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REVIEW ARTICLE

# Ribonucleoprotein multimers and their functions

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**Abstract**

Ribonucleoproteins (RNPs) play key roles in many cellular processes and often function as RNP enzymes. Similar to proteins, some of these RNPs exist and function as multimers, either momomeric or heteromeric. While in some cases the mechanistic function of multimerization is well understood, the functional consequences of multimerization of other RNPs remain enigmatic. In this review we will discuss the function and organization of small RNPs that exist as stable multimers, including RNPs catalyzing RNA chemical modifications, telomerase RNP, and RNPs involved in pre-mRNA splicing.

**Keywords:** *Ribonucleoprotein; multimerization; box C/D sRNP; box C/D snoRNP; telomerase; U4/U6 di-snRNP; U4/U6.U5 tri-snRNP; U11/U12 di-snRNP*



## Introduction

Estimates are that 35% of all cellular proteins are oligomers, with the average oligomerization state being a tetramer (Ali *et al.*, 2005). Oligomerization plays an important role in the function of proteins and, in particular, of enzymes, and can provide several functional advantages. First, oligomerization can be used as a means to control the function of proteins, including activation or inactivation dependent on the oligomeric state as well as enabling allosteric regulation and cooperativity between subunits. Second, oligomerization of proteins provides multivalency and can increase the binding strength for substrates. Third, oligomerization can create new active sites at interfaces between individual subunits. Fourth, oligomerization can stabilize protein structures, especially in hyperthermophylic organisms (Ali *et al.*, 2005).

While the formation of oligomeric complexes is a characteristic of many proteins, RNA–protein complexes can oligomerize as well. Similar to proteins, ribonucleoproteins (RNPs) can form homomeric or heteromeric oligomers. RNP multimerization can occur via RNA–RNA, RNA–protein and/or protein–protein interactions, with the multimerization state being determined by the number of RNA molecules within the RNP. In this review we will focus on small RNPs that stably accumulate as multimers, including RNPs that guide methylation of cellular RNAs, RNPs that extend telomeres, and RNPs that contribute to pre-messengerRNA splicing.

## Dimeric box C/D s(no)RNPs

### *Functions of box C/D s(no)RNPs*

Nucleotides in ribosomal RNA (rRNA) and other RNAs are post-transcriptionally altered, leading to as many as 200 modifications in the human ribosome that cluster in phylogenetically conserved and functionally critical regions (Decatur and Fournier, 2002). While individual modifications are not essential, collectively they have been shown to be important for rRNA folding, ribosome stability, and optimal translation of mRNAs (King *et al.*, 2003; Liang *et al.*, 2007). In archaea and eukaryotes, these chemical modifications are performed by small ribonucleoproteins (sRNPs in archaea) and small nucleolar ribonucleoproteins (snoRNPs in eukaryotes), respectively. They can occur as two different types: pseudouridylations, catalyzed by box H/ACA s(no)RNPs (Ganot *et al.*, 1997; Ni *et al.*, 1997), and 2'-O-ribose methylations, catalyzed by box C/D s(no)RNPs (Cavaille *et al.*, 1996; Kiss-Laszlo

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*et al.*, 1996; Tycowski *et al.*, 1996; Omer *et al.*, 2000). The nucleotide to be modified is dictated by base pairing of the RNA component of the s(no)RNP with the substrate RNA. Because of these base pairing events, s(no)RNPs are also thought of as chaperones which facilitate optimal rRNA folding (Steitz and Tycowski, 1995). While the majority of snoRNPs act as RNP enzymes to catalyze chemical rRNA modifications, a subset of them, however, does not function in this way but is instead directly required for the cleavage events that process rRNA precursors during ribosome biogenesis (Matera *et al.*, 2007). One example is the U3 snoRNP, a box C/D snoRNP which is a central component of the SSU processome/90S pre-ribosome (Dragon *et al.*, 2002; Grandi *et al.*, 2002). In addition to rRNA, archaeal transferRNAs and eukaryotic small nuclear RNAs (snRNAs) and a few mRNAs are also substrates of snoRNPs (Tycowski *et al.*, 1998; Omer *et al.*, 2000; Huttenhofer *et al.*, 2001; Jady and Kiss, 2001; Ziesche *et al.*, 2004; Vitali *et al.*, 2005; Kishore and Stamm, 2006a; 2006b).

In the following paragraphs we will focus on the organization of box C/D s(no)RNPs because an archaeal box C/D sRNP has recently been shown to be a di-sRNP (Bleichert *et al.*, 2009). Currently, no evidence exist that box H/ACA s(no)RNPs undergo multimerization, hence we will not discuss them. For a recent review on box H/ACA snoRNPs see Kiss *et al.* (2010).

### *Box C/D s(no)RNP components*

Methylation guide box C/D s(no)RNPs consist of a small box C/D s(no)RNA, characterized by the conserved box C, C', D, and D' sequence elements with the consensus sequences of RUGAUGA (boxes C and C') and CUGA (boxes D and D') (Figure 1A) (Tyc and Steitz, 1989; Kiss-Laszlo *et al.*, 1996; 1998; Gaspin *et al.*, 2000; Omer *et al.*, 2000). The RNA also contains guide sequences between boxes C and D' and between boxes C' and D that base pair to substrate RNAs and thereby confer specificity to the methylation event. The conserved boxes fold into k-turn (box C/D motif) or k-loop (box C'/D' motif) structures and nucleate the binding of the box C/D core proteins, which are common to all box C/D snoRNPs (Watkins *et al.*, 2000; Klein *et al.*, 2001; Nolivos *et al.*, 2005). In archaea, these proteins include L7Ae, Nop5, and fibrillarin, and in eukaryotes, they are 15.5K, Nop56, Nop58, and fibrillarin. Fibrillarin, the methyltransferase in box C/D s(no)RNPs, methylates the target nucleotide base paired to the fifth nucleotide upstream of boxes D or D' using S-adenosylmethionine (SAM) as the methyl group donor (Kiss-Laszlo *et al.*, 1996; 1998; Wang *et al.*, 2000; Omer *et al.*, 2002). The methylation of RNA stabilizes RNA structures by increasing base stacking interactions, by stabilizing the C3'-endo sugar pucker, and by providing protection again hydrolysis (Ishitani *et al.*, 2008).

### *The initial model for archaeal box C/D sRNP architecture*

In contrast to eukaryotic box C/D snoRNPs, the archaeal complexes can be reconstituted *in vitro*, as first demonstrated by Omer *et al.* (2002); this has greatly facilitated biochemical and structural studies of the RNP and its components. L7Ae is the first protein that binds to the sRNA, specifically to the k-turn and k-loop structures formed by boxes C/D and C'/D', respectively, suggesting that one sRNA is bound by two L7Ae proteins in the sRNP. Mutational studies of conserved nucleotides in the C/D and C'/D' motifs confirmed the stoichiometry of RNA and protein in the sRNA–L7Ae complex, with mutations in the C/D motif abolishing L7Ae binding to this motif but not the C'/D' motif and vice versa (Omer *et al.*, 2002; 2006; Rashid *et al.*, 2003; Tran *et al.*, 2003; Singh *et al.*, 2008). Following L7Ae binding to the sRNA, Nop5 associates with the box C/D sRNA–L7Ae complex, which forms a composite binding platform for Nop5 (Omer *et al.*, 2002; Ye *et al.*, 2009). Interestingly, a similar topology is observed in the ternary complex of human 15.5K, Prp31, and a U4 snRNA fragment (Liu *et al.*, 2007). The third protein, fibrillarin, does not bind the sRNA or L7Ae directly, but instead directly interacts with Nop5 (Rashid *et al.*, 2003; Tran *et al.*, 2003; Zhang *et al.*, 2006). A crystal structure of the Nop5–fibrillarin complex showed that Nop5 homodimerizes via an extensive coiled-coil domain to form a Nop5–fibrillarin heterotetramer (Aittaleb *et al.*, 2003; Oruganti *et al.*, 2007). The homodimerization of Nop5 was confirmed in solution and has been suggested to be important for communication between the C/D and C'/D' motifs (Rashid *et al.*, 2003; Zhang *et al.*, 2006).

These results provided a framework for the initial model of archaeal box C/D sRNP architecture (Aittaleb *et al.*, 2003; Tran *et al.*, 2003; Reichow *et al.*, 2007). Archaeal box C/D sRNPs were suggested to contain one box C/D sRNA and two copies of L7Ae, Nop5, and fibrillarin (Figure 1B). One set of the core proteins associates with each of the C/D and C'/D' motifs of the sRNA, where the distance between the motifs is bridged by the homodimerized coiled-coil domains of two Nop5 proteins. While this bipartite organization of box C/D sRNPs, which we will refer to as the mono-sRNP model, is consistent with a variety of biochemical data on various RNA and box C/D protein mutants, it also fails to explain some experimental observations: (1) A guide:substrate RNA duplex cannot be easily accommodated by the mono-sRNP model. The estimated length of the guide:substrate RNA duplex, which is typically 10–12 bp in archaea, is too short to span the distance mandated by the dimerized Nop5 coiled-coil domains (Zhang *et al.*, 2006; Ye *et al.*, 2009); (2) In the mono-sRNP model, the catalytic sites of the RNP enzyme are too far from their site of action. The active

