1/D2 heterodimer the $\beta 5$ strand of SmD1 interacts with the $\beta 4$ strand of SmD2. Likewise, it is assumed that the $\beta 5$ strand of the Lsm10 protein interacts with the $\beta 4$ strand of Lsm11.

To additionally verify the explicit function of pICln in the release of the cognate heterodimer, I pursued two additional experimental strategies. First, I cloned a construct of Lsm11, which lacks the c-terminal β-strands 4 and 5 (Lsm11$\Delta\beta4\beta5$). Since β-strand 4 from Lsm10 is assumed to interact with β-strand 5 from Lsm11, this truncated form of Lsm11 is predicted to be unable to bind Lsm10. I expressed Lsm11$\Delta\beta4\beta5$ and pelleted the ribosomes from the extract (figure 4.12). As expected, Lsm10 did not co-sediment with Lsm11$\Delta\beta4\beta5$ (see the 1st pellet) and co-transfection of pICln did not release the Lsm11$\Delta\beta4\beta5$ as efficiently as the full-length protein due to the lack of direct interaction of pICln with Lsm11 (see the 2nd pellet and supernatant). However some Lsm11$\Delta\beta4\beta5$ was released in the presence of fifty times molar excess of pICln. These experiments indicated that formation of the Lsm10/11 heterodimer occurs in a similar fashion as SmD1/D2 and verified that Lsm10/11 heterodimer formation is essential for their release from the ribosome by pICln.
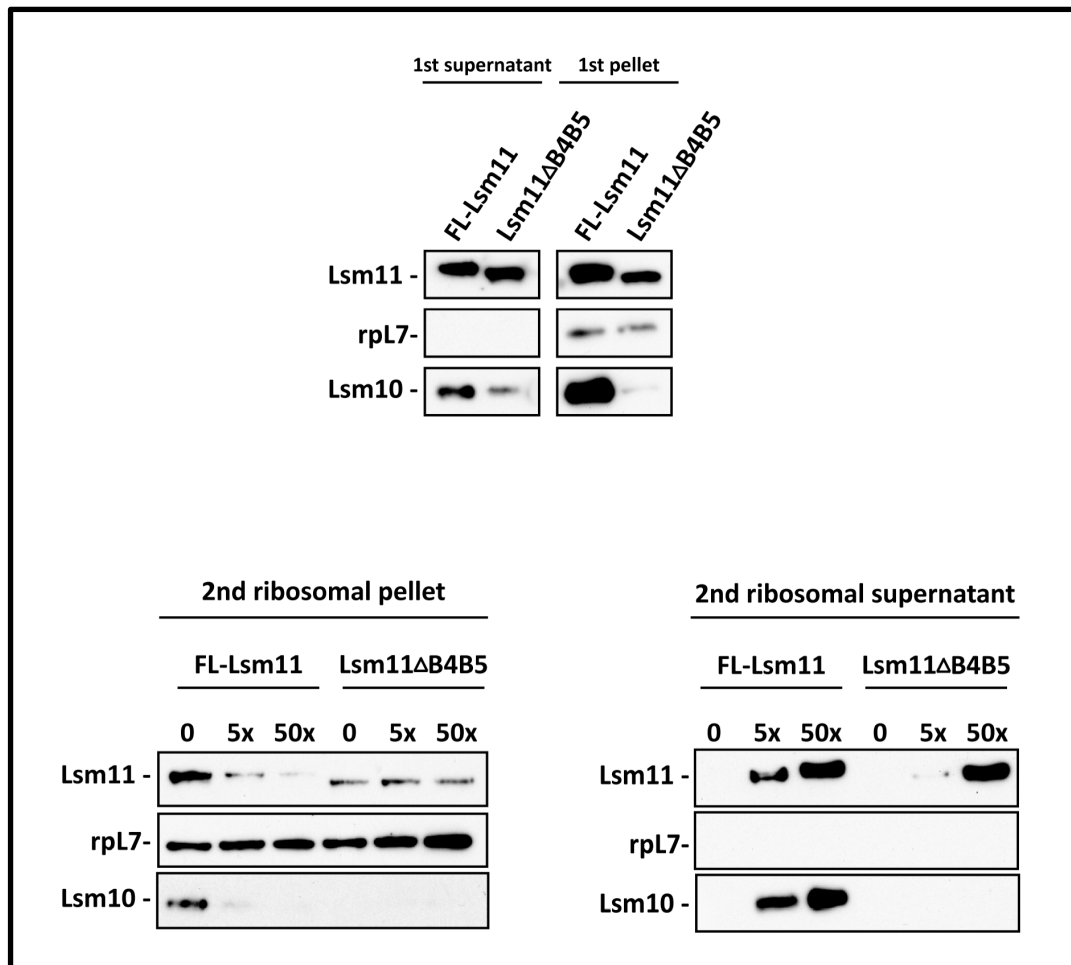
Figure 4.12. pICln releases the Lsm10/Lsm11 heterodimer from the ribosomes.

Full length (FL) and truncated (Lsm11Δβ4β5) forms of Lsm11 were co-expressed with Lsm10. Ribosomes were pelleted from the extract. Whereas, Lsm10 was pelleted along with full-length Lsm11 and ribosomes, truncated form of Lsm11 was unable to bind and co-pellet Lsm10 (See the 1st pellet). Increasing concentration of pICln led to the release of Lsm10/Lsm11 heterodimer in the case of the full-length protein. Release of truncated protein (Lsm11Δβ4β5) occurred only in the presence of very high concentration of the release factor, pICln.

Second, by sequence homology, SmD1 is assumed to be the cellular paralog of Lsm10, but is incapable of forming a heterodimer with Lsm11 as SmD1 is never found to be part of the U7 snRNP under any circumstances. Therefore, I co-transfected the cells either with Lsm11 and SmD1 (figure 4.13a) or Lsm11, SmD1 and pICln (figure 4.13b). I then prepared extracts from both transfected cells and investigated the sedimentation pattern of the exogenous, tagged Lsm11 and SmD1 proteins. As shown in figure 4.13,

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while Lsm11 co-sedimented with ribosomes in polysomal fractions as described above, SmD1 did not co-sediment with Lsm11 in ribosomal fractions. In contrast to the behavior of co-transfected Lsm10, SmD1 sedimented in fractions lighter than the ribosome, indicating that it does not form a heterodimer with Lsm11 on ribosomes. In agreement with this finding, in cells co-transfected with pICln, SmD1 and Lsm11, pICln was not capable of dissociating ribosome-bound Lsm11 (Figure 4.13b).

These results supported the notion that formation of a cognate heterodimer between Lsm10 and Lsm11 proteins is essential for their release in the presence of pICln.
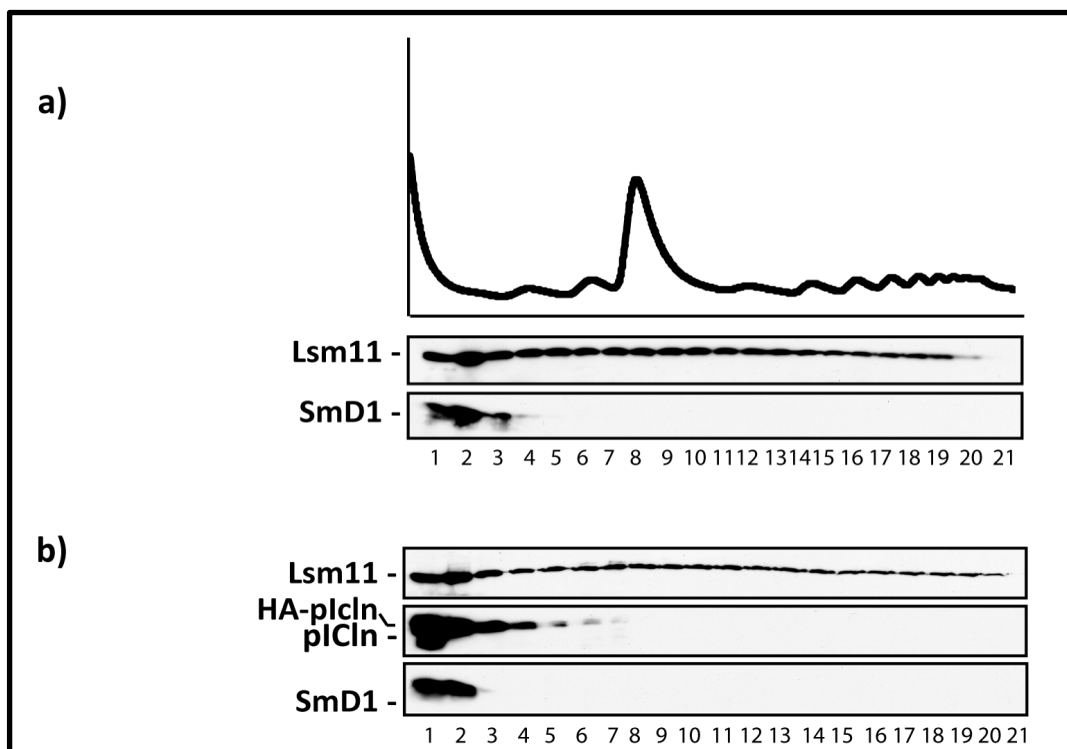
Figure 4.13. Formation of the cognate heterodimer is essential for the release of the heterodimer.

Extracts from transfected cells were fractionated on 5%-45% sucrose gradient. Western blot analysis was performed with anti-HA antibody. a) Cells transfected with Lsm11 and SmD1. Co-transfected SmD1 was not able to form a heterodimer with Lsm11 and consequently did not co-sediment with Lsm11 and ribosomes. b) Cells transfected with Lsm11, SmD1 and pICln. Co-transfected pICln was not able to release Lsm11 in the absence of Lsm10, which mediates the interaction of Lsm11 with pICln.

4.5 Transfected Lsm10 and Lsm11 integrate into the 6S- and 20S complexes

In the preceding sections, I investigated the initial phase of Lsm10 and Lsm11 proteins "handling" by the cellular translation machinery. In the next step, I wished to address the question whether the pICln-mediated release of Lsm10/Lsm11 heterodimer from ribosomes is essential for subsequent steps in U7 snRNP assembly. In this case, one expects that released Lsm10/Lsm11 integrate into complexes equivalent to 6S- and 20S assembly intermediates identified for spliceosomal snRNPs.

To address this issue, I transfected the cells with plasmids encoding Lsm11 alone, both Lsm10 and Lsm11 or Lsm10, Lsm11 and pICln. Extracts from these cells were then fractionated on a 10%-30% galactose gradient. In agreement with my previous observations (see figures 4.4b and 4.5b), a fraction of transfected Lsm11 was integrated into a complex of approx. 20S, whereas the majority of the protein was found in heavy fractions, containing polysomes (figure 4.14a). Co-transfection of Lsm10 led to an increased incorporation of Lsm11 into the complex with an S value of 20S (figure 4.14b). Only upon co-transfection of pICln, both Lsm10 and Lsm11 were integrated into assembly intermediates with S values of 6S and 20S (figure 4.14c).

