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Characterization of *Drosophila hibris*, a gene related to human nephrin

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SUMMARY

Hibris encodes a protein that is a newly identified member of the immunoglobulin superfamily and has homology to vertebrate Nephrins and *Drosophila* Sticks-and-Stones. The Hibris protein has eight Ig-like domains, a fibronectin domain and a 160 amino acid cytoplasmic tail. The hibris transcript is expressed in a broad range of tissues and across life stages. In the embryo, hibris transcript is present in the mesectoderm, then in a group of cells at the developing CNS midline and in a subset of glia. In the periphery, hibris is expressed by fusion competent myoblasts and the epidermal muscle attachment site cells. Deletion analyses show that loss of hibris does not visibly affect embryonic CNS or somatic muscle development. However overexpressing hibris in the somatic mesoderm disrupts myoblast fusion. Furthermore, when overexpressed in the epidermis, Hibris causes comprehensive derangement of muscle insertion locations. A similar myoblast fusion defect is observed when the *Drosophila* homologs of *DM-GRASP/BEN/SC1* (irregular chiasm-roughest and dumbfounded) are deleted together. Our S2 cell aggregation assays have revealed a heterotypic interaction between Hibris and Dumbfounded, but not between Hibris and Irregular Chiasm-Roughest. We propose that Hibris is an extracellular partner for Dumbfounded and potentially mediates the response of myoblasts to this attractant.

Key words: *Drosophila*, hibris, Nephrin, Mesoderm, Myoblast, Myotube, Muscle attachment sites, Mesectoderm, Dumbfounded, DM-GRASP, BEN, SC1

INTRODUCTION

Proteins of the immunoglobulin superfamily (IgSF) have been implicated in the development and maintenance of a diverse range of tissue types across a broad spectrum of organisms. In their roles as receptors and ligands, IgSF proteins underpin cell-cell adhesion and cell-cell signaling functions (Goodman, 1996; Simmons et al., 1997; Bracco et al., 2000; Kamiguchi and Lemmon, 2000). They can act both homotypically and/or heterotypically, and often interact with other IgSF members (Goodman, 1996; Brummendorf and Rathjen, 1996; Walsh and Doherty, 1997; Kamiguchi and Lemmon, 2000). Owing to both the diverse and overlapping activities of IgSF proteins, the result of the loss of function of an IgSF protein can range from catastrophic to negligible. Similarly, altered levels of expression or localization can have severe or minor effects on development and survival.

In the present study, we have examined the action of *Drosophila* Hibris (Hbs). Hbs is a new member of the recently identified Nephrin subfamily of the IgSF. The Nephrins are represented in organisms ranging from humans to worms (Kestila et al., 1998; Holzmann et al., 1999; Ahola et al., 1999; Teichmann and Chothia, 2000). In the *Drosophila* embryo, Hbs is now implicated in myoblast fusion and myotube guidance during somatic mesoderm development, influencing contact between mesodermal-mesodermal and mesodermal-epidermal cells. Until very recently, there has been extremely limited information on the extracellular molecules that facilitate these processes. With the gradually increasing identification of proteins that are involved in these events, several are now from the IgSF with two being from the Nephrin subfamily (Bour et al., 2000) (present study) and two from the DM-GRASP/BEN/SC1 subfamily (Ruiz-Gomez et al., 2000) (H. A. D. and H. S., unpublished).

During myoblast fusion in *Drosophila*, there are two identifiable populations of myoblasts – founder cells and fusion-competent myoblasts. The founder cells are specialized mesodermal cells that serve as a scaffold to which fusion competent myoblasts fuse (Bate, 1990). In order for fusion to occur, the fusion-competent myoblasts must find and recognize the founder cells. In *Drosophila* another Nephrin-like protein, Sticks and Stones (Sns), is present on the fusion competent myoblasts but not on the founder cells (Bour et al., 2000). In the absence of Sns, there is a comprehensive failure of myoblast fusion and the somatic musculature does not form (Paululat et al., 1999; Bour et al., 2000). This suggests that Sns has a role in the fusion-competent myoblasts-founder cell recognition and/or fusion initiation processes. Interestingly a phenotype identical to that for *sns* was reported for a deletion that affected the IgSF proteins Dumbfounded (Duf) and IrreC-Rst. In the absence of both *duf* and *irreC-rst*, the fusion competent myoblasts and founder cells fail to fuse. Returning *duf* expression to the mesoderm can largely rescue this combinatorial phenotype, and when ectopically expressed, Duf

can attract fusion competent myoblasts to novel locations (Ruiz-Gomez et al., 2000), which illustrates the attractant activity of Duf. While the role of *irreC-rst* in the deletion phenotype remains to be analyzed, overexpression in the mesoderm can block myoblast fusion (H. A. D. and H. S., unpublished). Therefore the IgSF proteins Sns, Duf and IrreC-Rst represented the first extracellular proteins involved in the myoblast-founder cell recognition/fusion process (Frasch and Leptin, 2000).

As fusion-competent myoblasts fuse to founder cells, the ends of the developing myotubes form structures highly reminiscent of the filopodia that are found on axonal growth cones (Bate, 1990). Just as growth cone filopodia search for guidance cues, so too the filopodia-like structures on myotubes are used to search for epidermal cells that will serve as sites for muscle attachment (Bate, 1990). An understanding of the molecular mechanisms that facilitate myotube guidance is still very much in its infancy. To date, the only extracellular molecules implicated in the guidance of myotubes are the ligand/receptor pair, Slit/Roundabout (Robo – an IgSF protein) (Kidd et al., 1998; Kidd et al., 1999), and the receptor tyrosine kinase Derailed (Callahan et al., 1996). In *slit* mutant embryos, a subset of muscle fibers is no longer attracted to its appropriate attachment sites, while overexpressing the Robo receptor in a myotube subset causes them to target attachment sites with high Slit levels (Kramer et al., 2001). Mutations that disrupt the derailed receptor tyrosine kinase gene also cause aberrant attachment site targeting by a small subset of myotubes (Callahan et al., 1996).

Clearly the small number of extracellular molecules involved in myoblast fusion or myotube guidance identified to date is insufficient to explain fully the molecular basis of coordinated fiber formation and correct localization of attachments. In the present study, we show that *hbs* is expressed in a diverse range of tissues, including fusion-competent myoblasts and the epidermal cells where the myotubes attach. In contrast to Sns, we find that the deleterious effects of altered Hbs levels arise when there is an excess of the protein. Overexpression of *hbs* in somatic mesoderm results in incomplete fusion between fusion-competent myoblasts and founder cells, thereby partially phenocopying the *sns* and the *duf/irreC-rst* deletion loss-of-function mutant phenotypes. When *hbs* is misexpressed in the epidermis, all myotube populations respond by going to aberrant locations. These results implicate Hbs as a component of the molecular machinery that facilitates both myoblast and myotube guidance. S2 cell aggregation assays suggest that this is mediated by a direct physical interaction with Duf. We also report on the effects of overexpressing *hbs* in adult tissues, implicating it in cell-cell contact events during eye, bristle sensory organ and wing development.

MATERIALS AND METHODS

Genetic stocks

Oregon R wild-type and *white*^{1118} flies were obtained from Corey Goodman. P-element lines *l(2)k08015, l(2)02064, l(2)k04218* and *scb*^{01288} are from the Bloomington Stock Center. Lines used for ectopic expression and overexpression experiments were *elav-GAL4* (Luo et al., 1994); *GMR-GAL4* (Freeman, 1996); *pnr-GAL4* (Calleja et al., 1996); *omb-GAL4* (Apidianakis et al., 1999); *da-GAL4* (Wodarz et al., 1995); *en-GAL4* (A. Brand, Wellcome/CRC Institute, Cambridge); *sca-GAL4* (Klaes et al., 1994); *twi-GAL4* on the third chromosome (Greig and Akam, 1993; Hacker and Perrimon, 1998); *UAS-sim* (Xiao et al., 1996); and *sim-GAL4* (Steve Crews). Mutant lines used were *sim*^{H9}*Kar/TM3 ftz-lacZ* (Nambu et al., 1990); *N*^{XK11} (Bour et al., 1995); *Df(1)w*^{67k30} (Ruiz-Gomez et al., 2000); *rst*^{CT} and *irreC*^{UB883} (Ramos et al., 1993); *sns*^{zf14}*/CyOwgenlacZ* (Susan Abmayr); *Df(3R)e*^{D7} *tin*^{re58}*/TM3 ftz-lacZ* (Jagla et al., 1997); and *Df(3R)e*^{F1}*/TM3 ftz-lacZ* (Mohler and Pardue, 1984).

