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# *Drosophila* Spag Is the Homolog of RNA Polymerase II-associated Protein 3 (RPAP3) and Recruits the Heat Shock Proteins 70 and 90 (Hsp70 and Hsp90) during the Assembly of Cellular Machineries\*<sup>♦</sup>

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**Background:** Mammalian RNA polymerase II-associated protein 3 (RPAP3) recruits heat shock protein 90 (Hsp90) to assemble cellular machineries such as RNA polymerases.

**Results:** *Spaghetti* encodes the *Drosophila* homolog of RPAP3. *Spaghetti* is essential for development. Spag protein binds and stimulates Hsp90 and Hsp70.

**Conclusion:** RPAP3 function is conserved among metazoans.

**Significance:** Our data suggest that Hsp70 assists RPAP3 in complex assembly.

The R2TP is a recently identified Hsp90 co-chaperone, composed of four proteins as follows: Pih1D1, RPAP3, and the AAA<sup>+</sup>-ATPases RUVBL1 and RUVBL2. In mammals, the R2TP is involved in the biogenesis of cellular machineries such as RNA polymerases, small nucleolar ribonucleoproteins and phosphatidylinositol 3-kinase-related kinases. Here, we characterize the *spaghetti* (*spag*) gene of *Drosophila*, the homolog of human

*RPAP3*. This gene plays an essential function during *Drosophila* development. We show that Spag protein binds *Drosophila* orthologs of R2TP components and Hsp90, like its yeast counterpart. Unexpectedly, Spag also interacts and stimulates the chaperone activity of Hsp70. Using null mutants and flies with inducible RNAi, we show that *spaghetti* is necessary for the stabilization of snoRNP core proteins and target of rapamycin activity and likely the assembly of RNA polymerase II. This work highlights the strong conservation of both the HSP90/R2TP system and its clients and further shows that Spag, unlike *Saccharomyces cerevisiae* Tah1, performs essential functions in metazoans. Interaction of Spag with both Hsp70 and Hsp90 suggests a model whereby R2TP would accompany clients from Hsp70 to Hsp90 to facilitate their assembly into macromolecular complexes.

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ATPases that associate in hexameric rings and are implicated in various cellular functions such as chromatin remodeling and snoRNP<sup>8</sup> biogenesis (2). Because of sequence similarity with the bacterial RuvB proteins, they were initially thought to be DNA helicases. However, biochemical studies have shown a very modest or no unwinding activity at all, and their molecular functions remain poorly understood. RVB1/2 associate with a number of protein complexes (2), and it is currently unclear in which case this relates to the R2TP function. In contrast, Pih1 and Tah1 activity seems to be restricted to the R2TP. In mammalian cells, R2TP contains similar proteins, namely RUVBL1, RUVBL2, RPAP3 (RNA polymerase interacting protein 3, also called Spagh, homologous to Tah1), and Pih1D1 (Pih1 domain containing 1) (3). In addition, R2TP co-purifies with a set of seven prefoldin and prefoldin-like proteins, which together form the R2TP/prefoldin-like complex (4). The role of these additional proteins remains elusive.

One of the first described role of R2TP concerns the biogenesis of the L7Ae ribonucleoproteins (RNPs). This family of structurally related RNPs includes the snoRNPs, necessary for ribosomal RNA maturation, the spliceosomal snRNA U4, and mRNP encoding selenoproteins. This function is conserved from yeast to mammals and requires a dedicated adaptor, called Rsa1 in *S. cerevisiae* and Nufip in mammals (3, 5). Later on, we showed that the R2TP is also involved in the early cytoplasmic steps of RNA polymerase II biogenesis (6). Finally, mammalian R2TP also stabilizes proteins from the PI3 kinase-like kinase family (PIKKs), including mammalian TOR and SMG-1, two regulators of protein synthesis (7). This function in PIKK stabilization is dependent on an adaptor called Tel2 (7). In all these processes, R2TP appears to stabilize newly synthesized proteins by recruiting Hsp90 and to assemble them into macromolecular complexes by yet poorly understood mechanisms (8).

These studies reveal that mammalian R2TP plays a role in the formation of cellular machineries that are necessary for cell growth and proliferation (8). Yet RPAP3 can be knocked down in cell lines without any gross effect on cell viability (6). In *S. cerevisiae*, R2TP function has been mostly documented for snoRNP biogenesis (5). Surprisingly, although snoRNPs are essential, the deletion of *TAH1* is viable, with no clear effect on cell growth, although that of *PIH1* results in thermo-sensitivity (5). Whether R2TP plays an essential or accessory role in metazoans and whether its clients would be conserved, besides snoRNP, remain open questions.

To address the role of the R2TP in a multicellular organism, we used *Drosophila melanogaster* as a model system to investigate the *spaghetti* gene (or *spag*) as it encodes a protein similar to the mammalian RPAP3. Homozygosity of a *P*-element insertion in the *spag* gene produces larval lethality. In mosaic flies, it gives rise to the formation of narrow strips of mutant cells in the

wings, hence the *spaghetti* designation for the gene (9). Therefore, it is of particular interest to determine whether the function of Spag could be similar to that of the mammalian RPAP3 and, if so, whether Spag would be part of a multimeric Hsp90 co-chaperone R2TP complex.

## EXPERIMENTAL PROCEDURES

**Animals**—All fly stocks were maintained on a standard *Drosophila* medium at room temperature, and the crosses were done at 25 °C. The *w*<sup>1118</sup> stock was used as a control. The *l(2)k12101* mutant line derives from a large *P*-element screen described previously (10). All second chromosomal mutations were further balanced with [CyO] or [CyO, *Dfd GFP*<sup>+</sup>] (kindly provided by M. Crozatier). Deficiency *Px*<sup>4</sup> was obtained from the Bloomington *Drosophila* Stock Center. Isolation of viable and lethal revertants was carried out as described previously (11).

We generated three different transgenes in the *CaSpeR4* *P*-element vector (9, 12) carrying different DNA fragments from the *spag* locus. The transgenic P[*DnaJ60*] is made of a 2.7-kb segment containing the *DnaJ60* gene (12); the transgenic fragment P[*spag*<sup>+</sup>] consists of a 4.3-kb DNA segment covering *spag* transcription unit located upstream from the *DnaJ60* gene, and the transgenic fragment P[*DnaJ60/spag*<sup>+</sup>] contains a 6.5-kb DNA segment encompassing both genes (Fig. 2A). Several transgenic lines were established for each construct. Transgenic flies expressing RNAi by the Gal4 upstream-activating sequence system were obtained from Vienna *Drosophila* RNAi Center and maintained at 25 °C: fly strains 23896 and 103353 were used to induce RNAi against *spag*, and strains 21784 and 106393 were used against *Nufip* (13).

**Protein Extracts, Immunoprecipitations, Western Blots, and Antibodies**—For protein extracts, 10 snap-frozen animals (larvae or pupae) were crushed, lysed in Laemmli buffer, boiled, and centrifuged to discard cell debris and lipids. For immunoprecipitations, Schneider's S2 cells were extracted in HNTG buffer (6). Following incubation at 4 °C for 10 min, extracts were centrifuged at 15,000 × *g* at 4 °C to sediment cell debris. Supernatants were collected and incubated for 1 h with agarose beads previously bound with serum or mouse monoclonal anti-Rpb1 antibody PB-7C2 (Euromedex, Souffelweyersheim, France). Bound complexes were then analyzed by Western blot.

Proteins separated by SDS-PAGE were transferred onto nylon or PVDF (small proteins) membranes, according to the size of proteins to be detected. Polyclonal antibodies against a 22-mer synthetic peptide corresponding to the C-end of Spag (CKNWPSKNPAVLNLFKEYGVA) were raised in rabbits. Polyclonal anti-dHsp90 antibody was kindly given by Renato Paro. Proteins were detected as follows: Rpb1 detected with mouse monoclonal PB7-C2 antibody; Rpb2 with goat S20 from Santa Cruz Biotechnology; Nop58 with polyclonal antibodies generated from rabbits immunized with an KKLQEVD-SLWKEFETPEK peptide (14); p70 S6K with monoclonal antibody SC-9027 from Santa Cruz Biotechnology; phospho-Thr398 p70 S6K with monoclonal antibody provided by Cell Signaling Technology (reference 9209); fibrillarlin with monoclonal antibody 5821 from Abcam; tubulin with monoclonal 12G10 (Developmental Studies Hybridoma Bank, Iowa City,

<sup>8</sup> The abbreviations used are: snoRNP, small nucleolar ribonucleoparticle; RNP, ribonucleoparticle; R2TP, complex of RVB1, RVB2, Tah1, and Pih1; RPAP3, RNA polymerase-associated protein 3; Pih1D1, protein interacting with Hsp90 domain containing 1; TOR, target of rapamycin; Hsp, heat shock protein; Hsc, heat shock constitutive; L7Ae, ribosomal protein L7 from archaea; PIKK, phosphatidylinositol 3-kinase-related kinase; Rpb1, RNA polymerase II subunit 1; aa, amino acid; TPR, tetratricopeptide repeat.



