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A role of *Ultrabithorax* in morphological differences between *Drosophila* species

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The mechanisms underlying the evolution of morphology are poorly understood[1,2]. Distantly related taxa sometimes exhibit correlations between morphological differences and patterns of gene expression[3–8], but such comparisons cannot establish how mechanisms evolve to generate diverse morphologies. Answers to these questions require resolution of the nature of developmental evolution within and between closely related species. Here I show how the detailed regulation of the Hox gene *Ultrabithorax* patterns trichomes on the posterior femur of the second leg in *Drosophila melanogaster*, and that evolution of *Ultrabithorax* has contributed to divergence of this feature among closely related species. The *cis*-regulatory regions of *Ultrabithorax*, and not the protein itself, appear to have evolved. This study provides experimental evidence that *cis*-regulatory evolution is one way in which conserved proteins have promoted morphological diversity[1].

In most species of the genus *Drosophila*, non-sensory microtrichiae, or trichomes, cover much of the posterior second femur, leaving a patch of naked cuticle near the proximal end (Fig. 1). The distribution of this naked cuticle varies between, and to some extent within, species. Of the three species studied here, *D. melanogaster* has a small naked patch, its sister species *D. simulans* has a larger patch, and the more distantly related *D. virilis* has no naked cuticle (Fig. 1).

In *D. melanogaster*, *Ultrabithorax* (*Ubx*) patterns unique morphological features from the second thoracic to the seventh abdomi-

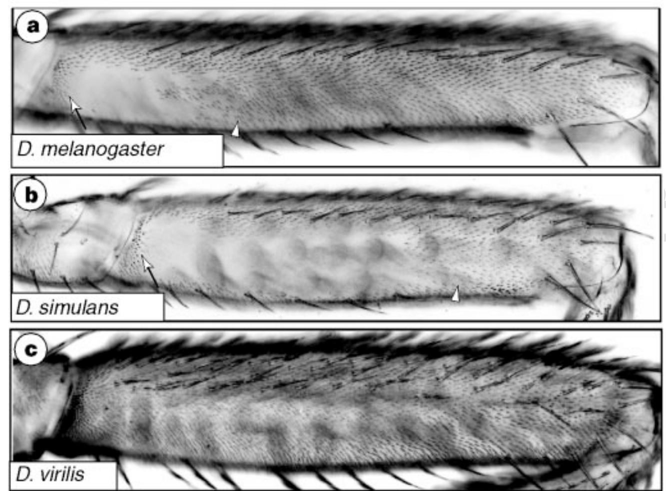

Figure 1 Trichome patterns on the posterior second femur vary among *Drosophila* species. **a**, *D. melanogaster* Oregon-R strain (mean naked cuticle length/femur length ± s.d., 0.31 ± 0.0035). **b**, *D. simulans* Tsimbazaza strain (0.61 ± 0.022). **c**, *D. virilis* Novosibirsk strain. The naked cuticle length was measured between the proximal (arrow) and maximum distal (arrowhead) extent of naked curticle.

