how Functions in Leg Development During Drosophila Metamorphosis

Tina M. Fortier,1 Runa Chatterjee,2,3 Susan Klinedinst,2 Eric H. Baehrecke,2 and Craig T. Woodard1*

The Drosophila how gene encodes a KH RNA binding protein with strong similarity to GLD-1 from nematodes and QK1 from mice. Here, we investigate the function of how during metamorphosis. We show that how RNA and protein are present in a variety of tissues, and phenotypic analyses of how mutants reveal multiple lethal phases and defects during metamorphosis. In addition to previously reported abnormalities in muscle and wing development, how mutants exhibit defects in leg development. how mutant leg imaginal discs undergo cell shape changes associated with elongation, but are oriented improperly, do not evert normally, and often remain incased in peripodial epithelium longer than normal. Consequently, how mutants exhibit short, crooked legs. Our findings suggest that how functions in interactions between imaginal epithelium, peripodial epithelium, and larval epidermal cells during imaginal disc eversion.


Key words: Drosophila; metamorphosis; development; leg morphogenesis; imaginal disc; peripodial epithelium; KH RNA binding protein

INTRODUCTION

The development of animal tissues is accomplished by a variety of cellular mechanisms. For example, the formation of epithelial structures requires the coordinated control of cell–cell interactions and the generation of intracellular forces by the cytoskeleton (Edwards and Kiehart, 1996; Halsell et al., 2000; Bayer et al., 2003; Pastor-Pareja et al., 2004). One such epithelial structure is the adult leg in the fruit fly, Drosophila melanogaster. Drosophila leg development involves changes in cell shape, interactions between cells, and inflation of structures by hydrostatic pressure (Bayer et al., 2003; Fortier et al., 2003; Chen et al., 2004). The legs and a number of other Drosophila adult structures originate from imaginal discs, which are epithelial sacs with two apposing surfaces, a columnar imaginal epithelium and a squamous peripodial epithelium (Gibson and Schubiger, 2001). The primordia of the thoracic imaginal discs originate during embryogenesis as groups of cells spanning the parasegment boundary. By 10 hours of development, the primordia of the dorsal (wing and haltere) and ventral (leg) imaginal discs form clearly recognizable groups of cells. During larval development, these primordia acquire their proper fates and positions, invaginate, and form sacs attached to the larval epidermis by a stalk (Cohen et al., 1993; Cohen, 1993; Fristrom and Fristrom, 1993).

A number of morphogenetic processes take place during the construction of the adult leg in Drosophila. Formation of the leg begins at the end of larval stages, when a high-titer pulse of 20-hydroxyecdysone (referred to here as ecdysone) triggers pupariation (puparium formation), which marks the beginning of the prepupal period and the onset of metamorphosis. This late larval ecdysone pulse directs the process of leg disc evagination, which transports the leg from the inside to the outside of the body, and includes two different morphological...
events: elongation of the appendage, and evasion of the appendage to the outside of the animal (Fristrom and Fristrom, 1993; von Kalm et al., 1995). During elongation, from approximately 6 hr before puparium formation until 6 hr after puparium formation (APF), morphogenesis of the leg disc begins with the telescoping of the flat disc into a tubular limb. Increase in length is partly accomplished by the unfolding of the epithelium and a selective change in cell shape on the apical surface, which increases the surface area of the disc (Condic et al., 1991; Fristrom and Fristrom, 1993). Elongation requires changes in cell–cell adhesions and transmission of signals from the cell surface to specific cytoskeletal elements. These cell–shape changes result from myosin-based shaping of the actin cytoskeleton, and contraction of the cortical belt of filamentous actin, in a response that involves the Rho signaling pathway (Edwards and Kiehart, 1996; Halsell et al., 2000; Bayer et al., 2003; Ward et al., 2003a; Chen et al., 2004).

During imaginal disc eversion, the peripodial epithelium is involved in the movement of the appendage to the outside of the larval epidermis (Fristrom and Fristrom, 1993; Gibson and Schubiger, 2001). Pastor-Pareja et al. (2004) have shown that, in a process requiring the JNK signaling pathway, imaginal disc eversion is driven by invasion of the larval epidermis by cells of the peripodial epithelium and stalk, followed by perforation of the peripodial stalk/larval bilayer, and protrusion of the imaginal epithelium. At approximately 10–12 hr APF, the prepupal pulse of ecdysone occurs, inducing the transition from prepupa to pupa. At this time, contraction of larval abdominal muscles drives head eversion as well as the inflation and extension of the legs and wings (Handler, 1982; Chadfield and Sparrow, 1985; Fortier et al., 2003). During the pupal stage, the final shaping of the appendages occurs, as well as differentiation of hairs and sensory structures, and deposition of the adult cuticle (Fristrom and Fristrom, 1993; Mirth and Akam, 2002).

