

Mutation of TweedleD, a member of an unconventional cuticle protein family, alters body shape in *Drosophila*

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Communicated by Dan L. Lindsley, University of California at San Diego, La Jolla, CA, September 6, 2006 (received for review July 17, 2006)

Body shape determination represents a critical aspect of morphogenesis. In the course of investigating body shape regulation in *Drosophila*, we have identified a dominant mutation, *TweedleD*¹ (*TwdD*¹), that alters overall dimensions at the larval and pupal stages. Characterization of the affected locus led to the discovery of a gene family that has 27 members in *Drosophila* and is found only among insects. Analysis of gene expression at the RNA and protein levels revealed gene-specific temporal and spatial patterns in ectodermally derived tissues. In addition, light microscopic studies of fluorescently tagged proteins demonstrated that Tweedle proteins are incorporated into larval cuticular structures. This demonstration that a mutation in a *Drosophila* cuticular protein gene alters overall morphology confirms a role for the fly exoskeleton in determining body shape. Furthermore, parallels between these findings and studies of cuticle collagen genes in *Caenorhabditis elegans* suggest that the exoskeleton influences body shape in diverse organisms.

arthropod | morphogenesis | tandem duplication | Tubby

Morphogenesis into a viable adult animal relies on developmental regulation of size and shape. In *Drosophila*, body size and body shape are controlled by correlated but distinct activities. For example, antagonistic cross-talk between the ecdysone and insulin signaling pathways regulates the final size of an individual fly without any accompanying variation in body shape (1). Furthermore, there are mutations that impart a short and wide body shape on larvae and adults but do not alter body mass. Although shaping processes such as convergent extension have been intensively studied in recent years (2, 3), there has been no broad investigation of shape-determining activities in the fruit fly.

The most extensive studies of body shape determination have been carried out with the nematode *Caenorhabditis elegans*. More than 50 *C. elegans* mutations that affect overall morphology have been described. Their phenotypes vary widely, as is apparent in characterizations that include blister, dumpy, and long, as well as left- and right-handed roller (4). Many of these mutations have been mapped, and the affected genes have been identified. More than half the mutations altering worm body shape (specifically the dumpy and long mutations) map to genes that encode cuticle collagens, the major components of the worm cuticle (5). Of those mutations causing a dumpy phenotype, a number disrupt the function of collagen-modifying enzymes, including prolyl-4-hydroxylases, a protein disulfide isomerase, and a procollagen C-proteinase (6–10). These data point to the structure of the exoskeleton as being a major factor in determining the overall shape of individual worms.

Like worms, fruit flies rely on a cuticular exoskeleton. However, whereas *C. elegans* has >170 cuticle collagen genes, *Drosophila* has very few genes encoding collagen molecules (4). Furthermore, none of the encoded collagens closely resembles the worm cuticle forms. The *Drosophila* cuticle is a stratified structure comprising an envelope layer, a proteinaceous epicuticle, and a procuticle rich in chitin (polymer of *N*-

acetylglucosamine) (11). The envelope functions as a support for subsequent cuticle deposition, and the epicuticle and the procuticle together stiffen the exoskeleton. The chitin microfibrils and associated proteins in the procuticle form a characteristic lamella, which is crucial for maintaining cuticle tension. In contrast, the epicuticle is composed of a large number of proteins (estimated in the hundreds) (12), which are cross-linked through sclerotization or melanization (13).

Mutations in *Drosophila* that disrupt the synthesis, modification, or deposition of chitin have been identified for a number of loci, including the genes for chitin synthase (*kkv*), UDP-*N*-acetylglucosamine pyrophosphorylase (*mummy*), and chitin-modifying proteins (*serpentine*, *vermiform*, *knickkopf*, and *retroactive*) (14–21). In each case, gene inactivation results in a dilated or deformed cuticle. For example, loss-of-function mutations in *knickkopf* or *retroactive* disrupt the structure of the lamella, leading to a loss of cuticle integrity and a bloated embryo appearance (20).

