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boudin is required for septate junction organisation in *Drosophila* and codes for a diffusible protein of the Ly6 superfamily

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The Ly6 superfamily, present in most metazoan genomes, codes for different cell-surface proteins and secreted ligands containing an extracellular motif called a Ly6 domain or three-finger domain. We report the identification of 36 novel genes coding for proteins of this family in *Drosophila*. One of these fly Ly6 proteins, coded by the gene *boudin* (*bou*), is essential for tracheal morphogenesis in the fly embryo and contributes to the maintenance of the paracellular barrier and the organisation of the septate junctions in this tissue. Bou, a glycosylphosphatidylinositol anchored membrane protein, is also required for septate junction organisation in epithelial tissues and in the chordotonal organ glial cells, but not in the central nervous system. Our study reveals interesting parallelisms between the Ly6 proteins of flies and vertebrates, such as the CD59 antigen. Similarly to this human protein, Bou travels from cell to cell associated with extracellular particles and, consistently, we show that it is required in a non-cell-autonomous fashion. Our work opens the way for future studies addressing the function of Ly6 proteins using *Drosophila* as a model system.

KEY WORDS: Ly6/uPAR, Three-finger toxin, Septate junctions, Paracellular barrier, Blood-brain barrier, Tracheal morphogenesis, *Drosophila*

INTRODUCTION

Model organisms, such as the fruit fly, are sophisticated tools that have contributed decisively to our understanding of genetic complexity, allowing functional characterisation of new genes and novel insight into many developmental processes. We have profited from the advantages offered by *Drosophila* to enlarge current knowledge about a poorly characterised family of proteins present in metazoan genomes, the Ly6 superfamily. The Ly6 proteins share an extracellular motif spanning about 100 residues known as a three-finger domain, three-finger snake toxin motif or Ly6/uPAR domain. This structure, first identified in the sea-snake erabutoxin b (Low et al., 1976), features a simple inner core stabilised by disulphide bridges, which supports three protruding loops or fingers. Besides a diagnostic set of 8 or 10 cysteines found in stereotyped positions, Ly6 primary sequences are poorly conserved, but they adopt remarkably similar three-dimensional structures (Kini, 2002; Ploug and Ellis, 1994). The Ly6 module is a structural domain involved in protein-protein interactions, tolerating an unusual degree of variation and binding with high specificity to a broad spectrum of targets.

The human genome codes for 45 members of the Ly6 superfamily (Galat, 2008). These include 12 TGFβ receptors, the ectodomains of which adopt the three-finger fold, but also many glycosylphosphatidylinositol (GPI)-anchored proteins and soluble ligands. Only a few of these proteins have been studied in detail, such as the urokinase plasminogen activator receptor (uPAR; PLAUR – Human Gene Nomenclature Database), which plays important roles in cell adhesion, proliferation and migration (Blasi and Carmeliet, 2002), and CD59, an inhibitor of complement activity (Davies et al., 1989). Other members, such as Lynx1 (Miwa et al., 2006) or the soluble SLURP proteins (Grando, 2008), act as regulators of nicotinic acetylcholine receptors, and are likely to be the ancestors of the snake neurotoxins. However, although they are often used as lymphocyte and tumoural markers (Bamezai, 2004), many Ly6 human and murine proteins have unknown roles.

We carried out a systematic search for members of the Ly6 superfamily in *Drosophila*, identifying 36 previously uncharacterised genes coding for one or more Ly6 motifs. We also explored the function of one of these proteins during *Drosophila* development, that encoded by the gene *boudin* (*bou*). Phenotypic analysis of *bou* mutants shows that this Ly6 protein participates in the formation of paracellular barriers in epithelial and neural tissues, physiological fences that regulate the passage of solutes between cells in both epithelial and glial sheaths (Banerjee and Bhat, 2007; Tepass et al., 2001). We show that *bou* is required for the organisation of septate junctions (SJs), invertebrate adhesion structures fulfilling an equivalent role to the vertebrate tight junctions. Differing from known SJ constituents, *bou* requirements are non-cell-autonomous, and, accordingly, we find that Bou can be released in extracellular particles and become incorporated into neighbouring cells. Altogether, our results indicate that *Drosophila* could be an attractive system in which to study the function and general properties of Ly6 proteins in a developmental context.

MATERIALS AND METHODS

Sequence analysis

We used the PSI-BLAST algorithm (Altschul et al., 1997) and the Rtv, CD59 and uPAR sequences as queries against the *Drosophila* RefSeq database (Pruitt et al., 2007). Newly identified Ly6 homologues were incorporated into the search matrix until no more members could be identified, typically after six to seven rounds of iterative search. Then, we used these sequences as novel queries. An identical strategy was used in the honeybee.

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Genetics

Full definitions of these stocks can be found in FlyBase (http://flybase.org/): *bou^{PG27} (bouGAL4), l(1)6Ea^{2} (bou^{let}), Dp(1;Y) ct^{+}y^{+}, rtv^{11}, nrg^{14}, NrgGFP, PdiGFP$^{74-1}$, UASApoLII-Myc, apGAL4, btlGAL4 UASActinGFP, nulloGAL4, enGAL4, ptcGAL4, dppGAL4, tubGAL4, hsFLP tubGAL80 FRT19A; UASmCD8GFP* and *GAL80^{ts}*. The *FM7c-Actin-lacZ* and *FM7c-KrGAL4UASGFP* balancers were used for genotyping. Temperature shifts at 18°C were done 24 hours before dissection in cultures containing third larval instars of the *bou^{PG27}/NrgGFP; UASHA-Bou/+; GAL80^{ts}/+* genotype. Mutant clones were induced in 48-hour larvae by 1 hour heat shock at 37°C, in *bou^{PG27} FRT19A/hsFLP tubGAL80 FRT19A; UASmCD8GFP/+; tubGAL4/+* larvae. A *ywFRT19* chromosome was used as control.

Dye injection

Dye diffusion into trachea and chordotonal organs was analysed injecting with a micromanipulator 10 mg/ml 10 kDa rhodamine dextran (Molecular Probes) into the body cavity of stage 16 (14- to 16-hour) embryos (Lamb et al., 1998). Diffusion into the nerve cord was monitored in 22-hour embryos. Samples were visualised with a Leica SP2 confocal microscope within 20-30 minutes of injection.

Molecular biology

Three independent PCR fragments containing the *bou* transcription unit were amplified from *bou^{let}* genomic DNA, cloned and sequenced. The HA-tag was introduced in frame within the Bou coding region by PCR, using specific oligonucleotides and the RE28342 cDNA (DRGC). The HA-BouΔC was generated substituting Gly128 for a stop codon. Both constructs were sequenced and subcloned into pAc5.1 (Invitrogen) for cell transfection or into pUAST (Brand and Perrimon, 1993) for transgenesis.

Cell culture and biochemistry

Cell culture, transfections and antibody staining were carried out as in Koh et al. (Koh et al., 2008). S2 cells co-transfected with pAcDMoe-GFP (kind gift from F. Payre, CBD, Toulouse, France) and pAcHA-Bou or pAcHA-BouΔC were fixed and stained in either permeabilising (PBS, 0.1% Triton-X100) or non-permeabilising (PBS) conditions. Transfected KcD26 (2×10^{6}) cells were incubated at 25°C for 1 hour in PBS, with or without 1 unit of phosphatidylinositol-specific phospholipase C (PI-PLC, Sigma). Cell proteins were extracted in 1× RIPA, whereas the extracellular medium was precipitated with TCA-DOC and resuspended in 50 μl of 1× loading buffer. For each condition, 20 μg of cell extracts and 25 μl of supernatant were run in an SDS-PAGE gel and blotted with anti-HA.

Immunohistochemistry

Sense riboprobes were generated from clone RE28342 for in situ hybridisation (Waltzer et al., 2003). Embryos and larval tissues were fixed for 20-30 minutes in PBS 4% paraformaldehyde. Blocking, washing and overnight incubation with primary and secondary antibodies were carried out in 0.05% Triton-X100 0.1% BSA. Primary antibodies include mouse anti-β-gal (Promega), rabbit anti-β-gal (Cappel), mouse anti-HA (Covance), rabbit anti-HA (Clontech), rabbit anti-GFP (Torrey), anti-NrxIV (gift of H. Bellen, Baylor College of Medicine, Houston, TX, USA), rat anti-Crb (gift of U. Tepass, University of Toronto, Toronto, Canada), and monoclonals 9E10 anti-Myc, anti-2A12, 4F3 anti-Dlg, DCAD2 anti-DECad, BP104 anti-Nrg and 7G10 anti-FasIII, all from DSHB. Secondary FITC and TRIT conjugated antibodies and streptavidin come from Molecular Probes. We also used CBP-FITC (NEB). Samples were visualised with a Leica SP2 confocal microscope.

RESULTS

The *Drosophila* genome codes for 41 Ly6 family members

In general, Ly6 domains share little sequence similarity, making their identification by genomic annotation algorithms difficult. For instance, the only known *Drosophila* proteins containing this domain are the five TGFβ receptors (*tkv*, *babo*, *sax*, *put* and *wit*) and the product of the gene *retroactive* (*rtv*) (Moussian et al., 2005). Using the iterative PSI-BLAST program (Altschul et al., 1997), we carried out a systematic search for Ly6 members in the fly genome, screening for domains of about 100 amino acids containing 10 cysteines, where Cys^{1} and Cys^{2} are always separated by two residues and an Asn residue contiguous to the last cysteine (canonical 10C motif). Alignment of the *Drosophila* Ly6 domains revealed the presence of short intervening distances between Cys^{8} and Cys^{9} (0-3 residues) and Cys^{9} and Cys^{10} (4-5 residues), confirming that they belong to the Ly6 family (see Fig. S1 in the supplementary material). Thus, besides the five TGFβ receptors, we have identified in flies 72 Ly6 canonical motifs and 14 related domains encoded by 36 different genes not previously ascribed to any known family (Table 1).

Ly6 motifs are never found in combination with other extracellular domains, a principle also valid in *Drosophila*, where a single Ly6 domain is the only module present in 28 proteins. In the other eight cases, multiple Ly6 motifs are found, as in the human uPAR and C4.4A proteins (Galat, 2008). We also identified three different types of Ly6-related domains lacking key features of a canonical domain. The first variant found was the 8C domain (11 motifs found in two proteins), with only eight cysteines. Interestingly, 8C domains are similar to the vertebrate uPAR domain I, which lacks both Cys^{7} and Cys^{8} and also the disulphide bridge formed by these residues. Nonetheless, the uPAR domain I also adopts a three-finger fold (Huai et al., 2006). Another variant is what we call 'atypical 10C' domain (a10C), found only in two proteins. This motif could have arisen by replacement of Cys^{8} by a new Cys placed two residues after the C-terminal Asn (see Fig. S1 in the supplementary material). Finally, we found a group of three contiguous genes coding for long stretches of repeated amino acids (mostly Ser, Thr and charged residues) in the region between Cys^{4} and Cys^{5} (see Fig. S1 in the supplementary material). We termed these long motifs 'disordered 10C' (d10C), as these repeats are predicted to form flexible regions of unstable conformation, called regions of intrinsic disorder (Dyson and Wright, 2002). Hence, it is not clear whether these proteins adopt a three-finger fold.

Vertebrate members of the Ly6 family are synthesised as propeptide precursors entering the endoplasmic reticulum thanks to an N-terminal signal peptide. They have often a second C-terminal hydrophobic peptide placed after their Ly6 domain, permitting the addition of a GPI anchor to an internal sequence of the precursor. In *Drosophila*, all the Ly6 genes code for an N-terminal portion of 25-35 residues and a 20- to 30-residue C-terminal stretch, raising the possibility that all could incorporate a GPI anchor.