Mutational analyses have provided insights into the molecular mechanisms underlying Drosophila leg development. A number of the genes implicated in directing leg morphogenesis are regulated by ecdysone, indicating that steroid hormonal signaling plays a key role in this developmental process. For example, Stubble (Sb), Broad Complex (BR-C), crooked legs (crol), and E74 mutants exhibit defects in leg disc eversion during the first 6 hr of metamorphosis (Beaton et al., 1988; Kiss et al., 1988; Fletcher et al., 1995; D’Avino and Thummel, 1998), while \( \beta \)FTZ-F1 mutants are defective in muscle-driven leg extension during the prepupal–pupal transition (Fortier et al., 2003). To date, mutants displaying defects specifically in leg imaginal disc eversion have not been described, and it remains unclear how the morphogenetic processes involved in leg development are coordinated.

The ecdysone-regulated held out wings (how) gene, also named \( \text{who} \), struthio (stru), and qkr93F, codes for two protein isoforms, How(L) and How(S), and functions in muscle and wing development in Drosophila (Baehrecke, 1997; Lo and Frasch, 1997; Zaffran et al., 1997; Nabel-Rosen et al., 1999, 2002). How(L) is localized in the nucleus, while How(S) is present in both the nucleus and the cytoplasm (Nabel-Rosen et al., 1999, 2002). Both How(L) and How(S) contain a KH RNA binding domain and are very closely related to two other proteins of this family, the mouse quaking gene product (Sidman et al., 1964; Ebersole et al., 1996) and the C. elegans gld-1 gene product (Francis et al., 1995) with the highest degree of similarity in the KH domain. Together, How(L) and How(S) act to control the transition from premature tendon precursors into mature muscle-bound tendon cells by regulating levels of Stripe, perhaps by binding to stripe RNA and thus controlling its stability or transport (Nabel-Rosen et al., 1999, 2002). Like integrin mutants (Newman and Wright, 1981; Brown, 1994; Wright et al., 1996; Prokop et al., 1998; Brown et al., 2000), animals that lack how function have defects in muscle attachment during embryogenesis, and possess wing blisters (Baehrecke, 1997; Lo and Frasch, 1997; Zaffran et al., 1997).

Here, we analyze how function during Drosophila metamorphosis. Different combinations of how alleles exhibit similar lethal phenotypes during metamorphosis, but the percentage of animals exhibiting a particular phenotype varies. In addition to the previously described how function in muscle, muscle attachment site, and wing development, we also observed defects in the development of legs. how mutant imaginal discs undergo cell-shape changes associated with elongation, but have defects in orientation and eversion, often remaining incased in peripodial epithelium. These defects result in a short, crooked leg phenotype. To our knowledge, mutants displaying specifically these abnormalities in imaginal disc development have not been described previously. Our findings show that how is essential for multiple developmental processes that involve interactions between cells and groups of cells.

**RESULTS**

**how Mutants Exhibit Distinct Lethal Phenotypes During Metamorphosis**

Animals with partial-loss-of-how function mutations either die during metamorphosis with their head stuck in their thorax or escape to adulthood, displaying a held-out-wings phenotype (Baehrecke, 1997; Zaffran et al., 1997). We were interested in investigating the function of how during metamorphosis. Complementation analyses of different combinations of how alleles show that animals with the strong how alleles in combination with how17 survive until metamorphosis and die as pupa (Table 1). how mutant animals die at various stages during metamorphosis, and four lethal phases were established. Based on our analyses, the strength of these alleles can be ordered how17, how94, and how17 from strongest to weakest (Table 2). Some how17/how94 animals that survive to metamorphosis die as pupae. These animals fail to completely evert their anterior spicules, and have an abnormal body shape (Fig. 1A and B, and Table 2). The second stage of pupal lethality is at the time of head eversion and results in a cryptocephalic phenotype (Fig. 1C and D). This phenotype is observed in how17/how94 and...
**TABLE 1. how Mutants Die at Distinct Stages During Development**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Early lethalb</th>
<th>% Metamorphosis lethalc</th>
<th>% Adultsd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3R)93F2       /Df(3R)93F2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>howe44 Df(3R)93F2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>howe44 howe44</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>44</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>4</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>4</td>
<td>87</td>
<td>9</td>
</tr>
</tbody>
</table>

*a*Homozygous or transheterozygous progeny from a cross were identified by the lack of dominant visible markers on the balancer chromosome TM6B, Hu e Tb. The percent lethality at a given stage represents the percent of the expected number of how mutant animals not seen (based on the expected Mendelian ratio of how mutant to how/TM6B, Hu e Tb) following analyses of 800–1,000 total progeny of each cross.