Mutations of a number of genes outside the chitin metabolic pathways alter body shape in flies. For instance, a recessive, hypomorphic allele of Calmodulin (*Cam*⁷) results in a squat pupal shape (22). There is evidence that the squat shape results from a hypercontraction of body wall muscle during pupariation, the larval–pupal transition. Similarly, a failure of muscles to contract properly during pupariation is thought to explain the extended, or twiggy, shape of pupae mutant for Mlp84B, a gene that encodes a muscle-specific LIM protein (K. A. Clark, personal communication). Alteration of body shape has also been observed for mutations in Toll pathway components (ref. 23 and S.A.W., unpublished results). Loss of function for Toll, Myd88, Tube, or Pelle causes a squat pupal shape, whereas inactivation of the inhibitor Cactus results in an elongated body shape. The exact relationship between Toll pathway function and body shape determination is unknown, but it may reflect the requirement for Toll in patterning and innervation of the larval musculature (24).

To further explore body shape determination in *Drosophila*, we conducted a genetic screen for dominant, radiation-induced mutations that cause a squat or twiggy pupal body shape. Among those identified was a mutation resulting in a body that is both shorter and wider than wild type. Characterization of the affected gene has provided insight into the role of a previously unrecognized gene family of cuticle components.

Results

The *TweedleD*¹ Mutation Alters Body Shape in Larvae and Pupae. During larval and pupal stages, the body shape of a fly can be conveniently described by the axial ratio (A.R., length/width) of

Author contributions: X.G., S.A., and S.A.W. designed research; X.G., B.W.M., and S.A. performed research; X.G., B.W.M., and S.A. contributed new reagents/analytic tools; X.G., S.A., and S.A.W. analyzed data; and X.G. and S.A.W. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: RFP, red fluorescent protein; A.R., axial ratio.

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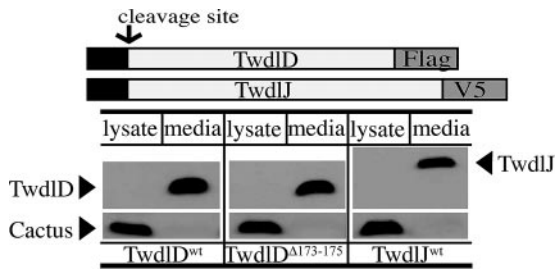


Fig. 3. TwdID and its homologue TwdIJ encode secreted proteins. S2 cells were transfected with constructs expressing Flag-tagged TwdID^{wt} or TwdID^{Δ173–175}, or with V5-tagged TwdIJ^{wt}. Media and cell pellets were separated by centrifugation, and equivalent amounts of media and total cell lysate were resolved by SDS/PAGE for immunoblotting. Proteins were detected with antibodies specific for their epitope tags. Blotting of a replicate gel with rabbit antiserum against Cactus, a cytoplasmic protein, controlled for separation of intact cells from media.

Apis, and *Tribolium* revealed several well conserved blocks (Fig. 4). None comprises a previously described motif. The positions of highly conserved amino acids within these blocks strongly suggest the presence of an internal repeat structure in each family member. In particular, blocks I and III contain a motif of the form KX_{2–3}YV (where X_{2–3} represents two or three non-conserved amino acids), whereas blocks II and IV contain a KX_{4–5}FIK motif. Furthermore, a secondary structure calculation made with the PHD program predicts a β-strand conformation for each of the four blocks (Fig. 4).

The region most conserved among all of the family members is that defined by an extended motif that spans the conserved block III and IV: YVLX_{20–23}KPEVYFiKY(R/K)t, where lower-case letters represent less strict conservation. Strikingly, the 9-bp deletion caused by the *TwdID*^{Δ173–175} mutation maps to this motif, eliminating the tripeptide KYR at positions 173–175 in the TwdID protein and the block IV. Consistent with the dominant nature of the Δ173–175 mutation, loss of these residues has no detectable effect on secretion or stability of the TwdID protein (Fig. 3).