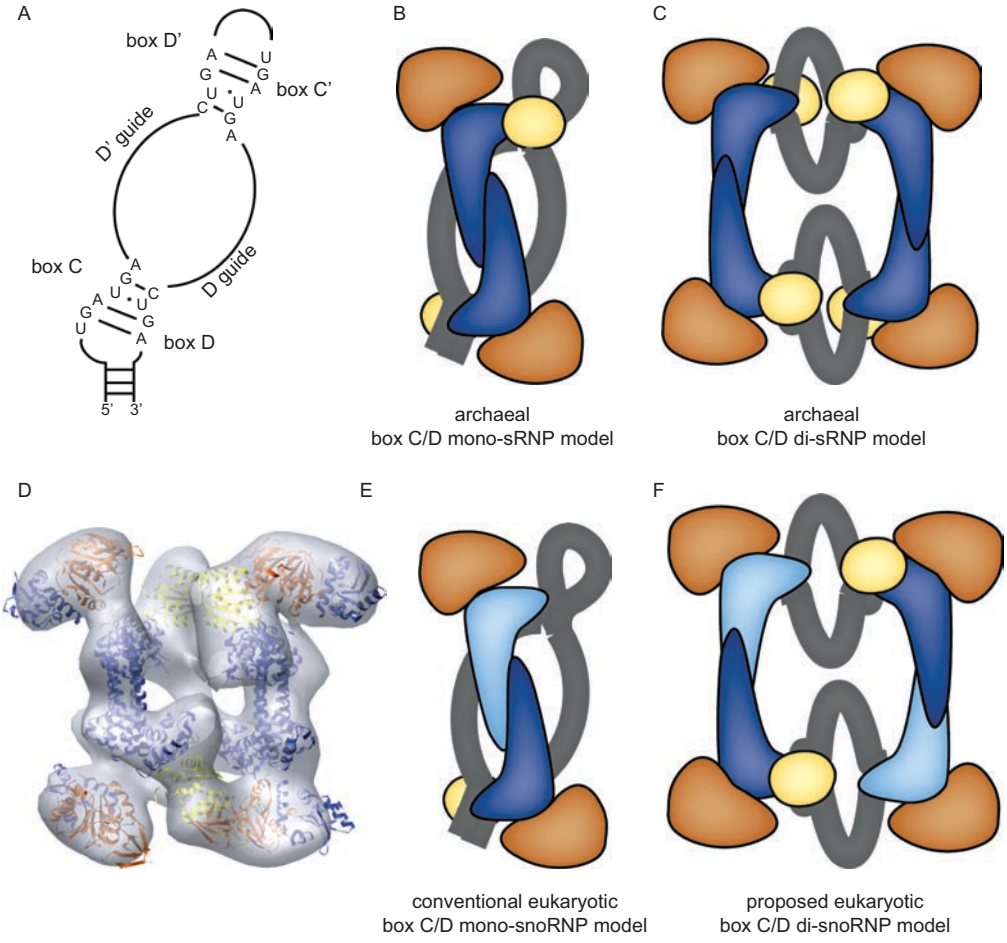


Figure 1. Architecture of box C/D s(no)RNPs. (A) Box C/D s(no)RNAs contain conserved sequences, named boxes C, D, C', and D'. The spacer sequences between boxes C and D' as well as between boxes C' and D contain guide sequences that are complementary to the substrate RNAs targeted for methylation. The 5' and 3' sequences usually form a terminal stem. (B) Conventional mono-sRNP model for archaeal box C/D sRNPs. According to this model, archaeal box C/D sRNPs are composed of one sRNA molecule and two copies of each of the three archaeal box C/D core proteins, assembled symmetrically on the sRNA. (C) Di-sRNP model of archaeal box C/D sRNP architecture. According to this structure-based model, archaeal box C/D sRNPs contain two RNA molecules and four sets of each core protein. Colors are: grey – RNA, yellow – L7Ae, blue – Nop5, orange – fibrillarin. (D) EM structure of a M. jannaschii box C/D sRNP (EMD-1636, Bleichert et al., 2009) with fitted crystal structures of core proteins (PDB 2nnw, Oruganti et al., 2007; PDB 1xbi, Suryadi et al., 2005). Figures 1A–D are reprinted from Bleichert et al. (2009). (E) and (F) Asymmetric assembly models of eukaryotic box C/D mono and di-snoRNP architecture, respectively. Colors are: grey – RNA, yellow – 15.5K/Snu13, light blue – Nop56, dark blue – Nop58, orange – fibrillarin.

site of fibrillarin, the methyltransferase, is not positioned in proximity to the nucleotide it modifies. To resolve these controversies, we sought to establish an accurate architectural model of methylation guide sRNPs based on the structure of enzymatically active box C/D sRNPs containing the full-length sRNA.

### *The di-sRNP model for archaeal box C/D sRNPs*

We determined the first structure of a catalytically active box C/D sRNP containing full-length sRNA and all core protein components by electron microscopy and single particle analysis at 27 Å resolution (Bleichert *et al.*, 2009). Unexpectedly, this structure showed that the *Methanocaldococcus jannaschii* sR8 sRNP is a dimeric RNP, containing two sRNA molecules and four sets of each protein component. Docking of crystal structures and direct localization of fibrillarin and the N-terminal domain of Nop5 provided a new model with a dimeric box C/D sRNP architecture (Figures 1C and 1D). Similar to the mono-sRNP model, each sRNA associates with two sets of each core protein, L7Ae, Nop5, and fibrillarin, each set assembling on the C/D and C'/D' motifs, respectively. However, in contrast to the proposed organization in the mono-sRNP, the guide sequences of the sRNA do not follow the direction of the Nop5 coiled-coil domain but connect two different Nop5–fibrillarin heterotetramers in the structure, resulting in two independent interfaces that stabilize the di-sRNP: the sRNA interface and the Nop5 interface. Thus, dimerization (from the perspective of the

sRNAs) is mediated by coiled-coil domain dimerization of two Nop5 proteins on either side of the sRNP. Currently it is not possible to assign a direction to the sRNAs or to differentiate the D and D' guide sequences due to the low resolution of the EM reconstruction.

The di-sRNP model derived from the EM structure resolves one of the clashes that the mono-sRNP model has with existing experimental results, but not the other. Because in the di-sRNP model the directionality of the dimerized Nop5 coiled-coil domains and the sRNA guide sequences are independent of each other, the guide:substrate RNA duplex should be easily accommodated. However, in the di-sRNP model the active sites of the fibrillarin catalytic subunits remain far from the guide sequences of the sRNAs. This suggests that substrate RNA binding induces conformational changes in the sRNP which brings the active site of the fibrillarin methyltransferase to the nucleotide to be methylated. Interestingly, crystal structures of Nop5 and fibrillarin from different archaeal species have revealed alternate conformations of Nop5 and fibrillarin, suggesting possible conformational changes in the context of the RNP that would be important for the catalysis of 2'-O-ribose methylation (Aittaleb *et al.*, 2003; Oruganti *et al.*, 2007; Ye *et al.*, 2009). Hinge motions of the fibrillarin-binding N-terminus of Nop5 with respect to the coiled-coil domain of Nop5 could move fibrillarin inwards and position it atop the substrate RNA nucleotide to be methylated.

Recently, a partial box C/D sRNP containing all core sRNP proteins and an RNA containing only the C/D motif and a D guide sequence instead of a full-length sRNA was crystallized (Ye *et al.*, 2009). This structure was interpreted in light of the mono-sRNP model. However, it is also consistent with the di-sRNP model. The organization of L7Ae, Nop5 and fibrillarin with respect to the C/D motif in the sRNA in this partial sRNP is similar to what is predicted from the EM structure of the fully assembled complex (Bleichert *et al.*, 2009; Ye *et al.*, 2009). Because the sRNA fragment used for RNP crystallization lacks the important C'/D' motif required for di-sRNP assembly, it would not be expected to assemble into the di-sRNP. We have tested this experimentally with a similar partial RNA construct and found it does not form the di-sRNPs (Bleichert and Baserga, submitted). Hence, while the crystal structure provides detailed high-resolution information on the interaction surfaces between the protein components and between the proteins and the C/D motif of the sRNA, it does not reveal how the guide sequences would be oriented in the context of the fully assembled sRNP. Thus, high resolution structural information on the oligomeric state of box C/D sRNPs awaits a crystal structure of a fully assembled and catalytically active complex.

In addition to providing the first structure of a box C/D sRNP containing the full-length sRNA, the EM structure and the derived di-sRNP model also explain the reduction in catalytic activity of sRNPs assembled with mutant sRNA lacking either a functional C/D or C'/D' motif (similar to the one used for partial box C/D sRNP crystallization discussed above) and also mutant Nop5 protein components (Omer *et al.*, 2002; 2006; Rashid *et al.*, 2003; Tran *et al.*, 2003; Zhang *et al.*, 2006). Interpretation of these results in light of the di-sRNP model suggest that the decrease in catalytic activity is caused by a lack of efficient di-sRNP assembly. Indeed, Nop5 coiled-coil domain mutants and sRNAs containing mutations in the conserved sequence elements that interfere with L7Ae binding assemble into RNPs that are distinct from the di-sRNP (Bleichert *et al.*, 2009; Bleichert and Baserga, submitted). This altered RNP structure likely interferes with the correct positioning of the guide:substrate RNA duplex with respect to the active site of fibrillarin.

Catalytically active box C/D sRNPs have been previously successfully assembled with RNA and protein components from other archaeal species as well, and biochemical studies aimed at deciphering the requirement for certain protein or RNA elements for sRNP assembly and enzymatic activity mostly yielded similar results (Clouet d'Orval *et al.*, 2001; Omer *et al.*, 2002; Rashid *et al.*, 2003; Tran *et al.*, 2003; Singh *et al.*, 2004). In addition, all common box C/D proteins are highly conserved between archaeal species, and crystal structures of homologous proteins or protein complexes revealed a similar topology (Aittaleb *et al.*, 2003; Oruganti *et al.*, 2007; Ye *et al.*, 2009). Taken together, this indicates that box C/D sRNPs from different archaeal species likely have a common 3D structure. We propose that this is the di-sRNP that we have observed with an *M. jannaschii* sRNP. Future analysis of sRNPs from different archaeal species will be necessary to test this hypothesis.