*b*Early lethal refers to any stage prior to metamorphosis. Previous reports indicate that the allelic combinations that result in 100% early lethality in this study are embryonic lethal (Baehrecke, 1997; Prout et al., 1997).

*c*Metamorphosis lethal includes any animal that dies during the prepupal or pupal stages.

*d*All how mutants that survive to adulthood exhibit wing-blist er defects.

**TABLE 2. Lethal Phases of Various how Mutant Combinations That Die During Metamorphosis**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Early prepupa</th>
<th>% cryptocephalic</th>
<th>% Head everted</th>
<th>% Pharate adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>howe44 / Df(3R)93F2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>4</td>
<td>55</td>
<td>9</td>
<td>32</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>0</td>
<td>18</td>
<td>39</td>
<td>43</td>
</tr>
<tr>
<td>howe44 / howe44</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*a*Homozygous or transheterozygous progeny from a cross were identified by the lack of dominant visible markers on the balancer chromosome TM6B, Hu e Tb. The percent lethality at a given stage represents the percent of those that die during the prepupal and pupal stages (Metamorphosis in Table 1).

howe17 / howe44 animals (Table 2). The third pupal lethal stage follows head eversion (Fig. 1E and F). These animals have lightly pigmented eyes, non-pigmented wings and have a distinc t head, thorax, and abdomen (49-57 hr APF). They die at this stage without any obvious defect in their body structure, but fail to develop further pigmentation of the eyes, wings, and body. This phenotype is observed in howe17/howe44 and howe17 / howe44 animals (Table 2). The last stage of lethality is the pharate adult stage (Fig. 1G and H). These animals have a completely developed head, thorax, abdomen, legs, and fully pigmented eyes and wings. Bristles are also observed on the thorax and abdomen. This phenotype is observed in howe17 / Df(3R)93F2, howe17 / howe44, howe17 / howe44, and homozygous howe17 animals that survive to metamorphosis and fail to eclose (Table 2). The small number of homozygous howe17 animals that form viable adults appear normal except that they all possess wings that are held-out and have blisters (Table 1).

**how RNA and Protein Are Detected in Various Tissues During Metamorphosis**

This and previous studies have provided evidence that how may be involved in processes other than wing and muscle development, and thus may possess pleiotropic function. how transcription is induced by edysone at the onset of metamorphosis (Baehrecke, 1997). In addition, analyses of the E7 3rd 4 enhancer trap line (Bier et al., 1989; Baehrecke, 1997) indicate that how is expressed at a low level in tissues other than muscles and muscle attachment site cells (data not presented). As a first step towards surveying the spatial pattern of how expression in tissues other than muscles and muscle attachment site cells, the levels of how RNA were analyzed in specific tissues during metamorphosis. Northern blots of RNA isolated from pooled imaginal discs, gut, fat body, salivary glands, and CNS from 0-hr prepupae and 12-hr prepupae (two stages when peaks in edysone occur at the onset of metamorphosis) were hybridized with a radiolabeled how cDNA probe. We detected how transcripts in imaginal discs, gut, fat bodies, and salivary glands at the 0-hr prepupal stage and reduced levels in the gut and salivary glands at the 12-hr prepupal stage (Fig. 2A). We detected no how transcripts in CNS (data not presented). Since we detected how mRNA in multiple tissues,
we examined the spatial expression pattern of How protein. Using an antibody against both the How(L) protein, which is localized to the nucleus, and the How(S) protein, which localizes to both the nucleus and the cytoplasm (Nabel-Rosen et al., 1999), we detected How protein in imaginal discs, salivary glands, gut, and fat bodies in animals aged 0 hr APF (Fig. 2B–D). In the imaginal discs, How expression is both nuclear and cytoplasmic. How expression in imaginal discs is concentrated in the folds corresponding to the segment boundaries, but is also present to a lesser degree over the entire apical surface of the disc (Fig. 2B). How protein is present in both the nucleus and cytoplasm of the salivary gland, though How expression in this tissue is concentrated in the nucleus (Fig. 2B). This may reflect a predominance of the How(L) isoform in the salivary gland. How expression in gut and fat body is distributed between the nucleus and the cytoplasm (Fig. 2C and D). Interestingly, although we detected no How transcripts in CNS at 0 and 12 hr APF, How protein appears to be present in nuclei and cytoplasm of this tissue (Fig. 2B). These findings further suggest that how may function in more than muscle development.