Next, I investigated the specific composition of the complexes formed by pICln and Lsm10/11 upon release from the ribosomes. Because Lsm10 and Lsm11 were found in complexes with S values of 6S- and 20S, it was plausible to assume that they formed assembly intermediates equivalent to the 20S- and 6S complexes identified in the assembly of spliceosomal snRNPs. For this purpose, I size-fractionated the extract from Lsm10/Lsm11/pICln co-transfected cells and pooled the fractions of 6S- and 20S peaks separately (see Figure 4.14c). Since Lsm11 was C-terminally Flag-tagged, an IP with anti-Flag antibody allowed the affinity-purification of Lsm11 containing complexes and their analysis by SDS-PAGE and Western blotting. As shown in figure 4.14e, the 6S complex contained pICln, Lsm10, Lsm11 and SmE/SmF/SmG but was devoid of SmD1/SmD2. This result proved the existence of a U7- specific 6S complex, where SmD1 and SmD2 from the spliceosomal 6S complex are replaced by Lsm10 and Lsm11, respectively.
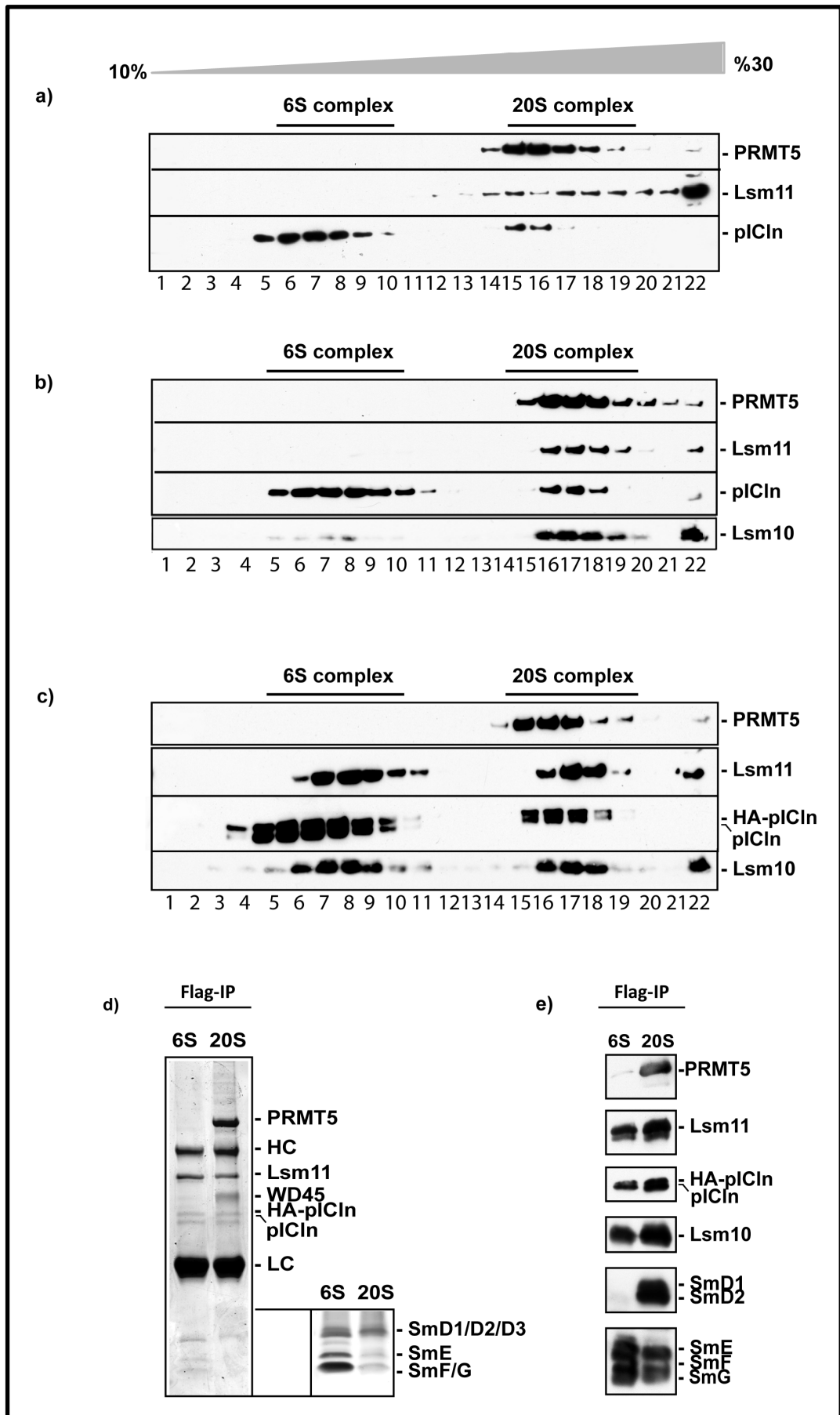
Figure 4.14. Released Lsm10/Lsm11 heterodimer is incorporated into the 6S- and 20S complexes.

Cells were transfected with a) HA-Lsm11-Flag, b) HA-Lsm11-Flag/HA-Lsm10 and c) HA-Lsm11-Flag/HA-Lsm10/HA-pICln. d) Coomassie stained gel of the anti-Flag purification. The lower part of the gel was also silver stained. e) Western blot analysis of the anti-Flag immunoprecipitation.

The purified 20S complex contained PRMT5, pICln, WD45, Lsm10, Lsm11 and all the common seven Sm proteins. I assumed that there exist two populations of PRMT5 complexes in the cell. One population contains all seven common Sm proteins and the other one contains the U7 snRNP specific Lsm/Sm proteins. Oligomerization of the two populations via PRMT5 can explain the existence of all the Lsm/Sm proteins in the purified 20S complex. Moreover, mass spectrometry analysis of the purified 6S- and 20S complexes confirmed the existence of an U7-specific 6S complex (Index 1).

Altogether, these results indicated that transfected Lsm10 forms a heterodimer with Lsm11 on the ribosome. The dimer then dissociates from the ribosomes in the presence of pICln and incorporates into the assembly intermediate 20S complex and then 6S complex, the latter being the dissociation product of 20S complex.

4.6 PICln functions as a general release factor for Lsm and Sm proteins

All members of Lsm/Sm protein family share similar structural features and assemble into toroidal complexes. This fact motivated me to speculate that the function of pICln on the ribosome is not restricted to Lsm10/Lsm11 but may also apply for the canonical Sm proteins (i.e. SmD1, D2, D3, B, E, F, and G). To test this hypothesis, I studied the role of pICln in the formation of the SmD1/SmD2 heterodimer and its release from the ribosome.

4.6.1 Role of pICln in the release of exogenous transfected SmD1/SmD2

To test for a potential role of pICln in the release of canonical Sm proteins, I knocked down pICln in 293T cells using siRNAs. This resulted in the reduction of the level of endogenous pICln to up to 80% (4.15a). Afterwards, the control and knock-down cells were transfected with plasmids coding HA-SmD1 and HA-SmD2 proteins and extracts prepared from these cells were fractionated on 5%-45% sucrose gradients. A significant increase in the association of either Sm protein with the ribosomes could be observed upon knock down of pICln (figure 4.15b). Co-transfection of pICln with SmD1/SmD2 led to the release of both proteins from the ribosomes, very similar to what was shown for the Lsm10/Lsm11 heterodimer (figure 4.15c). Together, these results strongly suggested that not only the Lsm10 and Lsm11, but also the canonical Sm proteins D1 and D2 require the assembly chaperone pICln in order to be released from the ribosome post-translationally.

4.6.2 Role of pICln in the release of endogenous SmD1/SmD2

The results presented above were based on transfection studies and hence overexpressed proteins. It was therefore important to investigate whether these results also applied to endogenous SmD1/SmD2 proteins. To address this issue, I knocked down endogenous pICln using siRNA. Extracts derived from these cells were then fractionated by gradient centrifugation followed by Western blot analysis with anti-SmD1/SmD2 antibodies. Interestingly, co-sedimentation of SmD1/SmD2 with the ribosomes was observed in both control and pICln depleted cells (figure 4.16a). Nevertheless, the amount of SmD1/SmD2 that co-sedimented with ribosomes increased significantly upon pICln depletion (figure 4.16a, +siRNA). Interestingly, over-expression of pICln led to the shift of Sm proteins to lighter fractions in both control and knock-down cells (figure 4.16b).
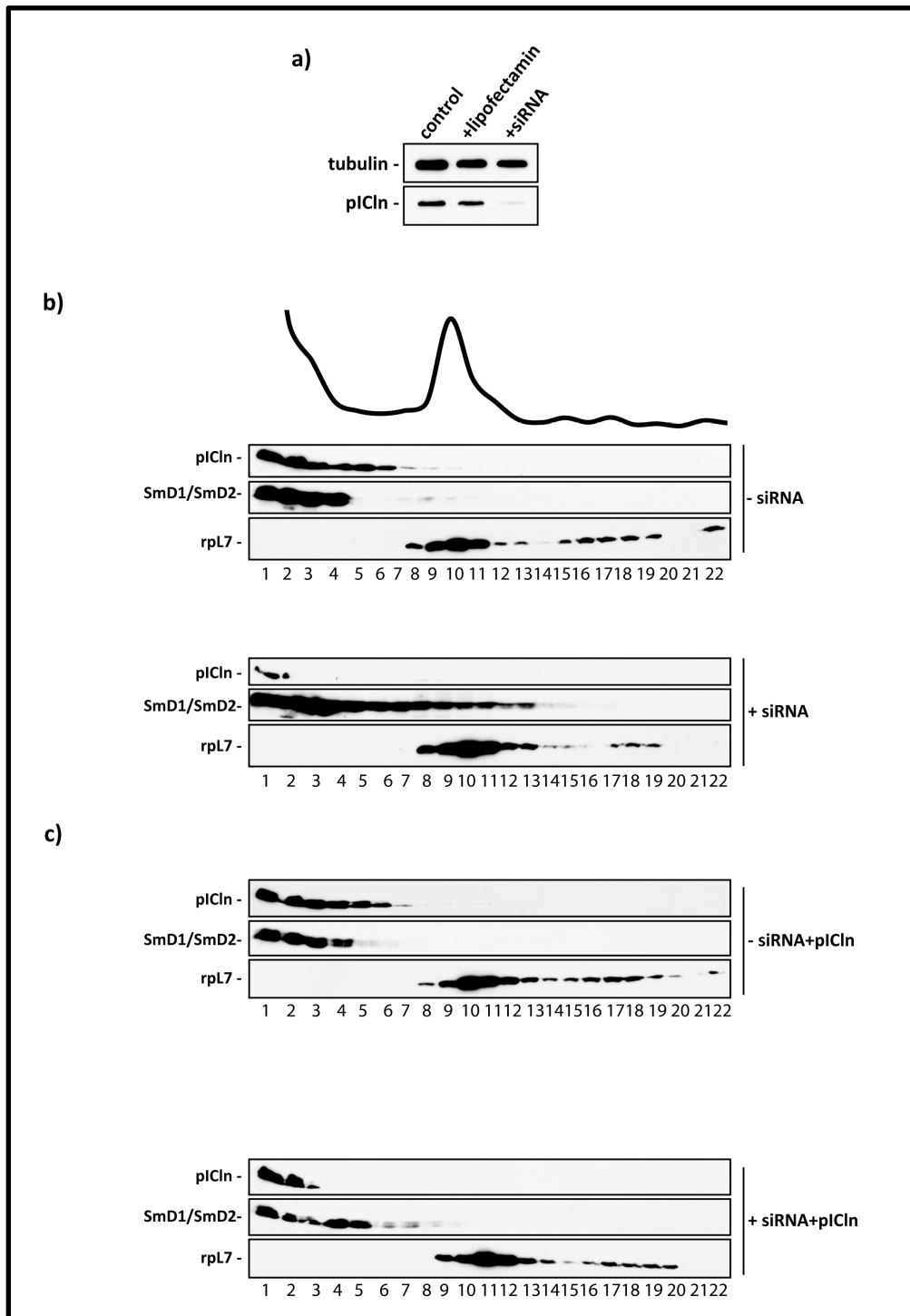
Figure 4.15. The role of pICln in the release of transfected SmD1/SmD2 heterodimer.

a) PICln was knocked down in 293T cells. Western blot analysis was performed with antibodies against pICln and tubulin as a loading control, b) Control and knock-down cells were transfected with plasmids coding HA-SmD1 and HA-SmD2. Extracts were fractionated on a 5%-45% sucrose gradient followed by Western blot analysis with anti-HA, anti-pICln and anti-rpL7 antibodies. Reduction in the level of pICln resulted in the shift of SmD1 and SmD2 to the ribosomal fractions. c) Co-transfection of pICln released the proteins from the ribosomes.
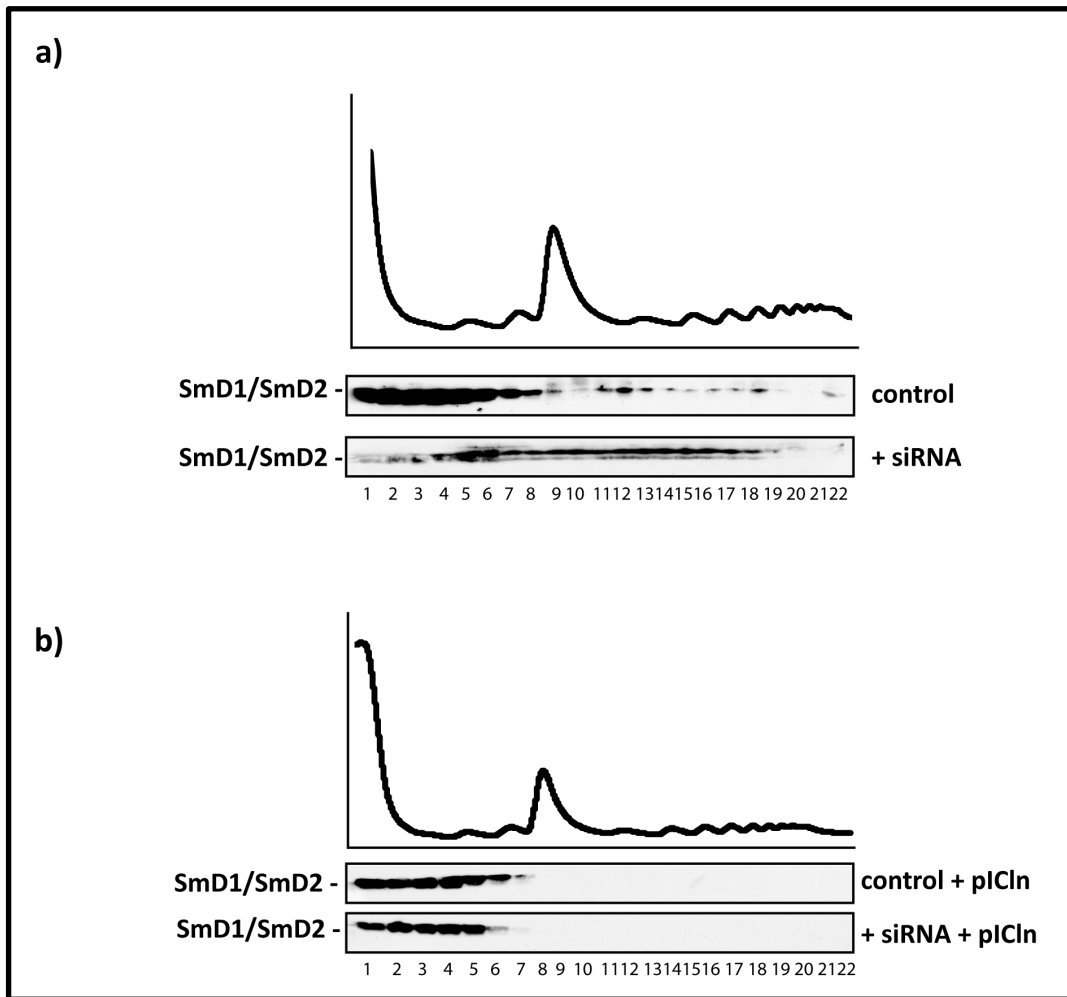
Figure 4.16. The role of pICln in the release of endogenous SmD1/SmD2.

a) Extracts from control and knock-down cells were fractionated on 5%-45% sucrose gradient and blotted against anti-SmD1/SmD2 antibodies. An increase in the association of SmD1/SmD2 with ribosomes upon knock down of pICln could be seen. b) Control and knock-down cells were transfected with pICln. In both cases, exogenous pICln released the endogenous SmD1/SmD2 heterodimer from the ribosomes.