### *Di-sRNPs in archaea* in vivo

Although archaeal box C/D sRNPs have been extensively studied *in vitro*, information on their composition *in vivo* is limited. This is due to the extreme growth conditions (i.e. high temperatures and anaerobic environment) of these organisms and the limited genetic tools available to manipulate protein expression. The results of a few *in vivo* studies with archaeal box C/D sRNPs, however, are consistent with a di-sRNP structure of these complexes. For example, in *Sulfolobus acidocaldarius* cell extracts, Nop5 and fibrillarin migrated 10–50 S in glycerol gradients (Omer *et al.*, 2000). While the lower end of this broad peak is in agreement with an archaeal box C/D di-sRNP, the larger complexes most likely reflect the association of box C/D sRNPs with pre-ribosomal RNAs. Isolation of these heterogeneous complexes and determination of their composition will be crucial to establish that the di-sRNP as observed *in vitro* is formed *in vivo* as well. On the other hand, it

has been suggested that archaeal box C/D sRNPs may only assemble transiently during ribosome biogenesis (Ciammaruconi *et al.*, 2008). To what extent these contradictory interpretations reflect different experimental approaches remains uncertain.

### *The prospect of eukaryotic box C/D di-snoRNPs*

The components and the methylation function of box C/D s(no)RNPs are more or less conserved between archaea and eukaryotes, yet eukaryotic box C/D snoRNPs have so far not been successfully reconstituted from individual components *in vitro*. This has greatly impeded structural studies of these macromolecules. Nevertheless, biochemical studies indicate that profound differences exist in the predicted RNP organization and functional RNP characteristics between the two kingdoms: (1) while in archaea boxes C' and D' are identical to boxes C and D, these sequence elements are more degenerate in eukaryotes and often not identical in sequence to boxes C and D (Kiss-Laszlo *et al.*, 1996; 1998; Omer *et al.*, 2000); (2) in archaea, the length of the spacer sequences between boxes C and D' and boxes C' and D, which harbor the guide sequences, is well conserved and constrained to about 12 nucleotides, whereas in eukaryotes these spacer sequences are usually longer and can contain more than one hundred nucleotides (Tran *et al.*, 2005); and (3) biochemical studies suggest differences in the organization of the archaeal and eukaryotic complexes. While all archaeal core proteins assemble symmetrically onto both the C/D and C'/D' motifs, the eukaryotic protein assemble asymmetrically (Cahill *et al.*, 2002; Szewczak *et al.*, 2002; 2005).

Crosslinking studies of box C/D snoRNAs containing a photoactivatable 4-thiouridine at different positions, which assembled into snoRNPs in Xenopus oocytes, demonstrated that Nop58 directly associates with the C/D motif, whereas Nop56 associates with the C'/D' motif. Fibrillarin, the methyltransferase, can be crosslinked to both motifs (Cahill *et al.*, 2002). In addition to the asymmetric assembly of Nop58 and Nop56, the L7Ae homolog, 15.5K, only binds to the terminal C/D motif but not the internal C'/D' motif (Szewczak *et al.*, 2002; 2005). Consistent with this asymmetric arrangement of Nop56, Nop58, and 15.5K in the RNP, only 15.5K and Nop58 are required for snoRNA stability *in vivo*, but not Nop56 (Lafontaine and Tollervey, 1999; 2000; Watkins *et al.*, 2000). Interestingly, Nop58 and Nop56, despite being highly homologous to each other and to archaeal Nop5, are both essential proteins in eukaryotes, indicating distinct functions of these proteins within the RNP (Gautier *et al.*, 1997). However, Nop58 and Nop56 most likely still form heterodimers within the RNP since they interact weakly with each other in the yeast-two-hybrid assay and since both proteins are able to associate with an RNA that contains the C/D motif but lacks the C'/D' motif (Watkins *et al.*, 1998; 2002; Boulon *et al.*, 2008).

Taken together, these results suggest a pseudosymmetric architecture of eukaryotic box C/D snoRNPs when compared to their archaeal counterparts. But is the dimeric organization of archaeal box C/D sRNPs conserved in eukaryotes as a di-snoRNP? While results of studies of eukaryotic box C/D snoRNPs to date are consistent or either a mono- and di-snoRNP architecture, we favor the di-snoRNP model (Figures 1E and 1F). Interestingly, in Euglena, box C/D snoRNPs sediment between 250 and 600 kDa in glycerol gradients, a size that is more compatible with a di-snoRNP than with a mono-snoRNP (Russell *et al.*, 2006). More careful sizing of these RNPs will be necessary to better discriminate between these possibilities. Eukaryotic scaRNPs (small Cajal body RNPs) often contain box C/D and box H/ACA hybrid RNAs, and hence function as 2'-O-ribose methylases and pseudouridylases of snRNAs (Jady and Kiss, 2001; Darzacq *et al.*, 2002). It will be interesting to see how the H/ACA RNP influences box C/D RNP architecture, mono- or di-snoRNP, and vice versa. A full understanding of the architectural differences between archaeal and eukaryotic box C/D RNPs and their functional consequences awaits detailed, high-resolution information on their structures with investigation of how disruption of the structure affects the function of these RNP enzymes.

### *Why a di-s(no)RNP?*

The dimeric structure of box C/D sRNPs raises questions regarding the advantages that a di-sRNP may provide over a conventional mono-sRNP. While the answer to this question is yet unknown, we can speculate about several different possibilities. Many archaeal organisms are extremophiles. Interestingly, the number of 2'-O-ribose methylations, and of predicted box C/D sRNAs, increases with higher optimal growth temperature of the organism (Dennis *et al.*, 2001). A dimeric sRNP structure may provide advantages in these conditions and increase the stability of box C/D sRNPs. In addition to complex stability, a di-sRNP structure could have other advantages *in vivo*. Because the di-sRNP has twice the number of sRNAs, it also has twice the number of guide sequences that a mono-sRNP would. This doubles the local concentration of substrate base paired to the guide sequences and could provide a kinetic advantage, especially if enzymatic activity were allosterically regulated. Besides their primary function of catalyzing 2'-O-ribose methylations, box C/D s(no)RNPs also participate in RNA folding, particularly of the pre-ribosomal RNA (pre-rRNA). Four guide sequences in a di-sRNP could also be advantageous in facilitating rRNA folding, since the number of regions of rRNA base paired to the sRNP would increase. One

intriguing possibility is that di-s(no)RNPs could contain two different s(no)RNAs and, hence, contain four different guide sequences that base pair to completely different regions in the rRNA, thereby bringing these regions into close proximity. However, this scenario also presents a challenge to cells, as they would need to control the pairing of different sRNAs in a di-sRNP to favor functionally valuable combinations. Whether this is indeed the case remains to be addressed in future studies.



## Is telomerase a di-RNP?

### *The function of telomerase*

With each cell division, the ends of eukaryotic chromosomes shorten due to the inability of conventional DNA polymerases to replicate the far ends of linear chromosomes, the telomeres (Watson, 1972; Olovnikov, 1973). To overcome this "end-replication problem" and to maintain chromosome termini, telomerase, a specialized ribonucleoprotein enzyme, elongates the ends of the linear eukaryotic chromosomes by adding tandem repeats of G-rich sequences to the 3' end of one strand of telomeres (Greider and Blackburn, 1985; 1987). While a telomerase-based mechanism to counteract telomere erosion is prevalent in eukaryotes, alternative telomerase-independent ways exist. *Drosophila*, for example, uses a retrotransposition mechanism to add repeat sequences to its chromosome ends (reviewed in Mason *et al.*, 2008). The repeat sequences added at telomeres by telomerase (TTAGGG in vertebrates, TTGGGG in Tetrahymena) are recognized by specific telomere-binding proteins, forming specialized nucleoprotein structures (reviewed in Palm and de Lange, 2008; Linger and Price, 2009). Besides regulating telomerase activity, and hence telomere length, telomere-binding proteins also distinguish natural chromosome ends from double-strand DNA breaks, thereby repressing DNA damage response and recombination. Thus, telomeres and telomere maintenance are essential for chromosome integrity and genome stability.

Telomerase was first discovered in the ciliate *Tetrahymena thermophila* in 1985 by Greider and Blackburn, who subsequently showed that this enzyme contained an integral RNA component that is essential for telomerase catalytic activity (Greider and Blackburn, 1985; 1987). Together with Jack Szostak, they received the 2009 Nobel Prize in Physiology and Medicine for their discovery. Research over the past decades identified the telomerase enzyme in other eukaryotes as well, including other ciliates, yeast, vertebrates and plants (for review see Autexier and Lue, 2006). A common feature of all telomerases is that the core enzyme components sufficient for enzymatic activity *in vitro* are telomerase RNA and telomerase reverse transcriptase (TERT), with the RNA providing the template for DNA synthesis by TERT (Greider and Blackburn, 1989; Singer and Gottschling, 1994; Feng *et al.*, 1995; Lingner *et al.*, 1997; Nakamura *et al.*, 1997; Weinrich *et al.*, 1997; Beattie *et al.*, 1998; Collins and Gandhi, 1998; Wenz *et al.*, 2001; Zappulla *et al.*, 2005). *In vivo*, optimal telomerase activity requires accessory proteins that are associated with the core enzyme to form the telomerase holoenzyme (Collins *et al.*, 1995; Lingner and Cech, 1996; Nugent *et al.*, 1996; Harrington *et al.*, 1997; Nakayama *et al.*, 1997; Mitchell *et al.*, 1999; Seto *et al.*, 1999; Chandra *et al.*, 2001; Chai *et al.*, 2002; Taggart *et al.*, 2002; Friedman *et al.*, 2003; Stellwagen *et al.*, 2003; Bianchi *et al.*, 2004; Chen and Greider, 2004; Ting *et al.*, 2005). These proteins mediate and regulate telomerase RNP assembly and stability, telomerase subcellular localization, telomerase association with telomeres, and enzyme processivity. While in unicellular eukaryotes telomerase is continuously expressed to ensure indefinite propagation, in multicellular organisms, including humans, telomerase is expressed in germline cells and certain stem cells but is undetectable in somatic cells (Counter *et al.*, 1992; Wright *et al.*, 1996; Meyerson *et al.*, 1997). Hence, telomeres of mortal somatic cells shorten with consecutive divisions, which contributes to replicative aging, providing a protective mechanism against cell immortalization and indefinite cell proliferation.