Cloning and molecular analysis of hibris

Primers were designed to PCR amplify a genomic DNA fragment that encodes part of the *hbs* ORF (sense strand primer: 5′-CCGTACTC-TGTAAGTATACGC-3′, antisense primer 5′-GCATGTAGAGTTCG-CACC-3′). The PCR fragment was amplified from adult fly genomic DNA and used to screen a *Drosophila* embryonic 9-12hour cDNA library (Zinn et al., 1988). cDNA inserts from positive plaques were sequenced (by Jackson Laboratories) and the data were organized and translated using Lasergene software (DNASTAR). BLAST searches of public databases were conducted to identify homologs in other organisms. To determine intron/exon boundaries, cDNA4 sequence was aligned with the genomic sequence for the region (Berkeley *Drosophila* Genome Project). cDNA4 (longest cDNA) was used to probe a northern blot using standard Church conditions (Sambrook et al., 1989).

Immunohistochemistry, in situ hybridization and eye sectioning

Oregon-R wild-type embryos were collected over a 24 hour period for analyzing the *hbs* wild-type expression pattern, and over 18 hours for analyzing expression in the *sim* loss- and gain-of-function experiments. Staging was in accordance with Hartenstein (Hartenstein, 1993). A non-radioactive antisense RNA probe was generated using cDNA4 as the template, and in situ hybridization for embryos followed the protocol of Kopczynski et al. (Kopczynski et al., 1998). For double-labeling with antibodies, embryos were washed when the desired intensity of the in situ expression pattern was attained, then stained in accordance with published protocols. In situ hybridization of wild-type third instar larvae imaginal discs followed the protocol available at http://www-bier.ucsd.edu/imagdisc.html. Adult eyes were fixed and sectioned as described (Tomlinson and Ready, 1987).

S2 cell aggregation assay

cDNA4 was subcloned into the *Eco*RI site of the RmHa3 vector (Bunch et al., 1988). Other constructs used included Sns-RmHa3 and Ha-Duf-RmHa3 (kind gifts from Malabika Chakravarti and Susan Abmayr); IrreC-Rst-RmHa3 (Schneider et al., 1995); Fasciclin II-RmHa3 (Fambrough and Goodman, 1996); and Side-RmHa3 (Sink et al., 2001). S2 cells were transfected using Superfect (Qiagen) in accordance with the manufacturer's directions. Induction of protein expression with copper sulfate and assaying of cell aggregation were carried out as previously described (Bieber, 1994). Cells were immunohistochemically processed to check for protein expression (Bieber, 1994). For mixed aggregation assays, cells were pre-labeled with DiI or DiO by adding 5 μl/ml of 2.5 mg dye/ml ethanol to the cells after transfection, shaking for 3 hours, then washing before induction.

Generation of polyclonal antibody and use

The *Sal*I/*Pst* fragment was subcloned into the *Sal*I/*Hin*dIII sites of the pQE31 expression vector (Qiagen) to generate a six-histidine tagged fusion protein. Five times at 21day intervals, two adult rabbits were immunized with 250 μg of fusion protein (at Covance Research Products). The antibody was concentrated over Protein A beads (Sigma) (Sambrook et al., 1989). We routinely preabsorbed the

antibody against 2 hours embryos overnight before use at a final concentration of 1:500.

Generation of X-ray deletions in the hibris region

Male *EP(2)2590* (Rorth et al., 1998) or *l(2)k04218* (Kania et al., 1995) flies were irradiated with 5000 rad for 10 minutes, then crossed to *w; ScO/CyO* virgins. Progeny were screened for the removal of the P-element, indicated by reversion to white eye color. In situ hybridization with cDNA4 was used to screen for the absence of embryonic *hbs* expression.

Generation of UAS-hibris transformants

cDNA4 was subcloned into the *Eco*RI site of pUAST (Brand and Perrimon, 1993). The transformant line was generated in a w^{1118} background and mapped to the 2nd chromosome. For the UAS-secreted Hbs construct cDNA4 was partially digested with *Eco*RI and completely digested with *Eco*NI, generating a new stop codon 5′ to the transmembrane domain. Six independent transformant lines were isolated.

RESULTS

Isolation of the hibris gene

hbs was initially identified in a search of the Berkeley *Drosophila* Genome Project (BDGP) database using another IgSF member – sidestep (Sink et al., 2001). PCR amplification of a genomic fragment containing part of the putative *hbs* ORF permitted the isolation of several cDNAs. Northern analysis revealed that the *hbs* transcript is a single band approximately 6.5 kb in size (data not shown), however our longest cDNA, cDNA4, is 4712 bp. Sequencing in from the ends of this cDNA revealed a poly-A^+ tail and part of the 5′ UTR.

Hibris is a member of the Nephrin subfamily of the immunoglobulin superfamily

BLAST searches of public databases with the cDNA4 sequence revealed it was in the GenBank database as *hibris* (*hbs*) (AF210316) (Artero and Baylies, 1999). The *hbs* gene encodes protein domains that are characteristic of members of the immunoglobulin superfamily (IgSF). Translation of the sequence (Figs 1, 2A) uncovered a characteristic conserved start site (Cavener, 1987) followed by a stretch of hydrophobic amino acids typical of signal peptides (von Heijne, 1990). Following the signal sequence are six consecutive immunoglobulin-like (Ig) domains. The next region has the tryptophan and potential 'second' cysteine characteristic of Ig domains, yet lacks the first conserved cysteine. This modified Ig domain is followed by two complete Ig domains, then a single fibronectin type-III (FN) domain. There are 12 potential sites for asparagine-linked glycosylation on the ectodomain. Following the FN domain there are the 26 hydrophobic amino acids of the transmembrane domain, then a cytoplasmic tail consisting of 160 amino acids (Figs 1, 2A) that contains 11 tyrosine residues. Analysis of the cytoplasmic tail with the PEST Sequence Utility program on the ExPASy Molecular Biology Server predicts a PEST sequence (Rogers et al., 1986) in the amino acids KSQSEAEPSNDDVYSK starting at amino acid 1078.

The type, number and order of the Hbs extracellular domains are conserved in the Nephrin protein subfamily (Fig. 1). Nephrins have been found in human (Kestila et al., 1998), mouse (Holzman et al., 1999), rat (Ahola et al., 1999) and *C. elegans* (Teichmann and Chothia, 2000). Another Nephrin subfamily protein, Sticks and Stones (Sns), was recently identified in *Drosophila* (Bour et al., 2000). The vertebrate Nephrin forms share close to ~80% amino acid identity with each other. By comparison, Hbs shares 23% amino acid identity with the ectodomain of human Nephrin, 20% with the ectodomain of *C. elegans* Nephrin and 46% overall with *Drosophila* Sns. While the endodomain of the Nephrins is highly conserved between the vertebrate forms (~80% identity between human and mouse), the fly forms differ greatly from the vertebrate forms (Hbs and vertebrates, ~10% identity), the worm (Hbs and worm, ~10% identity) and even each other (Hbs and Sns, ~27% identity).

Based on information from the BDGP (Kimmerly et al., 1998), *hbs* maps to 51D11-E1 on the right arm of the second chromosome. 51D11-E1 is covered by a contig of sequenced P1s. Alignment analyses showed that cDNA4 straddled a >40 kb region in P1s DS00087 and DS04940, with the bulk of the *hbs* ORF lying in a region of approximately 5 kb (Fig. 2B). Exons range from 172 bp to 647 bp. While most introns are small (23 bp to 130 bp), the intron between the first and second exon is almost 40 kb in size (Fig. 2B).