The data above suggested that reduction in the level of pICln led to a co-sedimentaion of SmD1/SmD2 proteins with ribosomes and addition of pICln to the system was able to reverse the observed co-sedimentation. In addition, I was able to reconstitute the release of endogenous SmD1/SmD2 as well as SmB/B' from ribosomes in a similar fashion as shown for Lsm10/Lsm11. For this purpose, ribosomes were pelleted from control and knock-down cells and analyzed by Western blotting for the presence of SmD1 and D2. As shown in Fig. 4.17, these Sm proteins could be efficiently

detected in the ribosomal pellets of both control and knock-down cells. To test whether the ribosome-bound Sm proteins can be released by pICln, ribosomal pellets were incubated at 4^0 C in the absence of pICln, 37° C in the absence of pICln and additionally at 37° C in the presence of pICln. Then ribosomes were pelleted for the second time through a sucrose cushion to separate the ribosomal bound proteins from the dissociated ones. Interestingly, the endogenous SmD1/SmD2 heterodimer as well as SmB/B' were released from ribosomes at 37° C and only in the presence of pICln (figure 4.17).

In sum, these experiments strongly suggested that similar to the Lsm10/Lsm11 heterodimer, pICln is essential for the release of fully translated SmD1/SmD2 and SmB/B' proteins from ribosomes.
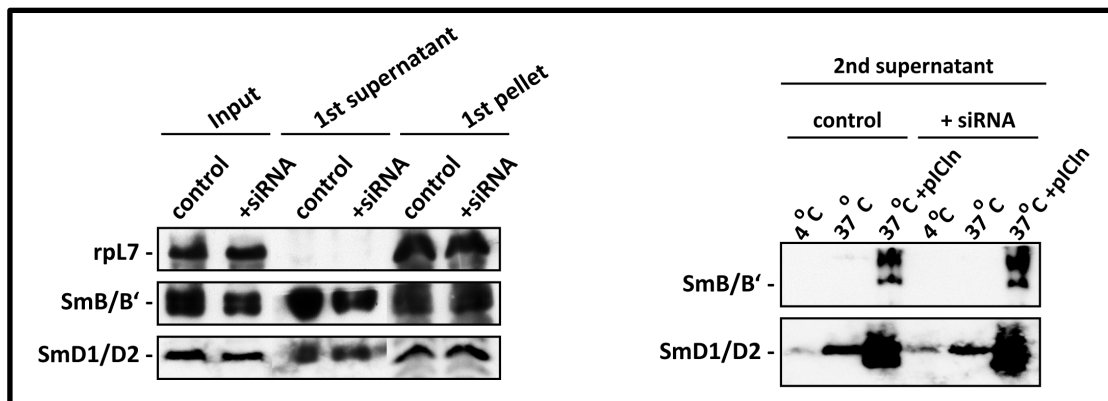
Figure 4.17. Reconstitution of endogenous SmD1/SmD2 heterodimer release from the ribosomes.

Ribosomes were pelleted from 293T cells through a sucrose cushion. Western blot analysis was performed with antibodies against rpL7, SmB/B', SmD1 and SmD2. Endogenous SmD1, SmD2 and SmB/B' were pelleted with the ribosomes in the control as well as knock-down cells (left). The supernatant of the second centrifugation is shown (right). The release of SmD1, SmD2 and SmB/B' proteins occurred only in the presence of pICln at 37° C.

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5   Discussion

5.1   Assembly of RNA-protein complexes

The formation of RNA-protein complexes (RNPs) with high accuracy imposes a high logistical demand on cells. The RNA and protein parts of these macromolecular complexes are synthesized in different cellular compartments. Thus, to ensure the accurate assembly of RNPs, the production of both components has to be synchronized. Moreover, subcellular transport events must ensure that both RNA and protein components are present in the same cellular compartment for assembly to initiate. Post assembly, an additional subcellular transport event carries most RNPs to their site of function (Chari and Fischer, 2010).

The fact that RNA and protein counterparts of RNPs are synthesized in different subcellular compartments brings up an additional problem for accurate RNP assembly. Within cells the total concentration of all macromolecules has been estimated to be up to 300 mg/ml. In such a "crowded" milieu, the random encounter of specific subunits by diffusion is significantly reduced. The newly synthesized protein subunits thereby collide with both the less abundant cognate RNA/protein, as well as the more abundant non-cognate RNA/protein molecules. In this situation, molecular crowding kinetically favors non-specific RNA-protein interactions leading to the production of dead-end as well as toxic products (Hall and Minton, 2003; Minton, 2000, 2001). This problem is overcome when the newly synthesized protein subunits are initially held in an inactive form by associating with assembly chaperones prior to assembly.

Small nuclear ribonucleoproteins (snRNPs) of the spliceosome are a resourceful system to study the assembly of RNA-protein complexes as their composition, structure and bi-phasic biogenesis pathway have been well studied. The assembly of these particles is an example of the situation, where synthesis of RNA and proteins appear not to be synchronized. In the cytoplasm, Sm proteins are found as hetero-oligomers that are unable to bind each other before newly synthesized

snRNA is exported from the nucleus. However, they might get involved with other abundant, non-specific RNAs such as tRNAs. To overcome this pitfall, the essential assembly chaperone pICln binds to Sm proteins and keeps them in an inactive form. Re-activation of Sm proteins is accomplished by the other essential assembly factor, the SMN-complex, which assembles the snRNP. After assembly the mature snRNP is transported to the nucleus, its site of function (Chari et al., 2008).

The recent years have led to the identification of a unique system for spliceosomal snRNP assembly and the elucidation of many mechanistic steps in this process. However two important questions, which I have addressed in my thesis, have remained unanswered: 1) How and when are Sm proteins transferred to the assembly machinery after their translation?, and 2) what is the mechanistic basis for the assembly of snRNPs, which contain variations in their RNA and protein counterparts?

5.1.1 The Assembly of the U7 snRNP

The U7 snRNP contains a unique Sm core, consisting of a distinct set of Sm and Lsm proteins. In the Sm core of the U7 snRNP, Lsm10 and Lsm11 replace SmD1 and SmD2 from the canonical Sm core of spliceosomal snRNPs (Schumperli and Pillai, 2004). A key question I could address in my thesis was, how this unique core is formed in vivo. I was able to show that U7 snRNP-specific 6S- and 20S assembly intermediates exist. The 6S complex in this case contains Lsm10, Lsm11, SmE, SmF and SmG but is devoid of SmD1 and SmD2. However, the 20S complex comprises of Lsm10, Lsm11, SmE, SmF, SmG, SmD3 and SmB (Figures 4.2 and 4.14). Although the Lsm10/Lsm11 heterodimer is not methylated (Azzouz et al., 2005), it is very interesting that they are found in the 20S complex. I speculate that the PRMT5 complex, in addition to the methylation of Sm proteins, functions as a platform for the formation of the 6S complex. Formation of the 6S intermediate on this platform could increase the efficiency and specificity of the reaction. Binding of pICln to the Lsm10/Lsm11 or the SmD1/SmD2 heterodimer appears to form the initial seed for the formation of individual 20S- and 6S assembly intermediates. SmD1/SmD2/pICln and Lsm10/Lsm11/pICln are likely to bind to the PRMT5 complex to form the

canonical- or U7- specific 20S complexes, respectively. Addition of SmE/SmF/SmG hetero-oligomer to SmD1/SmD2/pICln and Lsm10/Lsm11/pICln would then lead to the formation of the canonical- or U7- specific 6S complexes, respectively. In agreement with previous studies, I confirmed that U7 snRNP assembly occurs via a pathway that is very similar to that of spliceosomal snRNPs. This pathway involves the PRMT5 complex in the early and the SMN complex in the late phase. Consequently, I assumed that the recruitment of Lsm/Sm proteins into pICln containing assembly intermediates rather than a difference in the trans-acting assembly factors is the basis for the specificity of the assembly reaction towards the U7- or spliceosomal snRNP.

5.2 An unexpected association of Lsm proteins with the ribosome

At the beginning of this thesis it was entirely unknown how Lsm and Sm proteins gain access to this pathway. Specifically, the question remained unanswered whether newly translated Lsm/Sm proteins are released from the translation machinery as single proteins and subsequently bound by pICln by diffusion driven collision. An alternative mechanism was that Lsm and Sm proteins form already functional assembly intermediates with pICln at the ribosome and hence are never released as "unprotected" entities. The molecular dissection of the assembly of the U7 snRNP clearly supported the latter assumption. Exogenous, transfected Lsm11 was found associated with ribosomes and the co-transfection of Lsm10 with LSm11 resulted in the co-sedimentation of both proteins with ribosomes (Figure 4.5). Additionally, I was able to show that association of Lsm10 and Lsm11 with ribosomes was specific and occurred after translation termination (Figures 4.6, 4.7 and 4.9a).

Proteins are generally released from the ribosomes after translation termination and subsequently incorporate into their final complexes. This is the first example of a system, where the proteins are retained on the ribosomes upon completion of translation by yet unknown mechanism.

Based on the findings that Lsm11 does not bind to the ribosome through electrostatic or hydrophobic interactions (Figure 4.9b and c), I speculate that a putative factor yet to be identified could retain these proteins at the translation machinery. Altogether, I concluded that newly synthesized Lsm10 and Lsm11 form a heterodimer and remain bound to ribosomes upon the completion of translation. A possible explanation for this unexpected behavior is that ribosome-bound Lsm proteins might be shielded from non-specific interactions in the crowded environment of the cell.

5.2.1 The Assembly chaperone, pICln, releases Lsm10/Lsm11 from the ribosomes

The finding that newly synthesized Lsm10 and Lsm11 remain bound to the ribosome further favors the hypothesis of a coordinated transfer from the translation to the assembly machinery. At the next step, I aimed to determine the factor triggering the dissociation of the heterodimer from the ribosome. pICln was previously documented as the protein to bind earliest in the assembly pathway to Sm proteins. Also it has been documented as a canonical Sm proteins specific assembly chaperone. It therefore appeared plausible that pICln might represent the factor triggering the dissociation of the heterodimer from the ribosome. Interestingly, co-transfected pICln efficiently released the Lsm10/Lsm11 heterodimer from the ribosomes (Figure 4.10). Moreover, reconstitution of the release in vitro and in the presence of pICln confirmed that pICln alone is both necessary and sufficient for the release of the heterodimer (Figure 4.11a and b). In favor of this observation, my initial studies showed that the PRMT5 complex co-purifies (index 2) and components of SMN complex co-fractionate with ribosomes upon pICln depletion (index 4), which would facilitate the transfer of Lsm proteins to the PRMT5- and SMN complex. This direct transfer of Lsm10 and Lsm11 to the assembly machinery, which is mediated by the assembly chaperone pICln ensures that they remain shielded from engaging in non-specific reactions prior to insertion into the assembly line. Notably, this pICln-mediated release reaction is productive for subsequent assembly. My results indicated that the Lsm10/Lsm11/pICln hetero-

trimer released from the ribosomes is incorporated into the U7-specific assembly intermediates, 20S- and 6S- complexes respectively (Figure 4.14).

5.3 The cognate Lsm heterodimer are formed on the ribosome

Within cells, only hetero-oligomers of SmD1/D2, SmD3/B, SmE/F/G and Lsm10/11 are found. In the course of my thesis, one experimental observation indicated how the specificity for heterodimer formation might be ensured. The release of Lsm11 from the ribosome not only required the assembly chaperone pICln but also its cognate counterpart Lsm10. Furthermore, SmD1, the counterpart of Lsm10, which does not form a heterodimer with Lsm11 was not able to mediate the release of Lsm11 in concert with pICln. Thus the formation of a cognate heterodimer is a prerequisite, but not sufficient for the dissociation of newly translated Lsm proteins from the ribosome. However, their release occurs only in the presence of pICln (see Figure 4.13).

Further investigations enabled me to understand the mechanism of the heterodimer formation on the ribosomes. When Lsm11 is expressed alone, protein co-sediments with the ribosomes. In contrast, when Lsm10 protein is expressed in the absence of Lsm11, the protein is found as a free protein or a component of the assembly intermediate, the 6S complex (see Figures 4.4a and 4.5a). There are two explanations for this observation: 1) This occurs due to the lower concentration of endogenous Lsm10 in comparison to endogenous Lsm11. Transfected Lsm10, therefore, integrates into the 6S- and/or 20S complexes along with endogenous Lsm11. 2) Lsm10 is not able to have a stable and long-lived interaction with pICln in the absence of its interacting partner, Lsm11. Only upon binding to Lsm11, the complex of Lsm10 and pICln is stable. Consequently, in the absence of Lsm11, Lsm10 is found as a single protein or in a short-lived complex with pICln in the cytoplasm.

