The Hsp90 chaperone controls the biogenesis of L7Ae RNPs through conserved machinery

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RNA-binding proteins of the L7Ae family are at the heart of many essential ribonucleoproteins (RNPs), including box C/D and H/ACA small nucleolar RNPs, U4 small nuclear RNP, telomerase, and messenger RNPs coding for selenoproteins. In this study, we show that Nufip and its yeast homologue Rsa1 are key components of the machinery that assembles these RNPs. We observed that Rsa1 and Nufip bind several L7Ae proteins and tether them to other core proteins in the immature particles. Surprisingly, Rsa1 and Nufip also link assembling RNPs with the AAA + adenosine triphosphatases hRvb1 and hRvb2 and with the Hsp90 chaperone through two conserved adaptors, Tah1/hSpagh and Pih1. Inhibition of Hsp90 in human cells prevents the accumulation of U3, U4, and telomerase RNAs and decreases the levels of newly synthesized hNop58, hNHP2, 15.5K, and SBP2. Thus, Hsp90 may control the folding of these proteins during the formation of new RNPs. This suggests that Hsp90 functions as a master regulator of cell proliferation by allowing simultaneous control of cell signaling and cell growth.

Introduction

Noncoding RNPs are ancient devices that play fundamental roles in the cell. The L7Ae family of proteins comprises a set of related RNA-binding proteins that share a homologous RNA recognition domain (Koonin et al., 1994). Members of the L7Ae protein family occur in both Archaea and Eukarya. They have been found in numerous essential RNPs (Fig. 1), including the large ribosomal subunit, U4 spliceosomal small nuclear RNP (snRNP), box C/D and H/ACA small nucleolar RNPs (snoRNPs), small Cajal body RNPs (scaRNPs), telomerase, archaeal small RNP, and selenoprotein messenger RNPs (mRNPs). In humans, canonical members are ribosomal proteins L7A, L30, 15.5K, hNHP2, and SBP2 (Koonin et al., 1994; Watkins et al., 2000; Allmang et al., 2002).

The snoRNPs play essential roles in ribosomal RNA maturation both during the cleavage steps and nucleotide modifications (for reviews see Kiss, 2002; Matera et al., 2007). The snoRNPs are divided into two major classes: the box C/D snoRNPs that catalyze ribose 2′-*O*-methylation and the box H/ACA snoRNPs that mediate pseudouridine formation. Each snoRNP contains a small nucleolar RNA (snoRNA) and a set of common snoRNP proteins. The box C/D snoRNPs contain 15.5K (Snu13 in yeast), Nop56, Nop58, and the methyltransferase fibrillarin (Nop1 in yeast). Box H/ACA snoRNPs contain hNHP2, Gar1, Nop10, and the pseudouridine synthase dyskerin (Cbf5 in yeast). Vertebrate cells also contain related RNPs like the scaRNPs that also share box C/D or H/ACA RNP proteins. Although most scaRNPs are involved in the posttranscriptional modification of spliceosomal small nuclear RNAs (snRNAs), the telomerase box H/ACA scaRNP functions in telomeric DNA synthesis. Selenoprotein synthesis requires cotranslational recoding of in-frame UGA codons. In eukaryotes, this process involves the assembly of RNA–protein complexes at specific stem

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Abbreviations used in this paper: IP, immunoprecipitation; mRNP, messenger RNP; qPCR, quantitative PCR; scaRNP, small Cajal body RNP; SECIS, selenocysteine insertion sequence; snoRNA, small nucleolar RNA; snoRNP, small nucleolar RNP; snRNA, small nuclear RNA; snRNP, small nuclear RNP; TPR, tetratricopeptide repeat; Y2H, yeast two hybrid; Y3H, yeast three hybrid.

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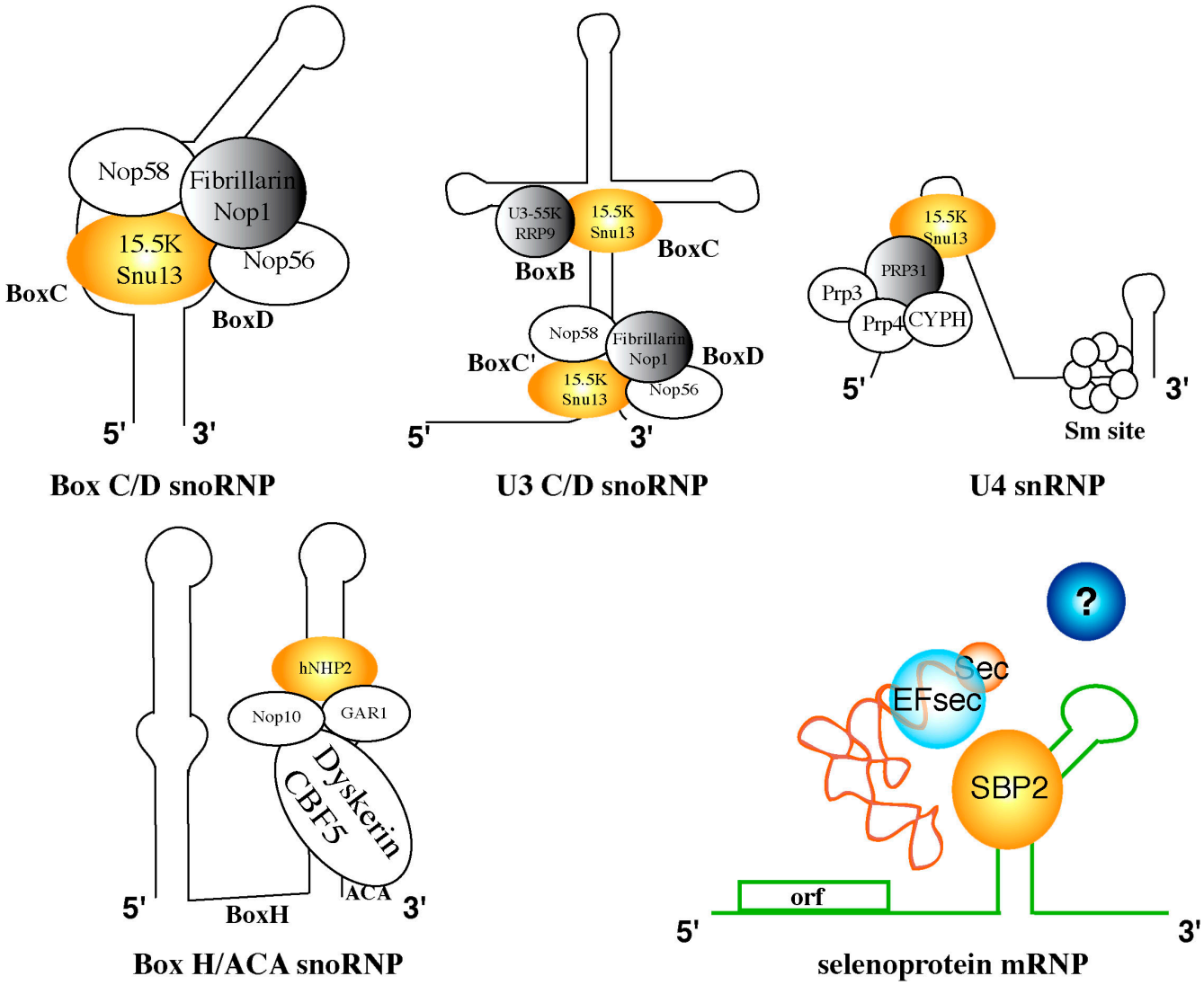

Figure 1. **Composition of L7Ae RNPs.** The various RNPs containing the L7Ae family members (yellow) studied here are depicted. The yeast names are indicated below the human names.

loops located in the 3′ untranslated region of selenoprotein mRNAs, termed the selenocysteine insertion sequence (SECIS). SBP2 specifically binds the SECIS element and recruits the specialized elongation factor EFsec (Copeland et al., 2000; Fagegaltier et al., 2000).

Detailed structural information is available for several members of the L7Ae family (Vidovic et al., 2000; Li and Ye, 2006). They bind to a common RNA structure called the K-turn or K-loop motif, and binding exposes further interaction surfaces on both the RNA and the protein. Thus, upon RNA binding, L7Ae proteins recruit additional factors to construct complex RNPs. Remarkably, some L7Ae proteins participate in several RNPs. This is the case for Snu13/15.5K, which is at the heart of the U4 snRNP, box C/D snoRNPs, and a specialized B/C structure of the U3 snoRNP (Watkins et al., 2000). Although Snu13/15.5K has structurally similar binding sites in these RNAs, it recruits different sets of protein partners (or core proteins), such as Nop56, Nop58, and fibrillarin in box C/D snoRNPs (Watkins et al., 2002), PRP31 and the cyclophilin H–hPRP4–hPRP3 complex for the U4 snRNP (Nottrott et al., 2002), and RRP9 (U3-55K in the human) for the B/C motif of the U3 snoRNP (Granneman et al., 2002). Importantly, the binding of Snu13/15.5K is required for association of the other core proteins. For instance, hPRP31 binds neither 15.5K nor U4 snRNA alone, but it does bind the 15.5K–U4 complex, in which it directly contacts both the RNA and protein (Liu et al., 2007).

Although some RNPs can be reconstituted in vitro, in living cells they are formed through assembly and maturation pathways that are far more complex than initially anticipated (Yong et al., 2004; for review see Matera et al., 2007). The number of assembly factors often exceeds the number of proteins present in the mature particle. Assembly of most RNPs requires dynamic remodeling of the maturing RNP particle, and it is often accompanied by a complicated series of transient intermolecular interactions involving stably associated core components and transiently interacting processing factors. Although RNP assembly requires the proper folding of both the RNA and protein components, protein chaperones have not yet been implicated

in this process. Interestingly, some factors involved in yeast snoRNP biogenesis have been independently identified as cochaperones for Hsp90. Indeed, the R2TP complex, which is composed of Tah1, Pih1, and the two AAA + ATPases Rvb1 and Rvb2, is a cochaperone for Hsp90 (Zhao et al., 2005), and both Rvb2 and Pih1 are involved in box C/D snoRNP biogenesis (King et al., 2001; Gonzales et al., 2005), raising the possibility that Hsp90 and the R2TP complex are involved in the production of box C/D snoRNPs. Although no human equivalent of the R2TP complex has been identified, it has been shown that immature human U3 particles contain the homologues of yeast Rvb1 and Rvb2 (hRvb1 and hRvb2, also called Tip48 and Tip49; Newman et al., 2000).

Hsp90 has attracted a lot of interest because it is involved in cancer and in the control of nuclear receptors and protein kinases (Pearl and Prodromou, 2006; Caplan et al., 2007). Hsp90 is highly conserved from eubacteria to eukarya and performs essential cellular functions. Compared with other chaperones, Hsp90 has two unique features. First, it usually binds target proteins at a late stage of their folding in a near-native state. Second, it appears as a specialized chaperone that is mainly involved in the control of signal transduction. Like other chaperones, Hsp90 recognizes its substrate through cochaperones and cofactors.