## Spaghetti/RPAP3 Function Is Conserved in *Drosophila*

IA); polyclonal anti-15.5K antibodies from Santa Cruz Biotechnology (SC-86760); polyclonal anti-P40 antibodies have been described previously (15), and anti-FLAG M2 monoclonal antibody was from Sigma.

**Tissue Analysis**—Larvae were dissected in PBS, and tissues were fixed with 4% paraformaldehyde and mounted in Vectashield mounting medium (Vector Laboratories).

**Northern Blots**—RNA from animals at the indicated stage was extracted, separated on agarose gel, and transferred to nylon membrane as described (12). The Northern blots were hybridized with <sup>32</sup>P-labeled *spag* or *DnaJ60* cDNA probes. <sup>32</sup>P-Labeled  $\beta$ -tubulin and actin cDNA probes were used as loading controls.

**Isolation of Spaghetti Interactants**—S2 cells were stably transformed with constructs in *pMT/V5-His* vector as follows: FLAG-Spag (aa 1–534), FLAG-Spag $\Delta$ TPR (lacking aa 96–197), or full size Spag without the FLAG epitope as a control. Cells were collected by centrifugation and washed with 20 mM Tris (pH 7.5), 50 mM NaCl and lysed in a buffer containing 20 mM HEPES (pH 7.2), 50 mM KCl, 2 mM MgCl<sub>2</sub>, and 0.2% Nonidet P-40 supplemented with 1 $\times$  protease inhibitor mixture (complete, Roche Applied Science) on ice. Insoluble components were separated by sedimentation for 15 min at 10,000  $\times$  g and the supernatant was centrifuged at 4  $^{\circ}$ C for 30 min at 100,000  $\times$  g. The FLAG-Spag or FLAG-Spag $\Delta$ TPR complexes were then separated by affinity chromatography on anti-FLAG M2 antibody-agarose column. After washing in lysis buffer, the complexes were released with 200  $\mu$ g/ml FLAG peptide in low salt lysis buffer, and the proteins were separated by PAGE. The most abundant protein bands were then analyzed by high mass accuracy matrix-assisted laser desorption/ionization (MALDI) peptide mapping.

Transient transfections in S2 cells were performed using Effectene (Qiagen), according to the *Drosophila* RNAi Screening Center (16) experimental procedures. 3 $\times$ FLAG-Pih1D1 was obtained by recombination of the pDON clone LD15349 in the pAFW vector (both from the *Drosophila* Genomics Resource Center, Bloomington, IN), using Gateway technology (Invitrogen).

**Yeast Two-hybrid Screen**—A yeast two-hybrid screen was carried out as described previously (15) using full-length ORF of a *spag*-cDNA fused in-frame into pBD(GAL4) vector (Stratagene) and transformed into YRG-2 yeast cells. The pBD(GAL4)-*spag* transformed yeast cells were retransformed with embryonic (0–5 h) or ovarian cDNA libraries, in Hibry-Zap II phagemid AD(GAL4) vector (15). Clones were selected for growth on –Leu, –Trp, and –His SD plates and  $\beta$ -galactosidase filter assay.

**In Vitro Assays**—For GST pulldown assays, ORF encoding *Drosophila* Hsp90 (aa 535–717), Spag, Spag $\Delta$ TPR (lacking aa 96–197), or Spag $\Delta$ 2Ct (lacking aa 231–490) were cloned into pGEX 4T-1 and transformed into BL21-CodonPlus-RIL competent cells (Stratagene). The induction was performed with 400  $\mu$ M isopropyl 1-thio- $\beta$ -D-galactopyranoside for 5 h, and GST fusion proteins were purified as described earlier (15). GST fusion proteins (~1–2  $\mu$ g) bound to ~20  $\mu$ l of glutathione-Sepharose 4B (GE Healthcare) were incubated with <sup>35</sup>S-labeled Spag, Spag $\Delta$ TPR, or Hsc70-3 previously synthesized by

TNT coupled reticulocyte lysate *in vitro* translation system (Promega) or with 2  $\mu$ g of unlabeled recombinant bovine Hsc70-4 protein (StressGen) in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, and protease inhibitor mixture (complete, Roche Applied Science) buffer for 3 h at 4  $^{\circ}$ C by rotation of the tubes. Following three 20-min washes with the same buffer, the resin was boiled in SDS-PAGE loading buffer. Two SDS-polyacrylamide gels were run in parallel and transferred to membranes. The first membrane was exposed to detect radioactive proteins and then incubated with anti-Hsp70/Hsc70 monoclonal antibody (StressGen, Enzo Life Sciences) followed by a second incubation with chemiluminescent antibodies. The second membrane was stained with Coomassie R-250.

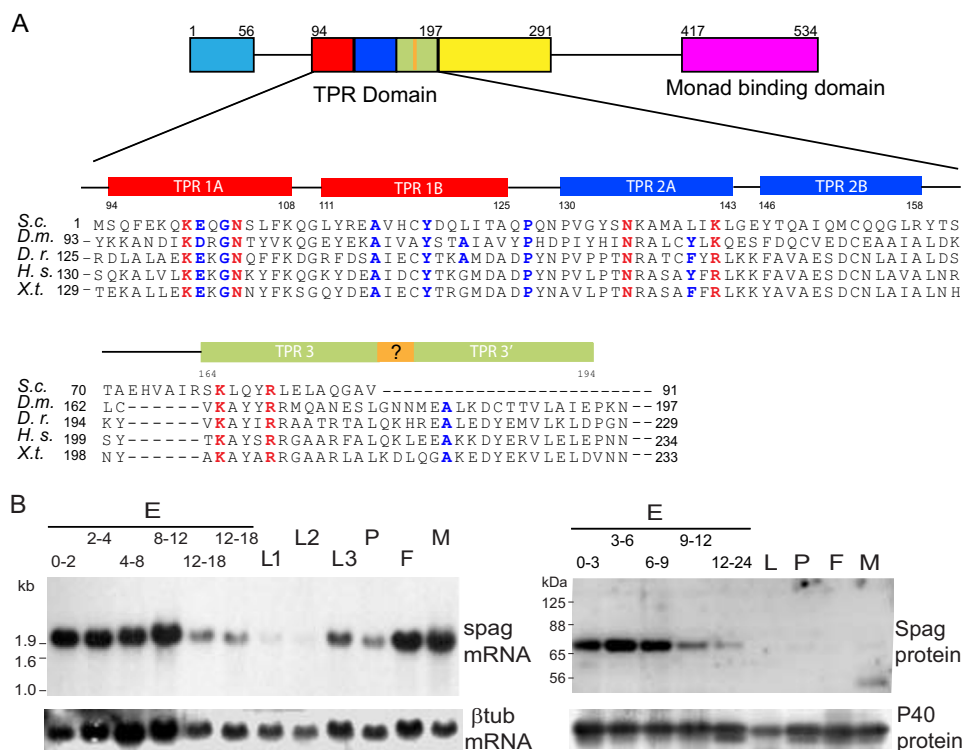
The Hsp70 ATPase measurements were performed as described (17, 18) using His-Spag and recombinant bovine Hsc70 and human Hsp40 available from StressGen/Enzo Life Sciences. 1  $\mu$ g of the indicated recombinant proteins was mixed in 10  $\mu$ l of reaction containing ATPase buffer. 2.5  $\mu$ l of ATP mix, including 50  $\mu$ M ATP and 2.5  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]ATP (3000 Ci/mmol, 10 mCi/ml; PerkinElmer Life Sciences), was added and incubated for 30 min at 30  $^{\circ}$ C. At the end, the reaction was loaded on a Micro-Spin G-50 column (GE Healthcare) and centrifuged for 2 min. 2- $\mu$ l aliquots of the G-50 spin column flow-through (containing the proteins and the bound nucleotides) were chromatographed on Polygram Cel 300 PEI/UV thin layer plate (Macherey-Nagel) using 1 M LiCl, 1 M HCOOH buffer. Unlabeled ATP and ADP were simultaneously run as standards and identified by UV light. The dried plate was exposed to X-ray film.