In contrast to Lsm11, the co-sedimentation of Lsm10 with ribosomes occurs only upon co-transfection with Lsm11 (Figures 4.4c and 4.5c). Two possible models are

able to explain this observation. The pre-formed complex of Lsm10 and pICln is stable upon association with Lsm11 on the ribosome (Figure 5.1a). However, co-sedimentation of both proteins with ribosomes in cells transfected with Lsm10 and Lsm11 favors yet another scenario, where Lsm10 and Lsm11 interact with each other prior to binding to pICln (Figure 5.1 b).

Under steady state conditions, pICln is an abundant protein in cells. Co-transfection of Lsm10 and Lsm11 or knock down of pICln leads to a condition, where pICln is limiting in comparison to Lsm protein heterodimers. It is unlikely that such an extreme situation occurs under steady state conditions in the cell. As a consequence, I assume that both hypotheses depicted in figure 5.1 and described in the previous paragraph, are possible and warrant further investigation. No matter which model turns out to be correct, either case leads to the production of the trimeric Lsm10/11/pICln complex that is released from the ribosomes and incorporated into the assembly intermediates.

5.4   Formation of Sm protein hetero-oligomers

The experiments presented in this thesis suggest that Lsm10/Lsm11 first encounter pICln on the ribosome and are handed over to the PRMT5/SMN assembly line upon dissociation. This finding favors a scenario in which the specificity in the assembly of spliceosomal- and U7 snRNPs is ensured at a very early stage. One prediction of this model is that spliceosomal Sm proteins would follow a similar route. I verified this by investigating the sedimentation pattern of both transfected and endogenous SmD1 and SmD2 under conditions where pICln level were reduced by RNAi. Interestingly, both exogenous and endogenous SmD1 and SmD2 are associated with ribosomes under pICln deficiency as is the case for Lsm10 and Lsm11 (see Figures 4.15b and 4.16a). Likewise to the situation with the Lsm10/11 heterodimer, pICln elicits the release of the SmD1/D2 heterodimer, both in vitro and in vivo (Figures 4.15c, 4.16b and 4.17).
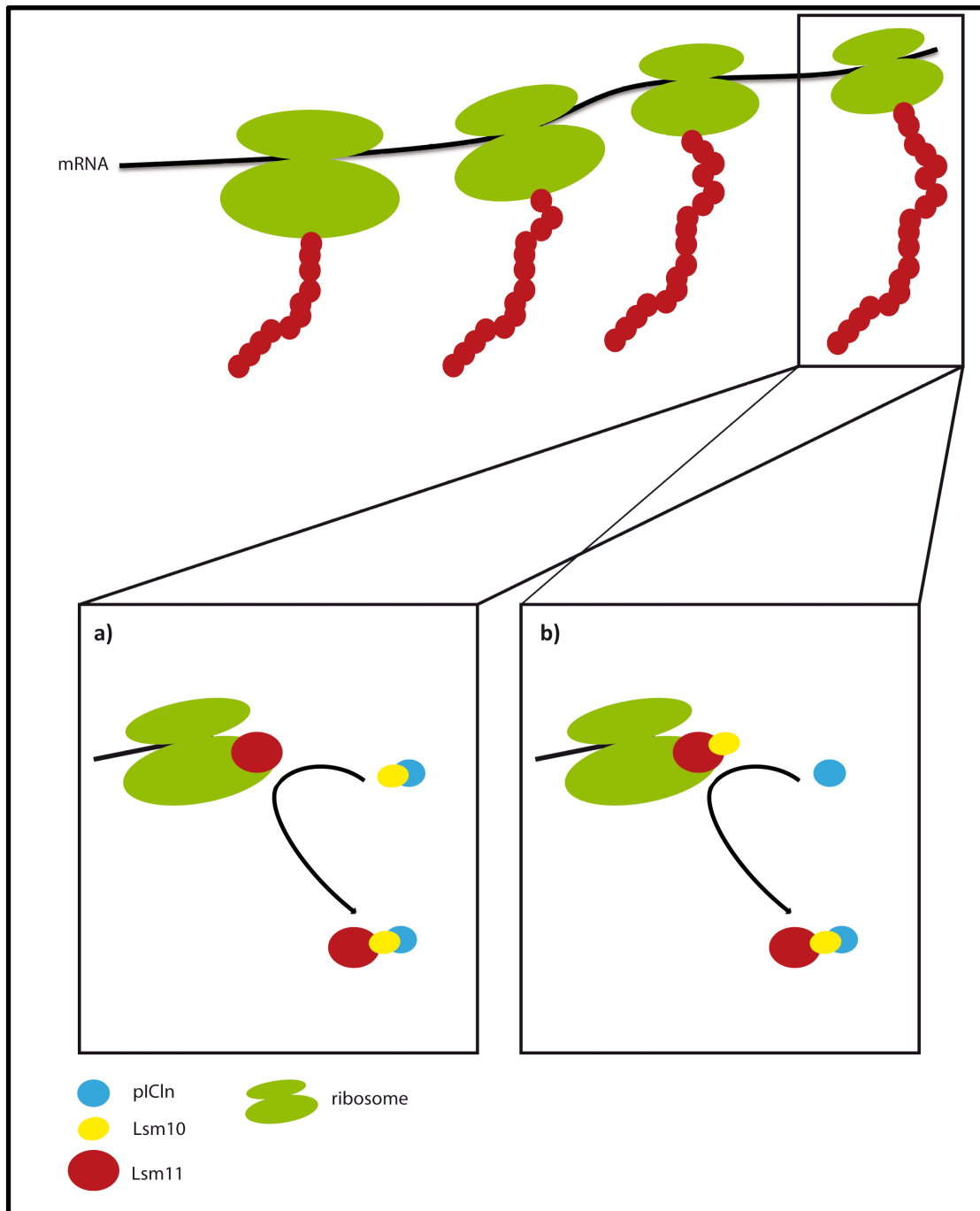
5.1. Proposed model for the formation and release of Lsm protein heterodimer from the ribosome

In this model Lsm11 is translated and released from the exit tunnel, but does not dissociate from the ribosomes. a) In this model, second member of the heterodimer, Lsm10, initially interacts with pICln. This complex then interacts with Lsm11 and triggers the dissociation. b) In this model, two Lsm proteins interact with each other on the ribosome prior to association with the release factor, pICln.

Accordingly, I conclude that cells assure the formation of the cognate heterodimer of Lsm/Sm proteins on the translational machinery as a universal mechanism. Only a cognate heterodimer is able to dissociate from the ribosomes by the action of pICln. For the first time, I have shown that Lsm/Sm proteins interact with their chaperone immediately upon completion of translation on the ribosomes and the assembly chaperone is employed as a release factor for Lsm/Sm protein heterodimers. Early employment of the assembly chaperone, pICln, assures the release of the cognate heterodimer and circumvents the need for diffusion-limited encounter of pICln with Lsm/Sm proteins in the cytoplasm. There is yet another principal conclusion from this observation. By employing pICln for the release, cells avoid using two different factors, one for the release of Lsm/Sm protein heterodimers and the other as an assembly chaperone for Lsm/Sm proteins.

5.5   Quality control check-point on the ribosomes

An immediate question that arises from the scenario described above is what happens if a cognate heterodimer is not formed on the ribosomes. Likewise, one may raise the question what happens when the heterodimer is formed but there is no pICln to elicit the release. Looking at cells over-expressing Lsm11 or Lsm10/Lsm11, I observed that protein(s) remained bound to the ribosomes in the absence of the interacting partner or pICln. I assumed these ribosomes would not be able to start a new round of translation, so I was expecting a reduction in the total translational efficiency of the cell. Performing metabolic labeling and Firefly luciferase assay, I analyzed the influence of Lsm11 or Lsm10/Lsm11 transfection on general translation of the cell. Interestingly, I did not notice any major reduction in translation (Figures 6.4 and 6.5). I speculate that the percentage of the cellular pool of ribosomes involved in translation of Lsm11 or Lsm10/Lsm11 is very small at any given time, so the possible effect on general translation (if there is one) would be marginal.

Further, I wondered what happens to the protein(s) caught on the ribosomes. I supposed the proteins, which were not able to dissociate from the ribosomes would be degraded and ribosomes would be released for a new round of translation. Mass

spectrometry analysis of the total cellular ribosomes under pICln deficiency showed an increase in the association of the proteasome machinery with the ribosomes (index 4). Consequently, I assumed that degradation of the Lsm/Sm proteins is carried out by proteasome. Although my initial studies, applying proteasome inhibitors, have not supported this assumption, the proteasome is still our best candidate to clear the proteins stalled on ribosomes (see Figure 6.3) and has been reported in the literature (Bengtson and Joazeiro, 2010; Defenouillere et al., 2013; Dimitrova et al., 2009).

5.6 Concluding remarks

In this thesis, I have elucidated several new aspects of the assembly of snRNPs: 1) The interaction of pICln with Lsm/Sm proteins on the ribosome guarantees the formation of cognate heterodimers early in the assembly pathway. 2) Employing pICln, cells prevent the Lsm/Sm proteins from pre-mature interactions in the cytoplasm and circumvent the necessity for their later diffusion-limited association with their partners as well as pICln in the cytosol. 3) SmD1/SmD2/pICln and Lsm10/Lsm11/pICln are core seeds for the formation of the canonical- and U7 snRNP-specific 20S- and 6S complexes, respectively. Therefore, they ensure the specificity of the assembly of two compositionally and functionally different snRNPs. 4) The SMN complex presumably plays an important role in the recognition of snRNAs. However, Lsm/Sm proteins themselves guarantee the specific association of spliceosomal- or U7- Sm core with the corresponding snRNAs.

In contrast to protein folding chaperones, the concept of the assembly chaperones is quite new to the field of biochemistry. Protein folding chaperones interact with their target proteins for a short time and assist them to reach their final, functional structure. Assembly chaperones interact with the target proteins and assist their integration into the higher ordered assemblies. Recent studies have characterized a group of protein folding chaperones, which mainly interact with components of macromolecular complexes, which are characterized by extensive protein/protein interactions and thus are prone to aggregation (Albanese et al., 2006; Willmund et al., 2013). These protein folding chaperones interact with the

nascent chains immediately upon emerging from the ribosomal exit tunnel and prevent them from intra- and inter-chain interactions and consequent aggregation. Interestingly, I have been able to uncover a new facet of the function of the assembly chaperone, pICln, which is conceptually very similar to the function of this group of folding chaperones. pICln is the first example of an assembly chaperone interacting with its substrates upon completion of translation on the ribosomes. I believe that similar to snRNPs, other macromolecular complexes utilize an assembly chaperone, which is functionally related to pICln. This chaperone mainly interacts with the subunits of the complex via hydrophobic interactions. Thus, the assembly chaperone prevents the subunit(s) from involvement in unwanted interactions and consequent aggregation. This assembly chaperone then guides the subunits to the site of the assembly and releases them only in the presence of remaining subunits. Upon assembly of the complex, the hydrophobic surfaces are involved in the interaction or buried inside the complex. Altogether, I assume that pICln is a member of a presumably growing list of assembly chaperones with similar functions in the assembly of cellular macromolecular complexes.

6 Outlook

In this section of my thesis, I have addressed several mechanistic aspects regarding the formation of Lsm/Sm protein heterodimers on the ribosome, namely the basis and significance. Although the following studies were not able to answer the existing questions, they have provided us with possible approaches for future investigations.

6.1 Factor-mediated association of Lsm11 with ribosomes

An interesting question, which remains to be answered, is the mechanism of association of Lsm/Sm proteins with the ribosome. As already mentioned in section 4.3, I was able to rule out that the association of Lsm/Sm proteins with ribosomes is mediated by electrostatic and hydrophobic interactions. Consequently, I had assumed that there exists a factor, which mediates the interaction of Lsm/Sm proteins with ribosomes. pICln most likely competes with this factor in the process of heterodimer release from the ribosomes. In this section, I will describe attempts to discover this factor.

6.1.1 Purification of Lsm10/Lsm11 complex with ribosomes

Initially, my experiments were based on the purification of the ribosome bound Lsm10/Lsm11. As Lsm11 is N-terminally HA-tagged, I performed the purification with anti-HA antibody. I hoped that identification of the co-purified proteins would lead me to this factor.