Testing Hibris binding ability

Proteins belonging to the IgSF are frequently implicated in cell-cell adhesion (Goodman, 1996; Brummendorf and Rathjen, 1996; Walsh and Doherty, 1997). The ability of Hbs, Sns, Duf, IrreC-Rst and Side to bind homotypically was tested with the S2 cell aggregation assay (Bieber, 1994). As a negative control, S2 cells were transfected with RmHa3 vector (Bunch et al., 1988), and as a positive control, S2 cells were transfected with Fasciclin II-RmHa3 (Fambrough and Goodman, 1996). Homotypic aggregation was observed for Fasciclin II and Duf. To test for heterotypic interactions, the S2 cells were labeled with either DiI (red) or DiO (green), and the aggregates were examined using confocal microscopy. When Fasciclin II-transfected cells (red) were mixed with RmHa3-transfected cells (green), all aggregates formed contained only red cells. Similarly, when Duf-transfected cells (red) were mixed with RmHa3-transfected cells (green), aggregates were again all comprised of only red cells (Fig. 2C). When Duf-transfected cells (red) were mixed with Hbs- (Fig. 2C) or Sns-transfected cells (green), the resultant aggregates all had both red and green cells, but when Duf-transfected cells (red) were mixed RmaHa3- or Irrec-transfected (green) cells, all the resultant aggregates contained only red fluorescent cells. A result matrix is presented in Fig. 2D.

hbs is expressed in a temporally and spatially complex manner

hbs is expressed in a highly dynamic manner across tissue types and life stages. At stage 5, along the dorsal surface of the cellularized embryo, a strong narrow band of *hbs* expression is present that extends along approximately two-thirds the length of the embryo. This band of expression broadens laterally, decreases in length, and becomes confined to the dorsal furrows (data not shown). By stage 8, dorsal expression is still present at the furrows, and *hbs* expression also begins ventrally, where it is associated with the mesectodermal cells. Expression strengthens in the mesectodermal cells as they move into close juxtaposition with one another at the ventral midline, forming

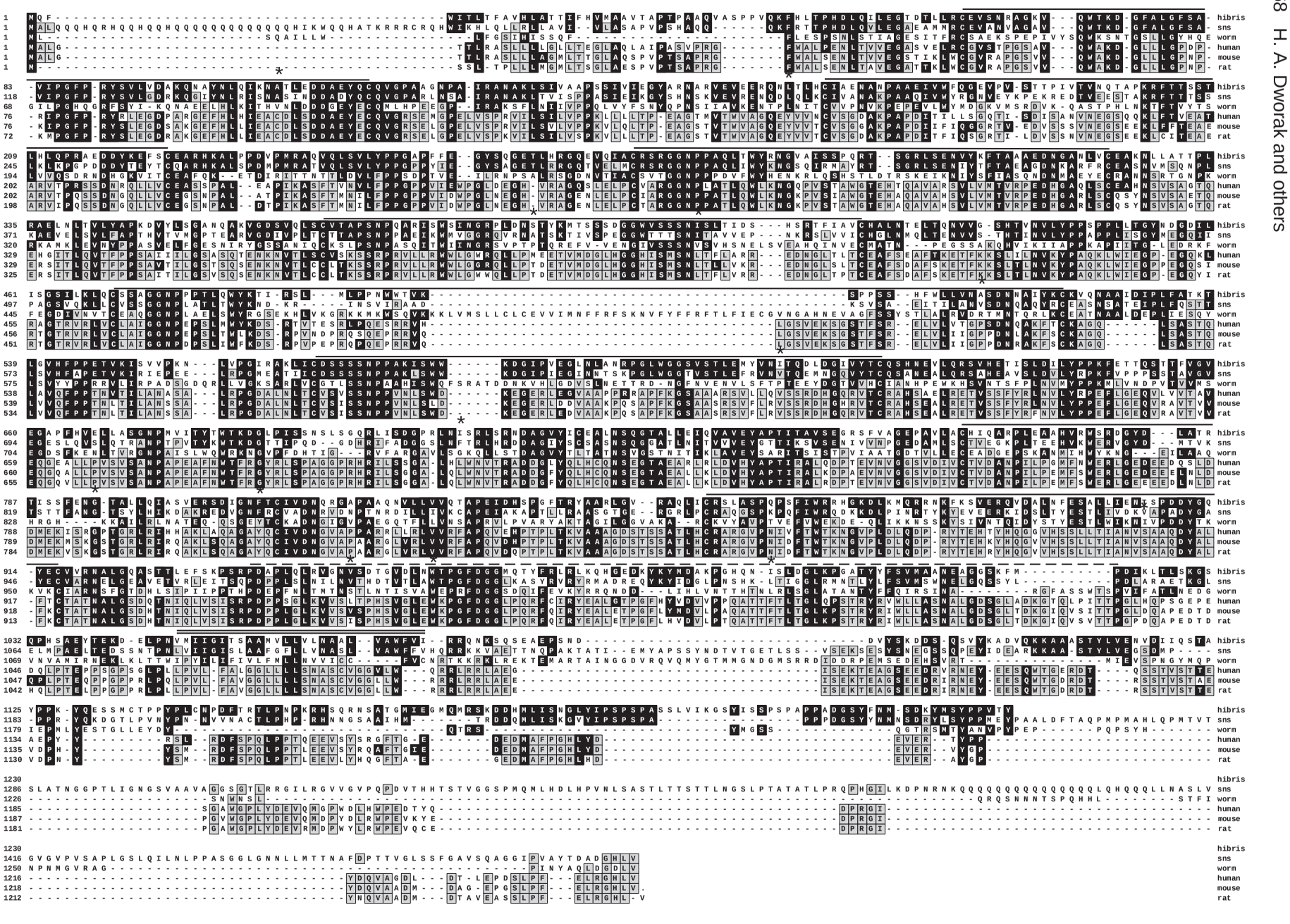

Fig. 1. Proteins of the Nephrin subfamily from *Drosophila* (Hibris and Sticks-and-Stones (Sns)), and worm, human, mouse and rat aligned with Clustal V. For Hbs, single lines lie over regions between conserved cysteines of Ig domains, the broken line is above the FN type-III like domain, the double line is over the putative transmembrane region and asterisks are over potential asparagine-linked glycosylation sites. The black indicates amino acid identity with respect to Hbs, while the gray indicates identity with respect to human Nephrin.

neighboring columns (across stages 9 and 10) (Fig. 3A,B). Expression continues during stage 11 as the mesectodermal cells intermingle, divide and move internally. By stage 12, as the midline axonal scaffold is forming (Klambt et al., 1991), a subset of midline cells posterior to the developing posterior commissure continue to express *hbs* (3C). The number of *hbs*-expressing cells at the midline decreases so that by late stage 14 there are two to three *hbs*-expressing cells below the posterior commissure (Fig. 3D). Expression in these cells is absent by stage 15. Double-labeling of glial cells with anti-Repo mAb revealed that a subset of the exit glia at the edge of the CNS were *hbs* positive from stages 12 to 15.

In the periphery during stage 10, laterally located clusters of cells begin expressing *hbs* and are distinct patches at stage 11 (Fig. 3B). These cells are visceral mesoderm, as their nuclei co-label with the anti-myocyte enhancer factor 2 polyclonal antibody (anti-MEF2) (Lilly et al., 1995). By late stage 11, the somatic and visceral mesoderm expresses *hbs.* During stage 12, *hbs* is clearly present in the fusion competent myoblasts (Fig. 3E). This expression is truly restricted to fusion competent myoblasts. In *Notch* (N^{XK11}) mutant embryos, all myoblasts adopt a founder cell fate (Corbin et al., 1991; Bour et al., 2000) and in N^{XK11} mutant embryos, there are no *hbs*-positive myoblasts (Fig. 3F). Similarly, co-labeling with anti-Kruppel antibody revealed that the fusion-competent myoblasts, but not the founder cells, were *hbs* positive (Fig. 3G). By stage 14, expression in the somatic mesoderm has ceased. As mesodermal expression decreases, epidermal expression begins. At stage 12, this expression is several cells broad and occurs at the segment boundary and in lateral patches. It becomes confined to the muscle attachment sites by stage 14 (Fig. 3H). Along the dorsal edges of the embryo lie some *hbs*-positive cells. These cells are MEF2 negative, identifying them as the pericardial cells (Ward and Skeath, 2000).

