

# *Drosophila* Topors Is a RING Finger-containing Protein That Functions as a Ubiquitin-protein Isopeptide Ligase for the Hairy Basic Helix-Loop-Helix Repressor Protein\*

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**Transcriptional repression plays an essential role in many aspects of metazoan development. *Drosophila hairy* is a primary pair-rule gene encoding a basic helix-loop-helix class transcriptional repressor that is required for proper segmentation. Previous characterization of Hairy-binding proteins has implicated two different classes of histone deacetylase as mediators of Hairy repression. Here, we present the characterization of a novel Hairy-interacting protein (dTopors) that binds specifically to the basic region of Hairy, but does not affect the ability of Hairy to bind DNA. By reducing the gene dose of *dtopors*, we demonstrate that it acts genetically as an antagonist of Hairy-mediated transcriptional repression. Consistent with this genetic interaction, we show that that recombinant dTopors protein possesses ubiquitin-protein isopeptide ligase activity *in vitro* and that dTopors mediates Hairy polyubiquitination and can lead to Hairy degradation. This work provides the first evidence that regulated proteolysis of Hairy is required for correct segmentation.**

Transcriptional repression is a critical regulatory mechanism for a vast number of developmental processes, including establishment of the *Drosophila* embryonic body plan. *Drosophila* segmentation occurs via sequential subdivision of the embryo, leading ultimately to the formation and determination of each body segment. This hierarchy of segmentation gene expression begins with the translation of maternally deposited transcripts (maternal genes), followed by the expression of the zygotic gap, pair-rule, and segment polarity genes. *hairy* is a pair-rule class gene that encodes a basic helix-loop-helix (bHLH)<sup>1</sup> transcriptional repressor that occupies a key position in the segmentation gene hierarchy: it is one of the first genes to show an overt reiterated periodicity that is central to the establishment of proper embryonic body plan throughout Metazoa (1–5). Hairy is one of a family of 13 structurally related bHLH transcriptional repressors that compose the HES family, which includes seven Enhancer of Split proteins E(spl)m3, E(spl)m5, E(spl)m7, E(spl)m8, E(spl)mβ, and E(spl)mγ and

Deadpan, which regulate neural cell fate and sex determination, respectively (6). Members of the HES class share several conserved functional motifs, including a basic DNA-binding region, an HLH dimerization domain, an Orange specificity region, and a C-terminal region required for binding the cofactor Groucho.

The isolation and characterization of the Hairy-binding proteins Groucho, dSir2, and dCtBP have suggested that Hairy represses transcription through a chromatin-remodeling mechanism. The conserved C-terminal WRPW residues of Hairy are required for the recruitment of the corepressor protein Groucho (7). Groucho is unable to bind DNA directly, but is able to repress transcription when recruited to a promoter by interacting with DNA-bound repressors (7, 8). Although Groucho has been proposed to utilize a chromatin-remodeling mechanism through its recruitment of histone deacetylases, the evidence for the significance of this interaction is mixed (9, 10). Groucho interacts genetically and physically with the *Drosophila* histone deacetylase Rpd3: interaction of Groucho with Rpd3 is necessary for efficient Groucho-mediated repression, and its activity is sensitive to the histone deacetylase inhibitor trichostatin A (9). However, the relatively mild phenotypic defects associated with the *Rpd3* (histone deacetylase-1) mutation led to the suggestion that Groucho does not function solely through recruitment of Rpd3 (11). Significantly, recruitment of Groucho is not the only mechanism by which Hairy is able to repress transcription, as deletion of the WRPW tetrapeptide reduces (but does not abolish) the ability of Hairy to repress transcription of target genes in *in vitro* and *in vivo* assays (12, 13). This Groucho-independent Hairy repression activity may be explained in part by the binding of a second histone deacetylase, dSir2 (14). dSir2, the *Drosophila* homolog of the yeast silencing protein Sir2, interacts directly with Hairy *in vitro* and behaves genetically as a Hairy corepressor protein. Hairy is therefore able to recruit two classes of histone deacetylase proteins: Rpd3 indirectly via Groucho and dSir2 by direct interaction. In contrast, the binding of the *Drosophila* homolog of the E1a C-terminal binding protein (dCtBP) acts to antagonize Hairy repression. Reducing the gene dose of *groucho* enhances a *hairy* embryonic segmentation phenotype, as would be predicted by its ability to act as a canonical corepressor (7). Conversely, reducing the gene dose of *dCtBP* suppresses a *hairy* embryonic segmentation phenotype, indicating that dCtBP acts to negatively regulate Hairy activity (13, 15–17). Consistent with this observation, overexpression of Hairy lacking its dCtBP-binding site has an enhanced ability to repress transcription *in vivo* (13). Genetic and molecular evidence suggests that the binding of dCtBP to Hairy antagonizes the ability of Groucho to recruit Rpd3, impairing the ability of Hairy to repress transcription (15). It is not yet known whether Hairy is able to simultaneously recruit all of these cofactors to any promoter target.

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<sup>1</sup> The abbreviations used are: bHLH, basic helix-loop-helix; E3, ubiquitin-protein isopeptide ligase; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein.

To further understand the molecular mechanisms of Hairy-mediated repression, we have characterized additional Hairy-binding proteins. Here, we describe a novel RING finger-containing Hairy-interacting protein, dTopors. Both the genetic and biochemical data presented are consistent with a model in which dTopors acts a ubiquitin-protein isopeptide ligase (E3) that negatively regulates Hairy function during embryogenesis.

#### MATERIALS AND METHODS

**Clones for Expressing Bacterial Proteins**—The GST-Hairy fusion proteins bHLH, Orange-WRPW, N30 (an N-terminal 30-amino acid fragment of Hairy), Basic (basic DNA-binding elements), and HLH have been described previously (5, 16). Dmp53 (obtained from M. Brodsky) is a BamHI/BglII fragment inserted in the BamHI site of pGEX2TK. pGST-topoisomerase I (original clone obtained from T. Hsieh) is a BamHI fragment cloned into pGEX2TK. His<sub>6</sub>-dTopors, pCS2-dTopors, and pCS2-dTopors(C102A) are BglII fragments cloned into the BamHI site of pRSETA (Invitrogen) or pCS2.

**Bacterial Expression and Purification of Proteins**—GST fusion proteins were made essentially as described by Van Doren *et al.* (5), except that the bacterial strain JM109 was used. In all cases, the yield of bacterially expressed protein was estimated by comparing the intensity of the purified GST-protein with known amounts of bovine serum albumin on a Coomassie Blue-stained gel. His<sub>6</sub>-dTopors in strain HMS174(DE3) was induced at A<sub>600</sub> = 0.7 with 0.4 mM isopropyl-β-D-thiogalactopyranoside and grown for 5 h at 30 °C. Cells were lysed in 30 mM Tris (pH 8.0), 500 mM NaCl, 5 mM β-mercaptoethanol, and 6 M guanidine HCl by sonication. Denatured His<sub>6</sub>-dTopors was bound to Talon beads (Clontech); washed with 30 mM Tris (pH 8.0), 100 mM NaCl, and 8 M urea; and then eluted with 200 mM imidazole. Eluted protein was dialyzed against 20 mM Tris (pH 8.0), 100 mM NaCl, 25 μM ZnSO<sub>4</sub>, and 20 mM β-mercaptoethanol to allow the protein to renature.