The Hsp90 ATPase measurements were performed with recombinant His-tagged *Drosophila* Hsp90 and Spag proteins using a previously described coupled enzymatic assay (19), with modified conditions (50 mM HEPES buffer (pH 7.5), KCl 50 mM, 2 mM ATP and 5 mM MgCl<sub>2</sub>). ATPase activity was evaluated using 5  $\mu$ M Hsp90. 20  $\mu$ M of Spag co-chaperone was added to the reaction, and background ATPase activity was determined by the addition of 15  $\mu$ M of geldanamycin. All experiments were carried out at 37  $^{\circ}$ C on a UVIKON spectrophotometer. Reagents were purchased from Sigma.

The aggregation of denatured rhodanese was determined according to Ref. 20. Bovine liver rhodanese (50  $\mu$ M, Sigma) was denatured in 6 M guanidine HCl, 30 mM MOPS-KOH (pH 7.2), and 2 mM DTT buffer for 30 min at room temperature. 2  $\mu$ l of the denatured rhodanese was added to 198  $\mu$ l of 20 mM MOPS-KOH (pH 7.2), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM DTT, 2.5 mM ATP buffer containing the proteins (His-Spag, bovine Hsc70, human Hsp40, and bovine serum albumin; 15  $\mu$ g of each) in the described combinations. Aggregation of the denatured rhodanese was determined at the indicated time points by measuring the light scattering (absorbance) at 320 nm. The measured time point values were subtracted with the corresponding to 0-min value.

## RESULTS

**Characterization of the Spaghetti Gene in *D. melanogaster***—The *spag* gene is referred to in FlyBase as *Dmel/CG13570* and encodes a protein of 534 residues (NCBI accession number



**FIGURE 1. Spag protein structure and patterns of gene expression and protein accumulation during *Drosophila* development.** *A*, schematic structure of the Spag protein and evolutionary conservation of the TPR region in Spag orthologs from yeast to human. Filled boxes represent predicted structural domains and lines unstructured domains. The three TPR motifs forming the TPR region are indicated as red, blue, and green boxes. TPR 3' is a putative helical domain absent in Tah1. Orange box represents a potential  $\alpha$ -helical junction between TPR 3 and TPR 3'. The potential monad binding motif (interPro 025986) overlaps with a predicted structured domain (magenta). Below are aligned the amino acid residues characterizing the three TPR motifs in *S. cerevisiae* (*S.c.*), *D. melanogaster* (*D.m.*), *Danio rerio* (*D.r.*), *Homo sapiens* (*H.s.*), and *Xenopus tropicalis* (*X.t.*). Conserved residues identified in Tah1 for Hsp90 binding are indicated in bold red (21). Blue indicates additional residues conserved in TPR domains and important for Hsp70/Hsp90 binding, as defined previously (23). *B*, expression pattern of *spag* mRNA (left panel) and pattern of Spag protein accumulation (right panel) during *Drosophila* development. RNAs and proteins were extracted from (E) embryos with age in hours after egg laying indicated above the lanes (L<sub>1</sub>, L<sub>2</sub>, and L<sub>3</sub>) first, second, and third instar larvae, (P) pupae, (F) female, and (M) male 3-days old imagos. The Northern blot was hybridized with either <sup>32</sup>P-labeled *spag* or  $\beta$ -tubulin cDNA. The Western blot was probed with anti-Spag or anti-ribosomal P40 protein polyclonal antibodies. The  $\beta$ -tubulin cDNA and anti-P40 antibodies were used for loading control.

NP\_524664.1). Spag protein displays one complete set of three adjacent tetratricopeptide repeats (TPR). TPR domains are responsible for Hsp binding and typically contain a triple repetition of 34 amino acids as follows: X<sub>5</sub>(WLF)X<sub>2</sub>(LIM)(GAS)-X<sub>2</sub>(YLF)X<sub>8</sub>(ASE)X<sub>3</sub>(FYL)X<sub>2</sub>(ASL)X<sub>4</sub>(PKE). *S. cerevisiae* Tah1 possess a single TPR domain, and residues responsible for its interaction with Hsp90 are conserved in Spag (21, 22). Additional residues important for the binding to the C-terminal EEVD motif in most Hsp70 and Hsp90 are also present (Fig. 1A) (23). Spag TPR domain is preceded by a structured domain at its N terminus, and followed by a second, incomplete TPR domain and a "potential Monad-binding" domain in the C terminus (InterPro 025986). Human RPAP3 (AAH5615.1) harbors two domains of triple TPR motifs and the potential Monad-binding domain at its C terminus (24). Such a potential Monad-binding domain is absent in Tah1 (Fig. 1A) (21).

*In vitro* translation of *spag* ORF produced a single peptide with an apparent molecular mass of ~68 kDa corresponding to the predicted molecular mass of Spag (data not shown). Polyclonal antibodies raised against a 22-mer synthetic peptide corresponding to the C-terminal end of Spag reacted on a Western blot with a protein of ~70 kDa (see below for the specificity of the antibodies). In embryo extracts, Spag migrated with a slightly higher mass than expected. Treatment with potato

acidic phosphatase (PAP) indicated that Spag is phosphorylated in embryos but not in larvae nor in S2 Schneider's cells (data not shown). Spag expression is at its highest level during early embryogenesis and then drops to a low level during the second half of embryogenesis to become barely detectable in successive developmental stages and in adult flies. This developmental profile partly reflects mRNA expression and suggests that *spag* may play a critical role during development (Fig. 1B).

The *spag*<sup>d(2)k12101</sup> mutation (hereafter termed *spag*<sup>k12101</sup>) was previously identified in a screen for *P*-element insertion causing tissue overgrowth during larval development (10). In this line, the *P-lacW*-transposon is inserted 52 nucleotides downstream from the *spag* ORF initiation codon (Fig. 2A-a). As a result, expression of both *spag* and its upstream gene *DnaJ60* mRNAs is reduced, whereas the expression of the neighboring genes *Dat* and *CG3328* is not (Fig. 2B, and data not shown) (12). Accordingly, we did not detect Spag mRNA nor protein in *spag*<sup>k12101</sup> animals, also demonstrating the specificity of the antibodies (Fig. 2, B and C).

Homozygous *spag*<sup>k12101</sup> larvae die 6–7 days after egg laying with overgrowth of the hematopoietic organs and occasional formation of melanotic pseudo-tumors (Fig. 2D, compare panel a with panel b) (25). Overgrowth of the hematopoietic organs