In this study, we describe a conserved molecular machinery for the assembly of RNPs of the L7Ae family. This machinery exists in yeast and human cells and is composed of the R2TP proteins and an adaptor called Nufip (Rsa1 in yeast). Remarkably, we show that Nufip holds together the core components of the mature RNP and directly links RNP assembly with Hsp90.

Results

Nufip and its yeast homologue Rsa1 bind 15.5K and Snu13 through a short peptide motif

To characterize new factors involved in box C/D snoRNP biogenesis, we performed yeast two-hybrid (Y2H) and yeast three-hybrid (Y3H) screens. First, we screened a yeast genomic library using a minimal U3 B/C motif as bait. Out of 10^4 clones screened, we found two positive ones that coded for Rsa1, a nuclear protein previously found in a synthetic lethal screen with Dbp6 (Kressler et al., 1999). Interestingly, when Rsa1 was tested in Y3H against various RNAs, its binding correlated with that of Snu13 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200708110/DC1), suggesting that it formed a complex with Snu13 bound to target RNAs (see the section Nufip and Rsa1 form a ternary complex…). Second, we screened a human liver library using 15.5K as bait. Upon screening of 10^7 clones, we detected 23 positives, three of which coded for Nufip, a nuclear RNA-binding protein previously shown to interact with nuclear isoforms of fragile X mental retardation protein (FMRP; Bardoni et al., 1999). No homology between Nufip and Rsa1 could be identified by BLAST search; however, a careful comparison of their sequence identified a conserved stretch of 32 amino acids (PEP; Fig. 2 A). Although no other homologies could be detected between the two proteins, BLAST searches performed with this oligopeptide sequence identified potential homologues in fungi, plants, and animals (Fig. S2). To identify the domains of Nufip involved in the interaction with 15.5K, we generated several mutants: an N-terminal fragment truncated before its Zn finger (Nufip-Nter), a central fragment containing the Zn finger and the PEP motif (Nufip-Zn), and a C-terminal region (Nufip-Cter). Y2H tests revealed that the 15.5K interaction domain matched the central region containing the PEP motif (Fig. 2 B). Remarkably, deletion of PEP (Nufip-ΔPEP) abolished binding, whereas the PEP motif alone was sufficient for interaction with 15.5K. To test whether Rsa1 also interacts with Snu13, full-length and mutant forms of Rsa1 were tested in Y2H assays (Fig. 2 B). We found that Rsa1 indeed interacted with Snu13 and that the interaction site mapped to its PEP motif.

To verify that Y2H interactions reflected a direct physical association of Nufip and Rsa1 with 15.5K and Snu13, in vitro binding assays were performed (Fig. 2 C). We found that 15.5K translated in vitro in a bacterial S30 lysate interacted with immobilized GST-Nufip and that recombinant Snu13p produced in *Escherichia coli* bound to GST-Rsa1p.

To test whether Nufip associates with 15.5K in human cells, we performed co-immunoprecipitation (IP) assays. A construct expressing GFP-15.5K was transfected into HeLa cells, extracts were incubated with anti-GFP beads, and the selected proteins were analyzed by Western blots. Nufip was found in the GFP-15.5K pellet but not in the control (Fig. 2 D). Collectively, these results show that Nufip associates with 15.5K in vivo and that Nufip and Rsa1 are related proteins that physically interact with 15.5K and Snu13.

Nufip binds hNHP2 and SBP2

The binding of Nufip to 15.5K prompted us to test whether it could also interact with other members of the L7Ae family. By using a Y2H system, we tested the interaction of Nufip with the box H/ACA snoRNP core protein hNHP2, with SBP2, which associates with mRNAs coding for selenoproteins, and also with the human ribosomal protein hL30, another member of this family. We found that Nufip interacted with hNHP2 and SBP2 but not with hL30 (Fig. 2 B). Although the PEP domain was required for these interactions, it was not sufficient to bind hNHP2, and it only weakly bound SBP2. In vitro binding assays confirmed that these interactions reflected direct physical associations (Fig. 2 C). Next, we transfected HeLa cells with a GFP-hNHP2 construct, and extracts were immunoprecipitated with an anti-GFP antibody. Western blot analysis revealed efficient co-IP of Nufip by GFP-hNHP2 (Fig. 2 D). Similarly, when nuclear HeLa cell extracts were passed through a column with immobilized anti-SBP2 peptide antibodies, we found that Nufip copurified with SBP2 (Fig. 2 D). These results show that Nufip associates not only with 15.5K but also with two other members of the L7Ae family, hNHP2 and SBP2.

Nufip and Rsa1 form a ternary complex with 15.5K/Snu13 and target RNAs

Rsa1 was initially found in a Y3H screen with an RNA bait that bound Snu13, suggesting that it interacted with an Snu13–RNA complex. To verify this, we reconstituted the complex in vitro using gel-shift assays (Fig. 3 A, left). Snu13 alone could bind

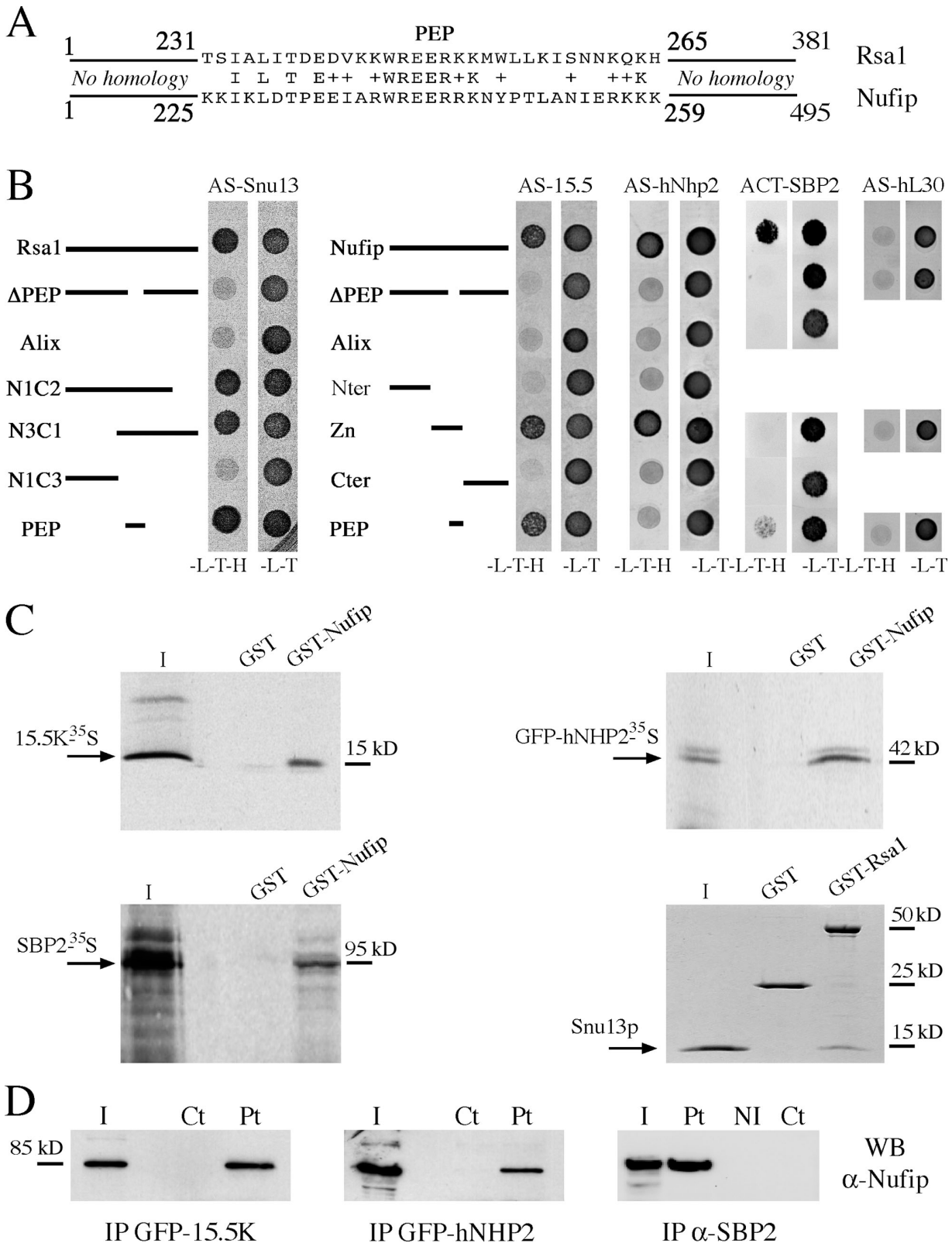

Figure 2. **Nufip and Rsa1 are related proteins that bind L7Ae family members through a short peptide motif.** (A) Alignment of Rsa1 with Nufip. The conserved motif, named PEP, is represented. (B) Y2H tests between the indicated proteins. Rsa1, Nufip, and their various domains are represented by lines. Growth on −Leu −His −Trp (−L −H −T) indicates an interaction. (C) GST pull-down experiments.15.5K and SBP2 were translated in vitro in bacterial S30 lysate and assayed for binding to the indicated recombinant GST fusions. GFP-hNhp2 was translated in rabbit reticulocyte lysate, but similar results were obtained with bacterial lysate (not depicted). Snu13 was purified from *E. coli* and stained with Coomassie blue. (D) In vivo co-IP assays. GFP-15.5K and GFP-hNHP2 were expressed in HeLa cells and purified with anti-GFP antibodies. Endogenous SBP2 was immunopurified from nuclear HeLa cell extracts using antipeptide antibodies. Bound proteins were analyzed by Western blots with anti-Nufip antibodies. I, input (10% of total); Pt, pellet; NI, preimmune control serum; Ct, control beads without antibodies. The sizes of the various products are indicated on the side of the gels.

U14 snoRNA, whereas two His-tagged fragments of Rsa1 (N3C1 and yPEP) did not. However, when Snu13 was added, complexes of higher molecular weight were obtained with both Rsa1 fragments. These complexes were supershifted by anti-His antibodies (Fig. 3 A), demonstrating that they contained N3C1 and yPEP. To check whether Nufip could also associate with Snu13–RNA complexes, we used Y3H assays (Fig. 3 A, right). As expected, we found that Nufip could specifically interact with the B/C motif in a PEP-dependent manner. These results demonstrate that Nufip and Rsa1 can form ternary complexes with Snu13 bound to RNA.