**GST Pull-down Assays**—GST pull-down assays were performed as described by Poortinga *et al.* (16). Full-length pCITE-Hairy has been described previously (16). *In vitro* translations were carried out according to the manufacturer's specifications using the Promega *in vitro* TNT kit in the presence of [<sup>35</sup>S]methionine. Full-length dTopors is a BamHI fragment cloned into pCITE4a (Novagen).

**Electrophoretic Mobility Shift Assay (EMSA)**—Complementary oligonucleotides containing the Hairy-binding site (C-box) from the *achaete* promoter were used for EMSA. The primer sequences for wild-type oligonucleotides are 5'-TAAACCGGTTGGCAGCCGGCAGCGACAGGGCCAGGTTTT and 5'-AAAACCTGGCCCTGTCGCGTGCCGGCTGCCAACCGGTTTA, where the Hairy-binding sites are underlined. Oligonucleotides with an altered Hairy C-box previously shown to disrupt Hairy DNA binding (5) are 5'-TAAACCGGTTGGCAGCCGGGACGCGACAGGGCCAGGTTTT and 5'-AAAACCTGGCCCTGTCGCGTGCCCGGCTGCCAACCGGTTTA, where the single base changes within the Hairy-binding site are in boldface. Annealed oligonucleotides were radiolabeled, and 0.4 ng of probe was used per binding reaction. Reactions contained 40 ng of GST-Hairy protein, 7% glycerol, 20 mM HEPES (pH 7.6), 50 mM KCl, 1 mM dithiothreitol, and 1 mM EDTA. For supershift experiments, 20 ng of His<sub>6</sub>-dTopors was added to each binding reaction. After a 15-min incubation at 25 °C, the reaction was run on a 4% polyacrylamide gel (37.5:1 acrylamide/bisacrylamide) with 0.5× Tris/borate/EDTA at 20 V/cm for 2.5 h at 4 °C.

For analysis of proteins within EMSA bands, EMSA was performed, and the wet gel was exposed to film. The region of the gel corresponding to the "supershifted" band was excised and electroeluted at 100 V for 16 h at room temperature, separated on an 8–16% gel (Invitrogen), and transferred to nitrocellulose. The resulting Western blot was probed with mouse anti-dTopors polyclonal antibody (1:1000 dilution) followed by horseradish peroxidase-coupled secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) and detected using ECL (Perkin-Elmer Life Sciences).

**In Vitro Ubiquitination Assays**—Ubiquitination assays were carried out in 50 mM Tris (pH 7.5), 2.5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 2 mM ATP, and 5 μg of ubiquitin in the presence of 100 ng of yeast ubiquitin-activating enzyme (E1), 100 ng of human UbcH5a (Boston Biochem), and 50–100 ng of His<sub>6</sub>-dTopors. Reactions were incubated at 25 °C for 90 min, stopped by addition of SDS, and run on an 8–16% or Novex acrylamide gel (Invitrogen). Proteins were transferred to nitrocellulose, probed with anti-ubiquitin monoclonal antibody P4D1 (1:500 dilution; gift from D. Gottschling) followed by horseradish peroxidase-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.), de-

tected using ECL, and exposed to film. For reactions containing substrate, 0.5 μl of *in vitro* translated <sup>35</sup>S-labeled protein was added to the ubiquitination reaction, and the products were separated on a 4–12% gel, dried, and exposed to film.

**Antibodies**—Anti-dTopors antibodies were raised against a GST-dTopors fusion protein encoding amino acids 494–919 of dTopors. A 1.3-kb EcoRI fragment was taken from pCITE-dTopors (including 24 bp of polylinker sequence) and subcloned into pGEX3x. GST-dTopors-(494–919) was bacterially expressed and purified as described for other GST proteins. Strain BALB/cByJ-Rb(8.12)5Bn r/J (Jackson ImmunoResearch Laboratories, Inc.) was used for antibody production. Mouse monoclonal antibody 9E10 and anti-Gal4 monoclonal antibody RK5C1 were obtained from Santa Cruz Biotechnology. The mouse monoclonal antibody that recognizes the His<sub>5</sub> epitope was obtained from QIAGEN Inc.

**Tissue Culture**—293T cells were transiently transfected using FuGENE 6 (Roche Applied Science). Co-immunoprecipitations using monoclonal antibody 9E10 were carried out by transfecting 50 ng of Myc-tagged Hairy (pCS2-Hairy) and Gal4-dTopors as previously described (15).

**Fly Strains**—A *dtopors* deletion was made by the imprecise excision of the *EP(2)2435* element inserted in the *proliferation disruptor (prod)* gene. *EP(2)2435/CyO* females were crossed with *wg<sup>Sp-1</sup>/CyO;Δ2-3Dr/TM6*, and non-Sternopleural (*wg<sup>Sp-1</sup>/Drop (Dr)* male flies were selected in the next generation. Male *EP(2)2435/CyO;+Δ2-3Dr* flies were then crossed with *Sco/CyO*, and flies that had lost the mini-*u+* marker from the *EP* and that did not have the *Δ2-3Dr* chromosome were crossed with *Sco/CyO*. *EP(2)2435<sup>REV</sup>/CyO* progeny from this cross were then interbred to generate a stock. The limits of deficiencies generated were mapped by Southern blotting and PCR sequencing across the deletion breakpoint. *dtopors<sup>AA</sup>* removes 4.1 kb including the *dtopors* promoter and translation initiation site, resulting in null mutations of both *prod* and *dtopors* (see Fig. 2A). A *dtopors*-specific mutation was generated by cloning a 5.1-kb *prod* genomic region from the start of *dtopors* transcript to a HindIII site 3' to the *prod* transcript into the pCaSpeR4 *P* element vector. Transgenic *P(prod<sup>+</sup>)* flies were crossed into the *dtopors<sup>AA</sup>* deletion background to generate flies of the genotype *dtopors<sup>AA</sup>/CyO; P(prod<sup>+</sup>)* (which we refer to as *dtopors<sup>AA+P</sup>*). *dtopors<sup>AA+P</sup>* flies are homozygous viable.