In third instar larvae, in the eye/antennal disc, *hbs* expression is strong behind the morphogenetic furrow, and also as clusters within the furrow (Fig. 3I). In the larval brain there is *hbs* expression in the optic lobes (Fig. 3J). Expression in the larval wing disc consists of a striking cruciform pattern (Fig. 3K), corresponding to the regions that abut the presumptive wing margin, and those areas destined to be wing veins L3 and L4. More proximally, is expression in the region destined to be wing veins L0 and L1. There is also light expression in the presumptive notum region. In leg discs expression is seen in concentric circles (Fig. 3L).

hbs mutant embryos do not display overt phenotypes

In order to determine the function of Hbs during embryonic development, a series of deletions were generated by irradiation of nearby P-elements (Fig. 4A). For *EP(2)2590*, over 50 w^- flies were isolated from the 71,700 progeny screened and 13 stable lines were successfully established, while for *l(2)k04218* 55,600 progeny were screened and 29 lines established. Deletion 12 removes *hbs* expression as assayed by in situ hybridization with cDNA4, and is lethal over

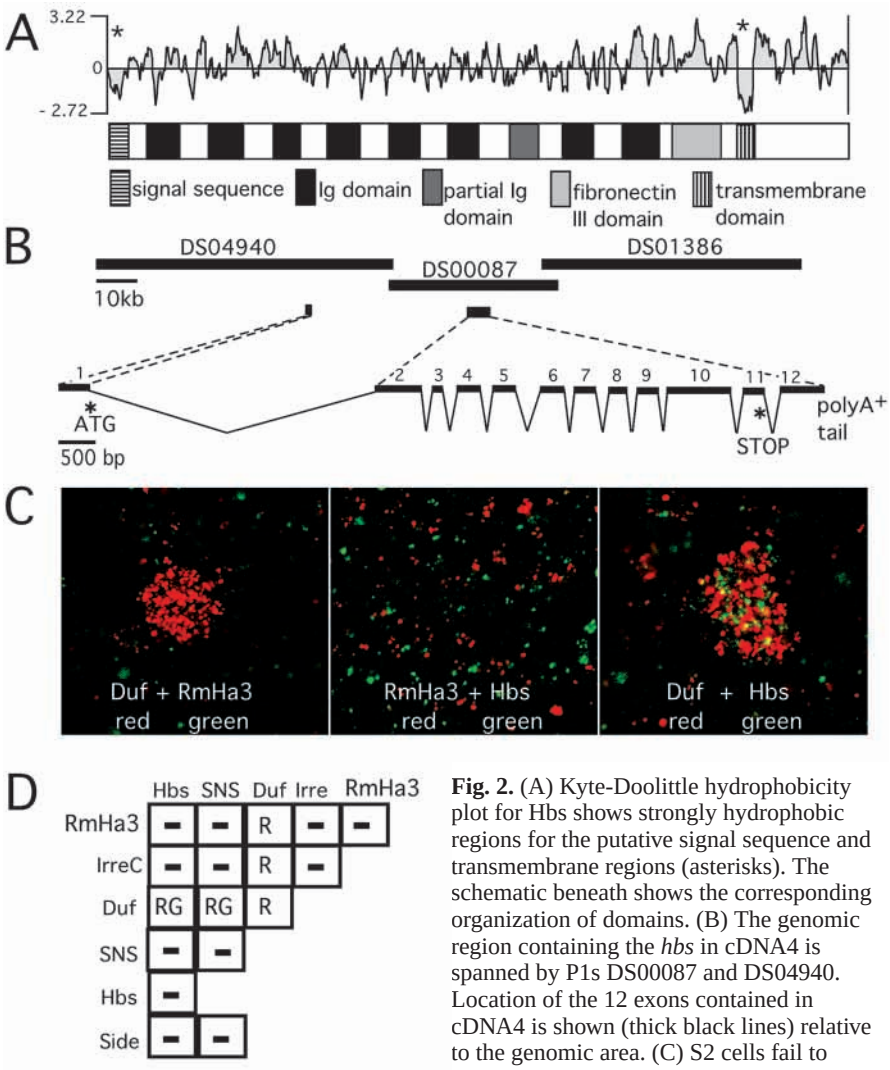

Fig. 2. (A) Kyte-Doolittle hydrophobicity plot for Hbs shows strongly hydrophobic regions for the putative signal sequence and transmembrane regions (asterisks). The schematic beneath shows the corresponding organization of domains. (B) The genomic region containing the *hbs* in cDNA4 is spanned by P1s DS00087 and DS04940. Location of the 12 exons contained in cDNA4 is shown (thick black lines) relative to the genomic area. (C) S2 cells fail to aggregate when Hbs-transfected and RmHa3-transfected cells are mixed, form aggregates containing only red cells when Duf-transfected (red) and RmHa3-transfected (green) cells are mixed, and form aggregates containing both red and green cells when Duf-transfected (red) and RmHa3-transfected (green) cells are mixed. (D) Matrix of S2 cell aggregation responses: –, no aggregation; R, aggregates with only cells of the one color; RG, aggregates with cells of both colors.

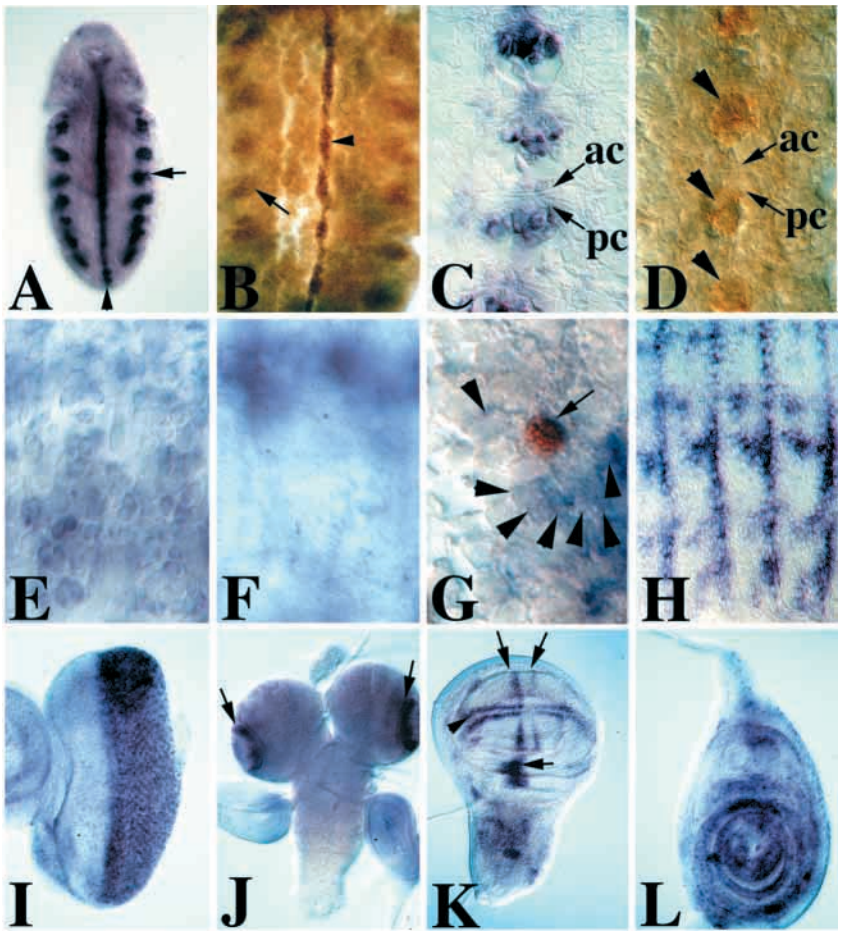

Fig. 3. Expression of *hbs* in embryonic tissues and larval imaginal discs. (A) A late stage 10 embryo has the mesectodermal cells aligned at the midline (arrowhead) and is *hbs* positive. Lateral patches of mesodermal cells (arrow) also express *hbs*. (B) This pattern is revealed at the protein level with the Hbs antibody. Arrow and arrowhead indicate equivalent tissues from A. (C) *hbs*-positive cells lie posterior and internal to the posterior commissure (pc) as it and the anterior (ac) are developing during stage 12. (D) By stage 14, only a subset of cells below the pc are still Hbs positive (arrowheads). (E) Stage 13 embryo. *hbs* is expressed by fusion competent myoblasts. (F) *hbs* is not expressed in muscle founder cells as shown in the age matched *Notch* mutant embryo. (G) At stage 11 *hbs* is expressed in fusion competent myoblasts (arrowheads) but not the Kruppel-positive founder cell (arrow). (H) Expression of *hbs* in the muscle attachment sites of a stage 14 embryo. (I) *hbs* is expressed in cells within and behind the morphogenetic furrow of the larval eye/antennal disc. (J) In the larval brain, *hbs* is expressed in the optic lobes in the locations where the photoreceptors terminate (arrows). In the wing disc (K), *hbs* expression is present in stripes that bound the presumptive wing margin (arrowhead), the stripes corresponding to wing veins L3 and L4 (downward arrows) and L0 and L1 (horizontal arrow). (L) In the leg disc, there are broad concentric circles of *hbs*.