**how Mutants Have Leg Defects**

Animals lacking how function have defects in muscle migration and attachment during embryogenesis, head eversion defects, and abnormalities in wing position and morphology, suggesting that how has a pleiotropic function during development (Bae-
has pleiotropic function during metamorphosis, we utilized several approaches. First, we carefully analyzed gross phenotypes of whole mounts of the mutant animals during metamorphosis. The only previously unrecognized defect we observed using this approach was malformed legs (Fig. 3A and B, Table 3). The legs of the transheterozygous how17/how44 and how17/how18r are short, with some of the segments twisted. Second, we analyzed sections of allelic combinations that die during metamorphosis. No clear defects in the muscle structure were observed in histological sections of the mutant animals (data not presented). Third, we generated somatic clones using the how18r allele. These mutants have a high frequency of wing blisters (data not presented), and some of the mutants exhibit a held-out-wings phenotype, but none of them have leg defects.

To investigate the nature of how mutant leg defects, we examined the leg imaginal discs of control and how mutant animals aged 6 hr APF, when initial leg elongation is completed in wild-type. Leg imaginal discs were dissected and visualized using light microscopy, or stained with phalloidin to visualize filamentous actin using confocal microscopy. The leg discs of how mutants at this stage show a range of phenotypes, from apparently normal to short and highly abnormal. Some of the how mutant leg discs remain incised in peripodial epithelium at 6 hr APF, indicating that they have failed to evert properly (Fig. 3C and D). Interestingly, the 6-hr APF leg discs that remain enveloped in the peripodial epithelium also range in length from short to apparently normal.

In some mutants, such as Stubble (Sb) and Broad Complex (BR-C), leg abnormalities result from defects in the cell-shape changes that contribute to leg disc elongation (Beaton et al., 1988; Kiss et al., 1988; von Kalm et al., 1995). We investigated the possibility that defective shape changes in early prepupal leg imaginal disc cells account for the abnormally short legs seen in how mutants. Phalloidin staining of dissected leg imaginal discs from control and how mutant 6-hr prepupae enabled us to visualize cortical filamentous actin. In all cases in which we could observe the first tarsal and basitarsal regions, a successful cell-shape transformation at the apical surface had occurred (Fig. 3E and F). These findings demonstrate that how is not required for the cell-shape changes in imaginal disc tissue that occur during leg elongation, but suggest that obstruction of elongation and/or extension by the peripodial epithelium contributes to the leg defects.

**how Mutant Leg Imaginal Discs Are Defective in Orientation, Eversion, and Extension**

In order to determine exactly when the defects in leg morphogenesis occur in how mutants, we examined leg development through the first 50 hr of metamorphosis in living animals. We generated time-lapse movies using data collected from control and how mutant animals that expressed GFP in the leg imaginal discs and legs (technique described in Ward et al., 2003b, and in Fortier et al., 2003). Low-level GFP expression was also detected in the wings, enabling us to visualize some aspects of wing development. These animals were collected together as 0-hr prepupae, and imaged side-by-side for a period of 50 hr at 19°C using a confocal microscope (see Experimental Procedures section). In these movies, the ventral side is up. QuickTime movies are available at http://www.mtholyoke.edu/courses/cwooddar/howmovies.html. In controls, the process of leg disc morphogenesis is consistent between animals both spatially and temporally. The second thoracic pair of leg discs (T2) is visible in the ventral plane of view at 00:00 (Fig. 4A, arrow), followed by the first thoracic pair of leg discs (T1) by 3 hr APF, and the third thoracic pair of leg discs (T3) by 5 hr APF. All leg discs are oriented with the most distal portion elongating toward the posterior end of the animal. The process of leg disc eversion in the control animals occurs between 4 and 5 hr APF, and all leg imaginal discs evert synchronously. Eversion is marked by the appearance of the distal portion of the developing legs folding back upon themselves (Fig. 4A, 04:30). At 6 hr APF, leg disc evagination is complete.