We could not establish orthology relationships between *Drosophila* and vertebrate Ly6 proteins due to their low degree of sequence similarity. However, for each *Drosophila melanogaster* protein we identified a putative orthologue in *Drosophila grimshawi*, a distant drosophilid species. We found that the organisation of canonical, 8C, a10C and d10C Ly6 motifs is also conserved in this species, despite 60 mya of separate evolution (Tamura et al., 2004) (Table 1). Therefore, the whole fly Ly6 family was already present in the drosophilid ancestor. We also performed a search for Ly6 members in the honeybee genome, finding only 14 genes coding for this motif. Among these, 12 are orthologues of *Drosophila* genes (sequence identity above 50%). Thus, several gene duplication events followed by rapid divergence occurred in the drosophilid lineage, which nonetheless conserved most of the ancestral Ly6 members. Intriguingly, as is also the case in humans and mice (Galat, 2008), the *Drosophila* genes coding Ly6 proteins are often contiguous in the genome, forming six clusters that group together 24 genes (Table 1).

Existing databases of gene expression patterns allowed us to visualise during embryogenesis the transcript distribution of 21 members of the *Drosophila* Ly6 family (Tomancak et al., 2002). They are expressed in a dynamic and tissue-specific pattern in a

Table 1. The *D. melanogaster* Ly6 genes ordered according to their cytological position, indicating inclusion in a genomic cluster (I-VI) and the number of residues of each protein

Dmel gene name	Length	Cytolocation	*Dmel* cluster	*Dgri* orthologue	*Amel* orthologue	Domain composition	Embryonic expression pattern
CG15773	478	5 B3	–	GH24088	–	4×10C + 1×a10C	NA
CG14430 bou	149	6 E4	–	GH24685	XP_001120415	1×10C	Trachea, fore and hindgut, salivary gland
CG15347	214	7 E11	–	GH12232	–	2×10C	Yolk nuclei, midgut
CG1397 rtv	151	10 A8	–	GH12509	NW_001253268.1	1×10C	Trachea, epidermis, head skeleton, pharynx
CG2813	153	21 E2	–	GH11235	XP_001120323	1×10C	Trachea, fore and hindgut, salivary gland
CG7781	147	29 A5	I	GH10175	XP_001120798	1×10C	No staining
CG14275	148	29 B1	I	GH11591	–	1×10C	Yolk nuclei, fat body
CG14274	136	29 B1	I	GH11592	–	1×10C	NA
CG14273	252	29 B1	I	GH11593	–	1×d10C	NA
CG7778	269	29 B1	I	GH11594	–	1×d10C	Late expression in head epidermis, hindgut, anal pad
CG31901	555	29 B1	I	GH11595	–	1×d10C	NA
CG9568	150	29 F7	II	GH13259	–	1×10C	Midgut, Malpighian tubules, gastric caecum
CG13102	150	29 F7	II	GH13260	–	1×10C	Midgut
CG6583	154	33 D2	III	GH11181	XP_001122840	1×10C	NA
CG17218	151	33 D2	III	GH11641	XP_393726	1×10C	Trachea, fore and hindgut, salivary gland
CG6579	185	33 D2	III	GH10139	–	1×10C	NA
CG15170	561	37 B8	IV	GH11596	–	3×10C + 1×a10C + 2×8C	NA
CG15169	345	37 B8	IV	GH10174	–	1×10C	NA
CG10650	425	37 B8	IV	GH10197	–	5×10C	Midgut
CG31676	159	38 F2	V	GH10589	–	1×10C	Gonad, prothoracic muscle, ring gland
CG9335	166	38 F2	V	GH10590	–	1×10C	Bolwig organ, ventral nerve cord, lateral glia
CG9336	148	38 F3	V	GH10591	–	1×10C	Dorsal vessel, ventral nerve cord, peripheral nervous system
CG9338	147	38 F3	V	GH10592	–	1×10C	Dorsal vessel, peripheral nervous system, trachea
CG31675	148	38 F3	V	GH10593	–	1×10C	Peripheral nervous system
CG14401	146	38 F3	V	GH10594	–	1×10C	NA
CG33472	158	47 F13	–	GH21388	NW_001253216	1×10C	NA
CG8501	152	49 A1	–	GH20694	–	1×10C	NA
CG3955	201	49 F2	–	GH21594	XP_623481	1×10C	NA
CG6329	155	50 C6	–	GH21037	XP_395132	1×10C	Ventral nerve cord
CG13492	2968	58 A2	VI	GH20775	–	27×10C + 7×8C	NA
CG34040	281	58 A2	VI	GH20774	–	2×10C	NA
CG4363	199	58 A2	VI	GH20773	–	2×10C	No staining
CG4377	231	58 A2	VI	GH20772	–	2×10C	NA
CG6038	158	68 D2	–	GH16386	NW_001253250.1	1×10C	Pharynx, hindgut, epidermis
CG8861	180	85 D8	–	GH22350	XP_397506	1×10C	Ventral nerve cord
CG31323	169	97 A2	–	GH19467	XP_001121813	1×10C	Midgut

A. mellifera and *D. grimshawi* accession numbers correspond to annotated proteins or contigs coding for the corresponding orthologues. Domain composition refers to the number of times (1×, 2×, etc.) a domain appears in a protein (10C, canonical domain; a10C, atypical domain; d10C, disordered domain; 8C, 8 cysteines domain; see text for definitions). Expression patterns descriptions are as in Tomancak et al. (Tomancak et al., 2002), except for *CG1397 rtv*, which is described by Moussian et al. (Moussian et al., 2005). NA, not available.

wide range of contexts, from the epidermis and its derivatives to the nervous system and the gut (Table 1). Thus, Ly6 genes can potentially participate in many different developmental and physiological processes.

The *bou* product is required for tracheal morphogenesis

We analysed the function of a new member of this family, the product of the *CG14430* gene, which we have called *boudin* (*bou*). The *bou* locus codes for a protein of 149 residues presenting all the typical features of Ly6 members. Bou is predicted to be a GPI-anchored protein by the Big-PI algorithm (Eisenhaber et al., 1998), which proposes Asn125 as the omega site of the mature protein, where the GPI moiety is attached (Fig. 1B). Unlike other members of the *Drosophila* Ly6 family, the Bou sequence appears conserved in other insect genomes, where we have identified clear orthologues (Fig. 1C).

The *bou* transcript was first detected by in situ hybridisation at the cellular blastoderm stage, first ubiquitously and then accumulating in the invaginating mesoderm (Fig. 1D-F). By stages 13 and 14, the hindgut, foregut, salivary gland and tracheal cells express high levels of *bou*, which is also present at lower levels in the epidermis (Fig. 1G,H). This pattern is maintained until the end of embryogenesis, although transcript levels start declining after stage 14 (Fig. 1I). We did not detect *bou* expression in the ventral nerve cord or in mesodermal derivatives, indicating that at late stages this gene is expressed only in ectodermal tissues.

In a genetic screen we recovered a GAL4 P-element embryonic lethal insertion in the 5′ UTR of *bou* (*bou*^{PG27}) (Fig. 1A) (Bourbon et al., 2002). Both remobilisation of this transposon or expression of an HA-tagged Bou form (HA-Bou), using *bou*^{PG27} itself as driver, restored the viability of *bou*^{PG27} flies. We used this allele to carry out complementation tests with lethal mutations mapping to the same

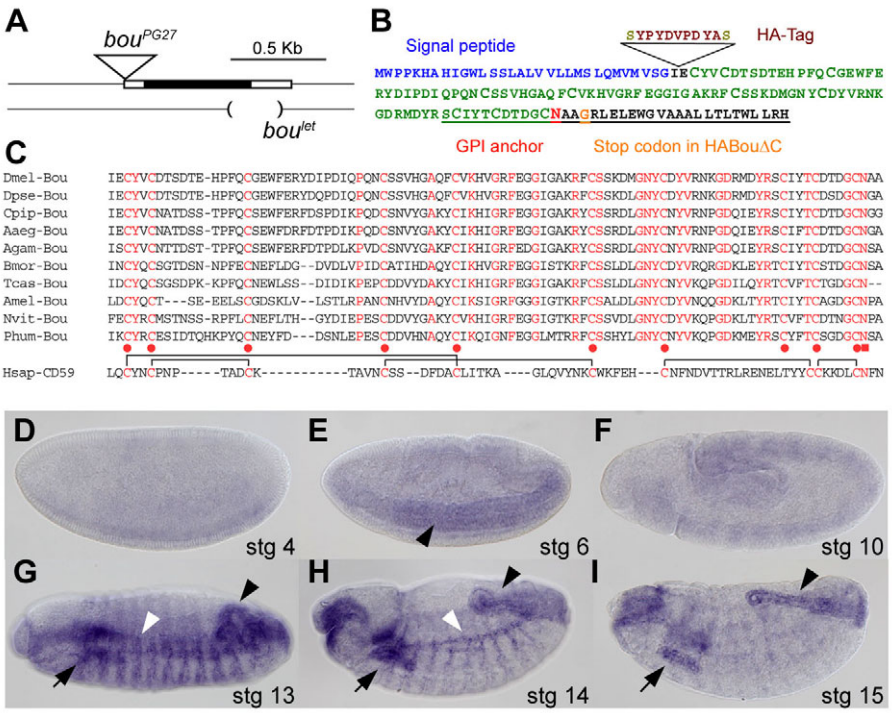

Fig. 1. *bou* codes for a conserved Ly6 protein. (**A**) The *bou* locus, indicating the *bou*^{PG27} and *bou*^{let} DNA lesions. (**B**) Bou precursor features: the signal peptide (indigo), the Ly6 domain (green) and the putative GPI anchor site (Asn125, red). The HA-tag position (brown) and the HA-BouΔC stop codon (orange) are also indicated. Residues deleted in the *bou*^{let} mutant are underlined. (**C**) Protein alignments of the Ly6 domain of insect Bou orthologues and human CD59, showing its stereotyped pattern of disulphide bridges. The 10 conserved Cys and Asn residues are indicated by red dots or a square, respectively. (**D-I**) *bou* expression during embryogenesis. *bou* mRNA is upregulated in the invaginating mesoderm (E, black arrowhead). At stages 13 and 14 (G-H), it accumulates in the hindgut (black arrowhead), salivary gland (black arrow) and trachea (white arrowhead), before its levels start decaying by stage 15 (I).

chromosomal region and identified a second *bou* lethal mutation, *l(1)6Ea*^{2} (*bou*^{let}) (Perrimon et al., 1989). Sequencing of the *bou* region in the *bou*^{let} chromosome revealed a deletion of 238 nucleotides encompassing the coding region and most of the 3′ UTR (Fig. 1A). We predict *bou*^{let} to be a null allele, as this deletion truncates the Ly6 domain and eliminates the C-terminus of the Bou precursor (Fig. 1B).

As *bou* is expressed in the tracheal cells, we first looked for morphological defects in this tissue. Staining with the 2A12 tracheal luminal marker and labelling of tracheal cells with ActinGFP revealed that *bou*^{PG27} and *bou*^{let} embryos display identical phenotypes, presenting tracheal tubes with abnormal shape and dimensions (Fig. 2A-H; and data not shown). At stage 16, the branch pattern of the tracheal network seemed normal, but the dorsal trunk appeared elongated and convoluted and we observed that the 2A12 luminal staining was interrupted along the dorsal branches and transverse connectives (Fig. 2B,E). These phenotypes point to tracheal lumen expansion defects (Beitel and Krasnow, 2000), and indeed, the tracheal dorsal trunk of stage 15 *bou* mutants did not present a uniform width (Fig. 2W), showing instead a series of bulging cysts resembling a string of sausages (hence the name *'boudin'*, a French black sausage).