Extracts from transfected cells were fractionated on a 5%-45% sucrose gradient. Afterwards, polysomal fractions were pooled and ribosomes were precipitated by the addition of 10% polyethylenglycol 6000. The "PEG pellet" was then re-suspended and an IP performed. As shown in figures 6.1a- 6.1b, using this strategy I was able to purify the Lsm10/Lsm11 associated ribosomes. The purified complexes were then used for mass spectrometry analysis. Looking at the long list of identified proteins, I tried to simplify the process. I speculated that this factor would be most abundantly present in the purification from Lsm10/Lsm11 co-transfected cells. Transfection of pICln led to

the release of the heterodimer and consequently the ribosome interaction-mediating factor. But the existence of Lsm11 and Lsm10 in the Lsm10/Lsm11/pICln co-transfected cells, means that there are residual amount of the factor in the last lane as well. Using the "Scaffold 3" program, I looked for all the proteins common between Lsm10/Lsm11 and Lsm10/Lsm11/pICln co-transfected cells. This brought me to a list of 165 proteins (Index 2). Then I searched for proteins, which are found in a higher concentration in Lsm10/Lsm11 than Lsm10/Lsm11/pICln transfected cells. A list of these proteins has been provided in index 3. Although this procedure has provided me with a considerably shorter list, I still have a number of possible factors, which have to be studied carefully. Consequently, identification of the factor awaits further biochemical studies, which far exceed the scope of this thesis.
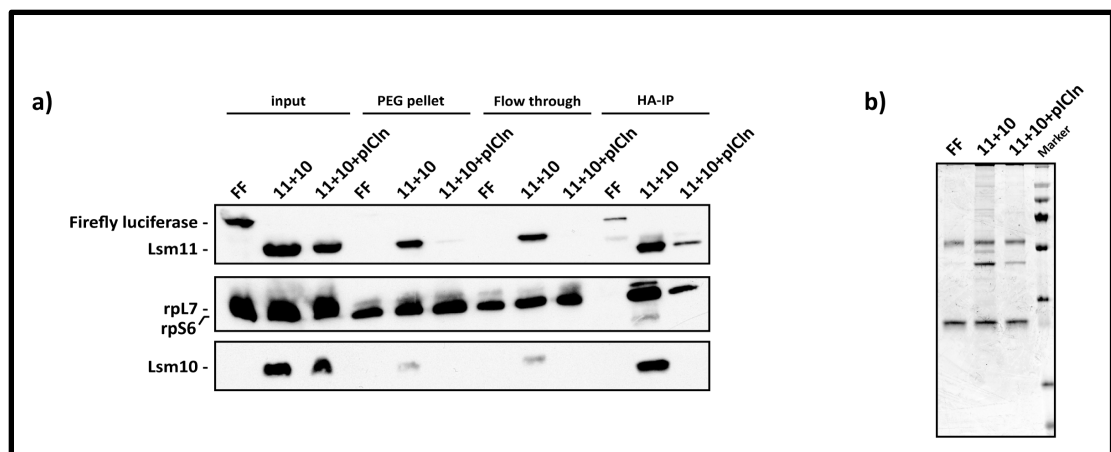
Figure 6.1. Purification of the Lsm10/Lsm11 complex with ribosomes

Extracts were prepared from cells transfected with FireFly luciferase (FF), Lsm10/Lsm11 or Lsm10/Lsm11/pICln. Immunoprecipitation was performed with the re-suspended PEG pellet. a) Western blot analysis was performed for the Input, PEG pellet, Flow through of the IP and the immunoprecipitates (HA-IP). b) SDS-PAGE of the immunoprecipitates.

6.1.2   Mass spectrometry analysis of total ribosomes of the cells

To find the factor mediating the association of Lsm/Sm proteins with ribosomes, I pursued yet another strategy. This was based on the assumption that the presumed factor will accumulate on ribosomes, when pICln is limiting in cells. Therefore, pICln was knocked down using siRNA and then ribosomes were pelleted from the extracts of

either control or knock-down cells (first pellet). The first pellets were re-suspended and equal amounts (equal units of OD_{260}) of first pellets were layered on sucrose cushions. Ribosomes were pelleted under high salt or low salt conditions (second pellet) (figure 6.2). Ribosomal pellets were re-suspended and used for mass spectrometry analysis (Index 4).
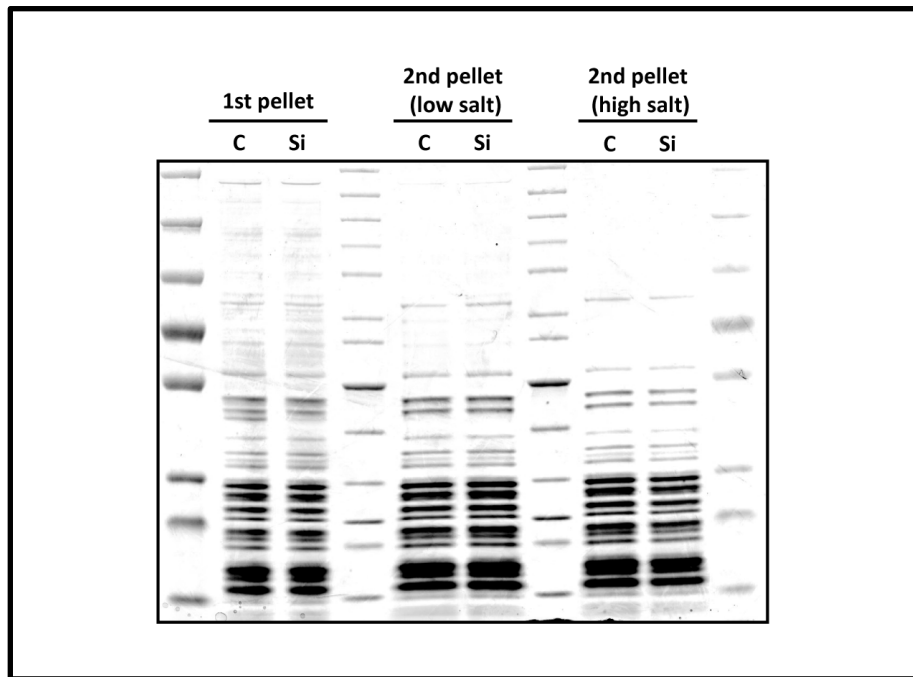
6.2. Ribosomal pellets from HEK 293T cells.

Ribosomes from control (C) and siRNA (against pICln)-transfected (Si) cells were pelleted on a 45% sucrose cushion. First pellets were re-suspended and pelleted for the second time under low salt or high salt condition.

Looking at the provided short list (index 4), one is able to clearly see an increase in the association of the ubiquitin-proteasome machinery with ribosomes upon reduction in the level of pICln. This increase indicates an essential demand for protein degradation on the ribosomes. I assume the elevation in the activity of proteasome is related to the increased ribosome- associated Lsm/Sm proteins. This assumption will be tested in section 6.2.

Interestingly, I observed an increase in the association of Gemin 5 and SMN, components of the SMN complex, with the ribosomes. The presence of these proteins in the vicinity of the ribosomes could provide a fast and accurate transfer of the

Lsm/Sm proteins onto the assembly machinery. The increase in the level of these proteins on the ribosomes upon reduction in the level of the release factor pICln might indicate a compensatory mechanism.

Additionally, several Sm proteins show higher levels on the ribosomes upon pICln knock down. In some cases the ribosomal association of the protein is salt sensitive while in the others it is not.

6.2 A novel quality control step

As discussed in previous chapters, I have been able to illustrate a new step in the biogenesis of Lsm/Sm cores. My results show that Lsm/Sm protein heterodimers form on the ribosome and pICln releases the heterodimers, which are later incorporated into the assembly intermediate, the 20S complex. Though pICln is an abundant protein, I asked "what happens to Sm proteins on the ribosomes in the absence of pICln?"

Based on my observations in chapter 6.1.2, the initial hypothesis was that Lsm/Sm proteins on the ribosomes will be immediately released by pICln and if not, degraded by the proteasome. To address this assumption, I transfected the cells with HA-Lsm11 and treated them with two different proteasome inhibitors prior to preparation of the extract. "MG-132" is a cell-permeable and reversible inhibitor, which effectively blocks the proteolytic activity of the 26S proteasome complex. Additionally it inhibits NF-κB activation and promotes apoptosis. "Bortezomib" binds the catalytic site of 26S proteasome specifically and prevents the degradation of the proteins by this complex.

As shown in figure 6.3, treatment of the cells with the aforementioned inhibitors had no effect on the level of ribosome-bound Lsm11. There is the possibility that cells have already degraded the majority of ribosome-bound Lsm11, so what I observed on the ribosomes is the basal level of ribosome-bound Lsm11 even in the presence of active proteasomes. The other possibility is that the proteasome does not play any role in the clearance of ribosome-bound Lsm proteins. We should also keep in mind that the results shown here are initial observations. One should use variable concentration of inhibitors or different incubation times for the treatment of cells with inhibitors to achieve the most accurate results. Another essential point is the extent of over

expression of proteins in cells prior to addition of proteasome inhibitors. One can only be sure about the role of proteasome in this quality control step only after including the above-mentioned parameters.
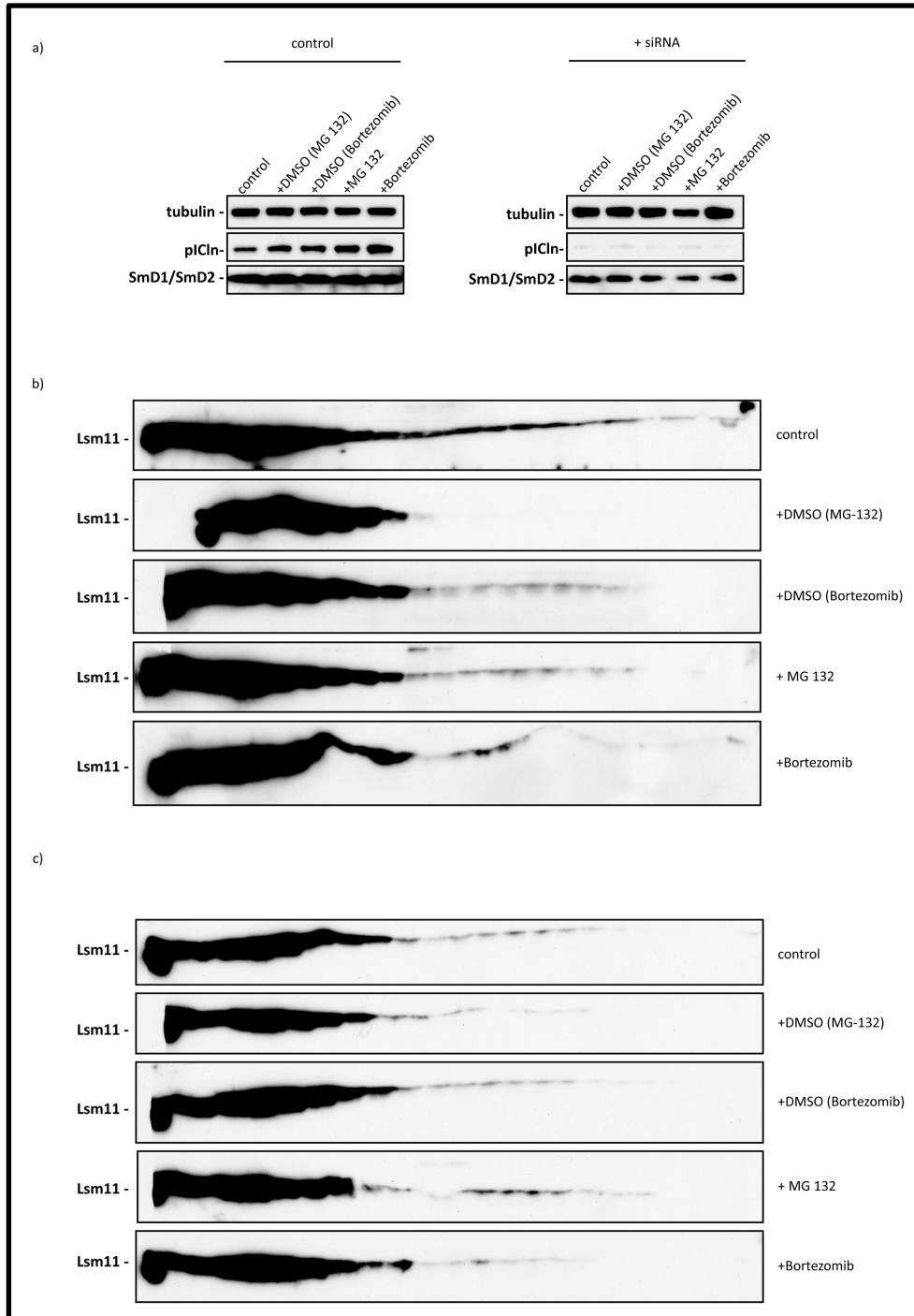
Figure 6.3. Effect of proteasome inhibitors on the association of Lsm11 with the ribosomes.

Extract from 293T cells transfected with HA-Lsm11-Flag was used in this experiment. a) Input of control- (left) or siRNA-transfected- (right) cells were checked by Western blotting. Cells were

treated with DMSO used for solving MG-132 (DMSO (MG-132)), DMSO used for solving Bortezomib (DMSO (Bortezomib)), MG-132 and Bortezomib, respectively. b) Sedimentation of Lsm11 in control cells was checked by Western blotting against anti-HA under different conditions. c) Sedimentation of Lsm11 in siRNA-transfected cells was checked by Western blotting against anti-HA under different conditions.

6.3   pICln shortage in the cell and general translation

As already shown, reduction in the level of pICln increases the association of Lsm/Sm proteins with the ribosomes. I presumed these ribosomes would not able to participate in and/or complete a new round of translation. Consequently, I expected that co-transfection of Lsm10/Lsm11 or knock down of pICln should lead to a reduction in the repertoire of free ribosomes and therefore general translation. To study this assumption, I employed two different methods, which are explained in the following sections.

6.3.1   Luciferase assay

In this study, stable cell lines expressing firefly luciferase were used to test the effect of Lsm10/Lsm11 co-transfection on general translation. As shown in figure 6.4b, co-transfection of Lsm10/Lsm11 led to a reduction in translation of firefly luciferase and possibly general translation. I expected that co-transfection of pICln, which releases the Lsm proteins and thus sets the ribosomes free, would increase the translation of firefly luciferase to the level of translation in control cells. In contrast, co-transfection of pICln, similar to Lsm10 and Lsm11, further reduced the translation of luciferase (figure 6.4b).