---

**Fig. 2.** how RNA and protein are present in various tissues during the onset of metamorphosis. A: Total RNA was isolated from imaginal discs, gut, fat body, and salivary glands of Canton-S 0- and 12-hr prepupae. Equal amounts of RNA isolated from each tissue were electrophoresed on formaldehyde-agarose gels, transferred to nylon, and hybridized to detect how RNA. This Northern blot has been used to analyze the pattern of other ecdysone-regulated genes (Baehrecke and Thummel, 1995). B–D: Imaginal discs, salivary glands, gut, and fat body dissected from Canton-S 0-hr prepupae and stained with primary antibody against both the How(L) and How(S) proteins. Tissues were incubated in Alexa Fluor 546 goat anti-rat IgG secondary antibody (Molecular Probes) and viewed on a confocal microscope. B: Asterisks highlight imaginal discs. SG, salivary gland. C: Gut. D: Fat body.
In controls, and all three pairs of leg discs are symmetrically aligned in an ordered array about the ventral midline (Fig. 4A, 05:54).

how mutants exhibit defects in leg development at 0 hr APF. At 00:00, the number of leg discs in the ventral plane of view ranges from the typical T2 pair of leg discs to all six leg discs (Fig. 4). Also at 00:00, the orientation of the leg discs ranges from apparently normal (Fig. 4A) to the most distal portion elongating toward the anterior end of the animal (Fig. 4B, arrow indicates direction of orientation). To determine if the defects in leg disc position could be detected early in development, we stained control and how mutant embryos with antibody to Distal-less (Dll) protein. Dll is expressed in the leg primordia of the thoracic segments of the embryo (Cohen, 1990; Vachon et al., 1992). We detected no differences in the Dll expression pattern in 5–5.5- to 13-hr-old how mutant embryos compared to controls (data not presented). This suggests that the defects seen in how mutant leg discs at 0 hr APF are not the result of defects in the spatial arrangement of leg primordia in embryogenesis.

how mutants exhibit defects in leg imaginal disc eversion. In how mutants, the process of leg disc eversion typically begins at approximately 6 hr APF. In 10 of the 25 how mutant time-lapse recordings analyzed, different discs within the same animal everted at different times, and eversion of some discs was never clearly evident (Fig. 4A). In some mutant individuals, it took longer than one hour for all six leg imaginal discs to evert (data not presented).

In control animals, an anterior leg translocation (Fig. 5A, 11:30) precedes leg extension, which begins at approximately 13 hr APF and occurs synchronously and uniformly in all six legs. Final leg length in the control is achieved by 15 hr APF and the legs extend nearly to the posterior end of the animal. Leg refinement begins at approximately 19 hr APF in controls. At 50:00, all control leg pairs are clearly segmented and uniformly aligned (Fig. 5A).

Leg extension is delayed, aberrant, and greatly reduced in how mutants. Unlike the dramatic event seen in con-

Fig. 3. how mutants exhibit leg defects. A: The leg of a Canton-S control animal has normal femur (fe), tibia (ti), and tarsal (ta) segments. B: The leg of a how+/how mutant animal has shortened and twisted segments. C,D: Low-magnification confocal images of 6-hr prepupal leg discs stained with fluorescent phalloidin. C: how+/+ control. D: how+/how mutant. Note that the how mutant disc remains incased in peripodial epithelium (arrow). E,F: High-magnification confocal images of 6-hr prepupal leg discs stained with fluorescent phalloidin show the successful cell-shape transformation in the first tarsal and basitarsal regions (arrows). E: how+/+ control. F: how+/how mutant.
trol animals, leg extension in the *how* mutant is typically marked by a subtle posterior shift of the legs at approximately 14 to 17.5 hr APF (Fig. 5A, 13:54). *how* mutant legs reach their final length, usually one-third the length of the animal, by approximately 18 to 21.5 hr APF (Fig. 5A, 18:12). Leg refinement begins much later in *how* mutants, at approximately 24 hr APF (data not presented). At 50 hr APF, when leg extension and leg refinement are typically completed, all *how* mutant legs are shorter than normal, and they are usually bent or twisted (Fig. 5). The most striking differences in *how* mutant leg development were observed in alignment, direction of lengthening, and in the degree of extension and refinement. These defects are most obvious at 50:00 (Fig. 5). Unlike in controls, in which all six legs are aligned and have extended toward the posterior end of the body by 50 hr APF, *how* mutants often fail to establish and maintain uniform alignment during leg development (Fig. 5B, arrow points to misaligned T1 leg disc). This is the same T1 leg disc oriented improperly at 0 hr APF in Fig. 4B). We also observed *how* mutant legs extending toward the anterior end of the animal (Fig. 5C). Final leg length varies between *how* mutants (Fig. 5), and may vary within an individual animal (Fig. 5C). The degree of leg refinement also varies between *how* mutants (Fig. 5), and may vary within an individual animal (data not presented). Interestingly, we also observed defects in wing orientation and extension in *how* mutants (data not presented).