The Ly6 genes *bou* and *rtv* regulate tracheal morphogenesis through different mechanisms

The defects observed in *bou* trachea were strikingly similar to those seen in mutant embryos for *rtv*, a gene coding for another Ly6 protein (Moussian et al., 2005). Rtv is required for the formation of an intraluminal chitin cable, which is essential for proper tube expansion of *Drosophila* trachea (Devine et al., 2005; Moussian et al., 2006; Tonning et al., 2005). To determine whether *bou* and *rtv* act by similar mechanisms, we monitored chitin cable integrity in stage 16 *bou* mutants, using a fluorescent chitin-binding probe (CBP). Chitin forms an organised filamentous structure in the lumen of wild-type trachea, but in the *rtv*^{11} null allele this structure is lost and CBP stains a diffuse luminal material (Fig. 2J-L) (Moussian et al., 2006). The chitin cable of *bou*^{let} mutants also loses its fibrous aspect, although the CBP staining is more intense than in *rtv*^{11} trachea (Fig. 2K,L). Thus, chitin cable formation is affected in both *bou* and *rtv* mutants, with *bou* presenting a weaker phenotype.

Mutations in different *Drosophila* SJ components also result in embryos with abnormal trachea, presenting the same cysts observed in *rtv* and *bou* mutants (Beitel and Krasnow, 2000; Wu and Beitel, 2004). As SJs are adhesion structures required for the establishment of paracellular barriers regulating molecular diffusion through epithelia, we examined the integrity of this barrier in both *rtv* and *bou* mutants. For this, we injected 10 kDa fluorescent dextran into the body cavity of live embryos and monitored the capacity of this molecule to enter the tracheal lumen (Lamb et al., 1998). At stage 16, both wild-type and *rtv*^{11} tracheal cells formed an efficient paracellular barrier, preventing dye diffusion into the lumen (Fig. 2M-R). By contrast, dextran was readily detected inside the *bou*^{let} tracheal tubes within 20 minutes of injection and we observed abnormal dye deposits trapped between contiguous cells (Fig. 2N,Q). Thus, the paracellular barrier is disrupted in *bou* mutants, suggesting that this gene is implicated in SJ organisation. To confirm this hypothesis, we examined the subcellular localisation of an SJ component, Fasciclin3 (Fas3), in tracheal cells (Beitel and Krasnow, 2000; Wu and Beitel, 2004). In wild-type and *rtv*^{11} tracheal cells, this marker accumulates in the most apical part of the lateral membrane, where SJs are present (Fig. 2S,U). By contrast, this apical accumulation was lost in *bou*^{let} embryos and Fas3 appeared uniformly distributed along the lateral membrane (Fig. 2T). Therefore, whereas *bou* is required for SJ maintenance, *rtv* seems dispensable for this process, indicating that these genes regulate tracheal morphogenesis by different mechanisms.

bou is essential for SJ organisation in *Drosophila* epithelia

To further characterise the *bou* phenotypes, we analysed the subcellular localisation of several SJ components, including Discs large 1 (Dlg1), Neurexin IV (Nrx-IV) and the protein-trap fusion

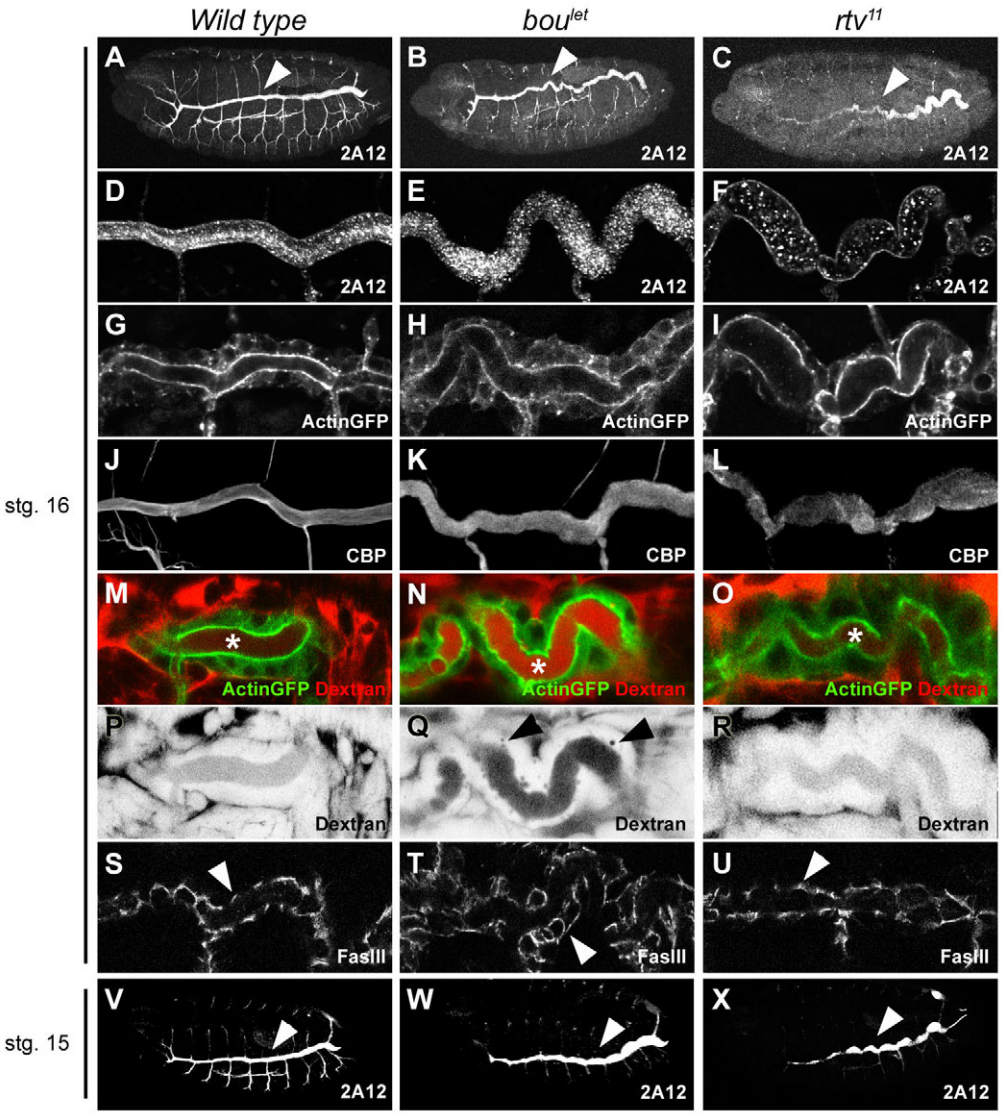

Fig. 2. *bou* and *rtv* regulate tracheal morphogenesis by different mechanisms. Projections (A-C, J-L and V-X) or single confocal sections (D-I and M-U) of embryonic trachea. (**A-I**) At stage 16, the dorsal trunk of both *bou*^{let} and *rtv*^{11} mutants displays an enlarged width and a convoluted shape, compared with wild type, as revealed by 2A12 luminal staining (A-F, arrowheads) or cell-contour labelling with ActinGFP (G-I). (**J-L**) CBP staining reveals luminal chitin cable disorganisation in both *rtv*^{11} and *bou*^{let} mutants. (**M-O**) Diffusion of 10 kDa dextran (red) into the trachea (marked by ActinGFP, green) of stage-16 wild-type and mutant live embryos. (**P-R**) Negative image in black and white of the dextran red channel. Dextran (black) diffuses into the tracheal lumen of *bou*^{let} (N,Q, asterisk) but not wild type or *rtv*^{11} embryos (M,P,O,R, asterisks). Notice abnormal dye deposits between *bou*^{let} contiguous cells (Q, black arrowheads). (**S-U**) Fas3 appears delocalised along the lateral membrane of tracheal *bou*^{let} cells (T, arrowhead) but localises to the apical part of both wild-type and *rtv*^{11} trachea (S,U, arrowheads). (**V-X**) Stage 15 *bou*^{let} and *rtv*^{11} embryos stained with 2A12, showing a series of cysts in their dorsal trunk (arrowheads).

NeuroglianGFP (NrgGFP) (Beitel and Krasnow, 2000; Wu and Beitel, 2004). Similarly to Fas3, all these markers appeared delocalised in the lateral membrane of *bou*^{let} tracheal cells (Fig. 3A,G-I,D,J-L). We also monitored the distribution of the cell polarity marker Crumbs (Tepass et al., 1990) and the apical junction component DE-Cadherin (Shotgun – FlyBase) (Oda et al., 1994). As in controls, these markers localised to the most apical part of the tracheal cells throughout development, indicating that *bou* specifically affects the SJ organisation rather than the general polarity of the cell (Fig. 3B,C,E,F). In addition, *bou* is required for the early establishment of SJ in this tissue (see Fig. S2 in the supplementary material), because a clear delocalisation of the Nrx-IV marker was already observed by stage 14, when pleated SJ begin to form (Tepass and Hartenstein, 1994).

Finally, we tested if *bou* is required for SJ organisation in other epithelial tissues, such as the epidermis, salivary gland and embryonic hindgut. Analysis of *bou*^{let} embryos showed that NrgGFP, Dlg1 and Nrx-IV are also delocalised in these tissues (Fig. 3M-R; and data not shown). Thus, *bou* is generally required for SJ organisation in embryonic ectodermal derivatives.

bou is required for SJ formation in a non-cell-autonomous fashion

Seeking to extend the characterisation of *bou* requirements to larval tissues, we analysed the contribution of this gene to the morphogenesis of imaginal discs, the epithelial precursors of the adult integument. For this, we studied mosaic individuals containing clones of homozygous *bou*^{PG27} cells, using the MARCM technique to positively label the mutant territories (Lee et al., 2000). We found that large *bou* wing clones generated early in larval development did not show any obvious growth defects. Moreover, the SJ marker Fas3 protein was correctly localised in *bou* mutant cells (Fig. 4A-A′). Thus, *bou* function could be restricted to the embryonic tissues or, more intriguingly, the surrounding cells could exert a rescuing activity upon the mutant territories.

