Physical and Functional Interactions between *Drosophila* Homologue of Swc6/p18\(^{\text{Hamlet}}\) Subunit of the SWR1/SRCAP Chromatin-remodeling Complex with the DNA Repair/Transcription Factor TFIIH*

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Mariana Herrera-Cruz, Grisel Cruz, Viviana Valadez-Graham, Mariana Fregoso-Lomas, Claudia Villicaña, Martha Vázquez, Enrique Reynaud, and Mario Zurita

From the Department of Developmental Genetics, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001, Cuernavaca Morelos 62250, México

**Background:** TFIIH interacts with multiple factors.

**Results:** The fly p8 subunit of TFIIH is encoded in a bicistronic transcript with the homolog of the Swc6/p18\(^{\text{Hamlet}}\) subunit of the SWR1/SRCAP complex and physically and genetically interacts with TFIIH.

**Conclusion:** There is a functional link between Swc6/p18\(^{\text{Hamlet}}\) and TFIIH.

**Significance:** This functional interaction opens new avenues to study how TFIIH modulates transcription and DNA repair.

The multisubunit DNA repair and transcription factor TFIIH maintains an intricate cross-talk with different factors to achieve its functions. The p8 subunit of TFIIH maintains the basal levels of the complex by interacting with the p52 subunit. Here, we report that in *Drosophila*, the homolog of the p8 subunit (Dmp8) is encoded in a bicistronic transcript with the homolog of the Swc6/p18\(^{\text{Hamlet}}\) subunit (Dmp18) of the SWR1/SRCAP chromatin remodeling complex. The SWR1 and SRCAP complexes catalyze the exchange of the canonical histone H2A with the H2AZ histone variant. In eukaryotic cells, bicistronic transcripts are not common, and in some cases, the two encoded proteins are functionally related. We found that Dmp18 physically interacts with the Dmp52 subunit of TFIIH and co-localizes with TFIIH in the chromatin. We also demonstrated that Dmp18 genetically interacts with Dmp8, suggesting that a cross-talk might exist between TFIIH and a component of a chromatin remodeler complex involved in histone exchange. Interestingly, our results also show that when the level of one of the two proteins is decreased and the other maintained, a specific defect in the fly is observed, suggesting that the organization of these two genes in a bicistronic locus has been selected during evolution to allow co-regulation of both genes.

Several multisubunit complexes participate in transcription in eukaryotic cells. TFIIH is an intriguing complex because it participates not only in transcription but also in nucleotide excision repair (NER)\(^3\) and cell cycle regulation (1, 2). TFIIH was discovered 20 years ago, and since then, the 10 subunits that form this complex have been characterized by biochemical, molecular, and genetic studies (2). In the cell, TFIIH subunits are found in a seven-subunit core complex composed of the XPD and XPB ATPases/DNA helicases together with p62, p52, p44, p34, and p8, which participate in NER. The cyclin-dependent kinase CDK7 along with cyclin H and MAT1 form the CAK complex, which, by itself, may also participate in cell cycle regulation. The core and the CAK complexes form the 10-subunit TFIIH complex, which participates in transcription mediated by RNA polymerases I and II (1–4). In addition, it has been suggested that the XPG endonuclease that participates in NER may interact with the 10 subunits of TFIIH and participate in transcription (5). Recently, a new putative subunit of TFIIH has been reported in yeast (6). This new subunit, called Tlb6, appears to be important for the dissociation of Ssl2 (XPB) from the rest of TFIIH after transcription initiation (6). Another important aspect of the study of TFIIH is that mutations in the XPB and XPD subunits are linked to three human syndromes: xeroderma pigmentosum (XP), Cockayne syndrome combined with xeroderma pigmentosum (CS/XP), and trichothiodystrophy (TTD). Whereas XP is caused by defects in NER, CS has been associated with defects in the mechanism of transcription-coupled repair, and TTD may be associated with deficiencies in transcription and DNA repair (1, 7). XP patients are hypersensitive to sunlight and have a high predisposition for skin cancer (7). Individuals suffering from CS have slow growth, cachexia, and defects in the nervous system. Patients with TTD have the characteristic manifestations of brittle hair, ichthyosis, and fragile nails (7). A particular form of TTD, termed TTD-A, is linked to mutations in the p8 subunit of TFIIH. Cells derived from patients with TTD-A have reduced levels of all of the

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Developmental Genetics, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001, Cuernavaca Morelos 62250, México. Tel.: 52-555-6227659; E-mail: mariozz@ibt.unam.mx.

[3] The abbreviations used are: NER, nucleotide excision repair; XP, xeroderma pigmentosum; CS, Cockayne syndrome; TTD, trichothiodystrophy; IP, immunoprecipitation; CTD, C-terminal domain.
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other TFIIH subunits, suggesting that p8 is important for maintaining the stability of the complex (8). The p8 subunit is a small, 71-amino acid protein that is highly conserved in all eukaryotes (8). The function of p8 has been linked to DNA repair in vitro (9). Tracking experiments in cultured human cells suggest that p8 is located in two different kinetic pools, one associated with TFIIH inside the nucleus and the other free of TFIIH in the cytoplasm (10). However, upon UV irradiation, the free pool is translocated into the nucleus, where it associates with TFIIH (10).

We have previously reported that in *Drosophila*, the overexpression of the p8 homolog (*Dmp8*) suppresses the phenotypes generated by mutations in other components of TFIIH, such as p52 (named *Dmp52* in the fly) and XPB (named *haywire* in the fly) (11, 12). In addition, overexpression of *Dmp8* generates flies that are more resistant to UV irradiation (12). In this study, we report that the *Dmp8* gene is encoded in a bicistronic transcript along with the fly homolog of the Swc6/p18Hamlet (referred to here as *Dmp18*) subunit present in yeast SWR1 and in human SRCAP chromatin remodeling complexes. In addition, p18Hamlet has been linked to the genotoxic stress response (9). Tracking experiments in cultured human cells suggest that p8 is located in two different kinetic pools, one associated with TFIIH inside the nucleus and the other free of TFIIH (12). In this study, we demonstrate that Dmp18 physically and functionally interacts with two components of TFIIH. Defects due to the depletion of Dmp8 and Dmp18 are synergistic and suggest a functional link between TFIIH and Dmp18, a subunit present in chromatin remodeling complexes.