## Spaghetti/RPAP3 Function Is Conserved in *Drosophila*

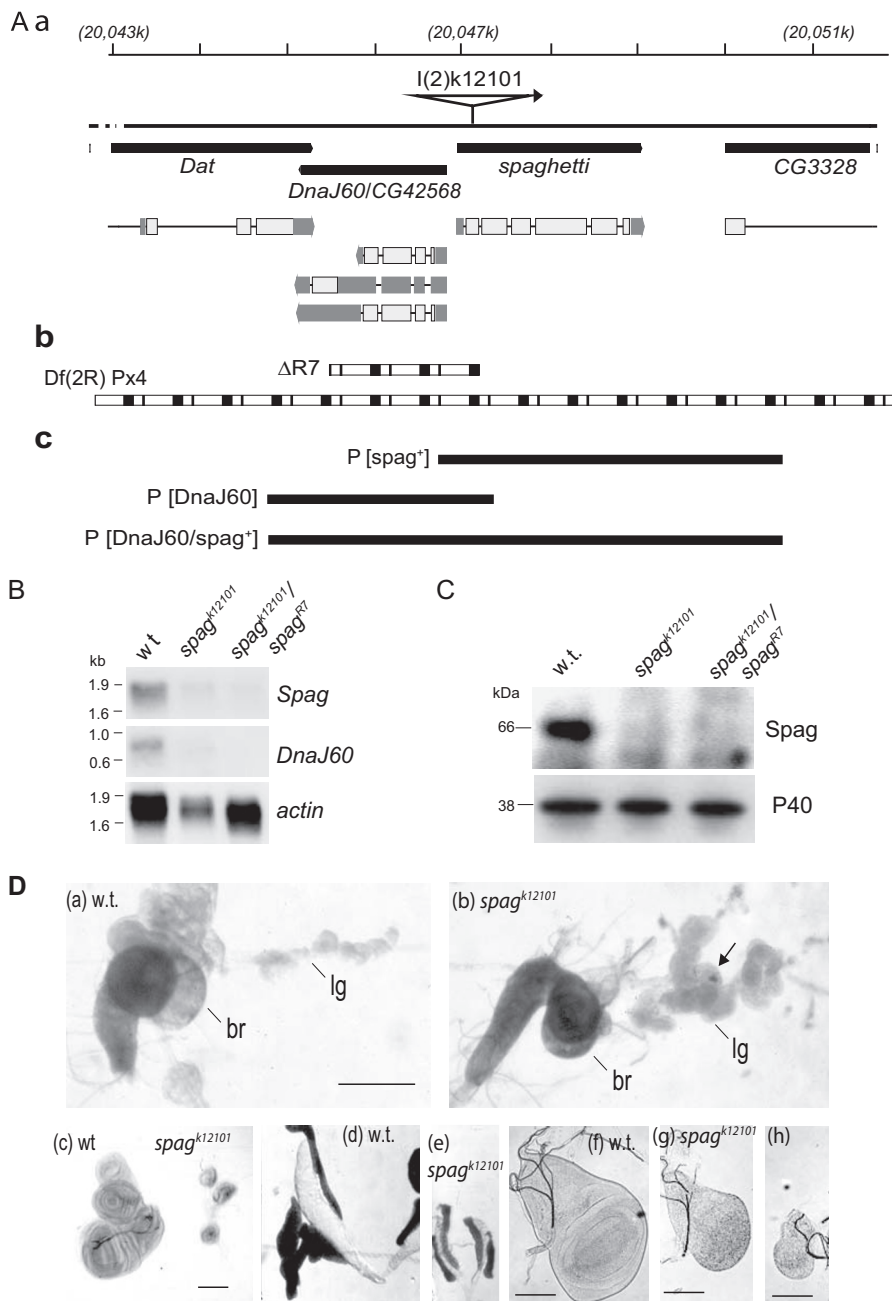


FIGURE 2. *A*, sketch of genomic map for the spaghetti locus. *A-a*, filled black boxes represent the genes, with the corresponding transcripts below. *P*-element insertion in the beginning of the *spag* ORF prevents mRNA accumulation of *spag* and its neighboring gene, *DnaJ60*. *A-b*, reversion of the *P*-element produced the lethal mutations R7, which is a deletion encompassing the *spaghetti* locus. Px4 is a wider deletion that encompasses the locus. *A-c*, three fragments encompassing either the *spaghetti* locus (P[spag<sup>+</sup>]), *DnaJ60* locus (P[DnaJ60]), or both loci (P[DnaJ60/spag<sup>+</sup>]) were used to generate transgenic flies for rescue of the null mutants. *B*, no mRNA for *spag* nor *DnaJ60* are detected by Northern blot in null mutants *spag*<sup>k12101</sup> nor in animals carrying this null mutation with the respective chromosomal deficiency *spag*<sup>k12101</sup>/*spag*<sup>R7</sup>, as compared with wild-type (Oregon-R). The Northern blots were hybridized with <sup>32</sup>P-labeled *spag* or *DnaJ60* cDNA probes. The <sup>32</sup>P-labeled  $\beta$ -tubulin mRNA was used as loading control. *C*, Western blot analysis shows a signal of ~70 kDa in extracts from wild type but not in homozygous null mutants *spag*<sup>k12101</sup> nor in *spag*<sup>k12101</sup>/*spag*<sup>R7</sup> animals. *D*, lymph gland (*lg*), but not the brain (*br*), is overgrown in the *spag*<sup>k12101</sup> larvae (panel *b*) as compared with the wild-type w<sup>1118</sup> (panel *a*). Arrow points toward a melanocytic tumor. In contrast, there is atrophy of the imaginal discs for the legs and halteres (panel *c*), the salivary glands (panels *d* and *e*), and the imaginal discs of the wings (panels *f-h*) in the *spag*<sup>k12101</sup> null mutants (panels *e, g*, and *h*). This effect is more pronounced at 25 °C (panel *h*) than 18 °C (panel *g*).

concerns more particularly the distal and the secondary lobes, which in wild-type larvae are usually the smallest. All other tissues such as imaginal discs (Fig. 2*D*, panels *c* and *f-h*) and salivary glands (Fig. 2*D*, panel *d* versus panel *e*) are reduced in size.

Following *P*-element excision, we recovered numerous white flies producing homozygous viable progeny, as well as

a series of 20 nonviable w<sup>-</sup> revertants with larval lethality. Molecular studies using Southern blotting and PCR analysis revealed that the majority of these revertants contained a partially excised *P*-element, whereas the lethal revertant line R7 displayed an interstitial chromosomal deficiency (Fig. 2*A-b*). These findings indicate that the *P*-insert is responsible of the lethality.



**TABLE 1****Identification of full-length Spag or Spag $\Delta$ TPR (lacking aa 96–197) interactants by proteomic analysis or yeast two-hybrid**

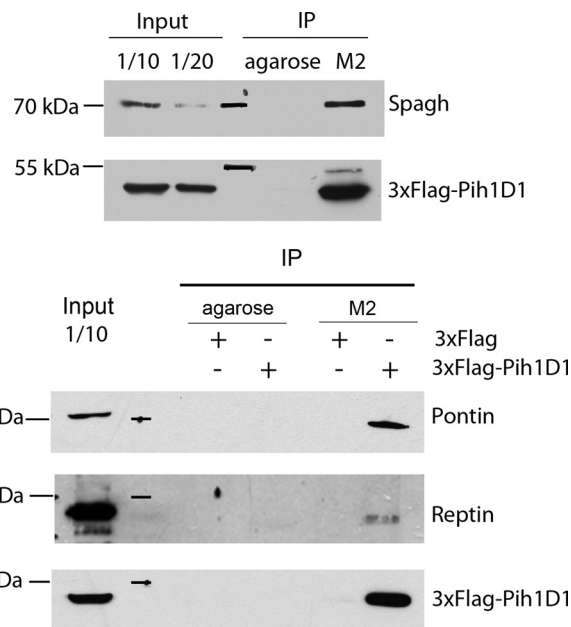
The number of prototypic peptides in the proteomic is indicated. ND = not detected. The presence of an EEVD motif at the C terminus of the Hsps is indicated.

Annotation symbol	Gene	FL Spag	Spag $\Delta$ TPR	C-terminal EEVD
<b>Proteomic data</b>				
CG13570	Spaghetti	116	29	
CG4003-PA	Pontin	8	ND	
CG9750-PA	Reptin	3	ND	
CG14207	Hsp20-like protein	4	ND	–
CG4460-PB	Hsp22	5	ND	–
CG4463-PA	Hsp23	10	ND	–
CG4183-PA	Hsp26	14	ND	–
CG4147-PA	HSC70-3	12	18	–
CG4264-PA	HSC70-4	19	7	+
CG31366	Hsp70Aa	6	5	+
CG8542-PA	HSC70-5	2	7	–
CG1242-PA	Hsp90/Hsp83	21	ND	+
CG5520-PA	Hsp90/GP93	19	ND	–
<b>Yeast two-hybrid</b>				
CG1242	Hsp90/Hsp83	+	–	+
CG13849	Nop56	+	–	
CG5792	Pih1D1	+	+	

In addition, transgenes encompassing the *spag* locus (transformation fragments P(*spag*<sup>+</sup>) and P(*DnaJ60/spag*<sup>+</sup>) from Fig. 2A-c) rescued the lethality of animals with either *P*-element insertion (homozygous *spag*<sup>k12101</sup>) or deletion in the *spag* locus (heterozygous *spag*<sup>k12101/spag</sup><sup>R7</sup>, *spag*<sup>k12101/Px</sup><sup>A</sup>, or *spag*<sup>R7/Px</sup><sup>A</sup> flies; see Fig. 2A-b for a representation of these deletions). In contrast, a *DnaJ60* transgene alone (P(*DnaJ60*) from Fig. 2A-c) was unable to rescue the development of the mutant animals. These data show that *DnaJ60* is dispensable, although *spag* is essential for the survival of the fly (12).