To discriminate between these possibilities, we sought to establish whether *bou* is required in larval tissues. To bypass embryonic lethality and recover *bou*^{let} mutant larvae, we expressed wild-type HA-Bou in *bou*^{let} embryos using *nulloGAL4*, a driver only active at the blastoderm stage (Coiffier et al., 2008). In this way, we obtained *bou*^{let} mutants now dying at pupariation and presenting discs with reduced size and abnormal shape. At the

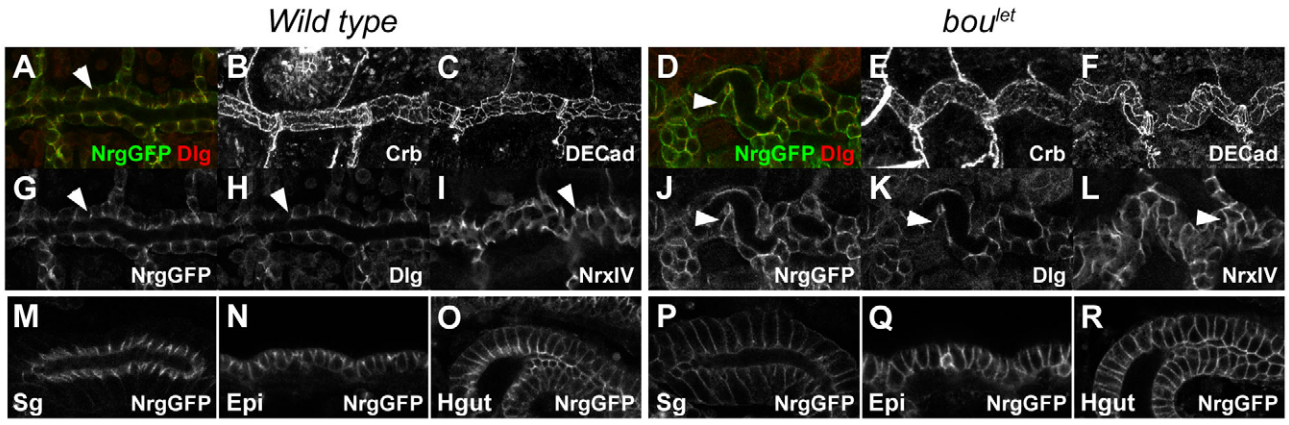

Fig. 3. *bou* is required for septate junction organisation in embryonic ectodermal derivatives. (**A-L**) Confocal sections of stage-16 tracheal dorsal trunks of wild-type and *bou*^{let} embryos stained as indicated. NrgGFP (G,J, green in A,D), Dlg1 (H,K, red in A,D) and Nrx-IV (I,L) distribute along the lateral membrane of *bou*^{let} mutant tracheal cells (D,J-L, arrowheads), differing from the control (A,G-I, arrowheads). The cell markers Crumbs (B,E) and DE-Cadherin (C,F) localise to the most apical part of tracheal cells in both wild-type and *bou*^{let} embryos. (**M-R**) Confocal sections of stage-16 embryonic salivary glands (M,P), lateral epidermis (N,Q) and hindgut (O,R) labelled with NrgGFP. In the wild type (M-O), these markers localise to the apical part of the cells (towards the lumen in hindgut and salivary glands; up in the epidermis) whereas in the *bou*^{let} mutant (P-R) they spread along the lateral membrane. Epi, epidermis; Hgut, hindgut; Sg, salivary glands.

cellular level, we observed that Crumbs localisation was not affected in *bou*^{let} wing cells. By contrast, the SJ marker Fas3 was delocalised and distributed uniformly along the basolateral membrane (Fig. 4C-F). Thus, the *bou* product is also specifically required for SJ organisation in imaginal epithelia. Consistent with the idea that *bou* phenotypes are not cell-autonomous, we recovered morphologically normal adult *bou*^{let} mutant flies expressing HA-Bou with *engrailed*, *patched* or *decapentaplegic GAL4*, three drivers with clear-cut regionalised patterns. Moreover, staining for Fas3 in *bou*^{let}*; enGAL4/UASHA-bou* mutant discs confirmed that SJs are normal throughout the disc and not only in the *engrailed* domain (Fig. 4B-B′). Therefore, cells expressing the Bou protein can rescue the mutant phenotypes in surrounding territories, confirming that *bou* acts non-autonomously.

One possibility is that Bou itself can travel from cell to cell. Indeed, some vertebrate members of the Ly6 family have the ability to diffuse, either as soluble ligands or coupled to lipid particles via their GPI anchor (Chimienti et al., 2003; Rooney et al., 1993). To gain insight into the Bou mode of function, we generated transgenic flies expressing a C-terminal truncated form of HA-Bou (HA-BouΔC), coding for an intact Ly6 motif but missing the last 22 residues of the precursor (Fig. 1B). We predicted this molecule would behave as an active soluble form, as the C-terminal region is necessary for GPI addition in other GPI-anchored proteins. Instead, we observed that HA-BouΔC expression did not rescue the *bou*^{PG27} lethality, indicating that the Bou C-terminus integrity is essential for its activity. Moreover, expression of HA-BouΔC in the tracheal cells driven by *breathlessGAL4* could not rescue Fas3 delocalisation in *bou*^{let} mutant trachea (Fig. 4J,N,R), whereas expression of a wild-type HA-Bou form not only rescued the *bou*^{let} phenotypes in the tracheal cells but also in the salivary gland and the hindgut, tissues not expressing HA-Bou in this genetic combination (Fig. 4I,M,Q; and data not shown). This finding indicates that Bou-targeted expression can elicit non-autonomous effects in other tissues, opening up the possibility that Bou could diffuse systemically.

Bou localisation is not restricted to SJ membrane areas

To characterise the Bou subcellular distribution, we first sought to confirm whether Bou is a membrane GPI-anchored protein. In *Drosophila* S2 cells, HA-Bou is observed in the cell body and also the plasma membrane, as confirmed by immunostainings carried out in non-permeabilising conditions (Fig. 5A,C). By contrast, the HA-BouΔC form could only be detected in internal cell compartments after permeabilisation, showing that the Bou C-terminus is essential for cell membrane insertion (Fig. 5B,D). HA-Bou is a GPI-anchored protein, because incubation of intact cells with phosphatidylinositol phospholipase C (PI-PLC) provokes its release to the extracellular medium (Fig. 5E).

Next, we studied the HA-Bou subcellular localisation in embryonic tissues and in the wing disc, activating its expression with tissue-specific drivers. As in cultured cells, HA-Bou appeared distributed homogenously throughout the tracheal cell body and did not accumulate in any particular structure (Fig. 6A). We found that the HA-BouΔC form has a more restricted localisation, as it was excluded from contact regions between adjacent cells (Fig. 6B). Co-staining with the SJ marker NrgGFP showed that HA-BouΔC was absent from the lateral membrane, whereas the HA-Bou staining overlapped with NrgGFP in the apical part of the cells (Fig. 6A,B).

In the wing disc, HA-Bou was also present in the cell body and throughout cell contact regions (Fig. 6C,E,G,H). By contrast, the HA-BouΔC form distributed like the disulphide isomerase PdiGFP, a resident enzyme of the endoplasmic reticulum (ER) (Bobinnec et al., 2003) (Fig. 6D,F,I,J). Thus, the HA-BouΔC form could not exit the ER, whereas the full-size HA-Bou reached membrane areas from which the ER is excluded (Fig. 6C,D,G,I). Co-staining with NrgGFP revealed that HA-Bou was present at the SJ level and accumulated in an apical region, placed above the SJ, that could correspond to a secretion compartment (Fig. 6E,H, see below).

To gain insight into the dynamics of HA-Bou protein localisation, we profited from the large size of the third-larval-instar salivary gland cells. Using the *bou*^{PG27} *GAL4* driver, we drove expression of HA-Bou and HA-BouΔC in this cell type, placing a *GAL80*^{ts} thermosensitive repressor in the same genetic background (McGuire

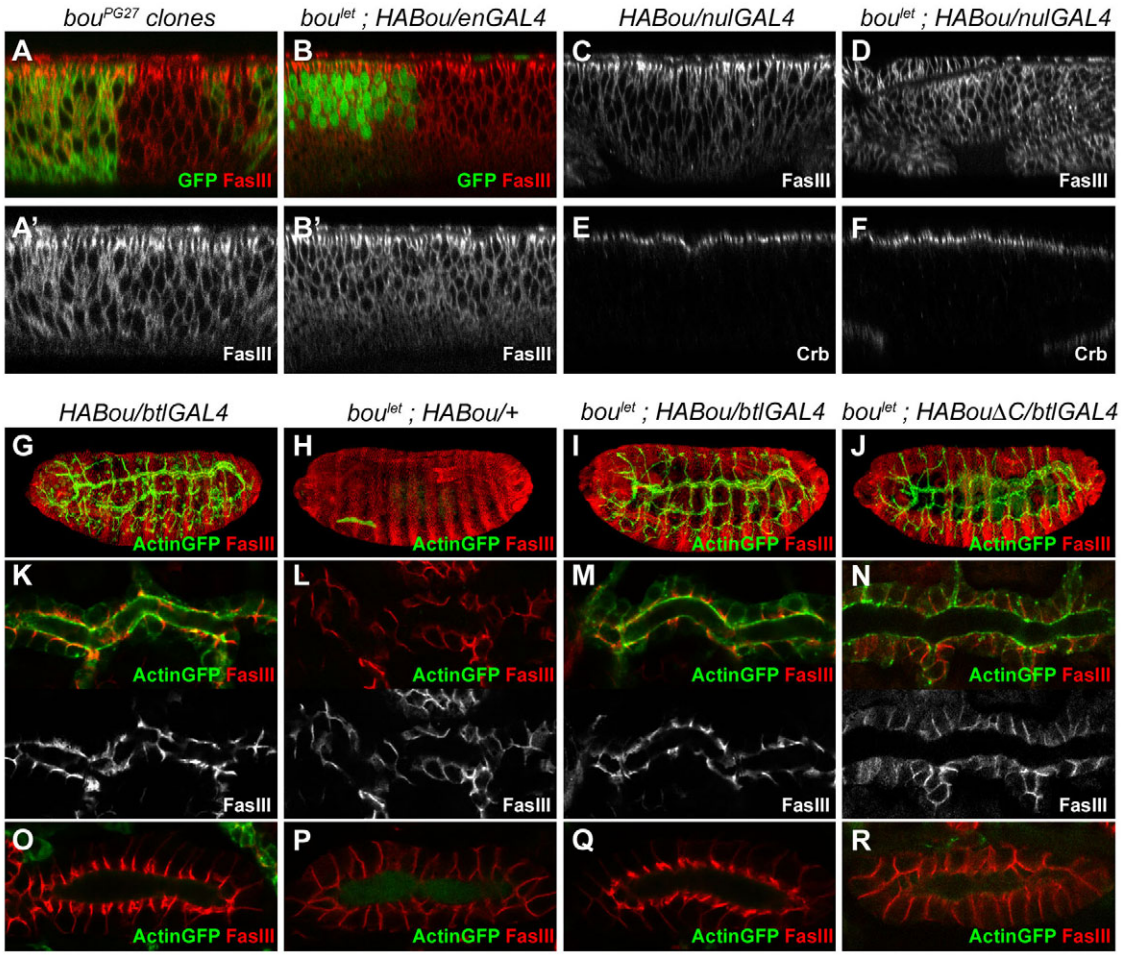

Fig. 4. *bou* SJ phenotypes are not cell-autonomous. (A-F) Confocal *z*-sections through the pouch of third-larval-instar wing discs. (**A**,**B**) Fas3 (red; white in A′, B′) is correctly localised in all the cells of mosaic wings containing GFP$^+$ *bou*^{PG27} mutant clones (A, green) or *bou*^{let} mutants expressing HA-Bou in *enGAL4* GFP$^+$ cells (B, green). (**C**,**D**) Fas3 distributes along the lateral membrane of *bou*^{let} mutant discs but localises apically in controls. (**E**,**F**) Crumbs localises to the apical region of both wild-type and *bou*^{let} mutant wing cells. (**G-R**) Confocal sections showing Fas3 localisation (red) in wild-type or *bou*^{let} embryos expressing HA-Bou or HA-BouΔC in the tracheal epithelium. Presence of ActinGFP (green) reveals *btlGAL4* driver activity. Only wild-type and HA-Bou-rescued *bou*^{let} embryos display normal accumulation of Fas3 in both trachea (K-N) and salivary glands (O-R). The green extracellular signal seen in salivary glands corresponds to unspecific background staining.

et al., 2003). At 25°C, the GAL80^{ts} repressor is inactive and we observed a strong accumulation of the HA-Bou forms in the salivary gland cell body (Fig. 6K,L). Then, we shifted the larvae at 18°C 24 hours before dissection, activating the GAL80^{ts} repressor and shutting down synthesis of HA-Bou protein. In these conditions, HA-Bou disappeared from the cell body and accumulated at high levels in the lumen of the salivary gland. In addition, we observed a weak but clear staining at the lateral membrane, coinciding with the NrgGFP SJ marker (Fig. 6M). As expected, the levels of HA-BouΔC decayed uniformly after the temperature switch (Fig. 6N). These results confirm that Bou associates with SJ membrane regions, although its localisation is not restricted to these membrane domains.