Because of the importance of telomerase in maintaining genomic stability and because of its connection to cell proliferation it is not surprising that dysregulation of telomerase expression or activity contributes to the pathogenesis of human diseases (Calado and Young, 2009). Most cancer cells, for instance, reactivate or upregulate telomerase to prevent cellular senescence and to allow continuous cell proliferation (Artandi and DePinho, 2010). On the other hand, Dyskeratosis congenita is a genetic disorder that is associated with insufficient telomerase activity (reviewed in Walne and Dokal, 2009 and Freed *et al.*, 2010).

In the following part of this review we will briefly discuss the organization of the core telomerase RNP, specifically focusing on the controversy about its multimerization properties and the resulting implications for the mechanism of telomerase action.

### *Telomerase components*

#### Telomerase RNA

Cloning of the core telomerase components from different species allowed reconstitution of this enzyme from recombinant components *in vitro*, helping to delineate the role of each component in RNP assembly and

catalysis. The telomerase RNAs vary greatly in sequence and length among eukaryotes, ranging from ~150 nucleotides in ciliates to > 1 kb in yeast. However, secondary structure determination of telomerase RNAs by phylogenetic comparison predicts common features that have subsequently been shown to be important for telomerase function (Romero and Blackburn, 1991; Chen *et al.*, 2000; Mitchell and Collins, 2000; Lai *et al.*, 2002; 2003; Chappell and Lundblad, 2004; Dandjinou *et al.*, 2004; Lin *et al.*, 2004; Zappulla and Cech, 2004). These include the template region, 5' boundary elements, a central pseudoknot structure, additional domains that bind TERT and are critical for catalysis (trans-activation domain), and species-specific domains. The latter regulates telomerase RNA processing, RNP assembly, localization, and stability *in vivo* but is dispensable for catalytic activity *in vitro*, e.g. the box H/ACA domain in vertebrates and the Sm-protein binding domain in yeast (for review see Theimer and Feigon, 2006). Besides providing the template for telomeric DNA repeat synthesis and binding sites for the TERT protein, telomerase RNA also participates in regulating catalytic activity and processivity of the enzyme (e.g. Prescott and Blackburn, 1997b; Roy *et al.*, 1998; Licht and Collins, 1999; Tzfati *et al.*, 2000; Bachand and Autexier, 2001; Comolli *et al.*, 2002; Chen and Greider, 2003; Lai *et al.*, 2003; Moriarty *et al.*, 2004).

**Telomerase reverse transcriptase (TERT)**

TERT contains a central reverse transcriptase (RT) domain that is essential but not sufficient for telomerase RNP assembly and activity (Figure 2A). Mutations in predicted active site residues in the RT motifs abolish telomerase activity both *in vivo* and *in vitro*, suggesting TERT is the catalytic subunit of the telomerase RNP (Lingner *et al.*, 1997; Nakamura *et al.*, 1997; Bryan *et al.*, 1998). Additionally, TERT can catalyze RNA- and template-independent DNA synthesis *in vitro* using its intrinsic terminal transferase activity, though the physiological importance of this activity is unknown (Lue *et al.*, 2005). Flanking the central RT domain of TERT proteins are the N-terminal and C-terminal domains, respectively, both of which confer telomerase-specific properties to the reverse transcriptase (Figure 2A). The N-terminal domain of TERT contains motifs conserved among ciliates, yeast, and humans (for reviews see Xia *et al.*, 2000; Autexier and Lue, 2006). Two different domains mediate telomerase RNA binding, although the regions of the RNA bound to TERT differ among species (Friedman and Cech, 1999; Bryan *et al.*, 2000b; Lai *et al.*, 2001; Moriarty *et al.*, 2002; Friedman *et al.*, 2003). The C-terminal domain of TERT is important for nucleotide and repeat addition processivity of telomerase (Peng *et al.*, 2001; Hossain *et al.*, 2002; Huard *et al.*, 2003). The latter has also been shown to be influenced by the N-terminus of TERT (Moriarty *et al.*, 2004). Interestingly, both termini of TERT have been implicated to promote telomerase multimerization (discussed below) (Beattie *et al.*, 2001; Arai *et al.*, 2002; Wang *et al.*, 2002). In addition to RNA binding, TERT also has affinity for DNA which seems important for stabilizing the association of the RNA template with the DNA primer during catalysis and allows efficient telomeric DNA elongation (Bryan *et al.*, 2000a; Hossain *et al.*, 2002; Wyatt *et al.*, 2007; Finger and Bryan, 2008).

***Structural studies of core telomerase components***

To fully understand how different parts of telomerase RNA and TERT mechanistically contribute to telomerase activity, structural information is necessary. While a structure of the core telomerase RNP is not yet available, significant progress has been made towards this goal by the determination of structures of telomerase subunits. RNA structures were determined by NMR and encompass regions of the CR4-CR5 domain and the conserved pseudoknot of human telomerase as well as helix II of *Tetrahymena thermophila* telomerase RNA, which serves as the 5' template boundary element (Leeper *et al.*, 2003; Leeper and Varani, 2005; Theimer *et al.*, 2005; 2007; Richards *et al.*, 2006; Kim *et al.*, 2008; Qiao and Cech, 2008). These structures provide a framework for interpretation of a variety of biochemical observation with mutationally altered telomerase components (reviewed in Theimer and Feigon, 2006; Sekaran *et al.*, 2010).

In addition, two different fragments of the N-terminal domain of *Tetrahymena thermophila* TERT, the far N-terminal domain (TEN) and the telomerase RNA binding domain (TRBD), have been crystallized (Figure 2A) (Jacobs *et al.*, 2006; Rouda and Skordalakes, 2007). The TEN domain represents a novel protein fold and is suggested to contact telomeric DNA in a sequence specific manner, rationalizing the importance of this domain in telomerase activity (Jacobs *et al.*, 2006). The TRBD RNA binding pocket is formed by the highly conserved T and CP motifs, and the structure suggests that it can bind both single and double stranded nucleic acids (Rouda and Skordalakes, 2007). Yet, the most striking advancement in understanding TERT function comes from the crystal structure of full-length TERT from the red flour beetle, *Tribolium castaneum* (Gillis *et al.*, 2008). Surprisingly, this structure revealed a ring-like arrangement of the different TERT domains, mediated by extensive interactions between the N-terminal TRBD domain and the C-terminal domain. In comparison to viral reverse transcriptases, RNA polymerases and some DNA polymerases, the TERT RT domain provides fingers and palm, and the TERT C-terminus corresponds to the thumb. The central ring contains the active site of TERT and is 26 Å wide, large enough to accommodate a DNA–RNA duplex. Indeed, in subsequent co-crystals of TERT and an RNA–DNA hybrid, the RNA–DNA duplex passes

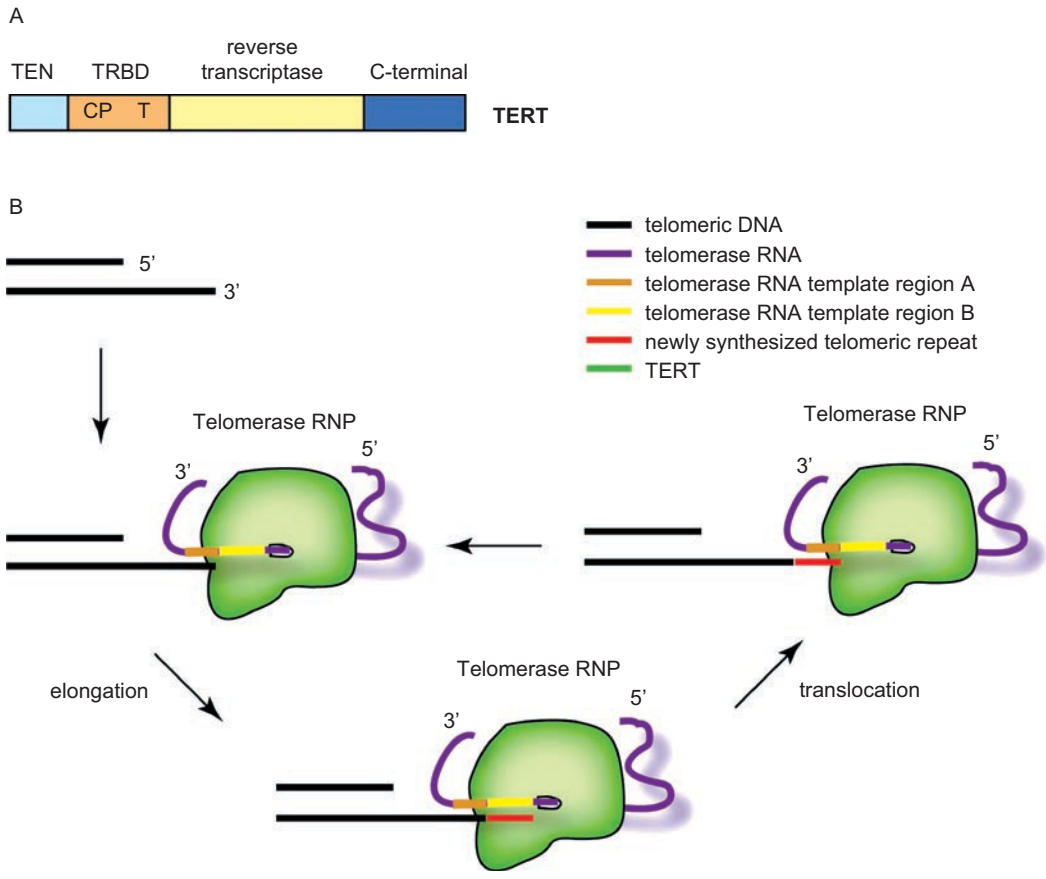


**Figure 2.** Telomerase RNP function. (A) Domain organization of telomerase reverse transcriptase (TERT). (B) Repeat addition cycle for telomerase. Telomerase RNA base pairs to 3' single stranded overhangs of chromosome ends and TERT reverse transcribes the template sequence into the telomeric DNA. Once the template boundary is reached, telomerase can translocate and catalyze the addition of another telomere repeat, which involves realignment of the 3' template sequence of telomerase RNA with the newly synthesized repeat sequence.