It seems that expression of any exogenous protein influences the general translation by engaging part of the translation machinery. It is hard to exclude this effect from the effect of pICln deficiency on translation. On the other hand, any impact resulting from pICln shortage might be dealt with within a very short time frame, which is very hard to capture by available biochemical approaches.
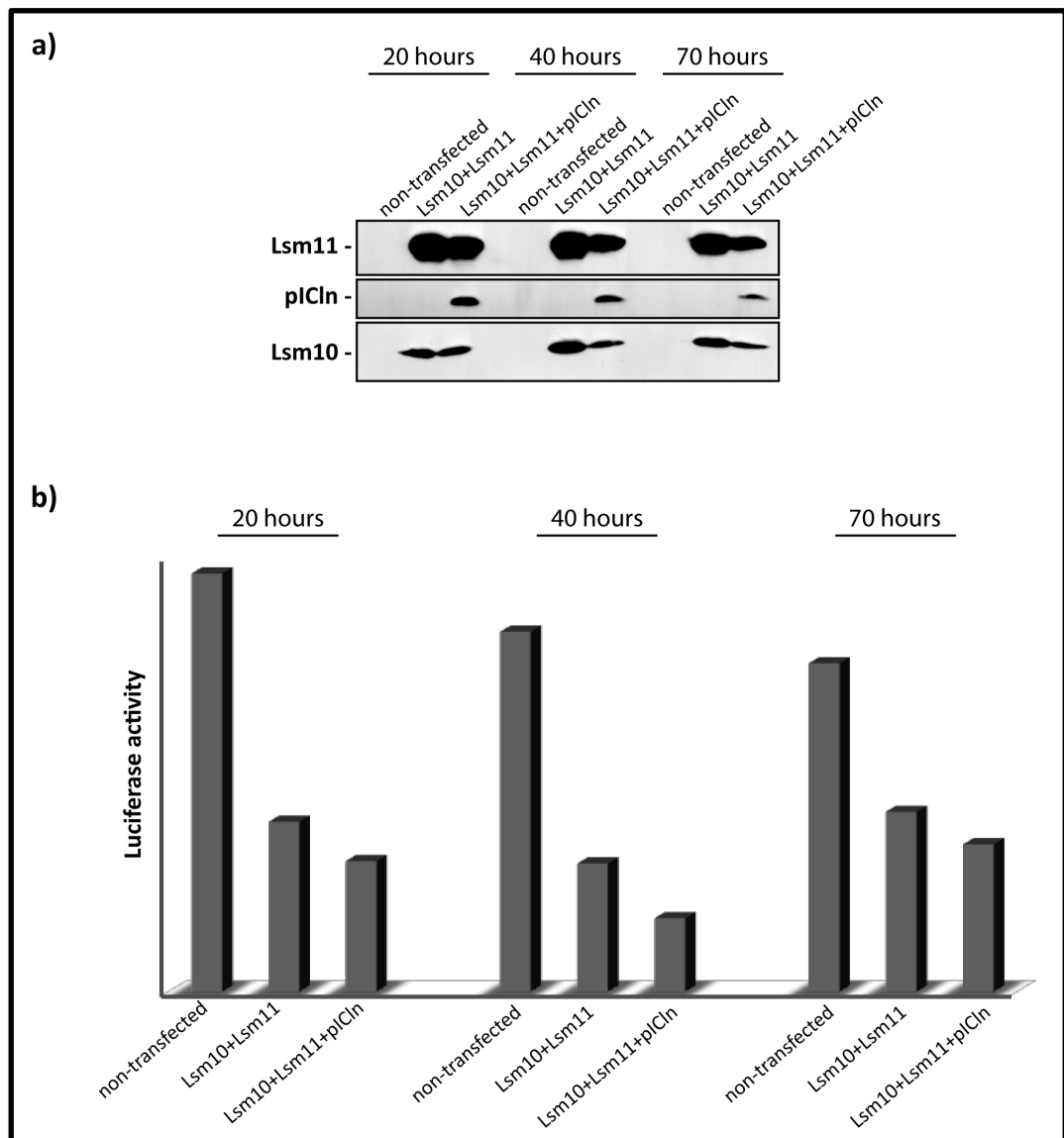
Figure 6.4. Luciferase assay.

Non-transfected, Lsm10/Lsm11- or Lsm10/Lsm11/pICln transfected HEK 293T cells were harvested 20 hours, 40 hours or 70 hours post transfection. Extracts were prepared and firefly luciferase activity was measured. a) Expression of proteins was confirmed by Western blot analysis with anti-HA antibody. b) The level of the activity of firefly luciferase has been shown in each sample.

6.3.2 Metabolic labeling

Labeling of proteins using radiolabeled amino acids enables us to study the translation of the whole or specific proteome in cells under different conditions. Using this method, I aimed to determine the influence of the association of Lsm/Sm proteins

with ribosomes on the general translation efficiency. I performed metabolic labeling in non-transfected cells and cells transfected with Lsm10/Lsm11 or Lsm10/Lsm11/pICln.

As shown in figure 6.5, in lanes 1-3 (control), transfection of cells with Lsm10/Lsm11 caused a reduction in total translation. Co-transfection of pICln was able to rescue this effect marginally. In contrast, in lanes 4-6 and 7-9, co-transfection of pICln led to further reduction in general translation. Comparing lanes 1, 4, and 7, transfection of cells with siRNAs against pICln has hardly any effect on general translation. Likewise, comparing lanes 2, 5, and 8 or lanes 3, 6 and 9, I did not observe any substantial influence on general translation resulting from reduction in the level of pICln or transfection of the exogenous pICln.

Altogether, I conclude that any reduction in the level of pICln has no major consequence on the total translation efficiency of the cells. Although Sm proteins are abundant proteins, the population of ribosomes engaging in the translation of these proteins forms a very small part of total cellular ribosomes. Consequently, I was not able to detect the influence of saturation of this specific population of ribosomes on the total translational efficiency of the cells. On the other hand, cells are presumably able to cope with this kind of saturation of ribosomes rapidly to liberate them for new rounds of translation.
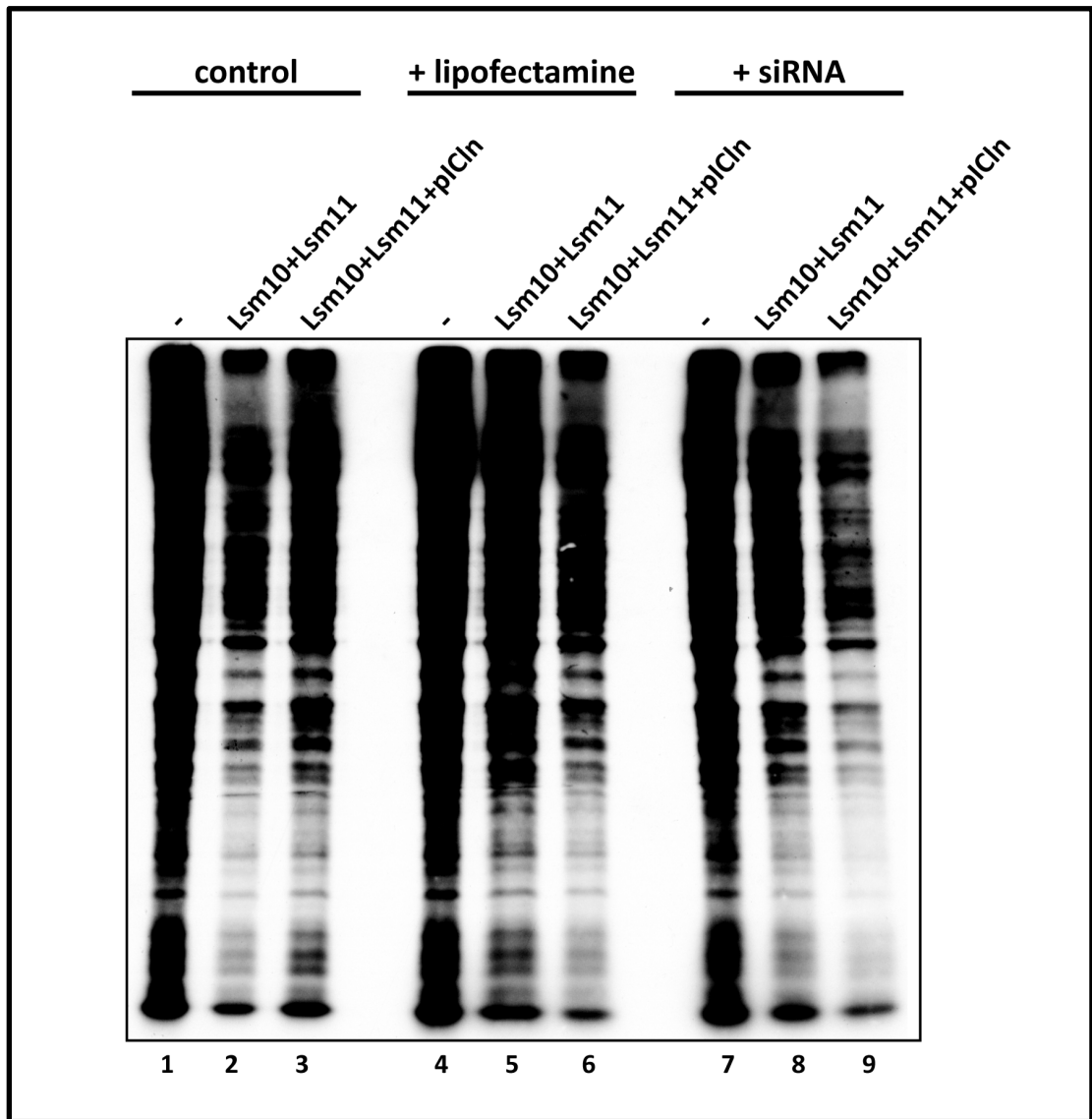
Figure 6.5. Radiolabeling of the total cellular proteins

Non transfected-, Lsm10/Lsm11- or Lsm10/Lsm11/pICln transfected HEK 293T cells were further treated with Lipofectamine or transfected with siRNA against pICln. Radiolabeling of the proteins was performed with ^{35}S-methionine for 2 hours. Then extracts were prepared and checked on a 12% Bis-Tris PAGE.

7   Appendix

Bio View: Identified Proteins (277) Including 8 Decoys	Accession Number	Mr	20S complex	6S complex
U7 snRNA-associated Sm-like protein LSm11 [Homo sapiens],	gi|27735089 (+1)	40 kDa	435	**359**
Protein arginine N-methyltransferase 5 isoform a [Homo sapiens],	gi|20070220	73 kDa	**631**	**17**
U7 snRNA-associated Sm-like protein LSm10 [Homo sapiens],	gi|14249632	14 kDa	254	**224**
Methylosome protein 50 [Homo sapiens],	gi|13129110 (+1)	37 kDa	**319**	**13**
Methylosome subunit pICln [Homo sapiens],	gi|4502891	26 kDa	159	**170**
Small nuclear ribonucleoprotein E [Homo sapiens] E	gi|4507129	11 kDa	92	**117**
Small nuclear ribonucleoprotein polypeptide F, isoform CRA_b [Homo sapiens]	gi|119617955 (+1)	8 kDa	42	**66**
Small nuclear ribonucleoprotein G [Homo sapiens]	gi|4507133	8 kDa	34	**62**
Small nuclear ribonucleoprotein Sm D2 isoform 1 [Homo sapiens]	gi|4759158	14 kDa	41	
Small nuclear ribonucleoprotein Sm D1 [Homo sapiens]	gi|5902102	13 kDa	22	

Index 1. Mass spectrometry analysis of purified 6S- and 20S complexes.

Major components are shown in this table. Protein arginine N-methyltransferase 5 (PRMT5) and Methylosome protein 50 (MEP50 or WD45) are mainly found in the 20S complex. SmD1 and SmD2 are exclusively found in the 20S complex. The numbers shown in the last two columns correspond to the individual peptides found in the respective samples.