Bou is secreted extracellularly

One way to explain the non-cell-autonomy of the *bou* phenotypes is that Bou could be secreted extracellularly. Consistently, we noticed the presence of extracellular particles containing this protein in the luminal surface of the wing disc, budding off from the apical HA-Bou-enriched domain (Fig. 6C; Fig. 7A,B). These particles were seen over *apGAL4*-expressing cells, but were also detected in other territories of the wing disc lumen, indicating that HA-Bou can diffuse (Fig. 7A). Interestingly, increasing the laser power of the confocal microscope, we observed a diffuse intracellular staining and the presence of dots containing HA-Bou in cells adjacent to the *apGAL4* territory, showing that the secreted protein is incorporated by neighbour cells (Fig. 7D). In addition, we observed intracellular vesicles accumulating high levels of HA-Bou within the *apGAL4* cells (Fig. 7C). As none of these structures was observed in HA-BouΔC-expressing discs (Fig. 7E-H), we conclude that they reflect the ongoing traffic of the HA-Bou protein in the wing epithelium. We tested if the Bou extracellular particles are lipophorin particles, as these lipid vesicles are known to contain GPI-anchored proteins (Panakova et al., 2005). However, co-expression of HA-Bou and ApoLII-Myc, the main protein component of lipophorin particles, revealed that these markers label different vesicle populations (Fig. 7I-K). Thus, HA-Bou extracellular transport is unlikely to rely on lipophorin particles.

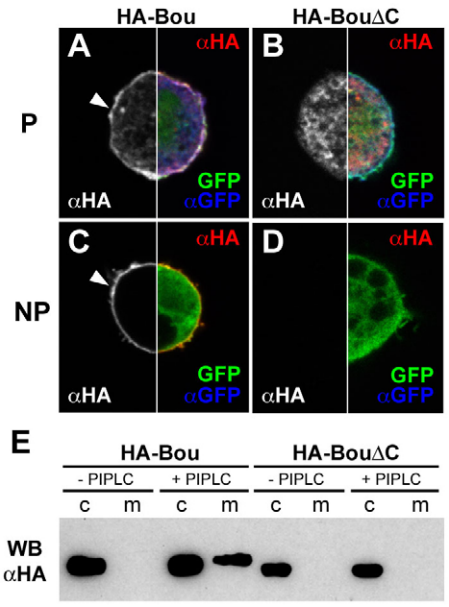

Fig. 5. Bou is a GPI-anchored membrane protein. (**A-D**) *Drosophila* S2 cells co-expressing Moesin-GFP (green, right panels) and HA-Bou (A,C) or HA-BouΔC (B,D). Staining with anti-HA (white, left panels; red, right panels) and anti-GFP (blue, right panels) was carried out in permeabilising (A,B) or non-permeabilising (C,D) conditions. White arrowheads indicate HA-Bou membrane accumulation. (**E**) Cellular fraction or culture medium of *Drosophila* Kc cells expressing HA-Bou or HA-BouΔC, blotted with anti-HA antibody. HA-Bou but not HA-BouΔC is released to the culture medium upon PI-PLC treatment. c, cellular fraction; m, culture medium.

bou function is required in a subset of neural tissues

We show that *bou* function is essential for SJ assembly in epithelial tissues. However, SJs also play a physiological role in the glial cells forming the blood-brain barrier and isolating the insect neural tissues (Banerjee and Bhat, 2007). This prompted us to examine if *bou* is involved in the maintenance of this barrier in the embryonic chordotonal organs. These sensory mechanoreceptors are made of five units, each formed by three glial cells protecting a sensory bipolar neuron. Two of these glial cells, the cap cell and the scolopal cell, form a luminal cavity encapsulating the neuron ciliar dendrite and forming SJs with each other to isolate this structure (Fig. 8A) (Carlson et al., 1997). The cell contacts between cap and scolopal cells accumulate SJ markers, such as Nrx-IV and NrgGFP (Fig. 8B-F) (Banerjee et al., 2006). We performed dextran injections in stage-16 wild-type embryos and confirmed that this dye is excluded from the chordotonal lumen (Fig. 8B-D). By contrast, the dye diffused into this structure in bou^{let} embryos of the same stage (Fig. 8H-J), and the SJ markers appear delocalised (Fig. 8H-L). Therefore, *bou* is also required for SJ organisation in the chordotonal organs.

The embryonic ventral nerve cord is also protected by a specialised layer of glial cells, the subperineural glia, which form an efficient paracellular barrier (Schwabe et al., 2005; Stork et al., 2008). Performing dye injections in 22-hour-old embryos (Fig. 8M-O), we observed that this barrier was still functional in bou^{let} mutants, whereas, as expected, the dye penetrated into the nerve cord of nrg^{14} mutants (Schwabe et al., 2005). Thus, the integrity of the central nervous system paracellular barrier does not depend on *bou* activity.

DISCUSSION

bou is required for SJ formation in different epithelial and neural tissues

Our results reveal that Bou plays an essential role in the organisation of SJs and the maintenance of paracellular barriers in *Drosophila* epithelia and chordotonal organs. Although some vertebrate members of the Ly6 family are known to participate in cell-adhesion processes (Bamezai, 2004), this is the first example showing that they are required for the formation of this type of cellular junction. As *bou* is well conserved in other insect genomes, its role in SJ organisation could have been maintained during evolution. Invertebrate SJs and vertebrate tight junctions are considered analogous structures because both participate in the establishment of paracellular barriers, although they present a different organisation. However, vertebrates have adhesion structures functionally, morphologically and molecularly similar to insect pleated SJs (Bellen et al., 1998): the so-called paranodal septate junctions, which are formed by neural axons and Schwann cells, at the level of the Ranvier's nodes (Schafer and Rasband, 2006). We show that Bou is necessary for SJ organisation in the embryonic peripheral nervous system, indicating that its activity is required in some neural tissues. Thus, our observations raise the possibility that some vertebrate Ly6 proteins could be involved in the formation of paranodal septate junctions, which are essential for axonal insulation and propagation of action potentials.

In insects, the epithelial and neural SJs share many components, so our observation that *bou* is not required for blood-brain barrier maintenance in the ventral nerve cord came as a surprise, revealing the existence of tissular and molecular heterogeneities in the organisation of these junctions. It will be interesting to establish whether these differences also determine different barrier selective properties. We speculate that other Ly6 proteins expressed in the nervous system could contribute to blood-brain barrier formation in the subperineural glia.

A secreted factor participating in SJ assembly?

Our results show that *bou* inactivation specifically perturbs the organisation of SJs. As these structures are large extracellular complexes including different transmembrane and GPI-anchored proteins (Wu and Beitel, 2004), one hypothesis is that Bou could be a membrane SJ component. Consistently, HA-Bou is found at lateral contact areas in tracheal, salivary gland and wing disc epithelia, overlapping with the membrane domains that contain SJ. However, this protein does not significantly accumulate in these membrane regions and is also seen in the most apical part of the cells, opening up the possibility that it could operate in other membrane areas or act as a signalling molecule. Indeed, studies in vertebrates indicate that Ly6 proteins can assume roles in both cell signalling and cell adhesion (Bamezai, 2004). Clearly, identification of the Bou molecular partners will be a crucial step in understanding how this protein exerts its activity.

In contrast with other genes required for SJ formation (Genova and Fehon, 2003), *bou* functions in a non-cell-autonomous way. Accordingly, the Bou protein is found in extracellular particles and can be captured by neighbouring cells, suggesting that its diffusion is responsible for the phenotypic non-autonomy. Although it is possible that Bou could act as a secreted ligand after release of its GPI anchor, a parallelism with other members of the family suggests that the full molecule could instead become incorporated into the membrane of neighbouring cells (Neumann et al., 2007). In fact, the mammalian Ly6 member CD59, a cell-surface antigen protecting host cells from the complement attack, travels coupled to

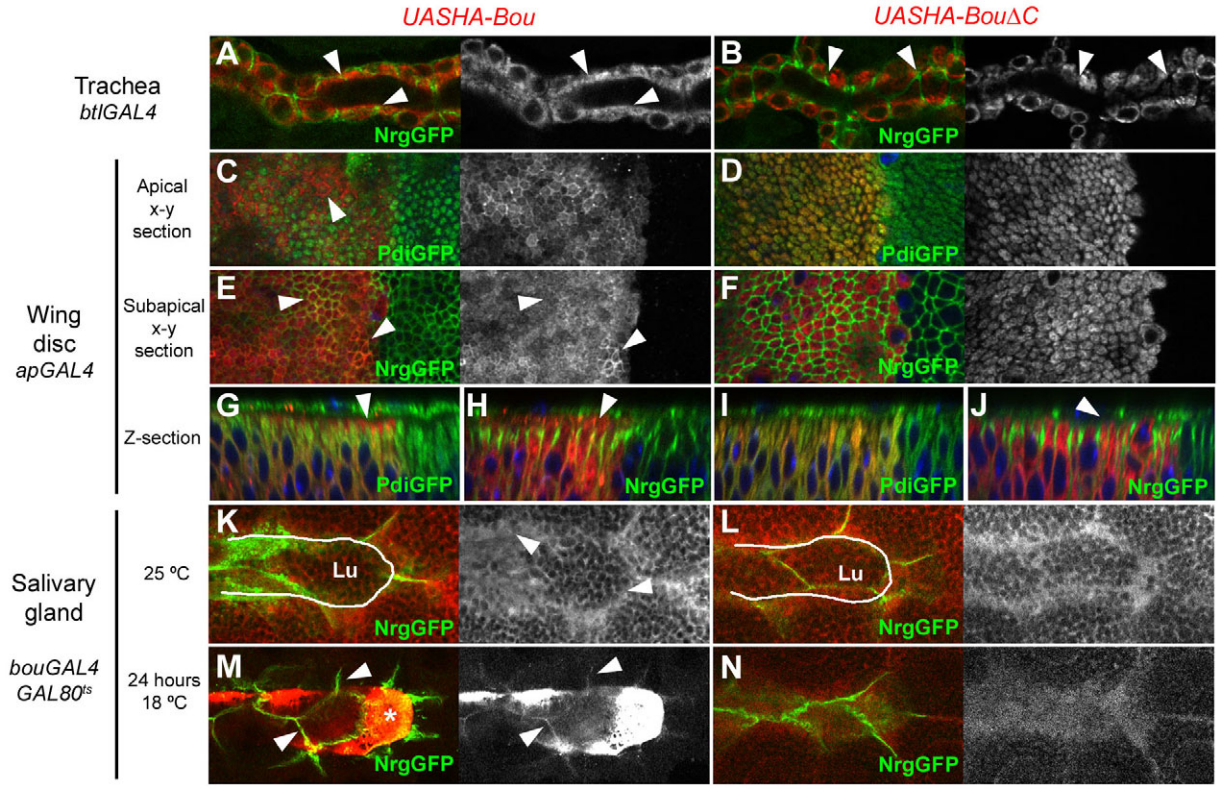

Fig. 6. HA-Bou is found in the membrane and is stabilised in SJ membrane areas. (**A-J**) Confocal sections showing HA-Bou and HA-BouΔC distribution (red) in tracheal cells (A,B) and wing discs (C-J) counterstained with NrgGFP or PdiGFP (green). HA-Bou overlaps with NrgGFP in membrane areas (A,E,H, arrowheads) and with PdiGFP in the cell body (C,G). HA-BouΔC colocalises with PdiGFP (D,I) but is excluded from membrane areas (B,F,J, arrowheads). (**K-N**) Single confocal sections featuring the luminal apical side of *bouGAL4/GAL80^{ts}* larval salivary gland cells expressing HA-Bou or HA-BouΔC. (K,L) At 25°C, both proteins stain the cell body. (M,N) Twenty-four hours after a shift to 18°C, the HA-Bou protein accumulates in the lumen surface (asterisk) and is seen colocalising with NrgGFP in lateral membrane regions (M, arrowheads), while HA-BouΔC levels decay uniformly (N). Lu, lumen.