through the hole and the 3' end of the telomeric DNA primer is placed at the active site (Mitchell *et al.*, 2010). Future structures of the complete telomerase core enzyme will undoubtedly bring more mechanistic insight into telomerase function and the associated conformational changes that must occur during nucleotide addition and telomerase translocation.

### *Evidence for telomerase multimerization*

Research over the past 20+ years has indicated that telomerase may function as a dimer. Certainly, the evidence for telomerase multimerization is compelling in some species. However, at the same time there is also some disagreement as to whether telomerase is a dimer or monomer, even in a single species. To what extent these discrepancies reflect different experimental approaches and conditions remains to be determined. In the following paragraphs we will discuss both the functional as well as physical evidence for and against telomerase RNP dimerization.

#### Functional evidence for telomerase multimerization

The first indication that telomerase may function as a multimer *in vivo* came from seminal observations by Prescott and Blackburn that certain mutations in the template region of the telomerase RNA rendered the yeast telomerase RNP inactive in the absence but not in the presence of wild type telomerase RNA (Prescott and Blackburn, 1997a; 1997b). The efficient co-purification of two DNA oligonucleotides complementary to the template region of telomerase RNA, one of which served as biotinylated bait, supported the conclusion that, in yeast, two telomerase RNAs function in a single complex. Therefore, telomerase could be a dimer (Prescott and Blackburn, 1997a). Subsequent studies with human telomerase yielded similar results. Truncated human telomerase RNAs that assemble into inactive RNPs were able to template DNA synthesis in the presence of wild type telomerase RNA (Tesmer *et al.*, 1999). Alternatively, two different inactive RNAs were able to functionally interact, restoring telomerase activity (Ly *et al.*, 2003).

Analogously, two different inactive TERT proteins could complement each other's function when co-expressed *in vivo* or *in vitro* (Beattie *et al.*, 2001). Moreover, some RNA or TERT mutants can also negatively affect the activity of wild type components of telomerase (Wenz *et al.*, 2001; Arai *et al.*, 2002).

**Biochemical sizing of ciliate telomerase RNPs**

In addition to the functional evidence for telomerase multimerization, sizing of telomerase RNPs provided direct biochemical evidence that the active telomerase complex from multiple species is a dimer (Table 1). Gel filtration chromatography of ciliate telomerases from *Euplotes crassus* and *Euplotes aediculatus* yielded a molecular weight that is consistent with a dimeric RNP architecture (Greene and Shippen, 1998; Wang *et al.*, 2002; Aigner *et al.*, 2003; Fouche *et al.*, 2006). Intriguingly, *Euplotes crassus* telomerase can be found in differently sized complexes with distinct biochemical characteristics depending on the developmental stage of the organism, ranging from 280–400 kDa in vegetative cells to > 5 MDa in cells undergoing macronuclear development (Greene and Shippen, 1998). While the exact composition or oligomeric state of these different RNPs is unknown, they all contain at least two active sites as judged by co-purification of regular DNA primers with biotinylated DNA primers complementary to the template region of telomerase RNA (Wang *et al.*, 2002). Unlike *Euplotes* species, *Tetrahymena thermophila* telomerase behaved as a monomer by gel filtration chromatography and was not able to bind two DNA primers simultaneously, making it likely that it contains only one active site (Bryan *et al.*, 2003).

**Biochemical sizing of human telomerase RNPs and determination of the copy number of telomerase RNAs per telomerase**

Sizing of the human telomerase RNP directly from cell lysates and after purification revealed that, while human telomerase can exist as a large holoenzyme *in vivo* with a molecular weight of at least 1000 kDa, purification of this enzyme resulted in dissociation of probably loosely associated components, leaving an enzymatically active core telomerase complex of 550–650 kDa (Schnapp *et al.*, 1998; Wenz *et al.*, 2001; Cohen *et al.*, 2007). This core RNP enzyme is composed of telomerase RNA, TERT, and dyskerin, a protein required for RNP assembly and stability (Mitchell *et al.*, 1999; Cohen *et al.*, 2007). Co-assembling telomerase RNPs with wild-type and mutant RNAs allowed quantification of the RNA content of these RNPs after purification, revealing directly for the first time two telomerase RNAs per RNP (Wenz *et al.*, 2001). Thus, the human core telomerase RNP is likely composed of each of two

**Table 1.** Summary of experimental size measurements of telomerase from different species.

| Species | Multimerization state | Measured molecular weight or S value | Source of telomerase | Method used | References |
|---|---|---|---|---|---|
| **Ciliates:** | | | | | |
| *Tetrahymena thermophila* | Monomer | 180–220 kDa | Recombinant | Gel filtration chromatography | (Bryan *et al.*, 2003) |
| *Euplotes crassus* | Dimer or higher order complex | 280–400 kDa, 550 kDa, 1.6 MDa,> 5 MDa[a] | Macronuclear extract | Gel filtration chromatography | (Greene and Shippen, 1998; Wang *et al.*, 2002) |
| *Euplotes aediculatus* | Monomer | 11.5–12.5 S or 230 kDa | Affinity-purified[b] or nuclear extract | Glycerol gradient centrifugation | (Lingner and Cech, 1996) |
| | Dimer | 450–500 kDa | Nuclear extract | Gel filtration chromatography | (Aigner *et al.*, 2003) |
| | Dimer | 400 kDa | Affinity-purified[b] | Gel filtration chromatography and EM images | (Fouche *et al.*, 2006) |
| **Vertebrates:** | | | | | |
| Human | Dimer | 550 kDa<br>1000 kDa | Affinity-purified<br>Nuclear extract | Glycerol gradient centrifugation | (Schnapp et al., 1998) |
| | Dimer | 600 kDa | Recombinant or affinity-purified | Gel filtration chromatography | (Wenz *et al.*, 2001) |
| | Dimer | 650–670 kDa | Whole-cell lysate or affinity-purified[c] | Glycerol gradient centrifugation | (Cohen *et al.*, 2007) |
| | Monomer | 277 kDa | Recombinant | Single-molecule fluorescence two-color coincidence analysis | (Alves *et al.*, 2008) |

[a] The size of the telomerase is developmentally regulated, with the 280 kDa complex found in the vegetative stage of the life cycle, and the larger complexes found during macronuclear development. The oligomeric state and composition of the larger complexes is unknown.
[b] Affinity-purified telomerase also contains p43 besides TERT (p123 in *E. aediculatus*).
[c] Affinity-purified telomerase also contains the protein dyskerin besides TERT.

copies of RNA, TERT, and dyskerin, which account for the observed molecular weight.

**Telomerase dimerization is not obligate for function**

While there is much evidence that telomerase can be found and can function as a dimer in yeast and humans, recent biochemical results suggest that telomerase multimerization may not be obligate. First, genetic and biochemical studies of some yeast telomerase RNA or TERT mutants indicated that telomerase may also exist as a monomer under some conditions (Livengood *et al.*, 2002; Friedman *et al.*, 2003). Moreover, reconstituted catalytically active human telomerase was recently found to be a monomer, containing one TERT and one RNA, by two color coincidence detection analysis, a single molecule fluorescence assay (Alves *et al.*, 2008). Thus, besides the apparent disparities in mulimerization states between telomerases from diverse ciliate species, i.e. a dimer in *Euplotes sp.* and a monomer in *Tetrahymena thermophila*, differences in multimerization states can also exist in a single species (i.e. humans). Resolving these apparent inconsistencies and determining the potential functional consequences of different telomerase multimerization states *in vivo* is an important goal for future studies.

**How is dimerization accomplished in the telomerase RNP?**

With the experimental evidence pointing towards telomerase being able to function as a dimer in several species, questions arise as to how dimerization is achieved and how components are organized within dimeric RNPs. Dimerization could occur via protein–protein interaction, RNA–RNA interaction, or by a combination of both. Here we will discuss these possibilities with respect to the current knowledge of TERT and telomerase RNA oligomerization.