**EXPERIMENTAL PROCEDURES**

*Fly Strains*—Fly stocks were maintained at 25 °C on standard food. *Oregon-R* and *w^1118* were used as the wild-type strains. The following alleles were obtained from the Bloomington stock center: *Df(2L)Exel7022, Df(2L)BSC110, P[GawB]BxM1096*, *Df(2L)BSC110*, and *P[GawB]BxM1096*.—RNA interference (RNAi) flies for *Dmp8* (P[GJD10407]v25909) were obtained from the Vienna stock center (39), and *P[lacW]v25909* strain was obtained from the *Drosophila* RNAi Center (39), and *P[lacW]v25909* was provided by the Vienna stock center. *In(2LR)Gla, wgGla-1 Bc EgfrE1* were obtained from the Bloomington stock center. The following alleles were obtained from the Bloomington stock center: *Df(2L)BSC110*, *Df(2L)BSC110*, and *P[GawB]BxM1096*.

**Immunostaining of Polytene Chromosomes**—Fixation and spreading of the chromosomes was made following the protocol reported (17). Co-staining of polytene chromosomes was performed using standard procedures. Immunodetection of co-IP was performed simultaneously.

**Expression Analysis**—Total RNA was isolated using TRIzol (Invitrogen). cDNA was prepared by reverse transcription of total RNA from adult flies or salivary glands. *Dmp8* and *Dmp18* transcript levels were assayed by reverse transcription (RT)-PCR, with specific primers: for *Dmp8*, 5’-GGCAAGACATGTTAAGGAGAA-3’ (forward) and 5’-CTAAGCTTTCGACACACAGGCTTGCTTGCAAGCCAGGAA-3’ (reverse); for *Dmp18*, 5’-ACAACCTGGGTTTCAACTCCACGAGGCTTGCTCTGCTGCAAGCCAGGAA-3’ (forward) and 5’-CTAAGCTTTCGACACACAGGCTTGCTTGCAAGCCAGGAA-3’ (reverse). The expression of the bicistronic mRNA was analyzed with the forward primers and reverse *Dmp8* primers. *rp49* was used as the control in these experiments. Transgene expression was verified by PCR from genomic DNA using a primer that specifically hybridizes with a sequence of pCasper-hsp83 vector (forward, 5’-CGATACCGCTCGACCTGAGGCTG3’- reverse), and a reverse primer specific for *Dmp8* or *Dmp18*.

**Transgenic Flies and Genetic Crosses**—Constructs encoding six histidines at the N or C terminus of Dmp8 (12) and a Myc PCR product of the complete open reading frame of *Dmp8* was sequenced and subcloned into the EcoRI-NolI sites of a modified pAc5.1/V5-His A vector to generate a Dmp8 protein with three repeats of the FLAG epitope at the C terminus (*Dmp8-FLAG*). The Dmp18 expression plasmid was constructed in a modified pAc5.1/V5-His A vector to produce the protein with the FLAG sequences at the N terminus (*FLAG-Dmp18*). *Drosophila* S2R+ cells were independently transfected with each of the recombinant expression vectors for transient expression of the Dmp8 or Dmp18 proteins by means of calcium phosphate. After 2 days, the transfected cells were harvested and lysed in 50 μl of lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 0.2 mM PMSF, pH 7.8) containing protease inhibitors. The protein concentration was determined with Bradford reagent and tested for expression by Western blotting. A fraction of the extract was saved as input. The remaining extract was precleared by incubating with 40 μl of protein G-Sepharose (Invitrogen) for 1 h at 4 °C. The first half of the precleared extract was incubated overnight in a cold room on a rotator with 2 μg of mouse monoclonal anti-FLAG antibody (Sigma)/mg of protein lysate. The remaining extract was incubated with an irrelevant antibody (anti-mouse IgG or anti-rabbit IgG) in the same conditions. 20 μl of protein G-Sepharose were added to each sample and incubated 2 h at 4 °C on a rotator. The samples were centrifuged, and the supernatant was saved as the unbound fraction. The immunoprecipitated fraction was washed four times in 1 ml of lysis buffer. Samples were separated by SDS-PAGE and blotted onto nitrocellulose membrane.

**Protein Detection**—Proteins were analyzed by immunoblotting using standard procedures. Immunodetection of co-IP complexes was performed using anti-FLAG M2 (1:10,000), rabbit polyclonal anti-Dmp8 (1:1000), affinity-purified rabbit-polyclonal anti-Dmp18 (1:1000), rat polyclonal anti-Dmp52 (1:500), anti-XPB (1:1000), anti-CDK7 (1:1000).

**Immunostaining of Polytene Chromosomes**—Fixation and spreading of the chromosomes was made following the protocol reported (17). Co-staining of polytene chromosomes using a rat polyclonal anti-XPD (16) and rabbit polyclonal anti-Dmp18 was performed simultaneously.

**Cell Transfection and Co-IP Assays**—The complete wild-type *Dmp8* and *Dmp18* cDNA sequences were obtained by reverse transcription-PCR and cloned in the pGEX4T-1 vector. The following anti-p8 antibodies were raised against a synthetic peptide corresponding to amino acids 92–108 (Sigma). Rat polyclonal antibodies against Dmp52 were generated using a peptide that corresponds to the last 15 amino acids of the protein as antigen (New England Peptides, Inc.). Polyclonal antibodies against TFIIH subunits (anti-XPB (15), CDK7 (ds-17), and XPD (S-19)) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies against β-tubulin (E7) were obtained from the Developmental Studies Hybridoma Bank. Mouse monoclonal anti-FLAG M2 (Sigma) and mouse monoclonal anti-V5 (ab27671, Abcam) were used in COIP experiments.

**Antibodies**—Two different polyclonal rabbit antibodies against Dmp8 were generated by New England Peptides, Inc., using synthetic peptides containing amino acids 45–59 and 62–73, respectively. Rabbit anti-Dmp18 antibodies were raised against a synthetic peptide corresponding to amino acids 45–59 (Sigma). Rat polyclonal antibodies against Dmp52 were generated using a peptide that corresponds to the last 15 amino acids of the protein as antigen (New England Peptides, Inc.). Polyclonal antibodies against TFIIH subunits (anti-XPB (15), CDK7 (ds-17), and XPD (S-19)) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies against β-tubulin (E7) were obtained from the Developmental Studies Hybridoma Bank. Mouse monoclonal anti-FLAG M2 (Sigma) and mouse monoclonal anti-V5 (ab27671, Abcam) were used in COIP experiments.