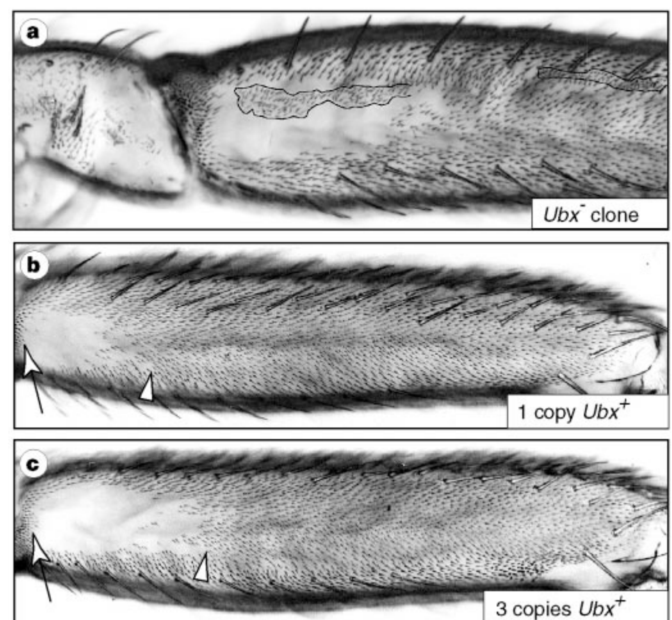

Figure 2 *Ubx* represses trichomes in the proximal naked cuticle in a dosage-dependent manner in *D. melanogaster*. **a**, A *multiple-wing-hairs* marked clone of *Ubx*⁻ cells (outlined) differentiated trichomes within the patch of naked cuticle. **b**, **c**, *Ubx* dosage altered the distribution of trichomes. Offspring from the cross $Df(3R)P9/Dp(3R)P5 \times st\,p^p\,e^{11}$ with one functional copy (**b**) and three functional copies (**c**) of *Ubx* are shown. (Mean naked cuticle length/femur length ± s.d.: $Df(3R)P9/st\,p^p\,e^{11} = 0.17 \pm 0.014$ versus $Dp(3R)P5/st\,p^p\,e^{11} = 0.25 \pm 0.014$; $t = 7.98$, d.f. = 5, $P = 0.0005$.) Arrows and arrowheads delineate the extent of naked cuticle.

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nal segment[9–13]. I tested the requirement of *Ubx* in patterning trichomes on the posterior second femur by generating clones of cells that lacked the ability to produce *Ubx* protein. When these clones were produced in the proximal patch of naked cuticle they differentiated trichomes; thus, *Ubx* is needed to repress trichomes in this region (Fig. 2a).

Three experiments indicate that the detailed expression pattern of *Ubx* is required to generate the specific morphology of a naked patch of cuticle. First, flies carrying three copies of the *Ubx* locus had significantly more naked cuticle than sibling flies carrying one copy (Fig. 2b, c). Second, expression of uniform, high levels of *Ubx* protein during pupal development repressed trichomes on most of the posterior second femur (Fig. 3). Maximal repression occurred between 20 and 28 hours after puparium formation (APF). Trichomes were repressed in a proximal to distal direction, so that expression before 18 hours APF or at lower levels (results not shown) repressed proximal, but not distal, trichomes. Finally, *Ubx* protein is expressed in a proximal–distal gradient (Fig. 4a, b), with