Human TERT can oligomerize both *in vitro* and *in vivo* in the absence of telomerase RNA (Beattie *et al.*, 2001; Arai *et al.*, 2002; Moriarty *et al.*, 2002). The interaction domains have been mapped to amino acids 301–538 in the N-terminal region and amino acids 914–1132 in the C-terminal region of human TERT, suggesting a tail-to-tail association of TERT molecules (Arai *et al.*, 2002). A second domain in the N-terminus, amino acids 1–200, has also been suggested to interact directly with the remainder of human TERT (Moriarty *et al.*, 2004). Likewise, *Euplotes crassus* TERT forms oligomers *in vitro* (Wang *et al.*, 2002). In addition to the head-to-tail association observed with human TERT, *Euplotes crassus* TERT is able to associate in a head-to-head and tail-to-tail fashion as well. This substantiates the hypothesis that telomerase dimerization could be mediated by association of two TERT proteins in these organisms. Interestingly, however, *Tribolium castaneum* TERT, the structure of which was determined by X-ray crystallography, is a monomer in solution, possibly indicating that the telomerase RNP may be also a monomer in this organism. Furthermore, the structure of *Tribolium* TERT shows that the N- and C-termini of the same molecule interact with each other to form a ring-like structure (Gillis *et al.*, 2008). Importantly, the amino acid residues involved in this ring formation reside in regions that correspond to the N (amino acids 301–538) - and C-terminal (amino acids 914–1132) fragments of human TERT that have been suggested to mediate oligomerization (Arai *et al.*, 2002). Whether the observed head-to-tail association of human TERT reflects a similar ring formation or true TERT dimerization remains to be determined.

In addition to TERT oligomerization, telomerase RNA oligomerization could mediate RNP dimerization. Indeed, human telomerase RNA can dimerize *in vitro* in the absence of TERT by forming an intermolecular pseudoknot, and mutations in telomerase RNA that prevent intramolecular pseudoknot formation, but allow trans-pseudoknot formation, assemble into active RNPs (Ly *et al.*, 2003). Although this suggests that this interaction is biologically relevant, an intermolecular pseudoknot is not absolutely required for telomerase activity (Chen and Greider, 2005). A second region that can promote dimerization is the J7b/8a loop near the 3' end of telomerase RNA, connecting the H/ACA and CR7 domains (Ren *et al.*, 2003). Interestingly, the Dyskeratosis congenita mutation C408G in the adjacent CR7 domain reduces dimerization of telomerase RNA *in vitro* (Ren *et al.*, 2003). In contrast to human and *Euplotes* telomerase RNA or TERT, *Tetrahymena thermophila* telomerase components do not oligomerize, which is consistent with a monomeric telomerase RNP in this organism (Bryan *et al.*, 2003). Taken together, at this point it is not well understood how telomerase di-RNP formation is achieved. Structures of the core telomerase RNP will be crucial for understanding how telomerase RNA interacts with TERT and whether and how multimerization is achieved.

### *The functional significance of telomerase multimerization*

Adding telomeric repeats to chromosome ends by telomerase requires annealing of the single stranded 3' telomeric DNA primer to the 3' region of the template sequence of telomerase RNA (Figure 2B). Telomerase then elongates this primer until the template boundary at the 5' end of the telomerase RNA template region is reached. Processive activity of telomerase requires translocation and reannealing of the newly synthesized telomeric sequence with the 3' end of the telomerase RNA template region, initiating synthesis of the next

repeat. Since a telomerase RNP monomer can complete such cycles, how could a dimerization of telomerase contribute to its function?

Several models of how a telomerase dimer may function have been proposed. The first model is the parallel extension model. This model suggest that the two active sites of dimeric telomerase function simultaneously, extending two different DNA strands (Prescott and Blackburn, 1997a; Wenz *et al.*, 2001; Ly *et al.*, 2003). The different DNA strands could be sister chromatids or telomeres from different chromosomes (Prescott and Blackburn, 1997a). In a second model, the template switching model, the two protomers in a telomerase dimer work cooperatively together to extend a single DNA substrate (Wenz *et al.*, 2001; Ly *et al.*, 2003). The active sites do not function at the same time but sequentially, associated with the transfer of the DNA substrate from one RNA template region to the other during telomerase translocation after addition of a telomeric repeat is complete. This could explain the observation that wild-type and mutant telomere repeat sequences are interdispersed in some organisms upon co-expression of telomerase RNAs with different template sequences (Yu and Blackburn, 1991; Prescott and Blackburn, 1997b). However, recent studies with mutant pseudoknot telomerase RNAs that assemble in obligate telomerase dimers which can only utilize one of the template sequences suggests that template switching is not essential for processive DNA synthesis (Rivera and Blackburn, 2004). Thirdly, the anchor site model proposes that the same protomer in a telomerase dimer is catalytically active at all times, whereas the other is inactive but instead binds to telomeric DNA 5' to the DNA primer site via an anchor site in TERT, thereby stabilizing the association of the DNA primer with the template RNA (Collins and Greider, 1993; Hammond *et al.*, 1997; Wenz *et al.*, 2001; Kelleher *et al.*, 2002; Jacobs *et al.*, 2006). Maintaining the anchor site interaction with DNA during translocation could prevent telomerase dissociation from the telomere and contribute to repeat addition processivity of telomerase. These models are not mutually exclusive.

## Multimeric snRNPs in pre-mRNA splicing

The coding regions of eukaryotic pre-mRNAs, exons, are interrupted by non-coding regions, introns, that need to be excised for faithful translation of mRNAs into proteins by ribosomes. The removal of intronic sequences and subsequent ligation of exons occur as two transesterification reactions, catalyzed by the spliceosome, a highly dynamic macromolecular machine containing five different small nuclear RNAs (snRNAs) and as many as 300 protein factors (reviewed in Jurica and Moore, 2003; Smith *et al.*, 2008; Wachtel and Manley, 2009; Wahl *et al.*, 2009). The snRNAs and their RNA–protein complexes, snRNPs, perform key functions during splicing and dynamically interact with each other through an intricate network of RNA–RNA or RNA–protein interactions. First, the U1 snRNP recognizes the 5' splice site by snRNA–mRNA base pairing whereas the U2 snRNP associates with the branch point in the intron to be excised forming the pre-splicesome, or complex A. Subsequently, U4, U5, and U6 snRNPs are recruited together as a preformed complex, the U4/U6.U5 tri-snRNP, which is assembled of the U4/U6 di-snRNP and the U5 mono-snRNP (Figure 3). Activation of this complex B results in formation of the catalytic center for the first transesterification reaction and requires substantial structural rearrangements. The RNA–RNA base pairing interactions between the U4 and U6 snRNAs in the di-snRNP and in the U4/U6.U5 tri-snRNP are unwound, allowing U6 snRNA to base pair with the U2 snRNA and with the 5' splice site of the pre-mRNA. The U1 and U4 snRNAs dissociate from the splicing machinery prior to the first splicing reaction, which results in cleavage at the 5' splice site and lariat formation of the intron. Alignment of the two exons by U5 snRNA ensures that both exons are joined and ligated during the second step of splicing.

While the vast majority of pre-mRNAs are spliced by the major spliceosome described above, a fraction of pre-mRNAs (< 1%) contain introns with non-canonical consensus sequences that are removed by a second spliceosome, the minor spliceosome (reviewed in Tarn and Steitz, 1997; Patel and Steitz, 2003). While the U5 snRNA is part of both types of spliceosomes, the other snRNAs of the major spliceosome are replaced by snRNAs of analogous functions, namely U11 (for U1), U12 (for U2), U4atac, and U6atac snRNAs. In contrast, many protein factors are shared between both types of spliceosome (Will *et al.*, 1999; Schneider *et al.*, 2002), and it is thought that catalysis in the minor spliceosome occurs by a similar mechanism to that in the major spliceosome. Interestingly, one of the main differences between minor and major spliceosome snRNPs is that, in contrast to U1 and U2 snRNPs, U11 and U12 snRNPs associate with the pre-mRNA as a preformed complex, a dimeric RNP (Wassarman and Steitz, 1992b).

In this last section we will discuss the composition and mutimerization of the dimeric and trimeric splicing snRNPs and how multimerization is thought to contribute to the function of these RNPs. We will focus on the U4/U6 di-snRNP and U4/U6.U5 tri-snRNP of the major spliceosome, but similar principles govern the assembly and function of the analogous RNPs in the minor spliceosome, namely U4atac/U6atac di-snRNP and U4atac/U6atac.U5 tri-snRNP (Schneider *et al.*, 2002). The U11/U12 di-snRNP is specific to the minor spliceosome, replacing the U1 and U2 snRNPs found in the major spliceosome.