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epitope at the N or C terminus of Dmp18 were cloned into the pCaSpeR-hsp83 and pUAST vectors. The constructions were sequenced to confirm their integrity. Transgenic flies were made by Genetic Services Inc. (18). All stocks were crossed with w1118 stock, and independent lines were established and balanced using CyO for the second chromosome and TM2 or MKRS for the third chromosome.

Rescue of viability, fertility, and hold-out wing phenotype of homozygous P(lacW)BxSH1279SH1279 flies was performed by crossing these mutants with the different pCaSpeR-hsp83 transgenic lines. Briefly, transgenic flies expressing Dmp8 or Dmp18 in the third chromosome and CyO balanced in the second chromosome were crossed with heterozygous P(lacW)BxSH1279SH1279 flies with third chromosome balanced with TM2 and MKRS; the F1 progeny was intercrossed to generate homozygous containing one or two copies of the Dmp8 or Dmp18 transgene.

Crossovers to overexpress the RNAi for Dmp18 (dsDmp18) in the adult wing were performed by crossing the Vienna Drosophila RNAi Center transgenic line with the w1118, P(GawB)BxMS1096 driver. To enhance the RNAi expression, a copy of Dicer-2 was added. The genetic interaction between Dmp8 and Dmp18 was evaluated in flies expressing the dsDmp18 induced by MS1096 driver plus one or two copies of the P(lacW) insertion.

The rescue of the genetic interaction was analyzed in RNAi-expressing flies where Dmp8-H6 or Myc-Dmp18 expression was also induced by the MS1096 driver. P(GawB)BxMS1096, Dmp8-H6 or myc-Dmp18/++; dsDmp18/;dsDmp18 males were compared with P(GawB)BxMS1096, +/++; dsDmp18/;dsDmp18.

Yeast Two-hybrid Interaction Assay—Yeast two-hybrid interaction analyses were made with the MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). pGBK7 or pGADT7 vectors were used for GAL4 DNA binding domain or GAL4 DNA activation domain fusion constructs, respectively. The full-length coding sequences of Dmp8, Dmp18, and Dmp52 were cloned into these vectors, and yeast strains AH109 and Y187 were transformed. Yeast mating was conducted, and colony selection was performed on minimal standard medium without His, Leu, and Trp (Clontech), supplemented with 2.5 mM 3-amino-1,2,4-triazole after 7 days. Finally, positive colonies were considered after α- and β-galactosidase enzymatic assays as reported previously (19, 20).

Northern Blotting—For Northern hybridization, 20 μg of total RNA per sample was separated on a 1% agarose gel. The RNA was transferred by capillarity onto a Hybond N+ membrane (Amersham Biosciences) using 20 × SSPE (3 M NaCl, 0.25 M NaH2PO4, 0.02 M EDTA, pH 7.4) as transfer buffer. UV cross-linking of the RNA to the membrane was performed by using the Auto cross-link mode of a UV Stratalinker 2400 (Stratagene). The membrane was prehybridized for 6 h at 42 °C with prehybridization buffer (0.25 M Tris, pH 7.5, 0.5% sodium pyrophosphate, 1% polyvinylpyrrolidone, 1% BSA, 1% Ficoll, 5% SDS, 1 M NaCl, 50% (v/v) formamide, 100 μg/ml denatured salmon sperm DNA). Hybridization was carried out overnight at the same temperature and with the same buffer as the prehybridization. DNA fragments for rp49 or the bicistronic transcript obtained by PCR with specific primers were used as probes after random prime labeling (Prime it II, Stratagene) with [α-35P]dCTP. Then the membrane was washed at high stringency with wash buffer (0.1% 20 × SSPE, 1% SDS), and the signal was detected by autoradiography.

**Functional Link between a SWR1/SRCAP Component and TFIH**

**RESULTS**

The Dmp8 Subunit of TFIH Is Encoded as a Bicistronic Transcript along with the Drosophila Homolog of the Swc6/p18Hamlet Subunit of the SWR1/SRCAP Complex—During the characterization of the Drosophila melanogaster Dmp8 gene, we noticed that it is encoded in a bicistronic locus (Fig. 1A). This bicistronic locus maps to chromosome 2L, and its transcript encodes two open reading frames (NM_135051 and NM_001144319; Fig. 1A). The bicistronic transcript has a small 58-bp intron located 35 bp downstream of the translation initiation codon of Dmp8 (Fig. 1A). As expected, the Dmp8 product is highly conserved, with the highest similarity in the N terminus of the protein (Fig. 1B) (8). The second ORF (named Dmp18 in this study) is highly similar to the human p18Hamlet/ZNHIT1 protein (Swc6 in yeast) that has been reported to be part of the chromatin remodeling complexes SWR1 in yeast and SRCAP in humans (Fig. 1B) (22–25). The SWR1 and SRCAP complexes catalyze the exchange of the canonical histone H2A with the H2AZ variant, which appears to be relevant for promoter activation and removal of phosphorylated H2AX after DNA damage (25–27). In addition, p18Hamlet has also been associated with genotoxic stress response as a substrate of p38α and p38β MAPKs and as a coactivator of p53-dependent transcriptional responses (22, 23). The particular gene organization
of Dmp8 and Dmp18 is intriguing because there are examples of bicistronic transcripts in eukaryotic cells where the two protein products are functionally related (28–33); in this case, the two factors are components of two different complexes involved in transcription and DNA repair.

Next, we determined whether this particular gene organization is also present in other Drosophila species. We found that these two genes are organized as a bicistronic locus not only in D. melanogaster but also in all other Drosophila species sequenced thus far (Fig. 1). Although information is available only from some Drosophila species expressed sequence tags, the same configuration of genes is found in all of the species, including the short intergenic sequence between the two ORFs (supplemental Fig. S1). Furthermore, this form of gene organization is also present in mosquitoes and other arthropods for which genome sequence and/or expressed sequence tags have been determined.
been reported (Fig. 1C and supplemental Fig. S1), suggesting that this configuration of gene organization has been evolutionarily conserved by more than 350 million years of evolution to ensure coordinated gene expression of the factors encoded by the bicistronic mRNA. Interestingly, the intergenic sequences between the two genes are of different lengths but are always very short (no more than 90 bp) and, in some cases, consist of no more than a dozen nucleotides (Fig. 1C and supplemental Fig. S1).