membranous vesicles called prostasomes with its intact GPI. These specialised vesicles are secreted into the seminal fluid by prostatic glands, and allow CD59 transfer to the sperm cells, which can then elude complement attack (Rooney et al., 1993). GPI-bound CD59 has also been found associated with human HDL apolipoproteins (Vakeva et al., 1994). However, we show that the Bou particles are not lipophorin vesicles, the insect equivalent to vertebrate apolipoproteins (Rodenburg and Van der Horst, 2005). Therefore, the fly wing epithelium could produce a different type of vesicle, possibly similar to prostasomes, which we propose to call 'boudosomes'. Unfortunately, we could not determine whether the Bou GPI anchor is required for incorporation into these particles,

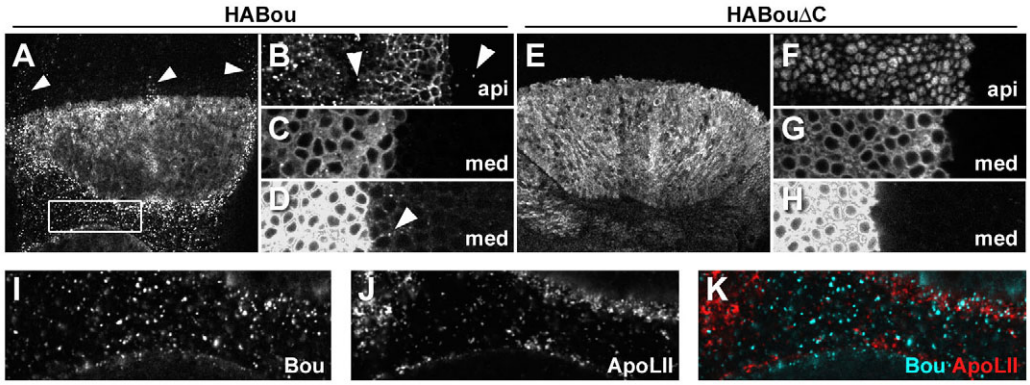

Fig. 7. The HA-Bou protein is present in extracellular particles. (**A-H**) Single confocal *x/y* sections of third-larval-instar wing discs stained with anti-HA antibody. HA-Bou accumulates apically in extracellular particles (A,B, arrowheads) and intracellular vesicles found in the medial regions of *apGAL4* cells (C) or contiguous cells (D, arrowhead). HA-BouΔC does not accumulate in any vesicular structure (E-H). (**I-K**) Higher magnification of framed area in A. HA-Bou (I, cyan in K) and ApoLII-Myc (J, red in K) label different populations of extracellular particles, as seen in merge channel (K). api, apical; med, medial.

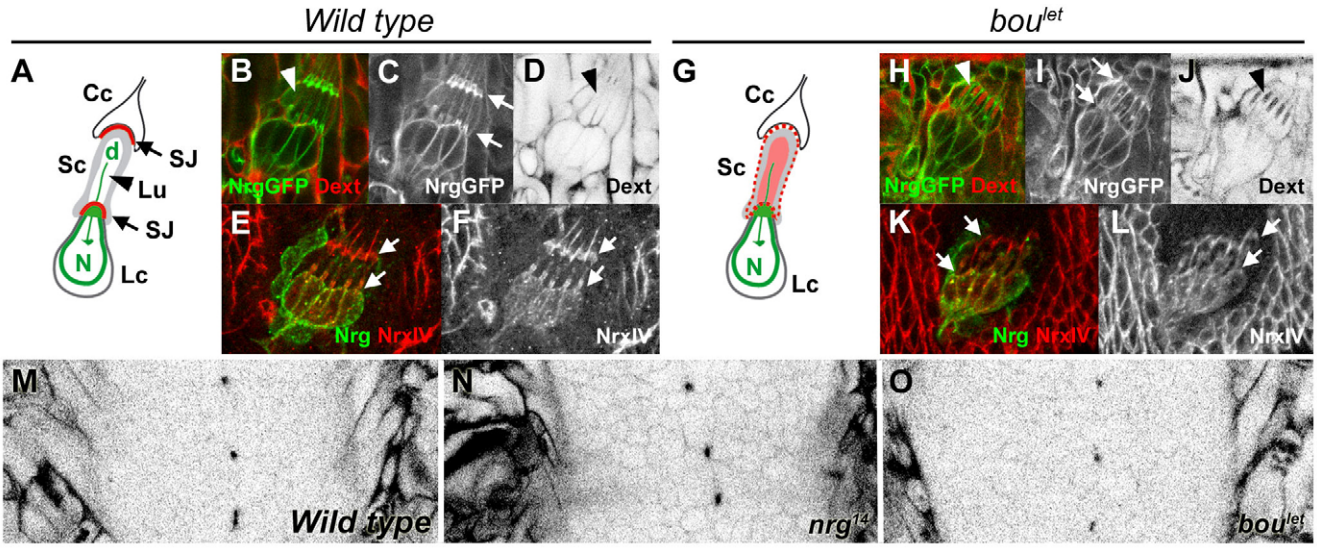

Fig. 8. The *bou* gene is required for blood-brain barrier maintenance in a subset of neural tissues. (**A-L**) Single confocal sections showing chordotonal organs of wild-type (A-F) and *bou*^{let} (G-L) stage-16 embryos. (A,G) Schematic representations of a single chordotonal organ unit. Injected dextran (red, B,H; black, D,J) diffuses into the lumen (arrowheads) of *bou*^{let} but not wild-type chordotonal organs. NrgGFP (green) and NrxIV (red) accumulate in wild-type SJ regions (B-F, arrows) but not in *bou*^{let} embryos (H-L). (**M-O**) Single confocal sections of ventral nerve cords of 22-hour live embryos injected with dextran (black). This dye fills intercellular spaces in *nrg*^{14} but not wild-type or *bou*^{let} embryos. Cc, cap cell; d, dendrite; Lc, ligament cell; Lu, lumen; N, neuron; Sc, scolopal cell; SJ, SJ contacts.

because the C-terminus of the protein seems essential for prior exit from the ER. Thus, future work will be needed to characterise the biochemical features of boudosomes and their function.

Little is known about how epithelial cells coordinate their activity to form efficient fences. As many SJ components are required in a cell-autonomous manner (Genova and Fehon, 2003), their simultaneous expression by each individual cell seems a prerequisite for barrier assembly. A component and/or SJ regulator shared by different cells could be an element coordinating the organisation of efficient barriers in a dynamic epithelium. Alternatively, Bou extracellular traffic could be a specialised feature of this GPI-anchored protein and not have functional relevance for SJ assembly during normal development.

The *Drosophila* Ly6 family boom

Besides Bou and the TGFβ receptors, the only member of the Ly6 fly family with a characterised role is the Rtv protein, which is also expressed in epidermal derivatives. We show that both *bou* and *rtv* mutants affect the organisation of the tracheal chitin luminal cable, although *rtv* mutants exhibit stronger phenotypes. However, SJ integrity is a prerequisite for proper assembly of the chitin cable (Swanson and Beitel, 2006), and we show that *rtv* is neither required for paracellular barrier integrity nor for SJ organisation. Thus, whereas our observations confirm that chitin cable deposition relies on the organisation of SJs, they demonstrate that these Ly6 proteins act in different processes.

We have carried out the first description of the Ly6 superfamily in the genome of an insect, identifying 36 new genes bearing this domain in *Drosophila*. The conservation of these proteins among the drosophilids indicates that the family was established before the evolutive radiation of this group. By contrast, we have identified only 14 genes coding for Ly6 domains in the honeybee genome. Most of these genes have fly orthologues, like *bou* and *rtv*, pointing out the existence in higher insects of a core of ancestral genes with potentially conserved roles. Thus, repeated events of gene duplication followed by rapid divergence of coding and regulatory sequences occurred in the drosophilid lineage. Indeed, the presence of genomic clusters grouping together different Ly6 genes is a novel evolutive acquisition, as the conserved genes tend to be in isolated positions (Table 1).

It seems that genes coding for a Ly6 motif are prone to sudden phases of extensive duplication and diversification in different phylogenetic groups. In fact, an interesting parallelism can be drawn with the evolution of three-finger elapid snake venoms. This large group of Ly6 secreted proteins operates using diverse strategies, such as forming membrane pores, targeting the activity of acetylcholine receptors, inactivating acetylcholine esterase or blocking platelet aggregation (Tsetlin, 1999). Moreover, crystallographic analysis has revealed that three-finger toxins can interact with their targets via virtually any part of their solvent exposed surfaces (Kini, 2002). Yet, most of them share a common ancestor (Fry et al., 2003). Given the broad diversity of expression patterns exhibited by the different *Drosophila* Ly6 members, it is likely that gene duplication has been followed by acquisition of new developmental and physiological functions. Analysis of this insect family from an evolutive perspective could be a way to enhance our understanding of the mechanisms underlying the generation of evolutive innovations.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/13/2199/DC1