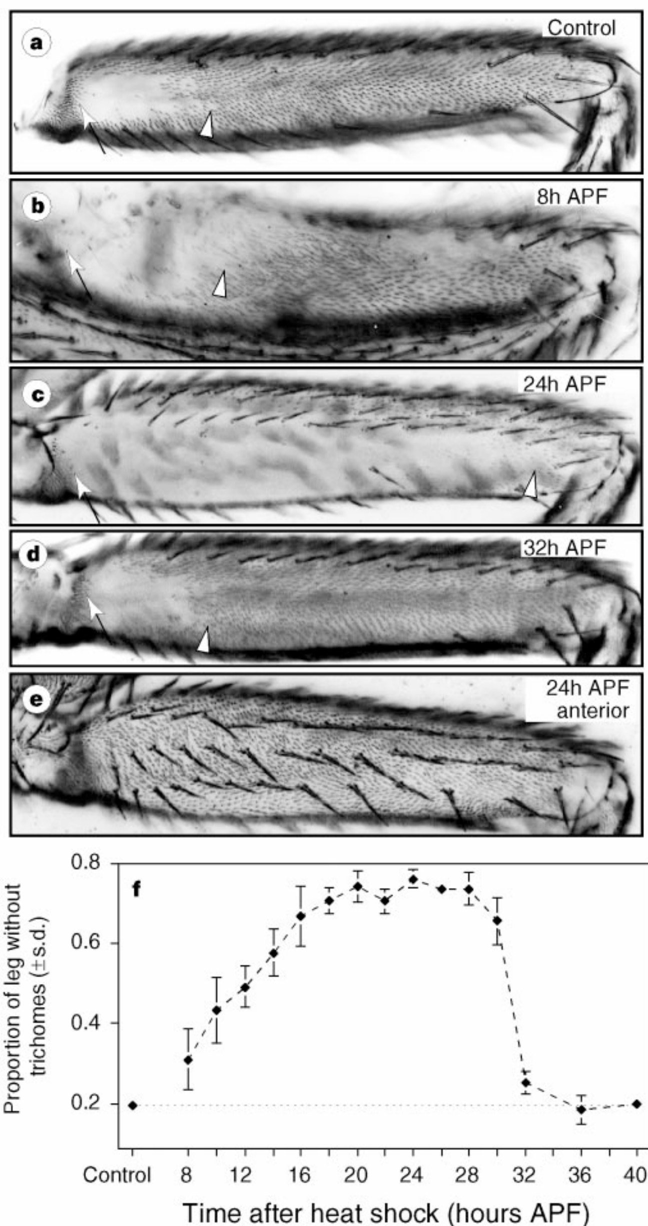

Figure 3 Uniform expression of *Ubx* in *D. melanogaster* represses trichomes on the posterior second femur during a short temporal window. **a**, Control flies. **b**, **c**, Files heat-shocked at 8 h APF (**b**) and 24 h APF (**c**) showed progressively larger patches of naked cuticle. **d**, Heat shocks at 32 h APF had little effect. **e**, Ectopic *Ubx* failed to repress anterior femur trichomes. **f**, Ectopic *Ubx* repressed posterior second femur trichomes most efficiently between 18 and 28 h APF. (After heat shock, increased amounts of *Ubx* protein remained detectable for 7 h (results not shown). This long perdurance, combined with the sharp drop in sensitivity to *Ubx* after 30 h APF, indicates that cells may respond to *Ubx* level during a shorter time window than is seen here.) Arrows and arrowheads in **a**–**d** delineate the extent of naked cuticle.

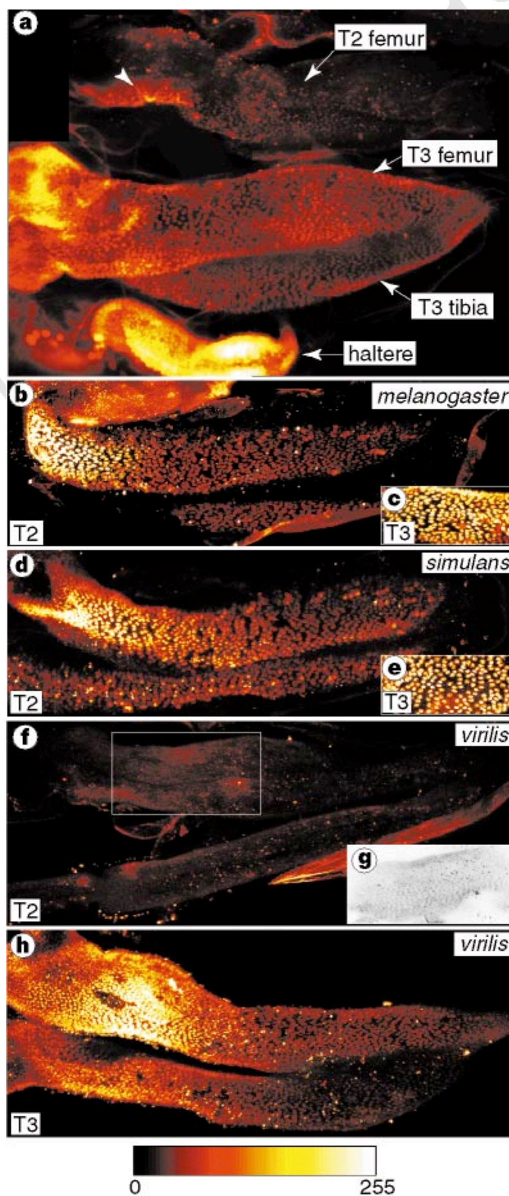

Figure 4 Distribution of *Ubx* protein in the posterior femurs of *Drosophila* species. **a**, Protein expression in the second (T2) and third (T3) leg and haltere in *D. melanogaster*. Proximal is to the left and the tibias fold back under the femurs. The second leg is twisted, providing an oblique view of the proximal femur (arrowhead) where *Ubx* is expressed at levels similar to those in the third femur. **b**–**e**, *D. melanogaster* (**b**) and *D. simulans* (**d**) show a proximal to distal *Ubx* expression gradient in T2. The approximate expression levels in the T3 femurs are shown for *D. melanogaster* (**c**) and *D. simulans* (**e**). **f**–**h**, *Ubx* expression in *D. virilis* is not visible in the second femur (**f**) at confocal settings that reveal high levels in the third femur (**h**). The boxed region of **f**, scanned at maximal sensitivity, shows that *Ubx* is expressed at low levels (**g**). Except in **g**, images were false-coloured with the scale shown below. Expression levels cannot be compared between **b**, **d** and **f**.