To confirm that in the fly, the two genes are transcribed into a single mRNA, we performed an RT-PCR experiment using oligonucleotide primers that flank the bicistronic loci (see “Experimental Procedures”). Fig. 1D shows that an RT-PCR product was obtained by this approach. The identity of the RT-PCR product was confirmed by DNA sequencing (data not shown). Using Northern blot analysis, we found that the bicistronic transcript is present in all developmental stages analyzed, and no other transcripts encoding Dmp8 and/or Dmp18 were detected (Fig. 1E). These data and the fact that all of the reported expressed sequence tags in Flybase (including organisms other than flies) encode for the two proteins in the same mRNA confirm that Dmp8 and Dmp18 are encoded in the same transcript. Moreover, there are no other copies of these two ORFs in the Drosophila genome (data not shown), suggesting that the two genes are functional.

The fact that both Dmp8 and Dmp18 are encoded in the same transcript does not necessarily indicate that both proteins are expressed in the fly. To verify the expression of both proteins, we generated antibodies specific for each protein (see “Experimental Procedures”) and analyzed the presence of both products by Western blot. A product of the expected molecular weight was identified for Dmp8 (Fig. 1F). In the case of Dmp18, two bands were recognized by the antibody, one corresponding to the expected size and the other of slower mobility in the gel (Fig. 1F). By biochemical cell fractionation experiments, we found that the protein corresponding to the expected molecular weight was preferentially localized in the nucleus, whereas the higher molecular weight protein was localized in the cytoplasm (Fig. 1F). We are confident that the antibodies recognize the expected proteins because these antibodies are capable of recognizing the GST fusion proteins, and, in competition experiments, antigenic peptides abolish the recognition of the specific bands (data not shown). Taken together, our results show that Dmp8 and Dmp18 are encoded in a bicistronic transcript and that both ORFs are expressed in the fly. The highly conserved configuration of the gene organization of Dmp8 and Dmp18 indicates that the co-transcription of these two genes may be related to their functions.

Dmp18 Physically Interacts with the Dmp52 Subunit of TFIIH and Co-localizes with TFIIH in the Chromatin—As mentioned previously, there are examples of functionally related eukaryotic proteins encoded in the same bicistronic transcript. Interestingly, in a yeast two-hybrid-based Drosophila protein interactome analysis, it was reported that the Dmp52 subunit of TFIIH interacts with the ORF that corresponds to Dmp18 (34). This information suggests that Dmp18 may interact with TFIIH. However, because Dmp8 is present in the same transcript with Dmp18 and because p52 has clearly been demonstrated to physically interact with p8 in humans and yeast (35, 36), there is a possibility that in the interactome assays, a part of Dmp8 was present in the same construct as Dmp18, which was later identified as interacting with Dmp52. To determine if Dmp18 can interact with Dmp52 or Dmp8, we performed a yeast two-hybrid analysis. Using stringent conditions for mutant complementation and β-gal activity, we found that Dmp18 interacts with both Dmp52 and Dmp8 and may have the ability to form a dimer (Fig. 2A). As expected, Dmp8 interacted with Dmp52 in this assay but only when Dmp8 was fused to the Gal4 activation domain (AD) and Dmp52 was fused to the Gal4 DNA-binding domain (BD) (Fig. 2A), indicating that the configuration of the fused proteins may affect their specific interactions.

To confirm the interaction between Dmp18 with Dmp52 and/or Dmp8, we made plasmid constructs for expression in Drosophila S2R+ cells that express either Dmp8 with a C-terminal FLAG tag or Dmp18 with a N-terminal FLAG tag (see “Experimental Procedures”). The constructs were transiently transfected into cells, and recombinant protein expression was analyzed by Western blot (data not shown). Co-IP experiments were performed using the FLAG antibody, and the co-immunoprecipitated proteins were analyzed by Western blot using antibodies against specific TFIIH subunits. Our results clearly show that Dmp8-FLAG co-immunoprecipitates with endogenous Dmp52, whereas endogenous Dmp18, DmXPB, and CDK7 do not (Fig. 2B). In cells transfected with FLAG-Dmp18, the FLAG antibody co-immunoprecipitates Dmp18 with endogenous Dmp52 but not with Dmp8, CDK7, or DmXPB. The fact that both FLAG-Dmp18 and Dmp8-FLAG proteins co-immunoprecipitate with endogenous Dmp52, but not with each other, suggests that only one protein may interact with Dmp52 at a time, although we cannot exclude the possibility that the presence of the FLAG tag on Dmp8 and Dmp18 may interfere with other potential protein-protein interactions. This result is important especially because Dmp8-FLAG does not co-immunoprecipitate with DmXPB, which is known to directly interact with Dmp52 in the context of TFIIH (37).

To support these results, we evaluated the co-localization of Dmp18 and TFIIH in chromatin. Immunostaining was performed in polytene chromosomes using Dmp18- and DmXPD-specific antibodies. Our results show that Dmp18 co-localizes with the TFIIH DmXPD subunit (Fig. 2D), which we have previously reported as co-localizing with other TFIIH components (16, 17), thereby supporting the idea that Dmp18 interacts with the TFIIH complex in the fly.

The results of the yeast two-hybrid assay-based fly protein interactome analysis (34) and the co-IP results presented here suggest that Dmp18 and TFIIH may interact via physical contacts with Dmp52 and/or Dmp8. To analyze this possibility, we performed in vitro pull-down assays using Dmp8, Dmp18, and Dmp52 proteins fused to GST (see “Experimental Procedures” and Fig. 3A). We also made GST fusion constructs that contained deletions in specific domains of Dmp52 and Dmp18 (Fig. 3A). These recombinant proteins were analyzed for specific interactions with in vitro-translated products of the full-length Dmp8, Dmp18, and Dmp52 cDNAs. We observed that Dmp18 directly interacts with Dmp52 through its C-terminal region.
(Dmp18(49–152)), which contains an HIT-zinc finger domain (Fig. 3B). The HIT-zinc finger is a sequence motif found in many proteins with important roles in gene regulation and chromatin remodeling and may mediate protein-protein interactions (the Dmp18 HIT-zinc finger is indicated in Fig. 1B). Interestingly, Dmp8 also interacts with the Dmp18 HIT-zinc finger domain (Fig. 3B). Next, we analyzed the regions of Dmp52 protein that interact with Dmp18. We found that Dmp18 specifically interacts with the C-terminal region of Dmp52 (amino acids 408–500) and also with the part of the protein that does not contain the C-terminal region (Fig. 3C), suggesting that Dmp52 may have two distinct Dmp18-binding sites.