l(2)k06403 but not *l(2)k04218*. As our smallest deletion removing *hbs*, this line has a phenotype where the ventral muscle pattern is abnormal in two to three hemisegments per mutant embryo. The abnormality consists of a loss of some muscle fibers from the ventral muscle group (Fig. 4B). In hemisegments where the muscle patterning is normal, motor innervation is also normal (Fig. 5D). Deletion 11 does not remove or disrupt *hbs* expression, yet also shows the ventral muscle defect that was seen in Deletion 12 (as does Deletion 6). Furthermore, in deletions 11 and 12 transheterozygotes, the muscle phenotype is present (Fig. 4C). As such, the ventral muscle phenotype maps to a gene(s) in the region other than

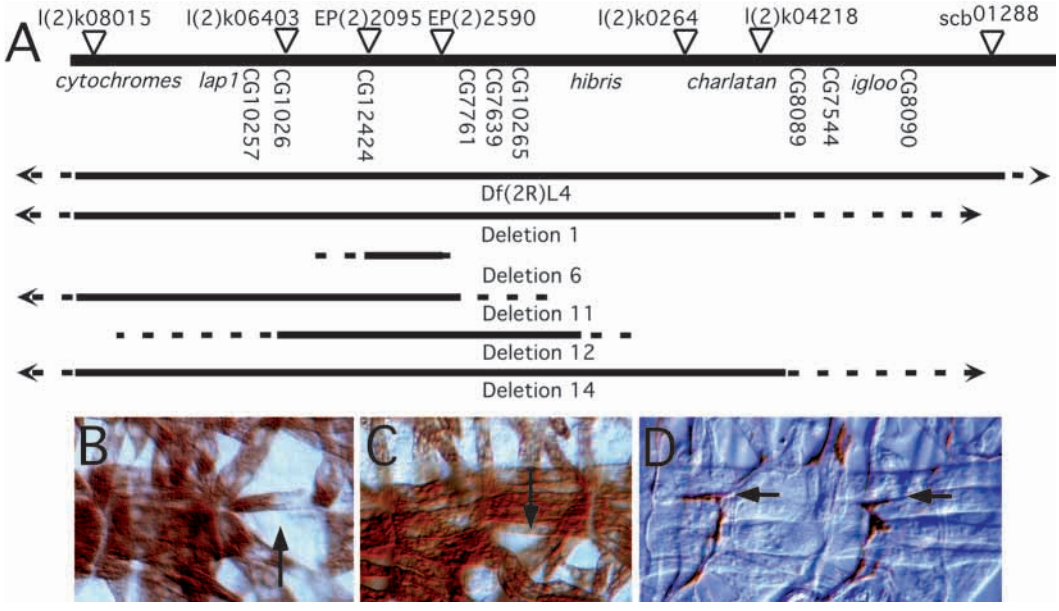

Fig. 4. (A) Map showing location of genes and P-elements in the region of *hbs*, and the regions removed by x-ray-induced deletion of *EP(2)2590*. Deletion 12 embryos show perturbed ventral musculature in a subset of hemisegments (B, arrow), and this phenotype is also observed in Deletion 11/Deletion 12 embryos (C, arrow). (D) Innervation of muscles is normal (arrows show innervation at muscle 12) in hemisegments without defects, and also in the muscles present in the disrupted regions.

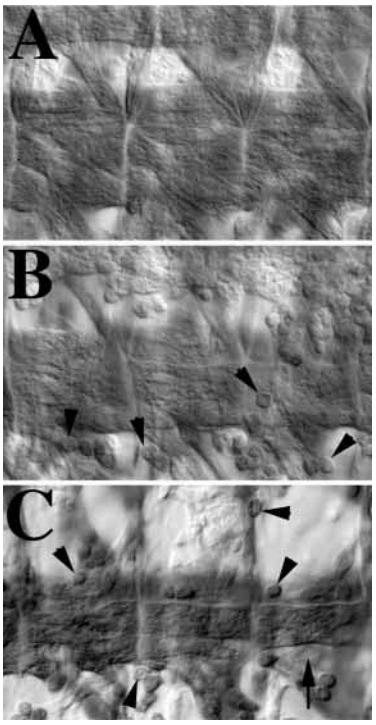

Fig. 5. Misexpression of *hbs* in the embryonic mesoderm disrupts myoblast fusion. (A) Wild type pattern of the ventral musculature. (B) *UAS-hbs;twi-GAL4* embryo showing partial failure of myoblast fusion. Arrowheads indicate unfused myoblasts. (C) *UAS-hbs/+;da-GAL4/+*showing both partial failure of myoblast fusion (arrowheads), and absence of a muscle fiber in the ventral muscle group (arrow). Preparations are stained with mAb FMM5.

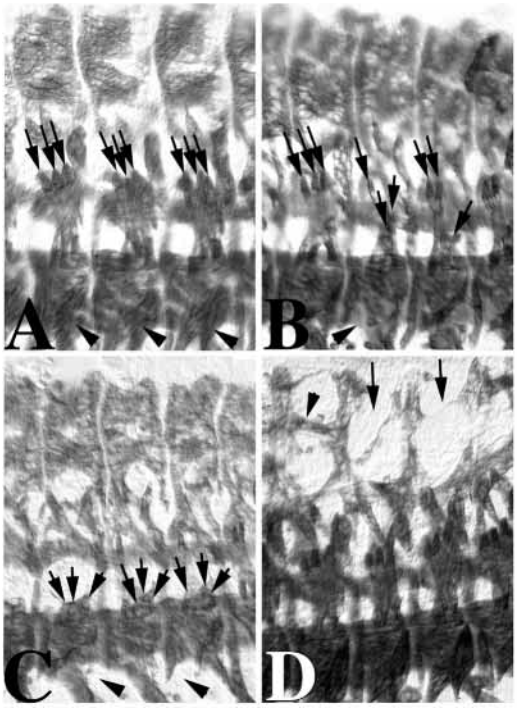

Fig. 6. Misexpression of *hbs* in the embryonic epidermis disrupts muscle attachment. (A) Wild-type embryonic somatic musculature in three hemisegments. Arrows indicate a segmentally reiterated group of three muscle fibers in the lateral musculature, while arrowheads indicate the external ventral muscles. (B) *UAS-hbs/en-GAL4* embryo show occasional failure of the three muscle fibers (shorter arrows) to insert at their dorsal attachment sites. More ventral muscles fibers are also occasionally defective (arrowhead). (C) The ability of the three lateral muscle fibers to insert at their dorsal attachment sites is comprehensively overturned in the *UAS-hbs/sca-GAL4* embryo (shorter arrows). Ventral muscles fibers sometimes no longer span the hemisegment correctly (arrowheads). (D) Overexpression of *hbs* in the dorsal epidermis with the *pnr-GAL4* driver results in dorsal muscle fibers failing to extend across the segment (arrows), or sending a thinned process (arrowhead). Many fibers remain aligned with the segment boundary. Preparations are stained with mAb FMM5.