To substantiate that the Δ173–175 deletion in *TwdID*^{Δ173–175} underlies the dominant squat phenotype, we carried out germ-line transformation experiments. Wild-type and Δ173–175 forms of TwdID were placed in a transformation vector downstream of the presumptive TwdID promoter. We examined four independent transgenic lines for wild-type TwdID; pupae from each exhibited a normal A.R. In contrast, pupae from each of three

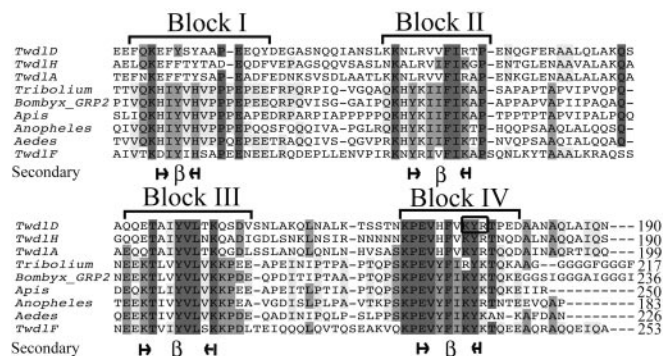


Fig. 4. Alignment of Tweedle family protein sequences. Amino acids are colored to reflect percent identity among the nine sequences: dark gray, conserved in eight or nine; medium gray, conserved in six or seven; light gray, conserved in four or five. The four β-strands predicted with the PHD program are shown. The amino acids KYR deleted in *TwdID*^{Δ173–175} are boxed.

<i>w¹¹¹⁸</i>	P{CG14243} transformants						<i>TwdID^{Δ173–175}/+</i>
	TwdID ^{wt}		TwdID ^{Δ173–175}				
	P.1 CyO	P.2 P.2	P.1 CyO	P.2 TM3	P.1 P.1	P.2 P.2	
	3.0	2.9	2.4	2.6	2.1	2.2	2.2

Fig. 5. Exogenous expression of TwdID^{Δ173–175} causes the *TwdID*^{Δ173–175} phenotype in transgenic flies. Numbers at bottom are mean A.R. derived from measurements of 40 pupae from each line. The standard deviation of the mean A.R. was 0.1 in all cases. For each construct, P.1 and P.2 represent a pair of independent isolates.

independent lines for TwdID^{Δ173–175} had a readily observable squat phenotype (Fig. 5). These findings demonstrate that the 3-aa deletion in TwdID is responsible for the dominant phenotype of the *TwdID*^{Δ173–175} allele.

In characterizing the transgenic pupae, we noted that two copies of the Δ173–175 transgene had a greater effect on body shape than a single copy. In particular, pupae carrying two copies of the mutant transgene had a mean A.R. of 2.1–2.2, similar to *TwdID*^{Δ173–175} pupae, whereas those with a single copy had an A.R. of ≈2.5, intermediate between wild-type and *TwdID*^{Δ173–175}. Because there are two wild-type copies of TwdID in the genetic background for the transgene experiments, these results suggest that the most severe phenotype is expressed only when the ratio of the mutant to the wild-type gene is 1:1 or greater.

TwdID belongs to the 97C Twdl gene cluster, which includes 13 of the 15 annotated genes in the 73.8-kb region within which *TwdID*^{Δ173–175} and *Tb*¹ map. To determine whether *Tb*¹, like *TwdID*^{Δ173–175}, mutates a Tweedle family member, we examined the *Tb*¹ genomic sequence for changes in the coding sequence of Tweedle genes. By this means, we found a deletion that removes residues 167–190 in the coding region of TwdIA (CG5480) (Fig. 4). This change eliminates all of block IV, as well as the linker between blocks III and IV, from TwdIA. Given the similarity of the TwdIA Δ167–190 deletion in *Tb*¹ to the Δ173–175 mutation in *TwdID*^{Δ173–175}, we propose that this change in TwdIA is responsible for the *Tb*¹ phenotype.

A New Family of Fly Cuticle Proteins. The observation that the *TwdID*^{Δ173–175} phenotype was apparent in newly hatched first-instar larvae provided good evidence for embryonic expression of TwdID. To determine when and where TwdID is expressed, and whether distinct family members differ in their expression patterns, we carried out *in situ* hybridization in embryos. For all seven of the genes tested, transcripts were detected at embryonic stages 13–16 (Fig. 6), but not earlier (data not shown). Expression of TwdID, as well as TwdIB (CG6478) and TwdIF (CG14639), was detected within the epidermis, with the expression of TwdID and TwdIB forming segmental stripes along the anteroposterior axis. In contrast, the transcripts of the remaining loci were each found in a more restricted domain of the embryo: the tracheal tree [TwdIβ (CG8986)], dorsal epidermis (TwdIA), and the foregut [TwdIC (CG14254) and TwdIE (CG14534)]. Although the patterns were varied, expression of all family members tested was confined to tissues that are ectodermal in origin.