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Accession	Protein Name
gi|126031226	Chain B, Structure Of Appbp1-Uba3~nedd8-Nedd8-Mgatp-Ubc12(C111a), A Trapped Ubiquitin-Like Protein Activation Complex
gi|27735089	U7 snRNA-associated Sm-like protein LSm11 [Homo sapiens]
gi|20070220	protein arginine N-methyltransferase 5 isoform a [Homo sapiens]
gi|119627667	poly(A) binding protein, cytoplasmic 4 (inducible form), isoform CRA_b [Homo sapiens]
gi|14249632	U7 snRNA-associated Sm-like protein LSm10 [Homo sapiens]
gi|13129110	methylosome protein 50 [Homo sapiens]
gi|1230564	Gu protein [Homo sapiens]
gi|119572744	ribosomal protein L18, isoform CRA_b [Homo sapiens]
gi|15080189	JUP protein [Homo sapiens]
gi|15431295	60S ribosomal protein L13 isoform 1 [Homo sapiens]
gi|189053683	unnamed protein product [Homo sapiens]
gi|223461341	PCM1 protein [Homo sapiens]
gi|4502891	methylosome subunit pICln [Homo sapiens]
gi|119598180	ribosomal protein L4, isoform CRA_b [Homo sapiens]
gi|119585653	guanine nucleotide binding protein-like 3 (nucleolar), isoform CRA_a [Homo sapiens]
gi|158260331	unnamed protein product [Homo sapiens]
gi|114205458	NEXN protein [Homo sapiens]
gi|119580717	ribosomal protein L3, isoform CRA_d [Homo sapiens]
gi|119589356	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R), isoform CRA_b [Homo sapiens]
gi|119613805	hCG1782414 [Homo sapiens]
gi|157830573	Chain A, Structure Of A Soluble, Glycosylated Form Of The Human Complement Regulatory Protein Cd59
gi|2105469	putative RNA binding protein KOC [Homo sapiens]
gi|14209614	DEAD-box corepressor DP103 [Homo sapiens]
gi|189053113	unnamed protein product [Homo sapiens]
gi|114647215	PREDICTED: 40S ribosomal protein S2-like isoform 5 [Pan troglodytes]
gi|111955084	5-azacytidine-induced protein 1 isoform a [Homo sapiens]
gi|10863945	X-ray repair cross-complementing protein 5 [Homo sapiens]
gi|189053499	unnamed protein product [Homo sapiens]
gi|119597983	hCG33299, isoform CRA_a [Homo sapiens]
gi|119593494	ribosomal protein L5, isoform CRA_b [Homo sapiens]
gi|4191610	IGF-II mRNA-binding protein 2 [Homo sapiens]
gi|14141193	40S ribosomal protein S9 [Homo sapiens]
gi|119577297	ribosomal protein S16, isoform CRA_b [Homo sapiens]
gi|13592009	60S ribosomal protein L10a [Rattus norvegicus]
gi|5803225	14-3-3 protein epsilon [Homo sapiens]
gi|14211540	putative helicase MOV-10 [Homo sapiens]
gi|158254664	unnamed protein product [Homo sapiens]
gi|119624101	ribosomal protein S18, isoform CRA_c [Homo sapiens]
gi|189502784	mitochondrial heat shock 60kD protein 1 variant 1 [Homo sapiens]

gi|157739942	**gem-associated protein 5 [Homo sapiens]**
gi|36796	**t-complex polypeptide 1 [Homo sapiens]**
gi|10835063	**nucleophosmin isoform 1 [Homo sapiens]**
gi|15082586	**Ribosomal protein L8 [Homo sapiens]**
gi|13385408	**60S ribosomal protein L11 [Mus musculus]**
gi|1699027	**nuclear corepressor KAP-1 [Homo sapiens]**
gi|119582037	**La ribonucleoprotein domain family, member 1, isoform CRA_b [Homo sapiens]**
gi|11067747	**cell division cycle 5-like protein [Homo sapiens]**
gi|119600034	**ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A, isoform CRA_c [Homo sapiens]**
gi|5730023	**ruvB-like 2 [Homo sapiens]**
gi|6005721	**erlin-2 isoform 1 [Homo sapiens]**
gi|15022507	**coactivator activator [Homo sapiens]**
gi|197692395	**RuvB-like 1 [Homo sapiens]**
gi|119583268	**chromosome 9 open reading frame 10, isoform CRA_b [Homo sapiens]**
gi|148746199	**60S ribosomal protein L31 isoform 2 [Homo sapiens]**
gi|13097600	**Similar to ribosomal protein L23 [Homo sapiens]**
gi|118582269	**serine/arginine-rich splicing factor 1 isoform 2 [Homo sapiens]**
gi|189053616	**unnamed protein product [Homo sapiens]**
gi|166064029	**angiomotin isoform 1 [Homo sapiens]**
gi|119568094	**hCG21078 [Homo sapiens]**
gi|1160963	**transmembrane protein [Homo sapiens]**
gi|119617762	**protein phosphatase 1, regulatory (inhibitor) subunit 12A, isoform CRA_e [Homo sapiens]**
gi|1136741	**KIAA0002 [Homo sapiens]**
gi|12643813	**RecName: Full=WD repeat-containing protein 6**
gi|13654270	**protein LAS1 homolog isoform 1 [Homo sapiens]**
gi|183182	**guanine nucleotide-binding regulatory protein alpha-inhibitory subunit [Homo sapiens]**
gi|119596872	**ARS2 protein, isoform CRA_b [Homo sapiens]**
gi|119581991	**enthoprotin, isoform CRA_c [Homo sapiens]**
gi|109255228	**centrosomal protein of 170 kDa isoform alpha [Homo sapiens]**
gi|119624259	**SFRS protein kinase 1, isoform CRA_b [Homo sapiens]**
gi|194386598	**unnamed protein product [Homo sapiens]**
gi|148727341	**serine-threonine kinase receptor-associated protein [Homo sapiens]**
gi|119576592	**ATPase family, AAA domain containing 3A, isoform CRA_c [Homo sapiens]**
gi|17225576	**progesterone-induced blocking factor 1 [Homo sapiens]**
gi|14141166	**poly(rC)-binding protein 2 isoform b [Homo sapiens]**
gi|119631003	**small nuclear ribonucleoprotein polypeptides B and B1, isoform CRA_c [Homo sapiens]**
gi|119580824	**Ran GTPase activating protein 1, isoform CRA_a [Homo sapiens]**
gi|119593532	**zinc finger protein 326, isoform CRA_b [Homo sapiens]**
gi|119584891	**leucine rich repeat (in FLII) interacting protein 2, isoform CRA_b [Homo sapiens]**
gi|119601416	**splicing factor, arginine/serine-rich 5, isoform CRA_e [Homo sapiens]**
gi|158257956	**unnamed protein product [Homo sapiens]**

gi|27597085	tropomyosin alpha-1 chain isoform 5 [Homo sapiens]
gi|117938759	protein ALEX XLas [Homo sapiens]
gi|179212	Na+ K+ ATPase alpha subunit [Homo sapiens]
gi|194387362	unnamed protein product [Homo sapiens]
gi|161172138	Chain A, Phosphorylation Independent Interactions Between 14-3-3 And Exoenzyme S: From Structure To Pathogenesis
gi|119581911	arginyl-tRNA synthetase [Homo sapiens]
gi|119581585	basigin (Ok blood group), isoform CRA_b [Homo sapiens]
gi|189069163	unnamed protein product [Homo sapiens]
gi|119620390	chaperonin containing TCP1, subunit 4 (delta), isoform CRA_a [Homo sapiens]
gi|119571516	ribosomal protein L23a, isoform CRA_a [Homo sapiens]
gi|16753227	60S ribosomal protein L6 [Homo sapiens]
gi|119596694	RNA binding protein, autoantigenic (hnRNP-associated with lethal yellow homolog (mouse)), isoform CRA_b [Homo sapiens]
gi|158256730	unnamed protein product [Homo sapiens]
gi|158254970	unnamed protein product [Homo sapiens]
gi|2914385	Chain C, Human Pcna
gi|189053699	unnamed protein product [Homo sapiens]
gi|108773810	leucyl-tRNA synthetase, cytoplasmic [Homo sapiens]
gi|23308577	D-3-phosphoglycerate dehydrogenase [Homo sapiens]
gi|119576060	flightless I homolog (Drosophila), isoform CRA_d [Homo sapiens]
gi|14124942	Similar to ribophorin I [Homo sapiens]
gi|119599447	mitochondrial ribosomal protein S22, isoform CRA_a [Homo sapiens]
gi|197692437	replication factor C 2 isoform 1 [Homo sapiens]
gi|8923579	ragulator complex protein LAMTOR1 [Homo sapiens]
gi|16550621	unnamed protein product [Homo sapiens]
gi|221042490	unnamed protein product [Homo sapiens]
gi|119580514	eukaryotic translation initiation factor 3, subunit 7 zeta, 66/67kDa, isoform CRA_b [Homo sapiens]
gi|119603388	hCG1782167, isoform CRA_a [Homo sapiens]
gi|4503483	elongation factor 2 [Homo sapiens]
gi|119571547	hCG1998851, isoform CRA_h [Homo sapiens]
gi|10441386	TPM4-ALK fusion oncoprotein type 2 [Homo sapiens]
gi|1107696	Mi-2 protein [Homo sapiens]
gi|12711291	dsRNA adenosine deaminase [Homo sapiens]
gi|14124984	Chaperonin containing TCP1, subunit 3 (gamma) [Homo sapiens]
gi|13259512	survival motor neuron protein isoform b [Homo sapiens]
gi|4506903	serine/arginine-rich splicing factor 9 [Homo sapiens]
gi|119575213	phosphoglycerate mutase family member 5, isoform CRA_b [Homo sapiens]
gi|126035028	liver histone H1e [Homo sapiens]
gi|125625324	nuclear RNA export factor 1 isoform 2 [Homo sapiens]
gi|119589043	hCG1784554, isoform CRA_a [Homo sapiens]
gi|11641247	Golgi-associated plant pathogenesis-related protein 1 [Homo sapiens]
gi|155030226	phosphatidylinositol 4-kinase alpha isoform 2 [Homo sapiens]

gi|4506681	40S ribosomal protein S11 [Homo sapiens]
gi|119585358	glutaminyl-tRNA synthetase, isoform CRA_b [Homo sapiens]
gi|114608952	PREDICTED: tyrosine-protein kinase Fyn isoform 4 [Pan troglodytes]
gi|19263767	Similar to cytoskeleton-associated protein 4 [Homo sapiens]
gi|223555917	protein LYRIC [Homo sapiens]
gi|158257374	unnamed protein product [Homo sapiens]
gi|119592989	ribosomal protein S5, isoform CRA_b [Homo sapiens]
gi|115206	RecName: Full=C-1-tetrahydrofolate synthase, cytoplasmic; Short=C1-THF synthase
gi|14043022	methionyl-tRNA synthetase, cytoplasmic [Homo sapiens]
gi|261399877	T-complex protein 1 subunit eta isoform d [Homo sapiens]
gi|5902102	small nuclear ribonucleoprotein Sm D1 [Homo sapiens]
gi|15010550	heat shock protein gp96 precursor [Homo sapiens]
gi|119627382	peroxiredoxin 1, isoform CRA_b [Homo sapiens]
gi|13544009	EPB41L3 protein [Homo sapiens]
gi|119581557	polypyrimidine tract binding protein 1, isoform CRA_b [Homo sapiens]
gi|19913428	V-type proton ATPase subunit B, brain isoform [Homo sapiens]
gi|119617955	small nuclear ribonucleoprotein polypeptide F, isoform CRA_b [Homo sapiens]
gi|112180796	Family with sequence similarity 98, member A [Homo sapiens]
gi|119573598	ubiquitin associated protein 2-like, isoform CRA_a [Homo sapiens]
gi|4506693	40S ribosomal protein S17 [Homo sapiens]
gi|119604207	coiled-coil-helix-coiled-coil-helix domain containing 3, isoform CRA_b [Homo sapiens]
gi|197692141	ATP-dependent RNA helicase DDX3X [Homo sapiens]
gi|127798841	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle [Homo sapiens]
gi|119594432	eukaryotic translation elongation factor 1 gamma, isoform CRA_d [Homo sapiens]
gi|5803227	14-3-3 protein theta [Homo sapiens]
gi|123291074	mitochondrial ribosomal protein S18B [Homo sapiens]
gi|60688557	Phosphoprotein associated with glycosphingolipid microdomains 1 [Homo sapiens]
gi|10716563	calnexin precursor [Homo sapiens]
gi|119600955	LIM domain 7, isoform CRA_a [Homo sapiens]
gi|13543657	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1) [Homo sapiens]
gi|40217805	polynucleotide 5'-hydroxyl-kinase NOL9 [Homo sapiens]
gi|119574079	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1, isoform CRA_c [Homo sapiens]
gi|307066	inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205) [Homo sapiens]
gi|119619436	hCG1983058 [Homo sapiens]
gi|119608226	SET translocation (myeloid leukemia-associated), isoform CRA_c [Homo sapiens]
gi|16552261	unnamed protein product [Homo sapiens]
gi|158259511	unnamed protein product [Homo sapiens]
gi|119610151	fatty acid synthase [Homo sapiens]
gi|1197636	p105MCM [Homo sapiens]
gi|189065517	unnamed protein product [Homo sapiens]
gi|4506583	replication protein A 70 kDa DNA-binding subunit [Homo sapiens]

gi|118196855	RBM25 protein [Homo sapiens]
gi|10801345	eukaryotic translation initiation factor 3 subunit K [Homo sapiens]
gi|313014	vacuolar proton ATPase [Homo sapiens]

Index 2. **List of proteins, which are associated with the ribosomes from both Lsm10/Lsm11 and Lsm10/Lsm11/pICln transfected cells.**

Ribosomes from HA-FireFly luciferase, Lsm10/Lsm11 and Lsm10/Lsm11/pICln transfected cells were purified. Further mass spectrometry analysis of the ribosomes led us a large list of proteins. The table above shows the proteins, which are only found in Lsm10/Lsm11 and Lsm10/Lsm11/pICln transfected cells.