Our pull-down results suggest that Dmp18 can interact with Dmp52 in two different regions of the protein, one of which includes the C-terminal domain. The C-terminal domain of p52 has previously been demonstrated to interact with p8 (35, 36). In fact, the C-terminal domains of p52 and p8 adopt a similar fold, forming a compact heterodimer that appears to stabilize p52 (35). Because we observed that Dmp18 also interacts with the Dmp52 C-terminal domain, we hypothesized that either these three proteins form a complex or Dmp8 and Dmp18 are unable to interact with Dmp52 simultaneously. To evaluate this hypothesis, we performed competition experiments using similar pull-down assays. The different GST constructs (GST-Dmp52 and GST-Dmp18) were co-incubated, keeping one of the in vitro-translated products constant and varying the concentration of the other in vitro-translated protein. We found that when the GST-HIT (GST-Dmp18(49–152)) domain of Dmp18 was incubated with Dmp52, both peptides interacted (Fig. 3D). However, when similar pull-downs were performed in the presence of increasing amounts of Dmp8, the amount of Dmp52 bound to the Dmp18-HIT motif decreased. A portion of Dmp52 was still bound to Dmp18, most likely due to the interaction of the Dmp18-HIT motif outside the C-terminal region of Dmp52 (Fig. 3D). Interestingly, under these conditions, we did not observe an interaction between Dmp18 and Dmp8, suggesting that Dmp8 preferentially interacts with Dmp52 (Fig. 3D). In contrast, when we performed the co-IP using the GST-fused C-terminal motif of Dmp52 (GST-
While keeping the Dmp18 amount constant and increasing Dmp8 amounts, we found that the Dmp18-Dmp52 interaction was lost. Taken together, these results indicate that although both Dmp18 and Dmp8 can bind to the C-terminal domain of Dmp52, this interaction is not simultaneously possible, at least in vitro. However, because Dmp18 also can interact with other regions of Dmp52, we cannot exclude the possibility that Dmp52 and Dmp18 might interact even in the presence of Dmp8. In summary, the results presented in this section strongly suggest that Dmp18 physically interacts with Dmp52, leading to its interaction with the TFIIH holocomplex.

**Genetic and Functional Interactions between Dmp18 and Dmp8**—The physical interaction between Dmp18 and TFIIH suggests that a functional relationship may exist between these factors. To address this question, we took advantage of the existence of a P element insertion (P[†acW] l(2)SH1279; Fig. 1A) in the middle of the Dmp8 ORF, that disrupts the second exon of the Dmp8 gene. This insertion was confirmed by genomic PCR using primers that flank the insertion site and adjacent DNA sequences (data not shown). It was reported that this insertion is homozygous lethal (FlyBase). In our experience, however, this line is semilethal, and 25% of the homozygous flies survive (Fig. 4, A and C). Interestingly, among the homozygous survivors, the males are sterile, the females have reduced fertility (lay very few fertilized eggs of which only very few develop), and all of the adults present a held-out wing phenotype as well as a slight reduction in the wing size (Fig. 4A). The held-out wing phenotype has been described for different
mutations in Drosophila, such as mutations in transcription regulators to genes that encode proteins involved in muscle formation and can be used to follow the interactions between functionally related genes (38).

Next, we analyzed the presence of the bicistronic transcript in adult homozygous l(2)SH1279 flies by RT-PCR. Using specific primer sets, we found that a transcript encompassing the Dmp8 gene or the bicistronic mRNA is not present in these flies (Fig. 4B). Interestingly, in the homozygous l(2)SH1279 organisms, a transcript encompassing the coding region of Dmp18 is still present (Fig. 4B), which may initiate transcription within the P element, suggesting that Dmp18 may be expressed in this homozygous survivor. Western blot analysis for Dmp8 and Dmp18 in this mutant fly indicates that Dmp18 is present in the homozygous organisms (Fig. 4B).

To confirm that the phenotypes observed in the flies containing the l(2)SH1279 mutation are due to the insertion in the Dmp8-Dmp18 bicistronic gene, we analyzed two deficiencies ( deletions) that lack the Dmp8-Dmp18 locus.

Df(2L)Exel7022 spans nucleotides 5,000,838–5,058,522 in chromosome 2L and deletes 17 genes, including the Dmp8-Dmp18 locus. Df(2L)BSC172 spans position 5,000,838–5,037,253 in chromosome 2L and deletes six genes. These two deficiencies were crossed with flies carrying the l(2)SH1279 mutant to verify if trans-heterozygous flies carrying the corresponding deficiency and the insertion generate the same phenotypes observed in the homozygous l(2)SH1279 flies. In both cases, flies with the genotypes Df(2L)Exel7022/l(2)SH1279 and Df(2L)BSC172/l(2)SH1279 were semilethal, the males were sterile, the females had reduced fertility, and all of the adult flies presented the held-out wing phenotype (Fig. 4C), indicating that these phenotypes are due to the l(2)SH1279 insertion. Interestingly, these heteroallelic organisms have a higher rate of survival than the homozygous l(2)SH1279 that express Dmp18. This result sug-