All fly strains were cultured and crossed on yeast/cornmeal/molasses/malt extract medium at 25 °C. The *hairy (h)* alleles used were the severe nonsense allele *h<sup>7H</sup>* (T72A) and the hypomorphic missense allele *h<sup>12C</sup>* (P336L) (18). The *prod* allele *prod<sup>1</sup>* was obtained from P. Bryant.

**Embryo Analysis**—Cuticle preparations were carried out as described by Wieschaus and Nüsslein-Volhard (19). Immunohistochemistry was performed as previously described (20) using alkaline phosphatase-coupled secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) and visualized with substrate kit II reagents (Vector Labs, Inc.).

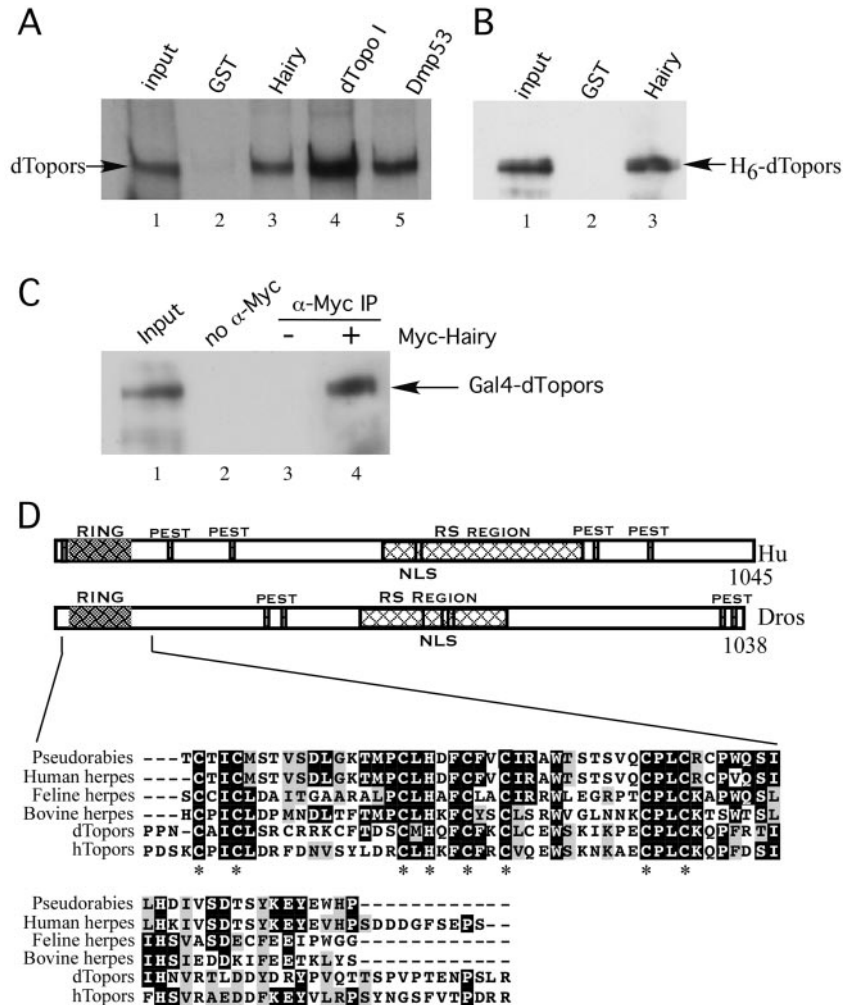
#### RESULTS

**dTopors Is a Hairy-interacting Protein**—We have previously used a yeast two-hybrid assay to isolate cDNAs encoding proteins that show Hairy-specific binding activity (16).<sup>2</sup> One of these cDNAs (CG15104) encodes a 1038-amino acid protein that interacts with Hairy in a GST pull-down assay using *in vitro* translated <sup>35</sup>S-labeled protein (Fig. 1A; see Fig. 3, A and B). In addition, recombinant His<sub>6</sub>-tagged CG15104 bound to GST-Hairy *in vitro* (Fig. 1B), demonstrating that this interaction is direct. Currently available anti-Hairy and anti-dTopors antibodies were unable to immunoprecipitate their cognate proteins from embryonic extracts (data not shown). Thus, to show that the interaction between CG15104 and Hairy can occur *in vivo*, Hairy complexes were analyzed by transfecting Myc-Hairy and Gal4-CG15104 into 293T cells. As shown in Fig. 1C, immunoprecipitation of Hairy using anti-Myc epitope antibody 9E10 and subsequent Western blotting with anti-Gal4 antibody showed the association of Hairy and CG15104 specifically when antibody 9E10 was present and both Gal4-CG15104 and Myc-Hairy were transfected into cells.

The conceptual open reading frame of CG15104 shows the greatest similarity to a human protein of unknown function

<sup>2</sup> S. M. Parkhurst, unpublished data.

**FIG. 1. dTopors interacts directly with Hairy *in vitro* and *in vivo*.** *A*, dTopors interacts with Hairy, *Drosophila* topoisomerase I (*dTopo I*), and Dmp53 in a GST pull-down assay. *In vitro* translated <sup>35</sup>S-labeled dTopors was tested for binding to GST (lane 2), GST-Hairy (lane 3), GST-topoisomerase I (encoding amino acids 1–440 of topoisomerase I; lane 4), and GST-Dmp53 (lane 5). 20% input is shown in lane 1. *B*, bacterially expressed and purified full-length His<sub>6</sub>-dTopors was incubated with GST (lane 2) or GST-Hairy (lane 3), and bound His<sub>6</sub>-dTopors was detected by Western blotting using anti-His<sub>5</sub> antibody. 20% input is shown in lane 1. *C*, Hairy and dTopors interact *in vivo*. Lysates from 293T cells transfected with Myc-Hairy and Gal4-dTopors or with Gal4-dTopors alone were incubated with anti-Myc antibody 9E10 or protein A-Sepharose beads, and Gal4-dTopors was detected by anti-Gal4 antibody Western blotting. Gal4-dTopors associated with Myc-Hairy (lane 4) was not precipitated in the absence of transfected Myc-Hairy (lane 3) or when no antibody 9E10 was added (lane 2). 5% input is shown in lane 1. *IP*, immunoprecipitate. *D*, the dTopors protein domain structure is presented. Shown is a schematic representation of the human (*Hu*) and *Drosophila* (*Dros*) Topors proteins and the amino acid alignment with herpes family ICP0 proteins over the 79-amino acid RING finger-containing region. In addition to an N-terminal RING finger, dTopors has four consensus bipartite nuclear localization sequences (*NLS*), 17 SR/RS dipeptides located predominantly within the RS region, and four PEST sequences. dTopors is 16% identical and 26% similar to human Topors (*hTopors*) over the entire protein and 42% identical and 65% similar within the extended RING finger region. Essential Cys and His residues required for formation of the RING finger are indicated by asterisks.