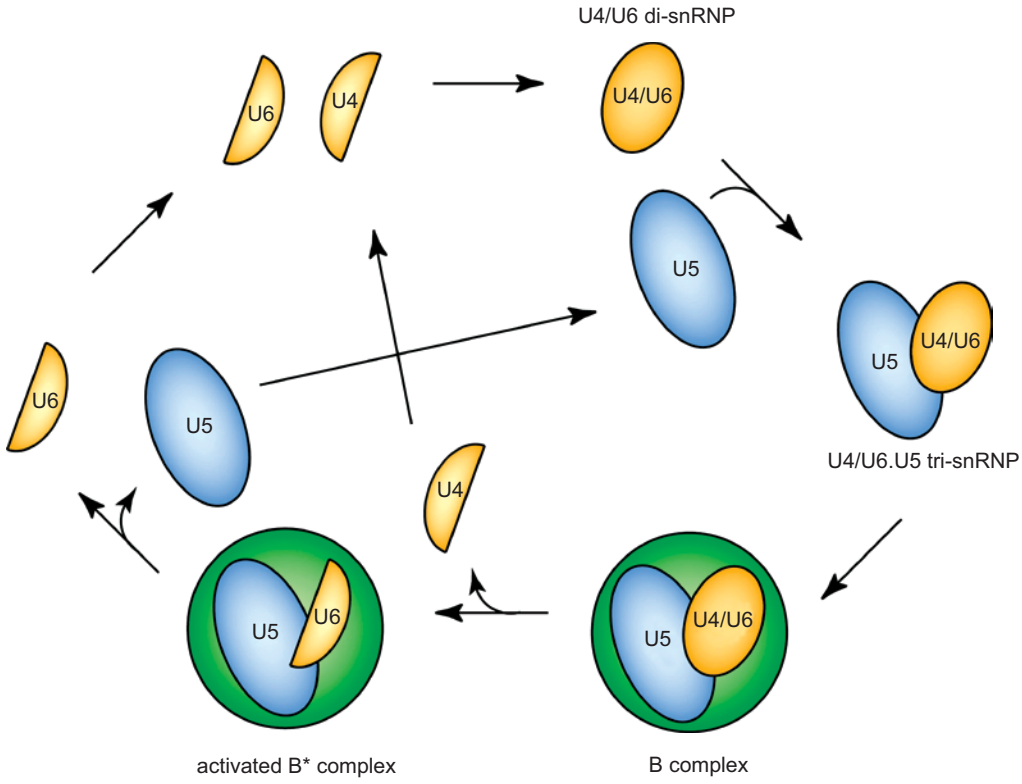


**Figure 3.** Assembly and recycling of U4/U6.U5 tri-snRNP during pre-mRNA splicing. The U4/U6 di-snRNP is formed by association of the U4 and U6 snRNPs through base pairing of their snRNA components. Subsequently, the U5 snRNP joins to form the U4/U6.U5 tri-snRNP. Recruitment of the U4/U6.U5 tri-snRNPs into the spliceosome results in formation of the B complex. Activation of the spliceosome requires structural rearrangements that result in release of the U4 snRNP. After splicing is completed, U5 and U6 snRNPs are recycled, joining the U4 snRNP to form U4/U6 di-snRNPs and U4/U6.U5 tri-snRNPs, respectively.



### *U4 and U6 snRNPs dimerize to form a U4/U6 di-snRNP*

The U4/U6 di-snRNP is composed of both the U4 snRNP and U6 snRNP monoparticles, and hence contains the snRNAs and protein components of both snRNPs (Figure 4).

The U4 snRNA associates with seven Sm proteins and, in humans, five specific U4 proteins to form the U4 snRNP. Two key elements in the U4 snRNA are crucial for recruitment of these proteins: (1) the Sm binding site in the 3' region of the U4 snRNA is bound by the Sm proteins SmB/B', D1, D2, D3, E, F, and G, forming a heptameric ring encircling the RNA (Kambach *et al.*, 1999; Urlaub *et al.*, 2001); (2) the 5' stem loop in the U4 snRNA is bound by 15.5K (Snu13 in yeast), which is a prerequisite for binding of all remaining U4-specific proteins (Nottrott *et al.*, 1999; 2002). The crystal structure of the U4 5' stem-loop bound to 15.5K demonstrated that 15.5K recognizes an RNA structural motif known as the k-turn motif. Together, the U4 snRNA–15.5K complex provides a composite binding platform for another U4-specific protein, hPrp31 (Makarova *et al.*, 2002; Liu *et al.*, 2007). Interestingly, 15.5K/Snu13 is also a component of box C/D sRNPs, and Nop56 and Nop58 are homologous to hPrp31, suggesting a common evolutionary ancestor of these proteins and a similar arrangement of these proteins in box C/D sRNPs as found in the U4 snRNP (Watkins *et al.*, 2000).

hPrp3, hPrp4, and the cyclophillin hCypH are also components of the U4 mono-snRNP and of the U4/U6 di-snRNP. They form a stable, RNA-independent heteromeric complex with each other (Horowitz *et al.*, 1997; Teigelkamp *et al.*, 1998). In this complex, hPrp4 directly interacts with both hPrp3 and hCypH (Teigelkamp *et al.*, 1998; Gonzalez-Santos *et al.*, 2002). In yeast, Prp4 also directly interacts with Prp3, but no cyclophyllin has been reported to be involved in splicing (Ayadi *et al.*, 1998). At this point it is not well understood how Prp3, Prp4, and hCypH are recruited to the U4 snRNAs. However, association with the U4 snRNA can occur independently of Prp31 but requires 15.5K and the 5' region of the U4 snRNA (Bordonne *et al.*, 1990; Xu *et al.*, 1990; Nottrott *et al.*, 2002).

The U6 snRNP has been purified from yeast and contains the U6 snRNA, seven Sm-like proteins, Lsm2–8, and Prp24 (Stevens *et al.*, 2001). Lsm2–8 bind to the 3' region of the U6 snRNA, and form a heptameric ring similar to the related Sm proteins found in U1, U2, U4,

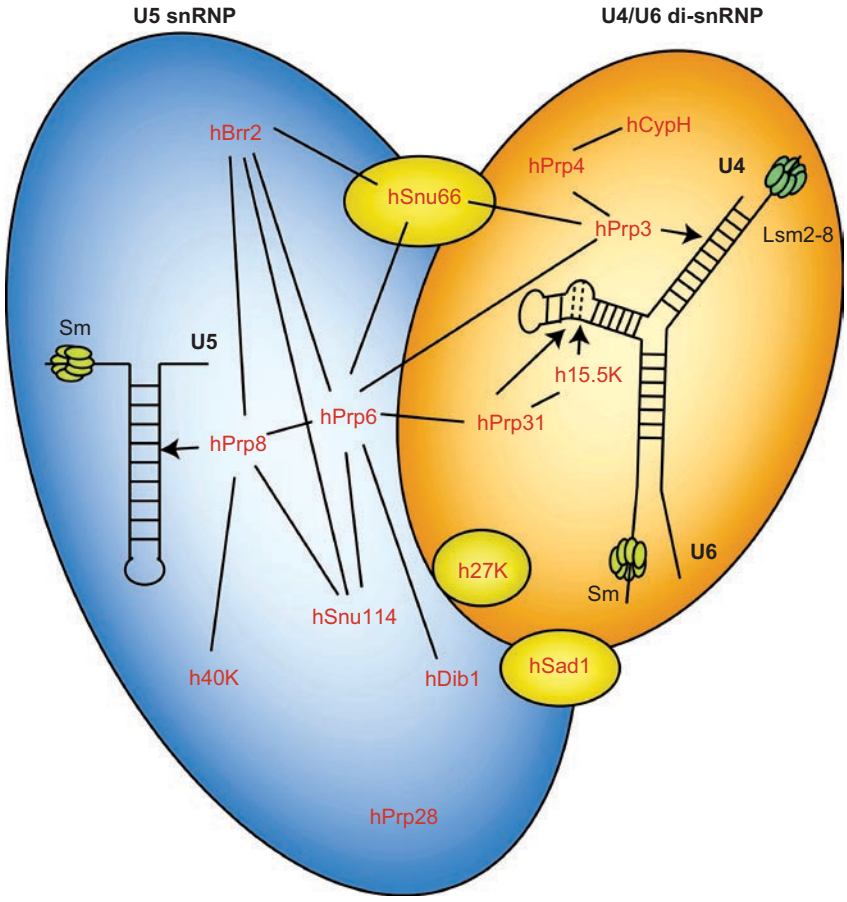


**Figure 4.** Composition of the human U4/U6.U5 tri-sRNP. U4/U6 di-snRNP (orange) and U5 snRNP (blue) associate to form the U4/U6.U5 tri-snRNP. Proteins specific to the tri-snRNP that are not found in the U4/U6 di-snRNP or U5 snRNP are shown in yellow. Protein–protein interactions within and between RNPs are indicated by solid lines. Known snRNA–protein interactions are indicated by arrows. The U4 and U6 snRNAs are base paired in both the U4/U6 di-snRNP and the U4/U6.U5 tri-sRNP. Proteins are conserved in yeast except for 27K, 40K, and hCypH.

and U5 snRNPs (Achsel *et al.*, 1999; Mayes *et al.*, 1999; Salgado-Garrido *et al.*, 1999; Karaduman *et al.*, 2008). Prp24 has been shown to directly contact the U6 snRNA and interacts with several Lsm proteins (Vidal *et al.*, 1999; Fromont-Racine *et al.*, 2000; Rader and Guthrie, 2002; Karaduman *et al.*, 2006). An important function of Prp24 is to promote U4/U6 di-snRNP formation (see discussion below).

Although U4 and U6 snRNPs exist as mono-snRNPs within cells, they assemble into higher-order complexes: U4 and U6 snRNPs dimerize to form the U4/U6-di-snRNP, which is subsequently joined by the U5 snRNP to form the U4/U6.U5 tri-snRNP, prior to their incorporation into the spliceosome. Heterodimer formation of U4 and U6 snRNPs is mediated by intermolecular base pairing between their respective snRNA components, resulting in two U4-U6 helices that are arranged in a Y-shaped structure (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Rinke *et al.*, 1985). The U6 snRNP components, Prp24 and the Lsm proteins, are essential for U4-U6 snRNP dimerization as they facilitate the annealing of U4 and U6 snRNAs (Ghetti *et al.*, 1995; Jandrositz and Guthrie, 1995; Raghunathan and Guthrie, 1998b; Achsel *et al.*, 1999; Rader and Guthrie, 2002). Interestingly, Prp24 is not a stable component of the U4/U6.U5 tri-snRNP or of spliceosomes, suggesting that it acts as a chaperone during U4/U6 di-snRNP assembly and then dissociates (Bell *et al.*, 2002). In addition to direct RNA–RNA interactions, RNA–protein interactions may also contribute to U4/U6 di-snRNP stability. Accordingly, Prp3, a U4-specific protein, could be crosslinked to the U6 snRNA in U4/U6 RNA duplex (Nottrott *et al.*, 2002). There is no evidence for protein–protein interactions between the U4 and U6 mono-snRNPs, suggesting that their dimerization is primarily mediated by RNA–RNA interactions.