FIGURE 4. Characterization and rescue of a mutant Dmp8 allele in the Dmp8-Dmp18 bicistronic locus that only affects Dmp8. A, the l(2)SH1279 allele is an insertion of a P[lacW] in the second exon of the Dmp8-Dmp18 locus (Fig. 1A). It is semilethal because about 25% of the homozygous flies survive, as is indicated in the table (C). All of the adult l(2)SH1279 homozygous flies have the held-out wing phenotype. B, RT-PCR experiment from total adult RNA to identify the presence of transcripts encoding for Dmp8 and/or Dmp18 in l(2)SH1279 homozygous flies shows that the Dmp8 mRNA encoding region as well as the bicistronic mRNA are not detected, whereas a Dmp18 transcript can be detected. Specific oligonucleotide primer sets to amplify the complete Dmp8-Dmp18 bicistronic mRNA, only the Dmp8 mRNA coding region, or only the Dmp18 mRNA coding region were utilized. As controls, total RNA from wild type and heterozygous l(2)SH1279/+ flies was used. The Western blot supports the existence of Dmp18 protein in l(2)SH1279 homozygous survivors, and Dmp8 protein was not detected. CDK7 and β-tubulin were used as loading controls. C, the reduced viability, disrupted fertility, and held-out wing phenotype observed in homozygous l(2)SH1279 flies are due to the insertion of the P element in the Dmp8-Dmp18 locus. The combination of l(2)SH1279 insertion with two different deficiencies that uncover the Dmp8-Dmp18 bicistronic locus generates the same phenotypes observed in the l(2)SH1279 homozygous flies. D, Dmp8, but not Dmp18, transgenic constructs are enough to rescue all of the phenotypes observed in l(2)SH1279 flies. The survival increase and the phenotypic rescue of l(2)SH1279 homozygous were evaluated by the expression of one or two copies of Dmp8-H6 or Myc-Dmp18 together with the l(2)SH1279 insertion. One copy of the Dmp8-H6 transgene was sufficient to increase the survival, abolish the held-out wing phenotype, and recover the fertility.
FIGURE 5. The knockdown of Dmp8 and Dmp18 in the wing of the fly produces deformed and small wings. A, the RNAi transgenic line dsDmp18 produces a dsRNA directed against a region of the Dmp18 mRNA. The dsDmp18 affects the complete bicistronic transcript, causing a decrease in Dmp8 and Dmp18 expression. B, total RNAs from third instar larvae with a salivary gland-specific driver (sgs3-GAL4; wt) or from larvae expressing the dsDmp18 RNA with this driver (RNAi) were analyzed by semiquantitative RT-PCR using specific oligonucleotide primers to amplify the Dmp8 or Dmp18 coding sequences. rp49 amplification was used as an internal control. The bands were quantified using the ImageJ program, and the numbers indicate the relative band intensity in the RNAi samples when compared with the WT RNA (value of 1). C, Western blot analysis of Dmp8 and Dmp18 protein levels in third instar larvae salivary glands expressing the dsDmp18 RNA as well as in wild type salivary glands. The expression of Dmp18 RNA generates a notable reduction of the Dmp8 and Dmp18 protein levels when compared with the wild type tissue. β-Tubulin was used as loading control. D, wing phenotype associated with the reduction of Dmp8 and Dmp18 by RNAi when the MS1096 driver is used is enhanced in a dose-dependent manner. Females with one copy of the driver and one copy of dsDmp18 show negligible wing defects, but the males with the same dose of RNAi but a major expression of the driver (which is located in the X chromosome, where dose compensation occurs) show a curly-like wing (indicated by the arrow). E, in females and males carrying the major possible doses of both the MS1096 driver and RNAi, the defects in the wing are enhanced, and a wing size reduction is observed. F, Dicer (Dcr-2) overexpression enhances the wing phenotype generated by the expression of the dsDmp18 RNA using the MS1096 driver. Note that the wings are practically absent in these organisms. This phenotype is very similar to the one observed in flies expressing RNAi against other TFIIH subunits.5

suggests that the ratio between Dmp8 and Dmp18 levels is important for maintenance of homeostasis during fly development (see below). Additionally, we crossed the l(2)SH1279 flies with a third deficiency Df(2L)BSC110, which contains the Dmp8-Dmp18 locus but lacks the genes that the other two deficiencies have lost, and the Df(2L)BSC110/l(2)SH1279 organisms were 100% viable and fertile (Fig. 4C).

The fact that the bicistronic transcript and mRNA encoding Dmp8 are not present in the homozygous l(2)SH1279 flies whereas a transcript encoding Dmp18 is still produced (Fig. 4B) suggests that the phenotypes observed in these flies are primarily due to the absence of Dmp8. To confirm this, we constructed transgenic flies that expressed either Dmp8 fused to a six-histidine tag (12) or Dmp18 containing the Myc epitope (see “Experimental Procedures”). These flies were crossed with the l(2)SH1279 flies to generate organisms that were homozygous for the l(2)SH1279 insertion with one or two copies of the transgene. We found that the presence of one copy of the transgene expressing Dmp8 with a six-histidine tag at the C terminus (Dmp8-H6) was sufficient to rescue viability and all of the other defects (sterility and held-out wing phenotype) observed in the homozygous l(2)SH1279 flies (Fig. 4D). Conversely, the transgenes expressing Dmp18 with a Myc epitope at the N terminus (Myc-Dmp18) were unable to rescue any of the phenotypes observed in the l(2)SH1279 homozygous flies (Fig. 4D). These results confirm that all of the phenotypes observed in the l(2)SH1279 mutant are due to the absence of Dmp8.

The next question was whether Dmp18 genetically interacts with TFIIH. To answer this question, we decided to use the l(2)SH1279 mutant because in these flies Dmp8 is affected, but Dmp18 is still produced. We also carried out an RNAi approach using a transgenic line from the Vienna collection (39) that expresses a dsRNA directed against the Dmp18 segment of the bicistronic transcript (dsDmp18; Fig. 5A) and is regulated by the UAS-GAL4 system (39, 40). The efficiency of dsDmp18 to reduce Dmp8 and Dmp18 levels was confirmed by semiquantitative RT-PCR and Western blot analysis (Fig. 5, B and C). The ubiquitous ectopic expression of dsDmp18 using actin or tubulin drivers was lethal for the fly (the flies die after the third instar larvae stage; data not shown). Therefore, we took advantage of
the wing phenotypes observed in the l(2)SH1279 homozygous flies as markers to analyze a possible genetic interaction between Dmp8 and Dmp18, and therefore we directed the dsDmp18 expression only to the dorsal compartment of the wing discs using the MS1096 driver (see “Experimental Procedures”). Heterozygous adult male organisms with one copy of the driver and one copy of the dsDmp18 transgene have a semi-curly-like phenotype in the wings (Fig. 5D). When the genetic dose of both the driver and dsDmp18 are increased, the wings developed blisters and were deformed and small (Fig. 5E). Increasing Dicer activity in these flies dramatically enhanced the wing defects (Fig. 5F), whereas expressing Gal80, which inhibits Gal4, prevented the appearance of the phenotypes (data not shown). These results indicate that the expression of the dsDmp18 RNA generates a detectable phenotype in the wing. Furthermore, the expression of two other dsRNA against Dmp52 and Dmp34 subunits of TFIIFH, using the same wing driver, showed the generation of very similar wing phenotypes, as deformed and smaller wings.\footnote{M. Herrera-Cruz, G. Cruz, V. Valadez-Graham, M. Fregoso-Lomas, C. Villicaña, M. Vázquez, E. Reynaud, and M. Zurita, unpublished data.} However, we did not detect the