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Ly6/uPAR motifs of proteins containing a single domain (25 proteins)

```
              1  2            3                        4                                5
CG14430       CYVCDTSDTEHPFQ--CGEWFERYDIPDIQPQN--------CSSVHGAQF------------------------CVKHVGRFEGGIGAKRF----------
CG1397        CYQCRSRGELGS----CKDPFTFNATDVEQEPGVAAIP---CASGW----------------------------CGKVIEGGGTYAIDDYDLAIQRM----
CG2813        CYVCSNQTGNTEK---CLNTIKT------------------CEPFENV--------------------------CGTEIRWGSQPYFSEGALKQYYVSKR-
CG7781        CFVCNSHKDAN-----CALDIPPDNLLKD------------CDEQYSSRGKGIPTY------------------CRKITQIIEFSVNSLPPDSRVIRT---
CG14275       CHQCNSHDNED-----CGGLVVNTPRAQRDNQYLTD-----CVPPSGEVAF-----------------------CRKTVINFEQNDERRIERS--------
CG14274       CYVCDSSDNPS-----CADLGSNSSIVAEE-----------CTLDKMKSLDTWLFDLNKFSYFDNGANKSPLMNCQKVVAKDPDTRKVVTARF--------
CG9568        CSKCTSPSG-------CKSPSSET-----------------CSNSTANANKEFLEGYHSNVPTVNGSLSFS---CANLTYYHAANYTHTFEFLG-------
CG13102       CYTCVTPKD-------CKSPKKVT-----------------CTNAAANETSYYLGVYHQNVGNLTSTRFD----CLALKYNWNNDVIHQLHG---------
CG6583        CYQCSSDQDRKGHDS-CGAYKRFNRTEHISIE---------CNSDESHMPGSF---------------------CMKVVQQGPRGFIWDGRWRQVIRR---
CG17218       CWDCRSDNDPK-----CGDPFDNSTLAITD-----------CQQAPELEHLKGVRPTM----------------CRKIRQKVHGEWRYFRS----------
CG6579        CYQCKSLTDPN-----CAKDKIDSASNIRAVD---------CDSVPKPNTMEQLQPVTR---------------CNKVVTSDRAGTIVSRD----------
CG15169       CYSCMF----------CNKTITNETKSN-------------CGPIPGKRNG-----------------------CRTILLNDPNVVPGKKYFLHRG-----
CG31676       CWRCSTDVSNGEF---CNDPFMPETISEQQRYWSYVN----CTYSVGAKSVNARPV------------------CKKLVQEVYGKRVISRS----------
CG31675       CYACESVYEAS-----CGDDFEVENHFKYD-----------CAFIAPPRFLENDLLSVNATA------------CLKRVFKENGVRKIVRG----------
CG9335        CWHCSSDTIGAEDF--CDVTFQEDNIPTDLIKERNINLLRSCNGTINSDHERAV--------------------CRKTVEENNGKLITKRF----------
CG9336        CYQCESLTMPK-----CGLKFEADETLLLD-----------CSRIGPPRYLQNFFPLRNATG------------CMKKTLESVAGHPQIVRS---------
CG9338        CYQCDSLTNSE-----CGKDIKSDSSLVLD-----------CTKMAPPRFLQNFFPVRNATG------------CMKQTIDIPGNPQIVRS----------
CG14401       CYECDSVNPG------CGERFVGDDISTTD-----------CDVVANMRSLGAEAT------------------CLTKYHEGMPGDTRFVRRS--------
CG33472       CYECDSWTDAR-----CKDPFNYTALPRDQPPLMT------CNGC-----------------------------CVKMVRHQRSPYEVVRRM---------
CG8501        CYECVDQETS------CGSADNSPGRVRE------------CPNSTM---------------------------CSTTMLTTMVNGNEWIRVRRG------
CG3955        CFACHTMDDGEA----CVDVAVRNDSALMKK----------CQGEEFI--------------------------CMVKRFSYTTSTENSTSSPKMWSLDRR
CG6329        CYDCNSEFDPR-----CGDPFEPYSIGEVN-----------CSKQEPLEHLKDKYKPTL---------------CRKTVQKIYGKTRIVRG----------
CG6038        CYRCTSATPG------CAEKFNWRGIGFLGEH---------CPEPDDI--------------------------CVKVTERRGARETITRD----------
CG8861        CHMCGQYNEGVGSITPCTNYTTDIAHLYLKE----------CTKKSEKF-------------------------CVKYVSELSTVRD--------------
CG31323       CFKCVSYNGANKA---CDDPFHNNYSTAILESP--------CMGGRKGRDGLFPATA-----------------CIKIAGYYDGTGETITVRG--------
```

```
            6                           7                                              8   9     10
CG14430     CSSKDMGNY-------------------CDYVRNKGDRMDYRS--------------------------------CIYTCDTDG-CN
CG1397      CVQRGPDDNMDR----------------CADTIYNYKKVYM----------------------------------CF--CQGDL-CN
CG2813      CMTKEQ----------------------CQSKRKRYMQLYCTHIWYEDWACNE----------------------C---CKGDR-CN
CG7781      CAYQNQTSTNY-----------------CYQRAGFGGRQVV----------------------------------CS--CDTDN-CN
CG14275     CGFIPEKIQNA-----------------CFTADNEGYKQII----------------------------------CT--CPDEG-CN
CG14274     CQLDTGDSDA------------------CEILRTKLRIPSPEEREQRNRNQNKRRKGHGQDAEEDDEISAEDAFFCGI-CKSHR-CN
CG9568      CVFNETNV--------------------CNLSLNNTASGWSKK--------------------------------CLQ-CGTDY-CN
CG13102     CVHPNVGA--------------------CSLALKPAYAHYNKTW-------------------------------CLT-CSGDK-CN
CG6583      CASVSDTGVVGV----------------CNWGVYENGVYWEE---------------------------------CY--CSSDS-CN
CG17218     CAYMGEPGIEGDERF-------------CLMRTGSYNIFMEF---------------------------------CT--CNSKDGCN
CG6579      CHFESIGQKDNE----------------CTVTHSRQVES------------------------------------CYT-CKGDL-CN
CG15169     CVSELDMELSRY----------------CAENEKL----------------------------------------CPT-CYEDN-CN
CG31676     CFYEDMDDSADK----------------CANDQTSSYIKTVY---------------------------------CRT-CTTDG-CN
CG31675     CYFGEVNATDVW----------------CKMDPTLSAVQNSS---------------------------------CHV-CDSENYCN
CG9335      CYYTNKSDPVEL----------------CNITSPEKNVRRIF---------------------------------CED-CLTDR-CN
CG9336      CYFGDINNIQAG----------------CQSDPSMPFVKQLG---------------------------------CDV-CTKDE-CN
CG9338      CYFGNIADTKVG----------------CQTDPSLTINKLLS---------------------------------CEV-CTEDE-CN
CG14401     CYFGDASPIGVS----------------CDDGPDPVVPFMNFLG-------------------------------CTL-CDTDL-CN
CG33472     CTSQLQINLFMVDHV-------------CMMESSGNGHM------------------------------------CF--CEEDM-CN
CG8501      CAKQVDHYFDYIGKHWEQKYRLMDLPEGCKKENGRMN--------------------------------------CN--CRGEL-CN
CG3955      CTAN------------------------CEPGCIIIGERTKLYSCTS----------------------------C---CEESF-CN
CG6329      CGYIPDENTDNK----------------CVRRSGTHDVAAIY---------------------------------CS--CTKDL-CN
CG6038      CLSALSFRKDIPADKYEG----------CRPAAHDEKLANYVNHTIKEHDVRRDYYTDTTF--------------CF--CFLDHRCN
CG8861      CATE------------------------CVEKEIWETQTY-----------------------------------C---CTEDG-CN
CG31323     CALDSGTLTTDTEIIRMSH---------CGKFYYDDKYVHG----------------------------------CLQSCSDADACN
```

Ly6/uPAR motifs of proteins with multiple domains (8 proteins)

```
               1  2         3             4             5                     6                     7             8  9          10
CG15773 1      CYACDSAEDSE--CATRPGQQLEVEE-CQQTGDE-------CVTSISAGLTRRG---------CLARLYPNGY------------CAAP----------CDRCNTSL------CN
CG15773 2      CYQCSGSD-----CIDVASRPQYLLP-CPVYQEDDR-----CYTNVVHLSNTLRG--------CEHTNLPTT-------------CPHV----------CLKCNYNG------CN
CG15773 4      CAACNSTANPG--CRAGRNLPTEK---CAAGTTA-------CYS-------------------CEQDTLVTHFPFIIPNTFLRRGCADADFAPAALGESCQLCRDADG-----CN
CG15773 5      CYRCNAQDQDSD-CAAMDLPTTMTTSNCSSPTEL-------CVSTVVSRLDGIYTVRG-----CEGQVPE---------------CSANDPY-------CVRCNGSL------CN
CG15347 1      CIQCNSKDNER--CATDPLSLLNRN--CSDGSSN-------CYSRVLDGYTIRG---------CAVDLDNATRNS----------CNNELL--------CQLCTYSEG-----CN
CG15347 2      CLQCTGNSTSSS-CATETYVSAKI---CPLYKFGDK-----CYIRNSNRTVDGSFQRG-----CLTSANARKQ------------CVKDGN--------CYTCEGRG------CN
CG10650 1      CYSCTSTTNDTGNCLTSPSTQTSIE--CDNE----------CYTLINAGTLTRG---------CLINGTA---------------CTLPS---------CSTCKEDN------CN
CG10650 2      CQQCLGEAN----CATTNVTDTQYNAVCPNNGQV-------CVNQLNDNKTVTRQ--------CGDP------------------CAAGTEST------CSSCSASL------CN
CG10650 3      CYNCSGEN-----CNAVSNTLVAG---CSQIDAG-------CFTTGTSASNMTRG--------CTSATTEIK-------------CASDSTDPS-----CLVCNSDF------CN
CG10650 4      CIICEN-------CAEQQVATNAKS--CGQAKYNQEVG---CYTMTSGTNVTRG---------CLNTLEAG--------------CSTTNA--------CTSCSENG------CN
CG10650 5      CITCISNEVSG--CWSAKYPDTLPLINCPNGT---------CYSGVWNELGVRG---------CFTAASHLMQYQ----------CNAKVEAHQ-----CELCTESK------CN
CG15170 4      CFTCNSVDQEL--CRTNITAIANST--CSSAEASS------CFS-------------------CEYSNWSIQRG-----------CGAPPENPQITK--CYECDEGAG-----CN
CG15170 5      CYQCSTDQAGAG-CANWESPGEIYIEECDEPAAP-------CVVVSFNNATTARG--------CQKSDFS---------------CASANVAS------CRLCEGSF------CN
CG15170 6      CHQCRGVQD----CEEISRGQTAMP--CPARANEPEDQELACLEYFDINSKEIVRG-------CRSNPELYYE------------CLLRSNHFDS----CRTCHSQA------CN
CG4363 1       CHTCEGAN-----CQRVQLSKTQT---CVDSLDY-------CVTIYDEAKVLFKG--------CSLEIPKDLRSK----------CNDNRS--------CHKCNTKE------CN
CG4363 2       CIQCDSSKDSN--CASNAAALEAAR--CSAPTAPNSY----CYVKSSGGSIVRG---------CSTTETDQQS------------CLNDAN--------CLLCSPGDIRN---CN
CG4377 1       CYSCEGIN-----CSRTTRQNATVS--CSDRLDV-------CVTIYEDFAVSERG--------CFSQISLAGQAK----------CAAKDDQ-------CQKCSGQL------CN
CG4377 2       CIQCIGSDSAD--CNKGATASLAATQ-CALPTSANSY----CYVKVVGDQKDSLQRG------CALSVKEQKA------------CLEDSK--------CSLCLPENSSDSVACN
CG34040 1      CIYCRDIN-----CQRSSYDADEQ---CSEKLDA-------CVSVFKAGVIQAQG--------CLESLEDDWREK----------CEDKDKGNEID---CEICVTER------CN
CG34040 2      CIQCNNTEDAQ--CAESPGLLTAVQ--CPIARSGRSF----CYASLVGDDLKRG---------CSLTLSDQVK------------CLADPN--------CHLCDPLEQPH---CN
CG13492 1      CYECSTLDE----CSAGSGVLRK----CLDNDGQS------CVAIFNSDGAVIERG-------CSDNLASV--------------CTESGDN-------CYECRSHG------CN
CG13492 2      CVVCDAQSDDN--CVFDIELVTERRK-CNQQ----------CMTALYPTNSEEGAPLELVRS-CLDDLDLDDREA----------CSEGSLEN------CVACSAAS------CN
CG13492 4      CKLCNSQTDED--CAVAPENVETNTQ-CESLVNTD------CYSRILDNGHTERG--------CLTSLEGEDYLD----------CLKDSNITK-----CSRCTGEL------CN
CG13492 5      CQSCSDAAS----CAASDLSNTT----CSSNSDY-------CVTVFSANGSVSAKG-------CSQEVEQTWASY----------CDANAGS-------CHNCNSNG------CN
CG13492 6      CIYCDYENED---CATNAANVKSRRL-CNGQ----------CFTGSRRRSSENFIYDTVRG--CLDDKDPEDQAA----------CIAGTDAA------CIACSGAN------CN
CG13492 8      CIVCDSSEDPN--CATDPSKITLIGN-CGVLPYTS------CQTRIISGRTQRS---------CLSSLEREQLQE----------CLDGTGN-------CVVCTGDR------CN
CG13492 9      CHRCNSVTDPT--CSSAPDAAEV----CPYYLEDEG-----CSAKLVDGETYRG---------CQREFT----------------CDDSDKQH------CRLCSGKDN-----CN
CG13492 10     CYTCNGTD-----CQGDSSGTLRK---CTGNDEQN------CGTVFDASGSVIFRG-------CSDTLYEDAELLQY--------CDENSAN-------CKFCKSSG------CN
CG13492 11     CLFCDGSDEAE--CVRKVGDITRTVS-CQGS----------CLTGLYPRNRSEANPVYDLARGCLDDLDYDDREA----------CSAGTLEN------CVSCSSAT------CN
CG13492 13     CNVCNSTYDDG--CVGGNSALATV---CEHYINPE------CYSYLNEDGVLLRG--------CLADTNDELFDE----------CSSDGSTN------CTICSTNN------CN
CG13492 14     CLRCDSASDAD--CALNPASYSSY---CRTYSEDDA-----CVTSLSNGRTRRG---------CQSELN----------------CDASQAGS------CRVCSEAN------CN
CG13492 15     CHVCSDAAS----CATVGTEITK----CEGNNKQT------CSTVFNSEGQVVARG-------CSDAVLAANVDY----------CDLNGDK-------CVQCKSDG------CN
CG13492 16     CYFCDAEDDVN--CAWEKPESTRK---CQGQ----------CMTGLYPRSSSWDSALLPTRG-CLDDLNEAERTS----------CAAGTHSN------CTACTGSL------CN
CG13492 18     CLQCTSTDDPR--CATNPNQLTTTNI-CSQIPYTE------CVTQIDSQGNTIRG--------CMSSLGGDDFYE----------CLTGDGKL------CETCTGER------CN
CG13492 19     CYQCNSTSDPN--CAASPATLESTV--CPIYSQDES-----CVTTLLNGITHRG---------CSSSLT----------------CSDPSDSRT-----CRVCSSADG-----CN
CG13492 20     CLVCSGTD-----CASSGGTLTK----CSGYDN--------CVTVFSDSGSVTQRG-------CSESVFEESDY-----------CDENPAY-------CPRCNSNG------CN
CG13492 21     CIVCDSSVDSN--CVSDPTQITKTRQ-CHER----------CISAFLPLFNETEDPSYALIRNCYDDLEKEDRDA----------CTAGSKRF------CATCEGTK------CN
CG13492 23     CTLCSSRTEAN--CAISPSSVVSSTD-CTLGGFPE------CYSRVLSDGSTERG--------CLSSLEDDEFLS----------CYNSTSAS------CNSCQGNK------CN
CG13492 24     CHVCNSATDAN--CENNPSSLTV----CYLYDADDQ-----CVTNLRNGVTYRN---------CASSLS----------------CEANAKT-------CVYCEGDG------CN
CG13492 25     CLTCEGTG-----CQEEEIASIK----CQNNNEQD------CLTVFGSDGTVTRRG-------CQDEVEADATISTY--------CGTNAGS-------CPSCKSNN------CN
CG13492 26     CIYCDSYKDSS--CLWEPTSASHRTRQCQGQ----------CMTALYGSADAGLDLIRT----CLDDKEASDQLI----------CANGSDST------CAACSGDS------CN
CG13492 28     CAVCDSREDPS--CATDAYAIGTFST-CSQLPHTG------CFTRLENDGSTQRG--------CLYDLDQTDFAA----------CLLGSDAN------CTVCSVDG------CN
CG13492 29     CYSCTSADDSS--CESDPSYTLA----CLWVSETET-----CKTLLSGNVTTRG---------CSSSVE----------------CDSSDFRN------CRSCSGSE------CN
CG13492 30     CHTCVGEH-----CLSSLGPAVT----CSLNLEQD------CKTVFEADGVSVRRRG------CSDDVDDYEDRY----------CRKNPAL-------CFTCKSNE------CN
CG13492 31     CTFCNSANKSS--CVTDPTESDLSKRNCKGQ----------CLVALSGEDLIRS---------CLDDKELYDRSD----------CTTDESGTN-----CATCSGAE------CN
CG13492 33     CLSCDSSKTLE--CAQDPTAVLTTIT-CHAPNSD-------CITRLENGHTHRG---------CRSEVSSTESSS----------CVADGT--------CSACSGAK------CN
CG13492 34     CYICNSIDDPL--CAQHPSSLAV----CPIYAANDE-----CVTSLNNGLLRRG---------CASELE----------------CEIDNEDH------CNKCSTDG------CN
```