### *U5 snRNP associates with the U4/U6 di-snRNP to form the U4/U6.U5 tri-snRNP*

Before incorporation into the spliceosome, the U4/U6 di-snRNP associates with the U5 snRNP to form the

U4/U6.U5 tri-snRNP. The U5 snRNP is the largest of the three snRNPs and is composed of the U5 snRNA, and seven Sm proteins as well as eight U5-specific proteins (Bach *et al.*, 1989; Fabrizio *et al.*, 1994; Stevens *et al.*, 2001). Four of them, Prp8, Brr2, Snu114, and the 40K (not present in yeast) protein form a stable complex in the absence of RNA, in which Prp8, the largest protein in the U5 snRNP, interacts directly with all the other proteins (Achsel *et al.*, 1998; Liu *et al.*, 2006; Pena *et al.*, 2007). Importantly, mutations in Prp8 that weaken the interaction with Brr2 and Snu114 have been linked to retinitis pigmentosa (Pena *et al.*, 2007). In addition, Prp8 also interacts with Prp6 and can be crosslinked to the U5 snRNA, underlining its central role in the U5 snRNP (Dix *et al.*, 1998; Liu *et al.*, 2006).

In contrast to U4-U6 snRNP dimerization, U5 snRNP association with the U4/U6 di-snRNP is mediated by protein–protein interactions (Figure 3). No direct interactions between the U5 snRNA and either the U4 or U6 snRNAs have been reported. Specifically, the U5-specific protein Prp6 directly interacts with the U4-specific proteins Prp3 and Prp31, acting as a bridge between the U4/U6 di-snRNP and the U5 snRNP (Liu *et al.*, 2006; Makarova *et al.*, 2002). Similar to mutations in Prp8, mutations in Prp31 have been linked to retinitis pigmentosa, and these mutations weaken the interaction of Prp31 with Prp6 (Liu *et al.*, 2007). Snu66, which is independently recruited to the tri-snRNP, also acts as a bridge by associating with Prp3, Prp6, and the U5-protein Brr2 (Liu *et al.*, 2006). One of the proteins found in the U5 snRNP, CD2BP2, is not found in the U4/U6.U5 tri-snRNP but dissociates during tri-snRNP assembly (Laggerbauer *et al.*, 2005). Since CD2BP2 directly interacts with Prp6, which is a central component of the U4/U6–U5 interface in the tri-snRNP, it could regulate their association (Laggerbauer *et al.*, 2005).

### *What is the role of U4, U6, and U5 snRNP multimerization in RNA splicing?*

U4, U5, and U6 snRNAs associate with the pre-mRNA together in the tri-snRNP. While the U5 and U6 snRNAs remain associated with the mRNA and the spliceosome during both catalytic steps, the U4 snRNA and associated U4-specific proteins are released prior to the first splicing reaction, suggesting that major structural rearrangements in the tri-snRNP and in the spliceosome occur before catalysis (Wahl *et al.*, 2009). The release of the U4 snRNP requires that the helices formed by the U4 and U6 snRNAs are unwound, which is performed by the helicase Brr2 and is controlled by Snu114 and Prp8 (Raghunathan and Guthrie, 1998a; Kuhn *et al.*, 1999). The U6 snRNA subsequently interacts with the U2 snRNA and replaces the U1 snRNA at the 5' splice site, positioning it at the heart of the catalytic site of the spliceosome (Datta and Weiner, 1991; Wu and Manley, 1991; Madhani and Guthrie, 1992; Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992a; Sun and Manley, 1995). The U6 snRNA is thought to play a key role during catalysis, coordinating a divalent metal ion and either independently or together with proteins catalyzing the transesterification reaction (Yean *et al.*, 2000; Valadkhan and Manley, 2001; Huppler *et al.*, 2002; Sashital *et al.*, 2004; Wachtel and Manley, 2009). Thus, U4-U6 snRNA base pairing as observed in the U4/U6 di-snRNP and U4/U6.U5 tri-snRNP is incompatible with catalysis, suggesting that the U4 snRNA acts as a chaperone, masking functionally critical regions of the U6 snRNA until it is certain that splice sites are accurately recognized. In addition, snRNP multimerization assists spliceosome assembly by forming stable multimolecular subcomplexes that are recruited to splice sites. Similar principles are observed in the assembly of other macromolecular machines, e.g. pre-ribosomal complexes that mature ribosomal RNAs (Staley and Woolford, 2009).

### *The U11/U12 di-snRNP of the minor spliceosome*

Splice sites of minor spliceosome introns, also called U12-type introns, differ in 5' splice site and branch site consensus sequences from the major spliceosome introns, the U2-type introns (Burge *et al.*, 1998). While the 5' splice site and branch point of U2-type introns are independently recognized by U1 and U2 snRNPs, respectively, the corresponding sequences in U12-type introns are recognized cooperatively by the U11 and U12 snRNAs and associated proteins, which bind as a preformed complex, the U11/U12 di-snRNP (Wassarman and Steitz, 1992b; Frilander and Steitz, 1999).

Purification of the 18S U11/U12 di-snRNP revealed that it contains both U11 and U12 snRNAs, protein components that are shared with the U2 snRNP, including the Sm proteins and the SF3b but not SF3a complex, and unique proteins not found in either U1 or U2 snRNPs (Table 2) (Will *et al.*, 1999; 2001; 2004). Interestingly, no U1 snRNP-specific proteins seem to be components of the U11/U12 di-snRNP but have been replaced by four novel U11-specific proteins, underlining differences in 5' splice site recognition between minor and major spliceosomes. One of the U11-specific proteins, U11-48K, directly interacts with the 5' splice site via a zinc-finger domain and has been proposed to stabilize U11-5' splice site base pairing (Turunen *et al.*, 2008; Tidow *et al.*, 2009). SF3b, which stabilizes U2 snRNA branch point interactions in the major spliceosome, may fulfill a similar function in the minor spliceosome, stabilizing the U12 snRNA interaction with the branch

**Table 2.** Protein composition of the 18S U11/U12 di-snRNP (Will *et al.*, 1999; 2001; 2004).

| |
|---|
| **Proteins shared with U2 snRNP or the major spliceosome:** |
| Sm proteins (SmB/B'. D1, D2, D3, E, F, G) |
| SF3b155 |
| SF3b145 |
| SF3b130 |
| SF3b49 |
| SF3b14a (p14) |
| SF3b14b |
| SF3b10 |
| Prp43 |
| YB-1 |
| Urp (U2AF35-related protein 2) |
| **U11/U12 di-snRNP-specific proteins:** |
| U11/U12-65K |
| U11/U12-31K (MADP1) |
| U11/U12-20K |
| **12S U11-snRNP proteins:** |
| Sm proteins (SmB/B'. D1, D2, D3, E, F, G) |
| U11-59K (ES18) |
| U11-48K |
| U11-35K |
| U11-25K |
| 9G8 |

point (Gozani *et al.*, 1996; Will *et al.*, 2004). EM reconstructions of isolated SF3b and U11/U12 di-snRNPs revealed different conformations of SF3b that may correspond to snapshots during branch point recognition (Golas *et al.*, 2003; 2005). One of the U11/U12-specific proteins, 65K, has been shown to directly bind the U12 snRNA (Benecke *et al.*, 2005). Currently it is not clear, however, which other proteins directly contact the U12 snRNA or how the U11 snRNA associates with proteins in the complex.

Early studies on U11/U12 di-snRNPs indicated that dimerization is likely mediated by protein–protein interactions rather than by RNA–RNA base pairing because the complex dissociates with increasing salt concentration (Wassarman and Steitz, 1992b). The U11/U12-65K protein seems to play a central role in bridging U11 and U12 snRNPs, since it binds to the U12 snRNA and also directly interacts with the U11 snRNP protein U11-59K (Benecke *et al.*, 2005). Whether additional protein–protein or RNA–protein interactions exist between U11 and U12 snRNP components remains a subject for future studies.

Why do U11 and U12 snRNPs function as U11/U12 di-snRNPs while the analogous components of the major spliceosome, the U1 and U2 snRNPs, function as mono-snRNPs? In the major spliceosome, SR proteins as well as Prp5 bridge the 5' splice site and the branch point, and hence the U1 and U2 snRNPs (Wu and Maniatis, 1993; Xu *et al.*, 2004). Prp5, however, is not part of the minor spliceosome, suggesting a distinct mechanism of intron bridging. Instead, dimerization of U11 and U12 snRNPs may replace Prp5 and directly contribute to intron bridging and accurate pairing of splice sites (Frilander and Steitz, 1999; Will *et al.*, 2004). Since the U11 snRNP associates with proteins distinct from those found in the U1 snRNP of the major spliceosome, the U11/U12 di-snRNP may also provide a target for independent regulation of splicing of U12-type introns. However, the mechanisms may differ between species because the U11 snRNA associated proteins are not conserved in *Drosophila*, indicating quite divergent mechanisms of U11/U12 snRNP dimerization, intron bridging, and 5' splice site recognition between different eukaryotes (Schneider *et al.*, 2004).

## Conclusions

The examples in this review illustrate that RNP multimerization is a prevalent form of RNP organization. Most likely many more multimeric RNPs exist and remain to be discovered. However, except for the U4/U6 di-snRNP involved in pre-mRNP splicing, we do not completely understand what advantages multimerization brings to RNP function. While models have been proposed for both dimeric box C/D s(no)RNPs and telomerase RNP functions, additional experiments are necessary to validate them.

## Declaration of interest

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