hbs. It appears that *hbs* mutant embryos do not have an overt muscle phenotype as: (1) the muscle phenotype in deletion 12 is no different from that in the transheterozygote deletions 11 and 12 embryos; (2) muscle number, insertion sites and innervation are normal in the unaffected hemisegments; and (3) unfused myoblasts are barely evident in late stage 16. We also assayed the nervous system across stages 12-17 with mAbs BP102, 1D4, 22C10, anti-wrapper and anti-Repo, and did not find any defects (data not shown). As such, Hbs appears functionally redundant in the development of the embryonic somatic mesoderm and central nervous system. Deletion 12 in *trans* with *sns*, *irreC-rst* or the *duf/irreC-rst* deletion did not produce phenotypes in the embryonic muscles or the adult.

hbs misexpression gives robust embryonic and adult phenotypes

In the embryo, overexpression of *hbs* in the CNS using the *sim-GAL4*, *elav-Gal4*, *da-GAL4* or *sca-GAL4* drivers did not disrupt axonal pathfinding (assessed with mAbs 1D4, BP102 and 22C10) or glial cell placement and number (analyzed with anti-Wrapper and anti-Repo mAbs) (data not shown). By contrast, overexpression of *hbs* in the mesoderm in homozygous *UAS-hbs;twi-GAL4* embryos partially disrupts myoblast fusion, but not muscle fiber number or sites of muscle attachment (Fig. 5B). This phenotype is evident in all hemisegments of all embryos. When *hbs* is misexpressed using the *da-GAL4* driver, unfused myoblasts are again present, and in all hemisegments, some muscle fibers are inserted at inappropriate attachment locations (Fig. 5C). *twi-GAL4* expression is restricted to mesoderm, while *da-GAL4* is expressed in all tissues, suggesting that aberrant muscle fiber attachments may be due to *hbs* misexpression in the epidermis. To further test this idea, we misexpressed *hbs* in the epidermis with additional GAL4 drivers. The expression patterns of drivers are consistent with the embryonic expression patterns described for the associated genes (H. A. D and H. S., unpublished). Driving in the engrailed pattern (Kornberg et al., 1985) in the epidermis with *en-GAL4* disrupted attachments made by several lateral muscle fibers in most hemisegments of all embryos (Fig. 6B). This was strongly exacerbated, occurring in all hemisegments of all embryos, by overexpressing more broadly and strongly in the lateral epidermis with *sca-GAL4* (Fig. 6C), a *GAL4* line that drives in the *sca* pattern (Mlodzik et al., 1990) in the epidermis and CNS. Driving with *pnr-GAL4* in the dorsal ectoderm (Winick et al., 1993; Heitzler et al., 1996), dorsal muscle attachments sites are radically disrupted, with the muscle fibers often failing to cross the segment and instead aligning with the segment boundary (Fig. 6D). Unfused myoblasts were also seen in the epidermal gain-of-function embryos.

The effects of increased *hbs* expression in imaginal discs were assessed using drivers giving general or specific domains of expression. The distal wing margin was abnormal in *omb-GAL4/+;UAS-hbs/+* and *UAS-hbs/+;da-GAL4/+* flies. There

Table 1. Misexpression of *hbs* in the wing disc does not alter microchaete number

Control		Misexpression	
	Average microchaete number (n=10)		Average microchaete number (n=10)
daughterless-GAL4	14.7	*UAS-hbs;daughterless-GAL4*	110.9
scabrous-GAL4	122.8	*UAS-hbs;scabrous-GAL4*	122.75
pannier-GAL4	96.1	*UAS-hbs;pannier-GAL4*	98.9

was a blistered appearance at the distal wing edge in all *omb-GAL4/+;UAS-hbs/+* flies (Fig. 7B), and blistered or notched distal wing margins in the *UAS-hbs/+;da-GAL4/+* flies (Fig. 7C). On the dorsal thorax (notum) the large anterior section (scutum) has two mechanosensory bristle populations: macrochaetes (large bristles) and microchaetes (small bristles) (Fig. 7D). Microchaetes on the central scutum are in 10 longitudinal rows and fairly constant numbers. Misexpression of *hbs* in the wing discs with *pnr-GAL4, sca-GAL4* or *da-GAL4* (Fig. 7E) perturbed the linear arrangement of the microchaetes, but their number was unaltered (Table 1).

Driving *UAS-hbs* with *GMR-GAL4* or *sca-GAL4* in the eye-antennal disc gave a rough eye phenotype (Fig. 7G,H) with disorganization of the ommatidia and bristles. Driving with the *da-GAL4* driver gave the strongest rough eye phenotype with occasional fusion of ommatidia (Fig. 7I). Examination with anti-Elav antibody showed that the photoreceptor clusters were irregularly placed (Fig. 7K-M), and pigment cells were absent at sites of ommatidia fusions (Fig. 7O-Q). Larval photoreceptor pathfinding and targeting appeared normal when examined with mAb 24B10 (data not shown).

Overexpressing a secreted form of Hbs with all of the aforementioned GAL4 drivers did not generate gain-of-function phenotypes (data not shown). In addition, the adult *hbs* gain-of-function phenotypes were not suppressed as transheterozygotes with *sns, irreC-rst* or the *duf/irreC-rst* deletion.

Correct *hbs* expression depends on *sim, Notch* and *bap*, but not *mef2*

hbs expression in the mesectoderm and developing CNS midline partially overlaps with the expression of the transcription factor *single minded* (*sim*) (Crews et al., 1988). In *sim* embryos, the mesectodermal progeny survive but fail to differentiate or migrate to appropriate locations (Nambu et al., 1990). In *sim* mutant embryos, *hbs* expression was abolished at the CNS midline (Fig. 8B). When *sim* was misexpressed in all neuroblasts with the *sca-GAL4* driver (Klaes et al., 1994), the domain of *hbs* expression at the CNS midline was expanded (Fig. 8C). Yet when *sim* was misexpressed in all post-mitotic neurons using *elav-GAL4* drivers (Luo et al., 1994) *hbs* expression was unaltered (data not shown).

Notch is pivotal in the development of multiple tissue types (Hoppe and Greenspan, 1986; Corbin et al., 1991; Hartenstein et al., 1992). At the developing ventral midline, Notch activity is essential for establishing *sim* expression (Morel and Schweisguth, 2000). Consequently, in N^{XK11} mutant embryos *hbs* expression is also lost at the CNS midline (Fig. 8D) at stage 12 and onwards, after depletion of the *Notch* maternal contribution. In addition, Notch is crucial for the development of fusion competent myoblasts (Corbin et al., 1991). In N^{XK11} mutant embryos, where myoblasts are transformed to founder cells (Corbin et al., 1991; Bour et al., 2000), *hbs* expression is absent in the mesoderm (Fig. 3F). We also examined whether *hbs* is downstream of two mesoderm-specific transcription factors, *bap* (Azpiazu and Frasch, 1993) and *mef2* (Bour et al., 1995; Lilly et al., 1995). *hbs* expression in visceral mesoderm was greatly decreased in *bap* mutant embryos (Fig. 8F), but unaffected in the *mef2* mutant embryos (Fig. 8E).

DISCUSSION

Hibris is a member of the Nephrin subfamily of IgSF proteins

Hibris (Hbs) is a new member of the Nephrin subfamily of the immunoglobulin protein superfamily. In vertebrates, Nephrin is a component of the slit diaphragm, the molecular sieve that facilitates ultrafiltration in the kidney glomeruli (Somlo and Mundel, 2000). In *Drosophila* the Nephrin-like protein Sns has a pivotal role in somatic myoblast fusion (Bour et al., 2000), mediating association between fusion competent myoblasts and founder cells. The present study shows that Hbs, the most recently identified member of the Nephrin subfamily, can be implicated in both myoblast fusion and myotube guidance during somatic muscle formation in the *Drosophila* embryo. In addition, several adult tissues are highly responsive to excess presentation of Hbs.

It was postulated that vertebrate Nephrins might bind homotypically (Tryggvason, 1999), as IgSF proteins often do (Goodman, 1996; Brummendorf and Rathjen, 1996; Walsh and Doherty, 1997). In the S2 cell aggregation assay, neither Hbs nor Sns mediated homotypic adhesion. Owing to the structural similarity between Hbs and Sns and their expression patterns they were also tested in S2 cells for ability to bind in trans and did not. This is the first evidence suggesting that Nephrin proteins interact heterophilically in *trans* with other potentially non-Nephrin extracellular partners.