\begin{figure}
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\caption{The overexpression of Myc-Dmp18 in the wing disc suppresses the phenotypes generated by the dsDmp18 RNA and generates the held-out wing phenotype. A, flies expressing the dsDmp18 RNA together with either Dmp8-H6 or Myc-Dmp18 (indicated in the genotype as UAS) in the wing by the MS1096 driver. In this context, the overexpression of Myc-Dmp18 results in significant suppression of wing defects caused by dsDmp18 RNA. On the other hand, when Dmp8-H6 is overexpressed, the rescue is not observed. The genotypes are indicated in the figure. B, flies overexpressing Myc-Dmp18 in the same domain of the wing disc where dsDmp18 is expressed present a variable expressivity of the held-out wing phenotype. A similar but slighter phenotype is observed in dsDmp18 flies that overexpress Dmp8-H6 (data not shown).}
\end{figure}

the held-out wing phenotype in any RNAi flies. The fact that Dmp8 and Dmp18 are encoded in a bicistronic transcript and that dsDmp18 RNA knockdown affects the expression of both genes (Fig. 5, B and C) may suggest that the held-out wing phenotype observed in homozygous l(2)SH1279 flies may be due to a change in the ratio of Dmp8 and Dmp18 levels.

When Dmp8-H6 was overexpressed in the wing disc of organisms homozygous for the wing driver and dsDmp18, a rescue of the wing defects was not observed (Fig. 6A). This finding indicates that the phenotype observed in flies expressing dsDmp18 in the wing is partially caused by depletion of Dmp18. Consistent with this hypothesis, overexpression of Myc-Dmp18 in flies homozygous for the wing driver and dsDmp18 resulted in significant rescue of wing size and shape defects (Fig. 6A). Interestingly, some of the flies that overexpress either Dmp8-H6 or Myc-Dmp18 together with the dsDmp18 RNA in the wing disc show different expressivity of the held-out wing phenotype (Fig. 6B). This may be due to a gradient expression of the RNAi and the transgenes, which deregulates the ratio of Dmp8 and Dmp18 levels.
Functional Link between a SWR1/SRCAP Component and TFIIH

To determine if Dmp18 and Dmp8 genetically interact, we performed crosses to obtain flies of the l(2)SH1279 background expressing the dsDmp18 RNA in the wing. Because RNAi only partially depleted the mRNA (Fig. 5B), the combination of dsDmp18 with the l(2)SH1279 mutant most likely abolished the expression of Dmp8 and partially down-regulated the expression of Dmp18. Interestingly, in homozygous flies for l(2)SH1279 and expressing dsDmp18, an observable reduction in overall wing size occurred (Fig. 7). In addition, wing deformation was enhanced, and the penetrance of the phenotypes was 100%. Even more, the homozygous flies for both the insertion and the RNAi were not born. These results strongly suggest that simultaneous reduction of Dmp18 and Dmp8 activity in the wing results in an additive effect on the wing growth and development, which indicates a genetic interaction between Dmp18 and Dmp8, which are encoded within the same transcript, and suggests a functional link between them.

DISCUSSION

In this study, we show that the Dmp8 subunit of TFIIH is encoded in a bicistronic transcript that also encodes another protein. This configuration of gene organization is conserved in other arthropods, indicating that the coordinated expression of these two genes has been conserved during evolution (Fig. 1C). Intriguingly, in some species, the intergenic sequence between the two ORFs is very short, and if both ORFs are translated from the same mRNA, it suggests the existence of an unknown, unique mechanism of translation. At some point, we must consider the possibility that Dmp8 and Dmp18 may be produced after proteolytic cleavage from a larger polypeptide. However, the detection of only one polypeptide using two independent antibodies against Dmp8 and the lack of polarity of the l(2)SH1279 insertion on Dmp18 discard this possibility.

The second gene (Dmp18) present in this bicistronic transcript appears to be the orthologue of SWC6/p18Hamlet/ZNHIT1, which has been described as a component of the SWR1 complex in yeast and the SRCAP complex in humans (22–25). Both complexes are composed of multiple subunits and catalyze the exchange of histone H2A with the histone variant H2AZ, which is predominantly located in nucleosomes around the transcription initiation site in yeast and in higher animals (41). In addition, SRCAP also catalyzes the removal of phosphorylated histone H2AX from sites of double-stranded breaks after DNA repair (42). Therefore, both TFIIH and SWR1/SRCAP participate in transcription and DNA repair. This observation, along with the evidence that, in some cases, the bicistronic transcripts encode for two proteins with related functions, suggests a link between TFIIH and Dmp18 and probably with a putative fly SWR1/SRCAP complex. In fact, using multiple approaches, we have shown that Dmp18 physically interacts with TFIIH mainly through a direct interaction with the Dmp52 subunit. However, it is intriguing that in the pull-down and yeast two-hybrid analysis, Dmp18 and Dmp8 interact, but this is not observed in the co-IP experiments or in the different conditions inherent to each type of assay. The discrepancy of these data can be explained by the presence of the FLAG tag epitope in the co-IP experiments, which could have interfered with the direct interaction between the proteins.