We then took advantage of two different strains carrying transgenes with inducible RNAi against *spag* (P(*GD8058*) and P(*KK100112*), hereafter referred to as *spag*<sup>RNAi1</sup> and *spag*<sup>RNAi2</sup>, respectively) (13). Induction of gene silencing was performed with the upstream activating sequence Gal4 system. RNAi expression driven by *Gal4* under either an *act5c* or a tubulin promoter resulted in pupal lethality shortly after metamorphosis. These animals die later than homozygous *spag*<sup>k12101</sup>, probably because gene silencing is a lengthy process, as compared with a genetic null mutation. This result further demonstrates that *spag* is necessary for fly development.

**Identification of Spag Partners**—The yeast Tah1 and mammalian RPAP3 act in the context of a multimolecular co-chaperone to assist client stabilization by Hsp90. To investigate the partners of *Drosophila* Spag, we isolated soluble, cytoplasmic complexes from Schneider's S2 cells expressing either FLAG-Spag (full length) or FLAG-Spag $\Delta$ TPR, in which the three TPR motifs were excised from amino acid residues 96–197 (Fig. 1A). Following binding to an anti-FLAG M2 antibody-agarose column, complexes were eluted with the FLAG peptide, to reduce contaminants. High mass accuracy matrix-assisted laser desorption/ionization (MALDI) peptide mapping identified numerous heat shock proteins, including Hsp90 and Hsp70 isoforms (Table 1). In the case of Hsc70-3 and Hsc70-5 (both devoid of a C-terminal EEVD), in contrast to the other Hsps, more peptides were detected with Spag $\Delta$ TPR than with full-



**FIGURE 3. Spag, dPih1D1, Reptin, and Pontin associate in *Drosophila* cells to form R2TP.** *Drosophila* S2 cells were transiently transfected to express 3 $\times$ FLAG-dPih1D1 or 3 $\times$ FLAG alone. Cell lysates were incubated with agarose or M2-agarose beads, and complexes were eluted with 3 $\times$ FLAG peptide. Western blot demonstrates an interaction of 3 $\times$ FLAG-Pih1D1 with endogenous Spag, Pontin, and Reptin. As controls, cell lysates (transfected or not) were incubated with agarose beads. 3 $\times$ FLAG-tagged dPih1D1 was detected using an anti-FLAG antibody. *IP*, immunoprecipitation.

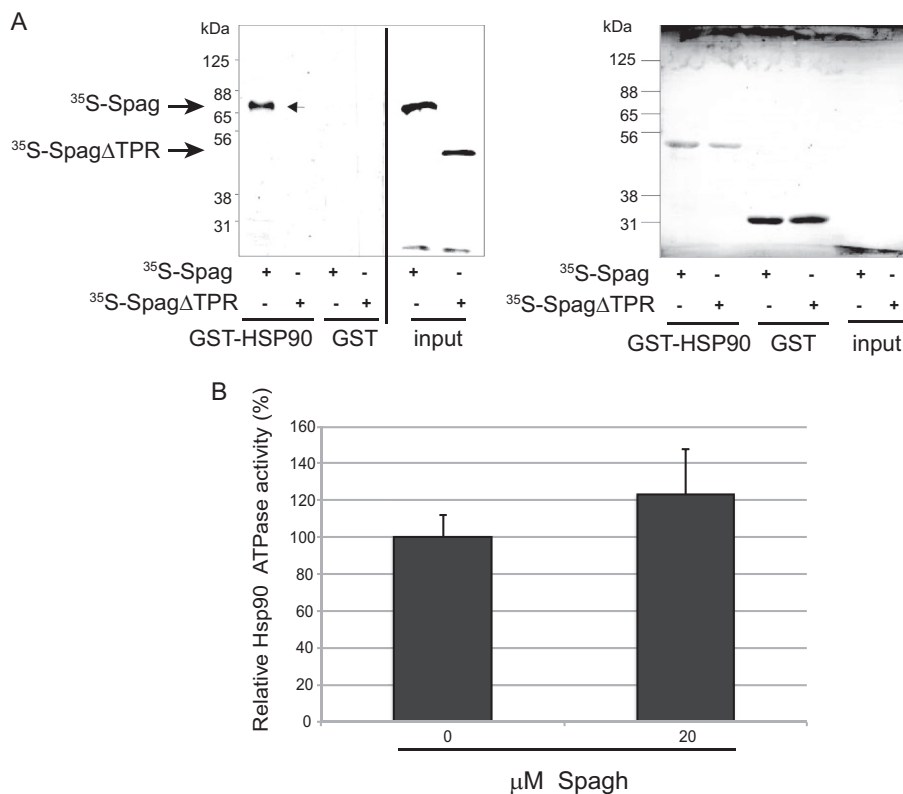
length Spag. In addition, we detected peptides for Pontin and Reptin in full-length Spag immunopurified complexes (Table 1).

We also performed a yeast two-hybrid screen using Spag protein fused to the DNA-binding domain of Gal4 as bait. We identified interacting clones corresponding to the genes *CG1242* (Hsp90), *CG5792*, in accordance with another two-hybrid screen (26), and *CG13849* (Nop56) (Table 1). Alternative splicing of *CG5792* gives rise to two transcripts, one of which (*CG5796-RA*) produces a polypeptide of 334 amino acids, which contains a Pih1 domain (*CG5792-PA* or NP-609590.1). It is thereafter described as D.mel/Pih1D1, as its likely ortholog. Interaction of Spag with Hsp90 parallels the proteomic data described above, as in both cases it required the presence of the TPR domain. This was also the case for Nop56, a core protein from the box C/D snoRNPs. *S. cerevisiae* Nop56 also interact with Tah1 in two-hybrid assays (3). In contrast, Spag interaction with dPih1D1 was independent from the TPR domain.

Co-immunoprecipitations in S2 cells, using a 3 $\times$ FLAG-tagged dPih1D1 confirmed that it binds to endogenous Spag, Pontin, and Reptin (Fig. 3). Altogether, these data indicate that the R2TP core complex is conserved in *Drosophila*.

**Spag Is a Co-chaperone of Hsp90**—To test whether the interaction between Spag and Hsps was direct, we used GST pull-down assays. Full-length *in vitro* translated Spag protein, albeit not Spag $\Delta$ TPR (lacking aa 96–197), could bind to immobilized C-terminal domain of Hsp90 (aa 535–717, encompassing the EEVD-binding motif for co-chaperones) (Fig. 4A). This finding is in accordance with the results from the proteomic and yeast

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**FIGURE 4. Spag is a genuine Hsp90 co-chaperone.** *A*, Spag binds Hsp90 directly through its TPR domain. The C-terminal domain of *D. melanogaster* Hsp90 (aa 535–717) was fused to GST in pull-down assays with rabbit reticulocyte <sup>35</sup>S-labeled proteins. Hsp90 retains Spag but not the truncated SpagΔTPR, which lacks the tripartite TPR domain (amino acids 96–197). Ponceau-stained filter shows that identical levels of the GST and GST-Hsp90 C terminus were retained on the column (*right panel*). *B*, ATPase activation of *Drosophila* Hsp90 by Spag. Relative levels of ATPase activity of 5 μM Hsp90 in the absence or presence of 20 μM of purified His-tagged Spag protein are comparable with that obtained for HSP90 with *S. cerevisiae* Tah1.

two-hybrid assays. In Tah1, the TPR domain is also responsible for binding to Hsp90 (21, 27).

To monitor the effect of Spag on Hsp90 function, we followed its effect on Hsp90 ATPase activity. We observed a weak stimulation in the presence of a 4-fold excess of Spag (Fig. 4*B*). The level of stimulation of Hsp90 ATPase by Spag appears similar to the effect reported for Tah1 on yeast Hsp82 (22, 27, 28).