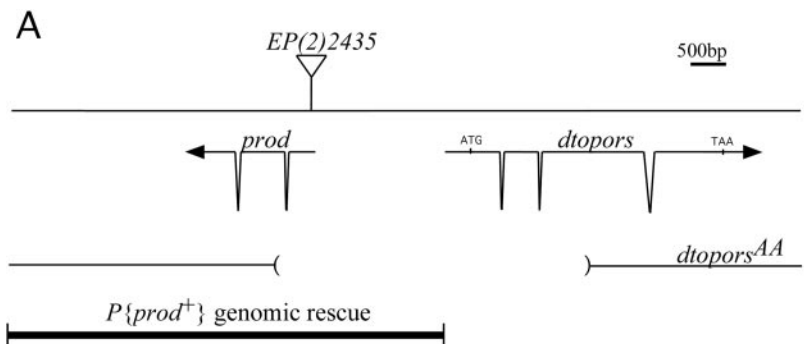
identified independently as a topoisomerase I-interacting protein, Topors (21); a p53-binding protein, p53BP3 (22); and a protein highly expressed in the lung that binds palindromic DNA sequences, LUN (23) (Fig. 1D). Like human Topors, CG15104 interacted with *Drosophila* topoisomerase I and p53 (Dmp53) *in vitro* (Fig. 1A; see Fig. 3A). On the basis of this similarity and the conserved binding activities, we have named this *Drosophila* protein dTopors.

The human and *Drosophila* Topors proteins share several structural motifs, including an N-terminal RING finger, a region rich in RS/SR dipeptide repeats, bipartite nuclear localization sequences, and PEST sequences (Fig. 1D). The N-terminal C<sub>3</sub>HC<sub>4</sub>-type RING finger of dTopors is most similar to the ICP0 family of viral proteins involved in activation of latent herpesvirus (24). The similarity between dTopors and ICP0 proteins is restricted to a 64-amino acid region that includes both the RING finger and adjacent residues that have been shown to contribute to ICP0 function (24). The central region of both human and *Drosophila* Topors proteins is rich in RS (or SR) repeats, containing 33 and 17 RS/SR dipeptides, respectively. Such SR/RS-rich regions are found in mRNA splicing factors, where they are targets of regulated phosphorylation (25, 26). However, dTopors has a reduced density of RS repeats and lacks the consensus RNA-binding domain characteristic of RS domain splicing factors. This suggests that, although the SR region of dTopors may be involved in protein-protein interactions or phosphorylated, it is unlikely to be a direct regulator of mRNA splicing. dTopors is also predicted to be a short-lived

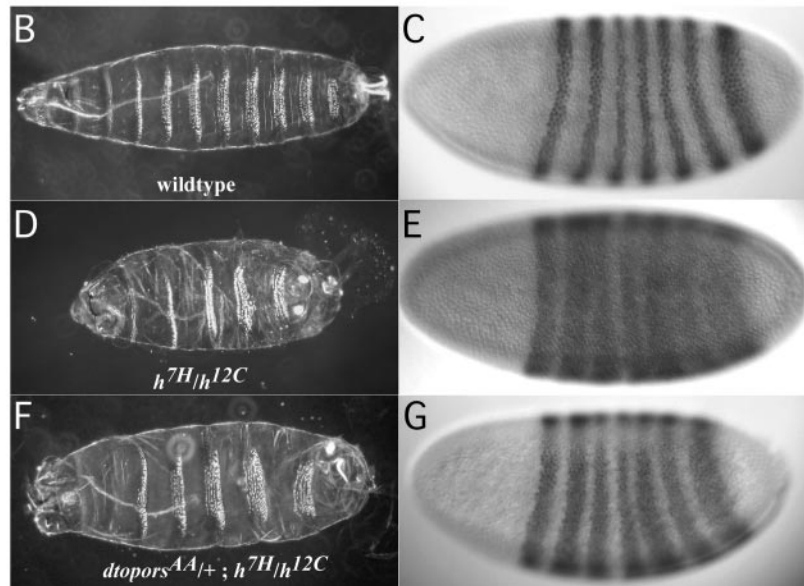
nuclear protein, as there are four partially overlapping bipartite nuclear localization sequences and four PEST sequences, which serve as proteolytic signals for a variety of cellular proteins (27).

*dtopors Genetically Interacts with hairy*—To investigate the functional interaction of dTopors and Hairy *in vivo*, we generated a *dtopors* mutant. A *dtopors* deletion was made by imprecise excision of a *P* element inserted in the gene adjacent to *dtopors*, *prod*. This deletion allele (*dtopors*<sup>AA</sup>) resulted in null mutations of both *prod* and *dtopors* (Fig. 2A). *dtopors*<sup>AA</sup> homozygotes die at the third larval instar and show phenotypes identical to those described for *prod* mutants (28). To generate a *dtopors*-specific mutant, a wild-type copy of the *prod* locus was introduced into *dtopors*<sup>AA</sup> deletion flies by *P* element-mediated transformation (*dtopors*<sup>AA</sup>; *P*[*prod*<sup>+</sup>]; see “Materials and Methods”). These flies, which we refer to as *dtopors*<sup>AA+P</sup>, are homozygous viable, demonstrating that zygotic dTopors function is not essential for viability.

We next examined the effect of reducing the gene dose of *dtopors* on a *hairy* embryonic pair-rule phenotype to determine whether *dtopors* influences Hairy-mediated repression. In wild-type embryos, Hairy acts indirectly to represses Fushi tarazu (*Ftz*) expression, resulting in seven *Ftz* stripes along the anterior-posterior axis of the embryo and correct determination of each larval segment (Fig. 2, B and C) (29, 30). Embryos *trans*-heterozygous for *h*<sup>7H</sup> and *h*<sup>12C</sup> show a pair-rule phenotype resulting from derepression of *Ftz* expression, leading to loss of every second segment (Fig. 2, D and E). This *hairy*



**FIG. 2. *dtopors* interacts genetically with *hairy*.** A, schematic representation of the *dtopors* and adjacent *prod* loci. *dtopors<sup>AA</sup>* is a deletion mutation with *dtopors* and *prod* removed. The *dtopors*-specific mutation *dtopors<sup>AA+P</sup>* was created by introducing a wild-type copy of the *prod* gene as a transgene into *dtopors<sup>AA</sup>* flies. The molecular limits of this *prod* rescue construct are indicated. B–G, reducing the gene dose of *dtopors* using *dtopors<sup>AA</sup>* suppresses the *hairy* mutant phenotype. Shown are larval cuticle preparations (B, D, and F) and Ftz staining (C, E, and G) in the wild type (B and C), *trans*-heterozygotes for the strong hairy allele *h<sup>7H</sup>* and the weak hairy allele *h<sup>12C</sup>* (D and E), and *dtopors<sup>AA/+</sup>;h<sup>7H/h<sup>12C</sup></sup>* (F and G). Note the suppression of the *hairy* mutant phenotype when the gene dose of *dtopors* was reduced (compare F and G with D and E).