Accession	Protein Name
gi|27735089	U7 snRNA-associated Sm-like protein LSm11 [Homo sapiens]
gi|119627667	poly(A) binding protein, cytoplasmic 4 (inducible form), isoform CRA_b [Homo sapiens]
gi|14249632	U7 snRNA-associated Sm-like protein LSm10 [Homo sapiens]
gi|1230564	Gu protein [Homo sapiens]
gi|15080189	JUP protein [Homo sapiens]
gi|15431295	60S ribosomal protein L13 isoform 1 [Homo sapiens]
gi|189053683	unnamed protein product [Homo sapiens]
gi|158260331	unnamed protein product [Homo sapiens]
gi|119580717	ribosomal protein L3, isoform CRA_d [Homo sapiens]
gi|119589356	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R), isoform CRA_b [Homo sapiens]
gi|119613805	hCG1782414 [Homo sapiens]
gi|14209614	DEAD-box corepressor DP103 [Homo sapiens]
gi|189053113	unnamed protein product [Homo sapiens]
gi|114647215	PREDICTED: 40S ribosomal protein S2-like isoform 5 [Pan troglodytes]
gi|189053499	unnamed protein product [Homo sapiens]
gi|119597983	hCG33299, isoform CRA_a [Homo sapiens]
gi|14141193	40S ribosomal protein S9 [Homo sapiens]
gi|119577297	ribosomal protein S16, isoform CRA_b [Homo sapiens]
gi|13592009	60S ribosomal protein L10a [Rattus norvegicus]
gi|14211540	putative helicase MOV-10 [Homo sapiens]
gi|119624101	ribosomal protein S18, isoform CRA_c [Homo sapiens]
gi|157739942	gem-associated protein 5 [Homo sapiens]
gi|13385408	60S ribosomal protein L11 [Mus musculus]
gi|119582037	La ribonucleoprotein domain family, member 1, isoform CRA_b [Homo sapiens]
gi|11067747	cell division cycle 5-like protein [Homo sapiens]
gi|5730023	ruvB-like 2 [Homo sapiens]
gi|15022507	coactivator activator [Homo sapiens]
gi|197692395	RuvB-like 1 [Homo sapiens]
gi|119583268	chromosome 9 open reading frame 10, isoform CRA_b [Homo sapiens]
gi|119568094	hCG21078 [Homo sapiens]
gi|119617762	protein phosphatase 1, regulatory (inhibitor) subunit 12A, isoform CRA_e [Homo sapiens]
gi|1136741	KIAA0002 [Homo sapiens]
gi|12643813	RecName: Full=WD repeat-containing protein 6
gi|13654270	protein LAS1 homolog isoform 1 [Homo sapiens]
gi|119596872	ARS2 protein, isoform CRA_b [Homo sapiens]
gi|119624259	SFRS protein kinase 1, isoform CRA_b [Homo sapiens]
gi|194386598	unnamed protein product [Homo sapiens]
gi|17225576	progesterone-induced blocking factor 1 [Homo sapiens]
gi|119631003	small nuclear ribonucleoprotein polypeptides B and B1, isoform CRA_c [Homo sapiens]
gi|119601416	splicing factor, arginine/serine-rich 5, isoform CRA_e [Homo sapiens]

gi|158257956	unnamed protein product [Homo sapiens]
gi|194387362	unnamed protein product [Homo sapiens]
gi|161172138	Chain A, Phosphorylation Independent Interactions Between 14-3-3 And Exoenzyme S: From Structure To Pathogenesis
gi|189069163	unnamed protein product [Homo sapiens]
gi|119571516	ribosomal protein L23a, isoform CRA_a [Homo sapiens]
gi|16753227	60S ribosomal protein L6 [Homo sapiens]
gi|119596694	RNA binding protein, autoantigenic (hnRNP-associated with lethal yellow homolog (mouse)), isoform CRA_b [Homo sapiens]
gi|158256730	unnamed protein product [Homo sapiens]
gi|158254970	unnamed protein product [Homo sapiens]
gi|189053699	unnamed protein product [Homo sapiens]
gi|119599447	mitochondrial ribosomal protein S22, isoform CRA_a [Homo sapiens]
gi|16550621	unnamed protein product [Homo sapiens]
gi|221042490	unnamed protein product [Homo sapiens]
gi|4506903	serine/arginine-rich splicing factor 9 [Homo sapiens]
gi|155030226	phosphatidylinositol 4-kinase alpha isoform 2 [Homo sapiens]
gi|119585358	glutaminyl-tRNA synthetase, isoform CRA_b [Homo sapiens]
gi|19263767	Similar to cytoskeleton-associated protein 4 [Homo sapiens]
gi|223555917	protein LYRIC [Homo sapiens]
gi|158257374	unnamed protein product [Homo sapiens]
gi|19913428	V-type proton ATPase subunit B, brain isoform [Homo sapiens]
gi|5803227	14-3-3 protein theta [Homo sapiens]
gi|189065517	unnamed protein product [Homo sapiens]

Index 3. **Short list of candidates for the factor mediating the association of Lsm/Sm proteins with the ribosomes.**

The proteins from "index 2" were studied and proteins, which are found in higher concentration in Lsm10/Lsm11 transfected cells in comparison to Lsm10/Lsm11/pICln transfected cells are listed above.

	Accession Number	Mr	first pellet (C)	first pellet (Si)	Low salt (C)	low salt (Si)	High salt (C)	high salt (Si)
26S proteasome subunit 9 [Homo sapiens]	gi|2150046 (+1)	47 kDa	5	11			78	95
26S proteasome non-ATPase regulatory subunit 6 [Homo sapiens]	gi|7661914	46 kDa	4	13			74	81
26S protease regulatory subunit 8 isoform 2 [Homo sapiens]	gi|312596881 (+1)	45 kDa	6	7			52	71
26S protease regulatory subunit 6A [Homo sapiens]	gi|21361144 (+1)	49 kDa	2	7			23	74
26S protease regulatory subunit 7 isoform 1 [Homo sapiens]	gi|4506209 (+1)	49 kDa	3	9			26	60
PREDICTED: 26S proteasome non-ATPase regulatory subunit 13 isoform 3 [Pan troglodytes]	gi|114635315	43 kDa	4	10			19	58
26S proteasome non-ATPase regulatory subunit 7 [Homo sapiens]	gi|25777615 (+2)	37 kDa	4	11			20	74
26S protease regulatory subunit 10B [Homo sapiens]	gi|195539395 (+1)	46 kDa	4	6			39	50
26S proteasome:SUBUNIT=5a	gi|1587697 (+1)	41 kDa		4			28	38
26S protease regulatory subunit 6B isoform 1 [Homo sapiens protein product [Homo sapiens]	gi|5729991	47 kDa		3			9	27
26S proteasome non-ATPase regulatory subunit 12 isoform 1 [Homo sapiens]	gi|4506221 (+1)	53 kDa	3	2			3	39
26S proteasome subunit p97 [Homo sapiens]	gi|1060888 (+4)	100 kDa	12	13				11
ubiquitin carboxyl-terminal hydrolase L5, isoform CRA_b [Homo sapiens]	gi|119611640 (+4)	44 kDa		3			5	21
proteasome subunit HSPC [Homo sapiens]	gi|4092058 (+1)	28 kDa	3	5				19
Proteasome (prosome, macropain) 26S subunit, ATPase, 1 [Homo sapiens]	gi|16741033 (+3)	49 kDa	9	9				13
proteasome subunit alpha type-6 [Rattus norvegicus]	gi|8394076	27 kDa	3	6				15
26S proteasome subunit p31 [Homo sapiens],	gi|1037164 (+2)	30 kDa	3	4				8
proteasome (prosome, macropain) subunit, alpha type, 5 [Homo sapiens]	gi|54696300 (+1)	26 kDa	4	3				10
proteasome subunit alpha type-2 [Homo sapiens]	gi|4506181 (+1)	26 kDa	3	7				9
proteasome subunit beta type-2 isoform 1 [Homo sapiens]	gi|4506195	23 kDa	3	5				5
26S proteasome non-ATPase regulatory subunit 14 [Homo sapiens]	gi|5031981	35 kDa	2	2				9
proteasome (prosome, macropain) subunit, alpha type, 3, isoform CRA_a [Homo sapiens]	gi|119601121 (+5)	24 kDa	3	2				7
ubiquitin carboxyl-terminal hydrolase 7 [Homo sapiens]	gi|150378533 (+4)	128 kDa	4	7		2		
proteasome (prosome, macropain) subunit, beta type, 1, isoform CRA_a [Homo sapiens]	gi|119567805 (+3)	24 kDa	2	8				2

Protein	Accession	MW						
proteasome subunit beta type-3 [Homo sapiens]	gi|22538465	23 kDa	**2**	**3**				**3**
Chain A, Crystal Structure Of The Human Y14MAGOH COMPLEX	gi|34810525 (+2)	17 kDa	**9**	**13**	9			**8**
gem-associated protein 5 [Homo sapiens]	gi|157739942 (+3)	169 kDa	29	32	**22**	**42**	26	**26**
Gem (nuclear organelle) associated protein 6 [Homo sapiens]	gi|17390437 (+1)	19 kDa	2					
gem-associated protein 7 [Homo sapiens]	gi|13376001 (+1)	15 kDa			3			**2**
survival motor neuron protein isoform b [Homo sapiens]	gi|13259512 (+4)	29 kDa	5	5	**5**	**7**		5
small nuclear ribonucleoprotein Sm D1 [Homo sapiens]	gi|5902102	13 kDa	5	4	6	6		
small nuclear ribonucleoprotein Sm D2 isoform 1 [Homo sapiens]	gi|4759158	14 kDa	6	8	7	4		**4**
small nuclear ribonucleoprotein polypeptide B'', isoform CRA_b [Homo sapiens]	gi|119630691 (+1)	28 kDa	6	6	4	4		
small nuclear ribonucleoprotein E [Mus musculus], small nuclear ribonucleoprotein E [Homo sapiens]	gi|312005 (+1)	10 kDa	**3**	**10**	7	8		**6**
small nuclear ribonucleoprotein polypeptide F, isoform CRA_b [Homo sapiens]	gi|119617955 (+1)	8 kDa	**4**	**6**	4	5		**5**
RecName: Full=Small nuclear ribonucleoprotein G-like protein	gi|205829943 (+2)	9 kDa	3	3	4	3		
protein LSM12 homolog [Homo sapiens]	gi|22748747 (+1)	22 kDa	**2**	**5**	**2**	**5**		**2**

Index 4. **Mass spectrometry analysis of total cellular ribosomes under different conditions.**

Four major groups of proteins, which are found in this analysis, are interesting. First group are components of the ubiquitin-proteasome machinery. Majority of these proteins are found in higher levels upon reduction in the level of pICln. Second group is MAGOH, which is component of the "Exon junction complex". Third group are components from the SMN complex. Fourth group are Lsm/Sm proteins. The proteins, which show an increase of at least 1.5 times upon knock down of pICln are shown in bold. The numbers shown in the last six columns correspond to the individual peptides found in the respective samples.

8   Abbreviations

α	alfa
β	beta
°C	Degree Celsius
C-terminal	carboxy terminal
E. Coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
g	gram
h	hour
HA	Hemagglutinin
HEK293	Human embryonic kidney 293 (cell line)
HeLa	Henrietta Lacks
min	minute
ml	milliliter
mRNA	messenger RNA
N-terminal	amino terminal
PCR	Polymerase Chain Reaction
PHAX	Phosphorylated adaptor for RNA export
PVDF	Polyvinylidene Fluoride
RanGTP	GTP-bound nuclear protein Ran
rpm	revolutions per minute
rRNA	ribosomal RNA
RNasin	RNase inhibitor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
Sm	Smith
SMA	Spinal Muscular Atrophy
SMN	Survival of Motor Neuron
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
tRNA	transfer RNA
µl	micro liter

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11 Publications

1) Ashwin Chari, **Elham Paknia**, Utz Fischer. *The role of RNP biogenesis in spinal muscular atrophy*. Curr Opin Cell Biol. 2009 Jun; 21(3).

2) Pasternack SM, Refke M, **Paknia E**, Hennies HC, Franz T, Schäfer N, Fryer A, van Steensel M, Sweeney E, Just M, Grimm C, Kruse R, Ferrándiz C, Nöthen MM, Fischer U, Betz RC. *Mutations in SNRPE, which encodes a core protein of the spliceosome, cause autosomal-dominant hypotrichosis simplex*. Am J Hum Genet. 2013 Jan; 92(1).

Abstracts

1) *Reconstitution of the U snRNP assembly machinery: Insights into mechanisms and spinal muscular atrophy.* Ashwin Chari, Nils Neuenkirchen, Clemens Englbrecht, **Elham Paknia**, Utz Fischer. 14th annual meeting of the RNA society. 26-30 May 2009, Madison, Wisconsin.

2) *Reconstitution of the U snRNP assembly machinery: Insights into mechanisms and spinal muscular atrophy.* Ashwin Chari, Nils Neuenkirchen, Clemens Englbrecht, **Elham Paknia**, Utz Fischer. EMB conference „the complex life of mRNA: From synthesis to Decay". 18-20 March 2010, EMBL Heidelberg, Germany.

3) *Mutations in SNRPE, which encodes a core protein of the spliceosome, cause autosomal dominant hypotrichosis simplex.* Pasternack S.M., Refke M., **Paknia E**., Hennies H.C., Franz T., Schäfer N., Fryer A., van Steensel M., Sweeney E., Just M., Grimm C., Kruse R., Ferrándiz C., Nöthen M.M., Fischer U., Betz R.C. 24th Annual Meeting of the German Society of Human Genetics. 20-22 March 2013, Dresden

4) *A case for cellular logistics: snRNPs biogenesis.* **Elham Paknia**. Hottest life science in town. 4 July, 2009, Wuerzburg, Germany.

5) *Subcellular trafficking of snRNPs.* **Elham Paknia**. Molecular basis of organ development in vertebrates. October 2009, Wuerzburg, Germany.

12   CURRICULUM VITAE

Publications

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