Nufip associates with U4 snRNA, box C/D and H/ACA snoRNAs, a minimal U3 B/C RNA, and SBP2 mRNPs

To test whether Nufip also associated with 15.5K RNPs in vivo, we performed a series of co-IP assays. First, we analyzed whether Nufip was associated with box C/D snoRNAs. HeLa cells were transfected with mouse U8, human U13, and rat U3 genes. Extracts were subjected to IP with anti-Nufip antibodies, and the pellets were analyzed by RNase protection with RNA probes specific for the 3′-terminal regions of the test RNAs. The U3, U8, and U13 snoRNAs are synthesized from their own promoters, and their precursors contain a short 3′ extension that is trimmed during RNP assembly (Verheggen et al., 2002; Boulon et al., 2004). Remarkably, we observed that Nufip was associated with all precursors and mature forms of U3, U8, and U13 (Fig. 3 C). Identical results that were obtained with GFP-Nufip and anti-GFP antibodies excluded nonspecific binding of the antibody. To test whether Nufip also binds snoRNAs processed from introns, we transfected HeLa cells with a construct expressing an artificial C/D snoRNA inserted in the second intron of the β-globin gene (dBB). The anti-Nufip antibody efficiently precipitated this snoRNA (Fig. 3 C), indicating that a C/D motif is sufficient to direct Nufip binding.

U3 contains two binding sites for 15.5K (Watkins et al., 2000): the C′/D motif that is equivalent to the C/D motif in other snoRNAs and the B/C motif that recruits U3-55K. IP experiments showed that Nufip bound a U3 mutated in its C′/D motif but not a U3 mutant in which both the B/C and C′/D motifs had been inactivated (Fig. 3 C). 15.5K is also found in U4 snRNP, where it interacts with hPRP31 (Watkins et al., 2000). Again, co-IP experiments found endogeneous Nufip and Nufip-GFP associated with both endogenous U4 and a transiently expressed tagged U4 RNA (Fig. 3 D).

The interaction of Nufip with hNHP2 suggested that it could also bind box H/ACA snoRNAs. RNAs immuno-precipitated with anti-Nufip antibodies from HeLa cell extracts were thus analyzed by RNase protection with probes specific for the U19 H/ACA snoRNA and for the U3 box C/D snoRNA as a control (Fig. 3 E). Nufip antibodies coprecipitated both U3 and U19 snoRNAs. Although this assay could not discriminate between mature and precursor forms of U19, the fact that Nufip is excluded from nucleoli in HeLa cells (unpublished data) is consistent with an association with forms of U19 not yet involved in rRNA maturation.

As Nufip was shown to interact with SBP2, we tested whether it could also bind selenoprotein mRNAs. Extracts were prepared from 293FT cells expressing GFP-Nufip and RNAs coimmunoprecipitated with anti-GFP antibodies and were analyzed by RT-PCR. Anti-GFP antibodies coprecipitated endogenous selenoprotein glutathione peroxidase 4 and type 2 deiodinase mRNAs but not the β-actin mRNA that was tested as a negative control (Fig. 3 F). These results demonstrate that Nufip associates with box C/D and box H/ACA snoRNAs, a B/C RNA, U4, and SBP2 mRNPs. Nufip binds precursor forms of C/D snoRNAs, and binding is dependent on the presence of a binding site for 15.5K. Finally, Rsa1 also interacts with U3 precursors in yeast (Fig. 3 B).

Nufip and Rsa1 are required for snoRNA biogenesis

To further document its role in snoRNA biogenesis, Nufip was depleted from HeLa cells using two different siRNAs (Fig. 4 A). Northern blot analysis showed that depletion of Nufip reduced the level of the endogenous U3 snoRNA by 50%. As demonstrated by quantitative PCR (qPCR) analysis, depletion of Nufip had little effect on accumulation of the U4 snRNA. Although its depletion slightly reduced the levels of the U14 box C/D and U19 box H/ACA snoRNAs, it had a more dramatic effect on the accumulation of telomerase RNA that carries an H/ACA domain.

Next, we investigated the effect of removing Rsa1 in yeast. Primer extension analyses showed that the levels of the U14 and U3 box C/D snoRNAs were reduced in the ΔRsa1 strain by 66% and 42%, respectively (Fig. 4 B). As in mammalian cells, the level of U4 remained almost unaffected, and a slight decrease was observed in the levels of box H/ACA snoRNAs. To gain further insights into the role of Rsa1, we analyzed a truncated version of the U3 snRNA (U3del) that is incorporated into snoRNPs with low efficacy and, therefore, accumulates precursor RNPs that are devoid of Nop1, Nop56, and Nop58 and are stabilized by La and LSm proteins (Kufel et al., 2000). The lack of Rsa1 resulted in a greater accumulation of U3 precursors (three- to fourfold when normalized to mature U3del levels; Fig. 4 C), indicating a role for Rsa1 in U3 assembly and maturation.

Nufip and Rsa1 tether 15.5K and Snu13 to the other core proteins of U4, box C/D, and box B/C RNPs

Because Nufip and Rsa1 bound Snu13 and 15.5K and were involved in RNP assembly, we tested whether they could interact with other core proteins contained in the mature RNP. In Y2H assays (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200708110/DC1), we observed that Rsa1 and Nufip associated with yeast and human PRP31, respectively. In addition, Rsa1 interacted with Nop58, and Nufip associated with U3-55K and fibrillarin. Binding of Nufip to the endogenous Snu13 did not contribute to these interactions because the C-terminal domain of Nufip that does not bind Snu13 and 15.5K also interacted with hPRP31, U3-55K, and fibrillarin. To verify these results, we performed GST pull-down experiments with in vitro–translated ^{35}S-labeled proteins. In agreement with the Y2H results, we found that Nufip directly bound hPRP31, U3-55K,

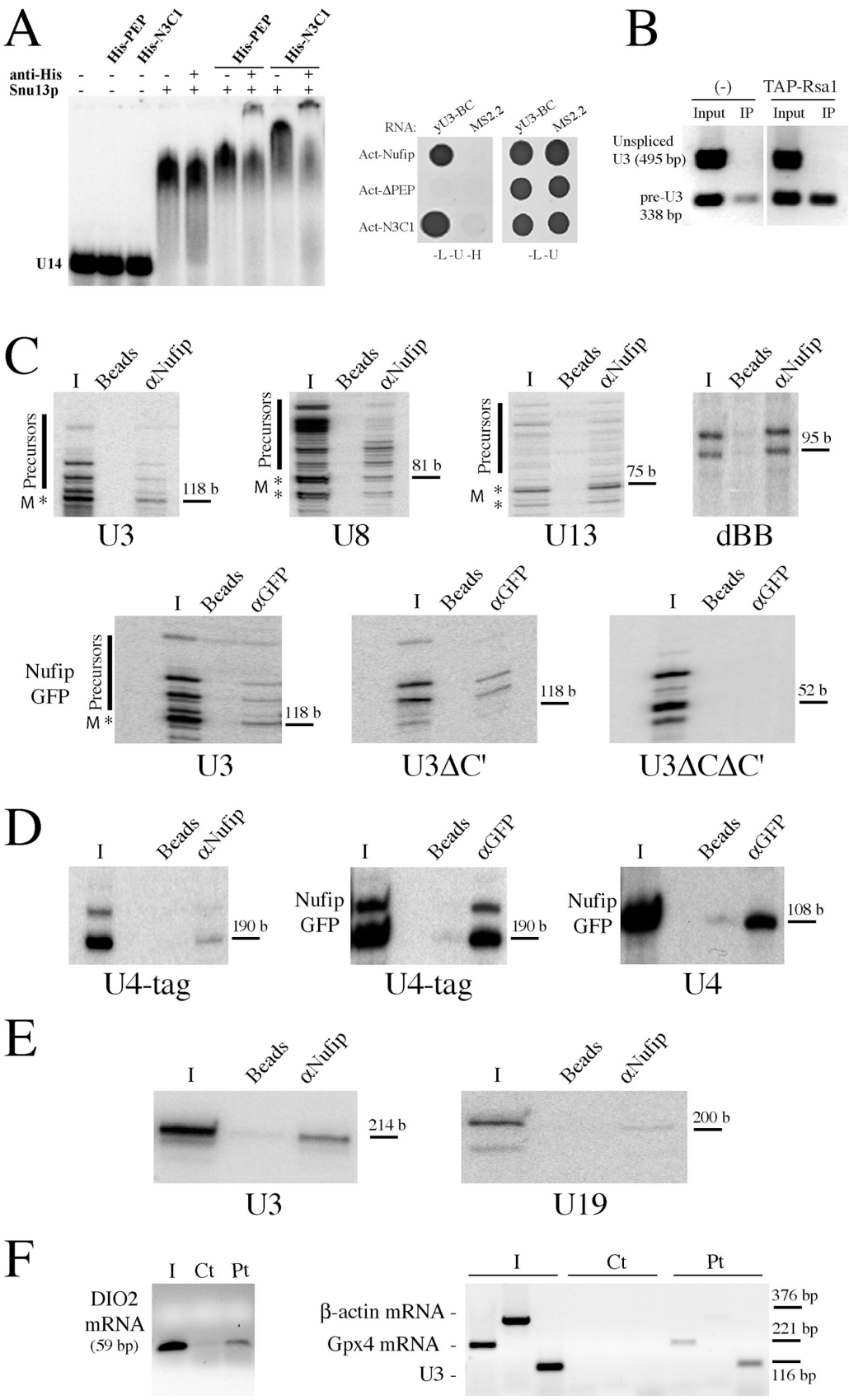

Figure 3. **Nufip binds box C/D and H/ACA snoRNAs, a B/C-containing RNA, U4 snRNA, and mRNAs coding for selenoproteins.** (A) In vitro interactions of Rsa1 and Nufip with Snu13–RNA complexes. (left) Gel-shift assays show that the Rsa1 N3C1 and PEP domains interact with Snu13 bound to RNA. Radiolabeled yeast U14 snoRNA was incubated with the indicated recombinant proteins and anti-His antibodies when indicated. (right) Y3H assays show that Nufip interacts with yU3-B/C RNA in a PEP-dependent manner. Plate −Leu −Ura (−L −U) shows the growth of the test strain. Growth on −Leu −Ura −His (−L −U −H)

and fibrillarin (Fig. 5, A and B). This suggested that Nufip can tether 15.5K to other core proteins. To test this more directly, we introduced it as a bridge in Y2H assays (Fig. 5 C). Although 15.5K fused to the Gal4 DNA-binding domain did not produce Y2H interactions with fibrillarin, hPrp31, or U3-55K fused to the Gal4-activating domain, introduction of Nufip into this strain allowed growth on selective media, indicating that the proteins formed a ternary complex. It was unlikely that endogenous yeast proteins contributed to the complex because little cross-reaction was observed between the yeast and human proteins in Y2H assays (unpublished data). In particular, ySnu13 did not interact with hPRP31 and fibrillarin. In addition, neither transformation of an empty vector nor expression of an Nufip-ΔPEP mutant produced an interaction. Thus, we conclude that Nufip can tether 15.5K with fibrillarin, hPrp31, and U3-55K.