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high levels proximally. Studies of the third leg support the hypothesis that high levels of *Ubx* repress trichomes. Clonal analysis showed that *Ubx* is required to repress trichomes on the posterior third leg (results not shown). In all three *Drosophila* species, most of the posterior third femur lacks trichomes and *Ubx* is expressed at high levels (Fig. 4a, c, e, h). The amount of expression in the posterior third femur is similar to that in the proximal patch of the posterior second femur, indicating that *Ubx* expression at or near these levels may repress posterior femur trichomes.

Patterns of *Ubx* expression correlate broadly with interspecific variation in trichome patterning. *Ubx* expression appeared to be similar in the two sister species *D. melanogaster* and *D. simulans* (Fig. 4b, d), both of which have a patch of naked cuticle. However, *D. virilis*, which has no naked cuticle (Fig. 1), expressed *Ubx* at levels far below those seen in the posterior third leg (Fig. 4f–h).

Existing methods of visualizing protein expression may be inadequate to detect the potentially small differences in the *Ubx* expression gradient between *D. melanogaster* and *D. simulans*. However, trichome production, which is a binary outcome of the amount of *Ubx* expression, may provide a more sensitive assay for differences in species' alleles. I therefore used a complementation test, in which a single functional *Ubx* allele from each species was tested in identical hybrid backgrounds. This approach bypassed the practical difficulties of analysis of F_2 generations of these species[14].

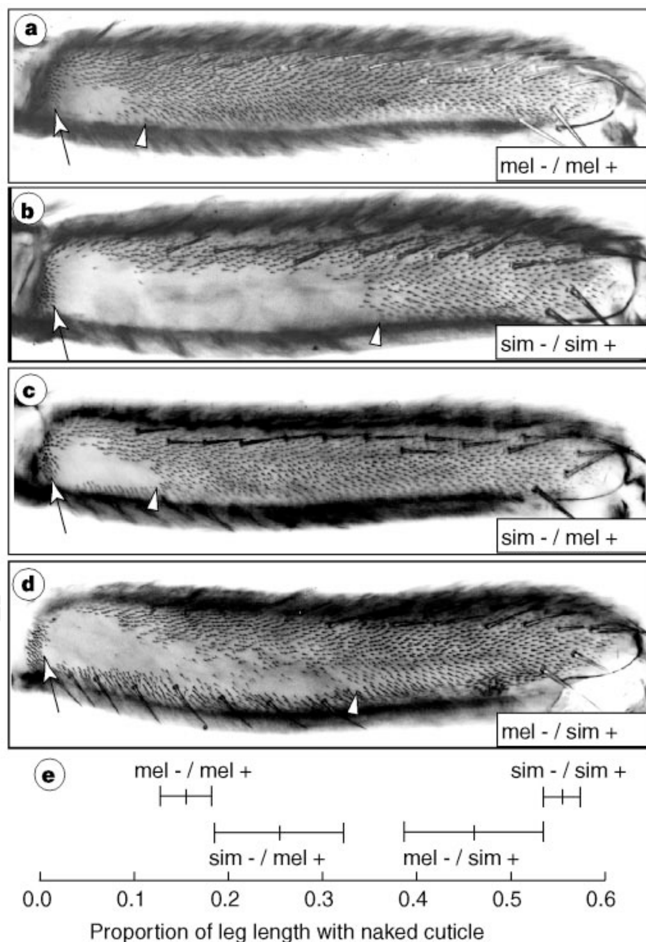

Figure 5 The *Ubx* alleles from *D. melanogaster* and *D. simulans* produce different trichome patterns in identical hybrid backgrounds. **a**, **b**, The posterior second femurs from *Ubx* heterozygous flies produced from parental strains of *D. melanogaster* (**a**) and *D. simulans* (**b**) in which new *Ubx* alleles had been induced. **c**, **d**, The posterior second femurs of the *Ubx* heterozygous F_1 offspring of the cross sim – /sim + x mel + /mel+ (**c**) and mel – /mel + × sim + /sim+ (**d**). **e**, The mean proportion of naked cuticle ± s.d. for the four genotypes. Arrows and arrowheads delineate the extent of naked cuticle. mel–, *D.melanogaster Ubx* null allele; mel+, *D. melanogaster Ubx* wild-type allele; sim–, *D. simulans Ubx* null allele; sim+, *D. simulans Ubx* wild-type allele.