Theoretically, an alteration in body shape could result from various causes, including neuronal malfunction, defective musculature, or structural abnormalities of the integument. To delimit the biological functions most likely affected by the *TwdID*^{Δ173–175} mutation, we designed experiments to monitor wild-type patterns of protein expression for TwdID and two other family

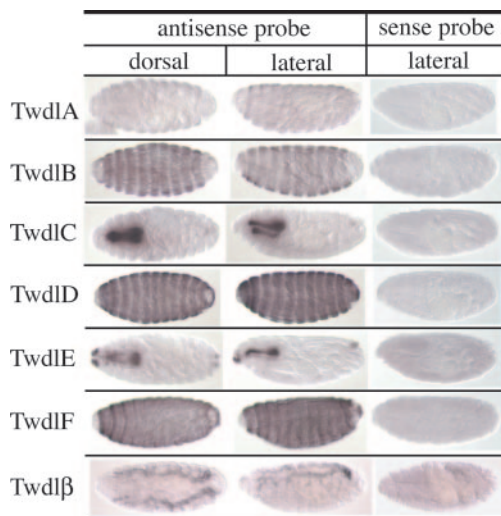


Fig. 6. Tweedle family genes are expressed in the hypodermis, foregut, or tracheal system of late-stage embryos. RNA expression of genes TwldA–TwldF as well as Twldβ was examined by *in situ* hybridization with antisense probes. All embryos are arranged with anterior to the left. Both dorsal (Left) and lateral (Center) embryo aspects are presented for each gene. A lateral (Right) view of embryos probed with sense strand control is also presented.

members. We generated fusion protein constructs in which sequences encoding monomeric red fluorescent protein (RFP) were introduced immediately 3' to the coding regions of TwldD, TwldF, and TwldH (CG31080). Each construct retained endogenous sequences representing the promoter, as well as the 5' and 3' UTR. Independent transgenic lines were generated by *P* element-mediated transformation.

The three family members exhibited distinct temporal and spatial localization patterns (Fig. 7). Expression of the TwldD–RFP fusion protein was strongest during the first and the second larval instars. During these stages, TwldD was detectable in the integument, as well as the tracheal tree (Figs. 7 and 8D). Signal in the integument was detected in 10 transverse stripes distributed along the anteroposterior axis. Expression in the integu-

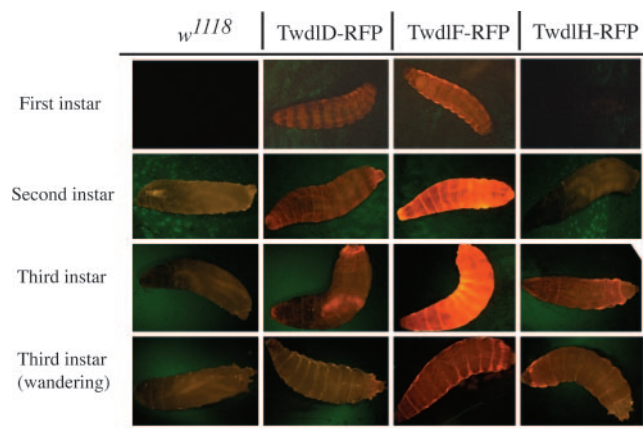


Fig. 7. Proteins of gene TwldD, TwldF, and TwldH show a distinct temporal and spatial localization pattern. The location of each protein in the transgenic flies was monitored by means of the RFP tag fused to the C terminus. The TwldD fusion protein is principally detectable during the first- and second-instar larval stages. The TwldF fusion protein is identified in a fine layer of the integument through the whole larval stages. The TwldH fusion protein is only visible in the third-instar larvae and within the segments close to the anterior or posterior ends.