Next, we tested whether Nufip could also link Snu13–RNA complexes to core RNP proteins. Using bridged Y3H assays, we found that Nufip could promote the interaction of hPRP31 and a fibrillarin C-terminal domain with yU3B/C RNA (Fig. 5 D). It should be noted that under in vitro conditions, B/C RNAs are not competent to form ternary complexes with 15.5K and either hPRP31 or fibrillarin (Schultz et al., 2006). This suggests that RNP complexes tethered by Nufip bind RNA primarily through Snu13/15.5K and that the other core proteins become associated by virtue of their binding to Nufip. Thus, we concluded that Nufip and Rsa1 could act as adaptors to bridge core RNP proteins to Snu13–RNA complexes.

Yeast R2TP proteins function in U3 assembly, and their human homologues, together with Hsp90, associate with immature U3 and U4 particles and SBP2 mRNPs

To define more precisely the function of Nufip and Rsa1, we characterized the composition of immature U3 and U4 particles in more detail. As described in the Introduction, yeast box C/D snoRNP accumulation and localization involve Rvb2 and Pih1 proteins that, together with Rvb1 and the yeast Hsp90 cochaperone Tah1, form the R2TP complex (Zhao et al., 2005). To confirm the role of Pih1 in the assembly of yeast U3 snoRNP and to demonstrate that Tah1 is also involved in this process, we analyzed the biogenesis of the truncated form of U3 (U3del) in strains lacking these proteins. As shown in Fig. 4 C, such strains had reduced levels of mature U3 but increased levels of U3 precursors, indicating a defect in the formation of U3 snoRNP.

The R2TP complex has not been identified in human cells, but a recent analysis of the human Hsp90 proteome showed that hRvb1, hRvb2, and the human homologue of Pih1 were associated with Hsp90 (Te et al., 2007). This raises the possibility that the R2TP complex may also occur and function as an Hsp90 cofactor in higher eukaryotes. The yeast Tah1 is a small protein mostly composed of two tetratricopeptide repeat (TPR) motifs. Given that the human genome encodes several TPR-containing proteins, a sequence comparison failed to identify the human functional homologue of Tah1. However, upon a closer examination of systematic interaction databases, we found that a *Drosophila melanogaster* TPR protein called Spaghetti had been reported to interact with both dHsp90 and dPih1 in Y2H screens (Giot et al., 2003). In addition, the human homologue of Spaghetti (FLJ21908, hereafter named hSpagh) was found in the Hsp90 proteome (Te et al., 2007), suggesting that it may represent the functional homologue of yeast Tah1. To test this possibility, we generated an antibody against hSpagh and performed IP analyses. Indeed, we found that hRvb1, hRvb2, and hPih1 were immunoprecipitated by this antibody (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200708110/DC1).

To determine whether these proteins associate with assembling U3 particles, we performed co-IP assays. Antibodies against Hsp90, hSpag, and hPih1 were used to immunoprecipitate their respective proteins from extracts prepared from HeLa cells transiently expressing rat U3 snoRNA. The coprecipitated RNAs were analyzed by RNase protection (Fig. 6 A). Hsp90, hSpag, and hPih1 bound precursor and mature forms of U3. Similar results were obtained with transiently expressed epitope-tagged proteins, confirming that they specifically interacted with U3 RNA (unpublished data). In similar experiments performed with U4 snRNA, we observed that antibodies against hPih1, hSpagh, Hsp90, hRvb1, and hRvb2 also coprecipitated the U4 snRNA (Fig. 6 B).

To test whether R2TP proteins can interact with other RNPs of the L7Ae family, we checked their association with SBP2. IP of Hsp90, GFP-Pih1, GFP-hRvb1, and GFP-hRvb2 coprecipitated SBP2 (Fig. 6 C). In addition, endogenous hRvb1 coimmunopurified with SBP2 from native HeLa nuclear extracts (Fig. 6 D). This suggests that these proteins are involved in the assembly of SECIS-containing RNPs. Collectively, these data provide a direct and remarkable link between the protein folding machinery and assembly of L7Ae RNPs. These observations also support the idea that the human homologues of yeast R2TP proteins function as Hsp90 cofactors during RNP assembly.

indicates a positive interaction. (B) In vivo association of Rsa1 with U3 precursors in yeast. Extracts from TAP-Rsa1 or wild-type (−) isogenic strains were purified on IgG beads and analyzed by RT-PCR with primers specific for U3 precursors. (C) In vivo interactions of Nufip with rat U3B.7 and other box C/D snoRNAs. HeLa cells were transfected with the indicated snoRNA gene either alone (top) or with an Nufip-GFP vector (bottom). Extracts were purified with anti-Nufip (top) or anti-GFP (bottom) antibodies or beads as a control, and bound RNAs were analyzed by RNase protection. U3ΔC′ and U3ΔCΔC′ are mutated in the C′ and in the C and C′ boxes. dBB is an artificial intronic C/D snoRNA (see Results). I, input (10% of total); M*, mature species. (D) In vivo association of Nufip with endogenous U4 snRNA (right) and a transfected, tagged U4 snRNA (left and middle). Legend as in C. (E) In vivo binding of Nufip with H/ACA snoRNAs. HeLa nuclear extracts were immunoprecipitated with anti-Nufip antibodies and analyzed by RNase protection with the indicated probes. Legend as in C. (F) Nufip associates with mRNAs coding for selenoproteins. Anti-GFP IP of extracts of 293FT cells transfected with SBP2 alone (lanes Ct) or together with Nufip-GFP (lane Pt). U3 and β-actin are positive and negative controls, and type 2 deiodinase and glutathione peroxidase 4 are two selenoproteins. Input, 10% of total.

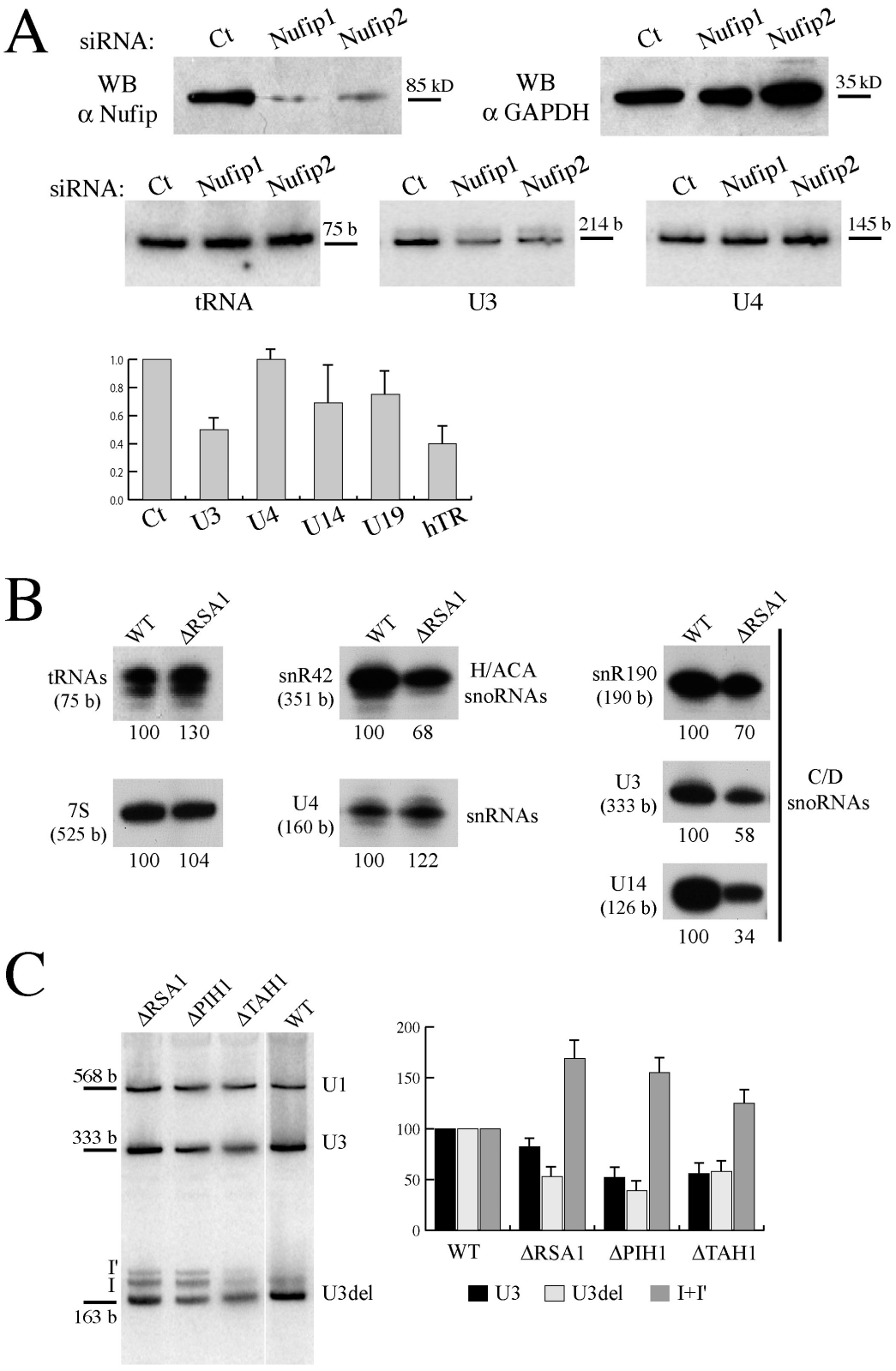

Figure 4. **Nufip and Rsa1 are required for the production of snoRNAs.** (A) Nufip is required for the production of U3. HeLa cells were treated with siRNA duplexes against Nufip. Nufip depletion was verified by Western blotting (top), and the levels of the indicated RNAs were monitored by Northern blotting (bottom) or by qPCR (graph). The qPCR values represent ratios of the indicated RNA to GAPDH mRNA and are expressed as fractions of the levels obtained with the control siRNAs (Ct). (B) Primer extension was used to measure the levels of small RNAs in yeast cells lacking Rsa1 (ΔRsa1). Numbers represent the amount of RNA relative to wild type (W303). (C) Processing of a modified U3del gene was analyzed in strains deleted for Rsa1, Pih1, and Tah1 (BY4741 background). This U3 gene accumulates precursor species (I and I'), and this defect is more pronounced in mutant strains. (left) Northern blot. (right) Quantification of each species after normalization to levels in wild-type cells. Error bars represent SD.

Inhibition of Hsp90 reduces the levels of newly synthesized U3, U4, and telomerase RNA and leads to the loss of 15.5K, hNHP2, SBP2, and hNop58

The aforementioned interactions suggested that Hsp90 could participate in RNP assembly; thus, we tested the effect of inhibiting Hsp90 on the production of L7Ae RNPs. The U3 and U4 RNPs have very long half-lives in human cells (Fury and Zieve, 1996), and they are essential RNPs, making the analysis of cells depleted from these RNPs problematic. In addition, inhibition of Hsp90 for long periods may result in indirect effects as a result of its pleiotropic functions. To specifically analyze newly synthesized RNAs, we thus resorted to a transient transfection approach. Human 293 cells were cotransfected with vectors encoding GFP as an internal control and either rat U3, tagged U4, or telomerase RNAs. 4 h after adding the DNA, cells were incubated with geldanamycin, a drug that inhibits the ATPase activity of Hsp90 and prevents folding of client proteins. After 16 h of treatment, the levels of transiently expressed RNAs were measured by RNase protection or qPCR. Remarkably, we found that the ectopically expressed RNAs under-accumulated in geldanamycin-treated cells (Fig. 7 A), indicating an important role for Hsp90 in the biogenesis of these RNPs. Interestingly, the transiently expressed U4 was more severely affected than the endogenous RNA, indicating that Hsp90 inhibition preferentially affected newly synthesized RNPs. This suggests that Hsp90 is involved in RNP assembly rather than in the maintenance of already formed RNPs.