pair-rule phenotype has been shown to be sensitive to the gene dosage of factors required for Hairy-mediated repression (7, 15, 16). Halving the gene dose of *dtopors* (*dtopors<sup>AA</sup>;h<sup>7H/h<sup>12C</sup></sup>* or *dtopors<sup>AA+P</sup>;h<sup>7H/h<sup>12C</sup></sup>*) resulted in suppression of the *h<sup>7H/h<sup>12C</sup></sup>* mutant phenotype, as shown by the increased number of denticle bands and more distinct Ftz stripes (Fig. 2, F and G). To ensure that the suppression of the *hairy* mutant phenotype is specific to *dtopors*, we verified that the original *EP* line from which the *dtopors<sup>AA</sup>* allele was generated did not genetically interact with *hairy* (data not shown). Suppression of the *hairy* mutant phenotype by *dtopors* indicates that dTopors negatively regulates the ability of Hairy to repress transcription *in vivo*.

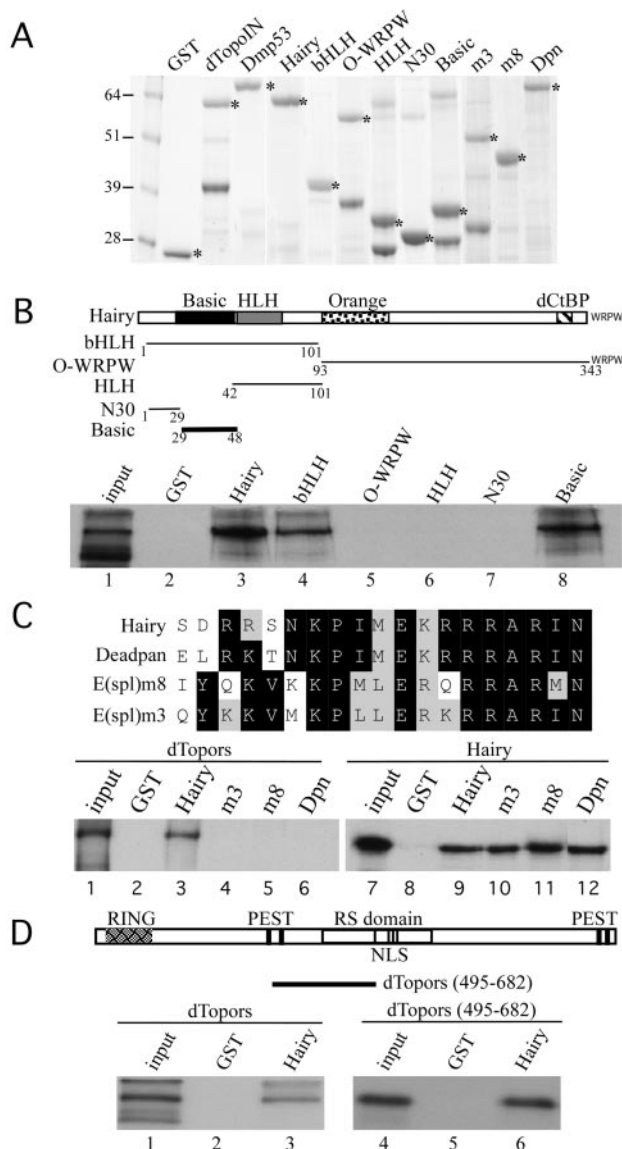
**dTopors Interacts with the Basic Domain of Hairy**—To investigate the molecular basis of the *dtopors* suppression of the *hairy* phenotype, we mapped the minimal regions within each protein required to mediate this protein-protein interaction. Truncated GST-tagged Hairy proteins were tested for their ability to bind full-length dTopors in GST pull-down assays. dTopors bound full-length Hairy and truncated bHLH and Basic, but not Orange-WRPW, HLH alone, N30, or GST alone (Fig. 3, A and B). Thus, the 20 amino acids of the basic domain previously shown to be required for DNA binding compose the minimal region of Hairy required for binding dTopors.

Because the basic region of Hairy that mediates dTopors binding is similar among the Hairy/E(spl) family of proteins (Fig. 3, A and C), we examined whether dTopors could bind the related E(spl)m3, E(spl)m8, and Deadpan proteins in a GST pull-down assay. dTopors bound specifically to Hairy, but not to E(spl)m3, E(spl)m8, or Deadpan (Fig. 3C). As a positive control, we confirmed the ability of the E(spl)m3, E(spl)m8, and Dead-

pan proteins to dimerize with Hairy. dTopors is therefore not a general cofactor for Hairy/E(spl) family proteins.

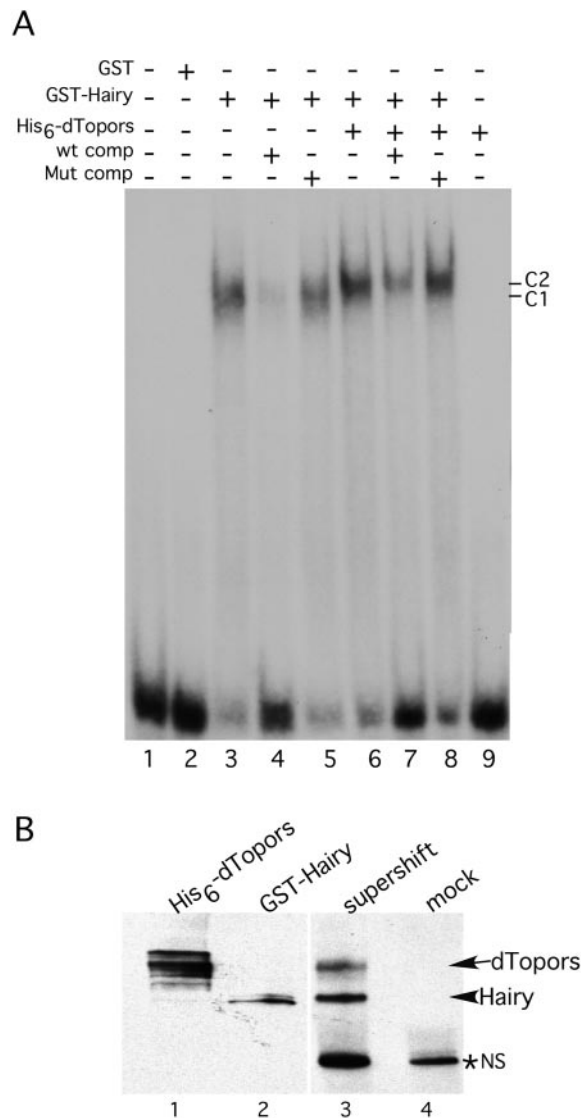
To determine whether Hairy binds to a conserved region within dTopors, truncated dTopors proteins were tested for Hairy binding activity in the GST pull-down assays described above (Fig. 3D). The minimal region of dTopors required for specific binding by GST-Hairy is a 187-amino acid region that includes a PEST sequence and eight RS repeats within the RS domain. Smaller protein fragments derived from this region were unable to bind Hairy, suggesting that the binding site may be bipartite or that the secondary structure of this region may be critical for Hairy interaction.