Disordered 10C domains (3 proteins)

```
                1  2    3           4                                5                6           7                8  9    10
CG14273         CYKCED--CDENTQLSEMEVCESPLM------- 118 residues -----FCYTVRLQLNESTITKRGCTTARRSNQTGGCDGLFENWTVAG-----CQLCQDDGCN
CG7778          CYVCDN--CAQLPKDAPLLACNEDFFNPGGS-- 132 residues --YTYHCYSVQVSVNGTMSTDRGCSRVSTMEGV--CEQLKIQNKNTELAN--CNPCSMNACA
CG31901         CYSCVGNECHVETVPTVT--CTLDDV------- 426 residues -YKIAACYSIYKDGELNRG----CVKVPEKHSG--CQAVRNELGITEDSADQCDICLTNLCN
```

Atypical 10C domains (2 proteins)

```
                1  2          3                4         5             6              7                 8     9  10
CG15773 1       CLQCTHNRLAPNPDCLRDQDPPAAVAEDQPKCSLSNSTVTHCVNKVMYGHREN--CFSYRNTQTEVLQRGCSTAMGFYPTGELTE---CHGEF-CNADC
CG15170 3       CATCDSAIGRG---CKIDLFQVNTGR-----CNVSLYEE--CQQDVLLGEQEDKYCFSFRRLSRVVRG--CSTKIPTDLEPYVEQLEKCNTSDHCNAGC
```

8C domains (2 proteins)

```
                1  2       3              4           5              6                    7    8
CG15170 1       CYHCDSIALPECSQTLGEVGVLPYKECATELT------CAMSIVDSITYRG--CGAETPIGATYSKT-------CSTNLCN
CG15170 2       CHHCAGQE---CVAAPASKPKP----CRYHLEEDQ---CYTDVISSSDAYRG-CTSEQNHTLSTSAQL------CEINGCN
CG13492 3       CNVCKGD----CSNPQSKT-------CRAVPSGDKPESCFIEFDESGAIYEMGCLSQYNVSDVTLLETNKQLWYCTGDNCN
CG13492 7       CYKCSGSD---CDDPKASQ-------CSQYSPDDR---CYILFDYNADITGMGCLSDLDEEYVDENFHSLLF--CDDNDCN
CG13492 12      CNVCEDDA---CETLTSQL-------CLGYRSGDQ---CYIHVGDLSIKAMG-CATDLQDSFLLTNRRDLYL--CSGDDCN
CG13492 17      CYTCKDPF---CEDPTTSK-------CVAYRENDQ---CYLAYDDSGVVAMG-CASEFEVQVIKELVAQQRLLLCSGQKCN
CG13492 22      CLVCQGDE---CQSPQASS-------CSNYREHDE---CYIQFDEERSITSLGCLSELSHDDIYLLKRSKRLLTCSDNDCN
CG13492 27      CYTCEGDD---CEDPQPKT-------CTIYKPEDS---CFLWVDEDNDLKQLGCLSSFRNQDLEAIIKTKRISVCNGTNCN
CG13492 32      CHTCLDDT---CSSSQSQA-------CLAYKTNDY---CFAKYATDGKVELMGCASSQNESSLEQWQEGNLLYSCQGSECN
```

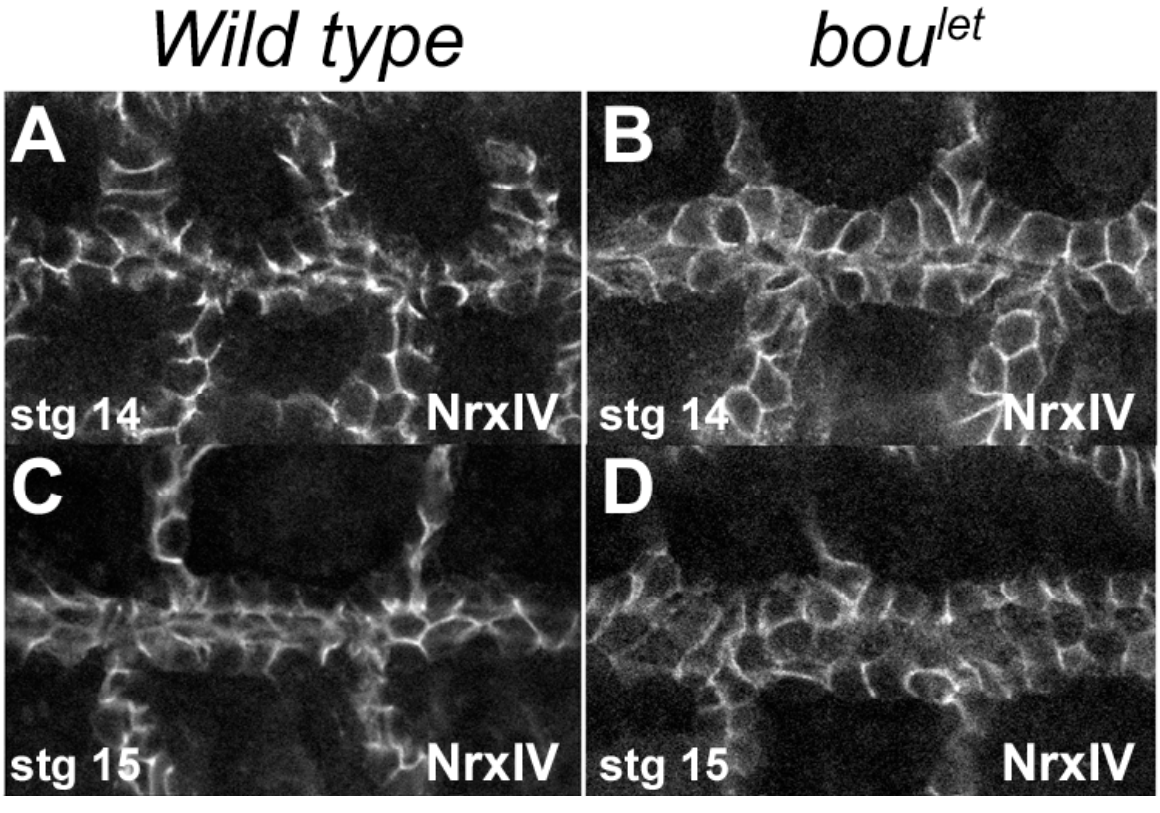
Ectodomains of TGFβ receptors type I (3 proteins) and II (2 proteins)

Type I receptors

```
                1  2   3         4       5                       6              7                      89     10
Babo            CH-CDT-CKESNNI---CETDGF--CFTSVEKNSDGSIIFSYR------CLHKSQIFPPGRSIWCNDGLHGGPTARPVGRNGAHA--CCKDRDFCN
Tkv             CY-CDGSCPDNVSNGT-CETRPGGSCFSAVQQLYDETTGMYEEERTYG-CMPPEDNGGFLM---CKVAAVPHLHGKNIV--------CCDKEDFCN
Sax             CYSCEPPCRDPYEFTHTCQNAIQ--CWKSRTRDADGQVQESRG------CSTSPDQLPMI----CSQNSLKINGPSKRNTGKFVNVVCCAGDY-CN
```

Type II receptors

```
                1                                         2         3                   4          5    6              789    10
Punt            CEHFDEKMCNTTQQCETRIEH---------------------CKMEADKFPSCYVLWSVNETTGILRIKMKGCFTDMHE----CNQTECVTSAEPRQGNIHF-CCCKGSRCN
Wit             CMSYQEDDNSFHDDDGDQDSSGELQEQQVESTPIPSEPHRRTCPDGYTF---CFTIWNQTANGARVVKQG--CWKDNTDRTSICSQSECTSSAPTSKTSSLYYCCCSGGVCN
```


Wild type
bou^let
A
stg 14
NrxIV
B
stg 14
NrxIV
C
stg 15
NrxIV
D
stg 15
NrxIV