Because Hsp90 is a protein chaperone, one likely possibility is that it acts by controlling the folding of core RNP proteins or assembly factors. Client proteins for Hsp90 often become unstable when its ATPase activity is inhibited by geldanamycin (Pearl and Prodromou, 2006; Caplan et al., 2007). To test which proteins could be Hsp90 substrates, we analyzed the effect of this drug on the accumulation of newly synthesized proteins using a similar transfection approach. Human 293 cells were cotransfected with vectors expressing GFP-tagged proteins and GFP alone as an internal control and were incubated with the drug for 4 h after adding the DNA, and protein levels were monitored after 16 h of expression. Remarkably, we found that some GFP-tagged proteins failed to accumulate when Hsp90 was inhibited (Fig. 7 B). This was the case for all L7Ae proteins (15.5K, hNHP2, and SBP2) and for hNop58. A mild effect was also seen for hNop56 and hPrp31, whereas U3-55K, fibrillarin, dyskerin, Nufip, hPih1, hRvb1, and hRvb2 appeared to be unaffected (unpublished data). Altogether, this indicates an essential role of Hsp90 in the biogenesis of C/D snoRNP, U4 snRNA, and telomerase RNPs. It also suggests that Hsp90 acts by controlling the folding of several core proteins during RNP assembly.

hSpagh links Hsp90 to hPih1 and Nufip

To understand how Hsp90 is recruited to assembling L7Ae RNPs and is presented to its potential clients, we analyzed the interactions of the proteins involved by performing systematic Y2H interaction assays with the human and yeast proteins (Fig. 8 A and Table S1). Consistent with previous results (Zhao et al., 2005), we found that yeast Hsp90 bound Tah1 that, in turn, associated with Pih1. Rsa1 appeared to make extensive contacts with the R2TP complex, as it interacted with Pih1, Rvb1, and Rvb2. For the human proteins, we defined a similar set of interactions. Hsp90 interacted with hSpagh, which bound hPih1. hSpagh was additionally connected to hRvb2 that bound hRvb1. Similar to Rsa1, Nufip was also tightly connected to the human homologues of the R2TP proteins because it interacted with hPih1, hRvb1, and hRvb2. Interactions between the human proteins were unlikely to be mediated by endogenous yeast factors because there was little cross-reaction between the yeast and human proteins in Y2H assays (unpublished data). To confirm that the Y2H interactions were direct, we performed GST pull-down experiments with proteins translated in vitro in a bacterial S30 lysate. The results confirmed the interactions of hSpagh with Hsp90, hPih1, and hRvb2 as well as the association of Nufip with hPih1, hRvb1, and hRvb2 (Fig. 8 A). We conclude that the human R2TP proteins form intermolecular interactions similar to those formed by their yeast counterparts and that hSpagh can bridge Hsp90 to hPih1 and Nufip.

hPih1 is associated with SBP2, hNop58, and hNop56

Because yeast Nop58 also forms a Y2H interaction with Pih1, we tested whether human Pih1 also binds snoRNP proteins. Indeed, hNop58 and hNop56 translated in vitro in a rabbit reticulocyte lysate-bound hPih1 in GST pull-down assays (Fig. 8 B). However, this was not the case when these proteins were translated in a bacterial lysate (unpublished data), suggesting that the interaction is indirect or requires protein modification. Interestingly, in Y2H assays, hPih1 was also able to interact with SBP2 (Table S1). The direct physical association of SBP2 and hPih1 was confirmed by in vitro binding assay. SBP2 translated in vitro in a bacterial S30 lysate interacted with immobilized GST-hPih1 (Fig. 8 B). Thus, hPih1 can also interact with hNop58, hNop56, and SBP2.

R2TP proteins associate with core snoRNP proteins in large cytoplasmic complexes

To test whether some assembly factors and core RNP proteins could associate together within large complexes, HeLa S100 extracts were separated onto linear 10–30% glycerol gradients and analyzed by Western blotting (Fig. 9 A and Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200708110/DC1). As previously described, hRvb1 peaked in the middle of the gradient in complexes >670 kD (Makino et al., 1998), and a similar pattern was found for hRvb2. Nufip, hSpagh, hNop58, and fibrillarin peaked in slightly lighter fractions but were also present in heavier fractions where hRvb1 and hRvb2 accumulated. To test whether this cosedimentation reflected a physical association, these fractions were immunoprecipitated with anti-fibrillarin antibodies. Indeed, hRvb1 was found to be associated with fibrillarin in these large complexes (Fig. 9 B).

Discussion

Assembly of L7Ae-type RNPs involves Nufip and a conserved set of proteins

In this work, we have characterized a conserved machinery that is involved in the assembly of several L7Ae RNPs, including box C/D

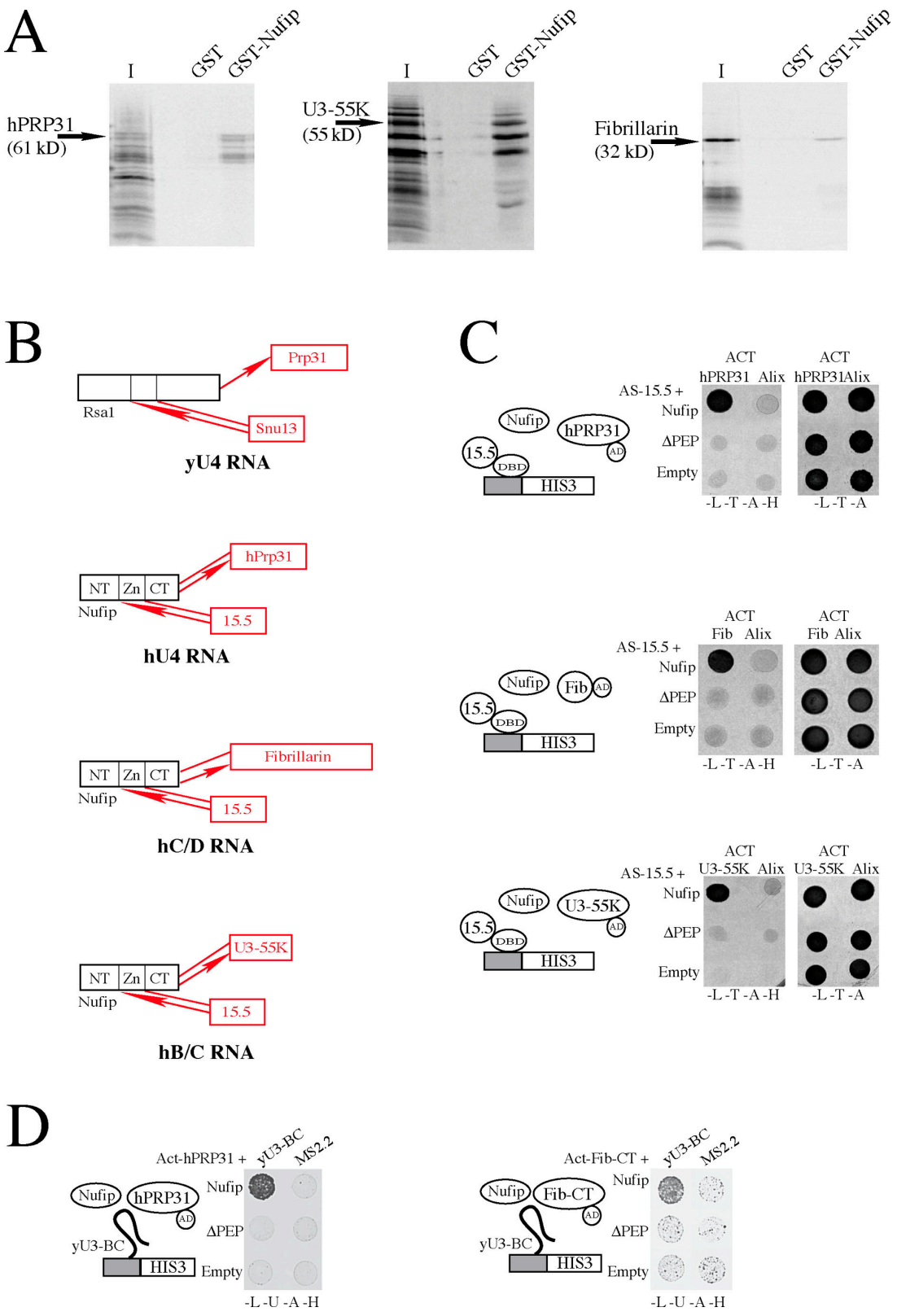

Figure 5. **Nufip tethers 15.5K to hPrp31, U3-55K, and fibrillarin.** (A) Nufip interacts with hPp31, U3-55K, and fibrillarin in GST pull-down experiments. The indicated proteins were translated in vitro in bacterial S30 lysate and assayed for binding to recombinant GST-Nufip. I, input (10% of total). Arrows indicate the full-length protein. The other bands represent incomplete and read-through translation products that occur in the bacterial lysate. (B) Summary of the interactions obtained between Nufip and U4 or box C/D core proteins. Y2H interactions are indicated by arrows, and physical interactions observed in GST pull-downs are indicated by straight lines. (C) Nufip links 15.5K to fibrillarin, hPrp31, and U3-55K in a bridged Y2H assay. Y2H strains containing the indicated pAS and pACT test plasmids were transformed with an ADE2 plasmid expressing Nufip, a mutant lacking the PEP domain, or no protein. Transformants were plated on triple or quadruple dropout media. Growth on −Leu −Trp −His −Ade (−L −T −H −A) indicates the formation of a