IgSF proteins and myoblast fusion

During normal development fusion competent myoblasts fuse to a specialized subset of myoblasts called ‘founder cells’. These founder cells act as scaffold for muscle formation (Bate, 1990). A crucial issue is the identity of the proteins that mediate the recognition/fusion event. The *Drosophila* homologs of the IgSF Nephrin subfamily and the DM-GRASP/BEN/SC1 subfamily provide the first putative links for the molecular pathways.

Both *Drosophila* Nephrin-like proteins, Sns and Hbs, are present on the fusion competent myoblasts but not the founder cells (Bour et al., 2000) (present study). In the *sns* mutant, fusion-competent myoblasts do not fuse to founder cells, and consequently normal muscle fibers fails to form (Bour et al., 2000). Potential roles for Sns are: (1) to recognize a founder cell derived attractant and facilitate fusion competent myoblast movement towards the founder cell; (2) to help form adhesive junctions at sites where fusion will be initiated; or (3) to function as the receptor/ligand that initiates myoblast fusion (Bour et al., 2000; Frasch and Leptin, 2000). In contrast to *sns*, in the absence of *hbs* myoblast fusion and muscle formation

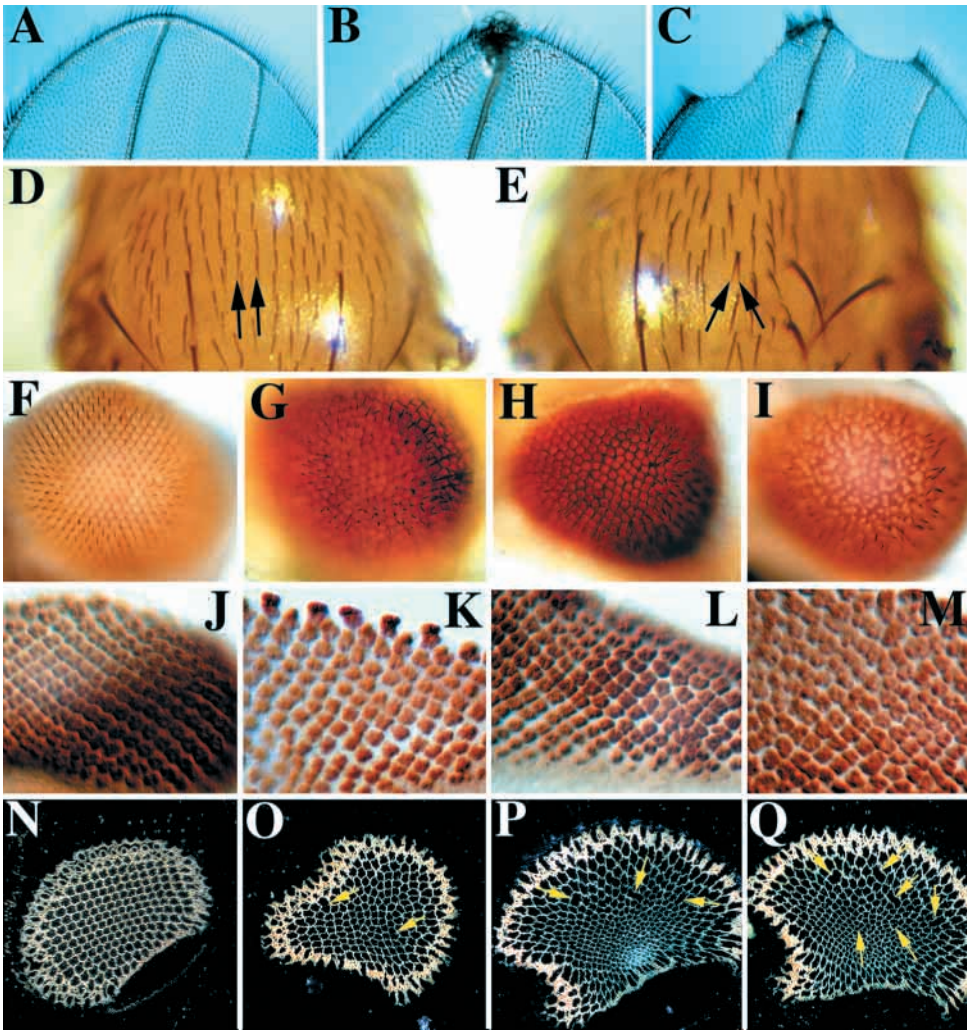

Fig. 7. Misexpression of *hbs* in imaginal discs leads to adult phenotypes. Distal wing margin in (A) wild type, (B) *omb-GAL4/+;UAS-hbs/+* and (C) *UAS-hbs/+;da-GAL4/+*. The misexpression conditions lead to abnormal development of the wing margin (B) and notching (C). (D,E) The microchaetes on the scutum of wild type flies are linear (D, arrows) but disorganized on *UAS-hbs/+;da-GAL4/+* flies (E, arrows). Wild-type flies show an orderly arrangement of eye structures (F,J,N), while in *UAS-hbs/GMR-GAL4* (G,K,O), *UAS-hbs/sca-GAL4* (H,L,P) and (I,M,Q) *UAS-hbs/+;da-GAL4/+* flies, patterning is disrupted. Gain-of-function flies show rough eye phenotypes (G-I) and disorganization of ommatidia with anti-Elav staining (K-M). Fusion of ommatidia is evident as absence of pigment cells under darkfield microscopy (O-Q). Arrows in O-Q indicate examples of fused ommatidia.

appear relatively normal when assayed with anti-muscle myosin antibody. Additionally, motor axon response to the muscles is normal, as judged by the pattern of connectivity. One interpretation of the failure to produce a loss-of-function phenotype is that Hbs is redundant. Alternatively, the function of Hbs may not be completely duplicated, but its absence may give a phenotype that is subtler than we can detect given our reagents and/or criteria.

With the identification of Sns and Hbs on fusion-competent myoblasts, the question arises as to what the corresponding extracellular partners may be on the founder cells/forming myotubes. IrreC-rst (Ramos et al., 1993) and Duf (Ruiz-Gomez et al., 2000) are the *Drosophila* members of the DM-GRASP/BEN/SC1 (Burns et al., 1991; Tanaka et al., 1991; Pourquie et al., 1992) subfamily of the IgSF. *duf* is expressed by the founder cells but not the fusion-competent myoblasts (Ruiz-Gomez et al., 2000), and *irreC-rst* is expressed in the embryonic mesoderm but the identity of the cells was not specified (Ramos et al., 1993). Myoblast fusion fails in the combined absence of *duf* and *irreC-rst* (Ruiz-Gomez et al., 2000). The return of *duf* expression to the mesoderm can rescue the phenotype; however, rescue with *irreC-rst* was not attempted. So the respective contribution of the two proteins to the fusion phenotype is uncertain. However, as Duf misexpression can guide myoblasts to novel locations, it is considered a founder cell-derived attractant (Ruiz-Gomez et al., 2000).

The similar fusion phenotype for the *sns* mutant and the *duf/irreC-rst* deletion and the attractive properties of Duf suggest Sns and Duf underscore a fusion-competent myoblast-founder cell attraction mechanism (Frasch and Leptin, 2000). Meanwhile, *hbs* overexpression in the somatic mesoderm partially phenocopies the *sns* loss-of-function mutant and the *irreC-rst/duf* deletion, suggesting reduced attraction of myoblasts to the myotubes. However myoblast fusion is also partially blocked when IrreC-Rst is overexpressed in the mesoderm (H. A. D. and H. S., unpublished), and myoblasts go to ectopic locations when Duf is presented in the epidermis (Ruiz-Gomez et al., 2000). So the Hbs gain-of-function phenotype could also be interpreted as the response of myoblasts to an imbalance of attractive forces.