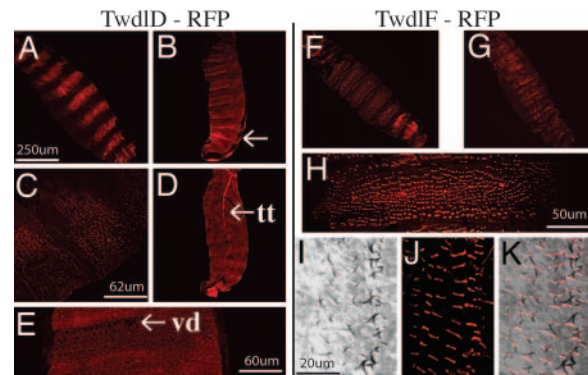


Fig. 8. Genes TwldD and TwldF encode cuticular proteins. Fixed first-instar larvae (2 h) were observed by confocal microscopy. (A) The TwldD–RFP fusion protein forms 10 stripes on the integument along the AP axis. (B) The TwldD–RFP protein is incorporated into the cuticle structure. The arrow in B shows a site where the larval body is detached from the cuticle. (C) TwldD–RFP fluorescence is apparent in cuticular dorsal hairs. (D) The TwldD–RFP protein is incorporated into the tracheal system. (E) The ventral denticles are not fluorescent in the TwldD–RFP transgenics. (F and G) The TwldF–RFP fusion protein is detectable within the cuticle. (H) TwldF–RFP fluorescence in dorsal hairs. (I–K) TwldF–RFP fluorescence in ventral denticles is confined to the basal portion. A differential interference contrast image (I), an RFP image (J), and a merged image (K) are presented. *tt*, tracheal tree; *vd*, ventral denticle.

ment was in fact cuticular, as demonstrated by the appearance of fluorescent signal within the cuticular structures known as dorsal hairs (Fig. 8C). Furthermore, that the striped expression pattern was associated with cuticle was apparent from examination of larvae in which the cuticle had been detached from the larval body during fixation and preparation (Fig. 8B). By the early third instar, TwldD expression was largely limited to the anterior and the posterior tips of the larvae. By late in the third larval instar (wandering larva stage), fluorescent signal was no longer detected.

As with TwldD, the RFP fusions for TwldF and TwldH displayed a pronounced cuticular localization. In the case of TwldF, the fusion protein was detected throughout larval development (Fig. 7). Fluorescence was associated with both dorsal hairs and ventral denticles (Fig. 8H and J). For the ventral denticles, signal was for the most part limited to the basal portion, resulting in a narrow bar of expression parallel to the cuticular surface (Fig. 8I–K). By comparison, TwldH had a much more limited pattern of expression. The TwldH–RFP fusion protein was detected only in third-instar larvae, where the signal was limited to the cuticle near the anterior and posterior ends (Fig. 7).

Although the three Tweedle proteins differ in the details of their expression patterns, all are cuticular proteins, consistent with a conserved role for the Tweedle family proteins in cuticle formation.

Discussion

The study of the *TwldD¹* mutant has led us to the discovery of a protein family, the Tweedle family. We observe ectodermal expression for all family members tested. In addition, we have shown that at least three of the family members are cuticular proteins. Our findings thus establish a connection between body shape regulation and structural proteins that contribute to the cuticle.

Role of Tweedle Proteins in Cuticle Assembly. Insects have evolved the chitin-based cuticle to protect them from the environment. The *Drosophila* larval cuticle is secreted by the underlying hypodermis at its apical face, and a new cuticle is generated for

each instar. The cuticle is attached to the hypodermis at multiple anchor sites (15). Extensive cross-linking is crucial in determining the mechanical properties of the cuticle. The *Twiddl¹* mutation, which does not change the stability or the secretion of the protein, is therefore very likely to affect either the conformation of the protein or its activity in forming or stabilizing cross-links.

The fact that the four most conserved blocks of amino acids in the Tweedle protein are predicted to form β -strands is intriguing. Previous studies of insect cuticle proteins have suggested that the barrel structure formed by multiple β -strands provides an interface for aromatic residues to stack with and bind to chitin (27, 28). In this regard, we note that several of the highly conserved residues in the Tweedle proteins that lie within these predicted β -strands have aromatic side chains: Y and H in block I, Y and F in block II, Y in block III and Y, and F and Y in block IV. We therefore postulate that the Twidl family proteins interact directly with chitin.