Table 1 Nucleotide divergence between *D. melanogaster* and *D. simulans* for *Ubx* exons and flanking regions

	Coding		Flanking	
	Differences*	Total sites	Differences	Total sites
Exon 1	11	766	1	53
Exon 2	0	51	5 (+ 1 insert/deletion)†	167
Exon 3	0	51	1	59
Exon 4	5	302	6	96
Total	16 (1.4%)	1,170	13 (3.5%) (+ 1 insert/deletion)	375

* All nucleotide differences in the coding regions were synonymous substitutions at third-base-pair positions.
† One 7-base-pair insertion/deletion was found downstream of exon 2.

To minimize the variation that arises from segregating modifier loci in existing stocks of *Ubx* mutants (results not shown), I generated new null alleles of *Ubx* in inbred lines of each species. Files heterozygous for each new *Ubx* mutation were then crossed to the original inbred line of the other species (Fig. 5). F_1 offspring carrying a null *Ubx* allele from one species had a wild-type allele from the other and should be identical hybrids at all other loci. These F_1 classes differed significantly from each other in the predicted direction; flies carrying the *D. melanogaster* wild-type allele of *Ubx* (Fig. 5c) had a smaller naked patch than flies carrying the *D. simulans* wild-type allele (Fig. 5d, $t = 11.4$, degrees of freedom (d.f.) = 43, $P < 0.0001$). Therefore, the *Ubx* locus has evolved differently in these species so that the *D. simulans* allele produces a larger naked patch than the *D. melanogaster* allele. These conclusions are supported by complementation tests done using previously existing *Ubx* alleles (results not shown). The presumptive *Ubx* protein in *D. simulans* is identical to the *D. melanogaster* protein (Table 1), indicating that *cis*-regulation of the *Ubx* protein may have evolved[15].

Ubx is not the only locus that influences this trait, because the hybrids were significantly different from the parental classes that carried the same wild-type allele of *Ubx* (Fig. 5; *D. melanogaster* null allele plus wild-type allele versus *D. simulans* null allele plus *D. melanogaster* wild-type allele: $t = -8.2$, d.f. = 58, $P < 0.0001$; *D. simulans* null plus wild-type alleles versus *D. melanogaster* null allele plus *D. simulans* wild-type allele: $t = -6.0$, d.f. = 25, $P < 0.0001$). The hybrids with a *D. simulans* wild-type allele also exhibited a dorsal–ventral pattern of trichome distribution (Fig. 5d): the ventral naked cuticle was longer than the dorsal cuticle. Therefore, at least one other gene, which is possibly involved in dorsal–ventral leg patterning, has contributed to the evolution of this trait.

This study illustrates one mechanism that contributes to a single difference between fly species. There are about 150,000 species of flies, all using roughly the same set of conserved genes to generate their morphology. How does such diversity arise from a conserved set of proteins? One possible answer, illustrated by my results, is that conserved proteins exhibit complex spatiotemporal regulation and that every element of this pattern is susceptible to subtle evolutionary manipulation[1,6–8,15–17]. □

Methods

Clonal analysis. Two clonal analysis[18] experiments were performed. First, I crossed males carrying the alleles *mwh jv st red sbd*^{2} *Ubx*$^{12.5}$ *e*^{11} *ro ca/TM1* to females carrying the alleles *f*^{36a};*M(3)w f*$^+$*87/TM3*. Legs of male offspring without balancer chromosomes were studied for *f*^{36a} bristles. Second, I crossed *mwh jv st red sbd*^{2} *Ubx*$^{12.5}$ *e*^{11} *ro ca/TM1* males to *Dp(3:3)S462, Cbx*^{1} *Ubx*^{1}*/TM6B* females. Legs of offspring without balancers were studied for *mwh* trichomes.

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Larvae were subjected to X-rays (1,000 rad) at 24–72 h after egg-laying.

Ectopic *Ubx* expression. White prepupae from the cross *st p^p e^{11}* × HSUbx − 1a (ref. 19) were aged at 25 °C. Separate samples were heat-shocked (at 37 °C) for 1 h at 2-h intervals from 0–48 h APF. Samples heat-shocked at 0–6 h APF and some heat-shocked at 8 h APF failed to differentiate cuticle, whereas all others developed to pharate adults but failed to eclose. Most anterior femur trichomes were not repressed, indicating that neither ectopic *Ubx* expression nor the heat shock itself interfered with the ability of cells to differentiate trichomes.