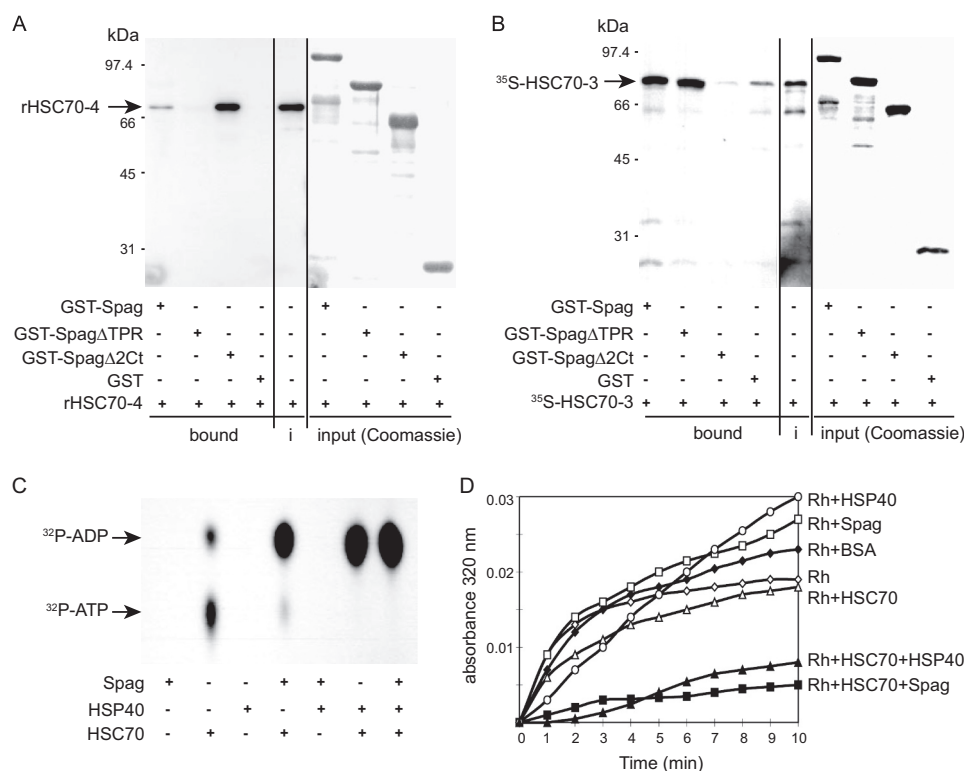
*Spag Is a Co-chaperone of Hsp70—In vitro* GST pull-down assay showed that binding to Hsc70-4 was also direct and depended on the TPR domain (Fig. 5*A*). This is consistent with the fact that Hsc70-4 harbors a C-terminal EEVD motif, known to bind to TPR domains of co-chaperones (5, 23, 27). Yet, in *S. cerevisiae*, Tah1 does not bind directly to Ssa-1, a cytoplasmic Hsp70 isoform (28). Conversely, direct binding of Spag to Hsc70-3, which lacks a C-terminal EEVD motif, was not dependent on the Spag TPR domain, further supporting the fact that deletion of the TPR did not induce a gross disorganization of the protein (Fig. 5*B*). Indeed, Hsc70-3 was unable to bind SpagΔ2Ct, which lacks aa 231–490. Altogether, our results indicated that Spag contains distinct domains able to bind to distinct chaperones.

To investigate the functional consequences of Spag binding to Hsp70, we first measured the *in vitro* ATPase activity of Spag in the presence of commercially available Hsc70 (HspA8, homolog of Hsc70-4). Neither Spag nor Hsp40 was able to hydrolyze ATP to ADP (Fig. 5*C*). In contrast, Spag could significantly enhance the intrinsic ATPase activity of Hsc70, to a level

comparable with that of the Hsc70-Hsp40 complex (Fig. 5*C*). Finally, we investigated the effect of Spag on rhodanese disaggregation. In this assay, rhodanese was first denatured to induce aggregate formation. Addition of Spag, Hsp40, or the control protein BSA alone exerted no effect on those aggregates, which were moderately dissociated by Hsc70. In contrast, combination of Hsc70 with either Hsp40 or Spag strongly enhanced rhodanese disaggregation by as much as ~75% (Fig. 5*D*). Hence, similarly to Hsp40, Spag can act as an Hsp70 co-chaperone. All these data show that Spag is also a *bona fide* Hsp70 co-chaperone.

*Conservation of the R2TP Clients*—Several substrates have been identified for R2TP. These include the box C/D snoRNPs (3, 5). Experiments in mammalian cells showed that accumulation of newly synthesized 15.5K and Nop58 depend on Hsp90 activity (3). We assessed the stability of Nop58 (also called Nop5 in *Drosophila*) (29) and 15.5K (Hoi encoded by the *hoipolloi* gene or *hoip* (9)) in *spag* null mutant larvae (*spag*<sup>k12101</sup>), as well as in pupae in which *spag* was knocked down by the induction of RNAi under the control of *Gal4<sup>act5C</sup>* driver. Western blot analysis showed a decrease in Nop58 and 15.5K content, in *spag*<sup>k12101</sup> larvae and *act5C>spag*<sup>RNAi</sup> pupae. Different protein turnovers in larvae and pupae could explain the differences observed for Nop58 and 15.5K destabilization in *spag*<sup>k12101</sup> or *act5C>spag*<sup>RNAi</sup> animals. In contrast, fibrillarin, another core protein of the C/D snoRNPs, was not affected (Fig. 6, *A* and *B*). This is coherent with results from mammals.





**FIGURE 5. Spag is a genuine Hsp70 co-chaperone.** Spag interacts differentially with different Hsp70 isoforms. Full-length or truncated Spag missing either the TPR domain (Spag $\Delta$ TPR lacking aa 96–197) or the C-terminal domain (Spag $\Delta$ 2Ct lacking aa 231–490) was fused to GST and immobilized on beads for pull-down assays. Prey input is detected in the *i* lane. Coomassie stainings of the gels (*right parts* of A and B) show identical amounts of baits on the columns. A, Hsc70-4 is retained by GST-Spag and GST-Spag $\Delta$ 2Ct, but not GST-Spag $\Delta$ TPR. B, in contrast,  $^{35}$ S-labeled Hsc70-3 is specifically retained on GST-Spag or GST-Spag $\Delta$ TPR but not GST-Spag $\Delta$ 2Ct. C, Spag stimulates ATP hydrolysis by Hsc70. Chromatography on PEI-cellulose thin layer plate shows that [ $^{32}$ P]ATP hydrolysis into ADP by Hsc70 alone is weak and undetectable in the presence of Spag or Hsp40 alone. ATP hydrolysis by Hsc70 is strongly enhanced by the addition of recombinant Spag, Hsp40, or both. D, combination of Hsc70 with either Hsp40 or Spag strongly reduces rhodanese aggregation by  $\sim$ 75%, as measured by light scattering at 320 nm. In addition, addition of Spag, Hsp40, or the control protein BSA alone exerts no effect on rhodanese aggregation, which is moderately suppressed when Hsc70 is alone. This experiment is representative of three.

Indeed, fibrillarin is incorporated at a latter step during snoRNP assembly, and it does not seem to be a client of R2TP (3, 30).

Interaction between R2TP and 15.5K requires Nufip as an adaptor (3). *CG4076* encodes a protein with 18% identity to mammalian Nufip. Induction of two different transgenic lines expressing RNAi against *CG4076/Nufip* driven by *Gal4<sup>act5c</sup>* produced early pupal lethality. In these animals, as in the case of *spag*, there is a decrease in endogenous Nop58 and 15.5K, as compared with controls (Fig. 6C). In conclusion, Spag and Nufip are required for the stabilization of the C/D snoRNP core proteins.