The Tweedle family members in the *Drosophila* genome form three major gene clusters. The 97C cluster, which includes the Twidd gene, consists of 14 family members. This cluster can be further divided in half, with the genes in each half all being transcribed from the same DNA strand. Why has the Tweedle gene family apparently undergone multiple gene duplication events? Our studies indicate that the expansion in gene number was accompanied by a differentiation of distinct patterns of expression. One possibility, therefore, is that each family member functions identically at the biochemical level, with the differences in expression determining the organization of the cuticle. Thus, for example, different levels of Tweedle protein at particular locations could determine the extent of cross-linking and, hence, flexibility. Similarly, differences in the timing of expression could dictate the order of assembly of cuticle at distinct locations. Alternatively, family members could differ in biochemical function, with the sequence differences seen between family members dictating local differences in cuticle composition and properties.

Although none of the 27 Tweedle genes in *Drosophila* has been studied previously, a recent report describes a characterization of a related gene in the silkworm *Bombyx mori* (29). This silkworm protein, BmGRP2, was detected in the cuticle layer of the wing tissue and in the trachea in the silkworm. The authors noted that BmGRP2 contains a glycine-rich domain that is present in cuticle and other structural proteins in many species, where such domains are proposed to provide flexibility. We note, however, that BmGRP2 also contains a sequence with substantial similarity to the Tweedle family signature motif YVLX₂₀₋₂₃KPEVvFiKY(R/K)t (see Fig. 4).

Like BmGRP2, some Tweedle proteins contain glycine-rich domains. However, the glycine-rich domain is absent in 21 of the 27 Tweedle genes in *Drosophila*, including the three studied here at the protein level: Twidd, Twidf, and TwidlH. Furthermore, many glycine-rich cuticle proteins lack the motif conserved in the Tweedle family. For these reasons, we speculate that the Tweedle motif and the glycine-rich domain have distinct and largely independent functions in cuticle formation.

Genetic Control of Larval and Pupal Body Shape. Although the *Twiddl¹* phenotype is mostly easily recognized during the larval and the pupal stages, Twidd gene expression begins in the latter half of embryogenesis (see Fig. 6) and is no longer detectable by the end of the last larval stage. The lack of any shape alteration in *Twiddl¹* embryos (data not shown) presumably reflects the fact that the surrounding eggshell is a protein-based extracellular matrix distinct from cuticle (30). Within the eggshell, however, the embryonic cuticle structure is clearly affected, as is evident upon examination of newly hatched first-instar larvae (see Fig. 1). A strong *Twiddl¹* phenotype observed during the pupal stage, after the cessation of gene expression, very likely repre-

sents residual effects during pupariation of the larval cuticle abnormality.

Two previously described dominant mutations, *Tubby¹* (*Tb¹*) and *Kugel^{Valencia}* (*Kg^V*), have phenotypes highly reminiscent of *Twiddl¹*. Like *Twiddl¹*, these mutations reduce A.R. at the larval and pupal stages. This similarity suggests that the three loci may act in the same pathway. We have mapped *Tb¹* to the same 73.8-kb region as *Twiddl¹* by *P*-induced male recombination, and we found a deletion within TwidlA that is the likely cause of the *Tb¹* phenotype. In the case of *Kg^V*, the mutation maps to the left side of the gene *Ki*, which is positioned at 83D–E on the polytene map. Although the mapping is less precise than that for *Tb¹*, this position is also roughly coincident with the location of a Tweedle gene cluster, the four Tweedle genes at 82A. We consider it very likely, therefore, that a mutation in this gene cluster is mutated in the *Kg^V* mutant.

People have known for a long time that the disruption of normal cuticle structure in *C. elegans* can cause the dumphy phenotype, which describes the shorter and wider morphology of the mutant worms. We have demonstrated in this report that the *Twiddl¹* mutation of the cuticular protein Twidd causes a similar morphology change in the fruit fly. The analogy between the two systems highlights the importance of a cuticle in maintaining the wild-type body shape in organisms with an exoskeleton.

Materials and Methods

Genetic Screen for Morphology Mutations. To screen for dominant mutations on the third chromosome affecting pupal shape, we crossed mutagenized males (4,000-rad γ -irradiation) to virgin females and assayed directly for altered A.R. in pupae (see below). From $\approx 25,400$ pupae, we identified two stable dominant mutations and characterized one, designated *TweedleD¹*.