Support for Hbs mediating an attractive function comes from the S2 cell assays. Under the given assaying conditions, neither Sns nor Hbs interacts homotypically, and Hbs does not bind to Sns in *trans*. These observations go against a model where Hbs might block in *trans* an Sns-mediated attraction between fusion competent myoblasts and bias the interaction towards the Duf-expressing founder cells. In the S2 cell aggregation assay,

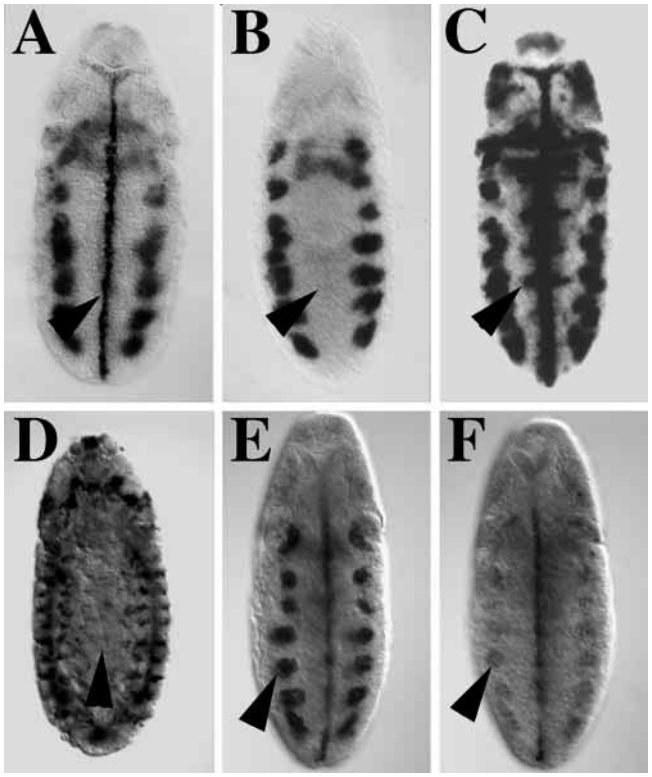

Fig. 8. *hbs* expression depends on *sim, Notch* and *bap*, but not *mef2*. (A) Wild-type stage 11 embryo showing characteristic line (arrowhead) of *hbs* expression in the mesectoderm. (B,C) This expression is absent in the *sim* mutant embryo (B, arrowhead), and expanded in *UAS-sim/sca-GAL4* embryos (C, arrowhead). (D) In a stage 12 *Notch* embryo, *hbs* expression is absent at the CNS midline (arrowhead; compare with Fig. 3F). (E,F) Stage 11 *mef2* embryos show normal expression of *hbs* in visceral mesoderm cells (E, arrowhead), but this is greatly reduced in *bap* mutant embryos (F, arrowhead).

both Hbs and Sns show an interaction with Duf, mediating heterophilic adhesion between S2 cells. But neither Sns nor Hbs induced aggregates in combination with IrreC-Rst or Side (other 5 Ig domain proteins in muscles) (Ruiz-Gomez et al., 2000; Sink et al., 2001). These results support a model where both Hbs and Sns facilitate the Duf-induced attraction of fusion competent myoblasts to founder cells. But the results do not rule out other interaction combinations between these different proteins. Further experiments are required to determine if they act in *cis* or in complexes, or whether they require different conditions for binding to one another in *trans* in the S2 cell assay (Bieber et al., 1994).

Hibris and myotube guidance

When *hbs* was globally expressed with the *da-GAL4* driver, the myoblast fusion defect was enhanced, and muscle fiber insertions were also misplaced. To determine whether the latter was due to *hbs* misexpression in the musculature or the epidermis, we misexpressed *hbs* in the epidermis with several GAL4 drivers. When misexpressed in the epidermis within a hemisegment, subsets of muscles fail to traverse the hemisegment and either bunch ventrally (*en-GAL4* and *sca-GAL4* drivers) or align with the segment boundary (*pnr-GAL4* drivers). As such, the misplaced muscle attachment phenotype observed in the *da-GAL4* gain-of-function condition is attributed to epidermally rather than mesodermally misexpressed *hbs*.

hbs is broadly expressed in the epidermis around and at the sites where muscles will ultimately attach, then becomes confined to the muscle attachment sites themselves. During normal development, Hbs may assist in slowing and constraining myotube exploration in the region where attachments must ultimately form. The data on myoblast fusion links Hbs to an attraction/adhesion mechanism. Furthermore, Duf is present in developing mytotubes (Ruiz-Gomez et al., 2000) and Sns is also present at the muscle attachment sites (Bour et al., 2000). Given these expression patterns and the heterotypic interaction of Duf with Hbs and Sns, it is possible that these proteins also interact during myotube guidance, serving to direct myotubes to their expidermal attachment sites.

Overexpression of *hbs* in the adult

hbs is also expressed in the larval imaginal discs. Misexpression of *hbs* in the wing disc yielded abnormal distal wing margins and disorganized microchaetes on the notum. At first glance, these phenotypes look like mild loss-of-function Notch defect; however, microchaete numbers did not deviate from normal. Hence, the microchaete phenotype appears to be due to displacement of cells, rather than changes in cell number caused by disturbance of lateral inhibition. This could arise if: (1) the cell-cell associations within proneural clusters are slightly perturbed, resulting in subtle changes in the location of the founder cells; or (2) founder cells are normally specified but subsequent alterations in cell-cell associations lead to them being slightly displaced.

Misexpression of *hbs* in the eye disc results in a rough eye phenotype, which is reminiscent of that seen when *irreC-rst* is absent or misexpressed in the eye disc (Ramos et al., 1993; Schneider et al., 1995). Yet neither gain-of-function eye phenotype was suppressed by a 50% decrease in the expression of the other gene, and no rough eye phenotype was observed in the *hbs* and *irreC-rst* transheterozygotes. As Hbs and IrreC-Rst did not give a positive result in the S2 cell aggregation assay, and the overexpression of *irreC-rst* in the wing imaginal disc caused a notal microchaete phenotype that differs from that for *hbs* (H. A. D. and H. S., unpublished), there is still no direct support that Hbs and IrreC-Rst interact directly with one another in a simple one-to-one *trans* binding relationship. More suitable interaction analyses await elucidation of whether Duf and Sns have roles in eye.

Regulation of *hbs* expression

The complex spatiotemporal expression pattern of *hbs*, added to the sensitivity of several tissues to abnormal *hbs* levels, brings into question the identity of the upstream regulators. We examined *hbs* expression in *Notch, mef2, bap* and *sim* mutant backgrounds.

Notch-mediated lateral inhibition defines which myoblasts will become founder cells (Corbin et al., 1991). *hbs* and *sns* are excluded from the founder cells and confined to fusion-competent myoblasts. As with *sns*, this exclusion of *hbs* from the founder cells occurs downstream of Notch. Thus Sns and Hbs represent two components of the molecular repertoire that distinguishes fusion competent myoblasts from founder cells

from one another. The transcription factor Mef2 regulates expression of muscle specific genes such as *myosin*, *MLP60A*, *tropomyosin I* and *muscleblind* (Bour et al., 1995; Ranganayakulu et al., 1995; Lin et al., 1996; Lin et al., 1997; Stronach et al., 1999; Artero et al., 1998) and its absence results in a myoblast fusion defect (Lilly et al., 1995). As for *sns*, *hbs* expression also appears unperturbed in the *mef2* mutant. So neither the loss of *sns* nor *hbs* expression contributes to the *mef2* myoblast fusion phenotype. In contrast to Mef2, the activity of the homeobox gene *bap* is crucial for correct levels of expression of *hbs* in the early visceral mesoderm cell clusters. Similarly the Sim helix-loop-helix transcription factor (Nambu et al., 1990; Nambu et al., 1991) is essential for *hbs* expression in the mesectoderm and CNS midline. Whether there a direct physical regulation of *hbs* expression by the Sim or Bap proteins awaits analysis of the *hbs* promoter region.

We have presented the first evidence of a direct physical interaction between extracellular molecules that are expressed on either fusion-competent myoblasts and at muscle attachment sites (Hbs and Sns), or on muscle founder cells and developing myotubes (Duf). Our observations suggest that adhesion between these molecules may aid recognition between fusion competent myoblasts and founder cells, and between myotubes and epidermal attachment sites. Whether the large cytoplasmic domains on these proteins have signaling abilities must now be determined.

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