### DISCUSSION

Previous studies have implicated *how* in the process of muscle cell migration and attachment to epidermal sites during embryogenesis in *Drosophila* (Baehrecke, 1997; Nabel-Rosen et al., 1999, 2002). The gross defects we observed in *how* mutants during metamorphosis suggest the possibility that *how* is also required for the development of adult muscles. However, no clear defects have been observed in muscle cell migration and attachment during metamorphosis in *how* mutants. By contrast, this study points to a role for *how* in leg development during metamorphosis.

Normal leg development during metamorphosis includes three major morphogenetic processes: leg disc elongation, leg disc eversion, and leg disc extension. *Sb* and *BR-C* mutants display defects in the cell-shape changes that contribute to leg disc elongation (Condic et al., 1991; Beaton et al., 1988; Kiss et al., 1988). In contrast, *how* mutant leg imaginal discs undergo normal changes in cell shape associated with elongation, but are oriented improperly, and do not evert normally. The leg imaginal discs of *how* mutants often remain incased in peripodial epithelium for longer than normal. This appears to contribute to failure of the legs to elongate and extend properly. Interestingly, we also observed similar defects in wing imaginal disc morphogenesis. This may contribute to the held-out-wings phenotype. While other mutations described to date affect the elongation step of imaginal disc evagination, our findings point to a role for *how* specifically in the eversion phase of imaginal disc development.

Our observations suggesting a role for *how* in imaginal disc eversion is interesting in light of recent findings implicating cell–cell interactions in this process. Pastor-Pareja et al. (2004) have proposed an interesting alternative to the long-held hypothesis that during imaginal disc eversion, contraction of the peripodial epithelium is the sole mechanism driving the movement of the appendage to the outside of the larval epidermis (Fristrom and Fristrom, 1993; Gibson and Schubiger, 2001). Pastor-Pareja et al. (2004) propose an updated model for imaginal disc eversion, which invokes additional cellular events. According to their model, imaginal discs evert by apposing their peripodial side to the

### TABLE 3. Frequency of Leg Defects Observed in *how* Heteroallelic Combinations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number analyzed</th>
<th>% Crooked legs</th>
<th>% Straight legs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>how</em>&lt;sup&gt;+/+&lt;/sup&gt;/Df&lt;sup&gt;3R&lt;/sup&gt;93F&lt;sup&gt;2&lt;/sup&gt;</td>
<td>114</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td><em>how</em>&lt;sup&gt;+/+&lt;/sup&gt;/Df&lt;sup&gt;3R&lt;/sup&gt;93F&lt;sup&gt;2&lt;/sup&gt;</td>
<td>102</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td><em>how</em>&lt;sup&gt;+/+&lt;/sup&gt;/Df&lt;sup&gt;3R&lt;/sup&gt;93F&lt;sup&gt;2&lt;/sup&gt;</td>
<td>121</td>
<td>46</td>
<td>54</td>
</tr>
</tbody>
</table>

*Transheterozygous mutant progeny that survived to the pharate adult stage were identified by the lack of dominant visible markers on the balancer chromosome TM6B, Hu e Tb.*

![Fig. 4. *how* mutants exhibit defects in leg imaginal disc orientation and eversion.](http://www.mtholyoke.edu/courses/cwooddard/howmovies.html)