Axial Ratio Determination. For pupae, A.R. (length/width) was measured by using a reticle in a stereo light microscope. For each genotype we measured at least 40 individuals and calculated the mean A.R. For larvae, A.R. was determined from digital photographs of cuticle preparations. At least two independent preparations were examined for each genotype, and 20 individual cuticles were measured for each preparation.

***P*-Induced Male Recombination Mapping.** *P* element-induced male recombination mapping was performed as described (26). Triply labeled chromosomes *Ly Tb¹ Dr* and *e Twiddl¹ Dr* were generated by meiotic recombination. *P* insertion lines BL12808, BL13710, BL20052, BL13022, BL10343, and BL11782 were obtained from Bloomington Stock Center (Bloomington, IN).

Sequence Analysis and Gene Assignments. Similarity searches used BLAST (www.ncbi.nlm.nih.gov/BLAST). The multiple protein alignment and similarity analysis were carried out by using CLUSTALW (www.ebi.ac.uk/clustalw). Detailed information on gene assignments for Tweedle family members is provided in *Supporting Materials and Methods* and Table 1, which are published as supporting information on the PNAS web site. The signal peptide prediction was made by using SignalP 3.0 (www.cbs.dtu.dk/services/SignalP) (31). Secondary structures were predicted with the PHD algorithm (32) (www.predictprotein.org) (33).

Protein Expression in S2 Cell Culture. The TweedleD coding region was fused to a FLAG tag at its C terminus and cloned into the pAc5.1/V5-His A vector (Invitrogen, Carlsbad, CA). The TweedleJ coding region was cloned into the same vector, where it was fused to the V5 epitope tag. S2 cell transfection and protein extract harvesting were as described (*Drosophila* Expression System; Invitrogen). For each plate of transfected cells, the media and the cell pellet were separated by centrifugation at $850 \times g$, and a 1/60th volume of the media and of the total cell lysate was each loaded

onto an SDS/PAGE gel for immunoblotting. Antibodies used in this experiment were as follows: anti-V5 antibody at 1:10,000 dilution (46-0705; Invitrogen), anti-FLAG M2 antibody at 1:1,000 dilution (200472-2; Stratagene, La Jolla, CA), and rabbit anti-Cactus antiserum at 1:10,000 dilution (34).

Embryonic *in Situ* Hybridization. Embryonic RNA expression patterns were investigated by *in situ* hybridization. The 3' UTRs of target genes were amplified from the *w¹¹¹⁸* genome by PCR and cloned into the pBluescript vector (Stratagene). Digoxigenin-11-UTP was incorporated into sense and antisense probes generated with T7 and T3 RNA polymerase, respectively. Alkaline phosphatase-conjugated anti-digoxigenin antibody (Fab fragments; Roche, Pleasanton, CA) was used at a 1:2,000 dilution.

RFP Constructs, Transgenic Flies, and Microscopy. Tweedle gene genomic fragments including 500 bp of presumptive upstream regulatory sequence were cloned by PCR from the *w¹¹¹⁸* genome. We used PCR sewing (35) to fuse the 3' end of each coding

sequence in frame with sequences for the monomeric RFP DsRed (Clontech, Mountain View, CA). The resulting DNA fragments were ligated into the pCaSpeR transformation vector (36). Three independent transgenic lines were generated for each construct. Eggs were collected at 25°C for 2 h for each transgenic line and aged for 22, 48, or 72 h to obtain the young first-, second-, and third-instar larvae, respectively. Two-hour-old first-instar larvae were fixed as described (37) and observed under a confocal microscope.

Note. We have confirmed that *TwddA* corresponds to the *Tubby* locus: Ten independent lines carrying a *TwddA* transgene generated from *Tb¹* have a squat pupal shape, whereas five lines carrying a *TwddA* transgene from *w¹¹¹⁸* have a wild-type body shape.

We thank Dan Lindsley for helpful discussions; Jim Posakony (University of California at San Diego) for the transformation vector and RFP isoform used in these studies; Ethan Bier, Raffi Aroian, and Bill McGinnis for sharing microscopy facilities; and Ethan Bier for comments on the manuscript. This work was supported by National Institutes of Health Grant R01 GM50545 (to S.A.W.).