**dTopors Is Able to Form a Stable DNA-binding Complex with Hairy**—The binding of dTopors to the basic domain of Hairy suggests that dTopors might either alter or preclude the ability of Hairy to bind DNA. To determine whether Hairy and dTopors form a complex on DNA *in vitro*, EMSA was carried out using bacterially expressed and purified GST-Hairy and His<sub>6</sub>-dTopors. GST-Hairy (but not GST alone) bound specifically to a 40-mer oligonucleotide probe derived from the promoter region of the Hairy target gene *achaete* (Fig. 4A). This binding was specifically competed by addition of a 100-fold molar excess of the wild-type 40-mer, but not by an oligonucleotide carrying a point mutation within the C-box sequence that was previously shown to abrogate Hairy binding (Fig. 4A) (5). Significantly, addition of His<sub>6</sub>-dTopors altered the mobility of the Hairy-DNA complex, and this activity was dependent on the presence of the Hairy protein (Fig. 4A). This supershifted band could be competed by addition of a 100-fold excess of the wild-type (but not mutant) oligonucleotide.



**FIG. 3. dTopors-Hairy interaction domain mapping.** *A*, Coomassie Brilliant Blue staining showing the purity and amount of each GST fusion protein used in GST pull-down experiments. Full-length GST fusion proteins are denoted by asterisks. Molecular mass standards are indicated on the left in kilodaltons. *dTopoIN*, *Drosophila* topoisomerase I; *O-WRPW*, Orange-WRPW; *m3*, E(spl)m3; *m8*, E(spl)m8; *Dpn*, Deadpan. *B*, dTopors interacts with the basic region of Hairy. Regions of Hairy tested for interaction with dTopors are indicated in the schematic. <sup>35</sup>S-Labeled dTopors (20% input; lane 1) was tested for binding to GST (lane 2), GST-Hairy (lane 3), GST-bHLH (lane 4), GST-Orange-WRPW (lane 5), GST-HLH (lane 6), GST-N30 (lane 7), and GST-Basic (lane 8). *C*, similarity between Hairy, Deadpan, and Enhancer of Split proteins E(spl)m3 and E(spl)m8 over the 19-amino acid region required for dTopors to bind Hairy. Identity between Hairy family proteins is depicted by black boxes, and similarity by gray boxes. <sup>35</sup>S-Labeled dTopors (20% input; lane 1) was incubated with GST (lane 2), GST-Hairy (lane 3), GST-E(spl)m3 (lane 4), GST-E(spl)m8 (lane 5), and GST-Deadpan (lane 6). <sup>35</sup>S-Labeled Hairy protein was used as a control for the integrity of purified GST-proteins, as Hairy did not bind GST (lane 8), but could homodimerize (lane 9) and heterodimerize with GST-E(spl)m3 (lane 10), GST-E(spl)m8 (lane 11), and GST-Deadpan (lane 12). *D*, the binding of Hairy to dTopors requires the 187-amino acid region of dTopors shown in the schematic. <sup>35</sup>S-Labeled dTopors (20% input; lane 1) did not bind GST (lane 2), but did bind GST-Hairy (lane 3). Similarly, dTopors-(495-682) (20% input; lane 4) did not bind GST (lane 5), but did bind GST-Hairy (lane 6). *NLS*, nuclear localization sequence.

To demonstrate that the supershifted band observed in EMSA represents a complex composed of both Hairy and dTopors proteins, the band was excised, and the proteins were



**FIG. 4. Hairy and dTopors form a stable complex on DNA *in vitro*.** *A*, EMSA using a 40-mer derived from the *achaete* promoter containing a C-box to which Hairy has been previously shown to bind (lane 1) (5). Full-length GST-Hairy was able to bind to the 40-mer probe (lane 3). This complex is dependent on Hairy binding the C-box, as addition of a 100-fold molar excess of the unlabeled wild-type 40-mer (lane 4), but not the mutant 40-mer containing a point mutation within the C-box (lane 5), competed for Hairy binding. Addition of His<sub>6</sub>-dTopors altered the mobility of the GST-tagged Hairy-DNA complex (lane 6), and this complex is dependent on the presence of the C-box, as it could be competed with a 100-fold excess of the wild-type (lane 7), but not mutant (lane 8), oligonucleotide. Neither GST (lane 2) nor dTopors (lane 9) bound to the wild-type 40-mer. *wt comp*, wild-type competitor; *Mut comp*, mutant competitor. *B*, electroelution of the proteins contained within the supershifted band. The supershifted band or a control region from a duplicate gel run in the absence of the 40-mer probe (mock) was excised and eluted as described under "Materials and Methods." Eluted proteins were detected by Western blotting using antibody raised against GST-dTopors that detects the GST moiety of GST-Hairy and His<sub>6</sub>-dTopors. Both GST-Hairy and His<sub>6</sub>-dTopors were detected in the supershifted band (compare lanes 1 and 2 with lane 3), but not in the band run under mock conditions (lane 4). A nonspecific (NS) band was also detected in both mock and supershifted elutions and is indicated by the asterisk.