Corresponding times are shown in the upper right corner in hr:min APF. **A:** In the control and *how* mutant, the second thoracic (T2) pair of leg discs is visible in the ventral plane of view at 0 hr APF and the leg discs are oriented with the most distal portion elongating toward the posterior end of the animal (arrows indicate leg imaginal discs). All control leg discs evert at 04:30, as marked by the appearance of the distal portion of the legs folding back upon themselves. Leg disc eversion in the *how* mutant occurs between 05:54 and 06:36. At 05:36, all three pairs of thoracic leg discs (T1, T2, T3) are in the ventral plane of focus. The first thoracic (T1) pair of leg discs begins the process of eversion at 05:54 (arrows). The second thoracic (T2) pair of leg discs begins evertting at 06:12 (arrows). By 06:36, eversion is complete in both the T1 pair and the T2 pair of leg discs. Eversion of the third thoracic (T3) pair of leg discs is never clearly evident. The curved nature of the T3 pair of leg discs persisted throughout development. **B,C:** *how* mutants exhibiting leg imaginal disc defects at 0 hr APF. **B:** The number of leg discs in the ventral plane of view deviates from the normal single T2 pair of leg discs. **C:** All six leg discs are in the ventral plane of view. Orientation of the leg discs may also vary at 0 hr APF from apparently normal to the most distal portion elongating in the opposite direction (B, arrow indicates direction of orientation), toward the anterior end of the animal.
Fig. 4. HOW FUNCTIONS IN LEG DEVELOPMENT
larval epidermis, and via invasion of the larval epidermis by cells of the peripodial epithelium and peripodial stalk. During these events, the peripodial stalk cells lose apical/basal polarity and cell–cell adhesion, show high cytoskeletal activity, and become motile and invasive. Zonula adherins (ZAs) are lost from the peripodial stalk cells leading the spreading of the discs over the larval tissues, as components of the ZAs delocalize from the leading edges of the cells and become cytoplasmic. The septate junction components, Corelak and Discs Large, are also missing from the leading front cells during this process. The peripodial stalk cells thus undergo a pseudo-epithelial-mesenchymal transition (PEMT), as seen in mesoderm and neural crest cells during vertebrate development (Savagner, 2001). This results in perforation of the peripodial stalk/larval bilayer, and protrusion of the imaginal epithelia. During this process, the JNK signaling pathway promotes the apposition of peripodial stalk and larval cells, determines the extent of PEMT and motility of the leading edge/peripodial stalk cells, and helps maintain adhesion between larval and imaginal tissue (Pastor-Pareja et al., 2004). how may play a role in directing interactions between the imaginal disc cells, the cells of the peripodial epithelium

Fig. 5. A: how mutants exhibit defects in leg imaginal disc extension, alignment, and refinement. Representative images of a control how+/+ (top) and a how/mutant (bottom) from the same time-lapse movie shown in Figure 4A. Corresponding times are shown in the top-right corner in hr:min APF. In the control, an anterior translocation of the legs at 11:30 precedes leg disc extension (which begins at 13 hr APF). Leg disc extension begins at 13:54 in the how mutant (arrow indicates posterior tips of legs). The developing legs reach their final length by 15 hr APF in control (data not presented) and 18:12 in mutant (arrow indicates most posterior point reached by extending legs). By 50:00, the mutant legs appear normally segmented but bent, and are shorter than control legs. B,C: how mutants exhibit defects in the alignment, extension, and refinement of the developing legs. Time-lapse movie still images of two how/mutants at 50 hr APF, when leg extension and leg refinement are typically completed. All how mutant legs are shorter than normal, and they are usually bent or twisted. B: how mutants often fail to establish and maintain uniform alignment during leg development (arrow points to misaligned T1 leg disc). This is the same T1 leg disc oriented improperly at 0 hr APF in Figure 4B. C: The leg imaginal disc outlined in white is extending toward the anterior of the animal, as indicated by the direction of the arrow. Final leg length and degree of leg refinement varies in how mutants and may vary within an individual animal.
and stalk, and larval epithelial cells during disc eversion. Perhaps how regulates expression of genes that play more direct roles in these cell–cell interactions.

The K homology (KH) domain is a small protein module consisting of 70 to 100 amino acids, which was originally identified as a repeated sequence in the heterogeneous nuclear ribonucleoprotein (hnRNP) K (Siomi et al., 1993). The KH domain is an RNA-binding motif that is thought to interact directly with RNA in different cellular processes. Proteins may carry either single or multiple copies of this domain (Siomi et al., 1993). Mutational analyses of genes encoding KH domain proteins in various species suggest an essential physiological role for this gene family. How may play a role in regulating expression of genes in the JNK signaling pathway, which has been shown to function in imaginal disc eversion (Pastor-Pareja et al., 2004). We demonstrate here that how function is required for proper imaginal disc eversion. The genes of the JNK signaling pathway, including hep and puc, function in imaginal disc fusion (Agnes et al., 1999; Martin-Blanco et al., 2000). However, we have thus far observed no abnormalities in imaginal disc fusion in how mutants. It will be interesting and important to carefully examine imaginal disc eversion in how mutants, focusing on expression of genes in the JNK pathway. Identification of the target RNAs that the how gene product binds to would help define its role in development. The pleiotropic function of how indicates that KH RNA proteins function in numerous developmental processes. Elucidating the role of how in Drosophila metamorphosis should provide valuable insights into the genetic and molecular mechanisms by which these proteins act to direct animal development.