- Colombani J, Bianchini L, Layalle S, Pondeville E, Dauphin-Villemant C, Antoniewski C, Carre C, Noselli S, Leopold P (2005) *Science* 310:667–670.
- Keller R (2002) *Science* 298:1950–1954.
- Ninomiya H, Elinson RP, Winklbaauer R (2004) *Nature* 430:364–367.
- Myllyharju J, Kivirikko KI (2004) *Trends Genet* 20:33–43.
- Kramer JM, French RP, Park EC, Johnson JJ (1990) *Mol Cell Biol* 10:2081–2089.
- Friedman L, Higgin JJ, Moulder G, Barstead R, Raines RT, Kimble J (2000) *Proc Natl Acad Sci USA* 97:4736–4741.
- Myllyharju J, Kukkola L, Winter AD, Page AP (2002) *J Biol Chem* 277:29187–29196.
- Winter AD, Page AP (2000) *Mol Cell Biol* 20:4084–4093.
- Eschenlauer SC, Page AP (2003) *J Biol Chem* 278:4227–4237.
- Novelli J, Ahmed S, Hodgkin J (2004) *Genetics* 168:1259–1273.
- Payre F (2004) *Int J Dev Biol* 48:207–215.
- Andersen SO, Hojrup P, Roepstorff P (1995) *Insect Biochem Mol Biol* 25:153–176.
- Schaefer J, Kramer KJ, Garbow JR, Jacob GS, Stejskal EO, Hopkins TL, Speirs RD (1987) *Science* 235:1200–1204.
- Ostrowski S, Dierick HA, Bejsovec A (2002) *Genetics* 161:171–182.
- Moussian B, Schwarz H, Bartoszewski S, Nusslein-Volhard C (2005) *J Morphol* 264:117–130.
- Moussian B, Soding J, Schwarz H, Nusslein-Volhard C (2005) *Dev Dyn* 233:1056–1063.
- Araujo SJ, Aslam H, Tear G, Casanova J (2005) *Dev Biol* 288:179–193.
- Tonning A, Helms S, Schwarz H, Uv AE, Moussian B (2006) *Development (Cambridge, UK)* 133:331–341.
- Luschnig S, Batz T, Armbruster K, Krasnow MA (2006) *Curr Biol* 16:186–194.
- Moussian B, Tang E, Tonning A, Helms S, Schwarz H, Nusslein-Volhard C, Uv AE (2006) *Development (Cambridge, UK)* 133:163–171.
- Devine WP, Lubarsky B, Shaw K, Luschnig S, Messina L, Krasnow MA (2005) *Proc Natl Acad Sci USA* 102:17014–17019.
- Wang B, Sullivan KM, Beckingham K (2003) *Genetics* 165:1255–1268.
- Letsou A, Alexander S, Orth K, Wasserman SA (1991) *Proc Natl Acad Sci USA* 88:810–814.
- Halfon MS, Hashimoto C, Keshishian H (1995) *Dev Biol* 169:151–167.
- Lindsley DL (1973) *Drosophila Inf Serv* 50:21.
- Chen B, Chu T, Harms E, Gergen JP, Strickland S (1998) *Genetics* 149:157–163.
- Hamodrakas SJ, Willis JH, Iconomidou VA (2002) *Insect Biochem Mol Biol* 32:1577–1583.
- Iconomidou VA, Willis JH, Hamodrakas SJ (1999) *Insect Biochem Mol Biol* 29:285–292.
- Zhong YS, Mita K, Shimada T, Kawasaki H (2006) *Insect Biochem Mol Biol* 36:99–110.
- Fakhouri M, Elalayli M, Sherling D, Hall JD, Miller E, Sun X, Wells L, LeMosy EK (2006) *Dev Biol* 293:127–141.
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) *J Mol Biol* 340:783–795.
- Rost B (1996) *Methods Enzymol* 266:525–539.
- Rost B, Yachdav G, Liu J (2003) *Nucleic Acids Res* 32:W321–W326.
- Reach M, Galindo RL, Towb P, Allen JL, Karin M, Wasserman SA (1996) *Dev Biol* 180:353–364.
- Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) *Gene* 77:51–59.
- Thummel CS, Boulet AM, Lipshitz HD (1988) *Gene* 74:445–456.
- Patel NH (1994) in *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology*, eds Goldstein LSB, Fyrberg EA (Academic, San Diego), pp 455–461.