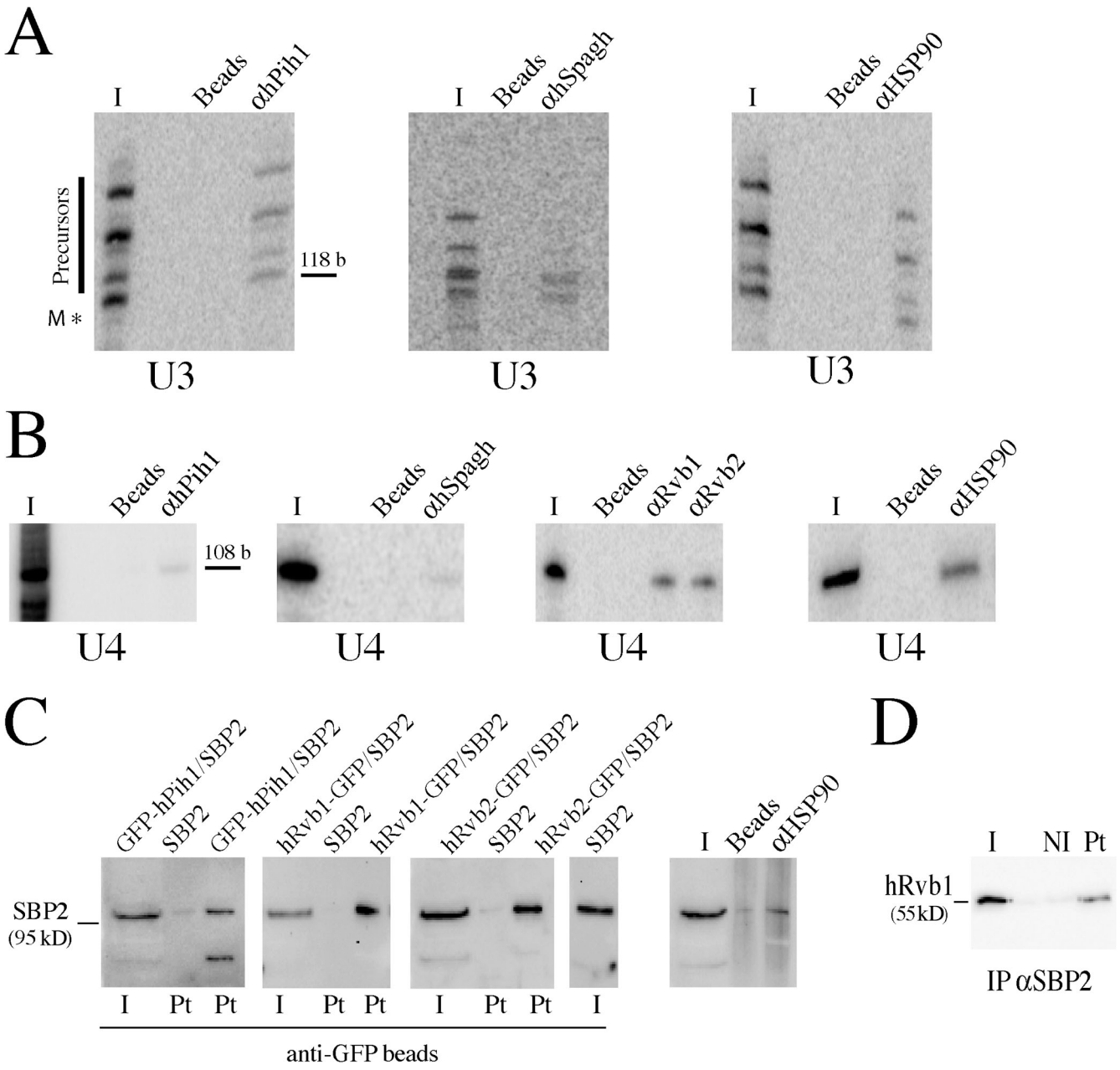

Figure 6. **Hsp90 and human homologues of R2TP proteins bind U3, U4, and SBP2.** (A) U3. Vectors expressing rU3B.7 were transfected in HeLa cells, extracts were immunoprecipitated with the indicated antibody, and RNAs bound to antibody-coated beads or beads alone as a control were analyzed by RNase protection. Precursors and mature forms of U3 are indicated by a bar and an asterisk, respectively. I, input (3% of total). (B) U4. Legend as in A except that binding was tested against the endogenous U4 RNA. I, input (10% of total). (C) SBP2. 293FT cells were transfected with vectors expressing SBP2 alone or with GFP-hPih1, hRvb1-GFP, or hRvb2-GFP, and extracts were immunoprecipitated with anti-GFP (left) or with anti-Hsp90 antibodies (right). Precipitates were analyzed with anti-SBP2 antibodies. I, input (5% of total); Pt, pellets. (D) hRvb1 is present in immunopurified SBP2 complexes. HeLa nuclear extracts were purified with anti-SBP2 antibodies (Pt) or nonimmune serum (NI) and analyzed by Western blotting with anti-hRvb1 antibodies. I, input (0.5% of total).

and H/ACA snoRNPs, the U4 snRNP, and, most strikingly, also the SECIS mRNPs that code for selenoproteins. In yeast, this machinery is composed of Rsa1 and four R2TP proteins: Tah1, Pih1, Rvb1, and Rvb2. In humans, it contains homologues of these proteins: Nufip, hSpagh, hPih1, hRvb1, and hRvb2. This conserved RNP assembly machine possesses several remarkable features: (1) it binds several L7Ae family members (Snu13/15.5K, hNHP2, and SBP2) through Nufip and Rsa1, and this directs assembly factors onto nascent RNPs; (2) it tethers L7Ae proteins to other core proteins, thus likely increasing the efficiency and specificity of their assembly; and (3) it is associated with chaperones of the Hsp90 family, indicating that RNP assembly is closely linked to protein folding.

complex between the indicated proteins. (D) Nufip tethers hPRP31 and the C-terminal domain of fibrillarin to yU3B/C RNA. Y3H strains deleted for Rsa1 were transformed with the indicated plasmid and an ADE2 vector expressing Nufip, a mutant lacking the PEP domain, or no protein. Growth on quadruple dropout media indicates the formation of a complex between the indicated proteins and yU3B/C RNA.

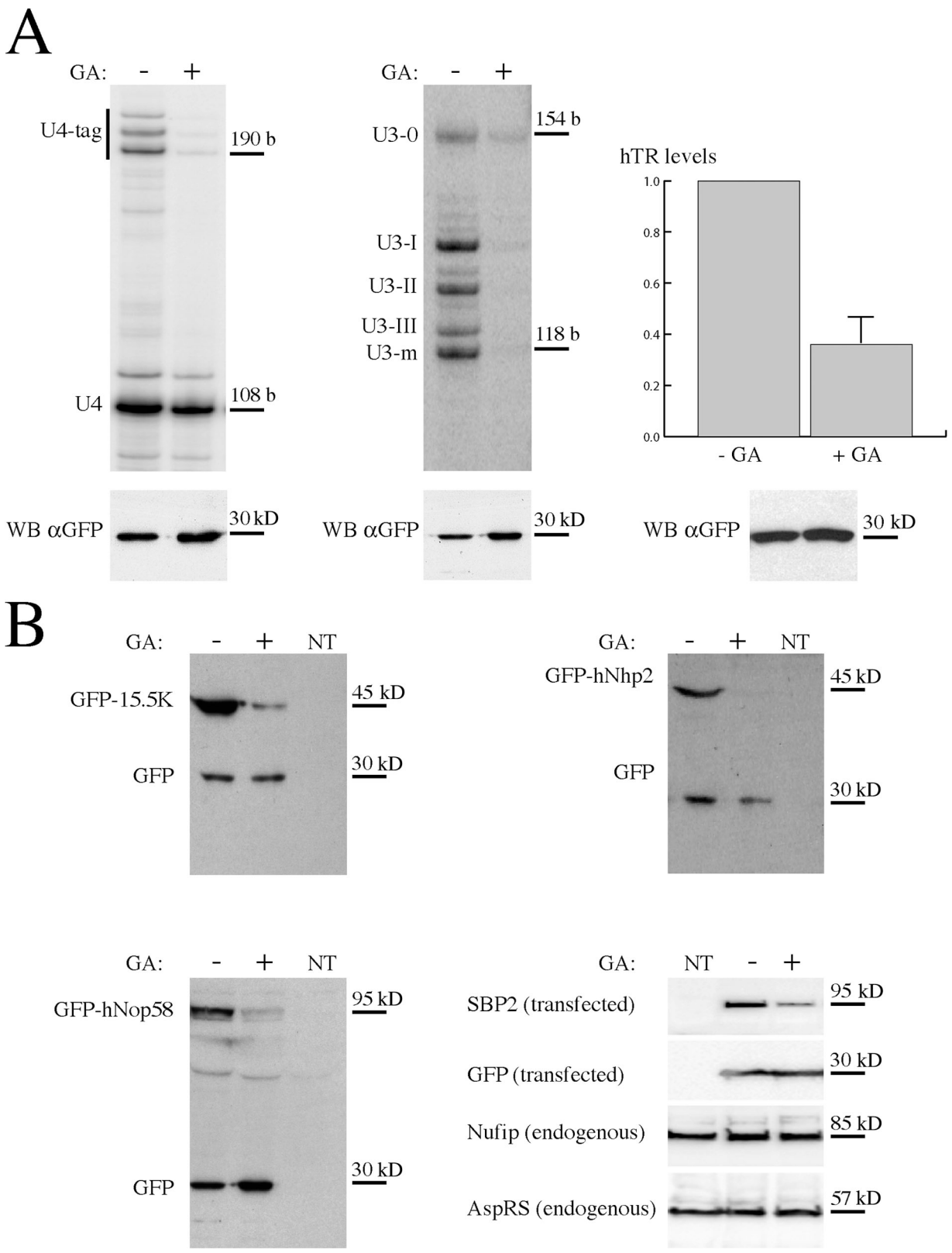

Figure 7. **Inhibition of Hsp90 prevents the production of L7Ae RNPs and destabilizes hNop58, 15.5K, hNHP2, and SBP2.** (A) Geldanamycin prevents the accumulation of U4, U3, and telomerase RNAs. HeLa cells were cotransfected with a GFP expression vector and either a tagged U4 gene (U4 tag), a rat U3 gene, or a human telomerase gene and were incubated with 2 μM geldanamycin for 16 h. The RNAs produced were then analyzed by RNase protection (left) or by qPCR (right). The different U3 precursors (U3-0 to U3-III) and the mature U3 (U3-m) are indicated. (bottom) Western blots against GFP as controls. The graph represents the levels of hTR RNA normalized to levels of GAPDH mRNA and expressed as the fraction of untreated cells. Values are the averages of three experiments. The error bar represents SD. (B) Geldanamycin inhibits the accumulation of GFP-15.5K, GFP-hNHP2, GFP-hNop58, and SBP2. 293 cells were cotransfected with vectors expressing the indicated proteins and GFP as a control and were incubated with 2 μM geldanamycin for 16 h. Cell extracts were analyzed by Western blotting with anti-GFP antibodies (left) or with the indicated combination of antibodies. NT, nontransfected cells.

Hsp90 is required for RNP biogenesis and acts by controlling protein folding during RNP assembly

Hsp90 plays an essential role in the formation of U3, U4, and telomerase RNP, as its inhibition with geldanamycin prevents their accumulation. Client proteins for Hsp90 are often degraded in cells treated by geldanamycin. We showed that this drug inhibits the accumulation of newly synthesized hNop58, 15.5K, hNHP2, and SBP2, thus suggesting that Hsp90 is required to chaperone their folding during RNP assembly. One possibility would be that these proteins adopt a particular conformation when present in the mature RNP complex but that this conformation is unstable or prone to aggregation when the proteins are unassembled and would thus require Hsp90. In this view, the role of chaperones and assembly factors would be to stabilize unassembled proteins, to bring them together on the nascent RNP, and to facilitate the transition to the mature complex. Thus, with the help of scaffolding factors, formation of an intermolecular complex would mimic intramolecular folding.