**EXPERIMENTAL PROCEDURES**

**how Lethal Phase and Phenotype Analyses**

All Drosophila melanogaster stocks were maintained on standard cornmeal/molasses/yeast medium at either 18° or 25°C. For analyses of lethal phase and phenotypes, mutant chromosomes were maintained in combination with the TM6B, Hu e Tb balancer chromosome (Lindsley and Zimm, 1992). The mutant chromosomes were used as Df/3R/93Bx2 (Baehrecke, 1997), how^{44}, how^{stru}, and how^{17}, how^{44} contains a mis-sense mutation in a conserved residue in the KH RNA binding domain (Baehrecke, 1997). how^{stru} was induced by X rays (Prout et al., 1997), and how^{17} was generated by excision of a P transposable element and has reduced RNA levels (Baehrecke, 1997). The wild-type Canton-S strain was used as a control. These flies were crossed to generate various chromosomal combinations. Lethal phases and phenotypes of the following transheterozygotes were studied: how^{17}/Df(3R) 93Bx2, how^{44}/how^{17}/how^{stru}, how^{17}/how^{44}, and how^{17}/how^{44}. These how heteroallelic combinations were selected based on their lethality during metamorphosis or the adult phenotype of held-out wings. Mutants were selected using the dominant pupal marker Tubby (Tb) and the dominant adult marker Humeral (Hu) to distinguish the transheterozygotes from how/TM6B, Hu e Tb animals. Lethality of a particular chromosomal combination was analyzed by calculating expected and obtained Mendelian ratios of transheterozygotes for each chromosomal combination. Whole mounts of animals at various lethal phases were analyzed for structural defects. The number of animals reaching metamorphosis was counted as well as the number of pupal lethal animals at the different stages during metamorphosis. The number of animals reaching eclosion (emerging as adults) was also determined. Samples of 800–1,000 animals were scored per cross to calculate Mendelian ratios. Developmental markers including head eversion (12–13.5 hr APF), yellow-eyes (49–57 hr APF), and pharate adults (90–103 hr APF) were used to stage animals and to identify the lethal phase as previously described (Bainbridge and Bownes, 1981).

**Expression of how**

Northern blots were simultaneously hybridized with ^32P-labeled how (Baehrecke, 1997) and rp49 (O’Connell and Rosbash, 1984) probes. The exposure of the film was normalized for equal levels of rp49. For immunostaining, tissues (imaginal discs, salivary glands, gut, and fat body) were dissected from 0-hr APF Canton-S control animals in phosphate-buffered saline (PBS), fixed and incubated in primary antibody overnight against both the How(L) and How(S) proteins, and diluted 1:1,000 (kindly provided by T. Volk). Tissues were rinsed and incubated in Alexa Fluor 546 goat anti-rat IgG secondary antibody (Molecular Probes, Eugene, OR), 1:200, for 2 hr, mounted and viewed on a Zeiss LSM 510 Meta confocal microscope.

**Phalloidin Staining of Leg Imaginal Discs for Cell-Shape Studies**

Fluorescent phalloidin (Alexa Fluor 488 from Molecular Probes) was used to stain filamentous actin and visualize leg imaginal discs and cell-shape outlines. Control how^{44}+/+ and mutant how^{44}/how^{17} 0-hr (white) prepupae were maintained at 25°C for a period of 6 hr, then dissected in oxygenated, sterile Robb’s saline (Robb, 1969) at room temperature. Leg discs were then treated and visualized as described (Fortier et al., 2003).

**Visualizing Leg Development in Living Animals**

In order to follow the progression of leg development in living prepupae and pupae, we generated animals expressing Green Fluorescent Protein (GFP) specifically in the leg imaginal discs, and the developing legs. Low-level GFP expression was also detected in the wings, enabling us to visualize some aspects of wing development. The control genotype was constructed by crossing w; P[UAS-GFP.S65T]T2 virgin females with w; P[w+; w+; w+/H11001] md23 CyO males. The how mutant genotype was made by crossing w; P[UAS-GFP.S65T]T2; how^{44}/TM6B, Hu e Tb virgin females with w; P[w+; w+; w+/H11001] md23 CyO; how^{17}/TM6B, Hu e Tb males. Animals of the appropriate genotype were collected as 0-hr prepupae and viewed as described (Fortier et al., 2003; Ward et al., 2003b). The corre-
sponding time for each image from the movies is represented as (hr: min). For example, 0 hr APF is (00: 00).

ACKNOWLEDGMENTS

We thank Laurie von Kalm for valuable discussion. We thank Talila Volk for anti-Ho antibody, and Dianne and Ian Duncan for anti-DII antibody. We thank Barbara Schreader and John Nambu for technical assistance, and Yuh-Nung Jan and Jim Fristrom for fly strains.

REFERENCES