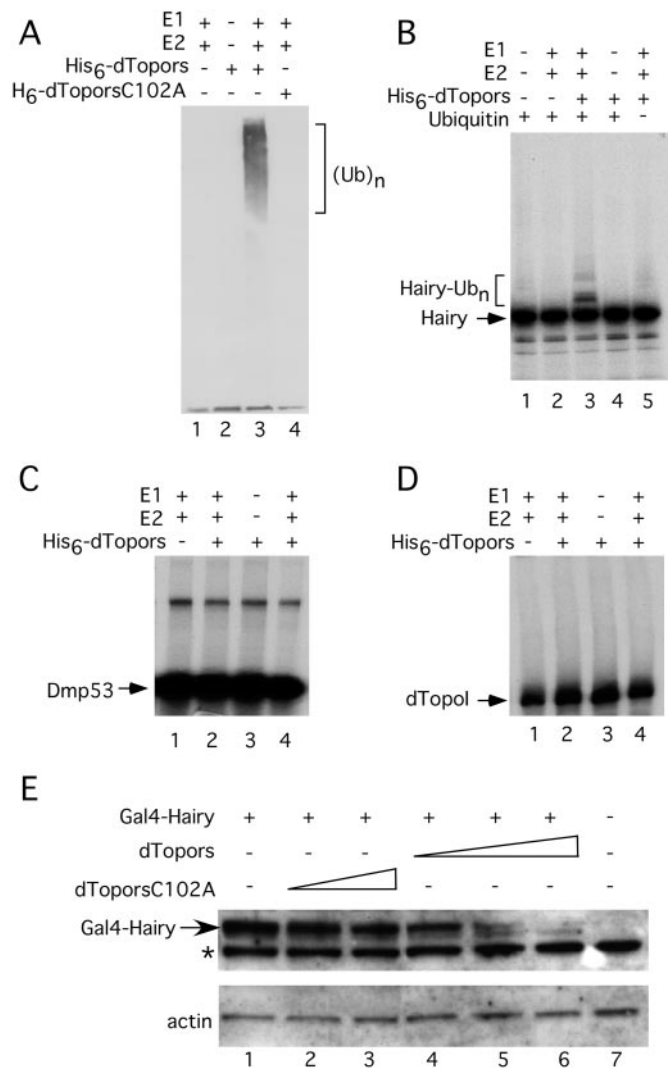
electroeluted and analyzed by Western blotting using an antibody raised against GST-dTopors that recognizes both His<sub>6</sub>-dTopors and the GST moiety of GST-Hairy. As shown in Fig. 4B, both GST-Hairy and His<sub>6</sub>-dTopors were present in the supershifted band, whereas neither were detected in a gel slice excised from an EMSA gel run in parallel in the absence of the

40-mer DNA probe. The supershift therefore represents a protein-DNA complex generated by the binding of both Hairy and dTopors. A lower molecular mass band was also detected in the electroeluted products of the supershifted band and in the mock lane (lane 4) and is labeled NS for "nonspecific" (Fig. 4B, *asterisk*). dTopors and Hairy are therefore able to form a stable complex on DNA.

**dTopors Is an E3 Ligase**—The sequence surrounding the RING finger of the *Drosophila* and human Topors proteins is most similar to the ICP0 family of viral proteins (Fig. 1A). During viral infection, ICP0 expression is correlated with the degradation of proteins such as the promyelocytic leukemia protein and the centromeric proteins CENP-A and CENP-C (24). This is likely to be a result of direct ICP0-mediated degradation, as recombinant ICP0 has been found to act as an E3 ligase *in vitro* (31). Several RING finger proteins, including ICP0, stimulate substrate-independent assembly of multiubiquitin chains in a reconstituted system containing E1 and an appropriate ubiquitin carrier protein (E2) (32). E2 enzymes fall into two classes: the Ubc class, which catalyzes the transfer of polyubiquitin chains to target proteins leading to proteasome-dependent degradation, and the UEV class, which catalyzes conjugation of polyubiquitin chains that do not necessarily lead to target protein destruction (33, 34). As ICP0 shows an E2 specificity for UbcH5a, this E2 enzyme was chosen to test whether dTopors acts as an E3 ligase. Purified recombinant His<sub>6</sub>-dTopors was observed to stimulate the formation of multiubiquitin chains in the presence of both E1 and the E2 enzyme UbcH5a (Fig. 5A). The first cysteine of the RING finger motif of c-Cbl is essential for its E3 activity (35). To demonstrate that the conserved RING finger domain of dTopors is essential for E3 activity, the corresponding mutation was made in dTopors (dTopors(C102A)). As expected, E3 activity was not detected using His<sub>6</sub>-dTopors(C102A) (Fig. 5A), demonstrating that the RING finger of dTopors is required for its E3 activity.

**dTopors Targets Hairy for Polyubiquitination**—The ability of *dtopors* to suppress the *hairy* mutant phenotype suggests that dTopors is a negative regulator of Hairy-mediated repression. Given that dTopors acts as an E3 ligase and that it binds directly to DNA-bound or free Hairy, we examined whether Hairy is a direct target of dTopors-mediated polyubiquitination *in vitro*. Addition of <sup>35</sup>S-labeled Hairy to the reconstituted E3 assay system described above resulted in the formation of multiple higher molecular mass forms of Hairy (Fig. 5B). These modified forms of Hairy were dependent on the E3 activity of dTopors, as neither E1 and E2 enzymes nor dTopors had any activity when assayed independently. In addition, elimination of ubiquitin from the reaction mixture prevented the formation of modified Hairy, demonstrating that the observed high molecular mass forms of Hairy are due to the conjugation of ubiquitin. Interestingly, dTopors did not catalyze the polyubiquitination of other known dTopors-binding proteins such as topoisomerase I and Dmp53 in this *in vitro* assay (Fig. 5, C and D). Therefore, dTopors acts as a Hairy-specific E3 ligase *in vitro*. The observation that dTopors polyubiquitinates Hairy using an E2 enzyme demonstrated to catalyze the formation of ubiquitin chains leading to proteasome-dependent degradation suggests that dTopors mediates Hairy destruction.

**dTopors Targets Hairy for Degradation**—Targeted ubiquitination of proteins has been shown to lead to numerous outcomes, one of which is degradation by the 26 S proteasome (33, 34). To investigate the biological outcome of ubiquitination of Hairy by dTopors, we examined the steady-state levels of exogenous Hairy in 293T cells in the presence of wild-type dTopors or dTopors(C102A), which lacks detectable E3 activity. Addition of increasing amounts of dTopors resulted in a



**FIG. 5. dTopors acts as a Hairy-specific E3 ligase *in vitro*.** A, anti-ubiquitin antibody P4D1 Western blot of E3 assay. Addition of E1 and the E2 enzyme UbcH5a did not stimulate substrate-independent formation of polyubiquitin chains (lane 1), nor did addition of bacterially expressed and purified His<sub>6</sub>-dTopors alone (lane 2). Addition of E1, E2, and His<sub>6</sub>-dTopors stimulated the formation of multiubiquitin chains (lane 3), and this was dependent on the RING finger of dTopors, as His<sub>6</sub>-dTopors protein harboring a cysteine-to-alanine point mutation in the RING finger (His<sub>6</sub>-dTopors(C102A)) was unable to catalyze polyubiquitination (lane 4). B, <sup>35</sup>S-labeled Hairy is a target for dTopors E3 ligase activity. Incubation of <sup>35</sup>S-labeled Hairy with E1, E2, and His<sub>6</sub>-dTopors in the presence of ubiquitin resulted in the conjugation of multiubiquitin chains to ~16% of the Hairy protein added (lane 3), whereas incubation in the absence of E1, E2, and dTopors (lane 1), the presence of E1 and E2 (lane 2), the presence of dTopors alone (lane 4), or without addition of ubiquitin (lane 5) did not. C, Dmp53 is not a target of dTopors E3 ligase activity. <sup>35</sup>S-labeled Dmp53 was incubated in the presence and absence of E1/E2 or His<sub>6</sub>-dTopors as indicated. D, *Drosophila* topoisomerase I (*dTopoI*) is not a target of dTopors E3 ligase activity. <sup>35</sup>S-labeled *Drosophila* topoisomerase I was incubated in the presence and absence of E1/E2 or His<sub>6</sub>-dTopors as indicated. E, overexpression of dTopors leads to Gal4-Hairy degradation. 200 ng of Gal4-Hairy DNA was transfected into 293T cells (lanes 1–6) alone (lane 1) or with 400 and 800 ng of pCS2-dTopors(C102A) (lanes 2 and 3, respectively), with 200, 400, and 800 ng of pCS2-dTopors (lanes 4–6, respectively), or with vector alone (lane 7). Gal4-Hairy was detected using anti-Gal4 antibody and is indicated by the arrow (upper panel). The asterisk denotes a cross-reacting band present in 293T cells. Actin was used as a loading control (lower panel).