A second class of clients described for mammalian R2TP are the PIKKs. Mammalian R2TP is recruited to these kinases via the adaptor Tel2, with a predominant effect on mammalian TOR and SMG1 stability (7). In *Drosophila*, proteomic data indicate that TOR similarly interacts with Pontin, Reptin, and Lqfr, a distant ortholog of Tel2 (31). To test whether these interactions are functionally relevant, we measured TOR activity in wild-type and Spag-deficient flies (as null mutant or transgenic RNAi). Phosphorylation of S6K is classically used as a surrogate marker of TOR activity, to circumvent the lack of antibodies to detect TOR in *Drosophila*. In animals where *spag* was inactive (as null mutant or transgenic RNAi), we detected a down-regulation of S6K phosphorylation (Fig. 7,

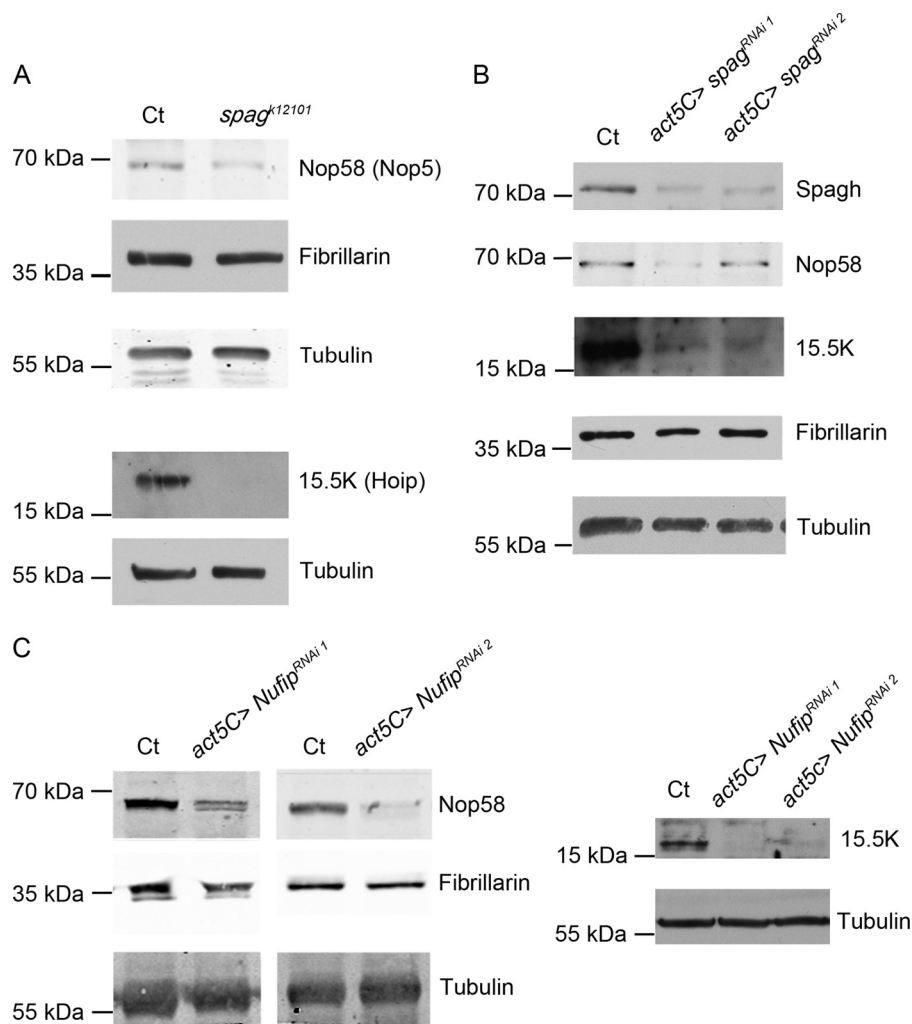
A and B). In contrast, phosphorylation of S6K was not affected upon *Nufip* inactivation (Fig. 7C). These data are consistent with a role for *spag*, but not *Nufip*, in the activity of TOR and are in accordance with a recent report in the *Drosophila* S2 cell line (4, 31).

Finally, in mammals, RNA polymerase II is also a substrate of R2TP (4, 6, 32). Indeed, human Spag binds to neo-synthesized Rpb1, the largest subunit of RNA polymerase II, to stabilize it before its association with the other polymerase II subunits. In flies devoid of Spag (*spag<sup>k12101</sup>*), we found a moderate decrease in total Rpb1 (RPII215). However no effect was seen in *spag<sup>RNAi</sup>* flies (Fig. 8A). Immunoprecipitation of soluble Rpb1 in S2 cells shows a tight interaction with Spag and Hsp90 (Fig. 8B). This suggests a role of Spag in RNA polymerase II assembly rather than Rpb1 stabilization.

## DISCUSSION

**Conservation of the R2TP Complex**—A multimeric Hsp90 co-chaperone known as R2TP was first identified in *S. cerevisiae* (1) and later in human cells (3, 4). In R2TP, Tah1/RPAP3 plays a central role, binding directly to Pih1/Pih1D1 and Hsp90 (3, 5, 27). In this study, we characterize *spaghetti*, a gene essential for *Drosophila* development. Spag interacts with Pontin, Reptin, and CG5792-PA, the *Drosophila* orthologs of the mammalian

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**FIGURE 6. *Spag* and *Nufip* are required for box C/D sno core protein stabilization.** *A*, Western blot analysis of protein extracts from third instar larvae *spag*<sup>k12101</sup>, as compared with wild-type *w*<sup>1118</sup> (Ct), showed a significant diminution in the content of Nop58 (Nop5) and 15.5K (Hoip) but not fibrillarin. Tubulin was used as a loading control. *B*, this phenomenon was also observed in pupae extracts from animals in which RNAi was induced by Gal4<sup>act5C</sup> to knock down *spag* (Gal4<sup>act5C</sup>/*spag*<sup>RNAi</sup> as compared with Gal4<sup>act5C</sup>/+). *C*, similar results were observed in pupae extracts from animals in which RNAi was induced against *Nufip* (C, Gal4<sup>act5C</sup>/*nufip*<sup>RNAi</sup> compared with *w*<sup>1118</sup>).

Pih1D1, in a complex very similar to the mammalian and *S. cerevisiae* R2TP.

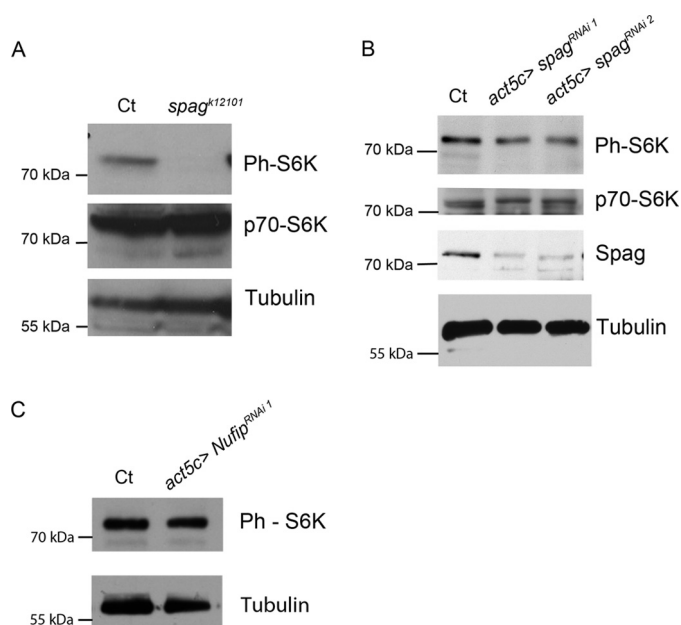
In mammals, R2TP associates with seven prefoldin-like proteins (4, 8). So far, only the association of Spag, Pontin, and Reptin with the product of the *CG14353* gene has been documented (31). The *CG14353*-encoded protein contains a domain with 56% sequence identity with the human prefoldin Monad, associated with the R2TP (24). Besides, an ortholog of Uri, also part of R2TP/prefoldin-like system (4), was identified as a specific interactor of protein phosphatase 1 $\alpha$  (33). dUri is cytoplasmic and is strongly expressed in embryo, pupae, and adult gonads. Interestingly, dUri co-localizes with RNA polymerase II on polytene chromosomes, yet its link with R2TP remains elusive. Conservation of the prefoldin-like association with R2TP in *D. melanogaster* thus remains open to question.

**Conservation of R2TP Clients across Metazoans**—In mammals, three types of clients have been found for the Hsp90/R2TP system as follows: PIKKs, snoRNPs, and RNA polymerases. In *Drosophila*, we detect a decreased activity for the

PIKK TOR when *spag* is inactivated. Proteomic experiments in Schneider S2 cells showed that dTOR interacts with Pontin, Reptin, Spag, and liquid facet-related, a distant homolog of Tel2 (31, 34). In mammalian cells, recruitment of the whole R2TP by Tel2 is necessary to stabilize mammalian TOR (7). Taken together, these data support the conservation of R2TP in the stabilization and assembly of PIKK complexes.

We found that the amounts of two box C/D snoRNP core proteins are diminished in animals devoid of Spag or Nufip. This is consistent with the role described for R2TP and Rsa1/Nufip in the biogenesis of snoRNPs in *S. cerevisiae* and mammals (3, 5). In *Arabidopsis thaliana*, *atNufip* hypomorph mutants are viable but show severe developmental defects, with a subclass of snoRNPs being affected (35). Yet, in this organism, no gene encoding products similar to Pih1D1 has been so far identified (36).