Antibody staining. White prepupae were aged at 25 °C for 20–30 h for *D. melanogaster* and *D. simulans*, and for 38–44 h for *D. virilis*. Dissected pupal legs were stained using standard techniques[20], except that the antibodies were applied at 4 °C for at least 12 h each. The two monoclonal antibodies Fp3.38 (for *D. melanogaster* and *D. simulans*) and Fp6.87 (for *D. virilis*) were used together with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody to detect *Ubx* protein[20,21]. Both of the monoclonal antibodies produced comparable results when tested in *D. melanogaster* (results not shown). Images were captured with a BioRad MRC-1024 confocal microscope.

Generation of new *Ubx* alleles. New alleles of *Ubx* were induced in the *st p^p e^{11}* *D. melanogaster* and Tsimbazaza *D. simulans* stocks by X-ray irradiation of 3-day-old virgin males with 4 krad. These males were mated with virgin 3-day-old females. Offspring were screened for enlarged halteres. One of 3,600 *D. melanogaster* and nine of 8,500 *D. simulans* flies had enlarged halteres, of which one and two flies of each species, respectively, were fertile. Only alleles that failed to complement *Ubx*, showed the appropriate null phenotype[9], and did not produce *Ubx* protein (as assessed by antibody staining) were used for the interspecific test.

DNA sequencing. The four *Ubx* exons were amplified by the polymerase chain reaction (PCR) from genomic DNA extracted from the Tsimbazaza strain of *D. simulans*, with the following primers: exon 1, 5′-GCCCGTCTCAGACGG AGCAC-3′ and 5′-TGGGATTTCGGGGGACTTTCAG-3′; exon 2, 5′-CCCTA CCACCAGATCCCCACGTACCC-3′ and 5′-GCCCCATTGATTCATGAATTT AGCACACC-3′; exon 3, 5′-TGAGGCATAATGACGTTCCTGGAC-3′ and 5′-CATAGGCAAAACTATAGGCTAAGGGTTTAC-3′; exon 4, 5′-ATGTATGTA TTTCGTCGATGCAGGTC-3′ and 5′-CCAATCCCACATACACCCTAC-3′. PCR fragments were cloned into pCRII (Invitrogen) and four clones of each exon were sequenced using Dye Terminator Cycle sequencing (Perkin Elmer).

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HMG-CoA reductase guides migrating primordial germ cells

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The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is best known for catalysing a rate-limiting step in cholesterol biosynthesis, but it also participates in the production of a wide variety of other compounds[1]. Some clinical benefits attributed to inhibitors of HMG-CoA reductase are now thought to be independent of any serum cholesterol-lowering effect[2,3]. Here we describe a new cholesterol-independent role for HMG-CoA reductase, in regulating a developmental process: primordial germ cell migration. We show that in *Drosophila* this enzyme is highly expressed in the somatic gonad and that it is necessary for primordial germ cells to migrate to this tissue. Misexpression of HMG-CoA reductase is sufficient to attract primordial germ cells to tissues other than the gonadal mesoderm. We conclude that the regulated expression of HMG-CoA reductase has a critical developmental function in providing spatial information to guide migrating primordial germ cells.

In many animals, including mammals and *Drosophila*, primordial germ cells (PGCs) form in a region of the embryo separate from the somatic portion of the gonad. Consequently, PGCs migrate through the embryo and make specific contacts with somatic cells to form the gonad[4,5]. In a genetic screen for mutations that disrupt this process in *Drosophila*, we identified 15 alleles of a gene that we called *columbus* (*clb*)[6]. We have cloned *clb* and find that it encodes a *Drosophila* homologue of HMG-CoA reductase[7] (*hmgcr*). Twelve ethyl methane sulphonate (EMS)-induced alleles and one transposon-induced allele of *clb* were examined and all show molecular defects in the *hmgcr* transcript (Fig. 1, and data not shown): we

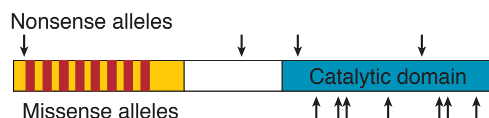

Figure 1 *columbus* is *Drosophila* HMG-CoA reductase. The HMG-CoA reductase open reading frame is shown schematically. The conserved amino terminus (yellow with transmembrane domains in red) and the conserved carboxy-terminal catalytic domain (blue) are 45% and 56% identical to human HMG-CoA reductase, respectively. Mutations found in 11 EMS-induced alleles are indicated by arrows (nonsense alleles above and missense alleles below the open reading frame). One EMS-induced allele has mutations in both a conserved amino acid in the catalytic domain and the transmembrane domain and is not represented in the figure.

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