decrease in Hairy protein, whereas addition of dTopors(C102A) had no effect (Fig. 5E). This is consistent with the targeted ubiquitination of Hairy by dTopors, resulting in degradation.

## DISCUSSION

We have shown a specific protein-protein interaction between the developmental transcriptional repressor Hairy and the RING finger protein dTopors. Although *dtopors* mutations are homozygous viable, reducing the gene dose of *dtopors* suppresses the *hairy* embryonic pair-rule phenotype, demonstrating that the interaction between Hairy and dTopors is functionally relevant *in vivo*. We have demonstrated that dTopors is an E3 ligase that is able to specifically catalyze the conjugation of multiubiquitin chains to Hairy, leading to Hairy degradation. Thus, dTopors represents a new class of Hairy-interacting protein that acts as a negative regulator of Hairy-mediated repression. Our findings are consistent with a model in which dTopors targets Hairy for ubiquitination and subsequent degradation and suggest that regulated proteolysis of Hairy is required for proper segmentation.

Previously, it has been shown that Hairy expression and, by association, Hairy-mediated repression are regulated primarily at the level of *hairy* transcription. During embryogenesis, the seven stripes of *hairy* expression are regulated by a complex set of upstream enhancer elements to which transcriptional activators and repressors, encoded by both maternal and gap genes, bind directly (4). This leads to an initial set of broad Hairy stripes, which are subsequently refined to the striped pattern characteristic of pair-rule genes. The genetic interaction between *dtopors* and *hairy* implicates dTopors in the regulation of Hairy protein levels during this period. dTopors may be involved in degrading Hairy during the process of refining Hairy expression from broad to distinct stripes or in the destruction of DNA-bound Hairy once it is no longer required for establishment of the embryonic body plan. Alternatively, dTopors may be important for degrading Hairy bound to C-boxes upstream of non-Hairy-regulated genes. This may be an important feature of repression in the early embryo, where it is important not only to stop transcription, but also to remove any residual protein quickly.

Whereas Hairy expression is restricted to a series of seven stripes, where it regulates Ftz expression, the Hairy cofactors Groucho, dCtBP, dSir2, and dTopors are ubiquitously expressed at this time during embryogenesis (7, 14, 16).<sup>3</sup> How then is the composition of Hairy-containing complexes regulated during development? One possibility is that ubiquitin modification of Hairy alters its ability to recruit its different cofactors. Another possibility is that the local concentration of individual Hairy cofactors within the nucleus determines the number of proteins that Hairy is able to bind at any given time. Consistent with this latter possibility, GFP-tagged human Topors is found in a "speckled" pattern within the nucleus in transient transfection assays (21, 22). These discrete nuclear speckles co-localize with promyelocytic leukemia bodies, which are linked to transcriptional regulation and cell growth control (36, 37). The Topors-binding protein p53 has also been shown to be a component of promyelocytic leukemia bodies, and this association directly affects p53-mediated transcriptional activation and cell survival (38, 39). The regulation of the local concentration of Hairy-binding proteins by their sequestration to subnuclear compartments may therefore prove crucial for the regulation of the ability of Hairy to repress transcription.

Post-translational modifications may also play a part in the regulation of Hairy complexes. In mammalian cells, phosphorylation of Gro/TLEs (transducin-like Enhancer of split; Groucho homologs) bound to the Hairy-related protein HES1 results in enhanced chromatin binding of the HES1-Groucho complex (40). Although the phosphorylation status of *Drosophila*

*ila* Groucho has not been characterized, post-translational modifications of Groucho may interfere with or assist the binding of other proteins, thereby altering the ability of Hairy to bind DNA or to repress transcription. Modification of dTopors may affect its ability to ubiquitinate Hairy in a similar manner. Phosphorylation of the SR region of SR family splicing factors regulates splice site recognition (25, 26). The SR region of dTopors may therefore act as a regulatory phospho domain to affect the E3 activity of dTopors.

Expression of Hairy outside its normal domain of expression can result in lethality (41). A protein predicted to be required for the regulated proteolysis of Hairy might therefore be expected to be an essential gene. *dtopors*-null mutants are viable, although these animals have a short life span and show a dramatic decrease in female fertility.<sup>3</sup> Significantly, the temporal and spatial expression of Hairy is not altered in embryos lacking maternal and zygotic dTopors function.<sup>3</sup> dTopors may therefore be redundant with another E3 ligase. The RING finger E3 ligases BRCA1 and BARD1 have been shown to act cooperatively to polyubiquitinate target substrates (42). Although both BRCA1 and BARD1 possess E3 activity, their enzymatic activity is dramatically enhanced when assayed together. Thus, although either dTopors or another RING finger protein may be sufficient for targeting Hairy for degradation, optimal regulation of Hairy protein levels would require both proteins. The characterization of other factors required for degradation of Hairy awaits genetic and biochemical characterization of additional RING finger proteins.

Although dTopors binds Hairy, Dmp53, and *Drosophila* topoisomerase I, only Hairy serves as a substrate for dTopors-mediated ubiquitination *in vitro*. This suggests that additional proteins or modifications are required for dTopors to ubiquitinate Dmp53 and *Drosophila* topoisomerase I or that dTopors does not function as an E3 ligase for these proteins. Mammalian Topors has been implicated as a regulator of transcription and as a palindromic DNA-binding protein *in vitro* (23). Topors has also been shown to bind to the adeno-associated virus-2 Rep78/68 proteins and to enhance adeno-associated virus-2 gene expression (43). Although it is unknown whether these transcriptional functions require the RING finger of Topors, it is likely that dTopors functions in addition to acting as an E3 ligase for Hairy. Further characterization of proteins that physically and genetically interact with *dtopors* will lead to the identification of additional components of dTopors complexes and pinpoint pathways that act in a redundant manner with dTopors. In addition to elucidating important information concerning dTopors function, this analysis will also provide insight into the protein-protein interactions that are required to control Hairy-mediated repression during embryogenesis.

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