Although our data are consistent with a role for Hsp90 during RNP assembly, it is equally possible that it exerts its essential folding activity at an earlier step, before the core proteins bind RNA. In this case, the proper protein fold may be stabilized by assembly factors such as hPih1 and Nufip. It is also possible that some proteins like 15.5K bind RNA first and that assembly factors and chaperones would then join the nascent RNP. Finally, the effect of Hsp90 could be indirect, and binding of Hsp90 to U3 precursors may not be functionally relevant or may even be caused by reassociation of the chaperone during the extraction or IP procedure (Kittur et al., 2006).

We found that hPih1 associates with hNop58 in reticulocyte lysates, which are cytoplasmic extracts devoid of nucleolar RNAs. Similarly, Nufip is bound to 15.5K in cytoplasmic S100 extracts (unpublished data). Thus, we favor a model in which core RNP proteins would follow an assembly line in which newly translated proteins would first bind chaperone and cochaperone complexes in the cytoplasm and then translocate into the nucleus, where they would assemble with nascent RNPs.

hSpagh, hPih1, and Nufip are probable cofactors for Hsp90

Hsp90 recognizes many of its client proteins through adaptors, which frequently interact with the chaperone through their TPR domains, as in the case of Tah1 (Zhao et al., 2005) and possibly hSpagh as well. We observed that in human cells, RNP assembly factors remain stable when Hsp90 is inhibited, indicating that they are Hsp90 cofactors rather than targets. Many such cofactors are not essential but merely facilitate the recognition of client proteins. In agreement, although Tah1, Pih1, and Rsa1 are all involved in U3 assembly in yeast (Fig. 4 C), they are not essential genes. Interestingly, strains lacking Pih1 and Rsa1 display a thermosensitive phenotype, suggesting that more chaperone activity might be required at higher temperature and may render these proteins essential. Thus, rather than being absolutely required for snoRNP biogenesis, Hsp90 might simply ensure efficient RNP formation.

Assembling L7Ae RNPs: a remodeling event that involves AAA + ATPases and Hsp90?

On the basis of the differential stability of immature and mature C/D snoRNP complexes toward salt, it has been proposed that pre-snoRNPs undergo a remodeling event to yield the mature particle (Watkins et al., 2004). Although Nufip is part of immature particles, it is absent from mature snoRNPs. Because Nufip is able to tether fibrillarin and hNop58 to 15.5K either directly or through hPih1, this suggests that fibrillarin and hNop58 are loaded on the assembling particle by virtue of their interaction with Nufip. Thus, the remodeling event that leads to the formation of the mature particle probably involves the transfer of hNop58 and fibrillarin from hPih1 or Nufip to 15.5K. Similarly, in the case of U4 and B/C RNPs, it is tempting to speculate that hPRP31 and U3-55K first interact with Nufip bound to 15.5K–RNA complexes and that remodeling transfers hPrp31 and U3-55K to 15.5K.

It is remarkable that the C-terminal domain of Nufip also binds the AAA + ATPases hRvb1 and hRvb2. This family of proteins is known to unfold client proteins, which can be presented by adaptors, and to drive the dissociation of protein complexes and protein aggregates (Hanson and Whiteheart, 2005). The C-terminal domain of Nufip also binds hPRP31, U3-55K, and fibrillarin, and hRvb2 itself binds fibrillarin (Table S1). Thus, one possibility would be that Nufip presents core RNP proteins to hRvb1 and hRvb2, which would partially unfold them to promote their transfer from Nufip to 15.5K–RNA complexes. Another contrasting possibility would be that hRvb2 brings fibrillarin to the nascent RNP and that interaction of this complex with Nufip would trigger its transfer to 15.5K–RNA complexes. Hsp90 may also participate in the process by controlling protein folding during remodeling.

Hsp90, L7Ae RNPs, cell growth, and cancer

Hsp90 is an essential and ubiquitous chaperone but with a rather specialized function in signal transduction (Pearl and Prodromou, 2006; Caplan et al., 2007). In this paper, we show that snoRNPs are targets of Hsp90 and that this is likely conserved from yeast to humans, which is indicative of an ancient function for Hsp90. Hsp90 is tightly linked to human cancer; its overexpression is a marker for cancer cells, and geldanamycin, a drug that specifically targets Hsp90, is a promising treatment for cancer (Pearl and Prodromou, 2006; Caplan et al., 2007). It was previously shown that Hsp90 binds and controls the activity of hTERT, the telomerase reverse transcription (Holt et al., 1999). Here, we show that Hsp90 is also required for accumulation of the telomerase RNA, a process that does not require hTERT. Thus, formation of an active telomerase is tightly controlled by Hsp90, as the chaperone is required at several steps during its biogenesis.

It has been proposed that Hsp90 behaves as a master regulator of cell proliferation by controlling many signal transduction pathways and that this underlies its role in cancers. Our results indicate that Hsp90 also controls cell growth through the synthesis of new ribosomes, replication through the production

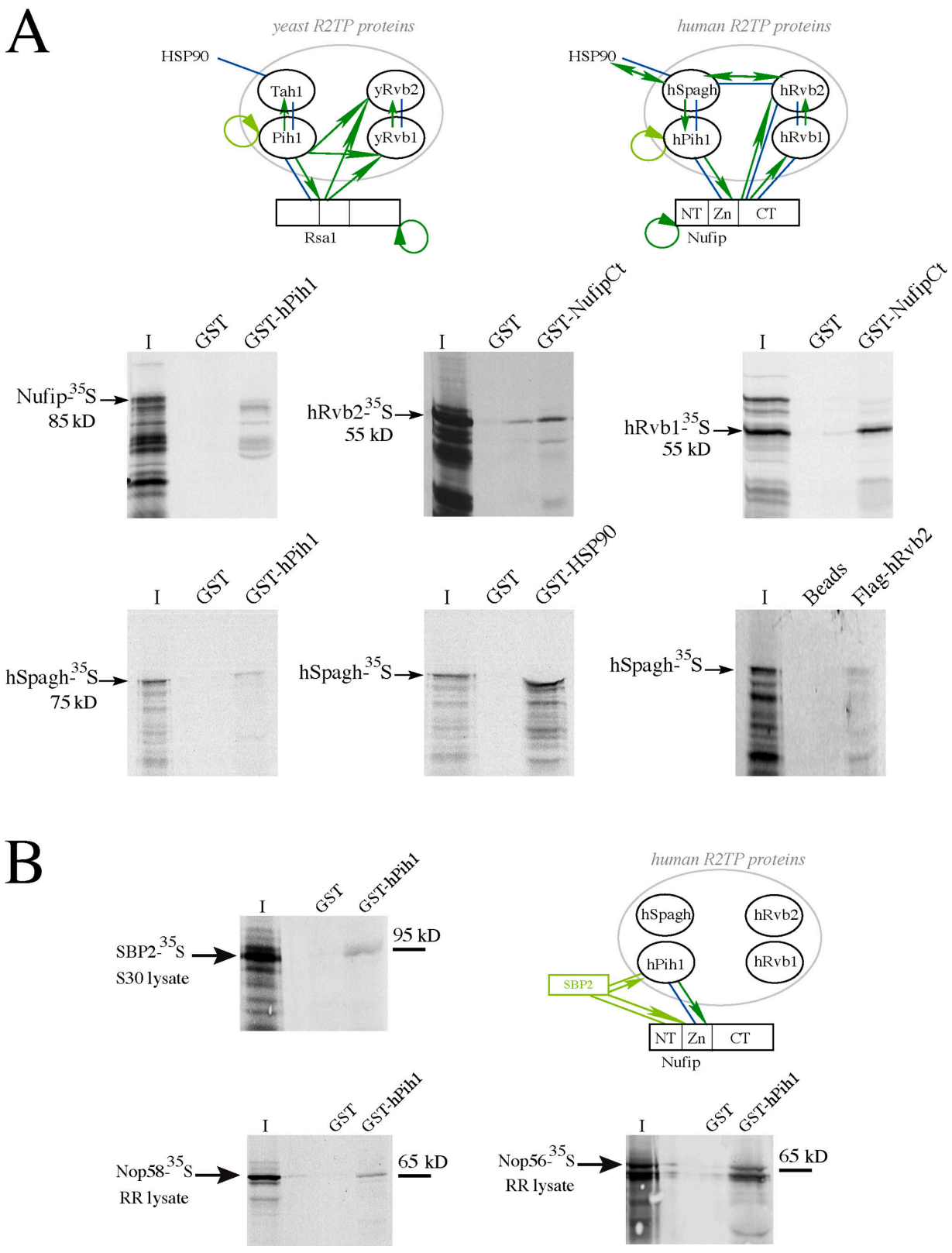

Figure 8. **hSpagh, hPih1, and Nufip mediate the interaction of Hsp90 with hNop58, 15.5K, hNHP2, and SBP2.** (A) Interactions between Rsa1, Nufip, and R2TP proteins. (top) Summary of interactions between yeast (left) and human (right) proteins. Y2H interactions are indicated by green arrows, and physical interactions observed in GST pull-downs are indicated by blue lines. (bottom) GST pull-down experiments. Legend as in Fig. 4. For the interaction between hSpagh and hRvb2, a FLAG-hRvb2 fusion was used. (B) SBP2, hNop56, and hNop58 interact with hPih1 in GST pull-down experiments. Legend as in A except that hNop56 and hNop58 were translated in rabbit reticulocyte lysates (RR). The schematic summarizes the interactions of SBP2 with Nufip and R2TP proteins (legend as in A).