The interaction of Dmp18 with Dmp52 is intriguing for several reasons. There are no reports indicating the existence of functional p52 free of TFIIH or associated with other proteins besides the ones forming this complex. In humans and yeast, p52 has been demonstrated to directly interact with p8 through the C-terminal domain of p52. This interaction is relevant for the stability of the complete TFIIH complex (36). Moreover, p52 is important for the assembly of XPB into the core of TFIIH and modulates XPB ATPase activity. This interaction occurs through two separate domains, one near the N terminus and the other near the C-terminal domain of p52 (43). Based on our results, Dmp18 may interact with Dmp52 at two different regions: at the C-terminal domain, which is also the Dmp8-interacting region, and outside the C-terminal domain. At least in vitro, it appears that Dmp8 has a higher affinity for the C-terminal domain of Dmp52 than Dmp18 does, suggesting that both proteins may not be simultaneously interacting with the C-terminal domain of Dmp52. However, because Dmp18 interacts with Dmp52 also in another region, it is possible that even when all 10 subunits of TFIIH are assembled, Dmp18 may still be interacting with Dmp52. This hypothesis is supported by the fact that Dmp18 co-localizes with TFIIH (DmXPD) in the majority of the immunostained sites within polytene chromo-
Functional Link between a SWR1/SRCAP Component and TFIIH

somes. Furthermore, these results are reinforced by a recent report showing that TFIIH (yeast RAD3/XPD) and SWR1 (Swr1) co-occupy the +1 nucleosome immediately downstream of the transcriptional start site where H2AZ resides (44).

In addition to the physical interactions described in this study, we found genetic evidence that supports a functional link between Dmp8 and Dmp18. A P element insertion that disrupts the Dmp8 coding sequence is semilethal because only a few organisms can develop to adulthood. This is interesting because some patients with TTD-A survive without functional p8/TTDA, although several TTD manifestations are present (8). Thus, in flies, a similar situation is found to occur. However, in these flies, the males are sterile, the females have reduced fertility, and all of the homozygous organisms that develop to adulthood have a held-out wing phenotype and a slight reduction in size. Sterility and reduction in the size of various structures in the adult flies are phenotypes that we have previously described for Dmp52 (marionette) and DmXPB (haywire) mutants (11, 45). However, it appears that the held-out wing phenotype is specific for this mutant, and interestingly, our data suggest that this phenotype may be due to an imbalance of Dmp8 and Dmp18 expression levels. Furthermore, the dsRNA-mediated depletion of Dmp18 in the wings combined with the presence of the l(2)SH1279 insertion, which only affects Dmp8, results in wing deformations and wing size reduction compared with flies that are homozygous for the disruption of Dmp8 or flies that only express the dsRNA against Dmp18. However, the held-out wing phenotype observed in l(2)SH1279 homozygous flies is not augmented in the flies that are homozygous for the l(2)SH1279 insertion and also express the dsDmp18 RNA. Thus, it appears that the held-out wing phenotype is only observed when a reduction or depletion of Dmp8 occurs together with an increase of Dmp18 level, suggesting that Dmp8 and Dmp18 levels need to be co-regulated for the adequate wing development. Therefore, the organization of these two genes in a bicistronic locus could be one way to ensure this co-regulation. This hypothesis is supported by evidence that the organization of Dmp8 and Dmp18 genes to allow the generation of a bicistronic transcript has been conserved in many insects and probably in several arthropods (Fig. 1C and supplemental Fig. S1) along 350 million years of evolution. Moreover, results from our genetic analysis and the evidence of physical interaction between Dmp52 and Dmp18 suggest a functional link between Dmp18 and TFIIH in Drosophila.

The results presented here raise the following question. What is the level of functional interaction between TFIIH and Dmp18? TFIIH participates in transcription and DNA repair. As mentioned previously, the yeast and human homologs of Dmp18 (SWC6 and p18Hamlet/ZNHIT1) have been described as being components of the SWR1 and SRCAP complexes, respectively, that play a role in both transcription and DNA repair and catalyze the exchange of histone H2A with H2AZ (46). In human cells and in Drosophila, protein subunits homologous to SWR1 together with subunits identified in the acetyltransferase complex NuA4 form the TIP60 complex (47, 48). In addition to its histone acetyltransferase activity, TIP60 also catalyzes the exchange of H2A for H2AZ (49, 50). The TIP60 complex has been characterized, and its subunits have been identified. However, neither fly Dmp18 nor human p18Hamlet/ZNHIT1 has been found in this complex. Interestingly, a complex similar to SRCAP has not been described in the fly. Thus, it is unknown whether Dmp18 in Drosophila is part of a complex involved in the H2A/H2AZ exchange or whether it has other functions independent of TIP60. On the other hand, although neither fly Dmp18 nor the human p18Hamlet/ZNHIT1 are found in this complex, sometimes proteins that behave as integral subunits of a complex in one organism are only interactors in another or are lost during the purification procedure. Therefore, it is still possible that Dmp18 may interact with TIP60 in the fly (49). However, consistent with our genetic interaction analysis, it has been reported that yeast SWC6 (the yeast Dmp18) genetically interacts with RAD3 and SSL1, the homologs of XPD and p44 subunits of TFIIH (51, 52), indicating that also in yeast there is putative functional interaction between p18 and TFIIH. Furthermore, because SWC6 is a bona fide component of SWR1, the fly and yeast genetic interactions between SWC6/Dmp18 and TFIIH subunits suggest that phenotypes observed may be the consequence of interactions between SWR1 and TFIIH. Based on previously published data and the results presented here, the future identification of the complex that contains Dmp18 and the demonstration that this complex directly interacts with TFIIH may explain the incorporation of the histone variant H2AZ in promoters of genes where TFIIH is recruited for transcription initiation. In contrast, Dmp18 alone may directly establish this interaction with TFIIH, resulting in a direct effect on transcription activation or repression. Consistent with this notion, p18Hamlet is phosphorylated by p38 MAPK in response to genotoxic stress in human cells and has been proposed to directly modulate the transcriptional activity of p53 (13–15). p18Hamlet has also been reported to function as a co-factor regulating the activity of nuclear receptors (53). Moreover, TFIIH also interacts with p53 in multiple ways in human cells (54) and in Drosophila (45). Therefore, a possible connection between these three factors should be taken into account in future analyses. In conclusion, we have demonstrated that the Drosophila homolog of SWC6/p18Hamlet/ZNHIT1 physically and functionally interacts with two components of Drosophila TFIIH, thereby opening up new avenues to study how TFIIH modulates transcription and DNA repair.

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