Mammalian RPAP3 stabilizes nascent Rpb1 prior to its association with other RNA polymerase II subunits (6). In *Drosophila*, *Spag* depletion only showed a mild destabilization of Rpb1 in the null mutant. Yet the strong binding of Spag to Rpb1



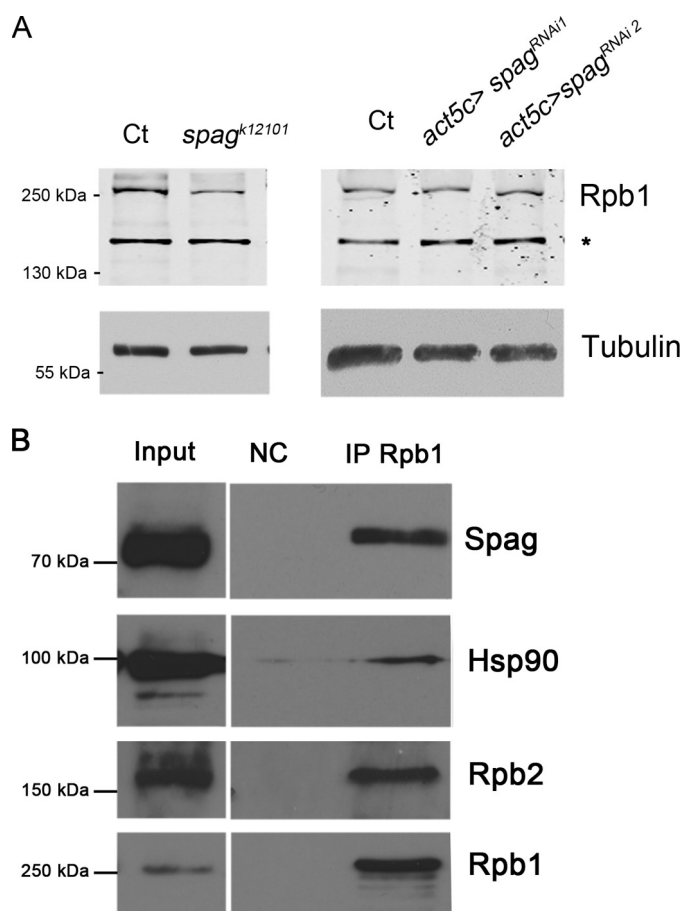
**FIGURE 7. Phosphorylation of p70 S6K, a readout of TOR activity, is decreased upon *spag* inactivation but not *Nufip*.** Western blot analysis showed a diminution of phospho-p70-S6K (*Ph-S6K*) but not total p70-S6K, as compared with tubulin. This was observed upon *spag* inactivation, either in *spag*<sup>k12101</sup> null mutants as compared with *w*<sup>1118</sup> (A) or in pupae with *spag* knockdown (B, *Gal4*<sup>act5c</sup>/*spag*<sup>RNAi</sup>). This was not the case, however, when *Nufip* was silenced (C, *Gal4*<sup>act5c</sup>/*Nufip*<sup>RNAi</sup> as compared with *w*<sup>1118</sup>) (C).

supports a role in RNA polymerase II assembly. Quantitative analysis of Hsp90 interaction with its clients demonstrates that some, but not all, are degraded upon Hsp90 inhibition (37). Hsp90 could act as a molecular tweezers, by maintaining Rpb1 via R2TP in a state amenable for interaction with Rpb2, as it maintains glucocorticoid receptors in a state compatible with ligand binding (38).

The phenotype observed in *spag* null mutant larvae, in which most organs are atrophied, could thus be explained by alterations in the functions of TOR, snoRNP, and/or RNA polymerase II, as all these machineries are required for cell growth (39, 40).

**Spaghetti Is a Co-chaperone for Both Hsp70 and Hsp90—*D. melanogaster*** *Spag* is able to bind directly through its TPR domain, with Hsp90 and Hsp70 isoforms containing a C-terminal EEVD. In addition, the conserved C-terminal domain is implicated in recruiting Hsc70–3, an Hsp70 isoform devoid of EEVD motif. Finally, *Spag* is able to stimulate Hsc70 activity, suggesting a role for Hsp70 in R2TP function.

Some co-chaperones such as CHIP, display a single TPR domain, which binds alternatively Hsp70 or Hsp90 (15, 17). In contrast, HOP harbors three TPR domains, which enable it to transfer clients from Hsp70 to Hsp90 (23). In invertebrates genomes, genes encoding proteins displaying both TPR and Monad-binding motifs can be identified, which are likely *Spag* homologs (41). Most insect *Spag*, including *D. melanogaster*, show a single TPR domain, although other insect *Spag* contain two such regions, as in vertebrates. *Spag* homologs with two TPR domains could bind simultaneously to Hsp70 and Hsp90 and transfer the client between these chaperones. In species with a single TPR domain (such as *D.*



**FIGURE 8. Spag interacts with Rpb1.** A, largest subunit in RNA polymerase II, Rpb1 (RpII215), showed a moderate decrease in extracts from *spag*<sup>k12101</sup> L3 and no sensible variation in *Gal4*<sup>act5c</sup>/*spag*<sup>RNAi</sup> pupae, as compared with matching controls. B, immunoprecipitation of Rpb1 in *D. melanogaster* S2 cells showed a robust interaction with *Spag* and Hsp90, as well as Rpb2.

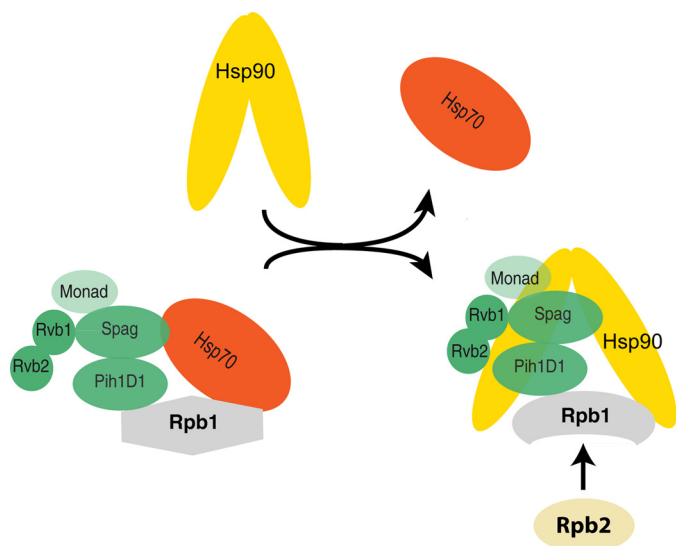
*melanogaster*), another co-chaperone or Hsp (such as Hsp22, -23, or -26) could assist the transfer of R2TP/clients from Hsp70 to Hsp90.

Intriguingly, the Ssa-1 and Ssb-1 proteins of *S. cerevisiae*, which are orthologs of cytoplasmic Hsp70, interact with Pih1 as well as the box C/D sno core proteins (42). This supports a role for Hsp70 in box C/D snoRNP biogenesis, and R2TPs function in general. Based on all these data, we propose a model whereby nascent R2TP substrates would first be pre-folded by Hsp70 and then transferred to Hsp90 to maintain them in a conformation amenable for integration into molecular complexes (Fig. 9).

**Conclusion—**In *S. cerevisiae*, single deletions of some R2TP components (namely Tah1 and Pih1) or adaptors (*Rsa1*) are not lethal unless cells are subjected to heat or nutrient stress (5). In particular, deletion of *TAH1*, the subunit that contacts Hsp90, generates only very mild phenotypes, thus suggesting that the chaperone plays an auxiliary role in the assembly of R2TP clients. In contrast, *spaghetti* is essential during fly development. This suggests that within the R2TP context, the role of Hsp90 might have evolved from a conditional machinery operating under stress conditions in yeast to a constitutive assembly fac-



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**FIGURE 9. Hypothetical model for R2TP function in complex assembly, such as RNA polymerase II.** Nascent Rpb1 could be tethered to Hsp70 via R2TP to undergo early folding steps, upon which it would be transferred to Hsp90, to maintain it in a conformation compatible with Rpb2 binding. Data from Ref. 31 suggest that an ortholog of the prefoldin-like Monad (light green) is associated to R2TP.

tor in metazoans. In addition, our data support a role for Hsp70 in R2TP-assisted complex assembly.

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