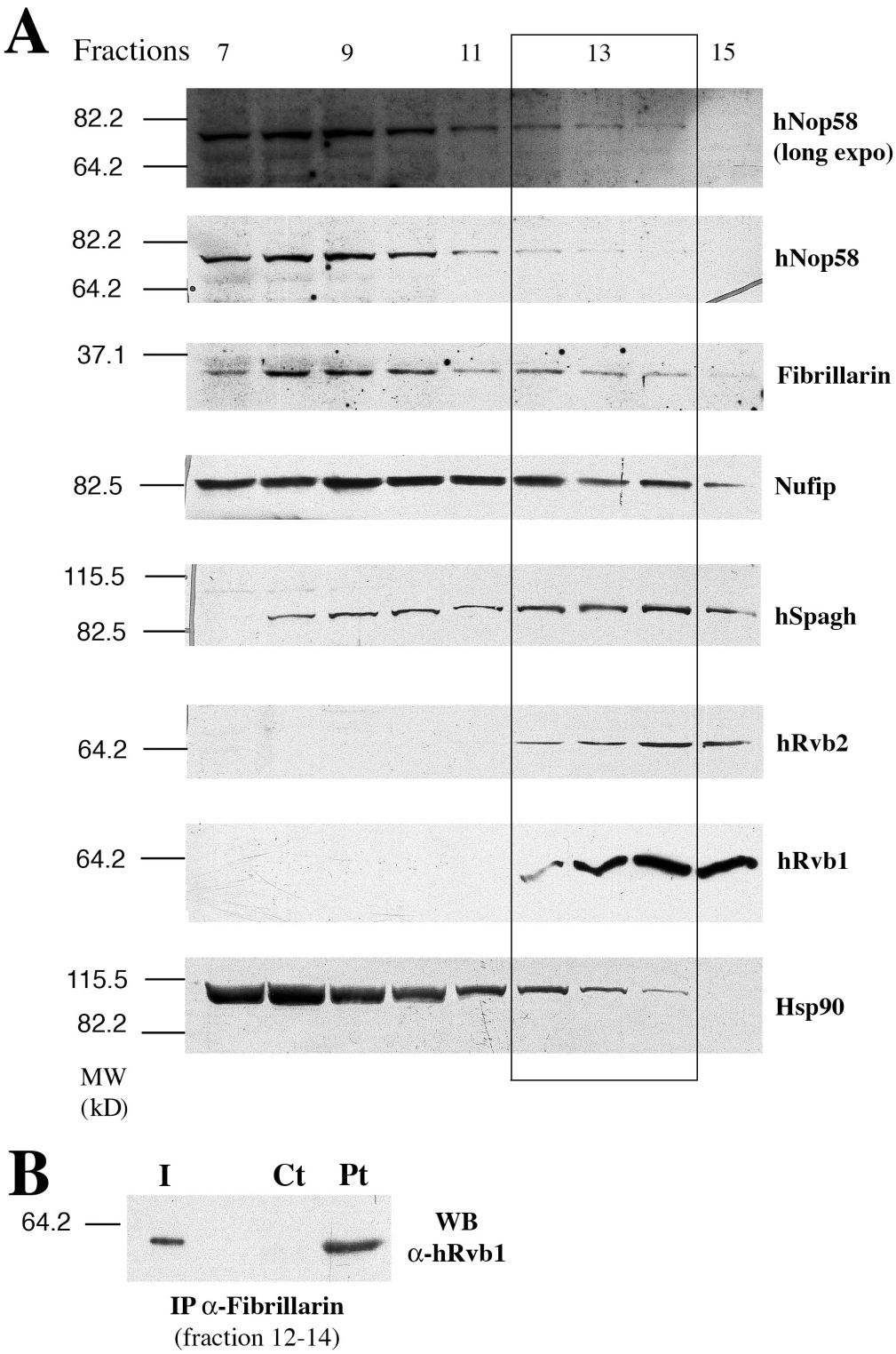

Figure 9. **Nufip and R2TP proteins are present within large cytoplasmic complexes.** (A) Sedimentation profile of Nufip, R2TP proteins, and core snoRNP proteins. S100 cytoplasmic extracts were fractionated on 10–30% glycerol gradients, and 23 fractions were collected. Fractions 7 (top)–16 (bottom) are shown. The boxed fractions contain all of the analyzed proteins. (B) Fibrillarin associates with hRvb1 within large cytoplasmic complexes. Fractions 12–14 were pooled, immunoprecipitated with fibrillarin antibodies, and analyzed by Western blots with anti-hRvb1 antibodies.

of telomerase RNA, and antioxidant defense through selenoprotein synthesis. Thus, Hsp90 may coordinate many of the events required for cell proliferation, explaining why its inhibition is so detrimental to tumor cells.

Materials and methods

Cells, plasmids, and antibodies

Plasmids were obtained by standard techniques and using Gateway technology (Invitrogen) with appropriate destination vectors (pACTII, pASIIΔΔ,

pDEST27, pET15b, pET32a, pGEX2T, and pSPOII-GFP). GFP-Nufip, yU3del, rat U3B.7, U8, and U13 were described previously (Bardoni et al., 1999; Boulon et al., 2004).

HeLa and 293 cells were cultivated at 37°C in DME containing 10% FCS. Transfections were performed with the calcium-phosphate procedure or with LipofectAMINE (Invitrogen) as recommended by the manufacturer. siRNAs against Nufip had the sequences 5′-GGAGCAGUAUUGACAACAA dT dT and 5′-GAGGAGAAACCACAACAUU dT dT. Cells were analyzed 48 h after transfection.

Antibodies against Nufip and fibrillarin were described previously (Bardoni et al., 1999; Verheggen et al., 2002). Anti-hRvb1 and anti-hRvb2 antibodies were purchased from ProteinTech Group. Anti-Hsp90 was monoclonal 3G3 (Affinity BioReagents). Polyclonal serum against hSpagh and hPih1 were produced by immunizing rabbit with peptides CTREENTKNRIKSYD-CONH2 and CIRSEQRPRIQELGDL-CONH2, respectively.

Electrophoresis mobility shift assays

Uniformly labeled yeast U14 snoRNA (yU14) was synthesized by T7 RNA polymerase in vitro transcription of a PCR fragment carrying the *Saccharomyces cerevisiae* U14 snoRNA sequence according to the conditions described previously (Marmier-Gourrier et al., 2003). For electrophoresis mobility shift assay, 200 nM of recombinant protein Snu13p, N3C1, or yPEP was mixed in various combinations at room temperature with ~5 fmol ^{32}P-radiolabeled yU14 RNA in 150 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 20 mM Hepes, pH 7.9. The RNA–protein complexes were resolved by native gel electrophoresis.

Yeast strains and measurements of RNA levels

Growth and handling of *S. cerevisiae* were by standard techniques. Total RNAs were extracted from exponentially growing yeast cultures. RNAs were analyzed by quantitative primer extension using 5 µg of total RNAs and 5 ng of appropriate radiolabeled oligonucleotide. Northern blot analyses were performed with oligonucleotides complementary to the RNA of interest. Sequences of oligonucleotides used as probes and in qPCR are listed in the section Oligonucleotides. qPCR was performed as described previously (Lutfalla and Uze, 2006).

Y2H, Y3H, and bridged assays

For Y2H assays, appropriate pACT-II and pAS2ΔΔ plasmids were introduced into haploid test strains (CG929 or Y190 and Y187), which were then crossed. Diploids were plated on double or triple selectable media (−Leu, −Trp, or −Leu, −Trp, and −His), and growth was assessed 3 d later. For Y2H tests involving pGBKT7-SBP2 or pGADT7-SBP2, plasmids were cotransformed into AH109 (Clontech Laboratories, Inc.). Diploids were plated on triple and quadruple selective media (−Leu −Trp −Ade or −Leu −Trp −Ade −His). For Y3H tests, L40-coat strains carrying appropriate pACTII plasmids were mated with R40-coat strains carrying the plasmid pIIIMS2-2::yU3B/C or pIIIMS2-2 as a control. Diploids were selected on −Trp, −Leu, and −Ura and were tested in –Leu −Ura −His. For the bridged Y2H and Y3H assays, bridge proteins were expressed from the ADE2 multicopy p422 plasmid.

Recombinant proteins and GST pull-down assays

Radiolabeled proteins were synthesized in the presence of [^{35}S]methionine, in *Escherichia coli* S30 lysate (Promega), or in rabbit reticulocyte lysate (TNT; Promega). Binding was performed with 5 µg GST-tagged protein in 20 mM Tris-HCl, pH 8, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1% NP-40, and 10% glycerol. Washing was performed with the same buffer but with 100 mM KCl instead of 40 mM and 5 mM $MgCl_2$ instead of 1 mM. GST- and His-tagged proteins were produced by standard procedure.

Co-IP assays

HeLa cytoplasmic S100 extracts were purchased from 4CBiotech (Belgium). Total extracts of human cells were prepared in HNTG (Girard et al., 2006) except in the experiment of Fig. 3 E, where it was prepared in NET-2 (Boulon et al., 2004). IP was performed as previously described (Boulon et al., 2004). For RNA analyses, TRIZOL (Invitrogen) was added to the beads, and RNAs were extracted and analyzed by RNase protection. For protein analyses, bound material was eluted in 1% SDS at RT, mixed with Laemmli buffer, and analyzed by Western blots. SBP2 co-IP was performed using µMACS Epitope Tag Protein Isolation kits (Miltenyi Biotec) according to the manufacturer's instructions.

For co-IP in yeast, total cellular extracts were prepared from exponentially growing cultures. Extracts were added to IgG–Sepharose beads (Sigma-Aldrich) and incubated at 4°C for 2 h in buffer IPP150 (NaCl 150 mM, 0.1% NP-40, and 10 mM Tris-HCl, pH 8). Beads were washed four times in the same buffer, and RNAs were extracted and analyzed by RT-PCR.

Purification of endogenous SBP2 complexes

HeLa cell lysates (a gift from R. Lührmann, Max Planck Institute of Biophysical Chemistry, Göttingen, Germany) were diluted in IPP250 (250 mM NaCl, 20 mM Hepes-NaOH, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.05% NP-40) and incubated for 2 h at 4°C with protein A–Sepharose (GE Healthcare) charged with affinity-purified anti-SBP2 peptide antibodies. Beads were washed with 120 column volumes, and bound proteins were eluted using IPP250 supplemented with 5% glycerol and 0.6 mg/ml peptide.

Oligonucleotides

The oligonucleotides used in this study for qPCR are as follows: U3 (5′-TTCTCTGAACGTGTAGAGCACCGA and 3′-GATCATCAATGGCTGACGGCAGTT), U4 (5′-GCTTTGCGCAGTGGCAGT and 3′-AGCAATAATCGCTCCTCGG), U14 (5′-CCAACATTCGCAGTTTCCACCAG and 3′-CTCACTCAGACATCCAAGGAAGG), hTel RNA (5′-CTAACTGAGAAGGGCGTAGGC and 3′-TGCTCTAGAATGAACGGTGGA), and U19 (5′-ATGTGGTGCCTGTGATGGTGTTAC and 3′-ACACTGCCCAAAGGTACTCAGCTA). The oligonucleotides used in this study for primer extension are as follows: RTU3 (GGGTACAAAGGTTAT), RTU14 (TCACTCAGACATCCTAG), RTsnR190 (CGAGGAAAGAAGAGACACCATTATC), RTsnR42 (TCAAACAATAGGCTCCCTAAAGCATCACAA), RTU4 (TAAATTTCAACCAGGGGAAACACAATCTCGGACGAA), RTtRNA (TGGACGCAACCGGAATCGAACCG), and RT7S (CAGGACAAATTTACGACGGAGGAA).

Online supplemental material

Fig. S1 shows Y3H interactions of Nufip with various B/C RNAs. Fig. S2 shows alignment of the Rsa1 PEP sequence with potential homologues in various organisms. Fig. S3 shows that hSpagh is associated with hRvb1, hRvb2, and hPih1. Fig. S4 shows cosedimentation of R2TP proteins and Nufip in glycerol gradients. Table S1 is a Y2H interaction map of yeast and human proteins. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708110/DC1.

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